Targeting IL-5 pathway against airway hyperresponsiveness: a challenge between benralizumab and mepolizumab

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Abstract

Background and Purpose Airway hyperresponsiveness (AHR) is a central abnormality in asthma. Interleukin-5 (IL-5) may modulate AHR in animal models of asthma, but inconsistent data are available on the impact of targeting IL-5 pathway against AHR. The difference between targeting IL-5 or IL-5Rα in modulating AHR remains to be understood in human airways. The aim of this study was to compare the role of the anti-IL-5Rα benralizumab and the anti-IL-5 mepolizumab against AHR, and to assess whether these agents influence the levels of cyclic adenosine monophosphate (cAMP). Experimental Approach Passively sensitized human airways were treated with benralizumab and mepolizumab. The primary endpoint was the inhibition of AHR to histamine; the secondary endpoints were the protective effect against AHR to parasympathetic activation and mechanical stress, and the tissue modulation of cAMP. Key Results Benralizumab and mepolizumab significantly ($P<0.001$ vs. positive control) prevented the AHR to histamine (maximal effect -134.14±14.93% and -108.29±32.16%, respectively), with benralizumab being 0.73±0.10 logarithm significantly ($P<0.05$) more potent than mepolizumab. Benralizumab and mepolizumab significantly ($P<0.001$ vs. positive control) inhibited the AHR to transmural stimulation and mechanical stress. Benralizumab was 0.45±0.16 logarithm significantly ($P<0.05$) more potent than mepolizumab against AHR to parasympathetic activation. The effect of these agents was significantly correlated ($P<0.001$) with increased levels of cAMP. Conclusion Targeting the IL-5/IL-5Rα axis is an effective strategy to prevent the AHR. Benralizumab resulted more potent than the mepolizumab and the concentration dependent beneficial effects of both these agents were related with improved levels of cAMP in hyperresponsive airways.

Abbreviations

ADCC: antibody-dependent cell-mediated cytotoxicity; AHR: airway hyperresponsiveness A kinase: cAMP-dependent protein kinase; ANOVA: analysis of variance; ASM: airway smooth muscle; cAMP: cyclic adenosine monophosphate; CRC: concentration-response curves; EC_n: concentration inducing n% E_{max}; EF_n: frequency inducing n% E_{max}; E_{max}: maximal effect; EFS: electrical field stimulation; FEV_1: forced expiratory volume in 1st second; FRC: frequency-response curves; GINA: Global Initiative for asthma; IC_{50}: half maximal inhibitory concentration; IgE: immunoglobulin E; IL-5: interleukin-5; IL-5Rα: IL-5 receptor α· KH: Krebs-Henseleit buffer solution; LAMA: long-acting muscarinic antagonist mAb: monoclonal antibody; MBP: major basic protein; NK cells: natural killer cells; PK: pharmacokinetic; QS: quick stretch; SEM: standard error of the mean; Th2:T-helper type 2.

Bullet point summary

What is already known

Targeting IL-5 pathways is an effective therapeutic strategy to treat severe asthmatic patients.

What this study adds

This is the first study that pharmacologically characterized the impact of benralizumab and mepolizumab in a human ex vivo model of AHR, by providing mechanistic evidences concerning the deleterious effect of IL-5 in isolated airways.

Clinical significance

Benralizumab and mepolizumab are effective in preventing AHR in a concentration dependent manner, with benralizumab being more potent than mepolizumab.

At the approved doses these agents only partially inhibit the parasympathetic hyperresponsiveness in human airways.

Introduction

Airflow limitation represents one of the most important and treatable characteristics in asthma. Together with other traits such as inflammation, the level of airflow limitation triggers the risk of asthma attack (Pavord et al., 2018). Airflow limitation is mainly due to repeated contraction of airway smooth muscle (ASM), inflammatory oedema of the airway wall and intraluminal factors (Pavord et al., 2018). Airway hyperresponsiveness (AHR) is a central abnormality in patients with asthma that induces enhanced sensitivity to a wide variety of stimuli, leading to an increased narrowing of the airways in vivo (Rabe, 1998).

In the recent years passive sensitization, a validated procedure that reproduces ex vivo the AHR typical of asthmatic airways in vivo (Mitchell et al., 1997; Rabe, 1998; Schaafsma et al., 2006; Schmidt et al., 1999), has been extensively applied to pharmacologically characterize the impact of several medications recommended in Step 1 – 4 therapy of asthma (Calzetta et al., 2018b; Cazzola et al., 2016b; Rogliani et al., 2020). Interestingly, passive sensitization has been used also to assess the effect of omalizumab, a monoclonal antibody (mAb) recommended in Step 5 therapy of asthma, on the contractile tone of human hyperresponsive isolated bronchi (Berger et al., 2007). Interleukin-5 (IL-5) plays a pivotal role in modulating AHR in vivo in animal models of airway sensitization and asthma (Hamelmann et al., 1997; Leckie et al., 2000), and passive sensitization of ASM cells elicits sequential autocrine and paracrine release of IL-5 resulting in altered contractility (Damera et al., 2011; Gounni et al., 2005).

Benralizumab, a humanized anti-IL-5 receptor α (IL-5Rα) mAb, blocks IL-5 signaling and hence type-2 inflammation while inducing antibody-dependent cell-mediated cytotoxicity (ADCC) of eosinophils and basophils (European Medicines Agency, 2018; US Food and Drug Administration, 2017). On the other hand mepolizumab, a humanized anti-IL-5 mAb that prevents IL-5 from binding its receptor on the surface of eosinophils and basophils, modulates type-2 inflammation occurring in approximately 50% of patients with asthma (European Medicines Agency, 2015; Farne et al., 2017; US Food and Drug Administration, 2015). The treatments with benralizumab and mepolizumab are recommended in Step 5 therapy of asthma and have been shown to induce clinical improvement in patients suffering from severe eosinophilic disease (Bleecker et al., 2016; FitzGerald et al., 2016; Nair et al., 2017; Ortega et al., 2014).

