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Rac1-mediated actin polymerization and Bcl-2 induction are critical for differentiation of CD4 single-positive T cells

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ABSTRACT

Rac1, one of the Rho family small GTPases, has been shown to work as a "molecular switch" in various signal transduction pathways. To directly assess the function of Rac1 in the positive selection process of T cell development, we utilized a CD4 CD8 double-positive cell line DPK, which can differentiate into CD4 single-positive (CD4-SP) cells after T cell receptor (TCR) stimulation *in vitro*. DPK cells expressing dominant-negative Rac1 (dnRac1) underwent massive apoptosis after TCR stimulation, the result being defective differentiation into CD4-SP cells. TCR-dependent actin polymerization was inhibited, whereas early activation of ERK was unaltered in dnRac1-expressing DPK cells. We found greatly suppressed TCR-dependent induction of Bcl-2 in dnRac1-expressing DPK cells, and this suppression was independent of actin rearrangement. Furthermore, introduction of exogenous Bcl-2 restored apoptosis and normalized defective generation of CD4-SP cells in the dnRac1-expressing DPK cell population. Collectively, these data indicate that Rac1 is critical for the positive selection of thymocytes through not only reorganizing the actin cytoskeleton but also preventing TCR-induced apoptosis via Bcl-2 up-regulation.

INTRODUCTION

Rac1 belongs to the Rho family of small GTPases, which plays key roles in actin cytoskeletal rearrangement in many cell systems. Rac1 is activated with a broad range of guanine nucleotide-exchanging factors and effectors, so that the molecule acts as a molecular switch in many aspects of signal transduction pathways. Recent studies using transgenic technology have revealed that Rho family GTPases play crucial roles in thymocyte development and T cell receptor (TCR)-mediated signal transduction [1]. Ectopic expression of bacterial C3T, which inhibits RhoA, RhoB, and RhoC, resulted in decreased numbers of CD4 CD8 double-positive (DP) cells in the thymus [2], whereas transgenic expression of constitutively active RhoA resulted in enhanced positive selection [3]. Constitutively active cdc42 induced massive apoptosis in DP thymocytes [4], which suggests that cdc42 is also involved in T cell development.

Rac consists of three independent genes, *rac1*, *rac2*, and *rac3*. Rac1 and Rac3 are ubiquitously expressed, whereas expression of Rac2 is restricted to hematopoietic cells. Rac2-deficient mice showed normal T cell development in the thymus, defective T_H1 differentiation caused by decreased interferon γ production [5], perturbed chemotaxis [6], and defective T cell activation accompanied by reduced activation of extracellular signal-regulated kinase (ERK) [7]. Rac2 is a component of the NADPH oxidase complex and plays a critical role in regulating reactive oxygen species in phagocytes [8]; Rac1 was recently shown to have a similar function in human macrophages [9]. Transgenic expression of constitutively active Rac1 (L61) generates DP thymocytes in a $RAG^{-/-}$ background [10] and converts positive selection to

negative selection [11], which indicates that Rac1 regulates the strength of TCR-mediated signal transduction. Rac1-deficient mice are embryonic lethal, and neutrophil-specific disruption of Rac1 was reported [12]. Recently, Rac1 conditional knockout mice in a Rac2$^{-/-}$ background were generated, and different crucial roles of Rac1 and Rac2 in growth and engraftment of hematopoietic stem cells [13-15], as well as in B cell development [16], were reported. However, the effect of Rac1 on T cell development is still unknown.

Because all of the three Rac gene products are expressed in T cells, a knockout strategy is not ideal for investigating the function of Rac proteins in T cell development. For this report, we studied the role of Rac in T cell development by using a dominant-negative strategy. We demonstrated, by means of DPK, which is a DP thymic lymphoma cell line capable of differentiation into CD4 single-positive (CD4-SP) cells after antigenic stimulation in vitro [17], that Rac is required for generation of CD4-SP T cells. We further established that Rac is critical in TCR-mediated Bcl-2 induction, which indicates that Rac is important in anti-apoptotic signal transduction in developing T cells as well as in inducing actin cytoskeletal reorganization.

MATERIALS AND METHODS

Construction of retroviral vectors

To construct the pMXs-PREP retroviral vector, a *Cla*I fragment of woodchuck hepatitis virus posttranscriptional regulatory element (PRE) sequence [18] and a *Sal*I fragment of puromycin resistance gene were inserted into *Cla*I and *Sal*I sites of pMXs-IRES-GFP [19], respectively. Polymerase chain reaction (PCR)-cloned Rac1N17 cDNA was inserted into pMXs-PREP to produce pMXs-PREP-dnRac1. Retroviral vector pMI.2, which contains the IRES-hCD2 sequence, was a kind gift from Dr. S. Levin (ZymoGenet Inc., WA), and PCR-cloned human Bcl-2 cDNA was inserted into the multi-cloning site of pMI.2 to construct pMI.2-Bcl2.

Retroviral transduction

Retrovirus-containing supernatant from cultures of vector (pMXs-PREP) or pMXs-PREP-dnRac1-transfected 293gp packaging cells [20] with vesicular stomatitis virus envelope plasmid were used for infection of DPK cells. Retrovirally transduced cells were selected with 1 μg/ml puromycin and were electronically sorted for GFP^{hi} cells without single-cell cloning using the fluorescence-activated cell sorter FACSVantage SE (Becton Dickinson, Palo Alto, CA).

In vitro T cell differentiation culture

A DPK differentiation assay was carried out as described elsewhere [17] with some modifications. Briefly, irradiated DC-I cells (E^k- and ICAM-1-transfected murine fibroblasts) were precultured in 6-well plates, at 9 X 10^5 cells per well, for 24 h with or without 100 ng/ml staphylococcal enterotoxin A (SEA; Toxin Technology, Sarasota, FL). During the last 2 h of the culture period, 4.5 X 10^5 DPK cells were added, and culture was allowed to continue for 3 more days at 37℃. Cells were harvested after the indicated time periods and were stained with anti-CD4-PE (GK1.5) and anti-CD8α-biotin (53-6.7) antibodies, followed by use of Streptavidin-PerCP-Cy5.5 for flow cytometry. All antibodies and staining reagents were purchased from PharMingen (Palo Alto, CA).

Cell cycle analysis

DPK cells were activated with plate-bound anti-CD3ε monoclonal antibody (50 μg/ml) for 16 h, fixed with 70% EtOH, and treated with RNase A (1 mg/ml). Fixed cells were stained with 50 μg/ml propidium iodide for 3 h at room temperature, followed by analysis with an FACScalibur (Beckton Dickinson).

Measurement of ERK activation

DPK cells were incubated with 10 μg/ml anti-CD3ε antibody at 4℃ and then cross-linked with anti-hamster secondary antibody for the indicated times at 37℃. After the indicated incubation periods, cells were lysed, and Western blotting was performed with anti-phospho-ERK and anti-ERK antibodies (Transduction Labs, Palo Alto, CA).

Staining of polymerized actin

DPK cells were placed onto coverslips coated with anti-CD3ε plus anti-CD28 monoclonal antibodies (10 μg/ml each) and were cultured for 15 min at 37℃. After supernatant was removed, cells were fixed with 4% paraformaldehyde followed by permeabilization with 0.3% saponin and staining with phalloidin-Alexa 594 (Molecular Probes, Eugene, OR).

Flow cytometry

Thymocytes or DPK cells were stained with various combinations of fluorescein isothiocyanate-conjugated anti-CD45.2 (clone 104), Phycoerythrin (PE)-conjugated anti-CD4 (GK1.5) and anti-CD5 (53-7.3), biotin-conjugated anti-CD8α (53-6.7), anti-TCRβ (597-H57), annexin V, anti-CD69 (H1.2F3), anti-CD45.1

(A20), and Allophycocyanin (APC)-conjugated anti-CD8α (53-6.7). Stained cells were analyzed by means of the two-laser FACScalibur (Becton Dickinson) for four-color FACS analysis.

Real-time PCR analysis

Total RNA was isolated from cells by using the RNeasy kit (Qiagen, Hilden, Germany), and real-time PCR was performed with the QuantiTect SYBR Green RT-PCR Kit (Qiagen) with specific primers for Bcl-2α (5'-cctgtggatgactgagtacct-3' and 5'-gagcagggtcttcagagaca-3') and Nor1 (5'-aagggcttcttcaagagaac-3' and 5'-tgaaatctgcagtactgacatc-3').

Western blotting

Bcl-2-transduced DPK cells (5 X 10^5) were lysed and underwent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Transferred membranes were Western blotted with rabbit polyclonal anti-Bcl-2 antiserum (PharMingen), and reactive proteins were visualized with ECL chemiluminescent substrates (Cell Signaling Technology, Beverly, MA).

RESULTS

Dominant-negative Rac1 (dnRac1) inhibited differentiation into CD4-SP cells

To analyze in detail the function of Rac in TCR-mediated signal transduction during positive selection, we utilized an in vitro experimental system representing CD4-SP cell differentiation. DPK is a naturally occurring DP thymic lymphoma from AND-TCR transgenic mice [17, 21]. This cell line differentiates into CD4-SP cells when co-cultured with antigen-loaded APC in vitro [17], and thus it has been used to study signal transduction during positive selection of immature thymocytes [22-24]. A dominant-negative (N17) Rac1 gene was PCR cloned into the newly established pMXs-PREP retroviral vector, containing an IRES-GFP cassette and an mRNA-stabilizing element (wPRE) sequence [18] for effective gene expression [25]. Although dnRac1-expressing DPK cells showed some increase in cell size, expression levels of CD4, CD8, and TCR were indistinguishable from those of the vector control-infected cells (Fig. 1a, b).

Figure 1b shows that CD8 expression by vector control-transduced DPK cells gradually decreased, so that almost 80% of cells differentiated into CD4-SP cells 3 days after antigenic stimulation. In contrast, generation of CD4-SP cells was almost completely abolished for dnRac1-expressing DPK cells (Fig. 1b).

This lack of CD4-SP cell generation for dnRac1-expressing DPK cells could be due to defective signal transduction or massive apoptosis after antigenic stimulation. We thus examined apoptotic cell death in cultures of DPK cells with or without TCR stimulation. Apoptotic cell death after 16 h of incubation was evaluated by using propidium iodide staining in cell cycle analysis to determine the proportion of cells in the $subG_1$ fraction (Fig. 2a). With no stimulation, 21% of control vector-expressing DPK cells were dead; this proportion did not increase significantly after TCR stimulation. However, TCR stimulation induced marked cell death of dnRac1-expressing DPK cells (56%), although even without stimulation this cell population had a larger proportion of dead cells. In addition, the number of annexin V-positive apoptotic cells dramatically increased after TCR stimulation in dnRac1-DPK cells compared with control DPK cells (Fig. 2b). Similar results were obtained with the trypan blue dye exclusion method for determination of cell viability (data not shown). These data collectively indicate that expression of dnRac1 in DP thymocytes leads to inhibition of positive selection and augmentation of TCR-induced apoptosis.

Early activation events were retained in dnRac1-expressing DPK cells

To investigate the function of Rac1 in TCR-dependent signal transduction during positive selection, early activation of ERK was evaluated. DPK cells were stimulated by beads coated with anti-CD3ε monoclonal antibody, and activation of ERK was determined by analysis of phosphorylation of ERK1 and ERK2 (Fig. 3a). The dnRac1 mutant had no effect on ERK activation, which indicates that TCR-stimulated activation of ERK was independent of Rac1. We also found that TCR-mediated up-regulation of CD69 and CD5 in dnRac1-DPK cells was indistinguishable from that of control DPK cells (Fig. 3b).

Rac1-mediated actin polymerization

We next examined TCR-dependent actin polymerization in dnRac1-DPK cells, because Rac is known to play a critical role in cytoskeletal reorganization. After 15 min of TCR stimulation, control DPK cells had accumulated polymerized actin, as detected by phalloidin staining, whereas TCR-dependent actin polymerization was abrogated in dnRac1-DPK cells (Fig. 4a). A requirement for actin reorganization during thymocyte development has not been determined directly. Thus, we examined the effect of an inhibitor of actin polymerization on the differentiation of DPK cells. Latrunculin A, which is such an inhibitor [26], completely

blocked antigen-induced generation of CD4-SP cells in the DPK in vitro differentiation system (Fig. 4b). These results suggest that a defect in actin polymerization could be at least part of the underlying mechanism of inhibition of positive selection in dnRac1-DPK cells.

