A DNA Vaccine Targeting Survivin Combines Apoptosis with Suppression of Angiogenesis in Lung Tumor Eradication

Rong Xiang,¹ Noriko Mizutani,¹ Yunping Luo,¹ Claudia Chiodoni,¹ He Zhou,¹ Masato Mizutani,¹ Yi Ba,¹ Juergen C. Becker,² and Ralph A. Reisfeld¹

¹Department of Immunology, The Scripps Research Institute, La Jolla, California and ²Universitaets Hautklinik, Wuerzburg, Germany

Abstract

A novel strategy achieved the eradication of lung tumor metastases by joint suppression of angiogenesis in the tumor neovasculature and induction of tumor cell apoptosis. This was accomplished by CTLs induced by a DNA vaccine encoding secretory chemokine CCL21 and the inhibitor of apoptosis protein survivin, overexpressed by both proliferating endothelial cells in the tumor vasculature and tumor cells. Oral delivery of this DNA vaccine by doubly attenuated Salmonella typhimurium (dam⁻ and AroA⁻) to such secondary lymphoid organs as Peyer's patches in the small intestine, elicited marked activation of antigen-presenting dendritic cells, and an effective CD8⁺ T cell immune response against the survivin self-antigen. This resulted in eradication or suppression of pulmonary metastases of non–small cell lung carcinoma in both prophylactic and therapeutic settings in C57BL/6J mice. Moreover, the suppression of angiogenesis induced by the vaccine did not impair wound healing or fertility of treated mice. It is anticipated that such novel DNA vaccines will aid in the rational design of future strategies for the prevention and treatment of cancer. (Cancer Res 2005; 65(2): 553-61)

Introduction

The effective suppression and treatment of metastatic non–small cell lung carcinoma remains a major challenge for oncology since clinical outcome is relatively poor. In fact, cancer immunotherapies designed for direct attacks on tumor cells face longstanding limitations, including poor immunogenicity of tumor self-antigens and the genetic instability of tumor cells which often combine to make tumor cells the most difficult and elusive targets for current immunotherapies (1–4). Recent efforts of Dario Altieri and other investigators provided an extensive amount of information on the structure and function of the inhibitor of apoptosis protein survivin, particularly its role in apoptosis and cell cycle regulation of cancer cells (14–23). In view of this large body of information, the small, 16.5-kDa inhibitor of apoptosis protein survivin is an almost ideal target for a DNA-based cancer vaccine (24). Thus, because survivin is overexpressed by essentially all solid tumors, it lends itself to broad therapeutic applications, especially because it is poorly, if at all expressed by normal adult cells and tissues. In fact, in gene-profiling studies, survivin was identified as the fourth “TRANSCRIPTOME” expressed in the most common human cancers but not in normal tissues (24). Survivin also was shown to be essential for cancer cell viability because it is one of the most important factors that regulate the balance between cell proliferation and programmed cell death during the cell cycle (15, 18, 24). In fact, survivin is highly regulated and optimally expressed in the G₂-M phase of the cell cycle, and its interaction with the mitotic spindle apparatus is essential for its antiapoptotic function (15, 17, 19). However, the complexity of the survivin pathway may extend beyond tumor cell populations, particularly because its increased expression by proliferating endothelial cells during the proliferative and remodeling phases of angiogenesis generates a cytoprotective mechanism for these cells (25, 26). Based on these facts, an attractive alternative to a direct attack solely on tumor cells will be to inhibit tumor growth and metastasis by simultaneously attacking both the tumor and its vasculature via an effective CTL response against survivin and thereby triggering tumor cell apoptosis and suppression of angiogenesis. This combined immunologic attack further extends the antiangiogenic intervention strategy pioneered by Folkman (27, 28) and offers an additional strategy for cancer therapy. To further enhance the efficacy of our survivin-based cancer vaccine and, in particular, to overcome the poor immunogenicity of this tumor self-antigen, we coexpressed the multifunctional murine chemokine CCL21 since it binds to CXCR3 in addition to its own CCR7 receptor (29, 30) and effectively chemoattracts activated antigen-presenting dendritic cells (DCs) and naïve T cells, bringing them together in lymphoid follicles and secondary lymphoid organs such as Peyer's patches for an effective T cell–mediated immune response (31, 32). Because CCL21 also binds to CXCR3 in the mouse, similar to the two angiostatic chemokines CXCL9 and CXCL10, its antitumor activity may be boosted in part by its angiostatic activity (33). This was shown by intratumoral (33), s.c. (34), or intralymphoid (35) injection of CCL21, which inhibited growth and metastasis of human tumor xenografts in SCID mice associated with a reduction in vascularity.
Polyubiquitination has been used for several of our DNA vaccines, particularly since we found in one of our initial studies that the presence of mutant polyubiquitin upstream of a DNA minigene encoding melanoma peptide antigens proved to be essential for achieving tumor-protective immunity (36). Based on the well-known role of polyubiquitin in protein processing by the proteasome, we assumed that this molecule was essential for optimizing antigen processing and ultimately effective antigen presentation in the MHC class I antigen pathway (36).

