Biomedicine & Pharmacotherapy 193 (2025) 118667

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Anti-inflammatory therapies from bedside to bench: Frameworks to support preclinical development

Cristina Cigana [*,1], Alice Rossi [1], Davide Gugliandolo, Ida De Fino, Alessandra Bragonzi [*]

Infections and Cystic Fibrosis Unit, Division of Immunology, Transplantation and Infectious Diseases, IRCCS San Raffaele Scientific Institute, Milano, Italy

ARTICLE INFO

Keywords:
Inflammation
Infection
Respiratory disease
Pre-clinical studies

ABSTRACT

The discovery of anti-inflammatory therapies is a top priority for people suffering from respiratory diseases. However, the limited clinical efficacy of new drugs—often not predicted in preclinical studies—underscores the need for improved evaluation criteria. This study retrospectively evaluates marketed anti-inflammatory therapies with different mechanisms of action (azithromycin, ibuprofen, and dexamethasone) to identify suitable preclinical models and biomarkers for efficacy testing. Lipopolysaccharide (LPS) exposure and both acute and chronic *Pseudomonas aeruginosa* lung infection models, using different treatment regimens, initiated either immediately or seven days after infection, were compared. Our results demonstrate that only the chronic *P. aeruginosa* infection model reliably detected therapeutic effects, making it the most appropriate for evaluating anti-inflammatory therapies in respiratory conditions. All tested drugs improved body weight and reduced inflammatory markers in the lung, including cell recruitment, myeloperoxidase, neutrophil elastase, and key cytokines, whereas blood cell measurements proved less reliable. Bacterial load was shown to improve alongside the amelioration of the immune response. In contrast, dexamethasone alone was effective in the LPS model, while the acute infection model showed worsening inflammation. These findings highlight the value of the chronic *P. aeruginosa* model, which remains underused in preclinical research, and support its broader adoption in testing anti-inflammatory therapies for respiratory diseases.

1. Introduction

Preclinical research follows the "bench-to-bedside" approach, translating basic scientific discoveries into clinical treatments. Anti-inflammatory drugs for respiratory conditions, such as those used in cystic fibrosis (CF), asthma, and chronic obstructive pulmonary disease (COPD), are approved through this process or via drug repurposing [1]. However, the uncertain efficacy of approved drugs, due to incomplete resolution of inflammation and side effects, along with the low success rate in developing novel therapies, has posed major challenges in the field. This issue reflects a broader problem in drug development, where preclinical models are often not predictive of clinical outcomes, regardless of the model used [2]. Despite efforts to improve the predictability of animal testing, the failure rate in clinical trials continues to rise, primarily due to insufficient clinical efficacy and unanticipated safety concerns [3–5]. Thus, designing reliable preclinical studies remains a major bottleneck in translating scientific discoveries into effective treatments.

A review of past preclinical studies of anti-inflammatory agents for respiratory diseases reveals a lack of established guidelines for the selection of appropriate animal models and biomarkers for efficacy testing [1,6]. This lack of standardized guidelines is not due to a lack of data, but because preclinical models, regardless of type, are not always predictive of clinical responses. Improved selection and rational application of these models could reduce failure rates and enhance the drug development process.

Inflammation plays a critical role in the progression of chronic respiratory diseases such as asthma, COPD, CF, and non-CF bronchiectasis, leading to lung destruction and loss of pulmonary function [7–9]. Many inflammatory processes are disrupted in respiratory conditions, whether due to intrinsic pathophysiological factors or acquired airway infections [1]. This complexity leads to numerous potential targets amenable to anti-inflammatory therapy [10]. Regardless of a drug's specific mechanism of action, it must ultimately affect cell-mediated responses,

* Correspondence to: Infections and Cystic Fibrosis Unit, Division of Immunology, Transplantation and Infectious Diseases, San Raffaele Scientific Institute, Milano, Italy.
E-mail addresses: cigana.cristina@hsr.it (C. Cigana), bragonzi.alessandra@hsr.it (A. Bragonzi).
[1] These authors share joint first authorship and are listed in alphabetical order.

https://doi.org/10.1016/j.biopha.2025.118667
Received 29 May 2025; Received in revised form 9 October 2025; Accepted 17 October 2025
Available online 29 October 2025

predominantly driven by neutrophils, or their products, to impact airway inflammation. Previous clinical trials have underscored the importance of selecting the right target and dosing to avoid excessive impairment of host defenses, which could increase the risk of pulmonary exacerbations [11,12]. Additionally, identifying biomarkers capable of monitoring disease activity and therapeutic response remains a critical challenge.

This study retrospectively evaluates three marketed anti-inflammatory therapies, which have different mechanisms of action and are used in both acute and chronic respiratory conditions. Dexamethasone (DEX) was chosen as an example of a steroid due to its potent anti-inflammatory effects, ibuprofen (IBU) as a non-steroidal gold standard for managing inflammation in people with CF and COPD, and azithromycin (AZM), a macrolide antibiotic, known for exerting its anti-inflammatory effects in people with CF and non-CF bronchiectasis [13,14]. We compared three commonly used preclinical mouse models: those induced with lipopolysaccharide (LPS), and acute and chronic *Pseudomonas aeruginosa* infections. Notably, although the chronic infection model more accurately reflects chronic respiratory diseases, it remains underutilized in preclinical studies despite its potential to provide more clinically relevant data [15–17].

This study (Fig. 1) aims to identify: (1) the most relevant preclinical models, and (2) biomarkers of inflammation, both local and systemic, as well as bacterial counts, for assessing the efficacy of anti-inflammatory agents with distinct mechanisms of action, to more accurately translate preclinical findings into clinical studies.

2. Results

2.1. AZM efficacy depends on treatment schedule in chronic P. aeruginosa lung infection and is ineffective in LPS and acute infection models

LPS from *P. aeruginosa* PAO1 and the *P. aeruginosa* multidrug-resistant (MDR)-RP73 strain, in both planktonic form and embedded in agar beads, were used to challenge C57BL/6NCrlBR mice via intratracheal instillation (i.t.) to induce lung inflammation or simulate acute and chronic infections [15,16,18]. AZM was administered orally by gavage at doses of 150 and 500 mg/kg before LPS and planktonic MDR-RP73 injection (Fig. 2). In the chronic infection model, mice received repeated drug administration for 7 days, either starting soon before infection ("early" treatment) or 7 days after infection ("late" treatment).

A single AZM dose at both concentrations did not significantly affect pulmonary or systemic inflammation in LPS or acute *P. aeruginosa* infection models. AZM and vehicle-treated mice showed no differences in bronchoalveolar lavage fluid (BALF) leukocyte levels (Fig. 2**A**), including neutrophils (Fig. 2**B**) and macrophages (Fig. S1A). Neutrophil infiltration and tissue damage markers, myeloperoxidase (MPO) and neutrophil elastase (NE), remained unchanged in both BALF (Fig. S1B**,C**) and lung tissue supernatants (Fig. 2**C, D**). Systemic levels of neutrophils, lymphocytes, and monocytes were also unaffected (Fig. S1D). Furthermore, AZM treatment did not reduce bacterial burden in the acute infection model (Fig. 2**E**).

