

ORIGINAL ARTICLE

Allergic sensitization is enhanced in early life through toll-like receptor 7 activation

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Summary

Background Prospective cohort studies suggest that children hospitalized in early life with severe infections are significantly more likely to develop recurrent wheezing and asthma.

Objective Using an inhalational mouse model of allergic airways inflammation, we sought to determine the effect of viral and bacterial-associated molecular patterns on the magnitude of the allergic inflammatory response and whether this effect was age dependent.

Methods BALB/c mice were sensitized by intranasal administration of endotoxin^{low} ovalbumin (OVA) in the absence or presence of viral single-stranded (ss)RNA, lipoteichoic acid or flagellin as neonates (within the first 24 h of life) or as weanlings (4 weeks of age). Mice were challenged four times with OVA at 6 weeks of age and end-points (bronchoalveolar lavage cytology, histology, antigen-specific T and B cell responses) determined at 7 weeks of age.

Results Inhalational sensitization (< 24 h or 4 weeks of age) and challenge with OVA induced a mild allergic inflammatory response in the airways as indicated by increased numbers of eosinophils and mucus cells, elevated serum OVA-specific IgG1, and production of T helper 2 (Th2) cytokines. Mice sensitized to endotoxin^{low} OVA at birth in the presence of ssRNA or lipoteichoic acid, but not flagellin, showed an increase in the numbers of airway and tissue eosinophils, mucus producing cells and antigen-specific production of IL-13 as compared with mice exposed only to endotoxin^{low} OVA. By contrast, all three TLR ligands failed to increase the magnitude of OVA-induced allergic inflammation in mice sensitized as weanlings.

Conclusions Recognition of distinct microbial-associated patterns in early life may preferentially promote the *de novo* differentiation of bystander, antigen-specific CD4⁺ T cells toward a Th2 phenotype, and promote an asthma-like phenotype upon cognate antigen exposure in later life.

Keywords allergy, asthma, eosinophil, IL-13, neonatal, sensitization, ssRNA, TLR2, TLR7, virus
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Introduction

Allergic asthma is a chronic inflammatory disease mediated by allergen-specific T helper 2 (Th2) CD4⁺ T cells which produce IL-4, IL-5, and IL-13 [1, 2]. Evidence from experimental mouse models has shown that IL-13 can promote the majority of the cardinal features of disease, including eosinophilia, airway remodelling, mucus cell hyperplasia and airways hyperreactivity, while IL-13-deficient mice are protected [3, 4]. In support of the concept that IL-13 is the central mediator of allergic

inflammation a recent clinical study demonstrated that blockade of the IL-4R α subunit, utilized by both IL-4 and IL-13 was efficacious in the treatment of asthmatic subjects [5]. However, although Th2 cytokines play a central role in mediating the hallmark pathologies of asthma, our understanding of why and how patients with allergic asthma preferentially generate Th2 responses remains rudimentary.

The activation of pattern recognition receptors (PRR), such as the toll-like receptor (TLR) family, by microbial-associated molecular patterns induces the production of

pleiotropic cytokines such as TNF, IL-6 and IL-12 family members, which direct the development of CD4⁺ T helper immune responses. For example, the development of Th2-driven allergic airways inflammation (AAI) following inhalational sensitization to ovalbumin (OVA) is dependent on the activation of TLR4, although this requirement can be overcome when exogenous TNF is administered during sensitization [6]. These findings suggest that Th2 aeroallergen sensitization is influenced by microbial-associated molecular patterns of bacterial origin, although whether the activation of other bacterial-associated TLRs, notably TLR2 and TLR5, promotes allergic sensitization remains to be shown. Significantly, severe infections with the single-stranded (ss)RNA virus respiratory syncytial virus (RSV) in infancy have been proposed to be a risk factor for asthma-like symptoms including wheezing and allergic sensitization [7, 8]; while experimental mouse models have shown that respiratory infections are able to break inhalational tolerance and promote allergic sensitization to bystander antigens [9–11]. Thus, the activation of TLR7 through recognition of viral ssRNA [12] may also be a contributory pathway that underlies the association between viral infection and the development of allergic sensitization.

In humans, primary exposure to allergens occurs in early life [13], yet the vast majority of experimental studies are performed on adult mice with mature immune systems. The importance of performing early life studies is highlighted by Culley et al. [14] who demonstrated that secondary exposure to RSV at 12 weeks of age is characterized by virus-specific Th2 cells when the primary infection occurred in the first week of life, whereas a Th1 response was generated when primary infection occurred at 8 weeks of age. Thus, the age of sensitization altered the type of adaptive immune response mounted against the virus. Although the adaptive immune response is well known to be immature at birth [15], recent evidence suggests that the magnitude of the innate response may also be differentially regulated in an age-dependent manner. This was elegantly demonstrated by Zhao et al. [16] who observed that TLR activation in the neonatal period leads to the hypersecretion of TNF and IL-6. In view of the prevalence of allergic asthma among young children, and the role of innate immunity in shaping T cell differentiation, these age-dependent findings have important ramifications for the study of allergic sensitization. Thus, we sought to determine whether the activation of other TLRs in addition to TLR4 were able to alter the type of T helper response generated to inhaled bystander antigens, and whether the age of allergic sensitization altered the magnitude of AAI to antigen challenge in later life. We observed that TLR activation, in particular TLR2 and TLR7, at the time of inhalational sensitization promoted the development of AAI, and that this phenotype was markedly greater when sensitization occurred in neonatal as compared with weanling mice.

