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High-level Constitutive Expression of Mouse CD4 and CD4/CD8α Hybrid Molecules in Transgenic Mice

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The CD4 and CD8 coreceptors, in conjunction with the T cell receptor (TCR), make important contributions to the differentiation of thymocytes. They have been shown to be involved in the clonal deletion and positive selection processes during T cell development in thymus. To further analyze the role of CD4 and CD8 proteins during T cell differentiation, we have generated transgenic mice constitutively expressing high levels of a native CD4 and a CD4/CD8α hybrid protein. The hybrid protein is composed of CD4 extracellular domain linked to the CD8α transmembrane region and cytoplasmic tail. The transgenes were driven by human beta-actin promoter, and therefore, they were expressed in all tissues examined including thymus, spleen, and lymph nodes. The resulting CD4 and CD4/CD8α transgenic mice were found to express the CD4 and CD4/CD8α, respectively, in developing thymocytes and peripheral T cells. The expression levels of transgenic proteins were 5-10 times higher than that of endogenous CD4 in thymus. However, total surface CD4 expression (CD4 or CD4/CD8α transgenic protein plus endogenous CD4) of the transgenic mice were maintained at similar levels compared to control littermates. Surface CD4 expression on CD8 T cells, however, was significantly lower than that on cells expressing endogenous CD4. These results suggest that a total avidity between developing thymocytes and thymic stromal cells is important for differentiation of thymocytes.

Mature T cells develop in the thymus through a series of regulated differentiation events. During the course of thymic development, selection events occur to eliminate self-reactive T cells (negative selection) and retain T cells restricted to self-major histocompatibility complex (MHC) molecules (positive selection) (Loh, 1991; von Boehmer, 1990). The T cell lineage branches into cells with αβ T cell receptor (TCR) and γδ TCR for antigen. Most mature T cells express αβ TCR and can be subdivided based on their expression of CD4 or CD8 membrane glycoproteins into two phenotypically and functionally different major subtypes; T cells expressing CD4 (CD4 T cells) whose clonotypic TCR recognizes antigenic peptides presented by MHC class II molecules and T cells expressing CD8 (CD8 T cells) whose TCR recognizes peptides presented by MHC class I molecules. CD4 T cells and CD8 T cells serve mostly as helper T cells and cytotoxic T cells, respectively (Swain, 1983).

A central feature of positive selection in the thymus, therefore, is the generation of T cells with matched TCR and coreceptor specificities, such that CD4 T cells express TCRs specific for MHC class II recognition and CD8 T cells express TCRs specific for MHC class I recognition. Recent studies have shown that the major lineage of lymphocytes expressing αβ TCR follows a distinct route upon entering the thymus. Precursor T cells which are $CD4^-CD8^-TCR^-$ begin expressing the CD4 and CD8 coreceptors to become $CD4^+CD8^+$ double-positive thymocytes (Fowlkes and Pardoll, 1989). During this time, their TCR genes finish rearranging and double-positive cells express between low to intermediate levels of TCR (MacDonald et al., 1988; von Boehmer et al., 1988). Thanks largely to TCR transgenic and gene-targeting systems, it is now evident that $CD4^+CD8^+$ thymocytes up-regulate their TCR levels following positive selection on appropriate self-MHC molecules expressed by thymic stromal cells and become mature $CD4^+CD8^-TCR^{hi}$ or $CD4^-CD8^+TCR^{hi}$ single-positive cells, or face programmed cell death (Kisielow et al., 1988; Berg et al., 1989; Kaye et al., 1989; Matzinger and Guerder, 1989; Scott et al., 1989). However, the mechanism(s) by which the double-positive intermediates become mature single-positive CD4 T cells or CD8 T cells is not yet clearly defined.

