CD8-Independent Tumor Cell Recognition Is a Property of the T Cell Receptor and Not the T Cell

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CD8-Independent Tumor Cell Recognition Is a Property of the T Cell Receptor and Not the T Cell

Jeffrey J. Roszkowski,* David C. Yu,* Mark P. Rubinstein,† Mark D. McKee,* David J. Cole,‡ and Michael I. Nishimura‡,*

The CD8 coreceptor enhances T cell function by stabilizing the TCR/peptide/MHC complex and/or increasing T cell avidity via interactions with the intracellular kinases Lck and LAT. We previously reported a CD4+ T cell (TIL 1383I), which recognizes the melanoma Ag tyrosinase in the context of HLA-A2. To determine whether CD8 independent tumor cell recognition is a property of the TCR, we used retroviral transduction to express the TIL 1383I TCR in the CD8− murine lymphoma, 58 α/β−. Immunofluorescent staining of TCR-transduced cells with human TCR Vβ subfamily-specific and mouse CD3-specific Abs confirmed surface expression of the transferred TCR and coexpression of mouse CD3. Transduced effector cells secreted significant amounts of IL-2 following Ag presentation by tyrosinase peptide-pulsed T2 cells as well as stimulation with HLA-A2+ melanoma lines compared with T2 cells alone or HLA-A2− melanoma cells. Further analysis of TCR-transduced clones demonstrated a correlation between T cell avidity and cell surface expression of the TCR. Therefore, the TIL 1383I TCR has sufficient affinity to mediate recognition of the physiologic levels of Ag expressed by tumor cells in the absence of CD8 expression. The Journal of Immunology, 2003, 170: 2582–2589.

T cells express Ag-specific receptors (TCRs) that mediate the elimination of tumor cells, tissue allografts, and virally infected cells by recognition of Ag in association with MHC molecules (1). T cells expressing CD4 coreceptors on their cell surface (Th cell) recognize peptide fragments associated with MHC class II molecules (class II restricted) (2–4). Conversely, cytotoxic T cells recognize antigenic peptides associated with MHC class I molecules (class I restricted) and express CD8 coreceptor on the cell surface (2, 4). Generally, TCRs bind to the peptide/MHC complexes with lower affinity than Ab/Ag interactions (5–7). One function of CD4 and CD8 is to enhance the overall affinity of the complex through binding to the β chain of MHC class II molecules and the α chain of MHC class I molecules, respectively (8, 9). CD4 and CD8 also provide signal transduction by association of their cytoplasmic domains with the tyrosine kinase Lck (10). These coreceptors therefore serve to enhance Ag recognition and avidity of T cells.

The model of MHC restriction described, while common, is not absolute. We recently described an MHC class I-restricted (HLA-A2), CD4+ T cell clone reactive to the melanoma Ag tyrosinase isolated from a melanoma patient (11). This tumor-infiltrating lymphocyte (TIL; 1383I) exhibits MHC class I-restricted Ag recognition and effector function in the absence of the CD8 coreceptor. We hypothesized that the CD8-independent function of these TILs is the result of a high affinity TCR capable of triggering T cell activation upon recognition of the peptide/MHC complex. The alternate hypothesis is that TIL 1383I represents a T cell clone highly sensitive to signaling through a normal TCR/peptide/MHC interaction.

These hypotheses were tested by expressing the genes encoding the TIL 1383I TCR in cells that lack human CD8. Using retroviral transduction, we inserted the 1383I TCR into the murine T cell lymphoma line 58 α/β− and verified receptor expression by immunofluorescence. TIL 1383I TCR-transduced cells recognized the peptide epitope in the context of HLA-A2 in a CD8-independent manner. Immunofluorescence analysis of TIL 1383I TCR-transduced 58 α/β− clones indicated that T cell avidity correlates with TCR expression level. These results confirm that CD8-independent tumor recognition by the TIL 1383I TCR is due solely to the TCR and is not a property of the T cells.

