Pulmonary adaptation to repeated poly(I:C) exposure is impaired in asthmatic mice: an observational study

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Abstract

Background While asthma exacerbations remain a major challenge in patient management, few animal models exist to explore the underlying mechanisms. Here, we established an animal model of asthma that can be used to study pathophysiological mechanisms and therapeutic strategies on asthma exacerbation.

Methods Female BALB/c mice were sensitized and exposed to PBS or *Dermatophagoides pteronyssinus* (DerP) extract for 11 weeks. Asthmatic phenotype was assessed through lung inflammation, bronchial hyperresponsiveness and bronchial smooth muscle remodeling. Asthmatic and control mice were exposed once or three times to poly(I:C) to simulate virus-induced inflammation.

Results Fourteen days after exposure to DerP, asthmatic mice showed resolution of inflammation with sustained bronchial hyperresponsiveness and bronchial smooth muscle remodeling compared to control. At this stage, when mice were subjected to a single exposure to poly(I:C), control and asthmatic mice were characterized by a significant increase in neutrophilic inflammation and bronchial hyperresponsiveness. When mice were repeatedly exposed to poly(I:C), control mice showed a significant decrease in neutrophilic inflammation and bronchial hyperresponsiveness, while asthmatic mice experienced worsening of these outcomes.

Conclusions This observational study report an asthmatic mouse model that can undergo exacerbation after repeated exposure to poly(I:C). Our findings on pulmonary adaptation in control mice may also pave the way for further research into the mechanism of adaptation that may be impaired in asthma and raise the question of whether asthma exacerbation may be a loss of adaptation.

Keywords Asthma, Exacerbation, Adaptation, Polyinosinic-polycytidylic acid, Neutrophils, Bronchial airway hyperresponsiveness, Bronchial remodeling, CCL20

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Background

Asthma pathophysiology is characterized by chronic bronchial inflammation, bronchial hyperresponsiveness and bronchial remodeling. Among the tissue remodeling, the increased bronchial smooth muscle (BSM) mass is an important factor in the development of asthma [1]. Noteworthy, the BSM mass has been found to be correlated with both lung function decline [2] and increased number of exacerbations [3, 4]. Asthma exacerbation has been defined by GINA (Global Initiative for Asthma) as "episodes characterized by a progressive increase in symptoms of shortness of breath, cough, wheezing or chest tightness and progressive decrease in lung function, i.e. they represent a change from the patient's usual status that is sufficient to require a change in treatment". Clinical observations link BSM remodeling to asthma exacerbation. For instance, reduction of BSM mass, using an invasive technique (i.e., bronchial thermoplasty), has been shown to reduce severe exacerbations, emergency department visits, and hospitalizations [5]. In asthma, rhinovirus infections are the leading cause of exacerbations [6], which is a major concern for patient care and a burden to the healthcare system. Contrary to what its name suggests, rhinovirus also chronically infects the lower respiratory tract [7]. A better understanding of respiratory virus-induced asthma exacerbations will pave the way for a new therapeutic strategy, with a non-invasive treatment for patients. Although there are animal models of asthma with respiratory virus infection [8], to our knowledge, none of them pay attention to the presence of BSM remodeling. Here, we report an animal model of asthma with inflammation, bronchial hyperresponsiveness and bronchial remodeling, that can be used to study pathophysiological mechanisms and therapeutic strategies on asthma exacerbation.

Methods

Animals

Female BALB/c mice were housed in a conventional animal facility at Bordeaux University. Six weeks old mice were challenged with either *Dermatophagoides pteronyssinus* extract (Stallergenes; lot: IHRP 099; 375 μg Derp1 / mg total protein and 102.5 μg Derp2 / mg total protein; reactivity index (100IR); 10 Endotoxin Unit/mg) in PBS or PBS only. On days 0 and 14, mice were sensitized with intraperitoneal injection of DerP (100ng/100μl) and on days 14 to 75, deeply anesthetized mice (Ketamine/Xylazine) were challenged with intranasal instillation of DerP (300ng/30μl). Fourteen days after DerP exposure, anesthetized mice (isoflurane) were challenged with intranasal instillation of poly(I:C) (85 μg/50μl) (Sigma), or PBS.

