A Dual-Function DNA Vaccine Encoding Carcinoembryonic Antigen and CD40 Ligand Trimer Induces T Cell-Mediated Protective Immunity Against Colon Cancer in Carcinoembryonic Antigen-Transgenic Mice[1]

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A carcinoembryonic Ag (CEA)-based DNA vaccine encoding both CEA and CD40 ligand trimer achieved effective tumor-protective immunity against murine colon carcinoma in CEA-transgenic mice by activating both naive T cells and dendritic cells. Peripheral T cell tolerance to CEA was broken in a prophylactic model by this novel, dual-function DNA vaccine, whose efficacy was further enhanced by boosts with a recombinant Ab-IL-2 fusion protein (huKS1/4-IL-2). These conclusions are supported by four lines of evidence. First, a lethal challenge of MC38-CEA-KS Ag murine colon carcinoma cells was for the first time completely rejected in 100% of experimental animals treated by oral gavage of this DNA vaccine carried by attenuated *Salmonella typhimurium*, followed by five boosts with huKS1/4-IL-2. Second, specific activation of dendritic cells was indicated by their marked up-regulation in expression of costimulatory molecules B7.1 (CD80), B7.2 (CD86), and ICAM-1. Third, a decisive increase over control values was observed in both MHC class I Ag-restricted cytotoxicity of CTLs from successfully vaccinated mice and secretion of proinflammatory cytokines IFN-γ and IL-12. Fourth, activation of CTLs was augmented, as indicated by up-regulation of activity markers LFA-1, CD25, CD28, and CD69. Taken together, these results suggest that a dual-function DNA vaccine encoding CEA and CD40 ligand trimer combined with tumor-targeted IL-2 may be a promising strategy for the rational development of DNA-based cancer vaccines for future clinical applications. *The Journal of Immunology,* **2001, 167: 4560–4565.**

One of the major obstacles for achieving a tumor-specific immune response is overcoming peripheral T cell tolerance against tumor self-Ags and inducing CTLs that effectively eradicate disseminated tumor metastases and subsequently maintain a long-lasting immunological memory preventing tumor recurrence (1–4). Human carcinoembryonic Ag (CEA)[3] is an oncofetal membrane Ag that provides a relevant tumor self-Ag target for the development of DNA vaccines for immunotherapy (5–7). A useful animal model for CEA-based vaccines was provided by the establishment of a mouse line that carries the genomic DNA transgene for human CEA (8, 9) and expresses CEA in a tissue-specific manner similar to humans. Following in vivo priming with CEA-transfected fibroblasts, anti-CEA $CD8^+$ T cells could be elicited in these transgenic mice that were tolerant to CEA in the $CD4^+$ T cell compartment (10). Studies in humans indicated that $CD8^+$ CTLs specific for CEA were not negatively selected, similar to findings obtained with transgenic mice (11, 12).

A large body of literature describes the biological roles of CD40 ligand (CD40L), particularly its interaction with CD40 expressed on APCs during costimulation of T cell activation (13–15). CD40L, a type II membrane protein of 35 kDa and a member of the TNF gene family, is expressed on T cells upon Ag recognition (13). Members of the TNF family are biologically most active when expressed as homotrimers. CD40L is no exception in this regard and consequently was expressed as a homotrimer (CD40LT) by modification of a 33-aa leucine zipper motif fused to the N terminus of the entire extracellular domain of this ligand (16). CD40LT DNA was found to enhance cellular immune responses such as induction of IFN-γ and cytolytic T cell activity when mice were vaccinated with DNA encoding the highly immunogenic model Ag β-galactosidase (17). CD40L is critically involved in the activation of T cells necessary to induce an effective protective immunity against tumor self-Ags. Once MHC class I Ag:peptide complexes are taken up by dendritic cells (DCs) and presented to naive T cells, the first Ag signal is delivered via TCRs, followed by up-regulation of CD40L. On the T cell surface, CD40L then induces costimulatory activity on DCs via CD40-CD40L interactions. Thus primed, these APCs now express costimulatory molecules B7.1 (CD80) and B7.2 (CD86), which send a second costimulatory signal to T cells via interaction with CD28, an event required for full activation of T cells to concurrently produce proinflammatory cytokines IFN-γ and IL-12 and to perform effector functions (18). The role of IL-2 targeted to the tumor

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[3] Abbreviations used in this paper: CEA, carcinoembryonic Ag; CD40L, CD40 ligand; CD40LT, CD40 ligand trimer; DC, dendritic cell; KSA, KS Ag.

