In Vivo Programming of Tumor Antigen-Specific T Lymphocytes from Pluripotent Stem Cells to Promote Cancer Immunosurveillance

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Abstract

Adoptive T-cell immunotherapy has garnered wide attention, but its effective use is limited by the need of multiple ex vivo manipulations and infusions that are complex and expensive. In this study, we show how highly reactive antigen (Ag)-specific CTLs can be generated from induced pluripotent stem (iPS) cells to provide an unlimited source of functional CTLs for adoptive immunotherapy. iPS cell–derived T cells can offer the advantages of avoiding possible immune rejection and circumventing ethical and practical issues associated with other stem cell types. iPS cells can be differentiated into progenitor T cells in vitro by stimulation with the Notch ligand Delta-like 1 (DL1) overexpressed on bone marrow stromal cells, with complete maturation occurring upon adoptive transfer into Rag1-deficient mice. Here, we report that these iPS cells can be differentiated in vivo into functional CTLs after overexpression of MHC I-restricted Ag-specific T-cell receptors (TCR). In this study, we generated murine iPS cells genetically modified with ovalbumin (OVA)-specific and MHC-I restricted TCR (OT-I) by retrovirus-mediated transduction. After their adoptive transfer into recipient mice, the majority of OT-1/iPS cells underwent differentiation into CD8+ CTLs. TCR-transduced iPS cells developed in vivo responded in vitro to peptide stimulation by secreting interleukin 2 and IFN-γ. Most importantly, adoptive transfer of TCR-transduced iPS cells triggered infiltration of OVA-reactive CTLs into tumor tissues and protected animals from tumor challenge. Taken together, our findings offer proof of concept for a potentially more efficient approach to generate Ag-specific T lymphocytes for adoptive immunotherapy. Cancer Res; 71(14): 4742–7. ©2011 AACR

Introduction

Adoptive cell transfer (ACT) of antigen (Ag)-specific CTLs is a promising treatment for a variety of malignancies (1). CTLs can target malignant tumors by T-cell receptor (TCR) and release cytotoxins as well as cytokines to kill tumor cells. However, ACT with these CTLs is often not feasible due to difficulties in obtaining such CTLs from patients. There is an urgent need to find a new approach to generate tumor-reactive CTLs for successful ACT-based therapies.

Several groups have generated induced pluripotent stem (iPS) cells from somatic cells by transduction of 1 to 4 transcription factors (2, 3). This approach provides an opportunity to generate patient- or disease-specific pluripotent stem cells (4). In addition, researchers have produced iPS cells that are safe for transplantation into patients (5, 6). Moreover, it has been reported that the combined iPS–gene therapy approach cures certain human genetic diseases in vivo (7). Because of the plasticity and potentially unlimited capacity for self-renewal, iPS cell-based therapies may have great potential in the treatment of diseases.

Previous studies have shown successful T-cell development from pluripotent stem cells (8, 9), and we have shown T lineage differentiation from iPS cells (10). However, it remains unclear whether iPS cells can differentiate into functional, Ag-specific CTLs. In this study, we adoptively transferred into mice iPS cells that were transduced with Ag-specific TCR genes. We found that these iPS cells differentiated into functional Ag-specific CTLs in vivo and significantly protected the hosts from a tumor challenge.

Materials and Methods

Cells and mice

The mouse iPS-MEF-Ng-20D-17 cell line was obtained from RIKEN Cell Bank on September 1, 2008. iPS-MEF-Ng-20D-17 cell generated from male C57BL/6 mouse embryonic fibroblasts by introducing the 4 factors (Oct3/4, Sox2, Klf4, and...
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c-Myc) is carrying Nanog promoter-driven green fluorescent protein (GFP)/internal ribosome entry site (IRES)/puromycin-resistant gene (11). Expression of Oct3/4, Sox2, Klf4, and c-Myc was confirmed by reverse-transcriptase PCR (RT-PCR), and GFP expression was confirmed by flow cytometry during the course of this study. The OVA-expressing E.G7 lymphoma cell line (E.G7-OVA) was purchased from American Type Culture Collection and was authenticated by flow cytometry before use. OT-I TCR- transgenic mice were purchased from The Jackson Laboratory. All experiments were approved by the Pennsylvania State University College of Medicine Animal Care and Use Committee, Hershey, Pennsylvania, and were in accordance with the guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care.

