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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

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Express Mail Label No. EL556129262US

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)

☐ *Additional inventors are being named on the _____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 8
☐ Drawing(s) *Number of Sheets*
☐ Application Data Sheet. See 37 CFR 1.76
☐ CD(s), Number
☐ Other (specify)

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Respectfully submitted,

SIGNATURE Marina T Larson

Date 05/28/2002

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REGISTRATION NO. *(if appropriate)* 32,038

Docket Number: MSK.P-058-PV

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MSK.P-058-PV
PROVISIONAL PATENT APPLICATION

Chimeric T Cell Receptors

This application relates to nucleic acids encoding chimeric T cell receptors (TCRs), to the chimeric TCRs, and to methods of using same to facilitate a T cell response to a selected target. Chimeric TCR's are known in the art, and are described, inter alia, in commonly assigned PCT Publication 07/36434 and US Patent Applications 08/940,544 and 09/786,502 which are incorporated herein by reference in their entirety.

In a first aspect of the invention, there is provided a nucleic acid encoding a chimeric TCR and the TCR encoded by it. The receptor comprises a TCR- zeta chain, CD28 signaling elements and a binding element which interacts specifically with the selected target. Suitable binding elements include, without limitation, members of immunological binding pairs, particular antibodies. Preferred antibodies are single chain antibodies, or scFv.

The chimeric TCR of the invention and the nucleic acid encoding it can be prepared in either of two orientations: zeta-CD28-binding element or CD28-zeta-binding element. As noted in the attached manuscript, the performance of the TCR in two orientations is not always identical . In the case where the binding element is an scFV directed to prostate-specific membrane antigen (PSMA), the preferred orientation is CD-28-zeta-binding element.

The target to which the chimeric T cell receptors of the invention are directed can be any target of clinical interest to which it would be desirable to induce a T cell response. This would include markers associated with cancers of various types, including without limitation prostate cancer (for example using a binding element that binds to PSMA), breast cancer (for example using a binding element that targets Her-2) and neuroblastomas, melanomas, small cell lung carcinoma, sarcomas and brain tumors (for example using a binding element that targets GD_2).

A second aspect of the invention provides a method for treating persons suffering from a disease condition wherein the disease is associated with a cellular marker which can serve as a disease preferential or specific target. In this method, a chimeric TCR is provided which comprises a zeta chain, CD-28 signaling elements and a binding element which specifically interacts with the cellular marker. T-lymphocytes from the individual to be treated, for example

a human individual, are transduced with the chimeric TCR. This transduction may occur *ex vivo*, after which the transduced cells are reintroduced into the individual. Selection procedures to increase the relative amounts of transduced cells may be employed. Thus, in addition to the zeta chain, CD28 and binding elements, the chimeric TCR may include a selection element. For example, dihydrofolate reductase (DHFR) may be included in the TCR to allow *ex vivo* or *in vivo* selection for transduced cells using methotrexate. (See commonly-assigned PCT Publication 9733988, which is incorporated herein by reference).

The invention is now described further with reference to the attached paper. All of the publications cited in the paper are incorporated herein in their entirety.

Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCRζ /CD28 receptor

John Maher, Renier J. Brentjens, Gertrude Gunset, Isabelle Rivière, and Michel Sadelain*

Artificial receptors provide a promising approach to target T lymphocytes to tumor antigens. However, the receptors described thus far produce either an activation or a co-stimulatory signal alone, thus limiting the spectrum of functions accomplished by the genetically modified cells. Here we show that human primary T lymphocytes expressing fusion receptors directed to prostate-specific membrane antigen (PSMA) and containing combined T-cell receptor-ζ (TCRζ), and CD28 signaling elements, effectively lyse tumor cells expressing PSMA. When stimulated by cell-surface PSMA, retrovirally transduced lymphocytes undergo robust proliferation, expanding by more than 2 logs in three weeks, and produce large amounts of interleukin-2 (IL-2). Importantly, the amplified cell populations retain their antigen-specific cytolytic activity. These data demonstrate that fusion receptors containing both TCR and CD28 signaling moieties are potent molecules able to redirect and amplify human T-cell responses. These findings have important implications for adoptive immunotherapy of cancer, especially in the context of tumor cells that fail to express major histocompatibility complex antigens and co-stimulatory molecules.

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The induction of potent tumor immunity presents a major challenge for cancer immunotherapy. Tumor cells have many properties that facilitate immune evasion[1–3]. Most tumor antigens characterized to date are self-antigens and are thus poorly immunogenic[4,5]. The paucity of target antigens, the difficulty of overcoming tolerance to self-antigens, and impaired antigen presentation also contribute to compromise T-cell priming in cancer-bearing hosts[1–3,6–10]. Furthermore, malignant cells may escape from tumor-specific effector T cells by downregulating major histocompatibility complex (MHC) and/or antigen expression, or by establishing an immunosuppressive microenvironment[1–3,11].

