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*J Immunol* 2004; 172:6634-6641; 
doi: 10.4049/jimmunol.172.11.6634
http://www.jimmunol.org/content/172/11/6634

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The Novel Murine CD4⁺CD8⁺ Thymocyte Cell Line Exhibits Lineage Commitment into Both CD4⁺ and CD8⁺ T Cells by Altering the Intensity and the Duration of Anti-CD3 Stimulation In Vitro

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A CD4⁺CD8⁺ double-positive thymocyte cell line, 257-20-109 was established from BALB/c mice thymocytes and used to analyze the requirements to induce CD4 or CD8 single-positive (SP) T cells. CD4SP cells were induced from 257-20-109 cells by anti-CD3 stimulation in the presence of the FcR-positive macrophage cell line, P388D1. During stimulation, maturation events, such as the down-regulation of CD24 and the up-regulation of CD69, H-2Dd, CD5, and Bcl-2, were recognized. Furthermore, these CD4SP cells appeared to be functional because the cells produced IL-2 and IL-4 when activated with phorbol ester and calcium ionophore. In contrast, CD8SP cells could be induced by stimulation with fixed anti-CD3 after removal of stimulation. To investigate the extent of signals required for CD4SP and CD8SP, the cells stimulated under either condition for 2 days were sorted and transferred to different culture conditions. These results suggested that the fate of lineage commitment was determined within 2 days, and that CD4 lineage commitment required longer activation. Furthermore, the experiments with subclones of 257-20-109 demonstrated that the lower density of CD3 did not shift the cells from CD4SP to CD8SP, but only reduced the amount of CD4SP cells. In contrast, when the 257-20-109 cells were stimulated by the combination of fixed anti-CD3 and anti-CD28, the majority of the cells shifted to CD4SP, with an enhancement of extracellular signal-regulated kinase 1 phosphorylation. Our results indicate that the signals via TCR/CD3 alone shifted the double-positive cells to CD8SP cells, but the reinforced signals via TCR/CD3 and costimulator could commit the cells to CD4SP. The Journal of Immunology, 2004, 172: 6634–6641.

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Received for publication July 7, 2003. Accepted for publication March 30, 2004.

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3 Abbreviations used in this paper: DP, double-positive; ERK, extracellular signal-regulated kinase; HSA, heat-stable Ag; MAPK, mitogen-activated protein kinase; SP, single positive.
with anti-CD3 Ab under different culture conditions. With this cell line we investigated the requirements for commitment to CD4SP or CD8SP. Our results support the strength of signal model, and we thus propose that the stronger and the longer TCR/CD3 signaling preferentially induces a CD4 lineage. In addition, we elucidated that the accessory molecule CD28 reinforces TCR/CD3 signaling and facilitates commitment to CD4SP.

Materials and Methods

Cell lines

257-20-109 (TCR- Vβ6) cells were established from a suspension culture of CD4+ thymic lymphoma, RVC, which had been induced by the injection of a radiation leukemia virus into neonatal BALB/c mice (21). The cells were cloned by three repeated cycles of limiting dilution. The subclones of 257-20-109, TA 17 (CD3high), TA 32 (CD3int), and TA 37 (CD3neg) were isolated when we tried to establish a tetracycline-dependent gene expression system by transfecting Tet-On vector (Clontech Japan, Tokyo, Japan). The DBA/2-derived macrophage cell line, P388D1 (22), and the BALB/c-derived B lymphoma, A20.2J (23), were supplied by Dr. H. Nariuchi (Institute of Medical Science, University of Tokyo, Tokyo, Japan). The B cell line, 83-12-5, was provided by Dr. Y. Katsura (Kyoto University, Kyoto, Japan).

Cell culture

The cells were cultured on a 24-well plate in RPMI 1640 medium supplemented with 10% FCS, 0.1% NaHCO3, 20 mM HEPES, 2 mM l-glutamine, 1 mM sodium pyruvate, 50 μg/ ml nonessential amino acid, and antibiotics. A 4-day culture of 257-20-109 cells (3 × 104/well) was conducted in the presence of P388D1 (1 × 104/well) or A20.2J cells (2 × 104/well) in 2 ml of medium. Before coculture with DP cell lines, P388D1 or A20 cells were treated with 80 μg/ml mitomycin C (Kyowa Hakko Kogyo, Tokyo, Japan) in a complete medium at 37°C for 30 min and washed three times with Hanks’ solution containing 2% FCS. P388D1 or A20 cells were seeded several hours before adding 257-20-109 cells and anti-CD3 (145-2C11) or anti-Vβ6 (44-22-1) Ab (5 μg/ml final concentration of each). When the Abs against Fcγ or class II were included, a 10% vol (200 μl) of culture supernatant of hybridoma (2.4G2 or M5/114) was added to the medium before the addition of anti-CD3.

