CD11c is an antigen receptor predominantly expressed on dendritic cells (DC), to which antigen targeting has been shown to induce robust antigen-specific immune responses. To facilitate targeted delivery of tumor antigens to DCs, we generated fusion proteins consisting of the extracellular domain of human HER or its rat homologue neu, fused to the single-chain fragment variable specific for CD11c (scFvCD11c-HER2/neu). Induction of cellular and humoral immune responses and antitumor activity of the fusion proteins admixed with DC-activating CpG oligonucleotides (scFvCD11c-HER2/neuCpG) were tested in transplantable HER2/neu-expressing murine tumor models and in transgenic BALB-neuT mice developing spontaneous neu-driven mammary carcinomas.

Vaccination of BALB/c mice with scFvCD11c-HER2CpG protected mice from subsequent challenge with HER2-positive, but not HER2-negative, murine breast tumor cells, accompanied by induction of strong HER2-specific T-cell and antibody responses. In a therapeutic setting, injection of scFvCD11c-HER2CpG caused rejection of established HER2-positive tumors. Importantly, antitumor activity of such a fusion protein vaccine could be reproduced in immunotolerant BALB-neuT mice, where scFvCD11c-neuCpG vaccination significantly protected against a subsequent challenge with neu-expressing murine breast tumor cells and markedly delayed the onset of spontaneous mammary carcinomas.

Conclusions: CD11c-targeted protein vaccines for in vivo delivery of tumor antigens to DCs induce potent immune responses and antitumoral activities and provide a rationale for further development of this approach for cancer immunotherapy.

Dendritic cells (DC) are key initiators and modulators of adaptive T-cell responses due to their outstanding ability to capture and process antigen, to present antigen-derived peptides in the context of MHC molecules to naive T cells, and to deliver appropriate costimulatory signals that dictate either immunogenic or tolerogenic T-cell stimulation (1, 2).

These unique features of DCs to regulate adaptive immunity suggest that significant clinical benefits could be gained from directly targeting antigens to DCs in vivo (3, 4). Coupling of antigens to ligands or antibodies that specifically bind to DC receptors has been widely investigated as a means of such targeting (3–5). The outcome of the immune response induced by targeting antigens to DCs depends on multiple parameters, including the specific receptor targeting, distribution of the targeted receptor among DC subsets, and the presence or absence of coadministered adjuvant (6–16). Using such an approach, a lowered requirement for antigen dose in stimulating immune responses in mice has been observed after targeting a variety of molecules, including MHC class II, CD11c, DEC205, DCIR2, Dectin-1/2, CD80/86, F4/80-like receptor, CIRE, mannose receptor, and CD36 (7–16). In addition, in vitro and in vivo studies have shown that antibodies specific for the mannose receptor or DC-SIGN can effectively deliver antigen to human DCs, indicating that this strategy will also be applicable to human vaccination (17, 18).

Overexpression of the HER-2 receptor tyrosine kinase has been found in various human malignancies, including breast, ovarian and gastric carcinomas, non–small cell lung cancer, and salivary gland cancers, and has been associated with poor prognosis (19, 20). Endogenous HER2-specific CD4+ T cells and antibodies have been detected in patients with different HER2-expressing cancers (21, 22), and in clinical trials, HER2-specific CD4+ and CD8+ T-cell responses could be induced by
peptide vaccination (23, 24). These studies provide support for HER2 being an important target for directed cancer therapy. The clinically approved HER2 immunotherapy is passive transfer of humanized HER2-specific monoclonal antibody (Herceptin; ref. 25). Although this has been shown to be effective at inhibiting tumor growth in a limited population of breast cancer patients, elicitation of an active and more comprehensive immune response that includes both antibody and T-cell responses may provide more effective antitumor immunity (26).

CD11c is a component of complement receptor 4, which, in the mouse, is expressed predominantly on DCs (27) together with some natural killer and CD8 T cells (28–30) and is present at high levels on all conventional DC subsets, including both CD8" and CD8" subtypes (6). Although the exact cellular role of CD11c is unclear, binding of monoclonal antibody (mAb) N418 has been reported to induce a rapid antibody or strong CTL responses after N418-carried antigen immunization (10, 31). Recently, Castro et al. showed that in vivo CD11c-targeted protein vaccines are effective tools for delivering tumor antigens to DCs to activate antigen-specific antitumor immunity and may be useful to improve current approaches for cancer immunotherapy.

