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Aberrantly glycosylated MUC1 is expressed on the surface of breast cancer cells and a target for antibody-dependent cell-mediated cytotoxicity

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Abstract Protein glycosylation often changes during cancer development, resulting in the expression of cancer-associated carbohydrate antigens. In particular mucins such as MUC1 are subject to these changes. We previously identified an immunodominant Tn-MUC1 (GalNAc-α-MUC1) cancer-specific epitope not covered by immunological tolerance in MUC1 humanized mice and man. The objective of this study was to determine if mouse antibodies to this Tn-MUC1 epitope induce antibody-dependent cellular cytotoxicity (ADCC) pivotal for their potential use in cancer immunotherapy. Binding affinity of mAb 5E5 directed to Tn-MUC1 was investigated using BiaCore. The availability of Tn-MUC1 on the surface of breast cancer cells was evaluated by immunohistochemistry, confocal microscopy, and flow cytometry, followed by *in vitro* assessment of antibody-dependent cellular cytotoxicity by mAb 5E5. Biacore analysis demonstrated high affinity binding (K_D=1.7 nM) of mAb 5E5 to its target, Tn-MUC1. Immunolabelling with mAb 5E5 revealed surface expression of the Tn-MUC1 epitope in breast cancer tissue and cell lines, and mAb 5E5 induced ADCC in two human breast cancer cell lines, MCF7 and T47D. Aberrantly glycosylated MUC1 is expressed on the surface of breast cancer cells and a target for antibody-dependent cell-mediated cytotoxicity suggesting that antibodies targeting glycopeptide epitopes on mucins are strong candidates for cancer-specific immunotherapies.

Keywords Cancer · Immunotherapy · Antibodies · Glycopeptide · MUC1 · ADCC · GalNAc · Glycans

Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
mAb	Monoclonal antibody
VNTR	Variable number tandem repeats
GalNAc	*N*-acetylgalactosamine
NeuAc	*N*-acetylneuraminic acid
CDC	Complement-dependent cytotoxicity
MAC	Membrane attack complex
Gal	Galactose
PBMC	peripheral blood mononuclear cells
NK	Natural killer
FcγR	Fc-γ-receptors

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Introduction

Immunotherapy using humanized antibodies that target tumor-associated antigens has the potential to selectively

target cancer cells and thereby limit damage to non-cancerous tissue. The MUC1 mucin is a large, heavily O-glycosylated membrane-associated protein consisting of three domains: a cytoplasmic domain involved in intracellular signaling [1], a transmembrane domain, and a large extracellular domain. The extracellular domain of MUC1 has a variable number of 20–120 tandem repeats (VNTR), each containing 20 amino acids with 5 potential O-glycosylation sites [2]. In healthy individuals, MUC1 is expressed on the apical surfaces of nearly all simple and glandular epithelial cells. During malignant transformation, MUC1 is greatly over expressed in 90 % of breast cancers [3] and in several other cancers, resulting in loss of its normal polarized distribution.

Malignant transformation of cells is accompanied by alterations in post-translational modifications of proteins [4, 5]. The abundant mucin-type O-glycosylation on MUC1 and other O-glycoproteins is a well-established example of a post-translational modification that undergoes tumor-associated changes. The biosynthesis of mucin-type O-glycosylation is a complex and regulated process that involves over 50 gene products [6]. A consistent feature of cancer cells is aberrant truncation of mucin-type O-glycans to short mono- and disaccharides, also known as the pan-carcinoma antigens Tn (GalNAcα-O-Ser/Thr) and STn (NeuAcα2-6GalNAcα-O-Ser/Thr) [7] (Fig. 1a). O-linked glycans are found on approximately 30 % of all cell surface proteins and, in particular, on the membrane-bound mucins overexpressed in cancer. Together with the loss of MUC1 polarization, this creates a diverse set of accessible cancer-specific glycopeptide epitopes on the surfaces of cancer cells and thus enables the induction of anti-MUC1 immune responses

