Brief Definitive Report

T Cell Receptor β Chain Lacking the Large Solvent-exposed Cβ FG Loop Supports Normal α/β T Cell Development and Function in Transgenic Mice

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Summary

The striking and unique structural feature of the T cell receptor (TCR) β chain is the bulky solvent-exposed FG loop on the Cβ domain, the size of almost half an immunoglobulin domain. The location and size of this loop suggested immediately that it could be a crucial structural link between the invariant CD3 subunits and antigen-recognizing α/β chains during TCR signaling. However, functional analysis does not support the above notion, since transgene coding for TCR β chain lacking the complete FG loop supports normal α/β T cell development and function.

Key words: T cell receptor • Cβ domain • mutagenesis • transgenic mice • T cell development and function

The α/β T lymphocytes use clonally distributed TCR to recognize cell-bound antigens, usually in the form of peptides embedded in MHC molecules. The α/β TCR is an oligomeric complex containing variable, covalently bound α and β chains responsible for antigen recognition and four noncovalently associated monomorphic subunits, CD3δ, γ, ϵ, and ζ chain. The invariant subunits are crucial for efficient assembly of the TCR and, hence, for surface expression (1). In addition, they couple extracellular ligand binding into cytoplasmic signaling machinery and, therefore, form an essential and the most proximal component of TCR signal transduction (2).

Although some of the sequential biogenesis steps of the TCR complex are quite well-characterized, the final complex on the cell surface is surprisingly poorly defined: not only is the overall topology of the complex unknown, but so is even the basic stoichiometry of the TCR, the most commonly proposed structure being $TCR\alpha\beta\zeta_2CD3\delta\gamma\epsilon_2$ (1).

Recently resolved three-dimensional structures of ectodomains of TCR β and α/β chains have now offered some potential insights into the puzzle of the TCR complex topology (3–6). The most striking feature of the structure of the Cβ domain is the large 14–amino acid long FG loop that protrudes freely into the solvent on the external face of the Cβ domain. It was soon proposed that this loop would interact with CD3 and, therefore, be part of the relay team in TCR signal transduction (3). Recent more detailed structural analyses and simple elegant antibody/epitope mapping of the TCR have added further details and suggested that the loop would form part of the interface between CD3ϵ and the Cβ domain (6, 7).

Here we report our finding that the TCR β chain lacking the complete 14–amino acid FG loop is able to support normal T cell development and function in transgenic mice.

Materials and Methods

TCR-β Mutagenesis. A retroviral expression vector LXSN coding for the wild-type TCR β chain (Vβ8.2-Jβ2.1) cDNA was used as template for mutagenesis. Deletion of the region corresponding to the 14–amino acid FG loop of the Cβ domain was performed by linking PCR. A 1:1 ratio of the products from PCR 1 (5′ oligo of Vβ8.2 GAATTCCTTGAGCTCAAGATGGGCTCCAGGCTCTTC [oligo A] and 3′ oligo spanning the deletion GTTCTGTGTGACCCCATGGAACTGCACTTGGCAGCG) and PCR 2 (5′ oligo spanning the deletion CAGTTCCATGGGGTCACACAGAACATCAGTGCAGAG and 3′ oligo containing the stop codon AGGATCCTCATGAGTTTTTTCTTTTGAC [oligo B]) was used as template for PCR 3 (oligo A and B). The PCR product was digested with EcoRI and BamHI and cloned into an EcoRI and BamHI–opened retroviral vector LXSN. Deletion (underlined amino acids 231–244) GLSEEDKWPEGSPKPV was then verified by DNA sequencing. Transgenic vectors were as described previously (8).

Transfection of Cell Lines. Infectious retroviral stocks were generated by transfecting packaging cell lines GP+E-86 (9) with retroviral expression vectors LXSN (neomycin resistant) coding for wild-type or mutant TCR β chain, or vectors LXSP (puromycin resistant) coding for wild-type TCR α chain (Vα4-Jα47). The supernatants from appropriately selected packaging cell lines were used to infect TCR^- hybridomas. The wild-type β or mutant β chain were first introduced into the hybridomas, and after neomycin selection (G418, 1 mg/ml) these lines were superinfected separately with TCR α chain as described previously (10).

