Chapter 21

Use of the Cockroach Antigen Model of Acute Asthma to Determine the Immunomodulatory Role of Early Exposure to Gastrointestinal Infection

Carolyn G. Durham, Lisa M. Schwiebert, and Robin G. Lorenz

Abstract

The increased incidence of asthma over the last 50 years in developed countries has been associated with a decrease in infections acquired early in childhood. These early infections are thought to shape subsequent immune responses. Although there have been multiple clinical associations between gastrointestinal infections and decreased asthma incidence, it has been difficult to move beyond a simple correlation when studying human patients. This section describes an acute asthma model in C57BL/6 mice designed to specifically evaluate the effect of prior gastric *Helicobacter* colonization and inflammation in a murine model of cockroach allergen-induced asthma.

Key words Asthma, *Helicobacter*, Gastritis, Murine, Cockroach antigen, Hygiene hypothesis, Toll-like receptors, Neonatal

1 Introduction

In 1989, Strachan proposed the hygiene hypothesis to answer the growing epidemiological trend of the rise of industrialization, decline in infectious diseases, and subsequent increase in diseases associated with a T-helper 2 (Th2) cell response, including asthma [1]. Despite the growing evidence for this hypothesis, the immunological mechanism by which decreasing infection rates and increasing Th2 diseases occur has not been elucidated [2]. Numerous studies have shown a role for viruses in asthma incidence. In fact, Strachan et al. showed that newborns who were exposed to respiratory viruses had an increased incidence of asthma [3]. However, there is a growing body of research that suggests that immune system development, spurred by early colonization with bacteria, decreases the rate of asthma development later in life [2]. For example, studies have shown that children reared in a household with a dog or whose bedrooms had high endotoxin levels were at

a lower risk for developing asthma [4, 5]. Additionally, numerous studies have shown that living on a farm dramatically reduces the risk for asthma, presumably because of the constant levels of bacterial exposure [6]. This clearly demonstrates the benefit of exposing young children to bacteria and bacterial components in reducing asthma incidence.

In developing countries, many of the infectious diseases are known to elicit a Th1 phenotype. One of these bacteria is *Helicobacter pylori*, which is endemic in many developing countries. As countries become more industrialized, antibiotic therapy is more widespread and these infectious diseases decline. This is especially noted when the incidence of infection is higher in adults, since they were young when the bacterial exposure was greater, than in children who were reared under more newly established standards of infection control [7]. Interestingly, the timing of bacterial exposure is critical in whether or not the microbial acquisition helps in shaping the immune system or causing a pathogenic problem. A study done with *Helicobacter pylori* infection in children and adults indicated that in children less than 10 years old, IL10 and IFNγ were significantly upregulated when compared to older children and adults [8]. The IL10 increase persisted past 10 years old and was higher than that of noninfected people, indicating that this early exposure promotes a Th1 immune response and that regulation of this response persists.

The bacterial exposure component of the hygiene hypothesis has gained acknowledgment and prompted members of the American Thoracic Society to set forth the notion that bacterial manipulation is a key factor in asthma prevention. Furthermore, they charge that bacterial exposure, especially through the gastrointestinal tract, is fundamental to properly developing a Th1-skewed immune system [2]. A key component of this immune system development is the body being able to detect the presence of the bacteria. Our lab has previously shown that, in C57BL/6 mice, toll-like receptors (TLRs) 2 and 4 are significantly upregulated 2 weeks after birth [9]. TLRs are pathogen-associated pattern receptors. TLRs 2 and 4 are specific for Gram-positive and Gram-negative bacterial ligands, respectively. However, in mice born to dames on a broad-spectrum antibiotic cocktail and that are then weaned onto this cocktail, this upregulation does not occur [9]. This indicates that the ability to sense bacteria is critical in immune system development. Our lab has also shown that mice that were born to parents that had been on broad-spectrum antibiotics and were, themselves, maintained on this regimen, had delayed Th1/17 development [9]. These data demonstrate the need for bacterial recognition and bacterial exposure in immune system development. To evaluate the role of bacteria in the hygiene hypothesis, we have developed a model where gastritis is induced by infection with the gastrointestinal bacteria *Helicobacter felis*, and subsequently asthma is induced using cockroach antigen (CRA).

