Cancer Immunotherapy Using *In vitro* Genetically Modified Targeted Dendritic Cells

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Abstract

Modest clinical outcomes of dendritic cell (DC) vaccine trials call for novel strategies. In this study, we have created a chimeric CD40 molecule that incorporates a single chain Fv (scFv) molecule specific for human ErbB2 antigen and fusing to the membrane spanning and cytosolic domains of murine CD40. After adenoviral transfer to bone marrow–derived DC, this chimeric receptor (CR) induced nuclear factor-κB (NF-κB)–dependent DC activation and effector function when cultured with immobilized ErbB2 protein or ErbB2-positive tumor cells in *vivo*. *In vitro* migration assays showed that ~40% injected CR-modified DC (scFv-CD40-DC) effectively migrated to ErbB2-positive tumors, where they were activated after ErbB2 antigen stimulation, and sequentially homed into the draining lymph nodes. In murine ErbB2-positive D2F2/E2 breast tumor (BALB/c) and EL4/E2 thymoma (C57BL/6) models, i.v. injection of 1 × 10^6 scFv-CD40-DC significantly inhibited tumor growth and cured established tumors. Importantly, the cured mice treated by injection of scFv-CD40-DC were effective in preventing both ErbB2-positive and parental ErbB2-negative tumor rechallenge. Analysis of the underlying mechanism revealed that i.v. infusion of scFv-CD40-DC elicited tumor-specific CTL responses, and the transfer of CTLs from scFv-CD40-DC–treated mice protected naive mice against a subsequent tumor challenge. These results support the concept that genetic modification of DC with tumor-associated antigen-specific CD40 chimeric receptor might be a useful strategy for treatment of human cancers. [Cancer Res 2008;68(10):3854–62]

Introduction

Effective immunization in the setting of cancer will require the induction of potent CD4+ Th1 and CD8+ CTL responses. Because dendritic cells (DC) are most potent antigen-presenting cells (APC) with unique ability to activate naïve CD4+ and CD8+ T cells (1, 2), immunization with *ex vivo* antigen-loaded DC could represent a potentially powerful method of inducing antitumor immunity (3, 4). In fact, it has been well-documented that antigen-loaded DC can generate antitumor immune responses capable of eradicating established tumors *in vivo* in a number of animal tumor models (5).

However, the limited success of recent clinical trials involving DC indicates that current strategies need further innovation (6).

Immunotherapy using *ex vivo* generated DC requires that the DC mature in culture to a stage in which they can migrate to the lymph node and activate the cognate T cells. DC activation can be divided into two stages. In the periphery, immature DC undergo a maturation process in response to inflammatory stimuli originating from pathogens or dying cells, collectively called “danger signals” (7). One important consequence of the maturation process is that DC up-regulate expression of CC chemokine receptor 7 (CCR7), resulting in acquisition of capacity to migrate to the lymph node. When reaching the lymph node, antigen-loaded mature DC undergo an additional activation step, termed “licensing” in response to various stimuli, notably CD40 ligand (CD40L). The licensed DC acquire the ability to stimulate CD8+ CTL (8–10). Although the list of agents that can affect DC maturation is long and growing, the exact sequence of events and composition of factors required for the efficient differentiation of the DC to become potent stimulators of CD4+ Th1 and CD8+ CTL are poorly understood. In addition, premature licensing of DC before their encounter with cognate T cells in the lymph node might be counterproductive. Interleukin (IL)-12 is a case in point. DC can be induced *in vitro* and *in vivo* to secret IL-12, but IL-12 expression is transient and DC become refractive to subsequent induction of IL-12, a phenomenon termed “exhaustion” or “paralysis” (11, 12). These findings underscore the need for innovative strategies capable of effectively regulating DC maturation or coordinating the DC activation windows with cognate T-cell engagement.

CD40, a receptor of the tumor necrosis factor (TNF) family, is a particularly excellent target for manipulation. CD40 is constitutively expressed on all of APCs and is up-regulated upon infection or inflammation (13–15). It binds to CD40L, a member of the TNF family, which is expressed mainly on activated CD4+ T cells. Stimulation of CD40 on DC, through either activated CD4+ T cells, soluble CD40L, or activating anti-CD40 antibody, up-regulates expression of costimulatory molecules CD80 and CD86, enhances production of cytokines (most notably IL-12), and promotes crosspriming to exogenous antigens (8, 16). *In vivo* CD40 stimulation increases the magnitude of CD4+ and CD8+ T-cell expansion, leading to enhanced tumor protection and conversion of steady-state tolerance into immunity (13, 17–19). Conversely, CD40 signaling blockade, mainly through anti-CD40L antibody, inhibits T-cell activation and results in immune tolerance, e.g., to transplants and autoantigens (20, 21). Consistent with a crucial importance of CD40-CD40L interactions in induction of immune responses, mice and humans that lack CD40 or CD40L genes have reduced antibody production and immunoglobulin class switching and are unable to mount effective responses against infectious agents. These observations have prompted therapeutic studies of

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CD40 stimulation. Strategies include systemic delivery of CD40-specific monoclonal antibodies or trimerized CD40L immunization with CD40-stimulated, antigen-loaded DC-based vaccines; and administration of genetically modified CD40L-expressing DC (22–24).

