RESEARCH ARTICLE

Anti-tumor Efficacy of a Hepatocellular Carcinoma Vaccine Based on Dendritic Cells Combined with Tumor-derived Autophagosomes in Murine Models

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Abstract

The majority of hepatocellular carcinoma (HCC) patients have a poor prognosis with current therapies, and new approaches are urgently needed. We have developed a novel therapeutic cancer vaccine platform based on tumor cell derived autophagosomes (DRibbles) for cancer immunotherapy. We here evaluated the effectiveness of DRibbles-pulsed dendritic cell (DC) immunization to induce anti-tumor immunity in BALB/c mouse HCC and humanized HCC mouse models generated by transplantation of human HCC cells (HepG2) into BALB/c-nu mice. DRibbles were enriched from H22 or BNL cells, BALB/c-derived HCC cell lines, by inducing autophagy and blocking protein degradation. DRibbles-pulsed DC immunization induced a specific T cell response against HCC and resulted in significant inhibition of tumor growth compared to mice treated with DCs alone. Anti-tumor efficacy of the DCs-DRibbles vaccine was also demonstrated in a humanized HCC mouse model. The results indicated that HCC/DRibbles-pulsed DCs immunotherapy might be useful for suppressing the growth of residual tumors after primary therapy of human HCC.

Keywords: Hepatocellular carcinoma - dendritic cells - autophagy - immunotherapy - tumor antigen - cross-presentation

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Introduction

Hepatocellular carcinoma (HCC) is a highly malignant type of cancer with a poor prognosis. It poses a threat to human health all over the world (Bosch et al., 1999). Curative resection or liver transplantation are the preferred treatments for patients with liver cancer, but because of multiple intra- and extra-hepatic metastasis, lack of donors, and various other factors, surgical treatment is possible in only a few newly diagnosed patients. In addition, the clinical evidence shows that the majority of liver cancer patients have poor responses to chemotherapy and radiotherapy. Therefore, new methods and means are needed for the clinical treatment of liver cancer. For example, biotherapy, which includes adoptive immunotherapy (Takayama et al., 2000), DNA-based immunotherapy (Grimm et al., 2000) or sorafenib (Llovet et al., 2008) is considered to have maximum specificity and minimum toxicity. Promising results of animal and preclinical studies have prompted interest in development of therapeutic vaccines based on tumor antigen pulsed dendritic cells (DCs) (Butterfield, 2004).

During the process of tumor progression, tumor antigens are not effectively presented to T cells and thus escape immune surveillance. This may be a consequence of weakened expression of tumor antigen or defects of antigen-presenting cells (APCs) in the host. Lack of an effective immune response leads to tumor formation and development. Currently, tumor antigen-based DC vaccines are considered to be one of the most effective cancer vaccines, and an enormous amount of basic research and numerous clinical trials are being undertaken (Gilboa, 2007). The functioning of DC vaccines depends on effective antigen presentation cells, and the full and effective expression of tumor antigens. The efficient cross-presentation of tumor associated antigen (TAA) is pivotal for the success of cancer vaccines. Cross-presentation involves antigen internalization, processing, and the presentation of peptides on MHC I molecules by DCs (Burgdorf et al., 2008).

Autophagy is a fundamental cellular process of packaging misfolded proteins or damaged organelles in autophagosomes and degrading them through fusion with lysosomes. We have previously shown that large numbers of autophagosomes containing abundant ubiquitinated antigens and short-lived proteins accumulated if

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autophagy was induced and protein degradation blocked through proteasome and lysosome inhibition. These autophagosomes were termed "Drips in blebbles" or "DRibbles" for defective ribosomal products (DRiPs) containing blebs (Li et al., 2008). In this study, we chose H22 and BNL hepatoma cell lines generated from BALB/c mice as target cells for production of DRibbles. Proteasome inhibitor was added to block the degradation of short-lived proteins and impel them to enter the degradation pathway of autophagy. An autophagy inducer was used to promote the formation and release of autophagosomes, and NH4Cl was added to inhibit lysosome functioning and prevent digestion of proteins in the autophagosomes. Natural and induced autophagosomes (DRibbles) were thus prepared and purified for use as "informational" antigen carriers.

The tumor cell derived DRibbles were tested as antigen carriers in cross-priming antigen-specific T cell responses to determine whether they were an optimal vaccine candidate. Additionally, to study its anti-tumor effect, a DCs-DRibbles vaccine was prepared and used in mouse subcutaneous H22 and BNL tumor models. Moreover, in order to mimic more ''human-like conditions,'' we established a humanized hepatocellular carcinoma model in nude mice to further test the anti-tumor efficacy of DCs-DRibbles vaccine by using the human HCC cell line HepG2 cells as the target cell. The objective was to establish the theoretical and experimental basis for a new biological treatment for liver cancer. We hope that this will prove to be a promising vaccine strategy for clinical study as a cure of hepatocellular carcinoma in the near future.