Genetic approaches offer a potential means to enhance immune recognition and elimination of cancer cells. One promising strategy is to genetically engineer T lymphocytes to express artificial TCRs that direct cytotoxicity toward tumor cells[12,13]. Artificial receptors typically comprise a tumor antigen–specific recognition element derived from a single-chain antibody variable fragment (scFv). When used to reprogram T-cell specificity, such fusion receptors permit MHC-independent recognition of native rather than processed antigen[12–14]. ScFv-based TCRs are engineered to contain a signaling domain that delivers an activation stimulus (signal 1) only[12–14]. The TCRζ cytoplasmic domain, which delivers a potent signal 1 in the absence of the remaining components of the TCR-CD3 complex[15,16], is well suited for activating cytolytic functions. The potential clinical utility of this strategy is supported by the demonstration that, despite fears about defective signaling in lymphocytes of tumor-bearing subjects[17], ζ-chain fusion receptors retain potent activity in cancer patient cytotoxic T cells[18].

However, while sufficient to elicit tumoricidal functions, the engagement of ζ-chain fusion receptors may not suffice to elicit substantial IL-2 secretion in the absence of a concomitant co-stimulatory signal[18]. In physiological T-cell responses, optimal lymphocyte activation requires the engagement of one or more co-stimulatory receptors (signal 2), the best characterized of which is CD28 (refs 19–22). Provision of signal 1 in the absence of CD28 signaling can result in a very poor T-cell proliferative response or in the induction of anergy or apoptosis[19–22]. Consequently, it may be extremely valuable to engineer human T cells so that they receive a co-stimulatory signal in a tumor antigen–dependent manner. An important development in this regard has been the successful design of scFv–CD28 fusion receptors that transduce a functional antigen-dependent co-stimulatory signal in human primary T cells, permitting sustained T-cell proliferation when both the endogenous TCR and the chimeric CD28 receptor are engaged[23].

In the present study, we combine activation and co-stimulatory functions within a single receptor by constructing scFv-based receptors that comprise both TCRζ and CD28 sequences. The function of these dual-fusion receptors was investigated in $CD4^+$ and $CD8^+$ primary T cells to fully assess proliferative and cytotoxic functions in biologically and therapeutically relevant cell types. These fusion receptors are specific for PSMA, a glutamate carboxypeptidase that is expressed on the surface of normal prostate epithelial cells and is overexpressed in the majority of prostate carcinomas[24]. PSMA is also present in the neovasculature associated with a broad range of solid tumors[25] and is therefore an attractive target for immunotherapy[26]. Here, we report that when the signaling domain of CD28 is placed proximal to that of TCRζ, the resultant P28z receptor confers upon human peripheral blood T lymphocytes (PBLs) the ability to produce IL-2, to proliferate,

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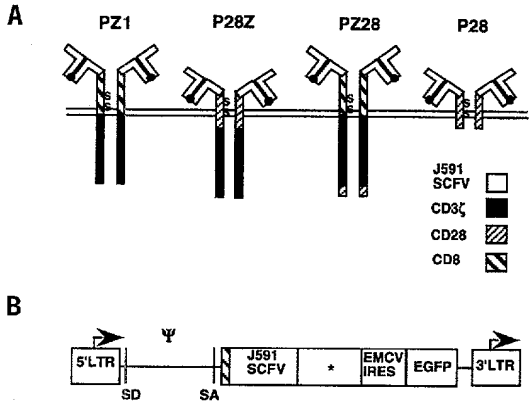

Figure 1. Structure of TCRζ–CD28 fusion receptors. (A) The PSMA-specific fusion receptors encompass an scFv derived from the J591 hybridoma, joining the V_H and V_L fragments through a serine/glycine linker (shown as the filled circle). In Pz1, a human CD8α hinge and transmembrane domain links the scFv to the intracellular domain of human TCRζ. In P28, the scFv is joined to the intracellular, transmembrane, and much of the extracellular portion of human CD28. In P28z, the intracellular TCRζ sequence has been joined to the C terminus of P28. Pz28 consists of the intracellular 41 amino acids of CD28 joined onto the Pz1 receptor. (B) All PSMA-specific receptors were expressed using the SFG onco-retroviral vector. The asterisk indicates the site of insertion of cDNA sequences that differed among the PSMA-specific fusion receptors. Bicistronic constructs encoding the receptor cDNA cloned upstream of the EMCV-IRES-eGFP cassette were used for all experiments. The long terminal repeat (LTR) promoter is arrowed, and the splice donor (SD) and splice acceptor (SA) sites flanking the packaging signal (ψ) are indicated.

and to kill in a PSMA-dependent manner. Our data demonstrate that a single molecule can deliver both functional and antigen-specific signals 1 and 2 in human primary T cells.

Results

Transduction and expression of PSMA-specific TCRζ–CD28 fusion receptors in primary human T lymphocytes. The goal of the present study was to genetically modify primary human T lymphocytes so that they acquire both a TCR-like and a co-stimulatory signal upon interaction with native PSMA. A series of receptors were generated that comprise a PSMA-specific scFv fragment coupled to signaling elements derived from TCRζ and/or CD28 (Fig. 1A). Pz1 (ref. 18) and P28 are designed to respectively deliver signals 1 and 2 in a PSMA-dependent manner. In P28z, the intracellular portion of TCRζ has been joined to the C terminus of P28 (ref. 23), while in Pz28, the CD28 signaling domain was added at the C terminus of Pz1. All chimeric complementary DNAs (cDNAs) were cloned in bicistronic onco-retroviral vectors upstream of enhanced green fluorescent protein (eGFP; Fig. 1B). Three days after transduction of mitogen-activated PBLs, gene transfer efficiency, as assessed by flow cytometry, ranged from 20% to 70% (Fig. 2A). $CD4^+$ and $CD8^+$ T-cell subsets were transduced at similar efficiencies (Fig. 2B), as reported elsewhere[18,19,27]. Expression of ζ-chain containing fusion receptors was also analyzed by western blotting, confirming homodimer formation and little, if any, heterodimerization with endogenous CD8 or CD28 (Fig. 2C).

