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ORIGINAL ARTICLE

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Synthetic double-stranded RNA poly(I:C) as a potent peptide vaccine adjuvant: therapeutic activity against human cervical cancer in a rodent model

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Abstract Due to the inherent lack of immunogenicity of peptides, it is generally recognized that the strong inflammatory signals that are required to elicit specific responses against peptide-based therapeutic tumor vaccines may not be provided by the standard/conventional vaccine adjuvants. In this study, we have demonstrated dsRNA in the form of synthetic pI:C as a potent adjuvant to enhance the specific anti-tumor immune responses against a peptide-based vaccine. When complexed with an MHC I-restricted minimal peptide epitope derived from the HPV 16 E7 protein, the resulting pI:C/$E7_{49–57}$ molecular complex induced strong $E7_{49–57}$-specific CTL responses that caused significant regressions of model human cervical cancer tumors pre-established in mice. In addition, although the proportion of DCs in tumor-bearing mice was significantly decreased when compared to that in naïve mice, immunization with pI:C/$E7_{49–57}$ restored the proportion of DCs in tumor-bearing mice. Double-stranded RNA may hold a great potential as an adjuvant to induce cellular immune responses for tumor immunotherapy.

Keywords Adjuvant · CTL · DC impairment · Cancer immunotherapy · TLR

Abbreviations CTL: Cytotoxic T lymphocyte · DC: Dendritic cells · LN: Lymph node · HPV: Human papillomavirus · TLR: Toll-like receptor · PAMP: Pathogen-associated molecular pattern · MHC: Major histocompatibility complex · pDC: Plasmocytoid DC · mDC: Myeloid DC · NK: Natural killer · pI:C or Poly(I:C): Polyinosine-polycytidylic acid · CFSE: 5-(and-6-)-carboxylfluorescein diacetate, succinimidayl ester · s.c.: Subcutaneous · HLA: Human lymphocyte antigen · IFN: Interferon · TAA: Tumor-associated antigens · TSA: Tumor-specific antigens · IFA: Incomplete Freund's adjuvant

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Introduction

Although vaccination with peptides derived from TAAs or TSAs appears to be an attractive approach to fight tumors, there are numerous barriers that have to date prevented its success in clinic trials [1]. Among these barriers is the inherent lack of immunogenicity of peptides. It is known that the activation of peptide-specific CTL responses requires the delivery of inflammatory signals to monocytes, lymphocytes, or granulocytes recruited at the site of vaccination; such signals may not be provided by the standard/conventional vaccine adjuvants. An efficient activation signal, however, may be provided by some non-conventional adjuvants, such as the recently identified ligands to TLRs.

Unmethylated CpG motifs with appropriate flanking sequences are well known to have potent adjuvant activity by functioning through the CpG/TLR9 signaling [2]. It has been used in recent years as an adjuvant for tumor immunotherapy. For example, Davila et al. [3, 4] reported that repeated CpG injections (100 μg daily for 9 days) in combination with protein- or peptide-based tumor vaccines were effective in delaying the growth of tumors and in extending the survival of melanoma tumor-bearing mice. Similarly, Miconnet et al. [5] reported that CpG oligos were efficient in inducing specific CTL responses against tumor-derived peptides. When mice transgenic for a chimeric MHC I molecule were immunized with a peptide analog of MART-1/Melan-$A_{26–35}$ in the presence of CpG oligos, a strong systemic CTL response that was able to recognize and kill melanoma cells in vitro was elicited. In a more recent study, it was shown that eight HLA-A2 melanoma patients who received four monthly low-dose of CpG oligos mixed with a Melan-A peptide in IFA all exhibited rapid and strong antigen-specific T cell responses [6].

Although co-administration of CpG oligos with peptide-based tumor vaccines improved the induction of antigen-specific, T cell-mediated immune responses, there are, unfortunately, species-specific differences between the cell type distribution of TLR9 in mice and humans. In humans, TLR9 is only present on pDCs and B cells [7]. Thus, only pDCs and B cells respond to TLR9 ligands. All other effect of TLR9 ligands on human immune cells seem to be indirect and depend on factors produced by pDCs and B cells. The situation in mice is significantly different because, not only pDCs and B cells, but also other DCs, as well as macrophages, express the TLR9, and thus respond directly to TLR9 ligation [7]. Therefore, it is often difficult to translate data obtained in murines into humans.

Unlike TLR9, TLR3 is expressed in mDCs and T cells as well as fibroblasts and other non-immune cells in humans [8]. Thus, its ligands, such as dsRNA [9], could be an excellent unconventional adjuvant to induce cell-mediated immune responses against peptide-based vaccines to fight cancers. In fact, a synthetic dsRNA, pI:C, had been established to have adjuvanticity in the 1960s–1970s [10, 11]; however, it was not until recently that scientists started to understand that it functions through TLR3 [9], and the interest in exploiting it as a vaccine adjuvant was revived again [12–15]. Poly(I:C) had been shown to induce specific humoral and cellular immune responses, including Th1 and CTL responses. It was shown to induce the stable maturation of functionally active human DCs [16]. Stimulation of highly purified NK cells with pI:C had been shown to significantly augment NK cell-mediated cytotoxicity [17, 18]. In vitro, pI:C was shown to directly promote the survival of activated $CD4^+$ T cells [19]. Similarly, it was shown that i.p. injection of pI:C into mice dramatically boosted the number of antigen-specific $CD8^+$ T cells against a s.c. injected peptide [20]. This increase in specific $CD8^+$ T cells was associated with an increase in $CD8^+$ T cell functions and an increased survival ability of the $CD8^+$ cells by inhibiting apoptosis [20], and thus a long-lasting memory response. Moreover, TLR3 was recently shown to promote cross-priming of virus-infected cells to DCs [21].

Cervical cancer is the second most common cancer among women worldwide [22]. HPV is generally understood to be the causative agent of cervical cancers. Nearly 100% of women with cervical cancer have evidence of cervical infection with HPV, typically of the "high risk" types 16, 18, 31, 45 or 58 [23]. The most common types of cervical cancer-associated HPV are types 16 and 18, which account for 50.5% and 13.1%, of all cases, respectively [24]. The early (E) proteins of HPV have proven to be tumorigenic [25, 26]. They are foreign to the host, specific to cervical cancer, and thus, would potentially be excellent antigens for the development of therapeutic cervical cancer vaccines. In fact, data from many studies have shown that immunization with peptide epitopes derived from E7 caused some extent of regressions of experimentally grafted E7-expressing tumors in mice. Several clinical trials have also been completed using peptide epitopes derived from HPV E7 protein [27–32]. For example, in a phase I trial, 18 women with high grade cervical or vulvar intraepithelial neoplasia (CIN) and positive for HPV-16 were given a HLA-A2-restricted peptide ($E7_{12–20}$) in IFA. DC infiltrates were observed in six out of six patients. CTL responses were observed in 10 out of 16 patients. Also, 3 out of 18 patients cleared their dysplasia after the vaccination [30].

To better use peptide epitope-based vaccines to treat cancers, we propose to exploit the dsRNA as an adjuvant to induce tumor-killing immune responses. We have complexed pI:C and an HPV 16 $E7_{49–57}$ peptide to form a molecular complex and demonstrated its efficacy in treating model human cervical cancer tumors in a murine model. This strategy is expected to be feasible for the therapy of other tumors as well. In future studies, pI:C and CpG may be used complementarily to induce an improved anti-tumor immune response in humans.

