Immunomodulation of Curcumin on Adoptive Therapy with T Cell Functional Imaging in Mice

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Abstract

Adoptive T-cell therapy involves the ex vivo expansion and subsequent transfusion of tumor-specific T lymphocytes to eliminate tumors. Using immune modulators to block immunosuppressive factors in the tumor microenvironment has emerged as a promising strategy to enhance T-cell–mediated tumor regression. Curcumin, a major component of turmeric, has been shown to possess antitumor and immunomodulatory effects by regulating a diverse range of molecular targets. Thus, we hypothesize that these beneficial effects of curcumin may improve the therapeutic efficacy of adoptive therapy. Here, we have shown that curcumin enhances cytotoxicity of CD8+ T cells toward tumors via alteration of the tumor microenvironment when combined with adoptive therapy. We found that T-cell accumulation and function were increased in combined treatment due to the blockade of different immunosuppressors, including TGF-β, indoleamine 2,3-dioxygenase, and regulatory T cells. Furthermore, bioluminescent imaging with a granzyme B promoter–conjugated optical reporter also reflected improved cytotoxicity of antigen-specific CD8+ T cells in tumor-bearing mice during treatment. These findings suggest that combination of multitargeting drugs, such as curcumin, with adoptive therapy may have potential for clinical application. In addition, using a granzyme B–specific imaging reporter to assess T-cell function may also be applied for the development and therapeutic evaluation of new immunotherapy in preclinical studies. Cancer Prev Res; 5(3); 444–52. ©2011 AACR.

Introduction

Adoptive T-cell therapy is a promising therapeutic strategy to treat malignancies and has provided impressive results in cancer patients (1, 2). However, this approach remains challenging because of the lack of persistence and trafficking, and the loss of effector function of transferred T cells (3). The immunosuppressive mechanism within the tumor microenvironment has been proposed as playing a critical role in mediating the antitumor effect of adoptive therapy (4–6). TGF-β is one of the most important immunosuppressors that cancer cells use to hamper the T-cell response. TGF-β has been shown to promote tumor growth through angiogenesis and to inhibit the activation and proliferation of CD8+ T cells by suppression of cytolytic gene expression, such as perforin, granzyme B, IFN-γ, and Fas ligand (7). Moreover, TGF-β is attributed to the development, maintenance, and recruitment of regulatory T cells (Treg) that actively attenuate tumor-specific activation of T cells (8, 9). Indoleamine 2,3-dioxygenase (IDO), another immunosuppressor with ability to catalyze the essential amino acid tryptophan via kynurenine pathway, is also a major impediment to successful adoptive therapy (7, 10). Because T cells are highly sensitive to tryptophan shortage, IDO has been proposed to inhibit T-cell proliferation and cause T-cell anergy by degrading tryptophan (11). Indeed, the expression of IDO is found in most human tumors, characterized by a lack of accumulation of CD8+ T cells, and serves as a significant predictor of poor prognosis in ovarian and colorectal cancers (12–14). Because IDO can be induced by IFN-γ, IDO-negative cancer cells may be initiated to express IDO when exposed to an inflammatory context in the tumor microenvironment, and thereby hinder CD8+ T-cell–mediated tumor regression before it can be effective (4, 5, 12). Recently, strong evidence has indicated that modification of the tumor microenvironment, such as the blockade of the TGF-β signaling pathway (15, 16),
inhibition of IDO (17, 18), or depletion of Tregs (19, 20) is able to augment the cytotoxicity of CD8+ T cells.

Curcumin, a natural compound derived from the plant Curcuma longa, has previously been shown to possess anticancer properties through mediating multiple molecular targets related to apoptotic and survival pathways in tumors (21). Other studies have also provided important insight into the relationship between curcumin and the immune system. Curcumin can inhibit the production of inflammatory cytokines and enzymes through inhibition of NF-kB-binding activity of innate and adaptive immunity and thus has potential against various autoimmune diseases and inflammations (22, 23). Moreover, curcumin has been found to mediate the growth and function of various immune cells (22, 24). Some studies show that curcumin can attenuate tumor-induced depletion of T cells and restore antitumor activity of CD8+ T cells in tumor-bearing animal models (25, 26). Although the connection between curcumin, the immune system, and growth inhibition of cancer needs to be further investigated, the above evidence suggests that the multitargeting benefit and immunomodulation of curcumin may render adoptive T-cell therapy more efficient in combination treatment.

