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Review article

Muscarinic receptor antagonists and airway inflammation: A systematic review on pharmacological models

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ABSTRACT

Airway inflammation is crucial in the pathogenesis of many respiratory diseases, including chronic obstructive pulmonary disease (COPD) and asthma. Current evidence supports the beneficial impact of muscarinic receptor antagonists against airway inflammation from bench-to-bedside. Considering the numerous sampling approaches and the ethical implications required to study inflammation in vivo in patients, the use of pre-clinical models is inevitable. Starting from our recently published systematic review concerning the impact of muscarinic antagonists, we have systematically assessed the current pharmacological models of airway inflammation and provided an overview on the advances in in vitro and ex vivo approaches. The purpose of in vitro models is to recapitulate selected pathophysiological parameters or processes that are crucial to the development of new drugs within a controlled environment. Nevertheless, immortalized cell lines or primary airway cells present major limitations, including the inability to fully replicate the conditions of the corresponding cell types within a whole organism.

Induced animal models are extensively used in research in the attempt to replicate a respiratory condition reflective of a human pathological state, although considering animal models with spontaneously occurring respiratory diseases may be more appropriate since most of the clinical features are accompanied by lung pathology resembling that of the human condition.

In recent years, three-dimensional organoids have become an alternative to animal experiments, also because animal models are unable to fully mimic the complexity of human pulmonary diseases. Ex vivo studies performed on human isolated airways have a superior translational value compared to in vitro and animal models, as they retain the morphology and the microenvironment of the lung in vivo.

In the foreseeable future, greater effort should be undertaken to rely on more physiologically relevant models, that provide translational value into clinic and have a direct impact on patient outcomes.

1. Introduction

Respiratory diseases pose a massive worldwide healthcare burden in terms of disability, premature mortality, and direct and indirect costs [1, 2]. According to the World Health Organization, an estimated 65 million people are living with moderate to severe chronic obstructive pulmonary disease (COPD), from which 3 million die each year, while asthma afflicts approximately 334 million people worldwide, making it the most prevalent chronic disease among children [1].

It is well established that airway inflammation plays a critical role in the pathogenesis of many respiratory diseases including COPD, asthma, pneumonia, and acute respiratory distress syndrome [3, 4, 5]. Lung tissue inflammation and anti-inflammatory reactions involve a complex interplay between and among structural lung cells and resident and infiltrating immune cell populations within the airways and lung parenchyma [6, 7].

Acetylcholine (ACh) is not conventionally considered an inflammatory mediator. Nevertheless, current evidence reports that, along with choline acetyltransferase (ChAT), ACh is expressed not only in neurons, but also at the level of inflammatory cells (i.e. lymphocytes, macrophages, mast cells, eosinophils, neutrophils) infiltrating bronchial tissue in course of chronic inflammatory disorders such as asthma and COPD [8,

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9]. Effectively, the activation of muscarinic M_3 receptors increases the levels of cytosolic Ca^{2+} and c-fos mRNA expression in human T- and B-cells and may regulate the cytotoxicity of T cells; on the other hand, the activation of lymphocytes increases the expression of ChAT mRNA and muscarinic receptors [10]. The expression of muscarinic receptors on inflammatory cells is altered in COPD, leading to significant release of leukotriene B_4 and activated the p42/p44 MAP kinase pathway [11]. Evidently, ACh may have a relevant role in airway inflammatory processes via autocrine/paracrine mechanisms.

The regulation of airway inflammation by muscarinic receptors is further corroborated by findings reporting that in human bronchi, especially at the level of small airways, non-neuronal ACh is extensively released by resident cells such as epithelial cells in response to inflammatory stimulation [12, 13, 14]. Interestingly, the activation of muscarinic receptors expressed on epithelium in small airways induces the release of chemotactic factors and the consequent migration of eosinophils, monocytes and neutrophils into the bronchial tissue [10]. Overall, these mechanisms related to the activation of muscarinic receptors by non-neuronal ACh are associated with inflammatory cells proliferation and increased release of cytokines leading to detrimental inflammatory effects in asthma and COPD. Moreover, and not less important, muscarinic receptors expressed on ASM cells may further support or initiate airway inflammation via the transcription of pro-inflammatory genes induced by increased sinusoidal length oscillation due to altered breathing cycles, a recurrent condition in chronic obstructive respiratory disorders [15].

The inhaled bronchodilators long-acting muscarinic receptor antagonists (LAMAs) are the mainstay therapy for symptom management of COPD and are recommended by the most recent Global Initiative for Chronic Obstructive Lung Disease (GOLD) report to patients categorized into GOLD groups A–D [16]. In asthma, LAMAs are indicated as alternative controller option to medium/high maintenance dose of ICS/LABA in Global Initiative for Asthma (GINA) Step 4 and as add-on therapy to high dose ICS/LABA as either a separate inhaler or a triple combination inhaler in GINA Step 5 [17]. Evidence coming from a recent systematic review supports the beneficial impact of muscarinic receptor antagonists and in particular LAMAs, against airway inflammation from bench-to-bedside, with a synthesis of results shown in Table 1 [18].

The assessment of airway inflammation in vivo in patients with chronic pulmonary diseases demands multiple sampling approaches [19, 20] These include the invasive method of bronchoscopy, the non-invasive collection of sputum [21], which is hampered by the difficulty in obtaining adequate amount of sample and by the high variability in cytokine concentrations [22], the breath analysis, and lung imaging [19, 20]. Besides, blood and urine sampling are required for the evaluation of systemic inflammatory markers [19, 20]. Moreover, considering the numerous ethical implications inherent to the studies on patients with asthma and COPD, the use of pre-clinical models to study inflammation is inevitable [23, 24].

Starting from our recently published systematic review [18], the aim of the present study was to systematically assess the currently employed pharmacological models used to study the impact of muscarinic receptor antagonists against airway inflammation and to give an insight on the current advances in preclinical respiratory research.

2. Materials and methods

2.1. Review question

The question of this systematic review was to assess the current evidence concerning the stimulation challenges used to evaluate the impact on airway inflammation of marketed or novel muscarinic antagonists.

2.2. Search strategy and study eligibility

The protocol of this systematic review was carried out in agreement with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses Protocols (PRISMA-P) [25]. The PRISMA 2020 flow diagram is shown in Figure 1. This study satisfied all the recommended items reported by the PRISMA-P checklist [25].

The patient problem, intervention, comparison, and outcome (PICO) framework was applied for the synthesis of literature search strategy and question, as previously described [26]. The "Patient problem" regarded airway inflammation; the "Intervention" included challenge models of inflammation to assess the effect of muscarinic receptor antagonists; the "Comparison" was performed with respect to unchallenged controls; the "Outcome" was the impact on inflammation.

A comprehensive literature search was performed for in vitro and in vivo studies conducted on research animals and patients, written in English and including a challenge model of airway inflammation used to evaluate the impact of muscarinic receptor antagonists. The search was performed in MEDLINE to identify relevant studies available from the last 10 years up to 5th June 2020. Clinical studies were searched with no time limitation. The expanded search string was: (("muscarinic antagonists"[Pharmacological Action] OR "muscarinic antagonists"[MeSH Terms] OR ("muscarinic"[All Fields] AND "antagonists"[All Fields]) OR "muscarinic antagonists"[All Fields] OR ("muscarinic antagonists"[Pharmacological Action] OR "muscarinic antagonists"[MeSH Terms] OR ("muscarinic"[All Fields] AND "antagonists"[All Fields]) OR "muscarinic antagonists"[All Fields] OR "antimuscarinic"[All Fields] OR "antimuscarinics"[All Fields]) OR ("tiotropium bromide"[MeSH Terms] OR ("tiotropium"[All Fields] AND "bromide"[All Fields]) OR "tiotropium bromide"[All Fields] OR "tiotropium"[All Fields]) OR ("glycopyrrolate"[MeSH Terms] OR "glycopyrrolate"[All Fields] OR "glycopyrronium"[All Fields]) OR ("gsk573719"[Supplementary Concept] OR "gsk573719"[All Fields] OR "umeclidinium"[All Fields]) OR "aclidinium"[All Fields] OR ("ipratropium"[MeSH Terms] OR "ipratropium"[All Fields]) OR ("oxitropium"[Supplementary Concept] OR "oxitropium"[All Fields])) AND ("inflammation"[MeSH Terms] OR "inflammation"[All Fields] OR "inflammations"[All Fields] OR "inflammation s"[All Fields])) NOT ("review"[Publication Type] OR "review literature as topic"[MeSH Terms] OR "review"[All Fields]).

Citations of previous relevant papers were assessed to identify any further pertinent study [27].

2.3. Study selection

This systematic review included in vitro, in vivo, and clinical studies using challenge models for investigating the effect of muscarinic receptor antagonists on airway inflammation. Research studies using a challenge method to evaluate the anti-inflammatory activity of a muscarinic receptor antagonist combined with another drug were not taken into account. Two reviewers independently checked the records resulting from MEDLINE database. The studies were selected according to the above mentioned criteria, and any difference in opinion was resolved by consensus.

2.4. Data extraction

Data concerning the year of publication, type of study, type of cells, animals or donors, pro-inflammatory stimuli, number of patients or donors or animals, sex, route of administration, type of biological samples, and investigated outcomes were extracted and checked from the included studies.

2.5. Endpoints

The endpoint of this systematic review was the impact of in vitro and in vivo challenges used to evaluate the airway anti-inflammatory effect of muscarinic receptor antagonists.

2.6. Strategy for data analysis

Results from reserch studies were extracted and reported through qualitative synthesis.

Table 1. Statistically significant beneficial effects of muscarinic receptor antagonists on humoral and cellular responses by using pre-clinical pharmacological models of airway inflammation, according to previous systematic review [18].

Drugs	Humoral factors resulting from in vitro or in vivo studies carried out on laboratory animals																			
	IL-1β	IL-4	IL-5	IL-6	IL-8	IL-13	CCL5	GN-CSF	IFN-γ	KC	MCP-1	MIP-1	MIP-2	MMP-1	MMP-2	MMP-9	NF-κB	PDCD5	TGF-β1	TNF-α
Aclidinium bromide	↓: in vitro	/	/	/	↓: in vitro	/	↓: in vitro	↓: in vitro	/	/	/	/	/	/	/	↓: in vitro	/	/	/	/
Anisodamine	/	↓: BALf	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
Bencycloquidium bromide	↓: BALf, lung	↓: BALf, lung	↓: lung	↓: nasal mucosa, NLF	/	↓: nasal mucosa, NLF	/	/	/	↓: BALf, lung	↓: BALf, lung	/	/	/	/	↓: lung	↓: nasal mucosa	/	/	↓: BALf, lung, nasal mucosa, NLF
Glycopyrronium bromide	↓: BALf, lung	/	/	↓: nasal mucosa, NLF	↓: in vitro	↓: nasal mucosa, NLF	/	/	/	/	↓: BALf, lung	/	↓: BALf	/	/	↓: lung	↓: nasal mucosa	/	↓: BALf, lung	↓: BALf, lung
Ipratropium bromide	/	/	/	↓: nasal mucosa, NLF	/	↓: nasal mucosa, NLF	/	/	/	/	/	/	/	/	/	↓: BALf	↓: nasal mucosa	/	/	↓: nasal mucosa, NLF
Mepenzolate	/	/	/	/	/	/	/	/	/	↓: BALf, lung	↓: BALf, lung	/	↓: BALf, lung	/	/	/	/	/	/	↓: BALf
Tiotropium bromide	↓: in vitro, BALf, lung	↓: BALf, lung	↓: in vitro, BALf, lung	↓: in vitro, BALf, lung	↓: in vitro, BALf	↓: in vitro, BALf	/	/	↓: in vitro, lung	↓: BALf, lung	↓: BALf, lung	↓: BALf	↓: BALf	↓: in vitro	↓: in vitro, BAL	↓: in vitro	↓: in vitro	↓: BALf, lung	↓: BALf, lung	↓: in vitro, BALf, lung
4-DAMP	/	/	↓: in vitro	↓: lung	/	↓: in vitro	/	/	↓: in vitro	↓: BALf	/	/	/	↓: in vitro	↓: in vitro	/	↓: in vitro, lung	/	/	↓: in vitro, lung

