

Diverse activation and differentiation of multiple B-cell subsets in patients with atopic dermatitis but not in patients with psoriasis

Tali Czarnowicki, MD, MSc,^a Juana Gonzalez, PhD,^b Kathleen M. Bonifacio, MD,^a Avner Shemer, MD,^c Peng Xiangyu, MSc,^d Norma Kunjraiva, MD,^a Dana Malajian, BA,^{a,e} Judilyn Fuentes-Duculan, MD,^a Hitokazu Esaki, MD,^{a,d} Shinji Noda, MD,^a Yeriel Estrada, BSc,^d Hui Xu, MSc,^d Xiuzhong Zheng, MSc,^a James G. Krueger, MD, PhD,^a and Emma Guttman-Yassky, MD, PhD^{a,d} *New York, NY, and Tel Aviv, Israel*

Background: Atopic dermatitis (AD) and psoriasis pathogenesis involve skin barrier impairment and immune dysregulation; however, the contribution of B-cell imbalances to these diseases has not yet been determined.

Objective: We sought to quantify B-cell populations and antibody-secreting cells in the blood of patients with AD, patients with psoriasis, and control subjects.

Methods: We studied 34 adults with moderate-to-severe AD (mean SCORAD score, 65), 24 patients with psoriasis (mean Psoriasis Area and Severity Index score, 16), and 27 healthy subjects using an 11-color flow cytometric antibody panel. IgD/CD27 and CD24/CD38 core gating systems were used to determine frequencies of plasmablasts and naive, memory, transitional, and activated B cells.

Results: We measured increased CD19⁺CD20⁺ B-cell counts in the skin and blood of patients with AD ($P < .01$). Significantly higher frequencies of chronically activated CD27⁺ memory and nonswitched memory B cells were observed in patients with AD

($P < .05$), with lower values of double-negative populations (4% for patients with AD vs 7% for patients with psoriasis [$P = .001$] and 6% for control subjects [$P = .02$]). CD23 expression was highest in patients with AD and correlated with IgE levels ($P < .01$) and disease severity ($r = 0.6$, $P = .0002$). Plasmablast frequencies and IgE expression were highest in all memory subsets of patients with AD ($P < .01$). Finally, CD19⁺CD24⁺⁺CD38⁺⁺ transitional and CD19⁺CD24⁻CD38⁻ new memory B-cell counts were higher in patients with AD versus those in patients with psoriasis (2.8% vs 1.4% [$P = .001$] and 9.2% vs 5.7% [$P = .02$], respectively).

Conclusions: AD is accompanied by systemic expansion of transitional and chronically activated CD27⁺ memory, plasmablast, and IgE-expressing memory subsets. These data create a critical basis for the future understanding of this debilitating skin disease. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: Atopic dermatitis, psoriasis, B cells

From ^athe Laboratory for Investigative Dermatology and ^bthe Translational Technology Core Laboratory, The Rockefeller University, New York; ^cthe Department of Dermatology, Tel-Hashomer hospital, Tel Aviv; ^dthe Department of Dermatology, Icahn School of Medicine at Mount Sinai, New York; and ^eColumbia University College of Physicians and Surgeons, New York.

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Corresponding author: Tali Czarnowicki, MD, MSc, Laboratory for Investigative Dermatology, The Rockefeller University, 1230 York Ave, New York, NY 10065.

E-mail: tczarnowic01@rockefeller.edu.

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Although they comprise only 1% to 7% of leukocytes,^{1,2} B cells play important roles in immune defense, including immunoglobulin production, antigen presentation, and cytokine secretion.^{3,4} Psoriasis and atopic dermatitis (AD), the 2 most common inflammatory skin diseases,⁵ are both characterized by immune dysregulation and barrier impairment.^{5,6} Immunologically, focus has been given to T-cell subset imbalances, whereas the B-cell contribution to AD and psoriasis phenotypes is unclear.

Previous skin studies showed some presence of CD20⁺ B cells in AD skin lesions, although this population accounts for a small minority of the dermal infiltrates (eg, 7 CD20⁺ B cells vs 165 CD3⁺ T cells).⁷ Decreases in B-cell infiltrates were correlated with AD improvement after topical tacrolimus or pimecrolimus treatments.^{8,9}

Additionally, roughly 80% of patients with AD have increased IgE levels.^{10,11} Despite the current dogma of IgE as a disease amplifier rather than a pathogenic factor in patients with AD, IgE levels were found to correlate with AD severity (SCORAD scores) in extrinsic patients.¹²

Mouse studies have shown that antigen presentation by antigen-primed B cells preferentially induces T_H2 and IL-4 production.^{13,14} Furthermore, the B-cell activation molecule OX40 ligand was shown to direct CD4 T cells to express IL-4.¹⁵ Another mouse study showed that depletion of CD19 improves cutaneous eczema and attenuates CD4⁺ T-cell proliferation¹⁶ and that in patients with allergic skin inflammation, B cells are required for antigen-specific T-cell activation.^{16,17}

Abbreviations used

AD:	Atopic dermatitis
ASC:	Antibody-secreting cell
CLA:	Cutaneous lymphocyte antigen
DN:	Double negative
IHC:	Immunohistochemistry
NSM:	Nonswitched memory
PASI:	<i>Psoriasis Area and Severity Index</i>
SwMe:	Switched memory
T _{CM} :	Central memory T
T _{EM} :	Effector memory T
TLR:	Toll-like receptor
Treg:	Regulatory T

Further evidence for the significance of B cells in patients with AD comes from the successful treatment of AD with the chimeric monoclonal anti-CD20 agent rituximab.¹⁸ In a study by Simon et al¹⁸ rituximab led to AD improvement, abolished B-cell counts, and decreased T-cell activation and IL-13 production in blood, whereas in skin CD20⁺, CD4⁺, CD8⁺, and epidermal CD1a⁺ counts and IL-5 and IL-13 mRNA expression were significantly reduced. Conversely, in patients with psoriasis, rituximab treatment largely did not lead to clinical improvement.¹⁹ These data suggest that B cells function beyond antigen presentation and are involved in shaping immune responses on many levels.^{20,21}

Together, these data suggest that B-cell dysregulation might affect the pathogenesis of T cell-mediated diseases, such as AD or psoriasis. Different classification schemes have been developed to define B-cell differentiation and development sequence^{22,23}; however, these subsets were not defined in patients with AD.

We sought to phenotype B cells in the circulation of adults with AD compared with those with psoriasis and control subjects. We showed that B-cell frequency is increased, memory cell and plasma cell counts are higher, and IgE production signatures are significantly enhanced in the blood of patients with AD but not in patients with psoriasis or control subjects.

METHODS**Patients' characteristics and blood samples**

Under an institutional review board-approved protocol, 34 patients with AD (mean age, 46 years; SCORAD score: range, 32-97; mean, 65), 24 patients with psoriasis (mean age, 46 years; *Psoriasis Area and Severity Index* [PASI] score: range, 9-34; mean, 16), and 27 control subjects (mean age, 40 years) were enrolled in this study.

No age ($P = .25$, 1-way ANOVA), ethnicity ($P = .8$, χ^2 test), or sex ($P = .6$, χ^2 test) disparities were observed between the groups.

Serum IgE levels in patients with AD (normal, <200 kU/L) ranged from 4 to 44,210 kU/L (mean, 4,655 kU/L). SCORAD and PASI scores were used to evaluate AD and psoriasis severity, respectively (see values above). Control subjects had no personal or family history of atopy. Demographic data are presented in Table 1.

Flow cytometric diagrams and nomenclature

Several flow cytometric approaches have been developed to address the diversity of peripheral B-cell subsets.^{2,22} the 2 most common methods use IgD and CD27 markers to characterize mature B cells or CD24 and CD38 to also account for premature cells.²³

The first classification uses CD27 as a general memory marker.^{24,25} After gating on CD19⁺ or CD19⁺CD20⁺ lymphocytes (to exclude plasmablasts, which are CD20⁻), IgD versus CD27 expression classifies circulating B cells into IgD⁺CD27⁻ (naïve), IgD⁺CD27⁺ (nonswitched memory [NSM]), IgD⁻CD27⁺ (switched memory [SwMe]), and IgD⁻CD27⁻ (double-negative [DN]) B cells (see Fig E1, A, in this article's Online Repository at www.jacionline.org).²⁶

A gating strategy with CD19⁺ as a B-cell lineage marker and the developmental markers CD24 and CD38 enable differentiation of further populations, including mature naïve B cells (CD19⁺CD38^{int}CD24^{int}), primarily memory B cells (CD19⁺CD38⁻CD24⁺), plasmablasts (CD19⁺CD38⁺CD24⁻), new memory B cells (CD19⁺CD24⁻CD38⁻), and transitional (CD19⁺CD38⁺⁺CD24⁺⁺) B-cell subsets, to better address B-cell subset complexity (see Fig E1, B).^{24,27,28}

We developed a combined flow cytometric panel that uses CD19 and CD20 as B-lineage markers and implements IgD, CD27, CD24, and CD38 as anchor markers to phenotypically characterize core B-cell populations. Our panel also included measurements of other surface markers, as detailed below.

