Anti-idiotypic single chain mimicking CA125 linked with tuftsin provides protective immunity against ovarian cancer in mice

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Abstract. The activation of effector cells by bifunctional proteins to kill target cells has great potential in the treatment of cancer. In this study, a recombinant fusion protein composed of an anti-idiotypic single chain mimicking CA125 connected with tuftsin by an artificial linker was constructed. The fusion protein was found to manifest a number of biological activities, including activation of macrophages and stimulation of the T-cell response against cancer. Compared with single-chain Fv without tuftsin, the fusion protein showed stronger immunogenicity triggering humoral and cellular immune responses in mice. Fusion of tuftsin to scFv resulted in enhanced production of anti-anti-idiotypic antibodies and T-cell response. Protection against tumor challenges may be achieved in animals immunized with fusion protein. These results raise the possibility of employing cancer immunotherapy by administration of fusion proteins composed of anti-idiotypic antibodies and tuftsin.

Introduction

CA125, also known as MUC16, is a mucinous glycoprotein, which was initially identified by Bast *et al* in 1981 (1). CA125 is a well-studied ovarian cancer antigen, which is elevated in 79% of all patients with ovarian cancer (2) and in 95% of patients with stages III and IV ovarian cancer (3), and remains an attractive target for active immunotherapy. One concept for immune therapy of cancer involves induction of antigen mimicking antibodies to trigger the immune system into a response against tumor cells. Anti-idiotypic antibodies (Ab2) directed against the antigen-combining site of other antibodies (Ab1) may functionally and even structurally mimic the antigen and induce an anti-anti-idiotypic immune response (4). Clinical trails of anti-idiotypic mAb-based vaccines in cancer patients have demonstrated the induction of immune responses against various types of cancer (5-7). In our laboratory, one monoclonal antibody WJ02 was generated as an anti-idiotypic mAb mimicking CA125. The antibody was considered to be an effective therapeutic agent, as it induced a protective cellular response against CA125-positive cancer cells (4).

Although mAb WJ02 is a promising candidate for ovarian cancer treatment, a possible problem exists, which may occur after applying it into clinical use. Human anti-mouse response (HAMA), which occurs during antibody therapies, may be the main factor limiting the use of repeated cycles of the antibody treatment. To avoid such unwanted immunoreactivity, a single-chain antibody fragment is considered to be employed rather than the full sequence of mAb. Since only the variable region of the anti-idiotypic antibody reflects the image of the antigen, vaccination using a single chain should reduce the non-specific immune responses.

According to this theory, a single-chain antibody of mAb WJ02, scFvWJ02, was constructed (8). However, due to reduced molecular weight, the use of scFvWJ02 in anti-idiotypic stimulation requires addressing a number of challenges, including decreased immunogenicity and rapid clearance from the body. To improve the immunoefficacy of single-chain antibodies, various immunostimulatory cytokines, growth factors or co-stimulatory antigens have been used following fusion or by co-administration with single-chain antibodies (9-11). In previous studies, we observed that immunization of scFv supplemented with cytokines was associated with improved anti-tumor responses compared with scFv alone (12).

In an attempt to develop an effective anti-idiotypic vaccine, we designed a single-chain fusion protein scFvWJ02/tuftsin by genetic engineering and demonstrated its capability to elicit a specific immune response against CA125 in mice. Tuftsin is a natural tetrapeptide (Thr-Lys-Pro-Arg) produced by enzymatic cleavage of the Fc-domain of the heavy chain of immunoglobulin G (IgG). Its biological activity is related primarily to the immune system function. Tuftsin binds to specific receptors on the surface of monocytes and macrophages, stimulating their migration, phagocytic, bactericidal

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and tumoricidal activity (13,14). In animal and clinical studies, tuftsin has displayed anti-tumor, anti-infection activity with no detectable toxicity (15). The anti-tumor potential may be very useful for targeted cancer therapy if it can be linked to a tumor-targeting agent, e.g., an antibody. Our *in vitro* and *in vivo* studies have shown that tuftsin fused to scFvWJ02 retains its biological activity and continues to mediate cellular cytotoxicity and anti-idiotypic response. The fusion protein may therefore be a valuable vaccine for an active specific immunotherapy of CA125-positive types of human cancer.

