

Immune-Reconstituted Influenza Virosome Containing *CD40L* Gene Enhances the Immunological and Protective Activity of a Carcinoembryonic Antigen Anticancer Vaccine

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The correct interaction of a costimulatory molecule such as CD40L with its contrareceptor CD40 expressed on the membrane of professional APCs, provides transmembrane signaling that leads to APC activation. This process can be exploited to significantly improve the efficacy of cancer vaccines and the outcome of a possible cancer vaccine-induced, Ag-specific CTL response. Therefore, we investigated whether a novel intranasal delivery of immune-reconstituted influenza virosomes (IRIV), assembled with the *CD40L* gene (*CD40L/IRIV*), could be used to improve protective immunity and the Ag-specific CTL response against carcinoembryonic Ag (CEA) generated with a novel vaccine constituted of IRIV assembled with the *CEA* gene (*CEA/IRIV*). Our results suggest that *CD40L/IRIV* was able to augment CEA-specific CTL activity and CEA-specific protective immunity induced by *CEA/IRIV* most likely through the induction of a CTL response associated with a Th1 phenotype. In conclusion, we provide evidence that *CD40L/IRIV*, by acting through the *CD40L/CD40* signaling pathway, acts as an immune-adjuvant that could increase the efficacy of a CEA-specific cancer vaccine, which could provide an efficacious new strategy for cancer therapy. *The Journal of Immunology*, 2005, 174: 7210–7216.

Active specific immunotherapy, or vaccine therapy is a rising new strategy for the treatment of human cancer. Several approaches are being evaluated in order for inducing an Ag-specific immune response with antitumor activity in cancer patients, some of which are currently being tested in clinical trials (1–4). It is already commonly accepted that it is possible to generate an efficient *in vitro* and *in vivo* immune response by delivering the target Ag directly to the APCs by using naked nucleic acids (DNA or RNA), or viral or bacterial recombinant constructs (5–8). In particular, DNA immunization has been shown to be capable of inducing *in vivo* an effective humoral response and a cellular response simultaneously *in vivo* (9), restricted by class I (10–12) as well as by class II MHC⁺ cells (13). We have previously demonstrated the possibility of using the immune-reconstituted influenza virosomes (IRIVs)² as a delivery system for plasmids expressing tumor-associated Ag. We have shown that this method is able to induce a specific CTL response with antitumor activity in an *in vitro* human model, and also *in vivo* using either BALB/c mice or HLA-A2.1 transgenic mice (HHD) (14). In the

present study, we investigated the possibility of enhancing the immunogenicity of a carcinoembryonic Ag (CEA) DNA vaccine by combining its administration with a plasmid expressing the coaccessory molecule CD40L in IRIV. This study was based on the fact that CTLs recognize protein Ags as small peptides produced from intracellular proteolysis operated by proteasomes bound to the class I MHC molecules on the membrane of target cells or APC. The TCRs on specific CTLs are engaged through a trimolecular interaction involving the same TCR, the peptide, and the specific MCH complex, which provides a first transmembrane signal that is necessary but not sufficient, to induce an efficient Ag-specific CTL activation with proliferation and consequent clonal expansion (15, 16). To obtain these events, it is necessary that the CTL precursors receive a second intracellular signal, which is mainly provided by a receptor/contrareceptor interaction of coaccessory molecules such as B7.1 or B7.2/CD28 or CD40L/CD40 (3, 14). CD40 is a molecule expressed on the cell membrane of partially primed APCs, which can bind to CD40L expressed on the membrane of activated CD4⁺ Th cells (17). This interaction is known to deliver a bidirectional second signal, which is able to simultaneously activate both the effector T cells and the APC, increasing their priming ability (17). Many researchers believe that the interaction between CD40 and CD40L is critical for helping the APC to efficiently prime CD8⁺-specific cytotoxic cells (17). It has already been reported that CD40L expressed as a homotrimer by a DNA plasmid can be used to enhance cellular immune response and cytolytic T cell activity in mice vaccinated with DNA encoding *CEA* gene (18, 19). In the present study, we investigated whether the intranasal (*i.n.*) administration of IRIVs containing the *CD40L* (*CD40L/IRIV*) gene plasmid could effectively improve the immunological and protective antitumor activity of a CEA-expressing IRIV vaccine, after a lethal challenge with CEA-expressing tumor cells, in an *in vivo* BALB/c model.

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² Abbreviations used in this paper: IRIV, immune-reconstituted influenza virosome; CEA, carcinoembryonic Ag; *i.n.*, intranasal(*ly*).