Here, we show for the first time the antitumor efficacy of a novel strategy employing two distinct but complementary pathways, one of apoptosis induction and the other of angiogenesis suppression, both triggered by CTLs against survivin evoked by an oral survivin-based DNA vaccine coexpressing chemokine CCL21.

Materials and Methods

Animals, Bacterial Strains, and Cell Lines. Female C57BL/6 mice, ages 6 to 8 weeks, were purchased from The Scripps Research Institute’s Rodent Breeding Facility and maintained at our animal facility. All animal experiments were done in compliance with the NIH Guides for the Care and Use of Laboratory Animals and approved by the Animal Care Committee of The Scripps Research Institute. The attenuated Salmonella typhimurium strain RE88 (dam- and Ara-) was provided by Remedeye Corp., (Santa Barbara, CA). Bacterial strain DH5α was purchased from Invitrogen, (Carlsbad, CA) and bacteria were routinely grown at 37°C in LB broth or on agar plates (EM SCIENCE, Darmstadt, Germany), supplemented, when required, with 25 μg/mL zeocin. The murine D121 lung cancer cell line was a gift from Dr. L. Eisenbach (Rehovoth, Israel).

Gene Cloning and Construction of Expression Vectors for DNA Vaccination. The full-length coding regions for murine survivin and CCL21 were amplified by reverse transcription-PCR of total RNA (1 μg) extracted from D121 mouse Lewis lung carcinoma cells and activated mouse splenocytes, respectively, Total RNA was extracted with the RNeasy Mini kit (Qiagen, Valencia, CA) and reverse transcription-PCR was done with a platinum quantitative reverse transcription-PCR thermoscript one-step system (Invitrogen) according to the manufacturer's instructions. Several constructs were made based on the pHudCE4.1 vector (Invitrogen) by using the PCR products, designed for independent expression of two genes from a single plasmid in mammalian expression vectors. The first of these constructs, pHud-CCL21 contains the CCL21 gene, which is inserted into the multiple cloning site A under the control of the P_CAV promoter. The second construct, pHud-survivin, contains mutant ubiquitin, inserted into the multiple cloning site B site under control of the PF_1a promoter, with the survivin gene being fused to the COOH-terminal of ubiquitin thus forming the polyubiquitinated protein. The last construct, pHud-survivin/CCL21, expresses both genes independently. The empty vector was generated as a control. These constructs are illustrated schematically in Fig. 1A. The sequences and protein expressions of all constructs were shown by sequence analysis and Western blotting (Fig. 1B).

Oral Vaccination and Tumor Challenge. C57BL/6J mice were divided into five groups for each experiment and were immunized thrice at 2-week intervals by gavage with 100 μL PBS, containing 1 × 10⁸ colony-forming unit’s attenuated S. typhimurium (RE88), harboring one of the following: empty vector pHud; individual expression vectors of either pHud-survivin/CCL21, pHud-survivin, or pHud-CCL21 along with a PBS control group. All mice used in the prophylactic experiments were challenged by i.v. injections of 1 × 10⁵ D121 murine Lewis lung carcinoma cells 1 week after the last immunization. In therapeutic settings, mice were first injected i.v. with 1 × 10⁹ D121 murine Lewis lung carcinoma cells and 5 days later subjected to three vaccinations, 5 days apart. Mice were examined daily, sacrificed, and examined for lung metastasis 28 days after tumor cell challenge in the prophylactic setting or 25 days after the initial tumor cell inoculation in the therapeutic model.

