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Glatiramer Acetate Stimulates Regulatory B Cell Functions

Kahina Amrouche,* Jacques-Olivier Pers,* and Christophe Jamin*,†

The control of the activities of regulatory B (Breg) cells in immune disorders is an emerging therapeutic strategy for the recovery of immune homeostasis. Manipulating B cells using numerous drugs in vivo affect their regulatory functions, although a direct link has not yet been demonstrated. Glatiramer acetate (GA) is a synthetic polypeptide that is used in the treatment of inflammatory and autoimmune diseases. We experimented on an in vitro coculture system to determine its direct effects on the Breg cell properties of human B cells. We found that GA improves the B cell–dependent control of T cells' immune responses. When B cells are stimulated by GA, the T cell proliferation and their Th1 IFN-γ production are further inhibited, whereas the B cell production of IL-10 is further enhanced. GA binds preferentially to the memory B cells and the activation of sorted B cell subsets shows that GA-dependent increased Breg cell activities are specifically supported by the B cells' memory compartment. Moreover, we found that the defective regulations that emerge from the B cells of systemic lupus erythematosus patients can be restored by GA stimulation. Overall, these data demonstrate that GA stimulates the Breg functions mainly by shifting the memory B cells known to contribute to the T cell–dependent inflammatory response into Breg cells. Our results also indicate that GA treatment could be a useful therapy for recovering the Breg cells in autoimmune situations in which their activities are defective. ***The Journal of Immunology*****, 2019, 202: 000–000.**

Regulatory B (Breg) cells, which were initially discovered in experimental allergic encephalomyelitis (EAE) (1), play a key role in the prevention and cure of many inflammatory and autoimmune diseases (2). In humans, several subpopulations have been identified and characterized as $CD24^{+}CD27^{high}$, $CD27^{+}CD38^{high}$, or $IgD^{+}CD24^{high}CD38^{high}CD5^{high}$ Breg cells (3–5). The ability to produce and secrete large amounts of IL-10 is a well-known feature of mouse Breg cells, which are responsible for the control of Th1 polarization (6–8). Human Breg cells can regulate both the proliferation of T cells and proinflammatory Th1 responses through distinct mechanisms. The regulation of T cell proliferation is IL-10 independent and occurs through the induction of $Foxp3^{+}$ regulatory T (Treg) cells, whose expansion is promoted by cell contact involving CD40 on B cells with CD40L on T cells. IL-10 produced by Breg cells plays a major role in the inhibition of Th1 polarization (9, 10). Furthermore, a functional impairment of Breg cells in some autoimmune conditions, such as systemic lupus erythematosus (SLE), has been described (3, 11). B cells are not sensitive to CD40 stimulation, are unable to regulate the proliferation of T cells, produce less IL-10, and are unable to efficiently control Th1 polarization (11, 12). The management of the Breg cell properties thus emerges as a promising therapeutic strategy to improve the treatment of autoimmune disorders (13).

Various drug treatments not predestined before can influence the production of IL-10 and could modulate the generation of Breg cells while also targeting different subpopulations of B cells (14). Among these drugs, synthetic polypeptide glatiramer acetate (GA) treatment increases the frequency of $CD5^{+}$ B cells in EAE mice and stimulates the production of IL-10 in vivo, and these activated B cells restrain the expansion of autoreactive T cells in vitro (15–17). In humans, GA could restore the production of IL-10 in the B cells of multiple sclerosis patients (18, 19). Taken together, GA appears to be an inducer of the Breg cell functions, although these in vivo observations do not indicate whether GA directly or indirectly affects B cells. The present study aims to first analyze the direct action of GA on the regulatory properties of normal human B cells on autologous T cell responses and, second, to assess its ability to modify the Breg cell activities in SLE patients, who are the prototypic model of Breg cell deficiencies (3, 11, 12). We demonstrated a direct potentiating action of GA on Breg cells. GA especially converts the phenotypic profile and stimulates the regulatory activities of memory B cells. Intriguingly, GA restores deficient Breg cells in SLE. GA-stimulated SLE B cells recover their control of T cell proliferation, upregulate their capacity to produce significant amounts of IL-10, and finally retrieve their inhibitory action on the Th1 proinflammatory response. These findings demonstrate that GA is a direct activator of Breg cells indicating that GA should be considered an important factor of targeted Breg cell therapies in autoimmune diseases.

Materials and Methods

Isolation of cells

Peripheral blood was collected from control laboratory staff donors and patients with SLE fulfilling the criteria of the disease. Tonsils were obtained from children undergoing routine tonsillectomies. All volunteers and parents of children gave informed consent in accordance with the Declaration of Helsinki, and the study was approved by the Institutional Review Board at the Brest University Medical School Hospital. The tissues were minced up and filtered to remove fragments and clumps. Blood samples and tonsillar cell suspensions were layered onto a Ficoll-Hypaque and centrifuged. Mononuclear cells were incubated with neuraminidase-treated sheep RBCs, and the T cells were depleted with a second 30-min round of centrifugation. B and T cells were

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K.A. and C.J. wrote the manuscript. K.A., J.-O.P., and C.J. planned the experiments. K.A. performed the experiments. K.A., J.-O.P., and C.J. performed data analysis. All authors reviewed and approved the manuscript.

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Abbreviations used in this article: Breg, regulatory B; EAE, experimental allergic encephalomyelitis; GA, glatiramer acetate; HD, healthy donor; PE-Cy7, PE linked to cyanin 7; SLE, systemic lupus erythematosus; Treg, regulatory T.