In several studies anti-MUC1 mAbs have been used to target tumor cells, either as unlabeled antibodies with the intent to induce immunological effector mechanisms such as ADCC or as carrier molecules for radioactive isotopes [8–10]. Furthermore, active vaccination trials with MUC1 tandem-repeat derived peptides have been performed in patients with breast, colon, ovarian, and other carcinomas [11–17]. Weak anti-MUC1 IgG and IgM could be induced in these patients, and the mAbs that were specific for tumor-associated antigens invoked some degree of ADCC. This demonstrates the potential of anti-MUC1 immune responses including anti-MUC1 mAbs to eliminate tumor cells. Most of these findings, however, are hampered by either the low affinity of the generated antibody or by lack of cancer specificity. MUC1 is expressed in various healthy tissues, including the apical surfaces of nearly all simple and glandular epithelial cells, but also in certain hematopoietic cells, including monocytes [18], subsets of B-lymphocytes [19], and activated T-lymphocytes [20]. In addition, MUC1 is shed into the serum of cancer patients in considerable amounts, which may interfere with the effects of therapeutic mAbs [21].

We previously identified an immunodominant O-glycopeptide epitope (Tn/STn-MUC1) generated by aberrant glycosylation of the VNTR domain of MUC1, which is not covered by immunological tolerance in MUC1 transgenic mice or in man [3, 22]. Thus, potent IgG Abs directed to this glycopeptide epitope were induced with Tn-glycopeptides coupled to KLH. We have also detected spontaneous IgG Abs to the epitope in a subset of breast cancer patients at time of diagnosis [23, 24]. These Abs are highly selective for the Tn/STn-glycopeptide epitope and do not react with the MUC1 peptide backbone or with the carbohydrate hapten Tn or STn alone. We have furthermore found that MUC1 shed into the circulation in late stage cancer patients is devoid of these aberrant glycoforms presumably due to selective clearance by innate immune lectin receptors [23]. Since the Tn/STn-MUC1 epitope is broadly expressed by cancer cells and not found in circulation it represents a prime target for therapeutic MAbs.

Here we demonstrate that the Tn/STn-MUC1 aberrant epitope is expressed on the cell surface of cancer cell lines, and that the mAb 5E5 Ab directed against the cancer-specific Tn-MUC1 glycopeptide epitope induce killing of human cancer cell lines by ADCC.

Materials and methods

Cell lines and tissues

The human breast cancer cell line MCF7 was cultivated in Dulbecco's Modified Eagle Medium (DMEM) 1885 supplemented with 5 % heat-inactivated fetal bovine serum (FBS) (BioWhittaker), 2 mM L-glutamine, and 10 μg/ml bovine insulin (Sigma Aldrich). T47D and HEK293 cells were cultured in DMEM 1965 supplemented with 10 % FBS and 2 mM L-glutamine. Paraffin-embedded sections (7 μm) from five human invasive ductal breast carcinomas were deparaffinized and microwave-heated prior to immunoflourescent staining. Additional sections were prepared from disease-free non-lactating and lactating breast tissue (n=2).

MUC1 mAbs and human serum samples

Murine MUC1 IgG1 mAbs used in the present study included 5E10 and HMFG2 (MUC1), 5E5 (Tn-MUC1), 2D9 (Tn-MUC1), and 1B9 (T-MUC1) (Fig. 1b) [3, 25]. 5E10 recognize a non-glycosylated epitope in the MUC1 tandem repeat sequence. 5E5 and 2D9 targets one immunodominant aberrant MUC1 glycopeptide epitope consisting of an O-linked GalNAc attached to the serine and/or threonine in the -GSTAP- region of the MUC1 tandem repeat (APPAHGVTSAPDTRPAPGSTAPP) [3, 25, 26]. A non-

