Targeting of Antigens to Activated Dendritic Cells *In vivo* Cures Metastatic Melanoma in Mice

Karsten Mahnke,[1] Yingjie Qian,[1] Sabine Fondel,[1] Juergen Brueck,[2] Christian Becker,[2] and Alexander H. Enk[1]

[1]Department of Dermatology, University of Heidelberg, Heidelberg, Germany and [2]Department of Dermatology, University of Mainz, Mainz, Germany

Abstract

Anti (α)-DEC-205 antibodies target to the DEC-205 receptor that mediates antigen presentation to T cells by dendritic cells. To exploit these properties for immunization purposes, we conjugated the melanoma antigen tyrosinase-related protein (TRP)-2 to αDEC-205 antibodies and immunized mice with these conjugates together with dendritic cell–activating oligonucleotides (CpG). Upon injection of the melanoma cell line B16, αDEC-TRP immunized mice were protected against tumor growth. Even more important for clinical applications, we were able to substantially slow the growth of implanted B16 cells by injection of αDEC-TRP2 conjugates into tumor bearing hosts. Approximately 70% of the animals were cured from existing tumors by treatment with αDEC conjugates carrying two different melanoma antigens (TRP-2 and gp100). This protection was due to induction of melanoma-specific CD4 and CD8 responses. Thus, these data show that targeting of dendritic cells *in situ* by the means of antibody-antigen conjugates may be a novel way to induce long-lasting antitumor immunity. (Cancer Res 2005; 65(15): 7007-12)

Introduction

Dendritic cells have been characterized by their exceptional T-lymphocyte stimulatory capacity that is owed to high expression of MHC molecules as well as to abundant expression of T-cell costimulatory molecules.

To exploit these immunostimulatory features of dendritic cells for immunization purposes, studies have been carried out that generate dendritic cells *in vitro* and load them with known peptides derived from tumor antigens or whole lysate of tumors, respectively. Thereafter, these tumor antigen–loaded dendritic cells were injected into tumor-bearing hosts where the injected dendritic cells eventually home into lymphoid organs and prime an antitumor response (1–3).

Despite some promising clinical trials, these experiments require extensive laboratory work and during *in vitro* culture, dendritic cells are either fed with known tumor peptides or lysate of whole tumors (4). During this culture, dendritic cells also take up all sorts of *in vitro* medium ingredients; therefore, the effective number of MHC-tumor antigen complexes expressed by the dendritic cells is difficult to estimate.

Moreover, antigens endocytosed via fluid-phase uptake are degraded to a large extend by immature dendritic cells instead of being loaded onto MHC molecules and only after terminal differentiation MHC-peptide complexes are generated in sizable numbers (5, 6). *In vivo*, dendritic cells take up antigens by the means of antigen receptors. One example is the DEC-205 receptor (CD205), which guides antigens into deeper endocytic vesicles containing MHC class II molecules. As a consequence of this unique intracellular targeting, antigens endocytosed by the DEC-205 receptor stimulate respective T cells up to 500-fold better than antigens taken up by pinocytosis or by other receptors (7). We could also show that injected anti-DEC-205 antibodies (αDEC) target to dendritic cells situated in the lymph nodes (8, 9). Henceforth, loading dendritic cells with tumor antigens via the DEC-205 receptor may result in effective production of MHC-tumor antigen complexes, which eventually lead to induction of protective antitumor immunity (10).

Therefore, we coupled the melanoma antigens tyrosinase-related protein-2 (TRP-2) and gp-100 to antibodies specific for the DEC-205 receptor (αDEC) and injected these αDEC-tumor antigen conjugates into mice. We show that anti-DEC conjugates load exclusively lymph node dendritic cells, leading to induction of an antitumor response, where up to 70% of the tumor bearing mice rejected the B16 melanomas after DEC-TRP treatment. Thus, these data show that targeting of protein derived tumor vaccines to maturing dendritic cells *in vivo* greatly enhances antitumor activity and may provide a tool for future cancer therapies.

Materials and Methods

Construction of recombinant proteins and coupling with antibodies. cDNA of mTRP2 (amino acid, aa 30-518) and enhanced green fluorescent protein (EGFP) were cloned into *Eco*RI sites of the vector pET43b (Novagen, Schwalbach, Germany). After cloning was confirmed by sequencing, vectors were transformed into Rosetta-gamiDE3 (Novagen) and proteins were isolated with Ni-NTA agarose columns (Qiagen, Hilden, Germany). Purified recombinant proteins were dialyzed against PBS overnight and coupled to anti-DEC-205 antibodies using the cross-linker Sulfo-SMCC (Pierce, Bonn, Germany).

Flow cytometric analysis. For the activation of dendritic cells, the following oligonucleotides were used: 5′-TCCATGA*CG*TTCCTGA*CG*TT-3′ (ARK Sigma, Deisenhofen, Germany). Mice were injected with PBS, 10 μg, 50 μg, and 100 μg CpG, respectively, and sacrificed 24 hours later and lymph node cell suspensions were prepared. Thereafter, $CD11c^+$ cells were prepared using MACS beads (Miltenyi, Bergisch Gladbach, Germany) according to standard protocols.