Materials and methods

Materials

Synthetic pI:C was purchased from GE-Amersham Healthcare (Piscataway, NJ). The pI:C 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 pI:C solution (2 mg/ml in endotoxin-free solution) was determined to be 2.4 ± 0.3 EU/ml using a Limulus lysate assay (Associates of Cape Cod, Inc. East Falmouth, MA). CFSE was purchased from Molecular Probes (Eugene, OR). FITC- or PE-labeled anti-mouse CD11c and PE-labeled anti-mouse CD86 Abs were purchased from BD Pharmingen (San Diego, CA). TC-1 cells were generously provided by Dr. T. C. Wu at the Johns Hopkins University. The cells were C57BL/6 mouse lung endothelial cells transformed with HPV 16 E6 and E7 oncogenes and an activated H-ras gene [33]. The 24JK tumor cell line was generated by Dr. P. Hwu in the National Cancer Institute. It is a weakly immunogenic tumor cell line derived from the MCA102 fibrosarcoma generated from C57BL/6 mice [34]. 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) and another 9 amino acid control peptide (NIVTFRAHY) were synthesized and purified ($> 80\%$) by the GenScript Corp. (Piscataway, NJ). CpG 1826 (5′-TCCATGACGTTCCTGACGTT-3′) was synthesized by the Integrated DNA Technologies, Inc. (Carolville, IA).

Preparation of pI:C/$E7_{49–57}$ complex

The pI:C/$E7_{49–57}$ complex was prepared by mixing equal volumes of pI:C and $E7_{49–57}$ solutions followed by gentle mixing. The mixture was allowed to stay at room temperature for at least 15 min prior to further usage. To characterize the stoichiometry between pI:C and $E7_{49–57}$ in the complex, pI:C solution (50 μg in 100 μl) was mixed with an equal volume (100 μl) of $E7_{49–57}$ solution, which contained either 5, 25, 50, 100, 150, or 200 μg of $E7_{49–57}$ peptide. Because of the opposite charges on pI:C and $E7_{49–57}$ and the polymeric nature of pI:C, the mixture of pI:C and $E7_{49–57}$ would aggregate and precipitate when an electro-neutral point was reached. By measuring the turbidity (OD655) of the mixture, the ratio of pI:C and $E7_{49–57}$ needed to reach the neutral point was estimated.

Tumor therapeutic experiments

Female C57BL/6 mice (6–8-week-old, Charles River Laboratories, Wilmington, MA) were used in all animal studies. National Institutes of Health (NIH) guidelines for care and use of laboratory animals were observed. Tumors were established by s.c. injecting TC-1 cells (5×10^5) in the flank of mice on day 0. Mice were then immunized on days specified later by s.c. injection (150 μl) of different peptide formulations, which included $E7_{49–57}$ alone (20 μg/mouse), pI:C alone (50 μg/mouse, ~0.5 nmol), pI:C complexed with $E7_{49–57}$, pI:C complexed with the 9 aa control peptide, CpG1826 (20 μg/mouse, ~1.5 nmol) admixed with $E7_{49–57}$, pI:C/$E7_{49–57}$ admixed with CpG1826, pI:C mixed with CpG, or CpG alone. One group of tumor-bearing mice was left untreated; another group was treated with $E7_{49–57}$ incorporated in a previously reported liposome-protamine-DNA (LPD) particle formulation as a positive control [35–37]. All formulations were in 5% dextrose to maintain its isotonicity. 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.

Of those mice whose tumors were eradicated by immunizing with pI:C/$E7_{49–57}$, 53 days after the initial TC-1 cell injection, they were re-challenged with either 24JK cells (5×10^5), TC-1 cells (5×10^5), or left untreated. The growth of the tumors was then monitored.

In vivo CTL assay

$E7_{49–57}$-specific CTL activity was measured using an in vivo CTL assay as described elsewhere [38]. Mice ($n=5$) were immunized on days 0 and 7. On day 20, splenocytes from naïve mice were harvested, pulsed with $E7_{49–57}$ (250 ng/ml) and labeled using a high concentration of CFSE (5 μM; $CFSE^{high}$). Same splenocytes without $E7_{49–57}$-pulsing were labeled using a low concentration of CFSE (0.5 μM; $CFSE^{low}$) as an internal control. Ten million cells of each population were mixed and injected into mice via the tail vein. The relative abundance of $CFSE^{high}$ and $CFSE^{low}$ cells in the spleen was determined by flow cytometry (BD LSR II laser benchtop, San Jose, CA) 3 h after the injection. Specific lysis was calculated according to the following formula: $\{1 - [\text{ratio of } CFSE^{low}/CFSE^{high} \text{ of naive mouse}]/[\text{ratio of } CFSE^{low}/CFSE^{high} \text{ of vaccinated mouse}]\} \times 100$.

Splenocyte proliferation and IFN-γ release assays

Splenocytes were prepared as previously described [35] and cultured (1×10^6 cells in 300 μl, $n=6$) in RPMI 1640 medium with 10% FBS, 50 U/ml penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mM non-essential amino acids, 40 U/ml IL-2, and 10 μg/ml of $E7_{49–57}$ for 48 h. The cells were spun down, and the concentration of IFN-γ in the supernatant was measured using an ELISA kit from Pierce (Rockford, IL).

In the splenocyte proliferation assay, splenocytes (1×10^5 cells/ml) were cultured as described above with $E7_{49–57}$ (0 or 100 μg/ml) for 5 days. Cell number was determined using an MTT test kit (Sigma-Aldrich). Proliferation index (ratio) was reported as the number of cells when stimulated with $E7_{49–57}$ over that without $E7_{49–57}$ stimulation.

Effect of pI:C on the proportion of DCs in mouse popliteal LNs

To evaluate the effect of pI:C on the proportion of DCs in local draining LNs, mice ($n=4$) were s.c. injected in their hind leg footpads with either pI:C (50 μg), pI:C/$E7_{49–57}$ (50/20 μg), lipopolysaccharide (LPS, Sigma-Aldrich, 1 μg), or sterilized PBS (10 mM, pH 7.4). Twenty-four hours later, popliteal LNs were removed; single cell suspension was prepared, stained with FITC-labeled anti-CD11c Ab, and analyzed by flow cytometry.

Effect of immunization with pI:C/$E7_{49–57}$ on the proportion of DCs in tumor-bearing mice

Mice were injected (s.c.) with TC-1 cells (5×10^5) on day 0. On days 4 and 7, they were immunized with pI:C/E7, pI:C alone, pI:C complexed with the 9 aa control peptide, or left untreated as described earlier. On day 25, mice ($n=5$) were euthanized; single LN and splenocyte suspensions were prepared, double-stained with FITC-labeled anti-CD11c and PE-labeled anti-CD86, and analyzed by flow cytometry.

Statistical analysis

Except where mentioned, statistical analyses were completed by performing one-way analysis of variance

(ANOVA) followed by pair-wise comparisons with Fisher's protected least significant difference (PLSD) procedure. The tumor regression curves were analyzed using the GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, CA). A p value of ≤ 0.05 (two-tail) was considered to be significant.

Results

Characterization of the pI:C/E7$_{49-57}$ complex

To characterize the binding between pI:C and E7$_{49-57}$, pI:C solution (50 μg in 100 μl) was mixed with an equal volume (100 μl) of E7$_{49-57}$ solution, which contained either 5, 25, 50, 100, 150, or 200 μg of E7$_{49-57}$ peptide. As shown in Fig. 1, significant precipitations were formed only when the pI:C solution was mixed with the E7$_{49-57}$ solution that contained 100 μg of E7$_{49-57}$ (Fig. 1), indicating that the electro-neutral point was reached at this ratio. At the neutral point, it was estimated that one pI:C molecule was associated with ≥89 E7$_{49-57}$ molecules. Thus, in the pI:C/E7$_{49-57}$ formulation we used to immunize mice in followed studies, where 50 μg pI:C (100 μl) was mixed together with 20 μg E7$_{49-57}$ (100 μl), one pI:C molecule should have bound to more than 18 E7$_{49-57}$ molecules. The size of this pI:C/E7$_{49-57}$ complex (pI:C/E7$_{49-57}$, 1:2, w/w) was determined to be 150 ± 25 nm using a dynamic light scattering particle sizer.