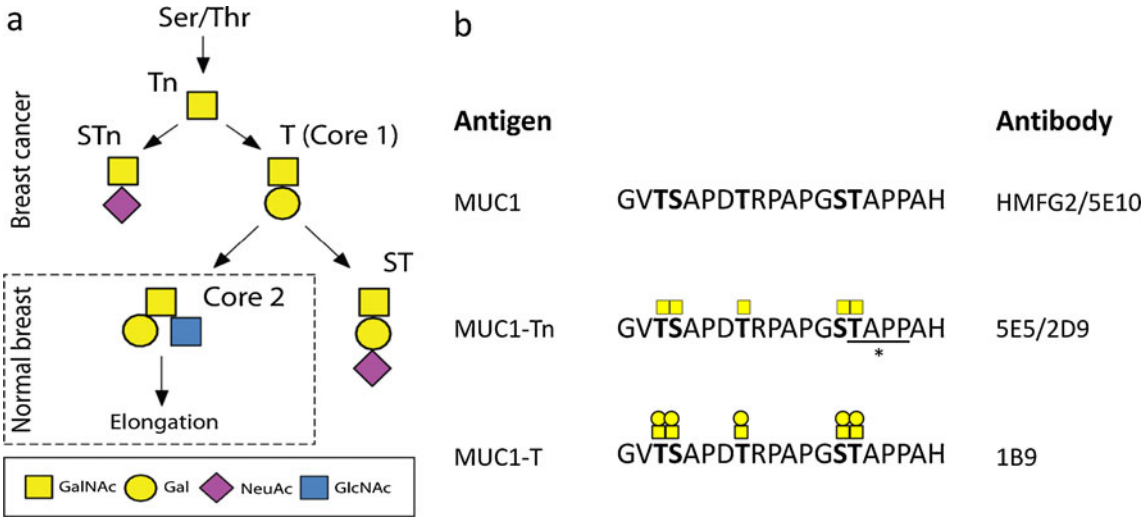

Fig. 1 O-glycosylation and MUC1 mAbs. **a** Initial steps of the biosynthesis of O-glycans in the mammary gland. O-glycosylation is initiated by the transfer of GalNAc to serine or threonine residues. The sialyl-Tn antigen is synthesized by the addition of a sialyl residue to GalNAc, whereas addition of a galactose residue to GalNAc results in core 1, the T antigen. The sialyl-T structure is synthesized by α2-3 sialylation of the T antigen. Further sialylation of sialyl-T can include α2-6 sialylation of GalNAc to form the disialylated T antigen and/or α2-8 sialylation of the α2-3 linked sialic acid residue. In a healthy mammary gland, a core-2 branch is introduced that is further elongated, sialylated, or fucosylated. **b** MUC1 mAbs and their antigens. MAbs 5E10 and HMFG2 react with the MUC1 peptide backbone independent of its glycosylation state, mAb 5E5 (* denotes epitope) and 2D9 reacts with Tn-MUC1, and mAb 1B9 reacts with T-MUC1

reactive murine IgG1 control antibody was used as a negative control (DAKO, Denmark).

Chemoenzymatic synthesis of glycopeptides

A MUC1 24-mer peptide containing the VNTR sequence was custom synthesized by Schafer-N (Denmark) and glycosylated *in vitro* using purified recombinant human uridine diphosphate (UDP)-GalNAc: polypeptide *N*-acetylgalactosaminyltransferases GalNAc-T1, T2, and T3 [27–30] as described previously [3]. From our previous studies GalNAc-T1, -T2, and -T3 is known to incorporate GalNAc residues at specific positions in the MUC1 tandem repeat resulting in the formation of the glycopeptide PPAHGVT (GalNAc)SAPDTRPAP**GS(GalNAc)T(GalNAc)A**PPAH (5E5 epitope in bold)[3, 27]. To create STn-MUC1, the GalNAc-substituted peptides were sialylated using purified recombinant human ST6GalNAc-I as described [31]. All glycopeptides were purified by high performance liquid chromatography (HPLC) on an 1100 Hewlet Packard system (Avondale, PA) using a Zorbax 300 SB-C18 column (9.4 mm×25 mm) (Agilent Technologies) with 0.1 % trifluoroacetic acid (TFA) and a gradient of 0–100 % acetonitrile. The reactions were monitored and the final products characterized by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry on a Voyager DE Pro (PerSeptive Biosystems) with 2,5-dihydroxybenzoic acid (Sigma-Aldrich, 25 mg/ml in 1:1 MeOH: MQ-H_2O) used as the crystallization matrix.

