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*Advances in Brief*

# Recognition of Tyrosinase by Tumor-infiltrating Lymphocytes from a Patient Responding to Immunotherapy

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## Abstract

**The observation that allogeneic melanoma cells matched for particular HLA class I alleles stimulate T-cells isolated from patients suggests that widely shared antigens exist on these tumors. A transient expression system was developed for screening a melanoma complementary DNA library using the highly transfectable human kidney cell line 293. Using this system, large numbers of complementary DNA clones can be rapidly screened for the expression of antigens which stimulate T-cells. Tumor-infiltrating lymphocytes from patient 888, which recognized melanoma in the context of HLA-A24, were used to screen a complementary DNA library made from the autologous melanoma. Our results demonstrate that these tumor-infiltrating lymphocytes recognize tyrosinase, a gene previously shown to be recognized by T-cells only in the context of HLA-A2. These data demonstrate that a single antigen can be recognized in the context of two different class I HLA alleles. In addition, this study suggests that recognition of tyrosinase by antigen-specific T-cells may be involved in tumor rejection.**

## Introduction

Evidence obtained from studies in experimental sytems using mouse tumors suggests that specific T-cells play an important role in tumor rejection (1–3). T-cells isolated from tumors as well as the peripheral blood of patients can recognize melanomas in a major histocompatibility complex-restricted fashion, indicating that these cells recognize specific tumor antigens (4, 5). The adoptive transfer of murine TIL[2] can mediate the regression of established metastases in a variety of murine tumor models (6, 7). TIL can also mediate cancer regression when adoptively transferred to the autologous patient (8).

T-cells isolated from cancer patients have been used to clone genes encoding antigens present on melanoma. One gene, termed *MAGE-1*, was cloned using PBL from a patient who had been immunized with autologous tumor. The protein encoded by this gene was shown to be recognized in association with HLA-A1 (9). *MAGE-1* lacked similarity to sequences in current databases and was found to be a member of a multigene family. The product of another member of the *MAGE-1* gene family termed *MAGE-3*, has also been shown to be recognized by HLA-A1-restricted T-cells (10). Tyrosinase, a previously described gene involved in melanin synthesis, was cloned using T-cells clones derived by repeated *in vitro* tumor stimulation of PBL from melanoma patients (11). Another previously described gene expressed in melanoma and melanocytes, gp100, has been found to be recognized by HLA-A2-restricted TIL (12, 13). Another gene, termed *MART-1*, has recently been cloned and was recognized by the majority of HLA-A2 TIL (14). This gene, which does not appear to be similar to any known genes, is also expressed in normal cultured melanocytes.

In this study, we used a patient's TIL population to isolate cDNA clones encoding tyrosinase from a library derived from the autologous melanoma. This gene product was recognized in association with HLA-A24 and may represent a tumor rejection antigen, since the patient showed a dramatic response to therapy when treated with the autologous TIL that were used to identify the tyrosinase gene.

## Materials and Methods

**Cell Lines.** The 888 TIL cell line was generated by culturing cells obtained from tumor suspensions with 6000 international units/ml of IL-2 (Cetus-Oncology Division, Chiron Corp., Emeryville, CA) for 30–70 days as described previously (8). The 888 TIL were incubated with flasks coated with anti-CD8 antibody (Applied Immune Sciences, Inc., Menlo Park, CA), and positively selected cells were isolated for use in screening assays. TIL were grown in AIM-V medium (Life Technologies, Inc, Gaithersburg, MD) containing 5% human AB serum and 6000 international units/ml IL-2.

The melanoma cell lines were established in this laboratory and were grown in RPMI containing 5% fetal bovine serum. The adenovirus-transformed human kidney cell line 293 was kindly provided by Dr. Joel Jesse (Life Technologies, Inc.). A stable transfectant of the 293 cell line expressing HLA-A24 was made by transfecting the A24 gene (isolated as described below) which had been cloned into the eukaryotic expression vector pCDNA3 under the control of the cytomegalovirus intermediate early promoter (Invitrogen, San Diego, CA) into 293 cells. Cells were transfected using Lipofectamine (Life Technologies, Inc.), and stable transfectants were selected with 0.5 mg/ml G418 (Life Technologies, Inc.). Transfectants of 293 cells expressing high levels of HLA-A24 were isolated after staining with the anti-HLA-A24 monoclonal antibody 138-HA-1 (immunoglobulin M; One Lambda, Canoga Park, CA), followed by incubation with a polyclonal anti-immunoglobulin M conjugated with fluorescein isothiocyanate (Vector Laboratories, Inc., Burlingame, CA). Cells expressing HLA-A24 were isolated using a FACStar plus (Becton Dickinson, Inc., Mountain View, CA). Melanoma cells were transfected with the same construct using Lipofectamine.

