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Human Antitumor CD8⁺ T Cells Producing Th1 Polycytokines Show Superior Antigen Sensitivity and Tumor Recognition

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Adoptive transfer of T cells expressing transgenic TCR with antitumor specificity provides a hopeful new therapy for patients with advanced cancer. To fulfill a large need for TCR with high affinity and specificity for various tumor entities, we sought to identify parameters for rapid selection of CTL clones with suitable characteristics. Twelve CTL clones displaying different Ag sensitivities for the same peptide-MHC epitope of the melanoma-associated Ag tyrosinase were analyzed in detail. Better MHC-multimer binding and slower multimer release are thought to reflect stronger TCR-peptide-MHC interactions; thus, these parameters would seem well suited to identify higher avidity CTL. However, large disparities were found comparing CTL multimer binding with peptide sensitivity. In contrast, CD8⁺ CTL with superior Ag sensitivity mediated good tumor cytotoxicity and also secreted the triple combination of IFN- γ , IL-2, and TNF- α , representing a Th1 pattern often missing in lower avidity CTL. Furthermore, recipient lymphocytes were imbued with high Ag sensitivity, superior tumor recognition, as well as capacity for Th1 polycytokine secretion after transduction with the TCR of a high-avidity CTL. Thus, Th1 polycytokine secretion served as a suitable parameter to rapidly demark cytotoxic CD8⁺ T cell clones for further TCR evaluation. *The Journal of Immunology*, 2012, 189: 598–605.

Adoptive transfer of T cells is an effective therapy for treatment of patients with life-threatening viral infections (1–3), various forms of leukemia and lymphoma (4), as well as some solid tumors (5–7). Successful therapy requires transfer of Ag-specific T cells with sufficient functional avidity to eliminate infected or transformed cells. Poor tumor recognition by lower avidity T cells can be bypassed using patient lymphocytes that are engineered to express high-affinity transgenic TCR (tgTCR) (8). Designer lymphocytes can be rapidly prepared for cancer patients based on matched HLA allotype and tumor profile. Adoptive transfer of designer lymphocytes with higher affinity tgTCR improved clinical efficacy (9, 10) but carried the risk for greater on-target toxicity, dependent on Ag specificity (11).

Combined use of tgTCR with different specificities will likely be needed to avoid selection of tumor escape variants (10).

Libraries of TCR with suitable specificities and affinities must be collected for different tumor entities to fulfill broad clinical needs. A critical bottleneck exists in obtaining TCR with sufficient affinities that are specific for nonmutated self-proteins. Several approaches can be used, including obtaining T cells from mice that recognize human target proteins (12); however, xenogeneic TCR may not persist in vivo (13). This hindrance can be bypassed with a transgenic mouse carrying the full human TCR repertoire (14). TCR can be genetically modified to improve their affinity (15, 16) or higher affinity TCR can be acquired from allo-restricted, peptide-specific T cells (17–19).

Regardless of approach, many T cell clones must be analyzed in a time-consuming and costly process, further complicated by variations in proliferation and survival of individual clones in vitro. Therefore, rapid screening assays and selection parameters are needed to identify T cell clones whose TCR merit more study, despite limited T cell growth in vitro. Various functional parameters (20–22) and structural MHC-multimer-binding properties (23, 24) have been used for T cell characterization. Systematic comparisons, however, have used only a few clones with different specificities, leaving gaps in understanding the best overall measures for T cell selection.

Virus-specific T cells of humans and mice had proinflammatory polycytokine profiles that were critical for virus control (22, 25). Importantly, polyfunctional CD8⁺ T cells displayed increased Ag sensitivity (22). In this study, we performed a systematic comparison of 12 CD8⁺ antitumor CTL clones of identical peptide-MHC (pMHC) specificity to determine whether particular polycytokine patterns would also correlate with greater Ag sensitivity and superior tumor recognition. Transfer of function was assessed using TCR cloned from lower and higher avidity CTL. Functional and multimer-binding characteristics were compared to define the parameters best suited for rapid T cell selection in primary screening assays.

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S.W. designed and performed the experiments, and helped in drafting the manuscript. D.S., M.L., and W.U. contributed retroviral transduction technology. B.F. helped in the characterization of the TCR. B.M. helped in new assay developments. D.J.S. designed the concept and prepared the final manuscript. All authors reviewed and edited the manuscript.

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Abbreviations used in this article: ATCC, American Type Culture Collection; EC₅₀, half-maximum cytotoxicity; ICS, intracellular cytokine staining; MFI, mean fluorescence intensity; PFA, paraformaldehyde; pMHC, peptide-MHC; tgTCR, transgenic TCR.

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Materials and Methods

Cell lines

The human melanoma cell lines Mel-A375 (HLA-A2⁺, tyrosinase⁻), CRL-1619, American Type Culture Collection (ATCC); Mel 04A12 (HLA-A2⁺, tyrosinase⁺), transduced to secrete IL-2 (gift of P. Schrier, Department of Immunohematology, Leiden University Hospital, Leiden, The Netherlands); Mel-624.38 (26) and SK-Mel-23 (both HLA-A2⁺, tyrosinase⁺; gifts of M.C. Panelli, National Institutes of Health, Bethesda, MD); SK-Mel-29 (HLA-A2⁺, tyrosinase⁺; gift of P. Rieber, Institute of Immunology, Technical University Dresden, Dresden, Germany); WM 266.4 (HLA-A2⁺, tyrosinase⁺), CRL-1676, ATCC; as well as the lymphoid cell line T2 (HLA-A2⁺), CRL-1992, ATCC were cultured in RPMI 1640 medium supplemented with 12% FCS, 2 mM L-glutamine, 1 mM sodium-pyruvate, and 1 mM nonessential amino acids.

Culture of tyrosinase-specific CTL

Effector CTL were derived by de novo priming with tyrosinase RNA-loaded dendritic cells, sorted using an HLA-A*02:01-tyrosinase₃₆₉₋₃₇₇ multimer (27), cloned by limiting dilution, and maintained in culture as previously described (19). All CTL are HLA-A*02:01 restricted and recognize the tyrosinase₃₆₉₋₃₇₇ peptide.

