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Targeting of Antigens to B Lymphocytes via CD19 as a Means for Tumor Vaccine Development

Yunfeng Ma,*†‡¹ Dong Xiang,*‡†¹ Jinwen Sun,*‡†¹ Chuanlin Ding,*† Min Liu,*† Xiaoling Hu,* Guoxin Li,* Goetz Kloecker, † Huang-ge Zhang,*† and Jun Yan*†‡||

Ab therapy against surface Ags on tumor cells has demonstrated significant efficacy for some cancers. However, it is costly and patients frequently develop acquired resistance over time. In cases of Ab therapy resistance, T cell responses have been shown to be essential in controlling disease progression. Thus, vaccination that generates a sustained Ab response as well as a T cell response may be more effective and economical. In this article, we have developed a vaccination strategy by targeting protein Ags to B cells via a CD19 single-chain variable fragment miniAb. Using the tumor-associated Ag her-2/neu extracellular domain, we showed that the engagement of CD19 and BCR induced full B cell activation to produce a high titer of Abs and enhanced CD4 Th2 response and CD8 T cell activation and differentiation. These Abs competitively inhibited humanized her-2/neu Ab binding and were capable of activating the complement and inhibiting human breast cancer growth in vitro. Therapeutic efficacy was demonstrated in vivo using murine mammary carcinoma models. Furthermore, four different extracellular domains of her-2/neu could be targeted to B cells to generate Abs against particular domains with different antitumor properties. This approach may offer a new avenue for vaccine development with significantly lower cost, which may be of use not only for cancer therapy but also for infectious agents. The Journal of Immunology, 2013, 190: 5588–5599.

Currently, great interest is being shown in activating the immune system for cancer therapy (1). However, developing effective cancer vaccines has proven to be a daunting task (2). The recent approval of the first therapeutic cancer vaccine by the U.S. Food and Drug Administration, sipuleucel-T, a vaccine for the treatment of asymptomatic, metastatic, castrate-resistant, advanced prostate cancer with a modest clinical benefit in some patients (3), has re-energized research into more potent cancer vaccine development. At this point, many cancer vaccine platforms have been evaluated in preclinical animal models or clinical trials (4), including protein or peptide-pulsed dendritic cell (DC)-based vaccines (5). The DC-based vaccine platform normally requires leukapheresis and further in vitro expansion of DCs. Drawbacks of this approach include large-scale preparation of clinical-grade DCs, the choice of DC subsets (6), and DC-related trafficking. In contrast, antitumor mAb therapy has achieved clinical promise and now is widely used in oncological patient care (7, 8). Thus, it would be desirable if tumor vaccines could elicit long-lasting antitumor humoral responses as well as T cell responses.

B cells are capable of eliciting antitumor responses by the production of Abs as well as serving as APCs to induce CD4 T cell responses (9, 10). In addition, B cells can present Ag to cross-prime CD8 T cells for expansion and activation (11). Ag activation of B cells has been shown to enhance the expression of costimulatory molecules, principally CD86, which is essential for the ability of B cells to break T cell tolerance. However, the role of B cells in tumor development has been controversial. Previous studies showed that therapeutic depletion of B cells enhances B16 melanoma growth in mice (12). In contrast, in a skin squamous carcinoma model, B cells, predominantly the Abs produced by B cells, promote tumor progression via triggering chronic inflammation (13). Nevertheless, the ability of B cells to induce both Ab production and T cell responses makes B cells an ideal cell subset for cancer vaccine development.

CD19 is a B cell–specific member of the Ig superfamily expressed at almost every stage of B cell development except after differentiation into plasma cells (14). CD19 is also considered a coreceptor for BCR; coengagement of BCR and CD19 reduces the B cell activation threshold (15). Our previous studies also showed that CD19 on the B cell surface is important for B cell Ag presentation (16, 17). Targeting of Ags to B cells via CD19 led to more efficient Ag presentation by B cells and potent CD4 and CD8 T cell activation. In addition, coligation of CD19 and the BCR potently activates B cells to induce Ag-specific Ab responses that may specifically target tumor cells (17).

Her-2/neu has been an attractive target for cancer immunotherapy (18). The her-2/neu chimeric humanized Ab trastuzumab (Herceptin) has been approved to treat metastatic her-2/neu–overexpressing breast cancers (19). Despite the great success of Herceptin therapy, the major limitation of immunotherapy with...
trastuzumab is the development of drug resistance usually within 1 y from the beginning of treatment, arising from various mechanisms (20–22). It appears that CD8 T cell responses are effective against these tumors (23). In addition, the cost of Herceptin per patient could be as much as $70,000 (U.S.) per year (24). Clearly, generating sustained and active immune responses to the her-2/neu protein is essential to this existing approach. In this study, we constructed CD19 single-chain variable fragment (scFv) miniAbs as a means to target Ags to B cells and found that this approach elicits not only augmented Ab responses but also T cell responses. More importantly, Herceptin-like Abs and enhanced CD8 cytolytic activity were elicited in immunized mice. These Ab and T cell responses could significantly delay tumor progression in both prophylactic and therapeutic settings and prolong the survival of tumor-bearing mice. In addition, this targeting approach could generate Ab responses against four different domains of Her-2/neu extracellular domains (ECDs) with different antitumor properties. Thus, this approach offers a new avenue for effective vaccine development.

