Gemcitabine enhances antitumor efficacy of recombinant lipoimmunogen-based immunotherapy

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ABSTRACT

Although immunotherapy is an attractive approach for cancer treatment, increasing evidence has shown that the combination of immunotherapy with other treatment modalities may improve the outcome of advanced malignancy. We combined the anticancer drug gemcitabine (Gem) with recombinant lipoprotein-based immunotherapy (rlipo-E7m/CpG) to treat advanced cancer. Mice bearing huge solid tumors (>12 mm in diameter) or orthotopic cervical cancer were treated with a therapeutic regimen consisting of rlipo-E7m/CpG and Gem. In addition, tumor-infiltrating immune cells were quantified by flow cytometry following the chemotherapy and/or immunotherapy. We observed the eradication of huge tumors following the administration of Gem on days 21, 24, and 27 or following rlipo-E7m/CpG therapy on day 30 post-tumor implantation. The combination therapy substantially reduced the number of immunosuppressive cells (CD11b\textsuperscript{+}Gr-1\textsuperscript{+}, CD11b\textsuperscript{+}F4/80\textsuperscript{+}, and CD4\textsuperscript{+}CD25\textsuperscript{+}FOXP3\textsuperscript{+}) and increased the number of tumor-infiltrating antigen-specific CD8\textsuperscript{+} T cells compared to Gem or rlipo-E7m/CpG monotherapy. Interestingly, the administration of Gem and rlipo-E7m/CpG reduced the quantity of programmed cell death protein 1 (PD-1)-expressing antigen-specific cytotoxic T lymphocytes (CTLs) in the regressing tumors. These findings demonstrated that Gem enhances the eradication of huge tumors by inhibiting a broad range of immunosuppressive cells when combined with immunotherapy. Based on the promising results from this animal study, Gem chemotherapy combined with recombinant lipoimmunogen-based immunotherapy represents a feasible approach for cancer therapy.

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Introduction

Therapeutic vaccination has increasingly become an appealing treatment modality for malignant diseases due to its high specificity for tumor cells. Immune adjuvant-based vaccines are promising immunotherapeutic agents that trigger endogenous immune responses against cancer. Of note, toll-like receptor (TLR) agonists are potential immunopotentiators that are used as adjuvants in the design of therapeutic vaccines to induce CTL responses against infectious diseases and/or cancers.\textsuperscript{1,3} We have employed platform technology to produce a recombinant lipoimmunogen (rlipo-E7m) comprising inactive HPV16E7 (E7m) and a bacterial lipid moiety displaying endogenous TLR2 agonist activity for the treatment of human papillomavirus (HPV)-associated cancers. Previously, we observed that single-dose injection of rlipo-E7m inhibited tumor growth accompanied by the induction of Th1-polarized immunity in a mouse model of cervical cancer.\textsuperscript{4} We also demonstrated the eradication of large tumors (0.6–0.8 cm diameter) and a reduction in the number of immunosuppressive cells following rlipo-E7m therapy in the presence of the TLR9 agonist Type B CpG Oligodeoxynucleotide 1826 (CpG ODN).\textsuperscript{5}

However, cancer immunotherapy and/or vaccines have faced some challenges due to a lack of clinical effectiveness.\textsuperscript{6} Notably, the development of tumor escape from immune surveillance may be a leading cause of the failure of cancer immunotherapy, thereby prompting the search for a superior treatment regimen for cancer. Accordingly, overcoming the barrier of tumor-associated immunosuppression (i.e., subsets of regulatory immune cells and/or inhibitory cytokine networks in the tumor milieu) is required for successful cancer treatment. It is clear that immunotherapeutics administered as a monotherapy are inadequate to obtain appreciable clinical benefits. Thus, a combination of immunotherapy with other treatment modalities, such as chemotherapy, radiation, or targeted therapy, is expected to represent an ideal therapy regimen for cancer treatment.

Chemotherapy is frequently used for the treatment of solid tumors in current clinical settings. In addition to their cytotoxic effects on dividing cancer cells, anticancer drugs act on immune system components, thereby contributing to their antitumor responses. Chemotherapeutic agents were demonstrated to elicit pro-inflammatory immune responses via the binding of TLR4 and its agonist, high mobility group box 1
protein (HMGB1), when released from dying tumor cells.\textsuperscript{7,8} Recently, accumulating evidence revealed that antigen processing and presentation by dendritic cells (DCs) were enhanced by certain chemotherapeutic drugs administered at low doses.\textsuperscript{9-11} Anticancer drugs also have been shown to possess adjuvant activity to stimulate tumor-specific immune responses via the accumulation of DCs at the tumor site and the activation of antigen-specific CTLs during cancer immunotherapy.\textsuperscript{12} Moreover, several clinical reports indicated that the combination of immunotherapy with chemotherapeutic agents led to considerable improvement in the survival rate of patients with cancer.\textsuperscript{13-15} These findings have inspired a great deal of enthusiasm for the use of combined treatment via chemotherapy and immunotherapy.

Gem is a nucleoside analog used to treat several cancers, including pancreatic, lung, breast, ovarian, and colon cancers.\textsuperscript{16-19} In addition to its direct cytolytic activity on tumor cells, Gem was shown to induce the enhancement of T-cell-mediated antitumor effects in pre-clinical studies.\textsuperscript{20} Previously, we observed that the administration of recombinant lipoprotein-based vaccination as a monotherapy was not sufficient for therapeutic success in mice bearing advanced-stage tumors (≥1 cm diameter). In this study, we aimed to augment the antitumor efficacy of recombinant lipoprotein-based therapy in mice bearing clinically relevant-sized tumors via Gem administration. Our findings demonstrate the eradication of advanced-stage tumors following combined treatment with lipoprotein-based immunotherapy and Gem in a mouse cervical cancer model. This study provides a conceptual framework in which chemotherapy may strengthen the therapeutic effects of immune-based monotherapy.

