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ORIGINAL ARTICLE

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Bronchiolitis and recurrent wheezing are distinguished by type 2 innate lymphoid cells and immune response

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Abstract

Background: Recurrent wheezing (RW) is frequently developed in infants that have suffered bronchiolitis (BCH) during first months of life, but the immune mechanism underlying is not clear. The goal was to analyze the innate immune response that characterizes BCH and RW.

Methods: Ninety-eight and seventy hospitalized infants with BCH or RW diagnosis, respectively, were included. Nasopharyngeal aspirate (NPA) was processed. Cellular pellet was employed to evaluate type 2 innate lymphoid cells (ILC2) by flow cytometry and mRNA expression assays by semi-quantitative real-time PCR (qRT-PCR). In supernatant, twenty-seven pro-inflammatory and immunomodulatory factors, as well as lipid mediators and nitrites, were evaluated by ELISA and Luminex.

Results: Bronchiolitis patients showed higher ILC2 percentage compared with RW ($P < .05$). Also, $ST2^+$/ILC2 percentage was higher in the BCH group than in the RW group ($P < .01$). *TLR3*, *IL33*, *IFNG*, *IL10*, and *FLG* mRNA levels were significantly increased in BCH vs RW ($P < .05$). In supernatant, no significant differences were reached, observing similar levels of parameters linked to vascular damage, monocyte activation, and fibroblast growth. Prostaglandin E2 and cysteinyl leukotrienes C4 were evaluated; a significant difference was only found in their ratio.

Conclusion: Bronchiolitis is associated with elevated nasal percentage of ILC2. This cellular population could be the key element in the differential immune response between BCH and RW which share some mechanisms such us monocyte activation, vascular damage, and fibroblast repair. Lipid mediators could play a role in the evolution of the disease later in life through innate lymphoid cells.

KEYWORDS

bronchiolitis, immune response, lipid mediators, recurrent wheezing, type 2 innate lymphoid cells (ILC2)

1 | INTRODUCTION

Respiratory disease morbidity in the neonatal period and infancy are one of the most important healthcare costs. More than three million worldwide hospital admissions are caused by bronchiolitis (BCH), with a 45% occurring in infants during the first 6 months of life.[1] Respiratory tract infections and wheezing episodes induced by virus particularly are an important risk factor for asthma in later life.[2] Although a relationship between viral BCH and recurrent wheezing (RW) and asthma exists, the exact mechanism is not clear.

Immune response in BCH and subsequent RW episodes is not fully understood. Several hypotheses have suggested the existence of a defective Th1 immune response in this first viral respiratory infection or that Th2 immune response takes precedence, exacerbating this response over later agents or antigens. Even, the enhancement of antiviral response through IFN-β administration in the cases of virus-induced asthmatic exacerbations has been postulated like a clinical approach.[3] Innate immune response could be the key element of immune system response in both pathological situations.

In this last decade, the innate lymphoid cells have been recognized as critical effectors of innate immunity, opening a new field of study in immunology area.

In this study, we determine the innate and adaptive immune responses that characterize viral BCH and wheezing episodes, trying to find the key immune mechanisms implicated in both scenarios that could help us to establish new clinical approaches to these respiratory diseases.

Key Message

This study represents the first comparative immune response analysis, including type 2 innate lymphoid cells evaluation, between bronchiolitis and recurrent wheezing using nasopharyngeal aspirates samples. The main difference between both diseases at immunological level resides in the increment of type 2 innate lymphoid cells observed in nasopharyngeal aspirates from bronchiolitis infants. New targets might help to establish different therapeutic strategies to prevent asthma development.

2 | METHODS

2.1 | Study design and clinical assessment

Prospective, cross-sectional, single-site recruitment study conducted at University Severo Ochoa Hospital (Leganés) between October 2016 and August 2017. The study populations comprised, as inclusion criteria, all infants younger than 24 months admitted with a diagnosis of bronchiolitis defined by the criteria of McConnochie,[4] who defined bronchiolitis as the first episode of acute-onset expiratory dyspnea with previous signs of viral respiratory infection, or recurrent wheezing (more than one episode of wheezing confirmed by a physician).[5] Signed informed consent was obtained from the parents or legal guardians. The study protocol was approved by the Ethics Committee of the hospital, and the study was conducted in accordance with the principles set forth in the Declaration of Helsinki.

During the hospital stay, a study questionnaire was filled out by a physician, providing information on epidemiological parameter and clinical variables during admission.

