Development of a new high-affinity human antibody with antitumor activity against solid and blood malignancies

Mouldy Sioud,*1 Phuong Westby,* Vlada Vasovic,† Yngvar Fløisand,‡ and Qian Peng†
*Department of Cancer Immunology, †Department of Pathology, and ‡Department of Hematology, Rikshospitalet-Radiumhospitalet, University Hospital, Oslo, Norway

ABSTRACT: mAbs have emerged as a promising strategy for the treatment of cancer. However, in several malignancies, no effective antitumor mAbs are yet available. Identifying therapeutic mAbs that recognize common tumor antigens could render the treatment widely applicable. Here, a human single-chain variable fragment (scFv) antibody library was sequentially affinity selected against a panel of human cancer cell lines and an antibody fragment (named MS5) that bound to solid and blood cancer cells was identified. The MS5 scFv was fused to the human IgG1 Fc domain to generate an antibody (MS5-Fc fusion) that induced antibody-dependent cellular cytotoxicity and phagocytosis of cancer cells by macrophages. In addition, the MS5-Fc antibody bound to primary leukemia cells and induced antibody-dependent cellular cytotoxicity. In the majority of analyzed cancer cells, the MS5-Fc antibody induced cell surface redistribution of the receptor complexes, but not internalization, thus maximizing the accessibility of the IgG1 Fc domain to immune effector cells. In vitro stability studies showed that the MS5-Fc antibody was stable after 6 d of incubation in human serum, retaining ~60% of its initial intact form. After intravenous injections, the antibody localized into tumor tissues and inhibited the growth of 3 different human tumor xenografts (breast, lymphoma, and leukemia). These antitumor effects were associated with tumor infiltration by macrophages and NK cells. In the Ramos B-cell lymphoma xenograft model, the MS5-Fc antibody exhibited a comparable antitumor effect as rituximab, a chimeric anti-CD20 IgG1 mAb. These results indicate that human antibodies with pan-cancer abilities can be generated from phage display libraries, and that the engineered MS5-Fc antibody could be an attractive agent for further clinical investigation.—Sioud, M., Westby, P., Vasovic, V., Fløisand, Y., Peng, Q. Development of a new high-affinity human antibody with antitumor activity against solid and blood malignancies. FASEB J. 32, 5063–5077 (2018). www.fasebj.org

KEY WORDS: immunotherapy · targeted therapy · phage display · immune effector cells

In addition to surgery, chemotherapy, and radiotherapy, immunotherapy represents a therapeutic avenue that has recently shown great promise in cancer (1). mAbs represent an example of successful targeted immunotherapy and are one of the fastest growing areas in oncology (2). Most commonly used mAbs in cancer immunotherapy are of the IgG class due to their long half-life and stability in serum. These therapeutic mAbs kill cancer cells via several mechanisms, including antibody-dependent cellular cytophagy (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP), and complement-dependent cytotoxicity. The best examples of antibody therapy come from the treatment of non-Hodgkin lymphoma (NHL) and HER2-positive breast cancer by rituximab (anti-CD20) or trastuzumab (anti-HER2), respectively (2, 3). However, the emergence of unresponsive tumors due to low or absent targeted cell surface receptors highlights the need to develop additional targeted options (3, 4). Moreover, in many cancers, no mAbs that target and kill tumor cells are yet available.

In addition to mAbs that specifically target cancer cells, a new type of mAbs called checkpoint inhibitors [e.g., ipilimumab (anti–cytotoxic T-lymphocyte–associated antigen 4) and nivolumab (anti–programmed cell death-1)] showed remarkable efficacy in skin and lung cancers by “releasing the brakes” on T cells (5). Unfortunately, these mAbs work best in tumors with high DNA mutations (neoantigens), such as melanoma, but are less successful in other tumors, such as prostate, breast, and blood cancers (6–9). Even in melanoma, the therapeutic benefit of ipilimumab, for example, has been limited to a fraction of

In vivo stability studies showed that the MS5-Fc antibody was stable after 6 d of incubation in human serum, retaining ~60% of its initial intact form. After intravenous injections, the antibody localized into tumor tissues and inhibited the growth of 3 different human tumor xenografts (breast, lymphoma, and leukemia). These antitumor effects were associated with tumor infiltration by macrophages and NK cells. In the Ramos B-cell lymphoma xenograft model, the MS5-Fc antibody exhibited a comparable antitumor effect as rituximab, a chimeric anti-CD20 IgG1 mAb. These results indicate that human antibodies with pan-cancer abilities can be generated from phage display libraries, and that the engineered MS5-Fc antibody could be an attractive agent for further clinical investigation.—Sioud, M., Westby, P., Vasovic, V., Fløisand, Y., Peng, Q. Development of a new high-affinity human antibody with antitumor activity against solid and blood malignancies. FASEB J. 32, 5063–5077 (2018). www.fasebj.org

KEY WORDS: immunotherapy · targeted therapy · phage display · immune effector cells

In addition to surgery, chemotherapy, and radiotherapy, immunotherapy represents a therapeutic avenue that has recently shown great promise in cancer (1). mAbs represent an example of successful targeted immunotherapy and are one of the fastest growing areas in oncology (2). Most commonly used mAbs in cancer immunotherapy are of the IgG class due to their long half-life and stability in serum. These therapeutic mAbs kill cancer cells via several mechanisms, including antibody-dependent cellular cytophagy (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP), and complement-dependent cytotoxicity. The best examples of antibody therapy come from the treatment of non-Hodgkin lymphoma (NHL) and HER2-positive breast cancer by rituximab (anti-CD20) or trastuzumab (anti-HER2), respectively (2, 3). However, the emergence of unresponsive tumors due to low or absent targeted cell surface receptors highlights the need to develop additional targeted options (3, 4). Moreover, in many cancers, no mAbs that target and kill tumor cells are yet available.

In addition to mAbs that specifically target cancer cells, a new type of mAbs called checkpoint inhibitors [e.g., ipilimumab (anti–cytotoxic T-lymphocyte–associated antigen 4) and nivolumab (anti–programmed cell death-1)] showed remarkable efficacy in skin and lung cancers by “releasing the brakes” on T cells (5). Unfortunately, these mAbs work best in tumors with high DNA mutations (neoantigens), such as melanoma, but are less successful in other tumors, such as prostate, breast, and blood cancers (6–9). Even in melanoma, the therapeutic benefit of ipilimumab, for example, has been limited to a fraction of
patients (5). Recent data suggest the involvement of pre-existing microbial T cells in antitumor immunity of checkpoint inhibitors (10). Whatever the origin of T cells, however, most tumors escape traditional T-cell killing through alteration of their antigen-processing machinery and/or down-regulation of major histocompatibility complex class I expression, rendering the neoantigens undetectable by patient T cells (11). Thus, novel treatment options are still needed that recruit and activate more immune effector cells (e.g., NK cells, macrophages, neutrophils) to kill tumor cells independent of their mutational load and major histocompatibility complex expression status. In addition, if antibodies could be engineered to recognize a common target for most cancer patients, the same antibody can be used for these patients, making the treatment widely applicable and hopefully cheaper.

