

# A role for gut-associated lymphoid tissue in shaping the human B cell repertoire

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**We have tracked the fate of immature human B cells at a critical stage in their development when the mature B cell repertoire is shaped. We show that a major subset of bone marrow emigrant immature human B cells, the transitional 2 (T2) B cells, homes to gut-associated lymphoid tissue (GALT) and that most T2 B cells isolated from human GALT are activated. Activation in GALT is a previously unknown potential fate for immature human B cells. The process of maturation from immature transitional B cell through to mature naive B cell includes the removal of autoreactive cells from the developing repertoire, a process which is known to fail in systemic lupus erythematosus (SLE). We observe that immature B cells in SLE are poorly equipped to access the gut and that gut immune compartments are depleted in SLE. Thus, activation of immature B cells in GALT may function as a checkpoint that protects against autoimmunity. In healthy individuals, this pathway may be involved in generating the vast population of IgA plasma cells and also the enigmatic marginal zone B cell subset that is poorly understood in humans.**

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Abbreviations used: ALDH, aldehyde dehydrogenase; BTK, Bruton’s tyrosine kinase; GALT, gut-associated lymphoid tissue; IGHV, immunoglobulin heavy chain variable regions gene; PBMC, peripheral blood mononuclear cell; SLE, systemic lupus erythematosus.

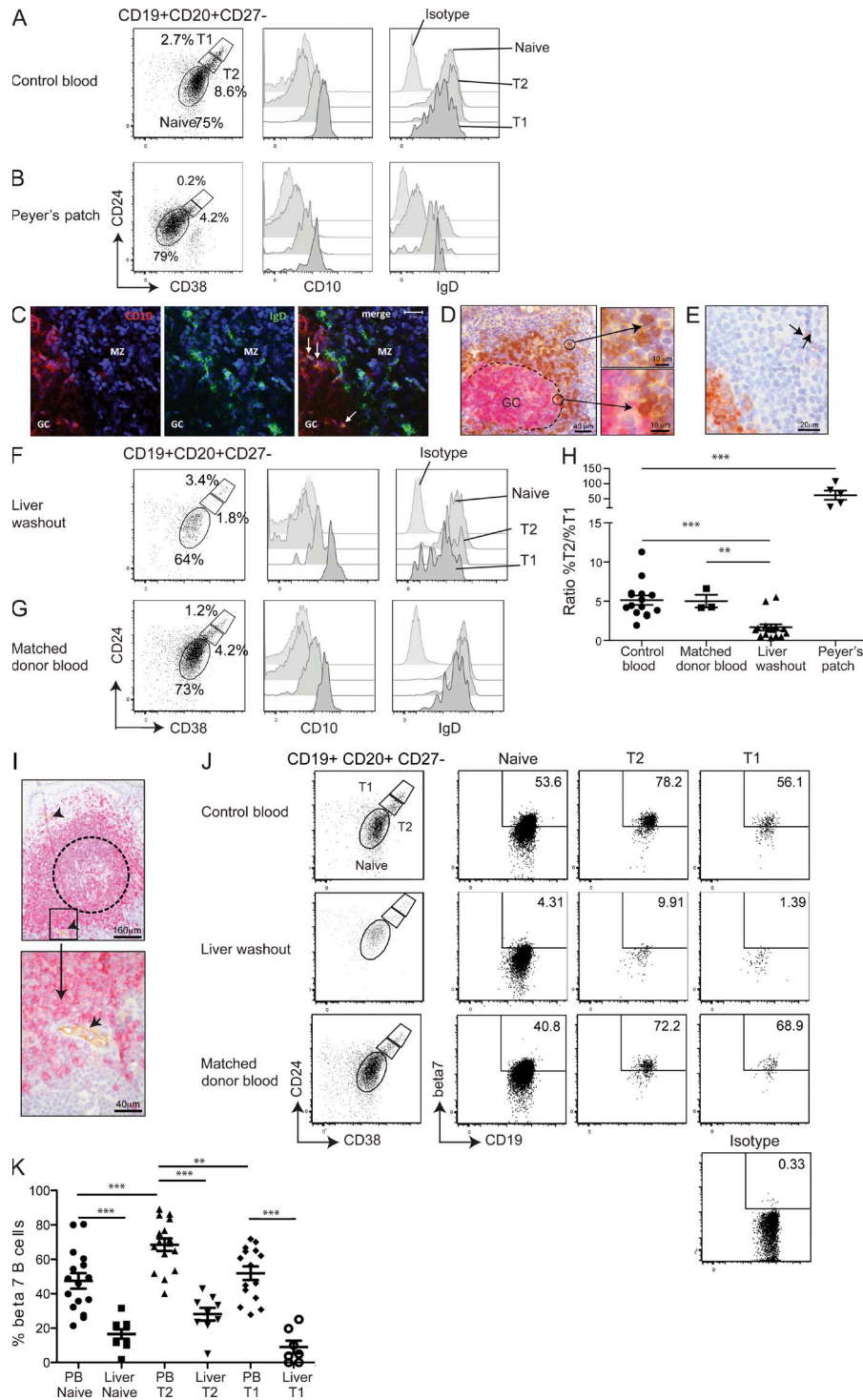
The success of the adaptive immune system in maintaining health is dependent on recognition of specific pathogens or vaccine epitopes by cell-unique antigen receptors of B and T lymphocytes. An inevitable drawback to a system that depends on extreme receptor diversity is the potential to break self-tolerance by binding autoantigens. Transitional B cells are bone marrow-derived immature B cells that continuously emerge into the blood-borne B cell pool throughout life. A relatively high proportion of transitional B cells express polyspecific immunoglobulin that may give rise to the autoimmune repertoire (Meffre and Wardemann, 2008; Mietzner et al., 2008). Transitional B cells can be divided into subsets based on stage of maturation; transitional 1 (T1) cells mature to T2 and possibly T3 before reaching maturity (Bemark

et al., 2012). In mice, transitional B cells mature in bone marrow and spleen through a process that involves a bifurcation in B cell fate to either circulating follicular B cells or splenic resident marginal zone B cells. Both are mature naive populations; the former generate conventional adaptive T cell-dependent B cell responses, whereas the latter are responsible for more innate type responses to T-independent antigens such as pneumococcal polysaccharide. This is unlikely to occur equivalently in humans where splenic zonal microanatomy is different (Mebius and Kraal, 2005; Vossenkämper and Spencer, 2011) and where there are fundamental differences in B cell subset biology. For example, mice have a self-renewing peritoneal B1 B cell population that humans do not have equivalently. In addition, the enigmatic human marginal zone

