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Identification and Characterization of a Human CD5⁺ Pre-Naive B Cell Population

Jisoo Lee, Stefan Kuchen, Randy Fischer, Sooghee Chang, and Peter E. Lipsky

We have identified a distinct pre-naive B cell population circulating in human peripheral blood that exhibits an intermediate phenotype between transitional and naive B cells. Like human transitional B cells, these cells express CD5 but have intermediate densities of CD38, CD10, and CD9, and the ABCB1 transporter compared with transitional and naive B cells. These pre-naive B cells account for a majority of circulating human CD5⁺ B cells. Importantly, CD5⁺ pre-naive B cells could be induced to differentiate into cells with a naive phenotype in vitro. CD5⁺ pre-naive B cells show only partial responses to BCR stimulation and CD40 ligation and undergo more spontaneous apoptosis and cell death than do naive B cells, whereas BAFF/BLyS (B cell-activating factor belonging to the TNF family) did not enhance their survival compared with naive B cells. In contrast, CD5⁺ pre-naive B cells carry out certain functions comparable to naive B cells, including the capacity to differentiate into plasma cells and the ability to function as APCs. Notably, an increased proportion of CD5⁺ pre-naive B cells were found in peripheral blood of patients with systemic lupus erythematosus. These results have identified a unique intermediate in human naive B cell development within the peripheral blood and derangements of its homeostasis in patients with systemic lupus erythematosus. The Journal of Immunology, 2009, 182: 4116–4126.

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FIGURE 1. Characterization of CD27+CD38intIgD+ B cells. A, Peripheral blood CD19+CD27− B cells were gated into CD38low (naive), CD38int (intermediate), and CD38high (transitional) B cell subsets and the differential expression of CD5 was assessed. Numbers represent mean fluorescence intensity of each population (naive, intermediate, and transitional B cells noted from left to right). Data are representative results from a total of 12 donors.

B, Expression of a variety of B cell markers by CD27+CD38intIgD− naive, CD27+CD38intIgD+ intermediate, and CD27+CD38highIgD+ transitional B cell populations. Numbers represent mean fluorescence intensity of each population (naive, intermediate, and transitional B cells noted from left to right). Data are representative results from a total of 12 donors. The mean (±SE) frequencies of naive B cells, intermediate B cells, and transitional B cells of the total CD19+ B cells from the 12 donors were 63.0 ± 2.8% (range, 44–82%), 7.0 ± 0.9% (range, 3–16%), and 2.0 ± 2.9% (range, 1–4%), respectively.

C, Expression of the ABCB1 transporter was assessed by R123 dye extrusion. CD27+CD38intIgD+ intermediate B cells express an intermediate degree of ABCB1 transporter activity between CD27+CD38lowIgD− naive B cells and CD27+CD38highIgD− transitional B cell populations. Data are representative results from three donors.
responded poorly to ligation of either CD40 or surface IgM. Of interest, this pre-naive B cell population accounted for the majority of circulating human CD5/ H11001 B cells, and an increase in the proportion of this population was found in peripheral blood of patients with systemic lupus erythematosus (SLE). Our data suggest that increased production or lack of further maturation of these pre-naive B cells can occur in autoimmune diseases, and may contribute to the development of autoimmunity.

Materials and Methods

Cell preparation

Blood samples are obtained from healthy adult donors of various ages, sexes, and races (39 from the National Institutes of Health, 10 from Ewha Womans University) and from 6 patients with active SLE (all from Seoul, Korea). The mean (±SD) ages of the normal donors were 41 ± 14 (range, 20–68) years, and 22 men and 27 women were included. SLE patients were all women, and the mean (±SD) age was 35 ± 10 (range, 26–50) years. At the time of the blood collection, three patients were untreated, and the other three were treated with hydroxychloroquine with or without low-dose glucocorticoids. Bone marrow aspirates were obtained from healthy donors. Cord blood was obtained from healthy newborns. These studies

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FIGURE 2. CD27/CD38int/IgD+ intermediate B cells are enriched in cord blood, but not in normal bone marrow. A, Adult blood and cord blood B cells were assessed for the distribution of transitional, intermediate, and naive B cells after staining for expression of CD19, CD27, IgD, and CD38. B, B cells from bone marrow of a normal donor were stained with anti-CD19, anti-CD27, anti-CD38, anti-IgD, anti-CD20, anti-IgM, and anti-CD5 to examine the frequency of naive, intermediate, transitional, immature, and pro- and pre-B cells and the differential expression of CD5 by each population.

