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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Proof of concept was established for the hypothesis driving this project indicating that effective suppression of tumor angiogenesis can be achieved with a DNA vaccine encoding murine VEGF receptor-2 (FLK-1) designed to induce CTL-mediated immune responses by targeting proliferating endothelial cells in the tumor vasculature. This vaccine effectively protected mice from lethal tumor cell challenges and reduced growth of established metastases. CTL-medicated killing of endothelial cells indicated breaking of peripheral immune tolerance against the FLK-1 self-antigen resulting in markedly reduced dissemination of metastases. Angiogenesis in the vasculature was suppressed without impairment of fertility, neuromuscular performance or hematopoiesis with only a slight delay in wound healing. In addition we constructed novel minigene-based DNA vaccines encoding multiple FLK-1 nonapeptides with either H-2K^d and/or H-2D^d anchor residues. Furthermore, a novel vaccine was developed against the fos-related transcription factor (Fra-1), co-expressing secretory IL-18, which was highly effective in suppressing or eradicating aggressive breast carcinoma metastases by inducing anti-angiogenesis coupled with pronounced activation of T-and NK cells.

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INTRODUCTION:

The objective is to induce effective regression in breast tumor growth and metastases in minimal residual disease settings by suppressing tumor angiogenesis with an orally delivered DNA vaccine carried by attenuated *Salmonella typhimurium* encoding murine VEGF receptor 2 (FLK-1) and CD40 ligand trimer (CD40LT) designed to induce effective antigen presentation by dendritic cells and cytotoxic T Cell (CTL) responses effective in killing proliferating endothelial cells in the tumor vasculature. This objective will be achieved with constructs of novel minigene-based DNA vaccines encoding multiple FLK-1 nonapeptides with H-2K^d and/or H-2D^d anchor residues and murine polyubiquitin to achieve optimal antigen processing in the proteasome and effective antigen presentation by dendritic cells.

BODY:

Task 1: (Months 1-15) outlined in the initial grant proposal was mostly completed. Importantly, proof of concept was established for the highly effective anti-angiogenic/anti-tumor effect of a DNA vaccine encoding murine VEGF receptor 2 (FLK-1). The results obtained are documented in a paper published in NATURE MEDICINE (Niethammer et al., 2002) which is added to the appendix. In addition metastatic breast cancer models were established and standardized. A dual-function DNA vaccine encoding FLK-1 and CD40LT has been constructed (Months 5-8) and is currently under evaluation for its anti-angiogenic activity (Months 9-15). In addition, work on Task 2 (Months 15-19) has already been partially completed as we finished constructs of minigene-based DNA vaccine encoding multiple FLK-1 nonapeptides with either H-2K^d and/or H-2D^d anchor

residues as well as polyubiquitin for optimal antigen processing. These constructs are depicted schematically in the Appendix, Fig. 1. Furthermore, we demonstrated anti-tumor effects against breast cancer by a novel DNA vaccine against the transcription factor Fra-1, overexpressed on highly aggressive, metastatic breast cancer cells. This vaccine co-expressed IL-18 and was highly effective against breast cancer metastases by inducing activation of T-and NK cells as well as anti-angiogenesis (see Appendix). Finally, the results published in our NATURE MEDICINE paper in December, 2002 which established proof of concept for the hypothesis driving this project were recognized widely, at both the national and international levels and stimulated several collaborations as well as numerous comments by science writers in leading newspapers, magazines and scientific journals throughout the world.

KEY RESEARCH ACCOMPLISHMENTS:

1) An oral FLK-1-based DNA vaccine exclusively targets genetically stable proliferating endothelial cells in the tumor vasculature characterized by markedly upregulated expression of FLK-1.

2) This vaccine effectively protected mice from lethal tumor cell challenges and reduced growth of tumor metastases in a therapeutic setting.

3) CTL-mediated killing of proliferating endothelial cells in the tumor vasculature indicated breaking of peripheral tolerance against the FLK-1 self-antigen were demonstrated.

4) This vaccine also induced markedly reduced dissemination of spontaneous and experimental pulmonary metastases.

5) Effective angiogenesis in the tumor vasculature was suppressed without impairment in fertility, neuromuscular performance or hematopoiesis and only a minor delay in wound healing.

6) Novel constructs of minigene-based DNA vaccines were completed encoding polyubiquitin and multiple FLK-1 nonapeptides with either H-2K^d or H-2D^d anchor residues.

7) An experimental metastasis model for D2F2 murine breast carcinoma was established and standardized to evaluate the efficacy of the minigene-based FLK-1 vaccine in suppressing tumor growth and metastasis.

REPORTABLE OUTCOMES:

Reportable outcomes and results after only 1 year of this grant are summarized in our NATURE MEDICINE paper published in December 2002 (see Appendix).

Funding from this award also stimulated an additional, anti-angiogenic therapy based on a novel DNA vaccine encoding murine fos-related transcription factor Fra-1, carried by doubly attenuated *Salmonella typhimurium* (DAM$^-$; AroA$^-$). This vaccine co-expressed secretory IL-18 and mediated killing via suppression of angiogenesis of aggressively metastatic murine D2F2 breast carcinoma cells. This led to tumor protective immunity against breast carcinoma cell challenges resulting in marked reductions in tumor size and a number of complete eradications of metastases vs. empty vector and PBS controls.

CONCLUSIONS:

The key finding of the first year of this grant was the proof of concept established for our FLK-1-based DNA vaccine. This proved the hypothesis driving this project to be valid, namely that T cell-mediated killing of proliferating endothelial cells lining blood vessels in the tumor vasculature which overexpress FLK-1, leads to the effective suppression of tumor angiogenesis and results in marked suppression of tumor growth and metastases. A key finding that is of critical importance was that this vaccine induces a long-term memory T cell response lasting up to 10 months after initial vaccination. An additional project produced the first DNA vaccine against a transcription factor (Fra-1), overexpressed on aggressively metastasizing breast carcinoma cells. Co-expression of secretory IL-18 of this vaccine resulted in activation of T- and NK cells as well as the suppression of angiogenesis in the tumor vasculature leading to either marked reduction or complete eradication of breast cancer metastases in experimental animals.

APPENDIX:

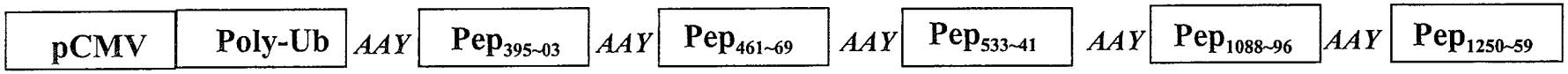
Figure 1, page 8

Publication: Niethammer, A.G., Xiang, R., Becker, J.C., Wodrich, H., Pertl, U., Karsten, G., Eliceiri, B.P. and Reisfeld, R.A. A DNA vaccine against vascular endothelial growth factor receptor 2 prevents effective angiogenesis and inhibits tumor growth. Nat. Med., 8:1369-1375, 2002.

Luo, Yunping, Zhou, He, Mizutani, Masato, Mizutani, Noriko, Reisfeld, Ralph A., and Xiang, Rong. Activated T-and NK cells combine with suppression of angiogenesis to protect against breast cancer growth. Journal of Clinical Invest., Submitted, 2003.

Animal Assurance/Certification Declaration

H-2K^d expression vector



H-2D^d expression vector

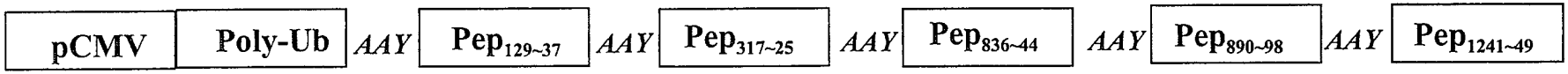


Figure 1: The construction of the mini-gene encoding the H-2Dd and H-2Kd epitopes
Four plasmids encoding the FLK-1 mini-genes have been constructed, which are derived from the entire FLK-1 gene with H-2Kd/Dd anchors ($AA_{395\text{-}403,\ 461\text{-}69,\ 533\text{-}41,\ 1088\text{-}96,\ 1250\text{-}59}$) and ($AA_{129\text{-}37,\ 317\text{-}25,\ 836\text{-}44,\ 890\text{-}98,\ 1241\text{-}49}$), respectively. Polyubiquitin was included in the pCMV vector with or without a myc tag; the former construct was used for detection of protein expression by Western blotting and the latter one for DNA vaccination.

A DNA vaccine against VEGF receptor 2 prevents effective angiogenesis and inhibits tumor growth

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Tumor cells are elusive targets for immunotherapy due to their heterogeneity and genetic instability. Here we describe a novel, oral DNA vaccine that targets stable, proliferating endothelial cells in the tumor vasculature rather than tumor cells. Targeting occurs through upregulated vascular-endothelial growth factor receptor 2 (FLK-1) of proliferating endothelial cells in the tumor vasculature. This vaccine effectively protected mice from lethal challenges with melanoma, colon carcinoma and lung carcinoma cells and reduced growth of established metastases in a therapeutic setting. CTL-mediated killing of endothelial cells indicated breaking of peripheral immune tolerance against this self antigen, resulting in markedly reduced dissemination of spontaneous and experimental pulmonary metastases. Angiogenesis in the tumor vasculature was suppressed without impairment of fertility, neuromuscular performance or hematopoiesis, albeit with a slight delay in wound healing. Our strategy circumvents problems in targeting of genetically unstable tumor cells. This approach may provide a new strategy for the rational design of cancer therapies.

The inhibition of tumor growth by attacking the tumor's vascular supply offers a primary target for anti-angiogenic intervention. This approach, pioneered by Folkman and colleagues[1–5], is attractive for several reasons. First, the inhibition of tumor-associated angiogenesis is a physiological host mechanism and should not lead to the development of resistance. Second, each tumor capillary has the potential to supply hundreds of tumor cells, so that targeting the tumor vasculature actually potentiates the antitumor effect. Third, direct contact of the vasculature with the circulation leads to efficient access of therapeutic agents[6].

Extensive studies by many investigators established that angiogenesis has a central role in the invasion, growth and metastasis of solid tumors[2,7–9]. In fact, angiogenesis is a rate-limiting step in the development of tumors since tumor growth is generally limited to 1–2 mm^3 in the absence of a blood supply[6,10]. Beyond this minimum size, tumors often become necrotic and apoptotic under such circumstances[11].

Because tumor cells frequently mutate in response to therapy and also downregulate major histocompatibility (MHC) antigens required for T cell–mediated antitumor responses[12,13], efforts have been made to eradicate tumors by therapies directed against the tumor microenvironment. One such report links calreticulin with a model viral tumor antigen, thus combining antitumor therapy with anti-angiogenesis[14]. Yet another approach is the administration of xenogeneic endothelial cells as a vaccine that yielded anti-angiogenic effects[15]. This approach differs from those of other investigators applying specific chemical or biological inhibitors of angiogenesis, which often require their constant administration at relatively high dose levels[16].

A more molecularly-defined alternative to xenoimmunization is offered by receptor tyrosine kinases (RTKs) and their growth-factor ligands required for tumor growth. Among these receptors, the vascular endothelial growth factor receptor 2 (VEGFR2, also known as FLK-1) that binds the five isomers of murine VEGF has a more restricted expression on endothelial cells and is upregulated once these cells proliferate during angiogenesis in the tumor vasculature. FLK-1 is strongly implicated as a therapeutic target, as it is necessary for tumor angiogenesis and has an important role in tumor growth, invasion and metastasis[7,8,17–24]. In fact, several approaches have been used to block FLK-1, including dominant-negative receptor mutants, germ-line disruption of VEGFR genes, monoclonal antibodies against VEGF and a series of synthetic RTK inhibitors[24,25].

Here we describe a novel strategy for achieving an antitumor immune response with a FLK-1-based DNA vaccine. Our vaccine causes the collapse of tumor vessels by evoking a T cell–mediated immune response against proliferating endothelial cells overexpressing this growth-factor receptor in the tumor vasculature.

A FLK-1 based DNA vaccine inhibits tumor growth

We tested our hypothesis by demonstrating that an effective antitumor immune response was induced against subcutaneous tumors by an orally administered DNA vaccine encoding murine FLK-1 carried by attenuated *Salmonella typhimurium*. To this end, we constructed the vector pcDNA3.1-FLK1 (Fig. 1*a*). Protein expression of FLK-1 was demonstrated by western blot-

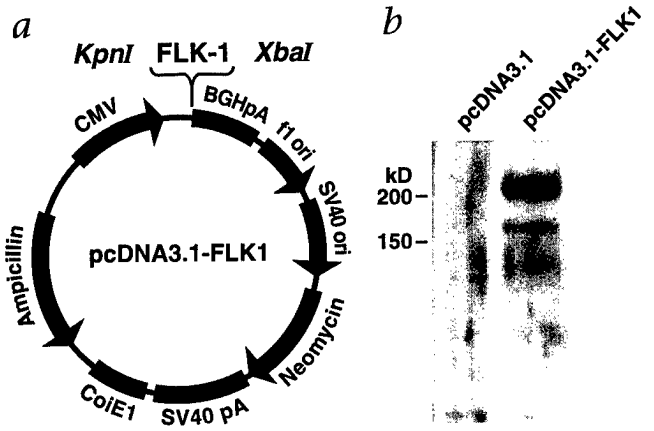

Fig. 1 Construction and functionality of expression vector. ***a***, The DNA encoding the entire murine Flk-1 gene was inserted into the pcDNA3.1 vector between the restriction sites *KpnI* (5′) and *XbaI* (3′) ***b***, This construct was verified by nucleotide sequencing and protein expression by western blots after transient transfection into COS-7 cells. The protein appears in the lysate in its glycosylated form at 220 kD and to a lesser extent in its unglycosylated form at approximately 150 kD.

ting of transfected COS-7 cells (Fig. 1*b*). We established the efficacy of gene transfer from attenuated *S. typhimurium* into Peyer's patches by GFP expression in the cells derived from Peyer's patches at different time points after oral administration of mice (data not shown).

Marked inhibition of subcutaneous (s.c.) tumor growth was observed in C57BL/6J mice challenged two weeks after the third vaccination with pcDNA3.1-FLK1 by s.c. injection of either B16G3.26 murine melanoma cells or D121 non–small cell Lewis lung carcinoma cells (Figs. 2*a* and *b*). In contrast, animals vaccinated with only the empty vector pcDNA3.1, carried by the attenuated bacteria, revealed uniformly rapid s.c. tumor growth.

Prolonged antitumor effects were demonstrated since C57BL/6J mice challenged s.c. with MC-38 colon carcinoma cells 10 months after their last vaccination revealed a marked decrease in tumor growth in all experimental animals compared with controls (Fig. 2*c*).

Protection against spontaneous pulmonary metastases

We noted a marked reduction in dissemination of spontaneous pulmonary metastases in all experimental animals following three immunizations with the FLK-1-based DNA vaccine. This became evident 30 days after surgical excision of the s.c. primary Lewis lung carcinoma tumors, as confirmed by visual examination of the lungs of these animals, which established their metastatic score (Fig. 2*d*), as well as by histological analyses (data not shown).