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**Figure 1. A subpopulation of transitional B cells is recruited to the GALT.** (A and B) Healthy peripheral blood and Peyer's patch B cells were analyzed by flow cytometry for the expression of CD19, CD20, CD27, CD24, CD38, CD10, and IgD. FACS plots display the percentages of T1, T2, and naive B cells, and histograms display CD10 and IgD expression for each subset, healthy control blood (A) and Peyer's patches (B). Plots show one of four experiments with similar results. (C) Confocal microscopy of human Peyer's patch. CD10<sup>+</sup> (red), IgD<sup>+</sup> (green) transitional B cells are highlighted by arrows at the periphery of the germinal center. GC, germinal center; MZ, marginal zone. One of three experiments with similar results is shown. (D) Immunohistochemistry of GALT identifies CD10<sup>+</sup> (pink) and IgD<sup>+</sup> (brown) transitional B cells in the periphery of the germinal center. One of three experiments with similar results is shown. (E) Immunohistochemistry of GALT showing CD10<sup>+</sup> cells (brown) scattered in the mantle zone of GALT. One of three experiments with similar results is shown. (F and G) T1, T2, and naive B cells were identified by flow cytometry (as in A and B) in F. Perfusates from normal liver grafts and matched donor blood (G) are shown. One of three experiments with similar results is shown. (H) Ratios of the %T2 to %T1 subpopulations in normal blood, and matched donor blood (G) are shown. One of three experiments with similar results is shown. (I) Histology of Peyer's patch. One of three experiments with similar results is shown. (J) Flow cytometry of beta7 expression in CD19<sup>+</sup> B cells from control blood, liver washout, and matched donor blood. One of three experiments with similar results is shown. (K) Percentages of beta7<sup>+</sup> B cells in peripheral blood (PB) and liver washout (Liver) for naive, T2, and T1 subsets. One of three experiments with similar results is shown.

B cell subset, while retaining the functional association with T-independent antigen, paradoxically has somatically hypermutated immunoglobulin heavy chain variable regions genes (IGHVs), suggesting a history of antigen exposure and germinal center transit.

Although neither the anatomical nor mechanistic bases for human B cell maturation after bone marrow exit are understood in humans, a checkpoint is known to exist that depletes cells with autoreactivity and polyspecificity from the bone marrow–emergent transitional B cell repertoire before differentiation to mature naive B cells. Failure of this checkpoint is apparent in systemic lupus erythematosus (SLE) where immature B cells are relatively abundant in blood and the polyspecific and autoreactive cells are not depleted from the mature naive repertoire (Yurasov et al., 2005; Meffre and Wardemann, 2008).

In mice, the fate of immature B cells is determined in spleen, but there is no evidence that this occurs in humans. However, in humans there is circumstantial evidence pointing to an association between gut-associated lymphoid tissue (GALT) and the spleen: first, the human splenic marginal zone B cell population expands in response to mucosal bacterial infection (Harris et al., 1996); second, low-grade malignancies of mucosal marginal zone B cells (mucosa-associated lymphoid tissue lymphomas), which parody the behavior of their normal healthy counterparts, migrate to the splenic marginal zone (Du et al., 1997); and third, marginal zone B cells in humans have evidence of previous antigen encounter (Weill et al., 2009). We therefore considered the possibility that human GALT could be involved in determining the fate of immature B cells and may influence repertoire development. The human gut is the largest immune organ in the body, with more effector cells, including plasma cells, than all other sites of immune expression in the body combined (Pabst et al., 2008). These plasma cells are generated in GALT that is maintained in a chronically activated state by the luminal microbiota.

## RESULTS AND DISCUSSION

### The T2 subset of human transitional B cells selectively localizes in GALT

We initially asked if GALT might recruit lymphocytes from the immature B cell pool. Subsets of B cells were analyzed in suspensions of mononuclear cells isolated from human Peyer's patches that were selectively sampled at endoscopy. The T2 subset of transitional B cells was consistently enriched in isolated Peyer's patch cell suspensions compared with the

T1 subset (Fig. 1, A and B; and Fig. S1). Consistent with this, cells with transitional B cell phenotype were identified on the periphery of GALT germinal centers (Fig. 1, C and D), and as scattered CD10<sup>+</sup> cells around the periphery of the mantle zone where it merges with the mucosal marginal zone (Fig. 1, D and E).

Recruitment of T2 cells into GALT would logically remove them from the blood circulation so that they would be selectively depleted from the venous blood that drains to the liver via the hepatic portal vein; most of the blood in the hepatic portal vein has previously passed through the gut. To confirm the selective recruitment of T2 cells to the gut, we analyzed the mononuclear cells obtained by vascular perfusion of normal liver before liver transplant. The T2 subset of transitional B cells was significantly reduced in the mononuclear cell population obtained by perfusion compared with paired donor blood samples (Fig. 1, F and G). The ratio of T2 to T1 transitional cells was higher in Peyer's patch cells and lower in liver perfusates compared with control and paired blood samples (Fig. 1 H), further confirming the selective recruitment of T2 transitional cells to the gut.