Results

Combination therapy with Gem and rlipo-E7m/CpG enhances therapeutic effects

In previous studies, we demonstrated that the antitumor effects of recombinant lipoprotein-based immunotherapy (rlipo-E7m/CpG) against large tumors had room for improvement because the regressed tumor subsequently regrew.\textsuperscript{2} To explore the possibility of eradicating large tumors, Gem was combined with rlipoE7m/CpG immunotherapy. Because a given dose and administration schedule of a chemotherapeutic drug can exert different effects as a combination therapy, several administration schedules and doses of Gem were tested. Fig. 1a shows tumor-bearing mice that received one dose of Gem on day 21, followed by immunization with rlipo-E7m/CpG on day 25 post-tumor implantation. We found that administration of Gem led to slightly delayed tumor growth compared to the control treatment. The tumors started to shrink 8 d following rlipo-E7m/CpG treatment, but relapse of the tumors was observed on day 55 post-tumor implantation. Enhanced therapeutic effects were observed in mice that received treatment with Gem (3 mg/mouse) and rlipo-E7m/CpG. In these mice, the tumor size was reduced from 812.5 ± 213 mm\textsuperscript{3} on day 31 to 106.4 ± 62.3 mm\textsuperscript{3} on day 52 post-tumor implantation (Fig. 1a). This initial experiment suggested that a single dose of Gem and rlipo-E7m/CpG as a combination treatment may not have been sufficient. To evaluate the effects of two-dose immunization with rlipo-E7m/CpG combined with a single Gem treatment, tumor-bearing mice were immunized on days 25 and 30. Fig. 1b shows the dramatic regression of large tumors following two injections of rlipo-E7m/CpG on days 25 and 30. The tumor size shrank from 1307.4 ± 142.1 mm\textsuperscript{3} on day 32 to 288 ± 102.5 mm\textsuperscript{3} on day 50 post-tumor implantation. The combination of one dose of Gem with two doses of rlipo-E7m/CpG exerted similar effects to those of two injections of rlipo-E7m/CpG; for the former treatment, the tumor size was reduced from 926.3 ± 102.1 mm\textsuperscript{3} on day 32 to 117.8 ± 24.5 mm\textsuperscript{3} on day 53 post-tumor implantation (Fig. 1b). Thus, Gem administration did not increase the therapeutic effects of treatment with two doses of rlipo-E7m/CpG.

Additionally, the antitumor effects of the combined regimen were evaluated by performing immunization on day 21 followed by administration of various dosages of Gem (3 mg/mouse, 6 mg/mouse, or 9 mg/mouse) on day 50, as shown in Fig. 1c. The tumor size started to shrink 8 d following immunization with rlipo-E7m/CpG. These therapeutic effects were enhanced when Gem was administered on day 50. Interestingly, the administration of a high dose of Gem (6 mg/mouse or 9 mg/mouse) did not result in enhanced antitumor effects compared to the administration of a low dose of Gem (3 mg/mouse). The tumor volume at day 60 was approximately 1061 ± 120.3 mm\textsuperscript{3}, 473 ± 91.8 mm\textsuperscript{3}, 568 ± 136.8 mm\textsuperscript{3}, and 584.6 ± 106.2 mm\textsuperscript{3} in mice treated with rlipo-E7m/CpG alone, rlipo-E7m/CpG and 3 mg Gem, rlipo-E7m/CpG and 6 mg Gem, and rlipo-E7m/CpG and 9 mg Gem, respectively. These results indicated that the administration of Gem enhanced the therapeutic effects of rlipo-E7m/CpG treatment.

Performing multiple injections of Gem in combination with rlipo-E7m/CpG immunotherapy eradicates large tumors

Although one injection of Gem in conjunction with rlipo-E7m/CpG therapy led to the evident regression of large tumors, recurrence of the tumors was observed approximately 50 d post-tumor implantation, eventually resulting in the death of the mice. Therefore, we tested multiple dose regimens of Gem in combination with the immunotherapy to enhance the antitumor effects. We initiated the treatment of the tumor-bearing mice via immunization with rlipo-E7m/CpG on day 21, followed by three injections of Gem (3 mg, 6 mg, or 9 mg per mouse) on days 40, 43, and 46 post-tumor implantation (Fig. 1d). Tumor regression was observed following rlipo-E7m/CpG monotherapy or Gem and rlipo-E7m/CpG combination therapy (Fig. 1d). Surprisingly, the eradication of large tumors was observed in mice receiving the combined treatment (Fig. 1d). The tumor volume on day 60 was approximately 1073.4 ± 313.98 mm\textsuperscript{3} under rlipo-E7m/CpG treatment but was reduced to 153.5 ± 90.47 mm\textsuperscript{3}, 44.3 ± 19.94 mm\textsuperscript{3}, and 46.7 ± 24.67 mm\textsuperscript{3} under combined treatment with 3, 6, and 9 mg of Gem, respectively. Furthermore, we evaluated the therapeutic effects of these three doses of Gem prior to rlipo-E7m/CpG administration. Tumor-bearing mice were injected on days 21, 24 and 27 with Gem (3 mg/mouse) and then received a single injection of rlipo-E7m/CpG on day 30. The tumor volume shrank from 252 ± 56.8 mm\textsuperscript{3} on day 21 to 94.6 ±
29.2 mm³ on day 30 in the Gem monotherapy group, and tumor relapse occurred on day 35 post-tumor implantation (Fig. 1e). Tumor regression was not observed in mice immunized with rlipo-E7m/CpG on day 30 post-tumor implantation. Notably, the tumors completely regressed in mice that received three injections of Gem (3 mg/injection) on days 21, 24, and 27 followed by immunization with rlipo-E7m/CpG on day 30 post-tumor implantation (Fig. 1e). Combination therapy also induced higher levels of CTLs responses (Fig. S1). These results demonstrated that combination therapy consisting of Gem and rlipo-E7m/CpG enhanced the antitumor effects on mice bearing large tumors compared to either treatment alone.

