Pharmaceutical Research, Vol. 24, No. 9, September 2007 (© 2007)
DOI: 10.1007/s11095-007-9293-5

Research Paper

Learning from Viruses: The Necrotic Bodies of Tumor Cells with Intracellular Synthetic dsRNA Induced Strong Anti-tumor Immune Responses

Zhengrong Cui,[1] Uyen M. Le,[1] Fu Qiu,[1] and Dalia S. Shaker[1]

Received November 28, 2006; accepted March 8, 2007; published online April 3, 2007

Purpose. Coaxing dead tumor cells to induce specific immune responses is an attractive tumor therapy. However, there continues to be a need for adjuvants that can promote the cross-presentation of the dead tumor cells to induce specific anti-tumor response. Viral dsRNA has multiple mechanisms to promote the cross-presentation of viral antigens in virus-infected cells. We propose to learn from viruses by generating dead tumor cells having synthetic dsRNA delivered inside them to allow the dsRNA to promote the cross-presentation of dead tumor cells.
Materials and Methods. Using synthetic dsRNA, poly(I:C), and the TC-1 cervical cancer model, we evaluated the extent to which the poly(I:C) can promote the necrotic bodies of TC-1 cells to induce specific anti-tumor immune response. The poly(I:C) was either simply mixed with the dead TC-1 cells or pre-loaded inside them.
Results. Immunization of tumor-bearing mice with the necrotic bodies of tumor cells admixed with poly(I:C) significantly inhibited the tumor growth. More importantly, immunization with the necrotic bodies having poly(I:C) pre-loaded inside led to a significantly stronger anti-tumor response than when the necrotic bodies were simply admixed with the poly(I:C), apparently through a $CD8^+$ CTL response-mediated mechanism.
Conclusions. These findings are expected to be clinically relevant for devising improved whole cell-based tumor vaccines.

KEY WORDS: CTL; intracellular dsRNA; TLR3; tumor immunotherapy.

INTRODUCTION

Double-stranded RNA is produced by many viruses during their replicative cycle. It is a strong inducer of type I interferons (IFN-α/β) (1), which have been shown to be immunostimulatory (2) and anti-proliferative (3). Some synthetic dsRNAs, such as the polyinosinic-cytidylic acid [poly(I:C)], have similar effects as viral dsRNA (4–6). The immunostimulatory activity of synthetic dsRNA molecules was originally documented in the 1960–1970s (7, 8). However, it was not until recently that Toll-like receptor 3 (TLR3) was identified as a receptor of dsRNA (9), and the interaction between dsRNA and TLR3 was shown to play an important role in the immunostimulatory activity of dsRNA. To summarize recent findings related to the effects of dsRNA on the immune system, dsRNA was shown to induce stable maturation of functionally active dendritic cells (DCs) (10), to augment natural killer (NK) cell-mediated cytotoxicity (11, 12), to directly promote the survival of activated $CD4^+$ T cells *in vitro* (13), and to boost the number and survival of antigen-specific $CD8^+$ T cells (14), although $CD4^+$ T helper response was still required for the dsRNA to promote the induction of a functional $CD8^+$ T cell memory response (15).

We are interested in exploiting the immunostimulatory activity of dsRNA to promote dead or dying tumor cells to cross-prime anti-tumor immune responses. Viral dsRNA has been shown to have multiple mechanisms to promote virally infected professional antigen-presenting cells (APCs) or non-APCs to prime or cross-prime specific immune responses against viral antigens expressed in virus-infected cells. The cross-presentation of antigens associated with dead or dying tumor cells resembles the cross-presentation of viral antigens expressed in dead or dying virus-infected cells. Antigen cross-presentation may occur after professional APCs take up soluble molecules or particular matters, including cells. Dead or dying tumor cells can be presented by APCs to T cells to induce anti-tumor immune responses (16). However, a cross-presentation of an antigen does not necessarily lead to the cross-priming of an immune response. The APCs need to receive both antigens and appropriate activation signals in order to successfully cross-prime an immune response. Otherwise, a cross-presentation may lead to tolerance.

Double-stranded RNA has been shown to be such a proper activation signal. Following are two recently identified mechanisms on how dsRNA promotes antigen cross-priming;

[1] Department of Pharmaceutical Sciences, College of Pharmacy, Oregon State University, Corvallis, OR 97331, USA.
[2] To whom correspondence should be addressed. (e-mail: zhengrong.cui@oregonstate.edu)

(a) dsRNA can promote cross-priming through the induction of type I IFNs. Viral dsRNA induces infected cells to produce type I IFNs, which then promote the cross-priming of $CD8^+$ T cells by stimulating the maturation of antigen-presenting DCs (17). Similar to viral dsRNA, some synthetic dsRNA are also potent inducers of type I IFNs (18, 19), and thus, can promote antigen cross-priming. (b) Viral dsRNA can also promote cross-priming of T cell responses towards viral antigens expressed in infected cells, independent of the contribution from type 1 IFNs. Recently, it was shown that $CD8\alpha^+$ DCs were activated by viral dsRNA present in virally infected cells, but not by uninfected cells (20). Similarly, the $CD8\alpha^+$ DCs were activated by irradiated Vera cells pre-loaded with poly(I:C), but not by irradiated Vera cells in the absence of poly(I:C). This DC activation required the phagocytosis of the infected cells or the Vera cells by the DCs, followed by TLR3 signaling (20). More importantly, immunization of mice with the apoptotic bodies of virus-infected cells or irradiated Vera cells pre-loaded with poly(I:C) and a model protein antigen led to a striking increase in the cross-priming of cytotoxic T lymphocyte (CTL) response against viral antigens in virus-infected host cells or the model antigen loaded into the Vera cells (20). This activity was largely dependent on the expression of TLR3 by the APCs (20).

In the present study, we tested the feasibility of using synthetic dsRNA to promote the cross-priming of specific immune responses against tumor cells by mimicking the promotion of the induction of anti-viral immunity by viral dsRNA. We evaluated the ability of a synthetic dsRNA to cross-present dead tumor cells, particularly when the dsRNA was pre-loaded inside the dead tumor cells, to induce specific anti-tumor immune responses. The model cervical cancer TC-1 tumor cell line, which expressed the human papillomavirus (HPV) type 16 E6 and E7 gene, was used in this study. It was previously shown that, in the TC-1 tumor model, tumor antigen-specific $CD8^+$ CTL responses were necessary and sufficient to kill the tumors (21, 22). Using this model, we have demonstrated that treatment of tumor-bearing mice with the necrotic bodies of tumor cells admixed with poly(I:C) significantly inhibited the tumor growth. More importantly, the immunostimulatory activity of the poly(I:C) was more potent when it was pre-loaded inside the dead tumor cells than when simply admixed with them. These findings are expected to be pertinent in the development of human tumor vaccines. Dead tumor cells with a known antigenic profile could be pre-loaded *in vitro* with synthetic dsRNA and injected into patients having tumors with a similar antigenic profile to induce an immune response towards the tumor cells (23).