MHC-multimer staining

T cells and tgTCR-modified PBL were incubated with 100 pg/ml multimer (kindly provided by D.H. Busch, Institute of Medical Microbiology and Hygiene, Technical University Munich) in PBS + 0.5% human serum for 20 min. Allophycocyanin-labeled, CD8-specific Ab (Sk-1; BD) was added for a further 25 min. After washing, cells were analyzed on a FACSCalibur (BD Biosciences). MHC-multimer on rates were made at 4°C using titrated multimer (6–100 pg/ml). For assay of MHC-multimer off rates, cells were washed after multimer binding at 4°C, resuspended in FACS buffer containing BB7.2 mAb (HB-82; ATCC), which captures detached multimers to prevent rebinding. Cells were incubated at 4°C, and samples were removed, fixed, and analyzed after 0–2 h by flow cytometry. PE-labeled HLA-B7 or HLA-A*02:01 multimers loaded with pp65 peptide served as specificity controls (data not shown).

Peptide loading of T2 cells

T2 cells (1×10^6 /ml) were incubated at 37°C and 5% CO₂ for 2 h with 10 μg/ml human β₂-microglobulin (Calbiochem) and titrated (10^{-5} – 10^{-11} M) tyrosinase₃₆₉₋₃₇₇ peptide (YMDGTSQV; Metabion). After washing, T2 cells were used in cytotoxicity, cytokine release, or intracellular cytokine staining (ICS) assays.

Cytokine assays

CTL (2×10^3 cells/100 μl) or TCR-modified PBL (5×10^4 cells/100 μl) were incubated with the melanoma cell lines (1×10^4 cells/100 μl). PMA (5 ng/ml) and ionomycin (750 ng/ml; both Sigma) were used for TCR-independent stimulation. Culture supernatants at 24 h were assessed by standard ELISA (OptEIA Human IFN-γ Set; BD Biosciences). Data represent mean values. Multiplex protein assays for IFN-γ, IL-2, TNF-α, IL-5, IL-13, GM-CSF, MIP-1β, IL-4, IL-6, and IL-12p70 were measured in supernatants of CTL cocultures with or without tyrosinase₃₆₉₋₃₇₇-pulsed T2 cells (10^{-5} M), as specified by the manufacturer (Bio-Rad Laboratories). Standard curves and concentrations were calculated with Bio-Plex Manager 4.1.1, as described previously (28). Th1 cytokines (IFN-γ, IL-2, and TNF-α) released by tgTCR-transduced PBL after coculture with tumor cells or peptide-pulsed T2 cells were measured by Cytometric Bead Array as specified (BD Biosciences). FACSCalibur data were acquired and analyzed with FlowJo software (Tree Star) and FCAP Array Software (BD Biosciences).

CD107a/b mobilization and ICS

CD107a/b mobilization and ICS assays were performed as described previously (29) using FITC-labeled Abs against CD107a and CD107b (clones H4A3 and H4B4; BD) and Golgi-Stop (brefeldin A at 10 μg/ml and monensin at 25 μM; both Sigma) at 37°C and 5% CO₂ for 6 h with CTL and unpulsed cells or T2 cells pulsed with 10^{-5} , 10^{-7} , and 10^{-9} M tyrosinase₃₆₉₋₃₇₇ peptide at a ratio of 2:1. Washed CTL were stained with CD8-AMcyan Ab (Sk1; BD) and 7-aminoactinomycin (Sigma) or live/dead fixable blue (Invitrogen) for 25 min at 4°C to detect dead cells. After washing, CTL were fixed with 1% paraformaldehyde (PFA) in PBS for 20 min at 4°C, washed thrice, and stored in PBS/1% FCS overnight. Cells were permeabilized by successive washes with 0.1 and 0.35% saponin

(Sigma). ICS used V450-conjugated IFN-γ (B27; BD Biosciences), allophycocyanin-labeled IL-2 (MQ1-17H12; BD), A700-conjugated TNF-α (MAb11; BD), and PE-labeled TCRβ (BMA031; Immunotech) or murine TCRβ (H57-597; BD) Abs for 20 min. Data acquisition and analysis used an LSRII (BD Biosciences) and FlowJo software (Tree Star). CD8⁺ TCRβ⁺ cells and CD8⁺ murine TCRβ⁺ cells were gated for CTL and TCR-transduced PBL, respectively.

Cytotoxicity assay

Cytotoxic activity was analyzed in a standard 4-h [⁵¹Cr]-release assay as published previously (19), using melanoma cell lines and peptide-loaded T2 cells as target cells. Percentages of specific, relative cytotoxicity and half-maximum cytotoxicity (EC₅₀) values were calculated as described previously (19).

