Effective Genetic Vaccination with a Widely Shared Endogenous Retroviral Tumor Antigen Requires CD40 Stimulation during Tumor Rejection Phase[1]

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Endogenous retrovirus (ERV) products are recognized by T lymphocytes in mice and humans. As these Ags are preferentially expressed by neoplastic tissues, they might represent an ideal target for active immunization by genetic vaccination. However, i.m. inoculation of plasmid DNA encoding mouse gp70 or p15E, two products of the *env* gene of an endogenous murine leukemia virus, elicited a weak Ag-specific T lymphocyte response and resulted in partial protection from challenge with mouse tumors possessing these Ags. Depletion experiments showed that CD8$^+$, but not CD4$^+$, T lymphocytes were crucial for the antitumor activity of the vaccines. Systemic administration of agonistic anti-CD40 mAb increased the therapeutic potential of genetic vaccination, but only when given during the tumor rejection phase and not at the time of immunization. This effect correlated with a dramatic increase in the number of ERV-specific CD8$^+$ T lymphocytes. Adjuvant activity of CD40 agonists thus seems to be relevant to enhance the CD8$^+$ T cell-dependent response in tumor-bearing hosts, suggesting that sustaining tumor-specific T lymphocyte survival in subjects undergoing vaccination might be a key event in the successful vaccination with weak tumor Ags. *The Journal of Immunology*, 2003, 171: 6396–6405.

Endogenous retroviruses (ERV)[3] exist as proviruses in the host's germline DNA and are transmitted from parent to offspring. These sequences represent ~1% of the human genome and derive from ancestral encounters with retroviruses (1, 2). During evolution, mutation accrual has rendered them defective in the capability to generate complete infectious virions. The relationship between ERV and the development of human tumors or autoimmune diseases has been extensively investigated, but the field remains rather controversial (3). Nonetheless, these sequences may hold some relevance for tumor therapy. Members of the human and mouse endogenous retrovirus (HERV and MERV, respectively) families, in fact, are preferentially expressed in fresh tumors and cell lines rather than in normal tissues (4–7), thus representing a class of potential tumor-associated Ags (TAA). Although many ERV sequences are unable to give rise to complete proteins, processing of MERV and HERV partial products can still originate antigenic peptides, which can be exploited for the active immunotherapy of cancer. Their relevance as TAA has been confirmed by several studies. The main antigenic activity of a mouse thymoma was encoded by the *env* gene of the endogenous mammary tumor virus (8). The epitope AH1 from a colon carcinoma (CT26), chemically induced in BALB/c mice, is derived from gp70, one of the *env* gene products of endogenous murine leukemia virus, and adoptively transferred T cells specific for gp70 could treat mice bearing the CT26 tumor (6). MC38 and B16 tumor cell lines derived from C57BL/6 mice were also shown to express *env* transcripts and CTLs specific for the transmembrane p15E (the other major *env* gene product) were detected in tumor-infiltrating lymphocyte cultures; moreover, p15E-specific CTL could be generated from cultures of naive splenocytes by in vitro peptide stimulation and recognized various p15E-positive tumors in a K^b-restricted fashion (9, 10). Recently, an epitope encoded by a short open reading frame in the *env* sequence of a spliced HERV-K-related transcript was demonstrated to be recognized by melanoma-specific CTL in melanoma patients (11).

Various evidence indicates that the *env* gene products are not good immunogens, a likely consequence of their self-antigenic nature. Mice immunized with a modified AH1 peptide of gp70 could be protected from subsequent tumor challenge (12). The amino acid substitution in the adapted epitope resulted in greater binding of the peptide-L^d class I complex to the TCR of the T lymphocytes and was required to induce a strong immune response in vaccinated mice, suggesting that the T lymphocyte repertoire in normal mice might comprise only low affinity TCR to the natural ligand. To break tolerance against this weak Ag and elicit an effective active immunization against gp70, a few other strategies have been successfully exploited: the combination of the AH1 peptide with a putative epitope of gp70 recognized by CD4$^+$ T lymphocytes, the expression of the AH1 epitope in recombinant vaccinia virus (rVV), and pulsing of in vitro preactivated dendritic cells (DC) with the unmodified AH1 peptide (13, 14). All these results point to a relevant requirement for CD4$^+$ Th lymphocytes and professional APCs to elicit, and possibly maintain, an immune reactivity to gp70/p15E epitopes.

A circuit that powerfully modulates APC and Th functions involves CD40 and its ligand, CD40-L (CD154), a member of the TNF family that is biologically active as a homotrimer. These molecules exert pleiotropic activities, such as B cell activation, production of Th1cytokines by Th cells, and generation of effector/ memory CTL. CD40 is expressed on B lymphocytes, DC, activated macrophages, follicular DC, and endothelial cells, whereas CD154 is mainly present on activated CD4^{+} T lymphocytes (15). The activation status of APC-presenting self Ags can determine the choice between tolerance and autoimmunity, because resting APC induce tolerance, whereas APC activated by Th lymphocytes unleash autoimmune responses (16). By activating professional APC, CD40-stimulating agents were shown to bypass the CD4^{+} T cell requirement and convert the CD8^{+} T cell response to tumor Ags from tolerization to full activation, thus unmasking their antitumor activity against established tumors (17, 18). The adjuvant activity of CD40 to the antitumor response was also established in genetic vaccination, because mixtures of plasmids coding for CD40L trimer and a model tumor Ag could significantly increase antitumor efficacy (19). However, some cautions in the use of CD40-activating agonists were raised by recent observations of the opposing effects elicited by CD40 stimulation in vivo on the survival and function of tumor-specific CD8^{+} T cells. In a mouse melanoma model, in fact, anti-CD40 Ab treatment in tumor-bearing mice accelerated the deletion of tumor Ag-specific T cells (20).

In the present manuscript we exploited genetic vaccination against MERV as a tool to induce antitumor immunity against various tumors in H-2[b] and H-2[d] haplotype mice. Effective vaccination required the construction of DNA vaccines optimized to attain the best Ag presentation in vivo. In both mouse strains, however, genetic vaccination resulted in a weak response that could be enhanced by the use of CD40 agonists. The timing of CD40 activation was crucial because it was necessary during the rejection of the tumor, whereas it could even be deleterious when given during priming of the immune response.

Materials and Methods

Mice and cell lines

C57BL/6 (H-2[b]) and BALB/c (H-2[d]) mice (8-wk old) were purchased from Charles River (Calco, Como, Italy). Animal care and procedures conformed to institutional guidelines in compliance with national and international regulations. Mice used for the in vivo tumor growth experiments were examined every other day and were euthanized when the tumor became ulcerated or one of two perpendicular diameters reached 10 mm and their product was >50 mm^2. CT26, a BALB/c carcinogen-induced, undifferentiated colon carcinoma, was previously described (21). The 293-L[d] cell line is a human embryonic kidney cell line stably transfected with pL[d].444 plasmid expressing the H-2 L[d] class-I molecule (provided by Dr. U. D'Oro, National Institutes of Health, Bethesda, MD). B16 LU8, a metastasizing melanoma cell line of C57BL/6 (H-2[b]) and the 293-K[b] cell line were provided by Dr. J. C. Yang (Surgery Branch, National Institutes of Health). E88 and T4905 CTL clones recognize the L[d]-restricted epitope of gp70 (AH1) and the K[b]-restricted epitope of p15E, respectively, and were provided by Dr. M. Colombo (Istituto Nazionale Tumori, Milan, Italy). Cell lines were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 2 mM L-glutamine, 10 mM HEPES, 20 μM 2-ME, 150 U/ml streptomycin, 200 U/ml penicillin, and 10% heat-inactivated FBS (Invitrogen).

