

Predominant Role of T Cell Receptor (TCR)- α Chain in Forming Preimmune TCR Repertoire Revealed by Clonal TCR Reconstitution System

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Abstract

The CDR3 regions of T cell receptor (TCR)- α and - β chains play central roles in the recognition of antigen (Ag)-MHC complex. TCR repertoire is created on the basis of Ag recognition specificity by CDR3s. To analyze the potential spectrum of TCR- α and - β to exhibit Ag specificity and generate TCR repertoire, we established hundreds of TCR transfectants bearing a single TCR- α or - β chain derived from a cytotoxic T cell (CTL) clone, RT-1, specific for HIVgp160 peptide, and randomly picked up TCR- β or - α chains. Surprisingly, one-third of such TCR- β containing random CDR3 β from naive T cells of normal mice could reconstitute the antigen-reactive TCR coupling with RT-1 TCR- α . A similar dominant function of TCR- α in forming Ag-specific TCR, though low-frequency, was obtained for lymphocytic choriomeningitis virus-specific TCR. Subsequently, we generated TCR- α and/or - β transgenic (Tg) mice specific for HIVgp160 peptide, and analyzed the TCR repertoire of Ag-specific CTLs. Similar to the results from TCR reconstitution, TCR- α Tg generated CTLs with heterogeneous TCR- β , whereas TCR- β Tg-induced CTLs bearing a single TCR- α . These findings of Ag recognition with minimum involvement of CDR3 β expand our understanding regarding the flexibility of the spectrum of TCR and suggest a predominant role of TCR- α chain in determining the preimmune repertoire of Ag-specific TCR.

Key words: antigen recognition • CDR3 • CTL • HIV gp160 • selection

Introduction

Recognition of Ag peptide assembled within the MHC groove by TCR initiates and maintains Ag-specific immune responses. TCR is composed of TCR- α and - β chains, which are both generated by somatic rearrangements of germline-encoded V, D, and J gene segments (1). TCR- α and - β chains both contain three complementarity-determining regions (CDRs), which exhibit extreme variability and are responsible for specificity. While CDR1 and 2 are encoded by germline V gene segments, CDR3 is somati-

cally created by rearrangement of V, (D), and J segments and provides major contributions to TCR diversity (1–3). It has been theorized that the diversity of TCR- $\alpha\beta$ can reach 10^{15} by random rearrangement and nucleotide addition (1), and that a single peptide/MHC complex positively selects at least 10^5 different V β rearrangements (4). Recent estimations of the functional repertoire of peripheral T cells by extensive sequencing of CDR3 regions have suggested that actual diversity is more limited than the theoretical assumption and that the size of the V β repertoire is $5\text{--}10 \times 10^5$ and the total clone size will be as small as 2×10^6 (5–7).

The diversity of TCR- α is shaped by numerous V α and J α genes and one N region, while the TCR- β chain is created by smaller numbers of V β and J β , two C β , but a greater contribution of two N regions. During T cell development, TCR- α rearrangement takes place in thy-

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mocytes which had undergone TCR- β rearrangement. This order of TCR rearrangement results in a significant difference between TCR- α and - β on the peripheral TCR repertoire at a single cell level. T cells bearing the same TCR- β and varied TCR- α chains could be generated because immature T cells expressing a TCR- β chain and a preT cell receptor exhibit proliferation before TCR- α rearrangement. On the contrary, a given TCR- α could not associate with multiple TCR- β chains under physiological condition. This may lead to the hypothesis that TCR- α exhibits a greater diversity than TCR- β chains and plays more important roles in the recognition of foreign antigen. In addition, a considerable percentage of T cells expresses two rearranged TCR- α chains (8), probably as a result of thymic selection of TCR, which may possibly occur through receptor editing (9, 10). Recently, several different class I-restricted TCRs and one class II-restricted TCR have been crystallized in complex with corresponding peptide/MHC (11–16). In most cases, it was shown that TCR- α has more contacts with peptide than TCR- β , pointing to the possibility that peptide recognition predominantly depends on TCR- α .

In addition to these structural analyses, analysis of single TCR-transgenic (Tg)* mice has provided additional insight into the functional aspects of the structure of Ag recognition and TCR repertoire (17). Brandle et al. analyzed the TCR repertoire of lymphocytic choriomeningitis virus (LCMV)-specific CTLs upon virus infection in TCR- α or TCR- β Tg mice and found that CTLs used highly restricted VJ α and more diverse VDJ β junctional regions (18). Furthermore, several experiments have analyzed the responses to altered peptide ligands in these Tg mice (3, 19–21) and demonstrated that every mutation in Ag peptide resulted in a change in the CDR3 sequences of TCR- α and - β chains, which supported the model in which CDR3 loops are laid directly upon the peptide bound to the MHC groove.

Nevertheless, these analyses using Tg mice have suffered from obvious limitations in determining the potential repertoire of TCR- $\alpha\beta$ pairs, because TCRs with strong (autoreactive) affinity, or without, to self-MHC were excluded through thymic selection. In addition, since TCR- α expression is not allelically excluded, unlike TCR- β and T cells express two TCR- α chains, simple sequencing of TCR- α cDNA cannot determine which combination of a TCR- $\alpha\beta$ dimer exhibits Ag specificity.

To overcome such limitations and analyze the preselection repertoire, we developed a novel transfection system that determines the precise mechanism of Ag recognition and allows analysis of the functional TCR repertoire at the clonal level. This system enabled us to systematically analyze hundreds of individual TCR- $\alpha\beta$ pairs for Ag-specific recognitions without the influence of thymic selection. In this study, we analyzed the repertoire of endogenous TCR associated with transgenic TCR chain in HIVgp160-specific CTLs in vitro using our transfection system as well as in vivo

by creating single TCR-Tg mice. We found that in order to recognize the HIVgp160 peptide/H-2D^d complex, CTLs have to possess a single TCR- α chain but can use a variety of TCR- β chains. Our results with two Ag systems suggest that recognition of foreign antigens is predominantly dependent on TCR- α chain and that the diversity of TCR develops in accordance with the heterogeneity of TCR- α , which would preclude the problems of autoimmunity and unwanted deletion of useful T cell clones.

Materials and Methods

Mice, Cells, Peptides, and Reagents. BALB/c and C57BL/6 mice were purchased from Shizuoka Laboratory Animal Corporation (Hamamatsu, Japan). RT1 is an HIV gp160 env P18IIIB-specific CTL clone as described previously (22). TG40 is a variant T cell hybridoma cell line lacking the expression of TCR- α and - β chains that has been used as recipient cells for TCR transfection (23). The sequences of Ag peptides are as follows: P18IIIB (315–329:RIQRGPGRAFVTIGK); P18MN (315–329:RIHIGPGRAFYTTKN); and LCMV p33 (KAVYNFATM).

Subcloning of TCR. CD8⁺ T cells or CD69⁺ CD8⁺ blast T cells were purified by FACStar™ sorting with a purity >98%, and total cellular RNA from these cells was extracted as described previously (24). cDNA was synthesized with random hexamer primers and Superscript II cDNA synthesis kit (GIBCO BRL).

