Differential synergy of Notch and T cell receptor signaling determines αβ versus γδ lineage fate

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Thymic precursors expressing the pre–T cell receptor (TCR), the γδTCR, or the αβTCR can all enter the CD4+8+ αβ lineage, albeit with different efficacy. Here it is shown that proliferation and differentiation of precursors with the different TCRs into αβ lineage cells require Notch signaling at the DN3 stage of thymic development. At the DN4 stage, Notch signaling still significantly contributes to the generation of αβ T cells. In particular, in αβ lineage commitment, the pre-TCR synergizes more efficiently with Notch signals than the other two TCRs, whereas γδTCR-expressing cells can survive and expand in the absence of Notch signals, even though Notch signaling enhances their proliferation. These observations suggest a new model of αβ versus γδ lineage choice in which lineage fate is determined by the extent of synergy between TCR and Notch signaling and in which the evolutionarily recent advent of the cell-autonomously signaling pre-TCR increased the efficacy of αβ T cell generation.

The mammalian immune system contains several T lymphocyte lineages of which the αβTCR lineage and the γδTCR lineage diverge relatively early in T lymphocyte development, resulting in CD4+/8+ cells expressing an αβTCR and CD4−8− double negative (DN) cells with a γδTCR.

The gene segments encoding the γδTCR rearrange slightly before or at the same time as the Tcrb v-gene segments that encode the TCRβ chain. T cells expressing the αβ T cell receptor develop from precursors that first rearrange the Tcrb locus (1, 2) and express TCRβ chains in association with the invariant pre-TCRα (pTα) chain (3, 4). The pre-TCR is required for the rescue of CD25+44− (DN3) cells from programmed cell death (5) and is essential for extensive proliferation of the rescued cells that eventually become CD4+CD8+ double positive (DP) cells that rearrange the Tcra locus (6). TCRα chains when compared with the pTα chain compete favorably for covalent pairing with the TCRβ chain (7), resulting in expression of the αβTCR. The αβTCR is essential for the rescue of DP cells from programmed cell death, which requires binding with relatively low avidity to intrathymic MHC ligands and results in the generation of CD4+ helper and CD8+ cytolytic T cells depending on the binding of the αβTCR to class II and class I MHC molecules, respectively (8–10). This binding also results in termination of Ptcra gene transcription (11).

Although the above scenario represents the most effective pathway of generating αβ lineage cells, it does not represent the only one. In Ptcra knockout mice, it was found that not only the pre-TCR but also γδTCRs or some prematurely expressed αβTCRs in DN3 cells could rescue the generation of bona fide αβ lineage cells (12): Ptcra−/− Tcrd−/− mice are unable to make either the pre-TCR or the γδTCR. In these mice, it was shown that prematurely expressed αβTCRs could rescue αβ T cell development by facilitating the generation of DP cells that all contained productively rearranged TCRβ chains (12). In Ptcra−/− Tcra−/− mice, γδTCRs could rescue the development of αβ lineage DP cells of which only 15% expressed TCRβ chains in the cytoplasm (12). The latter observation is well in line with

data showing that in pre-TCR–deficient mice, such as *Ptcra*^{−/−} and *Tcrb*^{−/−} mice, γδTCR-expressing cells can enter the αβ lineage, abolish γδTCR expression, and rearrange the *Tcra* locus (12, 13). However, only a fraction of (DN) cells with αβTCRs or γδTCRs become αβ lineage DP cells because many of the DN cells expressing a γδTCR or αβTCR become functionally mature CD4^{−}8^{−} cells in which TCR ligation results in cytokine secretion (13, 14). Such functionally mature CD4^{−}8^{−} cells, however, are not found among pre-TCR–expressing cells. These experiments suggest that the type of TCR expressed in immature precursors can contribute to lineage choice but that in all likelihood other factors have a decisive role as well. Here we have asked in which way can DN3 precursor cells that do not express the pre-TCR, i.e., DN3 cells with γδTCRs or αβTCRs, enter the αβ lineage.

In the context of these questions, it is of interest that experiments addressing the role of Notch in αβ versus γδ T cell lineage decision concluded that higher levels of Notch1 receptors on T cell precursors favored the development of αβ lineage over γδ lineage cells under conditions where *Notch1*^{+/+} cells were competing with *Notch1*^{+/−} precursors and in which more αβ lineage cells had the *Notch1*^{+/+} than the *Notch1*^{+/−} genotype (15). It was not clear from these data whether Notch favored survival and/or proliferation of committed αβ lineage precursors or whether Notch signaling was required for αβ lineage choice. Subsequent studies concerning the role of Notch in T cell development using knockout strategies revealed that Notch signaling played an essential role at an earlier stage in T versus B lineage com-

mitment (16), whereas elimination of the Notch-dependent transcription factor CSL (RBP-Jκ) at a later stage was associated with impairment of αβ lineage development (17). Again, the latter in vivo experiments did not establish whether Notch contributed to the survival and/or proliferation of cells after lineage commitment or whether it was directly involved in lineage commitment. This question was analyzed in more detail in a recently developed culture system, which revealed that Notch receptor Delta-like 1 (DL-1) ligands were required for early T lineage commitment as well as differentiation of CD44⁺25^{−} (DN1) cells into CD44⁺25⁺ (DN2) and CD25⁺44^{−} (DN3) cells, representing sequential steps of development before TCR expression (18). Also, at the DN3 stage, pre-TCR–expressing cells required Notch ligands to further develop into αβ lineage cells (19). By analysis of DN3 cells from RAG-deficient mice that are unable to rearrange TCR loci, it was found that DL-1 ligands contributed a survival signal at this checkpoint (20).

Here we have investigated whether Notch contributes to the entry into the αβ lineage of pre-TCR–, γδTCR-, and αβTCR–expressing precursors at the DN3 stage of development and whether there is differential synergy of Notch signals with signals generated by different TCRs. To this end, we cultured DN3 cells from mice that can express various TCRs in the presence or absence of Notch DL-1 ligands and have quantitated the requirement for Notch signals by inhibiting Notch signaling with a γ-secretase inhibitor (GSI). Furthermore, we analyzed whether Notch signaling contributes to the proliferation and differentiation of cells at the DN4
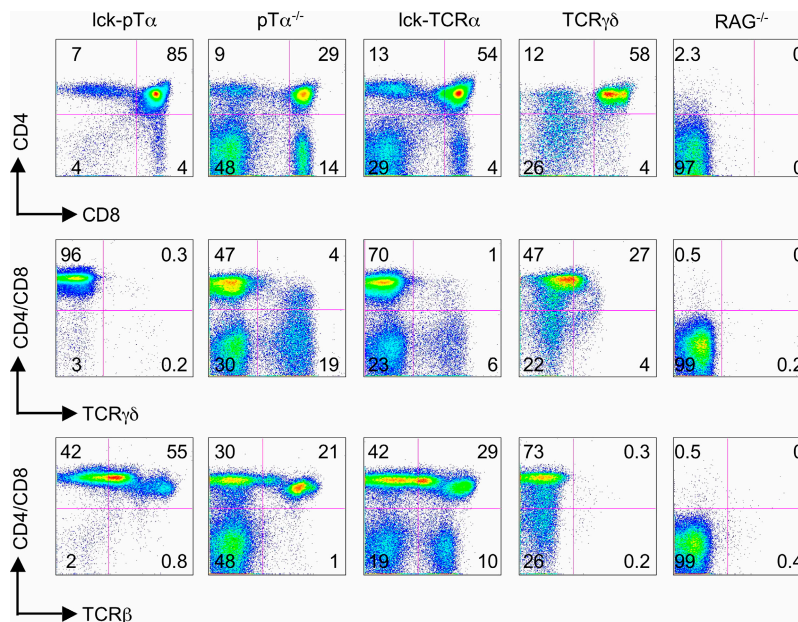
Figure 1.   Phenotype of mice with different TCRs. Thymocytes from lck-pTα transgenic mice on the *Ptcra*^{−/−} background (lck-pTα), *Ptcra*^{−/−} mice (pTα^{−/−}), lck-TCRα mice on the *Ptcra*^{−/−} background (lck-TCRα), TCRγδ transgenic mice on the *Rag-1*^{−/−} background (TCRγδ), and RAG-1^{−/−} mice (*Rag*^{−/−}) were stained with CD4, CD8, TCRγδ, and TCRβ antibodies. Numbers in each quadrant indicate the percentage of cells.

stage of development. The findings suggest a novel model of αβ T cell lineage commitment in which the extent of synergy between Notch signaling and TCR signaling determines lineage fate and in which different TCRs make a definite contribution to lineage choice.