Materials and methods

Animals

BALB/c mice were used in all experiments and obtained from the John Curtin School of Medical Research (Australian National University, Canberra, Australia) and housed in a Specific Pathogen Free Facility. Mice were treated according to Australian National University Animal Welfare guidelines.

Sensitization protocol

Adult mice were timed mated and pregnant mice identified by plugging. Pregnant mice were checked daily for litters so that newborn mice were sensitized within the first 24 h of life. Ovalbumin (OVA, Grade V; Sigma, St. Louis, MO, USA) was depleted of contaminating endotoxin using a Detoxi-GelTM Endotoxin Removing Gel (Pierce, Rockford, IL, USA) and is described as endotoxin^{low} OVA hereafter. Residual lipopolysaccharide (LPS) activity, measured by limulus amoebocyte assay (BioWhittaker, Vic., Australia), was <~1 ng/100 µg OVA. Mice were sensitized to endotoxin-free saline (vehicle), commercial (c) OVA (not endotoxin depleted; ~7 ng/100 µg OVA), endotoxin^{low} OVA, or endotoxin^{low} OVA mixed with TLR ligand. Neonatal mice (~1.5 g weight) were sensitized intranasally with 5 µg OVA/g body weight in 5 µL endotoxin-free saline (vehicle) on the first day of life. ssRNA based on a TLR7-active sequence identified by Heil et al. [12] ['ssRNA40/LyoVec', ssRNA40 5'-GsCsCsGsCsUsCsUsGsUsUsGsUsGs UsGsAsCsUsC-3' ('s' depicts a phosphothioate linkage); Invivogen, San Diego, CA, USA] was diluted in endotoxin-free water and mixed with OVA before administration and administered at 50 ng ssRNA/g body weight. LPS from *Escherichia coli* (O111:B4; Sigma), lipoteichoic acid from *Staphylococcus aureus* (LTA; Sigma) and flagellin from *Salmonella typhimurium* (generous gift from Dr. Andrew Gewirtz, Emory University) [17] were identically employed, but at 5 ng/g, 5 µg/g and 100 ng/g body weight, respectively. In some experiments (Fig. 4), mice were intranasally exposed to LPS at 500 ng/g or vehicle control 1 h before sensitization with OVA and ssRNA. Sensitization of weanling mice was performed under isoflurane-induced anaesthesia. Weanlings were intranasally sensitized at the same dose of OVA±TLR ligand dose/body weight as described for neonatal mice.

Airway challenge

Mice were anaesthetized with isoflurane and challenged intranasally with 25 µg endotoxin^{low} OVA in 40 µL vehicle on days 42, 43, 46 and 47 of life, and killed on day 49 of life.

Bronchoalveolar lavage

The trachea was pierced and a 21 G needle with syringe used to instill 0.75 mL phosphate-buffered saline (PBS) into the lungs. Following a second lavage, red blood cells were removed by hypotonic shock. Cells were then washed, counted and cytospin preparations made. Slides were stained with May–Grunwald–Giemsa solution and eosinophil counts obtained based on morphology and staining characteristics. Data represent total eosinophil numbers in bronchoalveolar lavage (BAL).

Characterization of lung eosinophilia and mucus cell hyperplasia

Lung tissue representing the central (bronchi–bronchiole) and peripheral (alveoli) airways were fixed in 10% phosphate-buffered formalin, sectioned and stained with Charbol's chromotrope 2R or periodic acid–Schiff (PAS) and counterstained with haematoxylin. Tissue eosinophils were identified by morphologic criteria and orange colouration and were quantified to a depth of 100 µm beneath the epithelial basement membrane (BM) as described previously [18]. Both eosinophils and mucus-secreting goblet cells were expressed as cells/100 µm BM length.

Stimulation of mediastinal lymph nodes

Mediastinal lymph nodes (MLNs) were excised and filtered through nylon mesh (70 µm). The filtrate was then centrifuged at 500 *g* for 5 min at 4 °C, red blood cell lysis performed and the resulting cells cultured at 10⁶ cells/well in complete medium in the presence or absence of 200 µg/mL endotoxin^{low} OVA for 6 days [19]. Cell-free culture supernatants were collected and stored at –70 °C until cytokine concentrations were determined.

Analysis of cytokines by enzyme-linked immunosorbent assay

The concentrations of IL-13 (R&D Systems, Minneapolis, MN, USA), IL-4, IL-5, and IFN-γ (BD Biosciences, Minneapolis, MN, USA) were determined in culture supernatants from OVA-stimulated MLN cells by ELISA according to the manufacturer's protocol.