The P28z and Pz28 fusion receptors promote PSMA-specific cytotoxicity. To confirm that the TCRζ–CD28 fusion receptors specifically engaged PSMA, cytotoxicity assays were performed three days after transduction. Pz1, which was previously shown to direct specific cytolysis against PSMA-positive tumor cells[18], served as positive control. Both P28z and Pz28 receptors, but not P28, mediated specific lysis of fibroblasts expressing human PSMA (Fig. 3A).

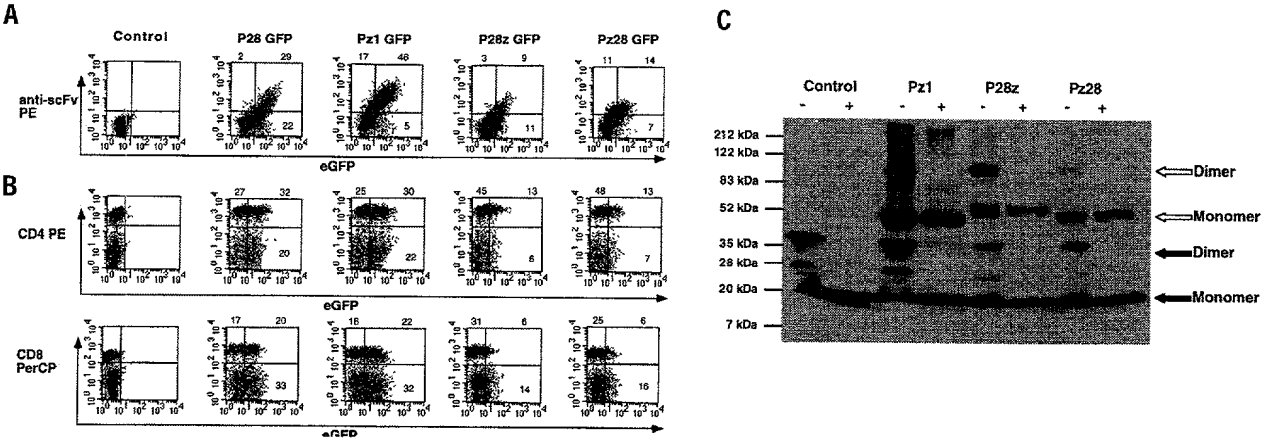

Figure 2. Expression of PSMA-specific TCRζ–CD28 fusion receptors in primary human T lymphocytes (PBLs). (A) PBLs were transduced with the indicated GFP-containing dicistronic retroviral constructs or an irrelevant control (SFG–c-*fms*). On day 7, gene transfer efficiency was assessed by flow cytometry, measuring GFP fluorescent emission and by staining with PE-conjugated antiserum reactive with the J591-derived scFv. (B) To determine the percentage transduction of T-cell subsets, samples were also stained with CD4 PE and CD8 PerCP antibodies and analyzed by three-color flow cytometry, using GFP emission to identify transduced cells. Quadrants were set using control samples so that 99% of events were negative for the marker of interest. Surface expression of Pz1 was typically greater than that of P28 or either of the TCRζ–CD28 fusion receptors. Mean fluorescence intensity when Pz1 expression was normalized to 100 was as follows: P28 = 35.1 ± 17.8 ($P < 0.05$); P28z = 29.6 ± 12.2 ($P < 0.01$); Pz28 = 25.9 ± 6.9 ($P < 0.01$) (n = 3–4 experiments). There was no significant difference in expression intensity between P28, P28z, and Pz28. (C) Lysates were prepared under reducing (+) and nonreducing (–) conditions from PBLs following transduction with Pz1 (54% GFP-expressing), P28z (21% GFP-expressing), and Pz28 (20% GFP-expressing). Untransduced PBLs were used as controls. Immunoreactive receptor bands were detected by western blotting using an anti-TCRζ monoclonal antibody. Filled arrows indicate the monomeric and dimeric forms of the endogenous TCRζ. Pz1 and P28z are predominantly expressed as homodimers, as would be expected from the design of the hinge regions of these molecules. However, Pz28 was found to dimerize less effectively in T cells and in PG13 cells (data not shown). No bands indicating productive heterodimerization with CD8α, CD8β, or CD28 were detected. The additional band seen under that corresponding to dimerized ζ is likely to be a degradation product of this dimer. Empty arrows show the positions of the monomeric and dimeric PSMA-specific fusion receptors. Molecular mass markers are indicated on the left of the panel.

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The fusion receptor P28z elicits IL-2 production upon engagement with PSMA. To assay the ability of the different receptors to signal for IL-2 production, transduced PBLs were co-cultivated with NIH3T3 cells expressing PSMA and/or B7.1 (refs 18,19) in medium lacking IL-2 (Table 1). Three receptors (Pz1, P28z, and Pz28) elicited IL-2 secretion in the presence of PSMA and B7.1. In the absence of co-stimulatory ligand, IL-2 production was only observed in cultures of P28z-transduced T cells. IL-2 levels were elevated, ranging within 40–55% of those obtained by co-culturing the same transduced T cells with the monolayer co-expressing PSMA and B7.1.

