Research Article

Production of Antibodies against Multipass Membrane Proteins Expressed in Human Tumor Cells Using Dendritic Cell Immunization

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Antibody mediated therapeutic strategies against human malignant tumors have been widely authorized and clinically applied to cancer patients. In order to develop methods to generate antibodies reactive to the extracellular domains of multipass plasma membrane proteins specifically expressed in malignant tumors, we examined the use of dendritic cells (DCs) for immunization. DCs were transduced with genes encoding the human six transmembrane epithelial antigen of prostate 1 (STEAP1), STEAP4, and seven transmembrane prostate specific G-protein coupled receptor (PSGR). Mice were immunized with these DCs and followed by repeated booster immunization with plasmids expressing each protein. The immunized mice produced significant amounts of antibodies against these proteins. Our results suggest that DC immunization is an effective method to produce antibodies reactive to extracellular regions of plasma membrane proteins with multiple-transmembrane domains, and may be useful to develop antibody mediated antitumor therapies.

1. Introduction

In the development of antibody-mediated therapies targeted to human cancer cells, including cancer stem/progenitor cells, the extracellular domains of plasma membrane proteins specifically expressed on cancer cells are promising candidates for antigens against which to be immunized [1], but the high homology of amino acid sequences between human antigens and their homologues in animals to be immunized often hamper efficient antibody production because of immunological tolerance. In the case of cellular membrane proteins having only a single transmembrane domain, recombinant protein with the extracellular domain fused to the Ig-Fc domain has been used as immunogen in many cases to generate antibodies reactive with the extracellular region [2]. However, in the case of plasma membrane proteins having multiple transmembrane domains, the three dimensional architecture of the protein outside the cell is expected to be composed of multiple extracellular domains, suggesting that construction of Ig-Fc fusion proteins for immunization would be difficult.

To obtain antibodies reactive to the native extracellular structure of such membrane proteins, immunization by injection of cultured cells expressing the antigen has been used [3]. However, large numbers of cells (typically 10^7-10^8 cells per animal) are usually needed to prepare for immunization and some modifications of the injected cells are required, for example, genes encoding immunomodulatory cytokines (interleukin-4, and others) or costimulatory molecules are expressed together with the antigen to obtain higher titers. Moreover, the cells expressing plasma membrane proteins having multiple transmembrane domains such as G-protein coupled receptors (GPCRs) are not always available for immunization. Therefore, development of a simple and successful protocol for immunization against human multi-pass membrane proteins is needed in antibody-mediated cancer research.

Dendritic cells (DCs) are the most potent antigen presenting cells and robustly induce adaptive immunity mediated by T cells and B cells [4, 5]. The central role of DCs in immunity may explain why DC-mediated vaccines have been used for induction of cellular immunity against

malignant tumor cells and infectious pathogens [6–8]. The potency of DCs was demonstrated in previous studies to disrupt immunological tolerance against a tumor antigen and induce tumor antigen specific T cells [9]. In addition, DCs also play a key role in induction of humoral immunity [10]. The activation of CD4^{+} T cells by DCs can exert helper functions to enhance efficient antibody production, production of high-affinity antibodies through somatic hypermutation, and class-switching of antibodies. DCs can also release exosomes containing intact antigen, which induces activation of antigen specific B cells antibody responses [11]. These observations strongly suggest that targeted expression of antigens in DCs to stimulate production of useful antibodies is a reasonable experimental approach; however, such attempts have been limited [9, 12].

In this study, we focused on an immunization method using DCs expressing human tumor transmembrane antigens. DCs can efficiently present antigen to B cells and CD4^{+} T cells because DCs express the antigen in intact form on the cell surface, to be recognized by antigen-reactive B cells, and in processed form in context with MHC molecules, to be specifically recognized by CD4^{+} T cells. These properties may offer many advantages in efficient generation of antigen-specific antibodies. The antigens used for immunization in this study were the human six transmembrane epithelial antigen of prostate 1 (STEAP1), human STEAP4, and the human prostate specific G-protein coupled receptor (PSGR) [13–17]. These antigens possess multiple transmembrane domains (6 in STEAPs and 7 in PSGR) and high degree of homology with the corresponding mouse proteins (82% in STEAPs and 92% in PSGR amino acid identity between human and mouse). The complex native extracellular structures and their high degree of homology imply that production of antibodies against such membrane proteins may be difficult. However, in this study, we show that immunization using DCs efficiently induced antibody production against these membrane proteins in mice, which could be used for antibody-mediated immunological assays, including flow cytometry, immnuofluorescent staining, and Western blotting.

2. Materials and Methods

2.1. Mice. BALB/C mice (6–8 weeks old) were purchased from Sankyo Labo Service Co. (Tokyo, Japan). All animal experiments were performed according to the guidelines of the Tokyo University of Science.

