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Activated CD4⁺ T Cells Dramatically Enhance Chemotherapeutic Tumor Responses In Vitro and In Vivo

Soroosh Radfar,¹ Yixiang Wang,² and Hung T. Khong³

Chemoimmunotherapy has been widely studied in melanoma, with various degrees of success. One of the most common approaches is the so-called biochemotherapy, which is associated with increased toxicities, but without overall survival benefit. Another conventional strategy is the use of chemotherapy as an immunomodulator to enhance the effect of cancer vaccines or adoptive cell transfer therapy. Based on this approach, recent studies using chemotherapy to prepare the host before the infusion of ex vivo-activated, melanoma Ag-specific tumor-infiltrating lymphocytes and high dose IL-2 resulted in an impressive response rate. However, the development of immunotherapy for the treatment of a broad range of cancer type is still lacking. In this study, we report the development of a simple yet universal approach termed “chemocentric chemoimmunotherapy” that has potential application in the treatment of all cancer types. This technique uses nonspecifically activated CD4⁺ T cells as a chemosensitizer before the administration of chemotherapy. Dramatic enhancement of the cytotoxic effect of chemotherapeutic drugs, either active or nonactive as single agents, was observed both in in vitro and in vivo human tumor xenograft models. Soluble factors secreted from activated CD4⁺ T cells, likely acting on the tumor and its microenvironment, were responsible for the observed effect. Although IFN-γ played a major role in the therapeutic outcome, it was consistently found to be inferior to the use of activated CD4⁺ T cells in tumor chemosensitization. Our model may provide a plausible mechanism to facilitate further understanding, design and development of improved chemoimmunotherapy in the treatment of cancer. The Journal of Immunology, 2009, 183: 6800–6807.

Currently, there is no cure for most patients with advanced or metastatic cancer. In addition, despite the effectiveness of conventional chemotherapy and targeted agents, many treated patients experience tumor progression and ultimately die of their disease. Therefore, there is an urgent need for better treatments, not only for advanced disease but also to prevent relapse.

Immunotherapy can play an important role in the treatment of cancer, and combinations of chemotherapy and immunotherapy have been investigated in many clinical trials. Chemoimmunotherapy or biochemotherapy has been extensively studied in melanoma. Conventional chemoimmunotherapeutic strategies for melanoma include two major categories. The first category uses cytokines such as IL-2 and/or IFN-α in combination with chemotherapy. This experimental strategy was not supported by strong preclinical evidence and has not been shown to be superior to chemotherapy alone (1–5). The second category is a strategy that we have termed “immunocentric chemoimmunotherapy”. In this approach, the primary or central focus is on immunotherapy, with chemotherapy playing a peripheral role in immunomodulation. Using this approach, melanoma patients have been successfully treated with nonmyeloablative, lymphodepleting chemotherapy and adoptive cell transfer with tumor Ag specific lymphocytes and high dose IL-2 (6, 7). Although this strategy is promising, it is not widely applicable to nonmelanoma cancers due to the difficulty in generating large number of tumor-Ag specific tumor-infiltrating lymphocytes.

Activated T cells elaborate cytokines that are essential for an effective immune response (8). These include IL-2, TNF-α, IL-21 (by activated CD4⁺ T cells), GM-CSF, IFN-γ, and other as yet identified cytokines. In addition to their immune modulatory activities, some cytokines can also have direct effects on tumor cells and/or tumor vasculature. TNF-α is known to induce hemorrhagic necrosis in tumors (9, 10). In addition, TNF-α has also been shown to induce apoptotic and necrotic tumor cell death in vitro (11–14). The hemorrhagic necrotic effect of TNF-α has been shown to be more potent when used in combination with chemotherapy in a rat sarcoma model (15). However, in these studies, TNF-α had no direct activity against tumor cell lines in in vitro assays, and demonstrated no synergy with chemotherapy (15), acting instead on the tumor vasculature (16, 17). The IFNs can exert direct effects on the proliferation, differentiation, and apoptosis of tumor cells (18–22). However, the response to the IFNs varies considerably, depending on the tumor histology, and resistance to the IFNs has been reported in several tumor types (23–26).

