A DNA Vaccine Targeting Fos-Related Antigen 1 Enhanced by IL-18 Induces Long-lived T-Cell Memory against Tumor Recurrence

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Abstract

A novel vaccination strategy induced specific CD8+ T cell–mediated immunity that eradicated spontaneous and experimental pulmonary cancer metastases in syngeneic mice and was also effective in a therapeutic setting of established breast cancer metastases. This was achieved by targeting transcription factor Fos-related antigen 1(Fra-1), overexpressed by many tumor cells, with an ubiquitinated DNA vaccine against Fra-1, coexpressing secretory IL-18. Insight into the immunologic mechanisms involved was provided by adoptive transfer of T lymphocytes from successfully immunized BALB/c mice to syngeneic severe combined immunodeficient (SCID) mice. Specifically, long-lived T memory cells were maintained dormant in nonlymphoid tissues by IL-18 in the absence of tumor antigen. Importantly, a second tumor cell challenge of these SCID mice restored both, robust tumor-specific cytotoxicity and long-lived T-cell memory, capable of eradicating established pulmonary cancer metastases, suggesting that this vaccine could be effective against tumor recurrence. (Cancer Res 2005; 65(8): 3419-27)

Introduction

The development of long-lived $CD8^+$ T-cell memory is a major goal of vaccination against tumors because it can provide continuous protection against their further dissemination and recurrence. In fact, successful protection against tumors critically depends on both, an increased number of tumor antigen–specific $CD8^+$ T cells in an immune host and the distinct capability of $CD8^+$ T memory cells to proliferate, secrete inflammatory antitumor cytokines, and repeatedly kill recurring tumor cells more effectively than naive $CD8^+$ T cells. Consequently, several ongoing efforts focus on the development of DNA-based cancer vaccines capable of inducing long-lasting immunologic memory responses endowed with specificity and a high potential to repeatedly kill tumor cells. Indeed, the establishment and continued long-term maintenance of immunologic memory has been the very key to successful tumor protective vaccination strategies (1–3).

Transcription factor Fos-related antigen 1(Fra-1), which is overexpressed by many human and mouse epithelial carcinoma cells (4–7), is involved in progression of various breast cancer cell types (8, 9) and thus represents a relevant, potential target for a breast carcinoma vaccine. Indeed, we previously showed that an oral DNA vaccine encoding murine Fra-1, coexpressing secretory murine interleukin 18 (mIL-18), induced an effective cellular immune response capable of eradicating established D2F2 breast cancer metastases in syngeneic BALB/c mice (10). IL-18 is a well-known multifunctional cytokine that was coexpressed in our DNA vaccine to enhance tumor antigen presentation by dendritic cells and to maintain an antitumor immune response. IL-18 was originally believed to elicit cytokine production by T and/or natural killer (NK) cells and to induce their proliferation and cytolytic activity, similar to an IFN-γ-inducing factor (11). The antitumor activity of IL-18 is now considered to be primarily mediated by T and NK cell activation and by enhancing cellular immune mechanisms via up-regulation of MHC class I antigen expression, favoring the differentiation of $CD4^+$ helper T cells toward the T helper 1 (Th1) subtype. In turn, Th1 cells secrete proinflammatory cytokines 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 (11, 12). In addition, IL-18 is an important mediator of memory $CD8^+$ T-cell proliferation and activation via bystander activation. This process was extensively studied by Sprent et al. (13, 14), who showed that administration of innate immune activators induces proliferation of memory $CD8^+$ T cell through a mechanism involving type I IFN, IL-12, IL-15, and IL-18, respectively. Here, we extended our prior studies on a Fra-1 based DNA vaccine coexpressing IL-18 in two breast tumor models as well as a non–small cell lung carcinoma model by investigating potential working mechanisms of this vaccine, focusing particularly on the generation, function, and long-term survival of $CD8^+$ memory T cells in tumor models of syngeneic BALB/c and severe combined immunodeficient (SCID) mice after adoptive transfer of T cells from successfully vaccinated mice. We also focused on $CD8^+$ T cells that could remain dormant at high frequency in nonlymphoid tissue after successful vaccination, because their ultimate presence in the periphery is important for eliciting resistance against secondary tumor cell challenges. These questions were addressed with a polyubiquitinated DNA vaccine encoding Fra-1, cotransformed with secretory murine IL-18, and carried by attenuated *Salmonella typhimurium*, which proved capable of inducing a long-lived $CD8^+$ T-cell response that eradicated recurring D2F2 breast cancer metastases in syngeneic BALB/c mice.

Materials and Methods

Animals, bacterial strains, and cell lines. Female BALB/c and C57BL/6 mice, 6 to 8 weeks of age, were purchased from the Scripps Research Institute Rodent Breeding Facility. Female SCID mice were obtained from the Jackson Laboratory (Bar Harbor, ME). These mice were maintained under specific pathogen-free conditions and used for experiments when 7 weeks old. All animal experiments were done according to the NIH Guides for the Care and Use of Laboratory Animals. The double-attenuated *S. typhimurium* strain RE88 (*aroA*$^-$; *dam*$^-$) was obtained from Remedyne Co. (Santa Barbara, CA). The murine D2F2 breast cancer cell line was kindly provided by Dr. Wei-Zen Wei (Karmanos Cancer Institute, Detroit, MI). The murine D121 non–small cell lung carcinoma cells were a gift from Dr. L.

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Eisenbach (Weizmann Institute of Science, Rehovot, Israel) and the murine 4T1 breast carcinoma was kindly provided by Dr. Suzanne Ostrand-Rosenberg (University of Maryland, Baltimore, MD).

Reverse transcription-PCR, Western blotting, and immunohistochemistry. *Reverse transcription-PCR (RT-PCR):* Total RNA was extracted with the Rneasy mini Kit or Rneasy tissue Kit (Qiagen, Valencia, CA) from 3 $\times$ 10^6 tumor cells of various origin: breast carcinoma cells D2F2, 4T1, and 4T07; colon carcinoma cells CT26; prostate carcinoma cells RM2; non–small cell lung carcinoma cells D121; and normal tissues from mouse spleen, liver, lung, and bone marrow. Reverse transcription was done with 1 μg of total RNA followed by PCR with the appropriate oligonucleotides. The following primers were used: ATGTACCGAGACTACGGGGAA (forward) and TCACAAAGCCAGGAGTGTAGG (reverse). The PCR was cycled 30 times at 52°C annealing temperature and quantities of RNA and PCR products were monitored for glyceraldehyde-3-phosphate dehydrogenase resulting in an 821-bp fragment. *Western blots*: Fra-1 protein expression was established in the above mentioned array of tumor cell lines and murine tissues. Western blot analysis was done with the total protein from cell lysate homogenates, using a polyclonal primary rabbit anti-murine Fra-1 antibody and anti-murine β-actin antibody as a loading control (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Specific protein was detected with a goat anti-rabbit-horseradish peroxidase (HRP)–conjugated IgG antibody (Bio-Rad, Richmond, CA). *Immunohistochemistry:* D2F2 tumor tissues were cut into sections and exposed to air until completely dry, fixed in cold acetone at −20°C for 10 minutes, and stained for Fra-1 with the DAKO immunostaining system (DAKO, Carpinteria, CA), using rabbit anti-murine Fra-1 antibody (Santa Cruz Biotechnology), diluted 1:1,000 or negative control reagents. This was followed by incubation at 4°C overnight. After three washes with PBS, a HRP-conjugated goat anti-mouse secondary antibody (DAKO) was used, and slides mounted with DAKO Faramount (DAKO). Cells were visualized microscopically, and images captured with a Nikon digital camera (Tokyo, Japan) linked to a workstation with Adobe Photoshop software (Adobe System, Inc., San Jose, CA).

