# Inhibition of Melanoma Growth by Targeting of Antigen to Dendritic Cells via an Anti-DEC-205 Single-Chain Fragment Variable Molecule

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**Abstract**

**Purpose:** Our goal was to target melanoma antigens to the dendritic cell-specific receptor DEC-205. DEC-205 is an antigen receptor expressed on dendritic cells and has been shown to guide antigens to MHC class I and II compartments for processing and presentation to T cells.

**Experimental Design:** The melanoma tumor-associated antigen (TAA), gp100, was fused to the single-chain fragment variable (scFv) specific for DEC-205. The binding capacity of the scFv was tested on lymph node–isolated $CD11c^+$ cells. Mixed lymphocyte reactions were carried out to show an increased proliferative capacity of gp100 antigen-specific CD4 and CD8 T cells. Furthermore the scFv-TAA was used in a therapeutic setting using two different melanoma mouse models.

**Results:** C57Bl/6 mice were injected with scFv-DEC-205-gp100, monoclonal antibody anti-DEC-205, or PBS. Using fluorescence-activated cell sorting, we showed that lymph node $CD11c^+$ dendritic cells stained positive for the binding of the scFv-mDEC-205-gp100 and the anti-DEC-205 monoclonal antibody, whereas the PBS-injected animals were negative. In mixed lymphocyte reactions, bone marrow–derived dendritic cells pulsed with scFv-mDEC-205-gp100 significantly increased proliferation of gp100-specific $CD8^+$ and $CD4^+$ T cells beyond gp100 peptide-pulsed or nonpulsed bone marrow–derived dendritic cells. Finally, in B16/F10 and RET models, a concentration-dependent suppression of tumor growth using scFv-mDEC-205-gp100 (66% reduction of tumor volume), in comparison with gp100 peptide vaccination, was observed.

**Conclusions:** Our results indicate that the scFv-mDEC-205-gp100 targets TAA to dendritic cells *in vivo* for presentation on both MHC class I and II molecules. *In vivo*, this leads to an improved immune response and a decrease in tumor growth rate.

The introduction of known antigens into an infected or tumor-bearing subject for the purpose of inducing an immune response has been the focus of many studies. Methods of delivery include techniques such as intramuscular DNA vaccination with electroporation, coupling of antigens to antibodies, and *ex vivo* loading of dendritic cells with tumor-associated antigen (TAA) or even with whole tumor cells (1–3). Each of these methods has disadvantages, including nonspecific targeting of antigens, dilution or degradation of antigens, and/or complex procedures for the generation of *ex vivo* antigen-loaded antigen-presenting cells (APC). Current approaches to the induction of efficient antitumor immune responses are focused on the targeting of TAA to dendritic cells, the immune systems premier APC. These approaches include targeting of antigens to CD40 (4) or DC-SIGN (5), but both approaches lack a singular specificity for dendritic cells without also targeting monocytes, eosinophils, or even fibroblasts (6). An alternative is the targeting of the dendritic cell-specific receptor DEC-205. DEC-205 is an endocytic receptor that belongs to the mannose receptor family and is highly expressed specifically on dendritic cells. We have reported previously that the chemical coupling of antigen to the monoclonal antibody (mAb) DEC-205 is effective in inducing antigen-specific immunity resulting in the eradication of established metastatic melanoma tumors in mice (3). When using the described methodology, although effective, it is difficult to achieve a consistent and efficient coupling of antigens and antibodies, which is a major concern for clinical applications. The goal of this study was to identify a single-chain fragment variable (scFv), containing only the variable heavy ($V_H$) and light ($V_L$) regions, to specifically target TAA to DEC-205 and therefore dendritic cells. In comparison with mAbs chemically coupled

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### Translational Relevance

The clinical use of a scFv molecule to specifically target tumor antigens to dendritic cells would provide several advantages. The range of tumor antigens that could be fused to the scFv is enormous and could therefore be applied to multiple forms of cancer. The lack of a Fc domain and high specificity of the DEC-205 scFv would lead to less dilution of the therapeutic drug through uptake by other cell types. This would correlate to lower dosages needed and possibly fewer adverse reactions. Beyond this, binding to FcγRIII has been shown to induce immunosuppressive signals, which the scFv would avoid. Dendritic cell treatments such as *ex vivo* loading of antigen are time-consuming and are associated with high costs. As a simple protein, the scFv cost of production would be lower; therefore, the availability to patients increased. By directly priming the bodies' own immune system, there should be less chance of recurrence from the same antigen-expressing cancer after primary treatment.

or fused with TAA, this type of minimized antibody should provide a targeting platform with fewer cross-reactions to nonspecific receptors or cell types while also increasing the efficiency and ease of production. In this study, we show that scFv-mDEC-205 binds to the mDEC-205 receptor with the same specificity as the anti-DEC-205 mAb. Moreover, we show that scFv-mDEC-205 targets dendritic cells within lymph nodes when administered in the context of an *in vivo* vaccination. Binding of the scFv to the DEC-205 receptor, with CpG oligodinucleotide and polyriboinosinic-polyribocytidylic acid [poly(I:C)] dendritic cell activation stimuli, leads to the internalization of the scFv antibody and an increase in the activation status of the dendritic cells, which can last up to 72 h. Finally, we used the scFv-mDEC-205-gp100 TAA fusion antibody with dendritic cell activation stimuli as a therapy for established transplantable melanoma tumor models B16/F10 and RET. In comparison with TAA peptide, nonbinding scFv, or activation stimuli-only therapies, the scFv-mDEC-205-gp100 significantly slowed tumor growth rates.

In summary, we have been able to show that the scFv-mDEC-205 is a viable targeting vector for bringing TAA specifically into dendritic cells where they are processed and presented to T cells in the context of MHC molecules.

## Materials and Methods

***Construction of scFv.*** Cells from the hybridoma cell line NLDC-145 for murine DEC-205 and hybridoma cell line GL117 for β-galactosidase (β-Gal) were cultivated in R5 [RPMI 1640 supplemented with 5% FCS, penicillin-streptomycin, and glutamine (all from PAA Biochemicals)] until confluent. Cells were then collected from a 75 $mm^2$ flask and total RNA was isolated using RNeasy kit (Qiagen) as per the manufacturer's protocol. Reverse transcription-PCR was then done using the degenerative primers as follows for both isolated RNA samples. The $V_H$ domains were amplified with the forward primer 5′-CTCGCGGCCCAGCCGGCCATGGCC-SAGGTSMARCTGVWRSARTCWGG-3′ and the reverse primer 5′-TGGTCCACTCGAGACGGTGACCGTGGTCCCTTGGCCCC-3′. The $V_L$ domains were amplified with a single forward primer 5′-GGCGGTAGTGCACAGATTGTGATRACMCARGATGAACTCTC-3′ used in combination with reverse primers 5′-TCATTCTGCGGCCGCCCCGTTTSAKYTCCAGCTTGGTSCC-3′ and 5′-TCATTCTGCGGCCGCCCCGTTTTATTTCCARCTTKGTCCC-3′ in equimolar ratios (all primers synthesized by MWG Biotech). The $V_H$ region was subcloned into the cloning vector pHEN3 in the *Sfi*I/*Xho*I restriction sites creating a pHEN3-$V_H$ plasmid. The $V_L$ domain was subcloned into the *Apa*LI/*Not*I sites of pHEN3-$V_H$ plasmid to produce plasmid pHEN3-scFv. The completed scFv region was cut from the plasmid pHEN3-scFv with *Sfi*I/*Not*I and subcloned into the bacterial expression vector pAB1. The tumor antigen portion of the scFv-mDEC-205-gp100 plasmid was synthesized within *Not*I/*Eco*RI restriction sites (Geneart) and subcloned into the same sites of the pAB1-scFv.