Determination of Antiangiogenic Effects. Mice were vaccinated as described above. Two weeks after the last vaccination, mice were injected s.c. in the sternal region with 500 μL growth factor-reduced Matrigel (BD Biosciences, La Jolla, CA.) containing 400 ng/mL murine fibroblast growth factor-2 (PeproTech, Rocky Hill, NJ) and D121 tumor cells (1 × 10⁷/mL) which were irradiated with 1,000 Gy. In all mice, except for two control animals, endothelial tissue was stained 6 days later by injecting into the lateral tail vein 200 μL of 0.1 mg/mL fluorescent Bandeiraea simplicifolia lectin I, Isolectin B4 (Vector Laboratories, Burlingame, CA); 30 minutes later, mice were sacrificed and Matrigel plugs excised and evaluated macroscopically. Lectin-FITC was then extracted from 100 μL of each plug with 500 μL of radio-immunoprecipitation assay buffer lysis buffer and then quantified by fluorometry at 490 nm. Background fluorescence found in the two non injected control mice was subtracted in each case.

Cytotoxicity Assay. Splenocytes were isolated from vaccinated mice 5 days after tumor cell challenge. Cytotoxicity was assessed by a standard 3H Cr release assay (36) against targets of either D121 tumor cells or murine endothelial cells overexpressing survivin. To determine specific MHC class I restriction of cytotoxicity, the inhibition experiments were done with 10 μg/mL anti-mouse MHC class I H-2Kβ/b monoclonal antibody (mAb; BD PharMingen, San Diego, CA).

Flow Cytometric Analysis. Activation markers of T cells and expression of costimulatory molecules on CD11c and MHC class II Ag-positive DCs were determined by two- or three-color flow cytometric analyses with a BD Biosciences FACSscan. T cell activation was evaluated by staining freshly isolated splenocytes from vaccinated mice with FITC-labeled anti-CD3e mAb in combination with PE-conjugated anti CD25, CD28, or CD69 mAbs. Activation of costimulatory molecules on APCs was measured with FITC-labeled anti-CD11c mAb and biotinylated anti-IAβ mAb, followed by streptavidin-allophycocyanin, and in combination with PE-conjugated anti-ICAM-1, CD80, or DEC205 mAbs. All reagents for these assays were obtained from BD PharMingen.

Cytokine Release Assay. Flow cytometry was used for detection of intracellular cytokines. To this end, splenocytes were collected from C57BL/6J mice 2 weeks after D121 tumor cell challenge and cultured 24 hours in complete T cell medium together with irradiated D121 cells as described previously (36). Preincubated cells were suspended with 1 μg purified 2.4G2 mAb to block nonspecific staining. The cells were washed and then stained with 0.5 μg FITC conjugated anti-CD4 or anti-CD8 mAbs. After washing twice, cells were fixed and stained with 1 μg/mL PE conjugated with either anti-IL2 or anti-IFN-γ mAbs for flow cytometric analysis. All mAbs were obtained from BD PharMingen.

Analysis of Tumor Cell Apoptosis. Apoptosis in D121 tumor cells induced by vaccination was measured at 3 and 24 hours, respectively. Both control and experimental animals were challenged i.v. with 1 × 10⁵ D121 cells 1 week after the last of three immunizations. Splenocytes were harvested from each individual mouse 1 week after tumor cell challenge and thereafter 2.5 × 10⁵ splenocytes were cocultured for 4 hours with 5 × 10⁵ D121 tumor cells in 6-well plates. Adherent tumor cells were easily separated from splenocytes in suspension. The ANNEXIN V-FITC apoptosis detection kit (BD Biosciences) was used for confirmation of early stage of apoptosis. To confirm later stage tumor cell apoptosis, 5 × 10⁵ D121 cells and 2.5 × 10⁵ splenocytes were cocultured for 24 hours and isolated tumor cells were then analyzed by fluorescence-activated cell sorting for apoptosis by the TUNEL assay with the APO-DIRECT KIT (BD Biosciences) according to the manufacturer’s instructions.

