Cancer-Associated CD43 Glycoforms as Target of Immunotherapy

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Abstract

CD43 is a sialoglycosylated membrane protein that is involved in cell proliferation and differentiation. CD43 glycoforms that are recognized by the UN1 monoclonal antibody (mAb) were expressed in lymphoblastoid T-cell lines and solid tumors, such as breast, colon, gastric, and squamous cell lung carcinomas, while unexpressed in the normal counterparts. The cancer association of UN1/CD43 epitope suggested the possibility to use the UN1 mAb for tumor diagnosis and therapy. In this study, we show that the UN1 mAb was endowed with antitumor activity in vivo because its passive transfer inhibited the growth of UN1-positive HPB-ALL lymphoblastoid T cells in mice. Furthermore, we demonstrate that tumor inhibition was due to UN1 mAb-dependent natural killer–mediated cytotoxicity. By screening a phage-displayed random peptide library, we identified the phagotope 2/165 as a mimotope of the UN1 antigen, as it harbored a peptide sequence that was specifically recognized by the UN1 mAb and inhibited the binding of the UN1 mAb to UN1-positive tumor cells. On the basis of sequence homology with the extracellular region of CD43 (amino acids 64 to 83), the 2/165 peptide sequence was likely mimicking the protein core of the UN1/CD43 epitope. When used as vaccine in mice, the 2/165 phagotope raised antibodies against the UN1/CD43 antigen, indicating that the 2/165 phagotope mimicked the UN1 antigen structure, and could represent a novel immunogen for cancer immunotherapy. These findings support the feasibility of using monoclonal antibodies to identify cancer-associated mimotopes for immunotherapy.

Introduction

CD43 is a highly sialylated and O-glycosylated membrane protein with the apparent molecular weight of 100 to 120 kDa, and is mostly expressed in hematopoietic cells, including stem cells (1). CD43 is involved in multiple functions, such as cell adhesion, apoptosis, and migration (2). The UN1 monoclonal antibody (mAb) was initially selected for a high reactivity against human immature thymocytes (CD₃imm; ref. 3), and recognized a CD43 epitope that included the monosaccharide GalNAc-O-linked to the polypeptide chain, named UN1/CD43 antigen (4). The UN1/CD43 antigen was expressed in human thymocytes, a subpopulation of peripheral blood CD4⁺ T lymphocytes, and some leukemic T-cell lines, such as HPB-ALL, H9, and MOLT-4 (3, 5). Moreover, the UN1/CD43 antigen was expressed at early stages of development in fetal tissues, including thymus, spleen, adrenal cortex, bronchial epithelium, and skin, and is downregulated in ontogeny (6). The involvement of UN1/CD43 glycoforms in oncogenesis was suggested by several findings. In fact, UN1/CD43 was detected in a variety of solid tumors, including breast, colon, gastric, and squamous cell lung carcinomas, whereas it was undetected in the relative normal tissues and benign lesions (6, 7). In particular, the expression level of UN1/CD43 glycoforms in breast cancer cells correlated with the progression stage of the disease (7). The evidence that the UN1-type CD43 membrane proteins were expressed in cancer tissues made the UN1 mAb an attractive tool for tumor detection and immunotherapy.
Several mAbs directed against surface antigens of tumor cells are currently used for cancer therapy (8). However, mAb-based therapy has many disadvantages, such as the relatively short-lived response, the development of therapy resistance and immune reaction overtime, and a high cost of production (9, 10). As alternative to the direct use of therapeutic mAbs, vaccine strategies using peptide mimics of specific epitopes to elicit a humoral and cellular immune response against tumor cells could be a more effective and economical approach (11). In this regard, phage-displayed random peptide libraries (RPL) have been used to select peptides that mimic natural epitopes, so-called mimotopes, even in the absence of knowledge on the antigen structure (12). Mimotopes have been used as immunogens for raising antibodies against the natural epitope, thus representing a suitable tool for immunotherapy (13–16). In particular, the mimotope-based immunization can overcome the major limitations of glycosylated antigens, including the poor immunogenicity, the inability to stimulate a long-lasting immune response, and the difficulty to synthesize and purify large quantities of glycosylated proteins for immunization (17).

In this study, we show that the passive transfer of the UN1 mAb inhibited the growth of UN1-positive lymphoblastoid T cells upon xenograft in mice, being this inhibition mediated by antibody-dependent cell-mediated cytotoxicity. By screening a phage-displayed RPL, we identified the phagotope 2/165 that expressed the peptide sequence mimicking the UN1/CD43 epitope, and we demonstrated that mouse immunization with this phagotope elicited antibodies that specifically recognized UN1-positive cancer tissues. These findings support the possibility to target cancer-associated CD43 glycoforms by using immunotherapeutic strategies based on UN1 mAb and UN1 mimotopes.