The primers used for cloning of V α 42H11⁺ TCR- α chain were: V α 42H11: ATGCTGATTCTAAGCCTGTT; and C α : TCAACTGGACCACAGCCT. The primers used for CDR3 β cloning were: common V β 8 family: GGGCTGAGGCTGATC-CATTA; and C β : CCAAGCACACGAGGGTAG. The primers for the full-length TCR- β cloning were: V β 8.1: ATGGGCTC-CAGACTCTTC; V β 8.3: ATGGGCTCCAGGCTCTTTCT; and C β : TCAGGAATTTTTTTTCTTGACCAT. These primers contained EcoRI and NotI sites at 5' ends to subclone into pMX-IRES-GFP (provided by Toshio Kitamura, Tokyo University, Tokyo, Japan.). Each cloned plasmid DNA was purified by Wizzard Plus SV DNA Purification System (Promega) and sequenced using BigDye Terminator Cycle Sequencing Ready Reagent (PE Biosystems) with an ABI 377 DNA sequencer.

Retrovirus-mediated Gene Transfer. A recipient T cell line for expression of a variety of TCR- $\alpha\beta$ dimers was prepared by electroporation of the expressible constructs of CD8 α and CD8 β in the pBCMGneo (provided by H. Nakachi, Tsukuba University, Tsukuba, Japan) into TG40 cells by GenePulser (Bio-Rad Laboratories). Various TCR- β chains in pMX-IRES-GFP vector, were transfected into a retroviral packaging cell line, Phoenix (from G. Nolan, Stanford University, Stanford, CA), with LipofectAMIN Plus Reagent (GIBCO BRL). The culture supernatant of Phoenix after 24 h culture was collected, centrifuged at 8,000 g for 16 h to concentrate the virus, and added to CD8 $\alpha\beta$ -expressing TG40 cells together with DOTAP Liposomal Transfection Reagent (Boehringer Mannheim). Transfection was monitored by the intensity of GFP and the cell surface expression of TCR by FACS[®] analysis. Transfection of a variety of TCR- β chains into CD8⁺ TCR- α ⁺ TG40 cells resulted in 25–40% of surface TCR⁺ cells.

IL-2 Production Assay. To analyze the IL-2 secretion from various TCR-transfected TG40 cells, 2 × 10⁴ TG40 transfectants were cultured with 10⁶ of irradiated BALB/c splenocytes in 200 μ l of complete RPMI 1640 medium in the presence of 4 μ M of various peptides in a 96-well flat-bottomed plate for 24 h. As a positive control for stimulation through the TCR complex, cells

*Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; Tg, transgenic.

were plated in 200 μ l of complete RPMI 1640 medium in a 96-well flat-bottomed plate precoated with 1 μ g/ml anti-TCR- β mAb, H57 (provided by R. Kubo, La Jolla Institute of Allergy and Immunology, San Diego, CA). The titer of IL-2 in the culture supernatant was determined by ELISA.

Establishment of Transgenic Mice. The transgenic TCR- α and - β genes were isolated from RT1. The TCR- α and - β genes responsible for the recognition of P18/D^d were first analyzed by PCR using specific primers for V α and V β , and C α and C β , respectively. The DNA sequences of the PCR products revealed that RT1-TCR- α was composed of V α 42H11 and J α 25 and the TCR- β chain of V β 8.1, J β 2.1, and C β 2. A full-length TCR- α was generated by inserting the junctional sequence into a TCR- α cDNA clone containing V α 42H11 (a gift from B. Huber, Tufts University, MA) at the site of SmaI and EcoRV. A full-length TCR- β chain was similarly constructed by recombinant PCR using pP142 β 8AR (TCR- β from a LCMV-specific CTL clone, P14, which was provided by H. Pircher, Freiburg University, Germany) as a template. The full-length cDNAs of RT1-TCR- $\alpha\beta$ genes were subcloned into the SmaI and BamHI sites of the expression vector pHSE3' under control of the H-2K^b promoter (provided by H. Pircher). The inserts of these expression constructs for each TCR- α and - β were injected into C57BL/6 oocytes, and two lines of Tg mice were generated. Tg lines with higher expression of TCR- α and - β were extensively analyzed. Tg mice were backcrossed with BALB/c mice for six generations for the experiments. Details of the establishment and characteristic of Tg mice will be described elsewhere (unpublished data).

Ag-specific Cytotoxicity Assay. Cytolytic activity of CTLs was measured by standard ⁵¹Cr release assay (25). Briefly, 5 \times 10⁶ freshly isolated splenocytes from RT1-TCR-Tg were stimulated in vitro with 2 \times 10⁵ of the gp160-transfected NIH 3T3 cells in 2 ml of complete RPMI 1640 medium containing 10% ConA supernatant. After 5-d culture, effector cells were mixed with peptide-pulsed NIH 3T3 target cells for 5 h and the ⁵¹Cr counts in the culture supernatant were measured. The percentage of specific cytotoxicity was calculated as 100 \times (experimental release - spontaneous release)/maximum release - spontaneous release.

Proliferation Assay. CD8⁺ T cells (>98%) were purified from splenocytes of RT1-TCR-Tg or non-Tg mice by cell sorting with FACStarTM plus (Becton Dickinson). 5 \times 10⁴ CD8⁺ T cells were cultured with 10⁶ irradiated BALB/c splenocytes in 200 μ l of complete RPMI medium with 4 μ M Ag peptide. Culture plates were pulsed with 2 μ Ci/well of [³H]-thymidine for 8 h on day 3, and the incorporated radioactivity was measured by Microbeta scintillation counter (Amersham Pharmacia Biotech).

Flow Cytometric Analysis. Cell surface expression of V β 8⁺ TCR- β chain was analyzed by staining with three different anti-V β 8 mAbs: F23.1 and F23.2 (provided by P. Marrack, National Jewish Center, Denver, CO) for V β 8.1+V β 8.2+V β 8.3 and for V β 8.2, KJ16 (Caltag) for V β 8.11V β 8.2, together with anti-CD8 α mAb (53-6.7) (BD PharMingen). 10⁶ cells were incubated with Ab for 40 min, followed with biotin anti-mouse IgG Ab (BD PharMingen) for 40 min. After blocking with mouse serum, cells were incubated with anti-CD8 α -FITC and streptavidin-PE (BD PharMingen) for 30 min. Stained cells were analyzed by flow cytometry with FACScaliburTM (Becton Dickinson).

Results

Clonal Analysis of Ag Recognition by Reconstituting Various TCR- $\alpha\beta$ Pairs. To estimate the functional TCR repertoire to a given peptide-MHC complex at the clonal level,

we developed a novel transfection system using retroviral infection. In this system, a single TCR chain (α or β) is transfected into a TCR-deficient cell line with a variety of the other TCR chains (β or α), and the specificity of a given TCR- $\alpha\beta$ pairs is analyzed. We focused on the Ag-recognizing repertoire by the TCR- α and - β chains derived from an HIV gp160-specific, H-2D^d-restricted cytotoxic T cell clone, RT1 (22). This clone recognizes the HIV env peptide P18IIIB. We first determined the usage of TCR- α and - β chains of this clone by RT-PCR and 5' RACE (data not shown). RT1-TCR- α chain was found to be composed of V α 42H11 and J α 25 and RT1-TCR- β chain of V β 8.1, J β 2.1, and C β 2. We designed a system to analyze the specificity of TCR- $\alpha\beta$ by creating a series of TCR- $\alpha\beta$ dimers either by reconstituting a TCR- $\alpha\beta$ dimer with the RT1-TCR- α chain and a variety of TCR- β chains or with the RT1-TCR- β chain and various TCR- α chains.

