

AD _____

Award Number: DAMD17-02-1-0562

TITLE: Blocking Blood Supply to Breast Carcinoma with a DNA
Vaccine Encoding VEGF Receptor-2

PRINCIPAL INVESTIGATOR: Rong Xiang, M.D., Ph.D.

CONTRACTING ORGANIZATION: The Scripps Research Institute
La Jolla, California 92037

REPORT DATE: April 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20040503 045

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE April 2004	3. REPORT TYPE AND DATES COVERED Annual (15 Mar 2003 - 14 Mar 2004)	
4. TITLE AND SUBTITLE Blocking Blood Supply to Breast Carcinoma with a DNA Vaccine Encoding VEGF Receptor-2			5. FUNDING NUMBERS DAMD17-02-1-0562	
6. AUTHOR(S) Rong Xiang, M.D., Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Scripps Research Institute La Jolla, California 92037 E-Mail: rxiang@scripps.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES Original contains color plates: All DTIC reproductions will be in black and white.				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) The major goal of cancer immunology is to induce a tumor protective immune response that could effectively eradicate growth and dissemination of metastases. In our second fiscal year, we demonstrated proof of concept indicating that effective suppression of tumor angiogenesis can be achieved with a DNA vaccine encoding either muring VEGF receptor-2 (Flk-1) or Flk-1 minigenes designed to induce CTL-mediated immune responses by targeting proliferation endothelial cells into tumor vasculature. In fact, CTL-mediated killing of endothelial cells indicated a breaking of peripheral immune tolerance against FLK-1 or fragments of this self-antigen resulting in markedly reduced dissemination of metastases. Angiogenesis in the vasculature was suppressed without impairment of fertility, neuromuscular performance or hematopoiesis and with only a slight delay in wound healing. Furthermore, a similar strategy was performed by targeting the transcription factor Fra-1 co-expressing secretory IL-18, in breast cancer model, which was highly effective in suppression or eradicating aggressive metastases by inducing anti-angiogenesis coupled with pronounced activation of T and NK cells. We demonstrated that this combination could not only suppress D2F2 breast cancer growth and metastases, but also induces a long-lived T cell memory.				
14. SUBJECT TERMS No Subject Terms Provided.				15. NUMBER OF PAGES 30
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	8
Reportable Outcomes.....	9
Conclusions.....	10
Appendices.....	11

INTRODUCTION:

Breast cancer kills over 40,000 women annually in the U.S. alone, largely due to metastasis or recurrence of the primary tumor. It is now clear that the immune responses against specific antigens and/or related molecules expressed by tumor cells and their neovasculature can both suppress primary tumors, block metastasis or tumor recurrence and induce antiangiogenesis as well as apoptosis. It is known that immune tolerance can be broken by activation of naïve T cells through over-expression and presentation of tumor antigens by a naked DNA vaccine.

Using gene transfer by double attenuated *Salmonella typhimurium* encoding murine Flk-1 or Fra-1 to express these genes in Peyer's patches can be a particularly effective approach. With such an appropriate gene delivery system, naïve T cells could be activated to elicit a robust immune response *in vivo*. This would simplify widespread clinical use, as a non-patient specific gene transfer system could be used. We have recently developed a novel DNA vaccine approach and a unique delivery system for this purpose, and propose to combine this strategy with recent advances in chemokine biology to develop *in vivo* immune prophylactic and therapeutic approaches for breast cancer. Our goals in the second fiscal year were to: a) Generate minigene DNA vaccines encoding mouse nonapeptides of Flk-1 with either H-2K^d and/or H-2D^d anchor residues. b) Evaluate the anti-angiogenic/anti-tumor activities of the DNA vaccine including Flk-1 minigenes in the breast cancer model D2F2 in syngeneic BALB/c mice. c) Test the possibility of using the Fra-1 DNA-based vaccine to induce both anti-tumor immunity and long-lived T cell memory after orally administered vaccination, delivered by double attenuated *Salmonella typhimurium*.

BODY:

As we mentioned above, our major task, i.e. task 2, was to determine anti-angiogenic/anti-tumor effects of minigene-based DNA vaccines encoding Flk-1 nonapeptides with either H-2K^d and/or H-2D^d anchor residues. This includes two subtype projects. First, construct the Flk-1 minigene DNA vaccines and determine their sequences and protein expression. Second, evaluate the anti-angiogenic/anti-tumor activities of the DNA vaccines including minigenes in experimental D2F2 mouse models in syngeneic BALB/c mice. In addition, I have also presented some additional new and encouraging data obtained in my breast cancer projects involving the Fra-1 DNA vaccine, which will further strengthen and enforce our concept regarding the breast cancer vaccine strategy.

Generally speaking, our outline in the initial grant proposal remained mostly on schedule. Four versions of our Flk-1 minigene-based vaccines were generated successfully with slight modifications. The construction, design and sequences are shown in Appendix 1. All the expression vectors encoding H-2K^d and D^d minigenes were confirmed by sequence analysis and Western Blotting assay. At first, to verify that a specific immune response was induced by the mini-gene vaccines, vaccination vectors were electroporated into doubly attenuated *Salmonella typhimurium* (*dam*⁻; *AroA*⁻) and used to vaccinate BALB/c syngeneic mice 3 times at 2-wk intervals. To this end, a survival experiment was performed with a lethal challenge of D2F2 breast cancer cells 1 wk after the last immunization. All experimental groups revealed a dramatic increase in percent survival, as compared with the control group (Fig. 1). Once we verified that protective immunity was induced by our DNA minigene vaccines, we looked into the mechanism of the immune response with a standard ⁵¹Cr release assay, using splenocytes isolated from immunized mice (Fig. 2).

In addition, a unique vaccine was developed against the Fos-related transcription factor, Fra-1 co-expressing secretory IL-18. The latter is highly effective in suppressing or eradicating aggressive breast cancer metastases by inducing anti-angiogenesis coupled with pronounced activation of T and NK-cells. In addition, based on recent publications, IL-18 can also maintain a T cell memory induced by specific tumor antigens and their relative molecules. We achieved this objective, using an approach similar to the Flk-1 DNA vaccine, by eradicating established D2F2 breast cancer metastases in BALB/c mice with an oral DNA vaccine delivered by attenuated *Salmonella typhimurium*. Importantly, for the first time, a humoral immune response was induced with this vaccination, suggesting that a combination immunity against breast cancer was achieved (Figs. 3 and 4). Furthermore, we expanded our findings further to study T cell memory using our Fra-1 DNA-based vaccine system (Figs. 5-9). Three lines of evidence indicate the efficacy of this novel DNA vaccine in inducing long-lived T cell memory protecting against growth and metastasis of D2F2 breast cancer cells. First, host immune cells, particularly CD8⁺T effector cells from successfully immunized mice can be passively transferred to naïve, syngeneic SCID mice resulting in a 2-3 fold increase in survival over controls, following lethal tumor cell challenge. Second, activation of these horizontally transferred CD8⁺T cells in SCID mice was indicated by up regulation of their activation markers and memory T cell markers CD44⁺high and CD122⁺ in both lymphoid tissue and tumor local tissue as well as by prolonged survival of these cells *in vivo*. Third, long-lived survival of memory T cells in SCID mice was indicated after tumor cell challenge, followed by their differentiation to CD8⁺T effector cells. This was evident from the increased release of IFN- γ and the effective lysis of D2F2 tumor cells *in vitro*.