2.2. Cell Culture. 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (FCS). PlatE cells were cultured in DMEM supplemented with 10% heat inactivated FCS, blasticidin (10 μg/mL) and puromycin (2 μg/mL). LNCaP and DU145 human prostate cancer cell lines were purchased from RIKEN BioResource Center (Tsukuba, Japan) and cultured in RPMI 1640 supplemented with 10% heat inactivated FCS. For androgen stimulation, 5-α-dihydrotestosterone (5-DHT) was purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.3. Generation of Mouse Bone Marrow Derived Dendritic Cells (BM-DCs). The culture of BM-DCs has been previously described [18]. Briefly, bone marrow cells from tibias and femurs of BALB/C mice were isolated, and 2×10^6 cells were plated in 10 cm bacterial dishes (IWAKI, Chiba, Japan) and cultured in RPMI 1640 supplemented with 10% heat inactivated FCS and 20 ng/mL recombinant murine GM-CSF (Peprotech, Rocky Hill, NJ) for 10 days. The suspension and loosely-adherent cells were collected as CD11b^{+}CD11c^{+} BM-DCs.

2.4. Construction of Expression Vectors. pCAGGS, and pCAGGS-HA containing cDNA encoding hemagglutinin (HA) of human influenza A virus (H1N1/PR8) was kindly provided by Dr. H. Hasegawa (National Institute of Infectious Diseases, Japan). The cDNA encoding human STEAPs (STEAP1 and STEAP4) and human PSGR were cloned from human spleen cDNAs (Clontech, Palo Alto, CA) using PCR and from human prostate RNA (Clontech) using RT-PCR, respectively. PCR products were inserted into pGEM T-easy vector (Promega, Madison, WI). The sequences of these cDNA fragments were confirmed using ABI Prism 3100-Avant DNA sequencer (Applied Biosystems, Foster City, CA). The cDNAs were excised from pGEM T-easy vector and inserted into either pMRX-IRES-GFP or pCAGGS. The plasmids were propagated in *E. coli* (DH5α) and purified using a kit (QIAGEN, Valencia, CA) for the following experimental procedures.

2.5. The Infection of BM-DCs with a Retroviral Vector. PlatE cells in 10 cm dishes were transfected with pMRX-hSTEAP1 (hSTEAP4, hPSGR)-IRES-GFP using Fugene 6 (Roche, Indianapolis, IN). The collected culture supernatants were added to BM-DC cultures on days 4 and 8 and incubated with 5 μg/mL polybrene (Sigma, St. Louis, MO) overnight. The medium was replaced by fresh medium after the overnight incubation. On day 10, BM-DCs were collected and analyzed using FACSCalibur (Becton Dickinson, San Jose, CA) to confirm GFP expression in BM-DCs. The percentage of GFP positive cells in BM-DCs was routinely 1–5% and the phenotype of the GFP positive BM-DCs was as same as non-infected BM-DCs.

2.6. The Immunization Protocol. The BM-DCs (2×10^6 cells/mouse) were injected via tail vein or directly into the spleen of mice (0 weeks). At 2 weeks, 4 weeks, and 6 weeks, gene transduction with plasmids expressing antigens (pCAGGS-hSTEAP1, -hSTEAP4, and -hPSGR; 5 μg of plasmids/mouse) was performed as a booster immunization using hydrodynamic delivery via the tail vein [19]. The sera at 8 weeks after DC injection were tested in following immunological assays.

2.7. Flow Cytometry Analysis. 293T cells were transfected with pMRX-hSTEAP1-IRES-GFP or pMRX-hSTEAP4-IRES-GFP using Lipofectamine LTX (Invitrogen, Calsbad, CA). Transfected 293T cells, LNCaP cells, and DU145 cells were detached from the dish by treatment with PBS/10 mM EDTA, collected, and resuspended in PBS/0.5% bovine

serum albumin (BSA). The immunized sera (1:100 diluted) were added to cells, followed by incubation on ice for 30 minutes. After washing, cells were incubated with PE conjugated goat antimouse IgG (Beckman Coulter, Miami, FL) and incubated on ice for 30 minutes. After washing, stained cells were measured using FACSCalibur and data analysis was performed using the CellQuest software (Becton Dickinson). To determine IgG1/2a ratio, the immunized sera are added to 293T cells transfected with pCAGGS-HA, followed by incubation on ice for 30 minutes. After washing, cells were incubated with PE conjugated goat antimouse IgG1 or IgG2a (Beckman Coulter). The mean fluorescence intensity of the stained cells was determined and IgG1/2a ratio was calculated.

2.8. Western Blotting. The proteins in cell lysates were separated by SDS-PAGE and transferred to Immobilon membranes (Millipore, Bedford, MA). The Block-Ace (Snow Brand Milk Products, Tokyo, Japan) solution was used for blocking the membranes, and membranes were then reacted with 1:500 diluted anti-STEAP1 serum for 1 hour at room temperature. After washing, the membranes were incubated with alkaline phosphatase conjugated goat antimouse IgG+M (Biosource, Camarillo, CA). Reactive bands were visualized using an NBT/BCIP substrate solution.

2.9. Anti-STEAP4 Monoclonal Antibody Production. Spleen and bone marrow cells removed from an immunized mouse were fused with SP2/0 myeloma cells using PEG. The fused cells were seeded in 96 well plates and cultured in HAT medium. Positive wells were identified by ELISA screening. Hybridoma cells producing anti-hSTEAP4 antibodies were cloned and established by limiting dilution and ELISA screening. The immunogloblin class of the antibodies produced from the hybridoma cells was determined using IsoStrip mouse monoclonal antibody isotyping kit (Roche).

