

ORIGINAL ARTICLE

Rhinitis, Sinusitis, and Upper Airway Disease

Sequential class switch recombination to IgE and allergen-induced accumulation of IgE⁺ plasmablasts occur in the nasal mucosa of local allergic rhinitis patients

Almudena Testera-Montes^{1,2} | Francisca Palomares² | Raquel Jurado-Escobar² |
 Ruben Fernandez-Santamaria²  | Adriana Ariza² | Jesus Verge³ | Maria Salas^{1,2}  |
 Paloma Campo¹ | Cristobalina Mayorga^{1,2,4} | Maria Jose Torres^{1,2,4,5}  |
 Carmen Rondon^{1,2}  | Ibon Eguiluz-Gracia^{1,2} 

¹Allergy Unit, Hospital Regional Universitario de Malaga, Malaga, Spain

²Allergy Research Group, Instituto de Investigación Biomédica de Málaga (IBIMA), RICORS "Enfermedades inflamatorias", Málaga, Spain

³ENT Unit, Hospital Clinico Virgen de la Victoria, Malaga, Spain

⁴Andalusian Center for Nanomedicine and Biotechnology (BIONAND), Malaga, Spain

⁵Universidad de Málaga (UMA), Málaga, Spain

Correspondence

Carmen Rondon, Allergy Unit, Hospital Civil, Hospital Regional Universitario de Malaga, pabellón 6, 1º planta, Plaza del Hospital Civil, Malaga 29009, Spain.
 Email: carmenrs61@gmail.com

Funding information

This work was supported by Instituto de Salud Carlos III (ISCIII) of the Spanish Ministry of Science and Competitiveness (grants co-funded by the European Regional Development Fund) through the research project PI20/01715.

This work was also supported by the Regional Ministry of Health of Andalucía through the research projects PI-0346-2016 and PI-0176-2018, and by the Regional Ministry of Education of Andalucía through the research project P20_00405. ATM holds a "Rio Hortega" contract (CM20/00160), IEG a "Juan Rodes" contract (JR19/00029) and RJE a P-FIS contract (FI18/00133), all granted by ISCIII. CM holds a "Nicolas Monardes" contract (RC-0004-2021) and AA a Senior Postdoctoral Contract (RH-0099-2020), both granted by the Regional Ministry of Health of Andalucía (co-funded by European Social Fund (ESF) "Andalucía se mueve con Europa"). FP holds a "Stop Fuga de Cerebros" contract

Abstract

Background: The involvement of allergen-specific (s)IgE in local allergic rhinitis (LAR) has been debated. Here, we investigate the effect of nasal allergen challenge with *Dermatophagoides pteronyssinus* (NAC-DP) in mucosal and peripheral B-cell subpopulations in LAR patients.

Methods: Nine LAR, 5 allergic rhinitis (AR), and 5 non-atopic healthy control (HC) individuals were subjected to a 3-day NAC-DP protocol, and nasal biopsies and blood samples were collected before and after provocation. Nasal biopsies were used for immunohistochemistry and gene expression studies, whereas the frequency of lymphocyte subsets and basophil activation test (BAT) were analyzed in blood samples by flow cytometry. sIgG was measured in sera.

Results: NAC-DP induced an increase in IgE⁺CD38⁺ plasmablasts in the nasal mucosa of LAR patients, but not in AR or HC individuals. Markers of sequential recombination to IgE (εCSR) (from IgG) were observed in 33% of LAR, 20% of AR, and 0% of HC subjects. NAC-DP increased the proportion of peripheral CD19⁺CD20⁺CD38⁺ plasmablasts in AR and LAR patients, but not in HC. Expression of the mucosal homing receptor CXCR3 in peripheral CD19⁺CD20⁺CD38⁺ plasmablasts from LAR, AR, and HC individuals was 7%, 5%, and 0.5%, respectively. In vitro DP stimulation increased proliferating CD19⁺CD20⁺CD38⁺ plasmablasts in LAR and AR patients, but not in HC. Serum DP-sIgG was higher in LAR and AR patients as compared to HC. BAT was positive in 33%, 100%, and 0% of LAR, AR, and HC subjects, respectively.

Testera-Montes, Palomares, Jurado-Escobar, Rondon and Eguiluz-Gracia contributed equally to this work.

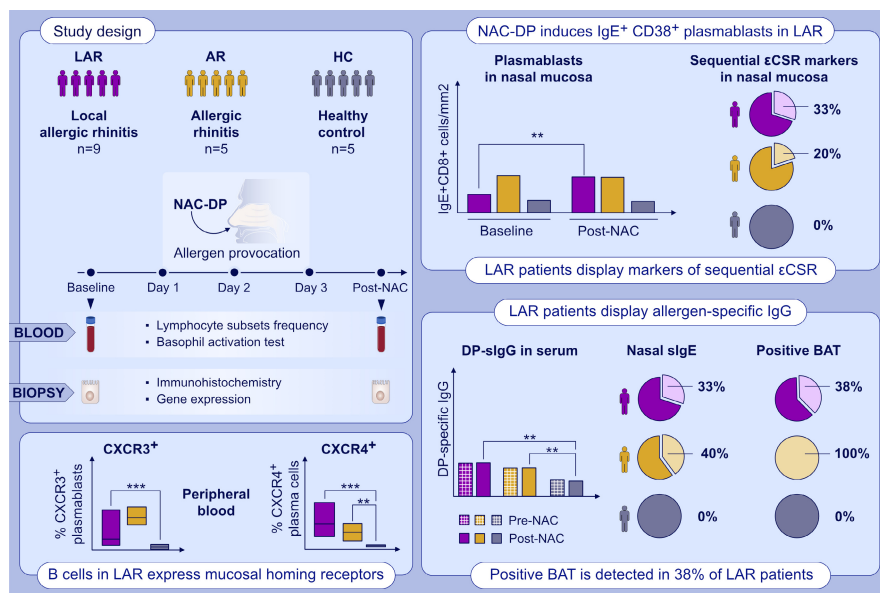
© 2022 EAACI and John Wiley and Sons A/S. Published by John Wiley and Sons Ltd.

(SFC-0002-2020) granted by Roche Pharma SA. This work was also supported by ISCIII (grants co-funded by the European Regional Development Fund) through its program of Redes Temáticas de Investigación Cooperativa en Salud (RETICS): Asma, Reacciones Adversas y Alérgicas-ARADyAL (RD16/0006/0001), and Redes de Investigación Cooperativa Orientadas al Resultado en Salud (RICORS): Enfermedades Inflamatorias (RD21/0002/0008)

Conclusion: These results suggest that allergen exposure induces the sequential ϵ CSR of IgG⁺CD19⁺CD20⁺CD38⁺ plasmablasts in the nasal mucosa of LAR patients.

KEYWORDS

basophil activation test, class switch recombination, IgE, local allergic rhinitis, plasma cell, plasmablast



GRAPHICAL ABSTRACT

Allergen exposure induces the accumulation of IgE⁺ plasmablasts in the nasal mucosa of LAR patients. LAR patients display markers of sequential class switch recombination to IgE (IgM→IgG→IgE) in the nasal mucosa. LAR patients have allergen-specific (s)IgE attached to the membrane of peripheral basophils and a higher level of serum slgG than healthy individuals.

Abbreviations: BAT, basophil activation test; ϵ CSR, class switch recombination to IgE; CXCR, C-X-C motif chemokine receptors; NAC-DP, nasal allergen challenge with *Dermatophagoides pteronyssinus*

1 | INTRODUCTION

Local allergic rhinitis (LAR) is a differentiated rhinitis phenotype defined by a clinical history suggestive of allergy, the negativity of atopy test (skin prick test (SPT) and serum allergen-specific IgE (slgE)) and the positivity of nasal allergen challenge (NAC).¹ LAR affects 16–25% of non-atopic children and adults with chronic nasal symptoms² and naturally evolves to the aggravation³ and the association of asthma (so-called local allergic asthma).⁴ Moreover, LAR and “atopic” allergic rhinitis (AR) can coexist in the same patient, and this clinical scenario has been termed dual allergic rhinitis (DAR).^{5,6}

Despite the advances in the clinical characterization, LAR pathophysiology remains elusive. Our group identified that in LAR patients NAC positivity is associated with an early peak of tryptase in the nasal secretions, followed by a progressive increase in eosinophil cationic protein (ECP) in the 24 h following allergen administration.^{7,8} Although increased numbers of eosinophils and mast cells (MC) were observed in the nasal mucosa of non-atopic rhinitis patients, no study has demonstrated to date the infiltration by eosinophils or the activation of MC in the nasal mucosa of NAC-positive non-atopic rhinitis patients

(confirmed LAR phenotype).⁹ Interestingly, these inflammatory phenomena are hallmarks of AR,¹⁰ a prototypical IgE-mediated condition.