Isolation of PBMCs. PBMCs were isolated from whole blood by using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). Briefly, the blood was laid under Ficoll gradient; after spinning, PBMCs were collected at the interface between the plasma and the Ficoll (see the Methods section in this article's Online Repository at www.jacionline.org).

PBMC cell-surface staining. PBMCs were stained with fluorochrome-labeled antibodies to cell-surface markers (CD19, CD20, CD24, CD25, CD38, CD27, HLA-DR, IgE, IgD, CD23, and Live/Dead). For further details, see the Methods section in this article's Online Repository.

Immunohistochemistry

Standard procedures were used for immunohistochemistry (IHC) staining, as previously described,²⁹ and are outlined in greater detail in the Methods section in this article's Online Repository. Frozen sections from normal ($n = 11$), AD lesional ($n = 32$), AD nonlesional ($n = 18$), psoriasis lesional ($n = 18$) and psoriasis nonlesional ($n = 11$) skin sections were stained with CD20.

Statistical analysis

Data were analyzed by using the Student t test to compare between variables. IgE values were log₁₀-transformed before analyses. Variables were correlated by using Pearson correlations. A P value of .05 or less was considered significant.

Means $\pm$ SEMs for all numeric results are presented in Table E1 in this article's Online Repository at www.jacionline.org.

RESULTS**Circulating and lesional B-cell frequency is highest in patients with AD**

Patients with AD had higher circulating CD19⁺ (14% vs 8%, $P = .01$; Fig 1, A) and CD19⁺CD20⁺ (13.5% vs 8%, $P = .02$; Fig 1, B) B-cell frequencies compared with patients with psoriasis. No correlation was found between CD19⁺ or CD19⁺CD20⁺ frequency and disease severity in patients with psoriasis or patients with AD ($P > .3$, data not shown).

To corroborate our blood findings, we performed IHC staining of lesional and nonlesional skin from patients with AD and those with psoriasis, as well as healthy control skin. Because CD19 staining was nonspecific and also stained keratinocytes, we used CD20 to define cutaneous B cells (Fig 1, C-J). Significantly higher CD20⁺ cell counts were found in lesional versus nonlesional skin of both patients with AD and those with psoriasis (20 vs 6, $P = .008$, and 9 vs 5, $P = .005$; Fig 1, E and H); however,

TABLE 1. Epidemiologic, laboratory, and clinical data for patients with AD, patients with psoriasis, and control subjects

Parameter	Patients with AD (n = 34)	Control subjects (n = 27)	Patients with psoriasis (n = 24)	P value
Age (y)	46 ± 2.1 (18-74)	40 ± 2.3 (19-66)	46 ± 3 (23-70)	.25
Sex, no. (%)				.6
Female	14 (41)	10 (37)	8 (34)	
Male	20 (59)	17 (63)	16 (66)	
Ethnicity, no. (%)				.8
Asian	1 (3)	1 (4)	0 (0)	
Black	4 (12)	3 (11)	2 (8)	
Hispanic	0 (0)	1 (4)	0 (0)	
White	29 (85)	22 (81)	22 (92)	
SCORAD or PASI score	65 ± 2.45 (32-97)	NA	16 ± 1.45 (9-34)	—
IgE (kU/L)*	4,655 ± 1,654 (4-44,210)	NA	NA	—
Eosinophil count (1000 cells/μL)†	0.45 ± 0.05 (0-1.2)	NA	NA	—

Data are presented as means ± SEMs, unless otherwise specified.

*Reference range: 0 to 200 kU/L.

†Reference range: 0 to 0.8 1000 cells/μL (absolute eosinophil count).

higher counts were documented in lesions from patients with AD compared with those with psoriasis or control subjects (20 vs 9, $P = .03$, and 20 vs 2, $P = .005$, respectively; Fig 1, J). In lesional skin of patients with AD, CD20⁺ cell counts could be further separated into 2 groups (high vs intermediate to low CD20⁺ cell counts; Fig 1, J). Comparing AD severity between these 2 groups showed significantly more severe disease in the group with high CD20⁺ cell counts (75 vs 58, $P = .02$; Fig 1, K). In this group (encircled in red in Fig 1, J and K) positive correlation was found between CD20⁺ cell counts and SCORAD scores ($r = 0.8$, $P = .02$; data not shown).

NSM B-cell frequency is increased in patients with AD

A IgD/CD27 gating strategy was used to define memory and naive subsets (Fig 2, A). In line with previous literature,^{30,31} CD27⁺ memory B-cell frequency was 43% in control subjects. Similar values were measured in the psoriasis group (45%), with a nonsignificant ($P = .2$) and slightly higher value observed in patients with AD (50%; Fig 2, B).

Conversely, the IgD⁺CD27⁺ NSM subset was significantly increased in patients with AD compared with control subjects (35% vs 27%, $P = .04$; Fig 2, B) and patients with psoriasis (35% vs 26%, $P = .01$; Fig 2, B).

CD27⁺IgD⁺ DN B-cell counts are lower in patients with AD

IgD⁺CD27⁺ DN cells, also considered CD27⁺ memory cells, represent approximately 5% of all B cells in healthy subjects.²⁴ Numbers were found to be similar between control subjects and patients with psoriasis ($P = .7$; Fig 2, B), whereas a significantly lower value was found in patients with AD (4% in patients with AD vs 7% in patients with psoriasis [$P = .001$] and 6% in control subjects [$P = .02$]; Fig 2, B). Yet, as detailed below, IgE production by this subset was highest in patients with AD.

IgE expression by SwMe, NSM, and DN B cells is highest in patients with AD

Patients with AD had the highest IgE expression across SwMe (3.6% in patients with AD vs 0.9% in patients with psoriasis

[$P = .0001$] and 1.4% in control subjects [$P = .001$]; Fig 3, A) and less strikingly among NSM cells (3.6% in patients with AD vs 1.4% in patients with psoriasis [$P = .02$] and 1.9% in control subjects [$P = .1$]; Fig 3, B) and DN cells (4.1% in patients with AD vs 1.7% in patients with psoriasis [$P = .01$] and 1.6% in control subjects [$P = .009$]; Fig 3, C). Nonsignificant IgE expression differences were observed between patients with psoriasis and control subjects ($P > .1$ for all; Fig 3, A-C).

Expectedly, highly significant correlations were found between total IgE levels (in serum) and IgE expression on SwMe ($r = 0.6$, $P = .0007$; Fig 3, D), NSM ($r = 0.6$, $P = .0003$; Fig 3, E), and DN ($r = 0.5$, $P = .002$; Fig 3, F) B cells.

CD27⁺IgD⁺ switched and CD27⁺IgD⁺ nonswitched memory HLA-DR activation is highest in patients with AD and correlated with T-cell activation

HLA-DR is a human class II MHC antigen that indicates chronic activation.³² Despite similar frequencies of SwMe B cells among the different groups, higher chronic activation was measured in patients with AD compared with those with psoriasis (5.3% vs 2.8% [$P = .0002$]; Fig 4, A). Accordingly, NSM cells were not only more frequent in patients with AD (as shown above) but also highly activated (6.3% vs 3.5% [$P = .05$]; Fig 4, B). No chronic activation differences were observed between groups in the DN or naive subsets (data not shown).

We have recently published T-cell memory subset (central memory T [T_{CM}] and effector memory T [T_{EM}] cells) activation data for these cohorts, showing that T-cell activation is highest in patients with AD.³³ To evaluate possible T- and B-cell interactions, we correlated these existing data with our current B-cell activation results. Positive correlations were observed between HLA-DR (chronic)³⁴ activation of skin-homing (cutaneous lymphocyte antigen [CLA]⁺) T_{EM} cells and HLA-DR activation of SwMe ($r = 0.35$, $P = .06$; see Fig E2, A, in this article's Online Repository at www.jacionline.org) and more significantly NSM ($r = 0.4$, $P = .03$; see Fig E2, B) B-cell subsets. Positive correlations were also detected for inducible T-cell costimulator intermediately activated³⁵ CLA⁺ T_{EM} cells, particularly with HLA-DR-activated NSM B cells ($r = 0.45$, $P = .01$; see Fig E2, C). These activated T- and B-cell correlations were not found in patients with psoriasis or healthy control subjects (data not shown).

