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Unexpectedly late expression of intracellular CD3ε and TCR γδ proteins during adult thymus development

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Abstract

During adult thymus development immature $CD4^-CD8^-$ [double-negative (DN)] precursor cells pass through four phenotypically distinct stages defined by expression of CD44 and CD25: $CD44^{hi}CD25^-$ (DN1), $CD44^{hi}CD25^+$ (DN2), $CD44^{lo}CD25^+$ (DN3) and $CD44^{lo}CD25^-$ (DN4). Although it is well established that the TCR β, γ and δ genes are rearranged and expressed in association with the CD3 components in DN thymocytes, the precise timing of expression of the TCR and CD3 proteins has not been determined. In this report we have utilized a sensitive intracellular (ic) staining technique to analyze the expression of ic CD3ε, TCR β and TCR γδ proteins in immature DN subsets. As expected from previous studies of TCR β rearrangement and mRNA expression, icTCR β^+ cells were first detected in the DN3 subset and their proportion increased thereafter. Surprisingly, however, both icCD3ε^+ and icTCR $\gamma\delta^+$ cells were detected at later stages of development than was predicted by molecular studies. In particular icCD3ε protein expression coincided with the transition from the DN2 to DN3 stage of development, whereas icTCR γδ protein expression was only detected in a minor subset of DN4 cells. The implications of these findings for αβ lineage divergence will be discussed.

Introduction

The two major lineages of T cells express mutually exclusive heterodimeric TCR (αβ or γδ) associated with the CD3 signaling complex. αβ and γδ T cells have been shown to arise from the same early thymic precursor population which has a surface phenotype $CD4^{lo}$, $CD90^{lo}$, $CD24^{int}$, $CD44^{hi}$, $CD117^+$ and class I^{hi} (reviewed in 1). During subsequent thymus development, these precursors differentiate along a specific pathway, in which four $CD4^-CD8^-$ [double-negative (DN)] subsets expressing high levels of CD24 can be defined by the differential expression of CD44, CD25 and CD117 as follows: $CD44^{hi}CD25^-CD117^+$ (DN1), $CD44^{hi}CD25^+CD117^+$ (DN2), $CD44^{lo}CD25^+CD117^-$ (DN3) and $CD44^{lo}CD25^-$ (DN4) (reviewed in 2–4). During this time, the TCR γ, δ and β genes are rearranged and expressed according to a precise developmental program (5–10). At the end of the DN stages, αβ precursors rapidly transit through a $CD4^-CD8^+CD3^{lo}$ immature single-positive (ISP) stage to the $CD4^+CD8^+$ [double-positive (DP)] stage where TCRα is rearranged and expressed and mature single-positive (SP) cells are produced. In contrast, although mature γδ T cells are also produced from the same $CD4^{lo}$ precursor population, little is known about the precise developmental pathway they follow (reviewed in 4). However, most of the DN populations have been shown either *in vivo* (1,5,11) or *in vitro* (11–13) to produce mature cells of both lineages.

One of the major problems that has hampered analysis of the early stages of αβ and γδ T cell development is the fact that readily detectable levels of TCR are only observed on relatively mature T cells. In the αβ lineage surface TCR expression can be reliably assessed by flow microfluorometry only on DP and SP subsets despite the fact that a pre-TCR complex (composed of a productively rearranged TCR β chain, an invariant pTα chain and CD3) is already present on the surface of DN3 cells (reviewed in 14). The situation in the γδ T cell lineage is even less clear, since only mature γδ cells express detectable levels of TCR on the cell surface, thus precluding any identification of putative γδ precursors.

To circumvent the lack of detectable surface TCR expres-

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sion on immature thymic precursor cells, many groups have utilized molecular methods to follow rearrangement and/or expression of TCR β, γ and δ genes (reviewed in 15,16) as well as CD3 genes (17) in defined subsets. Although these techniques have provided much useful information, they have two major drawbacks in that (i) they are generally not applicable to single cells and (ii) they do not measure the levels of the corresponding protein.

In this report we have attempted to overcome these technical problems by undertaking a detailed four-color flow cytofluorometric analysis of the expression of intracellular (ic) CD3ε, TCR β and TCR γδ proteins at defined stages of adult thymus development. Although similar techniques have been utilized previously in the context of fetal thymus development (18–20), the conclusions have been somewhat limited due to the general use of only two- (or occasionally three-) color analysis and to the fact that immature subsets are phenotypically less well defined in the fetal (as opposed to adult) thymus. Our data confirm and extend previous molecular analyses of the timing (and consequences) of icTCR β expression during adult thymus development. Moreover, they provide novel information concerning the timing of both icCD3ε and icTCR γδ protein expression.

Methods

Mice and cell suspensions

Six-week-old C57BL/6 female mice were obtained from Harlan Olac (Bicester, UK). TCR $\beta^{-/-}$ and TCR $\delta^{-/-}$ mice were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained in our animal facility. DN and DN CD25$^-$ thymocytes were prepared as described previously (7,21). Briefly, thymocyte cell suspensions were incubated at 4°C in DMEM/1% FCS containing the appropriate concentrations of IgM isotype antibodies against CD4 (RL172.4), CD8 (31M) and CD25 (7D4) for 20 m. Rabbit complement (Saxon Europe, Suffolk, UK) and DNase I (Boehringer Mannheim, Mannheim, Germany), were added, and the incubation continued at 37°C for 45 min. After washing, viable cells were recovered after density-gradient centrifugation through Lympholyte M (Cedarlane, Hornby, Ontario, Canada). During subsequent FACS analysis and/or sorting, contaminating CD4$^+$, CD8$^+$, CD25$^+$ (where required) and any CD3$^+$ (αβ$^+$ or γδ$^+$) cells were positively eliminated where appropriate by staining with a cocktail of FITC-conjugated mAb to these surface markers and 'live' gating out all FITC$^+$ cells.

