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### ► To cite this version:

Charlotte Vernisse, Edouard Tuaillon, Carey Suehs, Delphine Gras, Anne Sophie Bedin, et al.. Airway epithelial type-2 alarmin profiles: Blood eosinophil counts remain in memory. *European Journal of Immunology*, In press, 53 (4), pp.2250101. 10.1002/eji.202250101 . hal-03993667

**HAL Id: hal-03993667**

**<https://hal.science/hal-03993667>**

Submitted on 1 Jan 2024

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# **Airway epithelial type-2 alarmin profiles: Blood eosinophil counts remain in memory**

Charlotte Vemisse 1,2, 3, Edouard Tuallori, S, Carey Suehs<sup>2,6</sup>, Delphine Gras<sup>7</sup>, Anne Sophie Bedin<sup>4, 5</sup>, Jeremy Charriot 1.2, Lucie Knabel<sup>2</sup>, Isabelle Viachier 1.2,3, Pascal Chanez<sup>7,8</sup>, Aurélie Petit<sup>12.3</sup> and Arnaud Bourdin 1.2

1 Department of Respiratory Diseases, CHU Montpellier, University of Montpellier, Montpellier, France

2 PhyMedExp, CNRSINSERM, CHU Montpellier, University of Montpellier, Montpellier, France

3 Medicine Biology Mediterranee, Department of Respiratory Diseases and Addictology, Arnaud de Villeneuve Hospital, CHRU Montpellier, Montpellier, France

4 Pathogenesis and Control of Chronic and Emerging Infections, Inserm U1058, Université de Montpellier, Montpellier, France

5 Department of Virology, CHU de Montpellier, Montpellier, France

6 Department of Medical Information, Université de Montpellier, Montpellier, France

7 C2VN INSERM INRAE, UMR 1263, Aix Marseille Université, Marseille, France

8 Clinique des bronches, allergies, sommeil, APHM, Marseille, France

## **Keywords:**

Alarmins . Blood eosinophil counts . Epithelial cells . IL-25 . TSLP.

## **Abbreviations:**

ÀIJ: air-liquid interface . COPD: chronic obstructive, pulmonary disease . FDR: false discovery rate. HBEC: human, bronchial epithelial cell . TSLP: thymic stromal lymphopoietin

## **Correspondence to:**

Professor Arnaud Bourdin, Department of Respiratory Diseases, CHU Montpellier, Montpellier. France

## **e-mail.**

[a-bourdin@chu-montpellier.fr](mailto:a-bourdin@chu-montpellier.fr)

Epithelial cytokines are involved in the orchestration of T<sub>H</sub>2 inflammatory patterns. We question the persistence of this trait in air-liquid interface (ALI) epithelial cultures and whether this local orientation can be related to systemic patterns (e.g., blood eosinophil counts [BECs]). We investigated alarmin release related to high versus low T2 phenotypes associated with chronic airway diseases. ALIs were reconstituted from 32 control, 40 chronic obstructive pulmonary disease, and 20 asthmatic patients. Interleukin-8 (IL-8; a T<sub>H</sub>1-cytokine), IL-25, IL-33, and thymic stromal lymphopoietin (TSLP; T<sub>H</sub>2-alarmins) concentrations were assessed in subnatants at steady state and used to explain blood neutrophil and eosinophil counts. IL-25 and IL-8 levels were highest in asthma ALI-subnatants, whereas IL-33 was sparsely detected. Thymic stromal lymphopoietin levels were similar among groups. All asthma cell cultures were T<sub>H</sub>1-high/T<sub>H</sub>2-high, while chronic obstructive pulmonary disease and controls tended to be mixed. BECs were independently explained by both disease and in-culture T<sub>H</sub>2-alarmin levels, irrespective of the T<sub>H</sub>2-alarmin considered. The epithelial ALI-T<sub>H</sub>2 signature was more frequently high in patients with a BEC: > 300/mm<sup>3</sup>. Despite removal from an in vivo environment for ≥2 months, ALIs release disease-specific cytokine "cocktails" into their subnatants, suggesting continued persistence of alarmin orientation in differentiated cell line environments.

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## Introduction

The inflammatory cascades found in the airways of both asthma and chronic obstructive pulmonary disease (COPD) patients share a classic type 1 (T<sub>H</sub>1)/type 2 (T<sub>H</sub>2) inflammation paradigm [1, 2].