The nasal mucosa of AR patients is infiltrated by IgE-producing cells, as indicated by the expression of ϵ -germ line transcripts (ϵ -GLT) in nasal cells.¹¹ Interestingly, IgE immunobiology differs from that of other immunoglobulin isotypes. Most naïve B cells undergoing direct class switch recombination to IgE (ϵ CSR) in germinal centers cannot complete their somatic hypermutation, thus dying by apoptosis before exiting the secondary lymphoid tissues (SLT).¹² Nevertheless, IgG⁺ B cells primed at SLT can acquire expression of mucosal homing receptors (e.g., CXCR3 or CXCR4)¹³ and extravasate to the lamina propria where they mature to plasmablasts and undergo sequential ϵ CSR.¹⁴ This process will give rise to plasma cells (PC) producing high-affinity slgE.¹⁵ Both direct and sequential ϵ CSR are mediated through the expression of the enzyme activation-induced cytidine deaminase (AID) in the switching cell.¹¹ Gene rearrangement during ϵ CSR generates switch circles (ϵ SC) that differ between direct (IgM→IgE) and sequential (IgM→IgG→IgE) switching.¹⁶ Although both ϵ SCs will be ultimately eliminated, they can be detected transitorily.

Importantly, several studies demonstrate that allergen exposure induces the expression of sequential ϵ SCs in the nasal mucosa of AR patients.^{11,16,17} Direct ϵ SCs have also been detected occasionally, but their function is regarded as regulatory given the unlikelihood of functional IgE arising from direct switching at peripheral tissues.¹⁶ Interestingly, one study suggests that human IgA⁺ B cells can also undergo sequential ϵ CSR in the nasal mucosa.¹⁷ Moreover, recent data indicate that the sequential ϵ CSR at the mucosal level is the main source of sIgE in AR patients. The analysis of the specificity of local and peripheral IgE antibodies revealed that both repertoires show an oligoclonal relationship, with mucosal antibodies displaying a greater diversity.^{18,19} Importantly, one study demonstrated the presence of IgE directed against clinically relevant allergens bound to the membrane of mucosal cells in AR individuals.²⁰ Collectively, these findings suggest that most sIgE detected in sera from AR patients has been synthesized at the mucosal level with subsequent trafficking to the bloodstream.

Despite the clinical similarities with AR, the role of sIgE in LAR has been debated. Only 20%–43% of LAR patients display positive sIgE in nasal secretions.^{7,8,21–23} Conversely, the pooled analysis of LAR patients demonstrated a significant increase in sIgE in nasal lavages during the 24 h following positive NAC, although not every patient tested positive in at least one determination.^{7,8} Interestingly, a study using Merocel© sponges (a device growing into the nostril to perfectly adapt to the anatomy and scratching a significant amount of mucosal cells upon removal) found nasal sIgE in 93% of LAR patients.²⁴ Moreover, basophil activation test (BAT) experiments reveal that 50%–66% of LAR and DAR individuals have functional sIgE bound to the membrane of peripheral basophils.^{5,25–27} Finally, several clinical trials demonstrated the ability of allergen immunotherapy (a prototypical intervention for IgE-mediated conditions) to control nasal symptoms, improve quality of life, increase the allergen dose tolerated in NAC, and decrease the seasonal peak of nasal sIgE in LAR individuals.^{28–30}

Therefore, we hypothesize that the sequential ϵ CSR of IgG⁺ B lymphocytes occurs in the nasal mucosa of LAR patients. To investigate this hypothesis, we subjected 9 LAR and 5 AR patients due to *Dermatophagoides pteronyssinus* (DP), together with 5 healthy non-atopic controls (HC), to a 3-day NAC protocol and collected mucosal biopsies and blood samples before and after provocation. Nasal biopsies were used for immunohistochemistry and gene expression studies, whereas blood samples were stimulated in vitro with DP and subjected to flow cytometric analysis.

2 | METHODS

2.1 | Study individuals

Nine LAR patients ≥ 18 years were prospectively recruited from the Allergy Unit of Hospital Regional Universitario de Malaga between June and September 2017. All individuals fulfilled the following criteria: persistent moderate-to-severe rhinitis³¹ for ≥ 2 years, negative SPT to all relevant aeroallergens and positive NAC with DP. A

detailed description of their clinical features and of those of control individuals (5 AR and 5 HC subjects), together with exclusion criteria, can be found at the online repository and Table S1. The study was conducted according to the principles of the Declaration of Helsinki and approved by the Local Ethics Committee. All study participants signed informed written consent.

2.2 | Nasal allergen challenge protocol

NAC protocol is shown in Figure S1. A solution of commercially available DP extract (100 HEP/mL, LetiPharma, Tres Cantos, Spain) was administered once daily for three days into the right nostril with a hand-driven pump spray delivering a volume of 50 μ L (2 puffs/day). At day 1 (visit 1), patients were trained on how to administer the spray, and the first dose was given at the hospital by the investigators. Subsequent doses were self-administered by patients at home.¹⁰ The clinical effect was monitored daily by Lebel symptom score.³²

2.3 | Collection of nasal biopsies

At visit 1 (before NAC), a mucosal specimen was obtained under topical anesthesia (tetracaine/adrenaline) from the lower turbinate of the left nostril. Thereafter, NAC was performed on the right nostril. A second nasal biopsy was taken from the lower turbinate of the right nostril under topical anesthesia at day 4 (visit 2). The time interval between the last allergen dose and the second biopsy was 24 h.¹⁰ Biopsies were 2–4 mm wide. No biopsy-related complications occurred.

2.4 | Multicolor immunofluorescence staining

Mucosal samples were fixed in formalin overnight and subsequently embedded in paraffin. Multicolor immunostaining was performed as described elsewhere.¹⁰ The monoclonal antibodies (mAbs) used are listed in Table S2A and B. Concentration-matched irrelevant isotype antibodies were used as negative controls.

Tissue sections were examined with a fluorescence microscope blindly by 2 investigators (IEG and ATM) at $\times 40$ magnification. Cells were enumerated by superimposing a grid (250 $\times$ 250 μ m) parallel to the basement membrane of nasal epithelium to determine cell densities in the lamina propria. For illustration purposes and for analysis of CD63 expression pattern, a confocal microscope was used.

2.5 | Gene expression studies

From biopsies ≥ 3 mm, 1-mm piece was obtained for molecular biology experiments. After RNA extraction, ϵ -GLT was detected by PCR using primers homologous to ϵ -specific sequences in I exon and C_H

regions.¹¹ Conversely, semi-nested PCR was used to detect direct and sequential eSCs. To this end, primers homologous to sequences in Iε and CX (sets IεF1/CXR and IεF2/CXR) were utilized.^{16,17} A detailed description of PCR conditions and primers used is found at online repository and Tables S3 and S4.

2.6 | Flow cytometric analysis of peripheral B-cell subpopulations

Peripheral blood mononuclear cells (PBMC) were isolated from blood samples taken at day 1 (baseline sample) and 4 (post-NAC sample). The frequency of plasmablasts (CD19⁺CD20⁺CD38⁺ cells) and PC (CD19⁺CD20⁺CD38⁺) was expressed as proportion in B lymphocytes (CD19⁺ cells). The expression of chemokine receptors (CXCR3 and CXCR4) was expressed as proportion of positive cells in plasmablasts or PC. Results were analyzed by flow cytometry (FacsCanto II, BD Bioscience).

2.7 | Analysis of allergen-specific proliferation of peripheral B-cell subpopulations

Proliferation of PBMCs isolated from post-NAC samples was determined after in vitro stimulation with DP (10 μg in 250 μl of RPMI) for 10 days by using a 5,6-carboxyfluorescein diacetate N-succinimidyl ester (CFSE) dilution assay followed by flow cytometric analysis. Results were expressed as percentage of proliferating cells (CFSE_{low}) and as proliferation index (PI), with ≥2 cutoff being considered indicative of proliferation.³³ Moreover, PBMCs isolated from post-NAC samples were fixed, permeabilized, and stained with mAbs against the distinct immunoglobulin isotypes before being subjected to flow cytometry.³³ Results were expressed as mean fluorescence intensity (MFI) for the corresponding marker. Further details can be found at online repository, and the mAbs used are listed in Table S5.

2.8 | Determination of total and specific immunoglobulin

Culture supernatants of post-NAC PBMCs stimulated with DP were collected after 10 days, and immunoglobulin isotypes were measured by Procarta Plex Multiplex Immunoassays kit (Thermo Fisher Scientific, Waltham, MA). Data were represented as MFI.

To determine antibody specificity, serum DP-sIgE and DP-sIgG were quantified by commercial ImmunoCAP® (Thermo Fisher Scientific, Uppsala, Sweden) in sera from days 1 and 4. DP-sIgE was also measured in nasal secretions by commercial ImmunoCAP®²³ at day 4. Consistent with previous studies,²³ 0.35 and 0.1450 kUA/L were considered as cutoff for serum and nasal sIgE, respectively. Further details can be found at online repository.

2.9 | Basophil activation test

Blood samples collected at day 1 (before NAC) were also used to perform BAT with increasing concentrations of DP (Laboratorios Leti SL) as previously described.⁵ Basophils were identified as CCR3⁺CD203c⁺ cells by flow cytometry, and CD63 was used as activation marker for basophils. Further details can be found at online repository.