Vector construction and protein expression. Two constructs were made based on the vectors pcDNA3.1/Zeo and pSecTag2/Hygro (Invitrogen, San Diego, CA), respectively. The first construct, pUb-Fra-1, was comprised of polyubiquitinated, full-length murine Fra-1. The second construct, pIL-18, contained mIL-18 with an Igκ leader sequence for secretion purposes. The empty vector served as a control. Protein expression of Fra-1 and IL-18 were shown by Western blotting with a polyclonal rabbit anti-murine Fra-1 antibody (Santa Cruz Biotechnology) and a monoclonal anti-mouse IL-18 antibody (R&D Systems, Minneapolis, MN), respectively. IL-18 protein was detected in both cell lysates and culture supernatants.

Transformation and expression of *Salmonella typhimurium* with DNA vaccine plasmids. Attenuated *S. typhimurium* (dam^-; $aroA^-$) were transduced with DNA vaccine plasmids by electroporation. Briefly, a single colony of bacteria was inoculated into 3 mL of Luria-Bertani medium, harvested during mid-log growth phase, and washed twice with ice-cold water. Freshly prepared bacteria (1×10^8) were 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-Fra-1, pIL-18, or both pUb-Fra-1 and pIL-18 combined, indicated as pUb-Fra-1/pIL-18. After electroporation, resistant colonies harboring the DNA vaccine gene(s) were cultured and stored at −70°C after confirmation of their coding sequence.

Immunization and tumor cell challenge. *Prophylactic model*: BALB/c mice or C57BL/6 mice were divided into four experimental groups ($n = 8$) and immunized thrice at 2-week intervals by gavage with 100 μL 4.5% sodium bicarbonate containing 1×10^8 doubly mutated *S. typhimurium* harboring either empty vector, pUb-Fra-1, pIL-18, or pUb-Fra-1/pIL-18. All mice were challenged by i.v. injection with 5×10^5 D2F2 cells (BALB/c) or 2×10^5 D121 cells (C57BL/6) or fat pad injection with 7×10^3 4T1 cells (BALB/c), 1 week after the last immunization, to induce either experimental or spontaneous pulmonary metastases. The survival rate of mice, lung weight, and metastatic scores in experimental or control groups were observed. Two months after the first tumor cell challenge, the surviving mice from the treatment groups in only the D2F2 model were divided into two groups; one was reimmunized with pUb-Fra-1/pIL-18 and the other group received no reimmunization. All mice were rechallenged with 0.5×10^6 D2F2 cells 1 week after reimmunization and sacrificed at either 2 or 8 weeks after the first challenge or 1 week after rechallenge with tumor cells to determine the proliferation of $CD8^+$ T cells and IFN-γ release. *Therapeutic model:* BALB/c mice were divided into four experimental groups ($n = 8$) and injected i.v. with 0.5×10^6 D2F2 cells on day 0 and immunized with DNA vaccine thrice as described above. The experiment was terminated on day 28 to observe mouse lung weights.

Adoptive transfer of lymphocytes. BALB/c mice that served as donors of tumor-specific $CD8^+$ T cells for adoptive transfer experiments were those animals who were previously successfully immunized thrice at 2-week intervals with the pUb-Fra-1/pIL-18 vaccine. These animals were then challenged with 0.5×10^6 D2F2 tumor cells i.v. 1 week later and remained tumor free 2 months thereafter. Mice comprising the control group were immunized only with empty vector. The animals in all treatment groups were sacrificed 2 days after being reimmunized with the pUb-Fra-1/pIL-18 vaccine or after receiving no reimmunization. Lymphocytes were harvested via cannulation of the spleen and separated by Ficoll/hypaque gradient centrifugation ($600 \times g$, 20 minutes). Naive syngeneic SCID mice were reconstituted with a total of 4.5×10^7 lymphocytes by i.v. injection of 1.5×10^7 lymphocytes each, on days 0, 2, and 4. Their fate was then followed by flow cytometric analyses on days 7, 14, and 30, with anti-CD8 and anti-CD3 antibodies, respectively. After 2 days, individual groups of mice were challenged by i.v. injection of 0.5×10^6 D2F2 cells to initiate experimental pulmonary metastases. Tumor specific cytotoxicity and IFN-γ release, were determined 3, 7, and 30 days after tumor cell challenge and the survival rate of these SCID mice was observed.

Preparation of lymphocytes from nonlymphoid tissue. The lung vascular bed was flushed with 10 mL chilled HBSS (Life Technologies, Gaithersburg, MD) introduced via cannulation of the right ventricle. Lymphocytes were incubated for 60 minutes at 37°C in a solution of enzymes (i.e., 125 units/mL collagenase, Life Technologies; 60 units/mL each of Dnase I; and 60 units/mL of hyaluronidase, Sigma, St Louis MO). The cell suspension was layered over a lymphocyte-M (Cedarlane Laboratories, Hornby, Canada) density gradient (15), centrifuged at $600 \times g$ for 20 minutes at 25°C, and lymphocytes washed two to three times before further processing. Blood was flushed from livers by injecting 5 mL of RPMI 1640 through the portal vein. Leukocytes from the liver were then isolated by crushing this organ in a tissue grinder followed by incubation with the above enzyme solution and collection of the leukocyte layer from a Metrizamide (Sigma-Aldrich, St. Louis, MO) density gradient. Contaminating erythrocytes were removed from the leukocyte preparations by treatment with ACK lysis buffer (Cambrex Bio Science Walkersville, Inc., Walkersville, MD).

***In vitro* depletion of $CD4^+$ or $CD8^+$ T cells.** The depletion of $CD4^+$ or $CD8^+$ T cell *in vitro* was done as previously described (16). Briefly, splenocytes were isolated from C57BL/6 mice after vaccinations with experimental or control DNA vaccines, 2 weeks after challenge with D121 tumor cells. $CD4^+$ T-cell depletion was accomplished with 10 μg/10^7 splenocytes of anti-CD4 (derived from hybridoma GK1.5), and $CD8^+$ T cells were depleted with anti-CD8 antibody (derived from hybridoma 2.43) for 30 minutes at 37°C. Rabbit serum complement (1:6) was added with 1 mL diluted complement to 10^7 cells/mL. Cells were incubated for 30 minutes at 37°C, washed, and resuspended for the CTL assay. All antibodies were purchased from National Cell Culture Center (Minneapolis, MN). Rabbit serum complement was obtained from Serotec, Inc. (Raleigh, NC).

Cytotoxicity assay. Cytotoxicity was measured and calculated by a standard ^{51}Cr-release assay. Briefly, in the D2F2 tumor model splenocytes were harvested from BALB/c SCID mice at 3, 7, and 30 days after challenge with 5×10^5 D2F2 breast carcinoma cells after passive transfer of lymphocytes. In the D121 lung tumor model, splenocytes were obtained from C57BL/6 mice 2 weeks after challenge with 2×10^5 D121 tumor cells following either $CD4^+$ or $CD8^+$ T-cell depletion *in vitro*. These cells were then stimulated *in vitro* by irradiated (1,000 Gy) D2F2 cells or D121 cells for 4 days at 37°C in complete T-STIM culture medium (Becton Dickinson, Bedford, MA) containing 10% fetal bovine serum and recombinant IL-2 at 20 units/mL (PeproTech, Rocky Hill, NJ). These D2F2 or D121 target cells were then labeled with ^{51}Cr for 2 hours at 37°C and incubated with effector cells at

various effector-to-target cell ratios at 37°C for 4 hours. The percentage of specific target cell lysis was calculated by the formula $[(E - S) / (T - S)] \times 100$, where E is the average experimental release, S the average spontaneous release, and T the average total release.

