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Tumor progression-related transmembrane protein aspartate β -hydroxylase is a target for immunotherapy of hepatocellular carcinoma

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Abstract

Background/Aims—Hepatocellular carcinoma (HCC) has a poor survival rate due to recurrent intrahepatic metastases and lack of effective adjuvant therapy. Aspartate- β -hydroxylase (ASPH) is an attractive cellular target since it is a highly conserved transmembrane protein overexpressed on both murine and human HCC tumors, and promotes a malignant phenotype as characterized by enhanced tumor cell migration and invasion.

Methods—Dendritic cells (DCs), expanded and isolated from the spleen, were incubated with a cytokine cocktail to optimize IL-12 secretion and co-stimulatory molecule expression, then subsequently loaded with ASPH protein for immunization. Mice were injected with syngeneic BNL HCC tumor cells followed by subcutaneous inoculation with $5\text{--}10 \times 10^5$ ASPH loaded DCs using a prophylactic and therapeutic experimental approach. Tumor infiltrating lymphocytes (TILs) were characterized, and their role in producing anti-tumor effects determined. The immunogenicity of ASPH protein with respect to activating antigen specific CD4⁺ T cells derived from human peripheral blood mononuclear cells (PBMCs) was also explored.

Methods—We found that immunotherapy with ASPH-loaded DCs suppressed and delayed established HCC and tumor growth when administered prophylactically. Ex-vivo re-stimulation experiments and in vivo depletion studies demonstrate that both CD4⁺ and CD8⁺ cells contributed to anti-tumor effects. Using PBMCs derived from healthy volunteers and HCC patients, we showed that ASPH stimulation led to significant development of antigen-specific CD4⁺ T-cells.

Conclusion—Immunization with ASPH-loaded DCs has substantial anti-tumor effects which could reduce the risk of HCC recurrence.

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The authors have nothing to disclose.

Keywords

Hepatocellular carcinoma; immunotherapy; dendritic cells; aspartate- β -hydroxylase; CD4+ cells

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer [1] with an increasing incidence noted in the last two decades in the United States, Europe, and Japan. This increased rate is expected to continue over the next 10 – 30 years due to a large pool of individuals chronically infected with hepatitis B (HBV) and hepatitis C (HCV) virus. It is in the setting of persistent viral infection of the liver that these tumors most commonly arise. The five-year survival rate of patients in the United States is only 9%, [2, 3] and there is a recurrence rate of more than 70% following surgical resection [4]. Therefore new therapeutic approaches are needed [5].

We have studied the regulation, expression, and function of a highly conserved enzyme designated aspartate- β -hydroxylase (ASPH) that has been found to be overexpressed in HBV- and HCV-related HCC as well as murine hepatomas, and which may serve both as a biomarker and therapeutic target for this disease. ASPH is a ~86kD Type 2 transmembrane protein and member of the α -ketoglutarate-dependent dioxygenase family [6]. ASPH catalyzes post-translational hydroxylation of β -carbons of specific aspartate and asparagine residues in epidermal growth factor (EGF)-like domains in proteins such as Notch, and Jagged, which have known roles in cell growth, differentiation, cellular migration, adhesion, and motility [7–10]. The ASPH gene becomes overexpressed in HCC tumors and the protein translocates to the plasma membrane from the endoplasmic reticulum where it becomes accessible to the extracellular environment and could serve as a tumor associated antigen (TAA) target for immunotherapy.

We employed ASPH-loaded DCs to generate anti-tumor immune responses. DCs are antigen-presenting cells (APCs) with the unique ability to take-up and process antigens. DCs can induce effector CD4+ T helper cell (Th) responses. The Th1 response is characterized by the production of interferon (INF)-gamma (γ) which activates CTLs (CD8+) and induces cell mediated immunity [11]. These T cells are subsequently stimulated and polarized by APC-secreted cytokines such as IL-12 as well as by interaction with TCR co-receptors such as the B7 family of molecules. As a result, antigen-specific CD4+ and CD8+ T-cells may cooperatively eliminate TAA expressing tumors.

Previous investigations have demonstrated the importance of ASPH in the pathogenesis of HCC recurrence and progression via promotion of tumor cell migration and invasion [8, 12–14]. Most if not all HCC cells within a tumor express ASPH on the cell surface [15]. These findings generated a hypothesis that targeting of this overexpressed cell surface molecule by an immunotherapeutic approach may inhibit the development and progression of ASPH-expressing HCC tumors.

Methods

Recombinant human aspartate- β -hydroxylase

The full length human ASPH (GenBank accession no. 583325) was cloned into the EcoRI site of the pcDNA vector (Invitrogen). Recombinant ASPH protein produced in a Baculovirus system (Invitrogen) according to manufacturer's instruction.

Mice and generation of mature dendritic cells

Female 6 – 8-week-old BALB/c mice (Harlan Laboratories) were used. All animal protocols were approved by the Institutional Animal Care and Use Committee of Rhode Island Hospital. We isolated DCs as previously described [16]. This detailed method is further described in the Supplementary Methods.

