IN-DEPTH CHARACTERIZATION OF CD24^{high}CD38^{high} TRANSITIONAL HUMAN B CELLS REVEALS DIFFERENT REGULATORY PROFILES

Quentin Simon[1], MSc

Jacques-Olivier Pers[1], DDS, PhD

Divi Cornec[1,2], MD

Rizgar A Mageed [3], PhD, FRCP, FRCPath

Sophie Hillion[1*], PhD

[1]EA2216, INSERM ESPRI, ERI29, Université de Brest, Brest, France and LabEx IGO, Brest, France

[2]Rheumatology Department, CHRU Brest Morvan, France

[3]William Harvey Research Institute, Queen Mary University of London, EC1M 6BQ, UK

This work was supported by the LabEX IGO program via the investment of the future program ANR-11-LABX-0016-0

Disclosure of potential conflict of interest: The authors declare that they have no conflict of interest.

*Corresponding author: Sophie Hillion, Laboratoire d'Immunologie, CHRU Brest, BP824, 29609, Brest, France. Phone: 02-98-22-33-84; Fax: 02-98-22-38-47; sophie.hillion@univ-brest.fr

ABSTRACT

Background: $CD24^{high}CD38^{high}$ transitional B cells represent cells at a key stage in their developmental pathway. During this stage, B cells undergo peripheral tolerance and functional maturation. In addition, these B cells have been widely ascribed regulatory functions and involvement in the control of chronic inflammatory diseases. However, the phenotypic and functional overlap between these cells and regulatory B cells remain controversial.

Objective and Methods: In this study, we use multi-color flow cytometry in combination with bioinformatics and functional studies to show that $CD24^{high}CD38^{high}$ B cells can be distinguished into multiple subsets with different regulatory functions.

Results: The study reveals for the first time that human transitional B cells encompass not only transitional type 1 (T1) and T2 B cells, as previously suggested, but also distinct anergic T3 B cells as well as IL-10-producing $CD27^{+}$ transitional B cells. Interestingly, the latter two subsets differentially regulate $CD4^{+}$ T cell proliferation and polarization towards Th1 effector cells. Additional analyses reveal that the percentage of T3 B cells is reduced while the frequency of $CD27^{+}$ transitional B cells is increased in patients with autoimmune diseases compared with matched healthy individuals.

Conclusion: This study provides evidence for the existence of different transitional B cell subsets each displaying unique phenotypic and regulatory functional profiles. Furthermore, the study indicates that altered distribution of transitional B cells subsets highlights different regulatory defects in different autoimmune diseases.

Key messages: Our study reveals that different human transitional B cell subsets display different regulatory functions and that the frequency of such subsets is differentially altered in different autoimmune diseases.

Capsule summary: Recent studies of patients with autoimmune and inflammatory diseases indicate that B cells can prevent the development of adverse immune responses. This study documents the phenotype of human regulatory B cells and identifies potential cellular biomarkers that distinguish defects in immune regulation in patients with different autoimmune diseases.

Key words: Transitional B-cells; Immune regulation; chronic inflammatory diseases; Autoimmunity

Abbreviations used:

- Flow clustering without K (FLOCK)
- Healthy controls (HCs)
- Krome orange (KO)
- Mean fluorescence intensity (MFI)
- Mononuclear cells (MNCs)
- Pacific blue (PB)
- PE-linked to cyanin 5 (PC5)
- Population (pop)
- Primary Sjögren's syndrome (pSS)
- Rheumatoid arthritis (RA)
- Human immunodeficiency virus HIV
- Chronic antibody mediated rejection cABMR
- Standard error of the mean (SEM)
- Systemic lupus erythematosus (SLE)
- Type 1 (T1)
- Type 2 (T2)
- Type 3 (T3)

INTRODUCTION

Transitional B cells represent a central developmental stage in B cell maturation linking generation in the bone marrow with differentiation in the periphery.[1] The classification of murine transitional B cells into distinct type 1 (T1) and T2 subsets was subsequently reported, based on the expression of CD23, CD21 and the developmental marker CD24.[2] Studies of human B cells confirmed and extended the identification of transitional B cells in mice.[3,4] The early studies suggested that transitional human B cells can be subdivided into two major subsets: immature-T1 cells identified as $CD24^{high}CD38^{high}CD19^{+}CD10^{high}CD21^{low}IgM^{high}IgD^{low}CD27^{-}CD5^{+}$ cells and intermediate transitional cells (T2) that are $CD24^{high}CD38^{high}CD19^{+}CD10^{+}CD21^{+}IgM^{high}$ $IgD^{+}CD27^{-}$ $CD5^{low}$ cells.

The almost concurrent discovery of regulatory B cells (Bregs) drew interests towards potential links with transitional B cells due to phenotypic and functional similarities. Bregs were first reported to be IL-10-producing B cells in mice and termed B10 cells.[5] Although almost all human B cells have the capacity to produce IL-10, the available evidence indicates that the "most efficient" IL-10 producing B cells are Bregs that are similar to $CD24^{high}CD38^{high}$ transitional B cells.[6-9] Furthermore, associations between $CD24^{high}CD38^{high}$ B cells and immune regulation were noted in the favorable clinical outcome of patients with chronic inflammatory and autoimmune diseases. For example, long-term remission of patients with systemic lupus erythematosus (SLE) treated with B cell depleting biologic agents positively correlated with early reconstitution of transitional B cells after treatment.[10] Specific allergen immunotherapy drives suppression of IgE and promotion of IgG4 production by regulatory B cells.[11] These observations led investigators to draw parallels between $CD24^{high}CD38^{high}$ transitional B cells and Breg cells.[7,12-17] This, in turn, led to numerous

studies and the emergence of differing theories about the relationship between transitional and "real" Breg cells.

To increase understanding of $CD24^{high}CD38^{high}$ transitional B cells and potential overlaps with Breg cells in human, we developed a 10-color flow cytometry protocol for their phenotypic and functional characterization. The study reveals that $CD24^{high}CD38^{high}$ B cells incorporate phenotypically-distinct B cell subsets, each with distinct *in vitro* regulatory functions. Furthermore, the study reveals abnormal distribution of transitional B cell subsets in different autoimmune diseases, revealing new insights into Breg development and phenotype and aspects of defective immune regulation in different chronic inflammatory diseases.

METHODS

Detailed procedures and standard methods used in the study, such as cell enrichment, flow cytometry, apoptosis, ELISA, ELISPOT, Ca^{++} flux and cell culture are described in the Supplemental Materials.

Patients and controls

All patients and healthy controls (HCs) gave informed consent and the Ethics Committee at Brest University Medical School Hospital approved the study to be conducted in accordance with the Declaration of the Helsinki Principles. Table 1 summarizes the characteristics of patient groups included in the study.

Flow cytometry and cell sorting

All antibodies (Abs) were purchased from Beckman Coulter unless otherwise specified. All Abs used to make up the 10-color panel are listed in Table E1. Transitional B cell subsets and $CD4^+$ T cells were sorted with MoFlow XDP (Dako-Beckman Coulter). The purity of the sorted B cell subsets and T cells was greater than 98%.

Cell culture and proliferation assays

Sorted transitional B cells and $CD4^+$ T cells were co-cultured for 4 days in RPMI - 1640 complete medium as described.[18] For proliferation assays, sorted $CD4^+$ T cells were labeled with CellTrace^{TM} Violet reagent (Molecular Probes) before the co-culture. T cell proliferation was evaluated by flow cytometry using cell proliferation index in the FlowJo software (FlowJo, LLC).

Bioinformatic analyses and softwares

Bioinformatic analyses were performed with Flow Clustering without K online software (FLOCK). Files for each subject were uploaded to ImmPort (ImmPort, NIH website www.immport.org) and analyzed using FLOCK 1.0 to identify centroids for representative clusters. Markers listed in Table. E1 were used. CytoSpanning tree Progression of Density normalized Events (SPADE) analysis was carried out using Cytobank Inc. platform as described.[19]

Statistics

All data are expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism version 5.03 (GraphPad software). Non parametric Mann-Whitney *U*-test, or Wilcoxon *t*-test for paired observations were used and data were considered significant when $P<0.05$.

RESULTS

The frequency of CD24^{high}CD38^{high} B cells is variable in different diseases

High level co-expression of CD24 and CD38 identifies circulating transitional human B cells and is often used by investigators for quantifying Breg cells in peripheral blood. We have observed that the frequency of CD24^{high}CD38^{high} B cells in healthy controls (HCs) is 12.9±0.8% of total blood B cells but this frequency varies in different clinical settings (Fig 1, *A* and Table 1). The frequency of CD24^{high}CD38^{high} B cells in patients with primary Sjögren's syndrome (pSS) and SLE was significantly higher than in the HCs (15.5±1.2% and 17.9±1.8%, P=0.008, P=0.0037, respectively; Fig 1, *B*, left panel). This trend concurred with a similar increase in the absolute number of the transitional B cells (Fig 1, *B*, right panel). In contrast, patients with chronic antibody-mediated kidney rejection (cABMR) showed a significant decrease in the frequency of CD24^{high}CD38^{high} B cells compared with the HCs (2.4±0.4%, $P<10^{-3}$).[18] Interestingly, during the active course of HIV infection, patients manifested a dramatic decrease in transitional B cells (5.3±1.1% *vs* HCs, $P<10^{-3}$). These observations suggest that it is difficult to universally associate decreases/increases in the frequency/number of CD24^{high} CD38^{high} B cells with pathophysiological mechanisms.

Detailed characterization of CD19^{+} CD24^{high}CD38^{high} B cell subsets

In order to categorize CD24^{high}CD38^{high} populations in B cells, we carried out a 10-color flow cytometry analysis using the FLOCK (Flow clustering without K) software system.[20] First, we identified pre-gated transitional B cells as defined by high expression levels of CD24 and CD38 in 2D plots within CD19^{+} B cells (Fig 2, *A*). Next, we ran the FLOCK software on compiled flow cytometry data from 15 independent experiments using isolated B cells from the HCs. This enabled us to identify eight B cell clusters within the

$CD24^{high}CD38^{high}$ parent gate that partially overlapped in several Ab panels (Fig 2, *A* and Fig E1). Clusters 2, 3 and 5 were consistently found to be the three most abundant B cell clusters in $CD24^{high}CD38^{high}$ transitional B cells (cluster 2: 28.6±1.7%; cluster 3: 15.1±1.5% and cluster 5: 25.0±2.0%). In order to further characterize these subsets, we studied the expression level of additional different markers in each cluster. We observed that among the ten chosen markers, CD27, IgM and IgD were the best in differentiating B cell subsets within the 8 clusters based on their mean fluorescence intensity (MFI) (Fig 2, *B* and Fig E2, *A*). Cluster 6 displayed the highest MFI for CD27 expression (1639±46.7) compared with the other clusters ($P<10^{-3}$ *vs* cluster 1 and 3; others were negative for CD27). The MFI for IgM expression was higher in cluster 3 than in cluster 1 or 2 ($P<10^{-3}$). Cluster 2 had a significantly reduced level of IgD and IgM expression compared with the other clusters with none of the clusters expressing IgG or IgA (Fig 2, *C* and Fig E2, *B*). When the data were compiled together, clusters 1, 4, 5, 7 and 8 expressed significantly higher IgM MFI than cluster 2 and less IgD than cluster 3 and 6 ($P<10^{-3}$ and $P=0.037$, respectively) (Fig 2, *C*). Analysis of other markers confirmed the noted distinctiveness of clusters 2 and 3 (Fig E2, *A*). We further observed that CD10 expression was higher in cluster 3 than in the other clusters, while cluster 2 had an intermediate level of CD21 expression. Consequently, we congregated the 8 clusters into four different patterns based on CD27, IgM and IgD expression. Cluster 6 ($CD27^{+}$) was renamed population (pop) A. Cluster 3 ($IgD^{low}IgM^{high}$) was branded pop B. Cluster 2 ($IgM^{low}IgD^{+}$) was named pop C. Finally, we grouped clusters 1, 4, 5, 7 and 8 that expressed similar levels of IgM and IgD into pop D (Fig 2, *C*). Cross sample comparison of these four populations was highly consistent between different blood donors (Fig 2, *D*). Populations C and D represented the majority of $CD24^{high}CD38^{high}$ B cells (55.7±1.3% and 30.1±1.5, respectively) whereas pop B represented 7.3±0.8% and pop A 6.0±0.7% of all $CD24^{high}$ $CD38^{high}$ B cells.

