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Bacterial secretion of histamine within the gut influences immune responses within the lung

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Abstract

Background: Histamine is an important immunomodulator influencing both the innate and adaptive immune system. Certain host cells express the histidine decarboxylase enzyme (HDC), which is responsible for catalysing the decarboxylation of histidine to histamine. We and others have shown that bacterial strains can also express HDC and secrete histamine; however, the influence of bacterial-derived histamine on the host immune responses distant to the gut is unclear.

Methods: The *Escherichia coli* BL21 (*E coli* BL21) strain was genetically modified to express the *Morganella morganii* (*M morganii*)-derived HDC gene (*E coli* BL21_HTW). *E coli* BL21 and *E coli* BL21_HTW were gavaged to ovalbumin (OVA) sensitized and challenged mice to investigate the effect of bacterial-derived histamine on lung inflammatory responses.

Results: Oral administration of *E coli* BL21_HTW, which is able to secrete histamine, to wild-type mice reduced lung eosinophilia and suppressed ex vivo OVA-stimulated cytokine secretion from lung cells in the OVA respiratory inflammation mouse model. In histamine receptor 2 (H2R)-deficient mice, administration of histamine-secreting bacteria also reduced inflammatory cell numbers in bronchoalveolar lavage (BAL). However, the suppressive effect of bacterial-derived histamine on BAL inflammation was lost in HDC-deficient mice. This loss of activity was associated with increased expression of histamine degrading enzymes and reduced histamine receptor expression.

Conclusion: Histamine secretion from bacteria within the gut can have immunological consequences at distant mucosal sites, such as within the lung. These effects are influenced by host histamine receptor expression and the expression of histamine degrading enzymes.

KEYWORDS

gut-lung axis, histamine, inflammation, *Morganella morganii*, OVA mouse model

Abbreviations: BAL, bronchoalveolar lavage; DAO, diamine oxidase; DC, dendritic cell; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; GPCR, G protein-coupled receptor; H1/2/3/4R, histamine 1/2/3/4 receptor; HDC, histidine decarboxylase; HNMT, histamine-*N*-methyl transferase; IFN-γ, interferon-gamma; IL, interleukin; MIP-1, macrophage inflammatory protein; OVA, ovalbumin; PBS, phosphate buffered saline; RANTES, Regulated on activation, normal T-cell expressed and secreted; UPLC, ultra-performance liquid chromatography.

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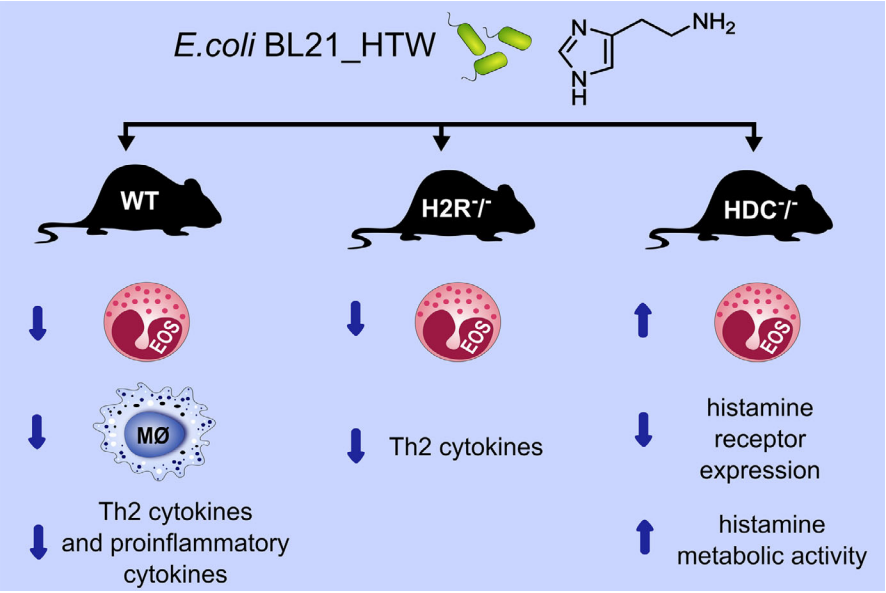


GRAPHICAL ABSTRACT

Bacterial secretion of histamine in the gut can influence allergic inflammation in the lung. Both H2Rdependent and -independent effects are observed, while murine expression of histamine metabolising enzymes may also alter the effects of histamine within the lung.

1 | INTRODUCTION

Asthma is a common chronic inflammatory disease, affecting 5%-10% of the population worldwide.[1] Despite the availability of several medications, which control asthma symptoms and reduce airway inflammation for many patients,[2-4] there is no curative treatment.[5] Histamine is a well-known mediator responsible for many of the acute symptoms associated with allergic responses, but it also plays a role in the long-term regulation of immune system responses.[6] Histamine is secreted by many innate and adaptive immune cells following decarboxylation of the amino acid L-histidine by the enzyme histidine decarboxylase (HDC).[7] The most common source of histamine among immune cells is mast cells and basophils. They are able to store large quantities of ready-to-use histamine, which is quickly released in response to stimuli. Other cell types such as dendritic cells (DC) and lymphocytes secrete histamine directly after synthesis.[8] In addition to mammalian cells, certain bacterial strains express HDC and secrete histamine.[9] Several studies have been performed to characterize histamine-secreting bacteria in food products, such as cheese, meat, vegetables, dairy products and also during beer and wine fermentation.[10-15] The most extensively examined histamine-releasing bacteria are the strains associated with scombroid food poisoning.[16-18] Recently, we reported the presence of histamine-producing bacteria in the gut microbiota of asthma patients and the levels of these bacteria were increased in asthma patients compared to healthy volunteers. Moreover, increased levels of *M morganii*, which secretes high amounts of histamine, were positively associated with asthma disease severity.[19] However, it is unknown if and how gut bacteria-derived histamine may influence allergic airway inflammation within the lung.

Histamine can regulate immune responses by signalling via one of its four receptors, which determine the type of responses triggered.[20,21] Histamine 1 receptor (H1R) is responsible for the classical immediate hypersensitivity response, whereas H2R typically antagonizes H1R-mediated effects. H2R has been shown to modulate DC responses to microbial ligands, prevent exaggerated invariant natural killer T-cell responses within the lung, can mediate bacterial-derived histamine effects within the gut and dampen the severity of colitis.[22-26] Histamine 3 receptor (H3R) is a neurotransmitter release controlling presynaptic receptor. Histamine 4 receptor (H4R) is involved in cytokine production and chemoattraction of innate immune cells, such as eosinophils, mast cells, basophils and DCs, as well as T cells.[7] Histamine activity is also tightly controlled via its degradation by two enzymes, diamine oxidase (DAO) and histamine-N-methyl transferase (HNMT). DAO, found in the gastrointestinal mucosa as well as the placenta and kidney, is secreted extracellularly, so it may play a role in scavenging extracellular histamine. HNMT is detected in a wider spectrum of tissues and remains in the cytosol; therefore, it is connected with metabolism and inactivation of intracellular histamine.[7,27,28] Thus, any potential systemic effect of histamine derived from bacteria within the gut of asthma patients will be influenced by histamine receptor expression on host immune cells and the ability of the host to metabolize and degrade excessive amounts of histamine.

In the present study, we generated a recombinant bacterial strain, which differs to the parent strain only in its ability to secrete histamine. We investigated the effect of this bacterium on airway inflammatory activity in wild-type, H2R knockout and HDC knockout mice. H2R knockout animals were examined to evaluate the role of this specific histamine receptor in mediating the effects of bacterial-derived histamine within the lung, while HDC knockout animals were evaluated to

determine if pre-exposure to host-derived histamine might influence the subsequent host response to bacterial-derived histamine. Bacterial secretion of histamine within the gut has significant effects on lung inflammatory responses, some of which are dependent on H2R, while other effects are influenced by the host's ability to secrete and degrade histamine.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and HDC cloning

Escherichia coli DH5α (Thermo Fisher Scientific, Waltham, USA) was used for all DNA manipulations.

Escherichia coli BL21 (DE3; Novagen, Darmstadt, Germany) was used for protein expression. All *E coli* strains were cultured in Luria-Bertani (LB) medium (MP Biomedicals, Santa Ana, California, USA) at 37°C and 250 rpm. When required, ampicillin was added to the medium at 100 μg/mL. pET17b (Novagen, Darmstadt, Germany) was used as the expression vector.