Phage display, which involves the cloning of the variable gene segments from heavy and light chains of IgGs in *Escherichia coli* and their display on filamentous bacteriophages, has been used for the selection and engineering of therapeutic mAbs with distinct functional properties (12). The goal of the present study was to investigate the possibility of selecting universal antitumor single-chain variable fragment (scFv) antibodies from a human scFv antibody library. By removing phage antibodies binding to common cell surface receptors expressed by normal cells and alternating cancer cells during the biopanning experiments, we have isolated cancer cell-binding scFv antibody fragments. Among the selected candidates, one variant (named MS5) recognized several types of cancer cells but did not, or weakly, recognize normal cells such as peripheral blood mononuclear cells (PBMCs). The MS5 scFv was fused to the human IgG1 Fc domain (hinge, CH2-CH3) resulting in the generation of an MS5-Fc antibody that efficiently induced ADCC and inhibited tumor growth in 3 different tumor xenografts, supporting its potential clinical use in cancer immunotherapy.

**MATERIALS AND METHODS**

**Cancer cell lines and blood cells**

Cancer cell lines used in this study (Table 1) were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured under recommended conditions. Human PBMCs were isolated from buffy coats by Lymphoprep density gradient centrifugation. Monocytes were enriched from PBMCs by using plastic adherence, and purification was verified by phenotypic analysis of the surface marker CD14+. To generate M2 macrophages, monocytes were cultured for 6 d in RPMI-1640 medium supplemented with 20 ng/ml of M-CSF. For full M2 polarization, IL-4 (20 ng/ml) was added during the last 48 h of culture (13). NK cells were purified from PBMCs by using an NK cell isolation kit and auto MAC Pro Separator according to the manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Purification was verified by phenotypic analysis of the surface marker CD56. T cells and B cells were purified from PBMCs by using either the Dynal CD4 or CD19 Positive Isolation Kit, respectively, as described in the manufacturer’s instructions (Invitrogen Dynal As, Oslo, Norway). PBMCs from patients with leukemia were isolated as noted earlier. Cells at 30°C were cultured in RPMI-1640 or DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics. Cell stimulation with the MS5-Fc antibody or Fc control was performed in X-VIVO 15 medium (Lonza, Basel, Switzerland). CD3+ cells were isolated from bone marrow (BM) mononuclear cell preparations as previously described (14). Approval for obtaining blood samples from patients with leukemia was granted by the Regional Committees for Medical and Health Research Ethics (REK 2017/1596).

**Antibodies and recombinant proteins**

Anti-CD56 and Anti-CD107a antibodies were purchased from BD Pharmingen (San Diego, CA, USA). FITC-conjugated anti-human Fc was purchased from MilliporeSigma (St. Louis, MO, USA). PE-conjugated anti-CD163, Pacific blue-conjugated anti-CD3, allophycocyanin-conjugated anti-CD19, PE-conjugated mouse anti-F88, and anti-NKp46 were purchased from BioLegend (San Diego, CA, USA). Anti-CD14 mAb, polyclonal rabbit anti-mouse Ig-FITC conjugated, and anti-human IgG-HRP conjugates were purchased from Dako (Glostrup, Denmark). PE-conjugated streptavidin was purchased from BD Biosciences. 5(6)-Carboxyfluorescein N-hydroxysuccinimide ester and 5(6)-carboxytetramethylrhodamine were purchased from MilliporeSigma (Oso, Norway). Recombinant heat shock proteins (HSPs) were purchased from ProSpec (Rehovot, Israel) and MilliporeSigma.

**Selection of scFv antibodies**

The synthetic human scFv library (Griffin-1 library) used in this study was a generous gift from Dr. Greg Winter (University of Cambridge, Cambridge, United Kingdom). The library was constructed by recloning synthetic heavy- and light-chain variable regions from the lox library vector (15) into the phagemid vector pHEN2. Before affinity selection, the library (1010 phage particles) was preabsorbed on normal human mammary epithelial cells (HMECs) (2×10⁶) and blood leukocytes (2×10⁵ cells) to remove most phages that bind to common receptors expressed by normal cells. The preabsorbed library was then incubated with the breast cancer cell line MDA-MB-453 (1×10⁵ cells) in PBS supplemented with 5% FCS for 1 h at room temperature with gentle mixing. Nonbound phages were removed by washing 10 times with 5 ml PBS (pH 7.5) containing 1% FCS and twice with 5 ml PBS (pH 6.5). Cell-bound phages were eluted by adding 500 µl 0.1 M Tris glycine pH 2.2 and then neutralized with 40 µl of 2 M Tris-base and then titrated. To amplify the recovered phages, 40 µl of exponentially growing *E. coli* TG1 cells were infected with 250 µl of the eluted phages, plated on 2xTY plates containing 100 µg/ml ampicillin and 1% glucose, and incubated overnight at 30°C. Bacterial cells were scraped from the plates, and phages were amplified, PEG purified, and titrated. For the second round of affinity selection, MDA-MB-453 amplified phages (1×10¹⁰ phages)

| Table 1. Enrichment of phage binders during the affinity selection experiments |
|-------------------------------|-----------------|-----------------|-----------------|
| **Cell line**                  | **Input phages** | **Output/eluted** | **Enrichment over** |
| MDA-MB453                     | 1×10¹⁰           | 2.1×10⁴          | 0               |
| PC3                           | 1×10¹⁰           | 8.5×10⁴          | 40              |
| SW900                         | 1×10¹⁰           | 1.7×10⁴          | 2               |
| U87MG                         | 1×10¹⁰           | 3.5×10⁵          | 2               |
| Ramos                         | 1×10¹⁰           | 4.5×10⁵          | 13              |

*Enrichment over the MDA-MB-453 eluted phages, which is considered as 0.*
were preabsorbed on normal cells and then affinity selected on prostate cancer cell line PC3 (1 × 10^7 cells) as described earlier. PC3-bound phages were eluted, titrated, amplified, and then 1 × 10^10 phages were affinity selected on lung cancer cell line SW900 (1 × 10^6 cells). Similarly, SW900-bound phages were eluted, titrated, propagated, and then 1 × 10^10 phages were affinity selected on glioma cell line U87MG (1 × 10^6 cells). Finally, 1 × 10^10 glioma-bound phages were affinity selected on Ramos lymphoma cell line (1 × 10^6 cells), and Ramos-bound phages were amplified and tested for binding to the 5 cell lines used in this selection protocol (Table 1). Moreover, single phage clones were picked randomly, and their binding to cancer cell lines was analyzed by using flow cytometry. Phages that bound to the 5 cell lines were selected, and their DNA sequences were determined by using the pHEN primer 5'-CTATGCGGCCCATTCGA-3' [German Neuroscience Society (NWG)]. To test for phage enrichment during the affinity selection protocol, 30 random phage clones from each output/eluted phage population were characterized by DNA sequencing. Plasmid or phagemid DNA was extracted by using the Wizard Plus SV Miniprep DNA Purification System (Promega, Madison, WI, USA) according to the manufacturer’s instructions.