### Materials and Methods

#### Cells

Human thymic acute lymphocytic leukemia HPB-ALL cells were obtained by the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) in 2003. The cell line was expanded and cryopreserved in liquid nitrogen in the investigators laboratory, and lastly verified in 2013 using the following tests: morphology was confirmed by flow-cytometric analysis of cell surface markers expression. Cells were maintained (maximum 25 passages) in RPMI-1640 medium supplemented with 1% l-glutamine and 10% fetal calf serum (FCS; Life Technologies).

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors by Ficoll–Paque gradient centrifugation (GE Healthcare Life Sciences), as previously described (27–29). Primary cultured natural killer (NK) cell populations were obtained from 10-day cocultures of PBMCs with irradiated Epstein–Barr virus-positive RPMI-8866 lymphoblastoid cell line, as previously described (30). HPB-ALL cells and primary cultured NK cells were maintained in RPMI-1640 supplemented with 1% l-glutamine and 10% FCS (Life Technologies; refs. 31, 32).

#### RPL, antibodies, and peptides

The 888–4/Cys5 phage-displayed peptide library (GenBank Accession AF246454) was kindly provided by Dr. George P. Smith (University of Missouri, Columbia, MI). This library contains random 15-mer peptides constrained at the N-terminus of the pVIII phage coat protein with a complexity of $5.9 \times 10^{5}$ primary clones (33).

The IgG1 isotype UN1 mAb was produced and purified by MAbTrap Kit (GE Healthcare), as previously described (3). Antibodies used as positive controls in antibody-dependent cell-mediated cytotoxicity (ADCC) were as follows: OKT3 mAb (eBiosciences), which recognizes a CD3 epitope that is strongly expressed on HPB-ALL cells; W6/32 mAb (eBiosciences), which recognizes a nonpolyorphic epitope commonly expressed in MHC class I, HLA-A, B, and C, on all human nucleated cells. Fluorescein isothiocyanate (FITC)-conjugated F(ab’)$_2$ fragment of rabbit anti-mouse immunoglobulins and immunoglobulin G (IgG) control were purchased from Dako. Peptides were purchased from Casio Laboratory ApS.

#### Cell proliferation, viability, cell-cycle, and apoptosis analysis

Cell proliferation was monitored by enumerating viable cells using a hemocytometer. Viability was determined by using Trypan blue dye exclusion. Cell-cycle and apoptosis analysis were performed as previously described (34–36).

#### In vivo tumor growth analysis

Six-week-old female NU/NU nude mice (BALB/c congenic Crl:NU-Foxn1nu, Charles River Laboratories) were engrafted with HPB-ALL cells ($5 \times 10^6$) by subcutaneous injection into the lateral flank. Then mice were randomly assigned to two treatment groups of 5 mice: one group was treated with control mouse IgG1 (Sigma-Aldrich), the second group was treated with the UN1 mAb. The UN1 mAb or IgG1 control (400 μg in PBS/mouse) were injected in the tail vein of mice the day after tumor cells engraftment and the inoculation was repeated at day 7 from xenograft. The tumor size was measured by caliper, three times per week, and the tumor volume was calculated by the modified ellipsoid formula $V = \frac{A \times B}{6}$, where $A$ is the longest and $B$ the shortest perpendicular axis of an assumed ellipsoid. Mice were killed when the tumor volume reached a size of 4 cm$^3$, according to the ethical guidelines.

#### Complement-dependent cytotoxicity

For assessment of cytotoxicity, triplicate HPB-ALL samples ($5 \times 10^6$ cells/well) were seeded in Terasaki plates in...
RPMMI-1640 and incubated with or without the UN1 mAb (200 μg/mL), or W6/32 mAb (100 μg/mL), or IgG1 (100 and 200 μg/mL), in the presence or absence of the complement for 30 minutes at 4°C. Then, cells were treated with rabbit complement for an additional hour at 37°C. Cells were analyzed by fluorescence microscopy to detect acridine orange-positive viable cells (green fluorescence) and ethidium bromide-positive dead cells (red fluorescence). Results were expressed as percentage of nonviable cells.