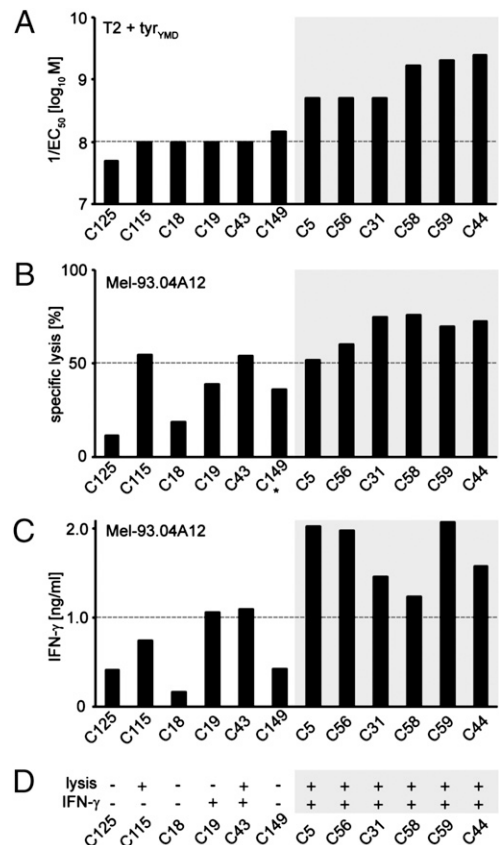


FIGURE 1. Functional characteristics of tyrosinase peptide-specific CTL. **(A)** Peptide concentrations required for EC₅₀ were determined in a 4-h [⁵¹Cr]-release assay against T2 cells loaded with different amounts of tyrosinase₃₆₉₋₃₇₇ peptide (10^{-11} – 10^{-5} M) at an E:T ratio of 10:1 (19). On the y-axis, peptide sensitivity is depicted as the reciprocal of the peptide concentration that was required to achieve EC₅₀ (1/EC₅₀). Each bar represents one T cell clone. Horizontal dashed line represents an EC₅₀ value of 10^{-8} M tyrosinase₃₆₉₋₃₇₇ peptide. Two groups are distinguished with respect to peptide sensitivity, with the higher avidity group indicated by a shaded background. The mean differences between groups are statistically significant (two-tailed Mann–Whitney *U* test, *p* < 0.01; *n* = 2). **(B)** The specific cytotoxicity of CTL measured in a 4-h [⁵¹Cr]-release assay against Mel-93.04A12 target cells (HLA-A2⁺tyrosinase⁺) at an E:T ratio of 10:1, except for C149*, which was assessed at 5:1 (*n* = 2; C56, C58, and C115: *n* = 3). Dashed horizontal line represents a cutoff of 50%. **(C)** CTL secretion of IFN-γ is shown after coculture with Mel-93.04A12 tumor cells (*n* = 4, except for C125 and C149 [*n* = 1], C18 and C19 [*n* = 3]). Dashed horizontal line represents a cutoff of 1.0 ng/ml. **(D)** Clones reaching cutoff values for specific lysis or IFN-γ release are marked with a plus sign; those falling below this cutoff are indicated with a negative sign.

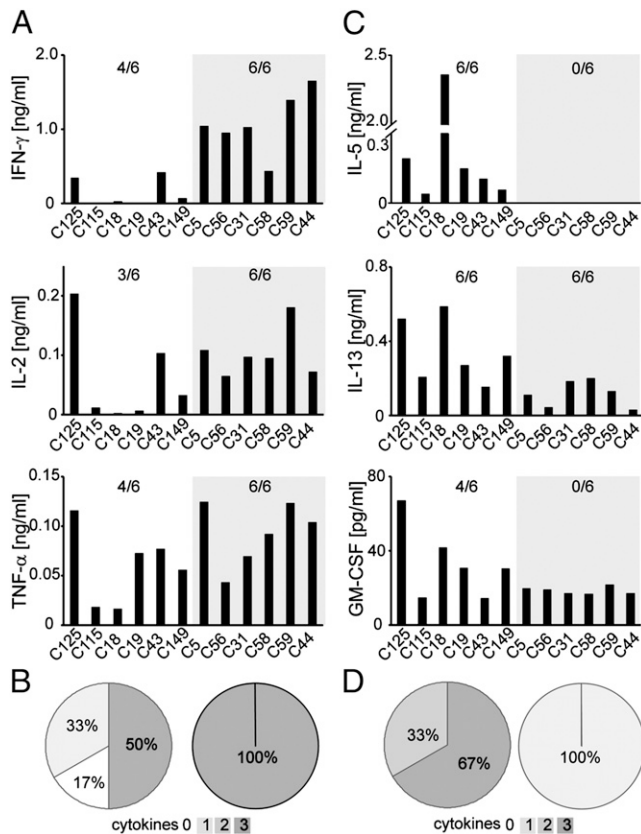
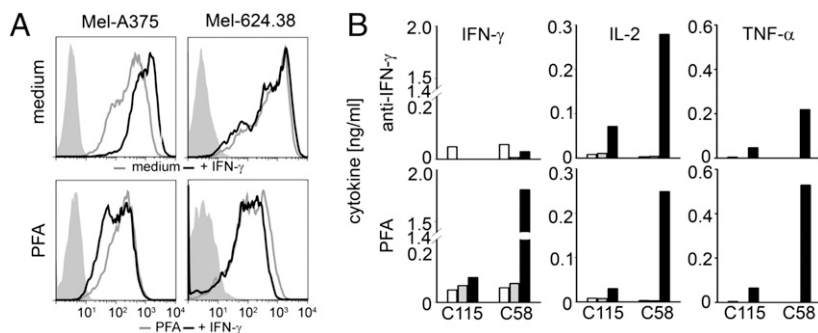


FIGURE 2. Capacity of CTL to simultaneously secrete multiple cytokines. Supernatants of cocultures of the 12 CTL with tyrosinase_{369–377} peptide-pulsed (10^{-5} M) T2 cells were harvested after 24 h and analyzed for cytokine content using a multiplex protein array. **(A)** Secretion is depicted for the Tc1 cytokines, IFN- γ , IL-2, and TNF- α after subtraction of background values secreted by cells stimulated with unloaded T2 cells ($n = 3$, except C125 and C149 [$n = 1$]). **(B and D)** Pie diagrams are depicted for the lower (*left*) and higher avidity (*right*) groups. CTL that secreted no cytokines above background levels are highlighted in white; CTL secreting one, two, and three cytokines above background levels are highlighted in increasing shades of gray. **(C)** Secretion of the Tc2 cytokines IL-5 and IL-13, as well as GM-CSF, is shown after subtraction of background secretion to unloaded T2 cells ($n = 2$, except C125 and C149 [$n = 1$]). Mean group levels of IFN- γ , IL-5, and IL-13 were significantly different (Mann–Whitney U test, $p < 0.01$).

