World Journal of Clinical Oncology

Online Submissions: http://www.wjgnet.com/2218-4333office
wjco@wjgnet.com
doi:10.5306/wjco.v1.i1.3

World J Clin Oncol 2010 November 10; 1(1): 3-11
ISSN 2218-4333 (online)

EDITORIAL

Cytotoxic T-cells as imaging probes for detecting glioma

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Author contributions: Arbab AS solely contributed to this paper.
Supported by NIH grants, No. R01CA122031 and No. 1R21-CA129801
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Received: May 4, 2010 Revised: September 4, 2010
Accepted: September 11, 2010
Published online: November 10, 2010

Abstract

Tumor vaccination using tumor-associated antigen-primed dendritic cells (DCs) is in clinical trials. Investigators are using patients' own immune systems to activate T-cells against recurrent or metastatic tumors. Following vaccination of DCs or attenuated tumor cells, clinical as well as radiological improvements have been noted due to migration and accumulation of cytotoxic T-cells (CTLs). CTLs mediated tumor cell killing resulted in extended survival in clinical trails and in preclinical models. Besides administration of primed DCs or attenuated or killed tumors cells to initiate the generation of CTLs, investigators have started making genetically altered T-cells (CTLs) to target specific tumors and showed *in vivo* migration and accumulation in the implanted or recurrent tumors using different imaging modalities. Our groups have also showed the utilization of both *in vivo* and *in vitro* techniques to make CTLs against glioma and used them as imaging probes to determine the sites of tumors. In this short review, the current status of vaccination therapy against glioma and utilization of CTLs as *in vivo* imaging probes to determine the sites of tumors and differentiate recurrent glioma from radiation necrosis will be discussed.

Key words: Cellular magnetic resonance imaging; Cytotoxic T-cells; Glioma; Glioma associated antigen; Imaging probes; Magnetic resonance imaging; Tumor vaccine

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Arbab AS. Cytotoxic T-cells as imaging probes for detecting glioma. *World J Clin Oncol* 2010; 1(1): 3-11 Available from: URL: http://www.wjgnet.com/2218-4333/full/v1/i1/3.htm DOI: http://dx.doi.org/10.5306/wjco.v1.i1.3

CELL BASED VACCINATION THERAPIES FOR GLIOMA

Tumor vaccination therapy has been used in active investigations for the last 50 years. Different strategies have been employed to activate or sensitize host T-cells against tumor antigen to eradicate malignant tumors. Investigators have used attenuated or irradiated intact tumor cells with or without adjuvant to initiate an immunogenic reaction[1-3]. These attenuated cells were injected subcutaneously or in the food pad of animals to initiate priming of antigen presenting cells (APC/s) and production of cytotoxic T-cells (CTLs) in the nearby draining lymph nodes. To attract more T-cells for the purpose of sensitization, different cytokines have already been tried during administration of attenuated cells or cell lysate[4-7].

In vitro priming of APCs is another popular way to activate immune systems against tumor antigens. These APCs are collected from peripheral blood, bone marrow or from cord blood[8-12]. APCs represent macrophages and mononuclear cells such as monocytes. These cells are converted to immature and mature dendritic cells (DC/s). During the process of making DCs, tumor specific antigen, either in the form of tumor cell lysate, apoptotic tumor cells or tumor-associated antigenic peptide or proteins are added to the media and then these cells, after priming, are administered systemically or locally to initiate the production of CTLs. Investigators have reported a varying degree of suc-

cess in treating recurrent, metastatic or primary malignant tumors of different origins both in preclinical models and in clinical trials.

Malignant glioma is one of the most aggressive tumors with a poor prognosis despite available treatments including surgery, chemotherapy and radiation therapy[13]. Standard treatment procedures, consisting of surgery and radiation therapy (followed by adjuvant chemotherapy), very often fail due to the inability to accurately delineate tumor margins[14-16], and the median survival time for patients with recurrent glioblastoma multiforme (GBM) is less than 1 year[17]. The infiltrative nature of GBM is considered to be one of the main factors impeding the complete removal of tumor mass by surgical procedure[18]. Following radiation therapy or surgery, recurrence is common and almost invariably occurs within < 2 cm of the prior resection line, which is due to leftover tumor or tumor cells. Dendritic cell-based vaccination therapy against recurrent glioma that utilizes the patient's own DCs which are pulsed, *ex-vivo*, with the derived glioma cell-lysate is currently in clinical trials[19-22]. In experimental glioma models, an increase in the number of CTLs compared to control or pre-vaccination levels is observed following the administration of glioma cell-lysate-pulsed DCs. Investigators have identified specific glioma-associated antigens, which are being used to pulse DCs[23-25]. There are many (at least 10) active clinical trials running, sponsored by the National Cancer Institute, and targeting primary as well as recurrent glioma that utilize primed DC-based vaccination (www.cancer.gov/clinicaltrials). Apoptotic tumor cells, tumor cell lysate and glioma-associated antigens (peptides) are being used to prime the DCs in these clinical trials.