Materials and Methods

Mice, cell lines, and cell culture

BALB/c mice (female, 6–8 weeks of age) and BALB/c-nu mice (female, 4 weeks of age) were purchased from Yangzhou University and maintained under specific pathogen-free conditions. All mice were maintained in accordance with the protocol approved by the Animal Care and Use Committee of the Southeast University Animal Center. The BNL cell line, a BALB/c mouse background hepatoma, was established by and kindly provided by Dr Shu-Hsia Chen (Mount Sinai School of Medicine, USA). The tumor cells were maintained in RPMI 1640 media (Gibico) supplemented with 10% FCS (Hangzhou Sijiqing Biological Engineering Materials Co), penicillin (100 units/ml), and streptomycin (100 μg/ml) (Beyotime Institute of Biotechnology) at 37˚C, 5% CO_2.

In vivo induction of DCs by hydrodynamic injection

Dendritic cells were induced in vivo through combined injection of GM-CSF and Flt3L expression plasmids. Flt3L (2–6 μg) and GM-CSF (2–6 μg) plasmids suspended in a volume of PBS equivalent to 10% of the mouse body weight were injected on day 1 and day 10 into a tail vein in 5–10 seconds. Spleens from injected mice were harvested on day 15, splenocytes were isolated and red cells lysed with Red Cell Lyse Buffer (Biyun Tian Biotechnology). Single-cell suspensions were washed in PBS and counted. Cells were frozen in medium containing 10% added DMSO (Dimethyl sulfoxide, Sigma) and stored in a Cryo1˚C Freezing Container (Nalgene; Thermo Fisher Scientific). The immediate yield of viable thawed cells was 80–90%.

Flow cytometry assay

The DRibbles derived from H22 cells were labeled with 10 μM CFSE (carboxy-fluorescein diacetate succinimidyl ester, BD) for 15 min in room temperature and then co-cultured with DCs at 37˚C. After 12 h of incubation, DCs were harvested, washed, and stained with PE (phycoerythrin)-conjugated CD11c antibody (BD PharMingen) for 20 min at 4˚C in the dark. The phagocytosis of DRibbles by DCs was analyzed by flow cytometry (BD FACSCalibur, and CellQuest Plot software, Becton Dickinson), measuring the mean fluorescent intensity of CFSE on $CD11c^+$ cells.

Preparation of DRibbles

Briefly, H22 and BNL mouse hepatocellular carcinoma cells were cultured in RPMI 1640 complete medium containing 10% FBS, 100 U/ml of penicillin, 100 mg/ml of streptomycin). HepG2 human hepatocellular carcinoma cells were cultured in DMEM complete medium containing 10% FBS, and treated with rapamycin (Enzo Life Sciences), bortezomib (Velcade, Millennium Pharmaceuticals) and NH4Cl for 16 to 24 h. The resulting suspension was pre-cleared by centrifugation at 1600 rpm for 10 min, and the supernantant was then separated from the crude autophagosome-containing large vesicles (DRibbles) consisting of cytosolic components by a 30 min centrifugation at 12,000 rpm followed by washing with PBS and a second 30 min centrifugation at 12,000 rpm. The DRibbles were than aliquoted into tubes for freezing. The total protein concentration was measured by BCA assay according to the manufacturer's protocol (BCA Protein Assay Kit, Thermo). DRibbles were resuspended by PBS with the total concentration of 1 mg/ml and kept at -20˚C for short-term storage (less than 1 month) or -80˚C for long-term storage.

Western blotting

H22-derived DRibbles and H22 cells (drug-treated or untreated) were lysed in RIPA buffer (Pierce). The lysates were mixed with 5X SDS sample loading buffer, and samples (15 μl each) were resolved by 15% SDS-PAGE (Invitrogen). Proteins were transferred to a nitrocellulose membrane, incubated with blocking buffer (3% BSA-TBST) for 1 h, incubated with primary antibody overnight, and with HRP-conjugated secondary antibodies for 1 hr. Protein bands were revealed by using chemiluminescent reagents (Pierce). The primary antibodies included rabbit anti-mouse LC-3II antibody (1:1,000, Sigma), The secondary antibodies were goat–anti-rabbit–HRP (1:5000, eBioscience).

Transmission electron microscopy

DRibbles containing autophagosomes were prepared from the culture media of murine H22 tumor cells treated with rapamycin, bortezomib and NH4Cl for 16 to 24 h as described above. DRibble samples were collected by centrifugation and fixed in 2.5% glutaraldehyde.

Samples were rinsed in water, dehydrated, infiltrated, and embedded in resin. After polymerization, and sectioning, 60–80 nm ultrathin sections were stained in lead citrate, rinsed, post stained in uranyl acetate, rinsed, and dried. Sections were photographed at original magnifications of 1,000× to 80,000×.

