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TCRs Genetically Linked to CD28 and CD3ε Do Not Mispair with Endogenous TCR Chains and Mediate Enhanced T Cell Persistence and Anti-Melanoma Activity

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Adoptive transfer of T cells that are gene engineered to express a defined TCR represents a feasible and promising therapy for patients with tumors. However, TCR gene therapy is hindered by the transient presence and effectiveness of transferred T cells, which are anticipated to be improved by adequate T cell costimulation. In this article, we report the identification and characterization of a novel two-chain TCR linked to CD28 and CD3ε (i.e., TCR:28ε). This modified TCR demonstrates enhanced binding of peptide–MHC and mediates enhanced T cell function following stimulation with peptide compared with wild-type TCR. Surface expression of TCR:28ε depends on the transmembrane domain of CD28, whereas T cell functions depend on the intracellular domains of both CD28 and CD3ε, with IL-2 production showing dependency on CD28:1CK binding. TCR:28ε, but not wild-type TCR, induces detectable immune synapses in primary human T cells, and such immune synapses show significantly enhanced accumulation of TCR transgenes and markers of early TCR signaling, such as phosphorylated LCK and ERK. Importantly, TCR:28ε does not show signs of off-target recognition, as evidenced by lack of TCR mispairing, as well as preserved specificity. Notably, when testing TCR:28ε in immune-competent mice, we observed a drastic increase in T cell survival, which was accompanied by regression of large melanomas with limited recurrence. Our data argue that TCR transgenes that contain CD28, and, thereby, may provide T cell costimulation in an immune-suppressive environment, represent candidate receptors to treat patients with tumors. The Journal of Immunology, 2014, 193: 5315–5326.

Metastatic melanoma is a highly lethal disease with an incidence that continues to increase. Adoptive transfer of tumor infiltrating lymphocytes or TCR-engineered T cells showed clinical success in the treatment of metastatic melanoma (1, 2). For example, T cells expressing TCR transgenes that were directed against the HLA-A2–restricted Ags MART-1, gp100, or NY-ESO-1 mediated objective clinical responses in 12–45% of patients with metastatic melanoma (3, 4). Despite these significant antitumor responses, clinical studies are hindered by both toxicity and the transient nature of tumor regression in the majority of patients.

Treatment-related toxicity became evident from studies with TCRs, in particular those with high affinity directed against Ags that are overexpressed on tumors but also expressed (in some cases a highly similar Ag) on healthy cells. Toxicities included severe, but treatable, inflammation of skin, eyes, and ears (for MART-1/HLA-A2, gp100/HLA-A2) and colon (CEA/HLA-A2) (5, 6). In addition, lethal neurologic toxicities were observed in two melanoma patients when targeting MAGE-A3/HLA-A2, and lethal cardiac toxicities were observed in one melanoma patient and one multiple myeloma patient when targeting MAGE-A3/HLA-A1 (7, 8). The observed toxicities can be addressed by using TCRs directed against Ags that are selectively expressed by tumor but not healthy tissues, such as neoantigens and potentially some defined and nonshared cancer testis Ags (described in more detail in Ref. 1). In addition to the above-mentioned toxicities, TCR gene engineering may result in recognition of self-peptides as a consequence of new TCR dimers that are formed between introduced and endogenous TCR chains (i.e., TCR mispairing). Although there has been no formal proof of TCR mispairing–mediated toxicity in patient studies, preclinical studies clearly demonstrated the destructive ability of T cells that express mixed TCR dimers toward healthy cells (9, 10). These findings warrant measures to prevent or limit TCR mispairing, such as genetic modification of TCR transgenes (11) or disruption of endogenous TCR chains via zinc finger nucleases (12).

The transient nature of tumor regression following T cell therapy became evident from observations that antitumor responses are initially significant yet not sustainable and most often are...
incomplete in 80–90% of patients (13, 14). Compromised anti-
tumor responses often coincided with a limited persistence of
transferred T cells (15). T cell persistence and antitumor activity
appear sensitive to T cell costimulation, as pointed out by a recent
clinical study in which T cells engineered with a CD19-specific
chimeric Ag receptor (CAR) that incorporated CD137 and CD3ζ
were used to treat patients with B cell lymphoma. In this study, T cell
persistence was significant (detectable up to 6 mo); complete clinical
responses were initially observed in two of three patients (16) and
subsequently confirmed in dozens of patients (17). Similarly,
clinical studies using CD19-targeted T cells with CARs that
incorporated CD28 and CD3ζ showed beneficial effects on long-
term T cell persistence and clinical responses (18, 19). In addition to
gene engineering, MART-1–specific T cells stimulated with
artificial APCs that expressed CD28 ligands and used to treat
patients with melanoma revealed enhanced T cell persistence and
clinical responses (20). Importantly, inclusion of T cell costimu-
lation in these clinical protocols eliminated the requirement for
patient preconditioning with chemotherapy and/or in vivo high-
dose IL-2 administration (16, 20).