Plasmid construction

The pcDL-Srd296-H52 (7) was a gift from Kanno (Center for Biomedical Science School of Medicine, Chiba University Japan). The *env* insert has been excised with *Acc*I, subcloned in pBluescript, and then cloned in pcDNA3 (Invitrogen) by the use of *Eco*RI and *Xho*I to produce the plasmid pcDNA3-Env. Functionality was checked by transient transfection, immunofluorescence and ELISA (data not shown). VR1055 (Vical, San Diego, CA) has been optimized for rapid cloning and in vivo expression as fol-

lows: a fragment between the *Eco*RI sites in pCR2.1 (Invitrogen) was excised with *Not*I and *Bam*HI and cloned in VR1055. The nonrelevant gene contained in the sequence was then excised with *Eco*RI, and the plasmid was purified by agarose gel extraction and self-ligated. To prepare the VR1055-P15 plasmid, two primers were designed to amplify by PCR the p15 region of *Env* gene (GenBank accession no. D10059): P15E-F (5′-GCG GAA TTC CAC CAT GGA CCC CGT CTC ACT AAC TCT GG-3′) with a restriction site for *Eco*RI, the Kozak sequence, and an ATG start codon in-frame; and P15E-R (5′-GCG CGA ATT CCC CCC TCT TTC TGT AAA CTG-3′) with a restriction site for *Eco*RI. Twenty nanograms of pcDNA3-Env was mixed with P15F and P15R primers (1 μM each), dNTPs (200 μM), buffer (1×), and 1 μl of Pfu-turbo (Stratagene, La Jolla, CA); after 5 min of initial denaturation, five cycles (95°C for 30 s, 57°C for 45 s, 72°C for 60 s) were followed by 25 cycles (95°C for 30 s, 62°C for 30 s, 72°C for 60 s) performed in a PTC-200 Peltier thermal cycler (MJ Research, Waltham, MA). Twenty microliters of the reaction was excised with *Eco*RI, purified by agarose gel, and cloned into VR1055. The correct orientation was checked by restriction analysis. For the VR1055-gp70 plasmid, two primers were designed to amplify by PCR the GP70 region of the *env* gene: GP70-F (5′-GCG CGA ATT CCA CCA TGG ATA CAC GCC GCC CAC G-3′) containing a restriction site for *Eco*RI, the Kozak sequence, and a ATG start codon in-frame; and GP70-R (5′-GCT CTA GAG CTC ATT ATC TCT TTT ATA TTT GGC TCG-3′) containing a restriction site for *Xba*I and two stop codons in-frame. Plasmid amplification was performed with the Endofree Plasmid Mega kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Gp70 expression analysis

Total RNA was extracted by TRIzol (Invitrogen) from cultured cells; its concentration and quality were assessed by $A_{260/280}$ absorbance and electrophoretic analysis. Two micrograms was mixed with 200 U of Moloney murine leukemia virus reverse transcriptase, 500 ng of oligo(dT)$_{15–18}$ primers, 20 U of RNAseOUT, and 500 μM dNTPs (Invitrogen) and incubated at 42 °C for 1 h. One-tenth of the reaction volume was used as a template for PCR with the specific primers for gp70, as previously described (7). After 5 min of initial denaturation, PCR was performed using recombinant *Taq*-DNA polymerase (Invitrogen) in 30 amplification cycles (95°C for 30 s, 62°C for 30 s, 72°C for 60 s), with a final extension at 72 °C for 7 min in a PTC-200 Peltier thermal cycler (MJ Research). One-tenth of the reaction volume was run on a 1.5% agarose gel. As control for retrotranscription, PCR with specific primer for β-actin gene was always performed.

DNA immunization

DNA immunization was performed according to commonly used protocols available at the DNA vaccine web site (http://dnavaccine.com/Protocols/protocol.html). Briefly, BALB/c and C57BL/6 mice were anesthetized by ethyl ether inhalation and injected i.m. with 250 and 100 pM cardiotoxin (Latoxan, Rosans, France), respectively. Five days later, mice were injected i.m. with 100 μg of plasmid DNA in 100 μl of saline.

In vivo Ab treatment

Mice were depleted of either CD4^{+} or CD8^{+} T cells by four i.p. injections of 200 μg of either GK1.5 (anti-CD4) or 2.43 (anti-CD8) mAbs. Depleting mAb were given 2 days before and 0, 4, and 8 days following s.c. challenge with tumor cells. The mAbs were produced from hybridomas (obtained from American Type Culture Collection, Manassas, VA) grown in ascites and purified by ammonium sulfate precipitation, followed by protein G-Sepharose affinity chromatography (Amersham Pharmacia Biotech, Uppsala, Sweden). Depletion was monitored by cytofluorometry of peripheral lymphocytes isolated from mouse blood and stained with FITC- or PE-conjugated anti-CD4, anti-CD8 (clones RM4-5 and 53.6-7, respectively; all from BD PharMingen, San Diego, CA). Depletion was consistently >98%. Anti-CD40 mAb were derived from clone FGK45.5 (provided by A. Groenewegen, Basel Institute for Immunology, Basel, Switzerland) (22). Activating Ab anti-CD40 was administered at the dose of 200 μg on days 0, 1, 2, and 4 after DNA vaccination or tumor challenge. As control, the anti-*Escherichia coli* β-galactosidase (β-gal; GL111.41, rat IgG2a) was used at the same dose.

Tumor challenge

BALB/c mice were inoculated s.c. on the right flank with 2.5 × 10^4 CT26 cells. C57BL/6 mice were inoculated i.v. with 10^5 B16LU8 (hereafter referred to as B16) cells. Tumor growth was monitored every 3 days by caliper measurement. Lungs were removed 14 days after challenge, and pulmonary metastases were counted in a blind fashion.

Mixed leukocyte peptide cultures (MLPC)

Splenocytes (2.5×10^7) were restimulated in vitro with the appropriate gp70 peptides (1 μM) for 5 days in 10 ml of DMEM-10% FBS in 25-cm^2 tissue culture flasks (Falcon; BD Biosciences, Lincoln Park, NJ) at 37°C in 5% CO_2.

Synthetic peptides

The MHC class I L^d-restricted peptides corresponding to aa 423–431 of gp70 (SPSYVYHQF, AH1 peptide) were synthesized by automatic solid phase procedures. The synthesis was performed on a 431 A model (PE Applied Biosystems Foster City, CA) peptide synthesizer using F-moc chemistry according to our protocols (23). After cleavage with trifluoroacetic acid, the peptides were purified to homogeneity by RP-HPLC on a prepNova-Pak HR C$_{18}$ column (Waters, Milford, MA) with a linear gradient of 10–45% acetonitrile at 12 ml/min. The molecular weights of the peptides were confirmed by mass spectroscopy using a matrix-assisted, laser desorption ionization, time-of-flight (MALDI-Tof) spectrometer (Maldi-1; Kratos-Schimadzu, Manchester, U.K.). Peptides corresponding to the aa 876–884 of β-gal protein (TPHPARIGL, MHC class I L^d-restricted peptide) and MHC class I K^b-restricted peptides corresponding to aa 604–611 of p15E protein (KSPWFTTL, P15E peptide), and aa 96–103 of β-gal protein (DAPIYTNV) were synthesized and purified by Technogen (Naples, Italy). They were >95% pure, as indicated by analytical HPLC. Lyophilized peptides were dissolved in DMSO (Sigma-Aldrich, St. Louis, MO; stock solution at 10 mM) and stored at −80°C before use.

Synthesis of MHC/peptide tetrameric complexes (TET)

DNA coding for residues 1–280 of the soluble domain of L^d H chain molecule was amplified from the pL^d.444 plasmid by PCR with the 5′ primer 5′-GGA ATT CCA TAT GGG CCC ACA CTC GAT GCG G-3′ and the 3′ primer 5′-CGG GAT CCG GAC GGA GGA GGC TCC CA-3′. The resulting fragment was cut with restriction enzymes *Nde*I and *Bam*HI and cloned into the expression vector pET-23a$^+$ containing the BirA substrate peptide (BSP; gift from Dr. J. D. Altman, Emory Vaccine Center, Atlanta, GA), thus resulting in the plasmid pET-23a$^+$-L^d-BSP. The expression plasmids pET-23a$^+$-L^d-BSP and pHN1$^+$-β_2-microglobulin (gift from Dr. F. Marincola, National Institutes of Health) were used to transform the *E. coli* strain BL21 (DE3) pLys S.