For detection of CD80, CD86, and MHC II, respective FITC labeled antibodies (all Becton Dickinson, Heidelberg, Germany) were used at 1:100 dilution and analysis was carried out using a FACScan (Becton Dickinson). For detection of antibodies in the serum of immunized mice, sera were collected and 1×10^6 B16 cells were suspended in 400 μL PBS, 1 mmol/L EDTA, 2% (v/v) FCS and incubated with the anti-sera at 1:100 dilution at 4°C for 30 minutes. Thereafter, FITC-labeled rat anti-mouse antibodies (Dianova, Hamburg, Germany) were added, and following two washes,

Note: K. Mahnke and Y. Qian contributed equally to this work.
Requests for reprints: Karsten Mahnke, Department of Dermatology, University of Heidelberg, Vosstrasse 3, 69115 Heidelberg, Germany. Phone: 49-6221-568170; Fax: 49-6221-561617; E-mail: karsten.mahnke@med.uni-heidelberg.de.

cell-bound antibodies were analyzed by fluorescence-activated cell sorting (FACS).

ELISA and ELISPOT. ELISA was carried out to detect antibodies specific for mTRP2; 96-well plates (Nunc, Wiesbaden, Germany) were coated overnight with recombinant mTRP2 protein (50 μL per well) in coating buffer [30 mmol/L Na_2CO_3, 70 mmol/L $NaHCO_3$ (pH 9.6)]. After three washes [2% bovine serum albumin (BSA), 0.01% Thimerosal, 0.5% Tween 20 in PBS], antisera were added and incubated at 4°C for 2 hours. After several washes, 100 μL per well rat anti-mouse peroxidase conjugates (Dianova) at a 1:3000 dilution were added and incubated at 4°C for 1 hour. Thereafter, 100 μL per well ABTS + 1N H_2O_2 (Sigma) were added for 10 minutes at room temperature and plates were evaluated at 405 nm with ELISA plate reader (Bio-Rad, Muenchen, Germany). For the detection of cytokines, T cells from immunized mice were isolated with anti-CD4 beads on MACS columns according to the manufacturer's protocol (Miltenyi). $CD4^+$ T cells were then restimulated with dendritic cell pulsed with recombinant protein mTRP2 *in vitro* for 3 days, and supernatants were collected and used for ELISA to detect IFN-γ, interleukin 2 (IL-2), IL-4, IL-10, tumor necrosis factor-α (TNF-α), production with standard ELISA kits (R&D Systems, Wiesbaden, Germany).

For detection of mTRP2-specific $CD8^+$ T cells, ELISPOTs were carried out as follows: Mice injected with conjugates or PBS were sacrificed and single spleen cell suspensions were prepared with standard protocols and cultivated in ELISPOT plates (Nunc) that had been precoated with 50 μL per well rat-anti-mouse-IFNγ antibodies in PBS (10 μg/mL; PharMingen, Heidelberg, Germany) overnight at 4°C.

For stimulation, mTRP2 peptide (aa 180-188) or the known $CD8^+$ T cell epitope (SVYDFFVWL) was added (1 μg/mL). β-Galactosidase (β-gal) peptide and RPMI alone served as controls. Plates were incubated at 37°C for 24 hours at 5% CO_2 and developed with 100 μL biotinylated rat-anti-mouse IFNγ (5 μg/mL) in assay buffer (0.5% BSA, 0.05% Tween 20 in PBS). Thereafter, 100 μL per well streptavidin-POD were added and incubated at room temperature for 30 minutes. Color was developed by addition of 50 μL per well of 3,3′-diaminobenzidine developer (peroxidase substrate kit; Vector, Birmingham, CA), and the number of mTRP2 peptide–specific $CD8^+$ T cells was evaluated by counting.

Vaccination and tumor challenge experiments. All animal protocols were approved under the guidelines of the animal protection act. For protective experiments, female B6 mice (6-8 weeks old) were injected s.c. with 10 μg DEC-205 conjugates in PBS plus 50 μg CpG twice at an interval of 1 week. One week after the second injection, each mouse was challenged for tumor growth by i.v. injection of 4×10^5 B16 cells in 100 μL PBS; after 2 weeks, mice were sacrificed and lung metastasis were countered. As control, 100 μL PBS + 50 μg CpG were injected.

For therapeutic experiments, tumor growth was induced by s.c. injection of 5×10^3 B16 cells in the right flank. After ~1 week when the tumor growth was first seen (diameter, 1 mm), 10 μg DEC-205 conjugates in PBS + 50 μg CpG per mouse were injected. The injection was repeated after 1 week and tumor growth was measured twice to thrice a week with a caliper ruler. In some experiments, $CD4^{-/-}$ and $CD8^{-/-}$ B6 mice were used.

