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Transitional B cell subsets in human bone marrow

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Summary

B cells originate from precursors in the bone marrow, and the first cells which migrate to the peripheral blood have been classified as 'transitional B cells'. Transitional B cells have been characterized in human blood with stage 1 (T1) and stage 2 (T2) subsets being proposed. In the present study, 27 normal human bone marrow samples were analysed for transitional B cell markers by eight-colour flow cytometry. T1 transitional B cells ($CD45^+CD19^+CD10^+IgM^+IgD^{lo}$) and T2 transitional B cells ($CD45^+CD19^+CD10^+IgM^+IgD^+$) were identified in normal bone marrow samples at a mean frequency of 3·2 and 3·1% of total B lineage cells, respectively. A majority of the bone marrow transitional B cells were $CD24^{hi}CD38^{hi}$, the phenotype of blood transitional B cells. Consistent with recent peripheral blood data, T2 B cells had a significantly higher CD21 expression compared with T1 B cells (72·4 *versus* 40·9%) in the bone marrow. These data raise the possibility that transitional B cells are capable of differentiating from T1 to T2 B cells within the bone marrow. Furthermore, transitional cells at either stages 1 or 2 might be capable of migrating out of the bone marrow.

Keywords: bone marrow, CD21, flow cytometry, human, transitional B cells

Introduction

B cells originate from haematopoietic stem cells present in bone marrow (BM) [1]. Developing B cells undergo several stages of differentiation in the BM before they enter the peripheral compartment [2]. Cells that are developmentally intermediate between immature BM B lineage cells and fully mature naive B cells in the peripheral blood (PB) and secondary lymphoid tissues are termed 'transitional B cells'. This term was first used in the mouse in 1995 [3] and three stages of murine transitional B cells, namely T1, T2 and T3, have been identified [4]. More recently, transitional B cells have been identified in humans, defined as new emigrant B cells, which have been selected for self-tolerance in the BM and have been exported to the periphery [5]. It is postulated that the transitional cells, after leaving the BM, are subjected to peripheral checks to prevent the production of autoantibodies [6]. Transitional B cells that survive selection against autoreactivity develop eventually into naive B cells [7–9]. Earlier reports described a $CD19^+$immunoglobulin (Ig)$M^+IgD^+CD24^{hi}CD38^{hi}CD27^-$ phenotype of transitional B cells, which is distinct from mature naive B cells that are $CD38^{lo}$, and both germinal centre and memory B cells that are $CD27^+$ [10–13].

Bright CD24 and CD38 co-expression have been proposed to be transitional B cell markers [5,10,12]. Studies on the PB [9,14] B cell compartment identified two transitional B cell subsets, T1 and T2 on the basis of CD24 and CD38 staining, with the former being brighter for both markers than the latter. A more recent publication used CD21 and IgD expression to divide peripheral blood transitional B cells into T1 and T2 subsets. T2 cells were reported to have a high expression of CD21 and IgD, whereas expression of these markers was low on T1 cells. Both these subsets displayed a $CD5^+CD10^+CD20^+CD27^-$ phenotype [7]. This report provided evidence that T2 ($CD21^{hi}$) B cells are more mature than T1 ($CD21^{lo}$) B cells, because the T2 subset had greater proliferation and Ig secretion *in vitro* than the T1 subset. Furthermore, following haematopoietic stem cell transplantation (HSCT), T1 B cells populated the PB before T2 B cells, suggesting that T1 B cells represent primary BM B cell emigrants [7].

Obtaining normal human BM is difficult, and thus few studies have searched for transitional B cells in this location.

Early BM studies identified transitional B cells using the $CD19^{+}CD24^{hi}CD38^{hi}$ classification [5,10], and a more recent study has identified T1 and T2 cells in four samples of human BM using a $CD19^{+}CD10^{+}CD24^{hi}CD38^{hi}IgD^{+}$ classification [9]. In the present study, we used eight-colour flow cytometry to provide a comprehensive immunophenotype of B lineage cells in 27 samples of normal human BM. We used this strategy to assess BM samples for the presence of T1 and T2 transitional B cell subsets.