Accordingly, as a potential surrogate for the epithelial orchestration of the latter, blood eosinophil counts became the peripheral biomarker of T<sub>H</sub>2 inflammation [3, 4]. Conversely, neutrophil counts are thought to be more related to low T<sub>H</sub>2 status. This epithelial origin/orchestration is purported to act through the release of epithelial derived cytokines, such as IL-33, IL-25, and thymic stromal lymphopoietin (TSLP) for high T<sub>H</sub>2 phenotypes and IL-8 for low T<sub>H</sub>2, though the latter is less firmly established [5, 6].

Airway epithelial alarmins may be sufficiently strong to drive eosinophil counts in the blood. The relationships between localized and circulating eosinophil population (i.e., which one is driving the other), especially when considering blood versus sputum eosinophilia, is therefore open for debate [7]. In therapeutic studies, the biologics targeting IL-5, IL-13, and/or IL-4 were found efficacious in controlling eosinophilic asthma (and to a lesser extent eosinophilic COPD), with further evidence forthcoming [8-13]. IL-8-targeting was found to have significant (but limited) benefits in patients not selected according to inflammatory traits [14,15].

Because cultured epithelia are reconstituted outside the physiological environment and its associated inflammatory cocktail, the persistence of a T<sub>H</sub>1/T<sub>H</sub>2 orientation during cell culture is considered a sign of a constitutive nature that will persist in the absence of local triggers. This is a distinct possibility as air-liquid interface (ALI) cultures have been shown to reliably reflect donor-specific characteristics [16, 17]. Assessing alarmin release in ALI culture subnatants can also be used to elucidate whether an expressed T<sub>H</sub>2 orientation is dominated by one such alarmin or is potentially the result of a cocktail phenomenon. Different alarmin

cascades may be observed for different patients but result in similar T1/T2 orientations (or not). Cytokine functions are multiple and redundant, and their up- or down-regulation may correspond to responses other than just eosinophil attraction, further entangling signaling pathways. We therefore hypothesized that alarmin levels at steady state were related to blood eosinophil counts irrespective of the underlying chronic airway disease. For testing this hypothesis, IL-33, IL-25, and TSIP for the T2 high inflammatory pattern and IL-8 for the low T2 pattern were assessed in ALI cultures derived from a large group of patents (with asthma or COPD) and healthy controls and compared to baseline clinical characteristics, including white blood cell counts.

**Table 1.** Patient characteristics

| Parameter  | Severe asthma          | COPD             | Healthy controls    |
|--|------------------------|------------------|---------------------|
| N  | 20                     | 40               | 32                  |
| Age in years   | 50 ± 11.22             | 62.55 ± 10.15    | 56.62 (29.67–67.87) |
| Smoking (never/past/active)  | 0/0/0                  | 0/27/13          | 0/1/0               |
| Pack years   | 0                      | 40 (30–45)       | 0                   |
| Body mass index (BMI) in kg/m <sup>2</sup>                         | 27.7 ± 5.55            | 25.07 ± 4.42     | 28.9 ± 5.95         |
| Forced expiratory volume in 1 s (FEV1) as % predicted              | 67.79 ± 20.78          | 55.38 ± 20.8     | 99.67 ± 16.22       |
| Forced expiratory volume in 1 s (FEV1)/forced vital capacity (FVC) | –                      | 0.53 ± 0.13      | 0.82 (0.78–0.9)     |
| Residual volume as % predicted                                     | 124.25 (108.95–208.32) | 186.67 ± 81.65   | 96.98 ± 20.79       |
| Blood eosinophil (cells/mm <sup>3</sup> )                          | 255.29 ± 175.65        | 190 (120–330)    | 155 (117–237.5)     |
| >300   | 35.0%                  | 22.5%            | 12.5%               |
| Blood neutrophil (cells/mm <sup>3</sup> )                          | 4200 (3315–7760)       | 4780 (3790–5430) | 4305 (3322.5–6335)  |
| No treatment   | 0.00%                  | 31.43%           | 100.00%             |
| Inhaled corticosteroid (ICS)/long-acting beta agonist (LABA)       | 100.00%                | 17.14%           | 0.00%               |
| ICS/LABA/long-acting muscarinic antagonist (LAMA)                  | 0.00%                  | 28.57%           | 0.00%               |
| LAMA/LABA  | 0.00%                  | 14.29%           | 0.00%               |
| LABA alone   | 0.00%                  | 5.71%            | 0.00%               |
| LAMA alone   | 0.00%                  | 2.86%            | 0.00%               |

Data for quantitative variables are summarized as mean ± SD for normally distributed variables or median (interquartile interval) otherwise. Qualitative variables are summarized as percentages. Missing data are indicated as "–".