2.10. Immunofluorescent Staining. LNCaP and transfected 293T cells were seeded in eight-well chamber slides (Nalge-Nunc, Naperville, IL). The cells were fixed with 3% paraformaldehyde and, when required, permeabilized with 0.1% Triton X-100 for 20 minutes at room temperature. After blocking, cells were incubated with 1:100 diluted serum or 4F2 (anti-STEAP4 monoclonal antibody) for 1 hour at room temperature. After washing, cells were incubated with FITC conjugated goat antimouse IgG (Jackson Immunoresearch, West Grove, PA) for 1 hour at room temperature. The stained cells were observed by confocal microscopy (LSM510, Carl Zeiss, Jena, Germany). In some cases, 293T cells transfected with pCAGGS-hSTEAP4 were cultured in regular medium for 30 minutes in the presence of 4F2. After culturing, medium was removed and the cells were washed by PBS, fixed and stained with FITC conjugated antimouse IgG.

3. Results

3.1. STEAP1 Immunization. We immunized mice with DCs expressing hSTEAP1. After 3 booster injections of pCAGGS-hSTEAP1, sera from immunized mice were subjected to flow cytometry analysis to check antibody production against native extracellular structure of hSTEAP1. The sera from two of the five immunized mice reacted with 293T cells expressing hSTEAP1 in a flow cytometry analysis (Figure 1(a)). As negative controls, we also immunized mice with DC not expressing hSTEAP1 with booster injections, or the hydrodynamic injections only ($N = 3$ for each group). No sera from these negative control groups stained 293T cells expressing hSTEAP1 (Figure 1(a)). The highest titer of anti-STEAP1 antibodies by flow cytometry analysis was 900. Moreover, immunized sera clearly reacted with hSTEAP1 protein (34 kd) in Western blotting, but negative control sera did not (Figure 1(b)). The previous studies [13, 17] demonstrate that human prostate cancer cell lines including LNCaP and DU145 express hSTEAP1 endogenously. Flow cytometry analysis showed that LNCaP cells were significantly stained with immunized serum, although DU145 cells stained very faintly (Figure 1(c)). These results demonstrate that immunized sera could react with STEAP1 in human prostate cancer cells. Collectively, DC immunization can induce production of antibodies reactive to the native extracellular domain of hSTEAP1.

3.2. STEAP4 Immunization. The frequency of antibody production against hSTEAP1 was relatively low; therefore, we investigated better administration routes for DC immunization of mice. We found that intrasplenic injection of DCs increased the frequency of antibody production relative to intravenous injections of DCs in HA and hSTEAP1 immunizations (data not shown). Thus, we immunized mice with hSTEAP4 expressing DCs using intrasplenic injection. The immunized sera clearly reacted with 293T cells expressing hSTEAP4 in flow cytometry (Figure 2(a)). Sera from all five immunized mice exhibited positive staining. In contrast, booster immunizations (by the hydrodynamics method) without DC injection of three mice resulted in no staining in sera from two mice and weak staining in serum from one mouse, suggesting that DC immunization is critical for efficient antibody generation against hSTEAP4, as well as hSTEAP1 (Figure 2(a)). The highest titer of anti-STEAP4 by flow cytometry analysis was 2700. The anti-hSTEAP1 serum did not react with 293T cells expressing hSTEAP4, and vice versa, demonstrating that there was no significant cross-reactivity between the hSTEAP1 and hSTEAP4 antibodies (Figure 2(b)).

A previous study showed that hSTEAP4 was expressed in LNCaP cells and increased upon androgen stimulation of the cells [16]. The hSTEAP4 immunized sera stained a small proportion ($\sim 1\%$) of LNCaP cells and staining in these cells increased following androgen (5-DHT) treatment of the cells (Figure 2(c)). The restricted expression of hSTEAP4 in LNCaP cells was also confirmed using immunoflorescent staining (Figure 2(d)). As a negative control, serum from mice immunized with DCs expressing HA as an irrelevant antigen was used, and only background staining was detected.

