Treatment with targeted vesicular stomatitis virus generates therapeutic multifunctional anti-tumor memory CD4 T cells

Y Gao1, P Whitaker-Dowling2, JA Griffin1 and I Bergman3

1Department of Pediatrics, University of Pittsburgh, Pittsburgh, PA, USA; 2Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA and 3Departments of Pediatrics, Neurology and Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

A generally applicable, easy-to-use method of focusing a patient’s immune system to eradicate or prevent cancer has been elusive. We are attempting to develop a targeted virus to accomplish these aims. We previously created a recombinant replicating vesicular stomatitis virus (VSV) that preferentially infected Her2/neu expressing breast cancer cells and showed therapeutic efficacy in an implanted Balb/c mouse tumor model. The current work shows that this therapy generated therapeutic anti-tumor CD4 T cells against multiple tumor antigens. CD4 T cells transferred directly from cured donor mice could eradicate established tumors in host mice. T cells were transferred directly from donor mice and were not stimulated ex vivo. Both tumors that expressed Her2/neu and those that did not were cured by transferred T cells. Analysis of cytokines secreted by anti-tumor memory CD4 T cells displayed a multifunctional pattern with high levels of interferon-γ, interleukin (IL)-4 and IL-17. Anti-tumor memory CD4 T cells traveled to the mesenteric lymph nodes and were activated there. Treatment with targeted recombinant replicating VSV is a potent immune adjuvant that generates therapeutic, multifunctional anti-tumor memory CD4 T cells that recognize multiple tumor antigens. Immunity elicited by viral therapy is independent of host major histocompatibility complex or knowledge of tumor antigens. Virus-induced tumor immunity could have great benefit in the prevention and treatment of tumor metastases.

Cancer Gene Therapy (2012) 19, 282–291; doi:10.1038/cgt.2011.90; published online 13 January 2012

Keywords: anti-tumor memory T cells; VSV; targeted virus; cancer therapy

Introduction

The immune system can rapidly destroy a large transplanted organ but directing this latent force to eradicate cancer tissue has been very difficult. Cancer immunotherapy began with bacterial vaccinations and now includes passive transfer of antibody or immune cells and tumor vaccination using a variety of strategies.1–3 Despite some successes, a reliable, safe, easy-to-use, reasonably priced, general immunological technique to treat or prevent solid tumor metastases is not available. We are developing a tumor-targeted replicating recombinant vesicular stomatitis virus (VSV) to be used both directly for immune-mediated tumor therapy and prophylactically to induce anti-tumor immunity. Prophylactic use would entail administering the virus to the primary tumor at the initial clinical presentation in order to prevent the later development of tumor metastases. The foundation for this strategy is established in part by the data in this paper, which shows that treatment with recombinant replicating VSV (rVSV) generates highly potent anti-tumor memory CD4 T cells.

VSV is an excellent candidate for development for cancer therapy because it is a safe oncolytic virus that can be genetically engineered.6 Normal tissues are protected from the virus by interferon (IFN) production but most human tumors are insensitive to the effects of IFN and are more susceptible to killing by VSV.7,8 Viral oncolysis releases multiple tumor antigens in the context of an antiviral inflammatory response. This inflammatory response stimulates an immune response instead of tolerance to the tumor antigens. The VSV genome can be altered to attenuate the virus or to have it express immune modifiers. Therapeutic effects in animal models have been observed using wild-type VSV (wt VSV), VSV modified with cytokines and VSV in combination with chemotherapy.9–12 A theoretical safety concern is whether the virus will be tolerated by cancer patients who are immunologically compromised. Several approaches to address this issue are being developed such as creating an rVSV expressing IFNβ, creating a VSV M-protein mutant that induced 20–50 times more IFN than wt VSV or administering IFN systemically during VSV infection.7 We improved the safety profile by creating a rVSV with an altered surface glycoprotein (gp) that targeted preferentially to breast cancer cells highly...
expressing the Her2/neu receptor, erbb2. The viral genome was also modified by the inclusion of genes expressing mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) and green fluorescent protein. We showed that this rrVSV selectively infected, replicated and killed cells expressing erbb2. We then showed that therapy with rrVSV expressing GM-CSF combined with anti-CTLA4 monoclonal antibody (MAb) could eliminate established macroscopic tumor implants. Successful therapy required both CD4 and CD8 T-cell responses in the treated animals. Surviving animals were resistant to tumor re-challenge suggesting a memory immune response.

We now sought to further characterize this anti-tumor memory immune response. We found that CD4 T cells were responsible for the immune memory response, were highly potent and could support a strategy of dealing with the problem of cancer metastases by immunoprevention. Anti-tumor memory T cells were obtained from donor animals whose established tumors were cured by therapy with rrVSV and anti-CTLA4 MAb. Cytokine production by these cells was studied using the Milliplex cytokine kit as well as EliSpot and intracellular IFN cytometry. Therapeutic efficacy was studied by transfer to host animals with implanted peritoneal tumors. We found that the memory CD4 T cells were powerful and could cure established tumors in host animals 40–50% of the time. T cells were transferred directly from donor to host mice. No ex vivo stimulation was required. As expected, we found that rrVSV treatment generated memory T cells to multiple tumor antigens. Unexpectedly, CD4 T cells alone mediated the memory anti-tumor effect. Transferring CD8 T cells, B cells or antibody from cured animals in addition to CD4 T cells did not improve outcome. Also unexpectedly, the memory anti-tumor CD4 T cells were not apparently restricted to Th1, Th2 or Th17 type but appeared multifunctional expressing a diverse array of cytokines including IFNγ, interleukin (IL)-4 and IL-17. Cytokine secretion of specific anti-tumor memory T cells was most effectively studied by in vivo tumor challenge and analysis of lymph node T cells.