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microenvironment by a recombinant Ab-IL-2 fusion protein (huKS1/4-IL-2) is to boost anti-tumor T cell responses either by acting as a second costimulatory signal in the activation of CTL (19) or by further activating preactivated DCs expressing IL-2Rs (20, 21).

An effective means of enhancing efficacy of DNA vaccines is to grow the plasmid encoding DNA in a nonreplicating strain of *Samonella typhimurium*, which can then be applied as an oral vaccine. The live, attenuated bacteria transport the DNA through the gastrointestinal tract and then through the M cells that cover the Peyer's patches of the gut. From there the attenuated bacteria enter APCs such as dendritic cells and macrophages where they die, because of their mutation, liberating multiple copies of the DNA inside the phagocytes (22).

Indeed, attenuated bacteria are believed to provide a danger signal (23, 24) and stimulate the innate immune system, producing proinflammatory cytokines such as IL-12 and mediators such as NO that enhance Ag presentation and promote Th1-type cellular immune responses associated with the eradication of tumors. In fact, attenuated *S. typhimurium* was found to be an effective carrier for an autologous oral DNA vaccine that protected against murine melanoma (25). A recombinant *Listeria monocytogenes* vaccine proved to be highly effective in mediating regression of primary murine melanoma and their established lung metastases (26). *L. monocytogenes* produces a strong cellular immune response, since unlike most other intracellular bacteria it escapes into the cytoplasm by disrupting the phagosomal membrane, thus allowing any protein it secretes to target both MHC class I and class II pathways of the infected cell for Ag presentation (27).

We previously achieved partial tumor protection against a lethal challenge of MC38 murine colon carcinoma cells, stably transduced with CEA and KS Ag (KSA), a human pan-epithelial cell adhesion molecule. Vaccinations were accomplished by oral gavage with a CEA-based DNA vaccine carried by attenuated *S. typhimurium*, which induced MHC class I Ag-restricted $CD8^+$ T cell responses, resulting in rejection of s.c. tumors. However, this occurred in only some of the experimental mice transgenic for CEA even when boosted with a recombinant Ab-IL-2 fusion protein that targeted IL-2 to the tumor microenvironment (28).

Here we extend these findings by achieving for the first time a $CD8^+$ T cell-mediated tumor-protective immune response against CEA self-Ag effective in 100% of experimental CEA-transgenic mice that completely rejected a lethal tumor cell challenge. This was accomplished with a unique dual-function DNA vaccine encoding CEA and CD40LT, activating both DCs and naive T lymphocytes, aided by boosts with huKS1/4-IL-2 fusion protein.

Materials and Methods

CEA-transgenic mice

C57BL/6J CEA-transgenic breeder mice were generated using a 32.6-kb *Aat*II restriction fragment containing the entire human CEA genomic region and flanking sequences isolated from a genomic cosmid clone. A mouse cell line (C57BL/6J-TgN (CEAGe) 18; FJP) was established (9) and CEA-transgenic mice were bred at The Scripps Research Institute's animal care facility. Mice were used between 6 and 8 wk of age. All animal experiments were performed according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

Tumor cell lines and bacterial strains

The chemically induced murine colon adenocarcinoma cell line MC38 was stably transfected with both CEA (C15-4.3 clone) and the epithelial cell adhesion molecule Ep-CAM/KSA (9, 29). The attenuated *S. typhimurium* $AroA^-$ strain SL 7207 was provided by Dr. B. A. D. Stocker (Stanford University, Stanford, CA). Chemically competent *Escherichia coli* were purchased from Invitrogen (Carlsbad, CA) and routinely grown at 37°C in Luria-Bertani broth or on agar plates (VWR, San Diego, CA), supplemented when necessary with 75 μg/ml ampicillin.

Construction of expression plasmids

We generated several distinct forms of expression plasmids to target CD40LT and CEA molecules to DCs or T cells, respectively. The plasmid used for immunization was pcDNA3.1/zeo$^+$ (Invitrogen). The pER-CEA control plasmid targeted to and retained in the endoplasmic reticulum and the pW-CEA plasmid targeted to the cell surface were described previously (28). The plasmid encoding the CD40LT gene (pCD40LT) contained a modified 33-aa leucine zipper motif to facilitate the formation of trimeric CD40L that was fused to the C terminus of the IL-7 leader sequence to direct protein expression to the cell surface or induce its secretion outside the cells (16). Detection of CD40LT by Western blotting was facilitated by incorporating a short antigenic sequence, Flag, detectable by specific mAbs. The plasmid pCD40LT-CEA contains the entire CEA extracellular domain fused to the C terminus of murine CD40L, thus generating a dual-function chimeric construct.

