TL Antigen as a Transplantation Antigen Recognized by TL-restricted Cytotoxic T Cells

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Summary

In contrast to broadly expressed classical class I antigens of the major histocompatibility complex, structurally closely related TL antigens are expressed in a highly restricted fashion. Unlike classical class I antigens, TL antigens are not known to be targets of cytotoxic T cells or to mediate graft rejection. Whereas classical class I antigens function as antigen-presenting molecules to T cell receptors (TCR), the role of TL is yet to be defined. To elucidate the function of TL, we have derived transgenic mice expressing TL in most tissues including skin by introducing a [TL gene, T3b of C57BL/6 mouse origin, driven by the H-2Kb promoter. By grafting the skin of transgenic mice, we demonstrate that TL can serve as a transplantation antigen and mediate a TCR-α/β + CD8 + cytotoxic T cell response. This T cell recognition of TL does not require antigen presentation by H-2 molecules. Furthermore, we show that C57BL/6 F1 mice develop CD8 + T cells that are cytotoxic for C57BL/6 TL + leukemia cells, providing further support for the concept that aberrantly expressed nonmutated proteins such as TL can be recognized as tumor antigens.

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mans and mice have over a dozen class I genes belonging to the MHC, the majority of which are "nonclassical" class I or class "Ib" genes (1-3). In contrast to well studied "classical" class I or class "Ia" genes, the function of nonclassical class I genes remains largely unexamined. Certain nonclassical class I genes have expression patterns similar to those of classical class I genes and have been shown to mediate graft rejection and CTL response, and even to present peptides to the TCR (4-7). It has been reported recently that Hmt, a member of nonclassical class I antigens, is specialized to present N-formyl peptides to TCR (8, 9) and that Qa-2, another nonclassical class I antigen, binds to a limited set of peptides (10). Some nonclassical MHC products have been speculated to present antigens to TCR-γ/δ (11-13). However, not all nonclassical class I genes are antigen-presenting molecules for TCR, e.g., a class I gene expressed in rat intestine functions as an Fc receptor of IgG (14).

TL represents one of the earliest and best defined cell surface antigenic systems coded for by nonclassical class I genes in the MHC locus (15, 16). Principal characteristics of TL include expression restricted to thymocytes during T cell development in TL + mice (e.g., A-strain and BALB/c), no expression in thymocytes of certain strains (TL - mice, e.g., C57BL/6 [B6]1 and C3H/He [C3H]), anomalous TL expression in T cell lymphomas arising in TL - mice, and a phenomenon termed antigenic modulation in which TL expression is downregulated in the presence of TL antibody (15-17). Until recently, TL expression was thought to be restricted to normal and leukemic cells of T cell derivation, but two reports (18, 19) have now shown that TL is expressed in the intestinal epithelium, raising the possibility that TL may be a presenting element for γ/δ T cells in the gut. In addition, our recent studies with TL transgenic mice showing arrest of T cell maturation suggest that TL plays a critical role in T cell development in the thymus (20, 21).

TL antigens are glycoproteins with a molecular mass of approximately 45,000 that are associated with β2-microglobulin (22, 23). Molecular analysis revealed that TL genes have an exon/intron structure very similar to that of classical class

1 Abbreviations used in this paper: B6, C57BL/6; cAb, conventional antibody; C3H, C3H/He.
I genes and share >70% identity in the nucleotide sequence of the coding region and in the putative amino acid sequence (16, 24–27). Despite these structural similarities, there has been no evidence that TL antigens are histocompatibility antigens or that they can induce a CTL response (15, 28), and this has led to the speculation that TL antigens have a function distinct from that of classical I antigens. To explore the role of nonclassical class I antigens with limited tissue distribution, such as TL, we have taken advantage of the newly derived transgenic TL strains to ask whether TL antigens can serve as transplantation rejection antigens and elicit a TL-restricted CTL response.