Measurement of bronchial responsiveness

Measurement of respiratory system mechanics was performed as previously described [9]. Briefly, anesthetized mice were connected to a mechanical ventilator (Flexi-Vent, Scireq, Montreal, QC, Canada), and aerosolized methacholine (Sigma-Aldrich, St. Louis, MO, USA) was administered at increasing concentrations (6.25–50 mg/ml).

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed with a single instillation of 1 ml of cold phosphate buffered saline with bovine serum albumin (PBS/BSA1%) via the tracheal cannula after the measurements of respiratory mechanics. The total number of cells was counted in a Neubauer chamber, and the differential cell count was determined after staining of cytospin slides with HEMA 3 STAT PACK (Fisher Scientific, Saint-Laurent, QC, Canada). The cell differential was determined from a count of 300 cells. The BAL supernatant was stored at -80 °C for further analysis.

Multiplex ELISA

A custom Bio-Plex Assay (BioRad, Hercules, CA) was performed to assess chemokine expression in bronchoalveolar lavage supernatants according to the manufacturer's instruction. Special plate reader (Bio-Plex MAGPIX™, BioRad) and software (Bio-Plex manager) were used. The assay running was based on the same principle as a classic ELISA, except that the assay was performed with washing steps using a wash station (Bio-Rad), equipped with a magnetic field that kept the microbeads to the bottom of the well while performing washing steps.

Immunohistochemistry

Mice were euthanized with an overdose of pentobarbital (CEVA Santé Animale, Libourne, France). The pulmonary circulation was flushed with sterile saline via the right ventricle until the effluent was clear. Lungs were inflated and fixed with 10% formalin, and embedded in paraffin. Adjacent tissue sections (4 μm) were deparaffinized in xylene (60 °C oven followed by 3 baths of 3 min), rehydrated through a decreasing ethanol gradient (2×3 min alcohol 100°; 3 min 95°; 3 min 70°, 3 min 50°; 3 min dH$_2$O). Tissue sections were processed for antigen unmasking in PT Link buffer pH 6 (20-minute cycle at 95 °C; and rinsed twice with warm PBS-Tween 0.05%). Saturation of non-specific sites was performed with incubation of Normal Goat Serum 4% for 30 min at room temperature. Without washing, monoclonal antibody for alpha-smooth muscle actin (clone 1A4; 1/1000; Sigma-Aldrich, Saint-Louis, MO) was applied for 40 min and then rinsed with PBS-Tween 0.05% (2×30 s). Goat anti mouse Alexa Fluor 488 (1/500) was applied

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for 40 min in the dark and then rinsed with PBS-Tween 0.05% (2×30 s). Slides were mounted with fluorescence mounting medium containing DAPI, incubated at 37 ℃ for approximately 15 min to allow polymerization and stored at 4 ℃ before viewing. Microscope slides were scanned at x20 (Nanozoomer, Hamamatsu) and blindly analyzed with Image J software. For each section, 6–10 bronchi were considered for the analysis (average bronchial perimeter analyzed: 569 µm±169 (SD)). The αSMA area was normalized by the bronchial perimeter (supplemental Fig. 1).

Statistical analysis

All statistical tests were performed on Graphpad Prism 5 software (Graphpad Software, San Diego, CA). Results were displayed as mean±SEM values of two independent experiments. Statistical tests used were ordinary one-way or two-way ANOVA with Bonferroni's multiple comparisons test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; for lung function, * compare poly(I:C) x1 or poly(I:C) x3 with PBS and $ compare poly(I:C) x1 with poly(I:C) x3) or Mann-Whitney test (#$p < 0.05$, ##$p < 0.01$). Results were considered statistically significant when $p < 0.05$.