Next, we tested AZM at repeated doses in the chronic *P. aeruginosa* infection model. Over 7 days of "early" treatment schedule, AZM-treated mice at both concentrations maintained healthier body weight and recovered faster than vehicle-treated mice (Fig. 2**F**). AZM-treated mice showed a significant dose-dependent reduction in BALF total cells, particularly neutrophils (Fig. 2**A, B**), with unchanged macrophage levels (Fig. S1A). MPO levels decreased in BALF and lung supernatants at both concentrations, with statistically significant differences reached only in the lung (Fig. S1B**,** Fig. 2**C**). A stronger effect was observed for NE, which was significantly reduced at both doses in both BALF and lung supernatants (Fig. S1C, Fig. 2**D**). Pro-inflammatory cytokines/chemokines IL-1β, KC, MIP-1α, and IL-6 were significantly reduced in a dose-dependent manner, while TNF-α and MCP-1 levels remained unaffected (Table 1). Hematological analyses evaluated the systemic immune profile (Fig. 2**G**). AZM-treated mice had significantly lower percentages of neutrophils and monocytes and higher percentages of lymphocytes and eosinophils, with unchanged basophil levels. In addition, AZM-treated mice exhibited antimicrobial effects, showing a significant reduction in pulmonary bacterial burden compared to vehicle (Fig. 2**E**).

When AZM treatment was initiated 7 days after infection ("late" treatment), there was no difference in body weight recovery between AZM- and vehicle-treated mice (Fig. 2**H**). AZM administered at this later stage failed to reduce BALF total and differential cell counts (Fig. 2**A, B,** Fig. S1A), as well as MPO (Fig. 2**C,** Fig. S1B**)**, NE (Fig. 2**D,** Fig. S1C**)**, and cytokines/chemokines levels (Table 1). Surprisingly, despite the lack of an anti-inflammatory effect, AZM treatment resulted in a significant

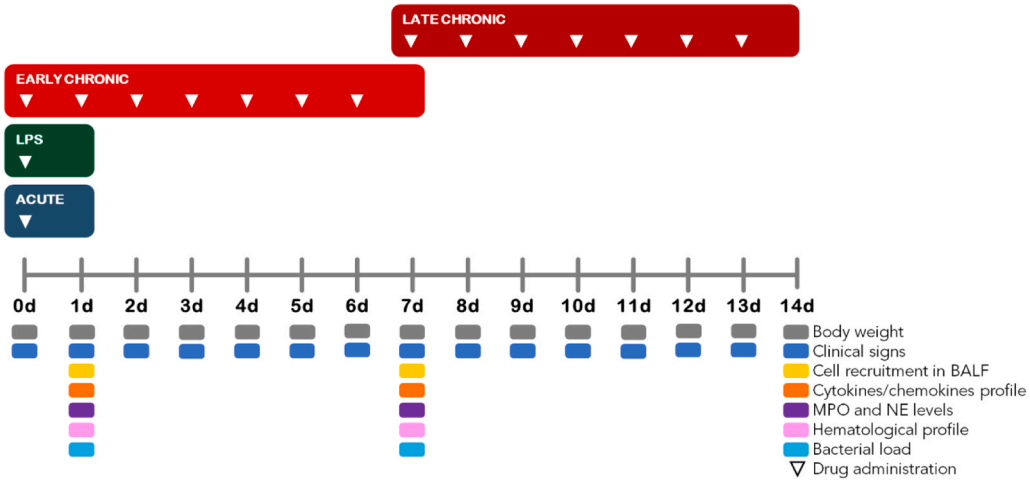
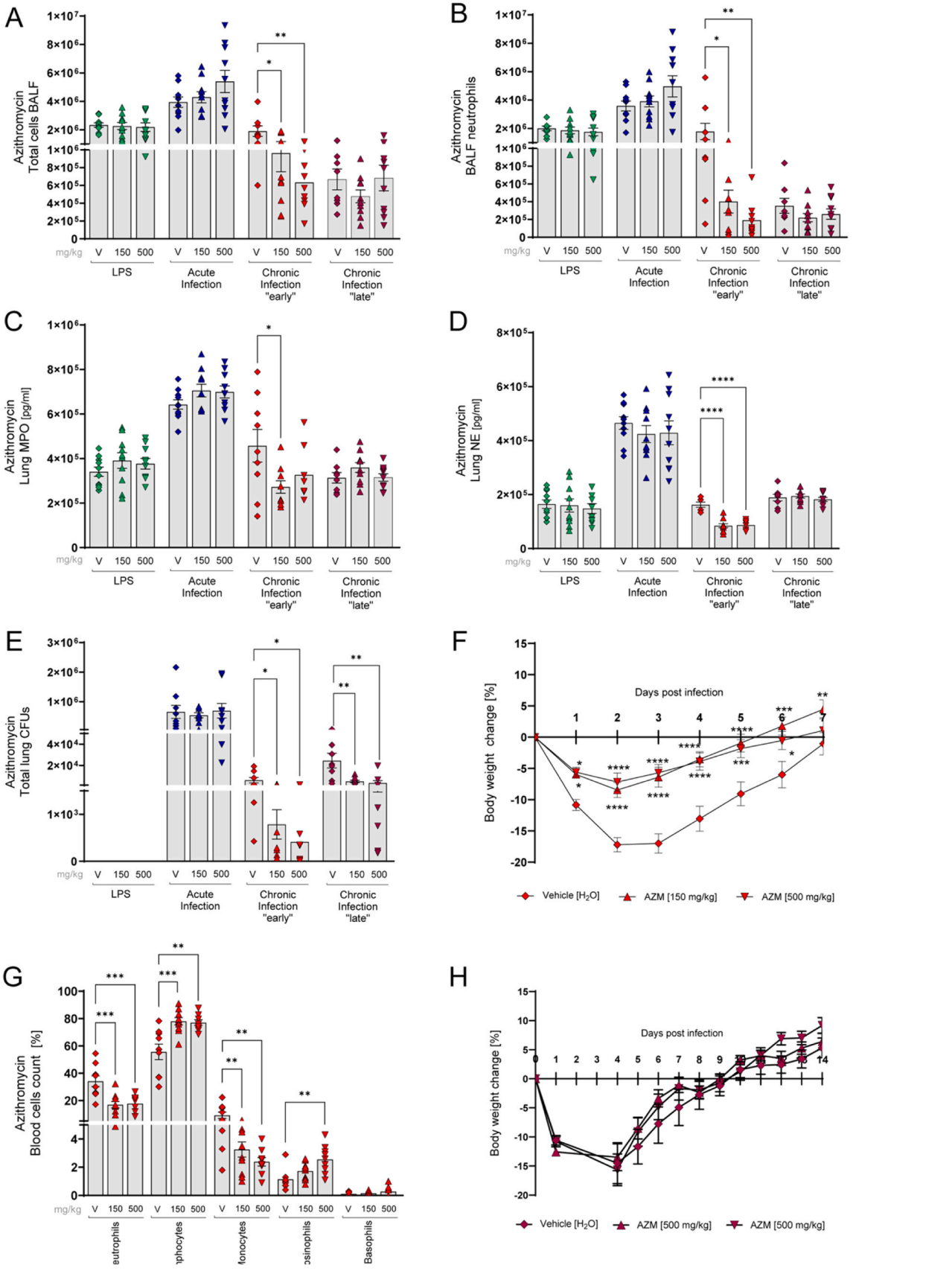


Fig. 1. A schematic representation of the anti-inflammatory treatment schedule and analysis in murine models of LPS-induced inflammation, and of acute and chronic *Pseudomonas aeruginosa* infection. On day 0, mice were treated with LPS or infected with *P. aeruginosa* planktonic cells to mimic acute infection, or with *P. aeruginosa* embedded in agar beads to induce a long-term chronic infection. In the LPS and acute infection models, a single dose of the anti-inflammatory treatment was administered orally 5 min before infection. In the chronic infection model, treatment was initiated either 5 min before infection ("early" treatment) or 7 days ("late" treatment) after infection, with daily doses administered over a 7-day period. Endpoints included body weight, clinical signs, CFUs, total and differential cell counts, hematological profiles, and cytokine/chemokine levels in BALF, assessed at 24 h post-LPS injection or infection. The chronic infection models were evaluated similarly after 7 days of treatment.