Helicobacter pylori infection is endemic in most developing countries, affecting at least 70 % of the population and colonizing about 20 % of the US population. Physiologically, the presence of *H. pylori* in the gut has been shown to elicit a strong IFNγ response, which has been shown to downregulate Th2 cells [10]. Specifically, administering the neutrophil-activating protein of *H. pylori*, both systemically and mucosally, reduces characteristic Th2 cytokines, IgE, and eosinophilia by activating the Th1 pathway [11]. These studies show that this bacterial infection causes an overwhelming Th1 response, one that epidemiologically and physiologically has been shown to directly affect the response of the Th2 pathway. In recent years, the role of Th17 cells in *Helicobacter* infections has been shown to be critical in pathogenesis. Shi et al. demonstrated that the Th17 cells are critical in acute infection, where it works with IL8 to recruit neutrophils and decrease bacterial burden [12]. Depleting these cells leads to proliferation of bacteria. Interestingly, if there is no control mechanism in place, such as regulatory T cells, IL17 is upregulated to the point where it is ineffective in pathogen clearance [13]. Data from our lab shows that in germfree mice, IL17 levels continue to climb after 8 weeks, while *H. felis* is never cleared. With conventionally reared C57BL/6 mice, IL17 increases initially, but decreases after 8 weeks [14]. Th1 and Th17 adaptive immunity plays a significant role in *Helicobacter* infections, though both must be tightly regulated.

Human studies demonstrate that polymorphisms in the TLR2 and TLR4 genes affect the pathogenesis of *Helicobacter* gastritis [15, 16]. However, there have been conflicting in vitro reports about which of these TLRs are needed to recognize the bacteria. Many epidemiological studies have shown an inverse correlation with this pathogen and asthma susceptibility. Chen and Blaser demonstrated that asthma onset in children younger than 5 years old was inversely associated with seropositivity for *H. pylori* (OR, 0.58; 95 % CI, 0.38–0.88). In the same study, seropositivity for *H. pylori* in children 3–19 years old was significantly inversely correlated with having a current case of asthma (OR, 0.41; 95 % CI, 0.24–0.69) [17]. Interestingly, with the rise of asthma, there has also been an increase in administering broad-spectrum antibiotics in small children. This illustrates that, not only is there a dysregulation in global bacteria but also that the bacteria, such as *H. pylori*, that were acquired during childhood that could potentially shape the immune system are being eliminated before their physiological effects can be properly achieved [18]. Additionally, *H. pylori* is associated with decreasing the severity of gastroesophageal reflux disorder, which is a positive correlate of asthma [10]. Immunologically, asthma is characterized as a Th2 disease, as asthmatic patients have increased serum IgE, production of which is known to be initiated by IL-4, 5, and/or 13 [10]. Interestingly, IFNγ production has been shown to hinder Th2 cytokine production;

IFNγ is a downstream target of NFκB, the transcription factor induced by TLR2 and 4 activation. Thus, activating TLR2 and 4 by environmental exposure to bacterial ligands could elicit a more Th1- and Th17-skewed adaptive response and result in the down-regulation of the Th2 phenotype in asthma patients [19].

Because asthma is a complex disease, various mouse models are used to elucidate various facets of the disease. Since mice do not spontaneously get asthma, the mice must be sensitized with a specific allergen and subsequently challenged with that allergen. Many labs use BALB/c mice for their studies because these mice have a Th2-skewed immune system and a robust asthmatic response upon asthma induction. However, this model does not parallel typical human disease since most people do not have a strongly Th2-skewed immune system. Therefore, the use of C57BL/6 mice is a good model because it has a Th1 bias, which is more like the human immune system. Using C57BL/6 mice is also beneficial when studying the bacterial colonization and/or sensing in asthma, as these mice respond more strongly to bacteria than the BALB/c mice [20]. Likewise, the allergens used in asthma studies vary greatly. Historically, ovalbumin has been used to induce the asthmatic phenotype because of its ability to produce strong Th2 responses [21, 22]. However, this allergen does not have clinical application, as ovalbumin is not a common allergen in humans. Therefore, more clinically relevant allergens, such as house dust mite and cockroach extracts, are becoming more frequently used [23, 24]. There are two categories of asthma models: acute and chronic. The acute models are used to study the onset and beginning stages of asthma, whereas the chronic models are used to study the long-term effect of asthma, such as airway remodeling. The chronic model is also helpful in studying therapies for asthma patients, since most patients already have remodeling taking place in their lungs [24].