MATERIALS AND METHODS

Materials

Poly(I:C) (or pI:C) was from GE Healthcare (Piscataway, NJ). It was a duplex polymer composed of a poly (I) strand (152–539 b) annealed to a poly (C) strand (319–1,305 b). The endotoxin level in the poly(I:C) solution (2 mg/ml in RNase- and endotoxin-free solution) was determined to be very low (2.4 ± 0.3 EU/ml) using a Limulus lysate assay (Associates of Cape Cod, Inc. East Falmouth, MA). The TC-1 cell line was engineered by Dr. T. C. Wu at the Johns Hopkins University. The cell line was generated by transducing C57BL/6 mouse lung cells with HPV 16 E6 and E7 oncogenes and an activated h-*ras* gene (24). The 24JK tumor cell line was generated by Dr. P. Hwu in the National Cancer Institute. It was a tumor cell line derived from the MCA102 fibrosarcoma, which was generated from C57BL/6 mice (25). Cells were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 U/mL of penicillin (Sigma-Aldrich, St. Louis, MO), and 100 μg/ml of streptomycin (Invitrogen). The HPV 16 $E7_{49–57}$ peptide (RAHYNIVTF) was synthesized and purified (>80%) by the GenScript Corp. (Piscataway, NJ). All lipids used to prepare the liposomes were from Avanti Polar Lipids, Inc. (Alabaster, AL). The CytoTox 96 Non-Radioactive Cytotoxicity Assay kit was from Promega Corporation (Madison, WI).

Animal Studies

Female C57BL/6 mice (6–8 weeks, Simonsen's Lab, Gilroy, CA) were used in all animal studies. National Institutes of Health (NIH) guidelines for care and use of laboratory animals were observed. TC-1 tumor cells (5×10^5) were s.c. injected in the flank of mice on day 0 to establish tumors. On days 3, 6, 9, and 12, mice were immunized by s.c. injection of different vaccine formulations, which included (a) phosphate buffered saline (PBS, 10 mM, pH 7.4, 0.2 ml) as a negative control, (b) the necrotic bodies of TC-1 cells alone (5×10^7) in PBS, (c) poly(I:C) alone (50 μg/mouse) in PBS, (d) the necrotic bodies of TC-1 cells (5×10^7) admixed with poly(I:C) (50 μg) in PBS, (e) the necrotic bodies of TC-1 cells (7×10^6/mouse) pre-transfected with poly(I:C) (50 μg/mouse). As a positive control, mice were also injected with poly(I:C) admixed with the $E7_{49–57}$ peptide (20 μg/mouse) in 5% dextrose (26). The necrotic bodies of tumor cells were prepared using the simple repeated freezing-and-thawing technique (27). Briefly, cells were gently spun down at 800–1,000 rpm using an Allegra X-12 bench-top centrifuge (Beckman-Coulter, Inc., Fullerton, CA), washed with cold PBS for three times, re-suspended into PBS, and then repeatedly frozen-and-thawed in −80°C for three times. Tumor size was measured using a caliper and reported by multiplying the largest dimension and the square of the second largest dimension of the tumors.

Transfection of TC-1 Cells with Poly(I:C)

Poly(I:C) was transfected into TC-1 cells using a previously reported cationic liposome formulation that had a final poly(I:C) concentration of 2 mg/ml (28). The poly(I:C) was 100% associated with the liposomes by electrostatic interaction (28). In order to quantify the liposomes taken up by the cells, the liposomes were labeled with 0.8% (molar ratio to the total lipids in the liposome) of diethylenetriaminepentaacetic acid α,ω-bis(8-stearoylamido-3,6-dioxaoctylamide) gadolinium (Sigma), and the final gadolinium concentration in the poly(I:C)-liposome preparation was 21 μg/ml. For the uptake study, TC-1 cells (1×10^6 in 0.5 ml) were seeded into 48-well plates. After overnight incubation, 0.2 ml of the poly(I:C)-liposome formulation was added into the wells, and the cells

were incubated in a 37°C CO_2 incubator for one more hour. The cells were washed with cold PBS (pH 7.4, 10 mM) for three times and lysed using a cell lysis buffer from Promega. The amount of the gadolinium recovered from the cells was determined using an inductively coupled plasma optical emission spectrometer (ICP-OES, Teledyne Leeman Labs, Hudson, NH) at 342.247 nm. The amount of gadolinium was then converted to the amount of poly(I:C) recovered from the cells.

To determine whether the transfection of the cells with the poly(I:C) was endocytosis-mediated, TC-1 cells were incubated with poly(I:C) in the liposome-based carrier either at 4 or at 37°C but in the presence of cytochalasin B (35 μmol, Sigma) for 2 h, and the amount of poly(I:C) associated with the TC-1 cells was determined. These data also allowed us to determine the proportion of poly(I:C) that was inside the TC-1 cells (~90%) (vs. the proportion that simply bound on the cell surface).

Splenocyte Proliferation Assay

Splenocyte suspension was prepared as previously described (26) and cultured (1×10^5 cells in 300 μl) in RPMI 1640 medium supplemented with 10% FBS, 50 U/ml penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mM non-essential amino acids, and 100 μg/ml of $E7_{49–57}$ for 5 days. Live cells were quantified using an MTT test kit (Sigma-Aldrich). The proliferation index was reported as the OD_{570}–OD_{630} of cells stimulated with $E7_{49–57}$ peptide over that without re-stimulation.

In Vitro CTL Assay

CTL activity was measured using the CytoTox® 96 NonRadioactive Cytotoxicity Assay Kit. Mice ($n = 5$) were immunized subcutaneously on days 0 and 7. On day 20, they were euthanized, and splenocytes were prepared and cultured in RPMI 1640 medium with 10% FBS, 50 U/ml penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mM nonessential amino acids, 40 units/ml of IL-2, and TC-1 cell lysate for 5 days. The TC-1 cell lysate was prepared by freezing-and-thawing TC-1 cells suspended in PBS and then forcing them through a needle. Effector cells were plated into 96-well plates at various effector:target (E:T) ratios (50:1, 25:1, and 12.5:1). Targets were TC-1 cells or 24JK cells. Before mixed with effectors, the targets were washed twice and re-suspended at a density of 2×10^5 cells per ml. The lysis reaction was carried out at 37°C for 4 h. The plates were centrifuged, and 100 μl of the culture medium was carefully removed from each well and assayed for lactate dehydrogenase (LDH) activity. The extent of specific lysis was calculated with the absorbance at 490 nm following the manufacturer's suggestion.

