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PRINCIPAL INVESTIGATOR: Ira Bergman

CONTRACTING ORGANIZATION:
University of Pittsburgh
Pittsburgh, PA 15224

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6. AUTHOR(S)
Ira Bergman (Initiating PI) Per Basse (collaborating PI),

E-Mail: Ira.Bergman@chp.edu basse@imap.pitt.edu

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14. ABSTRACT Purpose: To develop an immunologically based strategy for the treatment of breast cancer metastases to the brain (BM).
Scope: To establish an animal model of BM and to demonstrate that anti-tumor memory T-cells can be activated to enter and destroy BM by the following methods: 1. Viral infection of BM by a recombinant replicating vesicular stomatitis virus (VSV) targeted to the Her2/neu receptor and causing oncolysis and cytokine expression and 2. Adoptive transfer of cytotoxic, cytokine-producing natural killer (NK) cells capable of accumulating in smaller metastases of the brain.
Major Findings: A meningeal model was produced using the mammary cancer cell line, D2F2/E2. A parenchyma model was difficult to produce with this cell line. We showed that there is a blood-brain-barrier to bringing therapeutic memory T-cells to meningeal tumors. The barrier could be overcome by viral infection of the tumor. Viral infection of the meningeal tumors followed by memory T-cells transfer resulted in 89% cure of meningeal tumor in two different mouse strains. Viral infection produced increased infiltration and proliferation of transferred memory T-cells in the meningeal tumors. Following viral infection, the leukocyte infiltration in meninges and tumor shifted from predominantly macrophages to predominantly T-cells. Successful viral therapy of peritoneal tumors generates memory CD8 T-cells that prevent establishment of tumor in the brain meninges of these same animals. A-NK cells traffic to brain tumors but less well than seen in pulmonary and hepatic tumors.
Conclusions: These results support the hypothesis that a virally-based immunization strategy can be used to both prevent and treat brain metastases.

15. SUBJECT TERMS
Breast cancer metastases to brain; Meningeal metastases; Her2/neu targeted VSV; viral therapy; anti-tumor memory T cells; NK cells; cytokines and cytokine transgenes; effector cell traffic.

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Viral immunotherapy to eradicate subclinical brain metastases

INTRODUCTION

Subject: Breast cancer metastases to the brain.

Purpose: To develop an immunologically based strategy for the treatment of breast cancer metastases to the brain.

Scope: To establish an animal model of breast cancer metastases to the brain and to use this model to demonstrate that anti-tumor host memory T-cells can be re-activated to enter and destroy early BM by viral infection of Her2-positive breast BM by a recombinant vesicular stomatitis virus (VSV), which liberates tumor antigens by viral oncolysis and induces acute inflammation in BM and CSF via the cytokine transgenes it carries and/or by adoptive transfer of cytotoxic, cytokine-producing natural killer (NK) cells capable of accumulating in smaller metastases of the brain.

BODY OF REPORT

Grey text: Original SOW text
Black text: Work performed mainly in the **Bergman** laboratory.
Blue text: Work performed mainly in the **Basse** laboratory.

Tasks

1. **Demonstrate that anti-tumor memory T-cells are activated when tumor antigens and inflammatory mediators are delivered to CSF macrophages (Months 0-9)**

    a. Generate donor survivor animals by treatment with replicating recombinant Vesicular Stomatitis Virus (rrVSV)
        i. Months 0 to 24.
        ii. Bergman
        iii. These mice are generated weekly
        iv. 300 mice/year
        v. All donor mice are Balb/c
        vi. Tumor implants use the D2F2/E2 mammary cancer cell line

This task has been accomplished and works consistently. Tumors develop in 100% of animals implanted in the peritoneum with the D2F2/E2 mammary cancer cell line (Fig.1-Bergman). Therapy with rrVSV achieves > 90% cure rate. We are currently generating 5-10 cured mice per week and have available abundant donor animals required for our experiments. The availability of unlimited cured donor animals means that we can test different components of donor cells (CD4 T-cells, CD8 T-cells, B-cells or total spleen cells) in transfer experiments and use a large number of hosts each with a single experimental variable.

    b. Production of rrVSV and non-replicating VSV expressing various cytokines (IL-12, IL-1α, IL-6, TNFα, IL-4, IL-7, IL-15, IFNγ, IL-23, TGFβ and CD40L)
        i. Months 0-24

ii. **Bergman**

This task was technically challenging but has been accomplished for every cytokine. We have an extraordinary set of recombinant VSV expressing a wide variety of cytokines. These cytokines have a very broad range of inflammatory and immunologic effects that can now be manipulated in a controlled fashion in the local setting of the tumor.

c. Animal studies: Inject non-replicating VSV-infected tumor cells via cisterna magna (CM) into hosts with donor anti-tumor CD4 cells
   i. Months 1-9
   ii. Bergman
   iii. 100 mice

We performed the following experiment which provided a clear answer to the question posed by this task. Seven animals received IP D2F2/E2 tumor implants and were then cured of tumor by standard therapy with rrVSV. These cured animals were then challenged with D2F2/E2 tumor implants in the cisterna magna (CM). As shown in Figure 1, 5 of 7 animals survived and 2 died at 34 and 39 days after challenge. All control animals died 18 days after challenge. Challenge was then performed in cured animals that were depleted of CD4 T-cells, CD8 T-cells or both. Animals depleted of both CD4 and CD8 T-cells died promptly after challenge. Most animals with CD8 depletion also died promptly. Interestingly, animals with CD4 depletion usually survived indicating that the major memory cell type preventing meningeal neoplastic implantation in this model system was CD8 T-cells. In the absence of CD8 T-cells, CD4 cells could not prevent or treat neoplastic implantation.

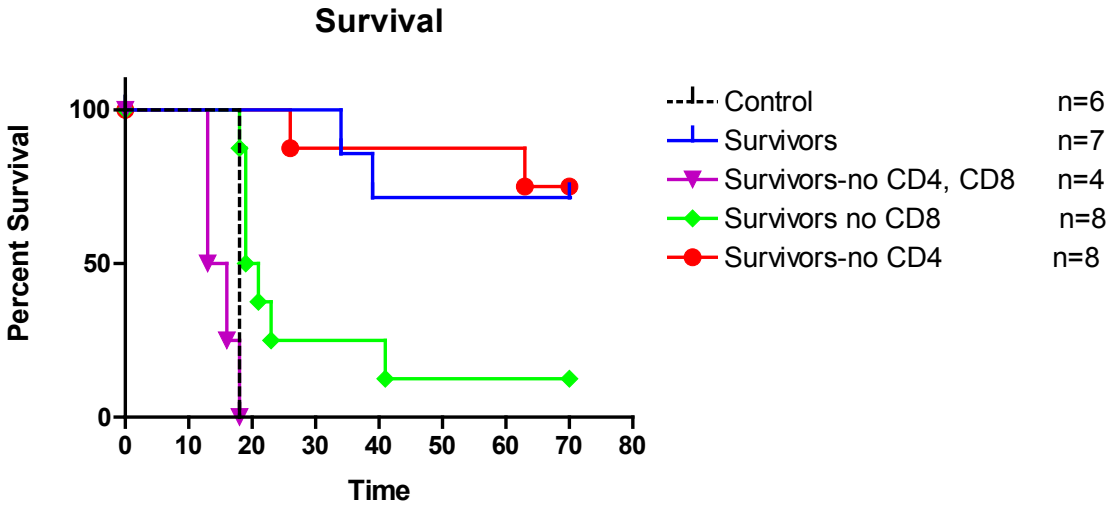


Fig. 1. Survival following CM challenge. Animals cured of IP tumors (survivors) using standard treatment with rrVSV, antiCTLA4 and CPM were challenged with CM tumor, as were controls. Anti-tumor memory CD8 T-cells prevented establishment of meningeal tumors in survivors.

It is therefore clear that cured animals not only develop immunity to the original tumor cells but that this immunity easily crosses the blood-brain-barrier and prevents growth of tumor in the meninges. This result is consistent with the clinical hypothesis of this project. We suggested a two-phased strategy to treat breast cancer metastases in the brain before they produce debilitating symptoms. The first phase was to generate anti-tumor memory T-cells when the patients first presented by treating the primary breast tumors with rrVSV. The second phase was to activate the patient's anti-tumor memory T-cells to enter the brain and find and destroy early metastases. In this experiment, implantation of the tumor cells may have provided sufficient inflammatory signals to bring memory T-cells to the tumor. In further experiments reported below, we explored methods to generate these beneficial signals when the tumors were growing in animals that were not pre-immunized.

d. Analysis of CSF: Number of cells and types of cytokines that mediate inflammation in the central nervous system (CNS)
    i. Months 1-9
    ii. Bergman

The first task was to create a robust model of meningeal tumor. Initially, the methods used to implant tumor cells into the cisterna magna did not produce consistent leptomeningeal (LM) tumors. We tried several techniques reported in the literature and generated LM tumors some of the time but felt that consistent implantation was required to compare experimental to control groups. In response, we improved both the method of implantation and the cells implanted. D2F2/E2 cells were implanted into the CM and new primary cultures were made from successful tumor implants. This was done several time and we now have a subclone, D2F2/E2-J9 that consistently implants in the LM. In addition, we developed our own very simple method for CM puncture that in combination with the adapted cell line currently yields 100% implantation (Fig. 1- Bergman). Tumors grew largest in the olfactory region but also grew in the meninges throughout the cerebrum and cerebellum (Fig. 2). Tumors grew rapidly and untreated average duration of survival was only 15-20 days.

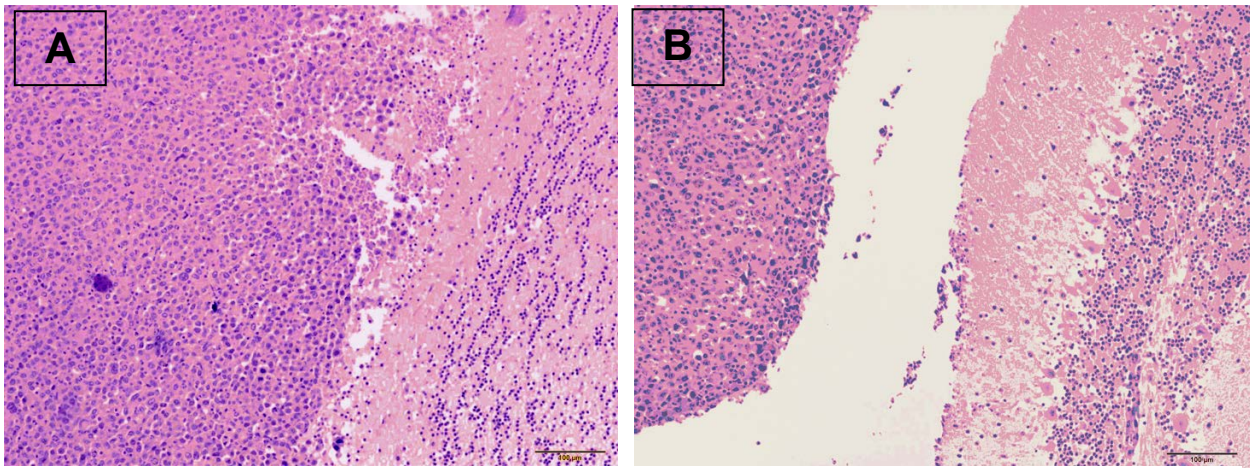


Fig. 2- Bergman. D2F2/E2-J9 brain tumors: D2F2/E2-J9 cells were implanted into the cisterna magna. The mouse was sacrificed 10 days later when it first manifested lethargy and weakness. The brain was fixed overnight in buffered formalin, cut in the sagittal plane and embedded in paraffin. Slides were cut at 5 µM and staied with hematoxylin and eosin.

A. A large nodular tumor is seen (left side of picture) in the meningeal space anterior to the olfactory bulb (right side of picture). Scale bar is 100 µM.
B. A nodule of tumor (left side of picture) is seen in the 4th ventricle adjacent to the cerebellar vermis (right side of picture). Scale bar is 100 µM.

To determine if the D2F2/E2 brain tumor cells expressed Her-2 also in vivo, fresh frozen sections were stained with antibody against human Her-2. We found a strong and almost homogenous expression of human Her-2 in all tumors analyzed (Fig. 3-Basse).

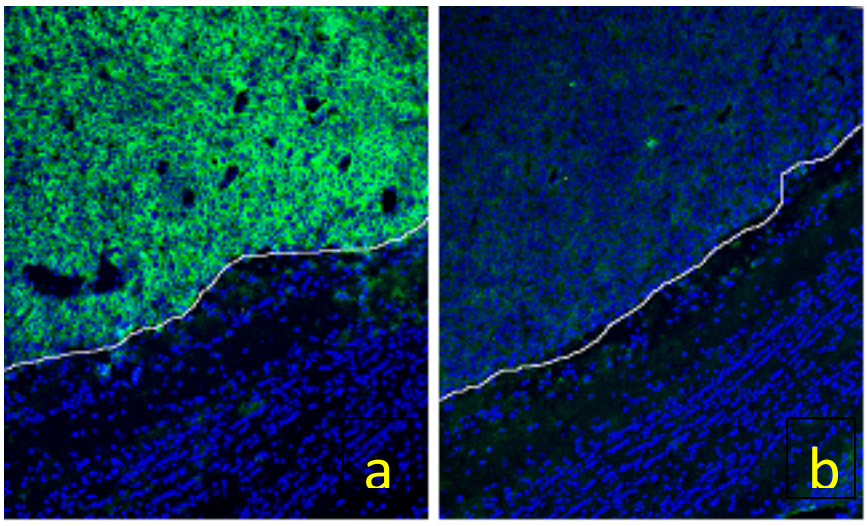


Fig. 3-Basse. Expression of human Her-2 by D2F2/E2 brain tumors. Sections of brain tumors were stained with humanized mouse anti-huHer-2 followed by FITC-labeled rabbit anti-human Ig. **a**) Section stained with both anti-huHer-2 (1:100) followed by FITC-labeled rabbit anti-human Ig (1:100). **b**) Section stained with FITC-labeled rabbit anti-human Ig (1:100) only. Nuclei stained with Hoechst (blue).

We have also adapted our simple method for CM puncture to be able to sample CSF percutaneously on living, lightly anesthetized animals. Surgery is not required and sample can be obtained on multiple different days. Only 2-6 µl of CSF can be obtained so analysis on any one sample is limited. The CSF can undergo cytospin for examination of cells or can be diluted for multiplex analysis of cytokines. Our major finding to date using this technique is that spleen cells from cured donor animals transferred intravenously to host animals bearing LM tumor can traffic to the CSF as illustrated in the figure below (Fig. 4- Bergman). The next step was to define the

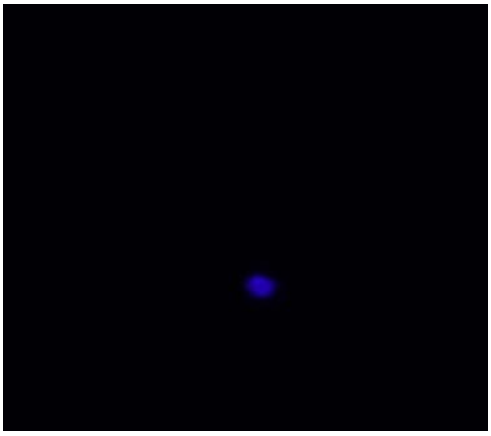


Fig. 4-Bergman. A host animal was implanted with D2F2/E2 cells in the cisterna magna (CM). Ten days later, spleen cells were harvested from donor animals cured of peritoneal D2F2/E2 using standard rrVSV therapy. Spleen cells were labeled with Hoechst 33342 and 1 x 10^8 cells were injected IV into the host animal. CSF was sampled 2 days later and this example clearly shows a Hoechst labeled donor cell. Scale bar is 20 µM.

conditions that allow and increase this trafficking and these studies are reported below. We did not require the analysis of CSF cytokines but instead use techniques of immunohistochemistry and flow cytometry.

One technical difficulty that we have not yet resolved is implantation of tumor by carotid artery injection. The surgical technique of isolating and injecting cells into the carotid artery of the mouse turned out to be very challenging. We can now accomplish this task consistently with the help of 34 gauge needles that we special order from Japan. The

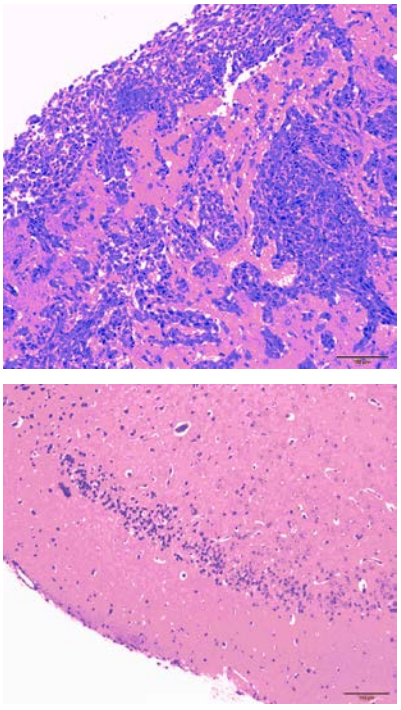


Fig. 5-Bergman. D2F2/E2 tumors induced by carotid injection. D2F2/E2 cells were implanted into the right carotid artery. The mouse was sacrificed 20 days later when it first manifested lethargy and weakness. The brain was fixed overnight in buffered formalin, cut in the coronal plane and embedded in paraffin. Slides were cut at 5 µM and stained with hematoxylin and eosin. Extensive tumor deposits are seen in the right hemisphere (top picture) in the meningeal and perivascular spaces and infiltrating the brain. Tumor is not visible in the left hemisphere (bottom picture).

remaining problem is that the tumor cells form growing tumors in the brain only about 30% of the time (Fig. 5-Bergman). As with meningeal tumor, we tried making primary cultures from tumors that grew in the brain to develop a sub-clone of D2F2/E2 that consistently implanted and grew in the brain following carotid injection. Unfortunately, the tumors continued to implant better in the eye than in the brain. In order to overcome this problem, we are trying two new approaches. In the first, we have now obtained the 4T1 mammary cancer line stably transduced to express Her2/neu (4T1-E2) from Dr. Michael Kershaw. This cell line grows well in the brain when implanted through the carotid artery or by cardiac injection. A set of experiments similar to those reported above including therapeutic trials, prevention trials and studies tracking localization and proliferation of anti-tumor memory T-cells will be used to extend therapeutic efficacy of memory T-cells from meningeal implants to brain implants derived by hematogenous spread.

In the second, we will attempt to inject tumor spheroids. From our previous work with a lung metastasis model, we learned that i.v. injection of a single tumor spheroid (composed of less than 50 tumor cells) consistently gave rise to one tumor metastasis, whereas we needed to inject at least 10,000 tumor cells to induce 1-10 metastases. Based on this observation, the Basse lab is generating D2F2/E2 tumor spheroids (diameter ~50 microns) of which we will inject 1-2 per mouse via the internal carotid artery.

In order to be sure that our therapeutic results, reported below, were generalizable we developed a second meningeal model using a mouse colon cancer cell line transfected to express human Her2/neu called MC38/E2. This cell line also reliably produced meningeal tumors following injection into the cisterna magna.

e. Analysis of tissues: Determine the density and state of activation of donor (especially CD4 T cells) and host immune cells in metastases and normal tissues
   iii. Months 1-9
   iv. Basse

As indicated in the grant, the first task was to determine optimal methods for labeling donor cells. A basic aim is to determine how anti-tumor memory T-cells from donor animals traffic to brain tumors and different methods of labeling the donor cells may be appropriate for different experiments. We have tested 3 methods of labeling and detecting donor cells: label with CFSE, label with Hoechst 33342 or use Thy 1.2 donors in Thy 1.1 recipients. We determined that Hoechst and CFSE were best visualized in brain tissue following fixation in 2-4% paraformaldehyde and dehydrating in sucrose. Thy 1.2 is best stained in flash frozen brain tissue. Hoechst and CFSE labeled cells are readily visualized in fresh CSF. The brightest staining is achieved with Hoechst and is detectable in pathological specimens for more than 3 days following cell transfer. Hoechst binds to DNA preventing cell replication and transcription. Trafficking of these transferred cells is therefore limited to properties they already possess at the time of labeling and not properties that they acquire in the host animal. CFSE labeled cells can replicate and transcribe new message but we found that label could only be clearly visualized within donor cells in the brain for 24 hours following cell transfer. Transfer of Thy 1.2 donor T-cells into Thy 1.1 hosts allows full replication, transcription and

activation of the donor cells. Being a genetic marker, Thy1 is not diluted by replication. We have therefore decided to use congenic Thy 1 as our primary method of visualizing transferred donor cells while still using Hoechst and CFSE for special purposes.

The need to use Thy 1.1 Balb/c mice created an additional Task. We had to establish a breeding colony for these animals because they are not available commercially. The first breeding pair did not produce a litter and consequently these experiments were slowed but we are proud to report that we now have a fecund breeding colony with an ample supply of Thy 1.1 mice.

Initial experiments showed that transfer of donor cells required intravenous administration and that intraperitoneal delivery did not yield satisfactory results. We attempted IP delivery because our previous work showed that IP delivery of anti-tumor T-cells from donor animals cured peritoneal tumors in host animals. Current work using flow cytometry quantified that the IV route delivered 5 to 18-fold more donor cells to the host spleen than the IP route (Fig. 6-Bergman).

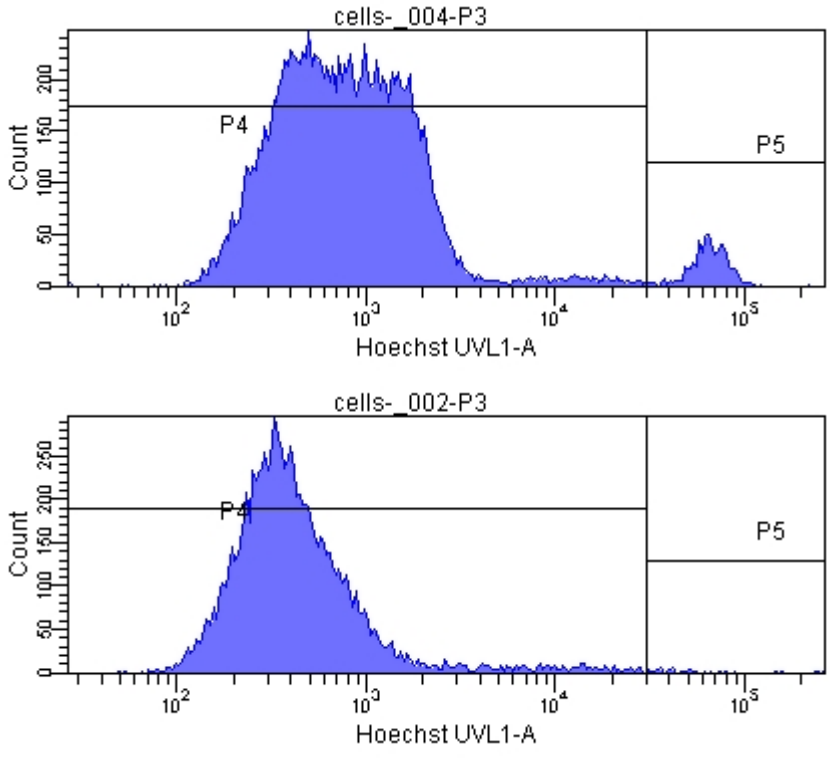


Fig. 6-Bergman. Hoechst-labeled spleen cells (5 x 10^7 cells) were transferred IV (top figure) or IP (bottom figure) to donor mice. Mice were sacrificed 24 hours later and spleen harvested, converted to single cell suspensions and analyzed by flow cytometry. There were 3% donor cells following IV transfer (top figure) and 0.2% following IP transfer (bottom figure).

Histo-pathological analysis of liver and spleen tissue (Fig. 7-Basse) similarly showed that cured spleen cells transferred i.p. to host animals inadequately reached the systemic circulation. We now transfer cells exclusively using the IV route.