The CD4 and CD8 coreceptor proteins have been

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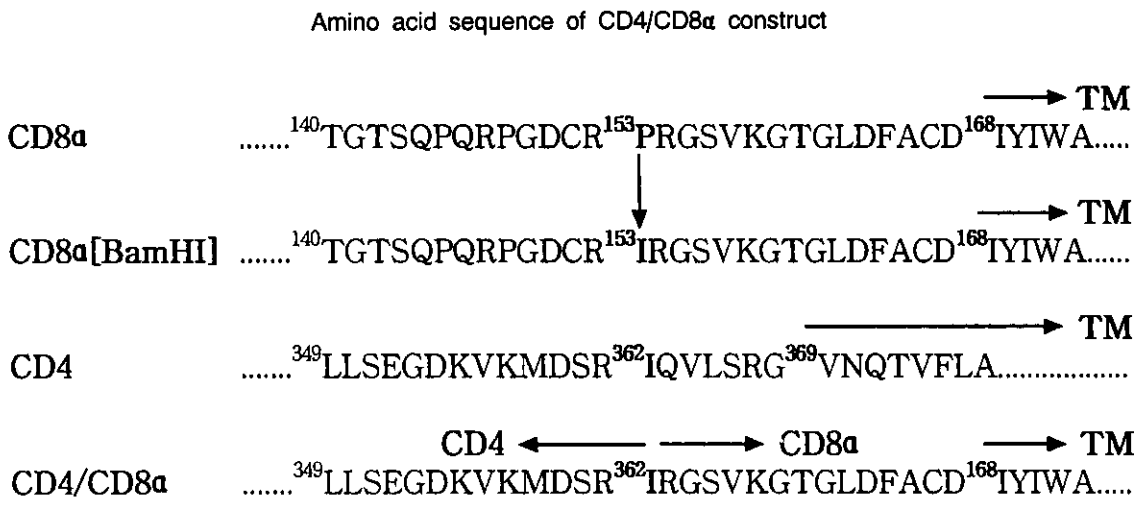

Fig. 1. CD4/CD8α hybrid protein of extracellular and transmembrane region of a native CD8α, mutated CD8α (CD8α[Bam HI]), and native CD4 sequences are shown. The resulting amino acid sequence of the CD4/CD8α chimeric protein is also shown.

shown to be involved in both clonal deletion and positive selection (Ramsdell and Fowlkes, 1989; Ingold et al., 1991) based on their ability to bind to defined nonpolymorphic regions of MHC class II or class I molecules, respectively (Connolly et al., 1990; Salter et al., 1990; Cammarota et al., 1992; König et al., 1992). They are able to complex with the TCR and function as coreceptors for recognition and activation by binding to the same antigen-bearing MHC molecules as the TCR (Connolly et al., 1990; Salter et al., 1990; Miceli et al., 1991; Janeway et al., 1992). At least two different mechanisms may be responsible for the effects of CD4 and CD8 on T cell development. One may be by an avidity enhancement between the cells involved in the selection processes, while the other may be by signal transduction through these coreceptors during thymocyte development (Seong et al., 1992; Parnes and Seong, 1994; Itano et al, 1996). Therefore, manipulation of CD4 and CD8 expression during thymocyte development represents a potentially useful approach to study the roles of these molecules during T cell development. In this study, to assess the roles of CD4 and CD8 during the differentiation of T cells, transgenic mice expressing a native CD4 and a CD4/CD8α hybrid protein composed of CD4 extracellular domain (EXT) and CD8α cytoplasmic tail (CYT) and transmembrane region (TM) were produced. These mice constitutively expressed the transgenic proteins at high levels in all cell types examined, including thymocytes and peripheral T cells. The expression profiles of the transgenic proteins on thymocytes and lymph node T cells were analyzed using flow cytometry.

Materials and Methods

Construction of CD4/CD8α hybrid cDNA

A Bam HI site was introduced into the CD8α cDNA at the sequence encoding amino acid 153 by site-directed mutagenesis. The sequence of the mutagenic oligonucleotide was 5'-gaa gat tgt **cgg a(c)t(c)c** cgt ggc tca gtg-3' (the nucleotide sequences in parentheses are wild type). The Bam HI site thus created was employed to link the CD8α cDNA fragment for the TM region and CYT to the mouse CD4 cDNA for EXT domain containing a nucleotide sequence for Bam HI site at amino acid 362. As shown in Fig. 1, the resulting hybrid cDNA encodes a chimeric protein whose EXT domain is derived from CD4, from amino acid 1 (Val) to amino acid 362 (Ile) (Littman and Gettner, 1987), and TM region and CYT are derived from CD8, from amino acid 154 (Arg) to amino acid 220 (Val) (Zamoyska et al., 1985).

