Dupilumab treatment decreases MBC2s, correlating with reduced IgE levels in pediatric atopic dermatitis

Brief report

Dupilumab treatment decreases MBC2s, correlating with reduced IgE levels in pediatric atopic dermatitis

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Background: A preference for type 2 immunity plays a central role in the pathogenesis of atopic dermatitis (AD). Dupilumab, an mAb targeting the IL-4 receptor α (IL-4Rα) subunit, inhibits IL-4 and IL-13 signaling. These cytokines contribute significantly to IgE class switch recombination in B cells, critical in atopic diseases. Recent studies indicate $IgG^+CD23^{hi}IL\text{-}4R\alpha^+$ type 2 memory B cells (MBC2s) as IgE-producing B-cell precursors, linked to total IgE serum levels in atopic patients. Total IgE serum levels decreased during dupilumab treatment in previous studies.

Objective: We sought to assess the effects of dupilumab treatment in comparison with alternative therapies on the frequency of MBC2s and the correlation to total IgE levels in pediatric patients with AD.

Methods: Pediatric patients with AD, participating in an ongoing trial, underwent randomization into 3 treatment groups: dupilumab (n = 12), cyclosporine (n = 12), and topical treatment (n = 12). Plasma samples and PBMCs were collected at baseline (T0) and at 6 months after starting therapy (T6). Flow cytometry was used for PBMC phenotyping, and ELISA was used to assess total IgE levels in plasma.

Results: Our findings revealed a significant reduction in MBC2 frequency and total IgE levels among patients treated with dupilumab. In addition, a significant correlation was observed between MBC2s and total IgE levels.

Conclusions: Systemic blocking of the IL-4Rα subunit leads to a decrease in circulating MBC2 cells and total IgE levels in pediatric patients with AD. Our findings unveiled a novel mechanism through which dupilumab exerts its influence on the atopic signature. (J Allergy Clin Immunol 2024;154:1333-38.)

Key words: *IgE, memory B cells, MBC2, atopic dermatitis, dupilumab, IL-4 signaling, cyclosporine*

INTRODUCTION

Atopic dermatitis (AD) is an inflammatory skin disease characterized by recurring red, itchy patches. AD exhibits an immune imbalance with an inclination toward type 2 immunity, marked by increased production of IgE antibodies, potentially leading to other atopic diseases such as food allergies, allergic asthma, and allergic rhinitis (referred to as the "atopic march").[1,2] In AD skin, keratinocytes and skin-resident innate immune cells are activated by invading pathogens and allergens, priming skin-resident dendritic cells and type 2 innate lymphoid cells to induce T_H2 cells. T_H2 cells release type 2 cytokines such as IL-4 and IL-13. The skin's type 2 inflammatory milieu fosters T follicular helper type 2 and T follicular helper type 13 differentiation in the draining lymph nodes, vital for B-cell selection and differentiation into IgE-secreting plasma memory B cells (MBCs) or long-lived MBCs in germinal centers.[3]

Treatment options for pediatric AD include the use of topical corticosteroids (TCSs) and emollients, the systemic administration of cyclosporine A (CsA) or dupilumab.[1,2,4,5] CsA, a calcineurin inhibitor, inhibits IL-2 transcription, essential for the activation and survival of T cells, especially the T_H cells.[6] Dupilumab is a fully human IgG4 mAb targeting the IL-4 receptor α (IL-4Rα) subunit, inhibiting the binding of IL-4 and IL-13, thereby suppressing type 2 immune responses.[7]

IgE^+ B cells can emerge as a result of both direct and sequential class switch recombination (CSR), during which naive B cells directly switch to IgE or switch to another isotype before IgE. Sequential CSR has been associated with high-affinity IgE production, whereas direct CSR appears to yield low-affinity IgE.[8] So far, no convincing evidence has been obtained supporting the existence of IgE^+ MBCs, and it has been proposed that allergen-specific IgE memory could be contained in B cells that underwent CSR to IgG (and potentially IgA1).[9] Recent studies have identified a population of $IgG^+CD23^{hi}IL\text{-}4R\alpha^+$ MBCs, in which germline IgE heavy-chain transcription was detected, named type 2 MBC (MBC2). This subset is suggested to serve

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Abbreviations used
AD: Atopic dermatitis
BCR: B-cell receptor
CsA: Cyclosporine A
CSR: Class switch recombination
FACS: Fluorescence-activated cell sorting
IL-4Rα: IL-4 receptor α
MBC2: Type 2 memory B cells ($IgG^+CD23^{hi}IL\text{-}4R\alpha^+IgD^-IgM^-$ B cells)
OD_{450}: Optical density at 450 nm
TCS: Topical corticosteroid

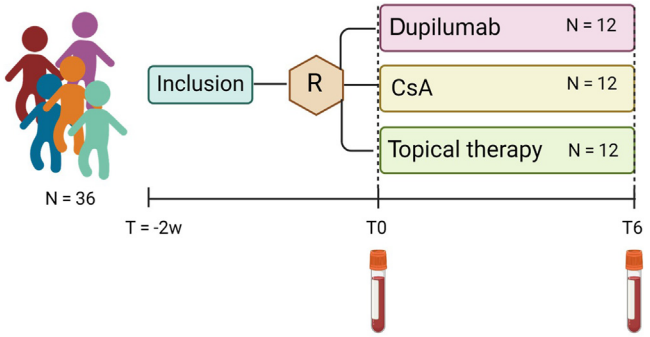

FIG 1. Schematic study overview of randomized controlled trial for pediatric patients with AD randomized into 3 treatment arms: dupilumab, CsA, and topical treatment. Blood was collected at baseline (T0) and at 6 months after starting therapy (T6). Figure was created with BioRender.com.