Statistical Analysis

Except where mentioned, statistical analyses were completed by performing ANOVA followed by pair-wise comparisons using Fisher's protected least significant difference procedure. The mouse survival curves were analyzed by completing a Kaplan–Meier survival curve analysis using the Prism 4 from the GraphPad Software, Inc. (San Diego, CA). A p value of ≤ 0.05 (two-tail) was considered to be significant.

RESULTS

The Necrotic Bodies of Tumor Cells, when Admixed with Poly(I:C), Significantly Inhibited Tumor Growth

To test whether poly(I:C) can help dead tumor cells to induce specific anti-tumor immune responses and thus inhibit tumor growth, we used the TC-1 cells in C57BL/6 mice as a model. Live TC-1 cells (5×10^5/mouse) were injected (s.c.) in the flank of C57BL/6 mice on day 0. Tumors were measurable on day 3. The mice were then divided into four groups ($n = 5$–8) and injected (s.c., distal to the tumor site) on days 3, 6, 9, and 12 with different vaccine formulations, and the tumor growth was monitored. As shown in Fig. 1a, although tumors in mice injected with PBS alone or the dead TC-1 cells alone grew rapidly, tumors in mice treated with the necrotic bodies of TC-1 cells admixed with poly(I:C) grew significantly slower (Fig. 1a). As we have previously shown, poly(I:C) admixed with the $E7_{49–57}$ peptide caused total regression of the tumors (Fig. 1a). The splenocytes isolated from mice treated with the necrotic bodies of TC-1 cells admixed with poly(I:C) also proliferated significantly when stimulated *in vitro* with the $E7_{49–57}$ peptide (Fig. 1b).

The Anti-tumor Response Induced by the Necrotic Bodies of Tumor Cells Admixed with Poly(I:C) was not Simply Due to the Non-specific Anti-tumor Activity of the Poly(I:C)

Because poly(I:C) alone was known to be anti-proliferative, an experiment was carried out to understand whether the anti-tumor activity induced by the necrotic bodies of TC-1 cells admixed with poly(I:C) was simply due to the non-specific tumor-inhibitory activities of the poly(I:C). Tumor-bearing mice ($n = 7$) were s.c. injected with either poly(I:C) alone or poly(I:C) admixed with the necrotic bodies of TC-1 cells, and the tumor growth was monitored. As expected, poly(I:C) alone slowed down the growth of the tumors, compared to tumor-bearing mice left untreated (Fig. 2a). However, the anti-tumor activity of the poly(I:C) alone was not as strong as that when it was dosed together with the necrotic bodies of TC-1 cells (Fig. 2a). In fact, 27 days after the injection of the tumor cells, only two of the seven mice treated with poly(I:C) alone were alive, while six of the seven mice treated with poly(I:C) admixed with the necrotic bodies of TC-1 cells were still alive ($p = 0.02$, Kaplan–Meier survival Analysis, Fig. 2 insert). As expected, none of the tumor-bearing mice that were left untreated survived for more than 24 days after the injection of live TC-1 tumor cells (data not shown).

The Immunostimulatory Activity of Poly(I:C) was more Potent when Pre-loaded Inside the Dead Tumor Cells than when Simply Admixed with the Dead Tumor Cells

We then tested whether the necrotic bodies of tumor cells pre-loaded with poly(I:C) would induce a stronger anti-

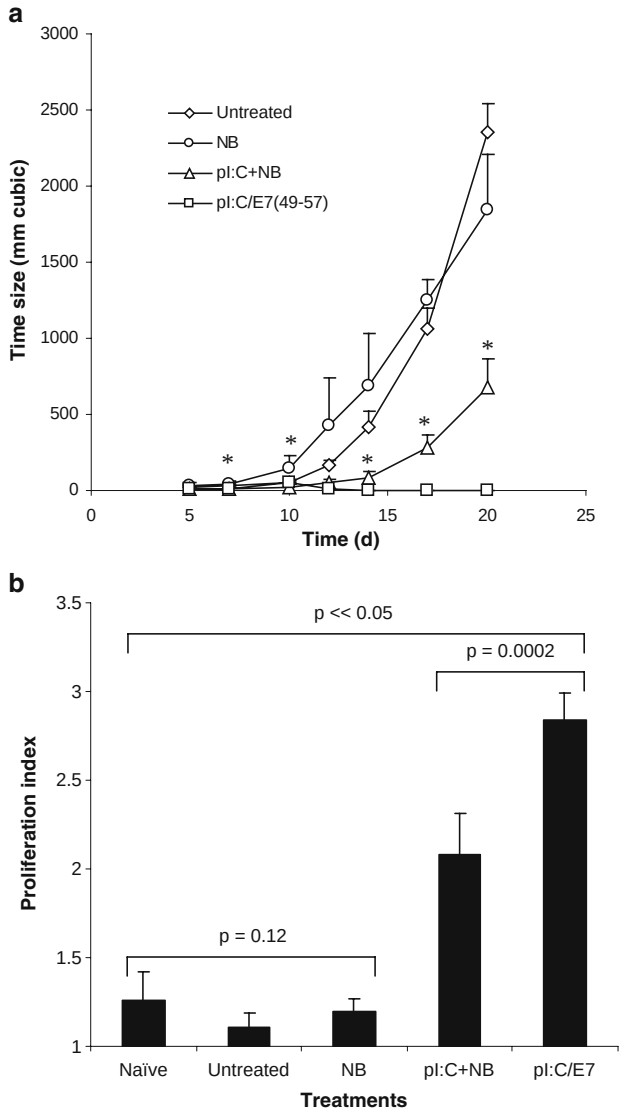

Fig. 1. Tumor cell necrotic bodies, when admixed with poly(I:C), significantly inhibited tumor growth. **a** Tumor growth kinetics. C57BL/6 mice were s.c. grafted with TC-1 tumor cells (5×10^5) on day 0. On days 3, 6, 9, and 12, mice were left untreated or injected (s.c., distal to the tumor site) with the necrotic bodies of TC-1 cells (NB, 5×10^7/mouse), or the NB admixed with pI:C (50 μg, pI:C+NB). As a positive control, mice were injected with poly(I:C) admixed with $E7_{49-57}$ (pI:C/E7). The *asterisks* (*) indicated that on days 7, 10, 14, 17, and 20, the values of pI:C/NB were different from that of the Untreated ($p < 0.05$). In all the days, the values of the NB were comparable to that of the Untreated. Data shown were mean ± S.E.M. This experiment was repeated twice, and similar results were obtained. The number of mice in each group ranged from 5 to 8. (**b**). The necrotic bodies of tumor cells admixed with poly(I:C) induced a splenocyte proliferative response. Mice were sacrificed 20 days after the tumor injection to measure the proliferation of splenocytes after *in vitro* re-stimulation with the $E7_{49-57}$ peptide. Data shown were mean ± S.D. ($n = 5$). The values of the pI:C+NB and pI:C/E7(49–57) were significantly different from each other ($p = 0.0002$) and were both greater than that of the others.

