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Annotating PDFs using Adobe Reader XI

Version 1.4 January 14, 2014

1. Update to Adobe Reader XI

The screen images in this document were captured on a Windows PC running Adobe Reader XI. Editing of DJS proofs requires the use of Acrobat or Reader XI or higher. At the time of this writing, Adobe Reader XI is freely available and can be downloaded from http://get.adobe.com/reader/

2. What are eProofs?

eProof files are self-contained PDF documents for viewing on-screen and for printing. They contain all appropriate formatting and fonts to ensure correct rendering on-screen and when printing hardcopy. DJS sends eProofs that can be viewed, annotated, and printed using the free version of Acrobat Reader XI (or higher).

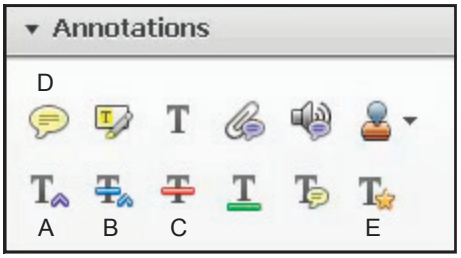
3. Comment & Markup toolbar functionality

A. Show the Comment & Markup toolbar

The Comment & Markup toolbar doesn't appear by default. Do one of the following:

- Select View > Comment > Annotations.
- Click the Comment button in the Task toolbar.

Note: If you've tried these steps and the Annotation Tools do not appear, make sure you have updated to version XI or higher.



A. Insert Text tool
B. Replace Text tool
C. Delete Text tool
D. Sticky Note tool
E. Text Correction Markup tool

B. Select a commenting or markup tool from the Annotations window.

Note: After an initial comment is made, the tool changes back to the Select tool so that the comment can be moved, resized, or edited. (The Pencil, Highlight Text, and Line tools stay selected.)

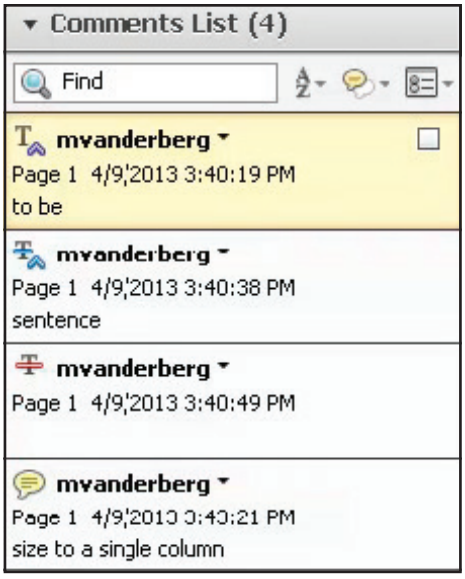
C. Keep a commenting tool selected

Multiple comments can be added without reselecting the tool.
Select the tool to use (but don't use it yet).

- Right Click on the tool.
- Select Keep Tool Selected.

4. Using the comment and markup tools

To *insert*, *delete*, or *replace* text, use the corresponding tool. Select the tool, then select the text with the cursor (or simply position it) and begin typing. A pop-up note will appear based upon the modification (e.g., inserted text, replacement text, etc.). Use the Properties bar to format text in pop-up notes. A pop-up note can be minimized by selecting the ⊟ button inside it. A color-coded ⊥ symbol will remain behind to indicate where your comment was inserted, and the comment will be visible in the Comments List.



5. The Properties bar

The Properties bar can be used to add formatting such as bold or italics to the text in your comments.

To view the Properties bar, do one of the following:

- Right-click the toolbar area; choose Properties Bar.
- Press [Ctrl-E]

... continues on page 2

6. Inserting symbols or special characters

An 'insert symbol' feature is not available for annotations, and copying/pasting symbols or non-keyboard characters from Microsoft Word does not always work. Use angle brackets < > to indicate these special characters (e.g., <alpha>, <beta>).

7. Editing near watermarks and hyperlinked text

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- Use the arrow keys to move the cursor beside the text to be edited.
- Hold down the shift key while simultaneously using arrow keys to select the block of text, if necessary.
- Insert, replace, or delete text, as needed.

8. Summary of main functions

A. Insert text - Use Insert Text tool (position cursor and begin typing)
B. Replace text - Use Replace Text tool (select text and begin typing)
C. Delete text - Use Strikethrough Text tool (select text and press delete key)
Note: The Text Correction Markup tool combines the functions of all three tools.
D. Sticky Note - Use Sticky Note tool to add comments not related to text correction.

9. Reviewing changes

To review all changes, do the following:

- Click the Comments button to reveal the comment tools
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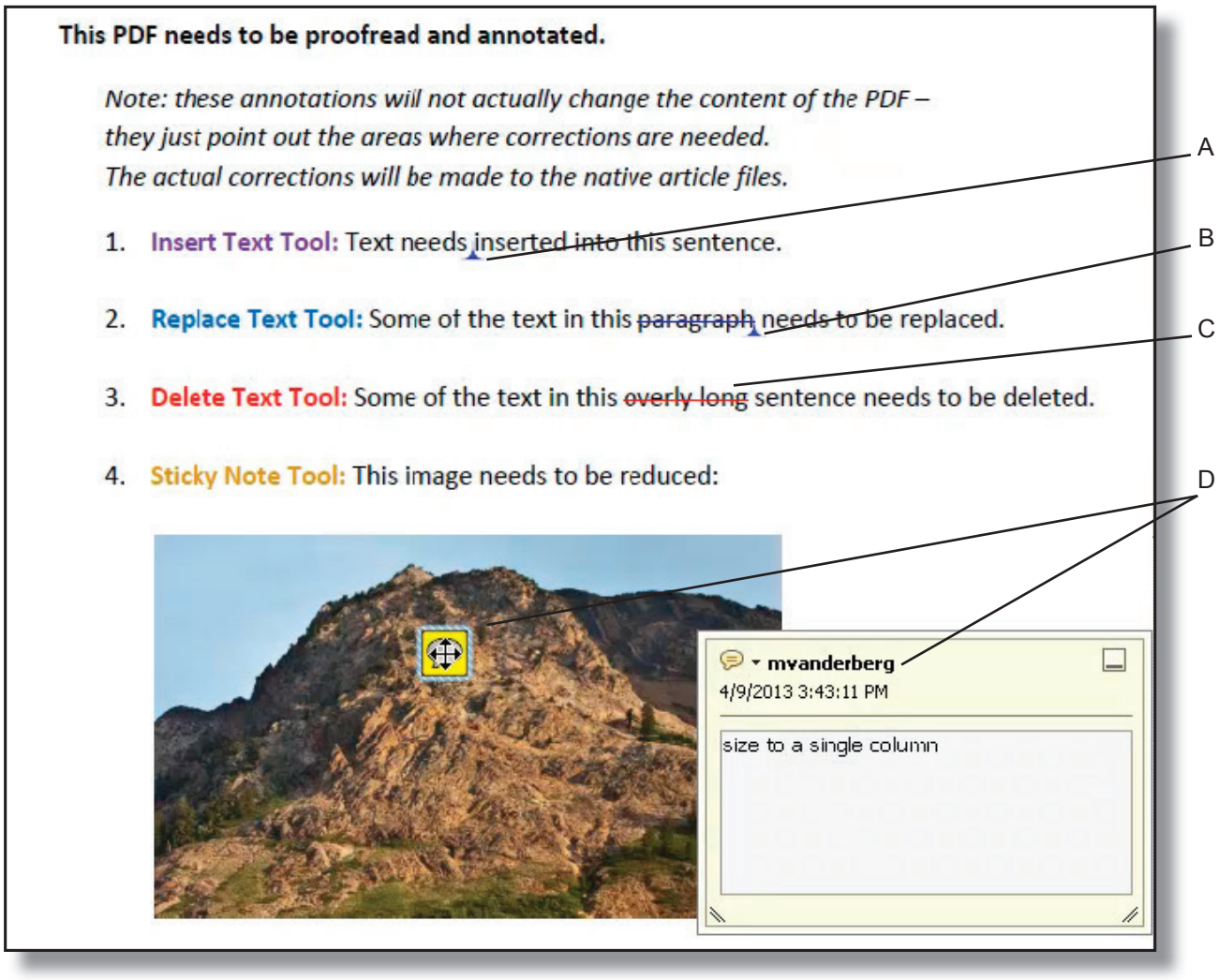

Critical Roles of Chemoresistant Effector and Regulatory T Cells in Antitumor Immunity after Lymphodepleting Chemotherapy

