European Review for Medical and Pharmacological Sciences 2015; 19: 2812-2817

The irradiated-fibroblasts elicit immune response against tumor in a murine colon cancer model

X.-Y. LI, L. XIAO, J.-H. ZHANG, L. CHEN

Medical Faculty of Kunming University of Science and Technology, Kunming, China

Abstract. – OBJECTIVE: The breaking of immune tolerance against tumor stromal fibroblasts should be a useful approach for cancer immunotherapy. The primary objective of this study was to verify whether irradiated-fibroblasts could induce an anti-tumor immune response *in vivo*.

MATERIALS AND METHODS: Normal fibroblasts were irradiated with 25Gy; their expressions of a-SMA, HMGB1 and calreticulin were assessed by Western blot. Mice were immunized with irradiated-fibroblasts and then challenged with CT26 colon carcinoma. Auto-antibodies were assessed by flow cytometric. The inhibition of proliferation of tumor cells by purified immunoglobulins was investigated.

RESULTS: The irradiated-fibroblasts expressed HMGB1, calreticulin and α-SMA. The irradiated-fibroblasts were effective in affording protection from tumor onset and growth. The immunized sera exhibited positive staining for tumor cells in flow cytometric analysis. The purified immunoglobulins from immunized mice inhibited the proliferation of CT26 tumor cells *in vitro*.

CONCLUSIONS: All these suggest that irradiated-fibroblasts could induce an anti-tumor immune response.

Key Words:
Irradiated-fibroblasts, Tumor immunotherapy, CT26 colon carcinoma.

Introduction

Stimulating the body's natural immune response against tumors has great potential to eliminate the cancer cells. Tumor immunotherapy often fails due to many reasons[1,2]. Interactions between cancer cells and surrounding stromal cells may play an important role in aggressive tumor progression[3]. Fibroblasts are the predominant cellular component of the tumor stroma in most types of cancers[4]. Stromal cells within tumors are diploid, genetically stable, and exhibit limited proliferative capacity; targeting the stroma could substantially reduce the incidence of immune evasion[5]. It has been shown that destruction of stromal cells within tumors by CTL is essential for eradication of large well-established solid tumors. The deleting a subpopulation of stromal fibroblasts arrests the growth of a solid tumor, an effect that depends on an immune response to the tumor[7]. These results agree with other studies suggesting that immunizing against fibroblasts in tumors can unmask an immune response to cancer[8].

Co-culture with irradiated-fibroblasts significantly increased the invasive ability of pancreatic cancer cells[9], and some studies showed that irradiated-fibroblasts results in enhanced tumor growth and invasion[10,11]. IMR90 cells (human fetal lung fibroblasts) expressed α-SMA when they received 8Gy irradiation[12], and *de novo* expression of α-SMA is the most commonly used marker for cancer-associated fibroblasts (CAFs)[13]. All these indicated that the irradiated-fibroblasts were different from the quiescent fibroblasts *in vivo*, and these irradiated-fibroblasts might have been activated. We hypothesized whether these irradiated-fibroblasts could elicit immune responses against tumor. To test this concept we immunized mice with irradiated-fibroblasts; we found that irradiated-fibroblasts could protect mice from tumor cells challenged by inducing humoral immune responses.

Materials and Methods

Animals and Cell Culture

BALB/c mice, 5 to 7 weeks old, were purchased from the HFK bioscience Co., LTD (Beijing, China). All mice were maintained in a pathogen-free animal facility for at least 1 week before each experiment. All studies involving mice were approved by the Institute's Animal Care and Use Committee. CT26 colon carcinoma (CT26; ATCC) cells were cultured in DMEM supplemented with 10% heat-inactivated fetal

Corresponding Author: Xiu-Ying Li, MD; e-mail: lixying98@163.com

bovine serum (FBS) and antibiotics. We prepared vaccines using proliferative fibroblasts cultured *in vitro*, fibroblasts were derived from normal mouse dermal about two weeks old and digested 2h in 1 mg/mL collagenase I (Gibco, Carlsbad, CA, USA), and cells were plated in Dulbecco's Modified Eagle Medium (DMEM containing 10% heat-inactivated FBS and antibiotics[14]. Fibroblasts were cultured for three passages and irradiated with 25Gy; two hour later these cells were injected s.c. into immunocompetent histocompatible mice.