To investigate whether specific targeting of tumor antigens to activated DCs via CD11c can induce potent antigen-specific immune responses, here we generated scFvCD11c-HER2 fusion protein consisting of scFvCD11c fused to the extracellular domain of human HER2. Immunization of BALB/c mice with scFvCD11c-HER2 admixed with DC-activating CpG oligonucleotides (scFvCD11c-HER2Cpg) resulted in enhanced HER2-specific immune responses and protection against challenge with transplanted HER2-expressing tumor cells. Furthermore, protective effects of a similar fusion protein consisting of scFvCD11c with the corresponding fragment of the rat homologue neu were investigated in immunotolerant BALB-neuT mice (34). In this model, scFvCD11c-neuCpg vaccination significantly protected against a subsequent challenge with neu-expressing tumor cells and markedly delayed the onset of spontaneous mammary carcinomas.

**Materials and Methods**

**Mice and cell lines.** Female BALB/c (H-2d) mice (ages 6–8 wk) were purchased from the Animal Experimental Center of the Second Military Medical University. BALB-neuT mice (H-2d) expressing a transforming neu under the control of mouse mammary tumor virus promoter were obtained from Charles River. Heterozygous 6-wk-old to 15-wk-old virgin females expressing rat neu as verified by PCR were used throughout this work.

Mouse breast tumor cell lines D2F2 and 4T1 and the 293T cell line were maintained in DMEM supplemented with 10% (v/v) FCS, D2F2, E2 and EL4/E2 stably expressing human wild-type HER2 were maintained in medium containing 20% FCS. All tissue culture reagents were purchased from Life Technologies unless described otherwise.

**Reagents.** Peptides used in this study were obtained from Sigma-Aldrich. All peptides were >95% pure, as indicated by the analytic high-performance liquid chromatography. Lyophilized peptides were diluted in DMSO and stored at −20°C until use. Recombinant HER2/Fc fusion protein was purchased from R&D Systems. CpG oligonucleotides containing unmethylated CG motifs (TCCAGACGTTCCTGAGCTT) was synthesized and phosphorothioate-stabilized by Invitrogen. The oligonucleotides were reconstituted in sterile pyrogen-free water and then diluted in PBS for in vivo injections.

**Fusion protein expression and purification.** The single-chain Fv antibody (scFv) gene obtained from N418 hybridoma (hamster anti-mouse CD11c) was engineered into a small immunoprotein (SIP) format by cloning them between the secretory signal sequence from a mouse immunoglobulin heavy chain and the third constant domain of human IgG1 (y1-CH3; ref. 35). The sequence encoding for the extracellular domain of human HER2 or rat homologue neu (amino acids 22-652) was cloned in-frame downstream of the y1-CH3 domain, followed by a His6 tag. As a control, the DNA fragment encoding for variable regions of anti–SM5-1 mAb was amplified and cloned into a similar SIP-HER2/neu format. SIP-HER2/neu-H6 proteins were transiently expressed in 293T cells after Lipofectamine 2000 transfection. Proteins were purified by affinity chromatography using a poly-H6 tag purification system (Novagen) and quantified by coomassie staining and Western blot using recombinant anti-HER2 (4D5) or anti-His mAb (ab1187, Abcam). The scFv-HER2/neu proteins were tested for the presence of endotoxin by the chromogenic Limulus amebocyte lysate assay (Associates of Cape Cod). The levels of endotoxin were below 0.05 endotoxin units per microgram in all of the scFv-HER2/neu proteins used in vivo or in culture.

**In vitro internalization assay.** Bone marrow–derived DCs were incubated at 4°C for 1 h with scFvCD11c-HER2, washed, and then either kept at 4°C or incubated at 37°C for 30, 60, or 120 min. For fluorescence-activated cell sorting analysis, the level of scFvCD11c-HER2 remaining at the cell surface was detected using FITC-conjugated anti-HER2 mAb. The median fluorescent values of HER2+ cells was...
CpG were injected. On day 14, mice were inoculated s.c. with 2 scFvCD11c-neu, scFvctrl-HER2, or recombinant HER2 in PBS plus 50 harvested and analyzed for the fluorescence of gated CD11c+ DCs by PFA, and permeabilized with 0.02% saponin. The scFvCD11c-HER2 and scFv ctrl-HER2 proteins (5 the manufacturing instructions (Molecular Probes). Then, labeled proteins were labeled with Alexa Fluor 488 fluorescence according to (Leica Microsystems).