(caption on next page)

Fig. 2. Efficacy of AZM in a mouse model of LPS-induced inflammation, acute, and chronic *P. aeruginosa* airway infection. Male C57BL/6N mice (8–10 weeks old) were challenged with planktonic *P. aeruginosa* MDR-RP73 (1×10^7 CFUs), with MDR-RP73 embedded in agar beads ($5–9 \times 10^5$ CFUs) or 20 μg/mouse of LPS from *P. aeruginosa* PAO1 by intratracheal instillation (i.t.). Five minutes before challenge with LPS or planktonic bacteria, AZM (150 or 500 mg·kg^{-1}) or vehicle (V) were administered via gavage. The treatment of mice challenged with bacteria embedded in agar beads was repeated daily for 7 days, starting the day of infection ("early" model) or seven days after infection ("late" model). At 24 h after the treatment, the mice were sacrificed, bronchoalveolar lavage fluid (BALF) was collected, and lungs were excised, homogenized, and plated onto tryptic soy agar to determine the bacterial burden. Each dot represents total cell count in BALF (A), neutrophil count in BALF (B), MPO (C), and NE levels in lung supernatants (D), and CFUs per lung (E) from one mouse. For the chronic "early" model, before each administration, mice were weighed, and the percentage change from the initial body weight was averaged for each group (F). Hematological analysis of specific populations in white blood cells (WBC) was performed (G). For the chronic "late" model, mice were weighed daily, and the percentage change from the initial body weight was averaged for each group (H). Data are presented as mean ± standard error of the mean (SEM), pooled from two independent experiments (n = 8–10). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

Table 1
Cytokine/chemokine concentrations following chronic *Pseudomonas aeruginosa* airway infection with "early" and "late" AZM and IBU treatment and upon LPS challenge following DEX treatment.

	Azithromycin					
	Chronic infection "early"			Chronic infection "late"		
	Vehicle	**150 mg/kg**	**500 mg/kg**	**Vehicle**	**150 mg/kg**	**500 mg/kg**
IL-1β	5.49 ± 0.94	**2.32 ± 0.42*****	**1.58 ± 0.20** ****	1.27 ± 0.09	1.43 ± 0.15	1.19 ± 0.07
IL-6	3.34 ± 0.67	**1.76 ± 0.35***	1.24 ± 0.12**	0.91 ± 0.04	1.16 ± 0.04	1.05 ± 0.12
TNF-α	6.61 ± 0.65	6.07 ± 0.52	6.05 ± 0.57	5.12 ± 0.22	6.34 ± 0.63	5.72 ± 0.32
MIP-1α	50.85 ± 13.03	**14.14 ± 3.34****	**12.35 ± 2.75*****	5.98 ± 1.02	5.35 ± 0.48	6.65 ± 0.78
MCP-1	142.3 ± 17.55	100.5 ± 8.92	186.7 ± 14.14	80.34 ± 6.84	90.8 ± 10.06	104.2 ± 10.53
KC	74.73 ± 13.97	**34.42 ± 4.57****	**28.97 ± 2.73*****	23.9 ± 2 05	25.83 ± 1.60	26.39 ± 1.45

	Ibuprofen				**Dexamethasone**		
	Chronic infection "early"		Chronic infection "late"		LPS-induced inflammation		
	Vehicle	**50 mg/kg**	**Vehicle**	**50 mg/kg**	**Vehicle**	**5 mg/kg**	**10 mg/kg**
IL-1β	7.08 ± 1.91			1.20 ± 0.09		10.91 ± 2.51	11.12 ± 1.94
		2.40 ± 0.30**	1.26 ± 0.09		16.21 ± 1.94		
IL-6	3.78 ± 0.59			0.91 ± 0.06		13.13 ± 2.79	10.81 ± 2.70
		1.38 ± 0.10 ***	0.91 ± 0.04		11.85 ± 1.83		
TNF-α	6.74 ± 0.51			5.07 ± 0.17		9.65 ± 2.45	10.63 ± 2.33
		6.14 ± 0.44	5.12 ± 0.22		11.76 ± 1.89		
MIP-1α	56.86 ± 10.03			6.35 ± 1.20		135.9 ± 37.48	134.5 ± 33.65
		13.9 ± 3.11 **	5.98 ± 1.02		228.8 ± 18.46		
MCP-1	176.9 ± 22.04			90.95 ± 9.08		405.1 ± 78.79	409.7 ± 55.47
		108.4 ± 7.24*	80.34 ± 6.84		410.3 ± 74.52		
KC	87.49 ± 12.15			44.77 ± 10.23		470.9 ± 84.67	472.6 ± 31.64
		40.46 ± 7.99*	23.9 ± 2.05		301.1 ± 28.56		

Concentration pg/700 pg of lung homogenate supernatant.
Data are presented as mean values ± standard errors of the means (SEMs) of results pooled from three independent experiments for LPS-induced inflammation model (n = 5) and chronic infection "early" (n = 8) and two independent experiments for chronic infection "late" (n = 7–8). Statistical significance was determined by one-way ANOVA with Bonferroni's multiple comparison test for AZM and DEX treatments and by Mann-Whitney T-test for IBU treatment, with significant differences between treatment and vehicle highlighted in bold. AZM: azithromycin; IBU: ibuprofen; DEX: dexamethasone; IL: interleukin; KC: keratinocyte chemoattractant; MCP: monocyte chemoattractant protein; MIP: macrophage inflammatory protein; TNF: tumor necrosis factor; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