Materials and methods

Patient samples

Bone marrow (BM) aspirate samples were obtained from patients as indicated by routine clinical care. Samples from patients who were receiving chemotherapy or those who had received a haematopoietic stem cell transplant were excluded. This study was approved by the St Vincent's Hospital Human Research Ethics Committee (file number 11/095) and signed informed consent was obtained from all patients, in accordance with the Declaration of Helsinki.

BM aspirate samples were assessed by microscopy and flow cytometry, and BM trephine samples taken at the same time were assessed by morphology and immunohistochemistry. Samples displaying no detectable abnormalities via this multi-disciplinary approach were classified as normal, and were included into the study. Twenty-seven normal adult BM samples (median age = 51 years; interquartile range = 43–63 years; 14/27 males) were collected.

Monoclonal antibodies

BM samples were collected in heparinized tubes. Immunofluorescence staining was performed with the following monoclonal antibodies (mAbs) and fluorochromes from BD Biosciences (San Jose, CA, USA): CD45 (V500), CD19 (allophycocyanin; APC), CD10 (phycoerythrin-cyanine 7; PE-Cy7), IgM (PE), IgD (peridinin chlorophyll protein-cyanine 5·5; PerCP-Cy5·5), CD5 (fluorescein isothiocyanate; FITC), CD20 (APC coupled with haemocyanine dye; APC-H7), CD27 (APC-H7) and CD21 (PE). The following mAbs from Biolegend (San Diego, CA, USA) were utilized: IgM (Pacific Blue), CD24 (FITC) and CD38 (PE).

Immunofluorescence staining

Eight antibodies (total volume of 44 µl) were added to each test tube in the combinations described in Table 1. The BM was washed twice in phosphate-buffered saline (PBS) and resuspended in 1% (v/v) PBS/fetal calf serum (FCS); 100 µl was added to each test tube to ensure that a minimum of 10 000 B cell events were recorded in all samples. The sample was vortexed and then incubated for 10 min at room temperature; 2 ml of FACS Lyse (BD Biosciences) was added to the sample and incubated further for 10 min at room temperature. Subsequently, 2 ml of PBS was added, and the sample was centrifuged (800 ***g*** at room temperature) for 5 min. The supernatant was discarded, and the cells were resuspended by vortex in 200 µl of stabilizing fixative (BD Biosciences). The sample was then acquired on a FACSCanto II flow cytometer (BD Biosciences) using 405, 488 and 633 nm lasers, and analysed using FACSDiva software (BD Biosciences). Positive staining was determined by a comparison with negatively stained cells in the same specimen.

Statistical analysis

Data analysis was performed with the assistance of GraphPad Prism version 5 software. Descriptive statistics were generated and *t*-tests were performed to compare means between two groups. Significant differences were classified as $P < 0{\cdot}05$. Data are expressed as mean ± standard error of the mean (s.e.m.).

Results

Strategy for identification of transitional B cell subsets in BM

Bone marrow aspirate cells were analysed by eight-colour flow cytometry to identify transitional B cells. The phenotypic criteria that were utilized are listed in Fig. 1a, and the flow cytometry strategy to identify these populations is presented in the remainder of Fig. 1. First, CD19 was used to identify cells of the B cell lineage. CD19 is present on all B cells except for the most immature cells of this lineage [15]. CD19-positive cells displaying low side-scatter (Fig. 1b) were selected for further analysis, excluding CD19-positive plasma cells with higher side-scatter. Next, the CD10 and CD45 staining properties of these B lineage cells were used to identify the two most immature B lineage populations (Fig. 1c). The earliest CD19-positive cells in the B cell lineage are $CD45^{dim}$ and $CD10^{bright}$, and have been termed pre-B-I cells [15]. At the next stage in maturation CD45

Table 1. Antibody panels for bone marrow samples.

Tube	Pacific blue	V500	FITC	PE	PerCP-Cy5·5	PE-Cy7	APC	APC-H7
1	IgM	CD45	CD5	CD21	IgD	CD10	CD19	CD27
2	IgM	CD45	CD24	CD38	IgD	CD10	CD19	CD27

FITC: fluorescein isothiocyanate; PE: phycoerythrin; PerCP-Cy.5: peridinin chlorophyll protein-cyanin 5.5; APC: allophycocyanin; Ig: immunoglobulin.

