Protective Immunity against Human Carcinoembryonic Antigen (CEA) Induced by an Oral DNA Vaccine in CEA-transgenic Mice


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Abstract

Peripheral T-cell tolerance toward human carcinoembryonic self-antigen (CEA) was broken in CEA-transgenic C57BL/6J mice by an oral CEA-based DNA vaccine. This vaccine, delivered by the live, attenuated AroA− strain of Salmonella typhimurium (SL7207), induced tumor-protective immunity mediated by MHC class I-restricted CD8+ T cells. Activation of these T cells was indicated by increased secretion of proinflammatory cytokines IFN-γ, IL-12, and granulocyte/macrophage-colony stimulating factor, as well as specific tumor rejection and growth suppression in vaccinated CEA-transgenic mice after a lethal challenge with murine MC38 colon carcinoma cells. These tumor cells were double transfected with CEA and the human epithelial cell adhesion molecule (Ep-CAM)/KSA and consequently served as a docking site for a recombinant antibody-IL2 fusion protein (KSI/4-IL2) recognizing KSA. Importantly, the efficacy of the tumor-protective immune response was markedly increased by boosts with this antibody-IL2 fusion protein, resulting in more effective tumor rejection coupled with increased expression of costimulatory molecules B7.2/B7.2 and intercellular adhesion molecule 1 (ICAM-1) on dendritic cells and intensified release of proinflammatory cytokines IFN-γ, IL-12, and granulocyte/macrophage-colony stimulating factor from T cells of successfully vaccinated CEA-transgenic C57BL/6J mice. Increased T-cell activation mediated by boosts with KSI/4-IL2 fusion protein after tumor cell challenge was further indicated by expanded expression of T-cell activation markers CD25, CD28, CD69, and LFA-1. The application of such CEA-based DNA vaccines and its further improved versions may ultimately prove useful in combination therapies directed against human carcinomas expressing CEA self-antigens.

Introduction

A new era for cancer immunotherapy emerged during the last decade based on the recognition that some tumors encode rejection antigens capable of inducing a tumor-protective immunity (1, 2). A number of immunotherapeutic modalities were developed for this purpose and designed to activate the cellular arm of the immune response, particularly because CTLs were effective in recognizing tumor cells as foreign and eradicating them (3). More recent efforts focused on the development of DNA-based cancer vaccines designed to overcome peripheral T-cell tolerance against tumor self-antigens, such as human CEA4 (4). This well-characterized oncofetal glycoprotein of Mr 180,000–200,000 is encoded by 29 genes located on the long arm of chromosome 19 that are part of the immunoglobulin supergene family (5, 6). CEA, as a membrane antigen, has served as a useful target for CEA-specific radioimmunoconjugates in clinical detection and therapy protocols (7–9) and was used more recently to construct DNA vaccines for immunotherapy (4, 10). A useful model for CEA-based vaccines was provided by the establishment of a mouse line that carries the genomic DNA transgene for human CEA (11, 12). Moreover, this CEA transgenic mouse expresses CEA in a tissue-specific manner, similar to that observed in humans in which the colon is the main site of CEA production. Importantly, anti-CEA CD8+ T cells could be elicited in these CEA transgenic mice after in vivo priming with CEA-transfected fibroblasts (13). Furthermore, studies in humans demonstrated that CD8+ CTLs with specificity for CEA were not negatively selected, similar to findings obtained with transgenic mice (14, 15). In contrast, anti-CEA antibody responses could not be detected in such transgenic mice unless an independent carrier was used, suggesting tolerance to CEA in the CD4+ T-cell compartment (13).

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4 The abbreviations used are: CEA, carcinoembryonic antigen; ICAM, intercellular adhesion molecule; ER, endoplasmic reticulum; CMV, cytomegalovirus; IL, interleukin; LFA, leukocyte function-associated antigen; mAb, monoclonal antibody; GM-CSF, granulocyte/macrophage-colony stimulating factor; GFP, green fluorescent protein.
On the basis of information obtained 20 years ago, healthy individuals and cancer patients were considered to be unresponsive to CEA, because most experimental data on host immunity to CEA were largely equivocal (16, 17). However, more recent studies indicate that CEA-based vaccines induce CEA-specific immune responses in CEA-transgenic mice and in humans (4). In fact, a wide range of CEA immunogens, including cDNA encoding the entire CEA gene or a variety of CEA peptides with dominant MHC class I anchor residues carried by a replication-defective vaccinia virus induced CEA-specific MHC-restricted CTL responses, T-cell proliferation, as well as CD4+ T-cell and antibody responses in both CEA transgenic mice (18, 19) and cancer patients (14).

