

## **Impaired NK1.1 T Cell Development in Mice Transgenic for a T Cell Receptor $\beta$ Chain Lacking the Large, Solvent-exposed C $\beta$ FG Loop**

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### **Summary**

A striking feature of the T cell receptor (TCR)  $\beta$  chain structure is the large FG loop that protrudes freely into the solvent on the external face of the C $\beta$  domain. We have already shown that a transgene-encoded V $\beta$ 8.2<sup>+</sup> TCR  $\beta$  chain lacking the complete C $\beta$  FG loop supports normal development and function of conventional  $\alpha/\beta$  T cells. Thus, the FG loop is not absolutely necessary for TCR signaling. However, further analysis has revealed that a small population of  $\alpha/\beta$  T cells coexpressing NK1.1 are severely depleted in these transgenic mice. The few remaining NK1.1 T cells have a normal phenotype but express very low levels of TCR. We find that the TCR V $\beta$ 8.2<sup>+</sup> chain lacking the C $\beta$  FG loop cannot pair efficiently with the invariant V $\alpha$ 14-J $\alpha$ 281 TCR  $\alpha$  chain commonly expressed by this T cell family. Consequently, fewer NK1.1 T cells develop in these mice. Our results suggest that expression of the V $\alpha$ 14<sup>+</sup> TCR  $\alpha$  chain is particularly sensitive to TCR- $\beta$  conformation. Development of NK1.1 T cells appears to need a TCR- $\beta$  conformation dependent on the presence of the C $\beta$  loop that is not necessarily required for assembly and function of TCRs on most  $\alpha/\beta$  T cells.

**Key words:** TCR • C $\beta$  FG loop • mutagenesis • NK1.1 T cells • V $\alpha$ 14

All crystal structures of the TCR  $\beta$  chain reported to date have shown that the constant and variable domains are closely associated, with a large, solvent-exposed loop of 14 amino acids protruding on the external face of the C $\beta$  domain (1–4). The location and size of this loop (almost half of an Ig domain) suggested that it could be the crucial link between TCR- $\alpha/\beta$  recognition of antigen and transmission of signals by the invariant CD3 (1, 4, 5). To study its function, we recently generated mice transgenic for a TCR  $\beta$  chain lacking the complete C $\beta$  FG loop. The TCR  $\beta$  chain (V $\beta$ 8.2-J $\beta$ 2.1) chosen for mutagenesis has been crystallized (1); it was cloned from T cell hybridoma 14.3.d, which expresses a TCR  $\alpha$  chain (V $\alpha$ 4-J $\alpha$ 47) and recognizes a PR8 influenza hemagglutinin peptide, HA 110–119, presented by the I-E<sup>d</sup> MHC molecule (6). Surprisingly, we found that development and function of conventional  $\alpha/\beta$  T cells was normal in mice transgenic for a TCR  $\beta$  chain lacking the C $\beta$  FG loop. Thus, the C $\beta$  FG loop is not absolutely required for transmitting the signal of antigen recognition by the TCR (7).

Further analysis revealed that a small population of  $\alpha/\beta$  T cells coexpressing NK1.1 is drastically diminished in these mice. Many features (8), including development, functional properties, and TCR repertoire, distinguish this latter population from conventional T cells. Development of NK1.1 T cells requires expression of the  $\beta_2$  microglobulin-associated, class Ib-like CD1d1 molecule (9, 10), which can restrict their

response to lipid ligands such as glycosylphosphatidylinositols or glycosylceramides (11, 12). NK1.1 T cells can readily produce large amounts of cytokines upon activation (13), and they have been implicated in tumor rejection (14, 15) and may also play a regulatory role in autoimmune manifestations (16–18). NK1.1 T cells express a limited V $\beta$  repertoire highly skewed toward V $\beta$ 8.2, V $\beta$ 7, and V $\beta$ 2 (8), and in transgenic mice expressing single V $\beta$ s such as V $\beta$ 3 and V $\beta$ 8.1, NK1.1 T cell development is totally abrogated (19). Furthermore, ~80% of NK1.1 T cells express an invariant TCR  $\alpha$  chain (V $\alpha$ 14-J $\alpha$ 281) (20, 21) that is required for their development (15).

