Modulatory effects of mycobacterial heat-shock protein 70 in DNA vaccination against lymphoma

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Background and Objectives. Pathogen-derived molecules are danger signals and are able to activate innate immunity that in turn controls and regulates generation of adaptive immune responses. *Mycobacterium tuberculosis* heat shock protein 70 (myc HSP70) has been shown to exert a potent adjuvant effect in vaccination against both infectious agents and solid tumors. Here we explore the use of myc HSP70, as an adjuvant, in DNA vaccination against lymphoma.

Design and Methods. We describe the effects of vaccination using myc HSP70 encoding plasmid (pHSP70) co-injected with idiotype encoding plasmid (pId), in the 38C13 murine lymphoma model. We dissect mechanisms of anti-tumor immune response and compared efficacy with that of other DNA vaccination strategies.

Results. We show that myc HSP70 plasmid prolongs survival of immunized mice challenged with a high number (2000) of tumor cells. The magnitude of the anti-tumor effect is comparable to that obtained using granulocyte-macrophage colony-stimulating factor (GM-CSF) in the same setting. Moreover, HSP-induced protection is independent from the generation of IgG1 and IgG2a antibodies. Instead, anti-idiotype antibodies of IgG2b subclass develop after vaccination with pHSP as well as with pId and Id-GM-CSF fusion plasmid (pId-GM).

Interpretation and Conclusions. Co-injection of HSP70 and Id plasmids induces a specific pattern of anti-idiotype immune response able to improve survival of immunized mice.

Key words: lymphoma, heat-shock protein, immunotherapy, vaccination.

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The variable regions of the immunoglobulin expressed by B-cell lymphomas contain tumor-specific idiotypic determinants and immunization with tumor immunoglobulin has given promising results in murine experiments and in clinical trials.[1-6] Because genes encoding immunoglobulin variable regions have been well characterized, customized DNA vaccines can be made; these are an attractive alternative to protein immunization against B-cell lymphomas.[7,8] However, the relatively low efficacy of DNA vaccines in inducing immune responses, especially in large animals and humans,[9] has impaired their practical use. Despite considerable effort to improve DNA vaccine delivery, only minute amounts of antigen are available for immune detection following DNA vaccination. Consequently, adjuvants have been used to increase and modulate the immune response induced by DNA vaccines through two complementary strategies: (i) supplementing DNA vaccines with plasmids encoding cytokines; (ii) genetically fusing the antigen to molecules (so-called danger signals) that are bound by many different receptors expressed by cells of the innate immune system.[10,11] In particular, pathogen-derived molecules activate innate immunity which, in turn, control and regulate adaptive immune responses.[12,13] *Mycobacterium tuberculosis* heat shock protein 70 (myc HSP70) has been shown to exert a potent adjuvant effect in models of infection and solid tumors.[14-16] Furthermore, the role of myc HSP70 has been mapped to a discrete domain.[17] Notably, CD40 has been shown to be a cellular receptor mediating myc *HSP70* stimulation of C-C chemokines.[18] Amongst cytokines, granulocyte-macrophage colony-stimulating factor (GM-CSF)

is a potent immunostimulatory product in multiple murine models and in clinical trials.[19,20] Since single vaccination protocols in animals cannot entirely represent the complex human scenario, we investigated the efficacy of GM-CSF-based DNA vaccination against lymphoma in different experimental settings and explored the use of myc HSP70 as an adjuvant. In particular, we studied the effects of co-injecting myc HSP70 encoding plasmid with the Id encoding plasmid in the 38C13 murine lymphoma model.

Design and Methods

Mice

Six-week old female C3H/HeN mice and four to six-week old (C3H/HeN×BALB/c)F1 mice were obtained from Charles River Laboratories (Lecco, Italy) and housed in the Animal Facility of the University of Perugia, Italy.

Cell lines

The carcinogen-induced murine B-cell lymphoma 38C13 expressing a clonal IgM/κ on its surface has previously been described.[1,21] All experiments were performed from a working cell bank of uniformly frozen 38C13 cells. Before tumor challenge, frozen 38C13 cells were cultured in RPMI 1640, supplemented with 10% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 50 μM 2-ME at 37°C in 5% CO_2 in a humidified incubator for 72 hours. The hybridoma 38C13/A1-2 is the product of fusion between a thymidine kinase mutant of 38C13 and the non-secreting BALB/c myeloma cell line P3/X63Ag8.653[22] it secretes large amounts of idiotype (Id) protein. The hybridoma was grown in ascites formed in pristane-primed (C3H/HeN×BALB/c)F1 mice.