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Antitumor immunity is augmented by cytotoxic lymphodepletion therapies. Adoptively transferred naive and effector T cells proliferate extensively and show enhanced antitumor effects in lymphopenic recipients. Although the impact of lymphodepletion on transferred donor T cells has been well evaluated, its influence on recipient T cells is largely unknown. The current study demonstrates that both regulatory T cells (Tregs) and effector $CD8^+$ T cells from lymphopenic recipients play critical roles in the development of antitumor immunity after lymphodepletion. Cyclophosphamide (CPA) treatment depleted lymphocytes more efficiently than other cytotoxic agents; however, the percentage of $CD4^+CD25^+$ Forkhead box $P3^+$ Tregs was significantly increased in CPA-treated lymphopenic mice. Depletion of these chemoresistant Tregs following CPA treatment and transfer of naive $CD4^+$ T cells augmented the antitumor immunity and significantly suppressed tumor progression. Further analyses revealed that recipient $CD8^+$ T cells were responsible for this augmentation. Using $Rag2^{-/-}$ mice or depletion of recipient $CD8^+$ T cells after CPA treatment abrogated the augmentation of antitumor effects in CPA-treated reconstituted mice. The transfer of donor $CD4^+$ T cells enhanced the proliferation of $CD8^+$ T cells and the priming of tumor-specific $CD8^+$ T cells originating from the lymphopenic recipients. These results highlight the importance of the recipient cells surviving cytotoxic regimens in cancer immunotherapies. ***The Journal of Immunology*****, 2015, 195: 000–000.**

Antitumor immunity has been well established to be augmented by cytotoxic regimens (1, 2). A number of studies have demonstrated that the antitumor efficacy of effector cells was greatly increased when they were adoptively transferred into tumor-bearing hosts that were lymphodepleted with cytotoxic agents or by whole body irradiation (3). In clinical settings, the transfer of effector T cells combined with lymphodepleting regimens has shown relevant antitumor effects (4). The transfer of not only effector T cells, but also naive T cells into lymphopenic tumor-bearing hosts enhances antitumor immunity (5). Transferred naive T cells rapidly proliferate and acquire memory-like functions in lymphopenic hosts (6, 7). The transfer of naive T cells following lymphodepletion induces antitumor effector T cells and inhibits tumor progression (5). Additionally, the antitumor effects of tumor Ag vaccination are augmented by lymphodepletion (8). Thus, the combination of cytotoxic regimens and tumor immunotherapy seems to be a promising approach.

Previous studies have demonstrated that transferred donor T cells play a critical role in the augmentation of antitumor immunity in lymphopenic hosts (5, 9, 10). However, the role of recipient cells from the lymphodepleted hosts remains poorly understood. We previously reported that the regulatory T cells (Tregs) from recipients significantly increased after irradiation (11). Radioresistant recipient $CD4^+CD25^+$ $Foxp3^+$ Tregs proliferated rapidly during recovery from lymphopenia and suppressed the development of antitumor immunity. The depletion of radioresistant Tregs following whole body irradiation and the transfer of naive T cells strongly inhibited tumor progression.

In previous studies, we sublethally irradiated mice to deplete lymphocytes and augment antitumor immune responses. In this study, to examine the effect of the combination of cytotoxic regimens and tumor immunotherapy in a clinical setting, we investigated several cytotoxic agents at sublethal doses for lymphodepletion. We found that cyclophosphamide (CPA) treatment efficiently depleted lymphocytes compared with other cytotoxic agents. Similar to whole body irradiation, CPA administration increased the percentage of $CD4^+CD25^+Foxp3^+$ recipient Tregs. The depletion of recipient Tregs combined with the transfer of naive T cells and CPA treatment significantly delayed tumor progression. We further investigated whether other recipient cells from CPA-treated mice were involved in this augmentation after combination therapy. We found that the depletion of $CD8^+$ recipient T cells abrogated the antitumor efficacy of the combination

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The online version of this article contains supplemental material.

Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; CPA, cyclophosphamide; LN, lymph node; MCA, methylcholanthrene; s.d., subdermal; TDLN, tumor-draining lymph node; Treg, regulatory T cell.

of Treg depletion, transfer of naive T cells, and CPA treatment. These findings indicate that both the effector T cells and the Tregs that survive CPA treatment play important roles in the development of antitumor immunity during recovery from lymphopenia.

Materials and Methods

Animals

Female C57BL/6N (B6) mice were purchased from CLEA Laboratory (Tokyo, Japan). Transgenic mice expressing GFP gene from *Aequorea victoria* were purchased from Japan SLC (Hamamatsu, Japan). Ly5.1 congenic B6 mice were from Sankyo Labo Service (Tokyo, Japan). $Rag2^{-/-}$ mice and OT-II transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were housed in a specific pathogen-free environment and used at an age of 8–12 wk. The experimental protocols were approved by the Niigata University Institutional Animal Care and Use Committee.

Tumors

The 3-methylcholanthrene (MCA)–induced fibrosarcoma cell lines MCA205 and MCA207, originally derived from B6 mice, were routinely passaged in vivo and were used between the fifth to eighth passage (12). Single-cell suspensions were prepared from solid tumors by digestion with a mixture of 0.1% collagenase, 0.01% DNase, and 2.5 U/ml hyaluronidase (Sigma-Aldrich, St. Louis, MO) for 3 h at room temperature. The cells were filtered through a 100-μm nylon mesh, washed, and suspended in HBSS for i.v. and subdermal (s.d.) inoculations.

Adoptive transfer

B6 mice were treated with CPA (Sigma-Aldrich), fludarabine (Wako, Osaka, Japan), cisplatin (supplied by Nippon Kayaku), etoposide (Sigma-Aldrich), paclitaxel (R&D Systems, Minneapolis, MN), or gemcitabine (R&D Systems). One day later, the mice were reconstituted i.v. with 40×10^6 spleen cells from normal mice. These mice were then inoculated s.c. with 1×10^5 MCA205 tumor cells along the midline of the abdomen. Tumor sizes were measured in two perpendicular dimensions two to three times per week with digital calipers and recorded as the tumor area (mm^2).

Cell separation

For the depletion of $CD4^+$ or $CD8^+$ cells from donor cells, naive spleen cells were suspended at 3×10^8 cells/ml in 2.4 ml MACS buffer (0.5% BSA in PBS with 2 mM EDTA) and then incubated at 4°C with CD4 MACS beads (Miltenyi Biotec, San Diego, CA) for 15 min. $CD4^-$ or $CD8^-$ cell populations were collected as flow-through cells from the MACS columns. In some experiments, $CD4^+CD25^+$ Tregs were purified using anti-CD4 mAb-coated Dynabeads and Detachabeads (Invitrogen, Carlsbad, CA), followed by positive selection with PE-CD25 mAb and PE-microbeads, as previously described (13).

Activation of tumor-draining lymph node cells

The generation of activated tumor-draining lymph node (TDLN) cells has been described previously (14). Briefly, B6 mice were inoculated s.d. with 3×10^6 MCA205 tumor cells on both flanks to stimulate TDLNs. Twelve days later, TDLNs (inguinal) were harvested, and single-cell suspensions were prepared mechanically. These TDLN cells were activated with anti-CD3 mAb (145-2C11) immobilized on 24-well plates for 2 d and expanded in complete medium containing 16 U/ml human rIL-2 (supplied by Shionogi) for 3 d. Complete medium consists of RPMI 1640 supplemented with 10% heat-inactivated FBS and antibiotics.

FACS analysis and in vivo proliferation

FITC-conjugated mAbs against CD25 (PC61) and BrdU (3D4); PE-conjugated mAbs against CD4 (RM4-5), CD8 (53-6.7), CD25 (PC61), and IFN-γ (XMG1.2); Cy-chrome–conjugated mAbs against CD4 (RM4-5), CD8 (53-6.7), Ly5.1 (A20), and Ki-67 (B56); isotype-matched mAbs; FITC-annexin V and 7-aminoactinomycin D (7-AAD) were purchased from BD Biosciences. PE anti-Foxp3 (FJK-16s) was purchased from eBioscience (San Diego, CA). The cell surface phenotypes were determined by direct immunofluorescence staining with conjugated mAbs and analyzed using FACSCalibur (BD Biosciences, San Jose, CA). Foxp3 staining was performed using the PE-Foxp3 staining set (eBioscience). For the BrdU incorporation assay, irradiated mice were injected i.p. with 1 mg BrdU (Sigma-Aldrich) for consecutive 3 d before harvesting. For the in vivo proliferation assay, spleen T cells from normal mice were labeled with CFSE (Molecular Probes, Eugene, OR). Briefly, T cells from the spleens of naive mice were suspended at 1×10^7 cells/ml and incubated with CFSE in HBSS for 10 min at 37°C. The labeling was stopped by adding ice-cold HBSS, and the cells were washed twice with HBSS before being transferred into irradiated mice.