Recently, Hanks and colleagues (25) developed a potent, drug-inducible CD40 (iCD40) receptor composed of a membrane-localized cytoplasmic domain of CD40 fused to drug-binding domains. These modifications permit prolonged activation of iCD40-expressing DC in vivo, resulting in more potent CD8+ T-cell effector responses and eradication of established solid tumors. Here, we design a novel DC vaccination strategy characteristic of modifying DC with a tumor-associated antigen (TAA)-inducible CD40 chimeric receptor (CR). The CR is composed of CD40 signaling domain fused to TAA-specific single chain Fv (scFv-CD40). By using an adenovirus vector to transfer and express the CR in murine immature DC, the present study shows that scFv-CD40 CR can target modified DC to specifically migrate to the TAA-positive tumors and mature in situ, induce tumor-specific cellular immunity, and suppress growth of preexisting tumors.

Materials and Methods

Mice and cell lines. Female BALB/c (H-2d) and C57BL/6 (H-2b) mice (6–8-wk-old) were purchased from the Animal Experimental Center of the Second Military Medical University. All animals in this study were housed in pathogen-free conditions and were maintained in accordance with the guideline of the Committee on Animals of the Second Military Medical University.

Mammary tumor cell line D2F2, fibroblast NIH3T3, thymoma cell line EL/4, melanoma cell line B16, and 293 cell line were maintained in DMEM supplemented with 10% FCS. D2F2/E2 and EL4/E2 stably expressing human wild-type ErbB2 were maintained in medium containing 0.4 mg/mL G418 (Sigma-Aldrich). All tissue culture reagents were purchased from Life Technologies unless described otherwise.

Construction and recombinant adenovirus production. The CR genes (scFv and scFv-CD40) were composed of the ErbB2-specific scFv (4D5), a c-myc tag, the hinge region of mouse CD8a (residues 105–165), and the transmembrane (CD40tm) and/or cytoplasmic region of the mouse CD40 molecule (scFv, residues 194–223; scFv-CD40, residues 194–289). The DNA encoding CD8a hinge, CD40tm, or CD40 were firstly amplified by PCR of BALB/c spleen cDNA and then, the CD8a hinge region was fused with CD40tm or CD40 using the overlapping PCR method to generate CD8a-CD40tm or CD8a-CD40 fragments. The PCR products were subcloned into pcDNA3.1(+) and sequence verified to create pcDNA3.1-CD8a-CD40tm or pcDNA3.1-CD8a-CD40. The scFv fragment removed from previously constructed vector pcDNA3.1-scFv was ligated into pcDNA3.1-CD8a-CD40tm or pcDNA3.1-CD8a-CD40 to generate pcDNA3.1-scFv-CD40 or pcDNA3.1-scFv-CD40. Finally, the scFv and scFv-CD40 CR genes were liberated from pcDNA3.1 vectors and ligated into the adenoviral shuttle vector pShuttleCMV. Construction and preparation of Ad-GFP, Ad-scFv, and Ad-scFv-CD40 vector and adenovirus titration were carried out as described previously (26).

Generation and adenoviral infection of murine DC. DC were generated from bone marrow as described previously (27). After 5 d of culture in granulocyte-macrophage colony-stimulating factor, immature DC were collected and replaced in serum-free RPMI 1640 at 105 cells/mL in 6-well plates (Corning). Cells were then infected with adenovirus at 100 multiplicity of infection (MOI) for 2 h. Subsequently, the adenovirus-containing medium was removed and DC culture medium was added back. The infected cells were left to express the transgenes of interest for another 24 to 48 h before further use. The infected DC were stained with PE-conjugated anti-c-myc monoclonal antibody (mAb; Sigma-Aldrich) and analyzed for the CR expression by flow cytometry.