Recently, another approach successfully amplified T-cell activation induced by CEA-based DNA vaccines by using either replication-competent vaccinia or replication-defective avipox vectors containing a triad of costimulator molecules, B7.1/ICAM-1/LFA-3 expressed by APCs. In each case, the degree of T-cell activation using vectors containing three costimulatory molecules was far greater than the sum of the constructs, each containing one costimulatory molecule. Significantly, this rTRICOM approach activated both CD4+ and CD8+ T-cell populations to levels greater than those achieved with only one or two of these costimulatory molecules and markedly enhanced antigen-specific T-cell responses in vivo (20). Furthermore, CEA-specific CTLs were elicited from colorectal cancer patients in Phase I trials by using a delivery-replicative-defective avipox virus as a delivery vehicle for genes encoding CEA. Although these vaccines were well tolerated, both quantity and quality of T-cell responses observed in these clinical trials have been modest, at best, and did not result in objective antitumor responses (21). Because CEA is a self-antigen, it can most likely evoke only weak or moderate T-cell responses. Consequently, successful efforts to overcome peripheral T-cell tolerance will require novel and diversified immunization protocols using more potent forms of immunogen, as well as effective vaccine adjuvants.

Here, we describe an initial effort to accomplish this task and demonstrate that an oral vaccine, containing the entire CEA gene carried by an attenuated strain of Salmonella typhimurium, protects C57BL/6J mice transgenic for CEA against a lethal challenge of murine colon carcinoma cells stably transduced with CEA and the human Ep-CAM/KSA. This tumor-protective effect, obtained in a prophylactic setting, was primarily induced by MHC class I-restricted CD8+ T cells. Importantly, the protective immunity evoked by this DNA vaccine could be substantially amplified after tumor cell challenge by boosts with a recombinant antibody-IL2 fusion protein, resulting in further T-cell activation, up-regulation of costimulatory molecule expression on APCs and increased release of proinflammatory cytokines from activated T lymphocytes.

Materials and Methods

**CEA Transgenic Mice.** Transgenic mice were generated by using a 32.6-Kb AatII restriction fragment that contains the entire human CEA genomic region and flanking sequences isolated from a genomic cosmid clone. A mouse line [C57BL/6J-TgN (CEAEg) 18; FJP] has been established from breeding with one of the founder mice. Male and female CEA transgenic mice were bred at Vanderbilt University’s animal care facility and have been described elsewhere (12).

**Cell Lines and Bacterial Strains.** The chemically induced colon adenocarcinoma cell line, MC38, stably transfected with CEA (C15–4.3 clone), has been described previously (12). This CEA cell line was also stably transfected with the EpCAM/KSA by cloning this antigen with PCR and expressing it in MC38-CEA cells using a retroviral vector, essentially as described previously (22). The attenuated S. typhimurium AroA strain SL7207 was kindly provided by Dr. B. A. D. Stocker (Stanford University, Stanford, CA). Bacterial strain DH5α was purchased from Invitrogen (Carlsbad, CA), and bacteria were routinely grown at 37°C in Luria broth or on agar plates (Sigma Chemical Co., St. Louis, MO), supplemented when necessary with 50 μg/ml ampicillin.

**Construction of Expression Plasmids Encoding the Entire CEA or Truncated CEA Genes.** Expression vectors encoding the intact CEA gene, based upon the pShooter subcellular targeting vectors (Invitrogen), were constructed to either direct CEA expression to the cell surface or the ER. The pW-CEA eukaryotic expression vector that targeted CEA epitopes to the cell surface was inserted into the pCMV/myc/cyto plasmid between XhoI and NotI restriction sites. It included the entire coding sequence of CEA containing both its endogenous leader and COOH-terminus anchor sequences. The construction of pW-CEA was by PCR amplification of a 2.1-Kb fragment encoding the entire CEA cDNA using primers 5′-CGGCTCTGGAGACGTGGTTAGGAGCTCTCCCGCCCCTC-3′ and 5′-TTTGCGGCCGCTATCAGAGCAACCCCAACCAGC-3′. Alternatively, a truncated CEA coding sequence was constructed in a similar way, however, lacking both the endogenous leader and COOH-terminus anchor sequences (pER-CEA). This construct was inserted into the pCMV/myc/ER plasmid. This created an extracellular CEA domain, fused at the NH2 terminus to an ER targeting signal and at the COOH terminus to a myc epitope tag, followed by the ER retention signal SEKDEL. The 1.95-Kb fragment encoding this truncated CEA gene was amplified by PCR, using primers 5′-CGGCTCGAGAGTGTTAGGAGCTCTCCCGCCCCTC-3′ and 5′-TTTGCGGCCGCTATCAGAGCAACCCCAACCAGC-3′. These constructs are depicted schematically in Fig. 1.