Intracellular IFN-γ staining

Intracellular IFN-γ staining was performed, as previously described (15). Briefly, activated T cells were stimulated with a single-cell suspension of either MCA205 or MCA207 tumor cells prepared from solid tumor tissues at a 1:1 ratio. Controls included stimulation with immobilized anti-CD3 mAbs. Brefeldin A (10 μg/ml; Sigma-Aldrich) was added at 6 h, and the cells were harvested at 24 h. The cells were then pretreated with FcR-blocking Abs, followed by staining for 30 min with Cy-conjugated anti-CD4 or anti-CD8 mAbs. Washed cells were fixed with 2% paraformaldehyde for 20 min, permeabilized with 0.3% saponin, and incubated for 40 min with PE-conjugated IFN-γ at 4°C. Unbound mAbs were removed by two washes with 0.3% saponin in PBS.

Statistical analysis

The significance of the differences between groups was analyzed using the Wilcoxon rank sum test or the Student *t* test. A two-tailed *p* value <0.05 was considered significant. All experiments were repeated at least twice.

Results

CPA treatment depletes lymphocytes and enhances antitumor immunity

We and others have previously reported that the transfer of T cells into lymphopenic hosts augmented antitumor immunity (1–5, 9, 14). Although the precise mechanisms underlying the enhancement of antitumor immune responses by lymphodepletion remain unclear, this augmentation might depend on the number of lymphocytes remaining after lymphodepletion (16–18). To examine which antitumor cytotoxic agent depleted lymphocytes and induced antitumor immunity efficiently, we administered several types of cytotoxic drugs into mice. We first determined the LD_{50} of the cytotoxic drugs in mice. Mice were injected i.p. with escalating doses of CPA, fludarabine, cisplatin, etoposide, paclitaxel, and gemcitabine. The calculated LD_{50} values were the following: 600 mg/kg CPA, 1200 mg/kg fludarabine, 8 mg/kg cisplatin, 280 mg/kg etoposide, 24 mg/kg paclitaxel, and 2000 mg/kg gemcitabine. Half doses of the LD_{50} were used to deplete lymphocytes in this study. Fig. 1A shows the number of spleen and lymph node (LN) cells at different time points after injection of the cytotoxic agents. CPA treatment decreased the number of LN and spleen cells more than the other cytotoxic agents. The number of $CD4^+$ T cells was decreased from $16.3 \pm 1.5 \times 10^5$ to $3.5 \pm 0.4 \times 10^5$ in the LNs and from $22.4 \pm 1.9 \times 10^6$ to $9.4 \pm 0.2 \times 10^6$ in the spleens 12 d after CPA treatment. CPA treatment also decreased the number of $CD8^+$ T cells from $11 \pm 1 \times 10^5$ to $1.8 \pm 0.2 \times 10^5$ in the LNs and from $9.6 \pm 0.8 \times 10^6$ to $4.1 \pm 0.1 \times 10^6$ in the spleens.

F 1

Next, we examined whether the administration of cytotoxic agents accelerated the proliferation of adoptively transferred T cells. Briefly, CFSE-labeled spleen cells (40×10^6) from naive Ly5.1 mice were transferred into treated or untreated Ly5.2 mice. Then, the Ly5.2 mice were injected s.d. with MCA205 tumor cells (3×10^6) to stimulate TDLNs. Twelve days later, TDLNs were harvested, and the proliferation of donor $CD4^+$ and $CD8^+$ T cells was assessed through CFSE dilution. Compared with the proliferation of T cells transferred into mice treated with other cytotoxic agents, rapid proliferation was observed in the T cells transferred into mice treated with CPA (Fig. 1B).

In previous studies, we reported that the percentage of Tregs was increased after lymphodepletion (11). Tregs survived sublethal irradiation and suppressed the development of antitumor immu-

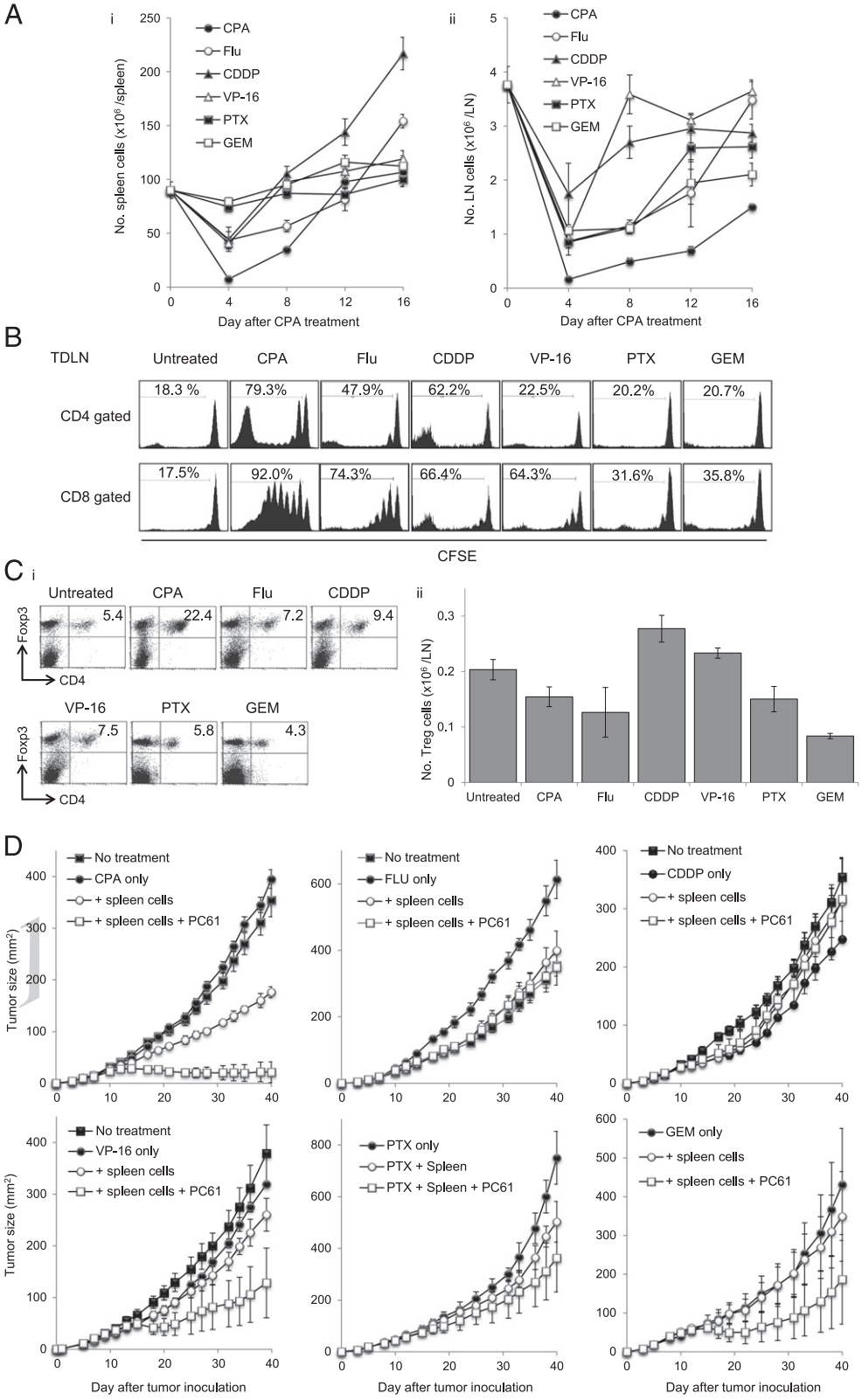

FIGURE 1. Administration of CPA efficiently depletes lymphocytes and augments the antitumor effects of naive T cell transfer and Treg depletion. (**A**) Kinetics of the absolute number of spleen cells (**i**) and LN cells (**ii**). Mice were injected i.p. with CPA, fludarabine (Flu), cisplatin (CDDP), etoposide (VP-16), paclitaxel (PTX), or gemcitabine (GEM). Spleens and inguinal LNs were harvested at different time points after chemotherapy. (**B**) Mice were treated with CPA, Flu, CDDP, VP-16, PTX, or GEM. One day later, 40×10^6 CFSE-labeled spleen cells from Ly5.1 congenic mice were transferred into chemotherapy- treated mice. These mice were then inoculated s.d. with 3×10^6 MCA205 tumor cells in the right flank. Twelve days after tumor inoculation, TDLN cells were harvested and analyzed for CFSE staining intensity within the $Ly5.1^+$ subset. A representative result from three independent experiments is shown. (**C**) The percentage (**i**) and the absolute number (**ii**) of $CD4^+CD25^+Foxp3^+$ cells in the LNs. LN cells were harvested from mice 12 d after the administration of cytotoxic drugs, and single-cell suspensions were prepared for FACS analyses. (**D**) One day after the injection of cytotoxic drugs, mice were transferred i.v. with 40×10^6 spleen cells from naive mice. These mice were inoculated s.d. with 1×10^5 MCA205 tumor (*Figure legend continues*)

nity during recovery from lymphopenia. Depletion of the surviving Tregs in lymphopenic mice significantly augmented antitumor immunity during the recovery from lymphopenia. To examine whether the treatment with cytotoxic drugs increases the percentage of Tregs, mice were injected i.p. with CPA (300 mg/kg), fludarabine (600 mg/kg), cisplatin (4 mg/kg), etoposide (140 mg/kg), paclitaxel (12 mg/kg), or gemcitabine (1000 mg/kg). Twelve days later, LN cells were harvested and stained for FACS analyses. A higher percentage of Tregs was observed in mice treated with CPA compared with the percentage of Tregs in mice treated with fludarabine, cisplatin, etoposide, paclitaxel, or gemcitabine (Fig. 1Ci). By contrast, the absolute number of Tregs was significantly decreased in mice treated with gemcitabine (Fig. 1Cii, $p = 0.0032$).

