Cancer Immunity, Vol. 3, p. 11 (14 August 2003) Submitted: 11 July 2003. Accepted: 30 July 2003.
Communicated by: GI Abelev

In vivo targeting of an anti-tumor antibody coupled to antigenic MHC class I complexes induces specific growth inhibition and regression of established syngeneic tumor grafts

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Keywords: mice, tumor antibodies, Fab immunoglobulins, immunoconjugates, immunotherapy

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Abstract

The concept of antibody-mediated targeting of antigenic MHC/peptide complexes on tumor cells in order to sensitize them to T-lymphocyte cytotoxicity represents an attractive new immunotherapy strategy. *In vitro* experiments have shown that an antibody chemically conjugated or fused to monomeric MHC/peptide can be oligomerized on the surface of tumor cells, rendering them susceptible to efficient lysis by MHC-peptide restricted specific T-cell clones. However, this strategy has not yet been tested entirely *in vivo* in immunocompetent animals. To this aim, we took advantage of OT-1 mice which have a transgenic T-cell receptor specific for the ovalbumin (ova) immunodominant peptide (257-264) expressed in the context of the MHC class I H-2K^b. We prepared and characterized conjugates between the Fab' fragment from a high-affinity monoclonal antibody to carcinoembryonic antigen (CEA) and the H-2K^b/ova peptide complex. First, we showed in OT-1 mice that the grafting and growth of a syngeneic colon carcinoma line transfected with CEA could be specifically inhibited by systemic injections of the conjugate. Next, using CEA transgenic C57BL/6 mice adoptively transferred with OT-1 spleen cells and immunized with ovalbumin, we demonstrated that systemic injections of the anti-CEA-H-2K^b/ova conjugate could induce specific growth inhibition and regression of well-established, palpable subcutaneous grafts from the syngeneic CEA-transfected colon carcinoma line. These results, obtained in a well-characterized syngeneic carcinoma model, demonstrate that the antibody-MHC/peptide strategy can function *in vivo*. Further preclinical experimental studies, using an anti-viral T-cell response, will be performed before this new form of immunotherapy can be considered for clinical use.

Introduction

Cancer immunotherapy by the systemic injection of high affinity monoclonal antibodies (mAbs) directed against cell surface tumor-associated antigens (TAAs) has demonstrated statistically significant tumor remissions in B cell lymphoma (1) and breast carcinoma patients (2). Despite these very encouraging results, the percentage of complete tumor remissions obtained by repeated injections of large amounts of mAbs, as a single-modality therapy, has remained low (1, 2). One of the numerous lessons learned from mAb treatments, including the injection of radiolabeled mAbs (3, 4), is that target TAAs do not need to be highly tumor-specific as long as they are easily accessible and overexpressed on tumor cells, and less so on vital normal cells. For instance, clinical immunoscintigraphy studies have shown that radiolabeled mAbs, or their fragments, directed against the CEA or A33 antigens can localize specifically into primary and metastatic colorectal carcinoma, while it is known that the CEA and A33 antigens are also expressed on the apical side of normal colon epithelium (3, 4). Furthermore, treatment with anti-CD20 mAb, known to react with both malignant and normal B-cells, is known to be clinically beneficial and very well tolerated (1).

The second and major approach of specific cancer immunotherapy consists in the active immunization of patients with antigens known to be recognized by and to activate T lymphocytes. In this regard, highly specific antigens recently identified include the MAGE group (5) and NY-ESO-1 (6), which all belong to the newly-defined cancer-testis antigen family since they are expressed only on tumor cells and on spermatogenic cells from the testis (7). Moreover, clinical studies using MHC-tetramer staining have demonstrated T lymphocyte responses against the immunizing tumor antigens in the course of vaccination. However, these promising clinical trials of active immunization suffered from a low percentage of tumor remissions (8, 9) and a surprising lack of correlation between clinical and T lymphocyte responses to the vaccine (9, 10, 11). Furthermore, this approach encounters the potential risk of selecting tumor cell variants which have undergone HLA loss (12).

The new cancer immunotherapy strategy described here has the potential to combine the advantage of the well-demonstrated tumor targeting properties of anti-TAA mAbs with the known efficient and specific cytotoxic activity of CD8 T lymphocytes directed against highly antigenic MHC/peptide complexes. It consists in using Fab' fragments from a high affinity anti-TAA mAb coupled to a MHC class I containing a selected antigenic peptide in order to target the active MHC/peptide complex on tumor cells and induce their lysis by specific CTLs. The group of Savage and our group (13, 14) first showed that biotinylated MHC/peptide multimerized on streptavidin coupled to an antibody to tumor cells could induce the T lymphocyte-mediated *in vitro* lysis of coated tumor cells. This approach became more realistic when we demonstrated that even monomeric HLA-A2/influenza (Flu) matrix peptide directly coupled to a specific Fab' antibody fragment was still active in killing various human tumor cell lines. It was in fact shown that anti-CEA, ErbB-2 or CD20 Fab' fragments coupled to HLA-A2/Flu matrix peptide could be oligomerized on the surface of different human tumor cell lines expressing the relevant TAA, and induce their specific and efficient *in vitro* lysis by Flu matrix peptide-specific HLA-A2-restricted T cell clones (15). More recently, two groups presented partially *in vivo* results of tumor graft inhibition, using either biotinylated MHC/peptide streptavidin complexes (16) or a recombinant fusion protein containing anti-tumor antibody and MHC/peptide (17). They showed in models of human tumor xenografts injected into immunodeficient mice that *in vitro* preincubation and coating of tumor cells with the antibody MHC/peptide conjugates, followed by coinjection of relevant activated T lymphocytes, could specifically prevent tumor development.

Here, we present the first entirely *in vivo* demonstration that the systemic injection of anti-TAA-MHC/peptide complexes can induce significant remission and regression of well-established tumor grafts in a model of subcutaneously transplanted syngeneic carcinoma. To ensure the presence of a sufficient T cell response against a well-defined MHC class I peptide epitope, we took advantage of OT-1 C57BL/6 mice in which most CD8 T lymphocytes express a transgenic T cell receptor (TCR) specific for the immunodominant ova peptide in

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the context of H-$2K^b$ (18). In a first approach, we subcutaneously transplanted a CEA-transfected syngeneic colon carcinoma cell line (MC38-CEA+) (19) in OT-1 mice and immediately treated them by injection of an anti-CEA-H-$2K^b$/ova conjugate. In a second series of experiments, spleen cells from OT-1 mice were adoptively transferred in CEA transgenic mice (19), followed by immunization with ovalbumin. Animals were then subcutaneously grafted with the same syngeneic colon carcinoma line and, only when the tumors were palpable, treated by systemic injection of the anti-CEA-H-$2K^b$/ova peptide conjugate or a control anti-CEA $F(ab')_2$ fragment without MHC.

Results

Synthesis and characterization of the anti-CEA Fab-H-$2K^b$/ova conjugate

Recombinant soluble class I H-$2K^b$ molecules containing a cysteine engineered at the carboxyl-terminus of the heavy chain (20), as well as a cysteine 121 to alanine substitution generated by site-directed mutagenesis, were produced in bacteria. Monomeric complexes of heavy chain, beta2-microglobulin (beta2M) and synthetic ova peptide (257-264) were refolded by dialysis and purified by size-exclusion chromatography as shown in Figure 1A. The MHC/peptide complex was then coupled to the Fab' fragment from the anti-CEA mAb (3, 21) by forming a thioether bond between the free thiol group of the terminal cysteine from the H-$2K^b$ heavy chain and the dimaleimide-derivatized cysteines from the Fab' fragment (15). The conjugate was then purified by size-exclusion chromatography as shown in Figure 1B. The fractions of the peak eluting with an apparent Mr of 95 kDa were further purified on an S200 fast performance liquid chromatography (FPLC) column (Figure 1C). About 5% of refolded H-$2K^b$ monomer was obtained from the total input protein, and the coupling efficiency with the antibody fragment was usually 30%. Purified H-$2K^b$/ova complex, Fab' fragment and the conjugate preparation were analyzed by SDS-PAGE (Figure 1D). Under non-reducing conditions, the 45 kDa H-$2K^b$/ova complex (lane 4) gives a major band of 33 kDa and a minor band of 12 kDa corresponding to the dissociated beta2M. The MHC coupled to the 50 kDa anti-CEA Fab' fragment (lane 3) forms a conjugate that gives a major band of about 83 kDa and a discrete beta2M band at 12 kDa (lane 5). Under reducing conditions, the MHC complex migrates similarly as expected (lane 4 and 7, respectively). The dissociation of the two chains from the Fab' fragments is only partial (lane 8) since some covalent thioether bonds may have been created between the chains after reduction and dimaleimide treatment. Thus, the conjugate gives a major band at 58 kDa resulting from the dissociation of the beta2M and the light chains of the Fab' fragment, and a second band at 83 kDa due to the non-dissociated light chain (lane 6).