Construction of hβAprCD4 and hβAprCD4/8α

The mouse CD4 cDNA was linked to human beta-actin promoter (hβApr) which drives the expression of the gene in all tissues. To provide 3'-untranslated region and poly(A) signal, 2.7 kb of Bgl II/Eco RI fragment of CD4 gene was added at the end of the cDNA. All these fragments were inserted into pBluescript II plasmid vector (Stratagene) and resulted in the hβ AprCD4 construct (Fig. 2). For the construction of the CD4/CD8α hybrid cDNA expression vector, the Bam HI fragment encoding the TM region and CYT of CD4 in hβAprCD4 cDNA was replaced with a cDNA fragment encoding the same regions of mouse CD8α, generating the hβAprCD4/8α construct (Fig. 2).

Mice

Transgenic mice expressing a native CD4 were produced by microinjection of the 8.4 kb Cla I/Spe I fragment of hβAprCD4 DNA into fertilized eggs of C57BL/6J. Transgenic mice expressing the CD4/CD8α

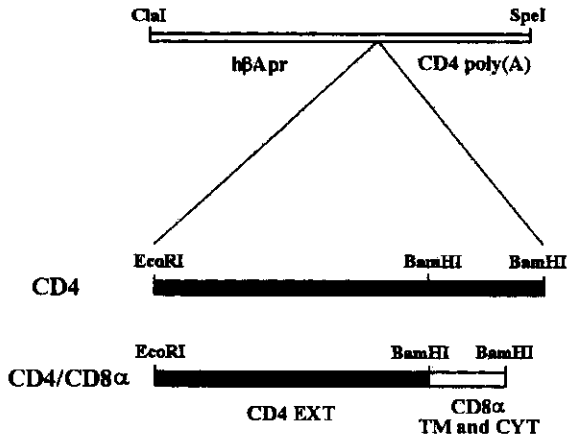

Fig. 2. Structures of the hβAprCD4 and hβAprCD4/CD8α. CD4/CD8α hybrid consists of CD4 EXT region and CD8 TM region and CYT which can associate with p56lck. The CD4 cDNA and CD4/CD8 hybrid cDNA were placed under the control of human β-actin promoter. Part of the 3' untranslated and flanking region of the mouse CD4 gene (2.7 kb of Bgl II/Eco RI fragment) was inserted at the 3' end of each cDNA to provide a poly (A) signal.

hybrid protein were produced also by microinjection of the 8.0 kb Cla I/Spe I fragment of hβAprCD4/CD8α DNA into fertilized eggs of (CBA/J x B6)F_2. Transgenic mice for the hybrid protein were mated to C57BL/6J or DBA/2 at least five times to give H-2^b or H-2^d MHC background, respectively, and were used for further experiments. DBA/2 (H-2^d), BALB/c (H-2^d), and C57BL/6J (H-2^b) were purchased from The Jackson Laboratory. All mice were housed and bred in the Institute for Molecular Biology and Genetics at Seoul National University. In all experiments, mice between the ages of 4-8 weeks old were used.

Screening with blood lymphocytes

Three- to four-week-old pups born after the microinjection were tail-bled, and about 0.1 ml of the blood was gently mixed with 0.5 ml of Alsever's solution (114 mM dextrose, 70 mM sodium chloride, and 30 mM sodium citrate) to prevent blood clotting. Each blood sample was washed twice with 10 ml of phosphate buffered saline (PBS) and then mixed with 2 ml of red blood cell (RBC)-lysis solution (160 mM ammonium chloride solution and 170 mM Tris-pH 7.65 buffer were mixed freshly before use at the volume ratio of 9 to 1). After incubation at room temperature for 2 min, each sample was rewashed with 10 ml of PBS and about 1×10^6 blood lymphocytes were stained with 1 μg of fluorescein isothiocyanate (FITC)-conjugated anti-CD4 monoclonal antibody (mAb) (GK1.5) at 4℃ for 20 min. After washing, stained cells were analyzed for the expression of CD4 using a FACStar Plus (Becton Dickinson).

