Potentiation of Tumor Eradication by Adoptive Immunotherapy with T-cell Receptor Gene-Transduced T-Helper Type 1 Cells

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ABSTRACT

Adoptive immunotherapy using antigen-specific T-helper type 1 (Th1) cells has been considered as a potential strategy for tumor immunotherapy. However, its application to tumor immunotherapy has been hampered by difficulties in expanding tumor-specific Th1 cells from tumor-bearing hosts. Here, we have developed an efficient protocol for preparing mouse antigen-specific Th1 cells from nonspecifically activated Th cells after retroviral transfer of T-cell receptor (TCR)α and TCRβ genes. We demonstrate that Th1 cells transduced with the TCR-α and -β genes from the I-Aβ-restricted ovalbumin (OVA)323-339-specific T-cell clone DO11.10 produce IFN-γ but not interleukin-4 in response to stimulation with OVA323-339 peptides or A20 B lymphoma (A20-OVA) cells expressing OVA as a model tumor antigen. TCR-transduced Th1 cells also exhibited cytotoxicity against tumor cells in an antigen-specific manner. Moreover, adoptive transfer of TCR-transduced Th1 cells, but not mock-transduced Th1 cells, exhibited potent antitumor activity in vivo and, when combined with cyclophosphamide treatment, completely eradicated established tumor masses. Thus, TCR-transduced Th1 cells are a promising alternative for the development of effective adoptive immunotherapies.

INTRODUCTION

Th1 helper type 1 (Th1)-dominant immunity, which is regulated by interleukin (IL)-12 and IFN-γ, plays a crucial role for the eradication of tumors in vivo (1, 2). However, the production of Th1 cytokines such as IL-2 and IFN-γ is markedly suppressed in the majority of tumor-bearing hosts (3, 4). Such defects in Th1-mediated immunity in tumor-bearing hosts have made it difficult to induce tumor-specific CTLs that promote tumor rejection (5). Therefore, it is critically important to develop methods that promote Th1-dominant immunity at the local tumor site.

In a previous study (6), we demonstrated that adoptively transferred Th1 cells exhibit strong antitumor activity in vivo and can eradicate an established tumor mass. Moreover, in contrast to Th2 cells, Th1-cell therapy is beneficial for inducing immunological memory in tumorspecific CTLs (6). These findings indicate that Th1-cell therapy is an effective strategy to introduce local T-cell help that overcomes strong immunosuppression in tumor-bearing hosts. However, the application of Th1 cells to adoptive tumor immunotherapy has been hampered by difficulties in inducing sufficient numbers of tumor antigen-specific Th1 cells.

Recently, it has become possible to introduce T-cell receptor (TCR)-α and -β genes into T cells by retroviral transduction (7–10). We used this technology to introduce TCR transgenes specific for the model tumor antigen ovalbumin (OVA) into anti-CD3-activated Th1-polarized CD4+ T cells. We show that TCR-transduced Th1 cells produce IFN-γ but not IL-4 in response to OVA323-339 peptides. Moreover, we show that adoptively transferred TCR-transduced Th1 cells, when combined with cyclophosphamide (CY) treatment, can completely eradicate A20-OVA tumor masses. These findings indicate that TCR transduction of nonspecific Th1 cells is an effective strategy for introducing local T-cell help in the tumor-bearing host.

MATERIALS AND METHODS

Mice. BALB/c mice were obtained from Charles River Japan (Yokohama, Japan). OVA323-339-specific I-Aβ-restricted TCR-transgenic mice (DO11.10) maintained on the BALB/c background were kindly donated by Dr. K. M. Murphy (Washington University School of Medicine, St. Louis, MO; Ref. 6). All of the mice were female and were used at 5–6 weeks of age.