Determination of ovalbumin-specific antibody titres by enzyme-linked immunosorbent assay

Briefly, flat-bottom 96-well microtitre plates were coated with OVA (2 µg/100 µL/well in NaHCO₃ buffer, pH 9.6) and incubated overnight at 4 °C. Plates were blocked with 3% BSA/PBS for 1 h at 37 °C. After incubation with dilutions of serum samples and standard murine IgG1 or IgG2a for

2 h at 37 °C, OVA-specific immunoglobulins were detected with biotinylated goat anti-mouse IgG1 or IgG2a (BD Pharmingen, San Diego, CA, USA). Plates were incubated with streptavidin-conjugated horseradish peroxidase (BD Pharmingen) for 1 h at 37 °C and TMB substrate solution (BD Pharmingen) was added. Reactions were stopped with 1 M sulphuric acid and the plates were read at 450 nm in a microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA).

Quantitative RT-PCR

Quantitative RT-PCR was performed as described previously [18]. Briefly, RNA from lung samples was extracted and transcribed into cDNA using random primers and Moloney murine leukaemia virus reverse transcriptase. The real-time PCR was performed with an ABI 7700 Sequence Detector System (Applied Biosystems, Scoresby, Australia). Cycling conditions were 50 °C for 2 min and 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and dissociation analysis. Expression of a housekeeping gene (HPRT) was also determined against plasmid controls to generate a standard curve. The expression of IL-13 was normalized against the expression of the housekeeping gene, which was not altered by the treatment. IL-13: For – agctgagcaacatcacacaagacc, Rev – tgggctacttcgatttgggtatcg. Comparative IL-13 expression levels were derived using the equation: $2^{-(\text{average } C_{T, \text{Target}} \text{ calibrator} - C_{T, \text{Target}} \text{ sample})} / (\text{copy number}_{\text{HPRT sample}} / \text{average copy number}_{\text{HPRT calibrator}})$, where the calibrator was the OVA-sensitized weanling mice.

Statistical analysis

Data presented are the means (±SEM). Datasets were analysed by unpaired Student's *t*-test. The software package GraphPad Prism 3.01 (GraphPad Software, San Diego, CA, USA) was used for all data analysis and preparation of graphs.

Results

The induction of inhalant OVA-induced AAI in mice is conditional on the activation of TLR4 by contaminating levels of endotoxin in commercial preparations of OVA [6]. To examine whether other microbial-associated molecular patterns promoted AAI, we first removed endotoxin from our commercial preparations of OVA using a polymyxin B column. To verify the findings of Eisenbarth et al. [6], we compared the effects of vehicle (saline), cOVA, endotoxin^{low} OVA and endotoxin^{low} OVA spiked with low dose LPS (5 ng/g body weight) on the induction of AAI. Mice were sensitized intranasally at 4 weeks of age, challenged four times with endotoxin^{low} OVA 2 weeks later, and euthanized at 7 weeks of age (Fig. 1a). As shown

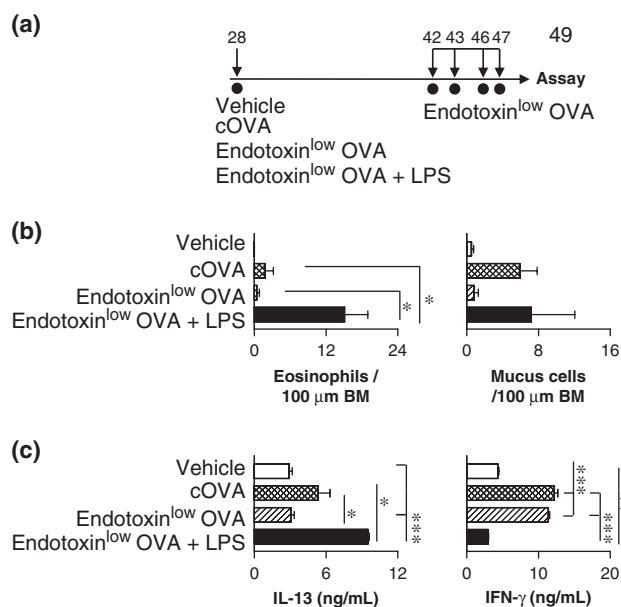


Fig. 1. Contaminating endotoxin in ovalbumin (OVA) preparations promotes features of allergic airways inflammation. (a) Study design. Weanling (28 days old) mice were sensitized to saline, commercial (c) OVA, endotoxin^{low} OVA, or endotoxin^{low} OVA mixed with lipopolysaccharide (LPS). Fourteen days later, mice were challenged four times with endotoxin^{low} OVA, and killed at 7 weeks of age (day 49). (b) Lung biopsies were stained with chromotrope 2R or periodic acid-Schiff (PAS) and eosinophil and mucus cell counts performed, and expressed per 100 μ m of basement membrane (BM). (c) Medial lymph node cells were seeded into 96-well plates at 10^6 /well and stimulated with endotoxin^{low} OVA for 6 days. IL-13 and IFN- γ production were measured by ELISA. Results are pooled from three experiments, each consisting of groups of four to six animals. Data are mean $\pm$ SEM; * P < 0.05, ** P < 0.01, *** P < 0.001.

