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$\alpha\beta/\gamma\delta$ Lineage Commitment in the Thymus of Normal and Genetically Manipulated Mice

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I. Introduction

The purpose of this article is to review the mechanism of $\alpha\beta/\gamma\delta$ T-cell lineage commitment in the thymus. As way of introduction, some basic and largely historical aspects of T-cell development will be reviewed briefly with particular reference to differences that might exist between the generation of $\alpha\beta$ versus $\gamma\delta$ cells. A more detailed analysis of some of these points can be found elsewhere (Distelhorst and Dubyak, 1998; Kang and Raulet, 1997; Kisielow and von Boehmer, 1995; Robey and Fowlkes, 1998; Rodewald and Fehling, 1998). Although natural killer (NK) T cells are discussed with particular reference to their presence in the thymus, detailed analysis of their phenotypic development is beyond the scope of this review.

A. Some Unique Features of the $\gamma\delta$ T-Cell Lineage

1. Identification of $\gamma\delta$ T Cells

The profound immunodeficiency observed in neonatally thymectomized mice (Miller, 1961) led to the realization that the thymus was a primary lymphoid organ responsible for the generation of thymus-derived, or T cells (Miller, 1979). Following these seminal observations, it was assumed that there was only one lineage of T cells. With the discovery that T cells were clearly part of the adaptive immune system, showing phenomena of specificity and memory, it seemed obvious that, like B cells, T cells should also express clonally distributed receptor molecules. However, the fact that T-cell receptor (TCR) molecules were not secreted meant that their identification was protracted and required the advent of more sophisticated biochemical (Allison and Lanier, 1987) and molecular biological (Hedrick *et al.*, 1984) approaches in the 1980s. It must be emphasized that it was only by such biochemical and molecular approaches that $\gamma\delta$ receptor molecules were first identified (Raulet, 1989). It would now appear that all jawed vertebrates (gnathostomes) generate two distinct types of T cells, which are characterized by the mutually exclusive expression of $\alpha\beta$ or $\gamma\delta$ T-cell receptor isotypes (Rast *et al.*, 1997). Cells expressing $\gamma\delta$ receptors were first identified by flow cytometry with monoclonal antibodies to hu-

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man CD3 and $\alpha\beta$ TCR as $CD3^+$, $\alpha\beta$ TCR^- cells (Brenner *et al.*, 1986). It was then shown that the mouse fetal thymus also contained $\gamma\delta$ cells that migrated to peripheral lymphoid organs prior to the generation of $\alpha\beta$ T cells (Fowlkes and Pardoll, 1989; Havran and Allison, 1988; Pardoll *et al.*, 1987).

2. *Obvious Functional Differences between $\alpha\beta$ and $\gamma\delta$ T Cells*

That neonatally thymectomized mice, which presumably contained peripheral $\gamma\delta$ T cells, nevertheless showed immunodeficiency (Miller, 1961) raises the important issue of the biological role of $\gamma\delta$ T cells. If one considers that the hallmark of cells of the classical adaptive immune system is (1) antigen specificity and (2) memory, then the position of $\gamma\delta$ cells in such a scenario is perplexing. Recognition of antigen by $\gamma\delta$ cells is dependent neither on CD4/CD8 co-receptor expression by the T cells themselves nor on expression of classical MHC molecules on the cells being recognized (Haas *et al.*, 1993; Schild *et al.*, 1994). Antigen recognition by $\gamma\delta$ T cells is quite different from that of $\alpha\beta$ T cells. Indeed, crystallographic analysis of $\gamma\delta$ TCR (Li *et al.*, 1998) appears to confirm the idea that recognition of antigen by $\gamma\delta$ TCR is more like that of antigen by immunoglobulins than that by $\alpha\beta$ TCR. This becomes particularly relevant when one considers the possible positive (Haas *et al.*, 1993) or negative (Boismenu and Havran, 1997) selection events affecting $\gamma\delta$ T-cell development in the thymus and the form, if any, of their selecting ligands.