Fluorescence microscopy

Smear preparations of DRibbles derived from H22 cells treated with or without drugs on glass slides were fixed in 4˚C acetone for 15 min and stained with FITC(fluorescein isothiocyanate) -LC3-II antibody at 37˚C for 30 min. Photographs of DRibbles were taken using a Zeiss inverted microscope capable of digital epifluorescence imaging.

Immunization and detection of immune responses

DCs (20 μl samples containing 106 cells) pulsed by H22-derived DRibbles (20 μg total protein) and the same volume of PBS were injected directly into both inguinal lymph nodes of BALB/c mice on day 1, and were boosted by 100μl DCs-DRibbles or PBS subcutaneously on day 2, day 3 and day 7. The mice were sacrificed on day 15, and both inguinal lymph nodes and spleens were collected. Red cells were lysed by Red Cell Lyse Buffer, lymphocytes were resuspended in RPMI 1640 complete medium and seeded into 24-well plates. H22-derived DRibbles (30, 10, 3, and 0 μg total protein/ml), inactivated H22 cells and H22 cell lysates (30μg total protein/ml) were added to the cell cultures. After 72 h of incubation, the cell culture supernatants were collected, and the concentration of IFN-γ was measured by ELISA (eBioscience). Similarly, the BALB/c mice were vaccinated with DCs loaded with DRibbles derived from BNL cells using the same schedule and dose. The lymphocytes were collected and restimulated with BNL-derived DRibbles, inactivated BNL cells or complete medium. IFN-γ was measured after 72 h. The lymphocytes were selected using $CD8^+$ cells and $CD4^+$ cells on magnetic beads (Dynabeads FlowComp Mouse CD8, Invitrogen) and co-cultured with different antigens. IFN-γ was measured after 72 h of co-incubation.

Immunotherapy experiments on mouse HCC models

For treatment of H22 primary tumors, BALB/c mice were immunized with DCs-DRibbles (2×106 DCs and 200μg DRibbles) subcutaneously into both flanks on day 1–day 3, and boosted on day 7. The mice were challenged with 106 H22 cells subcutaneously 1 week after vaccination. DC alone(2×106 DCs) and PBS were administered in the same way on day 1–day 3, and boosted on day 7 as controls. Tumor growth was measured three times a week. Six mice were included in each group.

For treatment of BNL primary tumors, mice bearing 5-day established subcutaneous BNL tumors (2×106) were vaccinated with DCs-DRibbles (2×106 DCs and 30 μg DRibbles) by intra-nodal injection on day 5 and boosted subcutaneously (2×106 DCs and 200μg DRibbles) on day 7 and day 9. Mice were also treated with DC alone or PBS in the same way by the same schedule to served as controls. Tumor growth was measured three times a week. Five mice were included in each group.

Preparation of hPBMCs and hDCs

hPBMCs were isolated by Ficoll–Hypaque gradient centrifugation from healthy donors, washed three times in Hanks' Balanced Salt solution (HBSS), and resuspended in complete RPMI 1640 medium (10% FCS, 2.5×106 cells/ml). hPBMCs were washed and resuspended with HBSS before use. For the generation of hDCs, the cells were adhered for 4 h (37˚C, 5% CO2) in culture flasks. Non-adherent cells were removed by rinsing with warm HBSS. The remaining adherent cells were cultured in complete RPMI 1640 medium with 100 ng/ml rhGM-CSF and 500U/ml rhIL-4. Fresh medium containing rhGM-CSF and rhIL-4 were added every other day. After 7–9 days of culture, the hDCs were collected for the next experiment.

Immunotherapy experiments in the humanized HCC mouse model

In the humanized immune reconstituted HepG2 HCC murine model, female BALB/c-nu mice were injected with 2×10^6 HepG2 cells subcutaneously in the lower left flank. On day 5, 30×10^6 hPBMCs (HLA-A*0201) were injected intravenously. On day 6, the mice were treated with hDCs (HLA-A*0201) loaded with HepG2-derived DRibbles (1×10^6 DCs and 30 μg DRibbles) by injection into the inguinal lymph nodes on both sides. DRibbles-pulsed hDCs (1×10^6 DCs and 200μg DRibbles) were injected subcutaneously into both flanks of the mice on days 8 and 10. Tumor tissue was removed on day 15 after vaccination, and anti-CD3 McAb was used to detect total T cells from hPBMCs by immunohistochemical staining using a streptavidin peroxidase-conjugated (SP) three-step method. Tumor growth was measured two to three times a week. Three mice were included in each group. Mice were sacrificed when the tumor area, determined by perpendicular diameters, was 150 mm2 or larger.

Statistical analysis

Graphpad Prism 5.0 (Graphpad software, San Diego, CA) was used for all statistical analysis. The mean ± S.E.M. was determined for each treatment group in the individual experiments, and the Student t-test was used to determine the significance of differences between treatment and control groups. *P*-values < 0.01 were significant.

