A Human Fab-Based Immunoconjugate Specific for the LMP1 Extracellular Domain Inhibits Nasopharyngeal Carcinoma Growth In Vitro and In Vivo

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Abstract
Nasopharyngeal carcinoma (NPC) is a major cause of cancer-related death in Southeast Asia and China. Metastasis and relapse are the primary cause of morbidity and mortality in NPC. Recent evidence suggests that the Epstein–Barr virus latent membrane protein 1 (LMP1) is exclusively expressed in most NPC and is a potential target for biotherapy. In this study, we successfully prepared a novel human antibody Fab (HLEAFab) against LMP1 extracellular domain, which was subsequently conjugated with mitomycin C (MMC), thus forming an immunoconjugate (HLEAFab-MMC). The effects of HLEAFab-MMC on proliferation and apoptosis in NPC cell lines HNE2/LMP1 and the inhibition rate of growth of NPC xenografts in nude mice were examined. The inhibition rate of HNE2/LMP1 cell proliferation was the highest for HLEAFab-MMC (76%) compared with MMC (31%) and HLEAFab (22%) at a concentration of 200 nmol/L and showed dose-dependent fashion. The apoptosis rate of HNE2/LMP1 cell lines was 13.88% in HLEAFab-MMC group, 3.04% in MMC group, 2.78% in HLEAFab group, and 2.10% in negative control group at the same concentration, respectively. In vivo, the inhibition rate of growth of NPC xenografts in nude mice was 55.1% in HLEAFab-MMC group, 26.5% in MMC group, and 5.64% in HLEAFab group. In summary, our findings show that HLEAFab-MMC is a unique immunoconjugate with the potential as a novel therapeutic agent in the treatment of LMP1-expressing NPC. Mol Cancer Ther; 11(3); 594–603. ©2011 AACR.

Introduction
Epstein–Barr virus (EBV) is a herpes virus prevalently associated with a variety of malignant diseases, such as nasopharyngeal carcinoma (NPC), Burkitt’s lymphoma, Hodgkin’s lymphoma, and some gastric carcinomas (1). An association of EBV with NPC has been consistently established by numerous studies. Various prints of EBV have been found in the tissues of NPC and antibodies against EBV present in serum of NPC patients. In NPC, most tumor cells are latently infected and express 3 latent membrane proteins (LMP): LMP1, LMP2A, and LMP2B, as well as EBV nuclear antigen 1 (EBNA1). Among these, LMP1 and EBNA1 have been detected in NPC biopsies by Western blotting analysis. LMP1 is considered to be the major EBV oncoprotein (2). Therefore, both EBNA1 and LMP1 are important tumor-specific markers for NPC. Furthermore, LMP1-positive NPCs are reported to be more progressive than LMP1-negative NPCs and show an increasing tendency of lymph node metastasis (3). Structurally, LMP1 is an integral membrane protein consisting of a short cytoplasmic N-terminus of 20 amino acids, a transmembrane domain with 6 membrane-spanning segments that anchor LMP1 in a patchy distribution along the plasma membrane, and a long cytoplasmic C-terminus of 200 amino acids (4–6).

In this study, we chose the extracellular domains of the oncogenic LMP1 as a target of therapy. We had screened a fully human naïve Fab phage library after panning against extramembrane domains (EMD) polypeptide, which consists of 3 EMDs proximal 16 amino acid, by panning on living cells and immobilized LMP1 (7), followed by characterizing its binding specificity and affinity to native LMP1 as well as the NPC cell line overexpressing LMP1. Using this strategy, we showed that this human anti-LMP1 EMDs antibody Fab fragment (HLEAFab) can bind to the extracellular domains of LMP1. We then conjugated

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this HLEAFab with mitomycin C (MMC), a chemotherapeutic drug, to generate a potential immunoconjugate agent. This study is the first to clone, express, and functionally validate the HLEAFab targeting the LMP1. The immunoconjugate, which consists of a Fab antibody fragment derived from a fully human Fab phage library and MMC, killed LMP1-positive NPC cell lines in vitro and suppressed NPC growth in a nude murine transplantation model.

