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AWARD NUMBER: W81XWH-04-1-0489

TITLE: An Oral DNA Vaccine Encoding Endoglin Eradicates Breast Tumors by Blocking Their Blood Supply

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REPORT DATE: May 2006

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;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE (DD-MM-YYYY) May 2006		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 12 Apr 05 – 11 Apr 06	
4. TITLE AND SUBTITLE An Oral DNA Vaccine Encoding Endoglin Eradicates Breast Tumors by Blocking Their Blood Supply				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-04-1-0489	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Ralph A. Reisfeld, Ph.D. E-Mail: reisfeld@scripps.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Scripps Research Institute La Jolla, California 92037				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. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT We extended our research efforts on the endoglin-based DNA vaccine for the treatment of breast cancer. We attained our goals for the second fiscal year, first, by constructing unique expression plasmids encoding endoglin and co-expressing IL-15 or encoding IL-7, the latter to be co-expressed with endoglin. Second, we verified the correct protein expression of those plasmids by Western blotting, assessed anti-angiogenic effects by imaging and Matrigel assays and optimized antigen processing and presentation. Third, we critically evaluated anti-angiogenic and anti-tumor efficacies of the DNA vaccines in vivo in orthotopic as well as spontaneous and experimental D2F2 mouse breast tumor metastasis models in syngeneic BALB/c mice.					
15. SUBJECT TERMS DNA vaccine; endoglin; anti-angiogenesis; suppression of breast tumor metastases					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	35	19b. TELEPHONE NUMBER (include area code)

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INTRODUCTION:

There is no doubt of the need to develop new and effective treatments for metastatic breast cancer since there are currently still 40,000 or more breast cancer related deaths per year among American women. Among these patients a majority with localized disease suffer from micrometastases. Since radiation or surgical methods do not sufficiently destroy such micrometastases that eventually can cause multi-organ dysfunction and death, therapies such as antiangiogenesis, blocking renewal of blood vessel growth in the tumor bed, have been proposed as suitable antitumor strategies. Endoglin (CD105) is a suitable target for such approaches since it is overexpressed on proliferating endothelial cells in the tumor neovasculature. Furthermore, once endoglin is prevented from binding transforming growth factor (TGF-beta) on such cells, and once such cells are preferentially killed by CD8⁺ T cells activated by the vaccine, effective blood flow to tumors is suppressed, resulting in tumor cell growth inhibition and apoptosis, i.e. programmed cell death. Once primed and activated, such T-killer cells are also capable of a memory response against their target and can kill cells expressing endoglin over prolonged time periods, thus preventing recurrence of cancer.

Among the strategies to be applied to optimize T cell-mediated killing of endoglin-positive proliferating endothelial cells in the breast tumor vasculature are DNA vaccines encoding the entire endoglin gene. To augment long-lived immunological memory of T-killer cells and to prevent recurrence of breast cancer, we plan to co-express cytokines IL-7 and/or IL-15 in the DNA vaccines. This strategy, combined with our targeted delivery of the vaccine's DNA to secondary lymphoid organs such as Peyer's patches by attenuated *Salmonella typhimurium*, should strongly enhance immunogenicity and induce a robust cytotoxic T cell response against endoglin. Our standardized orthotopic breast tumor models in syngeneic mice, which lend themselves to prophylactic and therapeutic evaluations, should facilitate a critical evaluation of antitumor efficacy and possibly lead to improved therapies for breast cancer.

BODY:

Our goals in the second fiscal year were to: a) generate several unique expression plasmids encoding endoglin and secretory IL-7 and IL-15 into multiple cloning sites of the pBUDCE4.1 vector under the control of different promoters; b) determine the protein expression of these constructs by Western blotting and assess antiangiogenic effects by imaging and Matrigel assays and optimization of antigen processing and presentation; c) evaluate antiangiogenic and antitumor activities of the DNA vaccine *in vivo* in orthotopic as well as spontaneous and experimental D2F2 mouse mammary tumor metastasis models in syngeneic BALB/c mice.

In general, our outline in the SOW remained on schedule. First, we generated several expression plasmids encoding the entire murine endoglin gene, including insertion of a gene encoding secretory IL-15 into multiple cloning sites (Fig. 1A). We also determined the protein expression of these constructs by Western blotting (Figs. 1B,C). Second, we determined their antitumor activities *in vivo* by assessing the increased life-span of immunized mice in Kaplan-Meier plots (Fig. 2) and *in vitro* by activity of CTLs from such mice against D2F2 breast cancer cells and MS-1 endothelial cells (Fig. 3). The marked anti-angiogenic activities induced by these constructs *in vivo* were demonstrated by Matrigel assays (Fig. 4) and T cell activation after endoglin-based vaccination was shown by ELISPOT analyses (Fig. 5)

KEY RESEARCH ACCOMPLISHMENTS:

- 1) Murine endoglin (CD105) was expressed in the vector PCM/myc/cyto and protein expression verified by Western blotting.
- 2) Endoglin expression was verified by confocal microscopy on CD11c⁺ dendritic cells (DCs) in Peyer's patches of vaccinated BALB/c mice.
- 3) Immunization with the endoglin-based DNA vaccine evoked a specific CTL response.
- 4) The vaccine-induced cytotoxicity was specifically directed against endoglin and target cells positive for endoglin were 2 times more sensitive to cytotoxic killing by T cells than wild-type tumor cells.
- 5) There was an increased incidence of pulmonary metastases of D2F2 breast carcinoma in mice that were depleted of CD8⁺ T cells. Depletion of CD 4⁺ T cells had no effect.
- 6) The mEndoglin vaccine induced activation of T cells and DCs was indicated by an increase in the expression of CD28 and CD80/86 on T cells and CD11c⁺ positive DCs, respectively.

REPORTABLE OUTCOMES:

- 1) A paper entitled “DNA vaccines suppress angiogenesis and protect against growth of breast cancer metastases” was published in BREAST DISEASE 20:81-91, 2005.
- 2) Another paper entitled “Endoglin (CD105) is a target for an oral DNA vaccine against breast cancer is in press in CANCER IMMUNOLOGY IMMUNOTHERPY and will be published in May 2006.
- 3) A poster presentation of our work on endoglin-based DNA vaccines was presented at the Department of Defense-sponsored Era of Hope meeting at Philadelphia, PA, June 8-9, 2005.
- 4) Based on our encouraging progress made thus far with the endoglin-based DNA vaccine, we plan further experiments in the third and last fiscal year with emphasis on assessing the effect of co-expressed IL-7 and/or IL-15, particularly on enhancing long-lived immune memory against the DNA vaccine. This approach looks most promising at this time for preventing breast tumor recurrence.

CONCLUSION:

Most of our objectives for the second fiscal year, outlined in the original SOW, were met as we performed additional critical evaluations of our mEndoglin-based DNA vaccine with emphasis on the mechanisms of the immunological response induced by the vaccine.

Also, we constructed a new endoglin-based DNA vaccine co-expressing IL-15 and performed some initial evaluations of its antitumor activity which turned out positive. After some initial problems in cloning murine IL-7, we were finally able to accomplish this and established its protein expression. This construct is now available to be co-expressed in the endoglin plasmid.

APPENDICES:

Fig. 1 p. 10

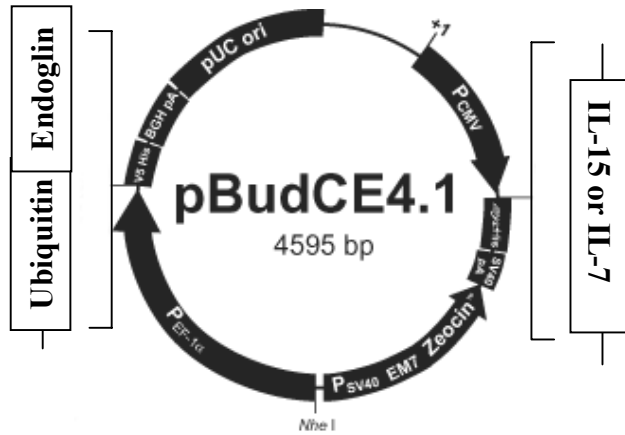
Fig. 2 p. 11

Fig. 3 p. 12

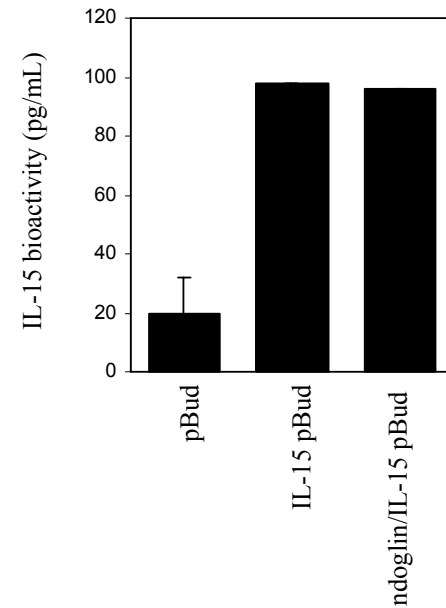
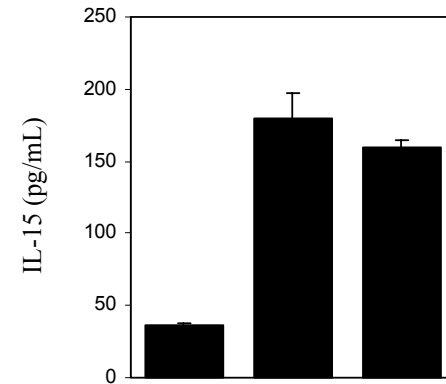
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Fig. 5 p. 14

A Vector construction



B Expression of IL-15



C

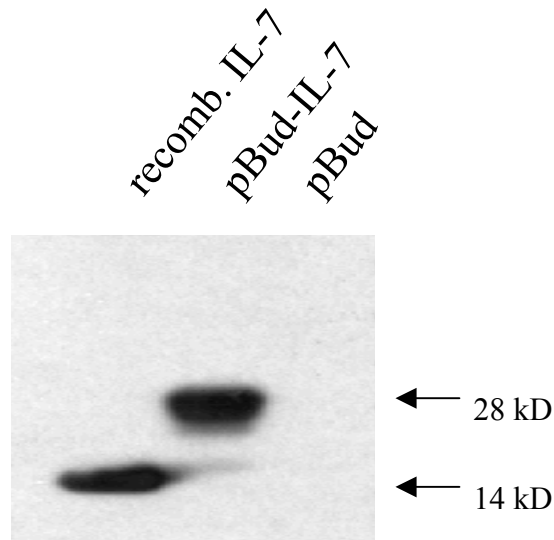


Fig.1. Construction and bioactivities of IL7 and IL15. A. schematic diagram of constructs; B. Protein secretion of recombinant IL-15 by ELISA (upper panel); Biological activity assay of recombinant IL-15 (lower panel); C. Protein expression of IL-7 by Western Blotting

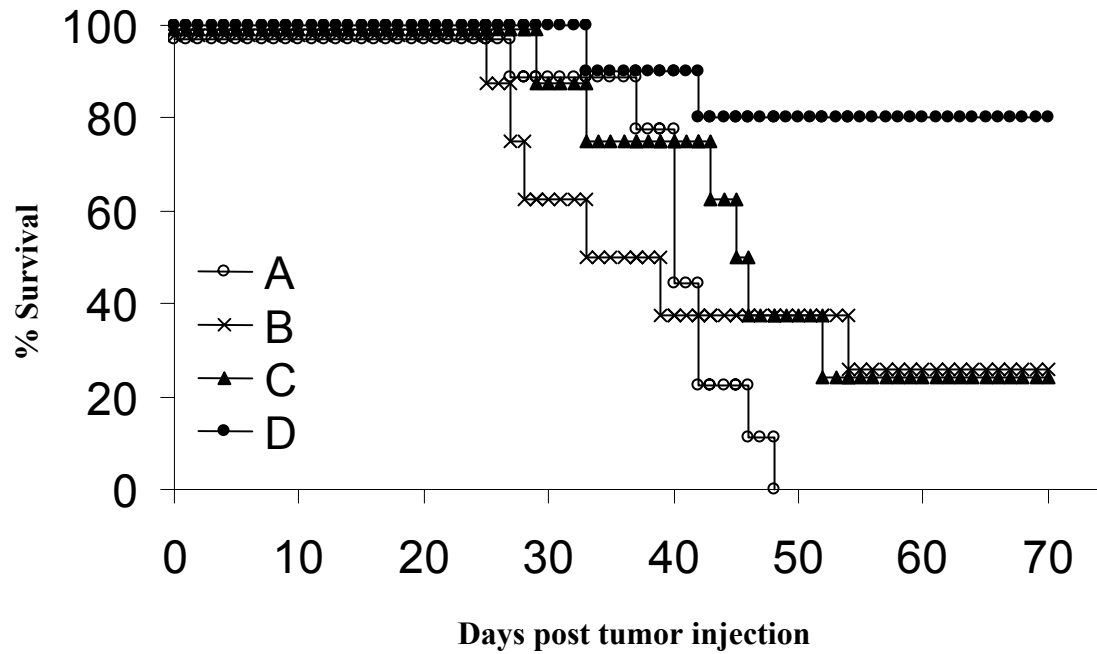


Fig.2. The Kaplan Meier Plot of successfully immunized mice. A. Empty vector; B. IL-15; C. Endoglin and D. IL-15-Endoglin