in Fig. 1b, mice exposed to cOVA developed a mild eosinophilia and mucus cell hyperplasia that was greater, without reaching significance, than mice exposed to endotoxin^{low} OVA. Consistent with this, MLN cells of mice sensitized with endotoxin^{low} OVA generated significantly lower amounts of IL-13 than cOVA-sensitized mice (Fig. 1c). In vehicle-treated mice, cytokine production from unstimulated and OVA-stimulated cells was not significantly different. Cytokine production from unstimulated cells did not differ between groups (data not shown). Of note, both endotoxin^{low} OVA and cOVA exposure induced significantly more IFN- γ , but not IL-13, as compared with vehicle-sensitized mice. In contrast, the co-administration of endotoxin^{low} OVA and LPS during sensitization resulted in a moderate but significant eosinophilia (but not neutrophilia), increased numbers of mucus cells and a switch from IFN- γ to IL-13 production (Figs 1b and c), confirming the findings of Eisenbarth et al. [6].

To determine whether the activation of other TLRs associated with bacterial infections also promoted allergic sensitization in an age-dependent manner, we administered the TLR2/6 ligand lipoteichoic acid (LTA) and the

TLR5 ligand flagellin. Sensitization to endotoxin^{low} OVA alone induced a mild airway and tissue eosinophilia irrespective of age (Figs 2a and b). Co-administration of OVA with LTA, but not flagellin, significantly enhanced the magnitude of tissue eosinophilia and goblet cell hyperplasia as compared with OVA alone in neonatal mice (Figs 2b–d). Notably, the degree of airway and tissue eosinophilia and mucus cell hyperplasia was not significantly different when weanling mice were sensitized with OVA/LTA or OVA/flagellin (Figs 2b–d). Consistent with the histological data, sensitization with LTA but not flagellin preferentially promoted the magnitude of Th2 cytokine production in an age-dependent manner, although significance was not reached (Figs 2e and f).

To determine whether viral-associated molecular patterns promote allergic sensitization, neonatal and weanling mice were sensitized with OVA in the absence or presence of ssRNA. In contrast to LPS (Fig. 1), OVA sensitization with ssRNA at 28 days of age did not significantly enhance the magnitude of eosinophilia. However, inoculation with viral ssRNA during sensitization in early life markedly enhanced both airway and tissue eosinophilia (Figs 3a and b). Assessment of mucus cell hyperplasia revealed the same pattern; neonatal mice sensitized in the presence of viral ssRNA developed a heightened mucus response as compared with mice sensitized as weanlings (Fig. 3c). Consistent with this, IL-13 mRNA expression was significantly greater in neonatal as compared with weanling mice sensitized in the presence of viral ssRNA (Fig. 3d). Th1 and Th2 immune responses promote class switching to immunoglobulin (Ig)2a and IgG1, respectively [20]. Inhalational OVA-sensitized/challenged mice generated significantly greater responses than vehicle-sensitized mice (data not shown). Notably, neonatal mice sensitized with OVA/ssRNA produced significantly less OVA-specific IgG2a (Th1) as compared with all other groups (Fig. 3e). In contrast, OVA-specific IgG1 was significantly increased by OVA/ssRNA treatment as compared with the OVA only control group, regardless of age of sensitization (Fig. 3f). OVA-specific IgE was undetectable.

To determine whether these responses were associated with altered T cell responses, MLN were excised and OVA-specific T cell cytokine production measured. Consistent with the inflammatory and humoral response, allergic sensitization in the presence of viral ssRNA in early life promoted IL-4 production (Fig. 3g), and significantly increased the amount of IL-5 and IL-13 (Figs 3h and i), while IFN- γ was unaffected (Fig. 3j). It is unclear why OVA-specific IFN- γ production was not decreased as shown with LPS (Fig. 1c), but it is possible that the greater concentration of Th2 cytokines produced in the LPS-treated group may have promoted greater negative cross-regulation of the Th1 subset.

To test whether the 'booster' OVA inoculation at 28 days of age (Fig. 2a) was responsible for the enhanced Th2