Materials and Methods

Phage library, helper phage, and bacterial strains

A human naive Fab phage library was constructed as previously described (8). Before the first-round panning, the library was titrated and 2 × 10^{13} phage clones were collected for panning. The VCSM13 helper phage and the E. coli strain XL1-Blue and another E. coli strain, Top 10 F’, for expressing the Fab, were saved and provided by the Key Laboratory of Cancer Biomarkers, Prevention & Treatment Cancer Center and The Key Laboratory of Antibody Technique of Ministry of Health, Nanjing Medical University, Nanjing, China. Both strains were tested to preclude any wild-type phage contamination.

Cell lines and purified EMDs of LMP1

Two cell lines were used for biopanning and Fab characterization as well as for in vitro bioassays: HNE2 is an EBV-LMP1–negative human NPC cell line, HNE2/LMP1 is a cell line constantly expressing LMP1 after the introduction of full-length LMP1 cDNA into HNE2 cells, purchased from Xiangya Central Experiment Laboratory, Hunan, China and were authenticated in this laboratory. The initial characterization of HNE2 and HNE2/LMP1 cells has previously been described (9). On the basis of PCR and Western blotting analyses for LMP1, HNE2/LMP1 cells are LMP1 positive, whereas HNE2 cells are EBV DNA negative. Both cell lines were cultured in a RPMI-1640 medium (GIBCO; Invitrogen) with 10% FBS, antibiotics. All of the cell lines are routinely screened for Mycoplasma species (GenProbe Detection Kit; Fisher). All cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO2. For the experiments, HNE2 and HNE2/LMP1 were used between passages 3 to 7. The EMDs of LMP1 and GST fusion protein, which was prepared by our research team previously (7), were affinity purified from Glutathione Sepharose 4B (GE product code:27-4574-01). The eluted LMP1 was verified by Western blotting and quantified by BCA Protein Assay (Bio-Rad).

Biopanning methods

To isolate phage that specifically bind to LMP1 on the cell surface, subtractive panning was carried out on both HNE2 cell line and HNE2/LMP1 cell line, as well as on immobilized LMP1 extracellular domains. Seven rounds of panning were carried out. The first, second, third, and sixth rounds of panning were done on the cells, and one million cells were used in each round. The cells were starved in serum-free medium for 12 hours, washed twice using pH 7.4 PBS, detached from the flask with cell dissociation buffer (catalog no. 13151-014; Invitrogen) at 37°C for 10 minutes, and washed twice in PBS. LMP1-negative HNE2 cells were resuspended and mixed with 10^{13} phagemids in 1 mL of 1% bovine serum albumin (BSA)-PBS, shaken at 37°C for 30 minutes, and centrifuged at 5,000 × g for 3 minutes. The supernatant was collected and mixed with HNE2/LMP1 and then incubated at 37°C for 1 hour. The cells were washed with RPMI-1640 and treated with 3 mL Trypsin-EDTA (Gibco, 25300.054) at 37°C for 5 minutes. The phages were used to infect E. coli. XL1-Blue for amplification and next-round panning. To increase binding specificity, as well as affinity to LMP1, the fourth, fifth, and seventh rounds of panning were carried out on immobilized LMP1, and the amount of coated LMP1 was gradient decreased in each of the 3 rounds (400 ng for fourth round, 200 ng for fifth round, and 100 ng for seventh round).

ELISA screening of LMP1–binding positive phage clones

Single phage clones from the E. coli XL1-Blue-infected by the seventh round of eluted phage were picked up and grown in 1 mL super broth (SB) medium containing 50 mg/mL carbenicillin and 1% glucose, with shaking at 37°C until the exponential phase. VCSM13 helper phage (1 × 108) was added to each vial. The culture was shaken overnight with 70 µg/mL kanamycin. Fifty microliters of supernatant from each vial was added to each well of 96-well ELISA plates coated with 100 ng GST-LMP1 fusion protein that had been preblocked with 5% milk blocking buffer (5% milk, 0.5% Tween-PBS). Purified GST protein was used as negative control. After incubation at room temperature for 1 hour, the plate was washed using 0.5% Tween-PBS; 50 µL of 1:4,000 diluted horseradish peroxidase (HRP)-conjugated anti-M13 antibody (Amersham code number: 27-942-01) in blocking buffer was added to each well and incubated for another hour and then the plate was washed again. Fifty microliters of HRP substrate solution (Prod# 34021; Pierce) were added and developed 30 minutes before stopping by addition of 1 mol/L H2SO4, and the absorbance value at 450 nm was read by Multiskan Spectrum Microplate (Themo Instruments Inc.). All ELISA assays were repeated at least 3 times.