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. Anti-CTLA4 (9H10) ascites was prepared from a hybridoma generously supplied by Dr James P Allison, Memorial Sloan Kettering Cancer Center, New York, NY or obtained commercially (BioXcell Fermentation/ Purification Services #BE0131, West Lebanon, NH). Cytoxan (cyclophosphamide (CPM), #NDC 0015-0502-42, Bristol-Myers Squibb, Princeton, NJ) was freshly diluted in sterile water to a stock concentration of 20 mg ml⁻¹. Stock solution of 125 μl was freshly diluted in 375 μl of phosphate-buffered saline (PBS) and administered intra-peritoneally (IP). All animal studies were conducted using female BALB/c mice, 8–20 weeks of age, weighing 20–25 g, obtained from Taconic (Hudson, NY). These animal studies were approved by the institutional Animal Research and Care Committee.

Recombinant replicating VSV
rrVSV targeted to cells expressing Her2/neu was created from vector components as previously described. In brief, 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. Vectors expressing Sindbis gp and Sindbis gp modified between amino acids 71 and 74 to express two immunoglobulin G-binding domains (Sindbis-ZZ) were generously supplied by Dr Irvin SY Chen, University of California, Los Angeles Medical School. A vector expressing a single chain antibody based on the 4D5 anti-erbb2 antibody was a generous gift by Genentech. As previously described, we used PCR to create a chimeric Sindbis gp, which consisted of the first 71 amino acids of the Sindbis E2 gp followed in order by a poly-glycine linker, single chain antibody to erbb2, CH1 linker, the remainder of the E2 gp and the entire E1 Sindbis gp. The gene for the native VSV-G gp was removed from the VSV genome (XN2) and replaced with a gene coding for the chimeric Sindbis gp. In addition, genes coding for enhanced green fluorescent protein and mouse GM-CSF were added to the VSV genome producing a genome of 14838 bases. Replicating recombinant VSV was created using standard techniques that expressed only the chimeric Sindbis gp on its surface and also expressed enhanced green fluorescent protein and GM-CSF. This rrVSV was then adapted to grow well on D2F2/E2 cells by serial passage in vitro on this cell line. rrVSV for animal trials was made by infecting D2F2/E2 cells at a multiplicity of infection = 0.001 in 168 cm² tissue culture flask (Corning/CoStar, Corning, NY) and harvesting supernatant 48 h later. Titers of rrVSV in the supernatant were typically 1–3 × 10⁸ per ml on D2F2/E2 cells assayed by counting green cells as previously described.

Cell collection
Animals were killed before cell harvesting. Spleens were harvested, minced and ground through a 70 μm nylon cell strainer (#352530, BD Falcon, Franklin Lakes, NJ). Red blood cell were lysed by incubating the cell suspension in 0.16 m tris-buffered NH₄CL for 5 min. Bone marrow cells were aspirated from both femurs and tibias, passed through the cell strainer and underwent red blood cell lysis. Mesenteric lymph node cells were collected by careful dissection of the lymph nodes in the peritoneum and grounding through the cell strainer. Red blood cell lysis was performed when necessary. Peritoneal washings were performed by injecting 10 ml of sterile PBS into the peritoneum through a 16 gauge needle, which was left in place. After 5 min, all the fluid that could be aspirated easily into the syringe was collected. Usually 9.0 ml was
collected. Mononuclear cells were collected, when necessary, by centrifugation over lymphocyte separation media (#25-072-CV, Mediatech, Manassas, VA). All cells were washed twice with PBS and re-suspended in PBS.

**T- and B-cell isolation**

Total T cells, B cells, CD4 T cells and CD8 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): CD90 (Thy1.2, #130-049-101), CD19 (#130-052-201), CD4 (L3T4, #130-049-201) and CD8a (Ly-2, #130-049-401).

**Flow cytometric intracellular cytokine analysis**

Intracellular staining for mouse IFNγ was performed using the Cytofix/Cytoperm Plus Kit with GolgiPlug (#555028, Becton Dickinson, Mountainview, CA) as recommended by the manufacturer. In all, 5 × 10^5 spleen cells per well were plated in 1 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum in Linbro 24-well tissue culture plates (#76-033-05, ICN Biomedicals, Aurora, OH) and incubated overnight at 37°C with 5% CO2. Following incubation with brefeldin A (final concentration at 10 μg ml⁻¹) for 4 h, T cells were isolated by positive selection as above. In total, 1 × 10^6 T cells were suspended in ice-cold PBS:0.1% bovine serum albumin/0.2% Azide and stained with phycoerythrin-conjugated antibodies to either CD4 or CD8 or isotype control antibody (eBioscience #12-0042-82, 12-0081-82 and 12-4321-82). The cells were fixed and permeabilized by incubation with 250 μl cytofix/cytoperm solution for 20 min on ice and stained with allophycocyanin-conjugated antibody to mouse IFNγ or isotype control antibody (eBioscience #17-7311 and 17-4714). Immunofluorescence was quantified using a FACStarPlus cytometer (Becton Dickinson).

**EliSpot**

EliSpot analyses for IFNγ producing T cells were performed using the mouse IFN-γ ELISpot PLUS kit with white precoated plates, horseradish peroxidase (#3321-4HPW-4, MBTec). Following in vivo challenge, 2 × 10^5 T cells from spleen, lymph nodes and peritoneal fluid were incubated in 0.1 ml media in separate wells without further stimulation for 48 h at 37°C in a 5% CO2 incubator. Exposure of the spots was performed according to the manufacturer's recommendations and the spots were visualized and quantified with an EliSpot microscope reader (Carl Zeiss MicroImaging, Thornwood, NY).