In addition, to evaluate possible side effects, we determined the effect of our vaccines on wounding, fertility and numbers hematopoietic cells as described previously. We have found that there were no significant differences between experimental and control groups of mice. The occurrence of common, FLK-1-positive progenitor cells for both endothelial cells and hematopoietic cells led us to evaluate peripheral blood samples of BALB/c mice after their last immunization. However, total blood counts and differentials did not indicate any decrease or compensating hematopoiesis (Fig10).

KEY RESEARCH ACCOMPLISHMENTS:

1. Flk-1 DNA minigene vaccines with H-2K^d and/or D^d effectively prolong the lifespan of mice after breast cancer challenge.
2. The minigene vaccine induced cytotoxicity against a Flk-1-positive endothelial cell line, but no significant cytotoxicity against Flk-1 negative tumor cells.
3. We demonstrated that an oral DNA vaccine encoding Fra-1 and IL-18 carried by an attenuated strain of *Salmonella typhimurium*, protected BALB/c mice against a lethal challenge of murine D2F2 breast cancer cells.
4. This DNA vaccine design could break T cell tolerance to a specific tumor antigen generating a long-lived T cell memory immune response against breast cancer, which was maintained in SCID mice in the absence of Ag.
5. We demonstrated that either entire gene or minigene vaccination can induce a strong immunity as well as suppress the breast cancer growth and dissemination of metastases.

REPORTABLE OUTCOMES:

The reportable outcomes and results from the second fiscal year of this grant are as follows:

1. A draft entitled “ Targeting of transcription factor Fos-related antigen 1 induces a long-lived protective memory T cell response against breast cancer” has been completed and will be sent out for publication shortly.
2. Funding for this award also stimulated an additional anti-angiogenic project, namely a naked DNA vaccine against breast cancer by targeting murine endoglin. The draft has been sent to “Cancer Research” for peer review.
3. Based on our preliminary data with the Flk-1 minigene vaccines, we plan to do further experiments to strengthen our results for a publication.

CONCLUSIONS:

Our objective was to induce effective regression in breast cancer growth and metastases by suppressing tumor angiogenesis and disseminating metastases with an orally delivered naked DNA vaccine carried by double attenuated *Salmonella typhimurium* encoding either Flk-1 or Fra-1 genes. The key finding from our second year was that our Flk-1 DNA minigene vaccines with H-2K^d and/or D^d anchor residues could effectively protect BALB/c mice from breast cancer challenge, as shown in a lifespan experiment. The immunity induced by both the entire Flk-1 gene or minigene vaccination was mediated by specific CD8⁺ T cells. In addition, we demonstrated that an oral vaccine encoding Fra-1 and IL-18 carried by an attenuated strain of *Salmonella typhimurium* protected Balb/c mice against a lethal challenge of murine D2F2 breast cancer cells. Our data revealed that not only a CD8⁺ T cell mediated tumor immune response was successfully induced coupled with a positive humoral response, but also long-lived memory T cells were generated in both syngeneic and SCID mice after tumor cell challenge. Importantly, we demonstrated for the first time the in depth mechanism that IL-18 plays a key role in maintaining T cell memory but does not induce it. Furthermore, we found that these long-lived memory T cells which were dormant inside non-lymphoid tissue after the first D2F2 challenge, then exhibited high cytotoxicity and quickly rebounded following a second tumor cell challenge, as compared with CD8⁺ T cells from secondary lymphoid tissue.

APPENDICES:

Fig. Legend, p 12

Fig. 1, p 19

Fig. 2, p 20

Fig. 3, p 21

Fig. 4, p 22

Fig. 5, p 23

Fig. 6, p 24

Fig. 7, p 25

Fig. 8, p 26

Fig. 9, p 27

Fig. 10, p 28

Figure legend

Figure 1: The lifespan was elongated by Flk-1 and minigene vaccination. Mice were vaccinated as described previously, and challenged one week later by intravenous (i.v.) injection of a lethal dose of syngeneic D2F2 breast carcinoma cells. The survival was monitored until all control animals died.

Figure 2. H-2 restricted Flk-1-specific T cells induced by the Flk-1 minigene vaccines are cytotoxic. Groups of BALB/c mice (n=4) were immunized 3 times at 2 wk intervals with attenuated *Salmonella typhimurium* harboring the vectors indicated. Mice were sacrificed 2 wk after the last immunization and isolated splenocytes were stimulated with irradiated HeVc-Flk-1 cells for 5 d. Cytotoxicity assays were performed using a standard ^{51}Cr -release assay.

Figure 3. Molecular expression of Murine Fra-1 in murine tissue and tumor cell lines and construction and functional assays of expression vectors encoding Fra-1 and IL-18

a) RT-PCR analysis of Fra-1 gene expression in tumor cell lines (D2F2, 4T1, 4T07, CT-26, RM2 and D121) and normal murine tissue (spleen, liver, lungs and bone marrow). Total RNA was extracted from cells growing at 70% confluence and normal murine tissue. GAPDH was used as a control for total RNA loading. b) Western blot analysis of Fra-1 protein expression in tumor cell lines (D121, 4T1, D2F2, CT-26 and RM2) and normal murine tissue (spleen, liver, lungs and bone marrow). Protein lysates were extracted from cells growing at 80% confluence and homogenized tissue. β -actin was used as a control for protein loading. c) Immunohistochemical analysis of Fra-1 in D2F2 breast cancer tissue. Paraffin sections from a D2F2 breast cancer tissue sample were analyzed by immunohistochemistry using an antibody raised against Fra-1 protein.

I. Immunochemical staining of D2F2 breast cancer tissue. II. Immunochemical staining of D2F2 breast cancer tissue without using primary antibody (anti-Fra-1). d) Vector construction map and protein expression. The coding sequence of full-length, murine Fra-1, fused with Tetanus Toxin

Fragment C (Tet1) at N terminus or only Tet1 was inserted into the pcDNA3.1/Zeo plasmid (pTet1, pTet1-Fra-1). A third plasmid, pIL-18, contained the entire coding sequence of murine IL-18 with an Igk leader sequence. Protein expression by pTet1-Fra-1 and pIL-18 was demonstrated by Western Blotting. Blots are shown for either pTet-Fra-1 (lane1) or pIL-18 (lane2) and of culture supernatant from pIL-18 transfected COS-7 cell (lane 3).