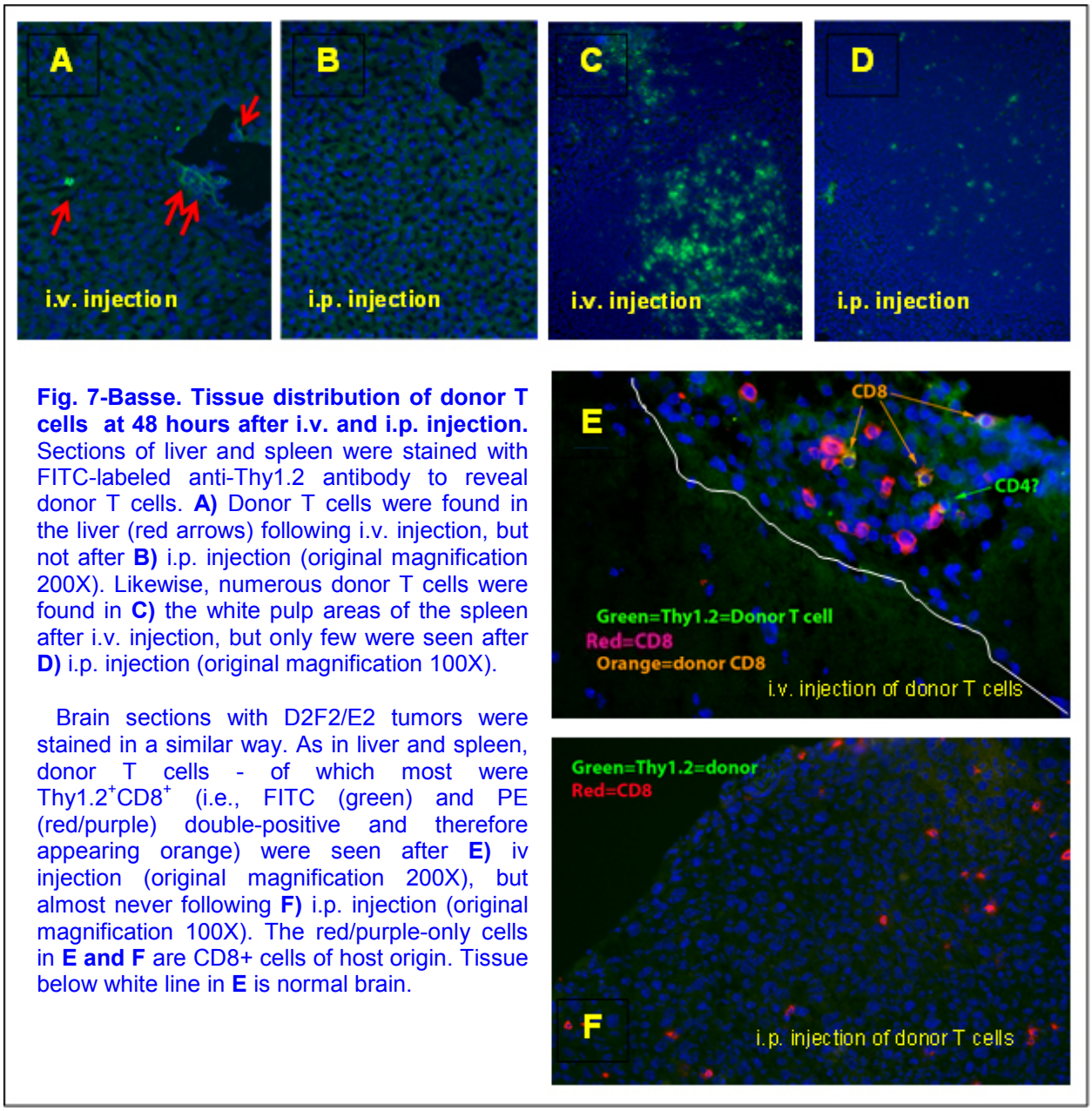


Fig. 7-Basse. Tissue distribution of donor T cells at 48 hours after i.v. and i.p. injection. Sections of liver and spleen were stained with FITC-labeled anti-Thy1.2 antibody to reveal donor T cells. **A)** Donor T cells were found in the liver (red arrows) following i.v. injection, but not after **B)** i.p. injection (original magnification 200X). Likewise, numerous donor T cells were found in **C)** the white pulp areas of the spleen after i.v. injection, but only few were seen after **D)** i.p. injection (original magnification 100X).

Brain sections with D2F2/E2 tumors were stained in a similar way. As in liver and spleen, donor T cells - of which most were $Thy1.2^{+}CD8^{+}$ (i.e., FITC (green) and PE (red/purple) double-positive and therefore appearing orange) were seen after **E)** iv injection (original magnification 200X), but almost never following **F)** i.p. injection (original magnification 100X). The red/purple-only cells in **E and F** are CD8+ cells of host origin. Tissue below white line in **E** is normal brain.

Early experiments with Hoechst-labeled donor cells showed that neither cells from naïve animals nor donor cells from animals that had been cured of peritoneal tumor with rrVSV therapy were seen in CSF or meninges of host animals bearing CM tumor. These findings are consistent with our initial hypothesis that anti-tumor memory T-cells would not find tumor in the brain unless there was an inflammatory reaction to bring them to the site. In a handful of animals, we have not seen Hoechst-labeled donor cells in meningeal tumors following infection of the tumor with pseudotype VSV. However, these Hoechst-labeled cells may not traffic to the brain because they cannot be

stimulated in local lymph nodes to become activated and divide due to the Hoechst binding to the cellular DNA. Using the congenic system, we were able to demonstrate homing of CD8+ donor T cells to brain tumors (as shown in Fig. 7E-Basse). However, the number is low, and homing of effector cells to these intracranial tumors must be improved. Much deeper explorations of these results was carried forward using congenic Thy 1.1/1.2 mice and reported below..

2. **Establish the ability of A-NK cells to infiltrate brain metastases of Her2/neu positive mammary cancer.**
    a. Generate animals with brain and leptomeningeal (LM) metastases using the D2F2/E2 mammary cancer cell line
        i. Months 1-24
        ii. Bergman
        iii. 60 mice
        iv. All host mice are Balb/c

As noted above, by improving the method of CM implantation and developing a sub-clone of D2F2/E2 that grows easily in the meninges, we are able to consistently produce an unlimited number of mice with LM metastatic implants. We have also produced a meningeal model using a mouse colon cancer cell line transfected to express human Her2/neu called MC38/E2. Parenchymal implants following carotid have so far shown only limited success and we are moving to a new cell line, 4T1-E2, which is known to grow in the brain following carotid artery and cardiac implantation.

    b. Generate A-NK cells: Adenovirally tansduced to express rmIL-2 and rmIL-12
        i. Months 1-6
        ii. Basse

To date, we have successfully transduced A-NK cells with two adenoviral vectors simultaneously (IL-2 and IL-12). Preliminary data suggest that even triple-transduction is possible, which would allow us to produce A-NK cells secreting, e.g., CTL-attracting chemokines or mediators of acute inflammation, in addition to IL-2 and IL-12 (Fig. 8-Basse).

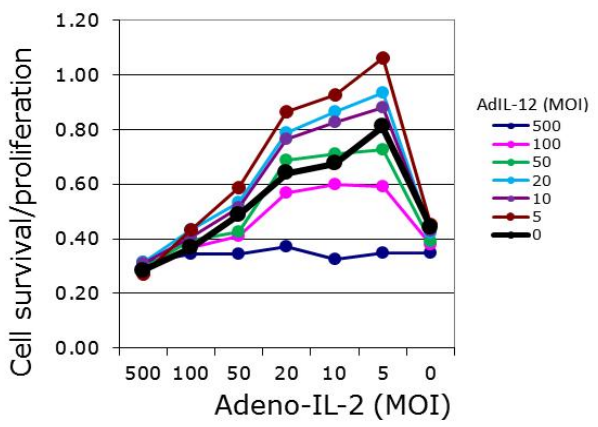


Fig. 8-Basse. Adenoviral transduction of IL-2 activated NK (A-NK) cells with adenoviral vectors containing the genes for IL-2 and IL-12. A-NK cells prepared from Balb/c splenocytes were incubated for 2 hours with adenoviral vectors – at different MOIs - containing the genes for IL-2 (AdIL-2) and IL-12 (AdIL-12). While AdIL-2 transduction at MOIs up to 20 provided growth/survival advantage, higher MOIs were inhibitory/toxic. While AdIL-12 at MOIs above 50 were clearly inhibitory, lower MOIs of AdIL-12 provided a synergistic effect when combined with AdIL-2. The optimal growth/survival of the A-NK cells were seen when an MOI of 5 of both vectors were used.

i. Months 1-6
ii. **Basse**
iii. 60 mice (same as 2. a. iii)

A-NK cells have been produced fromThy1.2+ Balb/c splenocytes and we have confirmed that they are cytotoxically active against tumor cells, which are resistant to non-activated NK cells. We have injected A-NK cells by the i.v. route into congenic, Thy1.1+ Balb/c mice with tumors in the brain and in the peritoneal cavity (Task 2a). So far, all the A-NK cells used for transfer have been non-transfected.

d. Analysis of tissue: Distribution of A-NK cells
i. Months 1-9
ii. Basse

We have determined the tissue distribution of intravenous (IV) injected A-NK cells in tumor-bearing animals using the Thy1.1/1.2 system. Thus, organs were removed at 3 and at 5 days after injection of the A-NK cells. Representative tissue sections or various organs were stained with antibodies against the congenic markers (host=Thy1.1 and donor cells = Thy1.2) and against NKp46 (a marker of NK cells). Thy1 is a marker of all

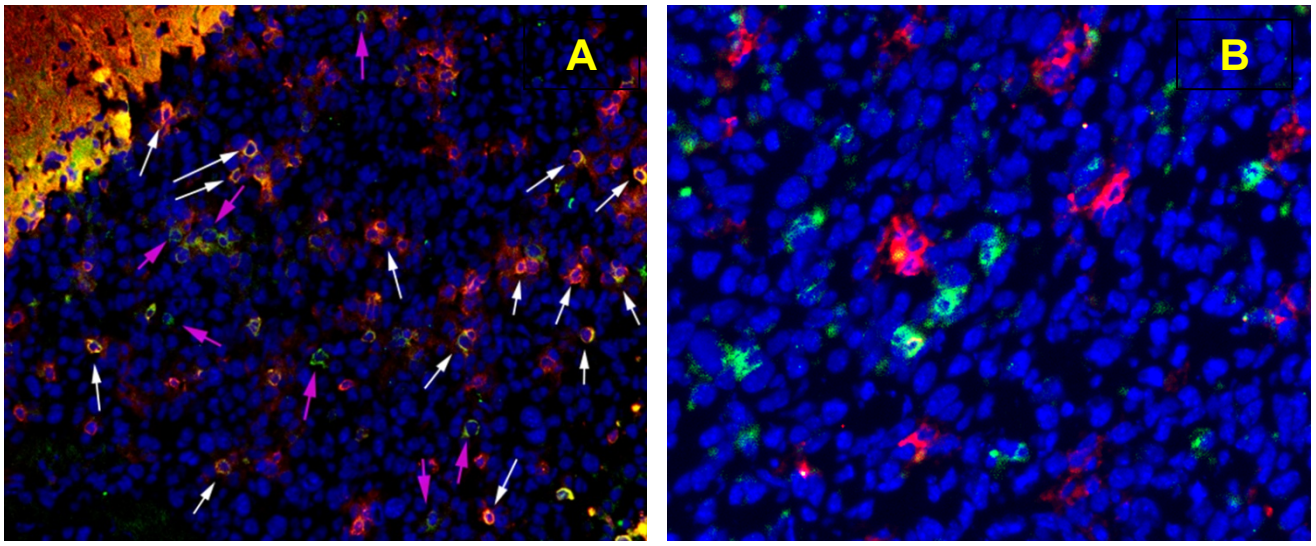


Fig. 9-Basse: Adoptively transferred and host NK cells in D2F2/E2 brain tumors. Animals with 9 day old D2F2/E2 brain tumors received 5 million Thy1.2^{+} A-NK cells via the IV route. Three days later, brains were removed and processed for immune-histology. **A)** Sections were stained with Alexa-488 (greenish) anti-NKp46 antibody to reveal NK cells and with PE (red/purple)-anti-Thy1.1 to reveal endogenous lymphocytes of T and NK cell origin. Several NKp46^{+}Thy1.1^{neg} A-NK cells are seen (at purple arrows), but also a substantial number of activated NKp46^{+}Thy1.1^{+} NK cells of host origin (at white arrows). Red-only cells represent host T cells. Original magnification; 200X. **B)** Brain sections from animals, which did not receive exogenous A-NK cells, revealed that endogenous NK cells also infiltrate the brain tumors (cells stained green). However, almost all of the tumor-infiltrating host NKp46^{+} cells were negative or only very weakly positive for the Thy1.1 marker, indicating that they are of a non-activated phenotype. The red/purple-only cells are host T-cells. Original magnification; 400X.

T cells as well as activated NK cells. Very few A-NK cells were found in any tissues at 5 days after injection. This was expected, since the A-NK cells in these animals were without IL-2 support from day 3 to day 5 after injection, resulting in their death by

apoptosis. However, at 3 days after injection, NKp46+/Thy1.2+ (not shown)/Thy1.1 negative cells – i.e., NK cells of donor origin, were found in tumor (Fig. 9A-Basse), liver and spleen (not shown). Somewhat surprisingly, a substantial number of NK cells of host origin were found in tumors from non-treated control mice (Figure 9B-Basse). However, in contrast to the host NK cells found in tumors of mice receiving A-NK cells (Figure 9A-Basse), the NK cells in the non-treated animals did not co-express NKp46 and Thy1.1, i.e., they did not appear to have an activated phenotype. Whether the host NK cells found in tumors from the A-NK cell treated animals are activated by IFNγ secreted by the Donor A-NK cells or by the exogenous IL-2 or both, is being investigated.

We are now repeating these experiments with AdIL-2+Ad IL-12 transduced A-NK cells which are injected without support by exogenous IL-2. We expect that these A-NK cells can support themselves with sufficient IL-2/IL-12 to survive in the tumors and we predict, according to our hypothesis, that they will secrete sufficient amounts of these cytokines to also support infiltrating anti-tumor CTLs and possibly infiltrating host NK cells.

3. **Determine that pseudotype VSV infection and A-NK cell infiltration of small brain and LM tumor nodules delivers tumor antigens to CSF macrophages and activates anti-tumor memory T-cells (months 6-21)**
    a. Generate donor survivor animals by treatment with rrVSV (same as 1.a)

This work has been fully accomplished as noted above.

    b. Generate animals with brain and leptomeningeal (LM) metastases using the D2F2/E2 mammary cancer cell line. Transfer T-cells from donor mice (from 3.a)
        i. Months 6-21
        ii. **Bergman**
        iii. 200 mice

As noted above, we are fully able to generate LM metastases using two cell lines and have a new cell line to develop parenchymal metastases via carotid implants.

We first used transferred T-cells to demonstrate that there is a blood-brain-barrier to bringing therapeutic memory T-cells to meningeal tumors. We showed that transferred anti-tumor memory T-cells cured peritoneal tumors more readily than brain meningeal tumors. Three days following tumor implantation in either peritoneum or cisterna magna, mice received spleen cells IV from cured donor animals (henceforth called cured donors). One donor was used per one recipient but all donor cells were pooled so recipient animals with peritoneal or meningeal tumors received donor cells from the same pooled collection. Each host received 4-6 x 10^7 donor cells. Memory T-cells were much more effective in eliminating peritoneal tumor than meningeal tumor (Fig. 10-Bergman; p=0.0003). Transferred anti-tumor memory T-cells increased survival by at least 25 days and cured 60% of mice with peritoneal tumors, but cured only 20% of mice with meningeal tumors and only increased survival by a few days. Transferred spleen cells from naïve animals (henceforth called naïve donors) were completely ineffective against peritoneal or meningeal tumors, as expected.

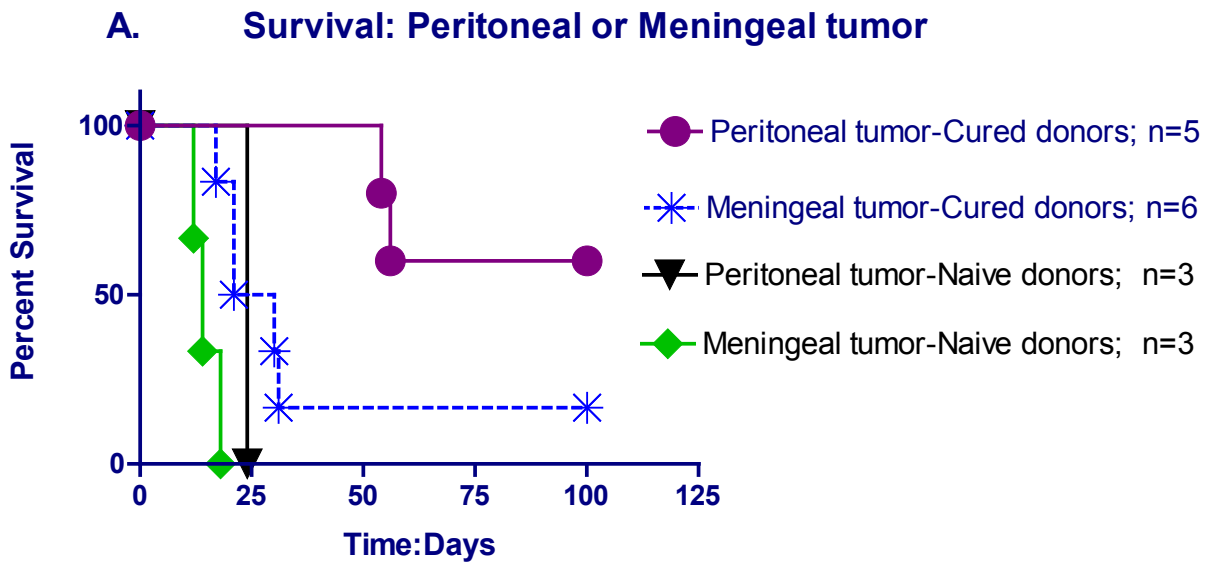


Fig. 10. Survival following treatment of peritoneal or meningeal tumors with cured donors. Mice were implanted with D2F2/E2 tumor cells in the peritoneum or the meninges and treated 3 days later with spleen cells from either cured or naïve donors. Cured donors significantly increased survival in peritoneal tumors compared with meningeal tumors (p=0.0003, log rank statistic). Naïve donors were not effective in either model.

These results support the idea that there is a relative BBB to cellular immune therapy. We next attempted to overcome this barrier by direct viral infection of the meningeal tumors.

c. Animal studies: Non-replicating VSV injected into tumor bearing animals (from 3b)
    i. Months 6-21
    ii. Bergman
    iii. 100 mice

Initial studies did not show an effective inflammatory response produced by non-replicating VSV and we therefore switched to replicating VSV. This change was not important scientifically because we were able to answer the question of whether an effective inflammatory stimulus could overcome the BBB to cellular immune therapy. In future work, we will explore multiple alternative non-infectious modes of generating an effective inflammatory response.

We were able to show that transferred anti-tumor memory T-cells cure leptomeningeal tumors after viral infection of the tumors. Meningeal tumors were established in Balb/c

mice and treated as above with the addition that one day after tumor implant the animals receive rrVSV CM (2 x 10^6 ID) and IV (2 x 10^7 ID). This therapy was remarkable effective resulting in cure of all 5 animals (Fig. 11) and significantly improved survival compared with treatment with virus alone (p=0.0016). All control animals who received virus alone developed neurological deficits and were sacrificed at a mean of 31.8 days. In order to prove that anti-tumor and not just anti-virus memory T-cells were critical to the therapeutic response, we treated one set of animals of 5 mice with donor cells from animals that had been infected with virus but never implanted with tumor. All animals died at a mean of 32.4 days, significantly worse than the experimental group treated with cured donors (p=0.0018), indicating that specific anti-tumor memory T-cells were necessary for successful therapy. Further evidence came from one donor whose IP tumor was cured by treatment with anti-CTLA4 and CPM alone but no virus. Transferred spleen cells from this animal combined with viral infection of the meningeal tumor cured meningeal tumor in one host animal indicating that anti-tumor memory T-cells without anti-virus T-cells were curative. These results generalized to a different mouse strain. Cures were achieved in C57/Bl6 mice who were implanted CM with MC38/E2 and treated as above with virus and spleen cells from cured donors; 2 from donors cured with virus plus anti-CTLA4 and CPM and 2 from donors cured with anti-CTLA4 and CPM alone and no virus. Three of 4 treated animals were cured. One treated animal died after 49 days. One control that received no treatment died at 28 days. In both strains of mice, cured animals behaved normally and showed no adverse effects of CM administration of rrVSV. We felt it most likely that viral infection of tumor was attracting circulating T-cells to the CSF and leading to the elimination of tumor by anti-tumor memory T-cells.

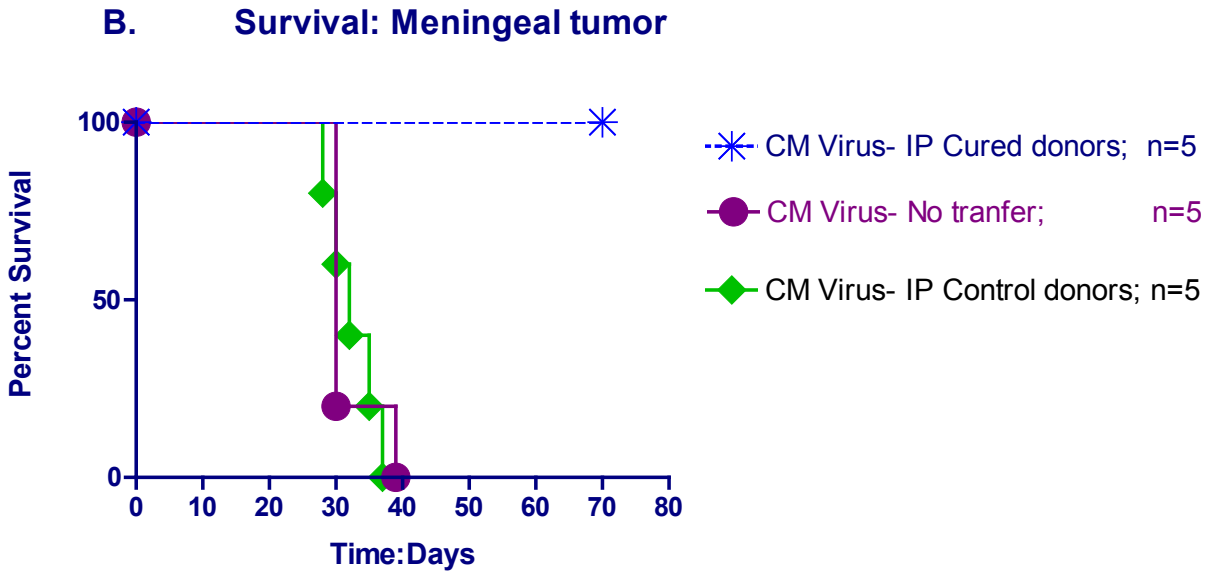


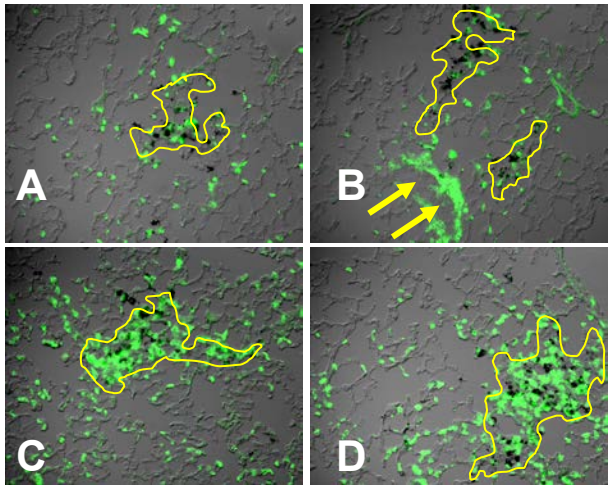
Fig. 11. Survival following treatment of meningeal tumors with cured donors combined with direct viral infection of the meningeal tumors. Experimental mice received meningeal virus one day and cured donors 3 days after tumor implantation. Control mice received no donor cells or donor cells from mice treated with virus but not implanted with peritoneal tumor. All animals received cyclophosphamide IP to facilitate cell transfer. N=5 for all groups. Cured donors with viral infection of the meninges

significantly increased survival compared with virus infection alone (p-0.0016) or virus infection with control donors (p=0.0018). Cured donors with viral infection of the meningeal tumors significantly increased survival of meningeal tumors compared with cured donors without virus infection (curve in 1A compared with curve in 1B; p=0.0086).

d. Animal studies: A-NK cells injected into tumor bearing animals (from 3b)
    i. Months 6-21
    ii. Basse
    iii. 100 mice

In a lung tumor model, we found indications that CTLs may trail IL-2+IL-12 double-transduced A-NK cells into tumors (Fig. 12). To determine if A-NK cells would be able to facilitate CTL-accumulation also in breast cancer metastases in the brain, mice were

FIGURE 12: Improved accumulation of tumor-specific CTLs in tumors of animals pretreated with IL2/IL-12 double-transduced A-NK cells. Four million *ex vivo*-primed CTLs were injected iv into mice with lung tumors. The day before the CTL injection, mice were pretreated with either 5 million mock (A+B) or IL2/IL-12 double-transduced A-NK cells (C+D). Note the better CTL infiltration ($FITC^+$ cells) of the tumors (yellow outline = tumor) in C+D compared to A+B. In B, also, note the rich CTL accumulation in the perivascular space around a smaller vessel (yellow arrows). CTLs (but never NK cells) accumulate in this location regardless of whether tumors are present or not.



implanted with D2F2/E2 tumor cells via the cisterna magnum and 3 days later injected i.v. with spleen cells from cured donors with or without IL-2 activated NK cells from naïve mice. Four-five days later, brains were removed and processed for cryosectioning and immunostaining (the results are described in Task 3-f).

e. Analysis of CSF: Number of cells and types of cytokines that mediate inflammation in the central nervous system (CNS)
    i. Months 6-21
    ii. Bergman

Initial studies reported above showed that we could obtain only 2-6 µl of CSF with cisterna magna puncture. We there modified previously reported techniques to harvest inflammatory cells from meninges and brain. The entire brain was harvested including cerebellum, brainstem and attached meninges, minced with scissors, ground through a 70 µM nylon cell strainer and washed with PBS. The cells were suspended in 20 ml of 30% Percoll (#17-0891, GE Healthcare, Uppsala, Sweden) and placed over 10 ml of 70% Percoll in a 50 ml conical centrifuge tube. The tube was centrifuged at 390g for 20 minutes at 4^{o}C and 5 ml was harvested from the Percoll interface and then washed twice with PBS.

