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Identification of a Precursor to Phosphatidyl Choline-Specific B-1 Cells Suggesting That B-1 Cells Differentiate from Splenic Conventional B Cells In Vivo: Cyclosporin A Blocks Differentiation to B-1

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The origin of B-1 cells is controversial. The initial paradigm posited that B-1 and B-2 cells derive from separate lineages. More recently it has been argued that B-1 cells derive from conventional B cells as a result of T-independent Ag activation. To understand B-1 cell differentiation, we have generated Ig transgenic (Tg) mice using the H and L chain genes (V \(_{H}\) 12 and V \(_{L}\) 4) of anti-phosphatidyl choline (anti-PtC) B cells. In normal mice anti-PtC B cells segregate to B-1. Segregation is intact in V \(_{H}\) 12 (6-1) and V \(_{H}\) 12/V \(_{L}\) 4 (double) Tg mice that develop large numbers of PtC-specific B cells. However, if B-1 cell differentiation is blocked, anti-PtC B cells in these Tg mice are B-2-like in phenotype, suggesting the existence of an Ag-driven differentiative pathway from B-2 to B-1. In this study, we show that double Tg mice have a population of anti-PtC B cells that have the phenotypic characteristics of both B-2 and B-1 cells and that have the potential to differentiate to B-1 (B-1a and B-1b). Cyclosporin A blocks this differentiation and induces a more B-2-like phenotype in these cells. These findings indicate that these cells are intermediate between B-2 and B-1, further evidence of a B-2 to B-1 differentiative pathway. The Journal of Immunology, 2000, 164: 2924–2930.
cells to B-1 cells (the induced differentiation hypothesis). To discriminate between these possibilities, we combined the anti-PtC Tgs with the X-linked immunodeficiency (xid) mutation (25), a null mutation of Bruton’s tyrosine kinase (26–29). This kinase is a component of the signaling pathway from the IgM receptor complex (30). The loss of Bruton’s tyrosine kinase results in impaired TI-2 responses and poor B-1 cell development (1, 31). Our analysis indicated that the majority of splenic anti-PtC B cells have the conventional B cell phenotype of xid mice (25), suggesting that B-0 cells are precursors to B-1 cells and that differentiation is dependent on signaling from surface IgM. The lineage hypothesis does not predict that anti-PtC B cells with the xid mutation would have a B-0 phenotype.

To identify precursors of B-1 cells in non-xid mice in vivo, we focus on this report on a population of PtC-specific B cells in double (Dbl) Tg mice that have an ambiguous phenotype (25). We demonstrate by adoptive transfer and treatment with cyclosporin A (CsA) that these cells are intermediate in differentiation between B-0 and B-1, indicating a line of differentiation between these two subsets consistent with a single B cell lineage.

**Materials and Methods**

**Mice**

The Dbl Tg mice used in this study were bred and maintained in our animal colony as described (15). C.B17 recipients were purchased from Taconic (Germantown, NY).

**Flow cytometry**

The Abs used for immunofluorescence were against IgMa (DS-1), IgMb (AF6-78), B220 (RA3-6B2), CD5 (53-7.3), CD43, and CD23, were obtained from PharMingen (San Diego CA), and were fluoresceinlabeled, biotinylated, or conjugated to PE. In three-color experiments, directly fluoresceinlabeled, PE-conjugated, and biotinylated Abs were combined. The biotinylated Ab binding was revealed by addition of streptavidin-RED670 (Life Technologies, Gaithersburg, MD). To detect PtC-binding B cells, liposomes encapsulating carboxyfluorescein were used as previously described (15). Contours are 5% probability.