**Antibody-dependent cellular cytotoxicity assay**

NK-mediated ADCC was determined using UN1-positive HPB-ALL cells. Target cells were labeled with 51Cr (100 μCi/1 × 10^6 cells) for 1 hour at 37°C, washed twice, and then incubated at room temperature for 20 minutes with the following IgG, isotype mAbs: UN1 mAb (0.25 μg/1 × 10^6), anti-MHC class I (W6/32; 2.5 μg/1 × 10^6), and anti-CD3 (OKT3; 0.25 μg/1 × 10^6). Serial dilutions of effector cells and 51Cr-labeled target cells (5,000/well) were plated in triplicates in round-bottomed 96-well plates to a final volume of 200 μL RPMI supplemented with 10% FCS and 10 mmol/L Hepes. After 4-hour incubation at 37°C, supernatant samples (30 μL) were collected from each well and counted by a β-counter instrument (TopCount, PerkinElmer Life and Analytical Sciences). Spontaneous release of 51Cr was evaluated by incubating the target cells with medium alone, whereas total release was evaluated by incubating target cells with SDS 10%. Percent-specific 51Cr release was calculated according to the following formula: percent-specific release = (experimental release - spontaneous release) / (maximum release - spontaneous release) × 100. Lytic units represent the number of effector cells required to mediate 20% lysis of target cells and were calculated by using the descending phase of curves generated by 51Cr release assay over the range of effector/target (E/T) cell ratios, as previously reported (37).

**Immunoscreeining of phage-displayed RPLs**

Specific phage clones for the UN1 mAb were isolated from the library by two rounds of affinity selection, as previously described (38, 39). To this end, the UN1 mAb (10 μg) was linked to streptavidin-conjugated magnetic beads (Promega), which had been coated with 10 μg of goat anti-mouse IgG (Fc-specific) biotin-conjugated Ab (Sigma-Aldrich) in 200 μL of beads suspension. A total of 3 × 10^10 transducing units of phages from the library were added and incubated for 16 hours at 4°C. After extensive washing, bound phages were eluted with 0.1 mol/L HCl/ glycine buffer, pH 2.2, 1 mg/mL bovine serum albumin (BSA), and neutralized with 1 mol/L Tris, pH 9.1. Eluted phages were amplified by infection of k91BK bacteria, and purified from plaques for a second round of affinity selection. Phage colonies were transferred according to an ordered grid on a lawn of K91BK cells on Luria-Bertani (LB) agar plates supplemented with 1 mmol/L isopropyl β-D-thiogalactopyranoside. Nitrocellulose filters were layered onto these plates and incubated overnight at 37°C. Filters were blocked for 2 hours with blocking buffer (1 × PBS, 5% nonfat dry milk, 0.1% NP40, 0.01% NaN₃) at room temperature and incubated O/N at 4°C with the UN1 mAb (1 μg/mL) in blocking buffer. Then, filters were washed with washing buffer (1 × PBS, 0.1% NP40) and incubated with alkaline phosphatase-conjugated anti-mouse IgG (Fc specific) secondary antibody (Sigma-Aldrich) at the dilution of 1:5000 for 4 hours at 4°C. After extensive washing, filters were incubated in developing solution (1-Step NBT/BCIP, Pierce, Thermo Scientific). Collected phage supernatants from positive clones were further analyzed by ELISA.

**ELISA**

For assaying phage reactivity to antibodies or sera, multwell plates (Immunoplates Maxisorp) were coated with 10 μg/mL anti-id bacteriophage Abs (Sigma-Aldrich) in 50 mmol/L NaHCO₃, pH 9.6, overnight at 4°C. After blocking with blocking buffer (1 × PBS, 5% nonfat dry milk, 0.05% Tween 20, 0.05% NaCl) for 1 hour at 37°C, the blocking buffer was discarded, and a mixture of 50 μL of blocking buffer and 50 μL of phage supernatant was added to each well. After incubation for 1 hour at 37°C, plates were washed six times with washing buffer (1 × PBS, 0.05% Tween 20, 0.05% NaCl). Then, UN1 mAb (5 μg/mL) or sera at the indicated dilution were resuspended in blocking buffer and added to the wells. After an O/N incubation at 4°C, plates were washed six times with washing buffer, and a 1:5000 dilution in blocking buffer of a goat anti-mouse IgG (Fc specific) alkaline phosphatase antibody (Sigma-Aldrich) was added to wells. Alkaline phosphatase was revealed by incubation with p-nitrophenyl phosphate (1 mg/mL) in 1 × diethanolamine substrate buffer (Pierce Thermo Scientific). Optical density (OD) at 405 nm (OD₄₀₅) and 620 nm (OD₆₂₀) were measured by an ELISA reader (Tecan), and values were expressed as difference between OD₄₀₅ and OD₆₂₀. Wild-type phage was used as a negative control (40).