In this study, we present evidence that the V $\beta$ 8.2 TCR  $\beta$  chain lacking the complete C $\beta$  FG loop cannot pair efficiently with the canonical V $\alpha$ 14<sup>+</sup> TCR  $\alpha$  chain. Consequently, NK1.1 T cell development is severely impaired.

### **Materials and Methods**

**TCR- $\beta$  Mutagenesis.** The wild-type TCR  $\beta$  chain (V $\beta$ 8.2-J $\beta$ 2.1) cDNA was used as a template for mutagenesis. Deletion of the 14 nucleotides forming the C $\beta$  FG loop has been described (7). Transgenic vectors have also been described previously (22).

**Transfection of Cell Lines.** Packaging cell lines GP+E-86 (23) were transfected with retroviral vector LXSXN expressing the V $\beta$ 8.2-J $\beta$ 2.1<sup>+</sup> TCR- $\beta$  or  $\beta$ -loop<sup>-</sup> chain or LXSP expressing the V $\alpha$ 4-J $\alpha$ 47<sup>+</sup> or V $\alpha$ 14-J $\alpha$ 281<sup>+</sup> TCR  $\alpha$  chain cDNA. The TCR  $\alpha$  chain (V $\alpha$ 14-J $\alpha$ 281) was cloned from NK1.1  $\alpha/\beta$ <sup>+</sup> T cell hy-

bridoma total RNA provided by R. MacDonald (Ludwig Institute for Cancer Research, Lausanne, Switzerland). After appropriate selection of the packaging cells, the infectious supernatants were used to infect TCR<sup>-</sup> hybridomas (24) as previously described (25). The TCR-β or β-loop<sup>-</sup> chain was first introduced into the hybridomas and, after neomycin selection (G418; 1 mg/ml), these cells were superinfected with TCR α chain by culturing them on packaging lines producing LXSP TCR-α Vα4-Jα47 or Vα14-Jα281. The hybridomas were then maintained in IMDM supplemented with 2% FCS, neomycin, and puromycin (10 μg/ml). TCR expression was tested by FACS™ as early as 4 d after selection. Stable transfectants were maintained in G418 and puromycin-containing medium.

**TCR Immunoprecipitation and Western Blot Analysis.** Hybridomas were lysed at 2 × 10<sup>7</sup> cells/ml in 1% Triton X-100 (Bio-Rad Labs.), 150 mM NaCl, 20 mM Tris/HCl, and 5 mM EDTA, pH 7.5, buffer containing complete protease inhibitors (Boehringer Mannheim) for 30 min at 4°C. Lysates cleared of cell debris were immunoprecipitated with purified mAb F23.1 (2 μg/ml) and protein G-Sepharose (Pharmacia). After washing with lysis buffer and PBS, the lyophilized pellets were resuspended in reducing SDS buffer, loaded on a 4–12% Bis-Tris precast gel (Novex), and transferred onto nitrocellulose membrane Hybond-C extra (Amersham). Blots were probed in PBS 6% blotting blocker nonfat milk (Bio-Rad Labs.) and 0.2% Tween with purified mAb H58 (anti-Cβ), followed by goat anti-hamster horseradish peroxidase-labeled mAb (Southern Biotechnology Associates, Inc.) or biotinylated F23.1 (anti-Vβ8) mAb followed by streptavidin-horseradish peroxidase (Southern Biotechnology Associates, Inc.). The proteins were detected with a chemiluminescent detection system (Pierce Chemical Co.).

**Mice.** BALB/c and C56BL/6 mice were purchased from IFFA-Credo. The TCR-β knockout mice have been described (26) and were bred in our specific pathogen-free animal facility with the TCR-β or TCR β-loop<sup>-</sup> transgenic mice.

**Cell Suspension, Flow Cytometry, and Antibodies.** Cell suspensions from thymi were depleted of CD8<sup>+</sup> T cells with anti-CD8 31M antibody (27) and complement treatment (Cedarlane Labs.), and liver cells were simply ficolled to eliminate red cells before immunofluorescence stainings, performed as previously described (28). Flow cytometric analyses were performed on a FACSCalibur™ equipped with CELLQuest software (Becton Dickinson). The reagents used were mAbs 145-2C11 (anti-CD3ε), NKR-P1C (anti-NK1.1), H57-597 (anti-Cβ), RM4-5 (anti-CD4), IM7 (anti-CD44, Pgp-1), TM-β1 (anti-IL-2R β chain), MEL-14 (anti-CD62L) (all seven mAbs purchased from PharMingen), biotinylated F23.1 (anti-Vβ8.1,2,3), and second step reagent streptavidin-allophycocyanin (Molecular Probes, Inc.).