2.10 | Statistical analysis

GraphPad Prism software (GraphPad5.0, La Jolla, California) was used for statistical analysis. P values ≤0.05 were considered significant. A detailed description of the analysis is found at online repository.

3 | RESULTS

3.1 | NAC induces eosinophil accumulation and MC activation in the nasal mucosa of LAR patients

NAC induced typical nasal and ocular symptoms in AR and LAR patients, but not in HC (Figure S2A). To investigate the immunological effect of NAC at the local level, we co-stained mucosal biopsies with IgE and FcεRI. MC and monocyte-derived dendritic cells represent the largest populations of FcεRI⁺ cells in the airway mucosa.¹⁰ As expected, the majority of FcεRI⁺ cells also expressed IgE. Moreover, NAC induced an increase in IgE⁺FcεRI⁺ cells in the nasal mucosa of AR and LAR patients, but not in HC individuals. After NAC, AR patients displayed more IgE⁺FcεRI⁺ cells than LAR and HC subjects, while LAR patients had more IgE⁺FcεRI⁺ cells than HC (Figure 1A). We next investigated the activation status of mucosal MC. CD63 expression in resting MC is restricted to intracellular granules, whereas it acquires a membrane pattern after MC activation.³⁴ Although the absolute number of tryptase⁺ MC did not change with the provocation in any study group (not shown), the proportion of membrane CD63⁺tryptase⁺ MC increased in AR and LAR patients after NAC, but not in HC (Figure 1B). Similarly, the number of mucosal eosinophils was higher at baseline in both rhinitis phenotypes than in HC. Moreover, NAC induced a significant increase in mucosal eosinophils in AR and LAR patients but not in HC (Figure S2B).

3.2 | NAC increases mucosal IgE⁺CD38⁺PC/plasmablasts in LAR patients

We next co-stained mucosal biopsies with IgE and CD38, which is primarily expressed by PC and plasmablasts.³⁵ The number of total CD38⁺ PC/plasmablasts did not increase with the provocation or differ among study groups at any time point (Figure S3A). Conversely,

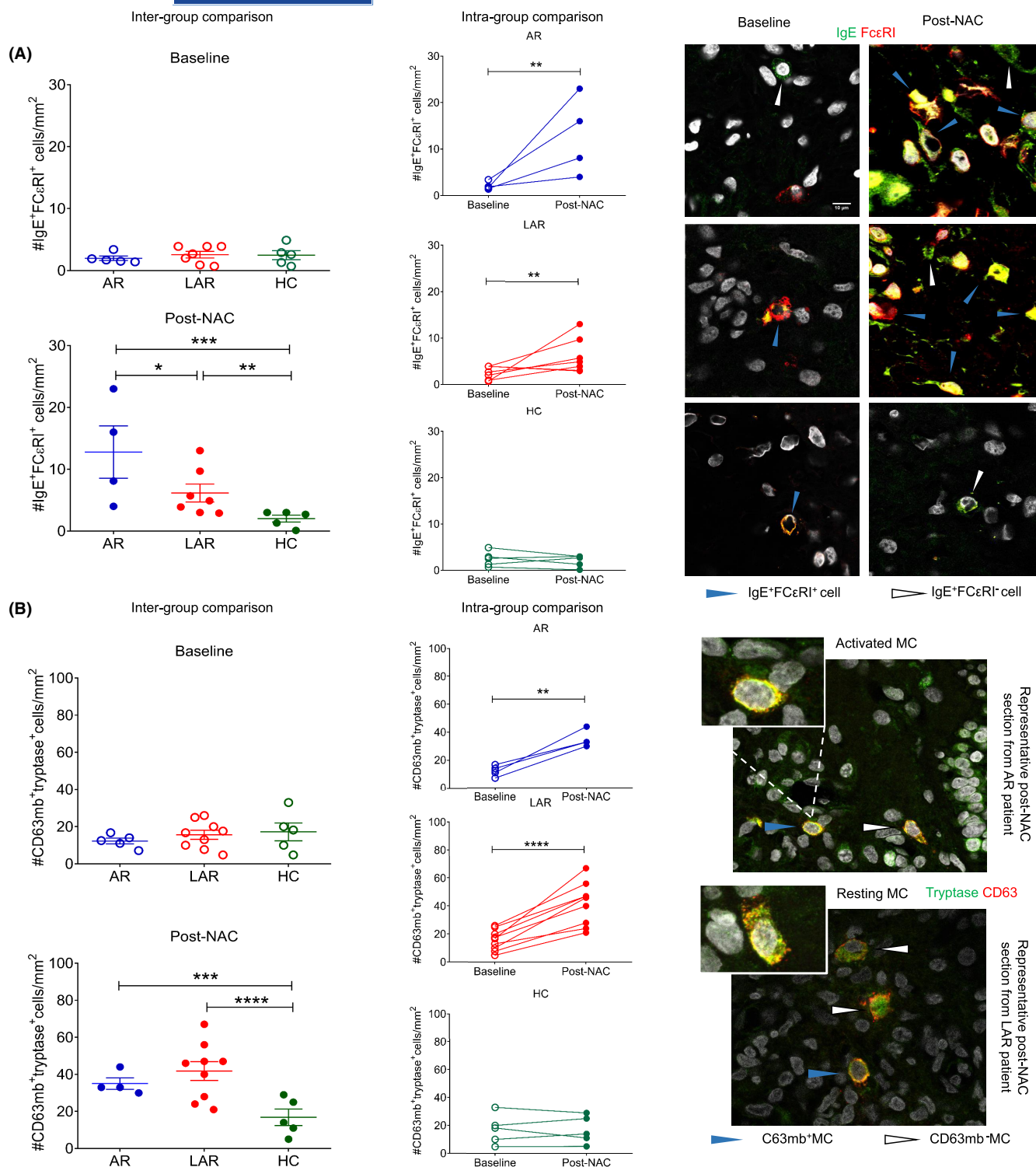


FIGURE 1 Nasal allergen challenge induces the increase in IgE⁺FcεRI⁺ cells and mast cell activation in the nasal mucosa of allergic rhinitis and local allergic rhinitis patients. (A) inter- and intra-group comparison of the density of IgE⁺FcεRI⁺ cells in the nasal mucosa before and after provocation. Blue arrows mark IgE⁺FcεRI⁺ cells, whereas white arrows mark IgE⁺FcεRI⁻ cells. (B) inter- and intra-group comparison of the proportion of activated (membrane expression pattern for CD63) mast cells in the nasal mucosa before and after provocation. Blue arrows mark CD63mb⁺tryptase⁺ cells, whereas white arrows mark CD63mb⁻tryptase⁺ cells. AR, allergic rhinitis; HC, non-atopic healthy control; LAR, local allergic rhinitis; MC, mast cell; NAC, nasal allergen challenge; CD63mb, membrane expression pattern for CD63. * $p < .05$; ** $p < .01$; *** $p < .001$

AR patients had more mucosal IgE⁺CD38⁺ PC/plasmablasts than LAR and HC individuals at baseline. Moreover, the provocation induced a significant increase in IgE⁺CD38⁺ PC/plasmablasts in LAR patients, but not in AR or HC subjects (Figure 2). Therefore, after NAC, the number of mucosal IgE⁺CD38⁺ PC/plasmablasts was comparable between the two rhinitis phenotypes and higher than in HC.

3.3 | LAR patients display markers of sequential ϵ CSR in the nasal mucosa

The accumulation of IgE⁺CD38⁺ PC/plasmablasts in LAR patients could be related to the maturation of directly switched IgE⁺ cells or to the sequential ϵ CSR of memory B cells. Therefore, we co-stained the mucosal samples with anti-IgE and CD20 antibodies. Importantly, CD20 is a marker expressed by B cells and plasmablasts, but not by PC.³⁵ Consistent with previous works,³⁶ we were not able to identify any IgE⁺CD20⁺ cell in any study subject (Figure S3D). Conversely, the number of mucosal CD20⁺ cells was higher in both rhinitis phenotypes as compared to HC after the provocation (Figure S3B). To investigate whether sequential ϵ CSR can occur in the nasal mucosa of LAR individuals, we performed gene expression studies using nasal biopsies. Because immunofluorescence was prioritized, only 11 pre-NAC (3 LAR, 5 AR, and 3 HC) and 11 post-NAC (8 LAR

and 3 HC) samples were available. Of note, ϵ -GLT was expressed in 3 of 8 post-NAC and in 1 of 5 pre-NAC mucosal specimens from LAR and AR patients, respectively, and in none from HC individuals (Figure 3A). We next investigated the expression of direct and sequential ϵ SCs in the nasal mucosa. Direct ϵ SCs were not observed in any sample (not shown), whereas sequential ϵ SC was detected in 2 of 11 pre-NAC (1 AR and 1 LAR) and 2 of 11 post-NAC (both LAR) samples (Figure 3B). Importantly, no ϵ SC was observed in HC at any time point. On the contrary, we were not able to establish reliable protocols for the identification of AID expression at either the gene or protein levels.