Forward (5′GGAATTCCATATGACTCTGTCTATCAATGATCAAAAC-3′) and reverse (5′GCCGCTCGAGTTATGCCGCGTGTAAGTTAAAATC-3′) primers were selected as flanking the HDC gene from *Morganella morganii* using CLC Main Workbench software (Qiagen, Hilden, Germany). Primers were generated by Microsynth AG (Balgach, Switzerland). PCR amplification was performed using 1 μg bacterial DNA, 5 μL 10x reaction buffer (Pfu buffer with $MgSO_4$; Thermo Fisher Scientific, Waltham, USA), 10 mmol/L of each dNTP, 0.5 μmol/L of each primer and 2.5 U of PfuDna Polymerase. Amplification was carried out using a PCR Thermocycler (Mastercycler gradient, Eppendorf, Hamburg, Germany) with the following conditions: denaturation at 95°C for 2 minutes, 30 cycles of 95°C for 30 seconds, 48.8°C for 30 seconds and 72°C for 2 minutes 19 seconds, followed by a final extension at 72°C for 10 minutes. The PCR product was purified using the Nucleo Spin® Gel and PCR clean-up Kit (Macherey Nagel AG, Oensingen, Switzerland). Restriction digestion of insert and pET-17b plasmid with XhoI and NdeI (Thermo Fisher Scientific) was performed at 37°C for 3 hours. Ligation was performed for 1 hour at 22°C. The T4 DNA Ligase was heat inactivated for 10 minutes at 65°C. The plasmid was purified with the NucleoSpin PCR Purification Kit (Macherey Nagel AG, Oensingen, Switzerland) and 1 μL was used for transformation. Plasmid pET-17b and pET-17bHDC were transformed to MAX Efficiency™ DH5α™ competent cells (Thermo Fisher Scientific) using Bio-Rad Gene Pulser™ Herkules, USA. The plasmid DNA was extracted from DH5α cells (GeneJET Plasmid Miniprep Kit, Thermo Scientific, Reinach, Switzerland). After confirming the DNA sequence, the plasmid was transformed to BL21 (DE3) 69450 competent cells (Merck Millipore), specialized in protein production, using a Gene Pulser™ (Bio-Rad, Herkules, USA). *E coli* BL21 transformed with the empty pET-17b plasmid (*E coli* BL21) was used as a control for *E coli* BL21 transformed with the pET-17b_*hdc* plasmid (*E coli* BL21_HTW).

2.2 | Confirmation of histamine secretion by transformed cells

The bacterial strains were cultured with 1% histidine and 0.005% pyridoxal-5-phosphate at 37°C. Bacterial supernatants were mixed with 20 μl of 5 g/l 1,7-diaminoheptane (Internal standard; Sigma-Aldrich, St. Louis, MD, USA), 80 μl of 2 M NaOH, 120 μl of saturated sodium bicarbonate solution and 800 μl of dansyl chloride (Sigma-Aldrich, Buchs, Switzerland) solution (10 mg/ml in acetone), and was then incubated at 40°C, 200 rpm, for 45 minutes. Residual dansyl chloride was removed by adding 40 μl of 25% ammonium hydroxide (Merck, Darmstadt, Germany). After 30 minutes at 25°C, the volume was adjusted to 2 ml with acetonitrile (Biosolve Chimie, Dieuze, France), centrifuged at 3000 *g* for 5 minutes, and supernatants were filtered (0.22 μm) prior to UPLC analysis. Separation was carried out by ultra-performance liquid chromatography (UPLC) on an ACQUITY UPLC H-Class Bio System (Waters Corp, Milford, MA, USA). Based on their different hydrophobicity, the dansylated biogenic amines were separated on an ACQUITY UPLC BEHC18 column (1.7 μm particle size, 2.1 mm × 50 mm, Waters Corp.) and the samples were eluted with a gradient elution of (A) acetonitrile (100%), (B) acetonitrile (50%) as follows: 0-0.72 minutes, A 40%, B 60%; 0.72-1.07 minutes, A 40%-80%, B 60%-20%; 1.07-1.42 minutes, A 80%-90%, B 20%-10%; 1.42-2.11 minutes, A 90%-95%, B 10%-5%; 2.11-2.46 minutes, A 95%-40%, B 5%-60%, 2.46-4.20 minutes, A 40%, B 60%. The flow rate was kept at 0.6 ml/min, column temperature at 25°C, injection of 1 μl, and detection wavelength was 217 nm.

2.3 | Animals

BALB/c female, wild-type mice aged 6-8 weeks were obtained from Charles River (Sulzfeld, Germany) and housed at the AO Research Institute Davos. BALB/c female H2R-deficient mice were obtained from Prof. Takeshi Watanabe (Kyoto University, Japan) and were bred at AO Research Institute Davos. BALB/c female HDC-deficient mice were provided by Department of Immunology (Medical and Health Science Centre, University of Debrecen, Debrecen, Hungary) and bred at the AO Research Institute Davos. Mice were housed at 6 animals per cage in individually ventilated cages in a 12-hour/12-hour light/dark cycle, with food and water available ad libitum. All experimental procedures were carried out in accordance with Swiss law and approved by the animal experiment commission of the canton Grisons, Switzerland. Mice were sensitized by intraperitoneal injection of 20 μg of ovalbumin (OVA) grade VI (Sigma-Aldrich, Buchs, Switzerland) emulsified in 500 μg alum (Pierce, Rockford, IL, USA) in 200 μL sterile 0.9% isotonic sodium chloride (NaCl) (Bichsel AG, Interlaken, Switzerland) on days 0, 7 and 21, followed by 20 minutes 1% OVA grade V (Sigma-Aldrich, St. Louis, MD, USA) aerosol exposures on days 26, 27 and 28. Negative control animals received NaCl and alum injections and were exposed to the OVA aerosolization. Analysis of mice occurred 24 hours after the last aerosol challenge. Between days 14 and 28, mice were daily gavaged with 200 μL NaCl (positive and negative controls), 200 μL BL21 *E coli*+ pET-17b (*E coli* BL21) or 200 μL BL21*E.coli*+ pET-17bHDC (*E coli* BL21_HTW).

2.4 | Bronchoalveolar lavage

Bronchoalveolar lavage was performed using 1 mL phosphate buffer saline (PBS) containing a 1× protease inhibitor cocktail (Roche, Mannheim, Germany). BAL was centrifuged for 2 minutes, 8000 *g*, at room temperature, and the pellet was resuspended in 1 mL PBS with 1% foetal calf serum. The total number of cells was counted using a Neubauer counting chamber. For differential cell counts, cytospin preparations were fixed and stained with Diff-Quick (Merz& Dade AG, Dudingen, Switzerland). Macrophages, lymphocytes, eosinophils and neutrophils were identified by standard morphologic criteria.

2.5 | Lung cell isolation and stimulation

To prepare single-cell suspensions from lung tissue, the lung dissociation kit for mouse and a gentleMACS™ device (Miltenyi, Bergisch Gladbach, Germany) were used according to the manufacturer's protocol. All flow cytometric analyses were performed on the Gallios Flow Cytometer (Beckman Coulter, Brea, CA, USA). Mouse anti-CD3, anti-CD4, anti-CD25, anti-Foxp3, anti-IL-17, anti-IFNγ, anti-IL-4, anti-IL-13, anti-F4/80, anti-CD11c, anti-CD86, anti-PDL1 and anti-PDL2 antibodies were obtained from BioLegend (San Diego, CA, USA). Mouse anti-MHC class II and anti-CD80 were obtained from Becton Dickinson (Franklin Lakes, USA). Cells were also stained with the fixable viability dye eFlour780 (eBioscience, Vienna, Austria). Kaluza software (Beckman Coulter, Brea, CA, USA) was used for data analysis.

Lung-derived single-cell suspensions were plated at a concentration of 1×10^6 cells/mL in complete RPMI (Sigma-Aldrich), were restimulated with 50 μg/mL OVA grade VI (Sigma-Aldrich) and incubated at 37°C and 5% CO_2. Supernatants were frozen at −20°C until the levels of cytokines and chemokines were determined by BioPlex (Bio-Rad, Hercules, USA).

