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High-Affinity TCRs Generated by Phage Display Provide CD4⁺ T Cells with the Ability to Recognize and Kill Tumor Cell Lines

Yangbing Zhao,* Alan D. Bennett,† Zhili Zheng,* Qiong J. Wang,* Paul F. Robbins,* Lawrence Y. L. Yu,* Yi Li,† Peter E. Molloy,† Steven M. Dunn,† Bent K. Jakobsen,† Steven A. Rosenberg,* and Richard A. Morgan2*

We examined the activity of human T cells engineered to express variants of a single TCR (1G4) specific for the cancer/testis Ag NY-ESO-1, generated by bacteriophage display with a wide range of affinities (from 4 μM to 26 pM). CD8⁺ T cells expressing intermediate- and high-affinity 1G4 TCR variants bound NY-ESO-1/HLA-A2 tetramers with high avidity and Ag specificity, but increased affinity was associated with a loss of target cell specificity of the TCR gene-modified cells. T cells expressing the highest affinity TCR (Kᵢ value of 26 pM) completely lost Ag specificity. The TCRs with affinities in the midrange, Kᵢ 5 and 85 nM, showed specificity only when CD8 was absent or blocked, while the variant TCRs with affinities in the intermediate range—with Kᵢ values of 450 nM and 4 μM—demonstrated Ag-specific recognition. Although the biological activity of these two relatively low-affinity TCRs was comparable to wild-type reactivity in CD8⁺ T cells, introduction of these TCRs dramatically increased the reactivity of CD4⁺ T cells to tumor cell lines. The Journal of Immunology, 2007, 179: 5845–5854.

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Abbreviations used in this paper: pMHC, peptide-MHC; wt, wild type; RCC, renal cell carcinoma cell line; IVT, in vitro transcribed; β₂m, β₂-microglobulin; IRES, internal ribosomal entry site; TIL, tumor-infiltrating lymphocyte; MSCV, murine stem cell virus; SCT, single chain trimer.
with Ag affinities of up to a million times higher than wild-type (wt) TCR (17). One of the panels of TCRs studied in this report was directed against the epitope of the cancer/testis Ag NY-ESO-1 (p157–165)/HLA-A2. The soluble form of these high-affinity TCRs bound only to NY-ESO-1/HLA-A2 and not to a panel of control peptide Ags, suggesting that the binding is highly Ag specific. Furthermore, high-affinity TCR monomers and tetramers showed no cross-reactivity to endogenous cellular pMHC Ags (17, 18).

To examine whether T cells expressing these high-affinity TCRs would exert enhanced reactivity toward tumor cells, we introduced a panel of these high-affinity TCRs with KD values of 4 μM, 450 nM, 84 nM, 5 nM, and 26 pM into stimulated human PBLs. Although CD8+ T cells expressing intermediate- and high-affinity IG4 TCR variants showed greatly increased binding to NY-ESO-1/HLA-A2 tetramers with high Ag specificity, increasing affinity was associated with a loss of specificity of the gene-modified cells. In contrast to CD8+ T cells, engineered CD4+ T cells expressing intermediate-affinity TCRs demonstrated a pronounced increase in sensitivity for recognition of target cells pulsed with the cognate peptide, and greatly enhanced recognition of appropriate tumor targets. These results suggest that genetically modified CD4+ T cells that express TCRs with intermediate affinity may be useful for TCR-based adoptive cancer therapies.

Materials and Methods

PBL, cell lines, and RNA electroporation

All of the PBLs used in this study were from metastatic melanoma patients treated at the Surgery Branch (National Cancer Institute (NCI), National Institutes of Health, Bethesda, MD). Melanoma cell lines 624.38mel, A375mel, 1390mel, 1363mel, SKmel22, 526mel, and 888mel were generated at the Surgery Branch as previously described (19). HLA-A2+ and HLA-A2 EBV-B and renal cell carcinoma cell lines (RCCs) were also generated at the Surgery Branch as described (20). All cell lines were cultured in medium consisting of RPMI 1640 supplemented with 10% heat-inactivated FBS (Biofluids), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen Life Technologies). Lymphocytes were cultured in AIM-V medium (Invitrogen Life Technologies) supplemented with 5% human AB serum (Valley Biomedical) and 300 IU/ml IL-2 (Chiron) at 37°C and 5% CO2. The MART-1 and gp100-specific T cell clones JFK6 and L2D8 were isolated from patient tumor-infiltrating lymphocyte (TIL) cultures (Surgery Branch, NCI). In vitro-transcribed (IVT) RNA electroporation of anti-CD3 Ab (OKT3) stimulated human PBLs and cell lines was conducted as described in our previous report (21).