Indeed, robust evidence generated by clinical trials consistently indicated that benralizumab (Bleecker et al., 2016; FitzGerald et al., 2016) is effective in improving lung function expressed as forced expiratory volume in 1st second (FEV_1). Conversely, conflicting data are available with respect to the impact of mepolizumab on lung function, with some studies reporting no effect on FEV_1 (Flood-Page et al., 2007; Haldar et al., 2009) and others showing some improvement in FEV_1 (Ortega et al., 2014; Pavord et al., 2012) that however was generally smaller than that induced by benralizumab (Bleecker et al., 2016; FitzGerald et al., 2016). A greater numerical effect of benralizumab compared to mepolizumab on $FEV_1$was reported also by a Cochrane analysis (Farne et al., 2017).

Beyond the effect of anti-IL-5 and anti-IL-5Rα mAbs on lung function, although it is recognized that IL-5 itself may modulate AHR, further inconsistent data are currently available also concerning the real efficacy of targeting IL-5 pathway in preventing AHR, at least in animal models of asthma. In this respect, while some studies indicated that acting on IL-5 pathway may inhibit AHR (Mauser et al., 1995; Nag et al., 2003; Shardonofsky et al., 1999), others failed to report any effect on abnormal ASM contractility (Eum et al., 2005; Mathur et al., 1999; Tanaka et al., 1998).

In this scenario the difference between targeting IL-5 or IL-5Rα in modulating AHR remains to be fully established. Therefore, also considering that unlike omalizumab (Berger et al., 2007) neither benralizumab nor mepolizumab have been pharmacologically characterized in human hyperresponsive airways, the aim of this study was to compare the efficacy and potency of benralizumab and mepolizumab in passively sensitized human airways, and to identify whether acting on the IL-5/IL-5Rα axis may protect against AHR by increasing the tissue synthesis of cyclic adenosine monophosphate (cAMP).

Materials and methods

Tissue collection and preparation

Regions of lungs were taken from uninvolved areas of neoplastic lesions and resected from 16 patients undergoing lobectomy surgery for lung cancer. Tissues were placed in Krebs-Henseleit buffer solution (KH) as previously described (Cazzola et al., 2011) and transported to the Laboratory of Respiratory Clinical Pharmacology at the University of Rome "Tor Vergata" (Italy) from a nearby hospital. None of the patients had been chronically treated with bronchodilators or corticosteroids, and serum immunoglobulin E (IgE) levels were in the normal range (<100 IU/ml). Preoperative lung function parameters were normal in all the patients who were not affected by chronic obstructive respiratory disorders. Detailed demographic and metric characteristics of donors are reported in e-Table 1.

In the laboratory, the airways were cut into rings (subsegmental bronchi: thickness 1-2 mm, diameter 4-6 mm) and transferred into a 10-ml High Tech 8 Channels Manual Compact Organ Bath system (Panlab Harvard Apparatus, Spain) containing KH buffer solution (37°C) and aerated with O_2/CO_2 (95:5%). Tissues were allowed to equilibrate and the KH buffer solution was constantly changed.

Passive sensitization

Isolated airways were rotated overnight at room temperature in tubes containing KH buffer solution in the presence of 10% vol^{-1} sensitizing serum (passively sensitized bronchi) or 10% vol^{-1} non-sensitizing serum (non-sensitized bronchi). Sensitizing and non-sensitizing sera were prepared by centrifugation from the whole blood. Sensitizing serum was obtained from a patient suffering from severe atopic asthma (total IgE 1,000 U ml^{-1} specific against common aeroallergens), whereas non-sensitizing serum was obtained from a non-atopic donor (total IgE 45 U ml^{-1}). The serum from the non-atopic donor was used as a negative control (C-). The subjects provided signed consent for serum donation. Sera were frozen at -80°C in 250 µl aliquots until required. The next morning bronchial tissues were transferred into the isolated organ bath system containing KH buffer solution (37°C) and continuously gassed with O_2/CO_2(95:5%). The passive sensitization is a model that closely mimics important functional characteristics of AHR in asthmatic patients, as previously reported (Schaafsma et al., 2006; Schmidt et al., 1999).

Transmural stimulation

Transmural stimulation, also called electrical field stimulation (EFS), was performed by placing tissues between two wire platinum electrodes (20 mm apart, Panlab Harvard Apparatus, Spain), connected to a 3165 multiplexing pulse booster stimulator (Ugo Basile, VA - Italy). Bronchial rings were contracted by EFS at increasing frequencies ($EFS_{1\text{-}50Hz}$, 10V, 10s, 0.5ms, biphasic pulse) in order to simulate the vagus nerve firing (parasympathetic pathway) observed in human in vivo and thus producing frequency-response curves (FRCs) via endogenous cholinergic contractile response (Cazzola et al., 2011).

Preparation of drugs

The following drugs were used: benralizumab (Creative Creative Biolabs Inc., NY, USA) diluted in distilled water, histamine (Sigma-Aldrich, Milan, Italy) diluted in distilled water, and mepolizumab (Creative Creative Biolabs Inc., NY, USA) diluted in distilled water. Compounds were stored in small aliquots at -80°C until their use.