Influence of IFN- γ on MHC expression and target cell recognition

Untreated and IFN- γ -stimulated (2 ng/ml; Imukin; Boehringer Ingelheim) melanoma cells were stained for MHC class I with W6/32 mAb (HB-95; ATCC) followed by detection with rat anti-mouse secondary Ab. Anti-IFN- γ Ab (50 μ g/ml; MMHG-1; Calbiochem) was added to cocultures of

FIGURE 3. Influence of IFN- γ on tumor cell recognition by CTL clones C115 and C58. **(A)** MHC class I expression on Mel-A375 and Mel-624.38 is shown in medium (*upper panels*) and after 1% PFA fixation (*lower panels*) after no (gray overlay) or with IFN- γ treatment (black overlay). Filled histograms depict the negative control. **(B)** Tc1 cytokine secretion is shown for C115 and C58 after coculture with Mel-A375 cells (light gray bars) or with Mel-624.38 cells (black bars). Background secretion of CTL alone is shown by white bars. *Top panels* show cocultures made in the presence of 50 μ g/ml anti-IFN- γ blocking Ab; *bottom panels* show cocultures made with PFA-fixed tumor cells.



CTL and melanoma cells to block CTL-derived IFN- γ . Alternatively, tumor cells were prefixed with 1% PFA to block response to IFN- γ .

Retroviral TCR gene transfer

Retroviral vector plasmids (19) were used for production of retroviral particles and subsequent transduction of T cells (30). Ten days after the second transduction, PBL were stained using allophycocyanin-labeled HLA-A2–tyrosinase_{369–377} multimer (Coulter) and FITC-labeled CD8-specific Ab (BD Biosciences). On day 15, TCR-transduced PBL were used in cocultures with target cells to assess cytokine secretion, cytotoxicity, and CD107a/b mobilization or ICS.

Statistical analysis

The nonparametric, two-tailed Mann–Whitney U test was used to evaluate statistical differences between data sets derived from the lower and higher avidity groups in functional assays using GraphPad Prism software version 5.0 for Windows (GraphPad). Statistical significance is indicated by * $p < 0.05$ and ** $p < 0.01$.

Results

MHC-multimer-sorted T cell clones vary in Ag sensitivity

We previously described 12 HLA-A2–restricted tyrosinase-specific CD8⁺ CTL clones derived through self- or allo-restricted dendritic cell priming in vitro (19). Specific CTL sorted with MHC-multimer and peptide screening confirmed that all CTL recognized the HLA-A2–tyrosinase_{369–377} pMHC epitope. The CTL displayed different peptide sensitivities in [⁵¹Cr]-release assays of T2 cells (HLA-A*02:01⁺) loaded with tyrosinase_{369–377} peptide. For reference, peptide concentrations yielding EC₅₀ are depicted in Fig. 1A. The CTL clustered in two groups, with six in a lower avidity group showing EC₅₀ values near 1×10^{-8} M peptide and six in a higher avidity group with EC₅₀ values between 2×10^{-9} and 4×10^{-10} M.

The CTL killed the melanoma cell line Mel-93.04A12 (HLA-A2⁺tyrosinase⁺) to varying degrees (Fig. 1B) but did not kill Mel-A375 cells (HLA-A2⁺tyrosinase⁻) (data not shown). Higher avidity CTL mediated $\geq 50\%$ tumor kill, whereas only two of the lower avidity CTL reached this level. IFN- γ secretion also varied, but always exceeded 1.0 ng/ml in the higher avidity group. Only two of the lower avidity CTL reached this level (Fig. 1C). Therefore, superior tumor cell recognition seen by greater cytotoxicity and higher IFN- γ secretion was associated with greater peptide sensitivity and distinguished the two groups, with only one exception (C43), when both parameters were used for CTL classification (Fig. 1D). Group differences in IFN- γ secretion were seen with additional melanoma cell lines (Supplemental Fig. 1A).

Higher avidity CTL secrete Th1 proinflammatory cytokines

Because many factors influencing variations in levels of pMHC on tumor cells may influence CTL recognition, we moved to a standardized assay using T2 cells pulsed with saturating amounts (10^{-5} M) of tyrosinase_{369–377} peptide. After 24-h coculture, cytokine

secretion patterns were measured with a multiplex protein array detecting IL-2, IL-4, IL-5, IL-6, IL-12p70, IL-13, GM-CSF, IFN- γ , MIP-1 β , and TNF- α . Only IFN- γ , IL-2, TNF- α , IL-5, IL-13, GM-CSF (Fig. 2), and MIP1- β (data not shown) were detected among the CTL. All higher avidity CTL made IFN- γ , but only four lower avidity CTL surpassed background levels (Fig. 2A). The mean group differences were significant (Mann-Whitney U test, $**p < 0.01$). Three lower avidity CTL secreted IL-2 and four released TNF- α above background, whereas all higher avidity CTL exceeded background for both cytokines. Although tending toward significance, these mean group differences did not reach significance because of the exceptionally high values of C125, which, however, showed very poor tumor cell kill (Fig. 1B).

The percentages of CTL in the two groups secreting one or more of the three Th1/Tc1 cytokines (hereafter Tc1-triplet) are depicted in Fig. 2B. All higher avidity CTL secreted the Tc1-triplet above background (Fig. 2B, right); only three lower avidity CTL produced this Tc1-triplet (Fig. 2B, left). All CTL released MIP-1 β , but in greater amounts by the higher avidity group (data not shown). Reciprocal patterns were seen for IL-5, IL-13, and GM-CSF (Fig. 2C). In particular, only lower avidity CTL produced IL-5 (Mann-Whitney U test, $**p < 0.01$), and levels of IL-13 levels were greater in this group (Mann-Whitney U test, $**p < 0.01$). GM-CSF release was very low throughout and was poor for discrimination. No higher avidity CTL showed a Th2/Tc2 (hereafter Tc2) pattern after peptide-specific stimulation (Fig. 2D). The CTL showed different capacities to release various cytokines after TCR-independent activation with PMA and ionomycin; these mostly followed the Tc1/Tc2-qualitative, but not -quantitative, patterns seen after TCR-dependent, peptide-specific stimulation (Supplemental Fig. 1B, 1C).