Suppression of TCR-mediated induction of Bcl-2

The massive apoptosis of stimulated dnRac1-DPK cells could be due either to an increase in death effectors or to a decrease in expression of anti-apoptotic proteins, because TCR ligation to DP thymocytes induces death effectors such as Nur77 and Nor1 [27], as well as anti-apoptotic molecules such as Bcl-2 and Bcl-xL [28]. To clarify the mechanism of such apoptosis, we examined TCR-dependent induction of an anti-apoptotic molecule in the DPK system. By real-time reverse transcriptase (RT)-PCR analysis, a two- to threefold increase in Bcl-2 mRNA was observed in control DPK cells after TCR stimulation, whereas stimulation-dependent induction of Bcl-2 was not seen in dnRac1-DPK cells (Fig. 5a). Furthermore, induction of Bcl-2 was independent of actin polymerization, because latrunculin A treatment of DPK cells did not inhibit TCR-dependent Bcl-2 induction (Fig. 5a). In contrast, TCR-dependent induction of Nor1 in dnRac1-DPK was comparable to that of control cells (Fig. 5b). These data strongly suggest that increased susceptibility to TCR-induced apoptosis in dnRac1-DPK cells is due to failed induction of the anti-apoptotic molecule Bcl-2 and not to increased induction of death effector molecules.

Restoration of generation of CD4-SP cells by Bcl-2

If defective positive selection observed in dnRac1-DPK cells is mainly due to massive apoptosis caused by insufficient induction of Bcl-2, overexpression of Bcl-2 in dnRac1-DPK should restore the defect. We therefore retrovirally introduced Bcl-2 cDNA into dnRac1-DPK cells (Fig. 6). Retroviral introduction of human Bcl-2 construct successfully induced expression of Bcl-2 protein (Fig. 6b). Introduction of Bcl-2 enhanced stimulation-dependent generation of CD4-SP cells (Fig. 6a), but more importantly, introduction of Bcl-2 almost completely restored the generation of CD4-SP cells in dnRac1-DPK cells (Fig. 6a and upper panel of Fig. 6c). In addition, stimulation-dependent apoptosis observed in the dnRac1-DPK cell population was also rescued by the introduction of Bcl-2 (Fig. 6c, lower panel). These results indicate that defective generation of CD4-SP cells in the dnRac1-DPK cell population is mostly due to the lack of TCR-dependent Bcl-2 up-regulation.

DISCUSSION

Although T cell development in constitutively active Rac1 mutant mice has been extensively studied by Cantrell's group [1, 10, 11], the effect of a loss-of-function Rac1 mutant on T cell development has not been investigated. Findings suggesting that active Rac1 enhances TCR signal transduction include the following: constitutively active Rac1 transgenic mice manifested conversion from positive selection to negative selection [11], restoration of the T cell defect in $Vav^{-/-}$ mice, and generation of DP cells in $RAG^{-/-}$ mice [10]. In the current report, we showed that Rac1 is required for positive selection in an in vitro model system (Fig. 1). That TCR expression in dnRac1-DPK cells (Fig. 1a) was indistinguishable from that in control cells indicates that Rac1 is not involved in assembly and trafficking of TCR components to the cell surface.

We found that early ERK activation was not inhibited by the presence of dnRac1 (Fig. 3a). The requirement for ERK activation in positive selection has been well established [29, 30], and recently PAK1, a major downstream target of Rac1, was shown to be involved in ERK activation [31, 32]. However, normal ERK activation in dnRac1-DPK cells indicates that TCR-mediated activation of ERK is independent of Rac1. Thus, the inhibitory effect of dnRac1 on positive selection cannot be attributed simply to inhibition of ERK activation.

Rac1 is generally recognized as a key molecule in actin reorganization processes [33]. It is thus not surprising that introduction of dnRac1 prevented TCR-mediated actin polymerization (Fig. 4a). The role of actin cytoskeletal reorganization in T cell development is still not clear, although complete abrogation of positive selection in dominant-negative WASP transgenic mice [34] and in $Vav^{-/-}$ mice [35] is certainly consistent with a requirement for actin polymerization during positive selection. We demonstrated that actin polymerization is required for positive selection in the DPK system by using an inhibitor of actin polymerization, latrunculin A (Fig. 4b). Thus, the lack of differentiation of dnRac1-DPK cells could be due partly to defective TCR-mediated actin reorganization. Recently, TCR-mediated Rac activation and immunological synapse formation have been shown to depend on DOCK2 [36]. Impaired positive selection in DOCK2-deficient mice [36] is consistent with our finding that Rac1 is critical in positive selection via actin cytoskeletal reorganization.

In the present study, we observed increased

TCR-mediated apoptosis in dnRac1-expressing DPK cells (Fig. 2b). We do not believe that introduction of dnRac1 merely increases susceptibility to cell death, because expression of dnRac1 (Rac1-N17) in Jurkat cells has been shown to protect cells from Fas-mediated apoptosis [37]. However, dnRac1-DPK cells did not show increased susceptibility to steroid-induced cell death (data not shown). The balance between TCR-mediated induction of pro-apoptotic and anti-apoptotic mediators is a key determining factor in positive and negative selection. TCR stimulation of DP thymocytes induced the orphan transcription factors Nur77 and Nor1 [27], which play major roles in negative selection [38, 39] by inducing expression of Bim [40, 41] and Fas-L [42]. Induction of the *Nur77* gene is positively controlled by MEF2 and negatively controlled by Cabin1 [43] and HDAC7 [44] via histone deacetylation. TCR signaling releases these repressors from MEF2 to activate *Nur77* transcription. We observed no effect of dnRac1 on induction of these pro-apoptotic mediators in the DPK system (Fig. 5b). In fact, TCR-mediated Nur77 induction was even lower in dnRac1-DPK cells compared with control cells (data not shown). These results clearly indicate that increased apoptosis in activated dnRac1-DPK cells is not due to increased expression of pro-apoptotic mediators.

In contrast, expression of dnRac1 did affect expression of the anti-apoptotic mediator Bcl-2 (Fig. 5a). Involvement of Rac1 in the Bcl-2-mediated survival response could be critical in the positive selection of thymocytes. Rac2-deficient mast cells have been shown to be defective in Akt activation and Bcl-xL expression, which resulted in impaired survival [45]. Thus, involvement of Rac proteins in mediating cell survival may be a more general phenomenon. In DP thymocytes, one anti-apoptotic signal is the exclusion of Nur77 from the nucleus by Akt-dependent phosphorylation of Nur77 [46, 47]. Although we have not examined the phosphorylation status of Nur77 in dnRac1-DPK cells, we believe that involvement of Rac1 in regulation of Nur77 phosphorylation is unlikely. TCR-mediated activation of Akt depends on phosphatidylinositol 3-kinase (PI3K), and generation of CD4-SP cells in the DPK cell in vitro differentiation system is resistant to the PI3K inhibitors wortmannin and Ly294002 (unpublished observation).

Although Bcl-2-deficient mice showed unimpaired T cell development [48], Bcl-2 is a major anti-apoptotic molecule in thymocytes and reportedly plays important roles in development and maintenance of thymocytes [49]. Its expression increases immediately after positive selection [50], and TCR stimulation induces Bcl-2 transcription in vitro as well [28]. Two promoter regions have been identified in the 5' regulatory region of the *bcl-2* gene, and NF-κB was shown to bind one of these promoter regions [51]. Other studies showed that the expression of Bcl-2 is positively regulated by NFAT4 (nuclear factor of activated T cells) [52] and NF-κB2 [53]. Thus, Rac1 may be involved in TCR-mediated activation of NFAT or NF-κB to induce Bcl-2 transcription. Consistent with this idea, Rac1 has been implicated in the activation of NFAT4 in FcR-mediated signal transduction in mast cells [54]. TCR-dependent induction of Bcl-2 was not inhibited in the presence of latrunculin A, an inhibitor of actin polymerization (Fig. 5a). However, latrunculin A did not inhibit TCR-dependent up-regulation of CD69 and CD5 (data not shown). These data suggest that Rac1 affects actin reorganization and Bcl-2 induction independently.

In summary, in the present study, we demonstrated that Rac1 is required for positive selection. By using an in vitro positive selection model system, we showed that Rac1 was critical for TCR-mediated actin cytoskeletal reorganization, as well as induction of the anti-apoptotic mediator Bcl-2. A Rac1-dependent pathway of Bcl-2 induction could be crucial during positive selection by preventing TCR-mediated apoptosis, and thus future studies are needed to elucidate details of the mechanism of Bcl-2 induction.

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REFERENCES

1. Cantrell, D.A. (2003) GTPases and T cell activation. *Immunol. Rev.* **192**, 122-130.
2. Henning, S.W., Galandrini, R., Hall, A., Cantrell, D.A. (1997) The GTPase Rho has a critical regulatory role in thymus development. *EMBO J* **16**, 2397-2407.
3. Corre, I., Gomez, M., Vielkind, S., Cantrell, D.A. (2001) Analysis of thymocyte development reveals that the GTPase RhoA is a positive regulator of T cell receptor responses in vivo. *J Exp Med*

194, 903-914.
4. Na, S., Li, B., Grewal, I.S., Enslen, H., Davis, R.J., Hanke, J.H., Flavell, R.A. (1999) Expression of activated cdc42 induces T cell apoptosis in thymus and peripheral lymph organs via different pathways. *Oncogene* **18**, 7966-7974.
5. Li, B., Yu, H., Zheng, W., Voll, R., Na, S., Roberts, A.W., Williams, D.A., Davis, R.J., Ghosh, S., Flavell, R.A. (2000) Role of the guanosine triphosphatase Rac2 in T helper 1 cell differentiation. *Science* **288**, 2219-2222.
6. Croker, B.A., Handman, E., Hayball, J.D., Baldwin, T.M., Voigt, V., Cluse, L.A., Yang, F.C., Williams, D.A., Roberts, A.W. (2002) Rac2-deficient mice display perturbed T-cell distribution and chemotaxis, but only minor abnormalities in T_H1 responses. *Immunol Cell Biol* **80**, 231-240.
7. Yu, H., Leitenberg, D., Li, B., Flavell, R.A. (2001) Deficiency of small GTPase Rac2 affects T cell activation. *J Exp Med* **194**, 915-926.
8. Kim, C., Dinauer, M.C. (2001) Rac2 is an essential regulator of neutrophil nicotinamide adenine dinucleotide phosphate oxidase activation in response to specific signaling pathways. *J Immunol* **166**, 1223-1232.
9. Zhao, X., Carnevale, K.A., Cathcart, M.K. (2003) Human monocytes use Rac1, not Rac2, in the NADPH oxidase complex. *J Biol Chem* **278**, 40788-40792.
10. Gomez, M., Tybulewicz, V., Cantrell, D.A. (2000) Control of pre-T cell proliferation and differentiation by the GTPase Rac-I. *Nat Immunol* **1**, 348-352.
11. Gomez, M., Kioussis, D., Cantrell, D.A. (2001) The GTPase Rac-1 controls cell fate in the thymus by diverting thymocytes from positive to negative selection. *Immunity* **15**, 703-713.
12. Glogauer, M., Marchal, C.C., Zhu, F., Worku, A., Clausen, B.E., Foerster, I., Marks, P., Downey, G.P., Dinauer, M., Kwiatkowski, D.J. (2003) Rac1 deletion in mouse neutrophils has selective effects on neutrophil functions. *J Immunol* **170**, 5652-5657.
13. Gu, Y., Filippi, M.D., Cancelas, J.A., Siefring, J.E., Williams, E.P., Jasti, A.C., Harris, C.E., Lee, A.W., Prabhakar, R., Atkinson, S.J., Kwiatkowski, D.J., Williams, D.A. (2003) Hematopoietic cell regulation by Rac1 and Rac2 guanosine triphosphatases. *Science* **302**, 445-449.
14. Cancelas, J.A., Lee, A.W., Prabhakar, R., Stringer, K.F., Zheng, Y., Williams, D.A. (2005) Rac GTPases differentially integrate signals regulating hematopoietic stem cell localization. *Nat Med* **11**, 886-891.
15. Jansen, M., Yang, F.C., Cancelas, J.A., Bailey, J.R., Williams, D.A. (2005) Rac2-deficient hematopoietic stem cells show defective interaction with the hematopoietic microenvironment and long-term engraftment failure. *Stem Cells* **23**, 335-346.
16. Walmsley, M.J., Ooi, S.K., Reynolds, L.F., Smith, S.H., Ruf, S., Mathiot, A., Vanes, L., Williams, D.A., Cancro, M.P., Tybulewicz, V.L. (2003) Critical roles for Rac1 and Rac2 GTPases in B cell development and signaling. *Science* **302**, 459-462.
17. Kaye, J., Ellenberger, D.L. (1992) Differentiation of an immature T cell line: a model of thymic positive selection. *Cell* **71**, 423-435.
18. Zufferey, R., Donello, J.E., Trono, D., Hope, T.J. (1999) Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J Virol* **73**, 2886-2892.
19. Kitamura, T. (1998) New experimental approaches in retrovirus-mediated expression screening. *Int J Hematol* **67**, 351-359.
20. Morita, S., Kojima, T., Kitamura, T. (2000) Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther* **7**, 1063-1066.
21. Kaye, J., Hsu, M.L., Sauron, M.E., Jameson, S.C., Gascoigne, N.R., Hedrick, S.M. (1989) Selective development of CD4+ T cells in transgenic mice expressing a class II MHC-restricted antigen receptor. *Nature* **341**, 746-749.
22. Shao, H., Kono, D.H., Chen, L.Y., Rubin, E.M., Kaye, J. (1997) Induction of the early growth response (Egr) family of transcription factors during thymic selection. *J Exp Med* **185**, 731-744.
23. Shao, H., Rubin, E.M., Chen, L.Y., Kaye, J. (1997) A role for Ras signaling in coreceptor regulation during differentiation of a double-positive thymocyte cell line. *J Immunol* **159**, 5773-5776.
24. Ochoa-Garay, J., Kaye, J., Coligan, J.E. (1998) Nuclear factor κB is required for peptide antigen-induced differentiation of

a CD4+CD8+ thymocyte line. *J Immunol* **160**, 3835-3843.