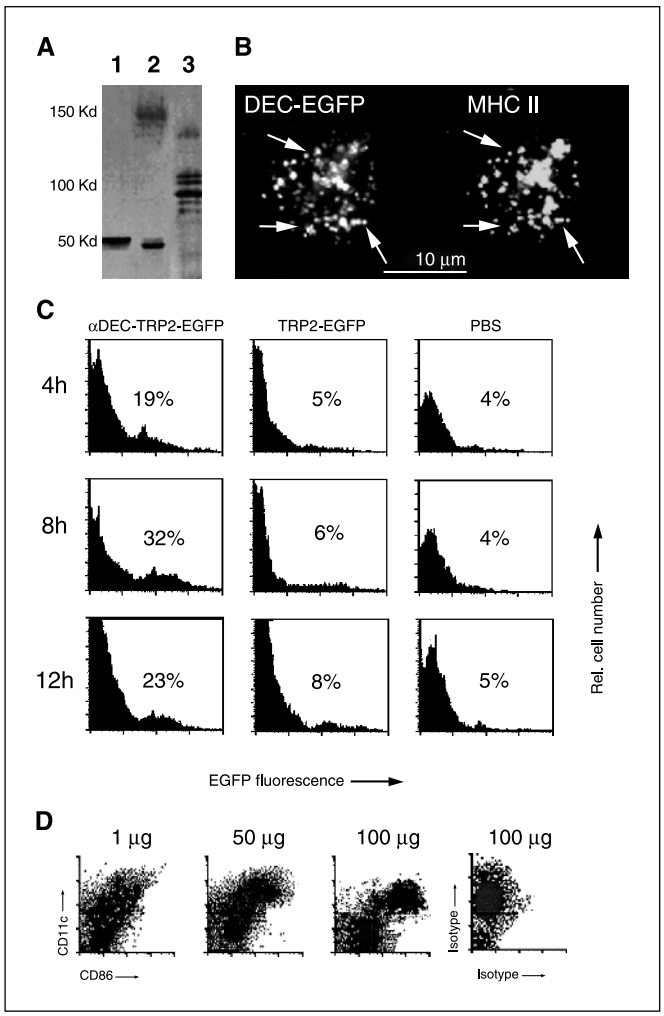

Figure 1. Targeting of αDEC-TRP conjugates to dendritic cells *in vitro* and *in vivo*. *A,* αDEC antibodies (*lane 1*) were coupled to recombinant TRP2 protein. This coupling resulted in aggregates of ~150 kDa in size (*lane 2*). As a comparison, purified TRP2 is shown in *lane 3*. *B,* bone marrow–derived dendritic cells were incubated with αDEC-EGFP for 2 hours and stained with Texas red conjugated anti-MHC class II antibodies. EGFP fluorescence (*left*); MHC staining (*right*). Examples of colabeling (*arrows*). *C,* αDEC-EGFP conjugates and controls as indicated were injected into mice and draining lymph nodes were removed 4, 8, and 12 hours thereafter and $CD11c^+$ cells were prepared. EGFP fluorescence was measured by FACS after gating onto the $CD11c^+$ cells. *D,* CpG at doses as indicated were injected s.c. into the hint flanks of mice, and lymph node cells were prepared and stained for CD11c in PE and CD86 in FITC 24 hours later.

Results

αDEC-TRP-2 conjugates target to dendritic cells *in vitro* and *in vivo*. To make DEC targeting applicable for many different tumor settings, we successfully set out to produce, purify, and couple known tumor antigens TRP-2 and gp-100 to αDEC antibodies (Fig. 1*A*).

To evaluate the intracellular targeting of αDEC-EGFP conjugates, bone marrow–derived dendritic cells were incubated with these conjugates for 2 hours and fixed and stained with Texas red coupled antibodies as indicated. Thereafter, specimens were examined by immunofluorescence microscopy. Figure 1*B* shows αDEC-EGFP proteins were efficiently taken up by the dendritic cells and double labeling with αMHC class II antibodies revealed colocalization. The punctuated pattern of EGFP and MHC staining was not restricted to the outline of the cells but is present throughout the cell body. This indicates intracellular localization of EGFP and MHC as observed previously (5). In contrast uncoupled EGFP was not taken up by the dendritic cells (data not shown). Thus, these data show that αDEC-EGFP conjugates gain access to MHC class II^+ antigen-processing compartments.

Next we investigated whether s.c. injection of the αDEC-EGFP also results in effective *in vivo* targeting to dendritic cells in regional lymph nodes. For this, αDEC-EGFP and respective controls were injected into mice and draining lymph nodes were removed as indicated. Figure 1*C* shows that 4 hours after injection ~19% and 8 hours later ~32% of the dendritic cells in the draining lymph nodes accumulated αDEC-EGFP protein. This is a rapid accumulation compared with cellular migration. Because it takes

Langerhans cells >6 hours to migrate from skin to regional lymph nodes, we conclude that αDEC-EGFP conjugates target to antigen presentation compartments in dendritic cells *in vitro* and *in vivo*. However, after injection of uncoupled protein some fluorescence slightly above background levels was detectable, resulting in only 6% to 8% of all dendritic cells being loaded (background level was 4%).

Dendritic cells are activated by CpG. Presentation of antigen(s) by immature dendritic cells leads to tolerance, whereas concomitant activation of the dendritic cells by αCD40 antibodies leads to induction of immunity. However, αCD40 antibodies are not approved for clinical trials in humans yet; therefore, we reasoned to inject CpG as dendritic cell activators, because this approach is currently under investigation in human trials (11).

To test the optimal concentration in our tumor model, mice were injected with different doses of CpGs and dendritic cells were purified 24 hours later. Thereafter, cells were stained for the maturation markers CD86 and CD80. Figure 1*D* shows, that injection of CpG increased dendritic cell maturation dose dependently as indicated by enhanced CD86 expression. We found that 50 μg CpG was optimal, as further increase of the CpG concentration did not result in enhanced CD86 expression. Therefore, in subsequent experiments, αDEC-TRP2 conjugates were injected together with 50 μg of CpGs (αDEC-TRP2$_{CpG}$).

Induction of antibody responses against tyrosinase-related protein. To test whether injection αDEC-TRP2$_{CpG}$ induces TRP-specific immune responses, we injected mice with αDEC-TRP2$_{CpG}$ and respective controls as indicated, and 14 days later, the serum was tested for TRP-2-specific antibodies. Figure 2*A* shows that mice injected with αDEC-TRP2$_{CpG}$ conjugates developed a more vigorous antibody response against the TRP-2 protein compared with controls. Moreover, no antibodies against TRP-2 were observed after immunization of mice with isotype conjugates.