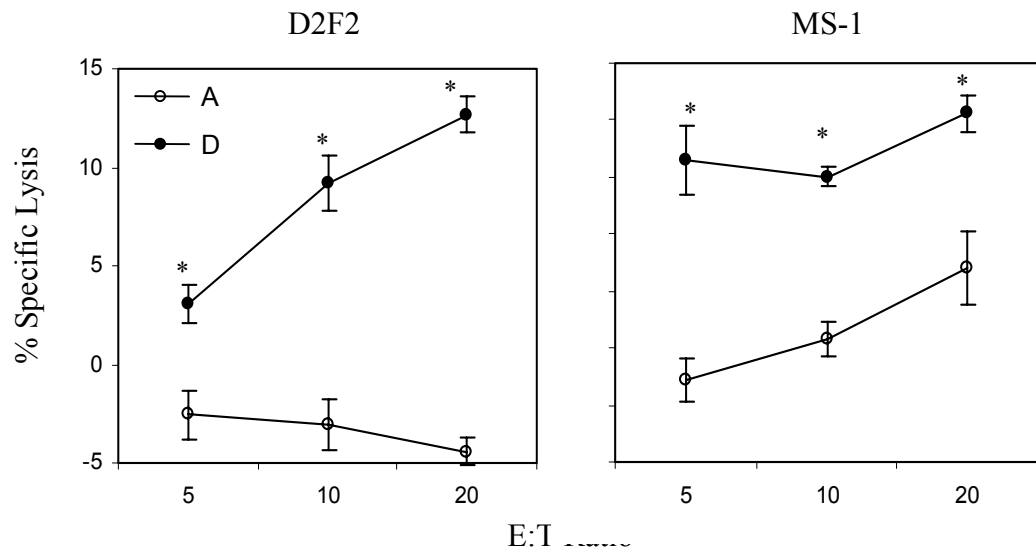


Fig.3. CTL activity against D2F2 breast cancer cells and MS-1 endothelial cells MS-1

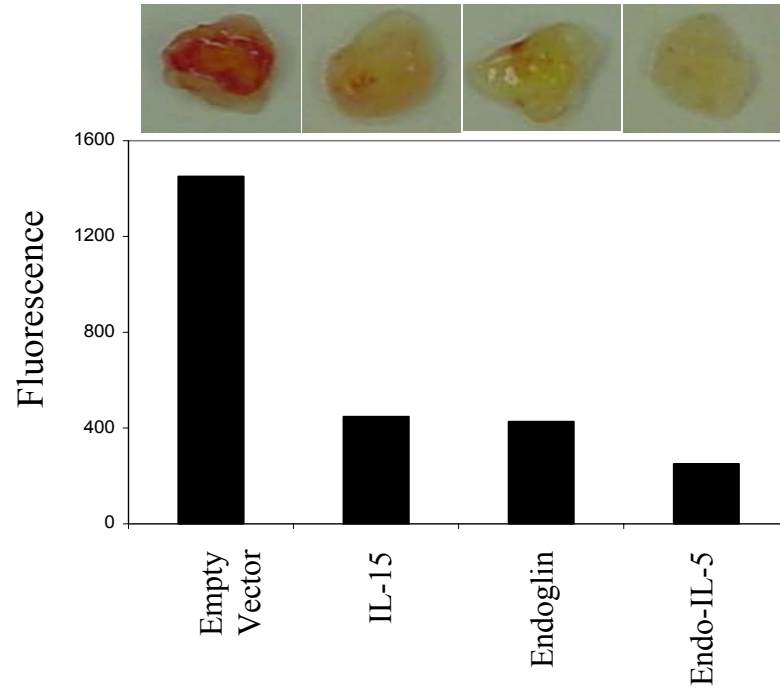
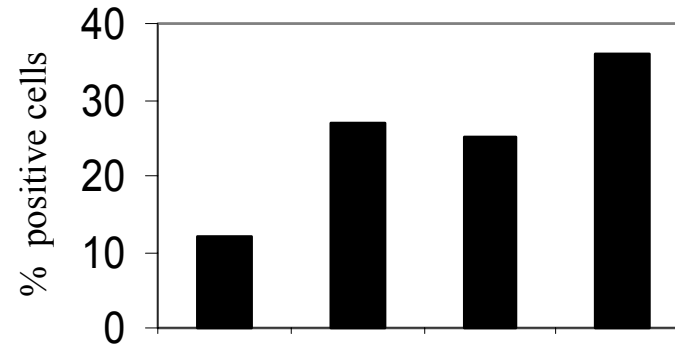


Fig.4. Antiangiogenesis effects against D2F2 breast cancer

A CD3+CD8+



B CD3+CD8+CD62L-

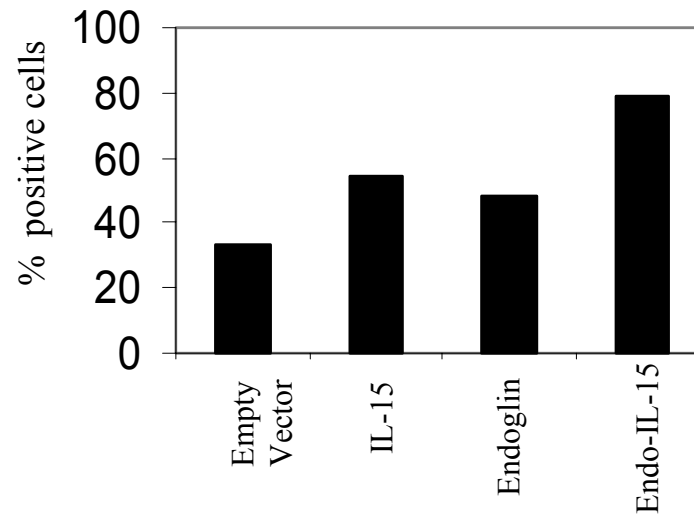


Fig.5. T cell activation after endoglin-based vaccination

DNA Vaccines Suppress Angiogenesis and Protect Against Growth of Breast Cancer Metastases

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Abstract. Two novel oral DNA-based vaccines provide immune protection against breast cancer in mouse model systems. These vaccines are delivered by attenuated *Salmonella typhimurium* to secondary lymphoid organs and are directed against novel targets such as transcription factor Fos-related antigen 1 (Fra-1) and endoglin (CD105). Both vaccines elicit suppression of angiogenesis in the breast tumor vasculature and break peripheral tolerance by eliciting potent cell-mediated protective immunity against these tumor self-antigens resulting in effective suppression of breast tumor growth and metastasis.

INTRODUCTION

A recent overview by Maurice R. Hilleman on the needs and realities for developing new and improved vaccines in the 21st century ends with the conclusion that “the development of multispecific vaccines that can be given orally, transdermally or mucosally may be the holy grail of future endeavor” [1]. In fact, our efforts, outlined in this chapter to develop novel DNA-based vaccines against breast cancer are very much guided by the principle of multifunctional DNA vaccines that are delivered orally to secondary lymphoid tissues by attenuated *Salmonella typhimurium*. Our goal is to induce the most effective and long-lived protective immune response possible against unique targets expressed by breast tumors and/or proliferating endothelial cells in their vasculature. In contrast to immunotherapy which relies on the administration of preformed effector mechanisms and is ideally suited to the treatment of established tumors, cancer vaccines are developed with an eye on prevention [2]. Based on successes achieved by vaccines in preventing infectious disease, our current efforts in cancer vaccines focus on the definition of unique vaccine targets not only expressed by tumors but also by their vasculature

or stroma, and on the development of immunization protocols that will be highly effective in priming the immune system to eliminate cancer before it manifests itself clinically or in delaying/preventing cancer recurrence. However, this entire approach is handicapped by the very fact that most tumor-associated antigens are poorly immunogenic self-antigens that necessitate the breaking of immunological tolerance against them by suitably designed immunological strategies, including powerful vaccine adjuvants. Among such adjuvants are cytokines or chemokines which we co-express as secretory components in our DNA vaccines to suitably enhance the activity of both, antigen presenting cells (APCs) and immune effector cells and thereby induce an optimal anti-tumor immune response. Here, we highlight two novel approaches for breast cancer vaccines based on results from our recent basic and pre-clinical studies that are providing support for preventive DNA-based breast cancer vaccines. First, a transcription factor Fos-related antigen 1 (Fra-1), over-expressed by D2F2 murine breast cancer cells, was demonstrated to be an effective target for a DNA-based breast cancer vaccine, which suppressed angiogenesis as well as tumor growth and dissemination, especially when co-expressed with secretory murine IL-18. Sec-

ond, endoglin (CD105) a binding protein of the transforming growth factor β receptor complex, overexpressed by proliferating endothelial cells in the D2F2 breast tumor vasculature, proved to be an excellent target for a DNA vaccine that was effective in suppressing tumor angiogenesis and subsequently tumor growth and dissemination in animal models.

Challenges for effective DNA-based cancer vaccines arise mainly from the poor immunogenicity of tumor-associated self-antigens. In fact, this necessitates the careful selection of the most effective target antigen; choosing the right delivery vehicle for DNA vaccines; and to design effective immunization protocols, including the most optimal vaccine adjuvants for the generation of a robust tumor protective immune response.

Selection of Effective Target Antigens

The major advantage which cancer immunotherapy has over other forms of therapy is its unique specificity whereby the immune response can recognize epitopes expressed by tumor cells or their microenvironment and target those cells for destruction without harming normal cells [3]. Research efforts in tumor immunology made during the last 20 years took full advantage of this specificity and identified a number of molecularly-defined tumor antigens which in pre-clinical studies elicited tumor-specific immunity and established long-term memory without inducing autoimmunity [3]. For example, breast cancer vaccine targets are composed of a number of well characterized epitopes such as HER2/Neu [4], mucin 1 [5], MAGE 3 [6] or carcinoembryonic antigen (CEA) [7], to name just a few. We selected two quite different antigens as respective targets for our breast cancer vaccines, namely transcription factor Fos-related antigen 1 (Fra-1) and endoglin (CD105) a binding protein of the TGF- β 1, and TGF- β 3 receptor complex, overexpressed by proliferating endothelial cells in the breast tumor vasculature but not expressed by breast tumor cells.

Our rationale for selecting Fra-1 as a vaccine target was based on several considerations. First, a limited number of transcription factors are generally overactive in most cancer cells which makes them appropriate targets for anticancer drugs, provided selective inhibition of transcription can be applied rather than general inhibition which is expected to be too toxic [8]. In fact, rather than selecting specific inhibitors of a transcription factor, we chose Fra-1, belonging to the transcription factor activating protein-1 (AP-1) family, since it defines tumor progression and regulates breast

cancer cell invasion and growth as well as resistance to anti-estrogens. In addition, Fra-1 is overexpressed by many human and mouse epithelial carcinoma cells, including breast cancer cells [9]. This overexpression of Fra-1 greatly influences these cells' morphology and motility, correlates with their transformation to a more invasive phenotype and is specifically associated with highly invasive breast cancer cells. These findings suggest Fra-1 to be a potentially useful target for active immunization against breast cancer [10].

The selection of endoglin (CD105) as a DNA vaccine target was based primarily on its overexpression by proliferating endothelial cells in the breast tumor vasculature which facilitates the induction of T cell-mediated suppression of tumor angiogenesis. This was already successfully demonstrated by us for a DNA vaccine against murine VEGF receptor-2 (FLK-1) [11]. Similar to endoglin, this receptor tyrosine kinase is also up-regulated by genetically stable proliferating endothelial cells in the tumor vasculature, but not expressed by genetically unstable, frequently mutating tumor cells. In fact, attacking the tumor's vascular supply to inhibit tumor growth, which was pioneered by Folkman and colleagues [12–15], is an attractive strategy for several reasons. First, angiogenesis is a rate-limiting step in the development of tumors since tumor growth is generally limited to 1–2 mm³ in the absence of a blood supply [16,17]. Beyond this minimum size, tumors frequently become necrotic and apoptotic under such circumstances [18]. Second, the suppression of tumor-associated angiogenesis is a physiological host mechanism and should not lead to the development of resistance. Third, each tumor capillary has the potential to supply many tumor cells, so that targeting the tumor vasculature potentiates the antitumor effect. Fourth, direct contact of the vasculature with the circulation leads to efficient access of therapeutic agents [15]. Importantly, targeting of genetically stable proliferating endothelial cells in the tumor vasculature does not down-regulate MHC class I and II antigens – an event that frequently occurs in most solid tumors, including breast cancer, which severely impairs T cell-mediated antitumor responses that are of paramount importance for DNA vaccine strategies. Additional advantages of this approach include the avoidance of immune suppression triggered by tumor cells at the cellular level; tumor independence of the therapeutic target, making this approach effective against a whole variety of malignancies; and ready availability of proliferating endothelial cells to lymphocytes in the circulation making it possible to reach target tissue unimpaired by anatomical barriers such as the blood-brain barrier or encapsulated tumor tissues [19].