Expression and purification of a soluble HLEAFab fragment

The recombinant HLEAFab was expressed in the E. coli Top 10 F’ strain. Briefly, the overnight culture of a single clone was re inoculated at 1:100 in SB medium with 50 mg/mL carbenicillin until the OD600 reached 1.0, induced by 1 mmol/L IPTG in the presence of 4% sucrose at 25°C and harvested 24 hours later. Western blotting was done on both bacteria lysate and sonicated supernatant to determine whether the HLEAFab was expressed after IPTG induction, with a purified human Fab as positive control.
control. The soluble HLEAFab was purified from the periplasm of the bacteria using Protein L (catalog no. L00239; GenScript Corporation) affinity purification.

**Cell ELISA**

NPC cell line (HNE2 and HNE2/LMP1) were cultured in complete medium in 96-well flat bottom plates (Corning) to form a subconfluent monolayer and further incubated overnight in RPMI-1640 medium containing 10% FBS. The cells were washed 3 times for 5 minutes each with PBS, pH 7.4 and cells were fixed by treatment with 4% paraformaldehyde for 10 minutes at 4°C. The endogenous peroxidase activity was blocked with 3% H2O2 in distilled water for 7 minutes at room temperature followed by 3 × 5 minutes washes in PBS, pH 7.4. Nonspecific binding was blocked with 3% BSA for 1 hour at room temperature. Cells were washed once in PBS and incubated with purified HLEAFab diluted in 1% BSA for 1 hour at room temperature. The unrelated antibody Fab fragment was used as the negative control antibody. After 3 × 10 minutes washes in PBS, HRP-conjugated anti-human IgG(Fab)' (catalog no. A0293; Sigma) was added for 1 hour at room temperature. The cells were washed again in PBS, following by TMB substrate solution, and incubated for 20 minutes at room temperature. The color reaction was stopped by adding 1 mol/L H2SO4 and the intensity was read at 450 nm in a Multiskan Spectrum Microplate (Thermo Labsystems; refs. 10–12).

**Fluorescence-activated cell sorting analysis**

HNE2 and HNE2/LMP1 cells were blocked using 1% BSA-PBS at 4°C for 30 minutes, then incubated with 100 μg/mL HLEAFab at 4°C for 30 minutes, then incubated with 100 μg/mL HLEAFab at 4°C for 45 minutes, and stained using 1:50 diluted fluorescein isothiocyanate (FITC)-labeled human anti-Fab IgG (catalog no. F5512; Sigma) at 4°C for 20 minutes. The unrelated antibody Fab fragment was used as the negative control antibody. Fluorescence intensity was analyzed with CellQuest software (Becton Dickinson Bioscience). The cells incubated only with secondary antibody were analyzed as controls.

**Conjugation of MMC to HLEAFab**

The principle used to conjugate small molecule anticancer drug MMC (Zhejiang Haizheng Pharmaceutical Co., Ltd.) to HLEAFab involved the activation of the amino group with N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP; Pierce), followed by disulfide exchange with iminothiolane-modified HLEAFab (Fig. 1; ref. 13). For activation, MMC was reacted with SPDP at 4°C for 10 hours and stored in small aliquots at −80°C until further use. HLEAFab in PBS was reduced by mixing with 2-iminothiolane for 1 hour under nitrogen. The reduced antibody was purified by size-exclusion chromatography using Desalting Column (Pierce) pre-equilibrated with

![Figure 1. Conjugation of anti-LMP1 antibody Fab and MMC. MMC was first conjugated with the SPDP linker (A), and Fab was reduced with 2-iminothiolane (B). The final immunoconjugate construct is shown in C.](https://example.com/figure1.png)
PBS. The conjugation of activated MMC with thiolated HLEAFab (MW: ~50 kDa) was done at room temperature for 1 hour under nitrogen using a 1:1 molar ratio. Excess free MMC–SPDP were removed using Desalting Column (Pierce) pre-equilibrated with PBS. HLEAFab-MMC products were filter sterilized using 0.2-μm Super Membrane (Pall). The dosage of the HLEAFab-MMC conjugate for cytotoxicity and in vivo experiments was calculated according to the total amount of MMC added. The conjugations of MMC with the unrelated Fab fragment were done the same way as described above.

