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The Binding of Thymus Leukemia (TL) Antigen Tetramers to Normal Intestinal Intraepithelial Lymphocytes and Thymocytes

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Thymus leukemia (TL) Ags belong to the family of nonclassical MHC class I Ags and can be recognized by both TCRαβ and TCRγδ CTL with TL, but not H-2 restriction. We previously reported that the CTL epitope is TAP independent, but the antigenic molecule(s) presented by TL has yet to be determined. In the present study, TL tetramers were prepared with T3β-TL and murine β2-microglobulin, not including antigenic peptides, and binding specificity was studied. CTL clones against TL Ags were stained with the T3β-TL tetramer, and the binding shown to be CD3 and CD8 dependent. Normal lymphocytes from various origins were also studied. Surprisingly, most CD8+ intraepithelial lymphocytes derived from the small intestines (iIEL), as well as CD8+ and CD4+CD8+ thymocytes, were stained, while only very minor populations of CD8+ cells derived from other peripheral lymphoid tissues, such as spleen and lymph nodes, were positive. The binding of T3β-TL tetramers to CD8+ iIEL and thymocytes was CD8 dependent, but CD3 independent, in contrast to that to TL-restricted CTL. These results altogether showed that TL-restricted CTL can be monitored by CD3-dependent binding of T3β-TL tetramers. In addition, CD3-independent T3β-TL tetramer binding to iIEL and thymocytes may imply that TL expressed on intestinal epithelium and cortical thymocytes has a physiological function interacting with these tetramer+CD8+ T lymphocytes. The Journal of Immunology, 2001, 167: 759–764.

Mice and humans have several MHC class Ib (or nonclassical MHC class I) molecules that are distinguishable from MHC class Ia (or classical MHC class I) by their limited polymorphism and unique expression patterns (1, 2). Mouse thymus leukemia (TL)3 Ags belong to the family of MHC class Ib and are restricted to the intestines in all mouse strains as well as the thymus of TL+ strains (e.g., A-strain and BALB/c mice) (3–6).

In our previous studies, we showed that TL can be recognized by both TCRαβ and TCRγδ CTL (7–9). Because the cytotoxic activity of these CTL is inhibited by TL, but not by anti-H-2 Abs, it was concluded that recognition of TL is direct without any requirement for Ag presentation by H-2 molecules. Recent studies conducted by ourselves and other investigators have demonstrated that TL molecules are transported to and stably expressed on the surfaces of TAP-deficient cells (10–13). We further showed that most TL-restricted CTL recognize the epitope(s), expressed on TL in a TAP-independent manner (10). However, it has not been determined whether TL can present the Ag molecule(s), as in the cases of MHC class Ia and II (14) and some MHC class Ib molecules (15).

Recent progress in molecular biology has allowed production of MHC tetramers as important tools to identify Ag-specific T lymphocytes by flow cytometry (16). The dynamics and magnitude of T cell responses in immunity against tumors and pathogens and in autoimmune diseases have thereby been characterized (17–19). Furthermore, this new tool has been shown to be useful in the search for natural ligands for MHC other than TCR (20, 21).

In the present study, we prepared TL tetramers consisting of T3β-TL and β2-microglobulin (β2m), not including antigenic peptides, and studied the binding specificity to TL-restricted CTL. Normal lymphocytes of various origins were also examined to identify natural ligand(s) for TL and elucidate the physiological function(s) of TL themselves and recognizing T lymphocytes.

Materials and Methods

Mice

The derivation of the transgenic mouse strains used in this study has been described previously (22, 23). Tg.Con.3-1, having a chimeric gene in which the T3β gene from C57BL/6 (B6) is driven by the H-2Kb promoter, expresses T3β-TL ubiquitously. Another strain, Tg.Tlaa-3-1, having a Tlaa-3 transgene (from A-strain) with its own promoter, expresses Tlaa-3-TL predominantly on thymocytes and intestinal epithelial cells. These transgenic mice were generated on a C3H background, without TL expression in the thymus, but with expression of T3β-TL in the intestine. C3H and B6 mice were purchased from Japan SLC (Hamamatsu, Japan), and CD8a knockout mice from The Jackson Laboratory (Bar Harbor, ME). DO11.10-TCR transgenic mice crossed with Rag2−/− mice (DO11.10/Rag2−/−; Refs. 24 and 25) were kindly provided by K. Iwabuchi (Hokkaido University, Sapporo, Japan).

