Visualization of CD4/CD8 T Cell Commitment

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Summary

A system to innocuously visualize T cell lineage commitment is described. Using a "knock-in" approach, we have generated mice expressing a β-galactosidase reporter in place of CD4; expression of β-galactosidase in these animals appears to be an accurate and early indicator of CD4 gene transcription. We have exploited this knock-in line to trace CD4/CD8 lineage commitment in the thymus, avoiding important pitfalls of past experimental approaches. Our results argue in favor of a selective model of thymocyte commitment, demonstrating a fundamentally symmetrical process: engagement of either class of major histocompatibility complex (MHC) molecule by a differentiating CD4+CD8+ cell can give rise to T cell antigen receptor (TCR)hi thymocytes of either lineage. Key findings include (a) direct demonstration of a substantial number of CD4-committed, receptor/coreceptor-mismatched cells in MHC class II-deficient mice, a critical prediction of the selective model; (b) highly efficient rescue of such "mismatched" intermediates by forced expression of CD8 in a TCR transgenic line, and an explanation of why previous experiments of this nature were less successful—a major past criticism of the selective model; (c) direct demonstration of an analogous, though smaller, population of CD8-committed mismatched intermediates in class I-deficient animals. Finally, we found no evidence of a CD4 default pathway.

Key words: homologous recombination • transgenesis • positive selection • CD4 • β-galactosidase

Most conventional T lymphocytes fall into two classes—MHC class II-restricted, CD4+ helper, and MHC class I-restricted, CD8+ cytotoxic cells. Precursors of both classes differentiate in the thymus, according to an elaborate program classically visualized by following changes in expression of cell surface markers, in particular the CD4 and CD8 coreceptors (1, 2). The most immature thymocytes express no (or very little) CD4 or CD8 and are thus termed double-negative (CD4-CD8-; DN) cells; they also display no α/β TCR on the surface. DN thymocytes differentiate into double-positive (CD4+CD8+; DP) cells, most of which express low levels of surface TCR. Only 3–5% of DP thymocytes survive the transition to end-stage single-positive (SP) cells (3, 4) committed to either the CD4+ or CD8+ lineage and expressing high levels of TCR.

Abbreviations used in this paper: β2m, β2-microglobulin; B6, C57Bl/6; βg4, β-galactosidase; DN, double-negative; DP, double-positive; ES, embryonic stem; FDG, fluorescein digalactopyranoside; I, MHC class I-negative; II, MHC class II-negative; P1/P2, MHC double-deficient; PGK, phosphoglycerate kinase; RT, reverse transcriptase; SP, single-positive; tg, transgenic.

Survival and commitment at the DP stage of thymocyte differentiation are dictated by positive selection events that depend critically on specific interaction between the thymocyte's TCR and MHC molecules expressed on thymic stromal cells. Experiments involving TCR transgenic (tg) mice have demonstrated that DP thymocytes expressing class I-reactive TCRs are selected to become CD8+ cells (5–7), whereas those displaying class II-reactive receptors differentiate into CD4+ cells (8, 9). Thus, lineage commitment is an inseparable by-product of the positive selection process, the specificity of the TCR being matched to a particular class of MHC molecule and thereby to a particular coreceptor.

The mechanism by which this match is achieved and the nature of the signals directing CD4/CD8 lineage commitment remain unclear. For several years, discussions have crystallized around the issue of whether lineage choice is primarily instructive (directed by the TCR–MHC interaction) or selective (decided after an initially noncommittal TCR–MHC interaction), or is some combination of the two. A series of studies on MHC-deficient and TCR tg mice had concluded that DP thymocytes expressing a class I-reactive TCR can commit to either the CD4+ or CD8+...
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Construction of the Repetitive Vector and Production of CD4\(^{+/+}\) Mice. To obtain CD4\(^{+/+}\) mice, we constructed a targeting vector as illustrated in Fig. 1. In brief, a 7.8-kb AvrII-SphI genomic fragment from the CD4 locus was subcloned. A 323-bp fragment containing most of exons 2 and 3 (including a 30-bp intron) was deleted, and three cloning sites (XhoI, EcoRI, and XbaI) were inserted by single-strand mutagenesis. The fragment’s coding portion was fused to the remainder of exon 3: 5\'-GGGGCAGCATGGCAAAGGTGTATTAATTAGAG-3\'.