Figure 4. Effective protective immune response of the pTet1-Fra-1/pIL-18 based DNA vaccine on tumor metastases. *a)* Vaccination schedule was designed with three immunizations at two week intervals and tumor cell challenge with 0.5×10^6 D2F2 tumor cells i.v., one week after the last immunization. Survival curve represents the results for eight mice in each of the treatment and control groups. Surviving mice were tumor free unless otherwise stated. Data are representative of three similar experiments. *b)* Cytotoxic activity was induced by DNA vaccine. Splenocytes were isolated from BALB/c mice after vaccination with experimental or control DNA vaccines 2 weeks after challenge with D2F2 tumor cells and analyzed for their cytotoxic activity in a 51 Cr-release assay at different effector-to target cell ratios. Specific lysis mediated by CD8⁺ T cells against D2F2 target cells. Each value shown represents the mean of eight animals. *c)* Production of IFN- γ was verified at single-cell level by measuring reproduction in individual T cells by the ELISPOT assay. A representative ELISPOT assay is shown as spot formation per well induced by three treatment groups (pIL-18 , pTet1-Fra-1 , pTet1-Fra-1/pIL-18 ). The mean spot distribution of each well in each experimental and control group is shown (n=4, mean $\pm$ SD). Differences between the control group (empty vector ) and the three treatment groups were statistically significant (**P<0.001, *P<0.05). *d)* Up-regulated expression of T cell activation molecules. Two-color flow cytometric analyses were performed with single-

cell suspensions of splenocytes obtained from immunized mice two weeks after D2F2 (0.5×10^6) cell challenge. Anti-CD25, CD28, CD69 and LFA-1 Abs were used in PE-conjugated form in combination with FITC-conjugated anti-mouse mAb directed against CD8⁺ T cells. Differences between the control group (empty vector) was statistically significant when compared with the three treatment groups (pIL-18, pTet1-Fra-1, pTet1-Fra-1/pIL-18) **P<0.001, *P<0.05.

Fig 5. T helper cell activation after vaccination with pTet1-Fra-1/pIL-18 DNA vaccine. *a)* Up regulated CD4⁺ marker. Lymphocytes were isolated from spleens of immunized mice two weeks after D2F2 (0.5×10^6) tumor cells challenge. Mice from experimental groups were treated with pIL-18, pTet1-Fra-1, or pTet1-Fra-1/pIL-18 and empty vector as a control. Two-color flow cytometric analyses were performed with single cell suspensions of lymphocytes. PE-labeled anti-CD3 Ab was used in combination with FITC-conjugated mAb anti-mouse CD4. Each value represents the mean of four mice. Differences between the control group (empty vector) was statistically significant when compared with the treatment groups P<0.05. *b)* IL-2 release from lymphocytes isolated from spleens two weeks after D2F2 tumor cells challenge. Production of IL-2 was detected at the single cell level by ELISPOT assay. Each value represents the lymphocytes from four mice. Differences between the control group (empty vector) was statistically significant when compared with the treatment group (pTet1-Fra-1/pIL-18), *P<0.05, **P<0.01. *c)* Antibody release induced by pTet1-Fra-1/pIL-18 DNA vaccine. Production of IgG2b and IgG3 antibodies was measured by Cytometric Bead Array. The mean counts of each experimental and control group is shown (n=8, mean±SD). Differences between the control group and three treatment groups (pTet1, pTet1-Fra-1 and pTet1-Fra-1/pIL-18) were statistically significant (*P<0.05, **P<0.001).

Figure 6. T cell activation in lymphoid and nonlymphoid tissue. *a)* Up-regulated CTL markers in different tissue. Lymphocytes were isolated from spleen, lungs, liver and blood of immunized mice two weeks (I) and eight weeks (II) after D2F2 (0.5×10^6) tumor cell challenge as well as one week after re-challenge (III) at fourteen weeks with surviving mice. Mice treated with empty vector were used as control. Two-color flow cytometric analyses were performed with single-cell suspensions of lymphocytes. PE-labeled anti-CD8 Ab was used in combination with FITC-conjugated anti-mouse CD3. Each value represents the mean for four mice. Difference between the control group (empty vector) was statistically significant when compared with the treatment group (pTet1-Fra-1/pIL-18), $P < 0.05$, especially for lymphocytes isolated from lungs, $P < 0.001$. *b)* Interferon- γ release from lymphocytes in different tissues. Lymphocytes were isolated from spleen, lungs and liver of immunized mice two weeks (I), eight weeks (II) after D2F2 (0.5×10^6) tumor cell challenge and one week (III) after re-challenge at fourteen weeks with surviving mice. Production of INF- γ was detected at single-cell level by ELISPOT assay. Each value represents the lymphocytes from four mice. Difference between the control group (empty vector) was statistically significant when compared with the treatment group (pTet1-Fra-1/pIL-18), $*P < 0.05$, $**P < 0.001$.

Figure 7. Maintenance of memory CD8⁺ T cells in the absence of tumor antigen. *a)* Vaccination schedule of donor mice was designed as described in Figure 2. Three immunizations at two weeks intervals and tumor cell challenge with 0.5×10^6 D2F2 tumor cell i.v., one week after last immunization. Lymphocytes were harvested from mice immunized with pTet1-Fra-1/pIL-18, two days after re-immunization with pTet1-Fra-1/pIL-18 or without re-immunization, when those mice were still tumor free 2 months following tumor cell challenge. Lymphocytes (4×10^7

/mouse) were horizontally transferred to BALB/c SCID mice. The mice were challenged with D2F2 (0.5×10^6), two days following the transfer. The surviving mice were tumor free unless otherwise stated. Mice that were transferred with lymphocytes harvested from empty vector immunized mice or only injected with PBS were used as controls. Data are representative of three similar experiments. *b)* Fate of CD8⁺ effector T cells after adoptive transfer into BALB/c SCID/ mice. Two color flow cytometric analysis of CD8⁺ (PE labeled), CD3⁺ (FITC labeled) splenic T cells adoptively transferred to BALB/c SCID mice cured from 7 days to 30 days to determine the effect of apoptosis on these cell after parking them in the syngeneic SCID mice. There is no difference between groups with and without re-immunization of donor mice with the DNA vaccine.

Figure 8. The memory CD8⁺ T cells go through rapid turnover after vaccination with pTet1-Fra-1/pIL-18. *a)* Three color flow cytometric analysis were performed on splenocytes obtained from immunized mice, stained with Cy-chrome labeled anti-CD8⁺, PE labeled anti-IL-2R β and FITC labeled anti-CD44⁺ and analyzed. The data shown are gated on live CD8⁺ T cells. *b)* The lymphocytes were isolated from Balb/c mice immunized with one of the treatment groups (pTet1-Fra-1 , pIL-18  and pTet1-Fra-1/pIL-18 ) or control groups (PBS  and empty vector ) at 24hr, 72hr, 1wk and 2wks after tumor cells challenge and analyzed with three color flow cytometric staining as described above. Each value represents the mean for eight mice. Differences between the two control groups (PBS and empty vector) were statistically significant when compared with the three treatment groups (*P<0.05, **P<0.001). *c)* The lymphocytes were isolated from different tissues (spleen, lungs, liver and blood) of Balb/c mice that were treated with either pTet1-Fra-1/IL-18 or empty vector eight weeks after tumor cell challenge or a control

group that only immunized with empty vector (I) or the lymphocytes were isolated from the different tissue of BLAB/c SCID/SCID mice that were transferred with either the lymphocyte from mice immunized with pTet1-Fra-1/pIL-18 or empty vector one week after tumor cells challenge (II). Three color flow cytometric analysis were performed, stained with Cy-chrome labeled anti-CD8⁺, PE labeled anti-IL-2R β and FITC labeled anti-CD44⁺ and analyzed. The data shown are gated on live CD8⁺ T cell as described above. Each value represents the mean for four mice. Differences between the control and treatment group were statistically significant for lymphocytes harvested from lungs, blood and spleens of Balb/c SCID mice following adoptive transfer and tumor cell challenge and also in lungs and liver of syngeneic Balb/c mice eight weeks after tumor cell challenge. (**P<0.05, *P<0.001).