Flow cytometry and mAbs

For flow cytometric analyses, single-cell suspensions of lymphocytes from thymic lobes or lymph nodes were stained at 4℃ for 20 min with Phycoerythrin (PE)-conjugated anti-CD4 mAb (L3T4), FITC-conjugated anti-CD8 mAb (53.6.7), and biotinylated anti-TCR mAb (H57.597) (Pharmingen). Cells were then washed with 1 ml of PBS and incubated at 4℃ for another 20 min with Quantum Red (QR)-conjugated streptavidin (Sigma). Stained cells were analyzed by using a FACStar Plus (Becton Dickinson). Typically, 2×10^4 cells out of $1\text{-}4\times10^6$ stained cells were collected and analyzed. Non-lymphocytes were removed from analyses by gating on both forward light scatter (FSC) and side light scatter (SSC) parameters. All the data were analyzed by using Lysis II software (Becton Dickinson).

Results

Constructions of hβAprCD4 and hβAprCD4/CD8α

The CD4/CD8α hybrid protein is composed of EXT domain of CD4 and TM region and CYT of CD8α. The resulting CD4/CD8α hybrid construct encodes the EXT region from the first amino acid (V) to amino acid 362 (I) of mouse CD4 and the TM region and CYT from amino acid 154 (R) to the C-terminus of mouse CD8α (Fig. 1). The hβAprCD4 and hβAprCD4/CD8α constructs were made by placing the CD4 and CD4/CD8α hybrid genes under the control of human β-actin promoter (Gunning, et al., 1987) (Fig. 2). Parts of the 3' untranslated region including poly(A) signal and the 3' flanking region of the mouse CD4 gene (Gorman et al., 1987) were inserted at the 3' end of the cDNA. Therefore it was expected that the transgenes would be expressed constitutively in all tissues including thymus, spleen, and lymph nodes. The DNA constructs were confirmed for their proper expression by transfection into COS 7 cells prior to microinjection (data not shown).

Generation of CD4 and CD4/CD8α transgenic mice

The 8.4 kb and 8.0 kb Cla I/Spe I fragments from hβ AprCD4 and hβAprCD4/CD8α constructs were isolated and used for microinjection into fertilized eggs. Mice expressing the transgenes were identified by testing blood cells collected by tail bleeding. Blood cells were stained with FITC-labeled anti-CD4 mAb and analyzed using FACS as described in Materials and Methods. Fig. 3 shows the surface CD4 levels of blood lymphocytes from normal littermates (A), CD4 transgenic (B), and CD4/CD8α transgenic (C) mice. In the normal mice, only cells expressing endogenous CD4 were stained with anti-CD4 mAb. However, in mice expressing the CD4 or CD4/CD8α transgenic proteins, all white blood cells were stained with anti-CD4 mAb. The mice identified as positive for the transgene expression were further confirmed for the presence of the transgenes by Southern analysis using isolated tail DNA (data not shown). Since mice expressing the transgenic CD4/CD8α were produced by injecting the DNA into