Cells

CTL clones against TL or H-2Kb were established as previously described (7–10). Intraepithelial lymphocytes (IEL) from the small intestines (iIEL)
(26) and lymphocytes from Peyer’s patches (26) and liver (27) were prepared as described.

Antibodies

Rat mAb against TL (HD168; Ref. 28) and mouse mAb to TL2 (TT213; Ref. 7) were described previously. The following mAbs were kindly provided by other investigators, as detailed earlier (7–10); hamster mAb against CD3 (145-2C11; Ref. 29), TCRαβ (H57-597; Ref. 30), and TCRγδ (3A10; Ref. 31); rat mAb against L3T4/L44 (GK1.5; Ref. 32) and Lyt-2/CD8α (53-6.7; PharMingen); hamster mAbs against anti-CD3 (145-2C11; PharMingen), TCRαβ (H57-597;Ref. 30) were used as the controls, and TCRγδ (G336-15; Cedarlane Laboratories); mouse mAb against Lyt-3.2/CD8a (53-19; PharMingen); and CyChrome-labeled mAb against Ly5-2/CD5 (5-6F11; PharMingen). Biotinylated mouse mAb against DO11.10-TCR (KJ1-26; Ref. 33) was kindly provided by K. Iwabuchi.

Construction of T3b-TL tetramers

Tetrameric T3b-TL/βm complexes were constructed as recently described (16, 35). A CDNA encoding a GlySer linker and a biotin-specific peptide (BSP) was fused to the 3’ end of the truncated T3b (encoding from the leader sequence to aa 279) by PCR with the 5’ primer GGAATTCTAG GTAGATGGGGCCAGATG and the 3’ primer GCGCAAGCTTTTATAAC GATGTTACACCATTTTTCTGTCAGAAATGATGCGGAG GATCCGTTGAGAAGCTTTCCCA, using the plasmid pUC19 (10) as the template. The 5’ and 3’ primers were tagged with EcoRI and HindIII sites, respectively. CDNA encoding mouse β2m was prepared by PCR with the 5’ primer GGGATCCATGGCTCGCTCGGTGACC and the 3’ primer CGGGATCCATGGCTCGCTCGGTGACC and the 3’ primer GCGCAAGCTTTTATAAC GATGTTACACCATTTTTCTGTCAGAAATGATGCGGAG GATCCGTTGAGAAGCTTTCCCA, using the plasmid pUC19 (10). The 5’ and 3’ primers were tagged with BamHI and HindIII sites, respectively.

Baculoviruses containing BSP-tagged soluble T3b or β2m CDNA were prepared using Bac-to-Bac baculovirus expression systems (Life Technologies, Gaithersburg, MD), according to the manufacturer’s instructions. Briefly, BSP-tagged soluble T3b and β2m CDNAs were inserted into EcoRI/HindIII and BamHI/HindIII sites, respectively, of the plasmid pFastBac1 and transferred into bacmid in DH10Bac competent cells. S9 cells were transfected with recombinant bacmid using CellFECTIN (Life Technologies) to prepare virus solution, and S9 cells were simultaneously infected with the viruses encoding BSP-tagged soluble T3b and β2m.

Soluble T3b-TL/β2m complexes were roughly purified from culture supernatants of infected S9 cells with DEAE cellulose (DE52; Whatman, Kent, U.K.) and gel filtration (Superdex 200 HR 10/30; Amersham Pharmacia Biotech). Concentrations of T3b/β2m complexes were approximately determined by sandwich ELISA using anti-TL mAbs (TT213 and HD168 for capturing and developing mAb, respectively). Affinity-purified T3b/β2m was used as the standards. In vitro biotinylation with BirA (Avidity, Boulder, CO), tetramerization by addition of PE-labeled streptavidin (Molecular Probes, Eugene, OR), and purification of tetrameric complexes by gel filtration (Superdex 200 HR 10/30) were performed as described (16, 35).