To determine the combined effects of curcumin with adoptive therapy, we first examined the function of CD8+ T cells and immunosuppressive factors in vitro and with an animal model. Because CD8+ T cells exert cytotoxicity through the perforin/granzyme B pathway, the expression of granzyme B signifies their full differentiation and acquisition of killing potential toward tumors. We have earlier validated an imaging strategy by linking a reporter gene to the granzyme B promoter coupled with CMV enhancer, allowing noninvasive monitoring of T-cell receptor (TCR)-based T-cell activation in living subjects using bioluminescent imaging (BLI; ref. 27). We herein utilized a E.G7/OT1 animal model that involved adoptive transfer of OVA-expressing E.G7+ T cells in tumor-bearing animal model. Because CD8+ T cells transduced with a granzyme B promoter–driven firefly luciferase and tomato fluorescent fusion reporter gene (pGBeLT) to evaluate the immunomodulatory effect of curcumin in OVA-expressing E.G7 tumor-bearing mice. Our results support the better understanding of curcumin-mediated immunomodulation and suggest the application of multitargeting drugs as a complementary strategy in adoptive therapy.

Materials and Methods

Animals and cell lines

Eight-week-old male C57BL/6 mice purchased from the National Laboratory Animal Center, Taiwan, and OT-1 transgenic mice obtained from the Jackson Laboratory were housed in the Laboratory Animal Center of National Yang-Ming University. All animal protocols were approved by the Animal Care and Use Committee at National Yang-Ming University. OVA-expressing E.G7 mouse lymphoma cell line from American Type Culture Collection (ATCC; Jan 2010) was maintained in RPMI-1640 (RPMI-1640, 10% FBS, 1% penicillin/streptomycin, and 25 mmol/L HEPES) with 400 μg/mL G418. Jurkat human T-cell leukemia cell line from ATCC (Jan 2010) was cultured in cRPMI-1640. Lentiviral-producing HEK-293FT cell line from Invitrogen (Jan 2010) was maintained in cDMEM [Dulbecco’s modified Eagle’s medium (DMEM), 10% FBS, 1% penicillin/streptomycin, 2 mmol/L L-glutamine, and 0.1 mmol/L MEM nonessential amino acids] with 500 μg/mL G418. All cell lines were authenticated by short tandem repeat profiling prior to their usage and passaged for less than 6 months in our experiments. Cell culture tested Mycoplasma-free routinely with MycoAlert Mycoplasma Detection Assay (Lonza).

Reagent preparation

Curcumin (Sigma-Aldrich) was dissolved in 0.5 N and 0.025 N NaOH at the stock concentrations of 60 mmol/L and 7 mg/mL for cell culture and in vivo studies, respectively, as previously described (28).

Isolation and culture of mouse T cells

Splenocytes from OT-1 transgenic mice were harvested, strained through a 40-μm filter (BD Biosciences), and subjected to erythrocyte lysis using an ACK lysis buffer. After washing, CD8+ T cells were isolated using a mouse T-cell enrichment kit (Stemcell Technologies). CD8+ T cells were prestimulated in cRPMI-1640 containing 2.5 μg/mL Concanavalin A (Calbiochem), 50 μmol/L β-mercaptoethanol (β-ME; Bio-Rad), 10 ng/mL interleukin (IL)-7 (R&D Systems) for 36 hours, and thereafter maintained in cRPMI in the presence of 50 μmol/L β-ME, 10 ng/mL IL-15 (R&D Systems), 10 U/mL IL-2 (R&D Systems), and 50 mmol/L α-methylmannoside (Calbiochem) for 24 hours.