RESULTS

Phenotype of mice with different TCRs

To study the impact of different TCRs on lineage development, we used the following mice: first, lck-pTα transgenic mice on the $Ptcra^{-/-}$ background that can mostly produce the pre-TCR as well as diverse γδTCRs and few diverse αβTCRs; second, $Ptcra^{-/-}$ mice that cannot make the pre-TCR but do make diverse γδTCRs and a few αβTCRs by rearrangement of endogenous genes; third, lck-TCRα mice on the $Ptcra^{-/-}$ background that instead of the pre-TCR express diverse αβTCRs at an early stage of development, exaggerating the few DN cells that express an αβTCR under physiological conditions (12); fourth, γδTCR transgenic mice on the $Rag-1^{-/-}$ background that express only one particular γδTCR; and fifth, $Rag-1^{-/-}$ mice that cannot make any TCRs of the adaptive immune system. Thus, all mice except for the lck-pTα mice are deficient in pre-TCR expression but express instead either αβTCRs or γδTCRs at the DN3 stage of development. It should be stressed that receptor expression in the lck-pTα and lck-TCRα mice is dependent on production of endogenous TCRβ chains, and thus, the

timing of receptor expression is equivalent to that seen in normal mice. The γδTCR transgenic mice express the transgenic TCR at the DN4 stage of development as it occurs in normal mice. Fig. 1 shows the phenotypic analysis of thymuses from these mice. Introduction of the pTα chain under the lck promoter into $Ptcra^{-/-}$ mice results in a normal thymic phenotype, with DP cells representing the vast majority of cells and a minor fraction of DN γδTCR- or αβTCR–expressing cells (21). $Ptcra^{-/-}$ mice have few DP cells and mostly DN γδTCR- and fewer DN αβTCR–expressing cells. Lck-TCRα mice on the $Ptcra^{-/-}$ background have a smaller proportion of DP cells than lck-pTα mice and an increased proportion of DN cells expressing γδTCRs and αβTCRs. TCRγδ transgenic $Rag-1^{-/-}$ mice have DP cells, some of which express low levels of the transgenic TCR, and few DN cells with low levels of the γδTCR when compared with γδ T cells from $Ptcra^{-/-}$ mice. Finally, development of $Rag-1^{-/-}$ thymocytes is arrested at the DN3 stage of development, and hence, these mice do not produce DP cells or cells that express any TCR. Perhaps the most unusual feature is the high proportion of DP cells in the γδTCR transgenic mice on the $Rag-1^{-/-}$ background. This phenotype, however, has been observed previously with γδTCR transgenes that are expressed at relatively low levels (22) and was attributed to weak γδTCR signaling that favors entry into the DP αβ lineage and prevents entry into the DN γδ lineage (22, 23).
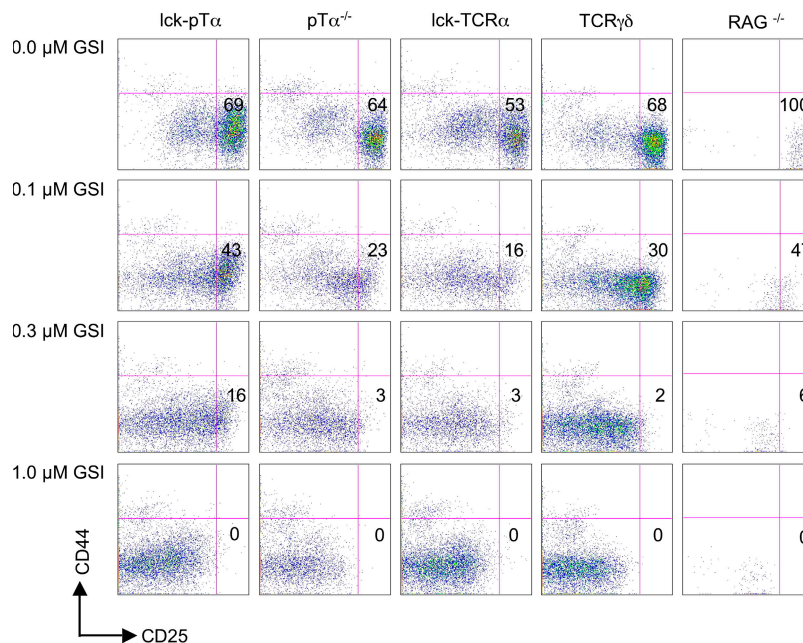
Figure 2. Survival of DN3 cells is dependent on Notch signaling and TCR expression. DN3 cells derived from lck-pTα, pTα$^{-/-}$ mice, lck-TCRα mice, TCRγδ mice, and RAG$^{-/-}$ mice were cocultured on OP9-DL1 monolayers with or without different c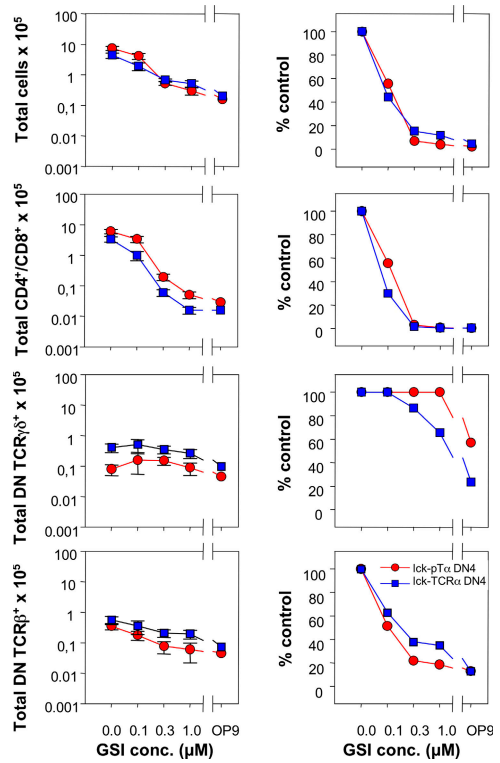oncentrations of GSI. Developmental progression of T lineage cells was examined on day 4 by flow cytometric analysis of CD25 and CD44 expression of electronically gated CD4$^-$CD8$^-$ DN cells. Data are representative of two to five independent experiments. Numbers refer to the percentage of DN3 (CD44$^-$CD25$^+$) cells in the quadrant.
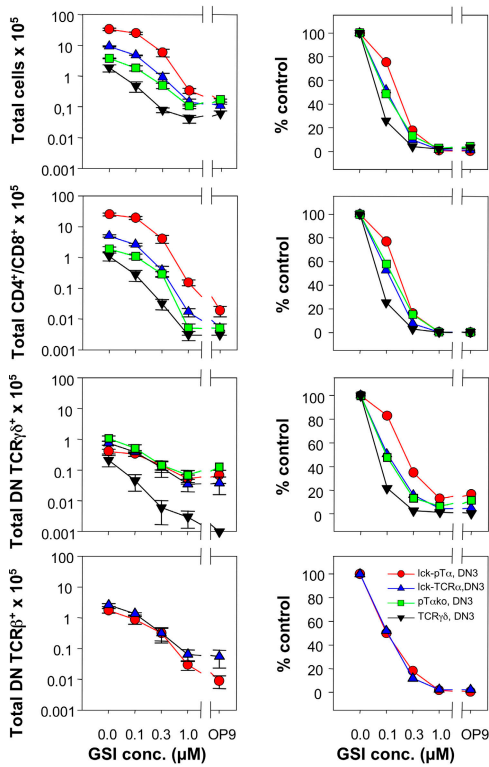
Figure 3. Analysis of proliferation and differentiation of DN3 cells with and without Notch signaling. Cell counts for DN3 cells derived from lck-pTα, pTα$^{-/-}$ mice, lck-TCRα mice, and TCRγδ mice cocultured on OP9-DL1 monolayers with or without different concentrations of the GSI or cocultured on OP9-control cells (OP9-GFP) are shown. Cells were plated at an initial density of 5×10^4 cells/well, cultured in the presence of DMSO or increasing concentrations of GSI, and analyzed after 8 d of coculture. Total cellularity, total CD4$^+$/CD8$^+$ cellularity, total DN TCRγδ cellularity, and DN TCRβ$^+$ cellularity are plotted as a function of GSI concentration. Data represent mean ± SEM of three to four independent experiments (left). Data are shown relative to the results obtained from coculture on OP9-DL1 cells without the addition of GSI (right).

Figure 4. Analysis of proliferation and differentiation of DN4 cells with and without Notch signaling. Cell counts for DN4 cells derived from lck-pTα mice cocultured on OP9-DL1 monolayers with or without different concentrations of the GSI or cocultured on OP9 control cells (OP9-GFP) are shown. Cells were plated at an initial density of 5×10^4 cells/well, cultured in the presence of increasing concentrations of GSI, and analyzed after 8 d of coculture. Total cellularity, total CD4$^+$/8$^+$ cellularity, total DN TCRγδ$^+$ cellularity, and DN TCRβ$^+$ cellularity are plotted as functions of GSI concentration. Data represent mean ± SEM of two (for lck-TCRα) and three (for lck-pTα) independent experiments (left). Data are shown relative to the results obtained from coculture on OP9-DL1 cells without the addition of GSI (right).