Flow cytometry and cell sorting

Thymocyte subsets were analyzed for simultaneous detection of both surface and intracellular proteins as described previously (22), using FACScan (three color) or FACSCalibur (four color) flow cytometers and sorted with a FACStar Plus (Becton Dickinson, San Jose, CA). Briefly, surface staining was performed as usual for one, two or three colours followed by fixation in 2% paraformaldehyde. After permeabilization with 0.5% Saponin, intracellular proteins were detected with mAb directly conjugated to phycoerythrin (PE) and/or CyChrome. Specifically, surface staining was performed using a pool of FITC direct conjugates as described above, together with either direct TriColor or CyChrome conjugates, such as TCR γδ–TriColor, CD4–TriColor and CD8α–TriColor (Caltag, Burlingame, CA), CD44–CyChrome (PharMingen, San Diego, CA), or biotinylated-CD25 revealed with TriColor–streptavidin, purchased from Caltag in FL3. For four-color staining, Cy5–CD25, Cy5–CD24 and CD117–allophycocyanin (PharMingen) were used in FL4. All FITC, biotin and Cy5 conjugates were purified and conjugated from hybridomas grown in this laboratory, and have been described elsewhere (7). PE- and/or CyChrome-conjugates of mAb to TCR β (H57-597), TCR γδ (GL3), CD3ε (17A2 and 145-2C11), and control rat and hamster Ig (PharMingen) were used for intracellular staining. PE conjugates of mAb to TCR V_γ1.1 [clone 11.2 (23)], TCR V_γ2 (UC3-10A6) and TCR V_δ4 (GL2) were prepared in this laboratory using the Phycolink PE-conjugation kit (Prozyme, San Leandro, CA). Surface TCR β, TCR γδ, TCR V_γ1.1, TCR V_γ2, TCR V_δ4 and CD3ε were pre-blocked with unlabeled purified mAb from the same clones as used for intracellular staining. Analysis was performed using Lysys II or CellQuest software.

Cell culture

Purified and FACS-sorted CD44^{lo}CD25$^-$CD3$^-$ DN thymocytes were cultured in DMEM supplemented with HEPES buffer, Glutamine, β_2-mercaptoethanol and 5% FCS for 4 h at 37°C. After harvesting and counting the cells were stained and analyzed by four-color FACS either for surface expression of CD4, CD8, TCR β and TCR γδ or for intracellular expression of TCR β and TCR γδ after gating out any mature cells (CD4, CD8, CD3ε, TCR β or TCR γδ) by surface staining with the FITC cocktail described above.

Cell cycle analysis

The DNA content of thymus subsets was determined by first sorting the selected populations defined by both surface and intracellular protein expression as described above. Propidium iodide (PI) staining of DNA was performed using standard procedures as described previously (22,24). Briefly, sorted cells were treated with 3N HCl to remove surface markers, washed in PBS and neutralized with 0.1 M $Na_2B_4O_7$. RNA was removed by incubation in RNase A at 37°C before the addition of PI. Analysis was performed on the FACScan flow cytometer using the doublet discrimination module.

Results

Intracellular CD3ε protein is first detected in CD25$^+$CD44^{lo} CD117^{lo} thymocytes

In order to establish the timing of CD3ε protein expression during adult thymus development, DN thymocytes were purified by depletion and surface stained in three colors with a cocktail consisting of mAb to CD4, CD8 and CD3 (to remove residual contaminating mature T cells) together with CD25 and either CD44, CD24 or CD117. The fourth color was used for icCD3ε. Surprisingly the CD25$^+$ DN population contained a well-defined subset of icCD3ε$^-$ cells (Fig. 1A). The proportion of icCD3ε$^-$ cells in the CD25$^+$ DN subset was highly reproducible (6 ± 1 %, N = 20) and did not depend upon the mAb used to detect CD3ε, since all four mAb used (145-2C11,

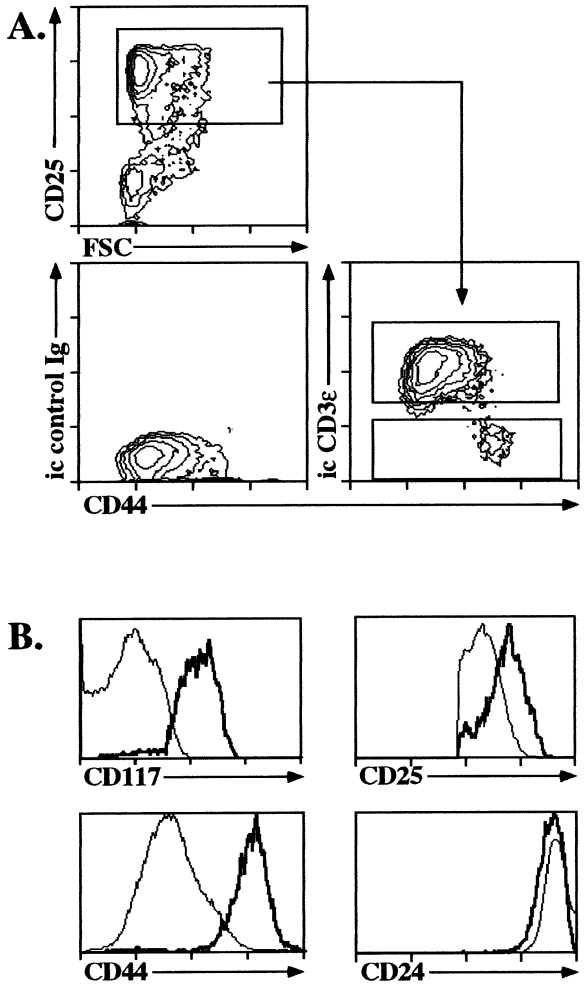

Fig. 1. Intracellular CD3ε expression marks the DN2 to DN3 transition in adult thymocytes. (A) Expression of icCD3ε and surface CD44 on total $CD25^+$ DN thymocytes. (B) Surface expression of CD117, CD44, CD24 and CD25 gated on $CD25^+icCD3\varepsilon^-$ (solid line) and $CD25^+icCD3\varepsilon^+$ (fine line) DN thymocytes.