Materials and Methods

Cell lines

All melanoma and TIL lines were established from surgical specimens obtained from melanoma patients undergoing immunotherapy in the Surgical Branch, National Cancer Institute. TIL 1235 (HLA-A2 restricted, MART-1:27–35 reactive), TIL 1383I (HLA-A2 restricted, tyrosinase:368–376 reactive), and TIL 1520 (HLA-A2 restricted, gp100:209–217 reactive) were maintained in AIM V medium (Life Technologies, Gaithersburg, MD) supplemented with 10% heat-inactivated pooled human AB serum (Valley Biomedical, Winchester VA), 100 U/ml penicillin, 100 µg/ml streptomycin, 2.92 mg/ml glutamine (Life Technologies), and 6000 IU/ml recombinant human IL-2 (Cetus, Berkeley CA) as previously described (12, 13). Melanoma cell lines 586 MEL (HLA-A2+), 624 MEL (HLA-A2+), 888 MEL (HLA-A2+), 1300 MEL (HLA-A2+), 1383 MEL (HLA-A2+), and SK23 MEL (HLA-A2+) were maintained in human complete medium (hCM), which consisted of RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Life Technologies), and 100 µg/ml penicillin, 100 µg/ml streptomycin, and 2.92 mg/ml glutamine (Life Technologies) as long terminal repeat; mCM, murine complete medium; MMLV, Moloney murine leukemia virus.
previously described (14). T2 and 293 GP cells were maintained in hCM. Murine cell lines 58 α/β and 172-10 cells were maintained in mouse complete medium (mCM), which consisted of RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Life Technologies), 100 μ/ml penicillin, 100 μg/ml streptomycin, 2.92 mg/ml glutamine (Life Technologies), and 5 × 10–3 M 2-ME (Life Technologies). All cells were incubated at 37°C in 5% CO₂ in a humidified incubator.

*TCR α/β-chain identification*

The TCR α- and β-chains from TIL 1383I were identified by RT-PCR using a panel of TCR α-chain V region (AV) and TCR β-chain V region (BV) subfamily specific primers as previously described (15). Briefly, total RNA was isolated from 1–5 × 10⁶ TIL 1383I cells using TRizol (Life Technologies) as previously described (16). First-strand cDNA was prepared from total RNA using SuperScript II reverse transcriptase (Life Technologies) and oligo(dT)₁₂₋₁₈ (Life Technologies) as previously described (16). Ten nanograms of cDNA was PCR amplified in a 50-μl reaction consisting of 1× PCR buffer (Life Technologies), 1.5 mM MgCl₂ (Life Technologies), 200 μM dNTP (Life Technologies), 400 nM TCR AV or BV subfamily-specific forward primer (Life Technologies), 400 nM TCR α-chain C region (AC)-specific or β-chain C region (BC)-specific reverse primer (Life Technologies), and 1 U of Taq DNA polymerase (Life Technologies). PCR amplification was performed using a thermocycler (MJ Research, Cambridge, MA) under the following conditions: 5 min at 92°C (one cycle) followed by 30 s at 92°C, 30 s at 58°C, and 1 min at 72°C (35 cycles), followed by 5 min at 72°C (one cycle). The resulting PCR products were separated on 1% agarose gels containing ethidium bromide (Life Technologies) and were visualized under UV light. The presence of a band of the appropriate size in a reaction indicated the presence of that TCR AV or BV subfamily in TIL 1383I.

*Construction of human/mouse (h/m) chimeric TCR chains*

A two-step PCR strategy was used to construct the TIL 1383I TCR α- and β-chains containing 3’ elements of the corresponding mouse constant regions. Forward cloning primers were designed from the genomic sequences of the TIL 1383I TCR α and β genes, which contained elements of the 5’-untranslated region, the ATG start codon, and UAA stop codon. Reverse cloning primers were designed from the genomic sequences of the mouse AC and BC regions, which contained the termination codon and SalI and XhoI sites, respectively, for subsequent subcloning (TIL 1383I α clone F and TIL 1383I β clone F). Reverse cloning primers were designed from the genomic sequences of the mouse AC and BC regions, which contained the termination codon and SalI and XhoI sites, respectively, for subsequent subcloning (M-Cα clone R and M-Cβ clone R). Two additional primers were designed to create a set of chimeric genes with the 5’ portion of the human TCR chains fused to the 3’ portions of mouse TCR. These primers have complementary sequences that contain 20 bp of the human AC or BC region fused to 20 bp of the adjacent mouse AC or BC region (h/m forward α chimera, 5’-CCGAAAGAAAACCTTGAACCAAGC-3’, h/m forward β F chimera, 5’-ACCATCCTCTAGAGATCGCT-3’).

Two separate RT-PCR reactions were performed to amplify the 5’ and 3’ fragments of the h/m hybrid TIL 1383I TCR as described above. PCR1 used the TIL 1383I α clone F and h/m α chimera R primers or the TIL 1383I β clone F with h/m β chimera R primers, respectively, to amplify the 5’ portions of the TIL 1383I TCR α- and β-chains. PCR2 used the h/m α chimera F and M-Cα clone R primers or the h/m β chimera F and M-Cβ clone R primers, respectively, to amplify the 3’ portions of the corresponding mouse TCR α and β C regions. The products of these PCR reactions have 40 bp of homology corresponding to the hybrid primer sequences. PCR 3 used these products from PCR1 and PCR2 as templates and the primers TIL 1383I α clone F and M-Cα clone R or TIL 1383I β clone F and M-Cβ clone R to complete the h/m hybrid TCR genes. The PCR fragments were ligated into the pCR 2.1 TA cloning vector (Invitrogen) and transformed into Escherichia coli DH5α competent cells (Life Technologies). Bacterial clones were subjected to PCR using the TIL 1383I α clone F and M-Cα clone R primers or the TIL 1383I β clone F and M-Cβ clone R primers to identify clones containing the inserts of the predicted size. DNA was isolated from these clones and their inserts were sequenced by cycle sequencing using BigDye Terminator Cycle Sequencing kits (PerkinElmer/PE Applied Biosystems, Foster City, CA) to ensure there were no PCR errors in the sequence.