**Cytokine and chemokine analyses**

Cytokine secretion was quantified using the Milliplex map mouse cytokine/chemokine kit (Millipore, Billerica, MA), which simultaneously measured the following cytokines: G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, KC, MCP-1, MIG, MIP-1α, RANTES and tumor necrosis factor-α. CD4 and CD8 T cells were fractionated as above and 1 × 10^6 cells suspended in 200 μl Dulbecco’s modified Eagle’s medium with 10% fetal calf serum were plated at 37°C in a 5% CO2 incubator in individual wells of a 96-well round bottom tray for 24 h (Corning). A Luminex xMAP reader (Bio-Rad, Hercules, CA) was used to measure cytokine concentration in 25 μl aliquots of supernatant from each well using standard samples supplied by the manufacturer and assayed according to the manufacturer’s instructions.

**In vitro** stimulation studies were performed by first incubating 1 × 10^5 dendritic cell (DC) with 1 × 10^5 tumor cells for 6 h in 200 μl media at 37°C in a 5% CO2 incubator. Tumor cells were either live, killed and disrupted by three rounds of freeze–thaw or killed by exposure to mitomycin (25 μg ml⁻¹). Then, 5 × 10^5 dendritic cell were incubated with 5 × 10^5 CD4 or CD8 T cells in 250 μl media in individual wells of a 96-well round bottom tray for 24 h. Cytokine concentration in supernatant was measured as above.

**Treatment trials**

Female BALB/c mice were implanted IP with 2 × 10^6 D2F2/E2 cells in 500 μl PBS. All viral and antibody treatments were administered IP. Adoptive cell transfer was usually administered IP except for two animals that received intravenous (IV) treatment as noted in the text and figure. 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 killed if they developed ascites, nodules or any of these signs. The animals were considered cured if they survived for 100 days after tumor.

**Statistics**

The log-rank statistic was used to compare survival among the treatment groups. The Mann–Whitney one-tailed test was used to compare cytokine secretion in the CD4 T cells from various treatment groups. This non-parametric test was used because some values in the Milliplex analyses were too low to be accurately measured and were arbitrarily assigned a value lower than the lowest recorded value. PRISM software was used to analyze the data (GraphPad Software, La Jolla, CA).

**Results**

We had previously used re-challenge experiments to demonstrate that successful viral therapy produced anti-tumor immunity. A Balb/c mouse mammary cancer cell line, D2F2, had been stably transfected by others with a vector expressing the human Her2/neu receptor creating the cell line, D2F2/E2.19 D2F2/E2 cells were implanted in the peritoneum and mice were treated 3 days later with rRVSV targeted to Her2/neu and anti-CTLA4 antibody. Animals surviving for 100 days were considered cured and then re-challenged with IP tumor. In a small series of animals, we showed that most animals were able to resist
re-challenge with first the Her2/neu expressing D2F2/E2 cells and then the non-Her2/neu expressing parental cell line, D2F2. We now confirmed this finding in a larger series of animals and used transfer experiments to identify the effective memory cells.

**Re-challenge cured animals**

Fifteen long-term survivors of D2F2/E2 implantation (including five previously reported) who had been treated with rrVSV and aCTLA4 MAb were re-challenged with D2F2/E2 cells. These mice did not receive any therapy. Fourteen survived > 100 days indicating the presence of immunity to this tumor cell line. One died 34 days after challenge. It was important to determine whether survival was determined by immunity only to the foreign Her2/neu receptor protein, which had been introduced into the D2F2/E2 cells or whether the immunity extended to the parent D2F2 cells. Twelve of these animals were then challenged with D2F2 cells. Nine never developed tumor thereby demonstrating that rrVSV therapy had resulted in immunity to the fully syngeneic D2F2 cells. Three animals died 31–40 days after challenge indicating partial immunity because the median time to death in naïve animals following tumor challenge has been 16–20 days. Five long-term survivors were challenged directly with D2F2 cells and all survived indicating that immunity to D2F2 antigens developed after initial viral therapy and did not require a first re-challenge with D2F2/E2.

**Transfer T cells from cured animals**

A series of transfer experiments was performed to prove that viral immunotherapy was generating anti-tumor memory T cells. In agreement with previous work, we found that transferred cells from cured donor animals were only effective when host animals were pre-treated with a single dose of CPM at 100–125 mg kg⁻¹. Pre-treatment with CPM is apparently required to make room for the transferred cells, to stimulate production of host cytokines such as IL-7 or IL-17 or to suppress inhibitory T-cell subsets.

The first experiment used spleen cells from cured donor animals to prevent tumor development. Spleen cells, 4–6 × 10⁶ cells, were administered IV (two animals) or IP (two animals) 1 day before IP tumor challenge (Figure 1). None of the animals given donor spleen cells ever developed tumors whereas the animals given CPM alone promptly developed tumors and were killed (median survival = 24.5 days). Thus, as reported by others, spleen cells from cured donor animals were able prevent tumor establishment.

Next, we attempted a more stringent model, which asked whether transferred cells could eradicate established tumors in host animals. Animals were implanted with tumor cells IP and treated 3 days later with cells transferred from cured mice. Spleen cells (mean: 2.7 × 10⁶ cells), bone marrow cells (mean: 2.4 × 10⁶ cells), lymph node cells (mean: 2.4 × 10⁶ cells) or cyclophosphamide (CPM) only (n = 5 for each group). 100 µl of serum from cured mice was added to one group that received spleen cells (n = 4). (Compared with CPM control, survival was significantly improved in the group treated with spleen cells, log-rank statistic P = 0.013 and the group treated with spleen cells and serum, log-rank statistic P = 0.0047).