The P28z fusion receptor promotes proliferation of genetically modified T cells in response to PSMA. To test if P28z could deliver combined and functional signals 1 and 2, transduced PBLs were plated on NIH3T3 cells expressing B7.1, PSMA, PSMA + B7.1, or on unmodified NIH3T3 cells. All cultures declined over one week in the absence of PSMA (Fig. 4A, B). When stimulated by a monolayer co-expressing PSMA + B7.1 (Fig. 4D), Pz1-transuced PBLs underwent expansion, as did PBLs transduced with P28z or Pz28, further establishing that both TCRζ–CD28 fusion receptors deliver a TCR-like signal. Control P28-transduced T cells did not expand under these conditions, indicating that neither co-stimulation alone nor adherence to the monolayer enhanced proliferation. When stimulation was provided by NIH3T3 cells expressing PSMA alone (Fig. 4C), T cells expressing Pz1 underwent limited expansion. Pz28-transduced cells also grew poorly, further indicating that this fusion receptor does not deliver a meaningful co-stimulatory signal. By contrast, P28z-transduced T cells consistently proliferated, corroborating observations by Eshhar *et al.* showing that immobilized hapten can induce proliferation in T cells that express a trinitrophenol-specific CD28–Fcγ fusion receptor[28]. P28z-transduced T cells markedly expanded, showing absolute increases in cell numbers (8.6- ± 5.2-fold over a seven-day period, $n = 8$ experiments). Taken together, these data strengthen the argument that P28z can provide both signals 1 and 2. Importantly, after seven days of co-culture onto a PSMA$^+$ fibroblast monolayer, T cells expressing the P28z fusion receptor retained the ability to specifically lyse PSMA$^+$ targets (Fig. 3B).

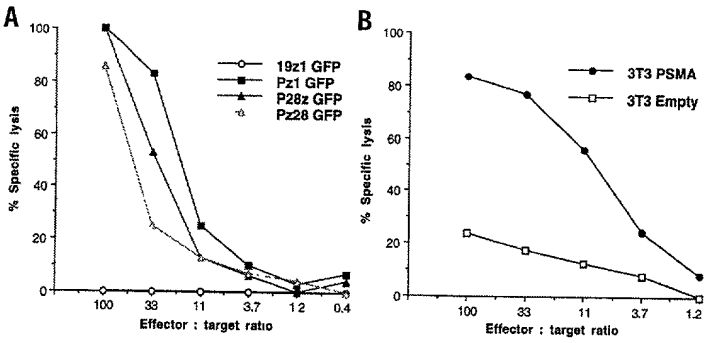

Figure 3. Specific target cell lysis by PSMA-redirected T cells. (A) T cells were transduced with 19z1 GFP (control), Pz1 GFP, P28z GFP, or Pz28 GFP. Three days after completion of gene transfer, 4 h CTL assays were established at the indicated ratios using as targets NIH3T3 cells expressing PSMA. No specific lysis was observed using untransduced NIH3T3 cells as control targets. The greater lytic activity of Pz1-transduced cells may reflect the higher cell-surface expression of this receptor, or, more likely, the greater proportion of transduced T cells (46% of T cells, of which 21% are CD8$^+$, compared with 25% P28z-transduced cells, including 12% CD8$^+$ cells, and 20% Pz28-transduced cells, including 10% CD8$^+$ cells). The control 19z1 receptor (specific for CD19; R.J.B. and M.S., unpublished results) did not effect lysis of PSMA-expressing targets, despite the presence of the same TCRζ chain in this molecule. (B) P28z-transduced T cells were stimulated on NIH3T3 cells expressing PSMA and, after one week, were established in 4 h CTL assays with NIH3T3 cells expressing PSMA or untransduced cells as controls. At this time, the T cells were 62% GFP$^+$ (of which 17% were CD8$^+$).

The P28z fusion receptor permits sequential re-stimulation of transduced human PBLs in response to PSMA. If P28z can provide co-stimulation in addition to a TCR-like signal, it would be expected that cells expressing this receptor should undergo further expansion upon secondary encounter with PSMA. However, if the co-stimulatory potency of this molecule is inadequate, sequential exposure to antigen could result in a poor proliferative response resulting from induction of anergy and/or apoptosis[20,21]. To test this, transduced PBLs stimulated on the different NIH3T3 monolayers were subjected to secondary re-stimulation after a seven-day interval. Pz1-transduced T cells expanded in response to primary encounter with PSMA. However, re-stimulation with PSMA resulted in a dramatic decline in the number of transduced cells (Fig. 5A, B). Importantly, the same T cells underwent brisk expansion after both primary and secondary stimulation if the fibroblast monolayer co-expressed PSMA and B7.1 (Fig. 5C and D, respectively). In contrast, the absolute number of P28z-transduced CD8$^+$ and CD4$^+$ T cells increased after primary stimulation and underwent further increase after re-stimulation on day 7, irrespective of the presence of B7.1. Expansion was indeed similar in response to PSMA alone or PSMA + B7.1, underscoring the relative potency of the co-stimulatory signal provided by P28z. Re-stimulation of P28z cultures with PSMA yielded a 4.0- ± 2.4-fold expansion in total cell number over a seven-day period ($n = 4$ experiments). Following another re-stimulation under the same conditions, the total cell number increased by more than 2 logs over a three-week interval (Fig. 5E). In this period, a progressive enrichment of transduced over nontransduced cells was observed, in keeping with the selective advantage conferred to cells expressing P28z (Fig. 5F). Together, these data provide conclusive evidence that P28z delivers a functional signal 1 and signal 2 upon interaction with PSMA. Importantly, the same result was obtained with another receptor, 19-28z, which was modeled on P28z. 19-28z-transduced PBLs showed the same ability to be re-stimulated by CD19$^+$ cells and to proliferate (unpublished observations), indicating that proliferative responses were achieved with receptors recognizing unrelated antigens.

