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<p>(54) Title: FUSION RECEPTORS SPECIFIC FOR PROSTATE-SPECIFIC MEMBRANE ANTIGEN AND USES THEREOF</p>		
<p>(57) Abstract</p> <p>A fusion receptor composition which is effective to promote a cellular immune response to prostate-specific membrane antigen (PSMA) <i>in vivo</i> when the fusion receptors is expressed by T lymphocytes has the structure: PSMA-scFv: connector: cytoplasmic domain. The PSMA-scFv in this structure is a single chain antibody cloned from the V region genes of a hybridoma specific for PSMA. The connector region is provided to give a spacing between the OSMA-scFv and the cytoplasmic domain, such that both can retain substantial function. A suitable connector is the CD8 hinge, although other connectors of greater or lesser length might be used. The cytoplasmic domain is included to direct the function of the fusion receptor. One exemplary cytoplasmic domain which can be used in the fusion receptor of the invention is a T cell receptor ζ-chain cytoplasmic domain. An expression vector encoding the fusion receptor is transduced into primary T lymphocytes obtained from an individual to be treated. The transduced lymphocytes are returned to the patient where cells expressing the fusion receptor secrete interleukin 2 and proliferate in response to PSMA-positive cells. The resulting cytotoxic lymphocytes specifically lyse cells expressing PSMA and thus can be used to target PSMA-positive tumor cells and neovasculature.</p>		

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- 1 -

FUSION RECEPTORS SPECIFIC FOR PROSTATE-SPECIFIC MEMBRANE ANTIGEN AND USES THEREOF

This application claims priority from US Provisional Application No. 60/099,138, filed September 4, 1998, which for purposes of the United States and countries allowing such incorporation, is incorporated herein by reference.

Field of the Invention

This invention relates to fusion receptors for prostate-specific membrane antigen (PSMA), and to uses thereof in the treatment of prostate cancer, other cancers expressing PSMA and tumor neovasculature. The invention provides fusion receptors, nucleic acids encoding these fusion receptors, and transduced cells expressing the fusion receptors, as well as methods of using the transduced cells.

Background of the Invention

A long-standing goal of cancer research has been to stimulate the immunological rejection of tumors. This goal is based on the hypothesis that many tumors express foreign or mutated forms of antigens that can potentially serve as targets for their destruction by the immune system. Cellular immunity plays the key role in this rejection, with both T helper cells and cytolytic T lymphocytes (CTLs) being involved (Greenberg, *Adv. Immunol.* 49: 281-355 (1991)).

There are several reasons why even those tumors that express rejection antigens can evade destruction by T cell immunity. Destruction of immunological targets requires T lymphocyte recognition via the T cell receptor (TCR) of antigenic peptides presented in the context of major histocompatibility complex (MHC) molecules on antigen-presenting cells (APC). Some tumors fail adequately to process and present antigens to T cells because of reduced expression of MHC class I molecules.

Many strategies have been devised to render tumor cells more immunogenic. One is based on the genetic engineering of tumor cells to stimulate the generation of tumor-specific effector T cells *in vivo*. This has been investigated by direct MHC class I gene transfection to enhance expression of MHC by introduction of the γ -interferon cDNA to

- 2-

upregulate endogenous class I antigens or by transfecting tumor cells with cytokines with the hope that interleukin paracrine secretion of lymphokines can substitute for T cell help, induce tumor specific cytotoxic T lymphocytes, and cause tumor rejection.

The molecular basis of T cell costimulation results from an interaction of the T cell surface receptor CD28 with the costimulatory ligand B7, which is primarily expressed on the surface of professional APCs and activated B cells leading to IL-2 secretion and clonal expansion of the activated T cells. *In vitro* and *in vivo* studies showed that signals transduced by the CD28 receptor determine whether TCR occupancy results in a productive immune response or clonal anergy. Therefore, one factor accounting for the poor immunogenicity of MHC-expressing tumors is that, despite presentation of potentially immunogenic peptides in the context of MHC molecules, tumors lack the costimulatory molecule B7, and thus fail to elicit a full activation of T cells and therefore an effective anti-tumor T cell response. Thus, the introduction of the B7 molecule (CD28 ligand) in tumor cells is one discussed therapy today (melanoma: Townsend et al, 1993; Chen et al, 1992; colon carcinoma: Townsend et al, 1994) to provide protective immunity by autologous CD8⁺ T cells which leads to a potent rejection of modified and unmodified tumor cells *in vivo*. CD4⁺ and CD8⁺ immunity induced by immunization with class II⁺B7-1⁺-transfected sarcoma cells are also widely discussed immunotherapy strategies.

Another approach is based on the manipulation of the effector cells, i.e., T lymphocytes, rather than the antigen-presenting cells or tumor cells. T cells can recognize and lyse tumor cells provided that they bind to the tumor cells and are appropriately activated. T cell activation operates according to the two signal model, which states that lymphocytes require for optimal activation both an antigen-specific signal delivered through the antigen receptor and a second antigen nonspecific or costimulatory signal. T cell costimulatory pathways determine whether TCR complex engagement results in functional activation or clonal anergy of CD4⁺ T cells.

One means of generating tumor-specific T lymphocytes is their modification by gene transfer of tumor-specific fusion molecules. The introduction of chimeric molecules in T cells combining tumor specific single chain variable fragment (scFv) with signal transduction domains of TCR related activation molecules is reported by a number of groups

(Eshar et al, *Springer Semin. Immunopathol.* 18: 199-209 (1993)). These genetically modified T cells are able to target tumor cells and to destroy them *in vitro*, but based on the two signal model for T cell activation, the reinfusion of these transduced T lymphocytes is limited by the incomplete activation signal after antigen recognition and clonal expansion *in vivo* is not successful.

