Vaccination with Human Pluripotent Stem Cells Generates a Broad Spectrum of Immunological and Clinical Responses Against Colon Cancer

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ABSTRACT

The history of immunizing with embryonic materials to generate an antitumor immune response dates back to a century ago. The premise is that cancer cells share the expression of oncofetal antigens with embryonic materials and that the immune response against these antigens in the embryonic tissues is cross-protective against cancer. However, such a practice has never advanced beyond experimental animal settings, because of lack of uniformed source tissues and ethical challenges. With the availability of well-characterized human pluripotent stem cells, it is now possible to ask whether tumor protective immunity could indeed be elicited with stem cells. Herein, we investigated whether vaccination with defined human embryonic stem cells (hESCs) or induced pluripotent stem (iPS) cells was effective against a colon carcinoma. We discovered that vaccination of mice with hESC line H9 generated consistent cellular and humoral immune responses against CT26 colon carcinoma. Protection correlated strongly with the expansion of tumor-responsive and interferon-γ-producing cells and the profound loss of CD11b+Gr-1+ myeloid-derived suppressor cells in the spleen. No evidence of autoimmunity was observed. We also compared the immunogenicity against colon cancer between a hESC line CT2 and an iPS cell line TZ1 that were generated in the same stem cell facility. We found that the iPS cell line was inferior to the hESC line in conferring tumor protection, suggesting that there is heterogeneity of expression of oncofetal antigens by hESCs and iPS cells. We conclude that the hESC-based vaccine is a promising modality for immunotherapy of cancer. STEM CELLS 2009;27:3103–3111

INTRODUCTION

Long before embryonic stem cells were cultivated and used for genetic and developmental studies [1], pathologists described cancers as anaplasia (Greek, “to form backwards”), which implied that cancer arose from mature, differentiated cells by a process of de-differentiation. This viewpoint is most likely incorrect, as there is strong experimental evidence for the notion that cancer develops from cancer stem cells, which is best exemplified by hematological malignancies including myelodysplastic syndrome and leukemias [2–4]. Nevertheless, cancer transformation is indeed intimately coupled with the “appearance” of stem cell-like features, in that (a) both express oncofetal developmental antigens; (b) both have the capacity of indefinite division; (c) both are able to engraft in immunocompromised hosts and to induce angio-genic growth factors; and (d) both maintain a certain degree of differentiation potential, given the correct developmental cues.

In the beginning of the 20th century, it was reported that prior injection of mice with fetal tissues led to rejection of transplantable tumors (reviewed elegantly in [5]). The tumor protection was later expanded to chemically induced cancers of a variety of types including cancer of the skin, liver, and gastrointestinal tract [6–11]. Moreover, tumor cells were
found to express a number of oncofetal antigens. In fact, a significant proportion of the human cancer vaccine trials to date are targeted against embryonic antigens such as carcinoembryonic antigen [12], cancer/testes antigen [13, 14], and α-fetoprotein [15]. Unfortunately, targeting one antigen alone is unlikely to generate effective antitumor immune responses to mediate tumor rejection because of rapid appearance of escape mutants and the general inefficiency of monovalent cancer vaccines [16, 17].

The use of fetal materials to vaccinate for tumor immunity has never advanced beyond animal models owing to its impracticality, the lack of understanding of molecular mechanisms, and its association with insurmountable ethical challenges. Recent interest in the potential of stem cells in regenerative medicine has made well-defined undifferentiated ESC lines widely available. Moreover, with surprisingly as few as four genes, including c-Myc, Sox2, Oct-4, and Klf4 [18–20], or the combination of OCT4, SOX2, NANOG, and LIN28 [21], somatic cells can be reprogrammed reproducibly into induced pluripotent stem (iPS) cells. These iPS cells are phenotypically and functionally similar to ESCs. In this study, we hypothesized that undifferentiated stem cells could immunize to generate immune response against a variety of antigens that are shared by colon carcinoma. We found, for the first time, that hESCs but not iPS cells were indeed able to induce a broad spectrum of both immune responses and clinical responses against colon carcinoma without evidence of inducing autoimmune diseases.

**MATERIALS AND METHODS**

**Mice**

Wild-type female BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME, http://www.jax.org) and were maintained by the Center for Laboratory Animal Care at the University of Connecticut Health Center using standard guidelines. Mice were used at 6-8 weeks of age. Severe combined immunodeficient (SCID)-beige mice were purchased from Charles River Laboratories (Wilmington, MA, http://www.criver.com). The animal experiments were carried out using a protocol approved by the Animal Care Committee of the University of Connecticut and the Embryonic Stem Cell Research Oversight Committee at Storrs-UConn (Storrs, CT).

**Cell Lines**

Human ESC line H9 was established in the University of Wisconsin [22]. The hESC line CT2 [23] and the IMR-90 fibroblast (ATCC, Manassas, VA, http://www.atcc.org)-derived iPSC cell line TZ1 were both generated, and their pluripotency was verified in the University of Connecticut Stem Cell Core under the direction of R.-H.X. Both CT1 and TZ2 were derived from females and the karyotype of 46, xx was confirmed (data not shown). At the time of immunization, CT1 and TZ2 were in passages 28-40 and 8-20, respectively. All cells were cultured on plates coated with Matrigel (BD Biosciences, San Diego, http://www.bdbiosciences.com) in human ESC medium, that is, Dulbecco’s modified Eagle’s medium/F12 containing 20% Knock Out serum replacer, 0.1 mM nonessential amino acids, 1 mM L-glutamine (all from Invitrogen, Carlsbad, CA, http://www.invitrogen.com), and 0.1 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com), which was conditioned on mouse embryonic fibroblast as feeders and then supplemented with 4 ng/ml basic fibroblast growth factor (Millipore, Billerica, MA, http://www.millipore.com) [24]. All three stem cells were irradiated (15 Gy) before immunization. CT26 colon adenocarcinoma cell line was obtained from P. Srivastava (University of Connecticut, Farmington, CT). CT26 cells are homogenous. Stem cells were also more than 95% pure without feeder cells.