Survival of DN3 cells as a function of TCR and Notch signaling

DN3 cells from various mice that were depleted of cells expressing TCRγδ and TCRβ were cultured on stromal cells expressing DL-1 Notch ligands (18) in the presence or absence of GSI to evaluate the contribution of Notch signaling on the survival of these precursor cells. Fig. 2 shows the proportion of CD25$^+$CD44$^-$ DN3 cells at day 4 of culture. Some DN3 cells from *Rag-1*$^{-/-}$ mice survive up to day 4. In the presence of increasing doses of inhibitor, they down-regulate CD25 levels somewhat (Fig. 2) and total cell numbers decline (Table S1, available at http://www.jem.org/cgi/content/full/jem.20060474/DC1). This confirms that the survival of receptor-less DN3 cells depends on Notch signaling. The results are different in cultures with DN3 cells from all other mice where some DN3 cells survive up to day 8 in the presence of Notch signals (not depicted). This indicates that the expression of TCRs further enhances

survival of DN3 cells. At day 4, however, survival of DN3 cells from all strains of mice is abolished by GSI because of strongly reduced proportions of DN3 cells (Fig. 2) as well as their total cell numbers, which are not compensated for by increasing numbers of CD25$^-$ cells (Table S1). This indicates that TCR expression is insufficient to keep DN3 cells alive. In addition, cells from TCR-competent mice down-regulate CD25 much more extensively than cells from TCR-deficient mice even in the presence of the inhibitor, indicating that some of these cells have received sufficient signaling from the TCR to differentiate by completely down-regulating CD25. The data show that both TCR expression as well as Notch signaling are required for the survival of cells with the DN3 phenotype. It should be pointed out that DN3 cells are depleted of TCR-expressing cells before culture. Nevertheless, some cells with low levels of the TCR may have received sufficient signaling in vivo to differentiate into CD25$^-$ cells (Fig. 2) in the absence of further Notch signaling (24).
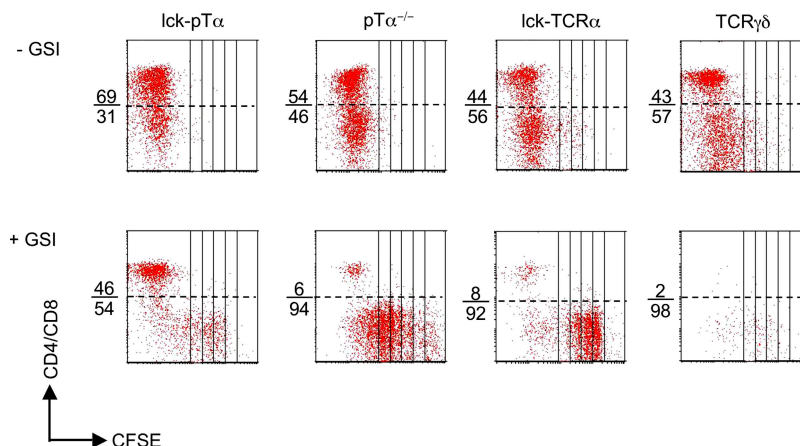
Figure 5. Proliferation and development of DN3 cells into αβ lineage DP cells require Notch signaling. DN3 cells derived from lck-pTα, pTα$^{-/-}$ mice, lck-TCRα mice, and TCRγδ mice were labeled with CFSE and cocultured on OP9-DL1 monolayers with or without 1 μM GSI. Proliferation and developmental progression of T lineage cells were examined on day 8 by flow cytometric analysis of CD4 and CD8 expression of CFSE-labeled cells. Data are representative of a minimum of three independent experiments. The number of cell divisions is depicted at the top of each panel. The two numbers next to each panel represent the proportion of CD4$^+$/CD8$^+$DP cells versus DN cells (percentage of surviving cells).

Inhibition of proliferation and differentiation by the GSI

When 5×10^4 DN3 cells from various mice are cultured on OP9-DL1 cells, there is expansion and differentiation within 8 d that results in the accumulation of different cell types. As shown in Fig. 3, pre-TCR–expressing precursors expand most extensively and generate the highest number of CD4$^+$/8$^+$ αβ lineage cells. The pre-TCR$^-$ precursors from $Ptcra^{-/-}$, lck-TCRα, and $Rag1^{-/-}$ TCRγδ transgenic mice expand less and generate fewer CD4$^+$/8$^+$ αβ lineage cells. On the other hand, pre-TCR–deficient precursors are at least as efficient as pre-TCR–competent precursors in generating DN TCRγδ-expressing cells, with the exception of the precursors that express low levels of a transgenic TCRγδ (see Fig. 1). Finally, DN3 precursors from lck-pTα and lck-TCRα mice generate DN cells that carry an αβTCR.

Both total cell numbers and numbers of CD4$^+$/8$^+$ αβ lineage cells declined with increasing doses of GSI by about 2–3 logs (Fig. 3), whereby at any dose cells derived from lck-pTα mice present with the highest number of total and CD4$^+$/8$^+$ αβ lineage cells. This indicates that the pre-TCR synergizes efficiently with DL-1–dependent Notch ligation in the generation of CD4$^+$/8$^+$ αβ lineage cells, more efficiently than the αβTCR or γδTCR, which are predominantly expressed in cells from lck-TCRα, $Ptcra^{-/-}$, and γδTCR mice. In contrast, GSI inhibits the number of DN cells expressing a TCRγδ or TCRαβ much less (by 1–2 logs; Fig. 3) and in fact such cells are present even in the absence of DL-1 Notch ligands. This indicates that the accumulation of these cells is much less dependent on Notch but that Notch signaling can help their expansion (see below).

The diagrams on the right hand side of Fig. 3 show the relative inhibition of different phenotypes developing in the cultures with increasing doses of GSI. There are definite differences with different precursors as detailed in Figs. 5–7 as well as Figs. S1 and S3, which are available at http://www.jem.org/cgi/content/full/jem.20060474/DC1.

Fig. 4 shows that even at the DN4 stage, Notch signaling still contributes to the generation of αβ lineage cells from precursors that either express the pre-TCR or the αβTCR. The comparison of precursors from these mice appears to be most meaningful because both the pre-TCR and αβTCR are expressed at the same time in development (25). As shown in Fig. 4, the diminution of total cell numbers by GSI is less severe with DN4 versus DN3 cells (Fig. 3), but there is still a profound suppression of the generation of CD4$^+$/8$^+$ αβ lineage cells, more so with cells from lck-TCRα than with cells from lck-pTα mice (Fig. 4 and Fig. S1). This suppression is clearly visible between days 4 and 8 of culture (Fig. S3). Thus, even at this stage, the generation of αβ lineage cells from precursors with different TCRs depends on Notch signals, implying a continuous requirement of Notch signals for proliferation and differentiation of αβ lineage cells. However, in contrast to cultures with DN3 cells, some nonproliferated cells can express CD4/8, indicating that these cells had received sufficient Notch signals at the DN3 and/or DN4 stage to express CD4/8. Thus, the better synergy of pre-TCR and Notch signaling compared with αβTCR and Notch signaling in the generation of αβ lineage is evident at the DN3 as well as the DN4 stage of development.

In contrast, at the DN4 stage, the number of DN TCRγδ or DN TCRαβ cells is much less dependent on Notch signaling when compared with the DN3 stage, indicating that at the DN4 stage, survival and proliferation of these cells depends much less on Notch than that of cells entering the αβ lineage. It should be noted that results obtained with lck-pTα mice are the same as those obtained with wt B6 mice (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20060474/DC1), which is the expected result because

pre-TCR expression in the lck-pTα mice depends on endogenous TCRβ rearrangement.

Proliferation and differentiation of DN3 cells from various mice were then directly assessed by CFSE labeling and monitoring CFSE dilution as well as the acquisition of CD4 and CD8 surface markers at the end of an 8-d culture period in the absence or presence of the highest dose (1 μM) of GSI (Fig. 5). As can be seen, DN3 cells from all strains proliferate extensively in the presence of Notch DL-1 ligands, and a higher proportion of cells from pre-TCR–competent than pre-TCR–incompetent mice acquires CD4 and CD8 surface markers as observed in vivo (Fig. 1). This indicates that the culture system as used here mimics developmental events observed in vivo, including the differentiation of γδTCR- or αβTCR-expressing cells into DP αβ lineage cells. The inhibition of Notch signaling with cultured DN3 cells shows that in all cases the CD4+/8+ cells represent the most divided cells and that their proportion declines in the presence of GSI, more so for the pre-TCR–incompetent and less so for the pre-TCR–competent precursors (Fig. 5). Fig. S3 shows that this inhibition is evident at day 4 of culture and more pronounced at day 8 of culture. These data reiterate the dependence on Notch signaling of αβ lineage cells with regard to both proliferation and differentiation as well as the fact that the pre-TCR synergizes better with Notch signals than the TCRγδ or TCRαβ because CD4+/8+ cells from the latter precursors are much more reduced than those derived from

pre-TCR–competent precursors. It is of interest to note that with increased doses of GSI in culture of DN3 cells, there is no increase in the proportion of CD4+/8+ cells that went through reduced numbers of divisions, indicating that Notch is required for both proliferation as well as differentiation of αβ lineage cells.