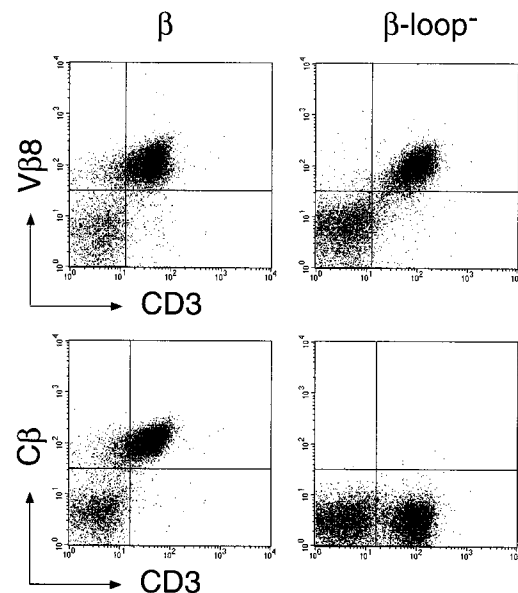
**Single-Cell Reverse Transcriptase-PCR.** Single NK1.1<sup>+</sup>CD3<sup>+</sup> cells were sorted into polycarbonated 96-well plates (one cell per well in 5 μl of PBS) and immediately frozen on dry ice and stored at -70°C. To prepare cDNA, the plate was heated up to 65°C for 1 min before adding into each well 10 μl of the reverse transcriptase (RT)-PCR mix (reverse transcriptase Superscript II; GIBCO BRL) for 1 h at 42°C under standard reaction conditions. After heat inactivation of the enzyme (2 min at 95°C), DNA amplification was carried out as described (29). 75 μl of a PCR mix containing Taq polymerase and the primers necessary for DNA amplification of the Vα14<sup>+</sup> TCR α chain (5' Vα14 CTAAGCACAGCAGCTGCACA [reference 20]; 3' Cα ATG-GATCCTCAACTGGACCACAGCCTCA) and Vβ8.2<sup>+</sup> TCR β chain (5' Vβ8.2 CTTGAGCTCAAGATGGGCTCCAGGCT-CTTC; 3' Jβ2.1 CTGCTCAGCATAACTCCCCG) were added to the wells for the first round of PCR (30 cycles). An aliquot from

this PCR (1 μl) was used for a second round of PCR (35 cycles) to individually reamplify the Vα14<sup>+</sup> TCR α chain or Vβ8.2<sup>+</sup> TCR β chain using the same specific primers.