Binding of the anti-CEA Fab-H-$2K^b$/ova conjugate to CEA-expressing cells demonstrated by cytofluorometry

The capacity of the conjugate to bind to the CEA-expressing, H-$2K^b$-negative, human LoVo carcinoma cell line, as well as the proper conformation of the refolded H-$2K^b$ molecule, were tested by cytofluorometry using a conformation sensitive anti-H-$2K^b$ mAb. The positive cytofluorometric profiles shown in Figure 2A demonstrate both that LoVo cells are efficiently coated with the conjugate and that the H-$2K^b$ complex has a native conformation.

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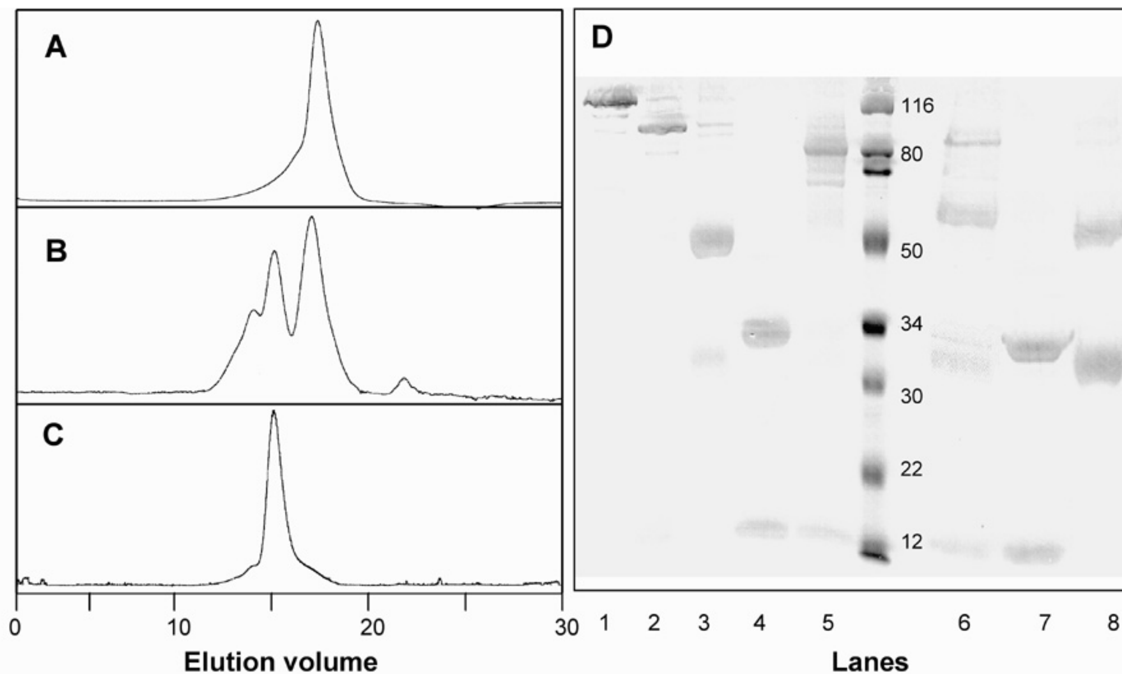

Figure 1. Characterization of the anti-CEA Fab-H-2K^b/ova conjugate. (A) Elution profile from a Superdex 200 FPLC column of the purified H-2K^b/ova peptide complex. (B) Elution on the same S200 column of the H-2K^b/ova complex after coupling to anti-CEA Fab' fragments. The conjugate is eluted after a peak of aggregates and is followed by a peak of uncoupled MHC I complexes and Fab' fragments. (C) Final S200 purification of the anti-CEA Fab-H-2K^b/ova conjugate eluted with an apparent Mr of 95 kDa. (D) SDS-PAGE showing the migration under non-reducing (lane 1-5) and reducing (lane 6-8) conditions of anti-CEA Fab-H-2K^b/ova conjugate (lane 5, 6) and its two main components: the H-2K^b/ova complex (lane 4, 7) and the anti-CEA Fab' fragment (lane 3, 8). The intact anti-CEA mAb (150 kDa, lane 1) and the anti-CEA F(ab')$_2$ fragment (100 kDa, lane 2) together with a high range molecular weight protein mixture were used as standards (central lane).

Specific CTL-mediated lysis of tumor cells coated with anti-CEA Fab-H-2K^b/ova conjugate

CTLs specific for the H-2K^b/ova complex were generated from the spleen of an OT-1 transgenic mouse after 5 days of culture with 1 µM ova peptide. Their capacity to specifically kill CEA-expressing target cells, either coated with the anti-CEA Fab-H-2K^b/ova conjugate or not, was tested at different T lymphocyte to target cell ratios. When coated with the anti-CEA-H-2K^b/ova conjugate, the MC38-CEA+ mouse colon carcinoma cells were specifically killed by the ova specific CTLs, with 45% lysis obtained at a ratio of 90:1, while no killing of the same target was observed in the absence of conjugate. Similarly, the CEA-expressing human colon carcinoma LoVo cells were killed by the murine ova specific CTLs, with up to 40% lysis at a ratio of 30:1, only when coated with the anti-CEA Fab-H-2K^b/ova conjugate (Figure 2B). These results demonstrate the ability of the Fab-MHC conjugate to coat tumor cells with defined MHC/peptide complexes that render them susceptible to lysis by peptide-specific T cells *in vitro*.

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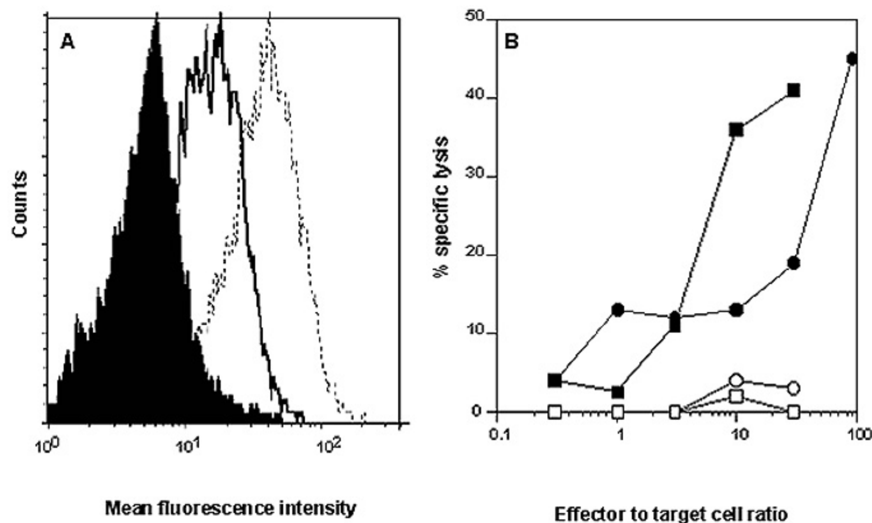

Figure 2. Biological activity of anti-CEA Fab-H-2K^b/ova conjugate. (A) Demonstration by cytofluorometry of the specific coating of CEA+ human LoVo colon carcinoma cells by anti-CEA Fab-H-2K^b/ova conjugate. The conjugate was revealed by a FITC-labeled anti-H-2K^b mAb (solid line). The negative control consisted of LoVo cells treated with the anti-H-2K^b and the FITC-labeled anti-murine IgG (black area), while the positive control consisted of LoVo cells coated with the anti-CEA mAb 35, followed by FITC-labeled anti-murine Ig antibody. (B) Specific lysis of ^{51}Cr-labeled CEA-expressing human LoVo colon carcinoma cells (black squares) or MC38-CEA+ colon carcinoma cells (black circles) precoated with the anti-CEA Fab-H-2K^b/ova conjugate and incubated with ova specific H-2K^b T cells at the indicated T cell/target cell ratio. Negative controls were uncoated LoVo (open squares) and murine MC38-CEA+ (open circles) cells.