We have developed a third strategy for combining chemo- and immuno-therapy, that we term “chemocentric chemoimmunotherapy”. In this model, chemotherapy plays the central effector role, while immunotherapy is used to sensitize the tumor and its microenvironment to the cytotoxic effect of chemotherapy. This strategy employs the use of nonspecifically activated CD4⁺ T lymphocytes (aCD4⁺) to presensitize tumor cells, followed by treatment with chemotherapeutic drugs. The rationale for this strategy

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4 Abbreviations used in this paper: aCD4, activated CD4⁺ T cell; CM, complete medium; TMZ, temozolomide; Carbo, carboplatin; Pax, paclitaxel; RFU, relative fluorescence signal; CI, combination index; AU, arbitrary unit.

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is the known ability of activated T cells to secrete multiple cytokines that can regulate proliferation and/or apoptosis of tumor cells, and the ability of activated T cells to exert direct activity on tumor cells through apoptotic pathways such as the Fas/Fas ligand pathway. This model does not depend on Ag-specific activation of T cells, and is therefore potentially applicable to all tumor types and all patients regardless of their HLA status, with the added advantage of ease of use and cost effectiveness for clinical use.

In the current study, we document that presensitization of tumor cells with aCD4 before the administration of chemotherapeutic drugs, either active or nonactive as single agents, dramatically enhances the cytotoxic effect of these drugs both in vitro and in vivo using human tumor xenograft models.

Materials and Methods

Cell lines and culture conditions

A375, 526mel, and 1938mel human melanoma cell lines were obtained from the Surgery Branch, National Cancer Institute. MCF7 and MDA-MB-231 human breast, DU145 human prostate, HCT116 human colon cancer cell lines were purchased from American Type Culture Collection (ATCC). All cell lines were cultured in RPMI 1640 (Mediatech) supplemented with 10% FBS (Atlanta Biologicals), 1 U/ml penicillin, and 1 µg/ml streptomycin (Mediatech) (hereafter referred to as “complete medium” or CM). All cell lines were maintained in a humidified incubator at 37°C with 5% CO2.

Chemotherapeutic drugs

All chemotherapeutic drugs were obtained through the medical oncology service (Mitchell Cancer Institute) and the University of South Alabama Medical Center pharmacy. Temozolomide (TMZ) was dissolved in DMSO, diluted in CM or PBS, and used at 750-1000 µM (in vitro use) or 100 mg/kg (in vivo use). Carboplatin (Carbo) was diluted in CM or PBS and used at 100–300 µM. Paclitaxel (Pax) was diluted in CM or PBS and used at 0.5–3 µg/ml (in vitro use). 10 µg/ml (in vivo use). 5-FU was diluted in CM or PBS and used at 400 µM.

PBMC and CD4+ T cell isolation and activation

Buffo cell preparations from healthy donors were purchased from the regional American Red Cross for in vitro use. PBMC were isolated from the buffy coat using Ficoll-Paque Plus (GE Healthcare) and frozen before use. For in vivo study, PBMC from melanoma patients was obtained from the Surgery Branch, NCI. CD4+ T cells were isolated negatively from PBMC, using the “CD4+ T cell isolation kit II” (Miltenyi Biotec). CD4+ T cells were activated immediately after isolation. The activation was performed overnight, using 24-well plate coated with 5 mg/ml anti-CD3 (OKT3, Orthobiotech) and 1 mg/ml anti-CD28 (EBioscience). aCD4 were harvested and used immediately for in vitro or in vivo experiments or labeled with a FITC-conjugated mouse anti-human CD4+ T cells (EBioscience) and analyzed for purity by flow cytometry (FACS Vantage, BD Biosciences). The activation of CD4+ T cells was determined by IFN-γ secretion in an ELISA. All ELISA reagents, mAbs, and recombinant cytokines were purchased from Pierce. The ELISA was performed according to the manufacture’s instructions.