P28z-transduced PBLs lyse PSMA$^+$ tumor cells and proliferate in response to LNCaP cells. We had previously shown that Pz1-transduced T cells specifically lyse LNCaP cells, a PSMA$^+$ human prostate cancer cell

Table 1. Interleukin-2 production by human PBL transduced with different CD3ζ–CD28 fusion receptors[a]

Fibroblast feeder	PSMA-specific receptor			
	P28	Pz1	P28z	Pz28
Unmodified NIH3T3	<50 (–)	<50 (–)	<50 (–)	<50 (–)
B7.1	<50 (–)	<50 (–)	<50 (–)	<50 (–)
PSMA	<50 (–)	<50 (–)	21,900 (1,153)	<50 (–)
PSMA + B7.1	<50 (–)	164,236 (3,285)	52,936 (2,786)	29700 (958)

[a]Three days after retroviral transduction, human PBLs expressing the indicated chimeric receptor were plated at 10^6 PBLs/ml on the specified NIH3T3 monolayers. Supernatants were harvested after 24 h and assayed for IL-2 content by ELISA. Data are expressed as mean IL-2 concentrations (pg/ml) from duplicate wells, followed, in parentheses, by IL-2 content normalized for gene transfer efficiency. Coefficients of variation between duplicates were all <2.5%. Normalized IL-2 values were determined by dividing the mean IL-2 concentration by the percentage transduced cells in the appropriate culture. Normalized IL-2 content measured in medium conditioned by P28z- and Pz1-transduced lymphocytes were comparable when the stimulus consisted of both PSMA and B7.1. Pz28-transduced T cells also produced readily detectable amounts of IL-2, but, interestingly, the magnitude of this response was consistently lower than that obtained with either Pz1 or P28z. Similar findings were observed in two independent experiments.

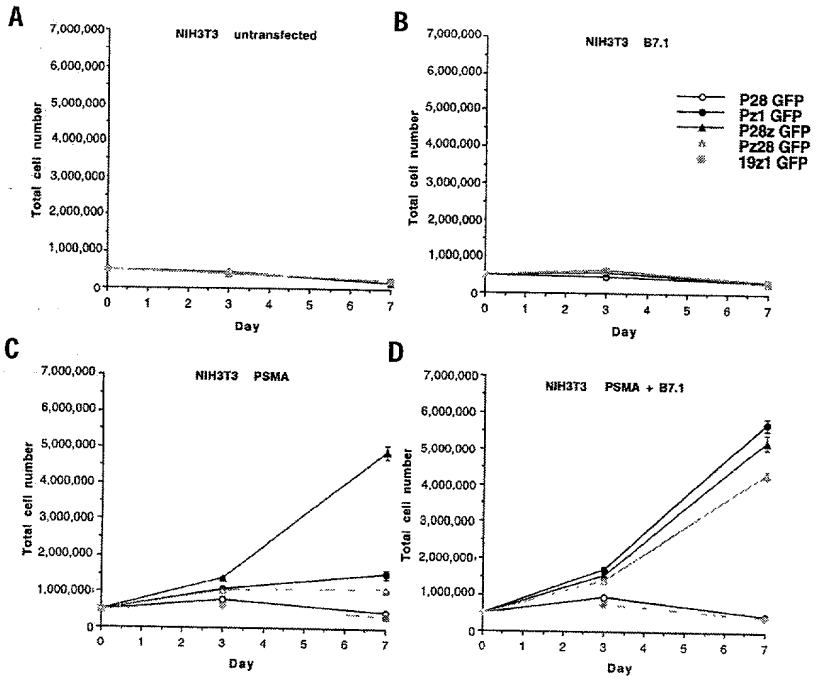

Figure 4. The P28z fusion receptor renders human T lymphocytes capable of PSMA-dependent expansion. Human T cells were transduced with the following retroviral constructs (gene transfer efficiency indicated in parentheses): SFG 19z1 (60%), SFG P28 (53%), SFG Pz1 (68%), SFG P28z (23%), and SFG Pz28 (32%). Three days later, 5×10^5 transduced T cells were co-cultured in 20 U/ml IL-2 with irradiated NIH3T3 feeder cells as follows: (A) unmodified (B) NIH3T3–B7.1 (C) NIH3T3–PSMA, or (D) NIH3T3–PSMA + B7.1. Cell numbers were counted on days 3 and 7, and data presented are mean ± s.d. of triplicate evaluations. Similar results were obtained in three experiments.

line, as well as PSMA-transduced PC3 and EL4 cells, which are respectively a human prostate cancer cell line and a murine thymoma[19]. Pz1, P28z, and Pz28 directed comparable and elevated cytolytic activity against LNCaP cells (Fig. 6A). Proliferative responses elicited by LNCaP cells expressing B7.1 were also comparable for these receptors (data not shown). Of the three receptors, however, only P28z could induce sustained proliferation during co-cultivation with LNCaP cells (Fig. 6B). The restimulated T cells preserved their tumoricidal activity (data not shown), corroborating findings obtained with PSMA$^+$ fibroblasts (Fig. 3B).