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The cell lines were then cultured in IMDM supplemented with 2% FCS, G418, and puromycin (10 μg/ml). TCR expression was tested by FACS® as soon as 4 d after selection. Stable transfectants were maintained in G418 and puromycin–containing medium.

Mice. BALB/c and C56BL/6 mice were purchased from IFFA-Credo. The TCR-β knock-out mice have already been described (11), and were bred in our specific pathogen–free animal facility with the wild-type TCR-β or mutant TCR-β transgenic mice.

Flow Cytometry and Antibodies. Immunofluorescence stainings were done as described previously (12). Flow cytometric analysis was performed with a FACSCalibur™ equipped with CellQuest software (Becton Dickinson). The reagents used were mAbs biotinylated 145-2C11 (anti-CD3ε), PE-labeled RM4-5 (anti-CD4) and FITC-labeled H57-597 (anti-Cβ) (13), B20.1 (anti-Vα2), RR3-16 (anti-Vα3.2), B21-14 (anti-Vα8), and RR8-1 (anti-Vα11.1, 2) (all seven mAbs purchased from PharMingen), Cy5-labeled 53-6.7 (anti-CD8), fluorescein-succinimidyl-ester (FLUOS)-labeled F23.1 (anti-Vβ8.1, 2, 3) (14), and second-step reagent streptavidin-allophycocyanin (APC) (Molecular Probes, Inc.).

T Cell Functional Assays. For T cell proliferation, 2×10^5 spleen cells were cultured in triplicate with various concentrations of staphylococcal enterotoxin B (SEB) and SEC 2 superantigens in 200 μl of IMDM supplemented with 10% FCS in 96-well flat-bottomed plates. Proliferative responses were assessed after 48 h of culture. Cultures were pulsed 8 h before harvesting with 1 μCi [^{3}H]TdR (40 Ci/nmol; Radiochemical Center, Amersham Pharmacia Biotech), and incorporation of [^{3}H]TdR was measured by liquid scintillation spectrometry. Helper T cell responses were tested by immunizing mice (three per group) with 100 μg of NIP-OVA in CFA in the tail base. For control, mice received PBS in CFA (referred to as CFA only in Fig. 3). After 14 d, sera from immunized mice were pooled and tested for the presence of anti-NIP IgG by ELISA as described (15). Plates coated with 5 μg/ml of NIP-BSA and then blocked with PBS/1% BSA received dilutions of the sera. Binding of the anti-NIP IgGs was revealed by alkaline phosphatase–conjugated goat anti–mouse IgG (Southern Biotechnology Associates). Allogeneic killer cells were generated as described previously (8). In brief, 10^7 responders (H-2^b splenocytes from wild-type TCR-β or mutant TCR-β transgenic mice) were cultured with 10^7 x-irradiated stimulators (H-2^d splenocytes from BALB/c mice). After 5 d, various numbers of responder cells (numbers used to calculate the E/T ratios) were cultured with 10^4 $Na_2{}^{51}CrO_4$-labeled target LPS blasts. After 4 h, supernatant was harvested. Some wells contained only labeled targets with or without 0.01 M HCl/10% SDS containing medium to determine maximum and spontaneous release, respectively. Data are presented as percentage of killing = [(experimental release − spontaneous release)/(total release − spontaneous release)] × 100.

