

GalNAc modification is the prime candidate for a clinically relevant Bet v 1 vaccine. However, the specific design will most likely be dependent on the biophysical and physiologic properties of the individual antigen. The described glycoengineered production platform will be useful for the design of next-generation therapeutic vaccines.

We acknowledge the Core Facility for Integrated Microscopy, Faculty of Health and Medical Sciences, University of Copenhagen, for assistance obtaining the confocal microscopy data.

Caroline B. K. Mathiesen, PhD^a
Michael C. Carlsson, PhD^a
Stephanie Brand, PhD^b
Svenning Rune Möller, MSc^c
Manja Idorn, PhD^d
Per thor Straten, MD, PhD^{d,e}
Anders E. Pedersen, MD, PhD, DSc^e
Sally Dabelsteen, PhD^a
Adnan Halim, PhD^a
Peter Adler Würtzen, PhD^b
Jens Brimnes, PhD^b
Henrik Ipsen, PhD^b
Bent L. Petersen, PhD^c
Hans H. Wandall, MD, PhD^a

From ^athe Copenhagen Center for Glycomics and Department of Cellular and Molecular Medicine, Faculty of Health Sciences, ^bthe Department of Plant and Environmental Biology, Glyco section, Faculty of Science, and ^cthe Department of Immunology and Microbiology and Department of Odontology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark; ^bGlobal Research, ALK-Abelló, Hørsholm, Denmark; and ^dthe Center for Cancer Immune Therapy, Department of Hematology, Herlev Gentofte Hospital, Herlev, Denmark. E-mail: hhw@sund.ku.dk.

Supported in part by the Danish Research Councils (1331-00133B); a program of excellence 2016 (CDO2016: 2016CDO04210) from the University of Copenhagen; the Danish National Research Foundation (DNRF107); the Carl Emil Friis og hustru Olga Doris Friis Foundation; the Lundbeck Foundation; the A.P. Møller og Hustru Chastine Mc-Kinney Møllers Fond til Almene Formaal, Kirsten og Freddy Johansen Fonden, Mizutani Foundation; and the Novo Nordisk Foundation.

Disclosure of potential conflict of interest: M. C. Carlsson is employed by Agilent. S. Brand, P. A. Würtzen, J. Bremnes, and H. Ipsen are employed by ALK-Abelló, a pharmaceutical company producing allergy vaccines. A. E. Pedersen is currently employed by Merck A/S. The activities of M. C. Carlsson and A. E. Pedersen are not related to the current project. The rest of the authors declare that they have no relevant conflicts of interest.

REFERENCES

- Jutel M, Kosowska A, Smolinska S. Allergen immunotherapy: past, present, and future. *Allergy Asthma Immunol Res* 2016;8:191-7.
- Kitzmüller C, Kalser J, Mutschlechner S, Hauser M, Zlabinger GJ, Ferreira F, et al. Fusion proteins of flagellin and the major birch pollen allergen Bet v 1 show enhanced immunogenicity, reduced allergenicity, and intrinsic adjuvanticity. *J Allergy Clin Immunol* 2018;141:293-9.e6.
- Macri C, Dumont C, Johnston AP, Minter JD. Targeting dendritic cells: a promising strategy to improve vaccine effectiveness. *Clin Transl Immunol* 2016;5:e66.
- van Die I, Cummings RD. Glycan gimmickry by parasitic helminths: a strategy for modulating the host immune response? *Glycobiology* 2010;20:2-12.
- Halim A, Carlsson MC, Madsen CB, Brand S, Möller SR, Olsen CE, et al. Glycoproteomic analysis of seven major allergenic proteins reveals novel post-translational modifications. *Mol Cell Proteomics* 2015;14:191-204.
- Unger WW, van Kooyk Y. "Dressed for success" C-type lectin receptors for the delivery of glyco-vaccines to dendritic cells. *Curr Opin Immunol* 2011;23:131-7.
- Sirvent S, Soria I, Cirauqui C, Cases B, Manzano AI, Diez-Rivero CM, et al. Novel vaccines targeting dendritic cells by coupling allergoids to nonoxidized mannan enhance allergen uptake and induce functional regulatory T cells through programmed death ligand 1. *J Allergy Clin Immunol* 2016;138:558-67.e11.
- Yang Z, Wang S, Halim A, Schulz MA, Frodin M, Rahman SH, et al. Engineered CHO cells for production of diverse, homogeneous glycoproteins. *Nat Biotechnol* 2015;33:842-4.

- Lo-Man R, Vichier-Guerre S, Bay S, Dériaud E, Cantacuzène D, Leclerc C. Anti-tumor immunity provided by a synthetic multiple antigenic glycopeptide displaying a Tri-Tn glycotope. *J Immunol* 2001;166:2849-54.

Available online August 17, 2018.
<https://doi.org/10.1016/j.jaci.2018.07.030>

Prophylactic and therapeutic inhibition of allergic airway inflammation by probiotic *Escherichia coli* O83



To the Editor:

Oral supplementation with probiotic bacteria is a promising alternative for allergy prevention. However, a recent meta-analysis has demonstrated that orally delivered bacteria only prevented the development of certain allergic manifestations, such as atopic eczema, but had limited efficacy on respiratory allergies.¹ Similarly, oral delivery of the gram-negative probiotic bacterial strain *Escherichia coli* A0 34/86 (*E coli* O83; serotype O83:K24:H31; a commercially available oral live vaccine, Colinfant Newborn) to high-risk infants reduced development of atopic diseases in later life but had no beneficial effects on respiratory allergies.²

Here, we address whether the delivery of *E coli* O83 directly through the airways might be more effective in preventing the development of allergic disease in the lung. To test this, mice were intranasally treated with *E coli* O83 or an attenuated α -hemolysin-deficient mutant *E coli* O83 before systemic sensitization and local airway challenge with ovalbumin (OVA). Detailed methods are provided in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Prophylactic intranasal treatment with *E coli* O83 during sensitization and challenge with OVA ([Fig 1, A](#)) completely inhibited airway hyperreactivity (AHR) to methacholine, as measured by using whole-body plethysmography ([Fig 1, B](#)) and invasive assessment of pulmonary resistance and compliance (see [Fig E1](#) in this article's Online Repository at www.jacionline.org). Treatment with *E coli* O83 reduced airway eosinophilia ([Fig 1, C](#)), T_H2 cytokine production (see [Fig E2, A and B](#), in this article's Online Repository at www.jacionline.org), and mucus secretion ([Fig 1, D](#), and see [Fig E2, E](#)) but did not induce pulmonary IFN- γ and IL-10 levels (see [Fig E2, C and D](#)) when compared with values in sham-treated/OVA-sensitized control mice. Of note, although OVA-specific antibody production was not influenced (see [Fig E2, F-I](#)), *E coli* O83 still inhibited the effector phase of the allergic response.