Ab-coated plate

Anti-CD3 or anti-Vβ6 Ab-coated plates were prepared by incubation with 20 μg/ml Ab in PBS at 4°C overnight. After removing the Ab, the culture plate was washed three times with 2% FCS Hanks’ solution and then was used for the assay. In this assay the culture was started with 3 × 104 cells/well. The plates used in Fig. 5 were pretreated with biotin-BSA (3 mg/ml) at 4°C overnight and washed three times with PBS. Next, the plates were treated with biotin-binding protein, NeutrAvidin (0.33 mg/ml; Pierce, Rockford, IL), at 4°C for 1 h. After washing with PBS, biotinylated Abs (5 μg/ml anti-CD3 and/or anti-CD24 or anti-CD28) were added to the plates and incubated further at 4°C for 1 h. The plates were washed with Hanks’ solution containing 2% FCS and then were used for the assay.

Pronase treatment

Pronase treatment was conducted as previously described (8). Briefly, the cells were washed extensively with PBS and suspended at a concentration of <2 × 106/ml in PBS containing 0.04% pronase (Calbiochem, San Diego, CA) and 50 U/ml DNase I (Roche Diagnostics, Basel, Switzerland). The cells were incubated at 37°C for 30 min, washed twice with medium containing 10% FCS, then used for culture.

Antibodies

FITC-conjugated anti-mouse CD4 (RM4-5), PE-conjugated anti-mouse CD8a (Ly-2, 53-6.7), PE-conjugated anti-mouse H-2Dd (34-5-8S), biotinylated anti-CD24 (M1/69), and biotinylated anti-CD69 (H1.2F3) were purchased from eBioscience (San Diego, CA). Anti-CD3 Ab (145-2C11) was purified from ascites and conjugated with FITC or biotin in our laboratory. Anti-mouse FcR Ab (2.4G2) (24), anti-mouse MHC class II Ab (M5/114) (25), anti-IL-2 (S4B6) (26), anti-IL-4 (11B11) (27), and anti-Vβ6 (44-22-1) Abs were provided from Dr. S. Saito (Jikei University, Tokyo, Japan).

Flow cytometric analysis

The expression of the cell surface molecules was analyzed by flow cytometry using FACSCalibur (BD Immunocytometry Systems, Mountain View, CA). Before being analyzed by the FACSCalibur, the cells were stained with 5–10 μl of propidium iodide (100 μg/ml) to exclude any apoptotic cells. When biotinylated Abs were included, streptavidin-PE-Cy5 was used for staining. The analysis of the data was performed using the WinMDI software package (e-mail address: trotter@scripps.edu). For sorting the cells, the cell suspensions of 257-20-109 cells with P388D1 or A20.2J cells were pretreated with a blocking agent (2.4G2 culture supernatant containing 20% normal mouse serum). Thereafter, the cells were stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8, and the CD4 or CD8 fraction was sorted using FACS-Vantage SE (BD Biosciences, Mountain View, CA).

II-1L/IL-4 production assay

CD4SP cells or original assay 257-20-109 cells were stimulated with 10 ng/ml PMA (Sigma-Aldrich, St. Louis, MO) and 100 nM calcium ionophore (A23187; Calbiochem, La Jolla, CA) for 24 h. The IL-2/IL-4 activity in the supernatant (final dilution, 1/20) was assayed with HT-2 cells (1 × 105 cells in 200 μl) in the presence of anti-IL-2 Ab (S4B6) and/or anti-IL-4 Ab (11B11) ascites fluid (final dilution, 1/400). Control cultures received a 1/20 dilution of medium containing 10 ng/ml PMA and 100 nM A23187. The uptake of [3H]thymidine by HT-2 cells was determined in the final 6 h of 24-h incubation.