Immunization

Mice were immunized by injecting $5 - 10 \times 10^5$ DCs or control HBSS into the base of the tail. Unless otherwise specified, mice were immunized twice spaced two-weeks apart. For the *in vitro* experiments, we studied the immunized mice two weeks after the last immunization. In other experiments, the immunization schedules are specified.

Enzyme-linked immunosorbent and in vitro cytotoxicity assays

See Supplementary Methods for details.

Tumor cell culture and inoculation

The BNL 1ME A.7R.1 (BNL) murine HCC cell line was purchased from ATCC BNL and its highly malignant and fast-growing subclone BNL3 as well as SP2-0 murine myeloma cells were maintained in DMEM with 10% FBS. The highly tumorigenic subclone of BNL3 cells was generated by three serial subcutaneous passages of parental BNL cells. It is noteworthy that BNL3 (1×10^3 cells) were capable of forming large subcutaneous tumors in incubated mice whereas 1×10^6 parental BNL cells were required. In these experiments, 1×10^6 BNL or 4×10^3 BNL3 cells were subcutaneously inoculated into the right flank and tumor size was measured every 7 or 3 days, respectively. Mice bearing tumors where the shorter diameter exceeded 10 mm were euthanized according to the requirements of the Animal Welfare Committee of the Rhode Island Hospital.

Evaluation of tumor-infiltrating lymphocytes and in vivo depletion of immune cells

See Supplemental Methods for complete description.

Human studies

The protocol for these experiments was approved by the Institutional Review Board of Rhode Island Hospital. Experiments were performed according to the methods published by Moser, et al [17]. Detailed protocols, *statistical analysis* and HCC patient characteristics are described in the Supplementary Methods (Table 1).

Results

Activation of DCs

Purified ASPH protein was obtained using a baculovirus expression system to yield a single band on SDS-PAGE which was immunologically confirmed by Western blot analysis (Fig. 1a). A previously described method was employed for isolation and purification of murine DCs; this technique combines three critical elements necessary for efficient DC-based immunization including maturation, enrichment and antigen targeting [16]. The percent of isolated DCs ingesting GFP-coated magnetic microbeads was $73.2 \pm 4.1\%$ (Fig. 1b). Fetal bovine serum was not used as a component of culture medium to avoid confounding non-specific immune responses [18, 19]. The combination of all four cytokines (IL-4, IFN γ , CD40L and GM-CSF) resulted in a high secretion of IL-12 by DCs (Fig. 1c). The CD8a⁺ DC subset has potent cross-presentation capabilities necessary for the induction of antitumor immunity [20]; the four cytokine cocktail increased the proportion of this subset in the DC

population (Fig. 1d). DC maturation markers including CD40, CD54, CD80, CD86, and I-A^d were significantly upregulated as well (Fig. 1e, f).

Effect of DC immunization on HCC growth in vivo

Immunization with ASPH-loaded DCs induced antitumor immunity against the syngeneic BNL 1ME ASPH expressing HCC cell line [21] [Supplementary Fig. 1]. An *in vitro* cytotoxicity assay was also performed using splenocytes derived from mice immunized with Hank's buffered salt solution (HBSS), green fluorescent protein (GFP) or ASPH-loaded DCs to demonstrate ASPH-specific cytotoxicity (Fig. 2a). Similar anti-tumor responses were obtained using a different ASPH-expressing murine SP2-0 myeloma cell line as target cells (Supplementary Fig. 2). Although the specific CTL activity is low (9%) due principally to reduced MHC class I expression on HCC cells compared SP2-0, the result is highly statistically significant. Irrelevant murine fibroblast target cells demonstrated no lysis. Immunization of mice bearing BNL-derived established tumors with ASPH-loaded DCs significantly suppressed tumor growth compared to immunization with HBSS and GFP-loaded DCs (Fig. 2b). Prophylactic immunization with ASPH-loaded DCs before inoculation of BNLT3 (a rapidly growing and highly malignant subline of BNL) also revealed delayed tumor growth and prolonged survival compared to control mice immunized with GFP-loaded DCs (Fig. 2c, d).

Generation of cellular immune responses to ASPH

Splenocytes derived from the ASPH-DC immunized mice were restimulated with ASPH protein and exhibited a high level IFN γ secretion as an index of an antigen specific immune response. In contrast, IL-4 secretion by the restimulated splenocytes was far lower in all study groups (Fig. 3a, b). When CD4⁺ T cells derived from immunized mice were restimulated with ASPH-loaded DCs, it was found that activated CD4⁺ cells expressing CD69 and IFN γ were generated (Fig. 3c and Supplementary Fig. 3). When splenocytes derived from mice immunized with ASPH-loaded DCs were restimulated with ASPH protein, IFN γ ⁺ and granzyme B^{hi} CD8⁺ cells were found to be increased as well (Fig. 3c). Thus, both antigen specific CD4⁺ and CD8⁺ T cells were generated, and may have contributed to ASPH-mediated anti-tumor immune responses. Staining of BNLT3 tumors (H&E) revealed focal areas of lymphocytic infiltration in tumors derived from mice immunized with ASPH-loaded DCs (Fig. 3d). More important, CD3⁺ T cells were increased in tumors from mice immunized with ASPH-loaded DCs (Fig. 3d, e). It was of interest that depletion of either CD4⁺ or CD8⁺ T but not NK cells abolished anti-tumor effects generated through immunization with ASPH-loaded DCs (Fig. 3f).