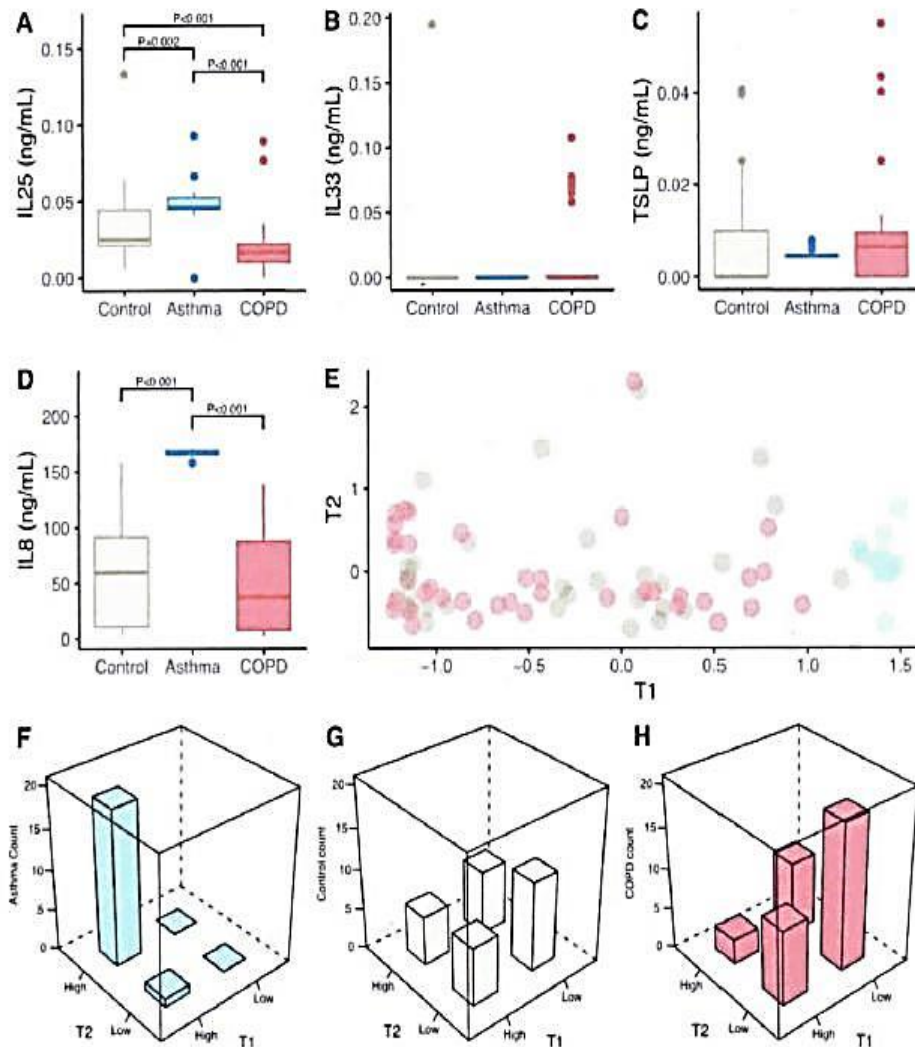
## Results and discussion

### Patient characteristics

Descriptive statistics for clinical characteristics are given in Table 1 and are largely in line with the classical features of the studied disease groups. Most asthma/COPD patients were considered as "severe" and required high treatment doses. Controls were subjects who required bronchoscopy (as confirmed by a senior pulmonologist) for foreign body removal, exploration of a peripheral nodule or other abnormal thoracic imaging findings (see the Supporting information for more details).