**Cell sorting and adoptive transfer**

Splenic B cells from Dbl Tg or BALB/c mice were stained with anti-B220-FITC and anti-CD23-PE and the B220+ CD23- cells and B220- CD23+ cells were sorted separately on a MoFlo (Cytomation, Ft. Collins, CO) high-speed sorter. A total of 2–4 × 10⁶ sorted Dbl Tg CD23-int cells or BALB/c CD23+ cells were injected i.v. into C.B17 mice that had been irradiated with 500 rad 2 days before cell transfer. The sorted Dbl Tg CD23-int cells were contaminated with 3–5% CD231 cells and often CD5-, CD43-, and CD5+, whereas B-1 cells are IgMab, CD23low, CD23+, and often CD43+/CD5+. In addition, B-1 cells are larger and more granular than B-0 cells. Although the majority of anti-PtC B cells in Dbl Tg mice have the typical B-1 phenotype (15), 5–20% have an unusual phenotype. These cells express CD23 typical of B-0 cells, albeit at lower levels, and express the B-1 cell markers CD5 and CD43 at levels equivalent to those of B-1 cells (Ref. 25 and Fig. 1). They are also intermediate to B-0 and B-1 in B220 expression (B220-int) and cell size, and they express less IgM than B-1 cells do. Thus, their phenotype suggests that they are cells in transition from B-0 to B-1 (B-1-int).

To test the possibility that B-1int cells are precursors to B-1, the B220?, CD23-int cells from Dbl Tg spleens were sorted (Fig. 2A) and adoptively transferred to subletally irradiated C.B17 mice. Three to five days later, spleen cells from recipient mice were analyzed by flow cytometry. The recovered donor cells were PtC-specific at all time points. At 3 days posttransfer, most transferred IgM+ B cells were similar in phenotype to the starting population, i.e., CD23-int, CD5+, and CD43-, although some had decreased expression of CD23 (Fig. 2B). However, by day 5 most transferred cells had lost CD23 expression. In addition, they had increased IgM expression and cell size, and some had decreased B220 (Fig. 2B).

Interestingly, a substantial fraction of the recovered B cells had lost expression of CD5 but retained expression of CD43. Thus, it appears that B-1int cells can give rise to both B-1a (CD5+) and B-1b (CD5-) cells. B-1 cells that contaminate the sorted B-1int population are not responsible for these results because transfer of B-1 cells at a number equal to that determined by flow cytometry analysis to contaminate (3–5%) the sorted B-1int cell populations yielded few or no recoverable cells 5 days posttransfer (data not shown). Thus, between 3 and 5 days posttransfer, B-1int cells had differentiated to B-1, supporting the claim that they are precursors to B-1. Sorted BALB/c splenic B-0 cells transferred to C.B17 mice did not change within this time period, indicating that the manipulations (staining with Abs, cell sorting, etc.) have not induced differentiation to B-1 (Fig. 2B).

**Results**

**The existence of a B-1 intermediate cell in VâH12 Tg mice**

B-0 and B-1 cells are distinguishable using an array of cell-surface markers. B-0 cells are IgMlow, B220high, CD23+, CD43-, and CD5+, whereas B-1 cells are IgMab, B220low, CD23-, and often CD43+ and CD5+. In addition, B-1 cells are larger and more granular than B-0 cells. Although the majority of anti-PtC B cells in Dbl Tg mice have the typical B-1 phenotype (15), 5–20% have an unusual phenotype. These cells express CD23 typical of B-0 cells, albeit at lower levels, and express the B-1 cell markers CD5 and CD43 at levels equivalent to those of B-1 cells (Ref. 25 and Fig. 1). They are also intermediate to B-0 and B-1 in B220 expression (B220-int) and cell size, and they express less IgM than B-1 cells do. Thus, their phenotype suggests that they are cells in transition from B-0 to B-1 (B-1-int).