First, in order to analyze Ag-recognition by TCR dimers composed of the fixed RT1-TCR- β chain with various TCR- α chains, \sim 30 TCR- α chains bearing V α 42H11 were isolated from unimmunized normal mice. These TCR- α chains contained random J and N region sequences (data not shown). Each V α 42H11⁺TCR- α chain was subcloned into a retrovirus vector and then transfected by retrovirus-mediated gene transfer into a TCR- $\alpha\beta$ -deficient recipient T cell hybridoma cell line, TG40 (23), in which RT1-TCR- β and CD8 $\alpha\beta$ had been transfected and expressed. Expression of each transfected TCR- α chain was monitored by the cell surface expression of the TCR-CD3 complex. Approximately 30-60% of the transfectants expressed the cell surface TCR complex, and the expression levels of the TCR complex were almost the same among these transfectants. A representative profile of such transfection is shown in Fig. 1. Functional specificity of the reconstituted TCR was assessed by measuring IL-2 production upon stimulation with specific Ag peptide, P18IIIB plus APC (D^d), or anti-TCR- β mAb cross-linking as the control. As postulated, all of the 29 different V α 42H11-bearing TCR- α chains with various junctional sequences isolated from unimmunized mice failed to reconstitute any TCR- $\alpha\beta$ complex reactive to the P18IIIB/H-2D^d complex when coexpressed with RT1-TCR- β chain and CD8, while all clones produced a similar level of IL-2 upon anti-TCR- β Ab cross-linking (Fig. 1).

We next performed a similar but opposite clonal analysis with the RT1 α chain and a variety of TCR- β chains derived from unimmunized naive T cells. More than 80 TCR- β chains expressing V β 8.1 and either of J β 2.1, J β 2.2, or J β 2.4 were randomly picked up. All of these TCR- β chains were transfected into TG40 cells expressing RT1 α and CD8. These TCR- β chains were able to pair with the RT1-TCR- α chain and were expressed on the cell surface in a similar manner to that shown in Fig. 1 (data not shown). These transfectants expressing RT1 α and various TCR- β were then tested for their reactivity to P18IIIB/H-2D^d for Ag-specific IL-2 production. To our surprise, an extremely high frequency of TCR- β chains

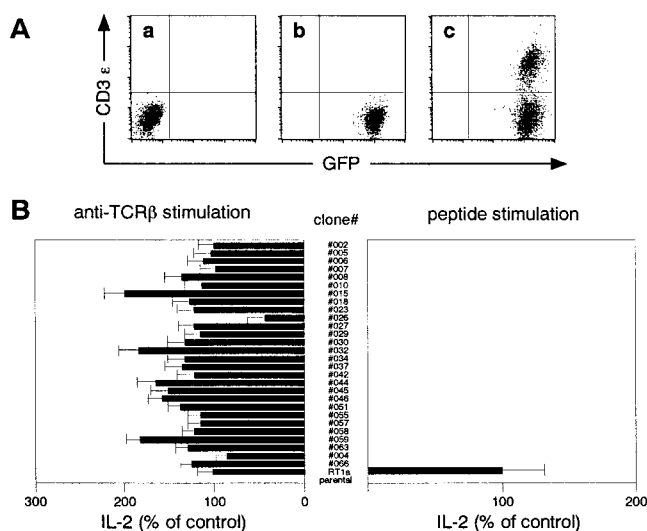


Figure 1. Transfectants expressing various TCR- α with RT1 β failed to respond to P18IIIB. (A) A representative FACS[®] profile for the expression of TCR and GFP in transfectants expressing RT1 β and a TCR- α chain. TG40, the TCR- $\alpha\beta$ -deficient T cell hybridoma cell line (a), was transfected with RT1 β chain, and GFP before TCR- α transfection (b). V α 42H11⁺ TCR- α chains were randomly isolated from naive CD8⁺ cells from wild-type BALB/c mice, and these TCR- α genes were transfected into TCR- β -expressing TG40 cells by retrovirus-mediated gene transfer. The expression of TCR was monitored by the intensity of CD3 ϵ -PE and 30–60% of the transfectant expressed the cell surface TCR complex (c). (B) IL-2 production of TCR- $\alpha\beta$ transfectants upon stimulation with Ag/MHC and anti-TCR- β Ab. The transfectants were stimulated with irradiated splenocytes and P18IIIB (right) or immobilized anti-TCR- β Ab, H57 (left) for 24 h. The concentration of secreted IL-2 was measured by ELISA and the data are presented as the percentages of RT1-TCR- $\alpha\beta$ -transfectant (RT1 β).

(45% [13/29]) of J β 2.1⁺, 54% [7/13] of J β 2.2⁺, and 18% [6/33] of J β 2.4⁺ TCR- β chains) could reconstitute TCRs with RT1-TCR- α as well as recognize P18IIIB/H-2D^d and secrete IL-2 (Fig. 2). As a sum, one-third (25 of 75) of randomly picked-up TCR- β chains from nonTg naive T cells could generate P18/D^d-specific TCRs when reconstituting a TCR with the RT1-TCR- α chain at the clonal level. As shown in Fig. 3, the analysis of amino acid sequences of the CDR3 regions of the TCR- β chains from these transfectants revealed no obvious difference either in the sequence or in the length of the CDR3 β regions (26) between the Ag-reactive and nonreactive TCR- β chains. To examine the functional sensitivity of the reconstituted TCRs to Ag, Ag dose-responses were analyzed on several representative transfectants expressing three different J β s, J β 2.1, J β 2.2, and J β 2.4 (Fig. 4). We found that the dose responses were clearly dependent on the structure of J β . While TCR reconstituted with J β 2.2-containing TCR- β chains were more sensitive than RT1-TCR- $\alpha\beta$, J β 2.1-positive TCR- β chains provided similar levels of response to RT1 β -TCR- $\alpha\beta$. In contrast, TCRs with J β 2.4-bearing β chains exhibited less sensitive dose-responses compared with RT1-TCR- $\alpha\beta$, though they were still within the physiological range (Fig. 4).

