New insights into the proliferation and differentiation of early mouse thymocytes

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Abstract

Early thymocyte development was compared in normal, recombinase-activating gene 2-inactivated (RAG-2 KO) and pre-T cell receptor alpha-inactivated (pre-Tα KO) mice, mutants representing either a complete (RAG-2 KO) or partial (pre-Tα KO) block in progenitor development. Using three colour analysis with antibodies to CD117, CD44 and CD25, cell numbers in each progenitor subset were quantified, demonstrating an accumulation of cells prior to the block. Progenitor number was influenced both by the nature of the genetic block and thymus size, as shown in the enlarged thymus of a transgenic mouse line. By four colour staining for CD3, CD117, CD44 and CD25 and deliberately not gating out CD3+ cells, a novel aspect of gamma delta T cell development in pre-Tα KO mice was identified. 5-bromodeoxyuridine labelling and subsequent four colour staining for BrdU, CD117, CD44 and CD25 showed firstly that DN1 cells were cycling, secondly that the developmental block in pre-Tα KO mice corresponded to a decrease in DN4 cell proliferation, and thirdly provided a novel ‘snapshot’ of T cell receptor beta-selected cells transiting the DN3 to DN4 compartment. Taken together, these results emphasise the need for a more detailed qualitative and quantitative analysis of the progenitor compartment in the thymus.

Introduction

The differentiation of T cells in the thymus is a carefully orchestrated process. Thymocytes expressing neither CD4 nor CD8, so-called double negative (DN) cells, represent ~5% of cells in the normal adult thymus and contain thymic progenitors (1–3). Using a combination of CD44 and CD25, DN cells can be subdivided into a developmental sequence from CD44+/CD25− (DN1), via CD44+CD25− (DN2), CD25+CD44− (DN3) to CD44−CD25− (DN4) cells (4). However, mature and immature members of other hemopoietic lineages can also be found among subpopulations of DN thymocytes and it has been rather difficult to clearly distinguish these contaminating cells from those involved in the major pathway of T cell differentiation, a topic that has recently been reviewed (5).

Based on CD44 and CD25 expression alone, the number of DN1 and DN4 cells is frequently over-estimated and DN2 cells not clearly identified. Strategies where contaminating cells are ‘gated’ out, using cocktails of monoclonal antibodies (mAb) to so-called lineage-specific markers, assume that early T progenitor cells entering the thymus do not express such markers. Some markers used, such as B220 (6) and Gr-1 (7) are not lineage specific whereas others, such as NK1.1, appear only on mature lineage cells (8). It was already known that early adult thymic T progenitors expressed CD4 (9) and that CD4 was passively acquired (10), and recent evidence suggests that bone marrow-derived progenitors entering the thymus express B220 (11).

In order to positively identify early T cell progenitors, expression of CD117 (c-kit) was incorporated into the DN differentiation scheme (12). The level of CD117 expression varies enormously, but DN1 and DN2 cells can be distinguished as CD117 bright cells, whereas expression progressively decreases on DN3 and DN4 cells (5,13). By combining CD117, CD44 and CD25 expression, the number of DN1 cells can be more accurately determined and the transition from DN2 to DN3 cells, otherwise only distinguishable as the appearance of cytoplasmic CD3, expression in DN3 cells (14), defined. However, this still leaves the correct identification of DN4 cells problematic.

Mouse mutants, in which genes crucial for the differentiation of DN thymocytes have been inactivated, have proven
instrumental in defining the different stages of T cell progenitor differentiation. Mice in which either of the recombinase activating genes RAG-1 (15) or RAG-2 (16) has been inactivated show a complete block in thymocyte development at the DN3 stage. At the DN3 stage, TCRβ genes rearrange, and if the rearrangement codes for a functional protein, the latter pairs with the so-called pre-TCR alpha (pre-Tα) protein (17) and is expressed on the cell surface, in association with CD3 proteins, as a pre-TCR complex. Efficient progression beyond the DN3 stage is contingent upon TCRβ expression and signalling through the CD3 components of the pre-TCR complex (18,19), a process that has been called TCRβ selection (20). Signalling of the CD3 components of the pre-TCR complex delivers a signal that down-regulates CD25 expression on DN3 cells and promotes their development, via DN4, to cells expressing both CD4 and CD8, so-called double positive (DP) thymocytes. Mice in which the gene encoding pre-Tα has been inactivated (21) show a marked, but incomplete, block in development at the DN3 to DN4 transition.

We have used the combined expression of CD117, CD44 and CD25 to re-investigate the DN compartment of normal, RAG-2 KO and pre-Tα KO mice and describe five novel findings. Firstly, we have quantified the number of cells in each DN subpopulation, thereby demonstrating an accumulation of cells at the stage preceding the developmental block and the depletion of DN1 and DN2 cells in RAG KO mice. Secondly, by analysing a transgenic mouse strain (VT4 mice) possessing deletion of DN1 and DN2 cells in RAG KO mice. Secondly, by analysing a transgenic mouse strain (VT4 mice) possessing depletion of DN1 and DN2 cells in RAG KO mice. Secondly, by analysing a transgenic mouse strain (VT4 mice) possessing depletion of DN1 and DN2 cells in RAG KO mice. Secondly, by analysing a transgenic mouse strain (VT4 mice) possessing depletion of DN1 and DN2 cells in RAG KO mice. Finally, the combined CD117, CD44 and CD25 distribution pattern of BrdU-labelled DN cells provided a novel 'snapshot' of T cell receptor beta-selected cells transiting the DN3 to DN4 compartment.