25. Tahara-Hanaoka, S., Sudo, K., Ema, H., Miyoshi, H., Nakauchi, H. (2002) Lentiviral vector-mediated transduction of murine CD34$^-$ hematopoietic stem cells. *Exp Hematol* **30**, 11-17.
26. Spector, I., Shochet, N.R., Kashman, Y., Groweiss, A. (1983) Latrunculins: novel marine toxins that disrupt microfilament organization in cultured cells. *Science* **219**, 493-495.
27. Cheng, L.E., Chan, F.K., Cado, D., Winoto, A. (1997) Functional redundancy of the Nur77 and Nor-1 orphan steroid receptors in T-cell apoptosis. *EMBO J* **16**, 1865-1875.
28. Groves, T., Parsons, M., Miyamoto, N.G., Guidos, C.J. (1997) TCR engagement of $CD4^+CD8^+$ thymocytes in vitro induces early aspects of positive selection, but not apoptosis. *J Immunol* **158**, 65-75.
29. Alberola-Ila, J., Forbush, K.A., Seger, R., Krebs, E.G., Perlmutter, R.M. (1995) Selective requirement for MAP kinase activation in thymocyte differentiation. *Nature* **373**, 620-623.
30. Alberola-Ila, J., Hernandez-Hoyos, G. (2003) The Ras/MAPK cascade and the control of positive selection. *Immunol Rev* **191**, 79-96.
31. Slack-Davis, J.K., Eblen, S.T., Zecevic, M., Boerner, S.A., Tarcsafalvi, A., Diaz, H.B., Marshall, M.S., Weber, M.J., Parsons, J.T., Catling, A.D. (2003) PAK1 phosphorylation of MEK1 regulates fibronectin-stimulated MAPK activation. *J Cell Biol* **162**, 281-291.
32. Eblen, S.T., Slack, J.K., Weber, M.J., Catling, A.D. (2002) Rac-PAK signaling stimulates extracellular signal-regulated kinase (ERK) activation by regulating formation of MEK1-ERK complexes. *Mol Cell Biol* **22**, 6023-6033.
33. Tapon, N., Hall, A. (1997) Rho, Rac and Cdc42 GTPases regulate the organization of the actin cytoskeleton. *Curr Opin Cell Biol* **9**, 86-92.
34. Zhang, J., Shi, F., Badour, K., Deng, Y., McGavin, M.K., Siminovitch, K.A. (2002) WASp verprolin homology, cofilin homology, and acidic region domain-mediated actin polymerization is required for T cell development. *Proc Natl Acad Sci U S A* **99**, 2240-2245.
35. Cantrell, D. (1998) Lymphocyte signalling: a coordinating role for Vav? *Curr Biol* **8**, R535-538.
36. Sanui, T., Inayoshi, A., Noda, M., Iwata, E., Oike, M., Sasazuki, T., Fukui, Y. (2003) DOCK2 is essential for antigen-induced translocation of TCR and lipid rafts, but not PKC-θ and LFA-1, in T cells. *Immunity* **19**, 119-129.
37. Brenner, B., Koppenhoefer, U., Weinstock, C., Linderkamp, O., Lang, F., Gulbins, E. (1997) Fas- or ceramide-induced apoptosis is mediated by a Rac1-regulated activation of Jun N-terminal kinase/p38 kinases and GADD153. *J Biol Chem* **272**, 22173-22181.
38. Calnan, B.J., Szychowski, S., Chan, F.K., Cado, D., Winoto, A. (1995) A role for the orphan steroid receptor Nur77 in apoptosis accompanying antigen-induced negative selection. *Immunity* **3**, 273-282.
39. Zhou, T., Cheng, J., Yang, P., Wang, Z., Liu, C., Su, X., Bluethmann, H., Mountz, J.D. (1996) Inhibition of Nur77/Nurr1 leads to inefficient clonal deletion of self-reactive T cells. *J Exp Med* **183**, 1879-1892.
40. Szegezdi, E., Kiss, I., Simon, A., Blasko, B., Reichert, U., Michel, S., Sandor, M., Fesus, L., Szondy, Z. (2003) Ligation of retinoic acid receptor α regulates negative selection of thymocytes by inhibiting both DNA binding of *nur77* and synthesis of Bim. *J Immunol* **170**, 3577-3584.
41. Bouillet, P., Purton, J.F., Godfrey, D.I., Zhang, L.C., Coultas, L., Puthalakath, H., Pellegrini, M., Cory, S., Adams, J.M., Strasser, A. (2002) BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. *Nature* **415**, 922-926.
42. Weih, F., Ryseck, R.P., Chen, L., Bravo, R. (1996) Apoptosis of *nur77/N10*-transgenic thymocytes involves the Fas/Fas ligand pathway. *Proc Natl Acad Sci U S A* **93**, 5533-5538.
43. Youn, H.D., Liu, J.O. (2000) Cabin1 represses MEF2-dependent Nur77 expression and T cell apoptosis by controlling association of histone deacetylases and acetylases with MEF2. *Immunity* **13**, 85-94.
44. Dequiedt, F., Kasler, H., Fischle, W., Kiermer, V., Weinstein, M., Herndier, B.G., Verdin, E. (2003) HDAC7, a thymus-specific class II histone deacetylase, regulates Nur77 transcription and TCR-mediated apoptosis. *Immunity* **18**, 687-698.
45. Yang, F.C., Kapur, R., King, A.J., Tao, W., Kim, C., Borneo, J., Breese, R., Marshall,

M., Dinauer, M.C., Williams, D.A. (2000) Rac2 stimulates Akt activation affecting BAD/Bcl-XL expression while mediating survival and actin function in primary mast cells. *Immunity* **12**, 557-568.

46. Pekarsky, Y., Hallas, C., Palamarchuk, A., Koval, A., Bullrich, F., Hirata, Y., Bichi, R., Letofsky, J., Croce, C.M. (2001) Akt phosphorylates and regulates the orphan nuclear receptor Nur77. *Proc Natl Acad Sci U S A* **98**, 3690-3694.
47. Masuyama, N., Oishi, K., Mori, Y., Ueno, T., Takahama, Y., Gotoh, Y. (2001) Akt inhibits the orphan nuclear receptor Nur77 and T-cell apoptosis. *J Biol Chem* **276**, 32799-32805.
48. Nakayama, K., Negishi, I., Kuida, K., Shinkai, Y., Louie, M.C., Fields, L.E., Lucas, P.J., Stewart, V., Alt, F.W., et al. (1993) Disappearance of the lymphoid system in Bcl-2 homozygous mutant chimeric mice. *Science* **261**, 1584-1588.
49. Korsmeyer, S.J. (1992) Bcl-2: a repressor of lymphocyte death. *Immunol Today* **13**, 285-288.
50. Punt, J.A., Suzuki, H., Granger, L.G., Sharrow, S.O., Singer, A. (1996) Lineage commitment in the thymus: only the most differentiated (TCRhibcl-2hi) subset of $CD4^{+}CD8^{+}$ thymocytes has selectively terminated CD4 or CD8 synthesis. *J Exp Med* **184**, 2091-2099.
51. Catz, S.D., Johnson, J.L. (2001) Transcriptional regulation of *bcl-2* by nuclear factor κB and its significance in prostate cancer. *Oncogene* **20**, 7342-7351.
52. Oukka, M., Ho, I.C., de la Brousse, F.C., Hoey, T., Grusby, M.J., Glimcher, L.H. (1998) The transcription factor NFAT4 is involved in the generation and survival of T cells. *Immunity* **9**, 295-304.
53. Viatour, P., Bentires-Alj, M., Chariot, A., Deregowski, V., de Leval, L., Merville, M.P., Bours, V. (2003) NF-κB2/p100 induces Bcl-2 expression. *Leukemia* **17**, 1349-1356.
54. Turner, H., Gomez, M., McKenzie, E., Kirchem, A., Lennard, A., Cantrell, D.A. (1998) Rac-1 regulates nuclear factor of activated T cells (NFAT) C1 nuclear translocation in response to Fcε receptor type 1 stimulation of mast cells. *J Exp Med* **188**, 527-537.

Figure Legends

Figure 1 dnRac1 blocked differentiation of CD4-SP.
(a) Expression of GFP and TCRβ in dnRac1-transduced DPK cells. DPK cells infected with pMXs-PREP-dnRac1 expressed dnRac1 protein as well as GFP. Sorted GFP^{hi} cells were analyzed by flow cytometry (FSC: forward scatter). (b) Vector (pMXs-PREP) control (Cont-DPK) and pMXs-PREP-dnRac1-transduced DPK (dnRac1-DPK) cells were co-cultured with DC-I and 100 ng/ml SEA. After the indicated culture periods, cells were harvested and expression levels of CD4 and CD8 were analyzed by flow cytometry. The phenotype of GFP-positive cells is shown.

Figure 2 Expression of dnRac1 increased TCR-induced apoptosis.
(a) Stimulation-dependent apoptotic cell death in suspension culture. Vector control-transduced (Cont-DPK) or dnRac1-expressing DPK (dnRac1-DPK) cells were cultured for 16 h with (stim) or without (non) plate-bound anti-CD3ε monoclonal antibody, and cell death was evaluated by analysis of DNA content with propidium iodide. Numbers are proportions (%) of cells in the G_1 subfraction in the cell cycle analysis. (b) GFP-positive dnRac1 or vector control-transduced DPK cells at indicated culture periods were stained with annexin V, and the proportions of stained cells in each group were plotted.

Figure 3 dnRac1 did not inhibit TCR-dependent early ERK activation.
(a) DPK cells were activated by anti-CD3ε antibody. After the times (in minutes) indicated, cell lysates were prepared and analyzed by means of Western blotting with anti-phospho-ERK (pERK) or anti-ERK antibodies. P+I = 10 ng/ml PMA and 1 μg/ml A23187. (b) Expression of CD5 and CD69 in dnRac1-DPK cells was analyzed by use of FACS analysis after 16 h of culture with APC in the absence (dotted line) or presence (solid line) of SEA.

Figure 4 Rac1 was critical for TCR-dependent actin polymerization.
(a) TCR-mediated actin polymerization. Vector control- or dnRac1-transduced DPK cells were cultured on coverslips coated with anti-CD3ε and anti-CD28 monoclonal antibodies for 15 min and then were fixed, permeabilized, and stained with Alexa 594-conjugated phalloidin to detect polymerized actin fiber. (b) Generation of CD4-SP cells. DPK cells were co-cultured with DC-I and SEA for 3 days in the presence of indicated amounts of latrunculin A, an inhibitor of actin polymerization.

Figure 5 Effect of dnRac1 on expression of apoptosis-related genes.
Changes in expression of (a) Bcl-2α and (b) Nor1 were determined by quantitative real-time RT-PCR in DPK cells (dnRac1-DPK and Cont-DPK) activated with plate-bound anti-CD3ε and anti-CD28 antibodies for 16 h with or without 150 nM latrunculin A. Results are presented as a ratio of expression of the indicated gene and the control housekeeping gene GAPDH.