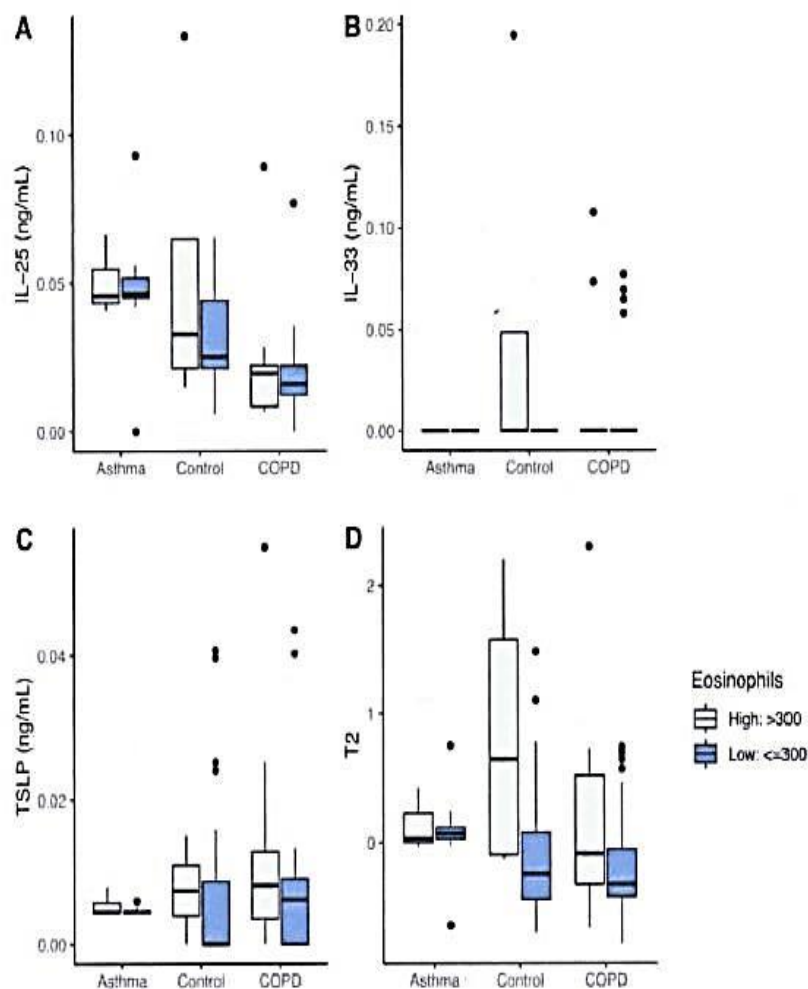
### Cytokine profile§ in chronic airway diseases

As demonstrated in Fig. 1, despite removal from an *in vivo* environment for over 2 months, ALI-cultured human bronchial epithelial cells (HBECs) release patient-specific and potentially disease-specific cytokine "cocktails" into their basal-state subnatants, suggesting persistent alarmin orientation in differentiated airway epithelia. The control, asthma, and COPD-derived cytokine profiles differed as follows: (i) significantly different IL-25 concentrations between all three groups (asthma > control > COPD; Fig. 1A), (ii) sparse IL-33 signaling detectable in only a few isolated individuals in the COPD group {FLg.18}, (iii) similar levels of TSLP for all groups (Fig. 1C), and (iv) higher IL-8 concentrations in the severe asthma group as compared to the other two (Fig. 1D). As concerns this last observation concerning IL8, which may appear unexpected, there are now many reports indicating that the view that eosinophils are restricted to asthma and neutrophils to COPD is a matter of debate [2, 12]. Whether low or non-T2 asthma exists is difficult to conclude [18]. Potentially, these patients may have hidden T2 phenotypes, in particular due to steroid exposure.



**Figure 1.** Airway epithelial release of alarmins. Cytokine profiles for control (N = 32, beige), asthma (N = 20, blue), and COPD (N = 40, red) patients as determined by sandwiched ELISA assays on subnatants of ALI-reconstituted airway epitheliums after 28 days of culture. The concentrations of IL-25, IL-33, TSLP, and IL-8 are displayed in panels A, B, C, and D, respectively (horizontal lines indicate medians, boxes interquartile ranges, whiskers nonoutlier ranges, and dots outliers). Panel E plots the T1 (scaled IL-8 concentration) versus T2 profile (average of IL-25, IL-33, and TSLP scaled concentrations) scores. *p*-Values indicate results from Conover's nonparametric all-pairs rank comparison tests with FDR corrections for multiple testing. Panels F, G, and H demonstrate T1 and T2 high/low profiles for the asthma, control, and COPD groups, respectively. High versus low status was determined according to median alarmin release.

