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CD40 triggering increases the efficiency of dendritic cells for antitumoral immunization

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Abstract

Recent studies have shown the importance of triggering CD40 molecules to enhance the efficiency of dendritic cells (DCs) as antigen-presenting cells (APCs). The P198 and P1A tumor antigens, which are expressed by mastocytoma P815, have been assessed for their immunogenicity using different modes of immunization. We measured CTL responses induced *in vivo* with antigenic peptides P198 and P1A loaded onto bone marrow-derived DCs that had matured as a consequence of CD40-CD40L interactions. CD40L-transfected 3T3 fibroblasts were used as a source of CD40L signal. Our results show that this mode of DC activation considerably improves their ability to induce CTLs against P198 and P1A antigens *in vivo* as compared to untreated DCs. We also show that immunizations carried out with CD40L-activated DCs loaded with the P1A peptide induce a very efficient protection against a lethal challenge with P815 tumor cells, which express P1A. Our results indicate that the efficiency of DC-based vaccines used in clinical trials of cancer immunotherapy could be increased significantly by triggering DCs via CD40 prior to immunization.

Introduction

In order to increase the capability of tumor cells to present tumor antigens in an immunogenic way to T cells, tumor cells have been submitted to mutagenic treatments ([1](#)) or transfected with genes encoding molecules involved in the development of T cell responses such as lymphokines or costimulatory molecules ([2](#)). These results suggested a direct presentation of these antigens by the tumor cells. But other reports have clearly shown that, in a number of *in vivo* situations, tumor antigens were presented indirectly by host APCs and probably mainly by dendritic cells (DCs) ([3](#), [4](#)). This antigen-presenting capacity of DCs appears to correlate with their

level of expression of B7-2 and MHC class II molecules (5). Different signals can upregulate the expression by DCs of molecules involved in the induction of CTL responses and antitumor protection. These signals consist mainly of inflammatory stimuli and of interactions that trigger CD40 (6, 7). Interactions with CD40 molecules leading to DC maturation have been shown to occur *in vitro* as well as *in vivo* through contacts with activated CD4 Th1 cells that express the CD40 ligand (8, 9, 10, 11), with CD40L-transfected tumor cells (12, 13), with CD40L-transfected fibroblasts (14, 15, 16) or with an activating anti-CD40 antibody (7, 17, 18, 19, 20, 21).

In studies performed in mice, tumor antigens were introduced into DCs by different means. One was to pulse DCs *in vitro* with protein (22), RNA (23) or DNA (24) derived from tumor cells. Alternative approaches were either to fuse tumor cells with DCs (25) or to load antigenic peptides onto DCs. In this last situation, immunizations were performed either with mixtures of peptides eluted from tumor cells expected to present tumor antigens (26, 27, 28) or with synthetic antigenic peptide(s) (29, 30, 31).

Among the different murine tumors used in such studies, mastocytoma P815 is the most thoroughly characterized. Five different antigens are recognized by CTLs on this tumor (32, 33). Among these antigens, the major target of the rejection response was shown to consist of a nonameric peptide encoded by gene P1A, which is silent in normal tissues and expressed in a number of mouse tumors (34). Antigen P198 is a different antigen which is expressed by an immunogenic variant obtained after mutagenesis of P815 tumor cells, and which consists of a peptide corresponding to a point mutation in a gene expressed ubiquitously (1, 35). Different modes of immunization with these two antigens have been tested for their efficiency to induce antitumor CTL responses or antitumor protection. Mice were injected with a recombinant adenovirus coding for P1A (36), with DNA encoding P1A (37), with L1210 cells expressing P1A and the B7-1 costimulator (38), with P198- or P1A-peptide pulsed APCs coinjected with IL-12 (39) or with P198 or P1A peptides in adjuvant with IL-12 (40). These different protocols allowed the induction of efficient antitumor CTL responses and/or antitumor protection. In the two protocols using tumor peptides, the use of IL-12 was found to significantly improve the efficiency of the immunization.

In this context, the aim of our study was to investigate T cell responses induced *in vivo* by P198 or P1A tumor antigenic peptides loaded on bone marrow-derived DCs that had matured as a consequence of CD40 triggering. Under these conditions of maturation DCs secrete IL-12. Our results indicate that CD40 triggering considerably enhances the ability of DCs to induce specific CTLs against the P198 or P1A peptides. We also found that with the P1A peptide this approach induces strong antitumor protection against P815 tumor cells.