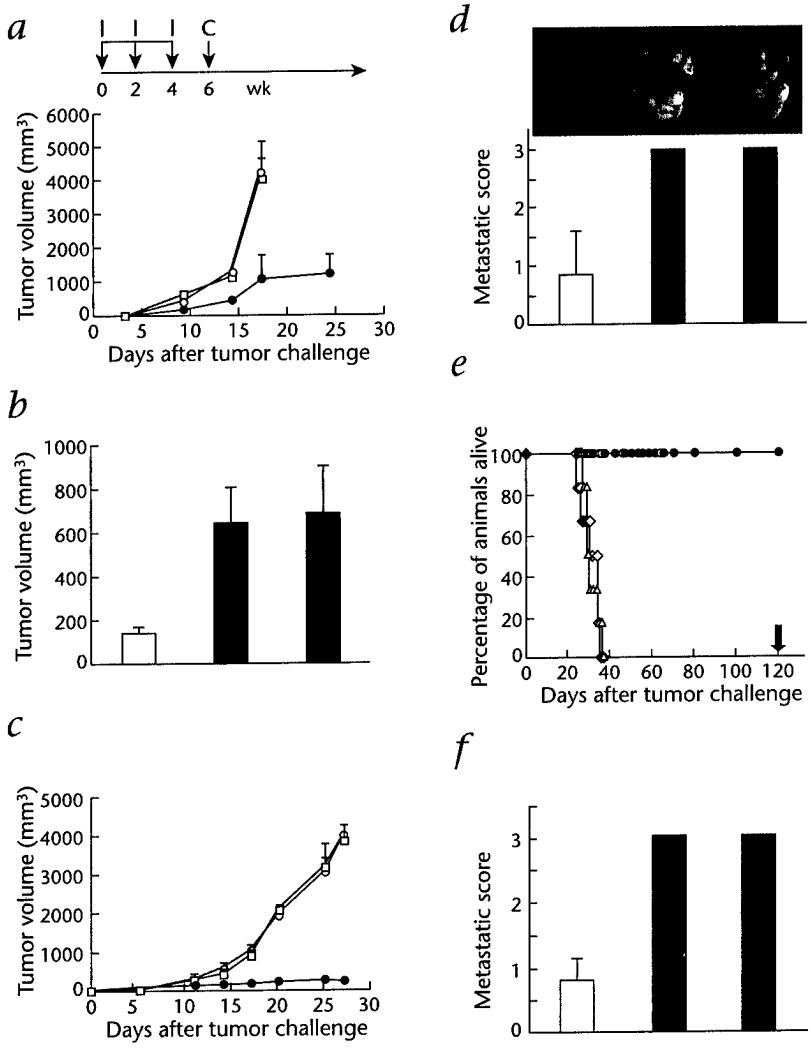

Fig. 2 Effect of the FLK-1 based DNA vaccine on tumor growth. ***a***, 2 wk after the last vaccination, mice were challenged (*c*). with a lethal dose of B16 melanoma cells. The average tumor growth of 8 mice is depicted. ●, immunization (I) with the vector encoding FLK-1; ○, control vector; □, PBS. $P < 0.01$. ***b***, C57BL/6J mice ($n = 8$) were challenged s.c. with a lethal dose of 1×10^5 D121lu Lewis lung carcinoma cells (□). Bar graphs indicate average tumor volume after 2 wk before tumor removal in comparison with mice that received only the control vector (■) or PBS (■). Error bars indicate s.d. ***c***, Long-term effect of the pcDNA3.1-FLK1 vaccination was tested by challenging C57BL/6J mice ($n = 8$) with MC-38 colon carcinoma s.c. 10 months after the third immunization. Symbols are as in *e*. ***d***, Representative lung specimens of mice challenged with D121 Lewis lung carcinoma cells 4 weeks after removal of the subcutaneous primary tumors. Bars are as in *b*. ***e***, Lifespan of BALB/c mice after i.v. challenge with CT-26 colon carcinoma cells. The lifespan of groups of mice ($n = 6$) are shown following tumor cell challenge after repeated vaccinations. Death occurred in control groups due to extensive metastatic dissemination throughout the lung. 5 of the surviving mice were rechallenged (black arrow) to test for possible resistance. ●, vaccine; ◇, empty vector control; △, PBS. ***f***, Inhibition of tumor growth in a therapeutic setting. BALB/c mice ($n = 5$) were challenged i.v. with CT-26 colon carcinoma, and 10 d later 1 dose of the vaccine was applied. Experimental groups were scored 28 d after challenge. Bars are as in *b*.

Vaccination prolongs the lifespan of mice

We found a fourfold increase in lifespan of BALB/c mice ($n = 6$), vaccinated as described above, and challenged two weeks later by intravenous (i.v.) injection of a lethal dose of autologous CT-26 colon carcinoma cells (Fig. 2*e*). Possible resistance against our therapy was ruled out by rechallenging survivors ($n = 5$) 120 days after their first tumor-cell challenge and collecting their lungs 30 days later. Four mice did not reveal any tumors, whereas the one remaining animal had less then 10% of its lung surface covered by metastases (data not shown).

Vaccination reduces growth of established metastases

We established that our FLK-1-based DNA vaccine is also effective in a therapeutic setting. This was shown by i.v. injection of BALB/c mice ($n = 5$) with CT-26 colon carcinoma cells and vaccination of these mice 10 days thereafter with pcDNA3.1-FLK1 when they had fully established pulmonary metastases. All such treated mice survived and showed only few small lung foci, whereas all control animals treated with the empty vector or PBS began to die 28 days after tumor cell challenge (Fig. 2*f*).

$CD8^+$ T cells are responsible for the antitumor response

There was a marked increase in T-cell activation markers in splenocytes from successfully vaccinated C57BL/6J mice after a 12-hour incubation with B16G3.26 melanoma cells that had been stably transduced to express murine FLK-1. This included increased expression of CD25, the high-affinity interleukin-2 (IL-2)-receptor α chain, CD69 an early T-cell activation antigen and LFA-2 (CD2) a lymphocyte function-associated antigen (Fig. 3*a*). This upregulation was clearly evident when compared with $CD8^+$ T cells from mice vaccinated with pcDNA3.1-FLK1 but incubated with wild-type B16G3.26 melanoma cells ($P \leq 0.05$). Specific recognition of FLK-1 was indicated as no increase in expression was noted following co-incubation of cells expressing FLK-1 with splenocytes from C57BL/6J mice vaccinated with the empty vector. No such upregulation could be observed for $CD4^+$ T cells (data not shown).

We demonstrated the involvement of $CD8^+$ T cells in the antitumor immune response given that *in vivo* depletion of $CD8^+$ T cells—before i.v. challenge of vaccinated mice with CT-26 tumor cells—resulted in the complete abrogation or severe impairment of the antitumor response. In fact, mice depleted of $CD8^+$ T cells died within 45 days after tumor-cell challenge due to extensive growth and dissemination of pulmonary metastases (Fig. 3*b*). However, *in vivo* depletion of $CD4^+$ T cells did not decrease the effectiveness of our vaccine (data not shown).

Cytotoxic T cells associate with tumor endothelium

To reveal the localization of $CD8^+$ T cells to their target site, we stained these cells with fluorescein isothiocyanate (FITC) and

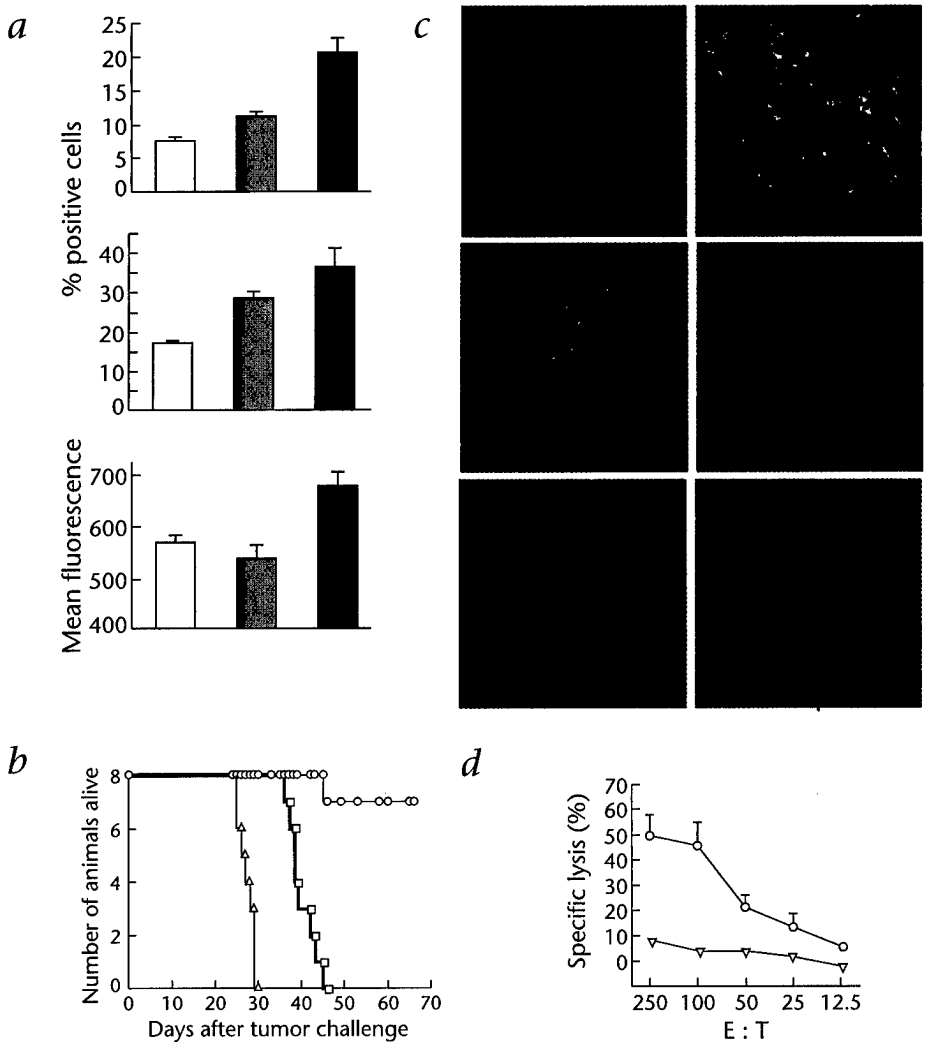

Fig. 3 Involvement of $CD8^+$ T cells. ***a***, Activation of $CD8^+$ T cells after *in vitro* co-incubation with cells expressing FLK-1. Shown is the increase in percentage of $CD8^+CD25^+$ (top) and $CD8^+CD69^+$ (middle) T cells, and increase in expression of CD2 on $CD8^+$ T cells (bottom) isolated from splenocytes of mice vaccinated with the vector pcDNA 3.1-FLK1 after cocultivation with cells expressing FLK-1 (■), or the identical cells lacking FLK-1 (■). A further control included $CD8^+$ T cells from mice immunized with the empty control vector and cocultivated with the B16 melanoma cell line expressing FLK-1 (□). Error bars indicate s.d. ***b***, Effect of $CD8^+$ T-cell depletion on lifespan. □, $CD8^+$ T-cell depletion during effector phase after vaccination with pcDNA3.1-FLK1; ○, no $CD8^+$ T-cell depletion of vaccinated mice; △, empty control vector. ***c***, Immunhistochemical analysis of $CD8^+$ T cells (FITC) and endothelial cells (rhodamine). Upper panels depict vascularized areas of CT-26 pulmonary metastases 4 mo after challenge and immunization with pc DNA3.1-FLK1. Left, ×20 magnification, 530 × 530 μm; right, ×40 magnification, 265 × 265 μm. Middle panels show Matrigel specimens after bFGF-induced vessel growth and prior immunizations with pcDNA3.1-FLK1 (left) and empty vector (right). Both panels, ×20 magnification. Lower panels reveal non-vascularized areas of tumor tissue (left) and adjacent skin tissue (right). Both panels, ×20 magnification. ***d***, Specific lysis of CT-26-FLK-1 cells by $CD8^+$ T cells from mice vaccinated with pcDNA3.1-FLK-1 (○) compared with $CD8^+$ T cells from control mice treated with empty vector (▽).

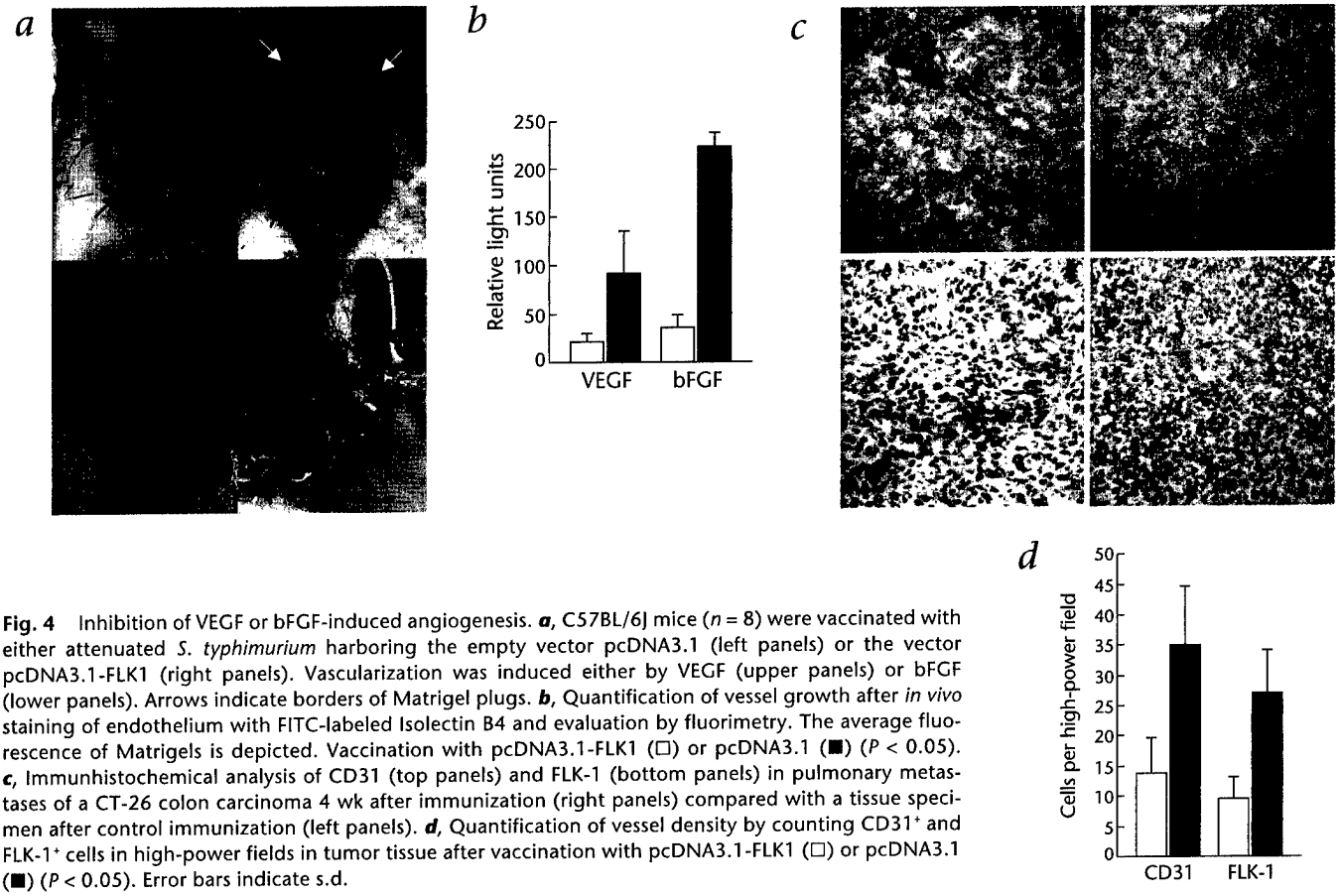

Fig. 4 Inhibition of VEGF or bFGF-induced angiogenesis. ***a***, C57BL/6J mice ($n = 8$) were vaccinated with either attenuated *S. typhimurium* harboring the empty vector pcDNA3.1 (left panels) or the vector pcDNA3.1-FLK1 (right panels). Vascularization was induced either by VEGF (upper panels) or bFGF (lower panels). Arrows indicate borders of Matrigel plugs. ***b***, Quantification of vessel growth after *in vivo* staining of endothelium with FITC-labeled Isolectin B4 and evaluation by fluorimetry. The average fluorescence of Matrigels is depicted. Vaccination with pcDNA3.1-FLK1 (□) or pcDNA3.1 (■) ($P < 0.05$). ***c***, Immunhistochemical analysis of CD31 (top panels) and FLK-1 (bottom panels) in pulmonary metastases of a CT-26 colon carcinoma 4 wk after immunization (right panels) compared with a tissue specimen after control immunization (left panels). ***d***, Quantification of vessel density by counting $CD31^+$ and $FLK\text{-}1^+$ cells in high-power fields in tumor tissue after vaccination with pcDNA3.1-FLK1 (□) or pcDNA3.1 (■) ($P < 0.05$). Error bars indicate s.d.

marked endothelial cells with rhodamine-conjugated antibody against CD31. Microscopic evaluation revealed an association of $CD8^+$ T cells with vessel structures throughout the tumor tissue or Matrigel sections of animals immunized with pcDNA3.1-FLK1. Almost no $CD8^+$ T cells were observed within non-vascularized, viable areas of tumor tissues even four months after tumor-cell challenge, nor were they associated with vessels in somatic tissues. Control vaccination did not induce any infiltration of cytotoxic T cells into tumor tissue or Matrigel (Fig. 3*c*).

Vaccination against FLK-1 induces T cell–mediated lysis

We demonstrated antigen-specific cytotoxicity against CT-26-FLK-1 cells with a standard 4-hour ^{51}Cr-release assay using splenocytes from BALB/c mice immunized against FLK-1 and challenged with CT-26 colon carcinoma cells. Immunizations with the vector encoding FLK-1 led to significant lysis of target cells by effector cells, in contrast to control immunizations (Fig. 3*d*). However, neither vaccination was effective in evoking any noticeable cytotoxicity against wild-type CT-26 cells not expressing FLK-1, thus excluding direct lysis of tumor cells (data not shown).

Reduction of neovascularization

We demonstrated distinct anti-angiogenic effects, independent of tumor cells, induced by the FLK-1-based DNA vaccine in a Matrigel assay. Differences were visible macroscopically, as shown in representative examples of Matrigel plugs removed six days after their installment (Fig. 4*a*). This was also evident from the extent of vascularization evaluated by relative fluorescence after *in vivo* staining of endothelium with FITC-conjugated lectin. There was a decrease in VEGF- or bFGF-induced neovascularization only after vaccination with the vector encoding FLK-1 but not with the empty vector (Fig. 4*b*). Immunohistochemical staining further revealed a decrease in vessel density among pulmonary metastases of CT-26 colon carcinoma after vaccination with pcDNA3.1-FLK1 as compared with tissue derived from control mice (Fig. 4*c*). Evaluation of high-power fields demonstrated decreased vessel density induced by the FLK-1-based vaccine (Fig. 4*d*).