Results and discussion

We first evaluated whether our model could recapitulate the main features observed in asthmatic patients (i.e., bronchial inflammation, bronchial hyperresponsiveness and bronchial remodeling) (Fig. 1A). Two days after the last allergen inhalation (DerP), mice showed a significant increase of inflammatory cells in the bronchoalveolar lavage (BAL) compared to control (Fig. 1B). Inflammatory cells observed were mainly monocytes/macrophages, eosinophils and to a lesser extent lymphocyte (Fig. 1C). Resolution of inflammation was observed 14 days after the end of DerP exposition (Fig. 1B and C). With regard to bronchial hyperresponsiveness in response to inhaled methacholine, both the total respiratory system Resistance (quantitatively assessing the level of constriction in the lungs) and the Newtonian Resistance (from the constant-phase model, representing the resistance of the central airways) increased significantly after the end of DerP exposure compared with the PBS control group at two-, eight-, fourteen- and twenty-one-days post exposure (Fig. 1D). In terms of bronchial remodeling, BSM area also increased significantly at the end of DerP exposure compared with the control (Fig. 1E). Previous study using long protocol of ovalbumin exposure (10.7 weeks) or house dust mite (6.5 weeks) also observed that mice develop BSM remodeling [10, 11]. In these models, the outcomes were measured 24–72 h after the last allergen challenge, when both bronchial inflammation and remodeling were increased, excluding the possibility of clearly defining which of these parameters was involved

in bronchial hyperreactivity. Here, we found that both bronchial hyperresponsiveness (BHR) and BSM remodeling remain significantly higher compared to control, even after the resolution of inflammation (day 14 and 21), suggesting that the measured BHR could be related to tissue remodeling rather than bronchial inflammation only. However, many aspects of airway remodeling could lead to BHR. Interestingly, a recent study found that a more muscular bronchial tree in male mice might contribute to their greater responsiveness to inhaled methacholine than that of female [12], suggesting that the BHR observed in female asthmatic mice might indeed be due to BSM remodeling in our study.