Materials and Methods

Mice. The derivation of transgenic mouse strains with the H-2K\(^b\)/T3\(^b\) chimeric gene has been described previously (20). Briefly, the chimeric gene in which T3\(^b\) gene is driven by the H-2K\(^b\) promoter was constructed as shown (see Fig. 1). By injecting DNA containing the chimeric gene into the pronuclei of fertilized eggs of C3H mice (TL\(^-\)/Tla', H-2\(^b\)), two transgenic founder mice were produced, Tg.Con.3-1 and Tg.Con.3-2, containing 23 and 19 copies of the transgene per haploid, respectively. The offspring of the founders were interbred to produce homozygous stocks, which were then maintained by brother–sister mating. The other transgenic mouse strains, Tg.H-2K\(^b\)-1 carrying H-2K\(^b\), and Tg.Tla-3-1 and Tg.Tla-3-2 carrying Tla-3, one of the three TL genes in A-strain mice, have been derived also on a C3H background and described elsewhere (20, 21). B6, C3H, and (B6 x C3H)F\(_1\) mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). Other mice were from our breeding colony at Aichi Cancer Center Research Institute.

Cells. The following TL\(^+\) and TL\(^-\) T cell lymphomas have been maintained either as in vivo passaged or in vitro cultured cell lines: ERLD (TL\(^-\)), B6RL \(\geq 6\) (TL\(^+\)), B6RV2 (TL\(^-\)), and B6RL \(< 6\) IL (TL\(^-\)) of B6; ASL1 (TL\(^+\)) and RADA1 (TL\(^+\)) of A-strain; and BALBRVE (TL\(^+\)) of BALB/c. These tumor lines are described in previous publications from our laboratories at Memorial Sloan-Kettering Cancer Center, and at Okayama University School of Medicine (15, 29).

Antibodies. The following mAbs or conventional antibodies (cAbs) were kindly provided by various scientists or developed in our laboratories. To characterize effector cells, rat mAb to Lyt-2 (57-3, obtained from Dr. N. Shinohara, Mitsubishi Kasei Institute for Life Science, Machida, Japan, [30]), rat mAb to L3T4 (GK1.5, obtained from Dr. N. Shinohara, Mitsubishi Kasei Institute for Life Science, Machida, Japan [31]), hamster mAb to TCR-\(\gamma\)-\(\delta\) (H57-597, a gift from Dr. R. T. Kubo, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO [32]), hamster mAb to TCR-\(\gamma\)-\(\delta\) (3A10, a gift from D. S. Tonegawa, Massachusetts Institute of Technology, Cambridge, MA [33]) and hamster mAb to CD3 (145-2C11, a gift from Dr. J. A. Bluestone, The University of Chicago, Chicago, IL [34]) were used. To define the target recognition, rat mAb to TL (HD 168, 24), mouse mAb to TL.2 (TT213, in this paper), cAb to TL.1.2.3 (B6 X A-Tla\(^b\) anti-ASL1; 15), cAb to TL.4 ([A X B6-Tla\(^b\)]F\(_1\) anti-ERLD; 15), rat mAb to H-2 (HD464, in this paper), cAb to H-2\(^b\) ([B6 X DBA/2 anti-CH3 mammary tumor, MM48], cAb to H-2\(^b\) (B6 anti-BALB/c), and mouse mAb to Qa (TT59, 35) were used.

RNA Blot Analysis. Total RNA prepared from tissues was fractionated in 2.2 M formaldehyde agarose gel and transferred to nitrocellulose filters. Blots were hybridized with \(\alpha\)-[\(\beta\]P]dCTP-labeled TL-specific probe, pTL1 (24). The same membrane was rehybridized with \(\beta\)-actin cDNA (36) to standardize the amount of transcripts. The intensity of bands was measured by densitometric analysis with a Fujix Bio-imaging Analysis System (Fuji Photo Film Co., Tokyo, Japan).

Immunohistochemistry. 5-\(\mu\)m frozen sections of the skin were air dried, fixed with cold acetone, and stained with rat mAb by the avidin–biotinylated horseradish peroxidase complex (ABC) method (Vectastain; Vector Laboratories Inc., Burlingame, CA).

Skin Grafts. Female C3H, (B6 x C3H)F\(_1\) and transgenic mice (6–10 wk-old) were used as recipients or donors. Full-thickness sections of skin (1-cm disk) were obtained from the abdomens of donors and were grafted to the back of recipients. Plaster casts were removed on day 10 and grafts were observed daily for at least 100 d.