ASPH-loaded DCs activate CD4⁺ cells in PBMC's

These experiments focused on the activation of CD4⁺ T cells following ASPH stimulation since it has been established that generation of such cells are critical for inhibition of tumor growth [22, 23]. Activated CD4⁺ T cells express CD154 [24], and we measured CD154 in combination with IFN γ expression as surrogate markers for antigen-specific activation of CD4⁺ T cells in PBMCs derived from normal controls and patients with HCC [17, 25, 26]. The CD4⁺ T cells were initially stimulated with autologous ASPH-pulsed DCs. Restimulation of the CD4⁺ T cells with ASPH-pulsed DCs resulted in a robust increase in the CD154⁺ cell sub-population compared to restimulation of the same cells with DCs pulsed with another HCC related protein biomarker such as AFP (Fig. 4 a,b). Thus, simultaneous comparisons were made regarding the immunogenicity of ASPH to that of AFP. Unlike ASPH, restimulation of AFP-stimulated CD4⁺ T cells with AFP-pulsed DCs exhibited a non-significant decrease in the CD154⁺ population when compared to the control (Fig. 4 a,b). However, stimulation with ASPH but not AFP activated antigen specific CD4⁺ T cells derived from normal volunteers. More important, the CD4⁺ T cell subset

expressing CD154 and IFN γ was significantly expanded when CD4⁺ T cells initially stimulated with ASPH-pulsed DCs were then restimulated with the same autologous ASPH-pulsed DCs (Fig. 4 **a,b**). Similar results were obtained in an experiment using ASPH-pulsed DCs or DCs (only) without antigen loading for restimulation (Supplementary Fig. 4). Moreover, it was also possible to activate CD4⁺ T cells to ASPH in PBMCs derived from patients with HCC (Fig. 4 **c,d**). Generation of antigen specific CD4⁺ cells following ASPH-DC but not AFP-DC immunization suggesting that this cell surface expression protein may have potential as an immunotherapeutic target for such tumors.

Discussion

Studies suggest that some “HCC associated antigens” including the cancer testis antigens (CT-antigens) are immunogenic and potential targets for vaccine development [27–32]. However subsequent host immune responses following vaccination have generally failed to control tumor growth. Inducible cellular immunity against α -fetoprotein, glypican-3, mutant p53, and proteins derived from HBV and HCV have been demonstrated in humans as well as in murine models [33–36]. Clinical investigation reveals improved survival when patients were immunized with tumor or formalin-fixed HCC tissue lysates [37, 38]. HCC tumor vaccine studies have been generally disappointing, and this lack of success may depend, in part on the characteristics of the immunizing antigen, as well as the type and robustness of the subsequent host cellular immune response.

We have identified ASPH as a potential candidate protein for immunotherapy of HCC. There are several attractive features of this target expressed on the surface of HCC cells. First, it is a transmembrane protein overexpressed in 70–90% of human tumors and all 7 HCC cells lines tested thus far [8, 12]. Second, ASPH is not expressed, or is expressed at very low levels in normal tissues [8, 12, 15, 39]. Third, functional analysis of ASPH reveals that it promotes HCC cell migration and invasion which may lead to tumor progression [12, 40, 41]. Fourth, clinicopathologic studies suggest that ASPH expression correlates with poorly-differentiated HCC tumors with intrahepatic metastasis which is a leading cause of post-surgical recurrence and reduced patient survival [14, 41].

In developing an immunotherapeutic strategy, DCs must be activated before they are capable of inducing anti-tumor immunity. As shown here, activated DCs have improved ASPH presenting abilities due to enhanced expression of T cell activating co-stimulatory molecules. Other observations suggest that it may be necessary to employ microbial products such as CpG motifs as adjuvants [42, 43] as well as cytokine additions such as IL-2 [44]. In addition, blocking cytotoxic T-lymphocyte antigen-4 (CTLA-4) may be necessary to achieve optimal cellular immune responses to TAAs [45, 46]. Blocking of CTLA-4 expression has resulted in the unmasking of TAA-specific immune responses by changing cytokine and chemokine profiles exhibited by mononuclear cells stimulated with TAA-related peptides in patients with HCC [47].