Lentiviral production and transduction

High-titer lentiviruses were produced using transient cotransfection of HEK-293FT cells with pGBeLT, the packaging vector pCMVΔ8.74, and the envelope-encoding vector pMD2.G as previously described (27). Titration of lentiviruses was carried out using Jurkat cells. Briefly, 1 × 105 Jurkat cells were transduced with serially diluted viruses for 3 to 4 hours and then activated with 1 μg/mL of anti-human CD3 and CD28 antibodies (R&D Systems) to trigger the expression of reporter gene. The titer of lentiviruses was determined using flow cytometry. In some experiments, mouse CD8+ T cells were prestimulated for 16 hours prior to lentiviral transduction (MOI = 5). Thereafter transduced CD8+ T cells were cultured in cRPMI with 50 μmol/L β-ME, 10 ng/mL IL-15, 10 U/mL IL-2, and 50 mmol/L α-methylmannoside for 24 hours prior to further study.

Western blot

Cell lysates were run on SDS-PAGE, then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk for 1 hour and probed with anti-mouse IDO monoclonal (Millipore), anti-mouse TGF-β monoclonal (Cell signaling), anti-mouse active caspase-3 (Millipore), and anti-mouse β-actin monoclonal (Millipore) antibodies (mAb) at 4°C overnight. Membranes were subjected to blocking for 1 hour and probed with anti-mouse TGF-β monoclonal (Cell signaling), anti-mouse active caspase-3 (Millipore), and anti-mouse β-actin monoclonal (Millipore) antibodies (mAb) at 4°C overnight. Membranes were subjected to blocking for 1 hour and probed with anti-mouse TGF-β monoclonal (Cell signaling), anti-mouse active caspase-3 (Millipore), and anti-mouse β-actin monoclonal (Millipore) antibodies (mAb) at 4°C overnight. Membranes were subjected to blocking for 1 hour and probed with anti-mouse TGF-β monoclonal (Cell signaling), anti-mouse active caspase-3 (Millipore), and anti-mouse β-actin monoclonal (Millipore) antibodies (mAb) at 4°C overnight. Membranes were subjected to blocking for 1 hour and probed with anti-mouse TGF-β monoclonal (Cell signaling), anti-mouse active caspase-3 (Millipore), and anti-mouse β-actin monoclonal (Millipore) antibodies (mAb) at 4°C overnight. Membranes were subjected to blocking for 1 hour and probed with anti-mouse TGF-β monoclonal (Cell signaling), anti-mouse active caspase-3 (Millipore), and anti-mouse β-actin monoclonal (Millipore) antibodies (mAb) at 4°C overnight. Membranes were subjected to blocking for 1 hour and probed with anti-mouse TGF-β monoclonal (Cell signaling), anti-mouse active caspase-3 (Millipore), and anti-mouse β-actin monoclonal (Millipore) antibodies (mAb) at 4°C overnight. Membranes were subjected to blocking for 1 hour and probed with anti-mouse TGF-β monoclonal (Cell signaling), anti-mouse active caspase-3 (Millipore), and anti-mouse β-actin monoclonal (Millipore) antibodies (mAb) at 4°C overnight. Membranes were subjected to blocking for 1 hour and probed with anti-mouse TGF-β monoclonal (Cell signaling), anti-mouse active caspase-3 (Millipore), and anti-mouse β-actin monoclonal (Millipore) antibodies (mAb) at 4°C overnight. Membranes were subjected to blocking for 1 hour and probed with anti-mouse TGF-β monoclonal (Cell signaling), anti-mouse active caspase-3 (Millipore), and anti-mouse β-actin monoclonal (Millipore) antibodies (mAb) at 4°C overnight. Membranes were subjected to blocking for 1 hour and probed with anti-mouse TGF-β monoclonal (Cell signaling), anti-mouse active caspase-3 (Millipore), and anti-mouse β-actin monoclonal (Millipore) antibodies (mAb) at 4°C overnight. Membranes were subjected to blocking for 1 hour and probed with anti-mouse TGF-β monoclonal (Cell signaling), anti-mouse active caspase-3 (Millipore), and anti-mouse β-actin monoclonal (Millipore) antibodies (mAb) at 4°C overnight. Membranes were subjected to blocking for 1 hour and probed with anti-mouse TGF-β monoclonal (Cell signaling), anti-mouse active caspase-3 (Millipore), and anti-mouse β-actin monoclonal (Millipore) antibodies (mAb) at 4°C overnight. Membranes were subjected to blocking for 1 hour and probed with anti-mouse TGF-β monoclonal (Cell signaling), anti-mouse active caspase-3 (Millipore), and anti-mouse β-actin monoclonal (Millipore) antibodies (mAb) at 4°C overnight.
were washed in Tris-Tween buffer saline and incubated with horseradish peroxide-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) at room temperature for 1 hour. Final detection was achieved by using an ECL chemiluminescent detection system (Millipore). ImageJ (NIH) was used for the quantitative analysis.