Plasmids

Production of plasmids encoding 38C13 heavy and light chain V regions (pId) and the chimeric Id-GM-CSF fusion product (pId-GM) has been described elsewhere.[1,23] Mycobacterial HSP70 encoding plasmid (pHSP) was generated by replacing the BglII-BamHI fragment of the pId vector with an HSP70 BamHI-BamHI PCR amplified fragment. We used pY3111/8 plasmid[14] as template and the following oligonucleotides as primers: oQH001:(5') ATAGTACTGGATCCATGGCTCGTGCGGTCGGGATCGACCTCGGG(3'); oJRO61:(5')GGGATCCCTATCTAGTCACTTGGCCTCCCGGCCGTC(3'). The recombinant plasmid was then transfected into the Phoenix cell line (*http://www.stanford.edu/group/nolan/retroviral_systems/phx.html*)[24] to assess HSP70 expression (see below). Figure 1 is a schematic representation of the three DNA vaccines. After transformation of competent bacteria (Top 10, Invitrogen, Milan, Italy), plasmids were grown from single colonies at 37°C in the presence of 100 μg/mL ampicillin for 20-24 hours. Closed circular plasmid DNA was isolated using an endotoxin-free plasmid purification kit (QIAGEN, Valencia, CA, USA). Purified DNA was re-precipitated, washed with 70% ethanol and resuspended in normal saline at a concentration of 1.0 μg/mL. The ratio of OD260/280 ranged from 1.8 to 2.0; each preparation was analyzed by restriction digest mapping. DNA was stored at –20°C in 1.0 mL aliquots.

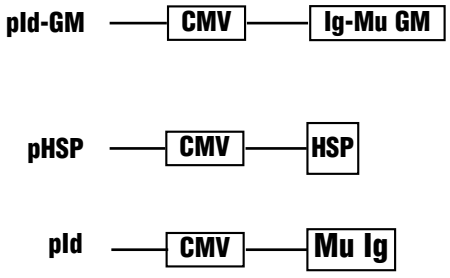

Figure 1. Schematic representation of DNA constructs. DNA fragments were inserted into a mammalian expression vector under a CMV promoter: pId encodes for murine tumor heavy and light variable regions; pId-GM encodes for Id-GM fusion fragment containing murine tumor heavy and light variable regions linked with murine GM-CSF; pHSP encodes for mycobacterial HSP.

Western blotting

Phoenix cells were collected 48 hours after transfection in boiling sample buffer. Cellular proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 8% acrylamide, and transferred to nitrocellulose membranes (0.2 μm, Schleicher & Schuell, Keene, NH, USA). Blots were incubated for 1 hour in Tris-buffered saline/Tween 20 (TBST) containing 10mM Tris-HCL pH 8, 150mM NaCl, 0.2%Tween 20 and 1% bovine serum albumin. After three washes with TBST, membranes were incubated overnight with the mouse anti-Mycobacterial HSP71 primary antibody (StressGen Biotechnologies, Victoria, Canada) (1 μg/mL in TBST) followed by 1 hours' incubation with horseradish peroxidase-conjugated goat anti-mouse (BioRad, Hercules, USA) (1:10000). Bound antibodies were detected using an electrochemoluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA), as shown in Figure 2.

Idiotype protein

Idiotype protein was purified from ascites formed in pristane-primed (C3H/HeN x BALB/c)F1 mice, by an IgM purification kit (PIERCE, Rockford, IL, USA) following the manufacturer's instructions. Isolated Id was estimated to be more than 90% pure by SDS-PAGE.

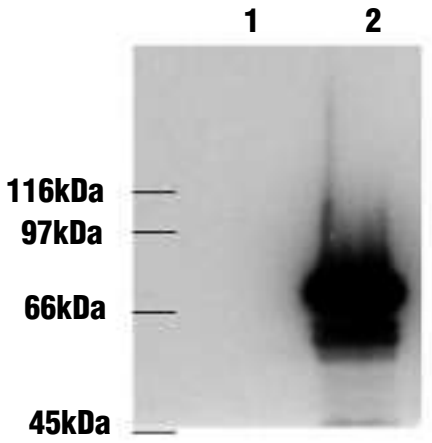

Figure 2. Expression of HSP70. Phoenix cells were transfected with pId (lane 1) or with pHSP (lane 2).