Materials and Methods

Plasmid construction

The *CEA* gene was amplified from the Colo 205 cell line (American Type Culture Collection) by means of RT-PCR starting from the specific mRNA by using the sense primer, 5'-AAAAGCTTATGGCAGAGCCACCC AAACCC-3', and the antisense primer, 5'-CCCGAATTCCTATATCA GAGCAACCCCAA-3', and cloned in *HindIII-EcoRI* sites of the pVax expression vector (Invitrogen Life Technologies) to obtain the recombinant plasmid GC115 (*CEA*). Murine *CD40L* gene was obtained starting from the mRNA of murine lymphocytes by RT-PCR. The primers were 5'-AAGCTTATGATAGAAACATACAGCCA-3' (sense) and 5'-GAAT TCTCAGAGTTTGTAGTAAGCCAAA-3' (antisense), and the amplicon was cloned in *HindIII-EcoRI* sites of the pVax expression vector (Invitrogen Life Technologies) to obtain the recombinant plasmid GC130 (*CD40L*), expressing the molecule. The constructs were grown in DH5 α cells (Invitrogen Life Technologies). Plasmid DNA was purified using the Qiagen Endo Free plasmid kit as described by the manufacturer and sequenced by DNA sequencing with a Sequenase kit (Amersham Biosciences). The influenza virosomes (IRIV) were prepared as described elsewhere (20). Nonencapsulated plasmids were separated by 0.1 gel filtration on a High Load Superdex 200 column (Amersham Biosciences) equilibrated with sterile PBS. The void volume fractions containing the virosomes and encapsulated plasmids were eluted with PBS and collected.

Transfection of cell cultures

P815 cells (1×10^3) (American Type Culture Collection) were grown in six-well microplates at 37°C and transfected with 1 μ g of plasmid DNA using the Effectene Transfection reagent (Qiagen) as described by the manufacturer. After 3 days, *CEA* or *CD40L* expression was analyzed by flow cytometer analysis by using a PE-conjugated anti-*CEA* mAb (Cymbus Biotechnology), and MR1 mAb (BD Pharmingen), respectively. Samples were analyzed on a FACScan (BD Biosciences), and data were analyzed using CellQuest software (BD Biosciences). P815 mastocytoma cells (American Type Culture Collection) were stably transfected with *CEA* (clone 13) and analyzed by FACScan, to evaluate the percentage of cells expressing *CEA*.

Mice immunization

Female BALB/c mice were purchased from Charles River Breeding Laboratories. They were used at an age of between 6 and 8 wk. Five groups of 10 mice were i.n. immunized. Group A received 20 μ l of pVax/IRIV (containing 10 μ g of pVax plasmid and 0.6 μ g of influenza hemagglutinin), group B received 20 μ l of *CEA*/IRIV (corresponding to 10 μ g DNA), group C received 20 μ l of *CD40*/IRIV, and group D received 10 μ l of *CEA*/IRIV in addition to 10 μ l of *CD40*/IRIV. Control mice (group E) were not treated. Each group of mice was inoculated four times, 3 wk apart. One week after the last boost, five mice of each group were challenged s.c. in the right flank with P815 stably transfected with *CEA* (clone 13) (2.5×10^6) tumor cells. Mice were examined daily, and 50 days later, they were sacrificed. The lungs, spleen, and liver were harvested, processed, and stained for histological analysis. All animal experiments were conducted according to the UKCCCR Guidelines (21).

Detection of *CEA* Ab response

Serum samples were collected from BALB/c mice before and 10 days after the last immunization and analyzed by ELISA. Briefly, 96-well microtiter plates were coated with 1 μ g/ml purified human *CEA* (Chemicon International) at 4°C overnight. Wells were washed with PBS-0.05% Brij 35 and blocked for preventing nonspecific binding by incubation with 5% heat inactivated FCS in PBS-Brij 35 for 2 h at room temperature. A 100- μ l aliquot of samples (diluted 1/40) were allowed to react for 1 h at 37°C. The plate was then washed, and 100 μ l of goat HRP-labeled anti-mouse IgG (γ) (1/30,000) (Bio-Rad) or IgG2a antiserum (1/1,000) (BD Biosciences) were added in duplicate, and the plate was incubated for 1 h at 37°C. A mouse anti-human *CEA* mAb, COL-1 (Zymed) was used as positive control (1:200). After washing, the substrate 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich) was added and allowed to react at room temperature for 30 min, and the reaction was stopped with 100 μ l of 0.5 N H₂SO₄. Colorimetric conversion for the substrate was measured in a microplate spectrophotometer at 450 nm (Molecular Devices). Positive sera were considered those showing an OD at least twice the value of the background represented by a pool of negative control sera. The concentration of *CEA*-specific Ig2a isotype was calculated against a standard curve of mouse Ig2a (Cappel/Organon Technica Corporation) and expressed as nanograms per milliliter.

CEA mRNA transcription in vivo

Lymph nodes were collected from mice 24, 48, and 72 h after administration of IRIV/*CEA* or PBS and were subjected to RNA extraction by using a Total RNA Isolation kit (Promega). Total RNA was amplified by RT-PCR; the reaction was performed at 37°C for 30 min, followed by 35 cycles (94°C for 1 min, 52°C for 30 s, and 72°C for 40 s). Sequences of the primers used to amplify a 499-bp fragment of *CEA* gene were forward primer, 5'-ATGGCAGAGCCACCCAAA-3' (nt 1054–1068), and reverse primer, 5'-CACTGGCTGAGTTATTGGCC-3' (nt 1529–1549). The amplified product was then subjected to a seminested PCR using the primers *CEA*, forward and reverse, 5'-TTCAGGATGACTGGGTCGCT-3' (nt 1306–1325) for 40 amplification cycles (94°C for 30 s, 58°C for 30 s, and 72°C for 40 s) to provide a 275-bp fragment. The products were subsequently gel purified and sequenced for confirmation. GAPDH served as an internal control for human cells and was amplified by using GAPDH forward primer, 5'-ATGGGGAAGGTGAAGTCCGG-3', and reverse primer, 5'-TTCTCCATGGTGGTGA-3'; mouse β -globin served as an internal control for murine cells using specific primers (Stratagene).

