# Memory Antitumor T-Cells Resist Inhibition by Immune Suppressor Cells

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**Abstract.** *Cancer immune therapy is difficult partly because several classes of suppressor cells, including regulatory T-cells and macrophage-derived suppressor cells, inhibit the antitumor T-cell response. We used treatment studies of implanted tumors in mice to demonstrate that the same inhibitory cells that abrogated an acute therapeutic T-cell response to established tumor did not inhibit the therapeutic response produced by memory T-cells. Generating antitumor memory T-cells may be a highly potent strategy against cancer with late developing metastases.*

New therapies for cancer are required because current radiotherapy and chemotherapies are often ineffective for metastatic disease and cause debilitating side-effects. In some circumstances, immunotherapies have been effective and safe but the overall therapeutic impact has been low because cancer is a chronic disease and mobilizes multiple mechanisms to inhibit the antitumor immune response (1, 2). Cellular inhibitors include regulatory T-cells (Tregs) and macrophage-derived suppressor cells (MDSC). In previous work we created a recombinant replicating vesicular stomatitis virus (rrVSV) that preferentially infected HER2/neu-expressing mammary cancer cells, eradicated peritoneal tumor implants in mice and generated anti-tumor memory T-cells (3-5). The current study sought to determine whether the treatment response could be improved by suppressing inhibitory cells with cyclophosphamide and whether anti-tumor memory T-cells were as sensitive to inhibition as acute effector T-cells.

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## Materials and Methods

*Cells, antibodies, chemicals and animals.* D2F2/E2 cells, a mouse mammary tumor line that has been stably transfected with a vector expressing the human Her2/neu gene and its parent cell line, D2F2 were a generous gift from Dr. Wei-Zen Wei, Karmanos Cancer Institute, Wayne State University, Detroit, MI. Monoclonal antibody (9H10) to cytotoxic T-lymphocyte antigen 4 (anti-CTLA4) was obtained commercially (BioXcell Fermentation/Purification Services #BE0131, West Lebanon, NH, USA). Cytoxan (Cyclophosphamide, #NDC 0015-0502-42, Bristol-Myers Squibb Co., Princeton, NJ, USA)(CPM) was freshly diluted in sterile water to a stock concentration of 20 mg/ml. Stock solution of 125 μl was freshly diluted in 375 μl of PBS and administered IP. All animal studies were conducted using female BALB/c mice, 8 to 20 weeks of age, weighing 20-25g, obtained from Taconic (Hudson, NY, USA). Animal studies were approved by the Institutional Animal Research and Care Committee, Protocol 12030356.

*Replicating recombinant vesicular stomatitis virus (rrVSV).* A replicating virus was created from vector components, as previously described (3), leading to the following properties: Preferential infection of cells expressing human HER2/neu, expression of mouse granulocyte-macrophage colony-stimulating factor (GM-CSF), and expression of enhanced green fluorescent protein (EGFP).

Vectors expressing the VSV genome (XN2) and the individual VSV genes P, L, N and G (pBS-P, L, N and G respectively) on a T7 promoter were a very generous gift of Dr. John K. Rose, Yale University School of Medicine, New Haven, CT, USA. Vectors expressing Sindbis glycoprotein (gp) and Sindbis gp modified between amino acids 71 and 74 to express two IgG binding domains (Sindbis-ZZ) were generously supplied by Dr. Irvin S. Y. Chen, University of California, Los Angeles Medical School, Los Angeles, CA, USA. A vector expressing a single chain antibody (SCA) based on the 4D5 anti-erbb2 antibody was a generous gift by Genentech Inc. South San Francisco, CA, USA.

*Treatment trials.* Female BALB/c mice were implanted intraperitoneally (IP) with $2\times10^6$ D2F2/E2 cells in 500 μl PBS. All rrVSV, anti-CTLA4 and CPM treatments were administered IP. Adoptive cell transfer was administered IP. Animals were assessed three times per week for ascites, abdominal nodules and signs of poor health such as low activity, poor grooming, rough coat,

hunched posture and dehydration and sacrificed if they developed ascites, nodules or any of these signs. The animals were considered cured if they survived for 100 days after tumor.

*Donor animals*. Memory cells were obtained from spleens of cured animals. These mice were produced by implanting female BALB/c Thy 1.2 mice intraperitoneally (IP) with $2\times10^6$ D2F2/E2 cells in 300 μl PBS. On day 3 they were treated with rrVSV, $1\times10^8$ IP, on day 4 with 200 μg anti-CTLA4 MAb and on day 5 with cyclophosphamide, ~100 mg/kg. The animals were considered cured if they survived for 100 days after tumor. Cells from all donor animals on any given day were pooled. In 1:1 transfers, cells from n animals were transferred to n hosts. In 1:2 transfers, cells from n animals were transferred to 2n hosts. As previously described, host animals were pre-treated one day before transfer of memory cells with a single dose of cyclophosphamide (CPM) at 100-125 mg/kg to facilitate cell transfer(4).