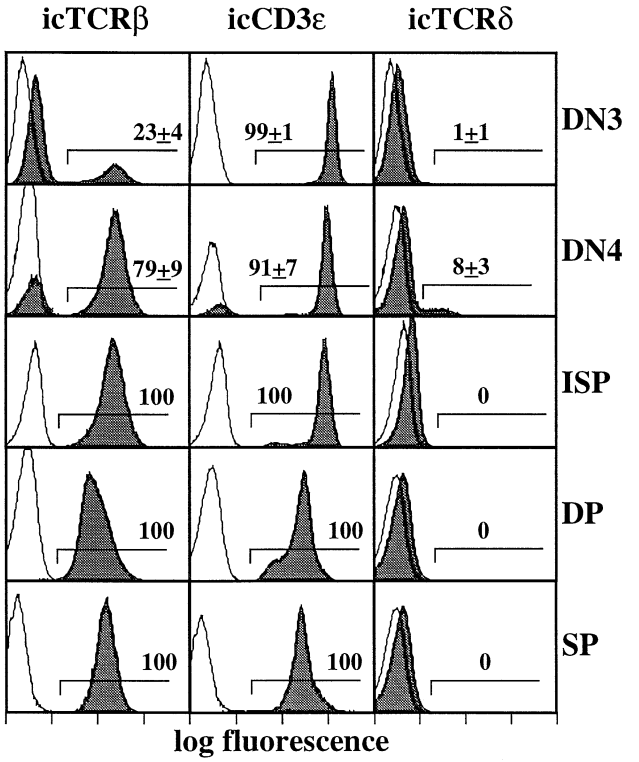

Fig. 2. Intracellular TCR β, TCR γδ and CD3ε expression in immature and mature thymus subsets. Purified DN cells were surface stained with a pool of mAb to remove $CD44^+$ early precursors and any $CD3^+$, TCR β^+ or TCR $\gamma\delta^+$ contaminants, together with CD25 to distinguish between DN3 and DN4 subsets. ISP, DP and mature $CD4^+CD8^-$ SP thymocyte subsets were directly gated from total thymus. Intracellular staining for TCR β, CD3ε and TCR γδ is shown on each gated subset. Surface TCR β, TCR γδ or CD3ε were pre-blocked with an excess of the appropriate unlabeled, purified mAb. Overlays are the same cell populations stained with control Ig. Numbers represent the percentage positive cells (± SD where shown).

17A2, 13D5 and 500-A2) yielded similar results (data not shown). By four-color analysis the $icCD3\varepsilon^-$ subset of $CD25^+$ DN cells homogeneously expressed much higher levels of CD44, CD25 and CD117 (c-kit R) as compared to the $icCD3\varepsilon^-$ $CD25^+$ population (Fig. 1B). However, expression levels of CD24 (HSA) were similar in both subsets. These data indicate that CD3ε protein is expressed later in adult thymus development than was originally believed based on mRNA expression studies (17). Indeed the homogeneous phenotype of $icCD3\varepsilon^-$ $CD25^+$ DN cells ($CD44^+CD117^+$) corresponds to the accepted definition of the DN2 subset (6), thus indicating that expression of CD3ε marks the DN2 to DN3 transition. Analysis of icCD3ε expression during subsequent stages of adult thymus development indicated that essentially 100% of cells were $icCD3\varepsilon^+$ as expected (Fig. 2), with the exception of a small subset of DN4 cells that probably do not belong to the T cell lineage (see below).

Intracellular TCR β protein is first detected in the DN3 subset

Semi-quantitative PCR and Southern blotting techniques have shown that TCR β rearrangement is initiated during the transition from the DN2 to DN3 stages of intrathymic development (6,10). However, the point at which productive TCR β protein can be first detected has been difficult to determine by conventional FACS analysis due to the extremely low amount expressed at the surface of immature thymocytes. In order to define the earliest DN subset in which icTCR β protein is expressed we have used four-color FACS analysis as described above. The results shown in Fig. 3 demonstrate clearly that icTCR β protein is first detected in a subset of DN3 thymocytes and not at all in DN2 thymocytes. These results confirm and extend previous studies (6,8,10,17), which show that full-length $VDJC_\beta$ transcripts can be first detected by Northern blot analysis in the DN3 subset. As development proceeds, the percentage of icTCR β^+ cells increases rapidly from ~25% in DN3 to 80% at the DN4 stage, after which essentially 100% of the cells show high levels of icTCR β (Fig. 2).

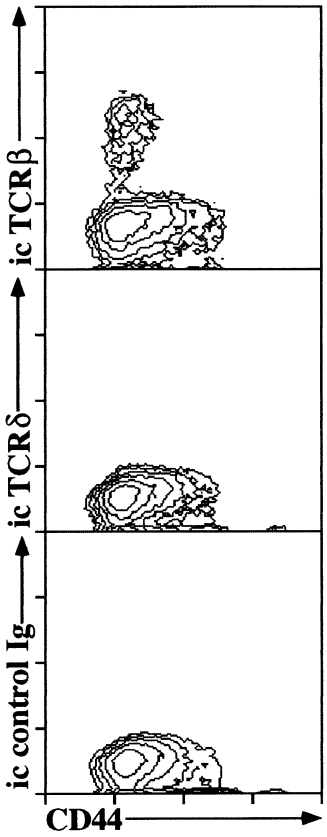

Fig. 3. Intracellular TCR β is first expressed in DN3 adult thymocytes. Expression of icTCR β or icTCR γδ and surface CD44 gated on total $CD25^+$ DN thymocytes.