**Teratoma Assay**

Human ESC/iPS cells from approximately 70% confluent 6-well plates were injected into the rear leg muscles of 4-week-old male SCID-beige mice (three mice per cell line). At 6-8 weeks after injection, the resulting teratomas were excised and examined histologically.

**Reagents and Antibodies**

Most antibodies used for flow cytometry and rat anti-mouse Foxp3 (FJK-16s) were obtained from eBioscience (San Diego, http://www.ebioscience.com). Other antibodies for immunohistochemistry and phycoerythrin (PE)-labeled anti-CD25 and PE-labeled anti-Gr-1 antibody for flow cytometry were obtained from BD Biosciences. Antibodies against SSEA4 and TRA-1-60 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, http://www.scbt.com). All other chemicals were obtained from Sigma-Aldrich unless specifically indicated.

**Immunofluorescence Analysis**

Cells were harvested, fixed with 4% paraformaldehyde for 10 minutes, and incubated in phosphate-buffered saline (PBS) containing 5% rabbit serum and 0.4% Triton X-100 for blocking and permeabilization, respectively. PBS containing 0.5% Tween 20 (PBS-T) was used to dilute antibodies and wash the cells in the following procedures. The cells were incubated with antibodies against SSEA4 or TRA-1-60 at 37°C for 1 hour, followed by washing with PBS-T three times. Afterward, the cells were incubated with fluorescent-conjugated, corresponding secondary antibodies at 37°C for 45 minutes and washed with PBS-T three times. Finally, the cells were examined under fluorescence microscope to capture both phase and fluorescent images.

**Immunizations Protocol, Tumor Challenge, and Evaluation of Antitumor Response**

Tumor rejection assays were typically performed using five BALB/c mice in each group as described previously [25]. Mice were immunized subcutaneously with $1 \times 10^6$ live irradiated hESCs (15 Gy) or $5 \times 10^3$ CT26 tumor cells (75 Gy) twice at a 1-week interval. At 1-4 weeks after immunization, mice were challenged with $5 \times 10^3$ live CT26 cells subcutaneously. Tumor growth was monitored every 3 days using digital calipers to measure both the longitudinal (a, mm) and transverse (b, mm) diameters. Tumor area (a x b, mm$^2$) was plotted. Mice were also monitored for the following general health indicators: overall behavior, feeding, neuromuscular tone, body weight, appearance of fur, and so on, particularly after immunization. The endpoint for this study was one diameter of tumor $\geq 15$ mm or tumor area of $\geq 225$ mm$^2$, at which point mice were euthanized. The primary tumor was also excised and weighed after the mice were sacrificed. Per our approved protocol and to ease the pain and suffering of tumor-bearing mice, no survival experiments were performed.

**Flow Cytometry**

Cell surface staining and analysis on a FACSCalibur (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com) were done as previously described [26] and results were analyzed with FlowJo software (TreeStar, Inc., Ashland, OR, http://www.treestar.com).

**Histology and Immunohistochemistry**

BALB/c mice were challenged with CT26 colon cancer cells. Mice were sacrificed, and the primary tumor was excised and snap-frozen in dry ice or fixed in 4% formaldehyde. Tissues were fixed and stained with hematoxylin and eosin by standard methods. Cryostat sections (5-μm each) were fixed in acetone and
stained with rat anti-mouse CD4 or CD8 antibody. Other sections were fixed in 4% formaldehyde, stained with anti-mouse Foxp3 antibody, and then sequentially incubated with biotinylated anti-rat secondary antibodies and horseradish peroxidase (HRP)-conjugated streptavidin. Sections were developed with H2O2 and diaminobenzidine (BD Biosciences).

**Immunofluorescence for Detection of Antinuclear Antibodies**

Antinuclear antibodies (ANAs) were measured by indirect immunofluorescence assay using Hep-2-coated slides (INOVA Diagnostics, San Diego, http://www.inovadx.com) as described previously [27]. Fluorescence intensity of ANA staining was graded in the single-blind fashion based on the following criteria: 0, negative; 1, lowest but discernibly positive; 2, positive; 3, clearly positive; 4, bright; and 5, brilliant.

**Western Blot**

H9 and CT26 cells were lysed on ice in lysis buffer (0.01 M sodium phosphate, pH 7.2, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P40, 1% sodium deoxycholate, 0.1% SDS, 2 mM 4-(2-aminophenyl) benzensulfonyl fluoride hydrochloride [AEBSF], 130 mM bestatin, 14 mM E-64, 0.3 mM aprotinin, and 1 mM leupeptin), followed by centrifugation to remove nuclei. The samples were separated by a 10% denaturing and reducing SDS-polyacrylamide gel electrophoresis, transferred onto an Immobilon-polyvinylidene fluoride membrane (Millipore, Billerica, MA, http://www.millipore.com), and incubated for 1 hour in a 1:50 dilution of sera from naïve or different cell-immunized mice. This was followed by incubation with HRP-conjugated antirat mouse IgG for 1 hour and then developed with a chemiluminescence reagent (Pierce, Rockford, IL, http://www.piercenet.com).