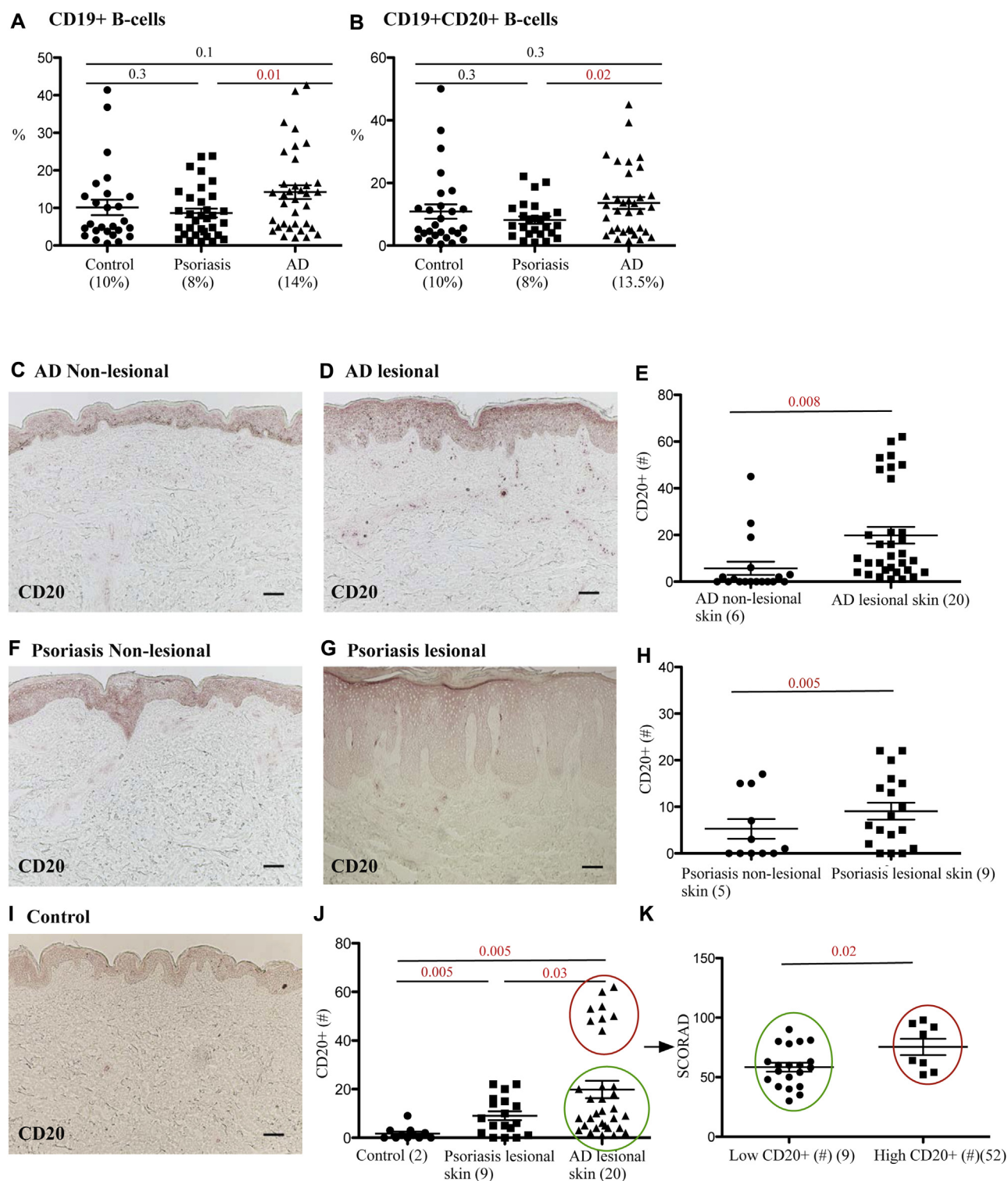
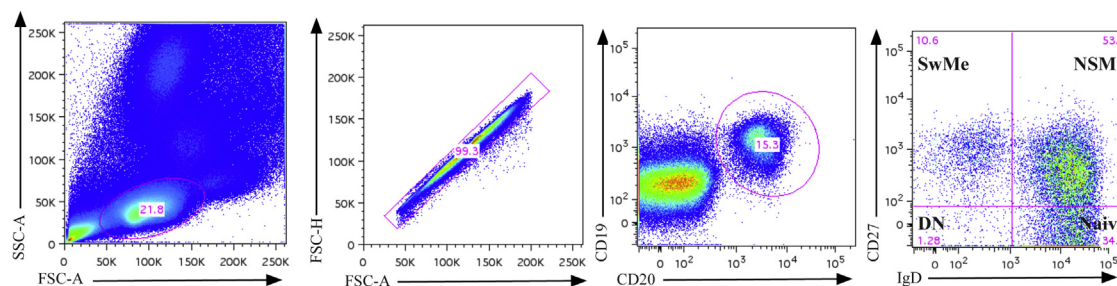
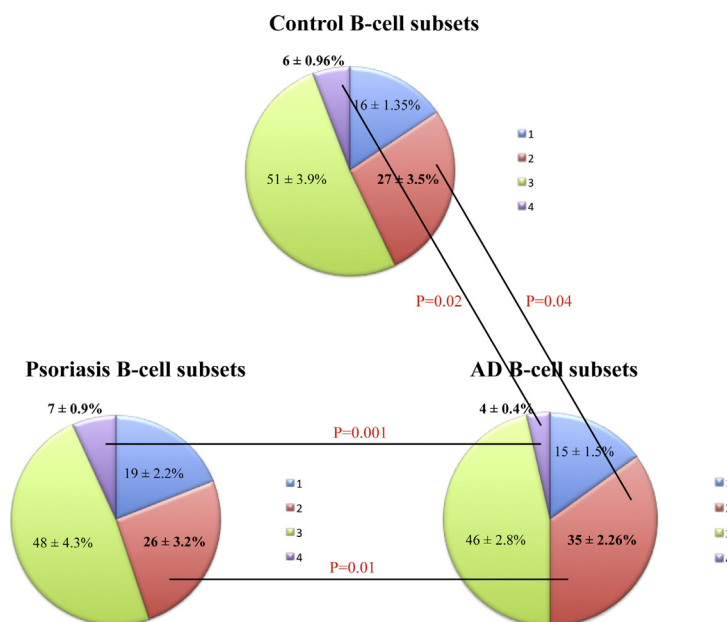


FIG 1. B-cell frequency in blood and skin of patients with AD versus patients with psoriasis and control subjects. **A** and **B**, Patients with AD had increased circulating CD19⁺ and CD19⁺CD20⁺ B-cell subsets, which were significant compared with those in patients with psoriasis. **C-G**, Representative IHC staining of CD20 in nonlesional (Fig 1, **C**) and lesional (Fig 1, **D**) skin from patients with AD showing significant increases in CD20 infiltrates characterizing lesional versus nonlesional skin of patients with AD (mean counts appears in parentheses; Fig 1, **E**) and skin of patients with psoriasis (Fig 1, **F** and **G**). **H**, Significant CD20 differences were observed between nonlesional and lesional skin of patients with psoriasis. **I** and **J**, However, lesional skin of patients with AD demonstrated the highest CD20⁺ B-cell counts. **K**, Higher SCORAD scores were observed among the AD group with high CD20⁺ cell counts. Bar plots represent means $\pm$ SEMs. IHC scale bar = 100 μ m.

A IgD/CD27 Gating strategy**B**

- 1= IgD-CD27+ Switched memory
2= IgD+CD27+ Non-switched memory
3= IgD+CD27- Naive
4= IgD-CD27- Double Negative

FIG 2. IgD/CD27 gating strategy and B-cell subset frequencies. **A**, IgD versus CD27 expression gating strategy classifies circulating B cells into IgD⁺CD27⁻ (naive), IgD⁺CD27⁺ (NSM), IgD⁻CD27⁺ (SwMe), and IgD⁻CD27⁻ (DN) cells. In the IgD/CD27 plot numbers represent the relative percentage of the specific subset. **B**, B-cell subset frequencies (by means $\pm$ SEMs) in the control, psoriasis, and AD groups, demonstrating higher NSM and lower DN B cells in patients with AD.

CD25 expression is lower in patients with AD, particularly among NSM B cells

In line with a previous report,³⁶ the mean CD25⁺ B-cell frequency (all subsets) in control subjects was 37%. Comparing patients with AD, patients with psoriasis, and control subjects, trends of lower values of CD25⁺ expressing B cells were observed in patients with AD for all subsets, most significantly among NSM cells (26% in patients with AD vs 40% in patients with psoriasis [$P = .02$] and 39% in control subjects [$P = .02$]; Fig 4, C).