The resulting vector was linearized and electroporated into D3 embryonic stem (ES) cells as described (25). Of the 100 G418-resistant D3 clones analyzed, 6 carrying the predicted integration by homologous recombination were identified. Using an Xhol-SphI probe, we confirmed the presence of the normal 15-kb band plus an additional 9.6-kb band after XhoI digestion and Southern blotting. The positive clones were further confirmed using an internal neo probe. Clone VE34 was expanded and injected into C57Bl/6 (B6) blastocysts, which were reimplanted into pseudopregnant females. Chimeras were crossed onto B6 mice to obtain germline transmission, and offspring carrying the mutation were maintained in a conventional animal facility.

“Homozygous” CD4\(^{+/+}\) mice were produced by crossing the CD4\(^{+/+}\) line with heterozygous CD4-deficient (CD4\(^{+/}\)) mice (26). Antibodies, cytometric analyses, and cell sorting. The following antibodies were used: FITC-labeled anti-CD8\(\alpha\), biotinylated anti-CD8\(\beta\), PE-labeled anti-CD4 (Catet Laboratories, Inc., Burlingame, CA); KT3, specific for CD3 (27); T3.70, specific for the \(\alpha\) chain of the transgene-encoded TCR (gift of H. van Boehmer, Institut N ecker, Paris, France [5]); and B-20.1, specific for \(\alpha\) (28). Texas Red-conjugated anti-rat antibodies and streptavidin-Cy5 were purchased from Jackson Immunoresearch Laboratories (West Grove, PA) or Catet Laboratories, Inc. Staining of thymocyte and lymph node suspensions was performed as described (25).

To obtain CD4\(^{+/+}\) mice with the LacZ gene targeted into the CD4 locus and experiments exploiting them to study CD4/CD8 lineage commitment.

Materials and Methods

Construciton of the Repetitive Vector and Production of CD4\(^{+/+}\) Mice. To obtain CD4\(^{+/+}\) mice, we constructed a targeting vector as illustrated in Fig. 1. In brief, a 7.8-kb AvrII-SphI genomic fragment from the CD4 locus was subcloned. A 323-bp fragment containing most of exons 2 and 3 (including a 30-bp intron) was deleted, and three cloning sites (XhoI, EcoRI, and XbaI) were inserted by single-strand mutagenesis. The fragment’s coding portion was fused to the remainder of exon 3: 5\'-GGGGCAGCATGGCAAAGGTGTATTAATTAGAG-3\'.
with 1 ml lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, plus protease inhibitors) on ice for 30 min. After lysis, supernatants were cleared of cellular debris by centrifugation at 13,000 rpm for 15 min. Cell lysates were preclearled twice for 30 min with 25 ml (50%) rat serum-coupled protein G-Sepharose 4FF (Amersham Pharmacia Biotech, Inc.). The first incubation was usually overnight. The precleared lysate was then preincubated for 1 h with protein G coupled to G1.5 (specific for CD4 [30]) or anti-βgal (Promega Corp., Madison, WI). As a control, precipitations were prepared with rat serum-coupled beads. Immunoprecipitations were washed three times with 1 ml lysis buffer (at 0.2% Triton X-100), then once with PBS.

25 μl of Læmmli buffer (60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 5% 2-ME, 0.001% bromophenol blue) was added to each immunoprecipitation. The samples were heated for 5 min at 100°C and then loaded and run on a 10% SDS-PAGE gel. Signals on the gel were enhanced using Enlightning (NEN Life Science Products, Boston, MA) after running and were quantified with a Bio Imaging Analyzer (Fuji Photo Film Co., Tokyo, Japan).

Reverse Transcriptase PCR. RNA was isolated from sorted cells by standard techniques. In brief, RNA was prepared by N-P-40 lysis from 1–5 × 10^6 sorted cells to which 10^6 HeLa cells were added as carrier. cDNA was synthesized by reverse transcriptase. Serial dilutions of the cDNA were then used as a template for PCR amplification with specific primers for CD8 (5'-ATGGAGGTCCACAGTGGGGTCAGC-3') and TCR-β (5'-GGCCAC-TGACCAGCACAGCATATAGGGTG-3'). After amplification (5 min at 94°C; 30 cycles of 94°C 30 s, 50°C 30 s, 72°C 1 min; 10 min at 72°C), the products were run on agarose gels, transferred, and detected by hybridization using CD8- or TCR-β10 min at 72°C (5 min at 94°C) PCR products, Boston, MA) after running and were quantified with a Bio Imaging Analyzer (Fuji Photo Film Co., Tokyo, Japan).