Contraction measurement

Bronchial rings were connected to isometric force transducers Fort25 (WPI, UK). The signal was amplified by a Powerlab 8/36 and Octal Bridge Amp system (AD instruments, UK), recorded and analyzed via the LabChart 7 interface software (AD instruments, UK). Tissues were mounted on hooks, and attached with thread to a stationary rod and the other tied with thread to an isometric force displacement transducer. Airways were allowed to equilibrate by flushing with fresh KH buffer solution. Passive tension was determined by gentle stretching of tissue (0.5–1.0 g) during equilibration. The isometric change in tension was measured by the transducer and the tissue vitality assessed by EFS_{25Hz}. These procedures allowed the bronchial rings to be correctly positioned between the hooks. When the passive contractile tone reached the plateau, rings were washed three times with fresh KH buffer solution and allowed to equilibrate for further 45 min.

Experimental design

Study characteristics

This study was designed as an ex vivo, prospective, randomized, negative and positive controlled, blinded, parallel groups, head-to-head comparison between benralizumab and mepolizumab.

Endpoints

The primary endpoint of this study was to assess the protective effect of benralizumab and mepolizumab against the AHR elicited by histamine in hyperresponsive human bronchi.

The secondary endpoints included the impact of benralizumab and mepolizumab against the AHR elicited by EFS and mechanical stress induced by quick stretch (QS), and the effect of these mAbs on the modulation of cAMP levels in hyperrespnsive bronchial tissue.

Study 1. Effect of benralizumab and mepolizumab on the CRC to histamine

Concentration-response curves (CRCs) to histamine were generated in isolated airways. The CRCs were produced via the cumulative administration of histamine. Airways were stimulated with histamine until a stable level of contractility was reached for each concentration, usually for 5-15 min. After that, the next concentration of agonist was administered. Experiments were performed in C-, passively sensitized airways (positive control, C+), and in passively sensitized airways that were incubated overnight with different concentrations of either benralizumab or mepolizumab during the sensitizing procedure.

Study 2. Effect of CRCs to benralizumab and mepolizumab on the contractile plateau to specific histamine concentrations

Histamine was administered at concentrations inducing different levels of contractile plateau to isolated airways that were previously incubated overnight with different concentrations of either benralizumab or mepolizumab during the sensitizing procedure. The concentrations of histamine used to elicit the specific contractile plateau were the half maximal effective concentration (EC_{50}), the 70% effective concentration (EC_{70}), and the 90% effective concentration (EC_{90}) with respect to the maximal contractile response [E_{max}] detectable in C+ isolated bronchi. The response to the different concentrations of each specific histamine concentration was recorded until the contractile plateau was reached, usually for 5-15 min. Experiments were performed also in C- and C+ tissues.

Study 3. Effect of benralizumab and mepolizumab on the FRC to EFS

FRCs to $EFS_{1\text{-}50Hz}$ were generated in isolated airways. Each EFS was delivered after that the contractile response induced by the previous EFS was terminated, usually in 3-5 min. Experiments were performed

in C- and C+ tissues, and in passively sensitized airways that were incubated overnight with different concentrations of either benralizumab or mepolizumab during the sensitizing procedure.

Study 4. Effect of CRCs to benralizumab and mepolizumab on the contractile response to specific EFS frequencies

Isolated airways were stimulated by EFS delivered at frequencies inducing different levels of contractile response to isolated airways that were previously incubated overnight with different concentrations of either benralizumab or mepolizumab during the sensitizing procedure. The EFS frequencies delivered to elicit the specific contractile responses were the half maximal effective frequency (EF_{50}), the 70% effective frequency (EF_{70}), and the 90% effective frequency (EF_{90}) with respect to the E_{max}detectable in C+ isolated bronchi. Each specific EFS frequency was delivered after that the contractile response induced by the previous EFS was terminated, usually in 3-5 min. Experiments were performed also in C- and C+ tissues.

Study 5. Effect of benralizumab and mepolizumab on the contractile response to QS

QS of 0.5 mm was elicited in isolated airways, as previously described (Mitchell et al., 1997). Briefly, a calibrated thumbscrew enabled the accurate measurement of the angular rotation of the threaded rod that was used to induce QS. Since the pitch of the screw was 1.0 mm, a rapid (<200 ms) 180° rotation was performed to elicited a QS of 0.5 mm on the bronchial rings. The resulting myogenic contractile response was recorded for 10 min. QS was performed in C- and C+ tissues, and in passively sensitized airways that were incubated overnight with different concentrations of either benralizumab or mepolizumab during the sensitizing procedure.

Quantification of IL-5 and cAMP

The supernatant of C- and C+ tissues was collected at different time-points during the overnight incubation in the presence of sensitizing and non-sensitizing sera in order to assess the release of IL-5 from the bronchial tissue. The concentrations of IL-5 were quantified by using human IL-5 ELISA kit (RayBiotech, Inc., GA, USA) according to manufacturers' instructions (available at: https://www.raybiotech.com/files/manual/ELISA/ELH-IL5.pdf).

At the end of experiments, isolated bronchi treated with either benralizumab or mepolizumab were collected along with C- and C+ airways in order to quantify the tissue levels of cAMP by using a cAMP ELISA kit (RayBiotech, Inc., GA, USA) according to manufacturers' instructions (available at: https://www.raybiotech.com/files/manua CAMP.pdf).

Time controls

For every seven bronchial rings mounted in the isolated organ bath system, one was used as a time control.