We found that following viral infection, the leukocyte infiltration in meninges and tumor shifted from predominantly macrophages to predominantly T-cells. Tumors were implanted CM and 7 days later experimental animals were treated with CM virus. Control animals received no treatment. Flow cytometry of brain and meningeal mononuclear inflammatory cells 5-6 days after viral administration showed that the percent macrophages were high in the brains and meninges of control animals but decreased markedly in experimental animals treated with virus ($p=0.0049$) (Fig. 13). Absolute numbers of macrophages were also 2.7 fold higher in control animals than virus treated animals. At the same time, the percentage of T-cells in the brain and meninges increased in virus treated animals ($p=0.0001$) (Fig. 13).

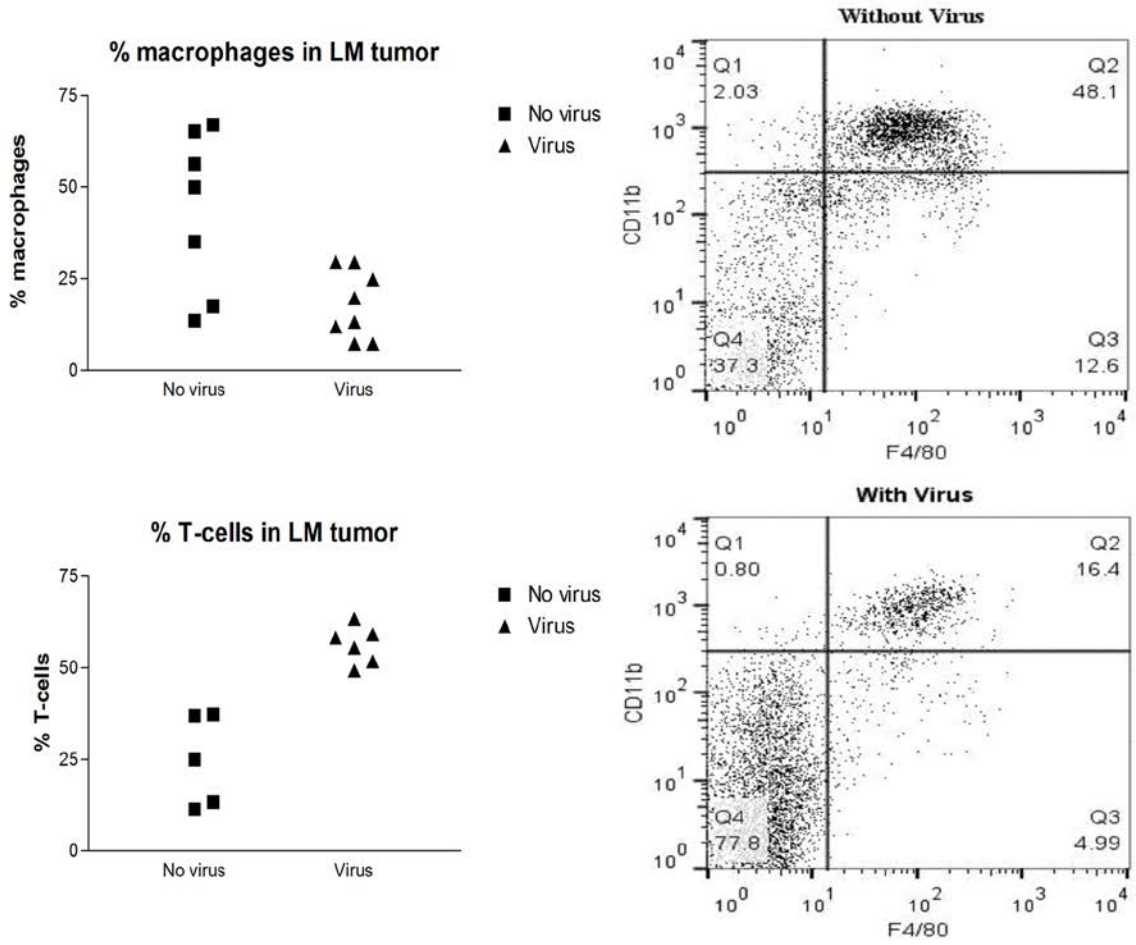


Fig. 13. Effect of CM viral infection on tumor associated meningeal macrophages. Seven days following CM tumor implants with D2F2/E2, experimental animals received CM virus and control animals did not. Brain and meninges were harvested 5-6 days later and analyzed for the presence of macrophages (F4/80 positive) and T-cells (Thy 1.2 or CD3e positive) by flow cytometry. Data on left shows significantly increased T-cells in the animals receiving virus and graphs on right are representative from 2 animals. An unpaired one-tailed t-test was used to compare percent accumulation of T-cells and macrophages in meninges, with and without virus administration.

Tumors in many tissues and especially in the brain have a large proportion of tumor-associated macrophages (TAM). TAM have an M2 phenotype which supports tumor growth and inhibits immunologic reaction against the tumor by a wide variety of mechanisms Further work is required to assess whether the therapeutic effect of viral infection in the meninges is in part due to reduction of the effects of suppressor macrophages. The work is complicated by the fact that macrophages in meninges and brain are a combination of yolk-sac derived microglia and bone marrow derived monocytes and current surface markers do not clearly differentiate these populations.

f. Analysis of tissues: Determine the density and state of activation of donor T-cells and host innate and adaptive immune cells in metastases and normal tissues
    i. Months 6-21
    ii. Basse

Brain tissue with D2F2/E2 tumors from animals receiving cured splenocytes and ex vivo activated A-NK cells (as described in 3-d) was stained with anti-NKp46 and anti-Thy1.2 antibodies to reveal transferred A-NK cells (double-positive) and T cells (Thy1.2 single-positive) from donors cured from ip D2F2/E2 tumors. Against expectations, relatively few of the transferred A-NK cells and T cells were found in the tumor tissue and the intratumoral density of donor T cells was not higher in animals receiving A-NK cells compared to animals receiving only the donor T cells. The A-NK cells in this experiment were not IL-2/IL-12 gene-transduced, but supported by exogenous IL-2. Since systemic IL-2 is known to reduce the number of circulating T cells, this may explain the low number of T cells from cured donors trafficking to the brain tumors. As we have previously seen a significant trafficking of adoptively transferred A-NK cells to tumors in the brain, we cannot, at this point in time, explain why it did not occur in this setting. It is possible, however, that the donor T-cells injected together with the A-NK cells consumed so much of the exogenous IL-2 given in support of the injected A-NK cells, that the A-NK cells received sub-optimal amounts of this cytokine.

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4. Establish that activated memory anti-tumor CD4 T-cells in the CSF target an immune response to tumor nodules (months 12-24)

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a. Generate donor survivor animals by treatment with rrVSV (same as 1.a)

This task has been accomplished as noted above.

b. Generate animals with brain and leptomeningeal (LM) metastases using the D2F2/E2 mammary cancer cell line. Transfer T-cells from donor mice (from 4.a)
    i. Months 12-24
    ii. Bergman
    iii. 300 mice

As noted above, we are fully able to generate LM metastases using two cell lines and have a new cell line to develop parenchymal metastases via carotid implants.

This task was accomplished with experiments that transferred T-cells with and without viral infection and all studies will be described in the next task.

c. Animal studies: Non-replicating VSV injected into tumor bearing animals (from 4b)
    i. Months 12-24
    ii. Bergman
    iii. 100 mice

Experiments utilizing both flow cytometry and immunohistochemistry (IHC) confirmed that viral infection of tumor recruited transferred memory T-cells to the meninges and tumor. This was the probably reason that viral infection of the tumors followed by transfer of memory anti-tumor T-cells produced cure of meningeal tumors, as reported above. Meningeal tumors were established in Thy 1.1 Balb/c mice and spleen cells were transferred from Thy 1.2 Balb/c cured donors. The experimental group of mice received CM virus on days 7-9 after tumor implant and transferred spleen cells from cured donors two days later. Control animals did not receive CM virus. Some animals also received IV virus which had no influence on transferred T-cells in the brain and was ignored in the analysis. Brains were harvested 3 days after cell transfer. In one set of experiments, flow cytometry was performed on mononuclear inflammatory cells isolated from whole brains by Percoll gradient separation. The virus treated group (n=4) had 17.9% transferred T-cells in brain inflammatory cells compared with 6.8% in the control group (n=6) (p=0.0005)(Fig. 14, top left). The percent transferred CD4 T-cells was 11.4% in the treated group compared with 4.1% in the control group (p=0.0005) (Fig. 14, middle left). The percent transferred CD8 T-cells was 4.0% in the treated group compared with 2.0% in the control group (p=0.013) (Figure 14, bottom left).

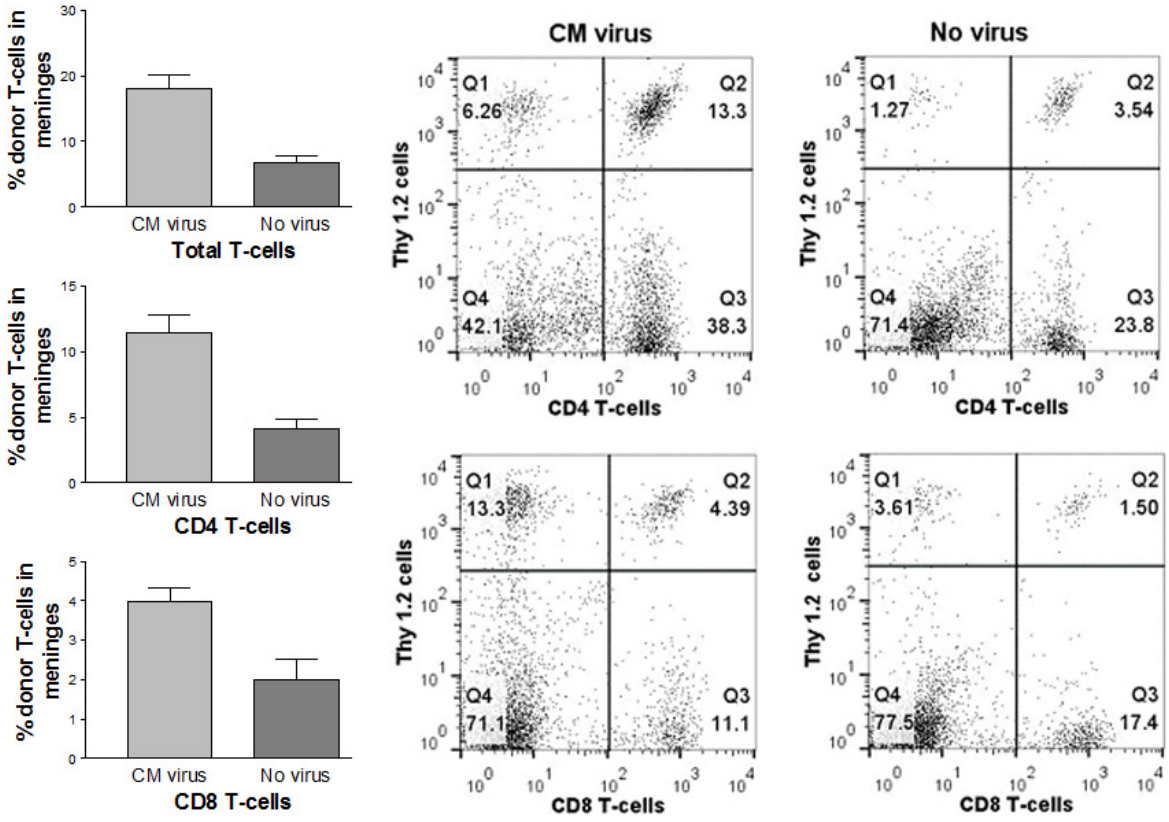


Fig. 14. Accumulation of donor T-cells in meninges following treatment with CM virus. Balb/c Thy 1.1 mice were implanted with CM tumor and 9-11 days later received spleen cells from cured Thy 1.2 donors. CM virus was administered 2 days before cells in the experimental (n=4) but not the control group (n=6). Mononuclear inflammatory cells were harvested from brain and meninges 3 days after cell transfer and flow cytometry used to determine the percentage of donor total T-cells, CD4 and CD8 T-cells (mean with SEM). Data on left shows significantly increased accumulation of total, CD4 and CD8 donor T-cells in CM virus treated animals than controls. Graphs on the right are representative from one experimental and one control animal.

In a separate experiment, transferred cells were counted in meninges and brain using immunohistochemistry (IHC). The cell density for transferred T-cells averaged 290.3/mm^2 in the CM virus treated group (n=3) which was significantly higher than the 44.0/mm^2 in the control group (n=5) (p=0.002). This histological experiment demonstrated that most of the transferred T-cells were in the meninges within or near tumor rather than spread randomly in meninges. Few if any transferred T-cells were in the tumor-free brain (Fig. 15).

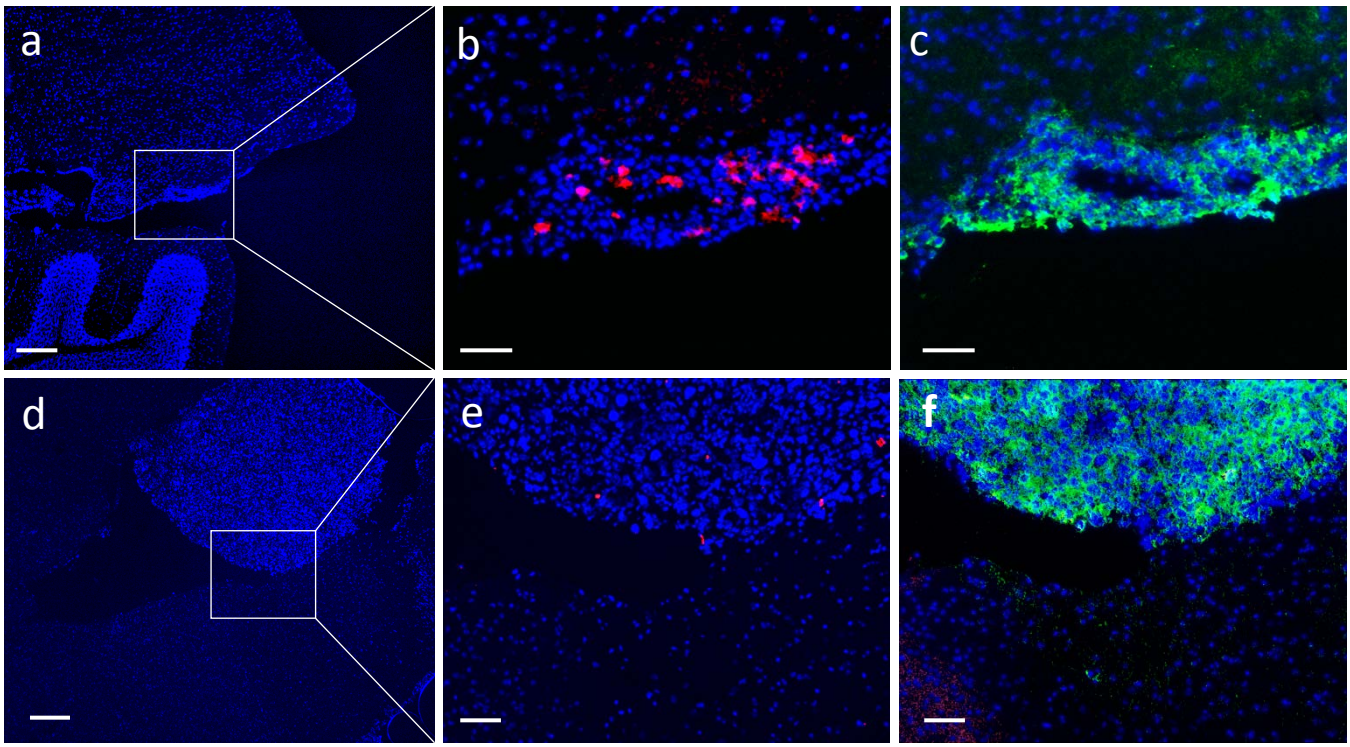


Fig. 15. Highly selective localization of adoptively transferred T cells into virally infected brain metastases. Spleen cells from cured animals were adoptively transferred into

animals with meningeal tumors. CM virus was administered 2 days before cells in the experimental (n=3) but not the control group (n=5). Animals were sacrificed 3 days after cell transfer. A significantly higher density of donor T cells was found in the meningeal tumors in animals treated with CM virus compared to control.
a-c: Sections of brain from virus-treated animals. Numerous PE-stained (red) donor T cells were found in most tumors growing in the meninges on the brain surface. Hardly any donor T cells were seen in the adjacent normal brain tissue.
d-f: Sections of brain from control animals. Only a few donor T cells (red) were found in brain tumors from control animals.
Bars in a and d = 200 microns. Bars in b, c, e and f = 50 microns.

The next set of experiments determined that following viral infection of CM tumors, transferred cells from cured donors were more likely to enter brain than transferred cells from naïve donors and that maximal entry to brain occurred 4 to 7 days following transfer. Similar preferential accumulation of transferred cells from cured mice was seen in the lungs and spleen but not the mesenteric lymph nodes. Meningeal tumors were established in Thy 1.1 Balb/c mice and treated as above with IV and CM virus and transferred spleen cells from either cured or naïve donor Thy 1.2 Balb/c mice. At various times after cell transfer, host animals were sacrificed and mononuclear inflammatory cells harvested from the entire brain, spleen and right lung. Total cell count was determined and flow cytometry was used to quantify the percent transferred CD4 and CD8 T-cells. Fig. 16 shows that CD4 T-cells from cured donors accumulated in brain, lung and spleen significantly more than T-cells from naïve donors; CD8 T-cells from cured donors accumulated in brain significantly more than T-cells from naïve donors (n=3 for most time points) ($p=0.04$ for brain CD4 T-cells; $p=0.03$ for brain CD8 T-cells; $p=0.0066$ for lung CD4 T-cells; $p=0.08$ for lung CD8 T-cells; $p=0.02$ for spleen CD4 T-cells; $p=0.05$ for spleen CD8 T-cells). As expected, no relative accumulation was noted in mesenteric lymph nodes (n=2 for almost all time points). Accumulation was apparent at 4 days after cell transfer, peaked at 7 days and was still present at 11 days.

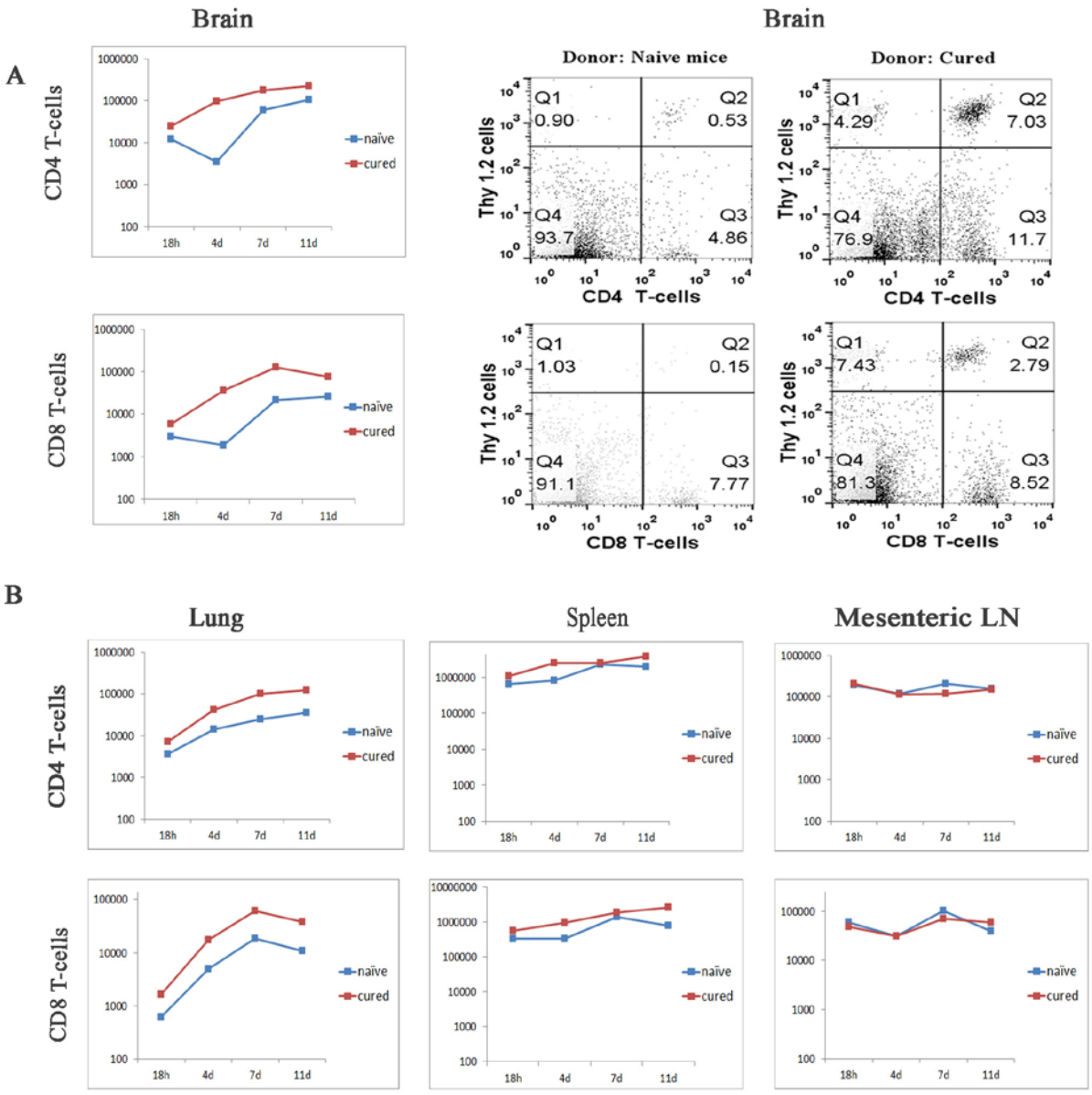


Fig. 16. Time course of accumulation of cured and naïve donor T-cells in brain, lung, spleen and mesenteric lymph nodes. Following CM implants of D2F2/E2 tumor, mice were treated with CM virus and then received either cured donor or cured naïve spleen cells. Animals were sacrificed at indicated times following cell transfer and inflammatory cells harvested from organs and counted. Flow cytometry was used to determine percentage of donor CD4 and CD8 T-cells. (n=3 for almost all time points except mesenteric lymph nodes. This organ had n=2 for all time points except 18h). A one-tailed t-test was used to compare area under the curve (AUC) for cellular accumulation over time in various tissues for experimental and control groups.

A. Mean data from brains of all animals showing significantly increased accumulation of CD4 and CD8 T-cells from cured than naïve donors. Representative graphs from 1 naïve and 1 cured donor are shown on the right.

B. Mean data from lung, spleen and mesenteric lymph nodes showing significantly increased accumulation of CD4 T-cells from cured than naïve donors.