As concerns combined "eT2" profiles (the average of scaled values for IL-25, IL-33, and TSIP as in [19]), there was a great deal of overlap between the three groups, while the "eT1"/IL8 profile singled out the asthma group (Fig. 1E). Among patients with asthma, all but one were characterized as being both eT1 and eT2 high (Fig. 1F). The distributions of T1/eT2 high/low categories for patients with asthma tended to be different from those of the control or COPD groups (false discovery rate [FDR] corrected Fisher's exact tests,  $p = 0.085, 0.092$ ). The T1:T2 high/low classification distributions were similar for the latter two groups (Fig. 1G-H), with a variety of T1/T2 high/low combinations found. Whether these persistent orientations are related to polymorphisms [20] affecting the expression of these cytokines would be ideally assisted by performing complimentary analyses, but unlikely able to provide a definitive answer given the limited sample sizes. Although relevant polymorphisms were found in 17q21 affecting TSLP and IL-33 and the IL-33 receptor ST2 in asthma, this finding was not reproduced in COPD [21].



**Figure 2.** T2 cytokine titres (A) IL-25, (B) IL-33, (C) TSLP, and (D) the T2 score (the average scaled value of IL-25, IL-33, and TSLP) according to a blood eosinophil count cut-off of  $300/\text{mm}^3$ . Horizontal lines indicate medians, boxes interquartile ranges, whiskers nonoutlier ranges, and dots outliers.

## Eosinophilia association with disease and T2 cytokine levels

Multiple regression indicated no associations between blood neutrophil levels, disease groups or IL-8, suggesting that airway epithelia are not a critical factor determining this trait. However, blood eosinophilia levels were significantly associated with group (asthma [coefficient  $\pm$  SD with significance level]:  $65.61 \pm 25.57$ ,  $p = 0.0110$ ; COPD:  $50.60 \pm 21.50$ ,  $p = 0.0195$ ) with a tendency for association with cytokine levels ( $17.30 \pm 9.06$ ,  $p = 0.0575$ ). However, the type of T2 cytokine considered was insignificant, suggesting that high levels of any T2 cytokine were associated with high eosinophilia levels. A summary figure demonstrating the relationship between high eosinophil status (here defined as those above a threshold of  $300 \text{ cells/mm}^3$ ) and T2 markers is presented in Fig. 2. Logistic regression similarly found two factors that independently predicted high eosinophil status: asthma status ( $1.26 \pm 0.43$ ,  $p = 0.004$ ) and the scaled value of any of the three T2 cytokines ( $0.36 \pm 0.14$ ,  $p = 0.011$ ).

## Study limitations

The limitations of this study include its small size and observational nature. In addition, the relationship found between the eT2 signatures and the blood eosinophil count deserves multiple comments: (i) this result should be taken cautiously as the correlation was far from perfect and was not consistent in all groups and the sample sizes were limited; (ii) we used the scaled values of the three classical T2 alarmins, whereas the biological reality behind this is unknown, (iii) highly sensitive IL-33 immunoassays based on a digital F.LISA methods (such as the kit recently launched by Quanterix) may improve detection of this cytokine in future ALI studies. If we accept these limitations, this relationship may indicate first that the airway epithelium is a major contributor to a systemic trait, thus suggesting that alarmin release is not restricted to the airway submucosa and diffuses beyond the nose and lungs in a way that affects all organs (at least for patients with respiratory diseases [22]). Second, it would be interesting to assess experimental epithelial injury and repair (though reproducible scratch challenges are difficult to reliably achieve in so many ALI cultures). Third, the fact that there was no single alarmin dependence may suggest that alarmin targeting may require personalization.

## Concluding remarks

Despite over 2 months in similar culture conditions, we observe continued persistence of alarmin orientation in differentiated lung epithelial cell lines from asthma, COPD, and control populations. Beyond group differences, the heterogeneity present in alarmin orientation suggests that different cytokines may be affecting different people.

# Materials and methods

Primary HBECs were obtained from biopsies specimens collected during fiberoptic bronchoscopy. Subjects (32 controls, 40 COPD patients, and 20 asthmatics) were recruited at two public hospitals in France, and HBECs were cultured as previously reported [16] (see Supporting information for more details). Clinical and demographic characteristics, including blood eosinophil and neutrophil counts assessed on the day of bronchoscopy, were recorded for all subjects.