Results and Discussion

Surface Expression of TCR Containing the Mutant, FG Loop–deleted β Chain. To test whether the deletion of the complete 14–amino acid FG loop in the Cβ domain would be deleterious for the TCR assembly and surface expression, we transfected TCR$^-$ T cell thymoma 58 with retroviral vectors coding for either a control or a mutant β chain together with a wild-type α chain (10). To our initial surprise, the TCR surface expression was only slightly lower in the mutant case (Fig. 1 A). However, we must point out that the observed 30–50% reduction in the surface expression represents a handicap in the TCR assembly which, although small, is real since we have used a very efficient retroviral transfection system that allows us to create bulk transformants containing thousands of individual clones and which, therefore, provides us with a reliable statistical average. Functional analyses of these transfectomas consistently showed that the cells transfected with the mutant TCR β chain responded slightly less (about threefold) to antigenic stimulation as exemplified here by the dose–response curves to influenza hemagglutinin peptide HA 110-119 (Fig. 1 B) or SEC 3 superantigen (Fig. 1 C).

Derivation of TCR β Chain Transgenic Mice. To more rig-

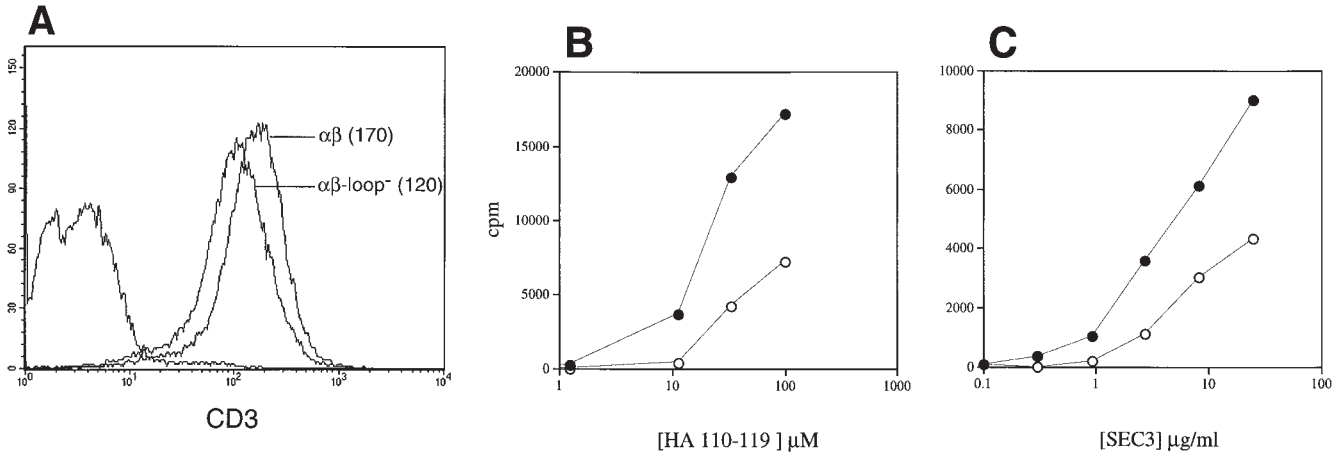

Figure 1. The mutant TCR β chain is functionally expressed on the cell surface. (A) Cells transfected with TCR α chain together with a control β (αβ) or mutant β chain (αβ-loop$^-$) were stained with biotinylated anti-CD3ε mAb followed by streptavidin-APC. Staining of cells transfected with mutant β chain only is shown as negative control. Numbers represent the mean of fluorescence intensity of CD3 staining. (B and C) Functional response of 10^5 TCR transfectants (αβ, ●; αβ-loop$^-$, ○) cultured with 10^5 irradiated antigen-presenting cells (B cell lymphomas A20) and the indicated concentrations of influenza hemagglutinin peptide HA 110-119 (B) or superantigen SEC 3 (C). After 20 h, the culture supernatant was collected and tested for the presence of lymphokines using the IL-2–dependent proliferation assay of HT2 cell lines.

orously assess the functional potential of the TCR containing the mutant β chain in normal physiological settings in vivo, we generated transgenic mice expressing either a wild-type or a loop-deleted version of the TCR β chain. The β transgenes, as in the above transfection studies, were derived from 14.3d T cell hybridoma expressing the TCR specific for influenza hemagglutinin peptide HA 110-119 in the context of I-E^d MHC class II molecules (16). In fact, it was the very same β chain (Vβ8.2-Jβ2.1) whose three-dimensional structure was first solved, thus providing us with the inspiration for the current study (3). Two characteristics of the transgenic lines used here were considered essential for straightforward interpretation of the data. First, the level of α/β TCR expression was identical in both lines (Fig. 2). Presumably the small handicap of the mutant β chain in the TCR assembly could be compensated by higher intracellular expression. Second, both transgenes were bred to TCR-$\beta^{-/-}$ background to avoid any contribution of endogenous β chains for the observed α/β T cell behavior (11).