Results

Generation of CD4^{+/L} Mice. To provide a convenient and harmless means of monitoring CD4 gene activity, we set out to confabulate a protein reporter with an identical pattern of expression. βgal seemed a good reporter candidate because it is easy to visualize, is inert, and, being cytoplasmic, possesses no signaling capacity, a disadvantage of most cell-surface reporters (31, 32). We chose a “knock-in” approach rather than transgenesis of a promoter-reporter construct in order to mimic as closely as possible the complex transcriptional controls on the native CD4 gene (33–37). Thus, mice expressing βgal in place of CD4 were generated by targeting a βgal coding region into the CD4 locus via homologous recombination in ES cells (Fig. 1). To ensure faithful expression of the βgal reporter (and to inactivate the CD4 gene at the same time), we introduced the βgal coding sequences exactly at the CD4 initiation codon in exon 2. The inserted sequence directs the translation of a fusion protein comprised of βgal and the neomycin (neo) resistance domain (βgeo, chosen because it is known to be expressed in normal T cells [23]); the insert also contains an expressible neo gene under an independent PGK promoter for selection of ES cell transfectants (Fig. 1).

The final construct was electroporated into ES cells, and G418-resistant clones carrying the specific homologous recombination event were selected. Chimeras generated from the injection of a positive clone into B6 blastocysts were mated with B6 females to obtain germline transmission of the knock-in mutation. The resulting mouse line was generally kept in heterozygous form (CD4^{+/L}; hereafter “+”) designates the wild-type CD4 locus, “L” our targeted CD4-βgal insertion, and “0” the original CD4 knockout mutation of Killeen et al. [26] used in some of the crosses. In the heterozygotes, both βgal and CD4 (at half normal levels; data not shown) should be simultaneously expressed under the influence of elements that control the CD4 locus.

It was first necessary to determine just how closely the expression of βgal matched that of CD4. Lymph node cells and thymocytes were stained with FDG to detect cytoplasmic βgal expression (29) in addition to antibodies against CD4, CD8, and CD3, and were analyzed by flow cytometry (Fig. 2). βgal expression was essentially superimposable.

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**Figure 1.** Production of CD4^{+/L} mice. A 326-bp fragment containing most of exons 2 and 3 (including a 30-bp intron) was deleted and replaced by the βgeo and neo cassettes. The 5' external probe, an XhoI-AvrlI fragment, detects an endogenous 15-kb XhoI band on Southern blots of DNA from wild-type D3 ES cells and a smaller 9.6-kb band on blots of DNA from cells carrying the knock-in mutation. Exons are marked with roman numerals, untranslated regions by open boxes, and translated regions by filled boxes. Arrows below the boxes represent their 5’ to 3’ orientation. Bent arrows mark the transcription initiation sites. Restriction enzyme sites are abbreviated as follows: X (XhoI), B (BamHI), A (AvrlI), S (SphI), H (HindIII), E (EcoRI), Sp (SpeI), Sm (SmaI), P (PstI), C (CfrI), X b (Xbal), Sa (SalI), Sc (SacI), and Bg (BglII).
with that of CD4 in both primary and secondary lymphoid organs. In the lymph nodes, CD4+/L cells expressed high levels of βgal, whereas CD8+ and non-T cells expressed low to background levels (Fig. 2 A). Similarly in the thymus, CD4 SP and CD4+CD8+ DP cells were βgal+, whereas mature CD8 (CD3hi) SP and immature DN cells were not (Fig. 2 B). βgal did not appear in only a select subset of CD4+ T cells for example, the minor (and peculiar) NK1.1+CD4+ subset of α/β T cells (38) was also βgal+ in thymic and spleen cell suspensions of CD4+/L mice (not shown). Importantly, βgal expression did not detectably alter thymus cellularity or population distributions, as assessed with a panel of markers including CD3, CD4, CD8, CD69, CD24, CD5, CD44 and peanut agglutinin-binding polysaccharides (not shown).

Upon closer examination, we noticed some interesting features of the expression of the βgal reporter. First, CD3− thymocytes within the CD8 SP population, the immediate precursors of DP cells, were βgal+, implying that enzymatic activity was detectable earlier than surface expression of CD4 (Fig. 2 B). Second, a proportion of the CD4− CD8−CD3hi thymocytes expressed low levels of βgal, suggesting that reporter activity was downregulated earlier than surface CD4 upon positive selection. These observations argued that βgal levels responded faster to changes in transcriptional activity at the CD4 locus than the level of CD4 protein itself. To test this hypothesis, we compared the turnover of CD4 and βgal proteins (Fig. 2 C ). In vitro metabolic labeling with [35S]methionine and precipitation with antibodies against CD4 and βgal revealed that the half-life of βgal (4.1 h) was significantly shorter than that of CD4 (6.2 h).