These results revealed that, although a certain structural

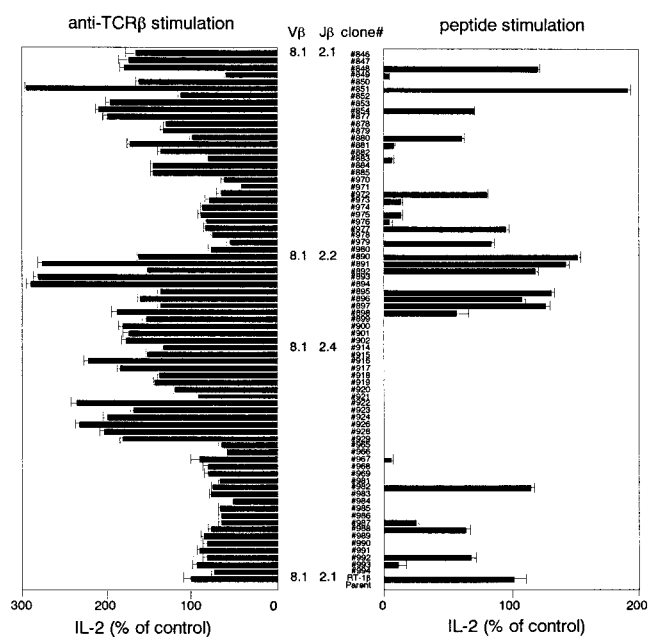


Figure 2. Ag-specific responses by transfectants expressing RT1-TCR- α and various TCR- β chains expressing random CDR3 β . V β 8⁺ TCR- β chains were randomly isolated from naive CD8⁺ cells from wild-type BALB/c mice. Their V β usage was V β 8.1, the J β usage was J β 2.1, 2.2, 2.4, and CDR3 β sequences were random. These TCR- β genes were transfected into RT1-TCR- α -expressing TG40 cells by retrovirus-mediated gene transfer, and these transfectants were stimulated with irradiated splenocytes and P18IIIB (right) or immobilized anti-TCR- β Ab, H57 (left) for 24 h, as for Fig. 1. The concentration of IL-2 was measured by ELISA and the data are presented as the percentages of that of RT1-TCR- β -transfectant (RT1 α).

constraint was present in the reconstitution of TCR- $\alpha\beta$ pairs reactive to Ag, a high frequency of irrelevant V β 8⁺ TCR- β chains can form a functional TCR- $\alpha\beta$ dimer with RT1-TCR- α chain so as to be able to respond to physiological concentrations of the HIVgp160 V3 loop peptide.

Requirement of Single TCR- α and Heterogeneous TCR- β for Ag Recognition in Single TCR Tg Mice. To examine whether the observed high frequency of TCR- β to constitute Ag-recognizing TCR with the RT1- α chain reflects the in vivo preimmune repertoire of peripheral T cells, we established transgenic mice expressing either RT1-TCR- α or - β chain (α Tg and β Tg), respectively, using an expression vector containing H-2^b promoter, pHSE3' (27). Immune responses of CD8⁺ cells from these mice to P18IIIB/H-2D^d were then analyzed. The expression of transgenic TCR- α and - β chains was confirmed by RT-PCR and also by cell surface staining of TCR- β chain with anti-V β 8 mAb and TCR- $\alpha\beta$ dimer with clonotypic mAb on T cells from each kind of Tg mouse (data not shown).

Ag-specific immune responses of CD8⁺ T cells from each of the single TCR- α and TCR- β Tg mice as well as from TCR- $\alpha\beta$ Tg mice were analyzed by measuring Ag-specific proliferation and cytotoxic function. CD8⁺ T cells from α Tg mice exhibited P18IIIB-specific proliferation (Fig. 5 A) as well as Ag-specific cytolytic activity against

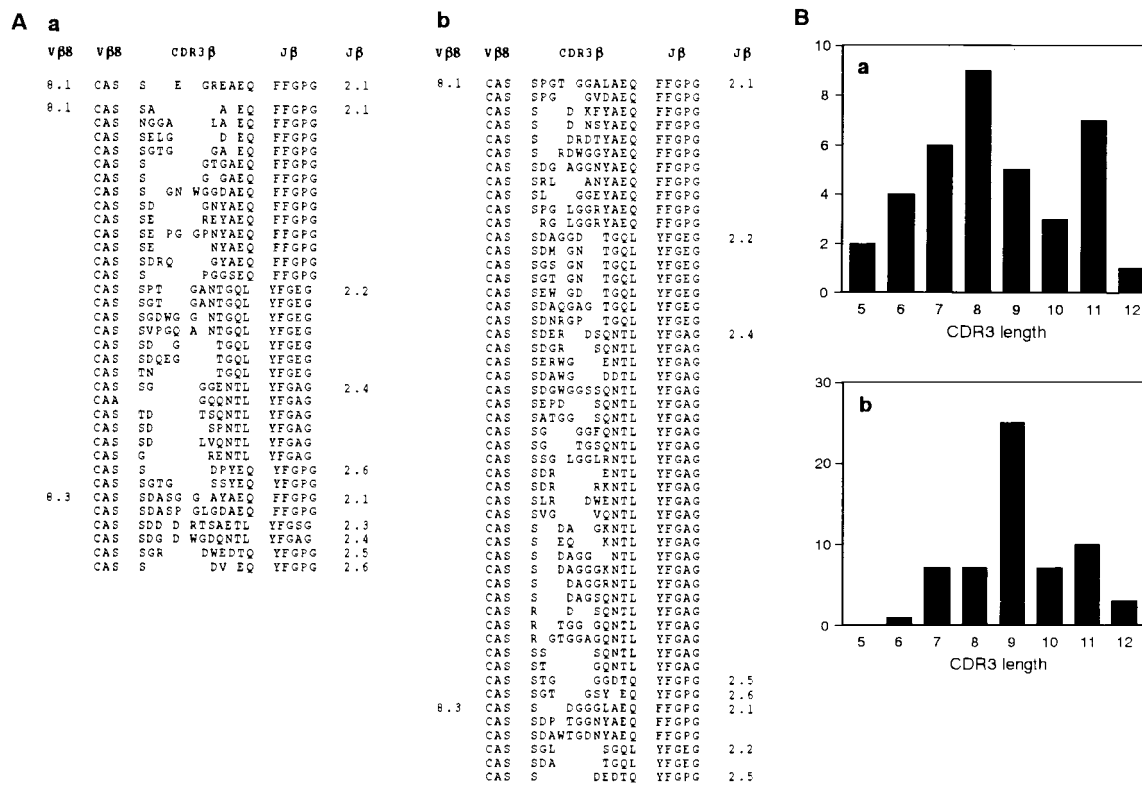


Figure 3. Amino acid sequences and the length of CDR3 of TCR- β chains. (A) Amino acid sequences of TCR- β chains used for transfection. (a) TCR- β chain capable of reconstituting the TCR complex to recognize P18IIIB/D^d with RT1-TCR- α . (b) TCR- β chain incapable of reconstituting the P18/D^d-reactive TCR complex. Amino acid sequences revealed no significant characteristics in either group. (B) Distribution of CDR3 length among the P18/D^d-reactive TCR- β (a) and nonreactive TCR- β (b).

P18IIIB-pulsed NIH 3T3 target cells (Fig. 5 B, a). As expected from the results of in vitro TCR reconstitution experiments, primary CD8⁺ T cells isolated from unimmunized α Tg mice showed strong proliferative responses to P18IIIB/H-2D^d, and the responses were more intensive than those from β Tg mice (Fig. 5 A). In contrast, CD8⁺ T cells from β Tg exhibited a significant, though weak, proliferative response to P18IIIB (Fig. 1 A). In accordance with the proliferation, CD8⁺ T cells from α Tg mice exhibited P18IIIB-specific cytotoxicity as strongly as that of α β Tg mice after 5-d culture with gp160-expressing cells (Fig. 5 B, b). Surprisingly, CD8⁺ T cells from β Tg mice showed significant cytotoxicity at approximately one-third of the magnitude of CTLs from α β Tg mice (Fig. 5 B, c). Collectively, these results demonstrated that TCR Tg could develop Ag-reactive CD8⁺ T cells upon Ag stimulation and that the actual TCR repertoire reactive to the antigen was much larger in α Tg mice than in β Tg, indicating that the Ag recognition of P18IIIB appeared to be mediated mainly by the TCR- α chain.