Oral immunization, tumor cell challenge, and Ab-IL-2 fusion protein boosts

CEA-transgenic C57BL/6J mice were divided into seven experimental groups ($n = 8$). Mice were immunized three times at 2-wk intervals by oral gavage with 100 μl PBS containing 1×10^8 attenuated *S. typhimurium* harboring empty vector (pcDNA3.1); individual expression vectors pER-CEA, pW-CEA, pCD40LT, or pCD40LT-CEA; or the latter followed by boosts with huKS1/4-IL-2. Control experiments included PBS, Ab-IL-2 fusion protein boosts without immunization by DNA vaccine and a group of mice immunized only with irradiated MC38 cells. All mice were challenged s.c. in the right flank with a lethal dose of 2.5×10^5 MC38-CEA-KSA cells 2 wk after the last immunization. Mice were examined daily until the tumor became palpable, after which its diameter was measured in two dimensions with a microcaliper every other day.

Construction of the huKS1/4-IL-2 fusion protein has been described previously (29). C57BL/6J mice transgenic for CEA that were immunized by oral gavage with attenuated *S. typhimurium* as described above received 5-μg boosts of huKS1/4-IL-2 fusion protein for 5 consecutive days starting 1 day after tumor cell challenge.

Cytotoxicity assay

Cytotoxicity was measured by a standard ^{51}Cr release assay (21). Splenocytes isolated from CEA-transgenic mice, 1 wk after tumor cell challenge, were subsequently cultured for 3 days at 37°C in complete T-STIM culture medium (BD Biosciences, Bedford, MA). MC38-CEA-KSA target cells (3×10^6), labeled with 0.5 mCi ^{51}Cr were incubated with effector cells at various E:T cell ratios at 37°C for 4 h.

Transfection and immunoblot assessment of protein expression

Lipofectamine was used for transient transfection of COS-7 cells according to the manufacturer's instructions (Invitrogen), seeding COS-7 cells at 2.5×10^5 cells/well in a six-well plate and adding, 24 h later, 1 μg DNA with 5 μl Lipofectamine in serum-free medium. Immunoblots were performed with equal quantities of protein (15 μg/lane), separated by SDS-PAGE under reducing and nonreducing conditions alongside a control lysate, and electroblotted onto a nitrocellulose membrane as described previously (21). After staining with mouse anti-human CEA mAb (ICN Pharmaceuticals, Aurora, OH) or anti-FLAG M2 mAb (Sigma, St. Louis, MO), followed by anti-mouse IgG-HRP, the blot was developed with ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) and X-OMAT-5 film (Eastman Kodak, Rochester, NY).

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-color flow cytometry analysis with a BD Biosciences FACScan. T cell activation was determined by staining freshly isolated splenocytes from successfully vaccinated mice with anti-CD8 FITC (53-6.7) in combination with PE-conjugated anti-CD25 (H129.19), LFA-1 (2D7), CD28 (37.51), and CD69 (H1.2F3) Abs. Activation of costimulatory molecules on APCs was measured with FITC-labeled anti-CD11c (HL-3) in combination with PE-conjugated anti-B7.1 (16-10A1), B7.2 (GL1), or ICAM-1 and biotinylated anti-IA^b (KH74) Abs, followed by streptavidin-allophycocyanin. All cytometric flow experiments were performed in the presence of 0.1 μg/ml propidium iodide to exclude dead cells. All reagents were obtained from BD PharMingen (La Jolla, CA).

Cytokine induction assay

Splenocytes were harvested from all experimental groups of mice 1 wk after s.c. lethal tumor cell challenge with 2.5×10^5 MC38-CEA-KSA cells. Lymphocytes were isolated on Ficoll-Hypaque (BioWhittaker, Walkersville, MD) and cultured for 24 h in complete T cell medium with 1×10^5 irradiated (15,000 rad) MC38-CEA-KSA cells. Supernatants were collected and stored at −70°C until use. Cytokines were analyzed for either IFN-γ or IL-12 with commercially available cytokine detection kits using a solid phase sandwich ELISA (R&D Systems, Minneapolis, MN).