GLIOMA ASSOCIATED ANTIGENS AND PRIMING OF DENDRITIC CELLS

Making DC-based vaccine using tumor cell lysate is relatively non-specific and primed DCs may initiate CTL production which may attack other normal cells and tissues in the body. Investigators have tried to find specific antigens that could be used to make primed DCs and specific CTLs after vaccination. Different antigens have been isolated from different malignant tumors which are expressed specifically for the types of tumors. Brugger *et al*[26] used MUC1 derived HLA-A2 restricted peptides to pulse DCs and produced CTLs *in vitro* to target blasts of acute myeloid leukemia (AML). The generated CTLs showed *in vitro* effectiveness against AML and multiple myeloma cell lines. Other investigators used leukemia-associated antigens as well as apoptotic cells to generate antigen-primed or tumor cell-primed DCs, respectively, for vaccination therapy in AML[27-31]. Malignant melanoma is one of the most widely investigated tumors for cell-based vaccination therapy[32-34]. The most widely used proteins/peptides to prime DCs are human melanoma antigen-A3 (MAGE-3), melanoma antigen MART-1/Melan-A (MART-1), gp100 and tyrosinase[35-37].

The method of priming DCs using tumor-associated antigens (TAA) is more specific than priming with whole tumor cell lysate. Investigators have identified tumor specific antigens (peptides) and these antigens can be used to pulse DCs to initiate antigen specific CTLs when administered into hosts. Zhang *et al*[25] have profiled the antigens in 20 different types of human glioma cell lines and concluded that all the cells exhibited multiple TAA which can be used to prime DCs to initiate production of CTLs. The authors identified a few important antigens, such as, melanoma-2 (Aim-2), B-cyclin, EphA2, GP100, h1, 6-N-acetylglucosaminyltransferaseV (GnT-V), IL13Ra2, Her2/neu, hTert, Mage, Mart-1, Sart-1, and survivin. Based on their results, Dr. Okada's group (at UPMC) and other investigators have identified three important antigens (EphA2, IL13Ra2, survivin) for priming DCs and used as vaccines for glioma treatment[38,39]. NCI sponsored clinical trials are underway to make GAA-pulsed DCs for vaccination in patients with recurrent glioma. In these proposals, the investigators prime the autologous DCs with specific peptides by simple incubation during the conversion of adherent peripheral blood mononuclear cells to mature DCs.

It is obvious from the above discussions that DC-based vaccine is about to be used in clinical practice as an adjuvant therapy for the treatment of different malignant tumors with immunogenic properties. Patient-specific or tumor-specific personalized DC-based vaccine can also be designed using mRNA loading[23,40,41]. However, there has been no discussion or report indicating how to utilize the DC-based technique to make tumor-specific CTLs *ex vivo* and use them as probes for imaging to identify recurrent or metastatic tumors or to differentiate glioma from radiation necrosis in the brain. The following paragraphs will discuss the methods to make CTLs *ex vivo* and to utilize them as probes for detecting tumors and differentiating from radiation necrosis.

MAKING OF CTLs AS PROBES FOR IMAGING

The making of CTLs *ex vivo* involves different stages of experimental procedures. First, autologous or allogeneic primed DCs need to be generated and then collected T-cells should be sensitized against target antigens or peptides. These CTLs can then either be tagged with radioisotopes for tracking with nuclear medicine imaging techniques or can be labeled with different MRI contrast agents to be tracked by MRI. The following describe the procedures for making primed DCs and CTLs for the purpose of using these CTLs as probes for imaging.

Making of primed dendritic cells

There are many ways to make primed DCs-pulsed with tumor-specific antigens. The most widely used method is to pulse autologous or allogeneic DCs with the tumor lysate that are generated from tumor tissues collected from patients[19,42,43]. In this process, the patent's peripheral blood mononuclear cells are collected and DCs are generated from either selecting CD14 positive cells or by selecting adherent cells in culture dishes or plates. These cells are cultured in the presence of granulocyte colony

