Novel Human Transitional B Cell Populations Revealed by B Cell Depletion Therapy

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Novel Human Transitional B Cell Populations Revealed by B Cell Depletion Therapy

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Transitional cells represent a crucial step in the differentiation and selection of the mature B cell compartment. Human transitional B cells have previously been variably identified based on the high level of expression of CD10, CD24, and CD38 relative to mature B cell populations and are expanded in the peripheral blood following rituximab-induced B cell-depletion at reconstitution. In this study, we take advantage of the gradual acquisition of the ABCB1 transporter during B cell maturation to delineate refined subsets of transitional B cells, including a late transitional B cell subset with a phenotype intermediate between T2 and mature naive. This late transitional subset appears temporally following the T1 and T2 populations in the peripheral compartment after rituximab-induced B cell reconstitution (and thus is termed T3) and is more abundant in normal peripheral blood than T1 and T2 cells. The identity of this subset as a developmental intermediate between early transitional and mature naive B cells was further supported by its ability to differentiate to naive during in vitro culture. Later transitional B cells, including T2 and T3, are found at comparatively increased frequencies in cord blood and spleen but were relatively rare in bone marrow. Additional studies demonstrate that transitional B cells mature across a developmental continuum with gradual up-regulation of mature markers, concomitant loss of immature markers, and increased responsiveness to BCR cross-linking in terms of proliferation, calcium flux, and survival. The characterization of multiple transitional B cell subpopulations provides important insights into human B cell development. The Journal of Immunology, 2009, 182: 5982–5993.
rituximab treatment (13, 14). We have taken advantage of this opportunity and the discriminatory power afforded by polychromatic flow cytometry to further define the characteristics of human transitional B cells. The data presented clearly demonstrate the presence of a new B cell population that represents a phenotypic and functional continuum between conventionally defined T1/T2 and mature naive B cells. This previously unrecognized late transitional B cell population is phenotypically indistinguishable from mature naive B cells by most conventional markers but revealed by the lack of expression of the ABCB1 transporter and represents the largest transitional fraction in normal PB.

### Materials and Methods

#### Sample procurement and cell isolation

Detailed written informed consent was obtained from all patients and healthy donors in accordance with protocols approved by the Human Subjects Institutional Review Board of the University of Rochester Medical Center. PBMCs from rituximab-treated patients (lupus, rheumatoid arthritis, or lymphoma) and healthy controls were isolated from heparinized blood by Ficoll-Hypaque density gradient centrifugation (Pharmacia Biotech). Patients were treated as part of routine clinical care with 1000 mg/m² rituximab every other week or 375 mg/m²every week (over several weeks) or as part of previously described clinical trials (15, 16). Tonsil samples were obtained from normal subjects undergoing routine tonsillectomy. Cord blood (CB), BM, and spleen samples were obtained from pathology specimens at board-approved protocols.

#### Flow cytometry and cell sorting

Immunofluorescence staining for flow cytometric analysis was performed by incubating PBMCs with excess mAb in PBS/1% BSA on ice for 20 min after blocking with 10 μg of human IgG for 20 min. Cells were washed in PBS/BSA and in some cases incubated with streptavidin conjugate. Cells were then washed and fixed in PBS/1% paraformaldehyde before analysis on a dual-laser FACS Calibur (BD Biosciences) or three-laser 11-color LSRII (BD Biosciences). For some experiments, B cells were purified using RosetteSep (Stem Cell Technology) and enriched for naive and transitional B cells by CD27 cell depletion using CD27 microbeads and magnetic sorting (Miltenyi Biotec). For purification of distinct transitional cell populations, RosetteSep-enriched B cells were sorted on a FACS Aria as further defined in the figure legends.

B cells were identified based on CD19 expression and additionally classified by multiparameter flow cytometry along a developmental pathway based on the expression of defined surface markers as follows: immature (CD38<sup>hi</sup>CD24<sup>int</sup>CD10<sup>hi</sup>IgD<sup>hi</sup>CD27<sup>−</sup>CD10<sup>−</sup>), transitional (CD38<sup>int</sup>CD24<sup>hi</sup>CD10<sup>−</sup>IgD<sup>−</sup>CD27<sup>−</sup>CD10<sup>−</sup>), naive (CD38<sup>−</sup>CD24<sup>hi</sup>CD10<sup>−</sup>IgD<sup>−</sup>CD27<sup>−</sup>CD10<sup>−</sup>), prec-BC (CD45<sup>hi</sup> tonsil), transitional (CD38<sup>hi</sup>CD24<sup>int</sup>CD10<sup>−</sup>CD10<sup>−</sup>), or memory B cells (CD38<sup>−</sup>CD24<sup>−</sup>CD10<sup>−</sup>CD10<sup>−</sup>). These thresholds of CD24/CD38 expression were defined as follows: BM pre-sorted below) and thresholds of expression of CD24 and CD38. Fluorescence minus one controls were used to define negative cell populations. Thresholds of CD24/CD38 expression were defined as follows: BM precursor populations including T1 (CD10<sup>+</sup>) have the highest expression (+ + +), T2 cells (also CD10<sup>−</sup> and R123<sup>−</sup>) have intermediate expression (+ +), and naive cells (R123 negative) the lowest expression (+). Thus, T1 (R123<sup>−</sup>CD38<sup>−</sup>CD24<sup>−</sup>CD10<sup>−</sup>IgD<sup>−</sup>CD27<sup>−</sup>), T2 (R123<sup>−</sup>CD38<sup>−</sup>CD24<sup>−</sup>CD10<sup>−</sup>IgD<sup>−</sup>CD27<sup>−</sup>), and T3 (R123<sup>−</sup>CD38<sup>−</sup>CD24<sup>−</sup>CD10<sup>−</sup>IgD<sup>−</sup>CD27<sup>−</sup>) cells were discriminated. In addition to examining a BM control with each experiment, a normal PBMC control was also included to further define the CD38/CD24 expression threshold on mature naive B cells. Transitional cells from multiple tissue sources were additionally examined for the expression of a variety of developmentally regulated and other markers (7, 8, 19).