Mutant TCR β Chain Supports Normal α/β T Cell Development. α/β T cell development proceeds undisturbed and similarly in both TCR β chain transgenic lines as shown by flow cytometric analysis of thymic and lymph node cells (Fig. 2). Even the skewing into single positive CD4 thymocytes, as noted earlier for our wild-type TCR-β transgenic mice (8), occurs to the same extent in both lines. As predicted, mAb H57-597 (anti-Cβ [13]) does not bind to mutant TCR β chain (Fig. 2 H [6]). Interestingly, mAb F23.1 (anti-Vβ8.1, 2, 3 [14]) binds equally well to both β chains, whereas mAb MR5-2 (anti-Vβ8.1, 2 [17]) fails to react with the mutant, suggesting that the FG loop may form part of the MR5-2 epitope (not shown). Since the cellularity of thymi is normal in both cases, we assume that pre-TCR–mediated T cell expansion occurs normally in these mice.

Normal α/β T Cell Responses in Mutant β Chain Transgenic Mice. Peripheral T cell responses were measured in several types of assays, and none of them, to our disappointment, showed any significant differences between mice of the different transgenic lines. The in vitro responses to anti-TCR antibodies (not shown) and to SEC 2 and SEB superantigens were repeatedly similar in all mice tested (Fig. 3, A and B). In addition, the in vivo $CD4^+$ T cell responses measured by T cell help for hapten-specific IgG production were basically indistinguishable between control and mutant mice (Fig. 3 C). Finally, α/β T cells from mutant TCR β chain transgenic mice made as vigorous cytotoxic T cell responses against allogeneic targets as their control counterparts (Fig. 3 D). We also monitored the representation of four different Vα families by flow cytometry in peripheral T cells in order to reveal any subtle in vivo biases, but none were found (Table I). In addition, limited DNA sequence analyses of Vα 2 and 8 families from single α/β T cells revealed no obvious "mutant"-specific features (data not shown).

Concluding Remarks. Thus far, we have found only a quantitative role in the TCR assembly process for the large solvent-exposed FG loop on the Cβ domain. In transfectants, the TCR will assemble in the absence of the loop in the β chain but slightly less efficiently compared with the wild-type structure. Of course, the reduced surface expression leads to somewhat impaired function. However, we were able to show in vivo that TCRs are functionally ex-