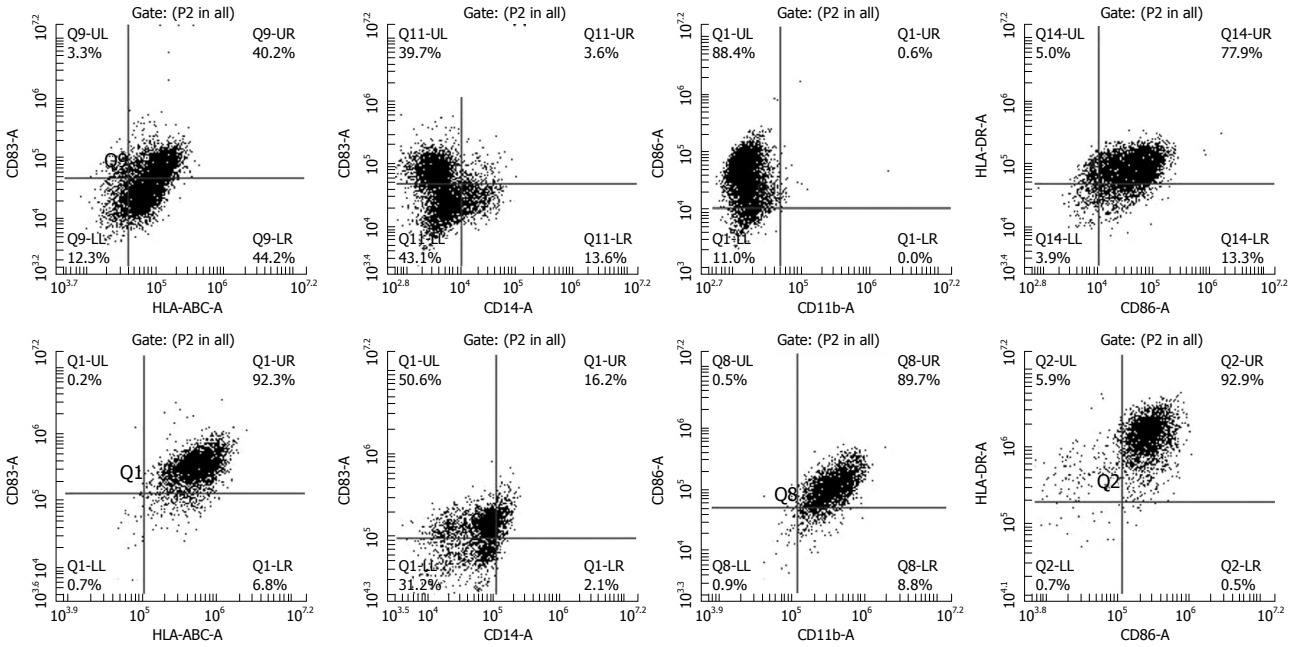

Figure 1 Phenotypic analysis of immature (upper panel) and mature dendritic cells (lower panel). CD14+ cells collected from cord blood were incubated in immature dendritic cell (DC) media (RPMI-1640 plus 10% FBS) containing granulocyte colony stimulating factor (G-SCF) and IL-4 for 8 d and then cells were incubated with tumor necrosis factor (TNF)-α in addition to G-CSF and IL-4. Cells show typical monocytic DC markers (low CD14, high HLA-ABC, HLA-DR, CD86, CD83 and CD11b). Note the increase in the population of CD83 positive cells, a marker of mature DCs, after addition of TNF-α.

stimulating factor (G-SCF) and interleukin-4 (IL-4), which makes immature DCs. To convert these immature DCs (which show high expression of CD86, HLA-DR, HLA-ABC and low expression of CD14) to mature DCs, cells are then cultured in the presence tumor necrosis factor (TNF)-α in addition to G-CSF and IL-4. During the process of making DCs, tumor lysate is added to the cultures. We have extensively studied the method of making tumor lysate-pulsed DCs to make CTLs *ex vivo* using cord blood derived CD14+ and CD2/3+ cells, respectively. Figure 1 shows the phenotypical changes that happen to CD14+ cells during the process of making tumor lysate-pulsed mature DCs. One of the phenotypical markers of mature DCs is CD83. DCs can also be primed using autologous or allogeneic killed or apoptotic tumor cells[44,45]. DCs can phagocytose the tumor cells and eventually express the antigen on the surface. Based on this idea, investigators also made hybrid cells combining DCs and tumor cells, and administered them into the host to initiate production of CTLs[46,47]. For quick and optimal expression of target antigen by DCs, electroporation methods are also used to prime the DCs[40,41,43]. From the clinical point of view, the electroporation method could be suitable for any good manufacturing practice (GMP) grade production of primed DCs for tumor vaccine therapy. A viral-based transfection technique has also been applied to transduce tumor mRNA into DCs for the purpose of vaccine[48-50].