Bone marrow cells, which include memory B cells, administered at about the same number as spleen cells (mean: 2.4 × 10⁶ cells) were ineffective. Finally, lymph node cells that contain about 2/3 T cells were also ineffective but the dose of transferred cells was 10-fold lower (mean: 2.4 × 10⁶ cells). In addition, it is possible that more anti-tumor memory T cells resided in the spleen than the lymph nodes in resting cured donor mice.

In order to determine whether memory T cells alone were responsible for cure or a combination of memory lymphocytes was required, we compared therapy using spleen T cells with therapy using a combination of T and B cells (Figure 3). We found no difference between the groups indicating that the anti-tumor effect was carried by the T cells alone.

We then asked whether the memory anti-tumor effect was mediated by CD4 T cells, CD8 T cells or both. We...
found that transfer of memory CD4 T cells produced cure in 37.5% of cases (Figure 4). Adding CD8 T cells to the CD4 T cells produced no significant additional benefit and CD8 T cells alone were not more effective than CPM alone. The larger number of transferred CD4 than CD8 T cells reflected the increased number of CD4 T cells harvested from individual mouse spleen.

In further extensions of these transfer experiments, we found that T cells from cured mice were able to eradicate not only 3-day old but also 5-day old established tumor in all five of a set of five host animals. More importantly, we proved that anti-tumor memory T cells recognized antigens distinct from Her2/neu. T cells from cured mice were administered to host animals bearing 3-day established tumor of the parent cell line, D2F2. Tumors were eradicated in 50% of animals ($n = 8$).

Long-lasting immunity was transferred from donor to host animals as demonstrated by re-challenge experiments (Table 1). Sixteen host animals cured by transfer of T cells from donor animals were re-challenged with D2F2/E2 cells. In all, 14 survived and 2 died at 22 and 38 days after challenge. Twelve of these animals were then re-challenged with D2F2 cells. Eight survived and four died at 24, 29, 29 and 63 days after challenge. It is most likely that transferred CD4 T cells survived in the hosts and were responsible for this permanent immunity but we did not pursue proof of this point.

**Characterize the anti-tumor memory T cells**

We sought to determine whether viral immunotherapy generated predominantly a single functional CD4 T cells response such as Th1, Th2 or Th17 or a multifunctional response. In addition, we wanted to clearly demonstrate that the memory CD4 T cells responded to native antigens from the parent D2F2 cells and not just the foreign Her2/neu receptor protein, which had been introduced into the D2F2/E2 cells. Cured animals, >100 days after tumor implant and viral therapy, were therefore re-challenged with either D2F2/E2 cells or D2F2 cells. After 3 days, CD4 and CD8 T cells were harvested from spleen, mesenteric lymph nodes and peritoneal lavage and allowed to secrete cytokines overnight without further stimulation. Supernatants were harvested and assayed for a wide variety of cytokines and chemokines. A standard control group consisted of naïve animals that were challenged with D2F2/E2 cells and killed 3 days later. However, we required a more stringent control group because the experimental groups received virus and anti-CTLA4 in addition to tumor and we wanted to be sure that we were assaying anti-tumor and not anti-viral T cells. The crucial control group, therefore, consisted of animals that did not receive tumor but were treated with virus and anti-CTLA4 antibody. After 60 to 100 days, they were challenged with D2F2/E2 cells just like the experimental group and T cells were harvested 3 days after challenge. The T cells were placed in wells without any stimulation and cytokine concentration was measured after 24 h of secretion. The primary question was whether the pattern of cytokine secretion in the experimental groups was different than the virus only control, indicating a specific memory anti-tumor response. A consistent anti-tumor memory response was found in

**Table 1** Re-challenge with either erbb2 expressing D2F2/E2 or parent tumor D2F2 in animals cured by T-cell transfer

| Cells          | Total | Survival |
|----------------|-------|----------|
| First re-challenge | D2F2/E2 | 16 | 14 (88%) |
| Second re-challenge* | D2F2   | 12 | 8 (67%)  |

*All of these animals survived a first re-challenge with D2F2/E2.
in vivo

following and CD8 T cells isolated from spleen and peritoneal cells
different in the experimental from the control group. CD4
not show a clear pattern of cytokine secretion that was
consistently

distinct patterns in the experimental group.

Unlike CD4 T cells, CD8 T cells from lymph nodes did not
show a clear pattern of cytokine secretion that was
different in the experimental from the control group. CD4
and CD8 T cells isolated from spleen and peritoneal cells
following in vivo tumor challenge also did not show a
pattern of cytokine secretion that was consistently
different in the experimental from the control groups.

Finally, cytokine concentrations in blood and peritoneal
fluid following in vivo tumor challenge did not show
distinct patterns in the experimental group.

Table 2 Cytokine production by CD4 T cells harvested from lymph
nodes in animals receiving various treatment

|          | A          | B          | C          | D          |
|----------|------------|------------|------------|------------|
| IFNγ     | 500        | 302        | 68         | 2          |
| IL-4     | 165        | 73         | 18         | 7          |
| IL-5     | 557        | 385        | 173        | 1          |
| IL-17    | 8          | 9          | 0          | 0          |
| IL-2     | 54         | 202        | 69         | 5          |
| IL-10    | 122        | 55         | 17         | 2          |
| GM-CSF   | 633        | 354        | 100        | 2          |
| IL-3     | 475        | 347        | 95         | 5          |
| MIP-1α   | 154        | 99         | 12         | 6          |
| Cured tumor | Yes       | Yes       | No         | No         |
| Virus    | Yes        | Yes        | Yes        | No         |
| Anti-CTLA4 | Yes       | Yes        | Yes        | No         |
| Re-challenge | D2F2/E2 | D2F2       | D2F2/E2    | D2F2/E2    |

Abbreviations: GM-CSF, granulocyte-macrophage colony-
stimulating factor; IFN, interferon; IL, interleukin.