**Gem reduces immunosuppressive cell numbers in tumor-bearing mice**

Our data showed that combination therapy dramatically inhibited TC-1 tumor growth. One explanation for this finding is that the TC-1 tumor is sensitive to Gem treatment (Fig. 1d and e). Further, because Gem is able to inhibit tumor-associated immunosuppressive cells [i.e., regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs)] in several murine tumor models and human cancers, we investigated whether the inhibition of immunosuppressive cells by Gem synergistically inhibits advanced tumor growth. To investigate the effects of Gem on immunosuppressive cells, mice received Gem on days 21, 24, and 27 after tumor implantation (Fig. 1e). Combination therapy also induced higher levels of CTLs responses (Fig. S1). These results demonstrated that combination therapy consisting of Gem and rlipo-E7m/CpG enhanced the antitumor effects on mice bearing large tumors compared to either treatment alone.
reduced to 10.98 ± 7.22% and 13.15 ± 5.15%, respectively, following treatment with Gem, compared to 28.31 ± 5.69% and 21.25 ± 4.65%, respectively, following the control treatment (Fig. 2c). The Treg population among TILs was decreased to 16.05 ± 8.51% of gated CD4+ cells following Gem treatment compared to 47.35 ± 10.39% following the control treatment (Fig. 2c). To clarify which subset of MDSCs among TILs was suppressed by Gem, the frequency of granulocytic MDSCs (G-MDSCs, CD11b+Ly6ClowLy6G+) and monocytic MDSCs (M-MDSCs, CD11b+Ly6ChiLy6G−) was determined via flow cytometry after treatment with Gem. We observed that the administration of Gem led to a decrease in the proportion of G-MDSCs (2.12 ± 1.22%) compared to the control treatment (7.03 ± 4.56%); however, no change in the frequency of M-MDSCs was observed (27.84 ± 12.79% vs. 30.61 ± 4.54%) (Fig. 2d). Gem treatment altered the frequencies of the CD11b and CD8+ populations among TILs (Fig. S2). These results indicate that the repeated administration of Gem concurrently reduced the numbers of systemic and intratumoral MDSCs, Tregs, and macrophages.

**Combination therapy increases the numbers of CD4+ and CD8+ T cells and decreases the numbers of immunosuppressive cells in tumor-bearing mice**

Next, we examined whether combination treatment with Gem and the recombinant lipoprotein immunotherapy increases effector cells and reduces immunosuppressive cells among splenocytes and TILs. The analysis of immune cell types was performed on day 43 after tumor implantation (Fig. 3a). We found that combination therapy, but not chemotherapy or immunotherapy alone, increased the frequency of splenic CD4+ T cells (12.38 ± 3.73%, 8.37 ± 3.16%, and 6.14 ± 4.14%, respectively)
Figure 3. Combination therapy with Gem and rlipo-E7m/CpG has a substantial impact on splenic and intratumoral immune cells. (a) Tumor-bearing mice received three injections of Gem (3 mg/injection) on days 21, 24, and 27 post-implantation of TC-1 cells (2 × 10^5/mouse). The mice were subsequently immunized with rlipo-E7m (10 μg/ml) in the presence of CpG OND (10 μg/ml) 3 d after the final Gem injection. (b) The percentages of splenic CD4^+ or CD8^+ T cells and immunosuppressive cells, including MDSCs, Tregs, and TAMs, were determined via flow cytometry on day 13 following immunization. (c) The percentages of tumor-infiltrating CD4^+ or CD8^+ T cells and immunosuppressive cells, including MDSCs, Tregs, and TAMs among infiltrating leukocytes (gated on CD45^+ cells), were determined via flow cytometry on day 13 following immunization. The data are presented as the mean number (±SD) of the indicated cell population. *p < 0.05; **p < 0.01; ***p < 0.001. The data were compiled from three independent experiments.

compared to the PBS control treatment (8.7 ± 4.34%) (Fig. 3b). Similarly, the frequency of splenic CD8^+ T cells was increased in mice that received combination therapy (11.26 ± 5.12%) compared to chemotherapy alone (5.02 ± 1.77%), immunotherapy alone (5.62 ± 3.9%) or PBS treatment (7.38 ± 2.64%). The combination therapy also led to a decrease in the percentage of splenic MDCSs compared to immunotherapy (12.86 ± 12.85% vs. 29.19 ± 17.71%, p < 0.01) or the PBS control (12.86 ± 12.85% vs. 27 ± 17.03%, p < 0.05). Interestingly, an increased percentage of splenic Tregs was observed in mice that received rlipo-E7m/CpG immunotherapy compared to Gem alone (19.36 ± 3.33% vs. 15.42 ± 2.25%, p < 0.05) or PBS treatment (19.36 ± 3.33% vs. 16.66 ± 2.71%, p < 0.05). In contrast, splenic Tregs were decreased in mice treated with the combined regimen (13.33 ± 3.9%) compared to immunotherapy alone or the control treatment. The percentage of macrophages in the rlipo-E7m/CpG group (8.55 ± 3.67%) was slightly higher than that in the Gem (4.72 ± 0.84%) and PBS groups (6.27 ± 3.04%) but was lower than that in the combined treatment group (4.97 ± 3.45%); however, these differences did not reach statistical significance (Fig. 2b). To assess whether these findings in the spleen were consistent with the characteristics of the tumor microenvironment, we examined the expression of T cells and immunosuppressive cells in tumor samples. Within the TC-1 tumor microenvironment, increased infiltrating CD4^+ T cells were observed in the Gem and rlipo-E7m/CpG group compared to the rlipo-E7m/CpG group (7.65 ± 2.98% vs. 4.16 ± 2.78%, P<0.01), but there was no significant difference between the Gem and rlipo-E7m/CpG group and the Gem (7.65 ± 2.98% vs. 6.16 ± 4.17%) or PBS group (7.65 ± 2.98% vs. 5.58 ± 2.58%) (Fig. 3c). Similarly, the frequency of infiltrating CD8^+ T cells was substantially increased in mice that received combined Gem and rlipo-E7m/CpG therapy compared to chemotherapy (26.28 ± 10.38% vs. 3.21 ± 1.34%, p < 0.001) or immunotherapy alone (26.28 ± 10.38% vs. 7.54 ± 3.63%, p < 0.001). Moreover, combination therapy markedly decreased the percentage of intratumoral MDSCs by approximately 2.5-fold and 2-fold compared to Gem alone and rlipo-E7m/CpG alone, respectively (the representative FACS picture is shown in Fig. S3a). In contrast to the results obtained in the spleen, the percentage of intratumoral Tregs in the rlipo-E7m/CpG group was decreased by 2.6-fold and 2.2-fold compared to PBS and Gem alone, respectively (Fig. 3c and S3b); there was no significant difference between rlipo-E7m/CpG and combined Gem and rlipo-E7m/CpG therapy. Additionally, the combination treatment significantly decreased tumor-infiltrating macrophages by 2.1-fold, 3.7-fold, and 3.2-fold compared to immunotherapy alone, the control group and Gem alone, respectively (Fig. 3c and S3c). These data indicated that the combination chemotherapy and immunotherapy alter the frequencies of immune cells in the tumor microenvironment in mice bearing large tumors (<500 mm^3). The increased numbers of effector cells and decreased numbers of immunosuppressive cells among TILs in mice receiving combination
therapy contributed to complete tumor regression, as shown in Fig. 1e.