CTL Assay. 4–8 wk after the rejection of grafted skin, spleen cells (3 x 10\(^7\)) from the mice were restimulated in vitro by culturing with 2 x 10\(^7\) Tg.Con.3-1 spleen cells pretreated with 50 \(\mu\)g/ml mitomycin C (Wako Pure Chemical Industries, Osaka, Japan). After 6 d in culture, effector cells were harvested to test for cell-mediated cytotoxicity. Target cells were blast cells or tumor cells. To generate blast cells, spleen cells were cultured for 3 d in the presence of Con A (Boehringer Mannheim GmbH, Mannheim, Germany) at a concentration of 5 \(\mu\)g/ml. Labeled target cells were prepared by incubating 10\(^7\) blast or tumor cells with 3.7 MBq of Na\(^{51}\)CrO\(_4\) (New England Nuclear, Boston, MA) in 0.2 ml of medium for 45 min, followed by washing three times with RPMI-1640. Target cells (2 x 10\(^6\)) were incubated with various numbers of effector cells. After incubation for 3 h, the supernatants were harvested using Supernatant Collection System (Skatron Inc., Lier, Norway) for measurement of \(^{51}\)Cr release. The percent specific lysis was calculated by the following equation: 100 \(\times\) (a-b)/c, where a is the radioactivity in the supernatant of target cells mixed with effector cells, b is the radioactivity in the supernatant of target cells incubated without effector cells, and c is the radioactivity in the supernatant after complete lysis of target cells with 2\% Triton X-100.

Blocking Test of CTL Activity by Antibody. Serially diluted antibodies were added to the mixture of effector cells and labeled target cells in CTL assays to characterize the effector cells and target molecules.

Results

TL Expression in the Skin of Transgenic Mice. Two transgenic mouse strains, Tg.Con.3-1 and Tg.Con.3-2 expressing TL antigen with the tissue distribution of H-2K\(^b\) antigen, have been derived by introducing a chimeric gene of T3\(^b\) with the H-2K\(^b\) promoter into C3H (TL\(^-\)) mice (Fig. 1; reference 20). The transcripts of the chimeric gene are present in almost all tissues. Serological analysis of lymph node lymphocytes showed that the chimeric gene expresses all expected TL antigenic determinants (TL.1.2.4) but no H-2K\(^b\) determinant. The skins of transgenic mice have the transcripts and TL antigenic determinants (TL.1.2.4) but no H-2K\(^b\) determinant. The skins of transgenic mice have the transcripts and TL antigenic determinants (TL.1.2.4) but no H-2K\(^b\) determinant. The skins of transgenic mice have the transcripts and TL antigenic determinants (TL.1.2.4) but no H-2K\(^b\) determinant.
Figure 1. Construction of the H-2Kb/T3b chimeric gene. The T3b gene is isolated from ERLD, a TL- B6 mouse. The H-2Kb/T3b chimeric gene (Construct 3) constitutes the 5' flanking region and exon 1 of H-2Kb (contained in the 4.7-kb EcoRI-Smal fragment), and exon 2-6 and the 3' flanking region of T3b (contained in the 5.3-kb FspI-KpnI fragment). Exons represented by numbered open (T3b) or filled (H-2Kb) boxes encode leader (L), extracellular (α1, α2, and α3), transmembrane (T), and cytoplasmic (C) domains of the proteins or the 3' untranslated region. Restriction enzyme sites: (E) EcoRI; (F) FspI; (G) BglII; (K) KpnI; (N) NarI; (Sa) SalI; (Sm) SmaI.

Figure 2. Transcripts of the H-2Kb/T3b chimeric gene in the skin of transgenic mice. Total RNA from the skin of Tg.Con.3-1 (4 µg), Tg.Con.3-2 (4 µg), C3H (40 µg), B6 (40 µg), and Tg.Tlaa-3-1 (40 µg) were analyzed with pTL1. Normal thymus of B6-Tla% a conventional TL+ mouse strain (40 µg) and ERLD (40 µg) were included for comparison. The same blot was hybridized with a β-actin probe to quantify the transcripts.

Figure 3. TL antigen in the skin of transgenic mice detected by immunohistology with the ABC method. The panels are Tg.Con.3-1 skin stained with rat mAb to TL (HD168); C3H skin with HD168, and C3H skin with rat mAb to H-2 (HD464). The expression pattern of TL in the skin of transgenic mice is identical to that of H-2 in C3H mice.