Alvarez-Vallina et al., *Eur. J. Immunol.* 26: 2304-2309 (1996) have shown that antigen dependent IL-2 secretion can be stimulated *in vitro* in Jurkat cells expressing a chimeric molecule formed from an antigen-specific single chain antibody variable fragment (scFv) and a truncated CD28 (amino acids 124-202; transmembrane and cytoplasmic domains), when the cells are exposed to the antigen in the presence of anti-CD3 or ionomycin as a costimulatory signal. Similarly, antigen dependent IL-2 secretion can be stimulated *in vitro* in Jurkat cells expressing a chimeric molecule formed from an antigen-specific scFv and the ζ -chain of CD3, when the cells are exposed to the antigen in the presence of anti-CD28 or ionomycin as a costimulatory signal. Cells expressing both chimeric molecules displayed responses to either antigen in the presence of appropriate costimulatory molecules. Alvarez-Vallina et al. suggests that these results offer the possibility that addition of antigen-specific CD28 mediated signaling could improve adoptive immunotherapies.

It is a substantial step, however, from such *in vitro* results to therapeutic efficacy *in vivo*. Importantly, while IL-2 secretion is suggestive of T cell activation, it can frequently be followed by T cell anergy or apoptosis (presumably as a result of inadequate costimulation) which results in the death of the T cells rather than the development of an immune response. There is no assurance that signaling species will be present *in vivo* to fulfill the role of the external costimulatory signals supplied by Alvarez-Vallina et al. in their *in vitro* experiments, or that these signals will result in the level of clonal expansion which is required to mount and maintain a therapeutically meaningful cytotoxic T cell response. Furthermore, artificial T-cell receptors have not been shown to function in human peripheral blood lymphocytes (PBL) and in particular in the T cells of actual cancer patients. The applicability of findings in normal T cells or carefully selected leukemic cells lines to T cells from cancer patients cannot be taken for granted given the signaling defects frequently observed in cancer patients and chronically tumor-bearing mice. Mizoguchi et al., *Science*

- 4 -

258: 1795-1798 (1992); Ochoa et al., in *Important Advances in Oncology*, J.B. Lippincott Co., Philadelphia (1995); Zier et al., *Hum. Gene Ther.* 6: 1259-1264 (1995). Some of these defects, which include ζ -chain, lck and ZAP-80 abnormalities, could limit the function of artificial TCRs in cancer patient T cells. Thus, there remains a need for a method for sustaining the formation of tumor-specific T lymphocytes which can be successfully reintroduced into a host organism, preferably a human being, for the *in vivo* generation of a cellular immune response to the tumor.

It is an object of the present invention to provide such a method, and to provide fusion proteins, and nucleic acid constructs encoding such fusion proteins which can be used in such a method.

It is a further object to provide such methods and fusion proteins specific for prostate-specific membrane antigen.

Summary of the Invention

The present invention provides a fusion receptor composition which is effective to promote a cellular immune response to a target antigen *in vivo* when the fusion receptor is expressed by T lymphocytes. By way of example, when the target antigen is prostate-specific membrane antigen (PSMA), the fusion receptor has the structure:

PSMA-scFv : optional connector : cytoplasmic domain.

The PSMA-scFv in this structure is a single chain antibody cloned from the V region genes of a hybridoma specific for PSMA. The optional connector region is provided to give a spacing between the PSMA-scFv and the cytoplasmic domain, such that both can retain substantial function. In cases where a connector is needed, a suitable connector is the CD8 hinge, although other connectors of greater or lesser length might be used. The cytoplasmic domain is included to direct the function of the fusion receptor. One exemplary cytoplasmic domain which can be used in the fusion receptor of the invention is a T cell receptor ζ -chain cytoplasmic domain.

In accordance with the method of the invention, an expression vector encoding the fusion receptor is transduced into primary T lymphocytes obtained from an individual to be treated, for example an expression vector encoding the PSMA-scFv containing fusion

- 5 -

receptor is suitably transduced into cells from a human patient who has been diagnosed with prostate cancer. The transduced lymphocytes are returned to the patient where cells expressing the fusion receptor secrete interleukin 2 and proliferate in response to PSMA-positive cells. The resulting cytotoxic lymphocytes specifically lyse cells expressing PSMA and thus can be used to target PSMA-positive tumor cells. Expression of the fusion receptor in CD4+ or CD8+ T-cells, natural killer (NK) cells or other immune effector cells allows these cells to target any tissue (including tumor tissue) expressing PSMA. Thus, such cells can be used to treat prostate cancer, other cancers expressing PSMA and tumor-associated neovasculature.

Brief Description of the Drawings

Fig. 1 shows the structure of a retroviral vector Pz-1 including a gene for a PSMA-specific fusion receptor in accordance with the invention;

Figs. 2 A-E show cytotoxicity of Pz-1 transduced PBL with respect to various target cells;

Fig. 3 shows the time course for cocultivation of transduced T cells with fibroblasts;

Fig. 4A shows T cells proliferation in coculture with various types of fibroblast cells;

Fig. 4B shows cell lysis by T cells after prior exposure to coculture conditions; and

Fig. 5 shows IL-2 production by transduced T cells in coculture with various types of fibroblast cells.

Detailed Description of the Invention

In a first aspect, the present invention provides fusion receptors which are useful in the generation of a cellular immune response to cells which express PSMA. Such fusion receptors have the general structure:

PSMA-scFv : optional connector : cytoplasmic domain.

- 6 -

This structure is produced by expression in transduced cells of a DNA sequence encoding the amino acid sequence of the fusion receptor.

In the general formula set forth above, PSMA-scFv is a single chain antibody cloned from the V region genes of a hybridoma specific for PSMA. A suitable hybridoma for this purpose is J591, which is described in Liu et al., *Cancer Res.* 57: 3629-3635 (1997), although other hybridomas which produce monoclonal antibodies specific to PSMA could also be employed. The production of such hybridomas has become routine, and the procedure will not be repeated here.