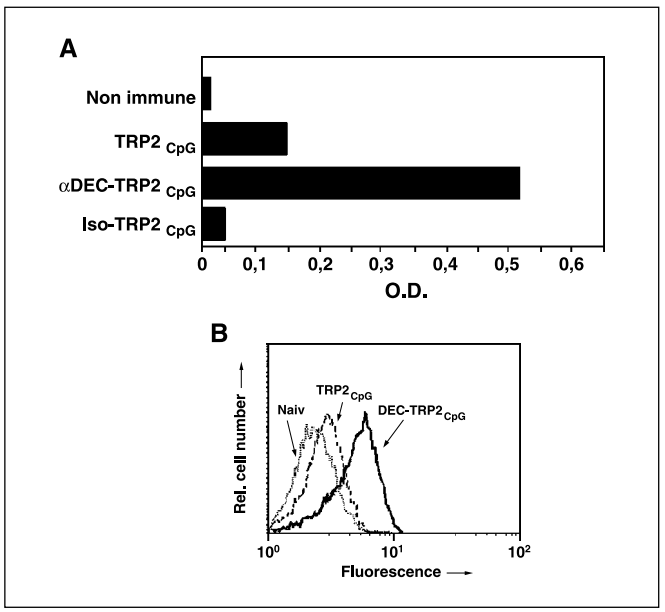

Figure 2. Induction of anti-TRP2 antibodies after αDEC-TRP$_{CpG}$ injection. Mice were immunized as indicated and sera were obtained 2 weeks after last immunization. *A,* thereafter, anti-sera were tested in ELISA plates, which had been coated with TRP-2 protein and respective controls. TRP-specific antibodies bound to the plate were then detected using horseradish peroxidase–coupled secondary reagents and colorimetric substrates. *Columns,* means of the absorbance (*O.D.*) of quadruplicates. *B,* B16 melanoma cells were incubated with antisera obtained from immunized mice as indicated and surface bound antibodies were detected using FITC labeled secondary reagents. Thereafter, samples were analyzed by FACS.

Recombinant proteins may differ in their secondary and tertiary structure from their natural counterparts; therefore, we additionally tested whether the sera of immunized mice contained antibodies binding to "natural" B16 melanoma cells. We therefore incubated viable B16 melanoma cells with the obtained antisera and analyzed the binding of antibodies by FACS analysis. In these assays (Fig. 2*B*), we detected B16-specific antibodies in sera derived from αDEC-TRP2$_{CpG}$ immunized mice, whereas no antibodies were observed in controls. The binding of antibodies in sera derived from TRP2$_{CpG}$ immunized mice was very weak compared with αDEC-TRP2$_{CpG}$ immunized mice. In aggregate, these assays show that mice immunized with αDEC-TRP2$_{CpG}$ conjugates developed antibodies that recognize epitopes expressed by "natural" B16 melanoma cells and thus may also confer protective activity against tumor growth *in vivo*.

Induction of TRP-2-specific CD4$^+$ T cells. To further test whether cellular responses (i.e., TRP-2-specific CD4$^+$ T cells) were induced by αDEC-TRP2$_{CpG}$ immunizations, we isolated CD4$^+$ T cells from immunized mice and restimulated with mTRP2-pulsed bone marrow–derived dendritic cells. Here (Fig. 3*A*), T cells obtained from αDEC-TRP2$_{CpG}$ and αDEC-TRP2 plus anti-CD40 antibody injected mice showed vigorous proliferation upon restimulation *in vitro*. In contrast, when mice were immunized with unconjugated TRP2$_{CpG}$, isotype controls, or αDEC-TRP2 without activation, no T-cell proliferation could be recorded.

To analyze the type of T-cell reaction induced by this immunization regimen, supernatants of stimulated T cells were tested for the presence of cytokines by ELISA. Here we show (Fig. 3*B*) that T cells recovered from αDEC-TRP$_{CPG}$-injected mice produced substantial amounts of TNFα and IFNγ, whereas only basal production could be recorded in control groups. The immunosuppressive cytokine IL-10 was not detectable in any group.

Induction of CD8$^+$ T cells. Because previous results have shown that fractions of antigens endocytosed by DEC-205 are also presented in context of MHC class I molecules (8), we next checked for induction of TRP-2-specific CD8$^+$ T cells. Therefore, we immunized mice with αDEC-TRP$_{CpG}$ conjugates and respective controls, isolated spleen cells 7 days after the second immunization, and restimulated the CD4-depleted T cells with the known MHC class I–specific TRP-2 peptide in ELISPOT plates. The quantity of spots as depicted in Fig. 3*C* shows that substantial numbers of IFNγ producing T cells were induced after immunization with αDEC-TRP$_{CpG}$. In contrast, after injection of TRP$_{CPG}$ or Isotype-TRP$_{CpG}$, no spots above background levels could be recorded. To further verify the antigen specificity, we stimulated spleen cells with the matched MHC class I peptide derived from the unrelated protein β-gal. In these controls, we could not detect any spots, indicating that the induced IFNγ producing CD8$^+$ T cells were specific for the TRP-2 peptide. Injection of recombinant adenovirus encoding for human TRP-2 served as a positive control (12). In summary, these data show that immunization of mice with αDEC-TRP2$_{CpG}$ conjugates leads to induction of antibodies as well as to induction of TRP-2-specific CD4$^+$ and CD8$^+$ T cells.