Western Blotting Analysis

The cells lysates of irradiated-fibroblasts, or unirradiated-fibroblasts were separated on SDS-polyacrylamide gels and transferred to a polyvinylidene fluoride (PVDF) membrane. After transfer, the membrane was blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) with 0.5% Tween 20 to block nonspecific protein binding, washed, and probed with interest proteins α-smooth muscle actin (α-SMA) (Abcam, Carlsbad, CA, USA), calreticulin (Cell Signaling, Beverly, MA, USA), and HMGB1 (Cell Signaling, ref 15) respectively. After several washing, PVDF membrane was incubated with horseradish peroxidase-conjugated secondary antibody (1: 5,000) in blotting buffer at room temperature for 2h. Then membranes were washed and protein antibody complexes were visualized by chemiluminescent detection using SuperSignal West Pico Chemilumenescent Substrate (Pierce, Appleton, WI, USA).

Induction of Protective Anti-Tumor Immunity

We immunized Balb/c (n=10 per group) subcutaeous on D0, D14 and D21 with 1.5×10^6 irradiated- fibroblasts, additional control animals were immunized with unirradiated-fibroblasts (control group) or normal saline alone (non-immunized). One week after the third immunization, mice were then challenged with 5×10^5 live CT26 colon carcinoma. Tumor growth was evaluated by measurement of tumor diameters every 3 days and tumor volume was determined by the following formula: tumor volume (mm^3) = 0.52× length (mm) × width $(mm)^2$.

Purification of Immunoglobulin, its Inhibition of Cell Proliferation in vitro

Immunoglobulins were purified from the pooled sera derived from the mice at day 7 after the third immunization or from control mice by affinity chromatography (CM Affi-gel Blue Gel Kit; Bio-Rad, Hercules, CA, USA). For determination of the effects of purified immunoglobulins on cell proliferation, exponentially growing unirradiated-fibroblasts or CT26 tumor cells (2×10^3 CT26/well in 96-well plates, 1×10^3 unirradiated-fibroblasts/well in 96-well plates) were exposed to various concentrations (50 μg/ml, 100 μg/ml and 150 μg/ml) of the immunoglobulins for 72 h of culture. Cytotoxicity was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described.

The Flow Cytometric Analysis

For the flow cytometric analysis, CT26 colon carcinoma cells were stained by an indirect method, using serum diluted 1:50, and then goat fluorescein isothiocyanate (FITC)-conjugated antibody against mouse IgG, IgM or IgA (Sigma, St Louis, MO, USA).

Satistical Analysis

SPSS 11.5 was used for statistical analysis. The statistical significance of results in all of the experiments was determined by ANOVA and Student's *t* test. Survival curves were compared using the log-rank test. $p < 0.05$ was deemed statistically significant.

Results

The Effects of Ionizing Irradiation on Exposure of Calreticulin, Release of HMGB1 and Expression of a-SMA

The fibroblasts which were irradiated by 25Gy expressed abundant α-SMA; ionizing irradiation could induce the exposure of calreticulin, and lead to release of HMGB1 from irradiated-fibroblasts; compared to unirradiated-fibroblasts (Figure 1; lane 1: irradiated-fibroblasts; lane 2: unirradiated-fibroblasts). All these indicated that the irradiated-fibroblasts were different from the normal fibroblasts *in vivo*; this might be the base that irradiated-fibroblasts could trigger immune response against tumor.

The Delay of Tumor Growth

Mice were immunized with irradiated-fibroblasts on D0, D14 and D21, and then challenged with CT26 tumor cells. Tumors grew progres-

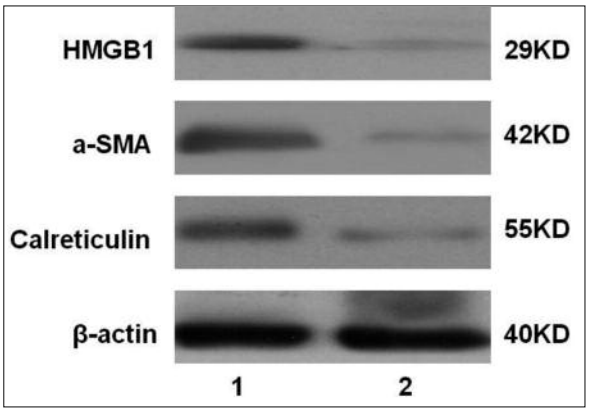

Figure 1. The ionizing irradiation exposure calreticulin, released HMGB1 and expressed α-SMA. The fibroblasts which were irradiated with 25Gy apparently expressed a-SMA; the irradiation could lead to the plasma membrane translocation of CRT, and the releasing of HMGB1, compared to the unirradiated-fibroblasts. Lane 1: irradiated-fibroblasts; Lane 2: unirradiated-fibroblasts.