We then analyzed the clonal basis of Ag-specific recognition by single TCR Tg mice by determining their TCR repertoire of specific CTLs. First, we analyzed TCR- β chain usage of Ag-specific CTLs generated from α Tg mice. In the FACS[®] analysis of V β repertoires, ~30% of unstimulated CD8⁺ T cells from α Tg mice expressed V β 8, similar

to normal mice, but the total of V β 8⁺ CD8⁺ T cells in α Tg mice was 92% after Ag stimulation, with an especially high expression of V β 8.1 (71%) (Fig. 6 A and B). Contrary to the strong skewing in the V β repertoire, the junctional sequences of V β 8⁺ TCR- β chains from Ag-stimulated CD8⁺ T cells from α Tg mice revealed no predominant usage of any single J β gene segment and no differences in the lengths and amino acid sequences of CDR3 β residues (Fig. 6 C). We next compared the junctional diversity of the V α 42H11⁺ TCR- α chains of Ag-stimulated CD8⁺ T cells from β Tg mice with naive β Tg mice. In sharp contrast, we found that only a single J α gene segment, J α 25, which is the same as the original RT1 clone, and almost the same CDR3 α sequences dominated in V α 42H11⁺ TCR- α chains from Ag-stimulated CD8⁺ T cells from β Tg mice (Fig. 7 A). It is unlikely that the restriction in TCR- α usage was due to thymic selection during T cell development, since J α and CDR3 α residues were found to be variable in nonstimulated CD8⁺ cells from β Tg mice (Fig. 7 B).

These analyses of the TCR repertoire in single TCR Tg mice demonstrate that, similar to the in vitro TCR reconstitution data, a single TCR- α chain and heterogeneous TCR- β chains were used to recognize P18IIIB/D^d in vivo.

Another TCR Model for Ag Recognition by a Single TCR- α and a Variety of TCR- β Chains. This unexpectedly high frequency of CDR3 β sequences giving rise to a functional

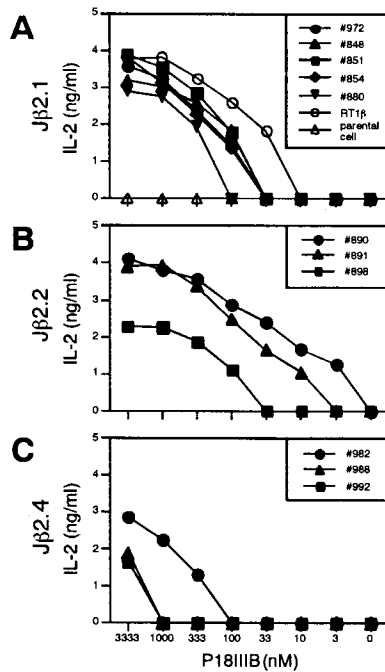


Figure 4. Ag dose responses by representative transfectants expressing a TCR dimer of a randomly picked-up TCR- β and the RT1-TCR- α . Transfectants reconstituted with TCR- β chains bearing either J β 2.1 (A), J β 2.2 (B), or J β 2.4 (C) together with RT1 α were stimulated with graded doses of p18IIIB peptide for IL-2 secretion in a similar condition as described in the legend of Fig. 5.

TCR- $\alpha\beta$ pair with a single TCR- α chain was not peculiar to the RT1-TCR and P18IIIB/H-2D^d system. To generalize from this observation, we used the same approach to the well-established P14-TCR, which exhibits LCMV-specific, H-2D^b-restricted Ag recognition. Brandle et al. reported that P14-TCR α -Tg mice, in contrast to P14-TCR β -Tg mice, were capable of responding to the LCMV glycoprotein peptide (GP33) in vitro, suggesting that the TCR- α chain plays a dominant role in GP33/H-2D^b recognition (28). We reconstituted the TCR- $\alpha\beta$ dimer by transfection of the P14-TCR- α chain and a variety of V β 8⁺ TCR- β chains isolated from naive T cells of non-Tg mice and measured the reactivity to the LCMV epitope GP33/H-2D^b. The results revealed that three out of 73 clones (4%) showed Ag-specific IL-2 production when expressed with the P14-TCR- α chain (Fig. 8 A). These three TCR β chains did not have any sequence similarity in the CDR3 β regions (Fig. 8 B). There was no positive response to either GP33 or P18IIIB when any one of the three β chains was expressed with RT1-TCR- α chain (data not shown). The frequency of functionally reconstituted TCR- $\alpha\beta$ dimers composed of P14-TCR- α and randomly cloned TCR- β was much lower than that in the case of RT1-TCR (Fig. 5). Nevertheless, since the frequency for a particular TCR chain to form an Ag-specific TCR dimer with a nonselected partner chain has been believed to be extremely low, the 4% level of randomly picked-up TCR- β chains was sufficient to illustrate high frequency.

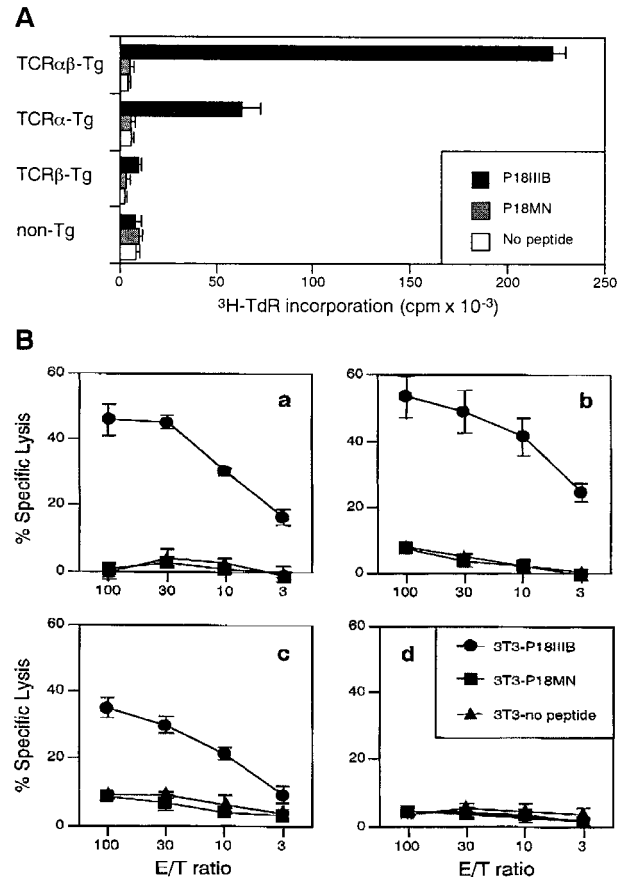
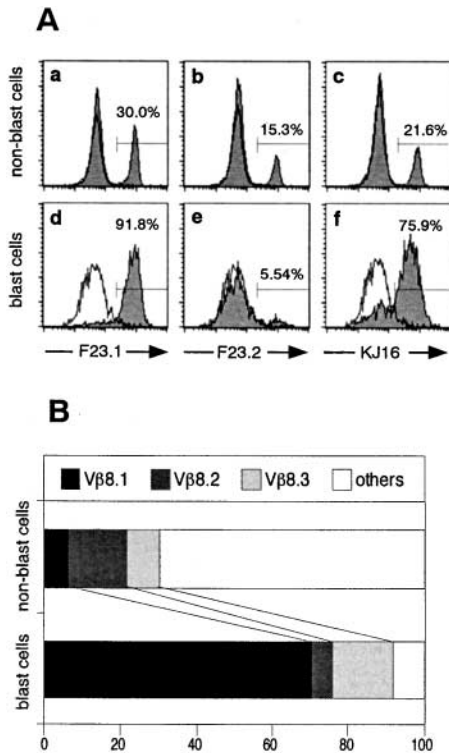


