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Experimental Models of Allergic Disease

Human β -defensin-2 suppresses key features of asthma in murine models of allergic airways disease

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Running Title: hBD2 as a novel asthma treatment

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Abstract (Word count 201, Max 300)

Background: Asthma is an airway inflammatory disease and a major health problem worldwide. Anti-inflammatory steroids and bronchodilators are the gold-standard therapy for asthma. However, they do not prevent the development of the disease and critically, a subset of asthmatics are resistant to steroid therapy.

Objective: To elucidate the therapeutic potential of human β -defensins (hBD), such as hBD2 mild to moderate and severe asthma.

Methods: We investigated the role of hBD2 in a steroid sensitive, house dust mite-induced allergic airways disease (AAD) model; and a steroid insensitive model combining ovalbumin-induced, AAD with *C. muridarum* (Cmu) respiratory infection.

Results: In both models we demonstrated that therapeutic intranasal application of hBD2 significantly reduced the influx of inflammatory cells into the bronchoalveolar lavage fluid. Furthermore, key type-2 asthma-related cytokines IL-9 and IL-13, as well as additional immunomodulating cytokines were significantly decreased after administration of hBD2 in the steroid sensitive model. The suppression of inflammation was associated with improvements in airway physiology and treatment also suppressed airway hyper-responsiveness (AHR) in terms of airway resistance and compliance to methacholine challenge.

Conclusions and Clinical relevance: These data indicate that hBD2 reduces the hallmark features and has potential as a new therapeutic agent in allergic and especially steroid-resistant asthma.

Keywords: Asthma, steroid sensitive, steroid resistant, human β -defensin-2

Abbreviations

AAD: Allergic airways disease

AHR: Airways hyperresponsiveness

AMP: Anti-microbial peptide

BALF: Bronchoalveolar lavage fluid

CD: Crohn's disease

CFA: Complete Freuds Adjuvant

Cmu: *Chlamydia muridarum*

DEX: Dexamethasone
H&E: Haemotoxylin and eosin
hBD: human beta-defensin
HDM: House dust mite
IBD: Inflammatory bowel disease
IFN: Interferon
IL: Interleukin
IN: Intranasal
KC: Keratinocyte chemoattractant
MCh: Methacholine
Ova: Ovalbumin
PAS: Periodic acid-schiff
RI: Airways
SPG: Sucrose phosphate glutamate buffer
T_H: T-helper
TNF: Tumour necrosis factor
UC: Ulcerative collitis

1 | INTRODUCTION

Asthma is an inflammatory disorder of the airways and a major public health problem affecting more than 300 million people worldwide.¹ It has considerably increased in prevalence over the past three decades, particularly in the Western World. Asthma is characterised by chronic inflammation, airway hyperresponsiveness (AHR) and by symptoms of recurrent wheezing, coughing and shortness of breath.^{1,2} It has a range of pathological features mediated by diverse cytokines and/or by different infiltrating and structural cell types. In asthmatic airway tissues CD4⁺ T cells generate an increased type-2 cytokine profile consisting of interleukin (IL)-4, IL-5 and IL-13.²⁻⁴

However, asthma is a heterogeneous disease with up to 25% of patients exhibiting more severe features of disease. These asthmatics are more commonly associated with aberrant type-1 and potentially type-17 immune responses and most importantly, corticosteroid therapies are ineffective.²⁻⁷ This group of steroid-refractory asthma patients takes up 50-80% of all asthma health care costs and represents the biggest issue in asthma management today.⁷

The respiratory and gastrointestinal tracts share common evolutionary developmental pathways, both emerging out of the endoderm, and there is now substantial evidence of immune and inflammatory crosstalk between the gut and the lung.⁸⁻¹¹ Indeed, the two major forms of inflammatory bowel disease (IBD): ulcerative colitis (UC) and Crohn's disease (CD) significantly increase the risk of developing asthma and *vice versa*.¹²

For decades it was thought that IBD that is characterised by chronic inflammation of the intestinal mucosa was a classical autoimmune disorder.¹³ However, the key focus in the understanding of the pathogenesis of IBD has changed from adaptive to innate immunity mediated by intestinal antimicrobial peptides (AMPs) and innate immune cells.¹⁴ Evidence also supports the paradigm shift in understanding of IBD to be a complex barrier defect disease that includes deficient antimicrobial mucosal barrier protection, which is caused by a variety of different mechanisms.¹⁵ This enables commensal bacteria to penetrate the barrier and drive mucosal and systemic inflammation.

Endogenous anti-microbial protection is mostly provided by AMPs, such as defensins. Human defensins are small cationic peptides that are divided into α - and β -

defensins based on the topology of their three intramolecular cysteine disulfide bonds.¹⁶ β -defensins are mainly produced by epithelial cells in various tissues and organs including the skin, airways, gastrointestinal tract, urogenital system, kidneys, liver and pancreas.¹⁷ They display chemotactic activity towards immature dendritic cells and T-cells thereby linking innate and adaptive immunity.¹⁸ The best characterised members of the human β -defensin (hBD) family are hBD1-4. hBD1 is produced constitutively, whereas the others are induced by pro-inflammatory cytokines or exogenous microbial products.¹⁹ Defensins serve to maintain a healthy microbiome allowing commensal bacteria to colonise but protect against potential pathogens.^{20,21} They have endogenous antimicrobial activity against Gram-positive and -negative bacteria, fungi, viruses and archae, as well as important immune modulatory activity.²²