Materials and Methods

Mice and cell lines

BALB/c, OVA TCR transgenic (Tg) OT-I, and OT-II Rag-deficient mice were purchased from The Jackson Laboratory (Bar Harbor, ME), C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). All experimental mice were housed under specific pathogen–free conditions in the animal facility of University of Louisville (Louisville, KY) and treated in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Louisville.

BALB/c mammary carcinoma cell D2F2/E2 expressing human her-2/neu was kindly provided by Dr. Wei-Zen Wei (Karmanos Cancer Institute, Detroit, MI). A human her-2/neu–expressing C57BL/6 mammary tumor line EO771 (EO771/E2) was generated by stable transfection with human full-length her-2/neu cDNA plasmid (25). Human breast cancer cell SKBR-3 and human ovarian cancer cell SKOV3 from American Type Culture Collection were maintained in DMEM supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Rat anti-mouse CD19 hybridoma (1D3) from American Type Culture Collection was maintained in MEM supplemented with 10% FBS.

Generation of CD19 scFv miniAb and fusion proteins

To generate CD19 scFv, total RNA was extracted from 1D3 and first-strand cDNA was synthesized. VH and VL were amplified using primers: V kappa start: 5′-GAAGATTCCTCCACCATGGACATTCAGCTGACCCAGTCTCC-A-3′; J kappa with linker: 5′-AGACCGCCATCCGGTTTGGTCCATGGCTCTGACATGCTGCTGTGGTCGCT-3′; VH start with linker: 5′-GGCCGAGGTTGGCT-CTARGTSMACRTGCVGWASRTGCGG-3′; and VH stop: 5′-ACTAGT-CGACTCTGAGGACACGGTACGCTTGGCCGCCC-3′. The single-chain Fv (VL–VH) was then synthesized by overlapping PCR. PCR product was sequenced and further cloned into pET-20b (+) vectors in an single-chain Fv (VL-VH) was then synthesized by overlapping PCR. PCR product was sequenced and further cloned into pET-20b (+) vectors in an

The full-length human c-ErbB-2 (Her-2/neu) cDNA was isolated from plasmid pCMV–ErbB-2 as a 4.4-kb EcoRI restriction fragment and was product was sequenced and further cloned into pET-20b (+) vectors in a

from the reducing agent over a desalting column. LPS-free recombinant protein P3–4 was activated with succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC; Pierce, Rockford, IL) according to the manufacturer’s protocol and mixed with the reduced mAb for 1 h at room temperature and then incubated overnight at 4°C. The un conjugated anti-CD19 mAb or free protein P3–4 was removed by size-exclusive column. The conjugates were examined for B cell binding, as assessed by flow cytometry.

Fusion protein binding assay and confocal microscopy

For in vitro binding assay, splenocytes were incubated with proteins scFv, P3–4, scFv-p3–4, OVA-biotin, scFv-OVA-biotin or biotin-labeled scFv-D1, scFv-D2, scFv-D3, scFv-D4, and then stained with Oregon green–labeled anti-Her2 Ab or PE-streptavidin and APC–anti-mouse B220. Cells were washed and assayed by flow cytometry. For the in vivo binding assay, mice were injected i.v. with biotinylated fusion proteins scFv, P3–4, scFv-p3–4, OVA, or scFv-OVA. Peripheral blood was drawn at 10 min after injection. Cells were stained with APC–anti-mouse B220 and PE streptavidin and assessed by flow cytometry.

For determination of protein (scFv, scFv-p3–4, parental CD19 mAb) Kd values, B cells were incubated with different concentrations of proteins and then assayed by flow cytometry. Kd values were calculated by the following equation: 1/F - Fback = 1/Kmax + (Kd/Fmax)(1/IFscFv), where F = fluorescence unit, Fback = background fluorescence, and Fmax was calculated from plot (26).

For colocalization assays, B cells were incubated with scFv-p3–4 for 30 min and stained with biotinylated anti–her-2/neu followed by Alexa Fluor 594 streptavidin and Alexa Fluor 488–anti-IgM for 30 min at 4°C. Cells were analyzed on a Nikon confocal microscope.

In vitro B cell culture

Purified B cells were stimulated with scFv, P3–4, scFv-p3–4, or CD19 mAb–conjugated P3–4 (1 μg/ml) for 24 h and then harvested to detect surface marker expression by flow cytometry. For cytokine assays, B cells were incubated for 48 h with different fusion proteins (20 μg/ml), and supernatants were harvested to measure cytokine levels by ELISA.

Detection of her-2/neu Abs by ELISA

First, 96-well plates were coated with recombinant her-2/neu P1–4 protein (1 μg per well) overnight at 4°C and blocked with 0.5% BSA/PBS. Pre- or postimmune sera from mice were diluted and further reacted with goat anti-mouse IgG or IgG HRP conjugates (Southern Biotech, Birmingham, AL). The assays were subsequently developed by the addition of ABTS One Component Microwell Substrate (BioFX Laboratories, Owings Mills, MD), and the OD405 nm was determined. To measure IgG isotype Abs, sera were diluted at 1:100 and further reacted with HRP-conjugated goat anti-mouse IgG1, IgG2a, and IgG2b (Southern Biotech). Ab concentrations were determined by generating a standard curve using purified mouse IgG.