To determine whether CTL-derived IFN- γ directly increased MHC class I on tumor cells, and thereby influenced recognition, we analyzed two CTL (C115 and C58) that released different amounts of IFN- γ after tumor cell contact (Fig. 1C). Direct stimulation with exogenous IFN- γ (2 ng/ml) did not alter Mel-624.38, but Mel-A375 had increased class I expression (Fig. 3A, top row). Tumor cells prefixed with 1% PFA were blocked for IFN- γ response but still retained their normal MHC expression (Fig. 3A, bottom row). Thereafter, C115 and C58 were cocultured with tumor cells, either in the presence of IFN- γ -blocking Ab (Fig. 3B, top row) or using PFA-fixed tumor cells (Fig. 3B, bottom row). IFN- γ could not be detected in cultures with IFN- γ -blocking Ab, but IL-2 and TNF- α secretion remained greater for C58 despite the IFN- γ block. Likewise, recognition of PFA-treated tumor cells was better with C58 for the full Tc1-triplet. Thus, superior recognition by C58 did not depend on its capacity to modulate MHC class I via higher IFN- γ secretion.

The TCR of a higher avidity CTL transfers superior tumor cell recognition

To determine whether characteristics of original CTL would be recapitulated by TCR gene transfer, we modified recipient PBL to express TCR of C115 or C58, as described previously (19). CD8⁺ cells expressing tgTCR were enumerated by HLA-A2-tyrosinase₃₆₉₋₃₇₇ multimer binding, and representative results for one matched pair are shown in Fig. 4A. EC₅₀ values of cytotoxicity against peptide-pulsed T2 cells were determined for matched pairs in independent experiments (Fig. 4B). PBL expressing TCR-C58 (PBL-TCR-C58) always displayed greater peptide sensitivity than PBL expressing TCR-C115 (PBL-TCR-C115). EC₅₀ cytotoxicity values differed by almost one log (mean 5.1×10^{-9} versus 3.7×10^{-8} M), but these only trended toward significance (Mann-Whitney U test, $p = 0.11$). This paralleled the original CTL, both

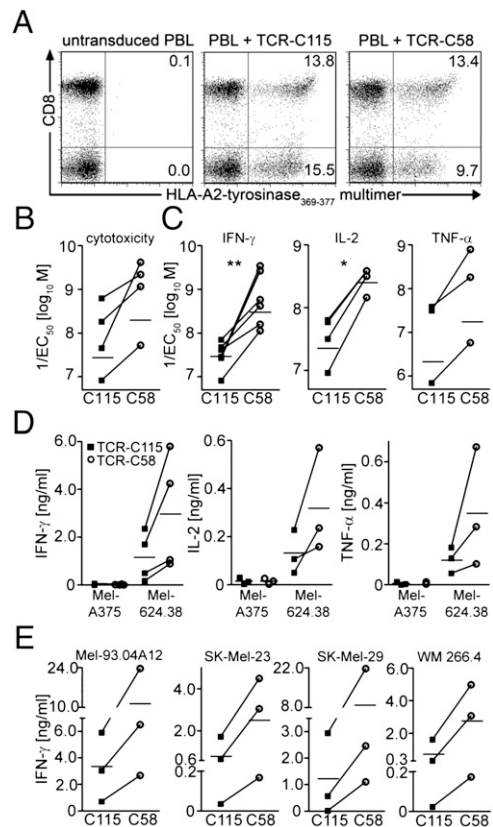


FIGURE 4. Functional characteristics of PBL transduced with two representative TCR. PBL of a healthy donor were transduced with a representative TCR of each group: C115 for the lower avidity group and C58 for the higher avidity group. These TCR represent D115 and T58 as reported previously (19). (A) On day 10 after retroviral transduction, PBL were stained using the HLA-A2-tyrosinase₃₆₉₋₃₇₇ multimer and a CD8-specific mAb. The percentage of CD8⁺multimer⁺ and CD8⁺multimer⁻ cells are shown in the upper and lower right quadrants, respectively. Results are representative of six independent experiments. (B) TCR-C115- and TCR-C58-transduced PBL were analyzed for their functional avidities as described in Fig. 1A. EC₅₀ cytotoxicity values were determined in a 4-h [⁵¹Cr]-release assay against T2 cells loaded with different amounts of tyrosinase₃₆₉₋₃₇₇ peptide (10^{-11} – 10^{-6} M) at E:T between 30:1 and 90:1. Filled squares (■) represent TCR-C115, and open circles (○) TCR-C58 transduced PBL from independent experiments ($n = 4$). (C) The 1/EC₅₀ values are depicted for cytokine secretion analyzed in cytokine bead assays using T2 cells loaded with titrated amounts of tyrosinase₃₆₉₋₃₇₇ peptide (10^{-11} to 10^{-6} M) as target cells. $*p < 0.05$ for IL-2 ($n = 3$), $**p < 0.01$ for IFN- γ ($n = 4$), n.s. for TNF- α ($n = 3$) (two-tailed Mann-Whitney U test). (D) IFN- γ ($n = 4$), IL-2 ($n = 3$), and TNF- α ($n = 3$) secretion in cocultures of PBL expressing TCR-C115 and TCR-C58 after stimulation with Mel-A375 melanoma cells (HLA-A2⁺tyrosinase⁻) or with Mel-624.38 cells (HLA-A2⁺tyrosinase⁺). Untransduced PBL showed no Ag-specific secretion (data not shown). (E) IFN- γ secretion is shown for TCR-transduced PBL cocultured with four different HLA-A2⁺tyrosinase⁺ melanoma cell lines ($n = 3$).

of which showed good tumor cell killing (Fig. 1B). In matched pairs, Tc1-triplet secretion was always induced in PBL-TCR-C58 with less peptide (Fig. 4C). The EC₅₀ values for IFN- γ ($**p < 0.01$) and IL-2 ($*p < 0.05$), but not TNF- α , were significantly different (Mann-Whitney U test). Only PBL-TCR-C115 produced IL-5 and IL-13 above background levels after peptide stimulation. They also secreted more GM-CSF (Supplemental Fig. 2A).