**MTT assay for antiproliferation of HNE2/LMP1 cells by HLEAFab-MMC**

MTT assay (14) was done on HNE2/LMP1 and HNE2 cells on a 96-well plate. A total of 5 x 10^3 cells were dispensed into each well and cultured for 12 hours. Following removal of cultured media, fresh medium containing MMC, naked antibody, or immunoconjugates (25–200 nmol/L) was added to each well for an additional 48-hour incubation. Subsequently, the culture medium was removed, and 100 μL of MTT (1 mg/mL in RPMI-1640) was added to each well after 5 hours of incubation with MTT at 37°C in 5% CO2 incubator, the supernatant was removed, and 150 μL of dimethylsulfoxide was added to each well followed by shaking at 150 rpm for 5 minutes. Absorbance at 490 nm was determined spectrophotometrically. The cells without any antibody treatment served as the negative control. MTT assay was repeated at least 3 times.

**Induction of apoptosis of HNE2/LMP1 cells by HLEAFab-MMC**

To investigate the apoptotic effects of HLEAFab-MMC on NPC cancer cells, Apo-Direct Kit (BD Pharmingen 556381) was used for detection and quantitation of apoptosis at a single cell level. Briefly, HNE2/LMP1 and HNE2 cells were cultured in RPMI-1640 medium at 37°C in a 5% CO2 incubator for 12 hours until all cells were attached to microwells. HLEAFab-MMC (200 nmol/L) was added to the attached cells for coincubation of 48 hours. As the negative control, MMC, HLEAFab, the unrelated Fab, and normal saline for negative control. The cells were allowed to grow for 14 days before treatment (15). To test HLEAFab-MMC on tumor growth inhibition, the mice with HNE2/LMP1 xenograft were given HLEAFab-MMC location injected (16, 17) at dose levels ranging from HLEAFab (8 mg/kg, 1.6 x 10^-5 mol/kg) in HLEAFab group, HLEAFab-MMC (8 mg/kg, 1.6 x 10^-5 mol/kg) in HLEAFab-MMC group, MMC (0.053 mg/kg, 1.6 x 10^-5 mol/kg) in MMC group on days 1, 4, and 7 including the unrelated Fab, the immunoconjugate of MMC with the unrelated Fab, and normal saline for negative control. The mice were followed for observation of xenograft body weight changes. At the end of the treatment period, the mice were killed, and the tumors were excised to determine tumor weight.

**Results**

**Selection of specific LMP1-binding phage and expression and purification of soluble HLEAFab**

After 7 rounds of panning, 45 single phage clones were randomly picked up and amplified to test for specific binding to LMP1 EMDs by phage ELISA. A ratio of sample OD_{530} versus the blank of greater than 2.5 was set as the standard for selecting positive clones. As shown in Fig. 2A, no. 36 clone with strongest binding to LMP1 EMDs were analyzed by DNA sequencing and were all identical (Table 1). Soluble expression of HLEAFab was induced overnight at low temperature (25°C). The expressed HLEAFab was found mainly in the periplasmic space of E. coli (Fig. 2B). SDS-PAGE and Coomassie blue staining showed equal expression of heavy and light chains. The purity was above 95% after Protein L affinity purification (Fig. 2C).