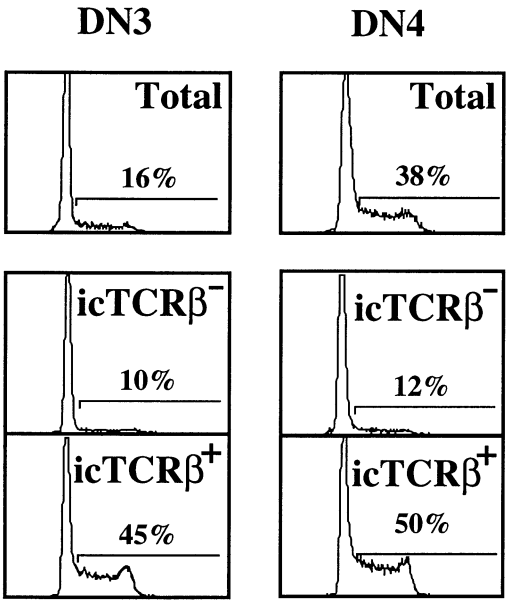

Fig. 4. Correlation between icTCR β expression and proliferation in immature thymus subsets. Purified DN thymocytes were stained as for Figure 2. DN3 and DN4 cells were sorted into icTCR β^+ or icTCR β^- subsets and the percentage of cells in S + G_2/M phases of the cell cycle was determined by PI staining. Control populations are total sorted DN3 and DN4 cells.

Immature DN thymocytes expressing intracellular TCR β are proliferating.

It is generally accepted that immature DN3 thymocytes which have undergone successful TCR β rearrangement express the pre-TCR (TCR β, pTα and the CD3 complex) and that subsequent signaling through this complex results in 'β-selection' (reviewed in 2–4,14,25). A major consequence of β-selection is proliferation. In the absence of any one of the components of the pre-TCR, this proliferative effect is absent or severely reduced (reviewed in 14–16). To determine directly whether expression of icTCR β protein correlates with proliferation, icTCR β^+ and icTCR β^- DN3 and DN4 thymocytes were purified by cell sorting and analyzed for DNA content by PI staining. As shown in Fig. 4 almost half (45%), of the icTCR β^+ cells in DN3 have a DNA content that corresponds to the S + G_2/M phases of the cell cycle compared with only 10% in the icTCR β^- DN3 population and 16% in the unfractionated DN3 population. Similar results were obtained for the DN4 subset where 50% of the icTCR β^+ cells are in cycle compared to only 12% in the icTCR β^- subset and 38% in the unfractionated population (Fig. 4). Thus there is a strict correlation between icTCR β expression and proliferation in both the DN3 and DN4 subsets of adult thymocytes. A similar correlation was observed previously for thymocytes developing in fetal thymic organ culture (FTOC) (12,26).

Intracellular TCR γδ protein is only detected in the DN4 subset

Until now, γδ lineage precursor T cells have not been able to be distinguished from their αβ counterparts by surface phenotype due to the lack of a unique surface marker. To search for possible γδ cell precursors we analyzed DN thymus subsets for icTCR γδ expression in the same manner as for icTCR β. As shown in Figs 2 and 3, in contrast to icTCR β expression, icTCR γδ protein is detected in only a small percentage (~7–10%) of the DN4 subset and is undetectable in any subset either earlier or later in thymus development. Since the GL3 mAb used to detect icTCR γδ protein in this study apparently only binds the TCR δ chain when associated with a TCR γ chain (27), we also stained DN3 and DN4 cells intracellularly with the available mAb against TCR V_γ and TCR V_δ chains (TCR V_γ1.1, V_γ2 and V_δ4). As shown in Fig. 5, a significant percentage of the icTCR $\gamma\delta^+$ cells in the DN4 subset can be stained with each of the anti-V region mAb tested. However, none of the mAb showed detectable staining of cells in the DN3 subset.

TCR $\gamma\delta^+$ thymocytes can be produced from DN4 icTCR $\gamma\delta^+$ thymocytes in vitro

In order to determine whether icTCR $\gamma\delta^+$ thymocytes are indeed precursors of TCR $\gamma\delta^+$ cells, purified and FACS-sorted DN4 thymocytes were cultured at 37°C for 4 h. As it is not possible to culture cells after intracellular staining due to the fixation and permeabilization protocol, DN4 icTCR $\gamma\delta^+$ cells could not be specifically isolated to do a direct precursor to product study. Therefore the cultured DN4 cells were monitored in parallel for the appearance of surface TCR $\gamma\delta^+$ cells and the disappearance of icTCR $\gamma\delta^+$ cells. Cell loss after 4 h