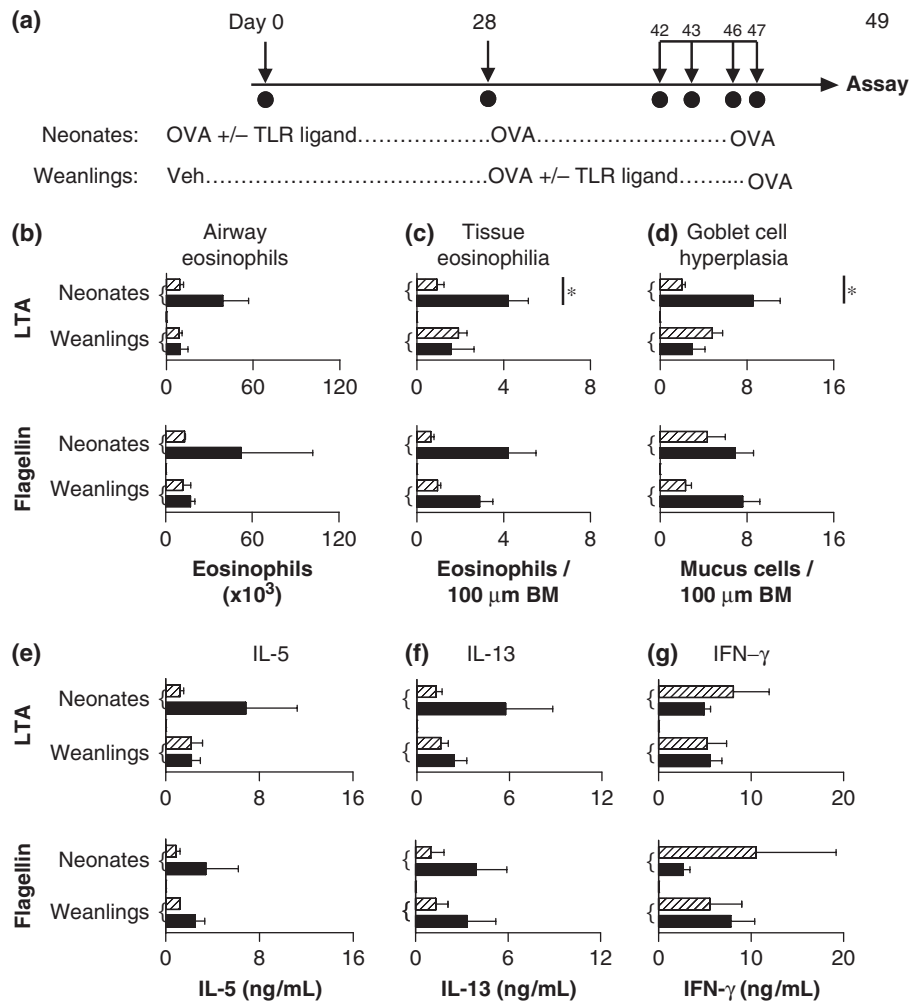


Fig. 2. Bacterial-associated molecular patterns weakly promote the development of ovalbumin (OVA)-induced allergic airways inflammation. (a) Study design. Mice were sensitized as neonates (within the first 24 h of life) or weanlings (28 days old) to endotoxin^{low} OVA in the absence (shaded bars) or presence (closed bars) of lipoteichoic acid (LTA) or flagellin. Allergen challenge occurred at 6 week of age and samples collected at 7 week of age. (b) Bronchoalveolar lavage (BAL) was performed and eosinophils identified using May–Grunwald–Giemsa stain. (c) Lung biopsies were stained with chromotrope 2R or periodic acid–Schiff (PAS), and eosinophil and (d) mucus cell counts performed, and expressed per 100 μm of basement membrane (BM). (e–g) Mediastinal lymph node cells were seeded into 96-well plates at 10⁶/well and stimulated with endotoxin^{low} OVA for 6 days. IL-5 (e), IL-13 (f) and IFN-γ (g) production were measured by ELISA. Results are pooled from 3 to 4 experiments, each consisting of groups of four to six animals. Data are mean ± SEM; **P* < 0.05.

response observed in neonatal mice, we omitted this step in a repeat experiment and measured T cell cytokine production. In agreement with our previous findings, sensitization with OVA and ssRNA led to the production of significantly greater concentrations of OVA-specific IL-13 but not IFN-γ in neonatal as compared with weanling mice (supporting information, Figs S1a and b).

The activation of selective combinations of TLRs can promote synergistic effects [21]. Notably, in an *in vitro* system, TLR4 and TLR7 stimulation cooperate to generate significantly greater quantities of T cell-active cytokines such as IL-12, IL-6 and TNF [21]. Since the F protein of RSV is known to activate TLR4 [22], we postulated that the allergic response would be enhanced by activating TLR4 shortly before sensitization with ssRNA/OVA. To investigate

whether such synergistic effects occurred *in vivo*, neonatal mice were exposed to LPS 1 h before sensitization with OVA/ssRNA. Although prior LPS exposure did not significantly increase the degree of tissue eosinophilia or mucus cell hyperplasia (Fig. 4a), the concentration of OVA-specific IgG1 in sera was significantly elevated (Fig. 4b). Furthermore, the production of OVA-specific IL-5 was increased, IL-13 was significantly increased, while the concentration of IFN-γ was significantly attenuated (Fig. 4c).

Discussion

The possible causal link between severe RSV infection in infancy and allergic sensitization led us to hypothesize that in early life, virus-induced activation of TLR7 at the