We also found that virus infection of meningeal tumor results in proliferation of anti-tumor memory T-cells in brain, lung and spleen. T-cell replication was assessed by labeling donor Thy 1.2 cells with CFSE, harvesting tissues at various times after cell transfer and using flow cytometry to identify replicated T-cells by diluted fluorescence and positive staining for Thy 1.2. T-cell proliferation was greater in donor cells from cured animals than from naïve animals indicating a rapid response from memory anti-virus and anti-tumor T-cells (n=2-6 for each time point) (Fig. 17). Proliferation was maximal 3 to 5 days following cell transfer and followed a similar temporal pattern in brain, lung and spleen suggesting memory T-cell division independently in each of these organs. The brain was the site of the tumor implant and viral infection. The spleen and lung are lymphoid organs that filter antigens released from the brain into the blood.

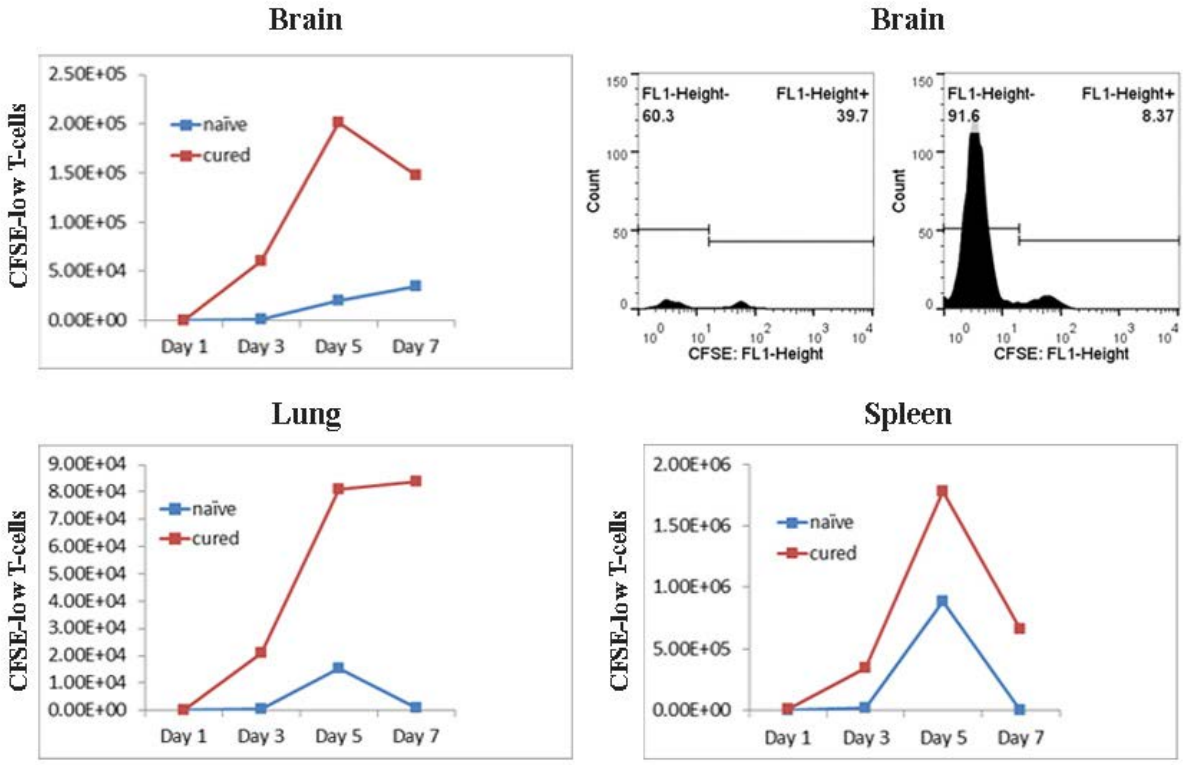


Fig. 17. Proliferation of transferred T cells from cured and naïve donor in tumor-bearing hosts. Meningeal tumors were established in Thy 1.1 Balb/c mice and treated with CM virus 7 days later. CFSE labeled spleen cells were transferred from either naïve mice or cured Thy 1.2 Balb/c mice 2 days after treatment with virus. Host animals were treated in matched pairs on the same day and always received the same number of cells (3.45 x 10^7 to 8.2 x 10^7 cells in experiments on different days). At various times after cell

transfer, host animals were sacrificed and inflammatory cells harvested from the entire brain, spleen and lungs. Flow cytometry was used to quantify CFSE signal in the Thy 1.2 gate. The total number of CFSE low donor T-cells are shown on the y-axis. (n=2-6 for each time point).

A. Mean data from brains of all animals. Representative graphs from 1 naïve and 1 cured donor are shown on the right.
B. Mean data from lung and spleen.

d. Animal studies: A-NK cells injected into tumor bearing animals (from 4b)
    i. Months 12-24
    ii. Basse
    iii. 100 mice

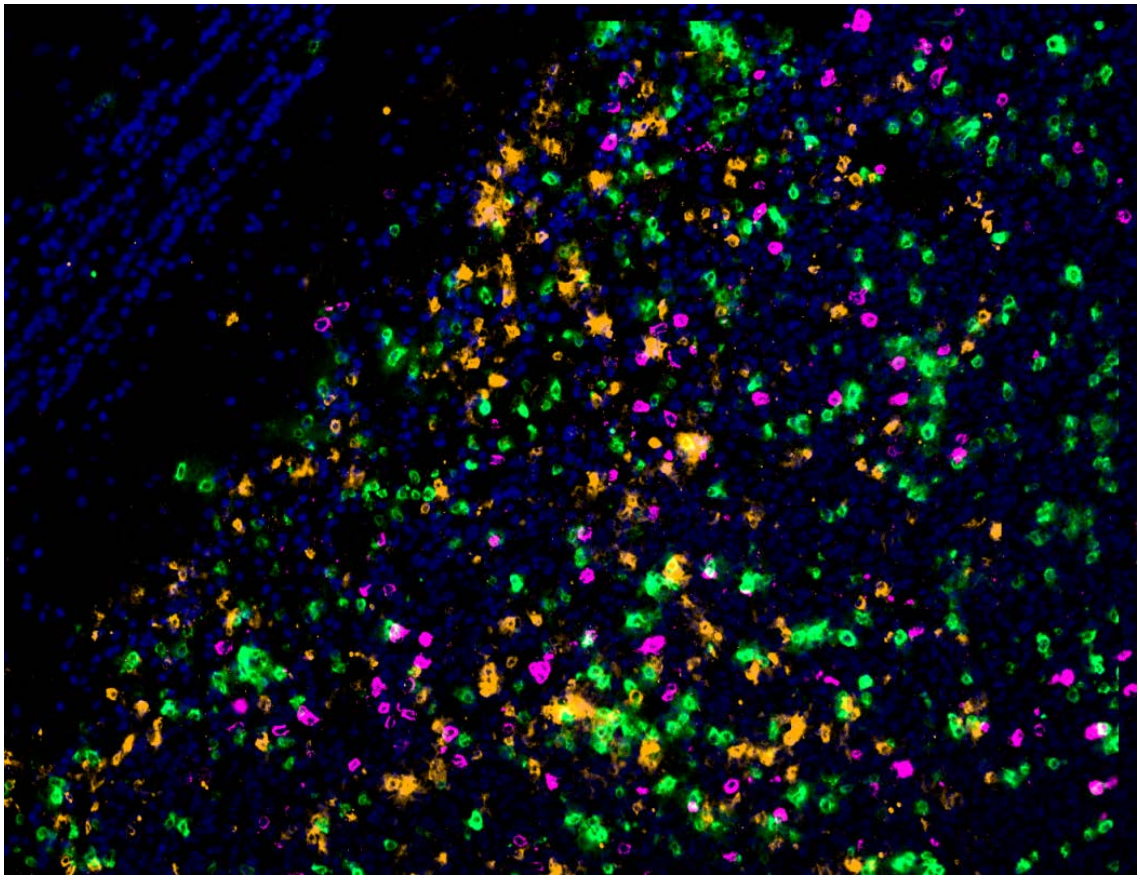
e. Animal studies: Non-replicating VSV injection followed by A-NK cells injection into tumor bearing animals (from 4b)
    i. Months 12-24
    ii. Basse and Bergman
    iii. 100 mice

Due to the promising results described in Tasks 3-e and 4-c, confirming the beneficial effect of VSV infection on the traffic of donor T cells to the brain tumors and the somewhat disappointing results of the experiments to determine the ability of A-NK cells to facilitate traffic of T cells from cured donors to the brain tumors (described in Task 3-f), we determined to focus on VSV injections alone to facilitate T cell traffic to the brain tumors. However, we decided to determine that ability of VSV infection alone to attract endogenous T cells and NK cells to brain tumors (Task 4-f)

f. Analysis of tissues: Determine the density of donor and host immune cells in metastases compared with normal brain
    i. Months 12-24
    ii. **Basse**

In this task, animals with D2F2/E2 brain metastases established as described in Task 1-d received CM injection of VSV on day 5 of tumor growth. The animals received no further treatment (i.e., no adoptive transfer of T and NK cells and no Cytoxan treatment). Brains with tumors were removed 4-5 days later and processed for cryo-sectioning and immuno-staining to reveal host lymphocytes (CD4+, CD8+ T cells and NKp46+ NK cells). While very few host lymphocytes were seen in non-treated control animals, the virus-infected tumors contained an unprecedented high number of all three types of lymphocytes (Fig. 18). In contrast, hardly any of these cells were found outside the tumor-areas. Clearly, infection with Her2-targeted VSV breaks down any barriers to lymphocyte homing to intracranial metastases. We are very excited by these findings and future studies will help us determine if the dramatic increase in host T and NK effector cells in the brain tumors is caused by augmented influx or increased intratumoral

survival and/or proliferation of the lymphocytes or – as we believe – by a combination of all these factors. In experiments planned for the near future, we will seek answers to maybe even more important questions, namely whether the impressive influx of host lymphocytes leads to reduction in tumor growth, whether the infiltrating T-lymphocytes recognize virus- or tumor-associated antigen, whether the many infiltrating NK cells are cytotoxically active (or whether their function is to secrete IFNγ to increase MHC-expression on the target cells) and finally, whether the massive infiltration by the host lymphocytes, despite their highly selective accumulation within the borders of the malignant tissues, induce unwanted inflammatory conditions in the brain which could lead to serious meningitis-like conditions resulting in increased intracranial pressure.



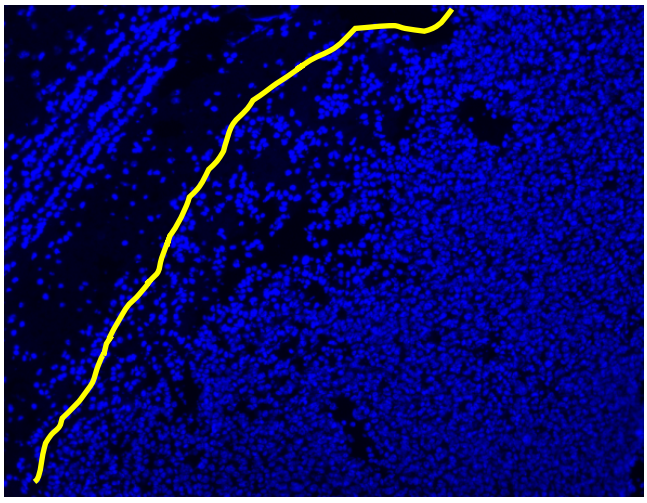


Figure 18: Dense infiltration of D2F2/E2 brain metastases at 4 days after intrathecal injection of VSV-HER2. CD8=green, CD4=orange, NKp46 = purple. Please note the very selective infiltration. Hardly any lymphocytes are found in the normal brain tissue to the upper left. Picture on the left: Hoechst staining. Yellow line separates tumor (right) and normal brain tissue (left).

g. Analysis of treatment effects: Survival curves
    i. Months 12-24
    ii. Basse and Bergman

These have been reported separately above but the distilled final results will be gathered together here. First, we showed that anti-tumor memory T-cells cure peritoneal tumors more readily than brain meningeal tumors (Fig. 10).

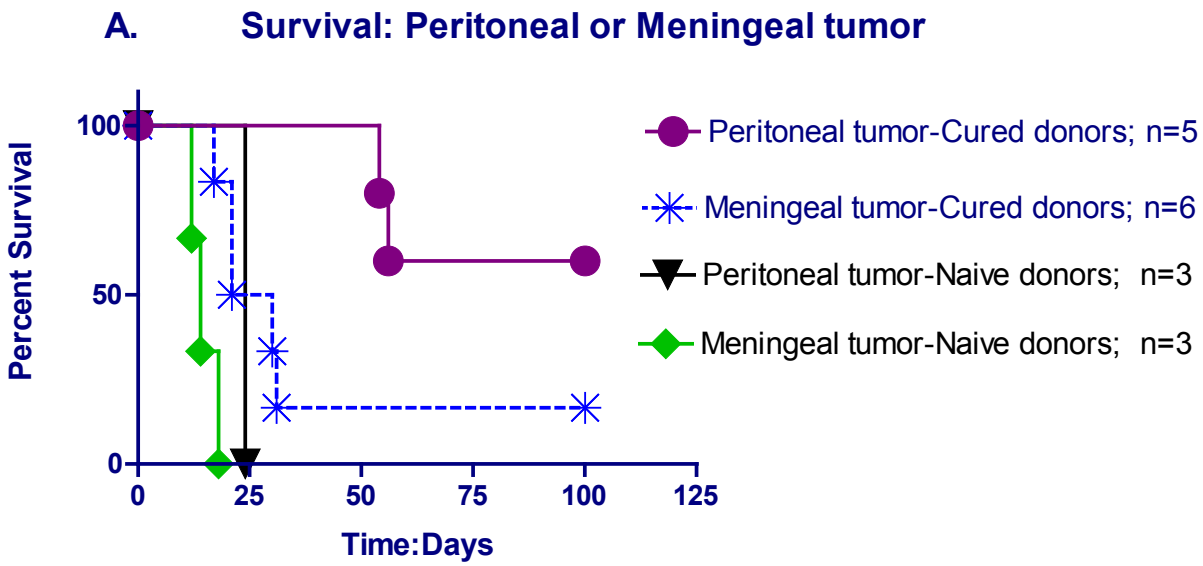


Fig. 10. Survival following treatment of peritoneal or meningeal tumors with cured donors. Mice were implanted with D2F2/E2 tumor cells in the peritoneum or the meninges and treated 3 days later with spleen cells from either cured or naïve donors. Cured donors significantly increased survival in peritoneal tumors compared with meningeal tumors ($p=0.0003$, log rank statistic). Naïve donors were not effective in either model.

Next, we demonstrated that this barrier could be overcome by direct viral infection of the meningeal tumors (Fig. 11).

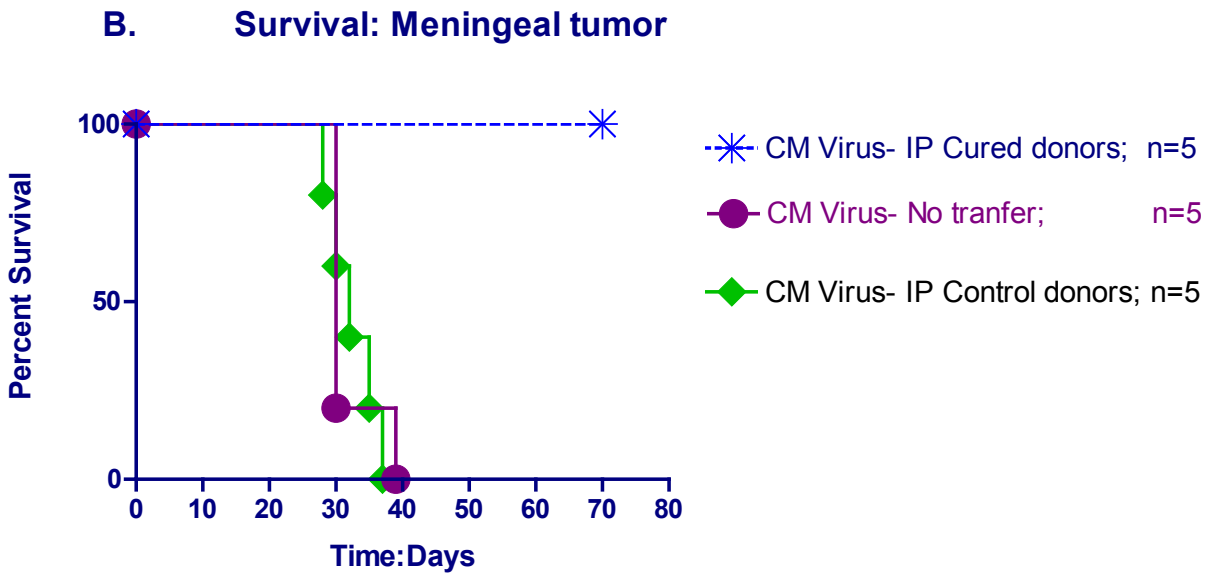


Fig. 11. Survival following treatment of meningeal tumors with cured donors combined with direct viral infection of the meningeal tumors. Experimental mice received meningeal virus one day and cured donors 3 days after tumor implantation. Control mice received no donor cells or donor cells from mice treated with virus but not implanted with peritoneal tumor. All animals received cyclophosphamide IP to facilitate cell transfer. N=5 for all groups. Cured donors with viral infection of the meninges significantly increased survival compared with virus infection alone (p-0.0016) or virus infection with control donors (p=0.0018). Cured donors with viral infection of the meningeal tumors significantly increased survival of meningeal tumors compared with cured donors without virus infection (curve in 1A compared with curve in 1B; p=0.0086).

Finally, we showed that successful viral therapy of peritoneal tumors generated memory T-cells that could prevent establishment of meningeal tumors without the requirement for viral infection. Prevention depended on memory anti-tumor CD8 T-cells but not CD4 T-cells (Fig. 1).

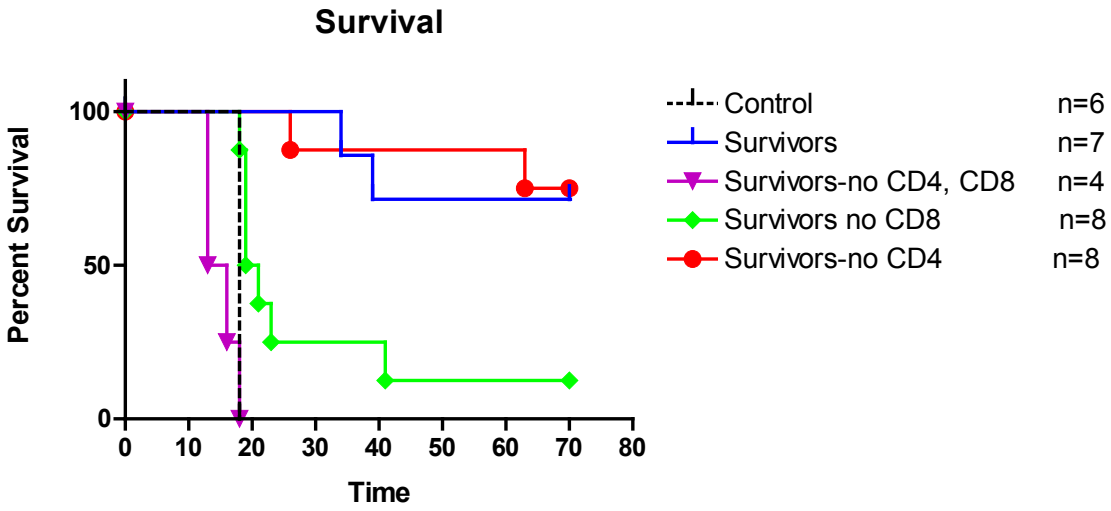


Fig. 1. Survival following CM challenge. Animals cured of IP tumors (survivors) using standard treatment with rrVSV, antiCTLA4 and CPM were challenged with CM tumor, as were controls. Anti-tumor memory CD8 T-cells prevented establishment of meningeal tumors in survivors.

KEY RESEARCH ACCOMPLISHMENTS

- New method of cisterna magna implant of cancer cells that produces meningeal tumors very easily, quickly and reproducibly.
- Breast cancer cell line (D2F2/E2) expressing Her2/neu shown to implant reproducibly in meninges and make excellent model of breast cancer meningeal metastases.
- New method of carotid artery implant of cancer cells that can be performed rapidly.
- VSV and natural killer (NK) cells containing cytokine transgenes successfully produced.
- Demonstrated that animals with peritoneal D2F2/E2 tumors can be cured reliably by treatment with VSV, cyclophosphamide, and anti-CTLA-4 and that anti-tumor memory T-cells can be harvested from the spleens of these cured animals.
- Targeted virus infection of tumor can generate therapeutic memory T-cells.
- The results support previous work by demonstrating experimentally that there is a relative barrier to therapeutic anti-tumor T-cells in the brain meninges. We show that the same anti-tumor memory T-cells that can cure peritoneal tumors are much less effective against leptomeningeal implants.
- Memory anti-tumor T-cells can treat established meningeal tumors following viral infection of the tumors. Importantly, this work is done in mice with a normal diversity of T-cells and should therefore be directly relevant to the clinical situation.
- Viral infection of the tumor was able to overcome the relative brain barrier to a cellular immune response probably by provoking an inflammatory response in the meninges. Time course studies showed that virus infection produced a 2.6-fold increase in total transferred T-cells in the meninges. The increase in memory T-cells must be much greater because of the diluting effect of the non-specific T-cells. This effect was seen on day 3 following cell transfer at the same time that cell proliferation of transferred memory cells was just beginning indicating that the increase at that time point was due to enhanced trafficking and accumulation and not enhanced local proliferation. Proliferation of memory T-cells was robust between days 3 and 5 after cell transfer and occurred simultaneously in brain, lung and spleen.
- Concomitant with the virus-induced increase of T-cells in tumor and meninges, we noted a decrease in the percent of macrophages. Tumors in many tissues and especially in the brain have a large proportion of tumor-associated macrophages (TAM). TAM have an M2 phenotype which supports tumor growth and inhibits immunologic reaction against the tumor by a wide variety of mechanisms. Further work is required to assess whether the therapeutic effect of viral infection in the meninges is in part due to reduction of the effects of suppressor macrophages.
- We found that mice cured of peritoneal tumors are resistant to re-challenge in the brain meninges providing hope that therapeutic vaccination at the time of primary tumor excision can not only produce memory T-cells that can be induced to travel to brain and cure growing metastatic lesions after they are discovered but that these T-cells might prevent establishment of brain metastases in the first place.
- Targeted virus infection of tumor attracts a small number of adoptively transferred A-NK cells to the tumor nodules but more impressively, attracts endogenous NK cells to the tumor in much higher than expected numbers. The next logical question is whether these endogenous NK cells are cytotoxic to tumor and therefore able to reduce tumor growth.

REPORTABLE OUTCOMES

1 Development of D2F2/E2-J9, a subclone of D2F2/E2 that consistently implants in the leptomeninges.

CONCLUSIONS

- We have produced a very robust experimental model system for preclinical studies of breast cancer metastases to the brain meninges and the ability of immune effector cells to reach these metastases.
- Targeted virus infection of tumor can generate therapeutic memory T-cells. In clinical practice this means that a single off-the-shelf reagent, the virus, can be used to treat any patient. Expensive individually-tailored therapy is not required. Following virus infection of tumor, each patient will generate the most potent immune response from their T-cell repertoire.
- The barrier to therapeutic anti-tumor memory T-cells can be overcome by viral infection of the brain implants which induces proliferation of anti-tumor memory T-cells and increases their accumulation in meningeal tumors. We show clearly that memory anti-tumor T-cells can treat established meningeal tumors following viral infection of the tumors. Importantly, this work is done in mice with a normal diversity of T-cells and should therefore be directly relevant to the clinical situation. In clinical translation, anti-tumor memory T-cells would be generated by viral infection of the initial breast tumor. Surveillance imaging would detect growing lesions and viral infection of the tumor in the central nervous system (CNS) would attract a memory response to the tumor. The results in this paper show that the memory T-cells are capable of finding multiple geographically-separated tumor collections of various sizes and completely eradicating them. This result was achieved in 2 mouse strains, Balb/c and C57/Bl6, with very different genetic immunologic biases. Certainly, there remain several pre-clinical steps to be accomplished before this therapy could be considered clinically. This model system is not fully syngeneic because the implanted mouse cells have been engineered to express the human Her2/neu receptor. The model is instructive because the animals do not generate an effective immune response and untreated tumor growth is progressive and lethal but translation to the clinic could founder on a weaker immune response. We are currently developing a fully syngeneic model system to more conclusively establish the concepts demonstrated in this paper. Although repeat viral infection in the CNS is possible because neutralizing antibodies formed in response to the initial infection cannot penetrate the BBB, we recognize that stimulating an immune response by infecting the brain with a replicating virus, albeit targeted and much attenuated, is not an ideal solution for the clinic. Further work is needed to develop an effective non-replicating pseudovirus or other immune stimulant to attract memory T-cells to the CNS. For maximum clinical utility, the results in this paper must be generalized to parenchymal brain tumors as well as meningeal tumors. We used a meningeal model in these studies to avoid the confounding factor of trauma at the tumor site. In this model, the tumors implant at multiple locations in the meninges far from the injection site. We are developing a model of parenchymal disease by carotid artery injection that will also avoid the problem of local trauma at the tumor site. Finally, toxicity studies will be required for all components of the proposed therapy.