**Oral Immunization and Tumor Cell Challenge.** CEA-transgenic C57BL/6J mice were divided in four experimental groups (n = 8) that received either 100 μl of PBS or were immunized three times at 2-week intervals by oral gavage with 100 μl of PBS containing 108 attenuated S. typhimurium harboring either empty vector (pCMV/cyto) or individual expression vectors (pER-CEA) and (pW-CEA), respectively. A control experiment included two s.c. injections, on days 0 and 7, of 2.5 × 105 irradiated (15,000 rads) MC38 cells that were doubly transfected with CEA and KSA. All mice were challenged s.c. in the right front flank with a lethal dose of 2.5 × 105 MC38-CEA-KSA cells 2 weeks after the last immunization. Mice were examined daily until the tumor became palpable, after which the tumor diameter was measured in two dimensions with a microcaliper every other day.
pW-CEA

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<tr>
<th>CEA Leader Sequence</th>
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pER-CEA

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Boosts with Recombinant Antibody-IL2 Fusion Protein. The construction of the KS1/4-IL2 fusion protein has been described previously (22). C57BL/6J mice transgenic for CEA that had been immunized by oral gavage with attenuated S. typhimurium harboring either empty vector (pCMV/cyto) or individual expression vectors (pER-CEA) and (pW-CEA), respectively, received 5-µg boosts of huKS1/4-IL2 fusion protein for 5 consecutive days starting 1 day after tumor cell challenge.

Antibody Immunoassay. A double antigen ELISA was used for the detection of anti-CEA antibodies using biotinylated CEA and anti-CEA mAb T84.1-E, as described previously (23).

Cytokine Release Assay. Splenocytes were collected 1 week after s.c. lethal tumor cell challenge with 2.5 × 10^6 MC38-CEA-KSA cells from all experimental groups of mice. Lymphocytes were harvested on Ficoll-Hypaque (BioWhittaker, OR), and coverslips were affixed to slides prior to sequential challenge were subsequently cultured for 3 days at 37°C in complete T-STIM culture medium (Becton Dickinson, Bedford, MA). MC38-KSA-CEA target cells (3 × 10^5), labeled with 0.5 mCi of ^{31}Cr, were incubated with effector cells at various E:T ratios at 37°C for 4 h. The percentage of specific target cell lysis was calculated using the formula: \[(E - S)/(T - S) \times 100\%\], where E is the average experimental release, S is the average spontaneous release, and T is the average total release.

Transfection and Immunoblot Assessment of Protein Expression. Lipofectamine was used for transient transfection of COS-7 cells according to the manufacturer’s instructions (Life Technologies, Inc., Grand Island, NY). Briefly, COS-7 cells were seeded at 2.5 × 10^4 cells/well in a six-well plate, and 24 h later, 1 µg of DNA was added with 5 µl of Lipofectamine in serum-free medium for each transfection. The serum-free medium was replaced by fresh, complete growth medium 5 h after adding the Lipofectamine reagent. Forty-eight h after transfection, parental or CEA-transfected COS-7 cells were rinsed briefly with PBS and lysed directly on the plate with lysis buffer. Insoluble material was removed by centrifugation, and the samples were assayed for protein content by the BCA protein assay (Pierce Chemical Co., Rockford, IL). Equal quantities of protein (15 µg/lane) were separated by SDS-PAGE under reducing conditions along side a control lysate and electroblotted onto a nitrocellulose membrane (Novex, San Diego, CA). The membrane was blocked overnight in TBS containing 5% skim milk and then incubated with 10 µg/ml mouse antihuman CEA mAb PARLAM-4 (ICN, Aurora, OH) in this buffer for 2 h at room temperature, washed vigorously with TBS containing 0.1% Tween 20 (TBS-T), and incubated at room temperature with horseradish peroxidase-labeled goat antimuscle antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). After further washing with TBS-T, the blot was developed with ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) and XOMAT-5 film (Eastman Kodak Co., Rochester, NY).