Three IgG monoclonal antibodies (one was IgG1, κ and two were IgG2a, κ) against hSTEAP4 were generated from an immunized mouse. In immunofluorescent staining,
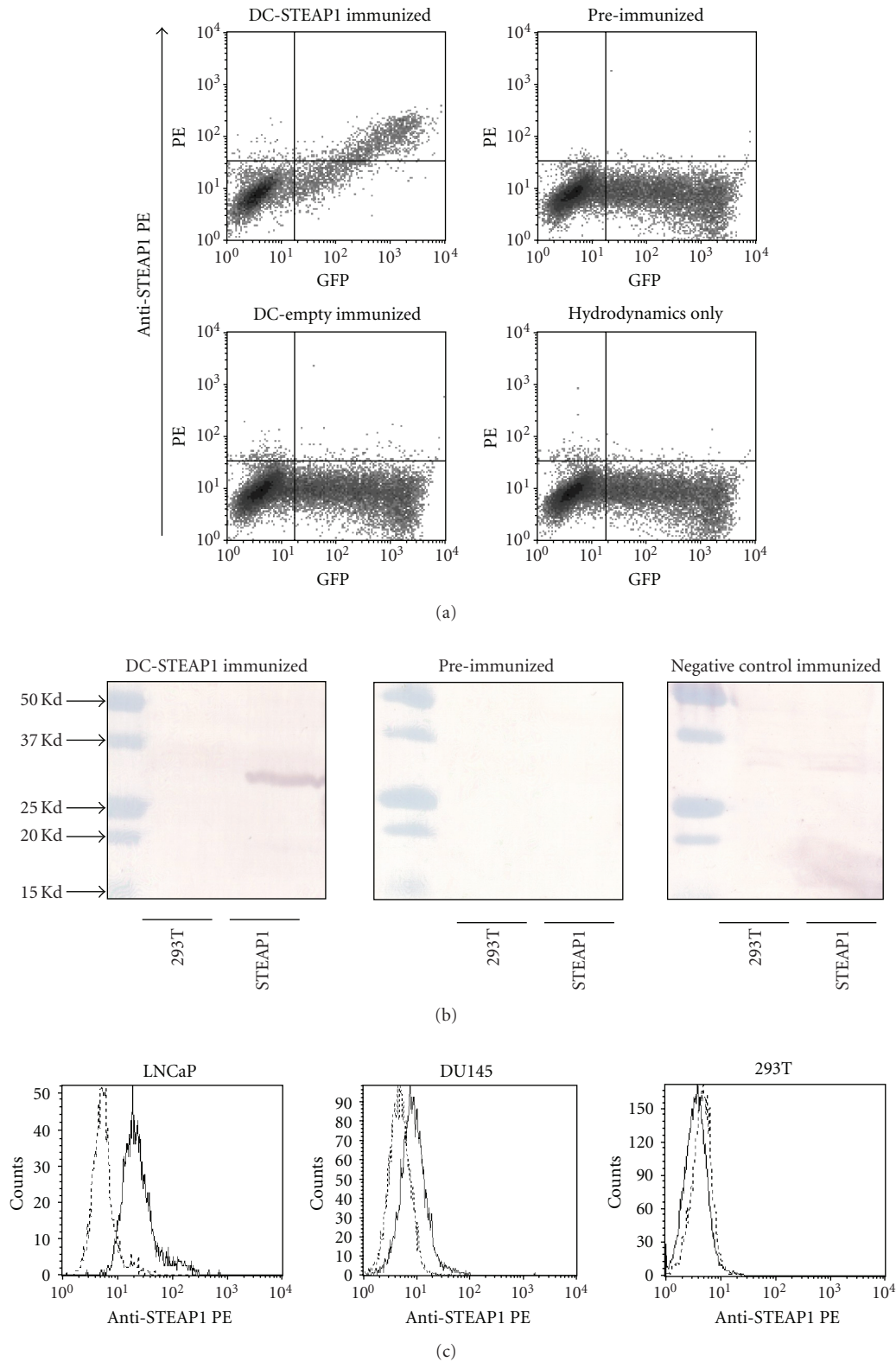
(a)

(b)

(c)