Abbreviations used in this paper: SLE, systemic lupus erythematosus; BAFF/BlyS, B cell-activating factor belonging to the TNF family.
have been approved by the National Institute of Arthritis and Musculo-
skeletal and Skin Diseases Institutional Review Board and the Ethics Com-
mitee of Ewha Womans University School of Medicine, and informed
cconsent was obtained according to the Declaration of Helsinki. B cells and
CD4+ T cells were enriched by negative selection from buffy coats or
leukapheresis using RosetteSep or StemSep B cell purification AB cocktails
following the manufacturer’s instructions (StemCell Technologies).

Flow cytometry and cell sorting
PBMC, enriched B cells, and postculture B and T cell subsets were
stained with various mAbs combinations for 20 min on ice in staining
buffer (PBS with 1% BSA). The directly conjugated mAbs used were
anti-IgD-FITC, anti-CD27-FITC, anti-CD10-PE, anti-CD95-PE, anti-
CD4-PerCP-Cy5.5, anti-CD38-PE-Cy7, anti-CD20-allophycocyanin-
Cy7, anti-CD11b-allophycocyanin-Cy7, anti-CD69-allophycocyanin-Cy7 (all
BD Biosciences), anti-CD21-PE, anti-CD5-PE, anti-HLA-DR-PE, anti-
CD19-PE-Texas Red, anti-CD19-PE-Cy5.5 (all Caltag Laboratories). Anti-
CD44-Alexa Fluor 647, anti-BAFF-R-Alexa Fluor 647, anti-CD29-Alexa
Fluor 647, anti-CD38-Alexa Fluor 647, and anti-CD27-Alexa Fluor 647
were labeled from purified mAbs (BD Biosciences) using the Alexa Fluor
647 mAb labeling kit (Molecular Probes/Invitrogen). Anti-IgM-Pacific
Blue, anti-CD27-Pacific Blue, anti-IgD-Pacific Blue, and anti-CD19-
Pacific Blue were prepared from purified mAbs (BD Biosciences) using the
Pacific Blue 647 mAb labeling kit (Molecular Probes/Invitrogen). An-
nexin-V-allophycocyanin (Caltag Laboratories) and the Live/Dead fixable
violet dead cell staining kit (Invitrogen) were used to assess apoptosis and
cell death. Stained cells were washed and fixed in 1% paraformaldehyde,
and data were collected by performing eight-color flow cytometry using the
eyeCy an multiparameter flow cytometer (Dako). Data were analyzed using
FlowJo software (Tree Star). B cell populations were sorted using the Dako
MoFlo cell sorter.

Cell culture
B cell populations were cultured in 96-well plates at 3 × 10^4 cells/ml in
100-μl volumes of culture medium (RPMI 1640 with 10% FCS, Gluta-
MAX, and gentamicin) at 37°C alone or supplemented with various cyto-
kines and stimuli: IL-21 (200 ng/ml; R&D Systems), IL-4 (100 ng/ml;
R&D Systems), IL-2 (200 ng/ml; R&D Systems), IL-7 (1 μg/ml), IL-15
(10 μg/ml; Jackson Immunoresearch Laboratories). To determine in vitro B cell dif-
erentiation, B cells were cocultured with human CD4+ T cells activated
by immobilized anti-CD3 as described previously (21). In brief, 96-well
plate bottom plates were coated with 10 μg/ml anti-CD3 clone UCHT1 (BD
Biosciences) overnight at 37°C in Tris buffer (50 mM (pH 7.4)). CD4+ T
cells were purified as described above and treated with a final concentra-
tion of 0.1 μg/ml mitomycin C (Sigma-Aldrich) for 30 min at 37°C. A total of
1 × 10^5 mitomycin C-treated CD4+ B cells and 3 × 10^4 sorted B cell
populations were cultured together in duplicates or triplicates in 200 μl on
the anti-CD3-precoated microtiter wells.

Cell proliferation and functional assays
To determine proliferation, purified B cells (2–5 × 10^7 cells/ml) were
labeled with CFSE (Molecular Probes) at a final concentration of 2.5 mM
for 8 min and cultured in medium supplied with various stimuli: anti-CD40
(100 μg/ml) and goat Fab’, anti-IgM (10 μg/ml) for 48 or 72 h. Prolif-
eration was analyzed by dilution of CFSE. CFSE-labeled B cells cocultured
with human CD4+ T cells activated by immobilized anti-CD3 as described
above were cultured for 96–144 h and analyzed for CFSE dilution. To
determine B cell-induced allogenic T cell proliferation, CFSE-labeled negativ-
ely selected CD4 T cells (1 × 10^5) were cultured alone or with an equi-
number of B cells with or without anti-CD40 (10 μg/ml) for 96 h, and the percentage of divided T cells was assessed.