The fact that the generation of DP cells from TCRαβ–expressing precursors requires more Notch signaling than generation of DP cells for pre-TCR–expressing precursors could suggest that in the former DP cells there would be stronger expression of Notch target genes, such as *Hes1*, when compared with DP cells generated from the latter precursors. When this was analyzed by real-time PCR, it was found that DP cells generated from DN3 cells at day 3 expressed marginally higher levels of HES1 when derived from αβTCR versus pre-TCR–expressing precursors (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20060474/DC1). In any case, the HES1 expression levels were lower in the DP than the DN3 cells, indicating that this Notch target is under more complex regulation (Notch signaling is also required to generate DN3 cells) that results from Notch signaling before and after TCR expression.

Relative independence of γδ lineage cells on Notch signals
In Fig. 6, various cultures with DN3 precursors from different mice were analyzed at day 8 for expression of TCRαβ or TCRγδ receptors as well as CD4 and CD8. As can be seen
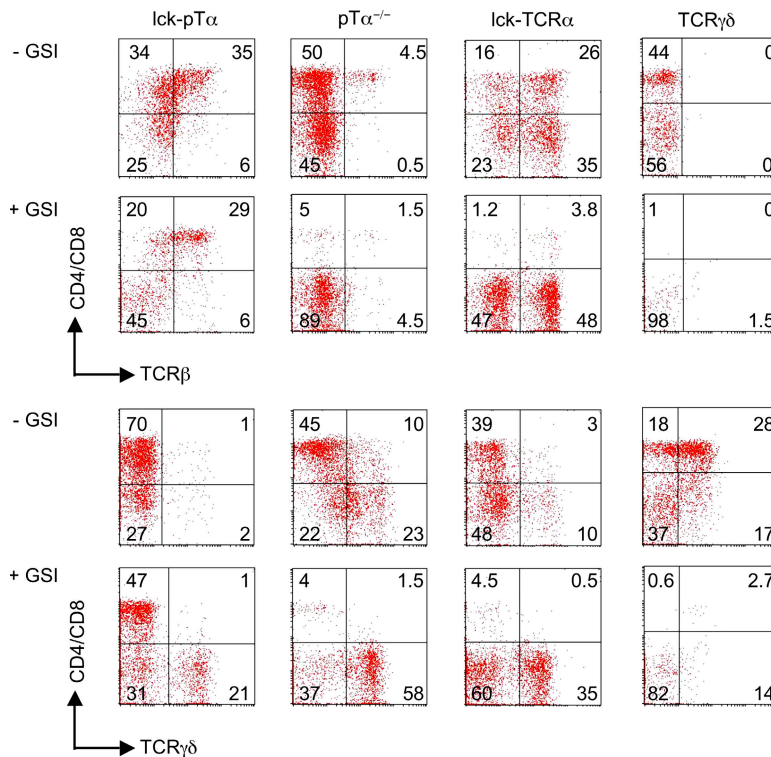
Figure 6.   Relative enrichment of TCR–expressing DN cells by inhibition of Notch signaling. DN3 cells derived from the indicated mouse strains were cocultured on OP9-DL1 monolayers with or without 1 μM GSI. At day 8 of coculture, T cell lineage cells derived from the various mice were analyzed for TCR expression by staining with CD4, CD8, TCRβ, and TCRγδ antibodies. Data are representative of a minimum of three independent experiments.
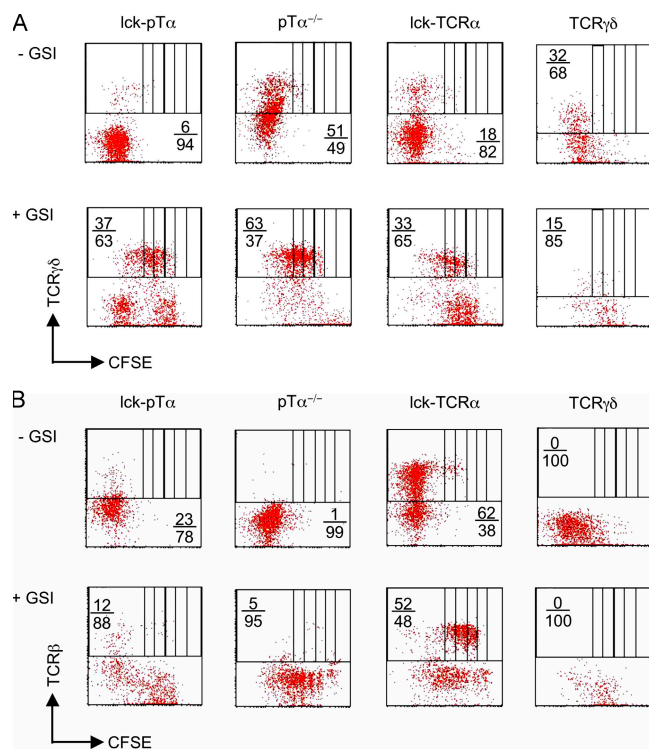
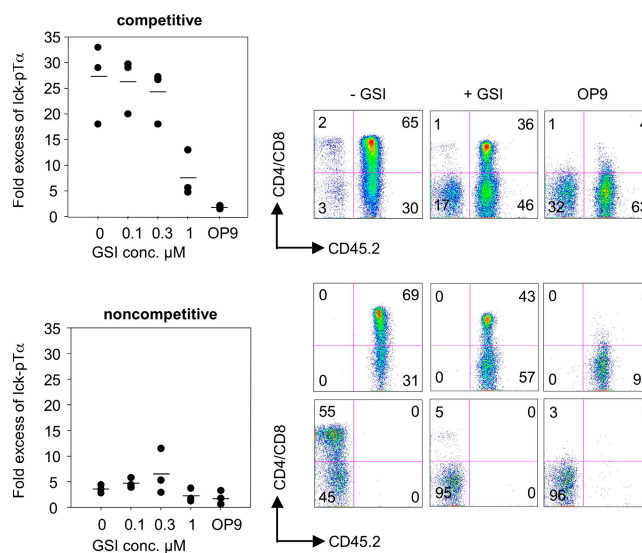**Figure 7. Proliferation of TCR–expressing DN cells is dependent on Notch signaling.** DN3 cells derived from the indicated mice were labeled with CFSE and cocultured on OP9-DL1 monolayers with or without 1 μM GSI. Proliferation and developmental progression of T lineage cells were examined on day 8 by flow cytometric analysis of TCRγδ- (A) and TCRβ- (B) expressing electronically gated CD4⁻CD8⁻ DN CFSE-labeled cells. Data are representative of a minimum of three independent experiments. The numbers of cell divisions are depicted at the top of each panel. Numbers in each panel represent the proportion of TCRγδ⁺ DN versus TCRγδ⁻ DN cells (A) and the proportion of TCRβ⁺ DN versus TCRβ⁻ DN cells (B), respectively.

Figure 8. Competition for Notch ligands by lck–TCRα– and lck–pTα–derived precursors. DN3 cells derived from lck-pTα (CD45.2⁺) mice and DN3 cells derived from lck-TCRα (CD45.2⁻) mice were cocultured separately (bottom) or together in a 1:1 ratio (top) on OP9-DL1 cells. The competitive advantage of lck-pTα precursors was examined by flow cytometric analysis of CD45.2 expression of surviving cells at day 7 of coculture on OP9-DL1 cells without GSI and in the presence of increasing concentrations of GSI as well as on OP9 control cells. Fold excess of lck-pTα precursors with (top) or without (bottom) competition is plotted as a function of GSI concentration. Each dot represents a single experiment, and the line represents the mean. Proportion of CD4⁺/8⁺ αβ lineage cells in coculture on OP9-DL1 cells without GSI (− GSI) in the presence of 1 μM GSI (+ GSI) and on OP9 control cells was examined by flow cytometric analysis of CD4 and CD8 expression of CD45.2⁻ and CD45.2⁺ cells.

in the two top rows, the generation of CD4⁺/8⁺ TCRβ-expressing cells is always reduced in the presence of 1 μM GSI and again more so with pre-TCR–incompetent than with pre-TCR–competent precursors. It is also evident that especially with DN3 cells from lck-TCRα mice, there is an increase in the proportion of DN TCRαβ-expressing cells; i.e., these cells are less dependent on Notch signals than DP αβ lineage cells. The same holds true for DN TCRγδ-expressing cells that are relatively enriched in the presence of the GSI inhibitor in all cultures except those with cells expressing the transgenic TCRγδ. The low level expression of this transgenic TCR permits the generation of TCRγδ-expressing CD4⁺/8⁺ αβ lineage cells (that cannot silence the expression of the transgenic TCRγδ), which is highly Notch signaling dependent, but not the generation of Notch-independent DN TCRγδ-expressing γδ lineage cells.