In the present investigation it was first determined if immunotherapy with ASPH-loaded DCs induces antitumor immunity against HCC grown in mice (Figs. 1–3). It should be noted that the syngeneic BNL3 cells used in the tumor prevention model (Fig. 2 **c,d**) were from a highly malignant, rapidly-growing sub-line that formed subcutaneous tumors in 100% of mice after inoculation of only 1×10^3 HCC cells. In contrast, in the treatment of established tumor models (Fig 3**b**) the BNL 1 ME A.7R.1 cells required 1×10^6 cells to establish slow-growing subcutaneous tumors in 100% of animals. Thus, the anti-tumor effects of immunotherapy with ASPH-loaded DCs was most striking with the BNL syngeneic HCC cell line.

Our findings indicate that ASPH may be a highly effective target protein for DC-based immunotherapy of HCC. Previous studies indicate that overexpression of ASPH increases cell motility and invasiveness of HCC and cholangiocarcinoma tumor cells [12,13] whereas gene knockdown strikingly reduces this phenotype. These findings have been complemented by a large-scale human study reporting that high level ASPH expression in HCC tumors was linked to tumor recurrence and poor patient survival following surgical resection [14]. In addition, high ASPH expression was also predictive of intrahepatic metastasis whereas lower levels of protein detection were related to a more differentiated tumor phenotype [14, 41]. These observations suggest that ASPH-DC immunotherapy could potentially delay recurrence of HCC following surgical resection. Antibody mediated therapy may also be useful as an anti-tumor approach since ASPH is a Type II transmembrane protein highly expressed on the cell surface and it accumulates during the transformation process [12]. Indeed, a human anti-ASPH monoclonal antibody has been generated to test this hypothesis [48].

Although cytotoxic CD8⁺ T cells play a central role in eliciting antitumor immunity, there is accumulating evidence to indicate that generation of antigen specific CD4⁺ T helper cells are absolutely required to augment the function of CD8⁺ T cells to maintain a sustained anti-tumor immune response. It has been demonstrated that CD4⁺ T cells will enhance CTL responses through clonal expansion, functioning as antigen-presenting cells for CTLs, and amplify clonal expansion at the tumor site; all such properties support subsequent humoral and cellular anti-tumor immune responses [49].

It is of interest that both antigen specific CD4⁺ and CD8⁺ T cells were generated and found necessary for anti-tumor activity following ASPH-DC immunization in the syngenic murine HCC model. Moreover, it was found that ASPH was a strong immunogen and primed antigen-specific CD4⁺ T cells derived from normal human and HCC patients PBMC's whereas AFP did not (Fig 4 **a, b, c, d**). The finding of weak immunogenicity for AFP is consistent with the observation that CD4⁺ T cells specific to AFP have not been detected in HCC patients immunized with this antigen although AFP-specific CD8⁺ T cells have been identified in some individuals with this disease [27]. Therefore, activation of both antigen specific CD4⁺ and CD8⁺ T cells may be required to generate optimal immunologic responses to ASPH expressing HCC tumors. This is the initial demonstration that ASPH-DC stimulation can lead to antigen specific CD4⁺ activation in PBMCs derived from HCC patients (Fig 4 **c, d**), and suggests that immunotherapy with ASPH-loaded DCs may be a novel approach to delay or prevent HCC recurrence following surgical resection.

In summary, the observations that ASPH-DC immunization, which appears to induce antitumor immunity mediated by both CD4⁺ and CD8⁺ T cells against HCC in preclinical animal models and generates antigen-specific CD4⁺ T cells in PBMCs derived from HCC patients, suggests that cell surface expressed ASPH may be an excellent therapeutic target. Because of its tissue specificity, association with tumor progression, and potent immunogenicity, this protein may represent an attractive candidate for a DC-based immunotherapeutic approach against HCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

HCC	Hepatocellular carcinoma
ASPH	Aspartate- β -hydroxylase
DCs	Dendritic cells
TILs	Tumor infiltrating lymphocytes
PBMCs	peripheral blood mononuclear cells
HBV	hepatitis B
HCV	hepatitis C
CTLs	cytotoxic T lymphocytes
TAA s	tumor-associated antigens
APCs	antigen-presenting cells
TCR	T cell receptor
NK	natural killer cells
CTLA-4	cytotoxic T-lymphocyte antigen-4
HBSS	Hank's buffered salt solution
DMEM	Dulbecco's modified eagle medium