Flow cytometric analysis and sorting

Cells were incubated with T3b-TL tetramers (10 μg/ml) at 37°C for 30 min and then incubated with fluorescein-labeled mAb at 4°C for 30 min. Flow cytometric analysis and sorting were performed on a FACSCalibur (BD Biosciences Immunocytometry Systems) using CellQuest software.

Results

TL-restricted CTL clones are stained with T3b-TL tetramers in a CD3-dependent manner

In a previous study, we showed most TL-restricted CTL clones to recognize TL expressed on insect cells (10). Based on this evidence, we have chosen soluble T3b-TL/β2m already assembled in insect cells as the source of tetramers. To preserve the association of T3b-TL and β2m (and a possibly presented antigenic molecule derived from insect cells), mild purification procedures were used, and the crude preparation of T3b-TL/β2m complexes was biotinylated with BirA biotin ligase. Most proteins biotinylated in these procedures were confirmed to be T3b-TL by SDS-PAGE (data not shown). After purification with gel filtration, the soluble T3b-TL/β2m complex was multimerized with PE-labeled streptavidin, and tetramerized fractions were isolated by gel filtration, as described (16, 35).

To investigate the binding specificity, TL- and H-2Kb-restricted CTL clones were stained with T3b-TL tetramers. As shown in Fig. 1, TL-restricted CTL clones, both TCRαβ and γδ phenotypes, were positive, whereas an H-2Kb-restricted CTL clone (2-8-2) was not. An additional five H-2Kb-restricted CTL clones were tested and found to be unstained (data not shown). Staining intensity roughly correlated with the cytotoxic activity of each CTL clone against TL+ target cells (9). Type I CTL clones that can kill any TL+ target cells were brightly stained, whereas type II CTL clones that can kill TL+ Con A blast cells, but not leukemia cells, were very weakly stained. To assess the binding specificity further, Ab-blocking tests were performed. As shown in Fig. 2, T3b-TL tetramer staining of TL-restricted CTL clone TC9-1 was inhibited by anti-TL, CD3, CD8α, and β2m mAbs. Similar results were also obtained with other CTL clones (data not shown). Because T3b-TL tetramer staining was not clearly inhibited by a mAb against TCR, a mAb against the Vβ chain was used for blocking experiments. T3b-TL tetramer staining of TC9-1 (expressing Vβ9) was weakly, but significantly inhibited by anti-Vβ9 mAb (Fig. 2). These results together suggested that the T3b-TL tetramer used in this study is recognizable by TCR/CD3 complexes expressed on TL-restricted CTL clones.

Subsequently, we enriched TL-restricted CTL using the T3b-TL tetramer. MLC to generate TL-restricted CTL was conducted as described previously (7–9), and thereafter, CD8+ cells were fractionated into two groups based on the T3b-TL tetramer reactivity, and their CTL activity against TL+ cells was analyzed. As shown in Fig. 3, only T3b-TL tetramer+ cells killed TL+ cells, demonstrating that TL-restricted CTL can be enriched with T3b-TL tetramer.

FIGURE 1. Binding specificity of the T3b-TL tetramer to TL-restricted CTL clones. TL (both type I and type II; Ref. 9) and H-2Kb-restricted CTL clones were incubated with the PE-labeled T3b-TL tetramer (10 μg/ml) at 37°C for 30 min and analyzed on a FACSCalibur.
restricted CTL clone. The TL-restricted clone TC9-1 (1 × 10⁵) was incubated with T3b-TL tetramer (10 μg/ml) at 37°C for 30 min in the presence or absence of various mAbs (100 μg/ml) and analyzed on an FACSCalibur.

Most CD8⁺ iIEL and CD8⁺ and CD4⁺CD8⁺ thymocytes are stained with T3b-TL tetramer in a CD3-independent manner

To investigate the tissue distribution of TL-reactive cells, T lymphocytes were prepared from various organs of C3H mice (TL⁻ strain) and stained with T3b-TL tetramer. Unexpectedly, large populations of iIEL as well as thymocytes were stained, whereas very few (<0.1%) CD3⁺ T lymphocytes from the spleen and peripheral lymph nodes were positive (Fig. 4). Intestinal IEL and thymocytes were also prepared from other strains, such as TL⁻ B6 and TL transgenic strains, Tg.Con.3-1 expressing T3b-TL ubiquitously, and Tg.Tla⁺-3-1 expressing Tla⁺-3-TL on thymocytes and intestinal epithelium, and then tested. Both iIEL and thymocytes were also positive (data not shown). When these cells were triple stained with T3b-TL tetramer/CD3/CD45, T3b-TL tetramer⁺ cells were almost exclusively CD3⁺CD45⁻, showing that other hemopoietic cells do not express the molecules interacting with TL (data not shown). Minor populations derived from Peyer’s patches and liver were also stained with the T3b-TL tetramer, but no further analyses were performed on these populations, because the intensity was weak (Fig. 4).

