ABSTRACT

CD4+ Th cells, in particular IFN-γ-producing Th1 cells, play a critical role in the activation and maintenance of Tc1 cells that are essential for tumor eradication. Here, we report the generation of artificial tumor-specific Th1 and Tc1 cells from nonspecifically activated T cells using a lentiviral transduction system. Anti-CD3-activated T cells from healthy human donors were transduced with a lentivirus containing a chimeric immunoglobulin T-cell receptor gene composed of single-chain variable fragments derived from an ant carcinoembryonic antigen (CEA)-specific monoclonal antibody fused to an intracellular signaling domain derived from the cytoplasmic portions of membrane-bound CD28 and CD3ζ. These artificial tumor-specific Tc1 and Th1 cells, termed Tc1- and Th1-T bodies, respectively, could be targeted to CEA+ tumor cells independently of MHC restriction. Specifically, Tc1- and Th1-T bodies demonstrated high cytotoxicity and produced IFN-γ in response to CEA+ tumor cell lines but not CEA− tumors. Although Th1-T bodies exhibited low cytotoxicity, they secreted high levels of IFN-γ and interleukin-2 in response to CEA+ tumor cells. Such CEA+ tumor-specific activation was not observed in mock gene-transduced nonspecific Tc1 and Th1 cells. Moreover, Tc1- and Th1-T bodies exhibited strong antitumor activities against CEA+ human lung cancer cells implanted into RAG2−/− mice. Furthermore, combined therapy with Tc1- and Th1-T bodies resulted in enhanced antitumor activities in vivo. Taken together, our findings demonstrate that Tc1- and Th1-T bodies represent a promising alternative to current methods for the development of effective adoptive immunotherapies.

INTRODUCTION

It has been well accepted that T cells, most notably CD4+ Th and CD8+ Tc cells, play an important role in cancer immunotherapy (1). T cells with antitumor activities recognize peptides derived from tumor rejection antigens (TRAs) bound by MHC molecules. Because genes encoding TRA were isolated, many investigators have tried to identify MHC class I- or class II-binding TRA peptides that can be used to develop tumor vaccines for treatment of cancer patients (2–5). However, although clinical studies have indicated that TRA peptides can elicit antigen-specific, tetramer-positive CTLs in vivo, the increased frequency of CTLs did not lead to tumor rejection in these patients (6). The low effectiveness of antitumor CTLs may be caused by a number of factors: (a) CTLs may be ineffective in reacting with tumor cells because such cells lack or down-regulate MHC and/or costimulatory molecules that are essential for inducing the full activation of T cells (7, 8); and (b) CTLs may fail to be fully activated in tumor-bearing hosts because such individuals often exhibit significant immunosuppression and defective helper T-cell function (9, 10).

To overcome these problems, we have developed an efficient method to prepare tumor-specific artificial Tc1 and Th1 cells from nonspecifically activated human CD8+ and CD4+ T cells using a lentiviral transduction system. We infected anti-CD3-stimulated CD4+ and CD8+ T cells isolated from human peripheral blood mononuclear cells (PBMCs) with a lentivirus containing a chimeric immunoglobulin T-cell receptor (cIgTCR) composed of single-chain variable fragments (scFv) derived from an anticarcinoembryonic antigen (CEA) monoclonal antibody (mAb) and an intracellular signaling domain derived from the cytoplasmic part of membrane-bound CD28 and CD3ζ. Such artificially generated antigen-specific T cells, which we refer to as T bodies, thereby possess the advantage of binding with Ag in an MHC-independent manner, while maintaining the capacity to efficiently activate the effector functions of T cells. Since T body technology was reported (11, 12), most investigators have focused on the cytotoxic function and targeting of these cells. The functional consequences of preparing T bodies with distinct effector functions but with the identical Ag specificity have not been evaluated. It is now well accepted that Th1-dominant immunity is important for successful induction of antitumor immunity in tumor-bearing hosts (13, 14). Moreover, the Th1/Tc1 circuit in tumor-bearing hosts is crucial for complete tumor eradication (15, 16). Therefore, we reasoned that an efficient method to prepare tumor-specific Th1- and Tc1-T bodies with the same antigenic specificity will be helpful for the development of novel adoptive tumor immunotherapies.