TL Antigen as a Transplantation Antigen. To test whether TL expressed in the skin can serve as a transplantation rejection antigen, skin graft experiments were carried out in various combinations of transgenic and inbred mice (Table 1). Skin grafts from Tg.Con.3-1 and Tg.Con.3-2 were rejected efficiently by recipient C3H mice. Skin grafts of the higher expressor, Tg.Con.3-1, were rejected faster than Tg.Con.3-2 grafts. Both transgenic strains accepted C3H skin, indicating no genetic drift between C3H and transgenic mice. Reciprocal grafts between the two transgenic strains were also compatible, thus removing the possibility that genes at chromosomal integration sites of the transgene are expressed in such a way
Table 1. Transplantation of Tg.Con.3-1 and Tg.Con.3-2 Skins Expressing TL Antigen

| Donor            | Recipient             | Total | Rejected | Crisis | Rejection time | Number of grafts |
|------------------|-----------------------|-------|----------|--------|----------------|------------------|
| Tg.Con.3-1       | C3H                   | 52    | 51*      | 1*     | 15.4 ± 8.3†    |                  |
|                  | Tg.Con.3-1            | 9     | 0        | 0      |                |                  |
|                  | Tg.Con.3-2            | 10    | 0        | 0      |                |                  |
|                  | Tg.H-2Kb-1            | 10    | 6        | 2      | 27.3 ± 24.9    |                  |
|                  | Tg.Tlaa-3-1          | 5     | 4        | 0      | 18.0 ± 3.2     |                  |
|                  | Tg.Tlaa-3-2          | 12    | 12       | 0      | 13.1 ± 1.4     |                  |
|                  | (B6 × C3H)F1         | 29    | 22       | 1      | 18.7 ± 4.8     |                  |
| Tg.Con.3-2       | C3H                   | 10    | 10       | 0      | 23.6 ± 18.6    |                  |
|                  | Tg.Con.3-1            | 5     | 0        | 0      |                |                  |
|                  | Tg.H-2Kb-1            | 9     | 3        | 6      | 17.7 ± 4.5     |                  |
|                  | (B6 × C3H)F1         | 9     | 0        | 4      |                |                  |
| Co-graft†: Tg.Con.3-1 | plus (B6 × C3H)F1 | 10    | 10       | 0      | 16.3 ± 2.8     |                  |
|                  | Tg.Con.3-2            |       |          |        |                |                  |
| C3H              | C3H                   | 6     | 0        | 0      |                |                  |
|                  | Tg.Con.3-1            | 10    | 0        | 0      |                |                  |
|                  | Tg.Con.3-2            | 10    | 0        | 0      |                |                  |
|                  | Tg.Tlaa-3-1          | 5     | 0        | 0      |                |                  |
|                  | (B6 × C3H)F1         | 5     | 0        | 0      |                |                  |
| Tg.H-2Kb-1       | C3H                   | 10    | 10       | 0      | 12.9 ± 0.9     |                  |
|                  | Tg.Con.3-1            | 5     | 5        | 0      | 13.0 ± 1.7     |                  |
|                  | Tg.Tlaa-3-1          | 5     | 5        | 0      | 14.6 ± 5.3     |                  |
|                  | (B6 × C3H)F1         | 5     | 0        | 0      |                |                  |
| Tg.Tlaa-3-1      | C3H                   | 10    | 0        | 0      |                |                  |
|                  | Tg.Con.3-1            | 5     | 0        | 0      |                |                  |
|                  | Tg.Tlaa-3-1          | 5     | 0        | 0      |                |                  |
|                  | (B6 × C3H)F1         | 10    | 0        | 0      |                |                  |

* Rejection was defined as a loss of >95% of the grafted tissue.
† Graft showed transitory signs of rejection and became smaller.
‡ Mean ± SD.
†† (B6 × C3H)F1 mice were engrafted simultaneously with both Tg.Con.3-1 and Tg.Con.3-2 skins on the right and left side of their back. The F1 mice rejected not only Tg.Con.3-1 but also Tg.Con.3-2 grafts.