RT-PCR

The CD4SP or CD8SP population on day 4 was used to observe the expression of CD4 and CD8 mRNA. To analyze the expression of granzyme B, the CD8SP population was stimulated beforehand with 10 ng/ml PMA and 100 nM A23187 for 24 h. The total RNA was extracted from the recovered cells by RNaseasy (Qiagen, Tokyo, Japan), and RT of the total RNA was conducted using the ThermoScript RT-PCR system (In vitrogen Japan, Tokyo, Japan) according to the manufacturer’s instructions. PCR was conducted using the following primer pairs: for granzyme B (272 bp), 5′-GAA GAT GAA GAT CCT CCT GC-3′ and 5′-TGT CGT GCT TCT CTC TT-3′; for β-actin (535 bp), 5′-GAA GTC TAG AGC AAC ATA GCA CAG CTT CTC-3′ and 5′-GTC GGA ATT CGT CAG AAG GCC TAC TAT GTC-3′; for CD4 (290 bp), 5′-TAC CCA ATG AGT CAG AGC AGC AGC GAG GAC TCC TAT GTC-3′ and 5′-AGG TGT TTG GAT GGT CCT TGT CG-3′; for CD8a (372 bp), 5′-AAG TGT TGG GGT CCTT TGG CC-3′ and 5′-AAT CTT CTG TGT GCT GGC GGT GCT G-3′; for hypoxanthine phosphoribosyltransferase (345 bp), 5′-GAT ACA GCC CAG ACT TGT TGT-3′ and 5′-GGT AGT CCG GCC TAT AGG GTT-3′. PCR was performed using TaKaRa En Taq (Takara Bio, Japan), and the conditions for granzyme B included denaturation at 95°C for 20 s, annealing at 55°C for 20 s, and extension at 72°C for 1 min in a total of 35 cycles. The PCR conditions for CD4/CD8 included denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and polymerization at 72°C for 1 min for a total of 35 cycles. Amplified products were analyzed on 1.5% agarose gel.

Western blot

257-20-109 cells were stimulated with fixed anti-CD3 (20 μg/ml), anti-CD3 (5 μg/ml) in the presence of P388D1 cells, biotinylated anti-CD3 (5 μg/ml), or biotinylated anti-CD3 plus anti-CD28 for 3 or 6 h. The cells were harvested and lysed with lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 1 mM PMSF, 0.1 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Na3VO4, 10 mM Na2P2O7, 25 mM β-glycerophosphate, and 20 mM NaF). In the case of a mixed culture with P388D1 cells, 257-20-109 cells were positively isolated using Dyna-beads mouse CD4 (L3T4; Dynal Biotech, Oslo, Norway), and the cells were then lysed as described above. After centrifugation, postnuclear supernatants were used by adding 3× SDS sample buffer (187.5 mM Tris (pH 6.8), 6% SDS, 30% glycerol, 150 mM DTT, and 0.03% bromophenol blue). Thirty micrograms of proteins (3 μg/lane for PMA-treated sample) were loaded in each lane on 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane. Blotted membranes were incubated with anti-phospho-p44/42 MAPK (The202/Tyr204) Ab (Cell Signaling, Beverly, MA), followed by goat anti-rabbit HRP secondary Ab. The signals were developed using ECL Plus Western Blotting Detection Reagents (Amersham Pharmacia Biotech, Piscataway, NJ). The membranes were reprobed with anti-extraacellular signal-regulated kinase 1 (anti-Erk1; C-16; Santa Cruz Biotechnology, Santa Cruz, CA) to account for loading variability. For the Bcl-2 Western analysis, cells were prepared by...
stimulation with fixed anti-CD3 and lysed on ice in lysis buffer. The samples were immunoblotted with anti-Bcl-2 (BD PharMingen) and anti-β-actin (Sigma-Aldrich) Abs.