**Methods**

**Mice**

Normal, C57Bl/6 female wild-type (WT) mice were obtained from IFFA CREDO (L’Arbresle, France) at 4 weeks of age, housed in the SPF animal facility at CEA-G and used 2–4 weeks later. C57Bl/6 RAG-2 gene-deleted mice (16) and C57Bl/6 transgenic mice containing the SV40 temperature-sensitive large T antigen (SV40Tts) transgene driven by control elements of the human vimentin promoter, so-called VT4 mice (22), were bred and maintained in the SPF animal facility at CEA-G. Pre-Tα gene-deleted mice on a C57Bl/6 background were originally obtained from Dr Hans Jorg Fehling (21) and bred on a homozygous background in a conventional animal facility at CEA-G. All mice were generally used at 6- to 8-weeks of age. Mice were administered BrdU by an initial intraperitoneal injection of 1 mg BrdU in 0.2 ml phosphate-buffered saline (PBS), followed by 1 mg/ml BrdU in the drinking water (24). Water bottles containing BrdU were protected from light and changed every 2 days. All animal experiments were carried out according to Institutional guidelines.

**Cell purification and staining**

Thymuses were removed and cell suspensions prepared in PBS, 1% fetal bovine serum (FBS) by gently pressing thymuses through a 70 µm pore size cell strainer (Becton-Dickinson Labware, B-D France, B, Pont de Claux, France). DN thymocytes from WT, pre-Tα KO and VT4 mice were obtained by a standard procedure (2,5) whereby thymocyte suspensions at 20 × 10⁶ cells/ml in PBS 1% FBS were incubated at 37°C in a 1:5 dilution of culture supernatants containing a mixture of rat IgM anti-mouse CD8 (clone 3.168.8.1) and anti-CD4 (clone RL172.4) antibodies followed after 10 min by addition of a 1:10 dilution of Low-Tox (Cedarlane Laboratories, Hornby, Canada) rabbit complement. After 45 min at 37°C and frequent gentle mixing with a Pasteur pipette, viable DN cells were recovered following centrifugation at 20°C over 1.077 g/ml density, mouse toxicity Lymphoprepᵀᴹ (AXIS-Shield PoC AS, Oslo, Norway); cell viability was generally >95%. The IgM anti-CD4 clone RL172 does not deplete CD4<sup>low</sup> DN1 and DN2 cells (data not shown).

For cell staining, 1 × 10⁶ cells in 50 µl staining buffer (PBS, 1% FBS, 0.01% NaN₃), so-called FACSwash, were incubated for 15 min at 4°C in the wells of round-bottom 96-well microtiter plates (B-D) containing saturating concentrations of labelled monoclonal antibodies (mAb) in 150 µl FACSwash (13). Labelled mAb were purchased from B-D Pharmingen and included CD3<sup>biotin</sup>, CD4<sup>PE</sup>, CD8<sup>FITC</sup>, CD69<sup>Chrome</sup>, CD25<sup>FITC</sup>, CD25<sup>APC</sup>, CD44<sup>Chrome</sup>, CD44<sup>APC</sup>, CD117<sup>PE</sup>, CD11c<sup>biotin</sup>, CD19<sup>biotin</sup>, CD117<sup>biotin</sup>, CD127<sup>biotin</sup>, B220<sup>biotin</sup>, DX5<sup>biotin</sup>, NK1.1<sup>biotin</sup> and TCRγδ<sup>biotin</sup>. Following washing in FACSwash, biotin-labelled antibodies were revealed with streptavidin-coupled Cyochrome (SA<sup>Chrome</sup>). For BrdU labelling experiments, cells surface-labelled with PE, Cyochrome and APC-labelled antibodies were fixed, permeabalized and stained with FITC-labelled anti-BrdU antibody using the BrdU Flow Kit (B-D Pharmingen) according to the manufacturer’s instructions.

Cell samples were analysed by three and four colour flow cytometry using a B-D FACSCalibur instrument. Cells were gated on a combination of FSC and SSC signals and list-mode data acquired on a minimum of 5 × 10⁵ events. In Figs 1–3, cytogram displays are from analysis carried out with age-matched mice, the same mixtures of mAb and instrument settings; instrument settings had to be varied with different batches of mAb. Data were analysed using either WinMDI 2.8 (Dr Joseph Trotter, Saik Institute) or FCS Express Version 2 (De Novo Software, Thornhill, Ontario, Canada) software. Two-dimensional displays are represented as either density plots or dot plots.

**Results**

**Double negative thymocytes of normal mice**

Double negative thymocytes from C57Bl/6 wild-type (WT) mice were stained for CD25<sup>FITC</sup>, CD44<sup>APC</sup>, CD117<sup>PE</sup> and additional biotin-labelled antibodies followed by SA<sup>Chrome</sup>. Gated total CD4<sup>+</sup>/CD25<sup>−</sup> DN1 cells (R1, Fig. 1A), which comprised 23.9% of all DN cells, were heterogeneous for many of the
markers tested but contained a distinct population of 2.75% (mean 2.6 ± 1.2%) CD117 ++ cells (Fig. 1B). Of particular note is the heterogeneous expression of B220 on thymic CD19 + B cells, which as previously shown (13) ranges from bright to negative. The CD117 versus CD44 cytogram display (Fig. 1C, where 7 × 10^4 events are represented) showed two major populations. Firstly, a heterogeneous population (R2) with increasing CD117 and CD44 expression and a second population, outside R2, of CD44 heterogeneous, CD117- negative cells (Fig. 1C, lower right panel). Within R2, 2.6 ± 1.2% (mean ± S.D) cells were CD117 ++. (D and E) The CD44 versus CD25 density plots of gated R3 and R2 cells, respectively. Around the displays are the names of the corresponding subpopulations and the percentage of cells in each quadrant is framed. (F) NK1.1 histograms of gated R2 (lower) and non-R2 (upper) cells as well as the percentage of positive cells.