Notch signaling enhances proliferation of γδ lineage cells

The data depicted in Fig. 7 A show the Notch-dependent proliferation of TCRγδ-expressing γδ lineage cells. With precursors from all mice, the TCRγδ-expressing cells represent a more divided population in the absence than in the presence of GSI. Thus, although there is a relative increase in γδ lineage cells in the presence of GSI due to the drastic reduction in αβ lineage cells, there is also some reduction of absolute numbers of γδ lineage cells (Fig. 3) due to the reduced proliferation. As shown in Fig. 7 B, essentially the same is true for DN TCRαβ-expressing cells. Thus, with cells from lck-TCRα mice, the only remaining cells in cultures with GSI are DN cells that express relatively high levels of the TCRαβ and TCRγδ (Fig. 6) but are less divided than the bulk of DN TCRαβ- and TCRγδ-expressing cells in the absence of GSI (Fig. 7).

Competition between pre-TCR and TCRαβ-expressing precursors

Previous in vivo experiments have shown that pre-TCR–expressing precursors out-compete TCRαβ-expressing precursors in that they generate an up to 100-fold excess of thymocytes when injected together at a 1:1 ratio with TCRαβ-expressing precursors, whereas the excess is only three- to fivefold when injected into separate hosts (25). Here we have analyzed whether this competitive advantage,
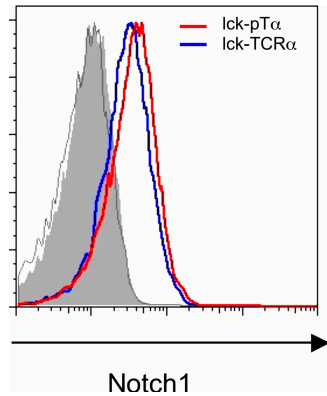
Figure 9.   Surface expression of Notch1 on lck–pTα– and lck–TCRα–derived precursors. Thymocytes were stained with anti-Notch1. Notch1 surface expression is shown for CD4$^-$CD8$^-$CD25$^+$CD44$^-$ (DN3) thymocytes. Gray histograms (filled, lck-pTα; line, lck-TCRα) represent isotype control staining.

which is likely to depend on the better synergy of the pre-TCR versus TCRαβ with Notch signals, can be reproduced in in vitro culture and if so, whether it can be abolished by interfering with Notch signaling. The CD45.2$^+$ marker was used to identify cells derived from the lck-pTα mice. Cells were either cultured separately (Fig. 8, bottom) or under competitive conditions (Fig. 8, top) in the absence or presence of various concentrations of GSI. The boxes on the left in Fig. 8 provide the fold excess in cell numbers derived from pre-TCR over TCRαβ precursors (each dot represents a single experiment, and the line represents the mean). The data clearly show (Fig. 8, left) that there is a competitive advantage of pre-TCR-expressing over TCRαβ-expressing precursors in cocultures that diminishes with increasing doses of GSI and is almost absent in the absence of Notch DL-1 ligands on feeder cells. The right hand side shows that the diminution of the competitive advantage by inhibition of signaling is accompanied by a diminution of the proportion of CD4$^+$/8$^+$ αβ lineage cells derived from precursors of both mice expressing either the pre-TCR (CD45.2$^+$) or the TCRαβ (CD45.2$^-$). Thus, the competitive advantage of pre-TCR–expressing precursors results from their more efficient use of Notch DL-1 ligands in the generation of αβ lineage cells when compared with TCRαβ precursors. In the absence of Notch ligands, there is only a minimal advantage in generating thymocytes of one over the other population.

Similar expression of Notch receptors on DN3 precursors with different TCRs

The better synergy of the pre-TCR versus the TCRαβ with Notch DL-1 ligand–dependent signaling may either depend on the possibility that αβ lineage development of pre-TCR–expressing cells requires less input from Notch or that these precursors express higher levels of Notch. To distinguish between these possibilities, DN3 cells from lck-pTα and lck-TCRα mice were stained with Notch 1 antibodies because

the dependency on Notch signaling is much more evident with DN3 than DN4 precursors from the different mice (Fig. 3). No significant difference was found (Fig. 9), indicating that precursors from pre-TCR–competent lck-pTα mice may in fact require fewer Notch signals to become αβ lineage cells than precursors from pre-TCR–incompetent lck-TCRα mice.

DISCUSSION

A variety of apparently conflicting findings with regard to the role of the γδTCR, αβTCR, and pre-TCR in the αβ versus γδ lineage decision has been published. Initially, it was found that DP αβ lineage cells of normal mice were selected against in-frame *Tcrg* or *Tcrd* rearrangements, arguing that αβ and γδ lineage cells were derived from a common precursor and that mostly cells that had failed to generate productive TCRγδ genes entered the αβ lineage (26, 27). After the discovery of the pre-TCR, it was found that in *Ptcra* knockout mice, the proportion of γδTCR-expressing cells with in-frame *Tcrb* rearrangement was increased, arguing that in wild-type mice the pre-TCR could divert TCRβ-expressing cells from the γδ lineage into the αβ lineage (28). Although these notions were compatible with a strict role of the γδTCR versus pre-TCR in instructing γδ versus αβ lineage commitment, other results were plainly inconsistent with that simple notion. In particular, it was shown in *Ptcra*$^{-/-}$ *Tcra*$^{-/-}$ mice that the γδTCR could support development of DP αβ lineage cells (12). It was also reported that in the absence of the pre-TCR in *Tcrb*$^{-/-}$ mice, γδTCR-expressing cells could become DP cells that were selected for in-frame *Tcrg* or *Tcrd* rearrangements (13). It had also been noted that in γδTCR transgenic mice, γδTCR-expressing DP cells could develop (29). These results showed clearly that the γδTCR could support the development of αβ lineage cells, but it remained elusive how this odd lineage decision was made.

With regard to the αβTCR, similarly confusing results were obtained. Although it was shown in *Ptcra*$^{-/-}$ *Tcrd*$^{-/-}$ mice that an early expressed αβTCR could rescue DP αβ lineage development (12), results in αβTCR transgenic mice expressing the αβTCR on a large number of DN cells showed that normal αβ lineage development was impaired but not abolished, whereas DN cells with a γδ lineage phenotype but αβTCR expression accumulated (30, 31). This suggested that a single αβTCR expressed early in development could do both; it could support some DP αβ lineage development as well as divert cells into the γδ lineage.

Collectively, these data showed that the type of TCR could influence lineage decision but that likely other factors contributed as well. Notch was a good candidate because data from various laboratories indicated that Notch signaling had some role in αβ lineage development (15, 17), even though it was not clear when and how Notch contributed to the development of αβ lineage cells from precursors expressing different TCRs.

The results described here suggest a novel model whereby differential synergy between TCR and Notch signaling

determines lineage fate. The model implies that Notch signaling at the DN3 stage of T cell development is not only required for DP αβ lineage commitment of pre-TCR–expressing precursors, but is also essential for the generation of DP αβ lineage cells from DN precursors expressing either a γδTCR or αβTCR. Furthermore, the data show that at the DN3 and DN4 stage, the αβTCR- and γδTCR-dependent pathways of DP αβ lineage development are more dependent on Notch signaling than the pre-TCR–dependent pathway. The former two are more severely affected by inhibition of Notch signaling than the latter. This could have been due to the fact that there are differences in the level of Notch receptors on the precursors with different TCRs or that cells with TCRs other than the pre-TCR require more Notch signals to differentiate along the αβ pathway. The data showing similar Notch levels on the precursors from the different mice favor the latter view.

The conclusion that proliferation and differentiation of αβ lineage cells of pre-TCR–expressing precursors require less input in terms of Notch signals is supported by the competitive advantage of pre-TCR⁻ over TCRαβ-expressing precursors when generating αβ lineage cells. The competitive advantage is gradually diminished in the presence of GSI and is almost entirely absent in the absence of Notch ligands. The data also indicate that the number of DN TCRγδ- or TCRαβ-expressing cells derived from ex vivo DN3 with low TCR levels is much less dependent on Notch signaling. Nevertheless, the proliferation of these cells and/or their precursors can be enhanced by Notch. The latter conclusion is in line with a recent report (32) that addresses the Notch dependence of γδ T cells in normal mice.

Our results agree with results from other studies in which DN3 cells from $Rag^{-/-}$ mice were retrovirally transduced with TCRγδ genes before culture in the absence of Notch ligands and which showed that TCRγδ-expressing cells could survive under these conditions (33). This does not exclude that Notch influences TCR⁻ precursors of γδ lineage cells, but it shows that unlike cells that express the pre-TCR, γδTCR-expressing cells do not require Notch to survive.