Western blots and real-time PCR. The Western blots and real-time PCR were performed as previously described (26). For nuclear Rel-B blotting, infected DC were stimulated in 24-well plates (104 cells per well) by various concentrations of immobilized bovine serum albumin (BSA), recombinant ErbB2 protein (R&D Systems), or 1 µg/mL LPS (Sigma-Aldrich) for 24 h. Nuclear lysates were prepared using the Transfactor Extraction kit (Clontech Laboratories) and probed with mouse Rel-B- and Sp1-specific mAb (Santa Cruz Biotechnology).

Flow cytometric analysis. To evaluate the phenotypic changes of infected DC after ErbB2 stimulation, the infected DC were cocultured with the irradiated D2F2/E2 or D2F2 cells (5,000 rad) at a DC to tumor ratio of 5:1 at 37°C. After 24 h, the cells were stained with the following mAbs: CD11c-APC (HL3), CD86-PE (GL1), CD40-PE (HM40-3), H-2Kd-PE (SF1-1.1), and 1-Aβ-PE (AMS-3.2) or isotype control mAbs (BD PharMingen). To evaluate antigen uptake, D2F2 or D2F2/E2 tumor cells were stained with Hoechst 33342 ( Molecular Probes) for 30 min before coculturing with infected DC for 24 h at a DC to tumor ratio of 5:1 at 37°C or 4°C. The cells were then counterstained with anti-mouse CD11c-APC and propidium iodide (PI). Viable DC (PI− Cytofluor 560) were gated and analyzed for uptake of Hoechst 33342 apostatic bodies on a FACS Calibur system with CELLQuest software.

Analysis of cytokines. Cell-free supernatants were harvested and analyzed for the production of TNF-α, IL-8, IL-12p70, and MCP-3 by using ELISA kits (R&D Systems) as described previously (28).

In vivo DC migration assays. The infected DC were labeled with 5 µM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) in PBS at 37°C for 15 min and washed twice immediately before i.v. injection into 8 d established D2F2/E2 tumor-bearing BALB/c mice. Twenty-four hours later, the tumors, the draining, and nondonoring inguinal lymph nodes were harvested and processed as described previously (29). The single cell suspensions were prepared, stained with CD11c-APC, CD86-PE, I-Aβ-PE, and CCR7-PE ( Tradeswell and analyzed by flow cytometry.

Tumor therapy studies. BALB/c or C57BL/6 mice received s.c. injection with 2 × 105 D2F2/E2 (n = 30), D2F2 (n = 30), EL4/E2 (n = 30), or EL4 (n = 30) cells in the left flank on day 0. On day 8, tumor size reached ~15 to 25 mm2. On day 8 and 11, mice were treated with i.v. immunization of 5 × 105 scFv-DC or scFv-CD40-DC in a total volume of 100 µL of PBS (n = 10 mice per group). Tumor size was assessed twice weekly and was documented as the average ± SE tumor area (mm2). If animals seemed moribund or the diameter of the tumors reached 15 mm, the mice were sacrificed and the data was recorded as the date of death for survival studies.

CTL assays. CTL assays were performed as described previously (30). Briefly, splenocytes were isolated from the mice bearing D2F2/E2 or EL4/E2 tumors 10 d after infected-DC treatment and restimulated at 3 × 105 cells/µL with 106 cells/µL irradiated tumor cells. After 5 d, viable cells were collected and tested in a 51Cr release assay for their ability to lyse tumor cells. Where indicated, mAb to CD4 (GK1.5) or CD8 (Lyt2) was included at a final concentration of 10 µg/mL.

Rechallenging experiment and adoptive transfer experiment. Tumor-free mice in scFv-CD40-DC treatment group (EL4/E2 tumor) were used for rechallenging and adoptive transfer experiments after 90 d of posttumor challenge. For rechallenging experiments, mice were implanted s.c. either with 2 × 105 EL4/E2, EL4, or 1 × 106 B16 tumor cells. Naïve C57BL/6 mice were used as controls and were challenged with the same dose of tumor cells.

For adoptive transfer experiments, splenocytes isolated from tumor-free mice were stimulated as described above. At the end of culture, CD4+ and CD8+ T cells at ≥90% purity were isolated by magnetic bead cell sorting as described previously (28). One × 105 purified T cells or total lymphocytes were then transfected i.v. into naïve C57BL/6 mice. Adoptive transfer of in vitro stimulated splenocytes from naïve mice served as control. After 1 d, mice in each of group were challenged s.c. with 2 × 105 EL4/E2 tumor cells.

Statistical analysis. Data are presented as mean ± SE. Statistical analysis was done using two-way ANOVA. Statistical significance was determined at the <0.05 level. Survival estimates were determined using the method of Kaplan and Meier. MLR assays, in vivo depletion experiments, and histology are described in detail in Supplementary Methods.  