Figure 5. Ag-specific cytotoxicity and proliferation of CD8⁺ T cells from RT1-TCR- α Tg and/or TCR- β Tg mice. (A) Ag-specific proliferation. Purified CD8⁺ spleen cells from RT1-TCR Tg or nonTg mice were cultured with irradiated spleen cells from BALB/c in the presence of 4 μ M of P18IIIB (closed bar), P18MN (hatched bar), or no peptide (open bar). Cultures were pulsed with [³H]-thymidine (TdR) for 8 h on day 3 of the culture and incorporated radioactivity was measured. (B) Ag-specific cytotoxicity. Spleen cells from unprimed RT1-TCR- $\alpha\beta$ Tg (a), TCR- α Tg (b), TCR- β Tg (c), and normal mice (d) were stimulated in vitro with HIV IIB gp160-transfected NIH 3T3 cells. After 5 d, the cytotoxic function of induced CTLs was assessed on NIH 3T3 target cells pulsed with P18IIIB (closed circles), P18MN (open circles), or no peptide (closed triangles) in a standard ⁵¹Cr-release assay.

Discussion

We developed a novel transfection system in order to determine the preimmune repertoire of Ag-specific TCR and the precise structure-function relationship of Ag recognition at the clonal level. In this system, a TCR- $\alpha\beta$ -deficient cell line was first transfected with a particular TCR- α , followed by further transfection with a variety of randomly cloned TCR- β chains, and each transfectant expressing a pair of TCR- $\alpha\beta$ dimers was assessed for its ability to recognize Ag peptide-triggered IL-2 production. Using this system, we were able to analyze hundreds of individual TCR- $\alpha\beta$ dimers for the specificity of Ag recognition. In general, the TCR repertoire has until now been analyzed by determining sequences of TCR after establishing T cell clones and isolation of cDNA. However, the finding that many T cells express two allelically unexcluded



C

vβ8	vβ8	CDR3β	Jβ	Jβ
8.1	CAS	SEG RE AEQ	FFGPG	2.1
8.1	CAS	SAPLGG AEQ	FFGPG	2.1
	CAS	SGT GD AEQ	FFGPG	
	CAS	SGT GV AEQ	FFGPG	
	CAS	SGG GP YAEQ	FFGPG	
	CAS	SET GGAQEAEQ	FFGPG	
	CAS	SDA GGE TAEQ	FFGPG	
	CAS	SGT GD EQ	YFGPG	2.6
	CAS	SAP GLG GHEQ	YFGPG	
	CAS	KS GN YEQ	YFGPG	
	CAS	SDP GVN QDTQ	YFGPG	2.5
	CAS	S SG GQNTL	YFGAG	2.4
	CAS	S DPGGTSQNTL	YFGAG	
	CAS	S DPGSSQNTL	YFGAG	
	CAS	T DNS QNTL	YFGAG	
	CAS	S GPG SAETL	YFGSG	2.3
	CAS	P PGGAPT GQL	YFGEG	2.2
	CAS	AGQP GQL	YFGEG	
	CAS	SDS NSDY	TFGSG	1.2
	CAS	SE EWQGPDY	TFGSG	
	CAS	SG PNSDY	TFGSG	
	CAS	SG GTSNSDY	TFGSG	
	CAS	SG QGNSDY	TFGSG	
	CAS	SG KANSDY	TFGSG	
	CAS	S DST NTEV	FFGKG	1.1
	CAS	S PH GTEV	FFGKG	
	CAS	I PPGA NTEV	FFGKG	
	CAR	QG HTEV	FFGKG	
	CAS	ID RV EV	FFGKG	
	CAS	SA V	FFGKG	
8.2	CAS	GD EGGGGEQ	YFGPG	2.6
8.3	CAS	SDAWTGDNYAEQ	FFGPG	2.1
	CAS	SDG GGL AEQ	FFGPG	
	CAS	SDA EGRMYAEQ	FFGPG	
	CAS	SDA SGGAYAEQ	FFGPG	
	CAS	SD D SLEQ	YFGPG	2.6
	CAS	SDA GTD YPEQ	YFGPG	
	CAS	SD ATEGIEQ	HFGPG	2.7
	CAS	S DASG ENTL	YFGAG	2.4
	CAS	SD EANSDY	TFGSG	1.2

Figure 6. Restriction of Vβ usage but random CDR3β in Ag-responding CD8⁺ cells from RT1-TCR-α Tg mice. (A) Naive and Ag-stimulated CD8⁺ cells from RT1-TCR-α-Tg mice were stained with F23.1 (a and d), F23.2 (b and e), and KJ16 (c and f). The upper histograms (a-c) show the profiles of TCR-β Tg naive cells and the lower ones (d-f) show Ag-stimulated blast cells that were gated as blastocytes. (B) Calculated percentages of Vβ8.1⁺, Vβ8.2⁺, and Vβ8.3⁺ cells from the FACS[®] data in (A) for Ag-responding (blast cells) and nonresponding (nonblast) cells. (C) Vβ8 junctional sequences of Ag-stimulated CD8⁺ T cells from RT1-TCR-α Tg mice.

functional TCR-α chains (8) made it difficult to determine the TCR-αβ dimer responsible for the Ag recognition by simple sequencing. Our system is able to analyze the specificity of the individual pair of TCR-αβ chains systematically, and further, it can analyze the repertoire without the influence of thymic selection.

When expressing various TCR-β chains together with the RT1-TCR-α chain, one-third of the randomly picked-up Vβ8⁺ TCR-β chains containing random CDR3β from naive T cells of nonTg mice could generate the specific TCR-αβ dimers that recognize the P18IIB/H-2D^d complex. The result that Ag-specific TCR can be reconstituted at such an extremely high frequency could not be expected except in the case of superantigen. Since the recognition is strongly restricted by Vβ8, Vβ may have contribution to the contact with MHC-peptide. Nevertheless, this result indicates that Ag recognition by RT1-TCR is not dependent on particular CDR3β. We applied a similar analysis to the LCMV-specific P14 Tg mouse, one of the widely used Tg mice.