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Specific tumor targeting of radiolabeled anti-CEA Fab-H-2K^b/ova conjugate in CEA transgenic and nude mice

To evaluate the capacity of anti-CEA Fab-H2-K^b/ova conjugate injected i.v. to target CEA-expressing tumors, purified conjugate was labeled with ^{125}I. Two micrograms of ^{125}I-labeled conjugate were then injected i.v. into either CEA transgenic C57BL/6 mice bearing a subcutaneous graft of the syngeneic MC38-CEA+ carcinoma or into nude mice bearing on one lateral side the same murine carcinoma graft, and on the other side, a xenograft of the CEA-expressing human carcinoma LS174T. Twenty-four hours after injection, the mice were sacrificed and the tumor and normal tissue dissected, weighted and the radioactivity counted. The results, expressed as the percentage of injected dose per gram of tissue (% ID/g), show that the ^{125}I-labeled conjugates target the MC38-CEA+ carcinoma grafts (Figure 3, panels A and B), as well as the human colon carcinoma xenografts (Figure 3B).

To further demonstrate the specificity of the targeting, two control F(ab')$_2$ fragments were labeled with ^{131}I and coinjected with the ^{125}I-labeled anti-CEA Fab-H-2K^b/ova conjugate. In the CEA transgenic mice the control fragment was an irrelevant F(ab')$_2$, while in the nude mice the control was an F(ab')$_2$ from an anti-human epidermal growth factor (EGF) receptor mAb. The measurement of radioactivity, performed in a dual-channel scintillation counter, shows that the irrelevant antibody fragment did not target in the MC38-CEA+ murine tumor (Figure 3A), while the anti-human EGF receptor F(ab')$_2$ targeted the human colon carcinoma xenograft LS174T, but not the murine carcinoma (Figure 3B). These double-labeling experiments (21, 22) confirmed the *in vivo* targeting specificity of the anti-CEA Fab-H-2K^b/ova conjugate in the CEA-expressing tumors.

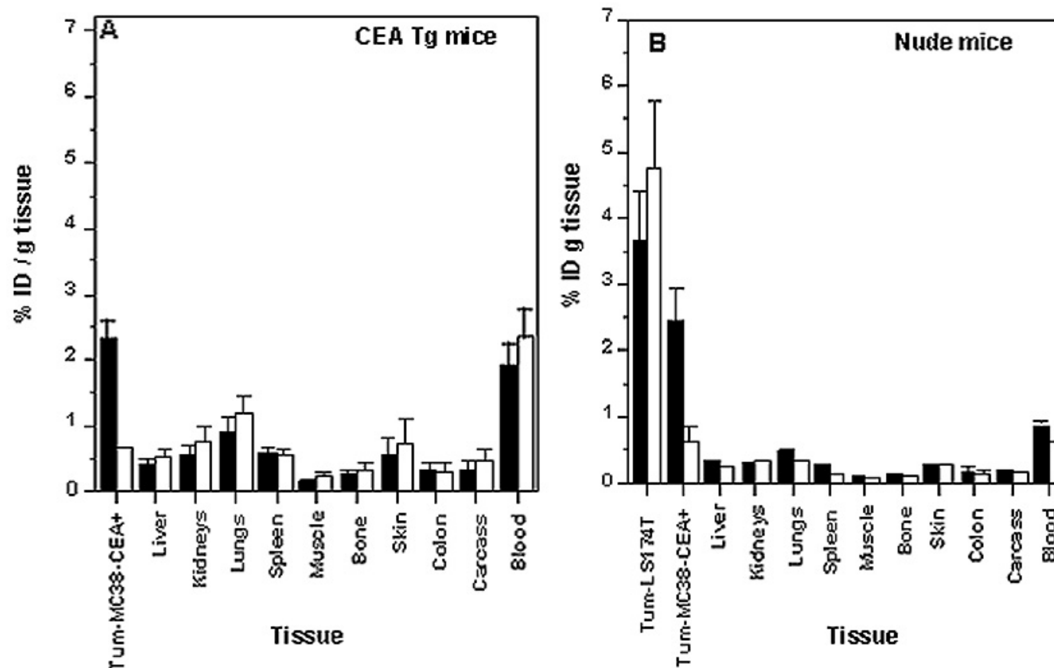

Figure 3. *In vivo* tumor targeting and biodistribution of radiolabeled anti-CEA Fab-H-2K^b/ova conjugates. Demonstration of the specific tumor uptake of radiolabeled anti-CEA-H-2K^b/ova conjugate, as compared with control F(ab')$_2$ fragments, injected i.v. into (A) three CEA transgenic C57BL/6 mice bearing a subcutaneous graft of the syngeneic colon carcinoma MC38-CEA+, and (B) four nude mice each bearing on one lateral side a subcutaneous graft of the same MC38-CEA+ cells and on the other side a second subcutaneous graft of the CEA-expressing human LS174T colon carcinoma cells. All mice were coinjected with 2 µg ^{125}I-labeled anti-CEA Fab-H-2K^b/ova conjugate and with a control F(ab')$_2$ fragment labeled with ^{131}I. For the CEA transfected mice the control F(ab')$_2$ had an irrelevant specificity and for the nude mice the control F(ab')$_2$ was derived from an anti-human EGF receptor mAb. Mice were sacrificed 24 h after injection and the radioactivity for both iodine isotopes was measured in tumors and normal tissues. Results expressed in percent of injected dose per g tissue (% ID/g) for anti-CEA Fab-H-2K^b/ova conjugate (black bars) and for the control F(ab')$_2$ fragments (open bars) show that the anti-CEA conjugate specifically targeted the CEA-expressing tumors while the irrelevant F(ab')$_2$ did not, and the anti-human EGF receptor targeted only the human colon carcinoma xenograft but not the MC38-CEA+ murine carcinoma.

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Systemic injection of anti-CEA Fab-H-2K^b/ova conjugate inhibits tumor growth in OT-1 transgenic mice

OT-1 transgenic C57BL/6 mice carry the TCR genes (Valpha2, Vbeta5.1) isolated from a T cell clone reactive with an H-2K^b/ovalbumin immunodominant octapeptide (257-264) (SIINFELK) (18). More than 90% of CD3-positive cells express the transgene, providing a large pool of specific CTL precursors to test our strategy. In a first approach, 7.5x10^5 syngeneic MC38-CEA+ colon carcinoma cells expressing human CEA were grafted subcutaneously in ten OT-1 mice. A group of five mice was injected i.v. with 20 µg anti-CEA-H-2K^b/ova conjugate on day 1, followed by 5 i.p. injections on days 4, 8, 18, 21 and 24. The control group of five mice was injected in parallel with 20 µg F(ab')$_2$ fragment from the anti-CEA mAb (having about the same Mr of 100 kDa as the conjugate, but without MHC/peptide complex). Tumor size was measured every two days. Figure 4A compares the individual tumor growth curves of all mice from the 2 groups and shows a significant delay in tumor development in the mice treated with the conjugate, as compared to the control group. Indeed, by day 25, of the

conjugate-treated group, two mice had no detectable tumor, one mouse developed only a tiny nodule and two mice had small tumors not exceeding 70 mm^3. In contrast, all mice in the control group had rapidly growing tumors ranging from 300 to 600 mm^3. Figure 4B shows the evolution of the mean tumor volume from all mice up to day 32 in the conjugate-treated and control groups. It demonstrates the effectiveness of the anti-CEA-H-2K^b/ova conjugate in inhibiting tumor growth, with no overlap of the standard deviation between the two groups. The results on day 28 for all mice showed a highly significant difference in tumor size between the two groups, with a mean tumor volume of 77 mm^3 for the conjugate-treated mice and 633 mm^3 for the control mice ($P = 0.0088$; Wilcoxon test). A second series of experiments on two groups of 5 mice entirely confirmed the results presented in Figure 4. This time, three mice out of five did not develop a tumor in the conjugate-treated group, while all five mice in the control group had large tumors 30 days after tumor graft (data not shown).

