
Dale I. Godfrey, Jacqueline Kennedy, Takashi Suda and Albert Zlotnik

*J Immunol* 2012; 189:4203-4211; ;
http://www.jimmunol.org/content/189/9/4203.citation
A Developmental Pathway Involving Four Phenotypically and Functionally Distinct Subsets of CD3−CD4−CD8− Triple-Negative Adult Mouse Thymocytes Defined by CD44 and CD25 Expression

Dale I. Godfrey, Jacqueline Kennedy, Takashi Suda, and Albert Zlotnik

DNA Research Institute, 901 California Ave., Palo Alto, CA 94304

ABSTRACT. We have subdivided mouse CD4+CD8+CD3− triple-negative (TN) thymocytes into four subsets based upon expression of CD44 and CD25, including CD44+CD25−, CD44+CD25+, CD44−CD25+ and CD44−CD25−. Characterization of these cells revealed several features distinct to each subset, in particular the expression of high levels of c-kit (the receptor for stem cell factor) by CD44+CD25−TN and CD44+CD25−TN but not by CD44+CD25+TN and CD44−CD25−TN. The CD44+CD25−TN subset also included the IL-7 and stem cell factor-responsive cells, whereas only minimal responsiveness was observed by the CD44+ populations. These subsets also showed differential cytokine production potential (CD44+CD25− > CD44+CD25+ > CD44−CD25+ > CD44−CD25−) after stimulation with calcium ionophore, PMA and IL-1. The repopulation potential of these subsets in 2-deoxyguanosine-treated fetal thymic lobes supports the following maturation sequence: CD44+CD25− → CD44+CD25− → CD44+CD25− → CD44−CD25−. Furthermore, the sequence of progression from CD44+CD25− to CD44−CD25− cells was confirmed by their TCR β-chain gene configuration. The former population exhibits germ-line TCR β-chain configuration, whereas the latter subset shows a rearranged pattern. Journal of Immunology, 1993, 150: 4244.

Intrathymic T cell development begins when fetal liver or bone marrow-derived precursor cells seed the thymus where they expand and differentiate into mature T cells. The earliest intrathymic precursors found within the adult mouse thymus have been defined as CD44+CD3− and Thy-1+ (1), and although no longer pluripotential, they maintain both T and B cell precursor activity (2). The subsequent steps include the T lineage commitment of these cells as they enter into the CD3−CD4−CD8−TN3 stage of development. This stage is characterized by extensive expansion and differentiation, as these cells begin to rearrange and express TCR genes in conjunction with accessory molecules such as CD4 and CD8. TN thymocytes have been reported to be heterogeneous, primarily based upon the expression of CD25-(IL-2Ra) and CD44(Pgp-1) (3, 4, and reviewed in Ref. 5). The generally accepted sequence of TN maturation proceeds from CD44+CD25− through CD4−CD25− to CD4+CD25−TN (6). The CD25− cells represent the most abundant of the TN subsets, and are commonly described as being CD4−, although some CD4+ cells

1 Abbreviations used in this paper: TN, CD4+CD8−CD3− triple-negative thymocytes; DN, CD4−CD8+ double negative thymocytes; SP, CD4+CD8+ or CD4−CD8− single-positive thymocytes; FTPC, fetal thymus organ culture; DP, CD4+CD8+ double positive thymocytes; PE, phycoerythrin; 2dG, 2-deoxyguanosine; CM, complete medium; HSA, heat stable Ag; SCF, stem cell factor.
within this subpopulation have been observed (4, 7). We have recently reported that a subset of CD25+TN thymocytes can differentiate in vitro into CD3+ DN cells (either bearing αβ or γδTCR), whereas the same population contains precursors that remain TN in cell suspension culture, but can differentiate into mature, CD4+ or CD8+ SP αβTCR+ thymocytes in FTOC (8, 9). Thus, it seems that subsets exist within the CD25+TN population that represent precursors to distinct intrathymic T cell lineages. The last stage of TN thymocyte maturation (CD44+CD25+) contains immediate precursors to the mainsteam thymocyte differentiation pathway, where these cells become CD4+CD8+ DP and express the CD3αβTCR complex. This progression from CD44+CD25+TN to CD3+DP occurs spontaneously in vitro, suggesting that these cells have already received the signal(s) that induce this differentiation (10, 11). In contrast, CD25+TN thymocytes remain TN in vitro (12).