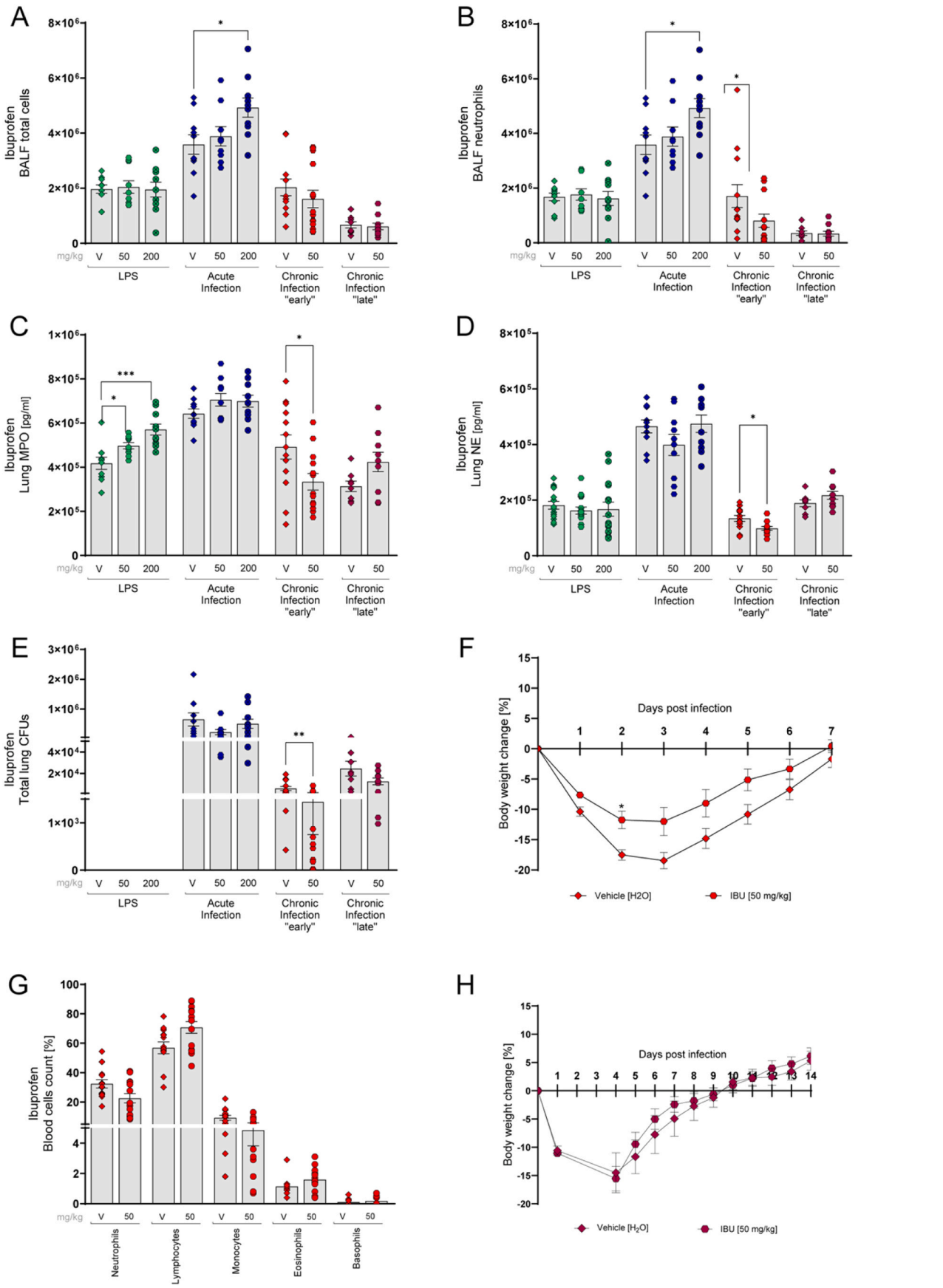
reduction in bacterial load compared to vehicle mice (Fig. 2**E**). These results prompted testing the minimum inhibitory concentrations (MICs) against the *P. aeruginosa* MDR-RP73 strain, which showed no direct effect of the drug (Table S1).

These findings highlight that the anti-inflammatory benefits of AZM are only evident when administered early in a chronic infection model. In addition to its anti-inflammatory effects, AZM treatment displayed antimicrobial properties, suggesting a general benefit in reducing infections when the immune system is balanced.

2.2. Early administration of IBU in the chronic P. aeruginosa lung infection model exhibits anti-inflammatory effects and reduces bacterial load but is ineffective in other models

The same LPS-induced, acute, and chronic infection *P. aeruginosa* MDR-RP73 infection models were used to evaluate IBU efficacy. IBU was administered orally by gavage at doses of 50 and 200 mg/kg before LPS and planktonic MDR-RP73 injection (Fig. 3). In the chronic infection model, only the 50 mg/kg dose was used, due to toxicity observed at higher doses (data not shown).

A single dose of IBU at both concentrations had no significant effect on pulmonary or systemic inflammation in the LPS model. Various parameters, including BALF cell recruitment (Fig. 3**A,B;** Fig. S2A), as well as MPO and NE (Fig. 3**C,D;** Fig. S2B,C), markers of inflammation, and cytokines/chemokines (Table 1), remained unchanged or even increased, as in the case of MPO, in IBU-treated compared to vehicle-treated mice. When IBU was administered in the acute *P. aeruginosa* infection model, the 50 mg/kg lower dose was ineffective, while the 200 mg/kg higher dose had a harmful effect, increasing BALF total cells, particularly neutrophils (Fig. 3**A,B**). IBU treatment did not reduce bacterial burden in the acute infection model (Fig. 3**E**). This indicates that the drug may have a deleterious effect in the presence of acute infection when administered at excessively high doses. Hematological analyses did not reveal any differences between IBU-treated mice and vehicle-treated mice in both the LPS-induced and acute *P. aeruginosa* infection models (Fig. S2D).



(caption on next page)

Fig. 3. Efficacy of IBU in a mouse model of LPS-induced inflammation, acute, and chronic *P. aeruginosa* airway infection. Male C57BL/6N mice (8–10 weeks old) were challenged with planktonic *P. aeruginosa* MDR-RP73 (1×10^7 CFUs), with MDR-RP73 embedded in agar beads ($5–9 \times 10^5$ CFUs) or 20 μg/mouse of LPS from *P. aeruginosa* PAO1 by intratracheal instillation (i.t.). Five minutes before challenge with LPS or planktonic bacteria, IBU (50 or 200 $mg \cdot kg^{-1}$) or vehicle (V) were administered via gavage. The treatment of mice challenged with bacteria embedded in agar beads was repeated daily for 7 days, starting the day of infection ("early" model) or seven days after infection ("late" model). At 24 h after the last treatment, the mice were sacrificed, bronchoalveolar lavage fluid (BALF) was collected, and lungs were excised, homogenized, and plated onto tryptic soy agar to determine bacterial burden Each dot represents total cell count (A), and neutrophil count in BALF (B), MPO (C), and NE levels in lung supernatants (D), and CFUs per lung (E) from one mouse. For the chronic "early" model, before each administration, mice were weighed, and the percentage change from the initial body weight was averaged for each group (F). Hematological analysis of specific white blood cell (WBC) populations was performed (G). For the chronic "late" model, before each administration, mice were weighed, and the percentage change from the initial body weight was averaged for each group (H). Data are presented as mean ± SEM, pooled from three independent experiments (n = 8–14). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

When IBU was tested at repeated doses, starting "early" in the chronic *P. aeruginosa* infection model, mice treated with 50 mg/kg of IBU maintained healthier body weight and recovered more quickly compared to vehicle-treated mice, although this difference did not reach statistical significance (Fig. 3**F**). IBU-treated mice showed a statistically significant reduction in several endpoints, including neutrophils in BALF (Fig. 3**B**), MPO, and NE in lung supernatant (Fig. 3**C,D**), as well as all cytokines/chemokines tested (IL-1β, KC, MCP-1, MIP-1α, and IL-6) (Table 1), with the exception of TNF-α. Although IBU does not have an antibacterial effect *in vitro* (Table S1), IBU treatment significantly reduced bacterial burden in the chronic infection model (Fig. 3**E**). Hematological analyses did not show a decrease in inflammatory cells in the blood (Fig. 3**F**).

When IBU treatment was initiated 7 days after infection ("late" treatment), there was no evidence of an anti-inflammatory effect, as indicated by no change in body weight recovery (Fig. 3**H**) and no difference in pulmonary inflammation (Fig. 3**A–D**; Fig. S2A–C) between IBU-treated and vehicle-treated mice.