**Fig. 1.** Double negative thymocytes from C57Bl/6 wild-type (WT) mice. Cells were four colour stained with CD25 FITC, CD44 APC, CD117 PE and NK1.1 biotin followed by Sacy. (A) CD44 versus CD25 density plot of 5 × 10^5 cells gated on a combination of FSC versus SSC signals; R1 is placed around all CD44+/CD25 - cells, representing 23.9% of cells. (B) Staining histograms with the indicated markers of gated R1 cells; in this instance, 2.75% of gated R1 cells were CD117 ++. (C) CD117 versus CD44 density plot of all cells. As described in the text, R2 is placed around cells showing a co-ordinated increase in CD117 and CD44 expression whereas cells outside R2 show increasing CD44 but remain negative for CD117. Within R2, 2.6 ± 1.2% (mean ± S.D) cells were CD117 ++. (D and E) The CD44 versus CD25 density plots of gated R3 and R2 cells, respectively. Around the displays are the names of the corresponding subpopulations and the percentage of cells in each quadrant is framed. (F) NK1.1 histograms of gated R2 (lower) and non-R2 (upper) cells as well as the percentage of positive cells.

levels of CD25 are found between DN3 and DN4 cells and called post-DN3 cells. As shown below, correct definition of DN4 cells is still problematic by this form of three colour analysis. However, our strategy is to ensure that CD117 staining intensity is maximized to ensure that any overlap between CD117 bright and CD44 dim DN4 cells is minimized. NK1.1+ cells, including CD3 +, CD127 + NKT and CD3 - NK cells were only found outside R2 (Fig. 1F). Consideration of cell numbers showed that there were 2 ± 0.4 × 10^4 DN1, 1 ± 0.2 × 10^4 pre-DN2, 8 ± 0.9 × 10^4 DN2, 121 ± 7 × 10^4 DN3, 30 ± 4 × 10^4 post-DN3 and 73 ± 6 × 10^4 total DN4 cells in the thymus of WT mice (Table 1).

**RAG-2 KO mice**

The utility of the above staining strategy is exemplified by the analysis of RAG-2 KO mice (Fig. 2) analysed in parallel with age-matched WT (Fig. 2) and pre-Tα KO mice (Fig. 3). Here,
cells corresponding to those in R2 and R3 of Fig. 1 can be clearly identified (Fig. 2C), but the proportion of CD117++ (R3) cells was reduced to 0.27% of R2. In (C), only $7 \times 10^4$ of the $5 \times 10^5$ total events are shown. Fig. 2(B) shows the phenotypic heterogeneity of the 6.4% total CD44+/CD25+/C255 cells; the major cell type being NK cells (28) expressing CD11c and the IL-7R (CD127). Note also that there are few CD117++ cells among gated CD44+/CD25+/C255 cells in RAG KO mice. Gated R3 cells comprised 18% DN1 and 82% DN2 cells with again some pre-DN2 cells (Fig. 2D). Gating for R2 (Fig. 2E), there were 0.04% DN1, 0.23% DN2; almost all (99.7%) cells were at the DN3 stage. Note that without gating for CD117 expression, the number of RAG-2 KO DN1 cells is vastly over-estimated (Fig. 2A). Importantly, CD44+/CD25− DN4 cells were not seen, confirming the complete block in thymocyte development at the DN3/4 transition in RAG-2 KO mice. Note also, as reported by others, the slight increase in CD25 staining intensity on DN3 cells of RAG-2 KO mice (15,29). In a series of three experiments, the mean fluorescence intensity (MFI) of CD25 expression by WT DN3 cells was 275 ± 26 whereas that of RAG-2 KO mice was 862 ± 11, a 3.1-fold increase in CD25 expression. Analysis of the cell numbers in the RAG-2 KO thymus (Table 1) showed that firstly, the absolute number of DN1 to DN2 cells was reduced by ~20-fold and secondly, there was an increase of ~2-fold in DN3 cells.

\[\text{Pre-Tα KO mice}\]

Because of their failure to express the pre-Tα protein, pre-Tα KO mice provide a model system for the analysis of a partial block in DN thymocyte development (19,21,30,31). Again, DN cells from pre-Tα KO mice show the presence of R2 and R3 cells and the 0.68% gated CD117++ R3 cells (Fig. 3B, showing $7 \times 10^4$ events) contain 24% DN1 and 76% DN2; pre-DN2 cells were clearly seen. When gated for R2, 0.4% of cells were DN1, 1.0% DN2, 86.5% DN3 and 12.1% DN4 (Fig. 3C). Therefore, the proportion of DN3 cells is increased in pre-Tα KO mice. Note that R2 cells do not contain NK1.1+ cells (Fig. 1D). The number of DN1, pre-DN2 and DN2 cells is similar to WT mice (Table 1) but the DN3 compartment is increased.
almost 5-fold; in contrast, the post-DN3 compartment is reduced ~2-fold (32,33). As reported by others (29) there is an increase in CD25 expression on DN3 cells in pre-Tα KO mice with CD25 MFI reaching 685 ± 632 versus 275 ± 26 in controls, a 2.5-fold increase.