Induction of protective antitumor immunity in mice. To investigate the effects of αDEC-TRP2$_{CpG}$ immunization in an *in vivo* tumor model, we immunized mice with the different αDEC-conjugates and respective controls twice in weekly intervals and

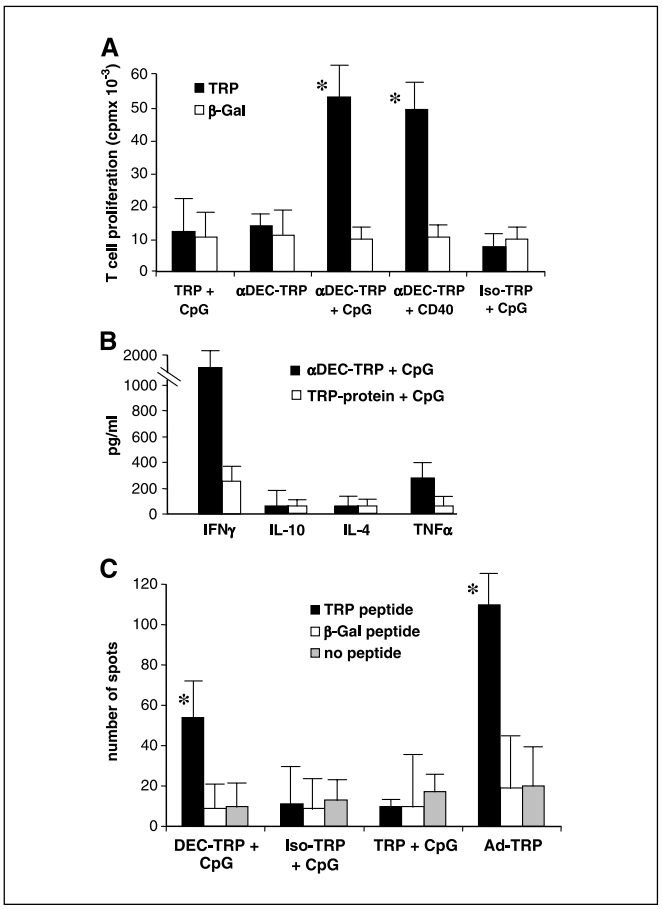

Figure 3. Induction of antitumor T-cell responses. *A,* mice were immunized with conjugates and respective controls as indicated on the *x*-axis. After 1 week, $CD4^+$ T cells from lymph nodes were prepared and restimulated in 96-well plates with either TRP or β-gal protein, respectively. T-cell proliferation was determined by [^{3}H]-thymidine uptake. *Columns,* means of triplicates; *bars,* ±SD. *, $P < 0.05$, significant difference to isotype-treated controls. *B,* mice were immunized with DEC-TRP conjugate or TRP protein as indicated. Thereafter, T cells were prepared from lymph nodes and restimulated *in vitro*. Supernatants were recovered and tested for cytokines as indicated using ELISA. *Columns,* means of triplicates; *bars,* ±SD. *C,* mice were immunized with conjugates and respective controls as indicated on the *x*-axis. Thereafter, CD4-depleted spleen cells were prepared and cultured in anti-IFNγ coated ELISPOT plates. Cells were pulsed with known CD8-specific peptides as indicated and plates were developed and spots were counted 48 hours later. *Columns,* means of triplicates; *bars,* ±SD. *, $P < 0.05$, significant difference to "no-peptide" controls. As a control for tumor protection (as published), mice were immunized with recombinant adenovirus-encoding human TRP-2 (Ad-hTRP).

challenged for tumor growth by i.v. injection of 4×10^5 viable B16 melanoma cells. After 2 weeks, mice were sacrificed, lungs were removed, and lung metastases were counted. In these experiments, we could show (Fig. 4) that ~80% of the mice immunized with αDEC-TRP2$_{CpG}$ conjugates were protected from tumor growth. In contrast, after immunization with TRP_{CpG} antigen alone or a mixture of αDEC antibodies and TRP_{CpG} respectively, no protection was apparent. In these instances, up to 100% of the mice had metastases developing in their lungs. Coupling TRP-2 to irrelevant antibodies, which may facilitate uptake by Fc receptors expressed by antigen presenting cells, was not sufficient to induce tumor protection, because RatIgG-TRP-2 conjugates as well as MHC II TRP-2 conjugates failed to induce protection.

In addition, we further confirmed that the activation status of dendritic cells is crucial for successful induction of immunity. When the dendritic cell–activating stimulus CpG was omitted, no protection could be generated after immunization with αDEC-TRP2 conjugates, supporting our previous results showing that activated dendritic cells are required for successful T-cell priming. To further monitor the contribution of $CD4^+$ as well as $CD8^+$ T cells to this protective effect, we immunized the respective knockout mice and challenged for tumor growth as described before. Here we show (Fig. 4*B*) that for successful priming of T cells for antitumor immunity, $CD4^+$ T cells seem crucial, because in CD4 knockout mice, the protective effect of αDEC-TRP_{CpG} treatment was completely abolished.

Treatment of developing melanoma by injection of αDEC-TRP_{CpG}. In clinical settings, a therapeutic approach (i.e., the treatment of tumor-bearing individuals) is desirable. Therefore, we investigated the effect of αDEC-TRP2$_{CpG}$ injection on already existing melanomas. We injected B16 melanoma cells first and waited until palpable tumors (~1-mm diameter) have developed. Thereafter, mice were treated by injection of 10 μg per mouse αDEC-TRP2$_{CpG}$. All mice received two injections of the conjugates in a 4-day interval and the growth of s.c. melanomas was measured by a caliper ruler. Here (Fig. 5), we could show that injection of αDEC-TRP2$_{CpG}$ substantially slowed melanoma growth and protected up to 50% of the mice from tumor growth. In contrast, injection of uncoupled TRP-2 protein or CpG alone as well as mixtures of TRP-2 and CpG did not affect tumor growth and nearly 100% of all melanoma-bearing mice died.

