Human Cancer Microarray Data Analysis

For human clinical samples, *IL-17D* gene expression was evaluated from NCBI GEO data sets from studies comparing primary and metastatic tumors (GDS2546) or low-grade versus high-grade glioma patient samples (GDS4467, GDS1976, and GDS1816) as described previously (Pachynski et al., 2012).

Generation of IL-17D-Deficient and Overexpressing Tumor Cell Lines

Cell lines were generated as described in the Supplemental Experimental Procedures.

Antibodies and FACS Analysis of Tumor Cells

For intracellular staining, cells were either incubated with or without 2 μM monensin (Sigma) and 1 μg/ml Brefeldin A (BD Biosciences) and then harvested by trypsinization, washed once with PBS, stained, and analyzed for intracellular IL-17D signal as described in the Supplemental Experimental Procedures.

Tumor Transplantation and TIL Analysis

Subconfluent tumor cell lines were harvested and injected subcutaneously into syngeneic recipient WT, RAG2$^{-/-}$, or RAG2$^{-/-}$ × γc$^{-/-}$ mice at either 1 × 10^6 cells/mouse (for all growth experiments) or 5–10 × 10^6 cells/mouse (for tumor-infiltrating leukocyte [TIL] analysis), as previously described (Bui et al., 2006). Tumor rechallenge was performed 3 months after mice had rejected transplanted tumors by injecting 1 × 10^6 cells per mouse subcutaneously with parental tumor cell lines. In vivo depletion of various immune subsets, doxycycline administration, and intratumoral injection of IL-17D are described in the Supplemental Experimental Procedures. Tumor growth and immune infiltration were analyzed as described in the Supplemental Experimental Procedures.

Mouse Air Pouch Experiments

C57BL/6 × 129/Sv F1 mice were injected subcutaneously with 3 ml of sterilized air filtered through a 0.2 μm Millipore filter (Bellerica) to form air pouches on day 0 and reinflated again on day 3. On day 7, 1 ml of LPS (1 μg/ml), IL-17A (5 μg/ml) (R&D Systems), IL-17D (5 μg/ml) (R&D Systems), IL-17D (5 μg/ml) (Mayfield Lab), MCP-1 (5 μg/ml) (Peprotech), or IL-17D (5 μg/ml) + anti-MCP-1 polyclonal antibodies (25 μg/ml) (R&D Systems) was injected into mouse air pouches 8 hr before air pouch harvest. Air pouches were lavaged with 2 ml PBS and centrifuged at 1,250 rpm for 5 min at room temperature. Supernatant was harvested and analyzed for chemokine protein levels using the mouse Chemokine FlowCytomix kit from eBioscience. Infiltrating air pouch cells were resuspended in FACS stain buffer, counted on a hemocytometer, and analyzed by cell-surface markers as described in the Supplemental Experimental Procedures.

Chemokine Secretion Assay

On days 7 and 14 posttransplantation, tumors were harvested and single-cell suspensions were prepared as described for the TIL analysis. Filtered tumor/immune cell suspensions were plated in triplicate wells at 40,000 cells per well in 100 μl for 24 hr at 37°C. Supernatant was analyzed for chemokines using the mouse chemokine flowcytomix kit from eBioscience.

Generation of cDNA and Quantitative PCR

Tumor cell lines were plated in triplicate at 6 × 10^4 cells/well in a six-well plate and incubated for 48 hr at 37°C. Supernatant was aspirated and cells were washed twice with PBS before addition of 1 ml TRIzol reagent (Invitrogen). CD31$^+$ and CD45$^+$ tumor-derived cell populations were washed twice with PBS before addition of 1 ml TRIzol reagent (Invitrogen). Details describing RNA extraction, cDNA preparation, quantitative PCR reactions, and analysis are described in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.03.073.