To further characterize our model and find conditions leading to exacerbation, we decided to simulate virus-induced inflammation by exposing mice with poly(I:C) (a synthetic analog of double-stranded RNA recognized by endosomal Toll-like receptor 3). It was important to start poly(I:C) exposure when the BAL inflammation induced by DerP was resolved in order to emphasize the potential role of tissue remodeling rather than allergic inflammation in the mechanism of exacerbation. Since respiratory virus may replicate in the airways after a single exposure, we also decided to run 3 inhalations of poly(I:C), in addition to only 1 exposure, to be closer to physiological conditions (Fig. 2A). We showed that both control and asthmatic mice had a significant increase of immune cell infiltration in the BAL after 1 exposure (Fig. 2B). Recruited immune cells were mainly neutrophils and to a lesser extent lymphocyte (Fig. 2C). But the most interesting result came with repeated inhalation of poly(I:C). Control mice had lower levels of neutrophils compared to 1 exposure, which was not observed in asthmatic mice. Moreover, the level of neutrophils was significantly higher in asthmatic mice compared to control mice after three exposures. This neutrophilic inflammation is of particular interest since rhinovirus induced NETosis (formation of neutrophil extracellular traps) has been involved in asthma exacerbation [13]. By contrast, the significant increase of lymphocytes observed in control mice was similar, after 1 or 3 exposures, whereas an additional increase was observed in asthmatic mice after 3 exposures. We did not explore the phenotype of these lymphocytes but it is interesting to note that rhinovirus infection may alter regulatory T cells function [14], which could explained this additional increase. When looking at bronchial reactivity in response to methacholine, we observed in control mice a significant increase of both Resistance and Newtonian Resistance 24 h after a single inhalation of poly(I:C) (Fig. 2D and E, left panels). Surprisingly, when control mice received 3 inhalations of poly(I:C), both Resistance and Newtonian Resistance were significantly decreased compared to a single inhalation of poly(I:C) (fold change of -0.27 for Resistance
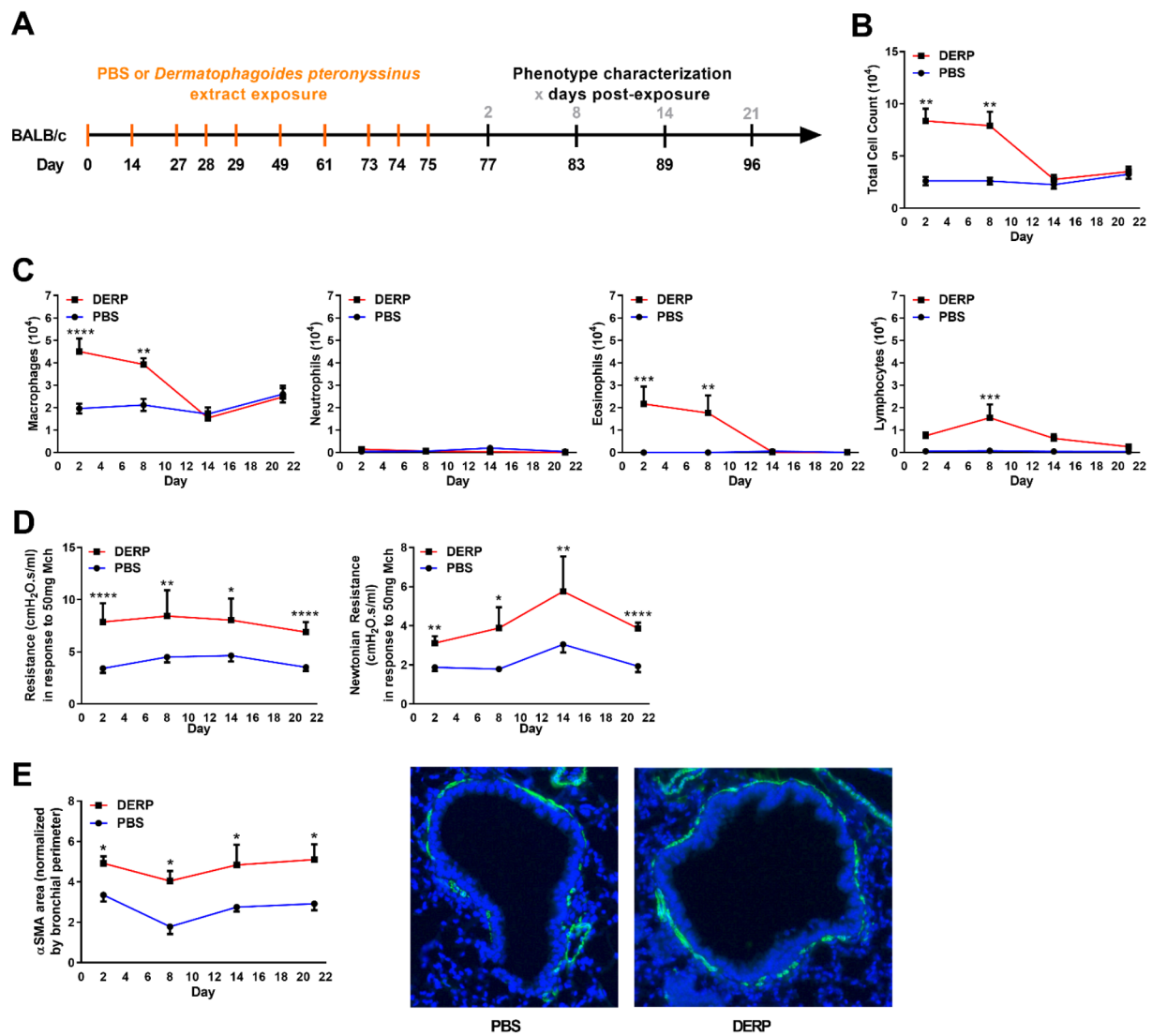
Fig. 1 Characterization of the asthmatic model. BALB/c mice were exposed to *Dermatophagoides pteronyssinus* (DerP) extract or PBS at the indicated days (**A**). Two-, eight-, fourteen- and twenty-one-days post exposure, bronchoalveolar lavage (**B** and **C**), lung function (**D**) and bronchial smooth muscle remodeling (**E**) were assessed (*n* = 5 mice per group at days 2 and 8, *n* = 6 mice per group at days 14 and 21, from two independent experiments). Pictures in E are representative staining of α-SMA in PBS and DerP mice at day 21