Ab competitive inhibition assay

Microtiter plates were coated with 1 μg per well of recombinant her-2/neu P3–4 for overnight and then blocked with 0.5% BSA/PBS for 1 h at room temperature. Serially diluted immune sera (1:20, 1:40, 1:80) were added into wells for 1 h at room temperature and followed by biotin-Herceptin. Preimmune sera were used as controls. The wells were incubated with streptavidin-HRP and ABTS One Component Microwell Substrate. OD405 nm was measured. The percentage of inhibition was calculated using the following formula: (ODpre - ODpost)/ODpre × 100%.

Complement activation assay

A total of 1 × 10⁶ SKOV3 tumor cells were suspended in 100 μl HBSS with calcium and magnesium and incubated with inactivated post- or preimmune sera (1:10 dilution) for 30 min at 37°C. SCID mouse sera as a source of complement were added and followed by FITC–anti-mouse C3 (MP Biomedical) and measured by flow cytometry.

In vitro tumor cell growth inhibition assay and WB

A total of 1 × 10⁵ SKBR-3 cells were placed into the wells of the ACEA 16-well plates for 24 h. Then 10 μl heat-inactivated pre- or postimmune sera were added to the wells and incubated for the indicated times. Herceptin was used as a positive control. The inhibition of tumor cell growth was calculated by measuring the relative decrease in current impedance among wells containing postimmune sera and wells containing preimmune serum only as described previously (27, 28). The percentage of inhibition was calculated using the following formula: (Cell indexpre - Cell indexpost)/Cell indexpre × 100%.
For WB, SKBR-3 cells were treated with medium, heat-inactivated immune serum (1:10), or Herceptin (2 μg/ml) for 3 h at 37°C. Cells were lysed, and immunoblot was performed with rabbit anti-phospho-Akt Ab (Cell Signaling Technology, Danvers, MA) or mouse anti–β-actin Ab (Sigma-Aldrich). The blots were visualized using the ECL Prime WB detection reagents (GE Healthcare Biosciences, Pittsburgh, PA).

**T cell proliferation assay**

For in vitro proliferation assays, splenocytes from OT-I or OT-II Tg mice were labeled with 10 μM CFSE (Molecular Probes, Carlsbad, CA) and then stimulated with varying concentrations of the fusion protein OVA or scFv-OVA. At 3 d later, turnover of T cells was examined by flow cytometry. For [3H]-thymidine incorporation assays, splenocytes from immunized mice were cultured in 96-well plates (5 × 10^5 cells per well) and stimulated with scFv–p3–4 (20 μg/ml) for 72 h. [3H]-thymidine was added 16 h before a 3-d culture, and proliferation was measured by a scintillation counter (Packard). A stimulation index was used to show fold increase. The stimulation index was calculated using the following formula: cpmexp/cpmcontrol. For in vivo proliferation assays, CD8 or CD4 T cells purified from OT-I or OT-II Tg mice were labeled with 10 μM CFSE. T cells (2 × 10^6 per mouse) were then adoptively transferred into recipient mice. OVA or scFv-OVA fusion proteins were injected into mice i.v. 24 h after adoptive transfer. Recipient mice were sacrificed after 3 d, and the turnover of T cells was examined by flow cytometry.

**Intracellular cytokine staining**

Intracellular cytokine staining was performed using the BD Cytofix/Cytoperm Kit with BD GolgiPlug (BD Pharmingen, San Diego, CA) according to the manufacturer’s protocol. For IFN-γ staining, cells were stimulated with OVA, scFv-OVA, or scFv–p3–4 for 3 d, and then restimulated with PMA plus ionomycin for 4 h in the presence of GolgiPlug, and then stained with APC- or FITC-conjugated mAbs against mouse CD8 or CD4 and PE-conjugated anti-mouse IFN-γ (BioLegend, San Diego, CA). For intracellular IL-4 staining, cells were stimulated with Con A (3 μg/ml) for 2 d, followed by mouse IL-2 (10 ng/ml) and IL-4 (50 ng/ml) for 3 d. Cells were restimulated with immobilized CD3 (10 μg/ml) and soluble CD28 Abs (2 μg/ml) in the presence of GolgiPlug for 5 h. Cells were surface stained with anti-mouse CD4–APC and intracellularly stained with anti-mouse IL-4–FITC (eBioscience, San Diego, CA).

**In vivo cytotoxicity assay**

B cells loaded with or without scFv–p3–4 were used as target cells for the in vivo cytotoxicity assay. In brief, B cells were pulsed with scFv–p3–4 (10 μg per million cells) and then labeled with 2.5 μM CFSE–p3–4. Unpulsed B cells were labeled with 0.25 μM CFSE (CFSEhigh). The mixed B cells at a ratio of 1:1 were injected into mice immunized with different regimens. Mice were killed after 24 h of target cell transfer. Specific cytotoxicity was determined by detecting the differentially fluorescent-labeled target cell populations by flow cytometry. The percentage of cytotoxicity was determined as follows: (1 – CFSEhigh/CFSElow) × 100%.

**Flow cytometry**

Splenocytes were incubated with anti-CD16/CD32 Fc receptor blocker for 10 min on ice and then washed and stained with the indicated fluorochrome-conjugated mAbs. Cells were collected with a FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA) and analyzed using FlowJo software (TreeStar, Ashland, OR).