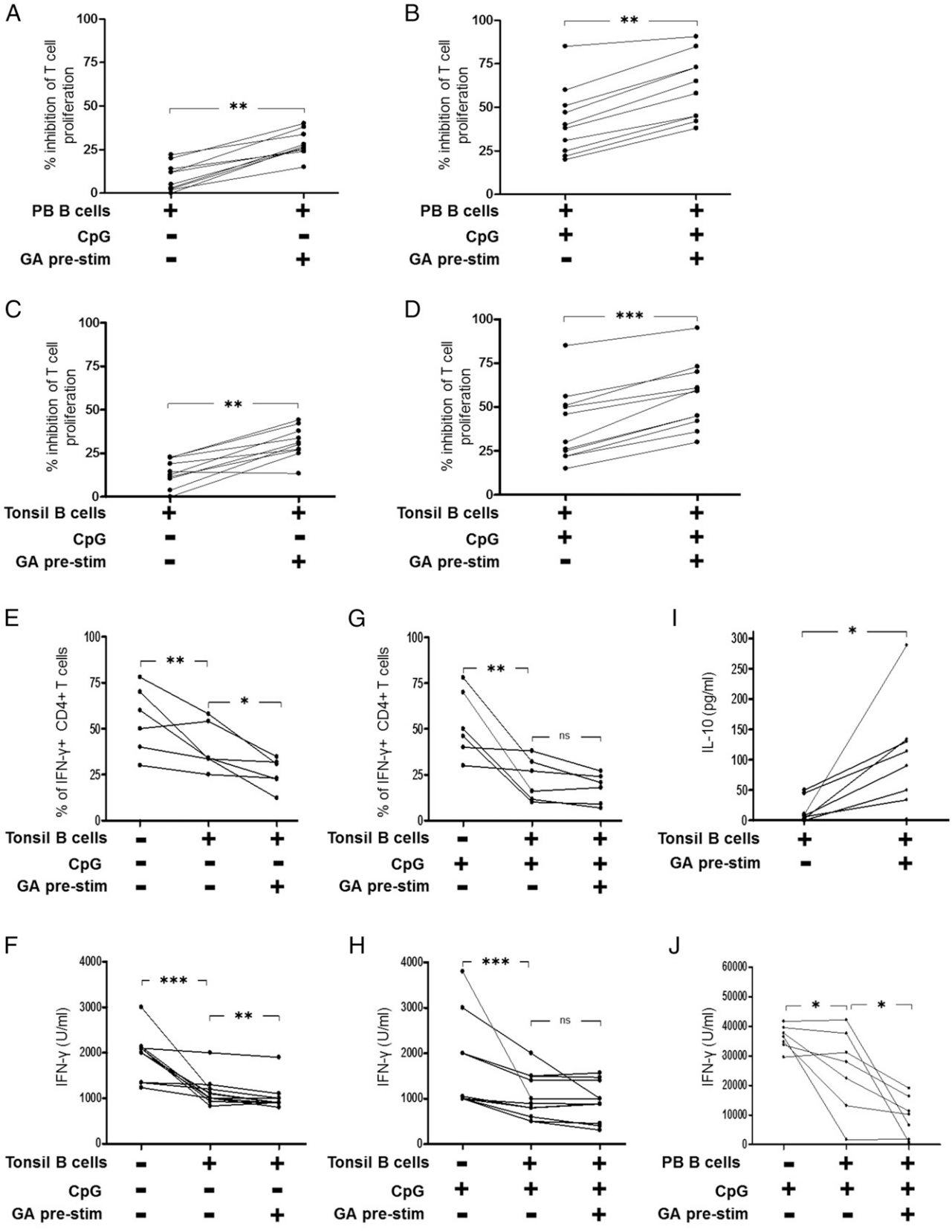

FIGURE 1. The regulation of T cell proliferation and Th1 polarization are enhanced by GA. T cells from the peripheral blood (PB) of HD (**A**, **B**, and **J**) and from tonsillar samples (**C**–**H**) were stimulated and cocultured with autologous B cells prestimulated or not with GA. The inhibition of T cell proliferation was evaluated without (A and C) and with (B and D) TLR9 activation of B cells with CpG/ODN. The level of IFN-γ production was evaluated without (E and F) and with (G, H, and J) TLR9 activation of B cells, with CpG/ODN as the percentages of IFN-γ+ cells among the $CD4^+$ T cells (E and G) and as the concentration of secreted IFN-γ in the supernatants of cultured and cocultured cells (F, H, and J). (**I**) Tonsillar B cells were activated or not with GA, and the production of IL-10 was measured. Significant differences were estimated using the Wilcoxon test. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. ns, not significant.

further purified using negative selection with cell-enrichment kits (StemCell Technologies) according to the manufacturer's procedure. All preparations were >95% pure $CD19^+$ B cells and >98% pure $CD19^-CD5^+$ T cells. Tonsillar B cells were sorted on the basis of CD19, CD38, and IgD surface expression using a MoFlo XDP cell sorter (Beckman Coulter) and then were washed in RPMI 1640 medium before culture. Naive mature $CD19^+IgD^+CD38^{+/-}$ and memory $CD19^+IgD^-CD38^-$ B cell subsets were >98% pure.

Conjugation of GA with FITC

For phenotypic studies, GA (Teva Pharmaceutical Industries) was conjugated with FITC using the standard procedure of Amine-Reactive Probes (Molecular Probes; Invitrogen) according to the manufacturer's instructions. Briefly, GA was dissolved in a serum-free RPMI 1640 medium to a concentration of 10 mg/ml and prepared for conjugation with FITC by dialysis in a carbonate/bicarbonate buffer (pH 9.5). Fluorescein-5-thiosemicarbazide (Molecular Probes; Invitrogen) was then added with 100 μg in DMSO for each 1 mg of GA for 1 h at 37°C. Excess FITC was removed by extensive dialysis against PBS. GA-devoid preparation was examined in parallel to guarantee that the fluorescence came from conjugated FITC/GA and not from dissociated FITC molecules. The preparation was assessed in a UV2 spectrophotometer (Unicam) by reading the absorbance at 488 and 518 nm. BSA that was similarly prepared was used as a negative control.

Cell culture

Freshly isolated B cells were cultured in 24-well plates in a RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine (Invitrogen; Life Technologies), 200 U/ml penicillin, and 100 μg/ml streptomycin. For stimulation, B cells or sorted B cell subsets were seeded at 1×10^6 cells/ml and cultured for 4 h alone or in the presence of 20 μg/ml GA. The cells were harvested and washed twice before the coculture experiments were conducted.

T cells were seeded at 1×10^6 cells/ml (tonsil samples) or 4×10^5 cells/ml (blood samples) on anti-mouse IgG/Fc-coated, 96-well plates in a complete RPMI 1640 medium (Jackson ImmunoResearch Laboratories, West Grove, PA). To induce their proliferation, the cells were stimulated for 4 d with 0.2 μg/ml anti-CD3 (clone OKT3) and anti-CD28 (clone CD28.2; Beckman Coulter, Marseille, France) mAbs. For the coculture experiments, B cells or sorted B cell subsets prestimulated or not with GA were added to T cells in the presence or not of 0.25 μM CpG/ODN 2006 (InvivoGen, San Diego, CA) for 4 d at a 1B:1T ratio.

Proliferation assays

Freshly isolated T cells or sorted B cells were labeled with 5 mM CFSE (Molecular Probes) before stimulation. A decrease of the CFSE mean fluorescence intensity is directly proportional to cell division. The level of proliferation was evaluated using a flow cytometer (FC500; Beckman Coulter) by analyzing the percentage of dividing cells at day 4. To induce their proliferation, the sorted B cells were stimulated for 4 d with 0.25 μM CpG/ODN 2006 and 1 μg/ml Human CD40-Ligand Multimer Kit containing the CD40 ligand and a cross-linking Ab for multimerization (Miltenyi Biotec, Bergisch Gladbach, Germany). Furthermore, in the coculture experiments, the suppressive effect of the B cells was appreciated by calculating the percent of the inhibition of the proliferation of T cells analyzed in the CD4-positive cells after staining with PE linked to cyanin 7 (PE-Cy7)–conjugated anti-CD4 mAb, according to the following formula: percentage of cells with more than three divisions in the T cells alone minus percentage of cells with more than three divisions in T cells in the presence of B cells divided by percentage of cells with more than three divisions in the T cells alone multiplied by 100.

