Excellence in research of Prof. Gaurisankar Sa

SYNOPSIS:

Our identified tumor-associated IL10-producing $CD19^{+}CD39^{-}$ immunoregulatory B cell subset (B-regulatory cell) was found to inhibit H-chain class-switch recombination and B cell differentiation by limiting autologous Tfh cell expansion and IL21 production.

SUMMARY

B cells are an essential component of humoral immunity, and their primary function is to mount an antigen-specific antibody response in order to remove invading pathogens. Despite an increase in B cell number, we found that serum-IgG levels were low in breast cancer patients. To solve this conundrum, we used high-dimensional flow-cytometry to analyze the heterogeneity of B cell populations and identified a tumor-specific CD19+CD24hiCD38hi IL10-producing B-regulatory (Breg) cell subset. Albeit IL10 is a defined marker for Breg cells, being an intracellular protein, it is of limited value for Breg cell isolation. Similarly, highly-expressed Breg cell surface proteins CD24/CD38 also impede the isolation of viable Breg cells. These are the major hurdles that prevent many functional aspects of Breg from being revealed. Our transcriptomic analysis identified, CD39-negativity as an exclusive, sorting-friendly surface marker for these tumor-associated Breg cells. The identified $CD19^{+}CD39^{-}IL10^{+}$ B cell branch is suppressive in nature as it limits T-helper cell proliferation, type-1 cytokine production, T-effector cell survival and $CD4^{+}FOXP3^{+}$ Treg cell generation. These tumor-associated immunoregulatory Breg cells were also identified to restrict autologous Tfh cell expansion and IL21 secretion, hence inhibiting germinal transcript formation and AID expression involved in H-chain class-switch recombination. This isotype-switching abnormality eventually hinders B cell differentiation into class-switched memory B cells and subsequent high-affinity antibody-producing plasma B cells, which collectively leads to the dampening of IgG-mediated antibody responses in cancer patients.

INTRODUCTION

In the immune system, B cells are an essential component of humoral immunity. They are classically positive modulators that regulate inflammation and immune responses. The solid tumor often contains significant B cell populations, suggesting B cells' role in influencing the tumor-microenvironment alone or in cooperation with other resident cells (1,2). Recently, research in B cell biology of cancer has witnessed contradictory reports. One side of the report describes B cells as the immune system's defensive powerhouse as they secrete antibodies and build up a memory B cell-mediated immune response against tumors (3). On the other hand, the tumor showed a reduction in metastasis during CD20 depletion from the total B cell pool (4,5). This observation leads us to identify a new pro-tumor B cell branch with a suppressive function besides the existence of well-known anti-tumoral B cell subsets like memory and plasma B cells. The newly observed immunosuppressive B cell branch was later designated as B-regulatory (Breg) cells (6,7).

Despite reaching a partial consensus on the IL10-mediated effector function of Breg cells, the field has yet to produce a unified view on the phenotypic status of Breg cells (8,9). Several IL10-producing Breg cell subsets with different surface markers have been described so far. Among them, $CD19^{+}CD24^{hi}CD38^{hi}$ B cells have a phenotype associated with transitional B cells (10), and $CD19^{+}CD24^{hi}CD27^{+}$, a phenotype associated with memory B cells (11), make up the majority of IL10-secreting Breg cells. It is now widely accepted that Bregs perform immunoregulatory functions primarily through the secretion of cytokines such as IL10, IL35, and TGFβ as well as other proteins such as granzyme-B (GrB) and PDL1 (7,12,13). Although the presence of suppressive B cells in various autoimmune disorders has been known for more than 30 years (14), very little is known about their presence and function in the field of tumor biology. Bregs appear to suppress T-helper cells, induce $FOXP3^{+}$ T-regulatory cells, and target other tumor-infiltrating lymphocytes such as myeloid-derived suppressor cells, macrophages, and natural killer cells to thwart anti-tumor immunity (15,16). Various studies have also shown that B-regulatory cells are closely associated with many clinicopathological factors in tumor patients and may be potential biomarkers for predicting patient survival (14). A study on gastric cancer found that Breg cells suppress the immune system by downregulating Th1 cells and increasing Treg generation (17,18). These findings suggest the potential therapeutic targets for B-regulatory cell-mediated future immunotherapy in cancer patients. Breg cells are widely known to be immunosuppressive B cell branch. However, other than intracellular IL10, no phenotypic surface signature has yet been assigned to this B cell branch in tumor microenvironment.

In this study, using high-dimensional flow-cytometry and transcriptome analysis, we identified a unique phenotypic-signature marker for tumor-associate IL10-producing Breg cells, which can be used to live-sort these cells. Our ex-vivo findings indicate that, in addition to their T-cell mediated immunoregulatory function, Breg cells play an important role in limiting the production of germline transcripts (GLTs), activation-induced cytidine deaminase (AID) expression

involved in class-switching recombination, B cell differentiation, and antibody production in the tumor microenvironment.

RESULTS:

Existence of B-regulatory cells in breast cancer patient

Tumor-infiltrating lymphocytes are known to be a critical modulator in controlling tumor progression (21,22). While the involvement of T cells has been extensively studied, the role of B cells tends to be overlooked in regulating anti–tumor immunity (23). Such a limited understanding of B cells' role in tumor immunology prompted us to explore the atlas of B cell subsets in the breast tumor-microenvironment.

We investigated B cell subset profiling, cytokine production, and antibody response in breast cancer patients and compared them to age-/sex-matched healthy individuals (Figure-S1a). We found a higher percentage of B cells in cancer patients based on our concatenated flow-cytometry data (Figure-1a) of lymphocytes obtained from healthy individuals and breast cancer patients. As B lymphocytes are primarily responsible for antibody production, we compared the serum-level immunoglobulins amongst them. Surprisingly, we found that the level of serum-IgG was significantly lower and the level of IgM was significantly higher in cancer patients than in healthy controls, while the level of IgA remained unchanged (Figure-1b). This has turned into a puzzle to solve.