#### Analysis of B cell proliferation and function

To determine proliferation, 1 × 10<sup>5</sup> B cells or purified subsets were labeled with CFSE (Molecular Probes) and cultured for 1–4 days in medium supplemented with various stimuli: F(ab<sup>−</sup>)<sub>2</sub> anti-IgM (10 μg/ml for all conditions, except in combination with CpG where 2.5 μg/ml is used; Jackson ImmunoResearch Laboratories) or without CpG oligodeoxynucleotide (7, 8, 19). Samples were analyzed for a 30- to 60-s baseline at 37°C followed by the addition of 20 μg/ml F(ab<sup>−</sup>)<sub>2</sub> goat anti-human IgM or anti-IgD.

#### ABC transporter activity

Naive B cells were distinguished from transitional cells and memory B cells by the expression of ABCB1 transporter activity and rhodamine 123 (R123) or Mitotracker dye extrusion as described previously (19). Cells were stained in culture medium at 37°C with R123 at 6 μM for 10 min and chased for 3 h before flow cytometry analysis (19). Dose-ranging studies were performed to determine the optimal loading concentrations and timing for extrusion. Alternatively, cells were stained and chased for 30 min at 37°C with Mitotracker. Equal loading of different B cell subsets from a variety of tissues and PB B cells from multiple normal controls and B cells reconstituting patients was demonstrated in control experiments (data not shown). B cells were gated as described above and dye extrusion examined. Specifically, gated memory B cells (CD27<sup>+</sup>) do not express the ABCB1 transporter and thus fully retain R123. In contrast, gated naive B cells (CD38<sup>−</sup>CD24<sup>−</sup>CD10<sup>−</sup>CD27<sup>−</sup>) effectively extrude R123, with the mean fluorescence intensity (MFI) for R123 on normal naive PB B cells (n = 7 donors) significantly different from the MFI for R123 on normal memory B cells (p = 3.0 × 10<sup>−5</sup>). Transitional B cells within the gated naive population were distinguished by the intermediate/high expression of R123.
**Statistical analysis**

Statistical significance of comparisons of mean values was assessed by a two-tailed Student’s *t* test or nonparametric Mann-Whitney *U* test using XLSTAT Excel software. Values of *p* \( \leq 0.05 \) were considered significant.

**Results**

**Developmental kinetics of human transitional B cells and demonstration of a late transitional subset**

We have previously reported an expansion of transitional B cells in diverse patient populations reconstituting after B cell depletion therapy, including lymphoma and autoimmune disease (lupus and rheumatoid arthritis) (13, 15). In this study, we sought to better delineate human transitional B cell subsets and the time course of B cell development through defined transitional B cell stages during B cell reconstitution. Circulating transitional B cells were initially analyzed based on surface phenotype: IgD⁺CD27⁻CD38highCD24high (Fig. 1A) (7). High expression of CD38 and CD24 was defined based on their level on the CD10⁺ PB B cells, a well-accepted marker expressed by most transitional cells (5). In normal PB, transitional B cells are detected at low frequency (Fig. 1A and Table II). To better discriminate distinct transitional B cell subsets, we compared B cells from BM and CB that contained increased proportions of less mature B cells (20). In the BM, there is a large population of cells very high for expression of CD38 and CD24 (++ +) and based on additional analysis of IgD and IgM correspond to pre/pro, immature, and T1 B cells. The corresponding T1 population is depicted in PB with the T2 subset intermediate for CD24/CD38 (++) compared with mature naive (+). Immune reconstitution after rituximab reveals an expansion of CD24highCD38high transitional B cells with progression from T1 to T2 to mature naive over time. Cells from normal and reconstituting PB were examined for R123 dye extrusion, which is reflective of expression of functional ABCB1 transporter by naive B cells. The gating strategy on the CD24 vs CD38 and CD27 vs IgD dot plots is shown in color-coded gates, with the respective R123 expression depicted in the histograms. The relatively inefficient R123 extrusion of the gated N population in the reconstituting subject compared with the normal naive population suggests an intermediate phenotype between T2 and mature naive. This population is also revealed in the CD38 vs R123 dot plots, with the gated T1 subset in blue and the CD27⁺ M in green.
represent mature recirculating B cells, but a relative paucity of cells with moderately high expression (+ +) (T2). In contrast, in PB CD38++CD24+++ T1 cells are sparse and CD38+CD24++ T2 cells more abundant. CB also contains a high proportion of T2 cells with levels of expression of CD38 and CD24 in between the position of the T1 and mature B cells (Fig. 2A).