Choosing a DNA Vaccine Delivery Vehicle

The potential use of attenuated strains of *Salmonella typhimurium* as a DNA vaccine carrier is based on initial findings by Hoiseth and Stocker [20] that an aromatic-dependent auxotrophic mutant of *Salmonella typhimurium* is non-virulent and effective as a live vaccine [21]. Later work with these attenuated (AroA) bacteria indicated that they could be used for oral somatic transgene vaccination [22] and to trigger the elicitation of antigen-specific humoral, T helper and cytotoxic responses against β -galactosidase, a model antigen [23]. Importantly, since professional antigen-presenting cells (APCs) play a key role in the induction of effective immune responses evoked by vaccination with plasmid DNA, the use of attenuated intracellular bacteria as delivery vehicle has the potential to efficiently target DNA vaccines to professional APCs.

We first demonstrated that the attenuated (AroA-) strain SL7207 of *Salmonella typhimurium*, made available by B.A.D. Stocker (Stanford University), was an effective carrier for oral delivery by gavage of an autologous DNA vaccine which effectively protected against challenge with murine melanoma cells. In fact, this vaccine broke peripheral T cell tolerance toward murine melanoma self antigens gp100 and TRP-2 containing the murine ubiquitin gene fused to minigenes encoding peptide epitopes gp100₂₅₋₃₃ and TRP-2₁₈₁₋₁₈₈ and induced a robust, tumor-specific CD8⁺ T cell response resulting in suppression of melanoma tumor growth [24]. Following gavage, these live, attenuated bacteria transport the DNA through the small intestine and then through the M cells that cover the Peyer's patches of the gut. From there the attenuated bacteria enter APCs such as dendritic cells and macrophages, which are plentiful in this secondary lymphoid tissue, where they die because of their AroA- mutation, liberating multiple copies of the DNA inside these phagocytes. There, the DNA is transcribed to protein which is then processed in the proteasomes of these APCs. This is followed by the formation of peptide-MHC class I antigen complexes which are ultimately presented by the APCs to the T cell receptor of naïve T cells. This entire process is depicted schematically in Fig. 1. We have successfully used attenuated *Salmonella typhimurium* as a carrier for several of our DNA vaccines encoding the human CEA gene effective in eliciting potent CD8⁺ T cell immunity in CEA-transgenic mice that eradicated growth and metastases of colon [25] and non-small cell lung cancer [26]. This same approach was also used successfully to deliver plasmids encoding genes for

VEGF-receptor 2 (FLK-1) to Peyer's patches resulting in a robust T cell mediated immune response against proliferating endothelial cells in the tumor vasculature in three different tumor models. This lead ultimately to effective suppression of angiogenesis and subsequent eradication of tumor metastases in both prophylactic and therapeutic settings [11].

Our rationale for delivering DNA vaccines orally by gavage with attenuated *Salmonella typhimurium* to Peyer's patches was strongly supported by results of a recent study by Maloy et al. [27] clearly indicating that intralymphatic immunization is the most effective means to strongly enhance DNA vaccination. This was evident from a comparison of conventional routes of immunization given i.d., i.m. or i.spl. with intralymphatic immunizations of DNA encoding CTL epitopes of the highly immunogenic LCMV glycoprotein. In this case, direct injection of the DNA into a peripheral lymph node enhanced immunogenicity by 100 to 1000-fold, inducing strong and biologically relevant CD8⁺ cytotoxic T lymphocyte responses.

We successfully transferred the plasmid encoding the Fra-1 gene by electroporation into doubly attenuated (dam-,AroA-) *Salmonella typhimurium* and then administered it as an oral vaccine by gavage to BALB/c mice. Here, two constructs were made based on the pIRES vector. As depicted in Fig. 2(A), the first, pUb-Fra-1, was comprised of polyubiquitinated, full-length murine Fra-1. The second, pIL-18, contained murine IL-18 with an Ig kappa leader sequence for secretion purposes. The empty vector with or without ubiquitin served as a control. By way of explanation, polyubiquitination has been used for many of our DNA vaccines, particularly since we found in one of our initial studies that the presence of ubiquitin upstream of a DNA minigene encoding melanoma antigens proved to be essential for achieving tumor-protective immunity [24]. Based on a vast body of literature on the role of ubiquitin in protein processing in the proteasome [28], we assumed that this molecule was essential for optimizing antigen processing and ultimately effective antigen presentation. Although ubiquitination does not invariably enhance CD8⁺ T cell responses, our work and reports by other investigators confirmed the important role played by ubiquitination in the MHC class I antigen presentation pathway [24].

As far as our Fra-1 vaccine is concerned, we demonstrated protein expression of pUb-Fra-1 and pIL-18 by transient transfection of each vector into COS-7 cells and by performing Western blots of the respective cell lysates (pUb-Fra-1 or pIL-18) and supernatant (pIL-

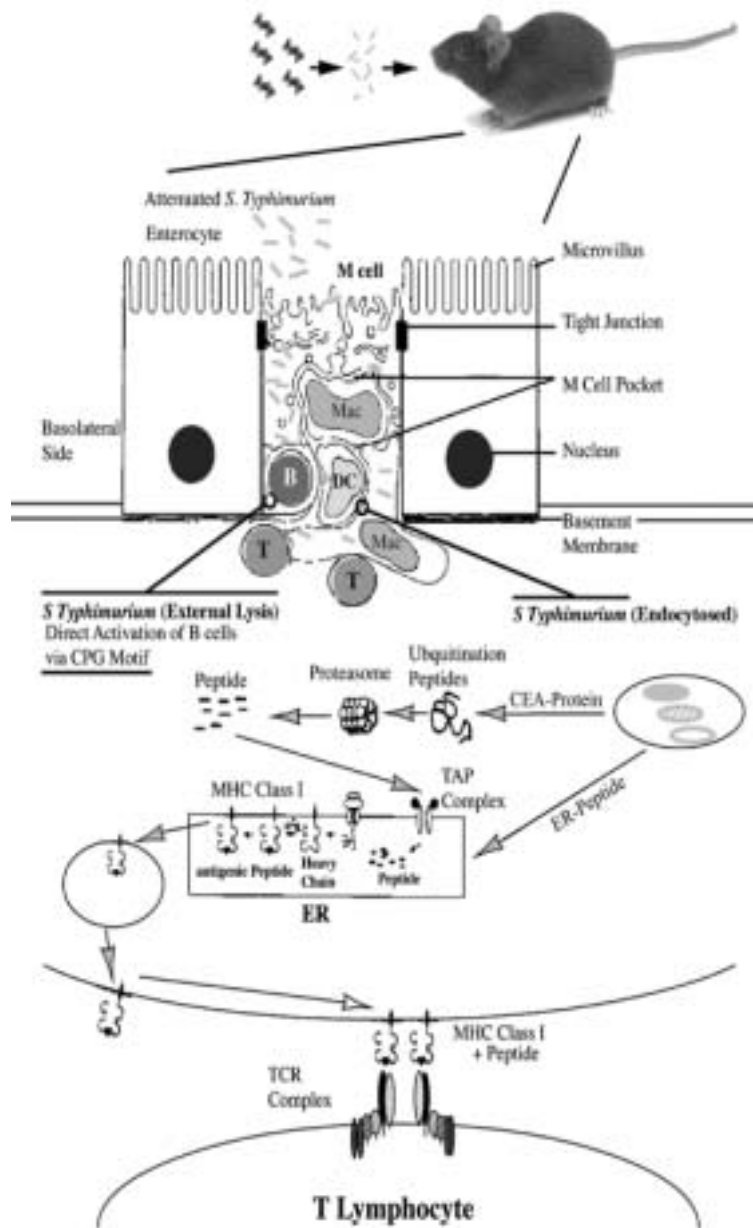


Fig. 1. Schematic diagram of mechanisms of action elicited by DNA vaccines delivered by attenuated *Salmonella typhimurium* to Peyer's patches.

18) with anti-Fra-1 and anti-IL-18 Ab, respectively. As shown in Fig. 2(A), all constructs produced protein of the expected molecular mass with IL-18 being expressed in its active form at 18 KDa (Fig. 2(A), lane 2) and Fra-1 as a 46 KDa protein (Fig. 2(A), lane 1). Protein expression of pIL-18 was also detected in the culture supernatant of transfected cells (Fig. 2(A), lane 3). Importantly, the biofunctional activity of IL-18 was clearly demonstrated by ELISA in supernatants of cells

transfected with pIL-18 (Fig. 2(B)).

Furthermore, the data depicted in Fig. 2(C) demonstrate that the attenuated *Salmonella typhimurium* successfully transferred expression vectors to mouse Peyer's patches. Thus, DNA encoding pUb-Fra-1 and pIL-18 was successfully released from the attenuated bacteria and entered Peyer's patches in the small intestine (Fig. 2(C)). The DNA was subsequently transcribed by APCs, processed in the proteasome, and presented as

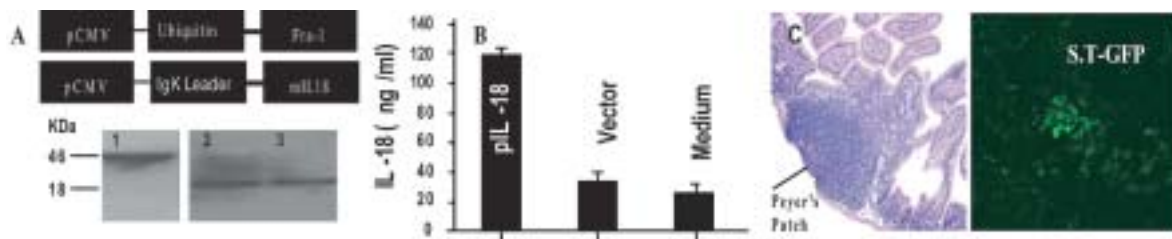


Fig. 2. Vector construction map, protein expression, bioactivity, and targeting of expression constructs. (A) The coding sequence of full-length, murine Fra-1, fused with polyubiquitin at the N terminus, was inserted into the pIRES plasmid (pUb-Fra-1). A second plasmid, pIL-18, contained the entire coding sequence of murine IL-18 with an Igk leader sequence. Protein expression by pUb-Fra-1 and pIL-18 was demonstrated by Western blotting. Blots are shown for either pUb-Fra-1 (lane 1) or pIL-18 (lane 2) and of culture supernatant from pIL-18-transfected COS-7 cells (lane 3). (B) Bioactivity of IL-18 (ng/ml) determined by ELISA in supernatants of KG-1 lymphoma cells that were transfected with pIL-18. The error bars indicate mean standard deviation of multiple assays. (C) Expression of EGFP activity in Peyer's patches was determined in mice immunized with 10^8 CFU of Aro-, dam- bacteria transformed with pEGFP (S.T-GFP) by gavage. Fluorescence expression of EGFP was detected by confocal microscopy (Right). Hematoxylin/eosin staining of mouse Peyer's patches is shown (Left).

MHC-peptide complexes to T cells. To this end, mice were administered by gavage 1×10^8 CFU of dam-, AroA- attenuated *S. typhimurium*. After 24 h these animals were killed and biopsies were collected from the thoroughly washed small intestine. In fact, the doubly attenuated bacteria harboring EGFP (S.T-GFP) exhibited strong EGFP fluorescence (Fig. 2(C)). This finding suggested not only that such bacteria can transfer the target gene to Peyer's patches, but also that plasmids encoding each individual gene can successfully express their respective proteins. Importantly, these doubly attenuated bacteria do not survive very long because neither EGFP activity nor live bacteria could be detected in immunized animals after 72 hours (data not shown). However, EGFP expression was detected in adherent cells, most likely APCs, such as dendritic cells and macrophages from Peyer's patches after oral administration of *S. typhimurium* harboring the eukaryotic EGFP expression plasmid. Taken together, these findings indicate that both plasmid transfer to and protein expression in eukaryotic cells did take place.

IMMUNIZATION WITH BREAST CANCER VACCINES

DNA Vaccine Encoding Fra-1/IL-18

There is a rationale for the development of prophylactic cancer vaccines since considerable data from experimental systems have shown that immunity can be activated to prevent tumors. Thus, there is a strong rationale for prevention since in such a setting one deals with an immune system which is unimpaired by immune suppression induced by tumors and/or treatment. Neither is there tolerance to tumor antigens that were

confronted in the absence of appropriate antigen presentation and costimulatory signals. In such a setting, the use of overexpressed growth factor receptors or transcription factor related antigens yields rational targets for specific immune prevention, also in individuals whose tumors were eradicated by standard therapies. The rationale for developing such prophylactic breast cancer vaccines has thus guided our research efforts described in this article.