***Protein production.*** The expression plasmid pAB1-scFv was transformed into *Escherichia coli* strain TG1. A 20 mL culture was grown overnight at 37°C in YT broth with 100 μg/mL ampicillin and 1% glucose. A 10 mL aliquot was transferred to fresh 1 L YT broth with 100 μg/mL ampicillin and 0.1% glucose followed by incubation at 37°C with shaking until an $A_{600}$ of 0.8 was achieved. The bacteria were briefly chilled on ice and then induced by the addition of 1 mmol/L IPTG and further cultured overnight at 22°C with shaking. The bacteria were pelleted (5,000 rpm, 15 min), the supernatant was removed, and the pellet was resuspended in 50 mL periplasmic resuspension buffer [30 mmol/L Tris (pH 8.0), 1 mmol/L EDTA, 20% sucrose]. Once resuspended, 0.25 mL freshly mixed 1 mol/L lysozyme was added and incubated for 15 min on ice with gentle agitation followed by the addition of 0.25 mL of 1 mol/L $MgSO_4$. The resulting slurry was centrifuged for 15 min at 12,000 rpm at 4°C. The supernatant was recovered and dialyzed against 10 mmol/L $K_2HPO_4$ overnight in snakeskin dialysis tubing (MWCO 10,000; Thermo Scientific). The supernatant was centrifuged again for 15 min at 12,000 rpm at 4°C and the cleared lysate was isolated. A Ni-NTA column was prepared from 2 mL Probond (Invitrogen) slurry in 1 cm diameter columns. The columns were washed with 1 column volume of native binding buffer [10 mmol/L imidazole, 50 mmol/L $NaH_2PO_4$, 500 mmol/L NaCl (pH 8.0)] followed by the addition of the degassed cleared bacterial lysate. The column was washed with two column volumes native wash buffer [30 mmol/L imidazole, 50 mmol/L $NaH_2PO_4$, 500 mmol/L NaCl (pH 8.0)] followed by collection of 1 mL fractions using elution buffer [250 mmol/L imidazole, 50 mmol/L $NaH_2PO_4$, 500 mmol/L NaCl (pH 8.0)]. The protein content of the elution fractions was determined by standard Bradford assay, and the positive fractions were pooled and dialyzed overnight at 4°C against PBS. Fractions were collected throughout the purification procedure for later analysis (all chemicals from Sigma).

***SDS-PAGE and Western blots.*** Protein production and purification steps were analyzed by SDS-PAGE. A 10% SDS-PAGE gel was loaded with fractions collected during the purification procedure. SDS-polyacrylamide gels run with samples from the purification procedure were blotted onto polyvinylidene difluoride membranes in transfer buffer containing 20% methanol overnight at 4°C and 30 V. Membranes were probed with anti-PentaHis antibody (1:800; Qiagen) followed by secondary anti-mouse horseradish peroxidase-labeled antibody (1:1,000; Dianova). Blots were developed with an enhanced chemiluminescence detection kit (GE Healthcare).

***Generation of cells and fluorescence-activated cell sorting.*** Murine bone marrow-derived dendritic cells (BMDC) were prepared according to standard methods. Briefly, bone marrow cells from naive C57Bl/6 mice were cultured in R5 supplemented with granulocyte-macrophage colony-stimulating factor for 6 days with refeeding. Thereafter, nonadherent cells were harvested and transferred to six-well dishes. Cytospins were prepared from BMDC by centrifugation in a Shandon Cytospin 2 at 300 rpm for 1 min onto Superfrost slides. Staining for the mAb DEC-205 was done with a 1:100 dilution of purified antibody from the hybridoma NLDC-145 followed by 1:500 dilution of FITC-labeled goat anti rat antibody (Jackson Immunoresearch). Staining for scFv was done with or without (depending on the injection of scFv or use in BMDC cultures) a 1:100 dilution of the scFv followed by 1:800

dilution of the biotin anti c-myc antibody (AbD Serotec) and 1:1,000 dilution of Alexa 488-labeled streptavidin (Invitrogen). $CD11c^{+/-}$ cells were prepared from single-cell suspensions of lymph nodes isolated from C57Bl/6 mice using MACS beads (Miltenyi) according to standard protocols. For detection of CD80, CD86, and MHC class II, respective FITC- or PE-labeled antibodies (all Becton Dickinson) were used at 1:100 dilutions and analysis was carried out using a FACScanto (Becton Dickinson).

***Staining of scFv in tissue sections.*** C57Bl/6 mice were injected with activation stimuli [consisting of 50 μg CpG (MWG Biotech) and 50 μg poly(I:C) (GE Healthcare)] and one of the following: PBS, mAb DEC-205, scFv-mDEC-205, scFv-mDEC-205-gp100, or scFv-β-Gal. At the set time point, animals were sacrificed and the lymph nodes were removed. Lymph nodes were frozen in OCT embedding medium and 8 μm sections were placed on Superfrost slides. Sections were rehydrated in PBS and then fixed in 4% paraformaldehyde followed by three washes with PBS. Sections were then stained for 30 min with biotin-labeled anti-c-myc antibody diluted 1:800 (AbD Serotec). Sections were washed in PBS and stained for 10 min with secondary Alexa 488-labeled streptavidin at a dilution of 1:1,000. Images were captured on a Leica DM5500B microscope using Metamorph software (Visitron Systems).

***Elispot assay.*** C57Bl/6 mice injected twice in weekly intervals with PBS, scFv-mDEC-205-gp100, or gp100 peptide (Activotec) plus activation stimuli [50 μg CpG and 50 μg poly(I:C)] were sacrificed 3 days after the last injection and single spleen cell suspensions were prepared with standard protocols and cultivated in Elispot plates (Nunc) that had been precoated with 50 μL/well rat anti-mouse-IFN-γ antibodies in PBS (10 μg/mL; Pharmingen) overnight at 4°C. For stimulation, gp100 peptide (amino acids 25-33) was used, whereas β-Gal peptide and RPMI alone served as controls. Plates were incubated at 37°C for 24 h at 5% $CO_2$ and developed with 100 μL biotintylated rat anti-mouse IFN-γ (5 μg/mL) in assay buffer (0.5% bovine serum albumin, 0.05% Tween 20 in PBS). Thereafter, 100 μL/well streptavidin-POD was added and incubated at room temperature for 30 min. Color was developed by addition of 50 μL/well of 3,3′-diaminobenzidine developer (peroxidase substrate kit; Vector), and the number of gp100-specific $CD8^+$ T cells was evaluated by counting.