	Inflammatory cells and markers resulting from in vivo studies carried out on laboratory animals						
	Eosinophils	Lymphocytes	Macrophages	Neutrophils	Total inflammatory cells	Total protein amount	Lung inflammatory score
Aclidinium bromide	↓: BALF	/	/	↓: lung	/	↓: BALF	/
Anisodamine	↓: BALF, lung	/	/	/	↓: BALF, lung	/	/
Bencycloquidium bromide	↓: BALF, lung, nasal mucosa	/	↓: BALF	↓: BALF, lung	↓: BALF, lung	/	↓: lung
Glycopyrronium bromide	/	↓: BALF	↓: BALF, lung	↓: BALF, lung	↓: BALF	↓: BALF	↓: lung
Ipratropium bromide	↓: nasal mucosa	/	/	↓: BALF	↓: BALF	/	↓: lung
Mepenzolate	/	/	/	↓: BALF	↓: BALF	/	/
Tiotropium bromide	↓: BALF, lung	↓: BALF	↓: BALF, lung	↓: BALF, lung	↓: BALF, lung	↓: BALF	↓: lung
V0162	/	/	/	/	↓: BALF	/	/
4-DAMP	/	↓: BALF	↓: BALF	↓: BALF	↓: BALF	/	/

BALf: bronchoalveolar lavage fluid; GM-CSF: granulocyte macrophage-colony stimulating factor; IFN: interferon; IL: interleukin; KC: keratinocyte-derived chemokine; MCP: monocyte chemotactic protein; MIP: macrophage inflammatory protein; MMP: matrix metalloproteinase; NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells; NLF: nasal lavage fluid; PDCD5: programmed cell death protein 5; TGF-β1: tumor growth factor-beta 1; TNF-α: tumor necrosis factor-alpha; 4-DAMP: 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide.

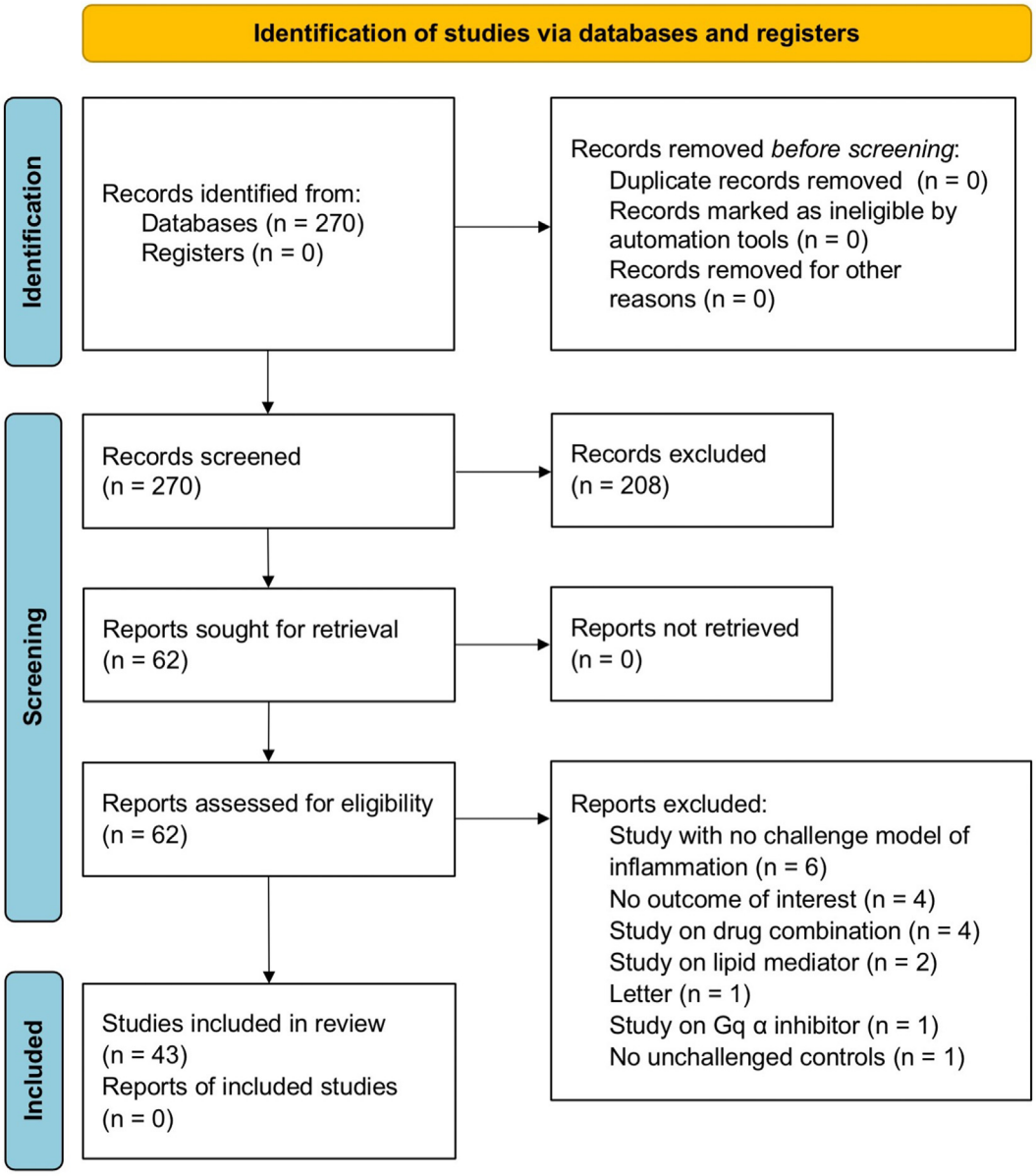

Figure 1. PRISMA 2020 flow diagram for the identification of studies included in this systematic review concerning the impact of challenge models of airway inflammation. PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses.

2.7. Quality score and risk of bias

The risk of bias of the included clinical studies was analyzed by using the Jadad score and the Cochrane Risk of Bias 2 (RoB 2), as previously described [28, 29].

Two reviewers independently assessed the quality of individual studies, and any difference in opinion about the quality score was resolved by consensus.

3. Results

3.1. Study characteristics

Of the 268 potentially relevant records identified in the initial search, 43 studies were deemed eligible for a qualitative analysis. Overall, this systematic review included data obtained from 28 in vivo studies performed on research animals [30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57], 11 in vitro studies [58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68], 3 studies conducted both in vitro and in vivo [69, 70, 71], and 1 clinical study performed in patients with COPD and then conducted ex vivo [72] (Table 2).

The following pro-inflammatory stimuli were used in the included studies: ACh, *Aspergillus fumigatus* extract, carbachol (CCh), CCh combined with interleukin (IL)-1β, cadmium chloride ($CdCl_2$), cigarette smoke (CS), CS extract (CSE), CSE combined with ACh, CS combined with H1N1 or respiratory syncytial virus infection, N-formyl-L-methionyl-L-leucyl-phenylalanine (fMLP), house dust mite extract, hydrochloric acid (HCl)-containing acidic solution, IL-1β, lipopolysaccharide (LPS), LPS with CCh or ACh or oxotremorine, muscarine or oxotremorine, organic dust (OD), ovalbumin (OVA), OVA combined with exposure to particulate matter (PM), platelet-derived growth factor-BB (PDGF-BB), phytohemagglutinin (PHA), porcine pancreatic elastase (PPE), resistive breathing, recombinant human IL-17A (rhIL-17A), supernatant of the sputum of COPD patients, rhinovirus type-14 infection, transforming growth factor β (TGF-β), TGF-β together with ACh, and TNF-α.

Table 2. Main characteristics of the studies included in the systematic review.

Study and year	Type of study	Type of cells, animals, and donors	Pro-inflammatory stimulus	Number of patients or donors or animals	Age	Male (%)	Comparator	Route of administration	Biological samples	Investigated outcomes
Ferraro et al., 2019 [58]	In vitro	16HBE human bronchial epithelial cells	CSE (3–20%)	NA	NA	NA	Unstimulated cells	Incubation	Cell culture supernatant	IL-8, HDAC, and SIRT1 expression
Kang et al., 2019 [30]	In vivo	BALB/c mice	OVA challenge (20 ng/50 μL)	5-8 per group	6 wks, 9 months, and 15 months	0.0	Unstimulated mice	Intranasal administration	BALf and lung tissue	IL-4, IL-5, IL-13, total inflammatory cell count, eosinophils, lymphocytes, and neutrophils
Neri et al., 2019 [59]	In vitro	16HBE human bronchial epithelial cells	ACh (1 mM)	NA	NA	NA	Unstimulated cells	Incubation	Cell culture supernatant	IL-8
Albano et al., 2018 [61]	In vitro	SV40 large T antigen-transformed 16HBE human bronchial epithelial cells	CSE (20%) or ACh (1 μM), or CSE (20%) + ACh (1 μM)	NA	NA	NA	Unstimulated cells	Incubation	Cell culture supernatant	IL-8
Hsiao et al., 2018 [69]	In vitro, In vivo	Human primary bronchial epithelial cells; C57BL/6J mice	CSE (3–20%) or ACh (100 nM); CS	10	8 wks	100.0	Unstimulated cells; unstimulated mice	Incubation; inhalation	Cell culture supernatant; BALf	IL-8; MCP-1, MIP-2, lung inflammatory score, total protein count, total inflammatory cell count, lymphocytes, macrophages, and neutrophils
Kurai et al., 2018 [31]	In vivo	BALB/c mice	Exposure to PM (0.1 mg/25 μL of NS) and OVA challenge (1% in NS)	6-8 per group	7 wks	100.0	Unstimulated mice	Ultrasonic nebulization	BALf	IL-5, IL-6, IL-13, IFN-γ, KC, total inflammatory cell count, eosinophils, macrophages, lymphocytes, and neutrophils
Gregory et al., 2018 [42]	In vivo	BALB/c mice	OD (1 mg/mL)	10 per group	8–10 wks	0.0	Unstimulated mice	Intranasal administration	BALf and lung tissue	IL-4, IL-6, IL-13, IL-17A, KC, MCP-1, MIP-2, and TNF-α, total inflammatory cell count, eosinophils, macrophages, lymphocytes, and neutrophils
Zhao et al., 2018 [51]	In vivo	Sprague–Dawley rats	$CdCl_2$ (0.1% solution)	68	NA	100.0	Unstimulated rats	Inhalation	BALf and histology	IL-1β, MMP-2, MMP-9, TNF-α, total inflammatory cell count, macrophages, neutrophils, total protein count, and inflammatory score
Toumpanakis et al., 2017 [52]	In vivo	Rats	40% and 50% IRB, 60% ERB or 40%/60% CRB	5-8 per group	8–12 wks	0.0	Unstimulated rats	NA	BALf and lung tissue	IL-1β, IL-6, total protein count, macrophages, and neutrophils, and inflammatory score

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Table 2 (*continued*)