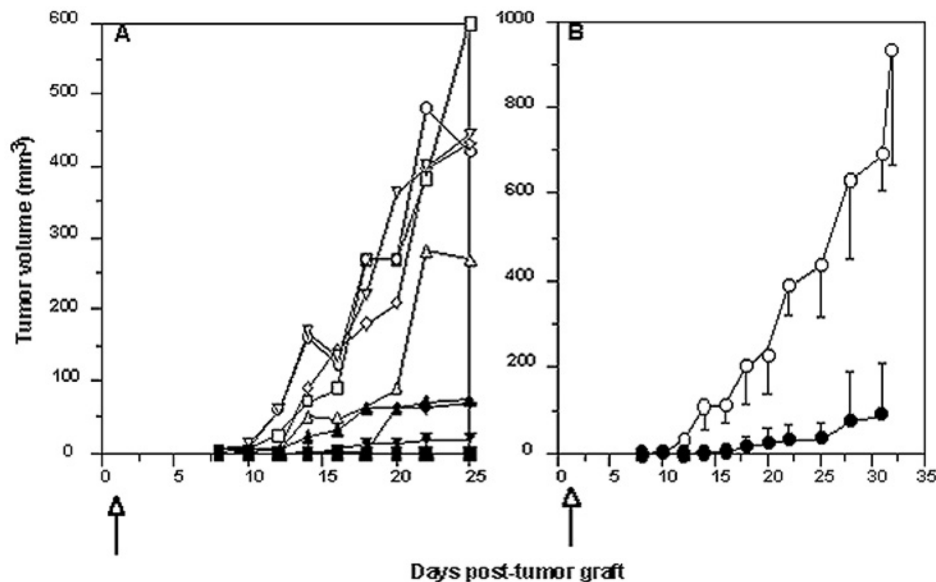

Figure 4. Systemic injection of anti-CEA-H-2K^b/ova conjugate inhibits tumor growth in OT-1 transgenic mice. Kinetics of MC38-CEA+ syngeneic colon carcinoma tumor growth during treatment with the conjugate (filled symbols) or the control anti-CEA F(ab')$_2$ fragment (empty symbols). Day 0 corresponds to tumor grafting and the arrow indicates the start of treatment on day one by injection of 20 µg conjugate i.v., followed by five repeated i.p. injections every four days. Tumor size was measured every two days with a caliper and expressed as a volume by the formula (length x width x height)/2. (A) Individual tumor growth curves for the five mice treated with conjugate and the five control mice over the first 25 days. (B) Means and standard deviations for the treated and control groups until the end of experiment when most control mice had tumors exceeding 1000 mm^3. A Wilcoxon test performed on the day 28 data, when all the mice were still present, gave $P = 0.0088$ for the comparison between treated and control groups.

Injection of anti-CEA Fab-H-2K^b/ova conjugate induces regression and growth inhibition of palpable tumors grafted in syngeneic mice adoptively transferred with OT-1 splenocytes

We also tested the model of adoptive transfer of OT-1 mouse splenocytes into CEA-transgenic mice for two reasons. First, this strategy gives the opportunity to immunize and actively stimulate the transferred ova-specific CTLs in a normal immune environment, which is closer to that of future real cancer immunotherapy conditions. Second, the CEA-transgenic mice are less likely to produce anti-CEA antibodies or T cells directed against the CEA expressed by the transfected tumor graft (19). Fifty million OT-1 splenocytes were transferred intraperitoneally into CEA transgenic C57BL/6 mice, which were immunized one day later with 200 µg ovalbumin

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in adjuvant. As shown in Figure 5A, the peak of activation of H-2K^b/ova specific T cells, determined by H-2K^b/ova tetramer plus CD8 double staining, was reached 10 days after immunization and their frequency ranged between 12 and 40% of total CD8 cells. Figure 5B gives a representative FACS dot blot on PBMCs at the peak of response. To exclude a non-specific tumor growth inhibition due to cytokines released during the peak of the primary immune reaction, 10^6 MC38-CEA+ carcinoma cells were grafted 19 days after ovalbumin immunization (Figure 5A).

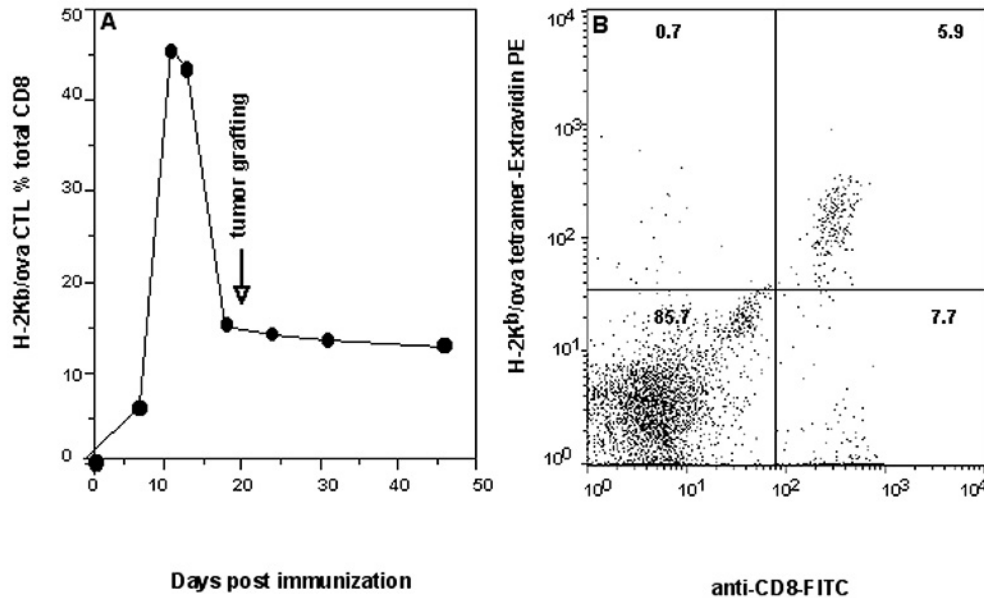

Figure 5. Immune response of CEA-transgenic mice transferred with OT-1 splenocytes and immunized with ovalbumin and adjuvant. (A) Kinetics of the immune response to ova peptide followed by double-color fluorescence staining with anti-CD8 FITC and H-2K^b/ova tetramer-PE. As indicated by the arrow, tumor grafting was performed 19 days after immunization. Results are expressed as the percentage of specific H-2K^b/ova over total CD8 T cells. (B) Representative FACS dot blot at the peak of response 11 days after injection.

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In contrast to the previous experiments in which conjugate treatment was started one day after tumor graft, here we waited until tumor nodules were palpable in the 10 mice (about 8 days). Two groups of five mice were then injected either with the anti-CEA Fab-H-2K^b/ova conjugate or the control anti-CEA F(ab')$_2$. In both groups, the first injections were given i.v. and the following injections i.p. every two days. Results from the treatment are shown in Figure 6. Panel A compares the tumor growth curves from individual mice in the two groups during the 24 days after tumor graft. On day 24, four out of five mice from the conjugate-treated group had tumor volumes smaller than 20 mm^3, while all mice from the control F(ab')$_2$ group had tumors larger than 200 mm^3, as did one of the conjugate-treated mice which escaped tumor treatment. On day 20 after tumor graft, a boost of 100 µg ovalbumin was given to all 10 mice and a smaller secondary response was observed (data not shown). Figure 6B shows the evolution over 36 days after tumor graft of the mean tumor volume in conjugate-treated and control groups, excluding the tumor that escaped therapy. The mean tumor volume on day 35 for the conjugate-treated mice (100 mm^3) was significantly smaller than for the control mice (676 mm^3), (P = 0.0209; Wilcoxon test). If the tumor which did not respond to therapy is included, the mean tumor volume in the treated mice increases to 227 mm^3, but the difference with the controls is still significant (P = 0.05). The results thus demonstrate that the anti-CEA Fab-H-2K^b/ova conjugate can induce tumor growth inhibition even when treatment is started 8 days after tumor grafting, at a time when the tumors are established and palpable.