It was recently shown that a subset of pre-TCR–expressing DN3 cells that have already received a signal in vivo can differentiate into DP cells in the absence of Notch ligands in a 3-d culture (24). This is consistent with our finding that when total DN3 cells are cultured for 4 or 8 d in the absence of Notch ligands, few DP cells are generated (Fig. 3 and Fig. S3). However, there is a dramatic 3-log increase in the number of DP cells in the presence of Notch ligands, indicating extensive synergy of Notch signaling with pre-TCR signaling at the DN3 stage in the generation of DP αβ lineage cells. This is even evident at the DN4 stage at which Notch signaling still makes a major contribution to the generation of αβ lineage cells. The culture system described by Taghon et al. (24) did not reveal the generation of DP cells by γδTCR-expressing cells from $Tcrb^{-/-}$ mice. However, $Tcra$-rearranging DP cells that are selected for in-frame $Tcrg$ and $Tcrd$ rearrangements are clearly observed in the thymus of

$Tcrb^{-/-}$ mice (13). This suggests that the 3-d culture system as described by Taghon et al. does not fully reflect conditions that control development in vivo, whereas the culture condition used here clearly permits γδTCR-expressing cells to develop into DP αβ lineage cells when Notch DL-1 ligands are provided, and raises questions with regard to lineage commitment of γδTCR-expressing DN3 cells.

At present, the molecular nature of the synergy of TCR signals with Notch signals in the generation of αβ lineage cells is not clear, in part because of the paucity of data on target genes regulated by the Notch IC-dependent transcription factor CSL (RBP-Jκ). However, several data obtained by overexpressing intracellular Notch may provide clues to how such synergy may take place at the DN3 and DN4 stage of development. Such overexpression has been shown to result in ERK phosphorylation as well as degradation of the $E2a$-encoded transcription factor complex (34). It has in fact been shown that pre-TCR signaling results likewise in activation of the Ras-dependent MAP kinase pathway (35, 36), including activation of ERK (37), which suffices to induce proliferation and differentiation into DP cells of DN3 cells in $Rag^{-/-}$ mice. Likewise, it was shown that E2A deficiency can result in differentiation of DN3 cells in $Rag^{-/-}$ mice, and it has been argued that pre-TCR signaling interferes with the function of basic helix loop helix E2A proteins (38). Thus, there is ample opportunity for synergistic action of Notch signaling and TCR signaling at the DN3 stage of T cell development. Perhaps the most intriguing observation with regard to the role of Notch at this stage of development is our observation that Notch IC overexpression can result in the accumulation of DP cells and generation of DP lymphoma cells in $Rag^{-/-}$ cells (39), suggesting that high levels of Notch IC can bypass the requirement for a TCR in T cell development at the DN3 stage.

Recently, it has been shown that Notch signaling can improve the survival of DN3 cells in a PI3 kinase–dependent manner (20). It is presently unclear whether this is one of several molecular pathways by which Notch contributes to the generation of αβ lineage cells. In any case, our data disagree with the conclusion that Notch signaling no longer contributes to the generation of αβ lineage cells after the early DN3 stage (24). Clearly DN4 cells expressing different TCRs profit from Notch signals when developing into DP αβ lineage cells.

The scenario of αβ versus γδ lineage commitment (Fig. 10) suggested by the data reported above extends an earlier proposal of the γδ versus αβ lineage decision in which the generation of all αβ lineage cells from precursors expressing different TCRs was postulated to depend on Notch signaling (40). The additional facts that different TCRs can differentially contribute to αβ lineage commitment were initially established with precursors expressing the αβTCR (12, 30) and recently likewise established with different γδTCRs. With regard to the role of TCRγδ, it was in fact shown that lack of ligation or low levels of expression both resulting in weaker signals supported the development of αβ lineage cells

more efficiently (22, 23). These results are well in line with our observation that low levels of the transgenic TCRγδ only permit the generation of DP cells with the TCRγδ. However, our results are in contrast with the previous interpretation of these results. Although it was hypothesized that weak γδTCR signaling may mimic pre-TCR signaling (22, 23), our results do not support this notion. Even precursors with low levels of the γδTCR that permit these cells to predominantly enter the αβ lineage require greater input from Notch signaling to enter the αβ lineage than pre-TCR–expressing cells do. Thus, pre-TCR and γδTCR signaling are different even under these conditions, thereby challenging the notion that weak γδTCR signaling equals pre-TCR signaling.

In summary, continuous Notch signaling at the DN3 and DN4 stage of T cell development is required for the optimal generation of αβ lineage from precursors expressing the pre-TCR but even more so for those precursors expressing a γδTCR or αβTCR. The fact that γδTCR-expressing cells are normally mostly DN cells and do not contribute much to the DP αβ lineage, but do in the absence of the pre-TCR, is due to the fact that they are more dependent on Notch signals to enter the αβ lineage than pre-TCR–expressing precursors. The same is true for cells that express an αβTCR early in development. Thus, in the presence of pre-TCR–expressing DN cells, precursors with other TCRs compete poorly for Notch ligands. It would appear, therefore, that the Notch–TCR synergy model as depicted in Fig. 10 can explain a variety of apparently contradictory data on the role of different TCRs in the γδ versus αβ lineage choice.

In evolutionary terms, we can hypothesize that the αβ lineage of the adaptive immune system was built on the γδ T cell lineage that is evolutionarily more ancient. The generation of the adaptive TCRαβ immune system required the development of the *Tcrb* locus as well as the *Tcra* locus and a signal that diverted cells from the γδ lineage. This signal involved the Notch-dependent activation of the CSL transcription factor such that γδTCR-expressing cells could enter the αβ lineage, which includes ∼10% of γδ T cells that exhibit productive *Tcrb* rearrangements. After such lineage diversion, the cells begin to rearrange the Tcra locus and thus generate some cells that can express an αβTCR. This ancient and inefficient pathway of generating αβ lineage cells was then made much more efficient by the development of the pTα chain that pairs with TCRβ chains but not, or only poorly, with TCRγ or TCRδ chains, thus generating the autonomously signaling pre-TCR (41, 42). The pre-TCR expands only cells with in-frame *Tcrb* rearrangement before the rearrangement of the bulk of Vα gene segments, thus permitting a much more efficient generation of a diverse αβ T cell repertoire through *Tcrb* selection and expansion of selected cells. This then became the most efficient pathway of generating αβ lineage cells because it requires less synergy with regard to Notch signaling than the "old" γδTCR–dependent pathway. The "old" pathway is also used by a few cells that express the αβTCR early in development due to *Tcra* rearrangement
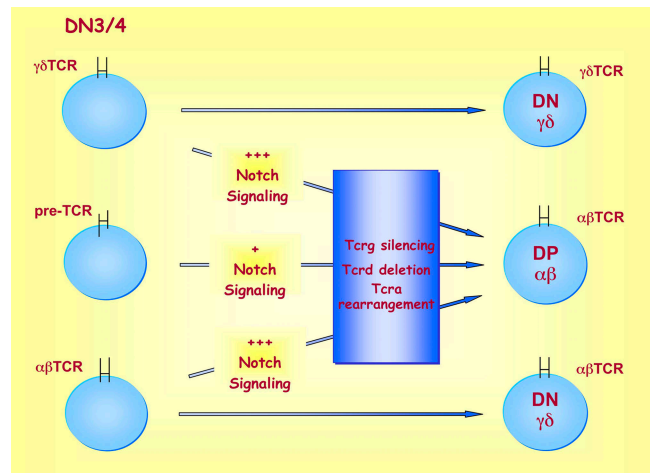
Figure 10. Notch–dependent differentiation into αβ lineage cells. DN3/4 cells expressing the γδTCR, pre-TCR, or αβ-TCR will differentiate into αβ lineage DP cells in the presence of Notch signaling, whereby differentiation of pre-TCR–expressing cells requires less Notch signaling than differentiation of cells with a γδTCR or αβTCR. Differentiation into γδ T cells or DN cells with a γδ lineage phenotype but αβTCR expression (references 30 and 31) does not require continual Notch signaling. Because of less of a requirement for Notch signaling of pre-TCR–expressing precursors, these cells usually out-compete cells with γδTCRs or αβTCRs for entry into the αβ lineage. Entry into the αβ lineage is associated with silencing of the *Tcrg* locus, deletion of the *Tcrd* locus, and *Tcra* rearrangement.

and/or TCRα expression that may depend on the Eδ enhancer before the Eα enhancer becomes active (12, 43). Thus, the αβ lineage cells generated by the synergy of γδTCR or αβTCR and Notch signaling represent remnants of a mechanism in place before the advent of the pre-TCR. This "old" pathway resulted in generation of bona fide αβ lineage cells through silencing of the *Tcrg* locus (29) and elimination of the *Tcrd* locus, thus terminating γδTCR expression (44). Silencing of the *Tcrg* locus and deletion of the *Tcrd* locus may likewise be instructed by the synergy of Notch and pre-TCR signaling that results in the generation of αβ lineage cells.