Results

Protein expression of CEA and CD40LT

We analyzed protein expression of plasmids pCD40LT, pCD40LT-CEA, and pW-CEA by transfection into COS-7 cells. Western blotting indicated that all constructs produced proteins of the expected molecular mass (~35, ~215, and 180 kDa, respectively), as shown by SDS-PAGE analyses of lysates from transfected cells analyzed under reducing conditions (Fig. 1). A plasmid encoding pCD40LT expressed proteins in the cell lysate indicative of monomeric, dimeric, and trimeric CD40L under nonreducing conditions (Fig. 1*B*). CD40L protein was also detected in supernatants of transfected cells under reducing conditions (data not shown).

Induction of tumor protective immunity by a dual-function chimeric molecule encoding both CD40LT and CEA molecules

A number of experiments performed, including several controls, indicated that the dual-function DNA vaccine targets CD40LT and CEA to DCs and T cells, respectively. Thus, C57BL/6J mice transgenic for CEA were immunized on days 0 and 7 by s.c. injections of 2.5×10^5 irradiated (15,000 rad) MC38 murine colon carcinoma cells. Challenge of these controls 2 wk later with a lethal s.c. dose of MC38-CEA-KSA cells resulted in rapidly developing tumors in all mice, indicating that MC38-CEA-KSA cells were not immunogenic per se (Fig. 2*A*). This was also found to be the case in three other key control experiments. Mice ($n = 6$) vaccinated three times at 2-wk intervals by oral gavage with 1×10^8 attenuated *S. typhimurium* carrying the empty vector, the pER-CEA plasmid exclusively targeted to and retained in the ER, or the pCD40LT construct alone all uniformly failed to elicit a protective immune response against a lethal s.c. tumor cell challenge and revealed rapid and uniform tumor growth (Fig. 2, *B–D*). In contrast, a group of mice treated with the same vaccination protocol, but receiving the DNA vaccine containing the pW-CEA vector, revealed a substantial decrease in tumor volume, with three of eight animals completely rejecting the tumor cell challenge (Fig. 2*F*). In mice vaccinated with pCD40LT-CEA, four of eight animals completely rejected the tumor cell challenge. In this case the remaining mice showed a dramatic suppression of tumor growth compared with controls ($p < 0.001$; Fig. 2*G*).

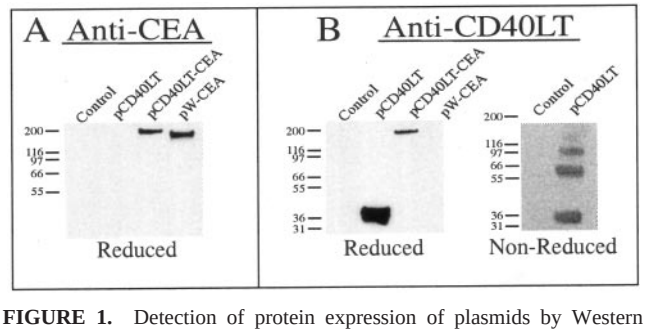

FIGURE 1. Detection of protein expression of plasmids by Western blotting. COS-7 cells were transfected with pCD40LT, pCD40LT-CEA, and pW-CEA plasmids, and cell lysates were analyzed under reducing or nonreducing conditions. *A,* The blot was probed with anti-human CEA under reducing conditions. *B,* The blot was probed with anti-FLAG M2 to show CD40L expression under reducing and nonreducing conditions.