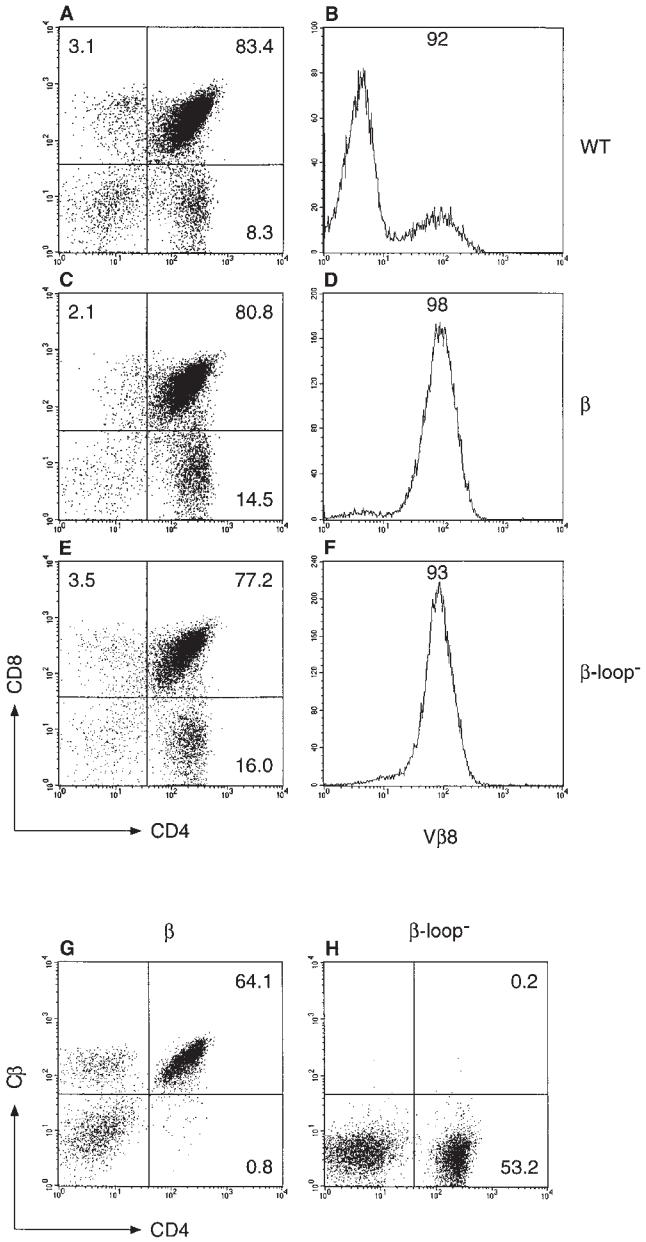

Figure 2. FACScan™ analysis of thymocytes or lymph node cells from mutant TCR β chain transgenic mice. Thymocytes from littermate control (A and B), wild-type TCR-β (C and D), or mutant TCR-β (E and F) transgenic mice were triple-stained with anti-CD8–Cy5, anti-CD4–PE, and anti-Vβ8 (F23.1-FLUOS) antibodies. Numbers in all dot plots are percentages of total cells. Histograms represent Vβ8 expression of gated single positive $CD4^+$ thymocytes, and numbers show the mean of fluorescence intensity of Vβ8 staining. Lymph node cells from wild-type TCR-β (G) or mutant TCR-β (H) transgenic mice were stained with anti-Cβ–FITC and anti-CD4–PE.