Materials and methods

Construction of fusion protein. The goal of this section of the study was to construct a fusion protein of scFvWJ02 linked to tuftsin and express it in the *Pichia pastoris* expression/secretion system (Beijing Zeping Bioscience and Technologies, Beijing, China), which has been described in detail elsewhere (16). Briefly, two constructs of scFvWJ02 derivatives with additional C-terminal extensions linking mouse tuftsin (pL-06), or a nonrelevant sequence (pL-01), were designed. To construct plasmids pL-06 and pL-01, DNA oligodeoxyribo nucleotides 5'-GAATTCTGGAGGTGGTACCCAGCCTAGG TAGC-3', and 5'-GAATTCAGCTGGAGGTGGTGGATGT GC-3' coding for the amino acid sequences N-SerGlyGlyGly ThrGlnProArg-C and N-SerAlaGlyGlyGlyGlyCysAla-C, respectively, were used by inserting fragments in *Eco*RI and *Eag*I sites of pPIC9-WJ02. The plasmid DNAs were transformed into competent GS115 cells by electroporation, and the resultant transformants were selected on histidine-deficient media, as described previously (8). All obtained positive clones were isolated, cultured in induction media, and analyzed for protein expression in SDS-PAGE. The obtained recombinant fusion protein pL06 was composed of scFvWJ02 connected with tufsin by an artificial linker (Ser Gly Gly Gly).

Characterization of scFvWJ02-tuftsin. Immunoreactivity of each fused domain of pL06 for specific targets was assessed by ELISA. Briefly, mouse macrophage cells J774A.1 (ATCC, Manassas, VA, USA) were seeded into 96-well plates at $3x10^3$ cells/well. The cells were cultured in RPMI-1640 complete medium supplemented with 10% FCS for 3 days and then blocked with 3% bovine serum albumin in phosphate-buffered saline (PBS). Various amounts of pL06 (10, 5, 2.5 and 1.25 μg) were applied to each well and incubated with the cells for 1 h. The plates were then washed three times with PBS and the bound pL06 was further incubated with mAb MJ01 (8 μg/well), which is an anti-CA125 antibody (IgG1) used to generate mAb WJ02. Following incubation for 1 h, the plates were washed three times, peroxidase-labeled goat anti-mouse IgG (Southern Biotech, Birmingham, AL, USA) was added and the cells were incubated for another hour. The plates were then washed three times and filled with 2,2'-azino-bis-(3-ethylbenzthiazolinesulfonate) (ABTS) substrate. The absorbance was read at 405 nm. Equivalent molars of pL01 and tuftsin were used as controls.

CTL assay. The mouse macrophage J774A.1 cells were cultured in RPMI-1640 complete medium supplemented with 10% FCS in the presence of 10 μg/ml of pL06, tuftsin or pL01, at 37˚C in a CO_2 incubator for 4 days. Approximately $5x10^5$ cells were collected for detection of MHC class II expression by flow cytometry. The rest of the cells were used for a ^{51}Cr-release assay for the measurement of *in vitro* cytotoxicity.

Flow cytometric analysis. The cells were incubated with 10 μl of mouse anti-MHC II IgG (Chemicon, CA, USA) for 30 min followed by a PBS wash. Then 10 μl of secondary antibody, goat anti-mouse IgG-FITC (Serotec, Oxford, UK), was applied and the cells were incubated for 30 min. Finally, cells were washed, fixed with 1% paraformaldehyde and analyzed by flow cytometry (Coulter Epics Elite Esp).

^{51}Cr-release assay. The CA125$^+$ ovarian carcinoma cell line OVCAR-3 and CA125$^-$ ovarian carcinoma cell line SKOV (both from ATCC) were used as target cells for the *in vitro* cytotoxic analysis. J774A.1 cells were added to the plates at $2x10^3$ cells/well with pL06 (10 μg/ml), tuftsin (10 μg/ml) or pL01 (10 μg/ml), respectively. Following a 4-day stimulation, T cells derived from mouse splenocytes by panning with anti-mouse Ig were washed with AIM medium and added to the culture for a 1-day incubation. OVCAR-3 or SKOV cells growing in a monolayer in tissue culture flasks were digested with a trypsin/EDTA dispersal solution and labeled with ^{51}Cr (China Isotope Co., Beijing, China) for 1 h at 37˚C. These target cells were washed three times in AIM medium and then seeded in microtiter plates with the above educated T cells at an effector/target ratio of 10:1. The plates were incubated for 6 h at 37˚C in a humidified atmosphere of 5% CO_2. After this incubation period, the supernatant was harvested and counted in a gamma counter (LKB, Turku, Finland). All tests were carried out in triplicate. The percentage of specific ^{51}Cr release was calculated according to the following formula:

% Specific lysis = (sample release cpm - spontaneous release cpm)/(maximum release cpm - spontaneous release cpm) x 100.