Immunofluorescense staining

Non-permeabilized cells were fixed with 4 % paraformaldehyde (PFA) for 30 min on ice, whereas permeabilized cells were treated with BD Cytofix/CytopermTM Fixation/Permeabilization Kit containing 4 % PFA and saponin. Permeabilized and non-permeabilized cells, as well as paraffin-embedded tissue samples were incubated with mAbs for 2 h at room temperature (RT), washed, and incubated with rabbit anti-mouse Ig-fluorescein isothiocyanate (FITC) (1:70, DAKO) for 45 min at RT. The stainings were examined using a Leica DM 4000 B microscope equipped with a Leica DC 300 FX digital or Zeiss LSM 710n camera after mounting with ProLong®Gold (Invitrogen). Localization studies were performed by confocal microscopy on a Zeiss LSM 710 confocal microscope. Alternatively, flow cytometric analysis was performed on a Cytomics FC 500 flow cytometer (Beckman Coulter), and the data were analyzed using CXP software (Beckman Coulter).

Biacore

The binding kinetics of the mAbs to the different MUC-1 peptides was studied by surface plasmon resonance using a Biacore 2000™ instrument (GE healthcare). For all experiments, 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005 % (v/v) surfactant P-20 at pH 7.4 was used as the running buffer. Purified preparations of mAbs 5E5 and 5E10 were immobilized in separate flow cells on CM5 sensor

chips (GE Healthcare) at surface densities of 22 fmol/mm^2 and 24 fmol/mm^2, respectively. Covalent linkage of the mAbs to the carboxylmethyl-dextran matrix on the CM5 sensor chip was performed with *N*-hydroxy-succinimide/*N*-ethyl-*N*′-[3-(diethylamino)propyl]-carbodiimide in 10 mM sodium acetate, pH 5.0, at a flow rate of 5 μl/min for 8 min. Serial 2-fold dilutions of the peptides MUC1 (4–2,000 nM), STn-MUC1 (4–2,000 nM), and Tn-MUC1 (4–2,000 nM) were injected at a flow rate of 50 μl/min at 20 °C into flow cells containing immobilized mAbs 5E5 and 5E10. Association data was recorded for 360 s and dissociation data for 750 s. After each run, the sensor chip was regenerated by a 30 s injection of 0.1 M acetic acid in 0.5 M NaCl, pH 2.5. The sensorgrams was processed by subtracting the signal obtained from a mock-coupled flow cell from the binding curves and selecting curves for kinetic analysis. The kinetic rate constants, k_{on} and k_{off}, were derived from the real-time analyses by fitting the association and dissociation phases to a bimolecular interaction model using the BIAevaluation 3.2 software. The equilibrium dissociation rate constant K_D was determined as k_{off}/k_{on}.

Evaluation of complement-mediated cytotoxicity (CDC) by Annexin V-FITC staining

Target cells (10^6 cells/ml) were trypsinized, washed in media, and suspended in media with human AB serum or in media with heat-inactivated (56 °C, 30 min) human AB serum. After incubation for 2 h at 37 °C with mAb or goat anti-human E-cadherin polyclonal Ab (R&D systems), complement-mediated cell death was evaluated using an Annexin V-FITC apoptosis detection kit (BD Pharmingen™). Flow cytometric analysis was performed on a Cytomics FC 500 flow cytometer (Beckman Coulter) and analyzed using CXP software (Beckman Coulter). Results were expressed as the percentage of dying cells, calculated according to the following equation: $(\text{experimental kill} - \text{spontaneous kill} \times 100)/(\text{maximal kill} - \text{spontaneous kill})$. To estimate antibody-induced membrane attack complex (MAC) formation on cancer cells, the antibody- and serum-treated cells were stained with 1 μg/ml mouse-anti human C5b-9 mAb (DAKO) diluted in 0.1 % BSA in PBS and analyzed by flow cytometry.