Quantitative analysis by real-time PCR

DNA was extracted from splenocytes by using the Qiamp DNA kit (Qiagen) according to the manufacturer's recommendations. One microgram of DNA was subjected to real-time PCR. Primer-probe combinations were designed using Primer Express software (Applied Biosystems).

The upstream and downstream primers were as follows: 5'-CGACTCGTCTTACCTTTCGGGA-3' and 5'-GTTCGACCTATATCAGAGCAACCCAA-3', respectively. The fluorogenic probe sequence was 5'-GTCAAGATCACAGTCTCTGCA-3'. PCR was performed by using the TaqMan Core reagent kit with AmpliTaq Gold (Applied Biosystems). Briefly, following the activation of AmpliTaq Gold at 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C were conducted in an ABI Prism 7700. Each sample was analyzed in triplicate. At least three negative water blanks were included in each test. Calibration curves were run in parallel, using a recombinant plasmid dilution series containing the *CEA* gene, ranging from 10 to 10⁸ copies per reaction. The threshold cycle values from the samples were plotted on the standard curve, and the copy number was calculated automatically by Sequence Detector, version 1.6 (Applied Biosystems), a software package for data analysis. The final results were expressed as copies of *CEA* gene per microgram of DNA. The detection limit was ~10 copies per microgram of DNA.

Cytokine assay

Splenocytes were drawn from immunized mice, and lymphocytes were collected by Ficoll-Hypaque (Pharmacia) gradient. Approximately 100 μ l of 2×10^6 unfractionated cells per milliliter in a complete RPMI 1640 plus 10% FCS were cultured in a total volume of 200 μ l with 10 μ g/ml *CEA* (Chemicon International) or Con A (Sigma-Aldrich) (2.5 μ g/ml) in a 96-well flat-bottom plate. Control wells received cell suspension only. Cell-free supernatants were harvested for the presence of IFN- γ , after 48 h. Samples were stored at -80°C. Measurements of cytokines were assessed by specific ELISA, using a solid-phase sandwich test (Pierce Biotechnology). The concentration of cytokines in samples was determined according to the standard curve.

Flow-cytometric analysis

Mice from each vaccination group were euthanized 7 days after the last boost, and cell suspension of spleens from each group was pooled. Splenocytes (10^6) were stained with FITC-conjugated (I-A^d) anti-class II and PE-conjugated Abs to B7.1 or B7.2 (BD Pharmingen) and analyzed on a FACScan flow cytometer (BD Biosciences). Cells displaying typical lymphocyte and macrophage scatter were gated, and two-color dot plots were generated using CellQuest software.

Cytotoxicity assay

Target cells, represented by *CEA* (clone 13)-transfected P815 cells, were labeled with 100 μ Ci of Na₂⁵¹CrO₄ (Amersham Biosciences) for 60 min at room temperature. Target cells (0.5×10^4) in 100 μ l of complete medium (see below) were added to each of the wells in 96-well flat-bottom assay plates (Corning Costar). The labeled targets were incubated at 37°C in 5% CO₂ before the addition of effector cells. The T cells were then suspended in 100 μ l of AIM-V medium (Invitrogen Life Technologies) and added to the target cells. The plates were incubated at 37°C for 6 h, and the supernatants were harvested for gamma counting with harvester frames (Skatron). Uninfected P815 target cells were used as control cells, whereas MHC class I CTL cytotoxic restriction was tested against P815 target cells (clone 13), as described above, and exposed to anti-MHC class I mAb

(H-2K^d/H-2D^d; BD Pharmingen) before the assay. The determinations were made in triplicate, and SDs were calculated. All of the experiments were repeated at least three times.

Specific lysis was calculated as follows: percentage of specific lysis = ((observed release (cpm) – spontaneous release (cpm))/(total release (cpm) – spontaneous release (cpm)) × 100).

Spontaneous release was determined from the wells to which 100 μ l of complete medium had been added instead of effector cells. Total releasable radioactivity was obtained after treating the target with 2.5% Triton X-100.

Histological analysis

The lungs, liver, and spleen of each mouse were fixed in 4% buffered formalin for 24 h, sectioned, and entirely embedded in paraffin. Four-micrometer-thick sections were cut from tissue blocks and stained with H&E.