2.6 | Gene expression analysis

Lung tissue was homogenized with the Precellys 24 homogenizer (Bertin Instrument, Montigny-le-Bretonneux, France) for 4000 rpm, 2 times for 90 seconds with 30-second breaks in between, using tissue homogenizing beads (Bertin Instrument, Montigny-le-Bretonneux, France). RNA was isolated using RNeasy Plus Universal Mini Kit (QIAGEN GmbH, Hilden, Germany). Reverse transcription was performed according to the manufacturer's instructions (Thermo Fisher Scientific), and qPCR was run using the Applied Biosystems QuantiStudio 7 Flex Real-Time PCR system (Thermo Fisher Scientific) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. The cycling conditions were as follows: 40 cycles of 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds and 60°C for 1 minute. Table S1 shows the primer sequences used.

2.7 | Statistical analysis

Graphical and statistical analyses were performed using Prism 7 (GraphPad software, San Diego, CA, USA). Data are expressed as mean±SEM and are analysed for significance using Student's *t* test or Mann-Whitney test.

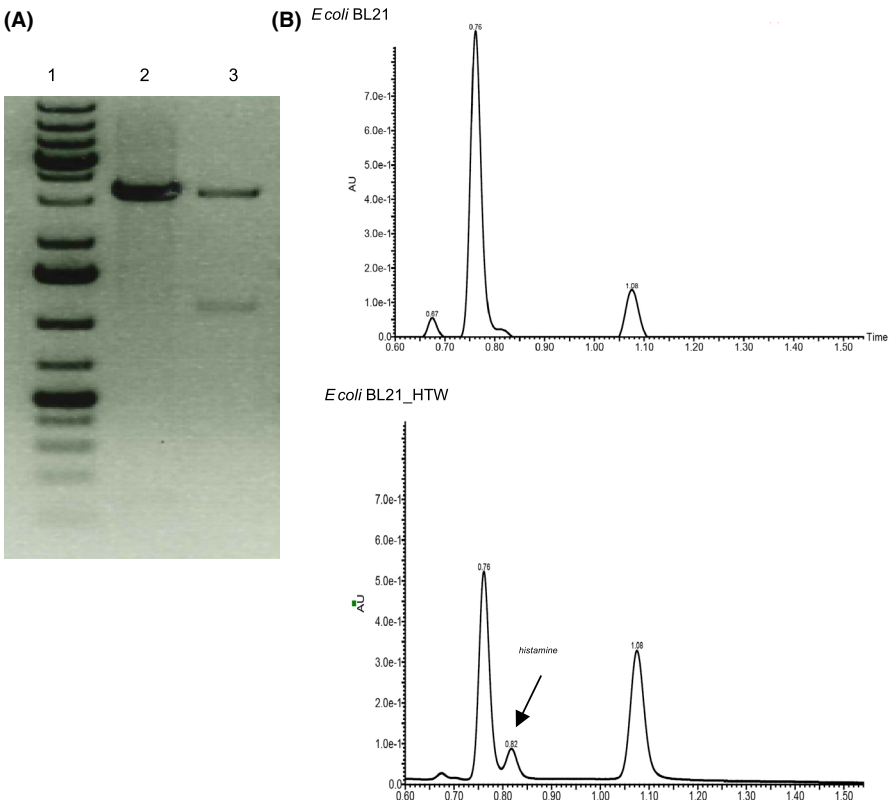


FIGURE 1 Genetically modified *Escherichia coli* secretes histamine. A, Representative gel demonstrating ligation of histidine decarboxylase enzyme (HDC) insert to pET-17b plasmid. A single band was observed after plasmid purification and digestion for pET-17b plasmid (lane 2) and a double band was observed for pET-17b plasmid with HDC insert (lane 3). B, Representative UPLC chromatogram illustrating the detection of bacterial-derived histamine in culture supernatants

3 | RESULTS

3.1 | Generation of an *E coli* strain that secretes histamine

To specifically assess the effect of histamine secretion from gut bacteria on lung immune responses, we inserted the gene for histidine decarboxylase into the non-histamine-secreting *E coli* BL21, in order to generate a recombinant strain that secretes histamine (*E coli* BL21_HTW). The *hdc* gene used for cloning was amplified from the 6 DT *M morganii* (KU612266) bacterial strain, which was originally isolated from an asthma patient[19] (Figure 1A). We confirmed by UPLC that *E coli* BL21_HTW was able to secrete histamine (Figure1B). Insertion of this plasmid encoding the *hdc* gene did not alter the morphology of the cells or its growth rate (Figure S1).

3.2 | Histamine from bacteria in the gut has an anti-inflammatory effect in the OVA respiratory inflammation mouse model

To investigate whether the severity of allergic airway inflammation was influenced by bacterial-derived histamine, we gavaged *E coli* BL21 or *E coli* BL21_HTW bacterial strains daily to OVA sensitized and challenged mice (Figure 2A). We observed a significant decrease in the number of inflammatory cells in BAL of *E coli* BL21_HTW treated animals, compared with positive controls, which was not observed for *E coli* BL21 treated animals (Figure 2B). The differences in BAL cell numbers were primarily driven by decreased macrophages and eosinophils in *E coli* BL21_HTW treated animals (Figure 2C). A small, but statistically significant, increase in BAL macrophages was observed in *E coli* BL21 treated animals, but not in those treated with *E coli* BL21_HTW (Figure 2C).

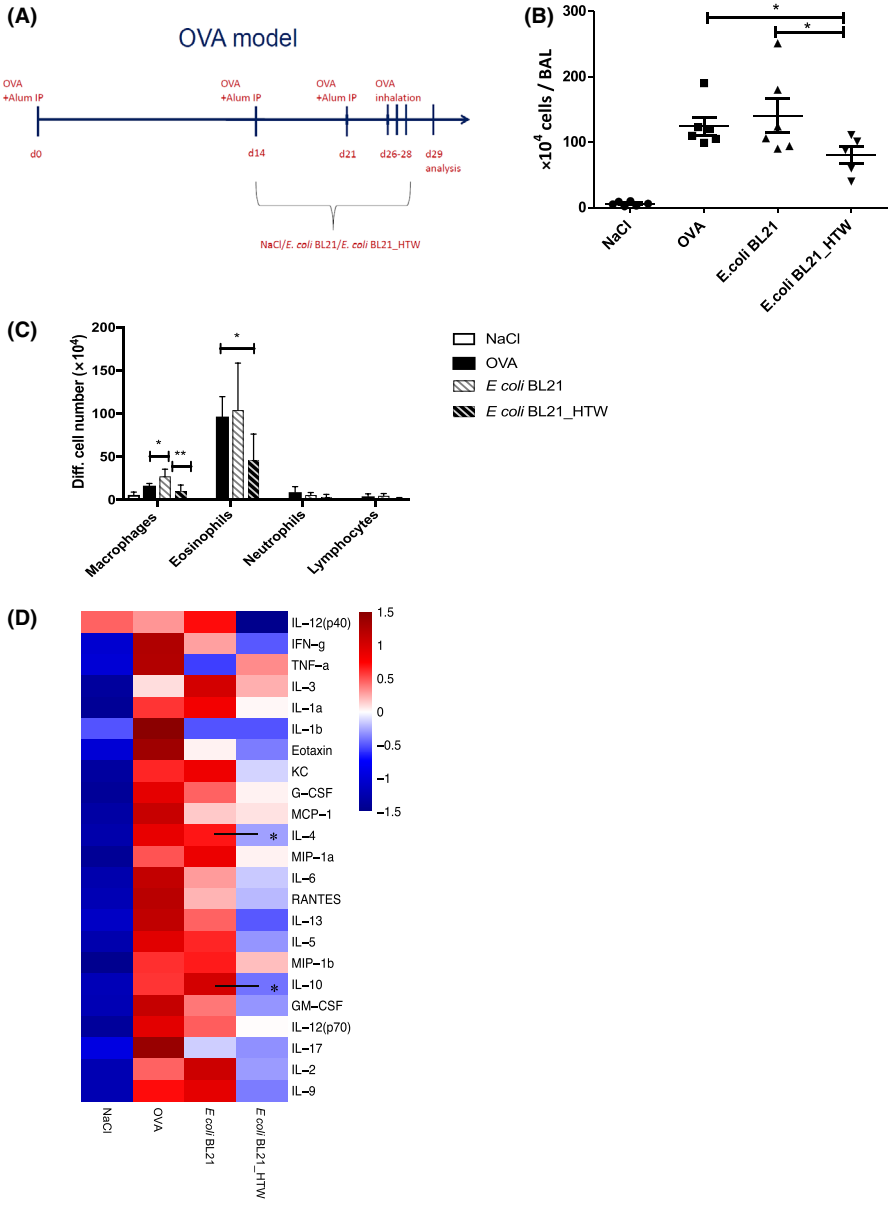


FIGURE 2 Oral application of *Escherichia coli* BL21_HTW to wild-type mice reduced severity of airway inflammation. A, OVA model design. B, Total BAL cell counts in OVA model. C, Differential BAL cell counts in OVA model. D, Heat map of 23 cytokines after OVA restimulation of lung-derived cells. *$P < 0.05$

After ex vivo OVA restimulation of lung single-cell suspensions, Th2 cytokine secretion (interleukin IL-4, IL-5 and IL-13) was reduced for *E coli* BL21_HTW treated animals (Figure 2D). Moreover, lower levels of secretion were observed for IL-1α, IL-2, IL-3, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-17A, eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MIP-1α, MIP-1β, RANTES, suggesting an overall anti-inflammatory effect of gut bacterial-derived histamine that was not restricted to modulation of Th2 cytokine responses. DAO gene expression in lung tissue was increased following induction of allergic airway inflammation, but was not further modulated by administration of either bacterial strain (Figure S2A). HNMT gene expression was not influenced compared to control animals under any of the conditions tested (Figure S2B).