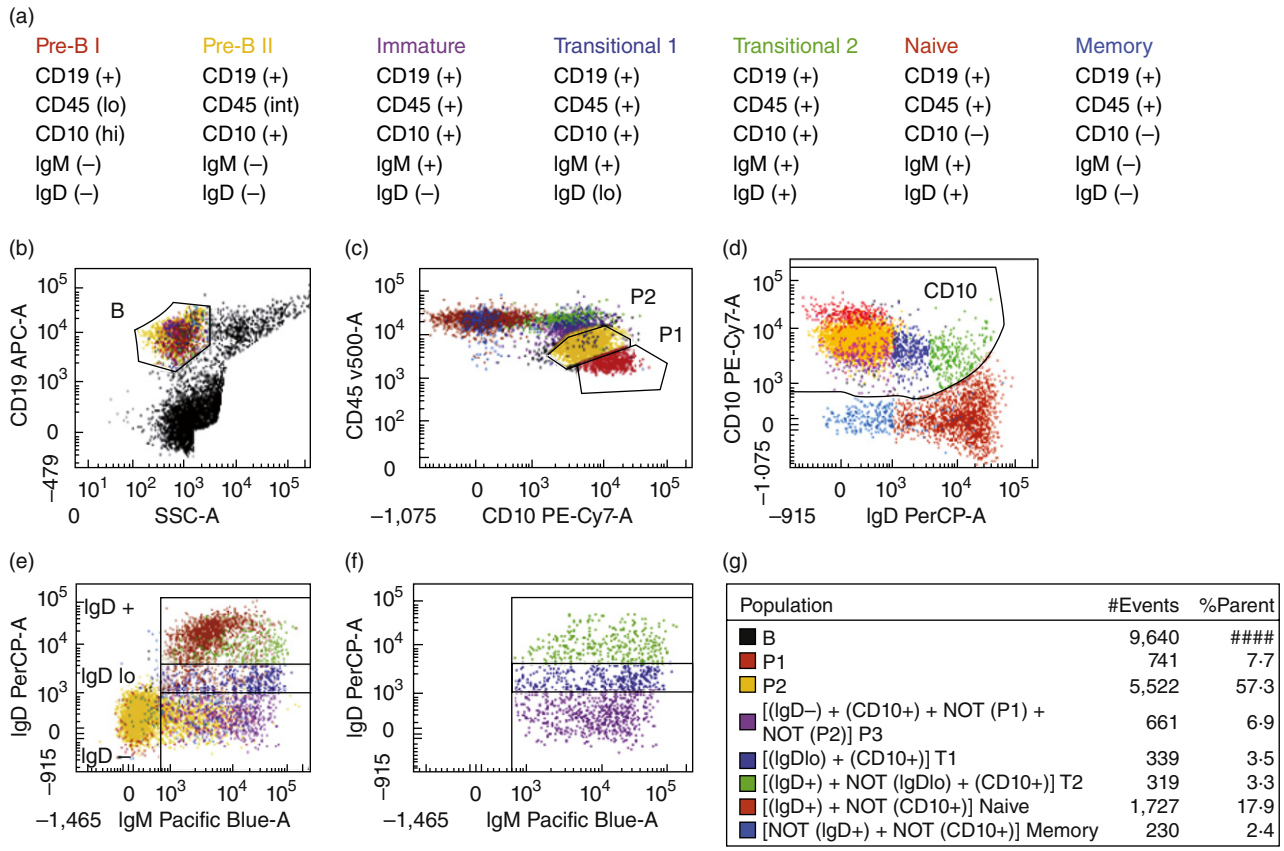

Fig. 1. Identification of transitional B cell subsets in normal human bone marrow (BM). The dot-plots presented display the flow cytometric approach used to identify various B cell subsets in a representative sample. Each B cell subset was assigned a particular colour. (a) Phenotypic criteria for the characterization of B lineage subsets (b) $CD19^+$ cells with low side-scatter (SSC) were gated as B cells. (b) All BM cells except those with high SSC. In (c–f), only B cells (gated in b) are shown. (c) Pre-B I cells (P1, red) were identified as $CD45^{lo}CD10^{hi}$, and pre-B II cells (P2, yellow) were identified as $CD45^{int}CD10^+$. (d) $CD10^+$ B cells were gated as shown. The $CD19^-$ T and natural killer (NK) cells (not shown) were used as a negative control to appropriately draw the $CD10^+$ gate. (e) Identification of B cell subsets. The most immature B cells [$CD19^+CD10^+$ immunoglobulin (Ig)D^-IgM^-] were used as a negative control to define the IgD^+ and IgM^+ cell populations. Gates were placed to identify the IgD^-, IgD^{lo} and IgD^+ cell populations; B cells with IgD intensities higher than pre-B cells ($CD45^{lo}$) and lower than mature B cells ($CD10^-$) were classified as low IgD intensity. (f) This is a duplicate of (e); however, only the $CD10^+IgM^+$ cells are shown. (g) The gating algorithms employed to derive B cell subsets are shown. Pre-B I cells (P1, red) were identified as $CD45^{lo}CD10^{hi}$ and pre-B II cells (P2, yellow) were identified as $CD45^{int}CD10^+$. All other subsets were $CD45^{bright}$. Immature (P3, purple) B cells were $CD10^+$ and IgD^-; T1 (dark blue) B cells were IgD^{lo} and $CD10^+$; T2 (light green) B cells were IgD^+ and $CD10^+$. Mature $CD10^-$ B cells were divided into IgD^+ cells (maroon; naive and natural effector B cells), and IgD^- cells (light blue; IgM only, and switched memory B cells).