To evaluate the antitumor efficacy of combination therapy with cytotoxic agents, T cell transfer, and Treg depletion, mice were injected i.p. with CPA. One day later, these mice were injected i.v. with spleen cells (40×10^6) from naive mice and then inoculated s.c. with MCA205 cells (1×10^5) along the midline of the abdomen. Tregs were depleted with anti-CD25 mAbs (PC61) on the same day. As shown in Fig. 1D, the retardation of skin tumor growth was observed in CPA-treated mice that were also injected with spleen cells ($p < 0.01$ versus no treatment or CPA alone on day 40). Although the depletion of Tregs delayed skin tumor growth in mice treated with etoposide, paclitaxel, or gemcitabine and reconstituted with spleen cells, CPA treatment followed by reconstitution and Treg depletion strongly inhibited tumor progression ($p < 0.01$ versus CPA and reconstitution alone on day 40).

Increase in Tregs after CPA treatment suppresses tumor-specific immune responses

To confirm that anti-CD25 mAbs efficiently prevented the increase in Tregs in lymphopenic mice, CPA (300 mg/kg)-treated mice were reconstituted with spleen cells (40×10^6) and injected with anti-CD25 mAbs. Twelve days later, LN cells were harvested and stained for FACS analyses. As shown in Fig. 2A, the percentage of $CD4^+CD25^+Foxp3^+$ Tregs was reduced in mice reconstituted with spleen cells, followed by injection with anti-CD25 mAbs (22.9–2.2%).

In previous studies, we demonstrated that TDLNs played a pivotal role in the development of antitumor immunity (9, 19). Tumor-specific effector T cells were primed in the TDLNs and showed antitumor effects after in vitro stimulation. To investigate whether the depletion of Tregs after CPA treatment enhances the induction of effector T cells in TDLNs, CPA-treated mice were reconstituted with spleen cells. These mice were inoculated s.d. with MCA205 tumor cells (3×10^6) to stimulate the TDLNs and then injected with anti-CD25 mAbs. Twelve days later, TDLNs were harvested, and the cells were activated in vitro with immobilized anti-CD3 mAbs for 2 d, and cultured in the presence of low doses of IL-2 (16 U/ml) for 3 d, as previously described (9, 14). We evaluated IFN-γ secretion from these activated TDLN cells after further stimulation with fresh MCA205 tumor digest. The tumor digest contains $CD11b^+$MHC-class II^+ APCs, as described in our previous study (10). The percentages of both $CD4^+$ and $CD8^+$ T cells responding to specific MCA205 stimulation were increased in reconstituted and Treg-depleted mice (Fig. 2B). By contrast, both $CD4^+$ and $CD8^+$ T cell responses to nonspecific stimulation were not affected by Treg depletion. To further examine whether the increase in Tregs in CPA-treated lymphopenic mice suppressed specific antitumor immune responses, tumor-specific T cells were generated from the TDLNs of normal mice that were inoculated s.d. with MCA205 tumor cells. These TDLNs included tumor-specific $CD4^+$ and $CD8^+$ T cells (20). Twelve-day TDLNs were harvested and stimulated in vitro using anti-CD3 and IL-2 in the absence or presence of $CD4^+CD25^+$ T cells (1:1) magnetically isolated from the CPA-treated mice. After further stimulation with MCA205 cells, the TDLN cells were analyzed for IFN-γ secretion. As shown in Fig. 2C, in the presence of Tregs, the percentage of $CD4^+$ and $CD8^+$ T cells responding to specific tumor stimulation was greatly decreased.

Proliferation and survival of Tregs after CPA treatment

After CPA treatment and reconstitution, the Treg population may consist of donor and recipient cells. To determine the origin of this increase in Tregs in lymphodepleted and reconstituted mice, CPA-treated mice were transferred with spleen cells from transgenic GFP mice. FACS analysis of day 12 LNs showed that 85.1% of the $CD4^+Foxp3^+$ Tregs were GFP^- recipient cells (Fig. 3A). Next, we analyzed the proliferation of Tregs in vivo after CPA treatment. CPA-treated mice were injected with BrdU for consecutive 3 d. Seven days after CPA treatment, LN cells were harvested and assessed for BrdU incorporation and Ki-67 expression. As shown in Fig. 3B, in untreated mice, $CD4^+Foxp3^+$ Tregs incorporated BrdU to a greater extent than their $CD4^+Foxp3^-$ counterparts (9.5% versus 2.2%). Ki-67 expression was also higher in the $CD4^+Foxp3^+$ Tregs compared with the $CD4^+Foxp3^-$ cells (34.8% versus 9.2%). Although CPA treatment increased both BrdU incorporation and Ki-67 expression in $CD4^+Foxp3^-$ cells (14.5 and 23.7%, respectively), the levels were still greater in $CD4^+Foxp3^+$ Tregs (32.5 and 78.7%, respectively). To evaluate the percentage of cell death in Tregs after CPA treatment, LN cells were harvested 7 d after CPA treatment. In untreated mice, the percentage of annexin V^+ apoptotic cells in $CD4^+Foxp3^+$ Tregs was higher than that in $CD4^+Foxp3^-$ cells (8.9% versus 4.5%). CPA treatment did not affect the percentage of annexin V^+ cells within the $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ subsets (9.1% versus 3.7%). Similar percentages of 7-AAD^+ cells were observed in the $CD4^+Foxp3^+$ cells and $CD4^+Foxp3^-$ cell subsets (7.1% versus 5.9%), and CPA treatment did not affect the percentage of 7-AAD^+ cells in either subset (7.6% versus 6.8%). These findings indicate that the recipient Tregs surviving CPA treatment rapidly proliferate during the recovery from lymphopenia.

Tumor-specific $CD4^+$ T cells are essential for antitumor immunity after CPA treatment

To determine whether the transfer of $CD4^+$ and $CD8^+$ T cells was responsible for the antitumor efficacy of the combination of CPA treatment, spleen cell transfer, and Treg depletion, we depleted $CD4^+$ cells, $CD8^+$ cells, or both $CD4^+$ cells and $CD8^+$ cells from donor spleen cells before transfer using magnetic beads. Although the depletion of $CD8^+$ cells from the donor spleen cells partially reduced the antitumor effects of this combination therapy, the depletion of $CD4^+$ cells completely abrogated the antitumor effects ($p < 0.01$ for the transfer of whole spleen cells versus $CD4^+$-depleted spleen cells or $CD4^+$- and $CD8^+$-depleted spleen cells on day 49; Fig. 4A). We next asked whether the transfer of

cells along the midline of the abdomen and then treated with anti-CD25 mAbs (PC61). The resulting skin tumors were measured in two perpendicular directions two to three times per week, and the tumor areas (mm^2) were recorded ($n = 5$/group). The combination of CPA treatment, spleen cell transfer, and PC61 treatment significantly suppressed skin tumor growth.