This was recorded as the date of death for survival studies. For determination and used to calculate the percentage of the remaining scFvCD11c-HER2 at the surface, with 1 h at 4°C incubated cells taken as 100%. For confocal analysis, after binding at 4°C for 1 h, cells were washed, plated on poly-l-lysine–treated coverslips, and incubated at 37°C for 30 min. Membranes were stained with 5 μg/mL PE-conjugated antimouse DC marker (33D1, BD Pharmingen), washed, fixed in 4% PFA, and permeabilized with 0.02% saponin. The scFvCD11c-HER2 protein was detected with FITC-conjugated anti-HER2 mAb. Then samples were analyzed using a Leica TCS SL laser scanning microscope (Leica Microsystems).

In vivo targeting assay. The scFvCD11c-HER2 and scFvKH-HER2 proteins were labeled with Alexa Fluor 488 fluorescence according to the manufacturing instructions (Molecular Probes). Then, labeled scFvCD11c-HER2 and scFvKH-HER2 proteins (5 μg/mice) were s.c. injected into mice, and the draining lymph nodes and spleens were harvested and analyzed for the fluorescence of gated CD11c+ DCs by flow cytometry at the indicated time point as described previously (36).

Protective and therapeutic vaccination. BALB/c or BALB-neuT mice (5-10 mice per group) were injected s.c. with 5 μg scFvCD11c-HER2, scFvKH-HER2, or recombinant HER2 in PBS plus 50 μg CpG in left flank on days 0 and 7. As control, 100 μL PBS or 50 μg CpG were injected. On day 14, mice were inoculated s.c. with 2 × 105 D2F2/E2, D2F2, or TUBO tumor cells. Naive BALB/c mice were used as controls.

Prevention of spontaneous tumors. Virgin female BALB-neuT mice were vaccinated at ages 8 and 10 wk as described above. Mammary glands were inspected every week to monitor the appearance of tumors. Measurable/palpable masses >2 mm in diameter were regarded as tumors. Data are reported as tumor multiplicity (cumulative number of tumors per number of mice in each group) and shown as mean ± SE. In all cases, when mice had tumors of >15 mm in the greatest dimension or when skin ulceration occurred, mice were sacrificed, and this was recorded as the date of death for survival studies.

Analysis of antibody responses. Peripheral blood was collected from the tail vein, and 1:100 dilutions of sera were analyzed by ELISA with recombinant HER2 protein or by flow cytometry using D2F2/E2 and TUBO tumor cells as described previously (36). Normal mouse serum served as negative control.

Evaluation of T-cell responses. For detection of HER2-specific CD4+ T cells, T cells from vaccinated mice were isolated with anti-CD4 beads on MACS columns according to the manufacturer’s protocol (Miltenyi). CD4+ T cells were then restimulated with bone marrow–derived DCs pulsed with recombinant HER2 or TRP2 protein in vitro for 3 d, and supernatants were collected and analyzed for production of IFN-γ, interleukin-4, interleukin-10, and tumor necrosis factor-α by ELISA kits (R&D Systems).

For detection of IFN-γ–producing CD8+ T cells, intracellular cytokine staining assays were done. Briefly, splenocytes harvested from vaccinated mice were restimulated in the presence or absence of HER2,53-71 peptide (TYLPTNASL; 10 μg/mL) for 6 h. During the final 4 h of incubation, 10 μg/mL brefeldin A (Sigma) were added. After surface staining with anti-CD8, cells were permeabilized and stained for intracellular IFN-γ as described previously (16).
For CTL measurements, $^{51}$Cr-release assays were done as described previously (37).

**Statistical analysis.** Differences between groups were analyzed using the Student’s $t$ test or ANOVA. Values of $P < 0.05$ were considered significant. For survival studies, Kaplan-Meier survival curves were plotted and analyzed using Prism software (GraphPad Software).