expression rises to intermediate and CD10, while still clearly positive, is less bright than on the pre-B-I stage. These cells have been termed pre-B-II cells [15]. These two populations are identified readily in Fig. 1c. Pre-B-I cells have considerable similarities to stage 1 haematogones and pre-B-II cells resemble stage 2 haematogones [16,17]. These two populations were negative for IgM and IgD (Fig. 1d,e), consistent with their classification as early B lineage cells.

The IgM^+ B cells were then divided into IgD^-, IgD^{lo} and IgD^+ subsets (Fig. 1e). B cells with IgD intensities higher than pre-B cells and lower than IgD^+ mature B cells were classified as IgD^{lo}. Figure 1f shows only the IgM^+ $CD10^+$ B cells. The three subsets in this figure are: IgD^- (immature B cells), IgD^{lo} (T1 cells) and IgD^+ (T2 cells). $CD10^+sIg^+$ cells have been described as stage 3 haematogones [16], but this term does not distinguish between the three $CD10^+sIg^+$ subsets displayed in Fig. 1f. The gating algorithms employed to assign a particular colour to each developmental B cell stage are shown in Fig. 1g.

Mature CD10-negative B cells were identified in Fig. 1d, and were divided further into subsets based on the intensity of IgD expression. $IgD^{lo/+}$ mature B cells include naive and natural effector memory B cells, while $CD10^-IgD^-$ B cells represent IgM-only, and switched memory cells [18–20]. Plasma cells in bone marrow typically display higher side-scatter than lymphocytes [21], and such cells were excluded by the side-scatter gating shown in Fig. 1b. However, plasma cells with low side-scatter might be present in bone marrow

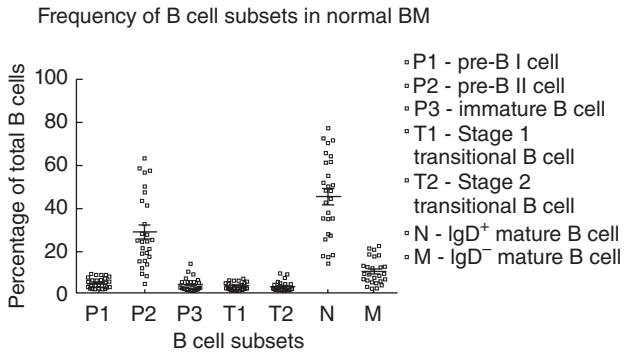

Fig. 2. Enumeration of B cell subsets in normal bone marrow (BM). The mean [± standard error of the mean (s.e.m.)] frequencies of various B cell subsets in the normal BM are presented in this chart ($n = 27$). The means (± s.e.m.) of each subset are as follows: pre-B I (4·7 ± 0·5%), pre-B II (28·7 ± 3·3%), immature (4 ± 0·6%), T1 (3·2 ± 0·3%), T2 (3·1 ± 0·4%), IgD^+ mature B cells (naive and natural effector) (45·2 ± 3·6%) and IgD^- mature B cells (IgM only, and switched memory) (10·2 ± 1·1%).

samples [15]. Any such cells are CD10- and IgD-negative [15,17] and would have been excluded from the transitional B cell populations.