Triton X-100 was used to obtain maximum release. The phagocytosis ability of macrophages by pL06 and pL01 was also assessed by ^{51}Cr-release assay and simplified by incubating ^{51}Cr-labeled OVCAR-3 cells with J774A.1 cells at a ratio of 1:10 in the presence of pL01 or pL06 (10 μg/ml). The formula with which to calculate '% of cell lysis' was the same as described above, in which 'spontaneous release cpm' was from the plates seeded by an identical number of ^{51}Cr-labeled target cells and incubated for 6 h at 37˚C.

Animal experiments. BALB/c female mice (Animal Center of CAMS), 6-8 weeks of age, were used in this experiment. As we disclosed previously, poly(lactic-*co*-glycolic acid) microspheres were applied as an adjuvant to encapsulate immunogens: tuftsin, pL01 or pL06 (12). The details of the encapsulation method were described elsewhere (17). A total of 24 animals were classified into three groups for immunization with tuftsin, pL01 or pL06, respectively. Following the collection of pre-immune serum samples, mice were administered with two subcutaneous (s.c.) injections of the immunogens on day 0 and day 14, followed by an additional intraperitoneal injection (i.p.) on day 21. The dose of immuno-

gens for each immunization was 50 μg. Blood samples from the tail vein were extracted periodically into Microtainer tubes (Becton Dickinson, Franklin lakes, NJ, USA) and frozen at -80˚C for later use.

Detection of humoral response. One week following immunization, the sera from immunized mice were screened for anti-tumor antibody production by an ELISA assay. CA125 (400 U/well, Shanghai Linc-Bio science Co. Ltd., China) was coated to the Nunc Maxisorb plates (Nunc, Roskild, Denmark) overnight at 4˚C. The plates were then blocked with 200 μl of 3% BSA in PBS per well for 2 h at room temperature. Following three washes with PBS, 100 μl of the serum samples was added to each well, which was diluted 100-fold in diluent (1% normal mouse serum in newborn calf serum) and incubated for 1 h at room temperature. The plates were then washed three times with PBS and the bound murine Igs were detected by incubation with peroxidase-labeled goat anti-mouse Ig (Southern Biotechnology Associates Inc., Birmingham, AL, USA) for 1 h at room temperature. Following three washes, 50 μl of ABTS substrate solution was added. The absorbance was measured at 405 nm.

Antigen-specific T-cell proliferation and expression of cytokine genes. Animals were sacrificed two weeks after the last immunization and spleens were removed under aseptic conditions. B cells were removed by panning with anti-mouse Ig. Antigen-specific T-cell proliferation was performed as follow: single-cell suspensions were prepared and $2x10^5$ cells/well were plated in 96-well plates. T cells were stimulated *in vitro* with CA125 and human albumin at a concentration of 50 μg/ml for 72 h. The cultures were pulsed with ^{3}[H]-thymidine (China Isotope Co., Beijing) at a concentration of 1 μCi/well for 18 h. The cells were harvested and thymidine incorporation was measured in a liquid scintillation counter (Beckman LS5801). Cytokine mRNA expression (IL-2, IL-4, IL-10 and IFN-γ) was detected by quantitative RT-PCR as follows: BALB/c mouse splenocytes were harvested and total cellular RNA was extracted using TRIzol reagent (Invitrogen, USA). cDNA was synthesized from the mRNA with the Reverse Transcription System (Promega Corp., Madison. WI, USA) according to the manufacturer's instructions. IL-2, IL-4, IL-10 and IFN-γ mRNA levels were quantified at least three separate times by real-time PCR using SYBR Premix EX Taq on ABI PRISM 7900 (Perkin-Elmer, Foster city, CA, USA) and normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as previously described (18,19).