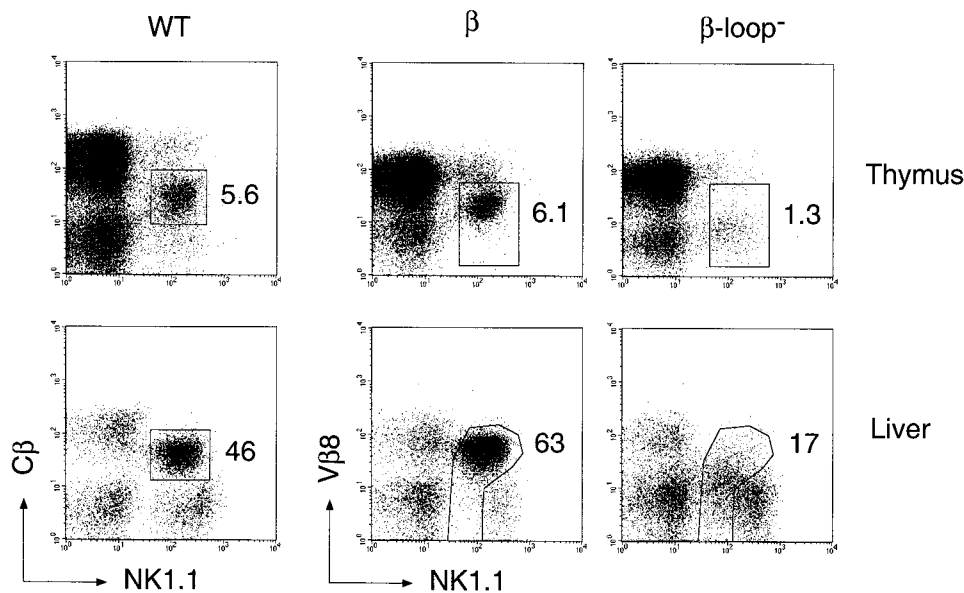
## Results and Discussion

To avoid any influence of the endogenous β locus on the expression of the mutated β chain, mice transgenic for the Vβ8.2<sup>+</sup> TCR β chain lacking the Cβ FG loop (β-loop<sup>-</sup>) were backcrossed to TCR-β<sup>-/-</sup> mice (26). T cell development in these mice was compared with that in wild-type Vβ8.2<sup>+</sup> TCR β chain transgenic mice, also with a β<sup>-/-</sup> background. As described in our previous study (7), peripheral T cells from mice transgenic for the TCR β or β-loop<sup>-</sup> chain express equal levels of the TCR-CD3 complex, and whereas the anti-Vβ8 F23.1 mAb recognizes all T cells, the Cβ-specific H57 mAb does not stain cells expressing the TCR β-loop<sup>-</sup> chain (Fig. 1; reference 4). It is worth pointing out that in the absence of the Cβ FG loop, the anti-CD3ε 2C11 mAb stains better, suggesting that the epitope recognized is more accessible, a result that might not be surprising, as one of the CD3ε chains is physically adjacent to the β chain in the TCR-CD3 complex (5).

We consistently found that TCR β-loop<sup>-</sup> transgenic mice have significantly fewer NK1.1 α/β<sup>+</sup> T cells in the thymus, liver, and spleen (data not shown) in comparison to TCR-β transgenic mice or wild-type littermates (Fig. 2). Thus, a mutation in the Cβ domain can notably alter development of NK1.1 α/β T cells. This result was puzzling, considering that conventional α/β T cells are normal in TCR β-loop<sup>-</sup> transgenic mice (7). Furthermore, the mutated TCR β chain uses Vβ8.2, a variable region that is usually expressed by >40% of NK1.1 α/β T cells (30). Hence, monoclonal expression of



**Figure 1.** T cells expressing the Vβ8.2<sup>+</sup> TCR β chain lacking the FG loop cannot be stained with the Cβ-specific H57 mAb. LN cell suspensions from TCR-β and β-loop<sup>-</sup> transgenic mice were stained with anti-CD3 together with anti-Vβ8 or anti-Cβ mAb.



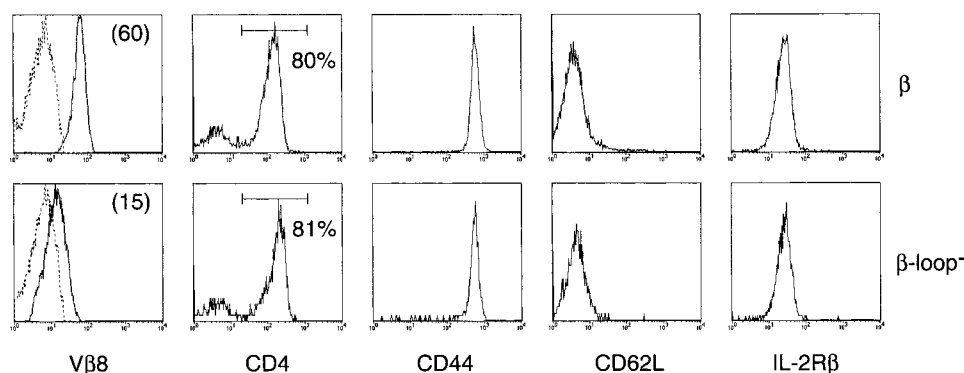
**Figure 2.** Decreased amount of NK1.1 T cells in TCR  $\beta$ -loop<sup>-</sup> transgenic mice. Cell suspension from thymi previously depleted of CD8<sup>+</sup> cells and livers from nontransgenic littermates (WT) or TCR- $\beta$  or  $\beta$ -loop<sup>-</sup> transgenic mice were double stained with anti-NK1.1 together with anti-C $\beta$  (to stain all T cells in WT mice) or anti-V $\beta$ 8 (which stains all T cells in transgenic mice) antibodies. Numbers express the percentages of total adjacent gated dots.