Wound healing is delayed after vaccination

We noticed a measurable prolongation in the time required to completely close a total of 24 circular wounds inflicted on the backs of 6 mice immunized with the FLK-1-based vaccine versus that of mice immunized with the control vector (14.75 days, s.d. 1.5 versus 13.3 days, s.d. 1.6; $P<0.01$. This was accompanied by macroscopically visible swelling and inflammation in 11 of 24 versus 4 of 24 cases among controls (Fig. 5*a* and *b*).

Further experiments revealed no impact on fertility of mice based on the time elapsed from start of cohabitation until parturition nor on the number of pups born (Fig. 5*c* and *d*). All females of each experimental group gave birth. Neuromuscular performance as determined by both the wire test and footprint test, as well as by body weight, overall behavior and balancing tests, did not demonstrate any impairment attributed to vaccination (data not shown).

The occurrence of common, FLK-1-positive progenitor cells for both endothelial cells and hematopoietic cells led us to evaluate peripheral blood samples of C57BL/6J and BALB/c

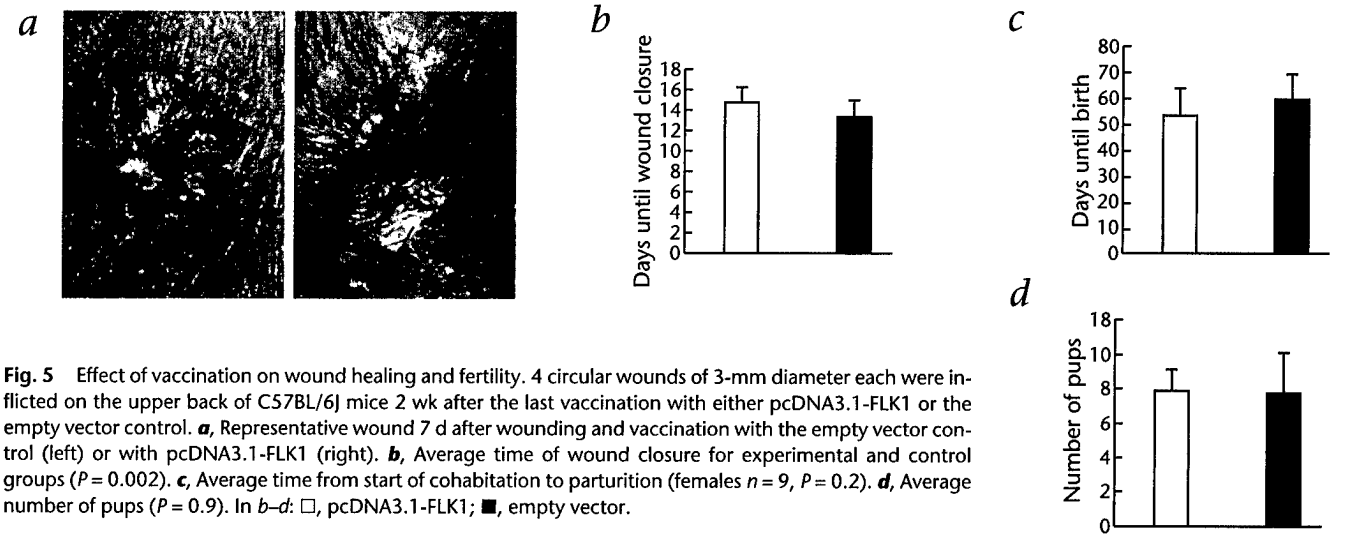

Fig. 5 Effect of vaccination on wound healing and fertility. 4 circular wounds of 3-mm diameter each were inflicted on the upper back of C57BL/6J mice 2 wk after the last vaccination with either pcDNA3.1-FLK1 or the empty vector control. ***a***, Representative wound 7 d after wounding and vaccination with the empty vector control (left) or with pcDNA3.1-FLK1 (right). ***b***, Average time of wound closure for experimental and control groups ($P = 0.002$). ***c***, Average time from start of cohabitation to parturition (females $n = 9$, $P = 0.2$). ***d***, Average number of pups ($P = 0.9$). In *b–d*: □, pcDNA3.1-FLK1; ■, empty vector.

mice up to ten months after their last immunization. However, total blood counts and differentials did not indicate any decreased or compensating hematopoiesis (data not shown).

Discussion

We developed a novel strategy that might overcome problems of tumor-cell heterogeneity and peripheral tolerance to self-antigens encountered in tumor cell–directed immunotherapy. We accomplished this by exploiting the obvious advantages of anti-angiogenic therapies developed by many other investigators[1–5]. In fact, there are several advantages of targeting $CD8^+$ T cells to proliferating endothelial cells in the tumor vasculature rather than directly to tumor cells. First, endothelial cells are genetically stable and do not downregulate MHC-class I and II antigens—an event that frequently occurs in solid tumors and severely impairs T cell–mediated antitumor responses[12]. In addition, immune suppression triggered by tumor cells at the cellular level can also be avoided by this approach. Second, the therapeutic target is tumor-independent, thus killing of proliferating endothelial cells in the tumor microenvironment can be effective against a variety of malignancies. Furthermore, proliferating endothelial cells are readily available to lymphocytes in the bloodstream. Consequently, the target tissue can be reached unimpaired by anatomical barriers such as the blood–brain barrier or encapsulation of tumor tissues[26].

Our studies show that peripheral T-cell tolerance against the murine VEGFR2 (FLK-1) can be broken by an oral DNA vaccine encoding autologous FLK-1, delivered by an attenuated strain of *S. typhimurium*. We induced antitumor immune responses in mouse tumor models of non–small cell lung carcinoma, colon carcinomas and melanoma, both against primary tumors and their respective spontaneous and experimental pulmonary metastases. Our DNA-vaccine was also effective in a therapeutic setting of established lung metastases. Additionally, we observed effective, prolonged antitumor effects evident after challenge up to ten months after the last immunization. Involvement of cytotoxic T cells in these events was suggested by marked upregulation of T-cell activation markers CD2, CD25, CD69 on $CD8^+$ T cells when co-incubated with cells expressing FLK-1. There was no apparent upregulation of these markers upon incubation with tumor cells. Furthermore, the effect of our vaccine was severely impaired in mice depleted *in vivo* of $CD8^+$ T cells throughout the effector phase. Depletion of $CD4^+$ cells was without effect. Importantly, angiogenesis was found to be effectively counteracted in a tumor cell–free, VEGF- or bFGF-induced Matrigel assay; both VEGF and bFGF upregulate the target antigen FLK-1 on endothelial cells[27]. Furthermore, *in vitro* cytotoxicity occurred only against target cells transduced to express FLK-1, but not against the identical wild-type cell line. We eliminated cytotoxicity mediated by $CD8^+$ T cells directly against tumor cells, as well as non-specific immune responses, as tumor protection was completely abrogated in mice that received attenuated *S. typhimurium* transformed with the empty vector lacking FLK-1.

We further demonstrated that fertility, neuromuscular performance and hematopoiesis of experimental mice remained unimpaired; anti-angiogenic effects induced by our DNA vaccine resulted in a slight but statistically significant delay in wound healing. However, the wounds of mice that were subjected to tumor excisions—including in some cases opening of the peritoneum—healed without any complications.

Together, our data show that the VEGFR2 is a suitable target for T cell–mediated immunotherapy within the tumor vasculature. Our findings may lead to a novel vaccine strategy for cancer therapy through the induction of an autoimmune response against self antigens expressed by proliferating endothelial cells. In fact, at least 46 transcripts are specifically elevated in the tumor-associated endothelium, thus providing a large array of potential candidates for this strategy[28]. This includes integrins or additional growth factor receptors and their ligands such as basic fibroblast growth factor or angiopoietin, as well as other molecules involved in their downstream signaling events[28]. It is also likely that DNA vaccines targeting proliferating endothelial cells could be used effectively in combination with specific inhibitors of angiogenesis, and chemotherapies or immunotherapies targeting the tumor cells themselves. Such combined approaches may ultimately lead to the rational design of novel and effective modalities for the treatment of cancer.

Methods

Animals, bacterial strains, and cell lines. Animal experiments were performed according to the National Institutes of Health *Guide for Care and Use of Experimental Animals* and approved by our Animal Care Committee (#ARC-43SEPT1). Attenuated *S. typhimurium* Aro/A (strain SL7207) was provided by B.A.D. Stocker. The D121 cell line was a gift from L. Eisenbach. Tumor tissues were screened for expression of FLK-1 by immunohistochemical staining and found to be negative; expression throughout the tumor-neovasculature was positive.

Construction of the expression vector encoding murine VEGFR-2 and transformation of *S. typhimurium*. DNA encoding murine VEGFR-2 (FLK-1) (provided by I. Lemischka) was cloned with the primers 3′-CCGGTA CCATGGAGAGCAAGGCGCTG-5′ and 5′-CCTCTAGACAGCAGCAC-CTCTCTC-3′ and inserted into the pcDNA3.1 vector (Invitrogen, San Diego, California) between the restriction sites *Kpn*1 and *Xba*1 generating pcDNA3.1-FLK1. Bacteria were electroporated as described[29,30].

Oral immunization and tumor-cell challenge. Mice were immunized by oral gavage 3 times at 2-wk intervals with 100 μl PBS containing 1×10^8 *S. typhimurium* transformed with pcDNA3.1-FLK1 or pcDNA3.1 as described[31]. C57BL/6J mice were challenged 2 wk later by s.c. injection of 1×10^5 B16G3.26 melanoma, MC-38 colon carcinoma or D121 lung carcinoma cells into the left front flank. Tumor volume was measured in 2 dimensions and calculated as follows: length/2 × width^2. Tumors of mice injected with D121 cells were excised after 2 wk to allow spontaneous dissemination to the lung. Metastatic scores were evaluated 4 wk later by the percentage of lung surface covered by fused metastases: 0% = 0; <20% = 1; 20–50% = 2; and >50% = 3. We injected CT-26 murine colon carcinoma cells (5×10^4) i.v. into BALB/c mice inducing experimental pulmonary metastases 2 wk after the last immunization. We tested our treatment in a therapeutic setting by vaccinating animals 10 d after i.v. injection of CT-26 cells.

Activation of CD8$^+$ T cells. We created the B16G3.26-FLK-1 melanoma and CT-26-FLK-1 colon carcinoma cell lines by retroviral transduction with FLK-1. One week after immunization, splenocytes were collected from C57BL/6J mice ($n = 4$), vaccinated with pcDNA3.1-FLK1 or the empty control vector. Cells were cocultured overnight with B16G3.26-FLK-1 or B16G3.26 tumor cells. Flow-cytometric analyses were performed using FITC-conjugated antibody to CD8 (#01044) in combination with PE-conjugated anti-mouse monoclonal antibodies to CD2 (#01175), CD25 (#01105A) or CD69 (#01505B) (BD-Pharmingen, La Jolla, California) as described[24]. We also used splenocytes in a standard 4-h ^{51}Cr-release assay to asses cytotoxicity against CT-26-FLK-1 and CT-26 target cells.

Evaluation of anti-angiogenic effects. C57BL/6J mice ($n = 8$) were injected into the sternal region with 250 μl growth factor–reduced Matrigel (#354230, BD Biosciences, Bedford, Massachusetts) containing 400 ng/ml murine VEGF (#450-32, PeproTech, Rocky Hill, New Jersey) or bFGF (#100-18B). Endothelium was stained 6 d later by i.v. injection of 200 μl (0.1 mg/ml) *Bandiera simplofica* lectin I, Isolectin B4 conjugated with fluorescein (Vector Labaratories, Burlingame, California). 30 min later, mice were killed and lectin-FITC was extracted from 100 μg per plug in 500 μl RIPA lysis buffer, centrifuged and its content in the supernatant quantified by fluorimetry (490 nm).

***In vivo* depletion of CD8$^+$ T cells.** We depleted CD8$^+$ T cells by weekly i.p. injections of 500 μg rat anti-mouse monoclonal antibody to CD8 (RH.495) as described[32]. Controls included non-depleted animals either vaccinated with pcDNA3.1-FLK1 or pcDNA3.1.

Immunohistochemistry. We stained cryosections (10 μm) fixed in paraformaldehyde. Antibody to CD31 (Pharmingen, San Diego, California) was incubated with rhodamine-conjugated secondary antibody, blocked with rat serum, followed by immunostaining with a FITC–conjugated antibody to CD8. Photomicrographs were captured with a laser scanning confocal microscope (Biorad, Hercules, California). Frozen tissue sections were stained with the Techmate Automate (Dako, Hamburg, Germany). Single stained serial sections were incubated for 30 min with biotinylated antibodies, followed by the streptavidin-peroxidase complex (DAKO) and the chromogen AEC (DAKO). Double stainings were performed as described[33]. Density of antigen-expressing cells was determined by counting of high-power fields.

Evaluation of possible side effects. To test wound healing, wounding was performed as described[34,35]. We inflicted 4 circular wounds of 3-mm diameter each on the upper back of C57BL/6J mice ($n = 6$), 2 wk after immunization with pcDNA3.1-FLK1 or the empty vector. Time until wound closure was noted. To evaluate fertility, 2 wk after the third immunization with either pcDNA3.1-FLK1 or with empty vector, female C57BL/6J mice ($n = 9$) were allowed to cohabitate with 3 males. The days until parturition and number of pups were noted. To test neuromuscular performance, we evaluated vaccinated and control mice by both the wire hang test and the footprint test[36,37] as well as by overall behavior and determination of body weight. To test hematopoiesis, animals were subjected to complete peripheral blood counts and differentials up to 10 mo after immunization.

Statistical analysis. The statistical significance of differential findings between experimental groups and controls was determined by Student's *t*-test and considered significant if two-tailed *P* values were <0.05.

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Competing interests statement

The authors declare that they have no competing financial interests.

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1. Folkman, J. Addressing tumor blood vessels. *Nature Biotechnol.* **15**, 510 (1997).
2. Folkman, J. Angiogenesis and angiogenesis inhibition: An overview. *EXS* **79**, 1–8 (1997).
3. Folkman, J. Antiangiogenic gene therapy. *Proc. Natl. Acad. Sci. USA* **95**, 9064–9066 (1998).
4. O'Reilly, M.S., Holmgren, L., Chen, C. & Folkman, J. Angiostatin induces and sustains dormancy of human primary tumors in mice. *Nature Med.* **2**, 689–692 (1996).
5. O'Reilly, M.S. *et al.* Endostatin: An endogenous inhibitor of angiogenesis and tumor growth. *Cell* **88**, 277–285 (1997).
6. Augustin, H.G. Antiangiogenic tumor therapy: Will it work? *Trends Pharmacol. Sci.* **19**, 216–222 (1998).
7. Eberhard, A. *et al.* Heterogeneity of angiogenesis and blood vessel maturation in human tumors: implications for antiangiogenic tumor therapies. *Cancer Res.* **60**, 1388–1393 (2000).
8. Folkman, J. Tumor angiogenesis and tissue factor. *Nature Med.* **2**, 167–168 (1996).
9. Goede, V. *et al.* Prognostic value of angiogenesis in mammary tumors. *Anticancer Res.* **18**, 2199–2202 (1998).
10. Folkman, J. Can mosaic tumor vessels facilitate molecular diagnosis of cancer? *Proc. Natl. Acad. Sci. USA* **98**, 398–400 (2001).
11. Heidenreich, R., Kappel, A. & Breier, G. Tumor endothelium-specific transgene expression directed by vascular endothelial growth factor receptor-2 (FLK-1) promoter/enhancer sequences. *Cancer Res.* **60**, 6142–6147 (2000).
12. Hicklin, D.J., Marincola, F.M. & Ferrone, S. HLA class I antigen downregulation in human cancers: T-cell immunotherapy revives an old story. *Mol. Med. Today* **5**, 178–186 (1999).
13. Ruiter, D.J., Mattijssen, V., Broecker, E.B. & Ferrone, S. MHC antigens in human melanomas. *Semin. Cancer Biol.* **2**, 35–45 (1991).
14. Cheng, W.F. *et al.* Tumor-specific immunity and antiangiogenesis generated by a DNA vaccine encoding calreticulin linked to a tumor antigen. *J. Clin. Invest.* **108**, 669–678 (2001).
15. Wei, Y.Q. *et al.* Immunotherapy of tumors with xenogeneic endothelial cells as a vaccine. *Nature Med.* **6**, 1160–1166 (2000).
16. Kisker, O. *et al.* Continuous administration of endostatin by intraperitoneally implanted osmotic pump improves the efficacy and potency of therapy in a mouse xenograft tumor model. *Cancer Res.* **61**, 7669–7674 (2001).
17. Cross, M.J. & Claesson-Welsh, L. FGF and VEGF function in angiogenesis: signaling pathways, biological responses and therapeutic inhibition. *Trends Pharmacol. Sci.* **22**, 201–207 (2001).
18. Dias, S. *et al.* Autocrine stimulation of VEGFR-2 activates human leukemic cell