Therapeutic experiments. A total of 50 8-week-old nude mice (Animal Center of CAMS) were randomly classified into 5 groups. Initially, the mice underwent i.p. injection with 2 ml of thioglycollate broth (Difco). One week later, mice were administered $5x10^6$ OVCAR-3 cells subcutaneously. The treatment was then initiated on the day of tumor cell inoculation. The mice were left untreated, or received an i.p. injection of PBS, 1 μg tuftsin microspheres, 50 μg of pL01 microspheres or pL06 microspheres (10 mice for each group), respectively. In 24 h, $1x10^7$ normal mouse splenocytes were transfused to these mice (i.p.). One day later the mice received another transfusion of $1x10^7$ splenocytes. Tumor development in mice was monitored every 3 days thereafter by measuring the tumor size with calipers and the tumor volume was calculated according to the formula: Volume (mm^3) = $0.5ab^2$, where a is the longest and b the shortest dimensions of the carcinoma, respectively. The experiment was terminated when the longest dimension of the tumor on the control mice reached 1.5 cm.

Statistical analysis. Statistical analysis was performed using Prism 5. Differences between groups of mice were evaluated by the Student's t-test. Differences were considered significant only when the P value of comparison was <0.05.

Results

Characterization of the bi-specific scFv. The strategies used to produce the scFv-tuftsin protein relied on the methylotrophic yeast *P. pastoris* expression/secretion system that has been developed for the overproduction of a variety of eukaryotic proteins with high secretion-efficiency (20). In this system, the pL06 or pL01 gene was inserted in the place of the *P. pastoris* alcohol oxidase (AOX1) gene and the expression of the cloned gene was thus under the control of the strong and methanol inducible AOX1 gene promoter. The resultant proteins were purified from the supernatant and characterized by SDS-PAGE, demonstrating that a protein of the expected molecular weight (28 kDa) had been isolated (data not shown).

Binding of pL06 to targets. To demonstrate that the binding capacities of domains scFvWJ02 and tuftsin were not disturbed by the cross-linking procedure, a cell ELISA was performed to detect the binding ability of pL06 to tuftsin receptor or mAb MJ01, an anti-CA125 antibody used to generate mAb WJ02. As previously reported, tuftsin receptors have been found on neutrophils, monocytes and macrophages (21); a mouse macrophage cell line J774A.1 was therefore used to capture the pL06 molecule. As shown in Fig. 1, pL06 had a considerably higher absorbance compared with pL01 or tuftsin in this system, which indicated successful binding of the fusion protein to the receptors on J774A.1 and idiotopes on mAb MJ01. By contrast, tuftsin and pL01 were not detected at the end of the experiment, in that they were unable to recognize mAb MJ01 or J774A.1, respectively.

Activation of J774A.1 by fusion protein. Following characterization of the binding ability to its receptors, the biological function of pL06 to activate macrophages was further detected. As tuftsin up-regulates class II MHC molecules on macrophages, and enhances their phagocytic activity and antigen-presenting capabilities (22), the expression of MHC class II molecules on J774A.1 prior to and following the stimulation of pL06 was detected by a flow cytometric assay (Fig. 2). Following a 4-day-stimulation by pL06, the expression of MHC II (99.19%) was significantly elevated compared to that by pL01 (81.03%). This result indicated that pL06 was capable of transforming macrophages into an activated state *in vitro*.

Fusion protein enhances specific and non-specific tumor cell lysis. The CTL response to tumor antigen has been elucidated to be a key feature in the protective immune response against tumor cells. In this regard, we analyzed whether the fusion

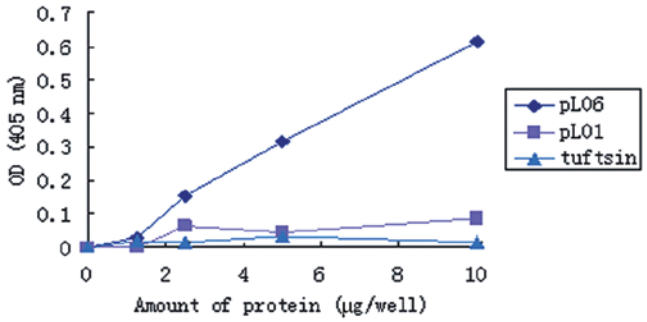

Figure 1. Functional characterization of pL06 fusion protein. Tuftsin receptor and Abl binding ability of pL06 was analyzed using a sandwich cell ELISA system. J774.1 cells were incubated for 1 h in the presence of increasing amounts of pL06. Then an Abl used to generate the scFv end of pL06 was added. Peroxidase-labeled goat anti-mouse IgG and ABTS were followed thereafter. The absorbance was read at 405 nm.