Peptide synthesis

Synthetic peptides used in this study were made using a solid phase method on a peptide synthesizer (Gilson) at the Surgery Branch (NCI). The quality of each peptide was evaluated by mass spectrometry (Biosynthesis). The sequences of the peptide used in this study are as follows: NY-ESO-1, 157–165 (165V) (SLLMWITQV); MART-1, 26–35 (27L) (ELAGIGILTV); gp100, 209–217 (210M) (IMDQVPFSV); Flu-MP, 58–66 (GIILGFVFTL).

High-affinity TCR α- and β-chain genes

High-affinity TCR genes were generated by bacterial plaque display (17). The IG4 c5b/c6 α TCR/β TCR in this publication is identical with “IG4c113” or “clone 113”; c10/c1 corresponds to “clone10” and c12/c2 corresponds to “clone 12” (17). The mutated TCR α-chain c5 and β-chain genes c59 and c100 were not previously reported (Y. Li and P. E. Molloy, unpublished observations).

IG4 wt and high-affinity mutant NY-ESO-1/HLA-A2-specific TCR genes were reconstructed as full-length TCR α- and β-chains (using the TCR β-chain 2 constant region) and were designed with native interchain disulfide bonds. The open reading frames and 5’ untranslated regions of these genes were optimized for maximal expression in human cells (22) by GENEART. Additional, nonoptimized, TCR genes for NY-ESO-1 (ET-8F) and gp100 were described previously (23, 24).

Single-chain trimer and retroviral vector construction

PCR primers were designed to amplify plasmid-encoded genes and introduce a T7 promoter at the 5’ end and a poly(A) tract at the 3’ for the α and β TCR chains, α and β CD8, HLA-A2 and both NY-ESO-1 (165V)- and MART-1(27L)-single chain (peptide-β2-microglobulin (β2m)/HLA-A2) trimers (SCT), respectively. Using these purified PCR products as templates, RNA was generated via IVT as described previously (21). SCT PCR templates for both NY-ESO-1 and MART-1 were generated by PCR as previously described (25). The pIRES.neo.OVA.hβ2m.A201 plasmid was obtained from T. Hansen (Washington University School of Medicine, St. Louis, MO). This plasmid encodes the following elements starting from the N terminus: the mouse β2m signal peptide, SFINFEKL (OVA-derived H2Kb-restricted epitope), the first flexible linker (G3ASG4SG4S), the mature portion of HLA-A201, and its 3’ untranslated region. The entire insert was then subcloned into the NotI and HindIII sites of pCR2.1 to create pCR2.1.OVA.hβ2m.A201. The region encoding the SFINFEKL epitope is flanked by unique AgeI site and Nhel sites within the β2m signal peptide and the first linker, respectively. For the final constructs, the SFINFEKL epitope was exchanged for the MART-1 or NY-ESO-1 HLA-A2-restricted epitope (details available upon request). Positive recombinants were identified by restriction mapping and confirmed by DNA sequencing.

The retroviral vector backbone used in this study, pMSGV, is a derivative of the murine stem cell virus (MSCV)-based splice-μg vector (MSGV), which uses a MSCV long terminal repeat and has been previously described (26). The TCR α-polio internal ribosomal entry site (IRES)-TCR β DNA fragment for each pair of wt or high-affinity IG4 TCR chains were generated and assembled by PCR and cloned into pMSGV (Xhol/EcoRI) to generate MSGV-TCR α-IRES-TCR β retroviral vectors. Generation of PGI3 packaging cell lines and retroviral transduction of stimulated PBLs were conducted as previously described (23).

FACS analysis, CD4/CD8 cell separation, and Ab-blocking assays

Cell surface expression of human CD3, CD4, and CD8 molecules on PBL was determined by specific Ab conjugate staining (FITC- or PE-conjugated Abs; BD Biosciences). Human Vβ8, Vβ13.1, and HLA-A2-specific mAb-PE conjugates were supplied by Immunotech. PE-labeled NY-ESO-1 (165V), MART-1 (27L), and gp100 (210M) peptide/HLA-A2 streptavidin tetrarmers were purchased from Beckman Coulter. Immunofluorescence, analyzed as the relative log fluorescence of ~1 × 106 live cells, was measured using a FACSCalibur flow cytometer (BD Biosciences).