Localization of Subcellular CEA Expression. COS-7 cells plated on coverslips were transfected with either the vector encoding the entire CEA coding sequence (pW-CEA) or the vector encoding the extracellular domain of CEA coupled to an ER retention sequence (pER-CEA), as described in the section concerning vector construction. Transfection was performed with Lipofectamine according to the manufacturer’s instructions (Life Technologies, Inc.). For identification purposes, the pW-CEA construct was cotransfected with a cytoplasmic GFP expression vector and the pER-CEA construct with a vector encoding GFP fused to an ER retention signal and at the COOH terminus to an ER targeting signal and at the COOH terminus to a myc epitope tag, followed by an ER retention signal (SEKDEL). This construct serves as a control and is designated pER-CEA because it directs CEA to be expressed and retained in the ER.

Localization of Subcellular CEA Expression. COS-7 cells plated on coverslips were transfected with either the vector encoding the entire CEA coding sequence (pW-CEA) or the vector encoding the extracellular domain of CEA coupled to an ER retention sequence (pER-CEA), as described in the section concerning vector construction. Transfection was performed with Lipofectamine according to the manufacturer’s instructions (Life Technologies, Inc.). For identification purposes, the pW-CEA construct was cotransfected with a cytoplasmic GFP expression vector and the pER-CEA construct with a vector encoding GFP fused to an ER retention signal (ER-GFP). Forty-eight h after transfection, cells were harvested, fixed, and permeabilized with 3.5% paraformaldehyde/0.1% Triton X-100 in PBS (pH 7.4). Cultures were subsequently blocked for 30 min with 3% BSA in PBS (PBS/BSA) prior to incubation for 1 h at room temperature with anti-CEA mAb (PARLAM 4). Cultures were washed extensively with PBS/BSA before reaction of the anti-CEA primary antibody with Texas Red-conjugated goat antimouse secondary antibody (Biosource International, Camarillo, CA) for 1 h at room temperature. Stained cultures were mounted with SlowFade Reagent (Molecular Probes, Eugene, OR), and coverslips were affixed to slides prior to sequential
expression. pER-GFP is shown to be retained in the ER (Fig. 2B). In contrast, pW-CEA/pC-GFP (Fig. 2A). In addition, Western blotting of lysates obtained from COS-7 cells transfected with either of the two mammalian expression vectors indicates that both express CEA protein at the correct molecular size of 180 kDa (Fig. 2C).

**Results**

**Subcellular Immunolocalization of CEA and Protein Expression.** We initially determined by immunofluorescence and scanning confocal microscopy that our subcellular targeting expression vectors pW-CEA and pER-CEA were correctly constructed to direct CEA to the cell surface or to the ER, respectively. Thus, in Fig. 2, where GFP exhibits its characteristic green fluorescence and CEA is indicated by staining with Texas red, confocal microscopy clearly indicates the cell surface expression of pW-CEA/pC-GFP (Fig. 2A). In contrast, pER-CEA/ 

**Detection of subcellular immunolocalization of CEA/GFP expression by confocal microscopy.** COS-7 cells (Cos) plated onto coverslips were cotransfected with either the pW CEA construct and a cytoplasmic GFP expression vector pC-GFP (A) or the pER-CEA construct and a vector encoding GFP fused to an endoplasmic reticulum retention sequence (pER-GFP; B). Forty-eight h after transfection, cells were fixed and processed for immunofluorescence, as described in “Materials and Methods,” with GFP exhibiting its characteristic green fluorescence and CEA indicated by staining in red. SDS-PAGE analysis of cell lysates from transfected pW-CEA, pER-CEA, or control COS-7 cells was performed, followed by detection of CEA by immunoblotting, as described in “Materials and Methods.”

**Induction of Protective Immunity against Colon Carcinoma Cells after Immunization with Eukaryotic Expression Vectors Encoding CEA.** These experiments were based on the working hypothesis that p.o.-administered attenuated S. typhimurium carrying expression vectors encoding CEA would pass through M cells in the small intestine into host Peyer’s patches, where these bacteria are taken up by phagocytes, such as macrophages and dendritic cells. These APCs are activated by the pathogen and start to differentiate, migrating to lymph nodes and spleen, a time during which the attenuated bacteria die because of their inability to synthesize aromatic amino acids. During this process, a large number of plasmids will be released to be subsequently transferred into the cytosol and nucleus of the infected cells. Eventually, the encoded genes will be expressed by these host APCs (24).