Analysis

Pharmacological analysis

The ASM contractile force in response to histamine, EFS, and QS was reported as mg normalized for 100 mg of bronchial tissue (mg/100mg). The protective effect of benralizumab and mepolizumab against AHR induced by contractile stimuli in passively sensitized airways was expressed as the percentage of the maximal AHR inhibition, that in turn was identified as the delta between the AHR induced in C+ airways and the normal contractile response elicited in C- airways. Since the effect of each single concentration of the mAbs investigated in this research was tested after overnight incubation during the sensitizing procedure, the CRCs to benralizumab and mepolizumab on the contractile plateau to specific histamine concentrations and EFS frequencies were generated by plotting in the same graph the data originated from different bronchial rings treated with different concentrations of the mAbs. Appropriate curve-fitting to sigmoidal models were used to calculate the effect (E), E_{max}, EC_{50}, EC_{70}, EC_{90}, EF_{50}, EF_{70}, EF_{90}, and the half maximal inhibitory concentration (IC_{50}). The equations used to describe the models used in this study were: Y=Bottom + (Top-Bottom)/(1+10^((LogEC$_{50}$_or_EF$_{50}$-X))) and Y=Bottom + (Top-Bottom)/(1+10^((X-LogIC$_{50}$))). EC_{50},

EF_{50}, and IC_{50}were transformed in pEC_{50} (-$LogEC_{50}$), pEF_{50} (-$LogEF_{50}$), and pIC_{50} (-$LogIC_{50}$) to perform the statistical analysis of the potency .

Data originated from ELISA kits were elaborated after normalization for the wet weight (100 mg) of isolated airways.

Group size, randomization, blinding, data and statistical analysis

No published data are currently available concerning the impact of benralizumab and mepolizumab against AHR in in human isolated bronchi. The only paper regarding the effect of a mAb on the AHR induced by histamine in passively sensitized bronchi has been performed on the anti-IgE mAb omalizumab (Berger et al., 2007). The results of that study reported that repeating experiments on n=4 different bronchi permitted to detect a small but significant reduction (-[?]30%, $p<0.05$) of histamine-induced AHR in passively sensitized bronchi treated with omalizumab, compared to control tissue (Berger et al., 2007).

In the present study it is not possible the calcutation of the sample size due by the fact that no studies have been conducted so far on neither benralizumab nor mepolizumab in an experimental setting by using passively sensitized human isolated bronchi. However we can set n=5 as the number of repetitions required to have >95% possibility ($p<0.05$) to detect, with at least 80% power, a relatively small but significant alteration of bronchial contractility induced by benralizumab and mepolizumab, accordingly with the results reported by Berger and colleagues (Berger et al., 2007). This sample size satisfies the guidelines of the British Journal of Pharmacology for preclinical studies, where n refers to independent values and not replicates (available at: https://bpspubs.onlinelibrary.wiley.com/hub/journal/14765381/author-guidelines.html).

The total number of bronchial rings necessary to complete the study was n=366, including C-, C+, and time control tissues. Isolated airways were collected from at least n=5 different donors. The treatment with each specific concentration of either benralizumab or mepolizumab was performed by using specimens collected from the same patient, and experiments were repeated 5 times in samples originating from 5 different donors. In the case the amount of samples from a subject did not permit to test all the benralizumab and mepolizumab concentrations in the same experiment, the remaining concentrations were assessed in specimens collected from other patients in parallel with further C-, C+, and time controls in order to provide controlled results not affected by potential bias.

The protective effects of different concentrations benralizumab and mepolizumab against AHR were compared each other and with C- and C+ tissues. Values were reported as mean+-standard error of the mean (SEM), the statistical significance was assessed by the Student's t -test and analysis of variance (ANOVA), and the level of statistical significance was defined as $P<0.05$. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). Detailed dataset of sectional tissues is reported in e-Table 2. Data were collected and managed in order to perform pre-specified statistical analysis; any so called "p-hacking" were avoided in order to prevent that any potential bias could affect the robustness of results, as reported by Head and colleagues (Head et al., 2015).

Each bronchial ring was randomly assigned to a specific treatment by using a computer generated sequence. All the study procedures were performed under blinded condition, in which both the operator and data analysis were blinded.

All data analysis was performed using computer software GraphPad Prism 5 (La Jolla, CA, USA) and OpenEpi (available at: https://www.openepi.com) was used for the power calculation and randomization.

Ethics approval and consent to participate

Ethical approval (R.S. 37/20, 2020; Independent Ethical Committee, Fondazione PTV Policlinico Tor Vergata) and informed consent were consistent with the National Committee of Bioethics and Committee for Bio-safety, Biotechnology and Life Sciences (available at: http://old.iss.it/binary/eric/cont/Informed_consent.pdf), the recommendations on the collection of biological samples for research purposes (available

at: https://search.coe.int/cm/Pages/result_details.aspx?ObjectID=09000016805d84f0), the ethical and legal recommendations concerning the biobank and the research biorepository (available at: https://www.oeci.eu/Documents/OEC Biobank.pdf), and the Comitato Nazionale per la Biosicurezza, le Biotecnologie e le Scienze per la Vita (Raccolta di campioni biologici a fini di ricerca, consenso informato, 2009; available at:http://bioetica.governo.it/media/3457/p2009 misto-2-raccolta-di-campioni-biologici-a-fini-di-ricerca-consenso-informato-it.pdf).

Results

Baseline characteristics of isolated airways

No significant differences (P>0.05) were found in the baseline characteristics of isolated airways used in this study. Passive sensitization induced significant AHR in C+ bronchi (P<0.05 vs. C-) and the maximal concentration of IL-5 was 12.21+-2.79 fold greater in C+ than in C- tissues (P<0.001, e-Figure 1). Further details are reported in the online supplement.