CD23 expression is highest in patients with AD

CD23 is the low-affinity receptor for the Fc region of IgE (FcεRII). Previous studies showed that FcεRII⁺ circulating cell

counts are increased in patients with AD and that CD23 is involved in the augmentation of IgE synthesis and responses.³⁷⁻³⁹

Consistent with these reports, CD23 expression was higher in patients with AD compared with those with psoriasis or control subjects in all B-cell subsets, including SwMe (20% in patients with AD vs 6.6% in patients with psoriasis [$P = .0002$] and 9% in control subjects [$P = .002$]; Fig 4, D), NSM (50% vs 29% [$P < .0001$] and 35% [$P = .003$], respectively; Fig 4, E), naive (66% vs 40% [$P < .0001$] and 48% [$P = .003$], respectively; Fig 4, F), and DN (27% vs 17% [$P < .01$], respectively; Fig 4, G) B cells.

CD23 expression was correlated with AD severity/SCORAD scores in all subsets (Fig 4, H-K); however, the most significant correlation was observed in the SwMe subset ($r = 0.6$, $P = .0002$; Fig 4, H).

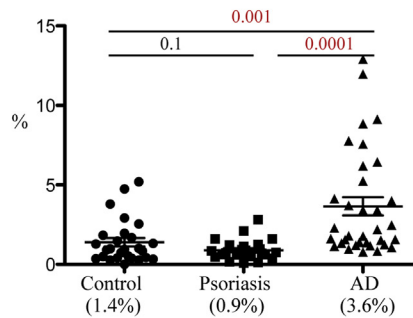
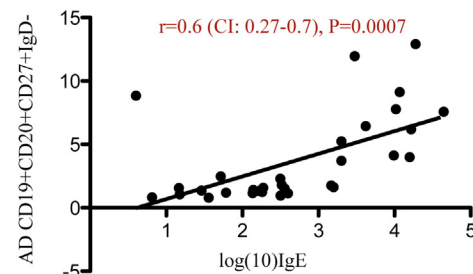
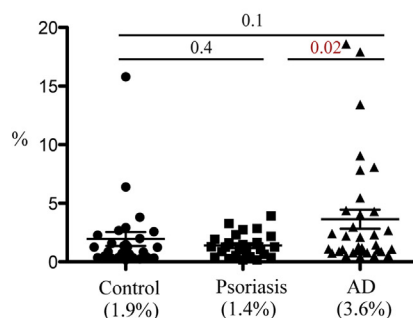
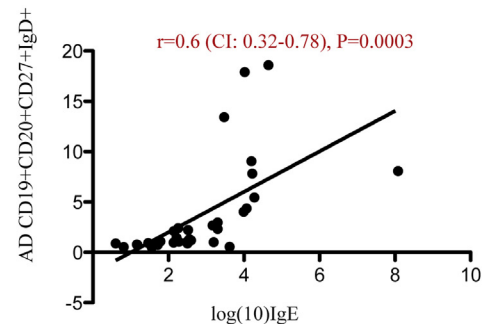
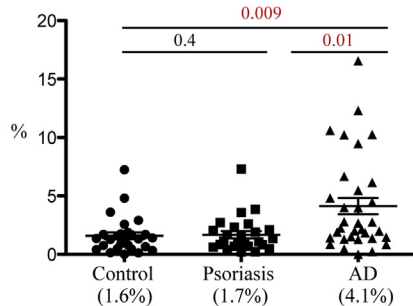
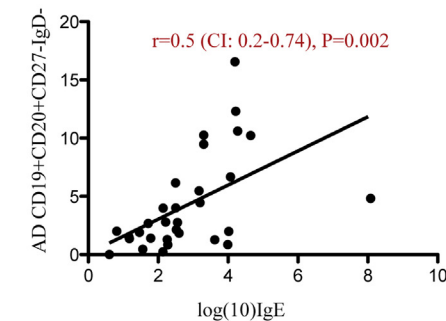
A IgE expression by CD19⁺CD20⁺CD27⁺IgD⁻ Switched-Memory B-cells**D****B** IgE expression by CD19⁺CD20⁺CD27⁺IgD⁺ NSM B-cells**E****C** IgE expression by CD19⁺CD20⁺CD27⁻IgD⁻ DN B-cells**F**

FIG 3. IgE expression by different memory B-cell subsets. **A-C**, Patients with AD had the highest IgE expression by CD19⁺CD20⁺CD27⁺IgD⁻ SwMe B cells (Fig 3, A), CD19⁺CD20⁺CD27⁺IgD⁺ NSM B cells (Fig 3, B), and CD19⁺CD20⁺CD27⁻IgD⁻ DN B cells (Fig 3, C). **D-F**, Significant correlations were obtained between total IgE levels and SwMe (Fig 3, D), NSM (Fig 3, E), and DN B-cell frequencies (Fig 3, C). Bar plots represent means $\pm$ SEMs.

Significant correlations were found between total IgE levels in serum and CD23 expression on SwMe ($r = 0.6$, $P = .0001$; see Fig E3, A, in this article's Online Repository at www.jacionline.org), NSM ($r = 0.65$, $P < .0001$; see Fig E3, B), naive ($r = 0.6$, $P = .0002$; see Fig E3, C), and DN ($r = 0.5$, $P = .003$; see Fig E3, D) subsets. Additionally, highly significant correlations were observed between IgE expression and CD23 expression in a given subset, including the SwMe ($r = 0.7$, $P < .0001$; see Fig E3, E), NSM ($r = 0.4$, $P = .009$; see Fig E3, F), and DN ($r = 0.37$, $P = .02$; see Fig E3, G) subsets.

CD19⁺CD24⁺⁺CD38⁺⁺ transitional B-cell counts are higher in patients with AD

Transitional B cells are the earliest functionally immature B-cell emigrants that can be found in blood.^{2,28,40} Similar to naive cells, transitional cells are CD27⁻, and in the IgD/CD27 scheme they colocalize with naive cells in the same quadrant. Thus for better identification of this subset, we used the CD24/CD38 gating strategy (Fig 5 and see Fig E1, B).

We found that although patients with psoriasis had lower transitional counts compared with those in control subjects (1.4% vs 2.3%, $P = .002$; Fig 5, A), patients with AD had significantly