Results

CD40L-activated DCs loaded with the P198 tumor peptide induce antitumor CTL responses *in vivo*

Tumor antigen P198 consists of an H-2K^d-restricted nonapeptide resulting from a point mutation. P198 tumor cells that express this antigen are strongly immunogenic. We tested the induction of P198-specific CTLs *in vivo* after immunization with P198 peptide-loaded DCs activated via CD40 triggering. We used 3T3 fibroblasts transfected with CD40L as a source of CD40L for DC activation. We first confirmed the efficiency of this activation by showing that bone marrow (BM) derived DCs collected after 7 days of culture with GM-CSF and coincubated with CD40L-transfected 3T3 cells during 4 days strongly upregulate expression of MHC class II and B7-2 molecules, whereas expression of B7-1 molecules did not change much (Fig. 1A). The fact that only a fraction of the DC population showed this activated phenotype could result from the low proportion (about 30%)

of transfected 3T3 cells that actually expressed CD40L. As expected, these DCs produce a significant amount of IL-12, whereas no IL-12 production was observed when untransfected 3T3 cells were used for the coculture or when DCs were cultured alone (Fig. 1B). The CD40 triggering also induces BM-derived DCs to develop larger veils and dendrites compared to controls (data not shown).

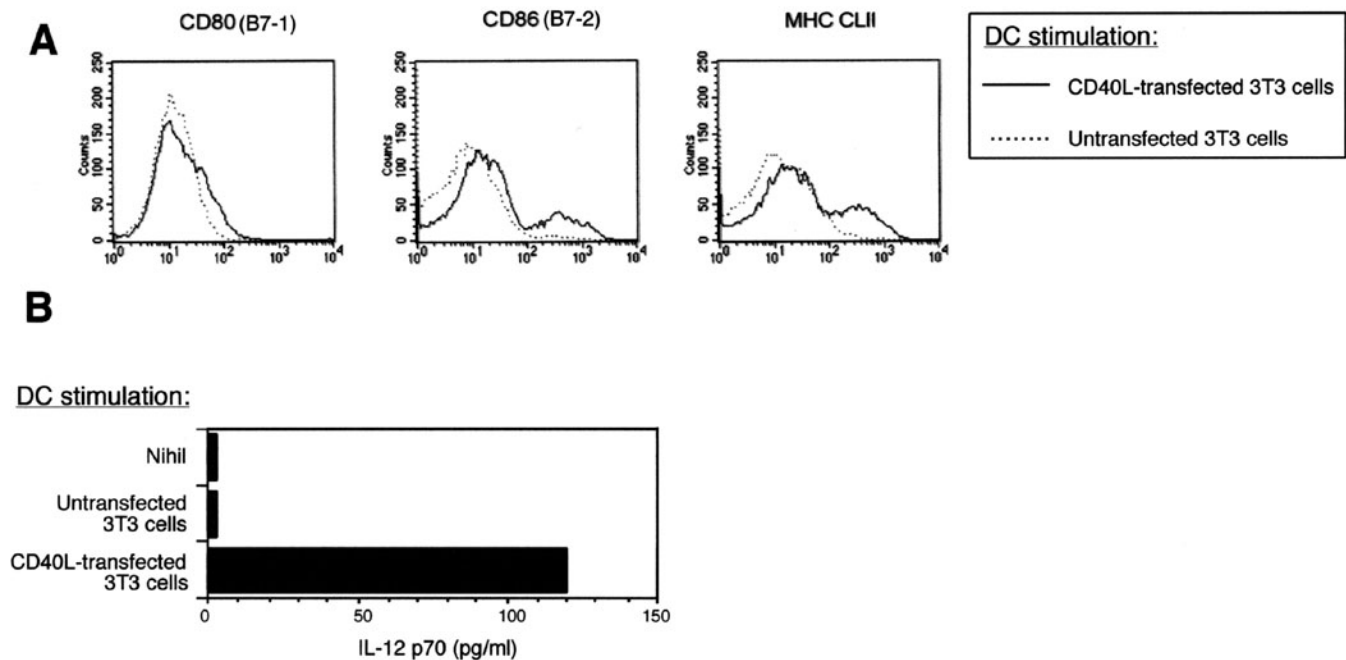


Figure 1. Upregulation of CD80 (B7-1), CD86 (B7-2) and MHC class II expression and induction of IL-12 production by CD40 triggering of bone marrow-derived DCs. BM-derived DCs collected after 7 days of culture with GM-CSF were cocultured for 4 days in GM-CSF either with CD40L-transfected 3T3 cells or with untransfected 3T3 cells. These cells were then processed for double staining using anti-CD11c (N418) mAbs followed by anti-B7-1, anti-B7-2 or anti-MHC class II mAbs. (A) Staining of the different mAbs on CD11c+ cells. (B) BM-derived DCs collected after 7 days of culture in the presence of GM-CSF were exposed to the indicated stimuli for 48 hours with GM-CSF and IL-12 p70 heterodimer production was determined by ELISA. Each value is the mean of 3 different experiments.