3.4 | NAC increases the proportion of peripheral CD19⁺CD20⁺CD38⁺ plasmablasts in LAR patients

Peripheral B-cell subpopulations were gated as shown in Figure S4. NAC induced a significant increase in the proportion of peripheral CD19⁺CD20⁺CD38⁺ plasmablasts and CD19⁺CD20⁻CD38⁺ PC in both rhinitis phenotypes but not in HC (Figure 4A). Therefore, we investigated the expression pattern of mucosal homing receptors on these cells.¹³ After NAC, the median of CXCR3⁺CD19⁺CD20⁺CD38⁺ plasmablasts from AR, LAR, and HC individuals was 7%, 5%, and 0.5%, respectively, although the proportions were significantly

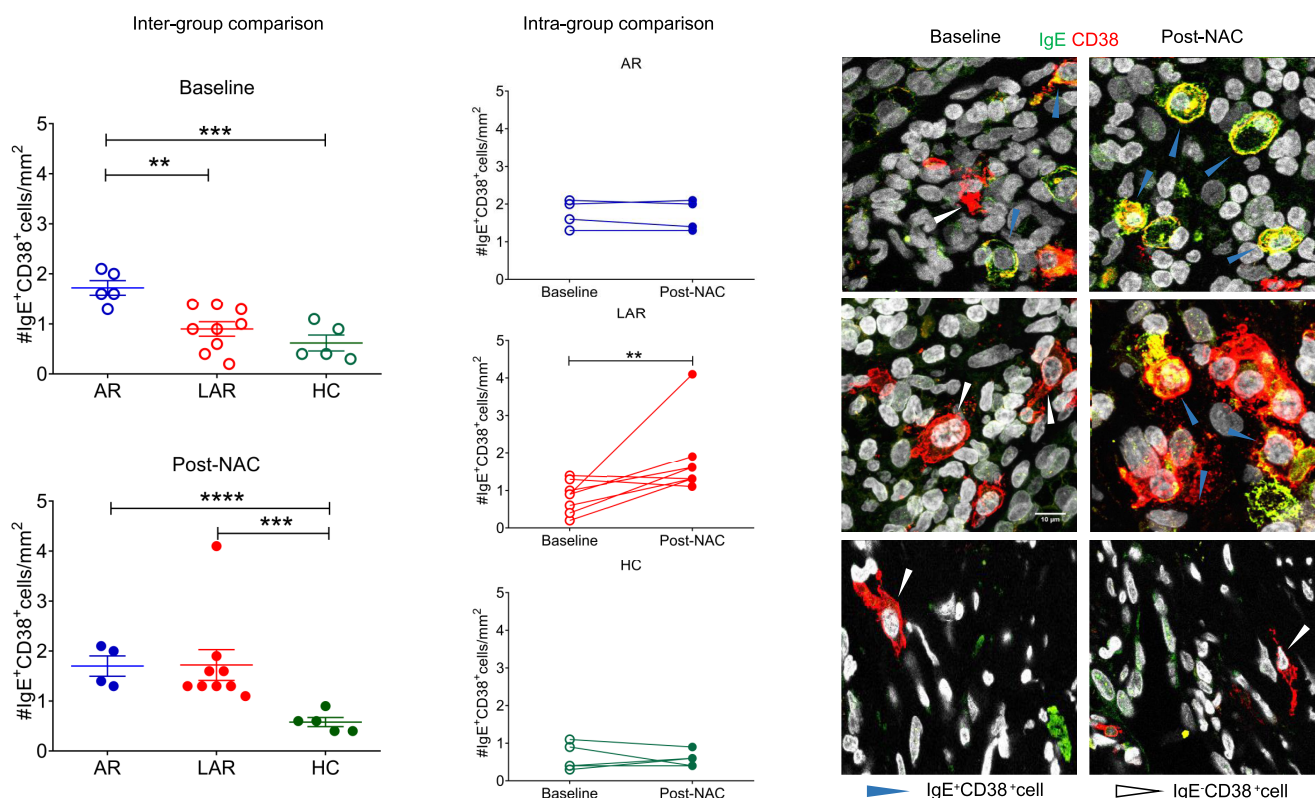


FIGURE 2 Nasal allergen challenge induces the increase in IgE⁺CD38⁺ plasmablasts/plasma cells in the nasal mucosa of allergic rhinitis and local allergic rhinitis patients. Inter- and intra-group comparison of the density of IgE⁺CD38⁺ cells in the nasal mucosa before and after provocation. Blue arrows mark IgE⁺CD38⁺ cells, whereas white arrows mark IgE⁻CD38⁺ cells. AR, allergic rhinitis; HC, non-atopic healthy control; LAR, local allergic rhinitis; NAC, nasal allergen challenge. **p* < .05; ***p* < .01; ****p* < .001

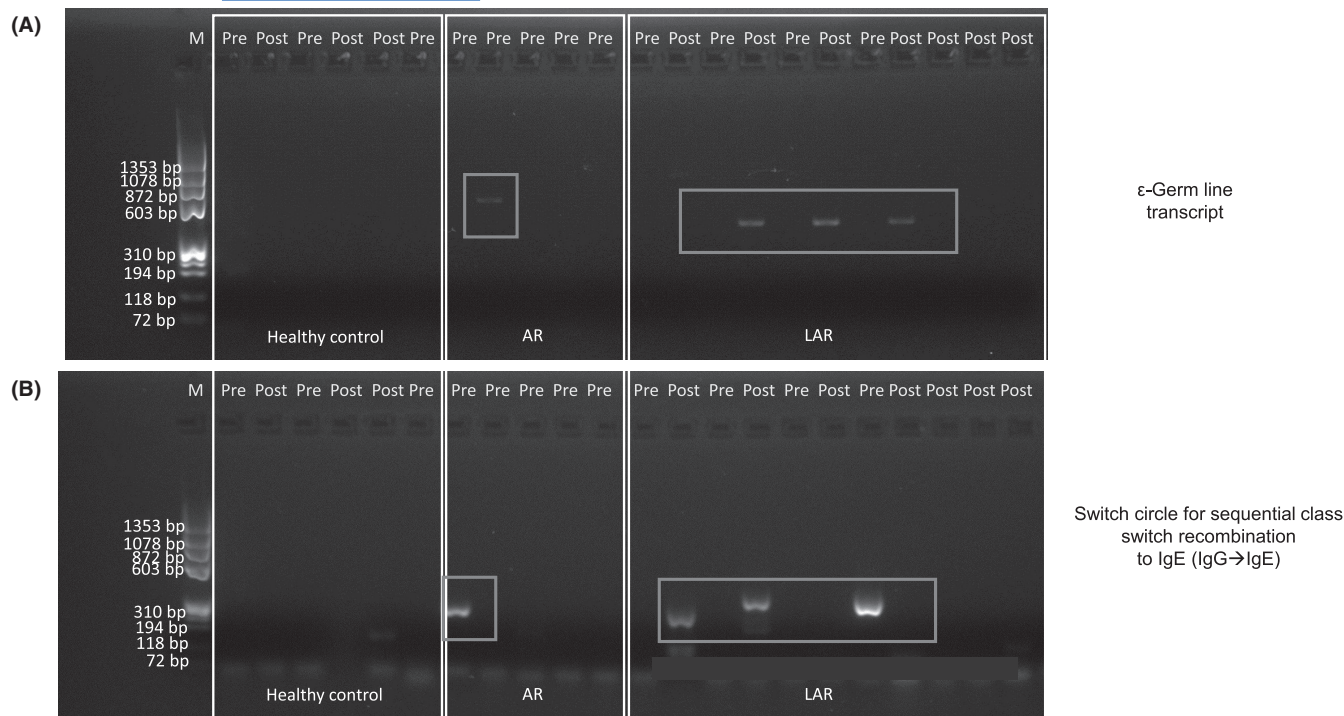


FIGURE 3 Sequential class switch recombination to IgE occurs in the nasal mucosa of local allergic rhinitis patients. (A) expression of cDNA for ϵ -germ line transcript in nasal biopsies taken from study individuals (size: 379 bp approx.). (B) expression of cDNA for sequential ϵ switch circle in nasal biopsies taken from study individuals (size: 339 bp approx.). Top rows in figures A and B refer to the time point when the sample was collected (pre- or post-challenge). PCRs products were fractionated and displayed in 2% agarose gel. Position 1: 1 μ l Marker Pm IX (Roche®). AR, allergic rhinitis; LAR, local allergic rhinitis; M, markers for molecular weight

different between AR and HC subjects only. Similarly, peripheral CD19⁺CD20⁻CD38⁺ PC from AR and LAR patients expressed significantly more CXCR4 than HC subjects (medians of 4%, 10%, and 1%, respectively) (Figure 4B).