In this study, we designed and generated a novel TCR genetically
linked to CD28 and CD3ζ (TCR:28ζ) to simultaneously address
TCR mispairing and enhance T cell costimulation. TCR:28ζ was
tested for two melanoma Ag specificities, MAGE-A1/HLA-A1
and gp100/HLA-A2, and resulted in maximum ability to bind
peptide–MHC, enhanced peptide responses, no TCR mispairing,
and no loss of Ag or peptide fine specificity. Moreover, T cells
expressing TCR:28ζ mediated highly active immune synapses and
eyt T cell signaling and resulted in improved T cell survival and
reduced numbers of melanoma recurrences in vivo.

Materials and Methods

Cells and reagents

T lymphocytes derived from healthy donors were isolated and expanded
using a feeder system, as described elsewhere (21), and cultured in HEPES-
buffered RPMI 1640 medium (BioWhittaker, Verviers, Belgium) supple-
mented with 10% human serum (Sanquin, Amsterdam, the Netherlands),
2 mM t-glutamine, 100 mg/ml streptomycin, and 100 U/ml penicillin. Jurkat
T cells expressing a single MelanA/HLA-A2–specific TCR (clone J.19
(22)), J.19 transfected with CD8a [J.19-CD8 (23)], the B cell lines APD
and BSM, and the TAP-deficient Tx hybrid T2 cells were cultured in
RPMI 1640 medium (BioWhittaker) containing 10% FBS (Stochem, Otsu, Japan) and pVSV-G ENV or pCOLT-GALV ENV in the case of Jurkat T cells or primary human T cells, respectively. The transduction protocol of human T cells was
optimized and described previously (30).

Flow cytometry and FACS

TCR-transduced T cells were washed and incubated with TCR mAbs or
peptide–MHC for 30 min on ice or 15 min at room temperature, respec-
tively, after which T cells were washed again and fixed with 1% PFA
(Brunschwig, Amsterdam, the Netherlands). To detect CD107α mobiliza-
tion, T cells were stimulated with target cells and analyzed as described
(31). T cells were gated according to forward and side scatter properties
using a FACSCalibur (Becton Dickinson, Alphen a/d Rijn, the Nether-
lands) equipped with CellQuest software (BD Biosciences). Enrichment of
T cells was performed by two-color FACS following staining with TCR-α
and TCR-β mAbs (for M1/A1) or peptide–MHC (for gp100/A2).

TCR gene constructs

The M1/A1 TCR was derived from CTL clone MZ2-82/30 (26, 27) and is
composed of TRAV19/J33/C and TRBV9/22/D/J2/C2 (25) with TCR-V(D)J
gene nomenclature according to http://www.imgt.org. The 28ζ cassette was derived via overlap PCR using human PBMC-derived template DNAs and covered the
transmembrane (TM) and intracellular (IC) domains of human CD28 (GL
338444, aa 153–220, numbering starting from first methionine), followed
by the IC domain of human CD3ζ (GI:4502670, aa 153–207). The 28ζ cassette was preceded by the amino acids GSPK (with GS covering a BamHI site) and cloned into both pBullet-TCRζ (TCR-ζ amino acids ending at SSP2) and TCRβ (TCR-β amino acids ending at WGRAD) via NotI and BamHI. Primer sequences used for cloning the TCR and 28ζ cassette can be provided upon request. All TCR constructs were sequence verified (Service X5, Leiden, the Netherlands). See Fig. 1A for a schematic
representation of the two-chain TCR:28ζ. TCR variants in which TM or IC
domains were replaced, deleted, or mutated were generated by overlap
PCR. See Fig. 3A for a schematic overview of these TCR variants and its
legend for the exact domain boundaries. TCR variants included TCR:TM-
ζ (TCR-ζ IC domain removed), TCR:TM2-ICζ (CD28 IC domain
removed), TCR:TM28-ICζζ (TCR:28ζ; described in Ref. 28), and TCR:TM28-ICζζ(mutε) [CD28 IC domain mutated to prevent LCK
binding, as described (29)].