LPS is a major constituent of the outer membrane of gram-negative bacteria, such as *E coli* O83, and potently stimulates the immune system through Toll-like receptor (TLR) 4. Studies on the effect of LPS in experimental models of asthma have produced conflicting results; some report a beneficial effect,³ and others show an exacerbation.⁴

To determine the role of TLR4 in *E coli* O83-mediated protection, we treated wild-type (WT) and TLR4-deficient (TLR4KO) mice with *E coli* O83 as in [Fig 1, A](#). Unlike in WT mice *E coli* O83-treated TLR4KO mice had strong eosinophilic airway

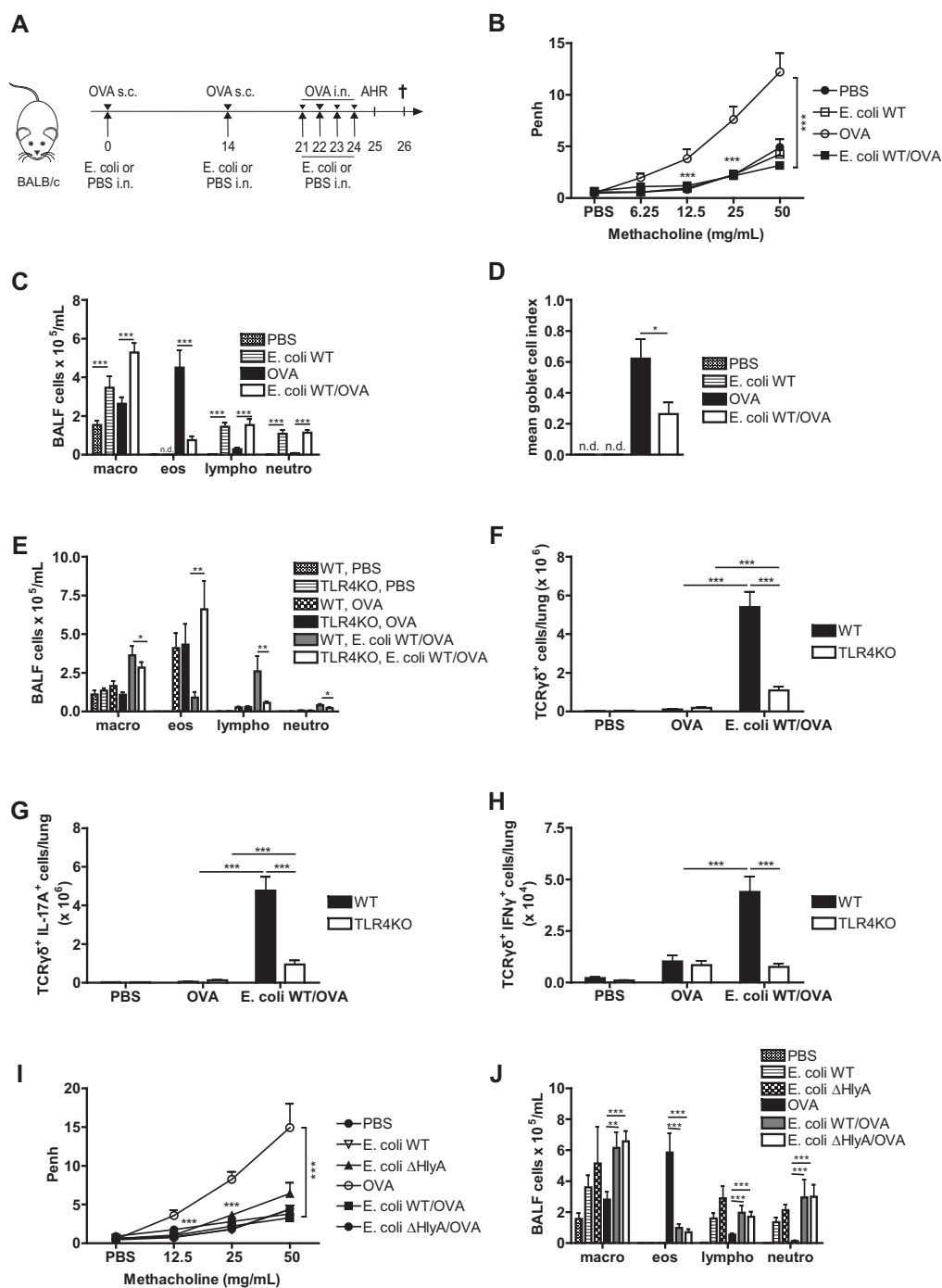


FIG 1. Intranasal exposure to *E. coli* O83 reduces AAI in WT but not TLR4KO mice. **A**, Experimental design. *i.n.*, Intranasal; *s.c.*, subcutaneous. **B**, AHR in response to methacholine. *Penh*, Enhanced pause. **C**, Differential cell counts in BALF. **D**, Mucus production in the airways. **E**, Differential cell counts in BALF. **F-H**, Absolute numbers of total TCRγδ⁺ T cells (Fig 1, *F*) and TCRγδ⁺ T cells producing IL-17A (Fig 1, *G*) or IFN-γ (Fig 1, *H*) in the lung. **I**, AHR in response to methacholine. **J**, Differential cell counts in BALF. Graphs show pooled results from 1 (Fig 1, *I*), 2 (Fig 1, *E-H* and *J*), or 3 (Fig 1, *B-D*) independent experiments with 4 to 6 (Fig 1, *I*), 4 to 11 (Fig 1, *E-H* and *J*), and 3 to 18 (Fig 1, *B* and *C*) mice per group. Error bars denote means ± SEMs. **P* < .05, ***P* < .01, and ****P* < .001. *eos*, Eosinophils; *lympho*, lymphocytes; *macro*, macrophages; *n.d.*, not detectable; *neutro*, neutrophils.

infiltration (Fig 1, *E*) and high levels of lung IL-5 and IL-13 (see Fig E3 in this article's Online Repository at www.jacionline.org). Mechanistically, *E. coli* O83 treatment led to increased accumulation of pulmonary T-cell receptor (TCR) γδ⁺ T cells, which

produced high levels of IL-17A and IFN-γ in a TLR4-dependent manner (Fig 1, *F-H*).