Phenotypic characterization of transitional B cell subsets identified by FLOCK

We next carried out detailed phenotypic analysis of these four defined populations using markers commonly ascribed to B cell-differentiation, survival or activation (Fig 3).

B cell-differentiation and activation markers

These analyses revealed that each cluster showed a unique B cell profile of differentiation (Fig 3, *A*) and activation (Fig 3, *B*). Pop A expressed CD27 and high levels of all of the other differentiation markers with the exception of CD23. This population, thus, expressed high levels of CD80, CD86, CD40, CD25 and TLR-9, depicting a high activation status. Pop B expressed lower levels of CD21, CD22, CD23, CD44, and CD62L than the other 3 populations but had high levels of CD10 and CD32 (Fig 3, *A*). The results also revealed lower basal expression levels of HLA-DR, CD80, CD86 and CD25 in pop C compared with pop D (Fig 3, *B*). This data suggests that pop C may be in a resting-state.

Mitotracker expression

The activity of the ATP-binding cassette transporter ABCB1 has been described as a functional characteristic that distinguishes transitional from mature naive B cells.[21] The level of MTG-Green MFI gradually decreased from pop B to pop D and C implying a developmental pathway through these subsets (Fig 3, *C*). Interestingly, pop A did not extrude the probe at all, supporting the likelihood that this subset has a memory-like phenotype.

By compiling these descriptive data, the results provide evidence that pop B presents a pattern of differentiation close to immature B cells as described for T1 B cells (Fig 3, *D*). Pop D, in contrast, showed a progressive pattern of upregulating maturity markers, IgD, CD21, CD44 and CD62L. The pattern observed in this population is in agreement with the previously defined T2 subset of B cells. In contrast, pop C displayed an intermediate profile of these markers consistent with been in a resting state described for T3 B cells.[22] Finally, pop

A represents an atypically-activated B cell with a memory-like phenotype, renamed $CD27^+$ transitional B cells (Fig 3, *D*).

Functional characteristics of T1, T2, T3 and $CD27^+$ transitional B cell subsets

To corroborate the phenotypic distinction between the transitional B cell subsets, functional characteristics of the subsets were investigated including analyzing survival and responses to BCR engagement.

Survival capacity

Reduced survival has been reported to be a functional characteristic of transitional B cells.[3,23] To compare and contrast this ability within the different subsets, we first analyzed viability of the different $CD24^{high}CD38^{high}$ B cell subsets in the absence of exogenous stimuli (Fig 4, *A*). The analysis revealed no significant differences between the subsets for early spontaneous apoptosis. Interestingly, $CD27^+$ transitional B cells showed a high necrotic rate in culture ($Draq7^+$ and Annexin V^+ cells). We next studied whether BCR engagement will modify survival of the transitional B cell subsets. Because staining B cells with FITC-conjugated anti-IgM Ab interferes with their response to BCR engagement, we developed an alternative gating strategy using CD27, IgD and CD32 (Fig 4, *B*). As depicted in the Figure, the newly-set gate for T1 ($CD32^+IgD^{low}$), T2 ($CD32^{high}IgD^{high}$) and T3 ($CD32^{low}IgD^{low}$) B cells perfectly matched our earlier phenotypic description of these subsets. BCR engagement increased apoptosis in the T1 B cell subset and did not increase survival of the other subsets (Fig 4, *A*).

Hyporesponsiveness of T3 B cells to BCR engagement

Low density surface IgM is a hallmark of anergic B cells in mice and humans.[24] Such anergic B cells have been shown to be over-represented in transitional B cells.[25] To determine the tolerance status of the B cell subsets identified in the current study, we next determined

the functional response of the $CD24^{high}CD38^{high}$ B cell subsets to BCR engagement. The ability of each subset to mobilize Ca^{2+} after BCR engagement was assessed by flow cytometry.[26] BCR engagement induced similar increases in [Ca^{2+}] influx in $CD27^{+}$ and T2 B cells (Fig 5 *A*). T3 B cells were unresponsive to BCR engagement as indicated by a failure to mobilize Ca^{2+} upon BCR engagement consistent with low levels of BCR expression.[24] To confirm this observation, we next analyzed the release of PIP3 and phosphorylation of PLCγ2.[27] According to their inability to mobilize Ca^{2+}, T3 B cells did not show an increase in PIP3 release (Fig 5, *B*, left panel) or increased PLCγ2 phosphorylation following BCR engagement (Fig 5, *B*, right panel). These findings indicate that the T3 subset of transitional B cells is refractory to BCR engagement consistent with a functional silencing similar to immunological anergy.

T1 and $CD27^{+}$ transitional B cells provide innate humoral immunity

Transitional B cells are capable of contributing to innate humoral immunity by differentiating to polyreactive IgM Ab-producing cells.[28] We, therefore, assessed whether differences existed between the transitional B cell subsets in their ability to produce natural IgM Abs. $CD24^{high}CD38^{high}$ B cell subsets were sorted by flow cytometry (purity indicated in Fig E3, *A*) and incubated for 4 days with CpG (to mimic T cell-independent stimulation). B cell differentiation was then analyzed by flow cytometry. The results revealed two differentiated populations: $CD27^{high}CD38^{high}$ plasmablasts and $CD27^{+}CD38^{-}$ memory B cells (Fig 5, *C*).[29] After 4 days of culture, $CD27^{+}$ transitional B cells differentiated significantly more to memory B cells (15.2±1.2%) than T1 B cells did (2.3±0.4%). A very small number of T2 B cells acquired a memory phenotype (0.66±0.1%) while almost none became plasmablasts. T3 B cells, consistent with their functional silencing, did not differentiate upon CpG stimulation.

We next analyzed the ability of the sorted transitional B cell subsets to secrete natural IgM using ELISPOT (Fig 5, *C*, lower panel). Interestingly, T1 and $CD27^+$ transitional B cells showed a high capacity to differentiate into IgM secreting cells. None of the populations switched Abs from IgM to IgG or IgA (data not shown).

All in all, the results demonstrate that transitional $CD27^+$ and T1 B cells are highly efficient producers of IgM Abs typical of innate immune responses consistent with an "innate memory" cell phenotype.

The regulatory functions of transitional B cell subsets

IL-10 production

Previous studies have revealed that B cell subsets producing IL-10 are consistently found within $CD24^{high}CD38^{high}$ B cells and $CD24^{high}CD27^+$ cells.[30] To further determine functional characteristics of the transitional B cell subsets, we next studied spontaneous IL-10 production *in vitro* without stimulation (Fig 6). Using a successive gating strategy (Fig 6, *A*) we observed that IL-10-producing B cells were mainly enriched in the T2 and $CD27^+$ B cell subsets (35.1±6.6% and 50.7±7.4% of $IL\text{-}10^+$ B cells, respectively) (Fig 6, *B*, left panel). Interestingly, the MFI for intracellular IL-10 expression was significantly higher in $CD27^+$ transitional B cells than in the other transitional subsets ($P<10^{-3}$, Fig 6, *B* middle panel). When the analysis was repeated but with B cells stimulated through the TLR9 for 24 hours with CpG ODN there was a dramatic increase in $IL\text{-}10^+$ B cells within the T2 and T3 subsets (Fig 6, *C*, left panel). Activation of the cells through TLR9 with CpG enhanced intracellular IL-10 expression (IL-10 MFI) in the pre-existing $IL10^+CD27^+$ transitional B cells (Fig 6, *C*, right panel and Fig E4). Finally, to objectively present the data on spontaneous IL-10 production by the different B cell subsets, we used Spanning-tree Progression Analysis of density-normalized Events (SPADE) software which maps cells in a hierarchical tree[19] (Fig 6,

D). To interpret the SPADE tree, we derived annotations according to the differential pattern of expression of each marker previously chosen to differentiate the various transitional B cell subsets: CD24, CD38, IgM, IgD, CD27, CD32 and CD19. The data confirmed that the $CD27^+$ and T2 transitional B cell subsets were the most inherently competent cells in producing IL-10. Interestingly, the SPADE analysis revealed that lower levels of IL-10 could also be produced by other B cell subsets.

Transitional $CD27^+$B cells inhibit TNFα and IFNγ production by T cells

The available evidence indicates that transitional human B cells were the most efficient subset of Breg cells in inhibiting TNFα and IFNγ production by T cells.[7,18] To determine which of the transitional B cells characterized in this study best manifests the ability to regulate cytokine production by T cells, we used a specially-adapted *in vitro* model system.[31] The transitional B cell subsets were FACS-sorted (purity >98% as depicted in Fig E3, *A*) and co-cultured with anti-CD3 and anti-CD28 Abs-activated $CD4^+$ T cells (purity >99% shown in Fig E3, *B*). All transitional B cell subsets were able to reduce TNFα production by $CD4^+$ T cells but the $CD27^+$ B cell subset was the most efficient (P=0.015 *vs* T1 B cells; P=0.015 *vs* T2 B cells; P=0.0078 *vs* T3 B cells) (Fig 7, *A*). Interestingly, $CD27^+$ transitional B cells were the only subset within transitional B cells capable of significantly suppressing both TNFα and IFNγ production by $CD4^+$ T cells (P=0.0078).

Anergic-like T3 B cells regulate T cell proliferation

In the next set of experiments, proliferation of the sorted $CD4^+$ T cells was studied by flow cytometry at day 4 in the presence or absence of sorted transitional B cell subsets (Fig 7, *B*). T1 and $CD27^+$ transitional B cells were unable to suppress T cell proliferation. In contrast T2 and T3 exhibited a significant ability to reduce $CD4^+$ T cell proliferation. Interestingly, the T3 B cell subset showed the highest reproducibility in the ability to reduce T cell proliferation

among different donors when compared with T2 B cells suggesting that the latter might be composed of different functional subsets.

These observations demonstrate that transitional B cell subsets have differential abilities to regulate T cell responses.

Altered distribution of transitional B cell subsets in patients with different autoimmune diseases

Results presented in Fig 1 provided evidence that the percentage of $CD24^{high}CD38^{high}$ B cells was significantly higher in patients with pSS and SLE compared with HCs. Based on these observations we investigated the distribution of each of the transitional B cell subsets characterized in the study in patients with these autoimmune diseases. For this purpose, we performed a FLOCK cross sample comparison of 10-color flow-cytometry data on enriched B cells from 10 HCs, 11 patients with pSS and 10 patients with SLE (Figs 7, *C* and *D*). The results showed that the frequency of $CD27^+$ transitional B cells was significantly increased in patients with pSS and SLE compared with the HCs (P=0.03 and P=0.0011, respectively; Fig 7, *C*). Interestingly, the frequency of T3 B cells was decreased in the patients compared with the HCs (P=0.006 in pSS patients and P=0.028 in SLE patients; Fig 7, *D*) whereas T1 and T2 B cells were similar. These findings suggest that T3 and $CD27^+$ transitional B cells may have been influenced by a breach in peripheral-tolerance in these patients.