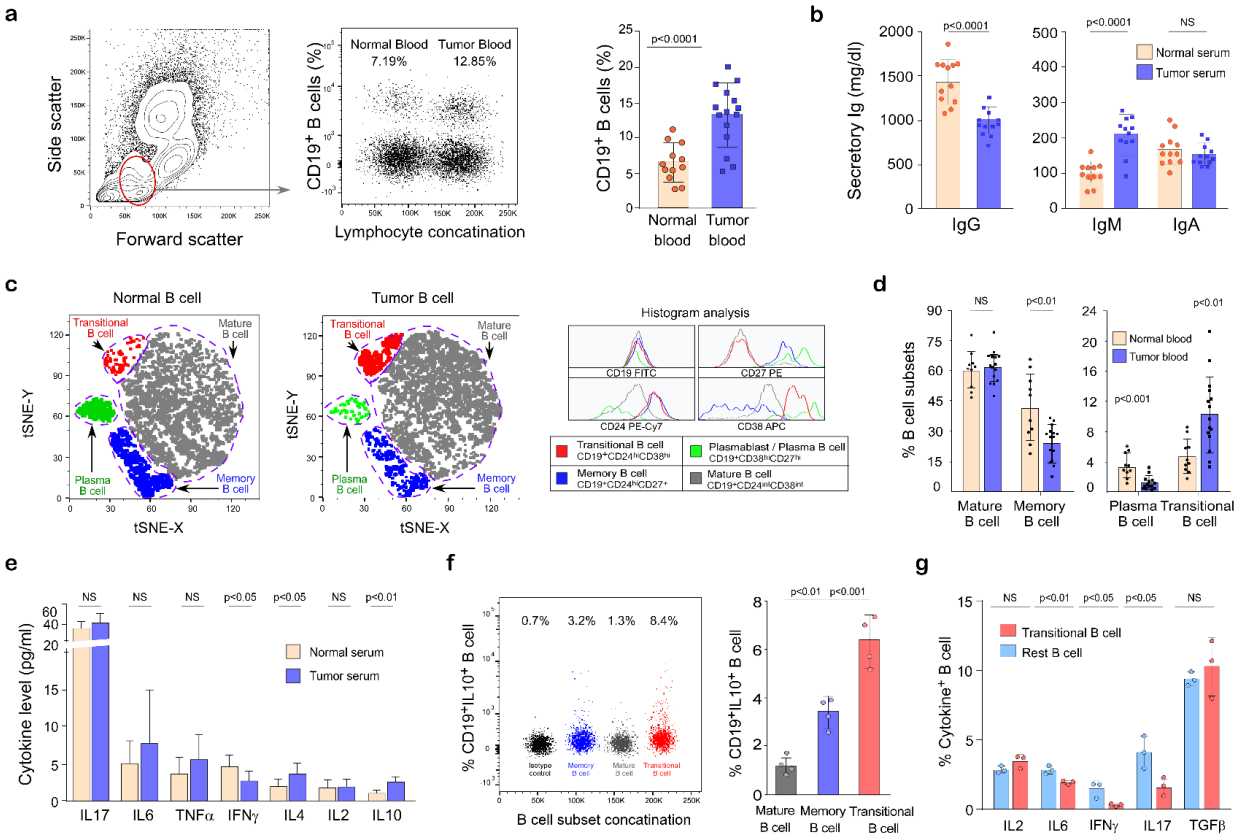

Figure-1. Prevalence of IL10-producing transitional B cells in breast tumor milieu. ***(a)*** *A representative flow-cytometry concatenated plot shows the percentage of CD19⁺ B cells in lymphocytes from the peripheral circulation of breast cancer patients (n =15) and healthy people (n =12).* ***(b)*** *The bar graph shows the levels of serum IgG, IgM, and IgA amongst breast cancer patients and healthy individuals.* ***(c)*** *The t-SNE cluster displays four major B cell subsets' distribution and compares the difference in frequency of these cell clusters amongst breast cancer patients and healthy individuals. The major B cell subsets are transitional B cells (red), plasma B cells (green), memory B cells (blue), and mature B cells (grey) within the CD19⁺ B cell compartment. Histogram plots show the comparison between surface marker (CD19, CD24, CD38, and CD27) expressions in CD19⁺ B cell populations in the gated regions.* ***(d)*** *Cumulative results of the mature, memory, plasma, and transitional B cell percentage within CD19⁺ B cell populations in breast cancer patients (n=15) and healthy individuals (n=10), are represented in the bar graph. Each dot represents data generated from an individual.* ***(e)*** *Simultaneous assessment of serum IL2, IL4, IL6, IL10, IL17, IFNγ and TNFα levels were performed by anti-human CBA array kit from breast cancer patient serum and healthy individual. Each point represents data generated from individual serum.* ***(f)*** *Representative flow-cytometric concatenated dot plot depicting IL10 expression in different B cell subsets: transitional (red), mature (grey), memory (blue) B cells and Isotype control (Black). The cumulative results from multiple samples are represented in a bar graph. The bar chart represents ±SDM of percent IL10⁺ B cells within different B cell compartments as shown in 4 sets of independent experiments.* ***(g)*** *The bar graph depicts the cumulative flow cytometric results of several cytokine expressions between transitional B cells and the rest of the B cells. Values are mean ±SD.*

To unravel this puzzle, a thorough investigation of B cell subsets and their roles in breast cancer dynamics was analysed using t-SNE (t-Distributed Stochastic Neighbour Embedding). The identification and differential expression of four key B cell subpopulations in the peripheral circulation of breast cancer patients were made possible by a detailed t-SNE analysis of $CD19^{+}$-gated B cells: plasma B cell/plasmablast ($CD19^{+}CD38^{hi}CD27^{hi}$), mature B cell ($CD19^{+}CD24^{int}CD38^{int}$), memory B cell ($CD19^{+}CD24^{hi}CD27^{+}$) and transitional B cell ($CD19^{+}CD24^{hi}CD38^{hi}$).

In cancer patients, the number of transitional B cells increased significantly, whereas the percentage of $CD27^{+}$ memory B cells and their eventual $CD27^{hi}CD38^{hi}$ plasma B cells decreased (Figure-1c). This finding was validated in a cohort of 10 healthy females and 15 breast cancer patients (Figure-1d). Aside from the predicted classical Th2-cytokine bias response (IFNγ skewed to IL4), cytometric bead array results revealed a significantly higher level of IL10 in breast cancer patients' serum (Figure-1e). (The generated cytokine standard curves are represented in Figure-S1b).

We investigated the intracellular level of IL10 in the tumor-associated transitional B cell subset because of the high frequency of transitional B cells in the peripheral blood, increased IL10 level in the serum of breast cancer patients, and previously reported IL10-positivity as a widely used marker for Breg cells identification (24). In the tumor microenvironment, intracellular IL10 labelling revealed that a considerably larger percentage of $CD19^{+}CD24^{hi}CD38^{hi}$ transitional B cells were IL10 positive than mature or memory B cells (Figures-1f and S1c). Aside from IL10, transitional B cells secret significantly less IFNγ, IL6, and IL17 than the rest of the B cell lineage (Figures-1g and S1d). All of this evidence confirmed that transitional $CD19^{+}CD24^{hi}CD38^{hi}$ B cells are more prevalent in cancer patients and contribute to a high amount of IL10 secretion. In the tumor microenvironment, these IL10-producing transitional B cells are B-regulatory cells.