Immunization of tumor-bearing mice with pI:C/E7$_{49-57}$ led to extensive regressions of tumors in mice

Tumors were established in C57BL/6 mice by s.c. injection of TC-1 cells (5×10^5) in the mouse flank on day 0. Tumors became visible on day 3 (~30 mm^3). On days 4 and 7, mice were immunized by s.c. injection with the pI:C/E7$_{49-57}$ complex, and the growth of the tumors was monitored. Figure 2 showed that although tumors in the un-immunized tumor-bearing mice grew continuously, those in mice immunized with pI:C/E7$_{49-57}$ regressed extensively. Treatment with pI:C alone did not lead to any tumor regression, but slowed down the growth, when compared to the growth of tumors in untreated mice (Fig. 2).

To evaluate the specificity of the anti-tumor responses induced by immunization with the pI:C/E7$_{49-57}$, 53 days after the initial tumor injection, mice whose tumor regressed after immunization with the pI:C/E7$_{49-57}$ were re-challenged by s.c. injection of either TC-1 cells (5×10^5) or 24JK cells (5×10^5), which was a sarcoma tumor cell line derived from C57BL/6 mice and did not express HPV 16 E proteins, and the growth of tumors was monitored. As shown in Fig. 3, TC-1 tumor cells were unable to grow; while 24JK cells grew freely and formed tumors of about 250 mm^3 12 days after the re-challenge. These data suggested that the tumor-killing activity induced by the pI:C/E7$_{49-57}$ was specific to the TC-1 tumor cells.

To further evaluate the anti-tumor activity induced by the pI:C/E7$_{49-57}$, the tumor immunotherapeutic

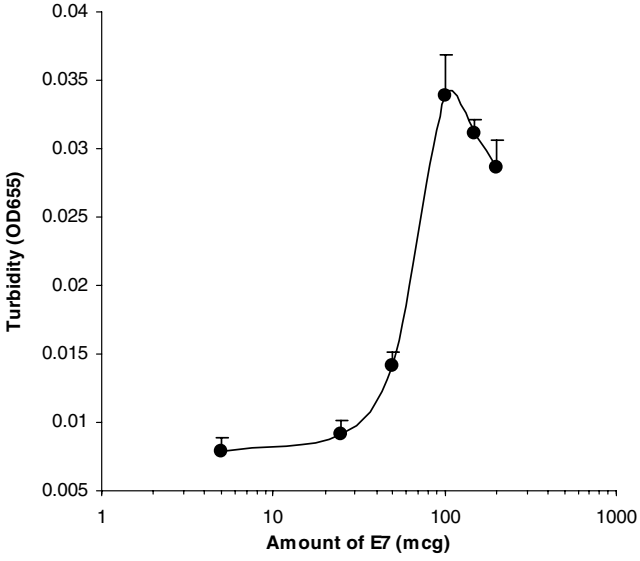

Fig. 1 Complexation of pI:C with E7$_{49-57}$. One hundred μl of pI:C solution (500 μg/ml) was gently mixed with an equal volume of E7$_{49-57}$ solution containing various amount of E7$_{49-57}$ peptide. After 15 min of incubation at room temperature, the turbidity of the suspension was determined (OD655). Data shown are mean ± S.D. ($n=3$)

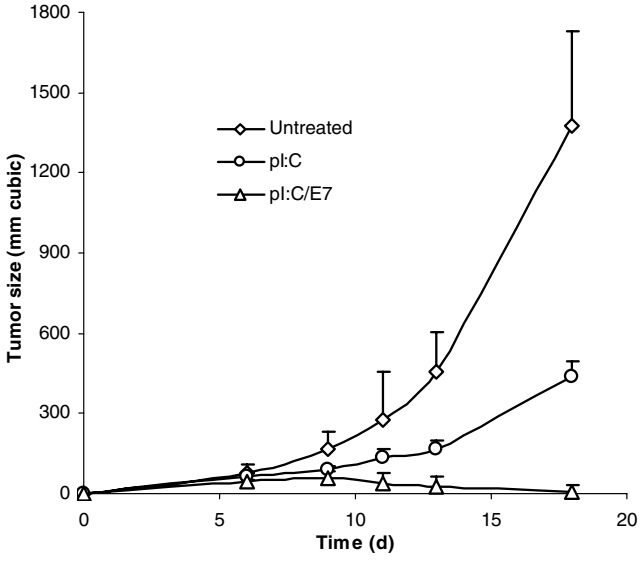

Fig. 2 Immunization of TC-1 tumor-bearing mice with pI:C/E7$_{49-57}$ caused extensive tumor regressions. C57BL/6 mice were s.c. injected in the flank with TC-1 cells (5×10^5) on day 0. On days 4 and 7, they were immunized by s.c. injection with pI:C/E7$_{49-57}$ ($n=10$) or pI:C alone ($n=5$). Another group of mice ($n=5$) was left untreated. The dose of E7$_{49-57}$ was 20 μg/mouse; pI:C was dosed at 50 μg/mouse. Data shown are one representative from two independent experiments, which had similar results

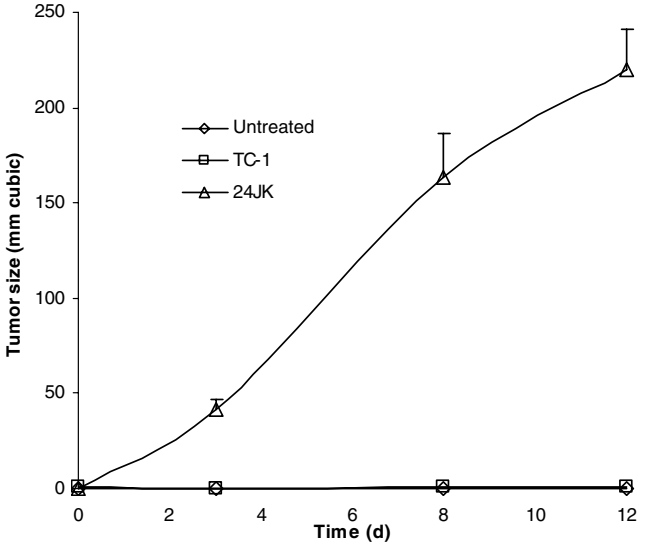

Fig. 3 The anti-tumor responses induced by pI:C/E7$_{49–57}$ was specific to TC-1 tumors. Mice were s.c. injected with TC-1 cells (5×10^5) on day 0. On days 4 and 7, they were immunized with pI:C/E7$_{49–57}$ ($n=10$). After the tumor regression (53 days post the initial TC-1 cell injection), mice were re-challenged with 24JK cells (5×10^5), TC-1 cells (5×10^5), or left untreated. Similar results were obtained when this experiment was repeated twice. Data shown are mean ± S.D. from one of the experiments

activity induced by it was compared to that induced by E7$_{49–57}$ incorporated into our previously reported LPD particles and to that induced by E7$_{49–57}$ admixed with CpG1826 oligos as an adjuvant. LPD/E7$_{49–57}$ was previously shown by one of us to be very effective in causing TC-1 tumor regressions in mice [35–37]. CpG motif-containing oligos have been shown by others to be an effective adjuvant for peptide-based tumor vaccines [3–6]. Moreover, CpG oligos are generally thought to exert their immunostimulatory functions by interacting with TLR9; while evidence supports that the strong immunostimulatory activity from pI:C is through the pI:C/TLR3 signaling [9, 20]. As shown in Fig. 4, the tumor therapeutic activity induced by the pI:C/E7$_{49–57}$ was comparable to that induced by the LPD/E7$_{49–57}$, and stronger than that induced by E7$_{49–57}$ admixed with CpG1826. Importantly, data in Fig. 4b demonstrated that both pI:C and E7$_{49–57}$ were required to induce an effective TC-1 tumor-killing activity, because when the E7$_{49–57}$ was replaced by another 9 aa peptide having identical amino acid composition, but different sequence, the pI:C/9 aa control peptide complex was not effective (Fig. 4b). In fact, the activity from the pI:C/9 aa peptide was comparable to that from pI:C alone in causing tumor regressions. Finally, the tumors in mice immunized with E7$_{49–57}$ peptide alone tended to grow faster than that in mice left untreated (Fig. 4b). Tolerance might have been induced by the pure E7$_{49–57}$ peptide [39, 40].