Results

Tumor cell production of DRibbles by induction of autophagy

Previous research showed that combined administration of velcade, rapamycin, and NH4Cl efficiently induced accumulation of autophagosomes and formation of DRibbles in melanoma and other tumor cell lines (Li et al., 2008). H22 mouse hepatocellular carcinoma cells were treated by the same method, and autophagosome-containing vesicles were isolated from the supernatant of treated cell suspensions by differential centrifugation. DRibbles derived from 107 cells typically contained about 100–200 μg total protein. The total protein was isolated from cell lysates for the detection of LC3-II antibodies. LC3 (microtubule-associated protein light

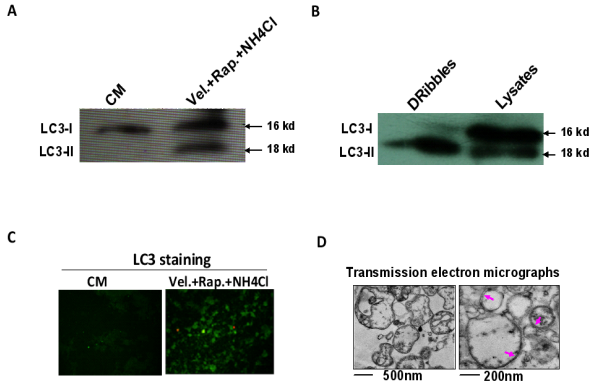

Figure 1. Characterization of Tumor Cell Derived DRibbles. H22 liver tumor cells were treated with rapamycin (20 nM), bortezomib (200 nM) and NH4Cl (10 mM) for 16–24 h. DRibbles and H22 cells were lysed in RIPA buffer. Lysates were prepared from both untreated and treated cells. (A) Cell lysates prepared from both untreated and treated cells. Fifteen micrograms of total protein were loaded on SDS PAGE gels for Western blot analysis and staining with rabbit anti-LC3 antibody. (B) DRibbles and lysates prepared from treated cells. Fifteen micrograms of total proteins were loaded on SDS PAGE gels for Western blot analysis and staining with rabbit anti-LC3 antibody. (C) The expression of LC3-II of DRibbles derived from untreated and treated cells stained by immunofluorescence. Green fluorescence represents FITC-labeled LC3-II. (D) Transmission electron micrographs of autophagosome-rich DRibbles harvested from mouse H22 liver tumor cells (80,000×). Vesicles were characterized by a unique double membrane structure and were 100–1000 nm in size. Data are representative of results from two to four independent experiments

chain 3) has both cytoplasmic LC3-I and membrane-bound LC3-II forms. LC3-II is known to be a specific marker of autophagy (Mizushima et al., 2004; Kabeya et al., 2004; Gutierrez et al., 2007; Karim et al., 2007). As shown in Figure 1A, the combined administration of velcade, rapamycin, and NH4Cl on H22 tumor cells resulted in accumulation of LC3-II, 18kd protein. Only LC3-I was observed in the cell lysates from untreated cells. In addition, as shown in Figure 1B, there was a much greater accumulation of LC3-II than LC3-I on H22 DRibbles following the induction of autophagy. Moreover, as shown in Figure 1C, fluorescence microscopy revealed very strong LC3-II expression in treated H22 cells, but nearly none in untreated cells. To further visualize the fine structure of DRibbles, H22-derived DRibbles were visualized by transmission electron microscope. As shown in Figure 1D, many vesicles were released from the treated H22 cells. Most vesicles were 100–1000 nm in size and characterized by a unique double-membrane structure. These results indicated that the combined use of three drugs on cultured H22 cells efficiently induced autophagy and the accumulation of autophagosomes.

DCs-DRibbles induced strong immune responses by lymphocytes of vaccinated mice

DCs-DRibbles vaccine effectiveness depends on cross-presentation of DRibbles antigens by APCs, and induction of specific cellular immune responses. The capacity of DCs to uptake DRibbles was examined by flow cytometry of CFSE-labeled cells. After 12 h of co-incubation, over 27% of cells were CD11c/+CFSE+, while