**Surface plasmon resonance**

Surface plasmon resonance (SPR) was performed using Biacore 3000 optical biosensor equipped with research-grade CM5 sensor chips (Biacore GE Healthcare), as previously described (41). Experimental details are provided in Supplementary Materials and Methods.

**Competition assay of UN1 mAb binding**

The UN1 mAb (0.37 μg/mL) was preincubated O/N at 4°C with CsCl-purified phages (3 × 10^11 up to 3 × 10^8) phage...
After washing, the cells were incubated for an additional 30 minutes at 4°C with FITC-conjugated rabbit anti-mouse immunoglobulins F(ab′)2, and analyzed with BD FACS-Calibur cytometer. Results were analyzed by CellQuest software (BD Biosciences; ref. 42).

**Mouse immunization**

Five- to six-week-old female Balb/c mice (Harlan) were immunized by intraperitoneal injection of CsCl-purified 2/165 phage particles or wild-type phage at weeks 0, 3, 6, 9, 12, 15, and 18. The animals were bled at day 0 and 7 to 10 days after the second, fourth, and sixth boost. Phages were injected as PBS suspension in CFA (Freund’s complete adjuvant) at week 0 or IFA (Freund’s incomplete adjuvant) at a concentration of $6 \times 10^{12}$ phage particles/mL. Serum IgGs from mice immunized with 2/165 phage particles or wild-type phage at weeks 0, 3, 6, 9, 12, 15, and 18 were assayed by ELISA (e-Biosciences), according to the manufacturer’s instructions.

**Immunoglobulin purification of antibodies**

Immunoglobulins were purified by incubating mice sera at 1:10 dilution in multwell plates coated with 5 x $10^{12}$/mL CsCl-purified phages. After extensive washing, the bound immunoglobulins were eluted with 0.1 mol/L glycine-HCl buffer, pH 2.7, 10 μg/mL BSA immediately neutralized with 2 mol/L Tris-HCl, pH 9.4, and concentrated using Microcon Ultracel YM-30 (Millipore).

**Immunohistochemistry**

Surgical specimens were derived from breast and gastric cancer tissues of the patients hospitalized at the clinical surgery Federico II University of Naples (Naples, Italy). The informed consent to research activity was expressed by patients at the time of surgery for excision of neoplasia, analyzed at the section of Pathological Anatomy (department of advanced biomedical sciences Federico II University of Naples). Immunohistochemical analysis was performed as previously described (6, 7). Experimental details are provided in Supplementary Materials and Methods.

**Statistical analysis**

Statistical analysis of tumor growth (Wilcoxon rank-sum test and Wei-Johnson test) and survival (long-rank Mantel–Cox test) were performed with GraphPad Prism 5 program (GraphPad Software Inc.). For ADCC assay, statistical analysis was performed using Prism software (GraphPad Software Inc.). Data were analyzed using the paired two-tailed Student t test. Differences were considered as statistically significant at the 95% level ($P < 0.05$).

**Ethics statement**

This study was carried out according to the recommendations of the Institutional animal care guidelines, Italian D.L. n. 116 of 27 January 1992 and European Communities Council Directive 2010/63EU.

**Results**

**UN1 mAb inhibited the tumor growth of UN1-positive leukemic T cells in nude mice**

On the basis of the evidence that the UN1 mAb specifically bound to UN1/CD43-positive neoplastic cells (6, 7), we addressed the question of whether it could interfere with the tumor growth in vivo. To this end, ten female 6-week-old NU/NU nude mice were tumor engrafted by subcutaneous injection of UN1-positive HPB-ALL cells into the lateral flank. Then, mice were randomly divided in two groups (5 animals/group) and treated with the UN1 mAb or IgG1 control (400 μg/mouse) by injection in the tail vein at day 1 and 7 after tumor engraftment. Tumor onset and volume were monitored from day 0 up to 31, when all mice of the IgG1 control group died. Tumor onset was observed at day 17 in IgG1-treated mice, whereas it was delayed up to day 24 in UN1 mAb-treated mice. A statistically significant inhibition of tumor growth was observed in UN1 mAb-treated mice as compared with IgG1-treated controls from day 17 to 31 ($P < 0.032$ by Wilcoxon rank-sum test and $P = 0.024$ by Wei-Johnson test; Fig. 1A). Mice survival was also significantly affected by the UN1 mAb treatment. In fact, the animal group treated with UN1 mAb showed 40% survival rates at day 30 as compared with the death of IgG1-treated control group ($P = 0.0031$ by log-rank Mantel–Cox test; Fig. 1B). These data showed that mAb UN1 treatment had an antitumor activity in the HPB-ALL tumor xenograft mice model.