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LETTER TO THE EDITOR

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Adoptive transfer of genetically engineered WT1-specific cytotoxic T lymphocytes does not induce renal injury

Hiroaki Asai[1], Hiroshi Fujiwara[1*], Sohei Kitazawa[2], Naoto Kobayashi[3], Toshiki Ochi[1], Yukihiro Miyazaki[1], Fumihiro Ochi[1], Yoshiki Akatsuka[4], Sachiko Okamoto[5], Junichi Mineno[5], Kiyotaka Kuzushima[6], Hiroaki Ikeda[7], Hiroshi Shiku[7] and Masaki Yasukawa[1*]

Abstract

Because WT1 is expressed in leukemia cells, the development of cancer immunotherapy targeting WT1 has been an attractive translational research topic. However, concern of this therapy still remains, since WT1 is abundantly expressed in renal glomerular podocytes. In the present study, we clearly showed that WT1-specific cytotoxic T lymphocytes (CTLs) certainly exerted cytotoxicity against podocytes *in vitro*; however, they did not damage podocytes *in vivo*. This might be due to the anatomical localization of podocytes, being structurally separated from circulating CTLs in glomerular capillaries by an exceptionally thick basement membrane.

Keywords: Immunotherapy, WT1, Podocytes, Cytotoxic T lymphocytes

Findings

Because WT1 is expressed in leukemia cells, including leukemia stem cells, the development of cell-mediated immunotherapy targeting WT1 has been an attractive translational research topic [1,2]. However, concern still remains about adverse events resulting from damage to normal tissues mediated by cytotoxic T lymphocytes (CTLs), since WT1 is also expressed in some lineages of normal cell as well as leukemia cells.

It is well known that WT1 is abundantly expressed in renal glomerular podocytes (or visceral epithelial cells) and that dysfunction of podocytes results in severe renal failure [3]. In addition, it has been recently reported that podocytes have functions of professional antigen-presenting cells [4]. Therefore, it seems important to clarify whether WT1-specific CTLs do not exert cytotoxicity against podocytes. In the present series of experiments, we examined in detail the cytotoxic effect of WT1-specific CTLs against podocytes using *in vitro* and *in vivo* systems.

Methods

WT1-specific and HLA-A*24:02-restricted CTLs were generated by *T-cell receptor* (*TCR*) gene transfer using the novel retrovirus vector [5] into peripheral blood CD8^{+} T cells, as described previously [6]. We used a mouse podocyte cell line, MPC-5 [7], as the target cells, since there is a high homology between the human and mouse WT1 amino acid sequences, and $WT1_{235-243}$ (CYTWNQMNL), which is the epitope of our WT1-specific CTLs, is completely conserved between the two species. The MPC-5 cells were transfected with the *HLA-A*24:02* gene, as described previously with a slight modification [8]. As shown in Figure 1A, *HLA-A*24:02* gene-transduced mouse podocytes expressed HLA-A24:02 molecules on their surface. We named this cell line MPC-5-A24.

*HLA-A*24:02*-transgenic mice were produced as reported previously [9]. All *in vivo* experiments were approved by the Ehime University animal care committee. As shown in Figure 1B, HLA-A24:02 molecules were expressed in the tissues of these transgenic mice, including glomeruli. HLA-A*24:02-transgenic mice were subsequently injected intravenously with 2.5×10^6 WT1-specific and HLA-A*24:02-restricted CTLs or non-gene-modified CD8^{+} T cells (control CTLs). As we reported previously