Figure 9. Active T cell response adoptively transferred to Balb/c SCID mice following challenge with D2F2 tumor cells. *a)* Up-regulated expression of T cell activation molecules. Two-color flow cytometric analyses were performed with single-cell suspensions of splenocytes obtained from adoptively transferred SCID mice one week after D2F2 tumor cell challenge. Anti-CD25, CD28, CD69 and LFA-1 Abs were used in PE-conjugated form in combination with FITC-conjugated anti-mouse mAb directed against CD8⁺ T cells. Difference between the control group (empty vector ) was statistically significant when compared with the treatment group (Tet1-Fra-1/IL-18 ) **P<0.001 except LFA. *b)* Interferon- γ release from CD8⁺ effector T cells adoptively transferred to Balb/c SCID mice at 2days, 7days and 30days following challenge with D2F2 tumor cells. Production of each well in each experimental and control group is shown (n=8, mean $\pm$ SD). Differences between the control group (empty vector) and treatment group (pTet1-Fra-1/pIL-18) are statistically significant (**P<0.001). *c)* Cytotoxicity induced by CD8⁺T

cells that were adoptively transferred to Balb/c SCID mice. Splenocytes were isolated from Balb/c SCID mice adoptively transferred with lymphocytes from Balb/c mice immunized with experimental DNA vaccines after two days, 7days and 30 days. Analysis of their cytotoxic activity in a ^{51}Cr -release assay at different effector to target cell ratio was performed. Each value shown represents the mean of four mice. Differences between the control group (empty vector) and treatment group were statistically significant from all three time points (* $P < 0.05$, ** $P < 0.001$).

Figure 10. Evaluation of possible side effects. To test hematopoiesis, animals were subjected to complete peripheral blood counts and differentials up to 3 months after immunization. The results revealed that total blood counts and differentials did not indicate any decrease or compensating hematopoiesis