2.2 | Sample collection and virus detection

Two nasopharyngeal aspirates (NPAs) were obtained at admission by a standard, routine technique (described in Appendix S1). One of the two samples was processed in the Respiratory Virus and Influenza Unit at the National Microbiology Centre (ISCIII) and could detect a total of 16 respiratory viruses, as it has been previously described.[6] The other sample was used for immunologic testing at the Immunology Department of IIS-Fundación Jiménez Díaz as described below.

2.3 | Nasopharyngeal aspirate (NPA) processing

Nasopharyngeal aspirates were processed; cells and supernatants were frozen at −80°C until use (Appendix S1). Samples with an elevated cellularity were used to evaluate type 2 innate lymphoid cells (ILC2).

2.4 | Immunologic analysis in NPA

Several genes were evaluated through their mRNA levels by semiquantitative real-time PCR (qRT-PCR) in purified total RNA from NPA cellular pellets extracted using the Chomczynski method.[7] All genes were examined for their relative expression which was calculated using the $2^{-\Delta C}$ and $2^{-\Delta\Delta Ct}$ method.[8]

In NPA supernatant, cytokines and chemokines linked to inflammation and immune responses were determined using a commercial Luminex and ELISA kit. Details are described in Appendix S1.

2.5 | ILC2 quantification by flow cytometry

Samples with a high number of cells were processed to ILC2 flow cytometry analysis as described in Appendix S1. The panel used consists of the following: Pe-Cy5-$CD45^{High}$/FITC-$Lineage^{-}$ (CD3/14/16/19/20/56)/PECy7-CD127/APC-Cy7-CD294/PE-ST2/IL-1R4 and isotype control (Figure S1) that was used to establish background fluorescence for each fluorochrome. The gating strategy used to ILC2 selection was as follows: $CD45^{High}$/$Lineage^{-}$/$CD127^{+}$/CD294 $(CRTH2)^{+}$.[9,10] (Figure 1A). Moreover, we established the $ST2^{+}$/ILC2 percentage.