Making of CTLs

CTLs sensitized to specific tumor antigen can be produced both *in vivo* and *in vitro* conditions. All of the clinical trials and animal experiments that utilized tumor specific antigen-pulsed DCs for vaccination, in fact, relied on the *in vivo* production of CTLs. The production of tumor-specific CTLs has been detected in all patients and animals. Investigators have pointed out that the tumor specific CTLs express different T-cells markers, such as CD25, CD4, and CD8[51-54]. We also have analyzed splenocytes collected from tumor (9L gliosarcoma) bearing Fisher-344 rats by flow cytometry, and increases in the population of CD4, CD8, CD80 and CD86 positive cells were observed[55]. The number of these cell populations was significantly different from the splenocytes collected from control (non-tumor bearing) rats, indicating the production of CTLs in tumor bearing rats. By co-incubating primed DCs with collected CD2/3 positive cells, tumor antigen specific CTLs can also be produced *ex vivo*. Our recent investigations showed that CTLs can be used to make imaging probes to detect tumors[55]. However, there has been no report showing the utilization of *ex vivo* produced CTLs for the purpose of diagnostic imaging to detect recurrent or residual tumors. Investigators have used genetically modified CTLs (*in vitro* manipulated) to detect the migration and accumulation in residual GMB[56] in humans and in a rat model of glioma[57]. Kircher *et al*[58] utilized B16-OVA-specific CD8+ to detect the migration and accumulation of magnetically labeled CTLs to the sites of B16-OVA melanoma. All these investigators used CTLs as probes for imaging, which were not collected from the host. We routinely produce CTLs *ex vivo* using cord blood derived primed DCs pulsed with tumor cell lysate and T-cells for the purpose of imaging to detect and differentiate different diseases. The following are the brief descriptions of our procedures: CD14+ and CD2+/CD3+ cells: Both types of cells are isolated from human cord blood under the approved IRB protocol. CD14+ positive cells are separated from other mononuclear cells (cord