sively in all control group mice, but there was an apparent protection from tumor onset and growth in mice immunized with irradiated-fibroblasts (Figure 2a). Furthermore, the lifespan of immunized mice was apparently prolonged compared with control mice (Figure 2b). The form-tumor rate of immunized group was 70 percent; the form-tumor rate of control group and non-immunized group was 100 percent.

Characterizations of Auto-Antibodies

In an attempt to explore the possible mechanism by which antitumor activity was elicited by irradiated-fibroblasts, we treated the unirradiated-fibroblasts and CT26 tumor cells with various doses of purified immunoglobulins isolated from mice immunized with irradiated-fibroblasts or unirradiated-fibroblasts, the immunized immunoglobulins resulted in apparent inhibition of proliferation of CT26 tumor cells (Figure 3a), and the proliferation of unirradiated-fibroblasts were only partly inhibited (Figure 3b); compared to the immunoglobulins which isolated from control group.

We stained CT26 tumor cells with sera isolated from irradiated-fibroblasts immunized mice or control group mice in flow cytometric analysis. CT26 tumor cells showed apparent positive staining for IgG (Figure 4a) and IgM (Figure 4b) of immunized sera.

Discussion

Cell-based cancer immunotherapy is one appealing strategy, which is designed to harness the patients' own immune system to induce a potent anti-tumor response. A wide array of cell-based immunotherapies utilizing T cells, NK cells, and dendritic cells have been established[16,17]. One key advantage of using cell-based vaccines is the convenience that tumor antigens do not have to be well defined. In addition, more potentially tumor antigens may be covered with this approach[18].

Our experimental data demonstrate that the irradiated-fibroblasts can serve as a vaccine for cancer immunotherapy. The vaccine could pro-

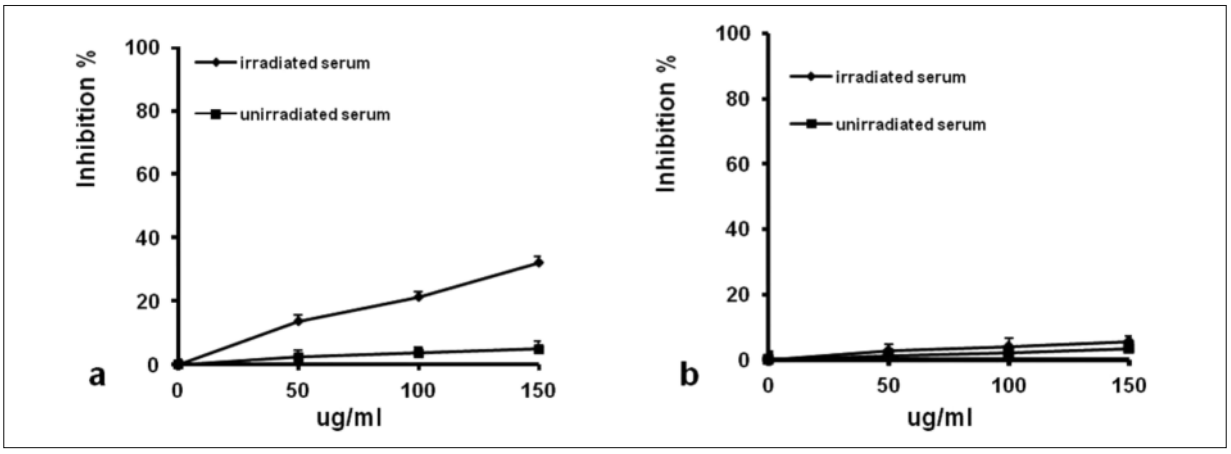

Figure 2. Induction of protective antitumor immunity. ***a,*** Mice (10mice/group) were immunized with irradiated-fibroblasts, unirradiated-fibroblasts, or normal saline alone on D0, D14 and D21. Mice were then challenged subcutaneously with 5×10^5 CT26 one week after the last immunization. There was an apparent difference in tumor volume between immunized and the control groups ($p < 0.01$). Results are expressed as mean ± SEM. Apparent increase in lifespan of immunized group, compared to the control groups was found ***(b)***.