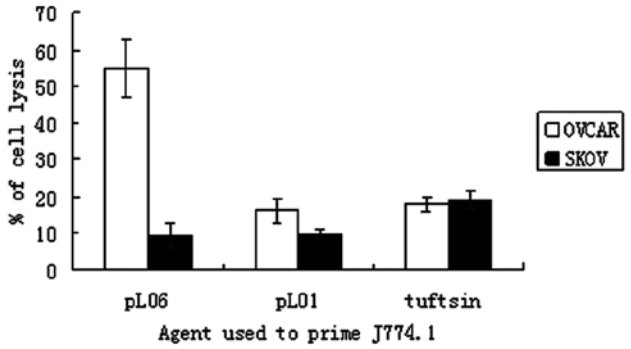

Figure 3. Induction of CA125-specific CTL activity by pL06. Mouse macrophage J774A.1 cells were cultured in RPMI-1640 complete medium supplemented with 10% FCS in the presence of pL06, tuftsin or pL01, at 37°C in a CO_2 incubator. On day 4, cytotoxic activity was determined by a 6-h ^{51}Cr release cytotoxicity assay using OVCAR-3 and SKOV as target cells. Each experiment was performed in triplicate, and representative results are shown.

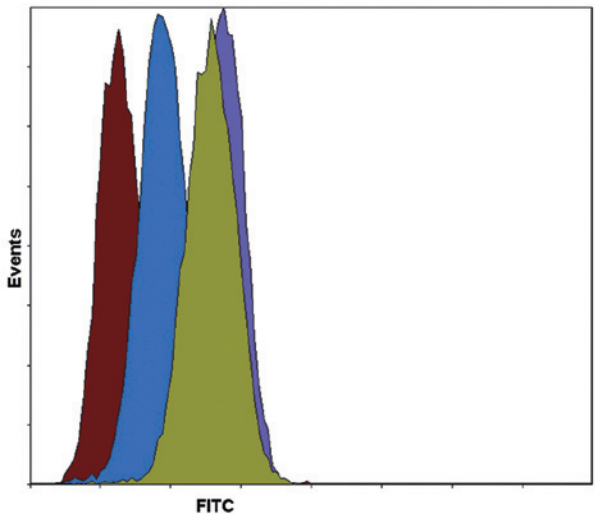

Figure 2. Expression of MHC class II by J774.1 cells. Mouse macrophage J774A.1 cells were cultured in RPMI-1640 complete medium supplemented with 10% FCS in the presence of pL06, tuftsin or pL01, at 37°C in a CO_2 incubator for 4 days. At the end of the culture, cells were stained and analyzed for the expression of MHC class II by flow cytometry. Results are a representation of three independent experiments. From left to right: control, pL01, pL06, tuftsin.

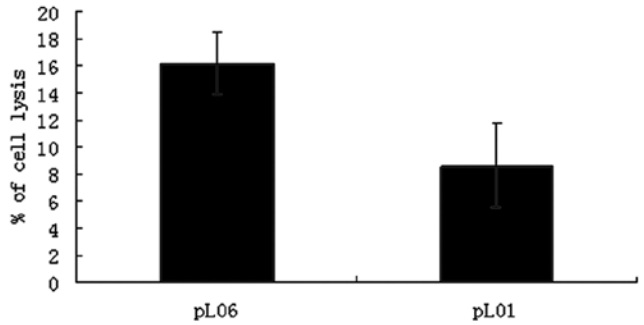

Figure 4. Enhanced phagocytosis ability of macrophages by pL06. Bar graph shows the comparison of phagocytosis ability of J774.1 after being primed by pL06 or pL01. The phagocytosis assay was measured by killing of chromium-labeled OVCAR-3 cells at target-to-effector cell ratios of 10:1.

protein was able to induce CA125-specific cytotoxic activity. Anti-idiotypic antibody WJ02 is the internal image of CA125, which has been proved to induce a CA125-specific immune response (7). As shown in Fig. 3, pL01, which has the variable region of mAb WJ02, marginally induced a CA125-specific cytotoxicity. However, the reaction was further enhanced by educating J774A.1 cells with scFvWJ02 fused with tuftsin, pL06, possibly due to the enhanced expression of MHC class II molecules on J774A.1 related to improved antigen presentation. This result suggests that scFvWJ02 induces CTLs, which recognize CA125-positive ovarian cancer cells in a MHC-restricted fashion. Without scFvWJ02, tuftsin alone enhanced the phagocytic ability of macrophages. Moreover, the cytotoxic function of tuftsin was not disturbed during the fusion process as indicated in the method, which led to a non-specific cell lysis of tumor cells (Fig. 4). Taken together, the fusion protein pL06 may enhance tumoricidal lysis specifically and non-specifically.