Expression of the scFv and scFv-CD40 CRs in murine DC. We generated two CR gene constructs composed of the scFv region of anti-ErbB2 mAb (4D5) linked via a mouse CD8α hinge to the transmembrane and/or cytoplasmic regions of mouse CD40 molecule (Fig. 1A). Recombinant adenovirus encoding the CRs (Ad-scFv and Ad-scFv-CD40) and a control green fluorescent protein (GFP) gene (Ad-GFP) were used to infect mouse bone marrow–derived immature DC (Fig. 1B). Surface expression of the CRs in the infected DC was determined by flow cytometry using an anti–c-myc mAb. As shown in Fig. 1C, consistent and equivalent levels of expression of scFv, scFv-CD40, and GFP were reproducibly detected in the infected DC with 100 MOI (n = 5). Expression of the CRs was also analyzed by immunoblotting. Under reducing conditions, two bands were observed in scFv-DC and scFv-CD40-DC but were absent in GFP-DC (Fig. 1D). Although the lower bands correspond well with the calculated size of the scFv and scFv-CD40 receptors (40 and 48 kd, respectively), the larger protein bands (~54 and ~62 kd, respectively) are most likely the result of complex glycosylation of the CRs within the antibody fragment and hinge region. Under nonreducing conditions, a series of additional bands were observed in lysates of the CR-modified DC representing CR homotrimers and perhaps heterotrimers of CR and endogenous CD40 chain.

The scFv-CD40 CR induces nuclear factor-κB–dependent DC activation and effector function after ErbB2 stimulation. Previous studies have indicated that CD40 activates the transcription
factor nuclear factor-κB (NF-κB). To test whether CRs function properly, we studied NF-κB signaling in infected DC by real-time PCR. As shown in Fig. 2A, ErbB2 protein, but not BSA, signaled a 2- to 4-fold increase in p50, c-Rel, and Rel-B mRNA in scFv-CD40-DC compared with that in GFP-DC or scFv-DC. Neither Rel-A nor p52 mRNA was induced by either stimulus. Lipopolysaccharide (LPS) stimulation induced an equivalent NF-κB mRNA elevation in all infected DC (data not shown).

Several recent studies have determined that the Rel-B subunit of NF-κB is a crucial mediator of DC maturation and survival (31, 32). Western blot analysis showed that ErbB2 ligation triggered Rel-B nuclear translocation in scFv-CD40-DC in a dose-dependent manner (Fig. 2B), which was comparable with that induced by LPS.

We next determined whether NF-κB signals induced DC activation. Culture with the irradiated D2F2/E2 cells, but not D2F2 cells, resulted in increased expression of CD86, K^d^, I-A^d^, and endogenous CD40 costimulatory molecules in scFv-CD40-DC, whereas culture with either cells failed to induce up-regulation of either molecules in GFP-DC and scFv-DC (Fig. 2C, I). Furthermore, the irradiated D2F2/E2 cells triggered the production of TNF-α, IL-8, IL-12p70, and MCP-1 in scFv-CD40-DC (Fig. 2C, II). No significant cytokines were detected in GFP-DC or scFv-DC after culture with the same cells. Importantly, the irradiated SK-BR-3 and MDA-MB-453 human breast tumor cells naturally expressing ErbB2 stimulated scFv-CD40-DC to secret TNF-α and IL-12p70 and up-regulate the costimulatory molecules expression (Supplementary Fig. S1). We also examined antigen presentation function of infected DC in allogeneic MLR assays. The scFv-CD40-DC prestimulated by the irradiated D2F2/E2 cells significantly induced allogeneic T-cell proliferation, which was comparable with that induced by LPS-treated DC (Supplementary Fig. S2).