Preclinical models highlight IBU's anti-inflammatory benefits in chronic infection when the drug is administered early. However, in advanced chronic infections, anti-inflammatory treatment remains challenging, though the model closely mimics human disease conditions.

2.3. Pre-clinical models of P. aeruginosa infection and inflammation exhibits poor predictive value when DEX is tested

The same inflammation and infection models were used to test DEX efficacy administered orally by gavage at doses of 5 and 10 mg/kg (Fig. 1). Unlike AZM and IBU, the chronic infection model, which received repeated administration of DEX for 7 days starting soon before infection ('early' treatment), did not respond to the drug, showing no effect on cell recruitment in the BALF (Fig. 4**A,B;** Fig. S3A) and other markers of inflammation, including MPO and NE (Fig. 4**C,D;** Fig. S3B,C), as well as cytokines/chemokines (Table 1). Hematological analyses also showed no changes under DEX treatment compared to the vehicle (Fig. 4**F**). Of note, repeated administration of DEX increased the bacterial load in the lungs, worsening the infection and potentially exacerbating inflammation (Fig. 4**E**).

A single dose of DEX at both concentrations had no significant effect on pulmonary or systemic inflammation in the acute infection model (Fig. 4**A–D**; Fig. S3A–D), confirming no predictive value for this model, as seen with AZM and IBU. However, the LPS model showed a reduction in specific parameters, such as total cells, particularly neutrophils, at the higher dose of DEX, and MPO and NE exclusively in the BALF, but not in the lungs (Fig. 4**A–D**; Fig. S3B–C). Other lung biomarkers, including cytokines/chemokines, were unchanged (Table 1). Overall, based on multiple readouts, we conclude that DEX exhibits poor predictive value in preclinical models tested.

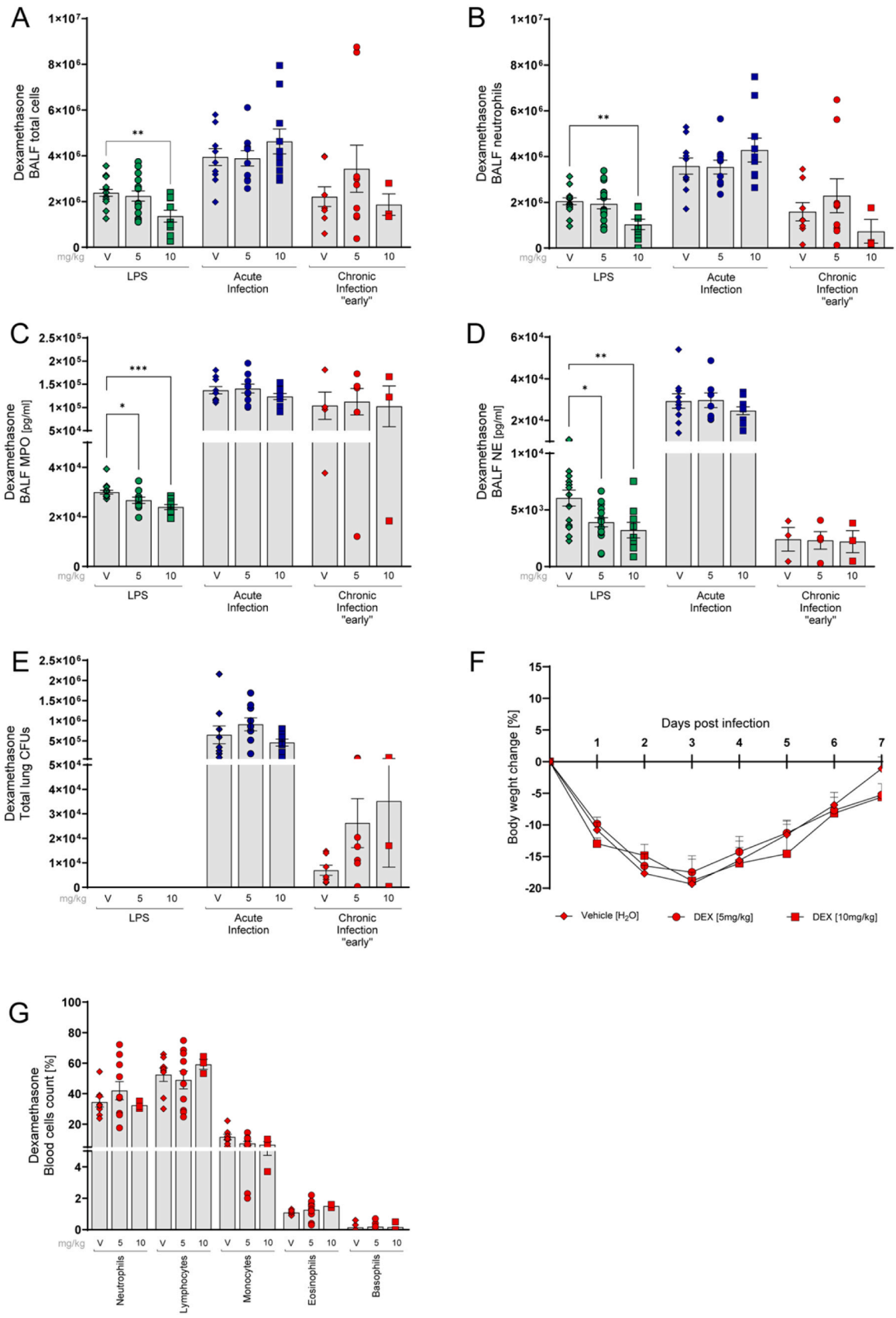
3. Discussion

This study marks the first retrospective evaluation of anti-inflammatory therapies with direct comparisons between different preclinical mouse models of respiratory disease, mediated by LPS stimulation, acute and chronic bacterial infections. Remarkable pathophysiological differences exist between the three models, leading to different host-pathogen interactions and responses that may ultimately affect the response to therapy [17]. LPS stimulates a mild, localized inflammation; acute infections result in rapid pathogen-induced injury to the host, with bacteria penetrating the parenchyma within hours; and chronic infections involve bacteria aggregating in a matrix, leading to a persistent pathogen-host response that stabilizes over days or weeks. Efficacy testing for anti-inflammatory therapies is typically conducted in a single preclinical model, often excluding a bacterial component, which limits its relevance to real-world clinical conditions. Our results showed that, for both AZM and IBU treatment, the chronic infection model was the only one demonstrating drug efficacy. This underscores the importance of incorporating chronic infection models in preclinical testing to better reflect chronic respiratory diseases and the clinical responses to anti-inflammatories observed in humans.

Repeated oral administration of AZM and IBU significantly improved general health and reduced several biomarkers of airway inflammation. Anti-inflammatory treatment initiated soon before infection ("early" treatment) resulted in significantly greater recovery and body weight gain compared to vehicle-treated mice. Total cell counts in the BALF were reduced, particularly the neutrophil load, with macrophages remaining unchanged. This is consistent with the inflammatory process in lungs affected by chronic respiratory diseases, which is largely driven by the accumulation of neutrophils and their associated mediators [19,20]. Pulmonary neutrophil NE and MPO, as key markers of neutrophil activity and inflammation, were decreased. Assessment of cytokine/chemokine profiles revealed significantly reduced levels of IL-1β, IL-6, MIP-1α, and KC in mice treated with AZM and IBU, strongly supporting the other read-outs. These immune mediators are known to be critical for neutrophil production, differentiation, and recruitment. The selection of this restricted panel from a broader cytokine/chemokine profile was sufficient to determine efficacy.