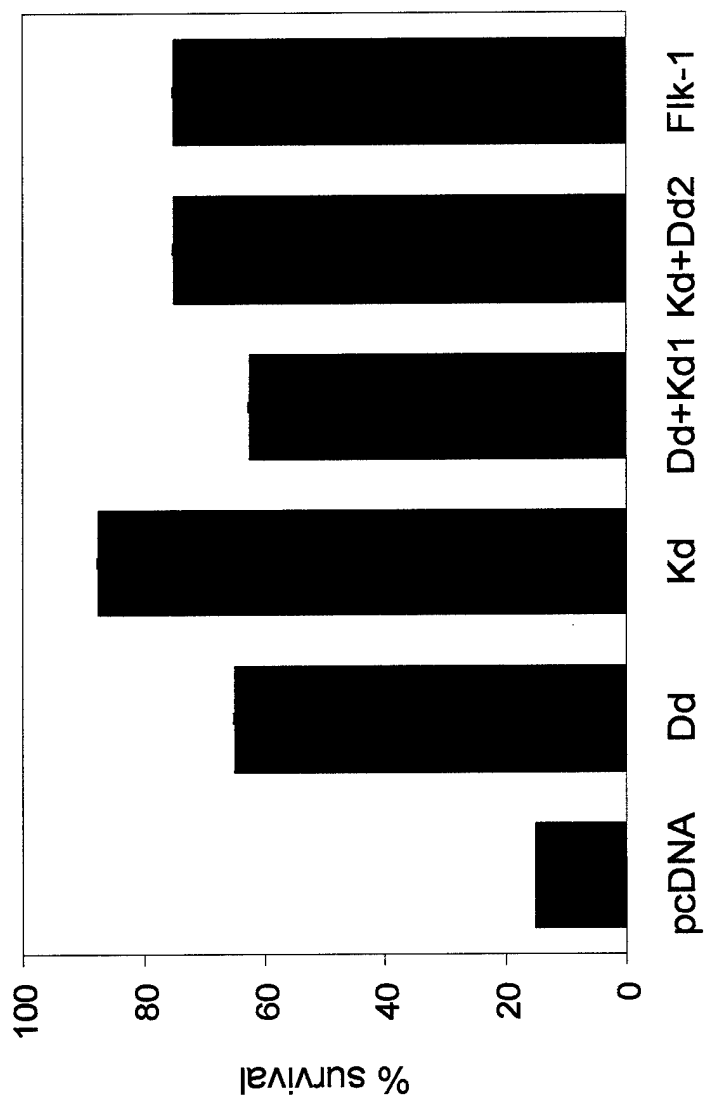


Fig. 1

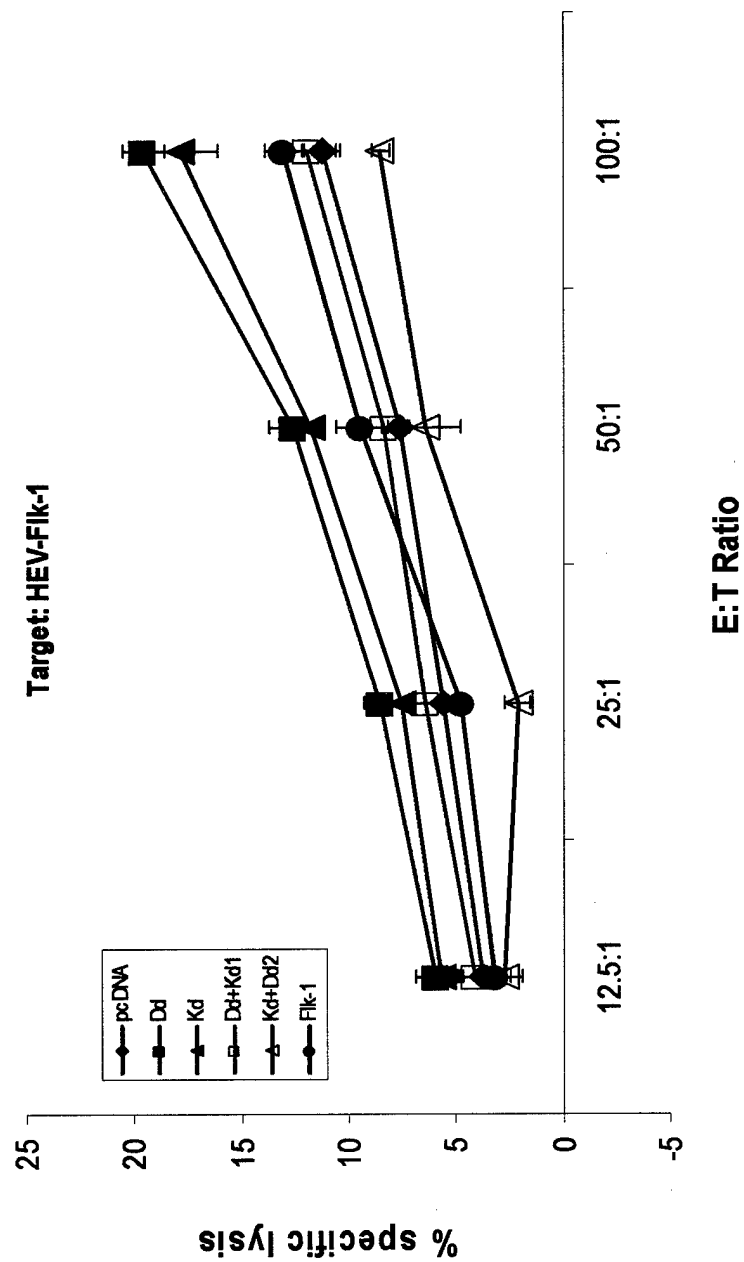
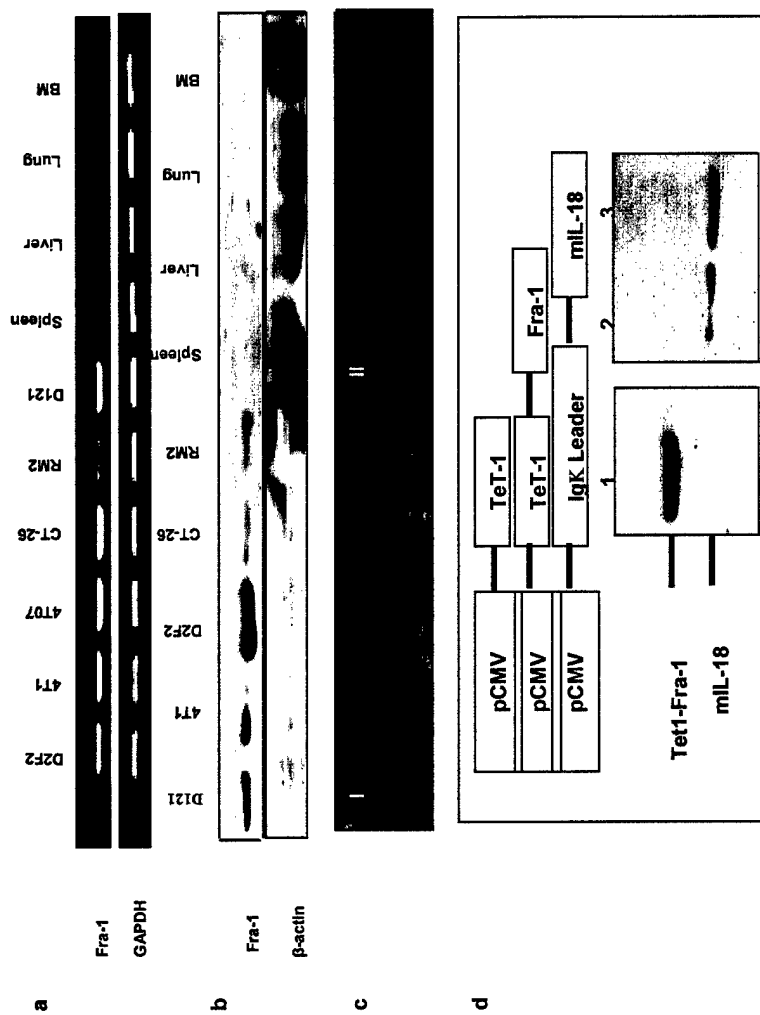
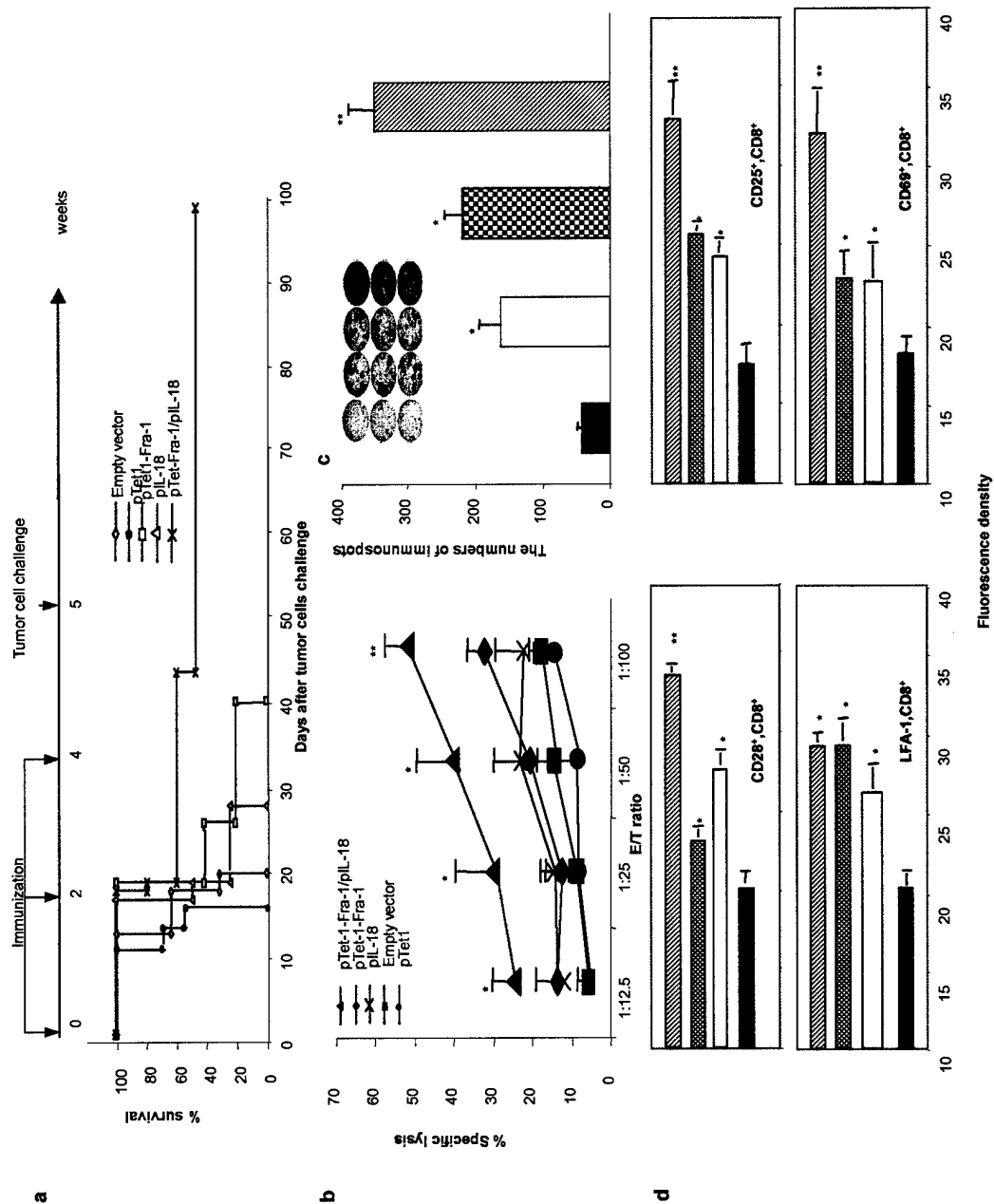