For immunohistochemical staining, the paraffin-embedded blocks were cut at 3 μ m, deparaffinized, and rehydrated. Immunohistochemistry was performed by means of the standard avidin-biotin complex (ABC)-peroxidase method (LAB Vision) and 3,3'-diaminobenzidine as chromogen. The following Abs were used: CEA (clone COL-1; 1:200; NeoMarkers) and mast cell tryptase (clone A1; 1:800; NeoMarkers). Microwave pretreatment was performed for mast cell tryptase by heating the deparaffinized and rehydrated sections immersed in 10 mM sodium citrate buffer (pH 6.0), in a microwave oven at 750 W for 5 min, three times.

Statistics

The mean differences were statistically analyzed using StatView statistical software (Abacus Concepts). The results were expressed as the mean \pm SD of three determinations made in three different experiments, and differences were determined using Bonferroni's (all-pairwise) multiple comparison test. A value of $\alpha = 0.05$ was considered statistically significant.

Results

Construction and characterization of CEA and CD40L IRIV

CEA and CD40L plasmids were synthesized as described in *Materials and Methods*, and analyzed by restriction analysis and DNA sequencing. We cloned only a fragment of the CEA sequence (accession no. M17303) encompassing nt 1054–2205 to avoid a possible cross-reaction with the nonspecific cross-reacting Ag, a member of the CEA Ag gene family, which shares its high level of homology (42%) in the upstream sequence (22).

This CEA amino acid sequence was also chosen, because it contained a large number of epitopes potentially able to bind the most common murine and human class I and class II MHC haplotypes according to Parker's and Remmensee's algorithms (23). The ability of IRIV to achieve APC *in vivo* has already been shown in previous studies (24–26). Both CEA and murine CD40L genes were expressed in transfected cells, as shown by cytofluorometric analysis (Table I). The ability of CEA/IRIV and CD40L/IRIV to deliver the respective genes *in vitro* was demonstrated on several target cells including murine spleen cells, human PBMC, human and murine DCs, and P815 cell lines by means of cytofluorometric analysis performed 24, 48, 72, and 96 h after transduction (data not shown). CEA *in vivo* expression was indirectly confirmed by the presence of anti-CEA Ab response in mice immunized with CEA/IRIV (Fig. 1), and it was supported by the presence of the specific mRNA in the lymph nodes of mice col-

Table I. Expression of CEA and/or CD40L molecules in transfected Vero cells

Transfection of Vero Cells	% of CEA-Expressing Cells \pm SD	% of CD40L-Expressing Cells \pm SD
IRIV	2.6 \pm 0.5	3.1 \pm 1.5
CD40L/IRIV	2.8 \pm 1	27.2 \pm 3.4
CEA/IRIV	35.5 \pm 5.4	2.4 \pm 1.9
CEA/IRIV + CD40L/IRIV	36.4 \pm 4.1	29.5 \pm 2.8

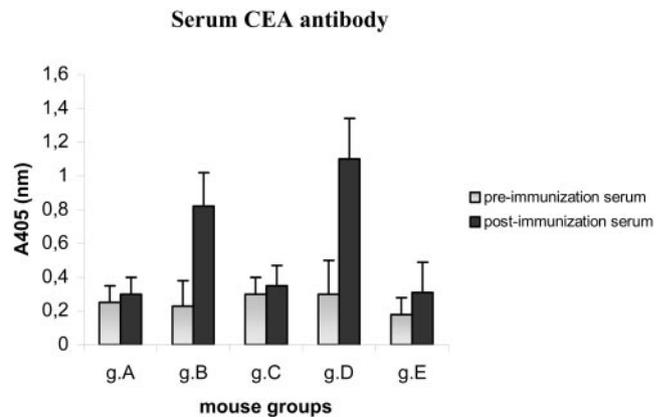


FIGURE 1. Representative experiment in which groups of mice received *i.n.* immunization as described in *Materials and Methods*. The relative anti-CEA Ab were determined by ELISA. The height of each bar depicts the mean OD of sera at a 1/40 dilution of each group \pm SD.

lected 24 and 48 h after *i.n.* administration of CEA/IRIV (Fig. 2). However, it was not possible to detect CEA transcripts 72 h after the mice inoculation.

Induction of protective immunity in mice receiving simultaneous administration of CEA/IRIV and CD40L/IRIV

We designed a cancer model by inoculating BALB/c mice with the autologous mastocytoma P815 cell line expressing CEA. The tumor cells were stably transfected with CEA and then cloned by limiting dilution. The clone expressing the highest percentage of CEA-positive subsets (clone no. 13; 42%) was expanded and used in this model. CEA expression on the membrane of these cells was evaluated by cytofluorometric analysis before inoculation in the mice (data not shown).