FIGURE 1: hSTEAP1 immunization. (a) Flow cytometry analysis to detect anti-hSTEAP1 in sera from immunized mice. Upper panels, DC-STEAP1 immunized (left), preimmunized (right). Lower panels, DC-empty immunized, with booster immunization (left), booster immunization only without DC immunization (right). 293T cells transiently transfected with pMRX-hSTEAP1-IRES-GFP were stained with sera, followed by PE conjugated antimouse IgG. (b) Western blotting analysis of hSTEAP1 immunization. The lysates of 293T cells and 293T cells transfected with pCAGGS-hSTEAP1 were probed with serum from immunized mice. (c) Anti-hSTEAP1 flow cytometry analysis in human prostate cancer cells. From left to right: LNCaP, DU145, and 293T cells. Solid line: anti-STEAP1 serum. Dashed line: preimmune serum.
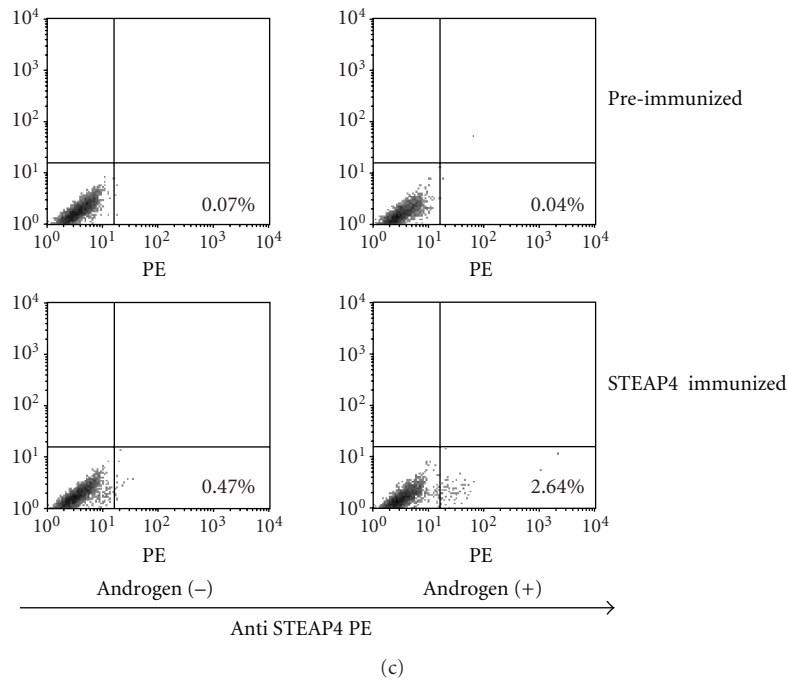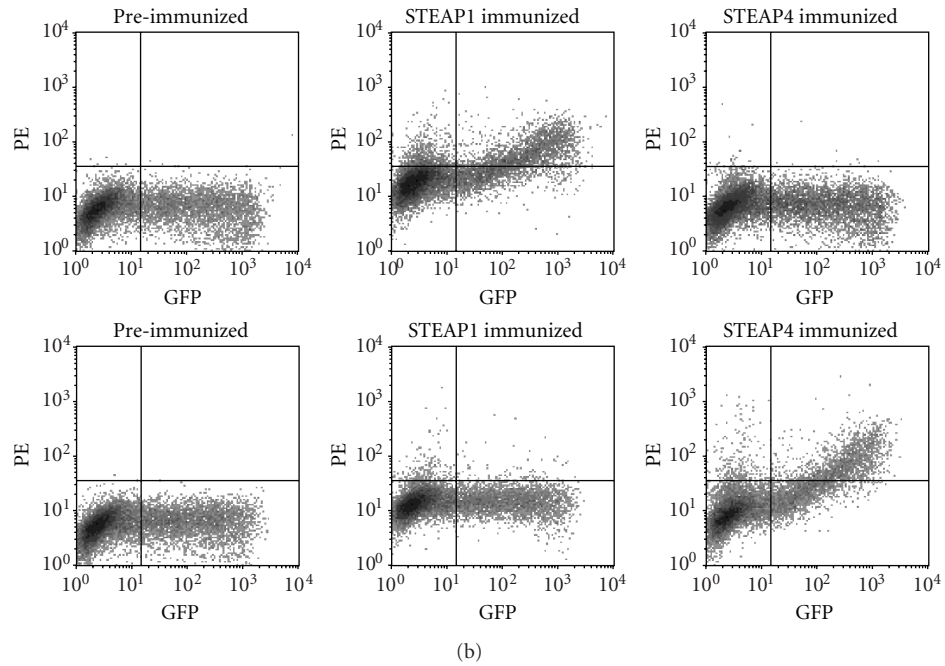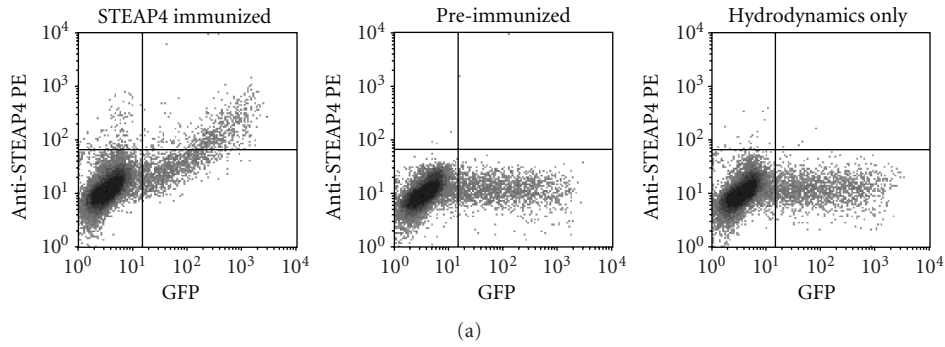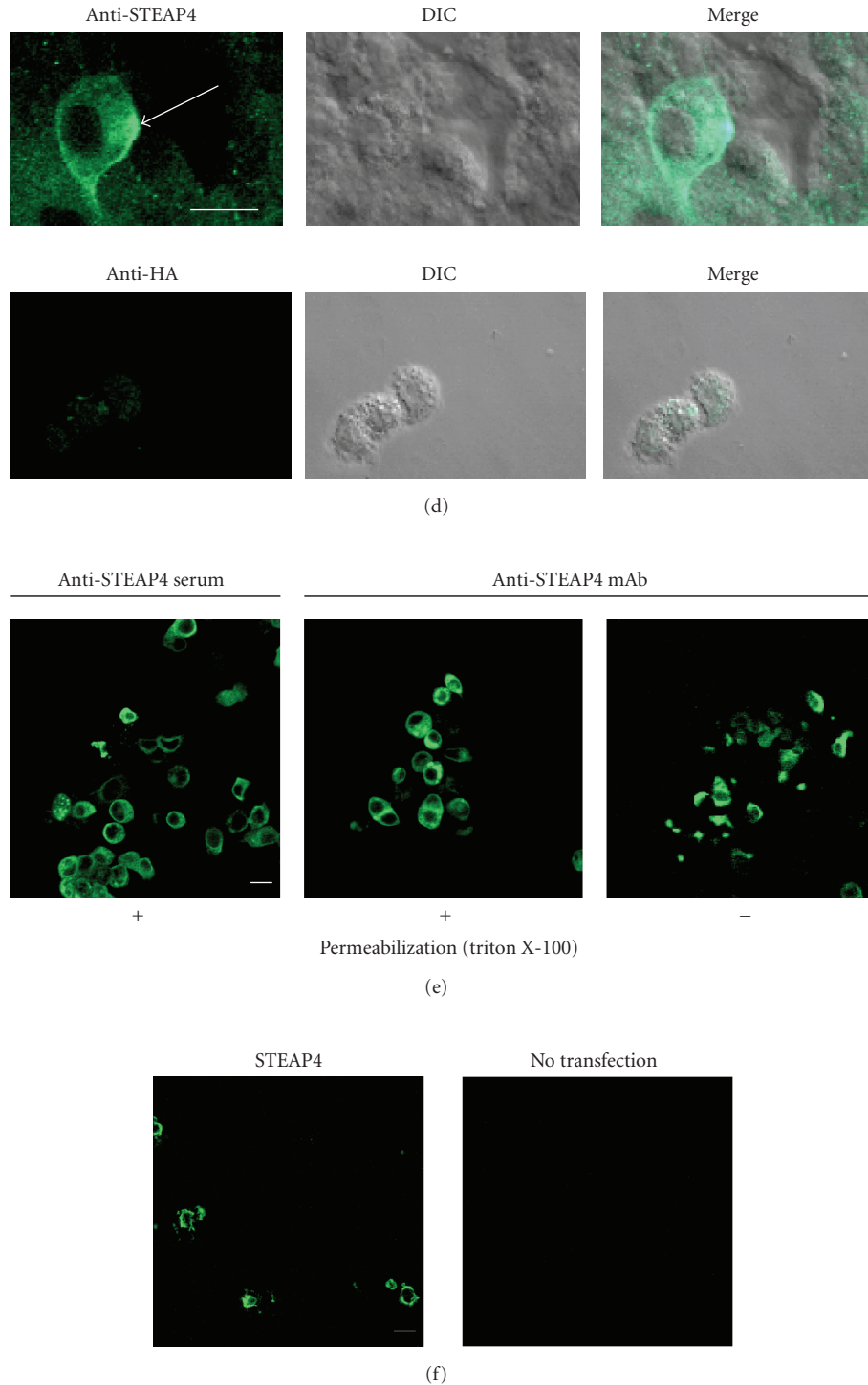
Figure 2: Continued.