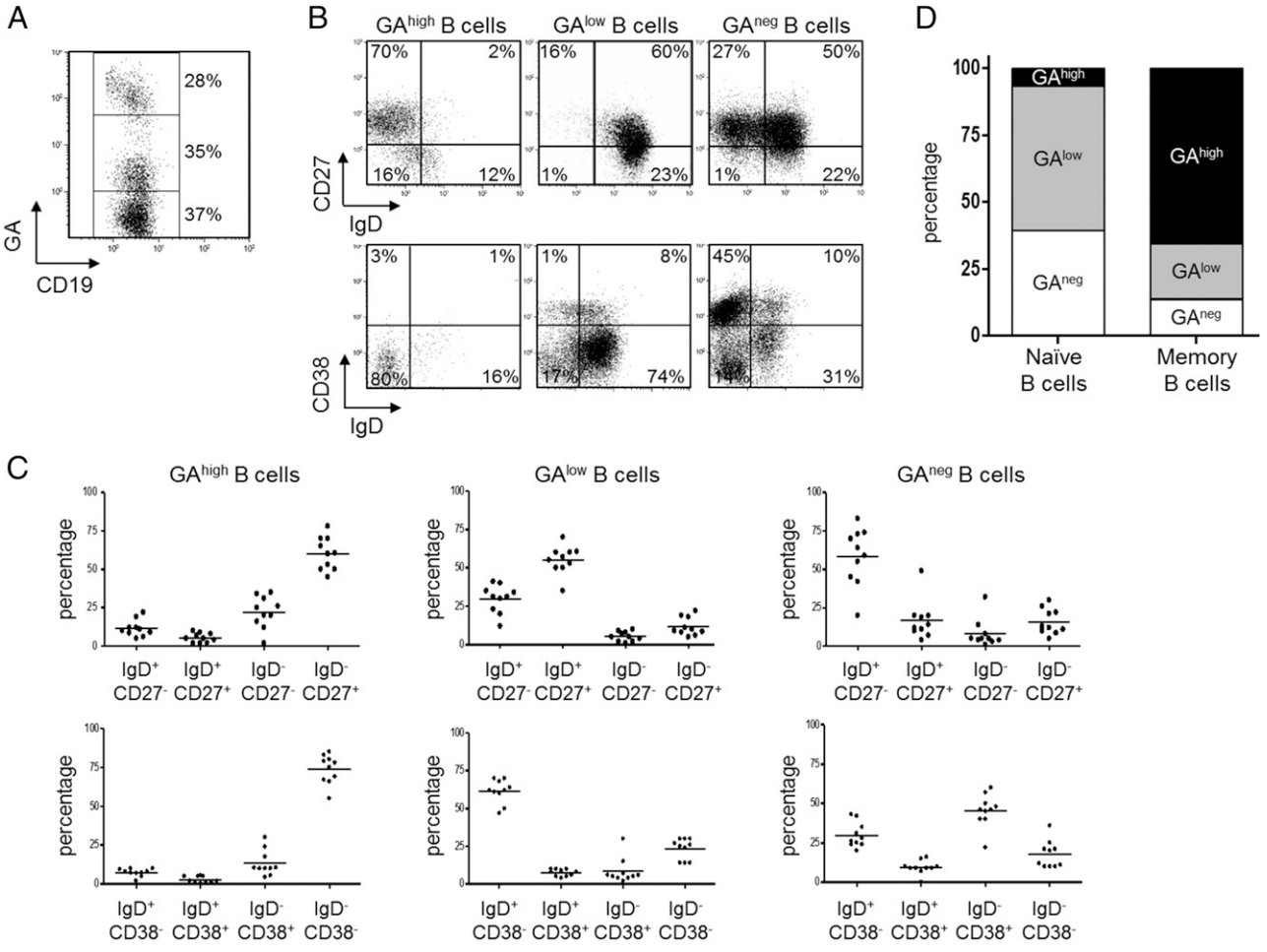

FIGURE 2. GA binds preferentially to the memory B cells of secondary lymphoid organs. Tonsillar B cells were analyzed using flow cytometry after staining with FITC-conjugated GA and (**A**) anti-CD19 mAb and (**B**) combinations of anti-CD19/anti-IgD/anti-CD27 and anti-CD19/anti-IgD/anti-CD38 mAbs. Representative examples of 10 independent experiments. (**C**) Distribution of the B cell subsets within GA^{high}, GA^{low}, and GA^{neg} B cells. (**D**) Distribution of GA^{neg}, GA^{low}, and GA^{high} cells among the naive and memory B cells. Mean of 10 experiments.

ELISA

Concentrations of IFN-γ and IL-10 in the supernatants of cultured and cocultured cells were measured with commercial ELISA kits using paired Abs according to the manufacturer's instructions (Beckman Coulter). RPMI 1640 complete medium was used as a negative control.

Flow cytometry

All mAbs were purchased from Beckman Coulter unless otherwise specified. PE/Texas Red (ECD)–conjugated anti-CD5 (clone BL1a), allophycocyanin-conjugated anti-IgD (clone IA6-2), PE-Cy7–conjugated anti-CD27 (clone 1A4CD27), PE-Cy5.5–conjugated anti-CD38 (clone LS198-4-3), allophycocyanin/Alexa Fluor 700–conjugated and PE-Cy7–conjugated anti-CD19 (clone J3-119), allophycocyanin/AF750-conjugated anti-CD24 (clone ALB9), and PE-conjugated anti–IL-21R (clone 17A12) were used for the phenotypic studies. For the intracellular detection of IFN-γ, the cells were first incubated for 4 h with the BD GolgiStop Protein Transport Inhibitor containing monensin (BD Biosciences). They were stained with PE-Cy7–conjugated anti-CD4 mAb and then fixed and permeabilized with the Cytofix/Cytoperm Kit (BD Biosciences) and incubated with FITC-conjugated anti–IFN-γ Ab (R&D Sytems). Cell viability was assessed using the DRAQ7 Far-Red Live-Cell Impermeant DNA Dye (Beckman Coulter).

Statistical analysis

All data were expressed as mean ± SEMs. Statistical analyses were performed with GraphPad Prism version 4 (GraphPad Software, La Jolla, CA) using Mann–Whitney *U* and Wilcoxon tests for comparisons of the unpaired and paired values, respectively. Significant differences were estimated at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results

The GA prestimulation enhances the suppressive properties of human B cells

We first explored the direct effect of GA on the Breg cell functions in healthy donors (HD). Without activation, the B cells had weak abilities to control the T cell responses, which were significantly improved when they were prestimulated with GA. Thus, the inhibition level of T cell proliferation increased from 9.3% ± 2.5 to 28.3 ± 2.3% for the peripheral blood B cells (Fig. 1A) and from 13.5% ± 2.8 to 31.5 ± 2.8% for the tonsillar B cells (Fig. 1C). GA also ameliorated the capacity to control Th1 polarization. The frequencies of IFN-γ^+CD4$^+$ T cells were attenuated from 56.3 ± 7.8 to 39.6 ± 5.3% in the presence of nonstimulated cells and further to 25.8 ± 3.3% in the presence of GA-prestimulated tonsillar B cells (Fig. 1E). This outcome was also associated with lower IFN-γ secretion in the supernatants, which was reduced from 1895 ± 205 to 1350 ± 116 U/ml and further decreased to 1000 ± 108 U/ml in the presence of nonstimulated B cells and GA-prestimulated B cells, respectively (Fig. 1F). These results establish that GA directly affects the human B cells from both peripheral blood and secondary lymphoid organs, stimulating their regulatory properties.