CD4+ and CD8+ cells were separated using a magnetic bead-based approach for both negative and positive selection of those population subsets (Dynal Biotech and Miltenyi Biotec).

In Ab-blocking experiments, peptide-pulsed T2 cells (5 × 105 cells/10 μl) were incubated with each mAb at a concentration of 10 μg/ml for 30 min at 37°C in a flat-bottom 96-well plate. T cells (5 × 105 cells/well) were then added and incubated with target cells overnight at 37°C. The supernatants were harvested and assayed for IFN-γ production by ELISA.

Cytokine release assays

PBL cultures were tested for reactivity in cytokine-release assays using commercially available ELISA kits (IFN-γ; Endogen). T2 cells were pulsed with peptide at indicated concentrations in R/10 medium for 2 h at 37°C, followed by washing (three times) before initiation of cocultures. Stimulator APCs and responder T cells were cocultured for 24 h. Cytokine secretion was measured in culture supernatants diluted to be in the linear range of the assay.

51Cr-release assays

The ability of the TCR-transduced T cells to lyse Ag-specific peptide-pulsed target cells was measured using a 51Cr-release assay as described previously (19). Briefly, 1 × 105 target cells were labeled with 1 × 106 cpm 51Cr sodium chromate (GE Healthcare). Labeled target cells (5 × 104) were pulsed with peptide for 1 h and incubated with effector cells at the ratios indicated in the text for 4 h at 37°C in 0.2 ml of R/10 medium. Harvested supernatants were counted using a Wallac 1470 Wizard gamma counter (PerkinElmer). Total and spontaneous 51Cr release was determined by incubating 5 × 104-labeled target cells in either 2% SDS or R/10 medium. The percentage of 51Cr release was calculated as follows: percent-specific lysis = ((specific 51Cr release – spontaneous 51Cr release)/(total 51Cr release – spontaneous 51Cr release)) × 100.

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Results

High-affinity TCRs bind pMHC tetramers with high Ag specificity

Previous studies demonstrated that soluble versions of high-affinity TCRs selected against NY-ESO-1 p157–165 Ag bound specifically to NY-ESO-1 peptide-HLA-A2 when assayed by Biacore surface plasmon resonance and flow cytometry (17, 18). A selection was made of genes encoding TCR/\lambda H9251/TCR/\lambda H9252 pairs with a wide range of monovalent in vitro Ag-binding kinetics to determine whether differing biological activities were observed in the multivalent cellular context (KD 4 \text{mM} to 26 \text{pM}, Fig. 1A). To test whether human primary T lymphocytes expressing these TCRs retained specificity, IVT RNA for both \lambda H9251- and \lambda H9252-chains from the wt 1G4 or mutant TCR pairs were coelectroporated into CD8/T cells and the transfected T cells were subjected to flow cytometry analysis by pMHC-tetramer staining. Both wt and high-affinity TCR-transfected CD8 T cells specifically bound to the NY-ESO-1 tetramer (they did not bind to control MART-1 and gp100 tetramers, Fig. 1B). The previously reported NY-ESO-1 TCR, ET-8F (23) bound less tetramer than the wt 1G4 (2.5 and 16%, respectively, background 0.3–0.4%) suggesting that 1G4 is a higher affinity TCR than ET-8F.

The percentage of specific tetramer binding of the mutant high-affinity TCR-transfected T cells were 5-fold higher than that of wt TCR-transfected T cells. The levels of staining observed when using an anti-V/\lambda H9252 13.1 Ab (IMMU222) were consistently lower in T cells transfected with the mutant TCR than those observed with cells that had been transfected with the wt TCR, while tetramer binding was significantly higher in the cells transfected with the mutant TCRs. These findings indicated that these mutations of the CDR3 region change the conformation of the TCR protein and therefore, its ability to be recognized by the anti-V/\lambda H9252 13.1 Ab, and suggest that the differences in transfection efficiency were not responsible for the enhanced tetramer binding observed with the high-affinity TCRs.