α-Hemolysin is an important virulence factor of pathogenic *E. coli* strains because of its cytotoxic activity and is also produced

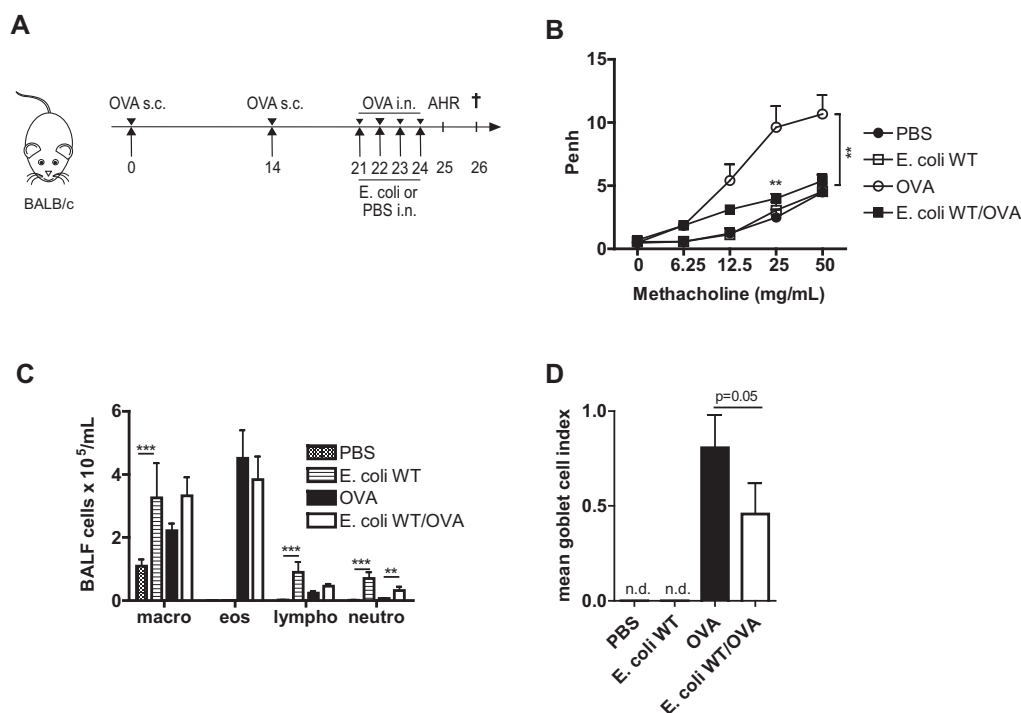


FIG 2. Therapeutic treatment of OVA-sensitized mice with *E coli* O83 inhibits development of AHR and mucus hyperplasia. **A**, Experimental design. *i.n.*, Intranasal; *s.c.*, subcutaneous. **B**, AHR in response to methacholine. *Penh*, Enhanced pause. **C**, Differential cell counts in BALF. **D**, Pulmonary mucus production. Graphs show pooled results from 2 independent experiments with 5 to 10 mice per group. Error bars denote means $\pm$ SEMs. ** $P < .01$ and *** $P < .001$. *eos*, Eosinophils; *lympho*, lymphocytes; *macro*, macrophages; *n.d.*, not detectable; *neutro*, neutrophils.

by the nonpathogenic *E coli* O83.⁵ To test whether *E coli* O83 α -hemolysin (HlyA) was required for protection, we treated mice with WT (*E coli* WT) or mutant *E coli* O83 lacking α -hemolysin (*E coli* Δ HlyA), as in Fig 1, A. Both *E coli* WT and *E coli* Δ HlyA reduced the development of AHR, airway eosinophilia (Fig 1, I and J), pulmonary IL-5 and IL-13 production (see Fig E4, A and B, in this article's Online Repository at www.jacionline.org), and mucus hypersecretion (see Fig E4, C and D) equally well. Thus, α -hemolysin-deficient *E coli* O83 could represent a safe prophylactic tool to inhibit allergic responses without loss of potency.

Next, we sought to test the therapeutic potential of *E coli* O83 applied after systemic allergic sensitization (Fig 2, A). Importantly, sensitized mice that received intranasal *E coli* O83 during allergen challenge were similarly protected from AHR (Fig 2, B), with reduced pulmonary IL-5 and IL-13 levels (see Fig E5, A and B, in this article's Online Repository at www.jacionline.org) and decreased mucus hyperplasia (Fig 2, D, and see Fig E5, C) compared with control mice. Interestingly, eosinophil levels in the lung were comparable with sham-treated allergic control values (Fig 2, C).

When applied to children, oral supplementation with *E coli* O83 reduced allergic sensitization but not asthma.² Here, we show that the intranasal route of application of *E coli* O83 prevented development of allergic airway inflammation (AAI) in mice, suggesting that local administration of probiotic bacteria targeting the mucosal surfaces of the lung, rather than the gut, might be an effective way to prevent/treat allergic lung disease

in human subjects. Extending this notion, we hypothesize that oral delivery of *E coli* O83 might be more potent at inhibiting food allergy rather than allergic disease in the lung; however, this has not yet been tested.

The role of TCR $\gamma\delta^+$ T cells in allergic patients remains unclear, with both pathogenic⁶ and protective⁷ roles reported. Here, intranasal exposure to *E coli* O83 increased the numbers of pulmonary TCR $\gamma\delta^+$ T cells, which were associated with suppression of AAI. *E coli* O83 induced IL-17A- and IFN- γ -secreting TCR $\gamma\delta^+$ T cells in a TLR4-dependent manner. However, antibody-mediated blockade of IL-17A did not affect the protective effect of *E coli* O83 (data not shown). Nembrini et al⁸ have shown that *E coli* DH5 α pDS prevents allergy in an IFN- γ -independent manner. However, the precise role of IFN- γ (alone or in combination with other factors) or TCR $\gamma\delta^+$ T cells in counterregulating allergic immune responses in the lung in our model remains to be determined.

Therapeutic application of *E coli* O83 to OVA-primed allergic mice reduced the development of several important features of AAI, suggesting that *E coli* O83 can suppress reactivation of established type 2 immunity and might be an interesting tool not only for prophylactic approaches but also for therapy of sensitized subjects.

Taken together, we have shown that intranasal delivery of *E coli* O83 suppressed cardinal features of AAI in mice in both prophylactic and therapeutic settings. Mechanistically, the suppressive effect was dependent on signaling through TLR4 and was associated with increased numbers of pulmonary IL-17A⁺ and

IFN- γ^+ TCR $\gamma\delta^+$ T cells. Importantly, intranasal application of an α -hemolysin-deficient strain, which presents a safer variant of *E coli* O83, had equally beneficial effects as the WT strain. This study contributes to further characterization of cellular and molecular pathways involved in regulation of AAI by probiotic bacteria.