**Mouse immunization and tumor challenge**

The 6- to 8-week-old BALB/c or C57BL/6 mice were immunized i.v. with scFv, P3–4, or scFv–p3–4 at 50 μg per mouse per injection on days 0, 7, and 14. A group of mice immunized with PBS was used as control. On days 7 and 21, the sera were collected for her-2/neu Ab measurement. For tumor therapy in the prophylactic setting, mice were immunized four times and challenged by s.c. injection in the flank with 1 × 10^6 D2F2/E2 (BALB/c) tumor cells. In the therapeutic setting, BALB/c or C57BL/6 mice were first challenged with 1 × 10^5 D2F2/E2 or 5 × 10^6 E0771/E2 tumor cells. When palpable tumors formed, mice were treated with different regimens for four times at 1-wk intervals. Tumor diameter was measured by calipers twice per week. Mice were killed when tumors reached 15 mm in diameter. In some experiments, survival was monitored up to 100 d beyond tumor implantation. In some experiments, mice were injected i.p. with CD8 mAb (clone 2.43; 500 μg per mouse) or CD4 mAb (clone GK1.5; 250 μg per mouse) or isotype control mAb (250 μg per mouse) 3 d prior to immunization.

**Statistical analysis**

Unpaired t test analysis was used to determine whether the differences between T cell- and B cell-mediated immune responses induced by scFv–p3–4 versus scFv or P3–4 were significant. A two-way ANOVA and Kaplan–Meier survival analysis were used to determine significance for in vivo tumor therapy. A p value < 0.05 was considered significant.

**Results**

**Generation of anti-CD19 scFv fusion proteins that specifically bind to B cells**

Our previous studies have demonstrated that targeting of Ags via CD19 led to enhanced Ag-specific T cell responses and broke immune tolerance (17). However, if whole Abs were used, these could potentially stimulate the production of inflammatory cytokines, resulting in serious adverse effects. Single-chain Ab V region fragments (scFvs) are potentially useful as therapeutic reagents (29–31) less likely to engender inflammatory responses. Single-chain miniAbs are recombinant monovalent Abs lacking the constant part of both heavy and light chains. These molecules retain their Ag recognition ability and can be easily expressed in a prokaryotic system or mammalian cell lines. We generated anti-CD19 scFv miniAb from 1D3 rat anti-mouse CD19 hybridoma cells. Candidate Ag genes such as her-2/neu ECD can be ligated with anti-CD19 scFv to make a fusion protein that specifically targets B cell CD19 and simultaneously engages the BCR (Fig. 1A). The tumor-associated Ag (TAA) her-2/neu ECD and surrogate Ag OVA were chosen to fuse with anti-CD19 scFv. Anti-CD19 scFv-her2/neu ECD cDNA constructs that contain cDNA encoding the Herceptin-binding domain (from residues 475–652 aa, designated as P3–4) were generated. Similarly, truncated OVA cDNA (residues 241–386) was also ligated with scFv plasmids. Subsequently, these recombinant proteins were produced, purified, and characterized. As indicated in Fig. 1B, recombinant her-2/neu P3–4, anti-CD19 scFv–p3–4 (scFv–p3–4) fusion proteins were blotted positively with both His-Tag and her-2/neu Abs. The recombinant anti-CD19 scFv miniAb protein was blotted positively with His-Tag Ab, but not with her-2/neu Ab or OVA Ab. Similarly, recombinant proteins OVA and scFv-OVA reacted with His-Tag Ab and OVA Ab, respectively (Fig. 1C).

To verify that the scFv, scFv–p3–4 and scFv-OVA proteins retained the Ag-binding activity of the parental Ab, we measured specific binding to B cells in vitro and in vivo. For the in vitro binding assay, splenocytes were incubated with protein scFv, P3–4, scFv–p3–4, biotin-OVA, or biotin-scFv-OVA. For the in vivo binding assay, biotin-labeled proteins were i.v. injected into mice. Peripheral blood was drawn 10 min after injection. The successful targeting of B cells was observed by the identification of double positive cells (Fig. 1D). These results indicate that anti-CD19 scFv miniAb, with or without tagged Ag, is capable of binding to B cells specifically. We also measured the protein Kd values by Lineweaver–Burk analysis. The results revealed that scFv and scFv–p3–4 proteins retained high binding affinity to B cells (Fig. 1E).

Fusion protein scFv–p3–4 activates B cells to produce low levels of proinflammatory cytokines

To determine whether Ag binding to B cells via anti-CD19 scFv was targeted to the BCR, B cells were incubated with anti-CD19 scFv–p3–4 fusion protein and stained with biotinylated anti–her-2/neu followed by Alexa Fluor 594–streptavidin (red) and Alexa Fluor 488–anti-IgM (green). As shown in Fig. 2A, Ag linked to anti-CD19 scFv colocalized with surface IgM on B cells. To examine whether the fusion protein scFv–p3–4 could activate B cells, purified B cells were incubated with fusion proteins scFv,