Flow cytometry. Activation markers of T cells were measured by two-color flow cytometric analysis with a BD Biosciences FACScalibur. T-cell markers were determined by staining freshly isolated lymphocytes from successfully vaccinated mice or from passively transferred SCID mice with anti-CD8 antibodies in combination with FITC-conjugated anti-CD3 antibody. Memory $CD8^+$ T cells bearing high levels of $CD44^{high}$ and $CD122^+$ (IL-2Rβ) were quantified by three-color flow analysis. Splenocytes were isolated from successfully vaccinated BALB/c mice or from passively transferred SCID mice and then stained with anti-CD8-Cy-Chrome, anti-CD122-PE and anti-CD44-FITS antibodies, followed by fluorescence-activated cell sorting (FACS) analyses. All antibodies were purchased from PharMingen (San Diego, CA). IL-2 release at the intracellular level was determined in lymphocytes of Peyer's Patches obtained 3 days after one time immunization and stained with APC-anti-CD4 or CD8 and combined with FITC-anti-CD69. Cells were fixed, permeabilized, and subsequently stained with PE-labeled anti-IL-2 antibodies to detect the intracellular expression of IL-2.

ELISPOT assay. ELISPOT assays were done to measure single cell release of IFN-γ. Splenocytes were collected 2 weeks after D121 tumor cell challenge from all experimental groups of C57BL/6 mice or 2, 7, and 30 days after lymphocyte transfer to SCID mice (only in the D2F2 tumor model), and splenocytes from control mice immunized only with the empty vector. After lysis of RBC with ACK lysis buffer, splenocytes were resuspended at a final concentration of 1×10^7/mL (D121 tumor model) or 2×10^6/mL (D2F2 tumor model), and 100 μL of this suspension was cultured for 24 hours in complete T-cell medium with or without 100 μL irradiated (1,000 Gy) D121 cells (1×10^5/mL) or D2F2 cells (1×10^4/mL). The assay was done according to instructions provided by the manufacturer (BD Bioscience, San Jose, CA). Plates were read by immunospot ScAnalysis and digitalized images were analyzed for areas in which color density exceeded background by an amount based on a comparison with experimental wells.

Statistical analysis. The statistical significance of differential findings between experimental groups and controls was determined by Student's t test. Findings were regarded as significant if two-tailed Ps < 0.05.

Results

Differential expression of Fra-1 in tumor cell lines and normal mouse tissues. To study the distribution and expression of the Fra-1 antigen in mouse tumor models, we examined its differential expression in normal and mouse tumor tissues by analyzing expression of mRNA with RT-PCR in normal tissues of spleen, liver, lungs, and bone marrow and in breast tumor cell lines D2F2, 4T1, 4T07, prostate carcinoma RM2, non–small cell lung carcinoma D121, and CT26 colon carcinoma cells. Expression of mRNA levels of Fra-1 was markedly increased in all of these tumor cell lines but was detectable only at very low levels in all normal tissues (Fig. 1*A*). This differential expression of Fra-1 was confirmed at the protein level by Western blotting, revealing high expression in D2F2 cells and somewhat lower expressions in RM2 and CT-26 cells. In contrast, Fra-1 protein level was uniformly expressed at very much lower levels in all of the normal murine tissues examined (Fig. 1*B*). Furthermore, immunohistochemical analysis indicated strong Fra-1 expression in D2F2 breast cancer tissue when paraffin-embedded sections were stained with anti-Fra-1 antibody (Fig. 1*C*, *I* and *III*) when compared with negative control sections stained without the primary anti-Fra-1 antibody (Fig. 1*C*, *II* and *IV*).

Fra-1/IL-18-based DNA vaccine induces effective antitumor immunity. We proved our hypothesis that an orally given DNA vaccine encoding murine Ub-Fra-1 and secretory IL-18, carried by attenuated *S. typhimurium*, can induce an effective antitumor immune response. We found an increase in life span of BALB/c mice (n = 8) vaccinated as described above and challenged 2 weeks later by i.v. injection of a lethal dose (5×10^5/mL) of D2F2 breast carcinoma cells. The life span of 62% of successfully vaccinated BALB/c mice (5 of 8) tripled in the absence of any detectable tumor growth up to 98 days after tumor cell challenge (Fig. 2*A*, *a*).

Vaccination reduces growth of established metastases. Marked inhibition of growth of established metastases was observed in C57BL/6 mice challenged by i.v. injection of D121 non–small cell lung carcinoma cells 2 weeks after the third vaccination with the Fra-1/IL-18-based vaccine as described above. In contrast, animals vaccinated with only the empty vector carried by the attenuated bacteria, revealed uniformly rapid metastatic pulmonary tumor growth of D121 non–small cell lung carcinoma (Fig. 2*A*, *b*). Our vaccine was also effective in a therapeutic setting. This was shown by an initial i.v. injection of BALB/c mice (n = 8) with D2F2 breast carcinoma cells and vaccination of these mice 5 days thereafter with our Fra-1/IL-18 vaccine when these mice had established pulmonary metastases, and by collecting their lungs 28 days later. All such treated mice showed lower lung weights and markedly reduced tumor burden, whereas all control animals treated with the empty vector revealed much increased lung weights and tumor burden (Fig. 2*B*).

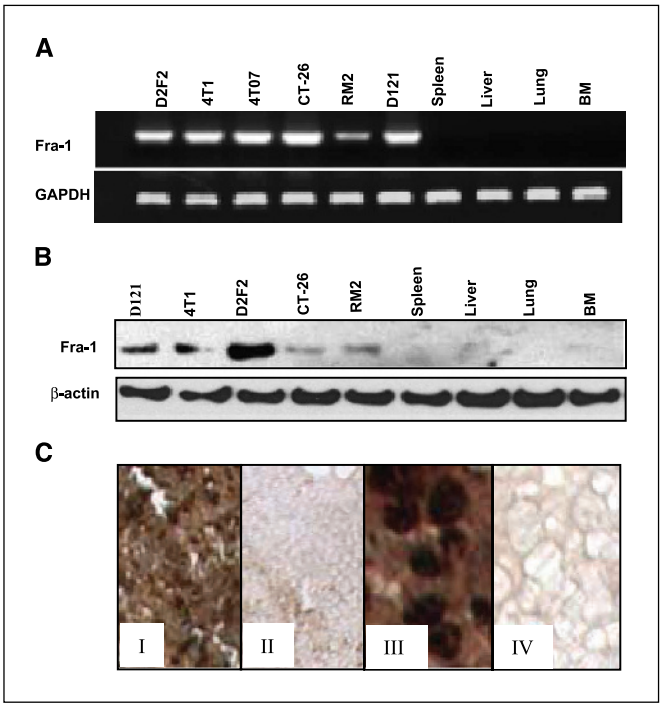

Figure 1. Expression of the murine Fra-1 molecule in normal mouse tissues and tumor cell lines. *A,* RT-PCR analysis of Fra-1 gene expression by carcinoma cell lines D2F2, 4T1, 4T07 (breast), CT-26 (colon), RM2 (prostate), and D121 (non–small cell lung) as well as normal murine tissues from spleen, liver, lungs, and bone marrow. Total RNA was extracted from cells growing at 70% confluence and from normal murine tissues. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a control for total RNA loading. *B,* Western blot analysis of Fra-1 protein expression in the above mentioned tumor cell lines and normal mouse tissues. Protein lysates were extracted from cells growing at 80% confluence. Homogenized normal tissues and β-actin were used as controls for protein loading. *C,* immunohistochemical analysis of Fra-1 in D2F2 breast cancer tissue. Paraffin-embedded sections from D2F2 breast cancer tissue samples were analyzed by immunohistochemistry using antibody against Fra-1 protein. Immunohistochemical staining of D2F2 breast cancer tissue with anti-Fra-1 antibody (*I*, ×10 magnification; *III*, ×40 magnification) and without using primary anti-Fra-1 antibody (*II*, ×10 magnification; *IV*, ×40 magnification).