**Isolation of the HLA-A24 Gene.** The HLA-A24 gene was isolated by reverse-transcribing polyadenylated mRNA from 888 melanoma cells with an oligodeoxythymidylate primer. A polymerase chain reaction was then carried out using HLA-5P2 and HLA-3P2 (15), two primers which flank the HLA coding region. A band of the appropriate size (1.1 kilobases) was isolated and cloned into the pT7Blue TA cloning vector (Novagen, Madison, WI). After digestion with a number of restriction enzymes, clones which demonstrated bands corresponding to the correct sizes for the A24 gene were partially sequenced using the dideoxynucleotide chain termination method with T7 DNA polymerase (Sequenase 2.0; United States Biomedical Corp., Cleveland, OH). This gene was identified as HLA-A2402.

**cDNA Library Construction and Screening.** A cDNA library was prepared from 2 μg of polyadenylated mRNA isolated from 888 melanoma using the PolyA Quik kit (Invitrogen). The RNA was reverse transcribed using an oligodeoxythymidylate primer containing a *Not*I restriction site, and cDNA was produced using the Riboclone cDNA Synthesis System (Promega, Madison, WI). The cDNA was then passed through an S400 column (Pharmacia, Piscataway, NJ) to remove small cDNAs, and *Bst*XI adaptors (Invitrogen) were ligated to the cDNA. The cDNA was then digested with

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[2] The abbreviations used are: TIL, tumor-infiltrating lymphocytes; PBL, peripheral blood lymphocytes; cDNA, complementary DNA; IL-2, interleukin 2; GM-CSF, granulocyte-macrophage colony-stimulating factor.

*Not*I, and the resultant product was run on a 1% agarose gel. The lane was excised, and DNA greater than 500 base pairs was isolated using the Prep-a-gene kit (Bio-Rad, Hercules, CA). The cDNA was then ligated to pCDNA3 (Invitrogen) which had been digested with *Bst*XI and *Not*I.

The ligated cDNA was transformed into DH5α cells (Life Technologies, Inc.), and cells were plated on ampicillin plates. Between 50 and 100 colonies were pooled together and grown for 3 to 5 h in Super Broth (Biofluids, Gaithersburg, MD). Plasmid DNA was then isolated using QIAprep-8 Plasmid kit (Qiagen, Chatsworth, CA). Transfection of 293-A24 cells was carried out by plating $10^5$ cells in a flat-bottomed 96-well dish in Dulbecco's modified Eagle's medium (Biofluids) without serum. DNA (200 ng) from each of the pools was then mixed with 2 μl of Lipofectamine in 100 μl of Dulbecco's modified Eagle's medium for 15–45 min and incubated with cells overnight. The next day, the transfection medium was removed, cells were rinsed once with medium, and TIL was added at a concentration of $10^6$ cells/ml in AIM-V medium containing 60 international units/ml IL-2. Supernatants were removed after 18–24 h and assayed for lymphokine production using a GM-CSF ELISA kit (R+D Systems, Minneapolis, MN).

## Results

A TIL line isolated from patient 888, typed as HLA-A1 and HLA-A24, lysed both fresh and cultured 888 tumor but not an Epstein-Barr virus line from patient 888. The 888 TIL line also lysed cultured melanoma lines matched for HLA-A24 but did not lyse lines lacking HLA-A24 (Table 1). In addition, allogeneic melanomas matched at the HLA-A24 locus stimulated the release of GM-CSF, γ-interferon, and tumor necrosis factor α from 888 TIL, whereas cells not expressing HLA-A24 failed to stimulate significant lymphokine release (16, 17). Of interest, however, non-HLA-A24 melanoma lines such as 397 mel appeared to contain the antigen recognized by TIL 888, since a stable transfectant of 397 melanoma expressing HLA-A24 stimulated cytokine secretion by TIL 888 (Table 2). These studies demonstrated that these TIL recognized a melanoma antigen in the context of HLA-A24.