Study and year	Type of study	Type of cells, animals, and donors	Pro-inflammatory stimulus	Number of patients or donors or animals	Age	Male (%)	Comparator	Route of administration	Biological samples	Investigated outcomes
John-Schuster et al., 2017 [53]	In vivo	C57BL/6J mice	House dust mite extract (10 μg)	12	8–10 wks	0.0	Unstimulated mice	Intranasal administration	BALf and lung tissue	Total inflammatory cell count, eosinophils, and inflammatory score
Anzalone et al., 2016 [62]	In vitro	SV40 large T antigen-transformed 16HBE human bronchial epithelial cells	Induced sputum supernatant from COPD patients (20%) or rhIL-17A (20 ng/mL)	NA	NA	NA	Unstimulated cells	Incubation	Cell culture supernatant	IL-8, HDAC expression, and IKKα
Bucher et al., 2016 [54]	In vivo	BALB/cAnNCrl mice	Exposure to CS and infection with H1N1 virus (50 μl) or RSV (50 μl)	4-8 per group	8–12 wks	0.0	Unstimulated mice	CS inhalation; H1N1 or RSV intranasal administration	BALf and lung tissue	IL-1β, IL-6, IFN-γ, KC, TNF-α, total inflammatory cell count, macrophages, and neutrophils
Kistemaker et al., 2016 [55]	In vivo	Hartley guinea pigs	OVA challenge (0.05–0.1% in saline)	3-8 per group	NA	100.0	Unstimulated pigs	Intraperitoneal injection	BALf and lung tissue	Total inflammatory cell count, eosinophils, lymphocytes, macrophages, neutrophils, and airway eosinophilia
Milara et al., 2016 [63]	In vitro	Neutrophils derived from patients with stable COPD (FEV_1 predicted: 53.2% and FEV_1/FVC: 50.1% at baseline)	LPS (1 μg/mL), CSE (5%), or CCh (10 μM)	52	65.1 years	67.3	Unstimulated cells	Incubation	Sputum and blood	CCL5, GM-CSF, IL-1β, IL-8, MMP-9, and HDAC expression
Devillier et al., 2015 [56]	In vivo	Guinea pigs	OVA challenge (200 μg in 1 mL of physiological serum)	7-9 per group	NA	100.0	Unstimulated pigs	Nebulization	BALf	Total inflammatory cell count
Long et al., 2015 [57]	In vivo	Sprague–Dawley rats	OVA (10%, 10 μL)	6 per group	NA	100.0	Unstimulated rats	Intranasal administration	NLF and nasal mucosa	IL-6, IL-13, TNF-α, eosinophils, and NF-κB subunits translocation
Zhang et al., 2015 [32]	In vivo	ICR mice	CS	10 per group	NA	0.0	Unstimulated mice	Inhalation	BALf and lung tissue homogenate	IL-1β, KC, MCP-1, TNF-α, total inflammatory cell count, lymphocytes, macrophages, and neutrophils
Costa et al., 2014 [64]	In vitro	Human primary lung fibroblasts derived from asthmatic patients (FEV_1 predicted: 87.8% at baseline)	IL-1β (10 ng/mL) in presence or absence of CCh (10 nM–10 μM), PDGF-BB 0.1–10 ng/mL, TNF-α 0.1–10 ng/mL	9	55.5	45.5	Unstimulated cells	Incubation	Cell culture supernatant	IL-6 and IL-8
Bosnjak et al., 2014 [33]	In vivo	BALB/c mice	OVA challenge (1% in PBS)	6-10 per group	8 wks	0.0	Unstimulated mice	Nebulization	BALf and lung tissue	Inflammatory cell count, eosinophils, lymphocytes, macrophages,

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Table 2 (*continued*)

Study and year	Type of study	Type of cells, animals, and donors	Pro-inflammatory stimulus	Number of patients or donors or animals	Age	Male (%)	Comparator	Route of administration	Biological samples	Investigated outcomes
										neutrophils, and inflammation score
Kolahian et al., 2014 [34]	In vivo	Domestic cats	CS	5 per group	NA	100.0	Unstimulated cats	Inhalation	BALf, serum, and lung tissue	IL-6, IL-8, MCP-1, TNF-α, total protein count, total inflammatory cell count, eosinophils, lymphocytes, macrophages, neutrophils, monocytes, basophils, and inflammation score
Shen et al., 2014 [35]	In vivo	ICR mice	CS	4-10 per group	NA	0.0	Unstimulated mice	Inhalation	BALf and lung tissue	IL-1β, MCP-1, MMP-9, TGF-β1, TIMP-1, TNF-α, total inflammatory cell count, lymphocytes, macrophages, and neutrophils
Smit et al., 2014 [36]	In vivo	Hartley guinea pigs	OVA (0.05% in saline)	5-6 per group	NA	100.0	Unstimulated pigs	Inhalation of aerosolized solution	BALf	Total inflammatory cell count, eosinophils, macrophages, and neutrophils
Dominguez-Fandos et al., 2013 [37]	In vivo	Hartley guinea pigs	CS	58	NA	100.0	Unstimulated pigs	Inhalation	Lung tissue	Eosinophils, neutrophils, and macrophages
Kistemaker et al., 2013 [38]	In vivo	C57BL/6NTac wild-type mice	CS	8-9 per group	10–12 wks	100.0	Unstimulated mice	Inhalation	BALf and lung tissue	KC, MIP-1α, MIP-2, TNFα, TGF-β1, total inflammatory cell count, lymphocytes, macrophages, and neutrophils
Tanaka et al., 2013 [39]	In vivo	DBA/2 and ICR mice	CS or PPE (100 μg in PBS)	4-8 per group	4–6 wks	NA	Unstimulated mice	CS inhalation; PPE intratracheal injection	BALf and lung tissue	KC, TNF-α, MCP-1, MIP-2, total inflammatory cell count, neutrophils, macrophages, HDAC, IκB-α, and NF-κB phosphorylation
Profita et al., 2012 [65]	In vitro	SV40 large T antigen-transformed 16HBE human bronchial epithelial cells and neutrophils from normal donors	Induced sputum supernatant derived from COPD patients (10%) or ACh (1 μM) or ACh (1 μM) in combination with rhTGF-β1 (5.0 ng/mL)	NA	NA	NA	Unstimulated cells	Incubation	Cell culture extract	Percentage of adhering neutrophils and MAC-1 expression
Kang et al., 2012 [40]	In vivo	BALB/c mice	OVA challenge (20 ng/50 μL in PBS)	8 per group	8–10 wks	0.0	Unstimulated mice	Intranasal administration	BALf	IL-4, IL-5, IL-13, total inflammatory cell count, eosinophils,

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Table 2 (*continued*)

Study and year	Type of study	Type of cells, animals, and donors	Pro-inflammatory stimulus	Number of patients or donors or animals	Age	Male (%)	Comparator	Route of administration	Biological samples	Investigated outcomes
										lymphocytes, macrophages, and neutrophils
Pera et al., 2011 [41]	In vivo	Hartley guinea pigs	LPS (5 mg/mL of sterile saline)	NA	NA	100.0	Unstimulated pigs	Intranasal administration	Lung tissue	Neutrophils
Santus et al., 2012 [72]	Clinical trial (performed ex vivo)	COPD patients (FEV_1: 62.0% of predicted and FEV_1/FVC: 60.5% at baseline)	Blood neutrophils from COPD patients challenged with fMLP (0.1 μM) and primed with GM-CSF	24	72.5	95.8	Unstimulated neutrophils	Incubation	Neutrophil supernatant	LTB_4 production
Buels et al., 2012 [43]	In vivo	Hartley guinea pigs	OVA challenge (5% in sterile PBS)	3-7 per group	NA	0.0	Unstimulated pigs	Inhalation	BALf and lung tissue	Eosinophils, lymphocytes, macrophages, and neutrophils
Xu et al., 2012 [70]	In vitro, in vivo	Alveolar macrophages; Kunming mice	LPS (1.0 μg/mL); LPS (4.0 mg/kg in 50 μL of saline)	7-10 per group	8 wks	100.0	Unstimulated macrophages; unstimulated mice	Incubation; intratracheal instillation	Cell culture supernatant; BALf and lung tissue	IL-6, TNF-α, total inflammatory cell count, neutrophils, and IκB-α
Yamaya et al., 2012 [66]	In vitro	Human tracheal surface epithelial cells	Rhinovirus type-14 (100 μL)	50 (patients who donated tracheas for cell cultures)	70.0	32.0	Unstimulated cells	Incubation	Cell incubation	IL-1β, IL-6, and IL-8
Vacca et al., 2011 [67]	In vitro	Alveolar macrophages from COPD patients	LPS (0.1–10 μg/mL); LPS (0.1–10 μg/mL) + ACh (0.1–1000 μM) or CCh or muscarin or oxotremorine (1–100 μM); ACh (0.1–1000 μM), or CCh or muscarin or oxotremorine (1–100 μM)	71	NA	35.2	Unstimulated macrophages	Incubation	Cell culture supernatant	IL-6, IL-8, TNF-α, LTB_4, GM-CSF and MIP-α/-β, and neutrophil migration rate
Cao et al., 2011 [44]	In vivo	ICR mice	OVA challenge (10 mg/mL in saline)	8-9 per group	NA	0.0	Unstimulated mice	Inhalation	BALf and lung tissue	IL-4, IL-5, IFN-γ, MMP-9, TIMP-1, eotaxin, total inflammatory cell count, eosinophils, lymphocytes, and macrophages
Suzaki et al., 2011 [68]	In vitro	BEAS-2B human bronchial epithelial cells and human lung fibroblasts derived from patients during pneumonectomy for tumor resection	LPS (1.0 μg/mL)	NA	NA	NA	Unstimulated cells	Incubation	Cell culture supernatant and extract	IL-8 and NF-κB
Xu et al., 2011 [45]	In vivo	BALB/c mice	OVA challenge (2% in 0.9% saline)	6-10 per group	10–12 wks	100.0	Unstimulated mice	Nebulization	BALf and histology	IL-4, IFN-γ, total inflammatory

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Table 2 (*continued*)

Study and year	Type of study	Type of cells, animals, and donors	Pro-inflammatory stimulus	Number of patients or donors or animals	Age	Male (%)	Comparator	Route of administration	Biological samples	Investigated outcomes
										cell count, and eosinophils
Ohta et al., 2010 [71]	In vitro, in vivo	PBMCs derived from asthmatic patients; spleen cells derived from BALB/c mice; BALB/c mice	PHA (1 μg/mL); OVA challenge (100 μg/mL); OVA challenge (2.5%)	8 per group	BALB/c mice: 6–8 wks	0.0	Unstimulated cells; unstimulated mice	Incubation; inhalation	Cell culture supernatant; BALf	IL-4, IL-5, IL-13, IFN-γ, TGF-β1, total inflammatory cell count, eosinophils, lymphocytes, and macrophages
Cui et al., 2010 [46]	In vivo	BALB/c mice	Acidic solution (0.3 mL; 1 N HCl and 0.5% pepsin)	11-17 per group	8 wks	100.0	Unstimulated mice	Esophageal instillation	BALf	IL-8, sICAM-1, total inflammatory cell count, lymphocytes, macrophages, and neutrophils
Damera et al., 2010 [47]	In vivo	BALB/c mice	*Aspergillus fumigatus* (25 μg in PBS)	12	12 wks	0.0	Unstimulated mice	Intranasal administration	BALf	IL-4, IL-6, IL-13, TNF, total protein count, eosinophils, macrophages, lymphocytes, and neutrophils
Zhang et al., 2010 [48]	In vivo	Sprague–Dawley rats	$CdCl_2$ (0.1% in saline)	114	NA	100.0	Unstimulated rats	Inhalation	BALf and lung tissue	IL-1β, MMP-2, MMP-9, TNF-α, total inflammatory cell count, macrophages, neutrophils, and inflammatory score
Zhang et al., 2010 [49]	In vivo	Sprague–Dawley rats	$CdCl_2$ (0.1% in saline)	113	NA	100.0	Unstimulated rats	Inhalation	BALf and lung tissue	IL-1β, MMP-2, MMP-9, TNF-α, total inflammatory cell count, macrophages, and neutrophils
Wollin et al., 2010 [50]	In vivo	C57BL/6J mice	CS or LPS (5 μg in 50 mL of 0.9% saline)	5-10 per group	NA	0.0	Unstimulated mice	CS inhalation or LPS intratracheal instillation	BALf and lung tissue	IL-6, KC, TNF-α, MCP-1, MIP-1, MIP-2, total inflammatory cell count, monocytic cells, and neutrophils
Asano et al., 2010 [60]	In vitro	Human lung fibroblasts derived from patients during pneumonectomy for tumor resection	TGF-β (1.0–10.0 ng/mL)	5	41.0–71.0	40.0	Unstimulated cells	Incubation	Cell culture supernatant	MMP-1, MMP-2, TIMP-1, and TIMP-2

ACh: acetylcholine; BALf: bronchoalveolar lavage fluid; $CdCl_2$: cadmium chloride; cAMP: cyclic AMP; CCh: carbachol; CCL5: chemokine (C-C motif) ligand 5; COPD: chronic obstructive pulmonary disease; CS: cigarette smoke; CSE: cigarette smoke extract; EBC: exhaled breath condensate; FEV_1: forced expiratory volume in 1 s; fMLP: N-formyl-L-methionyl-L-leucyl-phenylalanine; FVC: forced vital capacity; GM-CSF: granulocyte macrophage-colony stimulating factor; HCl: hydrochloric acid; HDAC: histone deacetylase; IFN: interferon; IκB-α: inhibitor of kB; IKKα: inhibitor kappa kinase alpha; IL: interleukin; KC: keratinocyte-derived chemokine; LTB_4: leukotriene B4; LPS: lipopolysaccharide; MAC-1: macrophage-1 antigen; MIP: macrophage inflammatory protein; MCP: monocyte chemotactic protein; MMP: matrix metalloproteinase; NA: not available; NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells; NLF: nasal lavage fluid; NS: normal saline; OD: organic dust; OVA: ovalbumin; PBMC: peripheral blood mononuclear cell; PBT: peripheral blood T; PBS: phosphate-buffered saline; PDCD5: programmed cell death protein 5; PHA: phytohemagglutinin; PM: particulate matter; PPE: porcine pancreatic elastase; rhIL-17A: recombinant human IL-17A; sICAM: soluble intercellular adhesion molecule; SIRT1: sirtuin 1; TGF-β1: transforming growth factor-beta 1; TIMP: tissue inhibitor of metalloproteinase; wks: weeks.