Christian Zwicker, MSc^a

Priya Sarate, MSc^a

Mirjana Drinić, MSc^a

Katharina Ambroz, BSc^a

Elke Korb, MSc^a

Ursula Smole, PhD^b

Cordula Köhler, BSc^b

Mark S. Wilson, PhD^c

Hana Kozakova, PhD^d

Peter Sebo, PhD^e

Miloslav Kverka, MD, PhD^e

Ursula Wiedermann, MD, MSc, PhD^a

Irma Schabussova, PhD^a

From the Institutes of ^aSpecific Prophylaxis and Tropical Medicine and ^bImmunology, Medical University of Vienna, Vienna, Austria; ^cImmunology Discovery, Genentech, South San Francisco, Calif; ^dthe Laboratory of Gnotobiology, Institute of Microbiology, Czech Academy of Sciences, Nový Hrádek, Czech Republic; and ^ethe Institute of Microbiology, Czech Academy of Sciences, Prague, Czech Republic. E-mail: irma.schabussova@meduniwien.ac.at.

Supported by Austrian Science Fund (FWF) grants SFB 4612 (to U.W. and I.S.), SFB 4609 (to U.S. and C.K.), and Doctoral Program MCCA DK W1248-B13 (to C.Z., P.S., U.W. and I.S.) and DK W1248 (to U.S. and C.K.), and OeAD grants CZ 15/2015 (to C.Z., P.S., M.D., H.K., and I.S.) and CZ 13/2016 (to C.Z., P.S., M.D., M.K., and I.S.).

Disclosure of potential conflict of interest: M. S. Wilson is an employee of Genentech and owns Genentech stock. P. Sebo is a founder and shareholder of Revabiotec SE, which develops a next generation of whole cell pertussis vaccines. U. Wiedermann is member of the SAB and supporting CSO of the biotech company Imugene, Australia, developing B cell cancer vaccines and is also member of the Austrian Advisory Board of Immunizations of the government of Health in Austria. The rest of the authors declare that they have no relevant conflicts of interest.

REFERENCES

- West CE, Jenmalm MC, Kozyskyj AL, Prescott SL. Probiotics for treatment and primary prevention of allergic diseases and asthma: looking back and moving forward. *Expert Rev Clin Immunol* 2016;12:625-39.
- Lodinoova-Zadnikova R, Prokesova L, Kocourkova I, Hrdy J, Zizka J. Prevention of allergy in infants of allergic mothers by probiotic *Escherichia coli*. *Int Arch Allergy Immunol* 2010;153:201-6.
- Rodriguez D, Keller AC, Faquim-Mauro EL, de Macedo MS, Cunha FQ, Lefort J, et al. Bacterial lipopolysaccharide signaling through Toll-like receptor 4 suppresses asthma-like responses via nitric oxide synthase 2 activity. *J Immunol* 2003;171:1001-8.
- Starkhammar M, Larsson O, Kumlien Georen S, Leino M, Dahlen SE, Adner M, et al. Toll-like receptor ligands LPS and poly (I:C) exacerbate airway hyperresponsiveness in a model of airway allergy in mice, independently of inflammation. *PLoS One* 2014;9:e104114.
- Sheshko V, Hejnova J, Rehakova Z, Sinkora J, Faldyna M, Alexa P, et al. HlyA knock out yields a safer *Escherichia coli* A0 34/86 variant with unaffected colonization capacity in piglets. *FEMS Immunol Med Microbiol* 2006;48:257-66.
- Zuany-Amorim C, Ruffie C, Haile S, Vargaftig BB, Pereira P, Pretolani M. Requirement for gammadelta T cells in allergic airway inflammation. *Science* 1998;280:1265-7.
- Lahn M, Kanehiro A, Takeda K, Joetham A, Schwarze J, Kohler G, et al. Negative regulation of airway responsiveness that is dependent on gammadelta T cells and independent of alphabeta T cells. *Nat Med* 1999;5:1150-6.
- Nembrini C, Sichelstiel A, Kisielow J, Kurrer M, Kopf M, Marsland BJ. Bacterial-induced protection against allergic inflammation through a multicomponent immunoregulatory mechanism. *Thorax* 2011;66:755-63.

Available online August 17, 2018.
<https://doi.org/10.1016/j.jaci.2018.07.029>

Fel d 1 and Fel d 4 levels in cat fur, saliva, and urine



To the Editor:

Cats are popular household pets and commonly cause allergies. Several cat allergens have been identified, including Fel d 1, an uteroglobin. More commonly, animal allergens are lipocalins, and the cat lipocalin allergen Fel d 4 has been identified.¹

Recently, Fel d 1- and Fel d 4-specific IgE was assessed in patients with pet allergy.² Of those with cat allergy, 94% had increased levels (>0.35 kU/L) of Fel d 1, and 49% had increased levels of Fel d 4. Older studies cited by Zahradnik and Raulf³ reported that Fel d 1 is produced in sebaceous and salivary glands and can be extracted from fur, skin, and saliva.⁴ Intact male cats were found to have more fur Fel d 1 than female cats. There appears to be no information on age- or breed-related differences in Fel d 1 nor is there information about sites of Fel d 4 production. Therefore Fel d 1 and Fel d 4 levels in fur, urine, and saliva were evaluated and compared in a cohort of domestic cats to determine which phenotypes would distinguish cats with higher levels of either antigen.

This study was performed in collaboration with a local cat hospital and enrolled cats undergoing routine procedures requiring anesthesia. Canadian Council on Animal Care guidelines were followed. After obtaining the owners' consent, a mixed cohort of 26 male and female cats older than 5 months that were neutered and intact and of different breeds was recruited. Cats with dental (abscess or cavity) or skin diseases or other health problems were excluded.

Samples of 100 mg or more fur cut close to the skin by using clippers were collected from either front leg at the site of anesthesia administration. Before use of anticholinergic medication and after anesthesia, 0.25 to 0.50 mL of saliva was pipetted from the cheek pouches. Approximately 2.5 mL of urine was collected by means of catheterization performed by a veterinarian or delegate. For analysis, fur samples were placed in clean plastic bags, stored at 4°C, and shipped at room temperature. Saliva and urine were placed in sterile cryovials, stored at -20°C, and shipped in dry ice. Fel d 1 and Fel d 4 were eluted, 3 aliquots from each sample were analyzed by means of ELISA, and the average was reported (Indoor Biotechnologies, Charlottesville, Va). Medians and ranges were calculated. Comparisons were made with a nonparametric test (Mann-Whitney). There was no attempt to account for multiple comparisons.