A technique which can be used for cloning the variable region heavy chain (V_H) and variable region light chain (V_L) has been described in Orlandi et al., *Proc. Natl Acad. Sci. (USA)* 86: 3833-3837 (1989). Briefly, mRNA is isolated from the hybridoma cell line, and reverse transcribed into complementary DNA (cDNA), for example using a reverse transcriptase polymerase chain reaction (RT-PCR) kit. Sequence-specific primers corresponding to the sequence of the V_H and V_L genes were used, for example Seq. ID. Nos. 1-4. Sequence analysis of the cloned products and comparison to the known for the V_H and V_L genes showed that the cloned V_H gene matched expectations. For the V_L gene, however, the cloned sequence did not match expectations, and contained a stop codon in the anticipated open reading frame. To correct this, corrective primers (Seq. ID. Nos. 5 and 6), containing inserted bases to correct the differences between the clone and the known sequence were used to amplify the cloned V_L gene to produce polynucleotides encoding the V_L gene.

The cytoplasmic domain portion of the general formula set forth above is selected to enhance the characteristics of the fusion receptor for purposes of promoting a cellular immune response to the antigen recognized by the scFv portion of the fusion receptor. In general, the cytoplasmic domain is the cytoplasmic domain of a molecule which functions as a transducer of a mammalian immune response in the presence of an MHC-peptide complex or costimulatory factor. Representative, non-limiting examples of cytoplasmic domains which may be employed in the present invention include the ζ -chain cytoplasmic domain, the CD28 cytoplasmic domain (particularly a fragment spanning amino acids 336 to 663 of CD28 cDNA), 41BB, CD40, ICOS and trance.

- 7 -

When the cytoplasmic domain is the ζ -chain derived the TCR complex, the fusion receptor of the invention closely mimics a native TCR. In this case, it might be expected that binding of an antigen to the scFv portion of the fusion receptor would result in changes to the ζ -chain which would trigger an intracellular phosphorylation cascade comparable to that observed when an antigen bound to an APC interacts with a native TCR. As noted above, however, this signal transduction is not sufficient to produce a complete cellular immune response, and a secondary signal must be provided to avoid anergy and premature cell death through apoptosis. In the art, this secondary signal was provided by a signaling antibody unsuited for use *in vivo*. The present invention provides for the first time the understanding that cells expressing PSMA and a costimulatory signal such as B7.1 provide sufficient secondary signal to maintain a stable population of PSMA-targeting T cells when the cells are transduced with the fusion protein of the invention.

Instead to the ζ -chain, the fusion receptors of the invention may include other cytoplasmic domains. For example, CD28 can be used as the cytoplasmic domain to enhance T-cell activation, survival and proliferation. A preferred CD28 moiety is one which spans amino acids 336 to 663 of CD28 cDNA, in which case no connector is needed to retain function. PSMA-fusion receptors incorporating 41BB as the cytoplasmic domain have also been prepared. Both the PSMA-CD28 and the PSMA-41BB fusion receptors have been made and tested in the same experimental model used with the PSMA- ζ chain fusion receptor. In both cases, sustained proliferation was observed in both human CD4⁺ and CD8⁺ primary T cells (PBL) in the presence of PSMA⁺ cells, with more sustained proliferation being provided by the PSMA-41BB fusion receptor. High production of IFN- γ and IL-2 was observed, for PSMA-41BB and PSMA-CD28 transduced, respectively. In each of the experiments performed, an external signal to complement the signaling of the fusion receptor was used. However, transfection of the PBL with fusion receptors encoding both the ζ -chain and either CD28 or 41BB or a comparable costimulatory molecule would eliminate this requirement. Thus, for example, PBL transduced with both the PSMA- ζ chain fusion receptor and either a PSMA-fusion receptor with a secondary signaling moiety would provide therapeutic efficacy for *in vivo* use.

Between the scFv and the cytoplasmic domain may be a connector. The function of the connector is to act as a spacer so that both the scFv and the cytoplasmic domain can be functionally oriented within the membrane of the transduced cell. One exemplary connector is the CD8 hinge, although other connectors of greater or lesser length could be used. In some cases, such as using the CD28 fragment described herein, no connector is required to permit the molecules to assume the desired orientation.

The chimeric fusion receptors are introduced into the individual to be treated (preferably a human) in one of two ways. Gene transfer can be carried out into bone marrow cells, either *in vivo* or *ex vivo*, or into immune effector/inflammatory cells such as T-lymphocytes or NK cells. Gene transfer may also be carried out into antigen presenting cells, particularly dendritic cells. In the case of dendritic cells, CD40 and trnce are the preferred cytoplasmic domain.

A preferred approach to this gene transfer is using retroviral vectors encoding the fusion receptor. A particularly preferred approach utilizes an SFG retroviral vector (Riviere et al., *Proc. Natl Acad Sci. (USA)* 92: 6733-6737 (1995)) transduced into patient PBL using gibbon ape leukemia virus (GALV) envelope-pseudotyped virions. (Gallardo et al., *Blood* 90: 952-957 (1997)).

The PSMA-specific fusion receptor of the present invention is useful in the treatment of prostate cancer. In addition, because PSMA is also found in the neovasculature of renal cell, urothelial, colon, breast and lung carcinomas, melanomas and some sarcomas, the PSMA-specific fusion receptor of the invention has broader applicability. Thus, in one aspect of the invention, the present application describes a method for treatment of cancers in which the cancer cells or neovasculature are characterized by expression of PSMA, comprising administering to a patient suffering from such a cancer patient-derived lymphocytes which express with a PSMA fusion receptor having the structure

PSMA-scFv : optional connector : cytoplasmic domain.