as a memory pool of B cells, functioning as precursors to IgE^+ B cells.[10-12] The fate of MBC2s on activation remains unclear; they may differentiate into IgE^+ plasmablasts or plasma cells rather than IgE^+ MBCs, because earlier studies identified circulating B cells with mature IgE B-cell receptors (BCRs) as plasmablasts or plasma cells.[13] MBC2s demonstrated a propensity to undergo CSR to IgE, and their frequencies were found to correlate with the total serum IgE levels in atopic patients.[10-12] Increased MBC2 frequency has been found in atopic individuals.[11,12]

Given the reported fact that dupilumab reduces serum IgE levels,[14,15] we hypothesized that dupilumab would reduce MBC2s and that this reduction would correlate with decreased total IgE levels in pediatric patients with AD. To study the effects of dupilumab, CsA, and topical treatment, we compared MBC2 and total IgE levels at baseline (T0) and at 6 months after starting therapy (T6) . For detailed methods, please see this article's Methods section in the Online Repository at www.jacionline.org.

RESULTS AND DISCUSSION

Pediatric patients with AD, participating in the ongoing NMF-CsA-Dupi trial (clinical trial registration: NCT04878770),[16] underwent randomization into 3 treatment groups: dupilumab, CsA, and topical treatment. Plasma samples and PBMCs were collected at T0 and T6. Thirty-six patients with a PBMC and/or plasma sample from T0 and T6 on the same therapy were included (Fig 1). Patient characteristics and available data are provided in Table I. Of each treatment group, 12 patients were included. The age of participants was significantly different between the groups. Because of prescribing restrictions for dupilumab, randomization was limited to those older than 6 years in the dupilumab group. The median age was 10.5 years in the dupilumab group versus 9 years in the CsA group as well as in the topical treatment group. Sex, total IgE at baseline, and Eczema Area and Severity Index (EASI) scores at baseline were not significantly different between the groups. Because of limited availability of study materials, not all assays were performed for every patient (for a detailed sample list, see Table E1 in this article's Online Repository at www.jacionline.org).

A part of the study population received dupilumab treatment, which binds to IL-4Rα, and therefore accurate quantification of IL-4Rα may be hampered by dupilumab occupying IL-4Rα. IL-4Rα could be present on the cell surface as either unoccupied by dupilumab (IL-4Rα(free)) or occupied by dupilumab (IL-4Rα(dupi)). Total IL-4Rα (IL-4Rα(total)) was defined as IL-4Rα(free) + IL-4Rα(dupi). To quantify IL-4Rα(total), an antibody recognizing the IL-4Rα(free) and an anti-IgG4 antibody recognizing the IL-4Rα(dupi) were required. In addition, the anti-IgG4 antibody used for the detection of dupilumab must not bind the $IgG4^+$ BCR (Fig 2, *A*). Heeb and Boyman[17] demonstrated this method to detect IL-4Rα in dupilumab-treated patients. We verified the specific binding of antibodies to IL-4Rα (to detect IL-4Rα(free): clone 209) and human IgG4 (to detect IL-4Rα(dupi): clone HP6025; and IgG4 BCR: clone SAG4). For this purpose, we treated PBMCs with dupilumab *in vitro* and compared IL-$4R\alpha(dupi)^+$, IL-$4R\alpha(free)^+$, and IgG4 BCR^+ populations between dupilumab-treated $CD19^+$ B cells ($dupi^+$) and non–dupilumab-treated $CD19^+$ B cells ($dupi^-$). For the gating strategy of $CD19^+$ and $CD19^+IgM^-$ B cells, see Fig 2, *B*. Nonspecific binding of clone HP6025 on non–dupilumab-stained B cells was negligible, verifying that this antibody is specific for IL-4Rα(dupi). The IL-$4R\alpha(free)^+$ B-cell population was higher in the $dupi^-$ B cells than in the $dupi^+$ B cells (65.9% vs 4.9%) (Fig 2, *C*), indicating that IL-4Rα(dupi) is not stained by clone 209, making clone 209 specific for IL-4Rα(free). The IgG4 BCR^+ population is equal in $dupi^+$ and $dupi^-$ $CD19^+IgM^-$ B cells (Fig 2, *D*), confirming that clone SAG4 does not bind IL-4Rα(dupi). Because the IL-4Rα(-dupi) background signal in $dupi^-$ IgM^- B cells is lower than in the IgG4 BCR^+ population (0.6% vs 4.2%) (Fig 2, *D*), we confirm that the HP6025 clone is specific for IL-4Rα(dupi).

We observed a significant increase in IL-$4R\alpha(dupi)^+$ and a significant reduction in IL-$4R\alpha(free)^+$ B cells in dupilumab-treated patients, but not in CsA-treated or topically treated patients (Fig 3, *C*). The total IL-$4R\alpha^+$ $CD19^+$ B-cell population also decreased significantly only in the dupilumab-treated group (Fig 3, *C*), previously reported by Heeb and Boyman[17] in an adult cohort with AD.[17] The impact of CsA treatment on IL-$4R\alpha^+$ cell frequency has not been studied *in vivo* and has shown conflicting results *in vitro*.[18,19] We observed a decrease in total IL-4Rα in the dupilumab-treated group among IgD^+CD27^- nonswitched B cells (Fig 3, *E*), in contrast to IgM^-IgD^- switched B cells (Fig 3, *G*).