**Phage amplification**

In brief, 40 ml of exponentially growing E. coli strain TG1 cells were infected with the eluted phages and grown in 2XY containing 100 μg/ml ampicillin and incubated for ~2 h at 37°C with shaking. The cells were infected with helper phage M13KO7 in a ratio of at least 20:1 (phage:bacteria) for 30 min at 37°C. The infected bacteria were centrifuged and resuspended in 100 ml 2XY containing 100 μg/ml ampicillin and 25 μg/ml kanamycin and incubated in a shaker at 30°C overnight. After the culture was centrifuged at 9000 g for 10 min, the phage particles were precipitated by adding 1/5 volume of PEG 6000/NaCl and then incubated for 2 h before centrifugation at 11,000 g for 30 min. Phage pellets were resuspended in PBS buffer and then titrated as indicated in the following section. Phages were also prepared from individual ampicillin-resistant colonies, PEG precipitated, and titrated; their binding to cancer cells was investigated by using flow cytometry.

**Phage titration**

Serial dilutions of eluted or amplified phages (10 μl/sample) were added to exponentially growing TG1 cells along with 3 ml top agar and then plated on agar plates. After incubation at room temperature for 1 h, plates were incubated overnight at 37°C, and the number of plaques was determined for each dilution.

**Cloning and expression of MS5-Fc fusion protein**

A synthetic gene coding for the scFv MS5 (VH-linker-VL-His tag) with appended EcoR1 and BglII restriction sites at the 5' and 3' ends, respectively, was made by GenScript. This DNA fragment was digested with EcoR1 and BglII and ligated into EcoR1-BglII-cleaved pFuse-hlgG1-e5-Fc2, pFuse-hlgG1-Fc2, or pFuse-mlgG2a-Fc2 vector in frame with IL-2 signal sequence and the Fc domain (hinge, CH2-CH3) of human IgG1 or mouse IgG2a (InvivoGen, San Diego, CA, USA). Positive clones were selected and verified by DNA sequencing. Fusion proteins and Fc controls were produced by transient transfection of the plasmids into HEK293T cells using the Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA). The cells were cultured in DMEM medium supplemented with 5% FCS, ultra-low IgG (Thermo Fisher Scientific), and antibiotics. On d 3, culture supernatants were collected, and Fc-fusion proteins or control Fc were affinity purified on a protein G column. Positive fractions and protein purity were determined by using 10% SDS-PAGE analysis with Imperial Protein Stain (Thermo Fisher Scientific) to visualize proteins. Positive fractions were collected, pH adjusted to 7.5, and then stored at −80°C until use. For conjugation to 5(6)-carboxyfluorescein N-hydroxysuccinimide or 5(6)-carboxytetramethylrhodamine ester, antibody purification and elution were performed in phosphate buffer, and pH was adjusted to 8.0. The final antibody concentrations were evaluated from OD_{520 nm} (NanoDrop, Saveen & Werner AB, Limhamn, Sweden) or by the Bradford protein assay.

**Size-exclusion chromatography**

The MS5-Fc antibody and Fc control proteins were analyzed by size exclusion chromatography using an ÄKTA Purifier (GE Healthcare, Waukesha, WI, USA) connected to a Superdex 200 Increase 10/3000 GL column (GE Healthcare). Proteins were injected at a flow rate of 0.5 ml/min. The used protein standard was conalbumin (76 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa), and aprotinin (6.5 kDa).

**Serum stability**

The MS5-Fc antibody (50 μg in PBS) was incubated in 40% human serum at 37°C in a humidified tissue culture incubator. The final volume was 350 μl. Aliquots (30 μl each) were collected at various days and immediately stored at −20°C until analysis by Western blots using an anti-His tag mAb. Densitometric quantification of the reacting full-length protein was calculated by using the Image Lab TM 4.1 Imaging System (Bio-Rad, Hercules, CA, USA).

**Flow cytometry analysis**

Binding of polyclonal and monoclonal scFv phage antibodies to human cells was determined by using flow cytometry. In brief, cells were gently detached from the culture dish by scraping, washed twice with PBS, and then aliquots of 10^5 cells were plated in a conical 96-well microplate in 100 μl staining buffer (PBS buffer + 2% FCS) and then incubated with phage antibodies (10^5 TU) for 30 min on ice. Cells were washed twice with 400 μl staining buffer and then incubated with biotin-conjugated anti-M13 mAb for 30 min at 4°C. After washing, cells were incubated with phycoerythrin (PE)-conjugated streptavidin, washed, and analyzed by using flow cytometry (Canto II; BD Biosciences, San Jose, CA, USA). Similarly, binding of MS5-Fc antibody and Fc control to cancer cells or normal cells was determined by using flow cytometry. Single-cell suspensions (10^5 cells) were incubated with the test molecules (5–10 μg/ml) for 30 min at 4°C in PBS buffer containing 2% human serum (staining buffer). After washing with staining buffer, cells were incubated with FITC-conjugated anti-human Fc IgG for 30 min on ice. Washing was repeated, and cells were resuspended in 300 μl staining buffer before being analyzed with the use of flow cytometry. In some experiments, cells were stained with biotin-labeled MS5-Fc antibody or Fc control followed by PE-conjugated streptavidin. All flow data were analyzed by using FlowJo software (FlowJo, Ashland, OR, USA).

**Affinity measurements**

Affinity measurements by ELISA for cell surface antigens were performed as described by Bator and Reading (16). Briefly, the antibody at various concentrations was mixed with cell suspensions incubated for 2 h on ice with occasional mixing before cells were pelleted by centrifugation. Supernatants were removed...
and retained for the quantification of unbound MS5-Fc antibody molecules using the human IgG ELISA Quantification Kit (Bethyl Laboratories, Montgomery, TX, USA). Bound MS5-Fc antibody concentrations were obtained by subtraction of unbound molecules from the initial antibody concentrations, and these values are then used to construct Scatchard plots.

**Fluorescence microscopy analysis**

Cancer cells were cultured in Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL, USA) for 24 h in RPMI-1640 or DMEM medium supplemented with 10% FCS. The medium was replaced with X-VIVO 15 medium supplemented with 2% human serum, and the cells were incubated with 5(6)-carboxyfluorescein-conjugated MS5-Fc antibody or Fc control for 10 μg/ml for 60 min at 4°C. Subsequently, the cells were incubated with Hoechst 33342 (1 μg/ml) for 5 min, washed, and then fixed with 4% paraformaldehyde for 30 min at 4°C. Slides were covered with Dako Cytomation fluorescent mounting medium before images were taken by using confocal microscopy (LSM 510; Carl Zeiss, Olympus, Tokyo, Japan). In some experiments, stained cells were incubated at 37°C for 3–6 h to allow receptor internalization and then processed as described earlier.