It has been proposed that both IgG⁺ and IgA⁺ B-cell subpopulations in humans are able to migrate to the nasal mucosa where they can undergo sequential ϵ CSR.¹⁷ However, we did not identify any IgA⁺ CD20⁺ cells at baseline or after the provocation in any study group (Figure S3E). Similarly, we did not find differences in the number of mucosal IgA⁺CD38⁺ cells at either the inter-group or intra-group comparisons (Figure S3C and F). Unfortunately, due to their ubiquity, we were not able to establish reliable immunohistochemistry protocols with either IgG or IgM.

3.5 | In vitro allergen stimulation induces peripheral IgG⁺CD19⁺CD20⁺CD38⁺ plasmablast proliferation in LAR patients

We next investigated the effect of in vitro allergen stimulation on peripheral B-cell subpopulations isolated from post-NAC samples. CFSE assay revealed that blood CD19⁺ cells from AR and LAR patients, but not from HC, proliferated after in vitro DP stimulation (Figure 4C). The flow cytometric analysis showed that the proportion of proliferating CD19⁺CD20⁺CD38⁺ plasmablasts in peripheral blood increased significantly in AR and LAR patients but

not in HC, after DP stimulation. Same trend was observed for CD19⁺CD20⁻CD38⁺ PC, although the changes were not significant in LAR subjects (Figure 4D).

Thereafter, we analyzed immunoglobulin isotypes in culture supernatants from DP-stimulated PBMCs. MFI for IgE was higher in LAR and AR patients than in HC after allergen stimulation, although the differences were significant between LAR and HC subjects only. Conversely, both AR and LAR individuals showed a significantly increased MFI for IgG as compared to HC. No significant changes were observed for IgA or IgM (not shown). Importantly, IgE was the most abundant isotype in culture supernatants from AR patients after allergen stimulation, whereas in LAR individuals comparable amounts of IgG and IgE were observed (Figure 5A).

To investigate the cell source of IgG and IgE, we next analyzed the effect of in vitro allergen stimulation on the immunoglobulin isotype synthesized by B-cell subpopulations. The allergen did not induce any significant change in the MFI for IgE or IgG of CD19⁺CD20⁺CD38⁺ plasmablasts in any study group, although MFI was significantly higher for both isotypes in AR and LAR patients, as compared to HC. Moreover, a preferential expression of IgG and IgE was observed for CD19⁺CD20⁺CD38⁺ plasmablasts from LAR and AR patients, respectively. Concerning CD19⁺CD20⁻CD38⁺ PC, the MFI for IgE was higher in AR patients than in LAR and HC individuals after allergen stimulation, whereas MFI for IgE was increased in LAR and AR subjects as compared to HC (Figure 5B).

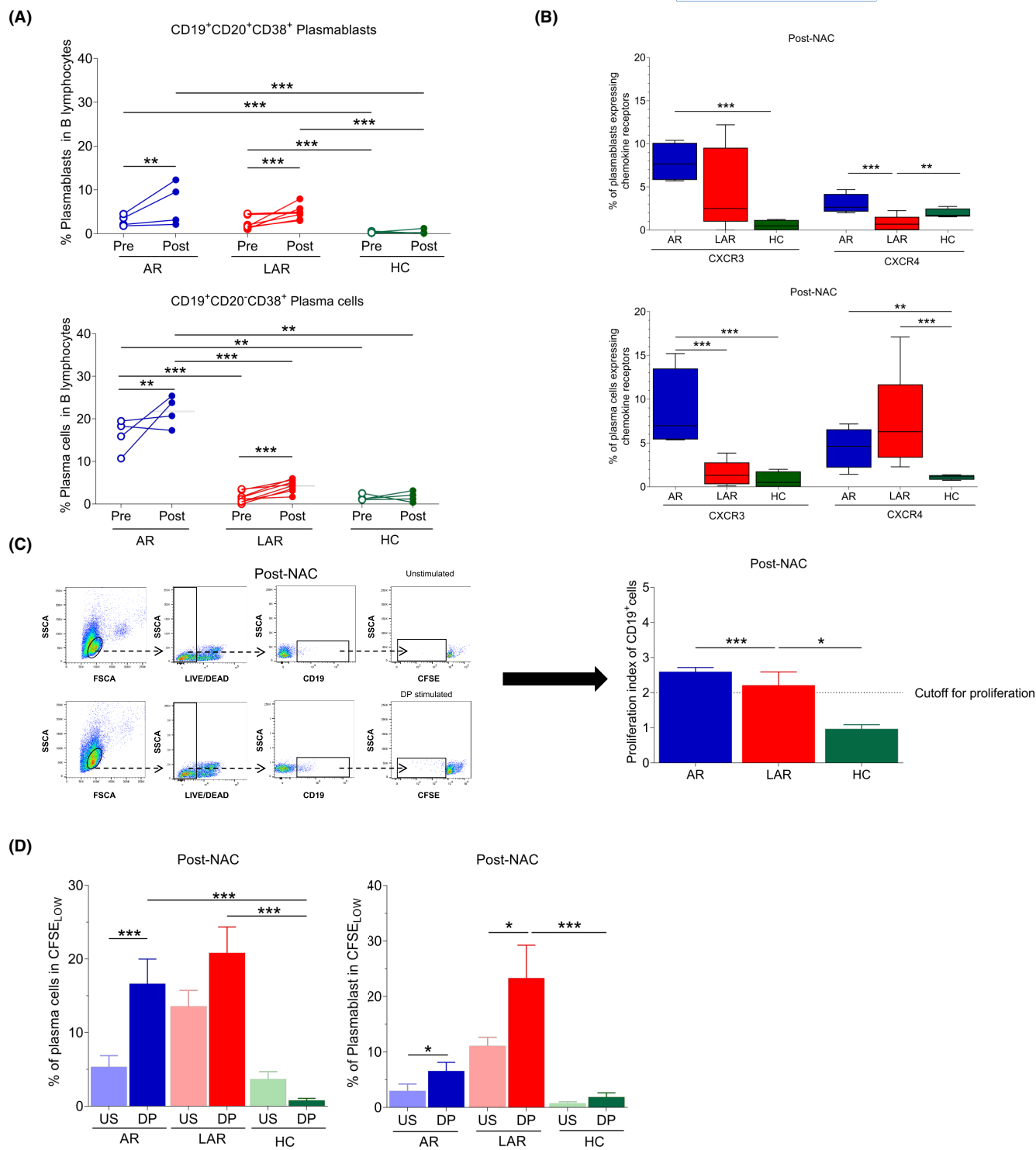


FIGURE 4 Nasal allergen challenge increases the proportion of peripheral CD19⁺CD20⁺CD38⁺ plasmablasts, and in vitro allergen stimulation induces the proliferation of peripheral CD19⁺CD20⁺CD38⁺ plasmablasts in local allergic rhinitis patients. (A) proportion of plasmablasts and plasma cells in peripheral B lymphocytes before and after the provocation. (B) proportion of peripheral plasmablasts and plasma cells expressing mucosal homing chemokine receptors after the provocation. (C) gating strategy for flow cytometry and proliferation index of CD19⁺ cells after in vitro allergen stimulation of peripheral blood mononuclear cells (PBMC) isolated from the blood sample taken after the provocation. The dotted line represents the cutoff indicative of proliferation. (D) proportion of proliferating (CFSE_{Low}) plasmablasts and plasma cells before and after in vitro allergen stimulation among PBMCs isolated from the blood sample taken after the provocation. AR, allergic rhinitis; DP, *Dermatophagoides pteronyssinus*; HC, healthy non-atopic control; LAR, local allergic rhinitis; US, unstimulated. **p* < .05; ***p* < .01; ****p* < .001