* Correspondence: yunarief@m.ehime-u.ac.jp; yasukawa@m.ehime-u.ac.jp
[1]Department of Hematology, Clinical Immunology, and Infectious Diseases, Ehime University Graduate School of Medicine, Toon, Ehime, Japan
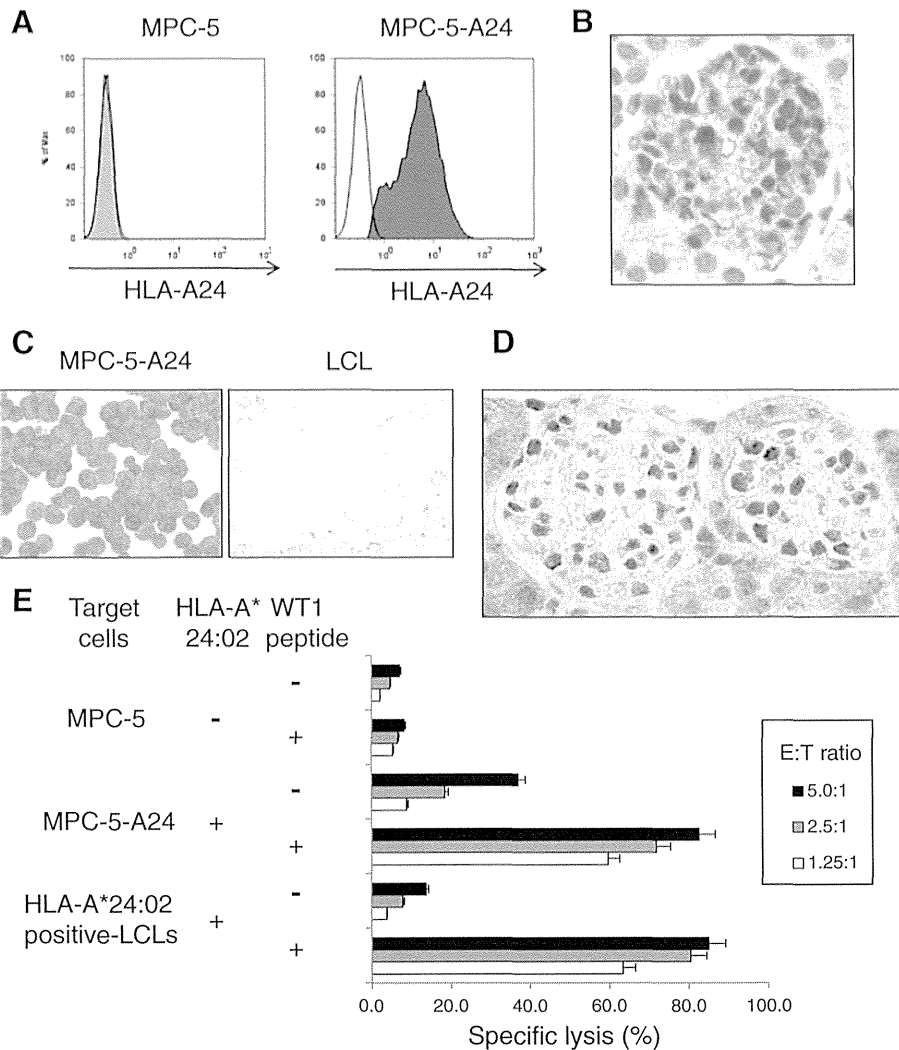Full list of author information is available at the end of the article

Figure 1 Cytotoxicity of WT1-specific and HLA-A*24:02-restricted CTLs against podocytes. (A) Expression of HLA-A24:02 on the *HLA-A*24:02* gene-transduced mouse podocyte cell line, MPC-5 (MPC-5-A24). Flow cytometric analysis was performed using anti-HLA-A24:02 monoclonal antibody (One Lambda, Canoga Park, CA, USA). **(B)** Expression of HLA class I in the glomerulus of a *HLA-A*24:02*-transgenic mouse (original magnification ×400). Immunohistochemistry was performed using an anti-HLA class I framework monoclonal antibody (Abcam, Cambridge, UK). **(C)** Expression of WT1 in the *HLA-A*24:02* gene-transduced mouse podocyte cell line, MPC-5-A24. MPC-5-A24 cells and LCL were stained with a rabbit anti-human and mouse WT1 polyclonal antibody (Santa Cruz Biotechnology, Dallas, TX, USA) (original magnification ×400). Notably, WT1 is abundantly expressed in MPC-5-A24 cells but not LCL. **(D)** Expression of WT1 in the glomerulus of a HLA-A*24:02-transgenic mouse (original magnification ×400). Immunohistochemistry was performed using a rabbit anti-human and mouse WT1 polyclonal antibody (Santa Cruz Biotechnology). **(E)** Cytotoxicity of WT1-specific and HLA-A*24:02-restricted CTLs against MPC-5, MPC-5-A24, and HLA-A*24:02-positive LCLs in the presence or absence of WT1 peptide at various effector:target cell ratios.