Administration of Gem prior to immunotherapy increases the number and function of intratumoral antigen-specific CD8\(^+\) T cells

Although our data showed that combination therapy increases CD8\(^+\) T cells in the tumor microenvironment, we needed to determine whether the infiltrating CD8\(^+\) T cells were functional. To this end, a fluorescent species-conjugated MHC class I tetramer containing an E7-derived H-2D\(^{b}\)-restricted CTL epitope was used to detect antigen-specific CD8\(^+\) T cells. Fig. 4a shows the tumor-infiltrating CD8\(^+\) T cells from TC-1 tumor-bearing mice receiving the indicated treatment. Chemotherapy with Gem did not impact the frequency of E7-specific CD8\(^+\) T cells. However, immunotherapy with rlipo-E7m/CpG increased the percentage of intratumoral E7-specific CD8\(^+\) T cells by approximately 18-fold and 11-fold compared to Gem administration alone and the control treatment, respectively (Fig. 4b). Gem and rlipo-E7m/CpG combination therapy significantly increased the percentage of intratumoral E7-specific CD8\(^+\) cells by 2-fold compared to rlipo-E7m/CpG immunotherapy (Fig. 4b). Additionally, we analyzed the phenotypes of E7-specific CD8\(^+\) T cells in the TILs of the combination therapy and rlipo-E7m/CpG immunotherapy groups by performing intracellular cytokine staining. The TILs were restimulated with the HPV16E7\(^{49-57}\) peptide. As shown in Fig. 4c, the percentage of IFN\(\gamma\)^+ CD8\(^+\) cells among the E7-specific CD8\(^+\) cells in the combination therapy group was higher than that in the immunotherapy group (77.62 ± 18.38% vs. 61.1 ± 12.8%, \(p < 0.05\)). Accordingly, the number of IFN\(\gamma\)-secreting T cells in the Gem and rlipo-E7m/CpG group was higher than that in the rlipo-E7m/CpG alone group (Fig. S4), but the percentage of IL-10\(^+\) CD8\(^+\) cells among the E7-specific CD8\(^+\) cells in the combination therapy group was lower than that in the immunotherapy group (47.64 ± 15.15% vs. 33.13 ± 12.28%, \(p < 0.05\)). These data suggest that combination therapy with Gem and rlipo-E7m/CpG may increase the quantity and activity of intratumoral Ag-specific CD8\(^+\) T cells in this subcutaneous tumor model.

Combination therapy decreases PD-1\(^+\) Ag-specific CD8\(^+\) T cells in regressing tumors

In addition to immunosuppressive cells, PD-1\(^+\) CD8\(^+\) T cells were increased after the induction of strong CTL responses; these cells consequently inhibited CTL functions\(^{21,22}\). Thus, we investigated PD-1-expressing CD8\(^+\) T cells among TILs. Fig. 5a shows a representative dot plot. We found that the percentage

Figure 4. The Gem and rlipo-E7m/CpG combination therapy induces dramatic intratumoral antigen-specific CTL responses. Mice bearing subcutaneous tumors received three injections of Gem (3 mg/injection) on days 21, 24, and 27 and/or rlipo-E7m/CpG immunization on day 30 post-tumor implantation. (a) Representative dot plots and (b) quantification of tumor-infiltrating tetramer\(^+\) CD8\(^+\) T cells (gated on CD45\(^+\) cells) as determined by flow cytometry on day 43 post-tumor implantation. (c) Quantification of intracellular IFN\(\gamma\)^+ or IL-10\(^+\) expression on tetramer\(^+\) CD8\(^+\) T cells in tumors from tumor-bearing mice that received the indicated treatment. The percentage of tumor-infiltrating IFN\(\gamma\)^+ or IL-10\(^+\) CD8\(^+\) T cells among tetramer\(^+\) CD8\(^+\) T cells was determined via flow cytometry on day 13 following immunization. The data are presented as the mean number (±SD) of the indicated cell population. *\(p < 0.05\); **\(p < 0.001\); ***\(p < 0.0001\). The data were compiled from three independent experiments.
of PD-1<sup>+</sup> CD8<sup>+</sup> cells among intratumoral CD8<sup>+</sup> T cells was moderate in the Gem alone group (14.5 ± 11.6%) or the control group (19.94 ± 12.61%). Surprisingly, the percentage of PD-1<sup>+</sup> CD8<sup>+</sup> cells in both the rlipo-E7m/CpG group (46.41 ± 20.8%) and the combination group (47.69 ± 17.31%) was increased by approximately 2–3-fold compared to the control group (Fig. 5b). Thus, immunotherapy may increase not only CD8<sup>+</sup> T cells but also negative regulatory PD1<sup>+</sup> CD8<sup>+</sup> T cells among TILs. We further analyzed the PD-1<sup>+</sup> cells in the antigen-specific CD8<sup>+</sup> T cell population. The data showed that combination therapy decreased the percentage of PD-1<sup>+</sup> E7-specific CD8<sup>+</sup> cells compared to immunotherapy alone (63.55 ± 21.7% vs. 90.18 ± 7.24%, p < 0.001) (Fig. 5c). These results indicated that combined therapy not only increased CTL infiltration into the tumors but also decreased the frequency of broad-range immunosuppressive cells in the tumor microenvironment. Based on these observations, we tested whether the administration of an anti-PD1 antibody enhances the efficacy of rlipo-E7m/CpG-based immunotherapy in the absence of the chemo- therapeutic drug. A monoclonal anti-PD1 blocking antibody or an isotype control antibody was administered following rlipo-E7m/CpG immunotherapy to evaluate the resulting antitumor effects. The tumor began to regress on day 28 (tumor size 955 ± 177.64 mm<sup>3</sup>) but began to re-grow by day 45 (605.74 ± 374.5 mm<sup>3</sup>) despite two injections of the anti-PD1 antibody into the mice following the rlipo-E7m/CpG therapy. There was no significant difference between the anti-PD1 and rlipo-E7m/CpG group and the rat IgG and rlipo-E7m/CpG group (Fig. 5d). These data indicated that the blockade of PD1 using a monoclonal antibody (mAb) did not enhance the rlipo-E7m/CpG immunotherapy-mediated antitumor effects in mice bearing TC-1 tumors. However, the decreased expression of PD1 on E7-specific CD8<sup>+</sup> cells may represent the state at which the immune checkpoint was reversed in the tumor microenvironment during combination treatment with Gem and rlipo-E7m/CpG.