Differences in peptide sensitivity for cytotoxicity and cytokine release could be directly ascribed to the tgTCR because they were introduced into the same recipient PBL populations, expressed at

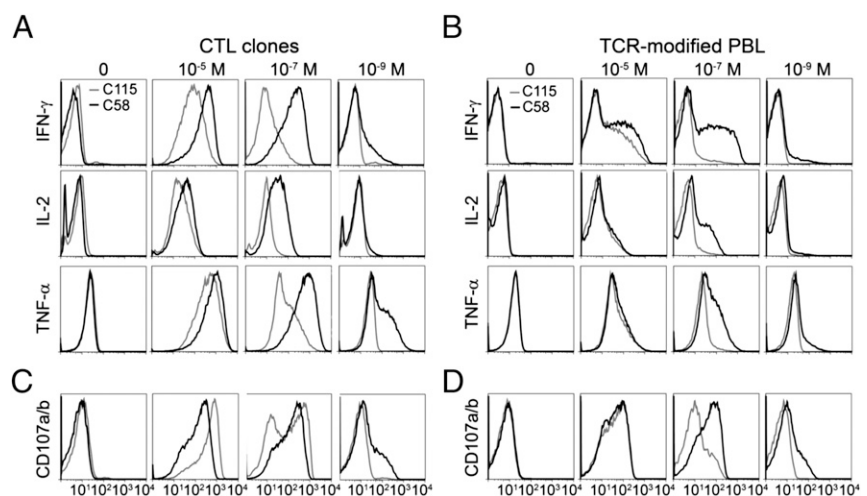
comparable levels, and assessed at the same time for each matched pair depicted in Fig. 4. Responses were Ag specific and required tgTCR expression because cytokine release required stimulation with specific tyrosinase_{369–377} peptide and unmodified PBL did not release these cytokines (data not shown).

PBL-TCR-C58 also secreted more Tc1-triplet cytokines than PBL-TCR-C115 after tumor cell stimulation. Again, recognition was tgTCR dependent and Ag specific because untransduced PBL did not respond and Mel-A375 (HLA-A2⁺ tyrosinase⁻) was not recognized (Fig. 4D). Differences were seen with additional melanoma lines (HLA-A2⁺ tyrosinase⁺; Fig. 4E), but these were not significant, perhaps because of wide fluctuations in pMHC. PBL-TCR-C115 also released more IL-5, IL-13, and GM-CSF after tumor cell stimulation (Supplemental Fig. 2B). However, mean differences were only significant when PBL expressing tgTCR were stimulated with specific peptide, likely because of complex variations in tumor cells. Importantly, however, was the finding that the reciprocal Tc1/Tc2 patterns of the original CTL were recapitulated by corresponding TCR transfer into PBL. TCR-transduced PBL showed comparable capacities to release Tc1 cytokines after TCR-independent activation with PMA and ionomycin (data not shown).

Higher avidity T cell clones display greater ICS after suboptimal peptide stimulation

ICS was tested as an alternative to analyze Tc1 polycytokine responses of the CTL, also allowing percentages of positive cells to be determined (Fig. 5, data not shown). Original C115 and C58 are shown in Fig. 5A, and PBL-TCR-C115 and PBL-TCR-C58 are shown in Fig. 5B, depicting the specific fraction of cells expressing the corresponding tgTCR. CTL were stimulated with T2 cells pulsed with excess (10^{-5} M) and two lower concentrations (10^{-7} and 10^{-9} M) of tyrosinase_{369–377} peptide. Background staining of nonstimulated cells served as a negative control. The original CTL stained for the Tc1-triplet after stimulation with 10^{-5} M peptide, with comparable mean fluorescence intensities (MFIs). In contrast, greater MFI was detected in C58 for the Tc1-triplet using 10^{-7} M peptide. Only a small fraction of C58 cells showed IFN- γ and TNF- α with 10^{-9} M peptide. PBL expressing the tgTCR showed similar trends, with no clear distinction at 10^{-5} M peptide and superior ICS for the Tc1-triplet in PBL-TCR-C58 at 10^{-7} M peptide. Minimal IFN- γ and TNF- α was seen in PBL-TCR-C58 at 10^{-9} M peptide, whereas no ICS was seen for PBL-TCR-C115.

FIGURE 5. ICS and CD107a/b mobilization of CTL C115 and C58, and the respective TCR-transduced PBL. **(A)** CTL C115 and C58 and **(B)** TCR-C115- and TCR-C58-transduced PBL were analyzed for simultaneous ICS for IFN- γ , IL-2, and TNF- α in coculture with T2 cells loaded with 10^{-5} , 10^{-7} , and 10^{-9} M tyrosinase_{369–377} peptide. Unpulsed T2 cells served as a negative control. Gating was performed on **(A)** living CD8⁺TCR β ⁺ cells and **(B)** living CD8⁺tgTCR⁺ cells. **(C)** CD107a/b mobilization is shown for CTL C115 and C58, and **(D)** for PBL transduced with TCR-C115 and TCR-C58 in coculture with T2 cells loaded with 10^{-5} , 10^{-7} , and 10^{-9} M tyrosinase_{369–377} peptide. Unpulsed T2 cells served as a negative control. Gating was performed as described in **(A)** and **(B)**. MFI values of CTL C115 and C58, and corresponding TCR-transduced PBL were not significantly different (two-tailed Mann–Whitney *U* test).



In addition, cytotoxic potential was measured by staining for CD107a/b (LAMP-1), a protein associated with release of cytolytic granules upon target cell contact (29). Original CTL expressed CD107 after stimulation with excess peptide (10^{-5} M), and C58 showed somewhat greater ICS at lower peptide concentrations (Fig. 5C). These distinctions were well recapitulated with the tgTCR populations (Fig. 5D). Although phenotypic shifts were seen in ICS and CD107 assays for both CTL and transgenic PBL, the MFIs were not significantly different (data not shown). Thus, measures of MFI in this assay did not match the significant quantitative differences detected by multiplex protein arrays.

T cell clones vary widely in MHC-multimer-binding characteristics

Because the CTL were isolated via HLA-A2-tyrosinase_{369–377} multimer, they could also be compared for multimer-binding parameters. Incubation with titrated amounts of multimer depicted multimer on rates. Loss of multimer over time, both with respect to percentage of positive cells and MFI, indicated the multimer off rates. All CTL showed variations in both measures (Supplemental Fig. 3, left). Importantly, ranking of CTL with the best to worst on or off rates did not correlate with their lower or higher avidity defined by peptide sensitivity (Supplemental Fig. 3, right). These results showed that multimer binding and functional parameters did not distinguish the same CTL groups.