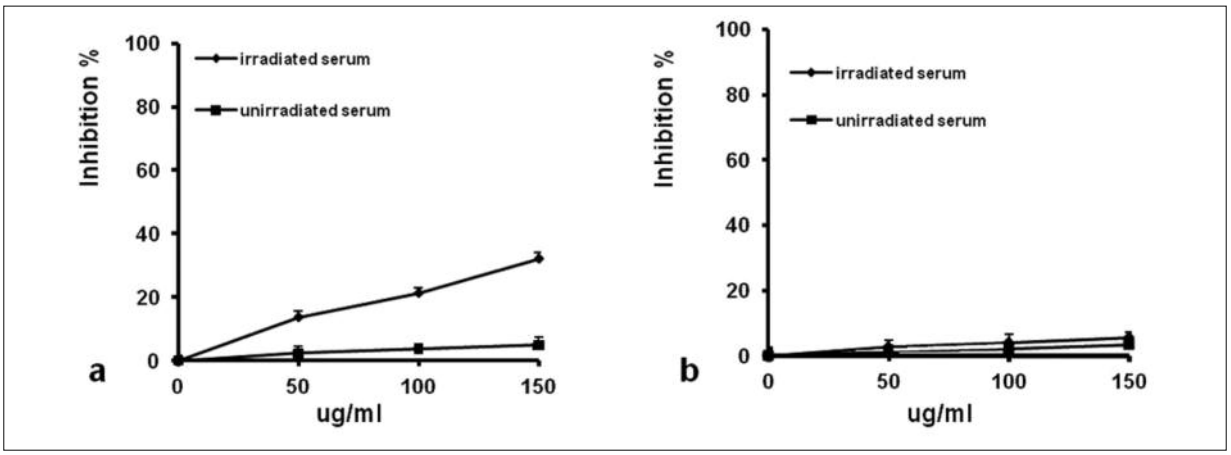

Figure 3. Inhibition of immunoglobulins-mediated cells' proliferation in vitro. Exponentially growing CT26 tumor cells ***(a)*** and unirradiated-fibroblasts ***(b)*** were treated with various concentrations of purified immunoglobulins from immunized or control sera. The immunized immunoglobulins resulted in apparent inhibition of proliferation of CT26 tumor cells, and the proliferation of unirradiated-fibroblasts were partly inhibited; compared to the immunoglobulins which isolated from control group.