**Tumor treatments and adoptive transfer**

Eight-week-old C57BL/6 male recipient mice were injected subcutaneously with 2 × 10⁶ E.G7 cells/100 μL PBS, and tumors were allowed to grow for 6 to 7 days. For drug and T-cell alone treatment, mice received 70 mg/kg/d curcumin intraperitoneally for 5 days and 10 × 10⁶ or 5 × 10⁶ CD8⁺ T cells intravenously, respectively. For combined treatment, one day after curcumin administration, tumor-bearing mice were intravenously injected with 5 × 10⁶ CD8⁺ T cells (see Fig. 3A for details). Tumor volumes were measured with a digital caliper and calculated according to the formula: length × width² × 0.523.

**Flow cytometry**

Tumors, spleens, and tumor-draining lymph nodes (TDLN) were harvested from mice, and single-cell suspensions were stained with the following conjugated Abs: anti-Vα2-FITC (BD Pharmingen), anti-Vβ5-PE (BD Pharmingen), anti-CD69-PerCP (Biolegend), and anti-CD8-APC (Biolegend) to determine the activation and percentage of adoptively transferred CD8⁺ T cells during the treatment. For the intracellular cytokine staining assay, cells were first stained for surface markers, fixed and permeabilized, and finally stained with intracellular IFN-γ–PerCP/Cy5.5 Ab (Biolegend). To identify Tregs, single-cell suspensions were stained with anti-FOXP3-Alexa Fluor 488/CD4-APC/CD25-PE Abs using a Mouse Treg Flow Kit (Biolegend) according to manufacturer protocols. Sample acquisition was done with a FACSCalibur flow cytometer (BD Biosciences), and the data were analyzed using FlowJo (Tree Star).

**Bioluminescent imaging**

Mice injected with pGBeLT-transduced CD8⁺ T cells received 150 mg/kg β-luciferin intraperitoneally and were anesthetized using 1% to 3% isoflurane 15 minutes before imaging. The photons emitted from tumors were detected by the IVIS50 Imaging System (Xenogen). All images were acquired for 5 minutes. Regions of interest from displayed images were drawn around the tumor sites and quantified as photons/s/cm²/sr using the Living Image software (Version 2.20; Xenogen).

**Confocal microscopy**

Tumors were removed from mice, frozen immediately with dry ice, and then embedded with Tissue-Tek OCT. Tissue microtome cryosectioning was prepared using a cryostat microtome (Leica) and fixed in acetone for 10 minutes. After washing, the slides were mounted in mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). The slides were scanned with Olympus FV1000 laser confocal microscopy (Olympus), and the fluorescent signal was analyzed using FV10-ASW 1.6 Viewer (Olympus).

**Statistics**

Student t test was used to evaluate the significance or P values between groups (*, P < 0.05; **, P < 0.01). SEM values were depicted as error bars in all figures.