It has been demonstrated that CTLs from P14-TCR-α Tg showed specific cytotoxic function, and T cells from P14-TCR-β Tg mice revealed a restricted repertoire of TCR-α chain (18, 28), suggesting that TCR-α plays a dominant role in Ag recognition. In our clonal TCR reconstitution analysis, transfection of various TCR-β chains containing random CDR3β together with the P14-TCR-α chain revealed that 4% of Vβ8⁺ TCR-β chains were

able to generate functional TCR-αβ recognizing GP33/H-2D^b. This percentage, though much smaller than that of RT1, still represents an extremely high frequency from the viewpoint of randomly rearranged TCRs. It was not at all expected that the combination of a particular TCR-α chain and randomly obtained TCR-β chains from naive mice that have not been selected with the TCR-α chain could generate an Ag-specific TCR-αβ dimer. Collectively, the predominant contribution of TCR-α chain may vary depending on the T cell clone. Ag recognition by RT1-TCR with a minimum involvement of CDR3β is probably an extreme case, and P14-TCR exhibits Ag recognition with a little less independence of CDR3β than RT1-TCR.

A number of analyses of the T cell repertoire have been based on the use of single TCR Tg mice. In some of them, TCR-α and -β appeared to contribute equally to Ag recognition (3, 29), whereas in other cases Ag recognition was profoundly dependent on TCR-α (18-20, 30, 31). We also analyzed the in vivo functional TCR repertoire of HIV-P18-specific CTLs by generating single TCR Tg mice to compare the endogenous TCR repertoires. In our transgenic systems, CTLs generated from RT1-βTg mice upon stimulation with Ag peptide exhibited weak but still significant Ag-specific cytotoxicity. Sequence analysis revealed that these CTLs expressed a homogeneous TCR-α chain, Vα42H11-Jα25, as did the original RT1-TCR-α chain.

A

V α 42H11	V α 42H11	CDR3 α										J α				
	C A M	R E D G G S G N K L	I F G T G													
V α 42H11.1	TGT GCT ATG	AGA GAG GAT GGG GGC AGT GGC AAC AAG CTC	ATC TTT GGA ACT GGC													
V α 42H11.1	TGT GCT ATG	AGA GAG GAT GGG GGC AGT GGC AAC AAG CTC	ATC TTT GGA ACT GGC	5/14												
V α 42H11.2	TGT GCT ATG	AGA GAG GAT GGG GGC AGT GGC AAC AAG CTC	ATC TTT GGA ACT GGC	3/14												
V α 42H11.2	TGT GCT ATG	AGA GAG GAT GGG GGC AGT GGC AAC AAG CTC	ATC TTT GGA ACT GGC	1/14												
V α 42H11.1	TGT GCT ATG	AGA GAA GAT GGG GGC AGT GGC AAC AAG CTC	ATC TTT GGA ACT GGC	1/14												
V α 42H11.2	TGT GCT ATG	AGA GAT GAT GGG GGC AGT GGC AAC AAG CTC	ATC TTT GGA ACT GGC	1/14												
V α 42H11.2	TGT GCT ATG	AGA GAG GAT GGG GGC AGT GGC AAC AAG CTC	ATC CTT GGA ACT GGC	1/14												
V α 42H11.1	TGT GCT ATG	AGA GAG GAT GGG AGC AGT GGC AAC AAG CTC	ATC TTT GGA ATT GGC	1/14												
V α 42H11.1	TGT GCT ATG	ATG AAT TAT GGG GGC AGT GGC AAC AAG CTC	ATC TTT GGA ACT GGC	1/14												

B

V α 42H11	CDR3 α	J α	J α	CDR3 α length
CAM	REDGSSGNKL	IFGTG	25	10
CAM	REDGSSGNKL	IFGIG	25	10
CAM	REGGNYAQLG	TFGLG	112.2	10
CAM	REGGNYAQLG	TFGLG		10
CAM	REGSNYAQLG	TFPLG		10
CAM	REGGNSAQLG	TFPLG		10
CAM	REGS YAQLG	TFPLG		9
CAM	RGG TGYQNF	YFGKG	65	9
CAM	REG AGYQNF	YFGKG		9
CAM	REGNTGYQNF	YFGKG		10
CAM	REGNTGYQNF	YFGKG		10
CAM	KN TGYQNF	YFGKG		8
CAM	RG SGYQNF	YFGKG		8
CAM	RDAGANTGKL	TFPHG	44	10
CAM	REAGANTGKL	TFPHG		10
CAM	RGGDSNYQ L	IWGS	18BBM142	9
CAM	REG GNYQ L	IWGS		18
CAM	SDMG YKL	TFGTG	8	7
CRM	RAGGSN AKL	TFPKG	34	9
CAM	REPSSGGKL	TLGTG	36	10
CAM	REG YGNEKI	TLGAG	40	9

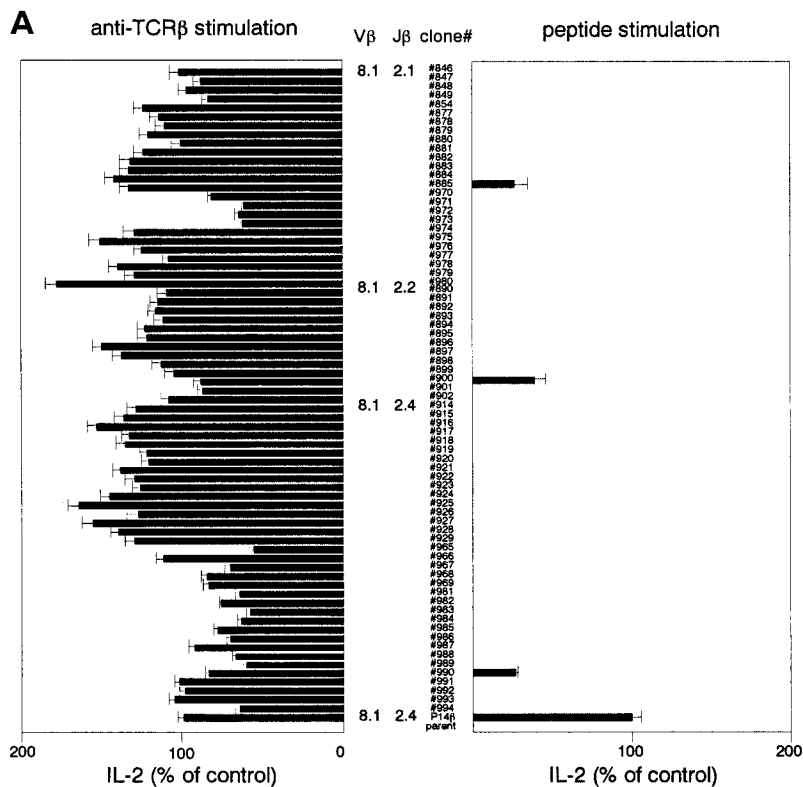
Figure 7. Dominant expression of a single TCR- α of Ag-stimulated CD8⁺ T cells from RT1-TCR- β Tg mice CD8⁺ cells derived from RT1-TCR- β Tg mice were stimulated or unstimulated with irradiated BALB/c whole spleen cells and antigen peptide for 3 d. RT-PCR analysis was performed with primers specific for V α 42H11 and C α , and CDR3 α sequences were compared. (A) CDR3 α junctional amino acid sequences of V α 42H11-bearing TCR- α chains of Ag-stimulated CD8⁺ cells from RT1-TCR- β Tg mice. (B) CDR3 α junctional amino acid sequences of V α 42H11-bearing TCR- α chains of naive CD8⁺ cells from RT1-TCR- β Tg mice.

