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A novel formulation of inhaled doxycycline reduces allergen-induced inflammation, hyperresponsiveness and remodeling by matrix metalloproteinases and cytokines modulation in a mouse model of asthma

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ABSTRACT

Background: In this study, we assess the effectiveness of inhaled doxycycline, a tetracycline antibiotic displaying matrix metalloproteinases (MMP) inhibitory effects to prevent allergen-induced inflammation, hyperresponsiveness and remodeling. MMPs play key roles in the complex cascade of events leading to asthmatic phenotype.

Methods: Doxycycline was administered by aerosols by the mean of a novel formulation as a complex with hydroxypropyl-gamma-cyclodextrin (HP-gamma-CD) used as an excipient. BALB/c mice (n = 16–24 in each group) were sensitized and exposed to aerosolized ovalbumin (OVA) from day 21 to 27 (short-term exposure protocol) or 5 days/odd weeks from day 22 to 96 (long-term exposure protocol).

Results: In the short-term exposure model, inhaled doxycycline decreased allergen-induced eosinophilic inflammation in bronchoalveolar lavage (BAL) and in peribronchial areas, as well as airway hyperresponsiveness. In lung tissue, exposure to doxycycline *via* inhaled route induced a fourfold increase in IL-10 levels, a twofold decrease in IL-5, IL-13 levels and diminished MMP-related proteolysis and the proportion of activated MMP-9 as compared to placebo.

In the long-term exposure model, inhaled doxycycline significantly decreased the extent of glandular hyperplasia, airway wall thickening, smooth muscle hyperplasia and subepithelial collagen deposition which are well recognized features of airway remodeling.

Conclusion: Doxycycline administered by aerosols decreases the allergen-induced airway inflammation and hyperresponsiveness and inhibits the development of bronchial remodeling in a mouse model of asthma by modulation of cytokines production and MMP activity.
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Abbreviations: BAL, bronchoalveolar lavage; FEV1, forced expiratory volume at the first second; HP-gamma-CD, hydroxypropyl-gamma-cyclodextrin; IL, interleukin; LPS, lipopolysaccharide; LTE, long-term exposure model; Mch, methacholine; MMP, matrix metalloproteinase; NaCl, sodium chloride; OVA, ovalbumin; PAS, periodic acid schiff; Penh, enhanced pause; SDS, sodium dodecyl sulfate; STE, short-term exposure model.

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1. Introduction

Asthma is an inflammatory disease of the airways. Assorted inflammatory cells infiltrate bronchial walls and bronchial hyperresponsiveness develops. During timelife, some morphological changes occur in the bronchial tree referred to as *bronchial remodeling*. Main changes described in bronchi of asthmatics are: (a) a sub-epithelial fibrosis mainly made of collagen (type I and III) and fibronectin, (b) some changes of the extracellular matrix composition, (c) a smooth muscle cell hyperplasia, (d) a ''Goblet cell'' hyperplasia, and probably (e) a perichondral fibrosis [1]. Despite controller medications available today, symptoms are not totally under control in many patients and current therapies are not able to stop the exaggerated decrease of FEV1 (forced expiratory volume at the first second) linked to bronchial extracellular matrix remodeling [2].

Cytokines (especially Th_2), and chemokines play an important role in the installation and persistence of inflammation in asthma [3–6]. Mechanisms of airway hyperresponsiveness, inflammation and remodeling can be approached using mice models of asthma based on either short-term exposure to allergens (allowing the study of hyperreactivity and acute inflammation) [7] or on long-term exposure to allergens (allowing the observation of an allergen-induced airway remodeling) [8].

Matrix metalloproteinases (MMPs) are zinc- and calcium-dependent enzymes involved in many inflammatory processes including asthma [9]. MMPs are regulators of extracellular matrix turnover and control critical steps of smooth muscle and endothelial cell migration and proliferation [10]. Increased levels of MMP-9 have been detected in bronchial secretions from asthmatics [11,12], and after allergen challenges [13,14]. MMP-9 gene deletion in mice inhibits allergen-induced airway inflammation and hyperresponsiveness in a murine model of asthma [7,15]. Similar results have been obtained in mice with chemical compounds displaying a wide spectrum of MMP inhibitory effects [16]. To date, none of the MMP-modulating compounds have been used as an inhaled therapy and a modulation of allergen-related bronchial remodeling by MMP inhibitors has never been reported.

Doxycycline belongs to the family of tetracycline antibiotics and inhibits bacterial protein synthesis. Doxycycline is an effective inhibitor of cell proliferation, migration, and MMP activity [17]. Those varied biological effects are ascribed to a MMP inhibition [18]. Mechanism(s) of MMP inhibition by doxycycline are not yet completely understood to date. Doxycycline could either bind directly Zn^{2+} or Ca^{2+}, directly target active site of MMPs, render pro-enzyme susceptible to fragmentation during activation process [19], induce a degradation of the pro-MMP zymogen [20] or provoke an inhibition of the transcription of MMP mRNAs. Doxycycline has been proposed as a therapy for many diseases implicating MMP-9, e.g. diseases of the ocular surface [21], aortic aneurysms [22], central nervous system disorder [18] and colorectal cancers [23]. It was also suggested that some tetracyclines display, *in vitro*, the ability to decrease IgE responses [24]. When considering doxycycline as an antibiotic, the minimal concentration inducing an inhibition of bacteria is generally considered to be about 2 mM while the MMP inhibitory effect is present for concentrations 100 times lower. For instance, concentrations required to inhibit 50% of the gelatinolytic activity (IC50) were found to be 30–50 μM [25,26].

Due to its intrinsic properties of MMP inhibitory agent and considering the role of MMPs in the pathogenesis of asthma, we hypothesised that doxycycline could be a disease modifying agent such as demonstrated for anti-tumor necrosis factor-alpha (TNF-alpha) in rheumatoid arthritis [27] and recently in asthma [28].

In this study, we developed a new formulation of doxycycline allowing aerosol administration and we demonstrate that a strategy based on MMP inhibition may lead to obtain a control of the allergen-induced airway inflammation, hyperresponsiveness and remodeling. We compared results with those obtained with an inhaled steroid routinely used for asthma therapy (fluticasone). The development of an aerosolized form of doxycycline was performed in accordance with our previously published data, assessing the toxicity of inhaled cyclodextrins in mice [29].

2. Materials and methods

2.1. *Doxycycline solutions and nebulization*

In the present study, we used doxycycline base USP XXIV (Kunsfan chem. and pharm factory, India) solutions buffered at pH 7.4. Unfortunately, in these conditions, doxycycline is not stable and has low solubility. In order to enhance doxycycline solubility and stability, inclusion complexes with HP-gamma-cyclodextrin (HP-gamma-CD) (Wacker, Germany) were investigated. Cyclodextrins are cyclic oligosaccharides consisting of alpha 1–4 linked glucopyranose units characterized by a lipophilic cavity and a hydrophilic outer surface. Their apolar cavity is able to include large organic molecules by non-covalent interaction forces, and thereby to improve their water solubility.

Phase diagram studies showed that HP-gamma-CD is able to improve the doxycycline solubility in buffered (pH 7.4) solutions. We have previously studied the feasibility of using CDs for the elaboration of pharmaceutical formulations designed for inhalation [29]. We described in this reference that depending on the CD concentration, the aqueous solutions can undergo aerosolization and that resulting droplet-size is compatible with pulmonary deposition. For HP-gamma-CD, the concentration was limited to 50 mM. At this concentration, HP-gamma-cyclodextrin allows to solubilize 7.5 mg/ml of doxycycline base.