3.2. In vitro challenges

An overview concerning the impact of pro-inflammatory stimuli on humoral and cellular responses in vitro is reported in Table 3 and a Venn diagram shown in Figure 2.

3.2.1. CSE

In bronchial epithelial cells, challenge with CSE 3%–20% for 1 day to 4 days increased the gene and protein expression of IL-8 and reduced the nuclear expression of sirtuin (SIRT1), histone deacetylase (HDAC)3 and HDAC2 [58,61,69]. Stimulation with CSE 5% for 6 h induced the release of IL-8 in sputum neutrophils of COPD patients [63].

3.2.2. CSE and ACh

Challenge with CSE 20% for 4 days and 48-hours stimulation with ACh 1 μM significantly ($P < 0.05$) increased the protein expression of IL-8 in bronchial epithelial cells [61].

3.2.3. LPS

In bronchial epithelial cells and human lung fibroblasts, incubation with LPS 1 μg/mL for 4 h increased the activity of nuclear factor-kappa B (NF-κB) by activating p50 and p65 subunits and the gene expression of IL-8, whereas incubation with LPS for 1 day increased the protein expression of IL-8 in bronchial epithelial cells [68].

In peripheral blood neutrophils of COPD patients, stimulation with LPS 1 μg/mL for 6 h increased the protein expression of IL-8, matrix metalloproteinase (MMP)-9, C-C Motif Chemokine Ligand 5, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-1β, with no impact on gene expression of HDAC2 [63].

In alveolar macrophages from COPD patients, challenge up to 20 h with LPS 0.1–10 μg/mL enhanced neutrophil migration rate in a concentration-dependent manner and the release of tumor necrosis factor (TNF)-α, IL-8, IL-6, leukotrienes B4 (LTB_4), GM-CSF and macrophage inflammatory protein (MIP)-α/-β [67]. Stimulation with LPS 1 μg/mL for 12 h significantly ($P < 0.05$) increased the level of TNF-α production in alveolar macrophage cells and induced IκB-α degradation [70].

3.2.4. CCh, ACh, muscarin or oxotremorine

Stimulation with CCh 10 μM for 6 h significantly ($P < 0.05$) increased the release of IL-8 in both sputum and peripheral blood neutrophils derived from COPD patients [63]. In lung fibroblasts of asthmatic patients, incubation with CCh from 10 nM to 10 μM for 1 day significantly ($P < 0.05$) increased the production of IL-1β-induced IL-6 and IL-8 [64].

Exposure of bronchial epithelial cells to ACh 100 nM increased the gene and protein expression of IL-8 [69].

In bronchial epithelial cells, 1 h-stimulation with ACh 1 mM caused a dose-dependent increase in the generation of microparticles, that are vesicles of 50–1000 nm in size expressing negatively charged phospholipids on the outer membrane and particularly implicated in acute exacerbations of COPD [59]. The ACh-induced microparticles generation significantly upregulated the synthesis of IL-8 [59].

Incubation with ACh 1 μM for 1 day significantly ($P < 0.01$) increased the number of adhering neutrophils to bronchial epithelial cells [65] and 48-hours of ACh challenge significantly ($P < 0.05$) enhanced the release of IL-8 in bronchial epithelial cells [61].

In alveolar macrophages derived from COPD patients, stimulation with 0.1–1000 μM of ACh or 1–100 μM of CCh, muscarin, or oxotremorine for 1 h did not induce the migration of neutrophils, nor potentiated the LPS-mediated effect [67].

3.2.5. TGF-β

Stimulation with recombinant human (rh)TGF-β1 5 ng/mL for 1 day significantly ($P < 0.01$) increased the number of adhering neutrophils to bronchial epithelial cells, which was further enhanced by the concomitant incubation with ACh 1 μM [65].

In human lung fibroblasts, 1-day stimulation with TGF-β 2.5 ng/mL significantly ($P < 0.05$) increased the level and activity of MMP-1 and MMP-2, and the gene expression of TIMP-1 and TIMP-2, whereas no modulatory effect was detected on MMP-9 levels [60]. Interestingly, TGF-β 1.0 ng/mL – 2.5 ng/mL enhanced the release of MMP-2, while at concentrations ≥5.0 ng/mL it exerted a suppressive effect on MMP-2 production [60].

3.2.6. PDGF

In lung fibroblasts of asthmatic patients, incubation with PDGF-BB 0.1–10 ng/mL for 1 day significantly ($P < 0.05$) increased the release of IL-6 and IL-8 [64].

3.2.7. TNF-α

Challenge with TNF-α 0.1–10 ng/mL for 1 day induced a significant ($P < 0.05$) enhancement in the release of IL-6 and IL-8 by lung fibroblasts of asthmatic patients [64].

3.2.8. PHA

In peripheral blood mononuclear cells of asthmatic patients, stimulation with PHA 1 μg/mL for 2 days increased the release of IL-5 and IL-13 in cell culture supernatant [71].

3.2.9. OVA

Challenge with OVA 100 μg/mL for 3 days induced the overexpression of IL-4, IL-5, IL-13, and interferon (IFN)-γ in spleen cells derived from OVA-sensitized BALB/c mice [71].

3.2.10. IL-1β

In lung fibroblasts of asthmatic patients, incubation with IL-1β 10 ng/mL for 1 day elicited a significant ($P < 0.05$) increase in the production of IL-6 and IL-8, further enhanced by the concomitant incubation with CCh 100 μM [64].

3.2.11. Induced sputum supernatant from COPD patients

In bronchial epithelial cells, stimulation with the supernatant of the sputum collected from COPD patients 20% for 4 h, significantly ($P < 0.05$) increased the gene and protein expression of IL-8, the concentration of IL-17A, the translocation of nuclear inhibitor kappa kinase alpha (IKKα), and reduced the expression and activity of HDAC2 and the activity of HDAC [62]. Stimulation with the supernatant of the sputum collected from COPD patients 10% for 1 day significantly ($P < 0.05$) increased the number of adhering neutrophils on bronchial cells, whereas challenge with sputum supernatant for 2 h produced a higher expression of MAC-1 on the neutrophils from healthy donors [65].

3.2.12. rhIL-17A

In bronchial epithelial cells, 1-day stimulation with rhIL-17A 20 ng/mL significantly ($P < 0.05$) increased the production of IL-8, the translocation of nuclear IKKα, and reduced the expression and activity of HDAC2 and the activity of HDAC [62].

3.2.13. Rhinovirus type-14 infection

In human tracheal surface epithelial cells, exposure to 100 μL of type-14 rhinovirus led to infection and significantly ($P < 0.05$) increased the secretion of IL-1β, IL-6, and IL-8 [66].

3.2.14. fMLP

According to the clinical trial by Santus et al. [72] which was performed ex vivo, blood neutrophils from COPD patients primed with GM-CSF and single challenged with fMLP 0.1 μM displayed a production of leukotrienes B4 (LTB_4) at the level of the neutrophil supernatant.

3.3. In vivo challenges

Impact of pro-inflammatory stimuli on cellular and humoral responses in vivo on laboratory animals is reported in Tables 4 and 5, respectively.

Table 3. Impact of pro-inflammatory stimuli on humoral and cellular responses in vitro.

Pro-inflammatory stimulus	Outcomes investigated in the in vitro studies*																				
	Type of analyzed cells	IL-1β	IL-4	IL-5	IL-6	IL-8	IL-13	IFN-γ	TNF-α	HDAC	SIRT1	NF-κB	CCL5	GM-CSF	Neutrophils	LTB_4	MIP α/β	MMP-1	MMP-2	MMP-9	TIMP-1	TIMP-2
CSE	Bronchial epithelial cells	/	/	/	/	↑ [58, 61, 69]	/	/	/	↓ [58, 61, 69]	↓ [58, 61, 69]	/	/	/	/	/	/	/	/	/	/	/
	Sputum neutrophils of COPD patients	/	/	/	/	↑ [63]	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
CSE + ACh	Bronchial epithelial cells	/	/	/	/	↑ [61]	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
LPS	Bronchial epithelial cells	/	/	/	/	↑ [68]	/	/	/	/	/	↑ [68]	/	/	/	/	/	/	/	/	/	/
	Human lung fibroblasts	/	/	/	/	↑ [68]	/	/	/	/	/	↑ [68]	/	/	/	/	/	/	/	/	/	/
	Peripheral blood neutrophils of COPD patients	↑ [63]	/	/	/	↑ [63]	/	/	/	≈ [63]	/	/	↑ [63]	↑ [63]	/	/	/	/	/	↑ [63]	/	/
	Alveolar macrophages of COPD patients	/	/	/	↑ [67]	↑ [67]	/	/	↑ [67, 70]	/	/	/	/	↑ [67]	↑ [67]	↑ [67]	↑ [67]	/	/	/	/	/
LPS + CCh or ACh or muscarin or oxotremorine	Alveolar macrophages of COPD patients	/	/	/	/	/	/	/	/	/	/	/	/	/	≈ neutrophilic migration rate [67]	/	/	/	/	/	/	/
CCh	Sputum and peripheral blood neutrophils of COPD patients	/	/	/	/	↑ [63]	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
	Alveolar macrophages of COPD patients	/	/	/	/	/	/	/	/	/	/	/	/	/	≈ neutrophilic migration rate [67]	/	/	/	/	/	/	/
CCh + IL-1β	Lung fibroblasts of asthmatic patients	/	/	/	↑ [64]	↑ [64]	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
ACh	Bronchial epithelial cells	/	/	/	/	↑ [59, 61, 69]	/	/	/	/	/	/	/	/	↑ percentage of adhering neutrophils [65]	/	/	/	/	/	/	/
	Alveolar macrophages of COPD patients	/	/	/	/	/	/	/	/	/	/	/	/	/	≈ neutrophilic migration rate [67]	/	/	/	/	/	/	/
		/	/	/	/	/	/	/	/	/	/	/	/	/		/	/	/	/	/	/	/

(*continued on next page*)

Table 3 (*continued*)