Thus, not only was βgal a faithful reporter of CD4 gene expression, it also appeared to be an early indicator.

βgal as a Marker for CD4 and CD8 Lineage Commitment in the Thymus. Since βgal appeared to be an early indicator of CD4 gene transcription, it represented a valuable marker of lineage commitment during the initial stages of T cell selection in the thymus. Thus, we stained CD4+/L thymocytes simultaneously for CD4, CD8, CD3, and βgal. In Fig. 3 A, CD3hi thymocytes are displayed in a CD4/CD8 plot, and show the customary boomerang-shaped distribution extending from the mature CD4 SP to the mature CD8 SP compartments. Several distinct gates were set within this boomerang to permit us to investigate the activity of the CD4 gene, as reflected by βgal staining, within different populations. The terminally differentiated populations (gates B and C) showed essentially homogeneous patterns of βgal staining: CD4 SP thymocytes were βgal+, CD8 SP cells βgal-. In contrast, most of the intermediate populations (gates E, G, and F) contained cells of both phenotypes, even the small CD3hi subpopulation which displayed high surface levels of CD4 and CD8 (gate D). These profiles confirmed the notion that surface coreceptor levels do not fully and immediately reflect lineage commitment (18, 21), and encouraged us to exploit the βgal marker for investigating lineage commitment ex vivo under unmanipulated conditions. However, it should be kept in mind that, although the βgal phenotype unambiguously denotes a shut-off of CD4 transcriptional activity in this context, and thus commitment to the CD8 lineage, βgal+ populations cannot be interpreted as readily, potentially including cells that have not yet committed to or those that are already committed to the CD4 lineage.

CD4+/L mice were crossed with MHC class I−negative (I10) animals (25) to allow us to evaluate the commitment status of MHC class I−reactive CD4+CD8+ thymocytes, cells proposed to be transitory intermediates committed to the CD4 lineage (10). Thymocytes from I10 CD4+/L mice were analyzed as above (Fig. 3 B). The CD4+CD8int popu-
lation (gate E) split clearly into two subsets, $\beta$gal$^+$ and $\beta$gal$^-$; there was a significant increase in the $\beta$gal$^-$ subset compared with the same transitional population in the class II$^+$-positive mouse. This result is consistent with the notion that at least some CD8-committed thymocytes transiently downmodulate surface CD8 levels after positive selection (18, 21), but also suggests that a sizeable contingent of CD4-committed cells can be selected in the absence of MHC class II molecules.

That CD4-committed thymocytes were selected on MHC class I (in the absence of class II) molecules is substantiated in Fig. 4, a five-color cytofluorometric analysis of the expression of CD4, CD8, CD3, $\beta$gal, and CD69, an early and transient marker of positive selection (14, 39–41). When CD69$^+$ thymocytes from II$^0$ mice were displayed in CD3/$\beta$gal plots, it seemed that CD4$^+$ and CD8$^+$-committed thymocytes could be traced by virtue of their very positions within this plot. The CD8$^+$-committed cells appeared to reside chiefly within the CD3$^+$/$\beta$gal$^+$ and $\beta$gal$^-$ gates; as they lost $\beta$gal expression, they also began to switch from the transitional CD4$^+$CD8$^+$ phenotype to the definitive CD4$^+$CD8$^+$ and CD8 SP phenotypes (compare the CD4/CD8 profiles, gates A and B of Fig. 4). In contrast, only CD4$^+$ thymocytes were found within the CD3$^+$/$\beta$gal$^+$ population (gate C), well separated from cells along the CD8 pathway.

Although rendered unlikely by the very appearance of the profiles in Fig. 4, a caveat to these interpretations is that CD4$^+$CD8$^+$ cells with high $\beta$gal expression may still have been destined for the CD8 lineage, but just had not yet downregulated $\beta$gal expression. To address this point, we purified CD4$^+$CD8$^+$CD3$^+$/$\beta$gal$^+$ and CD4$^+$CD8$^+$CD3$^+$/$\beta$gal$^-$ thymocytes, and compared their CD8 mRNA con-