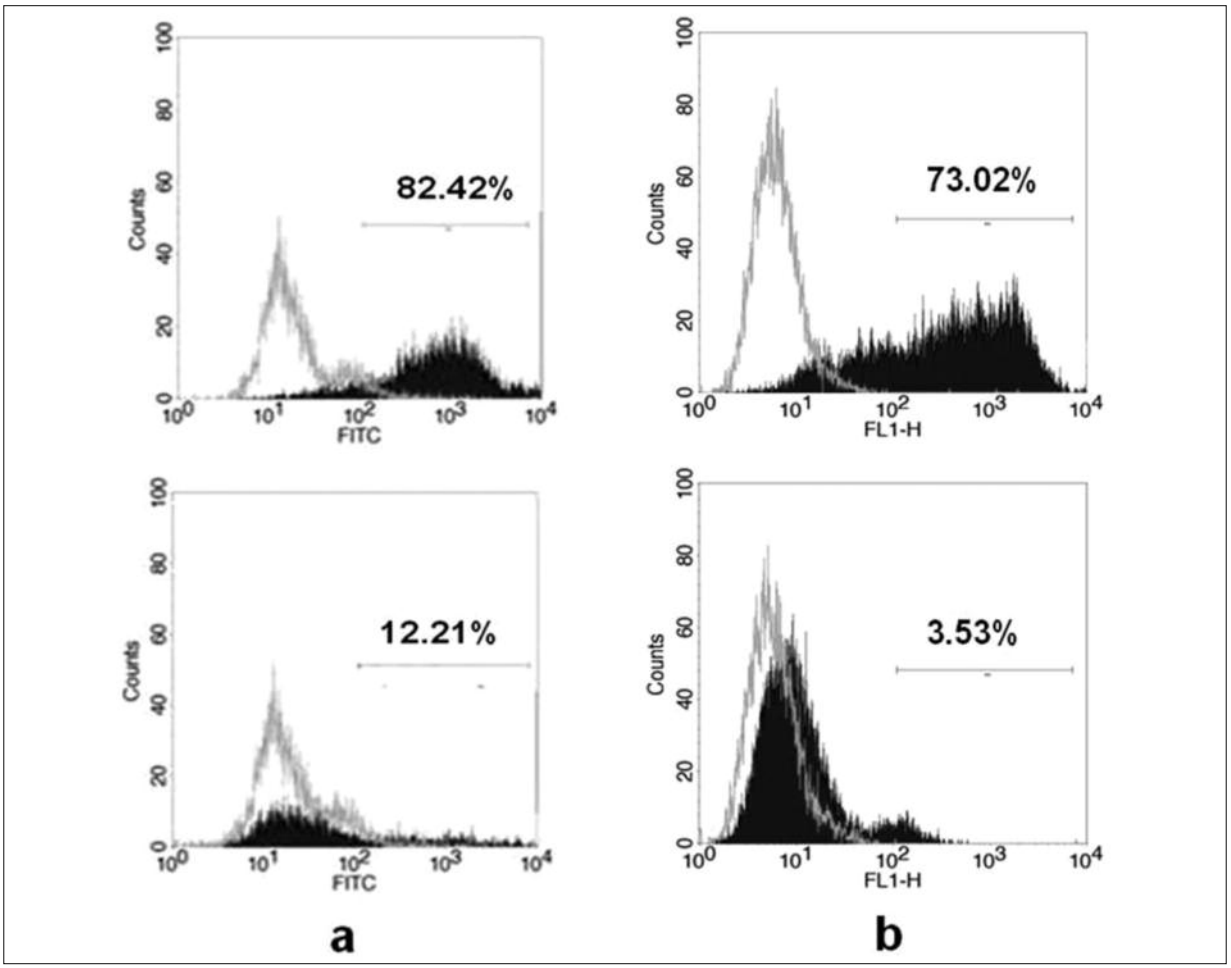

Figure 4. Identification of auto-antibodies which elicited by irradiated-fibroblasts. Cells were stained by an indirect method using 1:50 diluted sera and then FITC-goat anti-mouse IgG and IgM in flow cytometric analysis. CT26 tumor cells showed apparently positive staining for IgG ***(a)*** and IgM ***(b)*** of immunized sera, compared to only partly positive staining for control sera.

tect host from tumor onset, growth and prolonging survival of tumor-bearing mice in CT26 tumor models. Irradiated-fibroblasts could induce tumor-specific humoral immune responses, and some auto-antibodies in the immunized sera could react with CT26 tumor cells.

In normal cells, calreticulin (CRT) is a calcium-binding protein found predominantly in the endoplasmic reticulum, where it acts as a chaperone aiding proper folding of other proteins[19]. In line with previous studies that calreticulin can serve as an "eat me" signal when it is exposed on the cell surface of dying cells[20]. CRT also has adjuvant properties, the chimeric constructs encoding CRT and tumor antigens delivered with a viral vector[21] is more immunogenic than the tumor antigen alone; and CRT purified from tumors can elicit tumor-specific immunity[22]. CRT exposure is known to be induced by ultraviolet-C (UVC) light or γ-irradiation, and critical for the immunogenicity of cell death[23,24].

Radiation can increase the immunogenic properties of tumor cells by enhancing MHC class I expression[25], thereby increasing their vulnerability to CTLs. In response to ionizing, fibroblasts undergo immunogenic apoptosis, meaning that they could trigger a protective immune response when they are injected subcutaneously into immunocompetent mice. Dying cells can release adjuvant factors that amplify and sustain dendritic cell (DC) and T-cell-dependent immune responses[26,27]. The damage-associated molecular patterns (DAMP) released by dying tumor cells on chemotherapy or radiotherapy-induced distress has been identified as high-mobility group box 1 protein (HMGB1), a nuclear protein that is released passively during necrosis and actively during late-stage apoptosis[28]. HMGB1 is a critical mediator for the TLR4-dependent processing of exogenous tumor antigens by DCs[29]. HMGB1 can also act as chemotactic and/or activating factors for macrophages, neutrophils, and DCs[30].