Cytokines, mAbs, and Antigen. IL-12 was kindly donated by Genetics Institute (Cambridge, MA). IL-2 was supplied by Takuko Sawada (Shionogi Pharmaceutical Institute Co. Ltd., Osaka, Japan). Anti-IL-4 monoclonal anti-body (mAb; 1B11) was purchased from American Type Culture Collection (Manassas, VA). PE-anti-CD4 mAb, FITC-anti-CD45RB mAb, FITC-anti-CD8 mAb, purified anti-CD3 mAb, PerCP-anti-CD3 mAb, FITC-anti-IFN-γ mAb, and anti-IFN-γ mAb (R4–6A2) were purchased from PharMingen (San Diego, CA). KJ1–26 mAb was kindly donated by Dr. K. M. Murphy (Washington University School of Medicine, St. Louis, MO). Recombinant IFN-γ was purchased from Pepro Tech EC Ltd. (London, England). OVA323–339 peptide was kindly supplied by Dr. H. Tashiro (Fuiji Co. Ltd., Hadano, Japan).

Generation of OVA-Specific Th1 Cells from DO11.10 Mice. CD4+ CD45RB+ naive T cells were isolated from nylon-passed spleen cells from DO11.10 TCR-transgenic mice using FACS Vantage (Becton Dickinson, San Jose, CA) as reported previously (6). Purified CD4+ CD45RB+ cells were stimulated with 10 μg/ml OVA323–339 peptide in the presence of mitomycin C-treated BALB/c spleen cells, 20 units/ml IL-12, 1 ng/ml IFN-γ, 50 μg/ml anti-IL-4 mAb, and 20 units/ml IL-2 for Th1 development. At 48 h, cells were restimulated with OVA323–339 under the same conditions and were used at 9–12 days of culture.

Construction of Retrovirus Vectors. Complementary DNAs encoding MHC class II-restricted OVA-specific TCR-α and -β genes were amplified from cDNA of CD4+ T cells of DO11.10 mice by reverse transcription-PCR as described previously (7). TCR-α and -β cDNAs were inserted into EcoRI and NotI sites within the multiple cloning site of the pMCKvector, and were designated as pMX-DOTAE and pMX-DOTBE, respectively (7).

Preparation of Retroviruses and Infection of Nonspecific Th1 Cells. Recombinant retroviruses were produced as described previously (7). Briefly, pMX-DOTAE or pMX-DOTBE vector, which were kindly donated by Dr. T. Kitamura (Institute of Medical Science, University of Tokyo, Tokyo, Japan), were transfected into PLAT-E by using FuGENE 6 transfection reagent (Roche, Indianapolis, IN). Twenty-four h later, supernatants were replaced with fresh medium. After incubation for an additional 24 h, supernatants containing the retroviruses were harvested and stored at ~80°C until use. Nonspecific Th1 cells were induced from isolated CD4+ CD45RB+ naive T cells of BALB/c mice by stimulation with 2 μg/ml anti-CD3 mAb in the presence of mitomycin C-treated BALB/c spleen cells in Th1 cytokine conditions, as described above. Twenty-four h after stimulation, these CD4+ T cells were coinfected by retroviruses carrying pMX-DOTAE and pMX-DOTBE in 12-well plates coated with retinoic acid and anti-CD3 mAb. This

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infection procedure was carried out three times at 8-h intervals. At 4 days, the expression of DO11.10 TCR was examined by FITC-conjugated KJ1–26 mAb, which is a clonotypic mAb to DO11.10 TCR. These KJ1–26 cells were isolated with MACS beads (Miltenyi Biotec Inc., Auburn, CA) at over 98% purity and were expanded under Th1 conditions until day 6. More than 90% of Th cells, expanded in this Th1 condition, showed IFN-γ-producing ability but no IL-4-producing ability. As a control, polyclonally activated Th1 cells were transduced with pMX-internal ribosomal entry site (IRES) green fluorescent protein (GFP) vector and used as Mock-GFP gene-transduced Th1 cells.

**Flow Cytometry.** The phenotypic characterization of Th1 cells was carried out using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and Cell Quest software. Fluorescence data were collected with logarithmic amplification. For each sample, data from 10,000 volume-gated viable cells were collected. Mean fluorescence intensity was calculated using the Cell Quest program.