Induction of Tumor-Specific Protective Immunity

We tested the hypothesis that an orally administered DNA vaccine encoding murine, pUb-Fra-1 together with secretory pIL-18 (pUb-Fra-1/pIL-18), carried by attenuated *Salmonella typhimurium* can induce protective immunity against s.c. tumors and experimental pulmonary metastases of D2F2 breast carcinoma. Thus, Fig. 3(A) shows marked suppression of disseminated pulmonary metastases in BALB/c mice challenged 1 week after the third vaccination at 2 week intervals with pUb-Fra-1/pIL-18 by an i.v. injection of D2F2 tumor cells. A marked increase in tumor volume of s.c. D2F2 was also evident when this vaccine was applied and compared to a number of controls (Fig. 3(B)). Importantly, the life span of 60% of successfully vaccinated BALB/c mice (5/8) was tripled in the absence of any detectable tumor growth up to 11 weeks after tumor cell challenge (Fig. 3(C)).

Breast Cancer Cells are Killed *in vitro* by Tumor-Specific CTLs and NK Cells

The data shown in Fig. 4 indicate that $CD8^+$ T cells isolated from splenocytes of mice immunized with the vaccine encoding pUb-Fra-1/pIL-18 effectively killed D2F2 breast cancer cells *in vitro* in a ^{51}Cr -release assay.

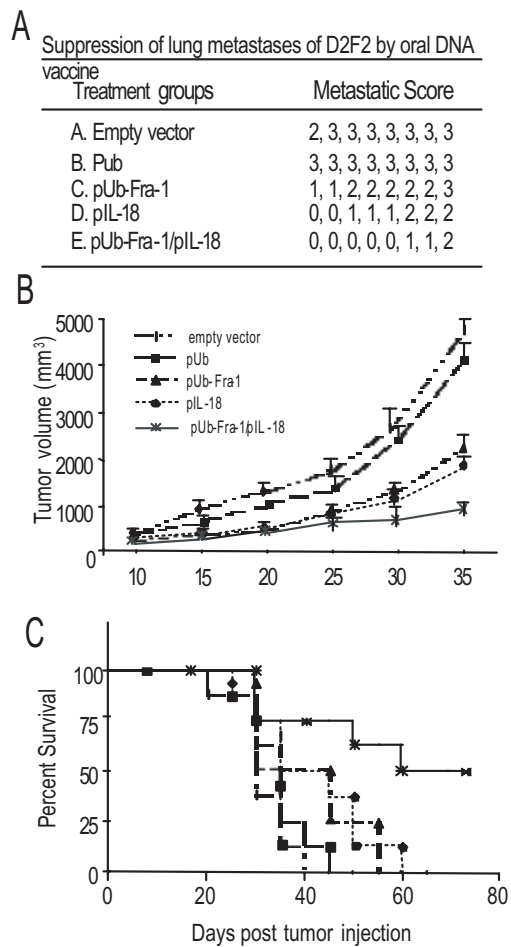


Fig. 3. Effect of the pUb-Fra-1/pIL-18 based DNA vaccine on primary tumor growth and metastases. Each experimental group ($n = 8$) of BALB/c mice was vaccinated by gavage. (A) Suppression of experimental pulmonary metastases of D2F2 breast carcinoma. Results are expressed as metastatic score, i.e. percentage of lung surface covered by fused tumor foci. (B) Tumor growth was analyzed in mice challenged s.c. with 1×10^6 D2F2 tumor cells 1 wk after the last vaccination in each treatment or control group. (C) Survival curves represent results for 8 mice in each of the treatment and control groups. Surviving mice were tumor free unless otherwise stated.

In contrast, such T cells isolated from control animals were ineffective. Thus CTL-mediated killing was specific since syngeneic prostate cancer target cells (RM-2) were not lysed (data not shown). The CD8⁺ T cell-mediated tumor cell lysis was MHC class I antigen-restricted since addition of anti-H2K^d/H2D^d Abs abrogated tumor cell lysis (Fig. 4). NK cells were also effective in killing D2F2 tumor cells in an assay against Yac-1 target cells in contrast to control immunizations which were ineffective (Fig. 4).

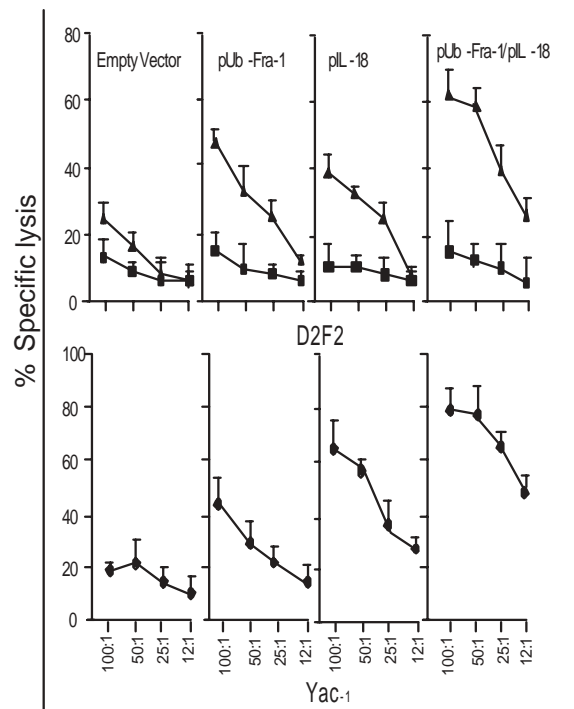


Fig. 4. Cytotoxicity induced by CD8⁺ T and NK cells. Splenocytes were isolated from BALB/c mice after vaccination with experimental or control DNA vaccines 2 wk after challenge with D2F2 tumor cells and analyzed for their cytotoxic activity in a ⁵¹Cr-release assay at different effector-to-target cell ratios. (Upper) Specific lysis mediated by CD8⁺ T cells against D2F2 target cells (▲), which was blocked by an anti-MHC-class I Ab (H-2K^d/H-2D^d) (■). (Lower) Lysis mediated by NK cells (●) against Yac-1 target cells. Each value shown represents the mean of 8 animals.

Specific Activation of T, NK and DCs by the DNA Vaccine

We could demonstrate that interactions between IL-18 and active T helper 1 and NK cells are critical for achieving both optimal Ag-specific T cell and NK cell responses. Thus, the pUb-Fra-1/pIL-18 vaccine or pIL-18 alone up-regulated the expression of the respective T and NK cell markers. This was evident from FACS analyses indicating a marked increase over the empty vector control in expression of CD25 (10.4%), the high affinity IL-2 receptor alpha chain, CD69 on early T cell activation antigen (14.1%), CD28 (12.2%) and CD11a (17.1%), all of which are important for initial interaction between T- and dendritic cells, as well as regular T cell markers CD4⁺ (14.5%) and CD8⁺ (16.1%). Since it is known that NK cells can also play a role in antitumor immune responses (29) we tested spleen cells from immunized and control mice with anti-DX-5Ab and found DX5 expression, important for NK cytotoxicity, to be markedly increased from 2% to 35.3% [10].

Increase of Costimulatory Molecules on DCs

It is well known that T cell activation depends on up-regulated expression of costimulatory molecules CD80 and CD86 on DCs to achieve optimal ligation with CD28 on activated T cells. Thus, we applied fluorescence activated cell sorting analysis (FACS) of splenocytes from successfully immunized mice and controls and found that expression of CD80 and CD86 was markedly upregulated by the vaccine on CD11c⁺ DCs by 10% and 9.5%, respectively [10].

Activation of T cells is Indicated by Increased Secretion of IFN- γ and IL-2

We analyzed for the release of these two proinflammatory cytokines from T cells since this is a well known indication of their activation in secondary lymphoid tissues. When analyzing for these cytokines both intracellularly with flow cytometry or at the single cell level by ELISPOT we found that vaccination with the pUb-Fra-1/pIL-18 plasmid and a subsequent challenge with D2F2 tumor cells resulted in marked increases of IFN- γ and IL-2 release over that induced by controls [10].

The pUb-Fra-1/IL-18 DNA Vaccine Suppresses Angiogenesis

We demonstrated distinct suppression of angiogenesis induced by our DNA vaccine in a Matrigel assay. Figure 5 shows a marked decrease in vascularization. This was evident from evaluation of fluorescence after *in vivo* staining of endothelium of mice with FITC-conjugated lectin. As shown in Fig. 5, such differences were visible macroscopically in representative Matrigel plugs removed from vaccinated mice 6 days after their injection. Suppression of angiogenesis is clearly demonstrated by FITC-lectin staining as shown by decreased vascularization in Matrigel plugs after vaccination with pUb-Fra-1/pIL-18 and to a lesser extent with pIL-18, but not with control vaccines [10].

DNA Vaccine Encoding Endoglin

Endoglin (CD105) which is part of the TGF- β_1 /TGF- β_3 receptor complex is overexpressed by proliferating endothelial cells in the D2F2 breast tumor vasculature, but not expressed by D2F2 tumor cells. Therefore, this antigen provides an effective target for a DNA vaccine encoding it which, in turn, induces a cytotoxic T cell mediated immune response that can destroy these endothelial cells and thereby suppress angiogenesis in the tumor cell vasculature resulting in suppression of breast tumor growth. This approach is in principle sim-

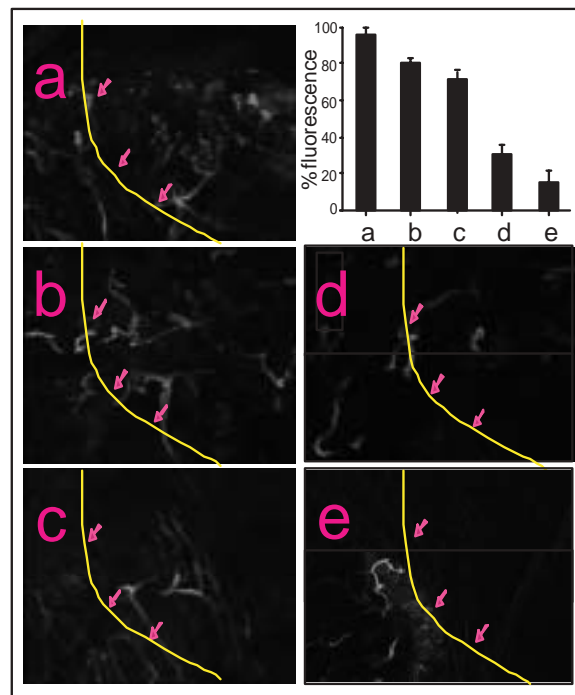


Fig. 5. Suppression of tumor angiogenesis. Antiangiogenesis was determined by the Matrigel assay. Quantification of vessel growth and staining of endothelium was determined by fluorimetry or confocal microscopy, respectively, using FITC-labeled Isolectin B4. The line and arrows (a-e) indicate the inside borders of the Matrigel plug. Matrigel was implanted into mice vaccinated with empty vector (a), pUb (b), pUb-Fra-1 (c), pIL-18 (d), or pUb-Fra-1/pIL-18 (e). The average fluorescence of Matrigel plugs from each group of mice is depicted by the bar graphs ($n = 4$; mean \pm SD). Comparison of control groups (a and b) with treatment groups (d and e) indicated statistical significance ($P < 0.05$).

ilar to the one we reported previously for a DNA vaccine encoding the murine vascular endothelial growth factor receptor-2 (FLK-1) which is also overexpressed on proliferating endothelial cells in the tumor vasculature, but not expressed by tumor cells. In this case, we could demonstrate a CD8⁺ T cell mediated immune response resulting in marked suppression of tumor angiogenesis in three mouse tumor models which resulted in eradication of tumor growth and metastases in both prophylactic and therapeutic settings [11].

For all the reasons that were cited above under "Selection of Target Antigens for DNA Vaccines", the genetically stable endothelial cells in the tumor vasculature offer a far better target for cytotoxic T cells than the genetically unstable, frequently mutating tumor cells which often down regulate critical MHC antigens and induce immune suppression at the cellular level. Here, we present some preliminary data providing evidence that a DNA vaccine encoding the entire murine en-

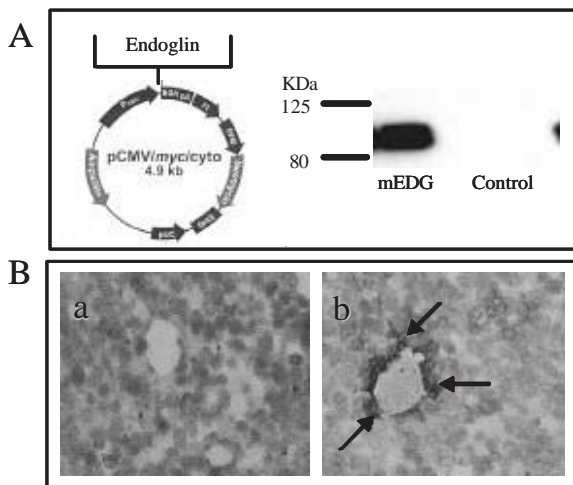


Fig. 6. Schematic of vectors encoding murine endoglin indicating that the entire gene was inserted into the pCMV/myc/cyto expression vector (A). Protein expression was demonstrated by Western blotting of cell lysates from COS-7 cells transfected with endoglin (lane 1)(B). Endoglin was detected by immunohistology in the D2F2 breast tumor vasculature by staining frozen sections of tumor tissue with anti-endoglin Ab showing endoglin lining a blood vessel (b) while a control reveals only background staining (a). Endoglin is not expressed by D2F2 tumor cells.

doglin gene can lower angiogenesis in the tumor vasculature and suppress pulmonary metastases of D2F2 murine breast carcinoma cells in BALB/c mice.