The 26 cats studied came to the clinic for procedures that required anesthesia, mainly dentistry and spaying (see Table E1 in this article's Online Repository at www.jacionline.org). They were 5 months to 12 years old, half were female, about half were of the domestic short-hair variety, and the majority (65%) had been neutered. Fur was collected from 26 cats, urine was collected from 20 cats, and saliva was collected from 17 cats. No adverse events were recorded from sample collection. Fel d 1 and Fel d 4 levels in fur, urine, and saliva are shown in Fig 1.

In fur, Fel d 1 levels (median, 12.24 μ g/g; range, 5.49-20.72 μ g/g) were significantly greater than Fel d 4 levels (median, 0.09 μ g/g; range, 0.03-0.19 μ g/g; $P < .001$). Conversely, Fel d 4 levels were greater than Fel d 1 levels in saliva (median of 7.62 μ g/mL [range, 1.41-17.42 μ g/mL] vs 2.45 μ g/mL [0.87-5.11 μ g/mL], respectively; $P = .039$). In urine, Fel d 1 (median,

METHODS

Mice

Female WT BALB/c mice aged 6 to 8 weeks were purchased from Charles River (Sulzfeld, Germany). TLR4KO mice on a BALB/c background were bred at the Institute of Microbiology, Czech Academy of Sciences, Prague, Czech Republic. All animals were kept under conventional housing conditions, and experiments were approved by the Animal Experimentation Committee of the Medical University of Vienna and the Austrian Federal Ministry of Education, Science and Culture (BMWFW-66.009/0358-WF/V/3b/2015).

Bacteria

WT *E coli* A0 34/86, serotype O83:K24:H31 (*E coli* O83; a gift from H. Tlaskalova, Institute of Microbiology, Czech Academy of Sciences, Prague, Czech Republic) or α -hemolysin-deficient *E coli* O83 (Δ HlyA)^{E1} was grown in Luria-Bertani medium (Sigma-Aldrich, St Louis, Mo) at 37°C. After 2 to 3 hours (OD, approximately 1) of incubation, bacteria were harvested, and colony-forming units were calculated based on a growth curve established in house. Bacteria were washed and resuspended in the appropriate concentration in PBS for *in vivo* use.

Experimental design and measurement of AHR

Mice were immunized subcutaneously with 10 μ g of OVA (grade V; Sigma-Aldrich) on days 0 and 14. On days 21 to 24, mice were anesthetized with 5% isoflurane (Isocare; Animalcare, York, United Kingdom) and treated intranasally with 100 μ g of OVA. Thirty minutes before each sensitizing dose or intranasal challenge with OVA, mice received 10⁶ colony-forming units of *E coli* O83 or *E coli* O83 Δ HlyA through the nasal route. AHR was determined by using unrestrained whole-body plethysmography (Data Sciences International, St Paul, Minn) 24 hours after the last intranasal application of OVA. For this purpose, mice were subjected to increasing doses (0–50 mg/mL) of methacholine (Sigma-Aldrich), and AHR was expressed as the dimensionless parameter enhanced value, as previously described.^{E2} The next day, organs, blood, and bronchoalveolar lavage fluid (BALF) were collected (day 26).

Mice were anesthetized intraperitoneally with ketamine (100 mg/kg; Ketasetol; AniMedica, Senden, Germany) and xylazine (10 mg/kg; Rompun-TS; Bayer Vital, Leverkusen, Germany), tracheotomized, and ventilated (Buxco FinePoint RC System; Data Sciences International) 24 hours after the final OVA challenge to determine lung resistance and compliance. Resistance and compliance were measured in response to 50 mg/mL nebulized methacholine. Baseline values were established by means of intratracheal application of aerosolized PBS. After measuring AHR, organs were harvested, and blood and BALF were collected.

Lung cell isolation and *in vitro* culture

Lungs were harvested from terminally anesthetized mice and processed, as previously described.^{E3} Briefly, lung lobes were minced and incubated in RPMI-1640 (Gibco, Thermo Scientific, Waltham, Mass) containing 0.05 mg/mL Liberase TL (Roche, Mannheim, Germany), and 0.5 mg/mL DNase (Sigma-Aldrich) for 45 minutes. Digested lung tissue was forced through a 0.70- μ m cell strainer, and red blood cells were lysed with ammonium-chloride-potassium buffer. After lysis, cells were resuspended in complete RPMI (10% FCS, 2 mmol/L mercaptoethanol, 2 mmol/L L-glutamine, and 100 μ g/mL gentamicin; Sigma-Aldrich) and plated in 96-well plates (5 $\times$ 10⁵ cells/well). Cells were incubated with 100 μ g/mL endotoxin-free OVA (EndoGrade; Hyglos, Bernried am Starnberger See, Germany) for 72 hours at 37°C in a 5% CO₂ atmosphere. Thereafter, supernatants were collected, and cytokines (IL-5, IL-10, IL-13, and IFN- γ) were analyzed by using commercially available ELISA kits, according to the manufacturer's instructions (Ready-SET-Go! Kit; eBioscience, San Diego, Calif).

Detection of OVA-specific antibodies

OVA-coated microplates (5 μ g/mL) were incubated with serum or BALF overnight at 4°C. The next day, the plates were washed and incubated with

anti-mouse IgG₁, IgG_{2a}, IgA, and IgE (1:500; BD Pharmingen, San Jose, Calif) overnight. Plates were washed again, and OVA-specific antibodies were detected by using a horseradish peroxidase-conjugated mouse anti-rat IgG (1:2000; Jackson ImmunoResearch, West Grove, Pa). For colorimetric measurement, ABTS buffer (1 mmol/L ABTS in 70 mmol/L citric-phosphate buffer; Sigma-Aldrich) was added, and plates were analyzed on a SparkControl Magellan plate reader (Tecan, Zurich, Switzerland).