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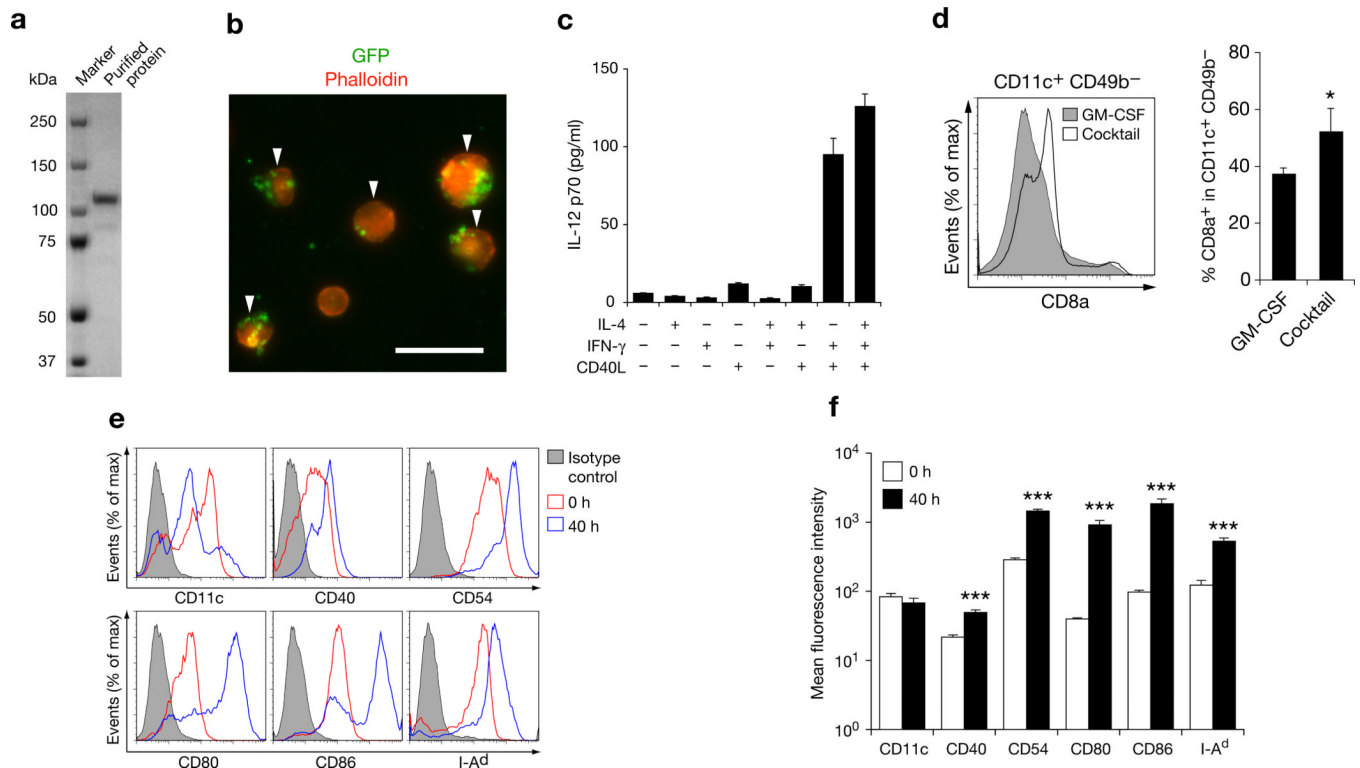


Figure 1. Characteristics of DCs used for immunization

(a) SDS-PAGE of purified recombinant ASPH used to pulse DCs. (b) Fluorescence microscopy demonstrating phagocytosis of GFP-coated microbeads by DCs (arrowheads). Bar scale 20 μ m. (c) IL-12 p70 secretion by DCs in the presence of cytokines; results were derived from triplicate cultures. (d) CD8a⁺ expression in CD11c⁺ CD49b⁻ cells derived from DCs cultured with GM-CSF or with GM-CSF, IL-4, IFN γ , and CD40L combinations (left panel). Percent of CD8a⁺ cells in the CD11c⁺ CD49b⁻ cell population. Results were derived from three independent experiments (right panel). (e) Flow cytometry of DCs before and after the 40-h culture with the cytokine cocktail demonstrating CD11c and five additional co-stimulatory DC maturation marker expression. (f) Changes in mean fluorescence intensities of DC cell surface marker expression generated from three experiments. All studies except for Western blot analysis were performed at least twice with similar results. Bar graphs are shown as means \pm S.D. * P < 0.05; *** P < 0.001.