Both AZM and IBU exhibited no direct antibacterial properties *in vitro*, as determined by MICs testing against the *P. aeruginosa* strain used *in vivo*. However, treatment of the mouse model showed a decrease in bacterial load indicating an improvement in infection. In our experience testing several anti-inflammatory compounds, this effect is reliably observed and linked to mechanisms of immune modulation [21–23]. The modulation of the immune response and balanced cytokine production improve the immune system's ability to fight the infection effectively, leading to an amelioration of the infection as a secondary outcome. Beyond direct pathogen–host interactions, host–microbiome dynamics are increasingly recognized as modulators of airway inflammation [24]. Although microbiota composition was not investigated here, our models could be applied in future studies to assess whether anti-inflammatory treatments influence such interactions.

The effect of AZM treatment was also evident in the blood, where neutrophils and monocytes significantly decreased, confirming that its anti-inflammatory action extends beyond the airways. This systemic effect is relevant because pulmonary inflammation is not confined to the lung but may trigger broader physiological responses, including potential impacts on the central nervous system through vagus



(caption on next page)

Fig. 4. Efficacy of DEX in a mouse model of LPS-induced inflammation, acute, and chronic *Pseudomonas aeruginosa* airway infection. Male C57BL/6N mice (8–10 weeks old) were challenged with planktonic *P. aeruginosa* MDR-RP73 (1×10^7 CFUs), with MDR-RP73 embedded in agar beads ($5–9 \times 10^5$ CFUs) or 20 μg/mouse of LPS from *P. aeruginosa* PAO1 by intratracheal instillation (i.t.). Five minutes before challenge with LPS or planktonic bacteria, DEX (5 or 10 $mg \cdot kg^{-1}$) or vehicle (V) were administered via gavage. The treatment of mice challenged with bacteria embedded in agar beads was repeated daily for 7 days, starting the day of infection ("early" model) or seven days after infection ("late" model). At 24 h after the last treatment, the mice were sacrificed, bronchoalveolar lavage fluid (BALF) was collected, and lungs were excised, homogenized, and plated onto tryptic soy agar to determine bacterial burden. Each dot represents total cell count (A) and neutrophil count in BALF (B), MPO (C), and NE levels in BALF supernatants (D), and CFUs per lung (E) from one mouse. For the chronic "early" model, before each administration, mice were weighed, and the percentage change from the initial body weight was averaged for each group (F). Hematological analysis of specific white blood cell (WBC) populations was performed (G). Data are presented as mean±SEM, pooled from three independent experiments (n = 3–15). *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

nerve–mediated pathways [25]. We did not investigate this aspect in the present work, but our models could be exploited in future studies to assess whether anti-inflammatory treatments influence such lung–brain communication and possibly affect other organs as well. In contrast to the systemic activity observed with AZM, IBU treatment was effective only in the lung, leaving this endpoint uncertain and indicative of the localized nature of IBU's effect. Neutrophil responsiveness is different in the lung and systemic circulation of children with CF, providing cumulative evidence that neutrophils can exhibit distinct functional capacities depending on their local environment [26]. Furthermore, we observed several unwanted effects of IBU, including gastrointestinal bleeding, a concern due to its high-dose usage in CF. Major side effects were observed at extremely high doses, but these were reduced at lower doses, indicating the need for careful dose selection. However, our study did not monitor gastrointestinal bleeding, which could affect systemic inflammation. While IBU showed a general improvement in health, measured by body weight gain, the increased recovery and weight gain were significant only during early treatment, but not during long-term treatment, compared to vehicle-treated mice.

Chronic and exaggerated inflammation in people with chronic respiratory diseases causes irreversible damage to lung tissues, eventually leading to respiratory failure. This condition, often driven by chronic infection with bacterial pathogens, is difficult to target, and treatment during advanced chronic infection focuses on preserving lung function and managing periodic exacerbations. When anti-inflammatory treatment was initiated seven days after infection ('late' treatment) in the chronic infection model, no weight recovery or reduction in inflammation, including cell recruitment in BALF, MPO, NE, cytokine/chemokine levels in the lungs, or bacterial burden, was observed with either AZM or IBU. These results underline the necessity of early intervention to achieve significant therapeutic outcomes. In addition, the data show that the advanced stage of lung pathology, induced by chronic bacterial colonization and major structural changes evidenced in this preclinical model (e.g., bronchus-associated lymphoid tissue-like structures, epithelial degeneration, collagen deposition, elastin degradation, and increased factors related to matrix remodeling) [27], represents a significant challenge in the treatment of inflammation. This highlights the complexity of treating chronic inflammation in respiratory diseases, further reinforcing the necessity of early intervention for meaningful therapeutic outcomes.

This study extends our understanding by comparing the chronic infection model with LPS to acute infection models. Overall, based on multiple readouts, we conclude that both AZM and IBU, administered as a single dose, were ineffective in reducing inflammation following acute inflammation and injury caused by acute infection. Occasionally, for IBU, increased cell counts in the BALF after acute infection and MPO activity after LPS stimulation were detected, but other parameters remained unchanged, showing no robust response for efficacy studies.

DEX was one of the anti-inflammatory treatments for CF, primarily used during exacerbations through inhaled or oral administration. Our data in mice suggest efficacy when inflammation is induced by LPS, but not by *P. aeruginosa*. Several parameters were improved, including reduced total cells, particularly neutrophils in the BALF, as well as decreased NE and MPO levels. However, no effect on cytokine/chemokine profiles was detected, indicating only partial efficacy. The systemic inflammatory profile was unaffected. The anti-inflammatory properties of DEX were not observed in the presence of *P. aeruginosa* infection, whether acute or chronic. Unlike AZM and IBU, repeated administration of DEX during chronic infection did not result in improvements in health or airway inflammation compared to vehicle-treated mice. On the contrary, DEX tended to increase bacterial load, which can be harmful for people with chronic respiratory diseases. Past experience suggests that caution should be taken when administering anti-inflammatory compounds to people with bacterial infections due to the risk of inducing bacteremia [11,12]. Like other corticosteroids, DEX use is limited to specific cases due to the occurrence of significant side effects and is not recommended for long-term treatment.

As observed, not all of the anti-inflammatory drugs tested in this study were equally effective in reducing all the inflammatory markers analyzed. However, they each demonstrated effectiveness in a specific manner, which can likely be attributed to the distinct mechanisms of action of each drug. It is important to emphasize that our primary goal was not to discover or validate the mechanisms of action of approved anti-inflammatory drugs, but rather to assess their effectiveness in preclinical models. These drugs are already in clinical use, and their efficacy in humans is well-established. We intentionally selected drugs with different mechanisms of action to evaluate which model best accommodates multiple anti-inflammatory drugs with varying mechanisms and which parameters are most reliable. Our objective was to gain insights into how different preclinical models can more accurately predict clinical outcomes, thereby helping us identify the most suitable model and parameters for testing diverse anti-inflammatory treatments with varying mechanisms.