Sterile, apyrogenic and isotonic solutions were prepared by dissolving HP-gamma-CD 50 mM and doxycycline base (7.5 mg/ml) in a 0.125 M potassium dehydrogenate phosphate buffer (pH 7.4). Cyclodextrins were tested following the Bacterial Endotoxin Test described in USP XXVI using Limulus Amebocyte Lysate (LAL). Endotoxin levels were <0.15 U/ml. Osmolality of every solution was measured by a Knauer Automatic semi-micro-Osmometer and adjusted to the value of 300 mOsm/kg by the addition of an adequate amount of NaCl. Solutions were stored at −20 °C until nebulization.

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2.2. Protocol of sensitization and allergen exposure

Care and use of experimental animals (BALB/c mice) were performed following "principles of laboratory animal care" formulated by the National Society for Medical Research (USA) and the experimental protocol was approved by the animal ethical committee (University of Liège) under the No. 03/158. Two different protocols were used in this study. We first designed a "short term" exposure protocol. In this protocol, 6–8 weeks old BALB/c males mice were sensitized on days 1 and 8, by intraperitoneal injection of 10 μg ovalbumin (OVA) (Sigma–Aldrich, Schnelldorf, Germany) emulsified in aluminum hydroxide (AlumInject; Perbio, Erembodegem, Belgium). Mice were subsequently divided in five groups: (1) a group of mice was only exposed to the inhalation of a PBS aerosol (sham challenge) and four other groups were subjected to ovalbumin (OVA) 1% aerosol for 30 min. Before the exposure to OVA, those mice were either exposed during 30 min to (2) PBS aerosol, (3) a HP-gamma-CD aerosol (50 mM), (4) a HP-gamma-CD-doxycycline complex (50 mM of HP-gamma-CD and 7.5 mg/ml of doxycycline), (5) a fluticasone aerosol (1 mg/ml) which was used in this study as an anti-inflammatory drug reference therapy. Aerosols were generated daily by ultrasonic nebulizer (Devilbiss 2000), from day 21 to 27. After determination of airway reactivity, sacrifice of mice was performed on day 28 as previously reported [7]. Three different experiments have been performed and measurements have been performed on cohorts of 7–14 mice per experimental condition.

A "long term" exposure protocol was designed and adapted from the short-term exposure protocol. Amendments were made to the short-term exposure protocol as follows: mice were sensitized by an intraperitoneal injection on days 1 and 11 and groups of mice were exposed to inhalation aerosols 5 days per week (odd week) from day 22 to day 96. No animal died during these experiments.

2.3. Determination of airway reactivity

Mice were anesthetized by intraperitoneal injection (200 μl) of a mixture of ketamine (10 mg/ml, Merial, Brussel, Belgium) and xylazine (1 mg/ml, VMD, Arendonk, Belgium). A tracheotomy was performed by insertion of a 20 gauge polyethylene catheter into the trachea. A ligature was performed around the catheter to avoid leaks and disconnections. Mice were ventilated with a flexiVent small animal ventilator® (SCIREQ, Montreal, Canada) at a frequency of 450 breaths per minute and a tidal volume of 10 ml/kg. A positive endexpiratory pressure was set at 2 h Pa. Measurement started after 2 min of mechanical ventilation. A sinusoidal 1-Hz oscillation was then applied to the tracheal tube and allowed a calculation of dynamic resistance, elastance, and compliance of the airway by multiple linear regressions. A second manoeuvre consisting in an 8-s forced oscillatory signal ranging frequencies between 0.5 and 19.6 Hz allowed measurement of impedance to evaluate tissue damping, tissue elastance, and tissue hysteresivity [30]. Following measurement of baseline lung function, mice were exposed to a saline aerosol (PBS) followed by aerosols containing increasing doses (3, 6, 9, 12 g/l) of methacholine (ICN Biomedicals, Asse Relegem, Belgium). Aerosols were generated by the mean of an ultrasonic nebuliser (SCIREQ, Montreal, Canada) and delivered to the inspiratory line of the flexiVent using a bias flow of medical air following the manufacturer's instructions. Each aerosol was delivered for 10 s and periods of measurement as described above were made at 1-min intervals following each aerosol. The mean airway resistance after methacholine exposure was the main parameter measured during the challenge.

2.4. Bronchoalveolar lavage (BAL)

After the sacrifice of mice, a cannula was placed in the trachea of mice and we performed a bronchoalveolar lavage by gentle manual aspiration using 4 ml × 1 ml of cold PBS-EDTA 0.05 mM (Calbiochem, Darmstadt, Germany) as previously described [7]. The bronchoalveolar fluid (BAL) was centrifuged at 4 °C for 10 min (1200 rpm). The supernatant was frozen at −80 °C for protein assessment and the cell pellet was resuspended in 1 ml PBS-EDTA 0.05 mM. Total cells counts were performed manually, using a thoma chamber. The differential cell counts were performed using morphologic criteria on cytocentrifuged preparations (Cytospin) after staining with Diff-Quick (Dade, Belgium). Differential cell counts were performed by one blinded observer unaware of the experimental treatment.

2.5. Pulmonary histology

After BAL, the thorax was opened and the left main bronchus was clamped. The left lung was excised and frozen immediately at −80 °C and then crushed in liquid N_2 using a Mikro-Dismembrator (Braun Biotech International, Gmbh Melsungen, Germany). This crushed lung tissue was incubated overnight at 4 °C in a solution containing 2 M urea, 1 M NaCl and 50 mM Tris (pH 7.5) and subsequently centrifuged 15 min at 16.000 × g for protein extraction [7]. The right lung was infused with 4% paraformaldehyde, embedded in paraffin and processed for histology. Sections of 5 μm thickness were cut off from paraffin and were stained with haematoxylin–eosin. The extent of peribronchial inflammation was estimated by a score calculated by quantification of peribronchial inflammatory cells (eosinophils, lymphocytes, macrophages, etc.), as previously described [7]. A value of 0 was adjudged when no inflammation was detectable, a value of 1 when there was occasional inflammatory cells, a value of 2 when most bronchi were surrounded by a thin layer (1–5 cells) of inflammatory cells and a value of 3 when most bronchi were surrounded by a thick layer (>5 cells) of inflammatory cells. Since 5–7 randomly selected tissue sections per mouse were scored, inflammation scores are expressed as a mean value and can be compared between groups. Peribronchial inflammation scores were assessed by two different observers blinded to the experimental treatment.