Thymuses with an increased number of progenitors
Young adult mice expressing the SV40Tt/s trangene driven by the human vimentin promoter, so-called VT4 mice (22), have a thymus that can contain $4 \times 10^9$ cells, twenty times that of WT mice. The enlarged thymus of 4- to 6-week-old VT4 mice contained the normal cellular subsets defined by CD4 and CD8 (Fig. 4A), 74.6% DP, 10% CD4SP and 6% CD8SP and a slight increase in the proportion of DN cells to 9.4%; the CD3 distribution on these subsets was normal (not shown). Purified DN cells contained 75.7% R2 cells, with an elevated (92.2%, mean 87.4%) proportion of DN2 amongst the mean 2.5% gated R3 cells (Fig. 4C). R2-gated cells were 0.4% DN1, 5.5% DN2, 8.4% DN3, 14% DN4 and 59% in controls.

**Fig. 3.** Analysis of pre-Tα KO thymocytes. (A–D) A similar analysis to that of WT (Fig. 1) and RAG KO mice shown in Fig. 2 (see the legend to Fig. 1 and the text for details).

**Table 1.** Subpopulations of DN cells

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Subpopulation</th>
<th>DN1</th>
<th>Pre-DN2</th>
<th>DN2</th>
<th>DN3</th>
<th>Post-DN3</th>
<th>DN4</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57Bl/6</td>
<td>%</td>
<td>1 ± 0.2</td>
<td>0.4 ± 0.07</td>
<td>3 ± 0.4</td>
<td>52 ± 2</td>
<td>13 ± 2</td>
<td>31 ± 3</td>
</tr>
<tr>
<td></td>
<td>#(×10⁶)</td>
<td>0.4 ± 0.04</td>
<td>1 ± 0.2</td>
<td>8 ± 0.9</td>
<td>121 ± 7</td>
<td>30 ± 4</td>
<td>73 ± 6</td>
</tr>
<tr>
<td>RAG-2 KO</td>
<td>%</td>
<td>0.04 ± 0.01</td>
<td>0.1 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>99 ± 0.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>#(×10⁶)</td>
<td>0.10 ± 0.03</td>
<td>0.2 ± 0.03</td>
<td>0.4 ± 0.2</td>
<td>266 ± 6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pre-Tα KO</td>
<td>%</td>
<td>0.43 ± 0.07</td>
<td>0.1 ± 0.03</td>
<td>1 ± 0.2</td>
<td>88 ± 0.4</td>
<td>2 ± 0.3</td>
<td>8 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>#(×10⁶)</td>
<td>3 ± 0.1</td>
<td>1 ± 0.2</td>
<td>1 ± 0.2</td>
<td>594 ± 15</td>
<td>14 ± 2</td>
<td>59 ± 4</td>
</tr>
</tbody>
</table>

Data represent the mean ± S.D. of six experiments.
DN2, 76.5% DN3 and 17.2% DN4 cells (Fig. 4D). In numerical terms, there were 115-fold more total DN thymocytes containing 55-fold more DN1, 110-fold pre-DN2, 176-fold DN2, 133-fold DN3, 159-fold post-DN3 and 63-fold DN4 cells. Crossing the VT4 transgene onto the CD3ε KO genetic background resulted in an enlarged thymus, containing $2.5 	imes 10^6$ cells with a block in differentiation at the DN3 stage which contained 20, 60 and 40-fold more DN1, DN2 and DN3 cells, respectively, than CD3ε KO mice (not shown). Therefore, under some circumstances, the space available for progenitor thymocytes can increase considerably and in VT4 CD3ε KO mice is independent of the presence of mature T cells.

Heterogeneity of DN4 cells and quantification of the DN3 to DN4 block

As outlined above, three colour analysis alone did not allow a correct identification of DN4 cells. Therefore, four colour analysis of DN cells from WT and pre-Tα KO mice was carried out including CD3ε and TCRγδ as additional markers. DN cells were stained with CD25, CD44 and CD117, and DN3 and DN4 cells analysed alone by excluding R3 (DN1+DN2 cells) from R2 as shown in Figs 1–4. Importantly, by deliberately not gating out CD3ε+ cells, some novel features of DN subpopulations in pre-Tα KO mice were revealed. Results of a typical experiment, one of six carried out, is shown in Fig. 5, and Table 2 summarizes these experiments.