**Antibodies**

Fluorescein isothiocyanate (FITC) anti-mouse Vβ5 TCR (MR9-4), R-Phycoerythrin (PE) or adenosomatous polyposis coli (APC) anti-mouse Vα2 TCR (B20.1) or interleukin (IL)-2 (JES6-5H4), and APC anti-mouse IFN-γ (XM61.2) were obtained from BD PharMingen. FITC or PE anti-mouse CD8 (6A242) were obtained from Biolegend. PE/Cy7 or APC anti-mouse CD69 were obtained from Biolegend. FITC-OVA (200-4233) was purchased from Rockland Immunochemicals.

**Cell culture**

iPS cells were maintained on feeder layers of irradiated SNL76/7 cells as previously described (10).

**Retroviral transduction**

Retroviral transduction was performed as described previously (12). Expression of DsRed was determined by flow cytometry gating on GFP+ cells. DsRed+ GFP+ cells were purified by cell sorting using a MoFlo high-performance cell sorter (Dako Cytomation).

**ACT and tumor challenge**

A total of 3 × 10^6 GFP+ DsRed+ iPS cells or bone marrow-derived CD117+ Lin- hematopoietic stem cells (HSC) from OT-I TCR transgenic mice in PBS were injected iv. into 4-week-old C57BL/6 mice. After 6 to 10 weeks, OVA-specific Vβ5+ CD8+ T-cell development in lymph nodes and spleen was determined by flow cytometry. For tumor challenge, 6 weeks after adoptive transfer, mice were challenged intraperitoneally with E.G7-OVA tumor cells as previously described (12). In some experiments, mice were challenged with tumor cells 1 day following iv. injection with CD8+ T cells isolated from OT-I TCR transgenic mice.

**Flow cytometric analysis**

On day 50 of tumor challenge, CD8+ T cells from spleens were stimulated with irradiated T-depleted splenocytes pulsed with 0.5 μmol/L OVA257–264 peptide (GenScript) for 7 hours. IL-2 and IFN-γ were analyzed by intracellular cytokine staining. Tumor tissue from the peritoneal cavity was prepared for a single-cell suspension and analyzed expression of Vα2 and Vβ5 by flow cytometry, after gating on CD8+ cells.

**In vivo proliferation/cytotoxicity assay**

Splenocytes from naïve C57BL/6 mice labeled with Carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) were used as targets. Cells labeled with 5 μmol/L CFSE (CFSEhi cells) were pulsed with 10 μg/mL OVA257–264 peptide, and cells labeled with 0.5 μmol/L CFSE (CFSElo cells) were not pulsed. A mixture of 2.5 × 10^6 CFSEhi plus 2.5 × 10^6 CFSElo cells were transferred by iv. injection into indicated recipients. After 16 hours, splenocytes were collected and analyzed as described previously (13).

**Histology and immunofluorescence**

**H&E staining.** Routine hematoxylin & eosin (H&E) staining was performed at an interval of every 5 serial sections.

**Immunologic staining.** Tissue sections were fixed with acetone and incubated with 3% bovine serum albumin to

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Figure 2. Ag-specific CD8⁺ T-cell development from iPS cells in vivo. GFP⁺ DaRed⁺ iPS cells were injected i.v. into C57BL/6 mice. After 6 to 10 weeks, OVA-specific Vβ5⁺ CD8⁺ T-cell development was determined. A, CD8⁺ Vβ5⁺ T cells from pooled lymph nodes and spleen were analyzed by flow cytometry. B, CD25 and CD69 expression was analyzed by flow cytometry, after gating on CD8⁺ Vβ5⁺ T cells (dark lines; shaded areas indicate isotype controls). C, IL-2 and IFN-γ production from the CD8⁺ Vβ5⁺ population (dark lines; shaded areas indicate isotype controls) was determined by intracellular cytokine staining. D, in vivo proliferation/cytotoxicity assay. CFSEhi (right peaks) and CFSElo (left peaks) target cells were pulsed with OVA257–264 peptide and the control, respectively, and were injected into mice 10 weeks after iPS cell transfer or 1 day after OT-I CTL transfer. Data are representative of 2 or 3 independent experiments.
block nonspecific protein binding. Sections were stained with PE anti-mouse TCR Vc2 and FITC-OVA.