In the present study, we document the generation and targeting of human tumor-specific artificial Tc1- and Th1-T bodies. Tc1-T bodies derived from anti-CD3 mAb-activated CD8+ T cells specifically lysed tumor cells and produced IFN-γ. Th1-T bodies produced high levels of interleukin (IL)-2 and IFN-γ in response to CEA+ tumor cells but showed limited cytotoxicity. Moreover, we demonstrate that Tc1- and/or Th1-T bodies exhibit strong antitumor activities against CEA+ human lung cancer cells implanted into recombination activating gene (RAG)2−/− mice. Thus, our novel and effective protocol for the preparation of Tc1- and Th1-T bodies may provide a new strategy for adoptive tumor immunotherapy.

MATERIALS AND METHODS

Cell Culture. The human tumor cell lines HLC-1 (lung cancer), Daudi (lymphoma), KATO-III (gastric cancer), SH10 (gastric cancer), and AZ521 (gastric cancer) were cultured in RPMI 1640 (Sigma, St. Louis, MO) containing 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.) and supplemented with 2 mM l-glutamine, 0.05 mM 2-mercaptoethanol (Sigma), HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate (RPMI-10S). 293T cells were maintained in DMEM (Sigma) containing 10% fetal bovine serum and supplemented with 2 mM l-glutamine, HEPES, penicillin, and streptomycin (DMEM-10S).

Animals. BALB/c background RAG-2−/− mice were donated by Dr. M. Ito (Central Institute for Experimental Animals, Kanagawa, Japan). All of the mice were male and used at 10–12 weeks of age.
clgTCR Gene and Lentiviral Vector. The cDNA F39scFv/CIR-2, containing the scFv derived from F11–39 mAb specific for CEA, the CD8α hinge lesion, the CD28 transmembrane and cytoplasmic domain, and the CD3ζ cytoplasmic domain, has been described (Refs. 17 and 18; Fig. 1). The third generation lentivirus vector system used in the experiments was developed by one of us (H. M). Plasmid vector CSII-EF-MCS-IRES-hrGFP (CSII-GFP) contains a multiple cloning site (MCS) and the gene encoding green fluorescent protein (GFP; Ref. 19). F39scFv/CIR-2 cDNA was inserted into the EcoRI and NotI restriction enzyme sites within the multiple cloning site of the CSII-GFP vector (CSII-CIR-GFP). As a control vector, CSII-GFP was used.

Preparation of Lentiviral Vectors. 293T cells (5 × 10^6) were plated on 24 10-cm dishes precoated with 0.002% poly-l-lysine (Sigma). Later (20–24 h), CS II, pMD.G, pMDL.gpRRE, and pRSV-Rev were cotransfected by calcium phosphate transfection (20). After 12–16 h, medium was aspirated and 7.5 ml DMEM-10% containing 10 μM forskolin (Sigma F3917) were added to each dish. Later (48 h), virus vector-containing supernatant was collected and passed through a 0.45-μm filter. Then, supernatant was concentrated by ultracentrifugation at 68,000 × g and stored at –80°C until use.

Generation of T Bodies. PBMCs (2 × 10^6) were isolated from healthy donors by density gradient centrifugation using Ficoll-Paque PLUS (Amersham Bioscience, Sweden). Purified cells were then cultured with immobilized anti-CD3 mAb (5 μg/ml; PharMingen, San Diego, CA) and Retronectin (25 μg/ml; Takara Biomedicals, Shiga, Japan) for 2 h at 37°C. After incubation, PBMCs were infected twice with lentivirus vectors at a 24-h interval by adding viral supernatants. The infected cells were expanded by culture with RPMI-10% medium containing IL-2 (100 units/ml), IL-12 (50 units/ml), IFN-γ (20 ng/ml), and anti-IL-4 mAb (2 μg/ml; PharMingen) for the generation of Th1- or Tc1-T bodies. Lentivirus supernatants were added at multiplicities of infection of 100–150.