$CD19^{+}CD24^{hi}CD38^{hi}$ Breg cells are tumor-specific

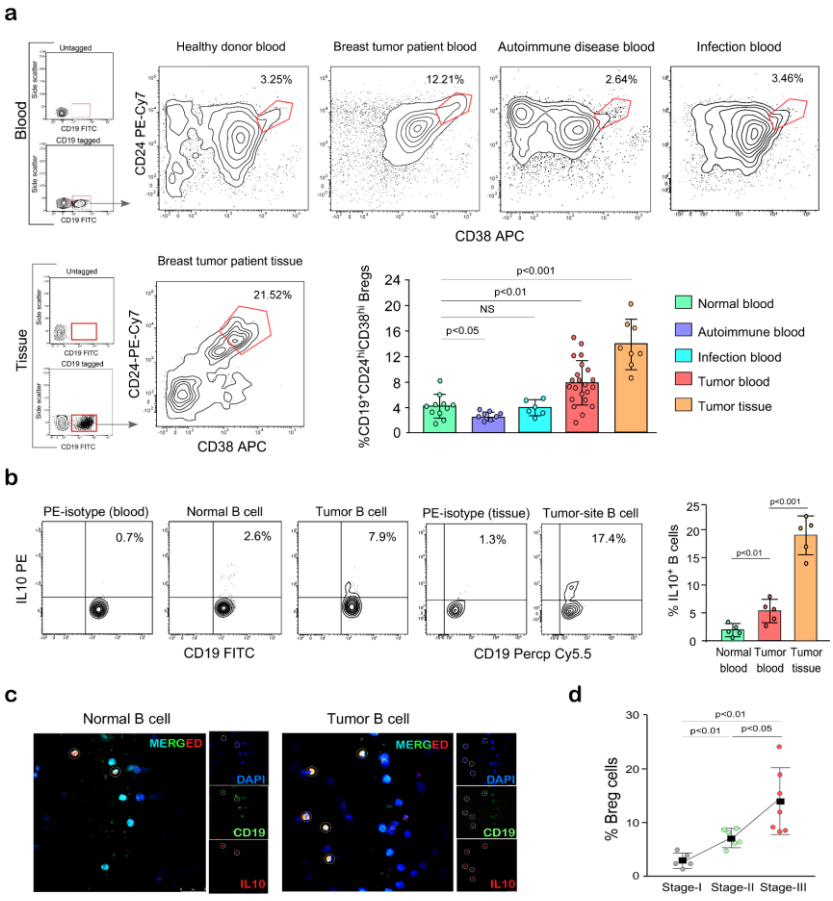

Figure-2. Phenotypic characterization of the IL10-producing Bregs in breast cancer. (a) *Flow-cytometric representation of CD19+CD24^{hi}CD38^{hi} B-regulatory cell populations in breast cancer patients, healthy people, autoimmune disorder patients, HBV infected patients, and tumor-infiltrating lymphocytes. The scatter plot shows the frequencies of CD19+CD24^{hi}CD38^{hi} Breg cell populations in breast cancer patients' blood (n =22), healthy individuals (n =11), autoimmune-disorder patients (n =8), HBV infected patients (n =6) and in tumor-infiltrating lymphocytes (n =8).* ***(b)*** *Representative flow cytometry data showing the percentage of $CD19^{+}IL10^{+}$ B cells in healthy people, breast cancer patients' blood, and tumor tissue. The cumulative results from multiple samples are represented in a bar graph. Values are mean ±SD or representatives of 5 sets of independent data points.* ***(c)*** *Confocal images showing the expression of IL10 in $CD19^{+}$ B cells in the peripheral circulation of breast cancer patients compared to healthy individuals.* ***(d)*** *A representative graph demonstrating the positive correlation between the percentage of Breg cells in tumor patients' peripheral circulation and tumor progression.*

The fact that breast cancer patients have a higher frequency of IL10-secreting transitional Breg cells encouraged us to investigate whether these $IL10^+$ Breg cells are tumor-specific or not. As a result, we used flow cytometry to investigate the frequency distribution of these cells in various pathophysiological conditions. Figure-2a shows that the percentage of $CD19^+CD24^{hi}CD38^{hi}$ Breg cells in the peripheral circulation (10-12%) and tissues (22%) of the breast cancer patient were higher than in healthy donor PBMCs. Pathological disorders such as autoimmune inflammatory disease (rheumatoid arthritis), on the other hand, showed lower levels of Breg cells than healthy individuals. B-regulatory cell percentage is reported to be low in autoimmune disorders (25). We also checked the percentage of $CD19^+CD24^{hi}CD38^{hi}$ Breg cells in HBV-infected patients and found that they had a similar expression level as healthy individuals. In Graves' disease and systemic lupus erythematosus patients, $CD19^+CD24^{hi}CD27^+$ B cells have been identified as Bregs (11,26). So, we investigated their status in tumor conditions and observed that the frequency of this cell is lower in breast cancer patients' blood than in healthy people's blood (Figure-S2a), implying that $CD19^+CD24^{hi}CD38^{hi}$ transitional B cell subsets are tumor-specific.

Flow cytometry was used to further investigate the interaction between tumor and IL10-producing Breg cells. When comparing cancer patients' blood and tumor tissue to their healthy counterparts, the percentage of $IL10^+$ B cells gradually increased, indicating a positive association between Breg cells and tumors (Figures-2b). We also used confocal microscopy to validate our findings (Figure-2c). Figure-S2b shows all of the necessary controls. We evaluated the percentage of $CD19^+CD24^{hi}CD38^{hi}$ Breg cells with the advanced stage of the tumor after finding an indication of a positive correlation between Breg cells and tumor. The percentage of Breg cells in the patient's blood was assayed using flow cytometry (Figure-S2c), and the multiple data points were then represented in a bar graph (Figure-2d). According to the results, it was found that the percentage of Breg was increasing as the tumor advanced. Together, our findings suggest that $CD19^+CD24^{hi}CD38^{hi}$ Breg cells are tumor-specific rather than disease-specific.

CD39-negativity as a key signature marker for tumor-associated Breg cells

Intracellular IL10-positivity is the hallmark for B-regulatory cell identification (27). Being an intracellular protein, IL10 only remains as the signature molecule for Breg cell identification rather than an isolation marker. However, because they have three highly-expressed cell surface markers, identified IL10-producing $CD19^+CD24^{hi}CD38^{hi}$ Breg cell branches have limitations in purifying functional B cells. These are the major reasons behind many functional aspects of the Breg still ill-defined.

We used data from the GEO database (GSE76272) to do a transcriptome analysis to find distinct Breg cell surface characteristic markers (10). At first, we shortlisted only significant ($p<0.05$) top 50 up-regulated and down-regulated genes in $CD24^{hi}CD38^{hi}$ Breg cell and compared them with $CD24^{int}CD38^{int}$ mature and $CD24^+CD38^-$ memory B cell subsets.

Only surface expressing genes were considered in ingenuity pathway analysis (IPA) (Figure-3a, right panel). CD39 (ENTPD1), an ATP-hydrolase that converts ATP to ADP/AMP, is one of the most promising surface markers, with minimal surface expression in $CD19^+CD24^{hi}CD38^{hi}$ Breg cells and high expression in both mature and memory B cell subsets. In their single-cell multi-omics investigation, Sean C. Bendall's group found a similar pattern of CD39 expression (28). Our multi-colour flow-cytometric dot-contour overlay analysis from healthy individual (Figure 3b, upper-panel) and tumor patient (Figure 3b, lower-panel) confirmed that the $CD39^-$ B cells are $CD24^{hi}CD38^{hi}$. $CD19^+CD39^-$ highlighted as red (red box) and the rest of the grey dots are $CD19^+CD39^+$. In the same CD38 *vs.* CD24 plot, we overlaid this compartment of cells. The majority of the $CD19^+CD39^-$cells (red) are $CD19^+CD24^{hi}CD38^{hi}$. This finding was confirmed in a cohort of breast cancer patients' samples, where there was no discernible difference in the frequency of expression between $CD19^+CD39^-$, $CD19^+CD24^{hi}CD38^{hi}$, and $CD19^+CD24^{hi}CD38^{hi}CD39^-$ B cells (Figure-3b, right-panel bar graph).

The high-dimensional flow-cytometric data were analysed using both the clustering (t-SNE) and visualising (FlowSOM) plugins. Within the entire breast cancer patients' B cell pool, $CD39^-$ B cells are limited in the same place as $CD24^{hi}CD38^{hi}$ cells, and their numbers are nearly comparable (Figures-3c). The FlowSOM analysis revealed a B cell branch with negligible CD39 expression but high CD24 and CD38 expression (Figure-3d). We analysed $CD19^+CD39^-$ Breg cell percentages in healthy donor and breast tumor patient blood for additional validation and found that $CD19^+CD39^-$ alias $CD19^+CD24^{hi}CD38^{hi}$ has a higher percentage in tumor samples than healthy equivalents (Figure-s3c). Furthermore, we looked into a variety of B cell developmental markers and found that $CD39^+$ markers are strongly associated with every significant B cell subset in cancers, including mature and, most notably, memory B cells and plasma B cells, whereas $CD39^-$ markers are not associated with any of the aforementioned subsets except for transitional B cells (Figure-3e & Figure-s3a). When isolated Breg cells were activated and cultured in the presence of LPS, the $CD39^-$ Breg percentage was maintained even after 7-days of culture (Figure-s3e). Aside from that, we also revealed that Breg cells never re-differentiate in post-Breg depletion samples (Figure-s6a) under exogenous stimulus. These shreds of evidence indicate that the $CD39^-$ Breg population is a stable, self-contained population and does not re-differentiate.