Figure 6 Overexpression of Bcl-2 restored generation of CD4-SP cells and TCR-mediated apoptosis in dnRac1-DPK cells.
(a) The vector (pMXs-PREP) (Cont-DPK) cells and pMXs-PREP-dnRac1/pMI.2-Bcl2-transduced DPK (dnRac1/Bcl-2-DPK) cells were co-cultured with DC-I and 100 ng/ml SEA. After 3 days, cells were harvested and analyzed via flow cytometry. Shown are CD4 and CD8 profiles of GFP-positive cells (Cont-DPK, dnRac1-DPK, and dnRac1/Bcl-2-DPK) and hCD2-positive cells (Bcl-2-DPK and dnRac1/Bcl-2-DPK). (b) Expression of Bcl-2 protein in transduced cells. Cell lysates of each transformant were subjected to SDS-PAGE, and Western blotting was performed with anti-Bcl-2 antibody. (c) The absolute number of CD4-SP cells (upper panel) and total number of cells (lower panel) after the indicated culture periods. The number of GFP-positive cells was counted.

Figure 1

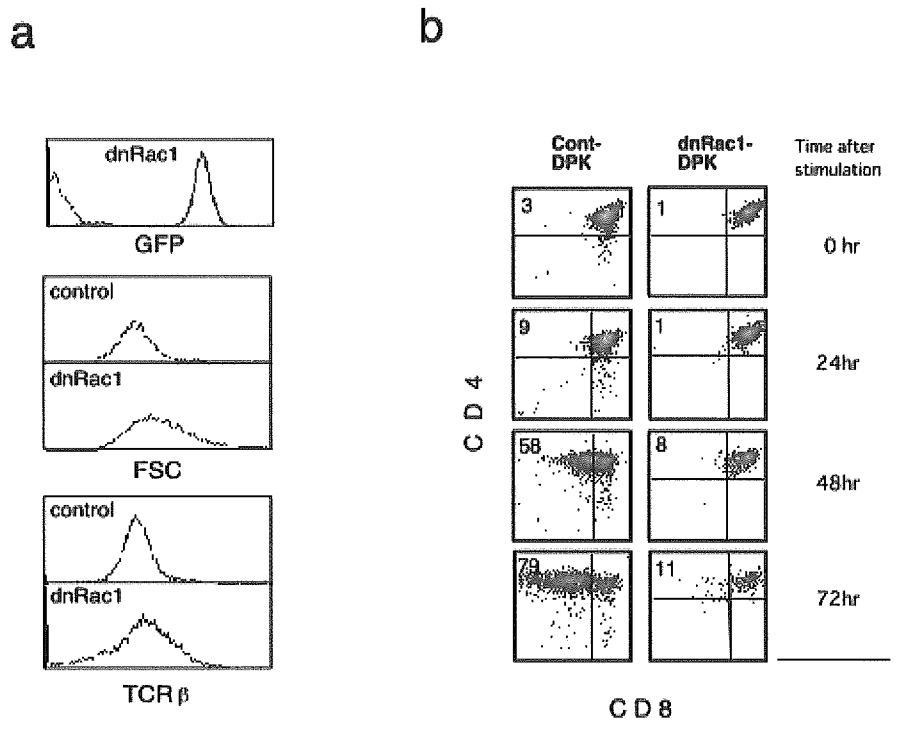

Figure 1 suzuki et al.

Figure 2

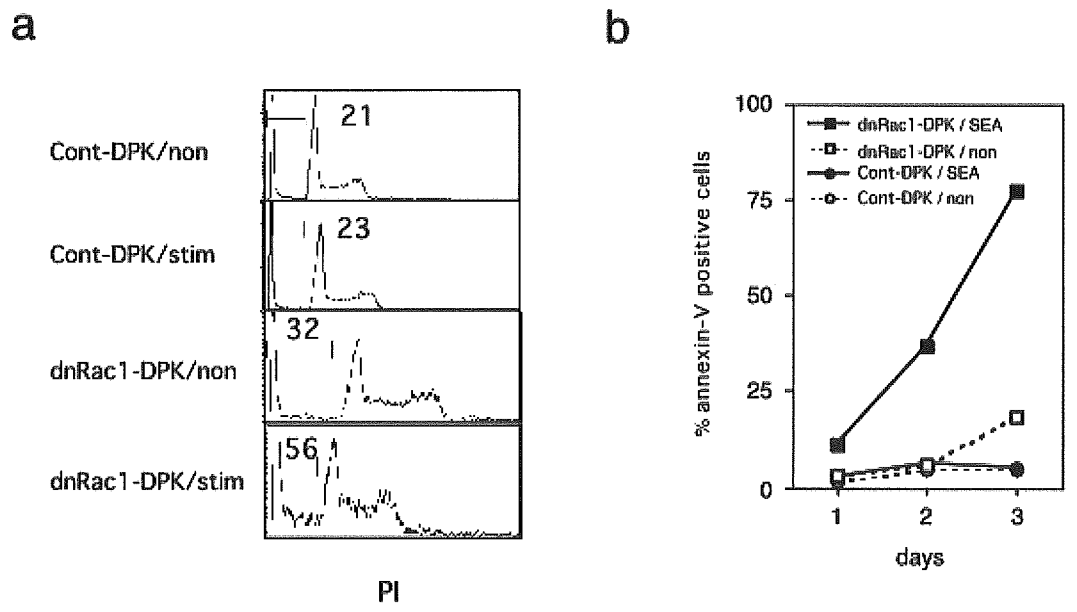

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Figure 3

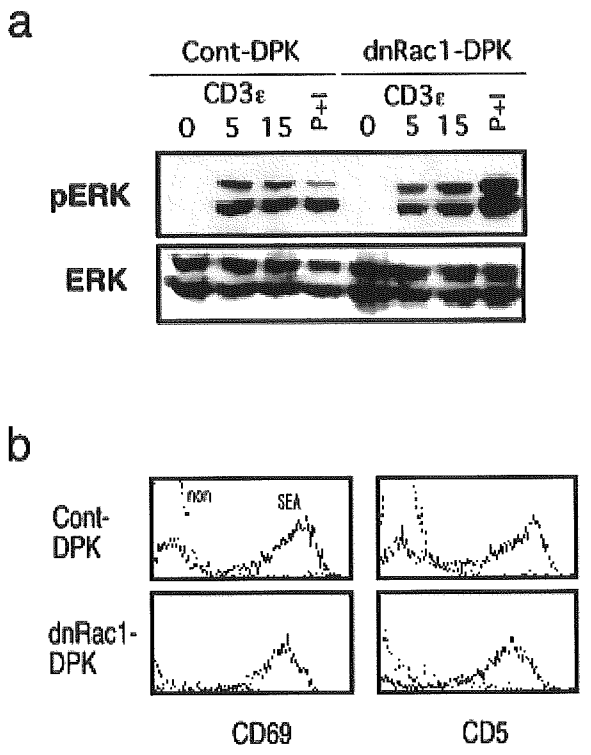

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Figure 4

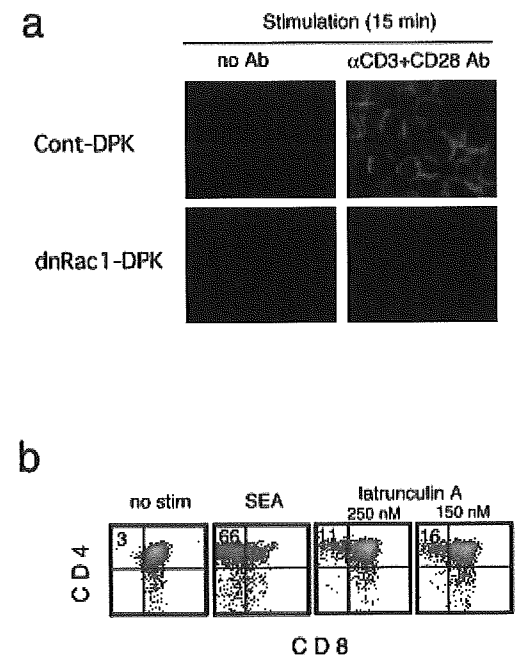

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Figure 5

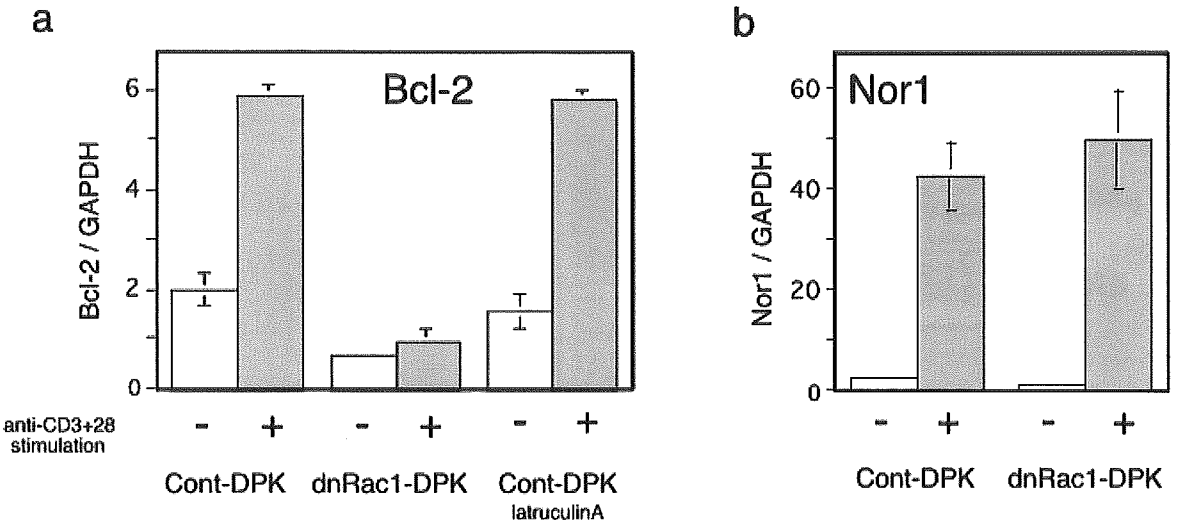

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Figure 6

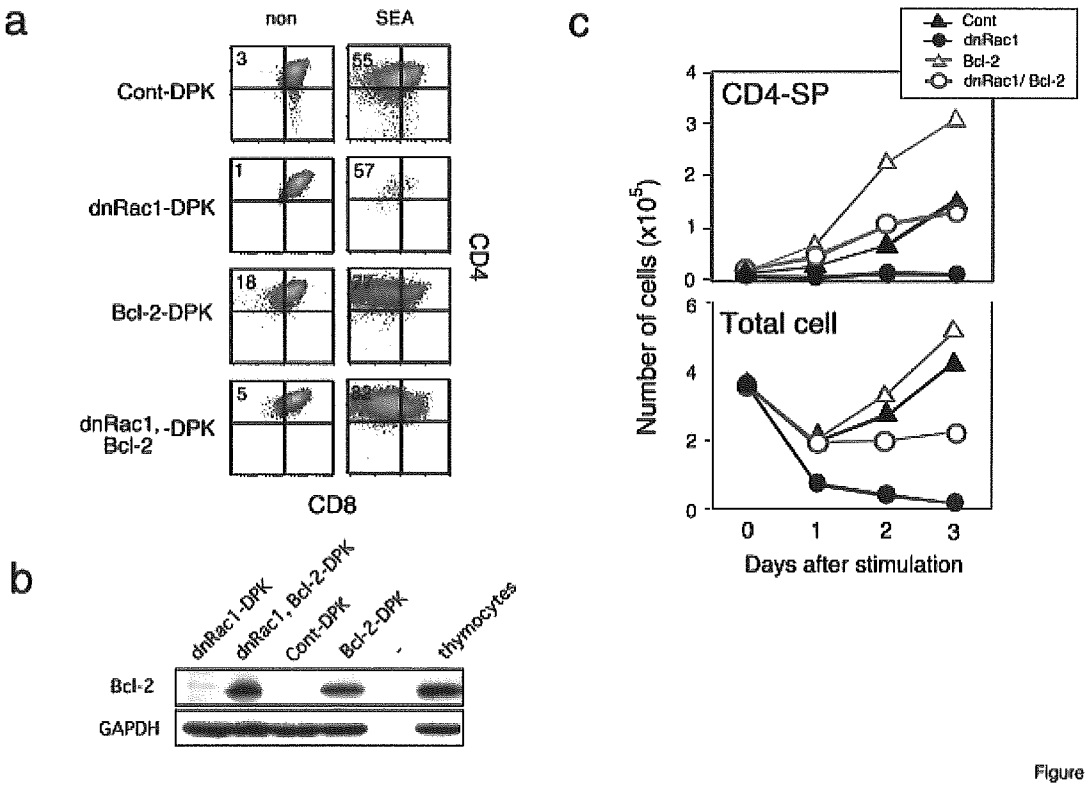

Figure 6 suzuki et al.