For the detection of $IgG^+CD23^{hi}IL\text{-}4R\alpha^+$ MBC2s, the total MBC2 population was defined as IgG^+CD23^{hi}IL-$4R\alpha(free)^+$ + IgG^+CD23^{hi}IL-$4R\alpha(dupi)^+$ (Fig 4, *B* and *C*). MBC2s are expressed as percentages of $CD19^+IgM^-IgD^-$ switched B cells. We found a significant decrease in the MBC2 population after dupilumab treatment in pediatric patients with AD, which did not occur after CsA or topical treatment (Fig 4, *D*). Furthermore, we observed a significant decrease in total IgE plasma levels after

TABLE I. Patient characteristics and available data per treatment arm

Characteristics	Dupilumab	Cyclosporine	Topical therapy	*P* value
Total no. of patients	12	12	12	
Age (y), median (p25-p75)	10.5 (9-16)	9 (3-12)	9 (5-14)	.02*
Sex (male/female)	6/6	7/5	9/3	.44†
Age (y)				.05†
2-5	0	6	4	
6-11	3	3	4	
12-18	9	3	4	
IL-4Rα data	11	11	11	
MBC2 data	9	9	9	
Total IgE data	11	12	12	
Mean EASI score at T0	30.77	29.13	28.70	.68*
Mean total IgE level at T0 (ng/mL)	12140.2	2007.2	5499.3	.75*

EASI, Eczema Area and Severity Index.
*Calculated using the Kruskal-Wallis test.
†Calculated using the χ^2 test.

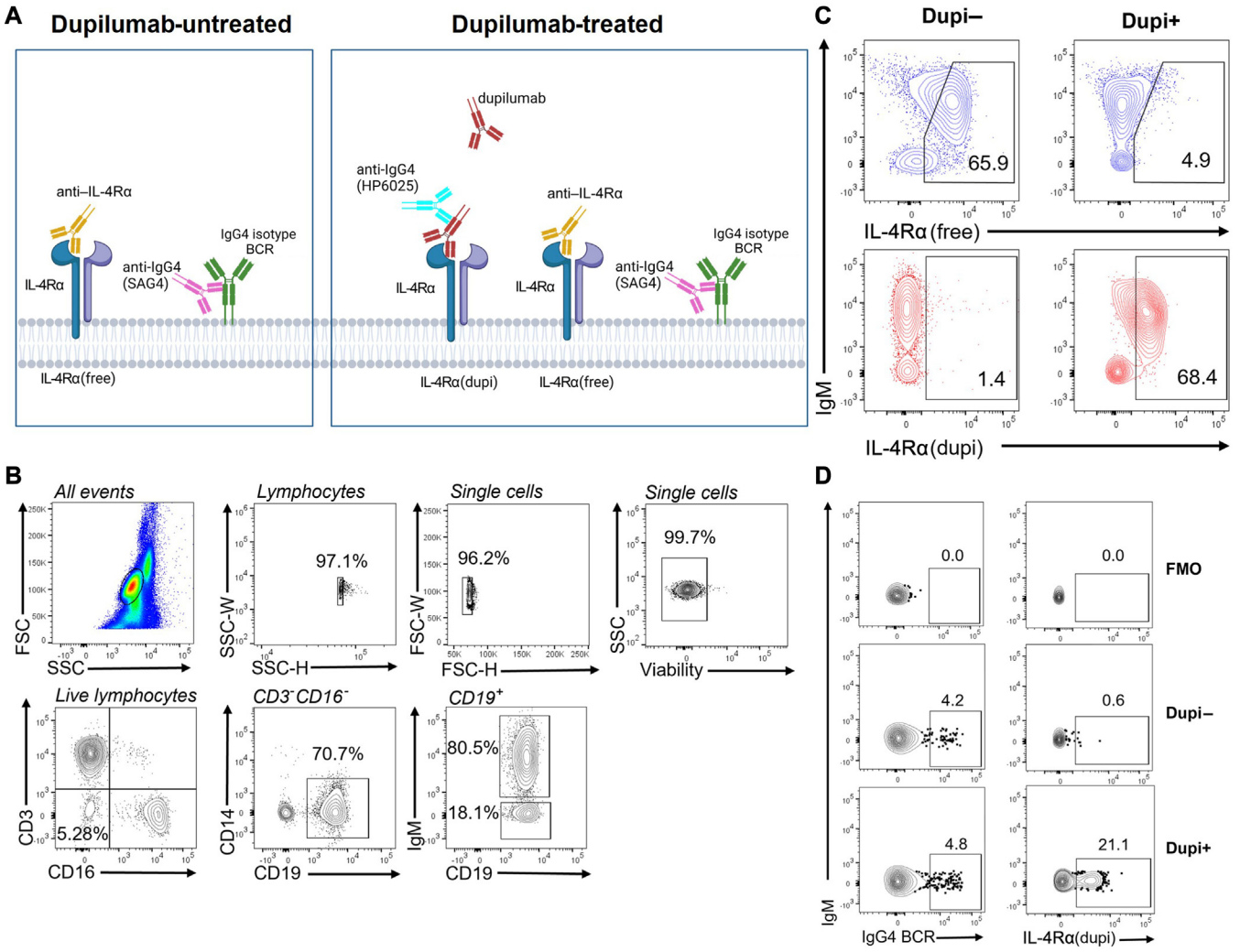

FIG 2. Quantification of IL-4Rα on the surface of *in vitro* dupilumab-treated or untreated B lymphocytes. **A,** Schematic illustration of IL-4Rα surface detection on dupilumab-untreated (*left box*) and dupilumab-treated B cells (*right box*). Dupilumab-bound IL-4Rα (IL-4Rα(dupi)) is detected by anti-IgG4 (clone HP6025) (*turquoise*); non–dupilumab-bound IL-4Rα (IL-4Rα(free)) by anti–IL-4Rα (clone 209) (*yellow*); and the IgG4 BCR by anti-IgG4 (clone SAG4) (*pink*). Figures were created with BioRender.com. **B-D,** Gating strategy for total $CD19^+$ and $CD19^+IgM^-$ B cells for detection of IL-4Rα(free) and IL-4Rα(dupi) in total $CD19^+$ B cells (Fig 2, *C*) and for detection of IgG4 BCR on $CD19^+IgM^-$ B cells (Fig 2, *D*). PBMCs were collected from a healthy donor, treated ($dupi^+$) or untreated ($dupi^-$) with dupilumab *in vitro*. *FMO,* Fluorescence minus one; *FSC-H,* forward scatter height; *FSC-W,* forward scatter width; *SSC-H,* side scatter height; *SSC-W,* side scatter width.