DISCUSSION

Phenotypic and functional features of transitional B cells have been extensively studied in mice leading to a qualified consensus that at least three major subsets of these cells exist.[2,22,25] In humans, however, the biology of transitional B cells remains controversial. T1 and T2 subsets have consistently been identified within $CD24^{high}CD38^{high}$ B cells.[32] In contrast, T3 B cells have been suggested to be part of the mature B cell pool.[4,21] In the current study, we identified the T1 and T2 subsets by their differential capacity to survive or undergo apoptosis with BCR engagement. *In vitro* and i*n vivo* studies revealed that whilst T1 B cells underwent apoptosis upon BCR engagement, T2 B cells proliferated and were resistant to apoptosis.[33,34] However, our study provides new evidence for the existence of two additional transitional B cell subsets with distinct phenotypic and functional properties. One subset, T3 B cells, expressed low levels of IgM, IgD and CD10 and displayed functional status similar to anergic cells. The second subset which includes $CD27^{+}$ transitional B cells have not been described before as transitional B cells because CD27 expression is generally considered a memory B cell marker. Nevertheless, we have demonstrated that 6.0±0.7% of $CD24^{high}CD38^{high}$ B cells express $CD27^{+}$. Furthermore, these cells responded rapidly to T cell-independent stimuli and secreted natural IgM Abs consistent with innate-like B cells.[35,36] $CD27^{+}$ transitional B cells may represent cells at a developmental stage in an alternative differentiation pathway that responds to TLR ligands. In mice, recent studies indicate that some transitional B cells have the potential to differentiate into plasmablasts and natural memory B cells.[37,38] The identification of multiple human transitional B cell subsets in the current study could, therefore, provide important new insights indicating the existence of multiple developmental programs through which human migrant B cells undergo tolerance and maturation. Based on the findings made in the study, we developed a new paradigm (Fig E5) that includes a novel developmental pathway for transitional B cell maturation in humans.

As a corollary to our hypothesis, we demonstrate that transitional B cell subsets identified within the $CD24^{high}CD38^{high}$ parent flow cytometry gate display differential regulatory abilities. Thus, we reveal that $CD27^{+}$ transitional B cells are specialized in suppressing the production of pro-inflammatory cytokines. This subset of transitional B cells has the capacity to produce high levels of IL-10 (Fig 6). Although the ability to produce IL-10 is often used as a marker of human Bregs, we show that this is a property that is not restricted to a specific B cell subset. Indeed, our flow cytometry and bioinformatics analyses revealed that IL-10 can be produced by transitional B-cells subsets, by $CD27^{+}CD24^{high}$ cells[30] and by $IgM^{+}IgD^{-}$ memory B cells[39] (Fig 6). Furthermore, our data are consistent with studies of B cells in mice showing that B10[30,40,41] progenitor and effector cells may coexist in human transitional B cells (Fig 6 and Fig E4). Interestingly, T3 anergic-like B cells possessed the best and most consistent ability to control T cell proliferation. The inconsistent suppressive ability of T2 B cells suggested that this subset may be composed of more than one functional subset. Indeed, the Flock analysis (Fig 2 and Fig E1) indicated that T2 B cells encompassed 5 different clusters of B cells. In this respect, several previous reports have suggested that T2 B cells are heterogeneous and include some follicular and marginal zone B cells.[42,43] The precise mechanism by which anergic B cells exhibit potent regulatory activities remains to be fully defined. In this respect, it has been suggested that B cells can be tolerogenic to T cells when presenting a limited number of antigen peptides/MHC complexes to T cells.[44] This characteristic was described for anergic B cells.[45] Additionally, the lack of costimulatory molecules (Fig 3) on the anergic-like B cells may induce anergy in the corresponding, antigen-specific, T cells.[46] Relevant to this observation, a recent study demonstrated that Ars/A1 anergic B cells are potent suppressors of humoral immunity in an IL-10 independent manner.[47]

These observations cast new lights on existing but rather conflicting data on the phenotype of Breg cells by underlying the differential ability of different B cell subsets to regulate distinct T cell responses. Thus, our study demonstrates for the first time that immune regulation by B cells is not confined to a "particular" B cell subset and also not in a functionally restricted manner. Instead, the data show that different B cell subsets can have different regulatory properties determined not only by their own developmental pathway and activation status but also by how the target cell is influenced by its microenvironment. In this regard, a recent report suggested a developmental link between human transitional B-cells and IL-10-producing plasmablasts.[38]

Using FLOCK cross-sample comparison analyses, the last part of our study revealed that patients with autoimmune diseases display abnormal distribution of transitional B cell subsets. Interestingly, $CD27^+$ transitional B cells were over-represented in patients with SLE and pSS whereas the frequency of anergic-like T3 B cells was decreased. An increase in transitional B cells in patients with SLE patients has been described before but this did not correlate with disease activity.[3] Reduction in the frequency of T3 B cells is consistent with the persistence of increased numbers of autoreactive mature B cells in such patients (25-31% in SLE patients *vs* 4-3% in HCs) as previously observed.[48] Furthermore, B cells from patients with SLE have been reported to be defective in their ability to suppress T cell proliferation.[49,50] However, further investigation of transitional B cell subsets would be required to accurately identify defective immune regulatory pathways in different diseases. Nevertheless, the study provides preliminary evidence that transitional B cells are not a homogeneous regulatory B cell population but rather represent a complex mixture of subsets from which different Bregs emerge.[51] The findings, thus, provide a new approach to define cellular biomarkers that identify specific defects in immune regulation in different diseases.

ACKNOWLEDGMENTS

399 The authors thank Simone Forest and Geneviève Michel for their secretarial
400 assistance.

401

REFERENCES

1. Carsetti R, Kohler G, Lamers MC. Transitional B cells are the target of negative selection in the B cell compartment. J Exp Med 1995;181:2129-40.

2. Loder F, Mutschler B, Ray RJ, Paige CJ, Sideras P, Torres R, et al. B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals. J Exp Med 1999;190:75-89.

3. Sims GP, Ettinger R, Shirota Y, Yarboro CH, Illei GG, Lipsky PE. Identification and characterization of circulating human transitional B cells. Blood 2005;105:4390-8.

4. Palanichamy A, Barnard J, Zheng B, Owen T, Quach T, Wei C, et al. Novel human transitional B cell populations revealed by B cell depletion therapy. J Immunol 2009;182:5982-93.

5. Fillatreau S, Sweenie CH, McGeachy MJ, Gray D, Anderton SM. B cells regulate autoimmunity by provision of IL-10. Nat Immunol 2002;3:944-50.

6. Duddy M, Niino M, Adatia F, Hebert S, Freedman M, Atkins H, et al. Distinct effector cytokine profiles of memory and naive human B cell subsets and implication in multiple sclerosis. J Immunol 2007;178:6092-9.

7. Blair PA, Norena LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, et al. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. Immunity 2010;32:129-40.

8. Bouaziz JD, Calbo S, Maho-Vaillant M, Saussine A, Bagot M, Bensussan A, et al. IL-10 produced by activated human B cells regulates CD4(+) T-cell activation in vitro. Eur J Immunol 2010;40:2686-91.

9. Flores-Borja F, Bosma A, Ng D, Reddy V, Ehrenstein MR, Isenberg DA, et al. CD19+CD24hiCD38hi B cells maintain regulatory T cells while limiting TH1 and TH17 differentiation. Sci Transl Med 2013;5:173ra23.

10. Anolik JH, Barnard J, Owen T, Zheng B, Kemshetti S, Looney RJ, et al. Delayed memory B cell recovery in peripheral blood and lymphoid tissue in systemic lupus erythematosus after B cell depletion therapy. Arthritis Rheum 2007;56:3044-56.

11. van de Veen W, Stanic B, Yaman G, Wawrzyniak M, Sollner S, Akdis DG, et al. IgG4 production is confined to human IL-10-producing regulatory B cells that suppress antigen-specific immune responses. J Allergy Clin Immunol 2013;131:1204-12.

12. Bao W, Zhong H, Manwani D, Vasovic L, Uehlinger J, Lee MT, et al. Regulatory B-cell compartment in transfused alloimmunized and non-alloimmunized patients with sickle cell disease. Am J Hematol 2013;88:736-40.

13. Landolt-Marticorena C, Wither R, Reich H, Herzenberg A, Scholey J, Gladman DD, et al. Increased expression of B cell activation factor supports the abnormal expansion of transitional B cells in systemic lupus erythematosus. J Rheumatol 2011;38:642-51.

14. Duggal NA, Upton J, Phillips AC, Sapey E, Lord JM. An age-related numerical and functional deficit in CD19(+) CD24(hi) CD38(hi) B cells is associated with an increase in systemic autoimmunity. Aging Cell 2013;12:873-81.

15. Franquesa M, Mensah FK, Huizinga R, Strini T, Boon L, Lombardo E, et al. Human adipose tissue-derived mesenchymal stem cells abrogate plasmablast formation and induce regulatory B cells independently of T helper cells. Stem Cells 2014.

16. Lin W, Jin L, Chen H, Wu Q, Fei Y, Zheng W, et al. B cell subsets and dysfunction of regulatory B cells in IgG4-related diseases and primary Sjogren's syndrome: the similarities and differences. Arthritis Res Ther 2014;16:R118.

17. Li X, Zhong H, Bao W, Boulad N, Evangelista J, Haider MA, et al. Defective regulatory B-cell compartment in patients with immune thrombocytopenia. Blood 2012;120:3318-25.

18. Nouel A, Segalen I, Jamin C, Doucet L, Caillard S, Renaudineau Y, et al. B cells display an abnormal distribution and an impaired suppressive function in patients with chronic antibody-mediated rejection. Kidney Int 2014;85:590-9.

19. Qiu P, Simonds EF, Bendall SC, Gibbs KD, Jr., Bruggner RV, Linderman MD, et al. Extracting a cellular hierarchy from high-dimensional cytometry data with SPADE. Nat Biotechnol 2011;29:886-91.

20. Qian Y, Wei C, Eun-Hyung Lee F, Campbell J, Halliley J, Lee JA, et al. Elucidation of seventeen human peripheral blood B-cell subsets and quantification of the tetanus response using a density-based method for the automated identification of cell populations in multidimensional flow cytometry data. Cytometry B Clin Cytom 2010;78 Suppl 1:S69-82.

21. Wirths S, Lanzavecchia A. ABCB1 transporter discriminates human resting naive B cells from cycling transitional and memory B cells. Eur J Immunol 2005;35:3433-41.

22. Allman D, Lindsley RC, DeMuth W, Rudd K, Shinton SA, Hardy RR. Resolution of three nonproliferative immature splenic B cell subsets reveals multiple selection points during peripheral B cell maturation. J Immunol 2001;167:6834-40.

23. Cuss AK, Avery DT, Cannons JL, Yu LJ, Nichols KE, Shaw PJ, et al. Expansion of functionally immature transitional B cells is associated with human-immunodeficient states characterized by impaired humoral immunity. J Immunol 2006;176:1506-16.