Evaluation of Possible Side Effects. Wound healing was tested by wounding as described (37, 38). Wounds of 3-mm lengths were inflicted on the upper backs of C57BL/6J mice (n = 4), 1 week after three immunization of each of the experimental groups. The time until wound closure was noted. To evaluate fertility, 1 week after the third immunization with either the survivin/CCL21 vaccine or PBS, female C57BL/6J mice (n = 6) were allowed to cohabitate with males, in a 3:1 breeding ratio. The days until parturition and number of pups were noted.
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 $P < 0.05$.

Results

A Survivin-Based DNA Vaccine Is Delivered to Peyer’s Patches. We tested the hypothesis that a DNA vaccine encoding the inhibitor of apoptosis protein survivin, overexpressed in both tumor cells and proliferating endothelial cells in the tumor vasculature, induces a T cell–mediated immune response that triggered both tumor cell apoptosis and suppression of angiogenesis which led to the eradication of lung tumor metastases. This vaccine, which also coexpressed the secretory chemokine CCL21, was delivered orally by attenuated Salmonella typhimurium (dam$^{-}$ and AroA$^{-}$) transformed with pEGFP. Mice were sacrificed at time points of 8, 16, and 32 hours and fresh specimens of small intestine were removed for analysis after thoroughly washing with PBS. Fluorescence expression of EGFP was detected by confocal microscopy (left). H&E staining of mouse Peyer’s patches (red arrows; right) for analysis of biopsies taken from mouse small intestine.

Vaccination Protects Against Pulmonary Tumor Growth and Metastases. We previously reported the effective in vitro DNA transfer from single mutant S. typhimurium (AroA$^{-}$), harboring the EGFP expression vector, to mouse primary peritoneal macrophages (36). Here, the new RE88 strain of doubly mutated S. typhimurium (dam$^{-}$ and AroA$^{-}$) was shown to successfully deliver plasmids to mouse Peyer’s patches in vivo (Fig. 1C). Such plasmids still expressed the relevant protein as determined by immunization of mice by gavage with doubly attenuated S. typhimurium, carrying the EGFP mammalian expression vector as a reporter gene. When such animals were sacrificed and the Peyer’s patches collected from the thoroughly washed small intestines, a strong fluorescence was observed by confocal microscopy (Fig. 1C). These data suggest that this doubly attenuated strain of Salmonella typhimurium not only transfers the respective plasmid to Peyer’s patches, but also that such plasmids encoding the target gene can still express the respective protein in vivo.
disseminated pulmonary metastases of D121 murine Lewis lung carcinoma were observed in mice vaccinated thrice at 2-week intervals and then challenged 1 week later by i.v. injection of tumor cells. Indeed, 6 of 8 mice completely rejected all pulmonary tumor metastases, whereas the remaining two animals revealed markedly reduced tumor metastases (Fig. 2A). In contrast, the survivin-based DNA vaccine lacking CCL21 was far less effective and induced complete suppression of metastases in only 1 of 8 animals, whereas all remaining mice showed extensive metastatic tumor growth. Additional animals that were treated only with control vaccinations of either PBS or empty vector revealed no tumor protection at all and died within 4 weeks after tumor cell challenge due to extensive metastases. Although immunization with doubly attenuated Salmonella carrying only the secretory CCL21 plasmid did not dramatically suppress tumor metastasis, it still resulted in statistically significant delays of metastases when compared with controls (Fig. 2A).