Immunization and tumor challenge

Plasmids (100 μg each time) were injected intramuscularly (i.m.) three times at 1-week intervals. Fifty micrograms of Id protein were injected intraperitoneally (i.p.) twice at a 1 week interval. Two weeks after the last immunization all mice were injected subcutaneously (s.c.) with either 1×10^3 or 2×10^3 38C13 tumor cells in RPMI 0.5 mL.

ELISA assay

Anti-idiotype antibodies were identified using the Mouse Typer Sub-Isotyping Kit (BIO-RAD, Hercules, CA, USA). Briefly, 96-well ELISA plates (Dasit, Milan, Italy) were coated with ascites-purified 38C13 IgM and incubated with serially diluted immune sera. As a control, we used saline injected mice sera. To establish specificity we coated plates with murine polyclonal IgM. Bound antibodies were separately detected with each of the rabbit anti-mouse IgG1, IgG2a and IgG2b. Immunoglobulin sub-classes were identified with conjugated goat anti-rabbit horseradish peroxidase (HRP), using the peroxidase substrate system. Absorbance was determined at 415 nm with a microplate reader (Model 680, BIO-RAD).

Cytotoxic T-lymphocyte (CTL) assay

After three i.m. immunizations, mice were sacrificed and spleens were harvested and prepared as previously described.[25] Splenocytes were incubated at 37°C in 5% CO_2, in a humidified incubator in c-RPMI-10 with irradiated 38C13 tumor cells (3000 rad) and 25 U/mL human interleukin-2 (IL-2) (added after 24 hours) at an effector: target (E:T) ratio of 100:1. Six days later, cells were washed and incubated with ^{3}H-thymidine-labeled 38C13 target cells (1×10^4 target cells/well labeled with 4 μCi/mL) for 12 h in 96-well U-bottom culture plates at E:T ratios of 40:1, 20:1, 10:1, 2:1. After four hours, cells were harvested onto glass fiber filter paper (FilterMat, Skatron Instruments AS, Norway) and radioactivity incorporation was measured in a scintilllation counter (LS 3801 Beckman Coulter, Milan, Italy).

Cellular proliferation assay

Immunized mice were sacrificed and mononuclear cells were obtained from spleens, by density gradient centrifugation. Cells were placed in 96-well U-bottom microculture plates (5×10^5 cells in 200 μL) with antigen at a concentration of 100 μg/mL. Cultures were mantained at 37°C in 5% CO_2 for 5 days. On the third day, interleukin-2 (IL-2) (100 U/mL) was added. Sixteen-eighteen hours before harvesting, 1 μCi of ^{3}H-thymidine (Amersham Pharmacia Biotech, Milan, Italy) was added. Incorporated radioactivity was measured by a scintillation counter (LS 3801 Beckman Coulter, Milan, Italy). All determinations were performed in triplicate.

Interferon-γ secretion assay

Murine interferon-γ secretion was measured using the mouse IFN-γ secretion assay cell enrichment and detection kit (Miltenyi Biotec, Bologna, Italy).[26]

After immunization with three i.m. injections, fresh mouse spleen cells were obtained under sterile conditions and resuspended in RPMI 1640 medium containing 5% murine serum. Cells were placed in 12-well U-bottom microculture plates (2×10^7 cells in 2 mL) and incubated for 16 hours with 100 μg/mL of antigen, in humidified 5% CO_2. Spleen cells that were not restimulated with antigen were used as negative control. Spleen cells that were incubated with the superantigen Staphylococcal enterotoxin B (Sigma, Milan, Italy), 10 μg/mL for 3 hours, were used as the positive control. After *ex vivo* stimulation, all cells were harvested and labeled for 5 minutes with 20 μL of Mouse IFN-γ Catch Reagent (PE) at a concentration of 10^7 cells/mL in ice cold medium. After labeling, cells were diluted with 37°C warm medium to a final concentration of 10^6 cells/mL and allowed to secrete interferon-γ for 45 minutes at 37°C. Cells were then centrifuged at 300×g for 10 minutes at 4°C, resuspended in cold buffer (phosphate-buffered saline pH 7.2, containing 0.5% bovine serum albumin and 2 mM EDTA) at a concentration of 10^7 cells/mL and stained with 20 μL of mouse IFN-γ detection antibody (PE), for 10 minutes on ice. Cells were washed and an aliquot was taken for flow cytometric analysis. In order to analyze the role of cellular immune response in mediating anti-tumor activity and to increase the sensitivity of the IFN-γ secretion assay, we enriched the antigen-specific T cells to allow detection of frequencies as low as one in a milion cells. Briefly, the remaining cells were resuspended in 80 μL of buffer per 10^7 total cells and magnetically labeled for 15 minutes at 4°C with 20 μL of anti-PE MicroBeads. After washing, cells were passed through an MS