FIGURE 2: hSTEAP4 immunization. (a) Flow cytometry analysis to detect anti-hSTEAP4 in sera from immunized mice. 293T cells transiently transfected with pMRX-hSTEAP4-IRES-GFP were stained with sera, followed by PE conjugated antimouse IgG. (b) Specific binding of anti-STEAP antibody in flow cytometry analysis. The upper panels show 293T cells transfected with pMRX-hSTEAP1-IRES-GFP. The lower panels show 293T cells transfected with pCAGGS-hSTEAP4-IRES-GFP. The cells were reacted with the indicated serum and stained with PE conjugated antimouse IgG. (c) Detection of hSTEAP4 expression in LNCaP cells using flow cytometry analysis. For androgen treatment, LNCaP cells were cultured with 5-DHT (10^{-9} M) for 24 hours. (d) hSTEAP4 expression in androgen stimulated LNCaP cells using immunofluorescent staining. Arrow indicates a stained cell. Scale bar = $20\,\mu$m. (e) hSTEAP4 proteins detected by anti-hSTEAP4 monoclonal antibody (4F2; IgG2a) in 293T cells transfected with pCAGGS-hSTEAP4. Scale bar = $20\,\mu$m. (f) Anti-hSTEAP4 monoclonal antibody bound to hSTEAP4 proteins on cell surface. The antibody (4F2) was added to 293T cells transfected with pCAGGS-hSTEAP4 and the cells continued to be cultured for 30 minutes. The cells were fixed and reacted with FITC conjugated antimouse IgG. Scale bar = $20\,\mu$m.

hSTEAP4 proteins were clearly detected in 293T cells transfected with pCAGGS-hSTEAP4 using these monoclonal antibodies as well as the immunized serum (Figure 2(e)). In Figure 2(e), 4F2 (IgG2a) was used as representative. The stained images demonstrated that hSTEAP4 was localized on the plasma membrane as well as intracellular structures, possibly endoplasmic reticulum, endosomes, and Golgi apparatus, as described previously [16]. Importantly, anti-STEAP4 monoclonal antibody (4F2) reacted with 293T cells transfected with pCAGGS-hSTEAP4 even without cell permeabilization treatment. The stained images showed extracellular domain of STEAP4 proteins on plasma membrane was recognized by anti-STEAP4 monoclonal antibody. Moreover, anti-STEAP4 monoclonal antibody (4F2) was added to 293T cells transfected with pCAGGS-hSTEAP4 followed by immunofluorescent staining. Figure 2(f) showed that anti-STEAP4 monoclonal antibody bound hSTEAP4 proteins on cell surfaces, suggesting that monoclonal antibody recognized the native extracellular domains of hSTEAP4. These results collectively suggest that antibodies specific for hSTEAP4 can be efficiently generated using DC mediated immunization.