Flow cytometry

Lung cells were washed with fluorescence-activated cell sorting buffer (0.1% BSA and 200 mmol/L EDTA in PBS), and Fc receptors were blocked by adding anti-CD16/CD32 (eBioscience) for 20 minutes at 4°C. Thereafter, cells were stained with mAbs for surface markers for another 20 minutes at 4°C, washed, and resuspended in fluorescence-activated cell sorting buffer. For detection of intracellular cytokines, cells were stimulated with 100 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich) and 1 μ g/mL ionomycin (Sigma-Aldrich) for 6 hours at 37°C in a 5% CO₂ atmosphere. For the last 4 hours, protein secretion was blocked by adding brefeldin A and monensin (Transport Inhibitor Cocktail; eBioscience). Cells were stained for surface markers and fixed and permeabilized with the intracellular fixation and permeabilization kit (eBioscience), according to the manufacturer's instructions. Intracellular cytokines were detected by incubating the cells with appropriate mAbs in Perm buffer for at least 30 minutes at room temperature. In all experiments dead cells were excluded by using the fixable cell viability dye eFlour 506 (eBioscience). Cells were analyzed by using a BD FACSCanto II, and data were processed with FlowJo software (version 10.0.7; TreeStar, Ashland, Ore). mAbs used included (all from eBioscience unless stated otherwise) CD3e (phycoerythrin [PE]; 145-2C11), CD4 (fluorescein isothiocyanate [FITC] or peridinin-chlorophyll-protein complex [PerCP]-Cy5.5; RM4-5), TCR β (FITC or PerCP-Cy5.5; H57-597, eBioscience or BioLegend, San Diego, Calif, respectively), TCR $\gamma\delta$ (PE-Cy7; eBioGL3), B220 (PerCP-Cy5.5; RA3-6B2), IFN- γ (PE; XMGI.2), and IL-17A (eFlour 450; eBio17B7).

Differential cell counts in BALF

Lungs were lavaged with 1 mL of PBS, and cells in BALF were obtained by means of centrifugation. The cell pellet was resuspended in PBS and spun onto microscopic slides (4 $\times$ 10⁴ cells; Shandon Cytospin; Shandon Southern Instruments, Waltham, Mass), air-dried, and subsequently stained with hematoxylin and eosin (Hemacolor; Merck, Darmstadt, Germany). A total of 200 macrophages, lymphocytes, eosinophils, or neutrophils per slide were counted under a light microscope ($\times$ 100 magnification; Nikon Eclipse; Nikon, Tokyo, Japan).

Lung histology

Formaldehyde (7.5%)–fixed lung tissue was paraffin embedded, and 5- μ m thick sections were stained with hematoxylin and eosin and periodic acid–Schiff (PAS; Sigma-Aldrich). Mucus production was evaluated by using a scoring system adapted from Zaiss et al.^{E4} The number of PAS-positive cells per 50 counted airway epithelial cells in 5 randomly selected regions from 1 section was determined and assigned a score as follows: 0, no PAS-positive cells; 1, less than 25 PAS-positive cells; and 2, 25 or more PAS-positive cells. If mucus occlusion of a bronchiole was detectable within a region, a score of 1 was added. The average of the sum of all 5 individual regions was calculated and expressed as the mean goblet cell index.

Statistics

Sample size determination was based on the primary end point of eosinophil counts, with 1 comparison (OVA vs *E coli*/OVA) or 3 comparisons (OVA WT vs *E coli*/OVA WT, OVA TLR4KO vs *E coli*/OVA TLR4KO, and *E coli*/OVA WT vs *E coli*/OVA TLR4KO) for the different experiments. The other experimental groups were included for validity checks only, and no statistical comparisons were planned for them. The effect size that should be detected at a power of 70% and a 2-sided significance level of 5% was set to a Cohen d value of

1.27 (5-fold difference and assuming an SD of $0.55 \log_{10}$ units). Between 9 and 15 animals per group are required to reach the power goal, depending on the type of experiment. Comparisons for the other end points must be considered explorative. A *post hoc* power consideration including the 3 other end points of primary interest (TCR $\gamma\delta^+$ cell counts, IL-17A $^+$ and IFN- γ^+ TCR $\gamma\delta^+$ T cells) revealed a power of 90% or greater for the effect size d value of 1.27 based on pooled variance estimates for these end points.

Data were statistically analyzed by using general linear models with the random factor “experiment” for the replications. Pooled results or representative examples are shown as means $\pm$ SEMs, and differences were considered significant at *P* value of less than .05. All analyses were performed with Stata 13.1 software (StataCorp, College Station, Tex), and graphics were prepared by using GraphPad Prism 6.0 software (GraphPad Software, La Jolla, Calif).

REFERENCES

- E1. Sheshko V, Hejnova J, Rehakova Z, Sinkora J, Faldyna M, Alexa P, et al. HlyA knock out yields a safer *Escherichia coli* A0 34/86 variant with unaffected colonization capacity in piglets. *FEMS Immunol Med Microbiol* 2006;48:257-66.
- E2. Webb DC, McKenzie AN, Koskinen AM, Yang M, Mattes J, Foster PS. Integrated signals between IL-13, IL-4, and IL-5 regulate airways hyperreactivity. *J Immunol* 2000;165:108-13.
- E3. Lajoie S, Lewkowich I, Herman NS, Sproles A, Pesce JT, Wynn TA, et al. IL-21 receptor signalling partially mediates Th2-mediated allergic airway responses. *Clin Exp Allergy* 2014;44:976-85.
- E4. Zaiss MM, Rapin A, Lebon L, Dubey LK, Mosconi I, Sarter K, et al. The intestinal microbiota contributes to the ability of helminths to modulate allergic inflammation. *Immunity* 2015;43:998-1010.

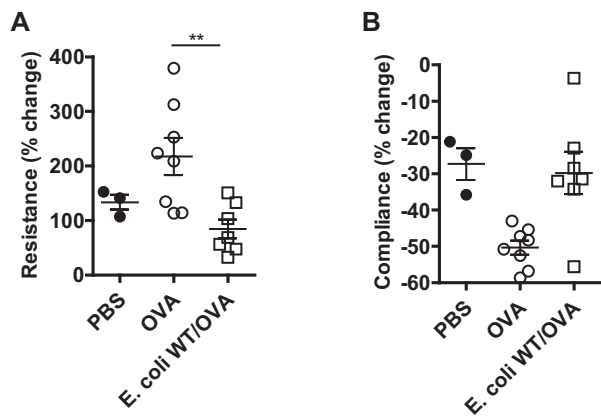


FIG E1. Intranasal treatment with *E. coli* O83 decreases lung resistance. Mice were treated as outlined in Fig 1, A. Twenty-four hours after the last allergen challenge, mice were killed, and AHR was assessed by means of invasive measurement of airway resistance (A) and compliance (B) in response to 50 mg/mL methacholine. Data are representative for one experiment with 3 to 8 mice per group. Error bars denote means $\pm$ SEMs. ** $P < .01$.