Enumeration of transitional B cells in BM

B cell subsets were characterized based on the phenotypic criteria presented in Fig. 1a. The mean frequencies of all the identified BM B cell subsets are presented in Fig. 2. A key finding of this study was that T1 and T2 populations were detected in all individuals. The mean cell frequency as a percentage of total BM B cells was 3·16 ± 0·34% (± s.e.m.) for T1 B cells, and 3·07 ± 0·45% for T2 B cells. Naive B cells and pre-B II cells were the most abundant B cell populations in the normal BM.

Further phenotypic features of transitional B cells in BM

The T1 and T2 subsets were assessed for bright CD24 and CD38 co-expression, features that have been used previously to define transitional B cells [5,12]. A majority of the T1 and T2 B cells identified by the strategy in Fig. 1 had the $CD24^{hi}CD38^{hi}$ phenotype, which provides strong evidence that these are indeed bona fide transitional B cell populations. The results showed that the expression of these two markers was significantly higher on the T1 B cells when compared to the T2 B cells (Fig. 3). Moreover, the differences remained significant when CD24 and CD38 were analysed individually (data not shown).

Furthermore, CD21 expression was examined on these T1 and T2 B cell populations. The results displayed a significantly higher CD21 expression on T2 B cells when compared to T1 B cells (Fig. 4). These phenotypes are consistent with the $CD21^{lo}$ (T1) and $CD21^{hi}$ (T2) transitional B cell subsets described recently in PB [7].

Interestingly, CD5 was not expressed uniformly on BM transitional B cells, as was expected in light of current definitions of transitional cells in human PB [22,23]. A significantly greater proportion of T2 B cells expressed CD5 compared to T1 B cells (Fig. 5).

Discussion

Our understanding of murine B cell lymphopoiesis is robust; however, this is not the case for human B cell development. In this study, transitional B cells were characterized in normal human BM. Obtaining normal human BM is challenging on both a practical and ethical front, which explains the low numbers of publications in this field [22]. In the few studies which have been performed, transitional B cells were identified as $CD24^{hi}CD38^{hi}$ B cells [5,10], with a later addition of CD10 positivity [9]. The experiments performed in the present study utilized an alternative classification ($CD45^+CD19^+CD10^+IgM^+IgD^{lo/+}$), as guided by research on PB [7,8].

In agreement with recent publications on PB cells, IgD was gained progressively on transitional B cells, thus enabling the identification of T1 (IgD^{lo}) and T2 (IgD^{hi}) B cell subsets (Fig. 1). Furthermore, CD21 expression was

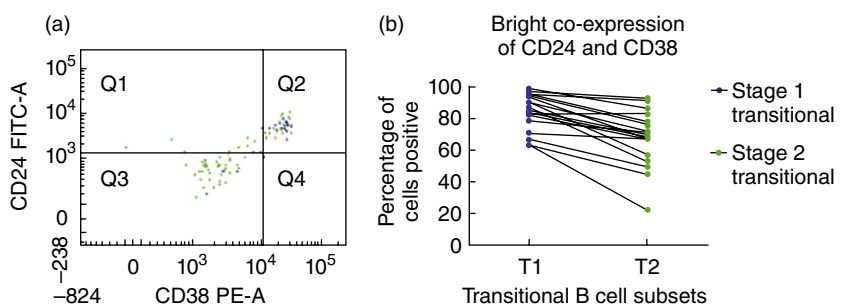

Fig. 3. Bright CD24 and CD38 co-expression on bone marrow (BM) transitional B cell subsets. (a) A representative normal BM sample is presented here, and only transitional B cell subsets are displayed [T1 (blue; $CD10^+IgM^+IgD^{lo}$) and T2 (green; $CD10^+IgM^+IgD^+$)]. (b) This chart displays the proportion of $CD24^{hi}CD38^{hi}$ cells in the T1 and T2 B cell populations. The mean (± s.e.m.) frequency of T1 and T2 B cells that were $CD24^{hi}CD38^{hi}$ in the normal BM was 85·2% (± 2·7) and 69% (± 4·1), respectively ($n = 19$). The lines join the two values in the same sample. CD24 and CD38 expression is significantly lower in the T2 B cells ($P < 0·001$).