Fig. 2





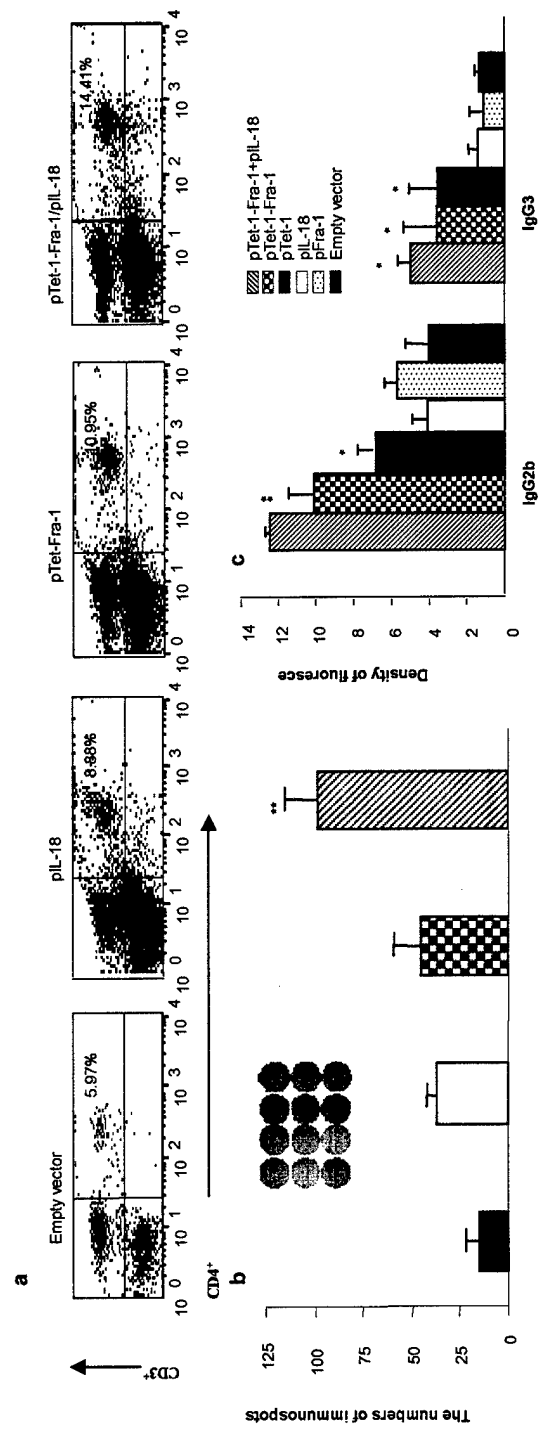


Fig. 5

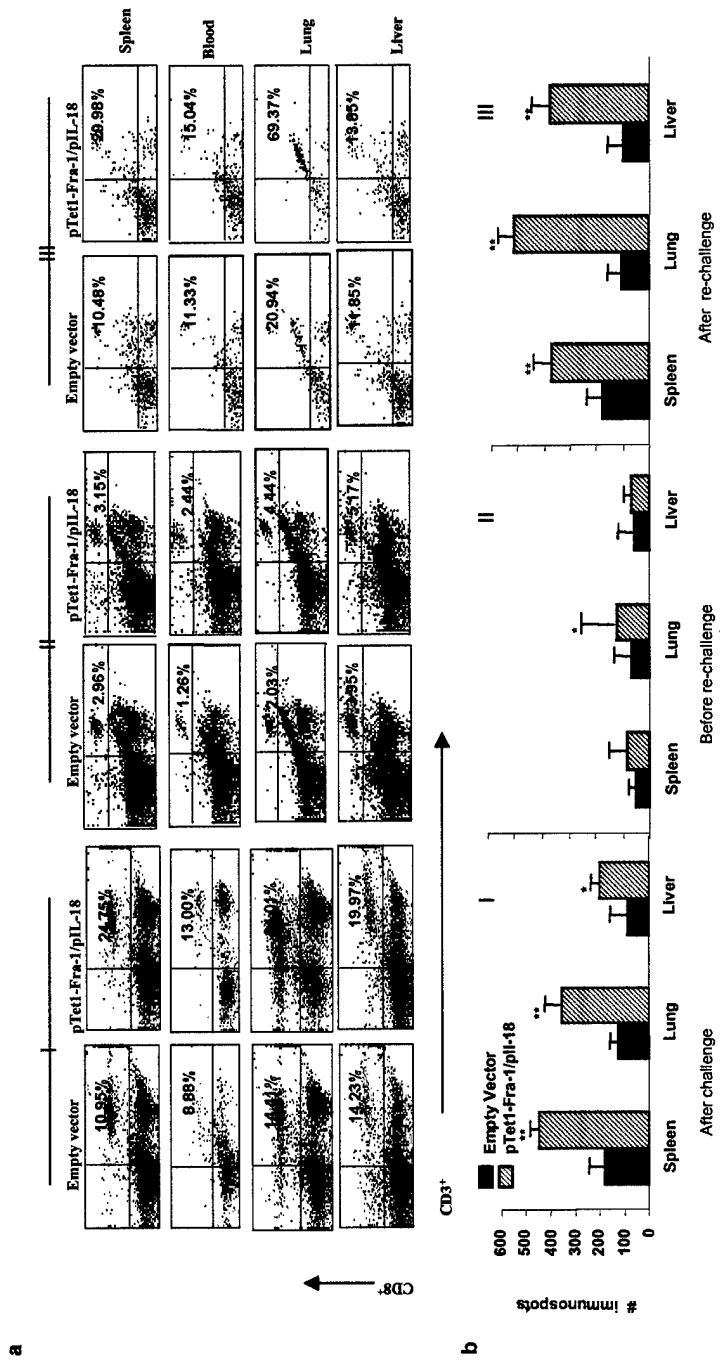


Fig. 6

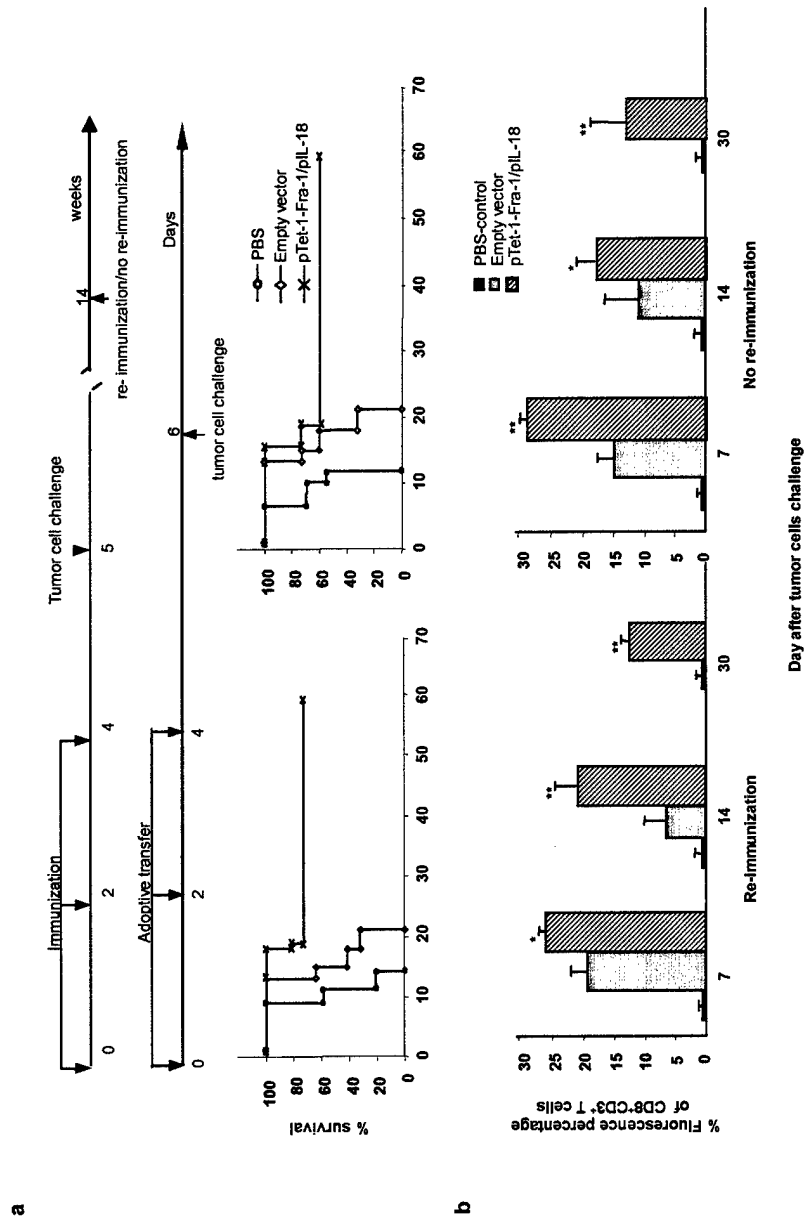


Fig. 7

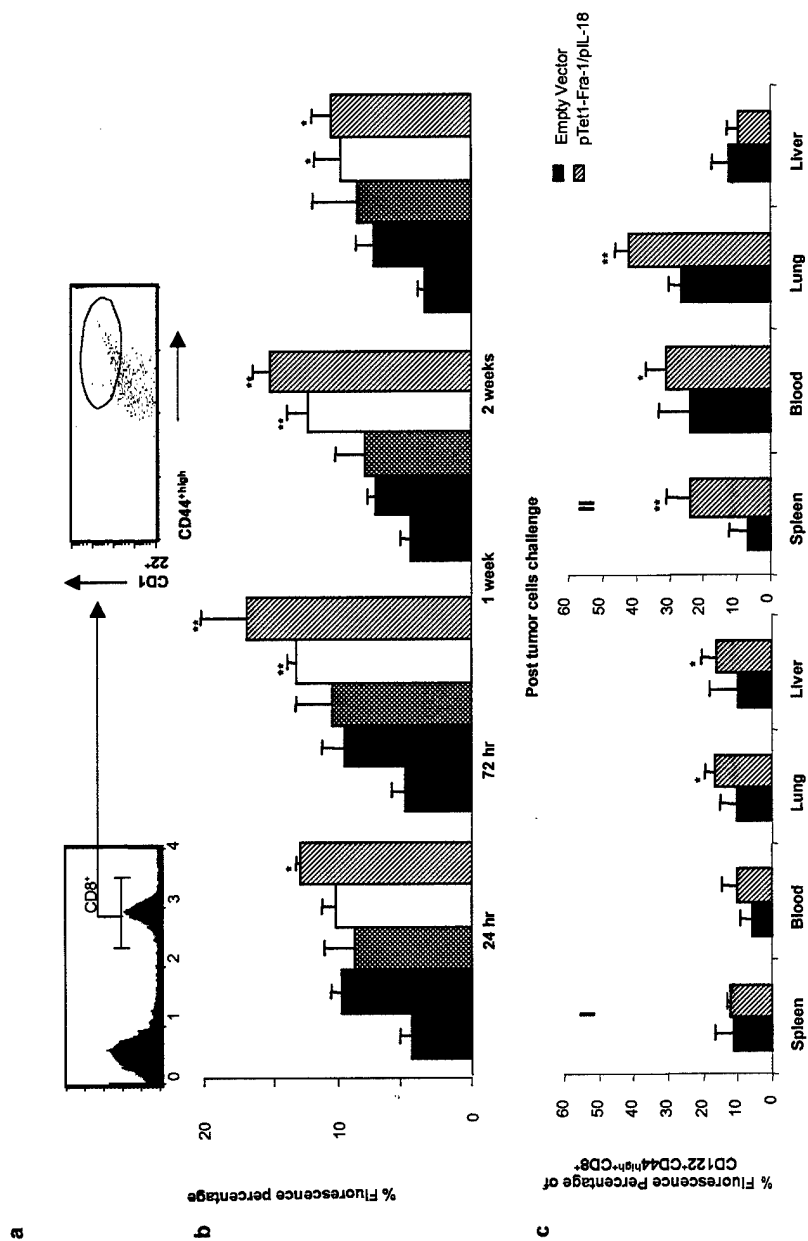


Fig. 8

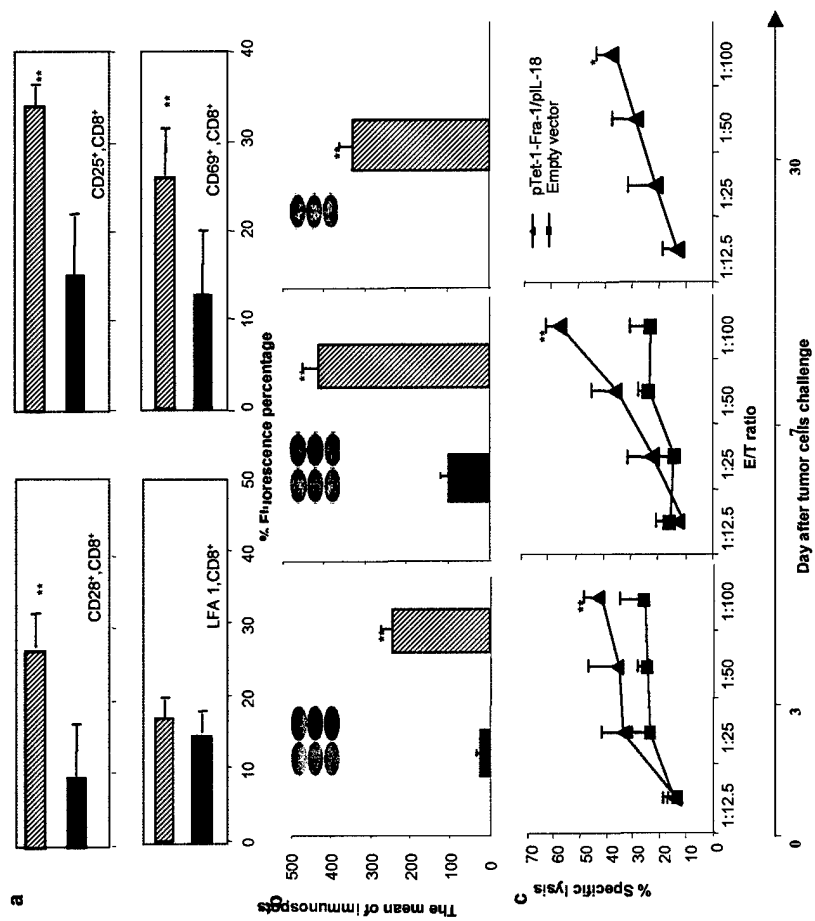


Fig. 9

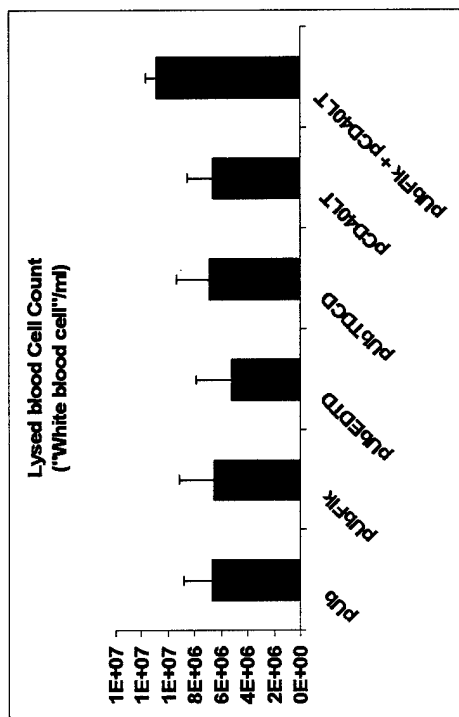
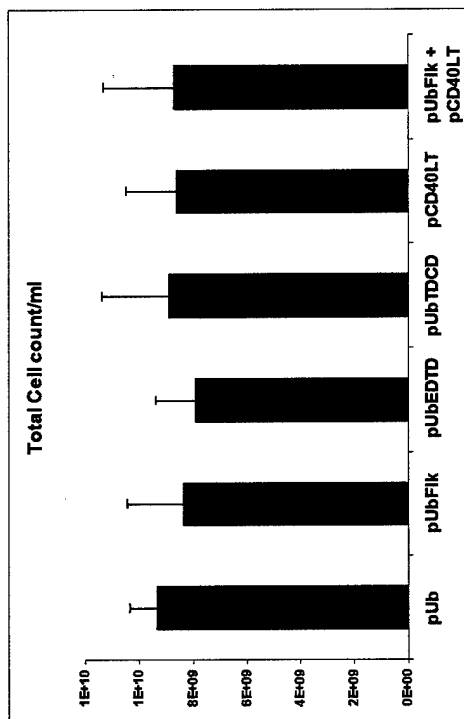


Fig. 10

ARC 67 JAN 4

ASSURANCE/ CERTIFICATION/DECLARATION

CARE AND USE OF ANIMALS

Animal Research Committee

☐ Grant

☐ Contract

☐ Fellow

☐ Other

The Scripps Research Institute

☐ New

☐ Competing continuation

☒ Noncompeting continuation

☐ Supplemental

1. DAMD17-02-1-0562

Department of Defense

☒ Original ☐ Follow-up

GRANT APPLICATION ID NO.

FUNDING SOURCE

Blocking Blood Supply to Breast Carcinoma with a DNA Vaccine Encoding VEGF Receptor-2

2. TITLE OF APPLICATION OR ACTIVITY

Rong Xiang, M.D., Ph.D.

3. PRINCIPAL INVESTIGATOR, PROGRAM DIRECTOR, OR FELLOW

POLICY:

Any research activity involving animals undertaken at The Scripps Research Institute (TSRI) must be reviewed and approved by our Institutional Animal Care and Use Committee (IACUC), the Animal Research Committee (ARC), in accordance with Public Health Policy regarding the care and use of laboratory animals. Applicants are required to submit to all granting agencies certification of ARC approval of compliance with the Guide for the Care and Use of Laboratory Animals, the American Association for the Accreditation of Laboratory Animal Care (AAALAC) standards, and the regulations set forth in the Animals Welfare Act (P.L. 89-544, as amended by P.L. 