and -0.40 for Newtonian Resistance between control poly(I:C) x1 and x3. These results, along with those of neutrophils, suggest that control mice developed pulmonary adaptation to poly(I:C)-induced chronic inflammation. Similar adaptation was observed in a mouse model of repeated chlorine gas exposure, also characterized by neutrophilic inflammation, where adaptation was mediated by a subpopulation of tolerogenic macrophages [15]. By contrast, asthmatic mice, showed no adaptation, but rather a worsening of outcomes (fold change of +0.20 for Resistance and +0.28 for Newtonian Resistance between asthma poly(I:C) x1 and x3, Fig. 2D and E, middle panels). Moreover, asthmatic mice with repeated poly(I:C)

challenge had a significant increase of both Resistance and Newtonian Resistance as early as 25 mg of methacholine demonstrating an increased sensitivity (Fig. 2D and E, middle and right panels). Interestingly, previous studies suggest that the amplitude of BHR may be "correlated" to the amount of neutrophils in the lung [16, 17], which is in line with our current observations. Worth noting, experimental rhinovirus infection in healthy and asthmatic human volunteers revealed that, in contrast to healthy participants, the state of asthmatics after rhinovirus infection more closely resembled that of other rhinovirus-infected asthmatics than their own state prior to rhinovirus infection. In other words, the lung function of
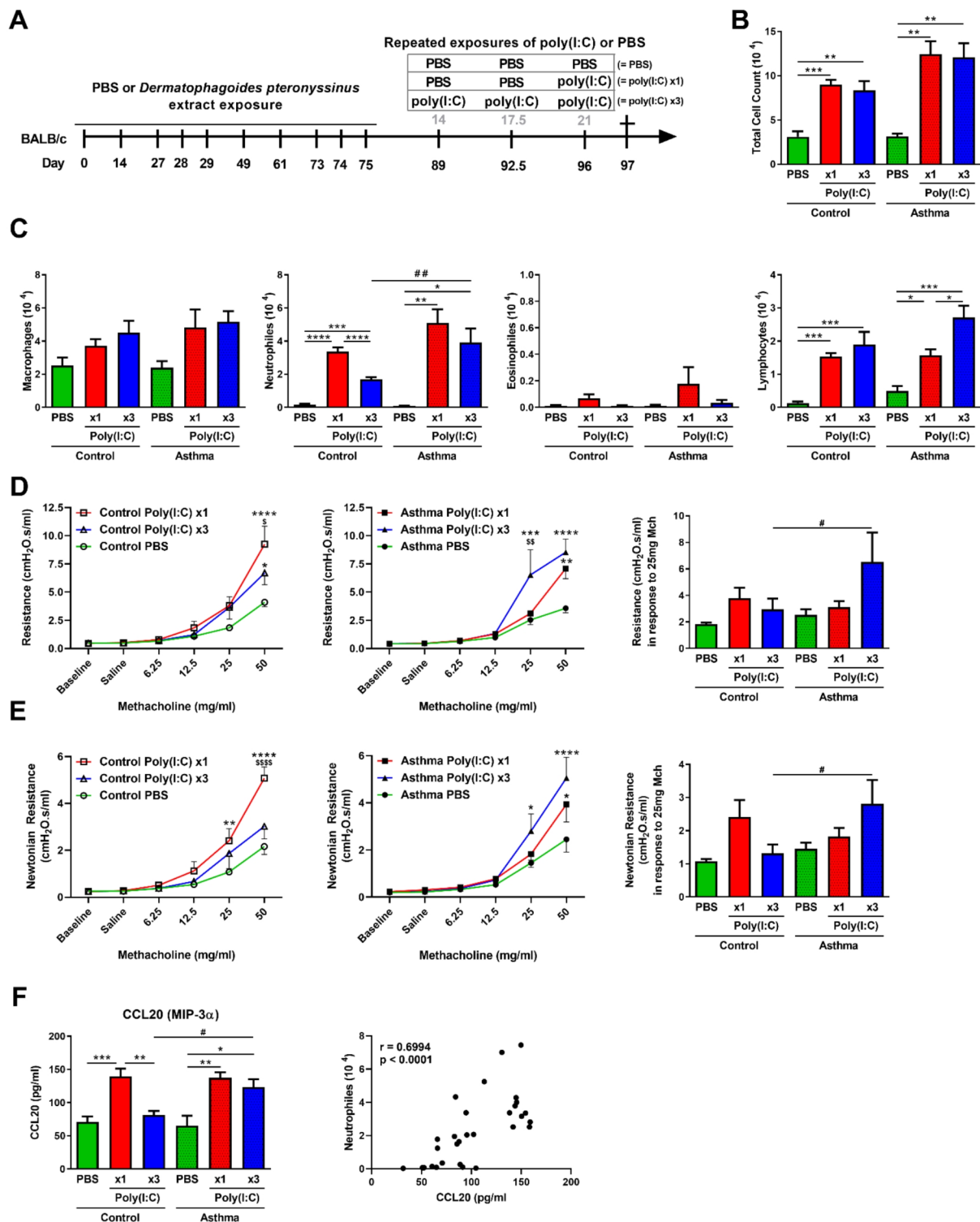
Fig. 2 Adaptation of control mice and exacerbation of asthmatic mice. Asthmatic and non-asthmatic mice were exposed to poly(I:C) or PBS as indicated (**A**). Twenty-four hours after the last exposure, bronchoalveolar lavage (**B** and **C**), lung function (**D** and **E**) and cytokine assay in bronchoalveolar lavage (**F**) were assessed (*n* = 5 mice for Control PBS, *n* = 6 mice for Control poly(I:C) x1 and x3, *n* = 4 mice for Asthma PBS, *n* = 5 mice for Asthma poly(I:C) x1 and x3, from two independent experiments)