**Vaccination Reduces Growth of Established Metastases.** The survivin- or CCL21-based DNA vaccine proved also to be effective in markedly suppressing the growth of already well established pulmonary metastases in all experimental animals in a therapeutic setting (Fig. 2B). In contrast, all mice receiving only the survivin- or CCL21-based vaccines per se, or empty vector and PBS controls, revealed disseminated pulmonary metastases of D121 non–small cell lung carcinoma in this experimental setting (Fig. 2B).

**CTL-Mediated Apoptosis Is Induced by Survivin Targeting.** A critical question was answered when we found that the antitumor immunity observed was induced by the triggering of CTL-mediated tumor cell apoptosis. In fact, results of two key experiments indicated that incubation of D121 tumor cells with splenocytes from mice successfully vaccinated, with the survivin/ CCL21 vaccine resulted in tumor cell apoptosis. Early apoptosis was detected at 3 hours by the ANNEXIN V assay and with a considerable further increase after 24 hours by the TUNEL assay as indicated by flow cytometric analyses (Fig. 3A). Thus, early stage apoptosis was up to 3–to 4-fold higher in groups of mice immunized with the survivin/CCL21 vaccine than in controls after splenocytes harvested from such mice were coincubated with tumor cells. Adherent tumor cells were easily separated from splenocytes in suspension. Importantly, a dramatic 85% increase in apoptosis was observed at 24 hours (Fig. 3B) only in mice immunized with the survivin/CCL21 vaccine, suggesting that the robust tumor cell immunity triggered this event.

A marked CTL response was induced by the survivin/CCL21 vaccine indicated by specific in vitro lysis of tumor cells which was mediated only by splenocytes isolated from such immunized mice. In fact, a standard 51Cr release assay revealed marked cytotoxicity induced by specific CD8+ T cells obtained from mice after vaccination and subsequent challenge with D121 Lewis lung carcinoma cells (Fig. 3C). In contrast, CD8+ T cells isolated from control animals were found to be completely ineffective in evoking any noticeable killing of tumor cells as they evoked only background cytotoxic activities (Fig. 3C). Characteristically, the T cell–mediated cytotoxicity observed was MHC class 1 antigen restricted because it cytotoxic activities (Fig. 3B). Taken together, these results suggest that CTL-mediated lysis plays a significant role in the increased apoptosis of D121 tumor cells.

**Vaccination Induces Suppression of Angiogenesis in the Tumor Neovasculature.** A key question of our study was answered when we found that the survivin/CCL21–based vaccine, which was already shown to be capable of triggering the induction of tumor cell apoptosis (Fig. 3A and B), also decisively suppressed angiogenesis in the tumor vasculature. This was shown by a significant decrease in tumor neovascularization indicated by Matrigel assays and their quantification by relative fluorescence measured after in vivo staining of mouse endothelium with FITC-conjugated lectin (Fig. 4A). These data were further corroborated by the detection of macroscopically evident differences among experimental groups and control groups of mice upon examination of representative Matrigel plugs removed 6 days after s.c. injection, following an i.v. injection of FITC-conjugated lectin (Fig. 4B). These experiments were repeated thrice with essentially the same results.

A possible mechanism involved in the suppression of angiogenesis in the tumor vasculature induced by the survivin/CCL21 DNA vaccine was shown by the finding that cultured murine endothelial cells expressing survivin were specifically lysed in vitro only by CD8+ T cells isolated from mice successfully immunized with the survivin- or CCL21-based DNA vaccine (Fig. 4C). Typically, this lysis