Cytokine array

The concentration of thirteen common cytokines secreted by aCD4 was analyzed. CD4+ T cells were activated overnight as described above, collected, washed, and transferred to new wells and cultured for another 48 h. The supernatants were collected and sent to LINCO Diagnostics Services for analysis of I3 common Th1 and Th2 proinflammatory and anti-inflammatory cytokines, including IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p40, IL-12p70, IL-13, IFN-γ, and TNF-α. The same supernatants were also used to determine GM-CSF concentrations, using the Human GM-CSF ELISA kit (Pierce) according to the manufacturer’s instructions.

Chemotherapy dose-response curves

Chemotherapy dose-response curves were established for each cancer cell line. In brief, cells were seeded in 96-well plates and kept overnight at 37°C. After 24 h, 48, and 72 h with WST-1 cell proliferation reagent (Roche) according to the manufacturer’s instructions. Finally, the absorbance was measured at 450 nm with an Opsys MR plate-reader (Dynex Technologies) and a killing curve was established. A suboptimal dose that decreased cell viability by 30–50% after 48-h treatment was chosen to use in additional experiments, to obtain a large window for clear observation of the effect, if any, of the experimental treatment. All experiments were performed in triplicate and repeated at least three times.

Tumor cell treatments and cell viability assay

Tumor cells were seeded in 96-well plates (1 × 10^4/well) and incubated at 37°C over night, followed by the addition of 2.5 × 10^4 aCD4 and cultured for an additional 24 h. Finally, the chemotherapeutic drugs were added. After another 24 h, the supernatants and aCD4 were removed, and the tumor cells were labeled with WST-1 cell proliferation reagent for 1 h to determine cell viability. In initial experiments, two approaches were used to eliminate any bias on the outcome of tumor cell viability assay that might have resulted from the presence of aCD4 in the coculture. The first approach was the removal of aCD4 before WST-1 cell proliferation assay. In the second approach, two additional controls, aCD4 alone with or without chemotherapy, were added. In addition, aCD4 were not removed before WST-1 cell proliferation assay. The second approach was designed to ensure that viability assay results were not falsely decreased because of accidental removal of tumor cells while removing aCD4 after the coculture period. The viability percentages from the two additional controls in the second approach were considered background noise and were subtracted from the viability percentages from treatment groups. In two independent experiments, there was no difference in the final results between the two approaches. Therefore, in subsequent experiments, we used the aCD4 removal method after the coculture period because it was a simpler approach for us. All experiments were performed in triplicate and repeated at least three times. Tumor cells without treatment, or treated with aCD4 alone, or treated with chemotherapeutic drug alone were used as controls.

Transwell assay

To determine the role of cell-cell contact vs soluble factors in our system, we used tissue culture treated HTS Transwell 96-well permeable support system with a 0.4-µm filter (Corning). In brief, 1 × 10^6 tumor cells were seeded in each well of the receiver and incubated at 37°C overnight. aCD4 cells were added to the inserts. After 24 h, chemotherapy drugs were added for another 24 h. Then, the inserts containing the aCD4 were removed from the receiver and tumor cells were labeled with WST-1 and their viability was measured as described above.

Apopotic assay

Cell death was determined in tumor cells presensitized (or not) with aCD4 and/or chemotherapeutic drugs, using a Cell Death Detection ELISA Plus kit (Roche), a photometric enzyme-immunnoassay for the qualitative and quantitative in vitro determination of cytoplasmic histone-associated-DNA fragments (mono- and oligonucleosomes). In brief, tumor cells were seeded (1 × 10^6/well) in a 96-well plate, pre-sensitized with aCD4, and/or treated with designated chemotherapeutic drugs for 12 h. Non-treated cells were used as negative control. The cells were lysed, and cell lysates were transferred to a streptavidine precoated microplate for ELISA analysis according to the manufacturer’s instructions. The absorbance was measured at 405 nm with an Opsys MR plate-reader (Dynex Technologies).