(A) Implanted with D2F2/E2. Treated with rVSV and anti-
CTLA4 MAb. Challenged > 100 days later with D2F2/E2 (erb2
expressing).

(B) Implanted with D2F2/E2. Treated with rVSV and anti-
CTLA4 MAb. Challenged > 100 days later with D2F2 (non-erb2
expressing).

(C) No tumor implanted. Treated with rVSV and anti-CTLA4
MAb. Challenged > 60–100 days later with D2F2/E2 (erb2
expressing).

(D) No tumor. No treatment. Challenged with D2F2/E2 (erb2
expressing).

Values are concentration of cytokine in supernatant, pg ml–1.

Means of four experiments.

Analysis by EliSpot supported the cytokine secretion
data. IFNγ-secreting CD4 T cells were assayed from
mesenteric lymph nodes following challenge with D2F2/
E2 or D2F2. The most positive cells were seen in the
experimental group challenged with D2F2/E2 but this
group and the group challenged with D2F2 had more
positive cells than the control groups challenged with
D2F2/E2 (Figure 5).

These experiments that assayed T cells after in vitro
stimulation were more successful in demonstrating a
memory T-cell response to tumor antigens than experi-
ments utilizing in vitro stimulation. In a single experiment,
we harvested T cells from spleens of cured animals,
challenged them with DCs loaded with tumor antigens
and assayed cytokine response using the Milliplex
cytokine kit. Tumor antigens were obtained from
freeze-thawed tumor cells, mitomycin-treated tumor cells
and live tumor cells. Results in the experimental animals
were not clearly different than the control animal who
received virus and anti-CTLA4 MAb but no tumor. The
in vitro conditions were not able to adequately simulate
antigen presentation as it occurs in the lymph nodes of
live animals challenged with tumor.

Attempts to identify and quantify the anti-tumor T cells
response at earlier times following therapy were clouded
by a very strong anti-viral T-cell response, as noted by
others.33 In one set of three independent paired experi-
ments, an experimental group implanted with D2F2/E2
and treated with virus and anti-CTLA4 was compared
with a control group receiving virus and anti-CTLA4 but
no tumor. All mice were challenged with D2F2/E2 30
days after treatment. After 3 days, mesenteric lymph
nodes were harvested and EliSpot analyses performed on
T cells. The mean number of activated IFNγ-secreting
CD4 T cells was 168 per 105 lymph node cells in the
experimental group (range 104–216) and 112 in the
control group (range 61–169). This difference was not statistically different. In another set of three independent experiments, experimental groups implanted with D2F2/E2 and treated with virus and anti-CTLA4 were compared with control groups receiving tumor and treated with virus alone, groups receiving tumor alone or groups receiving virus plus anti-CTLA4 but no tumor. Spleens were harvested 4 days after viral therapy and intracellular flow cytometric analyses performed on T cells. In all experiments, the experimental group had about 3% of CD4 T cells expressing IFN-γ (Figure 6) but values in the no tumor control group receiving virus and anti-CTLA4 varied from 1.6 to 3.6% and the differences were not statistically significant.

Discussion

Cancer therapy has been attempted using viruses and using immunotherapy. Passive immunotherapy using antibodies or ex vivo activated amplified CD8 T cells has had some success against specific cancers.\textsuperscript{4,27} Active immunization using a variety of techniques has had limited impact.\textsuperscript{3,28,29} Virus therapy alone has also been unsuccessful. Recently, viral therapy with immunotherapy has shown some clinical benefit.\textsuperscript{20} Our goal is to combine virus and immune therapy by developing a safe, easy-to-use targeted virus that consistently evokes anti-tumor immunity that eradicates or prevents tumor metastases. Our previous work has shown that a targeted rrVSV expressing GM-CSF combined with anti-CTLA4 MAb can eliminate established small macroscopic tumor implants through an immunologic mechanism. This paper proves conclusively that targeted rrVSV generates long-lived therapeutic anti-tumor memory CD4 T cells that recognize multiple tumor antigens. These CD4 T cells are capable of orchestrating a curative anti-tumor response that eradicates small established tumor implants.

A major advantage of using an oncolytic virus to induce active immunotherapy is that multiple tumor antigens are released in situ in the context of an anti-viral inflammatory response producing the ultimate personalized therapy. Each individual in the genetically heterogeneous human population will generate immunity to the antigen or antigens that best fit their unique major histocompatibility complex profile. This therapy requires no knowledge of specific tumor antigens and is independent of the patient’s major histocompatibility complex. The development of potent immune responses against multiple antigens was clearly shown in this model. As expected, the most potent immune response was generated against the foreign Her2/neu receptor protein as shown by cure rates in transfer experiments and analysis of cytokine secretion following in vivo tumor challenge. However, curative responses in transfer experiments and stimulation of tumor-specific CD4 T cells as shown by cytokine secretion were also found with challenge by D2F2 cells, which did not express Her2/neu. We recognize that it is important to confirm these findings in a fully syngeneic model and are currently performing studies in a transgenic mouse, which expresses the human Her2/neu receptor under the murine mammary tumor virus promoter.\textsuperscript{30} We will also attempt to treat spontaneous
tumors, which develop in 76% of animals in this model system.