## Results

**scFv\(^{CD11c}\)-HER2 targeted to DCs in vitro and in vivo.** To construct DC-targeted fusion protein, scFv\(^{CD11c}\) was engineered into the SIP format, a molecule that contains the third constant domain of human IgG1 (\(\gamma1\-CH3\)) downstream of the scFv, thus producing a dimeric scFv\(^{CD11c}\) (34). The COOH terminus of the SIP-scFv\(^{CD11c}\) was fused in-frame to the extracellular domain of HER2 (amino acids 22-652), followed by a His\(_6\) tag, to generate the dimeric recombinant protein that we named scFv\(^{CD11c}\)-HER2 (Fig. 1A, left). As a control, we constructed a similar recombinant containing an irrelevant scFv (scFv ctrl-HER2). Both proteins were produced by transient transfection in 293T cells (\(\sim 1 \text{ mg/L}\)) and purified from culture supernatants by anti-His\(_6\) tag affinity chromatography. Analyses by Western blot of the purified proteins revealed, for each of them, a single band of the expected molecular mass (monomer, \(\sim 150 \text{ kDa}\); dimer, \(\sim 300 \text{ kDa}\); Fig. 1A, right).

To study the endocytosis of scFv\(^{CD11c}\)-HER2, we did a flow cytometry–based internalization assay. As shown in Fig. 1B, scFv\(^{CD11c}\)-HER2 was rapidly internalized with \(\sim 70\%\) of the protein removed from the cell surface within 1 h at 37°C. The data were confirmed by immunofluorescence: scFv\(^{CD11c}\)-HER2 was efficiently internalized at 37°C (Fig. 1C).

We also investigated whether s.c. injection of scFv\(^{CD11c}\)-HER2 results in effective targeting to DCs in vivo. For this, Alexa Fluor 488–labeled scFv\(^{CD11c}\)-HER2 and respective controls were injected into mice and the draining lymph nodes and spleens were removed as indicated. At 4 or 8 h postinjection, \(\sim 55\%\) or \(\sim 88\%\) of DCs in the draining lymph nodes accumulated scFv\(^{CD11c}\)-HER2, respectively (Fig. 1D). Remarkably, \(\sim 80\%\) DCs in the spleens took up scFv\(^{CD11c}\)-HER2 protein 12 h after injection. However, after injection of scFv\(^{vitr}\)-HER2, some fluorences slightly above background levels were detectable, resulting in only \(\sim 3.2\%\) of all DCs being loaded (background level was 1%).

**Protection of mice from challenge with HER2-expressing tumor cells.** Procedures for targeting antigens to DCs in vivo should induce the concomitant activation of DCs because targeting antigens to immature DCs may lead to tolerance (3). Therefore, TLR9 ligand CpG was selected as DC-activating reagent in this study and, in subsequent experiments, scFv\(^{CD11c}\)-HER2 was injected together with 50 \(\mu\text{g}\) of CpG (scFv\(^{CD11c}\)-HER2CpG).

To investigate whether vaccination with scFv\(^{CD11c}\)-HER2CpG induces antitumoral immunity and protect animals from...
subsequent tumor challenge, BALB/c mice were vaccinated twice by s.c. injection of scFvCD11c-HER2CpG or respective controls as indicated on days 0 and 7. Seven days after the last vaccination, the mice were challenged with D2F2/E2 cells. All animals vaccinated with scFvCD11c-HER2CpG remained tumor free upon challenge with D2F2/E2 cells (Fig. 2A). In contrast, after vaccination with scFvctrl-HER2CpG, HER2CpG, or a mixture of CD11c antibodies and HER2CpG, no protection was apparent. In these instances, all animals developed continuously growing tumors and died by 6 weeks. Interestingly, vaccination with scFvCD11c-HER2 alone also moderately delayed tumor growth, although this preventive effect was not statistically significant.

To examine whether HER2-specific responses induced by the vaccines were responsible for protection, a similar experiment was done using parental, HER2-negative D2F2 cells for tumor challenge. Rapid tumor growth was observed in all animals regardless of either treatment (Fig. 2B), strongly suggesting that the observed rejection of HER2-expressing D2F2/E2 cells was due to HER2-specific immune responses induced by scFvCD11c-HER2CpG. Furthermore, we obtained a similar protective effect of scFvCD11c-HER2CpG in C57BL/6 mice using paired EL4 and EL4/E2 tumor models (data not shown).