tumor response than when the necrotic bodies were simply admixed with the poly(I:C). To introduce poly(I:C) into the necrotic bodies of tumor cells, we transfected TC-1 cells with poly(I:C) using a previously reported cationic liposome-based poly(I:C) carrier (28, 29). The poly(I:C) bound to the liposomes based on electrostatic interaction. Thus, it was reported that all the poly(I:C) molecules were associated with the liposomes (28, 29). As shown in Fig. 3, co-incubation of TC-1 cells with the liposome-based poly(I:C) carrier introduced the poly(I:C) into the TC-1 cells. The incubation of them in the presence of cytochalasin B as an endocytosis inhibitor or at 4°C without cytochalasin B significantly decreased the amount of poly(I:C) associated with the TC-1 cells (Fig. 3). This observation also indicated that the cell uptake was endocytosis-mediated. Data from a flow cytometry analysis using FITC-labeled liposomes also confirmed the uptake of poly(I:C) by the TC-1 cells (data not shown). After the TC-1 cells were incubated with poly(I:C) in the liposomal carrier for 1 h at 37°C, the cells were washed three times, and an estimated 7 μg of poly(I:C) was introduced into 1×10^6 TC-1 cells. The TC-1 cells were then frozen-and-thawed to prepare TC-1 cell necrotic bodies. These necrotic bodies with poly(I:C) inside were used to inject mice having pre-established TC-1 tumors. As a control, TC-1 cells that were incubated with the same liposomes that did not contain any poly(I:C) were killed by freezing-and-thawing to prepare poly(I:C)-free TC-1 cell necrotic bodies, which were then mixed with poly(I:C) to inject mice.

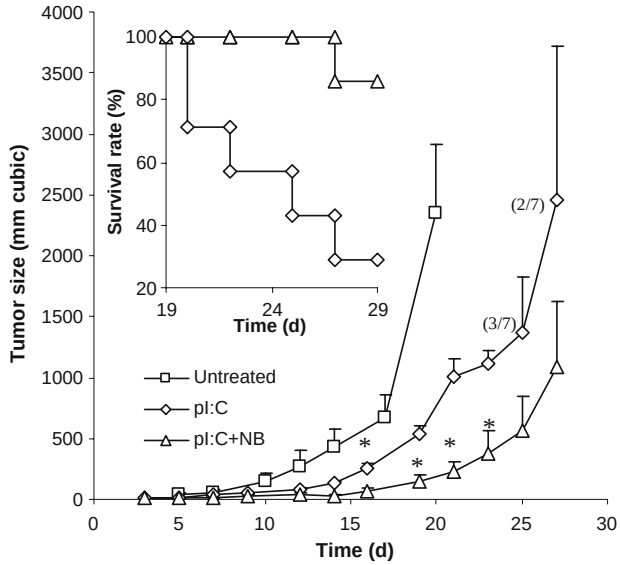

Fig. 2. The anti-tumor response induced by the necrotic bodies of tumor cells admixed with poly(I:C) was not simply due to the tumor-inhibitory activity of the poly(I:C). C57BL/6 mice ($n = 7$) were s.c. seeded with TC-1 tumor cells (5×10^5) on day 0. On days 3, 6, 9, and 12, mice were left untreated or injected (s.c.) with pI:C alone (50 μg) or pI:C admixed with the necrotic bodies of TC-1 cells (5×10^7/mouse). Data shown were mean ± S.E.M. ($n = 7$). Asterisks indicate that on days 17, 19, 21, and 23, the values of pI:C and pI:C+NB were different from each other ($p < 0.05$). This experiment was repeated twice and similar results were obtained. Statistical analysis was not done for the values on days 25 and 27 because the numbers of live mice in the group treated with poly(I:C) alone were only 3 (3/7) and 2 (2/7), respectively. Insert: Mouse survival curves. Kaplan–Meier survival curve analysis indicated that the two curves were significantly different from each other ($p = 0.02$). None of the tumor-bearing mice that were left untreated survived for more than 24 days.

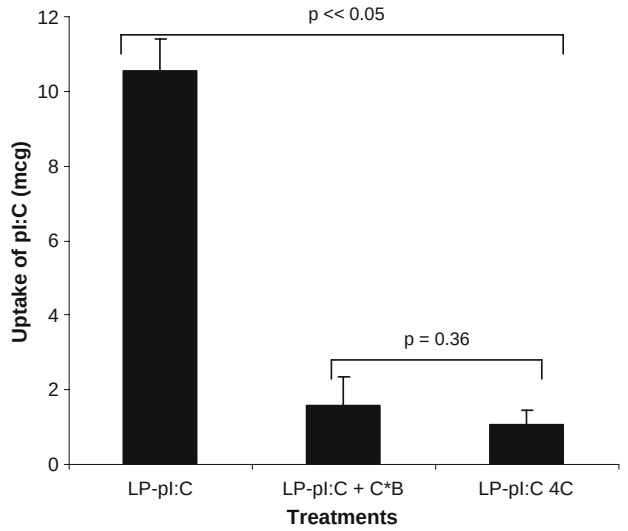

Fig. 3. Transfection of poly(I:C) into TC-1 cells. TC-1 cells (1×10^6) were incubated with the poly(I:C)-containing cationic liposome formulation (LP-pI:C) at 37°C for 2 h. The cells were immediately washed three times, and the amount of poly(I:C) recovered from the cells was estimated. As controls, the incubation was carried out at 4°C (LP-pI:C 4C) or at 37°C but in the presence of an endocytosis inhibitor (cytochalasin B, 35 mM, LP-pI:C C*B). Data shown were mean ± S.D. ($n = 6$). The value of LP-pI:C was significantly different from that of the other two ($p < 0.05$). The values of LP-pI:C C*B and LP-pI:C 4C were not different from each other ($p = 0.36$).