Discussion

The expansion of functional tumor-specific T lymphocytes is of central importance in tumor immunity. Whether in the context of *in vivo* immunization or *ex vivo* T-cell expansion, the biological requirements for T-cell priming and amplification have to be met to attain meaningful immune responses. Co-stimulation is crucial in this process[19–22] and is thus central to the development of effective adoptive immunotherapy of cancer[19,29]. Here we describe scFv-based chimeric receptors designed to provide both TCR-like and co-stimulatory signals upon binding of the tumor antigen PSMA. To achieve this, the intracellular domains of human TCRζ and CD28 have been fused in series within a single molecule, thereby recruiting these signaling motifs to the site of antigen engagement at a fixed stoichiometry of 1:1. Most important, our study was performed in human primary T lymphocytes—that is, in biologically and therapeutically relevant cells. The ability to sustain T-cell expansion and tumoricidal functions could therefore be evaluated, which is not possible in leukemic cells[30,31]. We show here that, following contact with cell-bound PSMA, activated human PBLs engineered to express the P28z receptor produce IL-2, undergo sequential rounds of expansion, and maintain thereafter their ability to execute specific lysis of PSMA-expressing target cells.

The most important finding in this study is the demonstration that expression of P28z enables T cells to undergo repeated rounds of antigen-dependent stimulation and expansion. This process was accompanied by a progressive increase in the proportion of transduced T cells within bulk cultures, consistent with the expected selective advantage conferred by the receptor. The capacity of P28z to deliver signal 1 is demonstrated by production of IL-2 and induction of cell proliferation upon stimulation with PSMA + B7.1, which are comparable to those obtained in T cells expressing Pz1 (which contains TCRζ but no CD28 sequences). Specific lysis of PSMA$^+$ targets also reflects functional activation through the TCR pathway. Importantly, the P28z fusion receptor can also provide potent co-stimulation (signal 2). Thus, in the absence of exogenous B7-driven co-stimulation, engagement of PSMA elicits IL-2 production and proliferation. Under the same conditions, Pz1-transduced cells fail to secrete IL-2 and proliferate, corroborating findings by Finney *et al.* obtained in Jurkat cells[31].

The relative positions of the TCRζ and CD28 signaling elements within the fusion receptor proved crucial. In P28z, the hinge, transmembrane, and proximal intracellular portions of the molecule were derived from CD28, followed by the signaling domain of TCRζ. When CD28 sequences were fused to the C terminus of TCRζ, as in Pz28, the functional activity was substantially compromised relative to P28z, particularly with regard to sustaining proliferation. This occurred despite comparable cell-surface expression of the two receptors. Pz28 retained the ability to deliver a TCR-like signal upon PSMA binding, as evidenced by cytolytic activity and B7.1-dependent proliferation and IL-2 production. However the co-stimulatory potency of Pz28, as evaluated in the absence of B7.1, was no better than that of Pz1.

One potential explanation for this finding is that the conformational integrity of the fusion receptor is disrupted when the CD28 signaling domain is placed downstream of TCRζ. It is noteworthy in this regard that western blotting analysis indicated that the Pz28 receptor exhibited less homodimerization in human T cells than either P28z or Pz1. An alternative explanation is that membrane proximity is more critical for CD28 than for TCRζ. Thus, placement of the CD28 moiety distal to TCRζ might impair its ability to associate with downstream signaling molecules, such as p56^{lck} (ref. 32), which reside in very close proximity to the cell membrane. A third possibility is that these fusion receptors differ in their ability to interact with negative regulators, for example, MAP kinase phosphatase-6 (MKP-6)[33]. It is plausible that the ability of P28z to bind MKP-6 might be impaired as a result of steric hindrance, thereby enhancing co-stimulatory activity. Conversely, in the case of Pz28, the binding of this phosphatase at the C terminus may adversely affect the signaling potency of this receptor. This hypothesis is supported by findings indicating that Pz28 was not only less active in eliciting IL-2 secretion than P28z, but also less active than Pz1. A final possible explanation for the superior function of P28z is that it contains the CD28 transmembrane domain, unlike Pz28 and Pz1. However, this is unlikely because the cytoplasmic portion of CD28 is sufficient for co-stimulatory activity[34].