Accordingly, there should be a strong induction of cytotoxic CD8+ T cells, because antigens are expressed in the cytosol, a cellular compartment for MHC class I presentation. This CD8+ T-cell response should induce a cell-mediated immune response in CEA-transgenic mice, breaking peripheral T-cell tolerance against a lethal challenge of MC38 murine colon carcinoma cells stably transfected with CEA. To this end, a number of experiments were performed, including three control experiments. C57BL/6J mice transgenic for CEA were immunized twice at 1-week intervals by s.c. injections with 2.5 × 10^8 irradiated (15,000 rads) MC38 murine colon carcinoma cells that had been stably transduced with CEA and the epithelial cell adhesion molecule KSA. When these mice were challenged 2 weeks later with a lethal s.c. dose of 2.5 × 10^8 MC38-CEA-KSA cells, these cells grew rapidly into tumors in all animals (n = 4), reaching a volume of >1000 mm^3 after 30 days (Fig. 3). This same observation was made in the other two control experiments. Specifically, three oral administrations, at 2-week intervals, of 1 × 10^8 attenuated S. typhimurium carrying either the empty vector (pCMV/cyto) or the CEA plasmid, targeted to and retained in the ER (pER-CEA), failed to elicit a protective immune response against a s.c. lethal challenge of 2.5 × 10^8 MC38-CEA-KSA administered 2 weeks after the last immunization. In fact, in each of these two groups of experimental animals (n = 6), there was uniformly rapid tumor growth in all mice (Fig. 3). In contrast, a group of mice (n = 8) that received three oral immunizations each with 1 × 10^8 S. typhimurium harboring the W-CEA expression vector (pW-CEA) revealed a dramatic decrease in tumor volume when compared with the animals of the three control experiments (P < 0.001). In fact, four of eight animals completely rejected the tumor challenge, indicating an effective tumor-protective immune response in this prophylactic setting.

**Recombinant Antibody-IL2 Fusion Protein Boosts Tumor-protective Immunity Induced by CEA-based DNA Vaccine.** These experiments were based on the working hypothesis that the KS1/4-IL2 fusion protein can specifically target this cytokine to the tumor microenvironment, thus activating and expanding CD8+ T-effector and memory cells and thereby increase the efficacy of this CEA-based DNA vaccine to induce tumor-protective immunity. To this end, after s.c. injection of a lethal challenge of 2.5 × 10^8 MC38-CEA-KSA cells, CEA-transgenic C57BL/6J mice (n = 8) that had received three immunizations with S. typhimurium carrying the pW-CEA expression vector were injected i.v. 1 day after tumor cell chal-
immunized with the DNA vaccine 6 days after tumor cell challenge. The CEA-based DNA vaccine in protecting CEA-transgenic huKS1/4-IL2 fusion protein can effectively boost the efficacy of initial working hypothesis regarding the mode of action of attenuated S. typhimurium-mediated Cytotoxicity Induced by a CEA-based DNA Vaccine (Fig. 3, A). An important control experiment indicated that the 5 μg of huKS1/4-IL2 fusion protein per se, when administered to naïve mice that only received the tumor challenge without immunization by the DNA vaccine (Fig. 3, FP). Taken together, these data suggest that the huKS1/4-IL2 fusion protein can effectively boost the efficacy of the CEA-based DNA vaccine in protecting CEA-transgenic mice against a lethal tumor cell challenge.

**Induction of MHC Class I-restricted CD8+ T Cell-mediated Cytotoxicity Induced by a CEA-based DNA Vaccine against MC38-CEA-KSA Colon Carcinoma Cells.** Our initial working hypothesis regarding the mode of action of attenuated S. typhimurium carrying an expression vector encoding CEA predicted the strong induction of cytotoxic CD8+ T cells, because ultimately antigens would be expressed in the cytosol after processing in the proteasome, transported to the ER by TAP and suitable processed peptides being transported by MHC class I molecules to the cell surface. This prediction was shown to be valid, because splenocytes isolated from mice immunized with the DNA vaccine 6 days after tumor cell challenge and/or boosts with huKS1/4-IL2 fusion protein specifically lysed MC38-CEA-KSA target cells (Fig. 4A). Splenocytes from mice that received the antibody-IL2 fusion protein boost were somewhat more effective in this assay than such cells obtained from immunized mice that did not receive this boost. In contrast, only background lysis was seen with splenocytes obtained from control animals, i.e., either naïve mice or those immunized with the vaccine containing pER-CEA targeted to and retained in the ER. This tumor cell lysis was specific, because the use of nonspecific B16 melanoma target cells lacking CEA expression resulted in a complete lack of cytolysis (data not shown).

The data depicted in Fig. 4B clearly demonstrate that the cytolytic response elicited by splenocytes from immunized mice against MC38-CEA-KSA tumor target cells was MHC class I restricted, because the presence of 50 μg/ml antibodies directed against H-2Kb/H-2Dd MHC class I antigens completely inhibited cytotoxic activities. We also determined whether there was any induction of specific anti-CEA antibodies by immunization...
with attenuated \textit{S. typhimurium} carrying either empty vector or the two CEA expression vectors. However, a sensitive ELISA for IgG and IgM anti-CEA antibodies indicated no measurable antibody titers in serum samples of treated mice (data not shown).