*Retroviral vector construction*

The SAMEN CMV/SRα retroviral vector was designed specifically for introducing TCR genes into alternate T cells. The structure of this vector is shown in Fig. 1. The 5’ long terminal repeat (LTR) in the SAMEN SRα backbone was replaced with a hybrid LTR consisting of human CMV enhancer and promoter fused to the Moloney murine leukemia virus (MMLV) 5’ LTR. This modification permits production of transient retroviral supernatants in 293 GP cells (17). Other key elements of the SAMEN CMV/SRα vector include an internal SRα promoter to permit the expression of multiple genes, unique SalI and XhoI restriction sites for ease of inserting TCR chains, and an internal ribosome entry site/neomycin cassette for G418 resistance. A rapid ligation strategy was used to subclone the 1383I TCR α- and β-chain genes into SAMEN CMV/SRα. TCR β-chain genes were excised from pCR 2.1 with XhoI and ligated into the SalI restriction site in SAMEN CMV/SRα using a mixture of T4 DNA ligase and SalI restriction endonuclease. The resulting SalI/XhoI hybrid restriction sites are resistant to digestion by SalI and XhoI restriction endonucleases. Ligation reactions were redigested with SalI, resulting in linearization of plasmids not containing the TCR β-chain insert, allowing for enrichment of recombinant clones. The ligation reactions were transformed into E. coli DH5α competent cells (Life Technologies), and bacterial clones were subjected to PCR using primers that flanked the cloning sites in SAMEN CMV/SRα. The DNA sequence of clones containing inserts was determined using BigDye Terminator Cycle Sequencing kits (PerkinElmer/PE Applied Biosystems) and analyzed using an ABI PRISM 310 Genetic Analyzer (PerkinElmer/PE Applied Biosystems) to ensure that TCR gene inserts were in proper orientation. This method was repeated to insert the TCR α-chain into the retroviral vector by ligating a SalI fragment containing the TIL 1383I α-chain into the XhoI site of SAMEN CMV/SRα. The resulting h/m hybrid TIL 1383I TCR containing SAMEN CMV/SRα retroviral vector was designated A9m.

*Generation of retroviral supernatants*

Tissue culture flasks (75 cm²) were coated with 0.02% type B bovine skin gelatin (Sigma-Aldrich, St. Louis, MO) in HBSS for 15 min at room temperature. 293 GP cells were seeded onto coated flasks at sufficient density to provide 50–60% confluence after 24 h. Monolayers were rinsed three times with PBS and transiently cotransfected using Lipofectamine Plus reagents (Life Technologies), 6.0 μg of the retroviral plasmid containing TCR genes, and 6.0 μg of the vesicular stomatitis virus envelope gene in 6.0 ml of serum-free DMEM supplemented with penicillin/streptomycin/glutamine. Following a 3-h incubation, 10 ml of DMEM supplemented

[FIGURE 1. Retroviral vectors used for TCR gene transfer. A. The SAMEN CMV/SRα backbone was modified from the SAMEN backbone that contains a MMLV LTR at the 5’ and 3’ ends, a psi packaging signal, the splice donor (SD) and splice acceptor (SA) sites, a multiple cloning site (MCS), and an internal ribosome entry site/neomycin cassette. The SAMEN CMV/SRαs was constructed by first inserting an internal SRα promoter between the SalI and XhoI sites in the MCS. The 5’ MMLV LTR was then replaced with the production of retroviral supernatants by transiently transfecting 293 GP cells. B. The TIL 1383I TCR retroviral vector (designated A9m) contained the TCR β-chain gene under control of the 5’ LTR, and the TCR α-chain gene under control of the SRα promoter.]
with 20% heat-inactivated FCS was added to flasks, and cells were incubated at 37°C. Medium was discarded after 24 h and replaced with 15 ml hCM. Retroviral supernatants were collected after 24 h, replaced with fresh hCM, and collected again after an additional 24 h. Retroviral supernatants were either used immediately or frozen at −70°C for later use.