Soluble H-2-peptide tetramers were produced using a previously described method (24). Briefly, recombinant K^b or L^d H chain, with a 15-aa substrate peptide for BirA-dependent biotinylation at its C terminus, and human β_2-microglobulin were produced as inclusion bodies in *E. coli* strain BL21(DE3)pLysS transformed with the expression plasmid pET 23a$^+$-Kb (gift from Dr. J. D. Altman, Emory Vaccine Center, Atlanta, GA) or pET-23a$^+$-L^d and pHN1$^+$-β_2-microglobulin. The inclusion bodies were purified and dissolved in urea denaturing buffer as previously described (25). Monomeric MHC-peptide complexes were formed by combining the K^b or L^d H chain, β_2-microglobulin, and the peptide in an arginine-folding buffer. The refolding reaction was dialyzed and concentrated on a Superdex 75 gel filtration column to purify the folded protein (Amersham Pharmacia Biotech). Soluble purified complexes were biotinylated using BirA enzyme (Avidity, Denver, CO). PE-labeled tetramers were produced by mixing the biotinylated complexes with Extravidin-PE (Sigma-Aldrich) and were validated by staining CTL clones with the appropriate specificity. Each tetramer batch was titrated and used at the optimum concentration (1–4 μg/ml) of K^b or L^d H chain.

Cell staining and flow cytometric analysis

Fresh or in vitro stimulated splenocytes (10^6/sample) were resuspended in 100 μl of FACS buffer (0.9% NaCl solution containing 2% BSA and 0.02% NaN$_3$; both from Sigma-Aldrich) with anti-mouse FcγR 2.4G2 mAb ascites (ATCC HB-197, American Type Culture Collection, Manassas, VA) for 10 min at room temperature to reduce the nonspecific staining. After washing, cells were resuspended in FACS buffer and labeled with either AH1 tetramer-PE (AH1-TET; 5 μg/ml) or p15E tetramer-PE (p15E-TET; 5 μg/ml) for 20 min at room temperature. Each sample was then stained at 4°C with rat anti-mouse CD8-Tri-color (0.1 μg/10^6 cells; clone CTCD8α; Caltag, Burlingame, CA) and with hamster anti-mouse CD3-FITC (1 μg/10^6 cells; clone 145-2C11; Caltag). Before analysis, cells were washed twice, resuspended in FACS buffer, and analyzed with a flow cytometer FACSCalibur (BD Biosciences, Mountain View, CA). Every sample was also stained with the control β-gal-TET according to the described protocols. Furthermore, untreated mice were used as negative controls for experimental samples. Data analysis was conducted using Cell Quest software (BD Biosciences).

ELISA

Splenocytes (10^5 cells) from MLPC were coincubated for 24 h with an equal number of target cells; the supernatant was then harvested and tested for released IFN-γ in a sandwich ELISA assay (Endogen, Boston, MA) following the manufacturer's instructions. Data were derived from triplicate wells.

Statistical analysis

The Wilcoxon-Mann-Whitney *U* test was used to examine the null hypothesis of rank identity between two sets of data. Kaplan-Maier plots and the Mantel-Haenszel test were used to compare survival of mice belonging to different treatment groups. All *p* values presented are two-sided.

Results

DNA immunization against MERV

We constructed the eukaryotic expression vector pcDNA3-Env, coding for the full-length gp70, to immunize against MERV. We injected BALB/c mice with the plasmid according to the protocol reported in *Materials and Methods* and evaluated whether immunization could protect from a lethal challenge with a gp70-positive tumor. Fig. 1*A* summarizes the results of six experiments in which treated mice (immunized with empty pcDNA3, pcDNA3-Env, or gamma-irradiated CT26 cells) or naive mice were injected with a lethal dose of CT26 cells. Although untreated mice died within 3 wk after challenge, complete protection against the lethal inoculum was achieved in mice vaccinated with the gamma-irradiated cell vaccine. Inoculation of the empty plasmid pcDNA3 did not affect overall survival, whereas immunization with *env*-encoding vaccine resulted in a significant prolongation of survival. Data evaluation yielded an overall rate of complete tumor prevention of ~24% in mice vaccinated with pcDNA3-Env (i.e., 14 of 58 mice were completely protected). We also observed an interexperiment variability that probably reflected the borderline efficacy of DNA vaccination against gp70 Ag (see Fig. 4).

To explore the role of T cells in the antitumor efficacy of genetic vaccination, mice that had been immunized with pcDNA3-Env received depleting doses of mAbs specific for either CD4$^+$ or CD8$^+$ T lymphocytes. The mAb administration schedule assured that the corresponding population was absent at the moment of tumor challenge and during the first 10 days of tumor growth, as assessed by cytofluorometry (data not shown). As shown in Fig. 1*B*, DNA immunization increased the survival of both normal mice and mice depleted of CD4$^+$ T lymphocytes, indicating that CD4$^+$ cells were not required to control CT26 tumor growth, at least in the effector phase. In contrast, mice depleted of CD8$^+$ T lymphocytes were no longer protected and succumbed to tumor challenge. Thus, CD8$^+$ T lymphocytes are critically required to reject CT26 colon carcinoma cells in immune mice.

DNA immunization against MERV can elicit an immune response against gp70 immunodominant epitope AH1

In a set of immunization experiments (described in Fig. 1), splenocytes from different groups of vaccinated mice were isolated 21 days after treatment and immediately tested to quantify AH1-specific T cells by staining with AH1-TET (Fig. 2, ex vivo). An aliquot of the same splenocyte preparation was stimulated in MLPC with the AH1 peptide; 5 days later, cells were stained with the same tetrameric reagents (Fig. 2, MLPC). Background staining with the irrelevant β-gal$_{876-884}$/L^d TET ranged from 0.05–0.14% of the CD3$^+$/CD8$^+$ T lymphocytes present in the spleen or in MLPC (not shown). Compared with the percentage of AH1-specifc T cells in untreated or mock-vaccinated mice, immunization with either pcDNA3-Env or gamma-irradiated CT26 cells did not result in a significant increase in AH1-specific T cells among freshly isolated splenocytes. However, AH1-TET$^+$ cells were
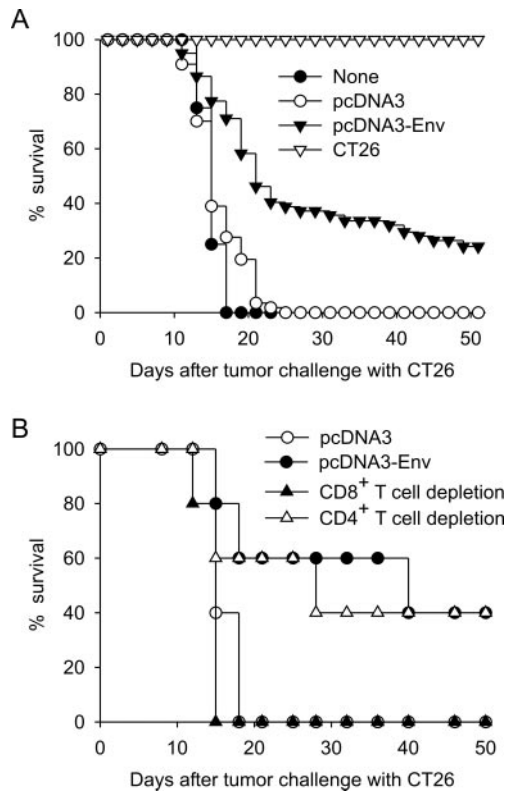
FIGURE 1. *A*, Immunization with gamma-irradiated CT26 or with a plasmid cDNA encoding *env* confers protection against a challenge with CT26 colon carcinoma cells. BALB/c mice were vaccinated either i.m. with 100 μg of naked pcDNA3-Env or s.c. with 1×10^6 gamma-irradiated CT26 cells. Control mice were either not treated or received the empty plasmid pcDNA3. Mice were challenged s.c. with 0.25×10^5 CT26 cells 21 days after vaccination. Euthanasia of mice bearing tumors >1 cm in the major diameter was used as the study end point. Data are cumulative from six different experiments. The survival rates of mice vaccinated with pcDNA3-Env and CT26 cells differ significantly ($p < 0.05$ and $p < 0.01$, respectively) from those of untreated and empty-vector treated mice. *B*, The protective effect of DNA vaccination is mediated by CD8^{+} T cells. BALB/c mice were vaccinated with empty vector or pcDNA3-Env, as described above, and then depleted of either CD4^{+} or CD8^{+} T cells by i.p. injection of 200 μg of mAb on days −2, 0, 4, and 8 from CT26 cell challenge. Results are from one representative experiment with five mice per group (with the exception of CD8-depleted mice, $n = 10$). Mantel-Haenszel statistics: mice vaccinated with pcDNA-Env vs mice vaccinated with pcDNA3-Env and depleted of CD4^{+} T cells, $p = 0.7$ (not significant); mice vaccinated with pcDNA-Env vs mice vaccinated with pcDNA3-Env and depleted of CD8^{+} T cells, $p = 0.0008$; mice vaccinated with pcDNA-Env and depleted of CD4^{+} T cells vs mice vaccinated with pcDNA3-Env and depleted of CD8^{+} T cells, $p = 0.04$.
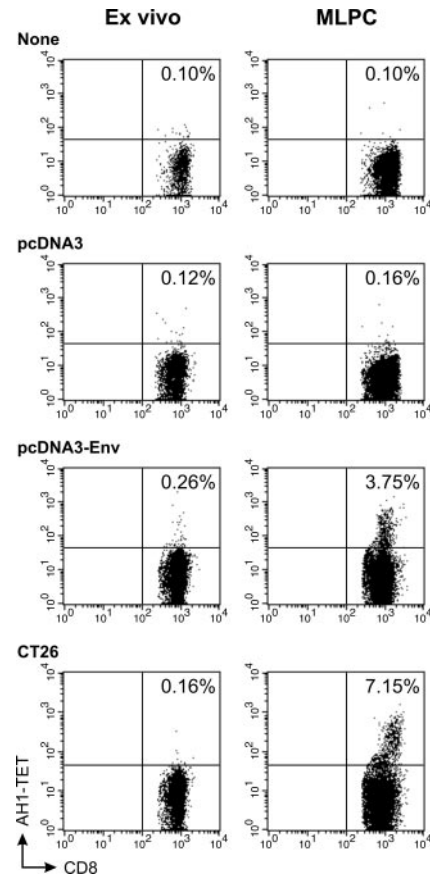