3.3 | Anti-inflammatory effect of bacterial-derived histamine is partially mediated through histamine 2 receptor

One potential mechanism by which bacterial-derived histamine might dampen inflammatory responses in the lung could be mediated by histamine signalling through H2R. To asses this hypothesis, we first measured the expression of histamine receptors in wild-type mice lung tissue. There was no difference observed in relative expression of these receptors between mice gavaged with *E coli* BL21 or *E coli* BL21_HTW (Figure 3).

To further investigate the potential role of H2R, we repeated the experiment described above with H2R-deficient animals. After oral administration of histamine-secreting bacteria to H2R knockout mice, a similar trend was observed as seen in wild-type animals on the number of inflammatory cells in BAL (Figure4A). The differences in BAL cell numbers were primarily driven by decreased eosinophils in *E coli* BL21_HTW treated animals (Figure 4B). After OVA restimulation of lung single-cell suspensions, KC, G-CSF, MCP-1, IL-4, MIP-1α, IL-6, RANTES, IL-13, IL-5, MIP-1β, IL-2 and IL-9 cytokine levels were also reduced in the group gavaged with *E coli* BL21_HTW. However, IL-12 (p40), IFN-γ, TNF-α, IL-3, IL-1α, IL-1β, eotaxin, IL-10, GM-CSF and IL-17 levels were no longer reduced in *E coli* BL21_HTW treated H2R-deficient animals (Figure 4C).

3.4 | Bacterial-derived histamine no longer reduces BAL inflammatory cell numbers in HDC-deficient mice

To further investigate the potential mechanisms of bacterial-derived histamine on lung inflammation, we administrated *E coli* BL21 and *E coli* BL21_HWT bacterial strains to HDC-deficient mice sensitized and challenged with OVA. These mice cannot produce histamine by decarboxylation of histidine, and we hypothesized that bacterial-derived histamine might have a different effect in a histamine-naive immune system. In contrast to the previous experiments, BAL cell numbers were no longer reduced in HDC-deficient animals treated with the histamine-secreting *E coli* BL21_HTW strain (Figure 5A). Indeed, there was a nonstatistically significant trend for increased numbers of eosinophils in *E coli* BL21_HTW treated animals (Figure 5B). In contrast to the BAL inflammatory cell numbers results, cytokine secretion following OVA restimulation of lung single-cell suspensions remained reduced after administration of *E coli* BL21_HTW compared to *E coli* BL21 treated mice (Figure 5C).

3.5 | Differential induction of immune cell activation, histamine receptors and histamine degrading enzymes within the lung of HDC-deficient animals

In order to further investigate the cellular mechanisms underpinning this difference in lung infiltration induced by *E coli* BL21_HTW administration to HDC-deficient mice, we analysed the levels of surface molecules expressed by macrophages and dendritic cells. Flow cytometric analyses of lung CD11c-F4/80+MHCII+ cells revealed that *E coli* BL21_HTW application increased significantly the percentage of CD80- and PDL2-positive macrophages, with a significant decrease in the percentage of PDL1-positive macrophages (Figure 6A). This change in macrophage phenotype was not observed for macrophages from wild-type animals

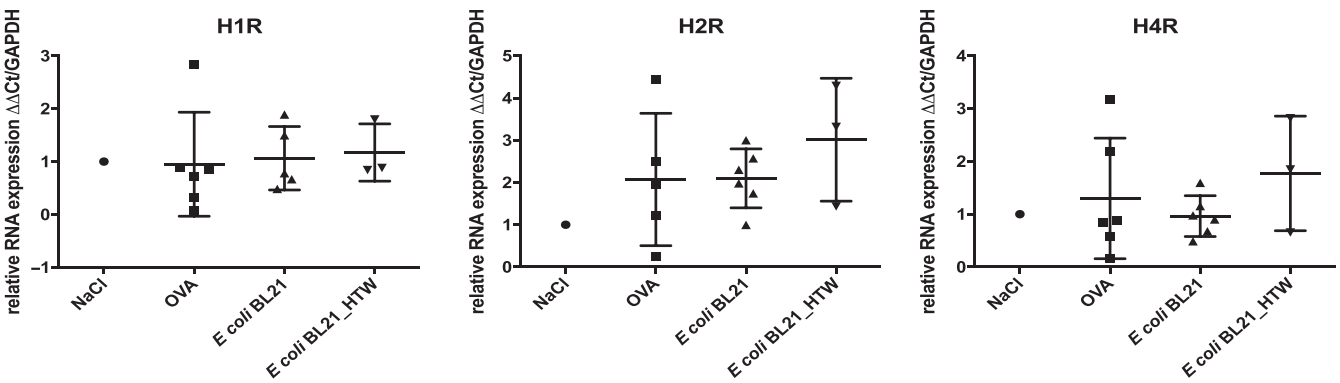


FIGURE 3 Histamine receptor expression in murine lung tissue. ΔΔCt values for H1R, H2R and H4R gene expression in lung tissue from wild-type mice following OVA challenge. The results were analysed using the negative control group (mice injected with NaCl and gavaged with NaCl) as a reference group and GAPDH as housekeeping gene

