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Priority Report

Cancer Research

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-I/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 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 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 adenomatous polyposis coli (APC) anti-mouse Vα2 TCR (B20.1) or interleukin (IL)-2 (JES6-5H4), and APC anti-mouse IFN-γ (XMG1.2) were obtained from BD PharMingen. PE/Cy7 or APC anti-mouse CD25 and APC/Cy7 or PerCP anti-mouse CD69 were obtained from Biolegend. FITC or PE anti-mouse CD8 (6A242) were obtained from Santa Cruz Biotech. 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 i.v. into 4-week-old C57BL/6 mice. After 6 to 10 weeks, OVA-specific $V\beta5^+$ $CD8^+$ 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 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 μmol/L $OVA_{257–264}$ 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 ($CFSE^{hi}$ cells) were pulsed with 10 μg/mL $OVA_{257–264}$ peptide, and cells labeled with 0.5 μmol/L CFSE ($CFSE^{lo}$ cells) were not pulsed. A mixture of 2.5×10^6 $CFSE^{hi}$ plus 2.5×10^6 $CFSE^{lo}$ cells were transferred by i.v. 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 albumen to

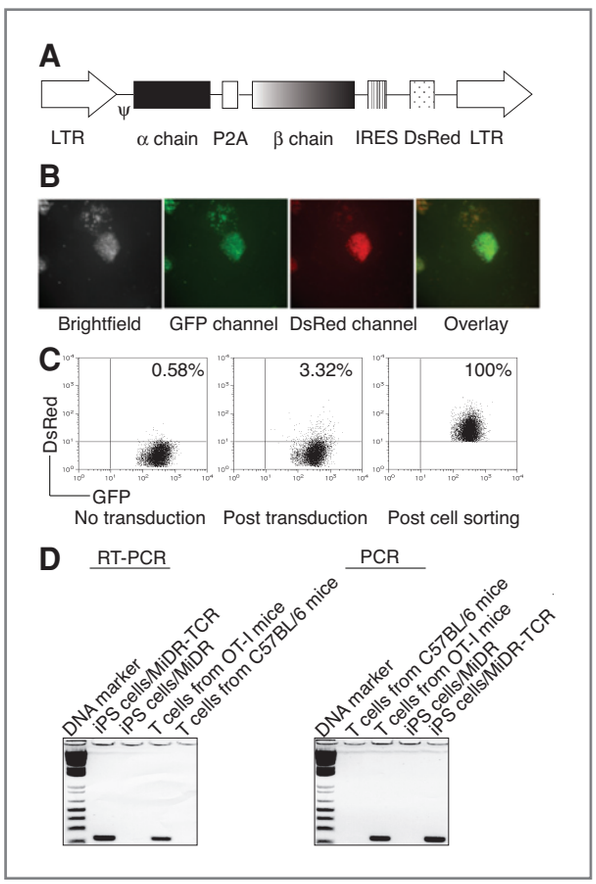

Figure 1. Retrovirus-mediated TCR transduction in iPS cells. iPS cells were transduced with the following retroviral constructs: vector control (MiDR) or OVA $_{257–264}$-specific TCR (MiDR-TCR). A, schematic representation of the retroviral construct. LTR, long terminal repeats. B, TCR-transduced iPS 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 Vβ5 gene expression by RT-PCR (left) and for the Vβ5 gene by PCR (right). The forward primer is ACGTGTATTCCCATCTCTGGACAT and the reverse primer is TGTTCATAATTGGCCCGAGAGCTG.