To test whether immunologic memory was developed in tumor-free mice initially vaccinated with scFvCD11c-HER2CpG, the animals were rechallenged with the D2F2/E2 cells 3 months
after initial tumor cell inoculation. The parental D2F2 cells or unrelated syngeneic 4T1 cells were used as controls. The mice rejected subsequent rechallenges with the D2F2/E2 cell and remained tumor free until the end of the experiment while being incapable of reject syngeneic unrelated 4T1 tumor (Fig. 2C). Interestingly, the mice also protected against outgrowth of the parental D2F2 cells with 60% of the mice not developing any tumors and the remaining animals displaying drastically slow tumor growth (Fig. 2D), suggesting that vaccination and initial tumor challenge can result in determinant spreading and subsequent immunity to an otherwise parental HER2-negative tumor variants.

**Induction of HER2-specific T cells.** To analyze the nature of the immune responses induced by scFv<sup>CD11c</sup>-HER2<sub>Cpg</sub>, CD4<sup>+</sup> T cells were isolated from the vaccinated mice and restimulated with HER2- or TRP2-pulsed bone marrow-derived DCs in vitro. As shown in Fig. 3A, CD4<sup>+</sup> T cells obtained from scFv<sup>CD11c</sup>-HER2<sub>Cpg</sub>-vaccinated mice showed vigorous proliferation upon restimulation with HER2-pulsed, but not TRP2-pulsed, bone marrow-derived DCs. In contrast, no evident T-cell proliferation could be observed when mice were vaccinated with scFv<sup>ctrl</sup>-HER2<sub>Cpg</sub>, HER2<sub>Cpg</sub> or scFv<sup>CD11c</sup>-HER2.

The supernatants of stimulated T cells were tested for the presence of cytokines by ELISA. T cells recovered from scFv<sup>CD11c</sup>-HER2<sub>Cpg</sub>-vaccinated mice contained populations of activated CD8<sup>+</sup> T cells that produced IFN-γ upon in vitro restimulation with HER2-derived synthetic p63-71 peptide TYLPTNASL whereas splenocytes from other group mice did not display an increase in CD8<sup>+</sup> IFN-γ+ cells. In addition, splenocytes from scFv<sup>CD11c</sup>-HER2<sub>Cpg</sub>-vaccinated mice exhibited significantly higher target cell killing than did those from other group mice (Fig. 3D, left). The specificity of the killing was shown by the inability of the splenocytes to kill parental D2F2 target cells and EL4/E2 target cells with a different H-2<sup>b</sup> background (Fig. 3D, right). The cytotoxic effect was mediated by CD8<sup>+</sup> CTLs, because the killing was inhibited by the anti-CD8, but not anti-CD4, antibody (data not shown). Taken together, the results indicate the superiority

**Fig. 4.** Vaccination with scFv<sup>CD11c</sup>-HER2<sub>Cpg</sub> induces HER2-specific antibodies. BALB/c mice were vaccinated with scFv<sup>CD11c</sup>-HER2<sub>Cpg</sub>, scFv<sup>ctrl</sup>-HER2<sub>Cpg</sub>, recombinant HER2<sub>Cpg</sub> or scFv<sup>CD11c</sup>-HER2 on days 0 and 7. On day 14, sera were collected from the vaccinated animals. A, HER2-specific total IgG and IgG subclass (IgG1 and IgG2a) antibodies in sera from the vaccinated animals after 1:100 dilution were determined by ELISA. The mean A405 values of pooled sera from each group (five mice per group) were presented. Bars, SE. The background A405 of normal mouse sera was <0.04. B, D2F2/E2 cells were incubated with the immune sera obtained from the vaccinated animals, and surface bound antibodies were detected using FITC-labeled secondary antibodies. Samples were then analyzed by flow cytometry. The data were represented as mean fluorescence intensity (MFI). Normal mouse sera served as control. All results are representative of three independent experiments. *, P < 0.01, **, P < 0.001, scFv<sup>CD11c</sup>-HER2<sub>Cpg</sub> compared with other groups.