- Viral infection of the tumor was able to overcome the relative brain barrier to a cellular immune response probably by provoking an inflammatory response in the meninges. Viral infection produced increased trafficking and accumulation of memory anti-tumor T-cells in the meningeal tumors. Proliferation of memory T-cells was robust between days 3 and 5 after cell transfer and occurred simultaneously in brain, lung and spleen. This suggests that antigen presentation was occurring simultaneously in these 3 organs. Antigen release from the brain and meninges occurs via CSF drainage into nasal lymphatics and into the dural venous sinuses. Lymphatic drainage into cervical lymph nodes was not assessed here but the results in this paper reinforce the view that the spleen and lung harvest brain antigens from the blood and present them effectively to circulating memory T-cells. This model system did not find evidence that T-cells must first become licensed in the lung before entering the brain. The results also support the view that local antigen presenting cells (APC) in the meninges stimulate memory T-cells that survey the CSF.
- Concomitant with the virus-induced increase of T-cells in tumor and meninges, we noted a decrease in the percent of macrophages. Tumors in many tissues and especially in the brain have a large proportion of tumor-associated macrophages (TAM). Further work is required to assess whether the therapeutic effect of viral infection in the meninges is in part due to reduction of the effects of suppressor macrophages.
- We found that mice cured of peritoneal tumors are resistant to re-challenge in the brain meninges providing hope that therapeutic vaccination at the time of primary tumor excision can not only produce memory T-cells that can be induced to travel to brain and cure growing metastatic lesions after they are discovered but that these T-cells might prevent establishment of brain metastases in the first place. The ideal clinical case would be to infect breast tumors before surgical removal and generate anti-tumor memory T-cells which prevent metastases from initially implanting in meninges or brain. The metastatic cells to be blocked could potentially come from subclinical sites in lymph nodes, lymph organs or lung.
- “So what now” : These results support the hypothesis that a virally-based immunization strategy can be used to both prevent and treat brain metastases. The brain and meningeal barriers to cancer therapy may be much more permeable to treatment based on cells than treatment based on drugs or molecules.

REFERENCES

NA

PERSONNEL RECEIVING PAY

Ira Bergman
Yanhua Gao
Judith Griffin
Per Basse
Lisa Bailey

APPENDICIES
Submitted paper:

Viral infection of implanted meningeal tumors induces anti-tumor memory T-cells to travel to the brain and eliminate established tumors

Yanhua Gao

Department of Pediatrics

University of Pittsburgh School of Medicine

Rangos Research Building

530 45th Street

Pittsburgh, PA 15201

Patricia Whitaker-Dowling

Department of Microbiology and Molecular Genetics

University of Pittsburgh School of Medicine

Pittsburgh, PA

3550 Terrace Street

Pittsburgh, PA 15261

Mamdouha A. Barmada

Department of Pathology and Laboratory Medicine

American University of Beirut Medical Center

American University Hospital, Third floor, Room 312 C-D

Rome and Cairo St – Hamra – Beirut – Lebanon.

Per H. Basse MD, PhD, DMSci

Departments of Immunology and Pathology

University of Pittsburgh School of Medicine

Hillman Cancer Center

5117 Centre Avenue

Pittsburgh, PA 15213

Ira Bergman[1]

Departments of Pediatrics, Neurology, and Immunology

University of Pittsburgh School of Medicine

Children's Hospital of Pittsburgh of UPMC

One Children's Hospital Drive

4401 Penn Avenue

Pittsburgh, PA 15224

Short title: Memory T-cells to treat brain tumors

[1]Corresponding author: Ira Bergman

Children's Hospital of Pittsburgh of UPMC

One Children's Hospital Drive

4401 Penn Avenue

Pittsburgh, PA 15224

Email: ira.bergman@chp.edu

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Conflict of Interest: IB and PWD: US Patent Application 7,429,481 entitled "Targeting viruses using a modified Sindbis glycoprotein" awarded September 30, 2008. The targeted virus is being tested in this animal work. IB and PWD have no affiliation with any company.

YG, MAB and PHB have no conflict of interest to report.

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Abstract:

Background: Brain and leptomeningeal (LM) metastases are a major public health problem. Patients receive successful therapy for their presenting cancers but then develop progressive metastatic disease in the brain. Current therapies are ineffective, toxic or both. We have developed a straightforward method to induce anti-tumor memory T-cells using a Her2/neu targeted Vesicular Stomatitis Virus (VSV). We now sought to determine whether viral infection of meningeal tumor could attract anti-tumor memory T-cells to eradicate the tumors.

Methods: Meningeal implants in mice were studied using treatment trials and analyses of immune cells in the tumors.

Results: This paper demonstrates that there is a blood-brain-barrier to bringing therapeutic memory T-cells to meningeal tumors. The barrier could be overcome by viral infection of the tumor. Viral infection of the meningeal tumors followed by memory T-cells transfer resulted in 89% cure of meningeal tumor in two different mouse strains. Viral infection produced increased infiltration and proliferation of transferred memory T-cells in the meningeal tumors. Following viral infection, the leukocyte infiltration in meninges and tumor shifted from predominantly macrophages to predominantly T-cells. Finally, this paper shows that successful viral therapy of peritoneal tumors generates memory CD8 T-cells that prevent establishment of tumor in the brain meninges of these same animals.

Conclusions: These results support the hypothesis that a virally-based immunization strategy can be used to both prevent and treat brain metastases. The brain and meningeal

barriers to cancer therapy may be much more permeable to treatment based on cells than treatment based on drugs or molecules.

Keywords:

Brain tumor

Meningeal tumor

Memory T-cells

VSV

Viral therapy

Introduction:

Brain metastases are common and lethal. Increasingly, patients receive successful therapy for their presenting cancers and appear well with excellent quality of life. However, years later they develop metastatic disease in the brain resulting in progressive physical and cognitive impairment and culminating in death. The incidence of brain metastasis is about 200,000 cases per year in the United States and most of these patients will die within months.[1] The unique devastation of brain metastases is highlighted in the case of breast cancer. Since the introduction of Herceptin, several groups have reported a high incidence of brain metastases, 28 to 43%, in treated patients.[2,3] The high incidence may reflect, in part, a greater affinity of HER2-overexpressing breast cancer for the brain[4,5] but is probably also due to the disparity of effectiveness of Herceptin in the brain versus

systemically. Herceptin is effective systemically and may prolong survival to such an extent that brain metastases, which manifest late in the clinical course, become apparent.[2] Herceptin is ineffective against brain or meningeal disease because it is an antibody which is unable to efficiently cross the blood brain barrier (BBB).

There is abundant empirical and experimental evidence that the BBB to molecules severely impedes chemotherapy of brain metastases. This barrier to molecules consists of tight endothelial cell junctions, limited pinocytosis in endothelial cells and efficient efflux transporters that move drugs from the brain to the blood side of the endothelial cell.[6] The end result is that most chemotherapeutic agents do not efficiently enter normal brain and do not enter small brain tumors or metastases in which the BBB is intact.[7,8] The brain barrier to cells is more complicated and much less studied. Important variables include immune cell type, activation state of the cells, cellular expression of selectins and integrins, secretion of cytokines and chemokines and anatomic compartment of the central nervous system. [9-11] The cellular and molecular mechanisms underlying immune surveillance of brain are just beginning to be elucidated and the application of this understanding to therapy of brain metastases has barely begun.[12]

The aim of this study was to determine whether there exists a brain barrier to immune cell therapy of brain and LM metastases and if so, whether viral infection of the metastases could overcome the barrier. Our long-term therapeutic goal is to generate anti-tumor immunity prior to surgical removal of the primary breast tumor by infecting the tumor mass with a replicating recombinant vesicular stomatitis virus (rrVSV) that preferentially infects Her2/neu expressing cells. We have previously shown that such therapy cures established peritoneal tumor implants and generates anti-tumor T-cells that

are curative when transferred to host animals with established peritoneal tumors.[13,14] In this paper we show that the same anti-tumor memory T-cells that can cure peritoneal tumors are much less effective against leptomeningeal implants demonstrating experimentally that there is a relative barrier to therapeutic anti-tumor T-cells in the brain meninges. However, this barrier can be overcome by viral infection of the brain implants which induces proliferation of anti-tumor memory T-cells and increases their accumulation in meningeal tumors. At the same time, there is a marked reduction in tumor-associated macrophages and an increase in the meningeal T-cell to macrophage ratio. The end result is elimination of the tumors and cure of the animals. Importantly, this work is done in mice with a normal diversity of T-cells and should therefore be directly relevant to the clinical situation. In addition, we find that mice cured of peritoneal tumors are resistant to re-challenge in the brain meninges providing hope that therapeutic vaccination at the time of primary tumor excision can not only produce memory T-cells that can be induced to travel to brain and cure growing metastatic lesions after they are discovered but that these T-cells might prevent establishment of brain metastases in the first place.

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. MC38/E2, a mouse colon carcinoma tumor

line that has been stably transfected with a vector expressing the human Her2/neu gene and its parent cell line, MC38 were a generous gift from Dr. Manuel Penichet, UCLA, LA, CA. Anti-CTLA4 (9H10) was obtained commercially (BioXcell Fermentation/Purification Services #BE0131, West Lebanon, NH). Anti-CD8 (2.43)(26) and anti-CD4 (GK1.5)(27) ascites were prepared from hybridomas obtained from the American Type Culture Collection (Rockville, MD). Animal studies with implanted D2F2/E2 cells were conducted using female BALB/c mice and studies with implanted MC38/E2 cells used female C57/Bl6 mice (Taconic). Mice were 8 to 20 weeks of age and weighed 20-25g. Thy 1.2 BALB/c were obtained from Taconic (Hudson, NY). A mating pair of Thy 1.1 BALB/c were purchased from the Jackson Laboratory (Strain name: CBy.PL(B6)-*Thy1*^{a}/ScrJ, Stock Number: 005443) and bred on site. Animal studies were approved by the institutional Animal Research and Care Committee.

rrVSV. rrVSV targeted to cells expressing Her2/neu was created from vector components as previously described and with generous contributions from Dr. John K. Rose, Dr. Irvin S. Y. Chen and Genentech Inc.[13]

Cell collection. Cells were harvested from spleens, lymph nodes and lungs by standard techniques. The entire brain was harvested including cerebellum, brainstem and attached meninges, minced with scissors, ground through a 70 µM nylon cell strainer and washed with PBS. The cells were suspended in 20 ml of 30% Percoll (#17-0891, GE Healthcare, Uppsala, Sweden) and placed over 10 ml of 70% Percoll in a 50 ml conical centrifuge tube. The tube was centrifuged at 390g for 20 minutes at 4^{o}C and 5 ml was harvested from the Percoll interface and then washed twice with PBS.

Depletion *in vivo* of T-cells. As previously described.[13]

Histopathology. Standard techniques of formalin fixation, paraffin embedding and H&E staining were used.

Immunohistochemistry (IHC). IHC was performed on whole brains that were harvested, embedded, sectioned and stained using standard techniques. At least 10 images of randomly chosen tumor tissue and surrounding normal brain tissue were acquired from each animal. The density – expressed as cells/mm^2 - of positively-staining cells in normal and malignant tissue was determined by image analysis (MetaMorph 7.2, Molecular Devices, Sunnyvale, CA).

Flow cytometry: As previously described.[14]

Cured animals and production of anti-tumor and anti-virus memory T-cells. Transfer experiments required spleen cells from cured mice. These mice were produced by implanting Female BALB/c Thy 1.2 mice intraperitoneally (IP) with 2 x 10^6 D2F2/E2 cells in 300 μl PBS. On day 3 they were treated with rrVSV, 1 x 10^8 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.

Meningeal Implants. Animals received isofluorane anesthesia. The hair was shaved from the posterior neck and the skin prepped with iodine and alcohol. The head was flexed and 20 μl of cells or treatment were inserted into the CSF of the cisterna magna slightly lateral to the midline just inferior to the occipital bone of the skull using an insulin syringe and needle (NDC # 08287-28, Los Angeles, CA).

Treatment trials. Peritoneal, meningeal or brain tumors were established as above. Adoptive transfer of splenocytes from naïve and cured animals was administered IV. Animals were sacrificed if they developed any signs of weakness or disability. The

animals were considered cured if they survived for 100 days after IP implants and 70 days after CM implants.

Statistics. The log rank statistic was used to compare survival among the treatment groups. A one-tailed t-test was used to compare area under the curve (AUC) for cellular accumulation over time in various tissues for experimental and control groups as noted in the text. An unpaired one-tailed t-test was used to compare percent accumulation of T-cells and macrophages in meninges, with and without virus administration. PRISM software was used to analyze the data (GraphPad Software, Inc., La Jolla, CA).

Results:

We have previously shown that rrVSV therapy of implanted peritoneal tumors generates therapeutic anti-tumor memory T-cells.[14] We now determined how to use these cells to eradicate brain meningeal tumors.

Transferred anti-tumor memory T-cells cure peritoneal tumors more readily than brain meningeal tumors. Peritoneal tumors were established as previously described.[13] Meningeal tumors were established by implanting cells percutaneously into the cisterna magna. Tumors grew largest in the olfactory region but also grew in the meninges throughout the cerebrum and cerebellum (Supplementary Fig. S1). Tumors grew rapidly and untreated average duration of survival was only 15 days.

Three days following tumor implantation, mice received spleen cells IV from cured donor animals (henceforth called cured donors). One donor was used per one recipient but all donor cells were pooled so recipient animals with peritoneal or meningeal tumors

received donor cells from the same pooled collection. Each host received 4-6 x 10^7 donor cells. As previously described, host animals, experimental and control, in all studies were pre-treated one day before transfer with a single dose of cyclophosphamide (CPM) at 100-125 mg/kg to facilitate cell transfer.[14] Memory T-cells were much more effective in eliminating peritoneal tumor than meningeal tumor (Fig. 1A; p=0.0003). Transferred anti-tumor memory T-cells increased survival by at least 25 days and cured 60% of mice with peritoneal tumors, but cured only 20% of mice with meningeal tumors and only increased survival by a few days. Transferred spleen cells from naïve animals (henceforth called naïve donors) were completely ineffective against peritoneal or meningeal tumors, as expected.

These results support the idea that there is a relative BBB to cellular immune therapy. We next attempted to overcome this barrier by direct viral infection of the meningeal tumors.

Transferred anti-tumor memory T-cells cure leptomeningeal tumors after viral infection of the tumors. Meningeal and brain tumors were established in Balb/c mice and treated as above with the addition that one day after tumor implant the animals receive rrVSV CM (2 x 10^6 ID) and IV (2 x 10^7 ID). This therapy was remarkable effective resulting in cure of all 5 animals (Fig. 1B) and significantly improved survival compared with treatment with virus alone (p=0.0016). All control animals who received virus alone developed neurological deficits and were sacrificed at a mean of 31.8 days. In order to prove that anti-tumor and not just anti-virus memory T-cells were critical to the therapeutic response, we treated one set of animals of 5 mice with donor cells from animals that had been infected with virus but never implanted with tumor. All animals

died at a mean of 32.4 days, significantly worse than the experimental group treated with cured donors ($p=0.0018$), indicating that specific anti-tumor memory T-cells were necessary for successful therapy. Further evidence came from one donor whose IP tumor was cured by treatment with anti-CTLA4 and CPM alone but no virus. Transferred spleen cells from this animal combined with viral infection of the meningeal tumor cured meningeal tumor in one host animal indicating that anti-tumor memory T-cells without anti-virus T-cells were curative. These results generalized to a different mouse strain. Cures were achieved in C57/Bl6 mice who were implanted CM with MC38/E2 and treated as above with virus and spleen cells from cured donors; 2 from donors cured with virus plus anti-CTLA4 and CPM and 2 from donors cured with anti-CTLA4 and CPM alone and no virus. Three of 4 treated animals were cured. One treated animal died after 49 days. One control that received no treatment died at 28 days. In both strains of mice, cured animals behaved normally and showed no adverse effects of CM administration of rrVSV. We felt it most likely that viral infection of tumor was attracting circulating T-cells to the CSF and leading to the elimination of tumor by anti-tumor memory T-cells.

Virus infection of meningeal tumor attracts anti-tumor memory T-cells to the meninges and tumor. Experiments utilizing both flow cytometry and immunohistochemistry (IHC) confirmed that viral infection of tumor recruited transferred memory T-cells to the meninges and tumor. Meningeal tumors were established in Thy 1.1 Balb/c mice and spleen cells were transferred from Thy 1.2 Balb/c cured donors. The experimental group of mice received CM virus on days 7-9 after tumor implant and transferred spleen cells from cured donors two days later. Control animals did not receive CM virus. Some animals also received IV virus which had no influence on transferred T-

cells in the brain and was ignored in the analysis. Brains were harvested 3 days after cell transfer. In one set of experiments, flow cytometry was performed on mononuclear inflammatory cells isolated from whole brains by Percoll gradient separation.[15] The virus treated group (n=4) had 17.9% transferred T-cells in brain inflammatory cells compared with 6.8% in the control group (n=6) (p=0.0005)(Fig. 2, top left). The percent transferred CD4 T-cells was 11.4% in the treated group compared with 4.1% in the control group (p=0.0005) (Fig. 2, middle left). The percent transferred CD8 T-cells was 4.0% in the treated group compared with 2.0% in the control group (p=0.013) (Figure 2, bottom left).

In a separate experiment, transferred cells were counted in meninges and brain using immunohistochemistry (IHC). The cell density for transferred T-cells averaged 290.3/mm^2 in the CM virus treated group (n=3) which was significantly higher than the 44.0/mm^2 in the control group (n=5) (p=0.002). This histological experiment demonstrated that most of the transferred T-cells were in the meninges within or near tumor rather than spread randomly in meninges. Few if any transferred T-cells were in the tumor-free brain (Fig. 3).

The next set of experiments determined that following viral infection of CM tumors, transferred cells from cured donors were more likely to enter brain than transferred cells from naïve donors and that maximal entry to brain occurred 4 to 7 days following transfer. Similar preferential accumulation of transferred cells from cured mice was seen in the lungs and spleen but not the mesenteric lymph nodes. Meningeal tumors were established in Thy 1.1 Balb/c mice and treated as above with IV and CM virus and transferred spleen cells from either cured or naïve donor Thy 1.2 Balb/c mice. At various times after cell transfer, host animals were sacrificed and mononuclear inflammatory cells harvested from the entire brain, spleen and right lung. Total cell count was determined and flow cytometry was used

to quantify the percent transferred CD4 and CD8 T-cells. Fig. 4 shows that CD4 T-cells from cured donors accumulated in brain, lung and spleen significantly more than T-cells from naïve donors; CD8 T-cells from cured donors accumulated in brain significantly more than T-cells from naïve donors (n=3 for most time points) ($p=0.04$ for brain CD4 T-cells; $p=0.03$ for brain CD8 T-cells; $p=0.0066$ for lung CD4 T-cells; $p=0.08$ for lung CD8 T-cells; $p=0.02$ for spleen CD4 T-cells; $p=0.05$ for spleen CD8 T-cells). As expected, no relative accumulation was noted in mesenteric lymph nodes (n=2 for almost all time points). Accumulation was apparent at 4 days after cell transfer, peaked at 7 days and was still present at 11 days.

Virus infection of meningeal tumor results in proliferation of anti-tumor memory T-cells in brain, lung and spleen. T-cell replication was assessed by labeling donor Thy 1.2 cells with CFSE, harvesting tissues at various times after cell transfer and using flow cytometry to identify replicated T-cells by diluted fluorescence and positive staining for Thy 1.2. T-cell proliferation was greater in donor cells from cured animals than from naïve animals indicating a rapid response from memory anti-virus and anti-tumor T-cells (n=2-6 for each time point) (Supplementary Fig. S2). Proliferation was maximal 3 to 5 days following cell transfer and followed a similar temporal pattern in brain, lung and spleen suggesting memory T-cell division independently in each of these organs. The brain was the site of the tumor implant and viral infection. The spleen and lung are lymphoid organs that filter antigens released from the brain into the blood.

An interesting corollary of the increased T-cells in the meninges and tumors was a corresponding decrease in the percentage of macrophages. Tumors were implanted CM and 7 days later experimental animals were treated with CM virus. Control animals received no

treatment. Flow cytometry of brain and meningeal mononuclear inflammatory cells 5-6 days after viral administration showed that the percent macrophages were high in the brains and meninges of control animals but decreased markedly in experimental animals treated with virus (p=0.0049) (Fig. 5). Absolute numbers of macrophages were also 2.7 fold higher in control animals than virus treated animals. At the same time, the percentage of T-cells in the brain and meninges increased in virus treated animals (p=0.0001) (Fig. 5).

Successful viral therapy of intraperitoneal tumors generates memory CD8 T-cells that prevent establishment of tumor in the brain meninges. The work reported above was done with transferred memory cells in an established tumor model. We now show that successful viral therapy of peritoneal tumors generated memory T-cells that could prevent establishment of meningeal tumors without the requirement for viral infection. Mice cured of peritoneal D2F2/E2 tumor with viral therapy as reported above were challenged with D2F2/E2 in the cisterna magna. As shown in Figure 6, 5 of 7 animals survived and 2 died at 34 and 39 days after challenge. All control animals died 18 days after challenge. Challenge was then performed in cured animals that were depleted of CD4 T-cells, CD8 T-cells or both. Animals depleted of both CD4 and CD8 T-cells died promptly after challenge. Most animals with CD8 depletion also died promptly. Interestingly, animals with CD4 depletion usually survived indicating that the major memory cell type preventing meningeal neoplastic implantation in this model system was CD8 T-cells. In the absence of CD8 T-cells, CD4 cells could not prevent or treat neoplastic implantation.

Discussion:

The results in this paper support the use of targeted viral infection of tumor to generate anti-tumor memory T-cells to prevent or treat leptomeningeal metastases. Current therapy is ineffective because surgery can rarely remove all metastases safely, radiation therapy has a poor therapeutic index in the brain and drug treatments are inhibited by a robust multilayered and multifunctional blood-brain-barrier to molecules.[1] In contrast, memory T-cells and other immune cells have no difficulty penetrating meninges and brain when attracted by the appropriate inflammatory signals.[10,16] This paper establishes several crucial proofs of principal as follows: 1. Targeted virus infection of tumor can generate therapeutic memory T-cells. In clinical practice this means that a single off-the-shelf reagent, the virus, can be used to treat any patient. Expensive individually-tailored therapy is not required. Following virus infection of tumor, each patient will generate the most potent immune response from their T-cell repertoire. 2. Memory T-cells can prevent establishment of meningeal tumors. The ideal clinical case would be to infect breast tumors before surgical removal and generate anti-tumor memory T-cells which prevent metastases from initially implanting in meninges or brain. The metastatic cells to be blocked could potentially come from subclinical sites in lymph nodes, lymph organs or lung. 3. Memory T-cells can treat established meningeal tumors following viral infection of the tumors. Once again, anti-tumor memory T-cells would be generated by viral infection of the initial breast tumor but in this case subclinical brain and meningeal metastases would already be established. Clinically, surveillance imaging would detect growing lesions and viral infection of the tumor in the central nervous system (CNS) would attract a memory response to the tumor. The results in this paper show that the memory T-cells are capable of finding multiple geographically-separated tumor collections of various sizes and completely eradicating

them. This result was achieved in 2 mouse strains, Balb/c and C57/Bl6, with very different genetic immunologic biases.[17]

There remain several pre-clinical steps to be accomplished before this therapy could be considered clinically. This model system is not fully syngeneic because the implanted mouse cells have been engineered to express the human Her2/neu receptor. The model is instructive because the animals do not generate an effective immune response and untreated tumor growth is progressive and lethal but translation to the clinic could founder on a weaker immune response. We are currently developing a fully syngeneic model system to more conclusively establish the concepts demonstrated in this paper. Although repeat viral infection in the CNS is possible because neutralizing antibodies formed in response to the initial infection cannot penetrate the BBB, we recognize that stimulating an immune response by infecting the brain with a replicating virus, albeit targeted and much attenuated, is not an ideal solution for the clinic. Further work is needed to develop an effective non-replicating pseudovirus or other immune stimulant to attract memory T-cells to the CNS. For maximum clinical utility, the results in this paper must be generalized to parenchymal brain tumors as well as meningeal tumors. We used a meningeal model in these studies to avoid the confounding factor of trauma at the tumor site. In this model, the tumors implant at multiple locations in the meninges far from the injection site. We are developing a model of parenchymal disease by carotid artery injection that will also avoid the problem of local trauma at the tumor site. Finally, toxicity studies will be required for all components of the proposed therapy.