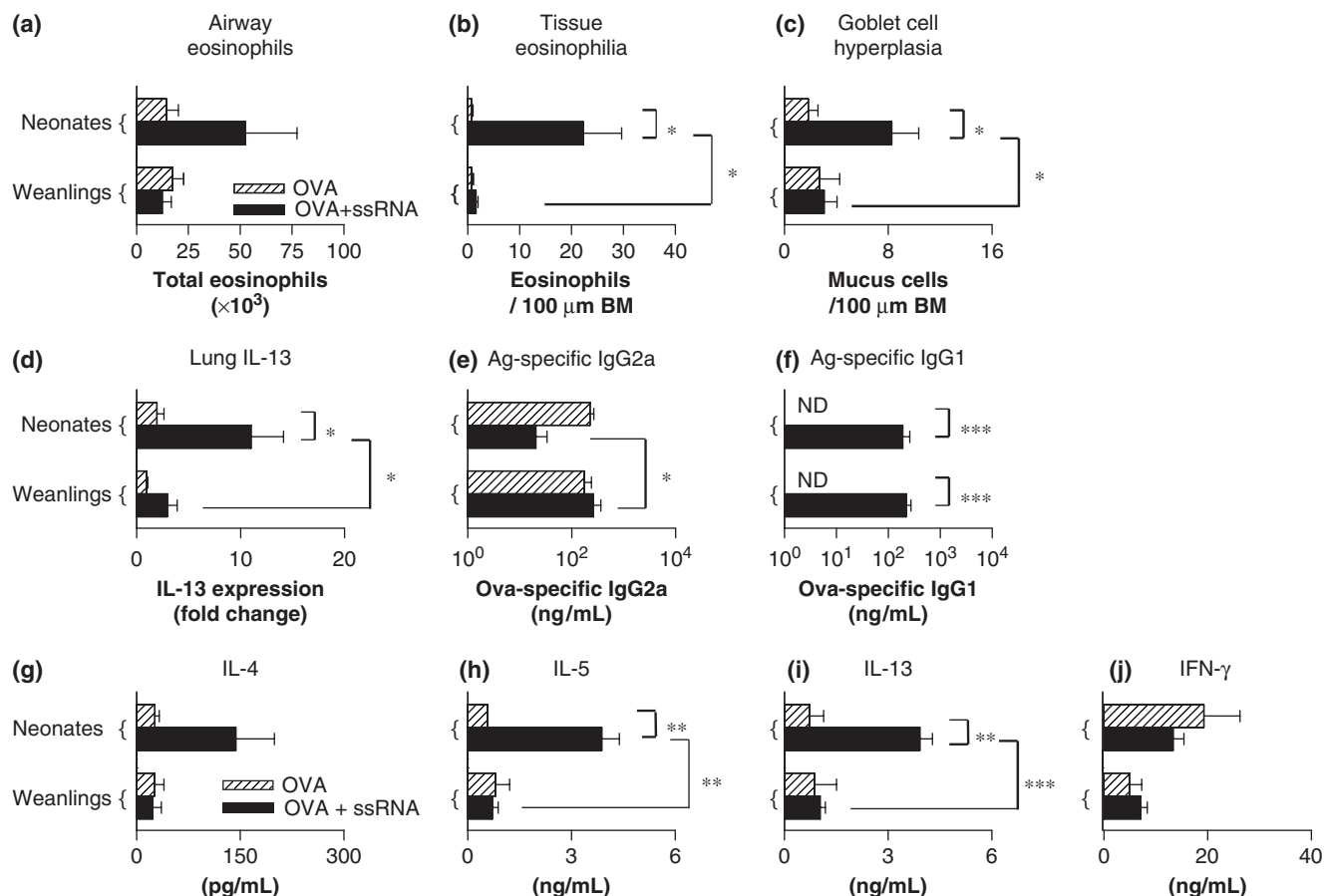


Fig. 3. The age of sensitization determines the magnitude of allergic airways inflammation and T helper responses to antigen exposure in later life. Mice were sensitized as neonates (within the first 24 h of life) or weanlings (28 days old) to endotoxin^{low} ovalbumin (OVA) in the absence (shaded bars) or presence (closed bars) of single-stranded (ss) RNA. Allergen challenge occurred at 6 week of age and samples collected at 7 week of age. (a) Bronchoalveolar lavage (BAL) was performed and eosinophils identified using May-Grunwald-Giemsa stain. (b) Lung biopsies were stained with chromotrope 2R or periodic acid-Schiff (PAS), and eosinophil and (c) mucus cell counts performed, and expressed per 100 μ m of basement membrane (BM). (d) qRT-PCR analysis of IL-13 mRNA expression in whole lung, relative to OVA-sensitized weanling mice. (e) Serum was collected and quantified for OVA-specific IgG2a and (f) IgG1. (g–j) Mediastinal lymph node cells were seeded into 96-well plates at 10^6 /well and stimulated with endotoxin^{low} OVA for 6 days. IL-4, IL-5, IL-13 and IFN- γ production were measured by ELISA. Results are pooled from three to four experiments, each consisting of groups of four to six animals. Data are mean $\pm$ SEM; * P < 0.05, ** P < 0.01, *** P < 0.001.

time of exposure to an innocuous antigen would promote the development of allergen-specific Th2 responses and associated Th2-like pathology. In this study, we found that the age of inhalational allergic sensitization, when promoted by the activation of viral-associated molecular patterns, critically altered the magnitude of the allergic response as measured by mucus cell hyperplasia, airway and tissue eosinophilia, allergen-specific IgG1 and Th2 cytokine production upon allergen challenge in later life. Since the hygiene hypothesis was first mooted by Strachan [23], many investigators have sought to investigate the relationship between infectious agents and the development of allergic asthma. Because the inhalational administration of the prototypic experimental allergen OVA promotes tolerance in the absence of an adjuvant or 'danger' signal [24, 25], the vast majority of animal models have used aluminium hydroxide as an adjuvant.