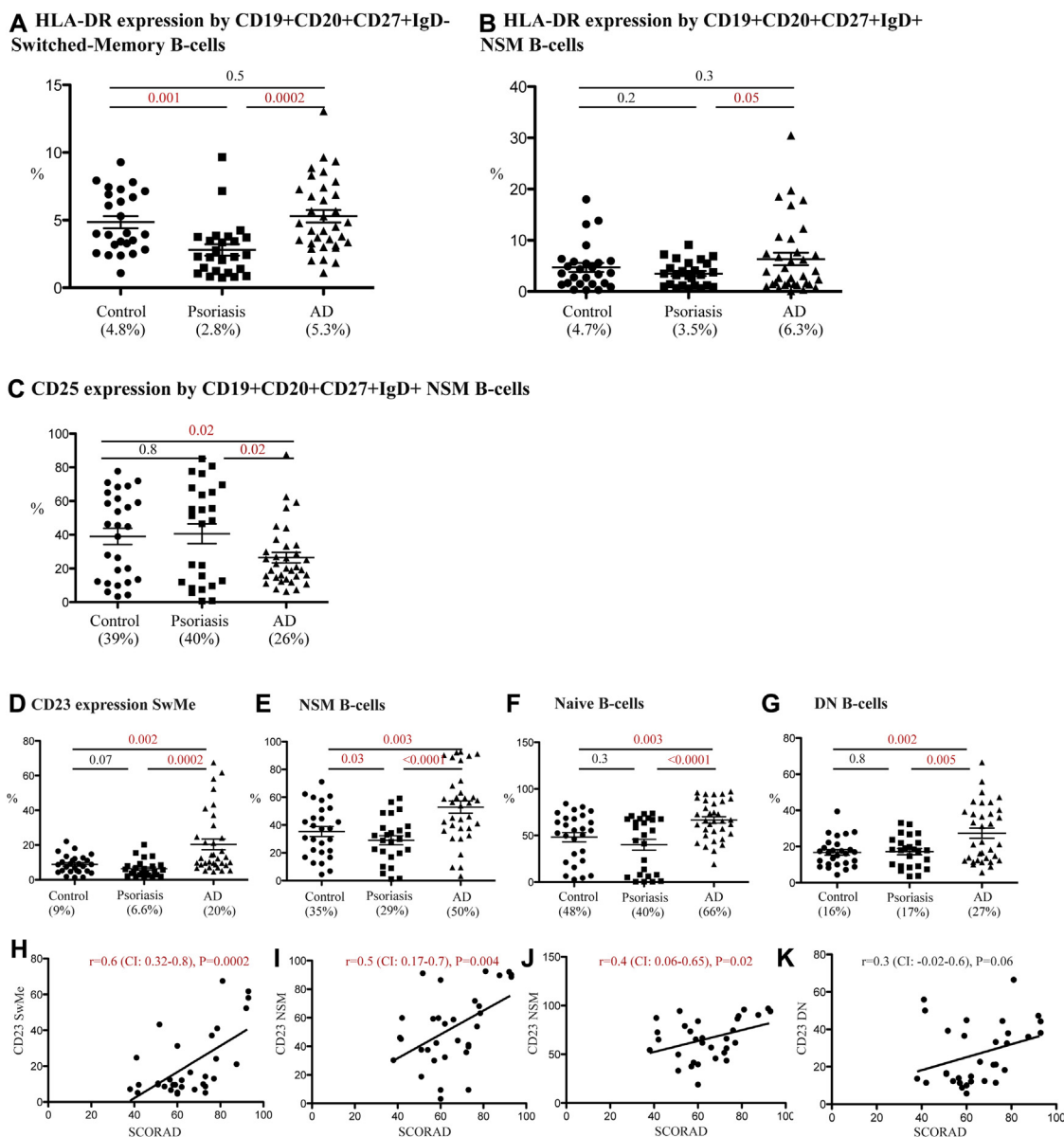


FIG 4. HLA-DR, CD25, and CD23 expression in control subjects, patients with psoriasis, and patients with AD. **A** and **B**, Patients with AD had higher HLA-DR expression compared with patients with psoriasis and control subjects in CD19⁺CD20⁺CD27⁺IgD⁻ SwMe (Fig 4, **A**) and CD19⁺CD20⁺CD27⁺IgD⁺ NSM (Fig 4, **B**) B cells. **C**, Lower CD25 expression was documented on CD19⁺CD20⁺CD27⁺IgD⁺ NSM B cells of patients with AD. **D-K**, CD23 expression was highest in all B-cell subsets of patients with AD (Fig 4, **D-G**) and correlated positively with disease severity (by SCORAD score; Fig 4, **H-K**). Bar plots represent means $\pm$ SEMs.

higher values compared with those in patients with psoriasis (2.8% vs 1.4%, $P = .001$; Fig 5, **A**), which correlated positively with IgE expression on NSM cells ($r = 0.5$, $P = .002$; Fig 5, **B**) and IgE levels in serum ($r = 0.6$, $P = .0008$; data not shown). Transitional cell frequency and disease severity were not correlated in either patients with psoriasis or those with AD ($P > .2$, data not shown).

Because we had existing cytokine expression among skin-homing (CLA⁺) cell data from another panel,⁴¹ we examined whether transitional B-cell counts were correlated with those cytokine subsets. In patients with AD, a trend toward negative correlation was found between transitional cell and IFN- γ ⁺/CLA⁺ frequencies ($r = -0.3$, $P = .1$; Fig 5, **C**), whereas

a positive trend was documented with IL-13⁺/CLA⁺ ($r = 0.3$, $P = .1$; Fig 5, **D**). In the psoriasis group a similar (although less significant) trend was documented for IL-13⁺/CLA⁺ cells ($r = 0.3$, $P = .14$; Fig 5, **E**), but negative correlation was not observed between transitional cells and IFN- γ ⁺/CLA⁺ cells in these patients ($P = .3$, data not shown).

CD19⁺CD24⁻CD38⁻ new memory B-cell counts are higher in patients with AD

CD19⁺CD24⁻CD38⁻ new memory B cells were first characterized by Buffa et al²⁷ as a cell population that is increased in the elderly and expanded in chronic stimulation states. Our

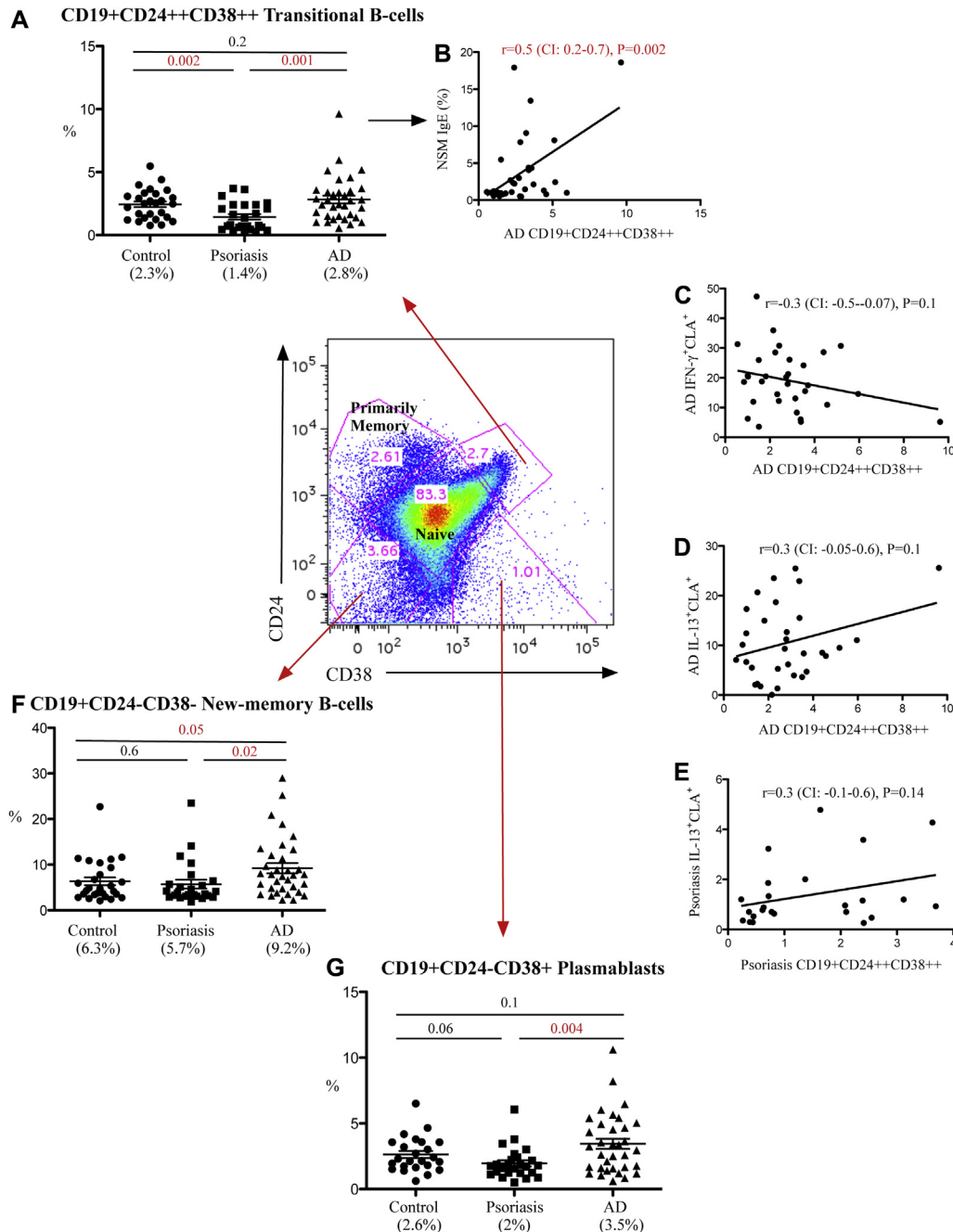


FIG 5. CD24/CD38 gating strategy and subsets. CD24 versus CD38 expression classifies circulating B cells into mature naive B cells (CD19⁺CD38^{int}CD24^{int}), primarily memory B cells (CD19⁺CD38⁻CD24⁺), plasmablasts (CD19⁺CD38⁺CD24⁻), and new memory (CD19⁺CD24⁻CD38⁻) and transitional (CD19⁺CD38⁺⁺CD24⁺⁺) B-cell subsets. **A-E**, Transitional cell counts were highest in patients with AD (Fig 5, A), positively correlated with IgE expression on NSM cells (Fig 5, B), negatively correlated with skin-homing (CLA⁺) IFN- γ T cells (Fig 5, C), and positively correlated with CLA⁺ T_H2 cell in patients with AD (Fig 5, D) and psoriasis (Fig 5, E). **F** and **G**, Patients with AD showed the highest frequency of new memory B cells (Fig 5, F) and plasmablasts (Fig 5, G). Bar plots represent means $\pm$ SEMs.

results showed that patients with AD had significantly higher frequencies of this subset compared with patients with psoriasis or control subjects (9.2% vs 5.7% and 6.3%, $P = .02$, and $P = .05$, respectively; Fig 5, F), but no correlations were found with disease severity (SCORAD/PASI scores) or IgE levels (not shown).