Mice were then injected subcutaneously with CD40L-activated DCs loaded with the P198 peptide. Spleen cells were harvested after the second injection and stimulated *in vitro* before being tested for P198-specific CTL activity. As shown in Figure 2, striking CTL responses against target cells that express the immunizing P198 peptide (P198 tumor cells or L1210 tumor cells loaded with the P198 peptide) were observed. In contrast, when untreated DCs loaded with the P198 peptide were used for immunization, most of the mice did not display any CTL activity against the P198 antigen.

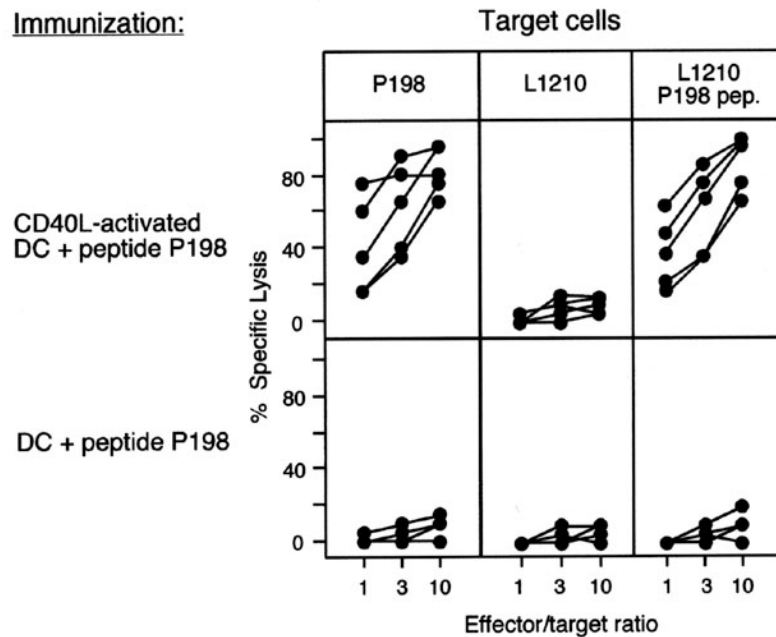


Figure 2. Induction of P198-specific CTLs by immunization with peptide-pulsed DCs activated by CD40 triggering. Mice were injected subcutaneously in the footpads on days 0 and 14 with 3×10^5 P198-loaded DCs either previously cocultured with CD40L-transfected 3T3 cells (CD40L-activated DC + peptide P198) or untreated (DC + peptide P198). Spleen cells from injected mice were harvested on day 21 and stimulated *in vitro* with P198 tumor cells. Six days later, these cultures were submitted to a second short-term stimulation (4 days) with P198 tumor cells and subsequently tested on ^{51}Cr -labeled P198 or L1210 tumor cells loaded or not with the P198 peptide in a three hours and half chromium release assay. Each curve gives the CTL response of spleen cells from an individually injected mouse. Data are from one representative experiment of 4 performed.

CD40L-activated DCs loaded with the weakly immunogenic P1A tumor peptide induce antitumor CTL responses *in vivo*

Antigen P1A is an H-2L^d restricted nonapeptide composed of two T cell epitopes. The gene encoding P1A is expressed in P815 tumor cells but not in normal cells except for testes. When injected into mice under various forms, the P1A peptide usually produces CTL responses that are significantly lower than those produced by the P198 peptide. In view of the strong anti-P198 CTL responses generated using CD40L-activated DCs, we tested whether CTL responses of similar levels could also be induced when these activated DCs were loaded with the P1A peptide. As shown in Figure 3, strong P1A-specific CTL responses were observed in the spleen cells of mice immunized with P1A-peptide pulsed CD40L-activated DCs. In contrast, when unactivated control DCs were used to present the P1A peptide for immunization, lower CTL responses were observed. Moreover, when the cytolytic activities were expressed in lytic units/ 10^6 effector cells, the killing capability of spleen cells derived from mice immunized with CD40L-activated DCs was found to be around 13 fold higher than the killing efficiency of spleen cells derived from mice immunized with the control DCs.