However, the use of aluminium hydroxide in models that seek to investigate the relationship between microbe-activated innate immune pathways and the development of allergic sensitization must be tempered by the recent discovery that this adjuvant mediates its effects by activating the innate PRR Nlrp3 [26]. In a notable study of inhalational sensitization, where aluminium hydroxide was not used, it was found that low dose LPS (in the order of 5 ng/g body weight) was necessary to promote the development of AAI upon allergen challenge [6]. Although we were able to confirm that spiking endotoxin^{low} OVA with low-dose LPS promoted a greater allergic response compared with endotoxin^{low} OVA alone, in our hands the magnitude of the inflammatory response never reached the scale observed by Eisenbarth et al. [6]. This may reflect variations in the grade of OVA used, background endotoxin levels in the OVA preparations or strain drift among

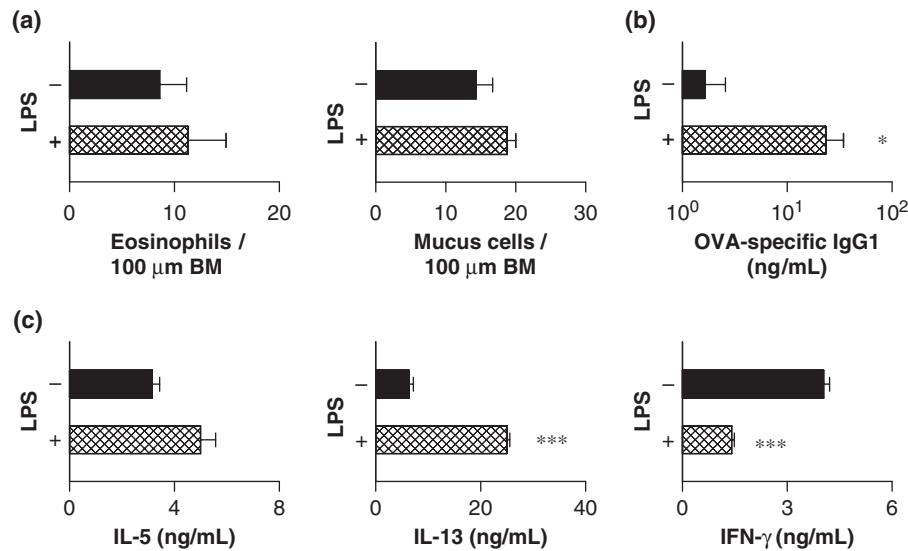


Fig. 4. Priming with lipopolysaccharide (LPS) before sensitization in early life enhances features of allergic airways inflammation to antigen exposure in later life. Neonatal mice were inoculated with LPS (hatched bars) or saline (closed bars) 1 h before sensitization with ovalbumin (OVA) and single-stranded (ss) RNA. Mice were challenged as described in Fig. 2a. (a) Lung biopsies were stained with chromotrope 2R or PAS and eosinophil and mucus cell counts performed, and expressed per 100 μ m of basement membrane (BM). (b) Serum was collected and quantified for OVA-specific IgG1. (c) Mediastinal lymph node cells were seeded into 96-well plates at 10⁶/well and stimulated with endotoxin^{low} OVA for 6 days. IL-5, IL-13 and IFN- γ production were measured by ELISA. Results are from one experiment consisting of groups of seven-ten animals. Data are mean $\pm$ SEM; *** P < 0.001.

inbred BALB/c colonies within different institutions. Nevertheless, we did observe that LPS-spiking of OVA led to increased Th2 cytokine production, tissue eosinophils and mucus secreting cells typical of AAI.

In humans, primary allergen exposure is likely to occur in infancy when the immune system is immature. Notably, the age of primary infection with RSV markedly affects the polarity of the T cell response upon secondary infection in adulthood [14]. Since severe RSV infections in early life have been proposed as a causal pathway for the development of asthma, we sought to explore whether allergic sensitization was promoted by an RSV-associated molecular pattern in an age-dependent manner. Like LPS, viral ssRNA (a TLR7 ligand) was able to enhance the magnitude of the allergic response to OVA in neonatal (1 day old) but not weanling (28 days old) sensitized mice. Similar to the findings obtained with low dose LPS, we observed markedly greater Th2 – but not Th1 – like responses when antigen sensitization occurred concurrently with ssRNA exposure during the neonatal period. Since initial allergen exposure likely occurs in the early postnatal period [13], while the majority of RSV infections occur between the ages of 2–6 months [27], our findings highlight the importance of studying the molecular and cellular processes that underlie allergic sensitization in appropriately aged mice. Intriguingly, a recent clinical study has demonstrated that the timing of birth in relation to the winter virus peak was a key predictor of asthma [28]. Thus, our model which more closely mimics the natural processes that occur during allergic sensitization supports the concept that respiratory viruses (or compo-

nents thereof), and the timing of viral exposure in early life, may dramatically modulate the development of allergic sensitization to bystander inhalant allergens. The importance of TLR7 is further underscored by a recent study investigating single nucleotide polymorphisms of TLR7 and TLR8 which demonstrated that these genes were linked to asthma and related diseases [29]. Although the TLR7 ligands R-848 and resiquimod have been shown previously to attenuate features of experimental asthma [30, 31], these data were obtained from mice sensitized by intraperitoneal injection with OVA, boosted with aluminium hydroxide, while R-848 was administered subcutaneously. We would advocate that studies aimed at investigating the mechanisms that bridge innate immunity with the development of allergic sensitization, employ experimental models where sensitization occurs in the neonatal period, is free of unnatural aluminium hydroxide-based adjuvants and occurs via the target organ [32].