Since unstimulated T cells have completely heterogeneous TCR- α similar to naive T cells, only the T cells expressing the single RT1-TCR- α chain were selected and expanded. Indeed, we observed that a majority of CD8⁺ T cell blasts became clonotype-positive after Ag-specific stimulation (data not shown). In contrast, CTLs from α Tg mice showed cytotoxicity as strong as $\alpha\beta$ Tg mice in spite of the random usage of J β and CDR3 β sequences. These results strongly suggest that RT1-TCR- α chain played a predominant role even in the *in vivo* recognition of P18IIIB/H-2D^d. Although the relative dependency on TCR- α chain in Ag recognition has been described in several systems (3, 19, 31–34), this is the first example of a single TCR- α being used to generate functional Ag-specific CTLs with various TCR- β chains.

The observation in single TCR Tg analyses that RT1-TCR- α chain could generate functional Ag-reactive TCRs with various V β 8⁺ TCR- β chains at a high frequency similar to the *in vitro* TCR reconstitution system indicates that the clonal size expressing RT1 α chain in preimmune repertoire is very small and only RT1 α ⁺ T cells expand after Ag stimulation. This result may reflect the fact that the functional assembly of TCR- α chains with a defined TCR- β is more easily formed than that of

TCR- β chains with a defined TCR- α in the physiological repertoire. These functional constraints of TCR assembly are created during selection of the TCR repertoires in the thymus.

Since TCR- α rearrangement takes place after TCR- β and the probability of generating identical V α -J α joints in a T cell expressing a rearranged TCR- β is thought to be virtually nil, the chance for a single TCR- α chain to pair with multiple TCR- β chains would be extremely small. In contrast, since extensive proliferation occurs after TCR- β rearrangement, each TCR- β would be expressed in numerous immature T cells and pair with multiple TCR- α chains. If a TCR- β chain had the capacity to exhibit Ag specificity with multiple TCR- α chains, Ag stimulation would activate a large number of T cell clones with a variety of avidities, including clones reactive to other Ags as well as possibly self-antigens. Moreover, negative selection in the thymus may delete otherwise useful TCR- $\alpha\beta$ pairs, as they also react to self-antigens. However, as shown in this study, a TCR- β chain paired with distinct TCR- α chains creates individually well-defined Ag specificities. This system reflects the mechanism for ensuring self-tolerance and generating the diversity of the T cell repertoire.



B

clone#	V β	CDR3 β	J β	J β	CDR3 β length
P14 β	CAS	SDA GGRNTL	YFGAG	2.4	9
#855	CAS	S RDWG GYAEQ	YFGAG	2.1	10
#900	CAS	SDA QGAGTQQL	YFGAG	2.2	11
#990	CAS	SDA GSQNTL	YFGAG	2.4	9

Figure 8. Ag-specific responses by transfectants expressing the P14-TCR- α chain and randomly selected V β 8.1⁺ TCR- β chains from naive T cells. (A) TCR- β chains were randomly picked up from naive CD8⁺ cells of normal mice similarly to Fig. 5. These TCR- β chains used V β 8.1-J β 2.1, 2.2, 2.4, and random CDR3 β segments. These TCR- β chains were transfected into P14-TCR- α -expressing TG40 cells and stimulated with APC and LCMV p33 peptide (right panel) or immobilized anti-TCR- β mAb H57 (left) for 24 h. The concentration of IL-2 was measured by ELISA and the data was presented as the percentages of that of P14-TCR- $\alpha\beta$ transfectant (P14 β). (B) The three TCR- β chains responding to p33/D^b did not have any sequence similarity to P14-TCR- β (P14 β).

In the *in vivo* peripheral repertoire, each TCR- α chain pairs in principle with a unique TCR- β chain because of the order of TCR rearrangement/selection, avoiding the problems of autoimmunity and unwanted deletion of useful T cell clones. The assembly of the same β chain with a distinct α chain may exhibit different Ag specificity. Therefore, analyzing a particular pair of TCR- $\alpha\beta$ cannot provide the potential capability of TCR- α to assemble with other β . The issue of whether the possible assembly of α chain with other β creates TCR with the same Ag specificity during T cell selection has been kept unsolved. Our present analysis could demonstrate for the first time by changing TCR composed of various TCR- β and - α defined TCR- α that multiple TCR- β can create the same Ag specificity if the rearranged β has a chance to assemble with a single TCR- α during generation of the T cell repertoire.

Recent crystallographic analyses of the trimolecular complex, TCR- $\alpha\beta$ and peptide-MHC revealed that CDR1, CDR2, and CDR3 $\alpha\beta$ regions generally contribute to the buried surface area in the interface, and subsequently a dominant role of the V α domain in peptide recognition was acknowledged (11–15). The distribution of the buried surface area by CDR3 α has been shown to be greater than by CDR3 β in 2C-TCR (12). In the case of D10-TCR, 23 of 27 atomic contacts with the peptide involve V α and only 4 involve V β (14). In addition, these reports suggested that the pivot point and the orientation angle between TCR and peptide/MHC might regulate the contact sites of CDR3 α to the peptide.

These results imply that TCR- α chain may present dominant contribution to Ag recognition, which is consistent with our results of extensive functional analysis of RT1- and P14-TCR. Although CDR3 β residues can be random for P18IIIB/H-2D^d recognition, the V β usage is restricted to be V β 8, suggesting that V β contributes to the contact with MHC and/or peptide. The actual structural basis of Ag recognition with random CDR3 β will have to wait for the crystallographic analysis of the RT1-TCR- $\alpha\beta$ /P18IIIB/H-2D^d trimolecular complex.

This study suggests the predominant role of TCR- α in the formation of the functional preimmune TCR repertoire. Numerous analyses of T cell clones and populations mostly by analyzing the junctional sequences of TCR have not been able to determine the functional contribution of TCR- α versus TCR- β in Ag recognition, as simple determination of each TCR sequence does not reveal functional TCR dimers. It has been postulated that CDR3 α and CDR3 β may equally contribute to most Ag recognition in general. Thus, the median of the dependency on CDR3 α versus CDR3 β for Ag recognition was postulated to be located in the middle of the distribution.

Our approach by functional and clonal TCR reconstitution unveiled the existence of TCR recognition with minimum involvement of CDR3 β and predominant dependency on TCR- α . Together with structural data, it can now be postulated that Ag recognition is mediated predominantly by TCR- α and the contribution of TCR- α versus TCR- β on Ag recognition may be shifted toward TCR- α . A considerable proportion of T cells expresses

two TCR- α chains after possible receptor editing during thymic selection (8). Although the physiological meaning of two TCR- α on a single T cell is not yet clear, one of the possibilities is a role in the shifting of the TCR contribution to Ag recognition toward TCR- α . Systematic analysis of functional repertoire as shown in the present study as well as structural determination of various TCR- $\alpha\beta$ dimers may provide the whole profile of the Ag recognition structure by TCR- $\alpha\beta$.

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