After Congo Red staining, the eosinophilic infiltration in the airway walls was quantified by manual count in randomly selected bronchi and normalized to the perimeter of corresponding epithelial basement membrane defining an eosinophilic inflammatory score. Glandular hyperplasia was observed using the Periodic Acid Shiff (PAS) staining in randomly selected bronchi. PAS stains mucus producing cells (Goblet Cells) and the results were expressed as percentages of

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epithelial cells positive for the PAS staining as counted by an observer blinded to experimental data. Masson's Trichrome staining was used to detect peribronchial collagen deposition. We developed software in Java (JDK 1.5) that allows one to annotate each image by drawing polygons on top of the image (by the means of line-drawing procedure between user-specified successive points and a close operation). Each polygon is labeled into a predefined class by the user (here bronchial tubes, vessel, collagen). The user saves the annotation, and then the software generates a bitmap image where each pixel within a polygon is colored by the color specific to its class. It then counts the number of pixels in the image for each class. The percentage of the pixels in the image for a class is returned as the quantification result for that specific class. The α-Smooth Muscle Actin (α-SMA) was detected by immunohistochemistry. Slides were treated successively by Trypsine 0.1% (Sigma–Aldrich, Schnelldorf, Germany), H_2O_2 3% (Merck, Darmstadt, Germany), Triton 1% (Merck, Darmstadt, Germany) and BSA 10% (Sigma–Aldrich, Schnelldorf, Germany) and then incubated 2 h at room temperature with mouse primary antibody anti-α-SMA-FITC (Sigma–Aldrich, Schnelldorf, Germany). Slides were washed with PBS and an antibody anti-FITC-POD (Roche, Mannheim, Germany) allows the detection of smooth muscle cells layer.

2.6. Measurement of allergen specific serum IgE

After sacrifice, blood was drawn from the heart for measurement of OVA specific serum IgE. Ninety-six well microtiter plates were coated with 300 μl/well of an OVA solution (5 mg/l). Serum was added and, after incubation and rinsing, followed by a biotinylated polyclonal sheep anti-mouse IgE (Calbiochem, Darmstadt, Germany) used at 1/1000. A serum pool from OVA-sensitized animals was used as internal laboratory standard; 1 U was arbitrarily defined as 1/100 dilution of this pool.

2.7. Cytokines measurements by ELISA

In the short-term exposure model, IL-5, IL-10, IL-13, IFN-γ and CCL-11 levels on lung protein extracts were assessed using commercial ELISAs (R&D systems, Abingdon, UK).

2.8. Measurement of MMP activity in lung tissue

General MMP activity was assessed using the EnzoLyte™520 Generic MMP assay Kit (AnaSpec, San Jose, CA). This kit is optimized to detect the activity of different MMPs such as MMP-1, -2, -3, -7, -8, -9, -12, -13 and -14, using a fluorescence-quenched substrate (5-FAM/QXL™520). Upon cleavage into two separate fragments by MMPs, the fluorescence of 5-FAM is recovered, and can be monitored at excitation/emission wavelengths 490/520 nm. Proteins extracted from the crushed lung tissue were incubated with 1 mM APMA (4-aminophenylmercuric acetate) during 1 h in order to activate MMPs. 50 μl of sample were incubated in a 96 well plate. The plate was incubated at room temperature for 45 min and protected from light. Stop solution from Anaspec (50 μl) was added in each well and the fluorescence intensity was measured at 490/520 nm.

2.9. Measurement of MMP-2 and -9 levels by zymography

Protein extracts were mixed with the same amount of sample buffer. Electrophoresis was carried out on a SDS–10% polyacrylamide gel containing 1 mg/ml gelatin. Gels were incubated for 30 min in 2% Triton X–100. After incubation in an activation buffer containing 100 mM $CaCl_2$ and 100 mM NaCl at 37 °C overnight, gels were rinsed and stained for 30 min in Coomassie blue. Gelatinase activity was detected as white lysis bands against blue background. Quantitative evaluation of the gelatinolytic activity was performed by scanning gel using an imaging densitometer (Bio–Rad Laboratories, Fluor-S™ Multimager, USA). On each gel, we used dilutions of culture medium conditioned by HT1080 cells, known to produce spontaneously high amounts of MMP-2 and -9, as an internal standard. Gelatinolytic activity of the murine MMP-2 and -9 was determined by the lysis band in the 72 kDa and the 95 kDa area, respectively.

2.10. Statistical analysis

All results were expressed as mean ± S.E.M. and the comparison between the groups was performed using non-parametric Kruskall–Wallis test followed by a Dunn's post-test. Kruskall–Wallis test was performed using GRAPHPAD INSTAT version 3.00 for WINDOWS 95 (GRAPHPAD SOFTWARE, San Diego, CA, USA, WWW.GRAPHPAD.com).

3. Results

3.1. Airway resistance measurement

Airway resistance was assessed in the short-term exposure model by using the FlexiVent System allowing the exposure of mice to inhaled methacholine (3–12 g/l). In the short-term protocol (7 days), allergen exposure did induce a bronchial hyperresponsiveness and inhalation of placebo or vehicle (hydroxypropyl-gamma-cyclodextrin (HP-gamma-CD)) 30 min before allergen did not affect this hyperreactivity to MCh (Fig. 1). On the contrary, the exposure to HP-gamma-CD-doxycycline aerosols or fluticasone aerosols 30 min before

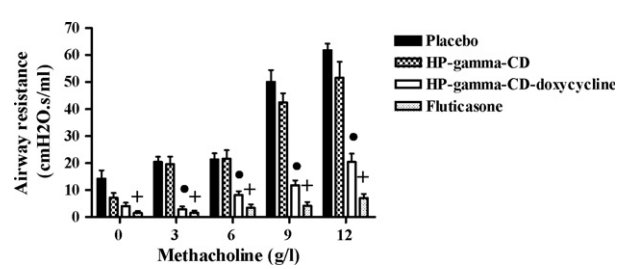

Fig. 1 – Effect of inhaled placebo, excipient, doxycycline, and fluticasone on bronchial reactivity after short-term allergen. Airway resistance was measured using FlexiVent System 24 h after the last allergen exposure in mice treated with placebo ($n = 5$), vehicle only (HP-gamma-CD) ($n = 5$), HP-gamma-CD-doxycycline complex ($n = 5$), and fluticasone ($n = 5$). $^{*}p < 0.05$ compared to HP-gamma-cyclodextrin; $^{+}p < 0.05$ compared to placebo.

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allergens did significantly reduce airway responsiveness to MCh ($p < 0.05$) (Fig. 1). This decrease of bronchial resistances measured in mice exposed to HP-gamma-CD-doxycycline complex or to fluticasone was observed for each dose of MCh used. These results clearly suggest that preventive inhalation of HP-gamma-CD-doxycycline complex leads to a decrease in bronchial hyperreactivity after allergen exposure.

3.2. *Bronchoalveolar lavage (BAL)*

In the short-term exposure model (STE), mice exposed to placebo and subsequently to OVA showed a large increase in eosinophil numbers when compared to OVA-sensitized sham-exposed mice ($p < 0.005$) (Table 1). Inhalation of the vehicle (HP-gamma-CD) 30 min before allergen exposure, did not cause any decrease of the allergen-induced eosinophil burden while the inhalation of HP-gamma-CD-doxycycline or fluticasone drastically reduced the BAL eosinophilia ($p < 0.05$) (Table 1). Total BAL cell number was also significantly decreased when mice were exposed to HP-gamma-CD-doxycycline and fluticasone 30 min before allergen ($p < 0.05$).

3.3. *Peribronchial inflammation*

A peribronchial inflammation score was evaluated for 5–7 bronchi per mouse. In the short-term exposure model (STE), this score was significantly increased by OVA exposure when compared to sham-exposed mice ($p < 0.001$) (Fig. 2). The exposure to HP-gamma-CD did not induce any change in peribronchial inflammation. Aerosols of HP-gamma-CD-doxycycline and fluticasone performed before OVA aerosols induced a significant decrease of the inflammation score ($p < 0.001$ and $p < 0.005$, respectively) (Fig. 2a).