Pro-inflammatory stimulus	Outcomes investigated in the in vitro studies*																					
	Type of analyzed cells	IL-1β	IL-4	IL-5	IL-6	IL-8	IL-13	IFN-γ	TNF-α	HDAC	SIRT1	NF-κB	CCL5	GM-CSF	Neutrophils	LTB_4	MIP α/β	MMP-1	MMP-2	MMP-9	TIMP-1	TIMP-2
Muscarin or oxotremorine	Alveolar macrophages of COPD patients														≈ neutrophilic migration rate [67]							
TGF-β	Bronchial epithelial cells	/	/	/	/	/	/	/	/	/	/	/	/	/	↑ percentage of adhering neutrophils [65]	/	/	/	/	/	/	/
	Human lung fibroblasts	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	↑ with TGF-β 2.5 ng/mL [60]	↑ with TGF-β 1.0–2.5 ng/mL [60], ↓ with TGF-β ≥5.0 ng/mL [60]	≈ with TGF-β 2.5 ng/mL [60]	↑ with TGF-β 2.5 ng/mL [60]	↑ with TGF-β 2.5 ng/mL [60]
TGF-β + ACh	Bronchial epithelial cells	/	/	/	/	/	/	/	/	/	/	/	/	/	↑ percentage of adhering neutrophils [65]	/	/	/	/	/	/	/
PDGF-BB	Lung fibroblasts of asthmatic patients	/	/	/	↑ [64]	↑ [64]	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
TNF-α	Lung fibroblasts of asthmatic patients	/	/	/	↑ [64]	↑ [64]	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
PHA	PBMCs of asthmatic patients	/	/	↑ [71]	/	/	↑ [71]	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
OVA	spleen cells from BALB/c mice	/	↑ [71]	↑ [71]	/	/	↑ [71]	↑ [71]	/	/	/	/	/	/	/	/	/	/	/	/	/	/
IL-1β	Lung fibroblasts of asthmatic patients	/	/	/	↑ [64]	↑ [64]	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
Supernatant of the sputum of COPD patients	Bronchial epithelial cells	/	/	/	/	↑ [62]	/	/	/	↓ [62]	/	↑ [62]	/	/	↑ percentage of adhering neutrophils, MAC-1 expression on neutrophils [65]	/	/	/	/	/	/	/
rhIL-17A	Bronchial epithelial cells	/	/	/	/	↑ [62]	/	/	/	↓ [62]	/	↑ [62]	/	/	/	/	/	/	/	/	/	/

(*continued on next page*)

Table 3 (*continued*)

Pro-inflammatory stimulus	Outcomes investigated in the in vitro studies*																		
	Type of analyzed cells	IL-1β	IL-4	IL-5	IL-6	IL-8	IL-13	IFN-γ	TNF-α	HDAC	SIRT1	NF-κB	CCL5	GM-CSF	Neutrophils	LTB_4	MIP α/β	MMP-1	MMP-2	MMP-9	TIMP-1	TIMP-2
Rhinovirus type-14	Human tracheal surface epithelial cells	↑ [66]	/	/	↑ [66]	↑ [66]	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
fMLP	Blood neutrophils from COPD patients primed with GM-CSF*	/	/	/	/	/	/	/	/	/	/	/	/	/	/	↑ [72]	/	/	/	/	/	/

* The study by Santus et al. [72] was performed ex vivo on human blood neutrophils.

3.3.1. CS

Exposure of DBA/2 and ICR mice to CS for 3 days significantly ($P < 0.05$) increased the number of total inflammatory cells, neutrophils, alveolar macrophages in the bronchoalveolar lavage fluid (BALf), reduced HDAC activity, and caused gene overexpression of MIP-2 and keratinocyte-derived chemokine (KC), but not of monocyte chemoattractant protein (MCP)-1 and TNF-α in lung tissue [39].

In ICR mice exposure to CS for 4 days increased the number of total inflammatory cells, neutrophils, macrophages, and lymphocytes, and produced the gene and protein overexpression of IL-1β, KC, and TNF-α, MCP-1, and TGF-β1 in the BALf and lung tissues [32, 35]. CS also increased the level of MMP-9 and reduced TIMP-1 in lung homogenate [35].

Exposure of C57BL/6 mice to CS for 4 days significantly ($P < 0.05$) increased the total and differential number of inflammatory cells in the BALf [38, 50] and lung tissue [38], and caused the overexpression of KC, TNFα, MIP-1α, and MIP-2 in the BALf [38, 50], while TGF-β1 release was only enhanced at the BALf level, but not in lung homogenates [38]. According to Wollin et al. [50], CS induced the overexpression of IL-6 and MCP-1 in the BALf, which is in contrast with the results by Kistemaker et al. [38], where CS did not modulate the levels of MCP-1 and IL-6 in the BALf [38]. A trend towards a higher number of LTB_4 and monocytic cells was detected in the BALf, while in the lung tissues, CS induced a diffuse perivascular inflammation and patchy alveolar infiltrates [50].

In C57BL/6J mice, exposure to CS for 1 month resulted in extensive inflammatory cells infiltration in the lung and increased the levels of total protein and cell counts, macrophages, lymphocytes, neutrophils, MCP-1, and MIP-2 in the BALf [69].

In BALB/cAnNCrl mice, 10 days-exposure to CS alone did not modulate cytokines expression in lung tissue and the level of inflammatory cells in the BALf [54]. Conversely, when mice were exposed to CS and infected by H1N1 influenza virus, the levels of IL-6, IFN-γ, TNF-α, IL-1β, and KC in lung tissue as well as the number of total inflammatory cells, neutrophils, and macrophages in the BALf were significantly ($P < 0.05$) increased [54]. The exposure of BALB/cAnNCrl mice to CS for 10 days with concurrent respiratory syncytial virus (RSV) infection significantly ($P < 0.05$) enhanced the expression of IL-6, TNF-α, IFN-γ, IL-1β, KC in lung homogenates, and increased the neutrophil cell count in the BALf [54].

In domestic cats, exposure to CS for 4 days significantly ($P < 0.05$) increased the concentrations of serum total protein and fibrinogen, the number of total inflammatory cells, macrophages, eosinophils, neutrophils, and lymphocytes in the blood and BALf, and induced the overexpression of IL-6, IL-8, MCP-1, and TNF-α in the BALf, as well as the perivascular, and peribronchiolar infiltration of inflammatory cells in lung tissues, thus causing an increase in the lung inflammation score [34].

In Hartley guinea pigs, gradually increased exposure to CS for 6 months, significantly ($P < 0.05$) elevated the number of infiltrating eosinophils, neutrophils, and macrophages in both alveolar septa and airways, as well as the number of pulmonary lymphoid follicles [37].

3.3.2. OVA

In BALB/c mice, challenge with 1% OVA in phosphate buffered saline (PBS) for 2 days (acute onset of allergic asthma), and rechallenge on 2 more consecutive days (relapse of allergic asthma) induced a significant ($P < 0.05$) airway eosinophilia at the level of lung parenchyma and BALf and an increase of the inflammation score [33]. In the acute allergic asthma model, OVA increased the number of total inflammatory cells, macrophages, and lymphocytes, but not neutrophils in the BALf, whereas in the relapse of asthma model, rechallenge with OVA enhanced the release of lymphocytes, with no modulatory effect on total inflammatory cells and macrophages, while neutrophil count was reduced [33].

In BALB/c mice, intranasal challenge with OVA 20 ng/50 μL in PBS for 1 month to 3 months increased the number of total inflammatory cells, eosinophils, neutrophils, and lymphocytes, the concentrations of IL-4, IL-5, and IL-13 in the BALf, and induced the infiltration of peribronchial inflammatory cells in lung tissue [30, 40].

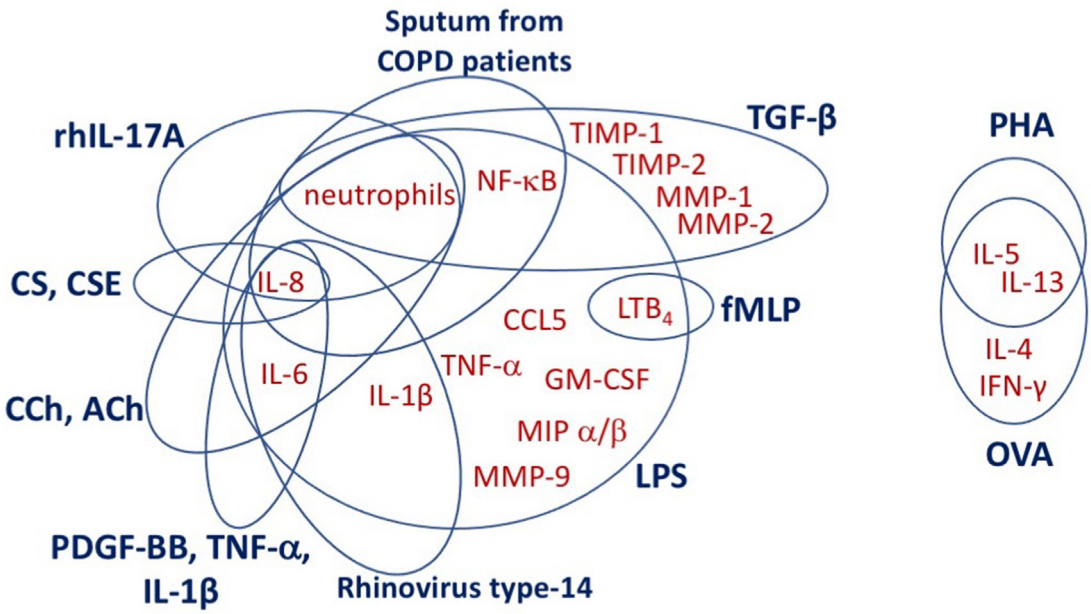

Figure 2. Venn diagram showing the overlap between the pharmacological models of airway inflammation (blue) and humoral or cellular responses in vitro (red). ACh: acetylcholine; CCh: carbachol; CCL5: C-C Motif Chemokine Ligand 5; CSE: cigarette smoke exposure; fMLP: formyl-L-methionyl-L-leucyl-phenylalanine; GM-CSF: granulocyte-macrophage colony-stimulating factor; IL: interleukin; LPS: lipopolysaccharide; LTB_4: leukotriene B4; MIP: macrophage inflammatory protein; MMP: matrix metalloproteinase; NF-κB: nuclear factor-kappa B; OVA: ovalbumin; PDGF-BB: platelet-derived growth factor-BB; PHA: phytohemagglutinin; TGF-β: tumor growth factor; TIMP: tissue inhibitor of metalloproteinase; TNF: tumor necrosis factor.

In BALB/c mice challenged acutely or chronically with OVA 2.5% for 3 days via inhalation, the BALf levels of total inflammatory cells, eosinophils, and macrophages increased, along with the release of IL-4, IL-5, IL-13, and TGF-β1 [71]. Lymphocyte count significantly ($P < 0.05$) increased only upon chronic challenge with OVA, while it remained unaffected by acute allergen exposure [71].

Challenge with OVA 2.0% in BALB/c mice for 10 days via inhalation significantly ($P < 0.05$) increased the BALf levels of total inflammatory cells and eosinophils, as well as the release of IL-4, while reducing the level of IFN-γ and IFN-γ/IL-4 ratio, and produced a peribronchial and perivascular infiltration of inflammatory cells in the lungs, especially eosinophils [45]. OVA did not modulate cell counts of neutrophils, lymphocytes, and macrophages in the BALf [45].

In BALB/c mice, challenge with nebulized 1.0% OVA for 6 days significantly ($P < 0.05$) increased the number of total inflammatory cells, eosinophils, lymphocytes, and neutrophils in the BALf, while macrophages were reduced [31]. When OVA challenge was followed by exposure to ambient PM for 11 days, total and differential leukocyte counts were significantly ($P < 0.05$) increased [31]. Stimulation with OVA alone or with subsequent exposure to ambient PM induced the overexpression of IL-5, IL-6, IL-13, and KC in the BALf and a greater peribronchiolar and perivascular inflammatory cell infiltration, while the level of IFN-γ remained unaffected [31].

In ICR mice, 1 week of challenge with inhaled OVA 10 mg/mL in saline significantly ($P < 0.05$) increased the total number of leukocytes, eosinophils, lymphocytes, macrophages, and the release of IL-4, but not IFN-γ in the BALf, whereas in lung tissues, it induced the gene overexpression of IL-4, IL-5, MMP-9, and eotaxin, with no modulatory effect on TIMP-1 and IFN-γ [44].

