



Office de la Propriété
Intellectuelle
du Canada

Un organisme
d'Industrie Canada

Canadian
Intellectual Property
Office

An agency of
Industry Canada

CA 2533705 A1 2007/07/13

(21) **2 533 705**

(12) **DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION**

(13) **A1**

(22) Date de dépôt/Filing Date: 2006/01/13

(41) Mise à la disp. pub./Open to Public Insp.: 2007/07/13

(51) Cl.Int./Int.Cl. *A61K 47/48* (2006.01),
A61K 38/08 (2006.01), *A61K 39/39* (2006.01),
A61K 9/127 (2006.01), *A61P 35/00* (2006.01)

(71) Demandeur/Applicant:
IMMUNOVACCINE TECHNOLOGIES INC., CA

(72) Inventeurs/Inventors:
DAFTARIAN, PIROUZ M., CA;
MANSOUR, MARC, CA;
POHAJDAK, BILL, CA;
BROWN, ROBERT G., CA

(74) Agent: SMART & BIGGAR

(54) Titre : UTILISATION DE LIPOSOMES DANS UNE EMULSION HUILEUSE OU DANS UN PORTEUR HYDROPHOBE
COMME VEHICULE DE TRAITEMENT DU CANCER

(54) Title: USE OF LIPOSOMES IN A WATER-IN-OIL EMULSION OR IN A CONTINUOUS HYDROPHOBIC CARRIER
AS A VEHICLE FOR CANCER TREATMENT



Use of liposomes in a water-in-oil emulsion or in a continuous hydrophobic carrier as a vehicle for cancer treatment

The present invention relates to the use of liposomes in a water-in-oil emulsion or in a continuous hydrophobic carrier as a vehicle for delivery of an agent in the treatment of cancer.

The invention is useful for the treatment of a broad range of cancers, including without limitation: cancers caused by human papilloma virus (HPV), such as, for example, cervical and/or vulvar cancer; cancers involving expression of tyrosinase, such as, for example, melanoma; cancers involving mutations or overexpression of the p53 gene product, such as, for example breast cancer or lymph node metastases; and other cancers like melanoma that express more than one tumor-associated protein simultaneously. Any cancer that has a cell surface component that is different in quantity or substance from the cell type from which the cancer is derived, is a candidate for treatment by the invention. In particular, p53 is a candidate target for broadly applicable cancer treatments (1, 2).

The subject to be treated may be any vertebrate, preferably a mammal, more preferably a human subject.

In one embodiment of the invention, the agent is a polypeptide or derivative thereof. The term "polypeptide" encompasses any chain of amino acids, regardless of length (e.g. at least 6, 8, 10, 12, 14, 16, 18, or 20 amino acids) or post-translational modification (e.g., glycosylation or phosphorylation), and includes e.g. natural proteins, synthetic or recombinant polypeptides and peptides, hybrid molecules, variants, homologs, analogs, peptoids, peptidomimetics, etc. In one embodiment, the polypeptides are derived from tumor-associated proteins and can be obtained by various methods including recombinant technology or chemical synthesis.

In an embodiment of the invention, the agent directs a specific T-cell response accompanied by other mechanisms, against a specific cancer. The agent may comprise a tumor-associated protein or a fragment thereof.

In one embodiment, the agent is an agent capable of inducing CD8⁺ cytotoxic T lymphocytes (CTLs). In this aspect of the invention, the agent may comprise an E7 H2-D^b CTL epitope, for example, the peptide RAHYNIVTF.

In one embodiment, the agent is an agent capable of stimulating IFN- γ producing cells, particularly TPR-2-specific IFN- γ producing cells. The agent may be a peptide that comprises a fragment of or is derived from tyrosinase-related protein (TRP-2), for example SVYDFVWL

In an embodiment, the agent is an agent capable of increasing p53-specific IFN- γ producing cells. The agent may comprise a fragment of p53, such as, for example, KYMCNSSCM.

The compositions of the invention may comprise more than one agent, such as two or more polypeptides, for example, a p53-derived polypeptide and a TRP-2 derived polypeptide.

The compositions of the invention comprise liposomes, together with agents that augment and/or target the treatment to a specific cancer type, suspended in a water-in-oil emulsion or hydrophobic carrier.

The compositions of the invention may comprise one or more T helper epitopes obtained from a variety of sources, including but not limited to, tetanus-derived epitopes, influenza-derived epitopes, or a universal T helper epitope such as PADRE, which is particularly useful in the invention. The helper epitope may be linked directly to the polypeptide agent or indirectly through an intermediate molecule.