Primary endpoint

Effect of benralizumab and mepolizumab on the AHR to histamine

Both benralizumab and mepolizumab inhibited the AHR mediated by the histaminergic tone in passively sensitized bronchi.

The E_{max} elicited by the CRC to histamine was significantly reduced when passively sensitized airways were incubated with benralizumab at concentrations [?]1 μg/ml (-1348.33±126.51 mg/100mg bronchial tissue, P<0.001 vs. C+; Figure 2A), whereas at least 3 μg/ml of mepolizumab were necessary to significantly inhibit the histamine mediated AHR (-954.59±333.13 mg/100mg bronchial tissue, P<0.001 vs. C+; Figure 2B). When either benralizumab or mepolizumab were administered at concentrations [?]10 μg/ml, the AHR induced by CRC to histamine in passively sensitized bronchi was completely prevented, with the contractile response of hyperresponsive ASM reduced at the same level of that detectable in C- airways (P>0.05 vs. C-), or even significantly lower for benralizumab administered at 100 μg/ml (P<0.05 vs. C-) (Figure 2A and B). Benralizumab administered at concentrations [?]10 μg/ml significantly (delta pEC_{50} 0.65±0.20, P<0.05 vs. C+) reduced the potency of histamine in passively sensitized airways, whereas 100 μg/ml of mepolizumab were necessary to elicit the same significant impact (delta pEC_{50} 0.65±0.22, P<0.05 vs. C+) on the pEC_{50} of histamine. Details on the effect of different concentrations of benralizumab and mepolizumab on the pharmacological characteristics of CRC to histamine (E_{max} and pEC_{50}) in passively sensitized bronchi are reported in Table 1.

The CRCs to either benralizumab or mepolizumab modulated the contractile plateau to specific concentrations of histamine in hyperresponsive airways (Figure 3A-F). Specifically, both benralizumab and mepolizumab prevented the AHR in passively sensitized airways contracted by histamine administered at EC_{50} (Figure 3A,D), EC_{70} (Figure 3B,E), and EC_{90} (Figure 3C,F) in a potent concentration-dependent manner. Benralizumab resulted 0.73±0.10 logarithm significantly (P<0.05) more potent than mepolizumab (Table 2).

Secondary endpoints

Effect of benralizumab and mepolizumab on the AHR to EFS

Both benralizumab and mepolizumab inhibited the AHR mediated by the parasympathetic tone in passively sensitized bronchi.

The E_{max} elicited by the FRC to EFS was significantly reduced when hyperresponsive airways were incubated with benralizumab at concentrations [?]1 μg/ml (-269.36±47.21 mg/100mg bronchial tissue, P<0.001 vs. C+; Figure 4A) or mepolizumab at concentrations [?]10 μg/ml (-271.59±47.28 mg/100mg bronchial tissue, P<0.01 vs. C+; Figure 4B). Benralizumab and mepolizumab both normalized the contractile response induced by FRC to EFS in hyperresponsive ASM when these mAbs were administered at concentrations [?]10 μg/ml, leading to not significantly different FRCs compared with that inducible in C- tissue (P>0.05; Figure 4A and B). Neither benralizumab nor mepolizumab significantly modulated the potency of FRCs to EFS in passively

sensitized airways (P>0.05 vs. C+). Details on the effect of different concentrations of benralizumab and mepolizumab on the contractile response to FRCs induced by EFS ($E_{max}$and pEF_{50}) in hyperresponsive airways are reported in e-Table 3.

The CRCs to either benralizumab or mepolizumab inhibited the contractile response to specific EFS frequencies in passively sensitized bronchi (Figure 5A-F). Specifically, both benralizumab and mepolizumab prevented the AHR in passively hyperresponsive airways stimulated by EFS delivered at EF_{50} (Figure 5A,D), EF_{70} (Figure 5B,E), and EF_{90} (Figure 5C,F) in a concentration-dependent manner. At EF_{90} benralizumab was significantly more potent (P<0.05) than mepolizumab (delta pIC_{50} 0.45±0.16) (Table E4).

Effect of benralizumab and mepolizumab on the AHR to QS

Both benralizumab and mepolizumab prevented the hyperresponsive myogenic tone induced by QS in passively sensitized bronchi.

Benralizumab administered at concentrations [?]10 μg/ml significantly reduced the AHR when compared with C+ bronchi (-499.43±9.98 mg/100mg bronchial tissue, P<0.01 vs. C+), conversely at least 30 μg/ml of mepolizumab were necessary to induce the same significant effect in hyperresponsive airways (-381.54±45.90 mg/100mg bronchial tissue, P<0.01 vs. C+) (Figure 6). Benralizumab at 10 μg/ml, but not mepolizumab, inhibited the contractile response to QS at a level significantly (P<0.05) lower than that detectable in C- tissue.

Effect of benralizumab and mepolizumab on the levels of cAMP in hyperresponsive airways

Both benralizumab and mepolizumab prevented the cAMP depletion elicited by passive sensitization in hyperresponsive bronchi, by normalizing the concentrations of cAMP when administered at 10 μg/ml (P>0.05 vs. C-). At concentrations [?]30 μg/ml both the mAbs restored the cAMP concentrations at levels significantly higher than those detected in C+ tissue (benralizumab +63.00±4.96 %, P<0.01 vs. C+; mepolizumab +47.05±4.66 %, P<0.05 vs. C+) (Figure 7A). Benralizumab at 100 μg/ml, but not mepolizumab, increased the cAMP concentrations at a level significantly (P<0.05) greater than that found in C- tissue.