As used herein, the concept of "administration" is intended to encompass both *in vivo* methods, in which the fusion receptor is introduced into the lymphocytes without first removing them from the patient, and *ex vivo* methods where the patient-derived lymphocytes

- 9 -

are obtained from the patient, transduced with the PSMA-specific fusion receptor and then reintroduced to the patient.

The transduced lymphocytes are introduced in an amount to provide therapeutic benefit. Where sufficient clonal expansion of the transduced lymphocytes occurs *in vivo*, a long-term immunity to the tumor cells may be induced after a single administration. If the transduced lymphocytes are less stable, multiple infusions may be required to obtain remission of a particular cancer, and long-term protection may not be achieved. In either case, the determination of the appropriate therapeutic regimen is a matter of routine developed in the course of clinical trials.

The invention will now be further described and illustrated with reference to the following non-limiting examples.

Example 1

PSMA-scFv was created by cloning the immunoglobulin genes from the J591 hybridoma encoding the variable region of the heavy chain (V_H) and the variable region of the light chain (V_L). The V_H and V_L genes were cloned using the technique previously described by Orlandi et al., *supra*. Briefly, mRNA was isolated from the J591 hybridoma cell line and reverse transcribed into cDNA using a reverse transcriptase polymerase chain reaction (RT-PCR) kit obtained from Pharmacia, Pisacatway, NJ. The V_H and V_L genes were cloned from the cDNA using the following degenerate primers:

V_H backward:

AGGTSMARCTGCAGSAGTCWGG

Seq. ID. No. 1

V_H forward:

TGAGGAGACGGTGACCGTGGTCCCTTGGCCCCAG

Seq. ID. No. 2

V_L backward:

GACATTGAGCTCACCCAGTCTCCA

Seq. ID. No. 3

- 10-

V_L forward:

TGCGGCCGCCCGTTTGATCTCCAGCTTGGTCCC

Seq. ID. No. 4

Sequence analysis of the V_H gene confirmed an appropriate open reading frame. However, the V_L gene sequence analysis revealed a stop codon in the anticipated reading frame. The sequences of the genes encoding the J591 monoclonal antibody heavy and light chain were compared to the sequences of the cloned products, and several discrepancies were noted between the V_L sequences. The major difference was that the primer pair used deleted a nucleotide from the actual sequence, resulting in an open reading frame shift that produced a stop codon. Nucleotide sequence corrections in the V_L product were made using corrective primers based on the actual sequences and using these primers in a second PCR amplification utilizing the obtained V_L sequence as a template. The corrective primers were:

V_L backward:GAAGAAGATCT***GTG***ACATTGTGATGACCAGTCTC***ACA***AAATTCATG

Seq. ID. No. 5

V_L forward:TGCGGCCGCCCGTTT***CAGGT***CC***AG***CATGGTCCCAGCACCG

Seq. ID. No. 6

wherein the bold and italicized letters indicate additions/substitutions to correct the obtained V_L sequence to the actual J591 V_L sequence.

Next, an oligonucleotide encoding the human CD8 leader sequence was cloned to the 5'-end of the V_H gene, and the 3'-end of the V_H gene was cloned to an oligonucleotide encoding a (gly-ser)₂ linker followed by the V_L gene, creating the PSMA-specific scFv. The scFv was then cloned to the CD8 hinge and transmembrane domains, followed by the T cell receptor ζ-chain cytoplasmic domain to create Pz-1, a PSMA-specific scFv/ζ-chain chimeric T-cell receptor. The Pz-1 fusion gene was then cloned into the SFG retroviral vector (Riviere et al, *supra*) as illustrated in Fig. 1.

Example 2

The SFG retroviral vector containing Pz-1 was transduced into PBL's harvested from five human patients suffering from prostate cancer with a variety of clinical stages using GALV envelope pseudotyped virions as previously described. Gallardo et al., *supra*. The clinical status of three representative patients is summarized in Table 1.

#	age	time since dx (mon)	GG	stage at dx	treatment	current stage	current PSA (ng/ml)
1	67	19	3+3	T1c	none	T1c	10.2
2	85	251	5+4	T3	hormonal	T4M+	501.2
3	54	7	4+5	T1c	RRP	pT3CNoMx rising PSA	4.0

In Table 1, the age is the current age of the patient, the time since dx is the time elapsed between date of diagnosis of prostate cancer and PBL harvest; GG is the Gleason grade of the patient's most recent prostate cancer pathology; stage at dx is the clinical stage at the time of original diagnosis; RRP in the treatment column stands for radical retropubic prostatectomy; current stage is the current clinical or pathological state and current PSA is the most recent serum prostate specific antigen (PSA) level. Normal PSA is in the range of 0-4 ng/ml. A rising PSA following RRP is biochemical evidence of residual prostate cancer, although the site of the residual disease is unknown.

After retroviral infection, the PBL were expanded 4 to 14 days in the presence of interleukin-2 (IL-2). Gene transfer efficiency was monitored by FACS analysis using a FITC-conjugated Pz-1 idio-type-specific antiserum. After incubation with the FITC labeled antiserum, the cells were washed incubated with 10% normal mouse serum, and stained with a PE-conjugated anti-CD8 mAb. The gene transfer efficiency observed varied between 20% and 50% in both CD8⁺ and CD4⁺ cells for Pz-1 and controls.