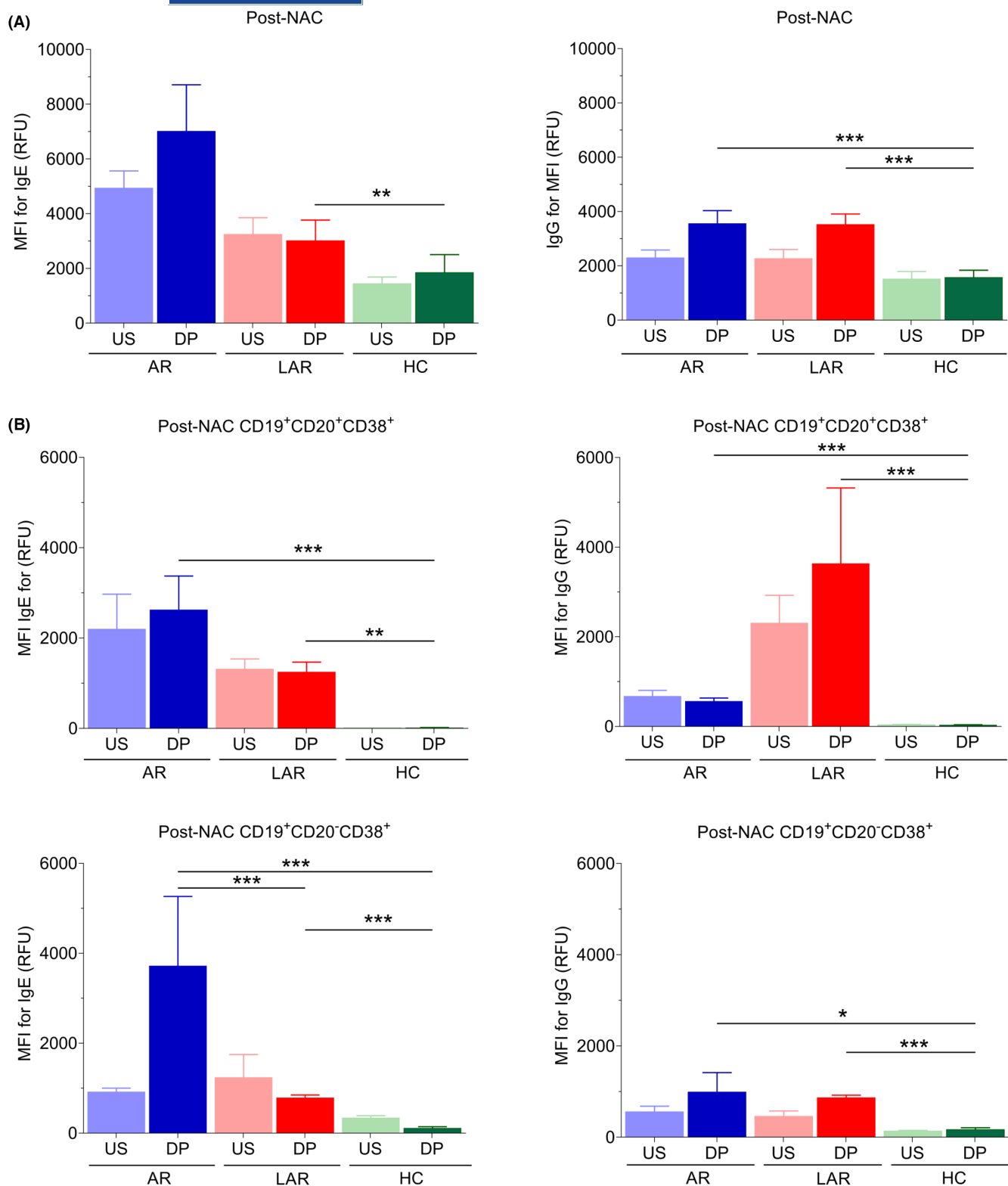


FIGURE 5 Peripheral plasmablasts from local allergic rhinitis patients preferentially express IgG after in vitro allergen stimulation. (A) MFI for IgG and IgE before and after in vitro allergen stimulation as analyzed by immunoassay in culture supernatants of peripheral blood mononuclear cells isolated from the blood sample taken after the nasal provocation. (B) mean fluorescence intensity (MFI) for IgG and IgE before and after in vitro allergen stimulation as measured by flow cytometry in peripheral plasmablasts and plasma cells (blood sample taken after the nasal provocation); AR, allergic rhinitis; DP, *Dermatophagoides pteronyssinus*; HC, healthy non-atopic control; LAR, local allergic rhinitis; RFU, relative fluorescence unit; US, unstimulated. * $p < .05$; ** $p < .01$; *** $p < .001$

3.6 | LAR patients display allergen-specific IgE bound to the membrane of peripheral basophils

After provocation, 2 of 5 (40%) AR, 3 of 9 (33%) LAR, and no (0%) HC individual displayed detectable DP-sIgE in nasal secretions (Figure 6A). Because IgE synthesized at the mucosal level might

travel to the bloodstream through the lymphatic vessels,⁵ we next investigated the presence of DP-sIgE bound to the membrane of peripheral basophils by means of BAT. Considering subjects with responder basophils, 5 of 5 (100%), 3 of 8 (38%) and 0 of 5 (0%) AR, LAR and HC individuals displayed positive BAT results, respectively. Moreover, experiments with the PI3K inhibitor wortmannin²⁵

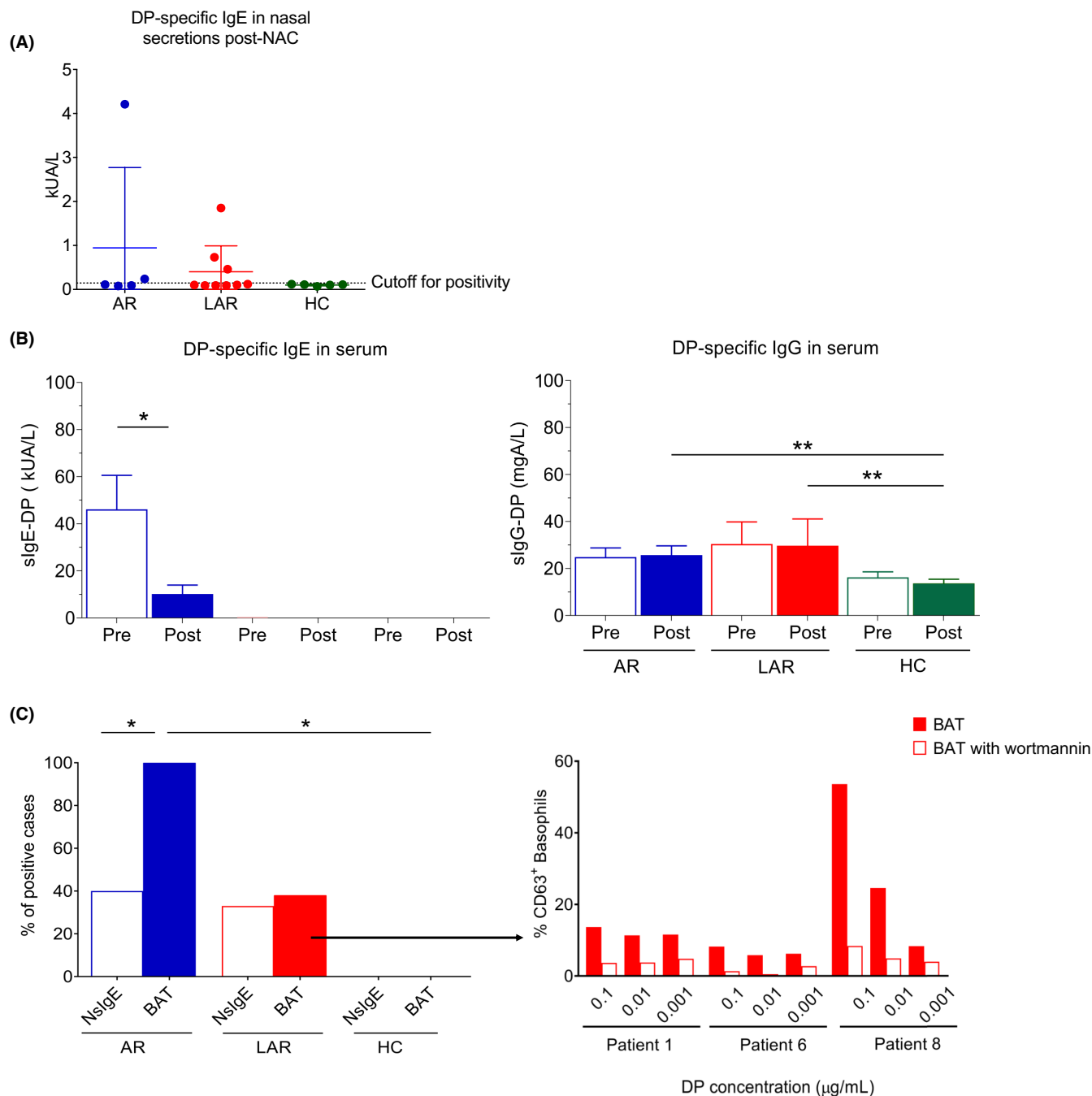


FIGURE 6 Local allergic rhinitis patients display allergen-specific IgG in serum and allergen-specific IgE attached to the membrane of peripheral basophils and in nasal secretions. (A) *Dermatophagoides pteronyssinus* (DP)-specific IgE (DP-sIgE) in the nasal secretions after the provocation. The dotted line represents the cutoff for positivity; (B) DP-sIgE and DP-sIgG in serum before and after the provocation. (C) proportion of study individuals with positive basophil activation test (BAT) and wortmannin experiments in the three local allergic rhinitis patients showing positive BAT results. AR, allergic rhinitis; HC, non-atopic healthy control; LAR, local allergic rhinitis. * $p < .05$; ** $p < .01$; *** $p < .001$

demonstrated that basophil activation in LAR patients was mediated by DP-sIgE (Figure 6B). Finally, we also measured DP-specific antibodies in sera. NAC decreased the level of serum DP-sIgE in AR patients, and DP-sIgE remained undetectable in sera from LAR and HC individuals. Conversely, serum DP-sIgG was significantly higher in both AR and LAR patients after NAC, as compared to HC (Figure 6C). NAC did not modify the level of DP-sIgG4 in any study group (Figure S5).