Column placed in the MiniMacs separation system (Miltenyi Biotec). Finally, the retained cells were eluted and counter-stained by FITC-labeled anti-CD4 and anti-CD8 monoclonal antibodies (Beckman Coulter, Inc. Fullerton, USA) and analyzed in a flow cytometer (Beckman Coulter).

Statistical analysis

Survival was estimated using the Kaplan-Meier method. Statistical significance was determined by the log-rank test.

Results

Plasmid encoding Id-GM-CSF induces tumor protection comparable to Id plasmid under stringent conditions

Vaccination with DNA plasmid encoding Id-GM-CSF fusion protein is efficacious, and is reported to improve survival after tumor challenge.[23] However, data obtained in a single protocol of vaccination may not always represent what happens in real patients, because of a number of experimental variables. In particular, the amount of DNA injected in humans is proportionally lower than in mice and established tumor masses are difficult to eradicate in the clinical setting. For this reason we investigated *in vivo* how, in GM-CSF-based DNA vaccine, tumor burden influences immune response to 38C13 murine B-cell lymphoma. The survival advantage deriving from pId-GM-CSF administration compared to pId alone (pId-GM-CSF vs pId, p <0.05) was measurable with tumor challenge of 1000 cells s.c. (Figure 3), but not with higher numbers of cells (e.g. 2000 cells s.c.) (*see below*).

Mycobacterial HSP70 encoding plasmids do not prolong survival

To exclude any potential non-specific immunological effect of the HSP encoding plasmid, we injected mice with HSP alone (i.e. without Id plasmid). The HSP survival curve (Figure 4) overlaps that of the saline injected mice (saline vs pHSP, p=0.67). We, therefore, concluded that activation of natural immunity, which is likely to follow HSP administration, has no role if the idiotype is not seen by the immune cells.

Co-injection of mycobacterial HSP70 encoding plasmid and idiotype encoding plasmid prolongs survival independently of IgG1 and IgG2a anti-idiotype antibodies

We investigated whether, under stringent conditions (challenge with 2000 tumor cells), pHSP injection acts as an adjuvant and possibly adds some benefit to pId administration. In our murine model, co-injection of mycobacterial HSP70 encoding plasmid with 38C13 idiotype encoding plasmid significantly prolonged the survival of mice after tumor challenge compared to the survival of both saline-injected mice and mice injected with pHSP alone (pHSP+pId vs saline, p=0.007 and pHSP+pId vs pHSP, p=0.009) (Figure 4). However, co-injection of pHSP and pId protected mice to a similar degree as did pId-GM and pId. We measured the humoral and cellular immune responses in the pHSP+pId injected mice and, surprisingly, we found no evidence of IgG1 and IgG2a antibodies. As expected, pId and pId-GM mice generate measurable IgG1 and IgG2a subclasses .[23] Moreover, we found that all three groups (pId, pId-GM and pHSP+pId) developed anti-idiotype antibodies of the IgG2b subclass (Figure 5) well above the background (i.e. saline injected mice, *data not shown*), reflecting the induction of a Th1-type

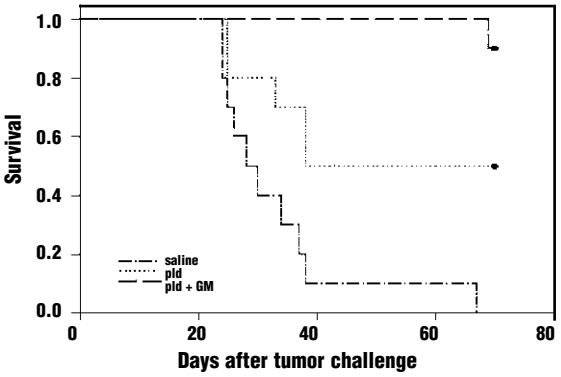

Figure 3. Survival following vaccination with pId, pId-GM and normal saline and challenge with 1000 tumor cells. Groups of 10 mice were immunized i.m. three times, at 1-week intervals, with 100 μg of plasmids. Two weeks after the last immunization, mice were injected s.c. with 1000 38C13 tumor cells and followed for survival. The curves were constructed according to the Kaplan-Meier method. Results are representative of 3 individual experiments.