**NK cell degranulation**

To measure surface expression of CD107a/LAMP-1, a surrogate marker for NK cell degranulation, NK cells (5 × 10⁶ in 200 μl X-VIVO medium) were incubated with the cancer cell–coated MS5-Fc antibody or Fc control for 5 h at 37°C. During stimulation, PE-Cy-conjugated anti-CD107a (2 μl/well) and monensin (0.2 μl/well) were added to the cell cultures. After incubation, the cells were harvested, washed, and stained with FITC-conjugated anti-CD56 before being analyzed with the use of flow cytometry. The data were analyzed by using FlowJo software.

**ADCC assay**

ADCC assay was conducted by using the Lactate Dehydrogenase Cytoxicity Detection kit (Promega) in accordance with the manufacturer’s instructions. Briefly, cancer cells (target cells) pretreated with either the MS5-Fc antibody or Fc control (10 μg/ml) were incubated with NK cells (effector cells) at various effector-to-target ratios for 18 h at 37°C. NK cells were prepared from human PBNCs. After incubation, plates were centrifuged at 300 g for 5 min, and 50 μl of supernatant from each sample was transferred to a 96-well plate to determine the amount of LDH released. The percentage of specific cell lysis was calculated by using the formula (W - L)/W × 100. The effector-to-target ratio (25:1) was determined from pilot experiments. In parallel experiments, culture supernatants were collected, and cytokine contents were measured by using ELISAs.

**ADCP assay**

To quantify ADCP, tumor cells were harvested by enzymatic dissociation, labeled with carboxyfluorescein succinimidyl ester (CFSE), washed with serum-free medium, and plated at a density of 5 × 10⁶ cells per well in 100 μl X-VIVO 15 medium in a 96-well plate. After incubation at 37°C for 4 h, tumor cells were opsonized by addition of the MS5-Fc antibody or Fc control for 30 min at room temperature. Human M2 macrophages were harvested by cell scraping and subsequently pelleted, washed, and added to opsonized tumor cells at a density of 5 × 10⁶ cells per well in 50 μl medium. Cells were incubated at 37°C for 10 h, pelleted, washed with X-VIVO 15 medium, and stained with anti-CD163 mAb. Samples were washed and then analyzed by using flow cytometry.

**Analysis of the tumor-associated MS5-Fc antibody by fluorescence microscopy**

Two weeks after tumor cell inoculation, 5(6)-carboxyfluorescein-labeled MS5-Fc antibody or Fc control were injected intravenously into separate animals using 200 μg of the conjugate in 100 μl of physiologic saline. Mice were euthanized 20 h after injection, and tumors and other organs (lungs, kidneys, and heart) were removed and incubated overnight in 4% paraformaldehyde solution and then embedded in optimal cutting temperature medium. Tumors and tissue sections (10 μm) were incubated with Hoechst 33342 for nuclei staining. Thereafter, the slides were covered with Dako Cytomation fluorescent mounting medium before being examined by using an epifluorescence microscope (Leica DM RHC; Leica Microscopy AS, Oslo, Norway). Samples were also examined by confocal microscopy (LSM 510; Carl Zeiss).

**Xenograft animal models**

Female BALB/c nude mice were produced at the animal core facility (Oslo University Hospital) and housed in microisolation cages during the course of the experiments. Mice were inoculated subcutaneously with cancer cells and then randomized into different groups (5–7 mice/group). Test molecules were administrated intravenously on various days after tumor cell inoculation (100 μg/mouse/injection). Animals were assessed daily for clinical symptoms and adverse effects. Tumor size was measured by using a caliper twice a week, and tumor volumes were calculated by using the formula (L × W³)/2, with L and W representing length and width, respectively. All animal studies were approved by the Institutional Animal Care Use Committee at Oslo University Hospital. Randomization was used in all animal experiments.

**Analysis of tumor-infiltrating macrophages and NK cells**

Detection of infiltrating immune cells in tumor tissues was performed according to standard immune-histochemical methods using 10 μm cryostat sections of tumors or organ tissues fixed with 4% paraformaldehyde and embedded in optimal cutting temperature medium. For detection of macrophages and NK cells, PE-conjugated anti-mouse F4/80 and PE-conjugated anti-NKp46 were used, respectively. Before incubation with antibodies, sections were blocked with 5% rat serum in PBS. Stained sections were analyzed with an epifluorescence microscope (Leica DM RHC; Leica Microscopy AS).

**Statistical analysis**

Results are reported as means ± SD. Statistical significance of differences was assessed by using Student’s t test. The antitumor effects of the MS5-Fc antibody vs. the Fc control were assessed by using a 2-tailed test. The level of significance was set at a value of P < 0.05.

**RESULTS**

**Isolation of cancer cell–binding scFv antibody fragments**

To determine the feasibility of selecting antibodies that recognize common cell surface receptors expressed by
cancer cells, a semisynthetic human antibody library was sequentially screened on different types of cancer cells. The library was first preabsorbed on PBMCs and normal HMECs to remove most of the phages that bind to receptors expressed by normal cells. Second, the preabsorbed library was sequentially affinity selected on breast MDA-MB-453, prostate PC3, lung SW900, glioblastoma U87MG, and lymphoma Ramos cancer cell lines (Fig. 1A). By alternating cancer cells during the selection process, the selected phage antibody fragments may bind to common receptors or carbohydrate/lipid structures expressed preferentially or exclusively by cancer cells. The results of phage enrichment during the affinity selection protocol are shown in Table 1. After selection on prostate cancer cell line PC3, the number of eluted phages increased from $2.1 \times 10^3$ to $8.5 \times 10^4$, a 40-fold enrichment. After selection on Ramos cells, the number of eluted phages also considerably increased from $3.5 \times 10^5$ (selection on U87MG cells) to $4.5 \times 10^6$. However, there was a moderate enrichment after selection on SW900 and U87MG cells, suggesting that these cancer cell lines may express fewer binding receptors. The overall enrichment was ~2080-fold.

We next examined the binding of Ramos-selected phages to the used cancer cell lines. The screening protocol resulted in the enrichment for phages binding to all 5 cancer cell lines relative to the unselected original library (Fig. 1B). No significant binding to BM CD34+ hematopoietic stem cells and normal HMECs was detected. Thus, specific scFv antibody fragments were enriched by the phage-display selection protocol used in this study. Analysis of individual random phage clones confirmed the strong binding of the selected phages (Fig. 1C). Most of the selected single phage clones did not bind to blood lymphocytes or BM CD34+ hematopoietic stem cells. Analysis of the DNA inserts of 50 single phage clones that recognized cancer cells revealed the presence of 3 dominant phage clones (Table 2). Importantly, 70% of all phage clones sequenced were found to be clone MS5, indicating that this clone was highly enriched. MS5 bound to most tested cancer cell lines, but weakly or not to peripheral blood lymphocytes and normal HMECs.