**Increased Expression of T-Cell Activation Markers Is Induced by the CEA-based DNA Vaccine and Boosted with Antibody-IL2 Fusion Protein.** On the basis of our working hypothesis and the finding of MHC class I-restricted, CD8$^+$ T cell-mediated \textit{in vitro} cytolysis of tumor target cells by splenocytes (isolated from mice immunized with DNA vaccines and boosted subsequently with antibody-IL2 fusion protein, huKS1/4-IL2), we analyzed the expression of well-recognized markers of T-cell activation. These included CD25, the high-affinity IL-2 receptor \(\alpha\) chain, CD69, an early T-cell activation antigen, and LFA-1, important for the initial interaction of T cells with APCs via intercellular cell adhesion molecules ICAM-1, ICAM-2, and ICAM-3. Importantly, these T-cell activation markers also included CD28, a member of the immunoglobulin superfamily, expressed on T cells and serving as the receptor for the costimulatory B7.1 and B7.2 molecules of APCs, the ligation of which with CD28 will costimulate growth of naive T cells. Our findings obtained with two-color flow cytometric analyses are depicted in Fig. 5 and demonstrate marked up-regulation of these T-cell activation markers. This became particularly evident by analyses performed after CEA-transgenic mice that were immunized with DNA vaccine and boosts with huKSI/4-IL2 fusion protein. Two-color flow cytometry analyses were performed with splenocytes obtained from these immunized mice at two time points, either after three immunizations with attenuated \textit{S. typhimurium} carrying the CEA gene or 1 day after receiving five booster injections with huKS1/4-IL2 fusion protein. Two-color flow cytometry analyses were performed with single-cell suspensions of splenocytes. Anti-LFA-1, anti-CD25, anti-CD28, and anti-CD69 were used in phycoerythrin-conjugated form in combination with FITC-conjugated anti-mouse mAb CD3e. Splenocytes were harvested after MC38-KSA-CEA tumor cell challenge from naive mice or from mice immunized with pER-CEA plasmid, with pW-CEA plasmid, or with pW-CEA plasmid, followed by boosts with huKS1/4-IL2 fusion protein (FP). Each value represents the mean for four animals; bars, SD.

**Expression of Costimulatory Molecules Is Increased by Immunization with DNA Vaccine and Boosts with Antibody-IL2 Fusion Protein.** Intercellular adhesion molecules, such as ICAM-1, which bind to the T-cell integrin LFA-1 are especially important in T-cell activation, as are the costimulatory molecules B7.1 and B7.2 expressed on APCs that ligate with CD28 on T cells. Consequently, we analyzed splenocytes from groups of mice immunized with either DNA vaccines, control vaccines, or vaccines combined with boosts of antibody-IL2 fusion protein for the expression of these costimulatory molecules by two-color flow cytometric analyses. The expression of B7.1 after immunization with DNA vaccine and tumor cell challenge increased 2-fold over controls; however, boosts with antibody-IL2 fusion protein did not result in any further increase in B7.1 expression. Expression of B7.2 and ICAM-1 were up-regulated 2- and 3-fold over that of controls, respectively, after DNA vaccination and tumor cell challenge. In each case, boosts with antibody-IL2 fusion protein resulted in an additional 40% increase in expression of these costimulatory molecules (Fig. 6).