An unexpected finding in these studies was the anti-tumor potency of memory CD4 T cells. Transfer of these cells alone was able to cure established tumors in host animals without apparent additional benefit by also transferring CD8 T cells or B cells. We were able to harvest and transfer more CD4 T cells than CD8 T cells from each donor animal and it is possible that transferring a larger number of CD8 T cells would have shown greater effect. A contributing role for memory CD8 T cells can therefore not be excluded. Importantly, host animals had endogenous CD8 T cells and B cells, which may have contributed to the therapeutic response but the only memory cells required from the donor mice were CD4 T cells. Most clinical programs of cancer immunotherapy attempt to generate or activate anti-tumor CD8 T cells.31,32 These T cells can directly attack tumor cells by attaching to their surface class I antigens. In contrast, CD4 T cells can not directly recognize tumor cells because tumor cells do not generally express class II antigens on their surface. Instead, CD4 T cells detect tumor cells indirectly via the antigens that they shed and are picked up and presented by DCs and macrophages. Previous work has shown that transferred CD4 T cells can eliminate tumors in host mice but these studies have either used T cells from transgenic mice that express a monoclonal CD4 T-cell population or activated the cells ex vivo or used additional therapy in conjunction with the transferred cells.33–40 A single patient has been treated successfully with autologous CD4 T cells activated ex vivo but other patients treated with the same protocol did not have a successful outcome.25,41 The anti-tumor memory T cells generated by therapy with rrVSV came from animals with a normally diverse immune system, were not stimulated ex vivo and did not require any adjunctive therapy to cure established tumors at least 5 days old and to cure tumors composed of fully syngeneic tumor cells. The location and mechanism of activation of transferred CD4 T cells has previously been questioned.33 This paper demonstrates that anti-tumor memory CD4 T cells traveled to the mesenteric lymph nodes and were activated there, presumably by DCs and macrophages that migrated with tumor antigens from the peritoneal implants of tumor.47,48,49 We did not explore the mechanisms by which CD4 T cells lead to tumor elimination but previous work in other model systems has shown dependence on macrophages, PMN and NK cells.44,45 These findings support the development of adoptive transfer of memory CD4 T cells for cancer therapy despite technical challenges such as expanding the number of cells without inhibiting their activity and dealing with the class II heterogeneity in the human population.25

Another unexpected finding was the apparent multifunctional nature of the anti-tumor memory CD4 T-cell response. The usual emphasis for tumor immunotherapy is to try to stimulate a Th1 response and thereby activate a cellular response, which can directly kill tumor cells.43 Recently, there is interest in activating a Th17 response, which is thought to be involved with autoimmunity and can be directed against the autologous tumor cells.44,45 Th2 responses are generally thought to counteract antitumor immunity28 although this has not been true in every model system.39 This study found that anti-tumor memory CD4 T cells stimulated by tumor antigens in the mesenteric lymph nodes secreted a heterogeneous group of cytokines characteristic of Th1, Th2 and Th17 cells. The key to generating a sustained immune response that eradicates all cancer cells may be a balance among the CD4 T-cell subsets.40 We recognize that further work is required to prove that each subset is not only present but necessary to achieve tumor cure and to characterize the balance required among the subsets, quantitatively, temporally and topographically.

Immunotherapy would benefit greatly from markers of anti-tumor immune response. In the clinic, survival as a measure of response can take years to quantify. Valid markers could identify ineffective therapies early and indicate the need for new tactics. In addition, markers that correlated with efficacy could help elucidate the mechanisms of successful immunotherapy. This study points out the difficulties in finding useful markers. As noted by others, viral therapy yields a strong anti-viral immune response, which overshadows the anti-tumor response.26 Equally problematic, the anti-tumor response may only be characterizable in tissues and under conditions that do not translate to the clinic. In our model, anti-tumor memory CD4 T cells were clearly present in the mesenteric lymph nodes following in vivo stimulation, as expected.46 However, they were not found in spleen and peritoneal fluid following in vivo stimulation and not found in spleen following in vitro stimulation. Cytokine response in blood and peritoneal fluid following in vivo stimulation also did not yield useful markers. A hopeful finding was that the Milliplex cytokine kit was more sensitive than Elispot or intracellular flow cytometry at detecting anti-tumor T cells and we are presently undertaking analysis of temporal patterns in CD4 T cells following acute therapy to identify valid markers of effector anti-tumor CD4 T-cell response. Interestingly, the cytokine with the greatest amplification in experimental animals compared with viral controls was MIP-1α (CCL3). This may be explained by a recent report showing that MIP-1α is secreted by activated CD4 T cells, which are in contact with DC.47 Assay of this chemokine at the appropriate time and location after viral therapy may provide a marker to the development of anti-tumor immunity.