Construction of Endoglin Expression Vector and Protein Expression

The entire gene encoding murine endoglin (CD105) was inserted into the pCMV/myc/cyto expression vector (Fig. 6(A)). Protein expression by endoglin was demonstrated by Western blotting of cell lysates from COS-7 cells transfected with endoglin (lane 1). In Fig. 6(B), endoglin is demonstrated by immunohistology in the D2F2 breast tumor vasculature by staining a frozen section of D2F2 tumor tissue with anti-endoglin Ab showing endoglin lining a blood vessel indicated by arrows while a control tissue reveals only background staining. FACS analyses showed the absence of endoglin expression by D2F2 mouse breast tumor cells (data not shown).

Suppression of D2F2 Pulmonary Breast Carcinoma Metastases

To test the efficacy of the vaccine, BALB/c mice were administered by gavage 10^8 CFU of doubly attenuated *Salmonella typhimurium* (dam⁻; AroA⁻) transformed by electroporation with the plasmid encoding endoglin. Vaccination was done by gavage in 100 μ l

sterile water, 3 times at 2 week intervals in BALB/c mice who received 15 minutes prior to vaccination 50 μ l of 7.5% sodium bicarbonate (SB) to neutralize the acid pH of the stomach which is detrimental to the bacteria. One week after the last vaccination, experimental pulmonary metastases were induced by i.v. injection of 5×10^4 D2F2 breast carcinoma cells. The experiment was terminated 28 days after tumor cell challenge and the weight of lungs and extent of tumor foci determined. Figure 7(A) depicting the lungs of mice receiving SB only (a) SB+ empty vector (b) and endoglin vaccine (c), clearly demonstrating that only the vaccine drastically suppressed pulmonary metastases. This is also shown in Fig. 7(B) indicating the weight of the lungs, (normal lung=0.2 g). The extent of metastases is also demonstrated in Fig. 7(C) which shows the metastasis score, i.e. the percent of lung cell surface covered by fused metastases: 0 = none; 1 = < 5%; 2 = 5–50% and 3 = >50% of lung surface covered by metastatic foci. Differences in metastasis scores and lung weights between mice treated with the vaccine and all controls were statistically significant ($P < 0.001$).

Cell-mediated Cytotoxicity Induced by the Endoglin DNA Vaccine Against Murine Endothelial Cells

To assess T cell mediated cytotoxicity, splenocytes of mice ($n = 8$) treated with either the endoglin vaccine or controls treated with SB+empty vector were isolated one week after tumor cell challenge, and analyzed for cytotoxicity in a 4-h ^{51}Cr release assay. Splenocytes from mice immunized with the endoglin vaccine revealed up to 45% cytotoxicity against murine endothelial target cells expressing endoglin when compared to those from controls which showed only background cytotoxic activity of 10% or less ($P < 0.005$).

Suppression of Angiogenesis

The DNA vaccine encoding endoglin, overexpressed by proliferating endothelial cells in the tumor vasculature, but not expressed by D2F2 breast tumor cells, suppressed angiogenesis in the tumor vasculature. This was evident when angiogenesis was measured by the Matrigel assay. In this case, one week after the last of 3 vaccinations with the endoglin vaccine, BALB/c mice were injected s.c. at the abdominal midline with Matrigel, supplemented with basic fibroblast growth factor. Six days later, the endothelium of these mice was stained by i.v. injection of Bandiera simplofica Isolectin conjugated with fluorescein, and 30 minutes thereafter matrigel plugs of 100 μ l were evaluated microscopically and then eluted with RIPA lysate buffer

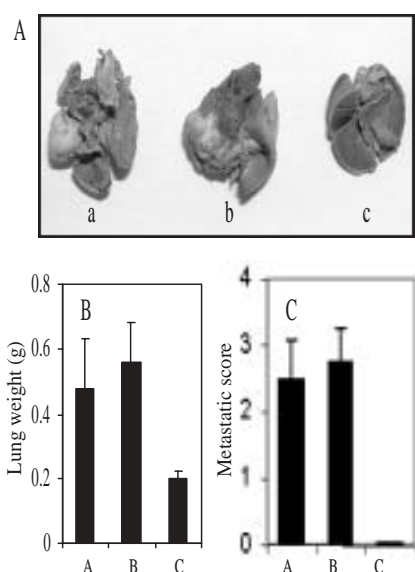


Fig. 7. Experimental lung metastases were induced by i.v. injection of D2F2 cells one week after the last of 3 immunizations administered at 2-week intervals with the endoglin vaccine. The vaccine was administered by gavage to BALB/c mice who received 15 min prior to vaccination 50 μ l of 7.5% sodium bicarbonate (SB) to neutralize the acid pH of the stomach. Results are shown in Fig. 7(A) depicting lungs of mice receiving SB only (a), SB + empty vector (b), and the endoglin vaccine (c). Figure 7(B) depicts the average lung weight of experimental mice ($n = 8$) in the same order as shown in Fig. 7(A). Figure 7(C) shows the metastatic score, i.e. the percent of lung cell surface covered by fused metastases: 0=none; 1 =<5%; 2=5–50% and 3=>50%.

to measure fluorescence. As depicted in Fig. 8 it is quite evident that the endoglin vaccine effectively suppressed angiogenesis in the tumor vasculature when compared to controls. Quantification of vessel growth and staining of endothelium was determined by fluorimetry or confocal microscopy. The average fluorescence of Matrigel plugs from each experimental group of mice is depicted by the bar graphs (Fig. 8) where differences between the vaccine and control groups were statistically significant ($n = 4$; $P < 0.05$).

CONCLUSIONS AND PERSPECTIVES

Taken together, our data suggest that peripheral T cell tolerance against the Fra-1 transcription factor was broken by a DNA vaccine encoding it, particularly when fused with polyubiquitin and modified by co-transformation with a gene encoding murine IL-18. Furthermore, a robust immune response was mediated by CD4⁺ T cells, CD8⁺ T cells and NK cells controlled by upregulation of IFN- γ . Our vaccine design

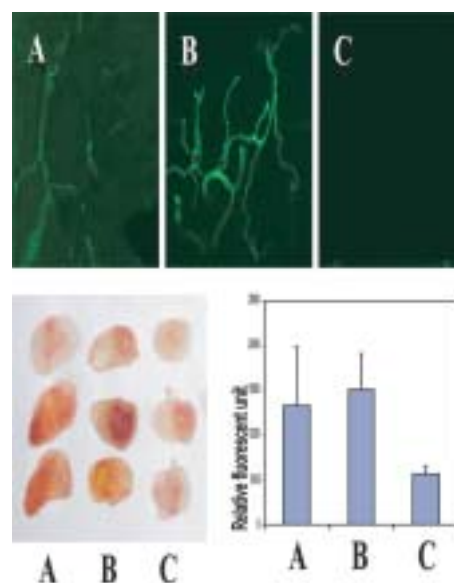


Fig. 8. Suppression of tumor angiogenesis by the DNA based vaccine encoding endoglin. Antiangiogenesis was determined by the Matrigel assay. Quantification of vessel growth and staining of endothelium was determined by fluorimetry or confocal microscopy, respectively, using empty vector (A), SB alone (B), SB+ empty vector (C), SB+endoglin vaccine. The average fluorescence of Matrigel plugs from each group of mice is depicted by the bar graphs ($P < 0.05$)($n = 4$; mean \pm SD).

was successful since activation of both T and NK cells was augmented as was that of dendritic cells as indicated by upregulation of CD80 and CD86 costimulatory molecules. We believe that our success in eliciting an active CD8⁺ T cell mediated tumor protective immune response with a completely autologous DNA vaccine, as well as the induction of antiangiogenesis was aided by our efforts to optimize antigen processing in the proteasome with polyubiquitination [10,11, 24–26]. Also, one of the more critical aspects of DNA vaccine design is the selection of an optimal carrier to deliver a target gene to secondary lymphoid organs, such as Peyer's patches. In this case, the live, attenuated *Salmonella typhimurium* harboring eukaryotic expression plasmids encoding Ag proved to be an effective vehicle for oral vaccine delivery, especially when combined with an adjuvant like IL-18.

Although the data obtained with the DNA vaccine encoding endoglin (CD105) are still preliminary, they suggest that a prophylactic vaccine capable of suppressing angiogenesis in the tumor vasculature could be of value for future clinical application.

As pointed out in this article the targeting of genetically stable proliferating endothelial cells rather than genetically unstable, often mutating tumor cells has its

advantages. The rationale for this approach was provided by our prior findings with a DNA vaccine against VEGF receptor 2 (FLK-1), overexpressed by proliferating endothelial cells in the tumor vasculature, which effectively suppressed angiogenesis and thereby tumor growth and metastasis in several solid tumor models [11]. Although the vast majority of immunotherapeutic regimens for breast cancer is directed against tumor cells per se, we believe it advantageous to also consider targeting proliferating endothelial cells in the tumor vasculature leading to suppression of both tumor angiogenesis and tumor growth.

As far as future perspective for breast cancer vaccines are concerned, it will be advantageous to direct DNA vaccines against inhibitors of apoptosis (IAPs), co-expressing secretory chemokines in order to promote tumor cell apoptosis, an effort currently ongoing in our laboratory with a DNA vaccine directed against the IAP Survivin which is overexpressed by most solid tumor cells. Since this IAP is also overexpressed by proliferating endothelial cells in the tumor vasculature, it is feasible for such a vaccine to both suppress angiogenesis in the tumor vasculature and induce tumor cell apoptosis, thereby concurrently producing a very potent anti-tumor effect. It is anticipated that such future research efforts will further contribute towards the rational design of DNA vaccines for the prevention and treatment of breast cancer in a setting of minimal residual disease.

ACKNOWLEDGEMENTS

We thank D. Markowitz and C. Dolman for advice and technical assistance and Kathy Cairns for preparation of this manuscript. Studies described in this chapter were supported in part by Department of Defense Grant DAMD17-02-1-0562 (to R.X.), Tobacco-Related Disease Research Program Grant 9RT-0017 (to R.A.R.) and E. Merck, Darmstadt-Lexigen Research Center (Billerica, MA.) Grant SFP1330 (to R.A.R.). This is The Scripps Research Institute's manuscript number 16204-IMM.

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Endoglin (CD105) is a target for an oral DNA vaccine against breast cancer

Received: 29 December 2005 / Accepted: 6 March 2006
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Abstract Endoglin (CD105), a co-receptor in the TGF- β receptor complex, is over-expressed on proliferating endothelial cells in the breast tumor neovasculature and thus offers an attractive target for anti-angiogenic therapy. Here we report the anti-angiogenic/anti-tumor effects achieved in a prophylactic setting with an oral DNA vaccine encoding murine endoglin, carried by double attenuated *Salmonella typhimurium* (*dam*⁻, *AroA*⁻) to a secondary lymphoid organ, i.e., Peyer's patches. We demonstrate that an endoglin vaccine elicited activation of antigen-presenting dendritic cells, coupled with immune responses mediated by CD8⁺ T cells against endoglin-positive target cells. Moreover, we observed suppression of angiogenesis only in mice administered with the endoglin vaccine as compared to controls. These data suggest that a CD8⁺ T cell-mediated immune response induced by this vaccine effectively suppressed dissemination of pulmonary metastases of D2F2 breast carcinoma cells presumably by eliminating proliferating endothelial cells in the tumor vasculature. It is anticipated that vaccine strategies such as this may contribute to future therapies for breast cancer.

Keywords DNA vaccine · Endoglin · Breast cancer · Anti-angiogenesis

Introduction

Angiogenesis, the growth of new capillary blood vessels from preexisting vasculature, is an essential feature

of tumor growth and metastasis. In fact, anti-angiogenic therapy, originally proposed by Folkman more than 30 years ago [10] has become a most attractive concept, receiving ever increasing attention during the last decade [12, 14, 34, 41]. The goal of this approach has been to deliver anti-angiogenic agents to appropriate targets in the tumor vasculature to eliminate or suppress blood supply to tumors, resulting in their ablation or growth suppression without seriously disturbing blood flow to normal tissues [3]. Several approaches have been reported to suppress murine tumor growth and metastasis through anti-angiogenesis by targeting specific molecules such as vascular endothelial growth factor receptor 2 (VEGF-R2) [31]. Our laboratory also demonstrated that a survivin-based oral DNA vaccine, coexpressing the chemokine CCL21, induced effective suppression of angiogenesis by triggering potent CTLs against tumor cells and proliferating endothelial cells expressing survivin, resulting in the suppression or eradication of metastases in a murine tumor model [47].