Recent studies show that β -defensins are associated with asthma in different ways. hBD1 gene polymorphisms are positively associated with asthma indicating a primary role in the disease process.^{23,24} Type-2 cytokines of the adaptive immune response are upregulated in asthma and suppress hBD2 mRNA levels in allergic airway inflammation.²⁵ In line with this observation, recent clinical evidence showed that the plasma concentration of hBD2 in asthmatic patients is reduced, which may suggest a protective role in the disease.²⁶

hBD2 is a prevalent β -defensin in the neonatal lung and its expression is developmentally modulated.²⁷ It can be induced by common pro-inflammatory innate cytokines such as IL-1 β , tumor necrosis factor (TNF)- α and interferon (IFN)- γ .^{28,29} In addition, the bacteria *Escherichia coli* Nissle 1917, probiotic *E. coli* strains, lactobacilli, rhinovirus and cholecalciferol (1,25-dihydroxyvitamin D3) promote hBD2 mRNA expression.³⁰⁻³⁴

In line with the protective role of this molecule as an integral part of mucosal defenses, we hypothesised that hBD2 might provide therapeutic benefit for inflammatory disorders, in particular those associated with mucosal barriers defects such as asthma. Here, we provide proof of concept of this potential by demonstrating that intranasal administration of hBD2 effectively suppresses hallmark features of disease in both steroid sensitive and steroid resistant experimental models of asthma. hBD2 significantly reduced the numbers of neutrophils in the bronchoalveolar lavage fluid (BALF) in steroid

sensitive, as well as steroid insensitive models of asthma. Key asthma cytokines and the severity of inflammation were reduced after intranasal administration of hBD2 in the steroid sensitive model. These effects were associated with treatment-induced reduction in the hallmark clinical features of both steroid sensitive and resistant AHR. These data indicate that hBD2 may be a promising new therapeutic agent in severe or refractory asthma by reducing disease mechanisms through immunomodulation.

2 | METHODS

2.1 | Mice

For the house dust mite (HDM)-induced, steroid-sensitive allergic airway disease (AAD) model, female BALB/c mice were purchased from Charles River (Italy). Experiments were conducted at Fidelta Ltd, Croatia, in accordance with the Tübingen Institutional Committee on Animal Research Ethics (CARE-Zg) 2010/63/EU and the National legislation regulating the use of laboratory animals in scientific research (Official Gazette 55/13). For the Ovalbumin (Ova)-induced, steroid-sensitive and steroid resistant AAD model, female BALB/c mice were purchased from the Australian Resource Centre, Perth, or the Central Animal House, University of Newcastle. Experiments were conducted in accordance with approval from the University of Newcastle Animal Ethics Committee. All mice were provided with food and water *ad libitum* and used for experiments between 7-8 weeks of age.

2.2 | Steroid sensitive AAD

On day 0 mice were sensitised subcutaneously to 100µg HDM (lot no 305469, Greer) in sterile saline/complete Freuds adjuvant (CFA, 200µl, 1:1); or were sham-sensitised without HDM. All mice were subsequently challenged on day 14 with intranasal (IN) administration of HDM (25µg, 50µl PBS) under light anaesthesia (1ml Narkamon, 0.5ml Rompun in 9ml saline). On day 14, mice received intranasal (IN) treatment with low dose at 1.2mg/kg (Novozymes, 0.4mg/kg, 3 times daily, 50µl PBS) or high dose at 5mg/kg (1.7mg/kg, 3 times daily) hBD2 or were sham-treated with PBS. Treatment occurred 1 hour prior to the challenge followed by two additional administrations at ~6-hour intervals

between doses. Features of AAD were then assessed 48 hours following HDM challenge (Figure 1A).

We also used Ova-sensitised and challenged groups without infection from our model of steroid resistant AAD (below). On day 0 mice were sensitised with ovalbumin (Ova; 50µg, intraperitoneal [i.p.] injection, Sigma-Aldrich, Sydney, Australia) in Rehydragel® (1mg, Reheis, Berkeley Heights, New Jersey, USA) in sterile saline (200µL).³⁵⁻³⁹ Non-allergic controls were sham-sensitised without Ova. All mice were subsequently challenged intranasally with Ova (10µg/50µL sterile saline) under isoflurane anesthesia on days 12-13 to induce AAD and again on days 33-34 to recapitulate AAD. On day 14 mice with established AAD were sham-inoculated with SPG (30µL). Mice in the treatment groups were administered dexamethasone (DEX, 2mg/kg, 50µL PBS) and/or high dose hBD2 (5mg/kg) once daily IN on days 32-34. Sham-treated controls received PBS. Features of AAD were assessed on day 35 of the study protocol (Figure 1B).³⁷⁻³⁹

2.3 | Steroid-resistant AAD

On day 0 mice were sensitised with Ova (50µg, i.p. injection, Sigma-Aldrich, Sydney, Australia) in Rehydragel® (1mg, Reheis, Berkeley Heights, New Jersey, USA) in sterile saline (200µL).³⁵⁻³⁹ Non-allergic controls were sham-sensitised without Ova. All mice were subsequently challenged intranasally with Ova (10µg/50µL sterile saline) under isoflurane anesthesia on days 12-13 to induce AAD and again on days 33-34 to recapitulate AAD. On day 14 mice with established AAD were inoculated IN under isoflurane anesthesia with the natural mouse respiratory pathogen *Chlamydia muridarum* (Cmu;100 IFU, ATCC VR-123, in 30µL sucrose phosphate glutamate buffer, SPG). Controls were sham-inoculated infected with SPG (30µL). Mice in the treatment groups were administered DEX (2 mg/kg, 50µL PBS) and/or high dose hBD2 (5mg/kg) once daily IN on days 32-34. Sham-treated controls received PBS. Features of AAD were assessed on day 35 (Figure 1B).³⁷⁻³⁹

2.4 | BALF inflammation

In HDM-induced steroid-sensitive AAD, airway inflammation was assessed in BALF. BALF samples were collected by three lung lavages totaling 1ml (0.4, 0.3 and 0.3ml, respectively) with PBS (4°C) *via* a cannula inserted into the trachea. BALF was centrifuged (5 mins, 1, 372xg, 4°C) and cell pellets resuspended in 600µl PBS. Total and differential cell numbers in BALF were determined on an automated hematological analyzer Sysmex XT-2000iV using morphological criteria.