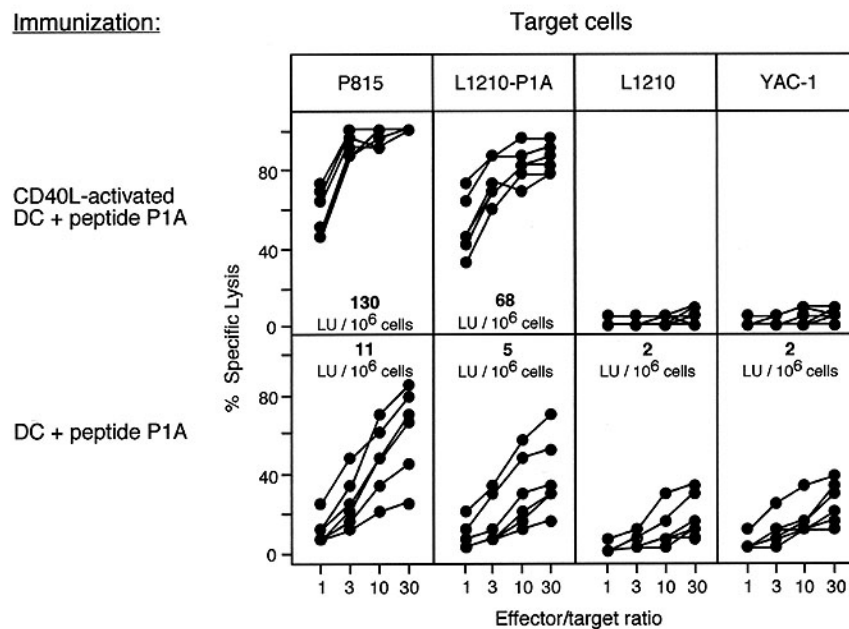
Immunization:

Figure 3. Induction of P1A-specific CTLs by immunization with peptide-pulsed DCs activated by CD40 triggering. Mice were injected s.c. in the 4 footpads on days 0 and 14 with 3×10^5 P1A-loaded DCs previously cocultured with CD40L-transfected 3T3 cells (CD40L-activated DC + peptide P1A) or with untransfected 3T3 cells (DC + peptide P1A). The spleen cells from injected mice were harvested on day 21 and stimulated *in vitro* with P815 tumor cells. Six days later, these cultures were submitted to a second short-term stimulation (4 days) with P815 tumor cells and subsequently tested on ^{51}Cr -labeled P815, L1210 tumor cells transfected or not with the P1A gene and YAC-1 tumor cells in a three hours and half chromium release assay. Each curve gives the CTL response of spleen cells from individually injected mice. Data are from one representative experiment of 4 performed. Lytic activities were also expressed in lytic units (LU) per 10^6 effector cells, where one LU is defined as the number of CTL required to lyse 50% of 10^4 target cells. Each result is the mean of the LU calculated for every mouse of a group.

CD40L-activated DCs loaded with the P1A peptide generate protective antitumor immunity

The efficiency of CD40L-activated DCs loaded with the P1A tumor peptide to prime specific CTLs *in vivo* prompted us to test whether this response correlated with the induction of a protective antitumor immunity. A total of 19 DBA/2 mice were immunized with peptide P1A loaded on CD40L-activated DCs as described above for the induction of CTL responses. One week after the second injection, DBA/2 mice were challenged with a lethal dose of P815 tumor cells injected into the peritoneal cavity. As shown in Figure 4, significantly prolonged survivals were observed, with 89% of mice surviving after 60 days. Examination of the peritoneal cavity following sacrifice of these survivors showed no evidence of residual tumor except in one mouse that was thoroughly investigated (discussed in the next section). In striking contrast, most of the unimmunized mice and most of the mice injected with P1A peptide-pulsed unactivated DCs died between days 27 and 40 after tumor challenge, with large numbers of tumor cells in their peritoneal cavity.