TLR9 stimulation is known to activate the B cells' regulatory functions (3, 20). The activation of B cells by CpG/ODN in the cocultures ameliorated their suppressive capacities, which were further enhanced by GA prestimulation. The inhibition of T cell proliferation was thus increased to 41 ± 5% and was further enhanced to 61 ± 6% for the peripheral blood B cells (Fig. 1B) and from 41.9 ± 6 to 61.5 ± 5.9% for the tonsillar B cells (Fig. 1D). Moreover, Th1 polarization was also further restrained for peripheral blood B cells. TLR9-stimulated tonsillar B cells reduced the frequencies of IFN-γ^+CD4$^+$ T cells from 52.3 ± 7.4 to 22.4 ± 4.7% and to 17.6 ± 3.3%; however, this was not significantly different when the cells were prestimulated with GA (Fig. 1G). This effect was associated with a decreased IFN-γ secretion from 1917 ± 178.9 to 1152 ± 372 U/ml in the presence of TLR9-stimulated tonsillar B cells but similarly to 1021 ± 164.9 U/ml following GA prestimulation (Fig. 1H). With TLR9-stimulated peripheral blood B cells, IFN-γ secretion was reduced from 36,160 ± 1509 to 25,135 ± 5319 U/ml and was significantly further inhibited to 9391 ± 2603 U/ml when the cells were GA prestimulated (Fig. 1J). Overall, these results indicate that GA acts synergistically with TLR9 on peripheral blood B cells and tonsillar B cells to increase the Breg cell effectiveness on the

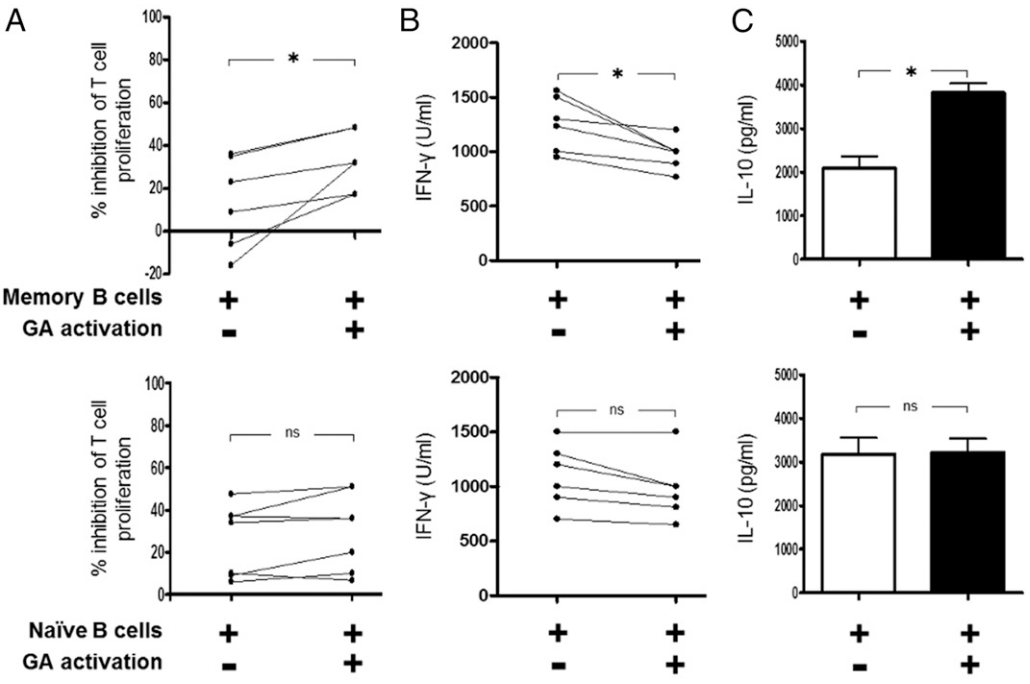

FIGURE 3. GA stimulates the regulatory activities of memory B cells. Memory and naive tonsillar B cells were sorted, activated by GA, and cocultured with stimulated autologous T cells. The inhibition of the T cell proliferative response (**A**) and the level of IFN-γ production (**B**) were evaluated. GA-activated sorted B cells were stimulated with CpG and human CD40 ligand, and the level of IL-10 production (**C**) was evaluated. Mean ± SEM of six experiments. Significant differences were estimated using the Wilcoxon test. $*p < 0.05$. ns, not significant.

control of T cell proliferation and on the peripheral B cells for the control of Th1 polarization. The B cell production of IL-10 was also significantly increased from 19.5 ± 9.3 to 133.3 ± 3 pg/ml upon GA prestimulation (Fig. 1I).

GA targets the memory compartment

To identify the populations supporting the effects of GA, tonsillar B cells were incubated with FITC-conjugated GA and analyzed using flow cytometry (Fig. 2A). Twenty-eight percent of the B cells strongly fixed GA (GA^{high} B cells), 35% weakly fixed GA (GA^{low} B cells), and the remaining cells did not fix GA at all (GA^{neg} B cells). Phenotypic analyses (Fig. 2B) revealed that the GA^{high} population mainly contained $CD19^+CD27^+IgD^-CD38^-$ memory B cells, whereas GA^{low} B cells mostly encompassed $CD19^+IgD^+CD27^{+/-}CD38^{+/-}$ transitional and naive B cells (Fig. 2C). Overall, $IgD^+CD38^{+/-}$ naive B cells included more than 53% of GA^{low} cells and fewer than 7% of GA^{high} cells, whereas IgD^-CD38^- memory B cells comprised more than 65% of GA^{high} cells and <21% of GA^{low} cells (Fig. 2D). It was recently demonstrated that memory B cells are not efficient in controlling T cell proliferation compared with transitional and naive B cells (20). To determine whether the GA-induced increasing suppressive functions were the result of specific population activation, memory and naive B cells were sorted, prestimulated with GA for 4 h, and cocultured with autologous T cells in the presence of CpG/ODN. GA improved the memory B cell control of the T cell proliferation, enhancing the inhibitory activity from 14.7 ± 9 to 34.6 ± 6%, but did not affect naive B cells, which remained stable (28 ± 6.5% versus 30.6 ± 6.6%) (Fig. 3A). The GA-dependent, increased regulation of Th1 polarization was also because of a specific effect on memory B cells that significantly reduced the IFN-γ production from 1257 ± 109 to 1030 ± 73.7 U/ml in cocultures, whereas IFN-γ secretion remained unchanged in the presence of naive B cells (1117 ± 122.7 versus 1002 ± 118 U/ml) (Fig. 3B). Finally, the production of IL-10 after GA prestimulation was assessed following 4 d of B cell stimulation with CD40L and CpG/ODN (Fig. 3C). Without GA prestimulation, memory B cells produced less IL-10 than naive B cells (2025 ± 4.2 pg/ml versus 3110 ± 3 pg/ml). After GA prestimulation, their production was significantly increased to 3755 ± 6.5 pg/ml, whereas that of naive B cells remained unimproved at 3130 ± 6 pg/ml. Moreover, subsequent to GA prestimulation, the proliferation of CD40/TLR9-stimulated memory B cells was repressed, whereas that of naive B cells was not affected (Fig. 4A). Cell survival without GA prestimulation was 86 ± 4 and 72.7 ± 2.5% for the memory B cells and the naive B cells and was sustained at 87 ± 2 and 74 ± 4% after GA prestimulation, respectively (Fig. 4B). These results exclude the possibility that GA-enhanced Breg cell activities result from an expansion or a higher viability of the memory B cell population, instead indicating that GA intrinsically increases the regulatory capacities of memory B cells.

GA modifies the phenotype of memory B cells

To decipher the GA-induced improvement of memory B cell regulatory properties, additional phenotypic analyses were performed. At the onset of the coculture (day 0), no tonsillar memory B cells and few naive B cells expressed CD5 molecules (Fig. 5A). After 4 d of coculture with T cells (day 4), CpG/ODN activation induced an increased frequency of $CD5^+$ cells in memory B cells and even more in naive B cells (35.7 ± 3% versus 61 ± 3.6%, respectively). Interestingly, the proportion of $CD5^+$ cells among memory B cells was significantly upregulated (53.5 ± 6.1%) after GA prestimulation (Fig. 5A, 5B), whereas that of naive B cells remained unchanged (60.8 ± 4.2%). At the onset of the coculture (day 0), neither the memory nor naive B cells expressed IL-21 receptors (Fig. 5C).