**Retroviral transduction**

Upon thaw, retroviral supernatants were diluted with a 50% volume of fresh hCM supplemented with 8 µg/ml polymyxin, 10 mM HEPES buffer, and 50 µM 2-ME and filter-sterilized. 58 α/β cells were suspended at 10⁶ cells/ml in retroviral supernatant. Cells (3 × 10⁶; 3 ml) were added to each well of a six-well culture plate, and the plates were centrifuged at 1000 × g for 2 h at 32°C. Transduced cells were incubated overnight, and retroviral transduction was repeated by resuspending cells from each well in 5 ml of fresh retroviral supernatant prepared as described above. Cells were incubated for 24 h, then resuspended in fresh complete murine medium at 10⁶ cells/ml and incubated for 1 day. Cells containing transduced genes were selected for by culture in mCM with 500 µg/ml G418. G418-resistant cells were subjected to limiting dilution cloning by diluting cells in mCM with G418 to a concentration yielding 0.3 cell/100 µl of a 96-well tissue culture plate. Positive growth wells were expanded for further analysis.

**Ag recognition assays**

G418-resistant TIL 1383I TCR-transduced 58 α/β cells were tested for reactivity to tumor Ag in cytokine release assays as previously described (18). Briefly, responders were cocultured with stimulator cells in a 1/1 ratio in a total volume of 200 µl of AIM-V medium supplemented with 50 µM 2-ME, 10 µg/ml of G418, and 96-well, U-bottom tissue culture plates. Actual cell numbers were 5 × 10⁵ each of stimulators and responders per well. As a positive control, TIL 1383I (5000/well) were cocultured with stimulators (5 × 10⁴/well) in 200 µl of AIM-V/well of 96-well, U-bottom tissue culture plates. Cocultures were incubated at 37°C in a humidified CO₂ incubator for 24 h. Supernatants were harvested, and the amount of cytokine released was measured by ELISA. Mouse IL-2 release by TIL 1383I TCR-transduced 58 α/β cells and human IFN-γ release by TIL 1383I were measured using ELISA reagents purchased from Endogen (Woburn, MA). Stimulators included a series of HLA-A2+ and HLA-A2− melanoma lines, fibroblast lines, and peptide-loaded T2 cells. T2 cells were loaded with antigenic peptides by incubating the cells (10⁶/ml) with the various concentrations of peptide (10−5000 nM) in complete medium for 2 h at 37°C. Pulsed T2 cells were washed three times with PBS before resuspension in AIM-V for coculture with responders. Peptides used in this study were obtained from Macromolecular Resources (Fort Collins, CO). The peptide sequences are as follows: gp100:209−217 (ITDQVPFSV) (19), MART-1:27−35, (AA 217 (ITDQVPFSV)), MART-1:27−35, (AA 217 (ITDQVPFSV)), MART-1:27−35, (AA 217 (ITDQVPFSV)), MART-1:27−35, (AA 217 (ITDQVPFSV)), MART-1:27−35, (AA 217 (ITDQVPFSV)), MART-1:27−35, (AA 217 (ITDQVPFSV)), MART-1:27−35, (AA 217 (ITDQVPFSV)).

**Immunofluorescence**

Cell surface expression of human TCR Vβ12 and mouse CD3 by TIL 1383I TCR-transduced 58 α/β cells and clones was measured using PE-labeled anti-human Vβ12 (Immunotech, Westbrook, MA), FITC-labeled anti-mouse CD3, and Ig controls (BD PharMingen, San Diego, CA). TIL 1383I were stained with PE-labeled anti-human Vβ12, and 172-10 cells were stained using FITC-labeled anti-mouse CD3 as positive controls. The relative log fluorescence of 10⁴ live cells was measured using a FACScan flow cytometer (BD Biosciences, Mountain View, CA).

**Results**

**TIL 1383I TCR expression in 58 α/β− cells**

We have previously demonstrated that TIL 1383I was a CD4+ T cell that recognized the 368–376 epitope from tyrosinase in the context of HLA-A2. Based on these results we speculated that the TIL 1383I TCR has sufficient affinity to recognize the physiologic level of tyrosinase:368−376 that is presented by HLA-A2 on the surface of melanoma cells without the need for CD8. To test this hypothesis a retroviral vector was constructed that could transfer the TIL 1383I TCR to the murine T cell lymphoma 58 α/β−. 58 α/β− cells were selected because they are readily transducible, up-regulate CD3 upon expression of an introduced TCR, lack expression of human CD8, and secrete IL-2 upon Ag stimulation.

The TCR AV4 and BV12S4 genes used by TIL 1383I were identified by RT-PCR using a panel of TCR AV and BV subfamily-specific PCR primers. Based on their DNA sequence, PCR primers were then designed to clone the full-length cDNAs that encode the TIL 1383I TCR. We and others have shown that human TCR genes cannot be expressed by murine T cells (22–24). However, by replacing the transmembrane and cytoplasmic regions of the human α and β C regions with the corresponding mouse sequences, human TCR genes can be expressed by mouse T cells (23, 24). Using this strategy, we constructed h/m hybrid TIL 1383I TCR α- and β-chains by PCR (not shown).