**In vitro *proliferation assay.*** To induce gp100-specific T cells, naive C57Bl/6 mice were injected with gp100 peptide (amino acids 25-33; Activotec) combined with adjuvant followed by a boost of gp100 peptide and adjuvant 1 week later. Lymph nodes were collected and cells were prepared as single-cell suspensions. Cells were then separated on MACS columns into $CD4^+$ and $CD8^+$ fractions and grown in R5 medium at 37°C, 5% $CO_2$. $CD4^+$ or $CD8^+$ cells ($1 \times 10^5$) were cultured with various ratios of BMDC that had been pulsed with the peptide gp100 (Activotec) for 16 h and proliferation was measured by thymidine incorporation.

***Tumor therapy.*** All animal protocols were approved under the guidelines of the animal protection act. For therapeutic experiments, tumor growth was induced by subcutaneous injection of $5 \times 10^5$ B16/F10 cells or $5 \times 10^5$ RET cultured melanoma cells in the shaved right flank. After 5 days, when the tumor growth was first seen (diameter, 1 mm), activation stimuli [50 μg CpG/10 μg poly(I:C)] with either PBS or 300 pmol scFv-mDEC-205-gp100 or 300 pmol gp100 peptide were injected in each mouse. Injections were administered on days 5, 8, and 12 and the tumor growth was measured manually using a caliper ruler.

## Results

***Development of a scFv for DEC-205.*** We have developed a scFv molecule that is specific for the murine endocytic scavenger receptor DEC-205. cDNA encoding the $V_H$ and $V_L$ domains of the mAb produced by the hybridoma cell line NLDC-145 were isolated via reverse transcription-PCR from total RNA using degenerative primers. The $V_H$ and $V_L$ domains separated by a $(G_4S)_4$ linker sequence were subcloned into the expression vector pAB1 in order that the scFv was followed in-frame by a TAA, c-myc epitope, and 6× histidine tag. Tumor antigens created by DNA synthesis were then subcloned 3′ of the $V_L$ domain (Fig. 1A). The protein production of the scFv was verified on several levels. First, the various fractions were collected throughout the Ni-NTA isolation process. These fractions were subjected to Bradford assay to determine total protein presence within the samples. Total protein was visible in the cleared lysate and the flow-through fractions but not in the final wash steps. Protein was again detectable in the elution fractions where the highest concentration of imidazole, with a higher binding coefficient, replaced the scFv on the Ni-NTA column (Fig. 1B). The fractions were analyzed using a 10% SDS-PAGE gel with total proteins visualized via Coomassie blue staining. A 33 kDa protein is clearly visible in the cleared lysate, which is absent in the flow-through and the wash steps but present as the strongest band in the dialyzed elution fraction (Fig. 1C). To clarify that the elution fraction contained a hexahistidyl-tagged protein, the fractions were blotted onto polyvinylidene difluoride membrane and probed with anti-PentaHis antibody followed by detection via enhanced chemiluminescence. His-tagged protein is detectable in the cleared lysate with low amounts visible in the flow-through but not the wash fractions and highly concentrated in the dialyzed pooled elution fractions (Fig. 1D). These figures are representative of all scFv produced in this study. These included a negative control, nonbinding scFv for β-Gal (scFv-β-Gal) as well as the scFv-mDEC-205 and scFv-mDEC-205-gp100 tumor antigen fusion molecule.

***Binding ability of the scFv-mDEC-205 to dendritic cells.*** To show the capacity of scFv-mDEC-205 to bind dendritic cells expressing the DEC-205 receptor, we cultured BMDC from C57Bl/6 mice. The BMDC phenotype was confirmed by $CD11c^+$ fluorescence-activated cell sorting analysis and then stained with either isotype control antibodies, the mAb for DEC-205, scFv-mDEC-205, or the nonbinding control scFv-β-Gal and analyzed using fluorescence-activated cell sorting. The mAb αDEC-205 stained 89% of the BMDC positive in comparison with its isotype control. The scFv-mDEC-205 stained 46% of the cells positive, whereas the scFv-β-Gal and isotype control showed negligible staining of BMDC (Fig. 2A; data not shown). The preincubation of BMDC with the mAb DEC-205 for 1 h shows a decreased staining of scFv-mDEC-205 on 6-day cultured BMDC in comparison with the staining without preincubation (Fig. 2B).

Cytospins of the BMDC were prepared and fixed in 4% paraformaldehyde. Cells were first stained with PE-labeled αCD11c and then stained with either mAb DEC-205, scFv-mDEC-205, or scFv-β-Gal followed by appropriate Alexa 488-labeled secondary antibodies. Fluorescence microscopy of cells stained by both αDEC-205 mAb and scFv-mDEC-205 revealed DEC-205 receptor-positive cells, whereas the control nonbinding scFv-β-Gal staining showed no green-fluorescing cells (Fig. 2C).

***scFv-mDEC-205 homes to the lymph nodes and remains resident in dendritic cells.*** To establish that the scFv-mDEC-205 can bind DEC-205-positive cells within an intact cellular environment and in the same manner as the mAb, we sectioned wild-type lymph nodes of C57Bl/6 mice and stained with the mAb or scFv-mDEC-205 and the appropriate FITC-labeled or Alexa 488-labeled secondary antibody. Both the mAb and

