In vivo programming of tumor antigen-specific T lymphocytes from pluripotent stem cells to promote cancer immunosurveillance

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Precis: Findings explore a novel approach to generate antigen-specific T lymphocytes from induced pluripotent stem cells, with implications to simply adoptive T cell therapies for cancer.

Running Title: Generation of antigen-specific T lymphocytes from iPS cells

Key Words: iPS cells, T cell differentiation, lymphoma, mouse model

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ABSTRACT

Adoptive T cell immunotherapy has garnered wide attention but their 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 (TCRs). In this study, we generated murine iPS cells genetically modified with OVA-specific and MHC-I restricted TCR (OT-I) by retrovirus-mediated transduction. After their adoptive transfer into recipient mice, the majority of OT-I/iPS cells underwent differentiation into CD8+ CTLs. TCR-transduced iPS cells developed in vivo responded in vitro to peptide stimulation by secreting IL-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.
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 iPS cells from somatic cells by transduction of one to four 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 vitro (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 demonstrated 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 Sep. 1, 2008. iPS-MEF-Ng-20D-17 cell generated from male C57BL/6 mouse embryonic fibroblasts by introducing the four factors (Oct3/4, Sox2, Klf4 and c-Myc), is carrying Nanog promoter-driven GFP/IRES/puromycin-resistant gene (11). Expression of Oct3/4, Sox2, Klf4 and c-Myc was confirmed by 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 ATCC, and was authenticated by flow cytometry before use. OT-I TCR-transgenic mice were purchased from Jackson Laboratory (Bar Harbor, ME). All experiments were approved by the Pennsylvania State University College of Medicine Animal Care and Use Committee and were in accordance with the guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care.

Antibodies

FITC anti-mouse Vβ5 TCR (MR9-4), PE or APC anti-mouse Vα2 TCR (B20.1) or IL-2 (JES6-5H4), and APC anti-mouse IFN-γ (XMG1.2) were obtained from BD PharMingen (San Diego, CA). PE/Cy7 or APC anti-mouse CD25, and APC/Cy7 or PerCP anti-mouse CD69 were obtained from Biolegend (San Diego, CA). FITC or PE anti-mouse CD8 (6A242) were obtained from Santa Cruz Biotech (Santa Cruz, CA). FITC-OVA (200-4233) was purchased from Rockland Immunochemicals (Gilbertsville, PA).

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, Fort Collins, CO).

**Adoptive Cell Transfer and Tumor Challenge**

3 x 10^6 GFP+ DsRed+ iPS cells or bone marrow-derived CD117+ Lin- HSCs from OT-I TCR transgenic mice in PBS were injected *i.v.* into 4-week old C57BL/6 mice. After six to ten weeks, OVA-specific Vβ5+ CD8+ T cell development in lymph nodes (LNs) and spleen was determined by flow cytometry. For tumor challenge, six weeks after adoptive transfer, mice were challenged *i.p.* with E.G7-OVA tumor cells as previously described (12). In some experiments, mice were challenged with tumor cells one day following *i.v.* 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 μM OVA257–264 peptide (GenScript) for 7 hrs. 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 naive C57BL/6 mice labeled with CFSE (Invitrogen) were used as targets. Cell labeled with 5 μM CFSE (CFSE\textsuperscript{hi} cells) were pulsed with 10 μg/ml OVA\textsubscript{257–264} peptide, and cell labeled with 0.5 μM CFSE (CFSE\textsuperscript{lo} cells) were not pulsed. A mixture of 2.5 x 10\textsuperscript{6} CFSE\textsuperscript{hi} plus 2.5 x 10\textsuperscript{6} CFSE\textsuperscript{lo} cells were transferred by i.v. injection into indicated recipients. 16 hr later, 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 five serial sections. Immunological staining: Tissue sections were fixed with acetone, and incubated with 3% BSA to block non-specific protein binding. Sections were stained with PE anti-mouse TCR V\textalpha\textsubscript{2} 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 were able to sort for DsRed+ GFP+ cells (Fig. 1c). Moreover, we confirmed 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 HSCs. In contrast, the CD8+ Vβ5+ cells were less than 2% in mice receiving control gene-transduced iPS cells (Fig. 2a and Supplementary Fig. 1). In addition, we examined 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 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 fifty days, we visualized an increased number of OVA-specific CD8+ T cells in the pooled LNs 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 prevent 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. 2). On day 50, we observed 100% survival of mice receiving TCR gene-transduced iPS cells, compared to 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).