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Cells at various stages of differentiation to B-1 appear to be evident in VâH12-only Tg (6–1) mice. In 6–1 mice, the transgene-encoded H chain can associate with multiple L chains and generate both PtC-binding and nonbinding B cells (15). Cells that stain...
brightly with liposomes (PtC bright) are B-1, whereas those cells that stain less brightly (PtC int) or not at all (PtC 2) are B-0 (15, 22). The difference in liposome binding ability is due to L chain use. The PtC bright cells predominantly use the V k 4/5H gene (15). This is the gene used by anti-PtC B-1 cell lymphomas and hybridomas, and it is the V k transgene in Dbl Tg mice (9, 15). Based on CD23 expression, there are three subpopulations of PtC bright cells: CD23 high, CD23 int, and CD23 low (Fig. 3). The CD23 low cells are the majority cell type in these mice and are B-1 (i.e., CD5 +, CD43 +, B220 low, and IgM high), whereas the CD23 int cells have a phenotype identical with the B-1 int cells of Dbl Tg mice (i.e., CD5 +, CD43 +, and B220 int). The CD23 high cells express CD23 at levels nearly equivalent to those of B-0 cells, but many are CD43 +, CD5 +, and B220 int (Fig. 3). However, a smaller proportion of CD23 high cells has this phenotype relative to the B-1 int population, suggesting that PtC bright CD23 high cells are at an earlier stage of B-1 cell differentiation than are B-1 int cells.

**FIGURE 2.** Adoptively transferred Dbl Tg B-1 int cells differentiate to B-1 cells. A. The CD23 + B-1 int cell population used in the cell transfer experiments is indicated. B. Sorted B-1 int cells were injected i.v. into sublethally irradiated C.B17 mice. After 3 or 5 days the transferred cells in the spleens of recipient mice were analyzed by flow cytometry. Shown is the phenotypic comparison of cells 3 and 5 days after transfer of Dbl Tg B-1 int cells with BALB/c CD23 + splenic B-0 cells taken 5 days posttransfer. C. B-1 int cells from Dbl Tg mice were transferred to sublethally irradiated mice that were (B-1 int CsA) or were not (B-1 int No CsA) treated daily with CsA beginning the day before transfer. The phenotypes of the cells of these populations are compared with that of B-1 cells from Dbl Tg mice (B-1 No CsA).

CsA blocks differentiation to B-1

TI-2 Ags are proposed to be responsible for driving differentiation to B-1 (19–21). Because B cell responses to CsA in vivo and in vitro (32, 33) can block TI-2 stimulation, we examined the effect of CsA treatment on B-1 cell differentiation. Dbl Tg B-1 int cells were transferred to sublethally irradiated C.B17 mice that received daily CsA treatment beginning the day before transfer. As shown in Fig. 2C, the transferred cells did not differentiate to B-1 after 4 days, even though B-1 int cells transferred into untreated mice differentiated to B-1. Relative to B-1 int cells transferred into untreated mice, CsA-treated cells showed essentially no change in CD23 levels, a decrease in CD5, CD43, and IgM expression, a decrease in cell size and granularity, and an increase in B220 expression. Thus, CsA blocks differentiation to B-1 and appears to cause B-1 int to acquire a more B-0-like phenotype, suggesting that continual Ag stimulation is required for differentiation to B-1.

**FIGURE 3.** 6-1 mice show a progression of differentiation of PtC bright cells from B-0 to B-1. 6-1 mice have multiple populations of B cells when stained with liposomes and CD23 as indicated by the boxes in the two-parameter histogram (left). These populations differ in phenotype as indicated by the one-parameter histograms to the right. The populations are coded as shown with the boxes in the two-parameter histogram.
Daily treatment of Dbl Tg mice with CsA for 2 wk results in the accumulation of PtC-specific cells that have a B-0-like phenotype. Dbl Tg mice treated daily with CsA or left untreated were compared by flow cytometry for phenotypic differences. Shown is the phenotype of the IgM<sup>+</sup>, CD23<sup>+</sup> population. For comparison, the CD23<sup>+</sup> cells of a non-Tg littermate are shown (Normal).

**FIGURE 4.** Long-term treatment of Dbl Tg mice with CsA leads to an accumulation of PtC-specific cells that have a B-0 phenotype. Dbl Tg mice treated daily with CsA or left untreated were compared by flow cytometry for phenotypic differences. Shown is the phenotype of the IgM<sup>+</sup>, CD23<sup>+</sup> population. For comparison, the CD23<sup>+</sup> cells of a non-Tg littermate are shown (Normal).