In this model, we chose CRA because the cockroach is a common household pest. However, this model has only previously been evaluated in adult mice, whereas we needed a model of childhood asthma induction if the effect of early gastrointestinal infections is to be adequately tested. Therefore, we utilized 2-, 4-, and 6-week-old mice to determine the induction of acute asthma by CRA. We concluded that, at 2 weeks, the newly weaned pups did not have a mature enough immune response to mount a Th2 acute asthma response. At 6 weeks, their immune reactions had switched to a more Th1/17 phenotype and the results were more variable. However, 4-week-old C57BL/6 mice had both a robust and consistent response to CRA and can now be established as an excellent model for acute sensitization and challenge with CRA to study the development and early stages of asthma (Figs. 1 and 2).
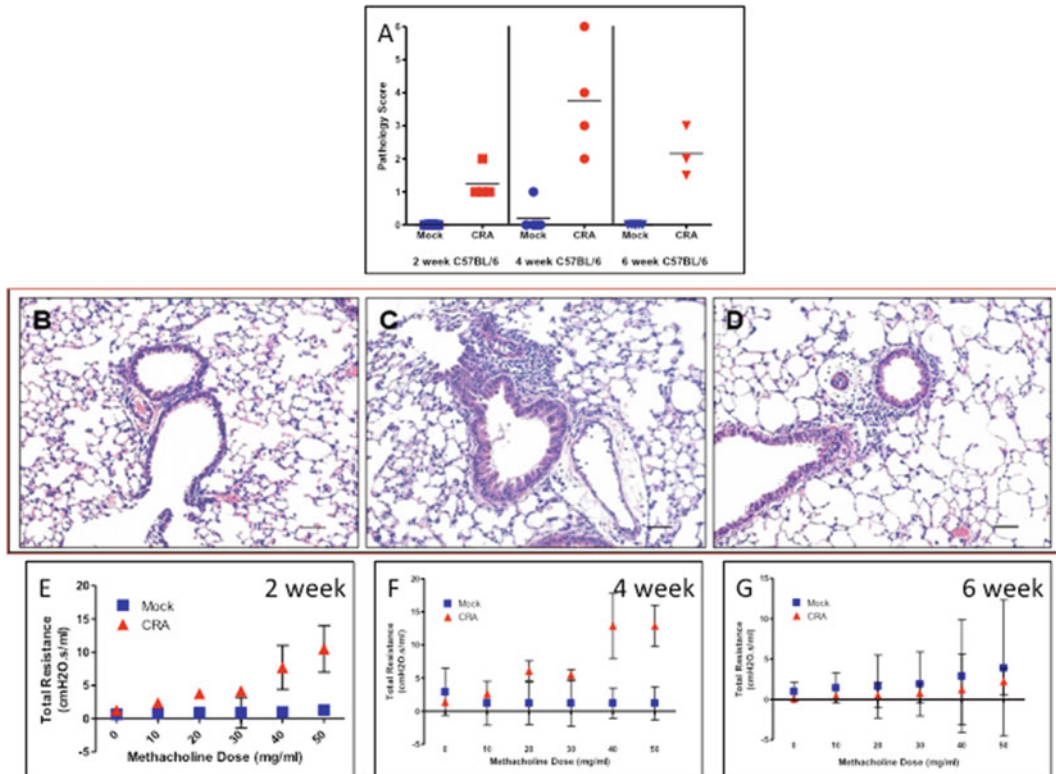
Fig. 1 Four-week-old mice have more robust pathology and airway resistance than 2- or 6-week-old mice. (**a**) Four-week-old mice developed more inflammation within their airways and vasculature. Although 6-week-old CRA-treated mice have moderate perivascular and peribronchial inflammation, the younger 4-week-old mice have much greater perivascular and peribronchial inflammation after asthma induction. The 2-week-old mice have only mild perivascular and peribronchial inflammation after CRA treatment. (**b**–**d**) Representative H&E images of the 2-week-old mice, 4-week-old mice, and 6-week-old mice, respectively. (**e**) Resistance (*R*) in the airways was consistently higher in the 4-week-old mice when compared with the mock-treated, as well as (**f**) the treated 2-week-old mice and (**g**) the treated 6-week-old mice

2   Materials

2.1   Helicobacter Components

1. C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME).

2. *Helicobacter felis* ATCC 49179.