These observations reflect the complexity of interactions that must occur during TN thymocyte development. Therefore, it is important to define these subsets to understand the mechanisms that control early thymocyte development. In this study, we have looked in detail at subsets of TN thymocytes defined by CD44 and CD25. We have confirmed the existence of a CD44+ subpopulation of CD25+TN, and show that these cells are phenotypically and functionally distinct from their CD44- counterparts. Furthermore, these cells are significantly less mature than the CD44+CD25+TN, as demonstrated by repopulation kinetics using FTOC and their pattern of TCR β-chain gene rearrangement. The cytokine production potential of these subsets was also tested and found to be highest in the most immature TN subsets. This potential decreased as the cells approached the DP stage, at which cells do not produce any of the cytokines tested (13, 14). Taken together, our results reveal at least four distinct TN developmental stages: CD44+CD25+ → CD44+CD25+ → CD44+CD25+ → CD44+CD25+.

Materials and Methods

Mice

Four to five-week-old male BALB/c mice (purchased from Simonsen Laboratories, Gilroy, CA) were used unless otherwise specified.

mAb, fluorescent labeling reagents, and flow cytometric analyses

TN thymocytes were isolated as previously described (8). Briefly, mAb used for cytotoxic depletion were anti-CD4 (clone RL172) and anti-CD8 (clone AD4; Cedarlane Laboratories, Hornby, Ontario). These were followed by treatment with low toxicity rabbit C (Cedarlane) and 20 μg/ml DNase I (Sigma Chemical Co., St. Louis, MO). Viable cells were subsequently stained with: anti-CD3-PE (clone 145–2C11, Pharmingen, San Diego, CA), anti-CD4-PE (clone, Pharmingen), anti-CD8-PE (clone 53–6.7, Pharmingen), anti-B220-PE (clone RA3–6B2, Pharmingen), anti-Mac-1-PE (clone M1/70, Caltag Laboratories, South San Francisco, CA), and anti-Gr-1-PE (clone RB6–8C5, Pharmingen) and PE-negative cells were sorted using a FACStar plus or FACS vantage (Becton Dickinson, Mountain View, CA). Purified TN thymocytes (>98% pure) were subsequently stained with anti-CD44-PE (clone IM7, Pharmingen), anti-CD25-Biotin (clone 7D4, a kind gift of A. Miyajima, DNAx), and a selection of FITC-labeled markers: anti-c-kit (clone ACK2; a kind gift of Dr. S.-I. Nishikawa, Institute for Medical Immunology, Kumamoto, Japan), anti-Thy-1.2 (clone 30-H.12, Becton Dickinson), anti-HSA (clone M1/69, Pharmingen), and rat IgG2b (Pharmingen) used as an isotype control. Anti-CD25-biotin was subsequently labeled with streptavidin-tri-color (Caltag Laboratories). Stained cells were analyzed using a FACScan (Becton Dickinson) and the data presented using LYSYS II software (Becton Dickinson).