Cellular Ag recognition by high-affinity TCR expressed on CD8\textsuperscript{+} T cells is affinity dependent

To test whether T cells redirected with high-affinity TCRs would convey better function than wt TCR-expressing T cells, we compared the function of CD8\textsuperscript{+} T cells expressing the highest affinity TCRs (monomer KD values ranging from 84 nM to 26 pM) with two wt TCRs by coculture of TCR-transfected cells with peptide-pulsed T2 cells (Table I, Expt. 1). Transfer of wt 1G4 TCR demonstrated specific cytokine production to 0.1 nM NY-ESO-1 peptide. As seen in tetramer-binding assays (Fig. 1B), CD8 T cells electroporated with wt ET-8F were less reactive (no reactivity observed below 10 nM peptide) than 1G4-engineered cells. In contrast to the wild-type TCRs, CD8 T cells expressing high-affinity TCRs exhibited cross-reactivity as demonstrated by recognition of MART-1 peptide-pulsed cells (Table I, Expt. 1). A lack of specificity was also observed in a second experiment conducted using CD8\textsuperscript{+} PBL cells from a different donor. In this experiment, T cells transfected with the highest affinity TCR, c58/c61 (26 pM), as well as the next highest affinity TCR, c5c/100 (5 nM) released equivalent levels of cytokine IFN-\gamma in response to culture
with unpulsed T2 cells (Table I, Expt. 2). The CD8+ T cells transfected with the TCR c12/c2 (450 nM), retained Ag specificity, but demonstrated reduced Ag reactivity relative to T cells transfected with the 1G4 wt TCR (Table I, Expt. 2). This lack of specific T cell recognition observed in cells transfected with the high-affinity TCR was not due to novel hybrid α TCRs/β TCRs formed between the endogenously expressed and the transfected TCR chains, as no reactivity was seen when either exogenous TCR α (c58) or β (c61) chain was separately transfected into CD8+ T cells (data not shown).

To determine whether the cross-reactivity observed for T2 cells was HLA-A2 restricted, a panel of HLA-A2+ or HLA-A2− target cell lines were tested (Table II). Five HLA-A2+ cell lines (CIR-A2, RCC1, RCC2, C1R, and C1R-A2) at different concentrations were tested with the c12/c2 TCR at varying concentrations of the NY-ESO-1 peptide 157–165 (165V) at the indicated concentrations. Control peptide was 1000 nM MART-1 27–35. Control TCRs were anti-NY-ESO-1 ET-8F wt and a gp100-specific TCR. Two independent electroporations were performed using different PBL donors (Expts. 1 and 2). Values in bold denote specific reactivity. Data are the mean values (picograms per milliliter) of triplicate samples, with SD in parentheses (representative of four experiments).

### Table I. Nonspecific production of IFN-γ by TCR-transferred CD8 T cells

<table>
<thead>
<tr>
<th>Expt. 1</th>
<th>1G4 wt (32 μM)</th>
<th>17,144 (1,575)</th>
<th>7,447 (818)</th>
<th>2,195 (338)</th>
<th>0</th>
<th>ND</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>c5c100 (5 nM)</td>
<td>2,735 (861)</td>
<td>25,569 (1,255)</td>
<td>22,266 (114)</td>
<td>23,847 (490)</td>
<td>ND</td>
<td>31,148 (11,244)</td>
<td></td>
</tr>
<tr>
<td>c5c100 (5 nM)</td>
<td>5,107 (288)</td>
<td>3,775 (192)</td>
<td>3,235 (86)</td>
<td>3,514 (177)</td>
<td>ND</td>
<td>4,722 (155)</td>
<td></td>
</tr>
<tr>
<td>c10c1 (84 nM)</td>
<td>5,961 (521)</td>
<td>5,611 (677)</td>
<td>3,011 (136)</td>
<td>3,553 (195)</td>
<td>ND</td>
<td>5,992 (219)</td>
<td></td>
</tr>
<tr>
<td>ET-8F wt</td>
<td>2,683 (771)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Expt. 2</th>
<th>1G4 wt (32 μM)</th>
<th>3,763 (67)</th>
<th>1,131 (28)</th>
<th>0</th>
<th>0</th>
<th>ND</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>c5c100 (26 pm)</td>
<td>4,356 (200)</td>
<td>3,212 (33)</td>
<td>4,189 (145)</td>
<td>4,205 (62)</td>
<td>3,809 (117)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>c5c100 (5 nM)</td>
<td>2,603 (38)</td>
<td>1,893 (41)</td>
<td>2,441 (45)</td>
<td>2,410 (180)</td>
<td>2,182 (91)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>c12/c2 (450 nM)</td>
<td>1,516 (13)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>ET-8F wt</td>
<td>381 (5)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>gp100 TCR</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