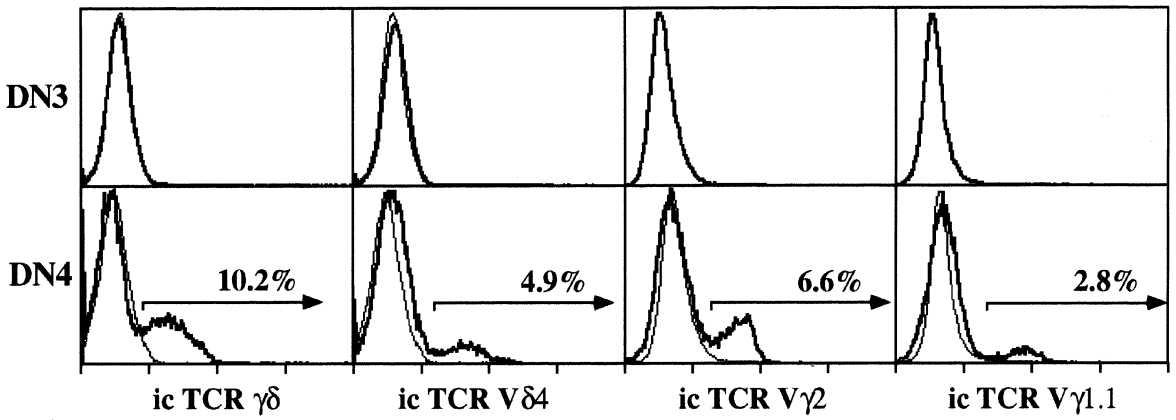

Fig. 5. Intracellular TCR V_γ and V_δ expression is first detected in DN4 thymocytes. Purified DN thymocytes were stained as for Fig. 2. Intracellular staining for TCR γδ, TCR $V_\delta 4$, TCR $V_\gamma 2$ and TCR Vγ1.1 is shown on gated DN3 and DN4 thymocytes. Overlays (thin lines) are isotype control stainings of the same gated subsets.

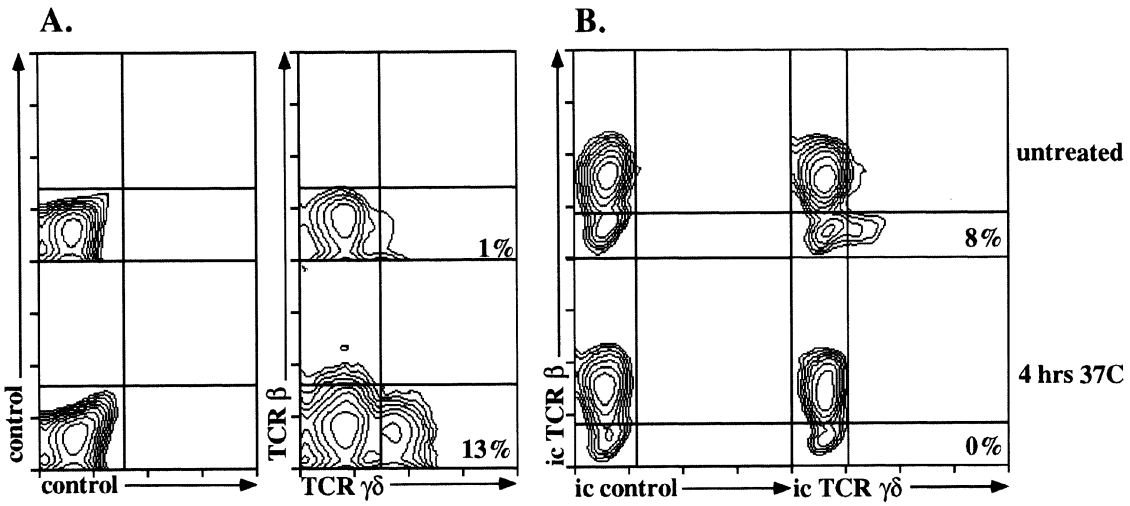

Fig. 6. Production of surface TCR $\gamma\delta^+$ cells from DN4 thymocytes *in vitro*. (A) Appearance of surface TCR $\gamma\delta^+$ cells from sorted DN4 thymocytes after 4 h in culture. Gated DN cells were stained for surface TCR β versus TCR γδ (or isotype controls). (B) Disappearance of icTCR $\gamma\delta^+$ cells from sorted DN4 thymocytes after 4 h in culture. Gated DN4 cells were stained for icTCR β versus icTCR γδ (or isotype controls). Untreated controls in both cases were FACS sorted DN4 cells incubated at 4°C.

in culture was minimal (~15%). As shown in Fig. 6, a significant proportion of surface TCR $\gamma\delta^+$ cells appeared after 4 h at 37°C and icTCR $\gamma\delta^+$ cells virtually disappeared. Importantly the absolute number of surface TCR $\gamma\delta^+$ cells produced in this time period corresponded to the number of icTCR $\gamma\delta^+$ cells that were present prior to culture and which had disappeared after 4 h at 37°C (Table 1). These data provide compelling evidence that icTCR $\gamma\delta^+$ DN4 cells are immediate precursors of thymic γδ T cells.

Most icTCR $\gamma\delta^+$ DN4 thymocytes are not 'β-selected'

To determine if icTCR $\gamma\delta^+$ cells within the DN4 subset also expressed icTCR β and could therefore be 'β-selected', we performed two-color intracellular staining for these two TCR proteins after gating out all non-DN4 and mature T cells with the third color. The contour plot shown in Fig. 7 demonstrates

Table 1. Quantitative conversion of icTCR $\gamma\delta^+$ DN4 thymocytes to surface TCR $\gamma\delta^+$ cells *in vitro*

Experiment	icTCR $\gamma\delta^+$ cells		Surface TCR $\gamma\delta^+$ cells	
	Pre	Post	Pre	Post
1	120,000	<10,000	–	126,000
2	151,000	15,000	–	152,000

DN4 thymocytes were purified by cell sorting and cultured for 4 h at 37°C in the absence of cytokines. The absolute number of ic TCR $\gamma\delta^+$ and surface TCR $\gamma\delta^+$ cells was calculated pre- and post-culture according to independent staining reactions (see Fig. 6). Data represent two independent experiments.