Inhibitory cells were obtained from spleens of 5 types of donors:
1) Standard therapy. Animals were implanted with D2F2/E2 IP on day 0 and received rrVSV on day 3 and anti-CTLA4 on day 4. Inhibitory cells were harvested on day 7.
2) Virus plus anti-CTLA4. Animals received anti-CTLA4 one day after virus. Inhibitory cells were harvested 5 days after virus administration.
3) Virus only. Inhibitory cells were harvested 5 days after virus administration.
4) Tumor only. Inhibitory cells were harvested 5 days after administration of IP D2F2/E2.
5) Naïve animals. No tumor or treatment was administered.

The number of animals in each experiment is detailed in the Figures.

*Spleen cell collection from donor animals*. Animals were sacrificed prior to cell harvesting. Spleens were harvested, minced and ground through a 70 μM nylon cell strainer (#352350, BD Falcon, Franklin Lakes, NJ, USA). RBC were lysed by incubating the cell suspension in 0.16M tris-buffered NH4CL for 5 minutes. All cells were washed twice with PBS and re-suspended in PBS.

*T cell isolation*. Total T-cells were isolated by positive selection using the autoMACS™ separator and the appropriate antibody microbeads according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA, USA): CD90 (Thy1.2, #130-049-101). Remaining cells were harvested and called non-T-cells.

*Statistics*. The log rank statistic was used to compare survival among the treatment groups. PRISM software was used to analyze the data (GraphPad Software, Inc., La Jolla, CA, USA).

## Results

In previous work, we created rrVSV that preferentially infected HER2/neu-expressing breast cancer cells. This rrVSV, in combination with anti-CTLA4 monoclonal antibody, called standard therapy, was able to eradicate established peritoneal tumor implants of a mouse mammary tumor cell line stably transfected to express HER2/neu by eliciting an anti-tumor CD4 and CD8 T-cell immunological response (5). In the current study we attempted to improve the cure rate with low-dose virus plus anti-CTLA4 by adding CPM (6) one day after the anti-CTLA4. This was called standard therapy plus CPM. Cure increased from 40% in animals that did not receive CPM to 90% in animals that did receive CPM ($p$=0.015; Figure 1). This improvement was not due to direct CPM killing of tumor cells because when CPM was administered to small tumors on day 2 after implantation, there was no improvement in survival (Figure 1). In fact, all treated mice rapidly died, indicating that CPM was abrogating the immune response which is essential for this viral oncotherapy.

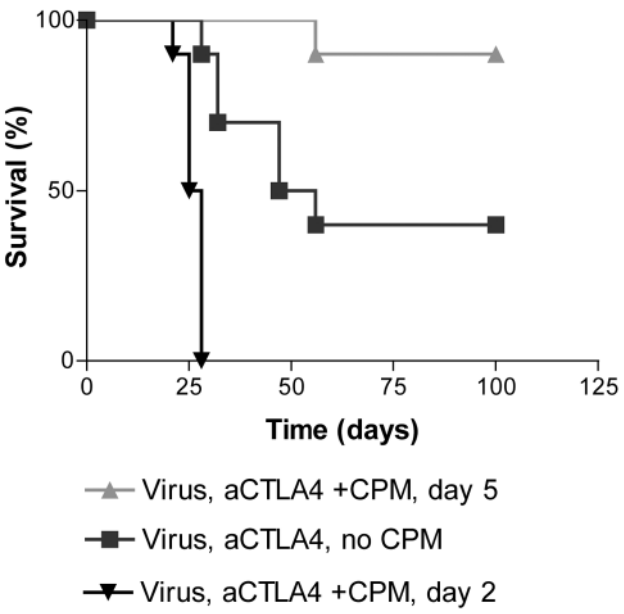


Figure 1. *Cyclophosphamide improves viral immunotherapy. Survival curves of D2F2/E2 implants treated with replicating recombinant vesicular stomatitis virus (rrVSV) and monoclonal antibody to cytotoxic T-lymphocyte antigen 4 (anti-CTLA4) with and without cyclophosphamide (CPM). Peritoneal tumors were treated 3 days after implantation with $1\times10^8$ ID of rrVSV. On day 4 after implantation, animals were treated with 200 μg of anti-CTLA4 MAb and on day 5 with 100 mg/kg CPM. Survival was significantly worse in control groups not receiving CPM (log_rank statistic p=0.015) or receiving CPM on day 2 after implantation (log-rank statistic p<0.0001) (n=10 for each group).*