FIGURE 2. Expansion of gp70-specific T lymphocytes in mice immunized with tumor cell or DNA vaccine. Mice were either untreated or treated with gamma-irradiated CT26, pcDNA3 empty plasmid, or pcDNA3-Env. Three weeks aftertreatment, spleen cells were stimulated in vitro for 5 days with peptide AH1 (MLPC). Day 5 was chosen because it coincided with the peak in TET positivity and functional activity of the lymphocytes in culture (not shown). For cytofluorometric analyses, cells were stained with PE-coupled L^d/AH1 tetramer (AH1-TET), anti-CD8-Tricolor (CD8), and anti-CD3-FITC mAb, either immediately after isolation from the spleen (ex vivo) or after 5 days of MLPC. TET and CD8 staining is shown on cells gated for CD3 expression. One mouse representative of each group is shown. The percent staining with the control β-gal$_{876-884}$/L^d TET for each mouse was: none, 0.06 and 0.09; pcDNA3, 0.05 and 0.09; pcDNA3-Env, 0.11 and 0.14; and CT26, 0.07 and 0.13 (for ex vivo and MLPC, respectively). The mean fluorescence intensity of PE-TET was similar to that obtained by staining the AH1-specific CTL clone E88 (not shown).

present in the spleen of immunized mice, as demonstrated by the expansion of these lymphocytes upon peptide stimulation (Fig. 2); in vitro expansion was greater in mice immunized with the irradiated cell vaccine (Figs. 2 and 3*A*).

To evaluate the T lymphocyte function induced by diverse immunization protocols, IFN-γ release assay was set up with splenocytes isolated from mice immunized with pcDNA3-Env or gamma-irradiated CT26 cells and stimulated with AH1 peptide. Lymphocytes from the MLPC of immunized mice released IFN-γ in the presence of either CT26 tumor cells or AH1 peptide-pulsed 293-L^d cells targets, thus indicating that the DNA and the cell-based vaccines were both capable of eliciting L^d-restricted CD8^{+} T lymphocytes specifically recognizing the AH1 epitope (Fig. 3*B*).

Consistent with staining and tumor protection data, the immune response elicited by DNA vaccination was persistently lower.

To clarify whether the inability to detect AH1-specific T cells in the spleen 3 wk after immunization depended on the kinetics of in vivo T cell expansion and/or the anatomical district examined, we decide to more deeply explore the AH1-specific immune response. Thus, BALB/c mice were vaccinated with pcDNA3-Env or gamma-irradiated CT26 cells and then challenged with CT26 cells 3 wk after vaccination, as previously described. Blood and spleen of these mice were sampled on days 7 and 14 after DNA vaccination and on day 9 after tumor challenge to enumerate the AH1-specific lymphocytes in these districts. Ag-specific response to both immunizing agents had similar kinetics, as AH1-TET^{+} cells were detectable in the spleen 7 days after immunization, then subsided to the baseline levels of nonimmune control mice (Fig. 3*C*, *upper panel*). As expected, AH1-TET[+] lymphocytes peaked in the blood
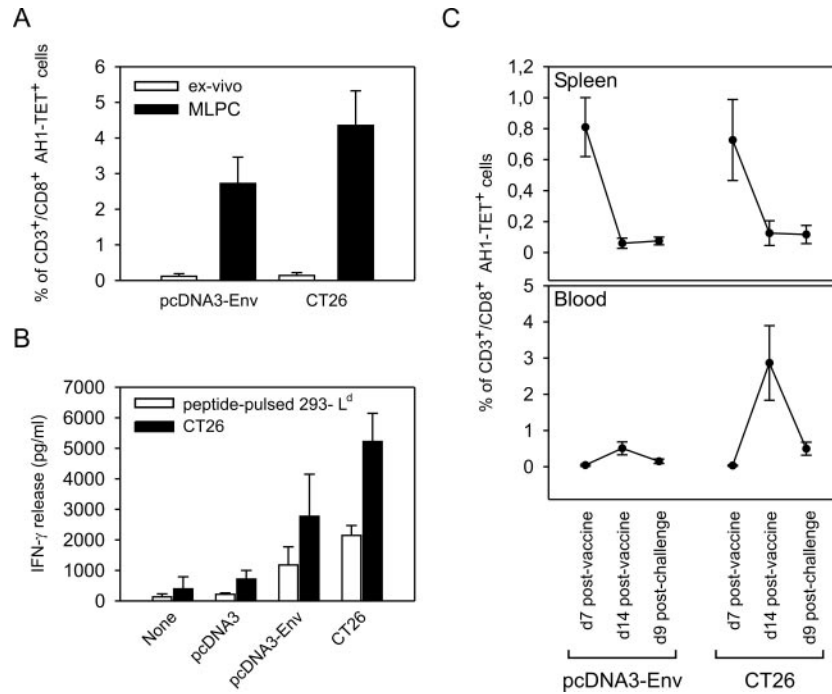
FIGURE 3. Vaccination elicits AH1-specific CD8$^+$ T cells. *A,* Spleens from mice vaccinated 21 days before, as described in Fig. 2, were surgically removed, and the AH1-specific T lymphocytes were identified by three-color staining with PE-AH1-TET, anti-CD8-Tricolor, and anti-CD3-FITC mAbs. A fraction of splenocytes was stimulated in vitro with 1 μM AH1 peptide and harvested 5 days later for FACS analysis. The percentages of AH1-TET-positive cells were calculated among gated CD3/CD8-positive cells after subtraction of the background staining given by control β-gal$_{876-884}$/L^d TET. Data are the mean $\pm$ SE, with five mice for each group. *B,* Splenocytes (10^5 cells) from MLPC were restimulated for 24 h in triplicate wells with an equal amount of 293-L^d cells, 293-L^d cells pulsed for 1 h with 1 μM AH1 peptide, or CT26 cells; the supernatant was then harvested and tested for released IFN-γ in a sandwich ELISA assay. Values of IFN-γ released in the presence of unpulsed cells were subtracted from values obtained with peptide-pulsed cells (AH1 peptide-pulsed 293-L^d). The SD of triplicate determinations for each effector/stimulator combination was <10%, and IFN-γ measured in control wells containing either effectors or stimulators alone did not exceed the lowest amount of IFN-γ detectable in our assay (i.e., 200 pg/ml, determined using serial dilutions of IFN-γ). IFN-γ released in presence of unpulsed 293-L^d was not different from that detected in wells containing effectors alone. *C,* BALB/c mice were vaccinated with pcDNA3-Env or gamma-irradiated CT26 cells and 21 days later were challenged with 0.25 $\times$ 10^6 CT26 cells. Blood mononuclear cells and splenocytes were sampled on days 7 and 14 after DNA vaccination (d7 and d14 postvaccine, respectively) and on day 9 after tumor challenge (d9 postchallenge). The percentages of CD3$^+$/CD8$^+$/AH1-TET$^+$ cells in spleen (*upper panel*) and blood (*lower panel*) are shown. Data are the mean $\pm$ SE, and the number of mice for each time point ranged from four to 10. The background staining given by control β-gal$_{876-884}$/L^d TET was subtracted for each determination.