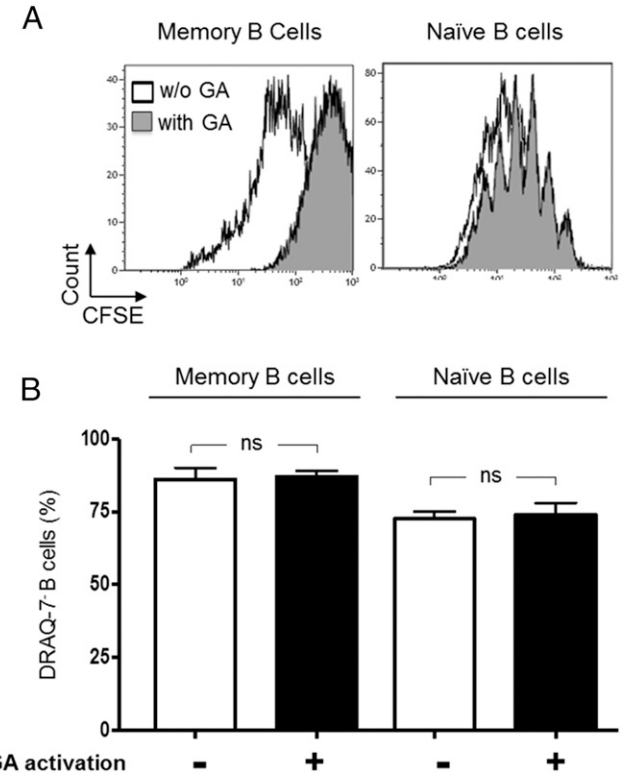

FIGURE 4. GA does not promote the proliferation and a higher survival of B cells. Memory and naive B cells were sorted and activated by GA for 4 h. The cells were stained with CFSE and stimulated with CpG and human CD40 ligand for 4 d. (**A**) B cell proliferation was evaluated with flow cytometry. Representative examples of six experiments. (**B**) B cell viability was assessed using DRAQ7 Far-Red Live-Cell Impermeant DNA Dye. Mean ± SEM of six experiments. Significant differences were estimated using the Wilcoxon test. ns, not significant.

After 4 d of coculture (day 4), the frequency of $IL\text{-}21R^+$ memory B cells was lower than that of naive B cells (17.3 ± 2.5% versus 42.1 ± 5.2%, respectively). Following the prestimulation with GA, a significant increased proportion of $IL\text{-}21R^+$ memory B cells was observed (28.7 ± 2.4%), whereas that of naive B cells remained unchanged (40.3 ± 5.5%) (Fig. 5C, 5D). Overall, these experiments demonstrate that the GA-specific effects on memory Breg cell functions are associated with specific enhanced CD5 and IL-21R expressions that are known to be associated with suppressive properties (21, 22).

GA also controls peripheral blood B cells

Similar to secondary lymphoid organ B cells, a subset of peripheral blood B cells from HD and from SLE patients highly bound the GA (17 and 25%, respectively), another subset bound weakly (47 and 43%, respectively), and a third subset did not bind the GA molecules (Fig. 6A). In-depth phenotypic studies (Fig. 6B, 6C) indicated that the GA^{high} B cells depicted a $CD19^+CD24^{low}CD38^{-/low}IgD^-CD27^+$ memory B cell profile and the GA^{low} B cells a $CD19^+CD24^{low/high}CD38^{low/high}IgD^+CD27^{+/-}$ transitional and naive profiles in HD B cells, as well as in SLE B cells (Fig. 6D).

Additionally, although CD5 was weakly expressed on HD and SLE B cells at the onset of the cocultures (day 0), CpG/ODN activation (day 4) promoted upregulation (Fig. 7A), showing a higher frequency of $CD5^+$ B cells in HD than in SLE patients (60.5 ± 2.2% versus 33.5 ± 5.4%) (Fig. 7B). In association with GA prestimulation (Fig. 7C), the proportion of $CD5^+$ cells increased to 65.1 ± 2.7% in HD and 44.5 ± 5.8% in SLE patients.

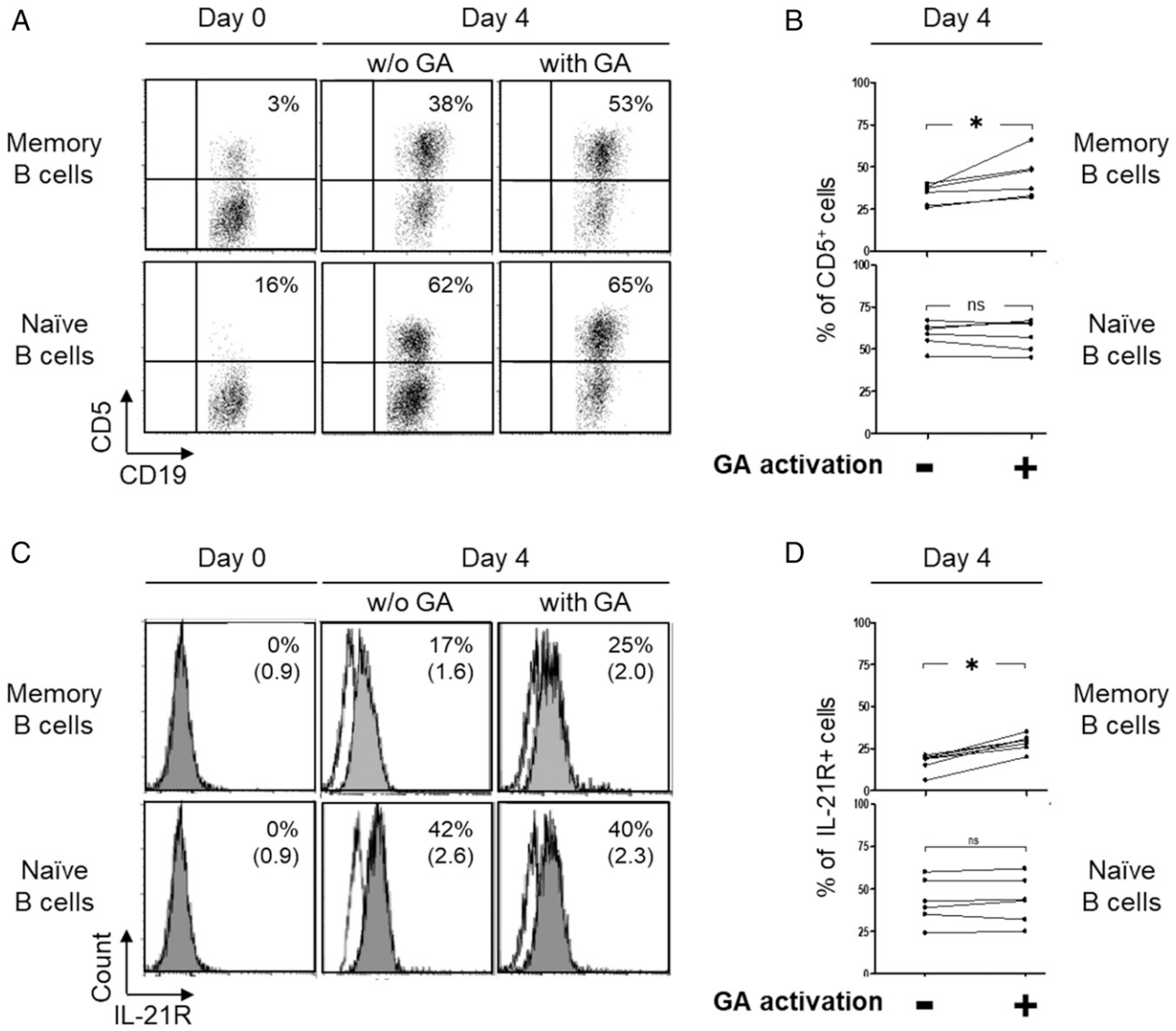

FIGURE 5. GA promotes a regulatory phenotype on memory B cells. Memory and naive tonsillar B cells were sorted, activated by GA, and cocultured with stimulated autologous T cells. The cells were stained and analyzed with flow cytometry using anti-CD19, anti-CD5 (**A** and **B**), and anti–IL-21R (**C** and **D**) mAbs. Representative example of six experiments (A and C) with isotype control shown as white histograms. Frequencies of $CD5^+$ cells (B) and IL-$21R^+$ cells (D). Significant differences were estimated using the Wilcoxon test. $*p < 0.05$. ns, not significant.