Cross-Positive Selection of Thymocytes Expressing a Single TCR by Multiple Major Histocompatibility Complex Molecules of Both Classes: Implications for $CD4^+$ versus $CD8^+$ Lineage Commitment[1]

Koji Eshima,* Harumi Suzuki,† and Nobukata Shinohara[2]*

This study has investigated the cross-reactivity upon thymic selection of thymocytes expressing transgenic TCR derived from a murine $CD8^+$ CTL clone. The Id^{high+} cells in this transgenic mouse had been previously shown to mature through positive selection by class I MHC, D^q or L^q molecule. By investigating on various strains, we found that the transgenic TCR cross-reacts with three different MHCs, resulting in positive or negative selection. Interestingly, in the TCR-transgenic mice of H-2^q background, mature Id^{high+} T cells appeared among both $CD4^+$ and $CD8^+$ subsets in periphery, even in the absence of RAG-2 gene. When examined on β_2-microglobulin$^{-/-}$ background, $CD4^+$, but not $CD8^+$, Id^{high+} T cells developed, suggesting that maturation of $CD8^+$ and $CD4^+$ Id^{high+} cells was MHC class I (D^q/L^q) and class II (I-A^q) dependent, respectively. These results indicated that this TCR-transgenic mouse of H-2^q background contains both classes of selecting MHC ligands for the transgenic TCR simultaneously. Further genetic analyses altering the gene dosage and combinations of selecting MHCs suggested novel asymmetric effects of class I and class II MHC on the positive selection of thymocytes. Implications of these observations in $CD4^+/CD8^+$ lineage commitment are discussed. ***The Journal of Immunology,*** **2006, 176: 1628–1636.**

The T cell clones are selected for compatibility to self Ag-presenting molecules (MHC molecules) after completing the rearrangement of TCR structural genes in the thymus. During this process, the choice of the coreceptor to be expressed is also made according to the class of the selecting MHC molecules. Analyses of a number of TCR-transgenic (Tg)[3] mouse lines and of mice deficient for MHC molecules clearly showed that CD4/CD8 double-positive (DP) cells expressing class I MHC-responsive TCR differentiate into $CD8^+$ T cells, while class II MHC-restricted DP cells differentiate into $CD4^+$ lineage. Recently, it was revealed that a zinc finger transcription factor Th-POK/cKrox plays a key role in the $CD4^+$ vs $CD8^+$ lineage commitment (1, 2). It still remains to be precisely clarified, however, how DP thymocytes undergoing positive selection could judge the class of MHC that they recognize, to differentiate into the appropriate lineages (for review, see Refs. 3 and 4).

Considering the size of the allelic heterogeneity of MHC molecules observed among wild mice, the chance of successful positive selection appears extremely low, even in the case with the full possibilities, i.e., heterozygotes of two full (I-E-positive) haplotypes. Nevertheless, it is also quite possible that a single TCR can find multiple different MHC molecules as compatible partners. In fact, T cell clones specific for a protein Ag presented by a self MHC molecule frequently show cross-reactivity to allogeneic MHC (+ peptide) (5–9). TCR may well show similar cross-reactivity (multiple specificity) at the level of positive selection in which lower affinity interaction is crucial. Such cross-reactions would minimize the wastefulness of the selection. The cross-reactivity in thymic selection can be best shown in TCR-Tg mice. In this study, an example of positive selection of a single TCR by multiple different MHC molecules (cross-positive selection) is shown. The cross-positive selection in this case involves even MHC molecules of different classes. This unique system provided us with an opportunity to verify several models proposed to date to explain $CD4^+$ vs $CD8^+$ lineage commitment.

To account for the mechanism by which MHC class specificity of TCR determines the lineage fate, two models were initially proposed: the instructive model and the stochastic/selective model (3, 4). The instructive model proposes that coreceptor molecules that assist with TCR in MHC recognition transduce signals that determine the lineage. Namely, recognition of class I MHC by TCR and CD8 delivers the signal(s) to shut off the CD4 gene and to initiate the differentiation into $CD8^+$ T cells, while TCR and CD4 coengagement by class II MHC sends signal(s) to silent CD8 gene and to induce $CD4^+$ lineage differentiation. In contrast, the stochastic/selective model postulates two-step differentiation of DP thymocytes. In this model, TCR-signaled DP cells differentiate into either $CD4^+$ or $CD8^+$ lineage in a stochastic way, following which only the cells with matched combinations of TCR specificity for MHC class and coreceptor expression would be filtered out by receiving the survival signals to mature.

After intensive assessments of these two models, two related models have been currently proposed, i.e., the strength of signal model and the kinetic signaling model. The strength of signal

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[3] Abbreviations used in this paper: Tg, transgenic; β_2m, β_2-microglobulin; DP, double positive; SP, single positive.

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model proposes that the strength or duration of the TCR signal dictates the fates of DP cells; stronger/longer signal would direct the precursor cells into $CD4^+$ T cells, whereas weaker/shorter signal would drive them into $CD8^+$ lineage (10–18). This model is supported by the fact that Lck, a *src* family tyrosine kinase that plays crucial roles in TCR-mediated signal transduction, is associated with CD4 at a much larger proportion than with CD8 molecule (19, 20). This fact may enable the MHC class II recognition by TCR and CD4 to trigger stronger signals than by TCR plus CD8. The kinetic signaling model proposes that the $CD4^+/CD8^+$ lineage choice would be influenced by the differential regulations in coreceptor gene expression. This is based on the observation that TCR-signaled DP cells terminate the CD8 gene expression transiently, which drives most of the positively selected cells into $CD4^+/CD8^{low}$ cells (21–26). At this stage, sustained TCR signal would be sent into class II MHC-restricted T cells and they become CD4 single-positive (SP) cells. In contrast, TCR signal may be ceased in class I-restricted DP cells by the loss of CD8, which would lead to Runx3-mediated CD8 reactivation and CD4 silencing, and consequently to $CD8^+$ lineage differentiation (27, 28).

Each of these models predicts different fates of the DP thymocytes expressing TCR that are responsive to both class I and class II MHC simultaneously in positive selection. The instructive model predicts that if TCR on DP thymocyte reacts with both classes of MHC, those cells would lose both of the coreceptor expressions and become CD4/CD8 double-negative T cells. From the stochastic/selective model, it is expected that both $CD4^+$ and $CD8^+$ subsets would mature in a mutually independent manner (without being affected with each other). In contrast, both the strength of signal model and the kinetic signaling model predict that those dual class-restricted DP cells would differentiate into $CD4^+$, rather than $CD8^+$ lineage.

In this study, we show that the DP cells expressing a single Tg TCR could develop into both $CD4^+$ and $CD8^+$ lineage when both classes of selecting MHCs are present in thymus. However, development of $CD4^+$ lineage was always dominant over CD8 lineage. In addition, by the use of mice with various H-2 haplotypes and of β_2-microglobulin (β_2m)-deficient mice, it was indicated that the selecting class II MHC was inhibitory to $CD8^+$ lineage differentiation, whereas the selecting class I MHC facilitated the maturation of $CD4^+$ lineage. These results did not perfectly match to the prediction from either model described above, suggesting that some modification(s) is required to be made to the models.

Materials and Methods

Mice

The TCR^{QM11}-Tg mouse was generated previously (29), and the progenies were typed by staining peripheral blood T cells using anti-Id Ab (29). B10.QBR ($H\text{-}2^{bq4}$), B10.S ($H\text{-}2^{s}$), and B10.D2 ($H\text{-}2^{d}$) mice were purchased from SLC; DBA/1 ($H\text{-}2^{q}$) and C57BL/6 ($H\text{-}2^{b}$) mice were obtained from Charles River Laboratories; and NOD/Shi ($H\text{-}2^{g7}$) and C3H/HeN ($H\text{-}2^{k}$) mice were from CLEA Japan. The $H\text{-}2^{b}$ mice deficient for RAG-2 (30) or β_2m (31) were kindly provided by Dr. M. Shimamura (Mitsubishi Kagaku Institute of Life Sciences, Tokyo, Japan). All mice used in this study were maintained in specific pathogen-free facility of Kitasato University School of Medicine. The experimental procedure was approved by the Animal Experimentation and Ethics Committee of the Kitasato University School of Medicine, and all animal experiments were conducted following the guidelines of the committee.

Reagents

Abs used in this study were as follows: FITC-labeled mAbs against CD4 (GK1.5), CD8α (53.6-7), CD69 (H1.2F3), and $I\text{-}A^{b}$ (AF6-120.1); PE-labeled Abs to CD4, CD8α, and CD154 (MR1); and PE-Cy5-labeled streptavidin were purchased from BD Pharmingen. Biotinylated anti-TCR^{QM11} Id Ab and FITC-labeled polyclonal rabbit Ab reactive to mouse Ig (but not to rat Ig) were prepared in our laboratory. Anti-D^q/L^q Ab (30-5-7, mouse IgG2a (32)) and anti-CD3ϵ Ab (145-2C11, hamster IgG) were prepared as ascitic form. PMA and the calcium ionophore ionomycin were purchased from Sigma-Aldrich.

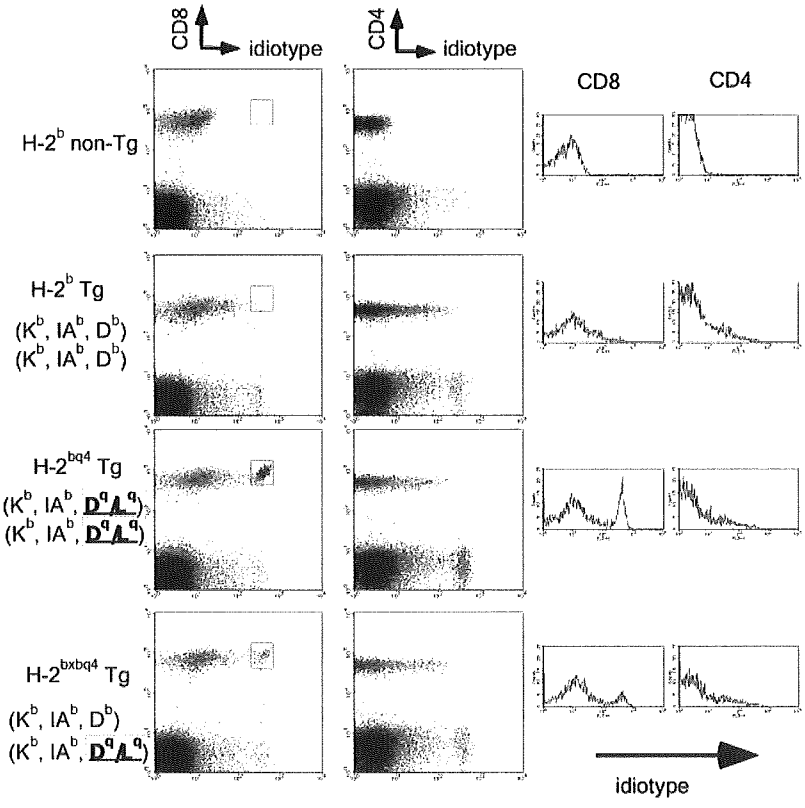

FIGURE 1. Positive selection of Id^{high+} cells by $H\text{-}2D^q/L^q$ molecule. Peripheral blood leukocytes from TCR^{QM11}-Tg or non-Tg mice of indicated H-2 haplotypes were stained with FITC-labeled anti-CD8α Ab, PE-labeled anti-CD4 Ab, and biotinylated anti-Id Ab, followed by PE-Cy5-labeled streptavidin. Stained cells were analyzed by three-color flow cytometry. Id expressions on $CD8^+$ and $CD4^+$ T cells are shown as histograms (*right panels*).