The second issue is that of memory within the $\gamma\delta$ T-cell compartment. Although there appears to be a proliferative response among $\gamma\delta$ cells to viral (Carding *et al.*, 1990), bacterial (Skeen and Ziegler, 1993), and parasitic (Rosat *et al.*, 1993) infections, whether a specific memory component is generated following this proliferative phase is less clear (Mombaerts *et al.*, 1993). This is in striking contrast to that seen in $\alpha\beta$ T cells where elegant studies have shown selection and expansion of cells with characteristic TCR clonotypes following immunization (Brawand *et al.*, 1998; MacDonald *et al.*, 1993; McHeyzer-Williams and Davis, 1995). In addition, primed $\alpha\beta$ T cells show modified activation thresholds upon rechallenge (Iezzi *et al.*, 1998), but such information for $\gamma\delta$ cells is sparse (Carena *et al.*, 1997). The question of the turnover and life span of $\gamma\delta$ cells in unimmunized normal and $\gamma\delta$ TCR transgenic mice has been addressed (Tough and Sprent, 1998). Thus, most thymic emigrant $\gamma\delta$ T cells appeared to have a restricted life span as naive cells. However, some $\gamma\delta$ cells converted to a memory phenotype as judged by acquisition of the $CD44^{high}$, $CD62L^{low}$, HSA^{low}, $CD45RB^{high}$ phenotype. It will be interesting to see if naive and memory phenotype $\gamma\delta$ cells in normal mice show different activation characteristics.

3. Anatomical Considerations with Regard to Thymic T-Cell Development

In the intervening period between the discovery of T cells and their corresponding receptor molecules, many studies addressed the issue of how the thymus generated T cells. In this regard, differences between αβ and γδ cells clearly exist. Combined histological and [^{3}H]thymidine labeling experiments indicated that the thymus was divided into two main anatomical regions (Metcalf, 1966). First, a predominantly outer cortex comprising 80–90% of cells and where both cell division and death occurred (McPhee *et al.*, 1979; Shortman and Jackson, 1974); cortical cells were smaller in diameter and in a compact organization. Second, a predominantly inner medulla where cell division and death were rare (Egerton *et al.*, 1990); medullary cells were larger in size and more widely spaced than in the cortex (Metcalf, 1966). From these combined studies, the notion was put forward that T cells were generated in the cortex and that cortical cells were the direct precursors of cells in the medulla (Shortman and Jackson, 1974). It was quickly realized that the vast majority of thymocytes were destined to die *in situ*, a finding that seemed perplexing before it was realized that the process of apoptosis is a major feature of both B and T lymphocyte development and is linked to the requirement for receptor selection (Kisielow and von Boehmer, 1995).

For the αβ lineage, more recent refinements of this cortical to medullary differentiation model certainly corroborate these earlier findings. However, the situation for γδ cells is less clear. By immunohistochemical analysis with antibodies to surface γδ TCR, the few γδ cells in the thymus are mostly found in clusters in the cortex (Farr *et al.*, 1990). Little information is available as to their subsequent transit through the thymus, although from labeling experiments it would seem that they probably migrate to the periphery from the medulla (Kelly *et al.*, 1993). Importantly, as discussed by Tough and Sprent (1998), the kinetics of αβ versus γδ thymocyte selection may be quite different, with γδ cells being generated and exported from the thymus more rapidly than αβ cells, which in the adult thymus require a prolonged sojourn in the medulla prior to emigration.

4. The Question of Positive and Negative Selection

The question of the developmental site and movement of γδ cells through the thymus during differentiation is not a trivial one because, for αβ cells, the transit from the cortex to the medulla is associated with receptor selection events. Positive selection takes place in the cortex whereas negative selection may take place in both cortex and medulla (Anderson *et al.*, 1997; Kisielow and von Boehmer, 1995; Merkenschlager *et al.*, 1997; Punt