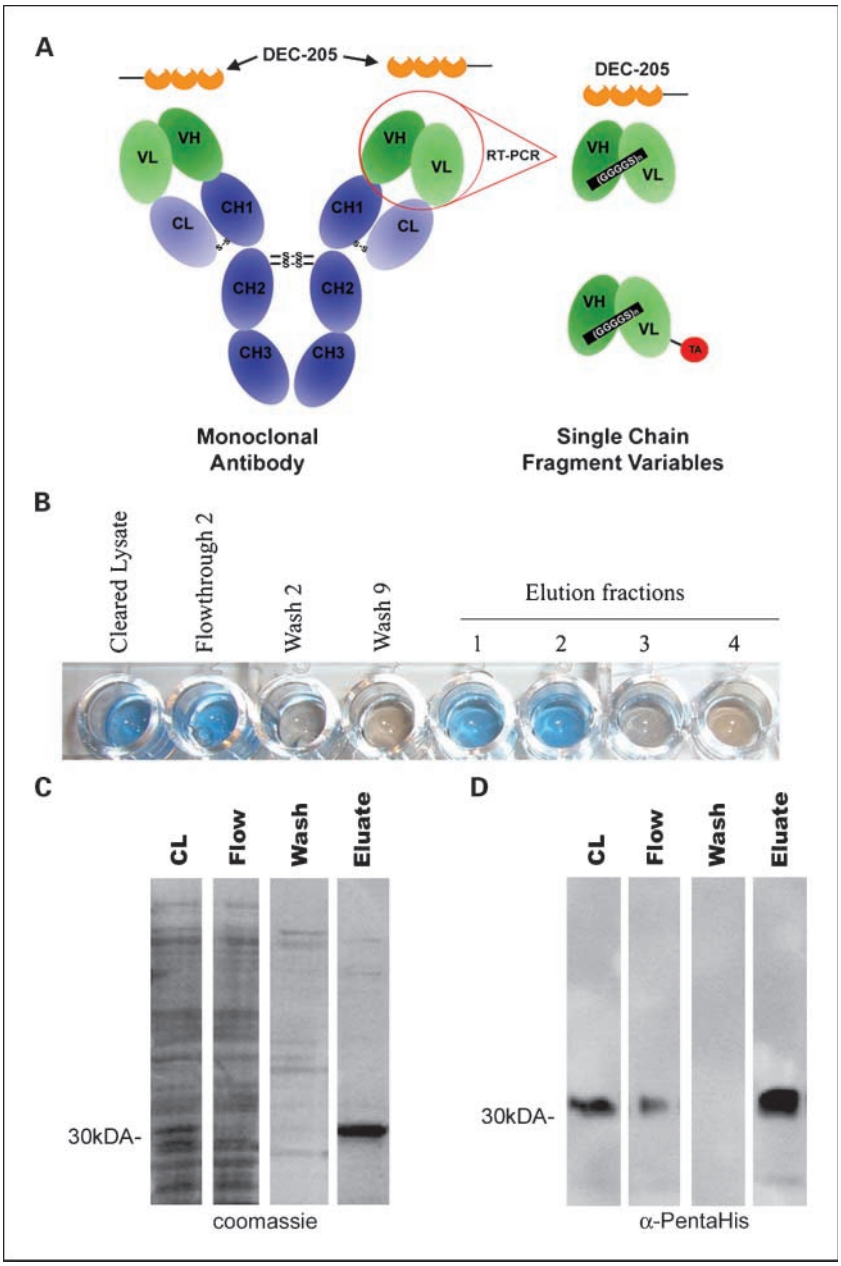


**Fig. 1.** Creation and isolation of scFv and scFv tumor antigens. *A,* schematic of the scFv consisting of the $V_H$ and $V_L$ domains from a mAb, connected via a poly-GS linker and various fusion protein possibilities. *B,* Bradford assay of protein fractions collected during protein production of scFv-mDEC-205. *C,* Coomassie blue-stained SDS-PAGE gel from protein production of the scFv-mDEC-205 isolated using Ni-NTA columns, left to right cleared bacterial lysate, second flow-through fraction, final wash step, and pooled elution fraction. *D,* polyvinylidene difluoride blot of scFv-mDEC-205 protein production stained with anti-PentaHis antibody and visualized via enhanced chemiluminescence. *CL,* cleared lysate; *flow,* column flow-through.

scFv-mDEC-205 display similar staining patterns of DEC-205-positive cells in these sections (Fig. 3A). Next, we established that the scFv has the ability to target dendritic cells *in vivo* by injecting naive C57Bl/6 mice subcutaneously in the footpad of the forelimb with one of the following: PBS, mAb αDEC-205, scFv-mDEC-205, scFv-mDEC-205-gp100, or scFv-β-Gal together with dendritic cell activation stimuli composed of CpG/poly(I:C). Mice were sacrificed after 24 h and the draining (proper axillary) and distant (subiliac) lymph nodes were removed for cryostat sectioning. Fluorescence microscopy of the sections showed that only those animals injected with the mAb, the tumor antigen-targeting scFv-mDEC-205-gp100 (Fig. 3B and C), or scFv-mDEC-205 (data not shown) were able to stain dendritic cells within the lymph nodes both draining and distant from the injection sight. The nonbinding scFv-β-Gal did not stain any cells positive within the lymph nodes (Fig. 3D). Positively stained cells in the draining lymph nodes could be seen as early as 6 h after injection of the scFv-mDEC-205 (data not shown).

To analyze how long the scFv remain resident in dendritic cells, we performed time-course studies followed by analysis via fluorescence-activated cell sorting. Naive mice were injected with dendritic cell activation stimuli composed of CpG/poly(I:C) and scFv-mDEC-205. At various time points, the mice were sacrificed and the lymph nodes were removed. Cells were then sorted by magnetic beads separation on MACS columns and the $CD11c^+$ and $CD11c^-$ fractions were stained with anti-c-myc and appropriate secondary antibodies. The $CD11c^-$ fraction of cells did not stain positive for scFv-mDEC-205 (Fig. 4) and showed minimal up-regulation of dendritic cell activation markers CD86 or CD80 over 72 h (data not shown). Conversely, the $CD11c^+$ fraction stained positive for

scFv-mDEC-205 after 24 h and staining was detectable up to 72 h after vaccination. The CD11c$^+$ fraction, which at day 0 showed low expression of the activation marker CD86, becomes highly activated after 24 h and retains an elevated level of expression for up to 72 h (Fig. 4). When scFv-mDEC-205-gp100 is injected without CpG/poly(I:C) activation stimuli, the activation marker CD86 remained at the same expression levels as unperturbed BMDC (data not shown).

***scFv-mDEC-205-gp100 elicits gp100-specific CD4 and CD8 T-cell proliferation.*** We immunized naive C57Bl/6 mice with gp100 peptide in adjuvant followed 1 week later by a boost of adjuvant and gp100 peptide to induce T cells specific for the endogenous tumor antigen gp100. The mice were sacrificed after 2 weeks and the CD4$^+$ and CD8$^+$ cells were separated via magnetic bead isolation on MACS columns. The CD4$^+$ and CD8$^+$ cells were then used for mixed lymphocyte reactions in which they were cultured with 6-day cultured BMDC from naive C57Bl/6 mice that had been pulsed with PBS, scFv-mDEC-205-gp100, or the peptide gp100 alone. A significant increase in T-cell proliferation was observed in CD4$^+$ and CD8$^+$ gp-100-specific T cells only when cultured with BMDC pulsed with scFv-mDEC-205-gp100 in comparison with T cells cultured with BMDC pulsed with gp100 peptide alone or non-pulsed BMDC (Fig. 5A and B).

***Elispot assay indicates an increase in CTL response after targeting with scFv-mDEC-205-gp100.*** We next carried out Elispot IFN-γ assays to assess the activation of a CTL response after vaccination with scFv-mDEC-205-gp100 in combination with dendritic cell activation stimuli. C57Bl/6 mice were injected twice in weekly intervals with dendritic cell activation stimuli of CpG/poly(I:C) and one of the following: PBS, scFv-mDEC-205-gp100, or gp100 peptide alone. Three days after the last vaccination, the mice were sacrificed and splenocytes were isolated. After isolation, the cells were cultured in Elispot plates with or without gp100 peptide stimulation and the number of IFN-γ-producing cells was determined. The splenocytes from animals that had received the vaccinations using scFv-mDEC-205-gp100 showed a significant increase in the number of IFN-γ-producing cells in comparison with cells exposed to peptide or dendritic cell activation stimuli alone or cells cultured in medium without additional peptide (Fig. 5C).