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FIGURE 1. CD19 scFv miniAb specifically binds to B cells with high binding affinity. (A) Schematic of fusion protein constructs. (B and C) Lysates of BL21(DE3)Plyss cells transfected with expression plasmids for CD19-scFv, her-2/neu P3–4, CD19–scFv–p3–4 (B), OVA, and CD19-scFv-OVA(C) were purified, and then WBs were done using Abs against His-Tag, her-2/neu, or OVA. (D) For in vitro B cell binding, splenocytes were incubated with scFv, P3–4, scFv–p3–4, biotin-OVA, biotin-scFv-OVA, followed by APC–anti-mouse B220 and Oregon green–Herceptin or PE-streptavidin. Cells were washed and assessed by flow cytometer. For in vivo binding, biotin-labeled proteins were i.v. injected into mice. Peripheral blood was drawn 10 min after injection. Cells were stained with APC–anti-mouse B220 and PE-streptavidin and assessed by flow cytometer. (E) Determination of protein K_D values by Line-weaver–Burk analysis.
Expression of surface markers on B cells was assessed by flow cytometry. Fusion protein scFv–p3–4, but not P3–4, significantly upregulated the expression levels of surface markers, including CD40, CD69, CD80, CD86, MHC class II, and MHC class I molecules (Fig. 2B). Further cytokine measurement indicated that scFv–p3–4 stimulated low levels of proinflammatory cytokines, including IL-6, IL-12p40, and TNF-α (Fig. 2C). scFv alone also stimulated moderate expression levels of CD69, CD86, and MHC class II, but the levels were significantly lower than those stimulated by scFv–p3–4. In addition, scFv alone stimulated IL-6 and TNF-α production, but not IL-12. These results suggest that Ag targeted to B cells via CD19 molecule can coengage the BCR and stimulate full B cell activation.

Targeting surrogate Ag OVA to B cells via CD19 scFv stimulates augmented Ag-specific CD4 and CD8 T cell responses

To determine whether targeting Ag to B cells increases CD4 and CD8 T cell responses, we generated the fusion protein scFv-OVA for Ag presentation. For an in vitro Ag presentation assay, OT-II CD4+ T cells or OT-I CD8+ T cells were used as readout of OVA Ag presentation. Indeed, CD4+ or CD8+ T cells underwent significantly more proliferation in response to scFv-OVA, compared with OVA stimulation (Fig. 3A). In addition, both CD4+ T and CD8+ T cells produced large amounts of IFN-γ upon scFv-OVA stimulation (Fig. 3B). Next, we examined whether this strategy would lead to enhanced T cell proliferation in vivo. Mice were i.v. administered 2 × 10^6 CFSE-labeled naive OT-I or OT-II cells. The next day, mice were injected with soluble OVA or scFv-OVA. As an additional control, PBS was injected into another group of mice. As shown in Fig. 3C, both CD4+ and CD8+ T cells underwent at least four divisions within the first 3 d of exposure to scFv-OVA in vivo. In contrast, OT-I or OT-II T cells responded significantly less to the same amount of soluble OVA. These results suggest that scFv-OVA fusion protein induces potent T cell proliferation and differentiation both in vitro and in vivo.

Herceptin-like antitumor Abs are elicited by immunization with fusion protein scFv–p3–4

We next tested whether targeting TAA her-2/neu Ag to B cells could elicit antitumor Abs. As depicted in Fig. 4A, mice immunized with scFv–p3–4 secreted large amounts of her-2/neu Abs. P3–4 or scFv protein immunization did not elicit any appreciable level of her-2/neu Ab. The Ab specificity was further confirmed with her-2/neu-expressing human ovarian cell line SKOV-3 (Fig. 4B). Next we determined whether immune sera are capable of activating complement, one of the mechanisms for antitumor Ab-mediated tumor killing. Immune sera from mice immunized with scFv–p3–4 showed potent complement activation (Fig. 4C). Because P3–4 contains the Herceptin-binding domain, we examined whether Abs from mice immunized with scFv–p3–4 have Herceptin-like activity. Competitive inhibition assay was performed in solid-phase immunoassay with recombinant her-2/neu protein (P3–4) as the target Ag. The results revealed that the

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**FIGURE 2.** Ag bound to B cells via CD19 is colocalized with the BCR, and coengagement of the BCR and CD19 activates B cells. (A) Purified B cells were incubated with scFv–p3–4 fusion protein for 30 min and stained with biotinylated anti-her2/neu followed by Alexa Fluor 594–streptavidin (red) and Alexa Fluor 488–anti-IgM (green). Cells were cytopun to slides, and images were acquired. (B) B cells were stimulated with fusion proteins, as indicated, for 24 h. Surface markers were assessed by flow cytometry. Data shown are one from three independent experiments. (C) Supernatants from B cells stimulated with fusion proteins scFv, p3–4, scFv–p3–4, or medium alone for 48 h were harvested and assayed for cytokines IL-6, IL-12p40, and TNF-α by ELISA. Data are representative of three experiments. Error bars represent SEM.
postimmune sera from mice immunized with scFv–p3–4, but not scFv or P3–4, were capable of competing with Herceptin binding (Fig. 4D). Approximately 50% inhibition was achieved when the immune sera were diluted at 1:20 (Fig. 4D).