**Figure 3.** Expression of the $\beta$gal reporter in thymocyte populations. (A) $\beta$gal expression in CD4$^{+/+}$ mice. CD3$^+$ thymocytes were gated according to their CD4/CD8 expression into distinct populations: CD4 SP cells ($4^+$/$8^+$), transitional CD4$^+$CD8$^+$ cells ($4^+$/$8^-$), CD4$^+$CD8$^+$ cells ($4^+$/$8^+$), intermediate CD4$^+$CD8$^+$ cells ($4^+$/$8^+$), and CD8 SP cells ($4^+$/$8^+$). Histograms represent the intensity of $\beta$gal expression within each of these CD4/CD8 gates. The mean fluorescence intensity (MFI) values for CD3 expression within the respective $\beta$gal$^+$ and $\beta$gal$^-$ subsets are shown above each histogram, except in cases where there are few or no cells within the $\beta$gal gates. The separate histogram at the upper right hand corner represents the intensity of $\beta$gal expression for total DP thymocytes (B) $\beta$gal expression in I$I^0$ CD4$^{+/+}$ mice. $\beta$gal expression within CD4/CD8 thymocyte subsets of CD4$^{+/+}$ mice on an MHC class II-negative background. Thymocytes were analyzed as in A.

**Figure 4.** Multiparameter visualization of lineage commitment. Thymocytes from a I$I^0$ CD4$^{+/+}$ mouse were analyzed after gating on CD69$^+$ cells. The lower left panel displays the $\beta$gal/CD3 profile of these cells, from which four populations can be distinguished: cells that express the highest levels of CD3 but have downregulated $\beta$gal expression (A); others that also express the highest levels of CD3, but have maintained CD4 gene activity, as evidenced by full $\beta$gal staining (C); intermediates in the process of downregulating $\beta$gal (B); and immature thymocytes that express low levels of CD3 and high levels of $\beta$gal (D). The CD4/CD8 profiles of these populations are shown in the smaller panels.
tent by reverse transcriptase (RT)-PCR (Fig. 5). If the CD4+CD8intβgalhi cells were CD8-committed or even uncommitted, their CD8 mRNA levels should be as high as those detected in the CD4+CD8intβгалo or DP populations. However, if the CD4+CD8intβgalhi thymocytes were CD4-committed, they should have downregulated CD8 transcription at least to some degree. The latter scenario appears to be the correct one: CD4+CD8βgalhi thymocytes expressed on average two- to threefold less CD8 mRNA than their βгалo counterparts, suggesting that many, if not all, of the CD4+CD8βgalhi thymocytes in II0 mice were CD4 committed.

Our original results (10) and those of van Meerwijk and Germain (16) suggested that a mirror-image population, committed to the CD8 lineage upon selection on MHC class II molecules, also exists. However, this interpretation was questioned in subsequent reports (21, 22, 42). Therefore, we analyzed β2-microglobulin (β2m)-negative (I0) mice (43) in which the CD4-βgal reporter had been introduced, allowing a distinction between true CD8-committed cells and other populations (i.e., DPs, CD4-committed). The cytometric analysis in Fig. 6 A illustrates that CD4+CD8+CD3hi cells did exist in class I-deficient mice, albeit at a much lower frequency than their counterparts in class II-deficient animals, and that they displayed the βгалo phenotype expected of the CD8 lineage. The low expression of βгал established that these cells were not contaminants from the neighboring CD4intCD8+CD3hi population, the great majority of which were βгалo. Our results also indicated that the CD4intCD8+CD3hi cells were a homogeneous set, as we did not detect CD4-committed cells within this population, a result consistent with recent findings from other groups (18, 21, 44, 45).

In the same series of experiments, MHC double-deficient (I0I0) mice with a CD4+I0 genotype were analyzed (Fig. 6 B): the CD3hi CD4+CD8int and CD4intCD8- thymocyte populations present in I0 and I0 mice, respectively, were not observed in MHC-deficient animals, in agreement with our previous findings (10); the rare CD3+ cells were all βгалhi and probably represent nonstandard T lineages (46). This result confirms that the intermediate populations discussed above require MHC engagement in order to be positively selected, indicating that a CD4 lineage default pathway in the absence of MHC recognition does not exist in vivo.