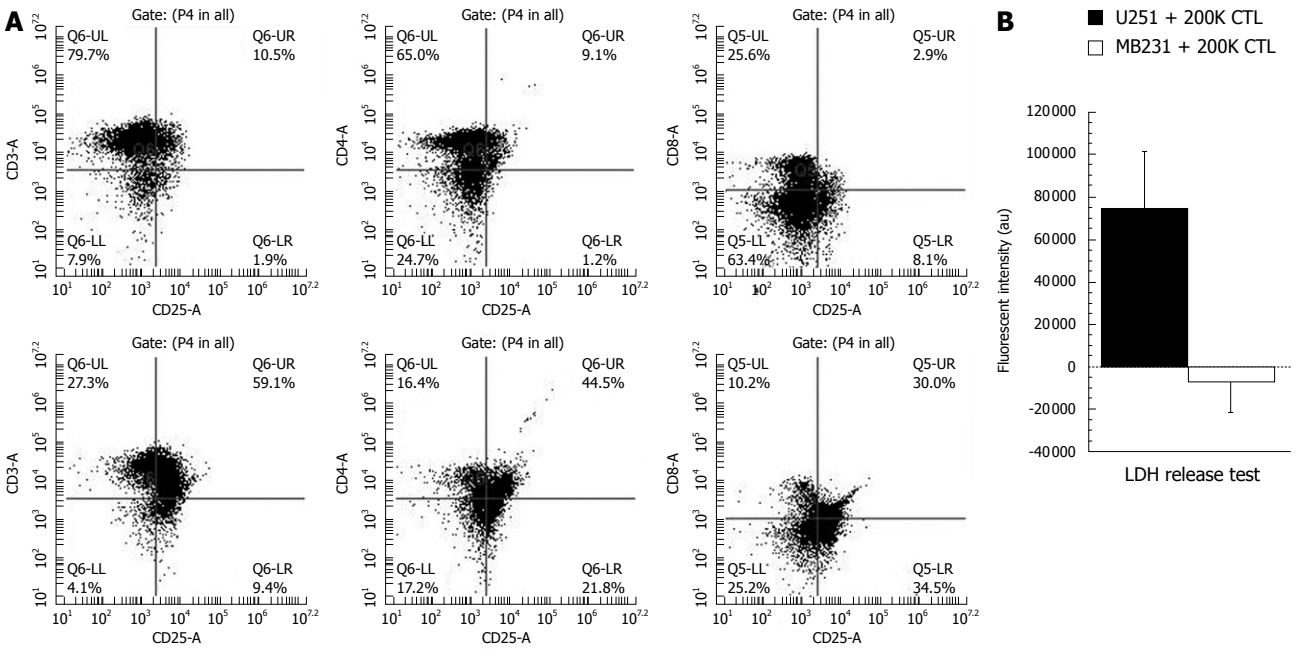

Figure 2 Phenotypic analyses of sensitized T-cells (cytotoxic T-cells) and cytotoxic activity of produced cytotoxic T-cells. A: Analysis of T-cell markers. Phenotypic analysis of control T-cells (upper panel) and T-cells co-incubated with tumor lysate-pulsed irradiated mature dendritic cells (DCs) for 4 d (lower panel). Note the increased number of CD25+ cells (activated T-cells) after sensitizing them with DCs; B: To determine the cytotoxic specificity of the produced cytotoxic T-cells (CTLs) to U251 cells, 200 000 (200K) CTLs (sensitized to U251 cell lysate) were co-cultured overnight with U251 (100 000 cells) or human breast cancer cells (MBA-MD-231, 100 000) and the released lactate dehydrogenase (LDH) was determined by a commercially available membrane integrity assay kit (Cyto Tox-ONE, Promega Corp, WI, USA). LDH levels indicate cytotoxicity since LDH is released to the media once cell membranes are damaged. Note the significantly ($P \leq 0.05$) increased LDH release from U251 cells indicating the specificity of produced CTLs.

blood mononuclear cells are obtained using a Ficoll gradient separation technique) by magnet activated cell sorter (MACS) using magnetic beads conjugated with anti-CD14 antibodies. CD14 depleted cells were further incubated with anti-CD2 antibodies conjugated to magnetic beads to separate CD2+/CD3+ cells. Collected CD14+ cells were further differentiated into DCs (as described below).

Preparation of tumor cell lysate-pulsed mature dendritic cells

CD14+ cells are resuspended at the concentration of 3-5 $\times 10^5$ cells/mL in RPMI 1640 media containing 10% FBS, 25 ng/mL of IL-4, 50 ng/mL of G-CSF (granulocyte colony-stimulating factor) and incubated in 5% CO_2/95% air at 37℃ in a humidified incubator for 4 d to make immature DCs. During this incubation period 1/2 of the media is replaced with 2/3rd of fresh media containing cytokines on day 3. On day 5, suspended and loosely adherent cells are collected, centrifuged and resuspended in fresh RPMI 1640 media containing 10% FBS, 25 ng/mL of IL-4, 50 ng/mL of GM-CSF and 50 μg/mL of tumor cell lysate at 5 $\times 10^5$ cells/mL. The cells are thoroughly mixed and further incubated for 4 d. At the end of the 4 d priming, cells are collected and resuspended in fresh media containing 10% FBS, 25 ng/mL of IL-4, 50 ng/mL of GM-CSF and 100 ng/mL of TNF-α and incubated for an additional 4 d. Expression of different markers specific for DCs (such as CD14, CD86, CD83, CD11b, HLA-ABC and HLA-DR) are assessed by flow cytometry before and after addition of TNF-α.

Sensitization of isolated T-cells

Either fresh or cryopreserved T-cells that are isolated from cord blood are cultured overnight in RPMI 1640 media containing 10% FBS, sodium pyruvate, non-essential amino acids, L-glutamine and 10 ng/mL of IL-2 and then co-cultured with irradiated (35 Gy) cell lysate-pulsed mature DCs for 5-6 d. The initial ratio of T-cell to DC was 10:1. T-cell proliferation is monitored every day and based on the cell density; fresh media is added to the co-culture. Phenotypical expression of different T-cell markers (CD3, CD4, CD8, and CD25) is determined by a flow cytometer before and after sensitization, as well as after the magnetic labeling of CTLs. Specificity of CTLs is also determined by a lactate dehydrogenase (LDH) release assay. Figure 2 shows the markers of CTLs and their cytolytic specificity.

Making of CTLs as probes

Commercially available, FDA-approved super paramagnetic iron oxides (SPIO) ferumoxides suspension (Feridex Ⅳ ®, Bayer-Schering Pharmaceuticals Inc, Wayne, NJ, USA) contains particles that are approximately 80-150 nm in size and has a total iron content of 11.2 mg/mL (11.2 μg/μL of iron). Protamine sulfate (American Pharmaceuticals Partner Inc. Schaumburg, IL, USA), supplied at 10 mg/mL, was prepared as a fresh stock solution of 1 mg/mL in distilled water at the time of use. We magnetically label CTLs using our published method, in brief ferumoxides (100 μg/mL) is directly added to the cell suspension in serum free media and then protamine sulfate (3 μg/mL) is added[59]. The FePro complexes are formed