**Enzyme-Linked Immunospot Assay**

Briefly, enzyme-linked immunospot assay (ELISPOT) plates (Millipore) were coated with rat anti-mouse interferon-γ (IFNγ) or interleukin-4 (IL-4) capture antibody (clone R4-6A2, BD Biosciences) and blocked with RPMI media supplemented with 10% fetal calf serum (Life Technologies, Carlsbad, CA, http://www.lifetech.com) followed by incubation with splenocytes (10⁶ cells per well) and stimulators, in triplicates, for 2 days. CD4+ splenocytes were separated using magnetic-activated cell sorting columns conjugated with antibody against mouse CD4 (L3T4; Millenyi Biotec, Auburn, CA, http://www.milenyi-biotec.com) according to the manufacturer’s protocol. Purity was approximately 90% as confirmed by flow cytometry (data not shown). Stimulators included irradiated autologous splenocytes (5 × 10⁵ per well) and irradiated CT26 cells (1 × 10⁶ per well). Concanavalin A (10 μg/ml; Sigma-Aldrich) was used routinely as a positive control for the assay. The biotinylated rat anti-mouse IFNγ or IL-4 antibody (clone XMG1.2; BD Pharmingen, San Diego, http://wwwbdbiosciences.com) and avidin-peroxidase complex (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com) were then added sequentially, followed by washing and development using 3-aminot-ethylcarbazole and H2O2 (Sigma-Aldrich). The spots were counted using a computer-assisted ELISPOT image analyzer (Immunospot, Cleveland, OH, http://www.immunospot.com) and the numbers of spots were expressed as the mean of triplicates.

**Statistical Analysis**

Most of the numerical values of the data were presented as mean ± SD. Comparisons between groups were done by Student t test or one-way analysis of variance tests, where appropriate. The dynamic tumor growth data were analyzed by multivariate general linear model. Differences were considered significant for p < .05. Statistical analysis was performed using SPSS statistical software for Windows (SPSS, Chicago, http://www.spss.com) or GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, http://www.graphpad.com).

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**RESULTS**

**Undifferentiated Human ESCs Exhibit Potent Antitumor Activity in a Murine Colon Cancer Model**

To study whether undifferentiated hESCs could stimulate the immune system to recognize shared oncofetal antigens to confer tumor protection, a well-established colon cancer (CT26) model was used [28, 29]. We immunized naïve immunocompetent BALB/c mice twice (1 week apart) with irradiated CT26 cells, H9 cells, or PBS control, followed 7 days later with challenge of mice with live CT26 cells (Fig. 1A). The kinetics of tumor growth was then closely monitored (Fig. 1B). As expected, tumors grew progressively in the PBS-immunized group, requiring euthanization of the tumor-bearing mice 20 days after tumor challenge. Strikingly,
immunization with both CT26 and H9 cells resulted in a dramatic retardation of tumor growth, with statistically significant differences in the average tumor size in each group compared with PBS group from day 6 on (*, p < .001) (Fig. 1B). Three weeks after tumor challenge, we sacrificed the mice, dissected the primary tumor, and measured average tumor weight, which was significantly decreased in mice immunized with both H9 and CT26 cells compared with the PBS group (Fig. 1C). The results suggested that the undifferentiated human ESC line H9 cells were able to immunize naive mice to generate potent primary antitumor activity against murine colon cancer, despite the xenogeneic nature of H9 and the incompatibility of the major histocompatibility complex (MHC) antigens between the immunizing hESCs and the host/cancer.

**Vaccination with hESCs Induced Both T-Cell and Antibody Response Against Colon Cancer**

The rejection of CT26 requires T-cell-mediated cellular immunity [29]. We then proceeded to determine whether H9 immunization resulted in expansion of tumor-specific cytokine-producing cells. After two weekly immunizations, BALB/c mice were sacrificed 10 days after the last immunization and the frequency of IFNγ- or IL-4-producing splenocytes against CT26 was enumerated by ELISPOT. We observed a significantly higher number of IFNγ-producing splenocytes in H9-immunized mice (5.81 ± 3.33 spots) compared with the PBS group (0.74 ± 1.20 spots; *, p < .05) (Fig. 2A). There was no significant difference in the frequency of IL-4-producing CD4+ cells in response to CT26 between H9-immunized mice and the PBS group (Fig. 2B). These data suggested that H9 immunization preferentially resulted in the generation of CT26 colon cancer-specific helper type 1 (Th1) T-cell immunity. As expected, CT26 immunization also induced significant accumulation of IL-4- and IFNγ-producing cells.

The Th1 response is essential for eliciting high-affinity IgG2a antibody response. To address whether cross-reactive antibody was generated between H9 and CT26, we tested the immunoreactivity of sera against both H9 and CT26 cell lysate from mice that were immunized with H9 and CT26, respectively. Sera from unimmunized naive mice did not react with either H9 or CT26. However, we demonstrated that sera from CT26-immunized mice were able to recognize multiple proteins in CT26 as well as H9 lysates by Western blot analysis (Fig. 2C). Moreover, a prominent ~70-kDa molecule in CT26 colon cancer cells was clearly recognized by sera from H9-immunized mice (Fig. 2C). We thus concluded that an antitumor antibody response was produced after H9 immunization and that there existed shared antigens between CT26 and undifferentiated H9 cells.

**hESC Immunization Results in Substantial Reduction of Gr-1+CD11b+ Myeloid-Derived Suppressor Cells in the Spleen**

To further study the cellular immune mechanism mediating the antitumor effect, we analyzed the phenotype of MDSCs in each group of mice. *, p < .05. Abbreviation: PBS, phosphate-buffered saline.