Purification of T Bodies. To determine the transduction efficacy, T cells expressing clgTCR were detected by GFP fluorescence using flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA). The GFP gene is expressed under the control of the internal ribosomal entry site sequence, which is inserted downstream of the clgTCR gene in the lentiviral vectors (Fig. 1). CD4+ or CD8+ cells were first separated by using immunomagnetic bead systems (MACS; Miltenyi Biotec, Glodbach, Germany) according to the manufacturer’s instructions. CD4+ GFP+ or CD8+ GFP+ cells were then separated by using a FACSVantage instrument (BD Biosciences). Isolated T bodies used in the following experiments were >95% pure.

Detection of Cytokine Activity. T bodies (5 × 10^6) and tumor cell lines (5 × 10^6) were cocultured in 96-well round-bottomed plates in 200 μl of RPMI-10% medium. After 24-h incubation, supernatants were collected, and IFN-γ and IL-2 levels were determined by ELISA using the OptEIA Human IFN-γ set (PharMingen) and Quantikine Human IL-2 Immunoassay kit (R&D Systems, Minneapolis, MN).

Determination of Cytotoxicity. The cytotoxicity mediated by T bodies was measured by 4-h 31Cr-release assays as described previously (21). Tumor-specific cytotoxicity was determined using human CEA+ cells lines HLC-1 and KATO-III as target cells. As a control, CEA+ human cell lines AZ521, SH10, and Daudi were used. The percentage of cytotoxicity was calculated as described previously (21).

Winn’s Assay. A mixture of HLC-1 with or without T bodies was injected intradermally into the abdominal skin of Rag-2–/– BALB/c mice. The tumor growth inhibitory effect of T bodies was determined by measuring change over time in the means of two perpendicular diameters of the tumor mass, as described previously (16).

RESULTS

Preparation of T Bodies. We first examined the efficacy of lentiviral transduction. PBMCs obtained from healthy donors were stimulated with immobilized anti-CD3 mAb and then infected with lentiviral vectors containing chimeric immunglobulin T-cell receptor-GFP or mock-GFP gene. Anti-CD3 monoclonal antibody-activated peripheral blood mononuclear cells were transduced with lentiviral vectors and expanded for 7–10 days under Th1-polarizing conditions as described in “Materials and Methods.” Cells were stained with phycoerythrin-conjugated anti-CD4 or -CD8 monoclonal antibody, and GFP expression on Th1 and Tc1 cells was investigated by flow cytometry (A–D). GFP expression on mock-GFP gene-transduced Th1 (A and E) and Tc1 (C and G) cells and chimeric immunoglobulin T-cell receptor-GFP gene-transduced Th1 (B and F) and Tc1 (D and H) is shown. The typical data in 16 different experiments were shown.

Fig. 2. Green fluorescent protein (GFP) expression of Th1 and Tc1 cells transduced with a lentivirus containing chimeric immunoglobulin T-cell receptor-GFP or mock-GFP gene. Anti-CD3 monoclonal antibody-activated peripheral blood mononuclear cells were transduced with lentiviral vectors and expanded for 7–10 days under Th1-polarizing conditions as described in “Materials and Methods.” Cells were stained with phycoerythrin-conjugated anti-CD4 or -CD8 monoclonal antibody, and GFP expression on Th1 and Tc1 cells was investigated by flow cytometry (A–D). GFP expression on mock-GFP gene-transduced Th1 (A and E) and Tc1 (C and G) cells and chimeric immunoglobulin T-cell receptor-GFP gene-transduced Th1 (B and F) and Tc1 (D and H) is shown. The typical data in 16 different experiments were shown.
were easily expanded by stimulation with immobilized anti-CD3 mAb.