The developmental relationships between the T1, T2, and mature B cell populations, as defined above, were further substantiated by the developmental kinetics during B cell reconstitution after rituximab. Notably, even more than 1 year after rituximab-induced B cell depletion, nearly all of the B cells in the PB display a transitional phenotype (Fig. 1C). As reconstitution continues, the PB B cells gradually down-regulate CD24 and CD38 and lose CD10 expression, shifting to an apparently mature naive phenotype with decreased fractions of transitional cells (15). A gradual progression from T1 to T2 to mature naive is evident (Fig. 1C).

To additionally distinguish transitional from mature naive B cells, we examined the activity of the ATP-binding cassette transporter ABCB1. Wirth and Lanzavecchia (19) have described this transporter as being up-regulated during the final differentiation from transitional B cells to mature naive and irreversibly lost on memory cell differentiation. We have shown that the ABCB1 is also lost in CD27-negative memory cells (21). When PB B cells are labeled with the vital dye R123 and then chased at 37°C for 3 h, a heterogeneous staining pattern is observed because some cells do not extrude the dye at all (memory and transitional B cells) and others extrude efficiently (naive cells). The R123 expression for each gated B cell population is depicted in the histograms in Fig. 1D. In this analysis, it is apparent that a fraction of the gated “naive” B cells (CD24int CD38intIgD−CD27−) do not extrude R123 efficiently, suggestive of a late transitional B cell phenotype that has down-regulated CD38 and CD24.

The identification of late transitional cells is best illustrated by the examination of B cells during the reconstitution phase in patients treated with rituximab. Such analysis readily detects an enrichment of cells with inefficient rhodamine extrusion within the CD24intCD38intIgD−CD27− gate (Fig. 1D). We postulate that these cells are in transition between T2 and mature naive, representing a population akin to the T3 subset described in mice (9). This developmental relationship is supported by the appearance of the T3 subset after T1 and T2 in diverse patient populations (lymphoma, SLE, and rheumatoid arthritis) reconstituting after rituximab (Table II) and its gradual replacement by mature naive B cells. It is further notable that the PB is enriched for this T3 population compared with normal adult PB even years after treatment.

Late transitional B cells are enriched in CB and spleen but not tonsil

Next, we examined different lymphoid tissue for late transitional B cells by combining surface markers (CD38, CD24, CD27, IgD) with dye extrusion (Fig. 2A). The naive surface phenotype (IgD−CD27−CD24−CD38−) cell population contains both naive and T3 cells (denoted N/T3). An increase in the proportion of the T3 subset is then revealed based on a higher R123 MFI for the N/T3-gated population (Fig. 2B and Table II) (an alternative gating strategy for the T3 subset is described in the next section). T1, T2, and T3 cells are equally dominant in CB (T1, 20.8 ± 9.5; T2, 28.2 ± 7.7; T3, 31.8 ± 11.1, p = 0.0002, <0.0001, and = 0.0007 for T1, T2, and T3 respectively, compared with PB), as revealed by the gated T1 and T2 populations (Fig. 2A) and the shift in R123 expression in the N/T3 population (Fig. 2B and Table II, lower M/N MFI). Based on this analysis, CB contains few if any mature naive B cells. Splenic B cells also display a shift toward poor R123 extrusion with enrichment for T2 and late transitional B cells relative to mature naive (Fig. 2B and Table II), suggesting that this is the site of their development at least under physiological circumstances.

There has been some confusion in the literature regarding the distinction between pre-GC cells and transitional B cells. This is an important distinction since the expansion of one or the other subset in the PB may not only reflect different immunological processes but also have separate biological consequences (22). To clarify this issue, we asked whether the CD38highBm2− (pre-GC) population in the tonsil (based on the Bm1–5 designation via CD38 and IgD expression) (17) overlaps with the CD38highCD24high subset that we are identifying as transitional B cells. In fact, it does not (Fig. 2C), given that the tonsil Bm2− population has decreasing expression of CD24 (Fig. 2D). Consistent with the identity of these cells as predominantly pre-GC, we found increasing expression of CD27 and CD77 (markers not found on transitional cells) (Fig. 2D). In contrast, the Bm2+ compartment in PB and BM overlaps