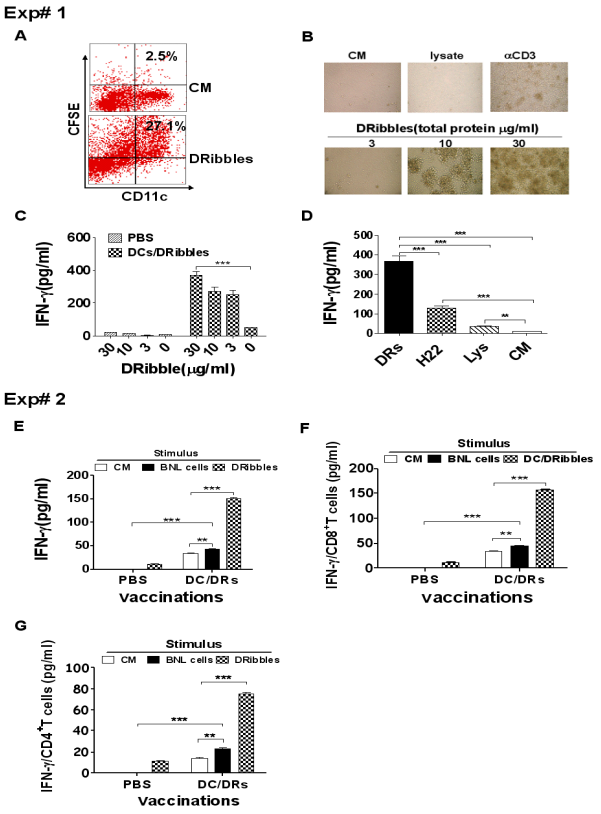

Figure 2. Immune responses of murine lymphocytes induced by DCs-DRibbles. (A) The uptake of DRibbles by DCs was evaluated by flow cytometry. DCs were co-incubated with CFSE-labeled DRibbles for 12 h, and the percentage of CD11c+/CFSE+ cells was determined. (B) Spenocytes were collected from DCs-DRibbles vaccinated BALB/c mice and were co-incubated with H22 derived Dribbles (30, 10, 3, 0 μg total protein/mL), cell lysates (30 μg total protein/mL) containing CD3 (10 μg/mL) were a positive control. Images were taken after 72 h of incubation (200×). (C) BALB/c mice were immunized with DCs-DRibbles or PBS by intra-nodal injection. Both lymph nodes and spleen of the mice were collected, processed into single cell suspensions and restimulated by DRibbles (30, 10, 3, 0 μg total protein/mL) derived from H22 cells. The IFN-γ produced by the responder cells was determined after 72 h by ELISA. (D) BALB/c mice were immunized with DCs-DRibbles by intra-nodal injection. Both lymph nodes and spleens of the mice were collected, processed into single-cell suspensions and restimulated by Dribbles (30 μg total protein/mL) derived from H22 cells, inactivated H22 cells, or H22 cell lysates (30 μg total protein/mL). The IFN-γ produced by the responder cells was determined after 72 h by ELISA. (E) BNL-derived DRibbles or BNL cells were used to restimulate lymph node cells isolated from DCs-DRibbles vaccinated mice or PBS-vaccinated mice. IFN-γ concentration in the culture supernatant was determined after 72 h of incubation ELISA. (F, G) The lymphocytes from DCs-DRibbles vaccinated mice or PBS-vaccinated mice were purified into CD8+ and CD4+ T cell preparations, which were stimulated by BNL-derived DRibbles or BNL cells. IFN-γ concentration in the culture supernatant was determined after 72 h of incubation by ELISA. Data are representative results of two to four independent experiments

the base line was only 2.5% (Figure 2A), which indicated that the CFSE-labeled DRibbles were efficiently taken up by CD11c+ cells within 12 h.

To evaluate the immune responses induced by DRibbles, we immunized BALB/c mice with DCs-

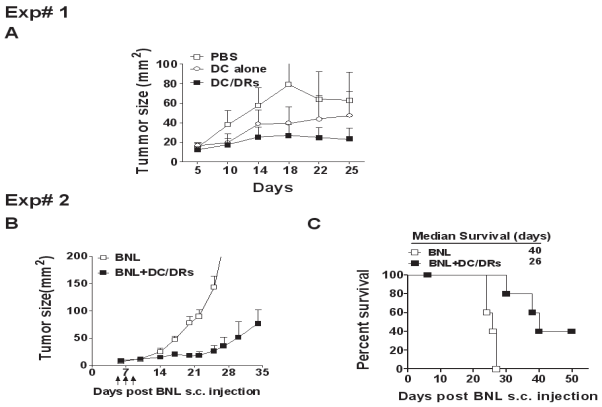

Figure 3. Anti-tumor Efficacy of DCs-DRibble Vaccine in H22 and BNL HCC models. The anti-tumor efficacy of DCs-DRibbles vaccine was evaluated in both H22 and BNL HCC murine models. (A) Mice were vaccinated with DCs with or without DRibbles derived from H22 cells and then challenged with H22 cells (1×10^6) 7 days later. PBS-treated mice were also challenged and served as a control. Tumor growth was measured three times a week. Six mice were included in each group. This data is from two experiments with similar results. (B, C) Mice bearing 5-day established BNL tumors (2×10^6) were vaccinated with DCs-DRibbles (2×10^6 DCs and 30 μg DRibbles). Mice treated with DCs alone (2×10^6 DCs) and PBS were served as controls. Tumor growth was measured three times a week. The median survival time of mice was recorded. Five mice were included in each group. Data are from three independent experiments