Protection against spontaneous pulmonary metastases. We noted a marked reduction in dissemination of spontaneous pulmonary metastases of 4T1 breast carcinoma cells after three immunizations with the Fra-1/IL-18-based DNA vaccine. This became evident 28 days after surgical excision of fat pads bearing primary 4T1 breast carcinoma and as confirmed by visual examination of the lungs of these animals for metastases, which established their metastatic score (Fig. 2*A*, *c*).

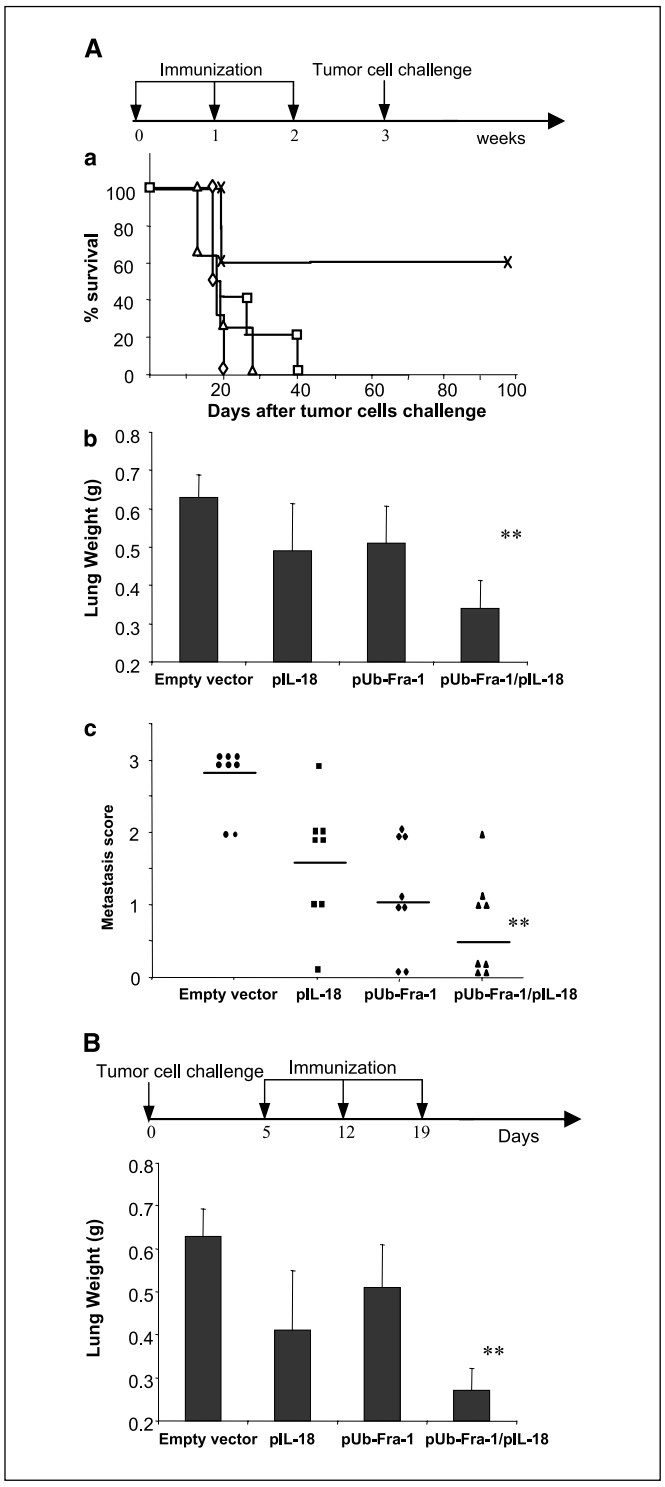

Figure 2. Effect of protective immunity induced by the pUb-Fra-1/pIL-18-based DNA vaccine on different tumor metastases models. *A,* prophylactic model. Vaccination schedule designed for three immunizations at 2-week intervals, followed by i.v. challenge with 0.5×10^6 D2F2 or 0.2×10^6 D121 tumor cells or fat pad injection with 0.7×10^4 4T1 tumor cells 1 week after the last immunization. Kaplan-Meyer plot (*a*) of the survival of eight mice in each of the treatment regimens (□) pUb-Fra-1, (△) pIL-18, (×) pUb-Fra-1/pIL-18, and (◇) control groups. Surviving mice were tumor free unless otherwise stated. Representative lung specimens of C57BL/6 mice ($n = 8$) were obtained 4 weeks after challenge with D121 non–small cell lung carcinoma cells (*b*). *Columns,* average lung weight (g) in each group. Normal lung weight is ~0.2g. **, $P < 0.001$, $P < 0.05$, $P < 0.05$ compared with empty vector, pIL-18, or pUb-Fra-1, respectively. Experiments were repeated three times with similar result. Representative lung specimens from BALB/c mice ($n = 8$) challenged with 4T1 breast carcinoma cells by fat pad injection 4 weeks after removal of primary tumor (*b*). Tumor metastasis scores on lungs were established by estimating the % surface area covered by metastases as follows: 0, no metastases; 1, <20%; 2, 20% to 50%; and 3, >50% represented by individual symbols for each treatment group. *Short lines,* average metastases score of each group. **, $P < 0.001$, $P < 0.05$, $P < 0.05$ compared with empty vector, pIL-18, or pUb-Fra-1, respectively. *B,* therapeutic model. Groups of BALB/c mice ($n = 8$) were initially injected i.v. with 0.2×10^6 D2F2 cells on day 0 and vaccinated with the DNA vaccine on days 5, 12, and 19, respectively. The experiment was terminated on day 28. *Columns,* average lung weight (g) in each group. **, $P < 0.001$, $P < 0.05$, and $P < 0.05$ compared with empty vector, pIL-18, or pUb-Fra-1, respectively.

CD8$^+$ T cells are responsible for the antitumor response. Evidence for an activated T-cell immune response was indicated by three lines of evidence. First, we found that only the vaccine encoding pUb-Fra-1/pIL-18 proved highly effective in markedly up-regulating IL-2 expression on CD69$^+$, CD4$^+$, or CD8$^+$ activated T cells during T-cell priming (Fig. 3*A*). Second, only lymphocytes isolated from mice immunized with this vaccine were effective in specifically killing D121 non-small cell lung cancer cells *in vitro* at different effector-to-target cell ratios. In contrast, lymphocytes isolated from vaccinated mice that were thereafter depleted of CD8$^+$ T cells *in vitro* failed to induce cytotoxic killing of D121 tumor target cells. However, *in vitro* depletion of CD4$^+$ T cells did not abrogate cytotoxic killing of these same tumor target cells (Fig. 3*B*). The same results were obtained in the D2F2 breast carcinoma model in BALB/c mice (data not shown). Third, release of IFN-γ from T cells, a well-known indication of T-cell activation in secondary lymphoid tissues, was found at the single cell level by ELISPOT (Fig. 3*C*) only after vaccination with the pUb-Fra-1/pIL-18 plasmid. In fact, subsequent challenge with tumor cells induced a dramatic increase in IFN-γ release when compared with that of splenocytes from control mice. Taken together, these data suggest that the activation of T cells was specific for Fra-1.