Western blot

Cells were washed three times with PBS and harvested in lysis buffer containing RIPA (Upstate Cell Signaling Solutions) and protease inhibitor mixture (Sigma-Aldrich). After incubation at 4°C for 1 h, the lysates were centrifuged at 14,000 g at 4°C for 10 min. Whole cell lysates were then incubated in SDS sample buffer (Boston Bioproducts) under reducing conditions at 100°C for 10 min. After SDS-PAGE on 4-20% gradient gel, proteins were electrotransferred onto membranes (ready gel blotting sandwiches, Bio-Rad). The membranes were then blocked with 5% skim milk in PBS-T (PBS plus 0.1% Tween 20). Membranes were incubated at 4°C overnight with one of the following Abs diluted in skim milk/PBS-T: P21 (1/2500) (BD Biosciences), Bcl-2 (1/1000) (Cell Signaling Technology). Immunoblots with anti-α-tubulin (1/3000) (Cell Signaling Technology) served as loading controls. Secondary Abs (goat anti-rabbit or goat anti-mouse; Cell Signaling Technology) were conjugated with HRP. Immunoreactive bands were detected with ECL (ECL Western Blotting Substrate, Pierce) and pictures were taken using a FujiFilm LAS 3000. The densitometric quantification of the immunoblots was performed with the AlphaEase FC Image Analysis Software (Alpha Innotech). Relative Bcl-2 expression was obtained by normalizing the densitometric value for Bcl-2 against that for α-tubulin.
Caspase-8 activity

The caspase-8 activity was measured using a caspase-8 activity assay kit (Calbiochem) according to the manufacturer’s instruction. In brief, A375 cells were seeded in 24-well plates and cultured for 24 h. They were then treated (or not) with aCD4 for an additional 24 h. TMZ was then added and 5–6 h later, the cells were harvested by scraping and analyzed immediately or frozen (−20 °C) for further analysis. Equal numbers of cells were used for each sample. Cells were lysed, and 50 μl of each lysate were transferred into a 96-well plate in which 50 μl of assay buffer and 10 μl of caspase-8 substrate conjugate were also added. The plate was read immediately, after 1 h and after 2 h with a fluorescent plate reader (Spectra Max M5, Molecular Devices) measuring excitation at 400 nm and emission at 505 nm. The results were expressed as the relative fluorescence signal (RFU) after subtraction of the relative signal of the appropriate buffer controls.

In vivo studies

Five × 10⁶ live A375 human melanoma or MDA-MB-231 human breast cancer cells were suspended in PBS (100–200 μl) and injected subcutaneously into right flank of 4- to 6-wk-old female athymic nude mice (nu/nu) (Charles River Laboratories). Tumors were allowed to develop to −3–5 mm. The mice were then randomized into groups of five to eight and were given no treatment, 50 μl PBS, or 0.5–1 × 10⁶ ex vivo activated human CD4⁺ T cells, re suspended in 50 μl PBS, intratumorally. Forty-eight hours later, they received either PBS or conventional chemotherapeutic agents i.p., in a volume of 100–200 μl. Forty-eight hours later, they were taken apart and analyzed immediately.

Animal studies were conducted in accordance with guidelines and approved by our institutional animal care and use committee. Mice were housed under standard conditions and received food and water ad libitum. All animals were observed at least once per day for morbidity, more if deemed necessary during the pretreatment and treatment periods. Signs of ill health were based on body weight loss, change in appetite, and behavioral changes such as altered gait, lethargy, and gross manifestations of stress. Animals were euthanized (CO₂ asphyxiation and cervical dislocation) if signs of severe toxicity, tumor area of 1 cm² or more, or tumor-related illness were observed. Each experiment was repeated once.

Statistics

All data are presented as mean values with SD, unless indicated otherwise. Statistical analyses were done with the Student’s t test. p < 0.05 was considered statistically significant. Drug synergy was determined by the combination-index (CI) methods using the CalcuSyn software (Biosoft). The CI is a method for quantifying drug cytotoxic synergism (27). Mean CI values from at least three independent experiments were subjected to a one-sided t test to identify synergism (CI < 0.9), additivity (CI between 0.9 and 1.1), or antagonism (CI > 1.1). Comparisons of tumor growth curves between the experimental group and control groups over time were performed using two-way repeated measures ANOVA.