3. ATCC Medium 260 Plates: Trypticase soy agar, defibrinated calf blood (5 % v/v) (Colorado serum Company, Denver, CO), trimethoprim (1 mg/ml), vancomycin (10 mg/ml), fungizone (1 % v/v). Resuspend 20 g of Trypticase soy agar in 500 ml of ultrapure water (e.g., Milli-Q or an equivalent) and heat with frequent agitation to boiling for 1 min to completely dissolve the powder. This should be autoclaved for 15 min at 121 °C and then cooled to 55 °C in a water bath. When cooled, add 25 ml of the defibrinated calf blood using sterile technique and
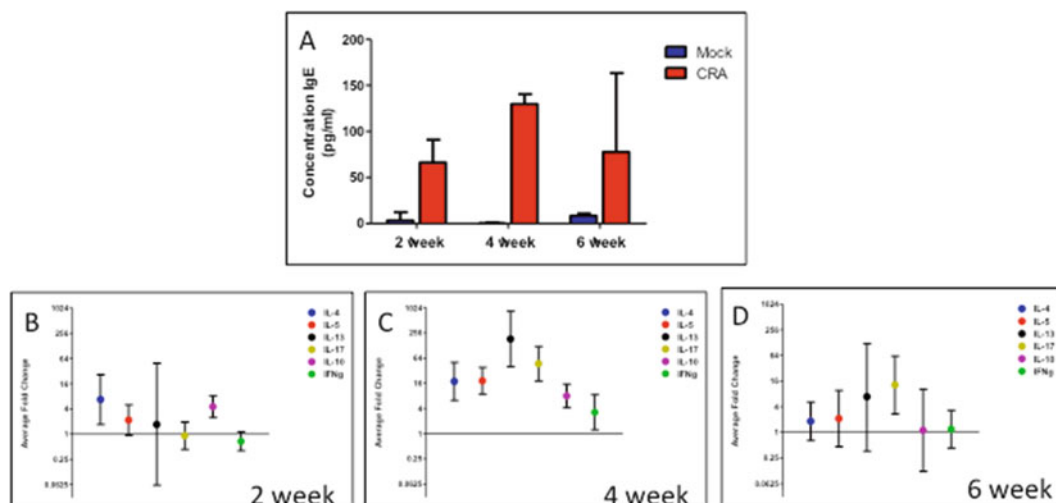
Fig. 2 Four-week-old mice developed higher serum asthma markers. (**a**) Serum IgE was consistently greater in 4-week-old mice than the other two groups. (**b**–**d**) The disease in the 4-week-old mice (**c**) appeared to be IL13 mediated, though all of the Th2 cytokines were elevated in these mice, as opposed to (**b**) the 2-week-old mice and (**d**) the 6-week-old mice

stir slowly to mix (*see* **Note 1**). Add 5 ml of trimethoprim, 1.5 ml of vancomycin, and 5 ml of fungizone (all previously sterilized) to the mix. Immediately pour plates in a sterile hood to approximately ½ full and flame the tops to get rid of any air bubbles. Allow the medium to solidify, and then store at 4 °C in a sealed container for no more than 1 month. This makes 20–25 plates.

4. Brain heart infusion (BHI) broth: Add 37 g of BHI broth to 1 L of deionized distilled water. Autoclave for 30 min on the liquid cycle. Let cool to 55 °C in a water bath and then add the following reagents: 3 μg/ml of vancomycin (3.0 ml of a 10 mg/ml stock); 10 μg/ml of trimethoprim (10.0 ml of a 1 mg/ml stock); 1 % fungizone (10 ml); and 5 % defibrinated fetal calf serum (50 ml).

5. BBL™ CampyPak™ Plus Microaerophilic System Envelopes with Palladium Catalyst (BD, San Jose, CA).

6. Histology sponges and cassettes.

7. Citrosolv (Fisher).

8. Pepsin (0.25 % in PBS).

9. Rabbit anti-*H. felis*/*H. pylori* antibody (Covance, Emeryville, CA).

10. Cy3 donkey anti-rabbit antibody (1:200 dilution, Jackson Immunoresearch, West-Grove, PA).

11. FITC-labeled lectin *N*-acetyl-D-glucosamine-specific *Griffonia simplicifolia* II (5 μg/mL, Invitrogen, Eugene, OR).

12. Hoechst 33258 (0.5 μg/ml of bis-Benzimide; Sigma, St. Louis, MO).

2.2 CRA Asthma Model Components	1. Cockroach antigen (Hollister-Stier, Spokane, WA) (*see* **Note 2**).

2.2 CRA Asthma Model Components

1. Cockroach antigen (Hollister-Stier, Spokane, WA) (*see* **Note 2**).
2. Incomplete Freund's adjuvant (IFA).
3. Phosphate-buffered saline (PBS).
4. 2-glass syringes (5 ml each).
5. Three-way stopcock.
6. 1 ml syringe with needle (32 G).
7. Eppendorf tubes (1.5 ml, one per mouse).
8. Isoflurane.
9. P20 and P200 pipette.
10. Plastic disposable pipette dropper (one per mouse).
11. 10 % buffered formalin.
12. Bouin's fixative solution: This is a picric acid–formalin–acetic acid mixture that can be either made within the lab (300 ml of saturated picric acid, 100 ml of formaldehyde, 20 ml of acetic acid) or purchased. This fixative allows better and crisper nuclear staining than 10 % neutral-buffered formalin. As picric acid is extremely explosive if allowed to dry out, it is usually safer to just purchase the Bouin's fixative solution.
13. RNAlater RNA Stabilization Reagent (Ambion, Austin, TX): This is an immediate RNA stabilization and protection reagent. It allows tissue archiving without the risk of RNA degradation.
14. Methacholine: Used in the methacholine challenge, in which the subject inhales aerosolized methacholine to determine the level of bronchial hyperreactivity.
15. Ketamine: Used for the induction and maintenance of general anesthesia.
16. Flexivent (Scireq, Montreal, Canada): This is a computer-controlled precision pump that controls mechanical ventilation while also obtaining measurements of respiratory mechanics.