**In vivo** ***tumor therapy with scFv-mDEC-205-gp100 slows tumor growth in transplantable melanoma models.*** Once we established that the scFv-mDEC-205-gp100 was able to specifically target dendritic cells in lymph nodes, we tested the efficacy of our scFv-mDEC-205-gp100 vaccination in a therapeutic setting. Tumor cells ($5 \times 10^5$) were injected subcutaneously on the shaved dorsal flank of naive mice. When the tumor was established (1 mm diameter), we vaccinated mice with dendritic cell activation stimuli of CpG/poly(I:C) and one of the following: PBS, scFv-mDEC-205-gp100, scFv-β-Gal, or gp100 peptide. In the B16/F10 model at day 7, there was only a subtle difference in the tumor growth between mice with varying vaccinations. Thereafter, the growth pattern of the tumors diverged. One week after the initial vaccination was administered, scFv-mDEC-205-gp100 started

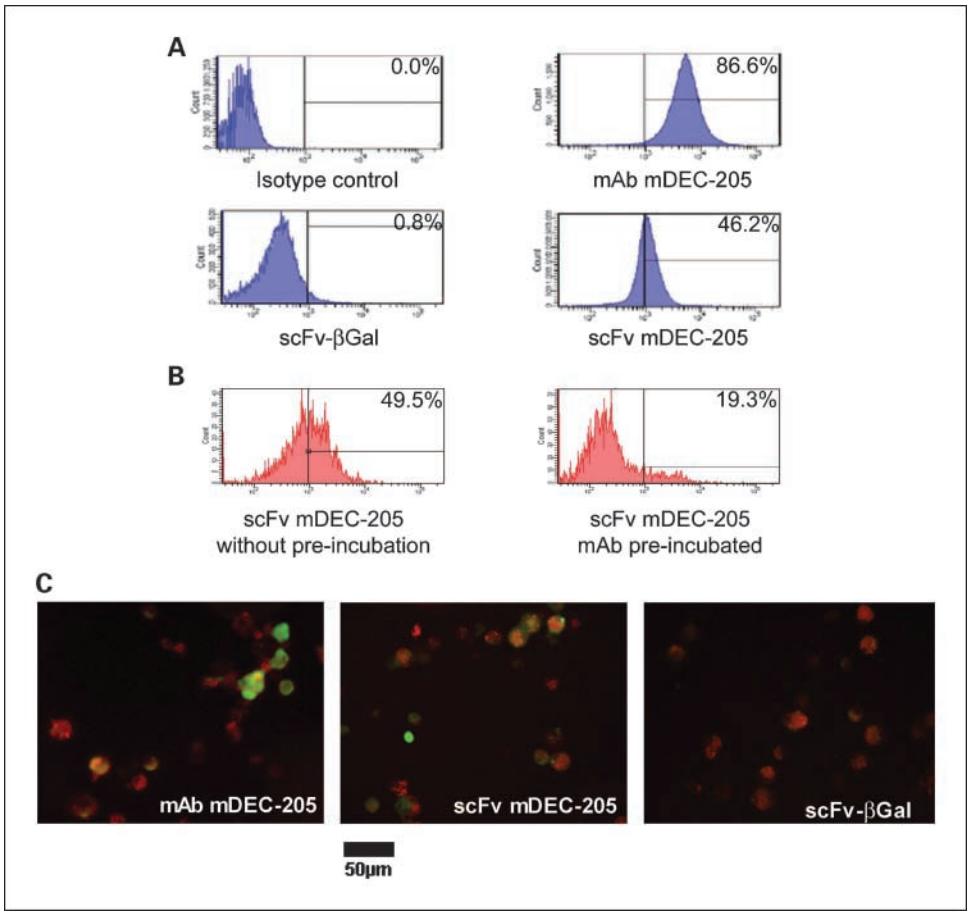


**Fig. 2.** scFv-mDEC-205 binding to BMDC. Fluorescence-activated cell sorting staining of 6-d murine cultured BMDC. *A,* comparison of staining by the mAb mDEC-205 with its isotype control and the scFv-mDEC-205 with a nonbinding scFv-β-Gal. *B,* blocking experiment where the BMDC are stained with or without preincubation of the mAb. *C,* immunohistochemistry of cytospins from BMDC fixed with 4% paraformaldehyde and counterstained with PE-labeled αCD11c. FITC-labeled or Alexa 488-labeled antibodies used for αmDEC-205 mAb, scFv-mDEC-205, and nonbinding control scFv-β-Gal. Magnification, ×20.

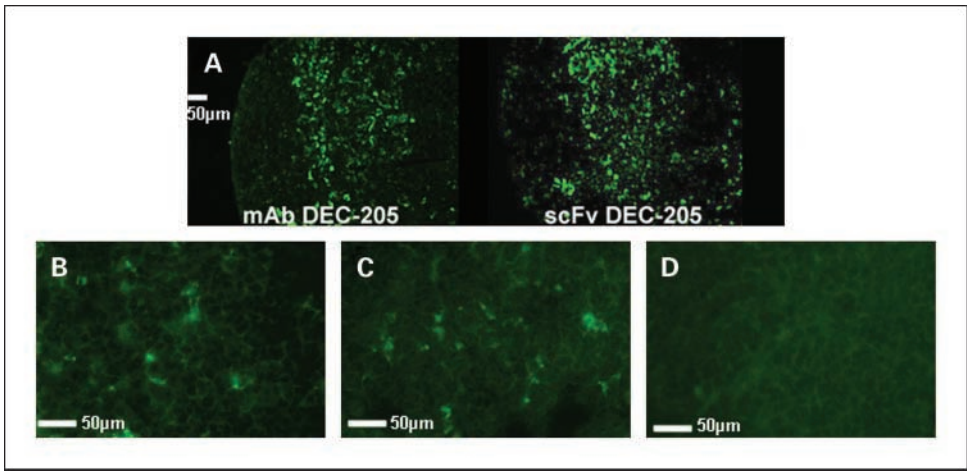


**Fig. 3.** scFv binding and targeting to lymph node-resident dendritic cells *in vitro* and *in vivo*. Lymph nodes from naive C57Bl/6 mice were cut in 8 μm frozen sections and fixed with 4% paraformaldehyde. *A,* sections were stained with the mAb αDEC-205 or scFv-DEC-205 and corresponding Alexa 488-labeled secondary antibodies. Magnification, ×20. *B* to *D,* C57Bl/6 mice were injected 24 h before sacrifice with activation stimuli CpG and poly(I:C) as well as the mAb DEC-205, scFv-DEC-205-gp100, or scFv-β-Gal. The lymph nodes were removed and snap-frozen and 8 μm cryostat sections were prepared and fixed in 4% paraformaldehyde. Sections were stained with biotin-labeled anti-c-myc and Alexa 488-labeled streptavidin. *B,* mAb αDEC-205, subiliac lymph nodes. *C,* scFv-mDEC-205-gp100, subiliac lymph nodes. *D,* scFv-β-gal-negative control, subiliac lymph nodes. Magnification, ×40.