24. Quach TD, Manjarrez-Orduno N, Adlowitz DG, Silver L, Yang H, Wei C, et al. Anergic responses characterize a large fraction of human autoreactive naive B cells expressing low levels of surface IgM. J Immunol 2011;186:4640-8.

25. Merrell KT, Benschop RJ, Gauld SB, Aviszus K, Decote-Ricardo D, Wysocki LJ, et al. Identification of anergic B cells within a wild-type repertoire. Immunity 2006;25:953-62.

26. Seite JF, Goutsmedt C, Youinou P, Pers JO, Hillion S. Intravenous immunoglobulin induces a functional silencing program similar to anergy in human B cells. J Allergy Clin Immunol 2014;133:181-8 e1-9.

27. Browne CD, Del Nagro CJ, Cato MH, Dengler HS, Rickert RC. Suppression of phosphatidylinositol 3,4,5-trisphosphate production is a key determinant of B cell anergy. Immunity 2009;31:749-60.

28. Ueda Y, Liao D, Yang K, Patel A, Kelsoe G. T-independent activation-induced cytidine deaminase expression, class-switch recombination, and antibody production by immature/transitional 1 B cells. J Immunol 2007;178:3593-601.

29. Carsetti R, Rosado MM, Wardmann H. Peripheral development of B cells in mouse and man. Immunol Rev 2004;197:179-91.

30. Iwata Y, Matsushita T, Horikawa M, Dilillo DJ, Yanaba K, Venturi GM, et al. Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. Blood 2011;117:530-41.

31. Nouel A, Pochard P, Simon Q, Segalen I, Le Meur Y, Pers JO, et al. B-Cells induce regulatory T cells through TGF-beta/IDO production in A CTLA-4 dependent manner. J Autoimmun 2015.

32. Benitez A, Weldon AJ, Tatosyan L, Velkuru V, Lee S, Milford TA, et al. Differences in mouse and human nonmemory B cell pools. J Immunol 2014;192:4610-9.

33. Petro JB, Gerstein RM, Lowe J, Carter RS, Shinners N, Khan WN. Transitional type 1 and 2 B lymphocyte subsets are differentially responsive to antigen receptor signaling. J Biol Chem 2002;277:48009-19.

34. Su TT, Rawlings DJ. Transitional B lymphocyte subsets operate as distinct checkpoints in murine splenic B cell development. J Immunol 2002;168:2101-10.

35. Genestier L, Taillardet M, Mondiere P, Gheit H, Bella C, Defrance T. TLR agonists selectively promote terminal plasma cell differentiation of B cell subsets specialized in thymus-independent responses. J Immunol 2007;178:7779-86.

36. Zhang X. Regulatory functions of innate-like B cells. Cell Mol Immunol 2013;10:113-21.

37. Giltiay NV, Chappell CP, Sun X, Kolhatkar N, Teal TH, Wiedeman AE, et al. Overexpression of TLR7 promotes cell-intrinsic expansion and autoantibody production by transitional T1 B cells. J Exp Med 2013;210:2773-89.

38. Matsumoto M, Baba A, Yokota T, Nishikawa H, Ohkawa Y, Kayama H, et al. Interleukin-10-producing plasmablasts exert regulatory function in autoimmune inflammation. Immunity 2014;41:1040-51.

39. Khoder A, Sarvaria A, Alsuliman A, Chew C, Sekine T, Cooper N, et al. Regulatory B-Cells Are Enriched Within the IgM Memory and Transitional Subsets in Healthy Donors but Deficient In Chronic Graft-Versus-Host Disease. Blood 2014.

40. Kalampokis I, Yoshizaki A, Tedder TF. IL-10-producing regulatory B cells (B10 cells) in autoimmune disease. Arthritis Res Ther 2013;15 Suppl 1:S1.

41. Tedder TF. B10 cells: a functionally defined regulatory B cell subset. J Immunol 2015;194:1395-401.

42. Pillai S, Cariappa A, Moran ST. Marginal zone B cells. Annu Rev Immunol 2005;23:161-96.

519 43. Meyer-Bahlburg A, Andrews SF, Yu KO, Porcelli SA, Rawlings DJ. Characterization
520 of a late transitional B cell population highly sensitive to BAFF-mediated homeostatic
521 proliferation. J Exp Med 2008;205:155-68.

522 44. Fuchs EJ, Matzinger P. B cells turn off virgin but not memory T cells. Science
523 1992;258:1156-9.

524 45. Kendall PL, Case JB, Sullivan AM, Holderness JS, Wells KS, Liu E, et al. Tolerant
525 anti-insulin B cells are effective APCs. J Immunol 2013;190:2519-26.

526 46. Dalai SK, Mirshahidi S, Morrot A, Zavala F, Sadegh-Nasseri S. Anergy in memory
527 CD4+ T cells is induced by B cells. J Immunol 2008;181:3221-31.

528 47. Aviszus K, Macleod MK, Kirchenbaum GA, Detanico TO, Heiser RA, St Clair JB, et
529 al. Antigen-specific suppression of humoral immunity by anergic Ars/A1 B cells. J Immunol
530 2012;189:4275-83.

531 48. Yurasov S, Wardemann H, Hammersen J, Tsuiji M, Meffre E, Pascual V, et al.
532 Defective B cell tolerance checkpoints in systemic lupus erythematosus. J Exp Med
533 2005;201:703-11.

534 49. Lemoine S, Morva A, Youinou P, Jamin C. Human T cells induce their own regulation
535 through activation of B cells. J Autoimmun 2011;36:228-38.

536 50. Gao N, Dresel J, Eckstein V, Gellert R, Storch H, Venigalla RK, et al. Impaired
537 suppressive capacity of activation-induced regulatory B cells in systemic lupus
538 erythematosus. Arthritis Rheumatol 2014;66:2849-61.

539 51. Rosser EC, Mauri C. Regulatory B Cells: Origin, Phenotype, and Function. Immunity
540 2015;42:607-12.

541

542

FIGURES AND LEGENDS

FIG. 1. **Comparison of $CD24^{high}CD38^{high}$ transitional B cell subsets in patients and healthy controls.** Blood from 33 HCs (■), 17 patients with pSS (○), 16 patients with SLE (●), 15 patients with RA (▽), 17 patients with cABMR (▲) and 26 patients infected with HIV (◊) were analyzed by flow cytometry. A, Representative dot plots of CD24 and CD38 expression and percentage of $CD24^{high}CD38^{high}$ cells in $CD19^{+}$ B cells. B, Percentages (left panel) and absolute numbers (right panel) of $CD19^{+}$ $CD24^{high}CD38^{high}$ B cells in each group.*P<0.05, **P<0.01, ***P<0.001.

FIG. 2. **Identification and phenotypic definition of transitional B cell subsets**. B cells from 15 HCs were labeled with 10 Abs with specificities listed in Table E1. A, Eight clusters of transitional B cells were identified using FLOCK analysis. Representative dot plots of IgM/IgD and CD21/CD32 are shown in the left panel. The percentage of each cluster is depicted in the right panel as individuals values in 15 HCs. B, CD27 (left), IgM (middle) and IgD (right) MFI expression on each cluster ***P<0.001. C, The grouping strategy for classifying cell clusters is presented for CD19/CD27 and IgM/IgD expression (two left panels) and their Mean±SEM of MFI (the two right panels). D, A supervised-FLOCK analysis according to the previous grouping strategy and percentages of defined pop A, B, C and D.

FIG. 3. **Broad phenotype of transitional $CD24^{high}CD38^{high}$ B cell subsets.** Representative histograms of the surface markers are indicated for each gated population: $CD19^{+}$ B cells (black), $CD19^{+}$ $CD24^{high}CD38^{high}$ cells (grey), pop A (green), pop B (red), pop C (blue) and pop D (yellow). A, Expression of the differentiation markers B, Expression of co-stimulatory and activation markers. C, ABCB1 transporter activity. Mature naive B cells were identified as $CD19^{+}IgD^{+}CD27^{-}$. D, A summary table indicating subset and population classification principles of transitional B cells and expression levels of the different markers.

FIG. 4. **BCR engagement induces apoptosis in T1 B cells while CD27+ transitional B cells are prone to die in culture by necrosis.** A, Percentages of apoptotic (Annexin V^+ $Draq7^-$) and necrotic cells (Annexin V^+ $Draq7^+$) are shown with and without BCR engagement for 8 hours (n=4), *P<0.05. B, Density of IgD and CD32 expression distinguishes T1 (IgD^{low} $CD32^+$), T2 (IgD^+CD32^+) and T3 (IgD^{low} $CD32^{low}$) in $CD27^-$ transitional B cells as does expression levels of IgD and IgM.

FIG. 5. **Transitional B cell subsets respond differently to BCR and Toll-like receptor 9 (TLR9) engagements**. A, Ca^{2+} flux after BCR engagement in different B cell subsets analyzed by flow cytometry. B, Intracellular staining for the level of PIP3 (left panel) and phosphorylated PLCγ2 (right panel) following BCR engagement (n=6) *P<0.05. C, In vitro plasmablast differentiation and total IgM secretion. Numbers under each of the developed membranes depict the number of IgM-secreting B cells. (n=3)

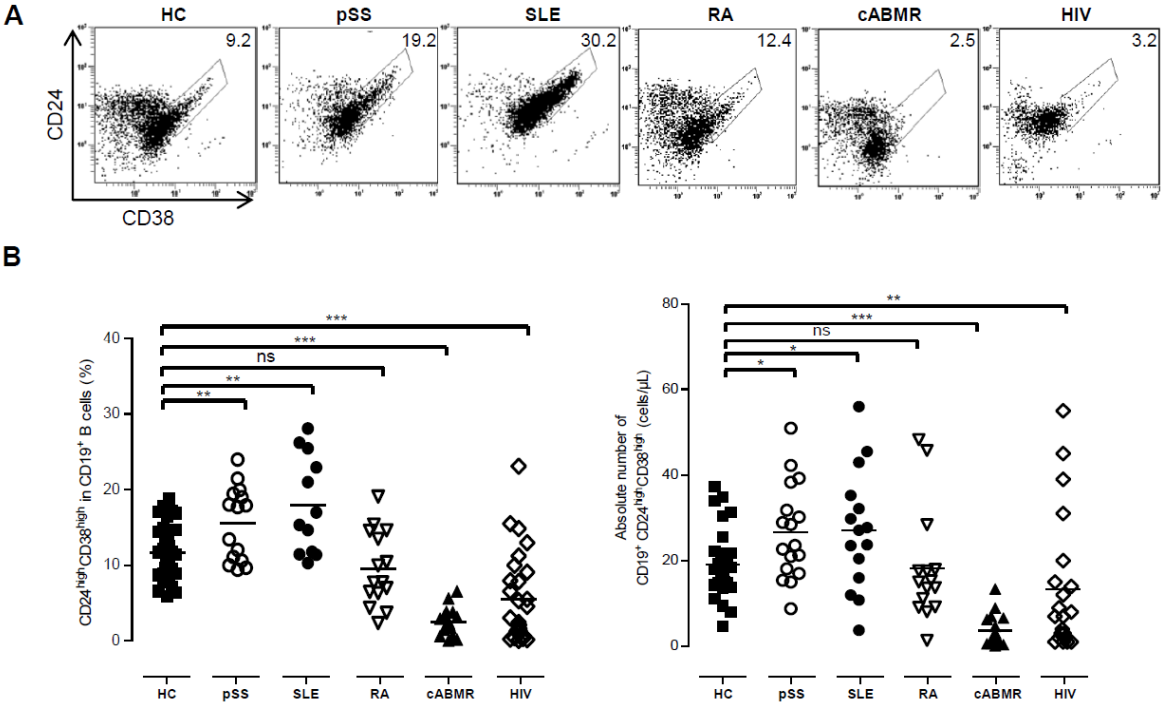
FIG. 6. **$CD27^+CD24^{high}CD38^{high}$ B cells are the most efficient spontaneous IL-10-producing transitional cells**. A, The gating strategy used to evaluate IL-10 secretion by transitional B cell subsets. B, Percentages of T1, T2, T3 and $CD27^+$ transitional B cells in $IL\text{-}10^+$ cells and $IL10^-$ cells. The middle panel depicts MFI for intracellular IL-10 in each transitional B cell subset within IL-10 positive cells (n=4) *P<0.05, **P<0.01. C, Comparison of Mean±SEM of each transitional B cell subsets (and IL-10 MFI) in IL-10 positive $CD24^{high}CD28^{high}$ B cells following 24 hours of stimulation with CpG (n=4) *P<0.05, **P<0.01. D, SPADE analysis of IL-10 density-based expression in all non stimulated B cell subsets.