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asthma patients after an infection does not recover and remains similar to that of an active infection. This suggests a loss of adaptive capacity and supports the concept that, asthma may alter the properties of a homeokinetic physiologic system, contributing to loss of control [18].

Finally, to further explore this model of exacerbation, a multiplex ELISA was performed on the BAL supernatants, with a particular interest in chemokines that could be involved in neutrophil recruitment. From the inflammatory mediators measured (Supplemental Figs. 2, 3 and 4), CCL20 was the only one showing (i) a significant increase after 1 exposure in both control and asthmatic mice, (ii) in the control group, a significant decrease between 1 and 3 exposures of poly(I:C) and (iii) a significant increase between control and asthmatic mice after repeated exposure (Fig. 2F). Notably, CCL20 followed the same pattern as neutrophils in BAL and a positive correlation between CCL20 concentration and neutrophil numbers was observed (Spearman: $r = 0.6994$; $p < 0.0001$). Although CCL20 is not the main neutrophil chemokine, it was recognized as a chemoattractant for neutrophils in a model of pneumococcal meningitis [19], but other chemokines, such as CXCL1 or CXCL5, were probably also involved in the neutrophil infiltration observed in this study. In particular, CXCL5 (also known as ENA-78), was significantly increased in asthmatic mice after 1 exposure of poly(I:C) compared to control, but not after 3 exposures (Supplemental Fig. 3). Interestingly, CCL20 has other interests in the pathophysiology of asthma exacerbation in addition to neutrophils. Elevated CCL20 production by asthmatic BSM cells may increase mucus production on the bronchial epithelium [20] and contribute to airway obstruction. More recently, using an advanced in vitro model of BSM cells co-cultured with bronchial epithelium, differentiated at the air-liquid interface, it was found that the chemokine CCL20 was extensively released from asthmatic BSM cells compared to non-asthmatic cells [21]. This observation was also confirmed ex vivo. Interestingly, CCL20 downregulated the activity of the PKR (protein kinase RNA-activated), an enzyme used by the cell to counteract RNA-viral infection, in bronchial epithelial cells. In this context, rhinovirus replication was dramatically increased in asthmatic bronchial epithelial cells. The ensuing inflammatory response was also increased [22, 23] and may be involved in exacerbation. The role of CCl20 in our study therefore deserves to be explored in greater detail in future studies. Regarding the other inflammatory mediators, it is important to note that several proteins are highly expressed following poly(I:C) exposure, independently of the phenotype (control or asthma) and the number of exposures. This is the case for CXCL11, CXCL13 and IL-16. These results suggest that although mice can develop an adaptation in terms of neutrophilic inflammation and bronchial hyperreactivity, several actors of the immune system remain functional and could have an impact on the pathophysiology of asthma. The large amount of CXCL13 after poly(I:C) exposure might be produced in response to type I interferon. This chemokine drives CXCR5-dependent recruitment of B cells to the lung, thereby supporting pulmonary germinal center formation [24]. Similarly, CXCL11, another interferon-stimulated gene secreted protein product, is highly expressed and has been described in lymphocyte trafficking from the interstitium to the airway lumen [25]. The amount of interleukin 16 was also dramatically increased after poly(I:C) exposures (similarly between 1 and 3 exposures) and this level was higher in asthmatic mice (mean: 1620 pg/ml in control vs. 2376 pg/ml in asthma). Interestingly, interleukin 16 may increase host susceptibility to influenza A virus infection, which is also known to trigger exacerbations [26]. Finally, it is also interesting to note that several inflammatory mediators (CCL3, CCL4, CCL11, CCL24, CXCL1 and GM-CSF) are significantly reduced after 3 exposures, regardless of the phenotype (asthma/control). The case of CCL24 is interesting because it is one of the mediators highly expressed after 1 exposure, with slightly higher expression in asthmatic mice (mean: 1335 pg/ml in control vs. 1689 pg/ml in asthma). In addition to eosinophil recruitment, CCL24 may contribute to airway fibrocyte recruitment in severe asthma, thereby contributing to bronchial remodeling [27].

Note that the aim of this study was not to explore the mechanisms of this adaptation/exacerbation, but simply to present its potential for further studies. Nevertheless, we can formulate several hypotheses to try and explain this non-adaptation in asthmatic mice. The most important lead would be to explain why neutrophilic inflammation persists in asthmatic mice. One may argue that, although resolution of inflammation was observed in the BAL prior to exposure to poly(I:C), it cannot be ruled out that immune cells present in the lung mucosa, probably with a T2 phenotype (such as group 2 innate lymphoid cells) [28], are also responsible for the exacerbation. The role of BSM cells in this non-adaptation will also have to be clarified. It is well known that asthmatic BSM cells may exhibit not only the "classical" contractile phenotype but also a proliferative-synthetic phenotype, which is capable of producing proinflammatory cytokines, chemotaxins, and growth factors [29]. It has recently been described that BSM cells can acquire an epigenetic memory that can perpetuate inflammatory reactions [30]. To fully assess the role of BSM cells in exacerbation, it will be interesting to deplete these cells in mice, which has never been done before. Future experiments are therefore necessary.