**Intracellular Cytokine Expression.** For the detection of cytoplasmic cytokine expression, cells stimulated with immobilized anti-CD3 mAb for 6 h in the presence of brefeldin A were first stained with FITC-conjugated anti-KJ1–26 mAb and PerCP-anti-CD4 mAb, treated with permeabilizing solution [50 mM NaCl, 5 mM EDTA, 0.02% NaN₃, and 0.5% Triton X-100 (pH 7.5)], and these cells were then stained with PE-conjugated anti-IL-4 mAb or anti-IFN-γ mAb for 45 min on ice. The percentage of cells expressing cytoplasmic IL-4 or IFN-γ was determined by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA).

**Cytokine Levels.** The levels of IFN-γ or IL-4 in culture supernatants were measured by OptEIA mouse IFN-γ and OptEIA mouse IL-4 (PharMingen), respectively.

**Cytotoxicity Assay.** The cytotoxicity mediated by Th1 cells was measured by 4-h ³¹Cr-release assays as described previously (11). Tumor-specific cytotoxicity was determined using A20-OVA (H-2b) as target cells. As a control, parental A20 cells (H-2b) were used. The percentage of cytotoxicity was calculated as described previously (11).

**Tumor Cell Therapy Using Th1 Cells Combined with CY.** A2-OVA cells (2 × 10⁶) were inoculated intradermally into BALB/c mice. When the tumor mass became palpable (6–8 mm), the tumor-bearing mice were treated with CY, CY + TCR-transduced Th1 cells, CY + mock-transduced Th1 cells, TCR-transduced Th1 cells, or mock-transduced Th1 cells. Th1 cells (5 × 10⁶ cells/mouse) were i.v. transferred into tumor-bearing mice 1 day after i.p. injection of CY (80 mg/kg). The antitumor activity mediated by TCR-transduced Th1 cells was determined by measuring tumor size in perpendicular diameters. Tumor volume was calculated by the following formula: tumor volume = 0.4 × length (mm) × width (mm)² (12). Tumor-bearing mice that survived for more than 60 days after therapy were considered completely cured. The mean of five mice per group is indicated in graphs.

**RESULTS AND DISCUSSION**

We have previously developed an adoptive tumor immunotherapy protocol, using Th1-polarized TCR transgenic T cells specific for the model tumor antigen ovalbumin (OVA) presented by I-A⁰. To evaluate the utility of retroviral gene transduction as a tool for generating tumor antigen-specific Th1 cells, we introduced the TCR-α and -β chain genes from the DO11.10 T-cell clone into polyclonally activated T cells by retroviral vectors. Specifically, we isolated CD4⁺CD45RB⁺ naive CD4⁺ T cells from wild-type BALB/c mouse spleen cells and activated these cells with anti-CD3 mAb under Th1-inducing conditions (IL-12 + IFN-γ + anti-IL-4 mAb) in the presence of mitomycin C-treated spleen cells as antigen presenting cells, which is essential for the cross-linking of soluble anti-CD3 mAbs. After 24 h of culture, the cells were retrovirally transduced with DO11.10 TCR-α and -β genes or a mock-GFP gene (three successive gene transductions 8 h apart). TCR- or mock-transduced Th cells were cultured for another 3 days in Th1-inducing conditions. Then, the expression of DO11.10 TCR on expanded cells was examined by flow cytometry. TCR expression was evaluated with the clonotypic KJ1–26 mAb, which stains most CD4⁺ T cells in DO11.10 transgenic mice (Fig. 1B), but very few T cells from normal mice (Fig. 1A). The majority (84%) of anti-CD3-activated CD4⁺ T cells successfully expressed DO11.10 TCR detected by KJ1–26 mAb (Fig. 1C) or a mock gene detected by GFP expression (Fig. 1E) after retroviral transduction.