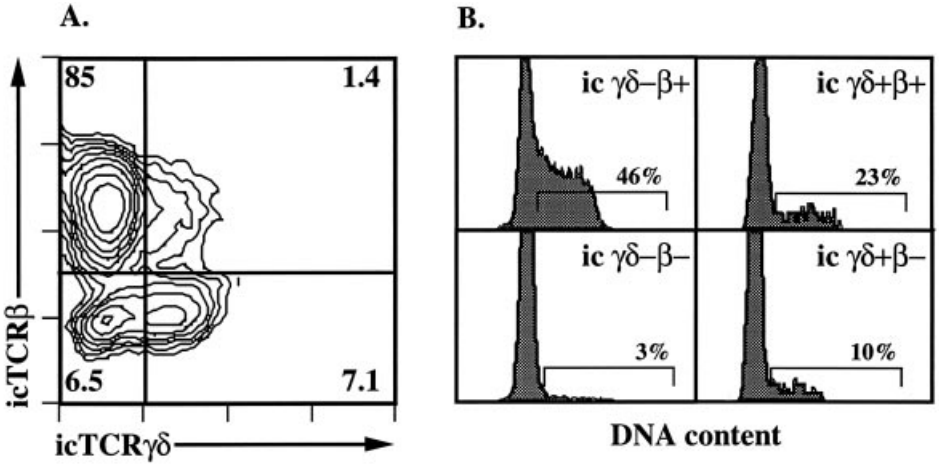

Fig. 7. Correlation of icTCR β and icTCR γδ staining of DN4 thymocytes with proliferation status. (A) Purified DN CD25⁻ thymocytes were surface stained with a pool of mAb to CD44, CD3ε, TCR β and TCR γδ to remove contaminating early precursors and mature T cells. The DN4 subset so defined was further stained with icTCR γδ together with icTCR β. Numbers are the percentage cells in each quadrant. (B) Percentage of cells in S + G_2/M phases of the cell cycle in the four subsets defined in Fig. 5(A).

that the majority of icTCR $\gamma\delta^+$ DN4 cells are icTCR β^-, although a small but reproducible fraction (~15%) are icTCR β^+. The four DN4 subsets defined by icTCR β and icTCR γδ staining were then sorted and analyzed for their cell cycle status by PI staining (Fig. 7). Interestingly, only 10% of icTCR $\gamma\delta^+\beta^-$ cells were found to be in the S + G_2/M phases of the cell cycle, whereas 23% of icTCR $\gamma\delta^+\beta^+$ cells were in cycle. As expected from analyses of the total DN4 subset (Fig. 4), 46% of icTCR $\gamma\delta^-\beta^+$ cells were in cycle as compared to 3% of icTCR $\gamma\delta^-\beta^-$ cells. Taken together these data indicate that icTCR $\gamma\delta^+$ DN4 cells are in general icTCR β^- and not cycling, so therefore not 'β-selected'.

Increased frequency of icTCR $\gamma\delta^+$ DN4 thymocytes in TCR β-deficient mice

Mice deficient for either the TCR β chain or pTα have essentially normal development of γδ cells despite a dramatic reduction (50- to 100-fold) in αβ T cell development (28,29). It was therefore of interest to investigate whether the percentage and absolute number of icTCR $\gamma\delta^+$ DN4 cells present in TCR $\beta^{-/-}$ mutant mice differs from wild-type animals. As shown in Fig. 8, the proportion of icTCR $\gamma\delta^+$ cells in the DN4 subset of TCR β-deficient mice is greatly increased (~58%). However, the number of DN4 thymocytes is correspondingly decreased in the absence of TCR β, such that the absolute number of icTCR $\gamma\delta^+$ DN4 cells is similar to that found in wild-type mice (data not shown).

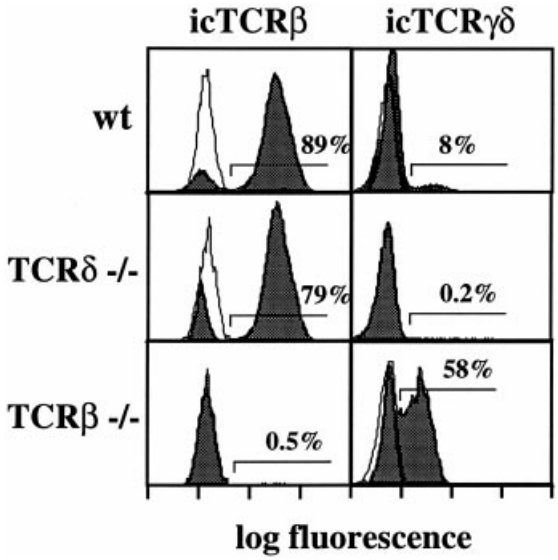

Fig. 8. Expression of icTCR β or icTCR γδ in DN4 thymocytes from normal or mutant mice. Purified DN CD25⁻ thymocytes from C57BL/6 (wt), TCR $\beta^{-/-}$ or TCR $\delta^{-/-}$ mice were surface stained as in Fig. 7, followed by staining for icTCR β or icTCR γδ (filled histograms). Negative controls (overlays) are the equivalent cells purified from TCR $\beta^{-/-}$ mice (icTCR β) or TCR $\delta^{-/-}$ mice (icTCR γδ) and stained in the same manner.

Discussion

Timing of CD3ε expression during normal adult T cell development

The data presented here demonstrate clearly that icCD3ε protein is not expressed at detectable levels until both CD44 and CD117 are down-regulated in $CD25^+$ DN adult thymocytes. Thus icCD3ε expression marks the transition between the DN2 and DN3 thymocyte subsets. Previous studies of icCD3ε expression in the fetal thymus have been controversial. Whereas essentially all $CD25^+$ fetal thymocytes were found to be $icCD3\varepsilon^+$ in one report (18), ~40% of $CD25^+CD117^+$ fetal thymocytes were shown to be $icCD3\varepsilon^-$ in another study (20). The apparent discrepancy between these data and our own may be related to differences in the timing of CD3ε expression in the fetal and adult thymus. Alternatively, since CD25 expression was not directly correlated with both CD44 and CD117 in the earlier studies, it is possible that a discrete

(but homogeneous) subset of icCD3ε$^-$CD25$^+$CD44$^+$ CD117$^+$ fetal thymocytes was overlooked.