Statistics
One-way ANOVA was used for the statistical analysis between groups and significance was set at 5%. Kaplan–Meier analyses were used to determine percentage of survival based on death of the animals due to tumor growth in the peritoneal cavity.

Results and Discussion

Generation of Ag-specific TCR gene–transduced iPS cells
We used the retroviral vector pMig in which MHC-I-restricted OVA–specific TCR α and β chain genes were linked with a 2A peptide (14). We replaced GFP with DsRed for monitoring gene integration and named the new vector as MiDR (Fig. 1A). After transduction, DsRed expression was visualized by fluorescent microscopy (Fig. 1B). Although the transduction efficiency was low, we could sort for DsRed+ GFP− cells (Fig. 1C). Moreover, we confirmed the expression of TCR Vβ5 mRNA and DNA integration in the sorted cells by RT-PCR and PCR (Fig. 1D).

TCR gene-transduced iPS cells differentiated into CTLs in vivo
We observed approximately 49% of CD8+ Vβ5+ cells in mice receiving TCR gene–transduced iPS cells or control cells. In contrast, the CD8+ Vβ5+ cells were less than 2% in mice receiving control gene–transduced iPS cells (Fig. 2A and Supplementary Fig. S1). In addition, we observed that most CD8+ Vβ5+ cells expressed CD25 and CD69 (Fig. 2B) and produced IL-2 and IFN-γ (Fig. 2C). Furthermore, we found that target cell lysis was approximately 90 times greater in mice receiving TCR gene–transduced iPS cells than in those receiving control gene-transduced iPS cells (94% versus 1%; Fig. 2D).

In vivo persistence of Ag-specific T cells derived from TCR gene-transduced iPS cells
After 50 days, we visualized an increased number of OVA-specific CD8+ T cells in the pooled lymph nodes and spleen cells in mice receiving TCR gene–transduced iPS cells than in mice receiving CD8+ T cells from OT-I TCR transgenic mice (52.7% versus 12.8%; Fig. 3A).

Adoptive transfer of TCR gene-transduced iPS cells prevents tumor growth
On day 30 after tumor challenge, we found fewer tumor cells in the peritoneal cavity of mice receiving TCR gene–transduced iPS cells than in mice receiving either CD8+ T cells from OT-I TCR transgenic mice or control gene-transduced iPS cells (Fig. 3B and Supplementary Fig. S2). On day 50, we observed 100% survival of mice receiving TCR gene–transduced iPS cells, compared with 55% survival of mice receiving CD8+ T cells from OT-I TCR transgenic mice (Fig. 3C). Moreover, we observed tumor-infiltrating OVA–specific CD8+ T cells in mice receiving TCR gene–transduced iPS cells (Fig. 4).
a number of HSCs or ESCs from cancer patients is often not feasible. Recent iPS cell technology can generate iPS cells from patients without any surgical approach. Thus, iPS cells have greater potential to be used in ACT-based therapies. Our study significantly facilitates this application.

Although TCR-transduced iPS cells need up to 6 to 8 weeks to develop into fully differentiated T cells, there are possibilities to enhance this development. Researchers have evaluated the efficacy of ACT therapy by transferring tumor-specific CD8$^+$ T cells at various stages of differentiation into tumor-bearing mice. These studies concluded that administration of naïve and early effector T cells, in combination with a lymphodepleting pretreatment regimen, γc cytokine administration, and vaccination, resulted in the eradication of established tumors (18–20). A conditioning treatment of mice (e.g., sublethal irradiation) prior to iPS cell transfer or cytokine treatment (IL-2 or IL-15) may benefit iPS cell-based therapies. This will be helpful for the translation of the studies for treatment of cancer patients.

Despite the observed control of tumor growth, we identified some limitations of ACT with TCR gene-transduced iPS cells. First, at least 6 weeks of in vivo development are essential for T-cell differentiation to occur from the transferred iPS cells. Although there are Ag-specific CD8$^+$ T cells presenting in lymph nodes and spleen 4 weeks after cell transfer, these cells are less than 3.55% of the total CD3$^+$ population. This will be helpful for the translation of the studies for treatment of cancer patients.

It has been previously shown that TCR-transduced bone marrow cells controlled the growth of human tumors in severe combined immunodeficiency mice (15). It has also been shown that TCR transduction of HSCs could mediate antitumor immunity (16, 17). However, the approach to obtain

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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