**Results**

**Establishment of CD4⁺CD8⁺ (DP) thymocyte cell line and induction to the CD4 lineage**

The CD4⁺CD8⁺ (DP) thymocyte cell line, 257-20-109 (CD3⁺, Vβ6⁺, CD24high, Thy1high, H-2Dlow, CD95⁻), was established from the suspension culture of thymic lymphoma that had been induced by injection of radiation leukemia virus into neonatal BALB/c mice (Fig. 1A). To determine whether the stimulation through TCR/CD3 complex can induce lineage commitment of the DP cell line, 4-day culture with anti-CD3 (145-2C11; 5 μg/ml) was conducted in the presence of the FcR-positive macrophage-like cell line, P388D1, or B cell lymphoma, A20.2J cells. A flow cytometric analysis revealed that the CD4SP lineage was derived from 257-20-109 cells 4 days after the cultivation (Fig. 1B). During the 4-day culture with anti-CD3 plus P388D1 cells, the number of 257-20-109 cells reduced from 3.0 × 10⁵/well to 1.4 × 10⁵/well (mean), whereas with P388D1 cells alone, 257-20-109 cells proliferated ~30 times in the cell number during the same period. The reduction of cell number was due to the induction of apoptosis during activation (our unpublished observations). During the culture, CD69 was expressed, and the levels remained high on days 1 and 2; thereafter the expression was gradually down-regulated (Fig. 1C, upper panel). CD24 (heat-stable Ag (HSA)) was reduced to intermediate, and the expressions of H-2Dd and CD5 were changed from low to high levels (Fig. 1C, lower panels). These alterations of the surface markers are similar to those observed in the differentiation of DP thymocytes in vivo. Furthermore, these CD4SP cells were functionally matured. The cells produced IL-2 and IL-4 when they were stimulated with PMA (10 ng/ml) plus A23187 (100 nM) for 24 h (Fig. 1D). In contrast, the original 257-20-109 cells could produce neither IL-2 nor IL-4.

To examine the requirement of anti-CD3 for induction of CD4SP, anti-FcR Ab (2.4G2) was added to the culture. Obviously, the lineage commitment to CD4SP was restrained, thus indicating that the stabilized anti-CD3 expression on the surface of P388D1 cells is important for the differentiation of DP cell lines (Fig. 1E). The addition of anti-MHC class II Ab, M5/114, which could block both I-A^d and I-E^d, did not affect the lineage fate of the 257-20-109 cells (Fig. 1E). CD4SP lineage was also induced by stimulation with anti-Vβ6, although the number of CD4SP cells on day 4 was less than that induced by anti-CD3 (Fig. 1F).

**Induction of CD8SP lineage from DP cell line**

As another activation method, 257-20-109 cells were stimulated with fixed anti-CD3 Ab on the plate. The cells were cultured for up to 4 days. About half the cells were found dead, and the rest of the
viable cells were assessed by FACS analysis. The CD4<sup>+</sup>CD8<sup>low-int</sup> population was observed on day 2 (Fig. 2A, upper panel), and thereafter the cells remained at the CD4<sup>+</sup>CD8<sup>low-int</sup> stage. When these cells were transferred to the culture without anti-CD3 stimulation, up-regulation of CD8 expression was observed (Fig. 2A, lower panels). Similarly, stimulation with fixed anti-Vβ6 also induced a CD4<sup>-</sup>CD8<sup>low-int</sup> population (Fig. 2B). Furthermore, cells from the CD8SP population expressed mRNA of granzyme B when they were stimulated with PMA plus A23187, thus indicating the functional maturity of the CD8SP population (Fig. 2C).

The lineage commitment could be defined as the selective termination of synthesis of CD4 or CD8 protein in a cell that expresses both CD4 and CD8 Ags. We isolated total RNA from the sorted population of CD4SP and CD8SP and confirmed that in the committed cells the expression of mRNA for either CD8 or CD4 was suppressed, respectively (Fig. 2D). In addition, the induction of Bcl-2 protein expression was reported to be correlated with the lineage commitment and maturation to SP phenotype (28). An increase in Bcl-2 protein expression was observed in the cells stimulated with fixed anti-CD3 Ab, as shown in Fig. 2E.