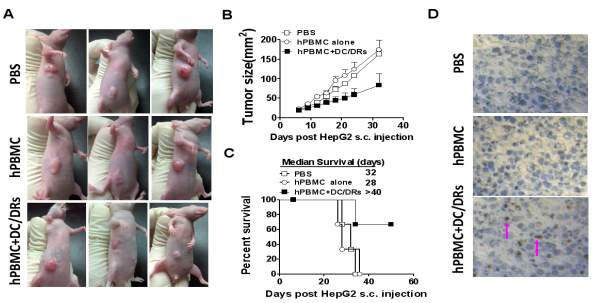

Figure 4. Anti-tumor Efficacy of DCs-DRibbles Vaccine in a Humanized Reconstituted HepG2 HCC Model. 2×10^6 HepG2 cells were injected subcutaneously into the lower left flank of Female BALB/c-nu mice. 30×10^6 hPBMCs (HLA-A*0201) were injected IV into BALB/c-nu mice on day 5. Mice were then given hDCs(HLA-A*0201) loaded with HepG2-derived DRibbles (1×10^6 DCs and 30 μg DRibbles) by intra-nodal injection on day 6. DRibbles-pulsed hDCs were injected subcutaneously into both flanks of the mice on days 8 and 10. Tumor growth was measured two to three times a week. (A) Tumor growth in nude mice treated with PBS, hPBMCs alone, and hPBMCs combined with hDCs/DRibbles 20 days after inoculation of HepG2 cells. (B) Tumor size of nude mice treated with PBS, hPBMCs alone and hPBMCs combined with hDCs/DRibbles. (C) The median survival time of nude mice treated with PBS, hPBMCs, and hDCs/DRibbles. (D) Immunohistochemical staining of tumor tissue removed on day 15 after vaccination with anti-CD3 monoclonal antibody. Anti-CD3 was used to determine total T cells from hPBMCs (400×). Three mice were included in each experimental group. Mice were sacrificed when the perpendicular diameters of the tumor reached 150 mm^2. Data are from three independent experiments

DRibbles or PBS as a control by intra-nodal injection. Both lymph nodes and spleen of the mice were collected and processed into single-cell suspensions. As shown in Figure 2B, after 72 h of co-incubation of lymphocytes and antigens, a dose-dependent trend of multiple cell clone formation was observed following stimulation by DRibbles. Almost no clones were observed by the stimulation of cell lysates or cell culture medium control, which indicated T cell activation by DRibbles. The lymphocytes from DCs-DRibbles vaccinated mice were highly efficient in producing IFN-γ in a dose-dependent manner. Lymphocytes from PBS-vaccinated control mice produced very little IFN-γ in response to stimulation by the same dose of DRibbles (Figure 2C). When restimulated by inactivated H22 cells, the lymphocytes from DCs-DRibbles immunized mice produced more IFN-γ than those restimulated by H22 cell lysates or CM controls. Moreover, when restimulated by H22-derived DRibbles, the lymphocytes from DCs-DRibbles immunized mice produced more IFN-γ than those restimulated by inactivated H22 cells or H22 cell lysates (Figure 2D). Taken together, these results showed that DRibbles, as a source of H22 tumor antigens, not only induced specific immune responses against H22 cells, but also activated antigen-specific T cell responses more strongly than inactivated whole tumor cells or tumor cell lysates.

To evaluate DCs-DRibbles induction of specific cellular immune responses in other mouse HCC cell lines, BNL-derived DRibbles or inactivated BNL cells were used to restimulate lymphocytes isolated from DCs-DRibbles vaccinated mice and un-vaccinated mice. As indicated in Figure 2E, IFN-γ was detected in the supernatant after 72 hours of incubation. The ELISA showed that lymphocytes from vaccinated mice produced more IFN-γ than un-vaccinated mice following restimulation by BNL-derived DRibbles and BNL cells. $CD8^+$ and $CD4^+$ T cells were further purified from the lymph nodes, and similar results were obtained after repeated experiments (Figure 2F, G). Higher levels of IFN-γ were detected in the $CD8^+$ cells than $CD4^+$T cells. These results suggested that DCs-DRibbles vaccine could induce antigen-specific cellular immune response in a mouse model and could prime strong $CD8^+$ T cell responses, supporting it as a tumor vaccine candidate.

DCs-DRibbles vaccine anti-tumor efficacy in the H22 and BNL HCC murine models

We evaluated the anti-tumor efficacy of the DCs-DRibbles vaccine in both the H22 and BNL HCC murine models. In the H22 HCC model, mice were vaccinated with DCs alone or DCs-DRibbles derived from H22 cells, and were challenged with H22 cells 1 week later. PBS-treated mice were also challenged by H22, and served as a control. As expected, vaccination with DCs-DRibbles significantly inhibited H22 tumor growth compared with DCs only or PBS (Figure 3A).