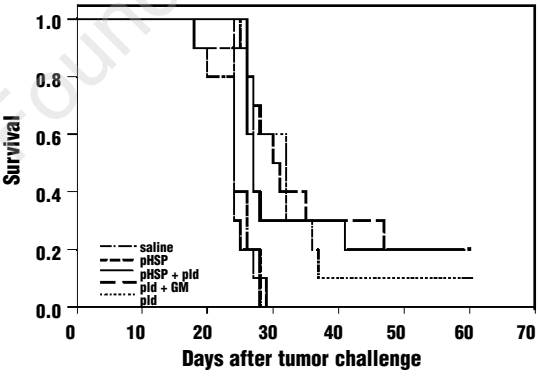

Figure 4. Survival following vaccination with 100 μg of pId, pId-GM, pHSP+pId, pHSP or normal saline and challenge with 2000 tumor cells. The curves were constructed according to the Kaplan-Meier method. Results are representative of 3 individual experiments.

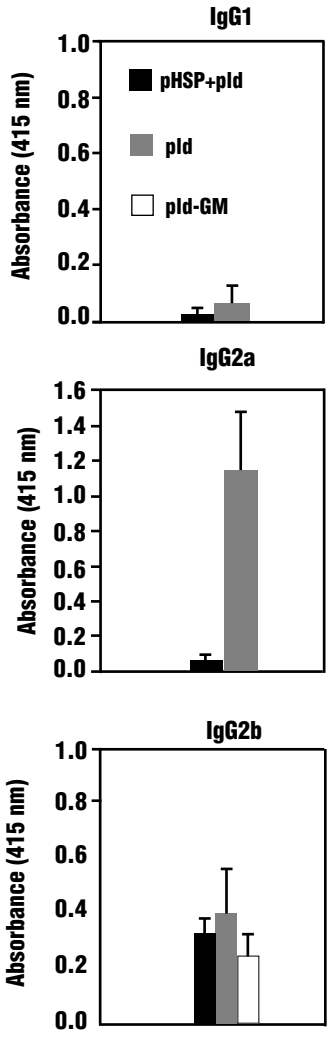

Figure 5. Humoral anti-idiotypic response after vaccination with pHSP+pId, pId and pId-GM. Fifteen days after the last inoculation, immune sera were collected and the identification of anti-idiotype antibodies was performed by ELISA using isotype-specific detectors. A: anti-idiotype IgG1 antibody titer; B: anti-idiotype IgG2a antibody titer; C: anti-idiotype IgG2b antibody titer. Sera were diluted 1:100. Error bars show the mean ± standard error. Results are representative of 2 experiments.

immune response.[27] We speculate that IgG2b antibodies could participate directly in tumor protection, through ADCC as well as complement fixation, and that HSP70 adjuvant activity could be at least in part mediated by IgG2b. *In vitro* cellular immunity assays did not detect either idiotype-specific proliferative or cytotoxic responses (*data not shown*). Since interferon-γ production is rapidly induced during the adaptive phase of immune responses in $CD4^+$ and $CD8^+$ T cells following antigen or mitogen stimulation of their T-cell receptors, we then tested T-cell secretion of IFN-γ by flow cytometry analysis after *in vitro* restimulation with the antigen. Confirming previous results, no secretory activity was detected (*data not shown*).

Discussion

Recombinant GM-CSF is the growth factor most widely used to augment immune responses in recombinant protein-based vaccination strategies.[28] However, development of anti-GM-CSF antibody has been reported in patients injected with human GM-CSF recombinant protein.[29,30] Moreover, immunosuppressive effects associated with the administration of GM-CSF protein at high doses have been described.[31]