1 wk later (Fig. 3*C, lower panel*). The early kinetics of the appearance of AH1-specific T cells in the spleen contrast with the 2–3 wk required to elicit an immune reactivity to conventional Ags such as OVA or β-galactosidase following DNA immunization, but were also observed with other self Ags, such as mouse tyrosinase-related protein 2 (data not shown). These kinetics are suggestive of a recall, rather than a primary, response. In this regard, it is singular that for tyrosinase-related protein 2 and *env*-encoded Ags, stimulation with immunodominant peptides of naive splenocytes is sufficient to increase CD8$^+$ effector T cells in vitro (9).

Although no difference was seen among the splenocytes, more AH1-specific T lymphocytes circulated in the blood of mice vaccinated with gamma-irradiated tumor cell vaccine. Apparently tumor challenge had no influence on the anti-AH1 memory response, because 9 days after tumor inoculum, it did not affect the number of AH1-specific lymphocytes in either blood or spleen (Fig. 3*C*). This suggests that in the absence of additional signals, growing tumors failed to effectively stimulate memory CD8$^+$ T lymphocytes.

In vivo activation of CD40 can increase the immunogenicity of MERV-based genetic vaccination

CD40 is expressed on APCs, and its stimulation by agonists, such as activating Abs or soluble CD40-L trimers, can deeply affect the functions of CD8$^+$ T lymphocytes. Particularly relevant to tumor immunology, anti-CD40 mAb administration was shown to increase the efficacy of peptide-based immunogens given with adjuvants by interfering with the deletion or anergy of tumor-specific T lymphocytes (17, 18). To overcome the intrinsic weakness of genetic vaccination to MERV, association of CD40 agonists with genetic vaccination appeared to be a promising approach. However, the timing of CD40 activation in vivo might represent a relevant issue, because it was recently shown that CD40 stimulation can paradoxically accelerate the deletion of tumor-reactive CD8$^+$ T lymphocytes in tumor-bearing mice (20). Thus, we decided to administer agonistic anti-CD40 mAb at the time of priming with pcDNA3-Env (before tumor challenge) or during the rejection phase (immediately after tumor challenge). The experiments reported in Fig. 4*A* represent the extremes of the previously mentioned variability in genetic vaccination efficacy. In experiment 1, in fact, DNA vaccination with pcDNA3-Env did not significantly prolong the survival of mice challenged with CT26 tumor compared with that of mice that received the empty pcDNA3 (the survival curve of mice treated with empty pcDNA3, identical with that of group receiving pcDNA3 and anti-CD40 mAb, was not included for simplicity); conversely, in experiment 2, DNA vaccination showed an efficacy comparable to that in the previously illustrated experiments. Anti-CD40 treatment during the priming

of immune response with pcDNA3-Env did not increase the overall protection rate (experiment 1) or was deleterious (experiment 2), as mice receiving the combination anti-CD40 mAb and pcDNA3-Env died earlier than those in the group immunized with pcDNA3-Env alone (experiment 2; $p < 0.01$). Unexpectedly, only CD40 activation at the time of tumor challenge resulted in a significant prolongation of survival in both experiments. Anti-CD40 mAb treatment alone (in conjunction with mock immunization) was not sufficient to affect tumor progression (Fig. 4A, pcDNA3 and anti-CD40), indicating that tumor-specific T lymphocytes elicited by genetic vaccination were necessary for the antitumor effect.

When we evaluated the effect of CD40 stimulation on AH1-specific CD8^{+} T lymphocytes in mice challenged with CT26 tumor, we detected a marked expansion in the percentage of Ag-specific T lymphocytes, consistent with the remarkable effect on tumor growth control (Fig. 4B, *left panel*). This difference was more noteworthy when the absolute number of AH1-specific T lymphocytes in the spleen was considered, given the cellularity increase in the spleens of anti-CD40 mAb-treated animals. There was, in fact, a 17-fold expansion of AH1-specific CD8^{+} T lymphocytes in mice that received anti-CD40 agonists (Fig. 4B, *right panel*). As expected, the majority of AH1-specific T cells had an effector/memory phenotype (CD62L low, CD44 high; not shown), and they were more prevalent in spleen rather than in blood (Fig. 4B).

Env gene expression by RT-PCR was found in most H-2[d] haplotype tumors tested (Fig. 5A). The list enclosed a plasmocytoma

(P815), colon carcinomas (CT26, C26, CA51), sarcomas (WEHI, MethA), a renal cell carcinoma (RENCA), and the mammary carcinoma TS/A. Indeed, only the fibroblast cell lines F1 and KF1/SV were found to be negative. However, among different tumors only the TS/A mammary carcinoma was recognized by the AH1-specific CTL clone E88 as well as CT26 tumor and the closely related C26 colon carcinoma (Fig. 5B), suggesting that not all gp70-positive tumors are able to process and present the AH1 peptide. Cell lines that are not recognized by E88 CTL, however, can be made susceptible to recognition by pulsing them with AH1 peptide (compare P815 with or without peptide in Fig. 5B). In different repeat experiments, genetic vaccination with *env*-encoding plasmid completely failed to protect mice from challenge with TS/A tumor cells (data not shown), unless combined with the administration of anti-CD40 mAb after tumor challenge (Fig. 5C), indicating that CD40 activation might be necessary to unmask the potential therapeutic activity of gene-based vaccines in the case of weakly immunogenic tumors such as TS/A carcinoma.

Generation of an antimestastatic immune response against the p15E-derived epitope of MERV

Env is endogenously expressed in H-2[b] haplotype tumors, such as B16 melanoma, MC38 adenocarcinoma, and MCA203 sarcoma cells, but not in T6215 fibroblasts derived from a syngeneic C57BL/6 mouse (Fig. 5A). Antimelanoma murine CD8^{+} T lymphocytes have been shown to recognize nonmutated epitopes derived from the p15E product of the *env* gene, and p15E-reactive T
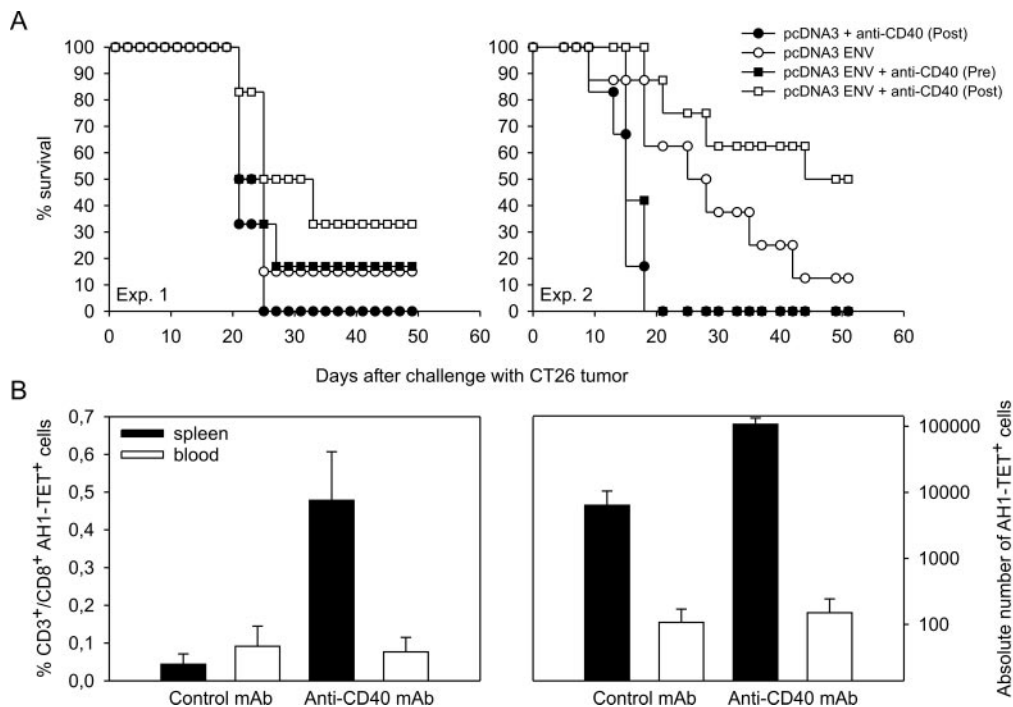