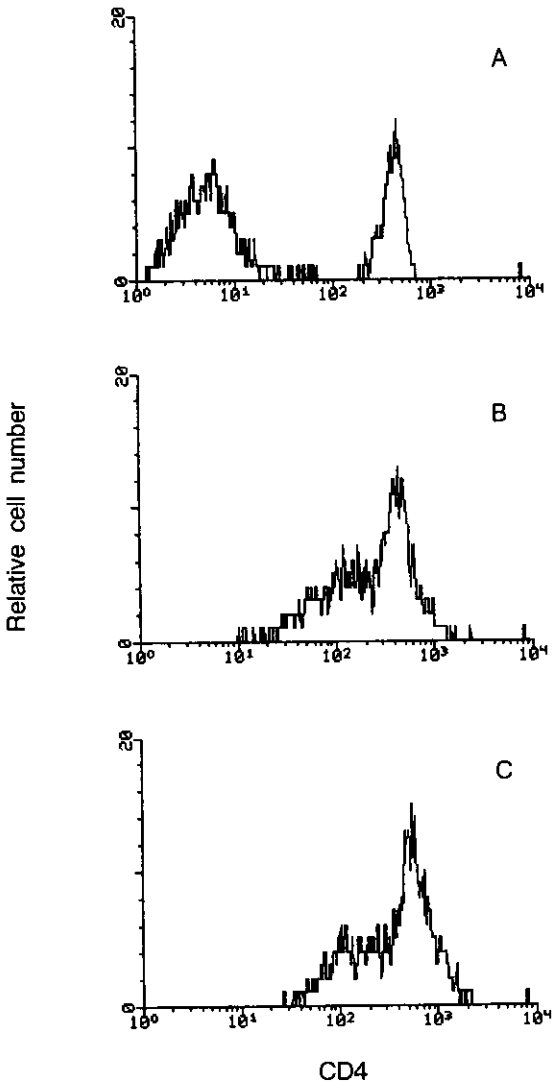

Fig. 3. FACS profiles of blood cells. Surface CD4 expression of blood lymphocytes from normal (A), CD4 transgenic (B), and CD4/CD8α transgenic (C) mice are shown. Anti-CD4 mAb detects extracellular part of CD4 protein, therefore, CD4/CD8α expression can also be detected by staining cells with the antibody.

fertilized eggs of (CBA/J × B6)F_2, the mice were further mated to C57BL/6J or DBA/2 to generate mice expressing the transgenes in H-2^b or H-2^d MHC background. One line for a native CD4 and two lines for CD4/CD8α hybrid transgenes in H-2^b background were established and further analyzed for the expression of the transgenes in thymus and lymph nodes.

Expression of transgenes on thymocytes and lymph node cells

Single cell suspensions of thymic lobes and lymph nodes from transgenic and control mice were prepared and stained with mAb against mouse CD4 or mouse CD8. The stained cells were analyzed using FACS

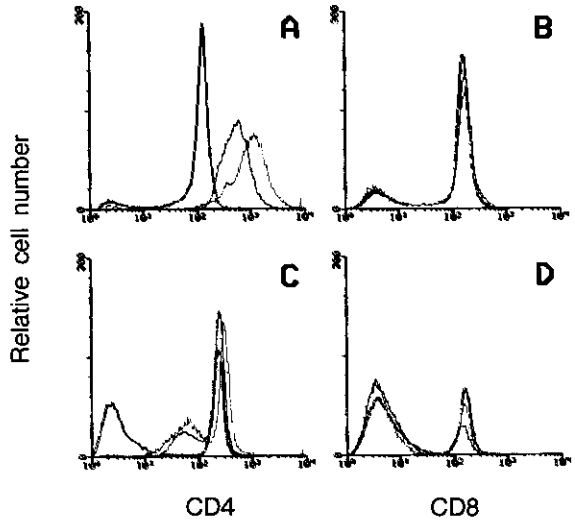

Fig. 4. Expression of surface CD4 and CD8 on thymocytes and lymph node cells. Single-cell suspensions of thymi (A, B) or lymph nodes (C, D) from normal (black), CD4 transgenic (green), and CD4/CD8α transgenic (blue) mice were stained with anti-CD4 (A, C) or anti-CD8 (B, D) mAb and analyzed using FACStar Plus.

and the results are shown in Fig. 4. Thymocytes (A, B) and lymph node cells (C, D) of litter-mate control, mice transgenic for native CD4, and for CD4/CD8α hybrid were analyzed for the expression of CD4 and CD8. Antibody against CD4 stains both endogenous CD4 and transgenic CD4 or CD4/CD8α hybrid proteins. Endogenous and transgenic CD4 proteins cannot be distinguished since there is no polymorphism for the CD4 molecule. Therefore, the level of surface CD4 (sCD4) consists of endogenous CD4 plus transgenic CD4 (in CD4 transgenic mice) or CD4/CD8α (in CD4/CD8 transgenic mice). However, the expression of the transgenic CD4 was easily seen by much higher level expression of CD4 proteins on thymocytes (Fig. 4A) and also by the expression of the CD4 on CD8 T lymphocytes as well as on non-T lymphocytes (Fig. 4C). The expression of CD4/CD8α hybrid protein can also be detected using mAb against CD4 since the EXT domain of the hybrid is CD4. Thymocytes from the CD4 transgenic mice expressed about 5 times higher level of sCD4 proteins than cells from the control littermate (Fig. 4A). Thymocytes from the CD4/CD8 transgenic mice also expressed about 10 times higher level of sCD4 proteins than control. However, no significant difference on the level of CD8 expression was detected among thymocytes from control, CD4 transgenic mice, and CD4/CD8α transgenic mice (Fig. 4B). In peripheral lymph nodes, the expression levels of sCD4 on cells from control, CD4 and CD4/CD8α transgenic mice were quite similar except the populations expressing low levels of transgenes in both transgenic mice (Fig. 4C). The populations that were not stained with mAb against CD4 in control mice were CD8 T and B lymphocytes. This was quite