To express these hybrid TCR genes in mouse T cells, we developed a second-generation retroviral vector to more efficiently express TCR genes. Starting with the SAMEN backbone used in other studies (22, 25), we replaced the 5′ MMLV LTR with a hybrid MMLV/CMV LTR. This hybrid MMLV/CMV LTR permits the production of high titer retroviral supernatants by transiently cotransfecting 293GP cells with the retroviral plasmid and a retroviral envelope cDNA (17). We then introduced an internal SRα promoter to the vector. This second promoter enabled us to express the TCR β-chain using the 5′ LTR and the TCR α-chain from the SRα promoter. The structure of the final retroviral vector containing the h/m hybrid TIL 1383I (designated A9m) is shown in Fig. 1.

A9m retroviral supernatants packaged using the vesicular stomatitis virus envelope were used to transduce 58 α/β− murine T lymphoma cells. Following G418 selection, transduced cells were stained with human Vβ12- and murine CD3-specific Abs to detect cell surface expression of TIL 1383I TCR on their cell surface. As shown in Fig. 2, TIL 1383I TCR-transduced 58 α/β− cells expressed both mouse CD3 and human Vβ12. CD3 expression was not due to re-expression of the endogenous mouse TCR, since these cells failed to stain with a pan mouse αβ TCR mAb (not shown).

TIL 1383I TCR-transduced 58 α/β− cells were tested for recognition of Ag by coculturing transductants with peptide-pulsed T2 APC as well as a panel of HLA-A2+ melanoma lines that were recognized by TIL 1383I. As shown in Table I, transduced cells secreted significant amounts of IL-2 (defined as twice background and at least 100 pg/ml) when cocultured with tyrosinase:368−376 peptide-loaded T2 cells compared with T2-loaded with a MART-1 peptide or T2 cells alone. TIL 1383I TCR-transduced 58 α/β− cells secreted significant amounts of IL-2 when stimulated with HLA-A2+, but not HLA-A2−, melanoma cells. These results indicate the A9m retroviral vector transferred a h/m hybrid TCR to murine cells, and the TIL 1383I TCR enabled T cells to recognize the physiologic levels of the tyrosinase:368−376 peptide presented by HLA-A2 on the surface of tumor cells.
with T2 cells alone or loaded with tyrosinase:secreted high, medium, and low amounts of IL-2 when stimulated with T2 cells alone or loaded with tyrosinase:368–376 370D or MART-1:27–35 peptides or with HLA-A2–MART-1:1–3 or HLA-A2–MART-1:1 melanoma cells. The amount of IL-2 released was measured by ELISA. Each value represents the mean of triplicate wells and is representative of three replicate experiments. Underlined values represent significant IL-2 release, which is defined as at least twice background and >100 pg/ml. Values represented as <31 were less than the lowest value on the standard curve.

To study the effect of TCR transgene expression on T cell avidity, a panel of TIL 1383I TCR-transduced 58 α/β–clones was generated by limiting dilution cloning. Clones were isolated that secreted high, medium, and low amounts of IL-2 when stimulated with T2 cells loaded with 1 μM tyrosinase:368–376 370D peptide (not shown). The relative avidity of each of the selected clones was tested by cytokine release following stimulation with T2 cells loaded with decreasing concentrations of tyrosinase:368–376 370D peptide (0.1–1,000 nM; Fig. 3). In these experiments we defined relative avidity as the minimum amount of peptide required to elicit significant IL-2 release (twice background and >100 pg/ml). Data were subjected to curve-fitting analysis using the PRISM statistical software package. Each clone required that T2 cells be loaded with between 1 and 55 nM peptide to stimulate significant IL-2 release. Clones B1 and B4 were observed to have the highest avidity (2–3 nM peptide), clone A4 had moderate avidity (11.5 nM peptide), and clones A3, A5, and A6 had low avidity (17–55 nM peptide) for tyrosinase:368–376 370D presented by T2 cells. Given the caveat that the 58 α/β–cells and the TIL 1383I cells are from different species (mouse and human, respectively), and different cytokines were used for the avidity measurements, we found that several clones (B1, B4, and A4) had similar avidity as the parental TIL 1383I cells. These cells required between 1 and 10 nM peptide loaded on T2 cells to elicit significant cytokine release (11). This implies that the transfer of TIL 1383I TCR to alternate effectors can lead to T cells with similar ability to recognize Ag as the parental T cell.