Next, the surface phenotypes of the T3b-TL tetramer-reactive thymocytes and iIEL were analyzed. Both CD4⁺CD8⁻ and CD8⁺ populations from the thymus were stained with the T3b-TL tetramer (Fig. 5). As shown in Fig. 6, T3b-TL tetramer-reactive iIEL almost exclusively expressed CD8α molecules, and the type of TCR (αβ or γδ) and CD8 (αβ or αα) and the expression of Thy-1 did not seem to correlate with T3b-TL tetramer binding. To analyze the binding specificity of the T3b-TL tetramer to these lymphocyte populations, Ab-blocking tests were performed. The results showed that the binding was inhibited by anti-CD8α, TL, and β₂m mAb, but not by anti-CD3 mAb (Fig. 7), in contrast to the binding to TL-restricted CTL, suggesting that T3b-TL tetramer binding to iIEL and thymocytes is CD8 dependent, but TCR/CD3 independent.

To analyze the binding specificity further, various mutant mice were used. First, to investigate whether the T3b-TL tetramer binds to CD8⁺ thymocytes and/or iIEL expressing irrelevant TCR to TL, DO11.10/Rag2⁻/⁻ mice (24, 25) were used. As shown in Fig. 8, thymocytes and iIEL from these mice expressing a monomorphic TCR (detectable with KJ1-26 mAb) were positively stained with

![FIGURE 3](https://example.com/figure3.png)

**FIGURE 3.** Enrichment of TL-restricted CTL using the T3b-TL tetramer. A, Sorting. A bulk CTL culture recognizing TL was prepared by MLC, as described previously (7–9). Cells from MLC were incubated with the PE-labeled T3b-TL tetramer (10 μg/ml) at 37°C for 30 min and then incubated with a FITC-labeled anti-CD8α mAb at 4°C for 30 min. Both tetramer⁺CD8⁺ (Fr.1) and tetramer⁻CD8⁺ (Fr.2) were sorted using a FACSCalibur, and their cytotoxicity was tested. B, Cytotoxicity against TL⁺ cells. Each sorted cell fraction was incubated with ⁵¹Cr-labeled T3b⁺ transfectants of RMA (10) or RMA target cells (2 × 10⁵) for 3 h.

![FIGURE 4](https://example.com/figure4.png)

**FIGURE 4.** Reactivity of the T3b-TL tetramer with T lymphocytes from various tissues. Cells were prepared as described in Materials and Methods, incubated with T3b-TL tetramer (10 μg/ml) at 37°C for 30 min, and finally incubated with CyChrome-labeled anti-CD45/leukocyte common Ag mAb or FITC-labeled anti-CD3 at 4°C for 30 min. CD45⁺ cells (bone marrow) and CD3⁺ cells (others) were gated and analyzed on a FACSCalibur. Cells from superficial cervical, axillary, superficial inguinal, or popliteal lymph nodes showed similar FACS profiles. Thus, results with the superficial inguinal lymph nodes are shown as representative.

![FIGURE 5](https://example.com/figure5.png)

**FIGURE 5.** Surface phenotype of thymocytes reacting with the T3b-TL tetramer. Thymocytes obtained from C3H mouse were incubated with the T3b-TL tetramer (10 μg/ml) at 37°C for 30 min and then incubated with FITC-labeled anti-CD4 and CyChrome-labeled anti-CD8α mAb at 4°C for 30 min. Stained cells were classified into four fractions based on CD4/CD8 expression, and their T3b-TL tetramer reactivity was analyzed on an FACS-Calibur. The percentage of each fraction is as follows: CD4⁺CD8⁺ double-positive (DP), 85.6%; CD4⁺CD8⁻ double-negative (DN), 3.9%; CD4⁺ single-positive (SP), 8.3%; and CD8⁻SP, 2.2%.
the T3^b-TL tetramer, suggesting that the binding is TCR/CD3-independent. Next, we used CD8α^−/− mice to investigate CD8 dependence of T3^b-TL tetramer binding to thymocytes and iIEL, because most of them express CD8α and the binding was blocked almost completely with CD8α mAb. As shown in Fig. 9, thymocytes and iIEL from CD8α^−/− mice were not stained with the T3^b-TL tetramer, suggesting that the CD8 molecule is indispensable for T3^b-TL tetramer binding.