To investigate the function of DO11.10 TCR-transduced Th cells, KJ1–26-expressing cells were enriched by MACS beads (Fig. 1D). As a control, GFP-expressing Th cells were isolated by fluorescence-activated cell sorting (Fig. 1F). As shown in Fig. 1, D and F, we could prepare retrovirally modified Th cells expressing DO11.10 TCR and mock-GFP Th cells that were >98% pure. We examined the cytokine producing ability of DO11.10 TCR gene-transduced Th cells by intracellular staining after stimulation with the cognate I-A⁻²-binding OVA 233-239 peptide. As shown in Fig. 2, B and F, DO11.10 TCR-transduced Th cells detected by KJ1–26 mAb produced IFN-γ but not IL-4 in response to OVA-peptide. In sharp contrast, mock-GFP-transduced Th1 cells produced neither IFN-γ nor IL-4 in response to stimulation with OVA 233-239 (Fig. 2, D and H). We further demonstrated that DO11.10 TCR-transduced Th1 cells exhibit high levels of
IFN-γ but not IL-4 production in response to stimulation with OVA-peptide-pulsed A20 tumor cells (Fig. 3, A and B). In addition to cytokine production, TCR-transduced Th1 cells, but not mock-transduced Th1 cells, exhibited strong cytotoxicity against antigenic peptide-pulsed A20 cells (Fig. 3C). When compared with Th1-polarized DO11.10 cells (DO11.10-Th1) from transgenic animals, the response of TCR-transduced Th1 cells to the cognate OVA peptide was lower than DO11.10-Th1 cells, although they exhibited a very similar response at a higher dose (2 μg/ml) of peptide (Fig. 3D). Such different responsiveness was also demonstrated when we used A20-OVA tumor stimulator, which exhibited lower stimulation activity compared with peptide-pulsed A20 tumor cells. As shown in Fig. 3, E and F, TCR-transduced Th1 cells showed lower IFN-γ production and cytotoxicity in response to A20-OVA tumor compared with DO11.10-Th1 cells. As shown in Fig. 1D, the expression intensity of genetically modified TCR is always lower than that of physiologically expressing DO11.10 TCR (Fig. 1B). Therefore, the lower responsiveness of TCR-transduced Th1 cells may be derived from lower expression intensity or affinity of genetically modified TCR compared with physiologically expressing TCR. In contrast to TCR-transduced Th1 cells, mock-transduced Th1 cells exhibited no significant IFN-γ production and cytotoxicity by stimulation with I-Aβ-binding OVA-peptide (Fig. 3, A–C).

Finally, we investigated the therapeutic activity of TCR-transduced Th1 cells against tumor-bearing mice. BALB/c mice were inoculated with A20-OVA tumor cells and, when the tumor mass became palpable (6–8 mm), TCR-transduced Th1, mock-transduced Th1 cells, or DO11.10-derived Th1 cells were i.v. transferred. Consistent with previous results (6), transfer of 2 × 10⁷ DO11.10-derived Th1 cells induced complete cure of all tumor-bearing mice. However, neither TCR-transduced Th1 cells nor mock-transduced Th1 cells exhibited significant antitumor activity in vivo, even when 2 × 10⁸ cells were transferred (Fig. 4A). This lower antitumor activity of TCR-transduced Th1 cells might be due to lower intensity or affinity of genetically modified TCR as described above. Alternatively, down-modulation of retrovirally transduced TCR expression by transcriptional silencing mechanisms (13) might influence the antitumor activity of TCR-transduced Th1 cells in vivo. As shown in Fig. 4B, we found that the therapeutic efficacy of Th1 cells was augmented by combination therapy with CY. Namely, the transfer of 5 × 10⁷ DO11.10-Th1 cells combined with CY pretreatment caused complete rejection of tumor, although Th1 cells alone exhibited no significant antitumor activity. Such potentiation by CY treatment was observed even when tumor-bearing mice were transferred with 2 × 10⁷ Th1 cells. Therefore, we examined whether therapeutic efficacy of TCR-transduced Th1 cells could be potentiated by combination with CY treatment. Neither TCR-transduced Th1 cells (5 × 10⁶) nor mock-transduced Th1 cells showed significant antitumor activity in vivo. However, when tumor-bearing mice were pretreated with CY (80 mg/kg) 1 day before the transfer of TCR-transduced Th1 cells (5 × 10⁶), tumor growth was strongly inhibited (Fig. 4C). All of the mice treated with CY plus TCR-transduced Th1 cells were completely cured from tumors (Fig. 4D). Such potentiation by CY was not observed when 2 × 10⁷ TCR-transduced Th1 cells were transferred into tumor-bearing mice. Therefore, physiological DO11.10 TCR-expressing Th1 cells appeared to exhibit 2-fold stronger antitumor activity in vivo compared with TCR-transduced Th1 cells. Thus, combined therapy with