Results

aCD4 chemosensitizes melanoma cell lines

Chemotherapeutic dose-response curves were generated for each tumor cell line. Treatment of tumor cells with TMZ or Carbo alone for 24 h yielded inconsistent results, with reduction in cell viability as determined by WST-1 assay in the range of 0–30%. In contrast, treatment for 48 h was found to yield more consistent results (data not shown); therefore, chemotherapy treatment duration of 48 h was initially chosen for all subsequent experiments. However, when cancer cells were pretreated with aCD4 followed by chemotherapy, maximal suppression of cell viability, as observed under the microscope, was achieved at 24 h of treatment. Therefore, the experiments were terminated at time (24 h), and WST-1 cell viability assay was performed for all groups. Based on these observations, chemotherapy treatment duration of 24 h was finally chosen for all subsequent experiments; even though the results for the chemotherapy alone groups at 24 h might be minimal and inconsistent.

Three doses of aCD4, 1 × 10⁴, 2.5 × 10⁴, and 5 × 10⁴, were used to presensitize A375 human melanoma cells for 24 h, followed by treatment with TMZ for another 24 h. The post treatment viability, as determined by WST-1 cell proliferation assay, of A375 cells treated with TMZ alone, as compared with nontreatment control, was 67.0% ± 1.0, and that of the three groups treated with various doses of aCD4 alone was 100% ± 1.7, 100% ± 1.6, and 69.9% ± 1.0, respectively. This was in contrast with the groups in which A375 cells were presensitized with aCD4, followed by treatment with TMZ. The viability of these cells in the three experimental groups was dramatically decreased and aCD4 dose dependent, at 18.5% ± 0.3, 6.7% ± 0.1, and 4.6% ± 0.1, respectively (Fig. 1A). Similar results were also obtained when Carbo was used as the chemotherapeutic agent (data not shown).

In addition, the reduction in A375 cell viability in this model was also found to be dependent on the doses of chemotherapy, TMZ (Fig. 1B), or Carbo (Fig. 1C). To investigate that the effect was not specific for A375 melanoma cell line, similar experiments were performed using two other human melanoma cell lines, 526mel and 1938mel, and similar results observed (Fig. 1D and data not shown). These data demonstrated that aCD4 sensitized melanoma cells to enhance the cytotoxic effect of TMZ, a drug clinically active in the treatment of melanoma, and Carbo, a drug with minimal activity against melanoma.

aCD4 chemosensitizes nonmelanoma tumor cell lines

To assess whether the chemosensitizing activity of aCD4 was unique to melanoma and/or to TMZ or Carbo, experiments were conducted using two breast (the hormonal sensitive MCF-7 and the hormonal resistant and aggressive MDA-MB-231), one prostate (DU-145), and one colon (HCT-116) cancer cell lines, and various chemotherapeutic drugs, including paclitaxel (Pax), Carbo, and 5-FU. Dramatic decrease in cell viability, as determined by WST-1 cell proliferation assay, was observed in all groups in which tumor cells were presensitized with aCD4 before treatment with chemotherapy (Fig. 2). Thus far, dramatic results were observed in 7/7 (100%) of the cancer cell lines of various histology (three melanoma, two breast, one colon, and one prostate cancer) that were tested. This effect was also seen with all chemotherapeutic drugs tested, including those that normally had minimal activity against these tumors. These data confirmed the role of aCD4 in sensitizing a variety of histologic tumor types to common chemotherapeutic agents.