the wild-type V $\beta$ 8.2<sup>+</sup> TCR  $\beta$  chain allows NK1.1 T cell development comparable to that of nontransgenic littermates. Characteristically, NK1.1 T cells express intermediate levels of TCR (8). Interestingly, in TCR  $\beta$ -loop<sup>-</sup> transgenic mice, TCR expression on the few remaining NK1.1 T cells is even lower than in control animals; these cells express about four times less TCR than do those in wild-type  $\beta$ -transgenic mice (Fig. 3). Otherwise, NK1.1 T cells in  $\beta$ -loop<sup>-</sup> transgenic mice express normal levels of CD4 and are CD44<sup>+</sup>CD62L<sup>-</sup> and IL-2R $\beta$ <sup>+</sup>, as expected for this T cell population (8). CD1d, a  $\beta_2$  microglobulin-associated molecule required for NK1.1 T cell development (9, 10), is also expressed at normal levels in TCR  $\beta$ -loop<sup>-</sup> transgenic mice (data not shown).

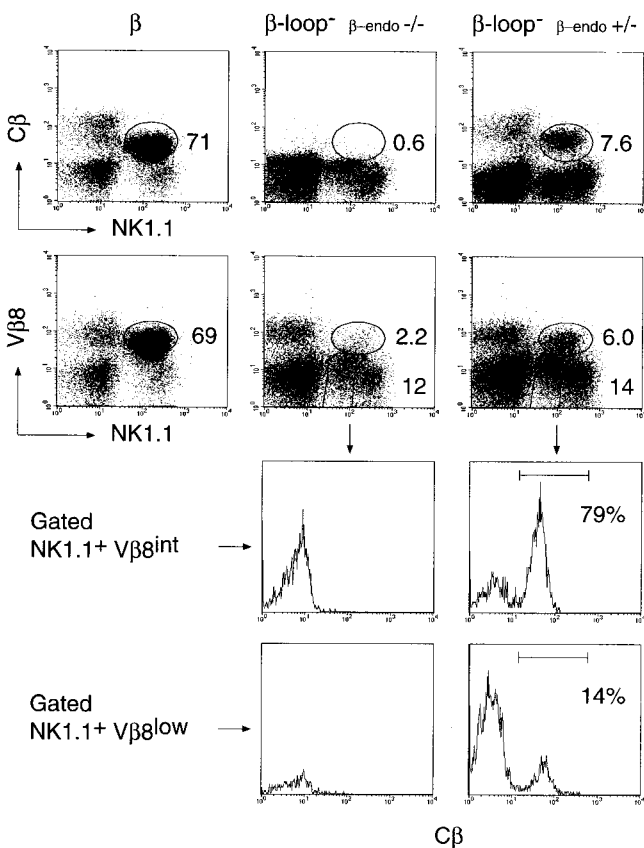
To determine if development of NK1.1  $\alpha/\beta$ <sup>+</sup> T cells could be rescued by the expression of endogenous  $\beta$  chains, as has been described for other TCR- $\beta$  transgenic mice (19), we studied NK1.1 T cell frequency in TCR  $\beta$ -loop<sup>-</sup> transgenic mice on a  $\beta$ <sup>+/-</sup> background. We have already observed that in these mice, inhibition of  $\beta$  rearrangements via allelic exclusion is not total, and ~10–20% of peripheral T cells can express endogenous  $\beta$  chains (data not shown). NK1.1  $\alpha/\beta$ <sup>+</sup> T cells expressing endogenous  $\beta$  and  $\beta$ -loop<sup>-</sup>