**Results**

**Augmentation of CD8⁺ T-cell function and cytokine production by curcumin in cell culture**

The expression of TGF-β and IDO in E.G7 cells treated with IFN-γ and/or curcumin was assayed to examine the effect of curcumin on immunosuppressive factors of tumor cells. The protein level of IDO was increased in response to IFN-γ induction but inhibited by the presence of curcumin (Fig. 1A). TGF-β was also suppressed by curcumin in a dose-dependent manner (Fig. 1B). To examine whether TCR-based T-cell function could be improved by curcumin via inhibition of TGF-β and IDO of tumor cells, CD8⁺ T cells derived from OT-1 transgenic mice, which expressed TCR specific to the chicken OVA peptide at residues 257–264 in the context of H2-Kb, were used. E.G7 cells as the target were pretreated with 10 μmol/L curcumin for 24 hours and pGBeLT-transduced CD8⁺ T cells were cocultured with the target at a 1:50 (E:T) ratio for 4 hours. The activation of T cells was assessed by detection of tomato fluorescent signal and IFN-γ production using flow cytometry. The amount of activated and IFN-γ-producing CD8⁺ T cells was significantly higher (about 2- and 1.5-fold, respectively) in curcumin-treated versus untreated E.G7 cells (Fig. 1C).

**Immunomodulation of curcumin in tumor-bearing mice**

To examine the immunomodulation of curcumin in vivo, E.G7 tumor-bearing mice were treated with 70 mg/kg/d curcumin for 5 consecutive days when tumor volume reached 150 mm³, and tumors were excised to analyze the expression of TGF-β and IDO with Western blot. No anti-tumor effect on E.G7 tumors and no significant change in body weight (<20%) were found at the end of curcumin treatment, suggesting that there was no general toxicity of curcumin in this study (data not shown). The expression of TGF-β and IDO from E.G7 tumors was suppressed by curcumin as compared with those of the control (Fig. 2A). Furthermore, the percentage of CD25⁺/FoxP3⁺ out of CD4⁺ T cells in TDLNs of curcumin-treated mice was 1.13 ± 0.15 versus 1.65 ± 0.17 of the control group, that is, 30% reduction (P = 0.043; Fig. 2B).

**The effect of curcumin on the infiltration and function of CD8⁺ T cells in tumors**

Because curcumin inhibits not only the expression of TGF-β and IDO but also the induction of Treg in the tumor microenvironment, it is noteworthy to explore whether a small number of CD8⁺ T cells can be used to enhance tumor targeting when combined with curcumin in living subjects.
According to our previous finding, \(10^6\) OVA-specific CD8\(^+\) T cells could lead to the complete tumor regression in E.G7 tumor-bearing mice and was considered as the regular dose in these studies (27). Here, E.G7 tumor-bearing mice were treated as the following 5 groups: control, 70 mg/kg/d curcumin, \(10^6\) CD8\(^+\) T cells (10 MT), \(5 \times 10^5\) CD8\(^+\) T cells (5 MT), and \(5 \times 10^5\) CD8\(^+\) T cells + 70 mg/kg/d curcumin (combination of 5 MT + 70 mg/kg/d curcumin) when tumor size reached about 150 mm\(^3\). Mice were intraperitoneally injected with curcumin one day before T-cell transfer, designated as day 0, for 5 days, and the treatment protocol is shown in Fig. 3A. Tumors, TDLNs, and spleens were harvested and subjected to flow cytometric analyses to determine the presence and activity of transferred V\(^a\)2\(^+\)V\(^b\)5\(^+\) CD8\(^+\) T cells on day 3 post adoptive therapy, the time at which there was no significant difference in tumor size among the 5 groups (Fig. 3B). The accumulation of V\(^a\)2\(^+\)V\(^b\)5\(^+\) CD8\(^+\) T cells in tumors and TDLNs of combination-treated mice was enhanced by 3- to 4-fold and 2-fold, respectively, as compared with those of the 5 MT group, and reached to about the same levels of the 10 MT group (Fig. 3C). The activity of V\(^a\)2\(^+\)V\(^b\)5\(^+\) CD8\(^+\) T cells was judged by CD69 expression. In Fig. 3D, the percentage of V\(^a\)2\(^+\)V\(^b\)5\(^+\) CD8\(^+\) T cells expressing CD69 in tumors from combined therapy was 60% higher than that of 5 MT group, but no expression of CD69 was seen in the spleens of all 3 groups. The results suggested that curcumin does not alter the function of transferred CD8\(^+\) T cells present in the spleen.