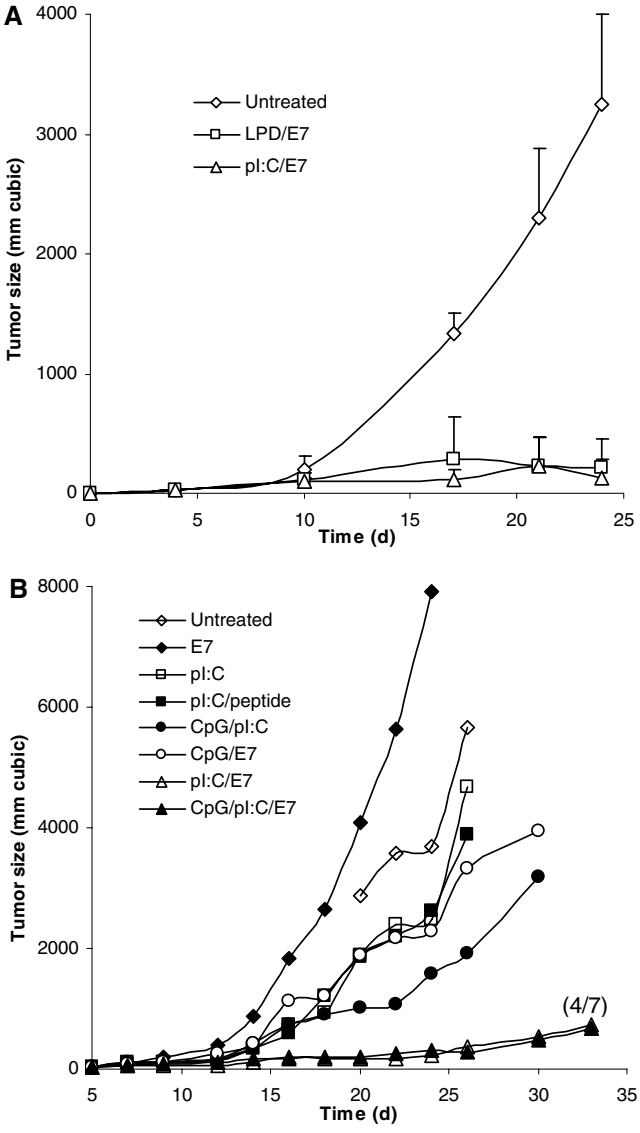

Fig. 4 **a** The tumor therapeutic activity induced by pI:C/E7$_{49–57}$ complex was comparable to that induced by E7$_{49–57}$ incorporated in LPD particles. Mice ($n=7$) were seeded with TC-1 tumor cells (5×10^5) on day 0. On day 6, they were immunized with either pI:C/E7$_{49–57}$, LPD/E7$_{49–57}$, or left untreated. On days 17, 21, and 24, two out of seven (2/7), 3/7, and 4/7 of mice in the pI:C/E7$_{49–57}$ immunized group became tumor-free. Data shown are mean ± S.D. **b** The tumor therapeutic activity induced by pI:C/E7$_{49–57}$ complex was stronger than that induced by E7$_{49–57}$ adjuvanted with CpG1826 oligos. Mice ($n=5$–7) were seeded with TC-1 tumor cells (5×10^5) on day 0. On days 4 and 7, they were immunized with either pI:C alone, pI:C/E7$_{49–57}$, pI:C complexed with a 9 aa non-specific peptide (pI:C/9 aa peptide), CpG1826 admixed with E7$_{49–57}$, CpG1826 and pI:C mixture, pI:C/E7$_{49–57}$ admixed with CpG1826, or left untreated. The dose for peptide, pI:C, and CpG1826 was 20, 50, and 20 μg/mouse, respectively. Mice immunized with pI:C/E7$_{49–57}$ or pI:C/CpG/E7$_{49–57}$ were monitored for 35 days, at which time all of them were still alive. Mice in other groups were monitored until their death. Only mean values of the tumor size are shown to clearly illustrate the trend of tumor growth. The (4/7) indicates that 4 out of 7 mice immunized with pI:C/E7$_{49–57}$ or pI:C/CpG/E7$_{49–57}$ were tumor-free on day 35

Immunization with pI:C/$E7_{49–57}$ induced $E7_{49–57}$-specific CTL responses

To further characterize the immune responses induced by pI:C/$E7_{49–57}$, the $E7_{49–57}$-specific CTL response induced was measured using an in vivo CTL assay. A CTL response (~63% specific lysis) was detected only in mice immunized with pI:C/$E7_{49–57}$ (Fig. 5). Moreover, when measured in tumor-bearing mice, CTL activity was again only detected in mice who were immunized with the pI:C/$E7_{49–57}$ complex, but not in those tumor-bearing mice who were treated with pI:C alone or left untreated (Fig. 6). All these findings suggested that the tumor-killing activity induced by pI:C/$E7_{49–57}$ was mainly due to the $E7_{49–57}$-specific CTL response.

Splenocytes isolated from mice immunized with pI:C/$E7_{49–57}$ proliferated and secreted IFN-γ after in vitro re-stimulation

Figure 7 showed that after in vitro re-stimulation with $E7_{49–57}$, significant IFN-γ secretion was detected only in the culture supernatant of splenocytes isolated from mice immunized with pI:C/$E7_{49–57}$. Moreover, only the splenocytes isolated from mice immunized with pI:C/$E7_{49–57}$ or pI:C/CpG/$E7_{49–57}$ proliferated after in vitro re-stimulation with $E7_{49–57}$ (Fig. 8).

Poly(I:C) enhanced the proportion of DCs in local draining LNs

As an initial step to identify the effect of pI:C on DCs, pI:C was s.c. injected into the hind leg footpads of mice. The proportion of $CD11c^+$ cells in the local draining popliteal LNs was measured 24 h after the injection. As shown in Fig. 9, injection of pI:C significantly enhanced the proportion of $CD11c^+$ cells in the popliteal LNs. Similar effect was observed after the injection of lipopolysaccharide (LPS), although LPS was more potent as expected.

Immunization of tumor-bearing mice with pI:C/$E7_{49–57}$ restored the proportion of DCs

To further identify the effect of pI:C on DCs, LN cells and splenocytes were isolated from tumor-bearing mice immunized with pI:C/$E7_{49–57}$, pI:C alone, pI:C/9 aa peptide, or left untreated (Fig. 4b) and stained with FITC-labeled anti-CD11c Ab and PE-labeled anti-CD86 Ab to evaluate the status of their DCs. As a control, LN cells and splenocytes from naïve mice of the same age, who have never been exposed to $E7_{49–57}$ or to tumor cells, were also prepared and stained. The proportion of CD11c and CD86 double positive cells in the LNs of mice whose tumors did not regress (tumor-bearing mice treated with pI:C, pI:C/9 aa peptide, or left untreated) was significantly lower than that in naive mice (Fig. 10). In contrast, the proportion of $CD11c^+$ and $CD86^+$ cells in the LNs of mice whose tumors regressed after immunization with pI:C/$E7_{49–57}$ was comparable to that in naïve mice. Similar results were observed in the splenocytes (data not shown). These data suggested that treatment with pI:C/$E7_{49–57}$ inhibited or reversed the dysfunction of DCs induced by tumors.

Discussions

After decades of debates over whether the immune system can fight tumors, growing and compelling evidence now suggests that the immune system plays an important role in controlling malignancy [41, 42]. However, there still are many major hurdles in developing efficacious therapeutic cancer vaccines, including the identification of TAAs or TSAs that can induce immune responses specifically targeting tumor cells without harming normal cells and the need for a powerful vaccine adjuvant to induce immune responses with a sufficient strength to eradicate tumors [43]. Cervical cancer is currently one of the few cancers, for which vaccine-based therapeutic strategies have the potential to significantly influence the incidence of the diseases. It is generally recognized that HPV is the caustic agent of cervical cancer, and that the E gene products of HPV, such as E6 and E7, are responsible for the tumorigenic activity of HPV [44–46]. The fact that these proteins are completely foreign to the host and are not presented on the surface of viral particles makes them excellent antigens for the development of therapeutic vaccines for cervical cancers. Thus, these cervical cancer-specific E proteins are ideal TSAs for researching tumor immunotherapy. Moreover, data from recent clinical trials using HLA-A2-restricted peptide epitopes derived from the HPV 16 E7 protein have clearly demonstrated the potential of such peptides in cervical cancer immunotherapy [30, 47]. However, due to its weak immunogenicity, there continues to be a critical need for a potent, unconventional vaccine adjuvant in order to induce stronger anti-tumor immune responses. The data in this present study clearly suggested dsRNA in the form synthetic pI:C as such an adjuvant.