Retroviral gene transfer into T cells

Melonery mucine leukemia retroviruses were produced by cocultures of
293T and Phoenix-A cells. Cells were calcium phosphate transfected with
TCR transgenes, the helper vectors pHT60 MLV GAGPOL, and either
pVSV-G ENV or pCOLT-GALV ENV in the case of Jurkat T cells or primary human T cells, respectively. The transduction protocol of human T cells was
optimized and described previously (30).
fixed and stained with TCR-Vß9-PE mAb and one of the following mAbs (labeled with Alexa Fluor 647 or followed by Goat-Alexa Fluor 647)—CD3ε, CD8α, CD45, p-CD3ζ, p-LCK, p-ERK, or p-ZAP70—covered with glycerol:PBS, and analyzed with a confocal microscope (LSM 510; Carl Zeiss, Jena, Germany). Data were collected using ImageJ software (National Institutes of Health, Bethesda, MD). Confocal images were taken in the donor, acceptor, and CFSE channels (the last channel for target cells) prior to and postphotobleaching. Fluorescence resonance energy transfer (FRET) efficiencies/pixel were calculated using the AccPbFRET program (32). The relative density of synaptic molecules was calculated as the ratio of mean fluorescence intensities (MFIs)/pixel inside versus outside the immune synapse. TCR-transduced Jurkat clone 19 T cells also were subjected to flow cytometric FRET. To this end, T cells were stained with TCR-Vß9-PE or TCR-Vß27-PE mAbs to provide donor fluorochromes and with nonconjugated anti-CD3ε-PE mAbs, or CD8α mAbs, followed by biotin-ConA-Cy5 mAb to provide acceptor fluorochromes, as described previously (22). Fluorescence intensities of donor, acceptor, and FRET signals were measured and collected on a FACS-Calibur. Data for viable T cells were analyzed with Reflex software on a per-cell basis (33).

Mice

Experiments were performed with HLA-A2–transgenic (tg) mice (kindly provided by Prof. François Lemonnier, Institut Pasteur, Paris, France and described in Ref. 34), in accordance with institutional and national guidelines, following approval by the Experimental Animal Committee of the Erasmus University Medical Center Cancer Institute.

Generation of mouse TCR T cells and human gp100/AA2* B16 melanoma cells

gp100/AA2-specific TCR genes were murinized (35), codon optimized (GeneArt, Regensburg, Germany), and cloned into the pMP71 vector (kindly provided by Prof. Wolfgang Uckert, Max-Delbrück Center, Berlin, Germany). TCRα and TCRß genes were separated by an optimized T2A ribosome-skipping sequence, resulting in pMP71: wild type (wt) TCRß–T2A–wt TCRα. gp100/AA2-specific murinized TCR:28ε genes were made by overlap PCR and insertion of a 28ε cassette (murine, codon-optimized) in pMP71:wtTCRß–T2A–wt TCRα (Ncol/MfeI flanking TCRß; MluI/EcoRI flanking TCRα). The murine 28ε cassette was synthesized in exact analogy to its human counterpart (TM+IC GSPK-CD28151-215 [GI:39852021]; IC CD3ε27-27 [GI:27965656]). The Ag gene (gp100/AA2) was obtained by inserting DNA that covered the leader sequence of HLA-A2, the gp100 peptide YLPEGVPYA, and a (G5S) linker, and was cloned via EcoRI into a pLXSN vector that already contained HHD. HHD cDNA was subcloned as an XhoI fragment into the retroviral vector pLXSN (Clontech Laboratories, Mountain View, CA).

To generate mouse splenocytes, mice were isolated, activated with Con A and recombinant human IL-2, and transduced with the retroviral supernatant containing TCR RNAs, as described by Pouw et al. (35). B16 cells were retrovirally transduced using a similar transduction protocol, with the exception that GALV-pseudotyped viruses and Polybrene (4 µg/ml) were used, and cells were incubated with the retroviral supernatant for 24 h, followed by a second incubation for 8 h. B16:gp200/AA2 cells were single-cell sorted for high HLA-A2–expressing cells on a FACSaria cell sorter using the anti–HLA-A2 Ab and cultured under neomycin selection. TCR surface expression, binding of peptide–MHC, and in vitro T cell functions were assessed as described in Supplemental Fig. 3.

Adaptive T cell therapy

For adoptive T cell therapy experiments, HLA-A2–tg mice were injected s.c. with 0.5 × 10⁶ B16 gp100/AA2 cells, and 10 and 11 d later, they received a total of two busulfan (Duchefa Farma, Haarlem, the Netherlands) injections i.p. (16.5 µg/kg each), followed 1 d later by a single i.p. injection (200 mg/kg) of cyclophosphamide (Sigma-Aldrich). Mice received 7.5 × 10⁴ murinized wt TCR or TCR:28ε T cells at day 13. Tumor growth was measured using a caliper three times a week, and tumor volumes were estimated as described (36). Peripheral blood was drawn at days 12, 26, and 40 after T cell transfer. TCR-transduced T cells were monitored with anti–TCR-Vß9 PE mAb (nomenclature according to http://www.imgt.org; clone CAS11.3, Beckman Coulter) and gp100/AA2 PE–PE-labeled peptide–MHC [gp100/AA2 peptide–MHC generated as described (37)] in combined TCR-Vß9-PE, CD8α, or CD8β mAbs, followed by PE- or PE-Cy5-labeled anti-CD8α or anti-CD8β mAbs using standard flow cytometry. Absolute T cell counts were determined using Flow-Count Fluorospheres. All samples were analyzed on a FACSCalibur using CellQuest software (BD Biosciences).

Statistical analysis

Statistically significant differences between TCR:28ε and wt TCR were tested by nonpaired and two-tailed Student t tests using GraphPad Prism4 software. Differences with p values < 0.05 were considered significant.