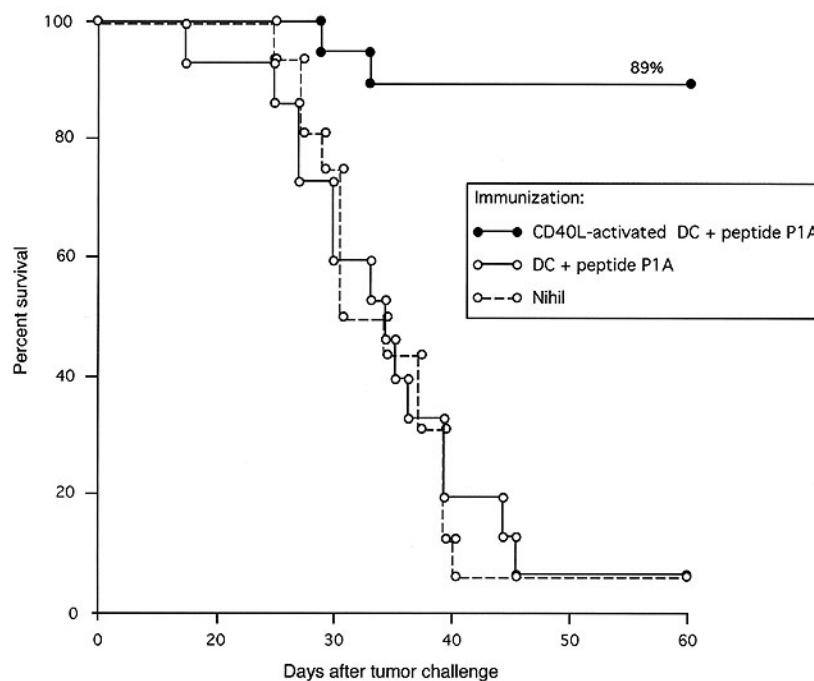


Figure 4. Immunization of DBA/2 mice with CD40L-activated DCs loaded with the P1A peptide induces protection from a lethal challenge with P815 tumor cells. Mice injected s.c. on days -21 and -7 with 3×10^5 P1A peptide-loaded DCs previously cocultured with CD40L-transfected 3T3 cells (19 animals) or with untransfected 3T3 cells (15 animals), were challenged with an i.p. injection of 4×10^5 P815 tumor cells on day 0. Normal mice injected with this lethal challenge of P815 tumor cells were used as controls (Nihil; 16 animals). The data shown are the cumulative survival results obtained from 3 independent experiments. Survival is reported as the percentage of surviving animals.

Mice immunized against P1A develop CTLs against additional P815 antigens upon rejection of a P815 tumor challenge

Since CTL responses appear in many instances to be involved in mediating antitumor effects, we tested spleen cells from protected mice sacrificed at day sixty after the tumor challenge for their ability to respond *in vitro* to P815 cells. As shown in Figure 5, after a 6-day culture period in the presence of P815 tumor cells, a striking CTL response was induced against tumor targets that express the immunizing P1A peptide (P815 tumor cells and L1210 tumor cells transfected with the P1A gene). Interestingly, cell line P1.204, a variant of P815 that does not express P1A but shares at least three other antigens with P815, was also significantly lysed by these spleen cells. This indicates that the interactions between anti-P1A CTLs and P815 tumor cells resulted not only in the observed tumor rejection, but also in the induction of CTLs against additional P815 antigens that were not used for the initial immunization. Such CTLs could play a role in the tumor rejections described. This phenomenon of epitope spreading during rejection of a P815 tumor challenge has previously been reported by Markiewicz *et al.* (41).