Our results support previous work showing that there is a relative brain barrier to a cellular immune response. [11,18-20] Viral infection of the tumor was able to overcome this

barrier probably by provoking an inflammatory response in the meninges that released chemokines attracting CD4 and CD8 T-cells and also by lysis of tumor cells releasing tumor and viral antigens which could activate memory T-cells in the draining lymphoid organs. Time course studies showed that virus infection produced a 2.6-fold increase in total transferred T-cells in the meninges. The increase in memory T-cells must be much greater because of the diluting effect of the non-specific T-cells. This effect was seen on day 3 following cell transfer at the same time that cell proliferation of transferred memory cells was just beginning indicating that the increase at that time point was due to enhanced trafficking and accumulation and not enhanced local proliferation. Proliferation of memory T-cells was robust between days 3 and 5 after cell transfer and occurred simultaneously in brain, lung and spleen. This suggests that antigen presentation was occurring simultaneously in these 3 organs. Antigen release from the brain and meninges occurs via CSF drainage into nasal lymphatics and into the dural venous sinuses. Lymphatic drainage into cervical lymph nodes was not assessed here but the results in this paper reinforce the view that the spleen and lung harvest brain antigens from the blood and present them effectively to circulating memory T-cellsprins [16,21] This model system did not find evidence that T-cells must first become licensed in the lung before entering the brain.[21] The results also support the view that local antigen presenting cells (APC) in the meninges stimulate memory T-cells that survey the CSF.[9]

Concomitant with the increase of T-cells in tumor and meninges, we noted a decrease in the percent of macrophages. Tumors in many tissues and especially in the brain have a large proportion of tumor-associated macrophages (TAM).[22 23] TAM have an M2 phenotype which supports tumor growth and inhibits immunologic reaction against the tumor by a

wide variety of mechanisms.[24-27] Further work is required to assess whether the therapeutic effect of viral infection in the meninges is in part due to reduction of the effects of suppressor macrophages. The work is complicated by the fact that macrophages in meninges and brain are a combination of yolk-sac derived microglia and bone marrow derived monocytes[28] and current surface markers do not clearly differentiate these populations.

In most transfer experiments, cured donor cells contained a mixture of anti-tumor and anti-virus memory T-cells. We were able to prove that anti-tumor memory T-cells were necessary and sufficient for the therapeutic response in some situations by 2 experiments. In one, donor cells from animals that were infected with virus but never implanted with tumor were not able to effect cure of CM tumor. In the second, donor cells from animals who were cured of IP tumors by aCTLA4 and CPM but no virus cured CM tumor in 2 animals and prolonged life in a third even though the donor animals had never received virus. These results do not exclude an important therapeutic role for the anti-virus memory T-cells in stimulating a strong pro-inflammatory response in the CSF in the D2F2/E2 model and further work is required to differentiate the effects of the anti-tumor and the anti-virus T-cells. We are confident that T-cells are the therapeutic cells in the donor spleen cells because we have shown that memory cells are required for cure, antibody from B-cells will not adequately cross the BBB to cure meningeal tumors, in previous work we showed that cure of IP tumors was achieved by transferring T-cells[14] and in the current work we show that prevention of CM implantation is abrogated by depletion of T-cells.

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Figures and Legends for Figures:

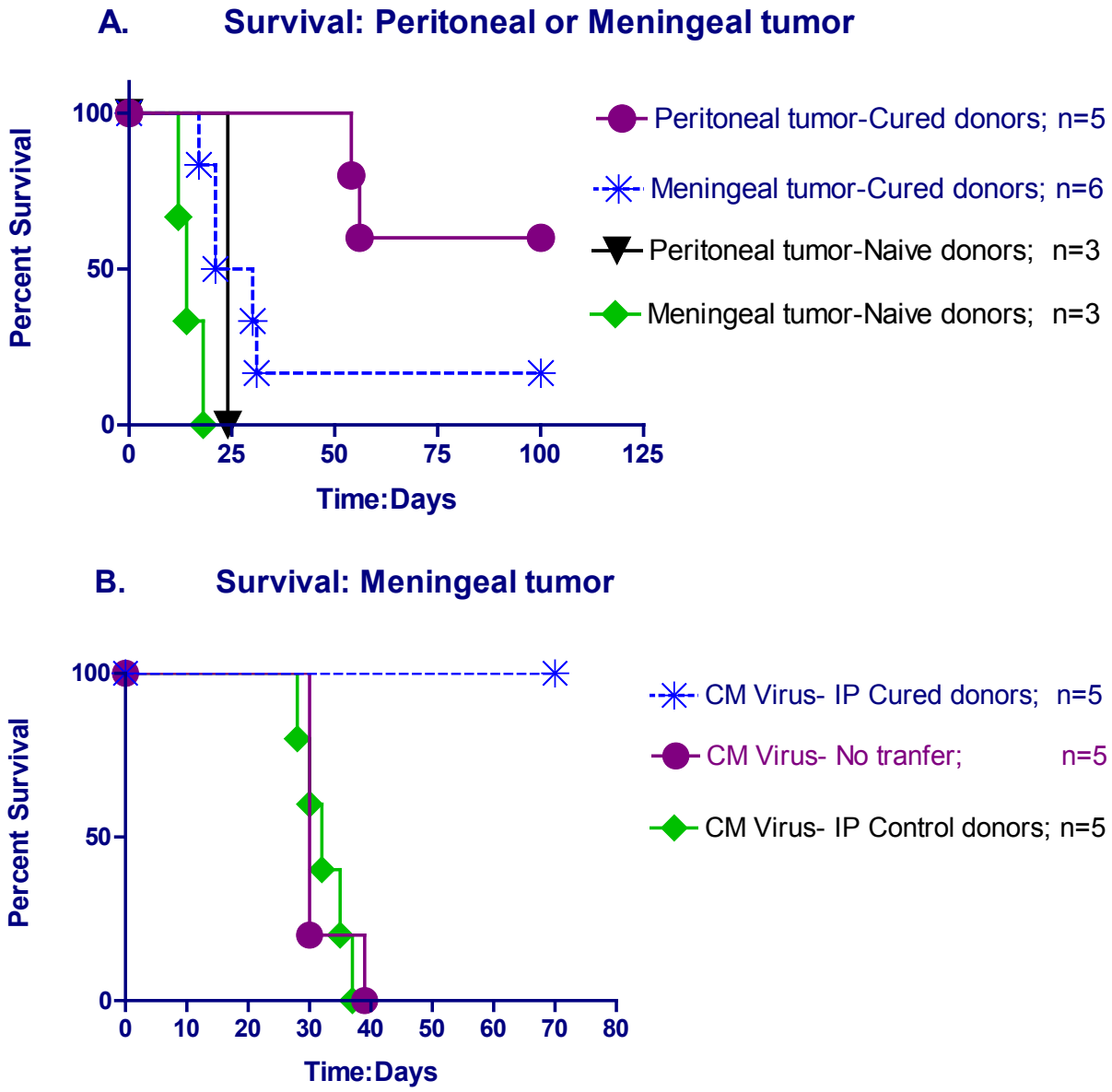


Fig. 1.

A. Survival following treatment of peritoneal or meningeal tumors with cured donors. Mice were implanted with D2F2/E2 tumor cells in the peritoneum or the meninges and treated 3 days later with spleen cells from either cured or naïve donors. Cured

donors significantly increased survival in peritoneal tumors compared with meningeal tumors (p=0.0003, log rank statistic). Naïve donors were not effective in either model.

B. Survival following treatment of meningeal tumors with cured donors combined with direct viral infection of the meningeal tumors. Experimental mice received meningeal virus one day and cured donors 3 days after tumor implantation. Control mice received no donor cells or donor cells from mice treated with virus but not implanted with peritoneal tumor. All animals received cyclophosphamide IP to facilitate cell transfer. N=5 for all groups. Cured donors with viral infection of the meninges significantly increased survival compared with virus infection alone (p-0.0016) or virus infection with control donors (p=0.0018). Cured donors with viral infection of the meningeal tumors significantly increased survival of meningeal tumors compared with cured donors without virus infection (curve in 1A compared with curve in 1B; p=0.0086).

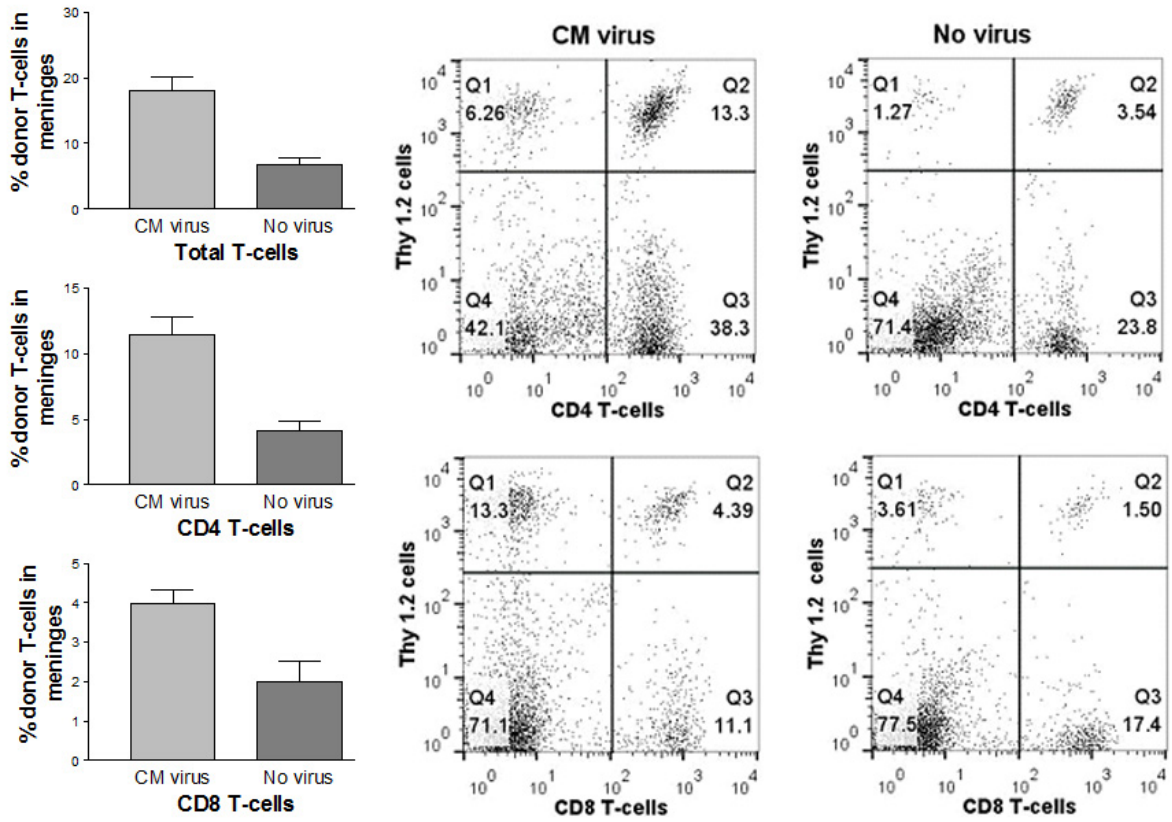


Fig. 2. Accumulation of donor T-cells in meninges following treatment with CM virus. Balb/c Thy 1.1 mice were implanted with CM tumor and 9-11 days later received spleen cells from cured Thy 1.2 donors. CM virus was administered 2 days before cells in the experimental (n=4) but not the control group (n=6). Mononuclear inflammatory cells were harvested from brain and meninges 3 days after cell transfer and flow cytometry used to determine the percentage of donor total T-cells, CD4 and CD8 T-cells (mean with SEM). Data on left shows significantly increased accumulation of total, CD4 and CD8 donor T-cells in CM virus treated animals than controls. Graphs on the right are representative from one experimental and one control animal.

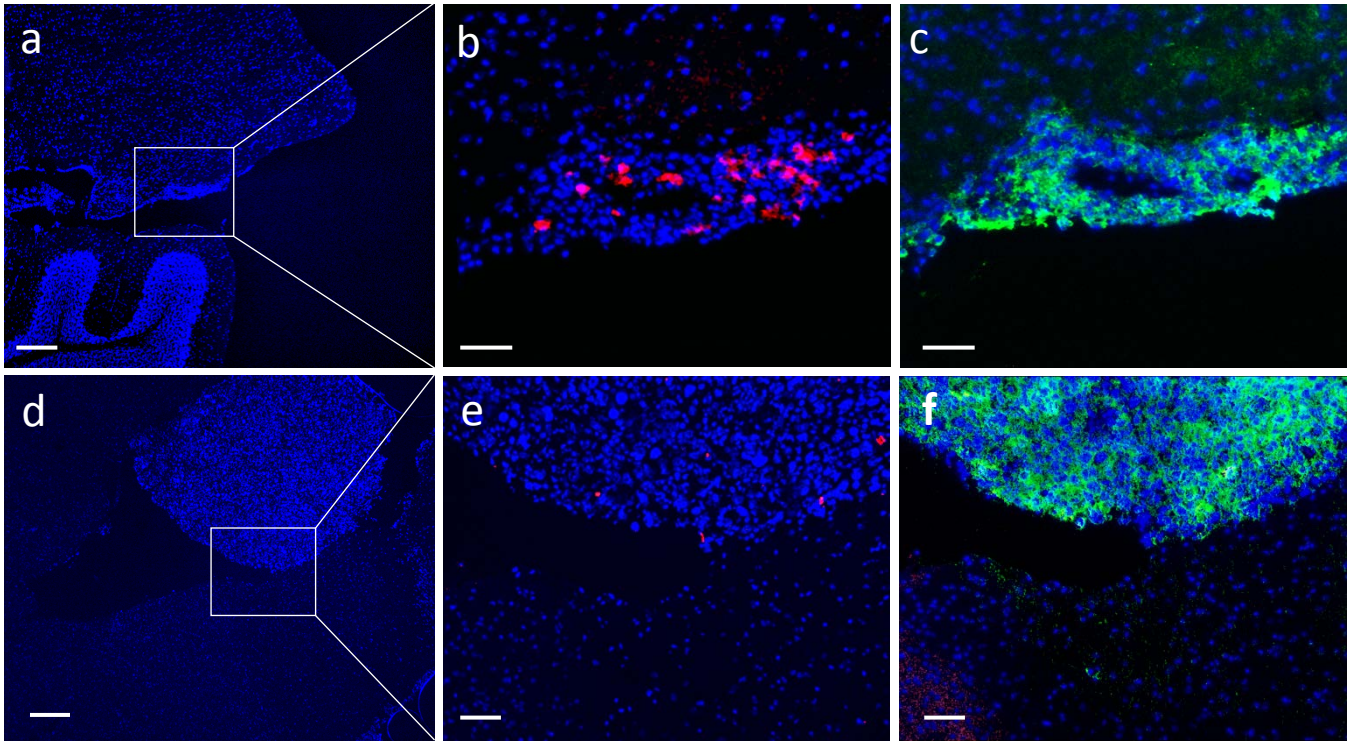


Fig. 3. Highly selective localization of adoptively transferred T cells into virally infected brain metastases. Spleen cells from cured animals were adoptively transferred into animals with meningeal tumors. CM virus was administered 2 days before cells in the experimental (n=3) but not the control group (n=5). Animals were sacrificed 3 days after cell transfer. A significantly higher density of donor T cells was found in the meningeal tumors in animals treated with CM virus compared to control.

a-c: Sections of brain from virus-treated animals. Numerous PE-stained (red) donor T cells were found in most tumors growing in the meninges on the brain surface. Hardly any donor T cells were seen in the adjacent normal brain tissue.

d-f: Sections of brain from control animals. Only a few donor T cells (red) were found in brain tumors from control animals.

Bars in a and d = 200 microns. Bars in b, c, e and f = 50 microns.

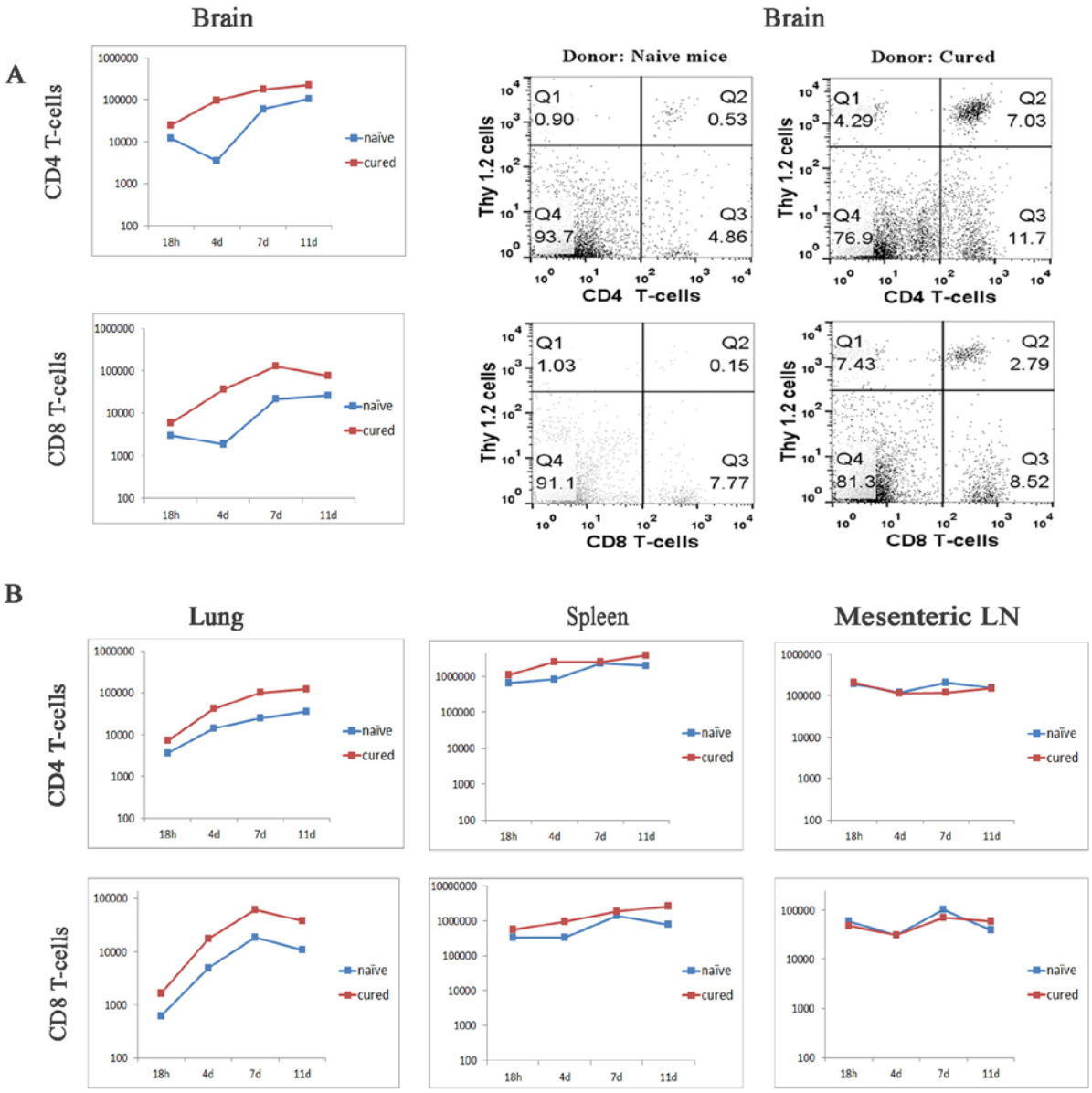


Fig. 4. Time course of accumulation of cured and naïve donor T-cells in brain, lung, spleen and mesenteric lymph nodes. Following CM implants of D2F2/E2 tumor, mice were treated with CM virus and then received either cured donor or cured naïve spleen cells. Animals were sacrificed at indicated times following cell transfer and inflammatory cells harvested from organs and counted. Flow cytometry was used to determine percentage of donor CD4

and CD8 T-cells. (n=3 for almost all time points except mesenteric lymph nodes. This organ had n=2 for all time points except 18h). A one-tailed t-test was used to compare area under the curve (AUC) for cellular accumulation over time in various tissues for experimental and control groups.

C. Mean data from brains of all animals showing significantly increased accumulation of CD4 and CD8 T-cells from cured than naïve donors. Representative graphs from 1 naïve and 1 cured donor are shown on the right.

D. Mean data from lung, spleen and mesenteric lymph nodes showing significantly increased accumulation of CD4 T-cells from cured than naïve donors.

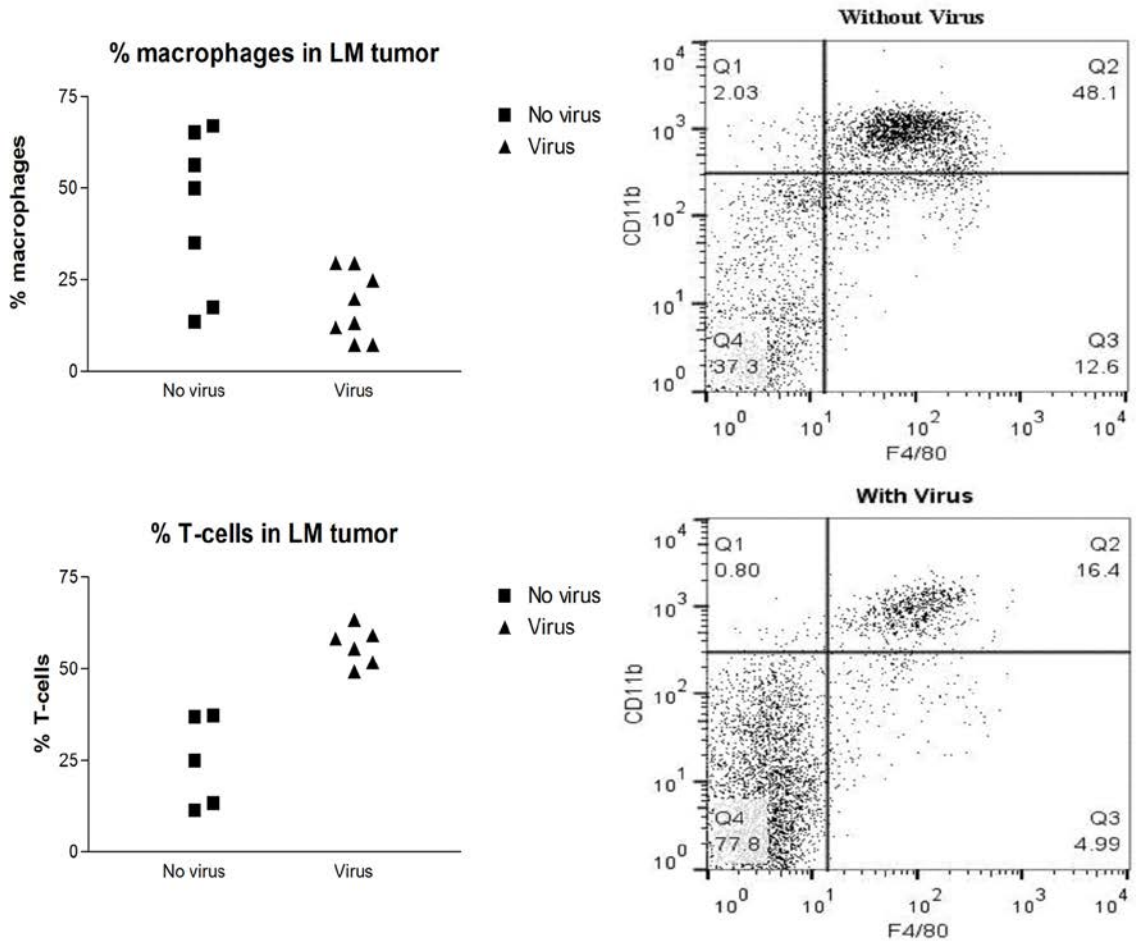


Fig. 5. Effect of CM viral infection on tumor associated meningeal macrophages. Seven days following CM tumor implants with D2F2/E2, experimental animals received CM virus and control animals did not. Brain and meninges were harvested 5-6 days later and analyzed for the presence of macrophages (F4/80 positive) and T-cells (Thy 1.2 or CD3e positive) by flow cytometry. Data on left shows significantly increased T-cells in the animals receiving virus and graphs on right are representative from 2 animals. An unpaired

one-tailed t-test was used to compare percent accumulation of T-cells and macrophages in meninges, with and without virus administration.