The mechanism underlying the age-dependent differential responses shown here is not known, although the Th2 bias in early life may result from the inability of neonatal dendritic cells to generate IL-12 in response to TLR agonists [33, 34]. Of note, the absence of IL-12 leads to both weaker IFN- γ responses, and enables IL-4-induced apoptosis of Th1 cells [34]. Additionally, the hyper innate inflammatory responses to various TLR stimuli and viral infection that occurs in the neonatal period [16] may enhance the magnitude of the Th2 bias that occurs at this time. Although much attention has focused on early life virus infections as a risk factor for allergic sensitization,

emerging evidence suggests that infections by intracellular pathogens, such as *Chlamydia* and *Mycoplasma*, may also cause wheezing in some individuals [35]. Consistent with the findings of other investigators [36, 37], we observed that sensitization with OVA in the presence of a TLR2 but not TLR5 ligand significantly heightened the magnitude of tissue eosinophilia and goblet cell hyperplasia. However, in our model the development of these pathologic features in response to OVA/LTA administration was only apparent in mice sensitized as neonates. These differences may be due to the different model systems and TLR2 ligands employed, while importantly in our study we focused on weanling and not adult mice. Notwithstanding these data, the ability of the bacterial ligands to induce AAI was markedly weaker than the viral-associated molecular pattern ssRNA, which may reflect the greater causal relationship between severe infections with the ssRNA viruses Rhinovirus and RSV in early life and the development of asthma and wheezing [7, 38].

Respiratory viral infections can increase susceptibility to various bacteria [39], and may delay clearance through an IFN- γ -mediated pathway [40] or TLR desensitization [41]. By contrast, simultaneous activation of selective prototypic viral and bacterial TLRs, for example TLR7 and TLR4, can lead to synergies that generate markedly greater proinflammatory responses from dendritic cells, although this has yet to be shown *in vivo* [21]. Since we had shown that co-administration of LPS or ssRNA with OVA during neonatal sensitization promoted Th2 responses in later life, and the fusion (F) protein of RSV is believed to activate TLR4 [22], we sought to determine whether the activation of TLR4 and TLR7 would synergize *in vivo* to enhance the magnitude of AAI. Intriguingly we observed that inoculation of LPS 1 h before neonatal OVA/ssRNA-induced allergic sensitization significantly increased the production of allergen-specific IL-13, raising the possibility that RSV may activate both TLR4 and TLR7 in early life to generate a cytokine milieu that favours Th2 differentiation. Future work will need to determine whether the activation of single or combinations of TLRs in early life promotes the expression of Th2-inducing factors such as IL-25 and thymic stromal lymphopoietin. Collectively, our data suggest that the activation of TLR2 and TLR7 in early life favours the development of Th2 cells, which promote AAI upon allergen challenge in later life. Thus, the hygiene hypothesis must be extended to embrace both the nature of the infection as well as the age of primary infection.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The age of sensitisation determines the magnitude of allergic airways inflammation to antigen exposure in later life. Mice were sensitised as neonates (within the first 24 h of life) or weanlings (28 days old) to endotoxin low OVA in the absence (shaded bars) or presence (closed bars) of ssRNA. Unlike the model employed in Fig. 2, neonatal mice did not receive a 'booster' OVA sensitisation on day 28. Allergen challenge occurred at 6 wk of age and samples collected at 7 wk of age. a and b) Mediastinal lymph node cells were seeded into 96 well plates at 106/well and stimulated with endotoxinlow OVA for 6 days. IL-13 and IFN- γ production were measured by ELISA. Shown are the results of one experiment consisting of groups of five animals. Data are mean $\pm$ s.e.m; * $P < 0.05$.

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Supplementary Fig. 1.

The age of sensitisation determines the magnitude of allergic airways inflammation to antigen exposure in later life.

Mice were sensitised as neonates (within the first 24 h of life) or weanlings (28 days old) to endotoxinlow OVA in the absence (shaded bars) or presence (closed bars) of ssRNA. Unlike the model employed in Fig. 2, neonatal mice did not receive a 'booster' OVA sensitisation on day 28. Allergen challenge occurred at 6 wk of age and samples collected at 7 wk of age. A and B) Mediastinal lymph node cells were seeded into 96 well plates at 10⁶/well and stimulated with endotoxinlow OVA for 6 days. IL-13 and IFN- γ production were measured by ELISA. Shown are the results of one experiment consisting of groups of five animals. Data are mean $\pm$ s.e.m; * $p < 0.05$.