DNA vaccines are easier to generate than hybridomas secreting protein against a lymphoma's idiotype (Id). DNA encoding GM-CSF protein has been previously shown to improve vaccine efficacy in B-cell lymphoma tumor models.[23] However, recent clinical data have failed to fulfill the earlier promises.[9] We investigated *in vivo* how tumor burden influences immune response to B-cell lymphoma in a GM-CSF based DNA vaccination model. We observed that beneficial effects of GM-CSF-containing plasmids compared to non-GM-containing ones are apparent only in specific settings. In fact, at higher tumor burden we did not observe a survival advantage deriving from the use of plasmid encoding for GM-CSF as compared to plasmid encoding for the idiotype alone. Cytokines form an extremely complex system and are redundant in multiple functions; therefore, it would be surprising that a single cytokine could generate anti-tumor immune responses efficacious in all situations. Other molecules need to be evaluated in this respect, aiming at exploiting possible synergistic adjuvant properties. Among molecules that could potentially serve as adjuvants, heat shock proteins (HSP) are certainly of great interest. HSP are a family of highly conserved proteins, present in all eukaryotic and prokaryotic cellular organisms, and play an important role in protein synthesis, folding and translocation. Moreover, they are upregulated by several forms of stress and have immunomodulatory effects, at the level of both innate and adoptive immunity. In fact, HSP are known to signal *danger* to antigen-presenting cells (APC) through CD14, Toll-like receptors and CD91.[32] Although self-HSP have been used to elicit immune responses,[33,34] there is a concern that self sequences can block, instead of stimulate immunity, at least in certain circumstances.[32] On this point, although bacterial HSP are known to be immunodominant antigens as a whole, HSP-epitopes homologous to endogenous host HSP-sequences induce T cells able to produce regulatory cytokines, such as interleukin-10.[35] *Mycobacterium tuberculosis*-derived HSP70 is well characterized as adjuvant that elicits both MHC class I-restricted $CD8^+$ and MHC class II-restricted $CD4^+$ T-cell responses when linked to antigenic peptides. Other studies of DNA vaccines in which myc HSP70 gene was fused to tumor antigens (others than idiotype) have shown that HSP70 can greatly enhance their potency, predominantly via CD8-dependent pathways.[14] We showed that HSP-encoding plasmid acts as an adjuvant and prolongs survival at high tumor burden (2000 38C13 tumor cells), as compared to saline injected mice. However, we found that HSP-injected mice behave similarly to those inoculated with both GM-CSF-containing plasmid and idiotype-containing plasmid alone. Despite this disappointing finding, we observed that survival was independent from IgG1 and IgG2a antibodies; instead, mice did produce antibodies of

IgG2b subclass. This is a quite peculiar pattern since GM-CSF-encoding plasmid induces IgG2a antibodies and idiotype alone has been shown to induce small amounts of IgG1 and modest amounts of IgG2a antibodies.[23] Indeed, we found that all groups (except saline-injected mice) produced IgG2b antibodies. We hypothesize that IgG2b could contribute to tumor protection both by acting directly through ADCC as well as complement fixation and by inducing a Th1 immune response. The results obtained in multiple assays testing several aspects of T-cell activation (proliferation, cytotoxicity and cytokine secretion) support our view that the survival advantage in mice immunized with mycobacterial HSP70 plus idiotype is independent of cellular immune responses.

Future studies, including *in vivo* depletion of T-cell subsets in different lymphoma models, will probably shed some light on the role of T-cell-mediated immunity. Nevertheless, in the attempt to evaluate the synergism of multiple adjuvants, co-injection of several molecules is a reasonable approach to achieve better immune control of cancer.[36] In particular, co-injection of HSP-encoding plasmid with fusion products (e.g. pId-GM-CSF) appears to be a relatively easy strategy to test in further studies in order to resolve whether HSP co-injection gives any advantage.

AL and RB were responsible for designing the study, performing animal experiments and immunological tests and for the analysis and interpretation of the data. MF was responsible for the construction of the pHSP, for growing the pHSP, pId, pId-GM and for critical revision of the article. AM was responsible for the construction of pHSP and for growing pHSP, pId and pId-GM. BF was responsible for obtaining funding, for critically reviewing the manuscript and for important intellectual content. We are very grateful to Dr. Ronal Levy (Stanford University, USA) for the generous gift of DNA plasmids and 38C13 cell lines. We also thank Mrs Geraldine Boyd for critically reading the manuscript. All the authors approved the article. The authors declare that they have no potential conflicts of interest. This work was supported by the AIRC. (Associazione Italiana per la Ricerca sul Cancro) and MIUR (Ministero Istruzione Università e Ricerca. RB is supported by the F.I.R.C. (Fondazione Italiana per la Ricerca sul Cancro).

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