**Figure 2.** Vaccination with hESCs induced both T-cell and antibody response against colon cancer. (A): Mice were sacrificed 10 days after the last immunization for the enumeration of the frequency of IFNγ- or IL-4-producing cells in response to CT26 tumor cells by enzyme-linked immunospot assay. Whole splenocytes were used as effector cells for the IFNγ assay, and purified CD4+ splenocytes were used for the IL-4 assay. Irradiated CT26 tumor cells served as target cells, along with culture medium as a negative control (no spots) and Concanavalin A as a positive control. There were five mice per group. *, p < .05. (B): Frequency of IL-4-producing CD4+ cells in response to CT26. p < .05. (C): Immunization with H9 generates cross-reactive antibody against CT26 colon cancer. Western blot analysis was performed with sera from immunized mice against total cell lysate of CT26 and undifferentiated H9 cells. Numbers indicate the molecular weight marker (kDa). Abbreviations: IFNγ, interferon-γ; IL-4, interleukin-4; PBS, phosphate-buffered saline.

**Figure 3.** hESC immunization results in substantial reduction of Gr-1+CD11b+ myeloid-derived suppressor cells in the spleen. (A): BALB/c mice were sacrificed on day 20; spleens were removed surgically and weighed. *, p < .05. (B): Representative fluorescence-activated cell sorting plots of splenic myeloid derived suppressor cells (MDSCs) in each group of mice. Numbers represent percentages of cells in each quadrant. (C): Absolute number of Gr-1+CD11b+ MDSCs in each group of mice. *, p < .05. Abbreviation: PBS, phosphate-buffered saline.
splenocytes from different groups of mice by flow cytometry. We found that there was a significant splenomegaly in tumor-bearing mice in the PBS group (Fig. 3A). The absolute number of B220⁺ cells increased in the PBS-immunized group compared with the H9-immunized group (supporting information Table 1). There was, however, no significant difference in the percentage and absolute number of CD4⁺ and CD8⁺ cells among the three groups of mice examined. We then turned our attention to two suppressor cell types that have been demonstrated to consistently impede tumor immunity: FoxP3⁺ CD4⁺ CD25⁺ regulatory T (Treg) cells [30] and Gr-1⁺ CD11b⁺ myeloid derived suppressor cells (MDSCs) [31]. No difference in the frequency of Treg cells was observed (supporting information Table 1). Strikingly, we found that MDSCs were significantly decreased in the spleen of mice after immunization with both H9 and CT26, compared with the PBS group (Fig. 3B, 3C). The reduction of MDSCs was in direct reverse correlation with the tumor burden of immunized mice.

Vaccination with Undifferentiated hESCs Generated Effective Memory Immune Response Against Murine Colon Adenocarcinoma

We next examined whether hESC immunization confers a memory antitumor immune response against CT26, by challenging mice with live tumor cells after a period of 4 weeks (Fig. 4A). We found that CT26-immunized mice were able to reject tumor challenge as efficiently as 1 week after immunization (Fig. 4B). Vaccination with undifferentiated H9 cells led to a modest but significant inhibition of tumor growth (p < .001) (Fig. 4B). Moreover, we found that hESC vaccination triggered significant tumor-specific immune responses. The frequency of both IFNγ-producing splenocytes (Fig. 4C) and IL-4-producing CD4⁺ cells (Fig. 4D) in response to CT26 was significantly increased in H9-immunized mice. We concluded that immunization with undifferentiated hESCs generated effective memory antitumor immune responses, although protective antitumor immunity appeared to wane over time.

Comparison of the Immunogenicity Between Undifferentiated hESCs and iPS Cells

Although hESC lines are considered similar in terms of self-renewal, expression of pluripotent markers, and the ability to differentiate, it is becoming more and more evident that differences between established stem cell lines do exist [32–36]. It is also unclear whether iPSCs are able to immunize against cancer, given a whole range of unresolved questions regarding the long-term stemness and pluripotency of iPSC cells. We thus decided to directly compare hESCs and iPSC cells in the same colon cancer protection model. To minimize the impact of culture conditions on the phenotype of these stem cells, we derived a hESC line (CT2) and an iPSC cell line (TZ1) in our stem cell core laboratory. Both cell lines, but not CT26 tumor cells, express typical stem cell markers including SSEA4 [22] and TRA-1-60 [37] (Fig. 5). In addition, the pluripotency of both TZ1 and CT2 could differentiate into neural epithelium (ectoderm), cartilage (mesoderm), and primitive gut (endoderm) was validated by the standard teratoma assay (data not shown). The primary antitumor responses of TZ1 and CT2 were evaluated according to the same immunization procedure as used for H9 (Fig. 1A). We demonstrated that immunization only with undifferentiated CT2 cells, but not with TZ1 cells, could inhibit tumor growth (Fig. 6A). Tumor burden at day 20 was also significantly decreased only in the CT2- and CT26-immunized groups (Fig. 6B). However, vaccination of both CT2 and TZ1 could trigger tumor-specific cellular immune responses, even though only CT2 induced protective antitumor activity (Fig. 6C, 6D). These results strongly suggested that the immunogenicity of hESCs differs from that of iPSCs. Furthermore, the fact that iPSCs were able to induce significant expansion of IFNγ- and IL-4-producing cells, but not tumor rejection, indicated that the mechanism of tumor rejection was not mediated simply by cellular immunity.