Figure 2. The scFv-CD40 CR induced NF-κB–dependent DC activation and effector function after ErbB2 stimulation. A, NF-κB mRNA levels from GFP-DC, scFv-DC, or scFv-CD40-DC stimulated by 1 μg/mL BSA or ErbB2 protein for 24 h were detected by real-time PCR. Results are normalized to glyceraldehyde-3-phosphate dehydrogenase and expressed as fold increase relative to uninfected DC. B, nuclear lysates from infected DC stimulated by titrated levels of ErbB2 protein (1, 100, and 1000 ng/mL) were prepared for RelB/Sp1-specific Western blots. Infected DC stimulated by 1 μg/mL LPS was included as a positive control. Infected DC were cultured with the irradiated D2F2 or D2F2/E2 tumor cells at a DC to tumor ratio of 5:1 at 37 °C. After 24 h, DC were analyzed for the expression of costimulatory and MHC molecules by flow cytometry. Results are normalized to uninfected-DC MFI. II, infected DC were treated as described in I before supernatants were collected and cytokine levels were determined by ELISA. D, infected DC were incubated with the D2F2 or D2F2/E2 tumor cells at a DC to tumor ratio of 5:1 at 37 °C for 24 h and cytoxicity was evaluated by MTT assay. Wells with medium alone were used as background control (BG) and wells for total viability/spontaneous death of untreated cells (TS) contained only medium and tumor cells. Experimental (Ex) wells contained tumor cells and infected DC. Percentage of cytotoxicity was calculated using the following formula: % cytotoxicity = [(TS – BG) – (Ex – BG)]/TS – BG) × 100%. II, D2F2 or D2F2/E2 tumor cells were stained with 4 μg/mL Hoechst 33342 for 30 min. Cells were washed and maintained in culture for additional 3 h before washing again and adding infected DC at a DC to tumor ratio of 5:1 at 37 °C or 4°C. After 24 h, the cells were harvested and stained with anti-mouse CD11c mAb and PI before flow cytometry analysis. Viable DC (CD11c^+^PI^-^) were gated for analysis, and MFI of Hoechst 33342 staining is reported. All results are representative of three independent experiments. *, P < 0.05; **, P < 0.01; NF-κB mRNA, costimulatory, and MHC molecules expression, cytokine levels, or tumoricidal activity significantly increased in scFv-CD40-DC after ErbB2 ligation compared with the controls (GFP-DC or scFv-DC).


Targeted Dendritic Cell Therapy
Several studies reported that human and murine DC mediate direct cytotoxicity on tumor cells in vitro and in vivo (33–36). We evaluated the in vitro tumoricidal activity of infected-DC against D2F2 or D2F2/E2 tumor cells using 24-hour 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. As shown in Fig. 2D (I) all infected DC displayed weak killing activity on D2F2 cells. In contrast, the scFv-CD40-DC significantly killed D2F2/E2 cells than GFP-DC or scFv-DC did. To evaluated antigen uptake capacity of infected DC, we cocultured the Hoechst 33342–labeled D2F2 or D2F2/E2 tumor cells with infected DC at 37°C or 4°C and, 24 hours later, harvested these cultures for flow cytometric analyses. The scFv-CD40-DC cocultured with D2F2/E2, but not D2F2 tumor cells, exhibited much higher mean fluorescence intensity (MFI) than GFP-DC or scFv-DC cultured with the same cells at 37°C (Fig. 2D, II). However, the infected DC displayed approximately basic level of the Hoechst MFI in all DC/tumor cell combination when cocultured at 4°C, which ruled out the possibility of nonspecific tumor cell binding. Also, we observed a similar effect when EL-4/E2 cells were used (data not shown). These suggest that scFv-CD40-DC exhibit enhanced effector function upon ErbB2 stimulation in vitro.

In vivo migration of the CR-modified DC. To trace the in vivo migration of the CR-modified DC, 1 × 10⁶ CFSE-labeled infected DC were injected i.v. into BALB/c mice, which were implanted s.c. with the D2F2/E2 tumor cells 8 days before. Twenty-four hours postinjection, the tumors, the draining, and nondraining inguinal lymph nodes were dissected and analyzed for the presence of infected DC by flow cytometry. The injected and resident tumor/lymph node DC were identified as CFSE⁺CD11c⁺ or CFSE⁻CD11c⁻ cells, respectively. Although scFv-DC and scFv-CD40-DC effectively migrated to D2F2/E2 tumors, only latter homed to the draining inguinal lymph nodes (Fig. 3A). We did not detect appreciable infected-DC in the nondraining lymph nodes. Based on the percentage of CFSE⁺CD11c⁺ in tumors and lymph nodes and the number of DC injected, it was possible to determine that <1% of GFP-DC migrated to the tumors and lymph nodes. However, ~40% of scFv-DC and scFv-CD40-DC migrated to the tumors. Importantly, only scFv-CD40-DC (~10%) migrated to the draining lymph nodes (Fig. 3C, left). We repeated the same experiment in D2F2 tumor–bearing mice. As expected, we detected no infected-DC enrichment in the D2F2 tumors, the draining or nondraining lymph nodes (Fig. 3C, right). We further analyzed the phenotype of scFv-DC and scFv-CD40-DC in the D2F2/E2 tumors. Compared with scFv-DC, the scFv-CD40-DC expressed high levels of MHC II, CD86, and CCR7 molecules (Fig. 3B).