In addition, after 4 d of cocultures with autologous T cells in the presence of CpG/ODN, flow cytometry analyses of the peripheral blood cells revealed an induced expression of IL-21R on HD, but not on SLE B cells (62.3 ± 4.3% versus 4 ± 1%) (Fig. 7D, 7E). Prestimulation with GA resulted in a slight 1.1 increase of IL-$21R^+$ B cell frequency in HD (67.83 ± 3%), but a 3.7 upregulation for SLE B cells (15.1 ± 1.2%) (Fig. 7F). Overall, these experiments demonstrate that the GA presensibilization of peripheral HD and SLE B cells induces the expression of the CD5 and IL-21R Breg cell markers.

The defective suppressive properties of SLE B cells are restored following GA presensibilization

We finally evaluated whether the GA-induced Breg cell markers on SLE B cells were associated with the appearance of efficient Breg cell functions. Without any activation, the inhibition of T cell proliferation was barely detectable (2.4 ± 0.4%). However, prestimulation using GA restored efficient Breg cell activity, enhancing the inhibition to 24.1 ± 3.5% (Fig. 8A). As previously described, TLR9 stimulation of SLE B cells by CpG/ODN did not induce any regulatory activity. Thus, the inhibition of T cell proliferation remained unchanged at 2.5 ± 0.4% (Fig. 8B), confirming SLE Breg cell deficiency. GA prestimulation of SLE B cells associated with TLR9 stimulation during coculture did not generate any synergistic effect because the inhibition of T cell proliferation increased only to the level reached without CpG/ODN (30.5 ± 5%) (Fig. 8B). Furthermore, GA prestimulation of SLE B cells caused a significant increase of IL-10 production from 224.9 ± 4 to 388.8 ± 46.1 pg/ml (Fig. 8C). The ability to regulate Th1 polarization was restored as well. The concentration of IFN-γ remained unchanged when the T cells were cocultured with B cells in the presence of CpG/ODN (24,478 ± 3405 U/ml versus 34,556 ± 5489 U/ml, respectively), but decreased when B cells were prestimulated with GA (14,577 ± 5278 U/ml) (Fig. 8D). These experiments demonstrate that GA can restore defective SLE Breg cell properties despite there being deficient TLR9 signals.

Discussion

It is now widely accepted that Breg cells are key elements in the control of several diseases and hence are promising therapeutic targets for the restoration of immune homeostasis (13). Numerous

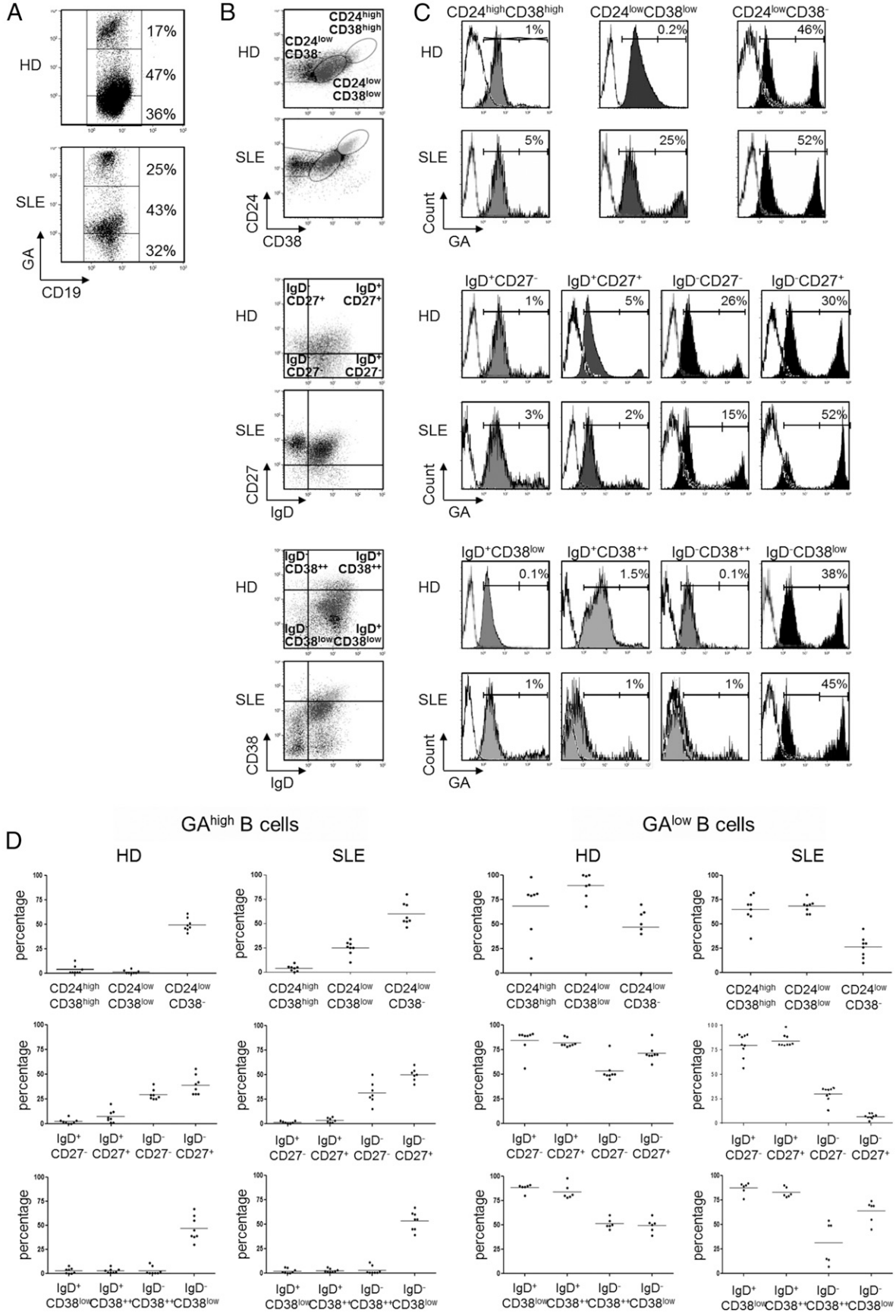

FIGURE 6. GA binds to the memory B cells of peripheral blood. Peripheral blood B cells from HD and SLE patients were analyzed after staining with FITC-conjugated GA and (**A**) anti-CD19 mAb and (**B**) combinations of anti-CD19/anti-CD24/anti-CD38, anti-CD19/anti-IgD/anti-CD27, and anti-CD19/anti-IgD/anti-CD38 mAbs. Representative examples of eight independent experiments. (**C**) Binding of FITC/GA on the B cell subsets. Representative examples of eight independent experiments with FITC-BSA negative control shown as white histograms. (**D**) Distribution of the B cell subsets within the GA^{high} and GA^{low} B cells.