Discussion

As reported for most MHC class I and class II tetramers containing appropriate antigenic peptides, the T3^b-TL tetramer used in this study also binds to TL-restricted CTL in a TCR/CD3-dependent manner. Because precise information about the nature of antigenic molecules presented by TL is not available, a soluble T3^b-TL molecule was prepared from insect cells transfected with the T3^b TL gene, not including the sequence encoding Ag peptides. However, the possibility still remains that T3^b-TL/β^m is associated with an antigenic molecule derived from insect cells. The present study demonstrated that the staining intensity of CTL clones roughly correlated with the cytotoxic activity against TL^+ target cells, and that TL-restricted CTL could be enriched by selecting the tetramer-positive population. These results altogether showed that the T3^b-TL tetramer prepared in this study is useful for monitoring the dynamics and magnitude of T cell responses against TL, similarly to other monitoring methods, such as cytoplasmic IFN-γ staining and CTL assays, as we reported previously (36), although blocking tests with anti-CD3 mAb should be performed to confirm the specificity of the tetramer binding. Studies are now in progress to compare these monitoring methods to determine which one is reliable to predict in vivo antitumor responses against TL-positive tumors.

In addition to the TCR/CD3-dependent binding of the T3^b-TL tetramer to TL-restricted CTL populations, we also showed its

![FIGURE 6](image) Surface phenotype of iIEL reacting with the T3^b-TL tetramer. iIEL obtained from C3H mouse were incubated with T3^b-TL tetramer (10 μg/ml) at 37°C for 30 min, and then incubated with CyChrome-labeled anti-CD8α mAb, and various mAb labeled with FITC at 4°C for 30 min. T3^b-TL tetramer-reactive cells were gated, and their surface phenotype was analyzed on a FACSCalibur.

![FIGURE 7](image) Ab-blocking test for the T3^b-TL tetramer binding to thymocytes or iIEL. Thymocytes or iIEL (1 × 10^6) from C3H mouse were incubated with the T3^b-TL tetramer (10 μg/ml) at 37°C for 30 min in the presence or absence of various mAbs (100 μg/ml) and analyzed on a FACSCalibur. A mixture of mAbs against TCRαβ and TCRγδ was used for blocking and indicated as anti-TCR.

![FIGURE 8](image) T3^b-TL tetramer binding to CD8^+ thymocytes and iIEL from TCR transgenic mice. For tetramer staining, thymocytes and iIEL (1 × 10^6) from DO11.10 TCR transgenic mice crossed with Rag2^−/− mice were incubated with the PE-labeled T3^b-TL tetramer (10 μg/ml) at 37°C for 30 min and then with an FITC-labeled anti-CD8α mAb at 4°C for 30 min. For DO11.10 TCR staining, cells were incubated with biotinylated anti-idiotype mAb (KJ1-26) at 4°C for 30 min and then with PE-labeled streptavidin at 4°C for 30 min. Cells were analyzed on a FACSCalibur, and the results obtained for CD8^+ populations are shown.

![FIGURE 9](image) No binding of T3^b-TL tetramer to thymocytes and iIEL from CD8α^−/− mice. Thymocytes or iIEL from CD8α^−/− mice were incubated with the PE-labeled T3^b-TL tetramer (10 μg/ml) at 37°C for 30 min and then incubated with an FITC-labeled anti-CD3 mAb and a CyChrome-labeled anti-CD8α mAb at 4°C for 30 min. CD3^+ cells were gated and analyzed.
CD8-dependent, but TCR/CD3-independent binding to thymocytes and iIEL. Because the binding to iIEL and thymocytes was almost completely inhibited by an anti-CD8α mAb, the CD8 molecule on T lymphocytes is indispensable for the binding. Thus, the important question needing to be answered is whether 1) the CD8 molecule alone is sufficient for the binding, or 2) CD8 plus another molecule expressed on TL tetramer-positive iIEL and thymocytes are necessary.