FIG. 7. **$CD27^+$ transitional B cells inhibit TNFα and IFNγ production by cultured T cells, whereas T3 B cells limit $CD4^+$ T cell proliferation and are differentially represented in patients with different autoimmune diseases.** A, Sorted CD4+ T cells were cultured with each sorted transitional B cell subset for during 4 days. TNFα and

IFNγ levels were measured in supernatants from the cultured cells using ELISA (n=7). B, Representative histogram of $CD4^+$ proliferating T cells cultured with or without sorted transitional B cell subsets (right panel). Mean±SEM of proliferation index of sorted $CD4^+$ T cells in the presence or absence of transitional B cell subsets (n=10, left panel). C, Percentages of T1 and $CD27^+$ transitional B cell subsets within $CD24^{high}CD38^{high}$ in HCs (■, n=9), patients with pSS (○, n=11) and SLE (●, n=10), after FLOCK cross-sample comparison. D, Percentages of T2 and T3 B cell subsets.*P<0.05, **P<0.01, ***P<0.001

619

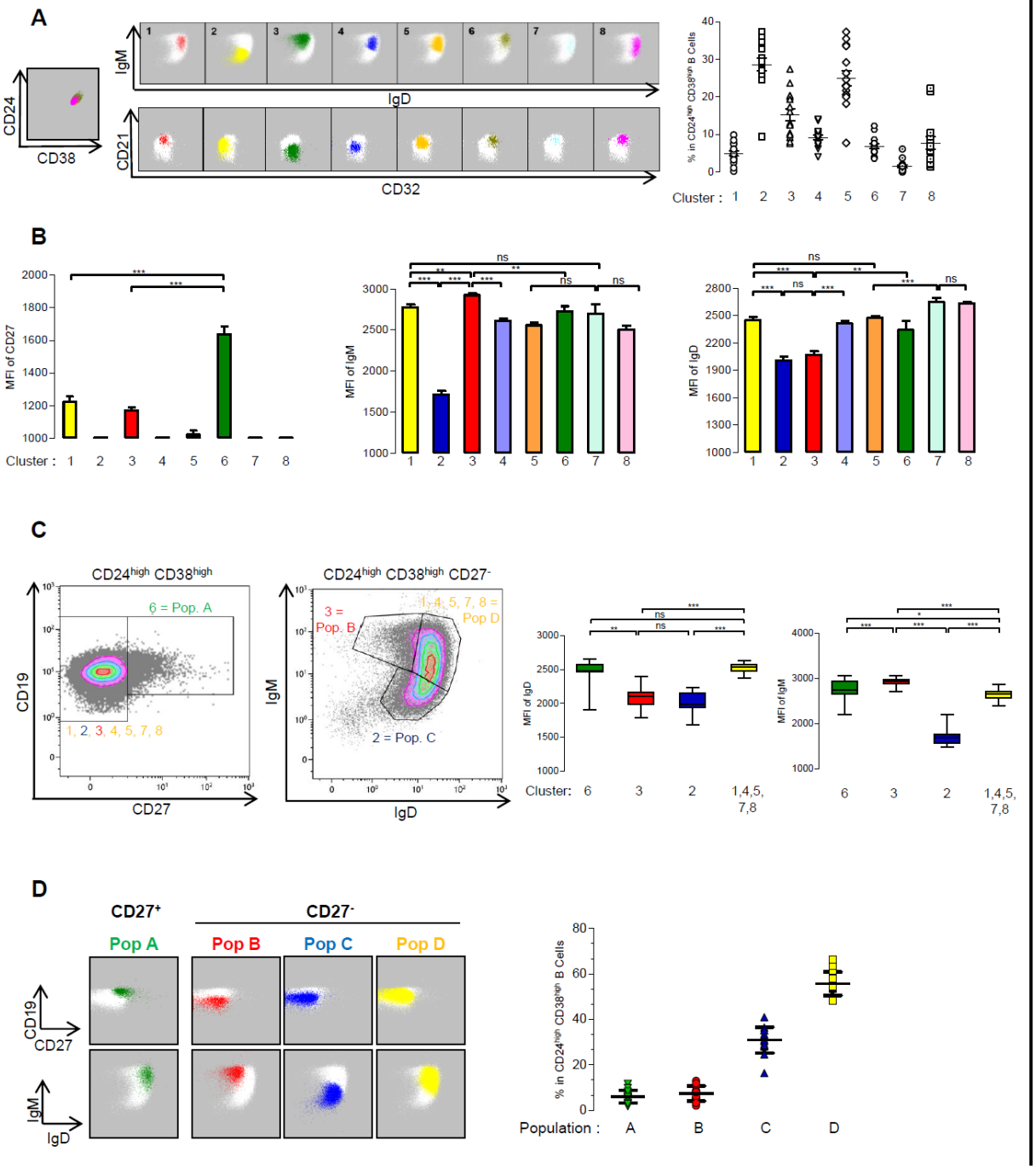
FIG 1



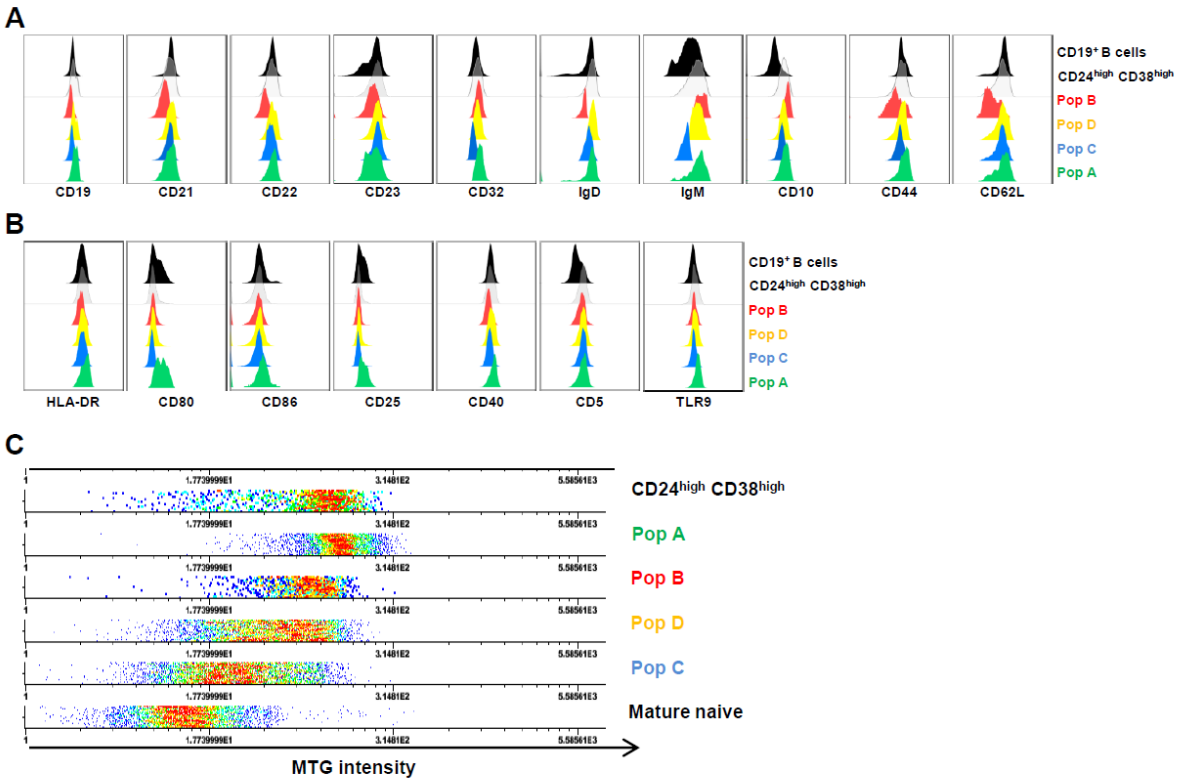
620

621

FIG 2.


A
IgM
IgD
CD24
CD38
CD21
CD32
% in CD24high CD38high B Cells
Cluster : 1 2 3 4 5 6 7 8
B
MFI of CD27
MFI of IgM
MFI of IgD
C
CD24high CD38high
6 = Pop. A
1, 2, 3, 4, 5, 7, 8
CD19
CD27
CD24high CD38high CD27-
3 = Pop. B
1, 4, 5, 7, 8 = Pop D
2 = Pop. C
IgM
IgD
Cluster: 6 3 2 1,4,5, 7,8
D
CD27+
CD27-
Pop A
Pop B
Pop C
Pop D
CD19
CD27
IgM
IgD
% in CD24high CD38high B Cells
Population : A B C D