It has been reported that a T cell’s avidity correlates with its ability to recognize tumor cells (25, 26). This hypothesis was tested using representative high, moderate, and low avidity 58 α/β–clones. Clones B4, A4, and A5 were stimulated with HLA-A2–, tyrosinase+ melanoma cell lines, and the amount of IL-2 released was measured. As shown in Fig. 4, the amount of IL-2 secreted by each TIL 1383I TCR-transduced clone when stimulated with HLA-A2+ melanoma lines was proportional to their avidity, as measured by peptide-loaded T2 cells. No cytokine release was detected from clones cocultured with HLA-A2– melanoma cells, HLA-A2–nonmelanoma cells, or T2 cells loaded with gp100:209–217 control peptide (data not shown). These results support the premise that the ability of a T cell to recognize tumor cells correlated with its relative avidity.

Construction of a hybrid TCR by fusion of the human extracellular domain to mouse transmembrane and cytoplasmic domains creates the possibility of subtle conformational changes that may impact the fine specificity of the receptor for peptide/MHC complex. To test the specificity of the both wild-type and chimeric

Table 1. Melanoma recognition by TIL 1383I TCR-transduced 58 α/β–cells

<table>
<thead>
<tr>
<th>Stimulators</th>
<th>HLA-A2 Expression</th>
<th>TIL 1383I TCR</th>
<th>Untransduced</th>
<th>Transduced</th>
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<tr>
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<tr>
<td>T2</td>
<td>N/A</td>
<td>&lt;31</td>
<td>&lt;31</td>
<td>T2 + MART-1:27–35</td>
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<tr>
<td>T2 + tyrosinase:368–376</td>
<td>+</td>
<td>&lt;31</td>
<td>4.934</td>
<td></td>
</tr>
<tr>
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<td>&lt;31</td>
<td>37.187</td>
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</table>

*The reactivity TIL 1383I TCR-transduced 58 α/β–cells was measured in cytokine release assays. TIL 1383I TCR-transduced 58 α/β–cells were stimulated with T2 cells alone or loaded with tyrosinase:368–376 370D or MART-1:27–35 peptides or with HLA-A2–MART-1:1 or HLA-A2–MART-1:1 melanoma cells. The amount of IL-2 released was measured by ELISA. Each value represents the mean of duplicate wells and is representative of three replicate experiments. Underlined values represent significant IL-2 release, which is defined as at least twice background and >100 pg/ml. Values represented as <31 were less than the lowest value on the standard curve.*

FIGURE 3. Relative avidity of TIL 1383I TCR-transduced 58 α/β–clones for peptide-loaded stimuliators. The relative avidity of several TIL 1383I TCR-transduced 58 α/β–clones was measured in IL-2 release assays using T2 cells incubated with different concentrations of tyrosinase:368–376 370D peptide. The amount of IL-2 released was measured by ELISA. Relative avidity was defined as the minimum amount of peptide loaded on T2 cells that could elicit significant cytokine release. Significant cytokine release was considered to be at least twice background and >100 pg/ml. Each point represents the average of triplicate wells and is representative of three replicate experiments.

FIGURE 4. Relative sensitivity of TIL 1383I TCR-transduced 58 α/β–clones for melanoma cells. The relative sensitivity of TIL 1383I TCR-transduced 58 α/β–clones to stimulation by HLA-A2–tyrosinase+ melanoma cell lines was measured in IL-2 release assays. Representative high (clone A5), medium (clone A4), and low (clone A5) avidity TIL 1383I TCR-transduced 58 α/β–clones were stimulated by autologous 1383I MEL, SK-23 MEL, and 624 MEL cells. Tyrosinase:368–376 370D-loaded T2 cells were used as stimulators to control for Ag recognition. Untransduced 58 α/β–cells were used as responders to measure background stimulation. The amount of IL-2 released was measured by ELISA. Significant cytokine release was considered to be at least twice background and >100 pg/ml. Each point represents the average of triplicate wells, and the data are representative of three replicate experiments.
TCR, TIL 1383I and TIL 1383I TCR-transduced clones of varying avidity were stimulated with T2 cells loaded with 1 μg/ml of the specified peptide. Cytokine release was measured by ELISA. The pattern of relative cytokine release (IL-2) by clone A4 in response to alanine-substituted peptides was consistent with cytokine release (IFN-γ) by TIL 1383I. Alanine substitution in positions 3 and 6 effectively eliminated recognition of peptide/MHC by both the chimeric TCR-transduced cells and TIL 1383i. The results represent the average of three triplicate wells and are representative of three independent experiments.