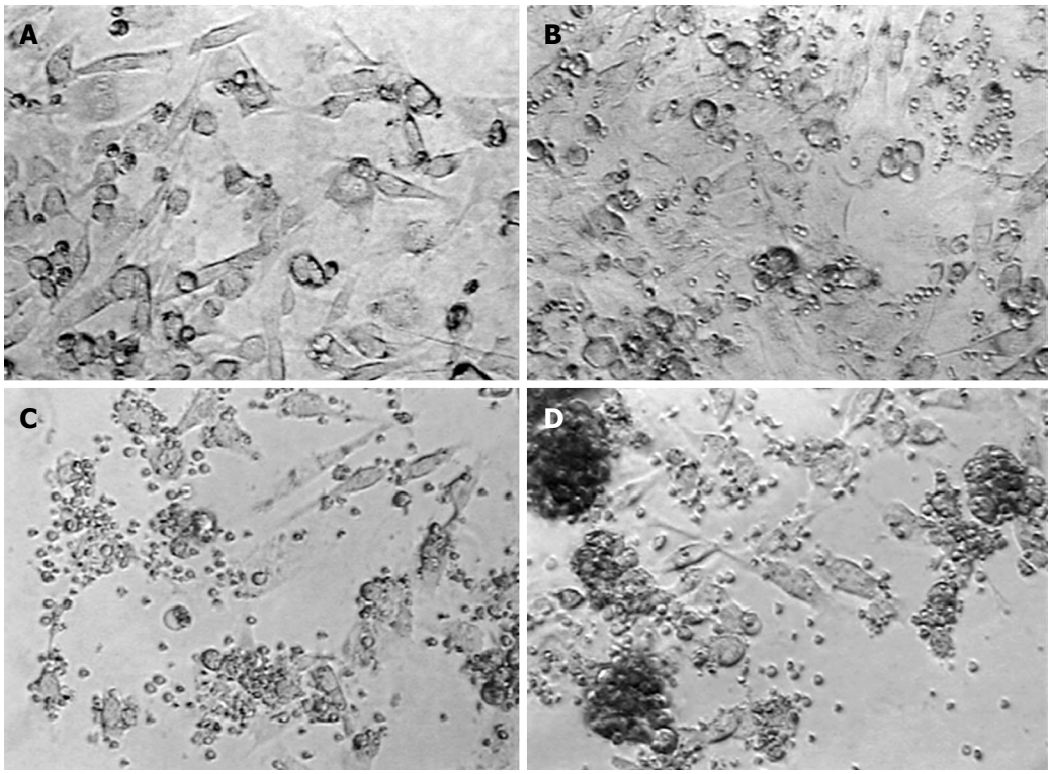

Figure 3 The interaction of cytotoxic T-cells (labeled and unlabeled) with U251 glioma cells. Cytotoxic T-cells (CTLs) were produced using U251 cell lysate-pulsed irradiated mature dendritic cells. A: Normal U251 cells; B: Control T-cells; C:Unlabeled CTLs targeting U251 cells; D:Labeled CTL U251 cells,ferumoxide-protamine sulfate (FePro) labeled CLTs were co-incubated with U251 overnight.