Immunization with Human Pluripotent Stem Cells Does Not Result in Significant Autoimmune Response

One important consideration for stem cell-based vaccine is the possibility of breaking immune tolerance against self-antigens and the emergence of significant autoimmune diseases. This question also has bearings on the application of stem cells for regenerative medicine in the immunocompetent host. As an index for systemic autoimmunity, we performed a semiquantitative assay for ANAs in the sera of mice that were immunized with PBS, or H9, CT2, TZ1, or CT26 cells, using serum from MRL/Lpr lupus mice [38] as a positive control (Fig. 7). HEp-2 cells are used widely as a

**Figure 4.** Vaccination with undifferentiated hESCs generated an effective memory immune response against murine colon adenocarcinoma. (A): Scheme of immunization. (B): Kinetics of tumor growth. *, p < .05. [staro], p < .001. (C, D): hESC vaccination triggers tumor-specific immune responses. Mice were sacrificed on day 20 and the frequency of IFNγ-producing splenocytes (C) or IL-4-producing CD4⁺ cells (D) were enumerated by enzyme-linked immunosorbent spot. *, p < .05. Abbreviations: IFNγ, interferon-γ; IL-4, interleukin-4; PBS, phosphate-buffered saline.
immunofluorescence assay substrate for the screening and titration of circulating ANAs in both human and mouse serum. We found that sera from both tumor- and stem cell-immunized mice had some background nonnuclear stain of HEp-2 cells compared with the PBS control. However, such staining at this low titer (1:80), which was primarily nonnuclear in the staining pattern, was clinically insignificant, since none of the postimmunized mice developed any clinical signs of autoimmune diseases such as alopecia, skin rash, arthritis, or any overt organ dysfunction (data not shown). Moreover, we also performed immunoblot with these sera against the lysates of kidney, spleen, and liver, and observed no reactivity (supporting information Fig. 1). We thus concluded that stem cell immunization did not pose a significant risk of inducing autoimmune diseases.

Figure 5. Expression of typical stem cell markers by both hESCs and induced pluripotent stem (iPS) cells but not CT26 tumor cell. Human iPS cell clone TZ1 was derived from IMR90 fibroblast with the Yamanaka factors (c-Myc, Sox2, Oct-4, and Klf4) in the University of Connecticut Stem Cell Core. H9, CT2, TZ1, IMR-90 fibroblast, along with CT26 tumor cells were stained for indicated stem cell markers and photographed under fluorescence microscopy (×20). The corresponding phase-contrast images under light microscopy were also indicated.

Figure 6. Comparison of the immunogenicity between undifferentiated hESCs and induced pluripotent stem (iPS) cells. (A): Primary antitumor response of the hESC line CT2 and the iPS cell line TZ1 was compared according to the same immunization protocol as H9 (Fig. 1A). *, p < .05. [staro], p < .001. (B): Comparison of primary tumor weight at 20 days after tumor challenge. (C, D): At 20 days after tumor challenge, the frequency of IFN-$\gamma$-producing (C) and IL-4-producing (D) cells against CT26 in the spleen was determined. *, p < .05. Abbreviations: IFN-$\gamma$, interferon-$\gamma$; IL-4, interleukin-4; PBS, phosphate-buffered saline.
The recent surge in interest in pluripotent stem cells arose from promising results in the area of regenerative medicine indicating that stem cell-derived adult cells may offer treatment options for a variety of degenerative diseases such as Parkinson’s disease, type I diabetes mellitus, and Alzheimer’s disease [19]. The other potential application of stem cells is in the area of immune-based therapy when various immune cells such as dendritic cells (DCs) can be differentiated and used for the treatment of immune disorders [39, 40]. In this study, we discovered yet another novel application of stem cells—that undifferentiated stem cells are powerful immunogens for generating effective immune response against histologically distinct, differentiation-divergent, and MHC-incompatible colon carcinoma. We found that both H9 and CT2, two embryonically derived human stem cells, were able to immunize naïve mice against challenge with a lethal dose of live colon carcinoma cells. Moreover, we demonstrated that these hESCs induced strong tumor-specific cellular immune responses and attenuated the expansion of MDSCs in the secondary lymphoid organs. We also showed that the immunogenicity of both H9 and CT2 dramatically rivals that of iPS cell line TZ1 in the same colon cancer model. Although more iPS cell lines need to be examined to make a general statement about their immunogenicity, our study does raise the possibility that there is a considerable degree of heterogeneity among stem cells in their expression of tumor antigens, even though the stemness and the pluripotency of these cells appear to be indistinguishable based on expression of typical stem cell markers and the differential potential in the standard teratoma assay.

CT26 is a widely used progressive and poorly differentiated colon carcinoma model in BALB/c mice [41]. In this system, as few as 1 × 10⁵ tumor cells given subcutaneously could lead to progressive local growth of the tumor and eventual death due, sometimes to distal lung metastasis [28]. We found that immunization with H9 cells, in the absence of any exogenous adjuvant, could lead to significant protection against live tumor challenge with high number of cells (5 × 10⁵). This observation was remarkable considering the report that hESCs were less immunogenic when graft rejection and immune cell infiltration were used as the criteria for immunogenicity [42]. Moreover, since the hESCs express a low level of human MHC (human leukocyte antigen) [43], the oncofetal antigens or other shared antigens in the hESCs must be cross-presented onto murine MHC or H-2 molecules to activate the host CD4⁺ and CD8⁺ T cells. Our study thus raises a question of the mechanism of antigen cross-presentation from hESCs by the host professional antigen-presenting cells such as DCs and subsequent cross-priming of antigen-specific T cells. We profiled the expression level of several major heat shock proteins (HSPs) in undifferentiated hESCs since HSPs are known for their ability to mediate cross-priming via both chaperoning antigenic peptides for cross-presentation to MHC and activating DCs [44–46]. We found that undifferentiated H9 cells, compared with the differentiated H9 cells, were indeed enriched with multiple HSPs including gp96, HSP86, HSP84, GRP78, HSC70, and calreticulin (supporting information Fig. 2). These data suggest that the abundance of HSPs in the undifferentiated state of hESCs may make antigens in the hESCs intrinsically more cross-presentable to the host DCs for generating protective antitumor activity. Such a model, although attractive, remains speculative.