Most strikingly, we were able to further increase the protective activity of αDEC conjugates by injecting mice with a fusion product of TRP-2 and gp100 protein. Using this fusion protein, up to 70% of the mice were protected from tumor growth in these "cure" experiments. Thus, these data indicate that αDEC targeting

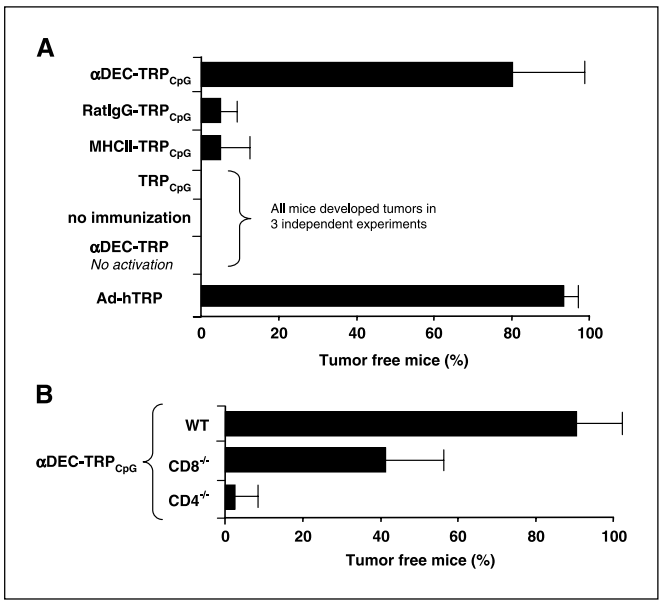

Figure 4. Vaccination with αDEC-TRP conjugates prevents tumor growth. *A,* groups of 5 C57/Bl6 mice were immunized twice with conjugates as indicated, and 1 week later, tumor growth was challenged by i.v. injection of 4×10^5 B16 melanoma cells. Fourteen days later, animals were sacrificed and lung metastasis were counted. *Columns,* % tumor-free mice of four independent experiments; *bars,* ±SD. As a control for tumor protection (as published), mice were immunized with recombinant adenovirus encoding-human TRP-2 (Ad-hTRP). *B,* groups of either CD8 knockout (*KO CD8$^{-/-}$*) mice or CD4 KO ($CD4^{-/-}$) mice were immunized and challenged as in (*A*). Wild-type mice (*WT*) served as controls. *Columns,* % tumor-free mice as in (*A*).

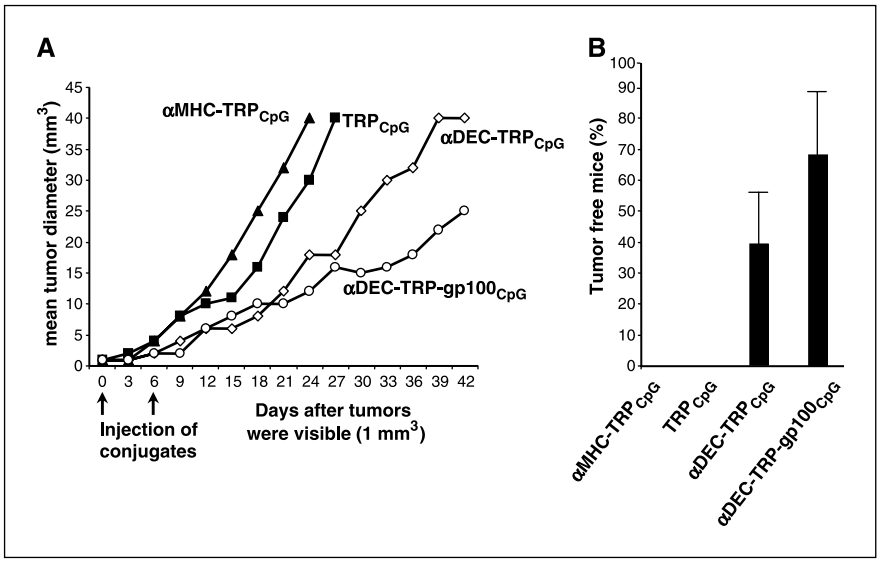

Figure 5. Treatment of developing melanomas by injection of αDEC-TRP conjugates. *A,* B16 melanoma cells were injected into the shaved abdomen of mice. After developing melanomas were visible (~1 mm^2), mice were treated as indicated and volumes of the melanomas were determined. *Points,* mean tumor volumes of groups of five mice in a typical experiment. *B,* in parallel, the number of tumor free mice was determined. *Columns,* means of 3 independent experiments; *bars,* ±SD.

offers the possibility not only to immunize against melanoma but also to treat tumor-bearing hosts successfully. By generating conjugates that harbor a combination of different relevant melanoma antigens, an even stronger tumor response could be induced that renders mice able to reject already existing melanomas.

Discussion

Tumor antigen–pulsed dendritic cells have been used in several clinical trials to induce tumor immunity with variable success (3, 4). Therefore, we reasoned to load dendritic cell directly *in vivo* with relevant tumor antigens by the means of antibody targeting.

For this aim, several prerequisites are mandatory. First, the antigen has to be targeted specifically to activated dendritic cells that are able to induce strong immunity *in vivo*. Second, the antigen has to be endocytosed by the dendritic cells to generate significant numbers of MHC-peptide complexes that eventually are presented to T cells *in vivo*.

Both criteria are fulfilled by αDEC-205 antibodies and here we show that αDEC-tumor antigen conjugates target to dendritic cells located in the lymph nodes. Upon injection, the αDEC coupled antigens were efficiently loaded onto dendritic cells *in vivo* and protective antitumor immunity was induced when conjugates were applied together with dendritic cell–activating stimuli. Even more interesting, we were also able to cure mice from B16 melanomas by the αDEC-tumor antigen treatment.