**The HLEAFab reacts with the extracellular domains of LMP1 in native conformation**

Further analysis of HLEAFab reactivity was carried out by comparing the binding ability of the antibody fragments to LMP1-positive (HNE2/LMP1) and LMP1-negative (HNE2) NPC cells using cell surface ELISA. Shown in Fig. 3A, the purified HLEAFab recognized LMP1 present in HNE2/LMP1 cells. We further evaluated whether the HLEAFab binds to extracellular domains of LMP1...
using a flow cytometric assay. Both HNE2/LMP1 and HNE2 cells were treated with or without HLEAFab before addition of fluorescence-labeled secondary conjugate. The result in Fig. 3B shows that the population of HLEAFab-treated HNE2/LMP1 cells was clearly separated from nontreated cells by fluorescent intensity, whereas no difference was noted between HLEAFab-treated and nontreated HNE2 cells, suggesting that the HLEAFab binds to LMP1-positive cell membranes only. Furthermore, to provide morphologic evidence that this HLEAFab binds to the cell membrane, a cell single staining experiment was conducted, and again, this HLEAFab stained only LMP1-positive NPC HNE2/LMP1 cells and not LMP1-negative NPC HNE2 cells (Fig. 3C).

Cytotoxicity and proapoptotic activity of immunoconjugates HLEAFab-MMC against LMP1-positive NPC HNE2/LMP1 cells in vitro

After confirming the binding capability of this HLEAFab, we conjugated it with MMC to investigate its antitumor effect in vitro. Mass spectrophotometer analysis showed that the molar ratio of HLEAFab to MMC was about 1:1 (average molecular weight of the conjugate increased 342 Da in comparison with HLEAFab, and the molecular weight of MMC is 334.3). The binding affinity of MMC-conjugated HLEAFab to LMP1 did not decrease significantly as compared with nonconjugated HLEAFab (data not shown), and the stability of MMC-conjugated HLEAFab is reasonable, as it was exposed at room temperature for days during conjugation reaction. To determine the efficacy of HLEAFab-MMC to specifically inhibit the growth of HNE2/LMP1 cells, MTT assays were carried out. Viable HNE2/LMP1 or HNE2 cells were

<table>
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<th>Table 1. HLEAFab variable region DNA and amino acid sequence</th>
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treated with different concentrations of HLEAFab-MMC, HLEAFab, MMC, unrelated Fab, or unrelated Fab-MMC. The results showed that HLEAFab-MMC was effective in inhibiting 76% cell proliferation at 200 nmol/L. However, MMC and HLEAFab yielded only 31% and 22% inhibition, respectively. In fact, the cell inhibition rate decreased as the concentration of HLEAFab-MMC declined, denoting a dose-dependent response. These data indicated that the cell cytotoxicity seen with HLEAFab-MMC is mediated by LMP1 specifically in a dose-dependent manner (Fig. 4A). MMC, naked antibody, or immunoconjugates exhibited very modest or no cytotoxicity at the highest dose tested (200 nmol/L) in HNE2 cells (Fig. 4B). By using TUNEL assay, inductions of apoptosis following treatments of cancer cells with HLEAFab-MMC were quantitatively determined. As shown in Fig. 4C, significant increases in apoptosis of treated HNE2/LMP1 cells were detected upon the treatment with HLEAFab-MMC (200 nmol/L), as compared with the negative control and HNE2 cells. The highest apoptosis rate of HNE2/LMP1 cell lines was observed in HLEAFab-MMC group (13.88%) compared with the MMC group (3.04%), HLEAFab group (2.78%), unrelated Fab (2.34%) or unrelated Fab-MMC (3.2%), or negative control (2.10%) at the same concentration. Characteristic morphologic changes of cell apoptosis were observed by fluorescence microscopy after HLEAFab-MMC acted on NPC HNE2/LMP1 cell lines. Normal cells were round, uniform, cells were stained green, the size and the shape were single (Fig. 4D1); necrotic cells have oval shaped cells stained orange, the size and shape were single (Fig. 4D2); early apoptotic cells were green, cell shape was irregular, like a crescent (Fig. 4D3); late apoptotic cell nuclei were orange, the nucleus broke into pieces, showing bud-like cellular processes (Fig. 4D4).