For sorting subpopulations of TN thymocytes, we used: anti-CD3-biotin (clone 145–2C11, Pharmingen), anti-CD4-biotin (clone, Pharmingen), anti-CD8-biotin (clone 53–6.7, Pharmingen), anti-B220-biotin (clone RA3–6B2, Pharmingen), anti-Mac-1-biotin (clone M1/70, a kind gift of A. O’Garra, DNAx), anti-Gr-1-biotin (clone RB6–8C5, Pharmingen), anti-CD44-PE (clone IM7, Pharmingen), and anti-CD25-FITC (clone 7D4, a kind gift of A. Miyajima, DNAx). The purity of sorted populations was generally greater than 98%, although in some, but not all, instances the distinction between CD44+CD25+ and CD44+CD25+ was as low as 95% because of the characteristic spread from one population to the next (see Figure 1). For multiparameter phenotypic analysis of TN thymocyte subpopulations after culture, we used anti-CD4-PE, anti-CD8-Biotin, and either FITC-labeled anti-αβTCR (clone H57–597, Pharmingen) or anti-γδTCR (clone GL3, Pharmingen). Rat IgG2b mAb labeled with FITC, PE, or Biotin (all from Pharmingen) were used as isotype controls. Streptavidin-triclor (Caltag), was used to detect biotinylated mAb. Data for analysis of TN thymocytes was acquired using a FACScan (Becton Dickinson) and analyzed using LYSYS II software (Becton Dickinson).

Culture media

CM consisted of RPMI-1640 medium supplemented with 20% FCS (JRH BioSciences, Lenexa, KS), L-Glutamine (200 mM), 5 × 10^{-5} M 2-ME, MEM amino acids, MEM-vitamins, sodium bicarbonate, penicillin, streptomycin (Sigma), and gentamycin (Schering Plough, NJ) as described previously (8).
In vitro culture of TN subsets in cytokines

Sorted TN subsets were cultured at 10^6 cells/ml in media alone, or supplemented with 200 U/ml mouse rIL-7 (a kind gift of A. Shanafelt, DNAX) and 50 U/ml mouse rSCF (a kind gift of F. Lee, DNAX). Viable cells were counted every day for 3 days, using a hemocytometer.

Fetal thymic reconstitution

Fetal thymic lobes were removed at day 15 of gestation and depleted of T cell progenitors using FTOC containing 2-dG as previously described (15). Each depleted lobe was combined with 5 x 10^5 - 1 x 10^6 of the chosen TN thymocyte subpopulation in 30 μl of CM in Terasaki (Nunc, Kamstrup, Denmark) plate wells. The plates were inverted to allow cells and lobes to meet at the bottom of the hanging drop (16). After 48 h, the recolonized lobes were placed back into FTOC for a further culture period (4, 10, 16, and 22 days). Media used for FTOC was the same as CM described above, except that it contained 20% rather than 10% FCS. At the end of the culture period, the thymocytes were released from the lobes by gently pressing under a coverslip in 100 μl of PBS containing 2% FCS and 0.02% NaN3 and passed through a nylon mesh to obtain single-cell suspensions for flow cytometric analysis.

Bioassays for cytokines

These assays have been described in detail previously (14). Briefly, IL-2 and IL-4 were assayed using the HT-2 cell line and either anti-IL-2 (clone S4B6) or anti-IL-4 (clone 11B11) neutralizing mAb and cell viability was measured using an MTT assay. Units were calculated by comparison to standard curves using purified recombinant cytokines.

IFNγ levels were measured by the induction of Ag-presenting ability in the thymic macrophage cell line IG18LA as described previously (17). Briefly, dilutions to be tested were incubated with the macrophage line for 24 h. The cells were then washed and incubated with the T cell hybridoma 3DO-18.3 (specific for I-Aκ/chicken ovalbumin) and chicken ovalbumin for a further 24 h. The resulting IL-2 production (indicative of successful Ag presentation) was measured using the HT-2 assay. IFNγ activity was confirmed using a neutralizing anti-IFNγ mAb (clone XMG1.2).

TNFα levels were measured by its ability to kill the WEHI 164.13 fibrosarcoma line as described previously (14, 18). Cell viability was measured using an MTT assay. This effect could be blocked by anti-TNFα-neutralizing antibody (R&D Systems, Minneapolis, MN).