**Fig. 5.** Immunization with scFv<sup>CD11c</sup>-HER2<sub>Cpg</sub> results in cures of tumor-bearing animals. BALB/c mice (10 mice per group) were inoculated s.c. with D2F2/E2 tumor cells in right flank. When tumors size reached ~ 40 mm<sup>3</sup> (day 8), animals were s.c. injected with scFv<sup>CD11c</sup>-HER2<sub>Cpg</sub>, scFv<sup>ctrl</sup>-HER2<sub>Cpg</sub>, or HER2<sub>Cpg</sub> in the vicinity of each tumor. Treatment was repeated on day 15. Control animals received PBS. Tumor developments were monitored, and animal survival was calculated. A, kinetics of tumor growth. B, survival of animals. The data are represented as the mean tumor volume (mm<sup>3</sup>) and representative of three experiments with comparable results. Bars, SE. *, P < 0.001, scFv<sup>CD11c</sup>-HER2<sub>Cpg</sub> compared with other groups.
Induction of HER2-specific antibodies. Sera were collected and tested for HER2-specific antibodies. In agreement with previous study (10), scFv CD11c-HER2 vaccination in the absence of adjuvant induced robust humoral responses with IgG1 being the predominant isotypes (Fig. 4A). Importantly, quantitatively and qualitatively enhanced humoral responses occurred in scFvCD11c-HER2CpG–vaccinated mice with significantly elevated titers of HER2-specific IgG2a antibodies, which is also consistent with the cytokine profile of splenic CD4+ T cells (Fig. 3B). However, scFvCD11c-HER2CpG–vaccinated or HER2CpG–vaccinated mice developed a mild humoral response.

Fig. 6. Vaccination with scFvCD11c-neuCpG protects against a subsequent challenge with neu-expressing tumor cells and delays onset of spontaneous mammary carcinomas in BALB-neuT mice. A, female BALB-neuT mice (five mice per group) were vaccinated with scFvCD11c-neuCpG, scFvCD11c-HER2CpG, or scFvCD11c-neu in left flank on days 0 and 7. Control animals received PBS or CpG. On day 14, mice were inoculated s.c. with neu-expressing TUBO cells in opposite flank. Tumor developments were monitored, and animal survival was calculated. B, in a separate experiment, female BALB-neuT mice were vaccinated as described above. On day 14, the splenocytes were isolated and restimulated in vitro with the irradiated TUBO cells for 5 d. The restimulated splenocytes were cocultured for 4 h with the 51Cr-labeled TUBO target cells. Percentages of target cell killing by the splenocytes from the vaccinated animals. Data represent the means of triplicate cultures. C, TUBO cells were incubated with the immune sera obtained from the vaccinated animals and surface bound antibodies were detected using FITC-labeled secondary antibodies. Samples were then analyzed by flow cytometry. The data were represented as mean fluorescence intensity (MFI). D, animals were immunized twice at weeks 8 and 10 as described above. Development of mammary tumors was monitored by manual examination of the mammary glands once every week. Measurable masses of >2 mm diameter were regarded as tumors. Points, mean number of tumors in each group (tumor multiplicity; left) and percentage of tumor-free mice (right); bars, SE. Arrows, day when the vaccine was given. All results are representative of two to three independent experiments. *, P < 0.01, **, P < 0.001, scFvCD11c-neuCpG compared with other groups.
We also tested whether the sera of immunized mice contained antibodies binding to “natural” D2F2/E2 cells. As shown in Fig. 4B, we detected D2F2/E2-specific antibodies in sera derived from mice vaccinated with scFvCD11c-HER2CpG or scFvCD11c-HER2, whereas no antibodies were observed in controls. The binding of antibodies in sera derived from scFvCD11c-HER2–vaccinated mice was significantly weaker compared with that from scFvCD11c-HER2CpG–vaccinated mice. In summary, these data show that scFvCD11c-HER2CpG–vaccinated mice developed antibodies that recognize epitopes expressed by D2F2/E2 cells and thus may also confer protection against tumor growth in vivo.