CD19⁺CD27⁺⁺CD38⁺⁺ plasmablasts and excess IgE production in patients with AD

Most antibody-secreting cells (ASCs) in the circulation are considered plasmablasts, as opposed to plasma cells, which are mainly located in the bone marrow.²³

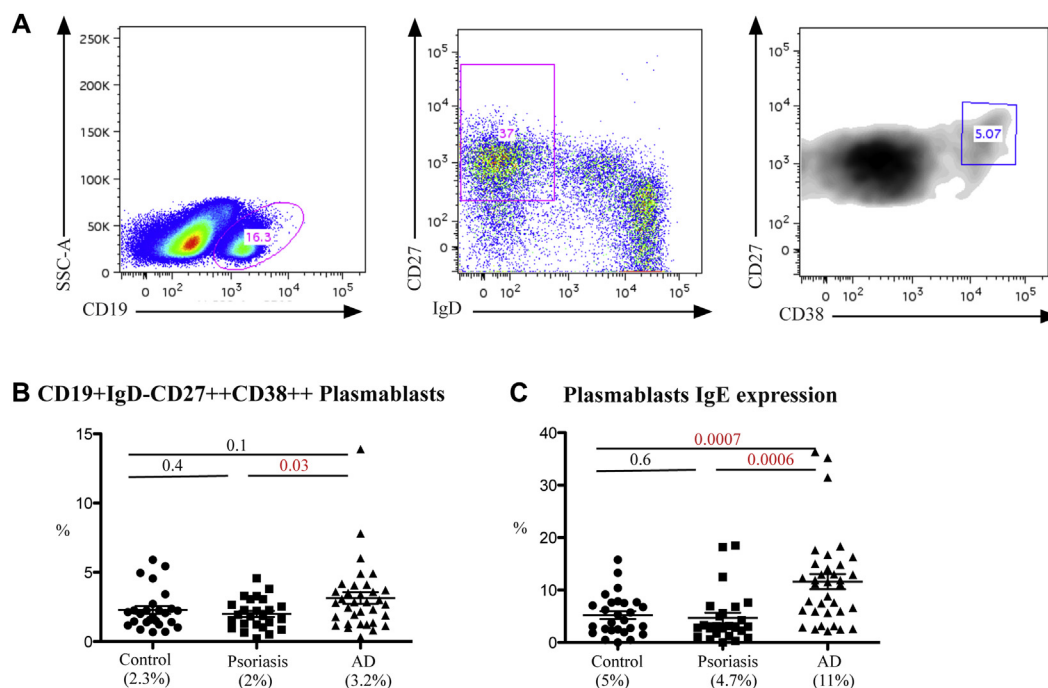


FIG 6. CD19⁺CD27⁺⁺CD38⁺⁺ plasmablast gating strategy, frequency, and IgE expression. **A**, CD19⁺CD27⁺⁺CD38⁺⁺ plasmablast gating strategy. **B** and **C**, Plasmablast frequency (Fig 6, **B**) and IgE expression (Fig 6, **C**) were highest in patients with AD. Bar plots represent means $\pm$ SEMs.

In the IgD/CD27 plot plasmablasts are embedded within the IgD⁻CD27⁺ quadrant, and thus different strategies are used to define these cells more accurately. The 2 most common approaches include (1) gating on CD19⁺ cells and then defining plasmablasts as CD24⁻CD38⁺ cells on the CD24/CD38 plot (Fig 5, **G**) or (2) gating on CD19⁺ cells and next defining CD27⁺IgD⁻ and CD27⁺⁺CD38⁺⁺ cells (Fig 6, **A**).

Because identification of this population might be challenging,²³ we have used both methods to classify plasmablasts. Our results showed that CD19⁺CD24⁻CD38⁺ or CD19⁺CD27⁺⁺CD38⁺⁺ plasmablast frequency was highest in patients with AD (3.5% vs 2% [$P = .004$; Fig 5, **G**] and 3.2% vs 2% [$P = .03$; Fig 6, **B**], respectively). Not only were these cells more common in patients with AD, but their IgE expression was highest (11% in patients with AD vs 4.7% in patients with psoriasis [$P = .0006$] and 5% in control subjects [$P = .0007$]; Fig 6, **C**).

DISCUSSION

B cells have traditionally been conceptualized as ASCs; however, they have diverse functions in the adaptive immune system, including antigen presentation, T-cell activation, and cytokine secretion. B cells are not only involved in T-cell activation and T_H2 production but can also differentiate into cytokine-producing cells that might alter effector T-cell differentiation.^{18,20,21}

Although prior mouse and human studies support the notion that B cells play a pathogenic role in patients with AD, no inclusive B-cell phenotyping has been published to date. Characterizing B-cell memory subsets, immunoglobulin switching, and premature populations is essential for a better understating of B-cell contributions to formation of the AD

immune profile. This is the first comprehensive study that phenotypes B-cell subsets in the circulation of patients with AD compared with those with psoriasis and control subjects, suggesting a unique B-cell signature in patients with AD. A similar B-cell profile between patients with psoriasis and control subjects supports the notion that B cells have limited contribution to the pathophysiology of skin lesions in patients with psoriasis.¹⁹

Higher percentages of chronically activated CD27⁺ memory B cells in patients with AD might relate not only to their high proliferative ability and enhanced T-cell stimulation^{31,42} but also to increased commitment toward plasma cell lineage differentiation⁴³ and a capacity to secrete 5- to 100-fold more immunoglobulins.^{31,44} This elicits higher IgE switching, as evidenced by the strong positive correlations observed with IgE expression. CD27⁺ memory B cells accumulate with cumulative antigen exposure,^{31,45} which might also account for their high numbers in patients with AD who have increased environmental and microbial antigen exposure.⁴⁶⁻⁴⁹ Positive correlation between high CD20⁺ cell counts in lesional skin of patients with AD and disease severity supports a possible role for B cells, and thus CD20 might serve as a biomarker in patients with severe AD.

There is a debate in the literature regarding the functional definition of IgD⁺CD27⁺ NSM cells.⁵⁰ They harbor somatic hypermutations and have characteristics of memory cells, but their development is independent of germinal center reactions,^{23,51} and they play a crucial role in secondary immune responses.^{25,52} Despite the fact that they are not generally considered an intermediate between naive and SwMe B cells,⁵² they represent a heterogeneous population, and at least some of these cells will class-switch into SwMe cells, whereas others retain the NSM phenotype.^{22,23}

Recent studies support NSM cell resemblance to IgG memory cells, and mouse studies have shown that these cells preserve long-term memory against protein antigens.^{53,54} According to the IgD/CD27 scheme provided here, NSM is the only memory subset that is expanded in peripheral blood of patients with AD. This might not only be attributed to chronic antigenic stimulation^{55,56} and enhanced immune responses in patients with AD⁵⁷ but also to higher plasticity of the immune system under a chronic inflammatory milieu.⁵⁸

Initial AD developmental mechanisms are still under investigation. It remains unclear whether enhanced T-cell activation³³ or aberrant B-cell stimulation with IgE switching⁵⁹ is the initial pathogenic mechanism. Significant correlations between T- and B-cell, particularly NSM B-cell, activation observed in patients with AD but not those with psoriasis or control subjects might not only emphasize NSM subset significance in AD pathogenesis but also suggest 2 consecutive pathways driving AD rather than a parallel chain of lymphocyte activation.

CD19⁺IgD⁻CD27⁻ DN memory cells are class-switched,⁶⁰ and in healthy subjects they mostly express IgG or IgA.⁶¹ Contrary to other memory subsets, their frequency is not increased with age, and their exact role is not entirely clear.² Our results show lower frequency of this subset in patients with AD. This might be secondary to their propensity to switch into IgA or IgG classes rather than IgE classes, levels of which have been shown to be increased among cells of this subset in our AD group.

CD20⁻CD27⁺⁺CD38⁺⁺ ASCs are mostly plasmablasts.²³ Although their numbers are physiologically low, they are increased in patients with certain diseases.⁶² As expected, we show here that patients with AD have higher plasmablast frequency accompanied by the highest IgE expression.

Past studies showed that CD23 expression is enhanced in patients with AD,⁶³ that it is induced by IL-4,^{64,65} and that these cells are important for IgE synthesis and augmentation of IgE responses.^{37,66} Consistently, and contrary to earlier reports in which CD23 did not correlate with IgE levels or AD severity,^{67,68} our results show higher CD23 expression in patients with AD, correlating with disease severity and IgE levels. This finding underlines the role of CD23 not only in IgE responses but also possibly in AD pathogenesis.