The HLEAFab-MMC immunoconjugate suppresses tumor development in a HNE2/LMP1 xenograft model

Mice models bearing HNE2/LMP1 xenografts were established and were confirmed for their LMP1-overexpressing by immunohistochemistry (IHC) method (Fig. 5A). As illustrated in Fig. 5B, HNE2/LMP1 xenografts mice treated with HLEAFab-MMC (1.6 × 10^{-5} mol/kg, MMC equivalent at 0.053 mg/kg HLEAFab equivalent at 8 mg/kg) achieved significant regressions from 20 to 40 days when compared with controls (P < 0.05). Although MMC (0.053 mg/kg) injection was potent to inhibit tumor growth, the efficacy was much lower than that of HLEAFab-MMC (MMC: 0.053 mg/kg), which suggested an extra advantage of LMP1-mediated specific tumor targeting. As shown in Fig. 5C, the HLEAFab-MMC immunoconjugate resulted in an average 55.1% inhibition of HNE2/LMP1 tumor growth in comparison with their controls (P < 0.05).
However, the MMC alone produced 26.5% inhibition, HLEAFab alone 5.64%, unrelated Fab 9.63%, and unrelated Fab-MMC 24.4%. So the HLEAFab-MMC immunoconjugate remarkably enhanced the tumor growth inhibition. During the entire treatment period, the antineoplastic antibiotic MMC, the antibody, and immunoconjugates administration were nontoxic according observation of weight, survival, and animal activities as well as pathologic examination results (Fig. 5D). No other complications such as anaphylaxis and skin necrosis were detected.

Discussion

In this study, we successfully prepared the immunoconjugate HLEAFab-MMC against LMP1-positive NPC. This involved the following procedures: (i) The HNE2/LMP1 cell line, which has high LMP1 expression, and the LMP1-negative HNE2 cell line were selected for subtractive pan-ning to increase the chances of capturing the phage that binds to native LMP1. (ii) We were able to generate a human HLEAFab fragment that binds to the LMP1-positive NPC cell lines. (iii) We showed that the major immunogenic region of the LMP1 extracellular domain can be used as an immunoconjugate target to kill LMP1-positive NPC cell lines in vitro. (iv) An antibody-based immunoconjugate HLEAFab-MMC killed LMP1-positive NPC cells in vitro and significantly slowed tumor growth in a mouse LMP1-positive NPC transplantation model, although HLEAFab or MMC alone was insufficient to generate comparable effects.
In our study, the repeated panning with living cells and coated LMP1 EMD protein in microtiter plates ensured the enrichment of specific LMP1 EMD–binding phage. After 7 rounds of panning, 1 of 45 randomly selected phage clones showed evidently positive results (Fig. 2). Moreover, the DNA sequencing results (Table 1) were identical to the phagemids that gave the strongest binding signal by ELISA, indicating that the phage with high specific binding to LMP1 were selected out from the panel. The ability of the expressed HLEAFab to specifically bind to native LMP1 on the HNE2/LMP1 cell surface was further confirmed by Cell ELISA, fluorescence-activated cell sorting, and fluorescence staining analyses (Fig. 3).

Antibody-based targeting immunotherapy is a well-established treatment for various tumor types, such as ErbB2-positive breast cancer (18), Hodgkin lymphoma, acute myeloid leukemia, colon cancer, lung cancer, melanoma, and some of head and neck squamous cell carcinomas (19–24). Immunoconjugate therapy has not yet been applied to NPC, although some antigens have been found since many years ago to expresses at high levels on the surface of these tumor cells (25, 26). This obstacle was overcome after we discovered that the LMP1 EMD is likely to be as a therapeutic target of the vast majority of NPC.

The LMP-specific monoclonal antibodies (mAb) CS1-4, S12, and other mAbs are known to recognize intracellular parts of LMP1 (27), and LMP antibody–positive sera stained our LMP-expressing cells in indirect immunofluorescence assays only when these cells were first permeabilized. These antibodies and sera, however, were unable to stain these cells without prior permeabilization, suggesting that these antibodies recognized only intracellular parts of the protein (28). Therefore these intrabodies are not fit for the preparation of antibody-based immunoconjugate unless they are internalized by the target cells (29). Accordingly, we selected LMP1 EMD for a specific target position as an extracellular domain antibody is an ideal carrier of antibody-based immunoconjugate.