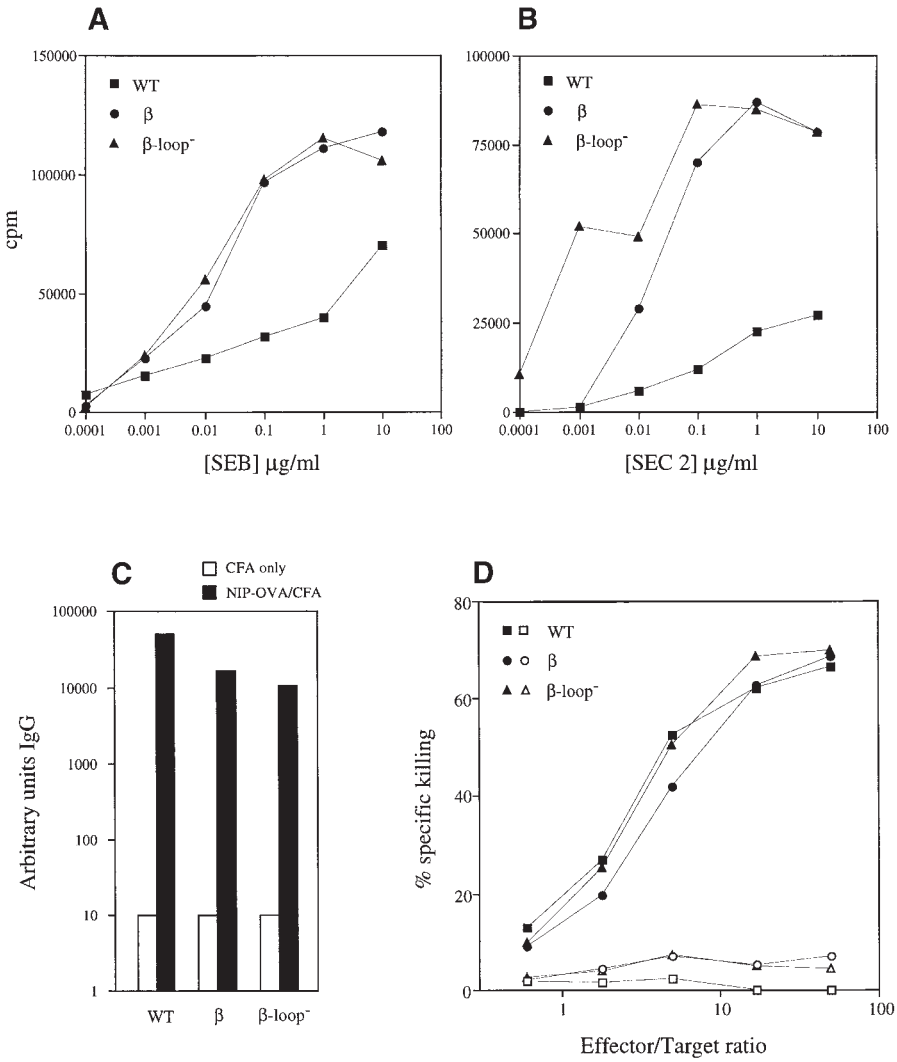

Figure 3. Functional analysis of peripheral T cells from syngeneic control mice (WT), wild-type TCR-β (β), or mutant TCR-β (β-loop$^-$) transgenic mice. Proliferative response to SEB (A) or SEC 2 (B) superantigens after 48 h of culture. Helper T cell responses (C) assessed 14 d after immunization with NIP-OVA/CFA. Sera from the immunized mice were tested for the presence of anti-NIP IgGs by ELISA on plates coated with NIP-BSA. (D) Allogeneic killer assay. Splenocytes from H-2^b control or transgenic mice were stimulated for 5 d with allogeneic H-2^d BALB/c cells. Cytotoxic function was then tested on syngeneic H-2^b (open symbols) or allogeneic H-2^d (filled symbols) $Na_2{}^{51}CrO_4$-labeled target LPS blasts.

pressed at the same level with or without the FG loop, and we did not find any qualitative or quantitative differences in their activity. This finding seems to rule out the models where the FG loop has an absolute role in TCR signaling. However, the apparent absence of any effect in vivo could also be due to the fact that some subtle compensatory mechanisms have been turned on in vivo (but not in cell lines), e.g., TCR affinities could be modulated, or new carbohydrate structures on the Cβ domain could partially replace the FG loop functionally. Interestingly, all nonmammalian species studied to date, including birds, amphibians, reptiles, and fish, do not have the FG loop on their Cβ domain (18); hence, our in vivo findings may not be that surprising.

Table I. *TCR Vα Usage in Mutant TCR-β Transgenic Mice*

	Percent of CD4$^+$				Percent of CD8$^+$			
	Vα2$^+$	Vα3.2$^+$	Vα8$^+$	Vα11$^+$	Vα2$^+$	Vα3.2$^+$	Vα8$^+$	Vα11$^+$
β	22.7 ± 1.0	0.7 ± 0.2	3.0 ± 0.1	1.5 ± 0.4	7.7 ± 1.8	1.0 ± 0.1	4.5 ± 0.1	1.2 ± 0.2
β-loop$^-$	22.7 ± 0.9	0.5 ± 0.1	2.6 ± 0.3	2.3 ± 0.3	7.6 ± 1.1	1.4 ± 0.6	3.6 ± 0.7	2.0 ± 0.3

Lymph node cells from TCR-β (β) or mutant TCR-β (β-loop$^-$) transgenic mice were triple stained with the FITC-labeled anti-Vα Abs, anti-CD4–PE, and anti-CD8–Cy5. Results (three mice per group) are expressed as the percentage of stained cells ± SD.

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