Activation of specific T cells in lymphoid tissue is followed by migration to nonlymphoid tissue. Interactions between IL-18 and active Th1 cells are believed to be critical for achieving both, optimal antigen-specific T-cell responses in lymphoid tissues and activated T-cell migration to nonlymphoid tissues in the local tumor microenvironment. To prove this contention in our models, we analyzed CD8$^+$ T cells in both lymphoid and nonlymphoid tissues. The vaccine encoding pUb-Fra-1/pIL-18 substantially up-regulated the CD8$^+$ T-cell populations 2 weeks after challenge with D2F2 tumor cells in both lymphoid tissues (spleen) as well as in blood and nonlymphoid tissues, especially in local lung tumor tissues (Fig. 4*A*, *I*). Eight weeks after tumor-cell challenge, the level of CD8$^+$ T cells in all these tissues declined and, in fact, this decline was more rapid in lymphoid tissues (spleen) than in nonlymphoid tissues (lungs; Fig. 4*A*, *II*). However, when 8 weeks after the initial tumor cell challenge, all surviving mice were rechallenged with D2F2 cells, CD8$^+$ T-cell populations were again dramatically up-regulated and these same

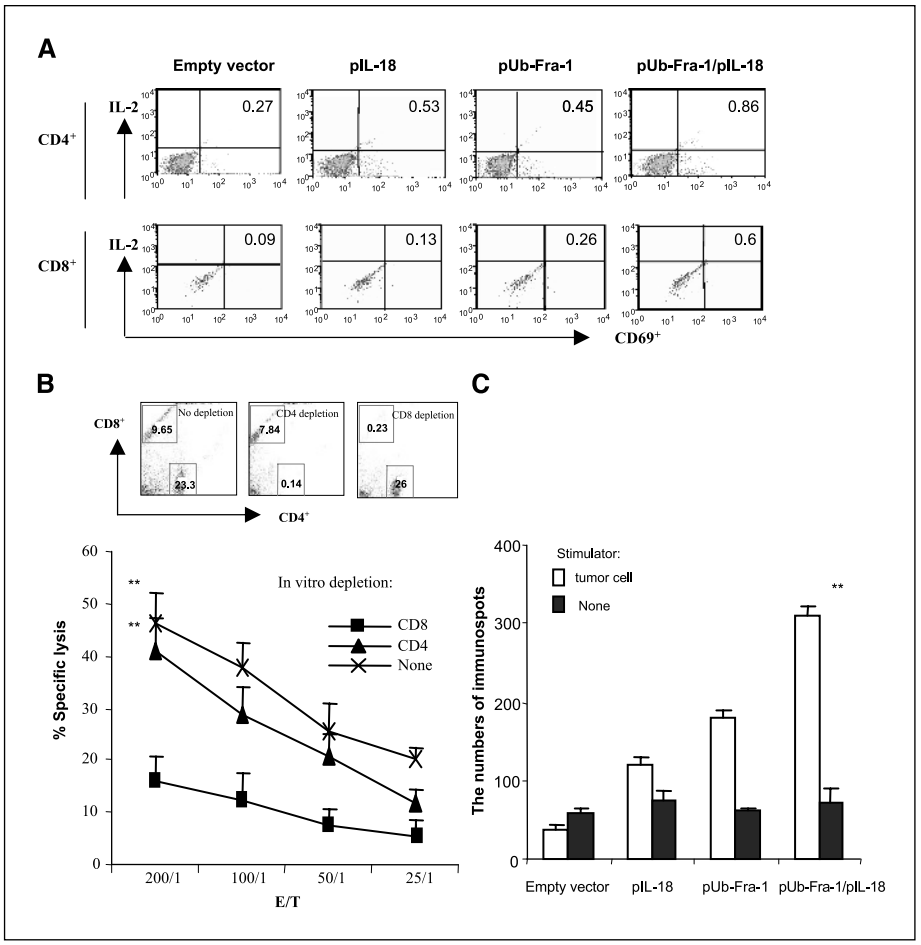

Figure 3. T-cell activation by the pUb-Fra-1/pIL-18-based vaccine in the non–small cell lung carcinoma model. *A,* up-regulated IL-2 expression of primed activated T cells. Three-color flow cytometric intracellular staining analyses were performed with single-cell suspensions of lymphocytes of Peyer's Patches obtained from immunized mice 3 days after one immunization. Cells were stained with APC-labeled anti-$CD8^+$ or anti-$CD4^+$ antibodies, FITC-labeled anti-$CD69^+$ antibodies, and PE-labeled anti-IL-2 antibodies and analyzed and gated on live $CD8^+$ or $CD4^+$ T cells. *B,* induction of $CD8^+$ T cell–specific cytotoxic activity. Splenocytes were isolated from C57BL/6 mice after vaccination with either experimental or control DNA vaccines 2 weeks after challenge with D121 tumor cells and analyzed for their cytotoxic activity in a ^{51}Cr-release assay at different effector-to target cell ratios. Specific lysis is shown mediated by $CD8^+$ T cell against D121 tumor target cells Depicted are cytotoxicity without depletion (×), specific lysis following depletion of $CD8^+$ T cell (■) or of $CD4^+$ T cells (▲). *Point,* mean of eight animals. **, $P < 0.001$ compared with no T-cell depletion. *C,* production of IFN-γ. This is indicated at the single T cell level either without (□) or with stimulation (■) as determined by the ELISPOT assay and depicted by the number of immunospots formed per well. Mean spot distribution of each well in each experimental and control group ($n = 4$, mean ± SD). **, $P < 0.001$, $P < 0.01$, and $P < 0.01$ compared with empty vector, pIL-18, or pUb-Fra-1, respectively.

cells increased even more rapidly in lung tissue than in lymphoid tissues (Fig. 4*A*, *III*). Furthermore, ELISPOT assay indicated IFN-γ release from these same lymphocytes and T-cell activation was confirmed by release of proinflammatory cytokine IFN-γ which increased in both lymphoid and nonlymphoid tissues 2 weeks after the first tumor cell challenge (Fig. 4*B*, *I*). However, 8 weeks after the initial tumor cell challenge, IFN-γ release decreased (Fig. 4*A*, *II*) but then increased again dramatically after rechallenge with D2F2 tumor cells, especially in lung lymphocytes (Fig. 4*A*, *III*).

A specific memory T-cell response is induced and maintained in the absence of tumor antigen. We tested the hypothesis that $CD8^+$ T cells, adoptively transferred from successfully immunized mice to syngeneic SCID mice, and parked there for 7 or 30 days, could maintain effective and long-lived memory in the absence of both tumor antigen and naive T cells. To this end, SCID mice were adoptively transferred with lymphocytes that were harvested from successfully immunized mice that had remained tumor free for at least 98 days after the initial tumor cell challenge and were then subjected to either reimmunization with the same DNA vaccine or to no reimmunization. The data depicted in Fig. 5*A* indicate that the life span of 75% (6 of 8) in the reimmunized group of mice and in 62% (5 of 8) in the nonreimmunized group of SCID mice was tripled in the absence of any detectable tumor growth up to 56 days after tumor cell challenge. Importantly, the continuous presence of tumor antigen was not required to maintain long-lived $CD8^+$ T-cell memory among $CD8^+$ T cells that were adoptively transferred into syngeneic SCID mice (Fig. 5*A*). Furthermore, we determined the fate of $CD8^+$ T effector cells in the absence of tumor antigen by adoptive transfer of lymphocytes from immunized BALB/c mice into SCID mice. Thus, when these animals' splenocytes were subjected to FACS analysis to detect the presence of $CD8^+$ T cells, there was a continuous decrease in the number of these cells for 30 days suggesting that the majority of these T effector cells gradually apoptosed (Fig. 5*B*).