Flow cytometry

Single cell suspension was prepared from spleens or thymi using frosted slide glasses (Matsunami Glass). Flow cytometric analyses were performed, as described previously (33). Briefly, one million cells were stained in and washed with ice-cold HBSS containing 0.5% BSA and 0.02% NaN_3. Secondary staining was performed in the same manner. Stained cells after washing were examined by flow cytometric analyses on FACSCalibur (BD Biosciences). Data were acquired and analyzed on CellQuest software.

Development and maintenance of T cell lines

The $CD4^+$ and $CD8^+$ T cell lines (designated as 4Rq11 and 8Rq11, respectively) were established from peripheral blood of H-2^q RAG-$2^{-/-}$ TCR^{QM11}-Tg mouse by stimulating peripheral blood T cells with irradiated splenocytes of C3H/HeN mouse after depleting $CD8^+$ or $CD4^+$ cells, respectively, by Abs and rabbit complement. The cell lines were maintained by biweekly stimulation with C3H/HeN splenocytes, in DMEM supplemented with 10% FCS and 2.5% conditioned medium prepared from 48-h culture of Con A-stimulated LEW rat splenocytes.

Cell-mediated cytotoxicity assay

Cytolytic activity of T cell lines was performed using anti-CD3ε mAb, 2C11 and FcR^+ tumors, P815 (H-2^d, mastocytoma, Fas^-), and A20.2J (H-2^d, B lymphoma, Fas^+) as targets. The target cells were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ for 1 h at 37°C in 10% CO_2 incubator. Washed target cells (2500 cells/well) were incubated with 40,000 effector T cells in round-bottom 96-well plate (Falcon) in the presence of gradually diluted ascitic form of 2C11 for 6 h in CO_2 incubator at 37°C. After incubation, supernatants were harvested, and the radioactivities in the supernatants were measured by gamma counter. Specific lysis was calculated as follows: percent specific release = 100 × (experimental release − spontaneous release)/(maximal release − spontaneous release). Spontaneous release or maximum release was determined from wells with target cells alone or wells in which target cells were lysed with 1% Nonidet P-40, respectively. Assays were performed at triplicates.

HT-2 assay

Forty thousand 4Rq11 or 8Rq11 were cultured with 8×10^5 irradiated (20 Gy) splenocytes from C3H/HeN or C57BL/6 mouse in a microtiter plate for 14 h. The amount of the cytokines released in the supernatants was examined for the ability to support the growth of HT-2 cells, as described previously (33). Briefly, 1×10^4 HT-2 cells were cultured in the presence of the serially diluted supernatants for 24 h, and [^{3}H]thymidine incorporation for the last 8 h was measured.

Bone marrow chimera

The H-2^q mice of $\beta_2m^{-/-}$ or $\beta_2m^{+/-}$ were obtained by using DBA/1 mouse (H-2^q) and H-2^b $\beta_2m^{-/-}$ mouse. The recipient mice were irradiated (8 Gy) using the x-ray irradiator MBR-1505R (Hitachi Medico) with a filter (Cu, 0.5 mm; Al, 2 mm). Fifteen million bone marrow cells from H-2^b RAG-$2^{-/-}$ TCR^{QM11}-Tg mouse were injected i.v. 20 h after irradiation. The reconstituted mice were analyzed 2 mo later.

Results

Description of the TCR^{QM11}-Tg mouse

In a previous study, we created a Tg mouse bearing genomic TCR α- and β-chain genes from a $CD8^+$, I-A^k-specific allogeneic CTL clone, QM11, which was established from a B10.QBR mouse (H-2^{bq4}: K^b, I-A^b, D^q/L^q) (29). Using highly specific anti-Id Ab, it was demonstrated that this clone (Id^{high+} cells) had been positively selected by D^q/L^q molecule to differentiate into $CD8^+$ cells. As shown in Fig. 1, Id^{high+} cells matured into $CD8^+$ subset (but not into $CD4^+$ cells) in periphery of Tg mouse of H-2^{bq4}. In contrast, Id^{high+} T cells were not observed either in $CD8^+$ or $CD4^+$ subset in B6 (H-2^b) background, which differs from B10.QBR (H-2^{bq4}) only at the H-2 D (L) region. The MHCs of H-2^b haplotype do not appear to have any effects on the selection of Id^{high+} cells (29), which was further supported by the observation that the thymi from β_2m-deficient and -sufficient H-2^b Tg mice contained similar percentage and number of DP cells (data not shown). The Id^{high+} cells are the ones that have been positively selected via TCR^{QM11}, and $Id^{low/-}$ cells have been shown to be Vα-double expressers selected by virtue of other pairs of TCRαβ (29). Interestingly, the number of $CD8^+$ Id^{high+} cells was larger in H-2^{bq4} Tg mouse than in H-2^{bxbq4} (B6 × B10.QBR)F_1 mouse (Fig. 1). This may indicate the gene dosage effect of D^q/L^q molecule on the efficiency of positive selection for $CD8^+$ Id^{high+} cells.

Cross-reactivity of Tg TCR to MHC molecules of other haplotypes in thymic selection

To investigate the cross-reactivity of this Tg TCR to other MHC molecules in thymic selection, we crossed this Tg mice to various strains of laboratory mice and examined the maturation of Id^{high+} cells. As shown in Fig. 2*A*, in H-2^{bxd} background, Id^{high+} cells differentiated into $CD8^+$ subset. This population was not observed in β_2m-deficient H-2^{bxd} mouse (data not shown), nor in H-2^{bxg7} mouse, which shares H-2K^d, but not H-2D^d (/L^d) molecule with

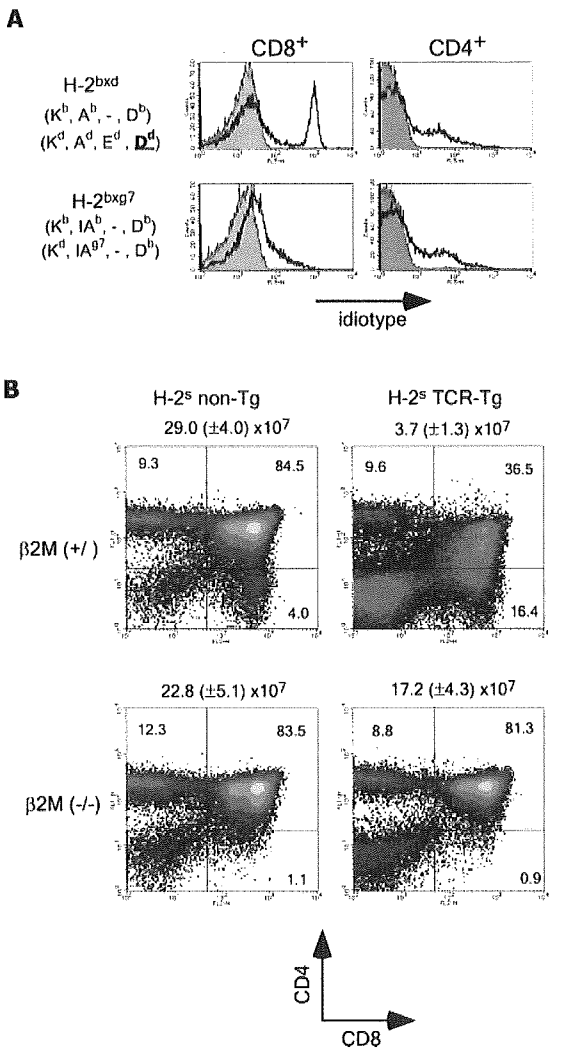

FIGURE 2. Cross-reaction of Tg thymocytes to class I MHC of H-2^d and H-2^s in thymic selection. *A*, PBL from TCR^{QM11}-Tg mice of (C57BL/6 × B10.D2)F_1 (H-2^{bxd}) or (C57BL/6 × NOD)F_1 (H-2^{bxg7}) background were stained with Abs to CD4, CD8α, or TCR^{QM11} Id. Id expressions on $CD8^+$ or $CD4^+$ cells are shown. Shaded histograms indicate negative staining. *B*, Single cell suspension of thymocytes from TCR-Tg (*right panels*) or non-Tg (*left panels*) deficient (*lower panels*) or sufficient (*upper panels*) for β_2m was stained with Abs to CD4, CD8, or Id, and expression of CD4 and CD8 is shown. The average number of total thymocytes (±SD, n = 3–4) was also shown on each panel.

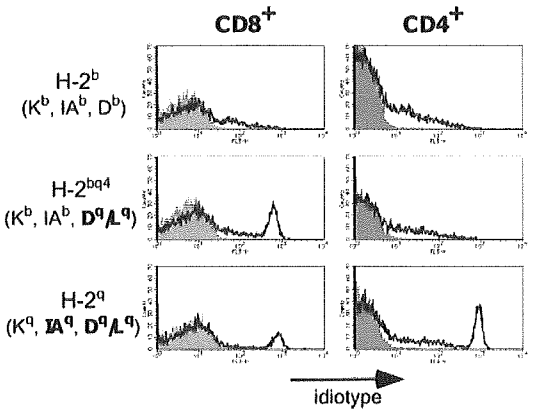

FIGURE 3. Maturation of Id^{high+} cells in both $CD8^+$ and $CD4^+$ subsets in mouse of H-2^q haplotype. Id expression on $CD8^+$ or $CD4^+$ cells in peripheral blood of TCR^{QM11}-Tg mice of indicated haplotypes was analyzed. Shaded histograms indicate negative staining.

H-2^{bxd} mouse, suggesting that D^d or L^d is the selecting element of H-2^d.

In H-2^s background, it was noticed that the percentage of peripheral blood T cells was decreased in the Tg mice ($CD8^+$, 2.6 ± 0.7%; $CD4^+$, 5.2 ± 2.3%; n = 11, 4–10 wk old) as compared with non-Tg littermates ($CD8^+$, 12.1 ± 1.4%; $CD4^+$, 18.1 ± 2.9%; n = 5, 4–10 wk old). Flow cytometric analyses of their thymocytes revealed that the proportion of CD4/CD8 DP cells was decreased in the Tg mice, and the total number of thymocytes was ~1/10 to 1/5 of non-Tg thymi (Fig. 2*B*). Instead, in the periphery, $CD8^{low+}$ Id^{high+} cells were present. This type of cell expressing Tg TCR is especially apparent in the mice in which the negative selection takes place (34). In the absence of β_2m, however, both the total number of thymocytes and the proportion of DP cells were restored to the comparable level as non-Tg littermates. These results indicated that the TCR^{QM11} also cross-reacts with H-2^s class I MHC with somewhat higher affinity, resulting in (partial) negative selection to Id^{high+} cells.

TCR^{QM11} could cross-react with class II MHC in thymic positive selection to generate mature $CD4^+$, Id^{high+} cells

Among various strains, it was found that, on H-2^q background, Id^{high+} cells differentiated into $CD4^+$ subset (Fig. 3). On this background, Id^{high+} cells were also found in $CD8^+$ population, although the number and the proportion of mature $CD8^+$ Id^{high+} cells in thymus or spleen were always smaller than those of mature $CD4^+$ Id^{high+} cells (data not shown). The maturation of $CD8^+$ Id^{high+} cells was most likely due to positive selection by the originally determined selecting class I MHC, D^q/L^q. The maturation of $CD4^+$ Id^{high+} cells was presumed to be dependent on $I\text{-}A^q$, because this molecule is the only class II MHC in H-2^q mouse. To examine this possibility, the TCR-Tg mice were crossed with β_2m-deficient mice to obtain H-2^q, $\beta_2m^{-/-}$ TCR-Tg mice, and the maturation of Id^{high+} cells in these mice was investigated. As shown in Fig. 4*A*, $CD8^+$ Id^{high+} cells disappeared in $\beta_2m^{-/-}$ TCR-Tg mouse, while $CD4^+$ Id^{high+} cells could still mature in the absence of β_2m. These results indicate that, in H-2^q TCR-Tg mouse, both class I and class II MHC mediate positive selection, resulting in maturation of $CD8^+$ Id^{high+} and $CD4^+$ Id^{high+} T cells. Interestingly, however, the generation of $CD4^+$ Id^{high+} cells in $\beta_2m^{-/-}$ mice appeared to be impaired as compared with in $\beta_2m^{+/-}$ background (Fig. 4*B*), suggesting that class I MHC could contribute to efficient maturation of $CD4^+$ Id^{high+} cells when selecting class II MHC molecules coexist.