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One of the limitations of our study may be the use of poly(I:C) rather than respiratory viral infection. In fact, what might at first appear to be a limitation could in fact be an advantage. The use of poly(I:C) rather than a viral infection will enable a greater number of laboratories to explore mechanisms of adaptation/exacerbation, for those who, like us, cannot handle viruses in their animal facilities. The choice of poly(I:C), a TLR3 agonist which mimic double-stranded RNA, was made because the infectious virions of positive-sense, single-stranded RNA viruses (such as rhinovirus or the pandemic SARS-CoV-2) transmit the viral genome as a single-stranded, messenger-sense RNA that is then replicated intracellularly via a double-stranded RNA intermediate [31, 32]. Thus, poly(I:C) is a synthetic analogue of RNA virus replication. However, studies have shown that rhinovirus infection act both through TLR3 and TLR7 activation [33]. Thus, to better mimic virus-induced inflammation, it may be interesting to activate additional pattern recognition receptors such as TLR7/8, which are activated by single-stranded RNA in endosomes. At the same time, it is important to note that some agonists have dual effects. In the case of TLR7 agonists, several studies have shown protective effects in various experimental models of asthma [34].

Conclusions

Taken together, this observational study describes an original concept of pulmonary adaptation of non-asthmatic mice in response to repeated inhalation of poly(I:C) and exacerbation of asthmatic mice. This model can be used to study the underlying mechanisms of asthma exacerbation and open up a new paradigm on exacerbation as an altered mechanism of pulmonary adaptation to chronic inflammation of viral origin.

Abbreviations

α-SMA	Alpha-smooth muscle actin
BAL	Bronchoalveolar lavage
BHR	Bronchial hyperresponsiveness
BSM	Bronchial Smooth Muscle
DerP	Dermatophagoides pteronyssinus
PBS	Phosphate Buffer Saline
Poly (I:C)	Polyinosinic-polycytidylic acid

Supplementary Information

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Supplemental Fig. 1. Illustration of αSMA area analysis

Supplemental Fig. 2. CCL family analysis from multiplex ELISA of bronchoalveolar lavage supernatants of asthmatic and non-asthmatic mice 24 h after the last exposure of either PBS, poly(I:C) x1 or poly(I:C) x3 (yellow block represents results outside the defined range)

Supplemental Fig. 3. CXCL/CX3CL family analysis from multiplex ELISA of bronchoalveolar lavage supernatants of asthmatic and non-asthmatic mice 24 h after the last exposure of either PBS, poly(I:C) x1 or poly(I:C) x3 (yellow block represents results outside the defined range)

Supplemental Fig. 4. Interleukins (and others) family analysis from multiplex ELISA of bronchoalveolar lavage supernatants of asthmatic and non-asthmatic mice 24 h after the last exposure of either PBS, poly(I:C) x1 or poly(I:C) x3 (yellow block represents results outside the defined range)

Author contributions

B.A. and P.B. conceived and designed research. B.A., O.O., Z.S., H.L., E.M. and M.C. performed experiments and analyzed data. B.A. interpreted results of experiments, prepared figures, drafted manuscript. B.A., T.T. and P.B. edited and revised manuscript, B.A., O.O., Z.S., H.L., E.M., M.C., T.T. and P.B. approved final version of manuscript.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All mice were treated in accordance with the guidelines of the Federation of European Laboratory Animal Science Associations, and protocols were approved by the local animal care committee (N°02712.02).

Consent for publication

Not applicable.

Competing interests

BA, OO, ZS, HL, EM, MC and TT declare no conflict of interest. PB is the medical coordinator of the French national cohort (i.e., COBRA), which received grants from AstraZeneca, GlaxoSmithKine, Sanofi, and Chiesi. Moreover, PB reports grants, personal fees and non-financial support from AstraZeneca, personal fees and non-financial support from Sanofi, personal fees from GSK, outside the submitted work. In addition, PB has 3 patents (i) (EP N°15152886.6 i.e., New compositions and methods of treating and/or preventing Chronic Obstructive Pulmonary Disease), pending; (ii) (EP N°3050574 i.e., Use of plerixafor for treating and/or preventing acute exacerbations of chronic obstructive pulmonary disease), delivered; (iii) (EP N°20173595.8 i.e., New compositions and methods of treating COVID-19 Disease), licensed.

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