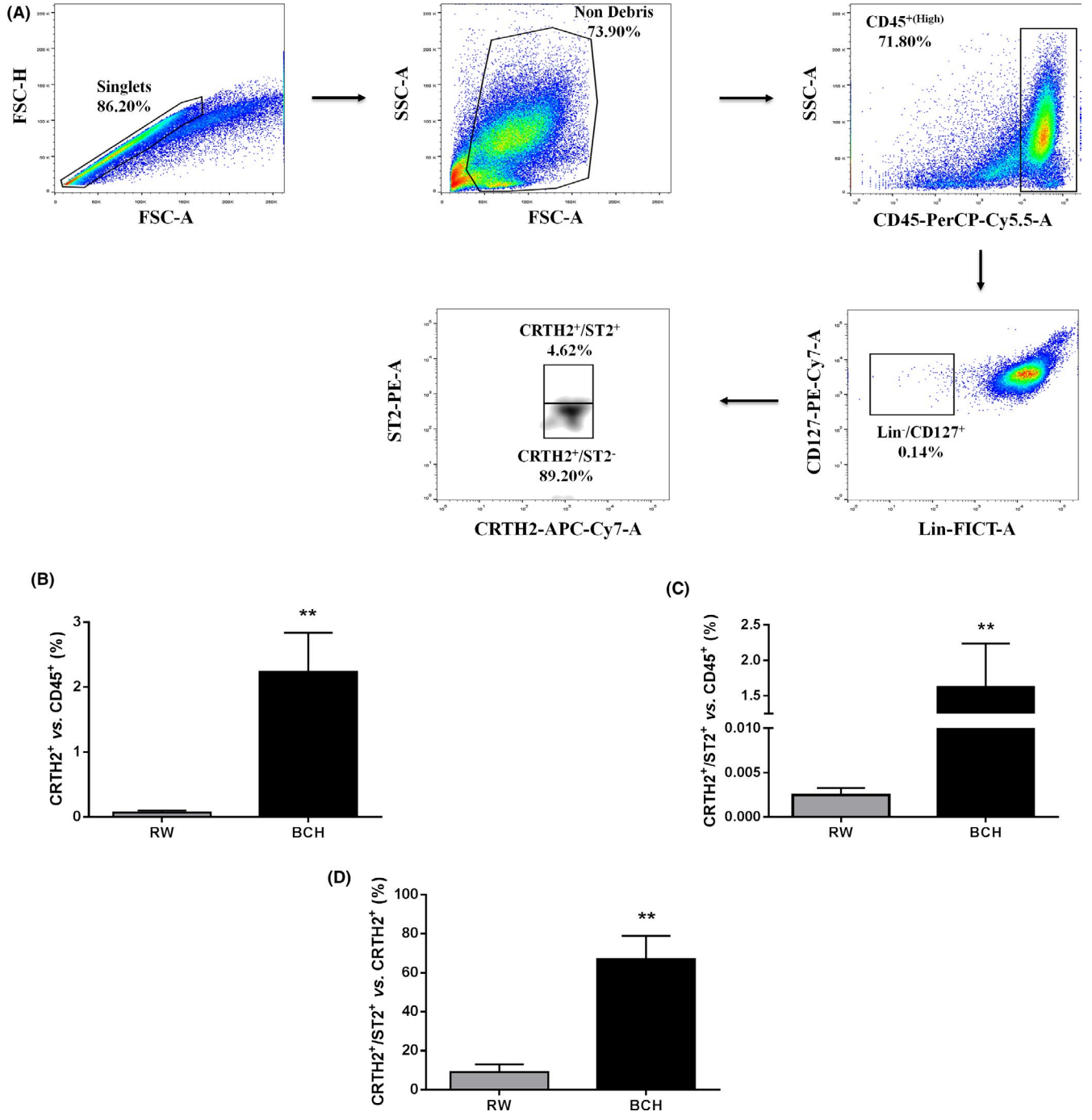

FIGURE 1 Analysis of type 2 innate lymphoid cell (ILC2) percentage in nasopharyngeal aspirate (NPA) from patients with bronchiolitis (BCH) or recurrent wheezing diagnosis by flow cytometry. A, Gating strategy analysis of ILC2 from NPA. B, ILC2 percentage relative to total leukocytes. C, ST2-positive ILC2 percentage relative to total leukocytes. D, ST2-positive ILC2 percentage relative to total ILC2. $^{**}P < .01$ [Colour figure can be viewed at wileyonlinelibrary.com]

2.6 | Determination of arachidonic acid pathway and nitric oxide (NO)

Prostaglandin E2 (PGE_2) and cysteinyl leukotrienes (LTC_4) were evaluated in the NPA supernatant of samples using ELISA (Enzo) following the manufacturer's instructions.

Nitrite determination was developed in supernatant of NPAs using Total Nitric Oxide and Nitrate/Nitrite Parameter Assay Kit (R&D System). Endogenous nitrites were subtracted from the total nitrite value.

2.7 | Statistical and bioinformatic analysis

Values are expressed as percentages for discrete variables or as mean and standard error of mean (SEM) or median and inter-quartile range

(IQR) for continuous variables. Comparisons used either chi-square or Fisher's exact test (two-tailed) for categorical variables and the Mann-Whitney *U* test for continuous variables. Correlations were determined using Spearman's rank correlation coefficients. Previous to mRNA expression performance evaluated by receiver operating curve (ROC) analysis, missing data were evaluated. Variables and patients with more than fifty percent missing data were removed from analysis. Odds ratio (OR) with 95% confidence interval (CI) was calculated for clinical variables and mRNA expression, comparing bronchiolitis and recurrent wheezing group data, establishing a cut-off based on Youden Index. Principal component analysis (PCA) was carried out with ClustVis bioinformatic tool.[11] $P < .05$ was considered significant. Statistical calculations and graphs were developed using GraphPad Prism 6 (GraphPad Software Inc). ROC analysis was performed using R (https://www.r-project.org/).

3 | RESULTS

3.1 | Clinical and epidemiological characteristics of the study population

The study populations were comprised by ninety-eight infants with BCH and seventy children with RW.

Epidemiological and clinical variables recorded during admission are shown in Table 1. Significant differences were observed in age between the groups as expected. Regarding hospital admission by BCH or RW, a significant increase in hospital stay and hypoxia duration in the BCH group was observed, whereas a higher proportion of children with fever was detected in the RW group.

Positive viral identification rate was very similar in both groups; however, respiratory syncytial virus (RSV) was significantly predominant in the BCH group ($P = .027$) with odds ratio (OR) value of 2.38 and 95% confidence interval (CI) of 1.14-5.0.

Several epidemiological characteristics such as type of feeding, atopy or asthma history from parents and siblings, tobacco exposure during pregnancy, or in-house environment were analyzed. Infants with RW had history of mother atopy (OR = 4.81, 95% CI: 1.51-15.35, $P = .0058$) or atopic dermatitis (OR = 7.81, 95% CI: 1.69-36, $P = .0027$) more frequently than BCH patients. No significant differences were observed in other clinical and epidemiological variables such as breastfeeding during infancy or tobacco exposure.