**UN1 mAb caused HPB-ALL cell lysis via antibody-dependent cell-mediated cytotoxicity**

To understand the mechanism of UN1 mAb inhibition of HPB-ALL tumor growth, we analyzed the direct effect of the UN1 mAb on cell growth by incubating the HPB-ALL cells with the UN1 mAb (1 up to 25 μg/mL), or IgG1-negative control. The UN1 mAb did not affect the proliferation rate, cell cycle, and the number of viable and apoptotic cells as compared with untreated or IgG-treated cells (Supplementary Fig. S1A–S1D). Furthermore, we analyzed whether the UN1 mAb could act via complement-mediated cell lysis. Cytotoxicity was assessed by incubating HPB-ALL cells with or without UN1 mAb, in presence or absence of the complement. W6/32 mAb and IgG were included as positive and negative controls, respectively. Differently from W6/32 mAb, the UN1 mAb did not affect cell lysis (Fig. 1C).
The ADCC is triggered by the binding of antibody-opsonized tumor cells to FcγRIIIA/CD16 of NK cells resulting in tumor cell lysis. Thus, we reasoned that ADCC could be a mechanism of UN1 mAb-dependent tumor inhibition. To evaluate whether the UN1 mAb induced CD16-mediated ADCC, HPB-ALL cells were opsonized with the UN1 mAb, or OKT3 or W6/32 mAbs as positive controls. Cultured primary NK cells from nine healthy donors were tested in a standard ADCC assay. A significant antibody-mediated lysis of tumor cells (P = 0.0026) was observed in the UN1 mAb-opsonized samples as compared with not-opsonized controls, being the UN1-opsonized targets were killed more efficiently in seven out of nine donors (Fig. 1D). Moreover, ADCC induced by UN1 mAb was slightly lower as compared with W6/32 mAb (mean 21.9% vs. 24.4%), or OKT3 mAb (mean 21.9% vs. 32.3%; Fig. 1D). The ability of UN1 mAb to induce ADCC was also supported by the analysis of lytic units within the same donor, which were calculated for the whole curve E/T cells ratio (Fig. 1E). For the UN1, OKT3, and W6/32 mAbs, the strength of binding to HPB-ALL cells directly correlated with their ADCC potency (Supplementary Fig. S2A), which was likely due to the expression levels of cognate antigens on cell surface.

**Identification of the UN1 mimotope by phage-displayed RPL**

On the basis of the UN1 mAb inhibition of UN1-positive tumor cells, we reasoned that the identification of the UN1/CD43 epitope recognized by the UN1 mAb could be useful for developing novel immunogens for cancer immunotherapy. To this end, we used the UN1 mAb to screen an f88–4/Cys5 phage-displayed peptide library by two rounds of affinity selection. A phage enrichment was observed during the selection as the output/input phage ratio increased from 1.1/C2 to 1.2/C2 after round II. After an immunoscreening step, 174 single phage clones were recovered and tested by ELISA for specific binding to the UN1 mAb and, among them, 153 clones were found to react with the UN1 mAb. Optical density values of positive phagotopes ranged between 4-folds and 40-folds higher than wild-type phage (Fig. 2A). On the basis of these results, 28 phage clones showing the strongest positive signal were selected for DNA sequencing, which yielded 11 different peptide mimotope sequences (Table 1). MUSCLE-based alignment analysis (http://www.ebi.ac.uk/Tools/msa/muscle/) showed a significant homology between the 11 peptides and the human protein CD43 (Fig. 2B), which is the natural antigen recognized by UN1 mAb (4). Indeed, the homology region of the 11 amino acidic sequences of
Two peptides were synthesized corresponding to the insert of the 2/165 phagotope (W15 peptide: TPH-TCKLLDECVPLW) and a longer synthetic peptide containing the W15 peptide sequence with additional amino acid residues flanking the insert of the phagotope 2/165 (G23 peptide: SFAATPHTCKLLDECVPLW-PAEG). As negative control, a scrambled peptide was synthesized (TCLAPDVPEPLSHCWAGETFKLA). By SPR, we measured binding affinity of the peptides W15 and G23 to the UN1 mAb. Sensorgrams showed that G23 and W15 peptides exhibited similar affinities for the UN1 mAb (KD 155 and 142 nmol/L, respectively), whereas the scrambled peptide failed to give the SPR signal (Fig. 2C).