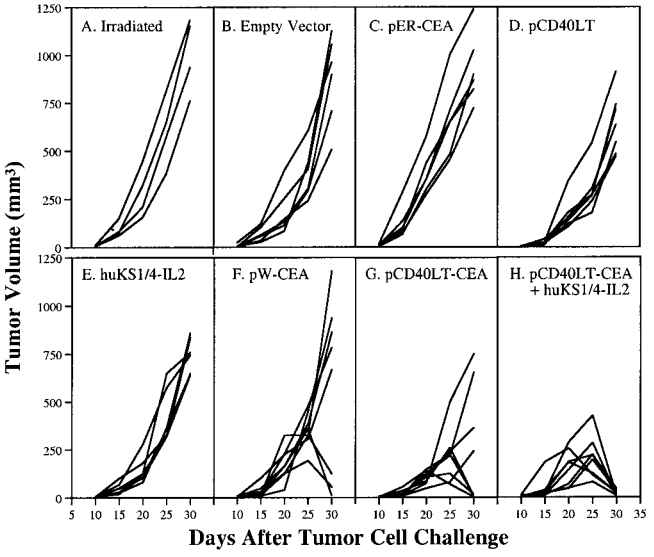

FIGURE 2. Growth prevention of MC38-CEA-KSA tumors by an oral DNA vaccine and boosts with huKS1/4-IL-2 fusion protein. Mice in group A were immunized with irradiated MC38-CEA-KSA colon carcinoma cells on days 0 and 7. Experimental animals in groups, B, C, D, F, G, and H were each immunized three times by oral gavage at 2-wk intervals with 1×10^8 attenuated *S. typhimurium* harboring the individual plasmids indicated. Two weeks later all mice were challenged s.c. with a lethal dose of 2.5×10^5 MC38-CEA-KSA cells. Group H was vaccinated and then boosted i.v. with five doses (5 μg each) of huKS1/4-IL-2 fusion protein, and naive mice (*E*) were treated only with fusion protein as a control. Animals were examined daily until the tumor became palpable, after which its diameter was measured with microcalipers in two dimensions every other day. The tumor growth of each mouse is depicted by a solid line.

Vaccination efficacy is amplified by boosts with Ab-IL-2 fusion protein

Boosts with small, noncurative doses of huKS1/4-IL-2 fusion protein targeted to the tumor microenvironment markedly increased the efficacy of the DNA vaccine. In fact, vaccination of CEA-transgenic mice by the same protocol described for the pCD40LT-CEA group, followed by i.v. injections of 5 μg huKS1/4-IL-2 1 day after tumor cell challenge for 5 consecutive days, resulted in complete rejection of the tumor cell challenge in eight of eight experimental animals (Fig. 2*H*). An important control experiment indicated that five injections of 5 μg huKS1/4-IL-2 fusion protein per se had essentially no effect on tumor growth when administered to naive mice that only received the tumor challenge without prior immunization by the DNA vaccine (Fig. 2*E*). The IL-2 fusion protein boost was specific, since boosting with a nonspecific fusion protein, hu14.18-IL-2, directed against ganglioside GD2 not expressed by M38 colon carcinoma cells was ineffective (data not shown).

Ag-specific CTL responses are increased by the pCD40LT-CEA dual-function vaccine

The application of the pCD40LT-CEA vaccine induced strong cytotoxic $CD8^+$ T cell priming with or without huKS1/4-IL-2 fusion protein boosts, as demonstrated in CEA-transgenic mice immunized with each of the individual plasmids (Fig. 3). CTLs from mice that received vaccinations with pCD40LT-CEA plus boosts

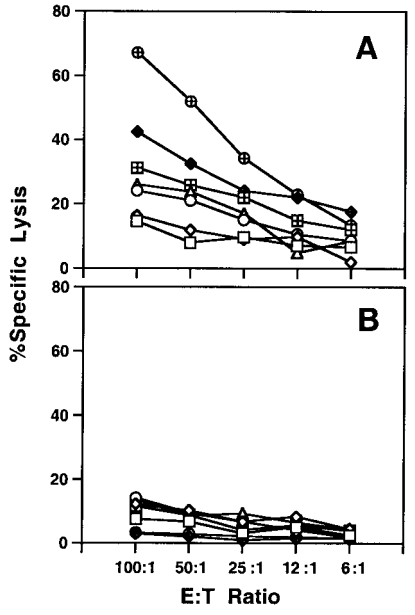

FIGURE 3. Priming of CTLs by DNA vaccine and functional amplification by huKS1/4-IL-2. C57BL/6J mice transgenic for CEA were vaccinated as described in Fig. 2. Splenocytes of mice were isolated 1 wk after tumor cell challenge, i.e., the second day after completion of boosts with fusion protein, and analyzed for their cytotoxic activity in a 4-h ^{51}Cr release assay at different E:T cell ratios. *A*, T cell-mediated cytotoxicity. MC38-CEA-KSA cells served as targets for splenocytes isolated from untreated tumor-bearing mice (□), mice treated only with fusion protein (◇), and mice immunized with plasmids pER-CEA (○), pCD40LT (△), pW-CEA (⊞), pCD40LT-CEA (♦), and pCD40LT-CEA plus huKS1/4-IL-2 boosts (⊕). *B*, Inhibition of MHC class I Ag-restricted lysis of tumor cells by CTLs. Blocking of cytotoxicity was performed in the presence of 50 μg/ml anti-MHC class I Ab (H-2K^b/H-2D^b) in the same groups of mice. Each value shown represents the mean of four mice.