Mice bearing 5-day established BNL tumors were vaccinated with DCs-DRibbles derived from BNL cells. Un-vaccinated mice or mice vaccinated with PBS were controls. As illustrated in Figure 3B,C, vaccination with DCs-DRibbles inhibited BNL tumor growth and remarkably improved survival compared to untreated group or the group treated by DC alone. Two of five treated

mice in the DC-DRibbles vaccinated group survived for more than 50 days. DCs-DRibbles delayed tumor growth and prolonged the median survival time of the mice (40 days for the vaccinated group vs. 27 days for DC alone controls and 26 days for PBS controls). These results showed that the DCs-DRibbles vaccine inhibited H22 and BNL tumor growth in both prophylactic and therapeutic murine HCC models.

DCs-DRibbles vaccine suppression of tumor growth in a humanized HCC mouse model

To establish a humanized immune reconstituted HepG2 HCC murine model, BALB/c-nu mice were given intravenous injections of hPBMCs 5 days after inoculation of HepG2 cells. Vaccination was with hDCs-DRibbles or PBS. As shown in Figure 4A,B, tumor growth was significantly suppressed by treatment with hPBMCs followed by hDCs-DRibbles, compared with untreated PBS controls or treatment with PBMCs alone after inoculation of HepG2 cells. As shown in Figure 4C, the median survival time of mice treated with hPBMCs followed by hDCs-DRibbles was significantly longer than in the other two groups. The tumors were removed on day 15 after vaccination for immunohistochemical staining. Anti-CD3 monoclonal antibody was used to detect the total T cells in hPBMCs. As shown in Figure 4D, only a few CD3+ cells were detected in tumor tissue from mice treated with hPBMCs only. In the combination treated group, there was a significant up-regulation of CD3 positive cells in the tumor tissue. These results indicated that vaccination with hDCs-DRibbles induced T cell activation and proliferation in hPBMCs, and inhibited HepG2 tumor growth in humanized immune reconstituted HepG2 murine HCC model. The result represents anti-tumor efficacy in a "human-like environment".

Discussion

Hepatocellular carcinoma is one of the world's deadliest cancers, ranking third among all cancer-related deaths. Potentially curative therapies can be offered to approximately 30% of patients, and are complicated by a high rate of recurrence (Farazi et al., 2006). There is thus an urgent need of multiple, successive treatment options for HCC, including immunotherapy. In a previous study, we documented the unique characteristics and potent anti-tumor efficacy of an autophagosome-based DRibble vaccine (Li et al., 2008). A recent study showed that DCs loaded with autophagosome-enriched DRibbles eradicated 3LL Lewis lung tumors and significantly delayed the growth of B16F10 melanoma (Li et al., 2011). The results presented here illustrate the potential of autophagosome-based DRibbles derived from HCC to act as a potent vaccine, inducing cellular immune responses in the cross-priming of antigen-specific T cells. The results also demonstrated the therapeutic efficacy of this vaccine candidate in the treatment of HCC in mice and humanized murine models.

Autophagy is one mechanism that cells use to degrade cytoplasmic proteins and organelles during the maintenance of cellular homeostasis (Klionsky et al., 2000). Autophagy has recently been proposed as a mechanism for MHC class I-mediated cross-presentation of exogenous antigens similar to tumor antigens (Li et al., 2008). During the process of cross-presentation, APCs such as dendritic cells (DCs) internalize and degrade antigens from the extracellular environment and display the resulting peptides in association with MHC class I molecules on their cell surfaces (Shen et al., 2006). The stimulation of naive $CD8^+$ T cells by these peptide–MHC class I complexes is known as cross-priming. A growing body of evidence has indicated that autophagy is a potential mechanism for MHC class I-mediated presentation of tumor antigens (Crotzer et al., 2009). Moreover, there is increasing evidence for autophagy in donor cells as an important mechanism for facilitating antigen delivery to conventional DCs (cDCs), which may contribute to a better understanding of how to optimize immunotherapy for treatment of cancer and chronic infectious disease (Joubert et al., 2012).

In a previous study, we collected large quantities of autophagosomes containing abundant tumor antigens by inducing autophagy and blocking protein degradation through proteasome and lysosome inhibition. Subsequent studies have shown that these autophagosomes, i.e., "DRibbles", are antigen carriers, and are efficient at cross-priming naive $CD8^+$ T cells by DCs both in vivo and in vitro. This makes them ideal candidates for tumor vaccine development in various cancer models (Li et al., 2009; Twitty et al., 2011; Joubert et al., 2012; Yi et al., 2012). In this study, we isolated DRibbles that were produced in H22 mouse hepatocellular carcinoma cells, BNL mouse hepatocellular carcinoma cells and HepG2 human hepatocellular carcinoma cells. Those antigen-containing vesicles had a unique double-membrane structure, were 100–1000 nm in size, and were further characterized by LC3-II, a specific marker of autophagosomes. LC3-II is known to be associated with autophagy in a few mouse tumor cell lines (Mizushima et al., 2004; Kabeya et al., 2004; Gutierrez et al., 2007; Karim et al., 2007), and similar results were also obtained in human HCC cells in our research (data not shown). The study thus showed that autophagosome and DRibble formation is possible in hepatocellular carcinoma cells.