In Sprague-Dawley rats, challenge with intranasal instillation of 10 μL of 10% OVA for 1 week significantly ($P < 0.05$) increased the release of histamine in the nasal lavage fluid (NLF), the number of eosinophils and mast cells in the nasal mucosa, the nuclear translocation of NF-κB p65 and p50 subunits, and induced the gene and protein overexpression of IL-6, IL-13 and TNF-α in NLF and nasal mucosa [57].

In Hartley guinea pigs, single challenge with inhaled OVA from 0.05% to 0.1% in saline significantly ($P < 0.05$) increased the BALf numbers of total inflammatory cells and eosinophils, and induced a non-significant increase in macrophages, lymphocytes, and neutrophils, whereas after 3 months of OVA challenge, guinea pigs showed airway eosinophilia in the submucosa and adventitia of non-cartilaginous airways as well as in the submucosa and adventitia of cartilaginous airways, although the increase in the latter was not significant [55]. A single challenge with nebulized OVA 200 μg/mL of physiological serum induced a massive infiltration of leukocytes in the BALf [56].

Hartley guinea pigs challenged once with 5% OVA by inhalation showed a not significant increase in the number of eosinophils in the BALf and no change was observed in levels of macrophages, lymphocytes, and neutrophils. In lung tissues of guinea pigs treated with insufflated lactose, challenge with OVA significantly ($P > 0.05$) increased the number of total and nerve-associated eosinophils [43].

Hartley guinea pigs showed a significant ($P < 0.05$) infiltration of total inflammatory cells, eosinophils, and macrophages in the BALf after a double challenge with inhaled OVA 0.05% in saline performed at one week interval, whereas the number of neutrophils was not significantly affected [36].

3.3.3. LPS

In C57BL/6J mice, single intratracheal administration of LPS 5 μg in 50 mL of physiological saline induced a prominent accumulation of total inflammatory cells and neutrophils, and significantly ($P < 0.05$) increased the levels of IL-6, KC, TNF-α, MCP-1, MIP-1 α, MIP-2, and LTB_4 in the BALf, with no modulatory effect on monocytic cells [50].

In Kunming mice, a single intratracheal administration of LPS 4 mg/kg significantly ($P < 0.05$) increased the histological injury score, TNF-α, and IL-6 in lung tissues, as well as the BALf levels of total inflammatory cells and neutrophils, and induced IκB-α degradation in lung tissues [70].

Repeated intranasal instillation of LPS 5 mg/mL in saline for 3 months significantly ($P < 0.05$) induced neutrophilia at the level of cartilaginous, and noncartilaginous airways, as well as parenchyma in a Hartley guinea pig model of COPD [41].

Table 4. Impact of pro-inflammatory stimuli on cellular responses in vivo on laboratory animals.

Pro-inflammatory stimulus	Outcomes investigated in the in vivo studies on laboratory animals									
	Type of analyzed animals	Total inflammatory cell count	Eosinophils	Macrophages	Neutrophils	Lymphocytes	Monocytes	Basophils	Total protein amount	Lung inflammatory score
CS	DBA/2 mice	BALf: ↑ [39]	/	BALf: ↑ [39]	BALf: ↑ [39]	/	/	/	/	/
	ICR mice	BALf: ↑ [32, 35, 39]	/	BALf: ↑ [32, 35, 39]	BALf: ↑ [32, 35, 39]	BALf: ↑ [32, 35]	/	/	/	Lung: ↑ [32]
	C57BL/6 mice	BALf: ↑ [38, 50, 69]	/	BALf: ↑ [38, 69]	BALf: ↑ [38, 50, 69]	BALf: ↑ [38, 69]	BALf: ≈ [50]	/	BALf: ↑ [69]	Lung: ↑ [69]
	BALB/cAnNCrl mice	BALf: ≈ [54]	/	BALf: ≈ [54]	BALf: ≈ [54]	/	/	/	/	/
	Domestic cats	BALf: ↑ [34]; Lung: ↑ [34]; Serum: ↑ [34]	BALf: ↑ [34]; Serum: ↑ [34]	BALf: ↑ [34]; Serum: ↑ [34]	BALf: ↑ [34]; Serum: ↑ [34]	BALf: ↑ [34]; Serum: ↑ [34]	Serum: ↑ [34]	Serum: ↑ [34]	Serum: ↑ [34]	Lung: ↑ [34]
	Guinea pigs	/	Lung: ↑ in alveolar septa and airways [37]	Lung: ↑ in alveolar septa and airways [37]	Lung: ↑ in alveolar septa and airways [37]	/	/	/	/	/
CS + H1N1 infection	BALB/cAnNCrl mice	BALf: ↑ [54]	/	BALf: ↑ [54]	BALf: ↑ [54]	/	/	/	/	/
CS + RSV infection	BALB/cAnNCrl mice	BALf: ↑ [54]	/	BALf: ↑ [54]	BALf: ↑ [54]	/	/	/	/	/
LPS	C57BL/6J mice	BALf: ↑ [50]	/	/	BALf: ↑ [50]	/	BALf: ≈ [50]	/	/	/
	Kunming mice	BALf: ↑ [70]	/	/	BALf: ↑ [70]	/	/	/	/	Lung: ↑ [70]
	Guinea pigs	/	/	/	Lung: ↑ in cartilaginous, non-cartilaginous airways and parenchyma [41]	/	/	/	/	/
OVA	BALB/c mice	BALf: ↑ in acute onset of allergic asthma [33], in acute and chronic challenge [40, 71], in advanced-age mice [30, 31, 45], ≈ in relapse of allergic asthma [33]; Lung: ↑ in advanced-age mice [30, 45]	BALf: ↑ in acute onset and relapse of allergic asthma [33], in acute and chronic challenge [40, 71], in advanced-age mice [30, 31, 45]; Lung: ↑ in parenchyma [33, 45]	BALf: ↑ in acute onset of allergic asthma [33], in acute and chronic challenge [40, 71], ≈ in relapse of allergic asthma [33, 45], ↓ [31]	BALf: ↑ in acute and chronic challenge [40], in advanced-age mice [30, 31], ≈ [45], ↓ in acute onset and relapse of allergic asthma [33]	BALf: ↑ in acute onset and relapse of allergic asthma [33], in acute and chronic challenge [40], in chronic challenge [71], in advanced-age mice [30, 31], ≈ in acute challenge [45, 71]	/	/	/	Lung: ↑ in acute onset and relapse of allergic asthma [33]
	ICR mice	BALf: ↑ [44]	BALf: ↑ [44]	BALf: ↑ [44]	/	BALf: ↑ [44]	/	/	/	/
	Sprague-Dawley rats	/	Nasal mucosa: ↑ [57]	/	/	/	/	/	/	/
	Guinea pigs	BALf: ↑ [36, 55, 56]	BALf: ↑ [36, 55], ≈ [43]; Lung: ↑ in submucosal and adventitia of non-cartilaginous airways and adventitia of cartilaginous airways [55], total and nerve-associated eosinophils [43]	BALf: ↑ [36], ≈ [43, 55]	BALf: ≈ [36, 43, 55]	BALf: ≈ [43, 55]	/	/	/	/

(*continued on next page*)

Table 4 (*continued*)

Pro-inflammatory stimulus	Outcomes investigated in the in vivo studies on laboratory animals									
	Type of analyzed animals	Total inflammatory cell count	Eosinophils	Macrophages	Neutrophils	Lymphocytes	Monocytes	Basophils	Total protein amount	Lung inflammatory score
OVA + exposure to PM	BALB/c mice	BALf: ↑ [31]; Lung: ↑ [31]	BALf: ↑ [31]	BALf: ↑ [31]	BALf: ↑ [31]	BALf: ↑ [31]	/	/	/	/
$CdCl_2$	Sprague-Dawley rats	BALf: ↑ [48, 49, 51]	/	BALf: ↑ [48, 49], ≈ [51]	BALf: ↑ [48, 49, 51]; Lung: ↑ in alveoli, peri-bronchial regions, and parenchyma [48, 49, 51]	/	/	/	BALf: ↑ [48, 49, 51]	Lung: ↑ [48, 49, 51]
OD	BALB/c mice	BALf: ↑ [42]	BALf: ≈ [42]	BALf: ≈ [42]	BALf: ↑ [42]	BALf: ≈ [42]	/	/	/	/
House dust mite extract	C57BL/6J mice	BALf: ↑ [53]	BALf: ↑ [53]	/	/	/	/	/	/	Lung: ↑ [53]
Aspergillus fumigatus	BALB/c mice	/	BALf: ↑ [47]	BALf: ≈ [47]	BALf: ≈ [47]	BALf: ≈ [47]	/	/	BALf: ↑ [47]	/
RB	Rats	BALf: ↑ 40% IRB, 50%IRB, and 40%/60% CRB [52], ≈60%ERB [52]	/	BALf: ↑ 40% IRB, 50%IRB, 60%ERB, and 40%/60% CRB [52]	BALf: ↑ 40% IRB, 50%IRB, 60%ERB, and 40%/60%CRB [52]	/	/	/	BALf: ↑ 40% IRB, 50% IRB, 60% ERB, and 40%/60% CRB [52]	Lung: ↑ 40% IRB, 50%IRB, 60%ERB, and 40%/60%CRB [52]
PPE	ICR mice	BALf: ↑ [39]	/	/	BALf: ↑ [39]	/	/	/	/	/
Acidic solution (with HCl)	BALB/c mice	BALf: ↑ [46]; Lung: ↑ [46]	/	BALf: ↑ [46]	BALf: ↑ [46]	BALf: ↑ [46]	/	/	/	/

BALf: bronchoalveolar lavage fluid; $CdCl_2$: cadmium chloride; CRB: combined resistive breathing; CS: cigarette smoke; ERB: expiratory resistive breathing; H1N1, influenza virus; HCl: hydrochloric acid; IRB: inhaled resistive breathing; LPS: lipopolysaccharide; NF-κB: nuclear factor-kappa B; OD: organic dust; OVA: ovalbumin; PM: particulate matter; PPE: porcine pancreatic elastase; RB: resistive breathing; RSV: respiratory syncytial virus.

3.3.4. $CdCl_2$

In Sprague–Dawley rats, a single or repeated exposure for 5 weeks to $CdCl_2$ 0.1% in saline by inhalation significantly ($P < 0.05$) increased the number of total inflammatory cells, neutrophils, total protein concentration, and the activity of MMP-2 and MMP-9 in the BALf [48, 49, 51]. $CdCl_2$ did not induce a significant variation in IL-1β [48, 49] and TNF-α release [48, 49, 51], although Zhao et al. [51] reported an increase in IL-1β BALf concentration after a single challenge. Macrophages count was generally increased after exposure to $CdCl_2$ [48,49,51], although not always significantly [51]. Single and repeated $CdCl_2$ inhalation also induced the infiltration of neutrophils and macrophages in the alveoli, peri-bronchial regions and parenchyma, thus increasing the inflammatory scores associated with the severity and extent of histological injuries [48, 49, 51].

3.3.5. OD

In BALB/c mice, intranasal instillation of OD 1 mg/mL for 3 days significantly ($P < 0.05$) increased the number of total inflammatory cells and neutrophils, the release of IL-4, IL-6, TNF, MCP-1, and MIP-1, and reduced the concentrations of IL-2 and IL-13 in BALf, with no change in macrophage, eosinophil, and lymphocyte counts. In lung tissues, exposure to OD significantly ($P < 0.05$) increased the release of IL-2, IL-6, TNF-α, KC, IL-17A, and MIP-1, with a trend towards higher levels of IL-4, while IL-13 concentration remained unaffected [42].

3.3.6. House dust mite extract

In C57BL/6J mice, challenge with intranasally administered house dust mite extract 10 μg in two periods of 5 days increased the number of total inflammatory cells and eosinophils in the BALf, as well as the inflammatory score in lung tissues [53].

3.3.7. Aspergillus fumigatus

In BALB/c mice, intranasal challenge with 25 μL of *Aspergillus fumigatus* extract in PBS for 3 days significantly ($P < 0.05$) increased the total protein content, the number of eosinophils, and the overexpression of IL-4, IL-6, IL-13, and TNF in the BALf. There was a trend towards higher levels in macrophages and neutrophils counts, while no change was detected in the number of lymphocytes [47].