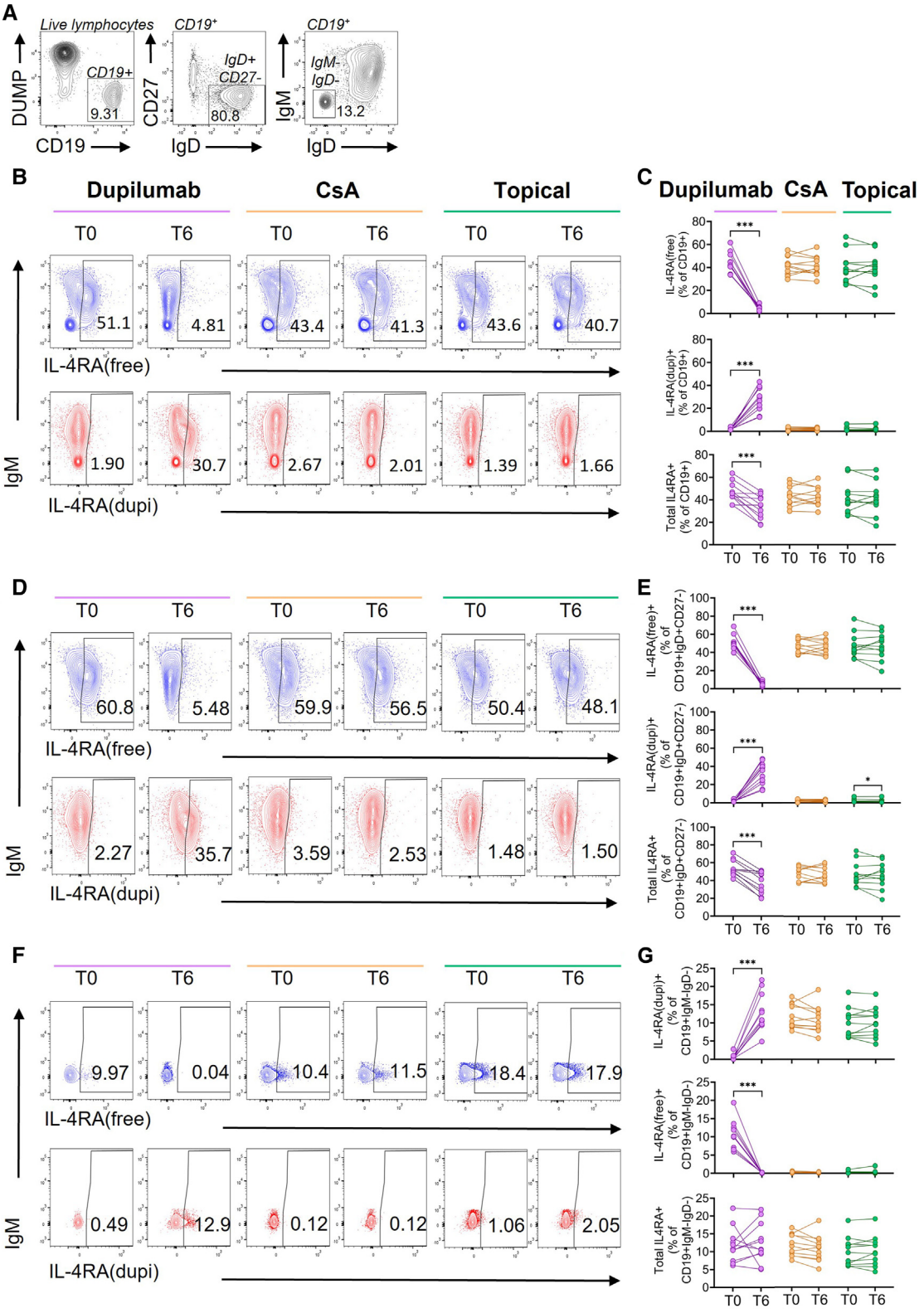

FIG 3. Quantification of IL-4Rα on total B cells, nonswitched B cells, and switched B cells using flow cytometry. **A-G,** Density plots showing gating strategies of total $CD19^+$ B cells (Fig 3, *A*), nonswitched $CD19^+IgD^+CD27^-$ B cells, switched $CD19^+IgM^-IgD^-$ B cells, and IL-4Rα$(free)^+$ and IL-4Rα(dupi) populations within total B cells (Fig 3, *B*), nonswitched B cells (Fig 3, *D*), and switched B cells (Fig 3, *F*). Before-after graphs comparing IL-4Rα$(free)^+$, IL-4Rα$(dupi)^+$, and IL-4Rα$(total)^+$ B cells at T0 to T6 for 11 patients in each treatment arm (N = 33) in total B cells (Fig 3, *C*), nonswitched B cells (Fig 3, *E*), and switched B cells (Fig 3, *G*). IL-4Rα(total) = IL-4Rα(free) + IL-4Rα(dupi). *DUMP,* CD3, CD14, CD16 and viability dye. $*P \leq .05$; $**P \leq .01$; $***P \leq .001$ (paired Wilcoxon signed-rank test).