Of particular interest were comparisons of C58 and C115, and the corresponding tgTCR-PBL (Fig. 6). Based on superior function, we expected C58 and PBL-TCR-C58 to have faster on rates and slower off rates. Surprisingly, opposite results were obtained whereby C115 and PBL-TCR-C115 showed faster on rates and slower off rates (Fig. 6A), indicating that they displayed greater TCR-pMHC stability (Fig. 6B, 6C).

Function, not phenotype, demarks optimal CD8⁺ CTL

The CTL were assessed for various surface markers, including CD3, CD25, CD28, CD40L, and CD62L, but discrete patterns that demarked the lower and higher avidity groups were not found (data not shown). Likewise, multimer staining (MFI) did not correlate with peptide sensitivity and superior tumor cell recognition. Rather, patterns of peptide-specific cytokine secretion and levels of tumor cell kill represented the prominent characteristics that distinguished the two groups, as illustrated in Table I. All higher avidity CTL simultaneously secreted the Tc1-triplet, but they produced no IL-5 and low IL-13 after peptide-specific stimulation. Fewer lower

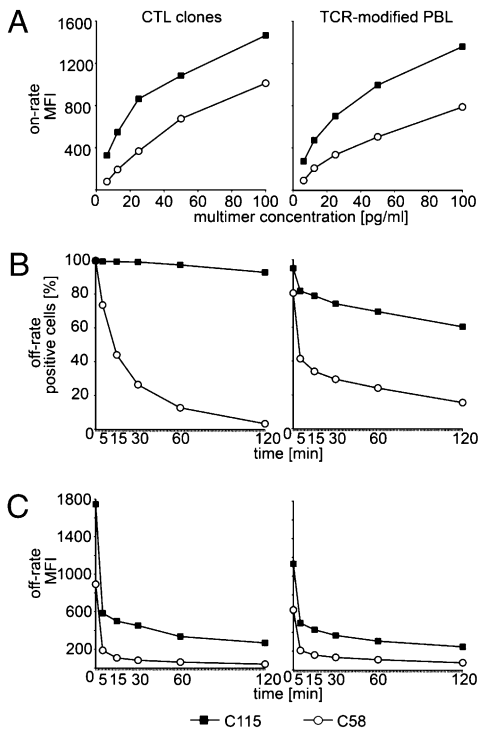


FIGURE 6. Multimer-binding analyses of CTL C115 and C58, and TCR-transduced PBL. Cells were analyzed for multimer binding using an HLA-A2–tyrosinase_{369–377} multimer. **(A)** For on-rate analyses, T cells were labeled for 45 min at 4°C with titrated concentrations of HLA-A2–tyrosinase_{369–377} multimer and fixed with 1% PFA. **(B and C)** For off-rate analyses, T cells were stained for 45 min at 4°C with HLA-A2–tyrosinase_{369–377} multimer, and either fixed immediately with 1% PFA (0 min) or after indicated time points at 4°C after cell washing to remove unbound multimers followed by addition of HLA-A2–specific Ab to prevent rebinding of dissociated multimers. Loss of multimer binding was assessed over time at 4°C as percentage positive cells (B) and MFI (C).

avidity CTL secreted the Tc1-triplet, but they always produced IL-5 and more IL-13 after peptide-specific stimulation. Also, higher avidity CTL mostly showed greater kill of tumor cells compared with lower avidity CTL. Thus, a functional biomarker of six parameters, including Tc1-triplet cytokine production with missing or modest Tc2 cytokine secretion, and greater cytotoxicity

served well to demark CTL of higher avidity. A summed rank analysis of the 12 CTL, combining Tc1/Tc2 cytokine patterns and cytotoxicity, led to clustering of the lower avidity CTL in the lowest six positions, whereas the higher avidity CTL ranked in the top six positions. The cytokine biomarker analysis could be performed using 8×10^3 CTL, which can normally be cultured in 3–4 wk, starting from a single cell. A second level screen for cytotoxicity requires 4×10^4 cells (Supplemental Table I).

Discussion

The goal of these studies was to identify functional or MHC multimer-binding parameters for primary screening to rapidly identify CD8⁺ T cell clones with superior antitumor function. We sought to determine whether the capacity of CTL to simultaneously produce particular cytokines would correlate with greater peptide sensitivity and improved tumor recognition. We also assessed the relationship between functional parameters and MHC-multimer binding. Furthermore, we determined whether characteristics of representative CTL would be recapitulated by TCR gene transfer, thereby improving development of designer lymphocytes. These questions were addressed using 12 CTL with identical pMHC specificity but varying Ag sensitivity that were previously characterized extensively for tumor specificity (19). The CTL were cryopreserved soon after isolation so that retrospective assays performed in this study reflect responses expected for newly emerging clones. The TCR of two CTL with different peptide sensitivities were used to assess transfer of function to recipient PBL.

Sensitive ELISA measuring either GM-CSF or IFN- γ have been used routinely to assess emerging T cell clones (16, 19, 22). In our studies, low levels of GM-CSF made it poorly suited to demark CTL with greater peptide sensitivity. IFN- γ secretion was more reliable, but inclusion in a multiplex-cytokine protein array delivered substantially more information. Comparisons of 10 factors showed that the Tc1-triplet of cytokines (IFN- γ , IL-2, TNF- α) successfully earmarked all higher avidity CTL but also identified several lower avidity CTL. Better group discrimination was achieved by including IL-5 and IL-13.