To gain a perspective on B-cell development and to create a basis for future pediatric studies, we also evaluated the premature transitional subset CD19⁺CD24⁺⁺CD38⁺⁺. Transitional cells represent approximately 1% to 2% of PBMCs in healthy subjects.⁶⁹ These are the youngest circulating B cells that eventually turn into mature competent naive cells²²; thus delineating their features is advantageous for understanding B-cell pathology. Transitional cells were shown to have regulatory capacity,⁶⁹ to inhibit T_H1 polarization of naive CD4⁺ T cells, and to help retain tolerance in immune-related diseases.⁶⁹⁻⁷¹ A positive correlation between transitional cells and IL-13⁺/CLA⁺ T-cell frequency in patients with AD is consistent with the positive correlation observed with IgE levels, supporting inhibited IFN- γ and an enhanced IgE-T_H2 axis in patients with AD.

Flores-Borja et al⁷² showed that in healthy subjects transitional B cells assist in converting effector CD4⁺ T cells into functional regulatory T (Treg) cells through IL-10 secretion. Recently, we have reported positive correlations between circulating Treg

cell counts in patients with AD and T-cell activation.³³ Moreover, although it is known that Treg cells in patients with psoriasis are dysfunctional,^{73,74} Ou et al⁷⁵ have shown that circulating Treg cells are functional in patients with AD. Thus in addition to possibly indicating higher systemic inflammation in patients with AD,³³ increased transitional B-cell counts might participate in converting CD4⁺ T cells into functional Treg cells, whereas their significantly low counts in patients with psoriasis and lack of IFN- γ suppression might indicate reduced suppressive capacity and contribute to the dysfunctional state of Treg cells in these patients.

Brisslert et al³⁶ showed that CD25⁺ expression on B cells is upregulated by Toll-like receptor (TLR) 2 and TLR4. These TLRs are an important part of the innate immune system.⁷⁶ TLR2, which is important not only for enhancing tight junction integrity⁷⁷ but also for defense against *Staphylococcus aureus* and herpes simplex virus infections, shows decreased expression in the epidermis of patients with AD.⁷⁷⁻⁸² In line with these data, our results show that CD25 expression is lower in patients with AD. Because CD25⁺ B cells have better antigen-presenting capacity and serve as a bridge between the adaptive and innate immune systems,³⁶ having lower CD25 expression adds to the reduced cutaneous immune defense that accompanies AD and might contribute to the propensity toward recurrent skin infections seen in this population.⁸³⁻⁸⁵

It has been formerly demonstrated that CD180 (RP-105/TLR-4 homologue) shows very low expression in new memory B cells (CD19⁺CD24⁻CD38⁻).²⁷ In patients with systemic lupus erythematosus, which, similar to AD, represents a state of chronic immune stimulation, B cells lack CD180 expression compared with those from control subjects.⁸⁶ Furthermore, when stratifying B-cell frequency according to age, the CD19⁺CD24⁻CD38⁻ population was found to be highest in the elderly, who have an exhausted immune system with low resistance to infections. Similarly, our results show that this cell population is significantly higher in patients with AD, further supporting the notion that the immune system of patients with AD is chronically stimulated, harboring a lower resistance against a myriad of infections. Finally, the CD24⁻CD38⁻ subset has been shown to induce TNF- α secretion and promote inflammation,²⁷ and thus its high frequency emphasizes the systemic inflammation that accompanies AD.

In conclusion, contrary to patients with psoriasis, in whom B-cell phenotyping to a large extent resembles that seen in healthy control subjects, a unique peripheral B-cell signature is seen in patients with AD. Higher percentages of certain B-cell subsets in patients with AD along with higher chronic activation support the findings of our recently published study showing higher immune activation in patients with AD versus those with psoriasis,³³ emphasizing the need for systemic therapeutic approaches in patients with severe AD.

Clinical implications: Because early B-cell stimulation might regulate pathologic T-cell activation in patients with AD, further studies would need to explore the sequence of B- and T-cell activation in young children.

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METHODS

Isolation of PBMCs and multiparameter flow cytometric blood analyses

PBMCs were obtained by means of gradient centrifugation with Ficoll-Paque Plus (Amersham Biosciences, Pittsburgh, Pa). Blood was laid under the Ficoll gradient; after spinning, PBMCs were collected at the interface between plasma and the Ficoll gradient and washed with PBS before staining for flow cytometry. For B-cell surface staining, PBMCs were washed and incubated for 30 minutes in ice with fluorochrome-conjugated mAbs to cell-surface molecules (CD19-V500, CD20-Brilliant Violet 650, CD24-Brilliant Violet 421, CD25-Pe CF 594, CD38-fluorescein isothiocyanate, CD27-Alexa Fluor 700, IgE-allophycocyanin, IgD-PeCy7, CD23-PerCpCy5.5, HLA-DR-allophycocyanin-H7, Live/dead-Blue Dye). Then the cells were fixed in ice with 4% paraformaldehyde (BD Biosciences, San Jose, Calif) for 20 minutes. Appropriate FMO-isotype controls were used.

The cells were acquired with the BD LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, Ore).

IHC

Frozen sections of normal ($n = 11$), AD lesional ($n = 32$) and nonlesional ($n = 18$), and psoriasis lesional ($n = 18$) and nonlesional ($n = 11$) skin biopsy specimens were blocked with 10% normal horse serum and stained with CD20 mouse mAb (Abcam, Cambridge, Mass). Biotin-labeled horse anti-mouse antibody (Vector Laboratories, Burlingame, Calif) was used to detect the mouse mAb. The staining signal was amplified with avidin-biotin complex (Vector Laboratories) and developed with chromogen 3-amino-9-ethylcarbazole (Sigma-Aldrich, St Louis, Mo). The number of positive cells per millimeter of epidermis surface length was counted manually by using computer-assisted image analysis (National Institutes of Health Image software 6.1; <http://rsb.info.nih.gov/NIH-image>).

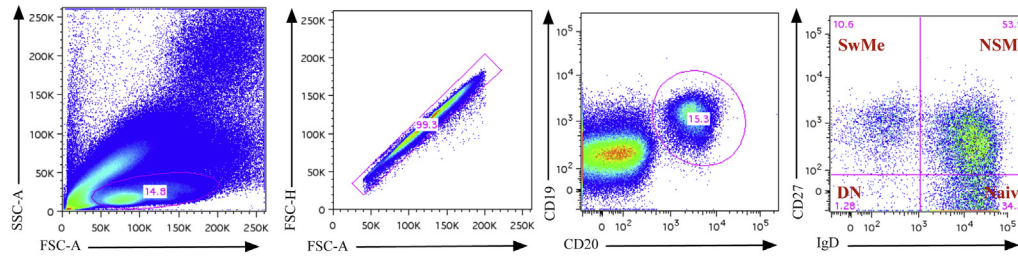
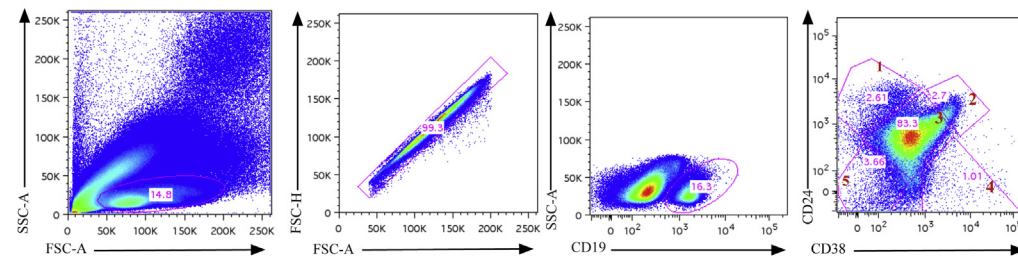
A IgD/CD27 Gating strategy**B** CD24/CD38 Gating strategy

FIG E1. B-cell gating strategies. **A**, IgD versus CD27 expression gating strategy classifies circulating B cells into IgD⁺CD27⁻ (naive), IgD⁺CD27⁺ (NSM), IgD⁻CD27⁺ (SwMe), and IgD⁻CD27⁻ (DN) subsets. In the IgD/CD27 plot numbers represent the relative percentage of the specific subset. **B**, CD24 versus CD38 expression classifies circulating B cells into (1) primarily memory B cells (CD19⁺CD38⁻CD24⁺), (2) transitional (CD19⁺CD38⁺CD24⁺), (3) mature naive B cells (CD19⁺CD38^{int}CD24^{int}), (4) plasmablasts (CD19⁺CD38⁺CD24⁻) and (5) new memory (CD19⁺CD24⁻CD38⁻) B-cell subsets. *FSC*, Forward scatter; *SSC*, side scatter.