PBMC isolation from human blood donors

Peripheral blood mononuclear cells (PBMC) that were isolated from the blood of healthy donors by density centrifugation with Lymphoprep (Nycomed Pharma Diagnostics, Oslo, Norway) were washed and tested for viability by eosin staining. We found that >95 % of the cells were viable. Purified PBMCs were incubated overnight in RPMI 1640 supplemented with 10 % human AB serum with or without recombinant human interferon (IFN)-α (5,000 U/ml, Schering-Plough, Kenilworth, NJ), interleukin (IL)-2 (1,000 U/ml, Chiron, Emeryville, CA), IL-15 (25 ng/ml, Peprotech), and IL-21 (25 ng/ml, Peprotech) and used as effector cells in the ADCC assay.

CDC and ADCC assays

Target cells were loaded with 100 μCi ^{51}Cr (PerkinElmer) for 1 h at 37 °C and washed 5× in culture media. For the CDC assay, the ^{51}Cr-labeled target cells were plated at 5,000 per well in a 96-well flat-bottom plate in media alone, media with human serum, or media with heat-inactivated (56 °C, 30 min) human serum. The indicated mAb was added and incubated with the cells for 2 h at 37 °C. For the ADCC assay, the target cells were plated at 5,000 per well in a 96-well flat-bottom plate and incubated for 1 h with the indicated mAb or with human serum samples (1:5) before the addition of effector cells at the indicated effector-to-target (E/T) ratio. After 4 h of incubation at 37 °C, 30 μl of supernatant was removed from each well and plated on a LumaPlate-96, dried, and counted on a Packard's TopCount®. Results were expressed as the percentage of specific release, which is calculated according to the following equation: $(\text{experimental release} - \text{spontaneous release} \times 100)/(\text{maximal release} - \text{spontaneous release})$. The percentage of Ab-specific release was defined as: % specific release with MUC1 Ab – % release without Ab.

Results

The Tn-MUC1 epitope is expressed on the surface of cancer cells in breast cancer tissue

In agreement with our previous findings mAb 5E5 stained essentially all of the breast cancers examined without cross-reactivity to healthy tissue. In contrast mAbs 5E10 and HMFG2 (not shown) known to react with the majority of MUC1 glycoforms [25] showed distinct binding to normal healthy breast tissue, in particular to the luminal surfaces of acini and ducts. MAbs 5E5 and mAbs 5E10 stained breast cancer tissue in a heterogeneous manner, showing high reactivity in a subset of cells (Fig. 2a). MAb 5E5 showed distinct intracellular staining along with weak surface staining of cancer cells (Fig. 2a). To verify the surface localization of the 5E5 epitope Tn-MUC1 on breast cancer tissue, co-staining was performed using an antibody against a surface marker, E-cadherin. Confocal imaging confirmed surface expression of Tn-MUC1, although the majority of staining was observed inside the cancer cells (Fig. 2b). The same reactivity pattern was observed with mAbs 5E10 (Fig. 2a) and HMFG2 (not shown). Based on the known up regulation of MUC1 in lactating women, sections from two