growth and migration. *J. Clin. Invest.* **106**, 511–521 (2000).
19. Drake, C.J., LaRue, A., Ferrara, N. & Little, C.D. VEGF regulates cell behavior during vasculogenesis. *Dev. Biol.* **224**, 178–188 (2000).
20. Ferrara, N. VEGF: An update on biological and therapeutic aspects. *Curr. Opin. Biotechnol.* **11**, 617–624 (2000).
21. Folkman, J. & D'Amore, P.A. Blood vessel formation: what is its molecular basis? *Cell* **87**, 1153–1155 (1996).
22. McMahon, G. VEGF receptor signaling in tumor angiogenesis. *Oncologist* **5 Suppl 1**, 3–10 (2000).
23. Ortega, N., Hutchings, H. & Plouet, J. Signal relays in the VEGF system. *Front Biosci.* **4**, D141–D152 (1999).
24. Strawn, L.M. *et al.* FLK-1 as a target for tumor growth inhibition. *Cancer Res.* **56**, 3540–3545 (1996).
25. Taraboletti, G. & Margosio, B. Antiangiogenic and antivascular therapy for cancer. *Curr. Opin. Pharmacol.* **1**, 378–384 (2001).
26. Ochsenbein, A.F. *et al.* Roles of tumor localization, second signals and cross priming in cytotoxic T-cell induction. *Nature* **411**, 1058–1064 (2001).
27. Hata, Y., Rook, S.L. & Aiello, L.P. Basic fibroblast growth factor induces expression of VEGF receptor KDR through a protein kinase C and p44/p42 mitogen-activated protein kinase-dependent pathway. *Diabetes* **48**, 1145–1155 (1999).
28. St Croix, B. *et al.* Genes expressed in human tumor endothelium. *Science* **289**, 1197–1202 (2000).
29. Niethammer, A.G. *et al.* An oral DNA vaccine against human carcinoembryonic antigen (CEA) prevents growth and dissemination of Lewis lung carcinoma in CEA transgenic mice. *Vaccine* **20**, 421–429 (2001).
30. Niethammer, A.G. *et al.* Targeted interleukin 2 therapy enhances protective immunity induced by an autologous oral DNA vaccine against murine melanoma. *Cancer Res.* **61**, 6178–6184 (2001).
31. Darji, A. *et al.* Oral somatic transgene vaccination using attenuated *S. typhimurium*. *Cell* **91**, 765–775 (1997).
32. Lode, H.N. *et al.* Gene therapy with a single chain interleukin 12 fusion protein induces T cell-dependent protective immunity in a syngeneic model of murine neuroblastoma. *Proc. Natl. Acad. Sci. USA* **95**, 2475–2480 (1998).
33. Schrama, D. *et al.* Targeting of lymphotoxin-α to the tumor elicits an efficient immune response associated with induction of peripheral lymphoid-like tissue. *Immunity* **14**, 111–121 (2001).
34. Kaesler, S., Regenbogen, J., Durka, S., Goppelt, A. & Werner, S. The healing skin wound: A novel site of action of the chemokine c10. *Cytokine* **17**, 157–163 (2002).
35. Werner, S. *et al.* Large induction of keratinocyte growth factor expression in the dermis during wound healing. *Proc. Natl. Acad. Sci. USA* **89**, 6896–6900 (1992).
36. Barlow, C. *et al.* Atm-deficient mice: A paradigm of ataxia telangiectasia. *Cell* **86**, 159–171 (1996).
37. Paylor, R. *et al.* Alpha7 nicotinic receptor subunits are not necessary for hippocampal- dependent learning or sensorimotor gating: A behavioral characterization of Acra7-deficient mice. *Learn. Mem.* **5**, 302–316 (1998).

Activated T-and NK cells combine with suppression of angiogenesis to protect against breast cancer growth

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Key words: DNA vaccine, breast tumor, suppression, growth, metastases

Abstract

Protection against breast cancer was achieved with a DNA vaccine against murine transcription factor, Fos-related antigen 1(Fra-1), overexpressed in aggressively proliferating D2F2 murine breast carcinoma. Growth of primary subcutaneous tumor and dissemination of pulmonary metastases was markedly suppressed by this oral DNA vaccine, carried by attenuated *Salmonella typhimurium*, encoding murine Fra-1, fused with mutant polyubiquitin and cotransformed with secretory murine IL-18. The lifespan of 60% of vaccinated mice was tripled in the absence of detectable tumor growth after lethal tumor cell challenge. Immunological mechanisms involved activation of T, NK- and dendritic cells indicated by upregulation of their activation markers and costimulatory molecules. Markedly increased specific target cell lysis was mediated by both MHC class I-restricted $CD8^+$ T cells and NK cells isolated from splenocytes of vaccinated mice, including a significant release of proinflammatory cytokines IFN-γ and IL-2. Importantly, fluorescence analysis of FGF-2 and tumor cell-induced vessel growth in Matrigel plugs demonstrated marked suppression of angiogenesis only in vaccinated animals. Taken together, this multi-functional DNA vaccine proved effective in protecting against growth and metastases of breast cancer by combining the action of immune effector cells with suppression of tumor angiogenesis.

Introduction

Breast cancer is one of the most common malignancies in women, and is the leading cause of death among women between the ages of 40 and 55 years in the U.S.A. (1, 2). During the last two decades, this neoplasm has been studied intensively, and recently new preventive measures and therapies have emerged, especially immunological and genetic treatments administered as adjuvant therapy after surgery, radiation, and chemotherapy. Biotherapy produced successful results in mice with mammary carcinoma, particularly with cellular vaccines (3), DNA vaccines (4-6), recombinant proteins (7, 8), and adoptive immunotherapy (9).

Progression of breast cancer is often accompanied by changes in gene expression patterns in cells of growing carcinomas, resulting in highly tumorigenic and invasive cell types (10). Thus, AP-1 transcription factor (Activating Protein-1) belongs to a group of factors, which define tumor progression and regulate breast cancer cell invasion and growth, as well as resistance to anti-estrogens (11, 12). In addition, Fra-1 (Fos-related antigen-1), a transcription factor belonging to the AP-1 family, is overexpressed in many human and mouse carcinoma cells, including those of thyroid (13), kidney (14), esophagus (15) and breast (16, 17). Overexpression of Fra-1 in epithelial carcinoma cells greatly influences their morphology, motility and invasiveness and activates the transcription of a number of genes. Overexpression of this transcription factor also correlates with transformation of epithelial tumor cells to a more invasive phenotype (18), and a close, specific association of Fra-1 expression with highly invasive breast cancer cells was reported (19, 20). Taken together,

these findings suggest that overexpressed Fra-1 can serve as a potential target for active vaccination against breast cancer (21).

IL-18 is a potent immunoregulatory cytokine that was initially described as an IFN-γ inducing factor (22, 23). This cytokine also enhances cytokine production of T and/or NK cells and induces their proliferation and cytolytic activity (24, 25). Tumor cells engineered to produce IL-18 are less tumorigenic (26-28) and systemic administration of IL-18 resulted in considerable therapeutic activity in several murine tumor models (29, 30). In addition, IL-18 enhances cellular immune mechanisms by upregulating MHC expression and by favoring the differentiation of $CD4^+$ helper T cells towards the Th1 subtype (31, 32). In turn, Th1 cells secrete IL-2 and IFN-γ, which facilitate the proliferation and/or activation of $CD8^+$ CTLs, NK cells and macrophages, all of which can contribute to tumor regression (33). In addition, IL-18 is a novel inhibitor of angiogenesis, sufficiently potent to suppress tumor growth by directly inhibiting fibroblast growth factor-2 (FGF-2)-induced endothelial cell proliferation (34). Recently, the role of recombinant IL-18 as a biological "adjuvant" has been evaluated in murine tumor models, and its systemic administration induced significant antitumor effects in several tumor models (30, 35, 36).

The induction of an efficacious Ag-specific immunity by DNA vaccines against self-Ag necessitates the optimization of vaccine design, including effective modalities of vaccine delivery, powerful adjuvants and optimal antigen processing. Such an approach is illustrated by an oral vaccine delivery system utilizing a doubly attenuated strain of *Salmonella typhimurium* (*dam*$^-$ *aroA*$^-$) (37). Thus, vaccination by oral gavage of these bacteria harboring plasmid DNA vaccines proved effective for DNA delivery to such secondary lymphoid

tissues as Peyer's Patches in the small intestine, with subsequent induction of immunity against antigens encoded by the plasmid (38). DNA immunization also can be enhanced significantly by exploiting natural pathways of antigen presentation. Thus, most peptides presented as complexes with MHC class I antigens that induce active CTLs are derived from cytosolic proteins degraded and processed by the proteasome. Protein is targeted to this organelle by polyubiquitin, a process in which many copies of this cellular protein are covalently attached to the target protein to markedly enhance its degradation by the proteasome (39, 40).

Here, we describe the anti-tumor activity and mechanism of action of a multi-functional DNA vaccine, encoding transcription factor Fra-1 and secretory IL-18, which effectively protects against primary breast tumor growth and metastases by suppression of tumor angiogenesis and activation of T, NK and dendritic cells.

Materials and Methods

Animals, Bacterial Strains and Cell Lines

Female Balb/c mice, 6-8 wk of age, were purchased from The Scripps Research Institute Rodent Breeding Facility. The attenuated *S. typhimurium* strain RE88 (*aroA*$^-$ *dam*$^-$) was kindly provided by Remedyne Corporation, (Santa Barbara, CA). Bacterial strain Top10 was purchased from Invitrogen, (Carlsbad, CA) and bacteria were grown routinely at 37°C in LB broth or on agar plates (EM SCIENCE, Darmstadt, Germany), supplemented, when required, with 50 μg/ml ampicillin. The murine D2F2 breast cancer cell line was obtained from ATCC (American Type Culture Collection USA) and cultured in DMEM supplemented with 10%

(vol/vol) FBS. All animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Construction of Expression Vectors

Two constructs were made based on the pIRES vector (Invitrogen). The first, pUb-Fra-1, was comprised of ubiquitinated, full-length murine Fos-related-antigen-1 (Fra-1). The second, pIL-18, contained murine Interleukin-18 (IL-18). The empty vector with (pUb) or without ubiquitin served as a control. Protein expression of Fra-1 and IL-18 was demonstrated by Western blotting, with IL-18 protein expression found in both cell lysates and culture supernatants. Bioactivity of murine IL-18 in cell supernatants was measured by an ELISA assay (RD systems, Minneapolis MN) using the production of IFN-γ in KG-1 lymphoma cells as an indicator (41).

Transduction and Expression of *S. typhimurium* with DNA Vaccine Plasmids

Attenuated *Salmonella typhimurium* were transduced with DNA vaccine plasmids by electroporation. Briefly, a single colony of bacteria was inoculated into 3 ml of LB medium, and then harvested during mid-log phase growth and washed twice with ice-cold water. Freshly prepared bacteria (1×10^8) were then mixed with plasmid DNA (2 μg) on ice in a 0.2-cm cuvette and electroporated at 2.5 KV, 25 μF and 200 Ω. The bacteria were transformed with the following plasmids: empty vector, pUb, pUb-Fra-1, pIL-18 or both pUb-Fra-1 and pIL-18 together, indicated as pUb-Fra-1/pIL-18. After electroporation, the bacteria were immediately removed from the cuvette and placed into a sterile culture tube containing 1 ml of LB broth medium and incubated with moderate shaking for 30 min at 37° C. The bacteria were centrifuged and then plated onto LB plates with 50 μg/ml ampicillin. Resistant colonies

harboring the DNA vaccine gene(s) were cultured and stored at -70° C after confirmation of the coding sequence.

Detection of EGFP Expression

EGFP expression by *aroA⁻ dam⁻ S. typhimurium* was used to obtain direct evidence for DNA transfer from the bacterial carrier to Peyer's patches and to establish that protein expression took place efficiently and successfully. EGFP expression was tested using the doubly attenuated strain *S. typhimurium* harboring the gene (S.T-GFP). Briefly, mice were administered 1×10^8 bacteria by oral gavage, and 24 h thereafter, these animals were sacrificed and biopsies collected from the small intestine washed thoroughly with PBS. The fresh specimens were checked for EGFP expression in Peyer's Patches by confocol microscopy or saved for further H&E staining.

Protein Detection by Western Blotting

To detect protein production, Cos-7 cells were transfected with the pUb-Fra-1 or pIL-18 plasmid using a calcium phosphate transfection kit based on the manufacturer's instructions (Invitrogen). After 24 h cells were harvested and lysed and protein concentrations determined with a BCA kit (Pierce, Rockford, IL). Protein (30 μg) of each sample was electrophoresed on 16% Tris-Glycine gels and then transferred onto nitrocellulose membranes (Invitrogen) that were subjected to 150 mA for 30 min. Membranes were blocked for 2 h by 5% nonfat dry milk in PBS containing 0.2% Tween 20. Western blot analysis was performed with anti-Fra-1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-mouse IL-18 mAb (MBL, Nagoya, Japan). Films were developed using a chemiluminescence protocol provided by the manufacturer (Pierce, Rockford, IL).

Oral Immunization and Tumor Cell Challenge

Balb/c mice were divided into five experimental groups (n=8) and immunized three times at 2 wk intervals by oral gavage with 100 µl PBS containing 1×10^8 doubly mutated *S. typhimurium* harboring either empty vector, pUb, pUb-Fra-1, pIL-18 or pUb-Fra-1/pIL-18. All mice were challenged s.c. into the right flank with a lethal dose of 1×10^6 D2F2 breast cancer cells or by i.v. injection with 0.5×10^6 of D2F2 cells 1 wk after the last immunization to induce primary tumor or experimental pulmonary metastases, respectively. In the s.c. tumor model, mice were examined twice each week until the tumor became palpable, after which its diameter was measured in two dimensions with a microcaliper every other day. In the pulmonary metastases model, mice were sacrificed 4 wk after i.v. injection. Metastasis scores were determined as percentage of lung surface covered by fused metastases as follows: 0=0%, 1=<20%, 2=20-50%, 3=>50%.

Cytotoxicity Assay

Cytotoxicity was measured by a standard ^{51}Cr-release assay. Splenocytes were harvested from Balb/c mice 2 wk after challenge with 0.5×10^6 D2F2 breast carcinoma cells and subsequently cultured for 3 d at 37° C in complete T-STIM culture medium (Beckton Dickinson, Bedford, MA). Both D2F2 and Yac-1 cells were used as targets. These cells were each labeled with 0.5 mCi of ^{51}Cr, and incubated at 37° C for 4 h with effector cells at various effector to target cell ratios. The percentage of specific target cell lysis was calculated with the formula $[(E-S)/(T-S)]\times100\%$, where E is the average experimental release, S is the average spontaneous release, and T is the average total release.

Flow Cytometric Analysis

Activation markers of T cells and NK cells as well as CD80 and CD86 costimulatory molecules were measured by two-color flow cytometric analysis with a BD Biosciences FACScan. T cell activation was determined by staining freshly isolated splenocytes from successfully vaccinated mice with anti-CD8-FITC or anti-CD3-FITC Ab in combination with PE-conjugated anti-CD25, CD11a, CD28, or CD69 Ab. Activation of NK cell markers was measured with FITC-labeled anti-NK-1.1 Ab in combination with PE-conjugated anti-DX5 Ab. Costimulatory molecules on APCs were detected by PE-conjugated anti-CD80 or CD86 Ab in combination with FITC-labled CD11c Ab. All reagents were obtained from BD Pharmingen (La Jolla, CA).

Cytokine Release Assay

We used flow cytometry for detection of intracellular cytokines and the ELISPOT assay to measure single cell cytokine release. To this end, splenocytes were collected 2 wk after D2F2 tumor cell challenge from all experimental groups of mice, and cultured for 24 h in complete T cell medium with irradiated D2F2 cells as described. Preincubated cells were suspended with 1 μg purified 2.4G2 Ab (BD Pharmingen) to block nonspecific staining. The cells were washed and then stained with 0.5 μg FITC conjugated anti-$CD3^+$ Ab. After washing two times, cells were fixed and stained with 1 μg/ml PE conjugated with anti-IL2 or anti-IFN-γ Ab for flow cytometric analysis. All Ab were obtained from BD Pharmingen. Immunospot plates (BD Bioscience, San Diego, CA) were coated overnight at 4°C with capture Ab specific for either IFN-γ or IL-2. The plates were then blocked with FBS (10% in RPMI 1640). D2F2 cells (1×10^4/ml) were irradiated with 1000 Gy, plated and stimulated

with mitogen. Splenocytes were collected 2 wk after intravenous D2F2 tumor cell challenge from all experimental groups of mice, and were plated in complete RPMI 1640 medium (1×10^6/ml). After overnight incubation, the cells were washed, first with deionized water, and then with washing buffer. Thereafter, Avidin-HRP (1:100) was added following incubation with biotinylated anti-mouse IFN-γ Ab (2 μg/ml) and IL-2 (2 μg/ml). The spots were developed with AEC development solution, and plates read by immunospot®ScAnalysis (BD Bioscience). Digitalized images were analyzed for areas in which color density exceeded background by an amount based on a comparison of experimental wells.