As a limitation of this study, we acknowledge that different *P. aeruginosa* strains may elicit diverse responses to treatments; however, we specifically chose a strain known to induce chronic persistence as a proof of concept for our approach. This strategy aims to demonstrate the relevance of our model and readouts, which could later be expanded to different strains with distinct characteristics. Additionally, we restricted our experiments to male mice in order to minimize hormonal variability that might confound inflammatory readouts. Still, the lack of female animals represents a limitation, and future studies in our models should address potential sex-related differences in disease course and treatment response. While it is paramount to develop models and readouts that capture patient heterogeneity, including differences related to bacterial strains and sex, our overarching goal remains to reduce the gap between preclinical studies and human outcomes.

4. Conclusions

Our study establishes criteria that help evaluate the efficacy of anti-inflammatory therapies (Fig. 5; Fig. S4). The selection of the appropriate mouse model and treatment regimen is crucial. We identify the *P. aeruginosa* chronic infection model as the most relevant and predictive mouse model for evaluating the efficacy of anti-inflammatory therapy. Despite the numerous potential targets suitable for anti-inflammatory therapy, this work establishes that consistent readouts, such as body weight, cell recruitment in the BALF, MPO, NE, and cytokine/chemokine levels in the lung, are critical. For highly effective drugs, monitoring these parameters may predict clinical efficacy; whereas,

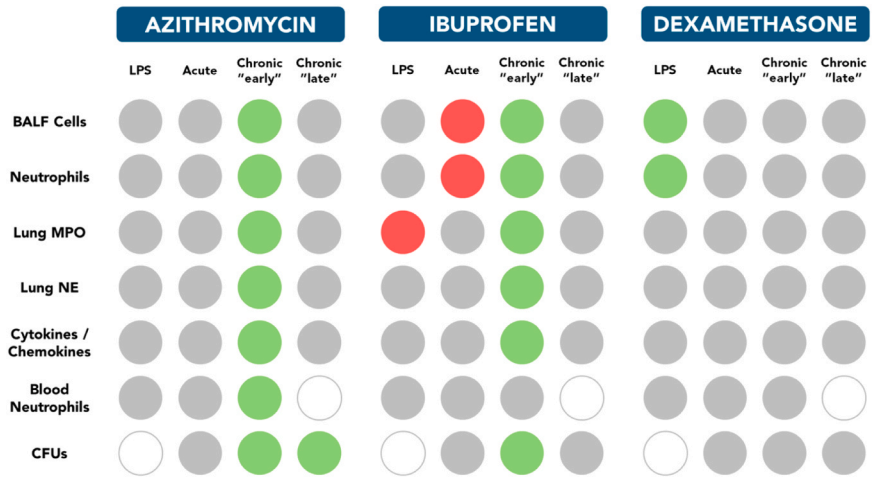


Fig. 5. Summary of anti-inflammatory efficacy testing in mouse models of respiratory inflammation and infection. The preclinical platform for testing anti-inflammatory efficacy includes LPS-induced inflammation, together with acute and chronic infection models. Multiple endpoints, including profiling of host and bacterial responses, are critical parameters for validating the efficacy of selected drugs. This scheme represents the positive or negative results of treatments in various infection models. Green dot: positive effect; red dot: negative effect; gray dot: no effect; white dot: not tested.

measuring cells in the blood proved to be more uncertain. Our ability to apply these standard models and their rational use will support the preclinical development of new drugs and enhance success in clinical studies.

Despite being sporadically used to test anti-inflammatory compounds, the chronic *P. aeruginosa* infection model is not routinely implemented in preclinical studies, even though it better mimics chronic respiratory diseases and provides more clinically relevant data. Our findings strongly support the broader adoption of this model in preclinical studies to improve predictability and translational success.

5. Methods

5.1. Ethics statement

Animal studies adhered to the Italian Ministry of Health guidelines for the use and care of experimental animals (IACUC #733 and #1438). Research with the *P. aeruginosa* MDR-RP73 isolate from a CF individual and storage of biological materials were approved by the Ethics Commission of Hannover Medical School, Germany.

5.2. LPS and bacterial strains

LPS is derived from *P. aeruginosa* (L9143; Sigma-Aldrich). *P. aeruginosa* MDR-RP73 strain was isolated from the airways of an individual with CF [28]. MICs for AZM, IBU, and DEX, in addition to the antibiotic tobramycin, as a control, were determined using the broth microdilution method in Mueller–Hinton cation-adjusted broth (MHB) according to CLSI guidelines [29].

5.3. Mouse models

Immunocompetent C57BL/6NCrlBR male mice (8–10 weeks; Charles River Laboratories, Calco, Italy) were used for the study because male animals exhibited less variability in phenotype. It is unknown whether the findings are relevant to female mice. Mice were challenged by intratracheal administration of 20 μg/mouse of LPS from *P. aeruginosa* to induce lung inflammation, or 1×10^7 colony-forming units (CFUs) of the planktonic MDR-RP73 strain for acute infection, or $5–9 \times 10^5$ CFUs of the MDR-RP73 strain embedded in agar beads for chronic infections [15,18].

Mice were treated with AZM, IBU, DEX, or vehicle (water) by oral administration using flexible polypropylene feeding tubes (FTP-20-34; 2Biol). Body weight was monitored daily.

Lung CFUs and cell counts in the BALF were analyzed as described previously [16]. Cytokine/chemokine levels were measured in the supernatant of lung homogenates by Luminex Assay (Bio-Rad Laboratories, Segrate, Italy). MPO and NE levels were evaluated in the supernatant of BALF and lung homogenates by DuoSet ELISA (Bio--Techne srl, Milano, Italy). Blood was collected from the retro-orbital plexus of anesthetized animals using Na-heparin-coated capillaries (Hirschmann Laborgeräte GmbH) and vials (Microvette® 200 K3 EDTA, 20.1288 Sarstedt). Hematologic parameters were evaluated using an automated cell counter (ProCyte Dx, IDEXX Laboratories, USA).

Additional details in accordance with the Animal Research: Reporting of In Vivo Experiments guidelines [30] are reported in the Supplementary material.

5.4. Statistics

Statistical analyses were performed with GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA) using a two-way ANOVA with Bonferroni's multiple comparison test for body weight changes and one-way ANOVA with Dunnett's multiple comparison test or Mann-Whitney *t*-test for the other readouts. Outlier data, identified by Grubbs' test, were excluded from the analysis.

CRediT authorship contribution statement

Cristina Cigana: Writing – review & editing, Supervision, Project administration, Investigation, Conceptualization. **Ida De Fino:** Writing – review & editing, Methodology, Investigation. **Alessandra Bragonzi:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Davide Gugliandolo:** Writing – review & editing, Methodology, Investigation. **Alice Rossi:** Writing – review & editing, Methodology, Investigation.

Support statement

Research in A. Bragonzi's laboratory is funded by the Italian Cystic Fibrosis Foundation (CFaCore). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank OSR Mouse Clinic, OSR animal biochemistry for hematological analysis.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2025.118667.

Data availability

Data will be made available on request.