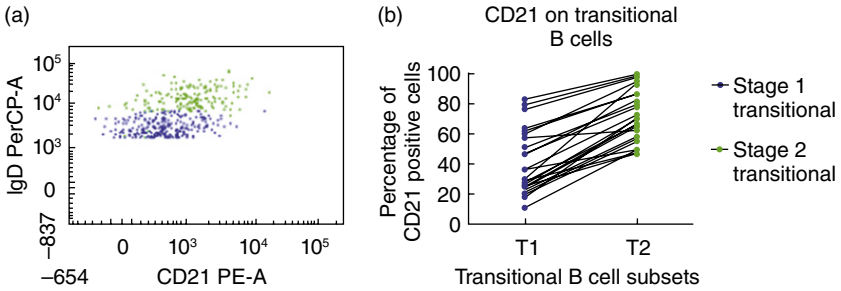

Fig. 4. CD21 expression on bone marrow (BM) transitional B cell subsets. (a) A representative normal BM sample is presented here, and only transitional B cell subsets are displayed [T1 (blue; $CD10^+IgM^+IgD^{lo}$) and T2 (green; $CD10^+IgM^+IgD^+$)]. (b) This chart displays the proportion of $CD21^+$ cells in the T1 and T2 B cell populations. The means [± standard error of the mean (s.e.m.)] of $CD21^+$ T1 and $CD21^+$ T2 B cells in the normal BM were 40·9% (± 4·2) and 72·4% (± 3·4), respectively ($n = 25$). A higher percentage of T2 B cells express CD21 compared with T1 B cells ($P < 0·0001$).

increased significantly on T2 B cells compared to T1 B cells (Fig. 4), providing compelling support for the recently published report that in human PB, T1 cells are $CD21^{lo}$ and T2 cells are $CD21^{hi}$ [7]. In another study, T1 B cells were noted to be the most abundant transitional subtype in bone marrow, and were reported to express dim CD21 [9]. However, detailed data on the BM T2 B cell phenotype was not presented, and only four BM samples were analysed.

In PB, approximately 75% of transitional B cells were reported to be of T2 ($CD21^{hi}$) stage [7]. These authors proposed that $CD21^{lo}$ T1 cells migrate from the BM and develop subsequently into $CD21^{hi}$ T2 cells in the periphery. This prompted the hypothesis that T1, and not T2, B cells would be found in the BM. Surprisingly, the results of the present study identified similar proportions of T1 and T2 B cell subsets in normal human BM (Fig. 2). These data suggest that transitional B cells might have the capacity to depart the BM at either the T1 or T2 stage, and that some T1 cells might be able to differentiate into T2 cells in the BM. Furthermore, it is possible that transitional cells departing the BM at different stages could differentiate into different B cell subsets. Moreover, transitional B cells could play a significant and currently unknown role in the human BM.

A majority of the transitional B cells identified in the BM displayed a $CD24^{hi}CD38^{hi}$ phenotype (Fig. 3), consistent with the initial phenotypic criteria proposed for human transitional B cells [12]. Interestingly, the results showed that a significantly greater proportion of BM T1 B cells displayed this phenotype compared with BM T2 B cells (Fig. 3). Moreover, when the markers were analysed individually, a significantly greater proportion of T1 B cells were $CD24^{hi}$ and $CD38^{hi}$ when compared to T2 B cells. These data are consistent with an earlier report on T1 and T2 cells in BM [9], and indicate that CD24 and CD38 are down-regulated simultaneously in the T2 B cell population.

Two recent publications have characterized a third (T3) $CD10^{-/lo}$ transitional B cell subset ($CD24^+CD38^+IgM^+IgD^+ABCB1^-$) [9,24]. In the present paper, positive staining for CD10 was utilized as a criterion for transitional B cell identification. As the published T3 populations exhibited reduced CD24 and CD38 expression, it is possible that the classifications used in the present study included the $CD10^{lo}$ T3 B cells with the T2 B cells. This could explain why a reduced number of BM T2 B cells displayed a $CD24^{hi}CD38^{hi}$ phenotype when compared to T1 B cells.