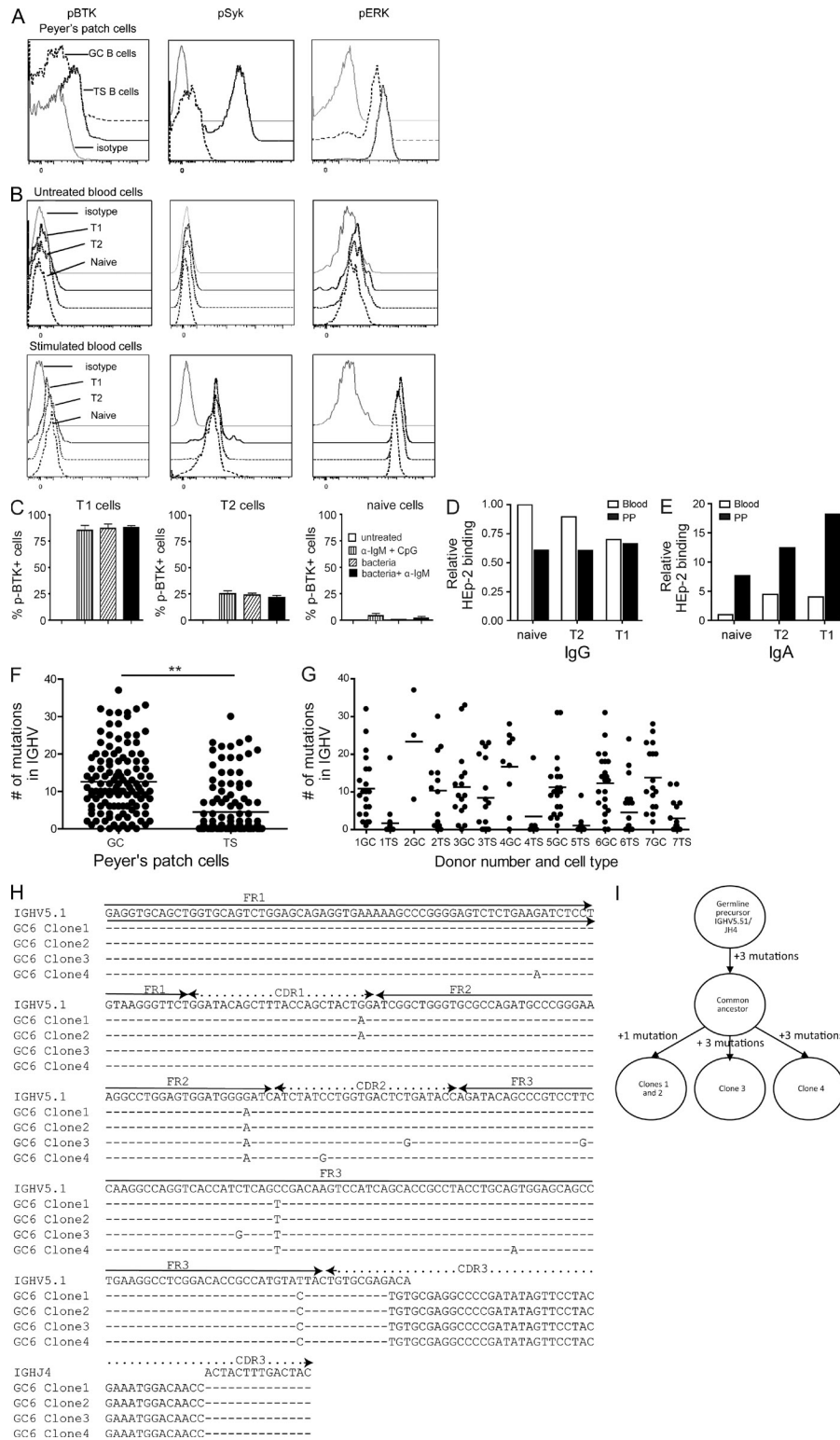
The interaction between integrin  $\alpha 4\beta 7$  on lymphocytes and the endothelial ligand MAdCAM-1 that is expressed by endothelium in GALT (Brandtzaeg et al., 1999; Fig. 1 I) mediates the localization of lymphoid cells in the gut. We therefore asked if the selective recruitment of T2 transitional B cells in the gut might be associated with a higher expression of  $\alpha 4\beta 7$ . A greater percentage of blood transitional B cells expressed high levels of  $\alpha 4\beta 7$  integrin ( $\beta 7$  expression considered representative for  $\alpha 4\beta 7$ ; Rott et al., 1996) than naive B cells, with the highest proportion of expression being within the T2 subset (Fig. 1, J and K). Residual T2 cells observed in liver perfusates were the T2 cells with lowest expression of  $\alpha 4\beta 7$  integrin, consistent with prior sequestration of T2 cells with higher  $\alpha 4\beta 7$  integrin expression in GALT (Fig. 1, J and K).

### The majority of T2 B cells in GALT are activated

Having observed that T2 cells are selectively recruited into GALT, we considered what the consequences of this might be. GALT is rich in exogenously derived bacterial antigens from the luminal microbiota. We therefore asked whether transitional B cells might be activated in the GALT microenvironment. Freshly isolated GALT transitional B cells were observed to have phosphorylated Bruton's tyrosine kinase (BTK), Erk, and Syk (Fig. 2 A), indicative of recent activation in vivo (Aoki et al., 1994). This frequency was significantly higher than that observed in other B cell subsets from GALT

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matched liver donor blood, liver graft perfusates, and normal Peyer's patches. Error bars show mean  $\pm$  SEM. Statistical test: one-way ANOVA.  $n =$  up to 15. \*\*\*,  $P < 0.0001$ ; \*\*,  $P < 0.003$ . (I) Immunohistochemistry showing MAdCAM (brown) and CD20 (pink). MAdCAM is seen on Peyer's patch vessels (marked by arrowheads). One of two experiments with similar results. (J and K) Healthy control blood, liver perfusates, and matched donor blood samples were analyzed by flow cytometry for the expression of CD19, CD20, CD27, CD24, CD38, and  $\beta 7$  integrin. One of three experiments with similar results is shown. (J) FACs plots display  $\beta 7$  expression on T1, T2, and naive B cells (K) Summary data showing percentages of naive, T1, and T2 B cells in normal peripheral blood and liver graft perfusates that express  $\beta 7$  integrin.  $n = 7-16$ . Error bars show mean  $\pm$  SEM. Statistical test: one-way ANOVA. \*\*\*,  $P < 0.0001$ ; \*\*,  $P < 0.0043$ .