**Imaging of adoptively transferred T-cell function in mice**

Evidence from preclinical studies indicates that alteration of the tumor microenvironment using TGF-\(\beta\) signaling inhibitor or IDO-blocking agent 1-methyl tryptophan (1-MT) results in the restoration of T-cell response (15–18).
In our preliminary experiments, we have tested whether BLI coupled with pGBeLT reporter is sensitive to TCR-dependent T-cell function during adoptive therapy combined with either TGF-β receptor I kinase (TβR-I) inhibitor or 1-MT–treated mice (Supplementary Fig. S1A and S1B). Flow cytometric analysis also confirmed more transferred T cells (~2-fold) present in E.G7 tumors from 1-MT–treated mice (Supplementary Fig. S1C).

Because curcumin efficiently increased the number and activation of adaptively transferred T cells within tumors according to flow cytometric analysis (Fig. 3), we further examined whether pGBeLT reporter constructed in these T cells could allow noninvasive and sequential tracking of T-cell targeting and activation, and reflect the improvement of TCR-dependent T-cell function. Similar to the treatment protocol in Fig. 3B, when E.G7 tumors reached about 150 mm³, mice were tail-vein injected with 10 × 10⁶ pGBeLT-transduced CD8⁺ T cells, and 5 × 10⁶ pGBeLT-transduced CD8⁺ T cells plus 70 mg/kg/d curcumin to compare the signal intensity of T-cell activation. As early as 4 hours after adoptive therapy, mice were subjected to BLI for baseline scans, and little bioluminescent signal was detected from E.G7 tumors (Supplementary Fig. S1A and S1B). Flow cytometric analysis also confirmed that the BLI signal of the combined group was significantly higher than that of the 10 × 10⁶ T cells group on day 5 postadoptive transfer (Fig. 4A). The photon signal of pGBeLT in the combined group was dramatically increased and peaked at day 3 postadoptive therapy similar to that of the 10 × 10⁶ T cells group. Sequentially, the decrease in BLI intensity paralleled the corresponding marked tumor shrinkage from day 3 to the end of the experiment (Fig. 4A and B). Because the release of granzyme B from CD8⁺ T cells could induce apoptosis of target cells, the expression of caspase-3 in E.G7 tumors on day 3 was examined. Caspase-3 expression was not obvious in the control and curcumin-treated groups but was increased in the 10 × 10⁶ T cells and combined groups (Fig. 4C). In addition, the BLI signal of the combined group was significantly higher than that of the 10 × 10⁶ T cells group on day 5 postadoptive transfer. We further examined whether curcumin could maintain antigen-specific CD8⁺ cells within tumors. Immunofluorescent staining confirmed the persistence of transferred CD8⁺ T cells in E.G7 tumor of the combined group (Fig. 4D).

Discussion

For decades, adoptive T-cell therapy has been proposed to mediate cancer regression, however, clinical trials highlight the need to continually improve this strategy mainly due to the suppressive nature of the tumor microenvironment (5, 6). Recent studies indicate that using specific inhibitors, mAbs, or siRNA to inhibit TGF-β signaling, IDO, and Tregs can reverse immune suppression in the tumor microenvironment, resulting in the augmentation of T-cell response, but the safety and effectiveness of such treatments have not been carefully evaluated in clinical trials. For example, small-molecule inhibitors, such as 1-MT may be toxic via promotion of inflammatory conditions (29). Although using siRNA to silence immunosuppressive genes of tumor cells is ideal, efficient siRNA delivery in vivo is still challenging and ongoing (18, 30). Specific mAb used to inhibit immunosuppressive factors may also bind to other cells and tissues expressing normal level of targeted proteins or receptors, for example, anti-CD25 Ab used for reducing Tregs may also target activated CD8⁺ T cells simultaneously (31, 32). Other studies show that combined adoptive T-cell therapy with chemotherapy may induce synergistic antitumor effect in animal models, nevertheless, the effect of combined treatment is not long lasting, and the toxicity of chemotherapy remains a valid concern (2, 33, 34).

Curcumin has been shown to regulate transcriptional factors, multiple protein kinases, antiapoptotic proteins, cytokine signaling receptors, and growth factors, which render it with antitumor and immunomodulatory effects (21, 22). However, little is known about the effect of curcumin when combined with T-cell therapy both in cell culture and in living subjects. In cell culture studies, we found that CD8⁺ T-cell activation was significantly increased when cocultured with curcumin-pretreated
E.G7 tumor cells, suggesting that inhibition of TGF-β and IDO of tumor cells could be a major contributor (Fig. 1).