Clear evidence that CPM was eliminating inhibitory cells came from transfer experiments showing that when cells from animals treated with standard therapy were added back to animals receiving standard therapy plus CPM, the therapeutic response was inhibited (Figure 2A). Test animals were treated with rrVSV, anti-CTLA4 and CPM. The expected cure rate was 90%. Control groups received no transferred cells or spleen cells from naïve mice. The experimental group received cells from animals that were treated with virus plus

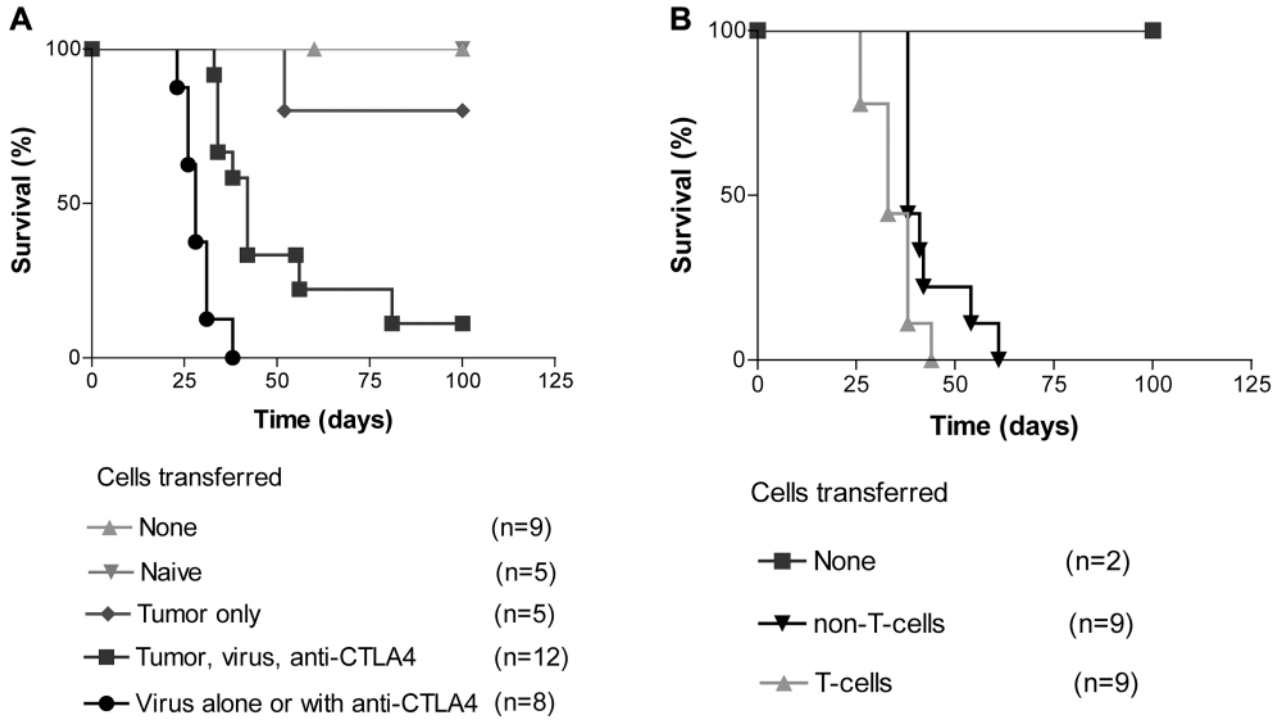


Figure 2. *Suppressor cells inhibit viral immunotherapy. Survival curves of D2F2/E2 implants treated with standard therapy plus cyclophosphamide (CPM) and various suppressor cells. Peritoneal tumors were treated 3 days after implantation with $1\times10^8$ ID of replicating recombinant vesicular stomatitis virus (rrVSV). On day 4 after implantation, animals were treated with monoclonal antibody to cytotoxic T-lymphocyte antigen 4 (anti-CTLA4) and on day 5 with 100 mg/kg CPM. A: Control groups that received no transferred cells or spleen cells from naïve mice achieved a 100% cure rate. The cure rate was 8.3% in animals who received suppressor spleen cells from animals implanted with tumor and treated with rrVSV and anti-CTLA4 (log-rank statistic p=0.0002) and 0% in animals who received suppressor cells from animals treated with rrVSV alone or rrVSV plus anti-CTLA4 (log-rank statistic p<0.0001). Transferred spleen cells from animals implanted with tumor but not receiving treatment had no significant inhibitory effect. B: The concurrent control group that received no transferred cells achieved a 100% cure rate as usual. Suppressor spleen cells were transferred from animals treated with rrVSV alone. Both T-cells and non-T-cells were effective suppressors, resulting in complete abrogation of the therapeutic response (log-rank statistic p=0.017 for T-cells and p=0.0168 for non-T-cells).*