**Increase in Secretion of Proinflammatory Cytokines from T Cells after Immunization with CEA-based DNA Vaccine Is Further Boosted by Antibody-IL2 Fusion Protein.** The release of proinflammatory cytokines, such as IFN-\(\gamma\), IL-12 and GM-CSF from T cells is a well-known indication of T-cell activation in secondary lymphoid tissues and spleen. Consequently, we used a solid-phase sandwich ELISA to analyze for these three cytokines in supernatants of various splenocyte preparations 24 h after being plated in the presence of irradiated (15,000 rads) MC38 tumor cells. As shown in Fig. 7, only background levels of IFN-\(\gamma\), IL-12, and GM-CSF were detected when analyzing supernatants of splenocytes obtained from either naive CEA-transgenic mice after challenge with MC38-CEA-KSA cells or from such mice immunized with the control DNA vaccine pER-CEA and challenged with tumor cells. In contrast, vaccination with the pW-CEA plasmid and challenge with tumor cells resulted in a 3-fold increase of IFN-\(\gamma\) release over these controls that was further augmented after boosts with huKS1/4-IL2 fusion protein. Production of IL-12 and GM-CSF was increased 2-fold over control values after vaccination with pW-CEA and tumor cell challenge. Boosts with antibody-IL2 fusion protein further increased production of GM-CSF ~2-fold. It was particularly striking, however, that boosts with huKS1/4-IL2 fusion protein increased production of IL-12 ~5-fold over control values, whereas vaccination with pW-CEA and tumor challenges exceeded controls by only 2-fold in the production of IL-12. There was no detectable production of either IL-4 or IL-10 (data not shown). Taken together, these data demonstrating increased release of three proinflammatory cytokines support the contention that DNA
Up-regulated expression of costimulatory molecules induced by DNA vaccines and boosts by KS1/4-IL2 fusion protein. C57BL/6J mice transgenic for CEA were immunized, challenged with tumor cells, and boosted with KS1/4-IL2 fusion protein in the same fashion, as described in the legend of Fig. 5. Two-color flow cytometric analyses were performed with single-cell suspensions prepared from splenocytes obtained 1 day after tumor cell challenge or fusion protein boosts. Anti-B7.1, anti-B7.2, anti-ICAM-1, and anti-CD11c were used in phycoerythrin-conjugated form in combination with FITC-conjugated antimouse mAb B220. A total of 10,000 labeled cells/sample were analyzed. Shown are cell surface expressions of costimulatory molecules B7.1, B7.2, and ICAM-1. Each value represents the mean for four animals; bars, SD.

Immunization coupled with boosts of antibody-IL2 fusion protein decisively increased T-cell activation in secondary lymphoid tissues and spleen.

Discussion

The present study was designed to determine whether peripheral T-cell tolerance to CEA, a human tumor self-antigen, could be overcome by a DNA vaccine containing the entire gene encoding CEA delivered p.o. by an attenuated *S. typhimurium* carrier to CEA-transgenic mice. It was of particular interest to assess whether this vaccine could induce a T cell-mediated, tumor-protective immune response that was effective in rejecting a lethal challenge of murine MC38 colon carcinoma cells, stably transduced to express CEA and the human epithelial cell adhesion molecule (Ep-CAM/KSA). It was also important to determine whether this protective immune response could be further improved by boosts with a recombinant antibody-IL2 fusion protein (huKS1/4-IL2) directed against the Ep-CAM/KSA.

Scanning confocal microscopy and Western blotting demonstrated that we could selectively obtain subcellular localization of CEA by designing two expression plasmids with completely opposite characteristics: (a) pW-CEA encoded the intact CEA gene that ultimately achieved expression of CEA epitopes on the cell surface; and (b) the other, pER-CEA, served as a control because it lacked both the endogenous leader and COOH-terminal anchor sequences, contained an ER targeting signal and the ER-retention signal, SEKDEL, thus causing its complete retention in the ER. Consequently, this truncated molecule could not be processed in the cytoplasm and proteasome and was retained in the ER, thus preventing suitable peptide epitopes from combining with MHC class I heavy and light chains for transport to the cell surface. Additionally, lysis of COS-7 cells transfected with either of these two expression vectors and subsequent Western blotting indicated that both expressed CEA protein of the correct molecular size of 180 kDa.

Importantly, it is evident from our experiments that the pW-CEA vaccine, when administered by oral gavage with attenuated *S. typhimurium*, was effective in eliciting an MHC class I-restricted, T cell-mediated, tumor-protective immune response. This resulted in the complete rejection of a lethal MC38-CEA-KSA tumor cell challenge in 50% of C57BL/6J mice transgenic for CEA, whereas the remaining animals revealed a marked, 3-fold suppression in tumor size compared with controls. All three control groups exhibited rapid and uniform tumor growth in all CEA-transgenic mice. In this regard, a prior report by others indicated that treatment of CEA-transgenic mice with a recombinant vaccinia virus expressing CEA (rV-CEA) also caused the rejection of MC38 tumor cells expressing CEA in 50% of experimental animals; however, in contrast to our data, the remaining 50% of mice did not display a 3-fold reduction in tumor size but exhibited large, s.c. tumors that were generally indistinguishable from controls (19). Also, the rV-CEA vaccine induced anti-CEA IgG antibody titers in CEA-transgenic mice that developed T helper 1-type, CEA-specific CD4+ T-cell responses. In contrast, our oral CEA
vaccine delivered by attenuated *S. typhimurium* did not elicit any detectable anti-CEA IgM or IgG antibody titers but induced MHC class I-restricted CD8⁺ T-cell responses in CEA-transgenic mice, resulting in effective tumor-protective immunity. The difference in the immune responses might be attributable to the different carriers used for the respective CEA vaccines. Thus, the live attenuated *S. typhimurium* may actually provide a "danger signal" (25, 26) and stimulate the innate immune system, possibly because of unmethylated bacterial CpG dinucleotide motifs (27), resulting in the production of cytokines, such as IL-12, which were released by activated T cells in our study.