For analysis of calcium signaling, purified B cells were stained with
anti-CD20-allophycocyanin-Cy7, anti-CD38-PE-Cy7, and anti-CD27-
Pacific Blue, resuspended in HBSS containing 1 mM Ca and Mg and 1%
FCS at a concentration of 2 × 10^6 cells/ml, and loaded with Fluoro-3 AM
(Molecular Probes; final concentration 4 μM) for 30 min at 37°C in the
presence of probenecid (Sigma-Aldrich; final concentration 4 mM).
CD20+CD27+CD38high and CD20+CD27+CD38−B cells were gated and
calculated responses were measured using a CyAn flow cytometer (Dako)
by Fluoro-3 fluorescence at 515–535 nm using linear amplification over 4–5
min. After 30 s, 10 μg/ml Fab’ anti-human IgM was added for BCR
crosslinking. Intensity of Fluoro-3 fluorescence was analyzed using FlowJo
software (TreeStar).

To determine ABCB1 transporter activity, PBMCs were prestained with
anti-CD20-allophycocyanin-Cy7, anti-CD38-PE-Cy7, and anti-CD27-
Pacific Blue and stained with R123 (Invitrogen) at 12.5 μM for 10 min at
37°C. R123 stained cells were chased for 3 h and analyzed for green flu-
orescence by flow cytometry.

Determination of Ig levels
Secreted Ig in the culture supernatant was quantitated by ELISA. Briefly,
MaxiSorp 96-well ELISA plates (Nalg Nunc International) were coated
overnight at 4°C with 1 μg/ml (50 ng/well) affinity-purified goat anti-
human IgG-Fc, IgM, or IgA Abs (all Bethyl Laboratories) in PBS (pH 7.4),
washed with PBS/Tween, and incubated with blocking solution (0.2% BSA
in PBS, pH 7.4) for 3 h at room temperature. B cell culture supernatants
and standards were diluted in blocking solution and incubated on coated
plates overnight at 4°C. Normal human IgG, IgM (both Caltag Laborato-
ries/Invitrogen) and human reference serum (Bethyl Laboratories) for IgA
ELISA were used as standards. Culture supernatants and standards were
removed by vacuum aspiration and plates were washed three times with
PBS/Tween. Alkaline phosphatase-conjugated affinity-purified goat anti-
human IgG, IgM, or IgA (all Bethyl Laboratories) in blocking solution
(200 ng/ml, 50 μl/well) were added as secondary Abs and incubated over-
night at 4°C. SigmaFast (Sigma-Aldrich) was used as alkaline phosphatase
substrate and specific absorbance measured at 410 nm using a PowerWave
X 96-well plate reader (Bio-Tek Instruments) and KC4 version 3.1 soft-
ware (Bio-Tek Instruments).

Statistics
Statistical analysis was performed using JMP 7. Comparison between two
groups was performed using unpaired Student’s t test, and comparison
between different conditions was performed using one-way analysis (error
bars represent SEM values). The Mann-Whitney U nonparametric test was
used for comparisons between patients and controls (data are expressed as
means ± SD). For analysis of correlation between variables, a linear re-
gression analysis was performed. Values of p < 0.05 were considered
statistically significant (*, p < 0.05; **, p < 0.005).

Results
We examined in detail the human CD27+CD38highIgD+ intermediate B cell
population in the peripheral blood to search for a CD5− B cell population
other than transitional B cells. Previous studies revealed that tran-
sitional cells and naive B cells in humans could be distinguished
by expression of CD38, in that transitional B cells expressed high
levels of CD38 compared with low levels in naive B cells (20).
This suggested that the CD38 expression profile might serve as a
marker of peripheral B cell maturation. We therefore performed
multicolor flow cytometric analysis of peripheral blood B cells
including staining for CD19, CD27, CD38, and IgD expression. As
shown in Fig. 1A, CD19+CD27+ B cells can be subdivided by CD38
expression into previously described CD38low (naive) and CD38
(high) (transitional) B cell populations. Additionally, we ob-
served another B cell subpopulation that expresses intermediate
levels of CD38. When differential expression of CD5 was assessed
on these populations, CD27+CD38highIgD+ (intermediate) B cells
were identified, as did CD27+CD38lowIgD+ transitional B cells,
and accounted for the majority of circulating CD5 B cells (87% of
total CD5+ B cells). Of the total 12 normal donors examined,
the mean (± SE) frequency of naive B cells was 63 ± 2.8% (range,
44–82%), intermediate B cells 7 ± 0.9% (range, 3–16%), and
transitional B cells 2 ± 2.9% (range, 1–4%) of the total CD19−
B cells. CD27+CD38lowIgD+ intermediate B cells accounted for
a mean (± SE) of 10 ± 1% of the total circulating CD19−CD27+B