Southern hybridization

High molecular weight DNA was prepared from sorted CD44^+CD25^- and CD44^+CD25^+ TN thymocytes (>99% pure) by standard methods. The DNA was digested to completion with HindIII and electrophoresed in a 0.8% agarose gel and then transferred to a Genescreen membrane according to manufacturer’s recommendations (DuPont Co., Wilmington, DE). The filter was hybridized at 65°C overnight with the 32P-labeled EcoRI fragment of the 86T5 mouse β-chain plasmid (a kind gift from C. Davis, Stanford, and described by Born et al. (19)) in 1% crystalline BSA (fraction V), 0.5 M NaHPO4, pH 7.2, 1 mM EDTA, and 7% SDS. The filter was washed in 0.2 x SSC/0.1% SDS at 50°C and exposed to Kodak XAR X-ray film (Kodak, Rochester, NY) at -70°C for 24 h.

**Results**

Expression of CD44 and CD25 define at least four distinct stages within TN thymocyte development

TN thymocytes were labeled with CD44 and CD25 as shown in Figure 1. As previously described, three major populations were apparent: CD44^+CD25^-, CD25^+, and CD44^+CD25^- (12). However, further analysis of this double-label profile revealed heterogeneity within the CD25^+TN population with regard to CD44 expression. These cells ranged from CD44^hi to CD44^lo, with the majority of these cells being CD44^lo. The existence of CD44^+CD25^+ thymocytes has been observed previously (4, 20), although no specific characteristics were ascribed to this population. Most studies (for example 6, 11, 13, 21) have treated CD25^+TN as a single population, generally referring to them as CD44^lo.
Phenotypic characterization of subsets of TN thymocytes defined by CD44 and CD25 expression

Several markers besides CD44 and CD25 have been used to further characterize TN thymocyte subsets; these include Thy-1, HSA, and more recently, c-kit (22). We phenotyped the four populations of TN thymocytes defined by CD44 and CD25 (Fig. 1) for these markers, as well as for forward scatter as an indicator of cell size (Fig. 2). The CD44+CD25+ population showed heterogeneity for most parameters tested. The CD44+CD25− cells were large (based upon forward scatter), Thy-1+, HSA−, and c-kit−. In contrast, CD44+CD25+TN cells were significantly smaller, expressed higher levels of Thy-1 and HSA, and were c-kit+. The changes in expression levels of these markers suggest a more mature phenotype for the CD44+CD25+TN compared with the CD44+CD25−TN, as both Thy-1 and HSA levels increase as TN thymocytes mature (23). c-kit is expressed by the earliest thymocyte precursors, and is down-regulated during the CD25+TN stage (22). The present data indicate that within CD25+TN, c-kit levels decrease in parallel with CD44, and provides further evidence that the CD44+CD25+TN are less mature than CD44+CD25−TN cells. The CD44+CD25−TN are larger than the CD44+CD25+ cells, and express high levels of Thy-1 and HSA, and are c-kit−

Reconstitution kinetics of each population of TN thymocytes in FTOC

To more directly assess the sequence of TN thymocyte maturation, we reconstituted 2-dg-treated fetal thymic lobes with each of the CD44/CD25 TN populations, and analyzed the resultant thymocytes from these lobes at 6, 12, 18, and 24 days of culture. Both total cell yields and phenotypic profiles with regard to CD4, CD8, αβTCR, and γδTCR expression were measured, revealing significant differences between the reconstitution potential of each precursor population. CD44+CD25−TN thymocytes did not give rise to CD4+CD8+ DP until day 18, whereas by day 6 (the earliest timepoint tested), the CD44+CD25−TN had already differentiated beyond the DP stage to mature αβTCR expressing CD4 or CD8 SP thymocytes. The reconstitution kinetics of the other TN populations fell between these two extremes. Figure 3 shows a representative experiment indicating the viable cell yield from lobes repopulated with each subset after 18 days of culture. The best cell recovery was from the CD44+CD25−TN repopulated lobes, whereas the lowest recovery was from the CD44+CD25−TN. The phenotypic profile of these cells is shown in Figure 4. The CD44+CD25−TN gave rise to very few αβTCR+ (SP or DN) thymocytes, whereas increased proportions of these mature cells were recovered from lobes that had been seeded with progressively more differentiated precursor cells. It is noteworthy that although the less-differentiated subsets (CD44+CD25−, CD44+CD25+, CD44−CD25−) also gave rise to significant populations of γδTCR+ (DN or CD8 SP) thymocytes, the CD44+CD25− precursors did not.