MATERIALS AND METHODS

Mice. *Rag-1*–deficient mice were bred and maintained in the animal facilities at the Dana-Farber Cancer Institute. pTα$^{-/-}$, lck-pTα, and lck-TCRα mice were described previously (5, 21, 25, 45). TCRγδ transgenic mice were generated in our laboratory using TCRγ and TCRδ cDNAs derived from the DTN40 TCRγδ hybridoma (46). TCRγδ cDNA was inserted into the TetO-SB vector that contains a tetracycline regulatable promoter based on the Tet operator. Inducible TCR expression was obtained by crossing these mice on the LTH-1 strain, in which the lck proximal promoter drives T cell–specific expression of the TetR-VP16 transactivator (47). These mice were bred on the *Rag-1*$^{-/-}$ background. For the described studies, the TCRγδ transgenic mice were kept in the absence of tetracycline to allow transgene expression. All mice were kept in specific pathogen-free animal facilities at the Dana-Farber Cancer Institute. All animal procedures were performed in compliance with the guidelines of the Dana-Farber Cancer Institute Animal Resources Facility, which operates under regulatory requirements of the U.S. Department of Agriculture and Association for Assessment and Accreditation of Laboratory Animal Care.

Cell lines. OP9 bone marrow stromal cells expressing the Notch ligand DL-1 (OP9-DL1) and OP9-control cells (OP9-GFP) were maintained in αMEM supplemented with 55 μM 2-mercaptoethanol, 10 mM Hepes, pH 7.5, 1 mM sodium pyruvat, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 50 μg/ml gentamycine, and 20% heat-inactivated FBS and passaged as described previously (18).

Flow cytometry and cell sorting. mAbs specific for CD4 (RM4-5 and GK1.5), CD8 (53-6.7), CD25 (PC61), CD44 (IM7), TCRβ (H57-597), TCRγδ (GL3), Gr-1 (RB6-8C5), CD19 (1D3), CD11c (HL3), CD11b (M1/70), pan-NK (DX5), CD45.1 (A20), and CD45.2 (104) were purchased from BD Biosciences and used as biotin, FITC, PE, peridinin chlorophyll protein, or allophycocyanin (APC) conjugates. The anti-Notch1, extracellular domain (rabbit polyclonal IgG) antibody was purchased from Upstate Biotechnology. Notch surface staining was performed using a secondary biotinylated goat anti–rabbit antibody. Purified normal rabbit IgG was included as staining control. APC- and PE-conjugated streptavidin was used to reveal staining with biotinylated mAb. Surface staining of thymocytes was conducted as described previously (12, 21). Four-color flow cytometry was performed on a FACSCalibur (Becton Dickinson). Data were analyzed with CellQuestPro software (BD Biosciences) and FlowJo (Tree Star). For analysis, dead cells and debris were excluded by appropriate gating of forward and sideward scatter and controlled by 7-AAD (BD Biosciences) staining. DN cells were isolated from total thymocytes by staining cell suspensions with a biotinylated lineage-specific antibody cocktail (CD4, CD8, CD19, DX5, Gr-1, CD11b, CD11c, TCRγδ, and TCRβ for DN3 cells and CD4, CD8, CD19, DX5, Gr-1, CD11b, and CD11c for DN4 cells, respectively), followed by incubation with streptavidin–conjugated magnetic beads (Dynal) and magnetic bead depletion of mature lineages. Enriched cell suspensions were surface stained with anti-CD44, anti-CD25, and streptavidin-APC. The DN3 or DN4 subsets were isolated by sorting lin$^-$CD44$^-$CD25$^+$ cells or lin$^-$CD44$^-$CD25$^-$ cells, respectively. Cells were sorted using a FACSAria (Becton Dickinson), and sorted cells were of ≥99% purity, as determined by postsort analysis.

OP9 cocultures. Before OP9 cocultures, sorted DN3 or DN4 cells were incubated with 1 μM CFSE (Invitrogen) at 1–5 × 10^6 cells/ml PBS, 0.1% BSA for 10 min at 37°C. Cells were washed extensively with OP9 medium to eliminate the remaining CFSE and plated onto subconfluent OP9-GFP or OP9-DL1 monolayers at 5 × 10^4 cells/well in a 24-well plate. For competition assays, DN3 cells derived from different transgenic mice were plated in a 1:1 ratio. All cocultures were performed in the presence of 1 ng/ml IL-7 and 5 ng/ml Flt3 ligand. Equal volumes of the GSI X (Calbiochem), serially diluted in DMSO or 0.1% DMSO carrier alone, were added to selected wells on day 0 and replaced together with the cytokines every 4 d. Cell division and developmental progression of CFSE-labeled cells were analyzed by flow cytometry on the indicated days of coculture. Contaminating OP9 cells were eliminated by filtering the harvested cocultured cells through a 70-μM cell strainer before flow cytometric analysis. In cases in which total cellularity is indicated, cell counts were performed by trypan blue exclusion.

Online supplemental material. By depicting total cell counts of DN3 cells from the different strains of mice, Table S1 shows that the survival of DN3 cells is dependent on Notch signaling and TCR expression. Fig. S1 shows by flow cytometric analysis of CD4 and CD8 expression of CFSE-labeled cells that proliferation and development of DN4 cells into αβ lineage DP cells require continuous Notch signaling. Fig. S2 shows the analysis of proliferation, survival, and differentiation of DN3 and DN4 cells from B6 control mice with and without Notch signaling. Fig. S3 demonstrates that proliferation and development of DN3 cells into αβ lineage DP cells require Notch signaling. Fig. S4 shows the quantitative RT-PCR analysis of HES1 expression in DN3 cells as well as αβ lineage DP cells that were generated from these DN3 cells during a 3- or 5-d culture period on OP9-DL1 cells.

REFERENCES

1. Snodgrass, H.R., Z. Dembic, M. Steinmetz, and H. von Boehmer. 1985. Expression of T-cell antigen receptor genes during fetal development in the thymus. *Nature*. 315:232–233.

2. Raulet, D.H., R.D. Garman, H. Saito, and S. Tonegawa. 1985. Developmental regulation of T-cell receptor gene expression. *Nature*. 314:103–107.

3. Groettrup, M., K. Ungewiss, O. Azogui, R. Palacios, M.J. Owen, A.C. Hayday, and H. von Boehmer. 1993. A novel disulfide-linked heterodimer on pre-T cells consists of the T cell receptor beta chain and a 33 kd glycoprotein. *Cell*. 75:283–294.

4. Saint-Ruf, C., K. Ungewiss, M. Groettrup, L. Bruno, H.J. Fehling, and H. von Boehmer. 1994. Analysis and expression of a cloned pre-T cell receptor gene. *Science*. 266:1208–1212.

5. Fehling, H.J., A. Krotkova, C. Saint-Ruf, and H. von Boehmer. 1995. Crucial role of the pre-T-cell receptor alpha gene in development of alpha beta but not gamma delta T cells. *Nature*. 375:795–798 (published erratum appears in *Nature*. 1995. 378:419)

6. Kishi, H., P. Borgulya, B. Scott, K. Karjalainen, A. Traunecker, J. Kaufman, and H. von Boehmer. 1991. Surface expression of the beta T cell receptor (TCR) chain in the absence of other TCR or CD3 proteins on immature T cells. *EMBO J*. 10:93–100.

7. Trop, S., M. Rhodes, D.L. Wiest, P. Hugo, and J.C. Zuniga-Pflucker. 2000. Competitive displacement of pT alpha by TCR-alpha during TCR assembly prevents surface coexpression of pre-TCR and alpha beta TCR. *J. Immunol*. 165:5566–5572.

8. Scott, B., H. Bluthmann, H.S. Teh, and H. von Boehmer. 1989. The generation of mature T cells requires interaction of the alpha beta T-cell receptor with major histocompatibility antigens. *Nature*. 338:591–593.

9. von Boehmer, H. 2004. Selection of the T-cell repertoire: receptor-controlled checkpoints in T-cell development. *Adv. Immunol*. 84:201–238.

10. Teh, H.S., P. Kisielow, B. Scott, H. Kishi, Y. Uematsu, H. Bluthmann, and H. von Boehmer. 1988. Thymic major histocompatibility complex antigens and the alpha beta T-cell receptor determine the CD4/CD8 phenotype of T cells. *Nature*. 335:229–233.

11. Groves, T., M. Parsons, N.G. Miyamoto, and C.J. Guidos. 1997. TCR engagement of CD4+CD8+ thymocytes in vitro induces early aspects of positive selection, but not apoptosis. *J. Immunol*. 158:65–75.