Patient 888 was treated by the infusion of $4.6 \times 10^{11}$ autologous TIL plus IL-2 and experienced a complete remission of multiple established lung, mucosal, and s.c. metastases (Fig. 1) that lasted for 3 years. She then recurred with a pelvic mass and was retreated with TIL plus IL-2 and again underwent a complete regression. She is now disease free 4.5 years after her initial treatment. Since this patient responded strongly to immunotherapy with TIL, an attempt was made to clone the antigen recognized by these TIL. A cDNA library was constructed from the 888 melanoma cell line, and stable transfectants of the 293 human embryonic kidney cell line expressing HLA-A24 were obtained (293-A24). The 293-A24 cell line was highly transfectable, and transfection could easily be carried out in microtiter trays, allowing rapid screening of cDNA pools. Transcription from the cytomegalovirus promoter, which drives expression of the cDNA inserts, has been shown to be enhanced in cells such as 293 which express adenovirus early proteins (18). In addition, the 293-A24 cell line was found to stimulate little release of GM-CSF from 888 TIL in the absence of any transfected gene (data not shown). To screen the library, pools of 50–100 cDNA clones were transfected into 293-A24 cells, TIL were added, and lymphokine release was assessed. After screening 160 pools (about 10,000 genes), two positive pools were found that stimulated GM-CSF release, and these pools were then subdivided into pools of 10 clones each. Following the identification of positive subpools, two individual positive clones were isolated.

The sequence of one of the positive clones corresponded to the tyrosinase gene which was previously described by Bouchard *et al.* (19) over the first three exons of the sequence but lacked exons four and five. The sequence of this gene, termed tyrosinase (1–3), diverged from the sequence of tyrosinase precisely at the intron-exon boundary (data not shown) and thus may represent an aberrantly spliced product

Table 1 *Specificity of lysis of TIL 888*

| Target | HLA-A24 expression | Effector[a] 40:1 | Effector[a] 10:1 |
|---|---|---|---|
| 888 TC mel[b] | + | 22 | 22 |
| 888 Fresh mel[c] | + | 29 | 30 |
| 888 EBV[d] | + | −2 | −1 |
| 938 TC mel | + | 36 | 18 |
| 1102 TC mel | + | 32 | 18 |
| 1195 TC mel | + | 11 | 11 |
| 501 TC mel | + | 10 | 10 |
| 501 EBV | + | 1 | −2 |
| 526 TC mel | − | 1 | 3 |
| 537 TC mel | − | −1 | 1 |
| 677 TC mel | − | 3 | 5 |
| K562 TC | − | 3 | −1 |
| Daudi TC | − | −1 | −7 |

[a] % lysis by TIL 888 at effector:target ratios of 40:1 and 10:1. All targets were lysed greater than 20% by lymphokine-activated killer cells at an effector:target ratio of 40:1.
[b] TC mel, tissue-cultured melanoma line.
[c] Fresh mel, cryopreserved, noncultured melanoma cells.
[d] EBV, Epstein-Barr virus-transformed cells.

Table 2 *Specific secretion of GM-CSF by TIL 888 is HLA-A24 restricted*

| Stimulator | Transfected gene | HLA-A24 expression | GM-CSF secretion (pg/ml)[a] |
|---|---|---|---|
| None[b] | None | − | <10 |
| 888 TC mel | None | + | 2600 |
| 938 TC mel | None | + | 250 |
| 1102 TC mel | None | + | 220 |
| 397 TC mel | None | − | 10 |
| 397 TC mel | HLA-A2 | − | <10 |
| 397 TC mel | HLA-A24 | + | 160 |

[a] All melanomas cultured in the absence of TIL secreted <10 pg/ml of GM-CSF.
[b] TIL 888 ($5 \times 10^5$/ml) were cultured alone or with the indicated stimulator cells ($5 \times 10^5$/ml).