Rapid turnover of memory $CD8^+$ T cells after vaccination and repeated tumor cell challenges. We determined that vaccination with the pUb-Fra-1/pIL-18 construct followed 2 weeks thereafter by a D2F2 tumor cell challenge, leads to a rapid turnover of $CD8^+$ T memory cells. This was indicated by up-regulated expression of $CD8^+$, $CD44^{high}$, $CD122^+$ memory T-cell markers at different time points in both lymphoid and nonlymphoid tissues (Fig. 6*A*). Thus, turnover of these memory T cells occurred just 24 hours after tumor cell challenge and reached a peak at 72 hours (Fig. 6*B*). Importantly, we could also verify that these putative, specific $CD8^+$ memory T cells can also effectively recognize a second challenge of D2F2 tumor cells. If fact, we not only detected increased expression of $CD8^+$, $CD44^{high}$, $CD122^+$ memory T cells in both lymphoid and nonlymphoid tissues 56 days after the first tumor cell challenge in syngeneic BALB/c mice (Fig. 6*C*, *I*) but also found the same up-regulation of these $CD8^+$ memory T-cell markers when these very same lymphocytes were adoptively transferred to SCID mice that were subjected 1 week thereafter to a D2F2 tumor cell challenge (Fig. 6*C*, *II*). Moreover, the $CD8^+$, $CD44^{high}$, $CD122^+$ memory T-cell expression was more pronounced

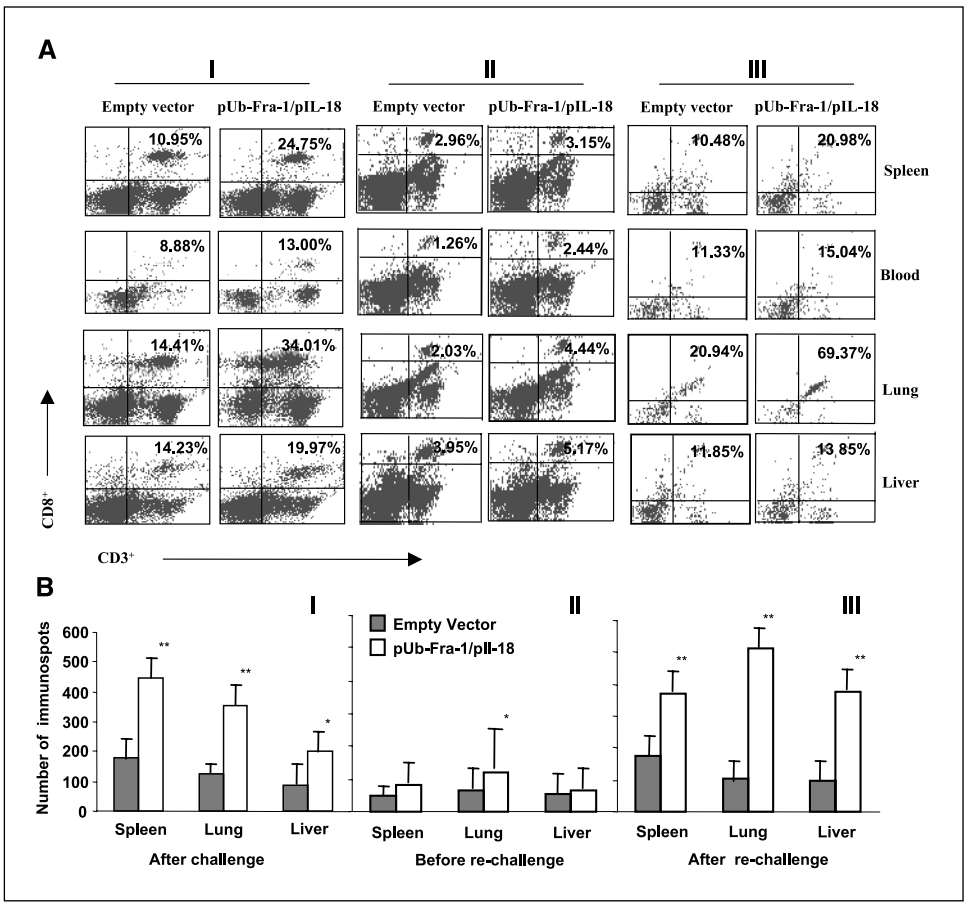

Figure 4. T cell activation in nonlymphoid tissue. *A,* up-regulated CTL markers. Lymphocytes were isolated from spleen, lungs, liver, and blood of immunized mice 2 weeks (*I*) or 8 weeks (*II*) after D2F2 (0.5×10^6) i.v. tumor cell challenge as well as 1 week after rechallenge (*III*) of mice surviving after 14 weeks. Mice treated only with empty vector served as controls. Two-color flow cytometric analyses were performed with single-cell suspensions of lymphocytes. PE-labeled anti-CD8 antibodies were used in combination with FITC-conjugated anti-mouse CD3 monoclonal antibody with each value representing the mean of four mice. Differences between the results obtained with the control group (empty vector) were statistically significant when compared to those of the treatment group (pUb-Fra-1/pIL-18). $P < 0.05$ and especially significant in the group of animals where lymphocytes were obtained from lungs ($P < 0.001$). *B,* IFN-γ release from lymphocytes in different tissues. Lymphocytes were isolated as described above. Production of IFN-γ was detected at the single T-cell level by the ELISPOT assay. *Column,* lymphocytes from four mice. Differences between the control group (empty vector) and the treatment group (pUb-Fra-1/pIL-18) were statistically significant. *, $P < 0.05$ and **, $P < 0.001$.

in local lung tumor tissues than in the spleen. Taken together, these data show that specific $CD8^+$ T memory cells rapidly turned over after a successful immunization with our vaccine and could again respond effectively to the next tumor cell challenge.

Cytotoxic T-cell response to a secondary D2F2 breast cancer cell challenge in severe combined immunodeficient mice. We could show that after vaccination with pUb-Fra-1/pIL-18 and subsequent tumor cell challenges, activated T cells can be successfully transferred adoptively to SCID mice where they also respond to a secondary challenge of D2F2 tumor cells. Specifically, SCID mice were challenged with D2F2 tumor cells after adoptive transfer of splenocytes from successfully immunized BALB/c mice. The data depicted in Fig. 7 indicate that putative $CD8^+$ T memory cells that had been parked for up to 30 days in SCID mice did effectively recognize a secondary challenge of D2F2 tumor cells. Furthermore, we observed that these activated $CD8^+$ T cells released increased amounts of IFN-γ (Fig. 7*A*) and were highly effective in cytotoxic killing of D2F2 breast cancer cells *in vitro* at different effector-to-target cell ratios (Fig. 7*B*).

Discussion

Fra-1, a transcription factor of the activator protein family, was shown previously to be involved in tumor cell progression and to be overexpressed in many human and murine tumor tissues (4–7). Here, we also provide evidence that Fra-1 is highly expressed in a variety of tumor cell lines at both the mRNA and protein levels. This finding suggests that Fra-1 could be linked to the malignancy of murine tumor cells and provides a potential target for immunotherapy of cancer, especially breast cancer cells. In fact, our previous work showed that a DNA vaccine targeting Fra-1 and coexpressing IL-18 could induce an effective cellular immune response, which led to the eradication of established D2F2 breast cancer metastases in syngeneic BALB/c mice (10). Here, we further extended our prior studies. Thus, we hypothesized that immunization with a DNA vaccine encoding murine Fra-1, fused to polyubiquitin and modified by cotransformation with a gene encoding secretory murine IL-18, will work effectively in two different breast carcinoma models as well as in a non–small cell lung carcinoma model and induce strong antitumor activity in syngeneic mice which can be maintained as a long-lived specific immune response against breast cancer cells. Meanwhile, IL-18 enhanced immune responses by activating T and NK cells while up-regulating MHC class I antigen expression and assisting the differentiation of $CD4^+$ T cells toward the Th1 subtype. Additionally, the effective generation and maturation of $CD8^+$ CTLs should result in an effective Th1 type immune response. Proof for this hypothesis was established by the induction of antitumor immune responses in three mouse tumor models, two of breast carcinoma (D2F2 and 4T1) and one non–small cell lung carcinoma (D121), both against primary tumors and their respective spontaneous and experimental pulmonary metastases. Our vaccine was also effective in a therapeutic setting of established pulmonary metastases. Three lines of evidence indicated that the effector cells responsible for this tumor protective immunity were primarily activated $CD8^+$ T cells. First, $CD8^+$ T cells isolated from splenocytes of specifically vaccinated mice specifically killed D2F2 and D121 target cells,