The exact antigens shared by hESCs and CT26 colon carcinoma remain to be identified. Most likely, the antigens that were reactive with the H9 immune sera were oncofetal antigens present in both CT26 and the hESCs. Since tumor cells such as myeloma cells do express stem cell-associated antigen Sox2 [47], stem cell immunization might trigger immune response against these gene products that are also expressed by tumor cells. Additionally, the immune response against H9 could lead to antigenic spread to induce protective immunity against CT26 unique tumor antigens, a concept akin to what was proposed to explain the efficiency of xenogeneic antigen immunization [48]. However, xenogeneity did not contribute to their observed immunogenicity, as a phenotypically, morphologically, developmentally, and molecularly similar human iPS cell line TZ1 was unable to induce any protective immunity in the same mouse colon cancer model. As the full functional potential of hESCs and iPS cells continues to be debated and resolved [35, 36, 49], our study suggested that the magnitude and the quality of the host immune response against these different sources of stem cells might not be the same. We suggest that a comprehensive and comparative analysis on how hESCs and iPS cells are dealt with immunologically in vivo is necessary before their clinical use for therapeutic purposes.

We have examined the potential mechanism of tumor rejection by stem cell-immunized mice. We observed both humoral and cell-based immunity, represented by production of

**DISCUSSION**

The recent surge in interest in pluripotent stem cells arose from promising results in the area of regenerative medicine indicating that stem cell-derived adult cells may offer treatment options for a variety of degenerative diseases such as Parkinson’s disease, type I diabetes mellitus, and Alzheimer’s disease [19]. The other potential application of stem cells is in the area of immune-based therapy when various immune cells such as dendritic cells (DCs) can be differentiated and used for the treatment of immune disorders [39, 40]. In this study, we discovered yet another novel application of stem cells—that undifferentiated stem cells are powerful immunogens for generating effective immune response against histologically distinct, differentiation-divergent, and MHC-incompatible colon carcinoma. We found that both H9 and CT2, two embryonically derived human stem cells, were able to immunize naïve mice against challenge with a lethal dose of live colon carcinoma cells. Moreover, we demonstrated that these hESCs induced strong tumor-specific cellular immune responses and attenuated the expansion of MDSCs in the secondary lymphoid organs. We also showed that the immunogenicity of both H9 and CT2 dramatically rivals that of iPS cell line TZ1 in the same colon cancer model. Although more iPS cell lines need to be examined to make a general statement about their immunogenicity, our study does raise the possibility that there is a considerable degree of heterogeneity among stem cells in their expression of tumor antigens, even though the stemness and the pluripotency of these cells appear to be indistinguishable based on expression of typical stem cell markers and the differential potential in the standard teratoma assay.

CT26 is a widely used progressive and poorly differentiated colon carcinoma model in BALB/c mice [41]. In this system, as few as 1 × 10⁵ tumor cells given subcutaneously could lead to progressive local growth of the tumor and eventual death due, sometimes to distal lung metastasis [28]. We found that immunization with H9 cells, in the absence of any exogenous adjuvant, could lead to significant protection against live tumor challenge with high number of cells (5 × 10⁵). This observation was remarkable considering the report that hESCs were less immunogenic when graft rejection and immune cell infiltration were used as the criteria for immunogenicity [42]. Moreover, since the hESCs express a low level of human MHC (human leukocyte antigen) [43], the oncofetal antigens or other shared antigens in the hESCs must be cross-presented onto murine MHC or H-2 molecules to activate the host CD4⁺ and CD8⁺ T cells. Our study thus raises a question of the mechanism of antigen cross-presentation from hESCs by the host professional antigen-presenting cells such as DCs and subsequent cross-priming of antigen-specific T cells. We profiled the expression level of several major heat shock proteins (HSPs) in undifferentiated hESCs since HSPs are known for their ability to mediate cross-priming via both chaperoning antigenic peptides for cross-presentation to MHC and activating DCs [44–46]. We found that undifferentiated H9 cells, compared with the differentiated H9 cells, were indeed enriched with multiple HSPs including gp96, HSP86, HSP84, GRP78, HSC70, and calreticulin (supporting information Fig. 2). These data suggest that the abundance of HSPs in the undifferentiated state of hESCs may make antigens in the hESCs intrinsically more cross-presentable to the host DCs for generating protective antitumor activity. Such a model, although attractive, remains speculative.

The exact antigens shared by hESCs and CT26 colon carcinoma remain to be identified. Most likely, the antigens that were reactive with the H9 immune sera were oncofetal antigens present in both CT26 and the hESCs. Since tumor cells such as myeloma cells do express stem cell-associated antigen Sox2 [47], stem cell immunization might trigger immune response against these gene products that are also expressed by tumor cells. Additionally, the immune response against H9 could lead to antigenic spread to induce protective immunity against CT26 unique tumor antigens, a concept akin to what was proposed to explain the efficiency of xenogeneic antigen immunization [48]. However, xenogeneity did not contribute to their observed immunogenicity, as a phenotypically, morphologically, developmentally, and molecularly similar human iPS cell line TZ1 was unable to induce any protective immunity in the same mouse colon cancer model. As the full functional potential of hESCs and iPS cells continues to be debated and resolved [35, 36, 49], our study suggested that the magnitude and the quality of the host immune response against these different sources of stem cells might not be the same. We suggest that a comprehensive and comparative analysis on how hESCs and iPS cells are dealt with immunologically in vivo is necessary before their clinical use for therapeutic purposes.