In C57Bl/6 mice (Fig. 5A), cells that have successfully completed TCRβ selection, and in transit from the DN3 to DN4 compartment via so-called post-DN3 cells, do not yet express sufficient CD3 molecules to be detected by conventional one-step immunofluorescence staining (34) and are seen in the left region of the cytogram displays as CD3ε/C255 cells. In a series of six experiments, $98.2\pm0.2\%$ DN3, $94.9\pm0.6$ post-DN3 and $62.5\pm1.2\%$ DN4 cells were CD3ε/C255 (left histograms). Thus, CD3ε cells constitute the major differentiation pathway from DN3 to DN4 cells in WT mice. Cells that share the CD44 and CD25 staining profile of DN3 and DN4 cells but that nevertheless express CD3 or TCRγδ are called for convenience CD3+ or TCRγδ+ ‘DN3’, ‘post-DN3’ and ‘DN4’ cells, respectively. Importantly, both CD3ε+DN4 (Fig. 5, lower FSC histograms) and post-DN3 cells (not shown) are relatively larger in size, as measured by FSC, and both are larger than either CD3low or CD3bright ‘DN4’ cells; TCRγδ+ ‘DN4’ cells have an intermediate cell size. Staining for TCRγδ cells indicated that $0.4\pm0.1\%$ ‘DN3’, $1.5\pm0.3\%$ ‘post-DN3’ and $8.6\pm1.4$ ‘DN4’ cells were TCRγδ+; only 23% (8.6/37.5; Fig. 5A) of CD3ε+ ‘DN4’ cells were TCRγδ+ and as shown (Fig. 1F) they were NK1.1+, thus they do not represent NK1.1+ TCRγδ cells.

**Fig. 4.** Phenotypic analysis of the enlarged thymus of VT4 mice. (A) CD4 versus CD8 density plot of all thymocytes; (B–D) are of purified DN cells. (B) Density plot of CD117 versus CD44 expression with R2 and R3 as described in the legend to Fig. 1. (C and D) CD25 versus CD44 density plots of gated R3 and R2 cells, respectively; the percentage of positive cells in each quadrant is framed.
Fig. 5. CD3 and TCRγδ expression by DN3 and DN4 cells. (A) C57Bl/6 and (B) pre-Tα KO mice. DN thymocytes were stained with CD25FITC, CD44APC, CD117PE and either CD3biotin or TCRγδbiotin followed by SAl. From CD117 versus CD44 cytogram displays (Fig. 1C and Fig. 3A), R2 cells were gated and R3 cells removed, thus leaving CD25+ DN3 and CD25− DN4 cells. The upper panels show the CD3 (left) and TCRγδ (right) density plots of gated DN3 and DN4 cells. The dotted box shows post-DN3 cells and the percentage of positive cells is shown in three of the four quadrants. Beneath each density plot are three histogram displays of CD3 (left) or TCRγδ (right) expression of gated DN3 (upper), post-DN3 (middle) or DN4 (lower) cells. The mean ± S.D% positive cells is shown in each histogram. At the bottom are histograms of forward scatter (FSC) signals from gated CD3+ and CD3− (left) or TCRγδ+ and TCRγδ− (right) cells.

Table 2. The DN3 to DN4 block in pre-Tα KO mice

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>DN3</th>
<th>Post-DN3</th>
<th>DN4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD3−</td>
<td>CD3+</td>
<td>TCRγδ</td>
</tr>
<tr>
<td>B/6</td>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>%</td>
<td>98.2</td>
<td>1.8</td>
<td>0.4</td>
</tr>
<tr>
<td>#</td>
<td>118</td>
<td>3</td>
<td>0.5</td>
</tr>
<tr>
<td>pre-Tα KO</td>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>%</td>
<td>98.5</td>
<td>1.5</td>
<td>0.6</td>
</tr>
<tr>
<td>#</td>
<td>586</td>
<td>9</td>
<td>3.6</td>
</tr>
<tr>
<td>Ratio</td>
<td>5</td>
<td>3</td>
<td>7</td>
</tr>
</tbody>
</table>

aData mean percent and cell number (×10⁴) based on a series of six experiments.

bRatio pre-Tα KO/B/6.
New insights into early thymocyte development

In pre-\(\text{T}_{\alpha}\) KO mice (Fig. 5B), like WT controls, DN3 cells were 98.5% CD3\(^{-}\). However, post-DN3 and DN4 cells were very different in that only 57 ± 3% of post-DN3 and 15.2 ± 0.6% DN4 cells were CD3\(^{+}\); the CD3 staining profile of post-DN3 and DN4 cells was triphasic. Thus, in pre-\(\text{T}_{\alpha}\) KO mice, despite the 5-fold increase in the number of CD3\(^{-}\) DN3 cells, there was a 3-fold decrease in CD3\(^{-}\) post-DN3 and 5-fold decrease in CD3\(^{-}\) DN4 cells (Table 2). Considering CD3\(^{-}\) cell numbers, the ratio of DN3 to post-DN3 was 4.1 (118/28.5) in WT mice, but 73 (585/8) in pre-\(\text{T}_{\alpha}\) KO mice, a 17-fold difference. For DN3 to DN4 cells, these values become 2.6, 65 and 25, respectively, a clear manifestation of the partial block in thymocyte development in pre-\(\text{T}_{\alpha}\) KO mice. Cell size analysis showed that unlike WT controls (Fig. 5A), post-DN3 (not shown) and DN4 cells (Fig. 5B, lower histograms) remained relatively small, presumably indicating the absence of a proliferative signal given to the few cells undergoing the DN3 to DN4 transition.