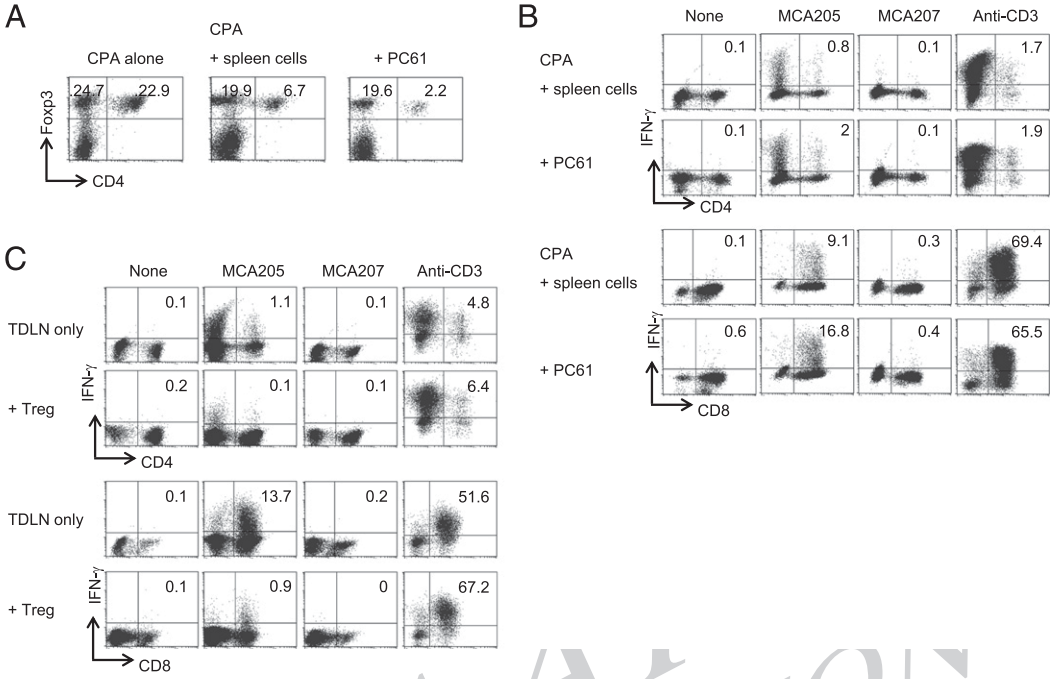

FIGURE 2. Depletion of Tregs following CPA treatment augments the induction of tumor-specific effector T cells in the TDLNs. (**A**) CPA-treated mice were transferred i.v. with 40×10^6 naive spleen cells and then treated with PC61. The PC61 treatment significantly decreased the percentage of $CD4^+CD25^+Foxp3^+$ cells. (**B**) CPA-treated and reconstituted mice were inoculated s.d. with MCA205 tumor cells and then treated with PC61 or left untreated. Twelve-day TDLN cells were harvested and activated in vitro using the anti-CD3/IL-2 method for 5 d. These activated TDLN cells were tested for IFN-γ secretion after specific or nonspecific stimulation. (**C**) Normal mice were inoculated s.d. with 1.5×10^6 MCA205 tumor cells. Twelve days later, TDLN cells were harvested and activated in vitro with the method of CD3/IL-2 in the absence or presence of $CD4^+CD25^+$ cells isolated from spleens of CPA-treated mice. These TDLN cells were tested for IFN-γ production after further stimulation, as indicated.

T cells that are capable of recognizing tumor Ags was necessary for the augmentation of antitumor effects in this model. Mice were treated with CPA and were reconstituted with spleen cells from OT-II transgenic mice, followed by the injection of anti-CD25 mAbs and inoculation with MCA205 tumor cells. The reconstitution with spleen cells from OT-II mice combined with CPA and anti-CD25 mAb treatment was associated with minimal antitumor efficacy ($p < 0.01$ versus spleen cells from wild mice with anti-CD25 mAb treatment on day 40; Fig. 4B). To examine whether effector T cells responding to tumor Ags are primed from the transferred donor $CD4^+$ T cells, mice were given spleen cells from GFP-transgenic mice after CPA treatment and were inoculated s.d. with MCA205 tumor cells. Twelve days later, TDLN cells were harvested, activated in vitro with CD3 and IL-2, and then further stimulated with specific MCA205 cells. FACS analysis revealed that 86.7% of the $CD4^+$ T cells that produced IFN-γ after specific tumor stimulation were from transferred donor cells (Fig. 4C). By contrast, 10.9% of the $CD8^+$ T cells responding to specific tumor Ags were from donor cells.

Donor $CD4^+$ T cells and recipient $CD8^+$ T cells are responsible for the augmentation of antitumor immunity in CPA-treated mice

As described above, recipient Tregs that survive CPA treatment suppress antitumor immune responses. We next examined whether

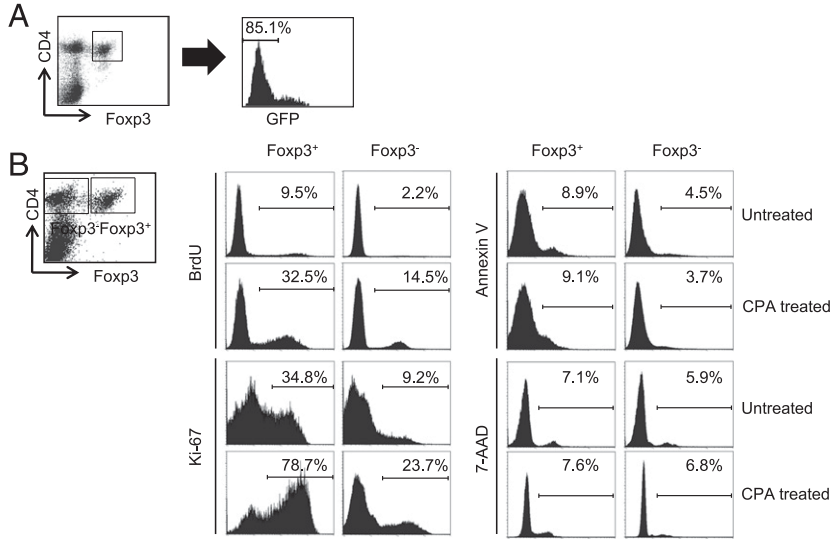

FIGURE 3. In vivo proliferation and apoptosis of Tregs after CPA treatment. (**A**) Mice were treated with CPA and then transferred i.v. with spleen cells from GFP-transgenic mice. Twelve days later, LN cells were harvested for FACS analysis. A histogram shows the percentage of GFP^- recipient cells among $CD4^+Foxp3^+$ cells. (**B**) CPA-treated or untreated mice were injected i.p. with 1 mg BrdU for consecutive 3 d before sacrifice. Seven days after CPA treatment, LN cells were harvested and analyzed by FACS. The percentage of $BrdU^+$, Ki-67$^+$, annexin V^+, and 7-AAD^+ cells was assessed on gated $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ subsets.