It has been previously demonstrated 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 anti-tumor immunity (16, 17). However, the approach to obtain 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.

Since TCR transduced iPS cells need up to six to eight weeks to develop into fully differentiated T cells, there are possibilities to enhance this development. It has been evaluated the efficacy for ACT therapy by transferring tumor-specific CD8$^+$ T cells at various stages of differentiation into tumor-bearing mice. These studies concluded that administration of naive 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 six-weeks of in vivo development is essential for T-cell differentiation to occur from the transferred iPS cells. Although there are Ag-specific CD8+ T cells presenting in LNs and spleen four weeks after cell transfer, these cells are less than 3.55% of total CD3+Vβ5+ population, which is not sufficient to generate efficient anti-tumor immunity. From wks 6 to 10 after cell transfer, there are approximately 24% of CD3+Vβ5+ population in LNs and spleen and more than 80% of these cells are CD8+ CD4- (Supplementary Fig. 1). Second, we noticed hair loss and bone softening in mice receiving TCR-transduced iPS cells. These effects may be caused by the generation of other immune cells from the transferred iPS cells. How such cells may be generated in vivo currently remains unknown. Nevertheless, we did not observe immunosuppressive cell subsets such as CD4+ CD25+ Foxp3+ cells develop from genetically engineered iPS cells (Supplementary Fig. 3).
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FIGURE LEGENDS

Figure 1. Retrovirus-mediated TCR transduction in iPS cells. iPS cells were transduced with the following retroviral constructs: vector control (MiDR), or OVA_{257-264}-specific TCR (MiDR-TCR). (a) Schematic representation of the retroviral construct. (b) TCR-transduced iPS cells were visualized by fluorescence microscopy. (c) GFP^{+} iPS cells (left) were transduced with the retroviral construct MiDR-TCR, and GFP^{+} DsRed^{+} iPS cells (middle) were analyzed by flow cytometry and sorted by a high-speed cell sorter (right). (d) GFP^{+} DsRed^{+} iPS cells were sorted and total mRNA and DNA were analyzed for V{\beta}5 gene expression by RT-PCR (left) and for the V{\beta}5 gene by PCR (right). The forward primer is ACGTGTATTCCCATCTCTGGACAT and the reverse primer is TGTTCTATAATTGCCCCGAGAGCTG.

Figure 2. Ag-specific CD8^{+} T-cell development from iPS cells in vivo. GFP^{+} DsRed^{+} iPS cells were injected i.v. into C57BL/6 mice. After six to ten weeks, OVA-specific V{\beta}5^{+} CD8^{+} T cell development was determined. (a) CD8^{+} V{\beta}5^{+} T cells from pooled LNs and spleen were analyzed by flow cytometry. (b) CD25 and CD69 expression was analyzed by flow cytometry, after gating on CD8^{+} V{\beta}5^{+} T cells (dark lines; shaded areas indicate isotype controls). (c) IL-2 and IFN-\gamma production from the CD8^{+}V{\beta}5^{+} population (dark lines; shaded areas indicate isotype controls) were determined by intracellular cytokine staining. (d) In vivo proliferation/cytotoxicity assay. CFSE^{hi} (right peaks) and CFSE^{lo} (left peaks) target cells were pulsed with OVA_{257-264} peptide and the control, respectively, and were injected into mice ten weeks after iPS cell transfer or one day after OT-I CTL transfer. Data are representative of two or three independent experiments.
Figure 3. Adoptive transfer of TCR-transduced iPS cells suppresses tumor growth and sustains mouse survival. GFP+ DsRed+ cells were adoptively transferred into C57BL/6 mice. One group of mice were injected with OVA-reactive CD8+ T cells from OT-I TCR transgenic mice, and one group of mice had no cell transfer. After either six weeks or on the following day after the cell transfer, mice were subjected to challenge with E. G7 tumor cells. (a) Ag-specific T-cell persistence. Seven weeks post tumor challenge or thirteen weeks without tumor challenge, CD8+ Vβ5+ T cells from the pooled LNs and spleen were analyzed by flow cytometry. (b) On day 20, tumor cells in the peritoneal cavity were enumerated. Data represent mean (±SEM) tumor cell counts from six individual mice. One-way ANOVA test was used for statistical analyses between two groups (*: p<0.05). (c) Mouse survival on day 50. Kaplan-Meier survival curves are shown (n=6). *: p<0.05; **: p<0.001, One-way ANOVA with Newman-Keuls Multiple Comparison Test. Data are representative of three independent experiments.