Phagotope 2/165 and UN1 peptide mimotopes competed the binding of UN1 mAb to tumor cells

Next, we determined whether the phage clone 2/165 and the peptides W15 and G23 mimic the structure of the UN1 epitope. To this end, we performed a competitive inhibition assay for the binding of the UN1 mAb to the HPB-ALL cells in the presence or absence of the phagotope 2/165 or the peptide mimotopes. The binding of UN1 mAb to HPB-ALL cells was significantly reduced in the presence of phage 2/165 (Fig. 3A and B), G23, or W15 peptides (Fig. 3C and D) in a dose-related manner. This effect was not observed in presence of wild-type phage or scrambled peptide (Fig. 3A–D). Conversely, the phage 2/165 and the peptides G23 and W15 did not compete the binding of W6/32 mAb, an antihuman HLA class I mimotopes falls in the extracellular domain of CD43 (amino acids 64–83), a region that undergoes highly O-linked glycosylation (4). Among the 11 selected clones, the phagotope 2/165 was chosen due to the strongest reactivity against UN1 mAb in ELISA (Table 1).

<table>
<thead>
<tr>
<th>Phage number</th>
<th>Peptide sequence</th>
<th>Frequency</th>
<th>OD&lt;sub&gt;405–OD&lt;sub&gt;620</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>SRPHCYPMDDCHPLW</td>
<td>4</td>
<td>1.137</td>
</tr>
<tr>
<td>1.201</td>
<td>LMSTCTWLDECFRPQ</td>
<td>6</td>
<td>1.025</td>
</tr>
<tr>
<td>2.16</td>
<td>PTPCHRGDECQPLW</td>
<td>5</td>
<td>0.703</td>
</tr>
<tr>
<td>2.165</td>
<td>TPHTCCKLLDECQPLW</td>
<td>3</td>
<td>2.925</td>
</tr>
<tr>
<td>2.171</td>
<td>TTVCTWLDCECPPWS</td>
<td>1</td>
<td>1.052</td>
</tr>
<tr>
<td>2.207</td>
<td>WSQMCESSWCKDFA</td>
<td>4</td>
<td>1.039</td>
</tr>
<tr>
<td>3.109</td>
<td>HTPWCSIADPCLWE</td>
<td>1</td>
<td>1.597</td>
</tr>
<tr>
<td>3.158</td>
<td>YHEPCLWATACPTTP</td>
<td>1</td>
<td>0.464</td>
</tr>
<tr>
<td>3.206</td>
<td>MQDMCNDDSCPLWS</td>
<td>1</td>
<td>0.481</td>
</tr>
<tr>
<td>2.110</td>
<td>CTHPCEPPPLWPIAP</td>
<td>1</td>
<td>2.082</td>
</tr>
<tr>
<td>2.8</td>
<td>PGPGCPYPAELWCTQ</td>
<td>1</td>
<td>0.336</td>
</tr>
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</table>

NOTE: Amino acid sequences of selected mimotopes. Frequency indicates the recurrence of the same sequence in different phage clones. OD<sub>405–OD<sub>620 refers to the ELISA results of phage reactivity to UN1 mAb. The phagotope 2/165, showing the strongest reactivity against UN1 mAb in ELISA, is highlighted in bold type.
As additional evidence of structural similarity to the UN1/CD43 epitope, the phage 2/165 and G23 competed the binding of UN1 mAb to UN1-positive breast and gastric cancer tissues, whereas the wild-type phage and scrambled peptide did not (Supplementary Fig. S3). These results indicated that the phagotope 2/165 as well as the derived G23 and W15 peptides mimicked the conformation of the UN1/CD43 epitope.

Mouse immunization with phagotope 2/165 elicited antibodies against the UN1/CD43 natural antigen

Filamentous phage can be used as immunogen as they elicit a strong antibody response in different animal systems (43, 44). Thus, we analyzed the immunogenicity of the phagotope 2/165 in mice. Balb/c mice (5 animals/group) were immunized with the phage 2/165 or wild-type phage, and the collected sera were analyzed for reactivity against the wild-type phage, G23, or scrambled peptide as control (Fig. 4). Both wild-type phage- and 2/165 phagotope-immunized mice appeared healthy and did not show any ill-effect. Moreover, both groups of immunized mice showed levels of serum inflammatory cytokine IL-1β, and hepatotoxicity marker GSH and LDH in the range of normality (Table 2), indicating that phage immunization was not toxic.