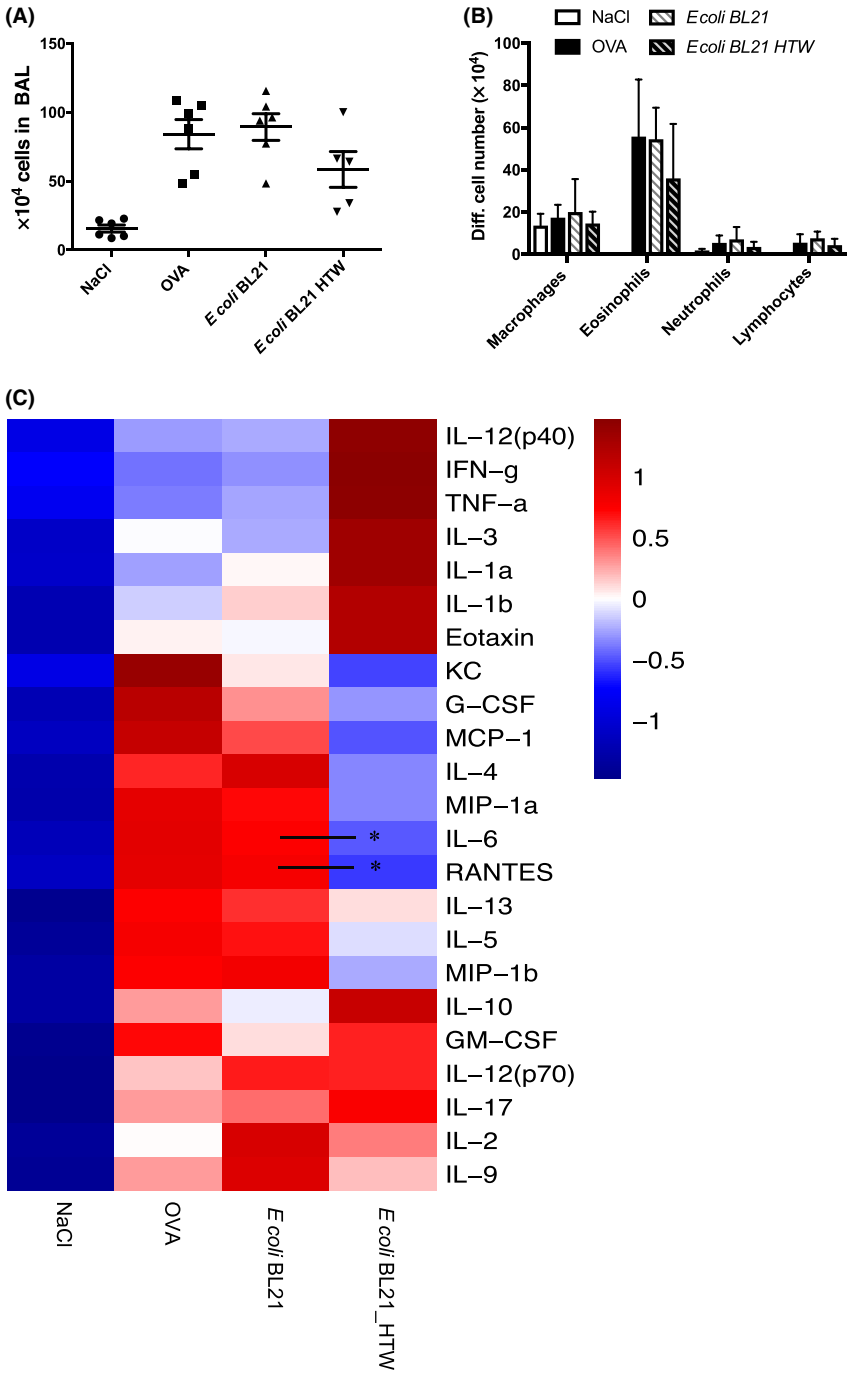


FIGURE 4 Oral administration of *Escherichia coli* BL21_HTW to H2R-deficient mice reduced the severity of airway inflammation. A, Total BAL cell counts in OVA model. B, Differential BAL cell counts in OVA model. C, Heat map of 23 cytokine after lung single-cell restimulation with OVA. *$P < 0.05$

(Figure 6B). In addition to increased macrophage activation, the proportion of lung lymphocytes expressing IFN-γ was increased in the HDC-deficient animals administered *E coli* BL21_HTW (Figure 6C), which was not observed in wild-type animals (Figure 6D). No differences were observed in dendritic cell CD80, PDL1 or PDL2 expression in HDC-deficient animals (Figure S3). Similarly, lymphocyte IL-4, IL-13, IL-17 expression or Foxp3+CD25+ lymphocytes were not influenced by *E coli* BL21_HTW in HDC-deficient animals (Figure S4).

Given these significant differences in responses to bacterial-derived histamine, we hypothesized that differences in histamine metabolism by histamine degrading enzymes or signalling through its receptors may play a role in HDC-deficient animals. We quantified the relative expression of DAO, HNMT, H1R, H2R and H4R in wild-type and HDC-deficient animals. Significant differences in receptor and enzyme expression were observed. DAO and HNMT were expressed at a significantly higher level in the lungs of HDC-deficient animals compared to wild-type mice (Figure 6E), suggesting an increased level of histamine metabolism and degradation. In addition, there was a significant trend for increased enzyme expression within small intestinal tissue (Figure S5). In contrast, all histamine receptors were expressed at a significantly lower level in HDC-deficient mice compared to wild-type animals (Figure 6F).

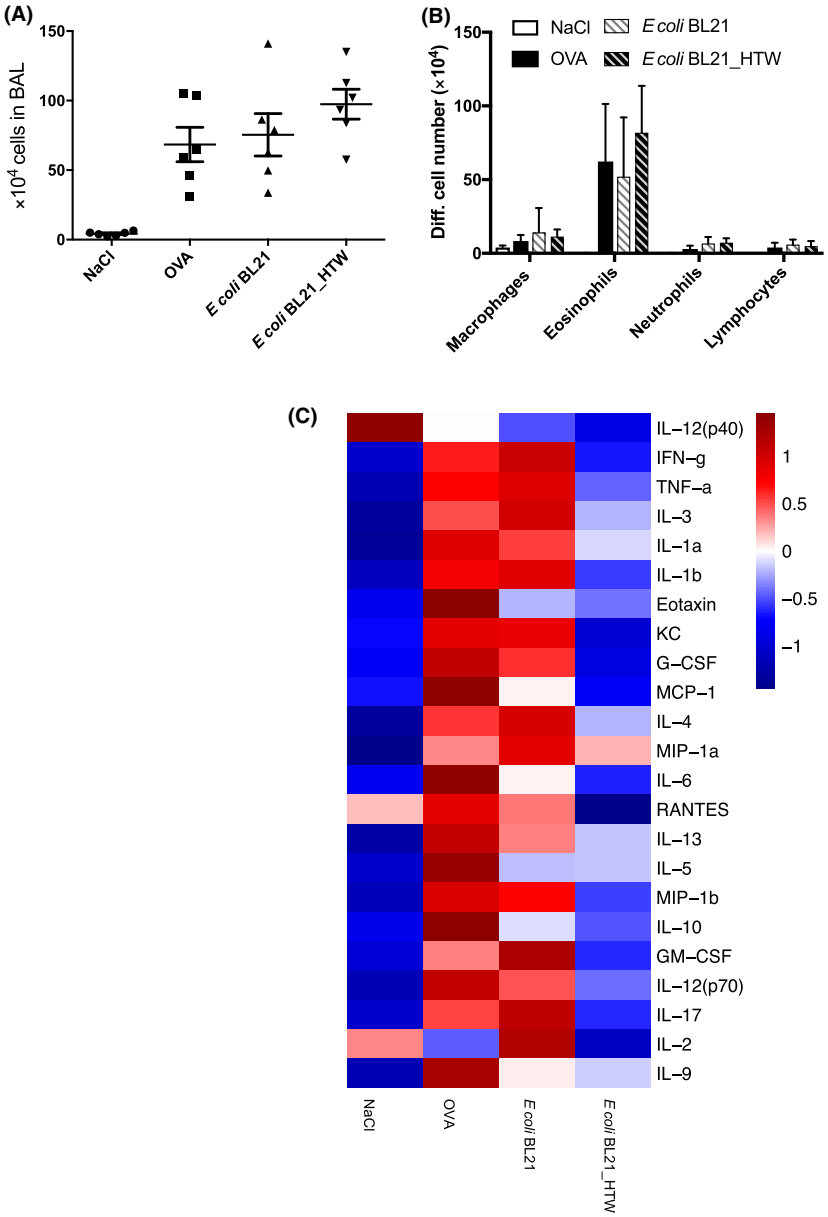


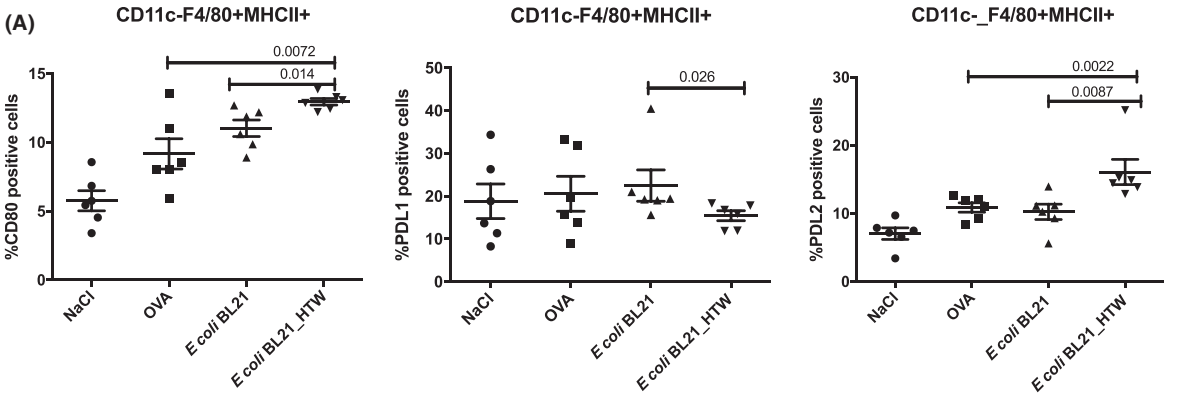
FIGURE 5 Oral administration of *E coli* BL21_HTW to HDC-deficient mice. A, Total BAL cell counts in OVA model. B, Differential BAL cell counts in OVA model. C, Heat map of 23 cytokines secreted by lung-derived cells following OVA restimulation ex vivo