Two Modes of CD8 Commitment. We wondered whether all T cells destined for the CD8 lineage transit through the CD4+CD8int stage, and just how early CD8-committed cells switch off CD4 gene activity. Therefore, we crossed the CD4+I0 line with two TCR tg lines expressing different MHC class I-restricted receptors: the “HY” TCR (selected on Dp) and the “OVA” TCR (selected on Kβ) (47, 48). Thymocytes from HY+CD4+I0 and OVA+CD4+I0 mice were stained for CD4, CD8, and βgal in addition to the appropriate V regions (Fig. 7; panels are gated on clonotypic cells, thereby focusing the analysis only on cells expressing the transgene-encoded receptor). In terms of βgal expression, the patterns were quite similar in the two types of animals: downregulation of the βgal marker began in the DP compartment, and the CD4+CD8int population contained both βгалhi and βгалo cells from the CD4int CD8int stage onwards, βgal expression was essentially shut off. Furthermore, both the timing and levels of TCR expression, as reflected by CD3 expression (not shown), were also similar for the two tg lines, not surprising since expression of both transgenes are driven by the same TCR Vβ promoter (Correia-Neves, M., unpublished results). However, the HY and OVA mice were strikingly different in their numbers of intermediate CD4+CD8int thymocytes. Consistent with a previous report (18), there were three to four times more transitional CD4+CD8int thymocytes in OVA than in HY mice. (Note that the OVA line used here expresses the same receptor as the OVA-TCR-1 line reported previously [48], but was generated using a different vector for transgenesis; that the two behave identically in having a large CD4+CD8int population indicates that this phenotype

![Figure 5. CD8 mRNA levels in transitional intermediates.](image)
more convoluted, generating large numbers of CD4<sup>+</sup> CD8<sup>int</sup> cells. This also appears to be true when endogenous TCR gene rearrangements in both transgenic lines are prevented by the SCID, RAG, or TCR Cα mutation (10; Heath, W., personal communication; Correia-Neves, M., unpublished results). Such a dichotomy suggests that there must be at least two modes (or a continuum) of class I–restricted CD8 lineage differentiation.

With both class I–restricted TCR tg lines, the CD4<sup>+</sup> CD8<sup>int</sup> populations contained βgal<sup>lo</sup> and βgal<sup>hi</sup> cells. βgal<sup>lo</sup> cells were most likely CD8-committed, in keeping with results from cell transfer experiments (18). A key question was whether the βgal<sup>hi</sup> cells were just precursors of the CD8-committed cells or whether they really included a CD4-committed population as we suspected. If there were CD4-committed cells destined to die because they bear mismatched T cell receptors and coreceptors, it should be possible to rescue them by restoring surface CD8 expression. Furthermore, the efficiency of the rescue should correlate with the ability of the TCR to generate the transitional CD4<sup>+</sup> CD8<sup>int</sup> βgal<sup>hi</sup> phenotype. Thus, one would predict that such a rescue experiment should be more successful in the OVA than the HY animals, which had previously yielded few rescued cells (49–51). We tested this prediction by crossing the HY and OVA lines with a transgenic mouse line expressing near-physiological levels of the CD8 α and β chains (CD8<sub>aβ</sub> [42]). Representative CD4/CD8 profiles of thymocytes and lymph node cells from these crosses are shown in Fig. 8. As observed previously (49–51), rescue of CD4<sup>+</sup>(tgCD8<sup>1</sup>) T cells was very limited in the HY mouse; in striking contrast, close to half of the peripheral clonotype<sup>hi</sup> lymphocytes in the OVA cross were of the CD4 lineage. Thus, there was a direct relationship between the number of CD4<sup>+</sup>CD8<sup>int</sup>βgal<sup>hi</sup> thymocytes and the number of rescued class I–restricted CD4<sup>+</sup>(tgCD8<sup>+</sup>) cells. We also observed a significant decrease in the positive selection of OVA<sup>hi</sup> thymocytes into the CD8 SP compartment. As the affinity of the OVA receptor may be quite high for its selecting ligand...
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it is possible that the CD8-committed cells were deleted due to the higher levels of CD8 expression. CD4 Lineage Cells in the Absence of CD4. Finally, we made use of the βgal reporter mice to examine the issue of CD4 lineage commitment in the absence of CD4. Previous studies had demonstrated a population of MHC class II-restricted, CD4⁺CD8⁻ TCR hi Th cells in CD4-deficient mice (26, 52–54). Although it was never formally demonstrated, these cells were quite logically considered to belong to the CD4 lineage.

We readdressed this issue by analyzing offspring from crosses of CD4-βgal knock-in mice with one of the original CD4 knockout lines (26), comparing profiles of βgal⁺ cells in heterozygous CD4⁺/L animals with those in fully deficient CD4⁻/L animals. In a four-color cytofluorimetric analysis using Fdg and antibodies against CD4, CD8, and CD3, the usual CD4⁺CD8⁻ profile can be substituted with a βgal⁺CD8⁻ profile (Fig. 9). In CD4⁺ mice, the βgal⁺CD8⁻ cells in the thymus and lymph nodes were essentially all CD4⁺CD3hi, as expected; on average, they amounted to 4.0 and 15.1% of total thymocytes and lymphocytes, respectively. When the same gate was applied to βgal⁺CD8⁻ cells from CD4-negative CD4⁻/L mice, the same populations of CD3⁺ T cells were present (now CD4⁻). The thymocyte population was only moderately diminished in number, whereas the reduction was more marked in the lymph nodes. The βgal⁺CD8⁻ cells were analyzed for various surface markers and for their TCR Vβ region usage. Preliminary results indicated that these cells were not particularly different in CD4⁺ and CD4⁻ mice, implying that the cells selected in the absence of CD4 were not a special subset of Th cells (not shown).