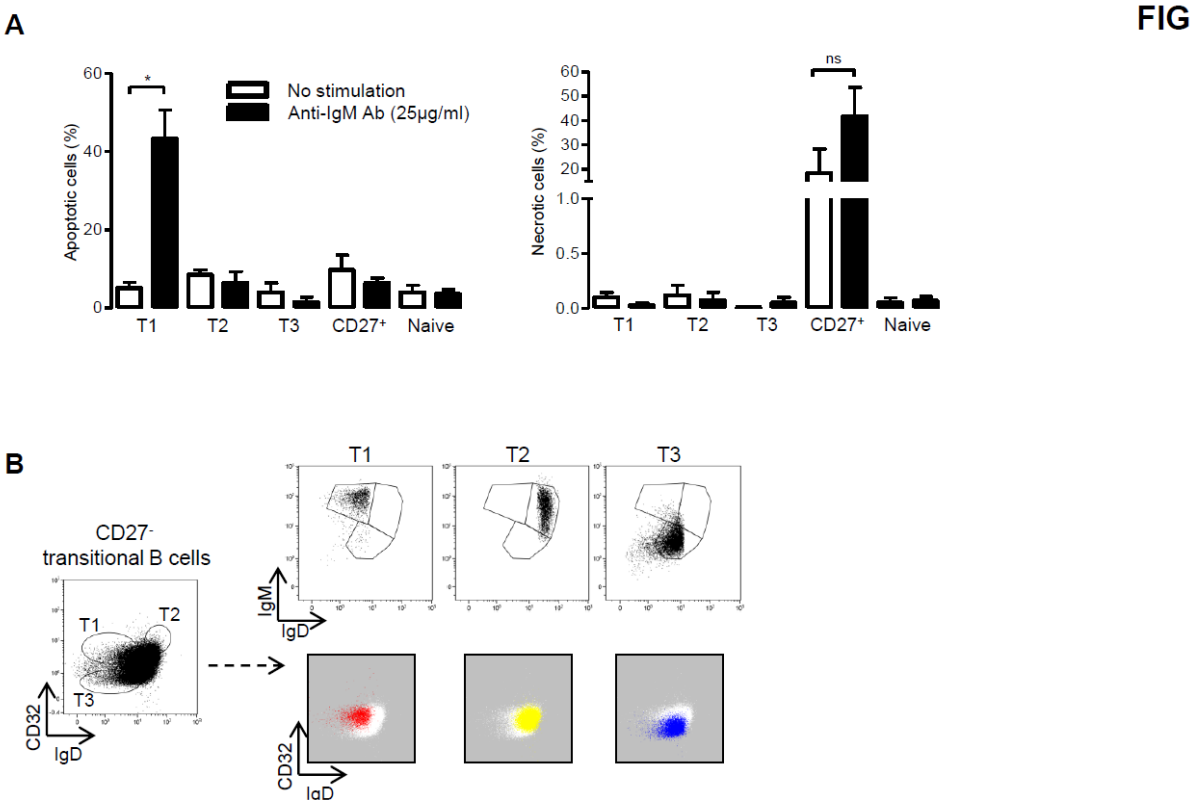
FIG



D

Population	Phenotype		B-cell subset
Pop A	$CD19^{++}$, $CD80^{++}$, $CD86^{++}$, $CD25^{++}$ and $CD40^{++}$ No ABCB1 transporter activity (MTG^{++})	Activated memory	$CD27^{+}$ transitional
Pop B	$CD10^{++}$, IgM^{++}, IgD^{low}, $CD32^{low}$ $CD21^{low}$, $CD44^{low}$, $CD62L^{low}$ and MTG^{+}	Immature	T1
Pop D	$CD10^{+}$, IgM^{+}, IgD^{+}, $CD32^{+}$, $CD21^{++}$, $CD23^{++}$ and MTG^{+}	Intermediate	T2
Pop C	$CD10^{low}$, IgM^{low}, IgD^{low}, $CD32^{low}$, $CD40^{low}$, HLA-DR^{low} and MTG^{low}	Resting state	T3

FIG


A
Apoptotic cells (%)
No stimulation
Anti-IgM Ab (25µg/ml)
*
T1
T2
T3
CD27+
Naive
Necrotic cells (%)
ns
B
CD27- transitional B cells
T1
T2
T3
IgD
CD32
IgM