### Table II. HLA-A2 restriction of TCR transfected CD8 T Cells

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>HLA-A2 (MFI)</th>
<th>Peptide conc.</th>
<th>1G4 wt (32 μM)</th>
<th>c5c100 (26 pm)</th>
<th>c5c100 (5 nM)</th>
<th>c10c1 (84 nM)</th>
<th>MART-1 wt/c59 (4 μM)</th>
<th>No RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIR-A2 (783)</td>
<td>100 nM</td>
<td>342 (26)</td>
<td>217 (15)</td>
<td>126 (35)</td>
<td>129 (2)</td>
<td>272 (78)</td>
<td>33 (25)</td>
<td></td>
</tr>
<tr>
<td>CIR-A2</td>
<td>10 nM</td>
<td>310 (20)</td>
<td>269 (2)</td>
<td>92 (12)</td>
<td>171 (42)</td>
<td>309 (118)</td>
<td>30 (7)</td>
<td></td>
</tr>
<tr>
<td>CIR-A2</td>
<td>1 m</td>
<td>172 (38)</td>
<td>279 (5)</td>
<td>106 (31)</td>
<td>218 (62)</td>
<td>161 (9)</td>
<td>26 (7)</td>
<td></td>
</tr>
<tr>
<td>CIR-A2</td>
<td>0.1 m</td>
<td>8 (3)</td>
<td>277 (22)</td>
<td>90 (20)</td>
<td>187 (3)</td>
<td>102 (8)</td>
<td>29 (3)</td>
<td></td>
</tr>
<tr>
<td>CIR-A2</td>
<td>0.1 nM</td>
<td>8 (3)</td>
<td>231 (21)</td>
<td>68 (1)</td>
<td>192 (42)</td>
<td>73 (7)</td>
<td>19 (6)</td>
<td></td>
</tr>
<tr>
<td>CIR</td>
<td>0</td>
<td>27 (9)</td>
<td>38 (4)</td>
<td>6 (2)</td>
<td>12 (9)</td>
<td>12 (6)</td>
<td>20 (2)</td>
<td></td>
</tr>
<tr>
<td>1558EBV</td>
<td>100 nM</td>
<td>1,288 (195)</td>
<td>880 (180)</td>
<td>1113 (181)</td>
<td>793 (174)</td>
<td>1437 (12)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1558EBV</td>
<td>100 nM</td>
<td>1,112 (169)</td>
<td>952 (192)</td>
<td>1049 (89)</td>
<td>563 (69)</td>
<td>1738 (119)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1558EBV</td>
<td>100 nM</td>
<td>1,112 (169)</td>
<td>952 (192)</td>
<td>1049 (89)</td>
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<td>563 (69)</td>
<td>1738 (119)</td>
<td>0</td>
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</tr>
</tbody>
</table>

### Notes
- IFN-γ production by purified CD8 T cells electroporated with IVT RNA for wt 1G4 or mutant high-affinity TCRs. Lymphocytes were cocultured overnight with T2 cells pulsed with serially diluted NY-ESO-1 peptide 157–165 (165V) at the indicated concentrations. Control peptide was 1000 nM MART-1 27–35. Control TCRs were anti-NY-ESO-1 ET-8F wt and a gp100-specific TCR. Two independent electroporations were performed using different PBL donors (Expts. 1 and 2). Values in bold denote specific reactivity. Data are the mean values (picograms per milliliter) of triplicate samples, with SD in parentheses (representative of four experiments).
1558EBV, 697EBV, RCC1, and RCC3) were pulsed with NY-ESO-1 peptide and used as stimulators for TCR-electroporated CD8 cells. As was observed with T2 cells, non-peptide-pulsed stimulator cells induced cross-reactive cytokine production from CD8 cells electroporated with IVT TCR RNA for wt 1G4, wt ET-8F, and four mutated TCRs; c58/c61 (26 pM), c5/c100 (5 nM), c10/c1 (84 nM), or wt α/ε59 (4 μM) TCR. T cells were cocultured with 3HCr-labeled peptide-pulsed T2 cells; 1 μM NY-ESO-1 or 1 μM Flu-MP and the amount of lysis was determined after 4 h. GFP RNA-transfected cells served as control. E:T was as shown; data are representative of two experiments.