4 | DISCUSSION

This study describes a novel mechanism by which bacteria in the gut can influence inflammatory responses within the airways. In addition, it supports the concept that there is significant immunological cross-talk between the gut and the lung. Indeed, it is already known that patients with asthma,[29,30] COPD[31] or patients with respiratory infections can display intestinal symptoms.[32,33] One potential mechanism associated with this gut-lung axis might be influenced by the gut microbiota.[34-37]

It is well known that host-derived histamine influences allergy and asthma symptoms.[38] In addition, there are many studies investigating bacterial secretion of histamine in foods.[10,11,39-41] We have previously shown that bacteria capable of secreting histamine[19] and other biogenic amines can be found in the human gut.[42] However, the potential influence of bacterial-derived histamine on immune responses in the respiratory tract has not been previously examined. Interestingly, we have observed a significant decrease in the total number of cells in BAL of wild-type mice following oral gavage with

FIGURE 6 Cell phenotypes, histamine degrading enzymes and histamine receptor expression in the lung. Quantification of CD80, PDL1, PDL2 expression by lung CD11c-F4/80+MHCII+ cells in HDC-deficient (A) and wild-type animals (B). Quantification of lymphocyte (CD3+CD4+) intracellular IFN-γ staining in HDC-deficient (C) and wild-type animals (D). E, DAO and HNMT relative gene expression within the lungs of wild-type and HDC-deficient mice. F, H1R, H2R and H4R relative gene expression within the lungs of wild-type and HDC-deficient mice. $*P < 0.05$; $**P < 0.01$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%CD80 positive cells
0.0072
0.014
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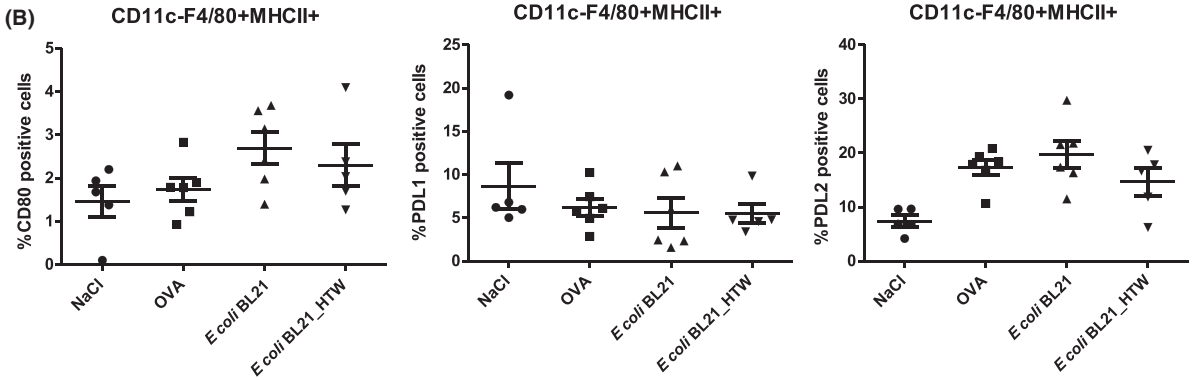
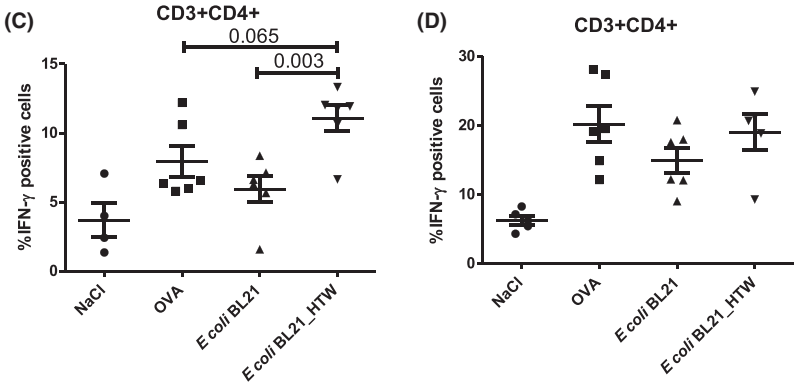
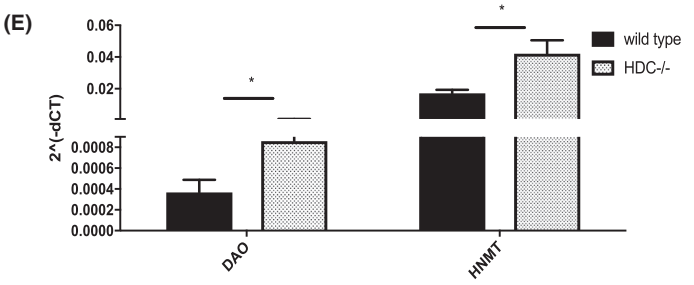
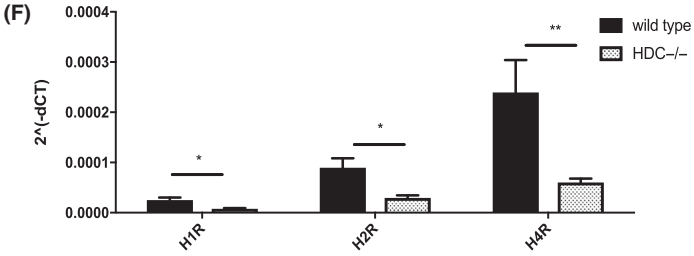
0.026
CD11c-_F4/80+MHCII+
%PDL2 positive cells
0.0022
0.0087
NaCl
OVA
E coli BL21
E coli BL21_HTW


(B)
CD11c-F4/80+MHCII+
%CD80 positive cells
CD11c-F4/80+MHCII+
%PDL1 positive cells
CD11c-F4/80+MHCII+
%PDL2 positive cells
NaCl
OVA
E coli BL21
E coli BL21_HTW


(C)
CD3+CD4+
%IFN-γ positive cells
0.065
0.003
(D)
CD3+CD4+
%IFN-γ positive cells
NaCl
OVA
E coli BL21
E coli BL21_HTW


(E)
2^(-dCT)
wild type
HDC-/-
DAO
HNMT


(F)
2^(-dCT)
wild type
HDC−/−
H1R
H2R
H4R

a histamine-secreting bacterium, while the parent bacterium, which does not secrete histamine, had no effect. Moreover, we observed a decrease in allergen-stimulated cytokine secretion. However, while we show an effect of gut bacterial-derived histamine on lung inflammatory responses, we do not know if bacterial-derived histamine also influences lung hyper-responsiveness.

Histamine stimulates immunomodulatory effects via the activation of its four receptors.[8] H2R is associated with anti-inflammatory effects as it promotes IL-10 production and enhances the suppressive effect of TGF-β.[43,44] We did not observe any differences in histamine receptor gene expression between mice treated with *E coli* BL21 or *E coli* BL21_HTW. In addition, the decrease in BAL cells was maintained in H2R-deficient animals, suggesting that H2R is not required for this effect. However, cytokine secretion was different to that observed for *E coli* BL21_HTW-treated wild-type mice, suggesting that H2R is involved in mediating modulation of Th1- and Th17-associated cytokines. Bacterial-derived histamine immunoregulatory effects were previously shown to be H2R-dependent when examining immune responses within the gut.[24,25] Our data suggest that the immunoregulatory effects of gut bacterial-derived histamine on distant organs such as the lung may involve H2R-dependent and independent mechanisms. In addition, these data suggest that inflammatory cell recruitment to the lung and ex vivo allergen-stimulated cytokine responses are independently regulated.

We observed that the protective effect of *E coli* BL21_HTW seen in wild-type and H2R-deficient mice was abrogated in HDC knockout mice. Moreover, increased numbers of lung macrophages expressed the costimulatory molecule CD80 and increased numbers of lymphocytes expressed IFN-γ in the *E coli* BL21_HTW treated HDC-deficient mice, which indicates their enhanced ability to stimulate the adaptive immune system while presenting antigen. Given that the immunomodulatory effects associated with histamine are known to be dose dependent,[22] one possible explanation for the lack of protection in the HDC-deficient mice is that the total amount of histamine present within the gut is not sufficient to induce anti-inflammatory effects, even in the presence of histamine-secreting bacteria. In addition, DAO and HNMT lung gene expression were increased, while histamine receptors gene expression was decreased in HDC-deficient animals. Thus, future experiments are required to determine if the differential response to histamine-secreting bacteria in these animals could be related to pre-existing levels of histamine, enhanced histamine metabolism or reduced histamine signalling through histamine receptors. It is also currently unclear exactly how histamine in the gut could influence immune reactivity in the lung. Our current hypothesis is that histamine alters immune cells in the gut, which communicate with and travel to the lung. However, future studies are required to fully elucidate the cellular and molecular mechanisms mediating communication between the gut and the lung. In addition, future studies will need to examine histamine secretion in vivo as in vitro secretion levels may not reflect the in vivo situation. Furthermore, bacterial-derived histamine may indirectly influence respiratory inflammation by modulating the composition of the gut microbiota and this will also require further study.