It became clear in recent years that some PAMP molecules are potent vaccine adjuvants [12, 13, 20, 48]. PAMPs are recognized by TLRs [9], and the recognition of PAMPs by TLRs triggers the activation of not only the innate immunity, but also the adaptive immunity. Unmethylated CpG motifs, a ligand for TLR9, have been extensively evaluated for its immunostimulatory activity [49, 50]. Double stranded RNA is produced by most viruses during their replication. It was shown to be a ligand/agonist for TLR3 and activate the NF-κB pathway, resulting in the activation of IFN-α and IFN-β, which have various activities, including being immunostimulatory [9, 51]. Poly(I:C) is a synthetic agonist of TLR3 [9]. In vitro, it is a very potent IFN inducer [52]. It

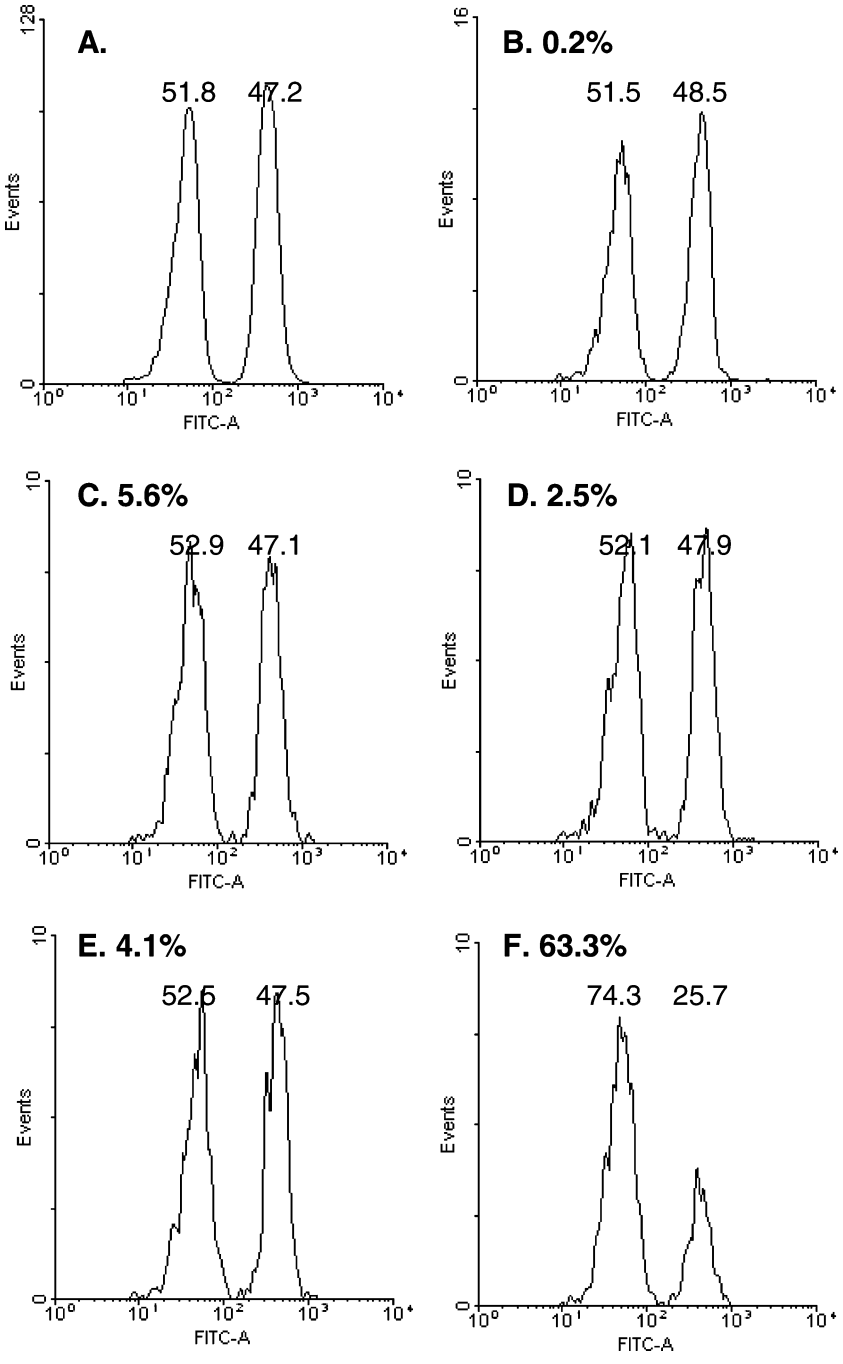

Fig. 5 Immunization with pI:C/$E7_{49–57}$ induced $E7_{49–57}$-specific CTL responses. Mice ($n = 3$) were immunized with pI:C/$E7_{49–57}$ (**f**), pI:C/9 aa peptide (**e**), pI:C alone (50 μg) (**d**), $E7_{49–57}$ alone (20 μg) (**c**), or left untreated (**b**) on days 0 and 7. On day 20, $E7_{49–57}$-specific CTL activity induced in the mice was assessed using an in vivo CTL assay. $E7_{49–57}$-pulsed, $CFSE^{high}$ and unpulsed, $CFSE^{low}$ splenocytes isolated from naïve mice (10×10^6 each) were injected into mice via the tail vein. Mice were sacrificed 3 h later, and their splenocyte suspension was prepared and analyzed by flow cytometry. One representative from three mice, which showed similar CTL activity, is shown. The experiment was repeated twice. In flow cytometry graphs, the peak in the left represents the unpulsed $CFSE^{low}$ splenocytes, and that in the right represents the $E7_{49–57}$-pulsed, $CFSE^{high}$ splenocytes. Shown in **a** was the $E7_{49–57}$-pulsed, $CFSE^{high}$ and unpulsed $CFSE^{low}$ splenocyte mixture prior to injection. Numbers shown in the left-upper corner of each graph were the % of $E7_{49–57}$-specific CTL killing activity. Numbers shown above each peak were the relative percent of $CFSE^{low}$ versus the $CSFE^{high}$ cells

can efficiently induce the maturation of DCs and the cross-presentation of antigens by DCs [53]. Data from recent studies have shown that pI:C can be used as an adjuvant to induce CTL immune responses [12, 13, 20]. In the present study, we have shown that the pI:C/$E7_{49–57}$ complex induced a strong $E7_{49–57}$-specific CTL response (Fig. 5) that caused the regression of E7-expressing TC-1 tumors pre-established in mice (Figs. 2, 3, 4). Also, when measured in tumor-bearing mice, an $E7_{49–57}$-specific CTL activity was only detected in mice who were immunized with the pI:C/$E7_{49–57}$ complex, but not in those tumor-bearing mice who were treated with pI:C alone, $E7_{49–57}$ peptide alone, or left untreated (Fig. 6). In Figs. 2 and 4b, tumors in mice injected with pI:C alone did not regress, but grew slower than in the tumor-bearing untreated mice, suggesting that pI:C may have induced some non-specific anti-tumor activities. This is understandable given that pI:C itself induces type I IFNs, which have immunostimulatory and anti-tumor activities. Moreover, pI:C had been shown to activate NK cells and enhance their cytolytic activity [53]. However, pI:C alone was apparently not sufficient to