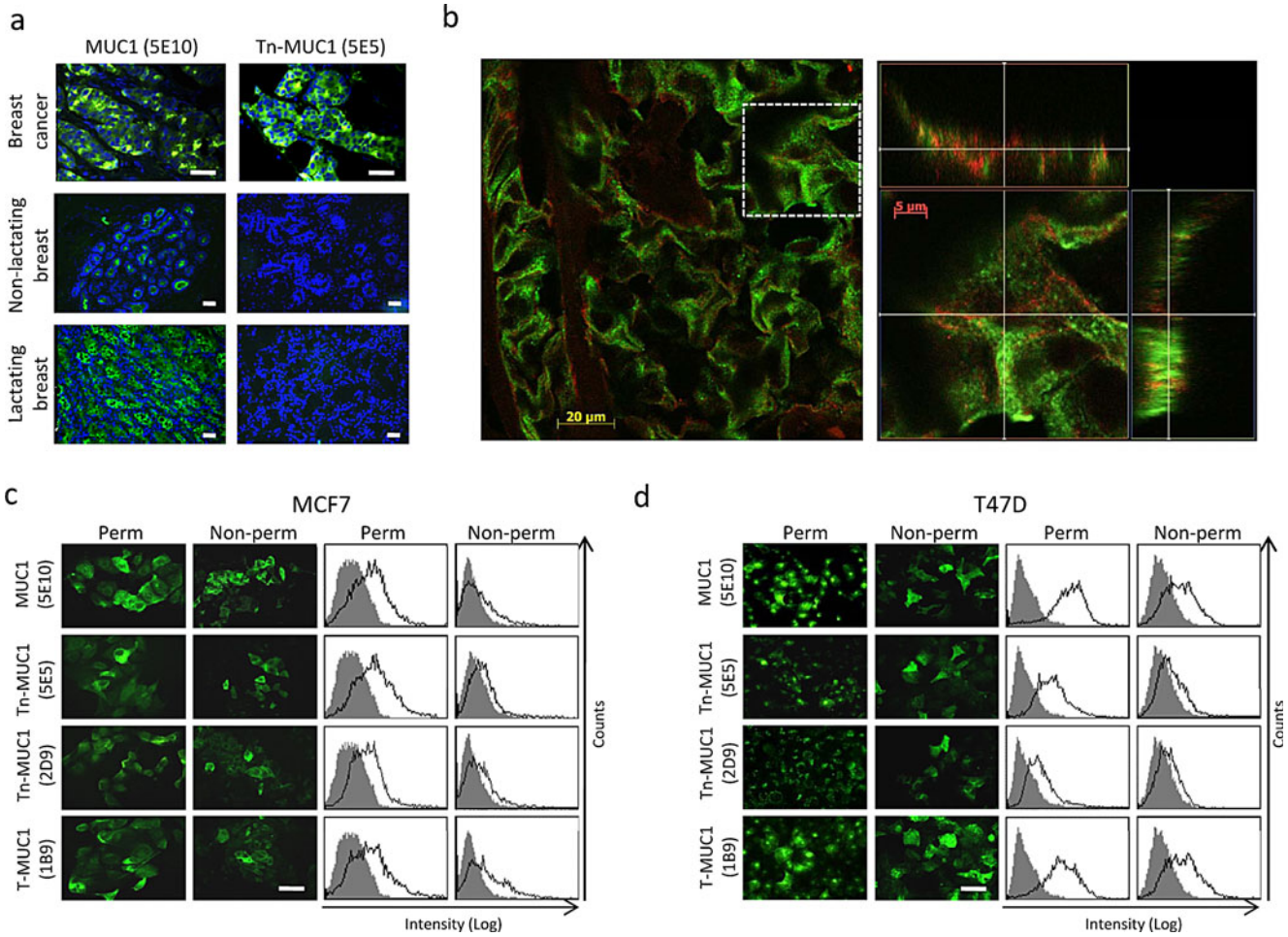

Fig. 2 Expression of Tn-MUC1 in breast cancer. **a** Immunofluorescence staining of invasive ductal carcinoma breast tissue and healthy lactating and non-lactating breast tissue by mAbs 5E10 (MUC1) and 5E5 (Tn-MUC1). **b** Multiple cross-sections were imaged using confocal microscopy technology. The left picture shows an overview. The right picture shows the X-Y axis analysis of 5E5 and E-cadherin, with colocalized 5E5 and E-cadherin shown in the vertical plane on top and intracellular accumulation of Tn-MUC1 (5E5; *green*) in the vertical plane shown to the right. The analyzed section is indicated with the *white square* in the left image. **c,d** Reactivity of mAbs 5E10 (MUC1), 5E5 (Tn-MUC1), 2D9 (Tn-MUC1), and 1B9 (T-MUC1) with two human breast cancer cell lines, MCF7 (**c**) and T47D (**d**), as visualized by immunofluorescence staining and flow cytometry (isotype-specific control antibody in *grey*)

specimens of lactating women were also evaluated. As expected, mAb 5E10 reacted strongly to epitopes in lactating breast tissue sections, while no mAb 5E5 reactivity was detected (Fig. 2a).