Cytokines, not cell-contact, mediate chemosensitization in tumor cells

To investigate the contribution of cell-cell contact vs soluble factor(s) in the chemosensitization of tumor cells, a permeable transwell system with a 0.4-μm filter was used to avoid contact between tumor cells and aCD4. There was no statistical difference in the chemosensitization with or without cell contact (Fig. 3A). To understand the soluble factors involved, the aCD4 supernatants were screened for the presence of 13 common Th1 and Th2 pro- and anti-inflammatory cytokines. IL-1β, IL-5, and IL-12p70 levels were negative or minimally expressed. For sensitization purpose, the aCD4 were replaced by a cytokine or combination of cytokines at concentrations equivalent to those found in a previously collected aCD4 supernatant: IL-2 (0.5 ng/ml), IL-4 (0.5 ng/ml), IL-6 (0.5 ng/ml), IL-8 (50 ng/ml), IL-10 (40 ng/ml), IL-12p40 (0.5 ng/ml), IL-13 (0.5 ng/ml), IFN-γ (5 ng/ml), TNF-α (7 ng/ml), and GM-CSF (4 ng/ml). The combination of IL-2 and IL-4 did not show any activity. The combinations of IL-6 plus IL-8 and IL-10...
plus IL-12p40 plus IL-13 each showed 7 and 8% enhanced activity upon TMZ, respectively. The combination of IFN-γ/H9253 plus TNF-α/H9251 plus GM-CSF enhanced the cytotoxic activity of TMZ by 17%, while the combination of all 10 cytokines or aCD4 yielded a 26 and 39% enhancement above chemotherapy alone treatment, respectively (Fig. 3B). Doubling the doses of cytokines had minimal impact on these percentages (data not shown). The results were expressed as percentage of cell viability, as determined by WST-1 cell proliferation assay, relative to no treatment control, of which viability was set at 100%. More than 95% viable cells were observed in the no treatment control in each experiment.

The chemosensitizing activity of cytokine combinations was primarily due to the effect of IFN-γ/H9253, and when IFN-γ/H9253 was used as a backbone for couplet combination with other cytokines individually (e.g., IFN-γ plus IL6, IFN-γ plus TNF-α, etc.), there was no difference in activity between these couplet combinations vs IFN-γ alone (data not shown). However, the combination of all 10 cytokines consistently yielded a better result compared with IFN-γ alone. Presensitization with aCD4 before chemotherapy was used as a positive control, yielding the best suppressive effect on cell viability (Fig. 3B).

**Apoptosis is markedly increased in aCD4-treated tumor cells treated with TMZ**

To understand the mechanisms that underlined the dramatic reduction in cell viability, as determined by WST-1 cell proliferation assay, A375 cells were presensitized with aCD4 via transwell system (to avoid contamination with aCD4 upon later harvesting), followed by treatment with TMZ for 12 h. Lysates from the treated A375 cells were quantitatively analyzed for cytoplasmic histone-associated DNA fragments as a measure of apoptosis. Melanoma cells treated with TMZ alone or aCD4 alone resulted in slightly higher levels of DNA fragments, 49 ± 0.1 arbitrary units (AU) and 54 ± 1.0 AU, respectively, as compared with nontreated cells, 23 ± 0.1 AU. However, when A375 cells were presensitized with aCD4 and treated with TMZ, the level of DNA fragments detected, 1041 ± 1.1 AU, was more than 21-fold over that of the TMZ alone group, 19-fold over the aCD4 alone group, and 45-fold over the nontreatment control (Fig. 4A).

In addition, significantly higher level of caspase-8 activity, 2341.8 ± 205.0 RFU, was detected in cells in the experimental group compared with the control groups, i.e., no treatment (524.4 ± 3.5 RFU), TMZ treatment only (508.6 ± 28.3 RFU), or aCD4 treatment only (392.7 ± 4.0 RFU) (Fig. 4B). Western blot analysis revealed a reduction in the relative expression of Bcl-2 in A375 cells treated with aCD4 alone or the combination of aCD4 followed by TMZ, but not in cells treated with TMZ alone. These data demonstrated that presensitization of tumor cells with aCD4 led to a decrease in Bcl-2, and that treatment with aCD4 and TMZ together resulted in enhanced caspase-8 activity, resulting in significant reduction in cell viability through an increase in apoptosis (Fig. 4C).
In vivo antitumor activity is markedly enhanced in human xenografts treated with aCD4 followed by chemotherapy