The relatively late expression of icCD3ε protein observed in this study is surprising in view of our previous analysis of CD3ε mRNA expression in adult thymus subsets (17). Whereas CD3ε mRNA could be detected in a large fraction of DN1 and DN2 thymocytes by single-cell *in situ* hybridization, no detectable icCD3ε protein can be observed prior to the DN3 stage. This discrepancy could reflect an intrinsic instability of the CD3ε protein in more immature thymocytes. Alternatively it is formally possible that icCD3ε protein can only be detected by the mAb used here (17A2 and 145-2C11) when complexed to other proteins first expressed at the DN3 stage such as TCR β and/or pTα. However, the latter explanation seems unlikely in view of the fact that icCD3ε can be readily detected in immature thymocytes of TCR β$^{-/-}$ and pTα$^{-/-}$ mice (data not shown).

Although icCD3ε protein expression strictly correlates with the DN2 to DN3 transition in the adult thymus it does not represent a critical check-point for further T cell development. Indeed in CD3ε-deficient mice development does not arrest until the DN3 stage, and TCR β, γ and δ genes undergo apparently normal rearrangement (30). Nevertheless it is possible that signaling via CD3ε may influence the level of expression of rearranged TCR β genes, as suggested by a recent report (31).

Timing of icTCR β expression during normal adult T cell development

Although it has been known for some time that icTCR β protein is expressed early during fetal thymus development (18,19,26), the data presented here provide the first quantitative analysis of icTCR β protein expression at defined stages of normal adult thymus development. Previous mRNA studies at the population level have shown that 1.3 kb TCR β transcripts (corresponding to VDJ_β rearrangements) are first detected at the DN3 stage (5,6,8,17), although shorter (DJ_β) and longer (presumably germline) transcripts can already be observed in DN2 cells (6,17). Intracellular staining directly confirms and extends these earlier studies to the single-cell level by demonstrating that TCR β protein is undetectable in DN2 cells but present in a fraction (~25%) of DN3 cells. Moreover, icTCR β$^+$ cells increase in frequency during subsequent thymus development, reaching levels of ~80% in DN4 cells, and 100% in the subsequent ISP and DP stages.

The heterogeneous expression of icTCR β protein observed in the DN4 subset reflects the presence of several distinct cell populations. In particular, the 20% icTCR β$^-$ DN4 cells are composed of ~10% icCD3ε$^-$ cells (presumably non-T cell contaminants resulting from the negative gating criteria used to define this subset) and ~10% icTCR γδ$^+$ cells, presumably γδ lineage precursors (see below). Thus it appears that essentially all αβ lineage-committed cells in the DN4 subset express icTCR β.

In contrast to DN4, the DN3 subset contains 75% icTCR β$^-$ cells that are homogeneously icCD3ε$^+$ and icTCR γδ$^-$. Many of these icTCR β$^-$ DN3 cells most likely represent αβ lineage-committed thymocytes that have either not yet attempted VDJ_β rearrangement or undergone non-productive rearrangement on one or both alleles. Since under normal circumstances 44% (four of nine) of precursor cells that attempt VDJ_β rearrangement on both alleles are unsuccessful, the simplest interpretation of our data is that ~45% of DN3 cells have already undergone VDJ_β rearrangement (25% productively and 20% non-productively) while the remaining 55% have not. Obviously this estimate does not take into account the dynamics of cell death and proliferation within the DN3 subset (9,32–34). In this context DN3 cells with non-productive VDJ_β rearrangements might be preferentially eliminated via programmed cell death, whereas selective proliferation of productively rearranged DN3 cells (see below) might lead to an overestimate of their frequency. Irrespective of these caveats it appears clear that VDJ_β rearrangement in immature thymocytes does not initiate synchronously during the DN2 to DN3 transition, but rather occurs progressively throughout the DN3 stage. Progression in VDJ_β rearrangement during thymus development has also been shown by quantitative Southern blot analysis using a V_β probe (10); however, the estimated frequency of VDJ_β rearrangements in DN3 cells in that study was too low to account for our observed frequency of icTCR β$^+$ cells.

Intracellular TCR β expression and 'β-selection'

The concept that productive TCR β rearrangement and the ensuing expression of a pre-TCR (composed of TCR β, pTα and CD3) allows progression of DN3 thymocytes to the DN4 stage as well as their subsequent expansion is based on two independent lines of evidence. First analysis of mutant mice lacking the ability to form a pre-TCR (TCR β$^{-/-}$, RAG$^{-/-}$, pTα$^{-/-}$ and CD3ε$^{-/-}$) has revealed that development is largely (or completely) blocked at the DN3 stage (28–30,35,36). Moreover re-introduction of a TCR β transgene in mice deficient for TCR β (TCR β$^{-/-}$ or RAG$^{-/-}$) releases this block and allows development to proceed (reviewed in 3,15). In a second approach PCR-restriction length fragment polymorphism analysis of the frequency of productive (in-frame) VDJ_β rearrangements in DN3 and DN4 cells has revealed a clear progression from essentially random to preferentially productive rearrangements (9,10,37). Moreover separation of DN3 thymocytes on the basis of forward scatter has revealed that large (cycling) DN3 cells exhibit preferentially in-frame VDJ_β rearrangements, whereas small (non-cycling) DN3 cells exhibit random VDJ_β joints (38). Taken together these results have led to the conclusion that DN3 thymocytes expressing productive TCR β rearrangements (and hence a pre-TCR) are selected to proliferate and proceed to the DN4 stage, a process which has been termed 'β-selection' (2).