Significantly, our data suggest immunological mechanisms responsible for the effective immunization with the pW-CEA vaccine. Thus, marked activation of T cells and dendritic-like cells (B220⁺; CD11c⁺) was indicated by the decisive up regulation in expression of the T-cell integrin, LFA-1, and the ICAM-1 (CD54). These two molecules are known to synergize in the binding of lymphocytes to APCs (28). In fact, the transient binding of naïve T cells to APCs is crucial in providing time for T cells to sample large numbers of MHC molecules on the surface of each APC for the presence of specific peptides. This might increase the chance of a naïve T cell recognizing its specific peptide:MHC ligand, followed by signaling through the T-cell receptor, and induction of a conformational change in LFA-1. This, in turn, will greatly increase its affinity for ICAM-1 and stabilize the association between the antigen-specific T cell and the cell-presenting antigen (29, 30).

In addition, the marked increase in the expression of CD28 on T cells and the B7.1 and B7.2 costimulatory molecules on dendritic-like cells observed after vaccination and tumor cell challenge is important, particularly because the activation of naïve T cells requires two independent signals: (a) binding of the peptide:MHC complex by the T-cell receptor transmits signals to T cells indicating antigen recognition; and (b) ligation of CD28 with B7.1 or B7.2 produces a second signal and thereby initiates T-cell responses and production of armed effector T cells (31, 32).

The increases over controls in expression of CD25, i.e., the high-affinity IL-2 receptor α chain and CD69, an early T-cell activation antigen, indicate that T-cell activation took place in secondary lymphoid tissues after vaccination and tumor cell challenge. Furthermore, the specific, marked elevation in expression of proinflammatory cytokines, IFN-γ, IL-12, and GM-CSF by such T cells is a well-known feature of their activation (33). The notion that secondary lymphoid tissue of mice that exhibited protective tumor immunity contained tumor-specific CD8⁺ T cells was further supported by the finding that splenocytes isolated from such mice specifically lysed MC38-CEA-KSA tumor target cells *in vitro* in an MHC class I-restricted manner. In contrast, splenocytes isolated from mice in all control groups failed to lyse these tumor target cells.

The pronounced 5-fold increase in the production of IL-12 by T cells induced by boosts with the antibody-IL2 fusion protein is particularly intriguing, because IL-2, *per se*, is not known to induce production of IL-12. However, because targeting of IL-2 to the tumor microenvironment activated dendritic-like cells, it is likely that this led to increased synthesis of IL-12 via CD40-CD40 ligand "licensing" of these APCs by activated CD4⁺ T cells. In this regard, we reported recently that help provided by CD4⁺ T cells via CD40/CD40L interactions in a melanoma tumor model was crucial for achieving effective immunotherapy with an antibody-IL2 fusion protein (34).

The rationale for using small boosts of huKS1/4-IL2 fusion protein to improve tumor-protective immune responses induced by our CEA-based DNA vaccine was based on results of our prior work, where this approach completely eradicated CT26 lung tumor metastases in 100% of syngeneic BALB/c mice (35, 36). In fact, injection of small, noncurative doses of huKS1/4-IL2 fusion protein shortly after tumor cell challenge also markedly improved the tumor-protective effect of our CEA-based DNA vaccine in CEA transgenic mice. Thus, six of eight vaccinated mice now completely rejected the tumor cell challenge, and the remaining two animals exhibited a marked suppression in tumor growth. Interestingly, this improvement in tumor-protective immunity correlated completely with the additional increases found in T-cell activation markers, costimulatory molecules, and proinflammatory cytokines.

In summary, we demonstrated that our p.o.-administered, CEA-based DNA vaccine induced effective tumor-protective immunity mediated by MHC class I-restricted CTLs. This antitumor effect correlated with the marked up-regulation of costimulatory molecules on APCs, markers of activation on T lymphocytes, and increased release of proinflammatory cytokines. Small, noncurative doses of huKS1/4-IL2 fusion protein administered after tumor cell challenge further increased these antitumor effects.

**References**


Protective Immunity against Human Carcinoembryonic Antigen (CEA) Induced by an Oral DNA Vaccine in CEA-transgenic Mice

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