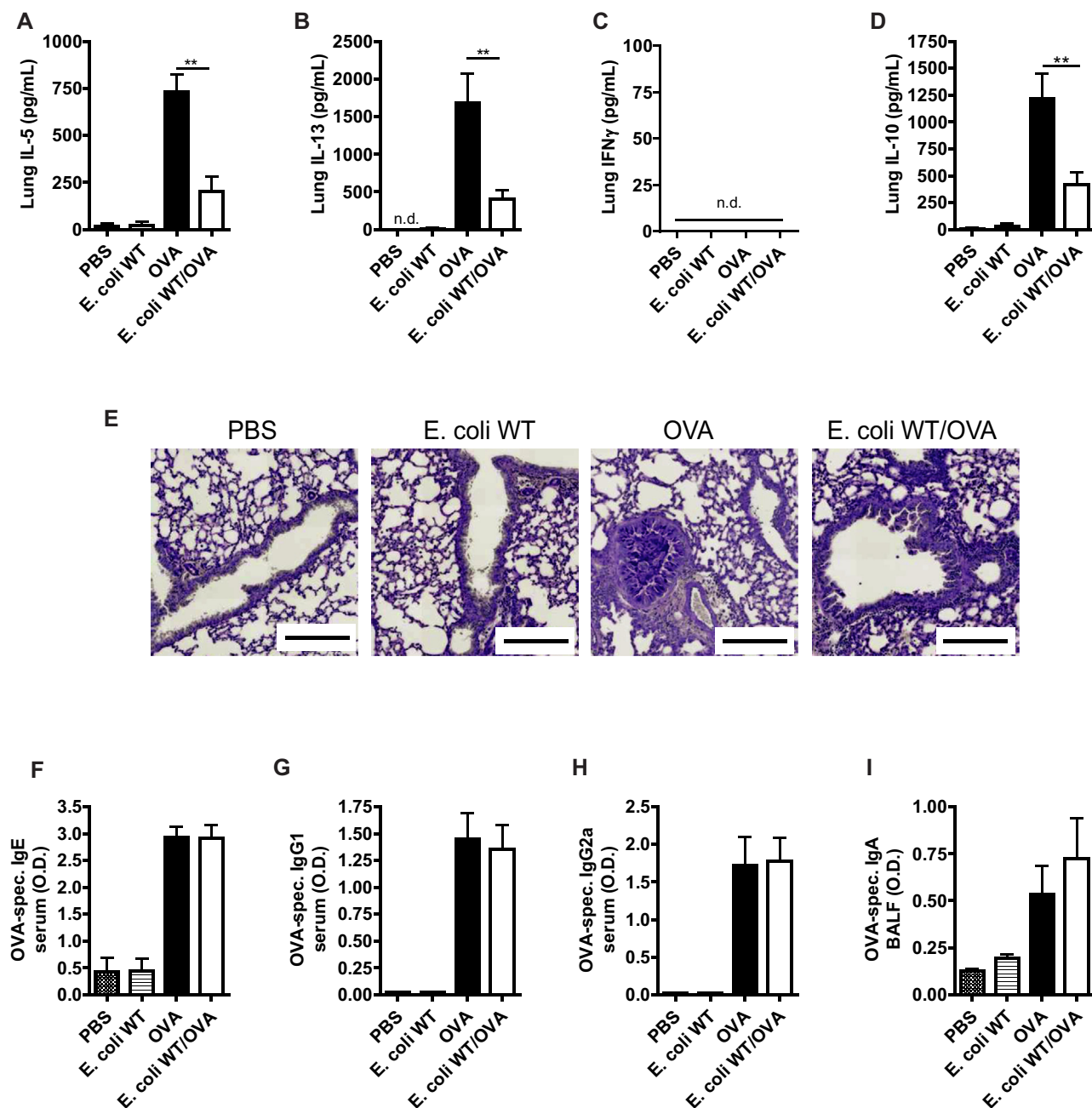


FIG E2. Intranasal treatment with *E. coli* O83 decreases cellular T_H2 responses in the lung but does not affect production of allergen-specific antibodies. **A-D**, IL-5 (Fig E2, A), IL-13 (Fig E2, B), IFN- γ (Fig E2, C), and IL-10 (Fig E2, D) levels after OVA restimulation of lung cells obtained from mice treated as in Fig 1, A. **E**, PAS-stained lung section from 1 mouse representative of each group. Scale bar = 200 μ m. **F-H**, OVA-specific IgE (Fig E2, F), IgG $_1$ (Fig E2, G), and IgG $_{2a}$ (Fig E2, H) in serum collected at the end of the experiment (d26). **I**, OVA-specific IgA in BALF. Graphs show pooled results from 3 independent experiments with 7 to 18 mice per group (Fig E2, A-D) or 1 representative example with 3 to 5 mice per group (Fig E2, F-I). Error bars denote means $\pm$ SEMs. ** P < .01. n.d., Not detectable.

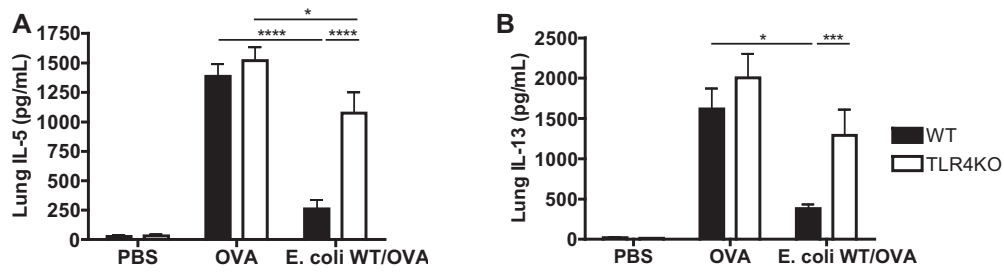


FIG E3. Recognition of *E coli* O83 by TLR4 is required for suppression of T_H2 responses. Lung cells derived from WT or TLR4KO animals treated as in Fig 1, A, were restimulated with OVA, and IL-5 (Fig E3, A) and IL-13 (Fig E3, B) levels were measured in supernatants. Graphs show pooled results from 2 independent experiments with 8 to 11 mice per group. Error bars denote means $\pm$ SEMs. * P < .05, *** P < .001, and **** P < .0001.