The effects of TCR affinity on target cell lysis were next investigated. The results demonstrated that CD8+ T cells transfected with the wt 1G4 as well as the wt ET-8F TCR lyse target cells pulsed with the NY-ESO-1 peptide but failed to lyse targets pulsed with the control influenza peptide (Fig. 2). Non-specific killing of peptide-pulsed T2 was observed for CD8+ T cells transfected with mutant TCRs at affinities ranging from 84 nM to 26 pM, while those transfected with the wt α/ε59 (4 μM) TCR lysed target cells pulsed with the cognate peptide but did not lyse control targets. The cell lysis assay results were thus consistent with data obtained from cytokine production assays and further demonstrated that the specificity of target cell recognition was dependent upon TCR affinity.

CD8 molecules affect the cross-reactivity of high-affinity TCR-expressing T cells

The CD8 coreceptor protein enhances the formation of stable TCR-pMHC complex as an active participant in the T cell recognition complex (27, 28) To examine the influence of CD8 molecules on the specificity of T cell activation observed with the 1G4 TCR variants, studies were conducted using purified populations of CD8+ and CD4+ T cells. In contrast to results in CD8+ T cells, CD4+ T cells engineered with c5/c100 (5 nM) and c10/c1 (84 nM) TCRs were Ag specific as evidenced by both IFN-γ and IL-2 production upon stimulation with NY-ESO-1-pulsed but not with MART-1 or gp100 peptide-pulsed T2 cells (Fig. 3A). As observed with the transfected CD8+ T cells, CD4+ T cells that expressed the highest affinity c58/c61 (26 pM) TCR demonstrated cross-reactivity as evidenced by recognition of target cells pulsed with control peptides (Fig. 3A). As previously noted, the secretion of IFN-γ by CD8+ T cells expressing intermediate-affinity c10/c1 (84 nM) and c12/c2 (450 nM) TCRs were significantly less reactive than the CD8+ T cells expressing 1G4 wt (Fig. 3A), but these same TCRs transfected into CD4+ T cells secreted both IFN-γ and IL-2 at higher amounts than when 1G4 wt TCR was transfected into CD4+ T cells (Fig. 3A, lower panels).

51Cr-release cell lysis assays were conducted to test whether CD4+ T cells expressing these high-affinity TCRs were capable of specifically killing target cells. CD4+ T cells transfected with the 1G4 wt TCR, as well as those transfected with the intermediate-affinity TCRs, specifically lysed T2 cells pulsed with the NY-ESO-1 peptide, whereas those transfected with the high-affinity c58/c61 TCR lysed target cells pulsed with both the NY-ESO-1 and control gp100 peptide (Fig. 3B).

To test whether partial inactivation of CD8 function could reduce this TCR cross-reactivity, TCRs were transfected into CD8+ T cells and cocultured with targets in the presence of CD8-specific Abs (Fig. 4A). In the presence of anti-CD8 Ab the TCR-transfected T cells were still capable of responding to NY-ESO-1 peptide-pulsed T2 cells but to a reduced level compared with the control Ab (or no Ab) groups. To further examine the role of CD8 molecules in the nonspecific activation observed in cells transfected with intermediate- and high-affinity TCRs, CD4+ T cells that had been retrovirally transduced with constructs encoding the 1G4 TCR variants were transfected with IVT RNAs encoding either the CD8 α-chain alone or were cotransfected with RNAs encoding
both the CD8 α- and β-chains. The results indicated that CD4⁺ T cells expressing TCRs c5/c100 (5 nM) and c1/c10 (84 nM), that were then transfected with the CD8 α- and β-chain constructs, released significant levels of IFN-γ in response to target cells pulsed with both the specific NY-ESO-1 and the control gp100 peptides (Fig. 4B).

FIGURE 3. Specificity of high-affinity TCR-transfected CD4⁺ T cells. A, CD8⁺ or CD4⁺ purified T cells were electroporated with IVT RNA from the TCRs as indicated and cocultured with 2T3 pulsed with serially diluted NY-ESO-1 peptide, or 100 nM MART-1 or gp100 peptide. IFN-γ production for both CD8⁺ and CD4⁺ cells, and IL-2 production by CD4⁺ cells is shown (nd, not determined; data are representative of three experiments). B, TCR-transfected CD4⁺ T cells were cocultured with ⁵¹Cr-labeled peptide-pulsed T2 cells; 1 μM NY-ESO-1 or 1 μM gp100 and the amount of lysis was determined after 4 h. E:T was as shown. GFP IVT RNA-transfected cells served as control; data are representative of two experiments.