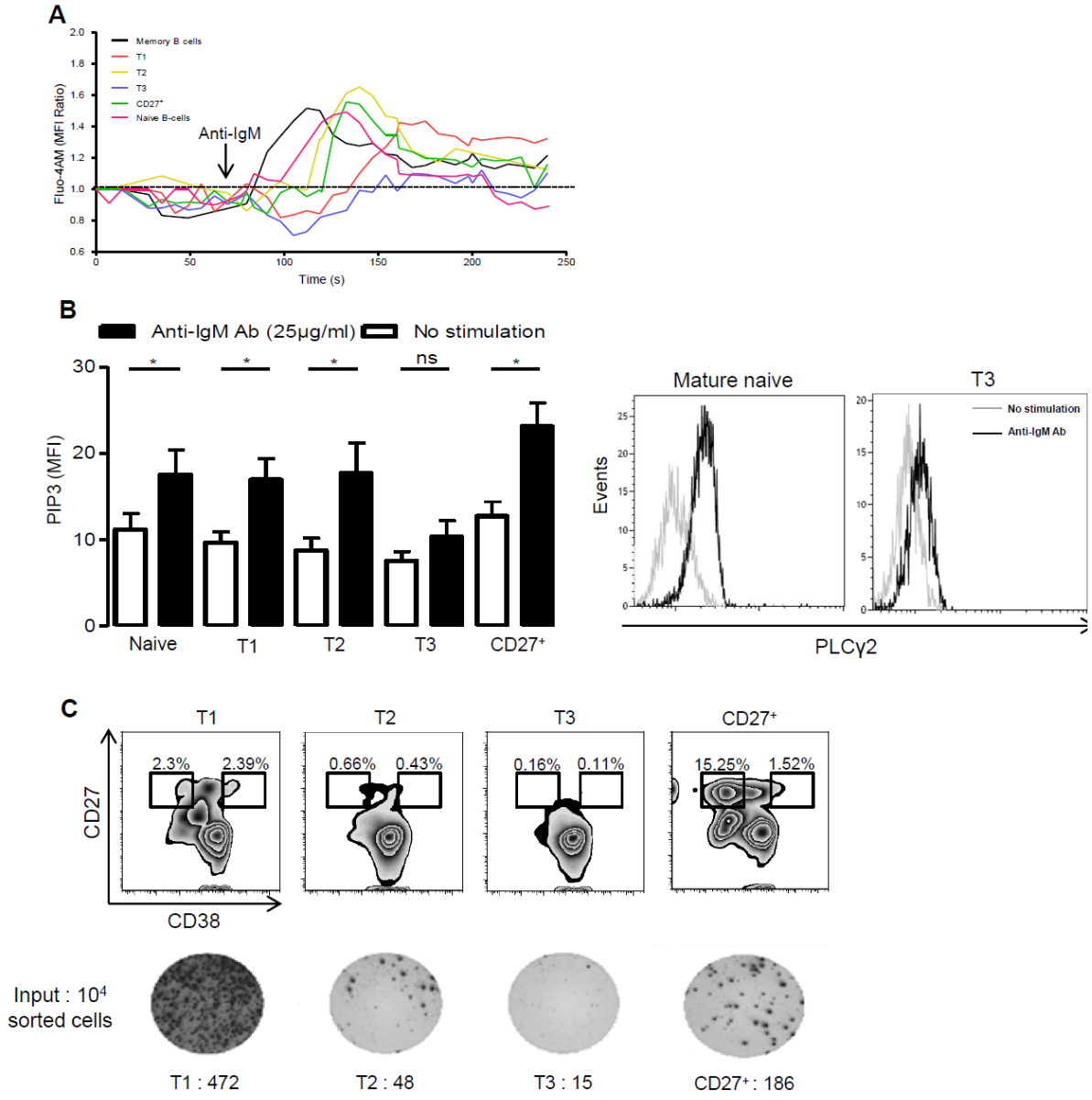
626

627

628

629

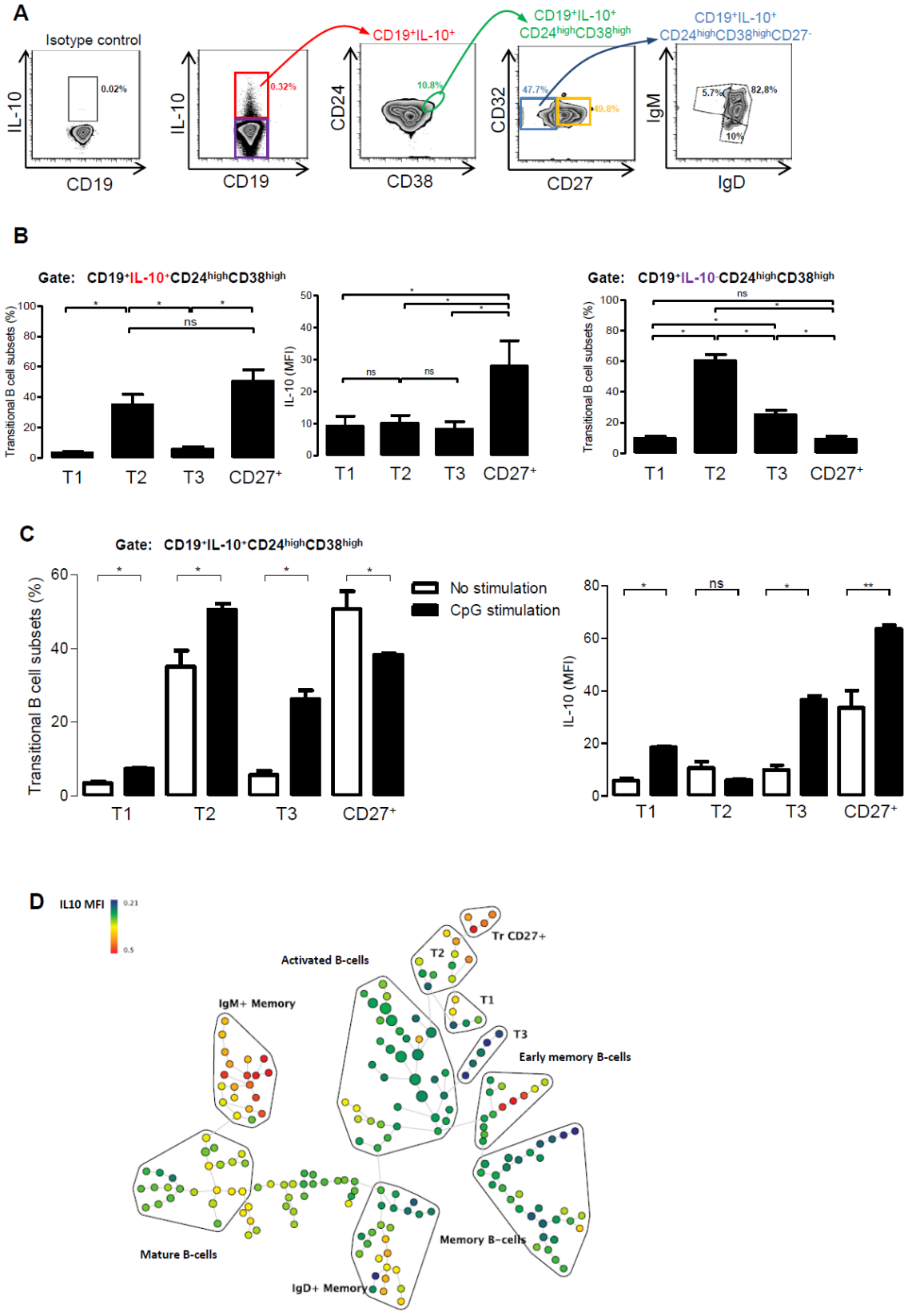
FIG 5



630

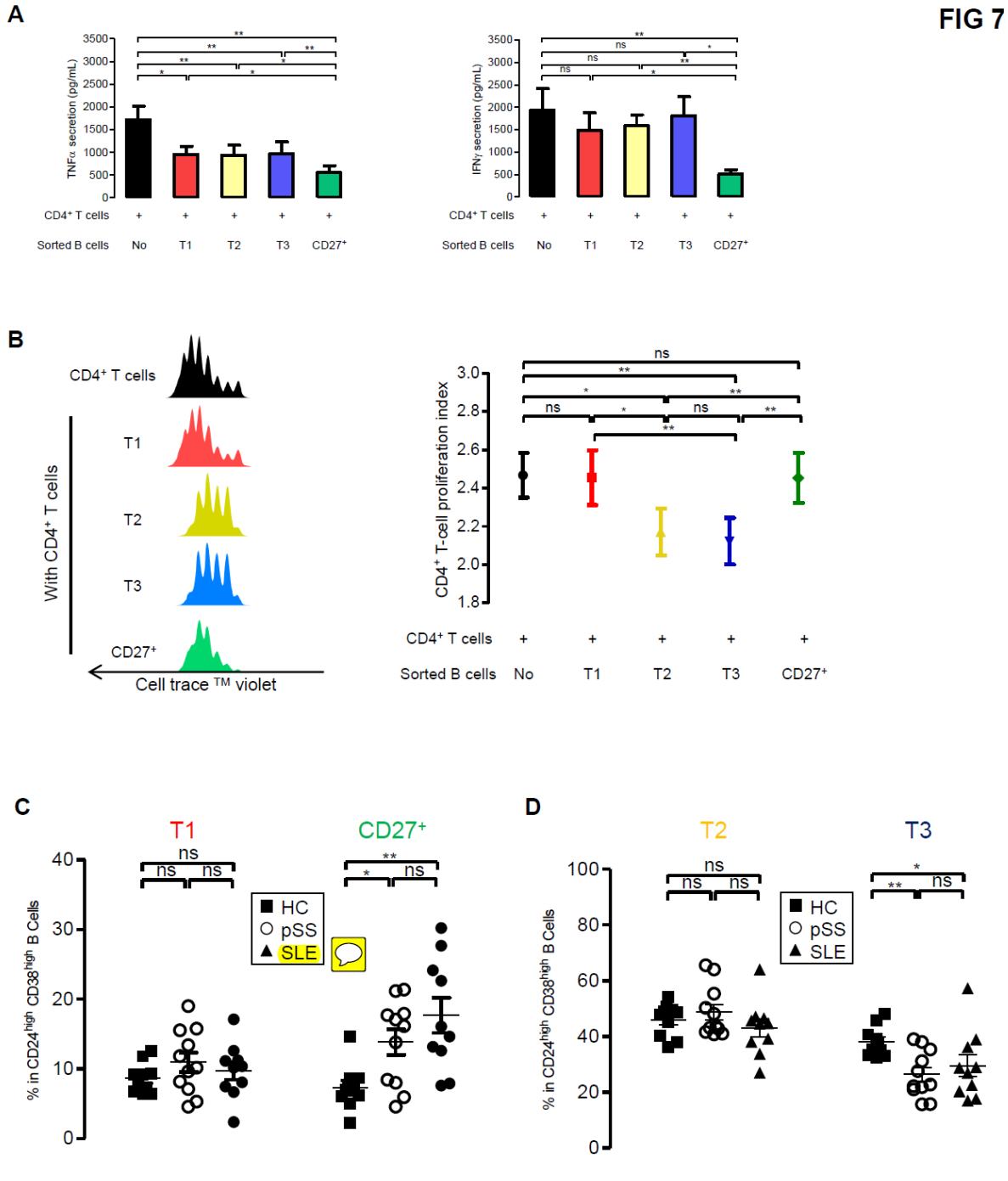
631

FIG 6


A
Isotype control
0.02%
IL-10
CD19
0.32%
CD19+IL-10+
10.8%
CD24
CD38
CD19+IL-10+
CD24highCD38high
47.7%
49.8%
CD32
CD27
CD19+IL-10+
CD24highCD38highCD27-
5.7%
82,8%
10%
IgM
IgD
B
Gate: CD19+IL-10+CD24highCD38high
Transitional B cell subsets (%)
ns
IL-10 (MFI)
T1
T2
T3
CD27+
Gate: CD19+IL-10-CD24highCD38high
C
Gate: CD19+IL-10+CD24highCD38high
No stimulation
CpG stimulation
D
IL10 MFI
0.21
0.5
Tr CD27+
Activated B-cells
IgM+ Memory
Early memory B-cells
Mature B-cells
Memory B-cells
IgD+ Memory

632

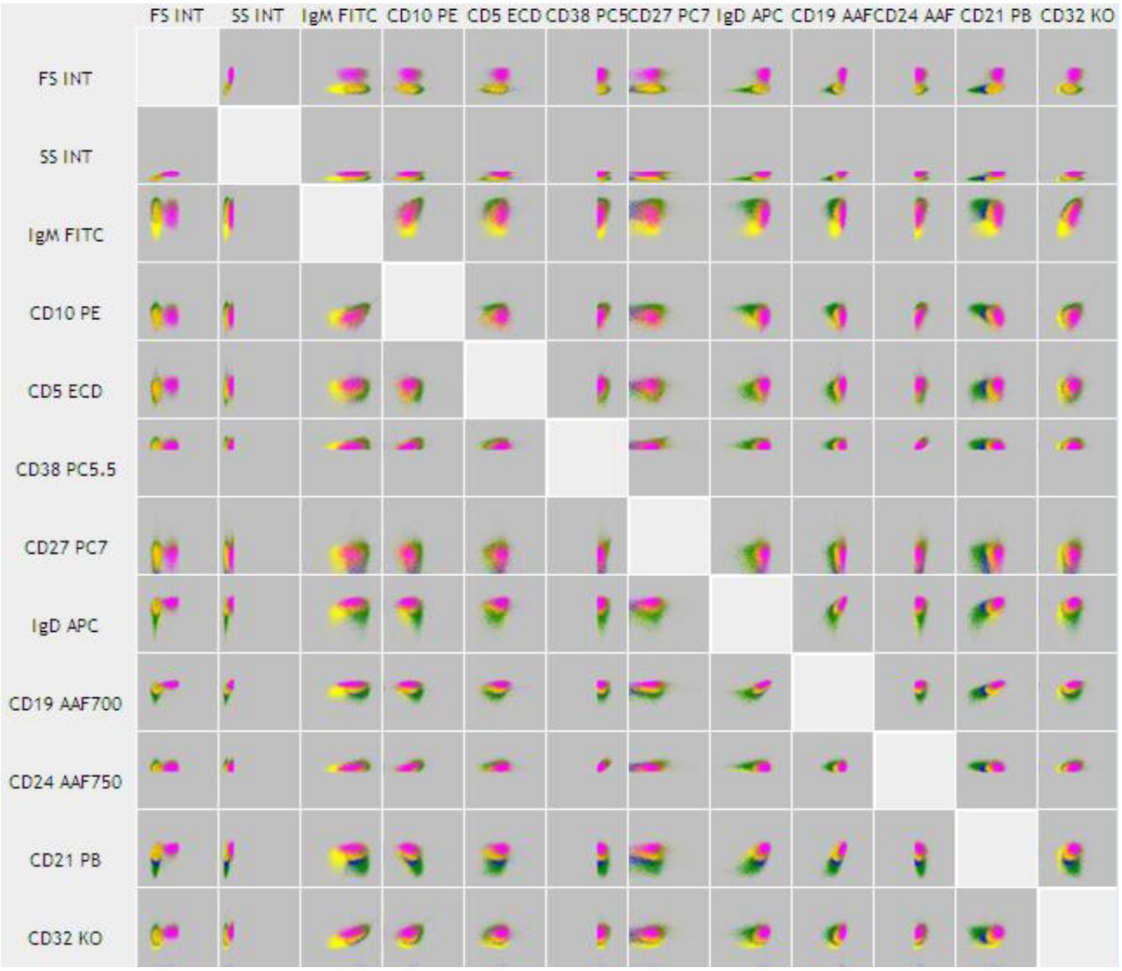
FIG 7


A
TNFα secretion (pg/mL)
IFNγ secretion (pg/mL)
CD4+ T cells
Sorted B cells
No
T1
T2
T3
CD27+
B
CD4+ T cells
T1
T2
T3
CD27+
With CD4+ T cells
Cell trace ™ violet
CD4+ T-cell proliferation index
C
T1
CD27+
% in CD24high CD38high B Cells
HC
pSS
SLE
D
T2
T3
ns

633

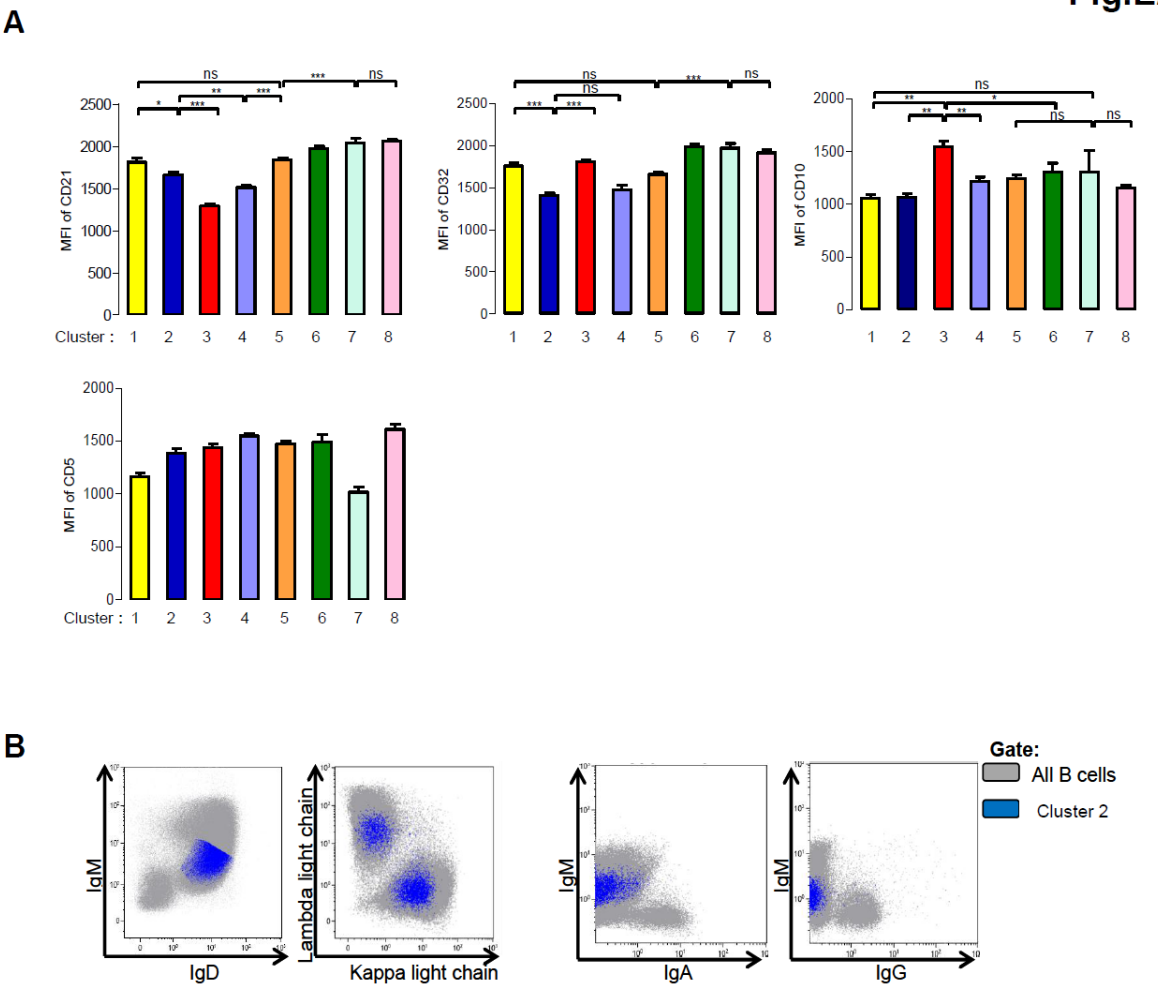
634

Fig.E1



	Cluster	%
	1	4.90±0.69
	2	28.60±1.72
	3	15.18±1.55
	4	9.19±0.69
	5	25.02±2.02
	6	6.87±0.64
	7	1.48±0.41
	8	7.70±11.6