**Figure 2. GALT transitional B cells are activated in vivo and in vitro in response to intestinal bacteria.** (A) Mononuclear cells were isolated from Peyer's patches and analyzed directly ex vivo by flow cytometry for their expression of CD19, CD10, IgD, and phospho-BTK, phospho-Syk, and phospho-ERK. Representative histograms displaying the degree of BTK, Syk, and ERK phosphorylation for CD19<sup>+</sup>CD10<sup>+</sup>IgD<sup>+</sup> transitional (TS) and CD19<sup>+</sup>CD10<sup>+</sup>IgD<sup>-</sup> germinal center (GC) B cells. Shown is one of three experiments with similar results. (B) PBMCs were stimulated polyclonally for 30 min with CpG, anti-IgM, and heat-inactivated intestinal bacteria as a positive control for B cell kinase phosphorylation, or left unstimulated. After stimulation, cells were analyzed by flow cytometry for the expression of CD19, CD20, CD27, CD24, CD38, and phospho-BTK, phospho-Syk, and phospho-ERK.

or blood (Barone et al., 2011; Fig. 2, A and B). The relatively low frequency of kinase phosphorylation in GC B cells is consistent with previous studies (Barone et al., 2011; Khalil et al., 2012).

The different percentages of cells detected by the different anti-kinase antibodies (Fig. 2 A) probably reflects the different signaling pathways that the kinases may contribute to and the different potential routes that might be involved in the activation of individual B cells, including activation through the BCR and TLRs. Higher frequencies of transitional and naive B cells with pBTK, pErk, and pSyk could be induced by polyclonal activation of peripheral blood mononuclear cell (PBMC) used as a positive control (Fig. 2 B).

To mimic the phenomena observed *in vivo*, T1, T2, and naive B cells were FACS sorted from blood (gating strategy shown in Fig. S2 A) and coincubated with washed heat-inactivated isolates of bacteria from the intestinal lumen. Phosphorylation of BTK in blood transitional B cells could be initiated *in vitro* by coincubation with washed heat-inactivated isolates of bacteria from the intestinal lumen (Fig. 2 C). Therefore antigens derived from the intestinal microbiome could potentially induce the activated state of transitional B cells *in vivo*. The relatively high activity of the T1 cells might be related to the expression of TLR9 and comparatively high frequency of cells expressing polyspecific receptors (Yurasov et al., 2005; Aranburu et al., 2010). Supernatant of T1, T2, and naive B cells isolated from Peyer's patches or blood that had been incubated with washed heat-inactivated isolates of bacteria from the intestinal lumen gave positive results for presence of autoantibody, according to cut off controls provided with the assay for clinical diagnoses (Fig. 2, D and E). Such studies of cells isolated from human GALT involved pooling of samples and, therefore, it was not possible to assess variability in these experiments. The production of autoantibodies may relate to a previously described ability of some antibodies produced by plasma cells in the gut, which are known to be derived from GALT, to cross react between autoantigens and gut bacterial antigens (Benckert et al., 2011).

Blood transitional B cells have immunoglobulin heavy chain variable region gene (IGHV) sequences that are unmutated from germline (Aranburu et al., 2010; Wu et al., 2010), consistent with their naive status. However, transitional B cells