To investigate the impact of the selection protocol on the enrichment of MS5 and MS10 phage clones, 150 phage clones were randomly picked from the eluted phages (30 phages/cell type) and sequenced, yielding 125 intact antibody clones. Analysis of the CDR3 region sequences of the heavy chains indicated a clear enrichment of MS5 and MS10 phages (Supplemental Table 1). The CDR3 sequences of the MDA-MB-453-binding phages were different (24 unique cell-binding scFv antibody fragments, including scFv MS5 and MS10). The frequency of MS5 and MS10 phages increased after affinity selection on prostate cancer cell line PC3. There was a modest enrichment of these 2 phage clones after affinity selection on SW900 and U87MG cancer cell lines. However, a high proportion (84%) of the eluted phages after affinity selection on Ramos cells was found to be either MS5 or MS10 phages. Indeed, 13 and 8 of the 25 intact scFv antibodies displayed the heavy chain CDR3 sequence of scFv MS5 or MS10, respectively. Some uncontrollable factors, such as receptor density on the different cell lines, may facilitate the enrichment of these 2 phage clones. In contrast to all sequenced CDR3 regions,
The data are from 1 single experiment and are representative of 8 independent experiments. Blue and orange histograms represent cells stained with the MS5-Fc antibody or Fc control, respectively. Red histograms indicate cells stained with only PE-streptavidin.

Moreover, gel filtration in nonreducing (NR) and reducing (R) conditions, it migrated as a band of 55 kDa monomers, whereas under nonreducing conditions, the fusion protein migrated as 100 kDa, corresponding to MS5-Fc antibody. The origin of the Fc domain does not affect the antibody binding profile.

**Generation and characterization of the MS5-Fc antibody**

Based on the flow data (Fig. 1C), we chose to focus on scFv MS5 for further study. Although soluble scFv antibody fragments have a wide range of applications in research, diagnostics, and therapy, the scFv-Fc format offers several advantages over candidate scFvs, including bivalent binding, longer half-life, and Fc-mediated effector functions (17). We therefore genetically fused the VH and VL gene segments of scFv MS5 to human IgG1 Fc domain (hinge, CH2-CH3) in frame with human IL-2 leader sequence (for secretion). The IgG1 domain contains the triple mutations DEL (S239D/I332E/A330L), known to enhance ADCC and ADCP via higher binding to Fcγ RIIa (18). Because of the hinge region, the scFv-Fc (MS5-Fc) fusion protein is expected to form an S-S linker dimer in solution. Recombinant proteins were produced in HEK293T cells and purified by using protein G-agarose chromatography. As a control, we purified the Fc domain of human IgG1 carrying the DEL mutations. The integrity of the MS5-Fc antibody and Fc control was confirmed by Coomassie Brilliant Blue staining of an SDS-PAGE gel (Fig. 2A).

Under reducing conditions, the fusion protein migrated as ~55 kDa monomers, whereas under nonreducing conditions, it migrated as a band of ~100 kDa, corresponding to the dimeric structure (12, 19). Moreover, gel filtration showed a single peak eluted at the position around the size of the dimer (90–100 kDa), indicating that MS5-Fc antibody does not form aggregates larger than the dimer in solution (Supplemental Fig. 1A–D).

We next assessed the binding of the MS5-Fc antibody to Ramos, MDA-MB-453, SW900, PC3, U87MG, and HL60 cancer cell lines. Consistent with the phage clone, the MS5-Fc antibody bound to the tested cancer cells (Fig. 2B). Its predicted binding affinity to MDA-MB-453, Ramos, and PC3 cells is high (5–10 nM). Table 3 shows the binding potency of the MS5-Fc antibody to a large panel of human cancer cell lines derived from solid and blood cancers. With respect to hematopoietic cells, the MS5-Fc antibody did not bind to hematopoietic stem/progenitor KG1a leukemic cells (20) or to Nalm-6, a pre-B acute lymphocytic leukemia. In line with this observation, the MS5-Fc antibody did not bind to normal BM CD34+ hematopoietic stem/progenitor cells. In contrast to either malignant or normal early progenitor cells, the MS5-Fc antibody bound to lymphoma and leukemia cell lines that are derived from intermediate and late myeloid or lymphoid precursors, respectively. Notably, the MS5-Fc antibody exhibited no binding to mature blood T cells and only a weak binding to B cells. Moreover, it did not bind to the myeloma U266 cell line, a cancer of mature B cells. Collectively, these cell-binding profiles suggest that the receptor for the MS5-Fc antibody is expressed on most malignant leukemia and lymphoma cells but not, or only weakly, on early progenitor cells and mature blood leukocytes.

**The origin of the Fc domain does not affect the antibody binding profile**

We next investigated whether MS5-Fc antibody fragment maintains its specificity when fused to a different Fc domain. Similarly, we genetically fused the scFv MS5 sequence with the sequence encoding the Fc domain of mouse IgG2a to generate MS5-mIgG2a fusion protein. We then produced the recombinant protein in HEK293T cells, followed by purification on protein G chromatography and characterization by Western blotting. Mouse IgG2a

**Table 2. Amino acid sequences of the heavy-chain and light-chain CDR3 regions**

| Clone | Variable heavy CDR3 | Variable light CDR3 | Frequency |
|-------|---------------------|---------------------|-----------|
| MS5   | GGGTLLR             | NSRDSGHNHV          | 30/50     |
| MS10  | TAPY                | QQARRKPKWT          | 18/50     |
| MS18  | SRP                 | LTFFGKT             | 2/50      |

Amino acid residues are indicated using a single-letter code.

the scFv MS5 has a longer synthetic CDR3 sequence, which is similar to natural antibodies.
The CD107a, which is a component of cytotoxic granules, accumulates on the surface of activated NK cells and cytotoxic T cells upon granulation/activation (21, 22). We therefore analyzed its surface display in response to the antibody treatment. MDA-MB-453, Ramos, and PC3 cells were used as target cells. As shown in Fig. 5A (representative examples), MS5-Fc antibody enhanced NK cell activation as revealed by the up-regulation of the CD107a marker. Indeed, it was able to activate NK cells in the presence of MDA-MB-453 cells (32 ± 5 vs. 10 ± 5%; P < 0.02), Ramos cells (44 ± 8 vs. 16 ± 4%; P < 0.02), and PC3 cells (35 ± 8 vs. 13 ± 3%; P < 0.02) compared with the Fc control. The effects were antigen-specific, as KG1a cells (which more likely lack the MS5 scFv receptor) were not affected. In the context of antibody treatment, NK cells are unique in that they express only the low-affinity activating Fc receptor CD64 (FcγRIIA) and no inhibitory receptors, underscoring a significant role in ADCC (23).