Up to the present, three groups of molecules other than the TCR/CD3 complex are reported to interact with MHC tetramers: 1) CD8 (37), 2) NK receptors (20), and 3) molecules belonging to the Ig supergene family (21). The recent report (37) that some human MHC tetramers bind to CD8+ T lymphocytes expressing irrelevant TCR in a dose-dependent manner suggests a possibility that the TL tetramer binds to CD8 without additional molecules. This possibility is supported by the finding that the binding affinity between murine MHC class I and murine CD8 is somehow stronger than that observed in human counterparts (reviewed in Ref. 38). TL molecule was also found to bind to human CD8-transfected Chinese hamster ovary cells (39). However, in the present study, the TL tetramer did not interact with lymphocytes from spleen and peripheral lymph nodes, even though they express almost the same amounts of CD8 on their cell surfaces. In addition, there is no difference in the intensity of T3β-TL tetramer staining between CD8αα and CD8αβ populations of iIEL, indicating that the composition of CD8 (αα vs αβ) does not affect the tetramer binding. Therefore, CD8 molecules expressed on iIEL and thymocytes must be distinct from those on peripheral T lymphocytes in their conformation and/or posttranscriptional modifications, if TL interacts with CD8 without additional molecule(s). Recently, Daniels and Jameson (40) reported that CD8 plays a critical role in interactions between TCR and MHC multimer, and that anti-CD8α mAbs can exert either blocking or augmenting influence, depending on the epitopes detected. Our present study showed that an anti-CD8α mAb (clone 35-17-2) blocked the binding of T3β-TL tetramer to iIEL, thymocytes, or CTL, but our preliminary results showed that binding to CTL was not affected or even slightly augmented by another mAb (clone 53-6.7), which was referred as an augmenting Ab by them. This may point to conformational differences in CD8 molecules expressed on TL tetramer-positive and -negative lymphocyte populations. However, at this moment there is no clear experimental evidence in support of this conclusion.

Another possibility is that CD8 plus another molecule expressed on TL tetramer-positive iIEL and thymocytes are necessary for the binding. A first candidate for our unknown molecule might be an NK receptor (20). However, our preliminary study showed that NK1.1+ cells from B6 mice were not stained with the TL tetramer, suggesting that typical NK receptors are probably not, although the coexpression with CD8 may facilitate binding to the TL tetramer. Another candidate group is that of Ig supergene family molecules, such as Ig-like transcript-2 and -4, which are the ligands for HLA-G (21). However, these molecules are reported to be expressed on blood monocytes, but not on lymphocytes in humans, suggesting that these mouse homologues are probably not the ligands for TL. Recently, Wang et al. (41) reported a novel, but enigmatic Qa-1-binding molecule on CD8+ cells, which seemed to be different from the possible TL ligand, because it is expressed by virtually all CD8+ cells derived from various lymphoid tissues, and its Qa-1 binding is not inhibited by an anti-CD8 mAb. To define TL-binding molecule(s), biochemical analyses have been attempted in our laboratory, but to date without success. This may be due to the low binding affinity between TL and its ligand itself, which can easily be speculated from the present findings that either the absence of CD8 on iIEL and thymocytes from the gene knock-out or anti-CD8 mAb blocking completely abrogates T3β-TL tetramer binding. We also plan to produce a mAb to inhibit TL tetramer binding to iIEL and/or thymocytes, and thereby detect molecules that are indispensable for the binding together with CD8.

TL is known to be expressed on intestinal epithelium of all mouse strains and cortical thymocytes of certain strains, but little is known about its function as an MHC class Ib molecule. Based on the present finding that the TL tetramer binds to iIEL and CD4+ CD8+ thymocytes, it is conceivable that these tetramer-positive lymphocyte populations interact in situ with TL expressed on intestinal epithelial cells and cortical thymocytes and play an important role in host defense. With the soluble TL molecule prepared in this study, it may be possible to define the Ag molecule presented by TL and elucidate the physiological function of TL in vivo.

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