The levels of cAMP were significantly correlated with the inhibition of AHR to QS induced by benralizumab and mepolizumab (y=1.52±0.12x+1.65; R^2 0.94; P<0.001) (Figure 7B).

Discussion and Conclusions

The results of this study demonstrate that targeting the IL-5 pathway with mAbs is a potent and effective strategy to prevent in a concentration dependent manner the AHR in passively sensitized airways, a condition leading to a massive production of IL-5 in bronchial tissue.

Benralizumab and mepolizumab reached the primary endpoint since both the agents flattened and shifted rightward the CRC to histamine. Lower concentrations of benralizumab than mepolizumab significantly reduced the AHR to histamine, with benralizumab being also most effective than mepolizumab by reducing the myogenic response at lower levels than that detected in non-hyperresponsive bronchi. The advantage of blocking IL-5Rα to IL-5 was further confirmed by the analysis of the potency, since benralizumab was [?]0.7 logarithm more potent than mepolizumab in reducing the contractile plateau generated by histamine in hyperresponsive airways.

Concerning the secondary endpoints, although the efficacy in preventing the AHR to parasympathetic stimulation was similar between the investigated mAbs, benralizumab was more potent than mepolizumab when the tissues were stimulated by higher EFS frequencies. Furthermore, lower concentrations of benralizumab than mepolizumab were sufficient to significantly counteract the FRC to EFS in hyperresponsive bronchi. Benralizumab and mepolizumab also prevented the hyperresponsive myogenic tone in response to QS, although only benralizumab reduced the ASM contractility at a level lower than that detectable in non-sensitized airways. Again, at lower concentrations (10 μg/ml) benralizumab but not mepolizumab was effective in inhibiting the AHR to QS.

As previously reported (Cazzola et al., 2016b), also in this study the passive sensitization altered the concentration of cAMP in hyperresponsive airways, a condition that was reverted in a concentration dependent manner by both benralizumab and mepolizumab. Benralizumab, but not mepolizumab, improved the cAMP concentrations at a level greater that that detected in non-sensitized tissue.

Certainly several complex indirect mechanisms (Molfino et al., 2012; Mukherjee et al., 2014) are involved in the prevention of AHR elicited by the inhibition of the IL-5/IL-5Rα axis in hyperresponsive airways. Whatever the pathways implicated, we have found that benralizumab and mepolizumab can restore at physiological levels the cAMP concentrations in passively sensitized airways. Increased intracellular concentrations of cAMP in ASM promote bronchodilation via activation of cAMP-dependent protein kinase (A kinase) (Rogliani et al., 2016). However, conflicting findings are currently available regarding the real impact of cAMP on airway inflammation, with some evidences suggesting for anti-inflammatory effects and other reporting increased cytokine synthesis and T cell adhesion to ASM cells induced by enhanced concentrations of cAMP (Black et al., 2012). Isolated airways include several residential cells such as ASM cells, fibroblasts, parasympathetic cells, epithelial cells, and inflammatory cells such as T-helper type 2 (Th2) lymphocytes, eosinophils, and mast cells, most of them stimulated or activated in response to passive sensitization (Schmidt et al., 2000). Therefore, in the isolated airways used in our experiments it was not possible to discern the exact origin of cAMP enhancement induced by the investigated mAbs. However, the strong correlation between the cAMP levels and the inhibition of AHR contractility induced by benralizumab and mepolizumab suggests that the improvement in cAMP concentrations represents a key mechanism on which the inhibition of IL-5/IL-5Rα axis may converge and, thus, protect ASM from hyperresponsiveness.

Unexpectedly, in passively sensitized airways benralizumab but not mepolizumab improved AHR and cAMP at levels greater than those detected in untreated non-sensitized tissue. Isolated airways are characterized by a certain degree of intrinsic tone mediated by the spontaneous generation of histamine and leukotrienes. Therefore, since eosinophil secretory granules contain both histaminase and leukotrienes (Bagnasco et al., 2017; Bandeira-Melo et al., 2002), it was expected that preventing the activation and degranulation of eosinophils by administering either benralizumab or mepolizumab would have led to the same effect. Perhaps, the difference in the efficacy between benralizumab than mepolizumab can be explained by considering that although both these mAbs counteract the IL-5 pathway, only benralizumab induces ADCC leading to the apoptosis of eosinophils and mast cells caused by natural killer (NK) cells (Kolbeck et al., 2010). Thus, the combination of IL5Rα blockade and ADCC activity of benralizumab may also support the greater potency of this mAb compared to mepolizumab against the AHR to histamine. Furthermore, while at low concentrations benralizumab can antagonize IL-5Rα and elicit ADCC (Kolbeck et al., 2010), low concentrations of mepolizumab could not be sufficient to neutralize the massive release of IL-5 in the sensitized tissue.

Such a condition can be translated to asthmatic patients before the next dose administration of a mAb. Accordingly with pharmacokinetic (PK) studies in healthy subjects (Martin et al., 2019; Shabbir et al., 2019) that received approved doses of mAbs (European Medicines Agency, 2015; European Medicines Agency, 2018; US Food and Drug Administration, 2015; US Food and Drug Administration, 2017), while the maximum plasma concentrations of benralizumab and mepolizumab were [?]3 μg/ml and [?]12 μg/ml respectively, the trough concentrations were [?]1 μg/ml and [?]5 μg/ml respectively. Indeed, our results indicate that at these concentrations both the agents are effective in submaximally inhibit the AHR to histamine in isolated airways. However, while the efficacy of the circulating levels of mepolizumab at trough could be neutralized by the cytokine storm during an asthma exacerbation (Borish, 2016), the protective effect of low concentrations of benralizumab can be preserved as it is directed on IL-5Rα and ADCC regardless of the the amount of circulating IL-5.