with the Ab-IL-2 fusion protein proved to be most effective, inducing up to 70% lysis compared with 45% lysis by such cells obtained from mice immunized with the same vaccine but without the fusion protein boost (Fig. 3*A*). In contrast, only background lysis was observed with splenocytes obtained from control animals. Tumor cell lysis was specific, since the use of nonspecific B16 melanoma cells lacking CEA expression as targets resulted in a complete lack of cytolysis (data not shown). Importantly, the data depicted in Fig. 3*B* clearly demonstrate that the cytolytic response elicited by splenocytes from mice immunized against MC38-CEA-KSA tumor target cells was MHC class I Ag restricted, since the presence of 50 μg/ml Abs directed against H2-K^b/H2-D^b MHC class I Ags completely inhibited cytotoxic activities. This inhibitory effect was specific, since the presence of nonspecific anti-H-2k^d and H-2D^d Abs did not inhibit cytolysis (data not shown).

Up-regulation of CTL activity markers by the dual-function DNA vaccine is enhanced by boosts with Ab-IL-2 fusion protein

The interaction between CD40LT on activated Th cells with its CD40 target on DCs is critical for achieving optimal Ag-specific T cell responses. We observed a correlation between the ability of the dual-function DNA vaccine to enhance T cell-dependent immune responses and the increase in expression of T cell activation markers. This was evident from increases in expression of CD25, the high affinity IL-2R α-chain, CD69, an early T cell activation Ag, and LFA-1, which is important for the initial interaction between T cells and DCs via the intercellular cell adhesion molecule, ICAM-1 (Fig. 4). Importantly, these up-regulated T cell activation markers also included CD28, a member of the Ig superfamily expressed on T cells that serves as the receptor for the costimulatory B7.1 and B7.2 molecules of DCs whose ligation with CD28, in turn, will costimulate growth of naive T cells (Fig. 4). Importantly, boosts with huKS1/4-IL-2 fusion proteins 24 h after tumor cell challenge further elevated the expression of these same markers by 20–35%.

Increased expression of costimulatory molecules by immunization with pCD40LT-CEA molecules and boosts by Ab-IL-2 fusion protein

T cell activation is critically dependent on up-regulated expression of costimulatory molecules B7.1 and B7.2 on DCs to achieve optimal ligation with CD28 expressed on T cells. Equally important is the up-regulation of ICAM-1 that binds the T cell integrin LFA-1. FACS analyses of splenocytes obtained from CEA-transgenic mice, successfully immunized with the DNA vaccine and boosted with Ab-IL-2 fusion protein, clearly indicated that we accomplished this particular task very effectively, as the expression of B7.1, B7.2 and ICAM-1 was up-regulated 1- to 2-fold over that of controls (Fig. 5). Boosts with Ab-IL-2 fusion protein resulted in an additional 20- 40% increase in the expression of both costimulatory and adhesion molecules (Fig. 5). These data provide evidence that vaccination with pCD40LT-CEA molecules induces and enhances the expression of costimulatory molecules on CD11c$^+$ and MHC class II Ag-positive DCs, suggesting that the capability of these APCs for tumor-specific Ag processing and presentation was significantly increased.

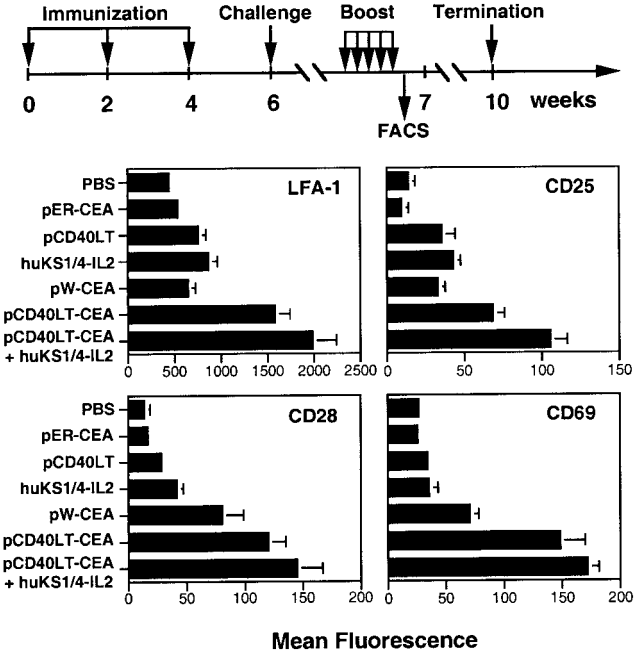

FIGURE 4. Up-regulated expression of T cell activation molecules. C57BL/6J mice transgenic for CEA were immunized with the DNA vaccine, then challenged with tumor cells and boosted with huKS1/4-IL-2 fusion protein as described in Fig. 2. FACS analyses were performed with splenocytes obtained from each experimental group at the time point indicated. Two-color flow cytometric analyses were performed with single-cell suspensions of splenocytes. Anti-LFA-1, CD25, CD28, and CD69 Abs were used in a PE-conjugated form in combination with FITC-conjugated anti-mouse mAb CD3e. Each value represents the mean and SD for four mice.