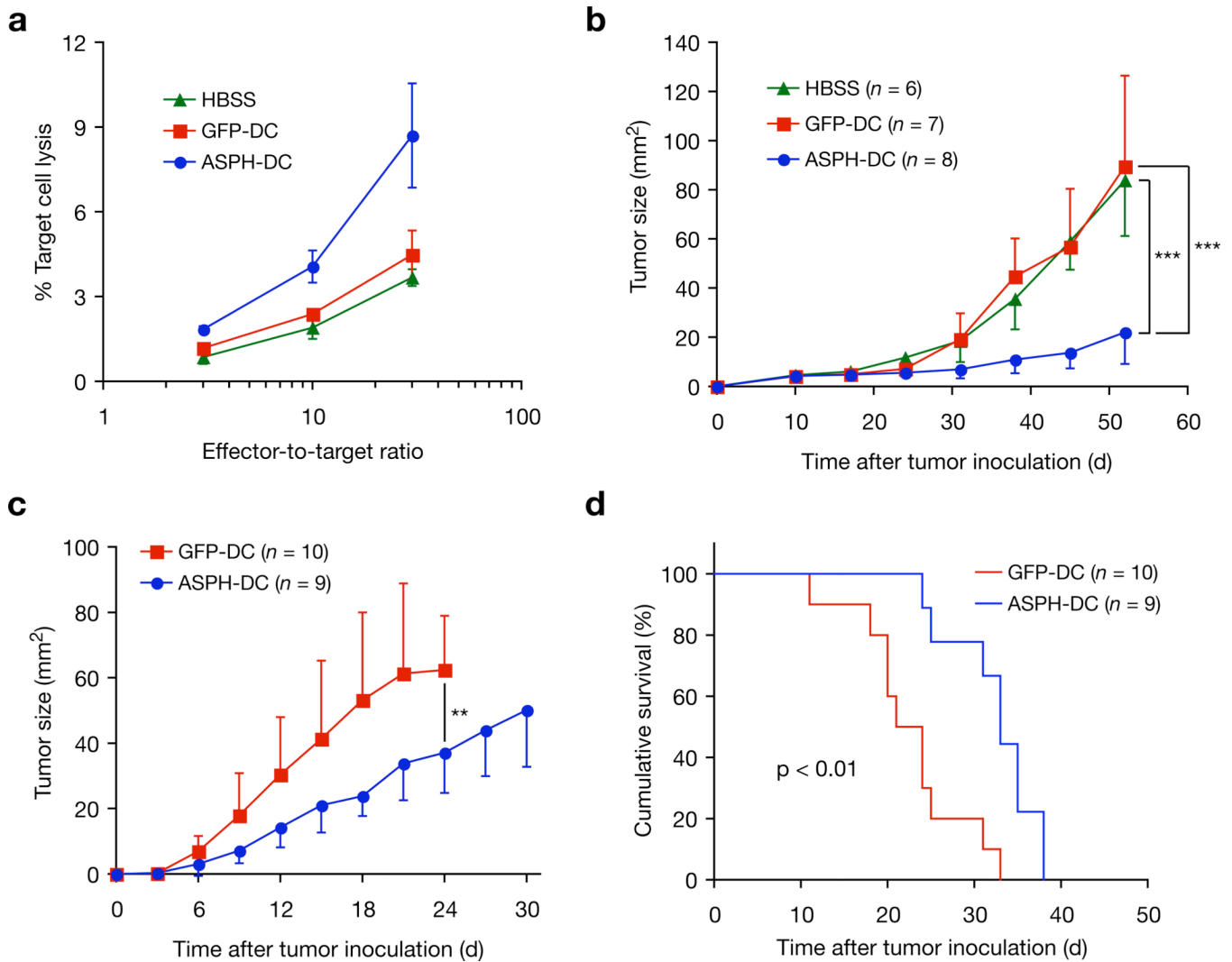


Figure 2. Antitumor immunity induced by immunization with ASPH-loaded DCs

(a) Results of *in vitro* cytotoxicity assays against BNL, a syngenic murine HCC cell line. Mean values of target cell lyses were derived from triplicate cultures; three independent experiments were performed with similar findings. (b) Effect of immunization with ASPH-DC on established tumors. After the subcutaneous inoculation of BNL (1×10^6 cells) on day 0, tumors were allowed to progress to about 0.5 cm in size. Tumor-bearing mice were immunized with HBSS, GFP-DC, or ASPH-DC on days 10 and 15 after inoculation of HCC cells. Mean tumor size (area mm²) are shown. Findings were representative of two experiments with similar results. (c) Effect of prophylactic immunization with ASPH-DC on subsequent HCC tumor growth by BNL3. Mean tumor size (area mm²) is shown. Graph is representative of two experiments with similar results. (d) Kaplan-Meie survival curves of the same experiment shown in (c). Results are presented as the mean \pm S.D. ** $P < 0.01$, *** $P < 0.001$.