In Ova-induced steroid-sensitive and steroid-resistant AAD, airway inflammation was assessed in cytospin preparations of cells from BALF. BALF samples were collected by two 1mL lung lavages with Hank's Balanced Salt Solution (Life Technologies, Australia). BALF was centrifuged (10 mins, 300xg, 4°C), red blood cells were lysed with lysis buffer (200µL, Tris-buffered NH₄Cl) and remaining cells were pelleted before total leukocyte numbers were determined using a haemocytometer. Cells were cytocentrifuged and stained with May-Grunwald-Giemsa. Differential leukocyte counts were enumerated (≈175 cells by light microscopy, x40 magnification).⁴⁰⁻⁴²

All samples were coded, and counts were performed in a blinded fashion.

2.5 | Whole lung and BALF cytokine levels

In HDM-induced steroid-sensitive AAD, lungs were removed, snap frozen in liquid nitrogen and stored at -80°C. Lungs were thawed and placed in PBS (1:5) with a protease inhibitor cocktail (Complete™, Mini Protease Inhibitor Cocktail, Roche) according to the manufacturer's specifications. Samples were centrifuged (10 mins, 16128xg, 4°C) and supernatants collected. Samples were immediately frozen at -80°C until analysis. Whole lung TNF-α, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IFN-γ and keratinocyte chemoattractant (KC) levels were measured by ELISA using mouse DuoSet® ELISA Development Systems (R&D Systems, MN, USA) as per the manufacturer's instructions.^{35,38,43} Optical absorbances were read on a TECAN INFINITE F500 microplate reader (450nm) and concentrations were calculated from optical densities and normalised to total lung protein.

In Ova-induced steroid-sensitive and steroid-resistant AAD, BALF supernatants were utilised to assess pro-inflammatory cytokines. KC, TNF-α and IFN-γ levels were measured by ELISA using mouse DuoSet® ELISA Development Systems (R&D Systems,

MN, USA) as per the manufacturer's instructions. Optical absorbances were read on a SpectraMax® M5 plate reader (450nm, Molecular Devices, CA, USA) and concentrations were calculated from optical densities and normalised to total lung protein.^{36,41,42}

2.6 | Histology

In HDM-induced steroid-sensitive AAD, lungs were inflated *in situ* with 10% buffered formalin, removed from the thorax, placed in 10% buffered formalin, paraffin embedded *in toto*, sectioned and stained with haematoxylin and eosin (H&E) or period acid-Schiff (PAS). Severity of the inflammation was evaluated in H&E-stained slides. To assess overall severity and distribution of inflammation a semi-quantitative scoring system was employed (Table 1). Six areas covering whole lungs were evaluated and individual scores were generated as median.^{41,44}

2.7 | AHR

In HDM-induced steroid-sensitive AAD, mice were anaesthetised with ketamine (100mg/ml, Narkamon, Bioveta, Czech Republic) and xylazine (2%, Rompun, Leverkusen, Germany) and cannulated (tracheostomy with ligation). A Buxco® FinePointe™ RC system (DSI, USA) was used to assess measurements of airway resistance (RI) to increasing doses of methacholine (MCh). Automated data acquisition software (Finepointe™, DSI) continuously recorded various parameters of lung function and automatically generated airway resistance (RI) and cadence (Cdyn) values, as well as statistical analysis.^{40,43,45} Data were calculated at each dose and values are represented as a curve for each group.

In Ova-induced steroid-sensitive and steroid-resistant AAD, mice were anaesthetized with ketamine (100mg/kg) and xylazine (10mg/kg, Troy Laboratories, Smithfield, Australia) and their tracheas were cannulated (tracheostomy with ligation). FlexiVent™ FX 1 apparatus (SCIREQ, Montreal, Canada) was used to assess airway-specific resistance (Rn; tidal volume of 8mL/kg at a respiratory rate of 450 breaths/min) in response to increasing doses of nebulized MCh (0-10mg/ml semi-log doses; Sigma-Aldrich, Sydney, Australia).^{36,41,42}

Assessments were performed at least three times per dose of saline/MCh and the average calculated.

2.8 | Data analysis and statistical evaluation

Comparisons between multiple groups used a one-way analysis of variance and an appropriate post-test or a nonparametric equivalent where appropriate. Lung function data were assessed using a two-way analysis of variance and an appropriate post-test or a nonparametric equivalent using GraphPad Prism software. Exclusion of outliers used Grubbs' test. Differences between groups were considered as statistically significant if $p < 0.05$.

3 | RESULTS

3.1 | IN treatment with hBD2 reduces inflammatory cell influx into the airways in steroid sensitive AAD

Asthma occurs in different phenotypes based on severity, responses to steroid treatment and immune phenotype. Distinctions are made between the subtypes of eosinophilic, neutrophilic, mixed granulocytic or without airway penetration of inflammatory cells.^{2,5,45,46} To investigate whether hBD2 is a potential treatment option in steroid-sensitive mild to moderate allergic asthma, we assessed its' effects on inflammation in the airways in HDM-sensitised and challenged mice with steroid sensitive AAD that recapitulates some hallmark features of the human phenotype. HDM challenge induced increases in the numbers of total leukocytes in the lung (Figure 2A), as well as increases in eosinophils (Figure 2B), neutrophils (Figure 2C), macrophages (Figure 2D) and lymphocytes (Figure 2E) in the airways of HDM challenged, vehicle-treated groups compared to non-allergic vehicle controls. hBD2 treatment suppressed total leukocytes, neutrophils and macrophages at low and high doses compared to HDM-challenged vehicle-treated groups. hBD2 treatment suppressed eosinophils at a low dose and trended towards a decrease at a high dose. These data show that hBD2 reduces the influx of inflammatory cells into airways in mild to moderate allergic AAD.