et al., 1997). The death of cells during intrathymic development is due either to absence of positive selection or to negative selection. Negative selection of developing $\gamma\delta$ cells (Dent *et al.*, 1990) seems to be generally accepted. Whether positive selection also takes place is less certain (Schweighoffer and Fowlkes, 1996; reviewed in Haas *et al.*, 1993; Robey and Fowlkes, 1998). The issue of positive selection is particularly pertinent with regard to those T-cell subsets expressing invariant $\gamma\delta$ receptors. These subsets include so-called dendritic epidermal cells (DECs), which are located in the skin and mostly bear a canonical Vγ3[1]/Vδ1 TCR, or $\gamma\delta$ cells in the reproductive tract, which express predominantly invariant Vγ4/Vδ1 TCRs. Experiments by Mallick-Wood *et al.* (1998) have revealed that mice lacking the Vγ3 chain due to targeted gene disruption are capable of generating almost normal numbers of DECs expressing a $\gamma\delta$ TCR with a similar, conserved conformational determinant (idiotype) as found in wild-type mice, despite the use of another nondeleted Vγ gene segment. This result provides convincing evidence for the positive selection of $\gamma\delta$ cells, at least with regard to this particular $\gamma\delta$ subset. At face value, these new findings seem to contradict earlier studies by Asarnow *et al.* (1993), who used transgenic TCRγ minigenes as artificial recombination substates to demonstrate that directed gene rearrangements—even in the absence of the possibility for selection—resulted in efficient formation of the invariant Vγ3 junctional sequence. However, both findings can be easily reconciled by assuming that the generation of the highly restricted TCR repertoire of dendritic epidermal cells is the result of two processes: (a) biased gene rearrangements mediated by the recombination machinery and (b) subsequent selection of cells bearing TCRs with the respective invariant determinant.

5. *Sensitivity to Glucocorticoids and Cyclosporin A*

Administration of glucocorticoids to mice results in a dramatic depletion of 100% of cortical and about 50% of medullary thymocytes 48 hr after drug administration (Blomberg and Andersson, 1971). From this observation, medullary cells, like peripheral "mature" T cells, are "resistant" to glucocorticoids and are therefore called "mature" thymocytes to distinguish them from their glucocortioid-sensitive cortical "immature" partners (Ceredig *et al.*, 1982). The glucocorticoid-mediated death of thymocytes is by apoptosis, and evidence implicates the purinergic receptor $P2X_1$ and an inositol 1,4,5-trisphosphate receptor (IP_3R) in mediating this process (Distelhorst and Dubyak, 1998). For $\alpha\beta$ cells, the transition from glucocorticoid "sensitive" to "resistant" occurs immediately post-TCR receptor selection

[1] Nomenclature throughout this article is according to Garman *et al.* (1986).

(Crompton *et al.*, 1992; Tolosa *et al.*, 1998). Little information is available on differences in glucocorticoid sensitivity between immature and mature γδ cells. Interestingly, the development of γδ but not αβ cells is largely resistant to the administration of cyclosporin A (Robey and Fowlkes, 1998). However, cyclosporin A does have an effect on the phenotypic maturation of intrathymic γδ cells (Leclercq *et al.*, 1993).

B. Arriving at the DN → DP → SP Model of Thymopoiesis

1. CD4 and CD8 as Useful, Developmental Stage-Specific Cell Surface Markers

The expression of serologically detectable markers was soon found to provide an important parameter for following T-cell development within the thymus. In general, expression of surface markers is used to define different stages within cell lineages, usually a valuable approach (for potential pitfalls, see Section I,B,4). The first such serological marker was the Thy-1 (CD90) antigen (Reif and Allen, 1964), which was considered to define cells of the T lymphoid lineage. By several criteria, CD90 was found not to be uniformly expressed on thymocytes, with small cortical cells expressing more CD90 than their larger medullary descendants (Ceredig *et al.*, 1982). CD90 expression is also low on the very earliest cells in the thymus and can be practically absent on some cells with T-cell characteristics, notably among intraepithelial lymphocytes (IEL) (Lefrancois and Goodman, 1989). This variation in CD90 antigen expression may be linked to the presence of multiple promoters within the CD90 gene (Spanopoulou *et al.*, 1991). There are also large species variations in the expression of CD90, with peripheral T cells in rats being mostly $CD90^-$ (Hosseinzadeh and Golschneider, 1993). However, at the time, with anti-CD90 reagents no clear dichotomy of peripheral T-cell subsets was observed.