The compositions of the invention may further comprise a CpG-containing oligodeoxynucleotide (CpG ODN). CpGs are species specific. The skilled person may select an appropriate CpG on the basis of the target species and efficacy. In place of CpG, a lipopeptide, such as Pam3Cys-SKKK or variants, homologs and analogs thereof may be used. In this regard, the Pam2 family of lipopeptides has been shown to be an effective alternative to the Pam3 family of lipopeptides. The purpose of these agents is to act as "danger signals" to the immune system. Any agent or combination of agents which have this function will serve in the invention in lieu of or in combination with the above mentioned agents.

The composition may further comprise one or more pharmaceutically acceptable carriers, adjuvants, excipients, etc., as are known in the art. See, for example, Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985) and The United States Pharmacopeia: The National Formulary (USP 24 NF19) published in 1999.

Any such additional components of the composition may be placed with liposomes in the water-in-oil emulsion or hydrophobic carrier.

Suitable methods for making liposomes and suspending them in a water-in-oil emulsion are also described in the examples of the invention included in this document.

The compositions of the invention may be formulated in a form that is suitable for oral, nasal, rectal or parenteral administration. Parenteral administration includes intravenous, intraperitoneal, intradermal, subcutaneous, intramuscular, transepithelial, intrapulmonary, intrathecal, and topical modes of administration. The preferred routes are intramuscular, subcutaneous and intradermal to achieve a depot effect.

The compositions of the invention may be effective when administered in a single application.

As can be understood by one skilled in the art, many modifications to the exemplary embodiments described herein are possible. The invention, rather, is intended to

encompass all such modification within its scope.

All documents referred to herein are fully incorporated by reference.

Although various embodiments of the invention are disclosed herein, many adaptations and modifications may be made within the scope of the invention in accordance with the common general knowledge of those skilled in this art. Such modifications include the substitution of known equivalents for any aspect of the invention in order to achieve the same result in substantially the same way. All technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art of this invention, unless defined otherwise.

The invention is further illustrated by the following non-limiting examples.

EXAMPLE 1

Eradication of cervical cancer.

Despite the encouraging development of preventative vaccines for human papillomavirus (HPV) induced cervical and vulvar cancer, for example, Gardasil and Cervarix, a therapeutic treatment for cervical and vulvar cancer remains a high priority. In this example, an E7 H2-D^b CTL epitope (RAHYNIVTF) was used to induce CD8⁺ cytotoxic T lymphocytes (CTLs). These CTLs need CD4⁺ T cell help for their differentiation and expansion, as well as their maturation into functional memory CTLs. To achieve a potent CTL response through CD4⁺ T cell help, the CTL epitope was fused to a universal T helper epitope known as PADRE yielding a fused peptide (FP). FP was encapsulated in liposomes together with synthetic deoxyoligonucleotides containing CpG motifs (CpG ODN) or lipopeptide (Pam3Cys-SKKKK) to act as a “danger signal”. The therapeutic formulation used a water-in-oil emulsion to deliver the therapeutic formulation in a single administration. Efficacy of the therapeutic treatment was demonstrated using HPV 16-expressing C3 tumor cells to challenge C57BL/6 mice in a well-described mouse model for pre-clinical cervical cancer research. Complete eradication of established, palpable tumors was demonstrated in all 10 mice in the group challenged with the C3 tumor then given the therapeutic treatment of the invention (Figure 1). In contrast, tumors in all 10 mice in the control group given all the ingredients of the therapeutic treatment except FP, continued to increase in size. (Details of the challenge studies are described following Figure 1).

Challenge studies

Cell Lines

The C3 cell line was maintained in Iscove Modified Dulbecco's Medium (IMDM; Sigma, St Louis, MO) supplemented with 10 % heat-inactivated fetal calf serum (Sigma, St Louis, MO), 2 mM L-glutamine (Gibco, Burlington, ON), 50 mM 2-mercaptoethanol (Gibco, Burlington, ON), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Burlington, ON). Cells were incubated at 37°C / 5 % CO₂.

The EL-4 cell line is a lymphoma cell line that originated in mice. The EL4 cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM; Sigma, St Louis, MO) with high glucose content containing 2 mM L-glutamine, and supplemented with 10 % heat-inactivated fetal calf serum (Sigma, St Louis, MO), 50 mM 2-mercaptoethanol (Gibco, Burlington, ON), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Burlington, ON). Cells were incubated at 37°C / 5 % CO₂.

Peptides

The HPV 16 E7 (H-2D^b) peptide RAHYNIVTF⁴⁹⁻⁵⁷ (R9F) containing a CTL epitope was fused to PADRE containing a CD4⁺ helper epitope by Dalton Chemical Laboratories Inc. (Toronto, ON). This peptide (FP) was used at 50 µg/dose. Where indicated, R9F was used as an antigen (25 µg/dose) or in cytotoxicity assays. The peptide KIMCNSSCM (Dalton) was used as an irrelevant control peptide.