that their products become transplantation antigens. Tg.H-2Kb-1 mice, C3H having H-2Kb as a transgene, also rejected Tg.Con.3-1 and Tg.Con.3-2 skin grafts, indicating that the leader sequence of the H-2Kb gene constituting a part of the chimeric gene cannot be the major antigen for rejection. C3H mice did not reject skin grafts from Tg.Tlaa-3-1, a transgenic mouse strain carrying the Tlaa-3 gene from A-strain, which like an A-strain mouse expresses high levels of TL antigen in the thymus, but not in the skin. From these results, we concluded that TL expressed in the skin functions as a transplantation rejection antigen similar to classical class I antigens. Furthermore, Tg.Con.3-1 grafts were rejected by Tg.Tlaa-3-1 and the related Tg.Tlaa-3-2 strains. Tlaa-3 encodes for a protein that, in comparison with the T3b product, has different amino acid residues at 33 sites (16). These "allogenic" differences in TL antigens apparently contribute to the rejection of Tg.Con.3-1 skin grafts by Tg.Tlaa-3-1 and Tg.Tlaa-3-2, again similar to classical class I antigens.

Generation of CTL against TL Antigen. We next analyzed the T cell response in C3H mice that had rejected Tg.Con.3-1
skin grafts. CTL from these mice lysed TL + blast target cells generated by Con A stimulation, but failed to lyse TL - blast cells (Fig. 4). The CTL response was characterized by blocking tests with various antibodies (Fig. 5), and also by flow cytometric analysis (data not shown). The results demonstrated that the CTL are CD4−/CD8+/CD3+/TCRαβ+/TCRγδ−, having the conventional CTL phenotype. The target molecule for TL-specific CTL was determined by blocking tests with various TL and H-2 antibodies. As shown in Fig. 6, none of the H-2 antibodies blocked cytotoxicity, whereas TL antibodies having serological specificities for different TL determinants blocked cytotoxicity. Thus, the recognition of TL by CTL does not require antigen presentation by H-2 molecules. This lack of H-2 involvement is further supported by the finding that TL-reactive CTL generated in C3H mice lyse TL + lymphomas of various H-2 haplotypes but not TL - lymphomas (Fig. 7).

Recognition of T3b-TL Antigen by B6F1 Mice. A single gene, T3b, encodes TL in B6 mice (24). The anomalous expression of TL in B6 lymphomas results from the transcriptional activation of T3b, which is silent in normal B6 T cells (15, 24). T3b isolated from a TL + B6 lymphoma, ERLD, contains no mutations in its coding region and is identical to T3b derived from normal B6 mice. TL antigens can elicit a high level of humoral immunity in TL - mice (38), and for this reason, TL antigens anomalously expressed in T cell lymphomas of TL - mice have long been regarded as tumor-specific antigens (15). However, this idea has been called into question with recent reports demonstrating TL expression in the intestinal epithelium of TL - strains, such as B6 mice, as well as in TL + strains (18, 19). We have confirmed this observation and have shown that TL transcripts and TL products are present in the intestine of B6, C3H, AKR, BALB/c, (B6 x C3H)F1, and also TL transgenic Tg.Tla-3-1 and Tg.Tla-3-2 mice (Obata, Y., T. Takahashi, E. Stockert, and L.J. Old, unpublished observations). To test the significance of these findings in relation to TL as a tumor antigen, the ability of TL - mice to mount a CTL response to the T3b-TL product was examined by grafting the skin of transgenic mice on (B6 x C3H)F1 mice (Table 1). Tg.Con.3-1 grafts were rejected by F1 recipients, whereas Tg.Con.3-2 grafts were not, although a crisis was observed in half of the transplants. To resolve this difference, (B6 x C3H)F1 mice were simultaneously engrafted with both Tg.Con.3-1 and Tg.Con.3-2 skins. In this experiment, not only Tg.Con.3-1 but also Tg.Con.3-2 grafts were rejected. The results clearly show that TL - mice can recognize the T3b-TL antigen as a transplantation antigen. The results also indicate that TL expression
above a certain level is required to mediate graft rejection. Mice grafted with Tg.Con.3-1 or Tg.Con.3-2 skin produced TL antibody (data not shown), and antigenic modulation, e.g., the reversible downregulation of TL antigen induced by TL antibody (17), may be the reason for the failure to reject grafts expressing lower levels of TL. CTL induced in (B6 x C3H)F1 mice lysed not only TL+ Con A blast cells but also TL+ B6 lymphomas, including ERLD (Fig. 8). No obvious histological abnormalities were observed in the intestine of immunized (B6 x C3H)F1 mice having TL-specific CTL. Thus, despite TL expression in intestinal epithelial cells, TL can be recognized as a transplantation rejection antigen in TL- mice, supporting the original notion that anomalously expressed TL behaves like a tumor-specific antigen in TL- mice. It was further established that once CTL are induced by immunization with a critical level of TL, even cells expressing smaller amounts of TL become targets for rejection.