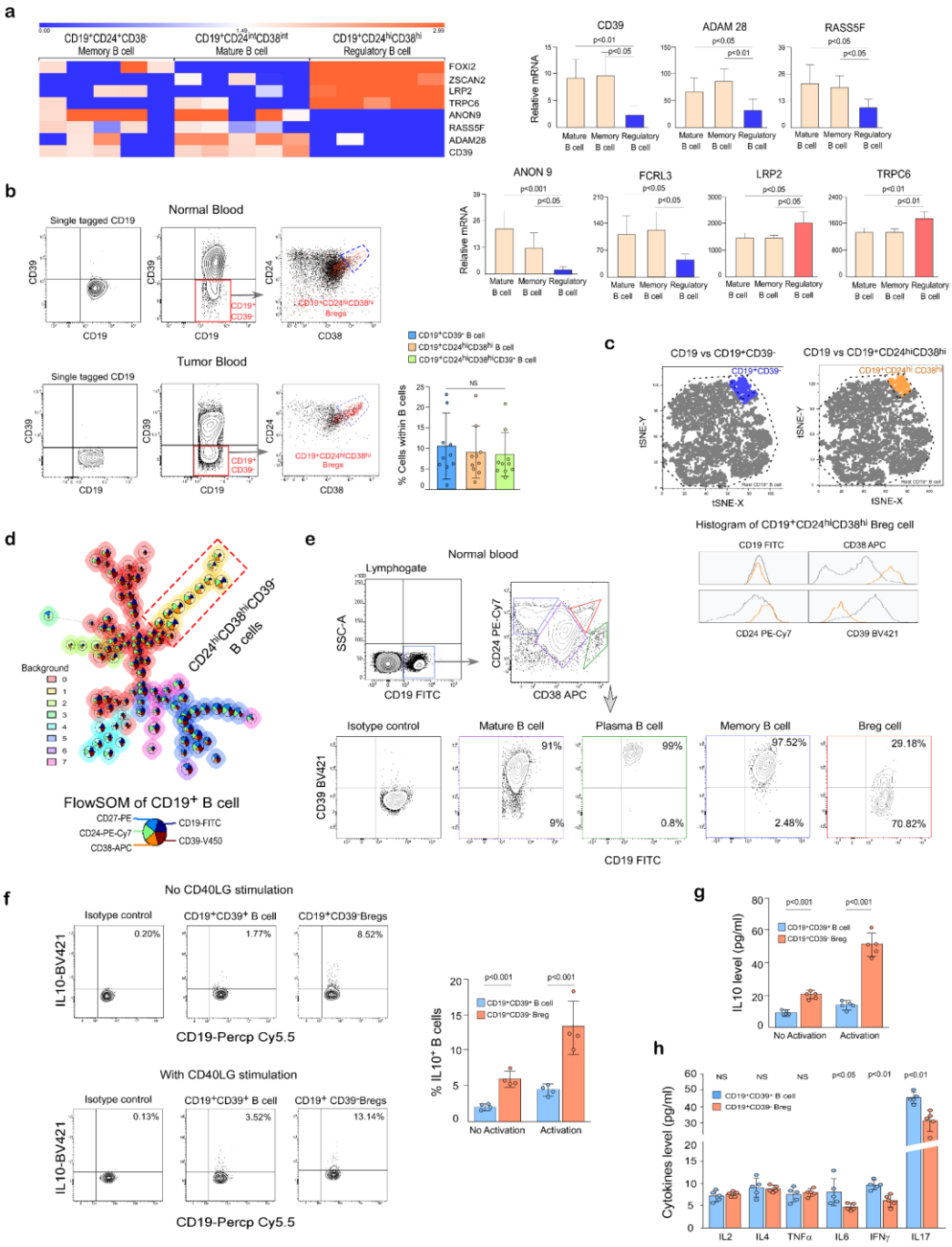

Figure-3. CD39-negativity is a signature marker for tumor-associated IL10-producing Breg cells. ***(a)*** *In-silico analysis of transcriptome data from five healthy donors' sorted CD19⁺CD24^{hi}CD38^{hi}, (regulatory) CD19⁺CD24^{int}CD38^{int} (mature), and CD19⁺CD24⁺CD38⁻ (memory) B cell subsets. Heatmap analysis depicts the up-and down-regulated genes (surface and transcription factors) in the three subsets of cells (left panels). The right panels demonstrated only surface-expressing genes. CD39 (ENTPD1) was found to be one of the top downregulated surface marker genes in the regulatory B cell (CD19⁺CD24^{hi}CD38^{hi}) compartment compared to the other two B cell subsets (right panels).* ***(b)*** *Flow-cytometric representation of CD39-negativity in total CD19⁺ B cell populations and status of CD24 and CD38 in CD39-negative populations in the peripheral circulation of a healthy person and a breast cancer patient. The bar graph depicts the cumulative results (n=10) from the percentage of CD19⁺CD39⁻, CD19⁺CD24^{hi}CD38^{hi} and CD19⁺CD24^{hi}CD38^{hi}CD39⁻ populations within total CD19+ B cells.* ***(c)*** *The t-SNE plot depicts the amount as well as the position of CD19⁺CD39⁻ (blue) and CD19⁺CD24^{hi}CD38^{hi} (orange) populations within total CD19⁺ (grey) B cell populations.* ***(d)*** *Representative FlowSOM analysis depicting seven different B cell branches in cancer patients. Clusters are represented as circles with star plots illustrating cluster median marker intensities. Meta-clustering is represented by the background colour. The identified yellow branch depicts CD19⁺CD24^{hi}CD38^{hi}CD39⁻ Breg cells.* ***(e)*** *Representative flow cytometry data show CD39 expression in various B cell subsets.* ***(f)*** *Flow-cytometry plot of IL10 positivity in CD19⁺CD39⁻ and CD19⁺CD39⁺ B cells with or without CD40LG (CD154) activation (left panels). Right panels demonstrated the cumulative results.* ***(g)*** *Supernatants from purified B cell subsets with or without CD40LG (CD154) activation were tested for the presence of IL-10 by ELISA. The data are shown as mean ±SD.* ***(h)*** *Anti-human CBA array kit was used to measure IL2, IL4, IL6, IL17, IFNγ and TNFα levels in sorted CD19⁺CD39⁻ Breg (n=5) and CD19⁺CD39⁺ Non-Breg (n=5) cell supernatants. Each point represents data generated from each sorted cell population.*

$CD19^+CD39^-$ was found as an alternate Breg isolation marker to $CD24^{hi}CD38^{hi}$ B cells based on transcriptome and flow-cytometric data. We evaluated their IL10 status to ensure that this $CD19^+CD39^-$ cell was a Breg cell. We used a customised magnetic bead-sorting method to separate $CD19^+CD39^+$ and $CD19^+CD39^-$ B cells from breast cancer patients' blood and activated them for 72 hours in the presence of CD40LG. Figure-S4b shows the gating criteria and post-sort purity of B cell subsets. $CD39^-$ B cells make up a higher percentage and secrete more IL10 than $CD39^+$ B cells, according to intracellular labelling from flow cytometry (Figure-3f) and cultural supernatants data from ELISA (Figure-3g). When CD40LG (CD40's counterpart) was involved, IL10 was elevated in the $CD19^+CD39^-$ Breg cells compartment. Apart from IL10, our cytometric bead array data from the cell supernatant of $CD19^+CD39^+$ non-Breg and $CD19^+CD39^-$ Breg cells cultured for 72 hours in the presence of CD40LG revealed that $CD39^-$ B cells have significantly lower levels of pro-inflammatory cytokines like IL6, IFNγ, and IL17 than $CD39^+$ B cells (Figure-3h).

Immunoregulatory functions of tumor-associated B-regulatory cells

The identification of suitable surface markers for Breg cells, as well as their prevalence in the breast tumor-microenvironment, prompted us to investigate the functional attributes of these $CD19^+CD39^-$ Breg cells. To assess this Breg cell's immunoregulatory function, we isolated them using our custom magnetic bead (Figure-S4b) and co-cultured them for 72h with purified autologous naïve $CD4^+$ T cells (Figure-S4c). Figure-S4a shows a schematic illustration of the co-culture experiment. Anti-CD3 and anti-CD28 mAbs were used to stimulate naïve $CD4^+$ T cells, and the frequencies of $CD4^+IFN\gamma^+$ cells were assessed using flow cytometry.