3.2 | IN treatment with hBD2 reduces asthma-associated cytokines in steroid sensitive AAD

Allergen specific T helper (T_H)2 cells but also other T cell subsets infiltrate the lung during the development of airway inflammation and produce a variety of effector cytokines in asthma. The type-2 cytokines IL-4, IL-5, IL-9 and IL-13 and type-1 cytokines IFN- γ , TNF- α and IL-6 play important roles in the pathogenesis of different phenotypes of asthma.^{2,4,44,46-48} The anti-inflammatory cytokine IL-10 may have protective effects. Consequently, we next assessed the effect of hBD2 treatment on key cytokines in steroid sensitive AAD in whole lung homogenates. HDM-sensitised and challenged, vehicle-treated groups had significantly elevated levels IL-4, IL-5, IL-9, IL-13, IFN- γ , TNF- α , IL-6 and IL-10 compared to non-allergic controls (Figure 3A-D and Figure S1A-D). Intranasal administration of low dose hBD2 did not reduce the concentration of IL-4 (Figure 3A) or IL-5 (Figure 3B) in lung homogenates. However, increases in the cytokines IL-9 (Figure 3C) and IL-13 (Figure 3D) were completely inhibited in hBD2 treated mice compared to HDM-challenged vehicle-treated controls. In fact, levels were reduced back to baseline to similar levels to those in groups without AAD. Among the other cytokines high dose but not low dose hBD2 suppressed IFN- γ , TNF- α , IL-6 and IL-10 back to basal levels (Figure S1A-D). These data demonstrate that hBD2 has anti-inflammatory effects by reducing key asthma-associated cytokines and thereby adaptive immune responses; and that high dose hBD2 administration may have utility for suppressing type-1 and/or -17 dominant factors which drive other endotypes of asthma.

3.3 | IN treatment with hBD2 reduces the severity of lung inflammation in steroid sensitive AAD

Asthma subtypes are characterized by the infiltration of various types of immune cells. We showed that the pulmonary inflammation in AAD is characterised by the infiltration of eosinophils and neutrophils in the BALF (Figure 2).^{45,46} To extend this analysis we next examined the effect of low dose treatment with hBD2 on lung histopathology, using an established scoring system to determine the severity of inflammation.^{44,49} HDM-sensitised and challenged vehicle-treated mice had focal to multi-focal moderate pulmonary inflammation characterised by substantial infiltration of inflammatory cells

around the blood vessels and in the airways compared to non-allergic controls (Figure 4A-D). Low dose hBD2 treatment substantially reduced the median histologic score and lung inflammation compared to vehicle-treated mice. Thus, our data show that hBD2 reduces the infiltration of immune cells in BALF (Figure 2), inflammatory cytokine release (Figure 3) and the severity of inflammation (Figure 4) in lung tissue in steroid sensitive AAD.

3.4 | IN treatment with hBD2 suppresses AHR in steroid sensitive AAD

Asthma patients are diagnosed by respiratory function tests that determine various characteristic features of asthma such as AHR.^{49,50} Pulmonary inflammation is associated with the induction of AHR. Thus, we next assessed the efficacy of hBD2 in suppressing the critical asthma feature of AHR in steroid sensitive AAD. HDM-sensitised and challenged vehicle-treated mice exhibited AHR, with increased airway resistance (RI) compared to non-allergic controls in response to increasing doses of MCh (Figure 5). hBD2 treatment of HDM-challenged animals suppressed MCh induced RI at both low and high doses. This was completely inhibited with levels returned to those in non-allergic controls. These data demonstrate that hBD2 suppresses AHR by reducing airway resistance that is associated with reduced pulmonary inflammation in steroid sensitive AAD.

3.5 | IN treatment with hBD2 suppresses eosinophil influx in the airways and AHR to equivalent extents as corticosteroid and inhibits neutrophil influx in the airways whereas corticosteroid does not, in steroid-sensitive AAD

We also assessed the effects of hBD2 treatment and compared them with corticosteroid therapy in a steroid-sensitive, Ova-induced mouse model of mild to moderate AAD. To match the assessment of the effects of hBD2 in our later infection-induced steroid resistant model, we used the same Ova sensitisation and challenge protocols without infection in that model. We show that hBD2 treatment (5 mg/kg) suppresses airway total leukocytes, eosinophils and notably neutrophils in BALF of non-infected, Ova-sensitised and challenged groups (SPG/Ova/Veh vs. SPG/Ova/DEX vs. SPG/Ova/hBD2; Figure 6A-

C). Total leukocytes and eosinophils were suppressed to the same level as with DEX, but DEX did not significantly suppress neutrophil counts whereas hBD2 did. Furthermore, hBD2 treatment completely suppressed AHR in Ova-sensitised and challenged groups back to baseline levels and to the same extent as DEX (SPG/Ova/Veh vs. SPG/Ova/DEX vs. SPG/Ova/hBD2; Figure 6D, E).

3.6 | IN treatment with hBD2 suppresses pulmonary inflammation and AHR in steroid resistant AAD

Corticosteroids are the first-line drugs for the treatment for asthma. However, steroid resistance or insensitivity is a major barrier to the therapy.^{2,5,6,50,51} This is indicative of more severe asthma that also often has a neutrophilic and type-1 inflammatory phenotype. To investigate the potential of hBD2 in treating this form of asthma, we used an established Ova- and Cmu infection-induced mouse model of severe steroid resistant AAD.^{36,41,42} Ova sensitisation and challenge without infection induced AAD with increases in the numbers of total leukocytes, eosinophils and AHR with increased RI compared to saline sensitized controls (Figure 7). These features were all steroid sensitive. As we showed previously combination of AAD with Cmu infection increased in the numbers of total leukocytes and neutrophils but reduced in eosinophils in BALF compared to Ova-sensitised and challenged and sham-inoculated groups. There were also increases in the type-1 cytokines KC, TNF and IFN- γ . All these features were steroid resistant. hBD2 treatment resulted in the significant reduction in steroid resistant neutrophils but not eosinophils, which was associated with the suppression in the levels of KC, TNF and IFN- γ compared to infected, allergic controls (Cmu/Ova/hBD2 vs. Cmu/Ova/Veh; Figure 7A-F). Combined AAD and infection also induced increases in steroid resistant AHR and RI (Figure 7G and H). Treatment with hBD2 (5 mg/kg/day) significantly reduced AHR and lung resistance, which was inhibited almost back to levels in saline challenged animals (Cmu/Ova/hBD2 vs. Cmu/Ova/Veh vs Cmu/Sal/Veh). These data show that hBD2 may effectively suppress steroid resistant airway neutrophilia and AHR in established disease.