**Culture period for lineage determination**

We next investigated the relationship between the period of anti-CD3 stimulation and the lineage commitment. The CD4<sup>+</sup>CD8<sup>low/med</sup> population appeared after 2-day culture with anti-CD3 plus P388D1 cells (Fig. 1B). The CD4SP fraction was sorted from this population, treated with pronase, and incubated without anti-CD3 stimulation. These sorted CD4SP cells on day 2 returned to DP cells when the cells were maintained for 3 more days without anti-CD3 stimulation (Fig. 3A, upper panels). In contrast, CD4SP cells obtained after the longer (4-day) culture with anti-CD3 plus P388D1 cells, remained at CD4SP at least 3 more days when the cells were transferred to the culture without stimulation (Fig. 3A, lower panels). Similar experiments were repeated with the cells activated for 2 days on the anti-CD3-coated plate. When the sorted CD8<sup>low-int</sup> cells were cultured 2 more days with medium alone, some cells remained at CD8SP, and some cells reverted to DP cells (Fig. 3B, lower left panel). The pattern did not differ whether the sorted cells were from day 2 (Fig. 3B) or day 4 (Fig. 2A) of culture. These findings support the idea that the CD4 lineage commitment requires a longer and more sustained activation than the CD8 lineage commitment. We also transferred the sorted cells on day 2 from the CD8SP-induced condition to the CD4SP-inducing condition. When the cells from the fixed anti-CD3 plates were further maintained in the presence of anti-CD3 plus P388D1 cells, the fractioned cells remained at CD4<sup>+</sup>CD8<sup>low</sup> and did not shift to CD4SP (Fig. 3B, lower right panel). These results suggest that lineage determination was completed within 2 days of activation.

**Influence of level of surface CD3 expression on lineage commitment**

During our efforts to establish a tetracycline-inducible system in 257-20-109 cells, we happened to isolate some cell lines that expressed different levels of surface CD3 expression. TA 17 (CD3<sup>high</sup> equivalent to 257-20-109), TA 32 (CD3<sup>int</sup>), and TA 37 (CD3<sup>low</sup>) were used to investigate the influence of CD3 expression on lineage fate (Fig. 4A). In the CD3<sup>high</sup> cell line, TA 17, the CD4SP lineage was induced by culture with anti-CD3 plus P388D1 cells, as observed in the original DP cell line, 257-20-109 (Fig. 4B). In contrast, the CD4SP populations in subclones, TA 32 (CD3<sup>int</sup>) and TA 37 (CD3<sup>low</sup>) cells, decreased compared with those of 257-20-109 cells after a 4-day culture with anti-CD3 plus P388D1 cells (Fig. 4B). Although DP and CD4<sup>+</sup>CD8<sup>low</sup> populations were increased in TA 32 and TA 37 cells, the preferential shift to CD8SP was not observed (Fig. 4B). Moreover, when TA 17 and TA 32 cells were activated with fixed anti-CD3, the appearance of a CD8<sup>low-int</sup> population was observed, and in TA 37 cells, the appearance of the CD8<sup>low-int</sup> population was decreased.
Influence of accessory molecules on lineage choice

To investigate the differences observed between fixed anti-CD3 and anti-CD3 plus P388D1 cells, we devised a sandwich method in which the plate was coated with an excessive amount of biotinylated BSA, followed by incubation with NeutraAvidin. On this plate we can activate the cells by using various combinations of biotinylated Abs. As shown in Fig. 5A, CD4^{-}CD8^{low-int} populations were obtained with biotinylated anti-CD3 (5 \mu g/ml) alone. However, when the cells were stimulated with biotin-anti-CD3 and biotin-anti-CD28, about two-thirds of the cells were found to be dead compared with \sim 50\% in the case of biotin-anti-CD3 alone, and the majority of the viable cells shifted to CD4SP populations 4 days after cultivation (Fig. 5A). In contrast, the coexistence of biotin-anti-CD24 did not change the pattern of commitment.

Regarding the differences in the signals that induce CD4 or CD8 lineage commitment, many reports have suggested that the activation of ERK kinase is stronger in CD4 lineage commitment than in CD8 lineage commitment (20). We compared the extent of phosphorylation of ERK between cells activated by different culture conditions. As shown in Fig. 5B, we detected stronger ERK activation in cells with anti-CD3 plus P388D1 cells compared with that in cells with fixed anti-CD3. Similarly, a stronger activation of ERK was observed in the cells activated together with anti-CD3 and anti-CD28 compared with anti-CD3 alone.