**Combination therapy with Gem and rlipo-E7m/CpG decreases immunosuppressive cells and increases Ag-specific CD8<sup>+</sup> T cells in an orthotopic cervical cancer model**

The administration of Gem chemotherapy followed by rlipo-E7m/CpG vaccination substantially decreased immunosuppression and increased antitumor immunity in the subcutaneous TC-1 model. To investigate whether these effects of this combination therapy also could be observed
in HPV-associated genital tumors, an orthotopic cervical cancer model was used. Groups of mice were intravaginally (IVAG) implanted with TC-1 tumor cells, followed by Gem and rlipo-E7m/CpG therapy. Intratumoral leukocytes were analyzed via flow cytometry, as depicted in Fig. 6a. Analysis of the genital tumors showed no significant change in the percentage of MDSCs after treatment with rlipo-E7m/CpG (42.43 ± 4.91%) or both Gem and rlipo-E7m/CpG (37.74 ± 11.16%) compared with PBS treatment (43.64 ± 8.4%, Fig. 6b, left panel). However, the frequency of Tregs was significantly decreased in both the rlipo-E7m/CpG group (20.52 ± 7.45%) and the Gem and rlipo-E7m/CpG group (15.04 ± 7.37%) compared with the PBS group (42.1 ± 7.4%), although there was no significant difference between the rlipo-E7m/CpG group and the Gem and rlipo-E7m/CpG group (Fig. 6b, middle panel). There were no significant differences in TAMs following treatment with rlipo-E7m/CpG (21.11 ± 7.15%) or both Gem and rlipo-E7m/CpG (15.16 ± 8.42%) compared with PBS treatment (24.55 ± 8.6%) (Fig. 6b, right panel). Analysis of the PD-1\(^+\)CD8\(^+\) T cell population among TILs indicated that the percentage of PD-1\(^+\)E7-specific CD8\(^+\) T cells was similar between the rlipo-E7m/CpG group and the Gem and rlipo-E7m/CpG group (80.18 ± 11.99% vs. 74.17 ± 13.82%). There was no significant difference in the E7-specific CD8\(^+\) T cell numbers between the groups (62 ± 17.57% vs. 71.24 ± 13.58%). Interestingly, the percentage of PD-1\(^+\)E7-specific CD8\(^+\) T cells in the rlipo-E7m/CpG group was higher than that in the Gem and rlipo-E7m/CpG group (91.45 ± 8.14% vs. 83.99 ± 7.27%, p < 0.01). These results indicated that Gem did not have an inhibitory effect on immunosuppressive cells in intravaginal cancer. However, Gem did reduce the number of PD-1\(^+\) E7-specific CD8\(^+\) T cells when combined with rlipo-E7m/CpG.

**Gem in combination with rlipo-E7m/CpG immunotherapy improves the survival of mice with established intravaginal tumors**

Having observed the regression of large tumors following combination therapy with Gem and rlipo-E7m/CpG in the subcutaneous TC-1 tumor model, we tested this approach in the orthotopic cervical cancer model. Mice with intravaginal tumors 50 mm\(^3\)–150 mm\(^3\) in volume were randomly allocated for treatment with both Gem and rlipo-E7m/CpG, rlipo-E7m/CpG alone, Gem alone, or the PBS control. We observed a significant increase in the overall survival of mice that received both Gem and rlipo-E7m/CpG compared to the control (p = 0.0052, log-rank Mantel-Cox). The survival rates on day 80 in the Gem and rlipo-E7m/CpG group and the rlipo-E7m/CpG group were 67% and 20%, respectively (Fig. 6d). These data