Using eight-color flow cytometry, we determined phenotypic
characteristics of the CD27+CD38highIgD+ intermediate B cells
(Fig. 1B). When expression of a variety of B cell markers was
analyzed, intermediate B cells displayed similar levels of expres-
sion of most B cell markers including CD23, CD21, CD24, CD44,
IgM, CD95, and CD11b as naive B cells. Both naive B cells and
CD27+CD38highIgD+ intermediate B cells could be distinguished
from transitional B cells by expression of CD23, CD21, CD24,
FIGURE 3. Intermediate B cells are functionally distinct. A. The CD27^+ CD38^{m} B cell population represents the naive B cell subset, and the CD27^+ CD38^{m} B cell population represents the intermediate B cell population. CD5 and IgD expression by each B cell population is shown. B. Proliferative responses of purified B cell subsets after incubation for 48 h and 72 h in medium alone, or with anti-IgM or anti-CD40. Proliferation was analyzed by dilution of CFSE and is representative of results from three experiments. C. Ca^{2+} mobilization of intermediate and naive B cells after crosslinking of BCR with 15 μg/ml anti-IgM. Ca^{2+} flux was measured by flow cytometry after loading cells with Fluo-3. D. Spontaneous apoptosis and cell death were analyzed in intermediate and naive B cells immediately ex vivo and after 48 h of culture. Apoptosis was measured by the frequency of annexin V-positive cells, excluding Live/Dead-binding cells. Cell death was measured by the frequency of Live/Dead-binding cells. Data shown are the means ± SEM of three experiments. **, p < 0.01 between naive and intermediate B cells. E. Intermediate and naive cells were cultured in medium alone or with either the addition of anti-IgM or anti-CD40. After 48 h, apoptosis and cell death were assessed by staining the cells with annexin V and Live/Dead as described in D. Data are the means ± SE of three experiments. *, p < 0.05 and **, p < 0.01 between culture medium alone and anti-IgM or anti-CD40. F. Sorted cells were cultured with medium alone or with various cytokines, including BAFF, IL-4, IL-21, and IL-10. After 48 h, apoptosis and cell death were assessed as described in D. Data are the means ± SEM of three experiments. ***, p < 0.01 between cultures with medium alone and various cytokines.
BAFF-R, CD44, IgM, CD95, CD11b, and CD86. However, the expression of B cell maturation markers CD9 and CD10 by CD27−CD38intIgD−/H11001 B cells was distinct from naive and transitional B cells, with intermediate levels of these markers noted. Additionally, ABCB1 transporter expression was assessed by R123 dye extrusion. CD27−CD38intIgD−/H11001 intermediate B cells also displayed an intermediate degree of R123 extrusion, whereas transitional B cells showed poor extrusion, and naive B cells showed full extrusion (Fig. 1C).

CD27−CD38intIgD+ intermediate B cells are increased in cord blood, but not in normal bone marrow

We next examined the distribution of intermediate B cells in human cord blood. As previously reported, transitional B cells were markedly increased in cord blood (20). Additionally, intermediate B cells were also significantly increased in cord blood (Fig. 2A). In contrast, intermediate B cells were infrequent in normal bone marrow (Fig. 2B). As in the blood, bone marrow intermediate B cells expressed CD5, as did transitional and immature B cells, but not pro- or pre-B cells.