Taken together the data from PK studies with those of our study, it is also evident that the concentrations of benralizumab and mepolizumab detectable in plasma can only partially prevent the AHR induced by parasympathetic activation. This translational evidence is of certain interest to optimize the pharmacological treatment of severe asthmatics in which a high intrinsic parasympathetic tone has been documented (Liccardi

et al., 2016). Probably the current Global Initiative for asthma (GINA) recommendations (GINA, 2019) should be improved by considering this specific asthma phenotype in Step 5, in which combining an anti-IL-5/IL-5Rα mAb with a long-acting muscarinic antagonist (LAMA) as preferred controller could lead to clinical and functional benefits.

Airway sensitization contributes to the adhesion of eosinophils to parasympathetic nerves, leading to their priming, activation and degranulation with consequent release of major basic protein (MBP). MBP increases acetylcholine release due to loss of function of the neuronal M_2 muscarinic autoreceptor expressed on postganglionic parasympathetic neurons (Drake et al., 2018). Since such an intimate interaction between eosinophils and airway cholinergic nerves contributes to the AHR in the course of tissue sensitization, favourable synergistic interaction could result by combining a LAMA, that inhibits the cholinergic transmission, with an anti-IL-5/IL-5Rα mAb that protects vagal fibres from the deleterious influence of activated eosinophils.

By a strict pharmacological viewpoint, the findings of our study suggest that the profile of loss of E_{max} to histamine and EFS in passively sensitized airways treated with either benralizumab and mepolizumab is due to an indirect inhibition of endogenous bronchoconstricting intermediaries release that, in turn, lead to the AHR. This evidence is supported by the fact that the AHR induced by histamine in passively sensitized bronchi is mediated not only by the direct activation of histaminergic receptors expressed on ASM, but also by the indirect facilitator effect of the acetylcholine release from the parasympathetic nerve (Cazzola et al., 2016a). Moreover, the AHR elicited by EFS is prevalently indirect and mediated by the sensitization of vagal fibres leading to increased release of endogenous acetylcholine from postganglionic parasympathetic nerves that in turn activate muscarinic M_3 receptor expressed on ASM (Ichinose et al., 1996; Mitchell et al., 1993).

This research provides also ancillary results, mainly concerning the validity of passive sensitization of human isolated airways as a suitable model of AHR in asthma. Although already demonstrated at human ASM cellular level (Grunstein et al., 2002; Hakonarson et al., 1999a; Hakonarson et al., 1999b), here we provide for the first time the evidence that passive sensitization of the whole human bronchial tissue induces an extensive synthesis of IL-5, and that IL-5 represents a key factor leading to the AHR in human airways. Certainly targeting the IL-5 pathway counteracts the AHR in human subsegmental bronchi, nevertheless Manson et al. (Manson et al., 2019) have recently demonstrated that IL-5 does not induces hyperresponsiveness in human small airways. Perhaps the lack of IL-5 mediated AHR in small airways could be due to the preponderance of ASM in the bronchioles wall and the significantly lower, and almost absent, number of eosinophils in bronchioles compared to medium bronchi we have used in our experiments (Faul et al., 1997; Hyde et al., 2009). Furthermore, a possible bias leading to the absence of AHR in the bronchioles used by Manson et al. (Manson et al., 2019) is that their tissues were not passively sensitized, a procedure that induces AHR via IgE in the presence of further serum factors (Ichinose et al., 1996; Mitchell et al., 1997; Schmidt et al., 2000; Schmidt et al., 1999). In our study we have also found that the passive sensitization of subsegmental bronchi (4-6 mm inner diameter) elicits AHR in response to EFS. Since no augmentation to EFS was detected in previous studies in smaller airways (2-3 mm inner diameter) regardless of the frequency delivered (Mitchell et al., 1997), it can be assumed that the distribution of functional parasympathetic innervation at the level of airways [?]3 mm is sparse and/or hyporesponsive to passive sensitization (Calzetta et al., 2018a), making these smaller airways not appropriate to reproduce ex vivo the AHR mediated by vagal activation.

The main limitation of this study is intrinsic to the isolated bronchial model itself, as it permitted to characterize the effect of benralizumab and mepolizumab against AHR in a sub-acute ex vivo experimental setting but not after a long-term exposure. In this regard, since AHR has been proposed to be a main treatable trait toward precision medicine in eosinophilic asthmatic patients (Agusti et al., 2016; Bel et al., 2017), well designed head-to-head clinical trials are needed to compare the efficacy of chronic treatment with benralizumab and mepolizumab specifically against AHR in severe asthma.

Taken together, the findings of this study demonstrate that passive sensitization induces a massive release of IL-5 from human airways, and that targeting the IL-5/IL-5Rα axis with mAbs prevent in a concentration dependent manner the AHR in response to histamine, parasympathetic activation, and mechanical stress. Benralizumab, by blocking the IL-5Rα, resulted more potent than the anti-IL-5 mepolizumab, and the

beneficial effects of both these agents were correlated with improved levels of cAMP in hyperresponsive airways. These observations also indicate that IL-5 is a key factor in determining AHR in passively sensitized airways. Further head-to-head studies evaluating the impact of long term treatment with benralizumab and mepolizimab against AHR are needed in severe asthmatic subjects, with specific focus on patients characterized by a high intrinsic parasympathetic tone.

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Tables

Table 1. Effect of overnight incubation with different concentrations of benralizumab and mepolizumab on the CRCs to histamine in passively sensitized bronchi.