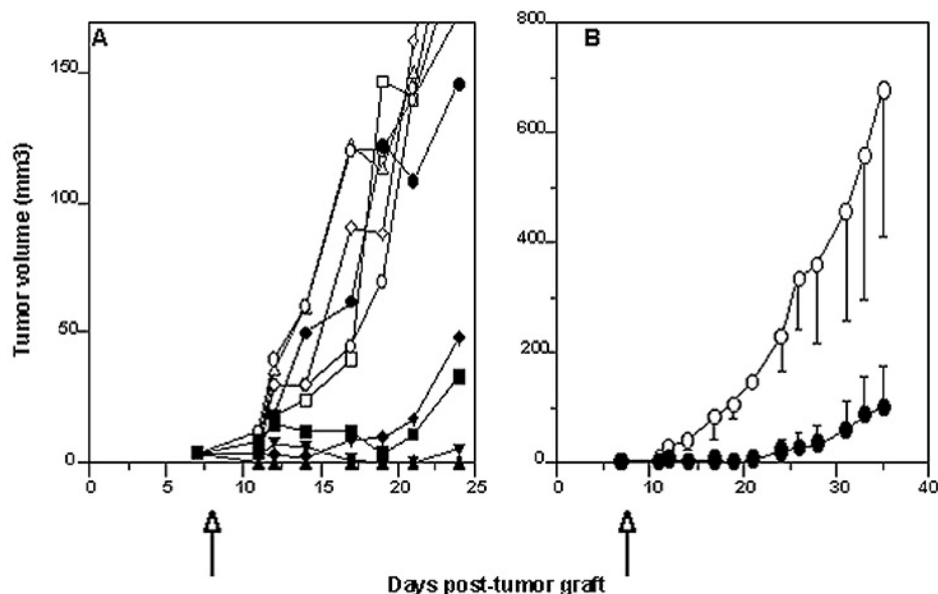

Figure 6. Injection of anti-CEA-H-2K^b/ova conjugate induces regression and growth inhibition of palpable tumors grafted in syngeneic mice adoptively transferred with OT-1 splenocytes. (A) Individual tumor growth curves of all five mice treated with the conjugate and five control mice during the first 24 days after tumor graft. Treatment was started on day 8 (arrow), when all ten mice had palpable nodules. Systemic injection of 20 µg conjugate (filled symbols) or anti-CEA F(ab')$_2$ fragment (empty symbols) was repeated every two days until the end of the experiment. The first three injections were i.v. and the following ones were i.p. (B) Mean values and standard deviations of tumor volumes from four treated and four control mice determined up to day 35. One mouse, which did not respond to treatment as shown in A (black circle), was excluded from the mean value, as was a control mouse which died on day 18, as shown in A (open triangle). On day 35, a Wilcoxon test was performed to compare the tumor volumes, giving $P = 0.0209$ for treated versus control groups, without including the non-responding tumor, and $P = 0.05$ when the non-responding mouse was included.

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Discussion

The purpose of this work is to demonstrate that the novel immunotherapy strategy consisting of targeting an antibody-MHC/peptide conjugate to tumor cells can function *in vivo* in a syngeneic solid tumor model. The conjugate has the property to bridge an antibody, specific for a defined antigen stably expressed on tumor cells, with a selected highly antigenic MHC complex recognized by cytotoxic T lymphocytes.

As preliminary steps, we showed that tumor cells coated *in vitro* with the newly synthesized anti-CEA Fab-H-2K^b/ova peptide conjugate were lysed by ova peptide-specific syngeneic T lymphocytes and that the conjugate was specifically targeted *in vivo* in CEA-expressing tumor grafts. Most importantly, we then demonstrated that both in OT-1 mice, transgenic for an ova peptide specific T cell receptor, and in CEA transgenic C57BL/6 mice, adoptively transferred with OT-1 spleen cells, the systemic injection of anti-CEA Fab-H-2K^b/ova peptide conjugate induced specific growth inhibition and regression of the subcutaneously grafted MC38-CEA+ syngeneic colon carcinoma line.

As a first experimental model, we took advantage of OT-1 transgenic mice in order to exploit their high number of specific T lymphocytes directed against the H-2K^b/ova peptide complex present in our conjugate. A significant

inhibition of tumor growth was observed following injection of the Fab-H-2K^b/ova peptide conjugate, as compared to control mice injected with anti-CEA F(ab')$_2$, and five out of ten conjugate-treated mice never developed tumors. If we consider that treatment was initiated on the first day after tumor graft, the *in vivo* effect of the conjugate, while highly significant ($P = 0.0088$), can be interpreted more as an inhibition of tumor graft than as a therapy of existing tumors. A limitation of the OT-1 model is that the abundant specific T cells present in these mice have a naive phenotype and are difficult to stimulate *in vivo*. This limited reactivity can be explained by the fact that these ova-specific T lymphocytes, when stimulated in the OT-1 environment, are prone to apoptosis (23) and do not proliferate upon antigen stimulation (24).

To benefit from a more normal environment, the second series of tumor therapy experiments was performed in C57BL/6 mice, transgenic for human CEA, which were adoptively transferred with OT-1 spleen cells. In this setting, immunization with ovalbumin induced a strong primary response. The tumor challenge, with the same syngeneic MC38-CEA+ colon carcinoma cells, was given a few days after the primary anti-ovalbumin response to avoid non-specific immunostimulation, but when enough ova peptide-specific memory T cells were still available. This time the treatment, consisting of systemic injections of anti-CEA Fab-H-2K^b/ova conjugate, was initiated 8 days after tumor grafts when all mice had palpable tumors. Four out of five mice responded very well to therapy, while a tumor in one mouse escaped treatment and rapidly grew like the tumors in the five control mice injected with anti-CEA F(ab')$_2$ fragments. Even if the mouse not responding to therapy is taken into account, the mean tumor volume on day 35 in the Fab-H-2K^b/ova conjugate treatment group was significantly smaller than that in the control group.

Interestingly, the significant tumor growth inhibition induced by repeated injections of antibody-MHC/peptide conjugates was obtained with no evidence of toxicity. None of the mice presented any abnormal behavior during or after injection of the conjugates. Even the OT-1 transgenic mice, which are known to have about 90% of their T lymphocytes reacting specifically with the injected H-2K^b molecules, did not present any symptom of toxicity. In fact, none of the 15 mice, which each received from 12 to 16 i.v. or i.p. injections of Fab-MHC/peptide complexes, died during therapy.

These first entirely *in vivo* results with a new immunotherapy strategy compare favorably with experimental results obtained with three other related tumor immunotherapy modalities involving anti-tumor antibodies and local T lymphocyte stimulation:

i) Treatment with anti-CEA single chain Fv antibody fragment fused to IL-2 was tested in the same tumor model as that used here of CEA transgenic mice grafted with an MC38-CEA+ syngeneic colon carcinoma line. This strategy gave some encouraging inhibition of tumor growth, especially when the fusion protein was injected i.v. every other day for 4 weeks starting one day after tumor grafting (25). However, no attempts were reported in this study to treat mice with established palpable tumors following tumor grafting.

ii) The use of bispecific antibody with one arm directed against a TAA and the other against a T cell receptor-associated protein, such as CD3 (26, 27), or an NK cell activating receptor, such as CD16 (28), was proposed eighteen years ago with elegant *in vitro* results. However this strategy has not led to convincing *in vivo* therapy results on solid tumors, most likely due to a lack of T cell activation by the bispecific antibodies. Indeed, when clinical trials of patients with peritoneal dissemination from ovarian carcinoma were performed, the patients' T lymphocytes were first activated *in vitro* and coated with the bispecific antibody, before being reinjected i.p. with the bispecific antibody (29). An alternative strategy was to coinject two types of bispecific antibody, such as anti-tumor/anti-CD3 and anti-tumor/anti-CD28, in order to activate T lymphocytes (30, 31).

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iii) The antibody-mediated targeting of a superantigen, such as staphylococcal enterotoxin A, on tumor cells (32) shares some similarities with our strategy, but in fact is closer to the field of immunotoxins. Indeed, superantigens are toxic molecules by themselves, while our antibody-MHC/peptide conjugates are physiological T cell antigens, which activate T lymphocytes only when oligomerized on target cells (15). Antibody-superantigen fusion proteins have been shown in mice to induce inhibition of lung metastases obtained after i.v. injection of B16 melanoma cells. However, it was observed that in addition to the toxicity, the repeated injections of antibody-SEA fusion protein induced the development of immunological unresponsiveness, requiring the additional injection of anti-tumor antibody-IL-2 fusion proteins (33).