Adjuvants

The appropriate species specific CpG ODN (Synthetic ODN 1826 with CpG motifs underlined 5'-TCCATGACGTTCCTGACGTT-3', 50 µg/dose) was obtained from Coley Pharmaceutical (Wellesley, MA). Lipopeptide (Pam3Cys-SKKKK, (100 µg/dose) was obtained from EMC Microcollections, Germany.

Treatments

Liposomes were prepared as follows; lecithin and cholesterol in a ratio of 10:1 (0.2 g lecithin and 0.02 g cholesterol/dose) were dissolved in chloroform/methanol (1:1; v/v) and the solution filter-sterilized using a PTFE 0.2 µm filter. Chloroform and methanol were removed under reduced pressure using a rotary evaporator and traces of the solvents were further removed from the resulting thin lipid layer *in vacuo*. For liposome encapsulation, FP with CpG was dissolved in sterile PBS and the resulting solution added to the thin lipid layer with mixing to form liposomes. The resulting suspension of liposomes was emulsified in IFA (Sigma, St Louis, MO) by adding the

liposome/PBS suspension to IFA to form a water-in-oil emulsion (PBS:IFA; 1:1,v/v; 100 μ l/dose). In some experiments, Montanide ISA51 (Seppic) was used in place of IFA as the oil carrier.

Pathogen-free C57BL/6 female mice, 6-8 weeks of age, were obtained from Charles River Laboratories (Wilmington, MA) and were housed under filter top conditions with water and food *ad libitum*. Institutional animal care and use guidelines were followed for all experiments. Mice were immunized subcutaneously (s.c.) by injection at the base of the tail. Unless stated otherwise, all immunizations were single administration and all treatment groups contained 10 mice. Mice that served as controls were injected s.c. with PBS or FP, R9F, CpG (or Pam3c), FP with CpG in PBS (100 μ l) or liposome encapsulated FP, R9F, CpG (or Pam3c) in a water-in-oil emulsion (PBS: IFA; 1:1,v/v, 100 μ l/dose).

C3 challenge and tumor implantation

C3 cells used in tumor implantation were grown to 95 % confluency and harvested with 0.05 % trypsin. To establish tumors in mice, mice were injected with 0.5×10^6 C3 cells s. c. in the left flank. Tumor sizes were determined every 4-5 days using the following formula: longest measurement x (shortest measurement)² divided by 2.

Cytotoxicity assays.

CTL assays, ELISPOT and intracellular staining for interferon (IFN)- γ showed the therapeutic response was specific for the selected E7 peptide since an irrelevant peptide did not elicit CTL activity or IFN- γ production above background. These studies indicate that increases in activated treatment-specific cytotoxic T-cells in splenocytes from mice given the therapeutic treatment correlates with tumor size reduction. Details of the procedures used are described below.

Lymphoblast generation and in-vitro stimulation (IVS). To examine the acute and memory CTL response, splenocytes from immunized mice were analyzed 7, 14 or 130 days post-immunization respectively, unless stated otherwise. Where stated, the cytotoxicity assay was performed upon one round of IVS. Briefly, three days before *in vitro* stimulation, naïve C57BL/6 mice were sacrificed by CO₂ asphyxiation and spleens were harvested and disassociated. Splenocytes were washed and counted in RPMI-10 where RPMI is supplemented with 10 % heat-inactivated fetal calf serum (Sigma, St Louis, MO), 50 mM 2-mercaptoethanol (Gibco), 100 U/ml penicillin and 100 μ g/ml streptomycin (Gibco). Splenocytes (10^6 cells/ml) were cultured with lipopolysaccharide (25 μ g/ml) and dextran sulphate (7 μ g/ml) treated lymphoblasts.

Syngeneic lymphoblasts were irradiated (by 4000 rad using a ¹³⁷Sc source for 15 minutes) and loaded with the R9F peptide (100 μ M). Peptide-loaded LPS activated lymphoblasts (3×10^6 cells/ml) were used to stimulate splenocytes of immunized mice in a ratio of 3:1 where effector cells were adjusted to 3×10^6 cells/ml, and T-stim (BD Biosciences, Mississauga, ON) was added to wells to obtain a final concentration of 20 %. Cells were incubated at 37°C /5 % CO₂ for 6 days.

JAM assay. EL-4 cells were labeled with 5 $\mu\text{Ci/ml}$ [*Methyl*- ^3H] thymidine (Amersham Pharmacia, Erlangen, Germany). The cells were incubated at 37°C / 5 % CO_2 for 24 hours then loaded with R9F or irrelevant peptides (10 $\mu\text{g/ml}$) for one hour. Suspensions of labeled target cells were then harvested, washed twice in RPMI-10, and seeded in 96-well U-bottom plates at a density of 2×10^3 cells/well. The effector cells were added by serial dilution starting at a concentration of 2×10^5 effector cells/well. The plates were incubated for 4 hours at 37°C / 5 % CO_2 . The cells were aspirated onto fiberglass filters and tritium counted using a Packard TopCount scintillation counter. The percent DNA fragmentation was calculated using the following formula: % DNA fragmentation = $(S - E)/E \times 100$, where S is retained DNA (counts) in the absence of treatment (spontaneous) and E is retained DNA (counts) in the presence of effector cells.