The advent of serology identified a series of T lymphocyte (Lyt) alloantigens, which for the first time subdivided mouse peripheral T cells into two phenotypically and functionally distinct populations, namely (1) Lyt-1 $(CD5)^+$, Lyt-2 $(CD8\alpha)^-$, Lyt-3 $(CD8\beta)^-$ "helper" and (2) Lyt-1^-, Lyt-2^+, Lyt-3^+ "cytotoxic cells" (Cantor and Boyse, 1975, 1977). CD5 was later shown to be expressed by a subset of B lymphocytes (Hardy *et al.*, 1994). Both subsets of peripheral T cells were derived from thymic precursors expressing all three Lyt alloantigens (Kisielow *et al.*, 1975).

Application of monoclonal antibody technology to human T cells and thymocytes resulted in the identification of two subpopulations of mature T cells expressing the antigens CD4 and CD8 in a mutually exclusive fashion (Reinherz *et al.*, 1980; Reinherz and Schlossman, 1980). This dichotomy of T-cell phenotype was particularly attractive given that there

appeared to be an association between CD4/CD8 phenotype and the specificity of MHC antigen recognition (Swain, 1980). When the human thymus was analyzed, cortical cells were found to express both CD4 and CD8 and were thus called double positive (DP), whereas medullary cells, like peripheral T cells, expressed either CD4 or CD8 and were called single positive (SP) (Janossy *et al.*, 1980). Combining the cortical to medullary anatomical model outlined earlier with the phenotypic results of CD4 and CD8 expression, the DP (cortical) to SP (medullary) transition of human thymocytes was proposed (Reinherz and Schlossman, 1980).

With a monoclonal antibody (GK-1.5) to the mouse CD4 antigen (Dialynas *et al.*, 1983), peripheral T cells and medullary thymocytes were found to be either CD4 or CD8 (SP) and cortical cells DP (Ceredig *et al.*, 1983). In addition, these studies identified a small subpopulation of cells in the thymus that expressed neither CD4 nor CD8, so-called double negative (DN) cells. Based on the observation that 100% of cells in the day 15 mouse fetal thymus were DN and that DP cells first appeared at day 16, 2 days before SP cells at days 18 to 19, for $\alpha\beta$ T cells the DN to DP to SP model of mouse thymocyte development was proposed (Ceredig *et al.*, 1983; Fowlkes and Pardoll, 1989) (see Fig. 1A). This scheme appears valid for "classical" $\alpha\beta$ TCR-expressing cells not bearing the NK-1.1 marker.

The DN to DP transition of thymocytes was directly demonstrated by *in vitro* culture (Ceredig *et al.*, 1983) and *in vivo* transfer experiments