Results

A signaling cassette consisting of CD28 and CD3ε results in improved surface expression and function of TCRβ-chains

We initially selected signaling cassettes that consisted of Fc(ε)RI, CD3ε, and CD3ζ accessory molecules, with or without the CD28 costimulatory molecule, according to their effect on the functional expression of single-chain TCR transgenes (Z. Sebestyen, M. Broerjtes, C. Govers, M. van Brakel, and R. Debets, manuscript in preparation). These data argued that the CD28-CD3ε (28ε) signaling cassette consistently provided T cells with the best binding of peptide–MHC, T cell cytotoxicity, and IFN-γ. We introduced the 28ε cassette into a two-chain TCR format (i.e., TCR:28ε; Fig. 1A), gene transduced primary human T cells with M1/A1 wt TCR or TCR:28ε, and used FACSort to obtain T cell populations with high and equal levels of TCR expression (>95%) (Fig. 1B). Following staining with TCRα and TCRβ mAbs, flow cytometry dot plots of TCR:28ε T cells revealed a typical diagonal with enhanced MFI values and equal staining intensities for TCRα— and TCRβ-chains. This staining pattern suggests correct pairing of TCRα and TCRβ transgenes and was similar to the pattern reported previously for TCR:ξ (22, 31). Following staining with peptide–MHC, TCR:28ε T cells demonstrated a significantly enhanced percentage and MFI over a range of peptide–MHC concentrations compared with wt TCR T cells (Fig. 1B, 1C).

To investigate T cell function, we stimulated TCR-engineered T cells with anti-TCR Abs, peptide-loaded cells, and native melanoma cells and analyzed activation of NFAT, mobilization of CD107a, and production of cytokines. Using a luciferase reporter assay with Jurkat T cells, we observed a significantly greater activation of NFAT upon stimulation of TCR:28ε T cells with TCR-Vß9 Β against the introduced M1/A1 TCR compared with wt TCR T cells (Fig. 2A). T cells transduced with wt TCR, but not with TCR:28ε, demonstrated decreased NFAT activation upon stimulation with TCR-Vß27 Β against the endogenous MelanA/A2 TCR. These findings suggest that enhanced functional expression of TCR:28ε does not occur at the expense of the functional expression of endogenous TCR. When assessing CD107a mobilization in primary human T cells, we observed that TCR:28ε showed enhanced performance upon stimulation with M1 peptide and equal performance upon stimulation with native, M1/A1* melanoma cells compared with wt TCR (Fig. 2B). Using TCRs directed against a second Ag (i.e., gp100/HLA-A2 A [gp100/A2]), we confirmed enhanced peptide–MHC binding (Supplemental Fig. 1A) and peptide-induced mobilization of CD107a by TCR:28ε (Fig. 2C). Enhanced responsiveness toward peptide-loaded targets cells of both M1/A1- and gp100/A2-specific TCRs also was observed upon assessing TCR:28ε T cells for their production of IFN-γ and IL-2 (see figure below and Supplemental Fig. 1B, 1C).

The CD28 TM domain determines surface expression of TCR:28ε, and both CD28 and CD3ε IC domains determine T cell functions

We generated and tested new TCR variants in vitro to discern the contributions of the TM and IC domains of CD28 and CD3ε to the above-mentioned TCR properties. A schematic representation of the TCR variants is given in Fig. 3A. We observed that the TCR variant not having the CD28 TM domain (TCR:TMζ-IC28ε) did
not express on the cell surface (Fig. 3A), whereas the TCRs not having CD28 or CD3ε IC domains or having a mutated CD28 IC domain [TCR:TM28-ICε, TCR:TM28-IC28ζ, and TCR:TM28-IC28(mut)ε] exhibited unaltered peptide–MHC binding compared with the parental TCR:28ε (Fig. 3B). These data suggest that the CD28 TM domain is of critical importance for the enhanced surface expression and peptide–MHC binding that was observed for TCR:28ε (Fig. 1B, 1C). Furthermore, we observed that TCRs lacking the CD28 or CD3ε IC domains mediated decreased CD107a mobilization and IFN-γ and IL-2 production in response to peptide (Fig. 3C–E). When the CD3ε IC domain was replaced by the CD3ζ IC domain (TCR:TM28-IC28ζ), all three T cell readouts were markedly decreased (~75% decrease compared with TCR:28ε). Interestingly, in the absence of the CD28 IC domain (TCR:TM28-ICε), the decrease in T cell CD107a mobilization was limited (~25%), the decrease in IFN-γ production was substantial (~50%), and the decrease in IL-2 production was most pronounced (~75%). The CD28 IC domain shows two main motifs, each with characteristic binding partners and downstream signal transduction (a simplified version of CD28 IC domain is presented in Fig. 3A). The testing of TCR:TM28-IC28(mut)ε, containing a mutated PYAPP motif and not able to bind LCK (Fig. 3A), revealed that the increased requirement for CD28 signaling with respect to
CD107a mobilization and IFN-γ and IL-2 production was accompanied by an increased requirement for LCK-mediated signaling (decreases were approximately 0% for CD107a, 25% for IFN-γ, and 50% for IL-2) (Fig. 3C–E).