3.2 | Type 2 innate lymphoid cells are highly expressed in NPA from BCH patients

We observed a significant increase of ILC2 population in the BCH group with respect to the RW one (Figure 1B; $P < .01$). This significant augment was also observed in the $ILC2^{+}/ST2^{+}$ population with respect to leukocytes ($CD45^{+}$) and total ILC2 population (Figure 1C,D, respectively; $P < .01$).

3.3 | Nasal cytokine and chemokine

Higher mRNA expression in BCH population with respect to the RW group was observed, being this increase statistically significant in *IL33* ($P < .01$), *TLR3* ($P < .0001$), *IFNG* ($P < .05$), *IL10* ($P < .05$), and *FLG* ($P < .01$) (Figure 2A) with more than twofold increase in all of these genes (Figure 2B). The rest of mRNA from different genes evaluated did not present significant differences (Figure S2).

However, in NPA supernatant, no significant differences in any of the twenty-seven molecules evaluated were observed (Figure S3).

Due to important increase in TLR3 and knowing that HRV binds to this receptor, we analyzed if virus strain has some relevance on this. No differences were observed when we classified patients according to HRV positive vs. rest of virus, or sub-classifying them by mono-infection or coinfection, or even intragroup analysis in the BCH group.

In order to better understand differential mRNA expression, we applied a statistical model analysis. The OR calculated for *TSLP*, *IFNG*, and *IL13* mRNA expression showed a significant higher frequency in the RW population than the BCH group (Figure 3).

To explore the association between the type of disease and the mRNA expression profile, we conducted a PCA on differential expressed genes. No clear clusters were observed, with the first and second principal components (PC1 and PC2) accounting for 60.1% and 19% of all the variance of the evaluated mRNA of specific genes, respectively (Figure S4).

In the ROC curve analysis of mRNA expression levels, only *IL33* and *TLR3* presented an acceptable ability to discriminate between BCH or RW, with values of area under the ROC curves over 0.7 (0.72 and 0.77, respectively) (Table S1).

Moreover, when the correlation between several studied genes was evaluated, only significant correlation was observed between *TLR3* and *IL33* mRNA expression ($\rho = .75$, $P < .001$). We have attached the heatmap with correlation data table in Appendix S1 (Figure S5). Rest of correlation between genes did not show association between analyzed genes (Figure S5).

3.4 | Determination of lipid mediators and nitrites

No significant differences were observed on LTC_4 or PGE_2 levels between both groups of patients (Figure 4A,B). However, when the ratio LTC_4/PGE_2 was analyzed to discern predominant status, a significant higher ratio was observed in the RW group (0.199 ± 0.044 vs 0.102 ± 0.024; $P < .05$), probably due to the low levels of PGE_2 detected in this group (Figure 4C).

COX-2 mRNA expression was also evaluated, and a similar expression pattern was observed in both groups (Figure 4D).

On the other hand, nitrite levels were determined in the supernatants of NPAs during the acute episode of BCH or RW. Similar levels were detected in the BCH and RW groups without reaching statistical differences (Figure 4E).

TABLE 1 Epidemiological and clinical characteristics of infants with bronchiolitis and recurrent wheezing episodes

	BCH (n = 98)	RW (n = 70)
Age (mo)[a]	3.25 (4.5)	23 (24.75)***
Male (%)	60/98 (61.2)	47/70 (67.1)
Prematurity (%)	11/97 (11.3)	7/66 (10.3)
Hospital stay (d)[b]	3.89 (0.23)	2.64 (0.17)***
Temperature > 37.9°C (%)	21/77 (27.3)	38/69 (55.1)***
Hypoxia ($SatO_2$ < 95%) (%)	75/98 (76.5)	47/69 (68.1)
ICU admission (%)	4/98 (4.09)	1/69 (1.44)
Neonatal admission (%)	4/61 (6.56)	7/62 (11.29)
Neonatal CPAP (%)	2/60 (3.33)	3/68 (4.41)
Antibiotic treatment (%)	17/98 (17.35)	13/68 (19.12)
Virus (%)	80/92 (86.96)	47/60 (78.33)
RSV	46/80 (57.5)	17/47 (36.17)*
HRV	38/80 (47.5)	22/47 (46.81)
Coinfection	28/92 (30.43)	10/50 (20)
Cesarean birth (%)	9/53 (16.98)	19/50 (38)
Feeding		
Exclusive breastfeeding (%)	41/60 (68.33)	40/65 (61.54)
Non-exclusive breastfeeding (%)	10/60 (16.67)	10/65 (15.38)
Artificial feeding (%)	9/60 (15)	15/65 (23.08)
Atopy		
Father (%)	13/72 (18.06)	10/67 (14.93)
Mother (%)	4/72 (5.56)	15/68 (22.06)**
Sibling (%)	6/59 (10.17)	2/43 (4.65)
Asthma		
Father (%)	8/72 (11.11)	2/66 (3.03)
Mother (%)	5/73 (6.85)	4/68 (5.88)
Sibling (%)	11/59 (18.64)	15/44 (34.09)
Atopic dermatitis	2/61 (3.28)	14/66 (21.21)**
Passive smoking (%)	24/72 (33.33)	24/68 (35.29)
Smoking during pregnancy (%)	17/72 (23.61)	8/61 (13.11)
Nursery school (%)	3/59 (5.08)	21/59 (35.59)***

Abbreviations: BCH, bronchiolitis; RW, recurrent wheezing.