**FIGURE 5.** PtC-specific B-0 cells from CsA-treated Dbl Tg mice differentiate to B-1 after adoptive transfer to sublethally irradiated mice. Shown are the phenotypic comparison of the CD23<sup>+</sup> cells from CsA-treated Dbl Tg mice (Sorted B-1<sup>int</sup> CsA) and the cells recovered from recipient mice 6 days later (Recovered Cells). For reference, the phenotype of B-1 cells (IgM<sup>+</sup> CD23<sup>-</sup>) from a Dbl Tg mouse that have not been treated with CsA (B-1 No CsA) is included.

**Discussion**

In this paper, we describe anti-PtC B cells that have the phenotypic characteristics of both B-0 and B-1 cells. Our analysis of these cells suggests that they are intermediate (B-1<sup>int</sup>) in a differentiative pathway from B-0 to B-1: they differentiate to B-1 upon adoptive transfer, and they acquire a more B-0-like phenotype upon exposure to CsA. However, CsA does not induce a complete loss of CD5 and CD43 expression in the transfer experiments (Fig. 2C), and CD43 expression is not completely lost by long-term CsA treatment (Fig. 4). We attribute this to the influence of constant Ag stimulation driving differentiation toward B-1, and to the short time frame of the transfer experiments. However, a more complete block in differentiation, mediated by the xid mutation, results in PtC-specific B cells that have the phenotype of conventional (B-0) cells of xid mice and that do not express either CD5 or CD43 (25).

**FIGURE 6.** Potentially useful targets for anti-PtC B cells include CD5, CD43, and IgM. Top: CD5 expression in CD23<sup>+</sup> CD43<sup>+</sup> B cells. Middle: CD43 expression in CD23<sup>+</sup> IgM<sup>+</sup> cells. Bottom: IgM expression in CD23<sup>+</sup> CD43<sup>+</sup> cells. Sorted CD23<sup>+</sup> CD43<sup>+</sup> cells were stained for CD5, CD43, and IgM expression. For comparison, the phenotype of the CD23<sup>+</sup> CD43<sup>+</sup> cells from a non-Tg littermate is shown (Normal).

Taken together, we conclude that anti-PtC B cells differentiate from B-0 to B-1 as a result of signaling through IgM. We further conclude, based on the effect of CsA on B-1<sup>int</sup> cells, that B-1<sup>int</sup> differentiation is reversible. Whether differentiation of cells that have reached the B-1 cell stage is reversible is presently unknown. This pathway, which is illustrated in Fig. 6, was initially proposed by Wortis and coworkers (19, 20) as a result of their observation that splenic B-0 cells can be induced to differentiate to B-1 in vitro by treatment with anti-IgM and IL-6.

Our findings have bearing on the relationship between B-1a and B-1b cells. Transferred B-1<sup>int</sup> cells give rise to both B-1a and B-1b cells. Thus, cells of these B-1 subsets must diverge late in differentiation from B-0. Moreover, because B-1<sup>int</sup> cells are CD5<sup>-</sup>, these data indicate that B-1b cells initially express CD5 and then lose CD5 expression during differentiation. The significance of CD5 loss to the function of these cells is unknown. One possibility is that CD5 may regulate the differentiation to Ab-secreting cells because CD5 is a negative regulator of IgM receptor signaling (34). Regardless of its role, because the B-1<sup>int</sup> cells in this study are all PtC-specific, the divergence to the B-1a and B-1b subsets must not be on the basis of specificity.