The ability of MS5-Fc antibody to induce ADCC was assessed by using the CytoTox Non-radioactive Cytotoxicity Assay (Promega) based on LDH release (24). MDA-MB-453, Ramos, and HL60 cells were used as target cells and freshly isolated human NK cells as effector cells. Fig. 5B presents a significant increase in cytolysis by 5 μg/ml of the MS5-Fc antibody at an effector-to-target cell ratio of 25:1, revealing 25 ± 5% cytolysis of MDA-MB-453, 30 ± 5% of Ramos, and 20 ± 5% of HL60 cells. The ADCC effect was antigen-specific, as KG1a cells were not affected. Similarly, autologous CD4+ T cells were not killed. In accordance with ADCC activity, cancer cell–coated MS5-Fc antibody induced IFN-γ and TNF-α production by NK cells (Fig. 5C).

**MS5-Fc antibody induces NK-cell activation and ADCC**
ADCP in vitro. Target cancer cells were fluorescently labeled with CFSE and then incubated for 30 min with either MS5-Fc antibody or Fc control before adding monocyte-derived M2 macrophages and further incubation at 37°C for 8 h. Thereafter, the cells were stained with APC-conjugated anti-CD163, a marker for M2 macrophages (25), before analysis by flow cytometry. Gated cell populations are indicated. B–F Binding of MS5-mIgG2a to purified blood T cells (B), purified blood B cells (C), early progenitor KG1a cells (D), Ramos lymphoma cells (E), and HL60 leukemia cells (F). Cells were incubated with MS5-mIgG2a fusion protein, followed with FITC-conjugated anti-mouse IgG, and analyzed by using flow cytometry (blue histograms). Control cells were incubated with mouse IgG2a (red histograms).

Figure 3. Binding of the MS5-mIgG2a fusion protein to blood leukocytes and cancer cells. A) Representative flow cytometry histograms showing the binding of the MS5-mIgG2a fusion protein to the PBMC lymphocyte population (R1 gate) or the monocyte population (R2 gate). Specific binding was compared vs. cells incubated with mouse IgG2a (red histograms) as isotype control. PBMCs were also stained with anti-CD3 (T cells), anti-CD19 (B cells), or anti-CD14 (monocytes) and then analyzed by using flow cytometry. Gated cell populations are indicated. B–F Binding of MS5-mIgG2a to purified blood T cells (B), purified blood B cells (C), early progenitor KG1a cells (D), Ramos lymphoma cells (E), and HL60 leukemia cells (F). Cells were incubated with MS5-mIgG2a fusion protein, followed with FITC-conjugated anti-mouse IgG, and analyzed by using flow cytometry (blue histograms). Control cells were incubated with mouse IgG2a (red histograms).

MS5 antibody binds to primary tumor cells and induces ADCC

The MS5-Fc antibody was further characterized by using primary malignant cells of patients with leukemia in a functional assay using allogenic human NK cells. Figure 7A shows the binding of the MS5-Fc antibody to blood cells from 1 patient with chronic myeloid leukemia (CML). In agreement with the data obtained with cancer cell lines, the MS5-Fc antibody bound to primary leukemic cells (R1 gate) but not to the normal lymphocyte population (R2 gate) from the same patient. Similar results were obtained with 4 additional PBMC preparations from 2 patients with CML and 2 patients with acute myeloid leukemia (AML). We next used the samples from patients with CML and AML and allogenic NK cells to analyze the ability of the MS5-Fc antibody to induce ADCC. Leukemia cell lysis was significantly enhanced in the presence of the MS5-Fc antibody compared with that obtained with the Fc control (P < 0.02) (Fig. 7B).

MS5-Fc antibody localizes into tumor tissues

We next investigated whether the MS5-Fc antibody could target tumor cells in vivo. Nude mice bearing subcutaneous MDA-MB-453 or Ramos tumors were injected intravenously with 5(6)-carboxyfluorescein–labeled MS5-Fc antibody. After 20 h, animals were euthanized to assess tumor tissues and normal organs for fluorescence (Supplemental Fig. 2). As opposed to the Fc control, the data show that the MS5-Fc antibody can target tumor cells in vivo. Confocal microscopic analysis of tumor sections showed the binding of the MS5-Fc antibody to tumor cells. Thus, the engineered antibody penetrated into tumor tissues. No significant fluorescence was detected in normal tissues such as the lungs. A nonspecific uptake of antibody-free fluorescence dye into the tumor is unlikely because different MS5-Fc-fluorochrome conjugates produced similar results (data not shown).
MS5-Fc antibody inhibits growth of human tumor xenografts in vivo

We next investigated the capacity of the MS5-Fc antibody to inhibit tumor growth in vivo. First, the antitumor activity was evaluated in a human breast cancer model in which MDA-MB-453 cells (1 × 10^7 cells/mouse) were inoculated subcutaneously into BALB/c nude mice. These mice bear monocytes/macrophages and NK cells capable of mediating ADCC and ADCP (26). The mice were treated intravenously either with MS5-Fc antibody or Fc control (100 μg/injection) on d 3, 7, and 10. Tumor growth was monitored, and tumors were collected and weighed at the end of the experiments (d 20). As shown in Fig. 8A, MS5-Fc antibody treatment inhibited tumor growth. At d 20, tumors in MS5-Fc antibody–treated mice (n = 6) reached a mean volume of 1641 mm^3, whereas the control mice (n = 7) developed tumors with a mean volume of 2754 mm^3 (P < 0.004). Consistent with tumor volume, the treatment reduced the mean tumor weight by 48% compared with the Fc control–treated mice.

An additional series of experiments following the same design were performed by using Ramos lymphoma cells. The cells (1 × 10^7 cells/mouse) were subcutaneously transplanted into nude mice (n = 5/group), and treatment was given intravenously on d 4, 8, 12, and 18. Mice treated with the MS5-Fc antibody displayed a significant reduction in tumor growth rate compared with those treated with the Fc control (Fig. 8B). At d 22,
tumors in MS5-Fc antibody–treated mice had reached a mean volume of 518 mm³, whereas those treated with Fc control reached 1200 mm³ ($P < 0.002$). In line with tumor growth curves, MS5-Fc antibody treatment reduced the mean tumor weight by 65% compared with Fc control–treated mice (d 22).

To extend the therapeutic use of the MS5-Fc antibody, we also evaluated its antitumor effect on leukemia cells. Nude mice ($n = 4$ /group) were subcutaneously challenged with HL60 cells ($1 \times 10^7$ cells/mouse) and then treated with either MS5-Fc antibody or Fc control on d 3, 7, and 11. Here, we decided to terminate the experiment at d 33 when tumors in the control group reached a mean size of 1200 mm³. Figure 8C shows tumor weights at d 33. Fc control–treated mice developed tumors with an average weight of 0.925 g, whereas those treated with MS5-Fc antibody developed smaller tumors with an average weight of 0.263 g ($P < 0.001$). One mouse in the MS5-Fc antibody–treated group did not develop a tumor.

Together, the data confirm the potential versatility of the MS5-Fc antibody in blocking tumor growth.