In the long-term exposure protocol (LTE), baseline inflammation seen in sham-exposed mice was slightly higher than in the short-term exposure model. OVA exposure increased peribronchial inflammation ($p < 0.001$) which was not modified by HP-gamma-CD aerosols while HP-gamma-CD-doxycycline and fluticasone significantly prevented the peribronchial inflammation ($p < 0.001$ and $p < 0.005$, respectively) (Fig. 2b). Representative examples of lung peribronchial inflammation after ovalbumin exposure (left panel) and HP-gamma-CD-doxycycline inhalation (right panel) are presented in Fig. 2c.

Eosinophil counts/mm of basement membrane were significantly reduced by HP-gamma-CD-doxycycline or fluticasone inhalation 30 min before allergen ($p < 0.05$) (Fig. 3a). In the long-term exposure protocol, the increase in eosinophil counts induced by OVA was smaller but similar results were obtained when considering different experimental groups (Fig. 3b).

3.4. *Effects of doxycycline on allergen-induced glandular hyperplasia*

Long-term exposure to allergen did induce a significant increase in the percentage of cells positive for PAS staining among epithelial cells. These PAS-positive cells had morphological features of Goblet cells (Fig. 4a). HP-gamma-CD aerosols did not modify that percentage significantly HP-gamma-CD-doxycycline or fluticasone inhaled before allergen did significantly reduce Goblet cell hyperplasia ($p < 0.05$ and $p < 0.01$, respectively) without any significant difference between these two groups (Fig. 4b).

3.5. *Effects of doxycycline on allergen-induced epithelium basement membrane thickening*

In the long-term exposure model, exposure of mice to allergen induced a significant increase of the average thickness of this membrane ($p < 0.001$) (Fig. 4c). HP-gamma-CD-doxycycline and fluticasone induced a significant inhibition of the allergen-induced basement membrane thickening ($p < 0.05$ and $p < 0.001$, respectively) (Fig. 4c).

3.6. *Effects of doxycycline on airway wall collagen deposition*

In the long-term exposure group, quantification of collagen deposition in bronchial walls was performed using the Java software and allowed us to demonstrate a significant increase of airway wall collagen deposition after OVA exposure as compared to sham-exposed mice ($p < 0.001$). Moreover, this increase in the surface occupied by collagen in bronchial walls was not influenced by inhalation of vehicle (HP-gamma-CD). On the contrary, HP-gamma-CD-doxycycline and fluticasone did prevent significantly this collagen deposition when compared either to placebo (PBS) or vehicle (HP-gamma-CD) ($p < 0.001$) (Fig. 4d and e).

Table 1 – BAL total and differential cell counts ($\times 10^4$/ml) in the short-term exposure protocol

Cell types	PBS ($n = 8$)	Placebo ($n = 25$)	HP-gamma-CD ($n = 22$)	HP-gamma-CD doxycycline ($n = 24$)	Fluticasone ($n = 17$)
Epithelial cells	6.5 ± 1.5	4.7 ± 0.5	2.8 ± 0.4[a]	4.3 ± 0.7	4 ± 0.5
Eosinophils	0.3 ± 0.2[b]	22.5 ± 3.8	25.4 ± 4.2	7.7 ± 1.2[a,c]	7.8 ± 1[a,c]
Neutrophils	1.1 ± 0.3	1 ± 0.2	1.9 ± 0.5	0.3 ± 0.09[a,c]	0.6 ± 0.3
Lymphocytes	1 ± 0.5	0.8 ± 0.3	0.6 ± 0.2	0.3 ± 0.08	0.2 ± 0.05
Macrophages	30.9 ± 6.6	28.4 ± 2.6	22.9 ± 2.5	25.6 ± 2.7	26.2 ± 2.2
Total cells	45.5 ± 13.2	56.8 ± 6	51.4 ± 6.1	38.3 ± 3.8[a]	39.7 ± 2.3[a]

[a] $p < 0.05$ compared with ovalbumin.
[b] Not increased in PBS group as compared to all groups ($p < 0.005$).
[c] $p < 0.05$ compared with HP-gamma-CD.

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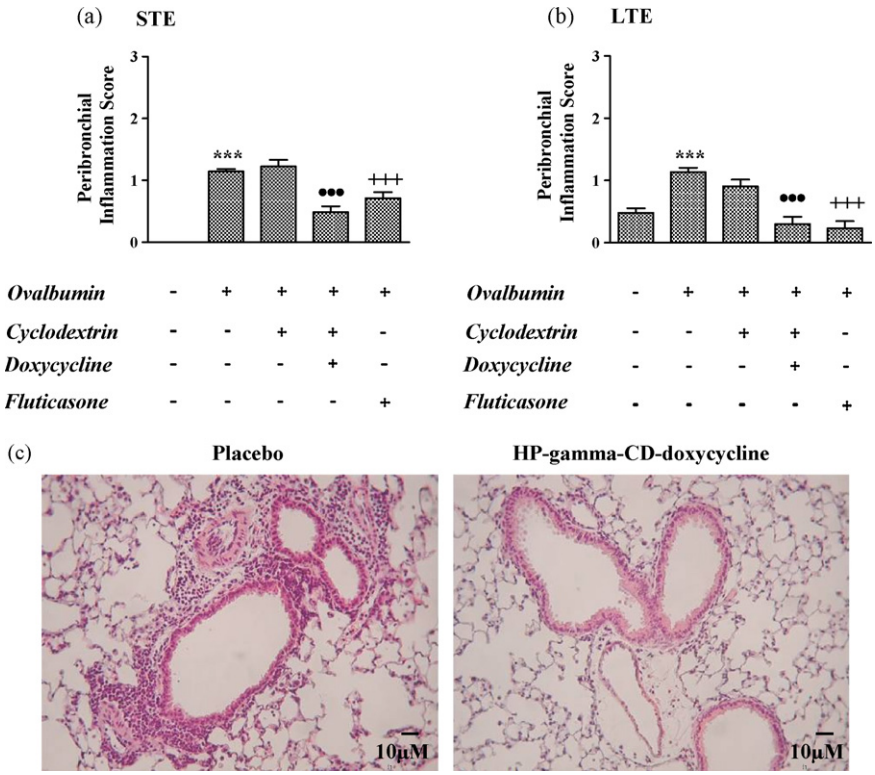

Fig. 2 – Peribronchial inflammation scores in the short-term allergen exposure model (STE) (a) and in the long-term allergen exposure model (LTE) (b). Peribronchial inflammation scores were calculated as described in Section 2. Results are expressed as mean and error bars represent the S.E.M. Numbers of mice per group: no therapeutic intervention and sham-exposed mice, $n = 8$ (row 1); placebo-exposed (PBS), $n = 25$ (row 2); HP-gamma-cyclodextrin (CD), $n = 22$ (row 3); HP-gamma-cyclodextrin-doxycycline (DO), $n = 24$ (row 4); fluticasone (FL), $n = 17$ (row 5). PBS, CD, DO, and FL groups were exposed to allergens (OVA). $^{***}p < 0.001$ compared to sham-exposed mice; $^{\bullet\bullet\bullet}p < 0.001$ compared to HP-gamma-cyclodextrin; $^{+++}p < 0.005$ compared to placebo. (c) Microphotographs (haematoxylin–eosin, 200×) of formaldehyde-fixed paraffin-embedded lung sections from mice exposed to ovalbumin and placebo (left panel) or to ovalbumin and HP-gamma-cyclodextrin-doxycycline complex (right panel) in the short-term exposure model.