Discussion

We have newly established a novel murine DP cell line, 257-20-109, which was able to differentiate into either a CD4SP or a CD8SP lineage in vitro by providing a different type of anti-CD3 stimulation. The cell line exhibited several maturation features, such as decreased expression of CD24 (HSA), expression of CD69, and up-regulation of H-2D^{b}, CD5, and Bel-2 as the lineage commitment. The lineage commitment was also reflected as down-regulation of expression of CD4 or CD8 genes depending on the stimulating conditions. Furthermore, cells that shifted to CD4SP or CD8SP were functionally mature, in contrast to the original cells.

Various models have been proposed to explain the lineage commitment, and among them evidence is now accumulating to support the strength of signal model (9–15). According to the model, stronger signals tend to induce DP cells to CD4SP, and weaker signals induce to CD8SP. This hypothesis is mainly based on the fact that the CD4 molecules isolated from DP cells are associated with more Lck than are CD8 molecules (14, 16–18). As a result, the experiments in which cytoplasmic domains of CD4 and CD8 coreceptors changed demonstrated that the lineage fate was largely dictated by the signaling end of coreceptors (29). In addition, thymocytes with class II-specific TCR were reported to develop into CD8 T cells when CD4 was absent (13). In addition, Ohoka et al. (12) demonstrated that different doses of phorbol ester and ionomycin selectively produce CD4SP or CD8SP cells. In our experiments the fate of 257-20-109 cells differed depending on whether the cells were activated on the fixed anti-CD3 or activated with anti-CD3 in the presence of the FcR\^+ macrophage-like cell line, P388D1, or the B cell lymphoma, A20.2J. Next, the question is what role these accessory cells play in the destination of DP cells. Anti-FcR Ab blocked the shift to CD4SP, and this indicates that the cross-linking of TCR/CD3 via FcR is a manifestation of these accessory cells. It is possible that the Fab region of anti-CD3 Ab could face toward the cells more effectively when anti-CD3 Ab is presented on the FcR compared with the Ab fixed on the plate. This difference in Ab presentation might result in the distinct strength and duration of the signals. However, even when the dose of anti-CD3 was further increased in the sandwich method, the pattern of CD8SP did not change (data not shown). In addition, experiments with TA subclones demonstrated that stimulation of the cells with a low density of CD3 did not convert the pattern from CD4SP to CD8SP, P388D1 cells or A20.2J cells express both class I and class II Ags (22, 23), and the CD4-class II Ag conjugation might facilitate the induction of CD4 cells. This possibility, however, was not the case in our system, because we could not block the shift with anti-class II Ab.

The accessory cells, such as P388D1 and A20.2J, have various other molecules to maintain contact with lymphocytes. Among them, CD28/B7 interaction promotes the formation of cell–cell interactions. In the present study we found that the addition of anti-CD28 Ab could shift the majority of cells from CD8SP to CD4SP cells. CD28 together with CD2 has been proposed to facilitate the formation of the T cell–APC contact zones, and the adhesive forces

FIGURE 3. The period of anti-CD3 stimulation required for lineage commitment. A, A CD4SP population, induced by 2-day (upper panels) or 4-day (lower panels) culture with anti-CD3 plus P388D1 cells as described in Fig. 1B, was sorted and treated with pronase. Next, the cells were cultured without anti-CD3 stimulation for another 3 days. After the culture, the expression of CD4/CD8 was examined by flow cytometry. B, A CD4^{-}CD8^{low} population, induced by 2-day (upper panel) culture with fixed anti-CD3 as described in Fig. 2A, was sorted, treated with pronase, and further cultured in the medium without anti-CD3 stimulation. In addition, the sorted cells were incubated for 2 more days in the presence of P388D1 cells plus anti-FcR or P388D1 cells plus anti-CD3.