[a]Median (IQR [inter-quartile range]).

[b]Mean (SEM [standard error of the mean]).

*$P < .05$.

**$P < .01$.

***$P < .0001$.

4 | DISCUSSION

This study sets out one of the widest comparative immune response profiles between viral bronchiolitis and recurrent wheezing at early life, focusing on innate immune response and, specifically, on the recently described ILC2. To our knowledge, this is the first study that ILC2 and $ST2^+$/ILC2 percentages in NPAs from patients with viral BCH or RW have been compared.

Viral BCH and RW together with asthma are very common diseases in childhood and they represent a huge clinical burden during infancy, existing an important relation among them; BCH may be an early marker of future wheezing episodes (although this does not always happen), and viral-induced wheezing in childhood is an important risk factor to later asthma development.[12,13] Some authors considered that the future effect depends on the dominant virus strain in the bronchiolitis episode, suggesting that RSV could be a marker of predisposition for asthma and atopy, whereas human rhinovirus (HRV) could have a greater causal role, especially in severe cases needing hospital admission.[14,15] However, the results of a recent study suggest that the cumulative number of respiratory episodes early in life is more important than the type of virus identified in the subsequent development of asthma.[16] Nowadays, demonstrated and confirmed results about this are not available.

Although the diagnosis of viral infections has been improved by the development of new molecular techniques, our understanding about the immune response in viral infections and linked illness such as wheezing is still poor.

Antiviral cytokines such as interferons or type 2 cytokines have been considered the main factors implicated in the early stages of allergic asthma and related pathologies. However, multiple studies have highlighted the key role that other elements such as ILCs could play in the pathogenesis and/or the evolution of these diseases.

In our study, the innate immune response in acute phase of both diseases was compared and a significant augment of ILC2 in NPA from patients with BCH compared with those in RW was observed.

ILC2 belong to the denominated helper-like innate lymphoid cells.[17,18] This specific cellular subpopulation is usually localized at barrier surfaces, and it is mainly induced, maintained, and regulated by the epithelium-derived cytokines denominated alarmins, such as IL-33, IL-25, and TSLP,[19] which are released by damaged tissue or by stressed cells after exposure to allergens or viral infection.[20] Other cells such as eosinophils, mast cells, or Th2 cells also secrete alarmins.[21] These cells are important in the maintenance of the homeostasis and tissue repair through ILC2-derived amphiregulin[20,22,23]; also, they have relevance in the induction of allergic inflammation.[24]

We observed a significant difference in the expression of IL-33 receptor (ST2) in the ILC2 population from NPA in the BCH group, reaching a fivefold increase. This expression receptor could be increased by the presence of IL-1β, IL-2, or some lipid mediators such as cysteinyl leukotrienes (LTC_4 or LTE_4) or prostaglandin D2 (PGD_2),[10] being the presence of PGD2 receptor (CRTH2) a characteristic feature of ILC2.

Linked to the augment of $ST2^+$/ILC2 percentage in the BCH group, a significant increase of *IL33* mRNA expression in the cellular sediment of these patients compared to the RW group was observed. However, the protein expression of IL33 in the NPA supernatant was similar between both groups. This dichotomy and potential disproof are explained by the results of some studies that show how the viruses that induce ILC2 through IL-33 synthesis by damaged epithelium, such as RSV or HRV, decreased GATA-3 expression, leading