2.3 ELISA Components

1. Immunlon 96 well plates (Thermo Fisher Scientific, Waltham, MA).
2. Goat Anti-Mouse IgE-UNLB (10 μg/ml; Southern Biotech, Birmingham, AL): For use in coating the ELISA plate.
3. Wash buffer (PBS, 0.5 % Tween-20).
4. Blocking buffer (PBS, 5 % bovine serum albumin (BSA)).
5. Diluent buffer (PBS, 1 % BSA).
6. Mouse IgE Standard (Southern Biotech).
7. Goat Anti-Mouse IgE-AP (Southern Biotech) (1:2,000 in diluent buffer): For detection of IgE in serum.
8. 3 N NaOH: 120 g in 1,000 ml of water.

9. SIGMA*FAST*™ *p*-Nitrophenyl phosphate Tablets (Sigma).

10. VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA).

2.4 RNA and QPCR Components

1. Applied Biosystems Assays-On-Demand primer/probe sets.

2. TaqMan Universal PCR Mix (PE Applied Biosystems; Foster City, CA).

3. Trizol® (Life Technologies, Grand Island, NY).

4. Turbo DNase Kit (Ambion, Austin, TX).

5. Roche Transcriptor First Strand cDNA Synthesis Kit (Roche, Penzberg, Germany).

6. Stratagene MX3000P Real-Time Cycler (Agilent Technologies).

3   Methods

3.1 Growing Helicobacter felis

1. One vial of *H. felis* (ATCC 49179) is inoculated onto an ATCC Medium 260 plate (*see* **Note 3**).

2. Incubate at 37 °C for 2 days in an anaerobic jar with a CampyPak (activate CampyPak as per instructions with water). Make sure that the container has an airtight seal (*see* **Note 4**).

3. Check *H. felis* viability by dropping one drop of the bacterial suspension onto a microscope slide and covering with a standard coverslip. Using a 20× or 40× objective lens on a light microscope, focus on the bacteria. Make sure that the majority of them are motile by watching them swim through multiple viewing fields.

4. If motile, using the broth from the plate, inoculate BHI broth (100 ml) in a flask. Secure in an anaerobic jar with a CampyPak (*see* **Note 5**).

5. Incubate at 37 °C for 18–24 h, with gentle shaking.

6. Check viability, as outlined in Subheading 3.1, **step 3**.

7. Determine the optical density of the bacteria culture (OD_{450}; 1 $OD_{450} \cong 10^9$ bacteria) (*see* **Note 6**).

8. Harvest bacteria by centrifuging at $3,000 \times g$ for 10 min, and then resuspend the pellet in glycerol:BHI freezing media (31 ml of glycerol:69 ml of BHI). Store aliquots of bacteria (2×10^9 CFU/ml) at –80 °C. The frozen stock should remain viable for ~4 months.

3.2 H. felis Inoculation

1. Days 0 and 3: Inoculate mice orally (per os (p.o.)) using a 200 μl pipette with 0.05 ml of *H. felis* (2×10^9 CFU/ml in glycerol:BHI) using a frozen aliquot.

2. Day 7: Inoculate mice p.o. as described in Subheading 3.2, **step 1**, using freshly grown *H. felis*. Use the same culture

technique as described above in Subheading 3.1, with the exception that a vial of frozen stock of *H. felis* can be used to start the culture instead of a new vial from ATCC (*see* **Note** 7).

3.3 Asthma Sensitization (Day 0)

1. CRA is resuspended in PBS to a final concentration of 4 mg/ml (*see* **Note** 2).

2. It is then put into one glass syringe. A second glass syringe is filled with an equal amount (v/v) of IFA.

3. Connect these two glass syringes to a 3-way stopcock, and then emulsify the IFA and CRA by syringe-extrusion (alternatively pushing the solution in each syringe through progressively smaller pore sizes in the stopcock) for 10–15 min (*see* **Note** 8).