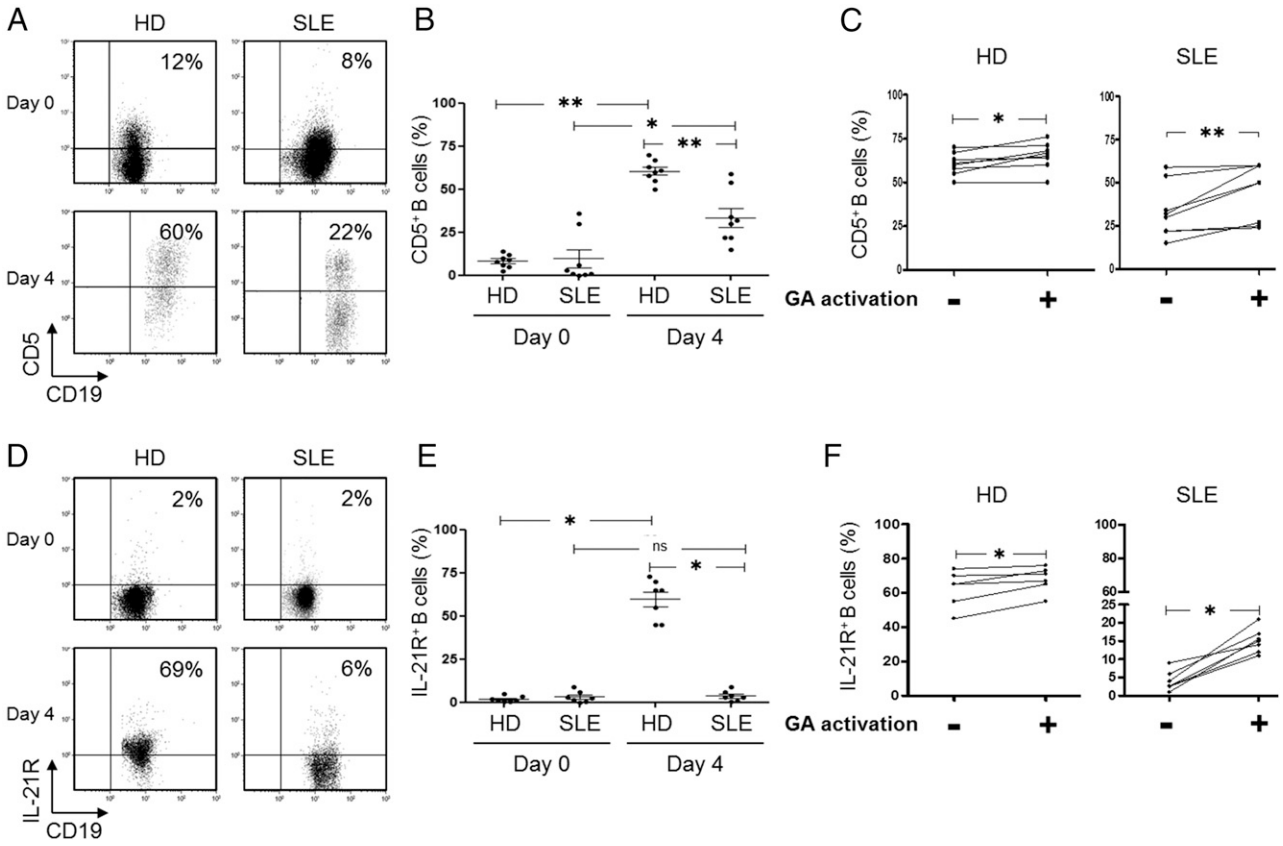

FIGURE 7. GA induces a regulatory phenotype on SLE B cells. Peripheral blood B cells from HD and SLE patients were activated or not by GA and then cocultured with autologous stimulated T cells. B cells were stained and analyzed by flow cytometry using anti-CD19, anti-CD5 (**A**), and anti–IL-21R (**D**) mAbs. Representative examples of eight independent experiments. Frequencies of $CD5^+$ (**B**) and IL-$21R^+$ (**E**) B cells from HD and SLE patients without GA prestimulation. Frequencies at day 4 of $CD5^+$ (**C**) and IL-$21R^+$ (**F**) B cells from HD and SLE patients activated or not with GA. Significant differences were estimated using the Wilcoxon test. $*p < 0.05$, $**p < 0.01$. ns, not significant.

treatments appear to influence Breg cell activity in vivo, although direct effects have never been clearly shown (14).

In the current paper, we demonstrate that GA ameliorates the regulatory functions of healthy B cells and restores the defective Breg cell properties of SLE B cells. Importantly, as previously observed by others (23), GA highly binds to and specifically targets the memory B cell compartment known to have weak regulatory capacities (24). GA stimulation of memory B cells induces a shift in their properties, leading to better control of T cell proliferation, to higher inhibition of Th1 polarization, and to enhanced IL-10 production. Because GA-stimulated memory B cells present neither higher survival rates nor increased proliferative responses, our observations indicate that GA intrinsically activates the regulatory machinery of memory B cells through yet-unidentified pathways. It has been previously shown that GA can induce the upregulation of genes and downregulation of others, such as those involved in inflammatory responses in PBMCs (25). The activation of cell surface molecules is likely involved in the induction of the Breg cell capacities (26). Thus, GA stimulation induces an upregulation of the IL-21R expression on the B cells, which is important for the development and function of Breg cells (21, 27). Furthermore, an increased expression of CD5 is also observed in GA-stimulated B cells. These findings indicate that IL-21R and CD5 molecules are not only Breg cell markers, but also might have functional roles in the switching of memory B cells into Breg cells in response to GA stimulation (21, 22, 27).

The GA-induced production of IL-10 by other cell types has been previously reported. Specifically, macrophages and dendritic cells produce increased levels of IL-10 when stimulated by GA (28). Anti-inflammatory type II monocytes activated by GA express reduced STAT-1 signaling, which might be responsible for increased IL-10 secretion (29). Similarly, dendritic cells activated by GA express lower RORγt nuclear receptor proteins, which reduce inflammatory IL-17 and enhance anti-inflammatory IL-10 productions (30). The mechanism by which GA activates IL-10 production associated with Breg cell activities remains unclear. CD5 expression on B cells induces STAT-3 and NFAT-2 upregulation and activates Erk1/2 kinase through TRPC1 upregulation, which are two distinct pathways responsible for IL-10 production (31, 32). Thus, it is likely that GA-dependent, increased CD5 expression might be associated with one or both pathways' activation, inducing the Breg cell machinery in the memory B cells and leading to IL-10 production.

Many innate or adaptive immune cells can potentially be direct or indirect targets for GA through a broad mechanism of actions. GA induces a shift of Th1 to Th2 T cell response (33), enhances the cytolytic capacity of NK cells toward dendritic cells (34), and increases the phagocytic activity of macrophages (35). However, GA can restrict the activation of monocytes and macrophages by increasing IL-10 and TGF-β and decreasing IL-12 and TNF-α secretion of type II monocytes (28). GA allows for the reconstitution of a naive Treg cell population and increases total Treg cells by converting $CD4^+CD25^-$ T cells into $CD4^+CD25^+Foxp3^+$ Treg cells (36), also improving their regulatory potential (36, 37). The newly described effects on Breg cell functions associated with all these observations indicate that GA can ameliorate or restore regulatory immune homeostasis.