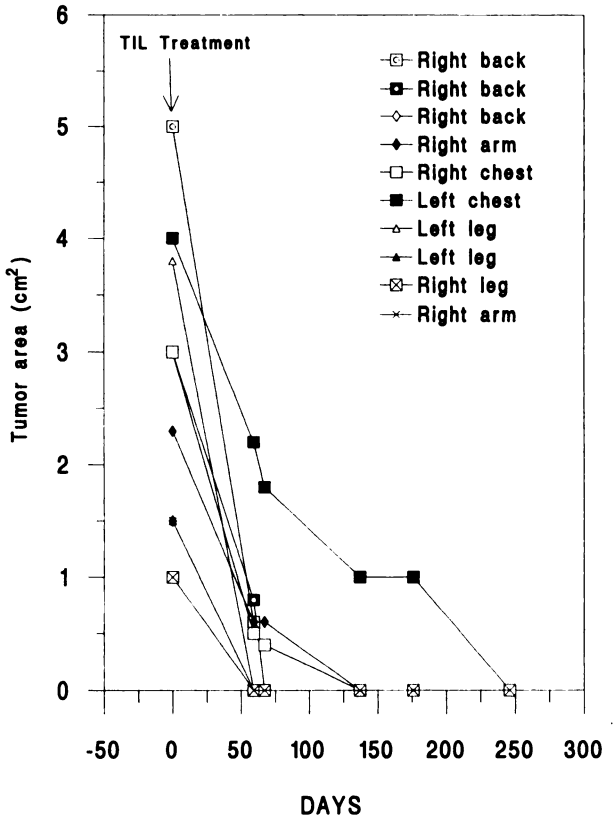


Fig. 1. Regression of s.c. melanoma metastases in patient 888 following treatment (on day 0) with TIL 888 plus IL-2. Some of the points overlap, but complete regression of all metastases was observed by day 250.

Table 3 *Stimulation of cytokine release by tyrosinase transfectants*

| Stimulator | Transfected gene | GM-CSF (pg/ml) Expt 1 | Expt 2 | Expt 3 |
|---|---|---|---|---|
| None[a] | None | 20 | 20 | 20 |
| 888 mel | None | 6600 | 6100 | 5400 |
| 293-A24[b] | Tyrosinase | 500 | 930 | 820 |
| 293-A24 | Tyrosinase (1–3)[c] | 490 | 1300 | NT[d] |
| 293-A24 | β-galactosidase | 20 | <10 | 20 |

[a] TIL 888 ($10^6$/ml) were cultured either alone or with the indicated stimulators ($5 \times 10^5$/ml).
[b] 293-A24 cell line was stably transfected with the HLA-A24 gene as described in text.
[c] Tyrosinase (1–3) represents the tyrosinase clone lacking exons 4 and 5.
[d] Not tested.

of the tyrosinase gene. The second clone isolated from the cDNA library was partially sequenced and found to contain the entire coding region of tyrosinase. An assay carried out with the 293-A24 cell line transfected with the two tyrosinase clones indicated that equivalent amounts of cytokine secretion by TIL 888 were stimulated by both clones (Table 3). Thus, the epitope recognized by TIL 888 appears to reside in the first three exons of this gene. In these as well as additional experiments, the 888 melanoma cell line always stimulated significantly more GM-CSF from TIL 888 than tyrosinase transfectants, which might indicate that other antigens are recognized by TIL 888. Alternatively, the level of antigen expression as well as differences in antigen processing in 293 cells and melanomas may be responsible for the lower level of cytokine stimulated by the tyrosinase transfectants. Upon further screening of the cDNA library, two additional pools were found to stimulate significant GM-CSF release from TIL 888. When a dot blot was carried out using a probe consisting of the tyrosinase coding region, both pools reacted (data not shown). Thus, tyrosinase may represent the predominant antigen recognized by TIL 888.

## Discussion

The tyrosinase gene has previously been shown by Brichard *et al.* (11) to be recognized by HLA-A2 T-cell clones derived from two melanoma patients. Three nucleotides differed between this sequence and the sequence reported by Bouchard (19), but these appear to represent allelic differences (20). This result, along with the observation reported in this paper, indicate that the product of an unmutated gene, expressed in normal melanocytes, is recognized by T-cells from patients with melanoma and can be recognized in association with two different HLA restriction elements, HLA-A2 and HLA-A24. Since the level of tyrosinase expression *in situ* in melanocytes is difficult to determine, quantitative variations in expression of tyrosinase in melanocytes and melanomas could be partially responsible for the apparent lack of self-tolerance to this enzyme. Cultured normal melanocytes do express high levels of tyrosinase (11), but this may not reflect their expression *in situ*. Vitiligo is also seen in some patients given TIL and could result from the reactivity of TIL with normal tissue antigens such as tyrosinase. Patient 888 did not show signs of vitiligo, however, and this issue is unresolved.

The demonstration that tyrosinase is recognized by T-cells from a patient who demonstrated a dramatic remission to TIL therapy suggests that tyrosinase may represent an important tumor rejection antigen. We have not yet, however, identified TIL from other patients which recognize melanoma in the context of HLA-A24. Identification of the immunodominant peptide in this protein will allow studies in which peptides are used to stimulate responses from PBL and TIL. If responses can be elicited, tyrosinase may be a good candidate for use in experimental anti-melanoma vaccine protocols.

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