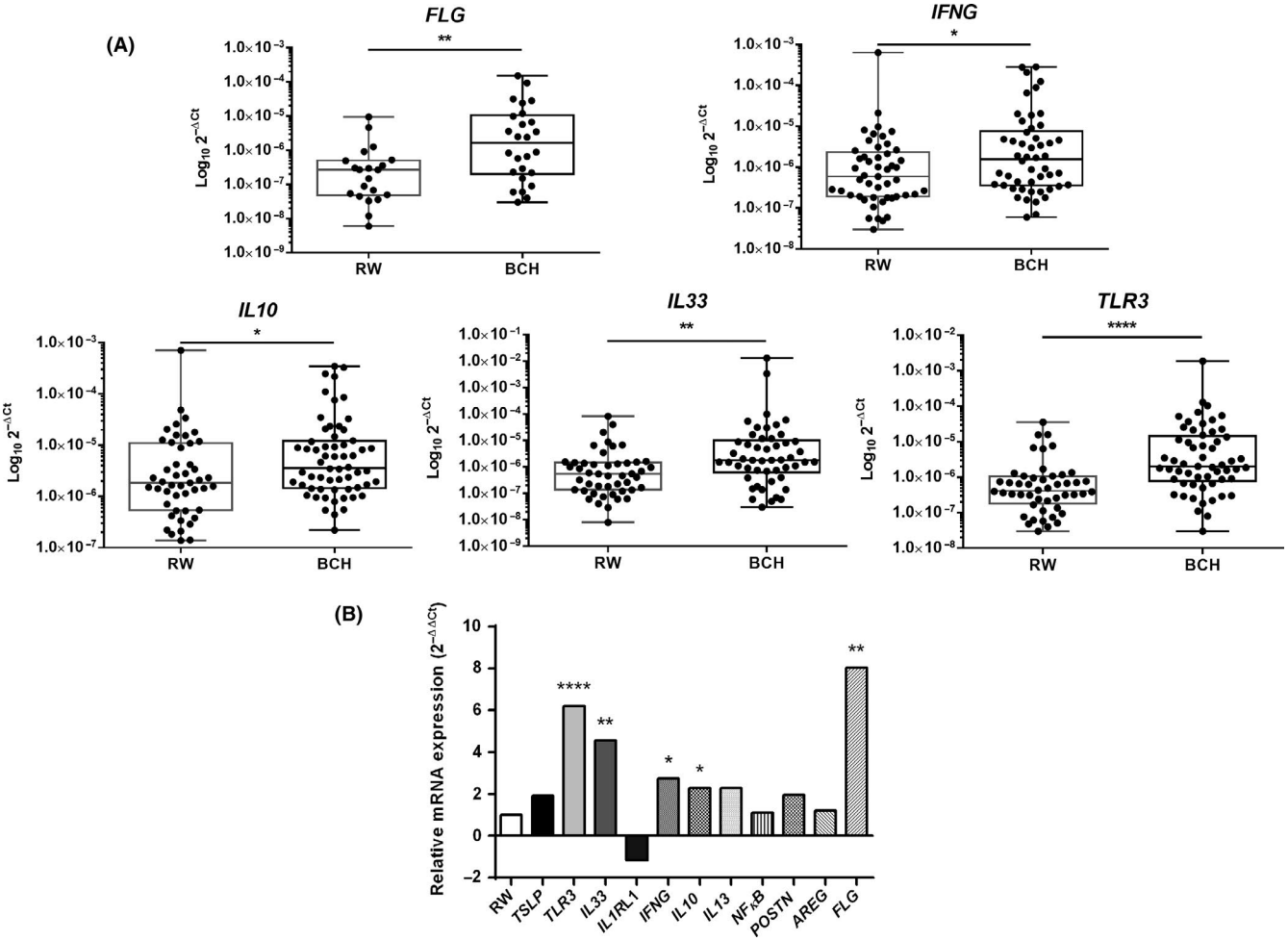

FIGURE 2 mRNA expression of several cytokines, factors, receptors, and transcription factors evaluated in cellular pellets of NPA in the acute stage of bronchiolitis or recurrent wheezing episode. A, *FLG*, *IFNG*, *IL10*, *IL33*, and *TLR3* are differentially expressed mRNA in NPA between both disease groups, BCH and RW. Data are expressed in $\log_{10}$ of $2^{-\Delta Ct}$. B, Data represent expression relative to recurrent wheezing values. mRNA relative expression has been evaluated through semi-quantitative real-time PCR, in purified total RNA from NPA cellular pellets. Their relative expression was calculated using the $2^{-\Delta C}$ and $2^{-\Delta\Delta Ct}$ method. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .0001$

to a reduction of ST2 expression on resident lung ILC2 surface in pulmonary diseases.[25,26] In this context, the viruses could act as mitigating elements of inflammation, in contradiction with other studies,[27] which suggest the role of viruses as important triggers of the Th2 immune response and inflammation through bronchial epithelial IL-33 production.