Fig. 3. T-cell receptor (TCR)-transduced T-helper type 1 (Th1) cells demonstrate both IFN-γ production and cytotoxicity in response to ovalbumin (OVA)–peptide-pulsed A20 tumor cells and A20-OVA tumor cells. Th1 cells transduced with DO11.10 TCR genes were induced as described in “Materials and Methods.” After culture for 7 days, KJ-1–26 TCR-transduced Th1 cells (TCR-Th1) were harvested and cultured with or without OVA23–35 peptide [with, OVA-pep (+); without, OVA-pep (−)] in the presence of mitomycin C-treated spleen cells to determine their ability to produce IFN-γ (A) or interleukin (IL)-4 (B). As negative control cells, Th1 cells transduced with a mock green fluorescent protein (GFP) gene (A, B, GFP-Th1) were used. C, cytotoxicity of TCR-Th1 (○) and GFP-Th1 cells (△, □) against unpulsed A20 tumor cells (○, △) or OVA23–35 peptide-pulsed A20 tumor cells (△, □) was measured by 4-h ⁵¹Cr-release assay. D, TCR-Th1 or Th1 cells induced from DO11.10 transgenic mice (DO11.10-Th1) were stimulated with three different doses of peptide (0, □; 0.02, △; 0.2, ○); 2 μg/ml; □). After 24 h, IFN-γ levels of culture supernatants were measured by ELISA. E, IFN-γ-producing ability of TCR-Th1 or DO11.10-Th1 by stimulation with A20-OVA (○) or A20 (□) tumor cells. F, cytotoxicity of TCR-Th1 (○, △) or DO11.10-Th1 (△, □) against A20-OVA (○, △) or A20 (△, □) tumor cells was measured by 4-h ⁵¹Cr-release assay. Bars, mean ± SE of triplicate samples. Similar results were obtained in three separate experiments.

Fig. 4. DO11.10 T-cell receptor (TCR)-transduced T-helper type 1 (Th1) cells produce IFN-γ but not interleukin (IL)-4 in response to stimulation with the I-Aβ-restricted ovalbumin (OVA)23–35 peptide. Th1 cells were transduced with DO11.10 TCR genes or with a mock green fluorescent protein (GFP) gene and induced into Th1 cells, as described in “Materials and Methods.” After culture for 7 days, KJ-1–26 TCR-transduced Th1 cells (A, B, E, and F) and GFP+ mock gene-transduced Th1 cells (C, D, G, and F) were stimulated with (B, D, F, and H) or without (A, C, E, and G) OVA23–35 peptide. After 24 h of culture, cytokine-producing ability of cells was determined by intracellular staining: IFN-γ producing ability of TCR-transduced Th1 cells (A, B) or mock gene-transduced Th1 cells (C, D). IL-4 producing ability of TCR-transduced Th1 cells (E, F) or mock gene-transduced Th1 cells (G, H). The figures in data represent the percentage of cytokine-producing cells. Similar results were obtained in three separate experiments.
CY might be better to induce the efficient therapeutic effect of genetically modified Th1 cells, although the transfer of large numbers of TCR-transduced Th1 cells might be effective. CY treatment alone or combined therapy with mock-transduced Th1 cells and CY failed to cure tumor-bearing mice, although these treatments showed a slight inhibition of tumor growth in vivo. Such antitumor activity by combination therapy with TCR-transduced Th1 cells and CY was not observed in A20 tumor-bearing mice (data not shown). Thus, these results clearly demonstrate that TCR-transduced Th1 cells induced from nonspecific Th1 cells exhibit specific antitumor activity in vivo. That combination therapy with TCR-transduced Th1 and CY can induce immunological memory in vivo was indicated by the fact that (a) all cured mice by treatment with TCR-transduced Th1 cells and CY could reject rechallenged A20-OVA tumor cells and (b) CD8+ and CD4+ CTLs were demonstrated in cured mice.