4. The solution is finished emulsifying when a drop of the solution does not disperse on the top of ice-cold water. Use the solution immediately (*see* **Note** 9).

5. Using a non-tuberculin syringe and a 32G needle, inject the mice intraperitoneally (50 μl) and subcutaneously (50 μl).

3.4 Asthma Induction via Intranasal Challenge (Days 14, 18, 22, 26) (See Note 10)

1. Mix CRA with PBS (0.075 mg/ml) (*see* **Note** 2).

2. Anesthetize mice individually using isoflurane (*see* **Note** 11).

3. Using a P20 pipette, drop PBS:CRA solution (10 μl, which equals 0.75 μg) onto the nostril. Once the mouse inhales the solution, drop another 10 μl onto the nostril (*see* **Note** 12).

3.5 Asthma Induction via Intratrachial Challenge (Day 28)

1. Mix CRA with PBS (0.12 μg/ml) (*see* **Note** 2).

2. Anesthetize mice individually using isoflurane (*see* **Note** 13).

3. When the mouse begins to awaken and gasp using its diaphragm, instill PBS:CRA (50 μl, 6.0 μg) solution into the mouse's throat between gasps with a P200 pipette (*see* **Note** 14).

3.6 Sacrifice (Day 29)

1. Anesthetize mice using isoflurane.

2. Perform cervical dislocation.

3.7 Tissue Collection

1. Mice are euthanized using 5 ml of isoflurane for approximately 30 s and followed by cervical dislocation.

2. Blood is collected via heart puncture, allowed to clot for 20 min at room temperature (r.t.), centrifuged for 10 min at $16,200 \times g$, and the serum removed and stored at –20 °C until analysis.

3. The stomach is removed and quartered. Each quarter is fixed in Bouin's fixative for histology, frozen at –80 °C (two quarters) for protein analysis, or stored in RNAlater for RNA analysis, as per protocol.

4. Place 1 stomach quarter flat between two thin histology sponges in a histology cassette. Store the cassette in Bouin's fixative for 24 h at 4 °C. Bouin's fixative is replaced with 70 %

ethanol every 24 h for 48 h (*see* **Note 15**). The tissue can be embedded in paraffin along its long, cut edge in order to get a cross section of the epithelium. One 5 μm slide is stained with hematoxylin and eosin and scored for pathology. Other slides can be stained with various antibodies to determine *Helicobacter* infection rates, presence of inflammatory cells, differentiated epithelial cells, proliferation, and/or apoptosis, as needed.

5. Freeze stomach quarters 2 and 3 at –80 °C for protein extraction, if needed.

6. Store stomach quarter 4 in RNAlater and process as directed by the manufacturer's protocol for future quantitative real-time RT-PCR (qRT-PCR).

7. The lungs are removed above the main stem branch directly below the larynx. The lungs are then separated directly below the main stem branch. Using a 25G needle and a 3 ml syringe, the left lung is perfused with 1–2 ml of formalin through the main bronchus until it is expanded, but not overextended. Pressure must be maintained on the fluid in the lungs by gripping the forceps around the needle inserted into the bronchus while gently applying pressure to the syringe. This is held for 1–2 min. Then, the lung is removed from the needle and placed into a histology cassette and stored in formalin.

8. The tissue in the cassettes is stored in formalin for 24 h. The formalin is then replaced with 70 % ethanol once every 24 h for 2 days. The tissue can be embedded in paraffin and stained with hematoxylin and eosin. Lung sections are then scored for pathology.

9. The right lung is stored in RNAlater as directed by the manufacturer's protocol for future qRT-PCR.

3.8 Airway Hyperresponsiveness

1. Mice are anesthetized with 450 mg/kg of ketamine, and a tracheotomy tube (18 G) is inserted and connected to the inspiratory and expiratory ports of a ventilator (Flexivent). Mice are ventilated at a rate of 160 breaths per minute at a tidal volume of 0.2 ml with a positive end-expiratory pressure of 2–4 cm water.

2. Increasing concentrations of methacholine (0, 10, 20, 30, 40, and 50 mg/ml) are administered via aerosolization. From 20 s to 3 min after each aerosol challenge, detailed measurements should be recorded continuously. The measurements should include resistance (R), compliance (C), and elastance (E) (*see* **Note 16**).

3.9 IgE ELISAs

1. Immunlon 96 well plates are coated with Goat Anti-Mouse IgE-UNLB (10 μg/ml) in PBS, overnight at 4 °C.

2. The next day, the plate is washed with wash buffer five times. The nonspecific binding sites are blocked for >1 h using blocking buffer.