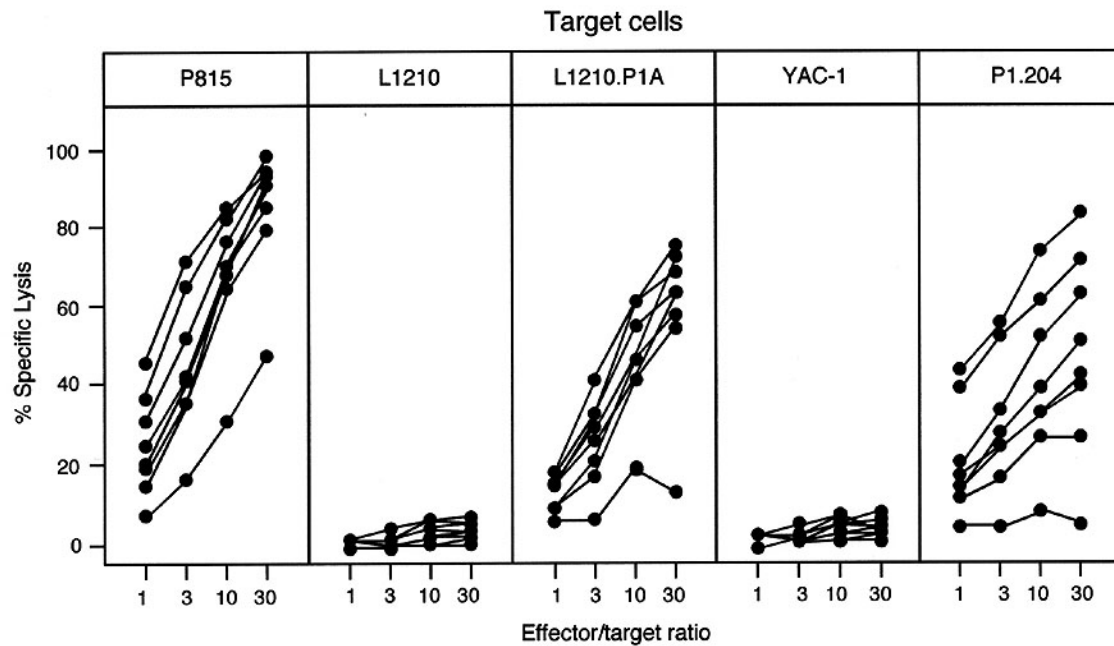


Figure 5. Lysis of different tumor cell lines by CTLs derived from mice surviving the P815 tumor cells challenge. Mice were injected s.c. with 3×10^5 CD40L-activated DCs loaded with the P1A peptide on days 21 and 7 prior to the challenge with an i.p. injection of 4×10^5 P815 tumor cells. At day 60, surviving mice (89%) were sacrificed and their spleen cells were stimulated *in vitro* with P815 tumor cells. After 6 days, these cultures were tested for their ability to lyse the indicated ^{51}Cr -labeled target cells. Chromium release was assessed after three hours and half. Each curve represents the lytic activity obtained with the spleen cells of one mouse. Data are from one representative experiment of 3 performed.

Among the surviving immunized mice sacrificed by day 60 after the P815 tumor challenge, one out of 17 displayed ascitic tumor cells. These tumor cells presumably had escaped the P1A-specific CTL response by losing expression of P1A, as they were found to be resistant to lysis by anti-P1A CTL populations (data not shown). However, this mouse also had CTLs against additional P815 antigens, and it is possible that, at the time of sacrifice, these CTLs were on their way to control the growth of P1A-negative tumor cells.

Discussion

In this study, we show that strong CTL responses can be induced *in vivo* against P198 and P1A antigenic peptides provided the DCs used as APCs are previously cocultured with CD40L-transfected 3T3 cells. About 90% of the mice immunized with P1A-peptide loaded CD40-triggered DCs were protected against a challenge with a lethal dose of P815 tumor cells injected into the peritoneal cavity. This compares favorably with previously reported protections observed after immunizations against P1A with various immunogens (37, 38, 39). Challenge injections with P815 tumor cells can be performed either subcutaneously or intraperitoneally. The subcutaneous

route appears less demanding as spontaneous regressions are occasionally observed (37). We used exactly the same dose of tumor cells and the same injection route (i.p.) for the tumor challenge as Brändle *et al.*, who reported about 50% of protected mice after immunization with living cells transfected with P1A and B7-1 (38). Therefore, our results indicate that P1A peptide-loaded DCs activated via CD40 are superior to living tumor cells expressing P1A and B7-1 in terms of inducing protective immunity.

The increased immunogenicity presumably results from the phenotypic and secretory modifications induced by CD40 triggering on DCs, such as the higher number of veils, higher levels of surface MHC class II and B7-2 molecules, and the secretion of IL-12, which is critical for the development of Th1-type immune responses (10, 14, 17, 42). CD40 triggering also appears to prolong DC survival, which may also contribute to increased immunogenicity (14, 42, 43). Our results corroborate recent findings showing that (a) DCs require CD40 triggering to evolve into efficient APCs (44), (b) CD40 ligands, compared to others stimuli, are very efficient in promoting DC maturation (5), and (c) it is possible to efficiently trigger DC maturation by using an activating antibody against CD40 (20, 21).

The antitumor responses that can develop in mice when mature DCs are used for immunization have prompted clinicians to test this type of immunization in cancer patients (28, 45). The clinical trials reported were based either on immature DCs or on DCs activated by other maturation protocols, including incubation with monocyte-conditioned medium (28, 45, 46). None of those clinical trials was based on the use of CD40L-activated DCs, although CD40 ligation has been shown to induce the maturation of human DCs more efficiently than LPS (16, 47). Indeed, monocyte-derived DCs stimulated by CD40 ligation but not by LPS produce IL-12 (47), which is critical for the differentiation of Th1 cells and can upregulate the expression of HLA class I, HLA class II and ICAM-1 molecules on human melanoma cells (48). Furthermore, CD40-triggered DCs are resistant to the immunosuppressive effect of IL-10, which can be produced by tumors (16).