Given the importance of innate immune cells in antibody antitumor effects, we examined whether MS5-Fc antibody treatment enhances their infiltration into tumor tissues. Figure 8D displays representative tumor sections stained with monoclonal antibodies specific for NK cells (NKp46) or macrophages (F4/80). The MS5-Fc antibody treatment increased the infiltration of both NK cells and macrophages into tumor tissues compared with those treated with the Fc control. A similar increase in the number of infiltrating NKp46+ and F4/80+ macrophages was also seen in MS5-Fc antibody–treated HL60 tumors (data not shown).

**Comparison of the MS5-Fc antibody antitumor effect vs. that of rituximab**

The CD20 mAb rituximab is incorporated into standard care for B-cell NHL (27). In the next experiments, the
The antitumor potency of MS5-Fc antibody was compared with that of rituximab. Ramos lymphoma cells (10^7 cells) were injected subcutaneously into the flank of BALB/c nude mice (n = 5), and antibody treatment (100 µg/injection) was given at d 3, 8, and 15. Tumor growth was monitored until d 22. As shown in Fig. 9A, the average tumor volumes (d 11, 14, 18, and 22) of mice receiving MS5-Fc antibody or rituximab were significantly smaller than those of Fc control–treated mice. At d 11, 14, or 18, there was no difference between either treatment. However, at d 22 (7 d after the last treatment), rituximab performed better than the MS5-Fc antibody (mean tumor volume 213 vs. 571 mm^3). Indeed, at d 22, MS5-Fc antibody treatment reduced the mean tumor weight from 0.70 to 0.20 g (P < 0.02), whereas rituximab treatment reduced the mean tumor weight from 0.70 to 0.06 g (P < 0.01). Notably, the level of CD20 expression on Ramos cells is in general higher than that for the MS5 binding receptor (Fig. 9B). Given the challenges involved in overcoming resistance to rituximab treatment (e.g., loss of CD20 from the cell surface) (4), the engineered MS5-Fc antibody could be an attractive means for treating patients with lymphoma.

**Figure 6.** Induction of ADCP by the MS5-Fc antibody. A) CFSE-labeled MDA-MB-453 breast cancer cell lines were incubated with M2 macrophages for 8 h (effector-to-target cell ratio, 1:1) in the presence of the MS5-Fc antibody or Fc control (5 µg/ml each). Subsequently, the cells were stained with anti-CD163 and then analyzed by using flow cytometry to identify phagocytosed target cells (dual staining). B) Experimental conditions were as in A. The percentages of phagocytosed cancer cells are presented. The results are representative of 4 independent experiments. M2, M2 macrophages. *P < 0.05, **P < 0.02.

**Figure 7.** Binding of MS5-Fc antibody to primary leukemia cells and induction of ADCC. A) Representative flow cytometry histograms showing the binding of the MS5-Fc antibody to freshly isolated mononuclear cells from a patient with CML (blue histograms). R1 gate contains most blood blasts, whereas R2 gate contains normal blood lymphocytes. Cells were incubated with biotin-conjugated MS5-Fc antibody or the Fc control (red histograms), washed, incubated with PE-conjugated streptavidin, and then analyzed by using flow cytometry. B) Induction of ADCC. Allogenic NK cells were incubated with leukemia cells (effector-to-target cell ratio, 40:1) of a CML patient (blast count > 67%) or an AML patient (blast count > 70%) in the presence of the MS5-Fc antibody or Fc control (10 µg/ml each). After 18 h of incubation, culture supernatants were collected and analyzed for LDH. The results are presented as means ± SD from triplicate determination and are representative of 3 independent experiments. Statistically significant differences between MS5-Fc antibody–treated cells and Fc control–treated cells are indicated by asterisks.
MS5-Fc antibody showed an enhanced stability in human serum

The stability in human serum is an important factor affecting the therapeutic efficacy of mAbs. Therefore, we investigated the stability of MS5-Fc antibody in 40% human serum. The antibody was incubated at 37°C for various time points, and the stability was assessed with Western blot experiments using an anti-His tag mAb to detect the full-length protein (Fig. 10A). Densitometric quantification of the signals indicates that ~60% of the antibody remained intact after 6 d of incubation (Fig. 10B), indicating that the engineered MS5-Fc antibody is not highly susceptible to serum proteases.

DISCUSSION

In the present study, we found that scFv antibody libraries provide a rich source of antibody fragments that specifically recognize receptors expressed by diverse tumor cells. One of the selected scFv antibody fragments was turned into a human IgG1 antibody that killed cancer cells in vitro and inhibited tumor growth in 3 different human xenograft models, suggesting that it could have widespread therapeutic use for a variety of cancers.

Consistent with its binding profile, the MS5-Fc antibody induced ADCC and ADCP against various cancer cell lines. The use of KG1a cells and blood T cells, more likely not expressing the MS5-Fc receptor, as targets revealed no significant effect of the MS5-Fc antibody on
NK cell activation and induction of ADCC, supporting the assumption that the antibody effects are receptor dependent. From the flow cytometry analyses, we can conclude that the receptor for MS5 scFv is expressed in lymphoma and leukemia cells but not in CD34+ hematopoietic stem cells, early hematopoietic progenitor cells, or mature peripheral blood leukocytes; a weak binding to B cells was detected, however. This binding profile is interesting because most, if not all, current therapeutic antibodies against lymphoma and leukemia cells also target normal blood leukocytes (2, 3). For instance, rituximab, which is part of the standard treatment regimen for B-cell lymphoma, kills both normal and malignant B cells, resulting in long-term profound deletion in circulating B cells. Thus, targeting proteins expressed in normal blood cells requires that the patient can do without the normal cells from which the cancer cell originates.

With respect to therapy, antibody internalization is an important issue to consider during antibody development projects. Tumor-specific antibodies that internalize efficiently provide a means for delivering cytotoxic drugs into target cells (28). However, for cancer immunotherapy relying on ADCC, ADCP, and/or complement-mediated cytotoxicity, noninternalizing antibodies are preferred. This approach would maximize the accessibility of the Fc domain to immune effector cells such as NK cells, macrophages, and neutrophils. Notably, antigen down-regulation and rapid internalization have been some of the main reasons for the limited therapeutic efficiency of several mAbs, including gemtuzumab (anti-CD33) and rituximab (4, 29–32). Under our experimental conditions, the confocal microscopic analysis showed that the MS5-Fc antibody is not internalized by the majority of examined cancer cell lines. Thus, it should be suitable for the activation of innate immune cells against tumor cells. Previous studies have shown that antibody internalization often depends on the target antigen and the epitope recognized by the antibody (31). Our data suggest that internalization may also depend on the cell types. Indeed, the prostate cancer cell line PC3 internalized the MS5-Fc-receptor complexes, but the breast cancer cell line MDA-MB-453 did not (Fig. 3).