Analysis of the cytokine production potential by each population of TN thymocytes

One of the characteristics we have defined for TN thymocyte subpopulations is their ability to produce high titers of cytokines upon activation with calcium ionophore, PMA, and IL-1 (required as a costimulant in the case of CD25+TN cells) (13, 14). Here, we tested the cytokine-producing potential of each of the defined TN thymocyte populations (Fig. 1), in the presence of calcium ionophore and PMA, with or without IL-1. As shown in Table 1, CD44+CD25−TN thymocytes produced high titers of IL-2, IFNγ, and TNFα. Furthermore, the presence of IL-1 during
the stimulation period resulted in a significant increase in the cytokine titers produced by these cells. Both CD44+CD25+ and CD44+CD25+TN thymocytes produced cytokines in an IL-1-dependent manner. However, CD44+CD25+ produced higher titers of IL-2, IFNγ, and (especially) TNFα than their CD44+CD25+TN counterparts. CD44+CD25+TN thymocytes failed to produce detectable cytokines as we have previously reported (13). While performing these experiments, an additional distinction was observed between the CD44+CD25+ and the CD44+CD25+TN subsets. CD44+CD25+TN thymocytes clumped together when activated in the presence of IL-1, whereas this phenomenon was not apparent with the CD44+CD25+TN thymocytes (data not shown). This observation indicates that this stimulation has specific effects on CD44+CD25+TN thymocytes, possibly through the induction of cell adhesion molecules.

IL-7 and SCF preferentially influence CD44+CD25+ but not CD44+CD25+TN thymocytes

We have previously reported that IL-7 is a maintenance factor for CD25+TN thymocytes (8), and that the cells maintained in vitro retain repopulation potential. More recently, we have shown that these cells respond by proliferation in the presence of IL-7 and SCF together (22). Here, we have tested the responsiveness of the TN subsets (from Fig. 1, with the exception of CD44+CD25+TN) to IL-7 in the presence or absence of SCF. As shown in Figure 5, all the populations tested were short-lived in media or IL-7 alone, except for the CD44+CD25+TN, which were maintained in the presence of IL-7. Furthermore, as may be predicted by the selective expression of high levels of c-kit by CD44+CD25+TN (Fig. 2), these cells showed an additional response to SCF over the 3-day culture period. Additionally, CD44+CD25+TN thymocytes cultured in IL-7 and SCF for 3 days maintained their reconstitution potential as assessed by their ability to repopulate 2-dG-treated fetal thymic lobes (data not shown).

TCR β-chain gene rearrangement parallels CD44 down-regulation on CD25+TN thymocytes

TCR β-chain gene rearrangement has first been detected within CD44+CD25+TN thymocytes with a second wave of rearrangement occurring within the CD25+TN (6). To investigate this further, we separated CD44+CD25+ and CD44+CD25+TN thymocytes and analyzed their DNA for TCR β-chain gene configuration. Figure 6 shows a Southern blot of HindIII digests that was hybridized with a Cβ probe. The germ-line configuration (Fig. 6, liver lane), gives two bands at 3 and 9 kb, whereas rearrangement results in disruption of the 9-kb band (Fig. 6, whole thymocytes lane). CD44+CD25+TN thymocytes showed a germ-line pattern at their TCR β-chain gene locus, whereas CD44+CD25+TN thymocytes exhibited TCR β-chain genes in a rearranged state. This observation indicates that CD44+CD25+TN are distinctly different and significantly less mature than CD44+CD25+TN.