**Cytokine Production by Th1- and Tc1-T Bodies in Response to CEA-Positive Human Tumor Cell Lines.** The capacity of T bodies to produce cytokines in response to CEA-expressing tumor cells was examined by measuring cytokine levels in the culture supernatant. T bodies or mock gene-transduced T cells were cocultured with CEA⁺ or CEA⁻ tumor cells for 24 h. Culture of CD4⁺ or CD8⁺ T-bodies by themselves caused no significant release of IFN-γ (data not shown). In contrast, CD4⁺ or CD8⁺ T-bodies produced high levels of IFN-γ when they were cocultured with CEA⁺ tumor cells (Fig. 3). These CD4⁺ or CD8⁺ T-bodies did not produce IL-4, IL-5, or IL-13 in response to stimulation with CEA⁻ tumor cells (data not shown), indicating that Th1- and Tc1-T bodies were successfully induced from nonspecifically activated T cells in Th1-polarizing conditions. Th1- and Tc1-T bodies did not produce IFN-γ in response to CEA⁻ tumor cells, indicating that IFN-γ production from T bodies was induced in an antigen-specific manner (Fig. 3). No significant IFN-γ production was detected in supernatants of control T cells cocultured with CEA⁺ or CEA⁻ tumor cells. We also evaluated the amount of IL-2 secretion from Th1- or Tc1-T bodies stimulated with tumor cells. As shown in Fig. 4, Th1-T bodies produced high amounts of IL-2 in response to CEA⁺ HLC-1 cells but not CEA⁻ Daudi cells, whereas Tc1-T bodies produced no significant IL-2. It was also demonstrated that Th1-T bodies produced high levels of cytokines independently on Class II and B7-signaling molecules, because HCL-1 cells expressed neither class II, B7-1, nor B7-2 on their cell surface (Fig. 3). Anti-CEA Antibody Blocks Cytokine Production by T Bodies Stimulated with CEA-Expressing Tumor Cells. To evaluate whether T bodies specifically recognize CEA antigen expressed on tumor cells, we examined the capacity of anti-CEA mAb to block cytokine production by Th1- or Tc1-T bodies cocultured with CEA⁺ tumor cells. As clearly shown in Fig. 5, IFN-γ production of T bodies was almost completely blocked by adding anti-CEA mAb, whereas the addition of control mAb caused no significant changes in IFN-γ production.

**Cytotoxicity Mediated by T Bodies against CEA⁺ Tumor Cells.** The cytotoxicity mediated by Th1- or Tc1-T bodies was examined by 4-h-³¹Cr-release assays. As shown in Fig. 6, Tc1-T bodies exhibited
a strong cytotoxicity against CEA\(^+\) tumor cells but not against CEA\(^-\) tumor cells. In contrast, Th1-T bodies showed marginal cytotoxicity against CEA\(^+\) tumor cells. In some experiments, Th1-T bodies exhibited low but significant cytotoxicity, but their cytotoxicity was always lower than that of Tc1-T bodies. Control Th1 or Tc1 cells lysed neither CEA\(^+\) nor CEA\(^-\) tumor cells. We further demonstrated that the cytotoxicity as well as cytokine production capacity of Tc1- and Th1-T bodies was strongly blocked by anti-CEA mAb (Fig. 7).

Thus, these findings clearly demonstrate that T bodies artificially generated from nonspecifically activated T cells exhibit cytokine production and cytotoxicity in an antigen-specific manner.