	C-	**C+**	**Benralizumab**	**Ben**
			1 μg/ml	3 μg
Histamine E_{max} (mg/100mg bronchial tissue)	996.80±100.00	1857.00±171.60 ###	1510.00±109.00 ***	1342
Histamine pEC_{50}	5.49±0.15	6.15±0.17 #	5.75±0.18	5.80

# P<0.05 and ### P<0.001 vs. C- (statistical analysis assessed by Student's t-test); *P<0.05, ** P<0.01, and *** P<0.001 vs. C+ (statistical analysis assessed by one-way ANOVA); data represent the mean±SEM of n=5 bronchial tissue from different subjects. C+: positive control, passively sensitized bronchi; C-: negative control, non-sensitized bronchi; CRC: concentration-response curve; EC_{50}: concentration inducing 50% E_{max}; E_{max}: maximal effect; pEC_{50}: -LogEC_{50}.

Table 2. Efficacy and potency of benralizumab and mepolizumab after overnight incubation on the AHR to different concentrations of histamine ($EC_{50\text{-}90}$) in passively sensitized bronchi. The pharmacological analysis was performed by assessing E_{max}as the difference in airway contractility between C+ and C- tissues.

	Benralizumab	Benralizumab	Benrali
Contractile tone to histamine at:	EC_{50}	EC_{70}	EC_{90}
Benralizumab or mepolizumab E_{max} (mg/100mg bronchial tissue)	-116.05±13.60	-111.90±13.28	-134.14±
Benralizumab or mepolizumab pIC_{50}	8.24±0.16 **	8.13±0.12 *	7.94±0.1

* P<0.05 and ** P<0.01 vs. mepolizumab (statistical analysis assessed by Student's t-test); data represent the mean±SEM of n=5 bronchial tissue from different subjects. AHR: airway hyperresponsiveness; C+: positive control, passively sensitized bronchi; C-: negative control, non-sensitized bronchi; EC_n: concentration inducing n% E_{max}; E_{max}: maximal effect; IC_{50}: concentration inducing 50% inhibition AHR to histamine in passively sensitized bronchi; pIC_{50}: -logIC_{50}.

Figures legends

Figure 1. Effect of overnight incubation with different concentrations of benralizumab (A) and mepolizumab (B) on the AHR to histamine CRCs in passively sensitized bronchi. ** P<0.01 and *** P<0.001 vs. C+ (statistical analysis assessed by two-way ANOVA); points represent the mean±SEM of n=5 bronchial tissue from different subjects. AHR: airway hyperresponsiveness; C+: positive control, passively sensitized bronchi; C-: negative control, non-sensitized bronchi; CRC: concentration-response curve.

Figure 2. Inhibition of AHR to different concentrations of histamine (EC_{50}: A and D; EC_{70}: B and E; EC_{90}: C and F) by overnight incubation with benralizumab (A-C) and mepolizumab (D-F) in passively sensitized bronchi. Points represent the mean±SEM of n=5 bronchial tissue from different subjects. AHR: airway hyperresponsiveness; C+: positive control, passively sensitized bronchi; C-: negative control, non-sensitized bronchi; EC_n: concentration inducing n% E_{max}; E_{max}: maximal effect..

Figure 3. Effect of overnight incubation with different concentrations of benralizumab (A) and mepolizumab (B) on the AHR to EFS FRCs in passively sensitized bronchi. ** P<0.01 and *** P<0.001 vs. C+ (statistical analysis assessed by two-way ANOVA); points represent the mean±SEM of n=5 bronchial tissue from different subjects. AHR: airway hyperresponsiveness; C+: positive control, passively sensitized bronchi; C-: negative control, non-sensitized bronchi; EFS: electrical field stimulation; FRC: concentration-response curve.

Figure 4. Inhibition of AHR to different EFS frequencies (EF_{50}: A and D; EF_{70}: B and E; EF_{90}: C and F) by overnight incubation with benralizumab (A-C) and mepolizumab (D-F) in passively sensitized bronchi. Points represent the mean±SEM of n=5 bronchial tissue from different subjects. AHR: airway hyperresponsiveness; C+: positive control, passively sensitized bronchi; C-: negative control, non-sensitized bronchi; CRC: concentration-response curve; EFS: electrical field stimulation; EF_n: frequency inducing n% E_{max}; E_{max}: maximal effect.

Figure 5 . Effect of overnight incubation with different concentrations of benralizumab and mepolizumab on the AHR to QS of 0.5 mm in passively sensitized bronchi. # P<0.05 vs. C-; * P<0.05 and ** P<0.01 vs. C+ (statistical analysis assessed by Student's t-test); bars represent the mean±SEM of n=5 bronchial tissue from different subjects. AHR: airway hyperresponsiveness; C+: positive control, passively sensitized bronchi; C-: negative control, non-sensitized bronchi; QS: quick stretch.

Figure 6. Effect of overnight incubation with different concentrations of benralizumab and mepolizumab on the cAMP concentrations in hyperresponsive bronchial tissue (A) and correlation between the cAMP concentrations and the modulation of myogenic tone to QS induced by the investigated treatments (B). ### P<0.001 vs. C-; * P<0.05, ** P<0.01, and *** P<0.001 vs. C+ (statistical analysis assessed by Student's t-test); bars and points represent the mean±SEM of n=5 bronchial tissue from different subjects. The colours of bars in A and the relative treatments are consistent with the colours of points in B. C+: positive control, passively sensitized bronchi; C-: negative control, non-sensitized bronchi; cAMP: cyclic adenosine monophosphate; QS: quick stretch.