**TCR:28ε induces immune synapses with enhanced accumulation of TCR, CD3ε, and CD8α and phosphorylated LCK and ERK**

The induction of immune synapses and their molecular profile are considered a critical measure of T cell activation. To study the induction ability and the composition of immune synapses, TCR-transduced Jurkat T cells were stimulated with M1 peptide–loaded target cells and analyzed by confocal microscopic FRET. We observed that TCR:28ε is able to induce immune synapses, and TCR:28ε present in immune synapses does not associate with CD3ε but does associate with CD8α (Supplemental Fig. 2A, 2B). In addition, the immune synapses induced by TCR:28ε and wt TCR are of similar size (Supplemental Fig. 2C). Notably, when TCR:28ε were stimulated with M1 peptide–loaded target cells and analyzed by confocal microscopic FRET, we observed that TCR:28ε mediated a significantly decreased accumulation of p-CD3ζ (~2-fold) but did not affect the accumulation of p-ZAP70 (Fig. 5B).

**TCR:28ε does not appear sensitive to off-target recognition in vitro**

To assess whether TCR:28ε is prone to off-target recognition, we performed two types of in vitro assays. First, we performed flow cytometric FRET to determine the extent of mispairing between introduced TCR:28ε and endogenous (wt) TCR chains. To this end, we transduced a Jurkat T cell clone already expressing the MelanA/A2 wt TCR and the CD8α coreceptor with the M1/A1 wt TCR or TCR:28ε gene. Measurements of flow cytometric FRET using MelanA/A2-TCR-β27–specific and M1/A1 TCR-Vα19–specific mAbs revealed significant energy transfer in the case of wt TCR but not TCR:28ε (values were <5% background signal) (Fig. 6A). Measurements of FRET using the M1/A1 TCR-Vβ9 and either CD3ε or CD8α mAb showed that TCR:CD3 associations were observed for wt TCR but not TCR:28ε, whereas TCR: CD8α associations were present for both TCR formats (Fig. 6A). Observations with flow cytometric FRET extend earlier observations with microscopic FRET using primary human T cells or Jurkat T cells (Fig. 4B and Supplemental Fig. 2B). These experiments show that TCR:28ε does not mispair with endogenous TCR chains.
FIGURE 3. Surface expression of TCR:28ε depends on TM domain of CD28, and T cell functions depend on IC domains of both CD28 and CD3ε. Primary human T cells were transduced with empty virus particles (no TCR transgene) or one of the following M1/A1 TCR variants: TCR:TM28-IC28ε, TCR:TMz-IC28ε, TCR:TM28-ICε, TCR:TM28-IC28z (TCR:28z), or TCR:TM28-IC28ε(mut)ε.

(A) Schematic representation of TCR variants, together with an illustration of CD28 IC amino acids. The CD28 IC domain shows two main motifs, each with characteristic binding partners and downstream signal transduction (for simplicity reasons only limited binding partners and signal transduction have been displayed, see text for details). Amino acids of the PYAPP motif that are mutated to prevent LCK binding are in black.

All TCR variants are derived from TCR:28ε, and the amino acid boundaries of domains are as follows: TM CD28153–179 (GI:338444) is replaced by TM CD3z31–51; IC CD28180–220 is removed or mutated at P208A, P210A, and P211A; and IC CD3ε153–207 (GI:4502670) is replaced by IC CD3z52–164. T cells were sorted by FACS with TCRα and TCRβ mAbs and analyzed by flow cytometry for M1/A1 peptide–MHC binding (B) or CD107α mobilization (C) or by ELISA for IFN-γ (D) or IL-2 (E) production. Assays and analyses were performed as described in Figs. 1 and 2. Bars represent measurements of PBMC (n = 3–5), and results with TCR:28ε were set to 100%. *p < 0.05, **p < 0.005, ***p < 0.0005 TCR:28ε versus wt TCR, Student t test. EC, extracellular; IC, intracellular; N.S., nonsignificant; TM, transmembrane.
nor does it associate with endogenous CD3 molecules, similar to observations reported for a TCR that is fused to a complete CD3ζ molecule (i.e., TCR-ζ) (22, 31). Second, we assessed T cell function in response to gp100 altered peptide ligands (APLs) to determine the extent to which peptide fine specificity of TCR:28ε is changed compared with wt TCR. To this end, we loaded T2 cells with gp100 APL, as described by Schaft et al. (25), and measured IFN-γ production following stimulation of TCR-transduced primary human T cells. We observed that both TCR:28ε and wt TCR mediated IFN-γ production to all APLs, except when alanine replaced glutamic acid at position 3 (Fig. 6B). This peptide response pattern was identical to that of the parental CTL-296 clone (25) and showed that incorporation of the 28ε cassette did not alter the peptide fine specificity of the parental TCR.