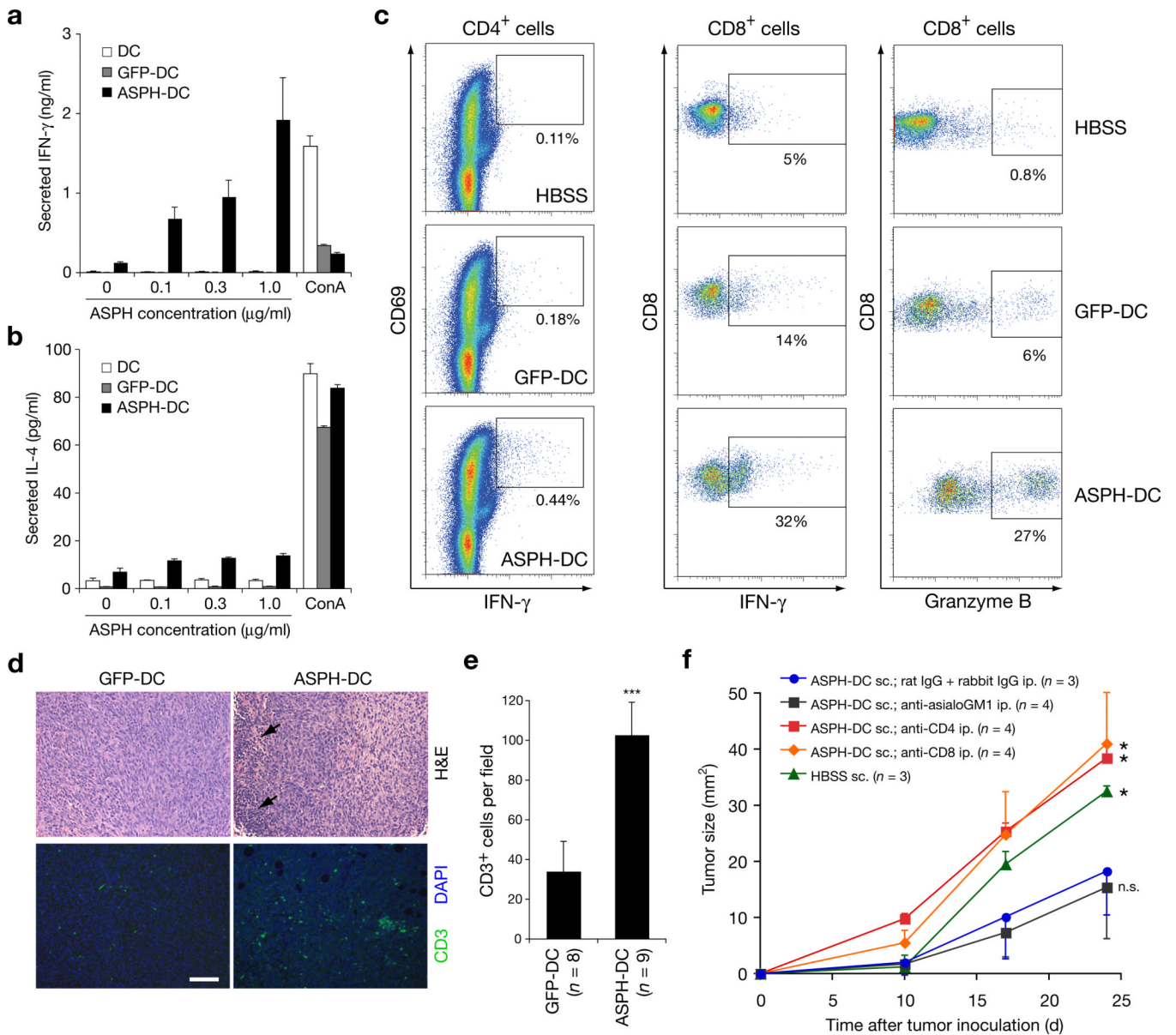


Figure 3. CD4⁺ and CD8⁺ T cells mediate antitumor immunity induced by immunization with ASPH-DC

(a,b) Cytokine production by splenocytes derived from immunized mice in response to ASPH restimulation. Splenocytes derived from mice immunized with DCs only (DC), or GFP-DC, or ASPH-DC were restimulated with 0 – 1 µg/ml ASPH or 10 µg/ml concanavalin A (ConA) as a positive control. Mean concentration of IFN γ (a) and IL-4 (b) produced from triplicate cultures are shown. (c) ASPH-specific activation of CD4⁺ T cells. Expression of CD69 and IFN γ in CD4⁺ cells are shown. (d) ASPH-specific activation of CD8⁺ splenocytes. Expression of IFN γ and granzyme B in CD8⁺ cells are shown. (d, e) Evaluation of tumor-infiltrating lymphocytes. Mice with established tumors were immunized twice with GFP-DC or ASPH-DC. Excised tumors were stained by H&E for histology and anti-CD3 antibody by immunofluorescence (d). Arrows indicate focal infiltration of mononuclear cells. Bar scale for d is 100 µm. (e) Mean level of tumor-infiltrating CD3⁺ T cells per field. Results were derived from two experiments. *** $P < 0.001$. (f) Effect of immune cell

depletion on tumor growth in mice immunized with ASPH-DC. Mean tumor size derived from each group and evaluated over time (days) is presented. All experiments were performed at least twice with similar results. * $P < 0.05$; n.s., not significant. All error bars are shown as mean \pm S.D.