Both $CD4^+$ and $CD8^+$ subsets matured in RAG-$2^{-/-}$ H-2^q TCR-Tg mice

Although T cells expressing only Tg TCR were readily identified as Id^{high+} cells and distinguished from Id^{low+} or Id^- cells that matured independently of Tg TCR, we confirmed that cells expressing a single TCR could truly differentiate into both $CD4^+$ and $CD8^+$ cells in TCR^{QM11}-Tg mice of H-2^q background. The TCR-Tg mice were crossed with RAG-2-deficient mice, and the T cell differentiation was investigated in H-2^q RAG-$2^{-/-}$ TCR-Tg mice. As shown in Fig. 5, both CD4 and CD8 SP subsets were observed as Id^{high+} cells in the thymi and in periphery of these mice. Once again, the number and the proportion of $CD4^+$ cells were larger than those of $CD8^+$ cells. Despite that the CD4/CD8 ratio varied among individuals, $CD4^+$ population was always dominant. This variation appeared independent of their sex or age (Fig. 5*B*, and data not shown).

To investigate whether these $CD4^+$ and $CD8^+$ cells in H-2^q RAG-$2^{-/-}$ TCR-Tg mice matured in normal functional T cells, their functional characteristics were examined. After establishing $CD4^+$ and $CD8^+$ cell lines from a RAG-$2^{-/-}$ H-2^q TCR-Tg

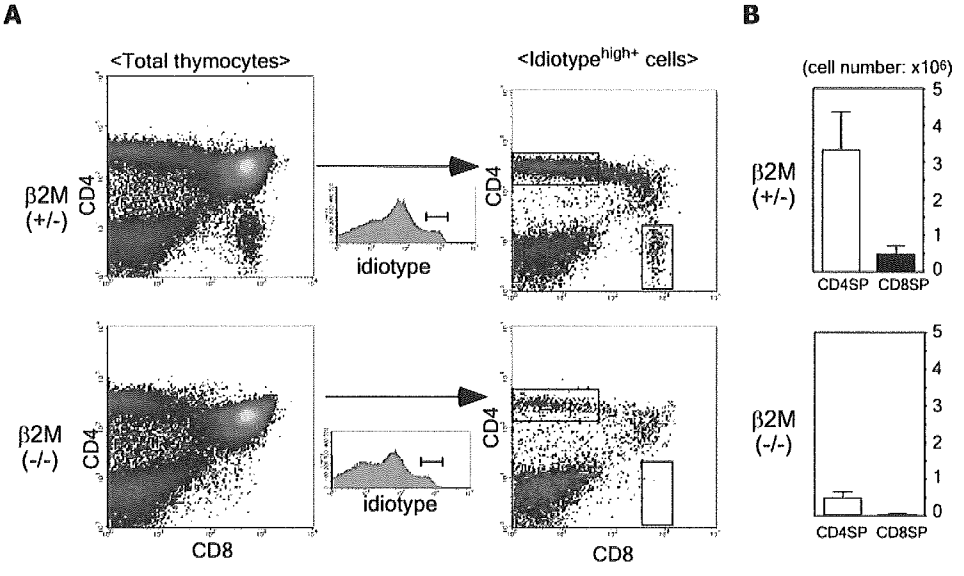

FIGURE 4. Maturation of $CD4^+$, but not $CD8^+$, Id^{high+} cells in H-2^q Tg mice deficient for β_2m. *A*, Single cell suspension of thymocytes from H-2^q TCR-Tg mice of $\beta_2m^{+/-}$ (*upper panels*) or $\beta_2m^{-/-}$ (*lower panels*) was stained with Abs to CD4, CD8, or Id. The expression of CD8α and CD4 on total (*left panels*) or Id^{high+} (*right panels*) is shown. *B*, The numbers of CD4 SP and CD8 SP Id^{high+} thymocytes from H-2^q Tg $\beta_2m^{+/-}$ (*top*, n = 5) or $\beta_2m^{-/-}$ (*bottom*, n = 6) mice are shown.

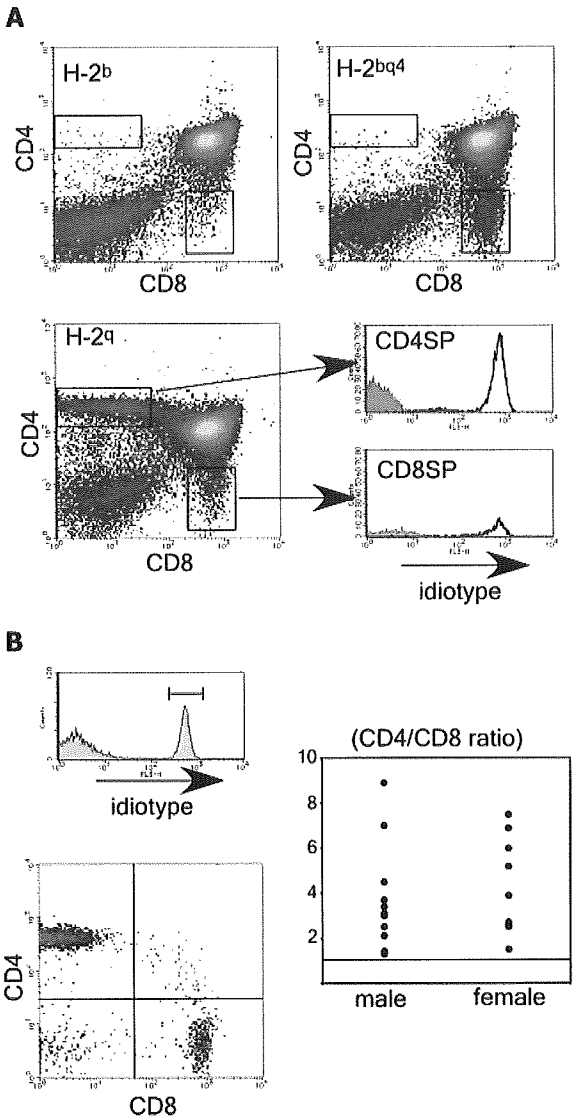

FIGURE 5. Differentiation of T cells in RAG-2-deficient TCR^{QM11}-Tg mice. *A*, Thymocytes from RAG-2-deficient Tg mice of indicated H-2 haplotypes were analyzed for the expression of CD4, CD8α, and Id. There were no significant differences in total thymocyte number among these mice. Id expression of CD4 SP or CD8 SP thymocytes from H-2^q mouse is shown as histograms. The shaded histograms show negative staining. *B*, PBL from H-2^q were stained with Abs to CD4, CD8α, or Id. The Id expression (*left histogram*) and CD4/CD8 expression on Id^+ PBL (*left bottom*) are shown. The ratio of $CD4^+/CD8^+$ Id^+ cells in PBL from H-2^q RAG-$2^{-/-}$ Tg mice (5–9 wk old) is shown in the *right graph.*

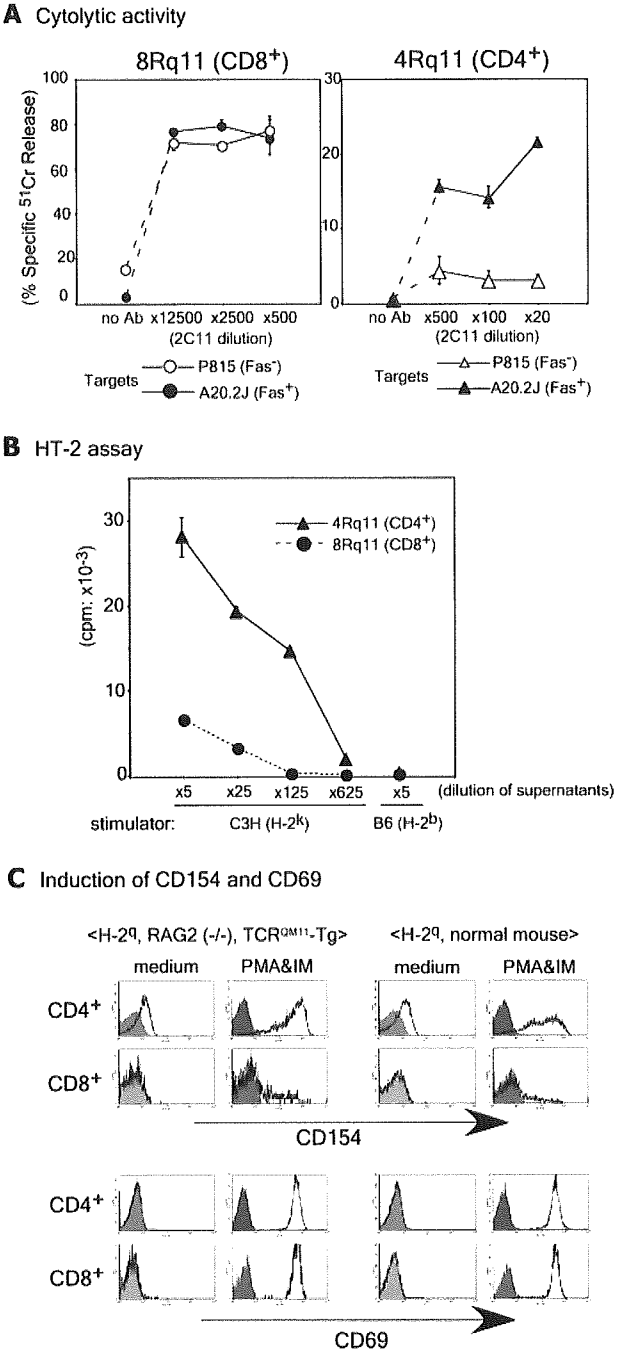

FIGURE 6. Functional characteristics of $CD4^+$ and $CD8^+$ cells matured in H-2^q, RAG-$2^{-/-}$, TCR^{QM11}-Tg mouse. *A*, $CD8^+$ (8Rq11, circle) and $CD4^+$ (4Rq11, triangle) T cell lines were established from a H-2^q RAG-2-deficient Tg mouse, and their cytolytic activities were examined using FcR^+ target cells and anti-CD3ϵ Ab, 2C11 (diluted ascites). Cytotoxic activity on Fas-negative P815 (open symbols) and on Fas-positive A20.2J (closed symbols) was shown as percent specific ^{51}Cr release. E:T ratio was 16. *B*, The lymphokine production by 8Rq11 (circle, dashed line) or 4Rq11 (triangle, solid line) was examined with IL-2/IL-4-dependent HT-2 cells. Shown were proliferative responses of HT-2 cells in the presence of diluted culture supernatants of the T cell lines stimulated with C3H (expressing $I\text{-}A^k$, for which TCR^{QM11} is specific) or B6 splenocytes. *C*, PBLs from H-2^q, RAG-$2^{-/-}$, TCR^{QM11}-Tg, or wild-type mouse were cultured in the presence (PMA/IM) or absence (medium) of PMA (10 ng/ml) and ionomycin (1 μg/ml). After 8-h culture, the cells were harvested, and the expression of CD154 (*upper panels*) or CD69 (*lower panels*) on $CD4^+$ and $CD8^+$ T cells was examined.

mouse, their $I\text{-}A^k$ specificity was confirmed by cytolytic assay using A20. 2J (B lymphoma, H-2^d) and its $I\text{-}A^k$ transfectant A20.$\alpha\beta$, as target cells (data not shown). It has been demonstrated that, in general, cytolytic activity of $CD4^+$ T cells (mainly Th1) is entirely dependent on Fas ligand/Fas pathway, whereas cytolytic $CD8^+$ T cells use both perforin/granzyme and Fas ligand/Fas pathways (35). This rule held true in the $CD4^+$ and $CD8^+$ cell lines, when their cytotoxic activities were tested on Fas^+ and Fas^- cells (Fig. 6*A*). Thus, $CD4^+$ line could kill Fas^+ target cells, but not Fas^- cells, whereas the $CD8^+$ line exerted efficient cytolytic activity on both Fas-positive and -negative target cells.

In contrast, as shown in Fig. 6*B*, the Ag-specific production of lymphokines that support the growth of HT-2 cells (IL-2 and/or IL-4) was observed in a larger amount from the $CD4^+$ line than

from the $CD8^+$ cell line. We also examined the expression of CD154 upon activation, which is normally found on $CD4^+$ T cells, but not on $CD8^+$ T cells. As observed in T cells from a normal mouse, the CD154 expression could indeed be induced preferentially on $CD4^+$ T cells, but not on $CD8^+$ T cells from peripheral blood of H-2^q RAG-$2^{-/-}$ TCR-Tg mouse (Fig. 6*C*). Taken together, these results indicate that T cells bearing a single TCR could differentiate into both $CD4^+$ and $CD8^+$ T cells with typical functional characteristics.