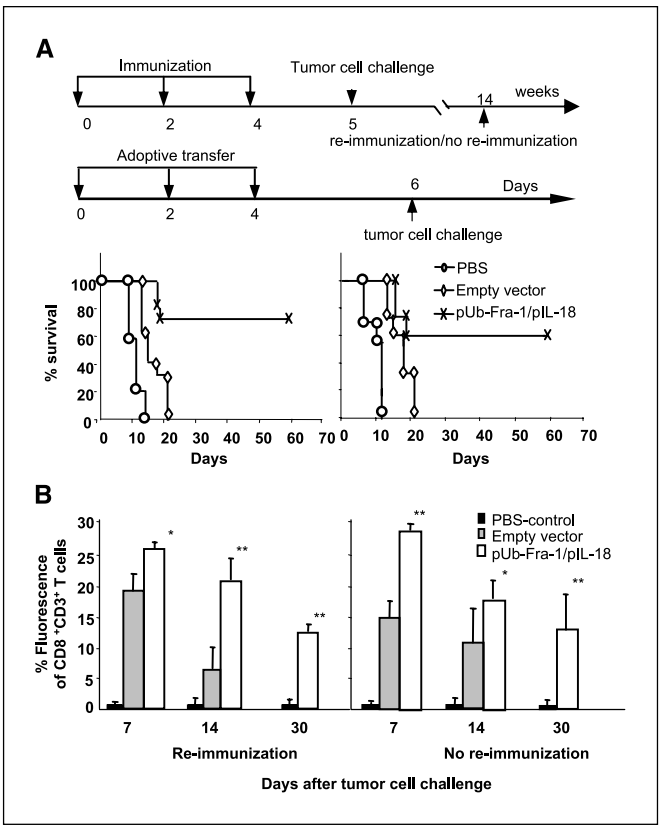

Figure 5. Maintenance of $CD8^+$ T memory cells in the absence of tumor antigen. *A,* vaccination schedule of donor mice was the same as that shown in Fig. 2 (i.e., three immunizations at 2-week intervals, followed by i.v. challenge with 0.5×10^6 D2F2 tumor cells 1 week after the last immunization). In the experimental group, 2 days after reimmunization at week 14 with pUb-Fra-1/pIL-18 or without reimmunization, lymphocytes were harvested from those mice that remained tumor free 2 months after the first tumor cell challenge. Lymphocytes (4×10^7/mouse) were adoptively transferred from BALB/c to SCID mice, and 2 days after transfer, these mice were challenged i.v. with 0.5×10^6 D2F2 tumor cells. Controls were mice adoptively transferred with lymphocytes harvested from control mice and immunized with only the empty vector or injected i.v. with PBS. Survival curves are representative of three separate experiments. *B,* fate of $CD8^+$ effector T cells after adoptive transfer into SCID mice was determined by two-color flow cytometric analysis of $CD8^+$ (PE labeled), $CD3^+$ (FITC labeled) splenic T cells, as well as by the effect of apoptosis on these cells after parking them in these mice 7 and 30 days, respectively. Differences between the two control groups (PBS and empty vector) and vaccine treatment groups were statistically significant. *, $P < 0.05$ and **, $P < 0.001$.

respectively in *in vitro* cytotoxicity assays. Second, the DNA vaccine did indeed activate $CD8^+$ T cells, because such cells isolated from splenocytes of successfully vaccinated mice, secreted the Th1 proinflammatory cytokine IFN-γ, and $CD8^+$ T-cell populations were markedly up-regulated. Third, $CD8^+$ T cells were activated both in lymphoid and nonlymphoid tissues, especially those located in tumor tissues of lung and liver. The mechanism of tumor protection is thought to depend on $CD4^+$ T cells and is potentially mediated by helper T cells, associated with effector functions and/or cytokine release which combine to break immunologic tolerance to tumor antigen. Our finding further supports this contention that both $CD4^+$ T cells and IL-2 release from these cells in the Peyer's Patches was markedly up regulated after one time vaccination.

The establishment and long-term maintenance of immunologic memory is a requirement for all protective vaccination strategies. Here, we showed that $CD8^+$ T cells isolated from splenocytes of successfully vaccinated BALB/c mice, when adoptively transferred to syngeneic SCID mice, maintained sufficient memory to markedly suppress dissemination and growth of a lethal challenge of D2F2 breast cancer cells. This finding was further supported by the markedly increased release of IFN-γ and $CD8^+$ T-cell cytotoxicity. Importantly, we found strong, local expression of $CD8^+$ T cells in tumor tissues, indicating that a $CD8^+$ T-cell response does not only occur in lymphoid tissues after successful vaccination, but that these T cells can also migrate to non-lymphoid tissues. This

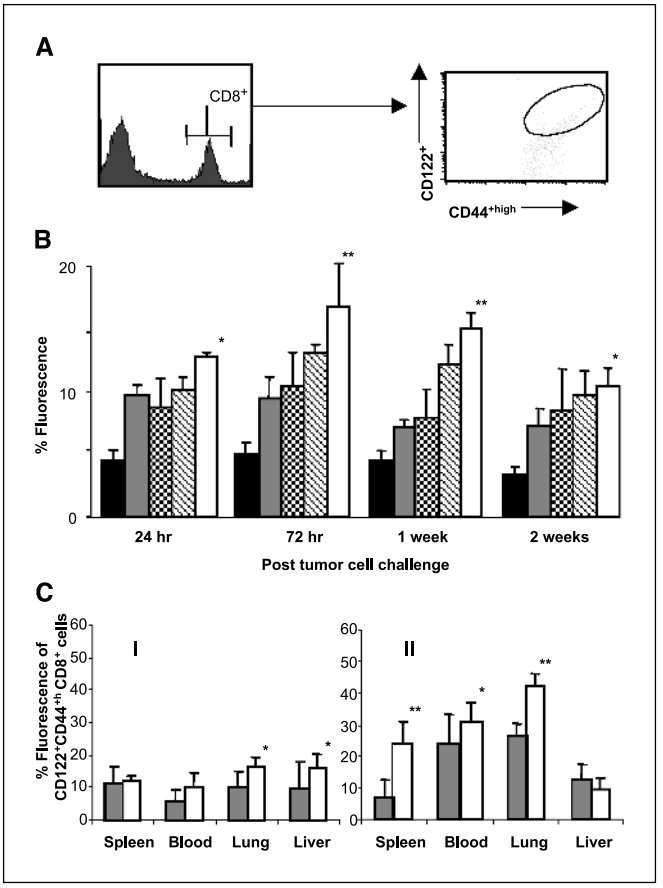

Figure 6. Turnover of memory $CD8^+$ T cells after vaccination with pUb-Fra-1/pIL-18. *A,* three-color flow cytometric analyses were performed of splenocytes obtained from immunized mice. Cells were stained with Cy-Chrome-labeled anti-$CD8^+$ antibody, PE-labeled anti-IL-2Rβ antibody, and FITC-labeled anti-$CD44^+$ antibody and analyzed and gated on live $CD8^+$ T cells. *B,* lymphocytes were isolated from groups of BALB/c mice treated with either pIL-18 (▨), pUb-Fra-1 (▩), and pUb-Fra-1/pIL-18 (□) or from groups of control mice treated only with PBS (■) or empty vector (▣) obtained at 24 and 72 hours, 1 and 2 weeks after tumor cell challenge and then analyzed by three-color flow cytometry, as described above. *Column,* mean for eight mice. Differences between the two control groups (PBS and empty vector) and the vaccine treatment groups were statistically significant *, $P < 0.05$ and **, $P < 0.001$. *C,* lymphocytes were isolated from spleen, lung, liver and blood of BALB/c mice of the treatment group pUb-Fra-1/IL-18 (□) 8 weeks after tumor cell challenge or from mice in the control group, that were only immunized with the (*I*) empty vector (■) or lymphocytes isolated from different tissues of SCID mice, adoptively transferred with either lymphocytes from mice immunized with pUb-Fra-1/pIL-18 (□) or with the empty vector (■) 1 week after tumor cell challenge (*II*). Three-color flow cytometric analyses were performed and cells stained with Cy-Chrome-labeled anti-$CD8^+$ antibody, PE-labeled anti-IL-2Rβ antibody, and FITC-labeled anti-CD44 antibody, gated on live $CD8^+$ T cells as described above. *Column,* mean for four mice. Differences between the control and treatment groups of mice were statistically significant when compared with lymphocytes from lungs, blood, and spleen of SCID mice after adoptive transfer following tumor cell challenge. This was also the case for lymphocytes from lungs and liver of syngeneic BALB/c mice obtained 8 weeks after tumor cell challenge. **, $P < 0.05$ and *, $P < 0.001$.