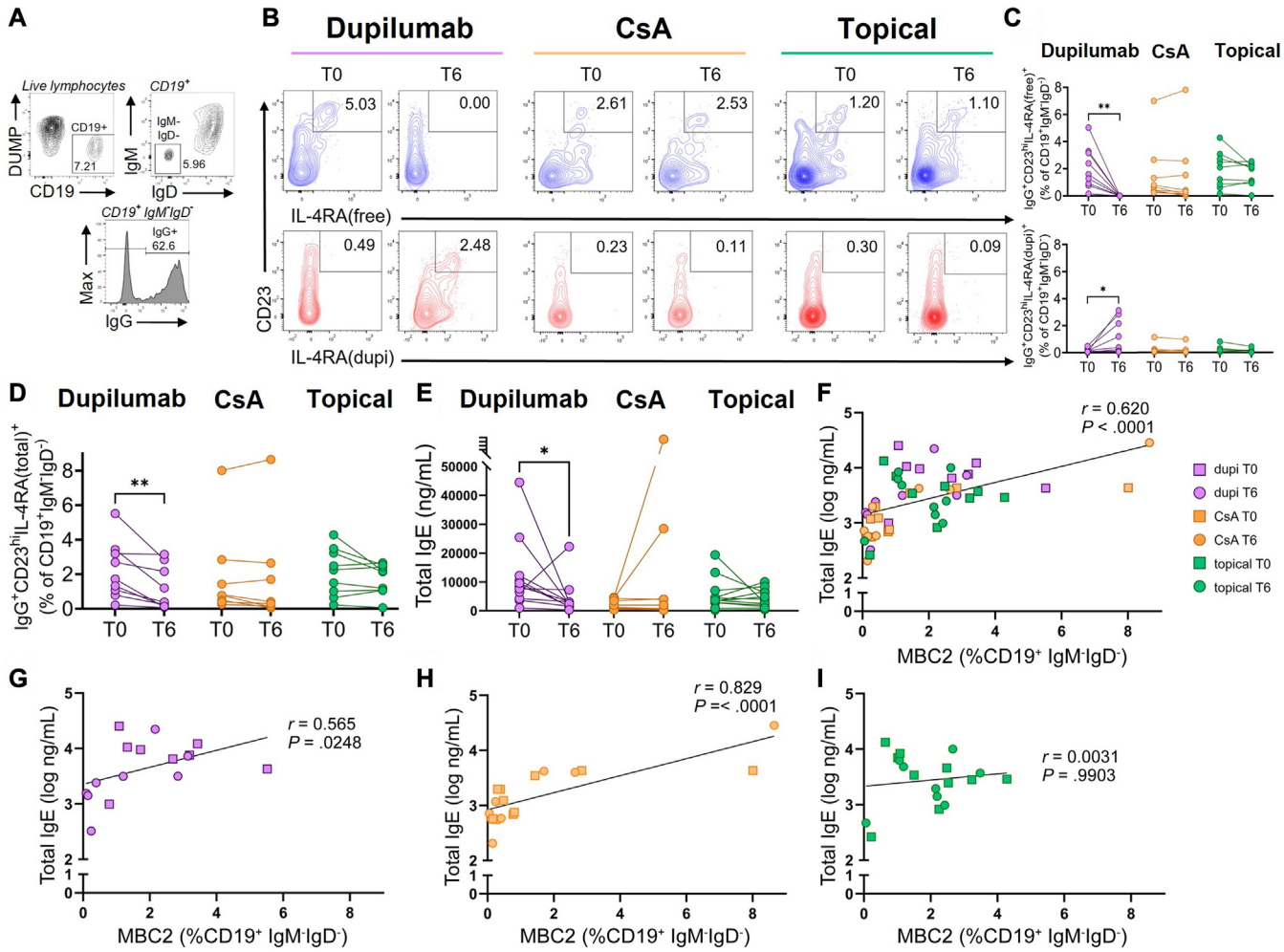

FIG 4. MBC2 frequency, total IgE levels, and correlation. **A,** Gating strategy for $CD19^+IgM^-IgD^-$ and IgG^+ switched B cells. **B-D,** Density plots showing the IgG^+CD23^{hi}IL-4Rα(free) and IgG^+CD23^{hi}IL-4Rα(dupi)$^+$ B-cell population as a percentage of $CD19^+IgM^-IgD^-$ switched B cells, compared between T0 and T6 in before-after graphs of 9 patients per group (N = 27) in Fig 4, *C*, together composing the IgG^+CD23^{hi}IL-4Rα(total)$^+$ cells (MBC2) in Fig 4, *D*. **E,** Before-after graphs comparing total IgE plasma concentrations (in ng/mL) between T0 and T6 in the dupilumab (n = 11), CsA (n = 12) and topical therapy (n = 12) groups (N = 35), using ELISA in duplex. $*P \leq .05$; $**P \leq .01$ (paired Wilcoxon signed-rank test). **F-I,** Correlation of MBC2 frequency in $CD19^+IgM^-IgD^-$ switched B cells (x-axis) and total IgE plasma levels (y-axis, log scale) for all patients and in the dupilumab (Fig 4, *G*), CsA (Fig 4, *H*), and topical (Fig 4, *I*) treatment groups separately (dupilumab group, n = 8; CsA group, n = 9; and topical therapy group, n = 9; N = 26). Spearman correlation coefficient, statistically tested (F): $r = 0.620$; $P < .0001$. *DUMP*, CD3, CD14, CD16 and viability dye.

dupilumab treatment (Fig 4, *E*), similar to observations in adult patients with AD.[14,15] We found that MBC2 frequency in $CD19^+IgM^-IgD^-$ B cells was strongly correlated with total IgE levels in pediatric patients with AD (Fig 4, *F*). On examining individual groups, significant correlations between total IgE levels and MBC2 frequency were observed in the dupilumab- and CsA-treated groups (Fig 4, *G* and *H*), but not in the topically treated group (Fig 4, *I*). Despite this, total IgE levels did not significantly decrease after 6 months of CsA treatment. Previous studies have not conclusively demonstrated the effect of CsA treatment on circulating IgE.[20,21]