[6,10], the dose of *TCR* gene-engineered T cells used in the present study is enough to show anti-leukemia effect *in vivo*. Mice that had received WT1-specific CTLs and control CTLs were sacrificed after 7 days, and the presence of tissue damage was examined morphologically. Trafficking of WT1-specific CTLs in *HLA-A*24:02*-transgenic mice was examined using *luciferase* gene-transfected CTLs in a bioluminescence imaging assay as reported previously [10]. Serial acquisition of luciferase photon counts using luciferin was carried out on days 1, 3, and 6 using

AEQUORIA (Hamamatsu Photonics, Hamamatsu, Japan), and analyzed using AQUACOSMOS software (Hamamatsu Photonics).

Results

As shown in Figure 1C, WT1 appeared to be abundantly expressed in the *HLA-A*24:02* gene-transduced mouse podocyte cell line, MPC-5-A24. We also confirmed that WT1 was abundantly expressed in podocytes of *HLA-A*24:02*-transgenic mice. (Figure 1D). Figure 1E shows

the cytotoxicity of WT1-specific and HLA-A*24:02-restricted CTLs against various target cells. WT1-specific CTLs showed strong cytotoxicity against WT1$_{235-243}$ peptide-loaded but not -unloaded HLA-A*24:02-positive LCLs. Notably, WT1-specific CTLs apparently exerted cytotoxicity against MPC-5-A24, and their cytotoxicity against WT1$_{235-243}$ peptide-loaded MPC-5-A24 appeared to be higher than that against WT1 peptide-unloaded MPC-5-A24. In contrast, WT1-specific CTLs did not show cytotoxicity against WT1 peptide-loaded or -unloaded MPC-5. These results showed that WT1-specific CTLs can lyse podocytes in an HLA-restricted manner through recognition of the WT1 epitope that is naturally processed from WT1 protein in podocytes and presented on the cell surface in the context of HLA class I molecules.

We monitored in detail the renal function of *HLA-A*24:02*-transgenic mice following transfer of WT1-specific CTLs. Body weight loss and severe proteinuria were not observed in mice that had received WT1-specific CTLs (data not shown). As shown in Figure 2A,

lymphocyte infiltration or glomerular injury was not detectable morphologically in WT1-specific CTL-transferred mice. Also, damage of other organs, including pleura, was not detectable (data not shown). Finally, we examined the kinetic distribution of WT1-specific CTLs in HLA-A*24:02-transgenic mice. As shown in Figure 2B, WT1-specific and HLA-A*24:02-restricted CTLs did not accumulate in kidneys.