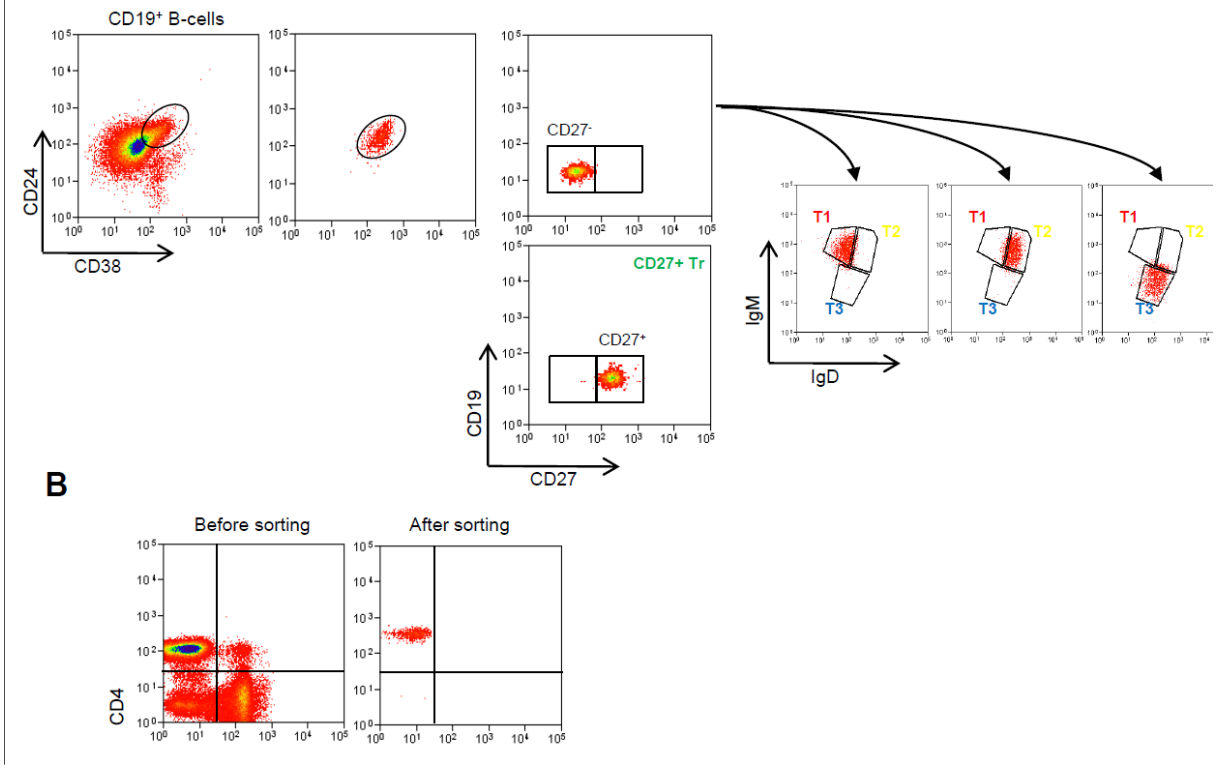
Fig.E2



637

638

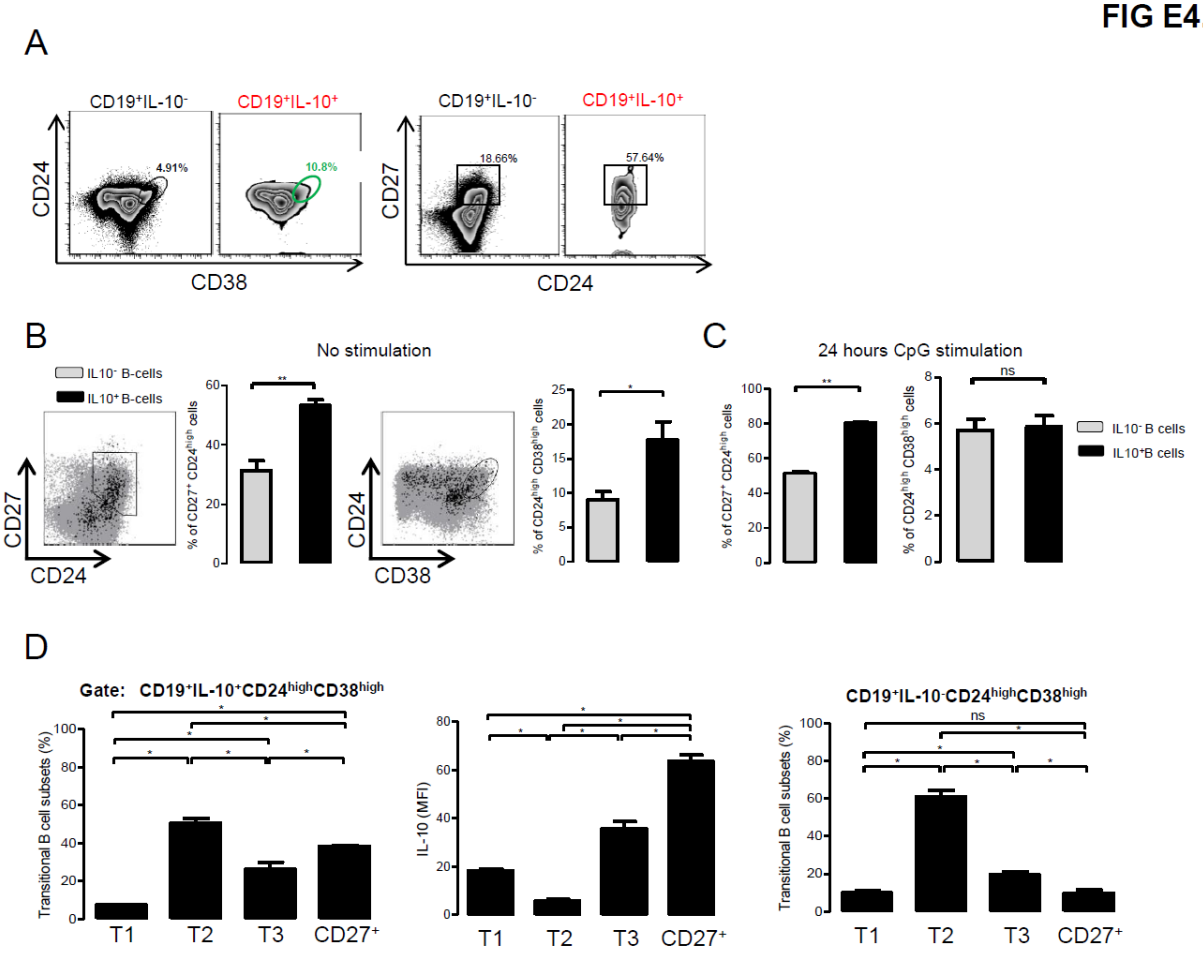
Fig.


A
CD19+ B-cells
CD24
CD38
CD27-
CD27+ Tr
CD27+
CD19
CD27
IgM
IgD
T1
T2
T3
B
Before sorting
After sorting
CD4

639

640

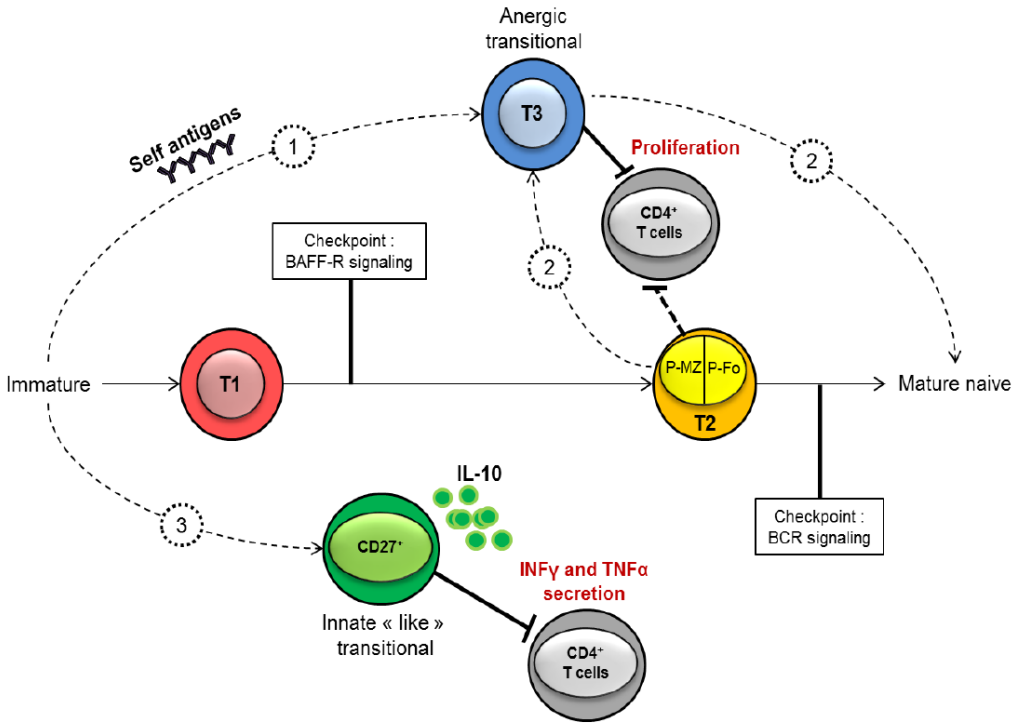
FIG E4.



641

642

Fig.E5



643

644

Table 1. Characteristics of patients included in the study.

Patients	Median age (range)	Sex ratio ♀/♂	Treatments
pSS (n=17*)	56 (29-77)	17/0	CTC=3 ; PLQ=4
SLE (n=16**)	41.5 (25-76)	14/2	CTC=4 ; PLQ=11, MPA=1 ; MTX=3 ; Thal.=2
RA (n=15)	51 (29-80)	8/7	CTC=12 ; PLQ=2 ; MTX=10 ; RTX=3 ; anti-IL-6=1 ; anti-TNFα=7
cABMR (n=17)	54.5 (27-73)	3/14	CTC=15/17 ; MPA=17/17 ; mTORi=2/17
HIV (n=26)	50 (37-62)	8/16	Not informed

Abbreviations: RA= Rheumatoid arthritis; cABMR= Chronic antibody-mediated kidney graft rejection; HIV= Human immunodeficiency virus; SLE= Systemic lupus erythematosus and pSS= Primary Sjögren's syndrome. CTC= corticosteroids; PLQ= Plaquenil; MPA= Mycophenolic acid; MTX= Methotrexate; Thal.= Thalidomide; RXT= Rituximab; mTORi= Mammalian target of rapamycin inhibitor receptor; TNFα= Tumor Necrosis Factor alpha. *11 patients with pSS and **10 with SLE were considered for the FLOCK cross sample comparison (Fig 7).

Table E1 List of fluorochrome-labelled antibodies used for the 10-color flow cytometry analysis.

Specificity	Clone	Fluorochrome	Functions
CD19	J3-119	APC-AF700	Lineage
CD21	BL-13	PB	Signal transduction
CD32	2E1	KO	
CD10	ALB1	PE	B cell growth
IgD	IA6-2	APC	Humoral response
IgM	SAD4	FITC	
CD27	1A4CD27	PE-Cy7	Memory
CD38	LS198-4-3	PE-Cy5.5	Proliferation
CD24	ALB9	APC-AF750	Proliferation
CD5	BL1a	ECD	Signal transduction, Activation

Abbreviations: AF: Alexa fluor; APC: Allophycocyanin; Cy: Cyanine; ECD: PE-Texas Red; FITC: Fluorescein isothiocyanate; PB: Pacific blue; PE: Phycoerythrin; KO: Krome orange.