**Discussion**

Several groups studying the affinity of the interactions between TCRs and their ligands have found that TCR affinity for peptide/MHC influences T cell activation by regulating the duration of TCR engagement (27–30). The slow off rate of a high affinity TCR permits sufficient time for adequate assembly of the T cell signaling complex for full activation of the T cell (30, 31). In practical terms, a TCR with high affinity for its ligand is predicted to increase the sensitivity (avidity) of the T cell, thus requiring lower levels of Ag for efficient target cell recognition. It has been reported that T cell avidity correlates with the elimination of tumor cells and viruses in vivo (25, 26). T cells bearing high affinity TCRs would be predicted to be better effectors than T cells bearing low affinity TCRs. However, high TCR affinity can be detrimental to T cell function. T cells expressing TCRs with extremely high affinity for peptide/MHC can undergo activation-induced cell death (AICD) upon encounter with Ag (32, 33).
Analysis of the TIL 1383 I TCR in this study adds new insight into the relationship among TCR affinity, T cell avidity, and T cell activation. Given that TIL 1383 I TCR-transduced cells recognize tyrosinase:368–376 peptide-loaded targets and HLA-A2+, tyrosinase+ melanoma cells in the absence of CD8 expression, the TIL 1383 I TCR must have high affinity for the tyrosinase:368–376 peptide/HLA-A2 ligand. Furthermore, the relative avidity of the TCR-transduced cells for peptide-loaded cells and melanoma cells was directly related to the levels of TCR expression. These results indicate that CD8-independent tumor recognition by TIL 1383 I was a property of the TCR and not of the parent T cell.

Despite the apparent high affinity of the TIL 1383 I TCR, the resulting TCR-transduced cells had relatively low avidity for the tyrosinase:368–376 peptide/HLA-A2 ligand. The highest avidity TIL 1383 I TCR-transduced 58 α/β clones B1 and B4 required between 1 and 5 nM peptide to elicit cytokine production, which is similar to the parental TIL 1383 I (11), whereas the low avidity clones required between 10 and 55 nM peptide (Fig. 3). With the caveat that these comparisons are made between a murine T cell lymphoma producing IL-2 and a human TIL producing IFN-γ in response to Ag stimulation, we can reasonably expect that transfer of the TIL 1383 I TCR to human T cells will result in cells of at least similar avidity to TIL 1383 I. This observation conflicts with reports demonstrating that TCR affinity influences T cell avidity and that high avidity T cells are required for efficient Ag recognition (25, 26).

We recently described isolating extremely high avidity gp100:209–217-reactive T cell clones for treating patients with metastatic melanoma (34). Each clone had high avidity (recognized <1 nM peptide loaded on T2 cells) and secreted large amounts of IFN-γ when stimulated with melanoma cells (34). Despite their high avidity, IFN-γ secretion by each clone was easily inhibited by anti-CD3 mAb (data not shown). Furthermore, a detailed analysis of the R6C12 clone using tetramers demonstrated that this clone had a low affinity TCR despite its high avidity (34). These observations further conflict with the idea that TCR affinity influences T cell avidity and that high avidity T cells are required for efficient Ag recognition. While it is clear that Ag recognition by T cells can be predicted based on TCR affinity and T cell avidity measurements, this study demonstrates that the expression of a high affinity TCR does not necessarily lead to a high avidity T cell. Similarly, high T cell avidity does not insures the expression of a high affinity TCR.

The analysis of TIL 1383 I TCR-transduced 58 α/β clones has also yielded results that conflict with the current models of AICD. The reduced IL-2 production by the TIL 1383 I TCR-transduced 58 α/β clones when stimulated by peptide-loaded stimulators relative to melanoma cells could be attributed to AICD (Table I). In this scenario, the micromolar amounts of Ag loaded on T2 cells would deliver a death signal rather than the stimulatory signal delivered by the physiologic amounts of Ag presented by tumor cells. However, the difference in the amount of IL-2 produced between peptide-stimulated cells vs melanoma cells cannot be explained by AICD, since the amount of IL-2 produced was proportional to the amount of peptide loaded on the stimulators (Fig. 3). Furthermore, no evidence of AICD was observed when the most avid clones (B1 and B4) were stimulated with amounts of Ag well above physiologic levels (>1 μM; Fig. 3). Therefore, reduced IL-2 production following stimulation with peptide-loaded cells relative to tumor cells must be due to factors other than AICD. There are three potential explanations for this observation. One possibility is that there are cell surface receptors (co-stimulatory or adhesion molecules) expressed on 58 α/β cells capable of being stimulated by ligands expressed by melanoma, but not T2 cells. Another possibility is that there is another epitope expressed by melanoma cells that is recognized by TIL 1383 I. This epitope would be a stronger agonist for the TIL 1383 I TCR than the tyrosinase:368–376 epitope. Alternatively, it is possible that additional post-translational modifications to the tyrosinase:368–376 peptide were not detected by Skipper et al. (21) when they eluted the peptide from the surface of melanoma cells. These modifications would be present in the natural, but not the synthetic, tyrosinase:368–376 peptides presented by HLA-A2. As a result, tumor cells would be better stimulators of cytokine release from TIL 1383 I TCR-transduced cells relative to peptide-loaded cells. Regardless of the mechanism, the TIL 1383 I TCR is capable of transferring efficient tumor recognition to other effectors.