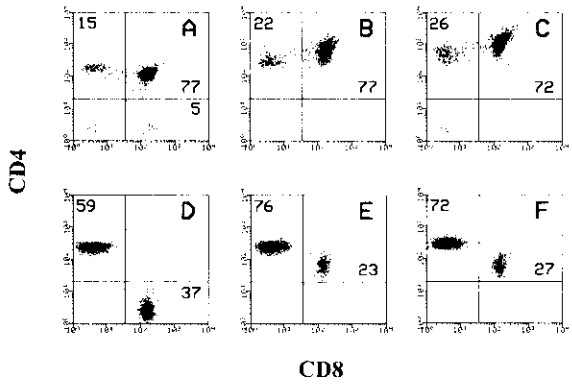

Fig. 5. FACS analysis of thymus. Thymocytes and lymph node cells from control littermate (A, D), CD4 transgenic mice (B, E), and CD4/CD8α transgenic mice (C, F) were stained with FITC-labeled CD8 and PE-labeled CD4. The stained cells were analyzed for the expression of CD4 vs CD8 using FACStar Plus. Numbers inside the quadrants are average percentages of three independent experiments, and one representative FACS staining result is shown.

in contrast to the staining profiles obtained from thymus in that the sCD4 expression on peripheral T cells was maintained at similar levels among these mice (Fig. 4B).

Flow cytometric analysis of thymocytes and lymph node T cells

To study the influence of transgene on the development of thymoytes, further analysis of thymocytes and lymph node T lymphocytes using three-color flow cytometry was performed. Thymocytes and lymph nodes cells from control and transgenic mice were stained with mAbs against TCR, CD4, and CD8α. FACS analysis of the control littermates showed typical staining profiles (Fig. 5A and D). Major populations of thymocytes (77%) expressed both CD4 and CD8 (double positive cells, DP: $CD4^+CD8^+$), and about 15% of total cells expressed only CD4, representing mature CD4 single positive (SP) thymocytes ($CD4^+CD8^-$). About 5% of cells were stained with CD8 only, representing mature ($CD4^-CD8^+$) and immature CD8 (TCR^-CD8^+) SP cells. Thymocytes expressing neither CD4 nor CD8 (double negative, DN: $CD4^-CD8^-$) were found to be about 3% of total population. In lymph nodes of the control mice, most cells were found to be T lymphocytes, expressing either CD4 (59%) or CD8 (37%). The rest (4%) of the lymph node population are mostly B lymphocytes. In CD4 and CD4/CD8α transgenic mice, an overall shift in sCD4 staining was observed because the transgenic mice constitutively expressed the CD4 or CD4/CD8α on all lymphocytes (Fig. 4B, C, E, and F). Therefore, the $CD4^-CD8^-$ DN and CD8 SP populations cannot be discriminated from those of CD4 SP and $CD4^+CD8^+$ double positive (DP), respectively, in these mice. In CD4 and CD4/CD8α transgenic mice (Fig. 5B and E), FACS profiles of thymocytes are similar to those of control except that all cells were shifted in CD4 expression. As shown in Fig. 4, all thymocytes expressed much higher level of sCD4 than control mice. It seems that the proportions of CD4 SP thymocytes increased in the transgenic mice (22% in CD4 transgenic mice and 26% of CD4/CD8α transgenic mice) compared to the control mice (15% plus 3-5% of DN cells now expressing transgenic CD4 or CD4/CD8α). This was consistent in three independent experiments. In the periphery, the FACS profiles of the control and transgenic mice are somewhat different. First, there is a clear increase in the CD4 population in the transgenic mice (76% in CD4 transgenic mice and 72% in CD4/CD8α transgenic mice) compared to control mice (59%). CD8 T cells also express the transgenic CD4 or CD4/CD8 proteins, but total levels of sCD4 in CD8 T cells were lower.