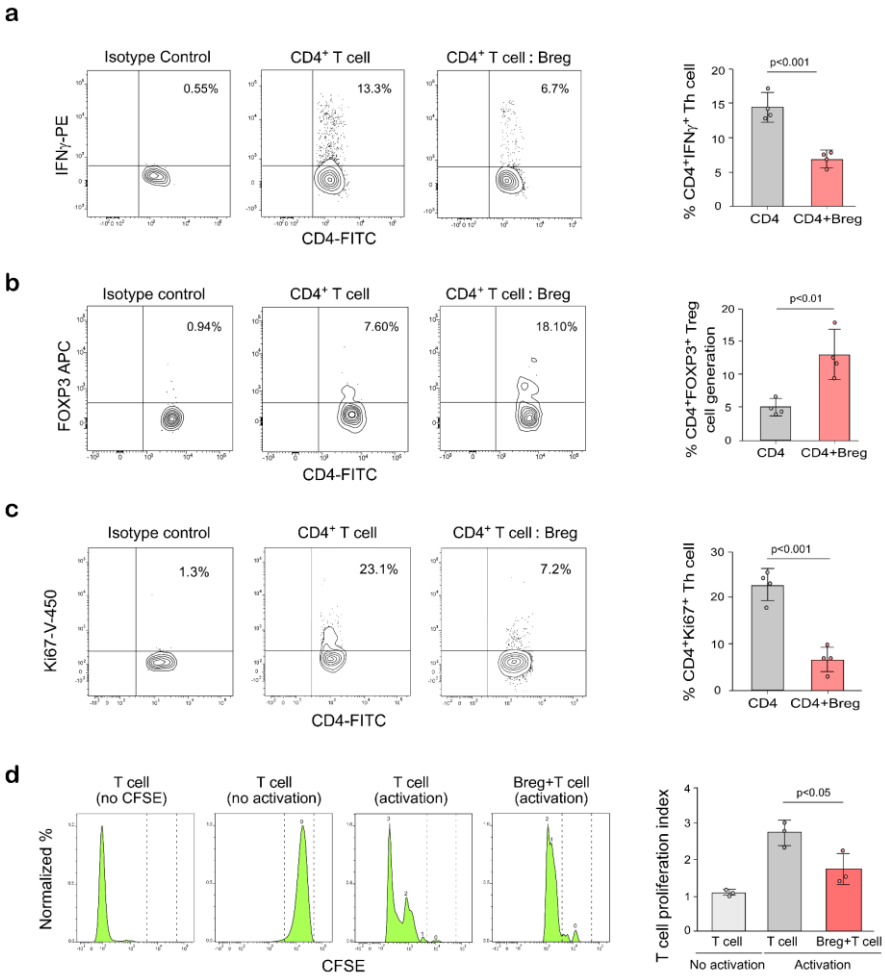

Figure-4. Suppressive function of tumor-associated $CD19^+CD39^-$ Breg cells. ***(a)*** *Representative flow-cytometry plot depicting the suppression of $CD4^+IFN\gamma^+$ Th1 cells when ex-vivo co-cultured with $CD19^+CD39^-$ Breg cells.* ***(b)*** *The percentage of $CD4^+FOXP3^+$ Treg cells generated from $CD4^+$ T cells when co-cultured ex-vivo with $CD19^+CD39^-$ Breg cells.* ***(c)*** *Flow-cytometry analysis of the inhibitory effects of Breg cells on $CD4^+$ T cell proliferation (Ki67-positivity).* ***(d)*** *$CD4^+$ responder T cell (stimulated with anti-CD3/-CD28 antibodies) proliferation was measured by a CFSE-dilution assay in the presence of $CD19^+CD39^-$ Breg cells. Cumulative results of the responder T cells' proliferation index from independent experiments are represented in the bar diagram. Values are mean ±SD or representatives of 3 sets of independent experiments. Cumulative results of the $CD4^+IFN\gamma^+$, $CD4^+FOXP3^+$ and $CD4^+Ki67^+$ percentage with or without the presence of Breg cells are represented in the bar graph. Each dot represents data generated from each experiment.*

When $CD4^+$ T cells were co-cultured with $CD19^+CD39^-$ Breg cells, it was revealed that Breg had an inhibitory effect on IFNγ production (Figure-4a). The inhibitory effect was also proportional to the cell ratio (Figure-S4e). Furthermore, the Ki67-positivity assay revealed that this subgroup suppresses the growth of activated $CD4^+$ T cells (Figure-4c). The Breg cell also induced effector T cell death when they were cultured together (Figure-S4d). When co-cultured *ex-vivo* with autologous activated T cells, these $CD39^-IL10^+$ Breg cells can transform them into suppressive $CD4^+FOXP3^+$ Treg cells (Figures-4b). Breg engagement resulted in reduced cell proliferation of activated $CD4^+$ T cells, as measured by the proliferation index level (CFSE dilution; Figure-4d). All of this data points to the $CD19^+CD39^-$ Breg cells that developed in the tumor-microenvironment being immunosuppressive and maintaining immunogenic tolerance in breast cancer patients.

CD39$^-$ Breg cells deregulate class-switch recombination

The adaptive immune response is characterized by somatic DNA recombination such as the class-switch recombination required to alter the antibody's effector function. H-chain class-switching occurs in three stages: germline gene transcription (GLT), DNA recombination, and B cell differentiation into memory B cells, followed by Ig-secreting plasma cells (Figure 5a) (29). We already saw in Figure-1 that Breg cell frequency is negatively related to memory and plasma B cells. From a cohort of 10 healthy individuals and 10 breast cancer patients, we observed that the frequency of Breg cells versus memory B cells and plasma B cells alters diagonally in breast cancer patients compared to their healthy counterparts (Figure-S5). As a result, Breg cells may be interfering with the establishment of memory B cells. Memory B cells are classified into two groups based on their IgG and IgM status. Non-class-switched memory B cells (CD19$^+$CD27$^+$IgD$^+$) do not undergo class-switching and generate more IgM and class-switched memory B cells (CD19$^+$CD27$^+$IgD$^-$) undergo class-switching recombination to produce IgG (Figure-5b) (30).