Anti-cancer response elicited by the pL06 vaccine. To examine whether the fusion protein generates an immune response against CA125, BALB/c mice immunized with pL06, pL01 or tuftsin were tested for antibody production, T-cell proliferation and expression of cytokine genes. Although pL06 and pL01 have a variable region of mAb WJ02, which functionally mimics cancer antigen CA125, the reduced molecular weight of the single-chain antibody may result in low immunogenicity. Our previous studies revealed that application of PLGA microspheres could trigger a better immune response than that by immunogen alone (12); microspheres were applied in this study to achieve a satisfactory immunogenicity of the single-chain fusion protein. Two weeks following the first immunization, production of the anti-anti-idiotypic antibody was observed in the animals treated with pL01 or pL06 (Fig. 5). These antibodies recognized the original antigen CA125, suggesting that Abl-like antibody had been developed following immunization with anti-idiotypic scFvWJ02. One week following the booster immunization, a

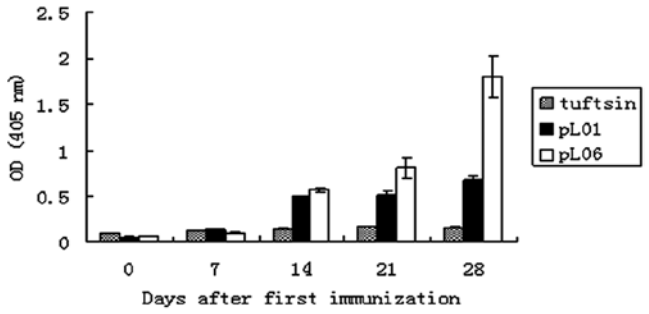

Figure 5. Time course of anti-CA125 antibody production in mice following immunization with the single chain antibody. Mice were immunized three times with tuftsin, pL01 or pL06, respectively, and blood was collected at indicated time points. Sera were diluted 1:100 with diluent and tested for the presence of the anti-CA125 antibody by ELISA. Data represent the mean ± SD of eight mice per group.

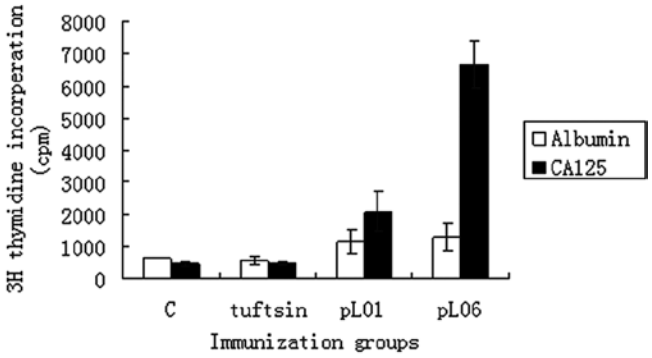

Figure 6. Proliferation of immunized spleen cells in response to antigen *in vitro*. Splenocytes were obtained from groups of mice immunized with tuftsin, pL01 or pL06, respectively. T-cell proliferation was determined by ^{3}[H]-thymidine incorporation in the presence of albumin and CA125.

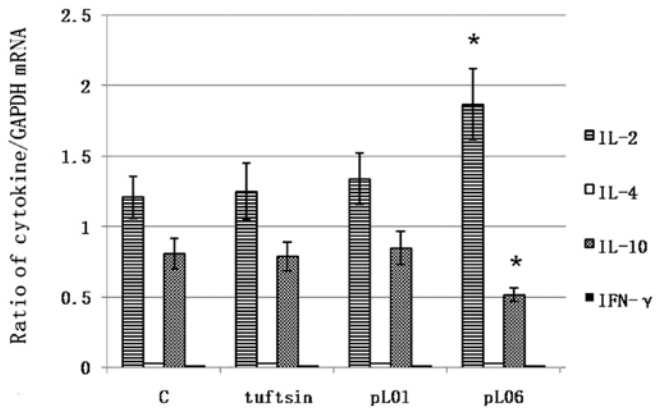

Figure 7. Expression of cytokine mRNA in spleen cells. IL-2, IL-4, IL-10 and IFN-γ mRNA levels were quantified in spleen cells derived from mice immunized with PBS, tuftsin, pL01 or pL06 by quantitative RT-PCR at least three separate times (*P<0.05). Bars show the means ± SD.