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**Figure 6.** Administration of Gem followed by rlipo-E7m/CpG therapy decreases PD-1 expression on infiltrating E7-specific CD8\(^+\) T cells and improves survival in an orthotopic tumor model. (a) Groups of mice were treated with Gem (3 mg/injection) on days 17, 20, and 23 via i.p. injection and/or were s.c. immunized with rlipo-E7m/CpG on day 25 after IVAG TC-1 implantation. Mice were sacrificed on day 35, and cells from the tumors were stained with antibodies against the indicated markers. (b) The percentage of tumor-infiltrating MDSCs, Tregs, and TAMs (gated on CD45\(^+\) cells) was determined via flow cytometry. (c) The percentages of tetramer\(^+\) CD8\(^+\) cells among total CD8\(^+\) T cells, PD-1\(^+\) CD8\(^+\) T cells among total CD8\(^+\) T cells, and PD-1\(^+\)-specific CD8\(^+\) T cells among total CD8\(^+\) T cells within the tumor infiltrate are shown for the indicated treatments. The data are presented as the mean number (±SD) of the indicated cell population. \(*p < 0.05; **p < 0.001; ***p < 0.0001.\) The data were compiled from three independent experiments. (d) Groups of mice were IVAG challenged with TC-1 cells according to the treatment schedule shown in (a). Overall survival of the mice bearing genital tumors is shown. Kaplan–Meier analysis was performed on the survival data (p < 0.05, rlipo-E7m/CpG vs. Gem and rlipo-E7m/CpG).
suggest that therapy consisting of three consecutive administrations of Gem in combination with rlipo-E7m/CpG immunotherapy represents a promising approach for inducing the regression of intravaginal cancer.

Discussion

Although rlipo-E7m/CpG immunotherapy leads to dramatic tumor regression in mice bearing large tumors, the resurgence of tumor growth reveals that additional treatment modalities are necessary. This study demonstrated the potential feasibility of combination therapy consisting of chemotherapy and recombinant lipoinmunogen protein-based immunotherapy for the treatment of advanced cancers. Our data showed that the combination of Gem and rlipo-E7m/CpG, but not Gem or rlipo-E7m/CpG monotherapy, led to tumor eradication (Fig. 1). As an explanation of the mechanisms underlying these impressive therapeutic effects, we propose that Gem administration alters the tumor microenvironment (Fig. 2) by decreasing the numbers of immunosuppressive cells, such as MDSCs, TAMs, and Tregs, thereby facilitating the eradication of tumor cells by a subsequent immunotherapy that induces efficient antigen-specific CTL responses. Similar to our observations, Kang, T. H. et al. demonstrated that cisplatin chemotherapy converted the immunosuppressive state in the tumor environment to a state permissive of the activation of antitumor responses mediated by peptide-based immunotherapy. Additionally, Fridlender et al. demonstrated that administering sequential courses of chemotherapy (cisplatin/Gem) after immunotherapy markedly boosted antitumor responses via the augmentation of the cross-priming of released antigen-specific CD8+ T cells in the tumor environment compared to immunotherapy alone.5

In this study, tumor eradication was observed in mice when Gem was administered prior to or after rlipo-E7m/CpG immunotherapy (Fig. 1d, e). These results suggest that the doses and schedules of combination therapy consisting of chemotherapy and immunotherapy should be optimized based on considerations of how the chemotherapeutic agent impacts vaccine-induced tumor immunity, which is an important issue for the design of combination regimens.

Our previous results showed that treatment with rlipo-E7m/CpG reduced the number of immunosuppressive cells and inhibited tumor growth in mid-sized tumors (6–8 mm in diameter).5 Unexpectedly, rlipo-E7m/CpG treatment of a huge tumor (<12 mm in diameter) did not result in any tumor regression despite the induction of significant intratumoral Ag-specific CD8+ T cells compared to Gem treatment alone or the control treatment (Fig. 1e and Fig. 4b). Combination therapy increased the number of IFNγ+ E7-specific CD8+ T cells and decreased the number of immune inhibitory IL-10+ E7-specific CD8+ T cells in the tumor environment compared to immunotherapy alone (Fig. 4c). Moreover, we observed that rlipo-E7m/CpG monotherapy on day 30 did not reduce the quantity of tumor-infiltrating MDSCs, splenic MDSCs, Tregs, or TAMs (Fig. 3). In contrast, the combination therapy reduced the percentages of splenic and tumor-infiltrating MDSCs, Tregs, and TAMs. These data indicate that the tumor subsists in a highly immunosuppressive state that might be reversed to an immunocompetent state via chemotherapeutic intervention. In addition to converting the immunosuppressive milieu, chemotherapy may impact the function of tumor-infiltrating CTLs, which underlies definitive tumor rejection. Interestingly, the combination therapy also displayed enhanced therapeutic effects in an orthotopic cervical cancer model (Fig. 6d), although only Treg cells were reduced in the orthotopic model after treatment with rlipo-E7m/CpG alone or in combination with Gem (Fig. 6b). The different inhibitory effects of Gem on the subcutaneous and orthotopic cancer models may be due to differences in tumor size. These data suggest that the reduction in Tregs and PD-1+ CD8+ T cells was more important in the orthotopic cervical cancer model (Fig. 6b and c).