(4C). These results suggest that the level of expression of TCR/CD3 with the same affinity would not be a critical factor in the lineage choice of DP cells, although certain levels of TCR/CD3 on the cell surface are still required to induce SP lineage. The accessory cells, such as P388D1 and A20.2J, have various other molecules to maintain contact with lymphocytes. Among them, CD28/B7 interaction promotes the formation of cell–cell interactions. In the present study we found that the addition of anti-CD28 Ab could shift the majority of cells from CD8SP to CD4SP cells. CD28 together with CD2 has been proposed to facilitate the formation of the T cell–APC contact zones, and the adhesive forces
exerted by CD28 and/or CD2 enhanced the signal production via TCR/CD3 (30). Actually, McKean et al. (31) reported that TCR plus CD28 coengagement did not invariably induce DP thymocyte apoptosis, but, depending on the intensity, CD28 costimulation could induce maturation of thymocytes. According to them, the engagement of costimulatory receptors enhances the distribution of receptors and the clustering of rafts at the T cell-APC interface. Thereby, CD28 helps to sustain Lck activation as reported by Holdorf et al. (32). Furthermore, inhibition of the CD28/B7 interaction was reported to result in impaired TCR-induced tyrosine phosphorylation of the signal-transducing ζ-chain and ζ-chain-associated protein of 70 kDa, followed by such diminished downstream signaling pathways as Ca\(^{2+}\)/calcineurin, ERK/MAPK, and c-Jun N-terminal kinase (33). Zhou and colleagues (34) have shown that the costimulation through CD28 as well as CD2 and CD5 facilitates the TCR/CD3-coupled signal and supports differentiation to the CD4 lineage. Collectively, it looks like CD28 contributes to the lineage commitment as a molecular amplifier extending TCR ligation and signaling capabilities. To date, however, we can not yet rule out the possibility that the CD28 signal pathway might play some specific role in facilitating the choice and not just reinforce the contact with TCR/CD3. Indeed, full phospholipase C\(_{\gamma1}\) activation has been reported to depend on the CD28-generated signal (35). In this study we showed that the enhancement of ERK1 signal was provoked in 257-20-109 cells by stimulation with anti-CD3 in the presence of P388D1 cells or with anti-CD3 plus anti-CD28 compared with anti-CD3 alone (Fig. 5B).

We are further investigating which signal is most relevant to CD4 lineage commitment by comparing the signal cascades between cells activated with anti-CD3 alone and cells activated with the combination of anti-CD3 plus anti-CD28.

One of the other findings of our experiments is that the commitment to CD4SP would require a longer duration of stimulation. The CD4 fraction obtained on day 2 returned to DP when the cells were maintained in medium without anti-CD3 stimulation. However, CD4SP cells obtained on day 4 remained at CD4SP even when cells were maintained without stimulation. In contrast, the CD8SP fraction obtained on day 2 or 4 demonstrated a similar pattern after removal of stimulation. These results correlate with previous investigations that pointed out the importance of time of stimulation for lineage choice (9–11). Moreover, our results clarified that the fate of lineage commitment was determined within 2 days of activation, even though the cells need a longer stimulation for the fixation of CD4SP.

To investigate the mechanism of apoptosis and the lineage commitment observed in DP cells, the application of lymphomas has been introduced (36–38). However, few such studies have yet been performed, and none of the papers have yet reported lymphomas that can induce both CD4SP and CD8SP cells. Our newly established lymphoma will be helpful to clarify the differences in the signal cascade required for the induction of CD4SP and

**FIGURE 4.** Different levels of surface CD3 expression on the lineage commitment of 257-20-109 cells. A, Three DP thymocyte subclones, TA 17 (CD3\(_{\text{high}}\)), TA 32 (CD3\(_{\text{int}}\)), and TA 37 (CD3\(_{\text{low}}\)), were established from the DP cell line, 257-20-109. The surface CD3 expression on these subclones was analyzed by flow cytometry. B, CD4/CD8 expression on the subclones after a 5-day culture with mitomycin C-treated P388D1 cells plus 5 μg/ml anti-CD3. C, CD4/CD8 expression on the subclones after a 4-day culture with fixed anti-CD3.
CD8SP cells. Furthermore, by treatment with mutants, lymphomas can easily obtain mutants that are defective in the commitment to CD4SP or CD8SP cells. With these mutants, we hope to be able to identify the genes responsible for such differentiation, and studies are presently being conducted along these lines in our laboratory.

Acknowledgments

We thank Dr. H. Nariuchi for providing P388D1 and A20.2J cells, Drs. S. Saito and Y. Katsura for mAbs, and Nagisa Sakamoto for excellent secretarial work.

References


28. Punt, J. A., H. Suzuki, L. G. Granger, S. O. Sharrow, and A. Singer. 1996. Lineage commitment in the thymus: only the most differentiated (TCR<sup>hi</sup>bcl-2<sup>hi</sup>) subset of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes has selectively terminated CD4 or CD8 synthesis. J. Exp. Med. 184:2091.