anti-CTLA4 but no CPM. As expected, all animals in the control groups were cured. On the other hand, cells from the experimental group significantly abrogated the therapeutic response ($p$=0.0002). Inhibitory cells were not tumor antigen-specific because cells from animals infected with virus but not implanted with tumor also abrogated the therapeutic response ($p$<0.0001) (7, 8). Some of these virus-infected animals also received anti-CTLA4 but cells from both groups behaved identically and were analyzed together. Cells from animals implanted with tumor but not receiving treatment had no significant inhibitory effect. We concluded that viral infection of tumor produced inhibitory cells that were eliminated by CPM treatment. Inhibitory cells were harvested 5 days after viral infection, indicating very early generation of these potent cells.

Immune suppressor cells are known to derive from the T-cell lineage, Tregs and the macrophage lineage, MDSCs. We, therefore, divided inhibitory cells into T-cells and non-T-cells and tested each group separately. We found that that each group alone abrogated the therapeutic response indicating that the inhibitory response following viral infection was pleomorphic and redundant (Figure 2B).

We had previously shown that animals with peritoneal implants cured with viral immunotherapy generated antitumor memory T-cells which prevented re-implantation in the cured animals and could also be transferred to cure 3-day established tumor in newly-implanted animals (4, 5, 9, 10). We now found that the very potent inhibitory cells described above had no inhibitory effect on memory T-cells. Survival curves following treatment of newly- implanted peritoneal tumors with memory T-cells with or without inhibition showed overlapping curves (Figure 3A). Initially, we treated 3-day implanted tumors with memory cells from the equivalent of one cured animal (see Material and