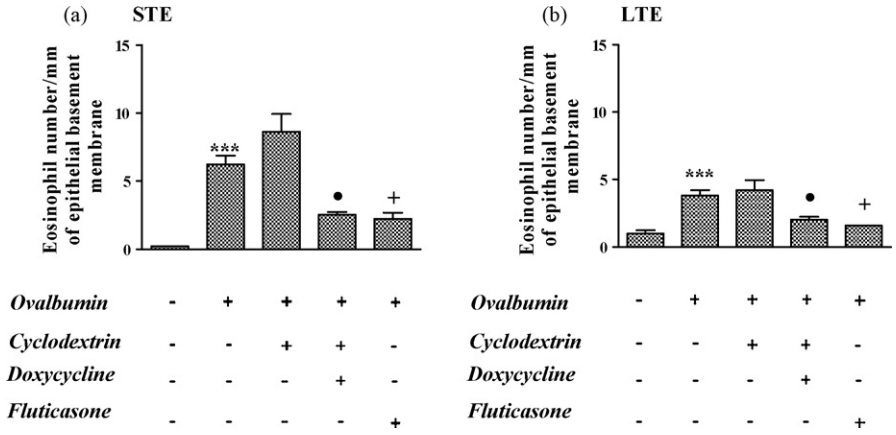

Fig. 3 – Measurements of eosinophilic infiltration in the bronchial tissue in short-term (a) and long-term (b) allergen exposure model. The bar graph represents the mean of three independent experiments and errors bars represent S.E.M. Numbers of mice per group: no therapeutic intervention and sham-exposed mice, $n = 8$ (row 1); placebo-exposed (PBS), $n = 25$ (row 2); HP-gamma-cyclodextrin (CD), $n = 22$ (row 3); HP-gamma-cyclodextrin-doxycycline (DO), $n = 24$ (row 4); fluticasone (FL), $n = 17$ (row 5). PBS, CD, DO, and FL groups were exposed to allergens (OVA). $^{***}p < 0.001$ compared to sham-exposed mice; $^{\bullet}p < 0.05$ compared to HP-gamma-cyclodextrin; $^{+}p < 0.05$ compared to OVA-exposed mice.

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3.7. *Effects of doxycycline on smooth muscle thickness*

Long-term allergen exposure induced an increase of smooth muscle layer thickness, measured by immunohistochemistry, around the bronchi when compared to sham-exposed mice ($p < 0.001$). As observed for other features of bronchial remodeling, HP-gamma-CD-doxycycline and fluticasone did prevent significantly this increase of smooth muscle cells thickness when compared either to placebo (PBS) or vehicle (HP-gamma-CD) ($p < 0.001$) (Fig. 4f).

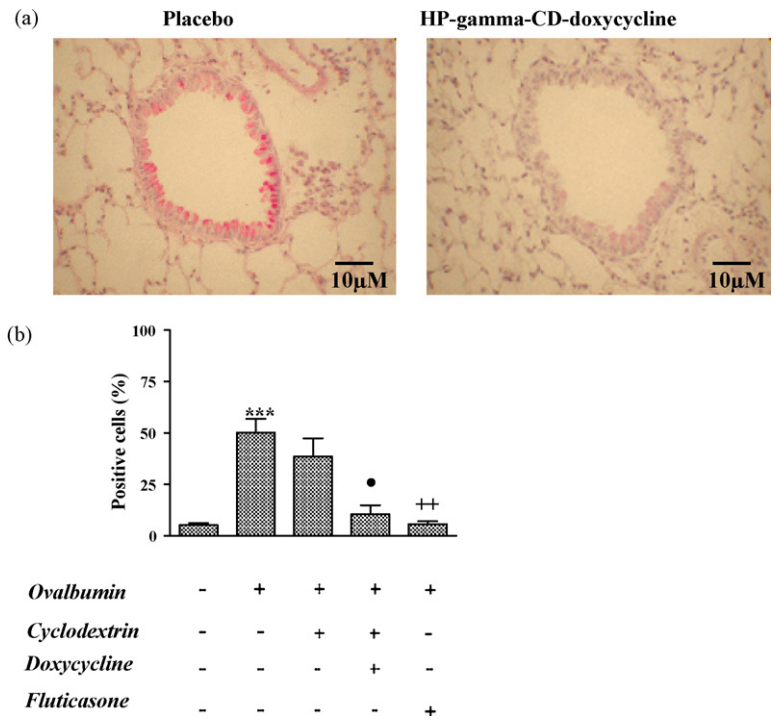

Fig. 4 – Determination of glandular hyperplasia by PAS staining in the long-term exposure model: (a) microphotographs of representative bronchial sections. Left panel: ovalbumin-exposed mice treated with excipient only (HP-gamma-CD) (400×). Right panel: ovalbumin-exposed mice treated with HP-gamma-CD-doxycycline (400×). (b) Quantitative assessment of mucus producing cells detected by PAS staining. Results are expressed as mean percentages of mucus cells in the epithelium. Error bars represent the S.E.M. Numbers of mice per group: no therapeutic intervention and sham-exposed mice, $n = 8$ (row 1); placebo-exposed (PBS), $n = 25$ (row 2); HP-gamma-cyclodextrin (CD), $n = 22$ (row 3); HP-gamma-cyclodextrin-doxycycline (DO), $n = 24$ (row 4); fluticasone (FL), $n = 17$ (row 5). PBS, CD, DO, and FL groups were exposed to allergens (OVA). $^{***}p < 0.001$ compared to sham-exposed mice; $^{\bullet}p = 0.05$ compared to HP-gamma-cyclodextrin; $^{++}p = 0.01$ compared to OVA-exposed mice. (c) Measurement of thickness of basement membrane in the long-term exposure model: effects of inhaled placebo, excipient, doxycycline, and fluticasone on allergen-induced thickening. The basement membrane was measured using ImageJ program (see Section 2). The bar graphs represent the mean and error bars correspond to S.E.M. Numbers of mice per group: no therapeutic intervention and sham-exposed mice, $n = 8$ (row 1); placebo-exposed (PBS), $n = 25$ (row 2); HP-gamma-cyclodextrin (CD), $n = 22$ (row 3); HP-gamma-cyclodextrin-doxycycline (DO), $n = 24$ (row 4); fluticasone (FL), $n = 17$ (row 5). PBS, CD, DO, and FL groups were exposed to allergens (OVA). $^{***}p < 0.001$ compared to sham-exposed mice; $^{\bullet}p < 0.05$ compared to HP-gamma-cyclodextrin-doxycycline complex; $^{+++}p < 0.001$ compared to OVA-exposed mice. (d) Microphotographs of lung sections (200×) stained with Masson's Trichrome in placebo-exposed mice (left panel) and HP-gamma-cyclodextrin-doxycycline complex (right panel). (e) Quantification of collagen deposition in the airways in long-term exposure model: effects of inhaled placebo, excipient, doxycycline, and fluticasone on allergen-induced collagen deposition. Results are expressed as mean ± S.E.M. Numbers of mice per group: no therapeutic intervention and sham-exposed mice, $n = 8$ (row 1); placebo-exposed (PBS), $n = 25$ (row 2); HP-gamma-cyclodextrin (CD), $n = 22$ (row 3); HP-gamma-cyclodextrin-doxycycline (DO), $n = 24$ (row 4); fluticasone (FL), $n = 17$ (row 5). PBS, CD, DO, and FL groups were exposed to allergens (OVA). $^{***}p < 0.001$ compared to sham-exposed mice; $^{\bullet\bullet\bullet}p < 0.001$ compared to HP-gamma-cyclodextrin; $^{+++}p < 0.001$ compared to OVA-exposed mice. (f) Measurements by using immunohistochemistry of the thickness of smooth muscle layer around the bronchi: effects of inhaled placebo, excipient, doxycycline, and fluticasone on allergen-induced thickening. Results are expressed as mean ± S.E.M. Numbers of mice per group: no therapeutic intervention and sham-exposed mice, $n = 8$ (row 1); placebo-exposed (PBS), $n = 25$ (row 2); HP-gamma-cyclodextrin (CD), $n = 22$ (row 3); HP-gamma-cyclodextrin-doxycycline (DO), $n = 24$ (row 4); fluticasone (FL), $n = 17$ (row 5). PBS, CD, DO, and FL groups were exposed to allergens (OVA). $^{***}p < 0.001$ compared to sham-exposed mice; $^{\bullet\bullet\bullet}p < 0.001$ compared to HP-gamma-cyclodextrin; $^{+++}p < 0.001$ compared to OVA-exposed mice.