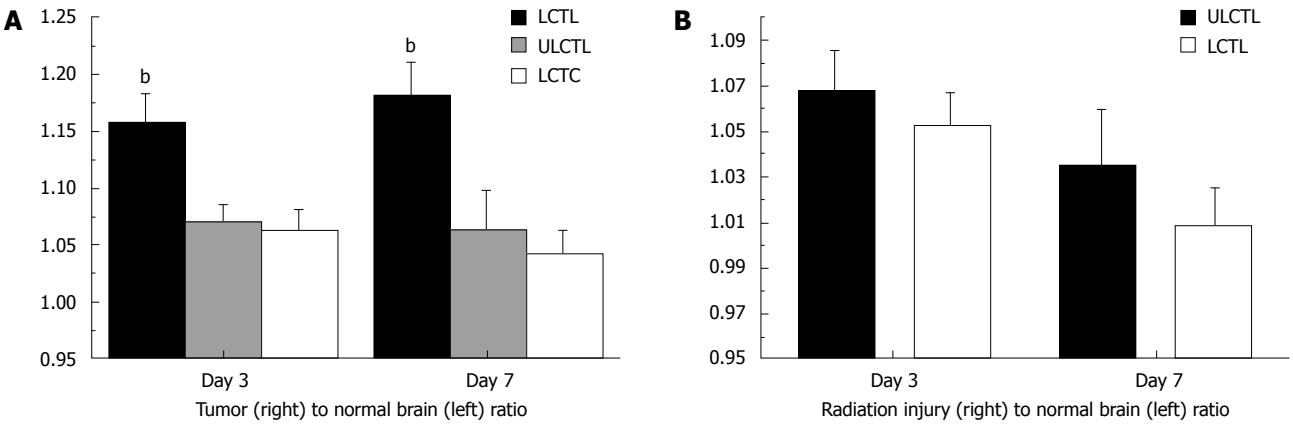

Figure 4 Accumulation of labeled cytotoxic T-cells in implanted U251 tumor and radiation injured sites in rat brain. A: Analyses of R2* values normalized to contralateral normal hemisphere (indirect indicator of the accumulation of iron positive cells) showed significantly higher ($^{b}P \leqslant 0.001$) accumulation of iron positive cells in tumor that received labeled cytotoxic T-cells (LCTL) compared to that of labeled control T-cells (LCTC) and unlabeled CTLs (ULCTL). The number of accumulated cells was higher at both day 3 and 7; B: Similar analyses of R2* values in radiation injured brain normalized to contralateral normal hemisphere showed no difference between the groups of animals that received labeled and unlabeled CTLs.

in the cell suspension. After 15 min of incubation in serum free media, an equal volume of complete media (containing serum) is added to the cell suspension and further incubated for 4 h.

Determination of specificity of labeled and unlabeled CTLs

To determine whether the produced CTLs have the specificity to target tumor cells *in vitro* and to determine whether FePro labeling alters this specificity, a specific number of CTLs (labeled or unlabeled) and control T-cells were co-incubated with target tumor cells. The interaction (accumulation of the added T-cells around tumor cells) of the added CTLs or T-cells with tumor cells was photomicrographed at 0 and 18 h of co-culture. Figure 3 shows the interaction of tumor cells and CTLs. We have not seen any changes in the specificity of CTLs following labeling with FePro for imaging purposes.