Ex-vivo analysis of antigen-specific T cells by ELISPOT. Activated antigen-specific cytotoxic T-cells in splenocytes harvested from immunized C57BL/6 mice were detected using the BD ELISPOT kit following the instruction manual (BD Bioscience, San Diego, CA). Briefly, on day 7 post-immunization a 96-well nitrocellulose plate was coated with the capture antibody, a purified anti-mouse IFN- γ antibody, and incubated overnight at 4°C . The antibody was discarded and the plate was blocked for 2 hours then the blocking solution was removed. Splenocytes were each added to their respective wells at an initial concentration of 1 million cells/well in a final volume of 100 μl followed by serial dilutions in subsequent wells of a row. The following stimulators and controls were added to 100 μl of media to obtain their desired final concentration. Either, C3 cells (5×10^5 cells/ml), the R9F peptide (10 $\mu\text{g/ml}$), the irrelevant peptide (10 $\mu\text{g/ml}$), or no peptides were added to the wells. PMA (5 ng/ml, Sigma), ionomycin (500 ng/ml, Sigma), served as positive controls and the irrelevant peptide and media alone served as negative controls. The plate was incubated overnight at 37°C / 5% CO_2 after which the detection antibody, a biotinylated anti-mouse IFN- γ antibody, was added for 2 hours at room temperature. Following the incubation period, the detection antibody was discarded and the enzyme conjugate (Streptavidin-HRP) was added for 1 hour and lastly the plate was stained with an AEC substrate solution for 20 minutes. The plate was washed and left to air dry overnight for visualization of spots using a magnifying lens.

Intracellular cytokine staining (ICS)

Splenocytes were retrieved from spleens of tumor-free mice as previously described, washed twice with RPMI-10 (500 x g, 5 minutes) and resuspended in RPMI-10 (10×10^6 cells/ml). Splenocytes (1×10^6 cells/well) were added to wells of a 96-well flat bottom plate and incubated with R9F or an irrelevant peptide at a final concentration of 3 $\mu\text{g/ml}$ in duplicate columns for each peptide. In experiments that used EL-4 cells to demonstrate the protective function of IFN γ -producing CD8 $^+$ cells, EL-4 cells (1×10^5 cells/well) loaded with either R9F or the irrelevant peptide were incubated for 6 hours at 37°C / 5% CO_2 before cytotoxicity measurements.

Intracellular cytokine staining was performed as described in the Cytofix/Cytoperm™ kit instruction manual (BD Biosciences, Mississauga, ON). In brief, after addition of stimulants, GolgiStop was added to each well and the plates were incubated (37°C / 5% CO₂) for 4 hours. Cells were washed with staining buffer then incubated (20 minutes at 4°C, in the dark) with anti-CD8 serum, washed again with staining buffer followed by incubation with anti-IFN-γ (30 minutes at 4°C in the dark). This was followed by washes with perm/wash buffer after which cells were resuspended with perm/wash buffer and transferred to FACS tubes (BD Falcon). Staining was assessed by FACSCalibur (BD Biosciences, San Jose, CA), and data were analyzed using CellQuest software.

EXAMPLE 2

A therapeutic treatment of melanoma.

Tyrosinase is a protein known to be overexpressed in melanoma. Peptides from tyrosinase protein are generally poor agents for treatment of melanoma. In the invention described herein, a peptide from tyrosinase-related protein (TRP-2; amino acids 181-188) that binds to murine MHC, H2K² and human HLA-A2.1 was used in a therapeutic treatment to stimulate production of IFN-γ producing cells. Stimulation of the number of TRP-2 specific IFN-γ producing cells indicates that a therapeutic effect directed specifically against melanoma can be anticipated.

In this example, C57BL mice were treated with the invention formulated to contain CpG and TPR-2 peptide fused to PADRE (a T helper epitope) encapsulated in liposomes. The liposomes were suspended in a water-in-oil emulsion and administered in a single application. Control treatments were the invention without CpG and the invention in which TPR-2 was replaced by an irrelevant peptide. *Ex-vivo* detection of IFN-γ producing splenocytes by ELISPOT indicated that the invention produced the greatest number of TPR-2 specific IFN-γ producing cells (Figure 2). The control treatment (invention without CpG) produced about one-half as many TRP-2 specific IFN-γ producing cells and replacement of TPR-2 by an irrelevant peptide produced background levels of TRP-2 specific IFN-γ producing cells. Of the formulations tested, the invention produced the most TRP-2 specific IFN-γ producing cells which are required to combat melanoma cancer.

EXAMPLE 3

A therapeutic treatment of breast cancer.