The data presented here on the relationship between proliferation and icTCR β protein expression in adult thymocyte subsets together with related studies carried out in FTOC (12,19,26) provide unequivocal evidence that 'β-selection' plays a critical role in thymus development. In particular icTCR β$^+$ cells in both DN3 and DN4 subsets were enriched by 4- to 5-fold in cycling cells as compared to their icTCR β$^-$ counterparts. Interestingly, the icTCR β$^+$ DN3 subset had a similar proportion of cells in cycle as the icTCR β$^+$ DN4 subset despite the fact that only a minority of DN3 cells express icTCR β. This result demonstrates that the consequences of 'β-selection' (at least insofar as cell cycle progression is concerned) are manifested rapidly as soon as

icTCR β is expressed. This in turn implies that icTCR β expression in DN3 cells is most likely the limiting component in the assembly of a pre-TCR complex and consequently that pTα must already be present. In this regard we have previously observed that pTα mRNA (assessed by Northern blot) is first detectable at the DN3 stage (17).

In contrast to αβ T cell development the potential significance of β-selection in γδ lineage cells remains controversial (reviewed in 4,39). In this context it is interesting to note that a small but distinct subset of icTCR $\gamma\delta^+$ DN4 thymocytes expresses TCR β. Moreover, the frequency of cycling cells in this icTCR $\beta^+\gamma\delta^+$ subset was 2-fold higher than in the corresponding icTCR $\beta^-\gamma\delta^+$ DN4 population. Assuming that icTCR $\gamma\delta^+$ DN4 thymocytes belong to the γδ lineage (see below), these data imply that expression of icTCR β protein confers a proliferative advantage on immature γδ cells. Interestingly a similar correlation between icTCR β expression and cell cycle has been observed for mature thymic γδ cells (22,40). Whether the increased cycling of icTCR β^+ γδ lineage cells reflects pre-TCR signaling or some other unknown function of the TCR β chain remains to be established. Clearly analysis of pTα expression in these cells will be required to resolve this issue.

αβ/γδ lineage divergence

An important new finding in this study is the identification of a distinct subset of thymocytes that express γδ protein intracellularly but not on the cell surface. Interestingly icTCR $\gamma\delta^+$ thymocytes are found exclusively in the DN4 subset. Although it is formally possible that icTCR $\gamma\delta^+$ DN4 thymocytes represent mature γδ T cells which have internalized the γδ TCR or 'δ-selected' αβ lineage-committed cells (41,42), that do not express a pre-TCR but have been rescued by expression of in-frame TCR δ and TCR γ rearrangements, we consider it much more likely that they are the immediate precursors of mature thymic γδ T cells for the following reasons. (i) Mature thymic γδ cells and icTCR $\gamma\delta^+$ DN4 cells are indistinguishable in surface phenotype (except for γδ TCR expression) (43). (ii) Although present in the DN4 subset, icTCR $\gamma\delta^+$ cells are completely absent in the subsequent ISP and DP subsets that are universally accepted to be αβ lineage committed. (iii) In contrast to what would be expected for 'δ-selected' αβ cells, icTCR $\gamma\delta^+$ DN4 cells contain a significant fraction of cells that actually co-express icTCR β. Importantly a similar proportion of icTCR β^+ cells (~10–15%) is found among mature γδ cells in both mice (22,40) and rats (44). (iv) Short-term culture of purified DN4 thymocytes results in the appearance of surface TCR $\gamma\delta^+$ cells and a concomitant reduction in the number of icTCR $\gamma\delta^+$ cells. The latter argument in particular provides compelling support for the hypothesis that a precursor–product relationship exists between icTCR $\gamma\delta^+$ DN4 cells and mature thymic γδ cells. If icTCR $\gamma\delta^+$ thymocytes are indeed the immediate precursors of γδ cells, it is of considerable interest that they are apparently restricted to the DN4 subset. In this regard, although the precise timing of TCR β, γ and δ rearrangements during adult thymus development remains controversial (reviewed in 1,4,10, 15,37,45), it is generally accepted that all three TCR loci are extensively rearranged in DN3 cells. Nevertheless no icTCR γδ protein was observed in the DN3 subset.

At least three hypotheses could explain our failure to detect icTCR $\gamma\delta^+$ DN3 cells. First, it is possible that the vast majority of DN3 cells represent αβ lineage-committed precursors that do not express TCR γ protein due to the activation of a lineage-specific transcriptional silencer (46–50). The few remaining *bona fide* γδ lineage-committed DN3 cells that productively rearrange and transcribe both TCR γ and TCR δ genes may be too infrequent to be detected by icTCR γδ staining. Alternatively the formation of a γδ TCR in γδ lineage-committed DN3 cells may signal a rapid down-regulation of CD25, thus resulting in the observed DN4 phenotype of icTCR $\gamma\delta^+$ thymocytes. This latter scenario would be consistent with the reported under-representation of productive TCR δ rearrangements in DN3 cells (51). A third formal possibility would be that γδ cells may not follow the same DN1 to DN4 developmental sequence as αβ cells. For example, immature γδ precursor cells could branch off from this pathway at the DN1 and/or DN2 stages. Following productive TCR γδ rearrangement and down-regulation of CD44 and/or CD25 these precursor cells would subsequently give rise to an icTCR $\gamma\delta^+$ DN4 developmental intermediate and ultimately to mature γδ cells.

Finally it should be noted that the unexpectedly late expression of icCD3ε protein during adult thymus development places certain constraints upon models of αβ/γδ lineage divergence that depend upon TCR signaling events. In this regard recent data indicate that TCR γ and TCR δ rearrangements initiate at the DN2 stage of adult thymus development (10,52,53). However, the absence of icCD3ε in the DN2 subset implies that such TCR rearrangements presumably cannot impinge upon the lineage commitment process until a later developmental stage.

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Abbreviations

ic	intracellular
DN	double negative
DP	double positive
FTOC	fetal thymic organ culture
ISP	immature single positive
PE	phycoerythrin
PI	propidium iodide
SP	single positive

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