Functional activity of CD27−CD38intIgD+ intermediate B cells

We next determined the in vitro functional responses of intermediate B cells to BCR ligation, anti-CD40, and various cytokines. For functional studies, we developed a strategy to sort naive and intermediate B cells without engaging receptors that could activate them. A combination of mAbs to CD20, CD38, and CD27 was employed that, as shown in Fig. 3A, could identify a unique population of CD20+CD38intCD27− B cells comparable to the CD19+CD38intCD27− IgD+ B cells previously analyzed. As also shown in Fig. 3A, these cells could be easily distinguished from CD20−CD27−CD38low naive B cells. These two populations were both IgD+, but only the CD20+CD38intCD27− population expressed CD5 (Fig. 3A). Naive B cells started to proliferate to both anti-BCR and anti-CD40 stimulation after 48 h of culture, and they proliferated robustly to both stimuli by 72 h of culture, whereas intermediate B cells showed no proliferative responses to either stimulus after 48 h, and by 72 h of culture, they exhibited a minimal proliferative response, but only to anti-CD40 stimulation (Fig. 3B). To characterize responsiveness of intermediate B cells, Ca2+ mobilization was measured after crosslinking of BCR with anti-IgM. The increase in intracellular Ca2+ of intermediate B cells in response to BCR crosslinking was significantly less than that of the naive B cells (Fig. 3C). To evaluate apoptosis and cell death, the frequencies of annexin V and Live/Dead violet stained positive cells were measured in both populations immediately ex vivo and after 48 h of culture. Intermediate B cells contained a significantly higher percentage of apoptotic cells and dead cells compared with the naive B cells (Fig. 3D). To evaluate apoptosis and cell death, the frequencies of annexin V and Live/Dead violet stained positive cells were measured in both populations immediately ex vivo and after 48 h of culture. Intermediate B cells contained a significantly higher percentage of apoptotic cells and dead cells compared with the naive B cells (Fig. 3D). Both intermediate and naive B cells were saved from undergoing apoptosis by anti-CD40 stimulation, but apoptosis of intermediate B cells was significantly increased by anti-IgM, whereas this was not observed in the naive B cells. When total dead cells were evaluated, intermediate B cells were rescued from cell death only by the anti-CD40 signal, whereas naive B cells were saved by both anti-CD40 stimulation and by anti-IgM to a lesser degree (Fig. 3E). Various cytokines

![FIGURE 4. Intermediate B cells can develop into naive B cells. A. Sorted CD27−CD38int B cells were cultured with mitomycin C-treated CD4+ T cells in anti-CD3-precoated microtiter wells and assessed for CD5 expression and CFSE dilution following 4 days and 6 days of culture. Data are representative of three experiments with similar results. B. CD5+CD27−CD38low B cells were sorted and cultured with anti-CD40 and IL-21. CD5 expression and CFSE dilution were assessed after 4 days and 6 days of culture. Data are representative of three experiments with similar results.](http://www.jimmunol.org/)

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were examined to determine the effect on survival of intermediate and naive B cells. BAFF had no significant effect on the survival of intermediate B cells, in contrast to its ability to rescue naive B cells from both apoptosis and cell death (p < 0.01). Of note, IL-4, IL-21, and IL-10 increased survival of both intermediate and naive B cells significantly (p < 0.01), with IL-10 having the least consistent effect (Fig. 3F).

CD20<sup>+</sup>CD38<sup>+</sup>CD27<sup>-</sup> intermediate B cells can develop into naive B cells via transition to CD5<sup>+</sup> naive B cells

Levels of expression of CD38, CD10, CD9, and the ABCB1 transporter by CD20<sup>+</sup>CD38<sup>+</sup>CD27<sup>-</sup> B cells that were between those displayed by transitional and naive B cells suggested that these B cells might be developmental intermediates between these two B cell populations. When we examined expression of CD5 on naive B cells, we noticed a small fraction of naive B cells that expressed CD5 (Fig. 3A). This suggested the possibility that CD20<sup>+</sup>CD38<sup>+</sup>CD27<sup>-</sup> B cells might mature into naive B cells and gradually lose CD5. To test this hypothesis, we attempted to induce differentiation of intermediate B cells and CD5<sup>+</sup> naive B cells into CD5<sup>-</sup> naive B cells by providing Ag nonspecific bystander T cell help conferred by activated T cells. As can be seen in Fig. 4A, activated CD4<sup>+</sup> T cells induced CD38<sup>+</sup>IgD<sup>+</sup> intermediate B cells to proliferate, and they differentiated into CD38<sup>+</sup>IgD<sup>+</sup> naive B cells, and this population subsequently lost expression of CD5. To ensure that T cell signals could also induce CD5<sup>-</sup> naive B cells to become CD5<sup>+</sup>, CD5<sup>-</sup> naive B cells were cultured with a combination of anti-CD40 and IL-21. As can be seen in Fig. 4B, such stimulation induced CD5<sup>-</sup> naive B cells to become CD5<sup>+</sup>.