scFvCD11c-HER2CpG immunization eradicated the established tumors. We also evaluated the therapeutic effect of scFvCD11c-HER2CpG vaccination on established tumors in D2F2/E2 tumor-bearing animals. BALB/c mice were s.c. inoculated with D2F2/E2 cells. On day 8, animals with tumors sizing 40 mm³ were immunized with scFvCD11c-HER2CpG or respective controls. Treatment was repeated on day 15. Administration of scFvCD11c-HER2CpG substantially slowed tumor development and protected up to 80% of the mice from tumor growth (Fig. 5). These long-term surviving mice also rejected D2F2/E2 cell rechallenges (data not shown). In contrast, scFvCD11c-HER2CpG or HER2CpG vaccination did not affect tumor growth and all animals died within 6 weeks. These results show that scFvCD11c-HER2CpG vaccination is also effective in a therapeutic setting, inducing tumor regression and long-lasting immunity in tumor-bearing animals.

Antitumoral activity of scFvCD11c-neuCpG vaccination in BALB-neuT mice. Although tumor models based on human HER2-expressing D2F2/E2 cells are useful to assess the basic functionality of cancer vaccines, such models do not fully reflect the situation of human cancer usually characterized by immunologic tolerance toward HER2. Hence, we further tested fusion protein vaccines in female BALB-neuT mice that reflect the situation of human cancer usually characterized by immunologic tolerance toward HER2. Therefore, the systemic distribution of fusion protein enables presentation by DC to both CD4+ and CD8+ T cells (40). CD11c-carried antigen can target DCs within 4 hours. Soon thereafter, fusion protein could be traced in spleen, illustrating the remarkable ability of the fusion protein to diffuse and bind specifically to distant targets. This is important, because cross-priming of soluble antigen mostly occurs in the spleen (39). CD11c-carried antigen can target CD11c+ cells in the marginal zone and to CD11c+ cross-presenting DC in the T-cell zone, which allows for antigen presentation by DC to both CD4+ and CD8+ T cells (40). Therefore, the systemic distribution of fusion protein enables loading of virtually all DCs in the body and provides a basis for the induction of a more powerful immune response compared with conventional immunization strategies.

Vaccination of mice with scFvCD11c-HER2CpG-induced HER2-specific CD4+ and CD8+ T-cell response, indicating antibodies delivered by CD11c enter into both MHC classes I and II processing and presentation pathways. Consistent with previous studies (41–43), HER2-specific CTLs recognized HER2 immunodominant epitope TYLPTNASL, specifically lysed HER2-expressing tumor cells, and were required to protect mice from growth of tumors expressing HER-2 (Supplementary Fig. S2). In addition, CD4+ T cells also played a crucial role as helper cells in the induction of antitumor immunity (Supplementary Fig. S2), in line with the early role of CD4+ T cells in conferring tumor protection in spontaneous HER2/neu transgenic tumor models (43). In this respect, our data contrast with another study from Demangel et al., in which vaccination with the plasmid encoding Ag85B fused to scFvCD11c, but not fused to scFvDIEC205, did not generate significantly stronger T-cell and antibody-specific responses compared with that elicited by the plasmid expressing Ag85B alone (44). In this study, naked plasmid was injected without CpG portion, and

In summary, these data show that scFvCD11c-antigen fusion proteins induced potent antigen-specific T-cell and antibody responses and protected mice from subsequent challenge with antigen-positive tumor cells. Furthermore, scFvCD11c-HER2CpG immunization eradicated established HER2-expressing tumors in a therapeutic setting. More importantly, scFvCD11c-neuCpG vaccination significantly protected against a subsequent challenge with neu-expressing tumor cells and markedly delayed the onset of spontaneous mammary carcinomas in immunotolerant BALB-neuT mice.

The results showed that scFvCD11c could mediate antigen to be effectively uptake by the DCs in vitro and in vivo. After s.c. injection, scFvCD11c-HER2 protein was present in lymph node DCs within 4 hours. Soon thereafter, fusion protein could be traced in spleen, illustrating the remarkable ability of the fusion protein to diffuse and bind specifically to distant targets. This is important, because cross-priming of soluble antigen mostly occurs in the spleen (39). CD11c-carried antigen can target CD11c+ cells in the marginal zone and to CD11c+ cross-presenting DC in the T-cell zone, which allows for antigen presentation by DC to both CD4+ and CD8+ T cells (40). Therefore, the systemic distribution of fusion protein enables loading of virtually all DCs in the body and provides a basis for the induction of a more powerful immune response compared with conventional immunization strategies.
this may make a difference. More important is the fact that the same research group showed that antigen-packed liposome modified with scFv<sup>CD11c</sup> was effective in stimulating strong antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses with a marked tumor protection in the presence of DC-activating reagents (31). Certainly, it is worth comparing CD11c-targeted and DEC-205–targeted antigen delivery, which will contribute to clarifying the differences.