To evaluate the antitumor effects of MS5-Fc antibody, 3 aggressive xenograft models were used. The MDA-MB-453 breast cancer cells do not respond to hormone therapy and are especially aggressive due to their high metastatic potential. The human Ramos Burkitt lymphoma is a type of high-grade NHL, and it is frequently used to assess the therapeutic performance of antibodies targeting lymphoma (33). The HL60 cells, perhaps the most widely used, best-known myeloid cell line, originated from a female patient with AML (34). Adults with AML have some of the

Figure 9. Antitumor activity of the MS5-Fc antibody and rituximab against lymphoma xenografts. A) BALB/c nude mice bearing Ramos xenografts were intravenously treated with MS5-Fc antibody or rituximab (100 µg/injection) at d 3, 8, and 15 after tumor cell implantation (10⁷ cells/mouse, n = 5). Average tumor sizes for each treatment group were calculated on d 4, 7, 11, 14, 18, and 22. B) Representative flow cytometry histograms showing the binding of the MS5-Fc antibody and rituximab to Ramos lymphoma cell line.

Figure 10. In vitro serum stability of the MS5-Fc antibody. A) The protein (50 µg) was incubated in 40% human serum at 37°C, and aliquots were collected on d 0, 1, 2, 3, 4, 5, and 6 (D0–D6) and analyzed by a Western blot using an anti-His tag mAb. The D0 sample was taken immediately after adding the serum. B) Densitometric quantification. Results indicate the percentage of intact MS5-Fc antibody. The signal in D0 was considered to be 100% intact antibody. Data are from 1 single experiment and are representative of 3 independent experiments using serum from different healthy donors.
highest unmet needs of all cancer patients (35). In these 3 different human tumor xenograft models, the MS5-Fc antibody exhibited significant antitumor activity. Indeed, only 3–4 intravenous injections of small amounts (100 µg/injection) of the antibody were sufficient to inhibit tumor growth, measured by using tumor size and/or tumor mass, compared with Fc control–treated mice. Moreover, the treatment was accompanied by infiltration of innate immune cells into tumor tissues. Preclinical and clinical studies support the role of effector cells, especially NK cells and macrophages, in the antitumor activity of several therapeutic antibodies such as rituximab, trastuzumab, and cetuximab (3, 36, 37).

The in vivo experiments also revealed that the MS5-Fc antibody and rituximab have comparable antitumor effects. Although comparable until d 18 post–cancer cell inoculation, the effect of rituximab was superior to that of the MS5-Fc antibody at d 22 (7 d after the last treatment). This enhanced activity could be due to rituximab’s higher serum half-life in mice and/or the high expression of the CD20 receptor on Ramos B-cell lymphoma. A half-life of 9.5 d was found for the full-length human IgG1, whereas that of scFv-IgG1-Fc fusion was ~4.5 d (38). Despite being effective in the treatment of B-cell lymphoma patients, ~50% of patients with relapsed/refractory CD20+ follicular lymphoma do not respond to initial therapy with rituximab, and close to 60% of previously rituximab-responsive patients will no longer benefit with retreatment with rituximab (27). Similarly, the clinical outcome of high fraction (40%) of diffuse large B-cell lymphoma remains unsatisfactory after rituximab treatment (39). There are currently no effective therapies for these patients, giving rise to a significant unmet medical need. With its antitumor effect, the MS5-Fc antibody could lead to novel therapeutic treatment for millions of patients with lymphoma or leukemia. This notion is further supported by the finding that MS5-Fc antibody can bind and kill primary leukemia cells in vitro. Moreover, it showed a significant serum half-life in vitro and exhibited no significant aggregation in solution. As discussed by Roberts et al. (40), protein aggregation presents a key challenge in the development of therapeutic proteins.

Screening of phage libraries on intact cells preserves the original conformation of cell surface proteins and protein–protein interactions that could be relevant in vivo. Although a number of selected antibodies and peptides from phage display libraries have been used for tumor imaging and/or drug delivery without knowledge of the cellular receptors, their clinical use will be further facilitated by the characterization of the binding receptors. Immunoprecipitation experiments followed by spectrometric identification of captured protein species identified HSPs, including HSP70 and GRP78, as potential MS5 interacting partners (data not shown). HSP70 and GRP78 are known to be expressed on the cell surface of most cancer cells but not normal cells (41). These expression patterns fit well with the binding profile of the MS5-Fc antibody to cancer cells. However, when we investigated the binding of the MS5-Fc antibody to recombinant HSP70 and GRP78 proteins in Western blot experiments, no reactivity was detected (data not shown). We believe that the receptor is formed by the interaction between at least 2 protein partners expressed on the cell surface of cancer cells. Hence, the binding may arise from the formation of a native conformational epitope involving the interaction of these protein partners. Such interaction is more likely to be lost upon the preparation of membrane proteins for affinity purification. Therefore, a combination of several cellular and molecular biology techniques will be needed to tackle this challenging question that is under investigation.

In summary, we demonstrate here that sequential affinity selection of an antibody library against a panel of human cancer cell lines can lead to the isolation of antibodies with pan-cancer binding abilities. The engineered MS5-Fc antibody showed in vitro and in vivo efficacy against cancer cell lines of different origins. Given the emergence of unresponsive tumors to the current immunotherapies and the fact that many cancer types still have no immunotherapy options available, the developed MS5-Fc antibody represents an attractive candidate for further development toward clinical studies. Moreover, the identification of antibodies that recognize common cell surface antigens/structures specifically or preferentially expressed by cancer cells may open the avenue to truly cheaper cancer immunotherapy.

ACKNOWLEDGMENTS

The authors thank the excellent technical assistance from Anne Mobergslien (Department of Cancer Immunology, Oslo University Hospital). They also thank Linn A. Rosenberg (Department of Cancer Immunology, Oslo University Hospital) for helping with the preparation of recombinant proteins, Ingrid Kjønsstad (Department of Cancer Immunology, Oslo University Hospital) for analyzing the antibody stability, Dr. Morten Oksvold (Department of Pathology, Oslo University Hospital) for assisting with the confocal microscopic images, Dr. Hans-Petter Hersleth (Department of Biosciences, Oslo University) for assisting with size-exclusion chromatography analysis, and Dr. Anne Dybwad (Norwegian Medicines Agency, Oslo, Norway) for critical reading of the manuscript. This work was funded by the Norwegian Cancer Society (Grant 182993) awarded to M.S. The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

M. Sioud designed the project experiments, supervised the entire project, performed the experiments, analyzed the data, and wrote the paper; P. Westby performed phage amplification and preparation, antibody purification, and Western blots and discussed the data; V. Vasovic performed the animal experiments and analyzed and discussed the data; Y. Fløisand provided patient samples and discussed the data; Q. Peng cosupervised with M. Sioud the animal experiments and discussed the data; and all authors approved the final version of the manuscript.

REFERENCES

1. Farkona, S., Diamandis, E. P., and Blasutig, I. M. (2016) Cancer immunotherapy: the beginning of the end of cancer? BMC Med. 14, 73
2. Strohl, W. R. (2018) Current progress in innovative engineered antibodies. Protein Cell 9, 86–120