We first evaluated whether this new delivery construct containing CD40L (CD40L/IRIV) was able to improve the protective activity of a CEA-directed cancer vaccine (CEA/IRIV) in mice *s.c.* challenged with the above-described CEA-expressing P815 cells (clone no. 13). A previous study revealed that this particular clone, once *s.c.* injected in BALB/c mice, rapidly gave rise to distant metastases with spleen involvement and did not result in solid tumor formation at the site of the inoculum. Thus, all animals were sacrificed 50 days after challenge to carry out an immunological

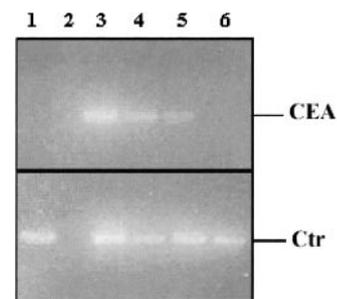


FIGURE 2. Detection of CEA expression by RT-PCR. The presence of CEA and housekeeping genes (Ctr: GAPDH for human cells and β -globin for murine cells) were determined using equivalent amounts of RNA isolated from 1×10^6 mononuclear cells of mouse draining lymph nodes 24 h after IRIV (lane 1) *i.n.* inoculation, or 24, (lane 4), 48 (lane 5), and 72 h (lane 6) after CEA/IRIV *i.n.* inoculation and from human Colo 205 cell line (lane 3), respectively. An additional control was represented by negative PCR control for RNA isolation (no template) (lane 2).

study and a histological analysis of the organs that could have developed tumor cells (lung, liver, bone marrow, and spleen).

Immunohistochemistry analysis revealed the presence of many CEA-positive cells in the spleens of mice immunized with pVAX/IRIV (group A), CEA/IRIV (group B), CD40L/IRIV (group C), and control mice (group E). No CEA-expressing cells, but only residual necrotic cells, were conversely detected in the spleens of mice that had received i.n. administration of either CEA/IRIV and CD40L/IRIV (group D) (Fig. 3). These results were confirmed in additional experiments where the copy number of the CEA recombinant plasmids (present only in the injected tumor cells) were evaluated in the metastasized spleen tissue of these mice. The lowest detectable number of CEA plasmid copies/microgram of DNA (mean \pm SD, 33 ± 11) was, in fact, found in mice immunized with CEA/IRIV together with CD40L/IRIV (group D), suggesting the occurrence of an efficient CTL-mediated destruction of CEA-positive tumor cells in the spleen tissue. Further results also demonstrated that this increased protective activity occurred only when CEA/IRIV and CD40L/IRIV were administered together. When one of the two constructs was administered 48–72 h before the other, the result was similar to that obtained with the CEA/IRIV alone (data not shown).

CEA-specific CTL response in immunized mice

We also evaluated whether the CD40L/IRIV construct was able to improve the ability of a CEA-directed cancer vaccine (CEA/IRIV) in inducing a CEA-specific CTL response. Spleens were taken from the mice vaccinated as described above, and spleen cells were isolated and pooled from the different mouse groups.

Pooled spleen cells were then *in vitro* stimulated with autologous irradiated splenocytes transfected with CEA gene and cultured for 10 days in a medium containing low-dose IL-2 before being tested in ^{51}Cr release assay against the P815 target cell clone expressing CEA. These experiments showed that the spleen cells from the mice that had received treatment with CEA/IRIV showed a significant CEA-specific antitumor activity. Our results also showed that the spleen cells from the mice that had received the combined treatment with CEA/IRIV and CD40L/IRIV elicited much higher lytic activity. Significantly different values (Bonfer-

roni's (with control) multiple-comparison test, $\alpha = 0.05$) were observed by comparing the results of CTL activity of mice immunized with CEA/IRIV (group B) and CEA/IRIV plus CD40L/IRIV (group D) against CEA-expressing cells vs the remaining groups. The lytic activity of these CTLs was CEA specific and class I MHC restricted, because they were not able to kill untransfected (not expressing CEA) P815 target cells and because their lytic activity was abrogated by the addition of anti-class I MHC mAbs in the cytotoxic assay (Fig. 4). The administration of IRIVs or CD40L/IRIV did not elicit any CEA-specific CTL response, and spleen cells from mice vaccinated with these constructs only gave rise to unspecific lytic activity that was not restricted by class I MHC molecules.

The CEA-CD40L combination enhances the expression of costimulatory molecules B7-1,2

CD40L binding to CD40 (receptor) activates intracellular signaling in the APCs and DCs that improves their presenting ability and leads to the up-regulation of the other adhesion/costimulatory molecules (27) on their surface. These latter molecules can provide the second signal required to activate naive T cells, amplify the immune response, and prevent anergy or tolerance induction. Therefore, we investigated whether our CD40L/IRIV construct could enhance, through CD40/CD40L interaction, the immune response against CEA, triggered by the CEA/IRIV vaccination. FACS analysis was performed from spleen cells isolated from the immunized mice administered with different IRIV constructs, and analyzed for the expression of B7.1 and B7.2 on class II MHC⁺ cells. The results of these experiments revealed a greater up-regulation of the B7.1 and, particularly, B7.2 molecules in mice receiving CD40L/IRIV and CEA/IRIV together (group D) ($\alpha = 0.05$) (Fig. 5). Moreover, besides the higher percentage of positive cells, a higher density of B7.1 and B7.2 molecules was expressed on the cells of group D mice. IRIVs alone were able to up-regulate the expression of B7.1,2 and MHC class II molecules on APCs, as described elsewhere (28), but immunization with this combination of two plasmids resulted in a much greater increase in B7 expression. On the contrary, the administration of CEA/IRIV (without the CD40L gene) to mice did not modify the level of B7 expression. These