For antigen targeting, the DEC-205 molecule is an ideal candidate, because this molecule mediates effective generation of MHC-peptide complexes and is almost exclusively expressed by dendritic cells (7, 13, 14). Some successful targeting of dendritic cells using antibodies against surface receptors with broader specificity (i.e., anti-FcR or anti-chemokine receptors) has been observed previously, but the effect on immunity was rather weak (15, 16). Presumably, the FcR conjugates were taken up by a variety of different lymphocytes, such as macrophages, B cells, and spleen dendritic cells, thus "diluting" the targeting effect (17, 18). The αDEC-205 conjugates in contrast, target specifically to dendritic cell *in vivo* without being "caught" and eliminated by binding to macrophages and/or B cells, respectively. Because dendritic cells are by far the most effective inducers of T-cell responses, dendritic cell–specific targeting contributes to the effective immunization against melanoma.

TRP-2 coupled to αDEC was present in lymph node dendritic cells within the first hour after injection (8). Soon thereafter, αDEC-coupled antigens could be traced beyond the regional lymph nodes. Because it takes skin-derived dendritic cells (Langerhans cells) >6 hours to migrate from the skin to regional lymphatics and migration beyond lymphatic organs of Langerhans cells does not occur, we conclude that the αDEC-antigen conjugates themselves reach the lymph nodes and were not transported from the skin via immigrating Langerhans cells. This broad loading of dendritic cell in virtually all lymph nodes is in contrast to results observed with *in vitro* generated, antigen-loaded dendritic cells. After injection of these dendritic cells, only 3% to 5% reach lymphoid organs and their migration is restricted to the regional lymph nodes only (19). Therefore, the systemic distribution of αDEC-antigen conjugates enables loading of virtually all lymph node dendritic cells in the body and provides a basis for the induction of a more powerful immune response compared with conventional immunization strategies.

The successive receptor internalization and intracellular sorting of the antibody-antigen conjugates further determines the generation of sizable numbers of MHC-peptide complexes that eventually are presented to T cells. In this regard, the novel intracellular targeting pathway of the DEC-205 receptor is crucial. Previous results indicate that DEC-205 targets directly into MHC class II compartments where novel MHC-peptide complexes are generated to stimulate $CD4^+$ T cells (7). CD8 responses were also induced, although results obtained with CD8 knockout mice indicated only a minor role in tumor protection. However, these results were expected because other TRP-2-derived tumor models suggest that $CD4^+$ T cells are crucial for providing "help" for the development of tumor-specific cytotoxic $CD8^+$ T cells (20). Thus, depletion of $CD4^+$ T cells prevents the mounting of an effective immune response because B cells, CTLs, and other cellular components are only partially activated.

Other receptors such as the FcR mainly feed into proteolytic pathways resulting in degradation and diminished MHC-peptide complex generation (18). In contrast, DEC-guided antigens

displayed a long half-life (8) indicating that the intracellular compartments served by DEC are only mildly proteolytic.

Furthermore, certain subtypes of receptors (e.g., FcγRII) possess signaling capacity leading to inhibition of immune responses via ITIM motives (21). Therefore, the intracellular "en route" targeting of the antibody-antigen conjugates guided by DEC-205 avoids the loss of putative antigenic peptides caused by protein degradation in lysosomal compartments and MHC-peptide complexes are produced with a high efficiency.

Generally, the targeting of antigens to dendritic cells does not activate immune responses per se, because several investigations have shown that concomitant activation of dendritic cell is crucial for induction of immune responses (22, 23). In this regard, the application of immune stimulatory substances such as CpG may have a 2-fold effect on the development of tumor immunity in our B16 model. First, as already mentioned, CpG ensures proper activation of the dendritic cells; second, it may activate the endothelium of tumor blood vessels. This may be important because Garbi et al. (24) have shown that proper activation of endothelium is crucial for the extravasation of tumor-specific T cells from the blood into cancerous tissues.

In contrast to "immunization" experiments, "cure" experiments are much more demanding, because the immune system has to fight cancer cells that are already proliferating. Despite this "head start" for the tumor cells, we had reasonable success by "curing" up to 70% of melanoma bearing mice by coupling two known tumor antigens (i.e., TRP-2 and gp100 together) to αDEC antibodies (25). In principle, the generation of fusion proteins that harbor even more then two melanoma epitopes is possible and even more interesting; one can speculate that also simultaneous expression of dendritic cell activating epitopes may be possible. That would obliviate the need to coinject dendritic cell activating stimuli and one can speculate that the coupling of such "multiepitope plus activation proteins" to αDEC antibodies may further improve the tumor therapy with αDEC-205 mediated *in vivo* targeting.

In summary, our results show that targeting of tumor antigens to dendritic cell *in vivo* may provide us with a tool to immunize against and to treat diseases. The use of αDEC targeting may not be restricted to tumor antigens, because for other infectious diseases or virally infections, protein antigens are characterized. Therefore, coupling of these antigens to αDEC may improve vaccination efficiency. Because the DEC-205 molecule is equally expressed by human dendritic cells (26) and shares a high (up to 90%) amino acid sequence homology with murine DEC-205, it is conceivable that human DEC-205 has similar properties as antigen receptor and therefore may be a useful target for developing vaccination strategies in human trials.

Acknowledgments

Received 3/21/2005; revised 5/2/2005; accepted 5/10/2005.