91-579 and P.L. 94-279) and other applicable federal, state and local laws, regulations and policies. The Scripps Research Institute currently has an assurance (A3194-01) of compliance on file with the U.S. Department of Health and Human Services (HHS) which covers the Institution's responsibility to monitor animal welfare. In the event that an application receives a pending approval from the ARC covering the proposed activity, certification of the ARC review and approval must be submitted to the granting agency within 60 days of filing the grant application.

4. HHS ASSURANCE STATUS

☒ The Scripps Research Institute has an approved assurance of compliance on file with HHS (A3194-01) which covers the care and use of laboratory animals and furthermore is accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

5. CERTIFICATION OF ARC REVIEW

☐ The applicant has sought approval of the portions of this research activity which include laboratory animals from the ARC of The Scripps Research Institute in accordance with HHS recommendations and AAALAC standards.

MAR 26 2004

(MO/DAY/YR) Date of ARC review and approval. (If approval is pending, write "pending". Follow-up certification is required within 60 days of filing grant application.)

☐ This activity contains multiple projects, some of which involve other institution(s) that have not been reviewed by the ARC. The ARC has granted approval on the condition that all projects involving laboratory animals will be reviewed and approved by an accredited Institutional Animal Care and Use Committee (IACUC) before they are initiated and that appropriate certification will be submitted by the responsible institution.