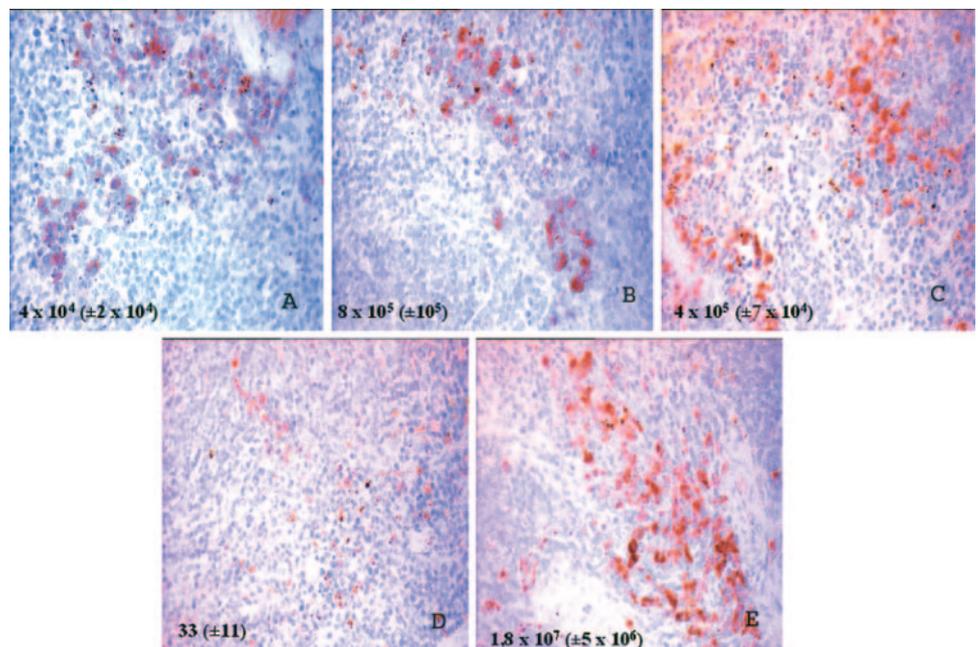
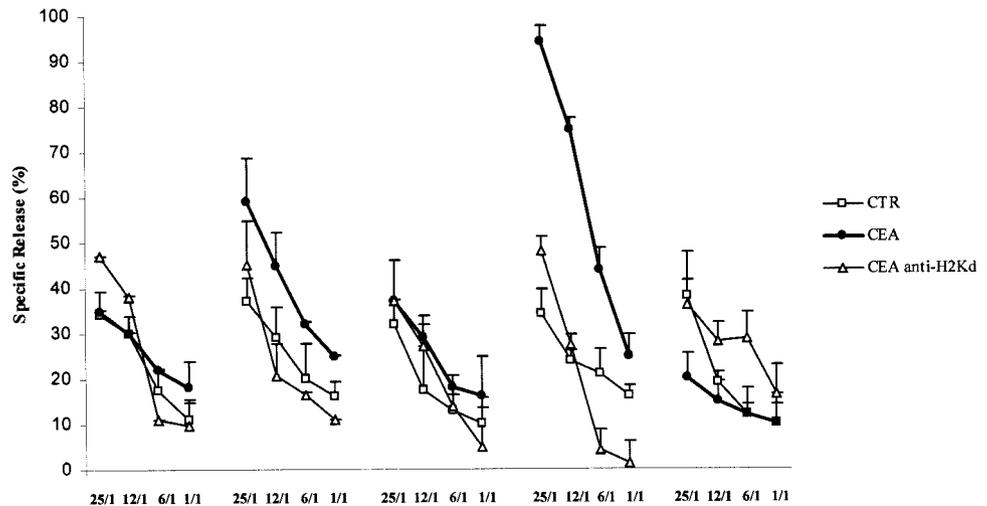


FIGURE 3. Immunohistochemical staining of splenic tissue, using anti-CEA mAb, from mice i.n. inoculated with IRIV (A), CEA/IRIV (B), CD40L (C), CEA/IRIV plus CD40L (D), and PBS (E). CEA immunohistochemistry exhibits tumoral positive cell clusters in the red pulp in mice of group A, B, C, and E; only residual necrotic cells are evident in mice of group D. On the bottom of each picture, the mean copy number of CEA plasmid/microgram of DNA (\pm SD) extracted from splenocytes is shown. Original magnification, $\times 400$.