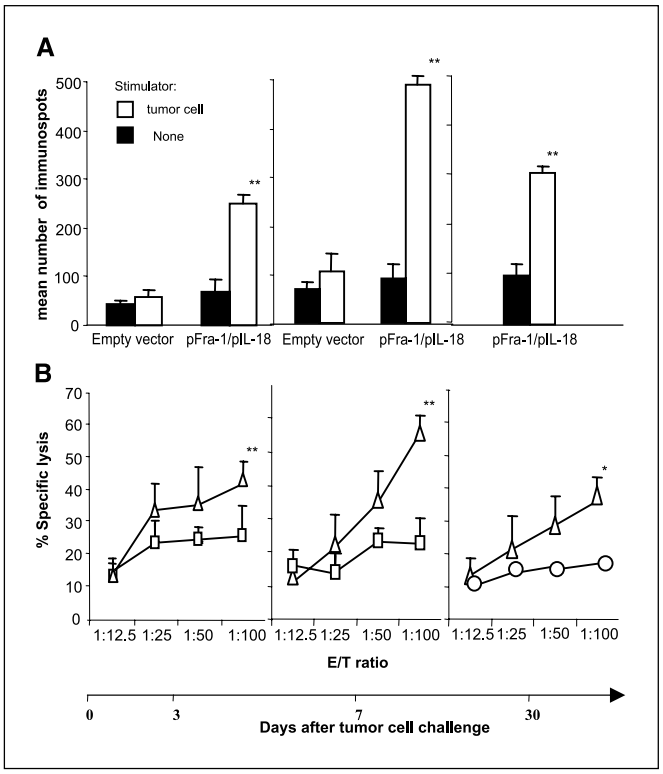

Figure 7. *A,* IFN-γ release from $CD8^+$ effector T cells, adoptively transferred to SCID mice as measured on days 2, 7, and 30 after i.v. challenge with D2F2 tumor cells. IFN-γ production of each experimental and control group of mice by ELISPOT assay is shown ($n = 8$, mean ± SD). Differences between the control group (empty vector) and the treatment group (pUb-Fra-1/pIL-18) are statistically significant **, $P < 0.001$. *B,* cytotoxicity induced by $CD8^+$ T cells, adoptively transferred to SCID mice. Splenocytes were isolated from mice, adoptively transferred with lymphocytes from successfully immunized BALB/c mice, and cytotoxity was measured on days 2, 7, and 30 after i.v. challenge with D2F2 tumor cells. Cytotoxicity was measured in a ^{51}Cr-release assay at different effector to target cell ratios. *Point,* mean of four mice. Differences between the two control groups of empty vector (□) and PBS (○) and the vaccine treatment group (△) were statistically significant *, $P < 0.05$ and **, $P < 0.001$.

occurs particularly in the tumor microenvironment where $CD8^+$ T cells react against antigen positive tumor cells. It is indeed relevant that some of these $CD8^+$ T cells were subsequently found as long-lived memory T cells that were ready to respond to the next stimulation upon re-encounter with the same tumor antigen. In this regard, it is well known that optimal T-cell activation results in clonal expansion, redistribution into nonlymphoid tissues and subsequent formation of memory (17, 18). It was also postulated that the immune response to foreign antigen is not necessarily limited to secondary lymphoid tissue. Importantly, nonlymphoid sites are essential for activated T-cell function and subsequent immunosurveillance. Most early studies of tumor antigen-specific T-cell responses were limited to analyses of lymph nodes, spleen, and blood. However, nonlymphoid tissues differ from organized secondary lymphoid organs in both, the quality and quantity of cytokines, lymphocytes, as well as immune accessory cells (19, 20). Importantly, we found that our DNA vaccine can induce protective antitumor immune responses, which not only occur in lymphoid tissues but also in nonlymphoid tissue near tumor sites and that, in addition, antigen-specific $CD8^+$ T cells can also migrate to nonlymphoid tissues and remain there for long periods of time as dormant memory cells. Strikingly, lymphocytes isolated from nonlymphoid tissues, such as lungs in our study, exhibited a greater release of IFN-γ and contained a higher percentage of $CD8^+$ CTLs as well as memory $CD8^+$ T cells than their splenic counterparts. These results point to the existence of a population of extralymphoid effector memory T cells poised for an immediate response to tumor-associated antigen. In fact, recent studies indicated that certain cytokines could induce bystander proliferation *in vivo* by T cells with a memory phenotype such as Type I IFN, IFN-γ, IL-15, IL-12, and IL-18. Moreover, it was found that injection of IL-18 stimulated a strong increase in the bromodeoxyuridine labeling of memory phenotype $CD8^+$ T cells *in vitro*. Furthermore, IFN-γ, which is inducible by IL-18, is also capable of promoting the turnover of memory phenotype $CD8^+$ T cells (21). Based on the finding that T-cell proliferation induced by IL-12 and IL-18 was dependent on IFN-γ (21), we examined the effect of IL-18 on T-cell turnover. Indeed, our results support the concept that coexpression of secretory IL-18 in our DNA vaccine induced the rapid turnover of $CD44^+$, $CD122^+$, $CD8^+$ T cells within 24 hours after immunization and that such $CD8^+$ T memory cells can be maintained in lymphoid tissues as well as locally in lung tumor tissues. Interestingly, $CD8^+$ T cells found in the lungs of our vaccinated mice were able to proliferate and acquire strong IFN-γ releasing capabilities after antigen exposure *in vitro*. Consequently, it is reasonable to conclude that persistently activated T cells and memory $CD8^+$ T cells in the lung can play a key role in the cellular immune response against tumor metastases.

The role of persisting antigen in T-cell memory cells and the requirement of such cells for chronic exposure to residual deposits of antigen for maintenance of $CD8^+$ T-cell memory have been the subject of much discussion and controversy (22–24). This is in contrast to $CD8^+$ effector T cells that absolutely do not require the presence of antigen. In fact, this is consistent with a decrease in adoptively transferred $CD8^+$ T cells observed in our studies in the absence of antigen (Fig. 5*A*). Indeed, a number of reports indicate that memory T cells survive poorly following adoptive transfer, unless accompanied by specific antigen (25–27). However, the contention that memory T cells require constant antigen stimulation has been challenged by reports demonstrating that $CD8^+$ memory cells can survive for prolonged periods of time after adoptive transfer in the absence of antigen (28–31). Our findings suggest that, at least for $CD8^+$ T cells, some memory T cells do not require continuous stimulation with antigen for survival.

In summary, we could show that an oral DNA vaccine, encoding Ub-Fra-1 and IL-18, carried by an attenuated strain of *S. typhimurium*, protected BALB/c mice against a lethal challenge of murine D2F2 and 4T1 breast cancer cells, and C57BL/6 mice against D121 lung carcinoma cell challenge. Moreover, this vaccine is capable of breaking T-cell tolerance to a self-antigen and generates a long-lived memory T-cell immune response against recurring breast cancer which could be maintained consistently in SCID mice in the absence of tumor antigen in both lymphoid and nonlymphoid organs.

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A DNA Vaccine Targeting Fos-Related Antigen 1 Enhanced by IL-18 Induces Long-lived T-Cell Memory against Tumor Recurrence

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