**Antitumor Activities of T Bodies in Vivo**

We next evaluated the antitumor activity of T bodies against human CEA\(^+\) tumor cells in vivo. Th1-T bodies, Tc1-T bodies, or both were mixed with CEA\(^-\) tumors and intradermally injected into RAG2\(^{-/-}\) mice (Fig. 8). Th1-T bodies alone were unable to completely eradicate tumors, despite the fact that these cells strongly inhibited tumor growth in vivo. Tc1-T bodies by themselves initially reduced tumors to a size that was not palpable or visible, but tumors recurred. However, when both Th1- and Tc1-T bodies were injected together with CEA\(^+\) tumor cells, RAG2\(^{-/-}\) mice completely rejected the tumors. Such antitumor activity was not induced when control T cells were mixed with tumors and transferred into RAG2\(^{-/-}\) mice. Neither Th1- nor Tc1-T bodies, either individually or in combination, exhibited antitumor activity in vivo against CEA\(^-\) tumor cells implanted into RAG2\(^{-/-}\) mice (data not shown). Thus, these results demonstrate that artificial human Tc1-T bodies have profound antitumor activity in vivo if they were combined with Th1-T bodies.

**DISCUSSION**

We have established an efficient method for the preparation of Th1- and Tc1-T bodies by lentiviral transduction of a clgTCR containing the scFv derived from an anti-CEA mAb fused with the intracellular signaling domains of membrane-bound CD28 and CD3\(\gamma\) molecules. These artificial tumor-specific Tc1- and Th1-T bodies, as compared with physiologically induced TRA-specific Tc1 and Th1 cells, have several advantages for tumor immunotherapy.

First, T bodies can be easily induced from anti-CD3 mAb-activated PBMCs (Fig. 2), and this technology is applicable to all tumor patients. Since the discovery of TRA peptides, it has become possible to expand tumor-specific CTLs in vivo using TRA-bound DC cells (2, 3). However, it still takes a long time to expand enough numbers of tumor-specific CTLs from PBMCs or tumor-infiltrating lymphocytes isolated from patients. Although some MHC class II-binding tumor rejection peptides were discovered, expansion of tumor-specific Th1 cells in vivo has proven very challenging (4). These difficulties in inducing and expanding tumor-specific Th1 and Tc1 cells have hampered their application to adoptive tumor immunotherapy. Our established method provides a rapid, easy, and general protocol for inducing and expanding tumor-specific IFN-\(\gamma\)-producing Tc1 and Th1 cells. This methodology should prove useful for adoptive immunotherapy of patients with tumors or infectious diseases.

Secondly, T bodies can recognize tumor-specific antigens via the Ag-binding domain of an scFv in an MHC-nonrestricted fashion. Therefore, T bodies can exhibit their functions in response to tumor cells even when tumor cells express low levels of MHC. It has been reported that MHC expression is down-modulated in tumor-bearing hosts (7, 22). Such down-modulation of MHC may result in the
escape of tumor cells from immunosurveillance mechanisms mediated by T-cell immunity. In addition to MHC molecules, the expression of costimulatory molecules, such as B7 and CD40, play a crucial role for long-lasting and effective T-cell activation (23–26). It has been demonstrated that FcRγ- and CD3ζ-mediated signaling is sufficient to induce cytotoxicity against tumor cells. However, cytokine production by T bodies equipped with T-cell receptor-mediated signaling was greatly enhanced by CD28-mediated signaling (27). We therefore prepared T bodies using clgTCR gene containing signal transduction units of CD28 and CD3ζ molecules, which are essential for induction of cytokine production. Consistent with previous results (28, 29), T bodies transduced with clgTCR gene exhibit a strong tumor-specific response to CEA+ tumor cells and show high levels of cytokine production and cytotoxicity, although HCL-1 cells expressed neither B7–1 nor B7–2. Moreover, HCL-1 tumor cells expressed class I but not class II molecules on their cell surface. However, Th1-T bodies produced high levels of IFN-γ in response to CEA+ HLC tumor cells, indicating that T bodies recognize tumor antigen in MHC-independent manner, and transduction units of CD28 in addition to CD3ζ may contribute to transduce activation signaling in response to B7-negative tumor cells (Fig. 3).