The biological properties of Herceptin were first described for their ability to inhibit her-2/neu–positive human breast cancer cell growth in vitro (32). Inhibition of tumor cell growth in vitro is also an early and important indication of efficacy in vivo. As shown in Fig. 4E, sera from mice vaccinated with scFv–p3–4 significantly inhibited growth of human breast cancer cell SKBR-3. In contrast, sera from mice immunized with scFv or P3–4 showed minimal inhibition of tumor cell growth. Taken together, these results suggest that targeting her-2/neu p3–4 to B cells via CD19 scFv could be established by this vaccination approach. For the tumor prophylactic experiment, BALB/c mice were immunized i.v. with scFv, P3–4, or scFv–p3–4 on days 0, 7, 14, and 21. Mice immunized with different regimens were then challenged on day 28 by s.c. injection in the flank with 1 \times 10^5 syngeneic D2F2/E2 tumor cells. As shown in Fig. 5A, mice immunized with scFv–p3–4 had a significantly delayed tumor progression compared with mice immunized with scFv, P3–4, or scFv–p3–4 on days 0, 7, 14, and 21. Mice immunized with different regimens were then challenged on day 28 by s.c. injection in the flank with 1 \times 10^5 syngeneic D2F2/E2 murine breast cancer cells that express human her-2/neu. As shown in Fig. 5C, postimmune sera from mice immunized with scFv–p3–4 secreted significantly more IL-4, compared with those from other fusion protein–immunized mice. In addition, fusion protein scFv–p3–4, but not scFv or P3–4, prompted enhanced T cell proliferation, as measured by [3H]-thymidine incorporation (Fig. 5C). To determine the cytolytic activity against her-2/neu–positive target cells, an in vivo cytotoxicity assay was performed. As shown in Fig. 5D, mice immunized with scFv–p3–4 exhibited the highest cytolytic activity (mean = 60%) versus < 20% cytotoxicity in mice immunized with scFv or P3–4 alone (p < 0.001).

**Vaccination with fusion protein scFv–p3–4 induces significant antitumor effects**

Because fusion protein scFv–p3–4 stimulated the her-2/neu Ab response as well as enhanced CD8 T cell responses in immunized mice, our next step was to determine whether antitumor immunity could be established by this vaccination approach. For the tumor prophylactic experiment, BALB/c mice were immunized i.v. with scFv, P3–4, or scFv–p3–4 on days 0, 7, 14, and 21. Mice immunized with different regimens were then challenged on day 28 by s.c. injection in the flank with 1 \times 10^5 syngeneic D2F2/E2 murine breast cancer cells that express human her-2/neu. As shown in Fig. 5C, postimmune sera from mice immunized with scFv–p3–4 secreted significantly more IL-4, compared with those from other fusion protein–immunized mice. In addition, fusion protein scFv–p3–4, but not scFv or P3–4, prompted enhanced T cell proliferation, as measured by [3H]-thymidine incorporation (Fig. 5C). To determine the cytolytic activity against her-2/neu–positive target cells, an in vivo cytotoxicity assay was performed. As shown in Fig. 5D, mice immunized with scFv–p3–4 exhibited the highest cytolytic activity (mean = 60%) versus < 20% cytotoxicity in mice immunized with scFv or P3–4 alone (p < 0.001).

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with fusion proteins scFv–p3–4, scFv, or P3–4, or with PBS, at 1-wk intervals. As shown in Fig. 6C, the tumor-bearing mice treated with scFv–p3–4 had a significantly lower tumor burden than did mice treated with scFv or p3–4, or PBS control mice. In addition, these mice achieved ~25% long-term, tumor-free survival at day 100 (Fig. 6D). To gain insight into the cellular mechanisms of this vaccine, CD4+ and/or CD8+ T cells were depleted before mice were vaccinated. Her-2/neu Ab was not formed when CD4+ T cells were depleted (data not shown). As shown in Fig. 6E, mice depleted of both CD4+ and CD8+ cells completely lost antitumor protection induced by scFv–p3–4. Mice depleted of CD4+ or CD8+ cells showed increased tumor burden, but not significantly different from that in isotype mAb-treated mice. These data suggest that both CD4+ and CD8+ are necessary for the antitumor immunity elicited by scFv–p3–4. To further confirm this therapeutic effect, C57BL/6 mice implanted with human her-2/neu–expressing murine mammary carcinoma EO771 were treated with different regimens. Similar to the D2F2/E2 BALB/C model, the tumor-bearing mice...
treated with scFv–p3–4 had a significantly lower tumor burden than did scFv- or p3–4–treated mice or PBS control mice (Fig. 6G). In addition, these mice achieved ∼20% long-term, tumor-free survival at day 100 (Fig. 6H).