Another essential limitation parameter in most experimental tumor immunotherapy models is the time of initiation of therapy. For instance, in elegant studies of adoptive transfer of T lymphocytes from transgenic mice bearing a T cell receptor specific for a rejection antigen expressed by a chemically-induced fibrosarcoma, it was shown that the transfer of as few as $3x10^4$ specific T cells could eradicate a fibrosarcoma subcutaneous graft of $3x10^6$ cells when injected 3 days after tumor challenge (34). However, on day 5 after tumor graft, adoptive transfer of $3x10^6$ specific T cells could eradicate the tumor in only three out of five mice and, on day 7 or later, a similar transfer of specific T cells could not prevent tumor growth in any mouse. These results suggest that when such a malignant tumor has reached a certain size, the same specific T cells which were very active at the early stage of tumor development can no longer attack the tumor.

In our model, we did not study the number of specific T cells necessary for adoptive transfer, but we showed that, in mice transferred with a high number of OT-1 spleen cells, injection of specific anti-tumor-MHC/peptide conjugates as late as 8 days after tumor graft was able to induce regression and inhibition of tumor growth. Thus, in comparison with related forms of immunotherapy, this first report of entirely *in vivo* testing of a new experimental treatment with anti-tumor antibody-MHC/peptide conjugates in a syngeneic carcinoma model is encouraging. The limitation of our results, however, is that in all mice treated at 8 days post-tumor graft, the tumors ultimately developed despite continued conjugate injection. The temporary nature of the therapeutic effect may be due to some limitations of our model:

a) The tumors were not well vascularized, as determined macroscopically by their white appearance and histologically by the limited number of blood vessels. Thus, the *in vivo* tumor localization of ^{125}I-labeled Fab-MHC/ova conjugates gave a highly specific, but relatively modest, % ID per g tumor. In previous experiments using a bispecific anti-CEA-anti-TNF antibody with the same monovalent anti-CEA Fab fragment, we were able to deliver more than 20% ID per g tumor in a human colon carcinoma xenografted in nude mice (22), whereas here the % ID per g tumor was approximately 2.5%. Thus, other experimental tumors and mAbs directed against different membrane antigens, such as mAbs A33, anti-Muc-I, anti-Her2 or anti-CD20 (35, 36), should also be tested for targeting MHC complexes on tumor cells.

b) While the specific T cell response to peptide or ovalbumin immunization was weak in OT-1 mice, the primary response to ovalbumin in adoptively transferred syngeneic mice was strong. However, in the latter, the secondary response was less significant and transient. In some of the treated mice, like the one which escaped therapy, the percentage of specific CTLs dropped to around 1-2% of total circulating CD8 T cells (data not shown), which may explain why at later time points the tumors no longer responded to antibody-MHC complexes therapy. In these early experiments no attempt has been made to inject IL-2, IL-12, or other cytokines that could increase the activation of specific T cells against the Fab-MHC /peptide-coated cells (37). However, this could be considered in further experiments.

Despite the limitations of this model, specific tumor regression and inhibition of tumor growth were obtained by the exclusive injection of antibody-MHC/peptide conjugate, which clearly demonstrates the feasibility of this new

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immunotherapy strategy. If one considers the potential clinical application of this immunotherapy strategy, there are definitive advantages in the antibody-mediated targeting of MHC complexes containing immunodominant viral peptides antigens, against which human beings have learned to develop high-affinity T cell clones, probably representing the strongest immune defences of our organism. Cancer patients could be injected with various anti-TAA mAbs conjugated to autologous MHC containing well-defined antigenic viral peptides derived from common viruses such as influenza, EBV, or CMV, against which they have an active T cell memory that could eventually be boosted by viral vaccination. The tumor targeting of these antigenic MHC complexes in patients will redirect the viral T cell response against the tumor cells and has a great chance to induce a more powerful anti-tumor response than the one observed in the first *in vivo* experiments described here.

Abbreviations

beta2M, beta2-microglobulin; CEA, carcinoembryonic antigen; EGF, epidermal growth factor; ID, injected dose; ova, ovalbumin; TAA, tumor-associated antigen

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Acknowledgements

We thank Pedro Romero and Hans Acha-Orbea for advice and suggestions, Gzime Idrizi for excellent technical assistance, Philippe Guillaume for help in MHC refolding, Alexis Kalergis and Stanley G. Nathenson for providing the C-terminal cysteine H-2K^{b} mutant gene construct, and Sally Betz-Corradin for reviewing the manuscript. This work was supported by grants from the Cancer Research Institute in New York and from the Swiss Cancer League.

References

1. McLaughlin P, Grillo-Lopez AJ, Link BK, Levy R, Czuczman MS, Williams ME, Heyman MR, Bence-Bruckler I, White CA, Cabanillas F, Jain V, Ho AD, Lister J, Wey K, Shen D, Dallaire BK. Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program. *J Clin Oncol* 1998; **16**: 2825-33. (PMID: 9704735)

2. Cobleigh MA, Vogel CL, Tripathy D, Robert NJ, Scholl S, Fehrenbacher L, Wolter JM, Paton V, Shak S, Lieberman G, Slamon DJ. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J Clin Oncol* 1999; **17**: 2639-48. (PMID: 10561337)

3. Delaloye B, Bischof-Delaloye A, Buchegger F, von Fliedner V, Grob JP, Volant JC, Pettavel J, Mach JP. Detection of colorectal carcinoma by emission-computerized tomography after injection of 123I-labeled Fab or F(ab')2 fragments from monoclonal anti-carcinoembryonic antigen antibodies. *J Clin Invest* 1986; **77**: 301-11. (PMID: 3484753)

4. Welt S, Divgi CR, Kemeny N, Finn RD, Scott AM, Graham M, Germain JS, Richards EC, Larson SM, Oettgen HF. Phase I/II study of iodine 131-labeled monoclonal antibody A33 in patients with advanced colon cancer. *J Clin Oncol* 1994; **12**: 1561-71. (PMID: 8040668)

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5. Boon T, Cerottini JC, Van den Eynde B, van der Bruggen P, Van Pel A. Tumor antigens recognized by T lymphocytes. *Annu Rev Immunol* 1994; **12**: 337-65. (PMID: 8011285)

6. Chen YT, Scanlan MJ, Sahin U, Tureci O, Gure AO, Tsang S, Williamson B, Stockert E, Pfreundschuh M, Old LJ. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc Natl Acad Sci USA* 1997; **94**: 1914-8. (PMID: 9050879)

7. Old LJ. Cancer/testis (CT) antigens - a new link between gametogenesis and cancer. *Cancer Immun* 2001; **1**: 1. (PMID: 12747762)

8. Marchand M, van Baren N, Weynants P, Brichard V, Dreno B, Tessier MH, Rankin E, Parmiani G, Arienti F, Humblet Y, Bourlond A, Vanwijck R, Lienard D, Beauduin M, Dietrich PY, Russo V, Kerger J, Masucci G, Jager E, De Greve J, Atzpodien J, Brasseur F, Coulie PG, van der Bruggen P, Boon T. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1. *Int J Cancer* 1999; **80**: 219-30. (PMID: 9935203)

9. Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, Restifo NP, Dudley ME, Schwarz SL, Spiess PJ, Wunderlich JR, Parkhurst MR, Kawakami Y, Seipp CA, Einhorn JH, White DE. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat Med* 1998; **4**: 321-7. (PMID: 9500606)

10. Lee PP, Yee C, Savage PA, Fong L, Brockstedt D, Weber JS, Johnson D, Swetter S, Thompson J, Greenberg PD, Roederer M, Davis MM. Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. *Nat Med* 1999; **5**: 677-85. (PMID: 10371507)

11. Parmiani G, Sensi M, Castelli C, Rivoltini L, Anichini A. T-cell response to unique and shared antigens and vaccination of cancer patients. *Cancer Immun* 2002; **2**: 6. (PMID: 12747751)

12. Ferrone S, Marincola FM. Loss of HLA class I antigens by melanoma cells: molecular mechanisms, functional significance and clinical relevance. *Immunol Today* 1995; **16**: 487-94. (PMID: 7576053)

13. Ogg GS, Dunbar PR, Cerundolo V, McMichael AJ, Lemoine NR, Savage P. Sensitization of tumour cells to lysis by virus-specific CTL using antibody-targeted MHC class I/peptide complexes. *Br J Cancer* 2000; **82**: 1058-62. (PMID: 10737389)

14. Robert B, Guillaume P, Luescher I, Romero P, Mach JP. Antibody-conjugated MHC class I tetramers can target tumor cells for specific lysis by T lymphocytes. *Eur J Immunol* 2000; **30**: 3165-70. (PMID: 11093131)