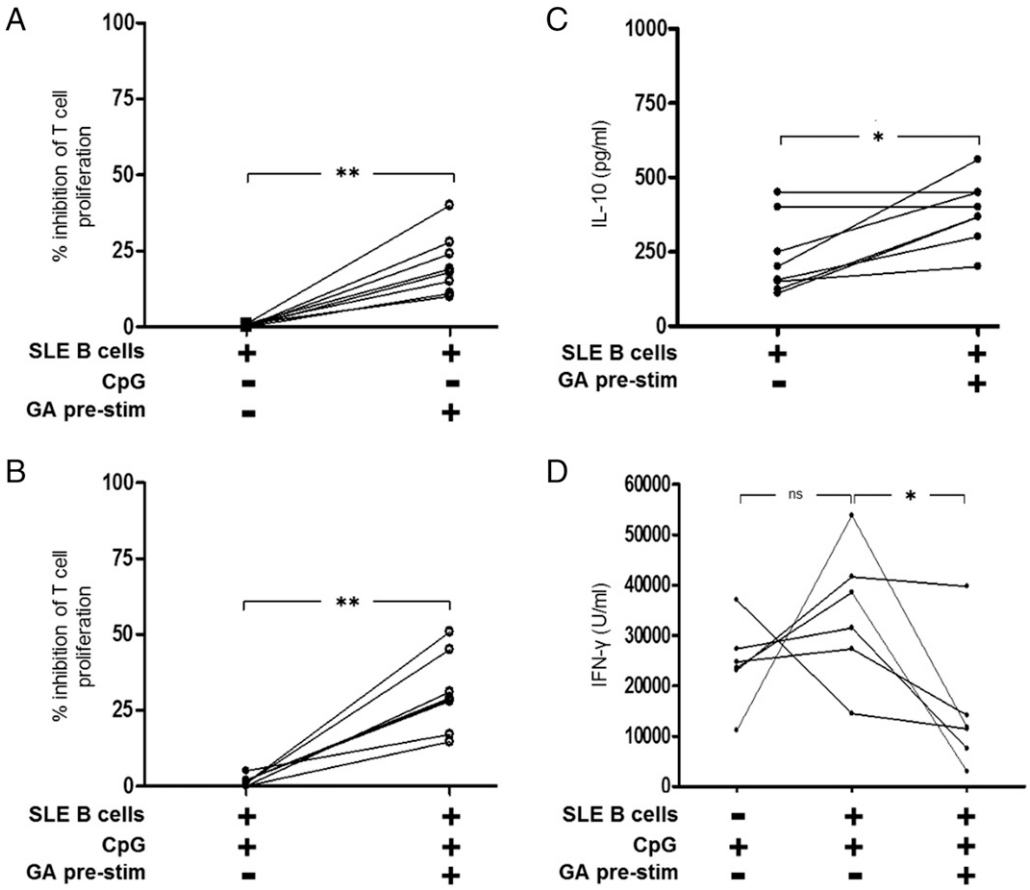

FIGURE 8. The regulatory activities of SLE B cells are restored by GA. T cells from SLE patients were stimulated and cocultured with autologous SLE B cells activated or not by GA. The inhibition of T cell proliferation was evaluated without (**A**) and with (**B**) TLR9 activation of B cells with CpG/ODN, and the secretion of IFN-γ (**D**) was evaluated. (**C**) SLE B cells were activated or not with GA, and the production of IL-10 was measured. Significant differences were estimated using the Wilcoxon test. $*p < 0.05$, $**p < 0.01$. ns, not significant.

We observed differential synergistic effects between TLR9 stimulation and GA stimulation for peripheral B cells and tonsillar B cells. The T cell proliferative response was synergistically controlled by the peripheral and tonsillar B cells, whereas Th1 polarization was synergistically controlled only by the peripheral blood B cells. The higher frequencies of $IgD^{-}CD38^{-}CD27^{+}$ memory cells present in the circulating B cell population (38) might be responsible for the TLR9 and GA-enhanced regulatory properties of the peripheral cells. Our observations may also have important issue in autoimmune situations. Lupus B cells are known to have defective regulatory properties (3, 11, 12, 39). Our experiments demonstrate that this is reversible. GA-stimulated SLE B cells trigger an efficient control of the T cell proliferative response, induce inhibition of Th1 polarization, and recover their capacity to produce IL-10. Interestingly, in contrast to their complementary effects observed on peripheral HD B cells, GA and TLR9 stimulation do not act synergistically on SLE Breg cell activities. SLE B cells remain refractory to TLR9 engagement, indicating that GA-induced Breg cell activation is independent of TLR9 activation pathways. Although deficient SLE Breg cell functions can be ascribed to defective CD40 pathways, hence leading to reduced STAT3 phosphorylation (11), aberrant CD40 activation of SLE B cells results in enhanced NF-κB activation (40) that can be modulated by GA (41). GA significantly reduces p65 NF-κB phosphorylation, which may play a role in the modulation of SLE disease. Associated with the IL-21R– and CD5-induced cascades, a complex interplay of various signaling pathways is likely activated by GA in SLE B cells to generate an overall response. Moreover, as previously described, SLE patients exhibit an abnormal distribution of peripheral blood B cell subsets that have a lower frequency of $CD27^{-}CD24^{low}CD38^{low}IgD^{+/-}$ naive B cells and a higher proportion of $CD24^{low}CD38^{-}CD27^{+}IgD^{+/-}$ memory B cells (42–44), which is correlated with the disease activity (45). Lupus-like models of mice highlight the variable protective role of B cells producing IL-10. In $CD19^{-/-}$ NZB/W F1 mice, there is a lack of B10 cells, less IL-10 transcription, and an exacerbation of the lupus-like disease (46). However, in $CD19^{Cre}$ $IL\text{-}10^{fl/fl}$ MRL.lpr mice, in which the IL-10 deficiency is restricted to B cells, the lupus symptoms are not worse (47), but this might be because of the persistence of residual B10 cells that inhibit disease severity. Overall, the production of IL-10 by B cells contributes to the control of the severity of the disease, but, depending on the animal models, the effectiveness may vary. Moreover, the activation of a protective regulatory control is constrained by the time course of the disease. Stimulation by IL-21 of EAE splenic B cells (21) or of MRL/lpr B cells (27) induces the expansion of B10 cells and the production of IL-10, indicating that IL-21R is required for the effective function of B10 cells. However, although early treatment of BXSB-Yaa lupus mice with soluble IL-21R/Fc molecules negatively affects the survival of the mice, treatment during the late phase of the disease ameliorates survival (48). This indicates a biphasic effect of IL-21 that is stimulating the Breg cells in the early phase of SLE, but promoting the pathogenic B cells in the late phase of the disease.

SLE patients have reduced frequencies of $IL\text{-}21R^{+}$ and $CD5^{+}$ B cell subsets correlated with faster disease progression (49, 50).

The overrepresentation of peripheral memory B cells in SLE likely unravels the global GA-specific restoration of the capacities of Breg cells that was observed in our in vitro models because of their shift from proinflammatory cells into regulatory cells.

Overall, our results show a direct effect of GA on the memory B cell compartment and establish that GA can restore Breg cell characteristics and functions in SLE patients. These results, which show the great promise of GA-based therapy, highlight the beneficial role that GA can provide in pathologic conditions associated with defective Breg cells.

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Disclosures

The authors have no financial conflicts of interest.

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