The p53 gene product is an ideal and widely expressed target for therapy of malignancies, in particular, breast cancer. A large portion of human cancers exhibit p53 mutations as an early event in tumorigenesis. Overexpression of p53 is an independent predictor of more aggressive cancer, lymph node metastases, failure of standard therapeutic regimens and ultimately of cancer-related mortality.

Mice treated with a single administration of the invention containing CpG and a peptide of p53 (KYMCNSSCM) fused to PADRE encapsulated in liposomes in a water-in-oil emulsion produced approximately 10 to 40 times more p53 peptide specific IFN- γ producing cells than mice given the invention in which the fused peptide was replaced by an irrelevant peptide or the above ingredients (p53-PADRE-CpG) were administered without the invention (Figure 3). Increased production of tumor specific IFN- γ producing cells is correlated with a reduction/eradication of cervical cancer tumors (see Example 1), therefore, one skilled in the art would predict a similar result for p53 bearing tumors.

EXAMPLE 4

Therapeutic cancer treatment against more than one target.

Some cancers express more than one tumor-associated protein simultaneously. Such cancers offer more than one target for therapeutic treatment. For example, melanoma cells overexpress both p53 and TRP raising the possibility that treatments aimed at both p53 and TRP simultaneously could be more effective and specific since cells expressing both p53 and TRP targets would be more vulnerable to the treatment.

Mice treated with a single administration of the invention containing a mixture of p53 and TRP-2 CTL peptides fused to PADRE together with CpG encapsulated in liposomes and delivered in a water-in-oil emulsion produced approximately equal numbers of both p53 and TRP specific IFN- γ producing cells (Figure 4). In contrast, mice treated with a single administration of a mixture of p53 and TRP-2 CTL peptides fused to PADRE together with CpG without the invention produced more TRP-2 than p53 specific IFN- γ producing cells. Production of p53-specific IFN- γ producing cells was at levels obtained with the control treatments (invention without CpG and TRP-p53-PADRE). These results indicate that mice treated with the invention mount a two-pronged attack against tumors bearing TRP-2 and p53 tumor-associated proteins. Without the invention, treated mice attack only one target, the TRP-2 tumor-associated protein despite being treated with both TRP-2 and p53 peptides.