As shown in Fig. 4, the necrotic bodies of TC-1 cells (7×10^6/mouse) with poly(I:C) (50 μg/mouse) inside them were more effective than the same number of the necrotic bodies (7×10^6/mouse) admixed with the same amount of poly(I:C) in inhibiting the growth of the TC-1 tumors. For example, 38 days after the injection of live TC-1 tumor cells, 80% of the mice treated with the necrotic bodies of TC-1 cells pre-loaded with poly(I:C) were still alive, while all mice treated with the necrotic bodies of TC-1 cells admixed with poly(I:C) died (Fig. 4b, $p = 0.02$, Kaplan–Meier survival analysis). Again, all tumor-bearing mice that were left untreated died in about 20 days after the injection of the live TC-1 tumor cells.

The Necrotic Bodies of Tumor Cells Pre-loaded with Poly(I:C) Induced a Stronger Specific CTL Response

The data presented above suggested that specific tumor-killing CTL responses were induced by the necrotic bodies of TC-1 cells with the help of poly(I:C). To confirm this, an *in vitro* CTL assay was carried out. Healthy tumor-free mice ($n = 5$) were immunized on days 0 and 7 with the necrotic bodies of TC-1 cells pre-loaded with poly(I:C) or with the necrotic bodies simply admixed with poly(I:C). On day 20, mice were sacrificed, and their splenocytes were used to evaluate the CTL activity against TC-1 cells or 24JK cells (as a non-specific target). As shown in Fig. 5, although no significant cytotoxicity was detected against the 24JK cells, a strong CTL activity against the TC-1 cells was induced by the necrotic bodies of TC-1 cells pre-loaded with poly(I:C). TC-1 cell-specific CTL activity, although weaker, was also detected in mice immunized with the necrotic bodies of TC-1 cells admixed with poly(I:C) (Fig. 5), suggesting that the ability of the necrotic bodies of tumor cells with poly(I:C) inside to induce tumor-specific CTL responses was responsible for their ability to inhibit tumor growth.

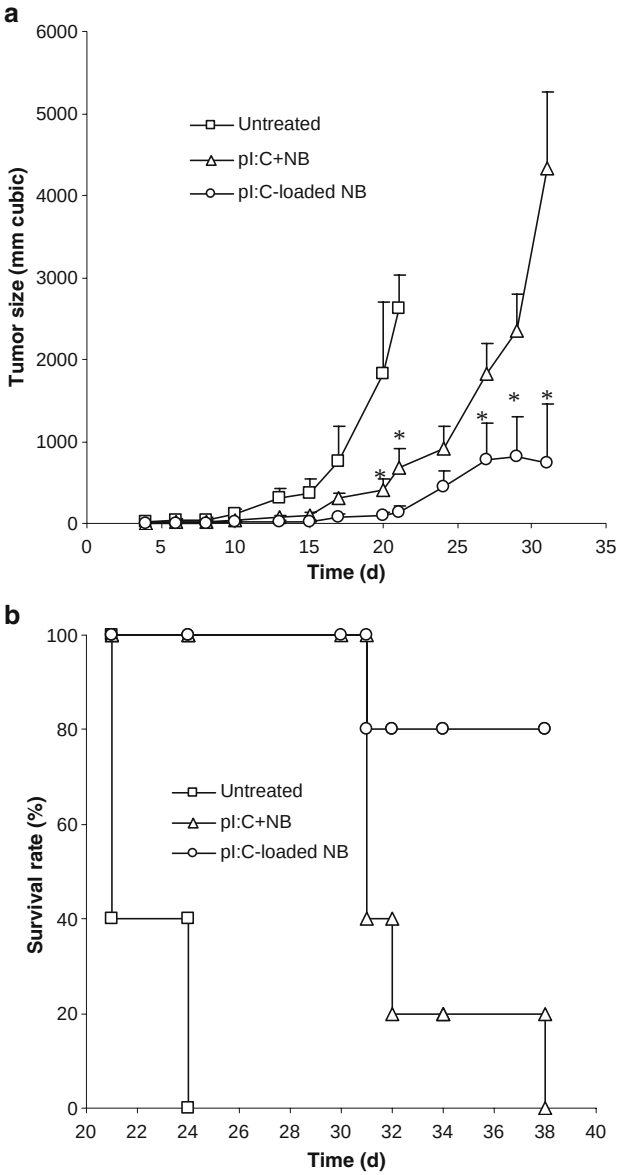

Fig. 4. The necrotic bodies of tumor cells loaded with poly(I:C) induced a stronger anti-tumor response than when the necrotic bodies were admixed with poly(I:C). C57BL/6 mice ($n = 5$) were s.c. injected with TC-1 tumor cells (5×10^5) on day 0. On days 3, 6, 9, and 12, mice were left untreated, injected with the necrotic bodies of TC-1 cells (7×10^6/mouse) pre-loaded with poly(I:C) (50 μg) (pI:C-loaded NB), or with the necrotic bodies of TC-1 cells (7×10^6/mouse) simply admixed with poly(I:C) (50 μg/mouse, pI:C+NB, which was different from that in previous experiments). **a** Tumor growth kinetics. Data shown were mean ± S.E.M. ($n = 5$). *Asterisks* indicate that on those specified days, the sizes of tumors in mice treated with pI:C+NB and in mice treated with pI:C-loaded NB were significantly different from each other ($p < 0.05$). This experiment was repeated independently twice. **b** Mouse survival curves. Kaplan–Meier survival curve analysis revealed significant differences between the survival curve of mice treated with pI:C+NB and that of mice treated with the pI:C-loaded NB ($p = 0.016$). In addition, both curves of the treated groups were significantly different from that of the untreated group ($p = 0.023$).