Evaluation of Anti-Angiogenic Effects

Balb/c mice were vaccinated as described above. Two wk after the last vaccination, mice were injected s.c. in the sternal region with 500 μl growth factor-reduced matrigel (BD Biosciences) containing 400 ng/ml murine FGF-2 (PeproTech, Rocky Hill, NJ) and D2F2 tumor cells (1×10^4/ml) which were irradiated with 1000 Gy. In all mice, except for 2 control animals, endothelium tissue was stained 6 d later by i.v. injection into the lateral tail vein with 200 μl of 0.1 mg/ml fluorescent *Bandeiraea simplicifolia* lectin I, Isolectin B4 (Vector Laboratories, Burlingame, CA). Thirty min later, mice were sacrificed and Matrigel plugs excised and evaluated macroscopically. Lectin-FITC was then extracted from 100 μg of each plug in 500 μl of RIPA lysis buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with the help of a tissue grinder. Solid materials were pelleted by centrifugation and the lectin-FITC content in the buffer quantified by fluorimetry at 490 nm. Background fluorescence found in the two non-injected control mice was subtracted in each case.

Results

Vectors Encoding Genes for Ub-Fra-1 or IL-18 Express the Respective Bioactive Protein

We successfully constructed the eukaryotic expression vectors based on the pIRES vector backbone, namely pUb-Fra-1 and pIL-18 (Fig. 1A). Protein expression of pUb-Fra-1 and pIL-18 was demonstrated by transient transfection of each vector into COS-7 cells, and by performing Western blots on the respective cell lysates (pUb-Fra-1 or pIL-18) and supernatants (pIL-18) with anti-Fra-1 and anti-IL-18 Ab. The results indicated that all constructs produced protein of the expected molecular mass with IL-18 being expressed in its active form at 18 kD (Fig. 1B, lane 2) and Fra-1 as a 46KDa protein (Fig. 1B, lane 1). Protein expression of IL-18 was also detected in the culture supernatant of transfected cells (Fig. 1B, lane 3). Importantly, the biofunctional activity of IL-18 was demonstrated by ELISA in supernatants of cells transfected with pIL-18. (Fig. 1C)

Salmonella typhimurium Transfer Expression Vectors to Mouse Peyer's Patches

We demonstrated that 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. 1D) to be subsequently transcribed by APCs, processed in the proteasome and presented as MHC-peptide complexed to T cells. To this end, mice were administered by oral gavage, 1×10^8 *dam*$^-$, *aroA*$^-$ attenuated *S. typhimurium*. After 24 h these animals were sacrificed and biopsies collected from the thoroughly washed small intestine. In fact, the doubly attenuated bacteria harboring EGFP (S.T-GFP) exhibited strong EGFP fluorescence (Fig. 1D), suggesting 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, because of the *aroA*$^-$ *dam*$^-$ mutation, these doubly attenuated bacteria do not survive very long since neither EGFP activity nor live bacteria could be detected in immunized animals after 72 h (data not shown). However, EGFP expression was detected in adherent cells, most likely APCs, such as DCs and macrophages from Peyer's Patches following oral administration of *Salmonella typhimurium* harboring the eukaryotic EGFP expression plasmid. Taken together, these findings suggest that both, plasmid transfer to and protein expression in eukaryotic cells did take place.

Tumor Specific Protective Immunity Against Breast Cancer is Induced by the DNA Vaccine

We proved our hypothesis that an orally administered DNA vaccine encoding murine Ub-Fra-1 and secretory IL-18, carried by attenuated *S. typhimurium*, can induce protective immunity against s.c. tumor growth and pulmonary metastasis of D2F2 breast carcinoma. Thus, we observed marked inhibition of both s.c. tumor growth and disseminated experimental pulmonary metastases in Balb/c mice challenged 1 wk after the third vaccination with pUb-Fra-1/pIL-18 by either i.v. (Fig. 2A) or s.c. (Fig. 2B) injection of D2F2 murine breast cancer cells. In contrast, animals vaccinated with only the empty vector (pIRES) or the vector encoding only ubiquitin (pUb), carried by attenuated bacteria, all uniformly revealed rapid s.c tumor growth and extensive dissemination of pulmonary metastases. Importantly, the lifespan of 60% of successfully vaccinated Balb/c mice (5/8) was tripled in the absence of any detectable tumor growth up to 11 wk after tumor cell challenge (Fig. 2C).

MHC class I-Restricted Tumor Specific CTLs and NK cells Kill D2F2 Breast Cancer Cells *In Vitro*

We demonstrated that immunization with our DNA vaccine induces tumor-specific immunity capable of killing breast cancer cells *in vitro* either by MHC class I Ag-restricted $CD8^+$ T cells or by NK cells. To this end, we isolated $CD8^+$ T cells from splenocytes of groups of Balb/c mice vaccinated as described above. The data depicted in Fig. 3 indicate that only $CD8^+$ T cells isolated from splenocytes of mice immunized with the vaccine encoding pUb-Fra-1/pIL-18 were effective in killing D2F2 breast cancer cells *in vitro* at different effector-to-target cell ratios. In contrast, controls such as $CD8^+$ T cells isolated from mice immunized with only the empty vector carried by attenuated *S. typhimurium* produced solely background levels of tumor cell lysis (Fig. 3). The $CD8^+$ T cell-mediated killing of D2F2 cells was specific because syngeneic prostate cancer target cells (RM-2) lacking Fra-1 were not lysed (data not shown). Importantly, the $CD8^+$ T cell-mediated tumor cell lysis was MHC class I Ag-restricted because addition of 10 μg/ml anti-H-$2K^d$/H-$2D^d$ Ab specifically inhibited lysis of D2F2 cells (Fig. 3).

We also examined whether NK cells were involved in tumor cell killing with a standard 4 h ^{51}Cr-release assay using NK-specificYac-1 cells as targets for splenocytes from Balb/c mice immunized and challenged with D2F2 breast cancer cells. Only immunization with the combined vectors, pUb-Fra-1/pIL-18 or pIL-18 alone led to significant lysis of Yac-1 target cells by NK cells in contrast to control immunizations which were ineffective (Fig. 3).

Activation of T Cells, NK Cells and Costimulatory Molecules

The interaction between IL-18 and active Th1 cells and NK cells is critical for achieving both optimal Ag- specific T cell and NK cell responses. The vaccine harboring either pUb-Fra-1/pIL-18 or pIL-18 alone was observed to upregulate the expression of T or NK cell activation markers, respectively. This was evident from an increase in expression of CD25, the high affinity IL-2R α-chain, CD69, an early T cell activation antigen, and CD11a, which is important for the initial interaction between T cells and DCs as well as regular T cell markers $CD4^+$ and $CD8^+$ (Fig. 4). Additionally, it has been known by published researchers that NK cells play a partial role in the process of anti-tumor immune response. For that reason, spleen cells obtained from mice successfully immunized with DNA vaccines along with the control groups were assayed with anti-DX5. As shown in Fig. 5, this regimen dramatically increased the DX5 expression on NK cells, which is especially important for NK cytotoxity.

Furthermore, T cell activation is critically dependent on up-regulated expression of costimulatory molecules CD80 and CD86 on DCs to achieve optimal ligation with CD28 expressed on T cells. In this regard, FACS analyses of splenocytes obtained from syngeneic BALB/c mice, successfully immunized with the DNA vaccine, clearly demonstrated that we accomplished this particular task successfully, as the expression of CD80 and CD86 was upregulated 2- to 3- fold over controls (Fig. 6).

T Cell Activation is Indicated by Increased Secretion of Pro-Inflammatory Cytokines

The release of pro-inflammatory cytokines IL-2 and IFN-γ from T cells is a well-known indication of T cell activation in secondary lymphoid tissues. Consequently, we measured

these two cytokines, both intracellularly with flow cytometry (Fig. 7), and at the single cell level with ELISPOT (Fig. 8). Indeed, vaccination with the pUb-Fra-1/pIL-18 plasmid and subsequent challenge with tumor cells resulted in a dramatic increase of IFN-γ and IL-2 release over that of splenocytes from controls by both experimental methods.

Suppression of Angiogenesis is Induced by DNA Vaccine

We could demonstrate distinct suppression of angiogenesis induced by the pUb-Fra-1/pIL-18 DNA vaccine in a Matrigel assay. Specifically, this was evident from the marked decrease in vascularization, following vaccination as evaluated by relative fluorescence after *in vivo* staining of endothelium with FITC-conjugated lectin. Differences were visible macroscopically, as shown in Fig. 9, depicting representative examples of Matrigel plugs removed from vaccinated mice 6 d after their injection. FITC-lectin staining clearly revealed suppression of angiogenesis indicated by a significantly decreased vascularization in matrigel plugs only after vaccination with the vector encoding pUb-Fra-1/pIL-18 and to a somewhat lesser extent with pIL-18 alone but not with vaccines encoding only pUb-Fra-1, pUb or the empty vector control (Fig. 9).

Discussion

The design of effective cancer vaccines remains a major challenge for tumor immunotherapy. The major objective of this study was to meet this challenge by developing a novel DNA vaccine encoding a transcription factor, Fra-1, which is overexpressed in breast cancer and reported to be significantly associated with invasion and growth of this neoplasm (19, 20). The results of our studies demonstrate that peripheral T cell tolerance against the Fra-1 transcription factor can indeed be broken by an oral DNA vaccine encoding full length

murine Fra-1, fused with mutant polyubiquitin, and further modified by co-transformation with a gene encoding secretory murine IL-18.

The immunological mechanisms and effector cells involved in the tumor protective immunity induced by our vaccine, clearly indicated a prominent cellular immune response by both T and NK cells. In this regard, it is well known that activation of immune effector cells is highly correlated with upregulation of IFN-γ. In fact, the regulation of IFN-γ expression is one of the most tightly controlled processes of the cellular immune response (42, 43). Production of IFN-γ, was induced by our DNA vaccine, and found to be essentially restricted to activated $CD4^+$ and $CD8^+$ T cells, as well as NK cells. For each of these cell types, IFN-γ secretion is further restricted by the availability of IFN-γ-inducing cytokines such as IL-2, IL-12 and TNF-α, which arise from accessory cells following activation. The discovery of IL-18, added a new molecule to the short list of IFN-γ regulators (24, 25). Furthermore, IL-18 was recently reported to be a potent antiangiogenic cytokine, both *in vitro* and *in vivo* (44). Consequently, we deliberately designed our multi-functional vaccine as a combination of Fra-1 with secretory IL-18. This design was successful as our data clearly demonstrate since activation of both T- and NK cells was significantly augmented after immunization with this vaccine, indicated by marked upregulation of a series of T- and NK cell activation markers. In this regard, it is well established that $CD8^+$ T cell activation is critically dependent on upregulated expression of costimulatory molecules CD80 and CD86 on APCs to achieve optimal ligation with CD28 expressed by T cells. Indeed, our data provide evidence that immunization with the pUb-Fra-1/pIL-18 DNA vaccine induces and enhances the expression of these costimulatory molecules on $CD11c^+$ and MHC class II Ag-positive APCs, suggesting

that the capability of these APCs for processing and presentation of tumor-specific Ag was significantly enhanced.

Moreover, the marked elevation in production of proinflammatory cytokines IFN-γ and IL-2 detected by intracellular cytokine staining and single cell cytokine release also demonstrated T cell activation after immunization with our vaccine. This was further indicated by the upregulation of CD25 especially since this occurred together with increased production of IL-2 by activated T-cells. Furthermore, tumor angiogenesis was found to be effectively suppressed only in experimental groups of mice which were immunized with pUb-Fra-1/pIL-18 and to a lesser extent with pIL-18 alone in the D2F2 breast cancer model as indicated by suppression of vessel formation and regression of growing blood vessels.

Our success in eliciting an effective $CD8^+$ T cell-mediated MHC class I Ag-restricted tumor protective immunity with a completely autologous oral DNA vaccine was most likely aided considerably by our efforts to optimize antigen processing in the proteasome by ubiquitination leading to more effective antigen presentation (45, 46). Support for this contention comes from our findings indicating that a DNA vaccine encoding murine Fra-1 lacking in ubiquitin was considerably less effective in inducing tumor protective immunity (data not shown). In contrast, the ubiquitinated DNA vaccine was clearly capable of inducing tumor protective immunity against a lethal challenge of D2F2 breast cancer cells. Our study further demonstrates the positive effects of ubiquitination in inducing T cell responses. These results are in agreement with our previous report that used modified ubiquitinated peptide antigens and confirmed the important role of ubiquitination in the MHC class I Ag presentation pathway (47).

One of the more critical aspects of DNA vaccine design is the selection of an optimally effective carrier to deliver the target gene to secondary lymphoid organs, such as Peyer's Patches, in the small intestine. This approach is designed to achieve a non-invasive administration as well as long-term protection by single or multiple vaccinations combined with ease of preparation, storage, and transport. In this regard, live, attenuated bacterial carriers that harbor eukaryotic expression plasmids encoding Ag, combined with powerful adjuvants, are attractive vehicles for oral delivery of vaccines. Current DNA vaccine delivery vehicles include replicating attenuated strains of intracellular bacteria like *Salmonella typhimurium*, *Listeria monocytogenes* and *Mycobacterium bovis* as well as *Bacillus Calmette Guerin* (BCG). These DNA vaccine delivery vehicles were reported to induce a broad spectrum of both mucosal and systemic immune responses. Moreover, the use of this natural route of entry could prove to be of benefit since many bacteria, like Salmonella, egress from the gut lumen via the M cells into Peyer's Patches (38) and migrate eventually into lymph nodes and spleen, thus allowing natural targeting of DNA vaccines to inductive sites of the immune system.

Our study made use of a novel, doubly mutated strain of *S. typhimurium* (*dam*$^-$, *aroA*$^-$) as a delivery vehicle for a DNA vaccine. The reasons for selecting this strain of bacteria are based on a number of advantages. First, DNA adenine methylase (*dam*$^-$) mutants of *S. typhimurium* were demonstrated to be highly attenuated and useful as live vaccines in a murine model of infection (48, 49). Additionally, *dam*$^-$ mutants do not cause a transient state of nonspecific immune suppression, indicating their potential usefulness as a vaccine carrier to deliver heterologous antigens to immune inductive sites (50). Although *dam*$^-$ mutants were

found unable to cause disease in mice, transient bacteria remained after several weeks in terminal organs (51). Thus, in order to completely abolish the systemic presence of the bacteria, a second mutation (*aroA*$^-$) was introduced which inhibits the synthesis of aromatic amino acids and causes the bacteria to die after just a few passages. *S. typhimurium aroA*$^-$ mutants have been characterized extensively in mice (37). The *dam*$^-$ *aroA*$^-$ double mutant which was undetectable in systemic tissues (data not shown), indicating a safer and less toxic salmonella, was consequently chosen as the vaccine carrier in our studies.

By using doubly mutated bacteria as a vaccine carrier, we not only demonstrated that the Fra-1 antigen targets appropriate pathways of major histocompatibility (MHC) class I Ag processing and presentation, but also that an adequate cytokine milieu is generated which effectively promotes Ag-specific responses. The most prominent advantage of this vaccine carrier vehicle is its capability to directly target DNA vaccines to Peyer's Patches which harbor immature dendritic cells, B cells, T cells and macrophages, i.e. most of the important immune effector cells necessary for an immune response induced by a DNA vaccine. Among these cells, DCs are the key antigen presenting cells that efficiently mediate Ag processing, transport and presentation to lymphoid tissues for the initiation of T cell responses. In this regard, Maloy et al (52) clearly demonstrated that intralymphatic immunization enhances DNA vaccination, increasing immunogenicity by 100- to 1000-fold and inducing strong and biologically relevant $CD8^+$ CTL responses.

Taken together, our studies demonstrate that the transcription factor, Fra-1, is a suitable target for induction of a T cell-mediated specific immune response against D2F2 breast cancer cells and that the design of a DNA vaccine, especially its ubiquitination and its attenuated bacterial

carrier lead to effective Ag processing and presentation. The coexpression of secretory IL-18 by our vaccine acts as a powerful and natural adjuvant for further activation of both $CD8^+$ and $CD4^+$ T cells as well as NK cells, leading to the production of IFN-γ and IL-2 as well as the suppression of angiogenesis in tumor tissues. It is anticipated that this multifunctional DNA vaccine might ultimately lead to the rational design of such vaccines for the immunotherapy of human breast cancer.

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Figure legends

Figure 1

Vector construction map, protein expression, bioactivity and targeting of expression constructs

A. The coding sequence of full-length, murine Fra-1, fused with polyubiquitin, was inserted into the pIRES plasmid (pUb-Fra-1). A second plasmid, pIL-18, contained the entire coding sequence of mouse IL-18.

B. Detection of protein expression by pUb-Fra-1 and pIL-18 was demonstrated by Western blotting. Blots are shown of cell lysates from COS-7 cells transfected with either pUb-Fra-1 (lane 1) or pIL-18 (lane 2) as well as from culture supernatant of pIL-18 transfected COS-7 cells (lane 3).

C. Bioactivity of IL-18 (ng/ml) was determined by ELISA in supernatants of KG-1 lymphoma cells that had been transfected with pIL-18.

D. Expression of EGFP activity in Peyer's Patches was determined in 6 wk old Balb/c mice immunized with 10^8 *aroA*$^-$ *dam*$^-$ bacteria transformed with pEGFP (S.T-GFP) by oral gavage per mouse. Mice were sacrificed 24 h later and a fresh specimen of small intestine was taken after thoroughly washing with PBS. Fluorescence expression of EGFP was detected by confocol microscopy (right panel). H&E staining of mouse Peyer's Patches is shown (left panel).

Figure 2

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 oral gavage as described in Materials and Methods.