3. After washing the plates five times with wash buffer, the serum samples are added. The serum from mice that received CRA is diluted 1:2 with diluent buffer, and the mock-treated mice serum samples are used neat. Mouse IgE-UNLB is diluted to a start concentration of 2,000 pg/ml, and then diluted 1:2 seven more times for the complete standard curve. The standards and samples are incubated overnight at 4 °C.

4. The next day, the plate is washed five times with wash buffer. Goat Anti-Mouse IgE-AP (1:2,000 in diluent buffer) is added and incubated at r.t. for 2 h.

5. After washing the plate five times with wash buffer, SIGMA*FAST*™ *p*-Nitrophenyl phosphate Tablets are dissolved in 20 ml of deionized water and added to the plate. Plates are incubated for 30 min at r.t. in the dark.

6. 3 N NaOH is added to stop the reaction, and the plate is read at 405 nm on a microplate reader.

3.10 RNA and RT-PCR

1. RNA isolation: The Trizol® (phenol and guanidine isothiocyanate) method can be used to isolate the total RNA from one quarter of each stomach and the left lung [25].

2. Before making cDNA, the RNA is processed to remove contaminating DNA using the Turbo DNase Kit. cDNA is made using the Roche Transcriptor First Strand cDNA Synthesis Kit with mRNA (2 μg) from each sample. Quantitative real-time reverse transcription polymerase chain reaction (QPCR) is performed on each sample. Primers/probes used are from Applied Biosystems Assays-On-Demand. These are used with TaqMan Universal PCR Mix. All RNA data is analyzed using the $-2^{\Delta\Delta Ct}$ relative quantitation method, described in the Applied Biosystems manufacturer's protocol (*see* **Note 17**) [25–27].

3.11 Pathology Scoring

1. Stomach: One-quarter stomach from each mouse that was stained using hematoxylin and eosin should be scored on a scale of 0–3 in each of the three categories. The scores from the three categories are then added together for a total score, with 0 being the lowest and 9 being the highest possible scores (Table 1) [28].

2. Lung: Each section should be scored using a method derived from Curtis et al. in which the inflammation around the vasculature and the bronchial is evaluated and added together for a total inflammation score, with 0 being the lowest and 6 being the maximum (Table 2) [29].

3.12 H. felis Staining and Quantification (See Note 18) [30]

1. Deparaffinize an unstained tissue section as follows: Wash the slides two times for 10 min per wash with Citrosolv; next, wash the slides three times for 10 min with isopropyl alcohol; and finally, rinse the slides for 5 min with running deionized water.

Table 1
Stomach pathology scoring

Score	Longitudinal extent of inflammation	Vertical extent of inflammation	Histological changes
0	None	None	None
1	Patchy	Basal lamina propria only	Mild loss of differentiated epithelial cells
2	<50 %	Transmural	Moderate loss of differentiated epithelial cells
3	>50 %	Both mucosa and submucosa involved	Severe loss of differentiated epithelial cells

H&E-stained stomach sections are evaluated based on the longitudinal extent of inflammation, the vertical extent of inflammation, and histological changes. Each parameter is scored on a scale of 0–3

Table 2
Lung pathology scoring

Score	Vascular	Bronchial
0	No inflammation	No inflammation
1	Occasional cuffing	Occasional cuffing
2	Most vessels surrounded by a thin layer (1–5) of inflammatory cells	Most vessels surrounded by a thin layer (1–5) of inflammatory cells
3	Most vessels surrounded by a thick layer (>5) of inflammatory cells	Most vessels surrounded by a thick layer (>5) of inflammatory cells

H&E-stained lung sections are evaluated based on the extent of inflammation around the vasculature and airways. Each parameter is scored on a scale of 0–3

2. After rehydrating the tissue in PBS, pepsin (0.25 % in PBS) is incubated on the slides for 10 min at r.t.

3. After rinsing the slides in PBS, to block nonspecific binding sites and to permeabilize the tissue, add PBS blocking buffer (1 % bovine serum albumin, 0.3 % Triton X-100) to each slide and incubate for 1 h at r.t.

4. The slides are then washed in PBS and the tissue stained with undiluted rabbit anti-*H. pylori* antibody to semi-quantitate *H. felis* colonization. This antibody is known to cross-react with *H. felis*.

5. After washing the slides in PBS, Cy3 donkey anti-rabbit antibody (1:200 dilution) and FITC-labeled lectin *N*-acetyl-D-glucosamine-specific *Griffonia simplicifolia* II (5 μg/mL) are added to the tissue and incubated for 1 h at r.t. for detection of *H. felis* and mucous neck cells, respectively.