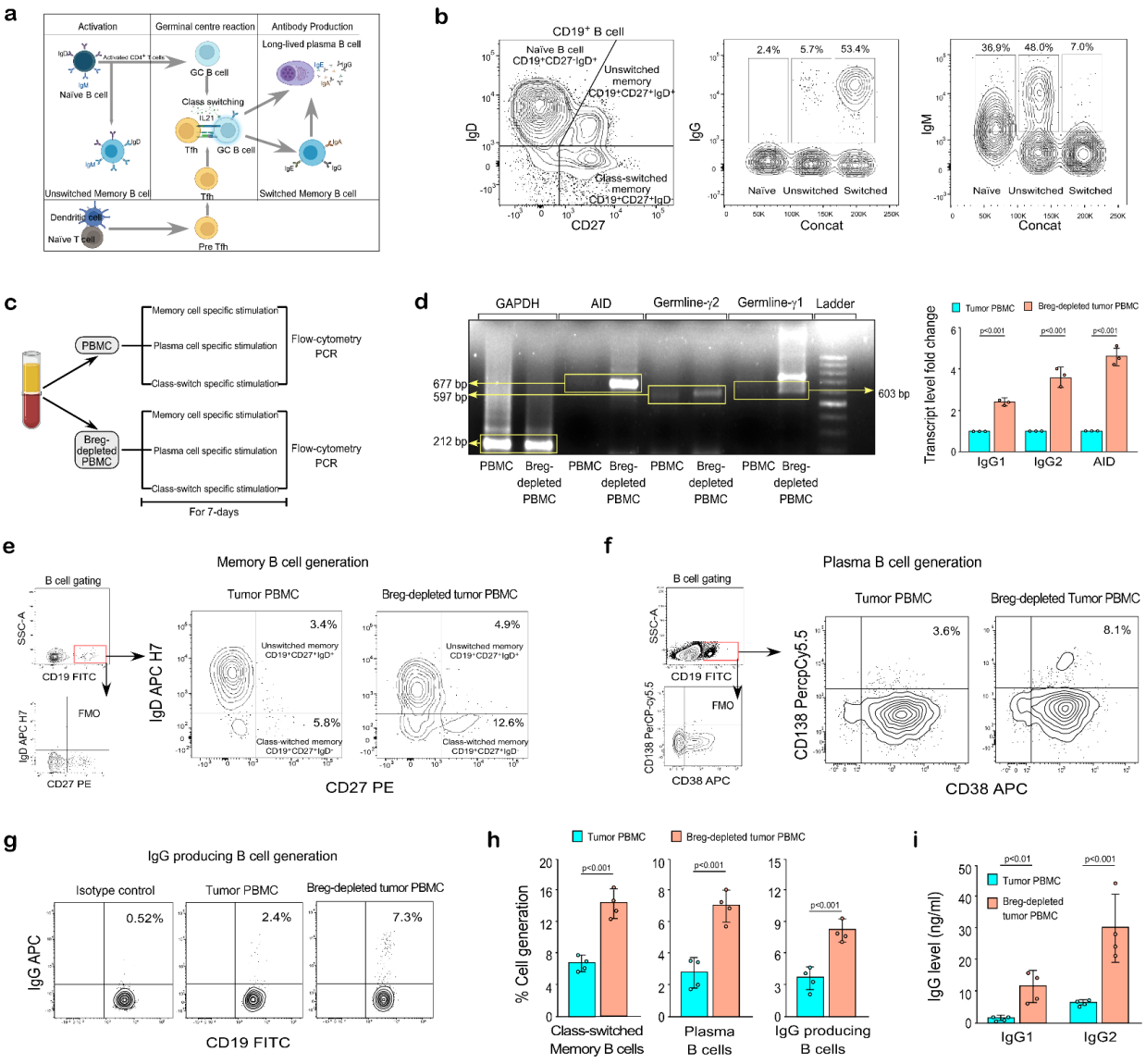

Figure-5. CD19$^+$CD39$^-$ Breg cells hinder B cell differentiation by limiting germline transcription involved in class-switch recombination for antibody production in cancer patients. ***(a)*** *A diagram of the stages of B cell development, from B cell activation to the formation of plasma cells that make antibodies* ***(b)*** *Flow-cytometric representation of the memory B cell subtype based on CD19, CD27, and IgD expression. Three B cell subpopulations are defined by the expression of IgD and CD27 within CD19$^+$ B cells: Naïve (IgD$^+$CD27$^-$), Unswitched memory (IgD$^+$CD27$^+$), and switched memory (IgD$^-$CD27$^+$). The expression of surface IgG and IgM in these B cells is represented in a concatenated format.* ***(c)*** *Schematic depiction of experiments for memory/plasma/CSR.* ***(d)*** *Representative DNA-gel electrophoresis of PCR product demonstrating the effect of Breg cell in the expression of germline-γ1, -γ2 and AID transcripts. The cumulative results from multiple samples obtained from Image J are represented in a bar graph. Values are mean ±SD or representatives of 3 sets of independent experiments (Right panel). Representative flow-cytometric plot depicting the ex-vivo differentiation of* ***(e)*** *memory B cells and* ***(f)*** *antibody-producing plasma B cells in Breg-depleted breast cancer patients PBMCs* ***(g)*** *and the ex-vivo development of IgG-producing B cells.* ***(h)*** *Results from multiple experiments are represented as ±SDM.* ***(i)*** *Supernatants from unaffected and Breg depleted PBMC were tested for the presence of secretory IGG1 and IGG2 by ELISA. Data are shown as mean ±SD.*

We depleted Breg cells from breast cancer patients' PBMC and cultured them *ex-vivo* for 7-days in media containing CD40LG+IL2+IL4 to generate memory phenotype, CD40LG+IL2+IL10 for plasma cell generation, and CD40LG+IL4+IL10 for germline-γ1/-γ2 and AID transcription to answer the enigma of Breg cells' impact on germline gene transcription (GLT), and B cell differentiation (Figure-5c). Flow cytometry was used to analyze the differentiation of memory B cells ($CD19^+CD27^+$), more precisely, class-switched ($CD19^+CD27^+IgD^-$)/non-class switched ($CD19^+CD27^+IgD^+$) memory B cells, and plasma B cells ($CD38^{hi}CD138^+$), and PCR was used to analyze class-switching recombination of germline-γ1/-γ2 and AID. Our flow-cytometry analysis demonstrated a rise in memory B cell percentages, specifically class-switched memory and plasma B cells in Breg-depleted conditions (Figure-5e, 5f and 5h). The class-switched transcripts germline-γ1/-γ2 and AID were also amplified in such conditions (Figure-5d). As a result, during the Breg-depleted condition, the surface level IgG (Figure-5g and 5h) and secreted antibodies (IgG1 and IgG2) (Figure-5i) both increased.