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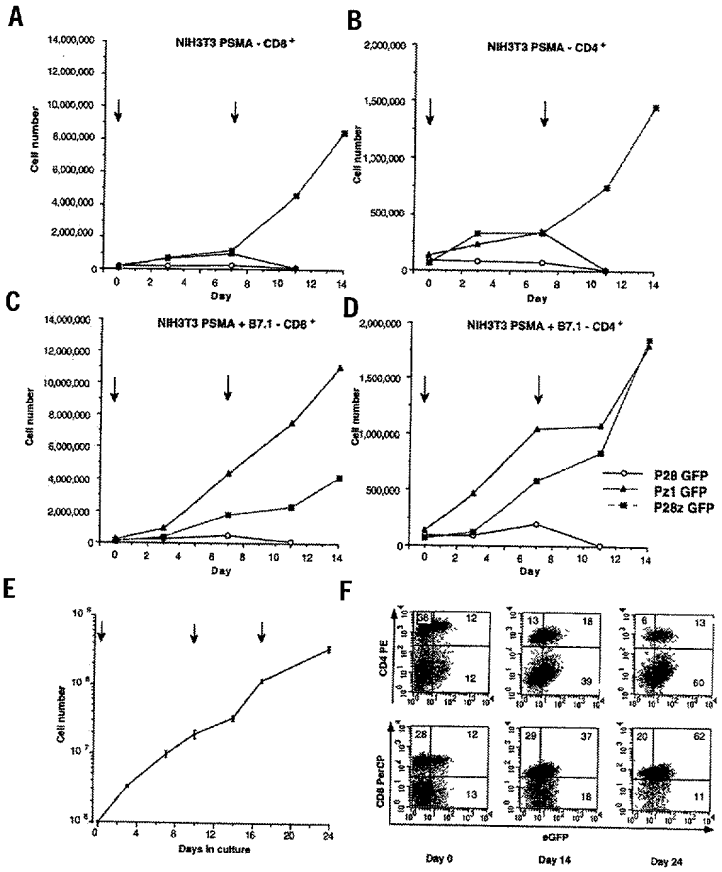

Figure 5. Primary and secondary stimulation of transduced T cells in response to PSMA. Peripheral blood T cells were transduced with the following retroviral constructs (gene transfer efficiency indicated in parentheses): P28 (27%), Pz1 (36%), or P28z (17%). Then the cells were subjected to two rounds of stimulation on NIH3T3 fibroblast feeder layers (indicated by arrows). For the primary stimulation, 1×10^6 transduced T cells were co-cultured in IL-2 (20 U/ml) with irradiated NIH3T3 cells expressing PSMA (panels A and B) or PSMA + B7.1 (panels C and D). On day 7, cultures were re-stimulated by co-culture with a similar monolayer. Absolute numbers of transduced $CD8^+$ (panels A and C) and $CD4^+$ T cells (panels B and D) were calculated as the product of percentage transduced (determined by flow cytometry) × total cell count. Co-culture of all transduced PBL populations with B7.1-expressing or unmodified NIH3T3 cells resulted in a progressive decline in total cell number and content of transduced T cells (data not shown). (E) P28z-transduced T cells were expanded by sequential re-stimulation on NIH3T3 PSMA fibroblast feeder layers, as indicated by the arrows. Cultures were maintained in IL-2 (20 U/ml), which was added every three days. The data represent the mean ± s.d. of six data points (triplicate cell counts from two separate cultures). These cultures were subjected to three-color flow cytometry at intervals to detect transduced ($eGFP^+$) cells of the $CD4^+$ and $CD8^+$ subsets. Similar data were obtained upon analysis of both cultures, and data shown are from one representative example (F).

How might adoptive transfer of cells expressing P28z be developed for therapy directed against PSMA-expressing tumors or tumor-associated vasculature? As this fusion receptor enables transduced T cells to proliferate in an antigen-dependent manner, this raises the prospect that these cells could be expanded both *in vitro*, before infusion, and, most importantly, *in vivo* in the tumor-bearing host. There is substantial preclinical evidence indicating that success of adoptive T-cell therapy depends largely on the relative numbers and growth kinetics of tumor cells and therapeutically administered T cells[35,36]. Consequently, treatment with T cells expressing a receptor like P28z may require smaller T-cell doses (and thus shorter *in vitro* culture periods) and allow for T-cell expansion following infusion. As P28z-transduced T cells expanded on PSMA-positive cells retained their specific cytolytic activity, such a cell culture procedure could provide a useful means to selectively expand transduced T cells. Importantly, P28z provides a means to activate and expand T cells upon engaging cells that lack MHC and/or co-stimulatory molecules, and may thus target the transduced lymphocytes to cells that escape immune recognition.

In summary, we have shown that artificial receptors based upon fusion of the signaling domains of TCRζ and CD28 can be used to redirect the specificity of primary human T cells to a tumor antigen. The transduced T cells undergo selective expansion following contact with cell-bound PSMA while maintaining the ability to mediate specific lysis of tumor cells. The availability of a single chimeric receptor providing both activation and co-stimulatory functions should greatly facilitate lymphocyte transduction and hence clinical applicability. These findings raise the prospect of a useful approach to cancer immonotherapy based on T cells that are genetically engineered to sustain proliferative and cytotoxic responses against tumor antigens.