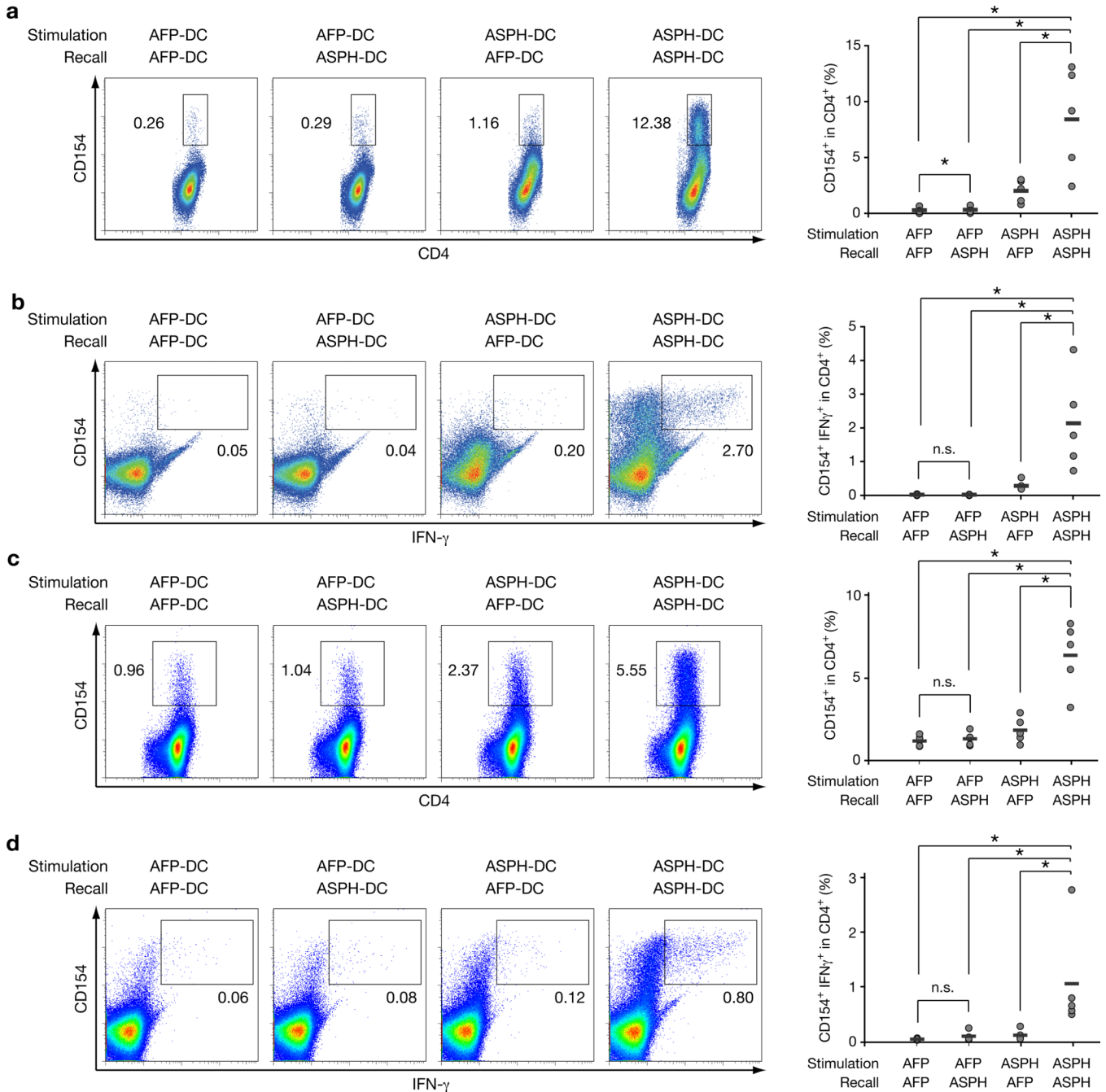


Figure 4. Generation of antigen specific CD4⁺ cells in humans
 CD4⁺ T cells purified from PBMCs were cocultured with monocyte-derived DCs (MoDCs) loaded with either recombinant α -fetoprotein (AFP) or ASPH for 8 days (Stimulation). The lymphocytes were then challenged by MoDCs loaded with either AFP or ASPH for 7 h (Recall). (**a,c**) Examples of the ASPH specific CD154⁺ population in CD4⁺ cells derived from healthy volunteers (**a,b**) and HCC patients (**c,d**). Analysis of CD154 expression generated by ASPH in PBMCs derived from five healthy volunteers (**a,b**; right panel) and five patients with HCC (**c,d**; right panel). The percent of CD154⁺ cells represented in CD4⁺ cell populations are shown. Analysis of the CD154⁺ IFN γ ⁺ subpopulation derived from CD4⁺ cells of healthy volunteers (**b**) and HCC patients (**d**) after stimulation with ASPH-

loaded DCs. Summary of CD154 and IFN γ expressing cells represented in PBMCs derived from five healthy human volunteers and five patients with HCC (**a,b,c,d**; right panels, respectively). The percent of CD154⁺ IFN γ ⁺ cells are shown. * $P < 0.05$; n.s., not significant. Note that AFP-loaded DCs were not immunogenic in developing antigen-specific CD4⁺ cells either in PBMC derived from normal human volunteers, or patients with HCC.