Figure 4. iPS cell-derived Ag-specific CTLs infiltrate into tumor tissues. On day 30 to 35 after tumor challenge, tumor tissues were examined for tumor-reactive T cell infiltration. (a) H&E staining. Inflammatory cells infiltrated in tumor tissues (↓). (b) Immunohistological staining. OVA-specific Vα2+ CTLs (red) infiltrated in OVA-expressing tumor tissues (green). (c) Single-cell suspensions from tumor tissues were analyzed for expression of Vα2+ and Vβ5+ by flow cytometry, after gating on the CD8+ population. Data are representative of three independent experiments.
Figure 1.

- **Figure 1a:** Diagram showing the genomic elements including LTR, a chain, P2A, b chain, IRES, DsRed, and LTR.

- **Figure 1b:** Images showing brightfield, GFP channel, DsRed channel, and overlay of GFP and DsRed channels before and after transduction and cell sorting.

- **Figure 1c:** Flow cytometry plots showing the percentages of DsRed-positive cells before and after transduction and cell sorting.

- **Figure 1d:** Gel images showing RT-PCR and PCR results with DNA marker, iPS cells from OT-I mice, T cells from C57BL/6 mice, and iPS cells from C57BL/6 mice.
Figure 2.

(a) Comparison of CD8+ T cell frequencies in MiDR vector/iPS cells, MiDR-TCR/iPS cells, and HSCs. The graph shows the percentage of CD8+ T cells in each group.

(b) Comparison of CD25 and CD69 expression in MiDR vector/iPS cells, MiDR-TCR/iPS cells, and HSCs. The graph depicts the percentage of cells expressing CD25 and CD69.

(c) Comparison of IL-2 and IFN-γ expression in MiDR vector/iPS cells, MiDR-TCR/iPS cells, and HSCs. The graph illustrates the percentage of cells secreting IL-2 and IFN-γ.

(d) CFSE dilution in MiDR vector/iPS cells, MiDR-TCR/iPS cells, and OT-I CTLs. The graph shows the percentage of cells remaining labeled with CFSE over time.
Figure 3.

**a**

![Graph showing CD8 and Vb5 expression with different treatments: OT-I CTLs + Tumor, IPS cells/MiDR-TCR + Tumor, and OT-I CTLs - Tumor.](image)

**b**

![Bar graph showing the number of tumor cells (x 10^6) with treatments: No cell transfer, IPS cells/MiDR, OT-I CTLs, and IPS cells/MiDR-TCR.](image)

**c**

![Line graph showing percent survival over days with treatments: No cell transfer, IPS cells/MiDR, OT-I CTLs, and IPS cells/MiDR-TCR.](image)
Figure 4.

(a) No cell transfer vs. iPS cells/MiDR-TCR

(b) No cell transfer vs. iPS cells/MiDR-TCR

(c) No cell transfer vs. iPS cells/MiDR-TCR

72.5% vs. 35.8%
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