Targeting different ECDs of her-2/neu to B cells elicits potent Ab responses

Because the her-2/neu ECD contains four different domains, we next investigated whether targeting different her-2/neu ECD domains to B cells could generate Abs specific to particular domains. Four different her-2/neu ECD domains were fused with CD19 scFv to generate fusion proteins. All fusion proteins blotted positively with His-Tag Ab, but only scFv-D4 domain fusion protein blotted positively with Herceptin Ab (Fig. 7A). This finding is consistent with a previous report that the Herceptin-binding domain is located in the her-2/neu ECD D4 domain (33). All fusion proteins bound to B cells with high affinity, except scFv-D3, which showed rather lower binding affinity to B cells as compared with other fusion proteins (Fig. 7B). Nevertheless, mice immunized with the four fusion proteins generated varying levels of IgG Ab levels with different isotypes (Fig. 7C). Of interest, in vitro human breast cancer growth inhibition assays indicated that sera from scFv-D3 and scFv-D4 immune mice were as effective as Herceptin in causing growth inhibition (Fig. 7D). Despite a high titer of IgG Ab levels in mice immunized with scFv-D1, the serum did not show any inhibitory effect directly on human breast cancer cells (Fig. 7D). Furthermore, SKBR-3 human breast cancer cells constitutively express high levels of phospho-Akt. Herceptin treatment significantly inhibited p-Akt levels (Fig. 7E). Immune sera from scFv-D4–immunized mice showed similar inhibitory effects. Sera from scFv-D2 and scFv-D3 also showed a significant inhibitory effect on p-Akt levels. However, sera from scFv-D1–immunized mice did not show any effect on p-Akt level (Fig. 7E). These data suggest that targeting different her-2/neu ECDs to B cells via CD19 is capable of generating Abs. However, these Abs could have differential biological effects.

Discussion

The goal of this study was to generate a sustained antitumor Ab response as well as potent T cell responses. Although targeting Ags to DCs via lectins such as DEC205 and Clec9A has been shown to induce potent T cell responses (34–36), desirable B cell responses...
to any given Ag require direct contact between naive B cells and intact Ag (37). Therefore, we targeted Ags directly to B cells via a CD19 miniAb. We showed that this approach generates not only augmented humoral response but also potent T cell responses. The efficacy of this B cell–based vaccine was demonstrated in murine breast cancer models. It appears that CD4+ and CD8+ T cells are both required for the vaccine to be effective. In addition, this strategy can be used to generate Ab responses against any Ags of interest.

The uniqueness of the B cell–based vaccine approach is that Ags targeted to B cells elicit exaggerated Ag-specific Ab responses. DCs are conventionally considered as more potent APCs to induce both CD4+ and CD8+ T cell responses (38). They also indirectly promote B cell humoral responses. However, when Ags enter into DCs for the induction of Ab responses, Ags are processed and dominant epitopes are presented on the surface in the context of MHC class I or class II molecules. Generation of blocking or neutralizing Abs requires the presentation of intact Ag to B cells.

FIGURE 6. Reduced tumor burden and enhanced tumor-free survival upon scFv–p3–4 vaccination. (A and B) BALB/c mice were vaccinated with scFv, P3–4, or scFv–p3–4 four times at 1-wk intervals. PBS-immunized mice were used as control. At day 28, immunized mice were challenged s.c. with 1 × 10^5 D2F2/E2 tumor cells. Tumor growth (A) and survival (B) were recorded. (C and D) BALB/c mice were challenged s.c. with 1 × 10^5 D2F2/E2 tumor cells. The mice were treated with scFv, P3–4, or scFv–p3–4 four times at 1-wk intervals when the tumor size (diameter) reached 2–3 mm. Tumor growth (C) and survival (D) were recorded. (E and F) BALB/c mice were first injected i.p. with anti-CD4 mAb (250 μg), anti-CD8 (500 μg), or isotype control mAb (250 μg) on day −3. Mice were then immunized with different fusion proteins. The depletion mAbs or isotype control mAb was further injected 3 d before boost immunization. After four immunizations, mice were challenged s.c. with 1 × 10^5 D2F2/E2 tumor cells. Tumor growth (E) and survival (F) were recorded. (G and H) C57BL/6 mice were challenged s.c. with 5 × 10^5 EO771/E2 tumor cells. Tumor-bearing mice were treated with scFv, P3–4, or scFv–p3–4 four times at 1-wk intervals when the tumor size (diameter) reached 2–3 mm. Tumor growth (G) and survival (H) were recorded.
We previously used intact CD19 mAb to target Ags specifically to B cells (16, 17). However, intact mAb conjugates could potentially induce severe inflammatory responses. Indeed, a comparison study showed that whole CD19 mAb conjugates induced much more proinflammatory cytokine (Supplemental Fig. 1). We therefore constructed a CD19 scFv miniAb and showed that these fusion proteins have high binding affinity to B cells, although $K_d$ values were lower than that with intact CD19 mAb. The coengagement of CD19 and the BCR by fusion protein CD19 scFv–p3–4 activates B cells to upregulate the surface molecules CD40, CD80, CD86, and MHC class I and II molecules that are critical for T cell activation and stimulation of low levels of cytokine production, including IL-6, TNF-α, and IL-12. Although Ag-specific B cells are normally scarce, non–Ag-specific B cells bound with fusion proteins via CD19 may serve as Ag-specific B cells for Ag presentation and T cell activation. However, it is unknown whether Ag internalization and further processing are required for B cell Ag presentation. In contrast, engagement of CD19 alone induces only moderate B cell activation, and the BCR alone did not significantly stimulate B cell activation. Thus, it appears that full B cell activation requires coengagement of CD19 and the BCR by the scFv–p3–4 fusion protein. Previous studies have shown that production of IL-6 correlates with B cell vaccine efficacy via direct stimulation of CD8+ T cell proliferation (39). IL-12 has also been shown to promote Th1 differentiation. Indeed, using the surrogate OVA Ag, we demonstrated that fusion protein