3.3.8. Resistive breathing

In healthy rats, induction of 6 h of inhaled resistive breathing (IRB) at a load of 40% and 50% of peak tidal inspiratory pressure (P_i) of maximum ($P_{i,max}$), expiratory resistive breathing (ERB) at 60% of peak tidal expiratory pressure (P_e) of maximum ($P_{e,max}$), and combined resistive breathing (CRB) at 40% of $P_{i,max}$ and 60% of $P_{e,max}$, produced a significant ($P < 0.05$) increase in the total protein content and in the number of macrophages and neutrophils in the BALf [52]. In lung tissues, an increase in the lung injury score and the overexpression of IL-1β and IL-6 were observed [52]. The level of total inflammatory cells significantly ($P < 0.05$) increased upon induction of IRB at 40% and 50% of $P_{i,max}$ and CRB at 40% of $P_{i,max}$ and 60% of $P_{e,max}$ in healthy rats, however ERB at 60% of $P_{e,max}$ did not alter the number of total inflammatory cells in the BALf [52].

3.3.9. PPE

In ICR mice, single intratracheal challenge with PPE 100 μg in PBS significantly ($P < 0.05$) induced the overexpression of TNF-α, MIP-2,

Table 5. Impact of pro-inflammatory stimuli on humoral responses in vivo on laboratory animals.

Pro-inflammatory stimulus	Outcomes investigated in the in vivo studies on laboratory animals																		
	Type of analyzed animals	IL-1β	IL-4	IL-5	IL-6	IL-8	IL-13	IL-17A	IFN-γ	NF-κB activity	MCP-1	MIP-1	MIP-2	KC	TNF-α	MMP-2	MMP-9	TGF-β1	TIMP-1
CS	DBA/2 mice	/	/	/	/	/	/	/	/	/	Lung: ≈ [39]	/	Lung: ↑ [39]	Lung: ↑ [39]	Lung: ≈ [39]	/	/	/	/
	ICR mice	BALf: ↑ [32, 35]; Lung: ↑ [32, 35]	/	/	/	/	/	/	/	/	BALf: ↑ [32, 35]; Lung: ↑ [32, 35], ≈ [39]	/	Lung: ↑ [39]	BALf: ↑ [32]; Lung: ↑ [32, 39]	BALf: ↑ [32, 35]; Lung: ↑ [32, 35], ≈ [39]	/	Lung: ↑ [35]	BALf: ↑ [35]; Lung: ↑ [35]	Lung: ↑ [35]
	C57BL/6 mice	/	/	/	BALf: ≈ [38], ↑ [50]	/	/	/	/	/	BALf: ≈ [38], ↑ [50, 69]	BALf: ↑ [50]	BALf: ↑ [50, 69]	BALf: ↑ [38, 50]	BALf: ↑ [50]	/	/	BALf: ↑ [38]; Lung: ≈ [38]	/
	BALB/cAnNCrl mice	Lung ≈ [54]	/	/	Lung ≈ [54]	/	/	/	Lung: ≈ [54]	/	/	/	/	Lung ≈ [54]	Lung ≈ [54]	/	/	/	/
	Domestic cats	/	/	/	BALf: ↑ [34]	BALf: ↑ [34]	/	/	/	/	BALf: ↑ [34]	/	/	/	BALf: ↑ [34]	/	/	/	/
CS + H1N1 infection	BALB/cAnNCrl mice	Lung: ↑ [54]	/	/	Lung: ↑ [54]	/	/	/	Lung: ↑ [54]	/	/	/	/	Lung: ↑ [54]	Lung: ↑ [54]	/	/	/	/
CS + RSV infection	BALB/cAnNCrl mice	Lung: ↑ [54]	/	/	Lung: ↑ [54]	/	/	/	Lung: ↑ [54]	/	/	/	/	Lung: ↑ [54]	Lung: ↑ [54]	/	/	/	/
LPS	C57BL/6J mice	/	/	/	BALf: ↑ [50]	/	/	/	/	/	BALf: ↑ [50]	BALf: ↑ [50]	BALf: ↑ [50]	BALf: ↑ [50]	BALf: ↑ [50]	/	/	/	/
	Kunming mice	/	/	/	Lung: ↑ [70]	/	/	/	/	/	/	/	/	/	Lung: ↑ [70]	/	/	/	/
OVA	BALB/c mice	/	BALf: ↑ in acute and chronic challenge [40, 71], in advanced-age mice [30, 45]	BALf: ↑ in acute and chronic challenge [40, 71], in advanced-age mice [30, 31]	BALf: ↑ [31]	/	BALf: ↑ in acute and chronic challenge [40, 71], in advanced-age mice [30, 31]	/	BALf: ≈ [31], ↓ [45]	/	/	/	/	BALf: ↑ [31]	/	/	/	BALf: ↑ in acute and chronic challenge [71]	/
	ICR mice	/	BALf: ↑ [44]; Lung: ↑ [44]	Lung: ↑ [44]	/	/	/	/	BALf: ≈ [44]; Lung: ≈ [44]	/	/	/	/	/	/	/	Lung: ↑ [44]	/	Lung: ≈ [44]
	Sprague-Dawley rats	/	/	/	NFL: ↑ [57]; Nasal	/	NFL: ↑ [57]; Nasal mucosa: ↑ [57]	/	/	NFL: ↑ [57]; Nasal	/	/	/	/	NFL: ↑ [57]; Nasal	/	/	/	/

(*continued on next page*)

Table 5 (*continued*)

Pro-inflammatory stimulus	Outcomes investigated in the in vivo studies on laboratory animals																		
	Type of analyzed animals	IL-1β	IL-4	IL-5	IL-6	IL-8	IL-13	IL-17A	IFN-γ	NF-κB activity	MCP-1	MIP-1	MIP-2	KC	TNF-α	MMP-2	MMP-9	TGF-β1	TIMP-1
					mucosa: ↑ [57]					mucosa: ↑ [57]					mucosa: ↑ [57]				
OVA + exposure to PM	BALB/c mice	/	/	BALf: ↑ [31]	BALf: ↑ [31]	/	BALf: ↑ [31]	/	BALf: ≈ [31]	/	/	/	/	BALf: ↑ [31]	/	/	/	/	/
$CdCl_2$	Sprague-Dawley rats	BALf: ↑ [51], ≈ [48, 49]	/	/	/	/	/	/	/	/	/	/	/	/	BALf: ≈ [48, 49, 51]	BALf: ↑ [48, 49, 51]	BALf: ↑ [48, 49, 51]	/	/
OD	BALB/c mice	/	BALf: ↑ [42]; Lung: ≈ [42]	/	BALf: ↑ [42]; Lung: ↑ [42]	/	BALf: ↓ [42]; Lung: ≈ [42]	Lung: ↑ [42]	/	/	BALf: ↑ [42]	BALf: ↑ [42]; Lung: ↑ [42]	/	Lung: ↑ [42]	BALf: ↑ [42]; Lung: ↑ [42]	/	/	/	/
Aspergillus fumigatus	BALB/c mice	/	BALf: ↑ [47]	/	BALf: ↑ [47]	/	BALf: ↑ [47]	/	/	/	/	/	/	/	BALf: ↑ [47]	/	/	/	/
RB	Rats	Lung: ↑ 40% IRB, 50% IRB, 60% ERB, and 40%/ 60% CRB [52]	/	/	Lung: ↑ 40%IRB, 50%IRB, 60% ERB, and 40%/ 60%CRB [52]	/	/	/	/	/	/	/	/	/	/	/	/	/	/
PPE	ICR mice	/	/	/	/	/	/	/	/	Lung: ↑ [39]	BALf: ↑ [39]	/	BALf: ↑ [39]	BALf: ↑ [39]	BALf: ↑ [39]	/	/	/	/
Acidic solution (with HCl)	BALB/c mice	/	/	/	/	BALf: ↑ [46]	/	/	/	/	/	/	/	/	/	/	/	/	/

BALf: bronchoalveolar lavage fluid; $CdCl_2$: cadmium chloride; CRB: combined resistive breathing; CS: cigarette smoke; ERB: expiratory resistive breathing; H1N1, influenza virus; HCl: hydrochloric acid; IFN-γ: interferon-gamma; IL: interleukin; IRB: inhaled resistive breathing; KC: keratinocyte-derived chemokine; LPS: lipopolysaccharide; MCP: monocyte chemoattractant protein; MIP: macrophage inflammatory protein; MMP: matrix metalloproteinase; NF-κB: nuclear factor-kappa B; NFL: nasal lavage fluid; OD: organic dust; OVA: ovalbumin; PM: particulate matter; PPE: porcine pancreatic elastase; RB: resistive breathing; RSV, respiratory syncytial virus; TGF: tumor growth factor; TIMP: tissue inhibitor of metalloproteinase; TNF: tumor necrosis factor.

MCP-1, and KC, increased the number of total inflammatory cells and neutrophils in the BALf.

In lung tissues, PPE increased the level of phosphorylated NF-κB and reduced IκB-α, the enzymatic activity of HDAC, and the gene and protein expression of HDAC2 [39].

3.3.10. Acidic solution

In BALB/c mice, esophageal instillation of an acidic solution containing HCl for 3 weeks, caused a marked infiltration of inflammatory cells in BALf and lung tissues, significantly ($P < 0.05$) increased the BALf number of total inflammatory cells, macrophages, lymphocytes, and neutrophils, and induced the overexpression of IL-8 and soluble intercellular adhesion molecule 1 (sICAM-1) in the BALf, as well [46].

3.4. Quality of evidence and risk of bias

The clinical trial by Santus et al. [72] included in this systematic review was ranked as being of low quality (Jadad score <3).

The traffic light plot for the assessment of the risk of bias of the included clinical study is reported in Figure 3A, and the weighted plot for the assessment of the overall risk of bias by domains is shown in Figure 3B.

The clinical trial had low risk of bias for missing outcome data, measurement of the outcomes, and selection of the reported results. There were some concerns for the risk of bias in the randomization process and no information concerning deviations from intended intervention.

4. Discussion

Airway inflammation plays a critical role in the pathogenesis of asthma and COPD [73], involving a complex interplay between and among structural lung cells and resident and infiltrating immune cell populations [6, 7]. The purpose of in vitro approaches and ex vivo models is to mimic certain pathophysiological parameters or processes that are likely to be important when developing new pharmacological options within a tightly controlled environment [74]. Human in vitro and ex vivo analyses enable to accomplish mechanistic studies that cannot be performed in vivo for ethical issues [75] and provide complementary information to animal experiments, yet in most cases, they do not offer feasible alternative approaches on their own, as sharply outlined in the latest statement of the European Respiratory Society on the optimization of experimental research in respiratory diseases [76].

Immortalized cell lines originating from human respiratory tract represent a simple and valuable tool to investigate airway cellular responses [77], however due to the transformation process and homogenous clonality [78], they lack all the underlying genetic or epigenetic hallmarks characterizing the original tissue [79].

By contrast, primary airway cells directly acquired from human donors are considered the gold standard to simulate pulmonary epithelia in vitro [80], as they more closely resemble the native in vivo microenvironment [78]. Major drawbacks include limited availability, a finite proliferative lifespan, and donor-donor variations [80, 81].

In recent years, considerable effort has been undertaken to develop three-dimensional (3D) organoids as alternatives to in vivo animal experiments [82]. On the one hand, this approach stems from ethical issues in agreement with the application of the "3Rs principles" (refinement, reduction, and replacement) to safeguard the highest standards of animal welfare in research and testing [83]. On the other hand, advances in leveraging in vitro approaches come as a response to the inability of animal models to fully mimic the complexity of human pulmonary diseases, with some questioning whether preclinical findings have a translational value for clinical application [84].