FIGURE 4. Effect of CD8 coreceptor on T cell activation of high-affinity TCR-transfected T cells. A, IFN-γ production by TCR, or GFP control, transfected CD8⁺ T cells were incubated with Abs as indicated and then cocultured with 100 nM NY-ESO-1 or gp100 peptide-pulsed T2 cells. Cytokine levels determined following overnight coculture; data are representative of two experiments. B, IFN-γ production by CD4⁺ T cells retrovirally transduced with TCR genes then electroporated with IVT RNA for CD8α, CD8α/CD8β or GFP. Engineered CD4⁺ T cells were cocultured overnight with peptide-pulsed T2 cells (100 nM NY-ESO-1 or gp100) and cytokine levels were determined. GFP-expressing retroviral vector MSGIN served as control.
Enhanced recognition of tumor lines by CD4$^+$ T cells expressing high-affinity TCR variants

One of the motivations for generating these high-affinity TCRs was their potential application in the T cell adoptive immunotherapy of cancer patients. To this end, we tested the recognition of a panel of melanoma cell lines by the variant TCRs transfected into CD8$^+$ or CD4$^+$ T cells. CD8$^+$ T cells transfected with 1G4 wt (32 $\mu$M) and c12/c2 (450 nM) TCRs recognized only the melanoma cell lines that are positive for both HLA-A2 and NY-ESO-1 protein, while the other three variant TCRs tested; c58/c61 (26 nM), c5/c100 (5 nM), and c10/c1 (84 nM) recognized both NY-ESO-1 protein positive and negative tumor lines (Fig. 5A).

The CD4$^+$ T cells transfected with the 1G4 wt TCR did not recognize any of the melanoma cell lines, presumably as a result of the relatively low levels of endogenously peptide presented on the surface of these target cells (Fig. 5B). In contrast, CD4$^+$ T cells expressing the intermediate-affinity c12/c2 (450 nM) and c10/c1 (84 nM) TCRs recognized the four tumor targets expressing both HLA-A2 and NY-ESO-1 protein positive and negative tumor lines (Fig. 5A).

Investigation into the basis of cellular cross-reactivity

The observation that RCCs that expressed relatively low levels of HLA-A2 failed to stimulate cross-reactive cytokine production by some of the high-affinity TCR-transfected CD8$^+$ T cells (Table II) suggested that HLA-A2 expression levels on stimulatig cells affected the specific activation of these T cells. To further test this hypothesis, varying amounts of IVT RNA-encoding HLA-A2 were transfected into the HLA-A2-negative cell line C1R (Fig. 6A). Using these cells as targets, we demonstrated that TCR c10/c1 (84 nM) transfected CD8$^+$ T cells manifest nonspecific T cell activation that was directly correlated with the HLA-A2 expression level (Fig. 6B).

The cross-reactivity displayed by high-affinity TCR-transfected CD8$^+$ T cells suggested that high-affinity TCRs could exhibit increased binding to some undefined endogenous Ag(s). To investigate this possibility, we transfected target cells with single-chain peptide-$\beta_2$ microglobulin-MHC trimers (SCT), which results in the preferential expression of a single peptide in association with the linked HLA-A2 molecule (25). HLA-A2 melanoma cell line 888mel was transfected, and similar levels of SCT expression were observed on the cell surface (Fig. 6D). The 888mel expressing a SCT for NY-ESO-1 p157–165 peptide was efficiently recognized by both 1G4 wt and by three high-affinity TCR-transfected CD8$^+$ T cells (Fig. 6C). In CD4$^+$ cells, the three mutated TCRs produced similar levels of cytokine when cultured with the NY-ESO-1 SCT-expressing cells, while the wt 1G4 TCR demonstrated minimal reactivity. Cells transfected with the c5/c100 (5 nM) and c10/c1 (84 nM) TCRs did not recognize control-transfected cells expressing MART-1 SCT or HLA-A2 alone in either CD8$^+$ or CD4$^+$ cells. Nonspecific activation was observed for the highest affinity TCR c58/c61 (26 nM) in both CD8$^+$ and CD4$^+$ T cells stimulated with both MART-1 SCT and HLA-A2 alone (Fig. 6C), but IFN-γ secretion was always less with the MART-1 SCT than with the

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HLA-A2-transfected cells. This suggests the existence of endogenous agonist peptide-HLA-A2 Ag(s) that were recognized by the very highest affinity TCR c58/c61 (26 pM) in the high-avidity cell-to-cell context.