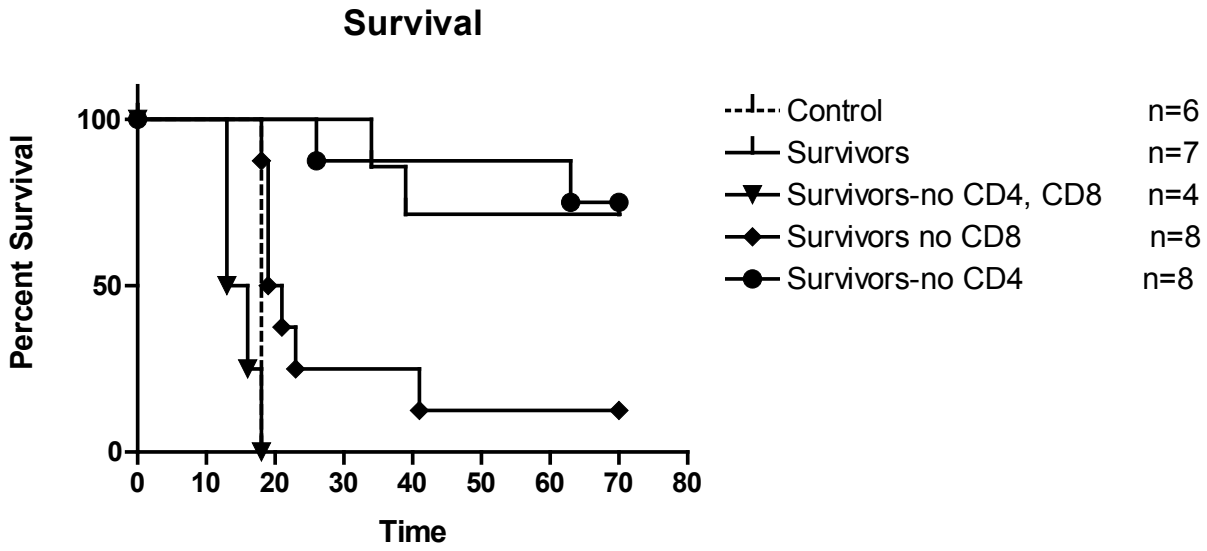
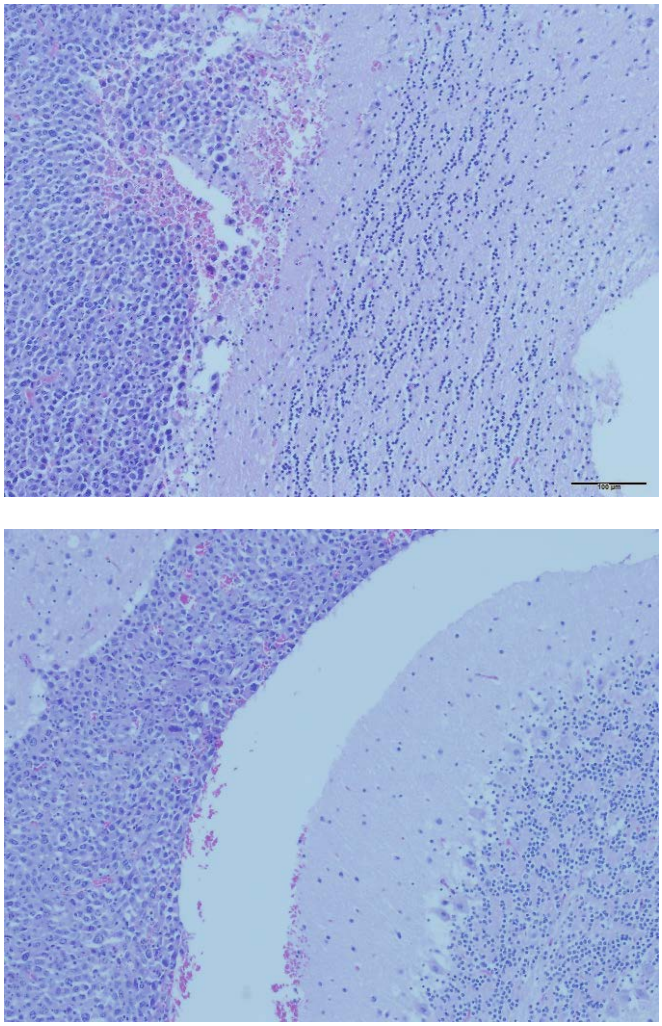


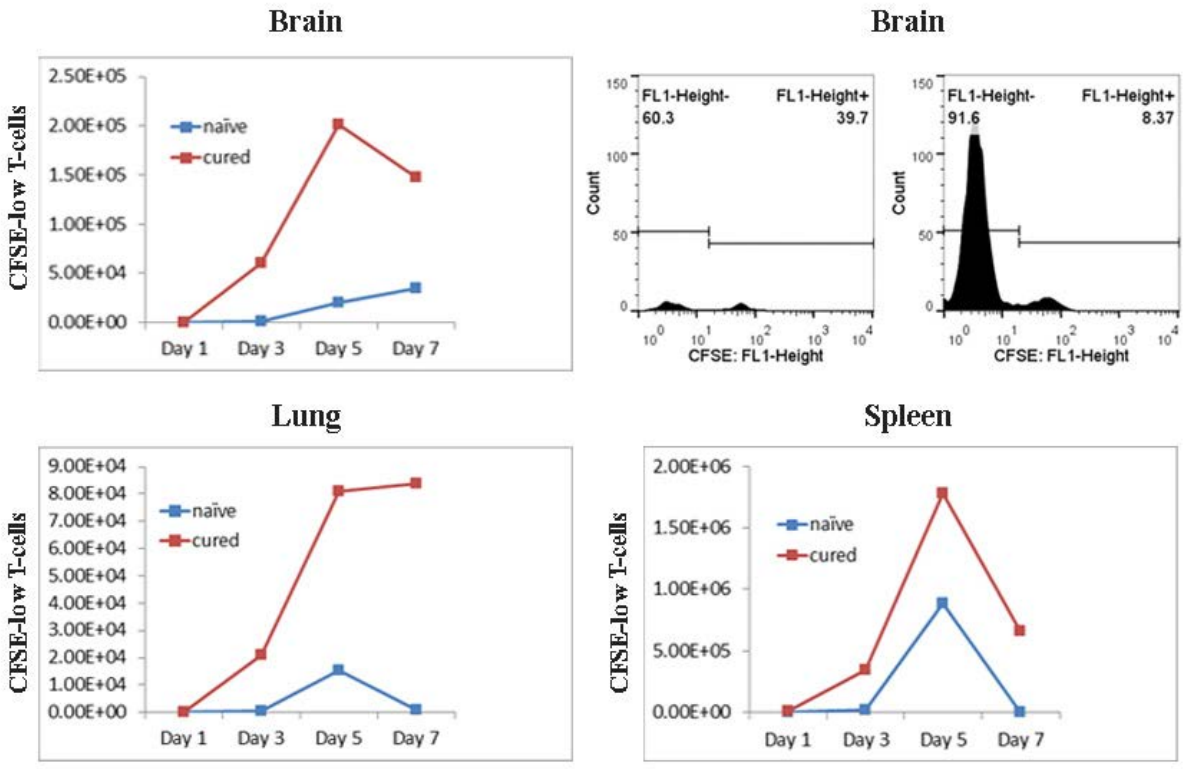
Fig. 6. Survival following CM challenge. Animals cured of IP tumors (survivors) using standard treatment with rrVSV, antiCTLA4 and CPM were challenged with CM tumor, as were controls. Anti-tumor memory CD8 T-cells prevented establishment of meningeal tumors in survivors.



Supplementary Fig. S1. Meningeal tumor. Sagittal sections of the brain in a mouse sacrificed 13 days after CM implantation of D2F2/E2 tumor cells (H&E stain, x10 objective; marker is 100 μM).

A. Meningeal tumor adjacent to olfactory lobe and beginning to invade parenchyma.

B. Tumor in the roof of the 4th ventricle adjacent to the cerebellum.



Supplementary Fig. S2. Proliferation of transferred T cells from cured and naïve donor in tumor-bearing hosts. Meningeal tumors were established in Thy 1.1 Balb/c mice and treated with CM virus 7 days later. CFSE labeled spleen cells were transferred from either naïve mice or cured Thy 1.2 Balb/c mice 2 days after treatment with virus. Host animals were treated in matched pairs on the same day and always received the same number of cells (3.45×10^7 to 8.2×10^7 cells in experiments on different days). At various times after cell

transfer, host animals were sacrificed and inflammatory cells harvested from the entire brain, spleen and lungs. Flow cytometry was used to quantify CFSE signal in the Thy 1.2 gate. The total number of CFSE low donor T-cells are shown on the y-axis. (n=2-6 for each time point).

C. Mean data from brains of all animals. Representative graphs from 1 naïve and 1 cured donor are shown on the right.

D. Mean data from lung and spleen.

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NK Cells in the Tumor Microenvironment

Stine K. Larsen[1,2], Yanhua Gao[3], & Per H. Basse[1,4,*]

[1]University of Pittsburgh Cancer Institute, University of Pittsburgh, Pittsburgh PA 15213, USA; [2]Department of Hematology, 54P4, Copenhagen University Hospital, Herlev, Denmark (current address); [3]Department of Pediatrics, University of Pittsburgh, Pittsburgh, PA 15224, USA; [4]Department of Immunology, University of Pittsburgh, Pittsburgh PA 15213, USA

***Address all correspondence to**: Per H Basse, University of Pittsburgh Cancer Institute, The Hillman Cancer Center, Research Pavilion G.17a, 5117 Centre Avenue, Pittsburgh PA, 15213; Tel.: 412 623 3236; Fax: 412 623 1119; Email: basse@imap.pitt.edu

ABSTRACT: The presence of natural killer (NK) cells in the tumor microenvironment correlates with outcome in a variety of cancers. However, the role of intratumoral NK cells is unclear. Preclinical studies have shown that, while NK cells efficiently kill circulating tumor cells of almost any origin, they seem to have very little effect against the same type of tumor cells when these have extravasated. The ability to kill extravasated tumor cells is, however, is dependent of the level of activation of the NK cells, as more recent published and unpublished studies, discussed below, have demonstrated that interleukin-2–activated NK cells are able to attack well-established solid tumors.

KEY WORDS: Natural killer cells; infiltration; tumor microenvironment; prognosis; IL-2; virus; anti-tumor effect.

ABBREVIATIONS: A-NK: activated natural killer cells; **Bid:** twice per day; **CAR:** chimeric antigen receptor; **CFSE:** carboxyfluorescein succinimidyl ester; **CM:** cisterna magna; **DAMP:** damage-associated molecular pattern; **E:T:** effector-to-target ratio; **Gy:** gray; i.p.: intraperitoneal; **i.v.:** intravenous; **IL:** interleukin; **IU:** international unit; **MHC:** major histocompatibility complex; **PAMP:** pathogen-associated molecular pattern; **Peg-IL-2:** Pegylated interleukin-2; **Poly I:C:** polyinosinic:polycytidylic acid; **Th1:** T-helper-1; **TLR:** Toll-like receptor; **VSV:** vesicular stomatitis virus

I. INTRODUCTION

The discovery of natural killer (NK) cells and naturally occurring cytotoxicity against tumor cells[1,2] immediately generated high expectations for the efficacy of immunotherapies based on these effector cells. An early finding in animal models indicated that NK cells participate in the defense against metastases. It was demonstrated that tumor cell clearance in the lungs, in large part, relied on NK cells and that the rate of the NK-cell–mediated clearance of tumor cells in the lungs inversely correlated with the number of experimental metastases that would develop in the lungs and most other organs following intravenous (i.v.) injection of tumor-cell suspensions.[3–6] Because the portal vein drains venous blood from the gut, pancreas, and spleen, it would seem beneficial if NK cells were present also in the liver to protect it from tumor cells that are released to the portal circulation. In fact, an inverse correlation between the rate of tumor cell clearance from the liver and the number of liver metastases has been demonstrated,[7,8] but more studies are needed to determine whether a similar inverse correlation between rate of clearance and number of metastases exists in other organs. Whether or not this turns out to be the case, an efficient NK-cell–mediated clearance of tumor cells in the lungs, through which all venous blood passes, is a logical way of protecting the host from metastasis. As early as 1990, Vaquer et al. found that a decrease in NK activity present in the blood from women with uterine cancer coincided with dissemination of the primary tumor.[9]

Numerous studies subsequently demonstrated that treatment of mice with NK activators, for example, Poly I:C (a TLR-3 agonist), substantially improved the mice's resistance to metastases.[10–16] However, because tumor cells may be shed and become blood-borne at any time, it is not simple, from a clinical point of view, to take advantage of this observation. To this end, the benefit of boost-

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ing the NK activity of cancer patients at least in those periods where a heightened number of circulating tumors cells or a reduction of the patient's NK activity can be expected (e.g., during surgical removal of tumors) has long been suggested[17–27] but has not yet been confirmed in clinical studies. However, more than a decade ago it was shown that preoperative NK-cell activity is a prognostic factor for distant metastasis following surgery for colon cancer.[28]

Disappointingly, most attempts to eliminate already established tumors and metastases by augmentation of host NK cell activity failed.[29–31] Due to these disappointments, it has long been the paradigm that, while NK cells are important as a first line of defense against metastasis from circulating tumor cells, they do not play any major role in the defense against tumor cells that reside in the tissues, i.e., outside the blood vascular system.

In this mini-review, we discuss the conundrum that, although NK cells efficiently kills circulating tumor cells of almost any origin, they seem to have very little effect against the same type of tumor cells when these have extravasated. We will also discuss newer published and unpublished findings that challenge the current paradigm that NK cells are incapable of attacking already established solid tumors.

II. THE DENSITY OF NK CELLS IN SOLID TUMORS CORRELATES WITH PROGNOSIS

The role of NK cells in cancer may not, as previously thought, be restricted to only blood-borne tumor cells; newer findings implicate NK cells in the control of extravascular tumor growth as well, e.g., in the early phases of oncogenesis. In a study by Guerra et al., tumors developed faster in models of spontaneous leukemia and prostate cancer in animals depleted of NK cells compared to animals with normal NK cells activity.[32] Although the presence of NK cells in the pre- and/or early malignant lesions was not investigated, fully developed tumors from NK-cell–deficient mice expressed ligands for the activating NK receptor NKG2D, whereas tumors from NK-competent mice did not express these ligands. This finding indicates that tumors developing in these animals (a process which, at least in the pancreas model, does not involve blood-borne tumor) had been edited by NK cells.

The prognostic significance of NK cells in patients with fully established colorectal carcinomas was first demonstrated by Coca et al.[33] Patients with little and moderate NK infiltration had significantly shorter survival rates (overall, and disease free survival) than those with extensive infiltration. In addition, the density of tumor-infiltrating NK cells appeared to be a prognostic factor in the survival of patients with squamous cell lung cancer.[34] However, in a recent study of 20 male patients who underwent surgery to remove a single cerebral metastasis from lung adenocarcinoma, there was no correlation between the degree of NK-cell infiltration within resected brain metastases and the period free of intracranial disease after surgery.[35] Nevertheless, the list of cancer types in which a correlation between intratumoral NK-cell density and prognosis has been found, is steadily growing (Table 1). These and similar studies show that NK cells indeed are able to not only localize into extravascular, solid tumors but that they also add functionally to the host–tumor relationship. While it seems very likely that host NK cells continue to kill pre- and/or early-malignant cells in small developing tumors until the tumor is either eliminated or until it has been edited to a point where it no longer expresses sufficient NK ligands to elicit NK-cell–mediated cytotoxicity, the causality between NK-cell density in larger tumors and prognosis remains an open question. Based on the available information, we have tried to estimate the densities of intratumoral NK cells, which, in the studies listed in Table 1, have been associated with favorable prognoses. In several studies, 30 or more NK cells mm^{-2} of tumor tissue was arbitrarily considered a high density. Assuming that the volume fraction of the malignant cells in most tumors is at least 25%, a minimum of 1,000 tumor cells will be visible per square millimeter of tumor in sections of standard thickness (5–8 μm). Thus, the estimated effector-to-target (E:T) ratio in high-NK-density tumor tissue is, at best, 1:35. It is doubtful whether

TABLE 1: Correlation between intratumoral NK-cell density and prognosis in various cancer types

Cancer type	Reference		
Colorectal carcinoma	Coca et al.	1997	33
Hepatocellular carcinoma	Taketomi et al.	1998	70
Gastric carcinoma	Ishigami et al.	2000	71
Adenocarcinoma lung	Takanami et al.	2001	72
Gastric carcinoma	Takeuchi et al.	2001	73
Leukemia	Lowdell et al.	2002	74
Squamous cell, lung	Villegas et al.	2002	34
Renal cancer	Cózar et al.	2005	75, 76
Squamous cell, esophagus	Hsia et al.	2005	77
Squamous cell, vulva	Sznurkowski et al.	2013	78

cytotoxicity or cytokine-secretion by NK cells present at such low densities has any significant impact on tumor growth. It is possible that the presence of a high number of NK cells in tumors indicates that the patient's NK cells are in a good shape overall and that they therefore are able to contribute to the host's anti-tumor immune responses, either by eliminating circulating tumor cells or by secreting the appropriate, perhaps Th1-stimulating, cytokines during cross talk with dendritic cells in the periphery and/or in the secondary lymphoid tissues.

III. ACTIVATED NK CELLS LOCALIZE EFFICIENTLY AT TUMOR SITES

The densities of NK cells found in well-established tumors in most animal tumor models are also very low. NK-cell density can be increased somewhat by treatment with TLR agonists[8,36,37] or with pro–NK-cell cytokines, particularly IL-2.[38,39] This may be a result of improved extravasation or retention, survival, or proliferation of NK cells at the tumor sites (or of course, a combination of all). In contrast, very impressive NK-cell densities are seen in tumors after adoptive transfer of *ex vivo* IL-2–activated NK (A-NK) cells (Fig. 1).[40–45] The density of NK cells reaches >500 cells mm^{-2} tumor tissue at 24 h after intravenous injection of $10{\times}10^6$ A-NK cells and increases to approximately 2,000 cells mm^{-2} by day 5.[46] In this period of time, the density of A-NK cells in the tumor tissue is, on average, 20-fold higher than the density of A-NK cells in the surrounding normal lung tissue. Using the same assumption as above, this translates into E:T ratios from 1:4 to better than 1:1. The highest A-NK-cell densities are found in lung tumors, but significantly higher densities of A-NK cells in tumors compared to the surrounding normal tissues have been observed in liver, adrenal glands, spleen, bone marrow, brain, and ovary (Fig. 1).[8] Interestingly, A-NK cells injected into the peritoneal cavity efficiently infiltrate tumors growing in the cavity; however, they seem to have some difficulty leaving the peritoneal cavity because lung tumors from animals receiving A-NK cells by the intraperitoneal (i.p.) route contain very few of the adoptively transferred cells at any time.[47]

To what extent these high intratumoral densities of A-NK cells are generated by a constant influx of A-NK cells or by proliferation of a few A-NK cells reaching the tumors (or both) is not fully elucidated. It is clear that proliferation of the A-NK cells, either in the tumor tissue or other places, is of major importance, because less than 250 A-NK cells mm^{-2} tumor tissue is found at 3 days after injection of irradiated (4 Gy) A-NK cells

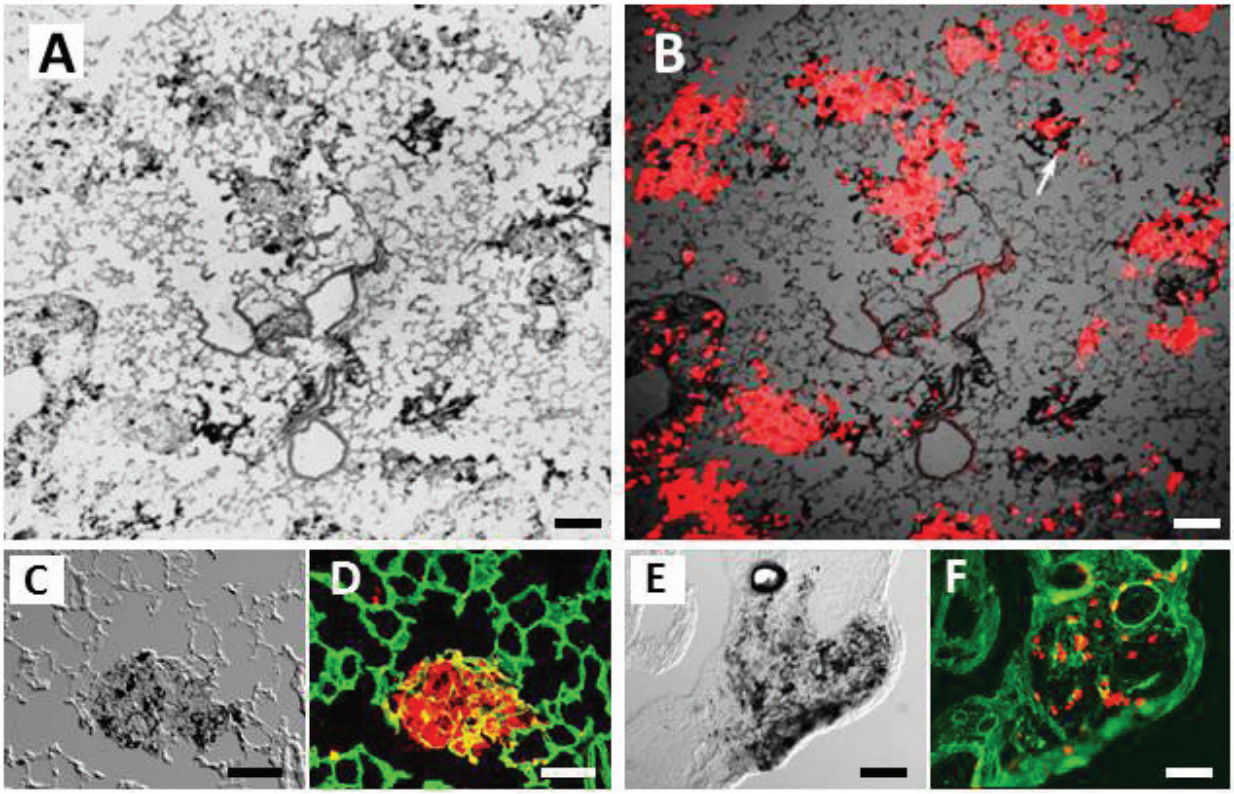

FIG. 1: Accumulation of IL-2–activated NK (A-NK) cells selectively at tumor sites. Flow-sorted NKp46+ splenocytes from congenic Thy1.1+ C57BL/6 mice were cultured with IL-2 for 5 days and injected i.v. into C57BL/6 mice (Thy1.2+) with 9-day-old B16 tumors. Each mouse received 5 million A-NK cells. 30,000 IU Peg–IL-2 was injected i.p. every 12 h (max. six injections). Organs were removed at 72 h after injection of the A-NK cells and fresh frozen. Eight micron cryosections were all stained with PE-conjugated anti-Thy1.1 antibodies (NK cells begin to express Thy1 within 24 h of IL-2 activation). Some sections were also stained with FITC-conjugated anti-laminin antibodies. (A) DIC picture of lung tissue with multiple black-pigmented B16 melanoma metastases. (B) Fluorescent photomicrograph of the same sections as in (A), showing a dense accumulation of PE-Thy1.1+ A-NK cells (red dots) selectively in the black-pigmented metastases. White arrow points to a single PE-Thy1.1+ A-NK cell. (C) and (D) same as (A) and (B), respectively, but at higher magnification and with staining of laminin (green fluorescence in (D)). Note the strong preference of the A-NK cells for the tumor tissue. (E) and (F) show a DIC and a fluorescent picture, respectively, of laminin-stained ovarian tissue (green in (F)) with a black-pigmented B16 metastasis infiltrated by PE-Thy1.1+ A-NK cells. Bars in A–B = 200 μm, Bars in C–F = 100 μm.

(Fig. 2). Furthermore, at 3 days after injection of non-irradiated, CFSE-labeled A-NK cells, hardly any of the A-NK cells contained enough CFSE for identification by fluorescence microscopy, indicating that the A-NK cells indeed continued to proliferate *in vivo*. The importance of cytokine-stimulation to maintain NK-cell proliferation is discussed later in this article.