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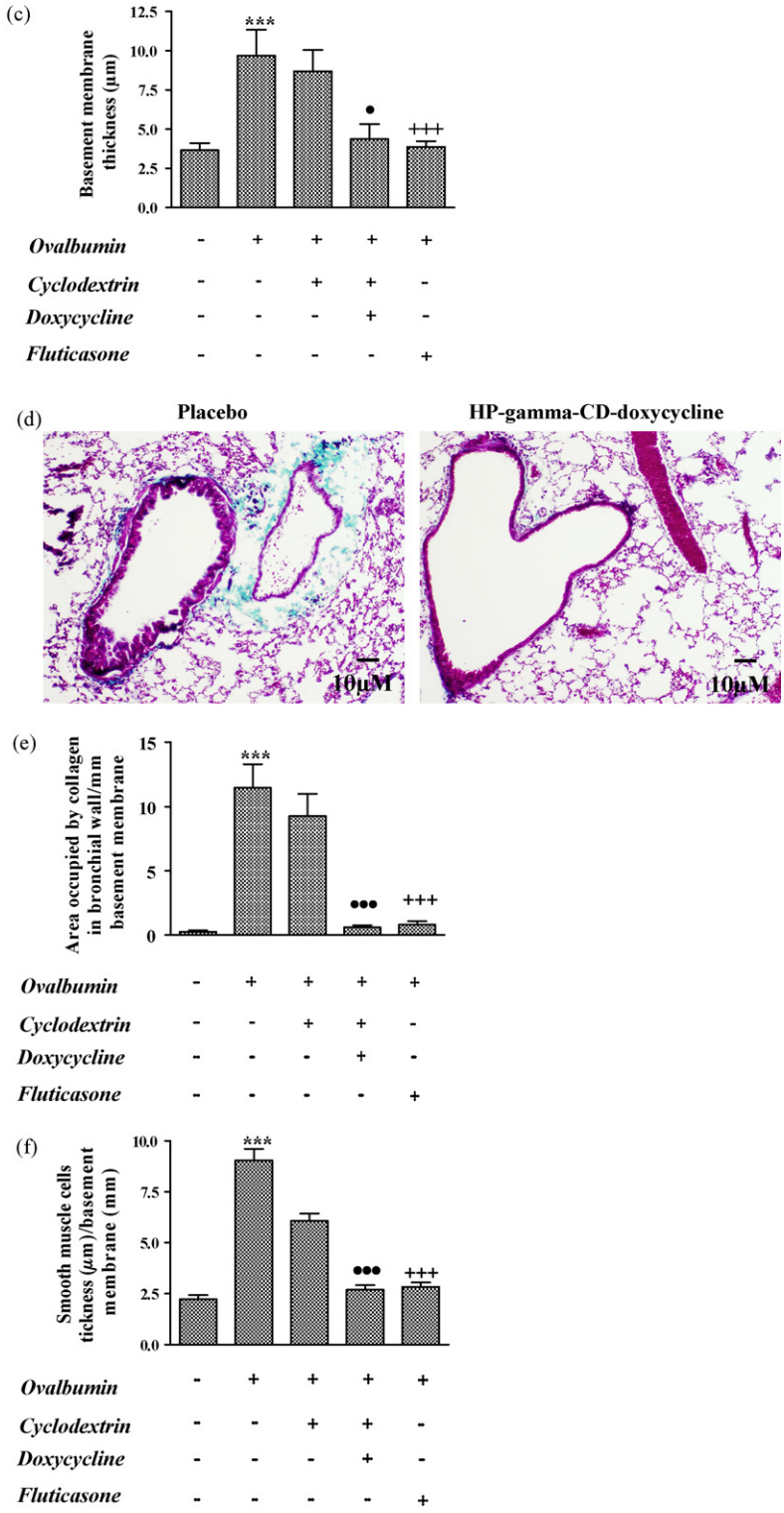

Fig. 4. (Continued).

3.8. *Measurements of allergen-specific IgE in serum and cytokine levels in bronchoalveolar lavage and lung protein extracts*

OVA-specific IgE levels were increased in serum after allergen exposure ($p < 0.05$) and there were no difference between the experimental groups exposed to allergen inhalation (data not shown). In the short-term exposure protocol, we measured levels of CCL-11 (eotaxin), IL-4, IL-5, IL-10, IFN-gamma and IL-13 in lung protein extracts. IFN-gamma was not detectable in almost all our samples. CCL-11 levels did not show any significant difference (data not shown). IL-13 and IL-5 were