In vivo antitumor effects of the CR-modified DC. We next assessed the efficacy of the CR-modified DC on tumor development in a therapeutic vaccination setting using two paired ErbB2-positive or negative tumor models. Two × 10⁵ D2F2/E2 and D2F2 or EL-4/E2 and EL-4 cells were injected s.c. into syngeneic BALB/c or C57BL/6 mice. On day 8, animals with tumors sizing 15 to 25 mm² were immunized with scFv-DC,

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**Figure 3.** In vivo migration of the CR-modified DC. A, the 8 d–established D2F2/E2 or D2F2 tumor–bearing BALB/c mice were infused i.v. with 1 × 10⁶ CFSE-labeled GFP-DC, scFv-DC, or scFv-CD40-DC. After 24 h, the mice were killed. The single-cell suspensions were prepared from harvested tumors, draining, and nondraining inguinal lymph nodes (LN) and stained with APC-conjugated anti-CD11c, PE-conjugated anti–I-Ab, CD86, CCR7, or isotype control mAb. The percentage of infused DC or endogenous DC that are CFSE⁺CD11c⁺ or CFSE⁻CD11c⁻ is indicated within each quadrant. Only representative plots from mice bearing D2F2/E2 were given. B, gated CFSE⁺CD11c⁺ scFv-DC or scFv-CD40-DC in D2F2/E2 tumors were analyzed for I-Ab, CD86, and CCR7 expression. C, the percentage of injected DC in indicated tissues versus total injected DC was shown. All results are representative of three independent experiments.
scFv-CD40-DC, or PBS. In preliminary studies, we determined i.v. scFv-CD40-DC injection twice on day 8 and 11 as an optimal vaccination schedule. Treatment of D2F2/E2 tumor–bearing BALB/c mice with 1 × 10^6 scFv-CD40-DC resulted in significant suppression of tumor growth and enhanced survival of the mice (Fig. 4A). There was no therapeutic effect in the control group. In EL-4/E2 tumors, mice receiving scFv-CD40-DC treatment showed significant inhibition of tumor growth, and the survival rate was higher than that in the control group (Fig. 4C). Accumulating data from several experiments suggested that tumor regressed completely in 53% (D2F2/E2, 16 of 30) and 40% (EL-4/E2, 12 of 30) of the mice, and those mice remained tumor free for >90 days when treated by scFv-CD40-DC. The H&E staining of tumor samples showed that inhibition of tumor development was accompanied by tumor necrosis and a marked lymphocyte infiltration (Supplementary Fig. S3). However, D2F2 or EL4 tumor–bearing mice die within 6 weeks regardless of either treatment (Fig. 4B and D).

**Induction of tumor-specific CTLs by i.v. injection of scFv-CD40-DC.** Effector cells were generated from the splenocytes obtained from the D2F2/E2 tumor–bearing mice 10 days after DC immunization by culture with the irradiated D2F2/E2 cells. Only cells from scFv-CD40-DC–treated mice exhibited specific lysis activity toward D2F2/E2 and D2F2 target cells, without CTL activity observed against syngeneic 3T3 cells (Fig. 5A–C), which indicates CTLs are tumor-specific. CTL activity against D2F2/E2 or D2F2 was significantly inhibited by preincubation of effector cells with anti-CD8 but not anti-CD4 mAb (Fig. 5D). In EL4/E2 tumors in C57BL/6 mice, CTL effector cells from scFv-CD40-DC–treated mice exhibited specific lysis of EL4/E2 and EL4 target cells without lysis of control B16 cells (data not shown).

**Figure 4.** In vivo antitumor effects of the CR-modified DC. A, D2F2/E2 tumors. BALB/c mice were injected s.c. in the left flank on day 0 with 2 × 10^5 ErbB2-positive D2F2/E2 mammary tumor cells. On day 8 and 11, 5 × 10^6 scFv-DC or scFv-CD40-DC were injected i.v. B, D2F2 tumors. BALB/c mice were injected s.c. on day 0 with 2 × 10^5 ErbB2-negative D2F2 mammary tumor cells. On day 8 and 11, 5 × 10^6 scFv-DC, scFv-CD40-DC was injected i.v. C, EL4/E2 tumors. C57BL/6 mice were injected s.c. on day 0 with 2 × 10^5 ErbB2-positive EL4/E2 thymoma cells. On day 8 and 11, 5 × 10^6 scFv-DC or scFv-CD40-DC were injected i.v. D, EL4 tumors. C57BL/6 mice were injected s.c. on day 0 with 2 × 10^5 ErbB2-negative EL4 thymoma cells. On day 8 and 11, 5 × 10^6 scFv-DC or scFv-CD40-DC was injected i.v. In all tumor models, mice injected with PBS were used as additional control. Tumor size was measured twice weekly and survival was monitored for 12 wk. *, P < 0.05; **, P < 0.01, tumor growth was significantly inhibited in the scFv-CD40-DC–treated group compared with the scFv-DC or PBS treatment group.