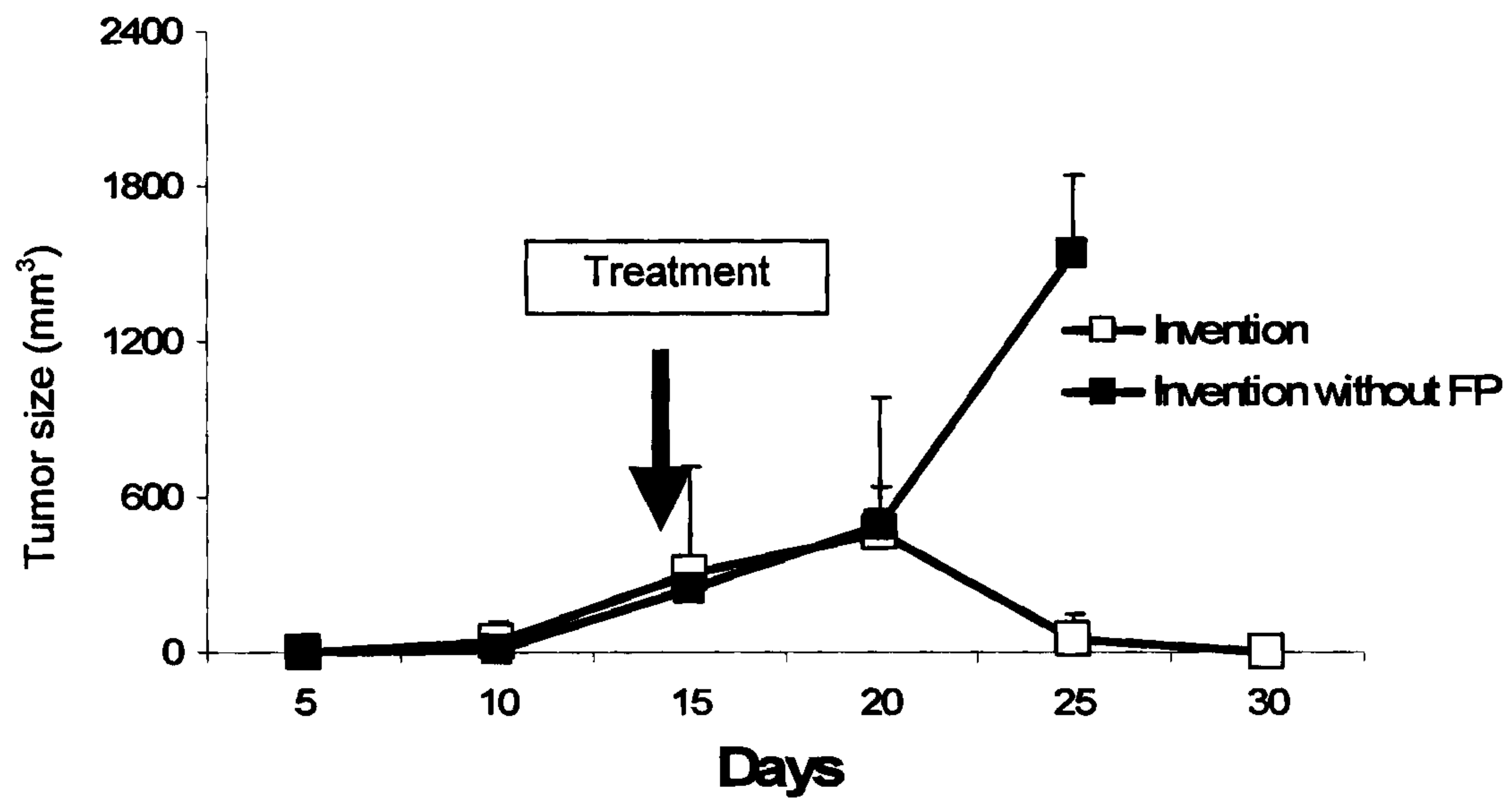


Figure 1. A single administration of the therapeutic treatment of the invention containing a E7 epitope fused to PADRE (FP, open squares) reduced the size of large C3 tumors so that tumors were undetectable by palpation by day 30. In contrast, C3 tumors in mice given a control treatment (invention without FP; closed squares) continued to increase in size. The difference in size of tumors in the two treatment groups was statistically significant ($P = 0.002$, day 25).