to show a slowing effect on tumor growth. Beyond this 12-day time point, the vaccinations with scFv-mDEC-205-gp100 caused a significant reduction in tumor size (34% tumor volume of WT) in comparison with the injection of peptide ($P \leq 0.05$) or scFv-β-Gal (80% or 88% tumor volume of WT animals, respectively; Fig. 6B). Furthermore, we established RET tumors in C57Bl/6 mice using $5 \times 10^5$ tumor cells and waited for the development of tumors 1 mm in diameter. The scFv-mDEC-205-gp100 vaccination therapy with dendritic cell stimulation again induced a significantly reduced tumor growth at days 17 and 21 in comparison with peptide-injected animals ($P \leq 0.05$). Tumor volumes of the scFv-mDEC-205-gp100 and gp100 peptide vaccinated animals were 50% and 122%, respectively, of the control group (Fig. 6C). Long-term survival comparisons were not carried out due to the high tumor burden of the control groups and gp100-immunized mice. Although mice from control groups had to be sacrificed in the third week after tumor challenge, the scFv-mDEC-205-gp100 immunization led to survival up to 35 days for mice in this group. These results taken together indicate that we could use

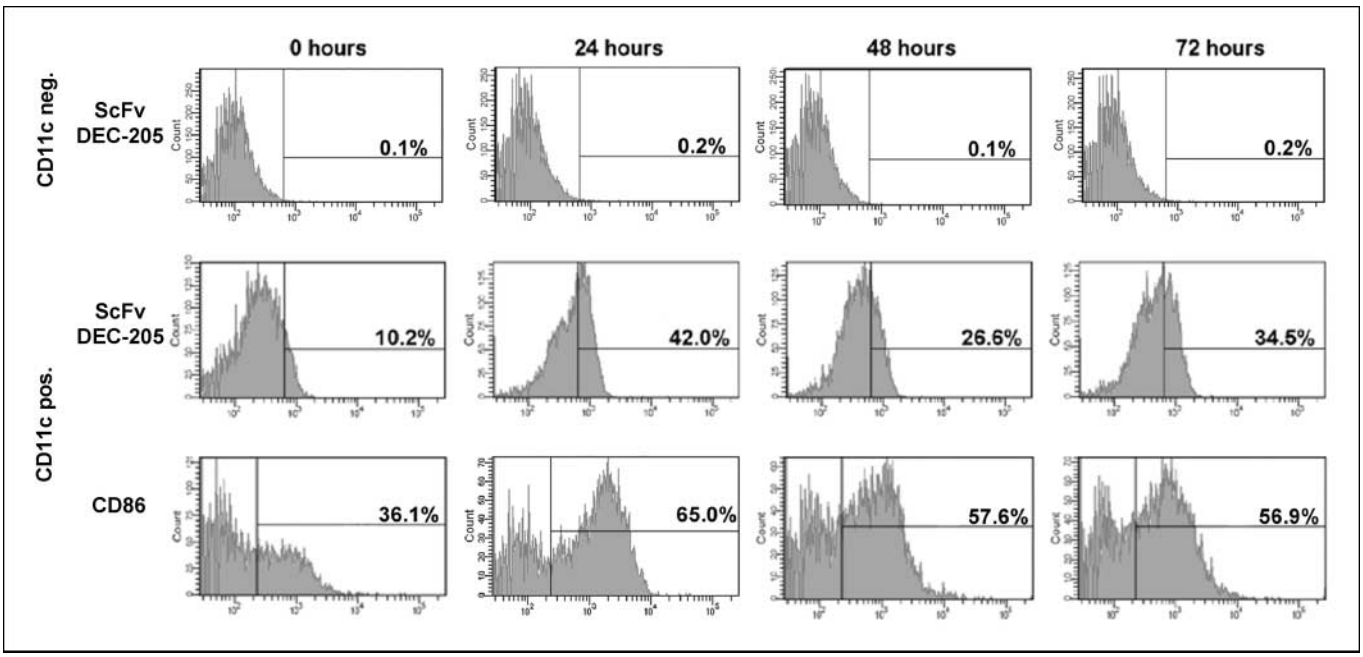


**Fig. 4.** scFv remains in the lymph node-resident dendritic cells up to 72 h. scFv-mDEC-205 was injected in naive C57Bl/6 mice with dendritic cell activation stimuli followed by sacrifice at defined time points. $CD11c^-$ and $CD11c^+$ cells were analyzed via fluorescence-activated cell sorting at various time points for the presence of scFv using secondary anti c-myc antibodies. scFv was detectable in $CD11c^+$ fraction, whereas it did not bind to non-APC cell types in the $CD11c^-$ fraction. $CD11c^+$ cells were also checked for activation status via CD86. scFv binding is detectable up to 72 h after vaccination. Activation is highest after 24 h and retains increase activation levels through 72 h. Representative of three experiments.