4 | DISCUSSION

In this study, we assessed the potential therapeutic applicability of the anti-microbial peptide hBD2 treatment in mild to moderate steroid-sensitive and severe steroid-resistant AAD. IN administration of hBD2 was highly effective in suppressing inflammation in three models and to an equivalent or greater extent than corticosteroid treatment in steroid-sensitive AAD, particularly, neutrophilic airway inflammation in the airways and lung tissue and the production of key asthma-associated cytokines. The anti-inflammatory effects exhibited by hBD2 were also associated with the complete inhibition of AHR, in both steroid sensitive and steroid resistant AAD, which highlights another potential major advantage over conventional corticosteroid use.

Currently, corticosteroids are considered the 'gold-standard' treatment for asthma.^{50,51} However, asthma is a heterogenous disease with many different phenotypes and endotypes observed.²⁻⁷ Critically, in the context of this research, no pharmacological agent with potent clinical efficacy has yet been approved to treat steroid-resistant asthma, which disproportionately accounts for more than 50% of asthma-associated healthcare costs.^{2,5,6} Recent studies have investigated the potential of macrolide therapy which has been shown to be beneficial in severe forms of asthma and in chronic obstructive pulmonary disease.^{41,51-53} However, prolonged macrolide therapy may induce bacterial resistance in patients, increasing the likelihood of opportunistic infections.^{51,52,54} Thus, the use of an endogenous agent such as hBD2 may provide a novel therapeutic approach without side-effects.

To model the impact of hBD2 therapy in mild to moderate allergic asthma we used an acute, HDM-driven model of steroid sensitive AAD. HDM has been extensively used to explore the effector mechanisms in rodent models of asthma.^{37,54,55} It is commonly used as a disease-inducing experimental antigen because it is also an aerosolised allergen that is frequently associated with human allergic asthma.⁵⁶ HDM challenge in our model induced a strong type-2 pro-inflammatory response with an increase in eosinophilic and neutrophilic inflammation in the airways, accompanied by increases in IL-4, IL-5, IL-9 and IL-13, and lung histopathology.^{37,44,49,55} These inflammatory processes were also associated with AHR. Thus, our HDM model is appropriate to model mild allergic asthma.⁵⁷⁻⁵⁹ Importantly, treatment with hBD2 potently suppressed inflammation in the airways, IL-9 and IL-13 levels and the degree of histopathology in the lungs. Furthermore, these reductions in inflammation and pathology were associated with

a reduction in AHR. These data are intriguing and indicate the potential use of small peptides as asthma treatments.

In the airways, β -defensins are secreted by endothelial cells and are electrostatically retained in surfactant, where they play an important role in the maintenance and defense of barrier functions of the lung.^{57,59-61} In this study we demonstrated that hBD2 contributes to lung homeostasis through its anti-inflammatory properties. Although the mechanisms of action of hBD2 in this context are unclear, it is possibly working to restore normal immune responses rather than having anti-microbial effects although this remains to be defined.²¹ The lung microbiome may be being affected that could also be having an effect. Indeed, the lung is dysbiotic in lung diseases including asthma.^{9,62-64} The constitutive defensin, hBD1, has previously been suggested to have diagnostic applications in asthma.²² It is conceivable that the treatment potential of anti-microbial peptides also includes related defensins and the data here provide a rationale for further therapeutic testing of other such factors.

We then performed a comparison of the effects of hBD2 (5mg/kg) with DEX (2mg/kg) using our established experimental mouse model of Ova-induced, steroid-sensitive AAD (Figure 1B).^{36,41,42} We previously performed a dose response study (unpublished) and showed that the 2 mg/kg dose of DEX used here is the maximally effective dose. We now show that hBD2 treatment suppressed inflammation (numbers of total leukocytes and eosinophils) and AHR, to the same degree as DEX. hBD2 treatment also inhibited neutrophil influx into the airways almost back to baseline whereas DEX had no effect. These data provide strong additional evidence of efficacy in a different, steroid-sensitive model performed in a different laboratory (Figures 2-5 Fidelta Ltd, Croatia, Figures 6 and 7 University of Newcastle/HMRI, Australia). It also further suggests that hBD2, but not corticosteroids, may be effective in neutrophil-dominated asthma endotypes.

To further our observations and assess the potential of hBD2 treatment in severe forms of asthma, we assessed the effects of treatment with hBD2 in an established model of infection-induced steroid resistant AAD that we developed.^{36,41,42} In our model, we superimpose Cmu infection on top of established Ova-induced AAD. Following clearance of infection, mice are challenged with Ova again, which induces neutrophilic inflammation, type-1 cytokines and AHR.^{36,41,42} Other studies provide evidence that Th17

responses may contribute to pathogenesis in a subset of patients with severe steroid-resistant asthma.⁵ The model of steroid-resistant asthma we have used is predominantly underpinned by NLRP3 inflammasomes and Th1 responses^{41,42}, which agrees with recent clinical evidence that shows that neutrophil-enriched, severe asthma is associated with increased NLRP3 and type 1 immunity.⁶⁵ It would be interesting to assess the impact of hBD2 on Th17 responses on future targeted studies with Th17 driven neutrophilic models.^{66,67} Steroid-resistant inflammation and AHR are the key functional readouts of steroid-resistant asthma, and our data definitively highlights the advantages of hBD2 over corticosteroids on these critical disease features.⁵ Most importantly, corticosteroid administration does not suppress the inflammation or AHR in this model and as such it represents a valuable model to assess the potential for therapeutic agents in severe asthma in a pre-clinical setting.^{36,41,42} In the current study, treatment with hBD2 suppressed neutrophilic inflammation in the airways in both steroid-sensitive and steroid-resistant Ova-induced, models of AAD; as well as levels of KC, TNF- α , IFN- γ to levels observed in non-infected, non-allergic controls. This contrasts with the lack of efficacy of the inhaled corticosteroid DEX. These data highlight the issues with corticosteroid treatment in severe forms of asthma. Furthermore, prolonged corticosteroid use has numerous local and systemic side-effects, and critically may increase the risk of respiratory infection.^{68,69}