In conclusion, this study suggests that the role of histamine in modulating immune responses in the lung needs to be re-evaluated. In addition, it also suggests that we cannot assume that increased numbers of histamine-secreting microbes within the gut would automatically lead to detrimental effects on the host. Host histamine receptor expression and histamine metabolism may be critical in determining the end responses to bacterial-derived histamine. Furthermore, our data provide strong support for the hypothesis that the gut microbiota influences inflammatory responses in the lung. Future diagnosis and treatment of asthma patients may be assisted by analysis of the composition and metabolic activity of an individual's microbiome.

CONFLICT OF INTEREST

LOM is a consultant to Alimentary Health Ltd and has received research funding from GlaxoSmithKline. CA has received research support from Allergopharma, Actellion, SNF and CK-CARE. The other authors have no relevant conflicts of interest.

AUTHOR CONTRIBUTIONS

LOM, WB, MS, CAl and CAk designed the studies and interpreted the data; WB, BP, MSB, PW, AR, DG, DvE, KK, RFr, RFe and MW performed the laboratory procedures. All authors contributed to the preparation and review of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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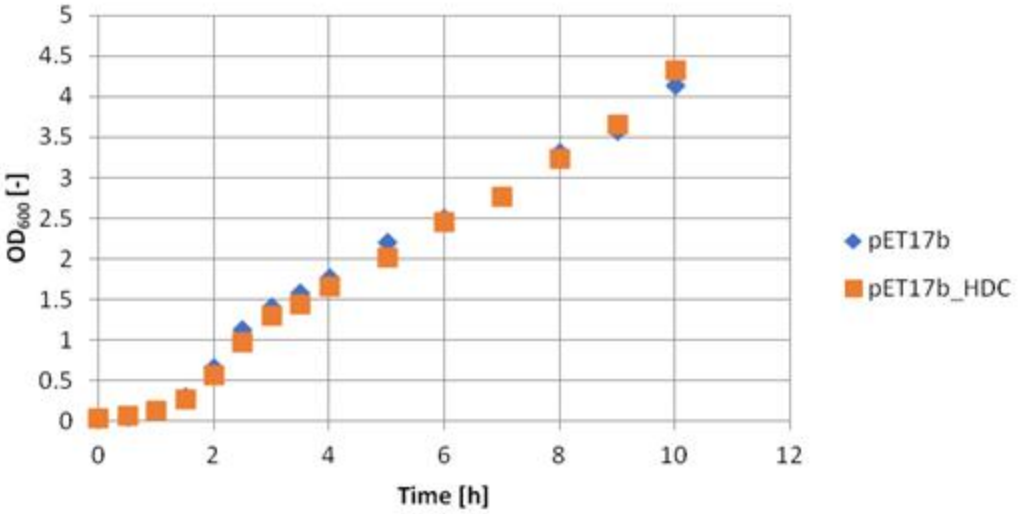
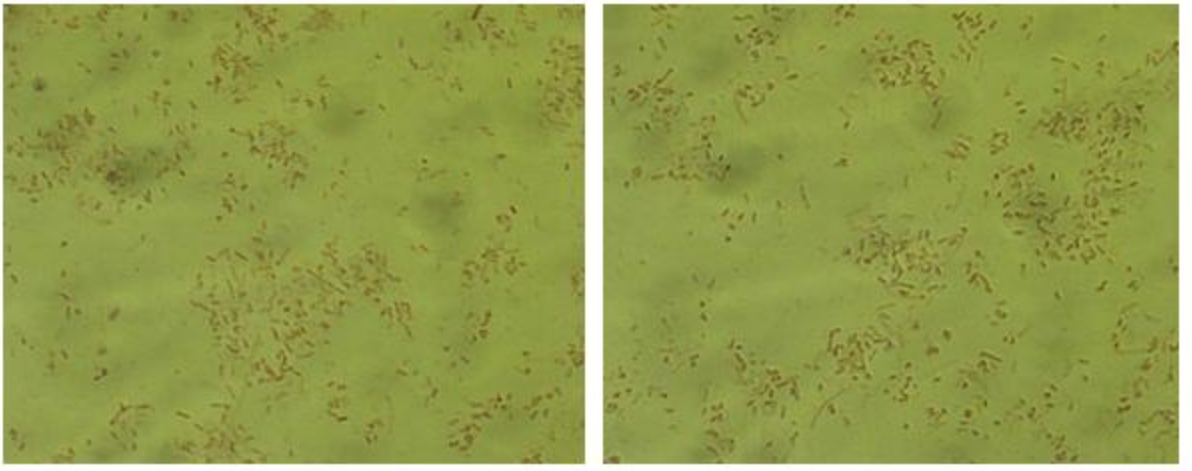
Supplementary Figure S1