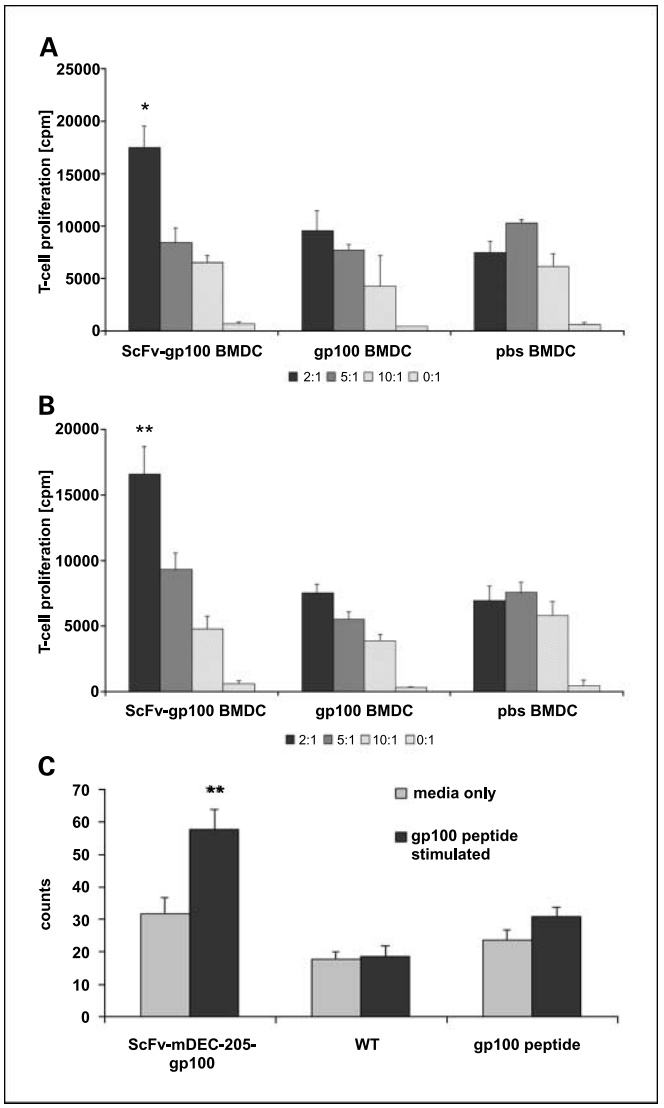


**Fig. 5.** *In vitro* proliferation of $CD4^+$ and $CD8^+$ T cells and CD8 Elispot assay. *A* and *B, in vitro* proliferation. Naive C57Bl/6 mice were injected with gp100 peptide combined with adjuvant followed by a boost of the peptide/adjuvant 1 wk later. CD4 and CD8 fractions were isolated from lymph nodes and tested for proliferation in mixed lymphocyte reactions with pulsed 6-day BMDC. CD4 (*A*) or CD8 (*B*) T cells ($10^5$) were cultured in triplicate with various ratios of BMDC that had been pulsed with gp100 peptide. Proliferation was measured by thymidine incorporation ± SD. *, $P \leq 0.05$; **, $P \leq 0.01$, compared with gp100-pulsed BMDC. Representative of two experiments. *C,* Elispot *in vivo* IFN-γ assay. C57Bl/6 mice were immunized twice over 7 d and CD8 cells were then isolated via MACS beads. Cells were plated in a multiscreen 96-well filtration plate, precoated with rat anti-mouse IFN-γ antibodies, in complete medium with or without gp100 peptide. After 24 h, plates were incubated with biotintylated rat anti-mouse IFN-γ followed by streptavidin-POD and color development with peroxidase substrate kit. The number of IFN-γ-producing cells was counted under a dissecting microscope ± SD. **, $P \leq 0.01$, compared with peptide-injected animals with gp100 peptide added to the medium. Representative of three experiments ($n = 8$).

the scFv-mDEC-205-gp100 fusion protein, in combination with activation stimuli, as a dendritic cell-specific TAA therapeutic vaccination to reduce the growth rate of already established melanoma tumors.

## Discussion

Dendritic cells are the most efficient APC described thus far and must therefore be included in therapies to induce antitumor immunity. Several clinical trials have used *ex vivo* loading of dendritic cells with tumor antigen followed by injection into diseased patients. However, this dendritic cell therapy has many technical complications, including *ex vivo* purification of dendritic cells, efficient loading with respective tumor antigens, and cultivation for prolonged time without the loss of dendritic cell functions (7). Once these obstacles have been overcome, the antigen-presenting lifespan of the reinfused dendritic cells is still reduced in comparison with natural *in vivo* antigen uptake. *In vivo*, only a fraction of the antigen-pulsed dendritic cells reach regional lymph nodes where cell-to-cell contact between dendritic cells and T cells is required for effective T-cell induction (8). To overcome these difficulties, several methods have been developed for targeting TAA to APC *in vivo* to modify T-cell responses toward cells expressing these surface antigens. Methodology ranges from the use of viral or liposomal targeting to the conjugation/fusion of antigen

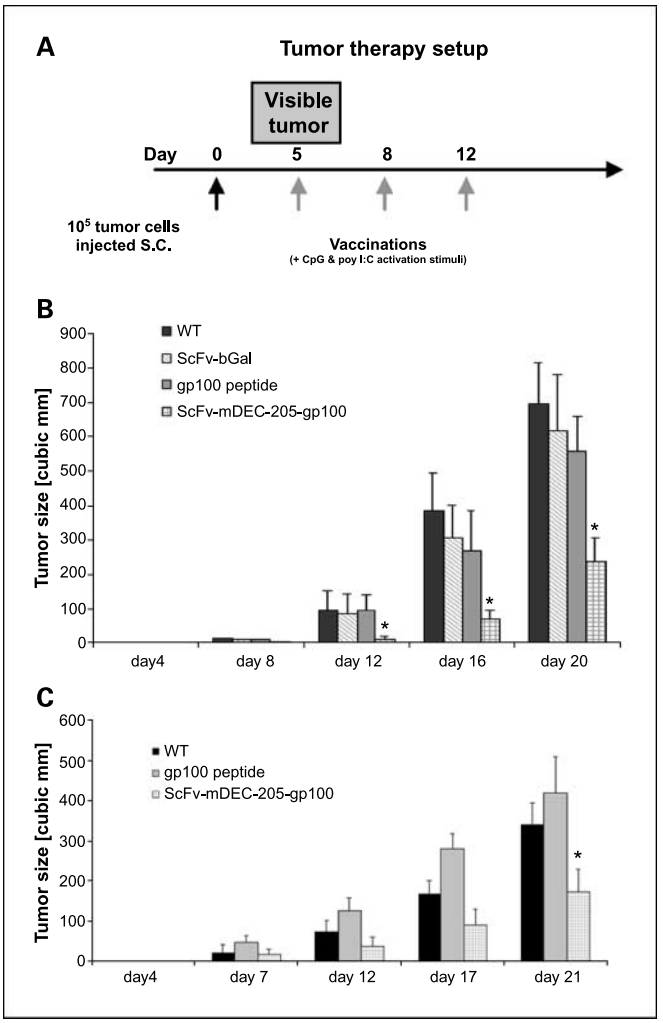


**Fig. 6.** Melanoma tumor therapies. *A,* melanoma tumor cells ($5 \times 10^5$ B16F10 or RET cell lines) were injected subcutaneously on the flank of C57Bl/6 mice. Vaccinations with PBS, gp100 peptide, scFv-β-Gal, or scFv-mDEC-205-gp100 plus activation stimuli were carried out after a visible tumor was established on days 5, 8, and 12. Tumor volume was measured over a period of 3 wk using calipers ($n = 5$). B16/F10 (*B*) or RET (*C*) tumor challenge. *, $P \leq 0.05$, compared with peptide-vaccinated animals. Representative of three independent experiments, including SD.