Mouse T cells expressing TCR:28ε limit and delay tumor recurrence and demonstrate enhanced peripheral T cell persistence

Finally, we set out to assess the in vivo behavior of T cells transduced with TCR:28ε. To this end, we constructed muri- nized gp100/A2-specific wt TCR and TCR:28ε (see Materials and Methods) and retrovirally transduced them into mouse spleno- cytes. Mouse T cells transduced with TCR:28ε showed enhanced surface expression and were able to kill gp100/A2+ B16 melanoma cells (Supplemental Fig. 3). For adoptive T cell therapy studies, HLA-A2–tg mice were transplanted with B16;gp100/A2 tumor cells; once tumors were palpable (in some cases >500 mm²), mice were conditioned with busulphan and cyclophosphamide and treated with 7.5 × 10⁶ T cells transduced either with TCR:28ε or wt TCR. Notably, T cells expressing TCR:28ε effectively limited tumor recurrence, because 4 out of 8 mice were tumor-free, whereas none of the mice treated with wt TCR T cells were tumor-free at 40 d posttreatment (Fig. 7A). Also, the day on which tumors recurred following treatment was significantly delayed in the case of TCR:28ε (Fig. 7C, day 35 versus 18). The effect on tumor recurrence was accompanied by a drastic increase in the numbers of peripheral CD8 T cells that bound peptide–MHC (in some cases >1500/μl blood) and remained stable until the end of the experiment (6 wk after treatment, Fig. 7B, 7D).

Discussion

In this study, we designed and generated a novel TCR geneti- cally linked to CD28 and CD3ζ (TCR:28ε). TCR:28ε was tested for two melanoma Ag specificities and provided T cells with the following beneficial properties for T cell therapy: enhanced pep- tide–MHC binding, more potent T cell responses upon stimulation with peptide and equal responses upon stimulation with Ag+ melanoma cells, formation of highly active immune synapses, no TCR mispairing and no change in peptide fine specificity, and improved T cell survival and reduced numbers of melanoma recurrences in vivo.

The enhanced ability of T cells transduced with TCR:28ε to bind peptide–MHC and respond to peptide is most likely a direct consequence of enhanced surface expression per cell (see MFI values in Fig. 1B, 1C, Supplemental Fig. 1A). In contrast to wt TCR (38), TCR:28ε does not compete for endogenous CD3 proteins to become surface expressed, as evidenced by the lack of association between TCR:28ε and CD3ζ (Figs. 3A, 5, Supplemental Fig. 2) and enhanced activation of NFAT when stimulating either TCR:28ε or the endogenous TCR in dual-TCR
and observed that CD28 TM domain affects surface TCR expression, whereas the combined actions of CD28 and CD3ε IC domains affect T cell functions (Fig. 4). The CD28 IC domain contains three main motifs that specifically associate with adapter proteins and kinases, which, in turn, initiate different signal-transduction cascades (39). The proximal YMNM motif, when phosphorylated, binds p85 (a subunit of PI3K) and GRB2. The two distal proline-rich motifs bind ITK (PRRP motif) and GRB2, as well as FILAMIN-A and LCK (PYAPP motif). CD28 signal transduction via PI3K and GRB2 results in downstream activation of AKT and PLCγ1, respectively, which then results in activation of NF-κB and NFAT transcription factors and, consequently, T cell survival and IL-2 production. CD28 signal transduction via LCK feeds predominantly into the activation of NFAT and IL-2 production, most likely via enhanced stability of IL-2 mRNA and its secretion. In addition to the CD28 IC domain, the CD3ε IC domain binds GRK2 and CAST, which results in downstream activation of NFAT and IL-2 production (40, 41). The use of TCR variants either lacking or containing an LCK-nonbinding mutant of CD28 IC domain demonstrated a hierarchy with respect to dependency on CD28 signaling, in particular LCK signaling for different T cell functions (CD107a < IFN-γ < IL-2). This observed hierarchy is in line with an earlier report using a peptide–MHC ligand with compromised TCR binding (a partial agonist of the human gp100280–288 peptide) (42). In fact, mobilization of CD107a generally requires limited T cell signaling and is pre-synthesized and ready to be mobilized, whereas production of cytokines requires de novo synthesis; in particular, IL-2 requires strong T cell signaling and NFAT activation.