3.3. PSGR Immunization. We demonstrated that DC immunization significantly induced antibody production against STEAP family proteins. These results prompted us to investigate whether DC immunization with hydrodynamics booster injections is applicable to other plasma membrane proteins. PSGR belongs to the olfactory receptor family and is an orphan GPCR specifically expressed in prostate in both normal human tissues and prostate cancers [14]. We, thus, examined DC mediated antibody production against hPSGR. To test immunized serum for reactivity to native extracellular domains of hPSGR, a flow cytometry analysis using LNCaP cells, which endogenously express PSGR [14], was conducted. The LNCaP cells were positively stained with sera collected from immunized mice (Figure 3(a)). Hydrodynamic booster immunization alone failed to induce a significant antibody response against hPSGR in flow cytometry analysis (data not shown). By immunofluorescent staining, PSGR expression was specifically detected on the cell surface of LNCaP cells (Figure 3(b)). The cell surface specific staining even without cell permieabilization treatment (Figure 3(b)) suggested anti-PSGR serum in DC immunization reacted with extracelluar domain of PSGR as well as flow cytometry analysis. Taken together, these observations indicate that DC immunization is effective in inducing antibody production against PSGR, in addition to STEAP family proteins.

3.4. The Antigen Specific IgG1/2a Ratio in DC Immunization. To further characterize the antibody response, we determined IgG1/2a ratio of anti-HA antibody. The ratio of IgG1/2a represents the balance of Th2/Th1 response. The anti-HA specific immunological response evoked by DC immunization was shown to be Th2 dominant because the ratio of IgG1/2a in DC immunization was 8.75 at average and anti-HA IgG1 was more abundant than IgG2a (Figure 4). The ratio of IgG1/2a in DC immunization was higher than hydrodynamics method (average of 4.17), although the difference was not statistically significant.

4. Discussion

In this study, we investigated whether DCs as immunogens elicited antibody production against multiple-transmembrane proteins expressed in human prostate cancer cells. BM-DCs stably expressing membrane proteins were used to immunize mice. Antigen specific antibodies reactive with native extracellular domains of membrane proteins were detected. Previous studies demonstrated that immunization of mice with antigen-pulsed DCs resulted in efficient induction of antibody response [20]. Accordingly, our results underline the utility of DC immunization in the induction of antibody response against the external domains of membrane tumor antigens that might be useful for development of antibody mediated treatment of cancer diseases.

The priming immunization with DCs was very effective, whereas plasmid-based immunization using hydrodynamic delivery alone was not sufficient to stimulate antibody generation, although hydrodynamics was an efficient method for booster immunizations (Figures 1 and 2). Moreover, DCs exerted superior activity in antibody production relative to BALB/C 3T3 fibroblasts in HA immunization (data not shown). These observations suggest that DCs possess strong priming activity to induce antibody production. In general, DCs are particularly known to induce Th1 immune response because of their ability to initiate adaptive cellular immune response through production of high amounts of IL-12. However, the high IgG1/2a ratio obtained following DC immunization indicated their potential effect in stimulation of antibody production as well. Actually, because HA antigen is highly antigenic, hydrodynamic immunization significantly could induce anti-HA humoral responses, but IgG1/2a ratio in DC immunization was higher than hydrodynamic immunization, suggesting that DC immunization can stimulate antibody response with Th2 profile. This strong immune response is particularly needed when using less immunogenic proteins such as STEAPs, which are highly homologous between human and mice.

The STEAP family (STEAP1-4) consists of a unique group of proteins: other proteins homologous to the STEAP family do not exist in higher mammals. The STEAP family proteins share a high degree of homology in amino acid sequence, but there may be functional differences between STEAP1 and STEAP4. STEAP1 is largely lacking a cytoplasmic N-terminal tail homologous to bacterial and archaeal FNO oxidoreductase, different from other STEAP proteins. STEAP4 exhibits a strong iron reductase activity, but STEAP1 showed no such activity [21]. In fact, coexpression of STEAP1 and STEAP4 may occur in some human normal and malignant tissues. The interplay between STEAP members to modulate their biological functions remains elusive. An antibody-based analysis will be critical for understanding STEAP function and the DC immunization protocol used in this study can provide such antibodies.