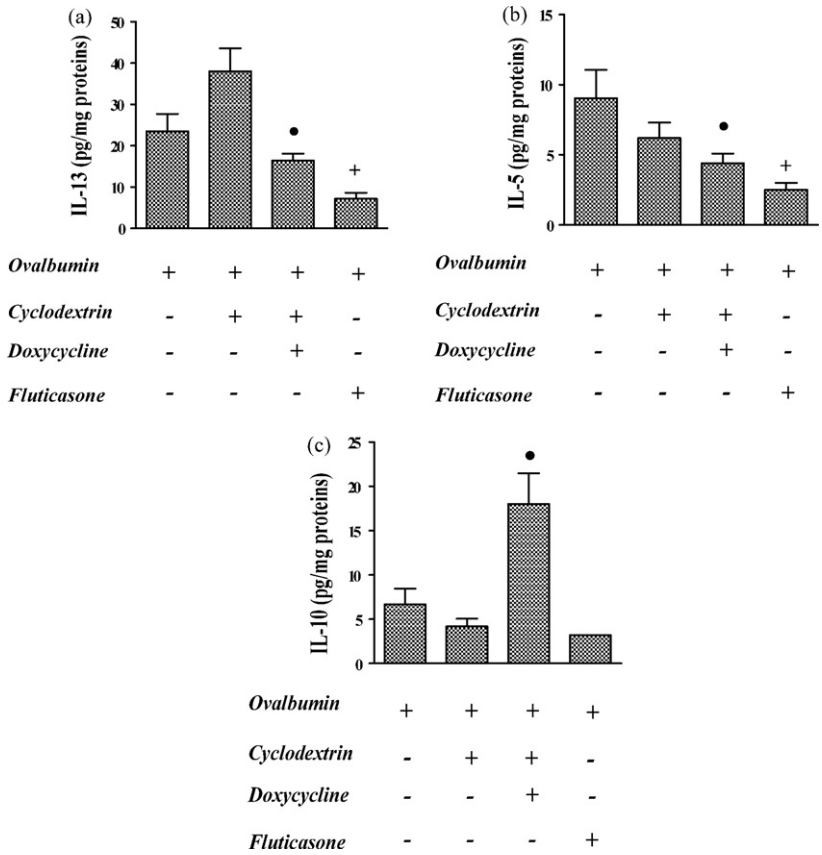

Fig. 5 – Levels of mediators in lung protein extracts after allergen exposure. ELISA measurement of IL-13 (a), IL-5 (b) and IL-10 (c) levels in the short-term allergen exposure model. Results are expressed as mean ± S.E.M. Numbers of mice per group: placebo-exposed (PBS), $n = 25$ (row 1); HP-gamma-cyclodextrin (CD), $n = 22$ (row 2); HP-gamma-cyclodextrin-doxycycline (DO), $n = 24$ (row 3); fluticasone (FL), $n = 17$ (row 4). PBS, CD, DO, and FL groups were exposed to allergens (OVA). $^{*}p < 0.05$ compared to HP-gamma-cyclodextrin; $^{+}p < 0.05$ compared to OVA-exposed mice.

both increased in the lung protein extracts of mice exposed to allergen and placebo or vehicle. Such an increase was inhibited by a previous exposure to HP-gamma-CD-doxycycline or fluticasone ($p = 0.05$ and $p < 0.05$ for IL-13 and IL-5, respectively) (Fig. 5a and b). Most importantly, we found an increase of IL-10 levels in lung protein extracts after the inhalation of HP-gamma-CD-doxycycline when compared to mice exposed to placebo (PBS) or to HP-gamma-CD ($p < 0.05$). In sharp contrast, IL-10 levels were not modified in the group treated by fluticasone (Fig. 5c).

3.9. *MMP activity and MMP-2 and -9 levels in lung proteins extracts*

Global MMP activity was assessed in crushed lung tissue by using a fluorescent substrate in the short-term allergen exposure group. MMP-derived enzymatic activity was significantly increased after allergen exposure as compared to sham-challenged mice. Exposure to inhaled doxycycline or fluticasone significantly reduced global MMP activity when compared to mice exposed to placebo or to vehicle alone ($p < 0.05$) (Fig. 6a). Similar results were found in the long-term exposure model. Mice exposed to OVA aerosols displayed higher MMP activity than sham-exposed mice. Doxycycline or fluticasone inhalation performed before each allergen exposure decreased global MMP activity as compared to mice exposed to placebo inhalation ($p < 0.05$) (Fig. 6b).

MMP-9 and -2 levels, in pro- and activated forms, were detected by zymography in the two protocols. Representative example of zymograms obtained in the long-term exposure protocol is shown in Fig. 6c. In long-term exposure model, levels of MMP-9 and the MMP-9/pro-MMP-9 ratio were drastically reduced after the treatment by HP-gamma-CD-doxycycline or fluticasone when compared to OVA-challenged placebo-exposed mice ($p = 0.005$ and $p < 0.01$, respectively) (Fig. 6d). This huge decrease was also found for pro-MMP-2 levels ($p = 0.001$ and $p = 0.005$, respectively).

4. Discussion

We demonstrate for the first time in the current study that inhaled doxycycline, in an adequate formulation with HP-gamma-CD, inhibits the allergen-induced airway reactivity, eosinophilic infiltration in BAL compartment and in bronchial walls, and peribronchial inflammation. Furthermore, we provide evidence for the first time that inhaled doxycycline prevents allergen-induced bronchial morphological changes

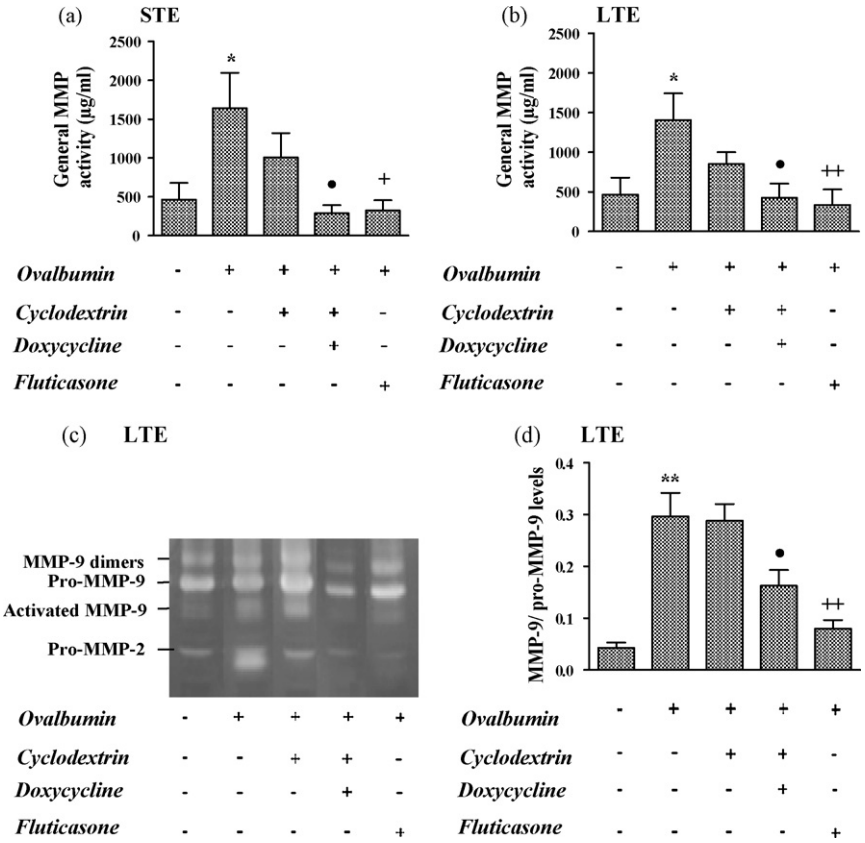

Fig. 6 – Measurements of MMP activity and MMP-2 and -9 levels in lung protein extracts. Quantification of general MMP activity in the short-term exposure protocol (a) and in the long-term exposure protocol (b). Data are a mean of value measured for 10 mice per group and are expressed in µg/ml. Representative examples of zymograms performed on lung protein extracts in the short-term exposure protocol (c). Quantification of MMP-9/pro-MMP-9 ratio in the long-term exposure protocol (d). Numbers of mice per group: no therapeutic intervention and sham-exposed mice, $n = 8$ (row 1); placebo-exposed (PBS), $n = 25$ (row 2); HP-gamma-cyclodextrin (CD), $n = 22$ (row 3); HP-gamma-cyclodextrin-doxycycline (DO), $n = 24$ (row 4); fluticasone (FL), $n = 17$ (row 5). PBS, CD, DO, and FL groups were exposed to allergens (OVA). $^{*}p < 0.05$ compared to sham-exposed mice; $^{\bullet}p \leq 0.05$ compared to HP-gamma-cyclodextrin; $^{+}p \leq 0.05$ compared to OVA-exposed mice; $^{**}p < 0.005$ compared to sham-exposed mice; $^{++}p < 0.005$ compared to ovalbumin.

and thus inhibits glandular hyperplasia, airway wall and smooth muscle layer thickening and peribronchial collagen deposition. Effects of doxycycline on tissue remodeling were comparable to those obtained after exposure to fluticasone, an inhaled steroid on the market used in the current study as a reference therapy. Mechanisms of pharmacological activity of doxycycline in the context of bronchial inflammation were investigated and the main findings are a doxycycline-induced MMP inhibition and impairment of Th_2 inflammation. Indeed, doxycycline exposure leads to a significant increase in the anti-inflammatory cytokine IL-10 and a decrease of IL-5 and IL-13 levels in the lung parenchyma. Doxycycline also reduced MMP-9 activation in the lungs from mice exposed to allergens.