By using OVA and gp100 as model antigens, Li et al. demonstrated that tumor cell derived DRibbles were efficient antigen carriers, and efficiently cross-primed OT-1 or pmel-1 cells in vitro or in vivo (Li et al., 2009). In another study using tumor cell lysates and inactivated human HCC cell lines (data not shown), DRibbles were highly effective in priming T cells and could stimulate anti-tumor responses. This study showed similar activity in H22 and BNL murine models. The study results found a trend toward higher production of IFN-γ by $CD8^+$ than by $CD4^+$ T cells from vaccinated mice, indicating the priming of tumor antigen-specific CTL responses by this novel form of antigen. Although previous studies have shown that autophagosomes function as efficient carriers of antigen from donor cells, it is not known how and where they interact with MHC class I molecules. Further study of the specific mechanisms by which autophagy is involved in presentation of tumor antigens by MHC class

I molecules is needed, and may lead to the development of new therapeutic strategies for the treatment of cancer (Mizushima et al., 2008).

The efficacy of a tumor vaccine depends on the optimal function of APCs. Among the various types of APCs, DCs are considered to be the most promising candidate for anti-tumor vaccines. Because less than 1% of DCs are found in the peripheral circulation, most clinical trials depend on DCs generated with standard protocols using GM-CSF plus a cocktail of maturation factors and require 8 to 10 days (Kvistborg et al., 2009). Although the resulting DCs do induce anti-tumor immune responses in vivo, this time span may not reflect the actual kinetics for DC differentiation from monocytes under physiological conditions. Monocytes represent a pool of circulating precursor cells capable of rapid differentiation into mature DCs after transit into inflamed or infected tissue (Randolph et al., 1998). Previous studies have shown that daily injection of human Flt3 ligand (Flt3L) into mice resulted in a dramatic increase in the number of cells co-expressing the characteristic class II MHC, CD11c, DEC205, and CD86 markers of DC. In mice treated with either GM-CSF, GM-CSF plus IL-4, c-kit ligand, or G-CSF, class II+ CD11c+ cells were not significantly increased (Maraskovsky et al., 1996). In a previous study, hydrodynamic intravenous injection of Flt3L and GM-CSF DNA plasmids resulted in dramatic splenomegaly in mice. The DCs generated in vivo were efficient in DRibbles uptake, and were used as the source of our DCs-DRibble vaccine. To develop a type of DC that can effectively cross-prime DRibbles and meet the requirements of a DCs-DRibble vaccine, we identified subsets of DCs with varying ability to uptake DRibbles and cross-present antigens to T cells. In mice, Clec9A, a novel C-type selectin receptor on a subset of DCs was shown to be a potent targeting receptor for tumor immunotherapy (Schreibelt et al., 2012). Hu et al. have shown high expression of Clec9A ligand on autophagosomes, and blockade of the interaction between the Clec9A ligand on autophagosomes and Clec9A on DCs significantly reduced antigen cross-presentation (Li et al., 2011). Additional studies on optimizing DC generation and identification of DC subsets that would be the best candidates for DRibbles vaccines are needed.

In previous studies by Li et al., mice bearing orthotopically injected 4T1 mammary tumors were treated by intra-nodal injection of DRibbles on day 13, when tumors were well established, and tumor cells had metastasized to the lungs, liver, and bone. Dramatic regression and cure of these well-established tumors were observed in 60% of the mice (Li et al., 2011). These encouraging results prompted us to test our therapeutic vaccine in murine liver tumor models. This study showed that DCs-DRibbles induced anti-tumor responses in prophylactic and in therapeutic liver tumor models using H22 or BNL cells. Vaccination with H22-derived DCs-DRibbles 1 week before challenge significantly inhibited H22 tumor growth compared to the un-vaccinated group. Similarly, vaccination with DCs-DRibbles inhibited BNL tumor growth in mice bearing established 5-day established BNL tumors, and remarkably improved survival compared to untreated mice. More importantly, the DCs-DRibbles vaccine derived from human HCC was found to have anti-tumor efficacy in a humanized immune reconstituted HCC murine model. Tumor growth was significantly delayed and median survival time prolonged by treatment with hPBMCs followed by hDC-DRibbles vaccine. These preclinical results support a novel strategy for developing an HCC immunotherapy, and support additional studies to increase the efficacy of DCs-DRibbles vaccine. On the basis of these preclinical findings, we began a phase I/II clinical trial of a DRibble vaccine in patients with HCC with the objective to develop a promising vaccine for the human cancer..

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