These results formally demonstrate that thymocytes can be selected into the CD4 lineage in the absence of cell surface CD4.

Discussion

The CD4-βgal knock-in mouse line provides an accurate, convenient, and harmless means of monitoring CD4...
gene activity directly ex vivo. Expression of the βgal reporter faithfully mimicked CD4 expression, was readily detectable, and had no detectable immunological consequences. The reporter could be used whether or not the CD4 molecule itself was present. The choice of reporter also proved advantageous because the half-life of the βgeo chimeric protein was significantly shorter than that of CD4, meaning that βgal activity more closely approximated CD4 gene transcription than surface display of CD4 itself. In this report, we have exploited the CD4-βgal line to study CD4/CD8 lineage commitment.

CD4-committed intermediates in the absence of MHC class II molecules. For some years, debate on the mechanism of CD4/CD8 lineage commitment has centered around the issue of whether commitment is essentially instructive or selective (49, 55, 56). A crucial distinguishing feature of these two models is the possibility of transitional populations, with mismatched receptors and coreceptors in the latter but not the former. Evidence for such populations was provided by studies on MHC-deficient and TCR tg mice and by rescue experiments based on forced expression of coreceptors (11, 12, 14, 15). However, a key finding in several of these studies was brought into question because of results from transfer experiments (18, 19) and coreceptor reexpression assays (21). In particular, these data were considered to invalidate the support given the selective model of lineage commitment by the demonstration of transitional intermediates in class II- and class I-deficient mice. We now exploit the βgal marker to establish that the CD4+CD8int population from class II-deficient mice includes cells committed to both the CD4 and CD8 lineages. CD4-committed cells maintain active CD4 gene transcription, whereas CD8-committed cells have turned off the CD4 gene, as reflected by expression of the βgal reporter (Figs. 3B and 5).

It might be argued that the CD4+CD8int-βgalhi thymocytes seen in class II-deficient mice are just precursors of the βgallo cells, having not yet shut down transcription of the CD4 gene. Several observations are inconsistent with this contention: the low level of CD8 mRNA in CD4+CD8int-βgalhi cells compared with their βgallo counterparts, as predicted if they are of the CD4 lineage; the very position of the βgalhi cells on the βgal/CD3 plot, visibly on a different branch than that of the βgallo cells (Fig. 4); the correlation between the numbers of CD4+CD8int βgalhi cells and the ability of the CD8 transgenes to rescue the CD4-committed cells in OVA and HY transgenic mice (Figs. 7 and 8). Finally, we have recently found that altering the avidity of TCR signaling by introducing a CD5 knockout mutation (57) increases the numbers of CD4+CD8int-βgalhi cells in II0 mice, without affecting CD4+CD8int βgallo numbers, a change in ratio incompatible with a simple precursor/product relationship (Chan, S., manuscript in preparation).

The data presented here do not really contradict those from the transfer and coreceptor reexpression studies (18, 19, 21, 22). The transfer experiments (18, 19, 58) demonstrated that the CD4+CD8int population from II0 mice contained CD8-committed thymocytes, but did not rule out that it also includes CD4-committed cells because, having mismatched receptors and coreceptors, these cells should not have survived in the host wild-type thymus any better than in the II0 donor. As for the coreceptor reexpression assays, the data actually did show that cells committed to both lineages were present within the CD4+CD8int population of II0 mice (Fig. 4A in reference 21). The existence of CD4-committed cells was discounted by these authors in favor of a CD4 “default selection” pathway, hypothesized to occur independently of MHC engagement. However, no evidence for such a pathway has been observed in several studies (22, 41; Fig. 6B), so that it appears reasonable to equate the CD4-committed CD4+CD8int cells described in this report with those that reexpressed only CD4 after pronase treatment.