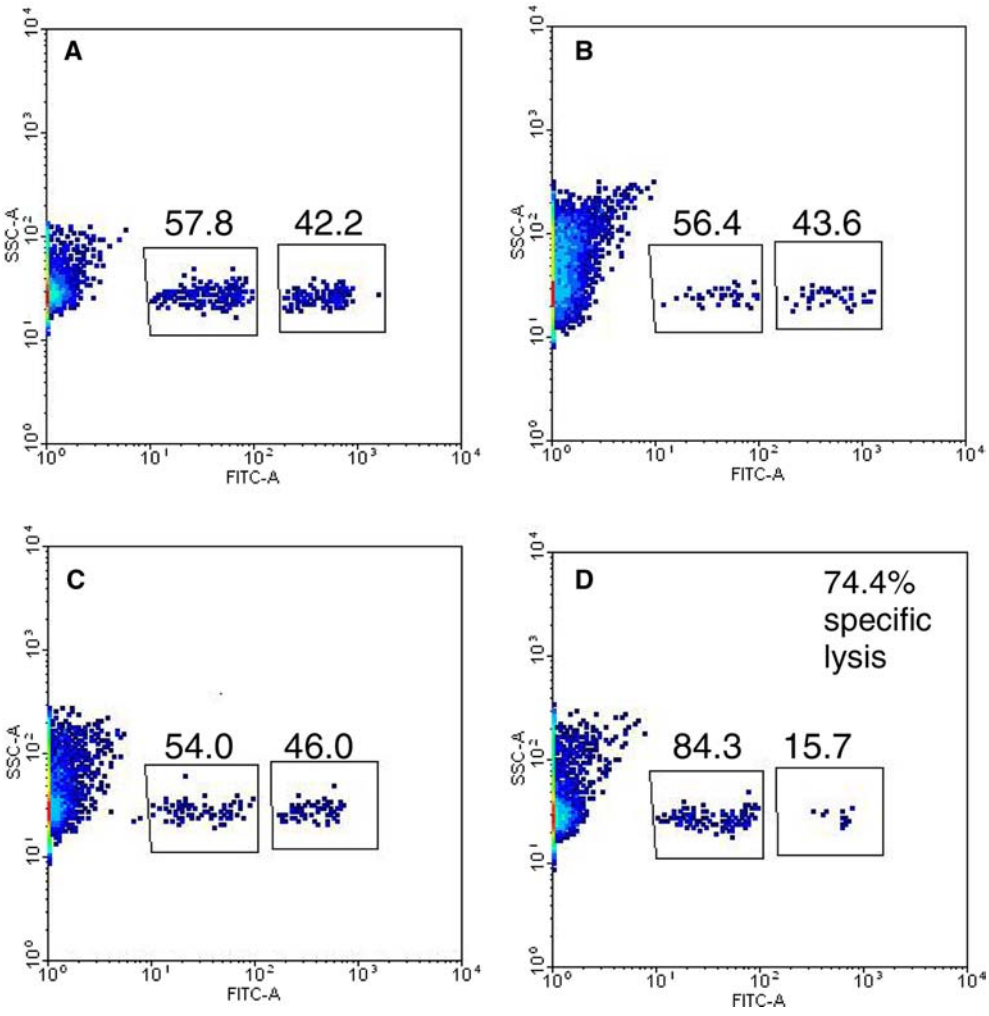

Fig. 6 $E7_{49–57}$-specific CTL responses were only detected in tumor-bearing mice treated with the pI:C/$E7_{49–57}$ complex. Mice ($n = 3$) were seeded with TC-1 cells (5×10^5) on day 0. On days 4 and 10, they were treated with pI:C (**c**), pI:C/$E7_{49–57}$ complex (**d**), or left untreated (**b**). On day 25, the $E7_{49–57}$-specific CTL activity in those mice was measured using an in vivo CTL assay by injecting them via the tail vein with $CFSE^{low}$ and $E7_{49–57}$-labeled $CFSE^{high}$ splenocytes (10×10^6 each). Mice were sacrificed 13 h later, and their splenocyte suspension was prepared and analyzed by flow cytometry. One representative from three mice, which showed similar CTL activity, is shown. The experiment was repeated twice. Shown in **a** was the flow cytometry graph of naïve mice that were not seeded with TC-1 cells. *Numbers* shown above each region were the relative percent of $CFSE^{low}$ versus the $CSFE^{high}$ cells. A significant $E7_{49–57}$-specific CTL activity was detected only in tumor-bearing mice treated with pI:C/$E7_{49–57}$

cause any tumor regression (Fig. 2, 4b). Instead, the $E7_{49–57}$ peptide epitope was required for the induction of a robust E7-specific CTL response to effectively kill tumor cells (Figs. 2, 4b). We suspect this strong immunostimulatory activity from the pI:C/$E7_{49–57}$ complex was partially due to the dsRNA/TLR3 signaling, which helped to mobilize DCs to the injection sites to pick up the pI:C/$E7_{49–57}$ complex and to induce their maturation for the successful presentation of the $E7_{49–57}$ peptide by the DCs to $CD8^+$ T cells in local draining LNs. Although we did not measure the expression of TLR3 on DCs and other lymphocytes after the injection of the pI:C/$E7_{49–57}$ complex, previous report have demonstrated a significant up-regulation of the mRNA of TLR3 and cytokines in the nasal-associated lymphoid tissues when an influenza vaccine was nasally co-administered with pI:C into mice [13].

The specificity of the anti-tumor activity induced by the pI:C/$E7_{49–57}$ was further confirmed by the observation that TC-1 tumor cells did not grow in mice whose TC-1 tumors had been eradicated by immunization with pI:C/$E7_{49–57}$; while 24JK tumor cells that do not express E7 protein grew freely in similarly treated mice to form visible tumors (Fig. 3). At this moment, it is difficult to eliminate the possibility that TC-1 cells injected in the first inoculation could have also induced some anti-TC-1 immune responses, which could have provided some extent of protection to mice in the second TC-1 tumor challenge. However, data from this re-challenge study (Fig. 3), the $E7_{49–57}$-specific CTL response (Figs. 5, 6), and the splenocyte proliferation and IFN-γ secretion after in vitro re-stimulation (Figs. 7, 8) altogether suggested that the specificity of the tumor-killing activity was originated by immunization with pI:C/$E7_{49–57}$.

One important finding in this study was that immunization with pI:C/$E7_{49–57}$ restored the proportion of DCs in tumor-bearing mice (Fig. 10). It is well known that tumor cells employ a variety of mechanisms to hide themselves from the immune system, and thus, escape elimination [54]. One of the mechanisms is to suppress DCs, resulting in a decrease in the proportion of DCs in the circulation and lymphoid organs, an increase in the population of immature DCs, and a decrease in the antigen presentation ability of DCs [55, 56]. Thus, it was not surprising to observe that, 25 days after tumor cell injection, the proportion of $CD11c^+$, $CD86^+$ cells

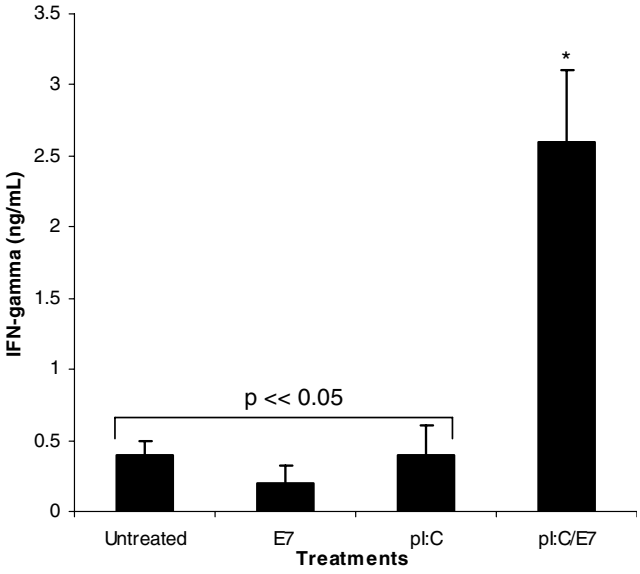

Fig. 7 Splenocytes isolated from mice immunized with pI:C/$E7_{49–57}$ secreted IFN-γ after in vitro re-stimulation. Mice ($n=3$) were immunized with pI:C/$E7_{49–57}$, pI:C alone (50 μg), $E7_{49–57}$ alone (20 μg), or left untreated on days 0 and 7. On day 20, splenocytes were isolated from them, and co-incubated with $E7_{49–57}$ (10 μg/1 × 10^7 cells) for 48 h. The concentration of IFN-γ in the culture medium was measured using ELISA. Data shown are mean ± S.E.M. ($n=3$). This experiment was repeated twice. Similar trend was observed. (*asterisks*) indicates that the result from pI:C/$E7_{49–57}$ was significantly different from that from other treatments ($P=0.002$ vs. untreated, *t* test). The values from mice treated with pI:C, $E7_{49–57}$, or left untreated are not different from each other