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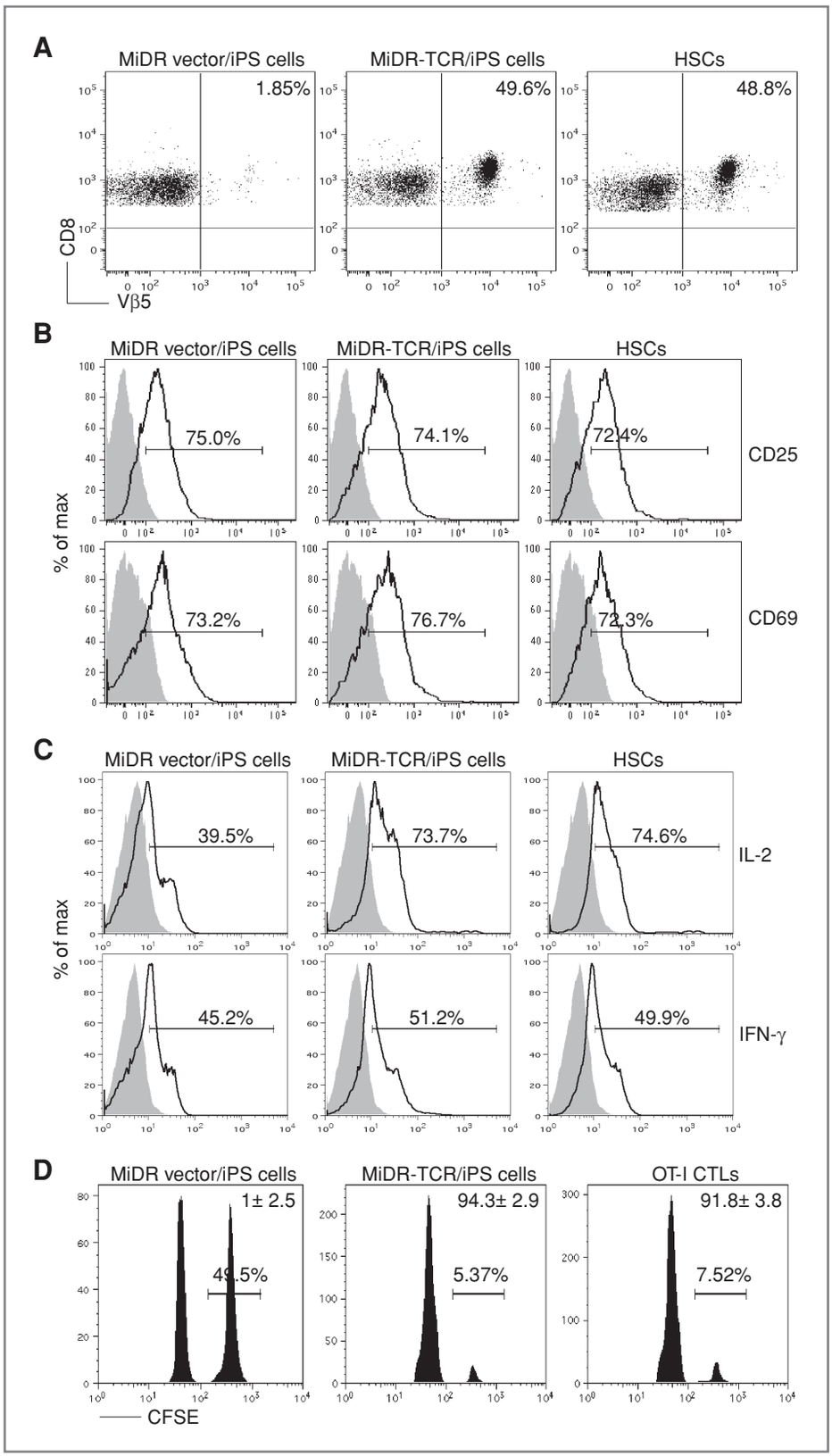

Figure 2. Ag-specific $CD8^+$ T-cell development from iPS cells *in vivo*. GFP^+ $DsRed^+$ iPS cells were injected i.v. into C57BL/6 mice. After 6 to 10 weeks, OVA-specific $V\beta5^+$ $CD8^+$ T-cell development was determined. A, $CD8^+$ $V\beta5^+$ T cells from pooled lymph nodes and spleen were analyzed by flow cytometry. B, CD25 and CD69 expression was analyzed by flow cytometry, after gating on $CD8^+$ $V\beta5^+$ T cells (dark lines; shaded areas indicate isotype controls). C, IL-2 and IFN-γ production from the $CD8^+V\beta5^+$ population (dark lines; shaded areas indicate isotype controls) was 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 10 weeks after iPS cell transfer or 1 day after OT-I CTL transfer. Data are representative of 2 or 3 independent experiments.