Cytolytic activity of Spleen cells from immunized mice

FIGURE 4. Cytotoxic activity from splenocytes cultured for 5 days at a density of 2×10^6 cells/ml with $1 \mu\text{g/ml}$ CEA. Target cells, a P815 cell clone expressing CEA, were mixed with effector cells for 6 h at 37°C at 25:1, 12.5:1, 6.25:1, 1:1 E:T ratio. A 6-h ^{51}Cr release assay was performed; results are presented as specific lysis. The data shown represent values averaged from five pooled mice of each group of immunized mice (A–E), with SEM for each E:T ratio. Blocking of cytotoxicity was performed in the presence of $50 \mu\text{l/ml}$ anti-MHC class I Ab (H-2K^d/H-2D^d) in the same groups of mice.



data suggest that CD40L/IRIV enhances the immunogenic potential of pCEA/IRIV by up-regulating the costimulatory molecules on APC and DCs, determining increased Ag-processing and -presenting capability of these cells.

CEA/IRIV and CD40L/IRIV induce the production of Th1 cytokines

Finally, we evaluated whether our treatments were also able to produce proinflammatory Th1 cytokines, such as IFN- γ . We examined the ability of the cultured spleen cells, pooled from the different groups of mice, to produce IFN- γ in response to CEA by a solid-phase sandwich ELISA. Interestingly, we found high levels of this cytokine only in the mice that had been immunized with the combination of CD40L/IRIV-CEA/IRIV (group D) (0.883 ± 0.3 ng/ml) (Fig. 6). The spleen cells from the other mouse groups, including those vaccinated with CEA/IRIV alone, did not produce a detectable level of IFN- γ in response to CEA. No significant differences in IFN- γ production were observed when spleen cells of the different groups were stimulated with Con A, indicating that CEA specifically induced the expression of IFN- γ . These data strongly suggest that the addition of CD40L/IRIV to CEA/IRIV promotes the differentiation of lymphocytes toward a protective Th1 phenotype. The result was further supported by an increase of CEA-specific IgG2a Ab titer in the group of mice immunized with CD40L/IRIV-CEA/IRIV (40 ± 2.32 ng/ml) in comparison with the titer of mice immunized with CEA/IRIV (15 ± 3.64 ng/ml) ($\alpha = 0.05$).

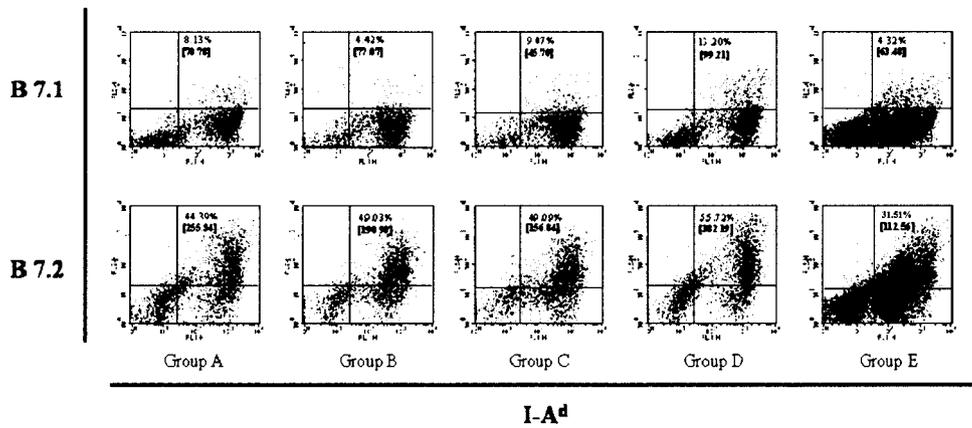
Discussion

The active specific immunotherapy targeted against cancer is aimed at mobilizing the immune system and destroying tumor cells (6, 29). Many tumor-associated Ags have been selected as possible targets for therapeutic cancer vaccines (3, 30), and the possibility of generating a CTL-mediated immune response directed at them, such as CEA, has been demonstrated through many different strategies (18, 31–35). Several hypotheses have been formulated to explain these deluding results; some are related to the lack of induction of an efficient immune response and some to mechanisms of escape, activated by tumor cells (36–39). The majority of these Ags are expressed at a low level in normal cells, but their immunogenicity is too weak to give rise to an efficient immune response. Thus, cancer cells can activate alternative escape mechanisms that

make them resistant to the cancer vaccine activated immune effectors. In this context, therapeutic immunization can also be hampered by inadequate activation or by a low number of professional APCs, as often observed in cancer patients and chronic diseases (40, 41).