### Table II. Transitional B cell fractions in different lymphoid compartments

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Naive</th>
<th>T3</th>
<th>T2</th>
<th>T1</th>
<th>M/N MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal PB (mean ± SD)</td>
<td>59.1 ± 12.3</td>
<td>11.2 ± 2.2</td>
<td>4.2 ± 1.6</td>
<td>0.9 ± 0.7</td>
<td>7.2 ± 2.2</td>
</tr>
<tr>
<td>After rituximab PB</td>
<td>31.8 ± 13.1</td>
<td>41.2 ± 12.9</td>
<td>26.6 ± 13.3</td>
<td>10.3 ± 11.8</td>
<td>2.5 ± 0.9</td>
</tr>
<tr>
<td>BM</td>
<td>39 ± 26</td>
<td>5.3 ± 3.7</td>
<td>2.6 ± 1.2</td>
<td>6.3 ± 0.9</td>
<td>7.1 ± 3.3</td>
</tr>
<tr>
<td>CB</td>
<td>19.2 ± 8.6</td>
<td>31.8 ± 11.1</td>
<td>28.2 ± 7.7</td>
<td>20.8 ± 9.5</td>
<td>2.0 ± 0.5</td>
</tr>
<tr>
<td>Spleen</td>
<td>27.7 ± 12.1</td>
<td>15.3 ± 6.1</td>
<td>5.4 ± 0.9</td>
<td>1.8 ± 0.7</td>
<td>2.2 ± 0.7</td>
</tr>
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</table>

* The fraction of each subset relative to the entire B cell population is shown (normal PB, n = 7; after rituximab PB, n = 8; BM, n = 4; CB, n = 5; spleen, n = 4). The post-rituximab subjects include lymphoma (n = 6), rheumatoid arthritis (n = 1), and SLE (n = 1) patients and were studied a mean of 16.5 mo after treatment. For analysis of T1 and T2 subsets after rituximab, an additional six lymphoma and SLE patients were available and included in the analysis. T1 cells were defined based on high expression of both CD24 and CD38 within the IgD−CD27− population, whereas T2 cells have intermediate expression of both of these markers. The gated naïve (N) B cell population (CD24−CD38−IgD−CD27−) actually contains both a R123 low true naïve (N) population and a R123 high late transitional (T3) population. The larger the T3 fraction of the gated N population (yielding a higher R123 MFI), the lower the M/N R123 MFI.

* p < 0.0001 compared to normal PB.
* p = 0.0002 compared to normal PB.
* p = 0.007 compared to normal PB.
* p = 0.0007 compared to normal PB.
* p = 0.0005 compared to normal PB.
* p = 0.0002 compared to normal PB.

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with the CD38<sup>high</sup>CD24<sup>high</sup> transitional B cell population. As described in the last section, among transitional B cells, T1 predominates in BM and T2 in PB, with both populations expressing IgD.

**Phenotypic characterization of distinct transitional B cell subsets**

We used 7- to 10-color flow cytometry to analyze simultaneously multiple surface markers to better define discrete human transitional B cell subsets. Fig. 3 depicts the gating strategy which examines dye extrusion in the IgD<sup>−</sup>CD27<sup>+</sup> population, with the dye-negative population representing the naive B cells and the dye-positive population further divided into T1, T2, and T3 based on the level of CD38/CD24 expression. By excluding the double-negative (IgD<sup>−</sup>CD27<sup>−</sup>) population, we avoid contamination with memory B cells which predominate in this minor subset in PB (21). Of note, the T3 subset represents the dominant transitional B cell population in PB (Fig. 3A). Overall, as cells mature from the T1 to mature naive B cell stage, there is an increase in the expression of mature markers (CD22, CD44, CD21, and CD23; data shown for latter two in Fig. 3 for PB and CB) and a gradual decrease in CD10, CD5, and IgM (Fig. 3B and C). In this analysis, T1 and T2 B cells are clearly distinguishable with the latter expressing lower levels of CD10, CD5, and IgM and higher levels of CD21. It is notable that most T3 cells do not express CD10, making this an imperfect marker for the delineation of all human transitional B cells, despite its wide use for that purpose. T3 cells are best distinguished from mature naive by higher expression of IgM and lower expression of CD23, especially pronounced in CB (Fig.
FIGURE 3. Phenotypic characterization of transitional B cells. A, CD19+ B cells from normal PB were examined for CD27, IgD, CD24, CD38, and Mitotracker extrusion to define the N, T3, T2, and T1 fractions shown. B, Expression of discrete markers on the four populations defined above in PB. C, Expression of discrete markers on the four populations in CB. Representative of more than five independent experiments.
3C) but also evident in PB (Fig. 3B). In BM, T1 cells similarly express high levels of CD10, CD5, and IgM and low levels of CD21 and CD23 compared with mature naive recirculating cells, although IgM expression overall was lower on BM T1 cells than on CB and PB T1 cells (data not shown). Notably, BM B cell precursor populations expressed the highest levels of CD10 but were distinguished from transitional cells by the lack of CD5 expression (data not shown).