$CD39^-$ Breg cells disrupt memory to plasma B cell differentiation pathway

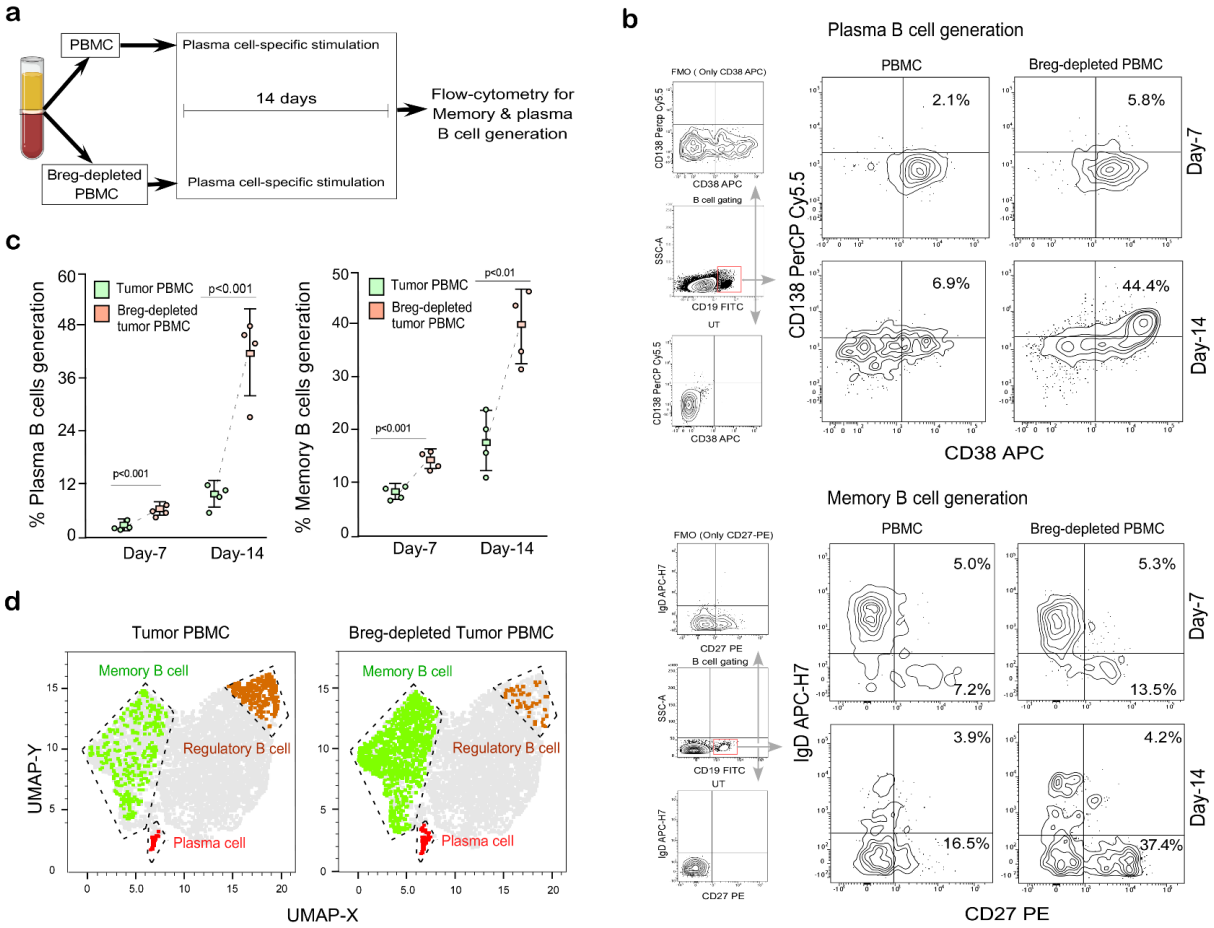

Figure-6. $CD19^+CD39^-$ Breg cells limit antibody responses by preventing the memory to plasma B cell differentiation pathway. ***(a)*** *Schematic representation of plasma B cell induction.* ***(b)*** *Flow-cytometric representation of plasma and memory B cell generation when Breg-depleted B cells were specifically treated with Plasma cell-specific induction.* ***(c)*** *The bar chart shows the effect of $CD19^+CD39^-$ Breg cells on the generation of memory and plasma B cells. Values are the mean ±SD of 4 sets of independent experiments.* ***(d)*** *A UMAP analysis sums up an inverse correlation between the memory (light green), plasma (red), and regulatory (orange) B cell subtypes in the peripheral circulation of breast cancer patients and Breg cell-depleted conditions. All these three subsets obtained from two conditions (PBMC and Breg depleted PBMC) are overlaid on B cells (light grey).*

Next, we generated plasma cells *ex-vivo* by incubating Breg-depleted patients' PBMC with plasma B cell-specific stimuli (Figure-6a) to investigate the role of Breg cells in the differentiation pathway of Ig-secreting plasma B cells. When Breg cells were eliminated from the cancer patient lymphocyte pool, the frequency of $CD38^{hi}CD138^+$ plasma B cells increased significantly (from 2% to 45%) (Figure-6b upper-panel, 6c), as did the frequency of class-switched memory B cells (from 7% to 37%) (Figure-6b lower-panel, 6c) after 14 days of culture. It's worth noting that Breg-depletion alone can cause memory B cell generation even in the absence of any memory B cell-specific activation. Our UMAP plots also demonstrate that memory and plasma B cell cluster frequencies increased in the Breg-depleted condition (Figure-6d).

All of this evidence supports the notion that the Breg cell has a negative impact on class-switch recombination, particularly at the level of germline transcripts and AID expression. As a result, the generation of class-switched memory B cells is halted, and the number of antibody-producing plasma B cells is reduced, resulting in a lower IgG level, which explains why there is less IgG in breast cancer patients' serum.

Breg inhibits antibody production by regulating Tfh responses

Previous investigations on B cell biology have demonstrated that T follicular helper cells (Tfh), are one of the important immune subsets that trigger class-switching induction by producing IL21 (Figure-5a)(31). As our data from Figure-5 and Figure-6 indicates that Breg prevents class switching recombination, therefore, we hypothesised whether Breg disrupts CSR by modulating Tfh responses or not. Purified Breg cells (Figure-S4b) were cocultured with naïve $CD4^+$ T (Figure-S4c) under Tfh-polarizing conditions to test the idea. During the Tfh cell differentiation process, we noticed that Breg had a considerable suppressive effect on $CXCR5^+PD1^{hi}$ Tfh development (Figure-7a), as well as the synthesis of the transcription factor BCL6 (Figure-7b). Because Tfh is a key source of IL21 from T cells (Figure-7c) (32), the percentage of $CD4^+IL21^+$ T cells was considerably lowered after Breg engagement (Figure-7d).

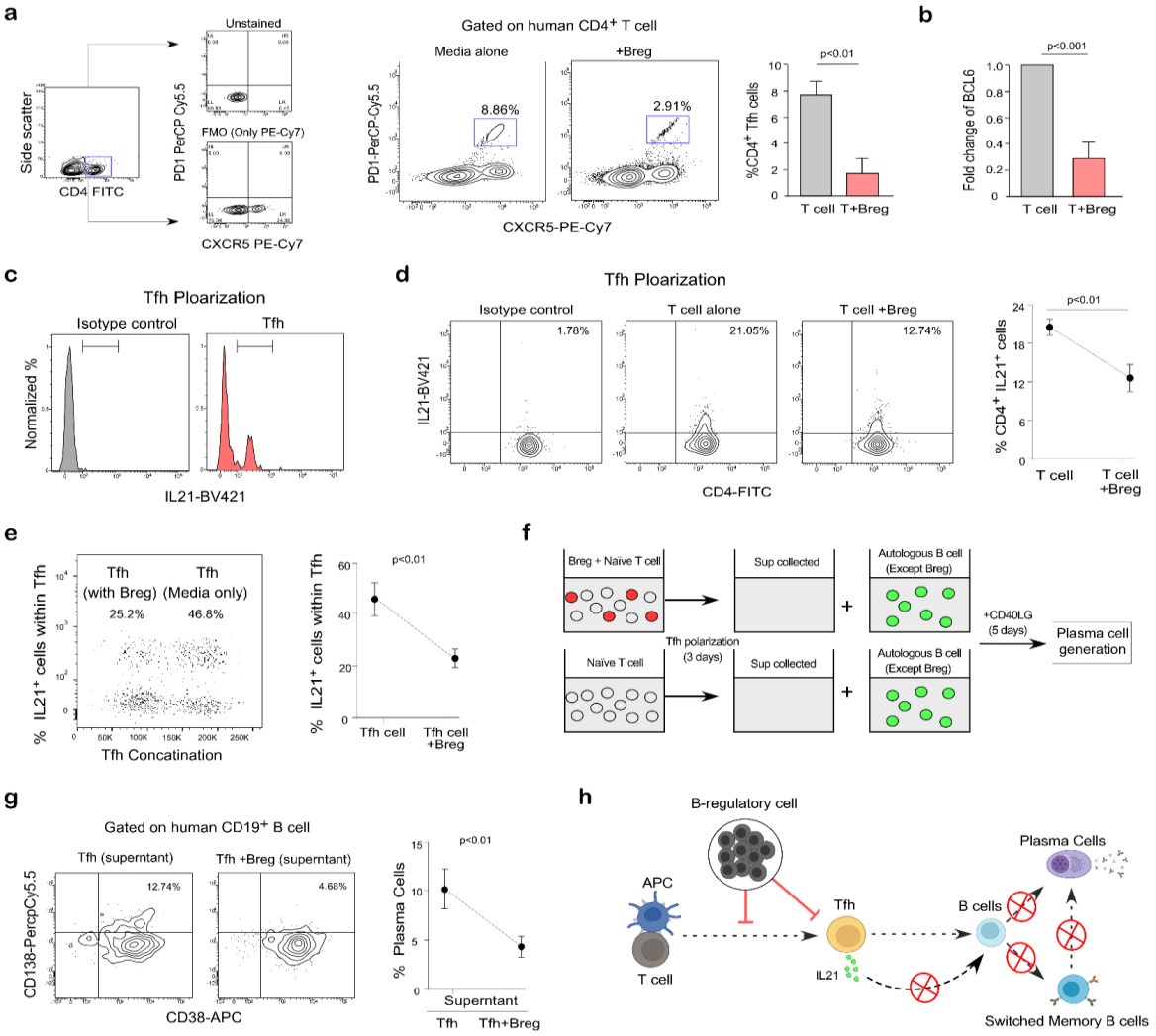

Figure-7. Breg inhibits Tfh differentiation and IL21 generation. ***(a)*** *Human $CD19^+CD39^-$ Breg cells from tumor patients cocultured with autologous naive $CD4^+$ T cells under Tfh-polarizing conditions. Tfh cell differentiation was analyzed using flow cytometry. Results from multiple experiments are represented as ±SDM (n=4).* ***(b)*** *Real-time PCR data demonstrate Bcl6 expression from the above-mentioned culture conditions.* ***(c)*** *Histogram analysis shows IL21 expression within in vitro generated Tfh cells.* ***(d)*** *The flow cytometric plot represents the expression of a reduced percentage of $CD4^+IL21^+$ T cells after engagement with Breg cells. The cumulative results from multiple samples are represented in a bar graph (n=3). Values are mean ±SD.* ***(e)*** *Percentage of IL21 expression among already differentiated Tfh cells from culture alone and Breg cultured is shown in a representative flow-cytometry concatenated plot. Cumulative results (n=3) are represented in a bar graph as ±SDM.* ***(f)*** *A schematic illustration of a culture condition in which ongoing Tfh differentiated cells were cultured with autologous breg cells for 72 hours and the collected cell supernatant was cultured with the same patient-derived unstimulated B cells (except the Breg) for another 5 days to assess plasma cell generation.* ***(g)*** *Generated plasma cells from the above-mentioned conditions were represented with a flow cytometry plot. Cumulative results (n=3) are represented in a bar graph as ±SDM.* ***(h)*** *A graphical representation of our findings that Breg prevents antibody response by controlling the Tfh differentiation process and IL21 generation from already generated Tfh cells.*