We have proposed that TCR gene transfer to normal PBL-derived T cells can represent a new treatment option for patients with malignancies and viral infections (35). Analysis of the TIL 1383 I TCR has provided useful information that will assist in successfully developing treatments for patients using TCR gene-modified T cells. Since we first reported that a retroviral vector could be used to transfer the antitumor reactivity of a MART-1-reactive TCR to naïve PBL-derived T cells (35), we and others have used similar approaches to engineer human and mouse T cells to recognize other Ags (36–41). However, a careful analysis of these studies indicates that it is often difficult to obtain TCR-transduced T cells with sufficient avidity to recognize tumor cells. There appear to be two major factors that dictate tumor recognition by TCR-transduced T cells. Given that T cell avidity correlates with tumor recognition in animal models (25, 26), high transduction efficiency and high level of TCR expression are critical for efficient tumor recognition. In studies with our anti-MART-1 TCR, we found that ~25% of the T cells contained the provirus before selection, and 80–90% of them contained the provirus after selection (35). Despite this high frequency of TCR-transduced cells, there were bulk cultures and individual clones that were reactive with peptide-loaded cells but not tumor cells. Given that α- and β-chains of the introduced TCR had to compete with α- and β-chains of the endogenous TCR, it requires relatively high transgene expression to insure that enough of the introduced TCRs assemble to enable the T cell to recognize tumor cells. Even though we had high levels of anti-MART-1 TCR α and β message, only 33–50% of the clones we isolated could recognize tumor cells. Therefore, the competition between the introduced and endogenous TCR α- and β-chains will always limit the avidity of the resulting TCR-transduced cells.

The second factor that influences tumor cell recognition by TCR-transduced T cells is TCR affinity. Since most tumor Ags are nonmutated gene products expressed by nonmalignant tissues (melanocytes, testes, etc.) (42, 43), immunologic tolerance should strongly influence the TCR repertoire of tumor-reactive T cells in the periphery of a cancer patient. Negative selection should delete all self-reactive T cells expressing high affinity TCRs (44, 45). This hypothesis is supported by the observation that high avidity tyrosinase-reactive T cells can be obtained from tyrosinase-deficient, but not tyrosinase-expressing mice (46). As a result, most of the TCRs available for TCR gene transfer will resemble our anti-MART-1 TCR that has low affinity for the peptide/MHC complex and requires CD8 for tumor recognition (35). While we and others have shown that tumor-reactive T cells can be obtained using these types of TCRs, it may be difficult to use low affinity TCRs to engineer T cells with similar avidity to naturally occurring T cells. Therefore, the affinity of most TCRs may limit the practical utility of TCR-transduced CD8+ T cells and make it impossible to engineer CD4+ T cells capable of recognizing tumor cells.
TCRs like the CD8-independent TIL 1383 TCR described in this study should circumvent many problems associated with most TCRs described to date. Tumor cell recognition by TIL 1383 TCR-transduced cells indicates that it is possible to provide patients with MHC class I-restricted T cell help. Furthermore, given the high affinity of the TIL 1383 TCR, it is likely that the number of correctly paired TCRs on CD8+ T cells required for tumor recognition would be lower than that for other TCRs. Given that our TIL 1383 TCR-transduced 58 αβ cells could recognize tumor cells despite expressing mouse costimulatory/adhesion molecules, a human CD8+ T cell expressing the TIL 1383 TCR would be predicted to have extremely high avidity and might be a more effective T cell in treating established tumors.

The only drawback to using a CD8-independent TCR might be AICD of the TCR-transduced T cells. Given the overwhelming evidence supporting AICD, TIL 1383 TCR-transduced CD8+ T cells may die upon contact with Ag, rendering them useless for adoptive immunotherapy. We believe it will be possible to engineer naïve CD8+ T cells to express the TIL 1383 TCR and survive encounters with Ag for the same reasons that may prevent CD8-dependent TCRs from being useful for adoptive immunotherapy. The high affinity of the TIL 1383 TCR mean that fewer correctly assembled TCRs will be required for tumor recognition. The clonal variation in TCR expression within a population of TCR-transduced T cells will work to our advantage using TCRs such as the TIL 1383 TCR. It should be possible to obtain T cells with sufficient avidity to recognize tumor cells, yet have insufficient TCR expression to induce AICD. Furthermore, since it will take fewer correctly assembled high affinity TCRs on each cell to permit tumor recognition, the degree of tumor recognition by a transduced T cell culture will not be limited to the same degree by transgene expression as it is with other TCRs. Therefore, we are optimistic that engineering CD8+ and CD4+ T cells with high affinity, MHC class I-restricted TCRs will enable us to provide a large number of patients with a source of autologous effector and Th cells reactive with any defined Ag regardless of their immune status.

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References