Example 3

Cytotoxic T lymphocyte (CTL) assays were performed on the human prostate cancer cell line LNCaP which abundantly expresses PSMA. In order to confirm that the

- 12-

cytotoxicity was PSMA-specific, PSMA was expressed in PC-3, a PSMA-negative human prostate cancer cell line and EL-4, a murine thymoma cell line. PBL from the three patients of Table 1 were transduced with either Pz-1 or NTP, a mutated human low-affinity nerve growth factor receptor used as a control cell surface marker. (Gallardo et al., *supra*). Transduction efficiency (%TR) measured as described above, and the fraction of CD8⁺ and CD56⁺ cells on the day of the CTL assay are reported in the common legend of Fig. 2. The cultured PBL, harvested 4 to 14 days after transduction, were incubated for 4 hours at different effector to target (E:T) ratios with 10⁴ ⁵¹Cr-labeled target cells. Percent specific lysis was determined for PSMA⁺ LNCaP cells (Fig. 2A), PC3 cells transduced with PSMA (Fig. 2B), wild-type, PSMA⁻ - PC3 cells (Fig. 2C); EL4 cells transduced with PSMA (Fig. 2D) and wild-type PSMA⁻ - EL4 cells (Fig. 2E). As shown, for all patients, PBL transduced with Pz-1 but not NTP effectively lysed PSMA⁺ targets. The same results were obtained using Pz-1 transduced PBL from two other patients with advanced, hormone refractory prostate cancer. The level of cytolytic activity did not closely correlate with the transduction efficiency achieved in each case. Background was variable on human target cells and uniformly low on murine cells. T cells transduced with an irrelevant scFv- ζ chain fusion receptor did not lyse target cells expressing PSMA above background levels. Thus, elevated PSMA-specific cytotoxicity was obtained in Pz-1 transduced T cells derived from five out of five prostate cancer patients tested, independent of their age or clinical stage of disease.

Example 4

To further assess the response of Pz-1 transduced primary T cells to PSMA, we investigated whether Pz-1⁺ PBL could undergo proliferation upon engagement with cell-bound PSMA and sustain thereafter their cytolytic potential. A cocultivation system was established in which transduced T cells were cultured with a layer of irradiated NIH3T3 fibroblasts expressing various combinations of PSMA and B7.1 for four days with periodic sampling to measure levels of IL-2 as illustrated in Fig. 3. FACS cell counts using FITC-conjugated Pz-1 idiotype specific antibody and with either anti-CD4 or anti-CD8 were performed after four days of co-cultivation, and again 4 days later. The transduced T cell count was derived by multiplying the percentage of CD4⁺Pz-1⁺ or CD8⁺Pz-1⁺ double positive

- 13-

cells by the number of viable cells. The results are shown in Fig. 4A, where /B7+PSMA, /PSMA and /B7 refer to the molecules expressed by the fibroblast layer.

PSMA induced proliferation of Pz-1 transduced T-cells, increasing the number of cells 6-8 fold after 4 days, and these transduced cells destroyed PSMA⁺ fibroblast layers within 48 hours, while the PSMA⁻ layers remained intact during the entire 4-day cocultivation. By day 8, however, the absolute number of Pz-1⁺ cells dropped to 2-3 fold above initial levels. To address whether costimulation could amplify proliferation, B7.1 (CD80) was transduced into PSMA⁺ and PSMA⁻ fibroblasts. After 8 days of cocultivation, Pz-1⁺ T cell counts were 5-8 fold higher in cultures with PSMA⁺ B7.1 fibroblasts than with PSMA⁺B7.1⁻ fibroblasts, and 25-36 fold higher than in cultures with PSMA⁻ B7.1⁺ fibroblasts, suggesting that Pz-1 signaling synergized with the B7.1 mediated costimulation. Fibroblasts expressing PSMA \pm B7.1 did no induce proliferation of T cells transduced with an irrelevant scFv- ζ chain fusion receptor.

Supernatants of the cocultured T cells were sampled after 24, 48 and 72 hours and assayed for IL-2 and interferon- γ (IFN- γ) secretion. Pz-1 transduced cells released significant amounts of IL2 (Fig. 4B) and IFN- γ 24 hours after exposure to PSMA⁺ fibroblasts. IL-2 was barely detectable in the supernatants of Pz-1⁺ cells cocultured with NIH3T3 fibroblasts expressing only B7.1, or in the absence of any feeder layer, nor was it detectable in any coculture condition with T cells transduced with a control irrelevant scFv- ζ chain fusion receptor. However, IL-2 and IFN- γ production was strongly potentiated in the presence of PSMA and B7.1, resulting in 10-fold higher IL-2 release after 24 hours (Fig. 4B). This confirms that B7.1-mediated signaling provided functional costimulation in Pz-1 transduced cells.

Example 5

To test whether cytotoxic T cells retain their cytotoxic potential after restimulation with antigen, T cells were harvested 12-17 days after the start of coculture with fibroblast monolayers expressing PSMA and B7.1 and retested in the CTL assay. As shown in Fig. 5, expanded Pz-1 transduced T cells remained fully capable of lysing PSMA⁻ target cells. Furthermore, the expanded Pz-1⁺ cells were capable of a second round of proliferation

- 14-

and IL-2 and IFN- γ secretion when reexposed to PSMA⁺B7.1⁺ fibroblasts. These findings indicate that at least a subset of the T cells transduced with the Pz-1 fusion receptor do not fatally undergo activation-induced cell death (AICD) or anergy, as they remain capable of proliferating and killing after contact with antigen. However, in the absence of costimulation, their proliferative potential appears limited.