To confirm the observed activity in an in vivo setting, two human tumor xenograft models were used. In the first model, athymic nude mice bearing human melanoma A375 xenograft, \( \sim 3 \) to 5 mm in diameter were injected intratumorally with activated human CD4\(^+\) T cells, followed 48 h later with intraperitoneal administration of TMZ. There was a pronounced delay in tumor growth compared with mice receiving no treatment or either treatment alone (Fig. 5A). The difference between aCD4 plus TMZ vs TMZ alone was statistically significant (\( p < 0.0001 \)). Similarly, mice bearing aggressive MDA-MB-231 human breast tumors treated with aCD4 followed by paclitaxel had significant delay in tumor growth compared with each of the control groups (Fig. 5B). The difference between aCD4 plus Pax vs Pax alone was statistically significant (\( p = 0.0004 \)).

Interestingly, aCD4 alone had a significant growth inhibition effect on the melanoma xenograft but not on the breast cancer xenograft. This was consistent with the known immuno sensitivity of melanoma compared with other solid tumors. To assess toxicity resulting from the experimental treatment, animal weights were measured and recorded during the course of the study. No statistical difference in weight was found between the experimental group and control groups. For the A375 melanoma study, \( p = 0.66 \) vs no treatment control group, 0.25 vs aCD4 only group, and 0.13 vs TMZ only group, at day 15 post initiation of treatment. For MDA-MB-231 breast cancer study, \( p = 0.58 \) vs no treatment control group, 0.50 vs aCD4 alone group, and 0.28 vs paclitaxel alone group, at day 16 post initiation of treatment. Similar results were obtained at day 30 (data not shown).

Discussion

We have demonstrated that presensitization of tumor cells with nonspecifically activated CD4\(^+\) T cells greatly enhanced the cytotoxic effect of chemotherapy, in both in vitro and in vivo models. This activity was observed in all seven tumor cell lines as well as all four chemotherapeutic agents tested. Soluble factors secreted from the activated CD4\(^+\) T cells were found to be responsible for the observed effect, with IFN-\( \gamma \) playing a major role in the chemosensitization of tumor cells. IFN-\( \gamma \) by itself, however, was consistently inferior to activated CD4\(^+\) T cells in the chemosensitization of tumor cells.

aCD4 seemed to accelerate the cytotoxic effect of chemotherapy by shortening the duration of chemotherapy treatment by at least 24 h. Based on our chemotherapy dose-response curves, treatment of tumor cells with chemotherapy alone, including TMZ or Carbo, for 24 h was suboptimal, and the effect on cell viability as determined by WST-1 assay was minimal and inconsistent, ranging...
from 0 to 30% suppression. In contrast, treatment with chemotherapy for 48 h was found to yield more consistent results; therefore, chemotherapy treatment duration of 48 h was initially chosen for all subsequent experiments. However, when cancer cells were presensitized with aCD4 then treated with chemotherapy, maximal suppression of cell viability was observed at 24 h of treatment. Therefore, the experiments were terminated at that time (24 h), and cell viability assay was performed for all groups. Based on these observations, chemotherapy treatment duration of 24 h was finally chosen for all subsequent experiments, even though the results for the chemotherapy alone groups at 24 h might be minimal and inconsistent. For breast and prostate cancer, the treatment duration was also shortened by 24 h, from 72 to 48 h, with the use of aCD4 presensitization.

The acceleration of chemotherapeutic activity by aCD4 was also observed using apoptosis assays. As described previously, the combination of aCD4 and chemotherapy yielded dramatic results at 24 h. To evaluate true pro-apoptotic potential of aCD4 presensitization, apoptosis assays were performed at earlier time points, 12 h instead of 24 h after treatment with chemotherapy. For caspase-8 activity, chemotherapy treatment was stopped after 5 to 6 h. Even at these early time points when aCD4 alone or chemotherapy alone had no discernable effects, the combination of aCD4 and chemotherapy resulted in a dramatic increase in apoptosis and caspase-8 activity.
Some recent studies may have helped shed light into possible mechanisms that underlie the dramatic chemosensitizing effect of aCD4. Inflammation and inflammatory cytokines, as represented by a mixture of IL-1β, TNF-α, and IFN-γ, were shown to generate NO through the induction of NO synthase, resulting in global inhibition of DNA repair activity in cholangiocarcinoma cells (28). Other studies demonstrated that IFN-γ or IL-24 could overcome temozolomide resistance in neuroblastoma and melanoma, respectively, through down-regulation of the DNA repair enzyme O6-methylguanine-DNA methyltransferase expression and activity (29, 30).