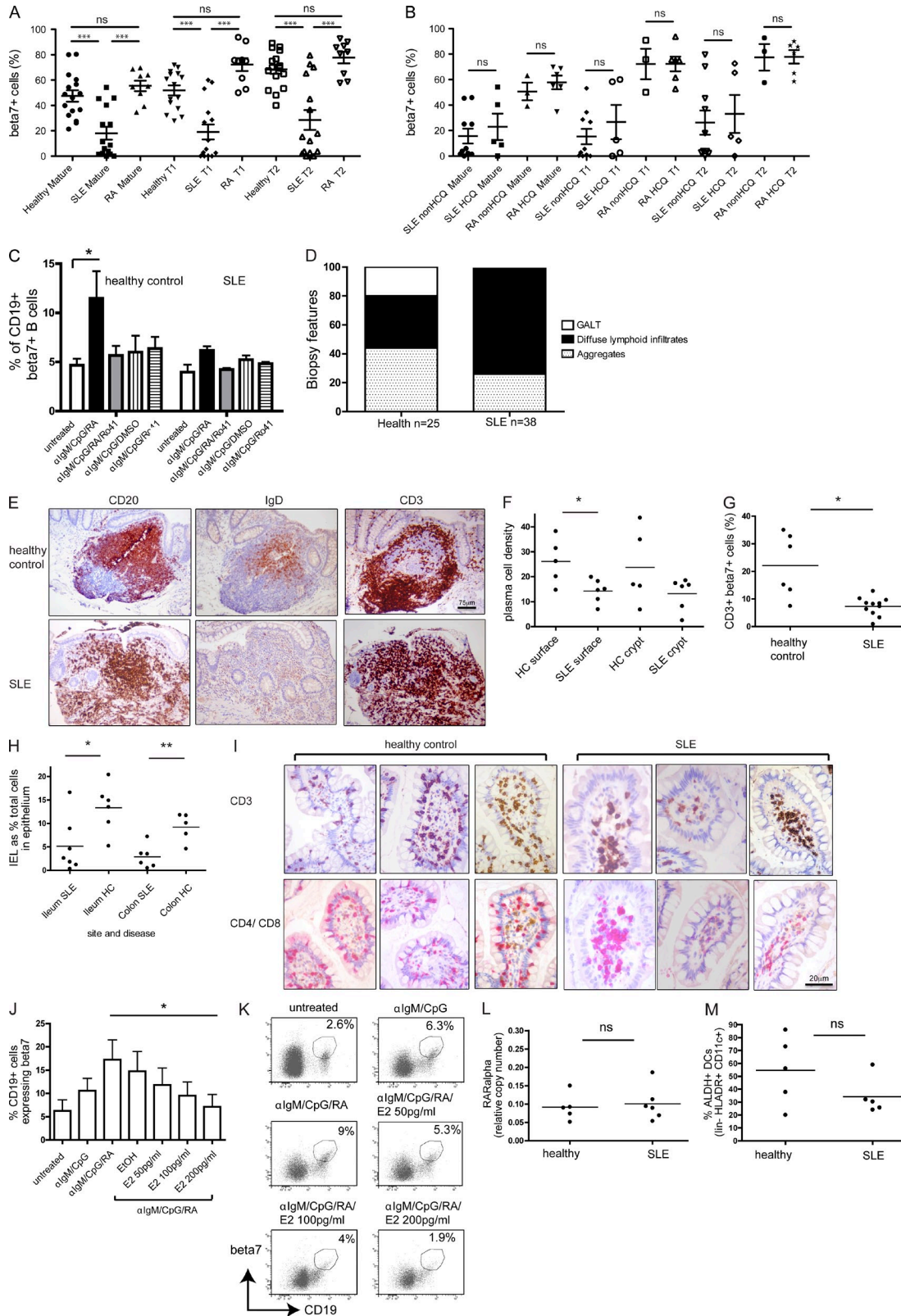
can acquire somatic mutations *in vitro* on activation (Aranburu et al., 2010). Because GALT transitional B cells are activated *in vivo* (Fig. 2 A), we asked whether they might have somatic mutations in their IGV genes. Transitional and germinal center B cells were isolated from GALT by a method that yielded populations with >99% purity (Fig. S2 B). Although the majority of transitional B cells had unmutated IGHV genes, 30% had three or more mutations in IGHV. The frequency of mutation was significantly lower than that observed in the GC (Fig. 2 F). This observation was consistent between seven individual donors (Fig. 2 G). We identified examples of clonal expansion in the GC B cells by the observation of the same IGHV rearrangements in different PCR reactions (Fig. 2, H and I). However, these clones were not observed in the transitional B cell samples from the same individual. No clones were observed in the transitional B cell samples. The different population profiles of germinal center cells and transitional B cells in terms of both population sort purity and features of the immunoglobulin gene sequences confirmed that we analyzed these two CD10-expressing populations independently and that the mutations identified in the transitional B cells were highly unlikely to be a result of germinal center cell contamination.

#### The intestinal immune system is depleted in SLE

Because T2 transitional B cells are selectively recruited to GALT where they acquire an activated state, we hypothesized that this could have a major impact on B cell repertoire development, as activation of transitional B cells by antigens in GALT would by definition remove them from the naive circulating pool. This could constitute a novel specificity checkpoint, failure of which may result in pathology, and might constitute a checkpoint that is not functional in an autoimmune disease such as SLE, which is reported to be B cell driven. We tested our hypothesis by studying SLE (Table S1; Yurasov et al., 2005, 2006; Meffre and Wardemann, 2008; Mietzner et al., 2008). We observed that lymphocytes in SLE, including transitional B cells, had significantly lower expression of  $\alpha\beta 7$  compared with healthy controls, implying a reduced potential to localize in the gut in SLE and reduced potential to access GALT (Fig. 3 A). Rheumatoid arthritis (RA) was studied as a disease control because it is also a

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Histograms display the degree of BTK, Syk, and ERK phosphorylation for T1, T2, and naive B cells. Histograms show one of three experiments. (C) FACS-sorted T1 cells (CD19<sup>+</sup> CD20<sup>+</sup> CD27<sup>-</sup> CD24<sup>++</sup> CD38<sup>++</sup>), T2 cells (CD19<sup>+</sup> CD20<sup>+</sup> CD27<sup>-</sup> CD24<sup>+</sup> CD38<sup>+</sup>), and naive mature B cells (CD19<sup>+</sup> CD20<sup>+</sup> CD27<sup>-</sup> CD24<sup>-</sup> CD38<sup>-</sup>) isolated from normal blood were incubated with combinations of heat-inactivated intestinal bacteria, CpG, and anti-IgM for 30 min, followed by intracellular staining for phospho-BTK. Mean values + SD from three independent experiments are shown. (D and E) Autoantibody secretion by *in vitro* stimulated peripheral blood and Peyer's patch B cells. T1, T2, and naive mature B cells were FACS sorted (sort strategy as in Fig. S2 A) from normal blood and Peyer's patches (one experiment with pooled cells from three donors) and were incubated with heat-inactivated intestinal bacteria for 8 d. Supernatants were screened for secreted autoantibodies by Hep-2 ELISA. Graphs show Hep-2 binding IgG (D) and IgA (E) autoantibodies. Data are standardized so that reactivity of supernatant from blood naive cells is 1 for each isotype. (F and G) IGHV genes were amplified by PCR from FACS-sorted TS and GC B cells isolated from Peyer's patches of seven different donors (gated and sorted as shown in Fig. S2 B) from two sorts. (F) Pooled data of the number of mutations in GC or TS IGHV genes. Mann-Whitney test,  $P = 0.0001$ . (G) Number of mutations in GC or TS IGHV genes segregated by donor. Horizontal bars show the mean number of mutations. (H and I) An example of mutations in clonally related germinal center B cells. (H) Examples of IGHV sequencing of germinal center B cells showing four clonally related sequences deriving from a common ancestor. (I) Graphical explanation of the relation of the clones in H to a common ancestor and their germline precursor.