Endoglin (CD105) is a 180-kDa homodimeric transmembrane glycoprotein, primarily expressed on endothelial cells. It acts as an auxiliary protein that interacts with the ligand-binding receptors of multiple members of the transforming growth factor beta (TGF- β) superfamily [4]. Studies have suggested that endoglin offers an excellent target for anti-angiogenic therapy since it is over-expressed on proliferating endothelial cells in blood vessels of tumor tissue. In fact, endoglin and its ligand, TGF- β , are significant modulators of angiogenesis [16, 19]. Moreover, endoglin expression on endothelial cells is up-regulated by TGF- β and hypoxic conditions [40]. In solid tumors such as breast carcinoma, endoglin is almost exclusively expressed on endothelial cells of both peri- and intratumoral blood vessels and on tumor stromal components [5]. Furthermore, a monoclonal endoglin Ab was reported to react with small and immature tumor blood vessels in prostate and breast cancer [46], and to strongly stain endothelial cells, but not smooth muscle cells associated with blood vessels

S.-H. Lee and N. Mizutani contributed equally to this manuscript.

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within all tumor lesions investigated [7]. In addition, quantifying tumor microvessel density with this same Ab also proved to be an independent prognostic parameter for survival of colorectal cancer patients [45]. Taken together, these data suggest the involvement of endoglin in tumor angiogenesis and point it as a candidate for vascular targeting in tumor therapy, especially since endoglin is not detectable in blood vessels within normal tissues [18, 20, 38]. In fact, Seon et al. [42] successfully applied an anti-human endoglin immunotoxin to inhibit growth of subcutaneous MCF7 human breast carcinoma in SCID mice. Recently, synergy was demonstrated between endoglin mAbs and TGF β in growth suppression of human endothelial cells in vitro, suggesting that TGF- β plays a key role by synergistically enhancing the anti-angiogenic activity of such antibodies [43]. In addition, endoglin-based xenogeneic vaccination was shown to effectively elicit both protective and therapeutic anti-tumor immunity in several mouse tumor models [44]. Preliminary data obtained in our laboratory suggested that a DNA vaccine encoding the entire murine endoglin gene suppressed angiogenesis and pulmonary metastases of murine breast carcinoma [29].

Here, we demonstrate that a DNA vaccine encoding murine endoglin was delivered orally by attenuated *Salmonella typhimurium* to secondary lymphoid organs such as Peyer's patches (PPs). This vaccine overcame peripheral T cell tolerance and induced a robust CD8⁺ T cell mediated immune response that inhibited angiogenesis, resulting in suppression of pulmonary breast tumor metastases and increased life-span of tumor bearing, syngeneic BALB/c mice in a prophylactic setting.

Materials and methods

Animals, bacterial strains, and cell lines

Female BALB/c mice, 6–8 weeks of age, were purchased from the Scripps Research Institutes (La Jolla, CA, USA) Rodent Breeding Facility. All animal experiments were performed according to the NIH Guides for the Care and Use of Laboratory Animals.

The double-attenuated *S. typhimurium* strain RE88 (*dam*⁻; *AroA*⁻) was obtained from Remedyne Inc. (Santa Barbara, CA, USA).

The murine D2F2 breast cancer cell line was kindly provided by Dr. W-Z. Wei (Karmanos Cancer Institute, Detroit, MI, USA) and cultured as previously described [49]. The murine high endothelial venule cell line (HEVc) was a gift from Dr. J.M. Cook-Mills (University of Cincinnati, OH, USA). The mEndo⁺-D2F2 cell, were obtained by transfecting vector encoding full-length endoglin into D2F2 cells with DMRIE-C Reagent (Invitrogen, Carlsbad, CA, USA). Endoglin positive cells were purified by FACSort and maintained in selection medium containing Neomycin (1 mg/ml).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted with the RNeasy mini kit or RNeasy tissue kit (Qiagen, Valencia, CA, USA) from D2F2, HEVc cells or normal mouse spleen or liver. Reverse transcription was performed with 1 μ g of total RNA followed by PCR with specific endoglin primers: TCG ATA GCA GCA CTG GAT (forward), and ATC TAG CTG GAC TGT GAC (reverse). Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as control.

Immunohistochemistry

Frozen sections were fixed and stained with anti-endoglin Ab (BD PharMingen, San Diego, CA, USA), followed by treatment with biotinylated anti-rat IgG Ab and HRP-conjugated streptavidin (Vector Laboratories, Inc, Burlingame, CA, USA). The DAB substrate (Sigma, St. Louis, MO, USA) was added and slides were examined microscopically.

Vector construction and Western blotting

Full-length murine endoglin was cloned from tumor tissue. The vector was constructed based on the pCMV vector (Invitrogen). The empty vector served as a control. Western blot analysis was performed with transiently transfected COS-7 cell lysates using monoclonal rat anti-mouse endoglin Ab (Cymbus Biotechnology, UK).

Transformation of attenuated *S. typhimurium* and expression of endoglin in vivo

Attenuated *S. typhimurium* (*dam*⁻; *AroA*⁻) were transformed with DNA vaccine plasmids by electroporation [26]. Freshly prepared bacteria (1×10^8) were mixed with plasmid DNA (2 μ g) on ice in a 0.2-cm cuvette and electroporated at 2.5 kV, 25 μ F, and 200 Ω . Resistant colonies harboring the vaccine vectors were cultured and stored at -70°C after confirmation of their coding sequence.

Peyer's Patches were dissected from the mouse small intestines [22] 24 h after vaccination. Frozen sections were fixed, blocked and stained with unlabeled anti-endoglin Ab and Alexa 568-conjugated goat-anti-rat Ab (Molecular Probes), followed by biotin labeled anti-CD11c Ab and streptavidin-Alexa 488 (Molecular Probe). Slides were air dried and mounted with Vectashield (Vector Laboratories) and analyzed by confocal microscopy with a Zeiss Axioplan/BioRad MRC 1024 confocal microscope.

Oral immunization and tumor challenge

BALB/c mice were divided into three experimental groups ($n=8$) and immunized three times at 1 week intervals by gavage with 100 μ l 5% sodium bicarbonate containing approximately 1×10^8 double attenuated *S. typhimurium* harboring either empty vector or murine endoglin vector (mEndoglin). All mice were challenged by i.v. injection of 1.5×10^5 D2F2 murine breast carcinoma cells 1 week after the last immunization. Mice were monitored and sacrificed as indicated.

In vivo depletion of CD4⁺ or CD8⁺ T cell populations

mAbs against CD8 (2.43: rat mAb, IgG_{2b}) or CD4 (GK1.5: rat mAb, IgG_{2b}) were purchased from The National Cell Culture Center (NCCC, Minneapolis, MN, USA). Immunized mice were injected i.v. with anti-CD4 or anti-CD8 mAb (0.5 mg/mouse) 1 day before D2F2 tumor cell challenge, followed by weekly i.p. injection of mAbs until sacrifice.

In vitro cytotoxicity assay

Cytotoxicity was performed by a standard [³⁵S] release assay [24, 25]. Splenocytes were prepared from immunized mice 14 days after tumor cell challenge, and restimulated in vitro for 4 days on a monolayer of irradiated (1,000 Gy) and mitomycin C-treated (80 μ g/10⁷ cells, 45 min at 37°C) mEndo⁺-D2F2 cells. Viable lymphocytes were separated by Lympholyte-M (Cendlerlane, ON, Canada) gradient centrifugation and mixed at different ratios with [³⁵S] methionine-labeled target cells for 5 h. Supernatants (100 μ l) were harvested and measured in a mixture with scintillation fluid. Percent specific lysis was calculated by the formula; $[(E-S)/(T-S)] \times 100$, where E is the average experimental release, S the average spontaneous release, and T the average total release.

Flow cytometric analysis

T cell activation was assessed by staining freshly isolated splenocytes from vaccinated mice with FITC-labeled anti-CD8 Ab in combination with PE-conjugated anti-CD28 Ab. DCs were analyzed by PE-conjugated anti-CD80/CD86 mAbs in combination with FITC-labeled anti-CD11c mAbs. All reagents were obtained from BD Pharmingen. D2F2 or HEVc cells were stained with PE-labeled rat anti-mouse endoglin mAb or isotype control Ab (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA). Flow cytometry were performed with a FACScan (Becton Dickinson, San Jose, CA, USA) and the data analyzed with FlowJo software (Tree Star, Inc, Stanford, CA, USA).

ELISPOT assay

The number of IFN- γ secreting cells was determined with an ELISPOT kit (BD Pharmingen) according to the manufacturer's instructions. Briefly, splenocytes were collected 10 days after the last immunization from all experimental groups. T cells were isolated from splenocytes on a Nylon Wool Column (Polysciences, Inc., Warrington, PA, USA). Purified T cells (2×10^5 /well) were cultured for 24 h with 2×10^4 /well of irradiated (1,000 Gy) D2F2 cells, mEndo⁺-D2F2 cells or HEVc cells.

Evaluation of anti-angiogenic activity

One week after the last vaccination, mice were injected s.c. near the abdominal midline with 500 μ l of growth factor reduced Matrigel (BD Pharmingen) containing 400 ng/ml bFGF (PeproTech, Princeton, NJ, USA). Mice were injected 6 days later with 200 μ l (0.1 mg/ml) isolectin B4 conjugated with fluorescein (Vector Laboratories) to stain the endothelium. Mice were sacrificed 15 min thereafter and Matrigel plugs were homogenized with RIPA lysis buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). After centrifugation, the fluorescein content in the supernatant was quantified by fluorimetry at 490 nm. Background fluorescence found in the non-injected control was subtracted in each case [1, 27].

Statistical analysis

The statistical significance of different finding between experimental groups and controls was determined by Student's t test and considered significance it two-tailed P values was < 0.05 .

Results

Determination of endoglin expression in vitro and in vivo

Endoglin expression by the murine breast tumor cell line D2F2, the murine endothelial cell line HEVc and normal mouse spleen and liver was assessed by RT-PCR. Results indicated that HEVc strongly express endoglin (Fig. 1a). However, endoglin is absent in D2F2 cells or normal liver tissue under these experimental conditions. Low levels of endoglin expression in spleen were also observed (Fig. 1a). We further confirmed this finding by FACS analysis: HEVc cells express endoglin on the surface, but endoglin is not detectable on D2F2 tumor cells (Fig. 1b). However, endothelial cells in metastatic D2F2 lung tumor tissue highly express endoglin, while endoglin expression is barely detectable in normal lungs (Fig. 1c). Thus, these results confirm that the expression

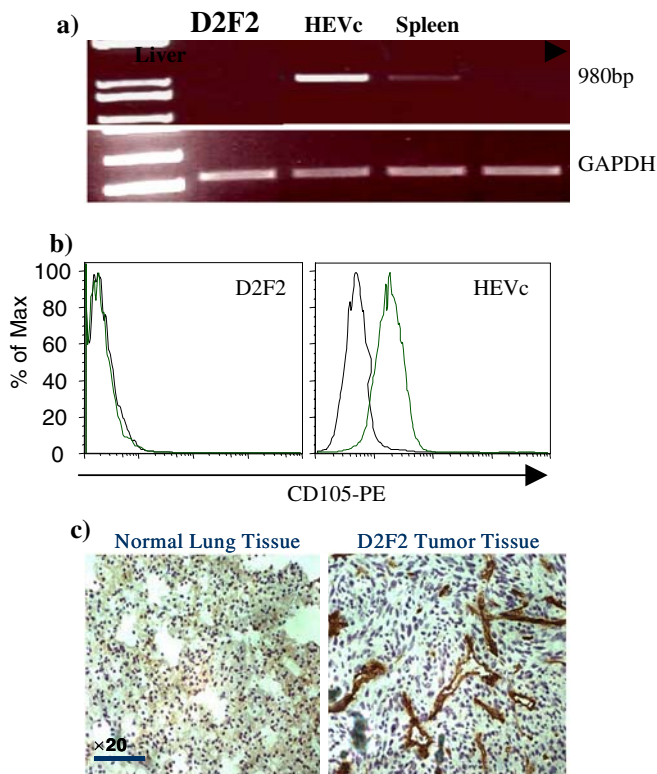


Fig. 1 Expression of murine endoglin by tumor cells lines and normal mouse tissues. **a** RT-PCR analysis of endoglin expression by wild-type D2F2 breast carcinoma cells, HEVc endothelial cells, as well as normal mouse spleen and liver. Total RNA extraction and RT-PCR were performed according to “[Material and methods](#)” and GAPDH was used as control for total RNA loading. **b** FACS analyses of endoglin expression on the cell surface of D2F2 cells and HEVc cells, both stained with PE-conjugated anti-endoglin Ab. PE-labeled Rat IgG Ab served as isotype control (*black line*). **c** Comparison of endoglin expression levels on normal lungs and lung metastases of D2F2 tumor induced by i.v. injection of breast tumor cells (1×10^6 cells/mouse). Mice were sacrificed when inoculated animals were moribund and frozen lung sections analyzed by immunostaining with anti-endoglin Ab ($\times 20$ magnification)

level of endoglin is significantly up-regulated on proliferating endothelial cells, despite the fact that D2F2 breast tumor cells themselves do not express detectable levels of endoglin [17].