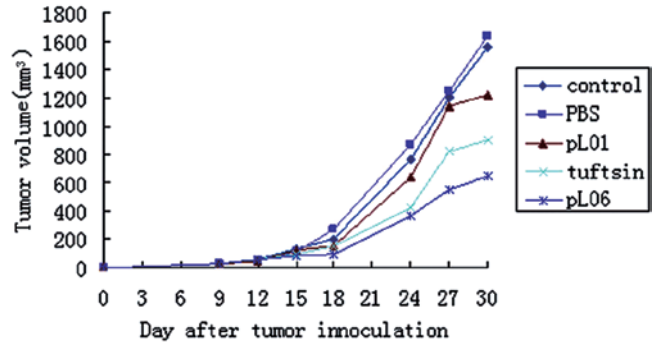

Figure 8. Anti-tumor immunity induced by pL06. Groups of mice received treatments of PBS, pL01, tuftsin or pL06 as outlined in Materials and methods. Additional splenocytes of healthy mice were administered to experimental mice by the i.v. route to mimic the presence of peripheral T cells. The volume of tumors was measured every 3 days after the prime treatment. The experiment was terminated when the longest dimension of the tumor on control mice reached 1.5 cm. Tumor growth curves derived from ten animals are shown.

significant difference in anti-CA125 antibody production was observed between the mice immunized with pL01 and pL06 ($p<0.01$). Fusing the recombinant single-chain variable fragment of mAb WJ02 to the murine tuftsin led to a significant elevation in the production of antibodies.

It is well believed that the ability of anti-idiotypic antibodies to invoke T-cell-mediated anti-tumor responses is more relevant to effective therapy than the ability to induce a humoral anti-tumor response (23). In this regard, we evaluated the protective T-cell response induced by pL06 immunization.

Splenocytes derived from mice immunized with tuftsin, pL01 or pL06 were challenged with either CA125 or an irrelevant protein (albumin). As illustrated in Fig. 6, T cells derived from mice immunized with pL06 produced a significantly increased proliferative response *in vitro* when re-challenged with CA125, while no specific response was observed when mice were immunized with tuftsin. Fig. 6 shows that pL01 primed T cells to induce a proliferative response to CA125, indeed this response was much weaker than that primed by pL06. We speculated that the adjuvant effect of tuftsin accounted for the differences observed. The significance of these results indicated that T cells primed and stimulated by pL06 were cross-reactive with CA125.

Cytokine mRNA expression (IL-2, IL-4, IL-10 and IFN-γ) was also examined by quantitative RT-PCR. As the results showed in Fig. 7, splenocytes derived from mice immunized with pL06 produced considerably high IL-2 mRNA expression than other groups. Furthermore, a significantly decreased IL-10 mRNA expression could be observed in the animals treated with pL06 in comparison with the other groups. These results indicated that pL06 was able to improve the activity of T cells effectively. Therefore, from a vaccination point of view, such T cells may recognize CA125 upon encountering tumor cells.

Anti-tumor effect elicited by pL06. The nude mouse xenograft model was used to determine the effect of pL06 treatment on the growth of ovarian cancer cells (Fig. 8). The results demonstrated that the fusion protein pL06 produced a more than 2-fold reduction in tumor volume as compared to the control groups ($p=3.6\times10^{-7}$). Furthermore, treatment by fusion protein was much more effective in retarding tumor growth than tuftsin ($p=0.006$) or pL01 ($p=8\times10^{-6}$).