**Figure 3. Expression of  $\beta 7$  integrin on TS B cells is impaired in SLE.** (A) PBMCs isolated from healthy donors and patients with SLE were analyzed by flow cytometry for the expression of CD19, CD20, CD27, CD24, CD38, and  $\beta 7$ . Graph displays the percentages of  $\beta 7^+$  healthy or SLE T1, T2, and mature B cells. \*\*\*,  $P < 0.001$  one-way ANOVA + Bonferroni's multiple comparison test. (B) PBMCs isolated from SLE and RA patients were analyzed by flow cytometry as in A. Graph shows percentages of  $\beta 7^+$  SLE and RA B cell subsets segregated into patients treated with or without hydroxychloroquine.

chronic inflammatory autoimmune disease treated with immunosuppressive drugs. Expression of  $\alpha 4\beta 7$  by B cells in RA was the same as in healthy controls (Fig. 3 A). To exclude the possibility that lower expression of  $\alpha 4\beta 7$  in SLE was a consequence of treatment with hydroxychloroquine (HCQ), which is known to interfere with TLR9 signaling, we divided patients according to whether they took HCQ medication or not. Expression of  $\alpha 4\beta 7$  directly ex vivo was not associated with HCQ and was not associated with any other immunosuppressive treatment (Fig. 3 B). Furthermore, we observed a failure to up-regulate  $\beta 7$  expression on SLE blood B cells compared with normal blood B cells after in vitro stimulation with CpG and retinoic acid, and this was irrespective of HCQ treatment (Fig. 3 C).

Having observed reduced expression of  $\alpha 4\beta 7$  by circulating B cell subsets in patients with SLE, we reasoned that if this is relevant to immune physiology by reducing immune access to the gut, then GALT should be depleted in SLE. We analyzed random histologically normal biopsies, including 25 from healthy controls and 38 from patients with SLE. Aggregates of lymphoid cells that did not show features of GALT were observed in biopsies from healthy and SLE donors (Fig. 3 D). However, whereas multiple examples of GALT were observed in healthy donors, no examples of GALT were observed in the sample of SLE biopsies (Fig. 3, D and E). A significantly lower density of IgA plasma cells was observed in the lamina propria in SLE compared with controls (Fig. 3 F). Reduced expression of  $\alpha 4\beta 7$  was also detected on blood T lymphocytes from SLE patients (Fig. 3 G). The intraepithelial T cell compartment was significantly depleted in the large and small intestines of patients with SLE (Fig. 3, H and I) irrespective of treatment. CD4 and CD8 cells were present in the lamina propria, but CD8 cells did not tend to localize in the epithelium in SLE as they do in health (Fig. 3 I).

The receptors for retinoic acid and estrogen are nuclear receptors, the signaling pathways of which have been observed to interact (Hua et al., 2009). RAR binding is highly coincident and overlaps with estrogen receptor  $\alpha$  binding. Further analysis showed that these two nuclear receptor–signaling systems could antagonistically regulate their target genes (Hua et al., 2009). Estrogens can also alter B cell behavior (Grimaldi et al., 2002; Hill et al., 2011). We added  $\beta$ -estradiol to lymphocytes at high and low physiological plasma concentrations. A consistent dose-dependent interference with the retinoic acid induction of  $\alpha 4\beta 7$  by  $\beta$ -estradiol was observed in healthy blood B cells (Fig. 3, J and K). We saw no evidence of differences in expression of retinoic acid receptor  $\alpha$ , or activity of retinaldehyde dehydrogenase between SLE and health (Fig. 3, L and M; and Fig. S3), and no evidence of elevated estrogens in the small sample of the SLE patients analyzed. The mechanism of dysregulation of  $\alpha 4\beta 7$  integrin in SLE is not known but may be related to signals downstream of estrogen binding (Grimaldi et al., 2005).

#### Transitional B cells activated in GALT could be precursors of plasma cells or marginal zone B cells

The fate of the T2 cells activated in GALT remains unknown, but we propose that they could be precursors of either marginal zone B cells, intestinal plasma cells, or they could die. The origin of human splenic marginal zone B cells has been a tantalizing unknown for many years (Weill et al., 2009). In mice, transitional B cells mature into either naive B cells or marginal zone B cells in the spleen. This does not happen in humans where the splenic anatomy is different and the marginal zone B cell population, in contrast to murine marginal zone B cells, has somatic mutation in IGV. Data presented here suggests that human splenic marginal zone B cells could arise from transitional B cells activated in GALT, two zones