As expected, both groups of immunized mice developed a strong and comparable antibody response against the wild-type phage, whereas control serum from a non-immunized mouse showed no reactivity (Fig. 4A and B, left). However, the phagotope 2/165-immunized mice sera showed a specific immune response against the UN1 mimotope G23 peptide (1:20,000 up to 1:100,000 antibody titer; Fig. 4A, middle), whereas they were unreactive against the scrambled peptide (Fig. 4A, right). As negative control, the preimmune and wild-type phage immunized mice sera did not show any detectable reactivity against the G23 (Fig. 4B, middle), or scrambled peptide (Fig. 4B, right). As further analysis of 2/165 phagotope-induced antibodies recognition of UN1 epitope, immunoglobulins were affinity purified from sera of 2/165 phagotope immunized mice or preimmune sera using the 2/165 phagotope or wild-type phage as ligand. The immunoglobulins (Ig) eluted from 2/165 phagotope (2/165 Igs) reacted with the G23 peptide, whereas immunoglobulins eluted from wild-type phage (wt Igs) did not (Supplementary Fig. S4A). Furthermore, by flow cytometry, we confirmed that the 2/165 Igs bound to the HPB-ALL cells (Supplementary Fig. S4B). On the basis of the reported reactivity of the UN1 mAb with tumor tissues (6, 7), we tested whether the 2/165 IgGs detected UN1-positive human breast and gastric cancer by immunohistochemistry. We found that 2/165 IgGs specifically reacted with breast and gastric cancer tissues with a pattern similar to the one observed with the UN1 mAb (Fig. 5). Conversely, no reactivity against the cancer tissues was observed using the wt IgGs or preimmune IgGs (Fig. 5). The binding specificity of the 2/165 IgGs to UN1-positive cancer
tissues was confirmed by competition assays. Indeed, the preincubation of 2/165 IgGs with the 2/165 phagotope or the UN1 mimotope G23 peptide inhibited the binding of 2/165 IgGs to UN1-positive tumor tissues, confirming the binding specificity of 2/165 IgGs to the UN1 epitope exposed on tumor cells (Fig. 6).

Discussion

CD43 is a transmembrane sialoglycoprotein that is mostly expressed in hematopoietic cells, and tumor cells of non-hematopoietic origin. CD43 signaling activates cellular pathways leading to activation of NF-κB and AP1 transcription factors that upregulate the expression of prosurvival and proliferation genes (45). Deregulation of CD43 signaling can promote cancer phenotype (45). We previously reported that specific CD43 glycoforms were recognized by the UN1 mAb and were highly expressed in lymphoblastoid T-cell lines and several solid tumors (4, 6, 7). In particular, the expression of UN1-type CD43 in breast cancer correlated with the grade of malignancy, suggesting that the specific CD43 glycoforms could promote an altered proliferation signaling and cause cancer progression (6, 7). On the basis of this evidence, the UN1 mAb was considered a suitable tool for cancer immunophenotyping.

The aim of this study was to evaluate the immunotherapeutic activity of the UN1 mAb based on the ability of this mAb to target specifically the cancer cells expressing the UN1-type CD43 glycoforms. We observed that the passive transfer of the UN1 mAb inhibited the growth of UN1-positive HPB-ALL lymphoblastoid T cells upon xenograft in mice, suggesting a possible use of this antibody for cancer immunotherapy. We excluded that tumor growth inhibition was due to direct cytostatic or cytotoxic activity of the UN1 mAb because the proliferation rate, cell cycle, and viability of HPB-ALL cells were unaffected by the UN1 mAb in cell culture. Conversely, the UN1 mAb induced the in vitro lysis of HPB-ALL cells via antibody-dependent NK-mediated cytotoxicity. On the basis of these findings, the UN1 mAb behaved similarly to the antibodies that are currently used in cancer therapy against specifically or differentially expressed tumor antigens, such as anti-CD20 (rituximab), anti Her-2/neu (trastuzumab), and anti-EGFR (cetuximab; ref. 46). It is worth mentioning that such validated anticancer therapeutic mAbs exhibited multiple effector mechanisms for their in vivo anti-tumor activity (46). Even though additional mechanisms of UN1 mAb action require further

Table 2. In vivo toxicity of phage immunization

<table>
<thead>
<tr>
<th>Group</th>
<th>IL-1β (pg/mL)</th>
<th>GSH (μmol/L)</th>
<th>LDH (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>58.0 ± 0.2</td>
<td>10.4 ± 1.2</td>
<td>65.4 ± 12.3</td>
</tr>
<tr>
<td>WT phage</td>
<td>59.7 ± 0.3</td>
<td>9.8 ± 0.8</td>
<td>60.0 ± 11.5</td>
</tr>
<tr>
<td>2/165 phage</td>
<td>58.2 ± 0.3</td>
<td>10.1 ± 0.9</td>
<td>52.9 ± 15.4</td>
</tr>
</tbody>
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NOTE: Balb/c mice (5 animals per group) were immunized with the phage 2/165, or wild-type phage, or left untreated, and the collected sera at days 7 to 10 from the sixth boost were analyzed for the indicated markers of systemic toxicity. Values represent the mean ± SEM of sample measurements.
investigation, such as receptor downmodulation, ligand blockade, or antibody-dependent cellular phagocytosis, the in vitro analysis performed in this study indicates that ADCC could be a major mechanism of in vivo antitumor activity.