detected in the LNs (Fig. 10) and spleens of tumor-bearing mice, whose tumors were not eradicated, was significantly lower than that in naive mice. However, it was interesting to find that the proportion of $CD11c^+$, $CD86^+$ cells in mice whose tumor regressed after immunization with pI:C/$E7_{49–57}$ was similar to that in naïve mice. Although there is not a single molecular marker that is DC-specific in mice (i.e., not all DCs are $CD11c^+$, and not all $CD11c^+$ cells are DCs), CD11c is generally used as a DC-restricted marker [57–65]. Thus, these findings suggested the restoration of the proportion of DCs, and probably their maturity as well as functionality, by immunization with pI:C/$E7_{49–57}$. In fact, it was also found that pI:C alone significantly enhanced the proportion of DCs in the popliteal LNs of mice (Fig. 9), which might be due to the migration of DCs into the popliteal LNs after stimulation. A comprehensive study has to be carried out in the future to fully elucidate the relationship between treatment with pI:C or pI:C/$E7_{49–57}$ and the functionality of DCs in both normal and tumor-bearing mice.

All these findings suggested that the pI:C is a very potent immunostimulatory molecule that can dramatically boost the CTL response to a peptide antigen. We have shown that the tumor-killing activity induced by the pI:C/$E7_{49–57}$ was comparable to that induced by $E7_{49–57}$ incorporated into LPD particles, which we have previously reported to be very efficacious in treating tumors pre-established in mice (Fig. 4a). However, the clinical applicability of the LPD particles may be limited by the toxicity from the cationic liposome component in the LPD particles. Moreover, pI:C/$E7_{49–57}$ induced a stronger tumor-killing immune response than $E7_{49–57}$ adjuvanted with CpG1826, which had also been shown in several previous studies to be a potent adjuvant for peptide-based tumor vaccines [3–6]. The response induced by CpG1826/$E7_{49–57}$ in this present study could have been stronger if more CpG1826 were more frequently dosed to mice. However, as described earlier, TLR9, the receptor for CpG motifs, is expressed only on pDCs and B cells in humans. But TLR3 is expressed in mDCs and T cells as well as fibroblasts and other non-immune cells in humans [8]. Moreover, it was shown that CpG oligos stimulated $CD11c^-$ type 2 DC precursors, and that pI:C stimulated $CD11c^+$ DCs to produce IFN-γ, respectively [66]. Thus, pI:C and CpG oligos together are expected to be more potent than each of them alone in humans by complementing each other. In this present study, pI:C/$E7_{49–57}$ and pI:C/CpG/$E7_{49–57}$ performed similarly in treating TC-1 tumors in mice (Fig. 4b). Increasing the dose and dosing frequency of

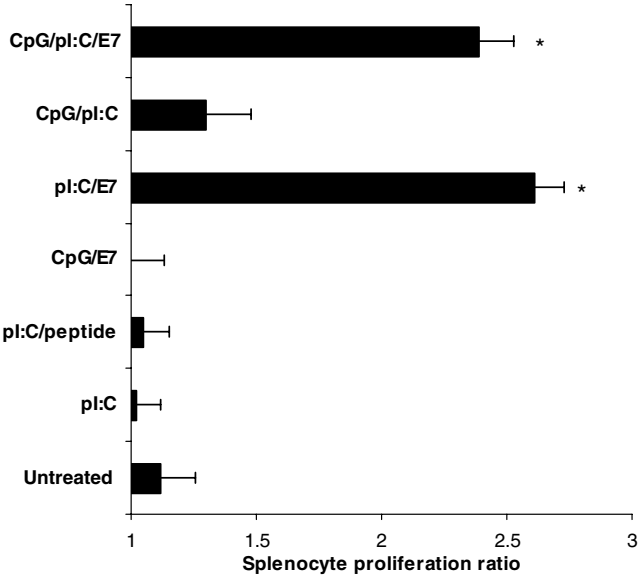

Fig. 8 Splenocytes isolated from mice immunized with pI:C/$E7_{49–57}$ or pI:C/CpG/$E7_{49–57}$ proliferated significantly after in vitro re-stimulation. Mice were seeded with TC-1 tumor cells (5×10^5) on day 0. On days 4 and 7, they were immunized with either pI:C alone, pI:C/$E7_{49–57}$, pI:C/9 aa peptide, CpG1826 admixed with $E7_{49–57}$, CpG1826 and pI:C mixture, pI:C/$E7_{49–57}$ admixed with CpG1826, or left untreated. The dose for peptide, pI:C, and CpG1826 was 20, 50, and 20 μg/mouse, respectively. On day 25, mice were euthanized; and their spleen was removed. Single splenocyte suspensions from each individual spleen were prepared and stimulated with $E7_{49–57}$ (0 or 100 μg/ml) for 5 days. The cell number was determined using an MTT test kit. *asterisks* indicates that the values from pI:C/$E7_{49–57}$ and pI:C/CpG/$E7_{49–57}$ were comparable, but significantly different from that of the other groups. This experiment was repeated twice. Similar trend was observed. Data shown are mean ± S.D. ($n=5$)