Discussion

The present *in vitro* and *in vivo* studies clearly showed that WT1-specific CTLs indeed exerted cytotoxicity against renal glomerular podocytes in an HLA-restricted manner; *in vivo*, however, podocytes were able to escape from the cytotoxicity of WT1-specific CTLs. This might be due to the anatomical localization of podocytes, being located outside the capillaries of the glomerulus. Because podocytes are completely separated from capillaries in which CTLs are circulating by a thick glomerular basement membrane which can inhibit the pass of blood
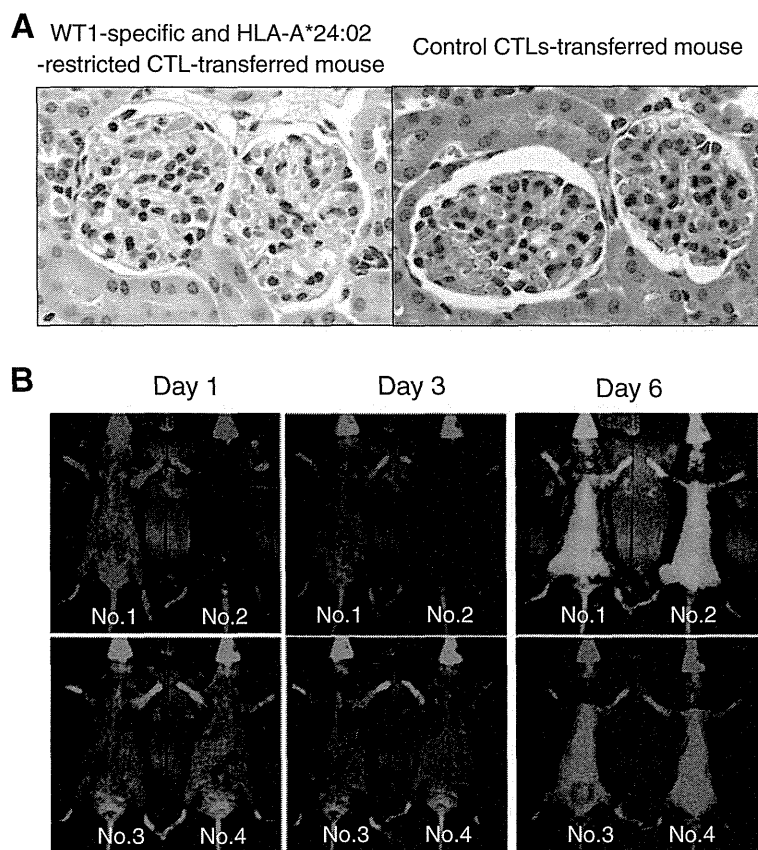

Figure 2 Monitoring of renal damage in *HLA-A*24:02*-transgenic mice following transfer of WT1-specific and HLA-A*24:02-restricted CTLs. (A) Histopathology of the glomeruli of *HLA-A*24:02*-transgenic mice that had received WT1-specific and HLA-A*24-restricted CTLs and control CTLs. (hematoxylin-eosin stain; original magnification x400). Notably, lymphocyte infiltration and tissue damage are not detectable in the glomerulus of the WT1-specific CTL-transferred mouse. **(B)** Trafficking of WT1-specific and HLA-A*24:02-restricted CTLs in *HLA-A*24:02*-transgenic mice. Four mice were transferred with WT1-specific and HLA-A*24:02-restricted CTLs. Notably, CTLs have not accumulated in specific organs, including the kidneys.

cells and even serum protein, CTLs cannot come into contact with podocytes under normal condition. However, in the patients with glomerulonephritis, the permeability of the glomerular basement membrane increases, resulting in proteinuria; therefore, CTLs may infiltrate through the basement membrane and damage podocytes. Therefore, in conclusion, adoptive transfer of WT1-specific CTLs in patients without renal failure is likely safe; however, it should be performed cautiously in patients with proteinuria.

Abbreviations
CTLs: Cytotoxic T lymphocytes; TCR: T-cell receptor.

Competing interests
The authors declare no competing interest.

Authors' contributions
HA, SK, TO, YM, and FO performed experiments and analyzed data. HF designed research and performed experiments. NK, SO, JM, KK, HI, and HS provided materials and discussed the experimental results. YA provided materials and performed experiments. MY designed research, wrote the manuscript, and provided financial support. All authors read and approved the final manuscript.

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Author details
[1]Department of Hematology, Clinical Immunology, and Infectious Diseases, Ehime University Graduate School of Medicine, Toon, Ehime, Japan. [2]Department of Molecular Pathology, Ehime University Graduate School of Medicine, Toon, Ehime, Japan. [3]Medical Education Center, Ehime University Graduate School of Medicine, Toon, Ehime, Japan. [4]Department of Hematology and Oncology, Fujita Health University, Toyoake, Aichi, Japan. [5]Center for Cell and Gene Therapy, Takara Bio Inc., Otsu, Shiga, Japan. [6]Division of Immunology, Aichi Cancer Center Research Institute, Nagoya, Aichi, Japan. [7]Department of Immuno-Gene Therapy, Mie University Graduate School of Medicine, Tsu, Mie, Japan.

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