An antibody per se can be a drug for immunotherapy. Indeed, several neutralizing antibodies have been approved by the U.S. Food and Drug Administration for clinical treatment of a variety of diseases. Besides, antibody can also be used as a carrier to deliver a broad range of therapeutics selectively to cancer cells by recognizing a specific tumor marker on the cell surface. The therapeutics might include nucleic acids, chemotherapy compounds, toxins, radionuclides, antibody, cytokine or antibody–ligand fusion proteins, and enzyme prodrugs. Such delivery may reduce or eliminate side effects caused by the...
drugs in normal cells. For the purposes of immunochemotherapy, an antibody capable of receptor binding is essential because, in that process, the antibody can help accumulate the conjugated molecules in the cancer cells (20, 30). LMP1 has been reported to be overexpressed in most solid tumors at various levels, making it an ideal target for antibody-directed drug delivery (31, 32). In this study, we selected MMC to be conjugated to an anti-LMP1 EMD HLEAFab and made an immunochemotherapeutic agent. Its biologic efficacy was tested in in vitro antitumor activity via cell proliferation inhibition and apoptosis assays (Fig. 4). The results showed that HLEAFab-MMC was effective in inhibiting 76% HNE2/LMP1 cell proliferation at 200 nmol/L, in contrast to the inhibitory rate of 31% by MMC and 22% by HLEAFab. The highest apoptosis rate of HNE2/LMP1 cell lines was observed in HLEAFab-MMC group (13.88%) compared with the MMC group (3.04%), HLEAFab group (2.78%), or negative control (2.10%) at the same concentration. These data suggest that the HLEAFab-MMC conjugate is more potent for treating LMP1-positive cancers and has less side effects relative to those seen with MMC alone.

The antitumor effect of this immunoconjugate was also assessed in vivo (Fig. 5). As illustrated in Fig. 5, 8 mice models with xenograft tumor were treated with HLEAFab-MMC (MMC equivalent 0.053 mg/kg, HLEAFab equivalent at 8 mg/kg) and achieved tumor inhibition and maintained tumor inhibitory effect for more than another 20 days. Although MMC injection was potent to inhibit tumor growth, the efficacy was much lower than that of HLEAFab-MMC, which suggested the great advantage of HLEAFab-mediated specific tumor targeting. To circumvent the relatively significant errors made by directly measuring tumor size, tumors were removed and weighed after the mice were sacrificed at the end of experiment (day 40). The average tumor weight of HLEAFab-MMC treatment group was much smaller than those from other groups. The tumors shrank to 51.1% compared with the control group (N.S. injected). In contrast, the MMC-alone treated tumors only shrank to 26.5%. These results clearly showed that HLEAFab-MMC can significantly inhibit tumor growth in vivo. HLEAFab-MMC significantly inhibits BALB/c nude mice transplantation tumor growth of human HNE2/LMP1 NPC in vivo.

Actually, different type of NPCs and different cells in the same tumor tissue are not always the same level of LMP1 expressing. Only 20% to 65% NPCs express LMP1 that can be detected by Western blot or immunohistochemical analysis and another (25, 33). Although the results presented here seem promising, the different level of LMP1 expressing in EBV-related carcinomas, may lead to the different biologic efficacy of this Fab-based immunoconjugate. To strengthen the antitumor biologic efficacy of HLEAFab-MMC to the low LMP1-expressing NPC, our next strategy is that the antibody will conjugate liposomes or polymer nanoparticles encapsulating more MMC to elevate potential efficiency and decrease clearance rate. The conjugation can kill not only the cancer cells of low-expressing LMP1 by targeted efficacy but also the cancer cells of LMP1 negative through a bystander effect. This phenomenon was also reported by Kovtun and his colleagues (34). On the whole, the evaluation of LMP1 expression in individuals is required before this therapeutic strategy is applied in NPC treatment.

In summary, our findings indicate that the LMP1 EMD is a specific marker of human NPC that can be recognized by a HLEAFab-based immunoconjugate (HLEAFab-MMC). The immunoconjugate, the first targeting agent based on a fully human antibody Fab fragment described so far, is effective both in vitro and in vivo against NPC cells and might represent a promising chemotherapeutics that merits further preclinical studies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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