In our study we used a lower dose of 1.2 mg/kg and a higher dose of 5 mg/kg. We previously conducted a series of dose finding studies where we identified 1.2 mg/kg split in one, two or three daily doses as the most efficient dose in several different indications and there were no differences between indications (colitis, necrotising enterocolitis, graft versus host disease).^{70,71} It is likely that a higher dose may be needed to suppress the more intractable neutrophilic and Th1 responses and so we used a higher dose of 5 mg/kg that is widely used to test experimental treatments. A low dose of hBD2 (1.2 mg/kg) was sufficient to suppress eosinophilic inflammation and Th2 responses in steroid-sensitive AAD. However, a higher dose was required to suppress neutrophilic and Th1 responses (Th2 responses were not assessed). Thus, different doses might be required for different asthma severities and/or dose-escalation treatment may be applicable. This will need to be determined in further development studies.

Our data provides proof of concept for the beneficial role of hBD2 in improving inflammation and AHR in different steroid sensitive and resistant and eosinophilic and neutrophilic asthma models. We demonstrate the beneficial effects of hBD2 on inflammation and AHR in two different models of steroid-sensitive AAD performed in two different laboratories (HDM Croatia and Ova Newcastle models) and a unique but established model of severe steroid-resistant AAD (Newcastle). This provides a high level of confidence in the beneficial effects of hBD2.

hBD2 suppressed type 2 and 1 eosinophilic and neutrophilic inflammatory responses showing efficacy in different phenotypes. The mechanisms of how this could occur remain to be elucidated. However, it is likely to occur through the suppression of inflammatory responses from innate (airway epithelial cells, macrophages) or adaptive (dendritic cells, T cells) immune responses. Furthermore, this could occur either by direct modulation of these cells or indirectly such as through modification of the microbiome. Defensins are potent immunomodulatory and are critical in maintaining immune balance at mucosal sites and have anti-inflammatory activity.⁷² hBDs are chemotactic for immune cells such as dendritic cells and T cells linking innate and adaptive immunity.¹⁸ hBD2 is also a prominent antimicrobial peptide and its normal function is to control pathogenic infections.⁷² Microbial populations in the gut and lung have components or release metabolites that have pro- or anti-inflammatory effects.^{9,62-64} Consequently, inhaled hBD2 may have direct anti-inflammatory effects on inflammatory and immune cells and/or modify the lung microbiome to indirectly suppress pro-inflammatory responses that drive AAD.

Strengthening mucosal barriers using hBD2 could be a stand-alone or supplementary therapy depending on the disease context. Future studies, should aim to assess hBD2 administration in appropriate human cell models such as primary respiratory cells.⁷³⁻⁷⁶ Treatment with protective endogenous molecules, like hBD2, provides a potential new treatment option that is not solely focused on targeting cytokines associated with the disease. This potential paradigm shift in asthma treatment is aimed to repair and restore the protective mucosal immune function evolutionarily provided by innate immunity. Such treatments with endogenous agents are expected to have minimal-side-effects compared to cytokine antagonising strategies, and therefore represent a promising new avenue of exploration.

CONFLICT OF INTERESTS

The authors have no financial conflict of interest.

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Table 1. Histology scoring system

Score	Description
0	None
1	Minimal focal
2	Minimal multifocal
3	Minimal diffuse
4	Moderate focal
5	Moderate focal and minimal diffuse
6	Moderate multifocal
7	Moderate diffuse
8	Marked focal
9	Marked focal and moderate multifocal
10	Marked multifocal
11	Marked focal and moderate diffuse
12	Marked diffuse

Figure Legends

FIGURE 1 Models of steroid-sensitive and steroid-resistant allergic airway disease (AAD).

A, Mice were sensitised with house dust mite (HDM) and complete Freund's adjuvant (CFA) intranasally (IN) on day 0 and then challenged with HDM IN at day 14. To assess the impact of hBD2 treatment, mice received low dose (1.2mg/kg) or high dose (5mg/kg) IN at day 14. Features of AAD were assessed 48 hours after challenge. **B**, Mice were sensitised with ovalbumin (Ova) and aluminium hydroxide intra-peritoneally (IP) on day 0 and then challenged at days 12-13 and days 33-34. To induce steroid resistance, mice were infected with *Chlamydia muridarum* (Cmu) on day 14. To assess the impact of corticosteroid treatment, mice received dexamethasone (DEX) on days 32-34. To assess the impact of hBD2 treatment, mice received hBD2 (5mg/kg) on days 32-34. Features of AAD were assessed 24 hours after challenge.

FIGURE 2 Treatment with hBD2 suppresses airway inflammation in steroid-sensitive allergic airway disease (AAD).

Mice with HDM-induced AAD were treated intranasally with low (1.2mg/kg) or high dose (5mg/kg) hBD2. Bronchoalveolar lavage fluid (BALF) was collected 48 h after challenge and differential cell counts were performed. A, Total leukocyte, B, eosinophil, C, neutrophil, D, macrophage, and E, lymphocyte numbers were determined using an automated haematological analyser Sysmex XT-2000iV. Data are from one experiment with $n=12$ mice for each group (mean $\pm$ SEM).