To exclude the possibility that T3 cells represent an activated B cell intermediate (either activated T2 or naive), activation markers were examined. In PB and CB, CD86, CD69 (Fig. 3, B and C), and CD80 (data not shown) are clearly not up-regulated on T3 cells compared with other transitional and mature naive B cells. CD95 also was not differentially expressed (data not shown). Given the role of BAFF in murine transitional B cell homeostasis, we also examined the expression of two BAFF receptors, BAFF-R and transmembrane activator and CAML interactor. Overall, expression of TACI was low on all subsets examined although higher on early transitional B cells. In contrast, BAFF-R was expressed at high levels on all transitional and mature B cell subsets (data not shown for memory B cells). In the BM, BAFF-R was also expressed at high levels by T1 and mature naive recirculating cells but not by B cell precursors (data not shown).

In spleen, transitional B cells similarly up-regulated mature markers with concomitant down-regulation of immature markers as they progress across the developmental continuum. In notable contrast to PB and CB, however, CD5 displayed heterogeneous expression on the T3 and mature naive subsets in the spleen (Fig.
I (CD27 not shown). Similar to fraction I in murine spleen, human fraction mature/transitional B cells is not expressed by human B cells; data CD38 utilized instead of AA4.1 (which in contrast to murine im-
divided based on differential CD23 and CD38 expression, with
the distribution of human splenic CD27 used in recent murine studies (Fig. 5) (3, 9, 10, 24). We compared
neously the surface markers used in the present study and those
the heterogeneity of splenic B cell populations was further exam-
ration and the apparent complexity of human B cell subsets here,
4). This suggests additional heterogeneity in these populations and,
indeed, human spleen is more complicated than PB and CB with a
sizable contribution to the B cell compartment by the MZ subset.
To exclude the possibility of a MZ contaminate in the T3 and naive
populations as an explanation for the heterogeneous CD5 expres-
sion, we examined the CD21highCD23low MZ for CD5, but its
expression was low (Fig. 4B). In contrast, MZ B cells express
CD1c and CD9, markers previously described on the MZ (23). The
expression of CD1c by transitional B cells suggests the inclusion of
a MZ precursor population within these subsets. We also con-
clude that the spleen contains a unique expansion of CD5+ B cells
compared with other lymphoid compartments, possibly analogous
to murine B1 cells (23). Another distinction in the spleen com-
pared with PB and CB is the up-regulation of activation markers
on a fraction of all B cell subsets, although most pronounced on the
expression was low (Fig. 4). This suggests additional heterogeneity in these populations and, indeed, human spleen is more complicated than PB and CB with a sizable contribution to the B cell compartment by the MZ subset. To exclude the possibility of a MZ contaminant in the T3 and naive populations as an explanation for the heterogeneous CD5 expression, we examined the CD21highCD23low MZ for CD5, but its expression was low (Fig. 4B). In contrast, MZ B cells express CD1c and CD9, markers previously described on the MZ (23). The expression of CD1c by transitional B cells suggests the inclusion of a MZ precursor population within these subsets. We also conclude that the spleen contains a unique expansion of CD5+ B cells compared with other lymphoid compartments, possibly analogous to murine B1 cells (23). Another distinction in the spleen compared with PB and CB is the up-regulation of activation markers on a fraction of all B cell subsets, although most pronounced on the T1 and T2 cells (Fig. 4B). This suggests that the spleen may be the site of activation and differentiation of early transitional B cells.

Given the critical role of the spleen in transitional B cell maturation and the apparent complexity of human B cell subsets here, the heterogeneity of splenic B cell populations was further examined using multiparameter flow cytometry to analyze simultaneously the surface markers used in the present study and those used in recent murine studies (Fig. 5) (3, 9, 10, 24). We compared the distribution of human splenic CD27+ and CD27+ B cells based on CD21 and IgM expression using initial gates to define three fractions (fractions I-III) similar to the recent description of murine splenocytes by Allman and colleagues (10). Focusing first on the CD27+ B cells, these three populations were further sub-
divided based on differential CD23 and CD38 expression, with CD38 utilized instead of AA4.1 (which in contrast to murine immature/transitional B cells is not expressed by human B cells; data not shown). Similar to fraction I in murine spleen, human fraction I (CD27+) contains immature B cells but also includes CD38−CD23− memory B cells (IgD−CD27−MTG-) and a subset of CD38+CD23+ naive cells (IgD+MTG-) (data not shown for MTG). Fraction II (CD27+) includes both CD23 higher follicular naive and CD23 lower T3 B cells. Approximately 50% of the cells within fraction III (CD27+) are CD21highCD23+ corresponding to the position of the recently defined murine MZ precursor pop-
ulation (10). Consistent with this phenotype is the high expression of IgD and CD1c on this population (Fig. 5B). The remainder of fraction III contains CD23intIgD+ B cells phenotypically similar to T3 cells with inefficient MTG extrusion and high CD5 expression (data not shown). The majority of the CD27+ B cells fall in the CD21highIgMhigh fraction III compartment and represent MZ B cells, although memory B cells are also represented within fraction II. In conclusion, this flow cytometric scheme resolves at least nine subpopulations of splenic B cells, including T1, T2, T3, and MZ precursor subsets.