The mEndoglin vaccine is delivered to PP

To test our hypothesis that an oral DNA vaccine encoding endoglin induces a T cell-mediated immune response, we first inserted the entire gene encoding murine endoglin into the pCMV/myc/cyto expression vector (Fig. 2a). Protein expression of endoglin was demonstrated by a single band of expected molecular weight (90 kDa) detected by Western blots of lysates of COS-7 cells transiently transfected with mEndoglin (Fig. 2a).

Our oral DNA vaccination strategy using double attenuated *S. typhimurium* (*dam*⁻; *AroA*⁻) is designed to achieve successful in vivo delivery of plasmids to sec-

ondary lymphoid organs, i.e. PPs, to facilitate subsequent priming of specific T cells. To confirm endoglin expression after vaccination, mice were sacrificed 24 h after oral vaccine administration and PP collected from the thoroughly washed small intestine. Confocal microscopy demonstrated that CD11c⁺ DC sub-populations expressed endoglin intracellularly in PP of mEndoglin-vaccinated mice (Fig. 2b). However, endoglin was not detected in CD11c⁺ DC cells from PPs of control mice.

The mEndoglin vaccine induces suppression of D2F2 breast tumor metastases

We tested the efficacy of mEndoglin vaccine in a prophylactic setting, in which disseminated pulmonary metastases were induced in mice challenged by i.v. injection of 1.5×10^5 D2F2 breast carcinoma cells 1 week after the last vaccination. Whenever control mice showed signs of morbidity, all animals were sacrificed and evaluated for lung metastases and lung weights. Results (Fig. 3a) indicated that all mice receiving either PBS or empty vector presented with extensive disseminated pulmonary metastases. In contrast, all mEndoglin-vaccinated mice exhibited significant suppression of pulmonary metastases when compared to control mice ($P < 0.05$). In addition, in survival studies, all control mice (PBS or CMV groups) died within 4 week after tumor cell challenge due to extensive metastases; however, mice immunized with the mEndoglin vaccine had a 60% prolongation in life span (Fig. 3b).

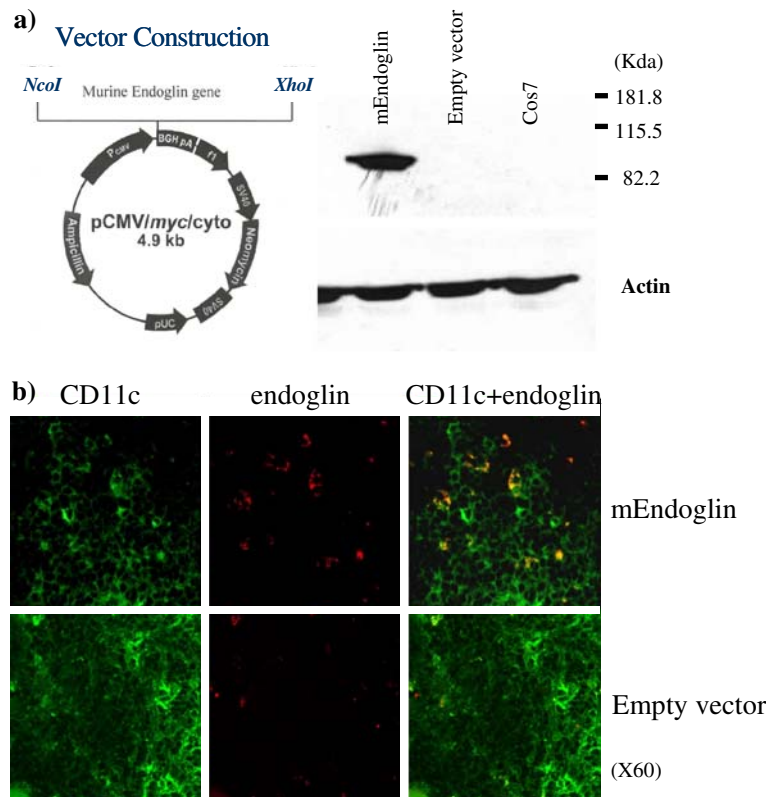
The anti-tumor effects induced by the mEndoglin vaccine are mediated by CD8⁺ T cells

To determine the roles of cell subpopulations played in mEndoglin vaccine-induced suppression of pulmonary metastases, in vivo depletions of CD4⁺ or CD8⁺ were performed (Fig. 4). We observed that non-depleted, vaccinated mice effectively suppressed D2F2 pulmonary metastases when compared to the empty control vector mice ($P < 0.05$); however, this suppression of pulmonary metastases was abrogated in mice depleted of CD8⁺ T cells (Fig. 4), indicating that CD8⁺ T cells play a major role in suppressing D2F2 pulmonary metastases. In contrast, in vivo depletion of CD4⁺ T cells did not significantly affect suppression of D2F2 pulmonary metastases, suggesting that CD4⁺ T cells do not play a major role in anti-tumor effects induced by the mEndoglin vaccine.

The mEndoglin vaccine induces T cell and DC activation

We then investigated whether the anti-tumor activity of the mEndoglin vaccine correlated with T cell acti-

Fig. 2 Schematic of a vector encoding murine endoglin and expression in vitro and in vivo. **a** Protein expression of endoglin was detected by Western blotting of endoglin-transfected COS-7 cell lysates with anti-endoglin Ab. **b** Endoglin expression of CD11c⁺ DCs in Peyer's patches isolated 24 h after vaccination. Sections were stained with Ab against CD11c (green) and Ab against endoglin (red), and examined for expression by confocal microscopy. Samples derived from mice administered with empty vector served as a negative control



vation. This was evident from the increased expression of CD28, an important marker of activated T cells (Fig. 5), especially since optimal T cell activation is critically dependent on the ligation of CD28 with costimulatory molecules CD86 and CD80 on DCs. In this regard, FACS analyses of splenocytes obtained from vaccinated mice clearly demonstrated that the expression of both CD80 and CD86 on CD11c⁺ DCs was up-regulated when compared with control animals (Fig. 5).

Immunization with the mEndoglin vaccine evokes endoglin-specific CTLs

In order to assess whether CD8⁺ T cells are able to specifically lyse endoglin-positive target cells, we generated endoglin-expressing D2F2 cells (mEndo⁺-D2F2) by transfection of D2F2 cells with the endoglin plasmid. These cells expressed endoglin on the surface (Fig. 6a), in comparison to wild-type D2F2 cells that did not express endoglin (Fig. 1b).

ELISPOT analysis for IFN γ secretion was performed to determine the frequency of endoglin-specific T cells in mEndoglin-vaccinated mice. The number of spots markedly increased when such cells were co-cubated with irradiated mEndo⁺-D2F2 cells as stimulators when compared to stimulation with wild-type D2F2 cells (Fig. 6b). These data indicate the success in expanding endoglin-specific cells in mEndoglin-vaccinated mice.

Furthermore, we determined whether such activated T cells could eliminate endoglin-expressing endothelial target cells. The results (Fig. 6c) indicate that endothelial HEVc target cells, which naturally express endoglin, are susceptible to lysis by effector cells obtained from mEndoglin-vaccinated mice. In contrast, T cells from control mice showed low level of killing (Fig. 6c).

We next examined the specificity of the vaccine-induced cytotoxicity. In fact, mEndo⁺-D2F2 target cells were two times more sensitive to CTL killing than wild-type D2F2 cells ($P < 0.05$, Fig. 6d). Moreover, mEndo⁺-D2F2 cells were more susceptible to lysis by effector cells obtained from mEndoglin-vaccinated mice than by those from control mice (Fig. 6d). These data indicate that the mEndoglin vaccine effectively induced the specific elimination of endoglin-positive target cells.

The mEndoglin vaccine elicits suppression of angiogenesis

We assessed whether the mEndoglin vaccine could suppress angiogenesis. In this regard, a Matrigel assay revealed a significant decrease in neovascularization only in mice immunized with mEndoglin vaccine (Fig. 7). In fact, quantification of relative fluorescence intensity, measured after in vivo staining of mouse endothelium with FITC-conjugated lectin, clearly indicated that the angiogenic process in such experimental animals decreased significantly in comparison to control mice ($P < 0.05$).

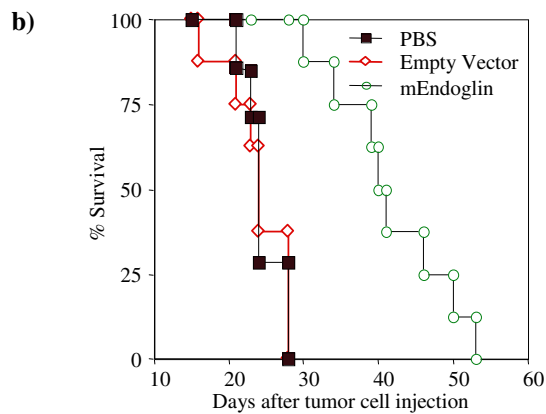
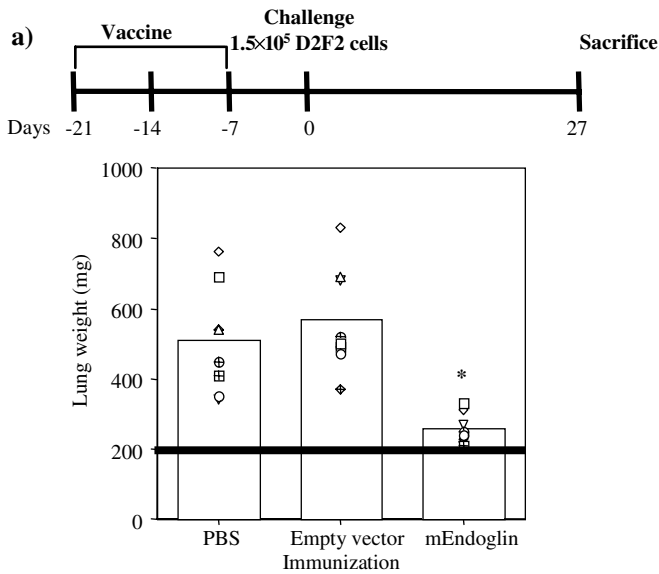


Fig. 3 Suppression of pulmonary metastases of D2F2 breast carcinoma by the mEndoglin vaccine in prophylactic tumor models. Lung metastases were induced by i.v. injection of 1.5×10^5 D2F2 cells, 1 week after the last immunization. Experiments were terminated 27 days after tumor cell inoculation and the extent of pulmonary tumor metastases evaluated based on lung weights. **a** Lung weights are depicted following immunization with PBS, empty vector or mEndoglin vaccine. Symbols within bar graphs of experimental groups each represent a single mouse ($n=8$). Normal lung weight is approximately 200 mg. * $P < 0.05$ when compared with either PBS or empty vector. **b** Kaplan–Meier survival plots of different groups of mice ($n=8$). Differences in survival times between the mEndoglin group and control groups were statistically significant according to Kaplan–Meier analyses ($P < 0.05$). Experiments were repeated three times with similar results

Discussion

An oral DNA vaccine encoding murine endoglin, which is primarily over-expressed by proliferating endothelial cells in the angiogenic tumor vasculature, effectively induced an endoglin-specific CD8⁺ T cell-mediated immune response. This immune response broke peripheral tolerance against the endoglin self-antigen and presumably suppressed tumor angiogenesis, resulting in the

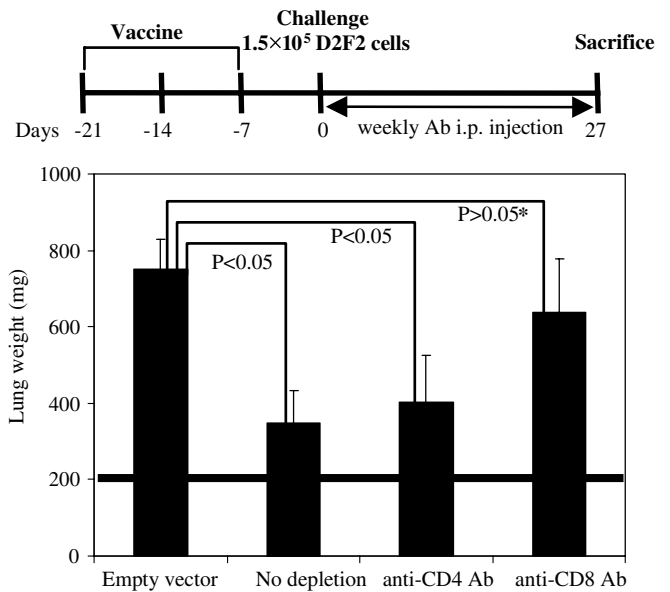


Fig. 4 Increased incidence of pulmonary D2F2 metastases of vaccinated mice that were depleted of CD8⁺ T cell populations. Mice were immunized orally three times at weekly intervals with either DNA encoding endoglin or the empty vector. Anti-CD8 mAb (2.43: mouse mAb, IgG2b) or anti-CD4 mAb (GK1.2: rat mAb, rat IgG2b), were each injected i.v. 7 days after the last immunization. The following day, experimental lung metastases were induced in a prophylactic setting by i.v. injection of 1.5×10^5 D2F2 cells followed by additional weekly i.p. injection of anti-CD8 or anti-CD4 mAb to maintain the depleted state of subset T cells until the termination of the experiments. Mice were sacrificed 27 days after D2F2 tumor cell challenge and lung weights established to determine the extent of pulmonary metastases. The data shown in bar graphs reveal the average \pm SD of lung weights in each experimental group ($n=8$). Statistically significant differences in lung weights between groups of mice treated with either the empty vector and non-depleted as well as CD4⁺ and CD8⁺ depleted T cells are shown. The data are representative of two independent experiments

suppression of pulmonary D2F2 breast carcinoma metastases in a prophylactic setting.