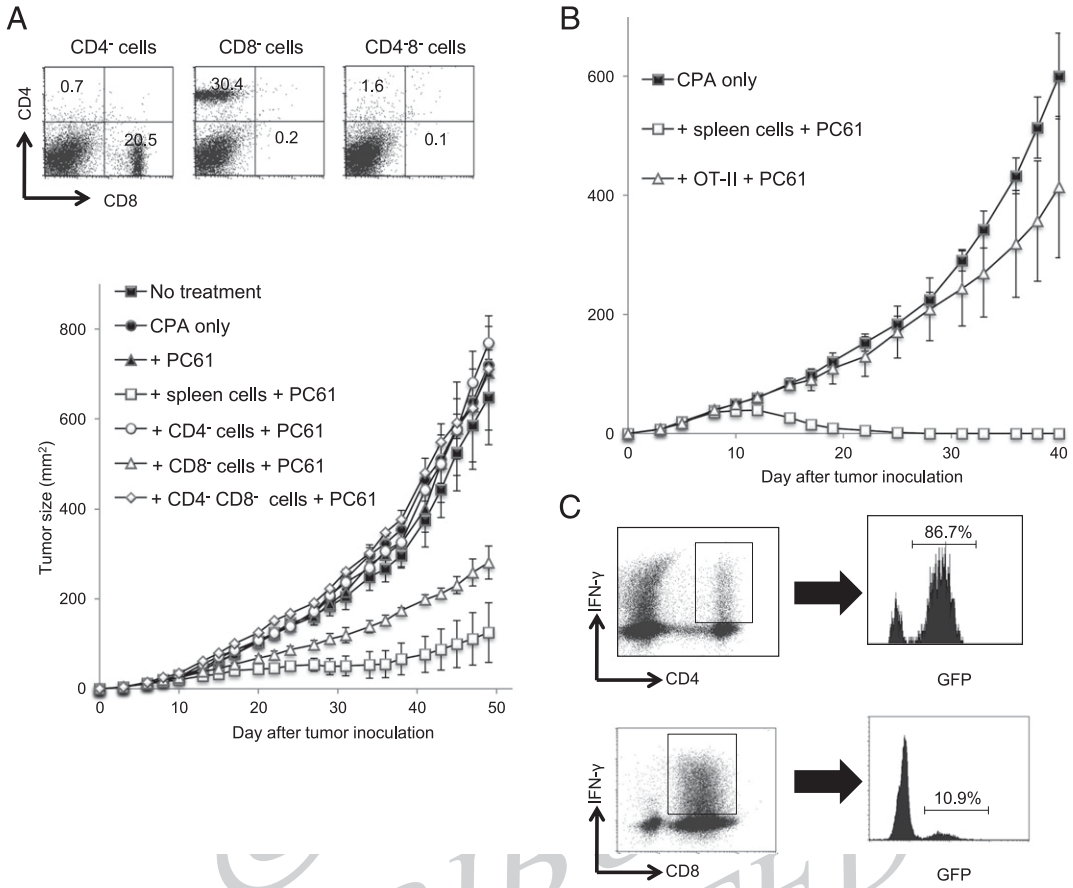

FIGURE 4. Donor CD4$^+$ T cells are required for the augmentation of antitumor immunity after CPA treatment. (**A**) CPA-treated lymphopenic mice were reconstituted with either CD4-, CD8-, or CD4- and CD8-depleted cell populations. Next, these mice were inoculated s.d. with 1×10^5 MCA205 tumor cells along the midline of the abdomen and then treated with PC61. The depletion of CD4$^+$ cells abrogated the antitumor effects of the combination of CPA treatment, spleen cell transfer, and PC61 treatment. (**B**) CPA-treated mice were transfused i.v. with 40×10^6 spleen cells from transgenic OT-II mice or normal mice. These mice were treated with PC61 following the inoculation of MCA205 tumor cells. The transfer of spleen cells from OT-II mice instead of the transfer of spleen cells from normal mice significantly decreased the antitumor effects of the combination therapy. (**C**) CPA-treated mice were transfused i.v. with 40×10^6 naive spleen cells from GFP-transgenic mice. These mice were inoculated s.d. with MCA205 tumor cells to stimulate TDLNs. Twelve-day TDLN cells were harvested and activated in vitro using anti-CD3 and IL-2. Activated TDLN cells were further stimulated with MCA205 tumor digests and stained for IFN-γ, as described in *Materials and Methods*. The majority (86.7%) of tumor Ag-specific CD4$^+$ cells was induced from the donor GFP$^+$ cells.

other recipient cells were involved in the development of antitumor immunity during recovery from lymphopenia. Rag2$^{-/-}$ mice were treated with CPA and reconstituted with spleen cells from wild-type mice. These mice were injected with anti-CD25 mAbs, followed by inoculation with MCA205 tumor cells. The augmented antitumor effects of the combination of CPA treatment, transfer of spleen cells, and Treg depletion observed in wild-type recipient mice were significantly decreased in the Rag2$^{-/-}$ mice ($p < 0.01$ on day 36; Fig. 5A). Previous studies demonstrated that CD8$^+$ effector T cells play an important role in antitumor immunity (3, 21). CD8$^+$ effector T cells are cytotoxic and eradicate tumor cells in vivo. To investigate the role of recipient CD8$^+$ T cells from CPA-treated mice in the development of antitumor immunity after lymphodepletion, we further depleted CD8$^+$ T cells with anti-CD8 mAbs (Lyt2) after CPA treatment and reconstitution with CD4$^+$ T cells. The depletion of CD8$^+$ T cells completely abrogated the antitumor effects observed after lymphodepletion, reconstitution, and anti-CD25 mAb treatment ($p < 0.01$ versus transfer of CD4$^+$ T cells into CPA-treated mice with anti-CD25 mAb treatment on day 50; Fig. 5B). As shown in Fig. 4A, the transfer of CD4$^+$ T cells was essential for the augmentation of antitumor immune responses after CPA treatment, followed by anti-CD25 mAb injection. To assess whether the transfer of CD4$^+$ T cells increased the number of CD8$^+$ effector T cells from the recipient mice, CPA-treated mice were reconstituted with CD8-depleted or CD4- and CD8-depleted spleen cells from Ly5.1 mice. These mice were inoculated with MCA205 cells and injected with anti-CD25 mAbs. Twelve-day TDLNs were harvested, activated with anti-CD3 and IL-2, and then stimulated with tumor cells. Although the depletion of CD8$^+$ cells from the donor spleen cells did not affect the percentage of CD8$^+$Ly5.1$^-$ recipient effector T cells (28.8 and 25.3%), the depletion of CD4$^+$ donor cells strongly decreased the percentage of CD8$^+$Ly5.1$^-$ recipient effector T cells (5.9%; Fig. 5C). Because the donor cells contain a small percentage of CD8$^+$ T cells after CD8 depletion in vitro, we confirmed the CD8$^+$ effector T cells were primed from the recipient mice in this model. CPA-treated mice were transferred ~~from~~ CD8-depleted spleen cells from Ly5.1 mice and inoculated with MCA205 cells. FACS analyses of 12-d TDLNs revealed that 99.4% of the CD8$^+$ effector T cells were from recipient cells (Fig. 5D). To evaluate whether the proliferation and survival of recipient CD8$^+$ cells were affected by the transfer of CD4$^+$ cells, magnetically isolated CD4$^+$ T cells from Ly5.1 mice were transferred into CPA-treated mice. Mice were administered BrdU for 3 d, and LN cells were harvested 7 d after the transfer. The transfer of CD4$^+$ T cells greatly increased BrdU incorporation (11.2–21.8%) and Ki-67 expression (29.8–

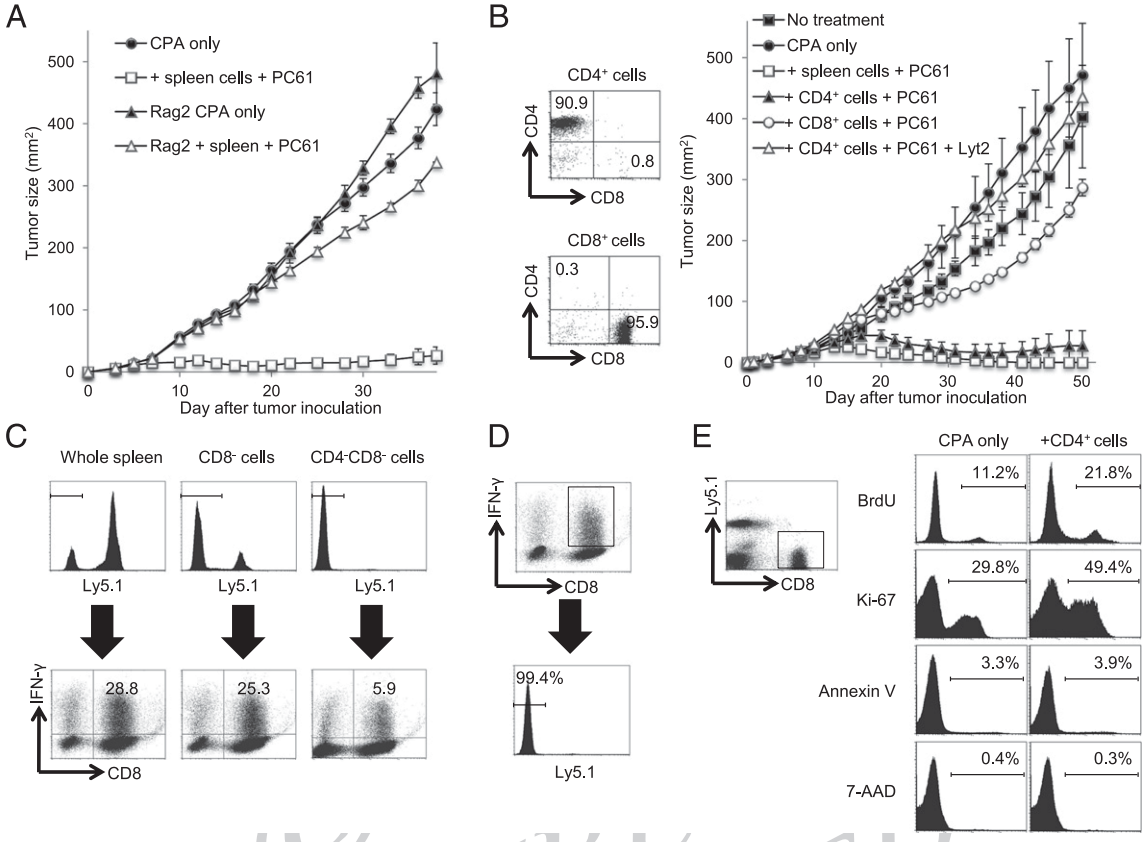

FIGURE 5. Effector $CD8^+$ T cells from CPA-treated recipients were essential for the augmentation of antitumor immunity. (**A**) $Rag2^{-/-}$ mice or wild-type mice were treated with CPA, followed by the transfer of spleen cells and PC61 treatment. Using $Rag2^{-/-}$ mice as recipients significantly reduced the antitumor effects of the naive T cell transfer and PC61 administration following CPA treatment. (**B**) CPA-treated wild-type mice were transfused i.v. with 40×10^6 whole spleen cells, 10×10^6 magnetically isolated $CD4^+$ cells, or $CD8^+$ T cells, and then injected s.d. with MCA205 tumor cells. Next, these mice were treated with PC61. Anti-CD8 mAbs (Lyt-2) were also administered to further deplete $CD8^+$ T cells from the recipients. The depletion of the recipient $CD8^+$ T cells completely abrogated the antitumor effects. (**C**) Mice were treated with CPA and reconstituted with either whole spleen cells, CD8-depleted, or CD4- and CD8-depleted cells from Ly5.1 congenic mice. These mice were then inoculated s.d. with MCA205 tumor cells to stimulate TDLNs. Twelve-day TDLN cells were harvested and activated with anti-CD3 and IL-2. The activated cells were tested for IFN-γ production after stimulation with specific MCA205 tumor cells. The depletion of $CD4^+$ T cells, but not $CD8^+$ T cells, from the donor cells greatly decreased the tumor-specific effector $CD8^+Ly5.1^-$ recipient cells. (**D**) CPA-treated mice were transferred i.v. with CD8-depleted cells from Ly5.1 mice and inoculated s.d. with MCA205 cells. Twelve-day TDLN cells were harvested and activated in vitro using anti-CD3 and IL-2. The activated cells were stimulated with MCA205 tumor cells and stained for IFN-γ. Histogram shows the percentage of $CD8^+Ly5.1^-$ recipient cells among $CD8^+$ effector T cells. (**E**) CPA-treated mice were left untreated or were transfused i.v. with $CD4^+$ T cells and then injected with BrdU for consecutive 3 d. Seven days later, LN cells were harvested and analyzed using FACS. Histograms show the percentages of $BrdU^+$, Ki-67^+, annexin V^+, and 7-AAD^+ cells among the $CD8^+Ly5.1^-$ recipient cells.