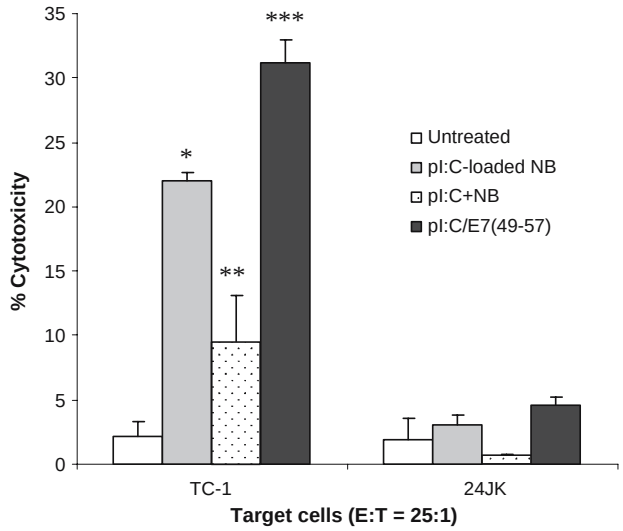

Fig. 5. Immunization of mice with the necrotic bodies of TC-1 cells pre-loaded with poly(I:C) induced a stronger specific CTL response. Tumor-free C57BL/6 mice ($n = 5$) were left untreated or immunized once a week for 2 weeks with the necrotic bodies of TC-1 cells (7×10^6/mouse) admixed with poly(I:C) (50 μg/mouse, pI:C+NB) or with the necrotic bodies of TC-1 cells (7×10^6) pre-loaded with poly(I:C) (50 μg) (pI:C-loaded NB). As a positive control, mice were immunized with poly(I:C) admixed with $E7_{49–57}$ peptide. Two weeks after the second immunization. Their splenocytes were incubated in the presence of TC-1 cell lysate for 5 days, and their ability to kill TC-1 cell (target) and 24JK cells (non-target) was determined. Data shown were mean ± S.D. ($n = 5$) at the effector to target ratio of 25:1. The data at the E:T ratios of 50:1 and 12.5:1 were trended similarly. *Asterisks* indicate that when the TC-1 cells were used as the target, the values of pI:C+NB, pI:C-loaded NB, and pI:C/$E7_{49–57}$ were different from one another and were all larger than that of the untreated control (pI:C/$E7_{49–57}$ vs. pI:C-loaded NB, $p = 0.001$, pI:C/$E7_{49–57}$ vs. pI:C+NB, $p = 0.0008$, pI:C-loaded NB vs. pI:C+NB, $p = 0.004$).

DISCUSSIONS

In the present study, we have evaluated the ability of a synthetic dsRNA to promote dead tumor cells to cross-prime specific anti-tumor immune responses. We have found that although injection of the necrotic bodies of tumor cells alone was unable to exert any significant anti-tumor activity, treatment with the tumor cell necrotic bodies admixed with a synthetic dsRNA significantly inhibited the tumor growth by an apparent immune-mediated mechanism. More importantly, we have found that the immunostimulatory activity of the dsRNA was more potent when pre-loaded inside the necrotic bodies of tumor cells than simply mixed with dead tumor cells. The dead tumor cell-based vaccines were not as effective as the $E7_{49–57}$ peptide-based vaccine. Taken together, these findings suggested the feasibility of devising new tumor vaccines by mimicking the promotion of the induction of immune responses against viral antigens expressed in infected cells by viral dsRNA. The dead tumor cells with poly(I:C) pre-loaded inside resembled viral infected cells containing viral dsRNA.

Although many tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs) that may be used to induce specific anti-tumor immune responses have been identified, immunization using whole tumor cell-based vaccines still represents an attractive and viable strategy. The potential advantages include the presentation and processing of multiple antigens and the applicability across a variety of patients. Moreover, the use of whole tumor cells is expected to provide both MHC class I and class II epitopes leading to a diverse immune responses including both CTL and CD4 activation (30). However, for a whole cell-based tumor vaccine to be effective, it requires the uptake of tumor cells by professional APCs and the activation of the APCs by proper molecules that can promote cross-priming. Viral dsRNA, a known pathogen-associated molecular pattern molecule, is used by host cells to detect the presence of viral infection. Moreover, similar to anti-tumor immune responses, an effective anti-viral immune response usually requires the activation of specific $CD8^+$ T cells in order to kill the virally infected host cells. Thus, we reasoned that one can design a tumor vaccine by mimicking the mechanisms used by viral hosts to raise immune responses against viruses. In fact, virus-infected tumor cells or the lysate of virus-infected tumor cells have been used to treat tumors before (31–33).

Viral antigens may be presented by APCs to T cells through at least three ways to prime anti-viral T cell responses. For those viruses that infect professional APCs directly, the virally infected APCs can process the viral antigens and present them directly to T cells. As mentioned earlier, for viruses that infect both APCs and non-APCs or only infect non-APCs, viral dsRNA can promote the cross-presentation of viral antigens in non-APCs through two recently identified mechanisms, the induction of type I IFNs (17) or the direct dsRNA-TLR3 interaction independent of the contribution from type I IFNs (20). Of course, during a natural viral infection, all these mechanisms are likely to synergize to promote the maturation of APCs and maximize the priming of virus-specific T cell responses (23).

The induction of specific T cell responses by dead or dying tumor cells involves the cross-presentation of antigens in tumor cells by professional APCs to T cells, which is similar to the cross-presentation of viral antigens in virally infected non-APCs by professional APCs to T cells. Some synthetic dsRNA molecules, such as the poly(I:C), have previously been shown to have activities similar to that of viral dsRNA (1, 8, 9, 34). We have found that immunization of tumor-bearing mice with the necrotic bodies of tumor cells admixed with poly(I:C) significantly inhibited the growth of model tumors (Figs. 1, and 2), very likely through an adaptive immune response-mediated mechanism. The necrotic bodies alone failed to exert any tumor inhibitory activity (Fig. 1a), likely because DCs may have taken up them, but without the provision of proper DC activation signals, the DCs were unable to cross-prime immune response against the tumor cells. As expected, treatment with poly(I:C) alone generated some tumor inhibitory activities (Fig. 2). Although poly(I:C) was known to directly induce tumor cells to undergo apoptosis, this mechanism was unlikely to have contributed significantly in the present study because the poly(I:C) needs to be in the tumor cells in order to interact with intracellular molecules, such as the dsRNA-dependent protein kinase (PKR) and 2,5-oligo A synthetase (34–36) to initiate pro-apoptotic signal cascades. We injected the poly(I:C) distal to the tumor site. Thus, we speculated that the non-specific

tumor inhibitory activity of the poly(I:C) alone was mainly due to its ability to activate the innate immunity (e.g., the activation of NK cells) (11, 12). In addition, the poly(I:C) might have induced the production of anti-proliferative cytokines such as type I IFNs (37).

When the necrotic bodies of tumor cells and the poly(I:C) were mixed together and injected into tumor-bearing mice, it was possible that both type I IFN-dependent (17) and the mechanism independent of the contribution of type I IFNs (20) had synergized to promote the cross-presentation of dead tumor cells. However, we speculated that the mechanism independent of the contribution of type I IFNs had played a rather limited role. Instead, it was very likely for cells in the close vicinity of the injection site to produce type I IFNs upon contact with poly(I:C), and the type I IFNs then helped DCs to cross-present the necrotic bodies to T cells (17).