Discussion

In this study we subdivided mouse TN thymocytes into four distinct populations based on their differential expression of CD44 and CD25. The phenotypic characterization with the differentiation markers, c-kit, Thy-1, and HSA, in addition to differential reconstitution kinetics, supports the following maturation pathway: CD44+CD25− → CD44+CD25+ → CD44+CD25+ → CD44+CD25+. This expands upon the commonly accepted sequence of CD44+CD25− → CD25+ → CD44+CD25+ (6, 12). CD44+CD25+ cells have been previously described (4, 7), and shown to have thymic reconstitution potential, as did the general CD25+TN population (20). In a later study, it was also demonstrated that during thymic reconstitution, the CD44+CD25+TN appear to develop before the CD44+CD25+TN (24). In these studies, however, no distinct characteristics were assigned to these cells, and subsequent reports on TN thymocytes have essentially ignored these subsets of CD25+TN (6, 12, 13, 21). We were therefore surprised to observe major differences between CD44+CD25+TN cells and CD44+CD25+TN with regard to reconstitution potential, c-kit expression, cytokine responsiveness, and cytokine production. However, the most definitive data that distinguishes between these two subsets is the status of TCR β-chain gene rearrangement, which appears to occur at some point between the former and the
FIGURE 4. Phenotypic analysis of cells recovered from fetal thymic lobes repopulated with TN subsets. Cells were recovered from 2-dG-treated lobes that had been repopulated with each of the TN subsets after 18 days. These were tested for expression of CD4, CD8, and either αβTCR or γδTCR, using triple-color labeling. CD4 versus CD8 expression is represented in the dot plots with percentages of cells included in the corner of each quadrant. The cells within each quadrant were also examined for expression of αβTCR (center block of four histograms) or γδTCR (right-hand side block of four histograms). The given profiles are representative of three separate experiments.

latter population. This finding conflicts with a previous report (6), where TCR β-chain rearrangement was observed at the earlier CD44⁺CD25⁺TN stage. However, data in this study also indicated that CD25⁺TN cells undergo a “second wave” of rearrangement before progressing to become CD44⁺CD25⁺TN thymocytes that exhibit complete rearrangement of the TCR β-chain locus. In light of our present results, we can reinterpret these data, because in that study (6) the CD25⁺TN were not separated into CD44⁺ and CD44⁻ subpopulations. When the minor subset of unarranged CD44⁺CD25⁺TN are pooled with the major subset of fully rearranged CD44⁻CD25⁺TN, we would predict the result observed in Pearse et al. (6) (approximately 75% rearrangement within the CD25⁺TN). This explanation can-
not, however, account for the partial (approximately 50%) rearrangement they observed in the earlier CD44^+ CD25^TN subset (6). The immediate precursors to the latter subset have recently been defined as CD44^Thy-1^CD44^+CD3^-CD8^- (1). These are the earliest thymocytes presently defined and, furthermore, are completely unarranged at the TCR β-chain gene locus (1). It is therefore inconsistent that their immediate progenitors would be approximately 50% rearranged (6), whereas the next population in the sequence would again show a germ-line configuration (the present study). A likely explanation for this discrepancy lies in the heterogeneous nature of the CD44^CD25^TN subset. We believe that the true pre-cursor thymocytes within this subset, which can be identified by c-kit expression (22), are probably unarranged.

The partial TCR β-chain gene rearrangement observed in Pearse et al. (6) may be the result of an as yet undefined population that shares the phenotype of the CD44^CD25^- subset (which would have been included in the study described in Ref. 6). Preliminary evidence from our laboratory suggests that this undefined population may be related to the αβTCR^DN (D. Godfrey and A. Zlotnik, unpublished observations).