15. Robert B, Guillaume P, Luescher I, Doucey MA, Cerottini JC, Romero P, Mach JP. Redirecting anti-viral CTL against cancer cells by surface targeting of monomeric MHC class I-viral peptide conjugated to antibody fragments. *Cancer Immun* 2001; **1**: 2. (PMID: 12747763)

16. Savage P, Cowburn P, Clayton A, Man S, Lawson T, Ogg G, Lemoine N, McMichael A, Epenetos A. Anti-viral cytotoxic T cells inhibit the growth of cancer cells with antibody targeted HLA class I/peptide complexes in SCID mice. *Int J Cancer* 2002; **98**: 561-6. (PMID: 11920616)

17. Lev A, Novak H, Segal D, Reiter Y. Recruitment of CTL activity by tumor-specific antibody-mediated targeting of single-chain class I MHC-peptide complexes. *J Immunol* 2002; **169**: 2988-96. (PMID: 12218113)

18. Hogquist KA, Jameson SC, Heath WR, Howard JL, Bevan MJ, Carbone FR. T cell receptor antagonist peptides induce positive selection. *Cell* 1994; **76**: 17-27. (PMID: 8287475)

19. Clarke P, Mann J, Simpson JF, Rickard-Dickson K, Primus FJ. Mice transgenic for human carcinoembryonic antigen as a model for immunotherapy. *Cancer Res* 1998; **58**: 1469-77. (PMID: 9537250)

20. Kalergis AM, Goyarts EC, Palmieri E, Honda S, Zhang W, Nathenson SG. A simplified procedure for the preparation of MHC/peptide tetramers: chemical biotinylation of an unpaired cysteine engineered at the C-terminus of MHC-I. *J Immunol Methods* 2000; **234**: 61-70. (PMID: 10669770)

21. Buchegger F, Haskell CM, Schreyer M, Scazziga BR, Randin S, Carrel S, Mach JP. Radiolabeled fragments of monoclonal antibodies against carcinoembryonic antigen for localization of human colon carcinoma grafted into nude mice. *J Exp Med* 1983; **158**: 413-27. (PMID: 6886623)

22. Robert B, Mach JP, Mani JC, Ychou M, Folli S, Artus JC, Pelegrin A. Cytokine targeting in tumors using a bispecific antibody directed against carcinoembryonic antigen and tumor necrosis factor alpha. *Cancer Res* 1996; **56**: 4758-65. (PMID: 8840995)

23. Kennedy NJ, Russell JQ, Michail N, Budd RC. Liver damage by infiltrating CD8+ T cells is Fas dependent. *J Immunol* 2001; **167**: 6654-62. (PMID: 11714837)

24. Attinger A, MacDonald HR, Acha-Orbea H. Lymphoid environment limits superantigen and antigen-induced T cell proliferation at high precursor frequency. *Eur J Immunol* 2001; **31**: 884-93. (PMID: 11241294)

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25. Xu X, Clarke P, Szalai G, Shively JE, Williams LE, Shyr Y, Shi E, Primus FJ. Targeting and therapy of carcinoembryonic antigen-expressing tumors in transgenic mice with an antibody-interleukin 2 fusion protein. *Cancer Res* 2000; **60**: 4475-84. (PMID: 10969795)

26. Staerz UD, Kanagawa O, Bevan MJ. Hybrid antibodies can target sites for attack by T cells. *Nature* 1985; **314**: 628-31. (PMID: 2859527)

27. Perez P, Hoffman RW, Shaw S, Bluestone JA, Segal DM. Specific targeting of cytotoxic T cells by anti-T3 linked to anti-target cell antibody. *Nature* 1985; **316**: 354-6. (PMID: 3160953)

28. Ferrini S, Prigione I, Miotti S, Ciccone E, Cantoni C, Chen Q, Colnaghi MI, Moretta L. Bispecific monoclonal antibodies directed to CD16 and to a tumor-associated antigen induce target-cell lysis by resting NK cells and by a subset of NK clones. *Int J Cancer* 1991; **48**: 227-33. (PMID: 1826900)

29. Bolhuis RL, Lamers CH, Goey SH, Eggermont AM, Trimbos JB, Stoter G, Lanzavecchia A, di Re E, Miotti S, Raspagliesi F, Rivoltini L, Colnaghi MI. Adoptive immunotherapy of ovarian carcinoma with bs-MAb-targeted lymphocytes: a multicenter study. *Int J Cancer* 1992; **Suppl 7**: 78-81. (PMID: 1428412)

30. Renner C, Jung W, Sahin U, Denfeld R, Pohl C, Trumper L, Hartmann F, Diehl V, van Lier R, Pfreundschuh M. Cure of xenografted human tumors by bispecific monoclonal antibodies and human T cells. *Science* 1994; **264**: 833-5. (PMID: 8171337)

31. Grosse-Hovest L, Brandl M, Dohlsten M, Kalland T, Wilmanns W, Jung G. Tumor-growth inhibition with bispecific antibody fragments in a syngeneic mouse melanoma model: the role of targeted T-cell co-stimulation via CD28. *Int J Cancer* 1999; **80**: 138-44. (PMID: 9935244)

32. Dohlsten M, Hedlund G, Akerblom E, Lando PA, Kalland T. Monoclonal antibody-targeted superantigens: a different class of anti-tumor agents. *Proc Natl Acad Sci USA* 1991; **88**: 9287-91. (PMID: 1924393)

33. Rosendahl A, Kristensson K, Carlsson M, Skartved NJ, Riesbeck K, Sogaard M, Dohlsten M. Long-term survival and complete cures of B16 melanoma-carrying animals after therapy with tumor-targeted IL-2 and SEA. *Int J Cancer* 1999; **81**: 156-63. (PMID: 1924393)

34. Hanson HL, Donermeyer DL, Ikeda H, White JM, Shankaran V, Old LJ, Shiku H, Schreiber RD, Allen PM. Eradication of established tumors by CD8+ T cell adoptive immunotherapy. *Immunity* 2000; **13**: 265-76. (PMID: 10981969)

35. Glennie MJ, Johnson PW. Clinical trials of antibody therapy. *Immunol Today* 2000; **21**: 403-10. (PMID: 10916144)

36. Mach JP. Tumours: Targeting of monoclonal antibodies for imaging and potential for therapy. In: Encyclopedia of Life Sciences. London: Nature Publishing Group; 2002. p. 1-13. URL: http://www.els.net

37. Colombo MP, Trinchieri G. Interleukin-12 in anti-tumor immunity and immunotherapy. *Cytokine Growth Factor Rev* 2002; **13**: 155-68. (PMID: 11900991)

38. Glennie MJ, McBride HM, Worth AT, Stevenson GT. Preparation and performance of bispecific F(ab' gamma)2 antibody containing thioether-linked Fab' gamma fragments. *J Immunol* 1987; **139**: 2367-75. (PMID: 2958547)

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Materials and methods

Animals and cell lines

OT-1 TCR transgenic mice of C57BL/6 background (18) and CEA transgenic mice [C57BL/6J-TgN (CEAGe)18FJP] (19) were bred at the animal facility of the Centre de Recherche en Cancerologie in Montpellier, France. The murine chemically-induced colon carcinoma cell line, MC38, transfected with human CEA (clone C15) and referred to as MC38-CEA+ (19) was maintained in DMEM supplemented with 10% FCS and 500 µg/ml G418. The human colon carcinoma cell lines, LoVo (ATCC CCL-229) and LS174T (ATCC CL-188), expressing CEA were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in RPMI 1640 supplemented with 10% FCS.

Monoclonal antibodies and fragments

Anti-CEA mAb 35A7 is a murine IgG1 specific for CEA and does not bind to cross-reacting antigens expressed by granulocytes (21). $F(ab')_2$ fragments were prepared by pepsin digestion (Sigma Chemical Co., St. Louis, MO) at a 3:100 (wt/wt) ratio of pepsin/IgG and incubated at 37°C in 0.2 M acetate buffer pH 4.0 for 15 h, followed by gel filtration on a Superdex 200 column (Pharmacia, Uppsala, Sweden) (15, 38). Fab' fragments were obtained by reduction of the $F(ab')_2$ with 5 mM beta-mercaptoethanol (Fluka, Buchs, Switzerland) for 30 min at 30°C in 0.15 mM phosphate buffer pH 6.5, followed by gel filtration on a Superdex 75 column (Pharmacia) (38).