Following that, we looked at whether, aside from Tfh differentiation and IL21 production, Breg had any negative impacts on IL21 levels from already generated Tfh. As a result, we ran another flow-cytometry experiment with the same number of Tfh cells from Breg culture or grown alone. This time, we observed that Breg can also inhibit IL21 from already differentiated Tfh cells (Figure-7e). Following that, we attempted to assess plasma cell generation responses in relation to the Tfh generating condition. We designed an experiment in which we grew ongoing Tfh differentiated cells

with Breg cells for 72 hours and collected cell supernatant, followed by a further 5-days of culture with autologous unstimulated B cells (excluding the Breg) (Figure-7f). Tfh cell-dependent stimulation of B cells, which is required for their final differentiation into plasma cells, was blocked in the presence of Breg cells, resulting in a significant reduction in plasma B cell generation (Figure-7g). All of these findings pointed to a scenario in which Breg prevents antibody response by limiting Tfh development and IL21 production from Tfh cells that have already been generated (Figure-7h).

DISCUSSION:

Antibody responses mediated by B cells can lead to tumor cell destruction, and IgG plays a key part in this process (33,34). Numerous studies in B cell biology have indicated that IgG levels in metastatic breast cancer patients are much lower and IgM levels are higher than in individuals without metastasis (34-37). Low IgG levels have also been associated with a poor prognosis (34). We noticed a similar pattern when we initially started working, but we couldn't figure out why.

To figure this out, we looked at the B cell atlas and noticed that in tumor conditions, there was an increase in IL10-secreting transitional $CD19^{+}CD24^{hi}CD38^{hi}$ Breg cells, which have immunosuppressive properties and showed an inverse relationship with a decreased number of switched memory and antibody-producing plasma B cells (38). This information suggests the possibility of transitional Breg cells' negative impact on B cell differentiation and antibody-mediated immunity. To test this hypothesis, we need to isolate $CD19^{+}CD24^{hi}CD38^{hi}$ viable Breg cells. As they contain three highly-expressed cell surface markers, these combinations of markers have limitations in isolating functional Breg cells, leaving flow-sorting as the only choice. Unfortunately, as the cell numbers are lower and they have to pass through the laser, it is very difficult to obtain sufficient unstressed viable Breg cells.

To address this issue, using high-dimensional flow cytometry and microarray analysis, we identified $CD19^{+}CD39^{-}$ as an alternate marker for $CD19^{+}CD24^{hi}CD38^{hi}$ B cells. In this context, it is worth mentioning that Figueiró et al. (39) reported $CD19^{+}CD39^{+}$ as a marker associated with B cells' immunosuppressive function but we noticed that the level of these cells is not increased in tumor conditions (Figure-S3c). CD39 expression is strongly associated with mature B cells, memory B cells, and plasma B cells. CD39-negativity, on the other hand, is not linked to any of the above subsets, except for transitional B cells that are $CD19^{+}CD24^{hi}CD38^{hi}$ (Figure-3e and Figure-S3a). CD39 is an E-NTPDase which hydrolyzes ATP to ADP/AMP and finally to adenosine by CD73 from activated B cells (Figure-s7b, 40). The released adenosine binds to the B cells and helps in differentiation into class-switched B cells (41). So, it can be assumed that in the absence of any of these cell-surface molecules, CD39 and CD73 or blocking in the pathway, adenosine synthesis may be hindered, leading to impaired class-switch recombination. Because our identified Breg cells lack CD39 on their surface, we observed that these cells produce less adenosine than $CD39^{+}$ B cells (Figure-S7a). To show that less adenosine impaired class-switching, we added adenosine to tumor B cells and observed that exogenous adenosine prompted class-switching even in the presence of Breg cells (Figure-S7c). So, in tumor conditions, due to a higher proportion of $CD39^{-}$ Breg cells failing to make adenosine, cannot help themselves or other B cells in class-switching.

The IL10-producing $CD19^{+}CD39^{-}$ tumor-associated Breg cells identified have various immunoregulatory functions including inhibiting Th cell proliferation, type-1 cytokine production, T-effector cell survival, and inducing the generation of $CD4^{+}FOXP3^{+}$ Treg cells. Studies indicate that IL10 is a characteristic molecule of transitional Breg cells that binds to T cells and promotes immunosuppression (13). The other immunosuppressive molecule like TGFβ has minimal or no effect on the suppressive activity of tumor-associated Breg cells since its expression has little to no change in comparison to non-Breg populations. Several studies have also revealed that interactions between CD80 and CD86 on Breg cells and CD28 and CTLA4 on $CD4^{+}$ T cells work synergistically to release IL10 from B cells, suppressing T cell pro-inflammatory cytokine production (13,42). PDL1 expressed by Breg (Figure-s3d), which is bound to PD1 on T cells, is also found to limit T helper cell responses (43,44). All of these immunosuppressive processes in cancer patients convert the environment from immunogenic to tolerogenic which helps the tumor to grow more aggressively.

To test the possibility that the Breg has a negative impact on antibody response, we depleted Breg cells from the PBMC of breast cancer patients. Breg depletion resulted in substantial germinal transcript formations and AID expression involved in H-class-switched recombination, and B cell differentiation into subsequent class-switched memory and antibody-producing plasma B cells. It is well established that Tfh cells, a well-known B cell-help provider, play an important role in the establishment of germinal centres (31,32). Breg was found to have a negative impact on Tfh cell generation and its IL21 production. As Breg prevents class-switch recombination by inhibiting the Tfh/IL21 axis, long-lived plasma cell generation is halted, resulting in lower IgG and a higher fraction of B cells unable to undergo CSR events, succeeding in higher IgM. Furthermore, CD39-negative transitional B cells (abundant in cancer patients) produce high-IgM/low-IgG because they do not participate in CSR due to their lower adenosine-generating capacity (Figure-S7). The concerted effects could be the reasons behind the high-IgM/low-IgG event in the cancer patient.

In this regard, it is worth noting that the molecule(s) responsible for Breg's suppression is still a topic of investigation. PDL1 could be the molecule expressed by the Breg that prevents class-switching (Figure-S3d). PDL1 may bind to PD1 on Tfh, inhibiting IL21 production and impairing both memory and plasma B cell development (44). In-depth studies using

single-cell genomics and conditional knockdown mouse models could provide more information about Breg's role in B cell-mediated immune regulation during tumor progression.

Our present work proposes that $CD19^{+}CD39^{-}$ is a key signature surface-marker for tumor-associated $IL10^{+}$ Breg cells, and their augmentation in cancer patients restricts Tfh cells expansion and IL21 secretion, hence inhibiting class-switch recombination to antibody responses. Breg depletion could therefore be a promising future strategy for enhancing the magnitude of antibody responses and the dynamics of memory and plasma B cell-mediated tumour immunotherapy.

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