We also explored the possibility to generate a mimotope of the UN1/CD43 epitope to be used as immunogen for eliciting highly specific Abs against the UN1-positive tumor tissues. By screening an f88–4/Cys5 phage-displayed peptide library with the UN1 mAb, we identified and characterized eleven peptide binders of the UN1 mAb. Among the selected phage clones, the peptide insert of phagotope 2/165 was a true UN1/CD43 epitope mimic because it was specifically recognized by the UN1 mAb and inhibited the binding of the UN1 mAb to the UN1/CD43 natural antigen, meeting the previously established guidelines for specificity and mimicry testing (47). Several studies have indicated that cysteine-constrained phage-displayed peptides are endowed with a stabilized conformation that allows the isolation of cyclic peptides with a higher affinity toward a bait as compared with non-constrained, linear peptides (48). Moreover, cysteine-constrained phage-derived peptides have been shown to bind the cognate ligand outside of the phage coat protein (38), either in a different protein backbone (49), or when coupled to therapeutic agents (50). In these settings, the 2/165 peptide could represent a flexible tool for the targeted therapy of UN1-positive tumor cells.

Bioinformatics-based analysis identified a significant sequence homology between the eleven UN1 peptide mimotopes and the amino acids 64 to 83 contained in the extracellular domain of CD43 that undergoes heavy O-glycosylation. These findings agree with our previous observation that the monosaccharide GalNAc-O-linked to the CD43 peptide core was an essential component of a higher affinity toward a bait as compared with non-constrained, linear peptides (48). Moreover, cysteine-constrained phage-derived peptides have been shown to bind the cognate ligand outside of the phage coat protein (38), either in a different protein backbone (49), or when coupled to therapeutic agents (50). In these settings, the 2/165 peptide could represent a flexible tool for the targeted therapy of UN1-positive tumor cells.

Figure 5. The 2/165 phage-induced antibodies detected UN1-positive human breast and gastric cancer tissues. Serial sections of surgical specimens derived from breast and gastric cancer tissues were stained with UN1 mAb, 2/165 IgGs (IgGs purified from sera of 2/165 phagotope-immunized mice), wt IgGs (IgGs purified from sera of wild-type phage-immunized mice), or control IgGs (IgGs purified from not immunized mice), according to peroxidase-antiperoxidase method. Original magnification × 200.

Figure 6. The 2/165 phagotope and G23 peptide inhibited the binding of 2/165 IgG to human breast and gastric cancer tissues. Serial sections of surgical specimens derived from breast and gastric cancer tissues were stained with 2/165 IgGs, preincubated overnight at 4 °C with the indicated phage (2.5 × 10¹¹ phage particles/mL) or peptide (500 μg/mL), according to peroxidase-antiperoxidase method. Original magnification × 200.
the UN1 epitope (4), and suggests the hypothesis that the UN1 peptide core overlaps the sequence from 64 to 83 amino acids of the CD43 extracellular domain.

For vaccine purpose, glycosylated antigens are difficult to synthesize and purify in large quantities, and usually induce a poor immune response with short-lived immunoglobulin M-type antibodies. Novel strategies of vaccine production are required to overcome these major limitations (17). Phage mimotopes of cancer antigens (18), (21–23) revealed a promising activity as immunogens by eliciting a strong cross-reactive antibody response against the selected natural antigens. Here, we have demonstrated that the phagotope 2/165 mimicked the cancer-associated UN1/CD43 epitope and induced in immunized mice specific antibodies against the UN1/CD43 antigen. In fact, purified immunoglobulins from 2/165 phagotope-immunized mice sera specifically reacted with several UN1-positive cancer tissues, as confirmed by competition assays. Furthermore, the UN1 mimotope-induced antibodies were IgG isotype, a peculiar feature of a long-standing immune response, indicating that the mimotope-based immunization can overcome the poor immunogenicity of purified glycosylated antigens.

On the basis of these results, the UN1/CD43 epitope may represent a suitable target for cancer immunotherapy, and UN1 mimotopes, such as 2/165 phagotope and derivative G23 peptide here described, are promising cancer vaccine candidates for UN1-positive tumors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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