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Statistics: Kruskal-wallis + Dunn's multiple comparison test. (C) Peripheral blood B cells from healthy individuals and SLE patients were cultured for 7 d with indicated treatments (10  $\mu\text{g/ml}$  anti-IgM, 1  $\mu\text{M}$  CpG, 100 nM RA, and 1  $\mu\text{M}$  Ro41). Graph shows  $\beta 7$  expression analyzed by flow cytometry. Means + SEM of three independent experiments are shown.  $P = 0.04$ , Mann-Whitney-test. (D) Intestinal biopsies were taken from eight healthy and eight SLE patients (up to eight biopsies per patient). The biopsies were stained by immunohistochemistry for CD3, IgD, or CD20 and were scored according to whether sections contained no lymphoid tissue (diffuse lymphoid infiltrates), diffuse lymphoid aggregates only (aggregates), or structured lymphoid tissue (GALT). GALT in biopsies from healthy individuals versus SLE  $P < 0.004$ ,  $\chi^2$ . (E) Representative histological pictures of a healthy biopsy displaying GALT and an SLE biopsy showing a diffuse lymphoid aggregate lacking GALT features. CD20, IgD, and CD3 are stained brown. Bar, 75  $\mu\text{m}$ . (F) Biopsies in D were stained for the presence of IgA<sup>+</sup> plasma cells identified by their morphology and cytoplasmic IgA. Graphs show mean plasma cell density. Each data point is a mean of several biopsies from each donor,  $P = 0.03$ , Mann-Whitney-Test. (G) PBMCs isolated from SLE patients and healthy controls were analyzed by flow cytometry for CD3 and  $\beta 7$  expression. Graph displays the mean percentage of CD3<sup>+</sup> T cells expressing  $\beta 7$ .  $P = 0.0078$ , Mann-Whitney-Test. (H) Sections of intestinal biopsies isolated from the ileum and colon of healthy controls and patient with SLE were stained with CD3. Graph shows mean percentages of intraepithelial lymphocytes (IEL) as percentage of total epithelial cells and IEL. \*,  $P = 0.03$ ; \*\*,  $P = 0.004$ , Mann-Whitney Test. (I) Representative examples of immunohistochemistry of intestinal biopsies from healthy controls or SLE patients, stained for CD3 (brown) or CD4 (brown) and CD8 (pink). Bar, 20  $\mu\text{m}$ . (J) Percent of  $\beta 7$ -expressing CD19<sup>+</sup> blood B cells after 7-d culture with indicated treatments ( $\alpha$ -IgM 10  $\mu\text{g/ml}$ ; CpG 1  $\mu\text{M}$ ; RA 100 nM; E50/100/200 =  $\beta$ -estradiol 50/100/200 pg/ml). Analysis was performed by flow cytometry. Shown are means + SEM of three independent experiments. \*,  $P = 0.02$ , two-tailed Student's *t* test. (K) Flow cytometry analysis of  $\beta 7$ -expressing CD19<sup>+</sup> blood B cells after 7-d culture with indicated treatments ( $\alpha$ IgM 10  $\mu\text{g/ml}$ ; CpG 1  $\mu\text{M}$ ; RA 100 nM; E50/100/200 =  $\beta$ -estradiol 50/100/200 pg/ml). The figure shows representative FACS plots of one of three experiments with similar results. (L) Transitional B cells were FACS sorted from the peripheral blood of healthy controls and patients with SLE based on their expression of CD19, IgD, and CD38. Sorted CD19<sup>+</sup>IgD<sup>+</sup>CD38<sup>+</sup> transitional B cells were analyzed for RAR $\alpha$  transcripts by RT-PCR,  $n = 6$ . The graph shows mean relative copy number for each group. No difference was observed. (M) Flow cytometric analysis of ALDH enzyme activity in blood myeloid dendritic cells (lineage<sup>-</sup> HLA-DR<sup>+</sup> CD11c<sup>+</sup>) from lupus and control blood samples using the Aldefluor reagent system. Mean percentages of myeloid DCs that were ALDH<sup>+</sup> are shown.  $n = 5$ .  $P = 0.3$ , two-tailed Mann-Whitney-test.

suggested to share a common pool of cells from studies of infection and malignancy (Harris et al., 1996; Du et al., 1997). Recruitment to the marginal zone by activation in response to mucosal microbiota could provide the explanation for the responsiveness of this population to bacterial antigens and also their prior acquisition of mutations in IGHV. It would also explain the appearance of the splenic marginal zone that is responsive to bacterial antigens over the first 2 years of life and splenic marginal zone expansion after bacterial colonization of the mucosa (Timens et al., 1989; Harris et al., 1996; Kruschinski et al., 2004).

The intestinal IgA-producing plasma cell population that is derived from B cells activated in GALT (Barone et al., 2011), while mostly antigen specific, includes a relatively high frequency of cells secreting autoreactive and polyspecific antibodies (Benckert et al., 2011; Scheid et al., 2011). This contrasts with the bone marrow IgG plasma cell population where autoreactivity is relatively rare. It is possible that recruitment of transitional B cells to GALT could enhance the antigen-binding spectrum of IgA antibody produced in the gut. IgA is transported into the intestinal lumen where antibody diversity is paramount but where autoreactivity is not a relevant issue. Continuous recruitment of T2 B cells could also contribute to the scale of IgA production that exceeds the production of all other isotypes in the body.

### The lymphoid interface with the microbiome: a novel checkpoint in B cell development?

We propose that selective entry and activation of T2 cells in human GALT, as demonstrated here, would affect the developing B cell repertoire by removing a proportion of B cells from the circulating pool before differentiation to mature naive B cells. We therefore suggest that this could constitute a novel checkpoint. Failure to access GALT efficiently in SLE may contribute to disease pathogenesis. SLE is a complex condition with diverse manifestations. It is not yet clear how our data relate to each individual phenotype associated with this disease.

## MATERIALS AND METHODS

**Reagents.** Reagents were purchased from Sigma-Aldrich if not stated otherwise. CpG DNA was purchased from Thermo Fisher Scientific. The retinoic acid receptor (RAR) alpha antagonist Ro41 was purchased from Enzo Life Sciences.

**Cell isolation and culture.** Mononuclear cells from human peripheral blood and GALT were isolated as previously described (Vossenkämper et al., 2010). An overview of the lupus patients is given in Table S1. Blood cells used as controls were obtained from healthy female individuals between the ages of 20 and 45. Peyer's patch biopsies were obtained during routine endoscopy. All patients gave informed written consent. The study was performed according to human experimental guidelines and with approval of the NRES Committee London-City, and East and KCL infectious diseases BioBank, Local Research Ethics Committee working under the authority of the Southampton and South West Hampshire Research Ethics Committee. Cells were cultured in RPMI medium supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-Glutamine, and 1% nonessential amino acids.

**Perfusate and donor PBMC collection.** Liver perfusates were collected from human liver grafts. During procurement of the donor liver, the aorta was clamped and the liver was perfused through the portal vein with 2 liters of University of Wisconsin (UW) solution to remove residual blood from the vasculature. After resection, the liver was placed in a bag and perfused with a further 1–2 liters of UW solution. After implantation of the liver, the ex vivo perfusate was retrieved. Mononuclear cells from the perfusate were isolated within 12 h by density gradient centrifugation using Ficoll Paque Plus (GE Healthcare). PBMCs were obtained from organ donors. All samples were stored in 10% DMSO-containing medium at  $-180^{\circ}\text{C}$ . The study protocol was approved by the Ethics Committee at King's College Hospital.