49.4%) in $CD8^+$ recipient T cells (Fig. 5E). By contrast, the transfer of $CD4^+$ T cells did not affect the percentage of annexin V (3.3–3.9%) or 7-AAD (0.4–0.3%) in the $CD8^+$ recipient cells.

Mice with a heavy tumor burden were successfully treated with a combination of CPA administration, spleen cell transfer, and anti-CD25 mAbs

To examine the efficacy of the combination of CPA administration, spleen cell transfer, and anti-CD25 mAb treatment, mice bearing 20-d skin tumors were treated. Although the CPA treatment retarded skin tumor progression, the transfer of spleen cells and anti-CD25 mAb injection following CPA treatment significantly suppressed skin tumor growth ($p < 0.01$ for the combination of CPA administration, spleen cell transfer, and anti-CD25 mAbs versus CPA treatment alone on day 50; Fig. 6A). Previous studies demonstrated that tumors induce suppressor cells to escape the attack by the immune system (22, 23). Furthermore, defects of TCR signal transduction in T cells from tumor-bearing hosts have been reported (24). We next evaluated the transfer of T cells from mice with a heavy tumor burden combined with CPA treatment and anti-CD25 mAb injection. Briefly, spleen cells were harvested from mice bearing 21-d skin tumors and transferred into CPA-treated mice also bearing 21-d skin tumors. These mice were treated with anti-CD25 mAbs. As shown in Fig. 6B, the transfer of spleen cells from mice with a heavy tumor burden combined with CPA treatment and anti-CD25 mAb administration significantly inhibited skin tumor growth ($p < 0.01$ for the combination of CPA administration, transfer of spleen cells from mice with a heavy tumor burden, and anti-CD25 mAbs versus CPA treatment alone on day 48). To further evaluate whether recipient Tregs from CPA-treated mice inhibit the augmentation of antitumor immunity during recovery from lymphopenia, we harvested spleens from mice bearing 21-d skin tumors and depleted $CD25^+$ Treg cells from spleen cells in vitro. CPA-treated mice bearing 21-d skin tumors were reconstituted with CD25-depleted donor cells and then treated with anti-CD25 mAbs to deplete residual recipient Tregs (Fig. 6C). Further depletion of recipient Tregs significantly delayed skin tumor progression compared with the depletion of donor Tregs alone ($p < 0.01$ for the combination of CPA administration, transfer of $CD25^-$ spleen cells from mice with a heavy tumor burden, and anti-CD25 mAbs versus the combi-

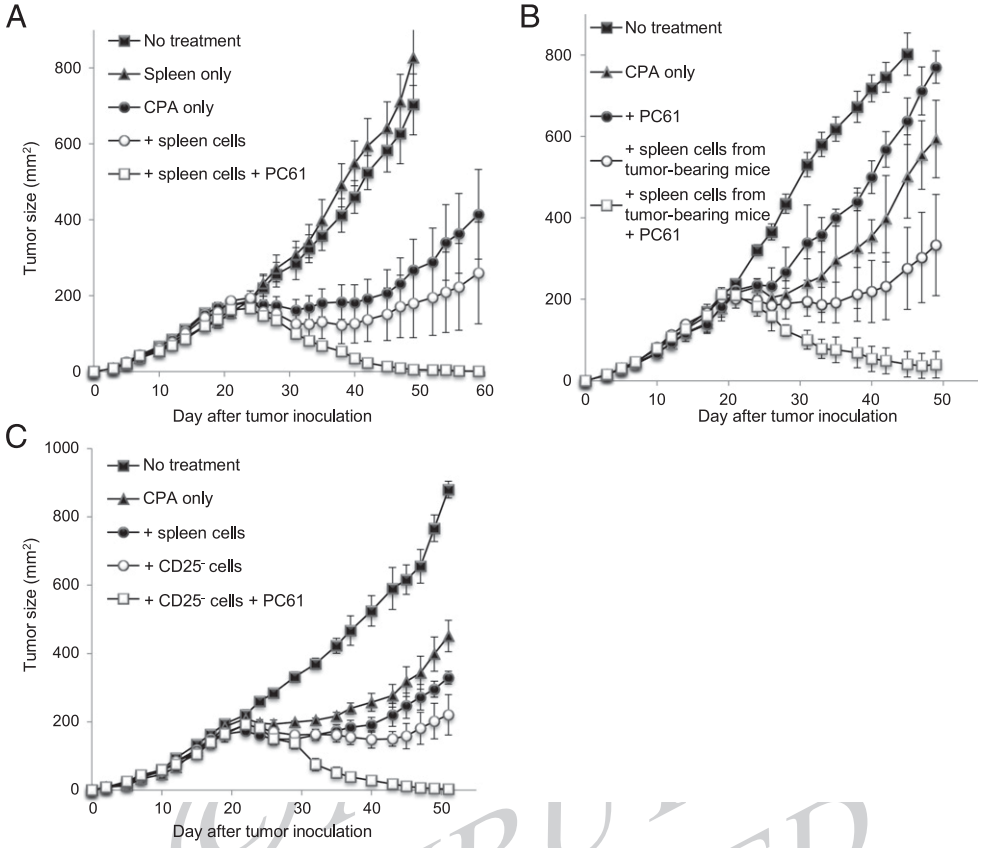

FIGURE 6. The combination of CPA treatment, T cell transfer, and Treg depletion successfully treated heavy tumor-bearing mice. (**A**) Mice were inoculated s.c. with 3×10^6 MCA205 tumor cells along the midline of the abdomen. Twenty days later, the mice were treated with CPA. Twenty-one days after tumor inoculation, the mice were transfused i.v. with 40×10^6 spleen cells and then treated with PC61. (**B**) Mice bearing 20-d skin tumors were treated with CPA. On day 21, these mice were transfused i.v. with spleen cells from mice bearing 21-d skin tumors and then treated with PC61. (**C**) Mice bearing 20-d skin tumors received CPA treatment. On day 21, these mice were reconstituted with $CD25^-$ spleen cells from mice bearing 21-d skin tumors and then treated with PC61 to further deplete the recipient Tregs. The depletion of recipient Tregs significantly inhibited tumor progression.

nation of CPA administration and transfer of $CD25^-$ spleen cells from mice with a heavy tumor burden on day 51).

Discussion

Lymphodepletion regimens, such as chemotherapy and radiotherapy, are well known to augment antitumor immunity. T cells transferred into lymphopenic hosts proliferate vigorously to restore the original pool size and acquire effector-like functions (25). The augmentation of T cell antitumor efficacy by lymphodepletion has been extensively studied, and it is now evident that lymphodepletion enhances the therapeutic efficacy of the adoptive transfer of effector T cells and naive T cells (1–5, 9, 14). Whole body irradiation, CPA treatment, and/or fludarabine treatment have been used to deplete lymphocytes. Recently, other cytotoxic agents have been reported to enhance antitumor immunity (26, 27). Several mechanisms are involved in the augmentation of antitumor immunity by cytotoxic agents, such as the direct stimulation of T cells, activation of dendritic cells, inhibition of suppressor cells, upregulation of MHC class I molecules on cancer cells, increase of the permeability of cancer cells to granzyme B, and triggering of immunogenic cell death (26, 27). In the current study, we investigated whether the administration of CPA, fludarabine, cisplatin, etoposide, paclitaxel, or gemcitabine augmented the antitumor efficacy of the adoptive transfer of naive T cells. Previous studies indicated that the elimination of lymphocytes competing for activation-inducing cytokines is one of the main mechanisms of the effectiveness of lymphodepletion (28, 29). An increase in the intensity of lymphodepletion was reported to be associated with the augmentation of the antitumor efficacy of adoptively transferred T cells (16–18). Therefore, we first determined the LD_{50} of these cytotoxic drugs. Half doses of LD_{50} were used for the lymphodepleting regimens thereafter. Although CPA treatment followed by reconstitution with naive T cells significantly delayed skin tumor progression, transfer of T cells after treatment with other cytotoxic agents did not inhibit tumor growth (Fig. 1D). Because the Treg depletion following transfer of T cells delayed tumor growth in mice treated with etoposide, paclitaxel, and gemcitabine, transfer of T cells after the administration of these drugs seemed to have minimal antitumor effects. CPA treatment deeply depleted endogenous lymphocytes, which are competing with transferred T cells for activating cytokines, and induced rapid T cell proliferation to a greater extent than the other cytotoxic agents. Tumor-specific T cells were suggested to acquire activation-inducing cytokines after CPA treatment, with these cells being primed more efficiently (Fig. 1A, 1B).