The *TLR3*, *IFNG*, and *IL10* mRNA expression values were also significantly enhanced in the BCH group compared to the RW one. These results are in concordance with mechanisms that are triggered in a viral infection context,[28] although their protein expressions in the NPA supernatant were not different among both populations. When we analyzed the OR, the presence of *IFNG* mRNA expression is higher in the RW group, although the mRNA expression level of this cytokine is higher in the BCH group than in RW population. This could be in agreement with the hypothesis that a fault in IFN-γ production could be the cause of a severe BCH evolution. In fact, in our BCH population the level of IFN-γ is lower (1.5-fold decrease) in patients with more severity status (necessity of high-flow oxygen therapy; Figure S6).

In spite of high expression of TLR3 mRNA, no link with HRV was observed. So, these results could point out that expression of TLR3 is not only due to virus strain and stimulus of immune environment or predominant cellular type could exert some effect on this TLR3 mRNA expression.

Perhaps the increased *IL33*, *IFNG*, and *IL10* mRNA expression could be indicative of the existence of a mixed Th1/Th2 immune response similar to that observed in several studies, mainly focussed on immunizations[29] or parasitic and viral infections.[30,31]

Filaggrin 1 mRNA expression was increased in BCH. It probably is part of the repair mechanism to maintain barrier function[32] triggered to respond to the epithelium damage caused by viral infection. Furthermore, the low expression of *FLG* mRNA expression in the RW group could be due to the increased atopic dermatitis percentage observed in this group compared with the BCH group. So, a decrease in this epithelial component supposes a disruption in skin barrier and promotes the epithelial damage characteristic of atopic dermatitis. Thus, these results are in agreement with the literature in which the association between the atopic march that usually starts with the development of atopic dermatitis and the loss of filaggrin or mutations on it is clear.[33,34]

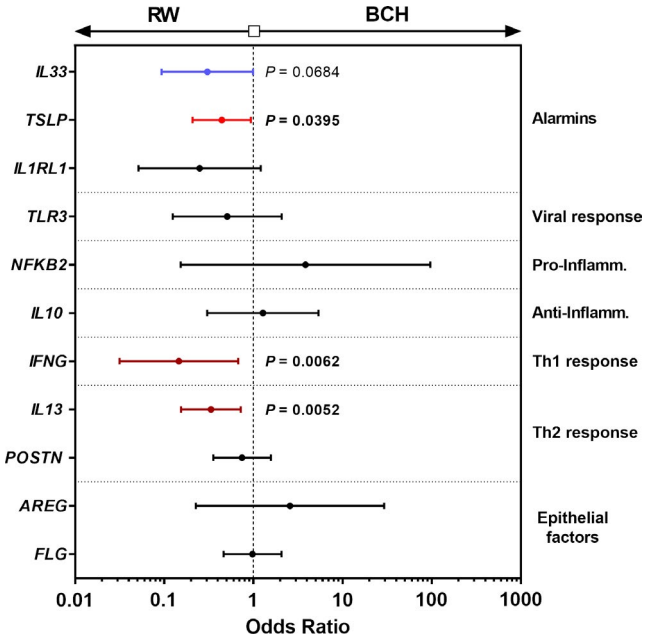

FIGURE 3 Odds ratio analysis of NPA evaluated genes linked to innate and adaptive immune response, epithelial damage, and viral infection. Odds ratio (OR) with 95% confidence interval (CI) was calculated, establishing a cutoff based on Youden Index. $P < .05$ was considered significant [Colour figure can be viewed at wileyonlinelibrary.com]

All these results are reflecting an important pro-inflammatory, antiviral, and epithelial repair response in the BCH group.

Previously, we have mentioned the role of lipid mediators on ILC2 expansion and activation. We have not found differences in the individual levels of LTC_4 and PGE_2. However, a decrease in the LTC_4/PGE_2 ratio has been shown in the BCH group. This result may be explained by the enhanced PGE_2 levels in this population. These raised levels could be a self-limited tool of the immune response since when this prostaglandin binds their EP2 or EP4 receptors on ILC2 surface and downregulation of GATA-3 (a main ILC2 inductor) occurs, while the rest of cytokines such as IL-25, IL-5, and IL-13 are linked to GATA-3 increment.[35]

It is the first time that the presence of ILC2 in nasopharyngeal secretions from infants with bronchiolitis and recurrent wheezing diagnosis has been compared, observing that the IL-33 receptor is in a higher proportion in the ILC2 from NPA of the BCH group. No differences in a wide range of innate, antiviral, and adaptive immune response were observed between both entities that could share some mechanisms such as monocyte activation, vascular damage, and fibroblast repair. ILC2 may be the key point discordant between the immune response triggered in both cases. Further studies are needed to clarify the exact role of ILC2 in viral respiratory infections in infants and its potential role as therapeutic target.