Intermediate B cells can manifest some functions of mature naive B cells

To determine whether intermediate B cells can perform normal mature B cell functions, we assessed their ability to differentiate and present Ag. Stimulation of both intermediate and naive B cells with anti-CD40 and IL-21 induced down-modulation of surface IgD and the differentiation of CD38<sup>+</sup>IgD<sup>-</sup> plasma cells (Fig. 5). Similar results were noted when intermediate B cells were stimulated by culturing them with activated CD4<sup>+</sup> T cells. As can be seen, by day 10 of culture, a large population of IgD<sup>-</sup> B cells was generated (Fig. 6A). All of these had proliferated, as gauged by dilution of CFSE (data not shown). Of these, a fraction was plasma cells. Plasma cells derived from intermediate B cells secreted mainly IgM (4.2–5.6 μg/ml), and less IgG (0.06–0.08 μg/ml) and IgA (0.24–0.53 μg/ml) than those from naive B cells, but the total amount of secreted Abs was 2- to 6-fold less than plasma cells derived from naive B cells (Fig. 6B).

The capacity of intermediate B cells to function as APCs was also examined. First, CD86 expression was assessed after stimulating intermediate and naive B cells with anti-CD40. As shown in Fig. 7A, intermediate B cells increased CD86 expression when stimulated with anti-CD40. In additional experiments, intermediate or naive B cells were cultured with allogeneic T cells to assess their capacity to stimulate the MLR. As shown in Fig. 7B, both populations induced comparable degrees of allogeneic T cell proliferation with or without stimulation by anti-CD40.

Increased frequencies of intermediate B cells are found in peripheral blood of SLE patients

Since SLE is associated with a loss of B cell tolerance and resultant disturbances in many B cell populations (22–25), we examined the frequency of intermediate B cells in the peripheral blood of SLE patients. The relative frequency of this B cell population in the peripheral blood of 10 healthy adult donors was compared with that of 6 active SLE patients. Intermediate B cells were found in increased proportions in patients with active SLE. Of note, the frequency of intermediate B cells was inversely proportional to the frequency of total B cells, suggesting that the expansion of the intermediate B cell population was related to
lymphopenia. Additionally, naive B cells of SLE patients also expressed CD5, suggesting a B cell population shift toward immature B cells (Fig. 8).

**Discussion**

We have defined a pre-naive B cell population that stands between transitional and naive B cells, comprising an additional definable subset during normal human B cell development in the periphery. This new subset accounted for the majority of the circulating CD5⁺ B cell population, and it was defined by both its phenotypic and functional characteristics. Although pre-naive B cells exhibit some capabilities of normal mature naive B cells, their main functional role under physiologic conditions appears to be as a developmental intermediate in maturation of human naive B cells, as shown for transitional B cells of both mice and humans (26, 27). However, their functional maturity suggests that under certain circumstances they could be inappropriately activated and contribute to autoimmune disease.

In mice, transitional B cells, which bridge immature B cells in the bone marrow and mature naive B cells in the periphery, can be subdivided into several developmental subsets. Initially, murine transitional B cells were separated into two maturational subsets, T1 and T2, based on expression of CD21 and IgD. Thus, CD24<sup>high</sup> transitional B cells could be subdivided into CD21<sup>low</sup>IgD<sup>low</sup> T1 and CD21<sup>high</sup>IgD<sup>high</sup> T2 cells. These two populations appear to have different population dynamics and require different maturation signals (28). Others have subdivided murine transitional B cells into three populations, that is, T1 (CD23<sup>+</sup>, IgM<sup>high</sup>), T2 (CD23<sup>+</sup>, IgM<sup>low</sup>), and T3 (CD23<sup>+</sup>, IgM<sup>low</sup>), based on CD23 and IgM expression (29). It is unclear how these subsets relate to stages of human B cell maturation. However, it is clear in both mice and humans, B cell development during the transition from immature to mature naive B cells is a multistep process, and at each step B cells are influenced by selection pressure to ensure tolerance before full maturation (29–31). It is notable that these populations reside in the murine spleen and are usually not found in other sites. A similar multistep developmental process is presumed to exist in...
Adult donors were stained with anti-CD19, anti-CD27, anti-CD38, and anti-IgD to examine the frequencies of CD27+ B cells in peripheral blood of healthy adult donors (n = 10) and in patients with active SLE (n = 6) was determined. Mann-Whitney U test was used to detect significant differences (***p < 0.01). B. Frequency of intermediate B cells is inversely proportional to the frequency of total B cells shown in a linear regression plot (r = -0.6363, p = 0.0048). Data include results from 6 SLE patients and 10 normal donors.