A. Suppression of pulmonary metastases of D2F2 breast carcinoma. Experimental lung metastases were induced by i.v. injection of 5×10^5 D2F2 cells 1 wk after the last vaccination. The experiment was terminated 28 d after tumor cell inoculation and the extent of tumor foci on the lung surface determined. Results are expressed as metastatic score, i.e. the % lung surface covered by fused tumor foci. 0=0%; 1=<20%; 2=20-50%; and 3=>50%.

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 of respective treatment or control groups. Tumor growth was determined by microcaliper measurements and tumor volume was calculated according to $0.5\times\text{width}^2\times\text{length}$.

C. Survival curves represent results for 8 mice in each of the respective treatment and control groups. Surviving mice were tumor free unless otherwise stated.

Figure 3

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 ^{51}Cr-release assay at different E:T cell ratios. The top panel depicts 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)(■). The bottom panel depicts lysis mediated by NK cells (●) against Yac-1 target cells. Each value shown represents the mean of 8 animals.

Figure 4

Upregulated expression of T cell activation molecules

Balb/c mice were immunized with the DNA vaccine, then challenged with tumor cells as described in Materials and Methods. FACS analyses were performed with splenocytes obtained from mice (n=4) of each experimental and control group of animals. Two-color flow cytometric analyses were performed with single-cell suspensions of splenocytes. Anti-CD25, CD69, CD28 and CD11a Ab were used in PE conjugated form in combination with FITC-conjugated anti-mouse mAb directed against CD8$^+$ T cells. PE-labeled anti-CD8 and anti-CD4 Ab were used in combination with FITC-conjugated anti-mouse mAb CD3. Each value represents the mean for 4 mice.

Figure 5

Increased expression of NK cell marker after administration of DNA vaccine

FACS analysis of splenocytes with anti-DX5 mAb revealed the activation of NK cells after DNA vaccination. The experimental setting is similar to that of Fig. 4. Percentages refer to cells gated scored positive for DX5 expression. A representative histogram plot is shown for each group with the value depicting the mean for 4 mice.

Figure 6

Upregulation of costimulatory molecules by DNA vaccine

The combined DNA vaccine, pUb-Fra-1/pIL-18 enhanced the expression of costimulatory molecules. In the same experiment as that depicted in Fig. 4, two-color flow cytometric

analyses were performed with single-cell suspensions prepared from mouse splenocytes obtained 30 d after tumor cell challenge. Splenocytes were stained with FITC-labeled anti-CD11c Ab in combination with PE-conjugated anti-CD80 or CD86 Ab. Shown is the percent fluorescence of cell surface expressions of these two costimulatory molecules in a representative mouse. The data from each group (n=4) is displayed in the bar graph (mean+SD).

Figure 7

Induction of intracellular cytokines

Balb/c mice were immunized and challenged with D2F2 tumor cells as described in Materials and Methods. Splenocytes were obtained 2 wk after tumor cell challenge and stained with FITC-anti CD4 or CD8 Ab. Cells were fixed, permeabilized and subsequently stained with PE labeled anti-IFN-γ or anti-IL-2 Ab to detect the intracellular expression of these cytokines. A representative dot plot is shown for each group with the value depicting the mean for 8 mice.

Figure 8

Cytokine production at the single T cell level

Single cell resolution of DNA vaccine induced IFN-γ and IL-2 production in freshly isolated splenocytes of mice, immunized and challenged with tumor cells as described in Materials and Methods was verified by measuring the cytokine production of individual T cells by ELISPOT assay.

A. A representative ELISPOT assay is shown as spot formation per well induced by empty vector (a), pUb (b), pUb-Fra-1(c), pIL-18 (d) and pUb-Fra-1/pIL-18 (e).

B. The mean spot distribution of each well in each experimental and control group is shown (n=4, mean +SD).

Figure 9

Suppression of tumor angiogenesis

Balb/c mice (n=8) were vaccinated 3 times at 2 wk intervals with doubly attenuated *Samonella typhimurium* harboring various experimental or control plasmids. Two wk after the last vaccination, Matrigel (0.5 ml) containing murine FGF-2 (400 μg) and D2F2 cells (1×10^5) were implanted s.c. into the sternal region of mice and plugs removed for evaluation 6 d later. Quantification of vessel growth and staining of endothelium was determined by fluorimetry or confocal microscopy, respectively, using FITC-labeled Isoletin B4. Martrigel implants were harvested from mice and photographed with the use of confocal microscope. The yellow line and red 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), pUb-Fra-1/pIL-18 (e). The average fluorescence of Martrigel plugs from each group of mice is depicted by the bar graphs ($P<0.05$) (n=4; mean+SD).

Reference List

1. Baselga, J. and Mendelsohn, J. 1994. The epidermal growth factor receptor as a target for therapy in breast carcinoma. *Breast Cancer Res. Treat.* **29**:127-138.

2. Harris J., Morrow, M. and Bodadonna, G. 1993. Principles and Practice of Oncology. *In Cancer of the Breast.* Devita V.T., Hellman S. and Tosenberg S. editor. Lippincott Co., Philadelphia 1264-1332.

3. Cefai, D., Morrison, B.W., Sckell, A., Favre, L., Balli, M., Leunig, M. and Gimmi, C.D. 1999. Targeting HER-2/neu for active-specific immunotherapy in a mouse model of spontaneous breast cancer. *Int. J. Cancer* **83**:393-400.

4. Niethammer, A.G., Xiang, R., Becker, J.C., Wodrich, H., Pertl, U., Karsten, G., Eliceiri, B.P. and Reisfeld, R.A. 2002. A DNA vaccine against VEGF receptor 2 prevents effective angiogenesis and inhibits tumor growth. *Nat. Med.* **8**:1369-1375.

5. Amici, A., Venanzi, F.M. and Concetti, A. 1998. Genetic immunization against neu/erbB2 transgenic breast cancer. *Cancer Immunol. Immunother.* **47**:183-190.

6. Reilly, R.T., Gottlieb, M.B., Ercolini, A.M., Machiels, J.P., Kane, C.E., Okoye, F.I., Muller, W.J., Dixon, K.H. and Jaffee, E.M. 2000. HER-2/neu is a tumor rejection target in tolerized HER-2/neu transgenic mice. *Cancer Res.* **60**:3569-3576.

7. Esserman, L.J., Lopez, T., Montes, R., Bald, L.N., Fendly, B.M. and Campbell, M.J. 1999. Vaccination with the extracellular domain of p185neu prevents mammary tumor development in neu transgenic mice. *Cancer Immunol. Immunother.* **47**:337-342.

8. Granville, D.J., Ruehlmann, D.O., Choy, J.C., Cassidy, B.A., Hunt, D.W., van Breemen, C. and McManus, B.M. 2001. Bcl-2 increases emptying of endoplasmic reticulum Ca2+ stores during photodynamic therapy-induced apoptosis. *Cell Calcium* **30**:343-350.

9. Granziero, L., Krajewski, S., Farness, P., Yuan, L., Courtney, M.K., Jackson, M.R., Peterson, P.A. and Vitiello, A. 1999. Adoptive immunotherapy prevents prostate cancer in a transgenic animal model. *Eur. J. Immunol.* **29**:1127-1138.

10. Fish, E.M. and Molitoris, B.A. 1994. Alterations in epithelial polarity and the pathogenesis of disease states. *N. Eng. J. Med.* **330**:1580-1588.

11. Liu, Y., Ludes-Meyers, J., Zhang, Y., Munoz-Medellin, D., Kim, H. T., Lu, C., Ge,G. Schiff, R., Hilsenbeck, S.G., Osborne, C.K., et al. 2002. Inhibition of AP-1 transcription factor causes blockade of multiple signal transduction pathways and inhibits breast cancer growth. *Oncogene* **21**:7680-7689.

12. Jochum, W., Passegue, E. and Wagner, E.F. 2001. AP-1 in mouse development and tumorigenesis. *Oncogene* **20**:2401-2412.

13. Chiappetta, G., Tallini, G., De Biasio, M.C., Pentimalli, F., de Nigris, F., Losito, S., Fedele, M., Battista, S., Verde, P., Santoro, M. and Fusco, A. 2000. FRA-1 expression in hyperplastic and neoplastic thyroid diseases. *Clin. Cancer Res.* **6**:4300-4306.

14. Urakami, S., Tsuchiya, H., Orimoto, K., Kobayashi, T., Igawa, M. and Hino, O. 1997. Overexpression of members of the AP-1 transcriptional factor family from an early stage of renal carcinogenesis and inhibition of cell growth by AP-1 gene antisense oligonucleotides in the Tsc2 gene mutant (Eker) rat model. *Biochem. Biophys. Res. Commun.* **241**:24-30.

15. Hu, Y.C., Lam, K.Y., Law, S., Wong, J. and Srivastava, G. 2001. Identification of differentially expressed genes in esophageal squamous cell carcinoma (ESCC) by cDNA expression array: overexpression of Fra-1, Neogenin, Id-1, and CDC25B genes in ESCC. *Clin. Cancer Res.* **7**:2213-2221.

16. Roy, D., Calaf, G. and Hei, T.K. 2001. Profiling of differentially expressed genes induced by high linear energy transfer radiation in breast epithelial cells. *Mol. Carcinog.* **31**:192-203.

17. Milde-Langosch, K., Bamberger, A.M., Methner, C., Rieck, G. and Loning, T. 2000. Expression of cell cycle-regulatory proteins rb, p16/MTS1, p27/KIP1, p21/WAF1, cyclin D1 and cyclin E in breast cancer: correlations with expression of activating protein-1 family members. *Int. J. Cancer* **87**:468-472.

18. Kustikova, O., Kramerov, D. Grigorian, M., Berezin, V., Bock, E., Lukanidin, E. and Tulchinsky, E. 1998. Fra-1 induces morphological transformation and increases in vitro invasiveness and motility of epithelioid adenocarcinoma cells. *Mol. Cell Biol.* **18**:7095-7105.

19. Zajchowski, D.A., Bartholdi, M.F., Gong, Y., Webster, L., Liu, H.L. Munishkin, A., Beauheim, C., Harvey, S., Ethier, S.P. and Johnson, P.H. 2001. Identification of gene expression profiles that predict the aggressive behavior of breast cancer cells. *Cancer Res.* **61**:5168-5178.

20. Bamberger, A.M., Methner, C., Lisboa, B.W., Stadtler, C., Schulte, H.M., Loning, T. and Milde-Langosch, K. 1999. Expression pattern of the AP-1 family in breast cancer: association of fosB expression with a well-differentiated, receptor-positive tumor phenotype. *Int. J. Cancer* **84**:533-538.

21. Darnell, J.E., Jr. 2002. Transcription factors as targets for cancer therapy. *Nat. Rev. Cancer* **2**:740-749.

22. Nakamura, K., Okamura, H., Wada, M., Nagata, K. and Tamura, T. 1989. Endotoxin-induced serum factor that stimulates gamma interferon production. *Infect. Immun.* **57**:590-595.

23. Okamura, H., Tsutsi, H., Komatsu, T., Yutsudo, M., Hakura, A., Tanimoto, T., Torigoe, K., Okura, T., Nukada, Y., Hattori, K. and et al. 1995. Cloning of a new cytokine that induces IFN-gamma production by T cells. *Nature* **378**:88-91.

24. Ushio, S., Namba, M., Okura, T., Hattori, K., Nukada, Y., Akita, K., Tanabe, F., Konishi, K., Micallef, M., Fujii, et al. 1996. Cloning of the cDNA for human IFN-gamma-inducing factor, expression in Escherichia coli, and studies on the biologic activities of the protein. *J. Immunol.* **156**:4274-4279.

25. Tsutsui, H., Nakanishi, K., Matsui, K., Higashino, K., Okamura, H., Miyazawa, Y. and Kaneda, K. 1996. IFN-gamma-inducing factor up-regulates Fas ligand-mediated cytotoxic activity of murine natural killer cell clones. *J. Immunol.* **157**:3967-3973.

26. Osaki, T., Hashimoto, W., Gambotto, A., Okamura, H., Robbins, P.D., Kurimoto, M., Lotze, M.T. and Tahara, H. 1999. Potent antitumor effects mediated by local expression of the mature form of the interferon-gamma inducing factor, interleukin-18 (IL-18). *Gene Ther.* **6**:808-815.

27. Heuer, J.G., Tucker-McClung, C. and Hock, R.A. 1999. Neuroblastoma cells expressing mature IL-18, but not proIL-18, induce a strong and immediate antitumor immune response. *J. Immunother.* **22**:324-335.

28. Fukumoto, H., Nishio, M., Nishio, K., Heike, Y., Arioka, H., Kurokawa, H., Ishida, T., Fukuoka, K., Nomoto, T., Ohe, Y. and Saijo, N. 1997. Interferon-gamma-inducing factor gene transfection into Lewis lung carcinoma cells reduces tumorigenicity in vivo. *Jpn. J. Cancer Res.* **88**:501-505.

29. Hashimoto, W., Osaki, T., Okamura, H., Robbins, P.D., Kurimoto, M., Nagata, S., Lotze, M.T. and Tahara, H. 1999. Differential antitumor effects of administration of recombinant IL-18 or recombinant IL-12 are mediated primarily by Fas-Fas ligand- and perforin-induced tumor apoptosis, respectively. *J. Immunol.* **163**:583-589.

30. Micallef, M.J., Tanimoto, T., Kohno, K., Ikeda, M. and Kurimoto, M. 1997. Interleukin 18 induces the sequential activation of natural killer cells and cytotoxic T lymphocytes to protect syngeneic mice from transplantation with Meth A sarcoma. *Cancer Res.* **57**:4557-4563.

31. Nakanishi, K., Yoshimoto, T., Tsutsui, H. and Okamura, H. 2001. Interleukin-18 regulates both Th1 and Th2 responses. *Annu. Rev. Immunol.* **19**:423-474.

32. Tatsumi, T., Gambotto, A., Robbins, P.D. and Storkus, W.J. 2002. Interleukin 18 gene transfer expands the repertoire of antitumor Th1-type immunity elicited by dendritic cell-based vaccines in association with enhanced therapeutic efficacy. *Cancer Res.* **62**:5853-5858.

33. Wigginton, J.M., Lee, J.K., Wiltrout, T.A., Alvord, W.G., Hixon, J.A., Subleski, J., Back, T.C. and Wiltrout, R.H. 2002. Synergistic engagement of an ineffective endogenous anti-tumor immune response and induction of IFN-gamma and Fas-ligand-dependent tumor eradication by combined administration of IL-18 and IL-2. *J. Immunol.* **169**:4467-4474.

34. Okamura, H., Tsutsui, H., Kashiwamura, S., Yoshimoto, T. and Nakanishi, K. 1998. Interleukin-18: a novel cytokine that augments both innate and acquired immunity. *Adv. Immunol.* **70**:281-312.

35. Osaki, T., Peron, J.M., Cai, Q., Okamura, H., Robbins, P.D., Kurimoto, M., Lotze, M.T. and Tahara, H.. 1998. IFN-gamma-inducing factor/IL-18 administration mediates IFN-gamma- and IL-12-independent antitumor effects. *J. Immunol.* **160**:1742-1749.

36. Micallef, M.J., Yoshida, K., Kawai, S., Hanaya, T., Kohno, K., Arai, S., Tanimoto, T., Torigoe, K., Fujii, M., Ikeda, M. and Kurimoto, M. 1997. In vivo antitumor effects of murine interferon-gamma-inducing factor/interleukin-18 in mice bearing syngeneic Meth A sarcoma malignant ascites. *Cancer Immunol. Immunother.* **43**:361-367.

37. Stocker, B.A. 2000. Aromatic-dependent salmonella as anti-bacterial vaccines and as presenters of heterologous antigens or of DNA encoding them. *J. Biotechnol.* **83**:45-50.

38. Darji, A., Guzman, C.A., Gerstel, B., Wachholz, P., Timmis, K.N., Wehland, J., Chakraborty, T. and Weiss, S. 1997. Oral somatic transgene vaccination using attenuated S. typhimurium. *Cell* **91**:765-775.

39. Rodriguez, F., Zhang, J. and Whitton, J.L. 1997. DNA immunization: ubiquitination of a viral protein enhances cytotoxic T-lymphocyte induction and antiviral protection but abrogates antibody induction. *J. Virol.* **71**:8497-8503.

40. Rodriguez, F., An, L.L., Harkins, S., Zhang, J., Yokoyama, M., Widera, G., Fuller, J.T., Kincaid, C., Campbell, I.L. and Whitton, J.L. 1998. DNA immunization with

minigenes: low frequency of memory cytotoxic T lymphocytes and inefficient antiviral protection are rectified by ubiquitination. *J. Virol.* **72**:5174-5181.

41. Kawashima, M., Yamamura, M., Taniai, M., Yamauchi, H., Tanimoto, T., Kurimoto, M., Miyawaki, S., Amano, T., Takeuchi, T. and Makino, H. 2001. Levels of interleukin-18 and its binding inhibitors in the blood circulation of patients with adult-onset Still's disease. *Arthritis Rheum.* **44**:550-560.