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**Figure 2.** Suppression of pulmonary metastases of Lewis lung carcinoma by the oral DNA vaccines in both prophylactic and therapeutic treatment models. Lung metastases were induced in the prophylactic setting by i.v. injection of 1×10^6 D121 cells 1 week after the last of three immunizations administered by gavage at 2-week intervals. Experiments were terminated 28 days after tumor cell inoculation and the extent of pulmonary tumor foci determined. A, tumor metastasis scores and lung weights following immunization with either PBS, empty vector, CCL21, survivin, or survivin/CCL21 vaccines, respectively. Results: metastasis scores expressed as the % lung surface covered by fused metastatic foci: 0, none; 1, <5%; 2, 5% to 50%; and 3, >50%. Differences in metastasis scores and lung weights between groups of mice treated with the survivin/CCL21 vaccine and all control groups were statistically significant (P < 0.001). B, inhibition of tumor growth in the therapeutic model. C57BL/6J mice were first injected i.v. with 1×10^6 D121 Lewis lung carcinoma cells and received 5 days later DNA vaccines by gavage as indicated. Lung weights of the various experimental groups and pictures of lung specimens of each group. (A–E) survivin/CCL21, survivin, CCL21, empty vector, and PBS. Normal lung weight = 0.2 g.
Figure 3. Analyses of apoptosis and in vitro cytotoxicity after immunization with survivin/CCL21-based DNA vaccines and various controls. All animals were challenged with D121 tumor cells after three immunizations with each individual DNA vaccine. Splenocytes were harvested 1 week after tumor cell challenge and incubated with $5 \times 10^5$ D121 tumor cells. A, adherent tumor cells were separated from splenocytes in suspension after a 3-hour coincubation and subjected to FACS analyses. ANNEXIN V-FITC was used to determine the percentage of cells within the population that are actively undergoing apoptosis at an early stage (3 hours). Propidium Iodide (PI) was used to distinguish viable from nonviable cells. B, late-stage tumor cell apoptosis (24 hours) induced by the survivin/CCL21 vaccine measured by the FITC-dUTP TUNEL assay. C, cell-mediated cytotoxicity induced by various DNA vaccines against D121 lung cancer cells. Splenocytes were isolated 4 days after vaccination and analyzed for their lytic activity in a 4-hour $^{51}$Cr release assay and D121 cells were used as targets for splenocytes obtained from mice treated with either PBS, empty vector, CCL21, survivin, or survivin/CCL21, respectively. Inhibition experiments were performed in the presence of 10 μg/mL mAbs directed against H2K$^d$/H-2D$^d$ MHC class I antigens. Points, mean for three mice.

Figure 4. Suppression of angiogenesis by the survivin/CCL21 vaccine. A, angiogenesis was determined by the Matrigel assay. Quantification of vessel growth and staining of endothelium were achieved by fluorimetry and confocal microscopy, respectively using FITC-labeled Isolectin B4. Confocal images depicted (a-d) are PBS, empty vector control, survivin, and survivin/CCL21. B, average fluorescence of 100 μg Matrigel plugs from each experimental group of mice is depicted by the bar graphs ($n = 4$; mean ± SD; $P < 0.01$) where a-d are the same as listed in A. C, CTL-mediated killing of murine endothelial cells. The murine endothelial cell line HEV expressing survivin (top) was used as a target for splenocytes obtained from mice treated with either the following: empty vector (c), CCL21 (c), survivin (d), and survivin/CCL21 (d), respectively. Inhibition experiments (bottom) with Abs against H2K$^d$/H-2D$^d$ MHC class I antigens similar, as described in Fig. 3C.
was also found to be MHC class 1 antigen-restricted since antibodies against H-2K<sup>b</sup>/H-2D<sup>b</sup> antigens completely ablated all cytotoxic activity (Fig. 4C).

**Vaccination Does not Impair Wound Healing or Fertility.** We did not observe any statistically significant prolongation in the time required to completely heal small wounds inflicted on the backs of mice immunized with DNA vaccines encoding either survivin/CCL21, survivin, or CCL21 versus that of mice given only PBS (Fig. 5A). There was also no detectable difference in macroscopically visible swelling and inflammation in the wound areas of the three experimental groups. Additional experiments did not show any impact of the survivin- to CCL21-based DNA vaccine on fertility of the treated animal (Fig. 5B and C). This was based on the time elapsed from the start of cohabitation until parturition and on the number of pups born.