According to conventional wisdom, it is counterintuitive to administer chemotherapy immediately after cell therapy because the transferred immune cells will be eliminated by chemotherapy. Although this may be true in the setting of conventional “immunocentric” chemimmunotherapy, where immunotherapy plays the major effector role and chemotherapy is used to prepare the host, this approach is rational in the context of “chemocentric” chemimmunotherapy, where chemotherapy exerts the principal effector function and immunotherapy is used transiently for the purpose of presensitization.

This model does not depend on the Ag-specific activation of T cells, and therefore may be applicable to all tumor types and all patients regardless of their HLA status. In addition, it will be much easier and much less expensive than specific activation of T cells for clinical use. The observed activity was dependent on soluble factors released from the activated CD4+ T cells but not on CD4+ T cell and tumor cell contact. Therefore, the source of CD4+ T cells is irrelevant because there is no need for specific ligand-receptor recognition. In most patients, autologous CD4+ T cells can be used. However, in patients with low number of white blood cell count or low number of CD4+ T cell count such as in heavily immunosuppressed cancer patients or in patients with HIV/AIDS, HLA-unmatched allogeneic donor CD4+ T cells could also be used. In our approach, the activated CD4+ T cells are relevant only during the first few days to presensitize the tumor to enhance the killing effect of chemotherapy and are dispensable after the initial few days of treatment. Hence, activated allogeneic CD4+ T cells from any donor could conceivably be irradiated before patient administration without fear of graft vs host disease because these cells will die of apoptosis in ~5 to 7 days (31, 32).

This concept may also expand the repertoire of chemotherapeutics that can be used effectively to treat cancer. One example is carboplatin. By itself, carboplatin has minimal to modest activity against melanoma, breast, or prostate cancer. However, using aCD4 to presensitize tumor cells resulted in a loss of tumor cell sensitivity to carboplatin. This may expand the therapeutic window of carboplatin and other chemotherapeutics.

### Figure 5

Activated CD4+ cells enhance in vivo antitumor activity of chemotherapy. Tumors were established by injecting 5 × 10^6 A375 human melanoma (A) or MDA-MB-231 human breast cancer cells (B) subcutaneously into the right flank of nude mice. The tumor-bearing mice were treated intratumorally with aCD4 (0.5–1 × 10^6/mouse) (arrows) 48 h before chemotherapy treatment using TMZ at 100 mg/kg daily for 5 days (A) or Pax at 10 mg/kg daily for 3 days (B). The treatments were repeated 1 wk later. The size of tumors was measured three times weekly and tumor volume was calculated. Data represent the average of tumor volume (n = 5) ± SEM. In A, the difference between aCD4 plus TMZ vs TMZ alone was statistically significant (p < 0.0001). In B, the difference between aCD4 plus Pax vs Pax alone was statistically significant (p = 0.0004). The p values in A and B are from repeated measures ANOVA (two-sided).
viability, as determined by WST-1 cell proliferation assay, of >95% with a single treatment using a suboptimal drug concentration. By improving the efficacy of already approved drugs, we can rapidly implement treatment in patients with advanced cancer who may not have enough time to wait for new drug development, which may have minimal or modest impact on their disease.

Several recent studies have reported improved response rate and survival with salvage chemotherapy in patients who previously received cancer vaccination and developed an immune response (33–37). Our model may provide a plausible mechanism to explain these observations as well as to facilitate further understanding, design, and development of improved methods for chemoimmunotherapy in cancer.

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Disclosures

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