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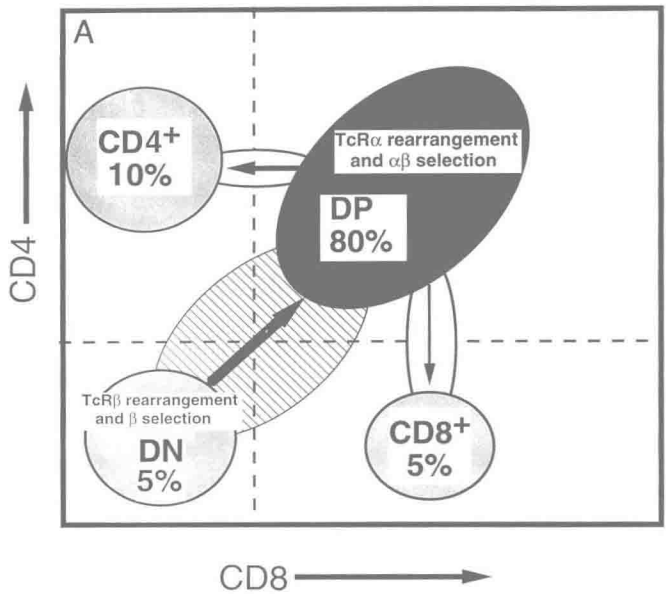
FIG. 1. (A) A Simplified schematic representation of adult mouse thymocyte differentiation. Thymocyte subpopulations are outlined based on their expression of CD4 and CD8 and their relative proportions indicated as a percentage. For conventional (non-NK1.1$^+$) $\alpha\beta$ T cells, development progresses from cells expressing neither CD4 nor CD8 (DN) (lower left) to DP cells expressing both antigens (upper right). Efficient transition from DN to DP is contingent on successful TCRβ rearrangement and pre-TCR expression (see text for details). Most cells transit directly; however, some DN cells proceed to DP via a CD8$^+$/CD4$^-$ intermediate; such cells have been called immature single positives (ISP). In some mouse strains, CD4$^+$ ISP can also be detected. Following $\alpha\beta$TCR receptor selection, DP cells become either CD4 or CD8 single positives (SP). (B) Subpopulations of mouse CD4$^-$/CD8$^-$ (DN) thymocytes. A schematic representation of DN thymocyte subpopulations defined by their expression of CD25 and CD44. CD44 expression varies from weakly positive ($-$/low) to bright ($++$) and, together with CD25, helps define four subsets of DN thymocytes that have been called CD25$^-$/CD44^{++} DN#1, CD25$^+$/CD44$^+$ DN#2, CD25$^+$/CD44$^{-/low}$ DN#3, and CD25$^-$/CD44$^{-/low}$ DN#4. This scheme highlights the heterogeneity of the DN#1 subset. B cells can be distinguished by expression of CD19, NK, and NK T cells by their expression of NK1.1 in appropriate mouse strains and by weak expression of CD117 (*c-kit*) and, finally, dendritic precursors by expression of CD11c and MHC class II. T precursor cells can be distinguished by bright expression of CD117. See text for further details.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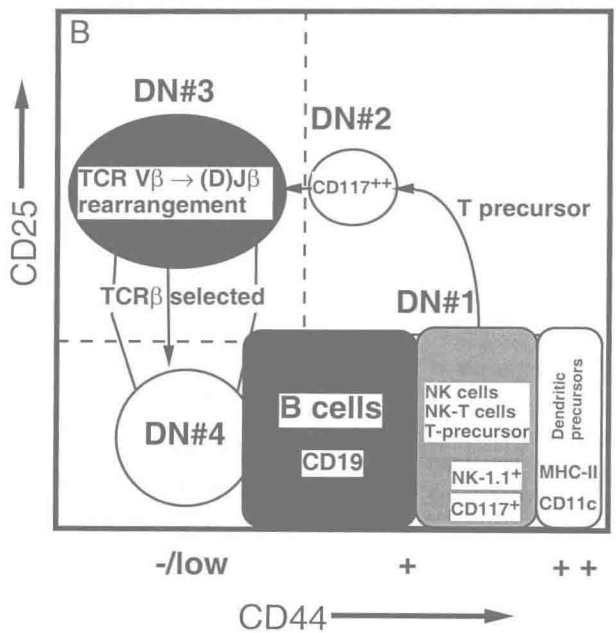
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CD25
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CD19
NK cells
NK-T cells
T-precursor
NK-1.1+
CD117+
Dendritic
precursors
MHC-II
CD11c
-/low
+
++
CD44

(Fowlkes *et al.*, 1985). Importantly, these *in vitro* experiments, when combined with the DNA-labeling technique (Ceredig and MacDonald, 1985; Ceredig *et al.*, 1983; Sekaly *et al.*, 1983), indicated that the differentiation to DP cells *in vitro* was a process independent of cell division. Later experiments showed that the transition from DN to DP *in vivo* at the population level was accompanied by a burst of rapid cell division (Hoffman *et al.*, 1996; Howe and MacDonald, 1988). However, whether all cells undergoing this transition do so by dividing has not been determined. It should be recalled that in other cell differentiation systems, e.g., gut epithelial cell development, cellular differentiation, as defined by changes in cell phenotype, may occur independently of cell division (Simon and Gordon, 1995). The transition of thymocytes from DP to SP was initially difficult to directly demonstrate *in vitro*, but has been subsequently confirmed by many groups. Several phenotypic changes are associated with the transition from DP to SP, including changing cell size, downregulation of CD24 and CD90, and upregulation of CD69, and is a topic that has been adequately reviewed elsewhere (Kisielow and von Boehmer, 1995).

2. *Heterogeneity of DN Thymocytes*

Phenotypic analysis of purified DN cells indicated that they were themselves heterogeneous for the expression of several markers, including CD3, TcR$\gamma\delta$, TcR$\alpha\beta$, CD25, and CD44 (Fowlkes and Pardoll, 1989). DN thymocytes depleted of CD3$^+$ $\alpha\beta$ and $\gamma\delta$ cells are called triple negative (TN) cells (Godfrey and Zlotnik, 1993). In the adult but not fetal thymus (Antica *et al.*, 1993), the earliest populations of TN cells are weakly CD4 positive, becoming negative at the CD25 stage (Wu *et al.*, 1991). It has been suggested, however, that the CD4 molecules on such cells are passively acquired, presumably from surrounding DP cells (Michie *et al.*, 1998).