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block nonspecific protein binding. Sections were stained with PE anti-mouse TCR Vα2 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 $CD8^+$ $V\beta5^+$ cells in mice receiving TCR gene–transduced iPS cells or HSCs. In contrast, the $CD8^+$ $V\beta5^+$ 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 $CD8^+$ $V\beta5^+$ 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).

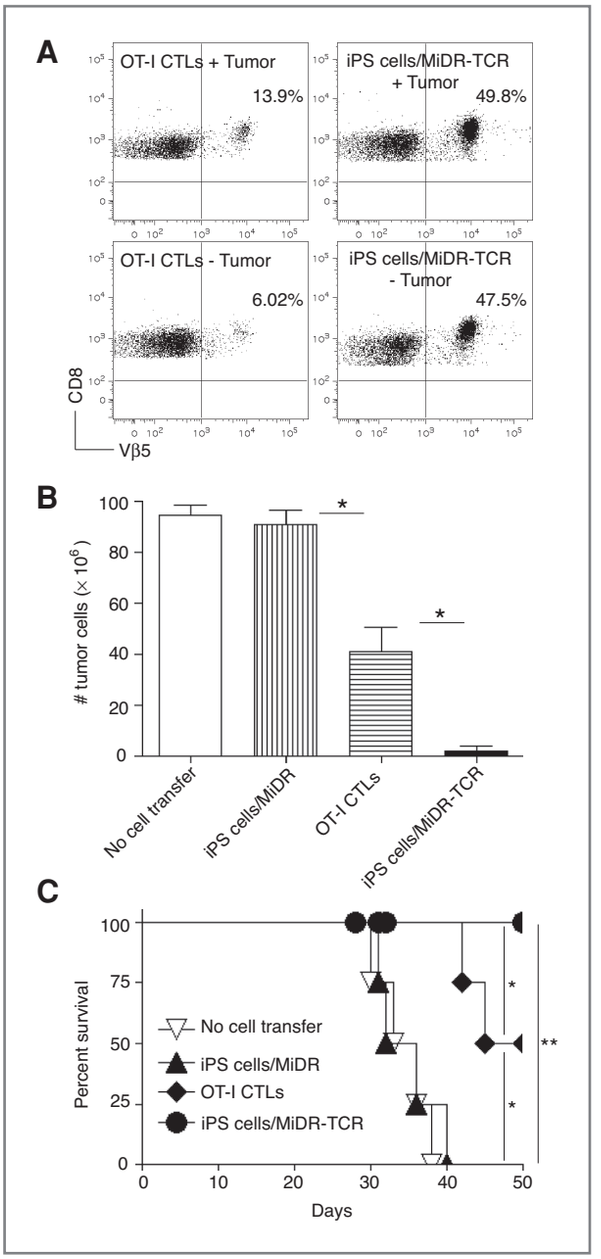

Figure 3. Adoptive transfer of TCR-transduced iPS cells suppresses tumor growth and sustains mouse survival. GFP^+ $DsRed^+$ cells were adoptively transferred into C57BL/6 mice. One group of mice was injected with OVA-reactive $CD8^+$ T cells from OT-I TCR transgenic mice, and 1 group of mice had no cell transfer. After either 6 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 13 weeks without tumor challenge, $CD8^+$ $V\beta5^+$ T cells from the pooled lymph nodes 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 6 individual mice. One-way ANOVA test was used for statistical analyses between 2 groups (*, $P < 0.05$). C, mouse survival on day 50. Kaplan–Meier survival curves are shown ($n = 6$). *, $P < 0.05$; **, $P < 0.001$, 1-way ANOVA with Newman–Keuls multiple comparison test. Data are representative of 3 independent experiments.