A B-0 to B-1 differentiation pathway provides a B cell activation framework for the interpretation of the numerous B cell receptor signaling and coreceptor knockout models that differentially affect B-1 and B-0 cells. For example, CD19 knockout and CD21 knockout mice lack B-1 cells (35–38), as do mice lacking the cytoplasmic kinases Vav and protein kinase C-β/II (39–41). Conversely, mice that overexpress CD19 and mice deficient in Src homology domain 2-containing phosphotyrosine phosphatase-1 have excessive numbers of B-1 cells (36, 37, 42). In light of our evidence of a B-0 to B-1 differentiative pathway, the effects of these signaling...
FIGURE 6. Schematic of B-1 cell differentiation based on the analysis of PtC-specific B cell differentiation. In this schematic, it is presumed that PtC-specific B cells pass through a transitional B cell stage before becoming B-0 cells that express CD23, although the existence of PtC-specific transitional cells has not been demonstrated. We propose that B-0 cells will differentiate to B-1 if stimulated by an appropriate Ag, presumably a TI-2 Ag. The mice in whom PtC-specific cells of each type have been observed are given across the top. Normal mice have a significant population of PtC-specific B-1 cells (12), and it is presumed that they have PtC-specific B-1int cells but that their number is too low to detect. xid blocks differentiation from B-0 to B-1int (25). Other mutations affect this pathway as discussed in the text. CsA blocks differentiation of B-1int to B-1 and probably blocks differentiation of B-0 to B-1int. It may also cause B-1int cells to revert to B-0. The effect of CsA on cells that have already differentiated to B-1 is not known, as indicated by the dotted reverse arrow.

The specificities known to be associated with B-1 are typically Ags that cannot elicit T cell responses (carbohydrates, lipids, and DNA (7, 10–14)), further supporting the idea that TI-2 Ags drive B-1 cell differentiation. Both self- and non-self-Ags can be involved in B-1 cell development; Hayakawa et al. (13) have shown that development of B-1 cells specific for a carbohydrate epitope on the self-Ag Thy-1 is dependent on the presence of Thy-1 itself, and Whitmore et al. (46) have shown that immunization with the non-self-Ag polyvinyl pyrrolidone (PVP) produces a population of anti-PVP B-1 cells.

A variety of factors that affect B cell specificity have been suggested to be key to the burst in B-1 cell differentiation in the neonate (20). These include the absence in neonatal lymphocytes of terminal deoxynucleotidyl transferase (47–51), which results in little or no N region addition among neonatal B cells (in contrast to adult bone marrow-derived cells) and a bias in the neonate relative to the adult in V\textsubscript{H} gene rearrangements (52–54). But other factors including the availability of Ags and other nonantigenic factors (e.g., cytokines or other cells) that drive B-1 cell differentiation or differences in the specificities produced as mice age could be involved. A recent report suggests that B cell differentiation in the neonate is less dependent on the efficient formation of a pre-B cell receptor than it is in the adult (55). Specifically, B cells expressing the V\textsubscript{H}11 gene were favored in the neonate over the adult. Because V\textsubscript{H}11 is also used to encode anti-PtC Abs (4, 5), this mechanism may favor the expression of cells that have B-1 specificities. Nevertheless, the neonatal repertoire appears to be biased by a variety of mechanisms that may be evolutionarily selected to rapidly fill the B-1 repertoire, leading to a rapid enlargement of the B-1 compartment relative to the B-0 compartment in neonates. In this regard, we have demonstrated that anti-PtC B cells are selected at multiple differentiative checkpoints to ensure that a high frequency of V\textsubscript{H}12-expressing B cells have an anti-PtC V\textsubscript{H}12CDR3 (56) and express V\textsubscript{4}/5H (22), and Booker and Haughton (57) have shown that the V\textsubscript{H}11 and V\textsubscript{H}12 genes are evolutionarily conserved. This
is evidence of strong evolutionary selection for the development of an anti-PC response, presumably to promote the survival of every individual.

B-1 cells may be receptive to T cell help (58). If this is a pathway generally open to these cells and they can be drawn into germinal centers, they may undergo affinity maturation through somatic hypermutation and selection as well as heavy chain class switch. Because there is a bias toward self-specificities among B-1 cells, they could be an important reservoir of anti-self B cells in autoimmune diseases. It is possible that B-1 cells are activated with T cell help and are driven to produce pathogenic Abs through somatic mutation and selection in a germinal center. In light of the evidence that B cells can move between subsets, an assessment of the potential of these B cells to be activated by T-dependent Ags in autoimmunity is warranted.

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References