**The Survivin Vaccine Activates Immune Effector Cells.**

We conclude that the antitumor response evoked by the survivin/CCL21 DNA vaccine, particularly the induction of an effective CTL response shown by the data depicted in Figs. 3C and 4C, suggests that the immune effector cells involved are activated. This contention was further supported by analyses of the activation markers of effector T cells at different levels of protein expression by double or triple staining during flow cytometric analyses. Three lines of evidence suggested that multiple effector cells and effector mechanisms are involved in this vaccine-induced activation. First, only the survivin/CCL21 vaccine per se was most effective in up-regulating the expression of CD25, CD28, and CD69 T cell activation markers (Fig. 6A). Second, the up-regulation of CD28 is of particular importance since its interactions with B7 costimulatory molecules on DCs is essential to achieve critical and multiple interactions between naïve T cells and antigen-presenting DCs. In contrast, the DNA vaccines encoding only survivin or CCL21 per se increased the expression of the T cell activation markers to a far lesser extent (Fig. 6A). Third, activation of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells by the survivin/CCL21 vaccine was also indicated by their increase in intracellular proinflammatory cytokines IFN-γ and interleukin 2. In comparison, PBS and empty vector controls as well as DNA vaccines encoding solely survivin or CCL21 were found to be less effective in inducing these cytokines (Fig. 6B).

The up-regulated expression of ICAM-1, CD80, and DEC205 on DCs, achieved by the survivin/CCL21-based DNA vaccine could be particularly important because it is well known that the activation of T cells critically depends on effective cell-cell interactions with these costimulatory molecules expressed on DCs in order to achieve optimal ligation with T cell receptors. Again, immunization with doubly attenuated *Salmonella typhimurium* carrying eukaryotic plasmids encoding survivin/CCL21 induced the most pronounced up-regulation of these activation markers which was up to 2- to 3-fold higher than those of controls (Fig. 7).

**Discussion**

We showed for the first time that an oral DNA vaccine against survivin, coexpressing chemokine CCL21, could induce a CTL response sufficiently effective to attack tumor cells as well as suppress angiogenesis in the tumor neovasculature and result in the eradication or suppression of pulmonary lung tumor metastases in prophylactic and therapeutic settings.

It is well established that the disregulation of apoptosis by inhibitors of apoptosis protein, resulting in increased resistance to programmed cell death, is a common feature of malignant cells and represents a significant obstacle for successful prevention and therapy of cancer (14, 16, 21, 22, 24). It is also well known, particularly through the pioneering efforts of Folkman et al., that the suppression of angiogenesis in the tumor neovasculature provides a strong stimulus for eradication of tumor growth and that this offers a powerful tool for improving both cancer prevention and therapy (24, 27, 28). The inhibitor of apoptosis protein survivin was strongly implicated as an effective target to overcome such obstacles to cancer prevention and treatment (16, 21, 24). Indeed, several approaches, other than DNA-based vaccines, have been reported to target survivin. These include the application of dominant-negative mutants to initiate apoptosis via the mitochondrial pathway and suppression of tumor-associated angiogenesis (24, 39), blockage of survivin expression by antisense constructs (40, 41), induction of a CTL response against peptides of the survivin molecule (42, 43), and interference with survivin signaling pathways by molecular antagonists (24, 44).

However, despite these intensive efforts and the considerable interest in survivin as a direct target for cancer therapy, some critical aspects involved in the genetic immunization against
survivin remain to be explored. In fact, we could show here that an orally delivered DNA vaccine encoding survivin and secretory chemokine CCL21 can indeed induce a T cell–mediated antitumor immune response against established pulmonary metastases of non–small cell lung carcinoma, sufficiently effective to cause their ablation in mouse tumor model systems through mechanisms involving both suppression of tumor angiogenesis and induction of tumor cell apoptosis. We conclude that the immunologic mechanism(s) involved in the suppression of tumor cell angiogenesis involves CD8+ T cell–mediated lysis that was likely facilitated by the overexpression of survivin by proliferating endothelial cells in the tumor vasculature triggered by vascular endothelial growth factor produced by tumor cells (25, 26). Two lines of evidence further support the contention that the suppression of tumor cell angiogenesis induced by our vaccine occurred via specific CTL-mediated killing of proliferating endothelial cells in the tumor vasculature. First, a distinct suppression of vascular endothelial growth factor–induced angiogenesis was shown in vivo by Matrigel assays and, second, specific CTL-mediated killing of both tumor cells and mouse endothelial cells overexpressing survivin was found repeatedly to be effective in vitro in cytotoxicity assays.