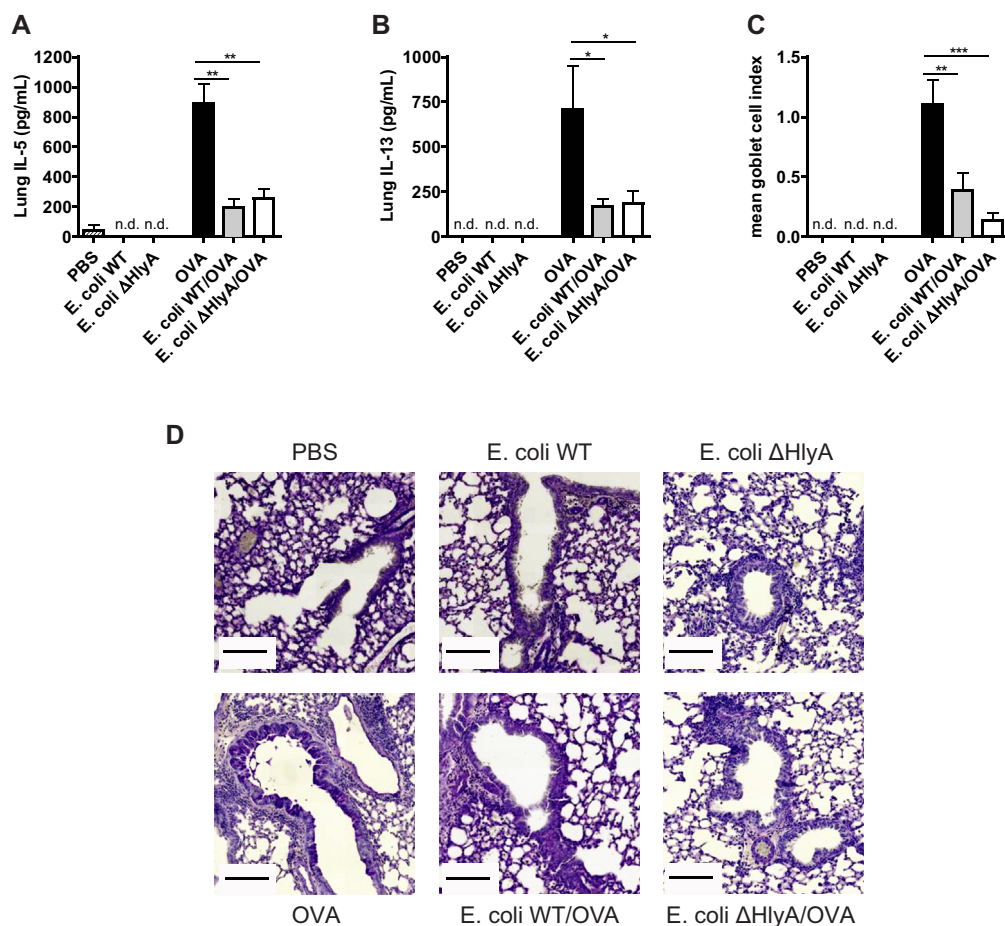


FIG E4. WT and hemolysin-deficient *E coli* O83 decreases cellular T_H2 responses and mucus hyperplasia in the lung. WT mice were treated with *E coli* O83 WT or *E coli* O83 lacking hemolysin (*E coli* HlyKO), as shown in Fig 1. **A** and **B**, Lung cells obtained from these mice were cultured in the presence of OVA, and levels of IL-5 (Fig E4, **A**) and IL-13 (Fig E4, **B**) were determined in supernatants. **C**, Quantification of mucus production by airway epithelial cells. **D**, One representative lung section per group stained with PAS. Scale bar = 200 μ m. Graphs show pooled results from 2 independent experiments with 4 to 11 mice per group. Error bars denote means $\pm$ SEMs. * P < .05, ** P < .01, and *** P < .001. n.d., Not detectable.

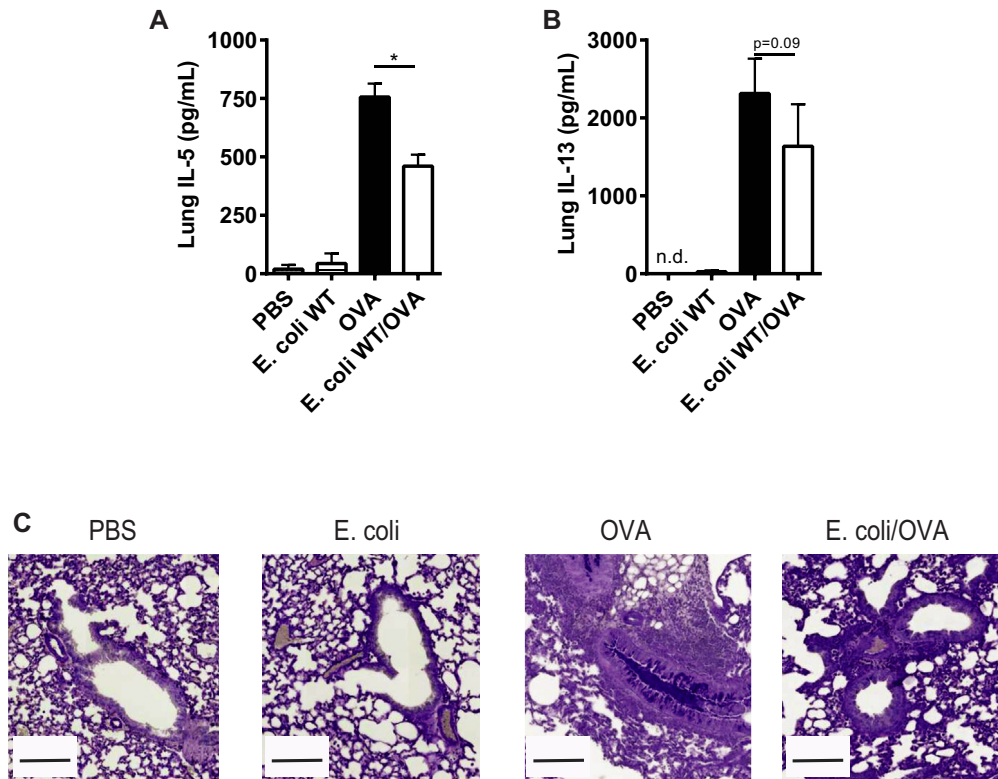


FIG E5. Therapeutic treatment of OVA-sensitized mice with *E. coli* O83 decreases cellular T_H2 responses and mucus hyperplasia in the lung. WT mice were treated with *E. coli* O83 WT, as shown in Fig 2. **A** and **B**, Lung cell cultures were restimulated with OVA, and levels of IL-5 (Fig E5, **A**) and IL-13 (Fig E5, **B**) were determined in supernatants. **C**, One representative lung section per group stained with PAS. Scale bar = 200 μ m. Graphs show pooled results from 2 independent experiments with 5 to 10 mice per group. Error bars denote means $\pm$ SEMs. * $P < .05$. n.d., Not detectable.