**Memory response induced in mice that have been cured of ErbB2-positive tumors.** The D2F2/E2 or EL-4/E2 tumor growth was completely eradicated in ≥40% of the mice, which received scFv-CD40-DC injection and survived for ≥90 days. To test whether immunologic memory was developed in these mice, the animals were subsequently rechallenged with the same tumor cells used for previous inoculation. The parental tumor cells or other syngeneic tumor cells were used as controls. Mice cured of EL-4/E2 tumors rejected subsequent challenges with the same tumor or parental EL-4 cells and remained tumor free until the end of the experiment, whereas the mice could not reject syngeneic unrelated B16 tumor (Fig. 6A). Interestingly, tumor-free mice also had protective immunity against subsequent challenge with the parental EL-4 cells, suggesting that mice developed immunity to other antigens expressed on EL-4/E2 and “shared” with the parental tumor cells, resulting in long-lasting memory against these tumors, which was consistent with results of CTL assays (Fig. 6B). All control mice died of progressive tumor growth, irrespective of challenge with EL-4/E2, EL-4, or B16 (Fig. 6B).

Finally, adoptive transfer experiment was done to determine whether this immunologic memory could be transferred into naive mice. Recipient mice (C57BL/6) were challenged with EL-4/E2 tumor cells 1 day after the adoptive transfer of in vitro stimulated splenocytes. Adoptive transfer of total splenocytes (>85% cells were CD4+ and CD8+ T cells) from tumor-free mice resulted in tumor protection in 100% of the mice, and these mice remained tumor-free until the end of the experiment (Fig. 6C). Interestingly, tumor-free mice also had protective immunity against subsequent challenge with the parental EL-4 cells, suggesting that mice developed immunity to other antigens expressed on EL-4/E2 and “shared” with the parental tumor cells, resulting in long-lasting memory against these tumors, which was consistent with results of CTL assays (Fig. 6B). All control mice died of progressive tumor growth, irrespective of challenge with EL-4/E2, EL-4, or B16 (Fig. 6B).
Discussion

In this study, we focused on the development of TAA-targeted DC vaccination strategy. The hypothesis is that TAA-targeted DC, when injected into tumor-bearing mice, would specifically migrate to and enrich in the TAA-positive tumor by scFv targeting, mature by CD40 signaling, and finally initiate a potent antitumor immunity. The results presented here support feasibility of this strategy.

The results show scFv-CD40 CR initiates a potent NF-κB signal, which resulted in DC activation only when cultured with immobilized ErbB2 protein or ErbB2-positive tumor cells, indicating that the activity of targeted DC was subtly and specifically controlled by scFv-CD40 CR. The observations also indicate that the crosslinking CR effectively couples with the necessary downstream effector signals. Initial studies showed CD40 ligation of DC alone is sufficient for the induction of DC maturation and an effective immune response (17), but more recent evidence indicates that microbial secondary signals are necessary for optimal DC activation (37). This is illustrated by the synergistic effect of combined Toll-like receptor (TLR)/CD40 stimulation on DC for IL-12 up-regulation and antigen-specific CD8+ T-cell expansion. Our study shows that scFv-CD40 CR can circumvent the requirement for these nonspecific TLR ligands. In fact, when cultured with D2F2/E2 or EL4/E2 cells, scFv-CD40-DC produce large quantities of cytokines, including IL-12. This observation is important because previous studies have found that in vitro transient production of IL-12 blunts the T-cell activation capacity of “exhausted” DC (11, 12). In this system, targeted DC do not produce IL-12 until they are encountered with cognate antigen in vivo, which will effectively avoid premature licensing and exhaustion of DC.

The results also show that scFv-CD40-DC exhibit direct cytotoxicity against ErbB2-expressing tumor cells in vitro, which resulted in enhanced apoptotic body engulfment by scFv-CD40-DC. The mechanisms underlying this tumoricidal activity may involve expression of TNF family ligands, including secreted or membrane-bound TNF-α, Fas ligand, TNF-related apoptosis inducing ligand, or lymphotoxin α1/α2 (33–35). The in-depth mechanisms are under investigation. As previously reported (36), this tumoricidal activity might greatly contribute to tumor antigen uptake and subsequent crosspriming of tumor-reactive T cells by targeted DC in vivo.