chains could be distinguished by the C $\beta$ -specific H57 mAb, which cannot stain T cells expressing the mutated  $\beta$  chain (Fig. 1). As shown in Fig. 4, expression of endogenous  $\beta$  chains can rescue NK1.1 T cell development to a certain extent. NK1.1 C $\beta$ <sup>+</sup> cells appear in the livers of TCR  $\beta$ -loop<sup>-</sup> transgenic mice on a  $\beta$ <sup>+/-</sup> background. Yet these cells only account for about one-third of the whole NK1.1 T cell population. The NK1.1 C $\beta$ <sup>-</sup> T cells are still predominant. There are two populations of NK1.1 V $\beta$ 8<sup>+</sup> cells, which express either intermediate or low TCR levels in TCR  $\beta$ -loop<sup>-</sup> transgenic mice on a  $\beta$ <sup>+/-</sup> background. Interestingly, expression of endogenous  $\beta$  chains accounts for most of the NK1.1 T cells expressing intermediate TCR levels. Thus, expression of endogenous  $\beta$  chains did rescue some NK1.1 T cell development and restore TCR expression to intermediate levels. This result strongly suggested that the C $\beta$  FG loop is needed for efficient TCR assembly in NK1.1 T cells.

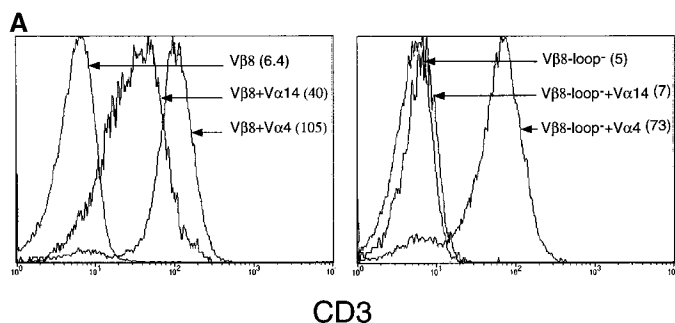
As most NK1.1  $\alpha/\beta$ <sup>+</sup> T cells express an invariant V $\alpha$ 14-J $\alpha$ 281 TCR  $\alpha$  chain (20, 21), and the mutant TCR  $\beta$  chain is expressed at normal levels by conventional  $\alpha/\beta$  T cells (Fig. 1) but not by NK1.1 T cells (Fig. 3), we assessed whether the mutant V $\beta$ 8.2<sup>+</sup> TCR  $\beta$  chain could still pair with the V $\alpha$ 14<sup>+</sup> TCR  $\alpha$  chain. TCR<sup>-</sup> hybridomas were



**Figure 3.** The remaining NK1.1 T cells in TCR  $\beta$ -loop<sup>-</sup> transgenic mice have a normal phenotype. Liver cell suspensions from TCR- $\beta$  or  $\beta$ -loop<sup>-</sup> transgenic mice were triple stained with anti-NK1.1, anti-V $\beta$ 8, and either anti-CD4 or anti-CD44 or anti-CD62L or anti-IL-2R $\beta$  mAbs. Histograms represent NK1.1<sup>+</sup>V $\beta$ 8<sup>+</sup> gated events. Numbers in parentheses represent the mean fluorescence intensity of V $\beta$ 8 staining. Negative controls of V $\beta$ 8 staining are shown (dashed lines).

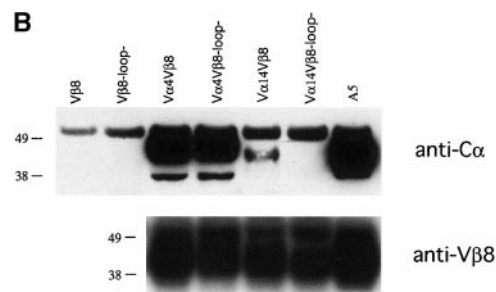


**Figure 4.** Expression of endogenous TCR  $\beta$  chains can rescue some NK1.1 T cell development. Liver cell suspensions from TCR  $\beta$ ,  $\beta$ -loop<sup>-</sup> transgenic with a  $\beta$ <sup>-/-</sup> background ( $\beta$ -loop<sup>-</sup>  $\beta$ -endo<sup>-/-</sup>) or  $\beta$ <sup>+/-</sup> background ( $\beta$ -loop<sup>-</sup>  $\beta$ -endo<sup>+/-</sup>) were triple stained with anti-NK1.1, anti-C $\beta$ , and anti-V $\beta$ 8 mAbs. Histograms represent expression of C $\beta$  (in  $\beta$ -loop<sup>-</sup> transgenic mice with a  $\beta$ <sup>-/-</sup> or  $\beta$ <sup>+/-</sup> background) for the NK1.1<sup>+</sup> gated population expressing either intermediate (NK1.1<sup>+</sup>V $\beta$ 8<sup>int</sup>) or low levels of TCR (NK1.1<sup>+</sup>V $\beta$ 8<sup>low</sup>).