4 | DISCUSSION

In this study, we show that allergen exposure induces significant inflammatory events in LAR patients. On the one hand, a 3-day NAC is associated with an increase in IgE⁺FcεRI⁺ cells in the nasal mucosa of AR and LAR patients. Because the total number of MC did not increase, the recruitment of monocytes and basophils probably accounts for this accumulation. Of note, monocytes acquire FcεRI expression during allergic inflammation and are massively recruited shortly after allergen re-exposure in AR individuals.¹⁰ We also observed significant MC activation and eosinophil accumulation following NAC in both rhinitis phenotypes, which is in line with previous studies showing an increase in nasal tryptase and ECP following positive NAC.^{7,8} Importantly, only allergic patients, regardless of their atopic status, experienced typical rhinitis symptoms which suggests that the inflammatory response is allergen-specific.

A significant increase in mucosal IgE⁺CD38⁺ PC/plasmablasts was observed in LAR patients after NAC, but not in AR subjects. This change resulted in a comparable number of nasal IgE⁺CD38⁺ PC/plasmablasts in both rhinitis phenotypes after NAC. Of note, previous studies show that AR patients react on average to lower allergen concentrations than LAR individuals.²² This finding could be related to a greater baseline infiltration of the nasal mucosa in the former group.

The increase in IgE⁺CD38⁺ PC/plasmablasts in the nasal mucosa of LAR individuals can arise from the maturation of directly switched IgE⁺ cells or from the sequential εCSR of IgG⁺ or IgA⁺ cells. Nevertheless, the absence of mucosal IgE⁺CD20⁺ cells together with the detection of sequential εSCs (IgM→IgG→IgE) makes the second scenario more probable. Interestingly, the local expression of εSCs and ε-GLT and the allergen-induced increase in IgE⁺CD38⁺ PC indicate that the nasal mucosa of LAR patients is an active site for IgE production. Although we only found εSCs in 3 of 9 LAR patients, this proportion is in line with previous works using nasal biopsies to investigate IgE synthesis in AR patients.^{11,16,17} Sequential εSCs were detected in both pre- and post-challenge samples. Despite significant seasonal variations, DP is detected yearlong in Malaga, and therefore, we cannot exclude that some patients were exposed to DP concentrations at baseline high enough to induce mucosal εCSR. Of note, sequential εSCs were reported in the nasal mucosa of AR patients both in- and out-season.¹⁶ In any case, this is the first study demonstrating that, similar to AR individuals, LAR patients can undergo sequential εCSR at the mucosal level. On the other hand,

we did not detect direct εSC in any study group. Although direct εSC were identified in AR explants after in vitro allergen stimulation, markers of direct εCSR were not found in nasal biopsies taken from AR patients in- or out-season.¹⁶ Similarly, future studies should investigate the occurrence of sequential switching from IgA1 (IgM→IgA1→IgE) in both AR and LAR phenotypes.

Interestingly, allergic patients, but not HC, experienced an allergen-specific response at the peripheral level both in vivo and in vitro, as illustrated by the increased proportion of CD19⁺CD20⁺CD38⁺ plasmablasts and CD19⁺CD20⁺CD38⁺ PC, and the significant proliferation of CD19⁺ cells, respectively. Moreover, a proportion of peripheral CD19⁺CD20⁺CD38⁺ plasmablasts from LAR individuals expressed homing receptors for the respiratory tract (especially CXCR3), which suggests that they are able to migrate to the nasal mucosa. Of note, after NAC, the preferential isotype of peripheral CD19⁺CD20⁺CD38⁺ plasmablasts from LAR patients was IgG. This finding, together with the lack of effect of NAC on the number of mucosal IgA⁺CD20⁺ cells, suggests that the accumulation of IgE⁺CD38⁺ PC/plasmablasts in the nasal mucosa of LAR patients derives from the sequential εCSR of IgG⁺ memory B cells/plasmablasts recruited to the nasal mucosa upon allergen exposure. This interpretation is supported by the high title of serum DP-sIgG observed in LAR individuals. In this regard, it is tempting to speculate that in LAR patients, allergen exposure induces the migration of peripheral DP-specific IgG-switched memory B cells/plasmablasts to the nasal mucosa, where they undergo sequential εCSR to give rise to PC releasing high-affinity DP-sIgE at the local level. Moreover, the presence of DP-sIgE attached to the membrane of peripheral basophils suggests that in some LAR patients DP-sIgE is enough to saturate the FcεRI receptor system of the mucosa and to traffic to the bloodstream through the lymphoid vessels. The high affinity of IgE for FcεRI ($K_d = 10^{-10}$)³⁷ would explain why DP-sIgE is not found at the free state in serum before the saturation of the whole FcεRI receptor system of the organism. Because bloodstream and nasal secretions are ultimately connected through the lymphoid vessels, this aspect would also account for the low proportion (33%) of LAR patients with detectable DP-sIgE in nasal secretions and its low values. On the contrary, we only detected nasal DP-sIgE in 40% of AR patients. This aspect is probably related to the low sample size ($n = 5$), since a study using the same methodology and inclusion criteria but evaluating a larger sample size found nasal DP-sIgE in 75% of AR patients.²³ Same rationale applies to the lower proportion of BAT-positive LAR patients as compared to previous studies,²⁵⁻²⁷ especially considering that the basophils of 1 of 9 LAR individuals were non-responder. In any case, our results suggest that the different status of the classical atopy biomarkers (SPT and serum sIgE) between AR and LAR phenotypes derives from the amount of sIgE that is left after the saturation of the FcεRI receptor system of the nasal mucosa.

Our study is limited by the small sample size and short in vivo provocation protocol (3 days). Nevertheless, the collection of nasal biopsies and the sustained administration of the allergen to allergic patients pose tolerability issues. This provocation period was

chosen as a compromise between patients' comfort and the certainty of providing the immune system with sufficient time to activate IgE synthesis.²¹ Conversely, PBMC were stimulated in vitro with DP for 10 days. This longer in vitro stimulation period was deemed necessary by previous studies investigating specific proliferative responses of peripheral lymphocytes obtained from AR patients.^{33,38} Moreover, we were not able to investigate the specificity of the antibodies bound to the membrane of mucosal cells. Further studies should consider using frozen biopsies to perform direct immune-staining with fluorescently labeled allergens.²⁰ On the contrary, our work is the first study investigating inflammatory events in mucosal biopsies taken from a group of well-phenotyped LAR patients.

In summary, our study shows that sequential ϵ CSR and IgE synthesis can occur in the nasal mucosa of LAR patients, as previously demonstrated for AR individuals.^{11,16,17} Moreover, allergen exposure induces the accumulation of mucosal IgE⁺ PC/plasmablasts and the proliferation of peripheral PC/plasmablasts, thus suggesting an allergen-specific effect. Finally, LAR patients show high titres of serum sIgG and can display sIgE bound to the membrane of peripheral basophils. Collectively, our results indicate that LAR is an IgE-mediated condition, a conclusion consistent with the clinical benefit associated with allergen immunotherapy in these patients.²⁸⁻³⁰ Further translational and clinical studies are warranted to elucidate whether LAR should become a new indication for allergen immunotherapy.

ACKNOWLEDGEMENTS

The present study has been performed in collaboration with the ICTS "NANBIOSIS", specifically with U28 Unit of the Andalusian Centre for Nanomedicine & Biotechnology (BIONAND). The authors would like to thank John Pearson (BIONAND Nanoimaging Unit) for his help with the confocal microscopy analysis, Veronica Prados and Ana Molina for their support with flow cytometric analysis, Carolina Lobo (SCAI-Central Research Support Services-Malaga University) for her assistance with the Bio-Plex analyzer, and the Biobank of IBIMA at Hospital Regional Universitario de Malaga for the storage of the samples. Finally, the authors also want to thank Philipp Starkl (University of Vienna) for his advice on mast cell biology.

CONFLICT OF INTEREST

The authors declare no conflict of interest in relation to this work.

AUTHOR CONTRIBUTIONS

CM, PC, MJT, CR, and IEG designed the study. ATM, MS, PC, MJT, CR, and IEG evaluated and recruited the study individuals. JV obtained the nasal biopsies. ATM, AA, and IEG performed the immunohistochemistry experiments, FP carried out the analysis of peripheral B-cell subpopulations and antibodies, RJE conducted the gene expression studies and the measurement of serum antibodies, and RFS performed the basophil activation test and measurement of nasal antibodies. CM, MJT, CR, and IEG supervised the work of

all the authors. AA, CM, MJT, CR, and IEG prepared the manuscript, which was reviewed by the rest of the authors.