Strikingly, 12.8 ± 0.9% ‘post-DN3’ cells were TCR\(\gamma\delta\)^+ cells, 8-fold more than in controls. However, given that DN3 numbers were increased 5-fold, the number of TCR\(\gamma\delta\)^+ ‘post-DN3’ cells was not significantly increased (Table 2). Because fewer DN3 cells differentiate to CD3\(^{-}\) DN4 cells in pre-\(\text{T}_{\alpha}\) KO mice, the alternative TCR\(\gamma\delta\) lineage pathway, known to be relatively increased and apparently originating in the DN3 compartment, can be clearly visualized. It has been reported that the proportion of NK1.1\(^{+}\) TCR\(\gamma\delta\) cells (so-called NKT cells) was increased in pre-\(\text{T}_{\alpha}\) KO mice (35). However, all R2 (Fig. 2D) and consequently all TCR\(\gamma\delta\)^+ ‘DN4’ cells, were NK1.1\(^{-}\) (Fig. 3) and HSA\(^{-}\) (not shown), indicating that they probably represent immature, conventional, TCR\(\gamma\delta\) cells.

BrdU labelling experiments

We wished to investigate the distribution of proliferating cells amongst the different DN subsets described above. To this end, mice were given BrdU and at various times, DN thymocytes stained for CD25\(\text{APC}\), CD44\(\text{Cy}\) and CD117\(\text{PE}\), fixed and permeabilized and their BrdU content determined with an anti-BrdU\(\text{FITC}\) antibody. Although FSC signals and immunofluorescent intensities were slightly modified by the fixation procedure, all subpopulations of DN cells shown in Figs 1–3 could be clearly identified. Figure 6(A) shows the results of a typical experiment where DN cells from WT mice were stained following a 4 h pulse with BrdU. After a 4 h pulse, 20% of total DN thymocytes were BrdU\(^{+}\) (upper histogram). Gated profiles show that almost all BrdU\(^{+}\) cells were contained within the R2 regions defined by CD117 and CD44 (not shown). The corresponding CD25 versus CD44 cytoflograms of all (upper cytoflograms) and R2-gated subpopulations (bottom cytoflograms) showed that BrdU\(^{+}\) cells were mostly found within the DN3 and DN4 compartments but importantly, some DN1 and DN2 cells are labelled at this time. Importantly, the CD25 versus CD44 distribution pattern of BrdU\(^{+}\) versus BrdU\(^{-}\) DN3 cells are different; BrdU\(^{+}\) DN3 cells appear to exclude a subpopulation in the upper left (CD25\(^{+}\) CD44\(^{-}\)) quadrant (see also below).

Figure 6(B) summarizes a series of experiments where groups of three WT, RAG-2 KO and pre-\(\text{T}_{\alpha}\) KO mice were BrdU labelled and analysed individually as shown in Fig. 6(A). For DN subpopulations from WT mice (hatched bars), there were clear differences in the proportion of labelled cells (upper panel). In percentage terms, post-DN3 cells contained the most BrdU\(^{+}\) cells, followed in decreasing order by DN4, DN2, DN1, DN3 and pre-DN2 cells. Importantly, unlike published results, CD117\(^{+}\) DN1 cells were indeed rapidly labelled with BrdU. The numbers of labelled DN3, post-DN3 and DN4 cells were about equal, but 10-fold greater than DN2 and ~100-fold that of DN1, pre-DN2 and DN2 cells (lower panels).

The same rank order of percent BrdU incorporation was not seen in RAG-2 KO mice (speckled bars, Fig. 6B). Thus, the proportion of BrdU\(^{+}\) DN1, pre-DN2 and DN2 cells was significantly increased; however, their number was nevertheless 10-fold lower than in WT mice. Thus, the few DN1 and DN2 cells in the RAG-2 KO thymus are actually enriched for proliferating cells. For DN3 cells, although the proportion of labelled cells was ~3-fold lower, because there were twice as many cells, their number was only slightly reduced. These results with RAG-2 KO mice confirm that most proliferation in the DN1 to DN3 compartments is independent of both TCR rearrangement and expression (36,37) and is presumably due to cytokine-mediated signals (38). Expression of CD117 (c-kit) (Figs 1–3) and CD127 ([32] and data not shown) by these cells suggest that c-kit ligand (stem cell factor) and IL-7 together support their proliferation.

For pre-\(\text{T}_{\alpha}\) KO mice, the labelling of DN1, pre-DN2 and DN2 cells was similar to, or slightly above that, of WT controls but for DN3, post-DN3 and DN4 cells, it was reduced by a half. When converted to cell numbers, the number of labelled cells was increased ~2-fold for DN3, but decreased ~3-fold for both post-DN3 and DN4 cells. Because the number of CD3\(^{-}\) post-DN3 and DN4 cells in pre-\(\text{T}_{\alpha}\) KO mice is 3- to 5-fold less than controls (Table 2), the number of BrdU-labelled CD3\(^{-}\) cells is proportionately much less.

Longer-term labelling experiments in WT and pre-\(\text{T}_{\alpha}\) KO mice (Fig. 7) are summarized in Table 3. Thus, at 48 h, 86% of WT (Fig. 7A) but only 64% of pre-\(\text{T}_{\alpha}\) KO (Fig. 7B) cells were BrdU\(^{+}\). The CD25 versus CD44 cytoflogram displays (middle panels) again showed a difference in distribution pattern between BrdU\(^{+}\) and BrdU\(^{-}\) cells. Some (~5%) DN3 cells in the upper left quadrant of the CD25 versus CD44 cytoflogram remained BrdU\(^{-}\) after 5 days of continuous labelling (not shown). The proportion of BrdU\(^{+}\) DN4 cells was decreased in pre-\(\text{T}_{\alpha}\) KO mice (Fig. 7B). Cell size analysis showed more blasts in WT mice and that gated blasts shared a similar CD25/CD44 distribution pattern to BrdU\(^{+}\) cells. There were far fewer blasts in pre-\(\text{T}_{\alpha}\) KO mice, but their CD25/CD44 distribution pattern was again similar to BrdU\(^{+}\) cells. Table 3 shows that in pre-\(\text{T}_{\alpha}\) KO mice, more DN3 but fewer DN4 cells became BrdU\(^{+}\) (21,37).