We confirmed that dupilumab binds to IL-4Rα on nonswitched and switched B lymphocytes, preventing IL-4 and IL-13 signaling, vital for T_H2 differentiation and IgE CSR. Multiple studies have identified IgG^+CD23^{hi}IL-4Rα$^+$ MBC2s as a subset of MBCs inclined to undergo CSR to IgE.[10-12] Koenig et al[10] revealed the requirement of IL-4 signaling for the differentiation of MBC2s in mouse studies, because MBC2s were absent in IL-4 deficient mice as a result of a homozygous replacement of the IL-4 locus.[10] However, MBC2s represent a small fraction of B cells. It remains unclear as to what extent IgE production in AD relies on sequential CSR through MBC2 intermediates. In patients with AD, most of the IgE is not specific for any of the most common allergens,[22] supporting the notion that a significant amount of IgE in these patients derives from direct CSR. Therefore, the decrease in MBC2 frequency may only partially explain the mechanism by which total IgE is reduced during dupilumab treatment. In this study, we did not measure antigen-specific IgE, but it has been reported by Spekhorst et al[23] that antigen-specific IgE decreases in food-allergic patients with AD treated with dupilumab. Another possibility is

that the reduction in total IgE levels contributed to the decreased detection of MBC2s, considering that the density of CD23 molecules on B cells is correlated with total IgE levels.[24] Koenig et al,[10] who identified MBC2s as $CD23^{hi}$, commented that in mice, neither the differentiation nor the phenotype of MBC2s relied on the presence of circulating IgE. However, we did not verify this in our study and therefore cannot rule out an effect of reduced circulating IgE on CD23 expression.

Our study had limitations, one of which was the inability to evenly distribute children across various age groups over the treatment groups, because of age restrictions for the prescription of dupilumab. Second, total IgE at T0, although not statistically significant ($P = .747$; Kruskal-Wallis test), differed per group, resulting in lower IgE levels at T0 in the CsA group. The overrepresentation of younger children, who typically have lower total IgE,[25] in the CsA group only partly explained the difference in mean total IgE at T0 between the groups. Excluding this age group did not eliminate the difference, suggesting that this limitation was mainly caused by unfortunate randomization of the naturally broad variance in total IgE levels between patients with AD.[14,15] Lastly, our study did not incorporate clinical outcomes, because those are the primary outcomes of the randomized controlled trial, which is still running at the time of publication.

Our results showed a significant decrease in MBC2 cells and total IgE in dupilumab-treated pediatric patients with AD, which did not occur after CsA or topical treatment, as well as a significant correlation between MBC2s and total IgE levels in the pediatric cohort with AD. Our findings suggested that MBC2 differentiation or survival could indeed depend on IL-4 signaling in patients with AD, revealing a novel mechanism of dupilumab's impact on the atopic signature.

DISCLOSURE STATEMENT

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Key messages

- **$IgG^{+}CD23^{hi}IL\text{-}4R\alpha^{+}$ MBC2s decreased during dupilumab treatment.**
- **MBC2s are dependent on IL-4 signaling in humans.**
- **MBC2 frequency is significantly correlated with total IgE in (pediatric) patients with AD during dupilumab treatment.**

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METHODS

Study design and subjects

Peripheral blood samples of a subset of patients included in the NMF-CsA-Dupi trial (clinical trial registration no. NCT04878770)[E1] were collected. In this randomized controlled trial, children 2 to 18 years old and diagnosed with moderate to severe AD who consulted at the Kinderhaven outpatient clinic of the Erasmus MC Sophia Children's Hospital in Rotterdam, the Netherlands, were considered and invited to participate in the trial. After inclusion, a 2-week washout period from current treatment followed, before randomization into 3 different treatment arms: dupilumab + topical treatment (systemic treatment group 1), CsA + topical treatment (systemic treatment group 2), and only topical treatment with emollients and TCSs (active control group) (see ClinicalTrials.gov for full study protocol). Dupilumab (Dupixent; Sanofi-Aventis, Bridgewater, NJ and Regeneron Pharmaceuticals, Tarrytown, NY) was administered as a solution by subdermal injection and dosed according to national guidelines, on the basis of age and body weight. Cyclosporine was administered orally and dosed at 4 to 5 mg/kg/d, tapered to 2 to 3 mg/kg/d depending on the (side) effects. Because dupilumab was not yet approved for children younger than 6 years at the time of starting this randomized controlled trial, only children aged 6 to 18 years were randomized into the dupilumab group. In addition to their assigned treatments, patients in the systemic treatment groups were allowed to use emollients and TCSs. PBMC and plasma samples were collected at baseline (T0) and at 6 months after starting therapy (T6). For this study, samples of the first 66 patients included in the NMF-CsA-Dupi trial were analyzed. Patient samples were included when there were PBMC and plasma samples available from T0 and T6 on continuous therapy.

Sample preparation

PBMCs and plasma were isolated from peripheral blood, collected in EDTA vacutainer blood collection tubes (Becton Dickinson, Franklin Lakes, NJ). EDTA tubes were centrifuged at 540*g* for 5 minutes. Plasma was collected and stored at −80C°. PBMCs were isolated using Ficoll-Paque Plus (Merck, Rahway, NJ) density gradient centrifugation in Leucosep tubes (Greiner Bio-One, Kremsmünster, Austria). PBMCs were then counted and frozen in RPMI, complemented with 20% heat-inactivated FCS and 10% dimethyl sulfoxide. The freezing vials were stored in liquid nitrogen until use. Frozen PBMC and plasma samples were thereafter transported from Rotterdam, the Netherlands, to Davos, Switzerland, on dry ice, with continuous temperature monitoring during shipment.