A

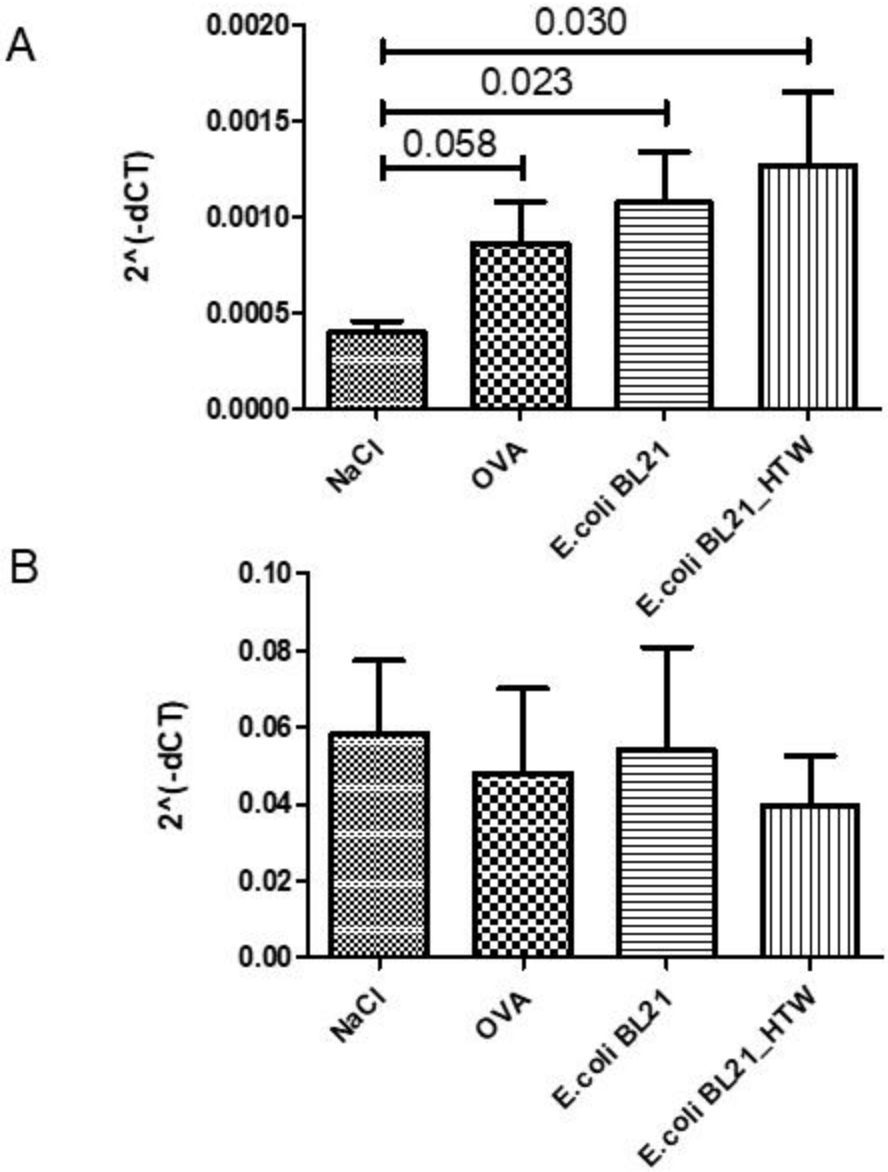


B

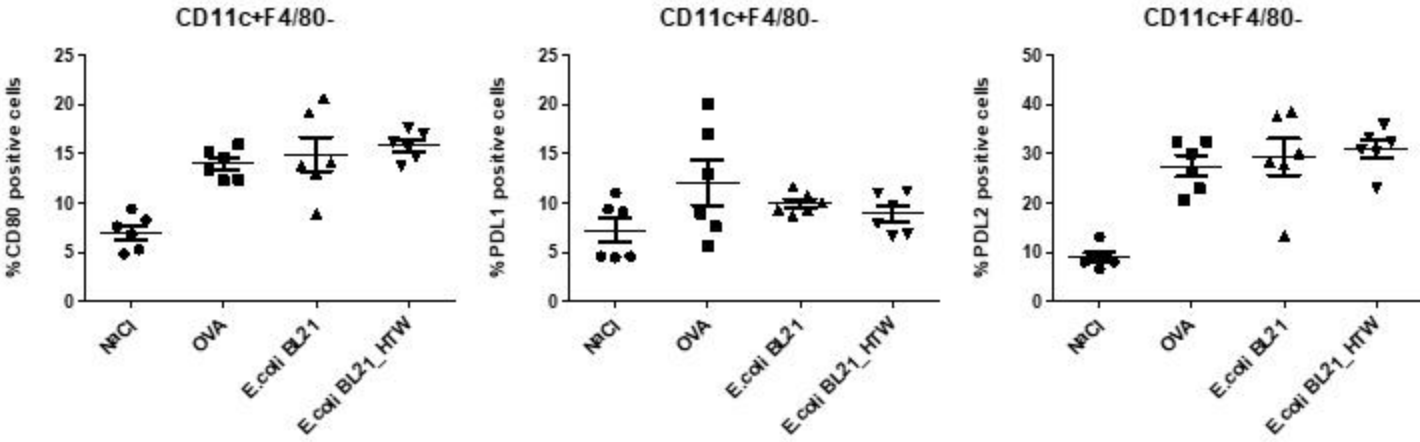


E.coli BL21 *E.coli* BL21_HTW

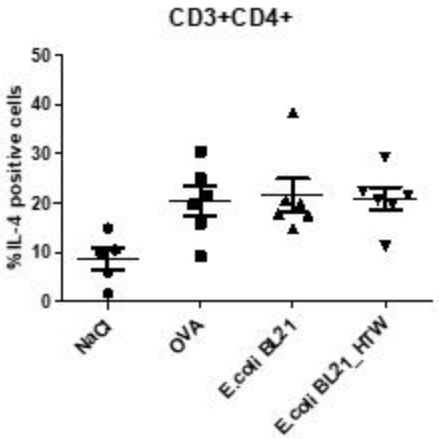
Supplementary Figure S2

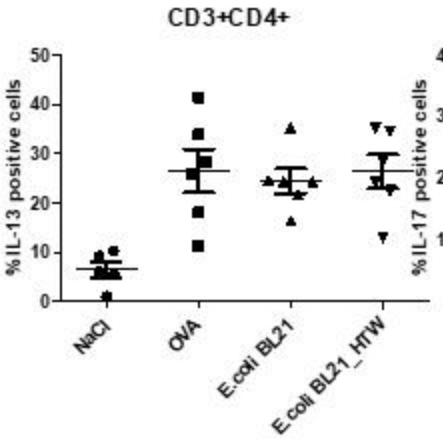


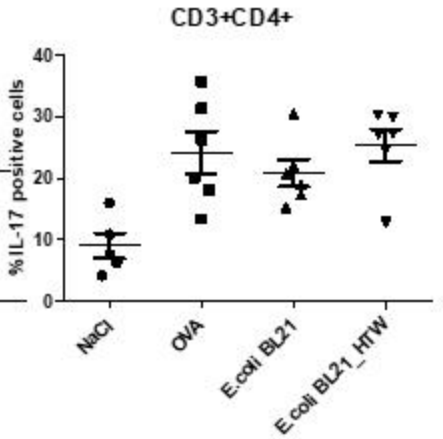
Supplementary Figure S3

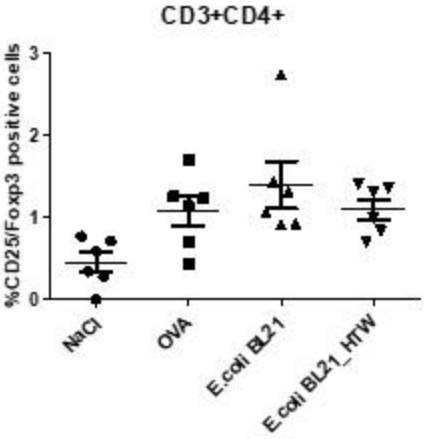


Supplementary Figure S4

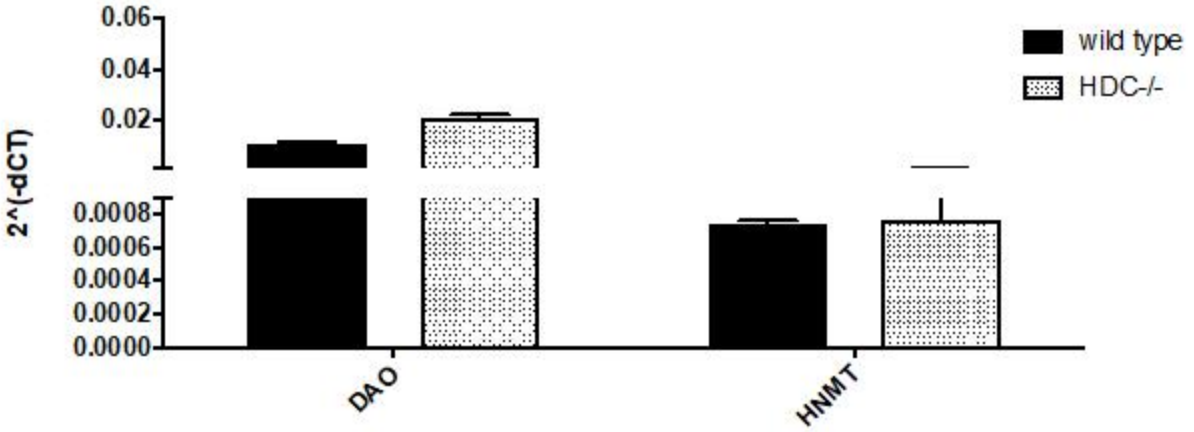








Supplementary Figure S5



Supplementary Table 1. Primer sequences used for quantitative real-time PCR analysis.

Primer set	Sense primer	Antisense primer
H1R_fw H1R_rev	5`-GTA TGT GTC GGG GTT GCA CTT	5`AAC CGA GAG CGG AAG GCA GCC A
H2R_fw H2R_rev	5`TGT TCG CGT TGC CAT CTC T	5`CTC TGG TCC CAT TTC TGC TGT T
H4R_fw H4R_rev	5`TGC CTT CAA AGT GGG TTC CT	5`GCT CTG CGT ACT CCC TTT GG
DAO_fw DAO_rev	5`GGT GGC AAG AGG AGT GGA TG	5`TGG GAT GAT GTA CGG AGA GTT G
HNMT_fw HNMT_rev	5`TCC GGG CAT AAT AGC AAG GAT	5`TTC AGC ACT TGG CTC AAC AAC
HDC_fw HDC_rev	5`GCA GGA TAG TAG GCG TGC AT	5`ACA GCT GGG ACA GCA TCT TT
GAPDH_fw GAPDH_rev	5`TGC ACC ACC AAC TGC TTA G	5`GGA TGC AGG GAT GAT GTT C

Figure S1. E. coli growth rate and morphology. (A) Growth rate measured by optical density for *E. coli* BL21 transformed with an empty plasmid (pET17b) or a plasmid containing *hdc* (pET17b_HDC). (B) Light microscopy showing the morphology of *E. coli* containing the empty plasmid or a plasmid containing *hdc*.

Figure S2. DAO (A) and HNMT (B) gene expression was quantified in lung tissue.

Figure S3. Flow cytometric quantification of lung-derived dendritic cell surface molecule expression.

Figure S4. Flow cytometric quantification of lung-derived lymphocyte subsets.

Figure S5. DAO and HNMT gene expression with gut tissue.