Discussion

In this report, we have used four colour flow cytometry, including measurement of BrdU incorporation, to analyse the subpopulation distribution and proliferation kinetics of DN thymocytes from mice with either a complete (RAG-2 KO) or partial (pre-\(\text{T}_{\alpha}\)-KO) block in T cell development. By this approach, the partial block in pre-\(\text{T}_{\alpha}\) KO mice can be quantified and some novel aspects of thymocyte development revealed for the first time.
The need for a more careful look at DN thymocytes has recently been reviewed (5). By gating on CD117++ cells, CD25/C255 DN1 and CD25+ DN2 cells can be more accurately identified, their numbers quantified and the transition from DN2 to DN3 cells pinpointed. Importantly, this analysis has permitted a better quantification of the different compartments of DN cells. The block in thymocyte development is complete at the DN3 stage in RAG-2 KO mice and without gating for CD117 expression, the number of DN1 cells is generally overestimated; as previously shown, most CD44+/CD25/C255 cells in RAG KO mice are mature NK cells (Fig. 2) (28). The absence

The need for a more careful look at DN thymocytes has recently been reviewed (5). By gating on CD117++ cells, CD25− DN1 and CD25+ DN2 cells can be more accurately identified, their numbers quantified and the transition from DN2 to DN3 cells pinpointed. Importantly, this analysis has permitted a better quantification of the different compartments of DN cells. The block in thymocyte development is complete at the DN3 stage in RAG-2 KO mice and without gating for CD117 expression, the number of DN1 cells is generally overestimated; as previously shown, most CD44+/CD25− cells in RAG KO mice are mature NK cells (Fig. 2) (28). The absence of mature T cells in RAG-2 KO mice and the consequent lack of development of thymic stromal (39,40) and endothelial elements (41) may explain why there are so few DN1 and DN2 cells and why the increase of DN3 cells is only 2-fold in RAG-2 KO mice. As shown by others, CD25 expression by DN3 cells from RAG KO and pre-Ta KO mice is really higher than controls (15,29), presumably due to the absence of pre-TCR signalling.

The block in thymocyte differentiation in pre-Ta KO mice is incomplete at the DN3 to DN4 transition (19,21,42) and our results indicate that this block is first manifest at the DN3 to
post-DN3 transition. Our quantitative data show that there are 5-fold more DN3 cells and that very few cells transit to the CD3+CD255− post-DN3 and DN4 compartments. BrdU labelling experiments tend to confirm this scenario. By multiplex PCR analysis, there was no clear increased TCRα rearrangement in DN3 and DN4 cells (N. Bosco, unpublished observation) confirming that the DN3 to DN4 transition in pre-Tα KO mice is not entirely due to premature TCRα rearrangement and TCRαβ expression (14,19).

The pre-Tα KO thymus, unlike the RAG-2 KO thymus, contains mature T cells (21) that can induce maturation of stromal and endothelial elements. This may account for the similarity between WT and pre-Tα KO mice in the number of DN1 and DN2 cells and the accumulation of DN3 cells (Table 1). In this regard, the block in T cell development in the thymus of pre-Tα KO mice resembles that in the BM of the k5KO mouse (43,44) where, because of available space, there is a 4-fold increase in pro-/pre-BI cells. For reasons outlined above, expansion of DN3 cell numbers in RAG-2 KO mice is less than expected. Analysis of VT4 mice clearly shows that the space for progenitors has the potential to increase enormously.

Because the CD3+DN3 to DN4 block is severe in pre-Tα KO mice, a minor differentiation pathway, whereby cells transit directly from DN3 to CD3+ TCRγδ cells, is phenotypically revealed (Fig. 5). Molecular analysis has indicated firstly, that complete TCRγ and TCRδ rearrangements can be detected in DN2 cells (45), prior to Vβ to DJβ rearrangements at the DN3 stage and secondly, that some TCRγδ cells can develop without

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Table 3. BrdU labelling kinetics

<table>
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<tr>
<th>Mouse strain</th>
<th>BrdU labelling</th>
<th>4 h</th>
<th>48 h</th>
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<tr>
<td></td>
<td>DN3</td>
<td>DN4</td>
<td>DN3</td>
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<tr>
<td>C57Bl/6</td>
<td>14 ± 3</td>
<td>47 ± 7</td>
<td>79 ± 3</td>
</tr>
<tr>
<td>RAG-2 KO</td>
<td>5 ± 0.4</td>
<td>35 ± 5</td>
<td>96 ± 3</td>
</tr>
<tr>
<td># (×10^6)</td>
<td>14 ± 1</td>
<td>1</td>
<td>242 ± 11</td>
</tr>
<tr>
<td>pre-Tα KO</td>
<td>14 ± 2</td>
<td>16 ± 2</td>
<td>68 ± 1</td>
</tr>
<tr>
<td># (×10^6)</td>
<td>81 ± 1</td>
<td>9 ± 0.1</td>
<td>405 ± 5</td>
</tr>
</tbody>
</table>

Values represent the mean ± S.D. of the percent and cell number from a series of six experiments.