FIGURE 4. Administration of CD40-activating mAb during the tumor rejection phase improves anti-gp70 response induced by DNA vaccination. *A*, The efficacy of *env*-based vaccination, associated or not with anti-CD40 mAb administration, was evaluated in two different experiments with six mice for each group. BALB/c mice immunized with DNA were injected with 200 μg of mAb anti-CD40 (FGK.45.5) or control Ab (GL111) on days 0, 1, 2, and 4 after either DNA vaccination (Pre) or tumor challenge (Post). The survival curves of some control groups (pcDNA3 alone, pcDNA3-Env plus control mAb) were not included for simplicity; however, they did not differ significantly from that in the group receiving pcDNA3 and anti-CD40 mAb. The *p* values for mice treated after tumor challenge with anti-CD40 mAb and immunized with either pcDNA3-Env or pcDNA3 were <0.05 and <0.01 for experiments 1 and 2, respectively. *B*, BALB/c mice immunized with DNA and challenged with CT26 tumor cells were treated with 200 μg of mAb anti-CD40 or the same dose of a control Ab on days 0, 1, 2, and 4 after tumor challenge. Spleens were surgically removed 9 days after the challenge and were evaluated by three-color cytofluorometry, as described previously. The percentages of AH1-TET-positive cells were calculated among gated CD3^{+}/CD8^{+} cells after subtraction of the background staining given by control β-gal$_{876-884}$/L[d] TET. Each group is composed of six (not pooled) spleens. Absolute numbers represent the results for 10^6 circulating mononuclear cells (*left panel*) or the total number of AH1-TET^{+} cells in the spleen (*right panel*). Note the logarithmic scale in the *right panel*.
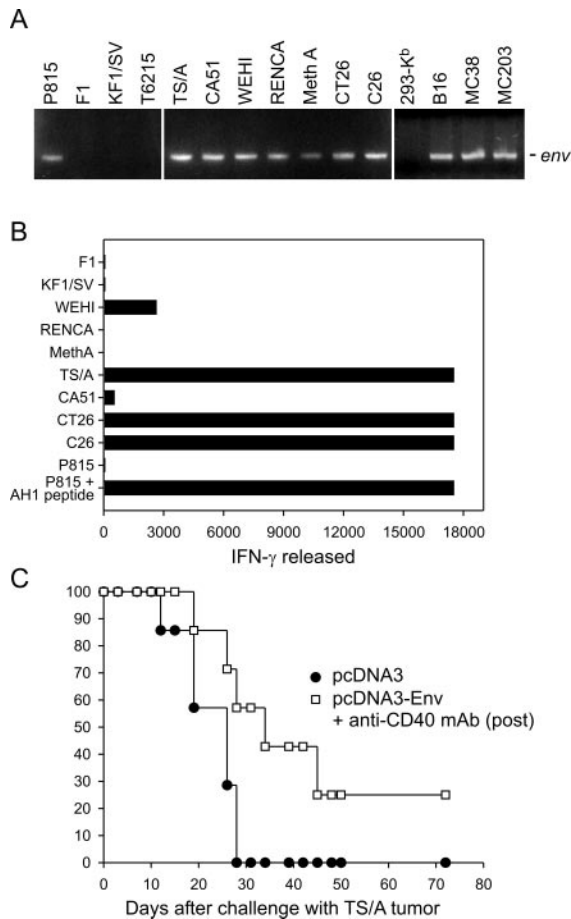
FIGURE 5. AH1 is a shared, but not widely recognized, TAA. Enhancement of the antitumor response by in vivo CD40 ligation is shown. *A*, Cell lines of the H-2^d or H-2^b haplotype were evaluated by RT-PCR for the presence of the *env* transcript. β-Actin amplification was performed as a control (not shown). *B*, Expression of membrane complexes between L^d class I molecule and AH1 peptide was evaluated by the release of IFN-γ (picograms per milliliter) from the CTL clone E88. Different cell lines, either unpulsed or pulsed with 1 μM AH1 peptide, were incubated for 24 h with the E88 CTL before harvesting the supernatant. IFN-γ measured in control wells containing either effectors or stimulators alone did not exceed the lower limit of detection (200 pg/ml). Data are from triplicate wells. *C*, The *env*-coding DNA vaccine associated with anti-CD40 mAb administration is effective against s.c. tumor challenge with 10^5 TS/A cells in BALB/c mice. Vaccination and Ab administration were described in previous figure legends for the CT26 tumor model. The experiment was conducted on seven mice in each group. The *p* value between the two treatment groups was <0.01.

cells with low avidity are readily induced from spleen cells of naive mice (9, 10). The B16 cell line is a high aggressive tumor with very low levels of MHC class I Ag and represents an interesting model of poorly immunogenic melanoma, as is the case of many human tumors. Vaccination with pcDNA3-Env, alone or associated with anti-CD40 mAb, failed to trigger an Ag-specific response and thus protect mice from challenge with B16 melanoma (data not shown). We speculated that this ineffectiveness might depend on the position of the p15E peptide in the polypeptide derived from *env* translation. It was previously shown, in fact, that COOH-terminal epitopes are inefficiently processed and presented, both in vitro and in vivo, and this defect could be restored by manipulation that enhanced the protein degradation, such as deletion of the NH$_2$-terminal signal sequence or construction of a ubiquitin fusion protein (26, 27). We created a novel series of DNA

vaccines based on the eukaryotic expression vector VR1055 to express the full-length gene (VR1055-Env) or the portion coding for gp70 (VR1055-gp70) or p15E (VR1055-p15E). VR1055 expression vector, optimized for rapid cloning and in vivo expression, was equivalent to pcDNA3 in our previous immunization studies with a different tumor Ag (28). Being of human origin, the 293 cells transfected with K^b allele (293-K^b) do not express MERV and are not recognized by the K^b-restricted T4905 CTL clone (Figs. 5*A* and 6*A*). In contrast, 293-K^b cells pulsed with p15E peptide are efficiently recognized by T4905 CTL as well as the 293-K^b cell transiently transfected with either VR1055-Env or VR1055-p15E, but not with VR1055-gp70, which does not contain the relevant epitope (Fig. 6*A*). However, whereas immunization with VR1055-p15E reduced the number of B16 pulmonary metastases in vaccinated mice, VR1055-env was completely inefficient (Fig. 6*B*) The therapeutic effect of VR1055-p15E was significantly enhanced by anti-CD40 mAb treatment (Fig. 6*B*). To analyze the p15E-specific response in the same experiment, blood was withdrawn from these mice 5 days after challenge with B16, and Ag-specific T lymphocytes were enumerated with p15E-K^b tetramer reagent able to bind specifically the T4905 CTL clone (not shown). Cumulative data indicated that, again, therapeutic effect of CD40 stimulation combined with genetic vaccination was associated with a relevant (5.3-fold; $p < 0.01$) expansion of p15E-specific circulating CD8$^+$ T lymphocytes (Fig. 6*C*).