FIGURE 3 Treatment with hBD2 reduces type-2 cytokines in steroid-sensitive allergic airway disease (AAD).

Mice with HDM-induced AAD were treated intranasally with low (1.2mg/kg) or high dose (5mg/kg) hBD2. Lung homogenates were processed 48 h after the final challenge. A, Interleukin (IL)-4, B, IL-5, C, IL-9 and D, IL-13 levels were determined by sandwich ELISA. Data are from one experiment with $n=6$ mice for each group (mean $\pm$ SEM).

FIGURE 4 Treatment with hBD2 improves lung histopathology profile in steroid-sensitive allergic airway disease (AAD).

Mice with HDM-induced AAD were treated intranasally with low (1.2mg/kg) or high dose (5mg/kg) hBD2. The severity of

inflammation was evaluated in lung histological sections. A, Six areas covering whole lungs were evaluated and individual scores for each animal were given as mean $\pm$ standard error of the mean. Representative photomicrographs of B, Sal/Veh, C, HDM/Veh and D, HDM/hBD2 (1.2mg/kg) are included (50x magnification). Data are from one experiment with $n=8$ mice for each group.

FIGURE 5 Treatment with hBD2 suppresses airway hyper-responsiveness (AHR) in steroid-sensitive allergic airway disease (AAD). Mice with HDM-induced AAD were treated intranasally with low (1.2mg/kg) or high dose (5mg/kg) hBD2. Airway resistance (RI) A, in response to increasing doses of methacholine (MCh) or B, at the maximal dose of 25 mg/ml. C, Dynamic compliance in response to increasing doses of methacholine (MCh) were measured 48hrs after challenge. Data are from one experiment performed with $n=6$ mice for each group (mean $\pm$ SEM).

FIGURE 6 Treatment with hBD2 suppresses eosinophil influx in the airways and AHR to equivalent extents as corticosteroid and inhibits neutrophil influx in the airways whereas corticosteroid does not, in steroid-sensitive ovalbumin (Ova)-induced allergic airways disease (AAD). Mice with Ova-induced AAD were sham-inoculated with sucrose phosphate glutamate (SPG) buffer, re-challenged with Ova, and treated intranasally with hBD2. Bronchoalveolar lavage fluid (BALF) was collected 24h after the final challenge and differential cell counts performed. A, Total leukocyte, B, eosinophil and C, neutrophil numbers in the airways. Airway resistance (Rn), D, in response to increasing concentrations of methacholine (MCh), or E, at the maximal dose of 10 mg/mL MCh. Data are from one experiment performed with $n=6$ mice for each group (mean $\pm$ SEM).

FIGURE 7 Treatment with hBD2 suppresses neutrophil influx and completely inhibits pro-inflammatory cytokine increases in the airways and airway hyper-responsiveness (AHR) in steroid-resistant allergic airway disease (AAD). Mice with Ova-induced AAD were infected with *Chlamydia muridarum* and re-challenged with ovalbumin and were treated intranasally with hBD2. BALF was collected 24 h after the final challenge and differential cell counts were performed. A, Total leukocyte, B,

eosinophil and C, neutrophil numbers in the airways. Lung homogenates were processed and D, keratinocyte chemoattractant (KC), E, tumour necrosis factor (TNF) and F, interferon (IFN)- γ levels were determined by sandwich ELISA. Airway resistance (Rn), G, in response to increasing concentrations of methacholine (MCh), or H, at the maximal dose of 10 mg/mL MCh. Data are from one experiment performed with $n=6$ mice for each group (mean $\pm$ SEM).

SUPPLEMENTARY FIGURE S1 Treatment with high, but not low, dose hBD2, suppresses non-type-2 cytokines in lung homogenates. Mice with HDM-induced AAD were treated intranasally with low (1.2mg/kg) or high dose (5mg/kg) hBD2. Cytokine concentrations were measured in the lung homogenates 48 h after the final challenge. A, tumour necrosis factor (TNF), B, interferon (IFN)- γ , C, IL-6 and D, IL-10 levels were determined by sandwich ELISA. Data are from two experiments with $n=12$ mice for each group (mean $\pm$ SEM).

Figure 1

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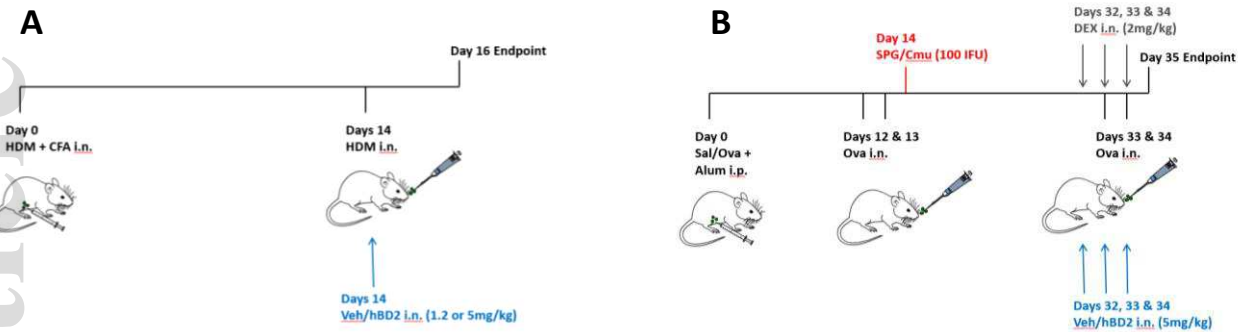