6. To counterstain the nuclei, the slides are incubated for 20 min at r.t. with Hoechst 33258 (0.5 μg/ml).

7. Colonization of the antrum with *H. felis* is evaluated on a scale of 0–4, where 0 = no bacteria per gland; 1 = 1–2 bacteria per gland; 2 = 3–10 bacteria per gland; 3 = 11–20 bacteria per gland; and 4 = ≥20 bacteria per gland.

4   Notes

1. Because the defibrinated calf blood is frozen, it must be thawed in a 37 °C water bath.

2. CRA is not stable after dilution. Therefore, all dilutions must be made fresh and used immediately.

3. The frozen aliquots of *H. felis* must be thawed at 50 °C.

4. When incubating the cultures, incubate with the solid agar on the bottom. Because the *H. felis* grows at the solid/liquid interface, turning the plate upside down will cause the liquid to spill and not be in contact with the solid media. This will result in no bacterial growth.

5. Because *H. felis* does not grow in colonies, remove the broth culture from the plate using a 5 ml pipette. Using a clean pipette, draw up 2 ml of BHI and put it on the used plate. Swirl the plate on a flat surface and then tilt it at a 45° angle to remove the liquid. Place this liquid into the fresh BHI broth. Repeat with a clean pipette and 2 ml of additional clean BHI.

6. If frequently taking aliquots for OD readings, replace the CampyPak microaerophilic packets every time you open the container.

7. In our lab, this infection scheme results in a 100 % infection rate.

8. During the emulsification process, the pore size in the 3-way stopcock must be made progressively smaller by closing the pore incrementally. This solution will be progressively more difficult to mix. This means that the emulsification is occurring.

9. If the solution is not completely emulsified, the antigen will disperse immediately after injection and will not induce the appropriate immune response.

10. The intranasal challenge causes proliferation of the CRA-specific T cells and elicits their migration into the airways, causing an asthmatic phenotype. Performing this procedure several days apart gradually builds up the asthmatic response in the mouse, similar to the development of asthma in children. The intratracheal challenge is designed to elicit a maximum number of T cells into the airways without causing the animal respiratory distress. The intratracheal challenge is much harder for the mouse to endure. Therefore, this route of administration is only conducted at the end of the procedure.

11. When performing the intranasal challenge, the mouse should only be exposed to the isoflurane for about 15 s, which should induce a low level of anesthesia.

12. The procedure must be done very quickly to get the "sniff" response to the drop of CRA, which ensures that it goes into their lungs and is not swallowed. During this procedure, the mouse will begin recovering from unconsciousness and the muscles will begin to tighten. Brace the lower jaw of the mouse with your thumb and the top of the mouse's head with your forefinger, wrapping your other fingers around its torso. In the event that the mouse awakens from the anesthesia before the procedure is complete, you will have a firm grip on the mouse.

13. The mouse should be exposed to the isoflurane for 30 s until completely limp with barely detectable breath movements in its chest. Wait to administer the challenge until the mouse begins to awaken and its diaphragm spasms.

14. The mouse should be held as previously mentioned upon being removed from the isoflurane through the entire procedure until it is fully awake. Often, if the mouse is not held it will die, possibly due to drop in body temperature. The pipette should be poised at the back of the throat, depressing the mouse's tongue. Ensure that the pipette is ready to deposit the antigen when the mouse awakens. The procedure goes very quickly and the mouse can awaken very rapidly, so care must be taken to continuously restrain the mouse while anesthetized and release the mouse if it awakens.

15. Tissue should not be fixed in Bouin's fixative for more than 24 h or pigments can begin to form. Excess fixative should be washed out of the tissue using the alcohol/water washes.

16. During this procedure, several measurements (including compliance and resistance) are taken; however, we only report the resistance measurement because this particular asthma induction protocol is not designed to dramatically affect other parameters, such as compliance. The acute nature of this model does not result in significant, long-term airway remodeling.

17. The housekeeping gene for comparison used in these experiments was the 18S gene because this gene has been determined to be relatively stable, even under inflammatory conditions [26, 31, 32]. This method uses the difference of the average crossing threshold (Ct) of the 18S gene from the average Ct of the target gene to determine the relative expression of the target gene within each group of animals (Ct). Next, the Ct is calculated determining the difference of the experimental Ct (*H. felis*-infected mice) from the control Ct (mock-infected mice). Finally, the average fold change of the gene is calculated with the following formula: $2^{-\Delta\Delta Ct}$. Using the standard deviation

of the Ct of the experimental group in the average fold change formula, the upper and lower limits are calculated.

18. A quarter of the stomach with the squamo-columnar junction and antrum from each mouse is deparaffinized, stained, and quantitated.

Acknowledgments

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