The existence of CD4-committed, but class I-reactive, intermediates is consistent with results from the older experiments forcing expression of coreceptors via transgenesis (11, 12, 14). Our data (Figs. 7 and 8) extend the older data by fulfilling, in two TCR transgenic systems, the prediction that the numbers of intermediates with mismatched receptors and coreceptors should correlate with their ability to be rescued to full maturity by artificially expressing the appropriate coreceptor. Our results also explain the range in observations made in previous rescue experiments, relatively efficient rescue being obtained in some cases (11, 14, 15, 59) but not in others (49, 50). Inefficient rescue has been cited repeatedly as evidence for instructive or hemi-instructive models (11, 12, 21). It is clear now that rescue experiments are not inherently inefficient but one must choose an appropriate TCR tg system that provides enough intermediates to be rescued.

The asymmetry of commitment. The present data reaffirm the existence of CD4 lineage-committed transitional intermediates that were nixed down the differentiation pathway by engagement of their TCRs by MHC class I molecules in the absence of MHC class II molecules. An analogous population of CD8 lineage-committed cells was hypothesized when CD4+CD8+ cells were found in class I-deficient mice (10, 16), but their existence has been questioned (20–22, 42). Our findings confirm that transitional intermediates committed to the CD8 lineage in the absence of MHC class I molecules do exist, as evidenced by the absence of βgal expression in CD4+CD8intCD3hi cells in I0 mice (Fig. 6A). This result is consistent with reports that transgenic mice expressing class II-restricted TCRs (17, 60) and MHC-deficient mice complemented with MHC class II genes delivered by an adenovirus vector injected intrathymically (Rokee, R., manuscript in preparation) can give rise to mature bona fide CD8+ cells. However, the numbers of CD8-committed transitional intermediates in class I-deficient mice are low: 2.3 ± 0.9% of the numbers in normal mice (n = 5), compared with 20 ± 5% the normal numbers for CD4-committed intermediates in class II-negative animals. The rarity of the CD4+CD8+ cells may explain why they were not detected by other groups—either because three-color bromodeoxyuridine la-
Asymmetry is also evident in the fact that no CD4-committed equivalent of CD8-committed CD4<sup>+</sup>CD8<sup>+</sup> thymocytes (waltzers) has been described. Here, we detected no βgal<sup>hi</sup> cells in the CD4<sup>+</sup>CD8<sup>+</sup> population, in good agreement with findings from several groups (18, 21, 22). Thus, while commitment to the CD8 lineage can launch at least two different modes of differentiation, either direct progression to the CD4<sup>+</sup>CD8<sup>+</sup> phenotype as seen for cells in the H<sup>2</sup>b and H-2<sup>m13</sup> backgrounds to different avidities for the negatively selecting ligands (13). At present, it is not clear whether stronger signals promote differentiation along the CD4 or CD8 pathway, as arguments have been presented on both sides (14, 49, 66). Thymocyte-extrinsic factors within the thymus milieu as well as intrinsic factors of a more generalized nature also play a role in determining cell fate. Concerning the former, it should be kept in mind that the thymic stroma is very heterogeneous, containing niches where the expression of self MHC molecules can vary markedly (4, 67); concerning the latter, recent results on N<sup>otch</sup> are intriguing (68).

Asymmetry is also evident in the fact that no CD4-committed equivalent of CD8-committed CD4<sup>+</sup>CD8<sup>+</sup> thymocytes (waltzers) has been described. Here, we detected no βgal<sup>hi</sup> cells in the CD4<sup>+</sup>CD8<sup>+</sup> population, in good agreement with findings from several groups (18, 21, 22). Thus, while commitment to the CD8 lineage can launch at least two different modes of differentiation, either direct progression to the CD4<sup>+</sup>CD8<sup>+</sup> phenotype as seen for cells in the H<sup>2</sup>b and H-2<sup>m13</sup> backgrounds to different avidities for the negatively selecting ligands (13). At present, it is not clear whether stronger signals promote differentiation along the CD4 or CD8 pathway, as arguments have been presented on both sides (14, 49, 66). Thymocyte-extrinsic factors within the thymus milieu as well as intrinsic factors of a more generalized nature also play a role in determining cell fate. Concerning the former, it should be kept in mind that the thymic stroma is very heterogeneous, containing niches where the expression of self MHC molecules can vary markedly (4, 67); concerning the latter, recent results on N<sup>otch</sup> are intriguing (68).

Conclusions. The results presented here argue that CD4/CD8 lineage commitment is fundamentally symmetrical, in that engagement of either class of MHC molecule by a differentiating DP thymocyte can give rise to transitional intermediates committed to either lineage. There does not appear to be a CD4 lineage default pathway, nor any special requirements to provoke commitment to the CD8 lineage. Along both routes of differentiation, the initial lineage choice is validated at a later stage, when only those cells expressing appropriately matched T cell receptors and coreceptors are permitted to survive.

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