**T3 cells are nonproliferative in vivo**

Since B cell depletion is associated with compensatory increases in BAFF levels and certain subsets of murine B cells are known to undergo homeostatic proliferation in response to BAFF (24), we examined whether transitional B cells express the proliferation Ag Ki-67. None of the transitional B cell subsets express significant Ki-67 in PB, CB, or BM (Fig. 6A). In contrast, positive controls including tonsil GC B cells and BM precursor B cells express significant Ki-67. Transitional B cells arising during B cell reconstitition after rituximab also do not express Ki-67 nor activation markers (Fig. 6B).

**Transitional B cells have reduced proliferative capacity**

B cell functional maturity can be assessed by examining the in vitro proliferative responses to BCR cross-linking (8, 9). Thus, the
proliferative capacity of CD38highCD24high B cells was compared with CD24highCD38low B cells by measuring the CFSE dilution in the corresponding subsets from reconstituting rituximab-treated patients and normal controls (PB) during in vitro culture. B cells did not proliferate when unstimulated or in response to anti-Ig alone, but with additional costimulation up to two rounds of division were induced at 2–3 days (CpG > BAFF > CD40L). The proliferative capacity of mature naive B cells was significantly greater than T2 cells \( (p = 0.05 \text{ for } T2 \text{ compared with naive}) \) which was greater than the T1 cells \( (p = 0.034 \text{ for } T1 \text{ compared with } T2; \text{ Fig. 7A}) \).

To define the proliferative capacity of the late transitional B cell population, we can compare the proliferation of IgD-CD27− CD24−CD38low peripheral B cells from normal controls and reconstituting rituximab patients. As described above, the latter group at early reconstitution time points has essentially all late transitional B cells (T3) within the phenotypically defined mature naive (N) compartment. Despite this, the proliferation of the N/T3 population was similar between controls and rituximab-treated subjects, suggesting that T3 cells proliferate at least as efficiently as naive cells. This result was confirmed by sorting naive and T3 subsets from normal PB and examining the proliferation profile of the cultured CFSE-labeled populations. Fig. 7B shows that the responder frequencies (100% at day 4) and proliferative capacities (PC) calculated from these CFSE profiles are similar (25).

**Transitional B cell subsets display reduced survival and calcium signaling**

When viability was assessed after 3 days of in vitro culture in the absence of any exogenous stimuli, survival was poor (Fig. 7C). Supplementing the cultures with anti-Ig and stimulatory CpG DNA, CD40L, or BAFF improved survival, with CpG providing the strongest survival stimulus. However, the survival of the T1 subset was still lower than the T2 subset which was lower than the mature naive subset (Fig. 7C). The survival effects of BAFF were more pronounced in the late transitional (T2, T3) and naive B cell subsets (T2, T3), consistent with the higher expression of BAFF-R based on CD24 and CD38 expression as in Fig. 1. The majority of CD24+CD38− (naive) B cells undergo one or two rounds of division, whereas the CD24−CD38− (T2) B cells proliferate less efficiently and the CD24−CD38− (T1) B cells even less so. This is reflected in the lower percent divided (responder frequency) and PC calculated from the plots according to the method of Wells et al. (25). The gray-filled line graph depicts nonproliferating cells cultured in medium alone. B. Response of sorted CFSE-labeled memory (M) (CD27+), naive (N), and T3 B cells to BCR cross-linking (anti-IgM) plus CpG plus IL-2 after 4 days of culture. B cell populations were sorted as described in Figs. 3 and 8 to >90% purity. Plots show resulting CFSE levels on live CD19+ cells at day 4 of culture under these conditions. C. B cells purified and gated as in A were cultured for 2.5 days in the presence of the indicated stimuli. For normal controls, survival of the N/T3 subset is greater than the T2 subset which is greater than the T1 subset. For early reconstituting subjects, the survival of the N/T3 B cells is significantly lower than T2 (+). D. Calcium responses in normal PB B cells after anti-IgD or anti-IgM stimulation. B cells were purified by negative selection and stained with CD2, CD27, CD3, CD14, and CD16 Abs (to gate out non-B cells and memory B cells). The expression of CD27, CD24, CD38, and Mitotracker extrusion was used to define the T1, T2, T3, and N populations as described in Fig. 3. The median responses are depicted for the gated B cell populations after the addition of 20 μg/ml F(ab')2, anti-IgM. Data are representative of at least three independent experiments for A–C.
portant as this complements the assertions based on phenotypic markers and R123 staining to define this novel subset. The enhanced apoptosis of the T3 population was confirmed with sort-purified and cultured naive, T3, and early transitional B cell subsets (data not shown).

The ability of CD24\textsuperscript{high}CD38\textsuperscript{high} B cells to flux calcium in response to BCR cross-linking was also significantly reduced compared with naive B cells (Fig. 7D). Of note, this was true for signaling through both IgD and IgM, despite the higher expression of IgM on the transitional B cells (data shown for the latter). This suggests an overall hyporesponsiveness of transitional B cells to cross-linking of the BCR. Upon further discrimination of mature naive, T3, T2, and T1 subsets, early transitional B cells (T1 and T2) displayed reduced calcium flux compared with both naive and T3 B cells (Fig. 7D).