Humans, but intermediate cell subsets during human peripheral B cell development have not been fully characterized. Importantly, intermediates in human B cell development can be found in the peripheral blood (20), raising questions about the site(s) of maturation of these cells in humans.

One of the difficulties in identifying human peripheral B cell subsets is that there are multiple phenotypic changes during development, and current understanding of the pattern of expression of these markers is remarkably limited. Previous studies revealed that transitional cells and naive B cells in humans could be distinguished by expression of CD38, among other phenotypic markers, in which transitional B cells express high levels compared with low levels in naive B cells (20). We found an additional B cell population that expressed an intermediate level of CD38 within the CD27− Ag inexperienced B cells, which were neither CD38low naive nor CD38high transitional B cells. Further analysis revealed that these cells were phenotypically more similar to the naive B cells and not to transitional B cells. In this regard, CD38intCD27+ intermediate B cells differed from transitional B cells by expression of CD23, CD21, CD24, IgM, CD95, and CD11b. However, these cells also differed in phenotype from naive B cells by expression of the immature B cell markers CD9 and CD10, and they were intermediate between transitional and naive B cells, distinguishing this CD38int B cell population as a distinct subset. Importantly, these cells expressed CD5, which is also characteristic of human transitional B cells and immature B cells in the bone marrow, but not of naive B cells, suggesting that CD5 might be an important development marker for human B cells. The expression of the ABCB1 transporter activity, which has been described as a definitive method to discriminate resting naive B cells from transitional B cells (32), also distinguished these cells from transitional and naive B cells. ABCB1 transporter expression, confirmed by R123 dye extrusion, showed that CD38int B cells have the ability to extrude the dye, but to a much lesser extent than do naive B cells, consistent with the conclusion that they are developmental intermediates. In summary, the phenotypic analysis indicated this newly identified subset is a pre-naive B cell population, which appears to be a developmental stage between transitional and naive B cells.

Fundamental functional differences between immature and mature B cells in the periphery have been described previously (33). In this regard, immature B cells have a shorter life span (34–36), and they undergo apoptosis rather than proliferation in response to BCR ligation (20, 28, 35–37). These functional characteristics of immature B cells were also found in the pre-naive B cell population, but to a lesser extent than manifested in transitional B cells. Pre-naive B cells were more prone to both spontaneous apoptosis and cell death than were naive B cells, but less so than transitional B cells (20). Additionally, BCR stimulation induced increased apoptosis compared with that found with naive B cells, suggesting that BCR engagement might be involved in negative selection at this developmental stage. It is notable that anti-CD40 and T cell-derived cytokines, including IL-4, IL-21, and IL-10, were able to enhance survival and prevent apoptosis of pre-naive B cells, but the known B cell survival and growth factor BAFF/BLyS was
unable to enhance survival or prevent these cells from undergoing apoptosis (20, 37).

Previously, it has been shown that human transitional B cells also did not respond to BAFF by increasing survival, although they expressed BAFF-R, but at a lesser density than that of mature naive B cells (20). Thus, during human B cell development, neither transitional B cells nor pre-naive CD38+CD27+CD5- B cells respond to BAFF with increased survival. Since both populations express BAFF receptors, failure of BAFF to promote survival of these populations cannot be explained by absence of these receptors, although downstream signaling events may be defective. Moreover, it is clear that a number of T cell-derived cytokines, such as IL-4 and IL-21, can promote survival of pre-naive B cells, whereas IL-4 can also promote survival of transitional B cells (20).

These results indicate that different signaling pathways than those activated by BAFF are involved in promoting survival of human pre-naive and transitional B cells. Finally, the lack of responsiveness of transitional and pre-naive human B cells to BAFF differs from findings in the mouse, in which B cell developmental stages from the T2 stage on are thought to be BAFF responsive (34).

Pre-naive B cells proliferated minimally to anti-CD40 or anti-IgM stimuli, and mIgM-induced Ca<sup>2+</sup> mobilization was much lower compared with that manifested by naive B cells. This may relate to the fact that CD5 is expressed by human pre-naive B cells. CD5 expression appeared to be characteristic of human immature B cells, being expressed on immature B cells, transitional B cells, and pre-naive B cells. In this regard, the most notable difference in phenotype between murine and human T1 B cells was reported to be the expression of CD5 (20). CD5 expression has been shown to be associated with negative regulation of BCR and TCR signaling, as well as maintenance of tolerance in anergic B cells (38–40).