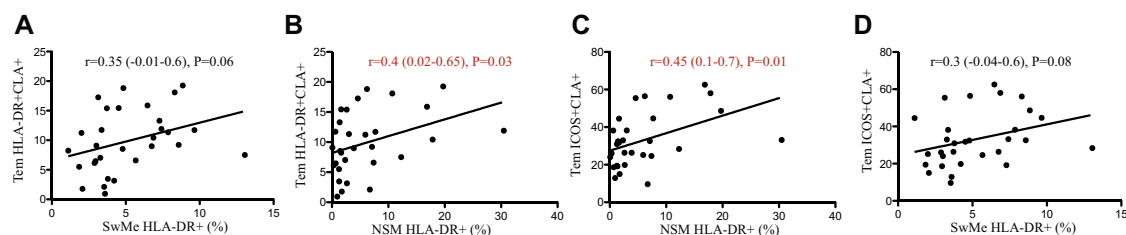


FIG E2. Activated skin-homing T_{EM} and T_{CM} T-cell correlations with activated SwMe and NSM memory B-cell subsets in patients with AD. **A** and **B**, Chronically HLA-DR-activated skin-homing T_{EM} cells were positively correlated with activated SwMe (Fig E2, **A**) and, more significantly, NSM (Fig E2, **B**) B cells. **C** and **D**, Intermediate inducible T-cell costimulator (*ICOS*)-activated skin-homing T_{EM} cells were positively correlated with activated NSM (Fig E2, **C**) and SwMe (Fig E2, **D**) B cells.

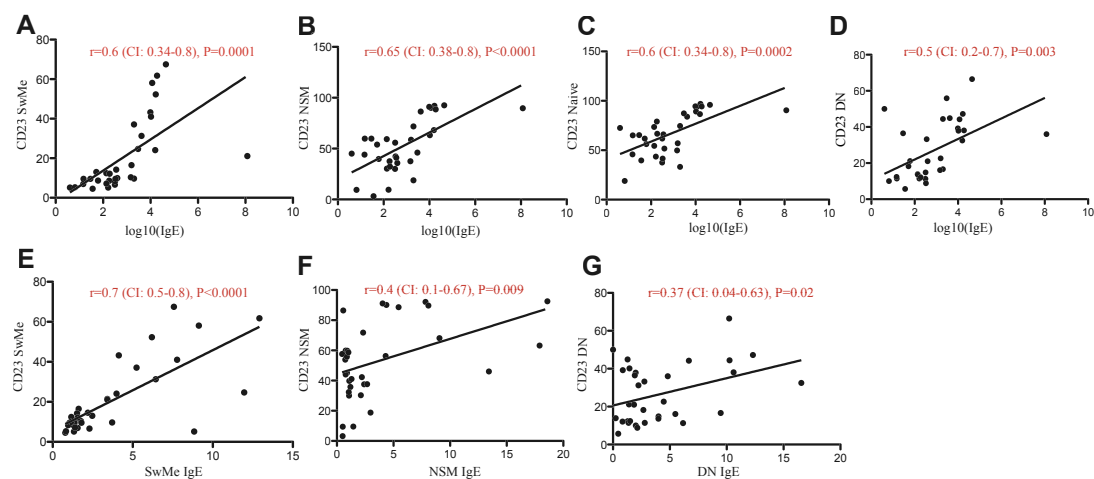


FIG E3. CD23 B-cell subset expression correlations with total IgE and IgE expression. **A-D**, CD23 expression was significantly correlated with total serum IgE levels in all B-cell subsets. **E**, Positive correlations were observed between CD23 and IgE expressions on SwMe NSM (**F**) and DN (**G**) B-cell subsets.

TABLE E1. Study data

B-cell subset	Control subjects (mean $\pm$ SEM)	Patients with psoriasis (mean $\pm$ SEM)	Patients with AD (mean $\pm$ SEM)
CD19 ⁺ cells	10 $\pm$ 2.046	8.231 $\pm$ 1.168	14.23 $\pm$ 1.848
CD19 ⁺ CD20 ⁺ cells	10.09 $\pm$ 2.306	8.1 $\pm$ 1.161	13.5 $\pm$ 1.888
CD19 ⁺ CD20 ⁺ IgD ⁻ CD27 ⁺ cells	16 $\pm$ 1.35	19 $\pm$ 2.2	15 $\pm$ 1.5
CD19 ⁺ CD20 ⁺ IgD ⁺ CD27 ⁺ cells	27 $\pm$ 3.5	26 $\pm$ 3.2	35 $\pm$ 2.26
CD19 ⁺ CD20 ⁺ IgD ⁺ CD27 ⁻ cells	51 $\pm$ 3.9	48 $\pm$ 4.3	46 $\pm$ 2.8
CD19 ⁺ CD20 ⁺ IgD ⁻ CD27 ⁻ cells	6 $\pm$ 0.96	7 $\pm$ 0.9	4 $\pm$ 0.4
CD19 ⁺ CD20 ⁺ IgD ⁻ CD27 ⁺ IgE ⁺ cells	1.4 $\pm$ 0.2623	0.9 $\pm$ 0.1248	3.6 $\pm$ 0.5674
CD19 ⁺ CD20 ⁺ IgD ⁺ CD27 ⁺ IgE ⁺ cells	1.9 $\pm$ 0.5941	1.4 $\pm$ 0.2040	3.6 $\pm$ 0.8120
CD19 ⁺ CD20 ⁺ IgD ⁻ CD27 ⁻ IgE ⁺ cells	1.6 $\pm$ 0.3030	1.7 $\pm$ 0.3071	4.1 $\pm$ 0.7045
CD19 ⁺ CD20 ⁺ IgD ⁻ CD27 ⁺ HLA-DR ⁺ cells	4.8 $\pm$ 0.4487	2.8 $\pm$ 0.4132	5.3 $\pm$ 0.4656
CD19 ⁺ CD20 ⁺ IgD ⁺ CD27 ⁺ HLA-DR ⁺ cells	4.7 $\pm$ 0.8701	3.5 $\pm$ 0.4891	6.3 $\pm$ 1.214
CD19 ⁺ CD20 ⁺ IgD ⁺ CD27 ⁺ CD25 ⁺ cells	39 $\pm$ 4.820	40 $\pm$ 5.806	26 $\pm$ 3.118
CD19 ⁺ CD20 ⁺ IgD ⁻ CD27 ⁺ CD23 ⁺ cells	9 $\pm$ 0.9984	6.6 $\pm$ 0.9634	20 $\pm$ 3.074
CD19 ⁺ CD20 ⁺ IgD ⁺ CD27 ⁺ CD23 ⁺ cells	35 $\pm$ 3.548	29 $\pm$ 3.295	50 $\pm$ 4.310
CD19 ⁺ CD20 ⁺ IgD ⁺ CD27 ⁻ CD23 ⁺ cells	48 $\pm$ 4.971	40 $\pm$ 5.844	66 $\pm$ 3.545
CD19 ⁺ CD20 ⁺ IgD ⁻ CD27 ⁻ CD23 ⁺ cells	16 $\pm$ 1.525	17 $\pm$ 1.701	27 $\pm$ 2.739
CD19 ⁺ CD24 ⁺ CD38 ⁺ cells	2.3 $\pm$ 0.2288	1.4 $\pm$ 0.2177	2.8 $\pm$ 0.3107
CD19 ⁺ CD24 ⁻ CD38 ⁻ cells	6.3 $\pm$ 0.8695	5.7 $\pm$ 0.9588	9.2 $\pm$ 1.117
CD19 ⁺ CD24 ⁻ CD38 ⁺ cells	2.6 $\pm$ 0.2709	2 $\pm$ 0.2356	3.5 $\pm$ 0.3906
CD19 ⁺ IgD ⁻ CD27 ⁺ CD38 ⁺ cells	2.3 $\pm$ 0.2823	1.99 $\pm$ 0.2189	3.2 $\pm$ 0.4257
CD19 ⁺ IgD ⁻ CD27 ⁺ CD38 ⁺ IgE ⁺ cells	5.1 $\pm$ 0.7535	4.68 $\pm$ 0.9911	11 $\pm$ 1.448
	AD, nonlesional	AD, lesional	
CD20 ⁺ IHC cell counts	5.8 $\pm$ 2.844	20 $\pm$ 3.567	
	Psoriasis, nonlesional	Psoriasis, lesional	
CD20 ⁺ IHC cell counts	5.2 $\pm$ 2.115	9 $\pm$ 1.816	
	Low CD20 ⁺ cell count	High CD20 ⁺ cell count	
SCORAD score	58.45 $\pm$ 3.741	75.38 $\pm$ 6.811	