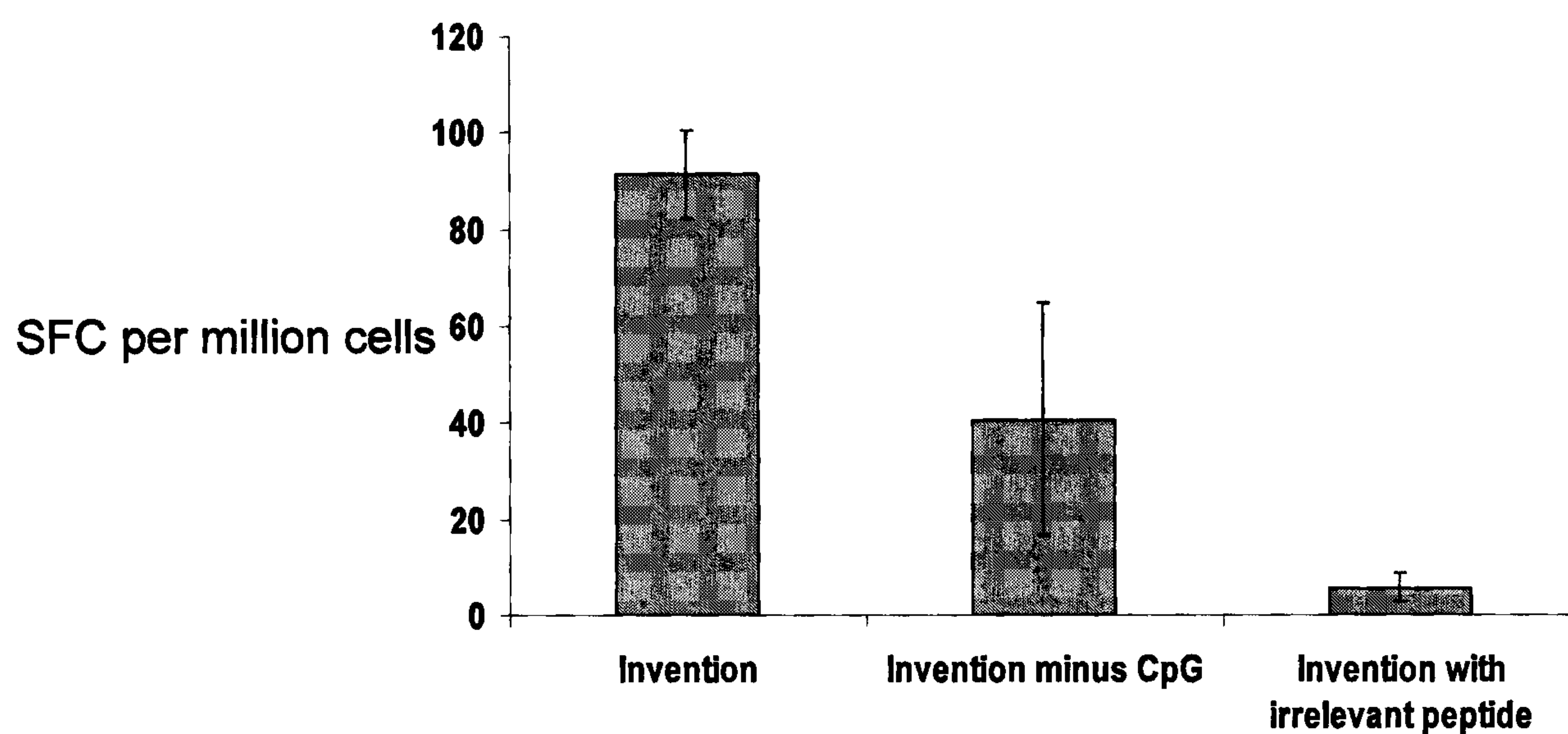


Figure 2. *Ex-vivo* detection of TRP-2 specific IFN- γ producing splenocytes (spot forming cells, SFC) in mice 8 days following a single treatment with the invention or the following control formulations; invention without CpG and invention with irrelevant peptide.

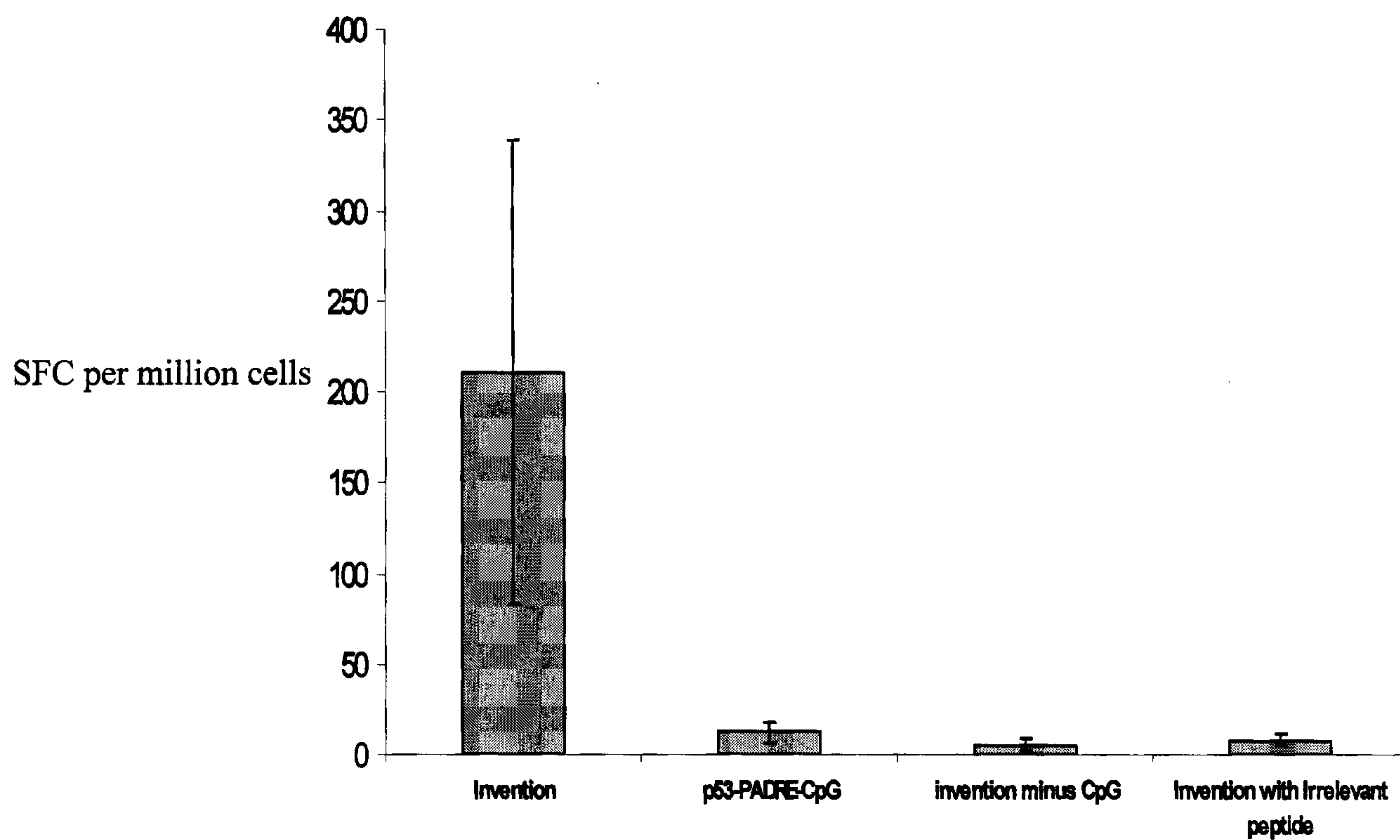


Figure 3. *Ex-vivo* detection of p53 specific IFN- γ producing splenocytes (SFC) in mice following a single treatment with the invention or control formulations as follows; p53 fused to PADRE with CpG, invention without CpG, and invention with irrelevant peptide.