## **Cytokine detection**

ALI epitheliums were considered as fully differentiated and representing a basal state on day 28. The concentrations of IL-25 (EK1179, Bioocean), IL-33 (435908, Biolegend), TSLP (434204, Biolegend), and IL-8 (851530005, Diaclone) in ALI culture mediums were determined by duplicate measurements on day 28 as previously reported [16], using sandwich enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's instructions. Color changes were spectrophotometrically detected at a wavelength of 450 nm, and sample concentrations were interpolated from standard curves.

## **Statistics**

Statistics and graphics generation were performed using the R programming environment. Epithelial T1 profiles (eT1) were determined by scaled IL-8 concentrations. Epithelial T2 profiles (eT2) were determined by the average of the scaled concentrations of IL-25, IL-33, and TSLP (as in [19]). Medians were used to separate "high" versus "low" eT1 or eT2 profiles. "High eosinophil" status was determined according to a 300 cells/mm<sup>3</sup> threshold [23].

Cytokine concentrations were compared among disease groups using Conover's nonparametric all-pairs rank comparison tests with FDR corrections for multiple testing.

Linear models were used to predict (i) blood neutrophil counts as a function of group (control, asthma, or COPD) and ALI subnatant cytokine scaled value; (ii) blood eosinophil counts as a function of group, type-2 cytokine identity (IL-25, IL-33, or TSLP) and ALI subnatant cytokine scaled value; and (iii) blood eosinophil counts as a function of group and the number of above-median type-2 cytokines in ALI subnatants. Finally, a logistic regression model was used to predict high eosinophil status as a function of disease group, type-2 cytokine identity, and (type-2 cytokine scaled value).

The distributions of T1 -high-T2-high, T1 -high-T2-low, T1 -low-T2-high, and T1-low-T2-low patients were compared between groups using Fishers exact tests with a FDR correction. The distributions of patients with 0,1, or 2 high-T2 cytokine status were compared between groups in a similar fashion.

## **Acknowledgment :**

This research is part of the PhD studies of Dr. Charlotte Vernisse, whose salary was provided via an industrial research training scholarship (CIFRE 2016/1000 between Medicine Biology Mediterranean [Isabelle Vachier], Montpellier, France and the PhyMedExp Laboratory, INSERM, CNRS, Université de Montpellier, France). Further support was provided by the Montpellier University Hospitals (No. 2013-A01405-40 [Principal Investigator (PI): Arnaud Bourdin] and No. 2018- A01530-S5 [PI: Arnaud Bourdin]), an inter-regional French grant (Direction Générale de l'Offre de Soins, PHRC (IR 2013-14) No. 2013-A00553-42 [PI: Pascal Chanez]), a French National Research Agency grant (ANR-13-BSV5-0015-02 - Mucocil [PI: Pascal Chanez]), the COBRA cohort (French National Institute of Health and Medical Research, INSERM, IDRGB 2008-A00284-51). and the Apard association (Adène Group). -

## **Conflict of interest**



CV ET, DG, ASB, LK, rv and IP report no conflicts of interest. CMS reports grants or personal fees from Astra Zeneca, GSK. JC has served as consultant, or lecturer, and has previously received honoraria from Astrazeneca, Chiesi, GSK, Sanofi. PC has served as an advisory board member, consultant, or lecturer. and has previously received honoraria or grants from ALK. Boehringer Ingelheim, Almirall, Centocor, GSK. MSD, Astrazeneca, Novartis, Teva, Chiesi, SNCF, and AMU. Arnaud Bourdin reports grants, personal fees, non-financial support, and/or other from GSK, Astra Zeneca, Boehringer Ingelheim, Novartis, Chiesi Farma, Teva, Sanofi Regeneron, Actelion/Jansen, United Therapeutics, Pulsar, and Roche.

### **Ethic approval**

Subjects were recruited under protocols approved by independent ethics committees (the COBRA cohort [24] for asthma patients [Comité de Protection des Personnes (CPP) Ile-de-France 1, 09-11962]) and the RhéMuc protocol for others (NCT03187860; CPP Es[-II,18/07/23/59436]); all patients gave written informed consent prior to enrolment.

### **Data availability statement :**

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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