Grant support: Deutsche Forschungsgemeinschaft grants Ma 1924/2-1 and SFB 405 and the Tumor-Center Heidelberg/Mannheim.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Jonuleit H, Giesecke-Tuettenberg A, Tuting T, et al. A comparison of two types of dendritic cell as adjuvants for the induction of melanoma-specific T-cell responses in humans following intranodal injection. Int J Cancer 2001;93:243–51.
2. Banchereau J, Palucka AK, Dhodapkar M, et al. Immune and clinical responses in patients with metastatic melanoma to CD34(+) progenitor-derived dendritic cell vaccine. Cancer Res 2001;61:6451–8.
3. Dhodapkar M, Steinman RM, Sapp M, et al. Rapid generation of broad T-cell immunity in humans after a single injection of mature dendritic cells. J Clin Invest 1999;104:173–80.
4. Nestle FO, Alijagic S, Gilliet M, et al. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med 1998;4:328–32.
5. Turley SJ, Inaba K, Garrett WS, et al. Transport of peptide-MHC class II complexes in developing dendritic cells. Science 2000;288:522–7.
6. Pierre P, Turley SJ, Gatti E, et al. Developmental regulation of MHC class II transport in mouse dendritic cells. Nature 1997;388:787–92.
7. Mahnke K, Guo M, Lee S, et al. The dendritic cell receptor for endocytosis, DEC-205, can recycle and enhance antigen presentation via major histocompatibility complex class II-positive lysosomal compartments. J Cell Biol 2000;3:673–84.
8. Bonifaz L, Bonnyay D, Mahnke K, Rivera M, Nussenzweig MC, Steinman RM. Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8(+) T cell tolerance. J Exp Med 2002; 196:1627–38.
9. Hawiger D, Inaba K, Dorsett Y, et al. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions *in vivo*. J Exp Med 2001;194:769–79.
10. Bonifaz L, Bonnyay P, Charalambous A, et al. *In vivo* targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. J Exp Med 2004;199:815–24.
11. Sparwasser T, Vabulas RM, Villmow B, Lipford GB, Wagner H. Bacterial CpG-DNA activates dendritic cells *in vivo*: T helper cell-independent cytotoxic T cell responses to soluble proteins. Eur J Immunol 2000;30: 3591–7.
12. Steitz J, Brueck J, Knop J, Tuting T. Adenovirus-transduced dendritic cells stimulate cellular immunity to melanoma via a CD4(+) T cell-dependent mechanism. Gene Ther 2001;8:1255–63.
13. Jiang W, Swiggard WJ, Heufler C, et al. The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. Nature 1995;375:151–5.
14. Witmer-Pack MD, Swiggard WJ, Mirza A, Inaba K. Steinman RM. Tissue distribution of the DEC-205 protein that is detected by the monoclonal antibody NLDC-145. II. Expression *in situ* in lymphoid and nonlymphoid tissues. Cell Immunol 1995;163: 157–62.
15. Schjetne KW, Gundersen HT, Iversen JG, Thompson KM, Bogen B. Antibody-mediated delivery of antigen to chemokine receptors on antigen-presenting cells results in enhanced CD4+ T cell responses. Eur J Immunol 2003; 3:3101–8.
16. Squire C, Studer E, Lees A, Finkelman F, Conrad H. Antigen presentation is enhanced by targeting antigen to the FceRII by antigen-anti-FcR-conjugates. J Immunol 1998;152:4388–95.
17. Serre K, Machy P, Grivel JC, et al. Efficient presentation of multivalent antigens targeted to various cell surface molecules of dendritic cells and surface Ig of antigen-specific B cells. J Immunol 1998; 161:6059–67.
18. Mellman IS, Plutner H, Steinman RM, Unkeless JC. Cohn ZA Internalization and degradation of macrophage Fc receptors during receptor-mediated phagocytosis. J Cell Biol 1983;96:887–95.
19. De Vries IJ, Krooshoop DJ, Scharenborg NM, et al. Effective migration of antigen-pulsed dendritic cells to lymph nodes in melanoma patients is determined by their maturation state. Cancer Res 2003;63:12–7.
20. Steitz J, Brueck J, Gambotto A, Knop J, Tueting T. Genetic immunization with a melanocytic self-antigen linked to foreign helper sequences breaks tolerance and induces autoimmunity and tumor immunity. Gene Ther 2002;9:208–13.
21. Billadeau DD, Leibson PJ. ITAMs versus ITIMs: striking a balance during cell regulation. J Clin Invest 2002;109:161–8.
22. Inaba K, Turley S, Iyoda T, et al. The formation of immunogenic major histocompatibility complex class II-peptide ligands in lysosomal compartments of dendritic cells is regulated by inflammatory stimuli. J Exp Med 2000;191:927–36.
23. De Smedt T, Pajak B, Muraille E, et al. Regulation of dendritic cell numbers and maturation by lipopolysaccharide *in vivo*. J Exp Med 1996;184:1413–24.
24. Garbi N, Arnold B, Gordon S, Hammerling GJ, Ganss R. CpG motifs as proinflammatory factors render autochthonous tumors permissive for infiltration and destruction. J Immunol 2004;172:5861–9.
25. Mendiratta SK, Thai G, Eslahi NK, et al. Therapeutic tumor immunity induced by polyimmunization with melanoma antigens gp100 and TRP-2. Cancer Res 2001; 61:859–63.
26. Guo M, Gong S, Maric S, et al. A monoclonal antibody to the DEC-205 endocytosis receptor on human dendritic cells. Hum Immunol 2000;61:729–38.

Targeting of Antigens to Activated Dendritic Cells *In vivo* Cures Metastatic Melanoma in Mice

Karsten Mahnke, Yingjie Qian, Sabine Fondel, et al.

Cancer Res 2005;65:7007-7012.