These findings suggest that during development from the immature through the pre-naive stages, human B cells may be inhibited from responding to BCR stimulation by expression of CD5. Presumably, this could contribute to the culling of autoreactive B cells during these stages of development, since a considerable percentage of immature B cells is known to be autoreactive (9). Interestingly, we have observed that activated T cell help caused down-regulation of CD5 on pre-naive B cells and induced proliferation and differentiation into naive B cells. These findings are consistent with a role for CD5 in limiting BCR stimulation and autoantigen-induced activation during B cell development. However, noncognate T cell help may permit autoreactive pre-naive B cells to expand and differentiate, thus bypassing this checkpoint.

As experimentally defined in our study, the pre-naive B cells comprise a population with the capacity to differentiate into plasma cells, and also the capacity to function as APCs. When these cells were stimulated with anti-CD40 in the presence of IL-21, a potent set of signals capable of inducing plasma cell differentiation, naive B cells exhibited the potential to undergo terminal differentiation into plasma cells, as previously shown for human peripheral blood naive B cells (41). Similarly, activated T cells induced naive B cells to proliferate and differentiate into plasma cells that mainly secreted IgM. We also found that anti-CD40 stimulation enhanced expression of CD86 by naive B cells and that pre-naive B cells could stimulate proliferation of allogeneic T cells in an MLR. These results clearly indicate that pre-naive B cells have acquired some of the functions associated with mature naive B cells. These findings also suggest the possibility that under certain circumstances, activation of pre-naive B cells, perhaps during non-cognate bystander interactions with activated T cells, could contribute to the functional activation and differentiation of these cells and the emergence of autoimmunity.

Human pre-naive B cells can be distinguished from the murine T2 B cells by several distinctive observations that may be associated with their functional role. In contrast to the murine T2 cells, which are found exclusively in the spleen, human pre-naive B cells were observed in the peripheral blood, suggesting that their maturation occurs at multiple sites. It is possible that human pre-naive B cells matured at different sites may give rise to different subsets of pre-naive B cells with different functions, as exemplified by marginal zone B cells and B-1 B cells in the murine system (14). However, phenotypic markers are not currently available that would permit examination of this question. Human pre-naive cells also seem to be phenotypically more mature than murine T2 cells, with most markers other than CD9, CD10, and the ABCB1 transporter assuming naive B cell density, whereas murine T2 B cells continue to express a more immature pattern of B cell markers, with the exception of IgD and CD21 (28). The expression of CD5 and the lack of responsiveness to BAFF are also characteristic of human pre-naive B cells but not murine T2 B cells, and suggest additional mechanisms to avoid responsiveness to autoantigens. These differences predict unique developmental functions for pre-naive B cells in humans that are different from the role of T2 B cells in the murine system.

We found that subjects with SLE have increased proportions of pre-naive B cells, and this expansion correlated with the degree of B cell lymphopenia. Previously, it had been observed in patients with SLE and Sjögren’s syndrome who had been treated with the B cell-depleting therapy rituximab that CD38<sup>+</sup>IgD<sup>+</sup>CD5<sup>-</sup> immature B cells repopulated during the recovery phase of B cell depletion, and this correlated with an increase in serum BAFF level (42, 43). Furthermore, an expanded population of CD38<sup>+</sup>IgD<sup>-</sup>CD5<sup>-</sup> B cells within the naive B cell compartment was found in peripheral blood of active SLE patients (44). These previously described B cell populations appear to fall within the pre-naive B cell subset characterized in our study. Collectively, these observations strongly imply that pre-naive B cells can be specifically amplified under certain conditions that can alter B cell homeostasis, including those characteristic of patients with active SLE and those recovering from B cell depletion. Characterization of this B cell population as pre-naive and demonstration that it has the capacity to respond to bystander T cell signals with differentiation into Ab-secreting plasma cells suggest that these cells may, under specific circumstances, contribute to autoimmunity.

In summary, human peripheral B cell development is a multistep process involving at least three developmental subsets: transitional, pre-naive, and naive B cells, and likely subsets within each stage. Pre-naive B cells possess a phenotype and function that are intermediate between transitional and naive B cells in that they have some of the characteristics of the more immature transitional B cells, but, at the same time, they have some of the functional capacity of the mature B cells to differentiate and present Ag. These immature features of the human pre-naive B cells may provide the basis for normal selection mechanism in the periphery at this stage of differentiation. However, the finding that human pre-naive B cells can proliferate and differentiate in response to non-cognate T cell signals suggests that under certain circumstances tolerance may be bypassed at this stage of development and autoimmunity could result.

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