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CONFLICT OF INTEREST

Victoria del Pozo has been a consultant/speaker for AstraZeneca and GSK. The rest of the authors declare that they have no conflicts of interest relevant to this article to disclose.

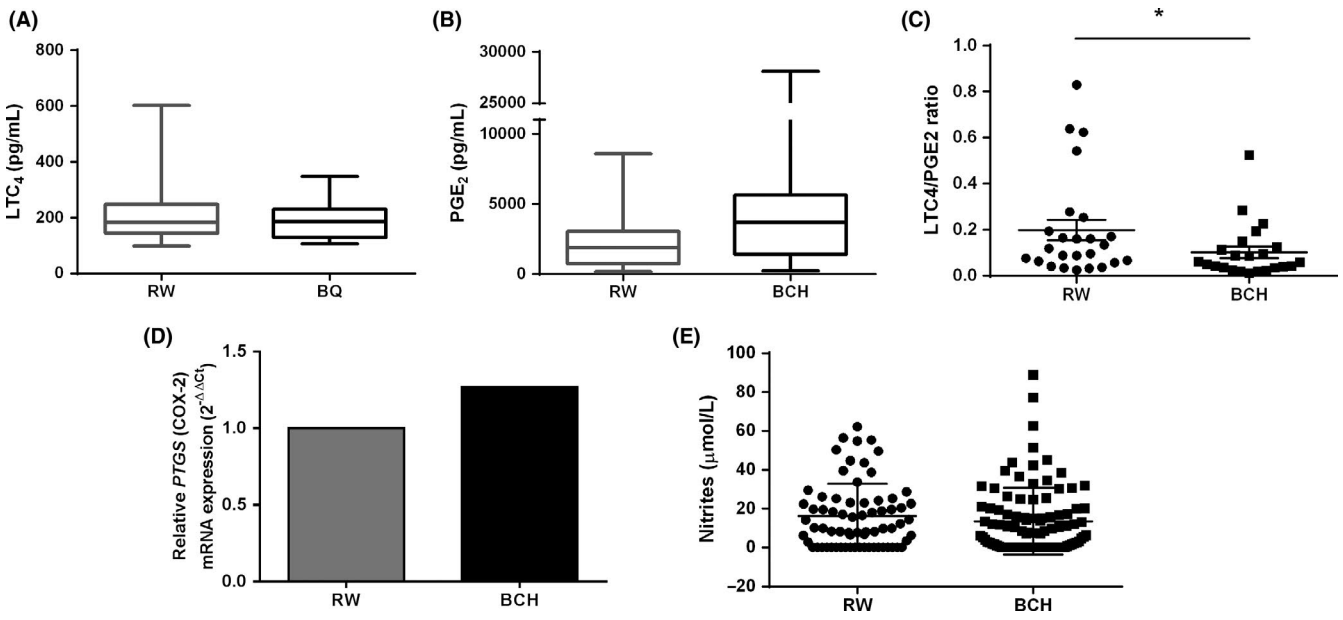

FIGURE 4 Levels of pro-inflammatory and lipid mediators in acute bronchiolitis and recurrent wheezing during acute period. A, LTC_4 levels in supernatant of NPA. B, PGE_2 levels in supernatant of NPA. C, LTC_4/PGE_2 ratio to determine the dominant pathway. D, Relative COX-2 mRNA (*PTGS2*) expression. E, Nitrite levels evaluated in supernatant of NPA. These lipid mediators have been evaluated through ELISA technique. $^*P < .05$

AUTHOR CONTRIBUTION

Beatriz Sastre: Conceptualization (lead); Formal analysis (lead); Investigation (equal); Supervision (lead); Validation (equal); Writing-original draft (lead). **Maria Luz Garcia-Garcia:** Conceptualization (lead); Formal analysis (equal); Funding acquisition (lead); Investigation (equal); Resources (equal); Supervision (equal); Validation (equal); Writing-original draft (equal). **Jose Antonio Cañas:** Data curation (equal); Formal analysis (equal); Investigation (equal); Validation (equal). **Cristina Calvo:** Conceptualization (equal); Investigation (equal); Validation (equal); Visualization (equal). **Jose Manuel Rodrigo-Muñoz:** Data curation (equal); Formal analysis (equal); Investigation (equal); Validation (equal). **Inmaculada Casas:** Investigation (equal); Methodology (equal); Resources (equal); Validation (equal). **IGNACIO MAHILLO:** Formal analysis (equal); Software (equal); Validation (equal). **Victoria Pozo:** Conceptualization (lead); Funding acquisition (lead); Project administration (lead); Supervision (lead); Validation (equal); Writing-original draft (lead).

PEER REVIEW

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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