transfected with cDNAs coding for either the wild-type TCR  $\beta$  or  $\beta$ -loop<sup>-</sup> chain together with the V $\alpha$ 14<sup>+</sup> TCR  $\alpha$  chain or V $\alpha$ 4<sup>+</sup> TCR  $\alpha$  chain (the original partner of the nonmutated  $\beta$  chain) cDNAs. As shown in Fig. 5 A, the TCR  $\beta$ -loop<sup>-</sup> chain clearly pairs with and is expressed on the cell surface with the V $\alpha$ 4<sup>+</sup> TCR  $\alpha$  chain but is barely detectable on the cell surface with the V $\alpha$ 14<sup>+</sup> TCR  $\alpha$  chain. In contrast, the wild-type TCR  $\beta$  chain is expressed on the cell surface with both  $\alpha$  chains (Fig. 5). However, the V $\alpha$ 14<sup>+</sup> TCR is expressed at lower levels than is the V $\alpha$ 4<sup>+</sup> TCR. This observation may reflect the *in vivo* situation in which a TCR on NK1.1 T cells is expressed at lower levels than on conventional  $\alpha/\beta$  T cells. To assess whether impaired cell surface expression of the TCR wild-type  $\beta$  and  $\beta$ -loop<sup>-</sup> chain together with the V $\alpha$ 14<sup>+</sup> TCR  $\alpha$  chain is due to a problem of pairing, TCRs from the transfectants were immunoprecipitated with anti-V $\beta$ 8 mAb. As can be seen in Fig. 5 B, the TCR  $\alpha$  chain can be coimmunoprecipitated with the TCR  $\beta$  chain in all transfectants expressing the TCR on the cell surface. In contrast, the V $\alpha$ 14<sup>+</sup> TCR  $\alpha$  chain cannot be coimmunoprecipitated with the mutant  $\beta$  chain in detectable amounts. This result implies that the V $\alpha$ 14<sup>+</sup> TCR  $\alpha$  chain pairs very poorly with the V $\beta$ 8<sup>+</sup> TCR  $\beta$  chain lacking the C $\beta$  FG loop. It is worth pointing out that in the hybridomas producing the wild-type  $\beta$  and V $\alpha$ 14<sup>+</sup> chains, many fewer assembled  $\alpha/\beta$  dimers can be immunoprecipitated compared with control V $\beta$ 8.2/V $\alpha$ 4 dimers. This latter result suggests that mere physical constraints on the assembly of the  $\beta$  chain with the V $\alpha$ 14<sup>+</sup> TCR  $\alpha$  chain exist and is consistent with the low TCR expression on normal NK1.1 T cells.

Next, we assessed whether the NK1.1  $\alpha/\beta$ <sup>+</sup> T cells that do develop in TCR  $\beta$ -loop<sup>-</sup> transgenic mice express the V $\alpha$ 14<sup>+</sup> TCR  $\alpha$  chain by performing RT-PCR on single NK1.1 CD3<sup>+</sup> T cells sorted from TCR- $\beta$  and  $\beta$ -loop<sup>-</sup> transgenic mice. As summarized in Table I, the frequency of NK1.1 T cells expressing V $\alpha$ 14 is not significantly decreased in TCR  $\beta$ -loop<sup>-</sup> transgenic mice in comparison to wild-type TCR- $\beta$  transgenic animals. One has to keep in



**Figure 5.** Inefficient pairing of the V $\alpha$ 14<sup>+</sup> TCR  $\alpha$  chain with the V $\beta$ 8.2<sup>+</sup> TCR  $\beta$  chain lacking the complete C $\beta$  FG loop. (A) TCR<sup>-</sup> hybridomas were transfected with either the V $\beta$ 8.2<sup>+</sup> wild-type TCR  $\beta$  or  $\beta$ -loop<sup>-</sup> chain, together with the V $\alpha$ 14<sup>+</sup> TCR  $\alpha$  chain or V $\alpha$ 4<sup>+</sup> TCR  $\alpha$  chain. Stable transfectants were stained with biotinylated anti-CD3 mAb, followed by streptavidin-allophycocyanin. Stainings of cells transfected with only the TCR  $\beta$  or  $\beta$ -loop<sup>-</sup> chain are shown as controls. Numbers in parentheses represent the mean fluorescence intensities of CD3 staining. (B) TCRs of transfected hybridomas or V $\beta$ 8<sup>+</sup> T hybridoma control (A5) (8.0, 8.0, 10.0, 14.0, 20.0, 20.0, and  $8.0 \times 10^7$  cells per lane, respectively, from left to right) were immunoprecipitated with anti-V $\beta$ 8 mAb (F23.1), electrophoresed on a 4–12% gel in reducing conditions, and blotted with anti-C $\alpha$  (H58) or anti-V $\beta$ 8 mAb as described in Materials and Methods. Numbers represent protein molecular mass (kD).