We have examined the potential mechanism of tumor rejection by stem cell-immunized mice. We observed both humoral and cell-based immunity, represented by production of
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CONCLUSION

In conclusion, we demonstrate the capacity of human ES cells to effectively immunize against murine colon cancer. This suggests the presence of shared embryonic antigens between hES cells and tumor cells. The potential application of ES cells in cancer immunotherapy warrants further investigation.

ACKNOWLEDGMENTS

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.


Human Stem Cells Vaccinate Against Cancer

CT26-specific antibody and IFNγ-producing cells, respectively. However, it is difficult to attribute responsibility for tumor rejection to a single mechanism, as the effector arm of tumor rejection is known to be a complex one that involves the coordinated roles of CD4+ cells, CD8+ T cells, antibody-mediated cytotoxicity, natural killer cells, phagocytic cells, and other soluble factors such as nitric oxide and cytokines (IFNγ, tumor necrosis factor α, etc.). Indeed, the field of tumor immunology is still in desperate need of a suitable immunological surrogate marker to predict the clinical effectiveness of cancer vaccines [50, 51]. It is thus not surprising that even the iPSC cell line TZ1 induced significant numbers of IFNγ- and IL-4-producing splenocytes against CT26, although no evidence of tumor rejection was seen. We have also histologically examined tumors for the presence of CD4+ CX3CR1+ macrophages [52]. In all groups, including the PBS-immunized group, infiltration with CD4+ and CD8+ cells was observed. The density of FoxP3+ cells in the tumor tissue also did not seem to correlate significantly with impending or ongoing tumor rejection. Remarkably, however, we did observe a consistent and significant inverse correlation between the number of MDSCs in the spleen and the tumor burden of immunized mice. MDSCs are increasingly recognized as a heterogeneous population of cells that expand during cancer with a remarkable ability to suppress T-cell responses through complex mechanisms [31, 52]. The accumulation of MDSCs in lymphoid organs has been observed in tumor-bearing individuals and is often associated with large tumor burdens. The function of MDSCs has been implicated in the suppression of T-cell responses and the induction of T-cell tolerance against cancer [53]. Thus the accumulation of MDSCs in both PBS- and TZ1-immunized groups may signal the collapsed functional state of host immunity and failure of tumor containment in our model.

We have not observed any significant autoimmunity in the hESC-immunized mice. Immunized mice were generally healthy without clinical evidence of autoimmune diseases. Serology study revealed evidence of low titers of ANAs, which was clinically insignificant. However, more follow-up study is needed before hESC-based cancer vaccines move into clinical testing.

In a broad context, our study has raised a number of intriguing questions that deserve further research. What are the critical tumor rejection antigens in hESCs that are able to trigger effective antitumor immunity? Could these antigens be present in other types of cancer? We did not apply any exogenous adjuvants in this study. Could the immunogenicity of hESCs be further improved by combining cells with known pro-inflammatory stimuli such as ligands of Toll-like receptors, cytokines, augmentation of costimulations, and/or ablation of Treg cells and MDCs? With further optimization, could a hESC-based vaccination strategy be effective against pre-established cancer? What is the molecular difference between iPSC and hESC that accounts for the failure of the former to elicit antitumor immunity in our system? Does the ability of hESCs to prime antitumor immunity fade or disappear during stem cell differentiation? With the availability of defined stem cells, these questions deserve quick answers before the launch of early phase clinical trials to test whether a stem cell-based vaccine is indeed a useful option for cancer immunotherapy.


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Supporting Information Table 1. Immunophenotypic analysis of splenocytes after immunization with cancer cells and hES cells. Twenty days after tumor challenge, splenocytes from mice of each immunized group were stained for the indicated antibody and subpopulations of cells were analyzed by flow cytometry.

Supporting Information Table 1a. Percentage of splenic cell subpopulation in total splenocytes in immunized mice.

<table>
<thead>
<tr>
<th>Subpopulation (%)</th>
<th>PBS</th>
<th>H9</th>
<th>CT26</th>
</tr>
</thead>
<tbody>
<tr>
<td>B220+</td>
<td>51.50±2.94</td>
<td>54.80±1.34</td>
<td>53.52±2.38</td>
</tr>
<tr>
<td>CD8+</td>
<td>9.20±1.22</td>
<td>10.12±0.85</td>
<td>10.93±1.50</td>
</tr>
<tr>
<td>CD4+</td>
<td>20.18±3.34</td>
<td>21.36±2.57</td>
<td>23.64±2.22</td>
</tr>
<tr>
<td>Gr-1+CD11b+</td>
<td>8.60±3.14</td>
<td>4.64±2.26</td>
<td>3.10±1.08</td>
</tr>
<tr>
<td>CD4+CD25+</td>
<td>3.35±0.28</td>
<td>3.27±0.40</td>
<td>3.28±0.22</td>
</tr>
</tbody>
</table>

Supporting Information Table 1b. Absolute number of splenic cell population in immunized mice.