TCR:28ε may be part of pre-existing clusters [as described for wt TCR/CD3 (43)] that are composed of dimers or oligomers of wt TCR/CD3 and TCR:28ε. The existence of such nanoclusters is suggested by the observed accumulation of endogenous TCR/CD3 complexes in immune synapses mediated by TCR:28ε (Fig. 6A). TCR/CD3 complexes may oligomerize via homotypic interactions governed by TCR-Cα and CD3ε (44, 45). According to such a model, TCR:28ε (encompassing TCR-Cα) and endogenous CD3ε are part of the same complex yet are interspersed with wt TCR and are not within 10 nm of each other (Figs. 3A, 5). We postulate that one important advantage of TCR:28ε over wt TCR is the formation of highly active immune synapses. Indeed, TCR:28ε-mediated, but not wt TCR-mediated, immune synapses were readily detectable in primary human T cells, showed enhanced accumulation of TCR, CD3, and CD8 (Figs. 5, 6), and are expected to facilitate Ag-dependent T cell activation (44, 46, 47). Notably, TCR:ζ mediates immune synapses in which densities of TCR, CD3, and CD8 are diluted over enlarged contact areas (23), whereas TCR:28ε mediates immune synapses in which densities of the aforementioned molecules are enriched in normal-sized immune synapses. We cannot exclude that TCR:28ε may differ from wt TCR with respect to its temporal organization of immune synapses. In fact, in primary human T cells we observed an association between the CD45 phosphatase and TCR:28ε, which may provide TCR:28ε with a kinetic advantage (Fig. 5B). In this respect, it is noteworthy that primary human T cells may require prolonged times to develop molecular clusters compared with Jurkat T cells (in which an association between CD45 and TCR:28ε was not observed) and may prove to be better suited for use in further experiments into the kinetics and stability of immune synapses (48, 49). Our results further argue that TCR:28ε facilitates the assembly of a molecular signaling scaffold at the immune synapse (Fig. 6). Enhanced densities of p-LCK within immune synapses may be a direct consequence of increased accumulations of TCR:28ε and CD8α, because both

Jurkat T cells (Fig. 2A). Unexpectedly, T cell responses toward Ag+ tumor cells were equal compared with wt TCR–transduced T cells. When testing TCR:28ε T cells in more detail, we observed a decreased effective dose of peptide yielding half-maximum response (Supplemental Fig. 4A), as well as a compromised response in the absence of CD8α binding (Supplemental Fig. 4B). These data, together with our observation that TCR:28ε does not show a decreased ability to associate with CD8α (Figs. 3A, 5A, Supplemental Fig. 2), suggest that TCR:28ε has a decreased affinity for peptide–MHC. The compromised peptide–MHC binding by TCR:28ε may be due to structural restraints of the fusion between TCR-Cα and the CD28 TM domains. Nevertheless, TCR:28ε enhances the strength of T cell responses at higher Ag concentrations (Supplemental Fig. 4A), which is most likely due to enhanced surface expression of TCR:28ε compared with wt TCR (Fig. 1B).

To dissect the contributions of TM and/or IC domains of CD28 and CD3ε to various TCR properties, we tested TCR:28ε variants

**FIGURE 5.** TCR:28ε T cells demonstrate enhanced synaptic accumulation of TCR, CD3ε, CD8α, p-LCK, and p-ERK. Jurkat T cells were transduced with M1/A1 wt TCR or TCR:28ε and sorted by FACS with TCRs and TCRβ mAbs, stimulated with APD B cells loaded with M1 peptide, and measured for densities of membrane proteins in immune synapses. T cells were stained with TCR–FITC–PE mAb and one of the following mAbs (labeled with Alexa Fluor 647 or followed by isotype-specific Gm–Alexa Fluor 647): CD3ε, CD8α, CD45, p-CD3ε, p-LCK, p-ERK, or p-ZAP70. T cells were analyzed by confocal microscopy. Data are densities of molecules (fold increase in signals inside versus outside immune synapses) + SEM (n = 3 independent measurements of 15 cells/measurement). TCR T cells stimulated with irrelevant peptide showed low background signals (data not shown). Data for wt TCR in (A) were modified from Roszik et al. (23). *p < 0.05, **p < 0.005, ***p < 0.0005 TCR:28ε versus wt TCR. Student t test. ns, nonsignificant.
CD28 (contained in TCR:28ε) and CD8α harbor a motif for LCK recruitment. Activated ERK can phosphorylate LCK at serine 59, preventing inactivation of p-LCK by the phosphatase SHP-1 (50), thereby providing an alternative reason, and possibly a positive-feedback loop, for the enhanced activation of LCK. In TCR:28ε T cells, levels of p-CD3ε were significantly lower compared with wt TCR T cells (Fig. 6B), which may be due to an inability of TCR:28ε to associate with endogenous CD3ζ and position this molecule sufficiently close to p-LCK to become phosphorylated. Despite the presence of fewer p-CD3ζ scaffolds, the accumulated levels of p-ZAP70 in TCR:28ε T cells were similar to those in wt TCR T cells (Fig. 6B), which may argue that ZAP70, even when docking to p-CD3ζ is hampered, becomes phosphorylated by the enhanced presence of p-LCK. The CD28 and CD3ζ IC domains are able to contribute to the reorganization of the cytoskeleton and formation of immune synapses. The CD28 IC domain, mainly through its PYAPP motif and activation of GRB2 and FILAMIN-A pathways, directs movement of CD28 to early immune synapses through its PY APP motif and activation of GRB2 and FILAMIN-A (39), whereas the CD3ζ IC domain, mainly through recruitment of NCK, leads to activation of the RAS/RAF/ERK pathway and formation of immune synapses (51, 52).