The goal for rrVSV therapy is to eradicate active, growing Her2/neu-positive tumor metastases and to prevent growth of occult, dormant metastatic collections. Local disease is not a clinical problem because it can be surgically extirpated. This study supports the development of rrVSV as an in vivo vaccine to prevent growth of tumor metastases. The plan would be to administer rrVSV to the primary tumor in order to stimulate a powerful anti-tumor memory T-cell response. Major obstacles hindering other cancer vaccines would be obviated because no knowledge of specific tumor antigens is
required and immunity would develop in patients with any set of major histocompatibility complex markers. The circulating memory CD4 T cells would orchestrate a curative anti-tumor response whenever occult metastases started growing and released tumor antigens to regional lymph nodes. Nascent metastases would be eliminated before they developed immnosuppressive properties. A good test case for these concepts would be advanced ovarian cancer. rVSV would be administered to Her2/neu-positive cancers at the time of initial tumor therapy in order to kill cancer cells that had implanted in the peritoneum and more importantly, to generate anti-tumor memory CD4 T cells, which would then prevent future outgrowth of IP and distant metastases.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This study was supported in part by NIH Grant number RO1 CA104404. The contents of this study are solely the official views of the granting institution. We thank Drs Wei-Zen Wei, John K Rose, Irvin SY Chen, James P Allison and Genentech who very generously supplied materials as noted in the text. We thank Erich Scheller for technical assistance.

References

1 Wei MQ, Mengesha A, Good D, Anne J, Wei MQ, Mengesha A et al. Bacterial targeted tumour therapy-dawn of a new era. Cancer Leti 2008; 259: 16–27.
2 Finn OJ, Finn OJ. Cancer immunology. N Engl J Med 2008; 358: 2704–2715.
3 Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. Nat Med 2004; 10: 909–915.
4 Dudley ME, Wunderlich JR, Yang JC, Sherry RM, Topalian SL, Restifo NP et al. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. J Clin Oncol 2005; 23: 2346–2357.
5 Drake CG, Drake CG. Prostate cancer as a model for tumour immunotherapy. Nat Rev Immunol 2010; 10: 580–593.
6 de Mattos CA, de Mattos CC, Rupprecht CE. Rhabdoviruses. In: Knipe D, Howley P (eds). Fundamental Virology, 4th edn. Lippincott Williams & Wilkins: Philadelphia, 2001, pp 1245–1277.
7 Stojdl DF, Lichte B, Knowles S, Marius R, Atkins H, Sonenberg N et al. Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. Nat Med 2000; 6: 821–825.
8 Obuchi M, Fernandez M, Barber GN. Development of recombinant vesicular stomatitis viruses that exploit defects in host defense to augment specific oncolytic activity. J Virol 2003; 77: 8843–8856.
9 Fernandez M, Porosnuc M, Markovic D, Barber GN. Genetically engineered vesicular stomatitis virus in gene therapy: application for treatment of malignant disease. J Virol 2002; 76: 895–904.
10 Diaz RM, Galivo F, Kotite T, Wongthida P, Qiao J, Thompson J et al. Oncolytic immunovirotherapy for melanoma using vesicular stomatitis virus. Cancer Res 2007: 67: 2840–2848.
11 Porosnuc M, Mian A, Barber GN. The oncolytic effect of recombinant vesicular stomatitis virus is enhanced by expression of the fusion cytotoxic deaminase/uracil phosphoribosyltransferase suicide gene. Cancer Res 2003; 63: 8366–8376.
12 Ebert O, Shinozaki K, Huang TG, Savontaus MJ, Garcia-Sastre A, Woo SL. Oncolytic vesicular stomatitis virus for treatment of orthotopic hepatocellular carcinoma in immune-competent rats. Cancer Res 2003; 63: 3605–3611.
13 Bergman I, Whitaker-Dowling P, Gao Y, Griffin JA. Preferential targeting of vesicular stomatitis virus to breast cancer cells. Virology 2004; 330: 24–33.
14 Pilon SA, Kelly C, Wei WZ. Broadening of epitope recognition during immune rejection of ErbB2-positive tumor prevents growth of ErbB-2-negative tumor. J Immunol 2003; 170: 1202–1208.
15 Demaria S, Kawashima N, Yang AM, Devitt ML, Babb JS, Allison JP et al. Immune-mediated inhibition of metastases after treatment with local radiation and CTLA-4 blockade in a mouse model of breast cancer. Clin Cancer Res 2005; 11: 728–734.
16 Bergman I, Griffin JA, Gao Y, Whitaker-Dowling P. Treatment of implanted mammary tumors with recombinant vesicular stomatitis virus targeted to Her2/neu. Int J Cancer 2007; 121: 425–430.
17 Lawson ND, Stillman EA, Whitt MA, Rose JK. Recombinant vesicular stomatitis viruses from DNA. Proc Natl Acad Sci U.S.A 1995; 92: 4477–4481.
18 Gao Y, Whitaker-Dowling P, Watkins SC, Griffin JA, Bergman I. Rapid adaptation of a recombinant vesicular stomatitis virus to a targeted cell line. J Virol 2006; 80: 8603–8612.
19 Wei WZ, Shi WP, Galy A, Lichlytre D, Hernandez S, Groner B et al. Protection against mammary tumor growth by vaccination with full-length, modified human ErbB-2 DNA. Int J Cancer 1999; 81: 748–754.
20 Gao Y, Whitaker-Dowling P, Griffin JA, Barnada MA, Bergman I. Recombinant vesicular stomatitis virus targeted to Her2/neu combined with anti-CTLA4 antibody eliminates implanted mammary tumors. Cancer Gene Ther 2009; 16: 44–52.
21 Bracci L, Moschella F, Sestili P, La SV, Valentini M, Canini I et al. Cyclophosphamide enhances the antitumor efficacy of adoptively transferred immune cells through the induction of cytokine expression, B-cell and T-cell homeostatic proliferation, and specific tumor infiltration. Clin Cancer Res 2007; 13: 644–653.
22 Taie J, Chaput N, Schartz N, Roux S, Novault S, Menard C et al. Chemoimmunotherapy of tumors: cyclophosphamide synergizes with exosome based vaccines. J Immunol 2006; 176: 2722–2729.
23 Viaud S, Flament C, Zoubir M, Pautier P, LeCesne A, Ribrag V et al. Cyclophosphamide induces differentiation of Th17 cells in cancer patients. Cancer Res 2011; 71: 661–665.
24 Liu Z, Noh HS, Chen J, Kim JH, Falo Jr LD, You Z et al. Potent tumor-specific protection ignited by adoptively transferred CD4+ T cells. J Immunol 2008; 181: 4363–4370.