\textbf{Developmental kinetics of transitional B cells}

The finding that the T3 population is prominent during immunological reconstitution and is gradually replaced by R123/Mito-tracker extruding naive B cells, first suggested to us that it represents a developmental intermediate. However, to further substantiate the developmental relationships between the transitional B cell subsets, we sort-purified T1/T2 and T3 cells (Fig. 8A) and cultured them in vitro (Fig. 8B). T3 cells displayed higher expression of CD24 and IgM relative to mature naive B cells at baseline, as expected (Fig. 8, A and B). Notably, T3 B cells gave rise to MitoTracker extruding naive B cells with progressive down-regulation of CD24 and IgM during culture (Fig. 8). Given that T1 and T2 B cells represent precursors of mature naive B cells, we reasoned that these cells should also give rise to a mature naive phenotype upon culture. Furthermore, if the T3 population is an intermediate in mature naive B cell development, then the T1/T2 cells should pass through this stage as they differentiate to mature naive. At baseline and day 1 of culture, the T1/T2 cells had significantly higher expression of CD10, CD24, CD38, IgM, and MitoTracker compared with T3 and naive cells. By day 3, the T1/T2 cells had differentiated to IgM\textsuperscript{low}CD10\textsuperscript{−}, overlapping in expression with the mature naive population. At earlier time points, the cells displayed an intermediate phenotype consistent with transit through a T3 stage en route to the mature naive B cell stage (Fig. 8B).

\textbf{Discussion}

Our data reveal several novel aspects of human peripheral B cell development. We show that human transitional B cells can be subdivided into multiple populations based on CD24/CD38 expression, a gradation of immature and mature surface marker expression, dye extrusion (reflecting the expression and functionality of the ABCB1 transporter), and functional responses. Notably, we identify a late transitional B cell population intermediate between T2 and mature naive stages that is a normal B cell developmental intermediate, the dominant peripheral transitional B cell population in healthy adults, and substantially increased in the peripheral compartment of patients reconstituting after B cell depletion therapy. Overall, our results indicate that human transitional cells exist along a phenotypic and functional continuum between immature and mature, both conferring and substantially extending the prior characterization of human transitional B cells (7, 8). Similar to the mouse, we suggest that immune competence is gradually acquired as immature B cells transit through the transitional cell stage toward mature naive (1). Thus, mature surface markers are gradually acquired, the ABCB1 transporter is expressed, and proliferation and survival begin to be favored over apoptosis in response to BCR stimulation.
There have been a number of recent reports defining human transitional B cell subpopulations. These studies have demonstrated a population of CD24-high CD38-high B cells with distinct phenotype and function compared with mature naive B cells. However, discrete developmental subsets have not been well characterized. Reduced proliferation, survival, differentiation, and chemotaxis of CD24-high CD38-high human transitional B cells have been demonstrated in vitro, but notably there are several discrepancies in the literature regarding the nature of the signals that mediate the survival and proliferation of distinct transitional B cell subsets. For example, one publication has proposed that human T1 cells have marked CD40L-enhanced proliferation but little response to BAFF stimulation (26). Other studies have demonstrated more of a gradation of response, with mature subsets simply having more proliferation (8) or entry into cell cycle in response to CD40L/anti-Ig than their transitional cell counterparts. Similarly, Sims et al. (7) found BAFF to be a relatively inefficient inducer of cell cycle entry in human B cells, but still with a trend toward greater induction in mature than transitional B cell populations. Our data are in accord with the view of mature human B cells having greater proliferative responses to a range of stimuli compared with transitional B cells. Thus, based on CFSE dilution, proliferation of the T1 subset is clearly induced by anti-Ig plus either CpG or BAFF, although to a lesser degree than in the T2 and mature naive subsets. It is of additional note in our study that CpG was the most highly effective stimulus for the proliferation and survival of human transitional cells, in accord with a recent publication (27), and raising the question of how autoreactive transitional cells escape such stimulation in vivo. We also demonstrate for the first time that human transitional B cells (T1/T2) have reduced calcium signaling compared with mature naive B cells.