42. Billiau, A. 1996. Interferon-gamma: biology and role in pathogenesis. *Adv. Immunol.* **62**:61-130.

43. Sad, S., Marcotte, R. and Mosmann, T.R. 1995. Cytokine-induced differentiation of precursor mouse CD8+ T cells into cytotoxic CD8+ T cells secreting Th1 or Th2 cytokines. *Immunity.* **2**:271-279.

44. Cao, R., Farnebo, J., Kurimoto, M. and Cao, Y. 1999. Interleukin-18 acts as an angiogenesis and tumor suppressor. *FASEB J.* **13**:2195-2202.

45. Finley, D. 2002. Ubiquitin chained and crosslinked. *Nat.Cell Biol.* **4**:E121-E123.

46. Hellmann, H. and Estelle, M. 2002. Plant development: regulation by protein degradation. *Science* **297**:793-797.

47. Xiang, R.,Lode, H.N., Chao, T.H., Ruehlmann, J.M., Dolman, C.S., Rodriguez, F., Whitton, J.L., Overwijk, W.W., Restifo, N.P. and Reisfeld, R.A. 2000. An autologous

oral DNA vaccine protects against murine melanoma. *Proc. Natl. Acad. Sci. U.S.A* **97**:5492-5497.

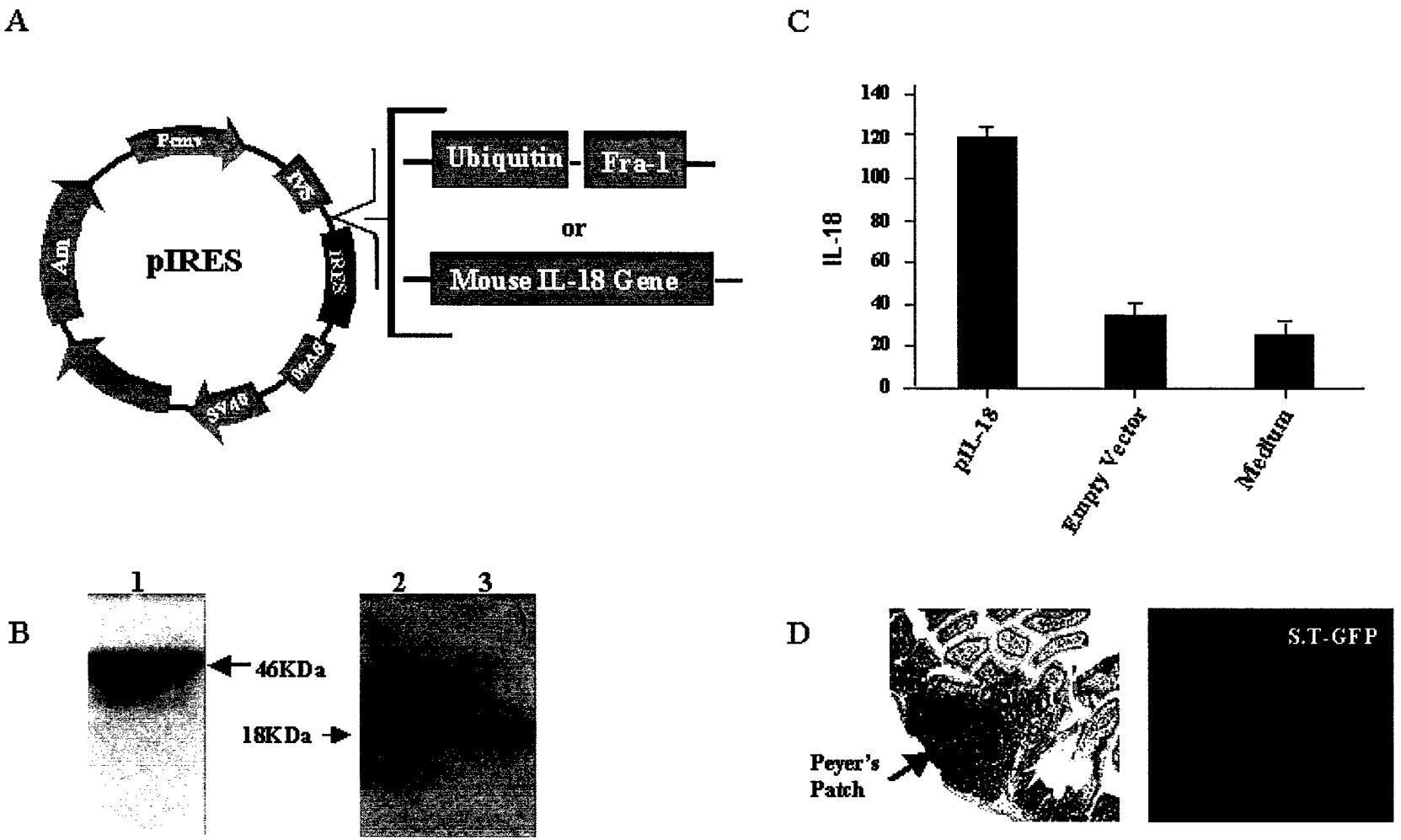
48. Heithoff, D.M., Conner, C.P., Hentschel, U., Govantes, F., Hanna, P.C. and Mahan, M.J. 1999. Coordinate intracellular expression of Salmonella genes induced during infection. *J. Bacteriol.* **181**:799-807.

49. Garcia-Del Portillo, F., Pucciarelli, M.G. and Casadesus, J. 1999. DNA adenine methylase mutants of Salmonella typhimurium show defects in protein secretion, cell invasion, and M cell cytotoxicity. *Proc. Natl. Acad. Sci.U.S.A* **96**:11578-11583.

50. Heithoff, D.M., Enioutina, E.Y., Daynes, R.A., Sinsheimer, R.L., Low, D.A. and Mahan, M.J. 2001. Salmonella DNA adenine methylase mutants confer cross-protective immunity. *Infect. Immun.* **69**:6725-6730.

51. Heithoff, D.M., Sinsheimer, R.L., Low, D.A. and Mahan, M.J. 1999. An essential role for DNA adenine methylation in bacterial virulence. *Science* **284**:967-970.

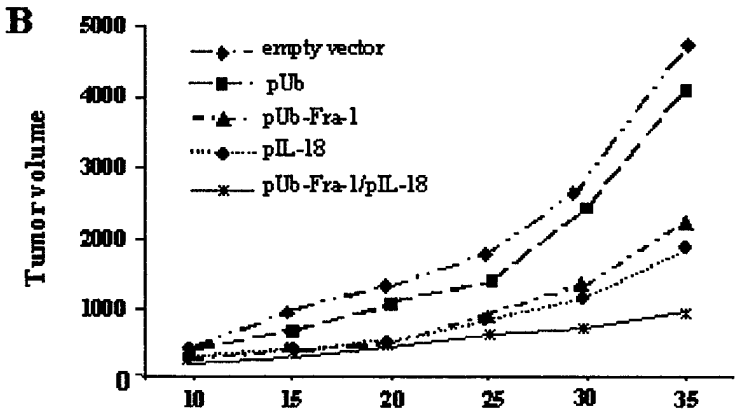
52. Maloy, K.J., Erdmann, R. Basch, V., Sierro, S., Kramps, T.A., Zinkernagel, R.M., Oehen, S. and Kuendig, T.M.. 2001. Intralymphatic immunization enhances DNA vaccination. *Proc. Natil. Acad. Sci. U.S.A* **98**:3299-3303..


A
pIRES
Fmv
Amp
IRES
SV40
Ubiquitin
Fra-1
or
Mouse IL-18 Gene
C
IL-18
140
120
100
80
60
40
20
0
pIL-18
Empty Vector
Medium
B
1
2
3
46KDa
18KDa
D
Peyer's Patch
S.T-GFP

A

Suppression of lung metastases of D2F2 by oral DNA vaccine

Treatment groups	Metastasis Score
A. Empty vector	2 3 3 3 3 3 3 3
B. pUb	3 3 3 3 3 3 3 3
C. pUb-Fra-1	1 1 2 2 2 2 2 3
D. pIL-18	0 0 1 1 1 2 2 2
E. pUb-Fra-1/pIL-18	0 0 0 0 0 1 1 2



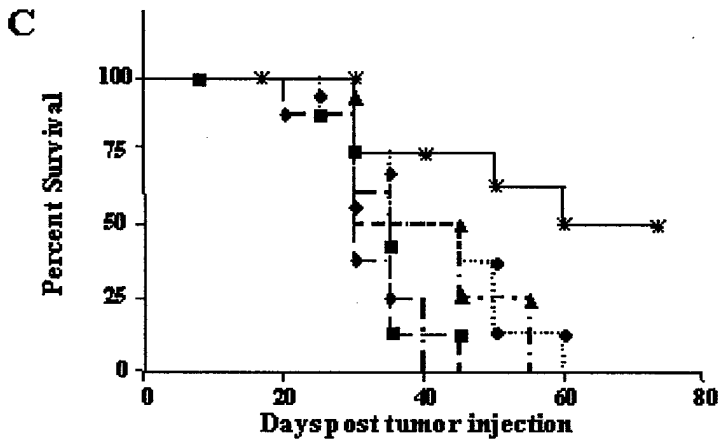
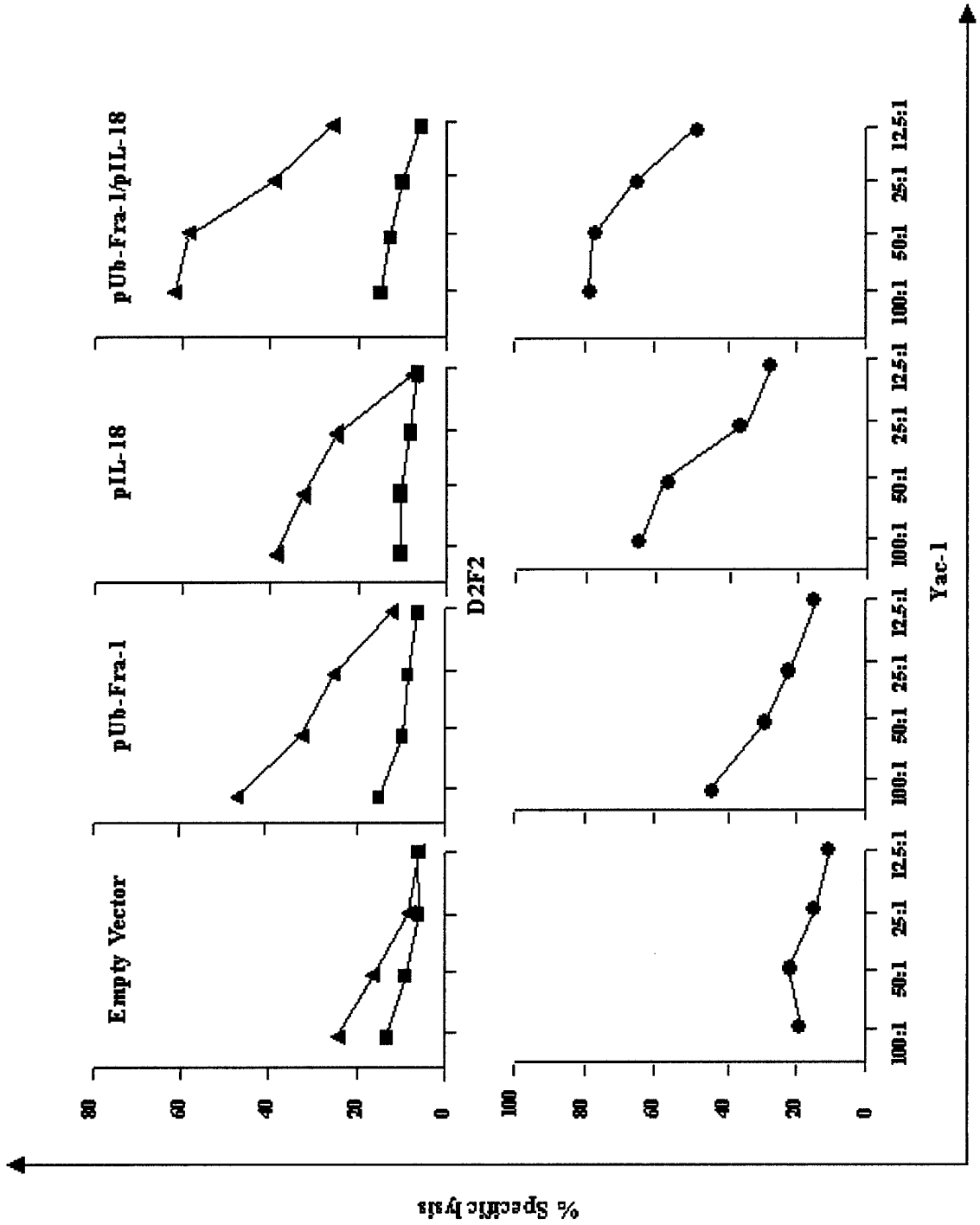
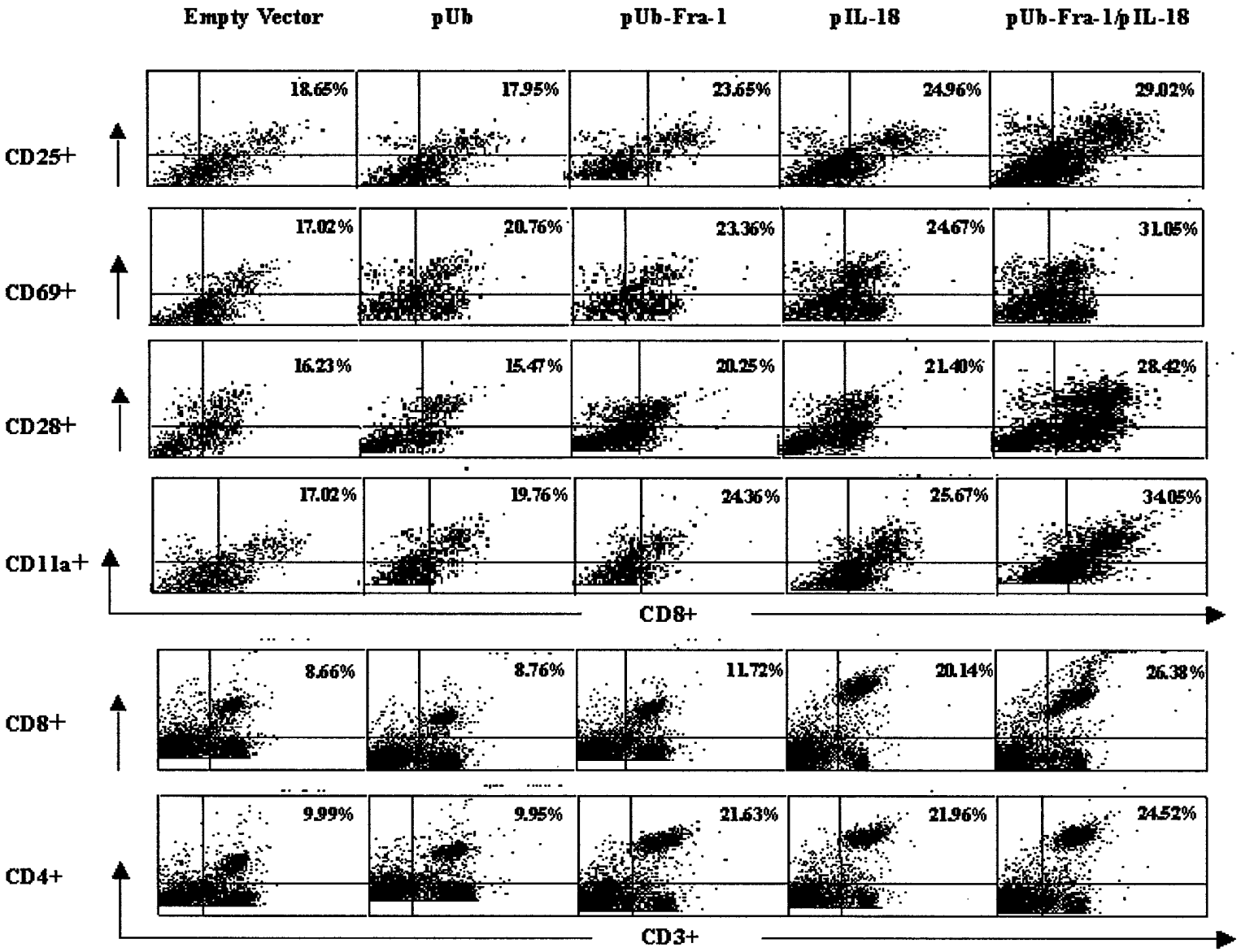


Figure 3


Empty Vector
pUb-Fra-1
pIL-18
pUb-Fra-1/pIL-18
D2F2
Yac-1
% Specific lysis
E:T Ratio
100:1
50:1
25:1
12.5:1


Empty Vector
pUb
pUb-Fra-1
pIL-18
pUb-Fra-1/pIL-18
CD25+
18.65%
17.95%
23.65%
24.96%
29.02%
CD69+
17.02%
20.76%
23.36%
24.67%
31.05%
CD28+
16.23%
15.47%
20.25%
21.40%
28.42%
CD11a+
17.02%
19.76%
24.36%
25.67%
34.05%
CD8+
CD8+
8.66%
8.76%
11.72%
20.14%
26.38%
CD4+
9.99%
9.95%
21.63%
21.96%
24.52%
CD3+