A strategic combination of cancer vaccines with conventional treatment modalities (especially chemotherapeutic drugs) has the potential to affect the local tumor microenvironment and immunity via ancillary and unappreciated immunological responses by the tumor-bearing host. Recently, the immune-checkpoint receptors PD-1 and CTLA-4 have been studied intensively in the field of cancer immunotherapy. Anti-PD1 or anti-CTLA4 therapy substantially enhanced the vaccine-induced antitumor effects on several low immunogenic cancers.24–27 Despite the striking clinical benefits observed from the use of immune checkpoint inhibitors to block PD-1 or CTL-4 and restrain T-cell-mediated antitumor immunity, a large proportion of patients with advanced cancer failed to respond to these immunotherapeutics.28,29 Thus, immune checkpoint blockade requires additional treatment modalities to improve antitumor efficacy. The increased PD-1 expression observed on intratumoral CD8+ T cells in our immunotherapy or chemoimmunotherapy group may reflect an exhausted or anergic state of the effector T cells; this finding prompted our investigations into the use of combinatorial immunotherapy with the rlipo-E7m/CpG cancer vaccine and an anti-PD1 mAb (Fig. 5b). Unexpectedly, the administration of an anti-PD1 blocking antibody did not enhance the therapeutic effects compared to rlipo-E7m/CpG therapy alone in our animal model of cervical cancer, whereas complete tumor rejection was observed in mice that received Gem and rlipo-E7m/CpG combination therapy (Fig. 5d and Fig. 1e). Although there was no significant difference in the percentage of PD-1-expressing CD8+ T cells between rlipo-E7m/CpG and combined Gem and rlipo-E7m/CpG, the percentage of intratumoral PD-1-expressing E7-specific CD8+ T cells was dramatically decreased in the combined Gem and rlipo-E7m/CpG therapy group (Fig. 5c). Cancer immunotherapy induces antitumor responses that may not lead to tumor regression owing to the upregulation of immune-checkpoint ligands—a process termed adaptive immune resistance.30 Therefore, the expression pattern of PD-1 ligands may be another determining factor of the suitability of anti-PD1 therapy because the anti-PD1 blocking antibody inhibits the lymphocyte by engaging PD-1 and its ligands. Accordingly, similar observations were found in an orthotopic cervical cancer model treated with combination therapy (Fig. 6). Our data provide new mechanistic insight suggesting that Gem administration may reduce cancer vaccine-induced PD-1 expression on specific CTLs to facilitate tumor rejection.
In addition to the Gem-induced reduction of tumor-infiltrating immunosuppressive cells, we noted that three doses of Gem were able to mildly inhibit TC-1 tumor growth (Fig. 1e). The tumor inhibitory effects of Gem may contribute another benefit to the combination therapy. Although we immunized mice on day 3 after Gem therapy, it appeared that the chemotherapeutic drug treatment did not suppress the effects of immunotherapy. This observation may be due to the rapid metabolic rate of Gem in the mouse.31 We do not know whether other chemotherapeutic drugs have effects similar to those of Gem. Our data demonstrated that treatment with Gem reduced tumor-infiltrating immunosuppressive cells and increased functional tumor-infiltrating CTLs. The identification of the optimal treatment schedule for combination therapy is critical for the successful eradication of the tumor. The combination therapy of Gem and recombinant lipoimmunogen-based therapy could be applied to future immunotherapy in human studies.

Materials and methods

Mice

Female 6-week-old C57BL/6 mice were obtained from the National Laboratory Animal Center, Taiwan. The mice were housed in the Animal Facility of the National Health Research Institutes. Protocols for the use of experimental mice were approved by the Institutional Animal Care and Use Committee (IACUC) of the National Health Research Institutes (NHRI). For experimentally induced neoplasia in mice, the allowable tumor burden and the criteria for euthanasia complied with the NCI Frederick ACUC Guidelines (Involving Experimental Neoplasia Proposals in Mice and Rats, 2006). The survival time of tumor-bearing mice was determined based on 20% weight loss, unexpected morbidity or an inability to obtain food or water.

Cell line

The TC-1 cell line (primary lung epithelial cells from C57BL/6 mice immortalized with HPV-16 E6/E7 and transformed with the c-Ha-ras oncogene) was obtained from Dr T.-C. Wu32 in 1997 and established in a master cell bank for tumor model in 2008. TC-1 cells were mycoplasma-free when the working cell bank was established in 2012. The expression of HPV-16 E7 and MHC class I molecule H-2D\(^b\) was confirmed in 2014. Tumor implantation was carried out within six subculture passages after thawing from the established working cell bank. Before the animal studies, TC-1 cells were confirmed as mycoplasma-free by PCR. The cells were grown in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, 50 units/mL penicillin/streptomycin, 0.5 mM sodium pyruvate, 20 mM HEPES (Biological industries, Beit Haemek, Israel), and 0.5 \(\mu\)M \(\beta\)-mercaptoethanol at 37°C in 5% CO\(_2\).

Reagents

The chemotherapeutic agent Gem was purchased from LC Laboratories\(^\circ\). CpG ODN was purchased from GeneDireX\(^\circ\). The CpG ODN consisted of a sequence of 5’-TCC ATG ACG TTC CTG ACG TT-3’ fused to a phosphorothioate backbone. The antibodies used in this study (with their respective clones in parentheses) were anti-CD16/32 (2.4G2), anti-CD4\(^+\) (GK1.5), anti-Ly-6C (HK1.4), anti-F4/80 (BM8), anti-Gr-1 (RB6-8C5), anti-CD11b (M1/70), anti-IFN\(\gamma\) (XMG1.1), anti-CD25 (PC61.5), anti-Foxp3 (FJK-16s) (all purchased from eBioscience\(^\circ\)), anti-CD8 (53-6.7), anti-Ly-6G (1A8) (BD Pharmingen\(^\text{TM}\)), and anti-CD45 (EM-05) (GeneTex, Inc.). The PE-conjugated HPV16E7\(_{19-57}\)/MHC I tetramer was purchased from Beckman Coulter, Inc. The LEAF\(^\text{TM}\) purified anti-mouse PD-1 antibody (RMP1-14) was purchased from Biolegend\(^\circ\). The synthetic peptide E7\(_{49-57}\) (RAHYNIVTF) corresponding to the H-2D\(^b\)-restricted epitope of HPV16-E7 was chemically synthesized by the peptide facility of NHRI (Taiwan). The peptides were dissolved in 50% DMSO (Sigma-Aldrich) in water at a concentration of 10 mg/mL and stored at −80°C until use.

In vivo tumor studies

Female, 6- to 8-week-old naïve C57BL/6 mice were subcutaneously implanted with TC-1 cells \((2 \times 10^5 \text{cells per mouse})\) into the left flank prior to chemotherapy or immunization. To evaluate the antitumor effects of the Gem and rlipo-E7m/CpG combination treatment, tumor-bearing mice were arbitrarily assigned to groups (six mice per group) and then administered Gem \((120 \text{mg/kg})\) via intraperitoneal (i.p) injection on days 21, 24, and 27 and/or subcutaneously (s.c.) administered recombinant lipoimmunogen (rlipo-E7m, 10 \(\mu\)g per mouse) in the presence of CpG ODN \((10 \mu\text{g per mouse})\) in a total volume of 100 \(\mu\text{L}\) in PBS into the dorsum of each mouse on day 30 after implantation with TC-1 tumor cells. To evaluate the antitumor effects of combined immunotherapy and anti-PD1 treatment, mice were s.c. immunized with rlipo-E7m/CpG on day 21 and/or administered an i.p injection of anti-PD1 (250 \(\mu\text{g per mouse})\) on days 24 and 28 post-tumor implantation. The tumors were measured using electronic calipers three times weekly to monitor tumor growth. The tumor volume was calculated using the following formula: length \(\times\) width\(^2\) \(/\) 2. The mice were euthanized when the tumors reached 2.0 cm in diameter.