References

[1] C. Mitri, Z. Xu, P. Bardin, H. Corvol, L. Touqui, O. Tabary, Novel anti-inflammatory approaches for cystic fibrosis lung disease: identification of molecular targets and design of innovative therapies, Front. Pharm. 11 (2020) 1096.
[2] A.A. Seyhan, Lost in translation: the valley of death across preclinical and clinical divide – identification of problems and overcoming obstacles, Transl. Med. Commun. 4 (18) (2019) 1.
[3] P. Bonniaud, A. Fabre, N. Frossard, C. Guignabert, M. Inman, W.M. Kuebler, et al., Optimising experimental research in respiratory diseases: an ERS statement, Eur. Respir. J. 51 (5) (2018).
[4] C.M.F. Carini, Andre van Gool, Lost in Translation – The Challenges with the Use of Animal Models in Translational Research, Chapman and Hall/CRC, New York, 2019.
[5] J.W. Scannell, A. Blanckley, H. Boldon, B. Warrington, Diagnosing the decline in pharmaceutical R&D efficiency, Nat. Rev. Drug Discov. 11 (3) (2012) 191–200.
[6] L. Perrem, F. Ratjen, Designing clinical trials for anti-inflammatory therapies in cystic fibrosis, Front. Pharm. 11 (2020) 576293.
[7] M.B. Long, S.H. Chotirmall, M. Shteinberg, J.D. Chalmers, Rethinking bronchiectasis as an inflammatory disease, Lancet Respir. Med. 12 (11) (2024) 901–914.
[8] K.F. Chung, I.M. Adcock, Multifaceted mechanisms in COPD: inflammation, immunity, and tissue repair and destruction, Eur. Respir. J. 31 (6) (2008) 1334–1356.
[9] M. Cohen-Cymberknoh, E. Kerem, T. Ferkol, A. Elizur, Airway inflammation in cystic fibrosis: molecular mechanisms and clinical implications, Thorax 68 (12) (2013) 1157–1162.
[10] C.M.P. Ribeiro, N.G. McElvaney, G. Cabrini, Editorial: novel anti-inflammatory approaches for cystic fibrosis lung disease: identification of molecular targets and design of innovative therapies, Front. Pharm. 12 (2021) 794854.
[11] G. Doring, A. Bragonzi, M. Paroni, F.F. Akturk, C. Cigana, A. Schmidt, et al., BIIL 284 reduces neutrophil numbers but increases P. aeruginosa bacteremia and inflammation in mouse lungs, J. Cyst. Fibros. 13 (2) (2014) 156–163.
[12] M.W. Konstan, G. Doring, S.L. Heltshe, L.C. Lands, K.A. Hilliard, P. Koker, et al., A randomized double blind, placebo controlled phase 2 trial of BIIL 284 BS (an LTB4 receptor antagonist) for the treatment of lung disease in children and adults with cystic fibrosis, J. Cyst. Fibros. 13 (2) (2014) 148–155.
[13] L. Saiman, M. Anstead, N. Mayer-Hamblett, L.C. Lands, M. Kloster, J. Hocevar-Trnka, et al., Effect of azithromycin on pulmonary function in patients with cystic fibrosis uninfected with Pseudomonas aeruginosa: a randomized controlled trial, Jama 303 (17) (2010) 1707–1715.
[14] N. Erdmann, T. Schilling, J. Hentschel, T. Lehmann, P. von Bismarck, T. Ankermann, et al., Divergent dynamics of inflammatory mediators and multiplex PCRs during airway infection in cystic fibrosis patients and healthy controls: serial upper airway sampling by nasal lavage, Front. Immunol. 13 (2022) 947359.
[15] C. Cigana, L. Curcuru, M.R. Leone, T. Ierano, N.I. Lore, I. Bianconi, et al., Pseudomonas aeruginosa exploits lipid A and muropeptides modification as a strategy to lower innate immunity during cystic fibrosis lung infection, PLoS One 4 (12) (2009) e8439.
[16] C. Cigana, S. Ranucci, A. Rossi, I. De Fino, M. Melessike, A. Bragonzi, Antibiotic efficacy varies based on the infection model and treatment regimen for Pseudomonas aeruginosa, Eur. Respir. J. 55 (3) (2020).
[17] A. Bragonzi, Murine models of acute and chronic lung infection with cystic fibrosis pathogens, Int. J. Med. Microbiol. 300 (8) (2010) 584–593.
[18] M. Facchini, I. De Fino, C. Riva, A. Bragonzi, Long term chronic Pseudomonas aeruginosa airway infection in mice, J. Vis. Exp. (85) (2014).
[19] A. Cantin, Cystic fibrosis lung inflammation: early, sustained, and severe, Am. J. Respir. Crit. Care Med. 151 (4) (1995) 939–941.
[20] T. Bonfield, J.R. Panuska, M.W. Konstan, K.A. Hilliard, J.B. Hilliard, H. Ghnaim, M. Berger, Inflammatory cytokines in cystic fibrosis lungs, Am. J. Respir. Crit. Care Med. 152 (6 Pt 1) (1995) 2111–2118.
[21] A. Rossi, A. Bragonzi, M. Medede, I. De Fino, G. Lippi, M. Prosdocimi, et al., Beta-sitosterol ameliorates inflammation and Pseudomonas aeruginosa lung infection in a mouse model, J. Cyst. Fibros. 22 (1) (2023) 156–160.
[22] M. Brindisi, S. Barone, A. Rossi, E. Cassese, N. Del Gaudio, A.J. Feliz Morel, et al., Efficacy of selective histone deacetylase 6 inhibition in mouse models of Pseudomonas aeruginosa infection: a new glimpse for reducing inflammation and infection in cystic fibrosis, Eur. J. Pharm. 936 (2022) 175349.
[23] C. Tupini, A. Chilin, A. Rossi, I. De Fino, A. Bragonzi, E. D'Aversa, et al., New TMA (4,6,4′-trimethyl angelicin) analogues as anti-inflammatory agents in the treatment of cystic fibrosis lung disease, Int. J. Mol. Sci. 23 (22) (2022).
[24] C. Rigauts, J. Aizawa, S.L. Taylor, G.B. Rogers, M. Govaerts, P. Cos, et al., R othia mucilaginosa is an anti-inflammatory bacterium in the respiratory tract of patients with chronic lung disease, Eur. Respir. J. 59 (5) (2022).
[25] D. Xu, M. Zhao, G. Liu, T. Zhu, Y. Cai, R. Murayama, et al., The vagus nerve-dependent lung-brain axis mediates brain demyelination following acute lung injury, Brain Behav. Immun. Health 44 (2025) 100966.
[26] H. Corvol, C. Fitting, K. Chadelat, J. Jacquot, O. Tabary, M. Boule, et al., Distinct cytokine production by lung and blood neutrophils from children with cystic fibrosis, Am. J. Physiol. Lung Cell Mol. Physiol. 284 (6) (2003) L997–L1003.
[27] C. Cigana, N.I. Lore, C. Riva, I. De Fino, L. Spagnuolo, B. Sipione, et al., Tracking the immunopathological response to Pseudomonas aeruginosa during respiratory infections, Sci. Rep. 6 (2016) 21465.
[28] I. Bianconi, J. Jeukens, L. Freschi, B. Alcala-Franco, M. Facchini, B. Boyle, et al., Comparative genomics and biological characterization of sequential Pseudomonas aeruginosa isolates from persistent airways infection, BMC Genom. 16 (1) (2015) 1105.
[29] CLSI. CLSI M100-S24. Performance Standards for Antimicrobial Susceptibility Testing; 24th Informational Supplement, Clinical and Laboratory Standards Institute, Wayne, Pa, 2014.
[30] C. Kilkenny, W. Browne, I.C. Cuthill, M. Emerson, D.G. Altman, NC3Rs Reporting Guidelines Working Group, Animal research: reporting in vivo experiments: the ARRIVE guidelines, Br. J. Pharm. 160 (7) (2010) 1577–1579.