Although curcumin exhibits promising results in various studies in vitro, poor absorption and rapid metabolism continue to be a major concern in preclinical models and clinical trials when it is taken orally (35). Pan and colleagues reported that low plasma concentration of 0.13 μg/mL was detected at 15 minutes, whereas a maximum plasma concentration of 0.22 μg/mL was obtained after 1 hour with oral gavage of 1.0 g/kg of curcuma in mice. After intraperitoneal administration of 0.1 g/kg, the plasma level peaked (2.25 μg/mL) at 15 minutes and declined rapidly within 1 hour (36). To avoid the low bioavailability of curcumin for the clinical application, various advanced drug delivery systems were developed.
systems, such as nanoparticles, liposomes, nanoemulsions, and the combination of the adjuvants have been designed, but their biopharmaceutical attributes need to be further validated (35, 37). In our E.G7/OT-1 animal model, we found that the critical time for T-cell activation is the first 3 days after adoptive therapy. To observe the biological effects of curcumin within this short period of time, we applied the intraperitoneal injection, instead of the oral administration, to achieve the higher maximum level in the blood circulation.

Other reports and our previous data have shown that multiple administration of 70 to 120 mg/kg curcumin slightly inhibits tumor growth and attenuates inflammation of normal tissues in small animal models, suggesting that this dosage has low to moderate toxicity to tumors and a moderate transferred CD8+ T cells was also slightly, but not significantly, elevated as compared with those of the 5 MT group. Furthermore, the augmentation of T-cell response by curcumin in mice was antigen specific because only the baseline number of transferred CD8+ T cells was detected in nontarget EL4 tumors (Supplementary Fig. S2). However, effects of curcumin on other immune cells may also exist. Dendritic cells (DC) are essential for the induction of adaptive immunity responsible for tumors, but also promote T-cell tolerance via inhibition of T-cell functions in certain circumstances (40). Although the exact mechanism to suppress T cells used by DCs remains poorly understood, some work has shown that murine and human IDO-expressing DCs in TDLNs are able to degrade tryptophan and create profound T-cell anergy (41, 42). Interestingly, curcumin also inhibits IDO significantly in IFN-γ–stimulated murine bone marrow–derived DCs in vitro, resulting in reversing IDO-mediated suppression of T-cell function (43). Therefore, the effect of curcumin on IDO-expressing DCs of the host may be another mechanism contributing to enhanced efficacy of adoptive therapy and is worthy of further study.

We observed the improvement in accumulation and activation of transferred T cells in target tumors by...
curcumin, but this important information from flow cytometric analysis only provided a snapshot of the immune response. Recent advances in molecular imaging, including MRI, PET, SPECT, and optical imaging, offer powerful strategies to examine immune cell trafficking patterns and their functional status in living organisms, providing dynamic information which otherwise is unavailable by conventional techniques, such as histology, reverse transcriptase PCR, and flow cytometry. However, most publications about T-cell imaging have been limited to invasive imaging with intravitral microscopy or noninvasive imaging of T-cell locations and then functional activity of transferred T cells is implied if tumor size is reduced (44–46). Recently, 18F-FAC, a new PET imaging probe, has been shown with increased accumulation in proliferating T cells through the deoxycytidine salvage pathway (47–49). Their results indicate that 18F-FAC is more sensitive to alterations in lymphoid mass and immune status than 18F-FDG. However, the retention in lymphoid organs and tumor uptake of 18F-FAC hampers the detection of weak immune responses at these sites (45). Ponomerev and colleagues created another PET imaging probe by placing GFP and herpes simplex virus type 1 thymidine kinase genes under the control of a nuclear factor of activated T cells enhancer element and successfully accelerated the study of adoptive immunotherapy in preclinical cancer models. In addition, our study provides the rationale that the combined therapy of CD8+ T cells with multitargeting drugs, such as curcumin, may open the possibility for clinical investigation in cancer patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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