**Flow cytometry and FACS.** Staining of cells for flow cytometry, intracellular phospho-staining, and FACS was performed as previously described (Barone et al., 2011). Phospho-staining in PBMCs was performed after 30 min of culture with heat-killed bacteria from human feces. Bacteria had been grown under aerobic and anaerobic conditions on blood-agar plates for 48 h and were then washed and heat-killed for 10 min at  $95^{\circ}\text{C}$ . Flow cytometry and FACS were performed using the LSR II analyzer and FACS Aria (BD). Antibodies were used at concentrations recommended by the manufacturers: phospho-BTK, phospho-syk, phospho-ERK, CD79b, CD10, IgD, HLA-DR, and CD11c (all BD); CD19, CD38, CD27, CD24, and IgM (all BioLegend); CD20 efluor 450 and  $\beta 7$  (eBioscience); and dendritic cell exclusion ("lineage") cocktail (anti-CD3/CD14/CD16/CD19/CD34; AbD Serotec). The specificities of phospho-BTK, phospho-syk, and phospho-ERK were confirmed by the manufacturers by methods including Western blotting.

**Immunofluorescence staining and immunohistochemistry.** Cryosections were fixed with 4% paraformaldehyde, followed by antigen retrieval in citrate buffer, pH 9, for 10 min at  $95^{\circ}\text{C}$ . For immunofluorescence, sections were incubated with the primary Abs (CD10 and IgD; Dako) for 1 h, and then washed and incubated with Alexa-fluorochrome-conjugated secondary Abs (Invitrogen) and DAPI. Specific staining was confirmed using isotype control Abs. Confocal images were acquired with the LSM 510 Meta, Plan-Neofluar 40 $\times$  oil/0.50 objective (Carl Zeiss). Immunohistochemistry of paraffin sections was performed by UCL Advanced Diagnostics, London as previously described (Barone et al., 2009). CD20 was purchased from Dako and MAdCAM-1 from AbD Serotec.

**RT-PCR.** Quantitative PCR was done as described previously (Barone et al., 2009). Primers and probes to quantify retinoic acid receptor expression were purchased from Applied Biosystems and data standardized to GAPDH expression.

**IGHV gene rearrangement analysis.** IGHV gene rearrangements in DNA from sorted cells were PCR amplified with primers to each of the IgVH families and a nested protocol used as described previously (Dunn-Walters et al., 1995). PCR products were cloned into the pGEM-T vector (Promega) and sequenced by Beckman Coulter Sequencing Services (Beckman Coulter Genomics). Alignments were made using the IMGT/V-quest database.

**B cell culture to assess autoantibody production.** B cell subsets were sorted by FACS from Peyer's patches or PBMCs from healthy individuals. Purified B cell subsets were cultured as previously described with minor alterations (Huggins et al., 2007). Cells were cultured at a density of  $5 \times 10^4$  cells/200-µl well in 96-well U-bottomed plates. The base cell culture media was IMDM (Invitrogen) with 10% human AB serum (Biosera), 50 µg/ml human transferrin, 5 µg/ml human insulin, 15 µg/ml gentamicin, and 100 U/ml penicillin-streptomycin. For the initial 5 d of culture, the base media was supplemented with 20 U/ml IL-2, 50 ng/ml IL-10, 10 ng/ml IL-15, and 5 µl/well of heat-killed bacteria from human feces. After 5 d, supernatants were removed and replaced with base cell culture media supplemented with 20 U/ml IL-2, 50 ng/ml IL-6, 50 ng/ml IL-10, and 10 ng/ml IL-15. Cells were then transferred to 200-µl wells in U-bottomed plates lined with irradiated mouse fibroblasts (L-cells) expressing human CD40L. Supernatants were collected after 3 d. Qualitative detection of autoantibodies



(i.e., immunoglobulin against Hep2 cell antigens) in cell supernatants was performed using the ANA-Hep2 Screen ELISA (IBL-America). To assess IgA content, the kit's anti-IgG-enzyme conjugate was substituted with an anti-IgA-peroxidase conjugate (Sigma-Aldrich). Results (in optical densities) of the Hep2 ELISA were standardized to immunoglobulin concentrations in the supernatants (TIG ELISA kit; BlueGene) and were adjusted to eliminate any contribution from the human AB serum.

**Aldefluor assay.** Aldehyde dehydrogenase (ALDH) activity in blood DCs (identified as CD3/CD14/CD16/CD19/CD34<sup>-</sup>, HLA-DR<sup>+</sup>, CD11c<sup>+</sup> population) was assessed by using the Aldefluor reagent system (STEMCELL Technologies). Aldefluor is a fluorescent substrate for ALDH. Cells expressing high levels of ALDH appear brightly fluorescent and were identified during flow cytometry by comparing the fluorescence in test samples to that in controls containing diethylaminobenzaldehyde (DEAB), a specific inhibitor of ALDH (Fig. S3).

**Statistical analysis.** Instat or Prism (GraphPad Software) was used to perform statistical tests. A two-tailed Student's *t* test, Mann-Whitney-test, and  $\chi^2$  test were used as appropriate. The one-way ANOVA test was used to compare normally distributed multiple groups. Kruskal-Wallis was used for non-normally/skewed distributed multiple groups. *P* < 0.05 was considered significant.

**Online supplemental material.** Fig. S1 shows full gating strategy for identifying transitional 1 (T1), transitional 2 (T2), and mature naive B cells based on their expression of CD19, CD20, CD27, CD24, and CD38. Fig. S2 shows FACS sort strategies for isolation of transitional, mature naive, and germinal center B cells from peripheral blood or Peyer's patches. Fig. S3 shows a B cell  $\beta$ 7 expression after in vitro stimulation ALDH enzyme activity in blood myeloid DCs. Table S1 shows demographic and clinical details of the SLE and RA patients enrolled in this study. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20122465/DC1>.

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