Discussion

In this study, we show that TL can mediate a transplantation rejection response and elicit CTL that are TL specific and independent of antigen presentation by classical class I molecules. With regard to classical class I antigens, the mechanisms for allo-recognition have been extensively studied and two distinct models have been proposed: (a) CTL recognizes allele-specific polymorphic residues on the MHC molecules (39-41); and (b) CTL recognizes endogenous peptides presented by allogeneic MHC molecules (42-45). Similar models can be proposed for TL recognition: CTL may recognize TL antigen per se or TL + X, where X are peptides presented by TL molecules. Structural comparisons reveal that TL molecules can form peptide-binding clefts similar to classical class I antigens (46, 47), and that these peptide-binding regions of TL, in contrast to classical class I antigens, have a very limited polymorphism, suggesting that TL binds only a limited repertoire of peptides. In this respect, Qa-2 has been reported to bind to a relatively small number of peptides as compared with H-2 (10). It will be important to isolate and identify the peptides eluted from TL molecules expressed on normal and leukemic TL+ cells in order to elucidate the function of TL as an antigen-presenting molecule and to determine the target specificity of TL-specific CTL.

Since the initial description of TL 30 years ago (38), numerous attempts have been made to induce CTL against TL, but without success. Our ability to generate TL-specific CTL in this study is most likely due to immunization with skin grafts from transgenic mice expressing abnormally high levels of TL. In support of this idea, we have found that immunization with spleen cells from TL-transgenic mice was far less efficient in CTL induction than immunization with skin grafts. Even after hyperimmunization with spleen cells from Tg.Con.3-1, (B6 x C3H)F1 mice generated CTL with only low CTL activity (~10% specific lysis of Tg.Con.3-1 blast cells). Because the skin consists of several different cell types with antigen-presenting characteristics, i.e., keratinocytes, Langerhans cells, and others, we are now attempting to identify which cells are responsible for TL-specific CTL induction and what costimulatory molecules may be involved.

The fact that TL can elicit humoral and cellular recognition in TL- mice such as B6 and B6F1, despite TL expression in intestinal epithelium, indicates that intestinal TL expression does not induce a state of systemic tolerance to TL. This is in contrast to TL+ mice, where TL expression in the thymus induces a tolerant state that precludes formation of TL-specific antibodies or CTL. No obvious histological abnormalities have been observed in the intestine of immunized (B6 x C3H)F1 mice with TL-specific CTL, suggesting (among other possibilities) that intestinal TL is inaccessible to cellular immune attack or that intestinal TL expression is downregulated via TL antibody-mediated antigenic modulation. In a typical graft-vs.-host reaction involving classical class I MHC mismatches, mice generally suffer from diarrhea and the small intestine shows pathological findings such as crypt hyperplasia (48, 49), reduction in the ratio of villus length to crypt length (48, 50), and lymphocytic infiltration of the epithelium (49).

Antigenic modulation has long been considered the reason that TL- mice with high levels of TL antibody are not resistant to challenge with syngeneic TL+ leukemias (17). However, we have recently observed that (B6 x C3H)F1 mice immunized with Tg.Con.3-1 skin showed heightened resistance to a challenge with a TL+ leukemia, but not a TL- leukemia. The poor capacity of lymphoid cells (in contrast to skin cells) to generate TL-specific CTL may account for the failure of past efforts to induce tumor resistance in TL-immunized mice. Understanding why skin-presented antigens are so effective in eliciting CTL to self-antigens like TL may provide new approaches to generating antitumor immunity.
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