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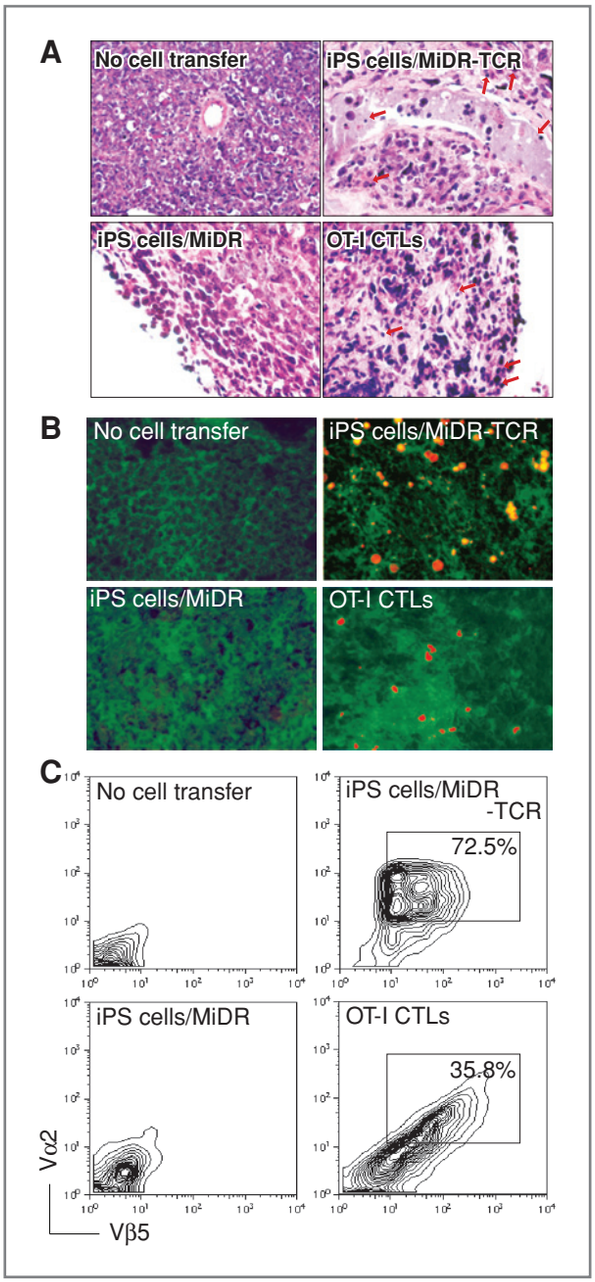

Figure 4. iPS cell-derived Ag–specific CTLs infiltrate into tumor tissues. On day 30 to 35 after tumor challenge, tumor tissues were examined for tumor-reactive T-cell infiltration. A, H&E staining. Inflammatory cells infiltrated in tumor tissues (↓). B, immunohistologic staining. OVA-specific $V\alpha2^+$ CTLs (red) infiltrated in OVA-expressing tumor tissues (green). C, single-cell suspensions from tumor tissues were analyzed for expression of $V\alpha2^+$ and $V\beta5^+$ by flow cytometry, after gating on the $CD8^+$ population. Data are representative of 3 independent experiments.

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 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, γ_c 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 $CD3^+V\beta5^+$ population, which is not sufficient to generate efficient antitumor immunity. From weeks 6 to 10 after cell transfer, approximately 24% of the $CD3^+V\beta5^+$ population are in lymph nodes and spleen, and more than 80% of these cells are $CD8^+$ $CD4^-$ (Supplementary Fig. S1). Second, we noted 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 that immunosuppressive cell subsets such as $CD4^+$ $CD25^+$ $Foxp3^+$ cells develop from genetically engineered iPS cells (Supplementary Fig. S3).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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