Figure 2

cea_13766_f2.pdf

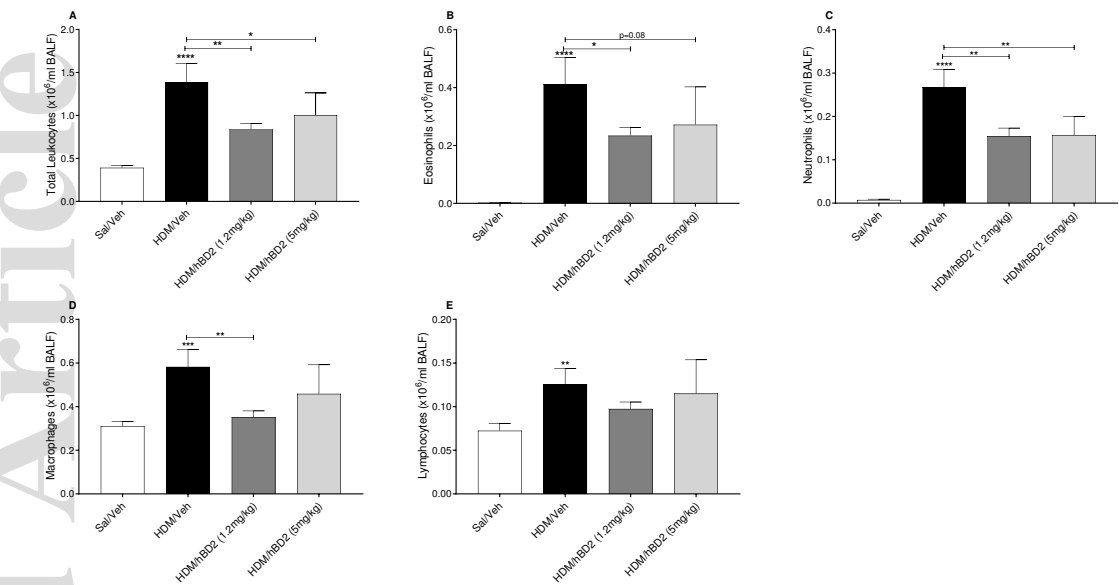
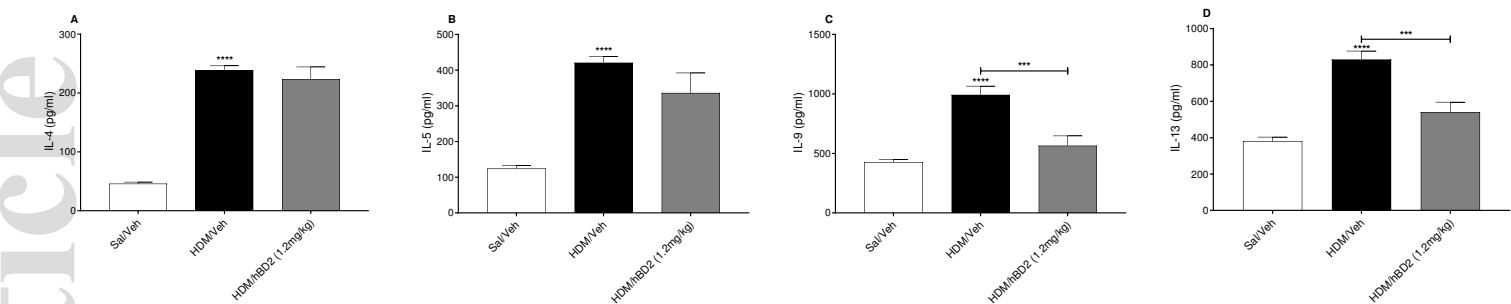


Figure 3

cea_13766_f3.pdf



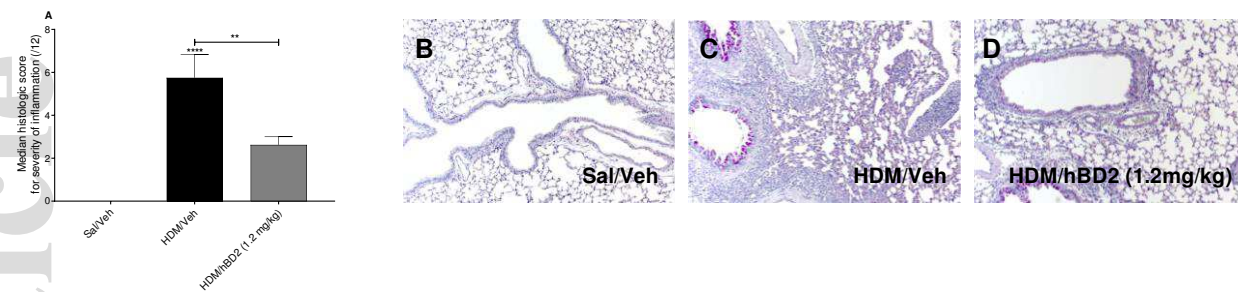


Figure 5

cea_13766_f5.pdf

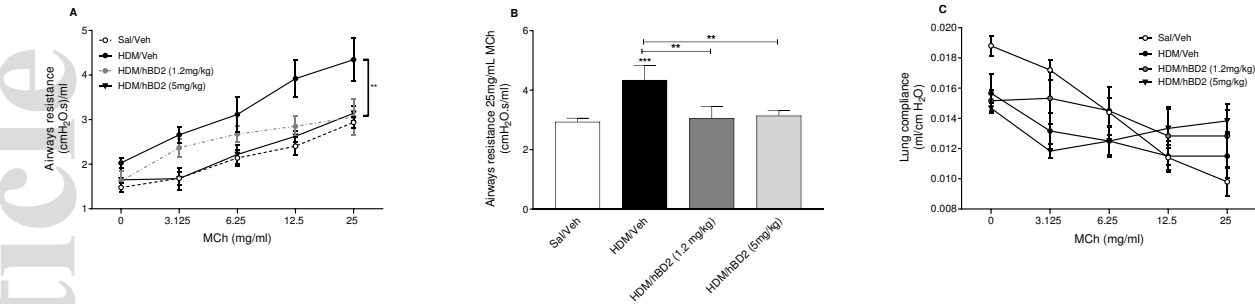


Figure 6

cea_13766_f6.pdf