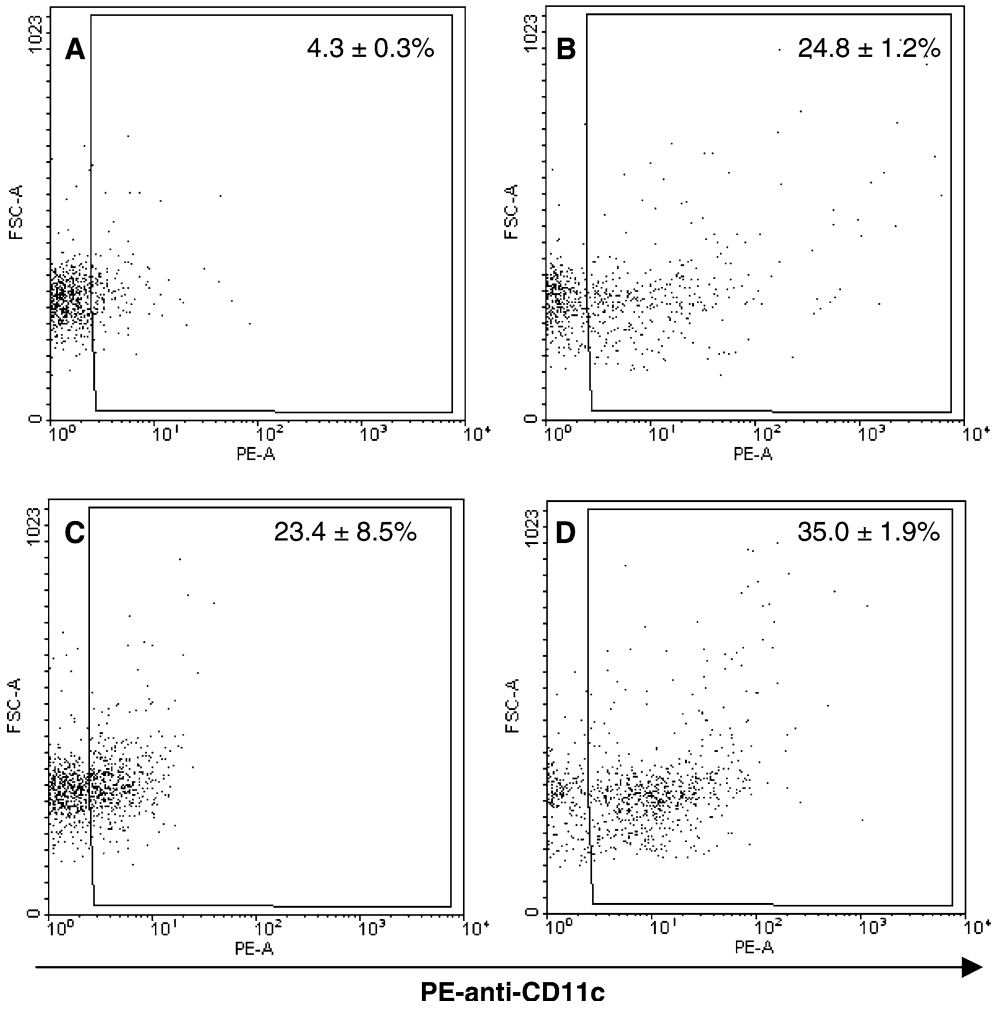

Fig. 9 Poly(I:C) enhanced the proportion of DCs in local draining LNs. Mice ($n=4$) were injected s.c. into their hind leg footpads with pI:C (50 μg in 20 μl) (**b**), pI:C/E7$_{49–57}$ (**c**), LPS (5 μg in 20 μl) (**d**), or sterile PBS (10 mM, pH 7.4) (**a**). Draining popliteal LNs were removed 24 h after the injection. Single LN cell suspension was prepared from LNs, stained with PE-labeled anti-CD11c Ab, and analyzed by flow cytometry. *Graphs* shown are one representative from four mice. *Numbers* in the graphs are the percent of LN cells that were CD11c$^+$ (mean ± S.D., $n=4$)

the CpG1826 would probably have made pI:C/CpG/E7$_{49–57}$ more effective.

The dose of pI:C in the present study was 50 μg/mouse. A dose response study has to be completed to determine the optimal dose for the pI:C as an adjuvant. In a separate study, we found that using pI:C and another protein antigen, increasing the dose of pI:C from 10 to 50 μg/mouse did not significantly change the resulting specific immune responses (Sloat and Cui, unpublished data). With the dose of 50 μg/mouse, no gross inflammatory, allergic, or toxic effects were observed when pI:C was injected (s.c.) in mice. Ichinohe et al. [13] reported that pI:C did not induce any detectable side-effect or toxicity when 25 μg/mouse/day was dosed intranasally or intracerebrally into mice daily for 9 days. Thus, it is expected that pI:C is safe, especially when used in a small quantity as an adjuvant. If needed, a modified form of pI:C, polyI:C$_{12}$U, which had been shown to have a much better safety profiles, may be used. PolyI:C$_{12}$U was generated by introducing unpaired uracil and quinine bases into pI:C [67]. Recently, it was shown to be as effective as pI:C in inducing in vitro maturation of human monocyte derived DCs [68].

Finally, only an H-2D^b-restricted peptide epitope from the HPV 16 E7 protein was used in the present study to complete the feasibility study. To develop an efficacious, therapeutic human cervical cancer vaccine, both MHC class I-restricted and class II-restricted epitopes from E7 and other E proteins may have to be included to form a multi-antigenic vaccine. The inclusion of MHC class II-restricted epitopes will be important because it had been shown that a CTL response generally has minimal durability without the presence of a cognate T helper response [3, 4, 69]. Moreover, due to the diversity of the HLA types in human population, multiple human HLA epitopes may have to be identified and included in the vaccine as well. The HLA restriction and epitope identification are among some of the pitfalls that need to be taken into consideration for the development of all peptide vaccines.

In conclusion, we have reported dsRNA in the form of synthetic pI:C as a potent adjuvant to boost the anti-tumor immune responses induced by a minimal MHC I-restricted peptide epitope. The strong immunostimulatory activity was likely to be related to its effect on DCs.

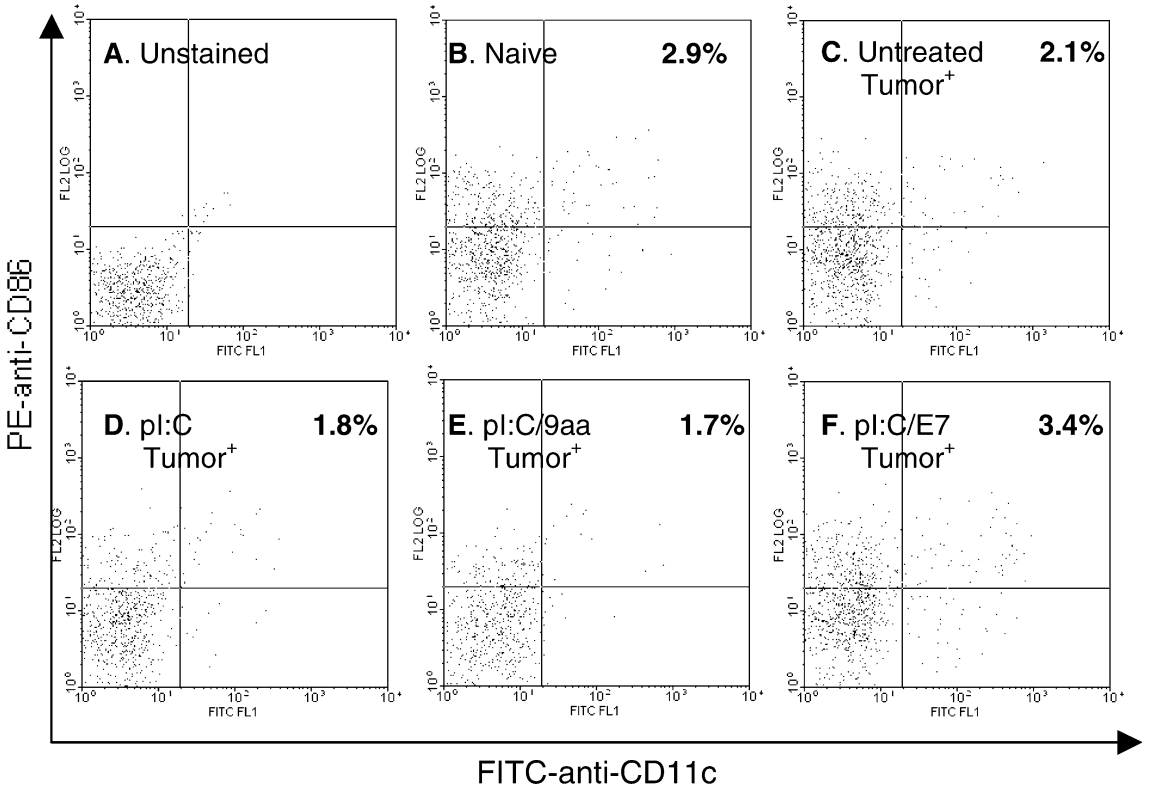

Fig. 10 Immunization of tumor-bearing mice with pI:C/$E7_{49–57}$ restored the proportion of DCs in their LNs. Mice were s.c. injected with TC-1 cells (5×10^5) on day 0. On days 4 and 7, they were immunized with pI:C (**d**), pI:C/9 aa peptide (**e**), pI:C/$E7_{49–57}$ (**f**), or left untreated (**c**). The dose of peptide was 20 μg/mouse; the dose of pI:C was 50 μg/mouse. LNs (popliteal, axillary, and inguinal) from five mice in each group were pooled. LN cells were stained with FITC-labeled anti-CD11c Ab and PE-labeled anti-CD86 Ab, and analyzed by flow cytometry. As a control, LNs from naïve mice (**b**) were also isolated and processed identically. *Numbers* in the graphs are the percent of $CD11c^+$, $CD86^+$ double positive cells. This experiment was repeated twice. In both times, the percentages of $CD11c^+$ and $CD86^+$ double positive cells in **c**, **d**, and **e** were significantly lower than that in **b** and **f**

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