Recent work demonstrated that loss of STEAP4 led to metabolic syndrome in mice [22]. We found that STEAP4
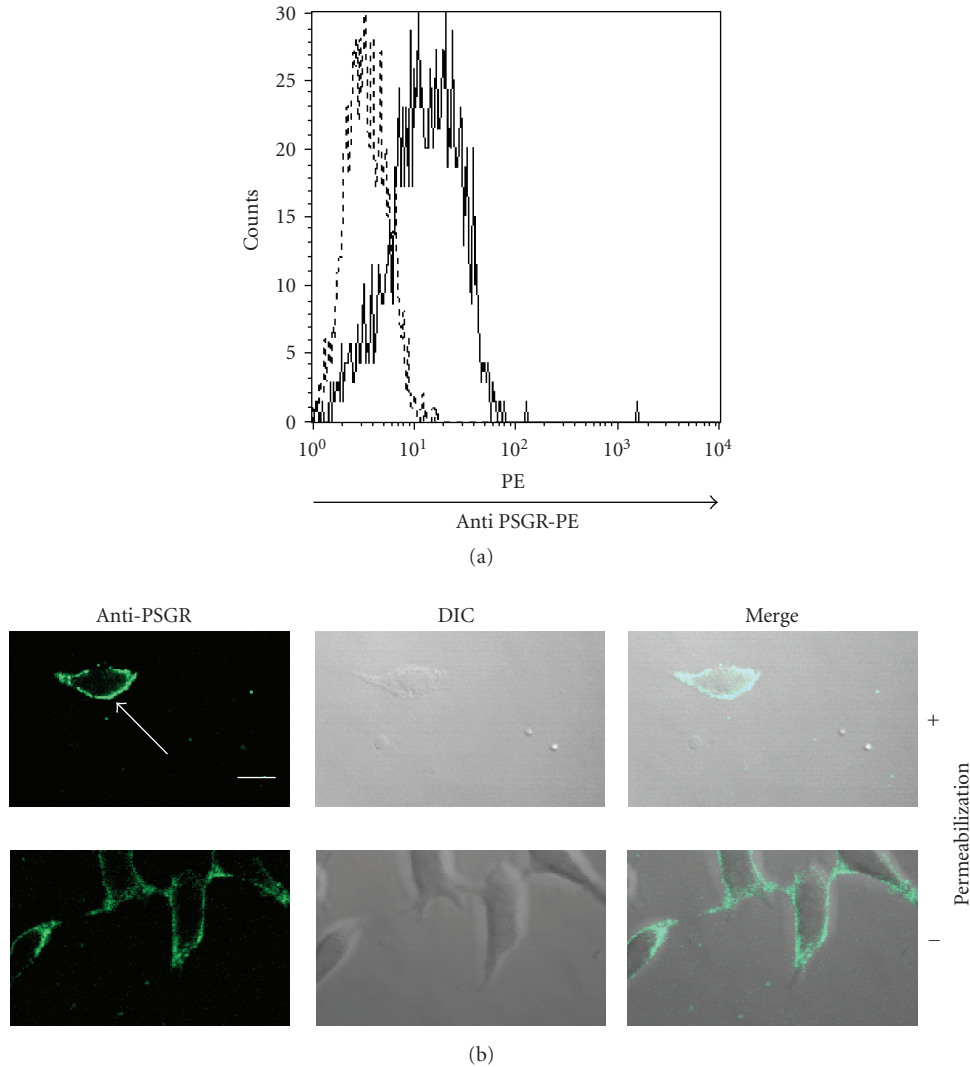
(a)

(b)

FIGURE 3: hPSGR immunization. (a) hPSGR expression in LNCaP cells using flow cytometry analysis. Solid line: anti-PSGR serum. Dashed line: preimmune serum. (b) Immunofluorescent staining of LNCaP cells using anti-PSGR serum. Arrow indicates a stained cell. Scale bar = 20μm.

expression was restricted to a small population of LNCaP cells. The characterization of these cells remains to be conducted. A flow cytometry analysis using anti-STEAP4 serum did not detect expression of STEAP4 in androgen independent DU145 cells (data not shown), which is consistent with previous results [16]. We are currently investigating whether STEAP4 function is involved in tumor progression. The monoclonal antibodies generated in this study were demonstrated to bind to the extracellular domains of hSTEAP4, suggesting that these antibodies could be functional to promote or inhibit the functions of STEAP4. Moreover, they could exert effector activities such as complement dependent cytotoxicity or antibody dependent cellular cytotoxicity. The monoclonal antibodies will be useful in these studies as well as in research on a role of STEAP4 in metabolic syndrome in mice and human.

PSGR expression is highly specific in normal prostate and prostate cancers, which makes it a very attractive target for antibody mediated therapy against prostate cancer. Numerous GPCRs, in addition to PSGR, are expressed in tumor tissues. DC immunization successfully induced antibody production against hPSGR, in this study. Using DC immunization, a variety of antibodies reactive with GPCRs can be prepared and can contribute to development of antitumor therapies.

The DC-mediated immunization protocol developed in this study is simple, and efficiently induced antibody production against the extracellular domains of several human multiple transmembrane proteins in mice. We also generated monoclonal antibodies from an immunized mouse. The development of larger scale DC culture protocols, or more efficient gene delivery into DCs will be important to improve immunization results. Furthermore, genetic manipulation of DC to confer immunomodulatory functions may be critical, depending on the immune responses evoked by particular antigens. We are further developing DC immunization
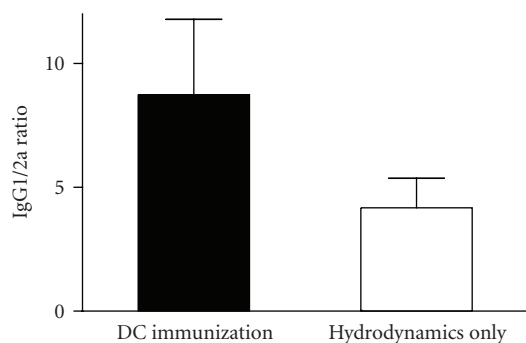
Figure 4: The ratio of anti-HA specific IgG1/2a in DC immunization. The BM-DCs expressing HA were injected into mice ($n = 5$). For control, hydrodynamic injection of pCAGGS-HA was carried out ($n = 4$). After booster immunization, the ratio of anti-HA specific IgG1/2a was determined using flow cytomery analysis. Data represent means $\pm$ SE of each group.

protocols to generate monoclonal antibodies that specifically react with cancer stem cells, or highly metastatic cancer cells, which will be useful for novel anticancer therapeutic strategies.

Acknowledgment

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