Previous studies have demonstrated that transfer of $CD4^+$ T cells inhibits tumor progression (30, 31). Because most solid tumors do not express MHC class II molecules, $CD4^+$ T cells are not able to directly recognize tumor Ags expressed on tumor cells. Professional APCs, such as dendritic cells, B cells, and macrophages, present tumor Ags bounding to MHC class II molecules to tumor-specific $CD4^+$ T cells. Stimulated tumor-specific $CD4^+$ T cells have helper function and improve antitumor effects of other immune cells, including $CD8^+$ T cells, NK cells, and macrophages. Moreover, recent studies have reported direct cytotoxic activity of

$CD4^+$ T cells (32, 33). Transfer of $CD4^+$ T cells from TCR transgenic mice into lymphopenic hosts bearing MHC class II–positive tumors resulted in tumor regression. These antitumor effects were independent of $CD8^+$ T cells. In our model system, the transfer of $CD4^+$ T cells is responsible for the enhancement of antitumor immunity in CPA-treated lymphopenic mice (Fig. 4A). Using $CD4^+$ T cells from OT-II transgenic mice abrogated the antitumor effect of the T cell transfer, suggesting that donor $CD4^+$ T cells that recognize tumor Ags mediate the augmentation of antitumor immune responses (Fig. 4B). Indeed, the majority of $CD4^+$ T cells responding to specific tumor Ag stimulation had a donor origin (Fig. 4C). Because MCA205 tumor cells do not express MHC class II molecules, APCs seemed to be involved in this augmentation of antitumor immune responses. Previous studies of adoptive cell therapy have largely focused on the transfer of antitumor effector $CD8^+$ T cells. It is evident that $CD8^+$ T cells are the most cytotoxic T lymphocytes that directly recognize tumor Ags and eradicate tumor cells (3, 21). In this study, we demonstrated that recipient $CD8^+$ T cells and donor $CD4^+$ T cells were also responsible for the antitumor effects observed after CPA treatment and reconstitution. If we used $Rag2^{-/-}$ mice as recipients or depleted $CD8^+$ T cells from the recipient mice, the antitumor effects were significantly decreased (Fig. 5A, 5B). Because a small percentage of effector $CD8^+$ T cells was primed from donor cells and transfer of $CD8^+$ T cells following CPA treatment delayed tumor progression, donor $CD8^+$ T cells may also be involved in the augmentation of antitumor immune responses (Figs. 4C, 5B). Collectively, the current study demonstrates that both $CD8^+$ T cells and Tregs surviving lymphodepletive CPA treatment play an important role in the development of antitumor immunity. Furthermore, the depletion of $CD4^+$ T cells from donor cells greatly decreased the percentage of $CD8^+$ effector T cells that were induced from CPA-treated recipient mice (Fig. 5C). An in vivo proliferation assay showed that the transfer of donor $CD4^+$ T cells following CPA treatment resulted in the proliferation of recipient $CD8^+$ T cells (Fig. 5E). Previous studies have shown that $CD4^+$ Th cells play a role in activating other immune cells, including $CD8^+$ T cells (3, 10, 34, 35). Our findings indicate that the donor $CD4^+$ T cells, which are capable of recognizing tumor Ags, enhance the induction of $CD8^+$ effector T cells from CPA-treated recipient mice.

Depletion of the induced Tregs in tumor-bearing hosts had been suggested to be crucial to the effectiveness of lymphodepletion (1, 2). Previous studies have demonstrated that CPA treatment decreases the number of Tregs and augments antitumor immune responses (36–42). Other investigators have also reported that the decrease of Tregs by CPA treatment increases immune responses and results in the exacerbation of autoimmune diseases (43–48). However, our previous study had shown that the percentage of Tregs was increased after sublethal whole body irradiation (11). These radioresistant Tregs that survive sublethal irradiation suppress antitumor immunity. In the current study, we also demonstrated that the percentage of Tregs increased in mice treated with CPA at a high dose (Fig. 1C). Depletion of these Tregs that survive CPA treatment significantly inhibited tumor progression (Fig. 1D). Furthermore, the in vivo depletion of Tregs following CPA treatment increased the percentage of tumor-specific effector T cells (Fig. 2B). Isolated Tregs from CPA-treated mice had suppressive functions against tumor-specific effector T cells in vitro (Fig. 2C). Previously, the rapid turnover of Tregs in the steady state had been reported (49, 50). Consistent with these studies, BrdU incorporation and Ki-67 expression assay revealed that Tregs in CPA-treated hosts proliferate more vigorously compared with their non-Treg counterparts (Fig. 3B). Additionally, an annexin V apoptosis assay showed that Tregs in the steady state and in CPA-treated hosts were more apoptotic; however, a 7-AAD assay demonstrated that a similar percentage of apoptotic cells was observed in Tregs and their non-Treg counterparts. These findings indicate that the recipient Tregs that survive CPA treatment recover rapidly and inhibit the development of antitumor immunity after lymphodepletion.

The ability of CPA to augment antitumor immunity has been extensively studied (26). Although CPA treatment for the conditioning of tumor immunotherapy seems to be a promising approach, the optimal doses of CPA for tumor immunotherapy have not been determined. The opposite effects of high- and low-dose CPA have been demonstrated in immune responses (51, 52). Administration of CPA at a high dose causes immunosuppression, whereas low-dose CPA stimulates immune responses. Zhao et al. (53) demonstrated that intracellular ATP level in Tregs was 8-fold lower than that in conventional T cells. Because CPA decreases intracellular ATP, Tregs have higher sensitivity to CPA and low-dose CPA could selectively deplete Tregs. Indeed, low-dose CPA significantly decreased the number of Tregs in LNs compared with high-dose CPA ($p = 0.0004$ for CPA at a dose of 100 mg/kg versus CPA at a dose of 600 mg/kg; ~~Supplemental Fig. 1B~~). There are several types of tumor immunotherapy, such as therapeutic cancer vaccines, nonspecific activation of the immune system against tumors, and adoptive cell transfer therapy. Different conditioning may be required to augment the antitumor effects of these tumor immunotherapies. Cancer vaccines demonstrate antitumor effects through the induction of tumor-specific effector T cells from naive T cells and restimulation of memory T cells. Considering the mechanisms of augmentation of antitumor immunity by cancer vaccines, the immunosuppression induced during lymphodepletion by CPA treatment at a high dose could work negatively for cancer vaccines. Indeed, previous studies reported that low-dose CPA treatment or metronomic administration of CPA augments the antitumor effects of cancer vaccines (37, 52, 54, 55). By contrast, high-dose CPA administration failed to enhance the antitumor effects as well as low-dose CPA treatment (54). However, there is a possibility that more immune ablation in tumor-bearing hosts could lead to the further enhancement of the antitumor effects of adoptive cell transfer therapy (16–18). CPA administration at a lymphodepletive dose followed by adoptive cell transfer therapy has shown durable clinical responses in patients with malignancies (56, 57). The current study also showed that lymphodepletion with high-dose CPA augmented the antitumor effects of transferred naive T cells. These findings suggest that different types of tumor immunotherapy require different doses of CPA to augment the antitumor effects. Furthermore, the direct cytotoxic effects of high-dose CPA treatment could be greater than those of low-dose CPA. CPA treatment at a high dose may enable tumor shrinking, deplete lymphocytes, and augment the antitumor effects of adoptive cell transfer therapy. Indeed, our results demonstrated that high-dose CPA treatment shrank 20-d established large tumors, and the combination of high-dose CPA treatment, adoptive transfer of T cells, and Treg depletion induced further tumor regression (Fig. 6A, 6B). High-dose lymphodepletive chemotherapy combined with adoptive cell transfer therapy seems to be a promising strategy for patients with advanced-stage cancer. Q:8

Disclosures

The authors have no financial conflict of interests.

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