Samples were thawed by submerging the cryovials in a 37°C water bath for 60 seconds and dropwise adding 1 mL of prewarmed RPMI (RPMI Gibco, Thermo Fisher Scientific, Waltham, Mass) supplemented with 1% penicillin/streptomycin (Sigma Life Science, Darmstadt, Germany) to a final concentration penicillin of 500 IU/mL and 0.1 mg/mL streptomycin, 1% kanamycin (Thermo Fisher Scientific) to a final concentration of 0.1 mg/mL, 1% Minimum Essential Medium (MEM) vitamins solution 100× (Sigma Life Science), 1% MEM nonessential amino acids solution 100× (Sigma Life Science), 1% Na-Pyruvat (Sigma Life Science) to a final concentration of 1 mM, and 10% heat-inactivated FCS (Sigma Life Science) to complete RPMI. The PBMC suspension was then diluted with prewarmed (37°C) complete RPMI to a total volume of 5 mL. PBMCs were left to rest for 5 minutes in the 37°C water bath, before centrifugation for 10 minutes at 330*g* at room temperature. Supernatant was removed by pouring, leaving a small volume behind in which the PBMCs were resuspended. Complete RPMI (1 mL) with 50 U/mL deoxyribonuclease (Life Technologies, Carlsbad, Calif) was added to each sample before incubation for 1 hour at 37°C, 5% CO_2. Cells were counted using a Neubauer chamber under a light microscope, and viability of the samples was determined using a LUNA-II Automated Cell Counter (Logos Biosystems, Villeneuve-d'Ascq, France). After 1 hour of incubation with deoxyribonuclease, PBMCs were used directly for flow-cytometric assays. Per sample, 1 to 1.3 million PBMCs were incubated for 30 minutes in 5% normal mouse serum (Jackson Immuno Research, West Grove, Pa) in fluorescence-activated cell sorting (FACS) buffer (1% FCS, 2 mM EDTA) for 30 minutes at room temperature to block free Fc receptors. The PBMCs were then incubated with fluorochrome-labeled antibodies (see Table E2 in this article's Online Repository at www.jacionline.org) for 30 minutes on ice. After incubation, cells were washed with FACS buffer and centrifuged at 330*g* for 5 minutes at room temperature. PBMCs were taken up in 200 μL FACS buffer and filtered in Falcon Round-Bottom Polystyrene Test Tubes with Cell Strainer Snap Cap, 5 mL (Corning Life Sciences, Durham, NC), before recording on the FACSAria III Cell Sorter (Becton Dickinson).

Total IgE detection with ELISA

For the detection of total IgE in plasma samples, an in-house developed assay was performed in duplex. High-binding 96-well ELISA plates (Thermo Fisher Scientific) were coated with 50 μL of (mouse IgG1) anti–human IgE 14-41 (gift from Christoph Heusser, Novartis, Basel, Switzerland) in a 1:1000 dilution in PBS (Thermo Fisher Scientific) and incubated at 4°C overnight. Plates were blocked with 200 μL of blocking buffer consisting of PBS containing 0.05% Tween 20 with the addition of 5% BSA (Sigma Life Science). The plates were then incubated for 2 hours with 50 μL of 1:80 diluted plasma samples and the 8-step standard curve with human IgE myeloma wild-type. Fifty microliters of biotinylated (mouse IgG1) anti–IgE 6-7 (clone MZ01; Christoph Heusser, Novartis) in a 1:5000 dilution (in blocking buffer) was then added to each well for the next incubation. The last incubation step was performed with 50 μL of 1:2000 diluted extravidin peroxidase (Sigma Life Science) in blocking buffer. All incubation steps were performed at 37°C for 1 hour, unless stated otherwise, and after each step the plates were washed 5 times with PBS containing 0.05% Tween 20. Plates were developed by adding 50 μL of TMB substrate mixture (1% 3,3′,5,5′-tetramethylbenzidine, 10% 1 mol citrate acetate buffer, 0.0125% of 30% H_2O_2, in double-distilled water). The colorimetric reaction was stopped by adding 50 μL of 1 mol H_2SO_4. The optical density was measured at 450 nm (OD_{450}) using a microplate reader (800 TS microplate reader, BioTek, Winooski, Vt).

Data analysis

Flow-cytometric data were analyzed using FlowJo version 10.8.1 (Becton Dickinson). B cells were manually gated into subpopulations. IL-4Rα and dupilumab$^+$ gating was performed

on all populations according to fluorescence minus one PBMC samples from a healthy donor. Total IL-4Rα surface detection was defined as IL-4Rα occupied by dupilumab + IL-4Rα unoccupied: IL-4Rα(total) = IL-4Rα(dupi) + IL-4Rα(free).[E2]

The OD_{450} values obtained from all ELISAs were corrected by subtracting the average value of the blank. A standard curve was included on each ELISA plate consisting of an 8-step dilution series. The log concentration of each dilution was plotted against the corresponding OD_{450} value to create a standard curve. Protein levels were thereafter extrapolated from the logistic regression analysis of the standard curve, followed by inverse log transformation. Concentrations were corrected for aforementioned dilution of the plasma samples.

Statistical analysis

Statistical analysis on baseline patient characteristics was performed using GraphPad Prism, version 8.0.2 for Windows (GraphPad Software, San Diego, Calif). Continuous data were analyzed using the Kruskal-Wallis test for nonparametric data, and categorical data were analyzed using the chi-square test.