In addition to the ability of TCR:28ε to activate T cell responses, we investigated how sensitive this TCR is at mediating off-target reactivities. We observed that TCR:28ε does not mispair with endogenous TCR, as evidenced by flow cytometric FRET (Fig. 3A), nor does it have a changed peptide fine specificity compared with wt TCR (Fig. 3B), making this receptor less prone to induce off-target toxicities (9, 10). Interestingly, the 28ε cassette does not harbor the extracellular cysteine of CD28 (at position 141), which normally facilitates homodimerization of CD28 at the cell surface. However, this cysteine is not required per se for dimerization and costimulatory function (53). In addition to TCR mispairing and peptide fine specificity, our studies provide the following lines of evidence that TCR:28ε does not hamper safety by constitutively activating T cells: we did not observe activation of NFAT following nonspecific TCR stimulation (Fig. 2A); we did not observe CD107a mobilization or production of IFN-γ and IL-2 in the absence of Ag (Figs. 2B, 2C, Supplemental Fig. 1B, 1C); and TCR:28ε T cells revealed no enhanced proliferation during routine culture conditions (data not shown).

Finally, we assessed the in vivo behavior of T cells expressing TCR:28ε. HLA-A2–tg mice bearing an established B16 tumor expressing human gp100 and HLA-A2 were conditioned with busulphan and cyclophosphamide and treated with T cells transduced either with TCR:28ε or wt TCR. T cells expressing TCR:28ε effectively limited tumor recurrence (Fig. 7A) and delayed the time point at which tumors recurred following treatment (Fig. 7C). Notably, the effect on tumor recurrence was
accompanied by a drastic increase in the numbers of peripheral CD8 T cells that bound peptide–MHC (Fig. 7B, 7D). In fact, mice showing >400 peptide–MHC–binding CD8 T cells/μL blood appeared protected from tumor recurrence within 40 d posttreatment. T cells generally demonstrate early and preferential accumulation within Ag+ tumors (54), but T cells appear to require sustained TCR- and CD28-mediated interactions to remain and accumulate in Ag+ tissue (55). CD28 signaling enhances T cell survival, most likely through prevention of T cell apoptosis via inhibition of FasL (56) and stimulation of Bcl-xL expression (57). These antiapoptotic pathways are most likely mediated through the CD28 motifs YMNM and PYAPP and binding of p85 and GRB2, with a dependence on NF-κB activation (39).

Most studies analyzing signaling cassettes were performed with CARs. Inclusion of CD28 into CARs, generally in combination with a CD3ζ domain, resulted in enhanced T cell activation and proliferation, in vivo T cell persistence, and antitumor effects. Signaling cassettes were tested further with other members of the CD28 family (58–62), such as CD137 (4-1BB) and CD134 (OX40), which showed promising results with respect to cytolysis, initiation or sustainment of an effective T cell response, and prevention of CAR-mediated proliferation of regulatory T cells (63). Importantly, clinical trials using CAR:CD28-CD3ζ or CAR:CD137-CD3ζ demonstrated significant objective responses in patients with B cell leukemia (16, 18, 19). In this study, we showed that a signaling cassette consisting of CD28 and CD3ε in the setting of TCRs improves the potency of T cell responses and peripheral T cell persistence without compromising Ag specificity and supports the further development of costimulatory TCRs for clinical testing.

Disclosures
The authors have no financial conflicts of interest.

FIGURE 7. T cells expressing TCR:28ε decrease or delay tumor recurrence and enhance T cell persistence. (A) HLA-A2-tg mice were transplanted s.c. with B16:gp100/A2 cells at day 0. The tumor was left to grow for 10 d, after which mice received conditioning with busulphan and cyclophosphamide and injection of 7.5 × 10⁶ T cells transduced either with TCR:28ε (lower panel) or wt TCR (upper panel). Tumor sizes were measured with a caliper and expressed as mm³/individual mouse; mice that were tumor-free at day 40 are indicated and the mean day that tumors started to recur following treatment is indicated by the dotted line. (B) Peripheral blood was collected from mice at the indicated days following treatment with T cells transduced either with TCR:28ε (lower panel) or wt TCR (upper panel), and absolute numbers of peptide–MHC–binding CD8 T cells were determined by flow cytometry. Data are T cell numbers/individual mouse, with numbering of mice as shown in (A). (C) Tumor recurrences from (A) are presented as mean day + SEM. (D) T cell numbers from (B) are presented as mean + SEM. *p < 0.05 TCR:28ε versus wt TCR, Student t test.
References


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