Orthotopic tumor model

Female 6- to 8-week-old C57BL/6 mice were synchronized in a diestrus-like state via s.c. injection with 0.1 \(\mu\text{g}\) \(\beta\)-estradiol (Sigma-Aldrich). The mice received 2 mg of Depo-Promone (Pfizer) diluted in 100 \(\mu\text{L}\) sterile PBS via s.c. injection one day after \(\beta\)-estradiol administration. Four days after Depo-Promone injection, the mice were vaginally treated with 50 \(\mu\text{L}\) of 4% nonoxynol-9 (N-9) (Santa Cruz\(^\circ\)) dissolved in 4% carboxymethylcellulose (Sigma-Aldrich) for 5–6 h. Then, the N-9 was removed by washing with 2 mL of PBS, and a cytobrush was inserted in the vagina and twirled clockwise and counterclockwise 10 times prior to inoculation with \(2 \times 10^5\) TC-1 cells in the cervix-vagina site of deeply anesthetized mice. To evaluate the antitumor effects of rlipo-E7m/CpG in combination with Gem on an orthotopic (IVAG) model of TC-1-bearing mice, the mice received three injections of Gem \((120 \text{mg/kg})\) on days...
17, 20, and 23 and/or were immunized with rlipo-E7m/CpG on day 25 post-tumor implantation.

Cell preparation and staining for flow cytometry

Cell surface molecule staining and flow cytometry were performed as previously described. Briefly, cell suspensions of RBC-lysed splenocytes or tumor cells derived from tumor-free and/or tumor-bearing mice were stained with FITC-conjugated anti-CD44, PE-conjugated anti-CD25 and PE-Cy5-conjugated anti-Foxp3 antibodies to quantify Tregs; PE-conjugated anti-CD11b and PE-Cy7-conjugated anti-Gr-1 antibodies to quantify MDSCs; FITC-conjugated anti-F4/80; and PE-conjugated anti-CD11b antibodies to quantify TAMs; FITC-conjugated CD8+ and PerCP-Cy5.5-conjugated PD-1 to quantify PD-1-expressing CD8+ T cells; and an APC-conjugated anti-CD45 antibody to quantify TILs. For the intracellular detection of IFN-γ- or IL-10-secreting CD8+ T cells in the tumor infiltrate, the tumor mass from Gem-treated (days 21, 24, and 27) and/or rlipo-E7m/CpG-immunized (day 30) C57BL/6 mice was mechanically disrupted into fragments on days 40–43 after tumor implantation. The obtained cell suspension was subjected to RBC lysis and filtration through a 0.7 μm strainer (Greiner Bio-One), followed by re-stimulation with 10 μg/mL E7 peptide (HPV16 E749-57) together with Brefeldin A solution (eBioscience®) and Ionomycin (Sigma Aldrich®) for 5 h. Then, the cells were harvested and incubated in the FITC-conjugated anti-CD8+ and PerCP-Cy5.5-conjugated anti-IFN-γ or anti-IL-10 antibody using a standard intracellular protocol. The TILs were primarily with a PE-conjugated H-2Dβ/RAH tetramer and the anti-CD8+–FITC antibody to quantify E7-specific CD8+ T cells. The percentage of H-2Dβ/RAH+ CD8+ T cells was determined via flow cytometry (FACSCalibur, BD Bioscience, San Jose, CA, USA). All data were acquired using a FACSCalibur device and were plotted using FCS express version 3.0 software (research edition, De Novo softwareTM). All analyses were conducted on a gated leukocyte population; the gating scheme is shown in Fig. S5.

In vivo cytolytic assay

To investigate the antigen-specific cytolytic activity of immunized mice in vivo, specific or irrelevant peptide-pulsed syngeneic splenocytes were used as target cells in this assay. The RBC-lysed splenocytes were counted and divided into two equal portions. These two portions were incubated at a density of 2 × 10^7 cells/mL with a specific peptide (HPV16 E749-57) or an irrelevant peptide (OVA257-264). After the splenocytes were washed in PBS, irrelevant and RAH peptide-pulsed cells were labeled at a final concentration of 0.5 μM or 5 μM of CFSE (Molecular Probes, Eugene, OR, USA) for 10 min at 37°C. The irrelevant and specific peptide-pulsed splenocytes were re-suspended in PBS at 2 × 10^6 cells/mL and remixed at a ratio of 1:1. To detect deviation from two peptide-pulsed target cells, the cell mixture was adoptively transferred into immunized mice via tail vein injection 10 d after the rlipo-E7m/CpG immunization. The experimental cells were harvested 18 h after adoptive transfer and analyzed using a FACSCalibur flow cytometer (BD Bioscience). The percentage of specific lysis was calculated using the following equation: % specific lysis = \left(1 - \frac{\text{CFSE} \text{ in target cells} \text{ without peptide} \text{ pulsed}}{\text{CFSE} \text{ in target cells} \text{ with peptide} \text{ pulsed}}\right) \times 100.

Statistical analyses

Statistical analyses were performed using Prism version 5.02 (GraphPad Software). Kaplan–Meier nonparametric regression analysis was performed to determine the survival rates of tumor-bearing mice; significance was determined using the log-rank test. The statistical significance of the differences between the groups was assessed using a two-tailed unpaired Students t-test. The differences were considered to be significant when p < 0.05.

Disclosure of potential conflicts of interest

SJ Liu and CH Leng are inventors of the patents (US7833776, US8426163, US8658176, and EP2445927) that related to the recombinant lipoinmunogen. All patents are held by the National Health Research Institutes. The authors have no other financial conflict with the subject matter or materials discussed in the manuscript.

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