<table>
<thead>
<tr>
<th>Number (x10^6)</th>
<th>PBS</th>
<th>H9</th>
<th>CT26</th>
</tr>
</thead>
<tbody>
<tr>
<td>B220+</td>
<td>32.18±2.61</td>
<td>28.36±1.62</td>
<td>29.99±8.30</td>
</tr>
<tr>
<td>CD8+</td>
<td>5.79±1.13</td>
<td>5.26±0.76</td>
<td>6.04±1.35</td>
</tr>
<tr>
<td>CD4+</td>
<td>12.75±2.98</td>
<td>11.12±1.94</td>
<td>13.28±4.14</td>
</tr>
<tr>
<td>Gr-1+CD11b+</td>
<td>5.45±2.19</td>
<td>2.37±1.06</td>
<td>1.71±0.62</td>
</tr>
</tbody>
</table>

a-b p=0.024; c-d p=0.022; c-c p=0.016
Supporting Information Figure 1. Lack of serological evidence of autoimmunity after stem cell immunization. Sera from mice immunized with the indicated cells or from a naïve mouse (1:50) was used to immunoblot cell lysates from the indicated organs: S-spleen, K-kidney, L-liver.
**Supporting Information Figure 2.** Expression profile of major heat shock proteins by H9 cells before (1) and after (2) spontaneous differentiation by Western blot. β-actin served as a loading control.
**Supporting Information Figure 3.** Immunohistochemistry of tumor-infiltrating CD4⁺, CD8⁺ and FoxP3⁺ cells after immunization with hES cells, iPS cells or cancer cells. Twenty days after tumor challenge, tumors from mice of each immunized group were removed, sectioned and stained for the indicated markers. One representative image from one mouse in each group is shown.
Stem Cell Vaccination Against Cancer: Fighting Fire With Fire?

Thomas P Zwaka

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A recent report suggests yet another application for embryonic stem (ES) cell technology: using such cells to immunize against cancer. However, before this novel strategy can prove useful, several issues need to be resolved. ES cells derived from preimplantation embryos and induced pluripotent stem (iPS) cells obtained by transient overexpression of specific transcription factors in somatic cells are frequently said to be an invaluable tool for the understanding and development of more efficient AAV vectors that can package desirable larger genomes for human therapy.

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antigens expressed by the primitive normal cells and the colon carcinoma cell line CT26—so-called oncofetal antigens. The authors report that vaccination of mice with the human ES cell line H9 induced both strong cellular and humoral immune responses against CT26 colon carcinoma that was manifested as a retardation of proliferation after injection of the cells into the experimental animal. On a more mechanistic level, they found that the protection correlated with an expansion of tumor-responsive and interferon-γ-producing cells and a profound loss of CD11b+Gr-1+ myeloid-derived suppressor cells in the spleen. Importantly, they found no evidence of any significant autoimmunity. Finally, the authors compared the immunogenicity of their human ES cell line against CT26 colon carcinomas with that of a newly established iPS cell line. Curiously, they obtained evidence that suggests that the iPS cell line was inferior to the human ES cell line in conferring tumor protection. By way of explanation, they proposed that oncofetal antigens were differentially expressed in human ES and iPS cells.

Three aspects of this study seem to deserve particular attention. The first is the concept of tumor stem cells. The authors reason that if the cancer stem cell concept is valid, then immunization with stem cells should prove valuable. However, it is possible to argue that human ES cells as a stem cell entity are defined merely on the basis of functional characteristics observed in vitro and that they are not likely to represent authentic stem cells found during embryogenesis or in adult somatic tissues. If this is the case, it would be difficult to accept at face value claims that the observed protective effect was attributable to specific immunogenicity against tumor stem cells. Second, the authors propose that the particularly primitive nature of human ES cells (given their embryonic origin) could form the basis for the specific effects against CT26. Although this may be partially true, for the most part the data presented do not directly address this. Instead, it seems that aberrant (and indeterminate) exposure to human antigens, regardless of the origin of the cell line, was critically responsible for the observed effects. Although they will no doubt be laborious, experimental strategies aimed at discerning the immunospecificity of the effects of human ES cells will be necessary to resolve this question. Finally, the apparent difference between iPS and ES cells clearly deserves attention; however, future studies must address this variability as more than a mere formality. The evidence that human ES and iPS cells are different in any substantial way is, at best, preliminary. Until the authors perform the requisite well-controlled experiments, which are not trivial, it would be easy to mistake inter-cell line variations for intrinsic biological differences.

There is a certain irony in the fact that human ES cells, which themselves possess many features of neoplastic cells—including sustained telomerase activity, formation of tumors after injection into mice, and infinite growth—would be exploited against cancer. By analogy, it is like fighting fire with fire. However, given the great uncertainty surrounding the nature of human ES cells, and thus the parameters that confer the ability to interact with the immune system, one must be cautious lest the positive analogy be replaced by the image of an 8-year-old boy playing with matches.

It is an obligatory exercise to dissect the specific effects of pluripotent cells and to determine how beneficial and safe they are relative to existing immunotherapeutic concepts. On balance, it is rewarding to see the potential spectrum of the application of human pluripotent stem cells growing far beyond what most of us have envisioned. This and other studies clearly indicate that the field of ES and iPS cells has entered a new, more mature era. As in most endeavors, timing is critical. But it is rewarding to see that the policy climate with respect to science in general, and ES cells in particular, seems to be moving in a more favorable direction worldwide, especially in the United States. Scientific discoveries as well as progress on the political–ethical front will guarantee that stem cell research will have a profound and positive impact on the future of biomedical research and therapy.

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In the Beginning: Reflections on the Genesis of Molecular Therapy

James M Wilson1

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On the occasion of the tenth anniversary of the inaugural issue of Molecular Therapy, I would like to share my recollections of the events that led to the creation of the Journal, which occurred under my watch as the second President of the American Society of Gene Therapy.

It all began with a series of phone calls from our colleague, George Stamatoyannopoulos, in the summer of 1996 to several of us working in the field of gene therapy. George proposed to get together to talk about creating a new professional society focused on gene therapy. He summoned us to a meeting at the Marriott Hotel in San Francisco, CA, on 29 October

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