directly to APC receptor-specific mAbs (1, 3, 4, 9–11). However, most of these various strategies lack the singular specificity for targeting only dendritic cells, as several antibodies used thus far also bind to macrophages or B cells. Moreover, Fc binding may further dilute the antibody-antigen conjugate and may convey immunosuppressive signals as shown for the FcγRIII (12). Among the surface molecules expressed by dendritic cells, DEC-205 may be in particular suitable for targeting of dendritic cells, as it has been shown to be an endocytic receptor of the macrophage mannose receptor family and it is expressed exclusively by dendritic cells (13, 14). Although a specific ligand for this receptor has not yet been elucidated, mAbs, both human and murine, have been used successfully to show the tissue distribution, confirming strong expression by lymph node dendritic cells. DEC-205 also uses a novel intracellular routing pathway, as it targets antigens directly to the MHC class II-positive late endosomal compartments. From here, the DEC-205 receptor recycles to the surface where it can repeat the antigen uptake process. Thus, this constant shuttling of antigen to MHC class $II^+$ compartments results in superior antigen presentation compared with other mechanisms such as pinocytosis or phagocytosis (15). Similar to most exogenous antigens, DEC-205-targeted antigens are presented on MHC class II molecules, but, in addition, the DEC-$205^+$ dendritic cell subset is able to cross-present DEC-205 antigens to $CD8^+$ T cells. Recently, Dudziak et al. have described two dendritic cell subsets: the first of which is DEC-$205^+$, whereas the second subset is $33D1^+$. They have shown that the DEC-$205^+$ but not the $33D1^+$ subsets have the ability to cross-present antigen via MHC class I molecules, thus inducing CTL responses as well as CD4 T-cell responses concomitantly (16). Therefore, our use of the hybridoma cell line NLDC-145 producing the mAb against DEC-205 was believed to be the best choice for the creation of a scFv molecule, which would be most advantageous for use in cancer immunotherapy.

Previous reports have had success in fusing viral peptides to mAb DEC-205 (2, 17) and scFv-mDEC-205 (17) or incorporation of bacterial epitopes into a scFv-mDEC-205 (10). These studies have focused on antigens that are highly immunogenic as they are purely exogenous. The source of infection generally produce large amounts of antigen through high levels of bacterial growth (18) or replication of antigen-expressing virions up to $1 \times 10^{12}$ per day (19). Still, these studies have validated the induction of immunity by targeting antigens to DEC-$205^+$ cells. Beyond these studies, we have developed scFv-mDEC-205 TAA targeting for melanoma. These tumors overexpress self-antigens such as gp100, TRP-2, or MART1; therefore, self-tolerance to endogenous antigen must first be broken before immunity can be achieved. In comparison with whole antibodies, the small size of the scFv allows for easier production of a protein fused to various and/or multiple epitopes of tumor antigens without the added reactivity of the Fc portion. In this study, we have created a minimal antibody fusion protein that retains the same binding ability of the standard mAb. This scFv is a stable protein at room temperature with a size of 33 kDa. As the size of the TAA that we have fused to the scFv is only 9 amino acids, there is minimal steric hindrance of binding and internalization of the fusion protein via the DEC-205 receptor and we have indeed shown that these scFv fusion proteins bind in a manner similar to the "native" DEC-205 mAb. The scFv-mDEC-205 and scFv-mDEC-205-gp100 not only home directly to lymph node-resident dendritic cells within 6 h of subcutaneous vaccination but also remain resident in the dendritic cells for up to 72 h. Moreover, we have not seen any uptake of the scFv by monocytes or B cells, indicating that that degradation of the scFv constructs by scavenging cells is minimized. This, together with the length of time that the scFv-mDEC-205 remains bound to dendritic cells in the lymph nodes, may also translate into an increase in the number of effector T cells, which see MHC class I and/or II processed tumor antigen.

The immunization protocols used in this study include the use of TLR-binding ligands poly(I:C) [poly(I:C)/TLR3] and unmethylated CpG oligodinucleotide 1826 (TLR9) to activate and mature dendritic cells (20). Experiments in which the scFv-mDEC-205-gp100 was injected without dendritic cell stimuli showed that the activation status of the dendritic cells remains relatively unperturbed as measured by surface expression of CD86. This is in agreement with previous reports that show the ligation of the DEC-205 receptor by DEC-205 antibodies conjugated to model antigens OVA or HEL do not mature dendritic cells as shown by activation markers CD86 or MHC class II (21, 22). Dendritic cell stimulation is required in order that antigen presentation by immature dendritic cells does not induce tolerance preferentially over immunity (23). Indeed, we have shown in mixed lymphocyte reactions that, using scFv-mDEC-205-gp100 pulsed BMDC and activated with CpG/poly(I:C), proliferative responses are induced in both $CD4^+$ and $CD8^+$ gp100-specific T cells. BMDC pulsed with gp100 peptide and activation stimuli or activation stimuli alone did not stimulate proliferation of $CD4^+$ or $CD8^+$ T cells. This confirms previous reports of cross-presentation of antigens by the DEC-205 receptor (16). That this cross-presentation was also effective *in vivo* was verified by IFN-γ Elispot assays. We could show that, after vaccination of mice with scFv-mDEC-205-gp100 and dendritic cell activation stimuli, significantly increased numbers of CTL were present, which may contribute to an improved tumor response.

In clinical settings, patients are treated for already existing tumors. Therefore, this study has concentrated on the development of a therapeutic approach to melanoma treatment. In our melanoma model systems, we have challenged C57Bl/6 mice with two different melanoma tumor cell lines and started the anti-DEC-mediated therapy after a sizable tumor had developed. The B16 model is a well-established and aggressive tumor model that has been developed through chemical induction. The RET model is a spontaneous melanoma model based on transgenic mice, which express the *ret* oncogene fused to the metallothionein promoter (24). In both systems, injection of scFv-mDEC-205-gp100 together with dendritic cell-activating stimuli gave a clear advantage to tumor-bearing mice in comparison with peptide, nonbinding scFv-β-Gal, or dendritic cell activation stimuli alone. This indicates that specific targeting of the endogenous melanoma antigen gp100 directly to dendritic cells increased the MHC class II antigen presentation to $CD4^+$ T cells and cross-presentation to $CD8^+$ T cells. The development of the scFv-mDEC-205-TAA is advantageous over other targeting methods, as it incorporates known TAA into a simple targeting vector (scFv) that includes fewer nonspecific binding elements and a high specificity for dendritic cells. Bivalent/trivalent molecules can also be developed with avidity for different ligands as has been shown previously (25).

It would therefore be possible to bind DEC-205 with a scFv joined to another scFv specific for example a toxin or an adenovirus. Further studies will examine the potential of using anti-DEC-205 in recombinant bivalent/trivalent antibodies for higher avidity binding to DEC-205 while at the same time eliciting either immunologic or tolerogenic effects dependent on the linked scFv specificity (26).

In summary, we have been able to produce a scFv molecule that is specific for DEC-205. This scFv stains dendritic cells with a pattern similar to the mAb from which it was derived. The scFv-mDEC-205 vaccination targets lymph node-resident dendritic cells *in vivo* and can be detected up to 72 h after vaccination while increasing the activation status of dendritic cells over the same period. The scFv-mDEC-205-gp100 enhances both CD4 and CD8 T-cell responses in comparison with peptide uptake under dendritic cells activating conditions. Finally, using a monomeric TAA fused to scFv-mDEC-205, we show that it is possible to achieve a therapeutic vaccination, which can slow the growth rate of an aggressive form of melanoma. This approach could be vital to the clinical treatment of malignant melanoma in which the 10-year survival rate of patients drops from 97% at stage IA to only 14% for patients at stage IV.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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