Recently, CD4+ T cells were demonstrated to play a crucial role not only in the induction of CD8+ CTLs via dendritic cell activation (14–16) but also for maintaining the number and function of CTLs (14–18). Coadministration of CD4+ T cells with CD8+ T cells resulted in increased CTL activity and enhanced infiltration of these cells into tumors; both facilitate CTL-mediated tumor eradication in vivo (19). The rationale for introducing CD4+ T-cell help for promoting antitumor immunity is supported by the fact that CD4+ T cells augment the therapeutic effect of tumor vaccines that are based on MHC class I-binding peptides or dendritic cells (19). An important role for CD4+ T-cell help has also been reported in tumor chemotherapy using CY (20). CY, in addition to its direct antitumoricidal activities, appears to act as an immunomodulator that facilitates the generation of antitumor effector T cells combined with CD4+ T-cell help.

In a prior report (6), we demonstrated that adoptive cell transfer of a large number (>2×10^7 cells) of Th1 cells is beneficial for inducing tumor-specific CTLs in vivo and for curing mice of established tumors. However, adoptive transfer of <10^7 Th1 cells was insufficient to cure tumor-bearing mice. This Th1-dependent antitumor immunity is completely dependent on the generation of host-derived tumor-specific CTL, because Th1 cell-therapy was unable to eradicate tumors in RAG-2−/− mice unless CD8+ T cells were cotransferred. Therefore, when adoptive immunotherapy using Th1 cells was combined with dendritic cell-based tumor vaccine therapy that promotes CTL generation, the number of required Th1 cells decreased to 5×10^6 cells per mouse (19). Here, we further demonstrate that the therapeutic effect of CY is greatly augmented by combination therapy with a small number (5×10^6) of TCR-transduced Th1 cells as well as DO11.10-derived Th1 cells (Fig. 4). The immunopotentiating mechanisms of CY remains unclear, but CY treatment appeared to enhance the migration of Th1 cells into tumor local site including draining lymph node. Then, the transferred Th1 cells exhibited proliferation and cytokine production by interacting with APC, which processed antigen of tumor cells destroyed by CY and/or Th1 cells. Thus, transferred Th1 cells introduce tumor local help that is essential for generating tumor-specific CTLs to eradicate established tumor.

The strategy used here for promoting tumor antigen-specific Th1 responses holds significant promise for tumor immunotherapy in human patients. A number of human tumor-antigen-specific T cells have been established. For example, several Th clones reactive against the class II-binding tumor rejection antigen peptide have been established (21–24). We have already cloned the TCR genes from a tumor-specific Th cell clone and introduced the TCR gene into polyclonally activated human T cells. These transduced T cells were able to produce cytokines in response to the relevant antigen. These findings illustrate the feasibility of applying antigen-specific Th1 cells
to adoptive tumor immunotherapy, either by inducing tumor-specific Th1 cells from patient samples or by TCR transduction, as described here.

Th1-dominant immunity plays a critical role in the induction of antitumor immunity at the local tumor site via multiple helper functions such as their capacity to enhance CTL priming of APC and to promote migration, proliferation, and cytotoxicity of tumor-specific CTLs (14–19, 25–27). Therefore, adoptive immunotherapy using tumor-specific Th1 cells combined with other therapeutic modalities such as (a) dendritic cell-based tumor vaccine therapy; (b) CD8+ T-cell therapy; (c) chemotherapy; or (d) radiation therapy (28, 29), may provide a more efficient strategy for tumor immunotherapy. Our protocol for inducing TCR-transduced tumor-specific Th1 cells should enable us to prepare tumor-specific Th1 cells even when tumor-specific Th1 cells from the patient cannot be obtained. This strategy should prove valuable for developing effective tumor immunotherapies. For application to clinical trial, the use of TCR-transduced Th1 cells comodified with thymidine kinase (TK) gene might be better strategy to prevent unwanted immune disorders induced by long-lived transferr Th1 cells (30).

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