25 Muranski P, Restifo NP, Muranski P, Restifo NP. Adoptive immunotherapy of cancer using CD4(+) T cells. Curr Opin Immunol 2009; 21: 200–208.

26 Cerullo V, Pesonen S, Diaconu I, Escutenaire S, Arstila PT, Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Muranski P, Restifo NP, Muranski P, Restifo NP. Adoptive immunity in cancer patients. Cancer Res 2010; 70: 4297–4309.

27 Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 2001; 344: 783–792.

28 Palucka K, Ueno H, Banchereau J, Palucka K, Ueno H, Banchereau J. Recent developments in cancer vaccines. J Immunol 2011; 186: 1325–1331.

29 Kantoff PW, Schuetz TJ, Blumenstein BA, Glode LM, Billhartz DL, Wyand M et al. Overall survival analysis of a phase II randomized controlled trial of a poxviral-based PSA-targeted immunotherapy in metastatic castration-resistant prostate cancer. J Clin Oncol 2010; 28: 1099–1105.

30 Finkle D, Quan ZR, Asghari V, Kloss J, Ghaboosi N, Mai E et al. HER2-targeted therapy reduces incidence and progression of midlife mammary tumors in female murine mammary tumor virus huHER2-transgenic mice. Clin Cancer Res 2004; 10: 2499–2511.

31 Morgan RA, Dudley ME, Rosenberg SA, Morgan RA, Dudley ME, Rosenberg SA. Adoptive cell therapy: genetic modification to redirect effector cell specificity. Cancer J 2010; 16: 336–341.

32 Rosenberg SA, Dudley ME, Rosenberg SA, Dudley ME. Adoptive cell therapy for the treatment of patients with metastatic melanoma. Curr Opin Immunol 2009; 21: 233–240.

33 Cohen PA, Peng L, Plautz GE, Kim JA, Weng DE, Shu S et al. CD4+ T cells in adoptive immunotherapy and the indirect mechanism of tumor rejection. Crit Rev Immunol 2000; 20: 17–56.

34 Muranski P, Boni A, Antony PA, Cassard L, Irvine KR, Kaiser A et al. Tumor-specific Th17-polarized cells eradicate large established melanoma. Blood 2008; 112: 362–373.

35 Lundin KU, Hofgaard PO, Omholt H, Munthe LA, Corthay A, Bogen B et al. Therapeutic effect of idiotype-specific CD4+ T cells against B-cell lymphoma in the absence of anti-idiotype antibodies. Blood 2003; 102: 605–612.

36 Nishimura T, Iwakabe K, Sekimoto M, Ohmi Y, Yahata T, Nakui M et al. Distinct role of antigen-specific T helper type 1 (Th1) and Th2 cells in tumor eradication in vivo. J Exp Med 1999; 190: 617–627.

37 Kennedy R, Celis E, Kennedy R, Celis E. Multiple roles for CD4+ T cells in anti-tumor immune responses. Immunol Rev 2008; 222: 129–144.

38 Liu Z, Tian S, Falo Jr LD, Sakaguchi S, You Z, Liu Z et al. Therapeutic immunity by adoptive tumor-primed CD4(+) T-cell transfer in combination with in vivo GITR ligation. Mol Ther 2009; 17: 1274–1281.

39 Perez-Diez A, Joncker NT, Choi K, Chan WF, Anderson CC, Lantz O et al. CD4+ T cells can be more efficient at tumor rejection than CD8 cells. Blood 2007; 109: 5346–5354.

40 Xie Y, Akpınarlı A, Maris C, Hipkiss EL, Lane M, Kwon EK et al. Naive tumor-specific CD4(+) T cells differentiated in vivo eradicate established melanoma. J Exp Med 2010; 207: 651–667.

41 Hunder NN, Wallen H, Cao J, Hendricks DW, Reilly JZ, Rodmyre R et al. Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1. N Engl J Med 2008; 358: 2698–2703.

42 Klebanoff CA, Gattinoni L, Torabi-Parizi P, Kerstann K, Cardones AR, Finkelstein SE et al. Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells. Proc Natl Acad Sci USA 2005; 102: 9571–9576.

43 Huang H, Bi XG, Yuan JY, Xu SL, Guo XL, Xiang J. Combined CD4+ TH1 effect and lymphotactin transgene expression enhance CD8+ Tc1 tumor localization and therapy. Gene Ther 2005; 12: 999–1010.

44 Martin-Orozco N, Muranski P, Chung Y, Yang XO, Yamazaki T, Lu S et al. Helper 17 cells promote cytotoxic T cell activation in tumor immunity. Immunity 2009; 31: 787–798.

45 Kryczek I, Wei S, Szeliga W, Vatan L, Zou W, Kryczek I et al. Endogenous IL-17 contributes to reduced tumor growth and metastasis. Blood 2009; 114: 357–359.

46 McKinstry KK, Strutt TM, Swain SL, McKinstry KK, Strutt TM, Swain SL. The potential of CD4 T-cell memory. Immunology 2010; 130: 1–9.

47 Castellino F, Huang AY, van-Bonnet G, Stoll S, Scheinecker C, Germain RN et al. Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell-dendritic cell interaction. Nature 2006; 440: 890–895.