**Table I.** Expression of the V $\alpha$ 14<sup>+</sup> TCR  $\alpha$  Chain by the Remaining NK1.1 T Cells in TCR  $\beta$ -loop<sup>-</sup> Transgenic Mice

	Number of NK1.1 <sup>+</sup> CD3 <sup>+</sup> cells	
	V $\beta$ 8.2 <sup>+</sup>	V $\alpha$ 14 <sup>+</sup>
$\beta$	27/27	20/27 (74%)
$\beta$ -loop <sup>-</sup>	30/30	21/30 (70%)

Liver cell suspensions from TCR- $\beta$  or  $\beta$ -loop<sup>-</sup> transgenic mice were double stained with anti-NK1.1 and anti-CD3 mAbs. Double-stained cells were sorted individually to perform RT-PCR as described in Materials and Methods. cDNA encoding the V $\alpha$ 14<sup>+</sup> TCR  $\alpha$  chain was amplified as well as the V $\beta$ 8.2<sup>+</sup> TCR  $\beta$  chain used as positive control for the reaction. Numbers in parentheses represent percentages of V $\beta$ 8.2<sup>+</sup> cells that were V $\alpha$ 14<sup>+</sup>. A representative experiment is shown here.

mind, however, that there are few NK1.1 T cells in the mutant mice, and these express much lower levels of TCR (Fig. 3). This, together with biochemical data, strongly suggests that in TCR  $\beta$ -loop<sup>-</sup> transgenic mice, both the impaired development of NK1.1 T cells and their weak TCR expression is due to the physical constraints on the assembly of the  $\beta$  chain lacking the C $\beta$  FG loop with the V $\alpha$ 14<sup>+</sup> TCR  $\alpha$  chain.

We have previously shown that in conventional T cells expressing V $\beta$ 8.2, deletion of the C $\beta$  FG loop has no effect on V $\alpha$  (7) and J $\alpha$  repertoire usage (our unpublished data). This result suggested that no drastic conformational changes in the TCR  $\beta$  chain were created by the mutation. However, in this study we clearly show that expression of the V $\alpha$ 14<sup>+</sup>  $\alpha$  chain is sensitive to deletion of the C $\beta$  FG loop. Therefore, deletion of the C $\beta$  FG loop must create some subtle change in TCR  $\beta$  chain conformation. It seems that expression of the V $\alpha$ 14<sup>+</sup>  $\alpha$  chain does not allow much structural flexibility of the TCR, as it is particularly sensitive to TCR  $\beta$  chain conformation. Its expression might impose stringent constraints on  $\alpha/\beta$  assembly. This could at least partially explain why the V $\beta$  repertoire of NK1.1 T cells is relatively limited (30). Pairing with the apparently conformation-sensitive V $\alpha$ 14-J $\alpha$ 281 TCR  $\alpha$  chain could be the initial pressure on V $\beta$  usage in NK1.1 T cells (19). Recently, results obtained by using V $\alpha$ 14-transgenic mice suggested that selection was the main force in shaping the NK1.1 T cell repertoire (31). Here we have shown that in addition to selection, differential V $\alpha$ -V $\beta$  pairing can also potentially influence the NK1.1 T cell diversity. In summary, our data show that subtle changes in the TCR  $\beta$  chain conformation (which do not seem to affect conventional V $\beta$ 8.2<sup>+</sup>  $\alpha/\beta$  TCRs) can substantially alter pairing with the V $\alpha$ 14<sup>+</sup>  $\alpha$  chain and impair NK1.1 T cell development.

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