These results are of critical importance to the utility of the invention in therapeutic applications. The acquisition of specific tumoricidal properties by autologous T cell is of only marginal importance if these cells are not able to carry out more than one cytotoxic hit. Indeed, physiological TCR engagement with peptide-MHC complexes result in T cell activation which, in the absence of costimulation does not result in complete activation and can eventually lead to anergy or apoptosis. This is especially the case in previously activated cells, such as T cells, cultured for the purpose of clonal expansion or retroviral transduction. Restimulation of antigen-specific CD4⁺ clones following their adoptive transfer in tumor-bearing animals has been suggested to cause clonal anergy.. In the case of T cells bearing artificial TCRs, the prospect of apoptotic cell death or anergy upon restimulation could be compounded by partial T cell activation if chimeric receptors fail to adequately recruit downstream signaling molecules. Faulty T cell activation could result in the induction of immune tolerance and the neutralization of the infused effector T cells. Such phenomenon could in part explain *in vivo* findings obtained with T cells expressing an ErbB-2 specific- ζ chain fusion receptor, required repeated high dose intra-tumoral administration to effectively eliminate established tumors. Altermshmidt et al., *J. Immunol.* 159: 5509-5515 (1997). The proliferation and cytokine release induced by PSMA⁺ murine fibroblasts together with the cytotoxicity achieved against PSMA⁺ murine EL4 cells suggest that the Pz-1 fusion receptor alone provides a sufficient signal to trigger T cell activation. However, although TCR signaling alone can induce lymphocyte proliferation, complete and more sustained activation generally requires that a costimulatory signal be provided in conjunction with TCR stimulation. The results of examples 1-5 show that Pz-1 receptor function is enhanced by costimulation and that costimulation can be provided by B7.1 expression on PSMA⁺ target cells.

- 15-

Example 6

In order to prepare a fusion receptor in which the cytoplasmic domain is derived from CD-28, the procedure of Example 1 was repeated only using a CD28 fragment in place of the ζ -chain segment. The CD28 cDNA fragment was obtained as follow.

A segment of the human CD28 cDNA that encodes part of the extracellular, the transmembrane, and the cytoplasmic domains (amino acids 336 to 663) was amplified by PCR from the plasmid pbsCD28, using the upstream primer

5'GCGGCCGCAATTGAAGTTATGTATCCT

SEQ ID No. 7

and the downstream primer

5'TCGAGGATCTTGTCAGGAGCGATAGGCTGC

SEQ ID No. 8.

These primers contain NotI and BamHI sites respectively for the insertion of the PCR product in the retroviral Vector SFG. Following digestion of the purified PCR product with NotI and BamHI, the CD28 fragment was ligated into the NotI and BamHI sites of the retroviral vector SFG, containing the CD8 α leader sequence, followed by the single chain gene, encoding the V_H and V_L domains of the PSMA-specific antibody

- 16-

CLAIMS

1. A fusion receptor composition having the structure:
PSMA-scFv : optional connector : cytoplasmic domain,
wherein PSMA-scFv represents a single chain antibody cloned from the V region genes of a hybridoma specific for prostate-specific membrane antigen, the cytoplasmic domain is the cytoplasmic domain of a molecule which functions as a transducer of a mammalian immune response in the presence of a costimulatory factor, and the connector is a region of one or more amino acids disposed between the PSMA-scFv and the cytoplasmic domain, said connector to be of sufficient length to allow both the PSMA-scFv and the cytoplasmic domain to retain function, whereby the fusion receptor is effective when expressed in a T-cell to promote a cellular immune response to prostate-specific membrane antigen.
2. The fusion receptor of claim 1, wherein the cytoplasmic domain comprises a ζ -chain of CD3.
3. The fusion receptor of claim 1, wherein the cytoplasmic domain is derived from CD28.
4. The fusion receptor of claim 3, wherein the cytoplasmic domain is a portion of CD28 cDNA spanning amino acids 336-663.
5. The fusion receptor of claim 1, wherein the cytoplasmic domain is derived from 41-BB.
6. The fusion receptor of any of claims 1 to 5, wherein the connector is a CD8 hinge.

- 17-

7. A method for treating a patient suffering from cancer, wherein the cells of the cancer or neovasculature associated with the cancer express prostate-specific membrane antigen, comprising the steps of:

- (a) preparing an expression vector comprising an expressible polynucleotide molecule encoding a fusion protein in accordance with any of claims 1 to 5;
- (b) transducing the expression vector into peripheral blood lymphocytes obtained from the patient to obtain transduced lymphocytes expressing the fusion protein; and
- (c) reintroducing the transduced lymphocytes into the patient, whereby said transduced lymphocytes respond to antigen on the surface of the cells of the cancer to generate a cytolytic immune response to the cells of the cancer.

8. The method of claim 7, wherein the expression vector is transduced into the peripheral blood lymphocytes in an *ex vivo* process.

9. The method of claim 7, wherein the expression vector is an SFG vector.

10. The method of claim 9, wherein the expression vector is transduced into patient PBL using gibbon ape leukemia virus envelope-pseudotyped virions.

11. The method of claim 8, wherein the expression vector is transduced into patient PBL using gibbon ape leukemia virus envelope-pseudotyped virions.

12. Peripheral blood lymphocytes transduced with and expressing a fusion receptor in accordance with any of claims 1 to 5.

13. An expression vector comprising a polynucleotide sequence encoding a fusion receptor in accordance with any of claims 1 to 5 and control sequences effective to promote expression of the fusion receptor in mammalian lymphocytes.

- 18-

14. The vector of claim 13, wherein the expression vector is an SFG vector.
15. The vector of claim 14, wherein the expression vector is packaged in gibbon ape leukemia virus envelope-pseudotyped virions.
16. The vector of claim 13, wherein the expression vector is packaged in gibbon ape leukemia virus envelope-pseudotyped virions.