Discussion

The progress in peptide synthesis and recombinant DNA technology hold the promise of a new generation of vaccines. As a result, more and more single-chain antibodies have been developed for various vaccine purposes (24). However, due to their reduced size, the elicited immune responses have been generally weak. The significance of this study is the linkage of tuftsin to an anti-idiotypic single-chain antibody to form a fusion protein, which resulted in a specific anti-tumor response. The single-chain antibody used in this study to construct a fusion protein was scFvWJ02, which is an anti-idiotypic protein with a similar antigenic construction of CA125. Since mAb WJ02 has previously been verified to be immunogenic by our laboratory (7), scFvWJ02, which preserved the ability to mimic the epitopes on CA125, was supposed to induce an anti-CA125 response upon immunization. However, the molecular weight of scFvWJ02 (only 28 kDa) was significantly reduced compared with the whole antibody, which indicated poor immunogenicity. As reported by Ivanov *et al* (25), antibody response to non-immunogenic peptides may be elicited by conjugation with polytuftsin. We, therefore, fused the single-chain antibody with a tuftsin molecule in terms of its ability to enhance immune response. The *in vivo* immune experiment showed that the single-chain itself was unable to produce an immune response. By contrast, when conjugated with tuftsin, equimolar amounts of the fusion protein pL06 under identical experimental conditions were found to generally improve humoral and cellular responses against CA125. Since this study was conducted in a non-tolerant animal model, the immunogenicity of the fusion protein is speculated to be stronger than that of the original antigen in the immunotolerant situation. Thus, additional studies are required to address this question.

Studies with various tuftsin analogs suggest that tuftsin is a natural stimulator, which activates numerous functions of monocytes and macrophages via its receptors (26). For synthesized tuftsin peptide, the binding ability to its receptors is the 'checkpoint' initiating downstream immune responses. Our *in vitro* ELISA assay demonstrated that the fusion protein pL06 could bind to macrophages successfully, suggesting that the end of the tuftsin domain preserved the ability to stimulate immune responses. This was further proved by the cytotoxic assay. As shown in Fig. 3, pL06 had greater anti-tumor activity in cytotoxic assays than each individual component. The specific cytotoxicity of pL06 was correlated with a higher level of MHC class II expression. A number of studies have demonstrated that the binding of antigenic peptides with MHC class-II molecules has a profound effect on T-cell proliferation (27). It is reasonable to speculate that the increased expression of MHC class II molecules induced by the tuftsin terminal of pL06 on antigen-presenting cells may lead to better antigen presentation to T cells. In the same experimental system, tuftsin showed certain cytotoxicity to the tumor cells. This was further confirmed in the non-specific release experiment, in which pL06 induced direct tumor cell lysis via macrophages (Fig. 4). These data implied the existence of non-specific killing by pL06 even in the specific release experiment, which provided anti-tumor activity through direct phagocytic mechanisms.

The following *in vivo* immune experiment was tested to examine whether pL06 generates an immune response against CA125, including antibody production, T-cell proliferation and expression of cytokine genes. These studies demonstrated that the fusion protein pL06 effectively stimulates the activation and proliferation of T cells specific for CA125. Therefore, pL06 was shown to be able to elicit a CA125-specific response in experimental animals and thus may have the potential to function as a vaccine in active immunotherapy against CA125-positive carcinomas. This hypothesis led us to determine the anti-tumor effects of pL06 on a xenograft tumor model. Since there is no allograft CA125-positive tumor model available, we recruited murine T cells to form immunocompetent mice. The immunization of pL06 inhibited the growth of OVCAR-3 cell xenografts in nude mice at a dose of 50 μg/mice over a period of 4 weeks. Additionally, pL06 was significantly more effective than pL01 single-chain alone in terms of tumor suppression, which supports our rational for augmenting immunogenicity of scFvWJ02 by linking with tuftsin. As an immunodeficient model was used in this study, the anti-idiotypic response was not included in the anti-tumor effect observed. A number of mechanisms may be envisioned for the generation of tumor lysis following immunization with pL06. First, pL06 enhanced MHC class II expression on macrophages and presented the antigenic anti-idiotope of scFvWJ02 to macrophages simultaneously. This education of macrophages aroused a high production of CA125-specific CTLs when transplanted splenocytes encountered these macrophages thereafter. It thus appears that scFv and tuftsin elicit their own signaling pathways that act simultaneously to trigger the CTL response in this situation. Another reason for the increased tumor protection by pL06 over pL01 may rely on the activated phagocytic ability of macrophages as proved *in vitro*, which resulted in an enhanced innate immune response against tumor cells (28).

Taken together, the results presented here are, to the best of our knowledge, the first to show the anti-tumor efficacy of an anti-idiotype/tuftsin fusion vaccine against human CA125. We propose that the application of pL06 in cancer patients should achieve a better anti-tumor effect than in the mouse, as in addition to CTL, the anti-idiotypic humoral response will play a role in tumor lysis. The present observations led us to conclude that the inclusion of an anti-idiotypic antibody and a natural immune potentiator, such us tuftsin, may be an ideal strategy for a cancer vaccine.

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