Discussion

Retroviruses belong to a group of genetic elements replicating via reverse transcription and infecting a wide range of vertebrate species. During vertebrate evolution, some of these genetic elements have been integrated into DNA of the host's germline and, thereby, transmitted to the next generation as a Mendelian trait (1). These genomes are known as ERV. It was advanced that the more pathogenic the ERV genome the less likely to be maintained in the host genome (2). However, it was demonstrated that ERV can contribute to pathogenicity, as collaborator in recombination events or via de novo insertion after mobilization, by allowing activation of cellular protoncogenes or causing disruption of tumor suppressor genes. Cell biology in eukaryotes can be influenced not only by serendipitous retrotransposition, but also by re-expression of viral proteins: accompanied by loss of immune tolerance, these proteins can induce autoimmune disease; some envelope proteins of retroviruses are immunosuppressive; the emergence of fusion proteins with viral and cellular determinants can direct oncogenic transformation (3). Despite these observations the real significance of ERV expression is far from clear.

ERV genome is transmitted as part of the host's normal genetic material so one could expect that the host itself is tolerant to ERV self-Ags, but many findings dispute this simplified consideration. Humoral and cellular immune responses to HERV proteins are detectable in patients with certain tumors (11, 29); similarly, an effective immune response has been described in mice that bear tumor expressing MERV proteins (6, 7, 9, 10). The well-documented expression of MERV proteins in many mouse tumors together with growing evidence of similar findings in human tumors support the attempt to exploit ERV as novel target for the immunotherapy of cancer. By targeting MERV, a nonmutated and widely shared TAA, we have shown in this study that DNA vaccination can elicit Ag-specific CD8$^+$ T lymphocytes that are responsible for the rejection of different histologic tumors. However, efficient engineering of DNA vaccine to optimize generation of the immunizing epitope in vivo and CD40 stimulation during tumor rejection represent critical requirements for a successful immunotherapy of tumors with MERV-based genetic vaccines.
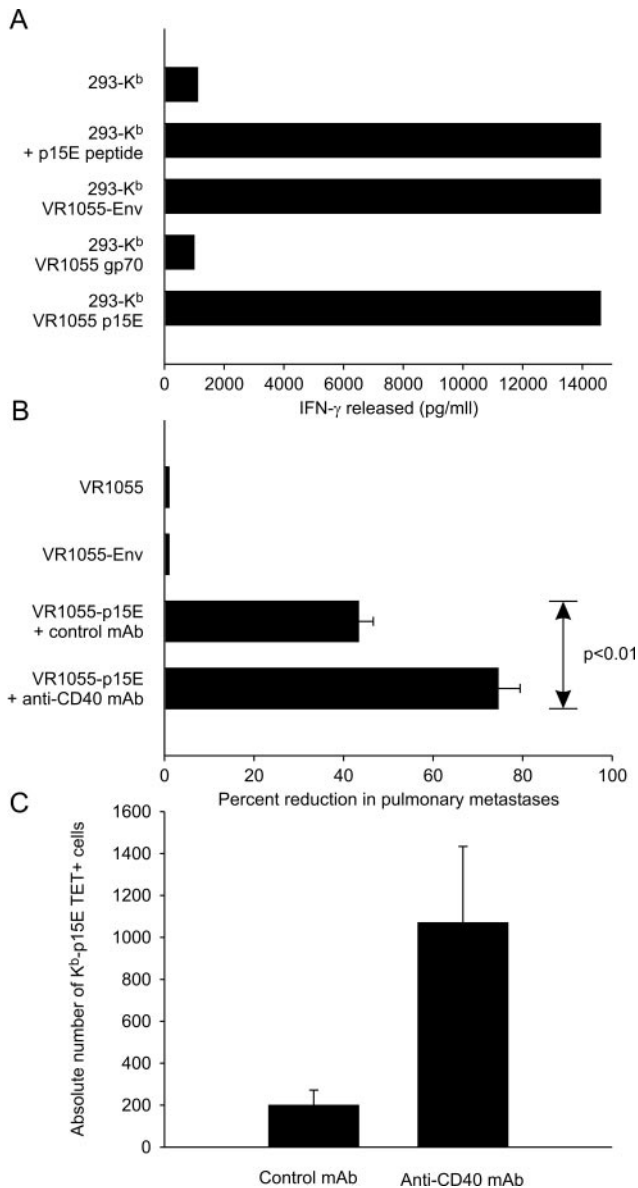
FIGURE 6. Plasmids encoding p15E are effective in inducing Ag-specific $CD8^+$ T lymphocytes capable of antimelanoma activity in vivo. *A*, 293-K^b cells were transiently transfected with plasmids encoding different portions of the *env* gene and were cultured for 24 h with T4905, a CTL clone specific for a K^b-restricted p15E epitope. The transfected 293-K^b cells were compared with 293-K^b cells pulsed with p15E peptide for the ability to induce IFN-γ release by T4905 CTL. *B*, C57BL/6 mice were vaccinated i.m. with plasmids encoding the *p15E* portion or the full-length *env* gene. Three weeks after vaccination, mice were challenged i.v. with 10^5 B16 melanoma cells. The group of mice treated with anti-CD40 or control mAb received Abs on days 0, 1, 2, and 4 after tumor challenge. As the number of metastases in untreated groups differed in various experiments, the efficacy of vaccination is expressed as the percent reduction ($\pm$SE) of metastasis number in treated groups compared with the untreated control group in each experiment. Results are cumulative for three separate experiments performed under the same experimental conditions. *C*, The absolute numbers of K^b-p15-TET-positive T lymphocytes in blood were calculated by three-color cytofluorometry in the same mice as those used in experiment illustrated in *A*, by sampling the blood on day 5 after tumor challenge. The figure shows the numbers ($\pm$SE) of p15E-specific T lymphocytes for 10^6 mononuclear cells present in the blood. The graph shows data from nine mice (three for each experiment) vaccinated with VR1055-p15E and treated with either anti-CD40 or control mAb.

It must be pointed out that even though *env* mRNA is detected in almost all the tumor cell lines we analyzed, the class I-restricted AH1 epitope is not presented to $CD8^+$ T lymphocytes by all of them. This failure is not dependent on major defects in the expression of the class I MHC molecules and, in fact, the majority of cell lines are efficiently recognized when the AH1 peptide is added exogenously (Fig. 5*A* and data not shown). The mechanisms underlying this deficiency are unknown and might involve different steps of the intracellular Ag processing. However, more intricate networks might be at work, because transfection with the genes of some alleles of H-2^b class I molecules interfered with the production of Emv-2 ectopic murine leukemia virus particles in a melanoma cell line (30). Knowledge of the exact pathways interfering with the generation of MERV-encoded epitopes might help broaden the list of tumors responsive to immunotherapy with MERV-based vaccines.

The *env* gene codes for a precursor protein (gp90) that is processed in Golgi complex in two viral proteins, gp70 and p15E, that are then assembled into a complex bound to the cell membrane. The processing of these Ags in vitro generates epitopes from both the NH2 terminus (gp70$_{423-431}$, L^d-restricted) and the COOH terminus (p15E$_{604-611}$, K^b-restricted) of the protein, which are recognized by Ag-specific CTL. The p15E-specific T4905 CTL clone is effective against the B16 tumor, indicating that B16 is capable of processing the p15 epitope from *env*. However, eliciting an immune reactivity against the p15E epitope by DNA vaccination in mice appeared to be more stringent, because a proper modification of the coding sequence, such as exclusion of the NH2-terminal portion of the *env* gene coding for gp70, was needed, indicating that processing and generation of relevant epitopes might be different in vivo and in vitro. One of the reasons for this discrepancy might be the expression levels of the *env* gene under different settings. B16 express gp70 on its surface at very high levels, to the point that this Ag is the exclusive target of Abs generated following immunization with B16 cells (31). Moreover, 293-K^b cells transfected with the plasmid VR1055 encoding the full-length *env* gene were able to process and present the peptide from the p15E portion that was thus recognized by the CTL T4905 (Fig. 5*A*). In contrast, 293-K^b cells transfected with the plasmid pcDNA3 encoding the full-length *env* gene were not recognized by the K^b-restricted clone T4905, whereas 293-L^d were recognized by CTL E88, although much less than when transfected with VR1055-Env (not shown). The plasmid pcDNA3 is less efficient than VR1055 in driving the synthesis of the heterologous gene. Therefore, under limiting expression conditions, such as following in vivo immunization, generation of the p15E epitope could be marginal.