Conclusions

Based on our findings mentioned above, we may rule out the possibility that antitumor activity of irradiated-fibroblasts may result from the nonspecifically augmented immune response against the tumor initiation and growth in host mice.

Acknowledgements

The authors gratefully acknowledge the Personnel Training Project of Kunming University of Science and Technology, Kunming, China (No. KKSY201460047).

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References

1) Drake CG, Jaffee E, Pardoll ADV. Mechanisms of immune evasion by tumors. Adv Immunol 2006; 90: 51-81.
2) Blankenstein T. The role of tumor stroma in the interaction between tumor and immune system. Curr Opin Immunol 2005; 17: 180-186.
3) Liotta LA, Kohn EC. The microenvironment of the tumour-host interface. Nature 2001; 411: 375-379.
4) Mueller MM, Fusenig NE. Friends or foes--bipolareffects of the tumour stroma in cancer. Nat Rev Cancer 2004; 4: 839-849.
5) Kerbel RS. A cancer therapy resistant to resistance. Nature1997; 390: 335-336.
6) Zhang B, Bowerman NA, Salama JK, Schmidt H, Spiotto MT, Schietinger A, Yu P, Fu YX, Weichselbaum RR, Rowley DA, Kranz DM, Schreiber H. Induced sensitization of tumor stroma leads to eradication of established cancer by T cells. J Exp Med 2007; 204: 49-55.
7) Kraman M, Bambrough PJ, Arnold JN, Robert EW, Magiera L, Jones JO, Gopinathan A, Tuveson DA, Fearon DT. suppression of antitumor immunity by stromal cells expressing fibroblast activation protein-alpha. Science 2010; 330: 827-830.
8) Liao D, Luo Y, Markowitz D, Xiang R, Reisfeld RA. Cancer associated fibroblasts promote tumor growth and metastasis by modulating the tumor immune microenvironment in a 4T1 murine breast cancer model. PLoS ONE 2009; 4: e7965.
9) Ohuchida K, Mizumoto K, Murakami M, Qian LW, Sato N, Nagai E, Matsumoto K, Nakamura T, Tanaka M. Radiation to stromal fibroblasts increases invasiveness of pancreatic cancer cells through tumor-stromal interactions. Cancer Res 2004; 64: 3215-3222.
10) Kalluri R, Zeisberg M. Fibroblasts in cancer. Nat Rev Cancer 2006; 6: 392-401.
11) Paquette B, Therriault H, Desmarais G, Wagner R, Royer R, Bujold R. Radiation-enhancement of MDA-MB-231 breast cancer cell invasion prevented by a cyclooxygenase-2. Br J Cancer 2011; 105: 534-541.
12) Tabata C, Kadokawa Y, Tabata R, Takahashi M, Okoshi K, Sakai Y, Mishima M, Kubo H. All-trans-retinoic acid prevents radiation- or bleomycin-induced pulmonary fibrosis. Am J Respir Crit Care Med 2006; 174: 1352-1360.

13) Erez N, Truitt M, Olson P, Arron ST, Hanahan D. Cancer-associated fibroblasts are activated in incipient neoplasia to orchestrate tumor-promoting inflammation in an NF-kappaB-dependent manner. Cancer Cell 2010; 17: 135-147.

14) Giannoni E, Bianchini F, Masieri L, Serni S, Torre E, Calorini L, Chiarugi P. Reciprocal activation of prostate cancer cells and cancer-associated fibroblasts stimulates epithelial-mesenchymal transition and cancer stemness. Cancer Res 2010; 70: 6945-6956.

15) Hayakawa K, Pham LD, Katusic ZS, Arai K, Lo EH. Astrocytic high-mobility group box 1 promotes endothelial progenitor cell-mediated neurovascular remodeling during stroke recovery. Proc Natl Acad Sci USA 2012; 109: 7505-7510.

16) Song GY, Srivastava T, Ishizaki H, Lacey SF, Diamond DJ, Ellenhorn JD. Reconbinant modified vaccinia virus Ankara (MVA) expressing wild-type human p53 induces specific antitumor CTL expansion. Cancer Invest 2011; 29: 501-510.