The most important finding in the present study is that immunization of mice with dead tumor cells pre-loaded *in vitro* with poly(I:C) induced a significantly stronger specific CTL response and exerted a stronger tumor inhibitory effect, when compared to immunization with the same number of necrotic bodies physically mixed with poly(I:C) (Figs. 4, and 5). This resembled the promotion of the cross-presentation of viral antigens in infected host cells by viral dsRNA inside the cells to prime T cell responses against the viral antigen (20). Very likely, when DCs picked up the injected tumor cell necrotic bodies, the poly(I:C) inside the dead cells interacted directly with TLR3 within the DCs and activated them. The activated DCs then processed the engulfed necrotic bodies and presented tumor antigens to T cells to prime specific anti-tumor immune responses. It needs to be pointed out that it was very unlikely that the liposome-based carrier per se was responsible for the different anti-tumor activities between the necrotic bodies of tumor cells pre-loaded with poly(I:C) and the necrotic bodies simply mixed with poly(I:C), because the necrotic bodies that were mixed with poly(I:C) were also treated with the same liposome carrier, free of poly(I:C). Moreover, the possibility that some secretory molecules or factors induced by the intracellular poly(I:C) were responsible for the enhanced anti-tumor immune response was also low, because the TC-1 cells were immediately washed three times after the 1 h incubation and then immediately killed.

Finally, our finding is in agreement with data from a recent study, showing that immunization of mice with irradiated Vera cells loaded with poly(I:C) and OVA protein induced a strikingly strong CTL immunity against the OVA protein (20). However, the authors did not show whether the Vera cells loaded with poly(I:C) were more effective than the Vera cells loaded with OVA and mixed with poly(I:C) in inducing OVA-specific CTL immune responses. Instead, they only reported that *in vitro*, the Vera cells loaded with poly(I:C) were 20–50-fold more effective in activating $CD8\alpha^+$ DCs than the poly(I:C) alone in solution (20). The superior ability of the intracellular dsRNA to activate DCs likely has contributed to the stronger immune responses induced by the necrotic bodies of tumor cells pre-loaded with poly(I:C).

In conclusion, we have found that immunization of tumor-bearing mice with the necrotic bodies of tumor cells admixed with poly(I:C) significantly inhibited the tumor growth. More importantly, immunization with the necrotic bodies having poly(I:C) pre-loaded inside led to a significantly stronger anti-tumor response than when the necrotic bodies were simply admixed with the poly(I:C), apparently through a $CD8^+$ CTL response-mediated mechanism. These findings are expected to be clinically relevant. We are currently test this immunization approach using model melanoma and prostate tumor cells in mice. If proven feasible in other tumors, this approach of immunization with dead tumor cells pre-loaded with synthetic dsRNA *in vitro* is expected to be applicable in clinical trials, in which autologous or allogeneic human tumor cells can be transfected with synthetic dsRNA *in vitro*, induced into necrotic bodies or apoptotic bodies by irradiation, and then used to immunize patients (23). Moreover, using the necrotic bodies or apoptotic bodies of tumor cells that have been modified to produce cytokines such as GM-CSF may further enhance the anti-tumor immune responses induced. Immunization with irradiated tumor cells that are infected *in vitro* with a detoxified virus that produces dsRNA and encodes genes such as GM-CSF should also be efficacious.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the Elsa U. Pardee Foundation and an OSU College of Pharmacy Translational Research Grant to Z.C. We would like to thank Malcolm Lowry for discussions.

REFERENCES

1. H. E. Kaufman, E. D. Ellison, and S. R. Waltman. Double-stranded RNA, an interferon inducer, in herpes simplex keratitis. *Am. J. Ophthalmol.* **68**:486–491 (1969).
2. G. R. Foster, and N. B. Finter. Are all type I human interferons equivalent? *J. Viral Hepatitis* **5**:143–152 (1998).
3. M. Chawla-Sarkar, D. J. Lindner, Y. F. Liu, B. R. Williams, G. C. Sen, R. H. Silverman, and E. C. Borden. Apoptosis and interferons: role of interferon-stimulated genes as mediators of apoptosis. *Apoptosis* **8**:237–249 (2003).
4. L. G. Guidotti and F. V. Chisari. Noncytolytic control of viral infections by the innate and adaptive immune response. *Annu. Rev. Immunol.* **19**:65–91 (2001).
5. G. R. Stark, I. M. Kerr, B. R. Williams, R. H. Silverman, and R. D. Schreiber. How cells respond to interferons. *Annu. Rev. Biochem.* **67**:227–264 (1998).
6. Y. L. Yang, L. F. Reis, J. Pavlovic, A. Aguzzi, R. Schafer, A. Kumar, B. R. Williams, M. Aguet, and C. Weissmann. Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase. *EMBO J.* **14**:6095–6106 (1995).
7. R. Herman and S. Baron. Immunologic-mediated protection of *Trypanosoma congolense*-infected mice by polyribonucleotides. *J. Protozool.* **18**:661–666 (1971).
8. J. H. Park and S. Baron. Herpetic keratoconjunctivitis: therapy with synthetic double-stranded RNA. *Science* **162**:811–813 (1968).
9. L. Alexopoulou, A. C. Holt, R. Medzhitov, and R. A. Flavell. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* **413**:732–738 (2001).
10. R. M. Verdijk, T. Mutis, B. Esendam, J. Kamp, C. J. Melief, A. Brand, and E. Goulmy. Polyriboinosinic polyribocytidylic acid (poly(I:C)) induces stable maturation of functionally active human dendritic cells. *J. Immunol.* **163**:57–61 (1999).
11. K. N. Schmidt, B. Leung, M. Kwong, K. A. Zarember, S. Satyal, T. A. Navas, F. Wang, and P. J. Godowski. APC-independent activation of NK cells by the Toll-like receptor 3 agonist double-stranded RNA. *J. Immunol.* **172**:138–143 (2004).