Despite the evidence (6) of a gradual progression of TCR β-chain gene rearrangement during TN thymocyte development, two recent reviews on this subject have suggested that TCR β-chain gene rearrangement occurs within the CD25^TN stage (25, 26), although as far as we know, ours is the first data that directly supports this (Fig. 6). The possibility that the onset of TCR β-chain gene rearrangement arises between the CD44^CD25^- and the CD44^CD25^-TN stages of thymocyte differentiation has important implications. It suggests that T lineage commitment may not occur until after the CD44^CD25^-TN stage.

---

**Table 1**

<table>
<thead>
<tr>
<th>TN Subset</th>
<th>IL-1^* (IU/ml)</th>
<th>IL-2 (IU/ml)</th>
<th>IFN-γ (IU/ml)</th>
<th>TNF-α (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44^CD25^-</td>
<td>8768</td>
<td>190</td>
<td>6200</td>
<td>813.3</td>
</tr>
<tr>
<td>CD44^CD25^-</td>
<td>38144</td>
<td>400</td>
<td>9800</td>
<td>7777</td>
</tr>
<tr>
<td>CD44^CD25^-</td>
<td>&lt;6.25</td>
<td>&lt;6.25</td>
<td>12</td>
<td>&lt;25</td>
</tr>
<tr>
<td>CD44^CD25^-</td>
<td>47360</td>
<td>&lt;6.25</td>
<td>800</td>
<td>625</td>
</tr>
<tr>
<td>CD44^CD25^-</td>
<td>&lt;6.25</td>
<td>&lt;6.25</td>
<td>0.71</td>
<td>&lt;25</td>
</tr>
<tr>
<td>CD44^CD25^-</td>
<td>14400</td>
<td>&lt;6.25</td>
<td>60</td>
<td>104.16</td>
</tr>
<tr>
<td>CD44^CD25^-</td>
<td>&lt;6.25</td>
<td>&lt;6.25</td>
<td>&lt;0.1</td>
<td>&lt;25</td>
</tr>
<tr>
<td>CD44^CD25^-</td>
<td>&lt;6.25</td>
<td>&lt;6.25</td>
<td>&lt;0.1</td>
<td>&lt;25</td>
</tr>
</tbody>
</table>

* 20 U/ml added during stimulation.
Indeed, Wu et al. (2) have shown that the earliest CD4^lo^ thymocytes, although no longer pluripotential, still have B cell progenitor activity. Furthermore, TCR β-chain gene rearrangement is likely to be an important event for the developing T cell, because mutant mice, e.g., SCID mice and RAG-1 or RAG-2 knockout mice (27–29), do not progress to the DP thymocyte stage, which suggests that they probably stop at the CD4^+^CD25^−^TN stage (as CD4^+^CD25^−^ normally are already programmed to become DP). This also supports our hypothesis that the CD4^+^CD25^−^TN is the first population during T cell development to undergo TCR β-chain gene rearrangement, and failure to do so successfully would trigger an abort program for that particular pre-T cell. Current experiments in our laboratory are aimed at investigating these hypotheses.