17) Pang J, Gao X, Liu X, Wang K, Zhang Y, Feng L, Zhan H. Enhanced antitumor effects by the coculture of allotumor RNA-pulsed dendritic cells with autologous cytokine-induced killer cells on hormone-refractory prostate cancer. Cancer Invest 2007; 25: 527-534.

18) Koido S, Hara E, Homma S,Torii A, Toyama Y, Kawahara H, Watanabe M, Yanaga K, Fujise K, Gong J, Toda G. Dendritic cells fused with allogeneic colorectal cancer cell line present multiple colorectal cancer-specific antigens and induce antitumor immunity against autologous tumor cells. Clin Cancer Res 2005; 11: 7891-7900.

19) Gelebart P, Opas M, Michalak M. Calreticulin, a Ca^{2+}-binding chaperone of the endoplasmic reticulum. Int J Biochem Cell Biol 2005; 37: 260-266.

20) Gardal SJ, Mcphillips KA, Frasch SC, Janssen WJ, Starefeldt A, Murphy-Ullrich JE, Bratton DL, Oldenborg PA, Michalak M, Henson PM. Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. Cell 2005; 123: 321-334.

21) Hsieh CJ, Kim TV, Hung CF, Juang J, Moniz M, Boyd DA, He L, Chen PJ, Chen CH, Wu TC. Enhancement of vaccinia vaccine potency by linkage of tumor antigen gene to gene encoding calreticulin. Vaccine 2004; 22: 3993-4001.

22) Basu S, Srivastava PK. Calreticulin, a peptide-binding chaperone of the endoplasmic reticulum, elicits tumor- and peptide-specific immunity. J Exp Med 1999; 189: 797-802.

23) Obeib M, Tesniere A, Ghiringhelli F, Fimia GM, Apetoh L, Perfettini JL, Castedo M, Mignot G, Panaretakis T, Casares N, Metivier D, Larochette N, van Endert P, Ciccosanti F, Piacentini M, Zitvogel L, Kroemer G. Calreticulin exposure dictates the immunogenicity of cancer cell death. Nat Med 2007; 13: 54-61.

24) Obeid M, Panaretakis T, Joza N, Tufi R, Tesniere A, van Endert P, Zitvogel L, Kroemer G. Calreticulin exposure is required for the immunogenicity of gamma-irradiation and UVC light-induced apoptosis. Cell Death Differ 2007; 14: 1848-1850.

25) Reit EA, Hodge JW, Herberts CA, Groothuis TA, Chakraborty M, Wansley EK, Camphausen K, Luiten RM, de Ru AH, Neijssen J, Griekspoor A, Mesman E, Verreck FA, Spits H, Schlom J, van Veelen P, Neefjes JJ. Radiation modulates the peptide repertoire, enhances MHC class I expression, and induces successful antitumor immunotherapy. J Exp Med 2006; 203: 1259-1271.

26) Tabeta K, Hoebe K, Janssen EM, Du X, Georgel P, Crozat K, Mudd S, Mann N, Sovath S, Goode J, Shamel L, Herskovits AA, Portnoy DA, Cooke M, Tarantino LM, Wiltshire T, Steiberg BE, Grinstein S, Beutle B. The Unc93b1 mutation 3d disrupts exogenous antigen presentation and signaling via Toll-like receptors 3, 7 and 9. Nat Immunol 2006; 7: 156-164.

27) Janssen E, Tabeta K, Barnes MJ, Rutschmann S, Bahjat KS, Schoenberger SP, Theofilopoulos AN, Beutler B, Hoebe K. Efficient T cell activation via a toll-interleukin 1 receptor-independent pathway. Immunity 2006; 24: 787-799.

28) Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 2002; 418: 191-195.

29) Apetoh L, Ghiringhelli F, Tesniere A, Criollo A, Ortiz C, Lidereau R, Mariette C, Chaput N, Mira JP, Delaloge S, Andre F, Tursz T, Kroener G, Zitvogel L. The interaction between HMGB1 and TLR4 dictates the outcome of anticancer chemotherapy and radiotherapy. Immunol Rev 2007; 220: 47-59.

30) Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, Mignot G, Maiuri MC, UllrichE, Saulnier P. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nature Med 2007; 13: 1050-1059.