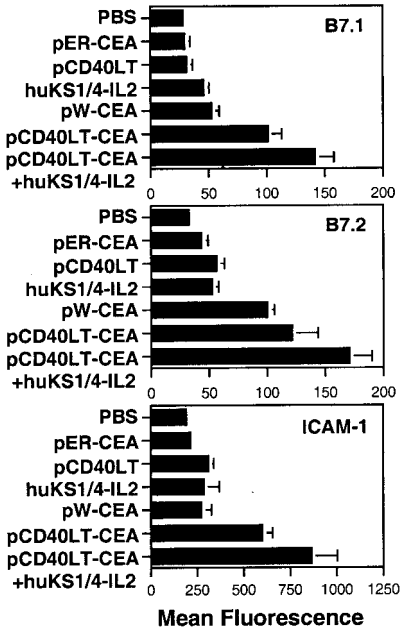

FIGURE 5. pCD40LT-CEA vaccination plus huKS1/4-IL-2 fusion protein boosts enhanced the expression of costimulatory molecules. In the same experiment as that outlined in Fig. 4, two-color flow cytometric analyses were performed with single-cell suspensions prepared from splenocytes obtained 1 day after fusion protein boosts. Splenocytes were stained with FITC-labeled anti-CD11c Ab in combination with PE-conjugated anti-B7.1, B7.2, or ICAM-1 Abs along with biotinylated anti-IA^b Ab, followed by streptavidin-allophycocyanin. Shown are cell surface expressions of costimulatory molecules B7.1, B7.2, and ICAM-1. Each value represents the mean and SD for four mice.

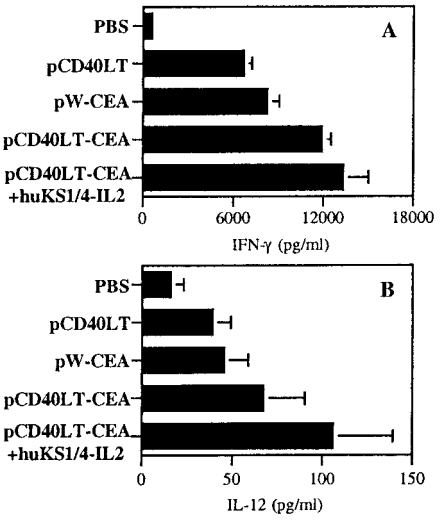

FIGURE 6. Induction of proinflammatory cytokines. C57BL/6J mice transgenic for CEA were immunized with the pCD40LT-CEA vaccine with and without boosts of huKS1/4-IL-2 fusion protein as well as with the pW-CEA vaccine and controls of pCD40LT and PBS and then challenged with MC38-CEA-KSA tumor cells. Splenocytes were obtained 1 wk after tumor cell challenge and plated in the presence of irradiated MC38-CEA-KSA cells. Culture supernatants were then harvested after 24 h and analyzed for the release of either IFN-γ (*A*) or IL-12 (*B*) by a solid-phase ELISA. Each value represents the mean and SD for four mice.

pCD40LT-CEA vaccination enhances production of cytokines boosted further by Ab-IL-2 fusion protein

The pCD40LT-CEA vaccine enhanced the release of proinflammatory cytokines IFN-γ and IL-12 from T cells, as indicated by a solid phase sandwich ELISA measuring their production in supernatants of various splenocyte preparations 24 h after being plated in the presence of irradiated (15,000 rad) MC38-CEA-KSA tumor cells. Only background levels of IFN-γ and IL-12 were detected when analyzing supernatants of splenocytes obtained from PBS-treated, CEA-transgenic control mice after challenge with MC38-CEA-KSA cells. However, if mice received the pCD40LT-CEA DNA vaccine, the production of IFN-γ and IL-12 increased by 75 and 50%, respectively, over levels observed in mice vaccinated with either pCD40LT or pW-CEA alone (Fig. 6). Production of IFN-γ was further augmented by 25% after boosts with huKS1/4-IL-2 fusion protein, while that of IL-12 increased 3-fold over control values and 100% over that observed after vaccination, but without the huKS1/4-IL-2 boost (Fig. 6). These data support the contention that DNA immunization coupled with boosts of Ab-IL-2 fusion protein decisively increased T cell activation in secondary lymphoid tissues.