We hypothesized that the immunogenic and therapeutic potential of Ag-directed vaccination could be significantly enhanced by the contemporary administration of agents that might increase the expression of coaccessory molecules on APCs and direct the activation and expansion of specific T cells. We believe that the CD40L molecule can modulate the immune response against human malignancies. In fact, it is known that CD40L retains a central role in initiating the immune response, although it is expressed transiently on a small proportion of cells, and it can produce long-lasting systemic immune responses, capable of blocking disease progression. CD40L, interacting with its contrareceptor (CD40), enhances the Ag-specific T cell growth by two distinct mechanisms: 1) it activates cultured DCs, which consequently express a higher amount of class I and II MHC and costimulatory molecules, and 2) it has direct stimulatory effects (coaccessory signal) on T cells. On this basis, our aim was to study the possibility of enhancing antitumor protective immunity in BALB/c mice by in vivo modulating the immune response with CD40L. In our model, we chose human CEA as the target Ag, because it has been widely tested (31–35), and because currently several clinical investigations are using a variety of different CEA-directed vaccine approaches. In the present study, we designed a DNA-based vaccine that is the CEA recombinant plasmid included in IRIV. This vaccine construct (CEA/IRIV) can be safely administered i.n. to mice, giving rise to an efficient CTL-mediated immune response. We also administered IRIV containing a plasmid expressing the murine CD40L, to improve the functional activity of the professional APC. Previous studies have already shown that IRIV are rapidly and efficiently taken up by many human and murine APCs, including DCs (24, 25), that they significantly up-regulate the expression of DCs maturation markers, such as MHC class I and II, ICAM-1, B7.1, B7.2, and CD40, and they are able to deliver DNA into these cells, which is then rapidly expressed. The results of this study support the hypothesis that the enhanced expression of CD40L on APCs definitely improves the protective antitumor activity of the CEA/IRIV vaccine with a mechanism that could most likely be related to a DC maturation process, due to the CD40L/

FIGURE 5. Expression of B7.1,2 costimulatory molecules on APC. Splenocytes of groups of mice i.n. inoculated with IRIV (A), CEA/IRIV (B), CD40L (C), CEA/IRIV plus CD40L (D), and PBS (E). A–E were stained with FITC-conjugated (I-A^d) anti-class II and PE-conjugated Abs to B7.1 or B7.2 and analyzed. In parentheses, the geometric mean fluorescence intensity value of B7.1,2 molecules.



CD40 interaction. CD40 activation on these cells might abolish their tolerogenic capacity or even trigger the potential for immunogenic presentation of the Ag (42, 43). In fact, we found a significant CEA-specific CD8⁺ T cell response in the mice coinoculated with CEA/IRIV and CD40L/IRIV, that achieved the best protective immunity against CEA⁺ P815 cell challenge. The splenocytes of these mice also produced high levels of IFN- γ in response to CEA exposure, suggesting the occurrence of a Th1 response, which could significantly improve the level and the efficiency of the immune response. Although a CEA-specific CD8⁺ T response was also detected in mice inoculated with CEA/IRIV alone, it was much less efficient in terms of cytotoxic activity and protective immunity. Moreover, the splenocytes of these mice did not produce IFN- γ in response to CEA exposure. As expected, no CEA-specific CTL activity or protective immunity was observed in mice administered with CD40L/IRIV or pVax/IRIV constructs. The histology and the molecular analysis of the tissues drawn from the animals administered with the combination of CD40L/IRIV-CEA/IRIV revealed the depletion of all cells expressing CEA, whereas the groups of mice immunized with CEA/IRIV, CD40L/IRIV, or pVax/IRIV developed much larger tumor overexpressing CEA molecules. This result appears most likely due to the immunomodulating activity of CD40L associated with CEA. Furthermore, CD40 signaling, necessary for induction of Th-dependent T cell responses induced the expression of IFN- γ when splenocytes of the immunized mice were pulsed with CEA, providing evidence of an Ag-specific T cell proliferation (44, 45). In fact, the CD40L-CD40 interaction controls the balance between helper and regulatory T cells in immune response, releasing immature DCs from the control of regulatory CD4⁺CD25⁺ T cells (46) and breaking the

immune tolerance against Ags. The increase of B7.1,2 expression on APCs of mice coimmunized with CEA/IRIV-CD40L/IRIV indicates that these cells have primed CEA-specific CD8⁺ T cells providing the secondary signals necessary to activate naive T cells and enhance the immune response against the tumor by these costimulatory molecules. In conclusion, we demonstrated that the i.n. administration of CD40L/IRIV in mice concomitantly with CEA/IRIV was able to induce an efficient tumor-protective immunity against CEA. CD40L/IRIV, by acting through the CD40L/CD40 signaling pathway, is a powerful immune adjuvant that was able to increase the efficacy of a vaccine specific for a poor immunogenic Ag, that could be an efficacious strategy for cancer therapy.

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Disclosures

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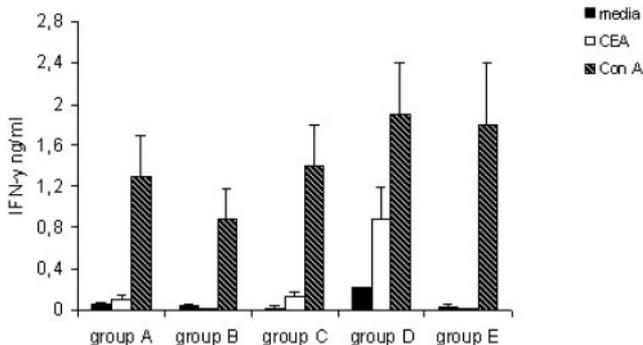


FIGURE 6. IFN- γ production by splenocytes of immunized mice (groups A–E) after stimulation with CEA (10 μ g/ml), Con A (2.5 μ g/ml), or nothing (medium). Data shown represent values average from five pooled mice.

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