The contention that activation of the tumor cell death machinery occurred via CTL-mediated tumor cell lysis induced by our vaccine is supported by our data indicating robust tumor cell apoptosis by Annexin V and TUNEL assays and by several recent reports in the literature. First, CTLs were found to induce apoptosis of tumor cells by releasing cytolytic granules containing the pore-forming protein perforin as well as granzyme serine proteases, both known to be involved in CTL-mediated tumor cell lysis (45). Second, several groups of investigators reported that T cells mount a vigorous cytolytic response against survivin peptides in vitro and in vivo (43, 46), and that HLA class I antigen–restricted T cells against survivin exist in patients with various malignancies (43). Recently, survivin also was found to be immunogenic in colorectal cancer patients and to elicit CD8+ and CD4+ T cell–mediated responses (47). Third, prior observations suggested that overexpression of survivin in proliferating endothelial cells in different vascular beds can result in cytoprotective mechanisms counteracting apoptosis by reducing the generation of active caspases and hence preservation of cell survival (24, 26). However, we cannot rule out the contribution of the apoptotic mechanism involving a mitochondria-dependent pathway with cytochrome c being released from these organelles.

**Figure 6.** Analyses of CTL activation markers. A, upregulated expression of T cell activation molecules following three immunizations of C57BL/6J mice with the various DNA vaccines and the control vaccine indicated, followed by challenge with 1 × 10⁵ D121 tumor cells. FACS analyses are depicted with splenocytes from such immunized mice 1 week after tumor cell challenge. Two-color flow cytometry analyses are shown with single cell suspensions of splenocytes. Anti-CD25, CD28, and CD69 mAbs were used in PE-conjugated form in combination with FITC-conjugated anti-mouse CD3 mAb. B, induction of intracellular cytokine release. Splenocytes were obtained 1 week after tumor cell challenge, stained with FITC-conjugated anti-CD4 or anti-CD8 Abs, and fixed, permeabilized, and subsequently stained with PE-conjugated anti-IFN-γ or anti-IL2 Abs. Cells stained with two colors were analyzed by flow cytometric analysis. Columns, mean for four animals; bars, SD. (a–e) PBS, empty vector, CCL21, survivin, and survivin/CCL21 vaccines. *, P < 0.05 compared with treatment groups.
into the cytosol, where it binds the protease activator, Apaf1, thus leading to the activation of caspase-9 (18, 24, 48).

In summary, our findings indicate that tumor cell death triggered by an oral, survivin-based DNA vaccine in both prophylactic and therapeutic settings, can be attributed to at least three mechanisms. First, proof of concept was established that the vaccine induced an effective CD8+ T cell–mediated tumor-protective immune response. Second, it was shown that oral delivery of both the DNA vaccine and its essential adjuvant, chemokine CCL21, to secondary lymphoid organs by attenuated S. typhimurium is required to attain the activation of both T and dendritic cells necessary to achieve an effective tumor-protective immunity. Third, and most important, our DNA vaccine could induce a CTL response sufficiently effective to jointly trigger suppression of angiogenesis in the tumor vasculature and induction of tumor cell apoptosis which then combined to eradicate or suppress established pulmonary metastases of non–small cell lung carcinoma. It is anticipated that novel strategies such as this will serve as a basis for the rational design of future strategies, which will ultimately lead to further improvements in the prevention and treatment of cancer.

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References


Figure 7. DNA vaccination enhances expression of costimulatory molecules by DCs. Similar as illustrated in Fig. 6, multicolor flow cytometric analyses were performed with single cell suspensions of splenocytes obtained 1 week after tumor cell challenge. Splenocytes are stained with FITC labeled anti-CD11c Ab, in combination with either PE conjugated anti-ICAM-1, CD80, or DEC205 Abs by VEGF in vascular endothelial cells. Biochem Biophys Res Commun 1999;264:781–8.
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