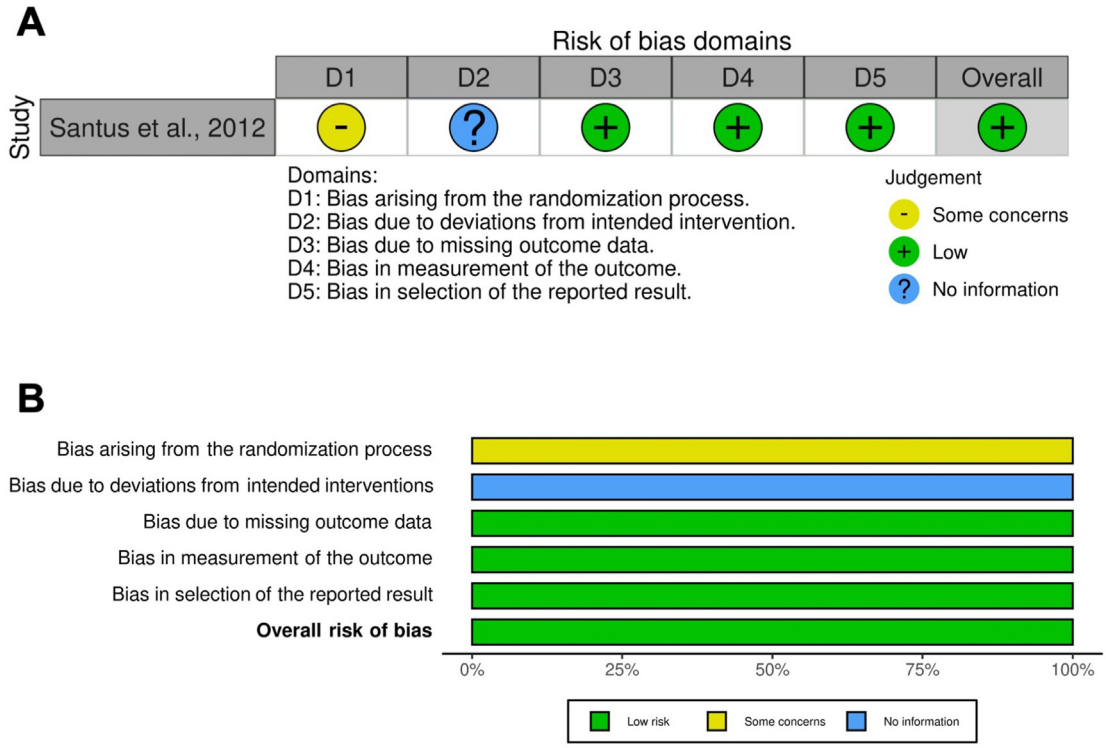

Figure 3. Assessment of the risk of bias via the Cochrane RoB 2 tool displayed by means of a traffic light plot of the risk of bias of each included clinical study (A), and weighted plot for the distribution of the overall risk of bias within each bias domain via the Cochrane RoB 2 tool (B) (n = 1 clinical study). Traffic light plot reports five risk of bias domains: D1, bias arising from the randomization process; D2, bias due to deviations from intended intervention; D3, bias due to missing outcome data; D4, bias in measurement of the outcome; D5, bias in selection of the reported result; Yellow circle indicates some concerns on the risk of bias and green circle represents low risk of bias. RoB: risk of bias.

Lung-on-chip models are micro-engineered cell culture devices that reconstitute in 3D the morphometric traits, the physiological functions, the microenvironment, and breathing motions of the human lung [85, 86]. Lung-on-chips are cost-effective, time-efficient, and highly sensitive, nevertheless they also present some disadvantages [87]. First, most models use immortalized cell lines instead of human primary cells and are devoid of several important morphological and immunological features [87], then the lack of universal protocols for the design, fabrication, and utility of these models make it difficult for researchers to adopt a specific combination of materials and settings as a conventional standard [87]. Despite advances in the field, few lung-on-chip studies have been designed to investigate respiratory diseases including asthma [88], pulmonary edema [89], and COPD [90] or to simulate a cellular microenvironment of a chronic lung disease state [91].

When it comes to complex diseases such as asthma and COPD, it is important to use animal models with an intact respiratory system for translation of in vitro findings from bench-to-bedside [92]. The idea of modelling human diseases in animals is a relatively recent advance, although the recognition of common features at the structural and functional level between humans and animals is ancient [93]. Especially since the adoption of the murine model as the preferred test system for preclinical research, Hau et al. [94] subdivided modern modelling into five categories: induced models, spontaneous models, genetically modified models, negative models, and orphan models.

Induced animal models are extensively used in pulmonary disease research and use healthy animals in the attempt to recreate a respiratory condition reflective of a human pathological state [93]. An example is represented by mice that do not spontaneously develop asthma, hence an asthma-like condition has to be artificially induced in the airways by using clinically relevant allergens such as chicken OVA and house dust mite [95]. It is true that mice offer several advantages including low handling costs, short breeding and gestational period compared to higher species, ease of genetic manipulation, and a wide array of immunological reagents for the analysis of allergic reactions [96, 97, 98]. However, mice present significant inter-species variations at the anatomical, genetic, and immunological level compared to humans [99].

In rodents, the release of mast-cell derived serotonin and the activation of airway parasympathetic nerves are essential to evoke bronchoconstriction in response to acute allergen challenge [100, 101, 102, 103]. By contrast, allergen-induced bronchospasm in humans and guinea pigs is mostly mediated by the activation of histamine H_1 and leukotriene $CysLT_1$ receptors in ASM, while mast cells store or release little if any serotonin, that has practically no effect on ASM [104, 105]. Moreover, murine airways are devoid of the bronchial vasculature and submucosal glands readily found in human and guinea pigs airways [106, 107] and the subtypes of β-adrenoceptors that can be activated therapeutically to reverse acute asthmatic bronchoconstriction are different in mice [57].

Compared to more conventional laboratory animal species, guinea pigs exhibit an anatomy and physiology of the lung, as well as receptor and mediator pharmacology that are comparable to humans [108, 109]. Interestingly, the allergen-induced airway obstruction in guinea pigs involves both early and late allergic response, that make it similar to humans and dissimilar from mice [110]. Although guinea pig models of asthma and COPD are clinically superior to rodents [111], their use is still limited due to the paucity of available reagents and lack of genetically manipulated strains [112].

Equine airways constitute a suitable ex vivo model to pharmacologically investigate the effect of treatments for human chronic obstructive pulmonary disorders, including asthma and COPD [113]. Equine recurrent airway obstruction (RAO), also called heaves or broken wind, is a chronic disorder of mature horses characterized by variable and episodic airflow limitation, AHR, and airway inflammation triggered by the exposure of susceptible animals to aeroallergens [114, 115]. The lung pathology of equine RAO shares remarkable homology with human asthma [115, 116], being the result of delayed lung's hypersensitivity response to inhaled antigens, AHR, and lower airway inflammation [117, 118]. Alternatively, the absence of an early phase response, the neutrophilic inflammation, the narrowing of the airways, and the limited bronchial reversibility make equine RAO comparable to COPD in humans. Moreover, equine RAO models have shown comparable responses to currently recommended treatments for asthma and COPD to the effect elicited in human patients [16, 17, 119]. As in human COPD, non-specific AHR in horses is predominantly caused by the release of neuronal and non-neuronal ACh that interacts with muscarinic M_3 receptors expressed on the ASM, leading to the activation of airway reflexes by inflammatory mediators, and a decrease in inhibitory mechanisms [14, 120, 121, 122]. Therefore, investigations in equine airways provide valuable translational results to human studies [113].

Most animal models of COPD use CS exposure as the primary testing approach for drug therapies and studies on the pathogenic mechanisms of the disease [123]. CS-induced model of COPD has shown to induce many of the features observed in humans, including lung infiltration of macrophages and neutrophils, fibrosis, emphysema, and airway and vascular remodeling in specific murine strains [93, 123, 124, 125]. A major advantage of CS-models is that the employed stimulus represents the main risk factor for the development of COPD in humans [123]. Nevertheless, there is no standardized protocol for the assessment of animal exposure to CS, therefore the type of cigarette used, the constituents of smoke, and the delivery systems vary across the studies [24]. Another important drawback of the model is that the lesions caused by CS do not appear to progress after cessation of exposure, therefore it fails to recreate the severe impairment observed in humans [123].

Emphysema can be modelled as well by exposing animals to the elastin-degrading elastase [126], yet some question the relevance of the model to human disease, as <1% of COPD patients show reduced elastin levels, as occurs with α1-antitripsin deficiency [93].

Genetically-modified animals are also routinely employed as models of COPD, for example transgenic collagenase overexpression induces histological alterations which reflect human emphysema [127].

Although these and related models of induced respiratory diseases have given the chance to shed light on the biological pathways of injury and repair, they are unable to recreate the overall complexity of humans [93]. Considering animal models with naturally occurring respiratory diseases may be more appropriate, as many of the clinical signs developed in these animals are accompanied by lung pathology similar to that of the corresponding human disease [93].

As no single animal model recapitulates all aspects of a respiratory disease clinical presentation but rather few features, it is necessary to utilize the complementarity between in vitro and in vivo animal models with translational approaches [76], first among all pre-clinical models employing human isolated airways ex vivo.

Passive sensitization is a validated ex vivo model that closely replicates important functional features of non-specific AHR, typical of asthmatic airways in vivo [128, 129, 130, 131, 132]. In agreement with this model, human isolated bronchi are incubated overnight at room temperature with Krebs-Henseleit (KH) solution and sensitizing serum from donors with atopic asthma during an exacerbation [128, 129]. Numerous studies performed ex vivo on passively sensitized human isolated bronchi have pharmacologically characterized the impact of ICS, bronchodilators, and monoclonal antibodies [12, 133, 134, 135, 136], as well as the bronchorelaxant effect of brain natriuretic factor on the ASM [137].

The overnight incubation of bronchial tissues with LPS administered at 100 ng/mL represents a validated ex vivo model of COPD mimicking the airways condition during an exacerbation in vivo [138, 139, 140]. Several studies employed this specific model to assess the anti-inflammatory effect of N-acetylcysteine [141, 142] while in others, acute administration of LPS 300 ng/mL was used to investigate the underlying mechanisms of AHR [140, 143].

Evidence resulting from ex vivo studies performed on human isolated airways have certainly superior translational value when compared with those obtained from animal models [144]. Indeed, serious biological differences between murine models and humans preclude the possibility

to fully replicate the results into clinical trials, including the phylogenomic distance [145], the complexity of human disease [146], and the different immune cellular composition [147].

Furthermore, ex vivo models of human bronchi are considered more physiologically relevant than in vitro cell-based models, as they retain the 3D structure and microenvironment of the lung, including ASM cells, neurons, epithelial cells, fibroblasts, inflammatory cells, and other residential cells [136].

Cell-based in vitro assays and murine models are extensively used to investigate the impact of muscarinic receptor antagonists on airway inflammation. Both options are cost-effective, easy to handle, and time efficient, although they also present major drawbacks, including the inability to replicate all pathophysiological features of a respiratory disease.

Overall, despite specific limitations, most pre-clinical pharmacological models performed in vitro or in vivo in laboratory animals could allow assessing the beneficial effect of muscarinic receptor antagonists against inflammatory response. However, in the last decade studies carried out on human isolated airways, both medium bronchi and small airways, have been performed and validated as suitable ex vivo models of asthma, COPD, and other respiratory disorders [12, 133, 135, 136, 141, 142, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157]. Interestingly, these models not only provide translational bench-to-bedside contributions by a functional and mechanistic point of view [148, 151, 153, 156], but they also strictly reproduced ex vivo the inflammatory profile detectable in patients suffering from stable COPD, acute exacerbation of COPD, and asthma [12, 141, 142, 158, 159, 160]. These characteristics make ex vivo experiments performed by stimulating human airways the most effective translational pre-clinical approach to investigate the impact of pharmacological treatments on the inflammatory profile of the airways in asthma and COPD. To date, only a model of asthma exacerbation is still missing, but it could be set up in the next future by challenging passively sensitized bronchi with platelet-activating factor, an ex vivo condition that allows activating airway tissue residential eosinophils in hyperresponsive human bronchi [130, 161, 162, 163, 164, 165]. In any case, great effort should be undertaken to rely on more physiologically relevant pre-clinical models, which provide translational value into clinic and have a direct impact on patient outcomes.

Declarations

Author contribution statement

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