The results with CD40-activating Ab are encouraging, although the mechanism of action is not completely understood. A direct activity of anti-CD40 mAb on the tumor is unlikely, because none of the tumor cell lines transplanted in vivo expressed CD40 (not shown). In another study administration of anti-CD40 mAb in tumor-bearing mice resulted in antitumor and antimetastatic effects that were reduced by NK cell depletion. Anti-CD40 mAb was shown to activate NK cells in vivo indirectly, via the systemic production of IL-12 and IFN-γ (32). Although the role of NK cells in tumor rejection was not investigated, we and others (17, 18, 20, 33) have not observed a substantial direct antitumor activity of CD40 agonists in the absence of immunization with TAA.

The most likely explanation for CD40 mAb effectiveness is that this agonist modulates APC activity, which, in turn, enhances the function of MERV-specific $CD8^+$ T lymphocytes in tumor-bearing mice. In this regard it is intriguing that vaccine formulation with gamma-irradiated CT26 cells induced a detectable expansion of AH1-specific $CD8^+$ T lymphocytes in alleged naive mice,

whereas the nonirradiated tumor growing in the host was not able to sustain a memory CD8$^+$ T cell response in the absence of CD40 stimulation (compare Figs. 3, 4, and 6). Anti-CD40 mAb treatment caused splenomegaly with a 4-fold increase in the number of splenocytes. Both the pool of total CD8$^+$ and the percentage of AH1-TET$^+$ T cells were interested by the anti-CD40-driven expansion, and the combined effects led to a relevant increase in the overall number of MERV-specific T lymphocytes. As reported for other tumor models, it is likely that CD8$^+$ T lymphocytes generated by DNA vaccination are tolerized in an Ag-specific fashion by the growing tumors (18, 34). In this context, CD40 activation of APC might supply the necessary signals to overcome this tolerance and assist the antitumor effector activity of memory CD8$^+$ T cells. An important consideration for the immunotherapy is that agonists acting on CD40 can be administered systemically, i.e., are not required to be delivered with the Ag to the same APC. Our study indicates that they can even be separated temporarily. We have not investigated whether the CD40-dependent stimulation of tumor-destroying CD8$^+$ T lymphocytes takes place outside the tumor environment (in lymph nodes draining the inoculation site) or within the tumor microenvironment. Nonetheless, the relevant issue is that the antitumor activity is elicited in the presence of a growing tumor. If confirmed by further observations, this strategy might be applicable to several clinical situations: vaccination in patients resected to the primary tumor could be followed by either systemic or local anti-CD40 activation during tumor recurrence.

CD40 stimulation can have a paradoxical effect on peripheral tolerance to Ags. CD40 stimulation alone in mice bearing a B16 melanoma carrying the OVA Ag as model tumor Ag resulted in the unexpected disappearance of OVA-specific CD8$^+$ T lymphocytes, and anti-CD40 treatment mediated T cell deletion in an autoimmunity model (20, 35). The negative effects seen in some experiments (Fig. 4A) when anti-CD40 was administered with the DNA vaccine at the time of priming are consistent with such enhanced systemic tolerance. All these paradoxical results can be interpreted under the perspectives of the Ag persistence. Vaccination of mice with a minimal CTL epitope from human adenovirus type 5 E1A administered with adjuvant readily induced CTL tolerance, resulting in the inability to control the growth of E1A-expressing tumors (17). Although efficient CTL priming was achieved when the peptide vaccine was combined with the systemic inoculation of anti-CD40 mAb, the CTL response waned quickly and failed to protect against tumor challenge. The fast disappearance of CTL was related to the systemic persistence of the peptide given with the adjuvant, because the same peptide administered in saline did not persist and, in fact, generated long term CTL immunity capable of rejecting Ad5E1A-positive tumors when combined with CD40 agonists (33). It is conceivable that DNA immunization can lead to prolonged expression of the MERV Ag in normal tissues devoid of APC properties, thus mimicking the situations of Ag persistence, a characteristic shared by Ag expressed by tumors, self tissues, and peptide emulsified in adjuvants.

Kedl and colleagues (20) showed that infection with rVV encoding OVA was able to synergize with anti-CD40 and supply an unknown inflammatory signal for the survival of OVA-specific T cells in tumor-bearing mice, whereas DNA vaccination with OVA-encoding plasmid was ineffective. Interestingly, association of OVA-encoding plasmid with a control VV and anti-CD40 treatment could fully replace OVA-rVV, indicating that Ag and the source of inflammatory signal could be physically separated. The idea of a third signal, in addition to the Ag and CD40 ligation, required to convert a tolerogenic encounter with self Ag to a fully immunogenic one is supported by other studies, and the cytokine IL-12 could be a potential candidate (36). In our system once the

CD8$^+$ T lymphocytes were generated by genetic vaccination, they did not require a third signal to eliminate the tumor, suggesting that the tumor inoculation itself was able to deliver such signal.

The complete protection from tumor take after immunization with a tumor cell vaccine (Fig. 1A) is not a novel finding (37) and cannot be used to claim the superiority of cell-based vaccines over DNA vaccination. CT26 express more than one TAA shared with other tumors. The weak cross-protective immune response to a fibrosarcoma and a mammary carcinoma following vaccination with CT26 expressing IL-12 was not associated with a CD8$^+$ T lymphocyte response to gp70 (38). Moreover, depletion of CD25$^+$ T regulatory cells was sufficient to allow rejection of a first inoculum of CT26 cells and conferred long term protection against CT26, A20, C26, and RENCA tumors despite the absence of any detectable anti-gp70 immunity (37). In contrast, vaccination with CT26 cells engineered to secrete GM-CSF induced strong anti-gp70 immunity, but did not protect mice from challenge with C26, a closely related colon carcinoma. These results indicate that cross-protection by CT26 tumor vaccination can depend on different Ags with a hierarchy of relevance that is somehow highlighted by the cytokine released from the cell vaccine.

The experiments reported in Figs. 2 and 3 also showed a slight superiority of irradiated CT26 over pcDNA3-Env in inducing an immune response against the AH1 epitope. This is somehow surprising, because DNA vaccination is regarded as a very effective strategy to elicit CTL in mice. However, previous attempts to immunize with single vaccines indicate that *env* gene products are poor immunogens; moreover, CD4$^+$ Th lymphocytes might be required to obtain an optimal immune reactivity to gp70/p15E epitopes (12–14). Being a complex antigenic mixture, irradiated CT26 might supply more than one epitope recognized by CD4$^+$ T lymphocytes, enclosing potential epitopes derived from serum proteins.

Our studies were performed in a preventive setting. Nonetheless, the findings that CD40 agonists are required during tumor rejection open the possibility of combining genetic vaccination with them in a full therapeutic setting. Unfortunately, the protocol of DNA vaccination used in our studies, which involves pretreatment with cardiotoxin 5 days before i.m. DNA inoculation, appears too slow to assist the therapy of rapidly growing mouse tumors. We are currently testing in vivo electroporation of the injected muscle as a way to augment the delivery of DNA vaccine. Results obtained with other TAA (39) encourage using this strategy in combination with anti-CD40 mAb administration. To date, the only successful therapeutic approach with MERV-based vaccination has been conducted by immunizing tumor-bearing mice with AH1 or p15E peptide-pulsed DC, activated in vitro with CD40-L and administered in conjunction with exogenous IL-2 (14). To apply this clinically on a large scale might be difficult, because it involves the skilled and intensive task of isolating and manipulating DC from each patient needing vaccination. Conversely, electroporation-enhanced genetic vaccination has been shown to be a valid system to allow contemporaneous expression of different genes, for example, Ag mixtures for polyimmunization (39) or combination of plasmids coding for Ags and immunostimulatory molecules.

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