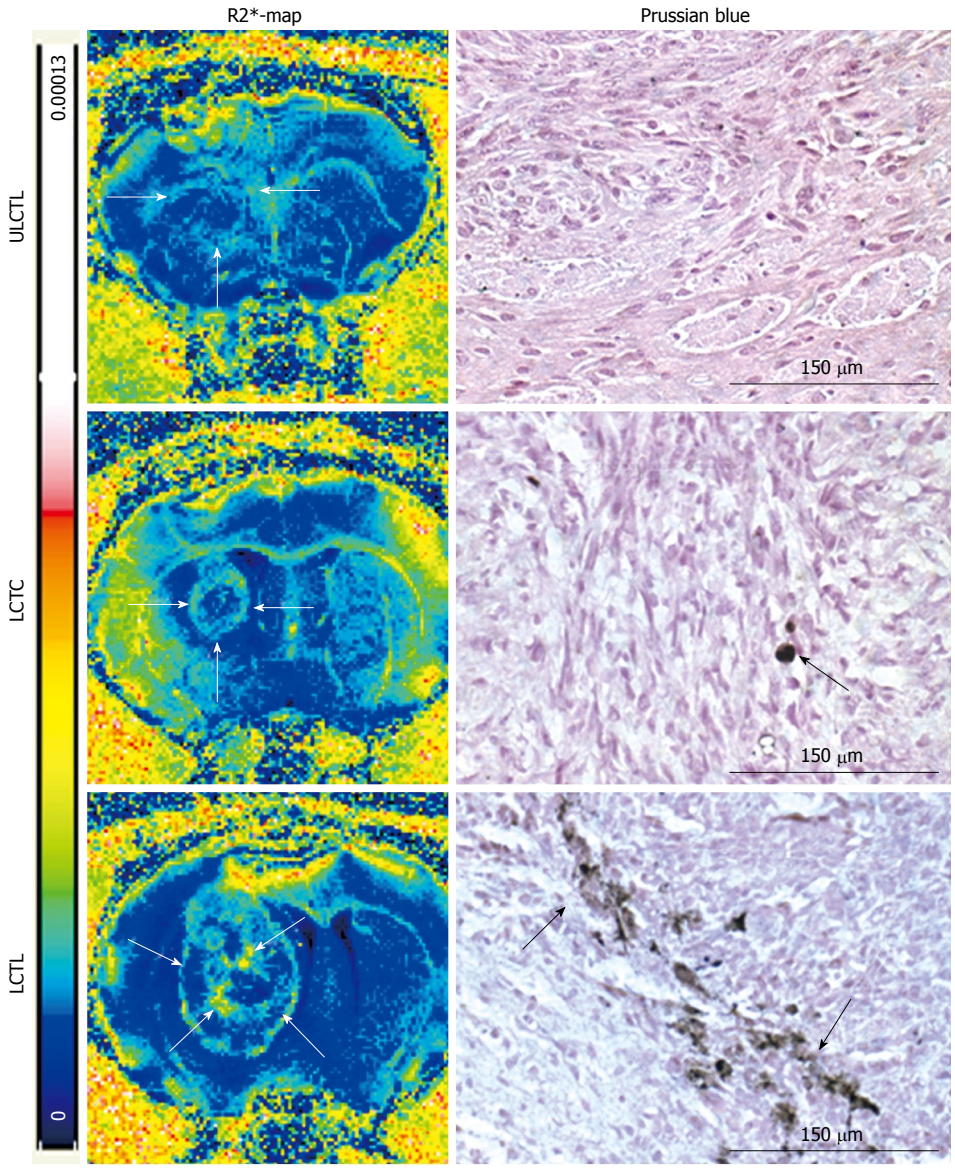

Figure 5 Magnetic resonance imaging relaxivity maps and Prussian blue staining. R2* maps and DAB enhanced Prussian blue staining from representative animals that received unlabeled cytotoxic T-cells (CTLs) (ULCTL, upper row), labeled control T-cells (LCTC, middle row) and labeled CTLs (LCTL, lower row). R2* maps show high signal intensity areas only in tumors that received LCTC and LCTL. Animals that received LCTL show high signal intensity areas both at the peripheral and central part of the tumors (arrows). Corresponding DAB enhanced Prussian blue staining show multiple Prussian blue positive cells in tumors that received LCTL (arrows). There are a few Prussian blue positive cells seen in tumors that received LCTC (arrow). No definite Prussian blue positive cells were seen in tumors that received ULCTL.

Applications of CTLs as imaging probes

Lymphocytes has been in use for decades to detect different disease conditions, such as xenografted tumor in rodents, renal allograft, autoimmune thyroid disease, metastatic melanoma, *etc*[60-63]. Autologous lymphocytes have been labeled with radioactive isotopes and accumulation at specific sites has been detected by nuclear medicine imaging[64,65]. Autologous lymphocyte labeling with radioactive isotopes, such as In-111-oxine and administration into patients are FDA approved procedures for diagnostic purposes. Chin *et al*[66] has reported the utilization of tumor infiltrating lymphocytes collected from resected tumor specimens and expanded *ex vivo* using recombinant IL-2 as imaging probes by labeling with In-111-oxine. However, the authors did not notice any accumulation of In-111 labeled lymphocytes at the sites of metastasis. Lymphocytes have also been labeled with iron oxides to track the migration by magnetic resonance imaging (MRI), however, there has been no report of making tumor specific CTLs *ex vivo* for the detection of tumors by *in vivo* imaging. Previously our group reported the making of sensitized splenocytes (CTLs) *in vivo* in syngeneic Fisher-344 rats by implanting 9L gliosarcoma cells. These CTLs were collected from spleen and used as imaging probes to detect the implanted tumor in another set of rats carrying the 9L glioma in the brain[55]. The *in vivo* produced CTLs showed specificity by accumulating in and around the implanted tumors, whereas splenocytes collected from control rats did not show significant accumulation in the implanted tumors. The CTLs (sensitized splenocytes) were also able

to differentiate implanted tumor from radiation necrosis as there was no accumulation at the sites of radiation injury (necrosis). Based on the results of sensitized splenocytes, we have started making CTLs in the *ex vivo* setting to sensitize T-cells against implanted U-251 glioma using glioma cell lysate-pulsed DCs. Our preliminary results are very encouraging (Figures 4 and 5) and can be translated to clinics after proper IND and FDA approval. In this study, we magnetically labeled *ex vivo* produced CTLs and injected them intravenously into rats bearing U251 glioma or radiation injury. Our main hypothesis was to prove that CTLs would specifically accumulate at the sites of glioma. We used MRI to detect the accumulated CTLs in the tumors. The results showed a significantly higher number of CTLs accumulated in U251 glioma and there was no significant accumulation of CLTs at the sites of radiation injury. The CLTs can be used to differentiate recurrent glioma from radiation necrosis. This study is underway.

CONCLUSION

An *ex vivo* technique at a GMP grade laboratory can be utilized to produce tumor specific CTLs and these CTLs can be used as cellular imaging probes to detect sites of recurrent or residual tumors. Personalized treatment is becoming a key word for current trends in cancer treatment. Because of polymorphism and the chance of mutation in tumor cells in an individual patient, our technique can be utilized to create cellular imaging probes to detect patient-specific recurrent or residual tumors and the treatment strategy can be changed based on the image findings.

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