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Fig. 7. Forty-eight hour BrdU labelling of C57Bl/6 and pre-Tα KO mice. (A) Results from DN thymocytes of C57Bl/6 and (B) pre-Tα KO mice. Above is the BrdU histogram of all cells and below the CD25 versus CD44 density plots of all (left) BrdU− (middle) and BrdU+ gated R2 cells; R2 represents cells with increasing CD117 and CD44 expression, as shown in Figs 1C and 3A. Below on the left are histograms of FSC signals from gated DN3 (upper) and DN4 (lower) cells and each histogram panel shows the FSC of gated BrdU+ and BrdU− cells. In the upper DN3 cell histograms, larger cells are labelled as blasts. The CD25 versus CD44 fluorescence density plots of gated blasts are shown in the right bottom panel.
apparently undergoing TCRβ selection (26,46), reviewed in (23). The small number of TCRγδ cells emanating directly from the DN3 compartment, made more conspicuous in pre-Tα KO mice by the relative absence of developing CD3+ DN3 cells (Fig. 5), perhaps represents such cells. Indeed, the thymus of mice homozygous for a mutation of the three C-terminal tyrosine residues of the so-called linker for the activation of T cells (LAT) molecule was recently found to contain CD25+ TCRγδ cells (47). Importantly, despite a partial block in the major TCRβγ lineage, deletion of the pre-Tα gene does not in itself directly promote TCRγδ development. From published data (18,19,48), it would be expected that cells with a functional TCRγδ receptor, generated at the DN2 stage (32,45) and expressed in association with CD3ε at the DN3 stage, would be capable of down-regulating CD25 expression and differentiating to CD25-‘DN4’ cells in the absence of pre-Tα expression.

BrdU labelling experiments were used to quantify cell turnover among DN subsets. Several results are worthy of note. Firstly, CD117++ DN1 cells proliferated extensively and proliferating CD117++ DN1 cells were enriched in RAG-2 KO mice. Previous studies had concluded that CD117++ DN1 cells were not cycling (24). Recent molecular evidence confirms that DN1 cells cycle (49). Most proliferation of DN1 to DN3 cells is TCRβ-independent (36,50) and is presumably mediated by cytokine signals (38,51).

For pre-Tα KO mice, twice as many DN3 cells, but at least 3-fold fewer post-DN3 and DN4 cells became BrdU labelled at 48 h, showing that the number of newly generated DN4 cells is considerably reduced. Combined staining with annexin V and propidium iodide showed that the number of apoptotic cells was not increased among DN4 cells of pre-Tα KO mice (N. Bosco, unpublished). Because of contamination by CD25+ TCRδ+ cells that were apparently undergoing TCRδβ selection (26,46), reviewed in (23), the BrdU labelling experiments were used to quantify cell turnover among DN subsets. Several results are worthy of note. Firstly, CD117++ DN1 cells proliferated extensively and proliferating CD117++ DN1 cells were enriched in RAG-2 KO mice. Previous studies had concluded that CD117++ DN1 cells were not cycling (24). Recent molecular evidence confirms that DN1 cells cycle (49). Most proliferation of DN1 to DN3 cells is TCRβ-independent (36,50) and is presumably mediated by cytokine signals (38,51).

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What was strikingly revealed by BrdU labelling experiments was the difference in CD25 versus CD44 staining profiles of BrdU+ and BrdU− DN3 cells (Fig. 7). Molecular analysis had indicated that small, non-cycling, DN3 cells contained random TCRβ rearrangements and retained RAG-2 expression, whereas large cycling DN3 cells contained predominantly in-frame TCRβ rearrangements and had down-regulated RAG-2 (25). Based on this, small DN3 cells were described as having undergone TCRβ selection whereas small RAG-2-containing DN3 cells were still capable of further TCRβ rearrangement. Because the CD25 versus CD44 staining profiles of gated blasts and BrdU+ cells were quite similar (Figs 5 and 6), it is tempting to speculate that what this distribution reflects is the profile of TCRβ-selected cells that rapidly differentiate through the DN3 into the DN4 compartment. Indeed, the staining profile of gated BrdU+ cells is reminiscent of that seen in TCRβ transgenic RAG-2 KO mice (52) where, because of premature TCRβ expression, cells down-regulate CD25 and rapidly transit the depleted DN3 compartment. In corollary, there is a corresponding co-localization of BrdU+ and small cells in the upper left quadrant of the CD25 versus CD44 cytograms, representing cells incapable of transiting the DN3 compartment because of either failed or incorrectly rearranged TCRβ genes. As revealed for the first time using this four colour analysis, the CD25 versus CD44 distribution pattern of BrdU+ cells may represent a ‘snapshot’ of TCRβ-selected cells transiting the DN3 to DN4 compartment in normal mice.

Acknowledgements

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Abbreviations

- BrdU: 5-bromo-2′-deoxyuridine
- DN: double negative (CD4/CD8-negative)
- DP: double positive (CD4/CD8-positive)
- MFI: mean fluorescence intensity
- pre-Tα: pre-Tα cell receptor alpha
- RAG-2: recombinase-activating gene 2
- SP: single positive (CD4 or CD8-positive)

References

New insights into early thymocyte development