Statistical analysis on immunophenotyping data and total IgE levels was performed using GraphPad Prism (GraphPad Software). Frequencies of populations and protein plasma concentrations were compared between T0 and T6. For all comparisons between T0 and T6, the paired Wilcoxon signed-rank test for nonparametric data was carried out. Differences with P values equal to or less than .05 were regarded as statistically significant.

REFERENCES

E1. NMF-CsA-Dupi trial. Bethesda, MD: US National Library of Medicine, National Institutes of Health; 2021.. 2021. Available at: https://classic.clinicaltrials.gov/ct2/show/NCT04878770. Accessed July 26, 2023.

E2. Heeb LEM, Boyman O. Comprehensive analysis of human IL-4 receptor subunits shows compartmentalization in steady state and dupilumab treatment. Allergy 2023;78:1073-87.

TABLE E1. Patient materials available per study objective

Patient (study no.)	Study arm	Sex	Age (y)	Flow data IL-4Rα	Flow data MBC2	IgE data T0-T6	Total IgE at T0 (ng/mL)	EASI score at T0
N001	Dupilumab	Female	16	Yes	No	Yes	44,479.52	41.00
N005	Dupilumab	Male	14	Yes	No	Yes	3,676.66	41.00
N008	Dupilumab	Female	17	Yes	Yes	Yes	6,510.00	54.70
N016	Dupilumab	Male	7	Yes	Yes	Yes	991.14	19.90
N017	Dupilumab	Female	9	Yes	Yes	Yes	12,191.56	18.90
N018	Dupilumab	Male	13	Yes	Yes	Yes	4,249.17	29.10
N021	Dupilumab	Female	9	Yes	Yes	Yes	25,425.61	26.30
N029	Dupilumab	Female	16	Yes	Yes	Yes	9,564.50	6.20
N044	Dupilumab	Female	16	Yes	Yes	Yes	10,522.73	8.80
N045	Dupilumab	Male	17	No	No	Yes	8,314.12	51.50
N047	Dupilumab	Male	13	Yes	Yes	Yes	7,616.87	38.40
N052	Dupilumab	Male	12	Yes	Yes	No	NA	33.40
N004	Cyclosporine	Female	3	Yes	No	Yes	618.19	28.60
N007	Cyclosporine	Female	6	Yes	No	Yes	4,644.57	32.60
N011	Cyclosporine	Male	13	Yes	Yes	Yes	689.86	34.00
N013	Cyclosporine	Female	17	Yes	Yes	Yes	4,273.89	42.10
N020	Cyclosporine	Male	4	Yes	Yes	Yes	3,488.94	40.20
N026	Cyclosporine	Male	3	Yes	Yes	Yes	754.62	20.70
N036	Cyclosporine	Male	4	Yes	Yes	Yes	1,227.92	15.40
N038	Cyclosporine	Female	5	Yes	Yes	Yes	559.27	20.70
N040	Cyclosporine	Male	16	Yes	Yes	Yes	4,329.88	33.20
N046	Cyclosporine	Female	2	No	No	Yes	354.77	21.60
N049	Cyclosporine	Male	7	Yes	Yes	Yes	1,964.98	27.20
N063	Cyclosporine	Male	9	Yes	Yes	Yes	1,179.24	33.20
N003	Topical	Male	8	Yes	No	Yes	4,439.09	30.40
N006	Topical	Male	17	Yes	No	Yes	3,317.34	45.80
N009	Topical	Male	9	Yes	Yes	Yes	2,817.70	38.80
N014	Topical	Male	16	Yes	Yes	Yes	3,422.82	27.90
N015	Topical	Male	15	Yes	Yes	Yes	2,892.13	13.40
N037	Topical	Female	5	Yes	Yes	Yes	262.84	22.90
N041	Topical	Male	4	Yes	Yes	Yes	824.16	19.20
N043	Topical	Female	12	Yes	Yes	Yes	13,289.50	41.40
N051	Topical	Male	5	No	No	Yes	19,413.08	12.80
N053	Topical	Male	8	Yes	Yes	Yes	4,589.31	24.60
N058	Topical	Female	4	Yes	Yes	Yes	7,022.57	30.20
N060	Topical	Male	9	Yes	Yes	Yes	3,701.37	37.00

EASI, Eczema Area and Severity Index; *NA*, not available.

TABLE E2. List of fluorochrome-labeled antibodies

Marker	Dye	Company	Catalog no.	Clone	Isotype	Final dilution
CD23	PC7	BioLegend	338516	EBVCS-5	Mouse IgG1, κ	1:200
IgM	PerCP/Cy5.5	BioLegend	314512	MHM-88	Mouse IgG1, κ	1:100
CD27	BV510	Sony	2114180	—	—	1:100
CD38	BV786	BioLegend	303530	HIT2	Mouse IgG1, κ	1:100
IgD	PE/CF594	Sony	2341200	IA6-2	Mouse IgG2a, κ	1:200
IL-4Rα	APC	Sino Biologicals	10402-R209-A	209	Rabbit IgG	1:100
IgG4	PE	Southern Biotech	9200-09	PH6025	Mouse IgG1, κ	1:50
IgG	BV421	BioLegend	410704	M1310G05	Rat IgG2a, k	1:1000
CD3	Biotin	BioLegend	317320	OKT3	Mouse IgG2a, κ	1:250
CD14	Biotin	BioLegend	301826	M5E2	Mouse IgG2a, κ	1:250
CD16	Biotin	BioLegend	302004	3G8	Mouse IgG1, κ	1:250
CD19	FITC	BioLegend	302206	HIB19	Mouse IgG1, κ	1:400
Viability dye	Zombie Yellow	BioLegend	423104	—	—	1:100

APC, Allophycocyanin; *FITC*, fluorescein isothiocyanate; *PE*, Phycoerythrin.