In a recent report, the effect of CD40 ligation on the efficiency of a DC-based tumor vaccine was studied in CD4-deficient mice (49). In such mice, CD40 ligation was shown to restore efficient CTL and protection responses against a CD4-dependent antigen, indicating that CD40 triggering was the main effector mechanism of CD4 T-cell help. However, in wild type mice, CD40 activation of the dendritic cells did not consistently improve tumor protection. In the model system we report here, which is based on a poorly immunogenic tumor antigen, the beneficial effect of CD40 activation is obvious in wild type mice. Our results therefore clearly support the idea of using CD40 triggering to improve the efficiency of DC-based vaccines used in clinical trials of cancer immunotherapy.

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Materials and methods

Mice

Male DBA/2 mice (H-2^d), between 8 and 12 weeks old, were used for the experiments. They were obtained from the animal facility of the Ludwig Institute for Cancer Research (Brussels).

Cell lines

Four subclones of mastocytoma P815 cells (H-2^d) were used in this study. P198 is an immunogenic variant obtained after a mutagenic treatment of P1 cells, a tumorigenic clonal line derived from P815 ([1](#)). P198.3 is an azaguanine-resistant variant of P198 tumor cells. P511 is an azaguanine-resistant variant of P1 tumor cells. P511 cells used in this study are referred to as P815 cells. P1.204 is a P815 variant carrying a deletion of gene P1A obtained from an *in vivo* escaping tumor population([32](#), [50](#)). The leukemia cell line L1210 is derived from a DBA/2 mouse. L1210.P1A is a transfectant that expresses antigen P1A ([34](#)). L1210.P1A.B7-1 is a transfectant obtained by electroporation of L1210.P1A cells with a cDNA encoding the murine B7-1 molecule ([51](#)). All these cell lines were kindly provided by T. Boon (L.I.C.R., Brussels). The transfectants were maintained in the selection antibiotics as recommended by the suppliers. YAC-1 cells that are NK sensitive targets were kindly provided by M. Moser (ULB, Belgium).

Peptides

Two tumor antigenic peptides were used in this study. They were kindly provided by T. Boon (L.I.C.R., Brussels). The single-letter code sequences are as follows: H-2K^d-restricted P198 14-22, KYQAVTTTL ([52](#)) and H-2L^d-restricted P815AB (P1A) 35-43, LPYLGWLVF ([50](#)).

Generation of dendritic cells (DCs)

DCs were prepared from bone marrow as previously described ([53](#)) but with minor modifications. Briefly, bone marrow cells that had not been depleted of non-DC precursors, were cultured in a 5% CO₂ atmosphere in 24-well plates (Nunc, Life Technologies) at 8 x 10⁵ cells/ml/well in RPMI 1640 medium supplemented with L-arginine (5.5 x 10⁻⁴ M), L-asparagine (2.4 x 10⁻⁴ M), L-glutamine (1.5 x 10⁻³ M), HEPES (10⁻² M), 2-mercaptoethanol (5 x 10⁻⁵ M), 5% heat-inactivated FCS and 500 U/ml murine GM-CSF. On days 2 and 4, cultures were depleted of nonadherent and loosely adherent cells (mainly granulocytes) and fed with fresh medium containing GM-CSF. On day 7, non-adherent and loosely adherent cells were harvested by gentle pipeting and assessed for their DC content. As immature DCs express CD11c, it was shown that DC preparations contained around 70% DCs by staining with monoclonal antibody N418 that recognizes CD11c (data not shown) and direct morphological evaluation. These cell populations enriched in DCs were referred to as DCs.

CD40-mediated activation

3T3 cells transfected with the gene encoding the ligand for murine CD40 molecules (CD40L.3T3 cells) were kindly provided by K. Thielemans (Vrije Universiteit Brussel, Belgium) and used to induce CD40 triggering of DCs. These transfectants were maintained in the selection antibiotic as recommended by the suppliers. Of these 3T3 cells 30% were CD40L positive. To induce CD40L-mediated activation, 8 x 10⁵ DCs collected at day 7 were cocultured on 10⁵ irradiated (100 Gy) CD40L.3T3 cells in 2 ml wells in culture medium supplemented with GM-CSF (500 U/ml) for 2 to 4 days. For the determination of IL-12 production, supernatants were recovered from the cocultures after 48 hours. For functional tests, i.e. their use as APCs for tumor antigenic peptides for *in vivo* immunizations, DCs were recovered after 4 days of coculture.