Discussion

The results from our present study support the hypothesis that raising the affinity of a TCR by directing the free energy of binding toward the MHC helices leads to a loss in peptide Ag specificity as may be seen in negative selection in the thymus (14, 29). Previous study using a soluble form of the highest affinity TCR, c58/c61 (26 pM), showed that this TCR bound to the surface of T2 APC cells in a totally MHC-specific and peptide-dependent manner (18). Our pMHC-tetramer-staining experiments also demonstrated specific binding to CD8$^+$ T cells expressing this TCR (Fig. 1) and yet there was a near complete lose of specificity when this same TCRs was put in the context of a native T cell (Table I). This dramatic difference in sensitivity and specificity between soluble TCR molecules binding to target cells and T cell-mediated recognition may be due to the cumulative effects of the avidity of multiple TCR-pMHC interactions, as well as to other additional interactions between T cells and target cells.

Our data indicate that the density of MHC molecules on target cell and the presence of the CD8 coreceptor on the effector T cell were important variables in the activation of T cells transfected with these TCRs. Multiple studies have indicated that CD8 molecules play an important role in T cell recognition by stabilizing binding of the TCR to pMHC complexes, as well as by enhancing intracellular signaling, and lowering the threshold for T cell activation (27, 30–32). In the current study, incubation with an anti-CD8 Ab increased the specificity of CD8$^+$ T cells transfected with the high-affinity TCRs (Fig. 4A). In addition, CD4$^+$ T cells that expressed the high-affinity 1G4 TCRs specifically recognized NY-ESO-1/HLA-A2 target cells, where CD8$^+$ T cells expressing the same TCR demonstrated a lack of specificity (Fig. 5). These observations provide evidence that the CD8 molecule plays an important role in mediating the cross-reactivity observed with the high-affinity 1G4 TCR.

The finding that enhanced Ag-specific recognition and cross-reactivity to self Ags, or even peptide-independent T cell triggering, could be achieved by increasing MHC class I density on target cells (Table II) indicates that TCR avidity for the MHC molecule is a significant arbiter of T cell specificity, and that TCR affinities for MHC and peptide maybe interchangeable (33, 34). This is of biological importance, because different sites and cells in the thymus express MHC at different levels to effect positive and negative selections (35, 36). The study by Sandberg et al. (34) on the immunization of $\beta_{2m}$-deficient transgenic mice, which expressed subnormal levels of MHC class I, with an H-2D$^b$-restricted immunodominant lymphocytic choriomeningitis virus gp33 peptide, showed that resulting CTL were specific for the gp33 epitope when loaded onto cells with low MHC class I expression. However, unlike gp33-specific CTLs generated from normal MHC class I-expressing mice, these CTLs also killed cells expressing high levels of self-MHC class...
NY-ESO-1 high-affinity TCRs, when transferred into CD4+ T cells, durable cancer regression in some patients (40), and these anti-recognition and killing abilities. We have recently shown that the additional power to eradicate tumor cells by their direct strong tumor class I-restricted TCR-expressing CD4+ T cells may have similar potential for use in clinical cancer immunotherapy.

Disclosures
Alan D. Bennett, Yi Li, Peter E. Molloy, Steven M. Dunn, and Bent K. Jakobsen are employed by MedGene Ltd., Oxon, UK.

References
12. Alexander-Miller, M. A., G. R. Leggatt, and J. A. Berzofsky. 2016. Selective expansion of high- or low-avidity cytotoxic T lymphocytes and affinity for adop-
l. 119: 135–145.
30. Davis, S. J., and P. A. van der Merwe. 2003. TCR triggering: co-receptor-de-
compatibility complex class I stabilizes T cell receptor-antigen complexes at the
33. Fooksman, D. R., G. K. Gronvall, Q. Tang, and M. Edidin. 2006. Clustering class
34. Sandberg, J. K., K. Karre, and R. Glas. 1999. Recognition of the major histo-
compatibility complex restriction element modulates CD8 T cell specificity and
compensates for loss of T cell receptor contacts with the specific peptide. J. Exp.
the level of expression of class I major histocompatibility complex proteins on
thymic epithelial and dendritic cells influence the decision of immature thymo-
cytes between positive and negative selection. Proc. Natl. Acad. Sci. USA 95:
5235–5240.
Cancer/testis antigens: an expanding family of targets for cancer immunotherapy.
40. Morgan, R. A., M. E. Dudley, J. R. Wunderlich, M. S. Hughes, J. C. Yang,
2006. Cancer regression in patients after transfer of genetically engineered lym-