THE OFFICIAL SIGNING BELOW CERTIFIES THAT THE INFORMATION PROVIDED ON THIS FORM IS CORRECT AND THAT OUR INSTITUTION ASSUMES RESPONSIBILITY FOR ASSURING REQUIRED FUTURE REVIEWS, APPROVALS, AND SUBMISSIONS OF CERTIFICATION.

Applicant Institution

The Scripps Research Institute
10550 North Torrey Pines Road
La Jolla, California 92037
(858) 784-8048

Curtis B. Wilson, M.D.
IACUC, Chairman

3-25-2004



**Institutional Animal Care and Use Committee
(IACUC)
Animal Protocol Review Form**

Protocol Number: ARC-67JAN4

Title: Blocking Blood Supply to Breast Carcinoma with a DNA Vaccine Encoding VEGF Receptor-2

Investigator: Rong Xiang

Dear Dr. Xiang:

The animal protocol mentioned above was reviewed by a designated reviewer. The protocol was **APPROVED**. No further responses are necessary; animals can be ordered and work can begin.

Protocol begin date: 3-20-04

Protocol expiration date: 3-24-05

- Please note that you are responsible for assuring that all individuals working on this project have appropriate levels of animal training.
- Please submit a copy of the grant and award statement so that it may be attached to the protocol on file in the IACUC Office. A copy of the grant is an integral part of the protocol and must be in the IACUC Office before final approval and release of animals is possible.

Please keep the Committee informed of any changes in studies covered by this protocol.

For the IACUC,

 3-25-2004

Curtis B. Wilson, M.D.
Chair, IACUC
Director of Animal Welfare