The BM phenotypic data generated by the present study correspond well with published human PB transitional B cell data, with the notable exception that CD5 expression was found to be lower in the BM in the present study (Fig. 5) compared with published reports on PB [5,9]. A limitation of the present study is that data for transitional cells in PB are not included. CD5 is expressed on certain human B cell subsets and functions to regulate B cell recep-

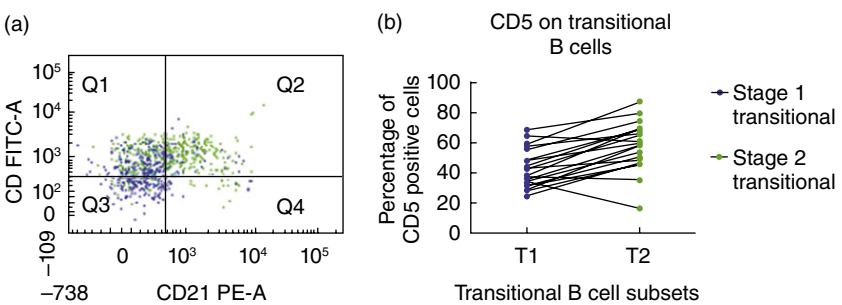

Fig. 5. CD5 expression on bone marrow (BM) transitional B cell subsets. (a) A representative normal BM sample is presented here, and only transitional B cell subsets are displayed [T1 (blue; $CD10^+IgM^+IgD^{lo}$) and T2 (green; $CD10^+IgM^+IgD^+$)]. (b) This chart illustrates CD5 expression on T1 and T2 B cells. The mean [± standard error of the mean (s.e.m.)] of $CD5^+$ T1 and $CD5^+$ T2 B cells in the normal BM was 43·1% (± 2·8) and 58·2% (± 3·6), respectively ($n = 20$). CD5 expression is significantly higher on T2 cells than T1 cells ($P < 0·001$).

tor (BCR) signalling negatively [25,26]. Moreover, CD5 is induced on B cells as a consequence of BCR engagement and cytokine-mediated activation [27]. Our results demonstrate that approximately 43% of BM T1 B cells and 58% of BM T2 B cells express CD5. It must be considered, however, that transitional B cells might only express CD5 adequately in the periphery after BCR engagement and/or interaction with microenvironmental factors.

A large proportion of the B cells identified in normal BM samples were of a naive B cell phenotype, which is due probably to the recirculating tendency of this population [28]. A small subset of unswitched memory B cells known as natural effector B cells is likely to have been identified as naive B cells because IgD, rather than CD27, was used to distinguish naive from memory B cells. It should be considered, however, that a proportion of B cells might not leave the BM until they have developed into naive B cells [17]. The CD4 : CD8 T cell ratio can be used as an indication of blood contamination, because CD8 T cells home preferentially to the BM [29]. In the 13 BM samples in which a PB sample was available for immunophenotyping at the time of BM investigation, the CD4 : CD8 T cell ratio was 1·1:1 in the BM and 1·9:1 in the corresponding PB sample. These data confirm the presence of BM tissue in the BM samples, although the possibility of blood admixture cannot be eliminated entirely.

Although the definition of 'normal' samples in this study is not absolutely ideal, the use of clinically indicated bone marrow investigations that showed no pathological abnormalities overcomes many practical and ethical issues, and has been employed by numerous other investigators [9,30–36]. Although patients receiving chemotherapy were excluded from this study, many variables such as oral medications and other medical conditions were not controlled for. Young and healthy volunteers provide a solution to this problem, and normal haematopoietic stem cell transplant donors have been recruited by certain centres [37–39].

These findings warrant further research into normal human transitional B cells. Transitional B cell definitions are being refined continuously, and studies are hence required to confirm their presence in normal human BM. Moreover, further PB characterization is essential, and other sites including the spleen, cord blood and coelomic cavities should also be analysed. The identification of differing transitional B cell frequencies in infants and adults could provide more clues about human B cell development. Finally, functional studies on transitional B cells will determine whether or not they play a distinct role in the BM, or indeed any other anatomical sites.

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Disclosure

The authors declare that they have no competing interests.

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