The rationale for using double attenuated *S. typhimurium* as a vaccine carrier is based on our prior data [30, 32, 33, 48], including the finding that transformation of such bacteria with a DNA plasmid encoding a tumor antigen and their subsequent oral administration by gavage leads to delivery of the vaccine via the small intestine and M cells into PPs. There, the attenuated bacteria are phagocytosed, primarily by DCs in the subepithelial dome of this secondary lymphoid organ [23], and then die due to their mutations and liberate the DNA. This is followed by transcription and translation of the DNA and processing of proteins or peptides in the proteosomes of these APCs, ultimately leading to the formation of antigen peptide/MHC class I Ag complexes in the cytosol, which are delivered to the cell surface and presented to T cell receptors. In this regard, intralymphatic immunization with naked DNA was reported to be most effective since it is 100–1,000-fold

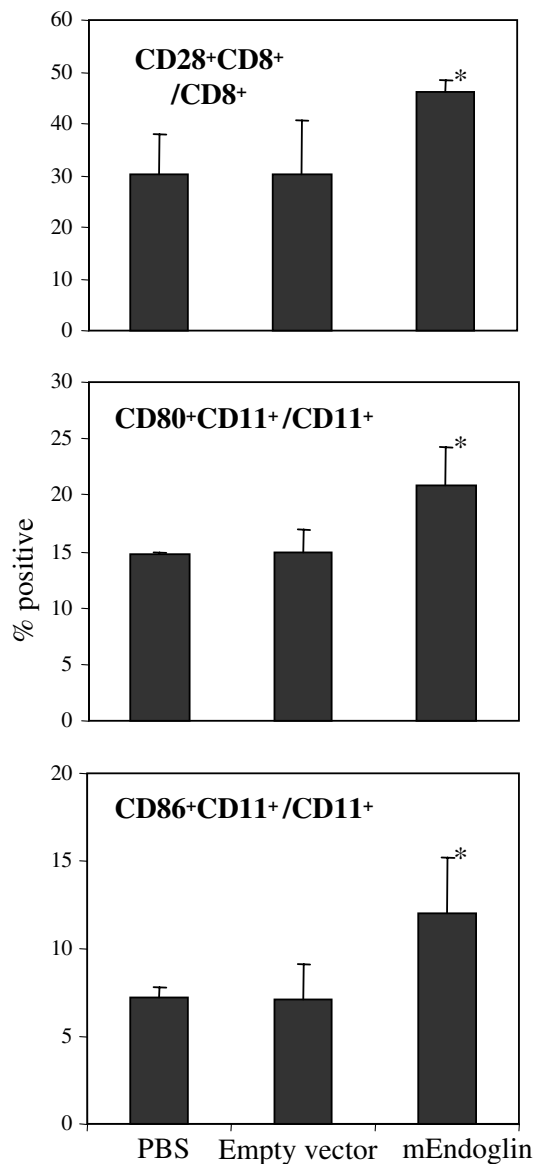


Fig. 5 The mEndoglin vaccine induced activation of T cell and DCs. Two-color flow cytometric analyses were performed with splenocytes from vaccinated mice 1 week after tumor cell challenge. For T cell activation, PE-conjugated anti-CD28 mAbs were used in combination with FITC-conjugated anti-mouse CD8 mAb (*upper panel*). For DC analyses, splenocytes were stained with FITC-labeled anti-CD11c mAb in combination with PE-conjugated anti-CD80 (*middle panel*) or anti-CD86 mAbs (*lower panel*). Y-axis represents % of double positive cells compared to the total CD8⁺ or CD11c⁺ cells (mean ± SD, *n* = 4). Differences between the control groups and the treatment group were statistically significant (*P* < 0.05)

more efficient inducing strong and biologically relevant CD8⁺ CTL responses over traditional i.m., s.c., or i.v. routes of immunization [28]. Consequently, vaccination with naked DNA appears to be optimal when targeted to secondary lymphoid organs such as PP. In addition, in this draining lymph node, effective cross-priming of CD8⁺ cells may also possibly be achieved without CD4⁺ T cell help [50].

Antiangiogenic therapies generally take two approaches: (1) targeting preexisting blood vessels or (2) preventing the development of the tumor neovasculature. Since our vaccination was performed in a prophylactic setting, where vaccination preceded tumor cell challenge, CD8⁺ T cell responses induced by our mEndoglin vaccine likely interfered with the development of the tumor angiogenic blood vessels which, in turn, prevented the establishment of D2F2 pulmonary metastases.

The well-established, high-level expression of endoglin by proliferating endothelial cells of both peri- and intratumoral blood vessels [6, 15, 39] makes endoglin an excellent target for antiangiogenic therapy, particularly in attempts to prevent the development of tumor blood vessels. Since in our experimental system, endoglin is only over-expressed by proliferating endothelial cells in angiogenic blood vessels (Fig. 1), targeting proliferating endothelial cells has several additional advantages over targeting tumor cells. These include the following: first, the avoidance of tumor antigen heterogeneity and the down-regulation of MHC class I antigens, both of which seriously limit effective T cell-mediated immune responses against tumor cells; second, the specific targeting of the antiangiogenic intervention to proliferating endothelial cells in the tumor neovasculature limits its toxicity; third, the direct contact of the vasculature with the circulation makes for efficient access of therapeutic agents since the target tissue can be reached unimpaired by anatomical barriers such as the blood-brain barrier or encapsulated tumor tissue [2, 9, 13, 37]; fourth, since the therapeutic target is tumor-independent, killing of proliferating endothelial cells in the tumor microenvironment could be effective against a variety of solid tumors [8, 11, 21, 35, 36].

Taken together, our data indicate that the endoglin-based DNA vaccine, delivered to PP in the small intestine by double attenuated *S. typhimurium*, evoked an effective CD8⁺ T cell-mediated anti-tumor immune response. Importantly, this response was shown to be specifically directed against endoglin expressed by proliferating endothelial cells, and presumably resulted in the suppression of angiogenesis in the breast tumor neovasculature. This included the ability of T cells from mEndoglin-vaccinated mice to specifically lyse both mEndo⁺-D2F2 and endothelial target cells, the latter which naturally express endoglin. The up-regulation of the T cell activation marker, CD28, and of co-stimulatory molecules CD80/CD86 on DCs provided further evidence for the activation of these cells. This type of activation is presumably of key importance to achieve a T cell-mediated immune response leading to the limitation of angiogenesis in the tumor vasculature, as well as to the suppression of breast tumor growth and pulmonary metastases in a prophylactic tumor model.

In conclusion, we anticipate that this novel, oral DNA vaccine targeting endoglin might ultimately lead to a successful clinical application aiding in the prevention and therapy of human breast cancer.

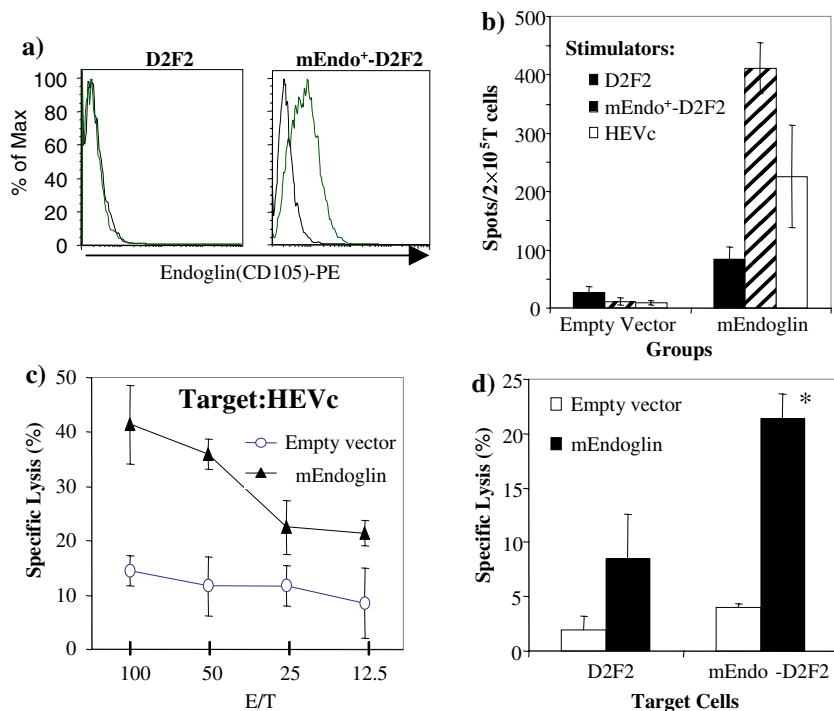


Fig. 6 Vaccination against endoglin induces specific T-cell responses. **a** FACS analyses of endoglin expression on endoglin-transfected D2F2 tumor cells (mEndo⁺-D2F2). Cells were stained with PE conjugated anti-endoglin Ab. Cells were stained with PE conjugated anti-endoglin Ab. Rat IgG Ab was used as an isotype control (*black line*). **b** ELISPOT analyses of IFN γ producing cells using different stimulator cells. Splenocytes, enriched for CD8⁺ cells, were isolated from vaccinated mice and incubated for 24 h with either irradiated wild-type D2F2 cells, mEndo⁺-D2F2, or HEVc endothelial cells. The mean spot number of each group is shown ($n=3$, mean \pm SD). **c** T cell-specific cytotoxicity against endoglin-positive HEVc endothelial target cells. Splenocytes were isolated from vaccinated mice 10 days after

tumor cell challenge. **A** [³⁵S]-release assay was performed at different effector-to-target cell ratios with splenocytes being restimulated with irradiated mEndo⁺-D2F2 cells for 5 days and [³⁵S] methionine labeled HEVc used as target cells. The data depict average \pm SD of triplicate wells. Similar results were obtained in three independent experiments. **d** Sensitivity of mEndo⁺-D2F2 and wild-type D2F2 cells (mEndo⁻-D2F2) to CTL killing. [³⁵S] methionine labeled wild-type D2F2 or mEndo⁺-D2F2 target cells were co-incubated with effectors at E/T = 1:12.5. Similar results were obtained in three independent experiments. * $P < 0.05$ compared with control wild-type D2F2 target cells

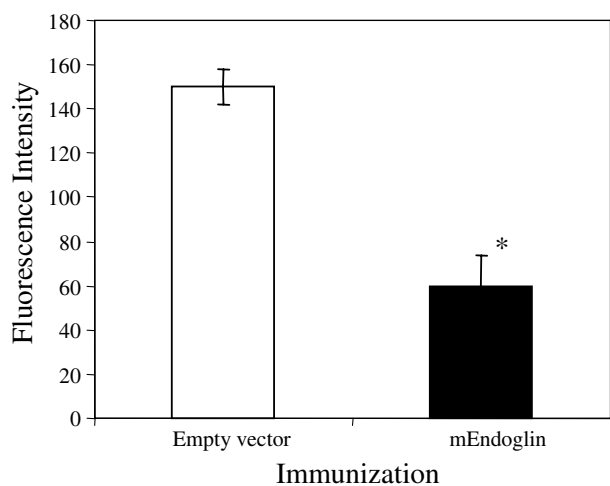


Fig. 7 Suppression of angiogenesis by the mEndoglin vaccine. One week after the last vaccination, Matrigel was implanted s.c. into the midline of the abdomen of either control mice ($n=7$) or vaccine-treated mice ($n=8$), and vessel growth quantified by staining of endothelium with FITC-labeled Isolectin B4 as described in [Material and methods](#). The average fluorescence of extracts is measured by fluorimetry at 490 nm and depicted by bar graphs (mean \pm SD; $P < 0.05$)

Acknowledgements We thank C. Dolman and D. Markowitz for excellent technical assistance, and Kathy Cairns for editorial help with manuscript preparation. He Zhou is supported by a post-doctoral fellowship from the Susan G. Komen Breast Cancer Foundation. This study was supported in part by Department of Defense grant BC 031079 (to R.A.R) and DAMD 17-02-1-0562 (to R.X.) and EMD-Lexigen Research Center Billerica, MA grant SFP 1330 (to R.A.R). This is The Scripps Research Institute's manuscript number 17620-IMM

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