Previous studies have showed that the number and maturation status of tumoral DC are important limiting factors in DC-based immunity (38, 39). The presence of immature DC in a wide range of tumors that are unable to stimulate T cells plays an important role in the failure of tumor-bearing hosts to mount an effective antitumor response (39, 40). Our results clearly show targeted scFv-DC and scFv-CD40-DC, but not control GFP-DC, can effectively migrate to ErbB2-positive tumors in vivo. Importantly, upon encounter with tumor cells, scFv-CD40-DC up-regulate MHC II, CD86, and chemokine receptor CCR7 expression and sequentially home into the draining lymph nodes. The observations indicate that targeted scFv-CD40-DC experience an in situ maturation process within the tumors. Thus far, most of DC vaccination trials have used an intradermal or s.c. delivery approach in human, whereby the overall number of DC reaching the draining lymph nodes is very small (c2–10%; ref. 41). By scFv specific targeting, however, we provide an effective approach of directing modified DC to tumors and the draining lymph nodes. We found that almost half of injected scFv-CD40-DC were located in the tumors and the draining lymph nodes within 24 h after injection. Compared with conventional DC vaccination strategies, this approach significantly enhances the ability of DC of migrating to the tumors and the draining lymph nodes.

I.v. injection of scFv-CD40-DC elicited therapeutic antitumor immunity in two paired murine tumor models that suppressed the growth of established tumors and enhanced survival of mice. Mechanistic investigation further indicated that administration of scFv-CD40-DC elicited tumor-specific CTL responses, mainly mediated by CD8+ T cells. Previous reports suggest that DC could not stimulate CTLs directly unless they are first stimulated via CD40 molecules on their surface. This is usually accomplished by CD40L expressed on CD4+ helper T cells. Theoretically, the targeted DC can directly stimulate infiltrated CD8+ CTLs in the absence of CD4+ helper T cells. This hypothesis was strongly supported by in vivo depletion experiments, in which depletion of CD8+ T cells had no influence on tumor regression (Supplementary Fig. S4). Interestingly, the CTL responses were directed to ErbB2-positive tumor cells and also parental ErbB2-negative tumor cells. In accordance with CTL responses, the tumor-free mice cured of EL4/E2 tumor rejected subsequent rechallenges with the identical tumor cells or parental tumor cells. Presumably, the development of long-term immunologic memory was not only dependent on the ErbB2 antigen but also on other unidentified antigens of EL4/E2 tumors. Because antigen-negative variants may arise after antigen-positive tumor cells are destroyed, immune responses to additional undefined TAs may be crucial to the ultimate success of DC.
vaccination. The results are also consistent with the reports using different DC vaccination strategies or non-DC vaccination protocols (42, 43).

Moreover, the ability to transfer immunity by the infusion of CTLs indicates the presence of tumor-specific T cells in tumor-protected mice. The adoptive transfer experiment suggests that both CD4+ and CD8+ T cells are required to achieve tumor protection. It is possible that the cytokines produced by the transferred CD4+ T cells is required for the maintenance of transferred CD8+ T cells and/or activation of host CD4+ or CD8+ T cells. Several recent studies have documented that CD4+ T-cell help is critical for the maintenance of CD8+ T-cell population (43–45).

Taken together, we designed a TAA-targeted DC activation system that couples DC maturation with DC migration in tumor. The immune responses induced by i.v. injection of targeted DC were effective in increasing the survival with complete tumor eradication in 53% and 40% of the mice, respectively, in the therapeutic settings. To our knowledge, this is the first proof of concept documenting the feasibility of TAA-targeted DC vaccination strategy in a clinically relevant mouse tumor model. Thus far, adenovirally engineered DC have been tested in patients with advanced digestive carcinomas and metastatic gastrointestinal malignancies, and it was shown that intratumoral injection of DC transfected with an adenovirus encoding IL-12 is feasible and well-tolerated (46, 47). To evaluate the clinical applicability of the presented strategy, we need to examine whether the modified human DC by this strategy would behave as mouse DC in vitro. At present, this work is under investigation in our laboratory. In addition, we will investigate the generality of this strategy through construction of other TAA-specific CR. Recently, Xu and colleagues (48) reported that a chimeric gene linking ErbB2-specific scFv to IRAK-1 signaling is able to mediate cytokine secretion and enhance T-cell stimulation capacity of DC cell line, which is consistent with our results. Perhaps integrating the components of TLR signaling into the CRs will reinforce the efficacy of targeted DC as reported recently by Lapteva and colleagues (49). Alternatively, targeted DC could be used in combination with adoptively transferred antigen-specific T cells, where they would effectively recruit and stimulate antigen-specific T cells. The findings from present study will provide a novel strategy for development of potent DC vaccine.

Disclosure of Potential Conflicts of Interest

No conflicts of interest were disclosed.

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