Thirdly, it is anticipated that coinjection of Th1-T bodies with Th1-T bodies may overcome immunosuppression in tumor-bearing hosts and facilitate the induction of antitumor immunity in vivo. It has been reported that T cells derived from tumor-bearing hosts show reduced immune responses as compared with healthy controls. Several factors may contribute to this immunosuppression, including defective Th cell function, overactivation of suppressive Treg cells, defective T-cell receptorζ signaling, and transforming growth factor-β production (9, 10, 30). Using an animal model of tumor adoptive immunotherapy, we have proposed that introduction of local help to CTL with Th cells, most importantly Th1 cells, is crucial for overcoming immunosuppression. This hypothesis is supported by a number of recent findings: (a) DC/Th1 cell–cell interactions are critically important for inducing the complete activation of CTLs and long-term maintenance of CTL memory in vivo (31–34); and (b) Th1/Tc1 circuits in tumor-bearing hosts are critical for inducing complete tumor eradication in vivo (14, 15, 19). As shown in Fig. 8, combined therapy with Th1- and Tc1-T bodies induced complete rejection of tumor cells, although Th1- or Tc1-T bodies alone were unable to completely reject coinjected tumor cells. Th1-T bodies showed the same levels of growth inhibition of tumor in vivo, although they exhibited lower cytotoxicity than Tc1-T bodies in vitro. This might be because of the potentiation of mouse natural effector cells (natural killer, Mφ, etc.) involved in antitumor immunity by cytokines produced by Th1-T bodies. Although Tc1-T bodies exhibit higher levels of cytotoxicity against tumor cells in vitro, they failed to reject tumor cells by themselves. This may be caused by the failure of Tc1-T bodies to produce IL-2, which is essential for long-lasting CTL functions in vivo. In contrast, Th1-T bodies produced both IFN-γ and IL-2 but showed low levels of cytotoxicity. Therefore, coinjection of Tc1-T and Th1-T bodies might be able to overcome the deficiency of Tc1-T bodies to produce IL-2 and exhibited the most effective antitumor activity in vivo.

The final goal of tumor immunotherapy is the development of an efficient strategy to enhance the concentration of tumor-specific Tc1 and Th1 cells at the local tumor site, either by tumor vaccine therapy or adoptive tumor cell therapy. To overcome the difficulties in inducing physiological tumor-specific Th1 and Tc1 cells, the TRA-specific immunoreceptor viral transduction strategy should prove highly effective. However, thus far, most investigators have focused on the cytotoxic function of T bodies or natural killer-T bodies. Moreover, nobody has tried to establish general protocol for preparing and expanding IFN-γ-producing Th1- or Tc1-T bodies, although there were some reports that CD4+ or CD8+ T cells were accidentally induced from PBMCs (35). However, in the present study, we have first succeeded in establishment of a general protocol to prepare pure IFN-γ-producing Th1-T and Tc1-T bodies, and we demonstrate that T bodies induce their effector functions in response to receptor engagement, in an antigen-specific but MHC-independent manner. The finding that combined therapy with Th1- and Tc1-T bodies induces complete rejection of tumor cells in vivo indicates that the efficient and effective protocol described here provides a novel strategy for adoptive tumor immunotherapy.

ACKNOWLEDGMENTS

We thank Dr. Luc Van Kaer (Vanderbilt University School of Medicine, Nashville, TN) for reviewing this manuscript. We also thank Dr. Michiko Kobayashi (Genetics Institute, Cambridge, MA) and Takuko Sawada (Shionogi Pharmaceutical Institute Co., Osaka, Japan) for their kind donations of IL-12 and IL-2, respectively.

REFERENCES


Generation and Targeting of Human Tumor-Specific Tc1 and Th1 Cells Transduced with a Lentivirus Containing a Chimeric Immunoglobulin T-Cell Receptor

Hiroshi Gyobu, Takemasa Tsuji, Yoshinori Suzuki, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/64/4/1490

Cited articles
This article cites 33 articles, 21 of which you can access for free at:
http://cancerres.aacrjournals.org/content/64/4/1490.full.html#ref-list-1

Citing articles
This article has been cited by 12 HighWire-hosted articles. Access the articles at:
/content/64/4/1490.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.