Flow cytometry analysis

A FACScan (Becton Dickinson and Co, Mountain View, CA) was used for flow cytometric analysis. Cells were processed for double staining using a FITC- or biotin-conjugated mAb N418 specific for CD11c followed by biotin-

conjugated mAb GL1 (rat IgG2a anti-CD86), biotin-conjugated 14.4.4 (murine IgG2a anti-IE^d) or FITC-coupled 16-10A1 (hamster Ig anti-CD80). Cells (10^6 per coloration) were washed in PBS supplemented with 20 g/l bovine serum albumin and 2 g/l sodium azide (FACS buffer) and incubated for 30 min at 4°C with 100 µl of FITC-conjugated mAb and/or biotin-conjugated mAb dilutions. The cells were then washed with FACS buffer and incubated, in the case of biotin-conjugated mAbs, with 50 µl of Streptavidin-Phycoerythrin solution (0.2 µg per coloration). After 20 min at 4°C, the cells were washed with FACS buffer and analyzed on a FACScan flowcytometer. These mAbs were kindly provided by Dr. M. Moser (ULB, Belgium).

Measurement of IL-12

Supernatants of DCs subjected or not to CD40-CD40L interactions were collected after 48 hours to determine IL-12 production and stored at -70°C until use. Cytokine levels in cell supernatants were measured by ELISA (ELISA kit for quantification of mouse IL-12 p70 purchased from Genzyme, Cambridge MA). This assay detects only the heterodimeric (p70) form of murine IL-12. Detection limit was about 5 pg/ml. Duplicate samples were analyzed in serial two-fold dilutions.

Loading of DCs with peptide

2×10^6 DCs were incubated in polypropylene tubes for 2 hours at 37°C in 5 ml RPMI 1640 medium supplemented with 5% FCS, 2-mercaptoethanol (5×10^{-5} M) and either the antigenic peptide P198 (5 µM) or P1A (10 µM). These peptide-loaded DCs were then extensively washed in the absence of FCS prior to their use for immunization.

Immunization protocol and generation of cytotoxic effector cells

On days 0 and 14 each mouse received subcutaneously 3×10^5 peptide-loaded CD40L-activated DCs or the same number of non-activated DCs split among the four footpads. Spleen cells from injected mice were harvested on day 21 and, subsequently stimulated once (at day 0) or twice (at days 0 and 6) in the conditions specified in the results. Briefly, responder cells (5×10^6) were cultured with 2×10^5 irradiated (100 Gy) stimulator cells in 2 ml of EHAA medium (Life Technologies) supplemented with 2-mercaptoethanol (5×10^{-5} M) and normal mouse serum (0.5%). At day 6 after the first *in vitro* restimulation (responding cell recovery ranged from 15 to 30% of the cells initially cultured), the cultures were tested for cytolytic activity on different ⁵¹Cr-labeled tumor targets. When the responding cells were submitted to a second short-term *in vitro* restimulation (for 4 days), the cultures were performed in round-bottom microtiter plates containing 2×10^5 responding cells and 10^4 irradiated stimulating cells prior to testing for cytolytic activity (responding cell recovery ranged from 30 to 50% of the cells introduced in the microwells).

In vivo tumor protection assays

One week after the second s.c. injection with P1A-loaded DCs, mice were injected i.p. with 4×10^5 P815 tumor cells in 500 µl RPMI medium. Protected mice were sacrificed 2 months after the tumor challenge and their spleen cells assessed for their ability to generate CTL responses against immunizing as well as non-immunizing tumor antigens expressed by the tumor cells used for the challenge.

Assay for CTL activity

The ⁵¹Cr-release assay was performed in conical microtiter plates as described previously (54). 2000 ⁵¹Cr-labeled tumor target cells were mixed with dilutions of spleen cells collected from the various cultures and incubated for 3 hours and half at 37°C. Some cytolytic activities were also expressed in LU per 10^6 effector cells,

where one lytic unit (LU) is defined as the number of cytolytic T cells required to lyse 50% of 10^4 target cells in 3 hours and half. This number was estimated by regression ($1-e^{-kx}$) from the specific chromium release obtained at three different E/T ratios chosen in the linear range of the lysis curve.

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