In line with our previous works demonstrating that MMPs and more specifically MMP-9 play a critical role in allergen-induced airway inflammation [7,12,13,15,31], we designed the present work as an attempt to control airway inflammation and remodeling by a molecule known to be a potent MMP inhibitor. Doxycycline was selected because its MMP inhibitory activity was confirmed by numerous studies and because this molecule was shown to have a very low toxicity in human adults. To our knowledge, this is the first demonstration that an inhaled non-steroidal drug (doxycycline) exerts an effect on both airway inflammation and airway remodeling features such as Goblet cells hyperplasia, collagen deposition and basement membrane and smooth muscle cells layer thickness. Other researchers have published an interesting report on the inhibition of toluene diisocyanate-induced airway inflammation by doxycycline [32]. In that paper, however, the drug was given orally at high dosages, higher than those currently recommended for antimicrobial therapy and remodeling parameters were not assessed. In the present study, mice were challenged with the allergen by an exposure to a new formulation given by aerosols and not by intratracheal instillation. Our model may therefore mimic more closely the clinical situation by the use of a well-characterized model of allergen-induced inflammation and airway remodeling in mice. By using a topical therapeutical approach allowed by an original galenic formulation, our study clearly shows that an inhaled doxycycline-based therapy for asthma is of potential

interest and is worth to be confirmed in other animal models and possibly in humans.

Doxycycline in solution without excipient has a very low pH and accidental tetracycline inhalation has been reported to be responsible for toxic pneumonia [33]. In addition, doxycycline is only poorly soluble at pH 7.4 and is not stable with a tendency to precipitate rapidly. We therefore used cyclodextrins (HP-gamma-CD) for increasing the solubility and stability of doxycycline at physiological pH. We did previously describe that inhaled HP-gamma-CD was non-toxic and did not provoke by itself some modifications of the airway architecture or induce inflammation [29]. The selection of this specific cyclodextrin is based on its higher potency to increase doxycycline solubility as compared to different related compounds (data not shown), and mainly on its safety profile [29]. In the present study, we demonstrate that HP-gamma-CD does not interfere with the inflammatory response and does not bear *per se* any pharmacological activity when used in a topical lung application in an inflammatory airway disease. Furthermore, we demonstrated previously that HP-gamma-CD does not modify the aerosol characteristics in terms of droplet size and nebulization speed in our experimental conditions [29].

To date, there are still controversies about the exact mechanism for MMP inhibition by tetracyclines. In the present study, we investigated whether the topical exposure by inhalation to doxycycline modulated the expression of key Th_2 and anti-inflammatory cytokines and we found IL-5 and IL-13 production being decreased and, interestingly, a large increase in the anti-inflammatory cytokine IL-10 production. To our knowledge, this is the first report of a decreased production of the Th_2 cytokines IL-5 and IL-13 after tetracycline exposure. Due to the properties of those two cytokines, this observation could explain at least in part the decreased airway responsiveness, reduced eosinophilic inflammation and airway remodeling prevention [5,6,34]. To date, the precise mechanism of action of the inhaled steroid fluticasone leading to control of inflammation is also not really well understood. However, our result suggests different mechanism of action between fluticasone and Doxycycline regarding the differences found in their respective effects on IL-10 production. The finding of an increased IL-10 production is of great interest since a relative IL-10 deficiency could favour asthma. Indeed, the number of IL-10-producing T cells has been reported to be lower in severe asthmatics when compared to milder patients [35] and IL-10 production is increased in the BAL cells of mice exposed to an immunotherapy protocol [36]. IL-10 has also been shown to decrease eosinophil survival and cytokine secretion [37] and could even mediate a part of the anti-inflammatory effects of steroid [38]. Finally, IL-10 interacts with the MMP metabolism since it inhibits MMP-2 and -14 transcription in some cancer cell lines [39,40]. Taken together, these data suggest that doxycycline-induced IL-10 production could inhibit the allergen-induced airway inflammation and airway remodeling (collagen deposition and membrane thickening) both indirectly by diminishing the venue of inflammatory cells and directly by inhibiting MMP transcription. Altogether, our data about cytokine measurements suggest an inhibition of Th_2 inflammation and a possible stimulation of T regulatory lymphocytes (T_{reg}) by doxycycline inhalation [32].

Doxycycline decreases the inducible nitric oxide synthase (NOS) activity as demonstrated *in vitro* [41] and in an animal model of LPS exposure [42]. As a correlation has been found between nitric oxide production and the extent of airway remodeling in humans [43], another possible explanation of our findings could reside in an inhibition of nitric oxide synthesis by tetracyclines.

Nevertheless, direct MMP inhibition by doxycycline is thought to be the main cause of many pharmacological effects of tetracyclines. Indeed, doxycycline has been proposed as a therapy to reduce MMP activation and expression (particularly MMP-2 and -9) in abdominal aortic aneurysms [44] and has been reported to decrease pathological remodeling after myocardial infarction in rat [45]. Doxycycline has the ability to inhibit MMPs belonging to the gelatinase subfamily [17,46] but this compound also inhibit other MMPs as MMP-3 and MMP-7 [47]. Moreover, doxycycline has been described to have putative inhibitory effects on members of collagenase subfamily of MMPs. Indeed, doxycycline is quite effective on MMP-8 and MMP-13 inhibition and, in a lesser extent, on MMP-1 [19,48]. In the present study, we can not rule out, however, that an inhibition of non-gelatinase members of the MMP family could cause some side effects. In the present work, mice exposed to doxycycline for up to 96 days did not display any sign of exaggerated inflammation or any visible toxicity. In the present study we demonstrate that, after doxycycline exposure, global MMP activity in lung tissue is decreased and that the proportion of activated MMP-9 is lower than measured in vehicle-exposed mice. This strongly suggests that, in our study, a significant part of the pharmacological effect obtained with doxycycline is linked to a direct MMP inhibition as the central role of MMP-9 in the allergen-induced inflammation has been described elsewhere using the same protocol of allergen immunisation and exposure.

In conclusion, the administration of doxycycline by aerosols in a complex with HP-gamma-CD prevents the allergen-induced airway inflammation and remodeling by modulation of both MMPs and interleukins. Doxycycline should be considered for other studies designed to confirm its disease modifying agent role in asthma. Our results suggest that inhaled doxycycline should be investigated as a potential new therapy for asthma.

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