12. Buer, J., I. Aifantis, J.P. DiSanto, H.J. Fehling, and H. von Boehmer. 1997. Role of different T cell receptors in the development of pre–T cells. *J. Exp. Med*. 185:1541–1547.

13. Kang, J., M. Coles, D. Cado, and D.H. Raulet. 1998. The developmental fate of T cells is critically influenced by TCRgammadelta expression. *Immunity*. 8:427–438.

14. Teh, H.S., H. Kishi, B. Scott, and H. Von Boehmer. 1989. Deletion of autospecific T cells in T cell receptor (TCR) transgenic mice spares cells with normal TCR levels and low levels of CD8 molecules. *J. Exp. Med*. 169:795–806.

15. Washburn, T., E. Schweighoffer, T. Gridley, D. Chang, B.J. Fowlkes, D. Cado, and E. Robey. 1997. Notch activity influences the alphabeta versus gammadelta T cell lineage decision. *Cell*. 88:833–843.

16. Radtke, F., A. Wilson, G. Stark, M. Bauer, J. van Meerwijk, H.R. MacDonald, and M. Aguet. 1999. Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity*. 10:547–558.

17. Tanigaki, K., M. Tsuji, N. Yamamoto, H. Han, J. Tsukada, H. Inoue, M. Kubo, and T. Honjo. 2004. Regulation of alphabeta/gammadelta T cell lineage commitment and peripheral T cell responses by Notch/RBP-J signaling. *Immunity*. 20:611–622.

18. Schmitt, T.M., and J.C. Zuniga-Pflucker. 2002. Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. *Immunity*. 17:749–756.

19. Ciofani, M., T.M. Schmitt, A. Ciofani, A.M. Michie, N. Cuburu, A. Aublin, J.L. Maryanski, and J.C. Zuniga-Pflucker. 2004. Obligatory role for cooperative signaling by pre-TCR and Notch during thymocyte differentiation. *J. Immunol.* 172:5230–5239.

20. Ciofani, M., and J.C. Zuniga-Pflucker. 2005. Notch promotes survival of pre-T cells at the beta-selection checkpoint by regulating cellular metabolism. *Nat. Immunol.* 6:881–888.

21. Aifantis, I., C. Borowski, F. Gounari, H.D. Lacorazza, J. Nikolich-Zugich, and H. von Boehmer. 2002. A critical role for the cytoplasmic tail of pTalpha in T lymphocyte development. *Nat. Immunol.* 3:483–488.

22. Hayes, S.M., L. Li, and P.E. Love. 2005. TCR signal strength influences alphabeta/gammadelta lineage fate. *Immunity*. 22:583–593.

23. Haks, M.C., J.M. Lefebvre, J.P. Lauritsen, M. Carleton, M. Rhodes, T. Miyazaki, D.J. Kappes, and D.L. Wiest. 2005. Attenuation of gammadeltaTCR signaling efficiently diverts thymocytes to the alphabeta lineage. *Immunity*. 22:595–606.

24. Taghon, T., M.A. Yui, R. Pant, R.A. Diamond, and E.V. Rothenberg. 2006. Developmental and molecular characterization of emerging beta- and gammadelta-selected pre-T cells in the adult mouse thymus. *Immunity*. 24:53–64.

25. Borowski, C., X. Li, I. Aifantis, F. Gounari, and H. von Boehmer. 2004. Pre-TCRα and TCRα are not interchangeable partners of TCRβ during T lymphocyte development. *J. Exp. Med.* 199:607–615.

26. Passoni, L., E.S. Hoffman, S. Kim, T. Crompton, W. Pao, M.Q. Dong, M.J. Owen, and A.C. Hayday. 1997. Intrathymic delta selection events in gammadelta cell development. *Immunity*. 7:83–95.

27. Livak, F., H.T. Petrie, I.N. Crispe, and D.G. Schatz. 1995. In-frame TCR delta gene rearrangements play a critical role in the alpha beta/gamma delta T cell lineage decision. *Immunity*. 2:617–627.

28. Aifantis, I., O. Azogui, J. Feinberg, C. Saint-Ruf, J. Buer, and H. von Boehmer. 1998. On the role of the pre-T cell receptor in alphabeta versus gammadelta T lineage commitment. *Immunity*. 9:649–655.

29. Bonneville, M., I. Ishida, P. Mombaerts, M. Katsuki, S. Verbeek, A. Berns, and S. Tonegawa. 1989. Blockage of alpha beta T-cell development by TCR gamma delta transgenes. *Nature*. 342:931–934.

30. Bruno, L., H.J. Fehling, and H. von Boehmer. 1996. The alpha beta T cell receptor can replace the gamma delta receptor in the development of gamma delta lineage cells. *Immunity*. 5:343–352.

31. Terrence, K., C.P. Pavlovich, E.O. Matechak, and B.J. Fowlkes. 2000. Premature expression of T cell receptor (TCR)αβ suppresses TCRγδ gene rearrangement but permits development of γδ lineage T cells. *J. Exp. Med.* 192:537–548.

32. Lehar, S.M., J. Dooley, A.G. Farr, and M.J. Bevan. 2005. Notch ligands Delta 1 and Jagged1 transmit distinct signals to T-cell precursors. *Blood*. 105:1440–1447.

33. Ciofani, M., G.C. Knowles, H. von Boehmer, and J.C. Zuniga-Pflucker. 2006. Stage-specific and differential Notch dependency at the ab/gd T lineage bifurcation. *Immunity*. In press.

34. Nie, L., M. Xu, A. Vladimirova, and X.H. Sun. 2003. Notch-induced E2A ubiquitination and degradation are controlled by MAP kinase activities. *EMBO J.* 22:5780–5792.

35. Swat, W., Y. Shinkai, H.L. Cheng, L. Davidson, and F.W. Alt. 1996. Activated Ras signals differentiation and expansion of CD4+8+ thymocytes. *Proc. Natl. Acad. Sci. USA*. 93:4683–4687.

36. Crompton, T., K.C. Gilmour, and M.J. Owen. 1996. The MAP kinase pathway controls differentiation from double-negative to double-positive thymocyte. *Cell*. 86:243–251.

37. Fischer, A.M., C.D. Katayama, G. Pages, J. Pouyssegur, and S.M. Hedrick. 2005. The role of erk1 and erk2 in multiple stages of T cell development. *Immunity*. 23:431–443.

38. Engel, I., and C. Murre. 2001. The function of E- and Id proteins in lymphocyte development. *Nat. Rev. Immunol.* 1:193–199.

39. Campese, A.F., A.I. Garbe, F. Zhang, F. Grassi, I. Screpanti, and H. von Boehmer. 2006. Notch1-dependent lymphomagenesis is assisted by but does not essentially require pre-TCR signaling. *Blood*. In press.

40. von Boehmer, H., I. Aifantis, O. Azogui, J. Feinberg, C. Saint-Ruf, C. Zober, C. Garcia, and J. Buer. 1998. Crucial function of the pre-T-cell receptor (TCR) in TCR beta selection, TCR beta allelic exclusion and alpha beta versus gamma delta lineage commitment. *Immunol. Rev.* 165:111–119.

41. Saint-Ruf, C., M. Panigada, O. Azogui, P. Debey, H. von Boehmer, and F. Grassi. 2000. Different initiation of pre-TCR and gammadeltaTCR signalling. *Nature*. 406:524–527.

42. Yamasaki, S., E. Ishikawa, M. Sakuma, K. Ogata, K. Sakata-Sogawa, M. Hiroshima, D.L. Wiest, M. Tokunaga, and T. Saito. 2006. Mechanistic basis of pre-T cell receptor-mediated autonomous signaling critical for thymocyte development. *Nat. Immunol.* 7:67–75.

43. Sleckman, B.P., B. Khor, R. Monroe, and F.W. Alt. 1998. Assembly of productive T cell receptor δ variable region genes exhibits allelic inclusion. *J. Exp. Med.* 188:1465–1471.

44. Chien, Y.H., M. Iwashima, K.B. Kaplan, J.F. Elliott, and M.M. Davis. 1987. A new T-cell receptor gene located within the alpha locus and expressed early in T-cell differentiation. *Nature*. 327:677–682.

45. Lacorazza, H.D., C. Tucek-Szabo, L.V. Vasovic, K. Remus, and J. Nikolich-Zugich. 2001. Premature TCR alpha beta expression and signaling in early thymocytes impair thymocyte expansion and partially block their development. *J. Immunol.* 166:3184–3193.

46. Azuara, V., M.P. Lembezat, and P. Pereira. 1998. The homogeneity of the TCRdelta repertoire expressed by the Thy-1dull gammadelta T cell population is due to cellular selection. *Eur. J. Immunol.* 28:3456–3467.

47. Labrecque, N., L.S. Whitfield, R. Obst, C. Waltzinger, C. Benoist, and D. Mathis. 2001. How much TCR does a T cell need? *Immunity*. 15:71–82.