ICS assays of Tc1 cytokines also showed differences in lower and higher avidity CTL, but only when at least two concentrations of peptide were compared: excess peptide (10^{-5} M) demonstrated CTL capacity to produce Tc1 cytokines, whereas 10^{-7} M peptide

Table I. Sum-rank analyses of lower and higher avidity CTL clones

Clones	Tc1 Cytokines, pg/ml ^a			Tc2 Cytokines, pg/ml ^a		CTX ^{a,b}	Sum ^c	Rank ^d
	IFN- γ	IL-2	TNF- α	IL-5	IL-13			
Lower avidity CTL								
C125	349 (5)	204 (12)	116 (10)	192 (2)	522 (2)	11 (1)	32	5
C115	0 (1)	13 (3)	18 (2)	40 (6)	210 (5)	55 (7)	24	4
C18	31 (3)	3 (1)	16 (1)	2360 (1)	589 (1)	19 (2)	9	1
C19	12 (2)	7 (2)	73 (6)	148 (3)	272 (4)	39 (4)	21	2
C43	426 (6)	104 (9)	77 (7)	104 (4)	156 (8)	54 (6)	40	6
C149	72 (4)	33 (4)	56 (4)	57 (5)	324 (3)	36 (3)	23	3
Higher avidity CTL								
C5	1044 (10)	109 (10)	125 (12)	0 (10)	113 (10)	52 (5)	57	10
C56	953 (8)	65 (5)	43 (3)	0 (10)	45 (11)	60 (8)	45	7
C31	1027 (9)	97 (8)	70 (5)	0 (10)	185 (7)	75 (11)	50	8.5
C58	435 (7)	95 (7)	93 (8)	0 (10)	201 (6)	76 (12)	50	8.5
C59	1394 (11)	181 (11)	124 (11)	1 (7)	133 (9)	69 (9)	58	11
C44	1660 (12)	72 (6)	104 (9)	0 (10)	32 (12)	72 (10)	59	12

^aNumbers in brackets represent rank (1–12) from lowest to highest value.

^bValues represent percentage specific lysis of HLA-A2⁺tyrosinase⁺ melanoma cells (Me1-93.04A12).

^cAddition of the individual ranks in brackets for Tc1 cytokines, Tc2 cytokines, and cytotoxicity (CTX) for each CTL.

^dFinal rank from lowest (worst) to highest (best) score based on the sum of individual ranks for each CTL.

showed greater ICS in higher avidity cells. CD107 mobilization also showed CTL differences after stimulation with 10^{-5} and 10^{-7} M peptide. Importantly, ICS and CD107 staining were recapitulated in transgenic PBL expressing TCR-C58 and TCR-C115, respectively. However, MFI values measured in ICS were not statistically significant, in contrast with values detected in multiplex assays. Based on this fact, as well as the need for many fewer cells, the multiplex protein array provided a faster and more reliable method for primary T cell selection. Preliminary studies suggest that further improvement is possible if two concentrations of specific peptide (10^{-5} and 10^{-7} M) are used for stimulation in multiplex assays (data not shown). Upon further expansion of Tc1-selected clones, a second level assay for tumor cell cytotoxicity would earmark CTL with presumed optimal antitumor functions.

Because these CTL were isolated by MHC-multimers, they could be compared for multimer-binding parameters. TCR-pMHC interactions can be judged by MFI of multimer binding as well as multimer on and off rates. All three measures correlated significantly with each other; however, none of these structural parameters correlated significantly with better CTL function assessed by the various functional assays (data not shown). This was clearly exemplified by analysis of C115 and C58, where C58 showed greater peptide sensitivity and superior function, but C115 had better multimer-binding parameters. Importantly, this reciprocal pattern was retained when the corresponding TCR were expressed in PBL. Multimer binding was useful for initial T cell isolation from priming cultures but failed to identify the best CTL with respect to Ag sensitivity and tumor recognition. Thus, functional parameters were needed for primary clone selection. New multimer-binding assays may overcome this deficit in the future. Biocore measurements are more precise (31, 32) but clearly not feasible for primary CTL screening. Others have also noted discrepancies in multimer binding and tumor cell recognition (33–36). Our studies add further support through analysis of multiple CTL with identical specificities. Preliminary live-cell imaging indicates that lower and higher avidity CTL differ in time to attachment and detachment from tumor cells, which may contribute to these discrepancies (data not shown).

The transfer of TCR from representative lower and higher avidity CTL into the same PBL populations allowed assessment of the therapeutic form needed for TCR gene therapy. Importantly, TCR-transgenic PBL recapitulated the properties of original CTL. Thus, polyfunctional Tc1 cytokine secretion rather than multimer-binding parameters served best for early clone selection and concurrently pinpointed important characteristics needed for designer lymphocytes. In this study, the capacity to secrete IL-2 may be particularly important because CD8⁺ TCR-transgenic PBL producing this IL may survive longer in vivo in the absence of CD4⁺ helper cells.

The association of Tc1- and Tc2 cytokine patterns with higher and lower avidity CTL, respectively, harken back to earlier studies of murine CD4⁺ T cells showing that potency of TCR-pMHC interactions regulated cytokine gene transcription and impacted on emergence of Th1 or Th2 responses (37–39). These original studies used altered peptide ligands to assess single transgenic TCR or mutants thereof. Surprisingly, our CD8⁺ CTL with naturally occurring TCR variations displayed Tc1 or Tc2 cytokine patterns after stimulation with an identical pMHC ligand, dependent on the peptide sensitivity of the individual CTL. These results suggest that selection of TCR based on peptide sensitivity may allow differential cytokine secretion to be manipulated in TCR gene therapy.

Studies of virus-specific CD8⁺ T cells demonstrated that poly-cytokine production of IFN- γ , IL-2, and TNF- α was associated with superior virus control in vitro and in vivo (22, 25, 40). Furthermore, the CD8⁺ HIV-specific clones that secreted these cytokines displayed greater peptide sensitivity (22). Our results extend these findings to antitumor responses, whereby greater peptide sensitivity and superior tumor recognition were also found for CTL that secreted this Tc1-triplet of cytokines. Thus, human CD8⁺ T cells with greater Ag sensitivity and superior antiviral or antitumor function share the common feature of Tc1 polycytokine secretion. Future clinical studies will show whether transfer of these characteristics to patient PBL via selected TCR will improve clinical outcomes in TCR gene therapy of tumors.

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Disclosures

The authors have no financial conflicts of interest.

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