Of note, even within the murine literature, there is some disagreement as to the precise delineation of transitional B cell subsets. Thus, Loder et al. (3) first proposed the partition of transitional B cells into two subpopulations based on CD21, CD23, and IgD: T1 and T2, with similar phenotypic properties to that described here with the exception of IgD expression on human T1 cells. A notable parallel between this classification scheme and our characterization of human transitional B cells is the increasing expression of CD21 on maturation from T1 to T2. However, Allman and colleagues (10) have described an alternate classification scheme using the developmental marker AA4.1 and variable expression of IgM and CD23 to delineate three transitional populations T1-T3. Similar to this analysis, we found higher expression of CD23 on human transitional B cells upon maturation from T1 to T2 and decreasing IgM expression. The T2 populations defined by these different murine classification schemes are likely distinct, with the CD21-high CD23+ T2 population of Loder et al. (3) recently postulated to be a MZ precursor (10). This is interesting since other data indicate that B cells transferred into lymphopenic murine hosts, a situation possibly recapitulated after B cell depletion therapy, undergo rapid activation and differentiation into cells phenotypically indistinguishable from purported MZ precursors and MZ B cells (10, 28). Our detailed characterization of human spleen defines for the first time an analogous MZ precursor population, although notably MZ B cells are not expanded in the PB following BCD or in the context of the PB lymphopenia associated with SLE (29).

As with the human T3 population characterized for the first time here, murine T3 cells would be included within the mature naive compartment by conventional gating strategies. Their presence in human PB is revealed here by the inability to efficiently extrude R123/MitoTracker, a higher CD24 and IgM expression, and a lower CD23 expression compared with conventional mature naive B cells, functional immaturity based on increased susceptibility to apoptosis, and a dramatic expansion during immunological reconstitution after B cell depletion therapy. The delineation of a T3 population in humans is an important departure from recent studies of human transitional B cells, where it has been suggested that human B cells may comprise only a single transitional stage or contiguous T1 and T2 stages. Regardless, it is clear that further identification of markers differentially expressed by subsets of human transitional B cells would greatly facilitate their resolvability. An additional layer of complexity has recently been proposed by Cambier and colleagues (12). Their results demonstrate that nontransgenic mature naive B cells can develop into anergic cells with a T3 phenotype in response to chronic antigenic stimulation (12). Along these lines is important to bear in mind that activated naive B cells may also lose expression of the ABCB1 transporter and become unable to extrude rhodamine, although this generally parallels the acquisition of CD27 expression (19). Hence, one possible alternative explanation for our results would be to postulate that PB B cells with a T3 phenotype represent mature anergic cells that have developed in response to repeated antigenic stimulation presumably by self-Ags. These interpretations are not mutually exclusive as it is possible that T3 cells could indeed contain a fraction of mature or immature anergic B cells. However, we believe that the dominance of a T3 phenotype within CB as well as the ability of these cells to differentiate to mature naive in vitro makes it unlikely that the majority of these cells would represent anergized mature cells rather than transitional cells.

The discrimination of multiple human transitional B cell subsets is meaningful in that it provides important insight into the developmental process followed by human B cells. Thus, it should be noted that in mice immature T1 cells home to the spleen which has been purported be the main or only environment for transitional cells differentiation (9). On the other hand, Lindsley et al. (30) have recently described a later murine transitional B cell subset that develops in the BM in parallel with peripheral splenic T2 development. The detection of abundant T2 and later transitional B cells in human PB after rituximab treatment indicates either substantially increased differentiation in and release from the BM or greatly increased generation in and recirculation from the spleen. Based on the fact that in normal subjects the BM contains mainly the T1 subset of transitional cells and the spleen is enriched for later T3 subsets and contains activated T1/T2 cells, we would suggest increased BM generation of T1 cells and differentiation in the spleen with subsequent recirculation in the PB. Extrasplicenic maturation of B cells may also take place given the expansion of T2 and T3 subsets in CB as well. Of note, the finding of late transitional B cells in human spleen and CB has been documented here for the first time.

The factors that govern the survival and selection of human transitional cells are important to further delineate, particularly given that such processes shape the mature B cell repertoire, deviations from normal regulation may precipitate autoimmunity, and the potential restoration of proper regulation will determine the long-term outcome of B cell depletion therapy. There has been consensus in the mouse literature that T1 B cells are a primary target of negative selection (4, 31). However, the factors governing selection in the later transitional stages (T2 and T3) in the periphery are more controversial (24). A general principle has existed that mature B cells are activated upon BCR stimulation, whereas the same signals on developing B cells lead to unresponsiveness or cell death. Critically, however, exactly where in the transitional cell spectrum this change in responsiveness occurs is unclear. Although it has been suggested that murine T2 B cells, when compared with T1 B cells, acquire responsiveness to T cell help signals...
other data support a more gradual acquisition of immune competence within the transitional B cell compartment (1). T2 and T3 subsets may also undergo negative selection vs positive selection depending on the combination of Ag-receptor mediated and non-Ag-receptor-mediated signals, the provision of T cell help, and cytokine stimulation (including BAFF) (24). Later transitional B cells may be particularly responsive to BAFF (24) and thus it is interesting to speculate whether the enhanced BAFF characteristic of a B cell-depleted state (33) may contribute to the prominent expansion of T3 cells during rituximab-induced B cell depletion and reconstitution. Further definition of the signals that mediate survival of human transitional B cells will elucidate our understanding of normal tolerance mechanisms and biological events underlying autoimmunity. Moreover, an improved understanding of the progressive maturation stage of human B cells will greatly enhance our ability to elucidate the precise checkpoints and mechanisms involved in the enforcement of B cell tolerance.

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