Cross talk of selecting class I and class II MHC signals in positive selection of Id^{high+} cells

The observations described above have shown that D^q/L^q and I-A^q are the selecting MHCs for TCR^{QM11}, and therefore that H-2^q mouse has both class I and class II, and H-2^{bq4} mouse has only class I-selecting MHC molecules for TCR^{QM11}. Using these mice as well as the neutral H-2^b mice, we next evaluated the effect of the density of selecting ligands on the lineage commitment of Id^{high+} cells. From the strength of signal model, it was predicted that the alteration of total ligand density would influence the ratio of $CD4^+/CD8^+$ Id^{high+} cells. We thus expected that in H-2^{bxq} mice, which have half densities of selecting MHCs of both classes as compared with H-2^q mice, the differentiation of $CD8^+$ Id^{high+} cells might be promoted even in the presence of selecting class II MHC. However, in H-2^{bxq} mice, the differentiation efficiency of Id^{high+} cells into both $CD8^+$ and $CD4^+$ subsets were similarly decreased (Figs. 7 and 8*A*). Interestingly, in H-2^{bq4xq} mice, as compared with H-2^{bxq} mice, the hemizygous increase of selecting class I ligand enhanced the generation of not only $CD8^+$ Id^{high+} cells, but also of $CD4^+$ cells, indicating that the selecting class I ligand could contribute to positive selection of $CD4^+$ T cells. This was consistent with the observation in Fig. 4, in which it was shown that in the absence of β_2m, the efficiency of positive selection of $CD4^+$ Id^{high+} T cells was decreased.

In contrast, the selecting class II MHC (I-A^q) seemed inhibitory to the optimal maturation of $CD8^+$ Id^{high+} cells. We compared the percentage and the number of $CD8^+$ Id^{high+} cells in three strains, H-2^{bq4}, H-2^{bq4xq}, and H-2^q, all of which have the same density of (homozygous) D^q/L^q molecules. As shown in Figs. 7 and 8, more $CD8^+$ Id^{high+} cells were observed in the thymus and in the periphery in mice lacking I-A^q molecules (H-2^{bq4} mouse) than in mice bearing I-A^q (H-2^{bq4xq} and H-2^q). Collectively, these results indicate that the class II MHC ligand may suppress the class I-dependent maturation of $CD8^+$ Id^{high+} cells, while the selecting class I could enhance the class II MHC-dependent differentiation of $CD4^+$ Id^{high+} cells.

Discussion

In the present study, the detailed analyses of TCR^{QM11}-Tg mice provided us with several important implications for the issues on thymic selection. 1) A single TCR on DP thymocytes could interact with multiple MHC molecules, irrespective of their classes, at thymic selection. 2) When both classes of selecting MHCs are present simultaneously, DP cells could differentiate into both

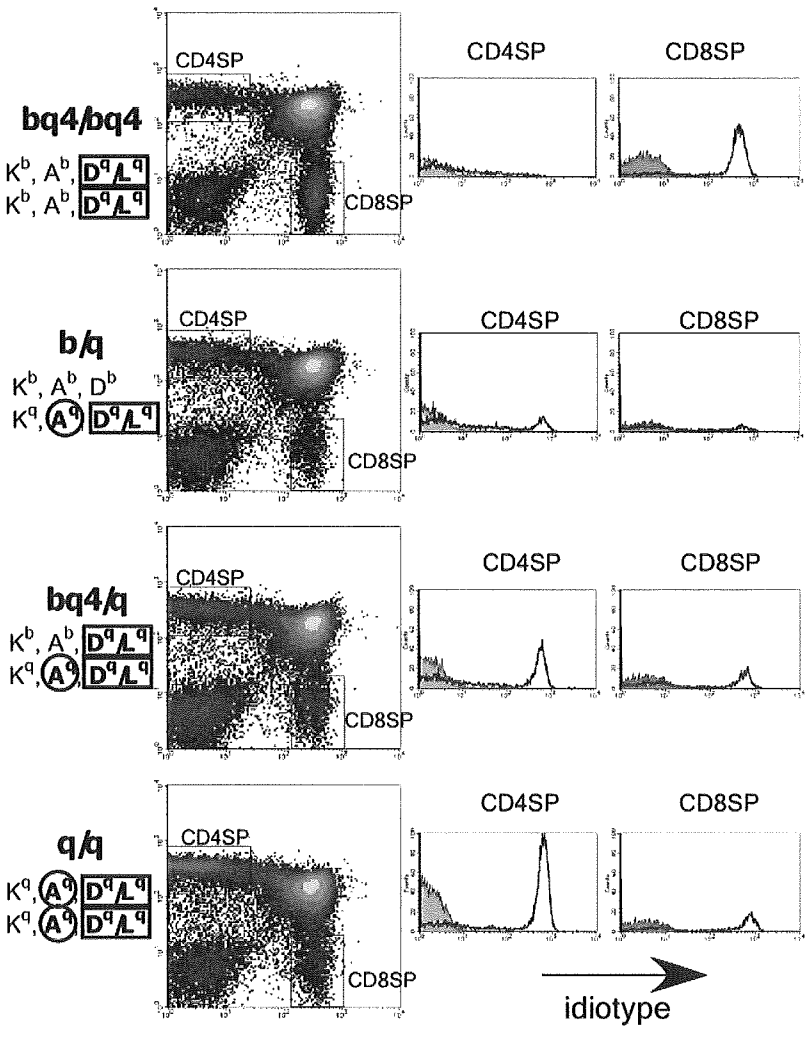

FIGURE 7. Id expression on CD4 SP and CD8 SP thymocytes matured in various H-2 circumstances. Thymocytes from Tg mice on indicated H-2 background were analyzed for the expression of CD4, CD8, and Id. The histograms show the Id expression after gating for CD4 SP or CD8 SP cells. The scales for *y*-axes (relative cell number) are same in all histograms. The shaded histograms indicate negative staining. The total thymocyte numbers were similar among the mice on these backgrounds at similar age.

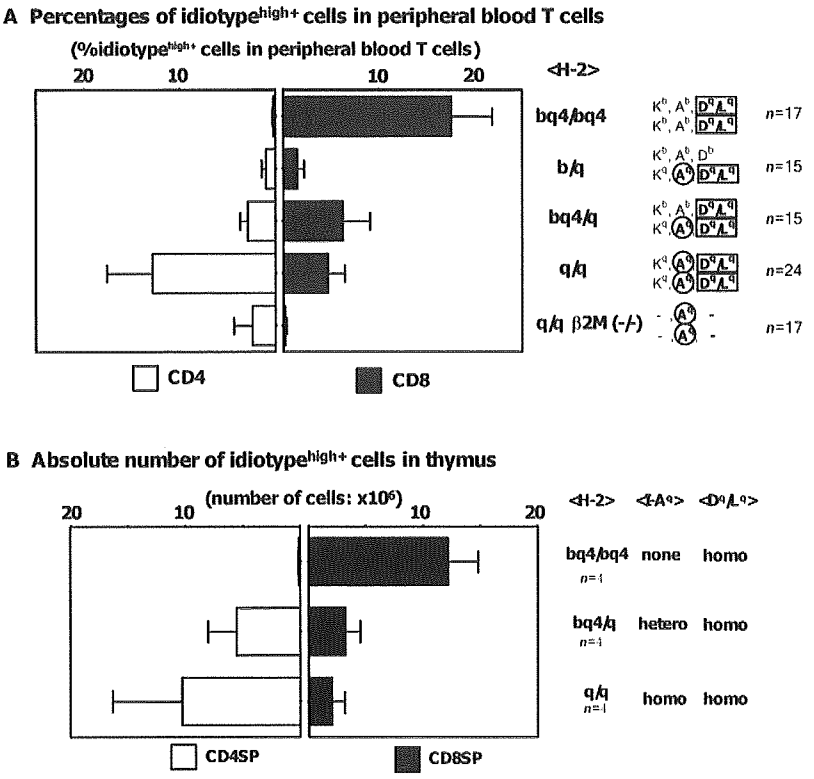

FIGURE 8. Effect of the density and combination of selecting MHC ligands on the differentiation of Id^{high+} cells. *A*, The percentages of $CD4^+$ Id^{high+} cells (□) and $CD8^+$ Id^{high+} cells (■) in peripheral blood T cells in the mice of indicated H-2 haplotypes were analyzed. *B*, Shown are the absolute numbers of CD4 SP and CD8 SP Id^{high+} cells in the thymi from the mice of indicated H-2 haplotypes, which were from breeding of male and female H-2^{bq4xq} TCR-Tg mice.

$CD4^+$ and $CD8^+$ lineage. 3) In the positive selection of dual class-responsive TCR, the effect of two classes of selecting MHCs was asymmetric. Namely, the selecting MHC class I was critical for generating $CD8^+$ cells, but also was supportive for $CD4^+$ differentiation. In contrast, selecting class II MHC was mandatory for $CD4^+$ lineage differentiation, but was inhibitory for $CD8^+$ differentiation.

The interaction of a single TCR with multiple MHC upon thymic selection

The analyses of TCR^{QM11}-Tg mice have revealed an example of cross-selection of a single TCR by multiple MHC products. In this Tg system, Id^{high+} cells were positively selected by D^q/L^q, D^d/L^d, and surprisingly I-A^q. Also, H-2^s class I (this study) and I-A^k (29) have been shown to function as negative selecting elements. Thus, there does not appear to be a clear border between class I and class II MHC products in terms of reactivity with TCR^{QM11}. At this point, it is not clear whether or not such borderless cross-reactivity is unique to TCR^{QM11}, as the Tg TCR was derived from a $CD8^+$ CTL clone specific for allo-class II MHC (29). However, we tend to speculate that such cross-reactivity might well be the case in general. Accumulated information on crystal structure of TCR/peptide/MHC complexes suggested that there are no conserved contact sites found on these molecules, despite the roughly similar docking orientation. In addition, basic structures of both classes of MHC proteins are very similar, except for the bound peptides being more exposed from the groove in class II MHC than in class I MHC (for reviews, see Refs. 36–38).

Thus, it is quite likely that class I and class II MHC molecules actually look alike to TCR. In fact, there are no critical (although some) differences in Vα/Vβ usage between class I MHC-restricted and class II MHC-restricted TCRs (39, 40). If this is the case, the coreceptor may well primarily determine the class restriction. Because mature T cells express exclusively one species of coreceptor molecules, such borderless cross-reactions ought not to be frequently observed. At the DP stage of thymocytes, however, T cell clones may well find multiple MHC products as their selecting elements in a borderless manner, owing to their hermaphroditic expression of both species of coreceptors. Very recently, Huseby et al. (41) reported that many clones derived from the mice in which negative selection is limited were very cross-reactive with allo-MHCs, and some of them even showed the reactivities against both classes of MHCs. These results indicate that preselected DP cells contain a significant portion of cells with dual class-responsive TCR. Thus, the process of positive selection of usable clones in the thymus may not be as wasteful as it appears.

Positive selection in the presence of both classes of selecting MHC: asymmetric effects of two classes of selecting MHC on the positive selection of dual class-responsive T cells

In the TCR^{QM11}-Tg mouse of H-2^q background, in which both class I- and class II-selecting MHC molecules were available, maturation of Id^{high+} cells was observed in both $CD4^+$ and $CD8^+$ subsets. The development of $CD4^+$ lineage was observed in H-2^q, $\beta_2m^{-/-}$ background, in which I-A^q may be the only MHC molecule, showing that the maturation of $CD4^+$ Id^{high+} cells was indeed I-A^q dependent. This was further confirmed by the analyses of thymocytes from bone marrow chimeras created using H-2^q $\beta_2m^{-/-}$ mice as recipients of bone marrows from H-2^b $RAG2^{-/-}$ TCR-Tg mice. Again, only CD4 SP, but not CD8 SP thymocytes, were found in these mice (our unpublished observation).

However, I-A^q molecule itself may not be a good selector of TCR^{QM11}-expressing cells. In the H-2^q $\beta_2m^{-/-}$ mice, differentiation of $CD4^+$ Id^{high+} cells was much less efficient when compared with that in class I-sufficient mice. This suggests that the selecting class I MHC could enhance the efficiency of class II-dependent differentiation into $CD4^+$ cells (Fig. 4). A similar observation was made in the comparison of peripheral Id^{high+} cells between H-2^{bxq} (heterozygous for both I-A^q and D^q/L^q) and H-2^{bq4xq} (heterozygous I-A^q and homozygous D^q/L^q) mice (Figs.