IV. TUMOR-INFILTRATING A-NK CELLS HAVE ANTI-TUMOR ACTIVITY

Experimental lung metastases in most murine models are quite heterogeneous with respect to a variety of factors. This includes permissiveness to A-NK-cell infiltration.[45] Thus, when comparing the fate of well-infiltrated lung tumors to that of poorly infiltrated lung

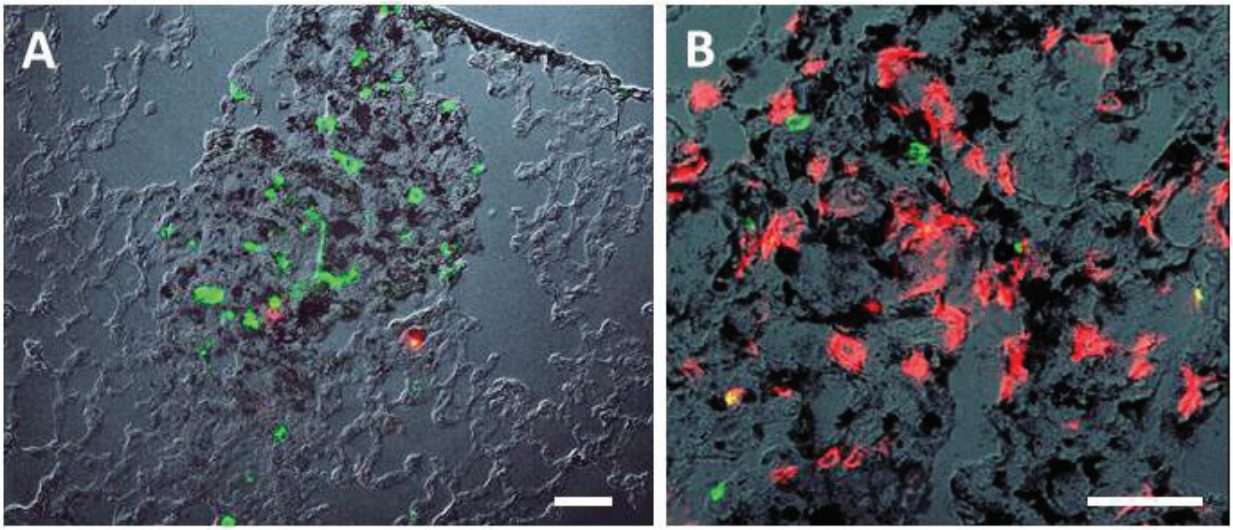

FIG. 2: In vivo proliferation increases the number of transferred A-NK cells found at tumor sites. Flow-sorted NKp46+ splenocytes from congenic Thy1.1+,CD45.2+ and congenic Thy1.2+, CD45.1+ C57BL/6 mice were cultured with IL-2 for 5 days and injected i.v. into C57BL/6 mice (Thy1.2+,CD45.2) with 9-day-old B16 tumors. Before injection either the Thy1.1+ or the CD45.1+ A-NK cells were irradiated (450 rad). Each mouse received a mixture of 2.5 million Thy1.1+ and 2.5 million CD45.1+ A-NK cells. 30,000 IU Peg–IL-2 was injected i.p. every 12 h (max six injections). Organs were removed at 72 h after injection of the A-NK cells and were fresh frozen. (A) B16 lung metastasis from an animal receiving non-treated Thy1.1+ and irradiated CD45.1+ A-NK cells. While many Thy1.1+ A-NK cells (stained with FITC-anti-Thy1.1 antibodies) are infiltrating the tumor, only few of the irradiated CD45.1+ A-NK cells (stained with PE-anti-CD45.1 antibodies) can be observed. (B) Close-up of a B16 lung metastasis from an animal injected with irradiated Thy1.1+ and non-treated CD45.1+ A-NK cells. While few of the irradiated Thy1.1+ A-NK cells (stained with FITC-anti-Thy1.1 antibodies) are infiltrating the tumor, many non-irradiated CD45.1+ A-NK cells (stained with PE-anti-CD45.1 antibodies) can be observed throughout the tumor nodule. Bars = 50 μm.

tumors following adoptive transfer of A-NK cells by the intravenous (i.v.) route, it became clear that significant size reductions occurred only among the well-infiltrated tumors (Fig. 3).[47] Furthermore, when the A-NK cells were injected using the i.p. route (hindering the A-NK cells in reaching any tumors except those growing in the i.p. cavity), a significant reduction of tumors in the i.p. cavity, but not of tumors in the lungs or any other organ, was observed. Thus, the ability of the A-NK cells to localize at tumor sites is not only impressive, but it is also a prerequisite for anti-tumor effect.

V. IN VIVO FUNCTION OF A-NK CELLS IS HIGHLY DEPENDENT ON CYTOKINE SUPPORT

Once activated with IL-2 or IL-15, *in vitro*–cultured A-NK cells become dependent on stimulation by these cytokines with respect to function, proliferation, and survival. Stimulation by just one of these cytokines is sufficient, regardless of which of them initially activated the NK cell. However, within just a few hours of deprivation of these cytokines, the proliferation of the A-NK cells slows down,[46] and within less than 24 h, most of the A-NK cells have or will begin to undergo apoptosis. *In vivo*, lack of cytokine-support is evident by poor tumor-localization, loss of anti-tumor function and rapid disappearance of the A-NK cells from the recipient. Thus, in all of the studies mentioned above, substantial amounts of exogenous IL-2 were given to maintain the functionality of the A-NK cells, both with respect to tumor homing and anti-tumor effect. Due to the short plasma half-life of IL-2 (5–10 minutes), it is difficult, especially in animal models, to maintain the necessary high plasma levels of IL-2

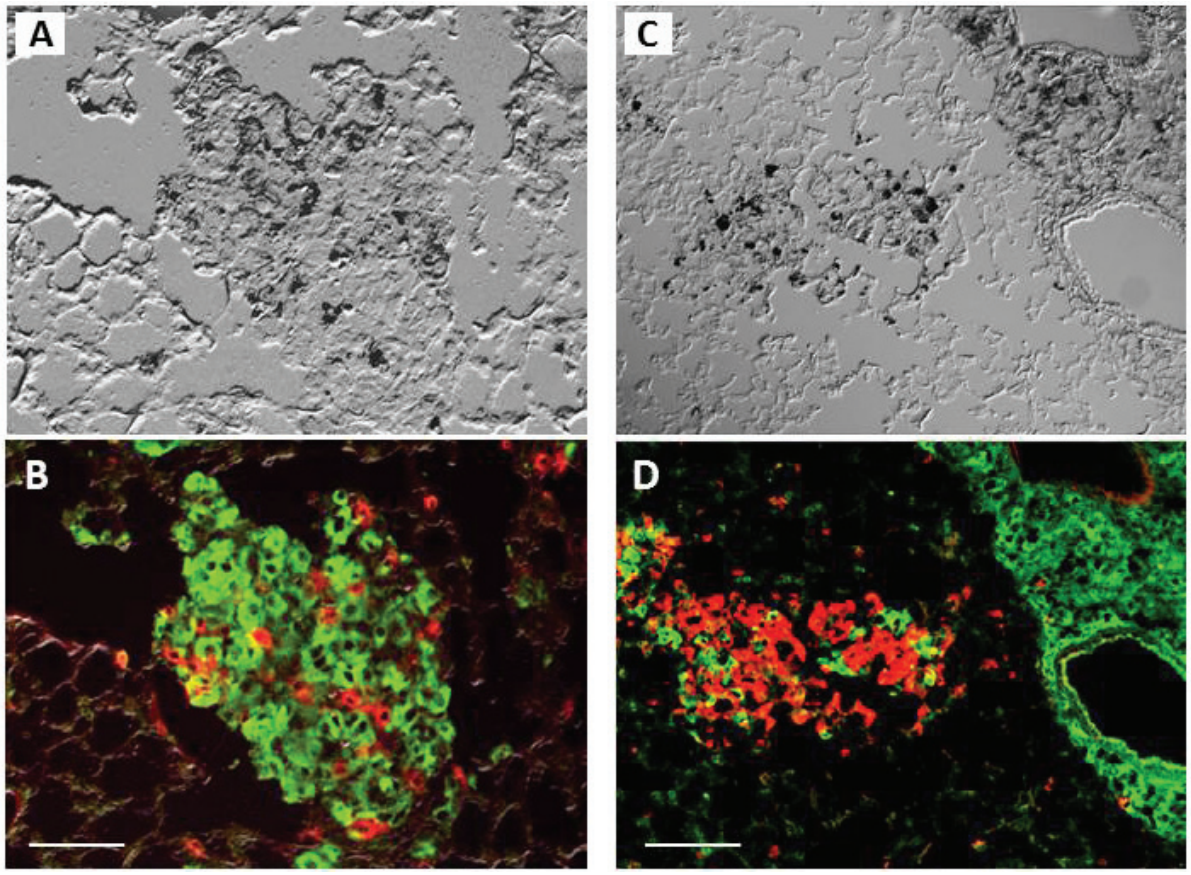

FIG. 3: Tumor-infiltrating A-NK cells eliminate tumor cells. Five million Thy1.1+ A-NK cells were injected into Thy1.2+ C57BL/6 mice bearing 9-day-old B16 melanoma lung metastases. 30,000 IU Peg–IL-2 was injected i.p. every 12 h (max. six injections). Organs were removed at 16 and 120 h after injection of the A-NK cells and were fresh frozen. (A) DIC picture of a B16 lung tumor from an animal injected with A-NK cells 16 h earlier. (B) Fluorescence picture of the tumor shown in (A) after staining with a 1:1 mixture of polyclonal rabbit anti-Tyrp114,15 and Pmel1716,17 antibodies (kindly provided by Dr. V. Hearing, NIH) and subsequently with FITC-conjugated anti-rabbit antibody. To reveal A-NK cells, the section was also stained with PE–anti-Thy1.1 antibodies. At this point in time, relatively few A-NK cells are found in the tumor, which is composed of B16 tumor cells (shoulder-to-shoulder). (C) DIC picture of B16 lung tumors from an animal injected with A-NK cells 120 h earlier. (D) Same area as in (C). Note that, while the tumor on the left is heavily infiltrated by A-NK cells (PE-Thy1.1+), which have almost replaced the tumor cells (FITCE+), the tumor on the right contains only few infiltrating A-NK cells but many tumor cells. Bars = 100 μm.

by bolus injections of this cytokine. This problem can be solved by the use of pegylated IL-2 (Peg–IL-2[48–51]) which, due to its greatly improved half-life (4–6 h[48]), needs to be injected just twice daily (approximately 30,000 IU bid,[47]). Unfortunately, the toxic side effects of Peg–IL-2 are enhanced in parallel with its beneficial effects on A-NK-cell homing and anti-tumor effect and the substantial toxicity of high-dose IL-2 treatment, especially the vascular leak syndrome, are well known.[52–55] These side effects have greatly reduced enthusiasm for clinical usage of Peg–IL-2. The toxicity of Peg–IL-2 has, also in animal models, interfered with measurement of the anti-tumor effect of adoptive A-NK cell treatment in terms of improved survival, because 3 days of Peg-IL-2 treatment can be fatal for animals with high

lung-tumor burdens. To circumvent this problem, NK cells have been modulated *ex vivo*, with vectors carrying genes for cytokines, which could enable the NK cells to produce their own IL-2[56] or to produce cytokines capable of synergizing with IL-2 in supporting the A-NK cells. One such cytokine is IL-12. A-NK cells pretreated with IL-12 or A-NK cells capable of IL-12–autostimulation via transgene IL-12 production need only 1/10–1/100 of the amount of IL-2 needed to maintain the same viability and functionality as A-NK cells that have not been stimulated with IL-12.[57] This is likely due to the IL-12–induced expression of the IL-2 receptor alpha chain by the A-NK cells, enabling them to express the complete IL-2α-β-γ, high-affinity IL-2 receptor. Thus, by adoptive transfer of IL-12 transduced A-NK cells supported by just two injections of Peg–IL-2 (each of 3×10^4 IU) given on the same day as the A-NK cells, a significant prolongation of survival was observed in both 3-day and well-established 7-day models of B16 and MCA205 lung metastases.[57,58] Although the A-NK-cell–produced IL-12 undoubtedly supported anti-tumor responses in addition to those generated by non–IL-12–producing A-NK cells, tumor homing by A-NK cells remained a very important and critical factor for the anti-tumor effect achieved by the IL-12 gene-transduced A-NK cells.[58]

VI. SURVIVAL OF A-NK CELLS IN VIVO

It is clear that both tumor homing and *in vivo* anti-tumor activity of A-NK cells are dependent on the continuous availability of IL-2 or IL-15, but it is less clear exactly which function(s) these cytokines support and which is most important. Possibly, they are causing changes not only in the NK cells but also in the tumor environment that are critical for the ability of the A-NK cells to sense the presence of the tumor cells, to extravasate, and to lyse the malignant cells. The answer may, however, be related to a more fundamental function, namely survival of the A-NK cells. It has long been known that lymph node–produced IL-15 is important for homeostasis of NK cells, i.e., if the NK cells are not frequently stimulated by IL-15, they rapidly die.[59] Although a variety of cell types can produce IL-15 and present it in trans (which may be the most effective way of presenting IL-15 to NK cells[60,61]), it is likely that the amounts of IL-2 or IL-15 necessary to keep NK and A-NK cells alive are never being produced in tumors, since these are characterized by chronic inflammation (i.e., DAMPs rather than PAMPs) and expression NK cell-suppressive cytokines. Thus, within hours of arriving at a tumor site, the NK cells must leave again to find a source of IL-2 or IL-15 (e.g., the lymphoid tissues) or, maybe more likely, they rapidly die at the tumor site, many of them before they have had a chance to kill more than a few (if any) tumor cells. This hypothesis is supported by experiments showing that A-NK cells, transferred into tumor-bearing animals without any support by exogenous IL-2, are found at much higher densities in tumors gene-transduced to produce small amounts of IL-2 than in mock-transduced tumors.[62] Likewise, adoptively transferred A-NK cells gene-transduced to produce just enough IL-2 to support their own survival in an intracrine fashion, i.e., with no detectable secretion of IL-2, were found in much higher numbers in tumors than mock-transduced A-NK cells (Fig. 4). Thus, it appears that, as long as the survival of the A-NK cells is ensured, they are able to traffic to and persist at tumor sites.

VII. NK-CELL HOMING TO SITES OF INFECTION

Under steady conditions, the survival of NK cells seems to be maintained by lymphoid tissue–produced IL-15.[59] However, as NK cells are believed to function as a first line of defense against especially intracellular infections, it would seem logical that, if cytokines are available at the site of infection, they are able to not only attract NK cells but also activate them and keep them alive. To test this, we established brain tumors in mice by injecting Her2-expressing D2F2/E2 mammary carcinoma cells into the cisterna magna (CM). Nine days later, when multiple tumors had formed in the brain parenchyma and the lepto-

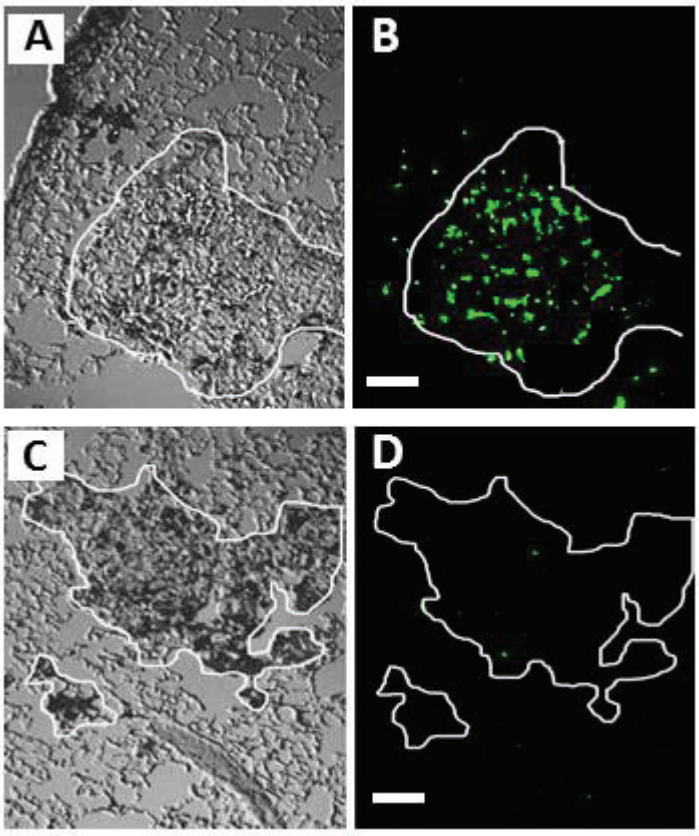

FIG. 4: Tumor-infiltrating A-NK cells depend on IL-2 for survival. GFP+ and GFP+,IL-2+ A-NK cells were produced by stable transduction of Mau-1 cells (a long-term A-NK-cell line (C57BL/6) developed in our lab) with adeno-associated virus containing the gene for GFP or the genes for both GFP and IL-2. (A) DIC picture of a B16 lung tumor from an animal injected i.v. with 5 million IL-2- and GFP-producing Mau-1 cells 72 h earlier. (B) Fluorescence picture of the tumor shown in (A) showing numerous GFP+ cells infiltrating the tumor. (C) DIC picture of a B16 lung tumor from an animal injected i.v. with 5 million GFP-producing Mau-1 cells 72 h earlier. (D) Fluorescence picture of the tumor shown in (A) showing very few GFP+ cells infiltrating the tumor. No exogenous IL-2 was given to support the injected Mau-1 A-NK cells. Bars = 100 μm.

meninges, targeted recombinant VSV with tropism only for Her2-expressing cells,[63–66] were injected into the CM. Three days later, the brains were removed, and sections of brain tissue with tumor were stained with NKp46 antibody to reveal host NK cells. As shown in Fig. 5, an unprecedented high number of NK cells were found in the tumor tissue from animals receiving the targeted recombinant VSV compared to controls (which were not given the VSV). Furthermore, infiltration by the host NK cells was strictly confined to tumor tissue. Studies are ongoing to determine the extent to which the tumor-infiltrating NK cells are activated compared to NK cells found in the periphery and whether the high density of NK cells in the infected tumor tissue is caused by better survival (and possibly better proliferation as well) of NK cells arriving at this site or whether it is caused by an infection-induced influx of NK cells fast enough to outpace the loss due to intratumoral death of the NK cells. These studies show that, compared to the microenvironment of tumors, the milieu of infectious foci appear to provide the right conditions for the generation of high-density NK cell infiltrates.

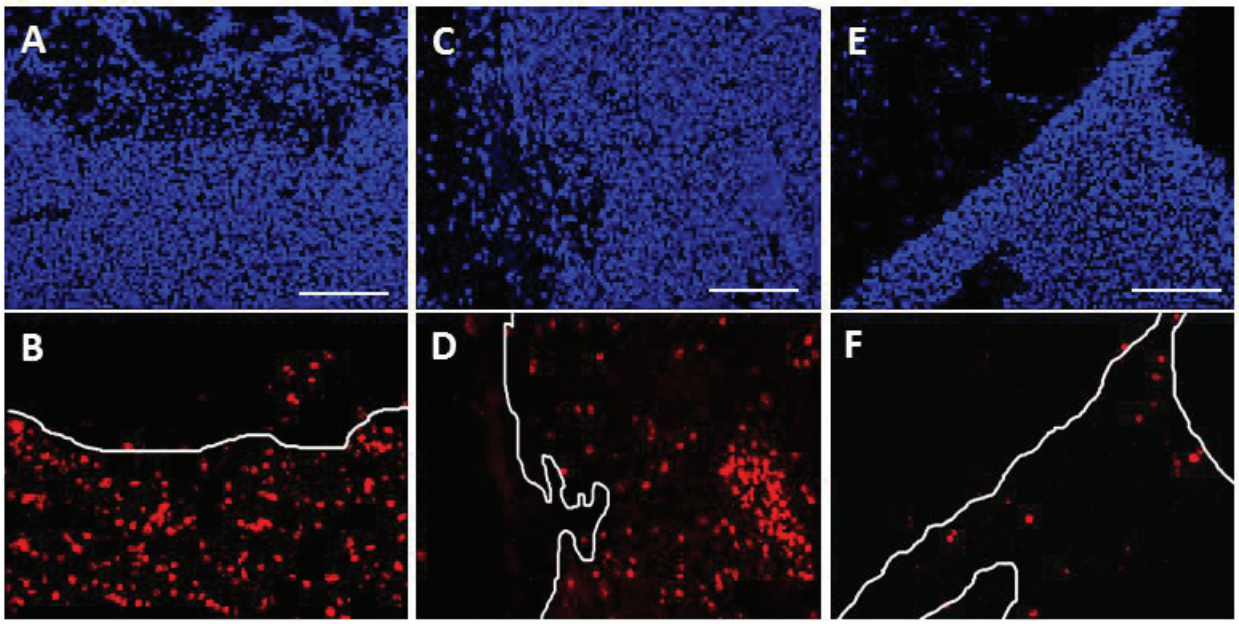

FIG. 5: Viral infection induces a vigorous tumor infiltration by host NK cells. Mammary brain and leptomeningeal metastases were induced by injection of Her2neu expressing D2F2/E2 cells into the cisterna magna (CM) of Balb/c mice. Nine days later, Her2-targeting VSV were injected into the CM. Three days later, brains were removed and sections of brain were stained with NKp46 antibody to reveal host NK cells. (A) Fluorescence picture of a Hoechst 33342-stained brain section with a large D2F2/E2 tumor (the nuclei-dense lower half of the picture) from an animal receiving Her2-targeting VSV 3 days earlier. (B) Same area as (A), showing a high number of PE-NKp46+ host NK cells infiltrating the D2F2/E2 tumor (below the white line). Note the low density of host NK cells in the normal brain tissue (above the white line). (C) and (D) show an D2F2/E2 tumor from another animal receiving Her2 targeted VSV 3 days earlier (to the right of the white line). Note the dense cluster of host NK cells to the right and the absence of host NK cells from the adjacent normal brain tissue (to the left of the white out-line). (E) and (F) show an D2F2/E2 tumor (below white out-line) from a control animal, which did not receive the Her2-targeted virus. Note the very low number of host NK cells in the tumor tissue. Bars = 200 μm.

VIII. CONCLUSIONS AND PERSPECTIVES

It seems clear that the density of NK cells in the tumor microenvironment is a useful prognostic indicator in a variety of cancers, although we do not fully understand why this is the case. It is also clear that number of NK cells needed at tumor sites to allow the NK cells to influence tumor growth and viability are not spontaneously generated; however, such high densities of NK cells may be created if the "flavor" of the tumor microenvironment is changed to mimic that of a virally infected tissue. Alternatively, non-myeloablatic lymphodepletion has been shown to increase effector-cell survival and the anti-tumor effect in the setting of adoptive T-cell therapy of cancer in humans. It has been suggested that the rapid induction and secretion of cytokines in the host, in particular IL-7, to restore the lymphocytic homeostasis also supports the transferred T-effector cells. The same may be the case for NK cells. In fact, in a recent trial of adoptive NK-cell therapy given after non-myeloablatic lymphodepletion, high numbers of viable, transferred NK cells were detected in the blood of the recipients for at least 7 days (and in some patients for many weeks). Despite this positive finding, no objective responses were observed in any of the eight patients receiving this treatment.[67] Thus, while the cytokine-response induced by the

non-myeloablatic lymphodepletion may be sufficient to prolong survival of adoptively transferred NK cells as they circulate in the blood vascular system, it may not be sufficient to support those NK cells that leave the blood vascular system to infiltrate the malignant tissues. Likewise, the amount of IL-2 that was given to the patients in this study to support the transferred NK cells (750,000 IU kg^{-1} every 8 h for at least 2 days) was at least 50-fold lower than the amount of IL-2 that, in most animal models, is needed to successfully support adoptively transferred A-NK and other lymphokine-activated killer (LAK) cells.[68] On this background, it is likely that the failure of adoptive NK cell therapy in this, as well as all other similar studies, is caused by insufficient availability, at the tumor sites, of cytokines capable of maintaining the NK cells' viability and anti-tumor functions. The relatively low doses of IL-2 and/or IL-15, which can be safely administered systemically, are far from sufficient to support intratumoral NK cells sufficiently. Therefore, better methods to ensure that the NK cells can produce their own IL-2 or IL-15 or strategies to increase the amounts of pro-NK cytokines in the microenvironment of tumors without simultaneously increasing their presence systemically, must be developed. In fact, we believe that similar strategies are needed to keep effector cells of T-cell origin alive at tumor sites. The superior survival of transferred CAR T cells, which incorporates the signaling portion of the 4-1BB receptor (ensuring that target-engagement of the CAR leads to IL-2 production by the CAR T cells[69]) strongly supports this notion. Because we strongly believe that the ultimate success of cellular immunotherapy will require the presence of both NK cells and T cells to eradicate both MHC class-I–positive and MHC class-I/ag–negative tumor cells, a strategy to ensure the survival of both NK cells and T cells selectively at the tumor site should lead to a substantial improvement in the efficacy of cell-mediated immunotherapy of disseminated cancer.

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