1/3

FIG. 1



FIG. 3

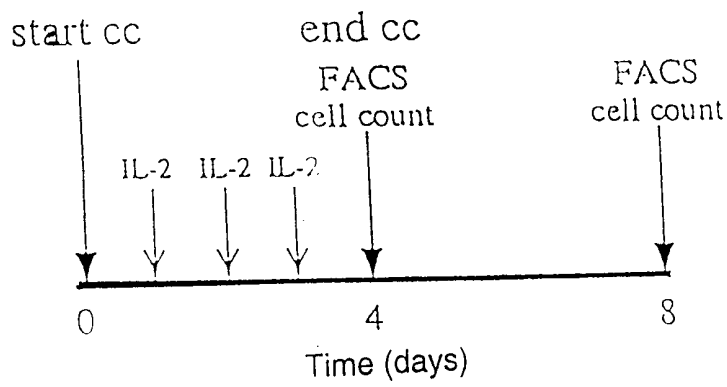


FIG. 5

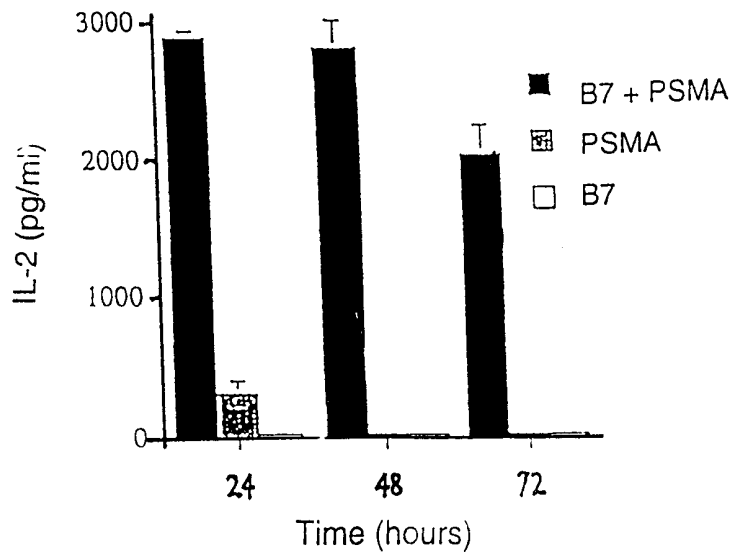


FIG. 2A

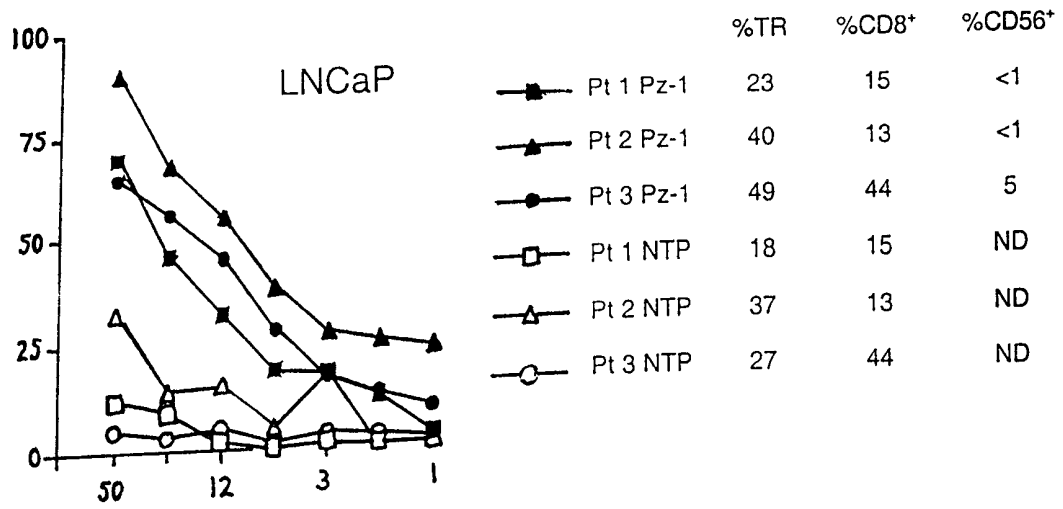


FIG. 2B

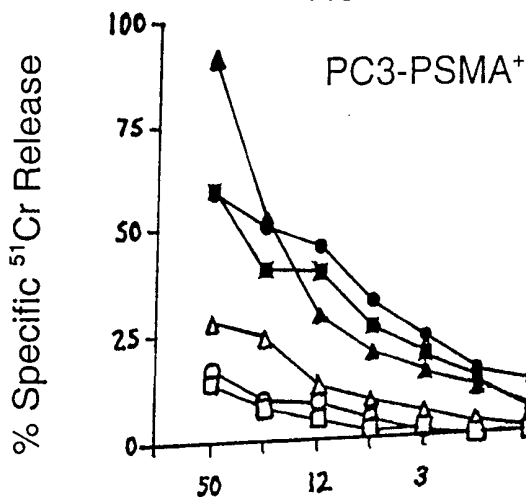


FIG. 2C

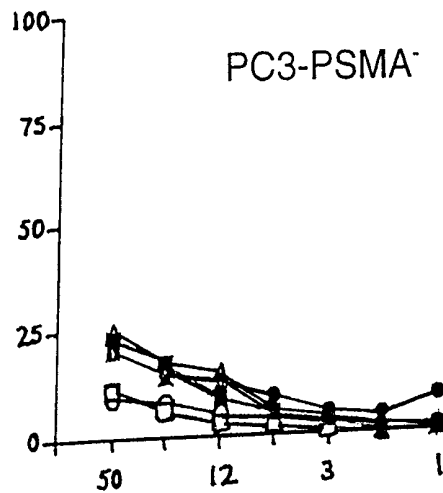


FIG. 2D

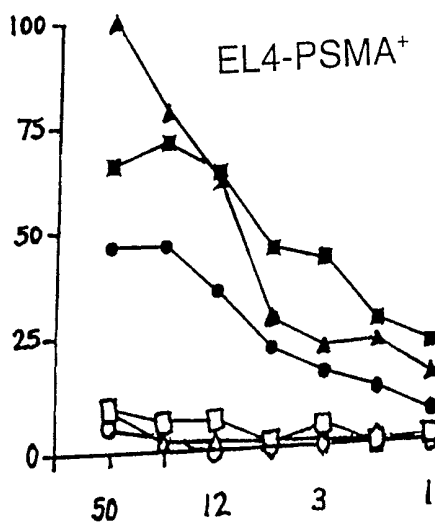
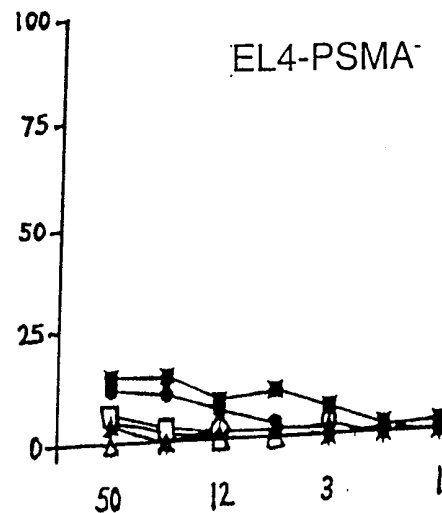


FIG. 2E



E:T Ratio

3/3

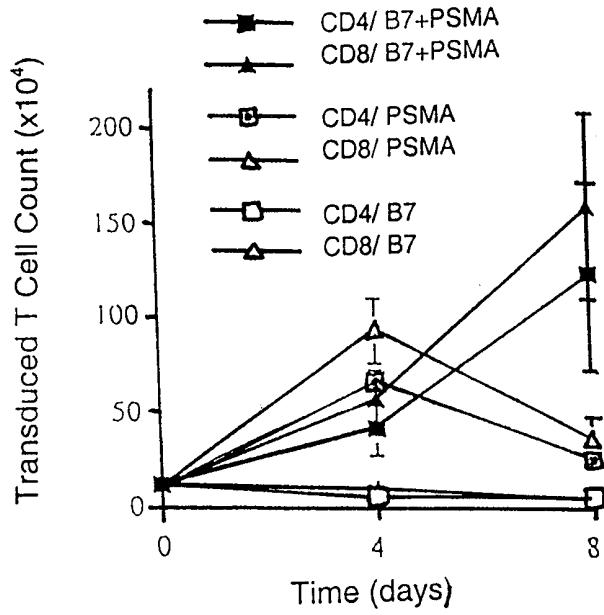


FIG. 4A

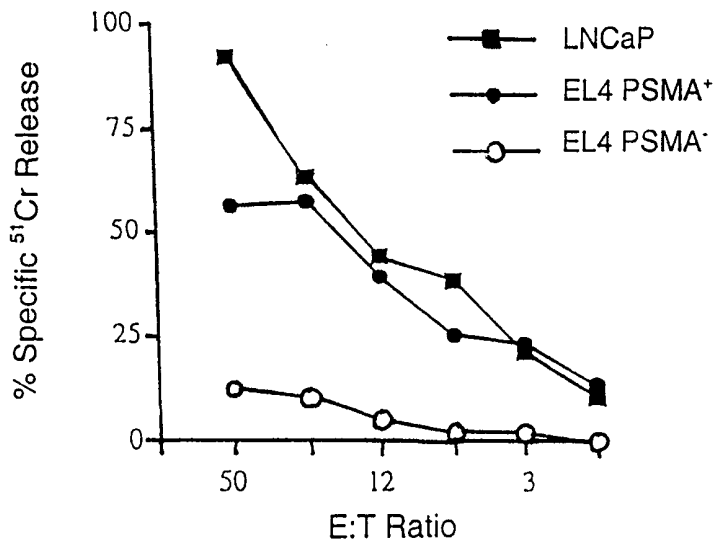


FIG. 4B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/20349

A. CLASSIFICATION OF SUBJECT MATTER
 IPC(7) : C12N 15/63+; A61K 48/00, 39/21, 48/00; C07K 14/00+
 US CL : 435/320.1; 514/44; 424/188.1; 424/93.6; 530/324
 According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
 Minimum documentation searched (classification system followed by classification symbols)
 U.S. : 435/320.1; 514/44; 424/188.1; 424/93.6; 530/324

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
 STN, Caplus, Biosis, Medline, WEST, dialog

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SALGALLER et al. Report of immune monitoring of prostate cancer patients undergoing T-cell therapy using dendritic cells pulsed with HLA-A2-specific peptides from prostate-specific membrane antigen (PSMA). Prostate. May 1998, Vol. 35, No. 2, pages 144-151, see entire document.	1-6, 12-13
Y	MURPHY et al. Phase I clinical trial: T-cell therapy for prostate cancer using autologous dendritic cells pulsed with HLA-A0201-specific peptides from prostate-specific membrane antigen. The Prostate. 1996, Vol. 29, No. 6, pages 371-380, see entire document.	1-6, 12-13

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* & * document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 29 DECEMBER 1999	Date of mailing of the international search report 03 FEB 2000
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/20349

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LINDEMANN et al. Versatile retrovirus vector systems for regulated gene expression in vitro and in vivo. Mol. Med. July 1997, Vol. 3, No. 7, pages 466-476, see entire document.	7-11,14-16
Y	WO 96/24676 A1 (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) 15 August 1996, all (pp. 23,line 22).	7-16
A,E	SIMMONS et al. GM-CSF as a systemic adjuvant in a phase II prostate cancer vaccine trial. Prostate. June 1999, Vol. 39, No. 4, pages 291-297.	1-16
A	LIU et al. Identification of rat prostate steroid-binding protein as a target antigen of experimental autoimmune prostatitis. Implications for prostate cancer therapy. J. Immuno. 1997, Vol. 159, No. 1, pages 472-480.	1-16