12. S. Sivori, M. Falco, M. Della Chiesa, S. Carlomagno, M. Vitale, L. Moretta, and A. Moretta. CpG and double-stranded RNA trigger human NK cells by Toll-like receptors: induction of cytokine release and cytotoxicity against tumors and dendritic cells. *Proc. Natl. Acad. Sci. U. S. A.* **101**:10116–10121 (2004).
13. A. E. Gelman, J. Zhang, Y. Choi, and L. A. Turka. Toll-like receptor ligands directly promote activated CD4+ T cell survival. *J. Immunol.* **172**:6065–6073 (2004).
14. M. L. Salem, A. N. Kadima, D. J. Cole, and W. E. Gillanders. Defining the antigen-specific T-cell response to vaccination and poly(I:C)/TLR3 signaling: evidence of enhanced primary and memory CD8 T-cell responses and antitumor immunity. *J. Immunother.* **28**:220–228 (2005).
15. Z. R. Cui and F. Qiu. CD4+ T helper cell response is required for memory in CD8+ T lymphocytes induced by a poly(I:C)-adjuvanted MHC I-restricted epitope. *J. Immunother.* **30**:180–189 (2007).
16. F. Henry, O. Boisteau, L. Bretaudeau, B. Lieubeau, K. Meflah, and M. Gregoire. Antigen-presenting cells that phagocytose apoptotic tumor-derived cells are potent tumor vaccines. *Cancer Res.* **59**:3329–3332 (1999).
17. A. Le Bon, N. Etchart, C. Rossmann, M. Ashton, S. Hou, D. Gewert, P. Borrow, and D. F. Tough. Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon. *Nat. Immunol.* **4**:1009–1015 (2003).
18. J. Frappa, P. Trotemann, Y. Beaudry, and R. Fontanges. Production of IFN beta with human fibroblasts in monolayer cultures. Comparative studies of induction with virus (NDV) and synthetic polynucleotides (poly I–poly C). *Dev. Biol. Stand.* **50**:185–192 (1981).
19. P. B. Sehgal and A. D. Sagar. Heterogeneity of poly(I) x poly(C)-induced human fibroblast interferon mRNA species. *Nature* **288**:95–97 (1980).
20. O. Schulz, S. S. Diebold, M. Chen, T. I. Naslund, M. A. Nolte, L. Alexopoulou, Y. T. Azuma, R. A. Flavell, P. Liljestrom, and C. Reis e Sousa. Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature* **433**:887–892 (2005).
21. G. J. Fernando, V. Khammanivong, G. R. Leggatt, W. J. Liu, and I. H. Frazer. The number of long-lasting functional memory CD8+ T cells generated depends on the nature of the initial nonspecific stimulation. *Eur. J. Immunol.* **32**:1541–1549 (2002).
22. G. J. Fernando, T. J. Stewart, R. W. Tindle, and I. H. Frazer. Th2-type CD4+ cells neither enhance nor suppress antitumor CTL activity in a mouse tumor model. *J. Immunol.* **161**:2421–2427 (1998).
23. M. Salio and V. Cerundolo. Viral immunity: cross-priming with the help of TLR3. *Curr. Biol.* **15**:R336–R339 (2005).
24. K. Y. Lin, F. G. Guarnieri, K. F. Staveley-O'Carroll, H. I. Levitsky, J. T. August, D. M. Pardoll, and T. C. Wu. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. *Cancer Res.* **56**:21–26 (1996).
25. P. Hwu, J. C. Yang, R. Cowherd, J. Treisman, G. E. Shafer, Z. Eshhar, and S. A. Rosenberg. *In vivo* antitumor activity of T cells redirected with chimeric antibody/T-cell receptor genes. *Cancer Res.* **55**:3369–3373 (1995).
26. Z. Cui and F. Qiu. Synthetic double-stranded RNA poly(I:C) as a potent peptide vaccine adjuvant: therapeutic activity against human cervical cancer in a rodent model. *Cancer Immunol. Immunother.* **55**:1267–1279 (2006).
27. N. Fujita, H. Kagamu, H. Yoshizawa, K. Itoh, H. Kuriyama, N. Matsumoto, T. Ishiguro, J. Tanaka, E. Suzuki, H. Hamada, and F. Gejyo. CD40 ligand promotes priming of fully potent anti-tumor CD4(+) T cells in draining lymph nodes in the presence of apoptotic tumor cells. *J. Immunol.* **167**:5678–5688 (2001).
28. H. B. Levy, G. Baer, S. Baron, C. E. Buckler, C. J. Gibbs, M. J. Iadarola, W. T. London, and J. Rice. A modified polyriboinosinic–polyribocytidylic acid complex that induces interferon in primates. *J. Infect. Dis.* **132**:434–439 (1975).
29. W. E. Houston, C. L. Crabbs, E. L. Stephen, and H. B. Levy. Modified polyriboinosinic–polyribocytidylic acid, an immunological adjuvant. *Infect. Immun.* **14**:318–319 (1976).
30. A. Michael, G. Ball, N. Quatan, F. Wushishi, N. Russell, J. Whelan, P. Chakraborty, D. Leader, M. Whelan, and H. Pandha. Delayed disease progression after allogeneic cell vaccination in hormone-resistant prostate cancer and correlation with immunologic variables. *Clin. Cancer Res.* **11**:4469–4478 (2005).
31. A. A. Green, C. Pratt, R. G. Webster, and K. Smith. Immunotherapy of osteosarcoma patients with virus-modified tumor cells. *Ann. N. Y. Acad. Sci.* **277**:396–411 (1976).
32. P. Hersey, A. S. Coates, W. H. McCarthy, J. F. Thompson, R. W. Sillar, R. McLeod, P. G. Gill, B. J. Coventry, A. McMullen, H. Dillon, and R. J. Simes. Adjuvant immunotherapy of patients with high-risk melanoma using vaccinia viral lysates of melanoma: results of a randomized trial. *J. Clin. Oncol.* **20**:4181–4190 (2002).
33. M. K. Wallack, M. Sivanandham, C. M. Balch, M. M. Urist, K. I. Bland, D. Murray, W. A. Robinson, L. Flaherty, J. M. Richards, A. A. Bartolucci, and L. Rosen. Surgical adjuvant active specific immunotherapy for patients with stage III melanoma: the final analysis of data from a phase III, randomized, double-blind, multicenter vaccinia melanoma oncolysate trial. *J. Am. Coll. Surg.* **187**:69–77, 77–79 (discussion) (1998).
34. G. P. Lampson, A. A. Tytell, A. K. Field, M. M. Nemes, and M. R. Hilleman. Inducers of interferon and host resistance. I. Double-stranded RNA from extracts of Penicillium funiculosum. *Proc. Natl. Acad. Sci. U. S. A.* **58**:782–789 (1967).
35. P. J. Farrell, G. C. Sen, M. F. Dubois, L. Ratner, E. Slattery, and P. Lengyel. Interferon action: two distinct pathways for inhibition of protein synthesis by double-stranded RNA. *Proc. Natl. Acad. Sci. U. S. A.* **75**:5893–5897 (1978).
36. E. F. Meurs, J. Galabru, G. N. Barber, M. G. Katze, and A. G. Hovanessian. Tumor suppressor function of the interferon-induced double-stranded RNA-activated protein kinase. *Proc. Natl. Acad. Sci. U. S. A.* **90**:232–236 (1993).
37. A. Shir, M. Ogris, E. Wagner, and A. Levitzki. EGF receptor-targeted synthetic double-stranded RNA eliminates glioblastoma, breast cancer, and adenocarcinoma tumors in mice. *PloS. Med.* **3**:e6, (2006).