Additional refinements to the TN developmental sequence have included c-kit (CD117), the stem cell factor receptor (Godfrey *et al.*, 1992; Matsuzaki *et al.*, 1993). Thus, in both fetal and adult thymus, the earliest (TN#1) subset is CD117$^+$/CD25$^-$/CD44$^+$, which then progresses through a CD117$^+$/CD25$^+$/CD44$^+$(TN#2) stage to CD117$^-$/CD25$^+$/CD44$^{-/low}$ (TN#3) and finally to CD117$^-$/CD25$^-$/CD44$^{-/low}$ (TN#4) cells (Fig. 1B). With sensitive flow cytometric techniques, purified TN cells do not show completely biphasic profiles with any of these markers. Indeed, expression of CD117 by TN#1 cells is quite heterogeneous in the thymus of recombination activating gene knock-out (RAG KO) mice, ranging from high on a small subset to low on a population of mature NK cells, which are found in the thymus of both RAG KO and normal mice (Carlyle *et al.*, 1998). In fact, CD117^{low} mature NK cells constitute the majority of TN#1 cells in adult RAG KO mice (R. Ceredig, unpublished data).

Several important events take place at the CD25 stage of thymocyte development. For instance, it was demonstrated that the Vβ to (D)Jβ rearrangement of TCR genes occurs among $CD117^-/CD25^+$ TN#3 cells and that subsequent transition to the $CD25^-$ TN#4 subset is contingent upon in-frame TcRβ rearrangements (Mallick *et al.*, 1993). This process has been called "TCRβ selection" and is mediated by the pre-TCR (reviewed by Fehling and von Boehmer, 1997; Rodewald and Fehling, 1998). It should be recalled that D to Jβ rearrangements are not unique to T cells and that the molecular indicator of T-cell commitment is the Vβ to (D)Jβ rearrangement. This is equally valid for B cells where the V_H to $(D)J_H$ rearrangement marks B-cell commitment. In the B lymphocyte lineage, CD25 expression is characteristic of pre-BII cells (Rolink *et al.*, 1994), a stage following successful Ig_H rearrangements at the $CD117^+/CD25^-$ pre-B1 cells (Osmond et al., 1998). Based on CD117 and CD25 expression, pre-B1 cells resemble TN#1 thymocytes, cells that contain little, if any, TCR Vβ→(D)Jβ rearrangements (Koyasu *et al.*, 1997). This differing pattern of CD25 expression by developing T and B cells indicates that there is, most likely, no physiologically relevant relationship between receptor gene rearrangement events and CD25 expression. In contrast, activation of CD25 transcription may be a completely fortuitous event due to the presence of a particular combination of transcription factors at a given developmental stage (Ivanov and Ceredig, 1992; Rothenberg and Ward, 1996).

Although changes in CD25 expression on maturing thymocytes are apparently of no functional importance, CD25 clearly provides a very useful developmental marker, particularly when used in combination with CD44. Heterogeneous CD25 and CD44 expression has therefore become the most frequently used marker system to subdivide the DN thymocyte population in a developmentally meaningful way. Figure 1B represents a scheme based on these two markers that illustrates the developmental progression of $CD3^-CD4^-CD8^-$ (TN) cells in the adult thymus along the four CD25/CD44-defined stages (TN#1–TN#4). The scheme also reveals the distinct heterogeneity of DN thymocytes in the adult mouse. The inclusion of a few additional markers leads to further refinement, allowing the attribution of most CD25/CD44-defined DN subsets to a distinct developmental stage or lymphoid cell lineage. Apart from conventional αβ and γδ T cells, the following cell types can be identified within the DN thymocyte subpopulation.

a. B Cells. The thymus contains a distinct population of B cells that can be phenotypically distinguished from peripheral blood B cells transiting the thymus (R. Ceredig, unpublished observations). To exclude thymic B