The immune responses induced by scFv<sup>CD11c</sup>-HER2<sub>Cpg</sub> vaccination were directed specifically against HER2-expressing tumor cells. Vaccinated animals were protected against HER2-expressing D2F2/E2 but not parental HER2-negative D2F2 tumor cells. In addition, scFv<sup>CD11c</sup>-HER2<sub>Cpg</sub> vaccination and tumor rejection induced immunologic memory, resulting in protection against subsequent rechallenge with D2F2/E2 and D2F2 cells. Presumably, the development of long-term immunologic memory was not only dependent on the HER2 antigen but also on other unidentified antigens of D2F2/E2 tumor.

Because antigen-negative variants may arise after antigen-positive tumor cells are destroyed, immune responses to additional undefined tumor-associated antigens may be crucial to the ultimate success of vaccination (37).

Antitumoral activity of CD11c-targeted proteins was also observed in immunotolerant BALB-neu<sup>T</sup> mice. Vaccination with scFv<sup>CD11c</sup>-neu<sub>Cpg</sub> significantly protected against a subsequent challenge with TUBO cells and markedly delayed the onset of spontaneous mammary carcinomas in BALB-neu<sup>T</sup> mice. However, the mechanisms of preventive immunity induced were different in these animals. Although comparable titers of neu-specific antibodies were found in scFv<sup>CD11c</sup>-neu<sub>Cpg</sub>-Vaccinated BALB-neu<sup>T</sup>, few CTL specific for the tumor or immunodominant TVVPANASL epitope could be detected. This is most likely due to central tolerance leading to the selective deletion of CTL clones reacting with the immunodominant epitope (45) and the suppressing activity of regulatory immune cells (46). Similar to CTLA-4–targeted plasmid vaccines (42), scFv<sup>CD11c</sup>-neu<sub>Cpg</sub> vaccination likely mediates antitumoral activity in BALB-neu<sup>T</sup> mice through neu-specific antibodies and possibly CD4<sup>+</sup> T cells (43). Recently, it was shown that depletion of regulatory T cells enabled neu-specific CTL responses after vaccination of neu-transgenic FVB/N mice with a cellular vaccine expressing neu and granulocyte-macrophage colony-stimulating factor (47). In BALB-neu<sup>T</sup> mice, regulatory T-cell depletion was able to break tolerance to the immunodominant TVVPANASL epitope (46) and combined with peptide vaccination and adjuvants markedly extended disease-free survival (48). Hence, it is likely that combination with regulatory T-cell–modulating reagents could further enhance the efficacy of CD11c-targeted vaccines. It also remains to be determined whether additional booster vaccinations would further delay, or ultimately, prevent the emergence of tumors in neu-transgenic mice.

Targeting of antigens to DCs does not activate immune responses per se. In this regard, the application of immune stimulatory agents such as Cpg may have a 2-fold effect on the development of tumor immunity in our tumor model. First, Cpg ensures proper DC activation; second, it may activate the endothelium of tumor blood vessels. This may be important because Garbi et al. have shown that proper activation of endothelium is crucial for the extravasation of tumor-specific T cells from the blood into cancerous tissues (49). The results are also in agreement with the recent two studies documenting that DNA or peptide vaccine in combination with TLR-9 agonists could effectively prevent and treat spontaneous mammary carcinomas in neu-transgenic mice (48, 50).

Taken together, our results show that vaccination with CD11c-targeted fusion proteins induces potent immune responses and provokes superior antitumoral effects. Thus far, in vivo DC targeting strategies using DC205 as a target molecule are being evaluated in clinical trials, thus, considering the CD11c molecule is equally expressed by human DCs, it is conceivable that human CD11c has similar properties as antigen receptor and therefore may be a useful target for developing vaccination strategies in human trials.

References


Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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