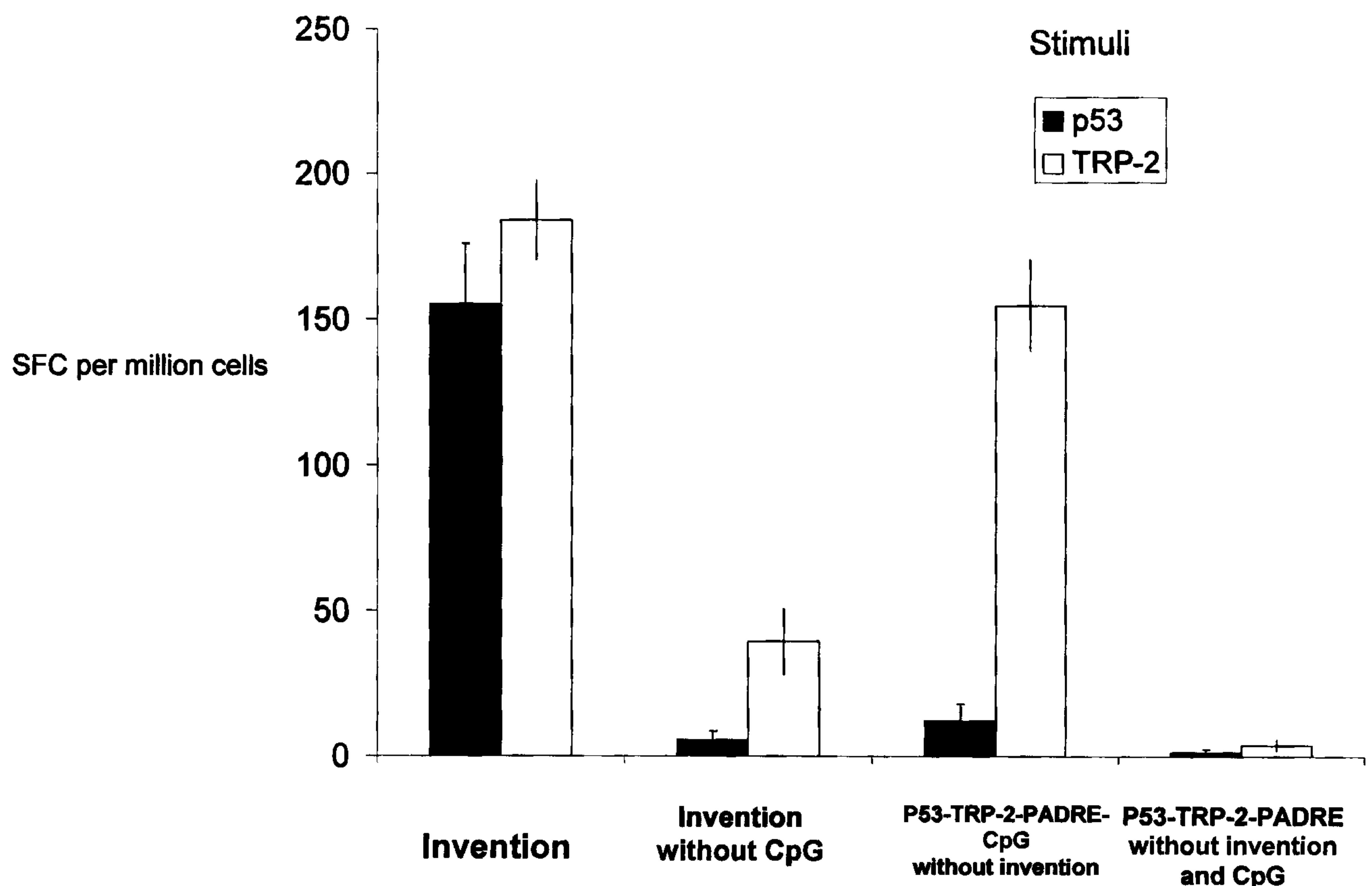


Figure 4. *Ex-vivo* detection of TRP and p53 specific IFN- γ producing splenocytes (SFC) in mice following a single treatment with the invention or control formulations as follows; invention without CpG, CpG with TRP-2 and p53 fused to PADRE without invention, and TRP-2 and p53 fused to PADRE without invention and CpG.

1. DeLeo, A. B. 1998. p53-based immunotherapy of cancer. *Crit. Rev. Immunol.* 18:29.
2. Vierboom, M. P. M., D. I. Gabrilovich, R. Offringa, W. M. Kast, and C. J. M. Melief. 2000. p53: a target for T-cell mediated immunotherapy. In *Peptide-Based Cancer Vaccines*. W. M. Kast, ed. Landes Bioscience, Georgetown, p. 40.