Discussion

The major objective of this study was achieved by breaking peripheral T cell tolerance against CEA, a human tumor self-Ag with a dual-function oral DNA vaccine encoding both CEA and CD40LT in CEA-transgenic mice. Importantly, a $CD8^+$ T cell-mediated rejection of a lethal challenge of murine colon carcinoma cells occurred that was completely effective in 100% of experimental mice in a prophylactic setting. Although we as well as other investigators (28, 30–32) previously reported that tumor-protective immunity was achieved with a CEA-based DNA vaccine in CEA-transgenic mice, this treatment was never completely effective in all experimental animals. We attribute the completely successful tumor-protective immunity achieved in all CEA-transgenic mice in this study to the combined action of the unique dual-function DNA vaccine and the huKS1/4-IL-2 fusion protein, which accomplished the concurrent activation of both Ag-presenting DCs and naive T cells. Possible mechanisms of action were suggested by the up-regulated expression of several receptor/ligand pairs known to critically impact effective activation of T cells following their interaction with DCs that present them with MHC:peptide complexes. These included CD40/CD40LT, LFA-1/ICAM-1, CD28/B7.1, CD28/B7.2, and CD25/IL-2 as well as the increased secretion of proinflammatory cytokines IFN-γ and IL-12. Several lines of evidence provided insights into the priming of $CD8^+$ T cells in vivo following immunization with the pCD40LT-CEA dual-function DNA vaccine and challenge with colon carcinoma cells. First, a marked activation of T cells and $CD11c^+$ dendritic-like cells was indicated by the decisive up-regulation in expression of T cell integrins LFA-1 and ICAM-1, which are known to synergize in the binding of lymphocytes to APCs (33). In fact, the transient binding of naive T cells to APCs is crucial in providing time for these cells to sample large numbers of MHC molecules on the surface of each APC for the presence of specific peptides. This mechanism would increase the chance of a naive T cell recognizing its specific MHC:peptide ligand, followed by signaling through the TCR and induction of a conformational change in LFA-1. This, in turn, will greatly increase LFA-1's affinity for ICAM-1 and stabilize the association between the Ag-specific T cell and the APC (34, 35). Second, the marked increase in the expression of CD28 on T cells as well as the costimulatory molecules B7.1 and B7.2 on DCs following vaccination and tumor cell challenge is particularly significant, since it provides the two signals required for activation of naive T cells. One signal indicates Ag recognition being transmitted to T cells after binding of the MHC:peptide complex to the TCR, and the other signal, ligation of CD28 with B7.1

and B7.2, initiates T cell responses and the production of armed effector T cells (13, 15, 18). Third, a clear indication of T cell activation in secondary lymphoid tissues was provided by marked increases in the expression of CD25, the high affinity IL-2R α-chain, and CD69, an early T cell activation Ag.

The significant elevation in the production of proinflammatory cytokines IFN-γ and IL-12 by T cells induced by our dual-function DNA vaccine suggests that a third signal may act directly on T cells (23, 24, 36). This danger signal was reported to be required for Th1 differentiation leading to clonal expansion of T cells (36). In fact, whenever T cell help is required to generate an effective $CD8^+$ T cell response against a tumor-self Ag like CEA, triggering of DCs is necessary before their encounter with an Ag-specific $CD8^+$ T cell (37). This effect is mediated by ligation of CD40 on the surface of APCs (35), with CD40L expressed on activated $CD4^+$ T cells. CD40LT expressed by our DNA vaccine probably acted as a surrogate for activated $CD4^+$ T cells, leading to maturation of DCs as indicated by their decisive up-regulation of B7.1 and B7.2 costimulatory molecules (38). In summary, we demonstrated that our orally administered, dual-function DNA vaccine containing genes encoding for both CEA and CD40LT induced a highly efficient tumor-protective immunity against CEA self-Ag in all experimental CEA-transgenic mice. It is anticipated that this strategy may ultimately lead to an improvement in the efficacy of DNA vaccines for cancer therapy.

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