ORCID

Ruben Fernandez-Santamaria  <https://orcid.org/0000-0002-0255-4280>

Maria Salas  <https://orcid.org/0000-0002-0583-9492>

Maria Jose Torres  <https://orcid.org/0000-0001-5228-471X>

Carmen Rondon  <https://orcid.org/0000-0003-0976-3402>

Ibon Eguiluz-Gracia  <https://orcid.org/0000-0002-3774-931X>

REFERENCES

- Eguiluz-Gracia I, Pérez-Sánchez N, Bogas G, Campo P, Rondón C. How to diagnose and treat local allergic rhinitis: a challenge for clinicians. *J Clin Med*. 2019;8(7):1062.
- Hamizan AW, Rimmer J, Alvarado R, et al. Positive allergen reaction in allergic and nonallergic rhinitis: a systematic review. *Int Forum Allergy Rhinol*. 2017;7(9):868-877.
- Rondon C, Campo P, Eguiluz-Gracia I, et al. Local allergic rhinitis is an independent rhinitis phenotype: the results of a 10-year follow-up study. *Allergy*. 2018;73(2):470-478.
- Campo P, Eguiluz-Gracia I, Plaza-Serón MC, et al. Bronchial asthma triggered by house dust mites in patients with local allergic rhinitis. *Allergy*. 2019;74(8):1502-1510.
- Eguiluz-Gracia I, Fernandez-Santamaria R, Testera-Montes A, et al. Coexistence of nasal reactivity to allergens with and without IgE sensitization in patients with allergic rhinitis. *Allergy*. 2020;75(7):1689-1698.
- Prieto A, Rondón C, Eguiluz-Gracia I, et al. Systematic valuation of allergic phenotypes of rhinitis in children and adolescents. *Pediatr Allergy Immunol*. 2021;32(5):953-962.
- Rondón C, Doña I, López S, et al. Seasonal idiopathic rhinitis with local inflammatory response and specific IgE in absence of systemic response. *Allergy*. 2008;63(10):1352-1358.
- López S, Rondón C, Torres MJ, et al. Immediate and dual response to nasal challenge with *Dermatophagoides pteronyssinus* in local allergic rhinitis. *Clin Exp Allergy*. 2010;40(7):1007-1014.
- Powe DG, Huskisson RS, Carney AS, Jenkins D, Jones NS. Evidence for an inflammatory pathophysiology in idiopathic rhinitis. *Clin Exp Allergy*. 2001;31(6):864-872.
- Eguiluz-Gracia I, Bosco A, Dollner R, et al. Rapid recruitment of CD14(+) monocytes in experimentally induced allergic rhinitis in human subjects. *J Allergy Clin Immunol*. 2016;137(6):1872-1881.
- Takhar P, Smurthwaite L, Coker HA, et al. Allergen drives class switching to IgE in the nasal mucosa in allergic rhinitis. *J Immunol*. 2005;174(8):5024-5032.
- Xiong H, Dolpady J, Wabl M, Curotto de Lafaille MA, Lafaille JJ. Sequential class switching is required for the generation of high affinity IgE antibodies. *J Exp Med*. 2012;209(2):353-364.
- Jeyanathan M, Afkhami S, Khera A, et al. CXCR3 signaling is required for restricted homing of parenteral tuberculosis vaccine-induced T cells to both the lung parenchyma and airway. *J Immunol*. 2017;199(7):2555-2569.
- He JS, Meyer-Hermann M, Xiangying D, et al. The distinctive germinal center phase of IgE+ B lymphocytes limits their contribution to the classical memory response. *J Exp Med*. 2013;210(12):2755-2771.
- Talay O, Yan D, Brightbill HD, et al. IgE+ memory B cells and plasma cells generated through a germinal-center pathway. *Nat Immunol*. 2012;13(4):396-404.
- Cameron L, Gounni AS, Frenkiel S, Lavigne F, Vercelli D, Hamid Q. S epsilon S mu and S epsilon S gamma switch circles in human nasal mucosa following ex vivo allergen challenge: evidence for

- direct as well as sequential class switch recombination. *J Immunol.* 2003;171(7):3816-3822.
17. Coker HA, Durham SR, Gould HJ. Local somatic hypermutation and class switch recombination in the nasal mucosa of allergic rhinitis patients. *J Immunol.* 2003;171(10):5602-5610.
 18. Eckl-Dorna J, Pree I, Reisinger J, et al. The majority of allergen-specific IgE in the blood of allergic patients does not originate from blood-derived B cells or plasma cells. *Clin Exp Allergy.* 2012;42(9):1347-1355.
 19. Wu YC, James LK, Vander Heiden JA, et al. Influence of seasonal exposure to grass pollen on local and peripheral blood IgE repertoires in patients with allergic rhinitis. *J Allergy Clin Immunol.* 2014;134(3):604-612.
 20. KleinJan A, Vinke JG, Severijnen LW, Fokkens WJ. Local production and detection of (specific) IgE in nasal B-cells and plasma cells of allergic rhinitis patients. *Eur Respir J.* 2000;15(3):491-497.
 21. Rondón C, Romero JJ, López S, et al. Local IgE production and positive nasal provocation test in patients with persistent non allergic rhinitis. *J Allergy Clin Immunol.* 2007;119(4):899-905.
 22. Rondón C, Fernández J, López S, et al. Nasal inflammatory mediators and specific IgE production after nasal challenge with grass pollen in local allergic rhinitis. *J Allergy Clin Immunol.* 2009;124(5):1005-1011.
 23. Campo P, Del Carmen P-S, Eguiluz-Gracia I, et al. Direct intranasal application of the solid phase of ImmunoCAP® increases nasal specific immunoglobulin E detection in local allergic rhinitis patients. *Int Forum Allergy Rhinol.* 2018;8(1):15-19.
 24. Meng Y, Wang Y, Lou H, et al. Specific immunoglobulin E in nasal secretions for the diagnosis of local allergic rhinitis. *Rhinology.* 2019;57(4):313-320.
 25. Gómez E, Campo P, Rondón C, et al. Role of the basophil activation test in the diagnosis of local allergic rhinitis. *J Allergy Clin Immunol.* 2013;132(4):975-976.
 26. Campo P, Villalba M, Barrionuevo E, et al. Immunologic responses to the major allergen of *Olea Europaea* in local and systemic allergic rhinitis subjects. *Clin Exp Allergy.* 2015;45(11):1703-1712.
 27. Duarte Ferreira R, Ornelas C, Silva S, et al. Contribution of In Vivo and In Vitro Testing for The Diagnosis of Local Allergic Rhinitis. *J Investig Allergol Clin Immunol.* 2019;29(1):46-48.
 28. Rondón C, Campo P, Salas M, et al. Efficacy and safety of D. pteronyssinus immunotherapy in local allergic rhinitis: a double-blind placebo-controlled clinical trial. *Allergy.* 2016;71(7):1057-1061.
 29. Rondón C, Blanca-López N, Campo P, et al. Specific immunotherapy in local allergic rhinitis: A randomized, double-blind placebo-controlled trial with *Phleum pratense* subcutaneous allergen immunotherapy. *Allergy.* 2018;73(4):905-915.
 30. Bożek A, Kołodziejczyk K, Jarzab J. Efficacy and safety of birch pollen immunotherapy for local allergic rhinitis. *Ann Allergy Asthma Immunol.* 2018;120(1):53-58.
 31. Bousquet J, Khaltaev N, Cruz AA, et al. Allergic rhinitis and its impact on asthma (ARIA) 2008*. *Allergy.* 2008;63:8-160.
 32. Lebel B, Bousquet J, Morel A, Chanal I, Godard P, Michel FB. Correlation between symptoms and the threshold for release of mediators in nasal secretions during nasal challenge with grass-pollen grains. *J Allergy Clin Immunol.* 1988;82(5 Pt 1):869-877.
 33. Gonzalez M, Doña I, Palomares F, et al. Dermatophagoides pteronyssinus immunotherapy changes the T-regulatory cell activity. *Sci Rep.* 2017;7(1):11949.
 34. Kraft S, Jouvin MH, Kulkarni N, et al. The tetraspanin CD63 is required for efficient IgE-mediated mast cell degranulation and anaphylaxis. *J Immunol.* 2013;191(6):2871-2878.
 35. Fernández TD, Gómez E, Doña I, et al. Differential Plasma-cell evolution is linked with Dermatophagoides pteronyssinus immunotherapy response. *Sci Rep.* 2015;5(1):14482.
 36. Gevaert P, Nouri-Aria KT, Wu H, et al. Local receptor revision and class switching to IgE in chronic rhinosinuitis with nasal polyps. *Allergy.* 2013;68(1):55-63.
 37. Kraft S, Kinet JP. New developments in FcεRI regulation, function and inhibition. *Nat Rev Immunol.* 2007;7(5):365-378.
 38. Gómez E, Fernández TD, Doña I, et al. Initial immunological changes as predictors for house dust mite immunotherapy response. *Clin Exp Allergy.* 2015;45(10):1542-1553.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Testera-Montes A, Palomares F, Jurado-Escobar R, et al. Sequential class switch recombination to IgE and allergen-induced accumulation of IgE⁺ plasmablasts occur in the nasal mucosa of local allergic rhinitis patients. *Allergy.* 2022;77:2712-2724. doi:[10.1111/all.15292](https://doi.org/10.1111/all.15292)