Figure 7. Abs elicited by targeting her-2/neu different ECD domains via CD19 scFV have differential antitumor effects in vitro. (A) Purified fusion proteins scFv-D1, scFv-D2, scFv-D3, and scFv-D4 were blotted with His-Tag mAb or her-2/neu Ab. (B) For in vitro B cell binding, splenocytes were incubated with biotinylated scFv-D1, scFv-D2, scFv-D3, and scFv-D4 followed by APC-anti-mouse B20 and FITC-streptavidin. (C) Mice were immunized with scFv-D1, scFv-D2, scFv-D3, and scFv-D4 (50 $\mu$g/mouse) four times at 1-wk intervals. Sera were collected at day 28 and then measured for her-2/neu-specific Abs by ELISA. (D) A total of 1 x 10^6 SKBR-3 cells were placed into the wells of the ACEA 16-well plates for 24 h. Heat-inactivated immune serum (1:20), preimmune serum (1:20), or Herceptin (10 $\mu$g/ml) was added to wells and incubated for the indicated times. The inhibition of tumor cell growth was calculated by measuring the relative decrease in current impedance among wells containing postimmune serum and wells containing preimmune serum. (E) Immunoblotting of phosphorylated Akt (p-Akt) in postimmune serum–treated (1:10 dilution), Herceptin-treated (2 $\mu$g/ml), or medium-treated SKBR-3 cells. β-Actin served as loading control. Densitometric quantification is also shown ($n=4$). *$p<0.05$, **$p<0.01$, ***$p<0.001$. 

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CD19 scFv-OVA elicited augmented CD4+ and CD8+ T cell proliferation as well as effector differentiation, as revealed by more IFN-γ production.

This B cell–based vaccine strategy was extended to use TAA her-2/neu. Anti–human her-2/neu Ab Herceptin has been widely used for care of patients with metastatic breast cancer (40). As noted earlier, it costs as much as $70,000 (U.S.) per patient per year (24). In addition, a subset of breast cancer patients is refractory to Ab therapy despite high levels of her-2/neu expression on tumor cells. Furthermore, many patients who initially respond to Ab therapy ultimately develop resistance, leading to disease progression (41, 42). Previous studies demonstrated that her-2/neu–specific CD8 T cell responses could eradicate drug-refractory tumors (43). Thus, it would be ideal if cancer vaccine against her-2/neu TAA could generate both Ab and T cell responses. The current study demonstrated that targeting her-2/neu TAA to B cells via CD19 scFv miniAb elicited potent Ab responses. These Abs competitively inhibited Herceptin-binding ability. More importantly, these her-2/neu Abs are capable of activating complement and inhibiting her-2/neu human breast cancer cell line SKBR-3 growth. In addition, her-2/neu–specific CD8 T cells were significantly enhanced in mice vaccinated with an scFv–p3–4 fusion protein. Although IFN-γ–producing CD4 T cells were not significantly different among all groups, IL-4–producing CD4 T cells were significantly increased in mice immunized with scFv–p3–4, which is consistent with a potent Ab response elicited in these mice. This finding is in contrast to a recent report in which human her-2/neu protein was targeted to DCs via DEC-205 (44). In this study, DEC–her-2 vaccination with polyI:C as adjuvant was included in the current studies. Because B cells T cells completely abrogated vaccine efficacy, suggesting that although coengagement of CD19 and BCR by targeting different her-2/neu ECD domains to CD19 scFv-OVA elicited augmented CD4+ and CD8+ T cell responses and breaks immune tolerance.

The therapeutic efficacy of B cell–based vaccine was demonstrated using two murine breast cancer models. Human her-2/neu protein was targeted to DCs via DEC-205 (44). Immunodominance is the concept that a property of an antigenic determinant causes it to be responsible for the major immune response in a host. Immunodominance can occur in T and B cells (46). Ab immunodominance is reflected in the fact that the IgG response normally is specific for a single epitope (46). However, combating pathogens or cancer may require Ab and T cell responses against multiple epitopes to circumvent immune selection and escape. Targeting different her-2/neu ECD domains to B cells via CD19 generated Abs against each domain to varying levels. This approach may also offer a new way to generate Abs. Of interest, these Abs have different antitumor properties. Thus, targeting selected, multiple epitopes to B cells may generate broader Ab and T cell responses that can clear pathogens or control tumor progression and recurrence. In summary, targeting Ags to B cells via CD19 miniAb generates both T and B cell responses. This vaccination approach provides a cost-effective way to generate a sustained Herceptin-like Ab response as well as antitumor T cell responses.

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Disclosures
The authors have no financial conflicts of interests.

References


Corrections


A source of funding was omitted in this article. The corrected funding information footnote should read “This work was supported by research funding from National Institutes of Health Grant R01 CA86412, American Cancer Society Grant RSG-09-190-01, and the Kentucky Lung Cancer Research Board (to J.Y.). H.-g.Z. is supported by a Research Career Scientist Award.”

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Figure S1. B cell surface marker expression and cytokine secretion stimulated by whole CD19 mAb conjugated with P3-4 or CD19 scFv-p3-4. (A) Purified B cells were stimulated with chemically conjugated whole CD19 mAb with P3-4 (CD19 mAb-p3-4) or CD19 scFv-p3-4 (scFv-p3-4) for 24 h and surface marker expression levels were assessed by flow cytometry. (B) IL-6 and TNF-α levels from 24 h B cell cultures under different conditions determined by ELISA.
Table S1. Primer sequences

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