Generation of an MHC-peptide mutant

The H-2K^b heavy chain construct with an engineered cysteine at the C-terminus was kindly provided by Alexis Kalergis (20) and was further modified by site-specific mutation to replace a natural free cysteine at position 121 by an alanine. Renaturation of the modified H-2K^b peptide complex was performed in bacteria and refolded by dialysis as described (20). Briefly, the H-2K^b heavy chain and beta2M were produced as inclusion bodies in *Escherichia coli*. The heavy chain, beta2M, and the chemically synthesized H-2K^b-restricted ova (257-264) (SIINFEKL) immunodominant peptide were refolded by dialysis and purified on a Sephacryl S100 column (Pharmacia).

Anti-CEA Fab' fragment-H-2K^b/ova peptide conjugate synthesis

The anti-CEA Fab' fragment was incubated with a 25 molar excess of the cross-linker N,N'-*ortho*-phenylene dimaleimide (Sigma) for 2 h at room temperature in 0.15 M phosphate buffer pH 6.5. Excess coupling reagent was eliminated by gel filtration on a Superdex 75 column (Pharmacia). After concentration of the two partners to 1 mg/ml, a 1.5 molar excess of the dimaleimide-derivatized 50 kDa anti-CEA Fab' fragment was immediately coupled to a freshly prepared H-2K^b/ova peptide complex followed by a 2 h incubation at room temperature. The conjugate was purified by FPLC on a Superdex 200 column and analyzed under non-reducing and reducing conditions by 12% SDS-PAGE. The concentration was determined by the Bradford method (Bio-Rad, Hercules, CA).

H-2K^b/ova tetramer

To obtain an H-2K^b/ova tetramer, freshly refolded and purified mutant H-2K^b/ova complexes were incubated with a 2.5 M excess of biotin-maleimide (Pierce Chemicals Co., Rockford, IL) for 2 hours at room temperature. After removal of the excess biotin on a S75 Superdex column, the biotinylated H-2K^b/ova complexes were tetramerized by stepwise addition of extravidin phycoerythrin (Sigma) at a ratio of 4:1.

Flow cytometry analysis

The CEA+ human colon carcinoma LoVo cells were incubated with the anti-CEA-H-2K^b/ova peptide conjugate at 10 µg/ml for 45 min at room temperature in 50 µl PBS, 2% BSA, 0.02% azide. After two washes, the conjugate was analyzed by a FACS Calibur instrument (Becton Dickinson, San Jose, CA) using a conformational sensitive anti-H-2K^b-FITC mAb (clone AF6-88.5; BD Pharmingen, San Diego, CA), added to the cells for 20 min at 4°C. As a positive control, the intact anti-CEA mAb 35A7 at 10 µg/ml was used, followed by a FITC-labeled anti-mouse IgG Fc specific (Sigma).

The kinetics of immunization of CEA transgenic mice adoptively transferred with OT1 splenocytes was followed by H-2K^b/ova tetramer and anti-CD8 (53.6.7-FITC, Pharmingen) double-staining on fresh PBMCs isolated from

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the tail vein. Briefly, after ammonium chloride treatment to remove red blood cells, the PBMCs were washed twice with PBS and incubated with the K^b/ova tetramer for 45 min at 4°C. Anti-CD8-FITC was then added to the cells without washing, for 20 min at 4°C. Samples were run on a FACSCalibur instrument (Becton Dickinson) and the data analyzed using CELL QUEST software.

Chromium-release cytotoxicity assay

The two target cells, the murine MC38-CEA+ carcinoma cells (after saturation of their free H-$2K^b$ MHC I molecules with 1 µM of an irrelevant VSV peptide (RGYVYQGL) for one hour at 37°C) and the CEA-expressing human LoVo carcinoma cells, were incubated for 45 min at 37°C with 10 µg/ml anti-CEA-H-$2K^b$/ova conjugate. During the same incubation, the tumor cells were labeled with ^{51}Cr. After three washings with DMEM, the target cells (2000 cells per well) were incubated for 4 h at 37°C at the indicated T cell to target cell ratios in V-bottomed microwell plates. The peptide-specific T lymphocytes were harvested from the spleen of an OT-1 mouse and cultured *in vitro* for five days with 1 µM ova peptide. Chromium release was measured and the percentage specific lysis was calculated as: 100 x [(experimental - spontaneous release) / (total - spontaneous release)]. As negative controls, the MC38-CEA+ tumor target cells preincubated with the irrelevant VSV peptide or the LoVo cells alone were tested with the same CTL clone.

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In vivo targeting of radiolabeled anti-CEA Fab-H-2Kb/ova conjugate

Three CEA transgenic C57BL/6 mice and four nude mice of Swiss genetic background (Iffa- Credo, L'Abresle, France) were subcutaneously grafted with the MC38-CEA+ colon carcinoma. The four nude mice were also grafted on the other lateral side with the CEA-expressing human colon carcinoma LS174T. Twenty micrograms of purified anti-CEA Fab-H-$2K^b$/ova conjugate were labeled with 20 µCi ^{125}I (1 Ci = 37 Gbq) by the Iodogen method (Pierce Chemicals). The size of the ^{125}I-labeled conjugate was controlled by filtration on a S200 FPLC column, where it eluted with a Mr of 95 KDa, as its unlabeled counterpart. The percentage of immunoreactivity of the ^{125}I-labeled conjugate was verified by a 3 h incubation at room temperature on an excess of CEA chemically coupled to CNBr-Sepharose (Pharmacia) and found to be 70 to 80%. Twenty micrograms of two control F(ab')$_2$ fragments, one with irrelevant specificity and one derived from a mAb reacting specifically with the human EGF receptor, were similarly labeled with 20 µCi of ^{131}I. Two micrograms of ^{125}I-labeled conjugate mixed with 2 µg of one of the ^{131}I-labeled control F(ab')$_2$ were coinjected i.v. in the tail vein of all mice. In the CEA transgenic mice, the control F(ab')$_2$ was that of irrelevant specificity, whereas in the nude mice it was the anti-human EGF receptor. After 24 h, mice were sacrificed and the radioactivity of both iodine isotopes in tumor, blood and normal organs was measured in a dual channel gamma counter. Results are expressed, for both iodine isotopes, as the percentage of injected dose per gram of tissue (% ID/g tissue).

Adoptive transfer and ovalbumin immunization

Fifty million OT-1 spleen cells were harvested, washed and injected i.p., in a volume of 0.5 ml DMEM medium, into unirradiated CEA transgenic mice. After 24 h, recipient mice were immunized by s.c. injection of 200 µg whole ovalbumin (Grade VI, Sigma) emulsified in Montanide ISA 720 adjuvant (Seppic, France).

Tumor immunotherapy

Inhibition of tumor growth in OT-1 mice

On day 0, ten OT-1 mice were subcutaneously grafted with 0.75x10^6 MC38-CEA+ syngeneic colon carcinoma cells in 200 µl PBS. Starting on day one, five mice were treated every four days by first i.v. and subsequently i.p.

injections of 20 µg of anti-CEA-H-2K^b/ova conjugate in 200 µl of PBS. As negative controls, the other five mice were injected by the same route with 20 µg anti-CEA $F(ab')_2$ fragments without MHC in 200 µl PBS.

Tumor treatment in adoptively transferred CEA transgenic mice

Ten CEA transgenic mice, adoptively transferred with fifty million OT-1 splenocytes 20 days previously and immunized with 200 µg ovalbumin in Montanide adjuvant the next day, were subcutaneously grafted on day 0 with 10^6 MC38-CEA+ cells in 200 µl PBS. On day 8, when tumor nodules were palpable in all mice, five mice were treated every two days with 20 µg anti-CEA Fab-H-2K^b/ova conjugate in 200 µl of PBS first by i.v. injections, and subsequently by i.p. injections. The other five mice were injected by the same route with 20 µg control anti-CEA $F(ab')_2$ fragments without MHC in 200 µl PBS. All mice were boosted with 100 µg ovalbumin in Montanide adjuvant on day 20 after tumor grafting.

Tumor growth was monitored every two days. The tumor size was determined by measuring the three orthogonal diameters with a caliper and using the formula: (length x width x thickness)/2. Mean tumor size and standard deviation were calculated for each group of mice at each time point.

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