It is important to point out that when CD4^+^CD25^−^TN were used to repopulate 2-DG-treated fetal thymic lobes, γδTCR^+^ thymocytes were not detected in these cultures (even after 24 days). This result suggests that γδTCR^+^ cells branch off at the CD4^+^CD25^−^TN stage, a logical assumption if this is the stage at which cells of the αβ lineage have rearranged their TCR β-chain genes. This is also consistent with our previous study, where we reported that CD25^−^TN thymocytes contain precursors for both αβTCR^+^DN and γδTCR^+^DN thymocytes (9). However, this contrasts with a recent report by Petrie et al. (30), which suggested that γδTCR^+^ cells branch off at the more mature CD4^+^CD25^−^TN stage. We have also seen the appearance of γδTCR^+^ cells in short term (3-day) cell suspension culture from CD4^+^CD25^−^TN (data not shown), yet these cells did not give rise to significant numbers of γδTCR^+^ cells in FTOC. These researchers, however, raised the possibility that γδ T cell development may parallel αβ T cell development during the early TN stages, and that the branching point may occur earlier than the CD4^+^CD25^−^TN stage. If the γδ T lineage does branch off at the CD4^+^CD25^−^TN stage (as our FTOC repopulation suggests), such parallel development would simply entail down-regulation of CD25 on both lineages before αβ or γδ TCR expression. This scenario would imply that CD4^−^CD25^−^TN would contain distinct precursors to either lineage, but have not yet acquired surface TCR and therefore appear to be a homogeneous population. Two possibilities may explain the differences in our FTOC repopulation results with the in vivo repopulation results reported by Petrie et al. (30). 1) The CD4^+^CD25^−^TN γδ-lineage committed precursors are able to expand in vivo but not in FTOC, and in the latter situation, most of the expansion of γδ T cell precursors occurs at the earlier CD4^+^CD25^−^TN stage; or 2) CD4^+^CD25^−^TN γδ-lineage-committed precursor cells are unable to seed the 2-DG-treated fetal thymic lobes.

In two previous studies (13, 14) we documented the cytokine production potential of various populations of thymocytes including several subsets (CD4^+^CD25^−^, CD25^−^ and CD4^+^CD25^−^) of TN thymocytes. These results showed that the cytokine-producing potential is highest in the most immature subset, decreased in the CD25^+^TN subset, and was completely diminished in the CD4^+^CD25^−^TN cells. In line with these findings, our current results demonstrate that the cytokine producing potential follows this sequence: CD4^+^CD25^−^ > CD4^+^CD25^+^ > CD4^+^CD25^−^TN > CD4^+^CD25^−^, which precisely parallels their developmental status. Interestingly, in our current results, we observed maximal cytokine production with all subsets studied when the cells were stimulated with calcium ionophore and phorbol ester in the presence of IL-1. This requirement for IL-1 has been previously reported for some TN subsets (13, 14, 31), but contrasts our previous report in that the production of cytokines by CD4^+^CD25^−^TN thymocytes was independent of the presence of IL-1 during the stimulation period (13). A likely explanation for this conflicting data lies in the exclusion of non-T lineage cells (using anti-B220, anti-Mac-1, and anti-Gr-1) during the isolation of TN subsets in the present report, but not in our previous studies. Because CD4^+^CD25^−^TN include many non-T lineage cells, including B cells and myeloid lineages (22), these may have provided an internal source of IL-1 during the stimulation. Taken together, our present results demonstrate that immature pre-T cell subsets that have not yet rearranged the TCR β-chain genes are capable of significant cytokine production. Although other reports have documented cytokine production by TN subsets, the present study is the first to pinpoint the precise cells capable of this cytokine production and the IL-1 dependent nature of their response. These cytokines may be necessary during early T cell ontogeny to establish an appropriate thymic microenvironment for T cell development to occur, e.g., induction of class I and class II MHC expression by thymic epithelial cells (32), although the nature of the physiologic signal(s) that may activate these cells in vivo remains obscure.

In summary, the present results provide a more detailed differentiation sequence within the immature TN thymocyte population. Characterization of these subsets has revealed both phenotypic and functional distinctions, and most importantly shows that CD25^+^TN thymocytes can no longer be treated as a single population. These observations have given us greater insight into the cytokine responsiveness and cytokine-producing potential, and has provided evidence that commitment to the αβ T cell lineage and divergence of the γδ T cell lineage may both occur at the CD4^+^CD25^−^TN stage.

Acknowledgments

We thank Melissa Fischer and Rachel Freiberg for technical support, and Dr. James Cupp, Josephine Polakoff, and Eleni Callas for their dedicated assistance with FACS sorting. We also thank Dr. Shin-ichi Nishikawa for his generous gift of anti-ε-kB mAb, Drs. Martin Pearse and Takashi Sada for helpful discussions, and Dan Finn for preparing the manuscript.
References