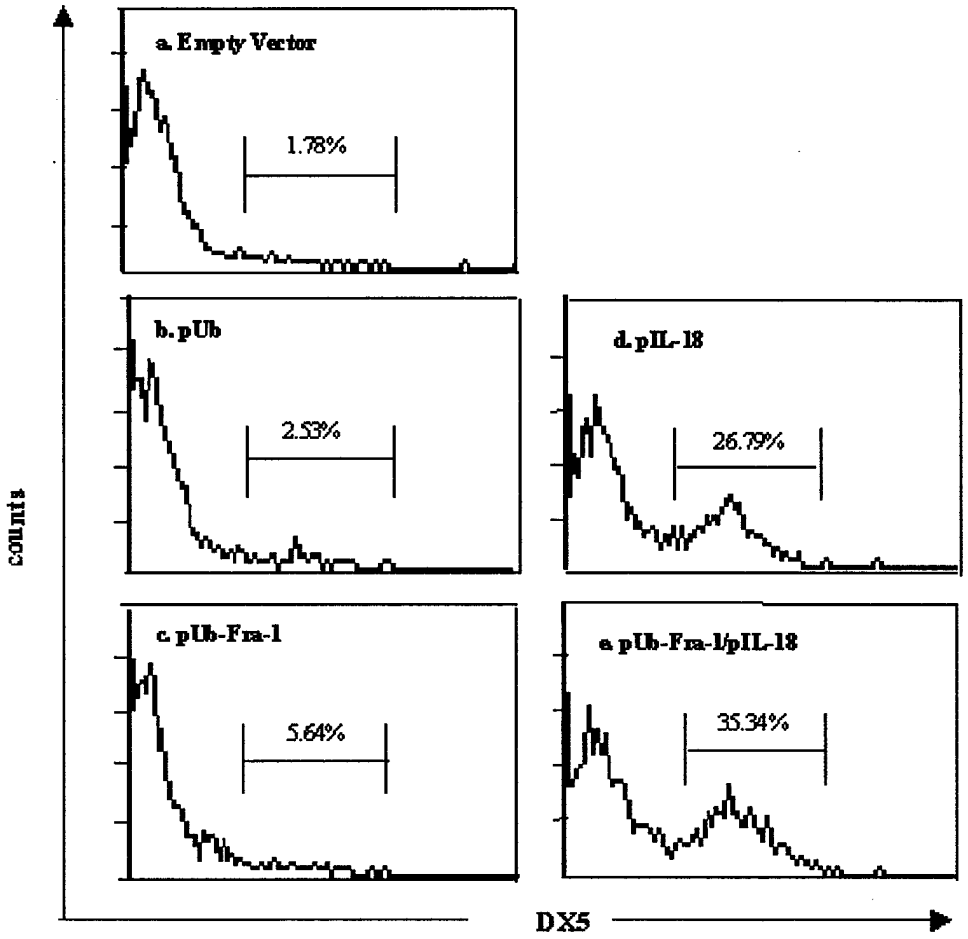
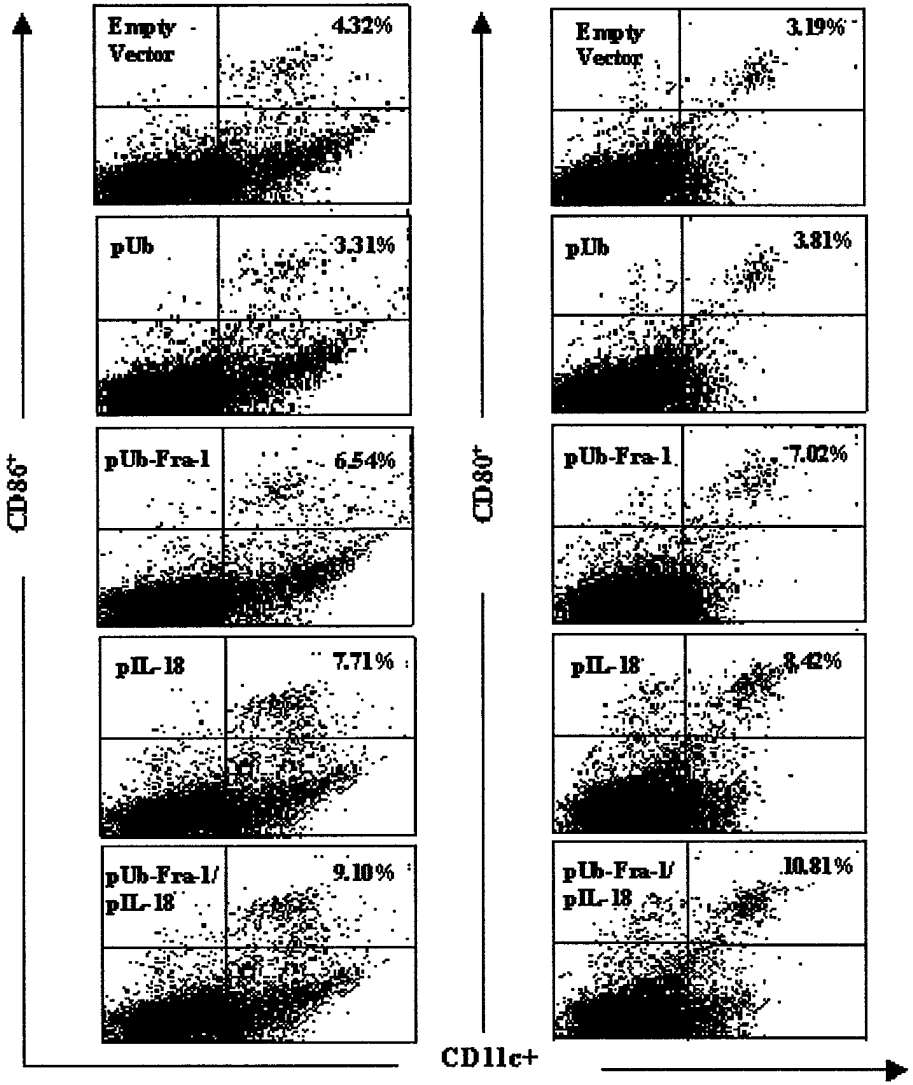

a. Empty Vector
1.78%
b. pUb
2.53%
d. pIL-18
26.79%
c. pUb-Fra-1
5.64%
e. pUb-Fra-1/pIL-18
35.34%
counts
DX5

Figure 6


Empty Vector
4.32%
pUb
3.31%
pUb-Fra-1
6.54%
pIL-18
7.71%
pUb-Fra-1/ pIL-18
9.10%
CD86+
Empty Vector
3.19%
pUb
3.81%
pUb-Fra-1
7.02%
pIL-18
8.42%
pUb-Fra-1/ pIL-18
10.81%
CD80+
CD11c+

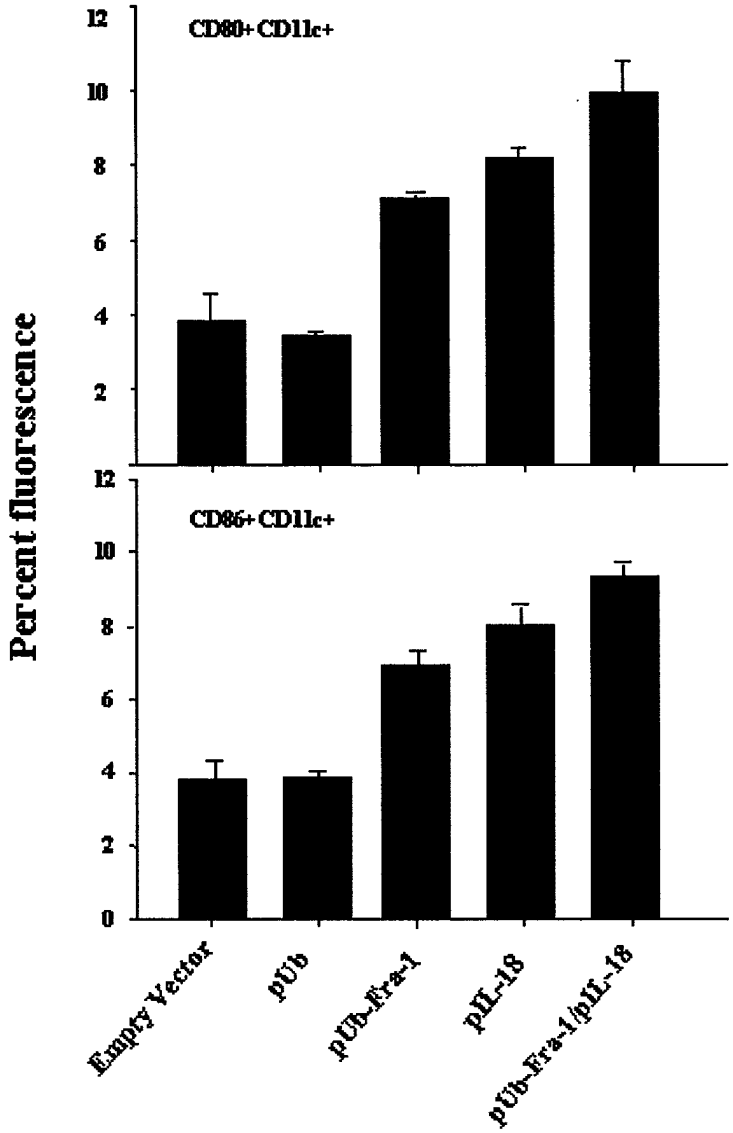
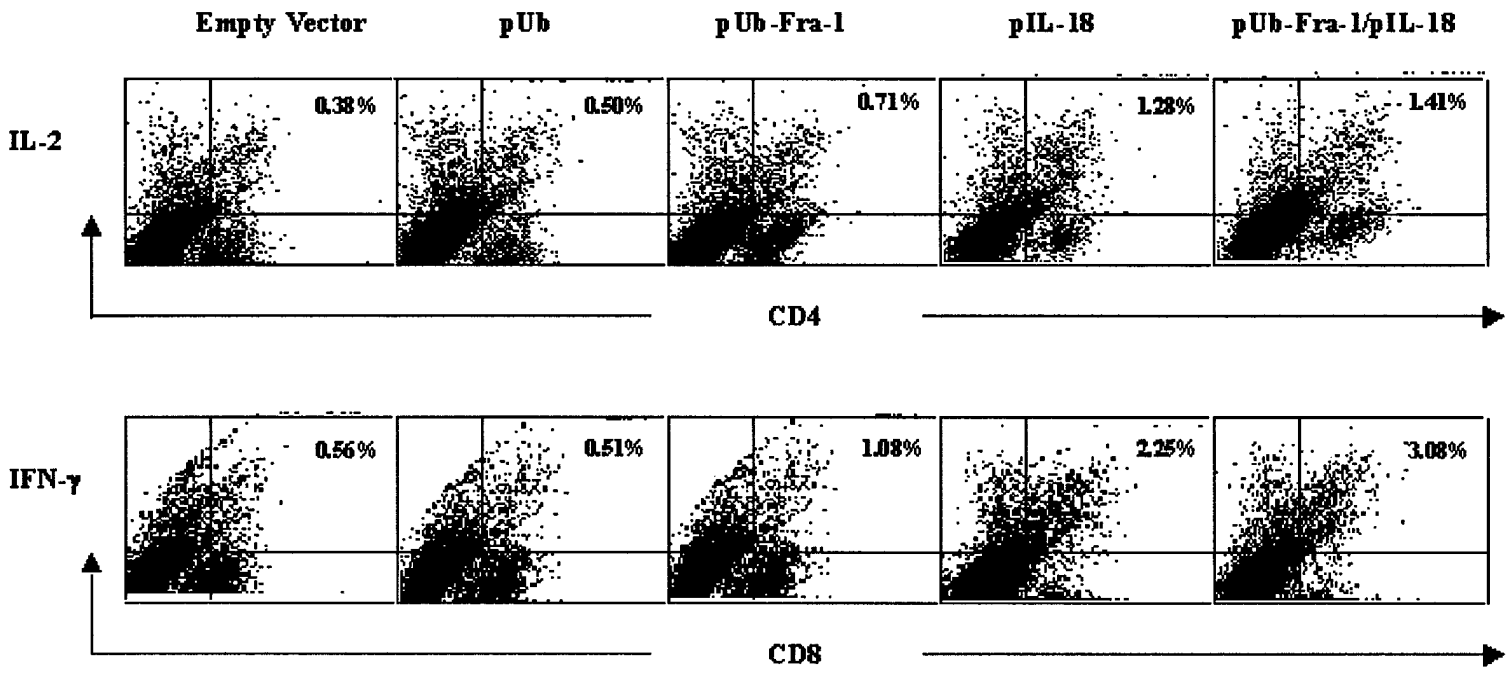

CD80+CD11c+
CD86+CD11c+
Percent fluorescence
0
2
4
6
8
10
12
Empty Vector
pUb
pUb-Fra-1
pIL-18
pUb-Fra-1/pIL-18

Figure 7


Empty Vector
pUb
pUb-Fra-1
pIL-18
pUb-Fra-1/pIL-18
IL-2
0.38%
0.50%
0.71%
1.28%
1.41%
CD4
IFN-γ
0.56%
0.51%
1.08%
2.25%
3.08%
CD8

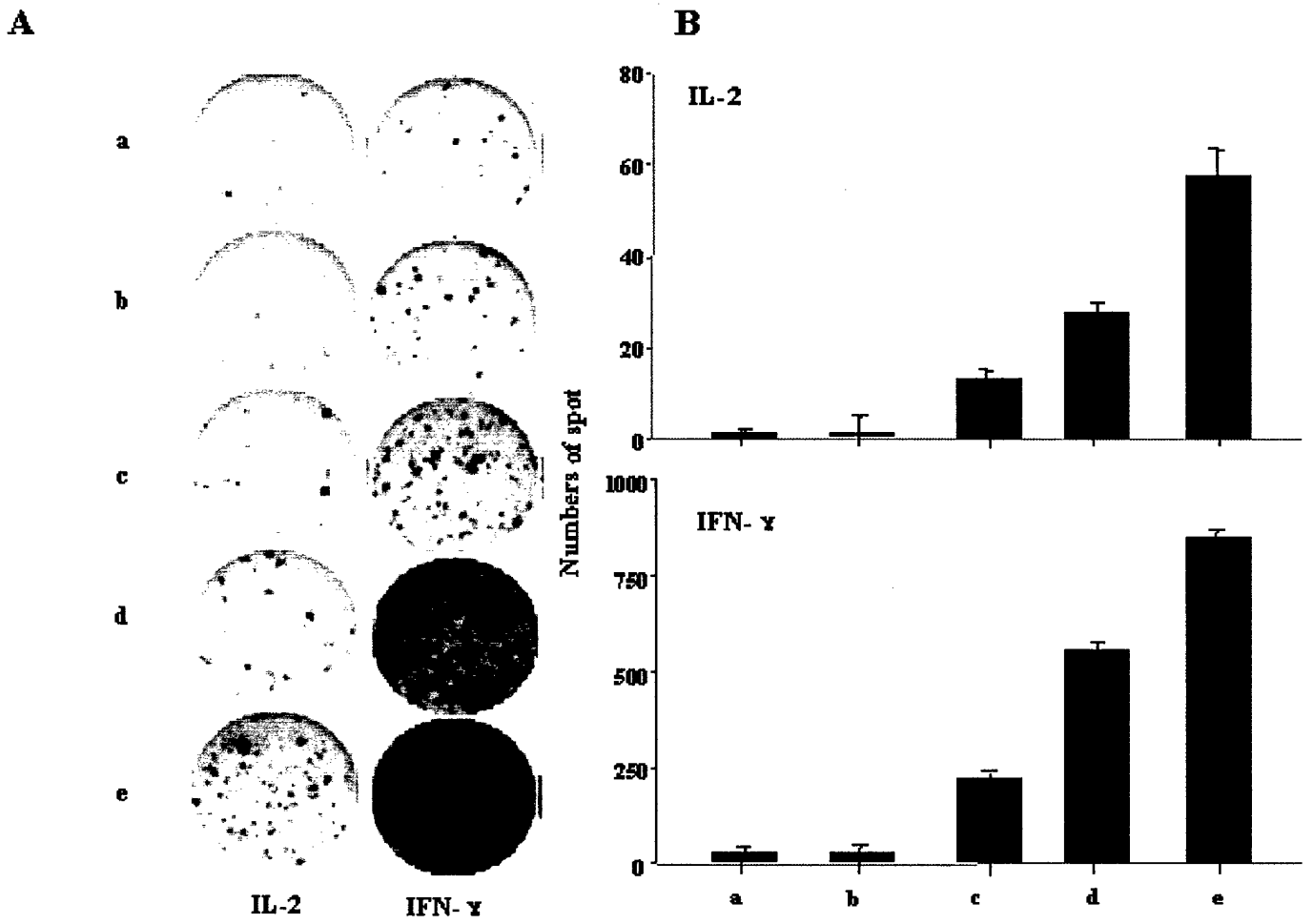

A
B
a
b
c
d
e
IL-2
IFN-γ
Numbers of spot
IL-2
IFN-γ
80
60
40
20
0
1000
750
500
250
0
a
b
c
d
e

Figure 9