Experimental protocol

Recombinant receptors and retroviral vectors. All fusion receptors contain a scFv derived from the J591 hybridoma[25] as described[18]. To facilitate detection of transduced cells, all constructs contained the encephalomyocarditis virus internal ribosome entry site (EMCV-IRES)[37] and the eGFP gene inserted in the SFG vector[38]. In Pz1, the J591 scFv is coupled through human CD8α hinge and transmembrane sequences to the intracellular domain of human TCRζ (ref. 18). P28 comprises a fusion of the J591 scFv to human CD28 as described[23,39]. To construct P28z, nucleotides 336–660 of CD28 were amplified using primers 1 (5′-GGCGGCCG CAATTGAAGTTATGTATC-3′) and 2 (5′-TGCGCTCCTGCTGAACTTCACTCTGGAGCGATAGGCTGCGAAGTCGCG-3′). The intracellular domain of TCRζ was amplified using primers 3 (5′-AGAGTGAAGTTCAGCAGGAGCGCA-3′) and 4 (5′-CTCGAGTGGCTGT-TAGCGAGG-3′). The products were fused in a separate PCR reaction driven by primers 1 and 4, A-tailed with *Taq* polymerase, and subcloned as a

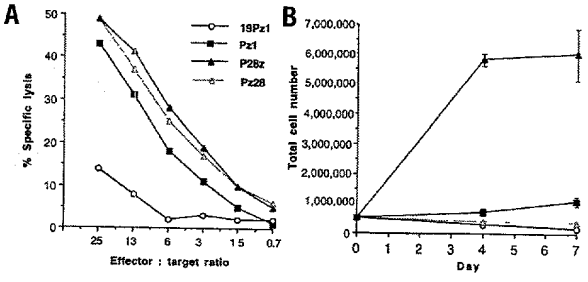

Figure 6. $PSMA^+$ tumor cells activate cytolytic and proliferative responses in P28z-transduced PBLs. (A) Specific tumor cell lysis by PSMA-redirected T cells. T cells were transduced with 19z1 (control), Pz1, P28z GFP, and Pz28 GFP. Four days after completion of gene transfer, equivalent numbers of transduced T cells were added to LNCaP human prostate cells. All PSMA-specific T cells (Pz1, P28z, and Pz28) demonstrated cytotoxic activity similar to that demonstrated against NIH3T3 $PSMA^+$ fibroblasts. Background cytotoxic activity seen with 19z1 control T cells may be due to alloreactivity (which is not seen with the murine NIH3T3 fibroblasts; Fig. 3). (B) The P28z fusion receptor renders T lymphocytes capable of PSMA-dependent, B7.1-independent expansion following co-cultivation with LNCaP tumor cells. 19z1-, Pz1-, and Pz28-transduced T cells did not expand.

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NotI/XhoI fragment into SFG-Pz1. To generate Pz28, the intracellular domain of CD28 was amplified using 5′-GCACTTCACATGCAGGCTCTGCCAC-CTCGCAGGAGTAAGAGGAGCAGGCTCCTGCAC-3′ and 5′-CGCTC-GAGTCAGGAGCGATAGGCTGCGAAGTCGCGT-3′ (two silent mutations introduced to interrupt cytosine repeats are underlined). The resultant PCR product represents a fusion of the distal nine codons of TCRζ (minus stop codon) to the intracellular domain of CD28 and contains a convenient 5′ *NspI* site. This fragment was subcloned, digested with *NspI/XhoI*, and ligated into SFG-Pz1. SFG–c-*fms* encodes the human macrophage colony-stimulating factor receptor (J.M. and M.S., unpublished results).

Culture and retroviral transduction of primary human T cells. Peripheral blood mononuclear cells from healthy donors were established in RPMI + 10% (vol/vol) human serum, activated with phytohemagglutinin (2 μg/ml) for two days, and transferred to non-tissue culture–treated plates (Falcon, Becton Dickinson, Franklin Lakes, NJ) precoated with retronectin (15 μg/ml; Takara Biomedicals, Shiga, Japan). Gibbon ape leukemia virus envelope–pseudotyped retroviral particles were generated as described[27,40]. Transduced cells were co-cultivated with NIH3T3 fibroblasts expressing PSMA and/or B7.1 as described[18,23]. For experiments with LNCaP cells, cells were admixed weekly at a T-cell:tumor cell ratio of 5:1.

Protein analyses. Flow cytometry was carried out using a FACScan cytometer with Cellquest software. Expression of PSMA-specific fusion receptors was directly demonstrated using phycoerythrin (PE)-conjugated goat anti-mouse antiserum[18]. CD4-PE and CD8-PerCP antibodies (Becton Dickinson) were used for T-cell subset identification. For western blot analysis, transduced T-cell samples were prepared as described[41]. Briefly, cells were suspended in radioimmunoprecipitation buffer at a concentration of 1×10^7 cells/ml. After 1 h incubation on ice, cells were boiled in 2× loading buffer under nonreducing or reducing conditions with 0.1 M dithiothreitol. Samples were run on 10–20% acrylamide gradient gels and transferred to polyvinylidene fluoride transfer membrane (NEN Life Science Products, Boston, MA). Fusion proteins were detected using the anti-human ζ-chain monoclonal antibody 8D3 (PharMingen, San Diego, CA) as described[41]. Immunodetection was performed using the ECL Plus western blotting detection system (Amersham, Buckinghamshire, UK).

Cytotoxicity assays. Cytotoxic T-lymphocyte assays were performed using a nonradioactive cytotoxicity detection kit (lactate dehydrogenase (LDH); Roche Diagnostics, Indianapolis, IN) as described[42] or ^{51}Cr-release assays performed as described[18].

Statistical analyses. The one-tailed Student's *t*-test was used.

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