Discussion

Differentiation of immature $CD4^+CD8^+$ thymocytes into mature $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells requires the expression of CD8 or CD4 coreceptor molecules to be terminated, respectively; this process is referred to as lineage commitment. Previous studies with TCR transgenic mice have demonstrated that thymocytes with class I-specific TCR develop in the CD8 lineage, while those with class II-specific TCR mature as CD4 cells (Berg et al., 1989; Kaye et al., 1989; Kisielow et al., 1988; Sha et al., 1988). However, recent experiments have demonstrated that thymocytes with mismatched TCR and coreceptor specificities can be generated, albeit in low numbers (Davis et al., 1993; Baron et al., 1994,; Chan et al., 1994; Itano et al., 1994; Robey et al., 1994). These results indicate that the TCR alone, in the lineage commitment, seems unlikely to produce distinct signal to lead immature DP cells into mature CD4 or CD8 cells. In this study, we produced transgenic mice expressing a CD4/CD8α chimeric protein composed of CD4 EXT domain linked to CD8 TM region and CYT and investigated the expression patterns of CD4 and CD8 on thymocytes and lymph node T cells. It was shown that while the sCD4 expression levels of thymocytes from CD4 and CD4/CD8α transgenic mice were 4-5 and 8-10 times higher than normal mice respectively, those of lymph node T cells were maintained at similar levels. In addition, the expression levels of sCD4 in CD4 and CD4/CD8α transgenic mice (in the periphery) were lower in CD8 T cells than cells expressing endogenous CD4. These results suggest that the total avidity conferred by sCD4 and CD8 strongly affect the differentiation of thymocytes. Even though the total avidity, especially the one contributed by CD4 and CD8, seems to be an important factor for determining thymocyte differentiation as shown also by others (Seong and Parnes, 1992; Itano et al., 1996), the signal mediated through the CD4 and CD8 CYT seems to be also important. It was previously reported that some peripheral helper T cells have developed by the expression of tailless transgenic

CD4 in CD4 knock-out mice (Killeen and Littman, 1993). However, while the expression of a native CD4 at the endogenous CD4 level in the CD4 knock-out mice restored the normal level of peripheral CD4 T cells very efficiently, about 10-fold overexpression of the tailless CD4 than the level of endogenous CD4 was required for the same effect. Therefore, the expression of the tailless CD4 was very inefficient to drive the differentiation of developing thymocytes into the CD4 lineage (Killeen and Littman, 1993). Recently, a quantitative signaling model for CD4 vs CD8 lineage commitment was suggested based on evidence that the MHC class II-specific T cells can develop in the CD8 lineage when CD4 is absent (Matechak et al., 1996). In this model, there are no distinct signals but rather it is the net quantity of TCR signal that directs the CD4 vs. CD8 lineage determination. Signals of strong intensity promote a CD4 fate, whereas weaker signals promote a CD8 fate. Therefore, from the data indicating that Lck binds better to the cytoplasmic domain of CD4 than to CD8 (Veillette et al., 1988; Weist et al., 1993; Ravichandran and Burakoff, 1994; Itano et al., 1996), a model was proposed suggesting that strong Lck signals promote CD4 lineage commitment, whereas weak Lck signals promote CD8 lineage commitment (Matechak et al., 1996). While the effects of transgenic CD4 and CD4/CD8α on T cell lineage commitment seemed similar in these experiments, it appeared not to be necessarily the case when we followed a differentiation of thymocyte population expressing a clonotypic TCR. Expression of these transgenes together with a transgene encoding a specific TCR will further clarify the difference and provide important informations about the signaling role of CD4 and CD8 coreceptors during T cell development.

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