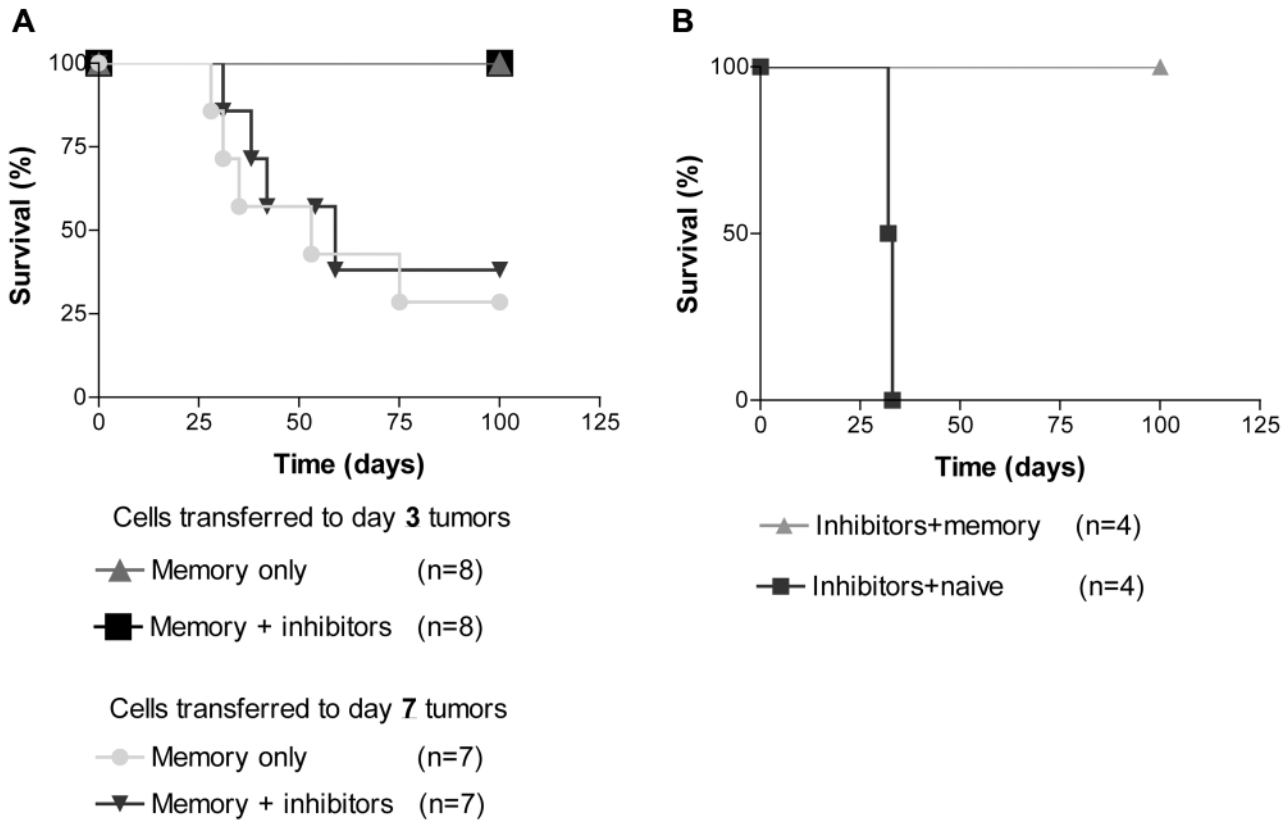


Figure 3. *Memory T-cells are not inhibited by suppressor cells. A: Survival curves of D2F2/E2 implants treated with transferred memory cells with or without transferred suppressor cells. Peritoneal tumors were treated 3 or 7 days after implantation with spleen cells from cured animals. The control animals received only memory cells and the experimental animals received memory and suppressor cells on the same day. Suppressor spleen cells came from animals treated with replicating recombinant vesicular stomatitis virus (rrVSV) alone and were found in concurrent experiments to be potent suppressors of the acute response (data not shown). Suppressor cells had no effect on the therapeutic response elicited by transferred memory cells. B: Survival curves of D2F2/E2 implants treated with rrVSV, monoclonal antibody to cytotoxic T-lymphocyte antigen 4 (anti-CTLA4) and cyclophosphamide (CPM), transferred suppressor cells and either transferred memory cells or transferred naïve cells. Memory cells completely rescued the inhibitory effects of the suppressor cells (log-rank statistic p=0.0084).*

Methods). Three animals received memory cells alone and three received memory and inhibitory cells. In this and all similar experiments, the same inhibitory cells were shown on the same day to inhibit the acute treatment response. All animals were cured. We then lowered the dose of memory cells to the equivalent of 0.5 cured animals and treated five animals with memory cells alone and with memory and inhibitory cells. Again, all animals were cured (Figure 3A). The inhibitory cells clearly did not abrogate the memory therapeutic response but it was possible that a partial inhibitory response was hidden by a super-therapeutic memory dose. We, therefore, treated 7-day tumors with a dose equivalent of one cured animal. This time the memory dose was not super-therapeutic and there was still no difference between the groups with and without inhibitory cells (Figure 3A). Surprisingly, memory cells were still able to cure some of these very extensive and large tumors.

In order to compare the effects of inhibitory cells on memory T-cells and acute effector T-cells under precisely the same conditions, mice were implanted with tumor and treated with standard therapy plus CPM. On day 7, they received inhibitory cells plus naïve cells or inhibitory cells plus memory cells. As expected, all animals receiving inhibitory cells plus naïve cells died from tumor, whereas all animals receiving inhibitory cells plus memory cells survived ($p$=0.008; Figure 3B).

## Discussion

The present study suggests that memory T-cells differ from naïve T-cells by resistance to cellular inhibition. Memory T-cells are known to differ from naïve T-cells by elevated frequency to previously encountered pathogens, rapid proliferation and secretion of cytokines, low activation

thresholds, response to multiple antigen-presenting cells, including B-cells, ready localization to non-lymphoid tissue, antigen-independent persistence and long lifetime (11-13). We propose that an additional critical property for antitumor effect is resistance to multiple types of immune inhibitors including both T-cells and non-T-cells. In this study, resistance was very strong because T-cell inhibitors alone and non-T-cells inhibitors alone were able to completely block the acute therapeutic immune response but both were unable to block the therapeutic memory response. Similar evidence for resistance to Tregs has been shown in alloreactive memory T-cells (14, 15). In future work, the mechanisms of resistance must be explored and are likely to be multiple because the suppressor cells were pleomorphic and comprised both T-cells and non-T-cells.

The implication of this observation is that there may be special utility for immune therapy that generates antitumor memory T-cells soon after detection of the tumor. If the tumor can be completely resected, the memory cells remain available and potent (16) to prevent or treat late metastases arising from sanctuary sites. In place of trying to boost the immune response by individually inhibiting known inhibitors, such as MDSCs and multiple types of Tregs, tumor control may be more easily achieved by generating memory cells that are therapeutically effective despite the presence of these known and other currently unknown inhibitors. The data from this study and in other studies suggest that VSV and with CPM are potent stimulators of memory T-cell development (11, 17).

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