We next verified the expression of the 5E5 epitope Tn-MUC1 on the surface of cells from two human breast cancer cell lines, T47D and MCF7. Both breast cancer cell lines showed high expression of Tn-MUC1 as evaluated by immunofluorescence staining and flow cytometry (Fig. 2c, d). Similar to the staining patterns observed in breast tumor tissue, both cell lines showed heterogeneous 5E5 reactivity, with 5–15 % of the cells showing stronger staining than the remaining cell population. Similar staining patterns were obtained with mAbs 2D9 having similar specificity as 5E5 [3] and 5E10. In accordance with the observations in breast cancer tissue, 5E5 reacted predominantly intracellularly as evidenced by low reactivity with non-permeabilized cells and strong reactivity with permeabilized cells (Fig. 2c, d). In both cell lines, high expression of T-MUC1 (Galβ1-3GalNAcα-O-MUC1) was detected with mAb 1B9 (Fig. 2c,d) [25]. 3 C9 reactivity was mainly on the cell surfaces, *i.e.* strong reactivity with and without permeabilization. This is in agreement with earlier observations that O-glycans expressed on MUC1 in breast cancer consist primarily of T and ST (sialylated T) [3].

Binding affinity of mAb 5E5

We next determined the affinity constants for selected anti-MUC1 mAbs with binding to MUC1 glycopeptide epitopes using surface plasmon resonance (Biacore). The glycopeptide target GalNAc-MUC1 (Tn-MUC1) was synthesized

chemoenzymatically by *in vitro* glycosylation of a synthetic 24-mer MUC1 peptide using site-selective recombinant polypeptide GalNAc-transferases, GalNAc-T1, -T2, and -T3. This produced the MUC1 glycopeptide PPAHGVT(GalNAc)-SAPDTRPAP**GS(GalNAc)T(GalNAc)A**PPAH (5E5 epitope in bold) [3, 27]. Further *in vitro* glycosylation with recombinant ST6GalNAc-I created the glycopeptide target NeuAc2-6αGalNAc-S/T (STn-MUC1) as confirmed by MALDI-TOF MS (not shown).

mAb 5E10 bound non-glycosylated MUC1 and Tn-MUC1 with a K_D of approximately 1 μM, demonstrating low affinity binding (Fig. 3), while no specific binding to STn-MUC1 was observed (not shown). In contrast, mAb 5E5 had high affinity for Tn-MUC1, with a K_D of 1.7 nM. Lower binding affinity was measured for STn-MUC1 (K_D~100 nM) (not shown), while no specific binding was observed to non-glycosylated MUC1 (Fig. 3).

Complement-mediated and antibody-dependent cellular cytotoxicity with breast cancer cells

The ability of mAb 5E5 to induce complement-mediated cytotoxicity (CDC) of breast cancer cells was tested by both ^{51}Cr-release assay and flow cytometry. Both assays demonstrated very low levels of CDC (Fig. 4a, b), regardless of antibody and serum concentration (not shown). Although, the Annexin V-based assay detected a slight increase (from 17 % to 21 %) in cells undergoing apoptosis with mAb 5E5 compared to control antibody, the ^{51}Cr release assay showed a non-significant increase from 1.5 % to 3 % in terms of complement-mediated lysis of target cells. MAC complex formation on the cancer cells was visualized, and mAb 5E5 failed to induce the incorporation of MAC into the target cell membrane (Fig. 4c); this contrasted with the positive control antibody to E-cadherin, demonstrating that the

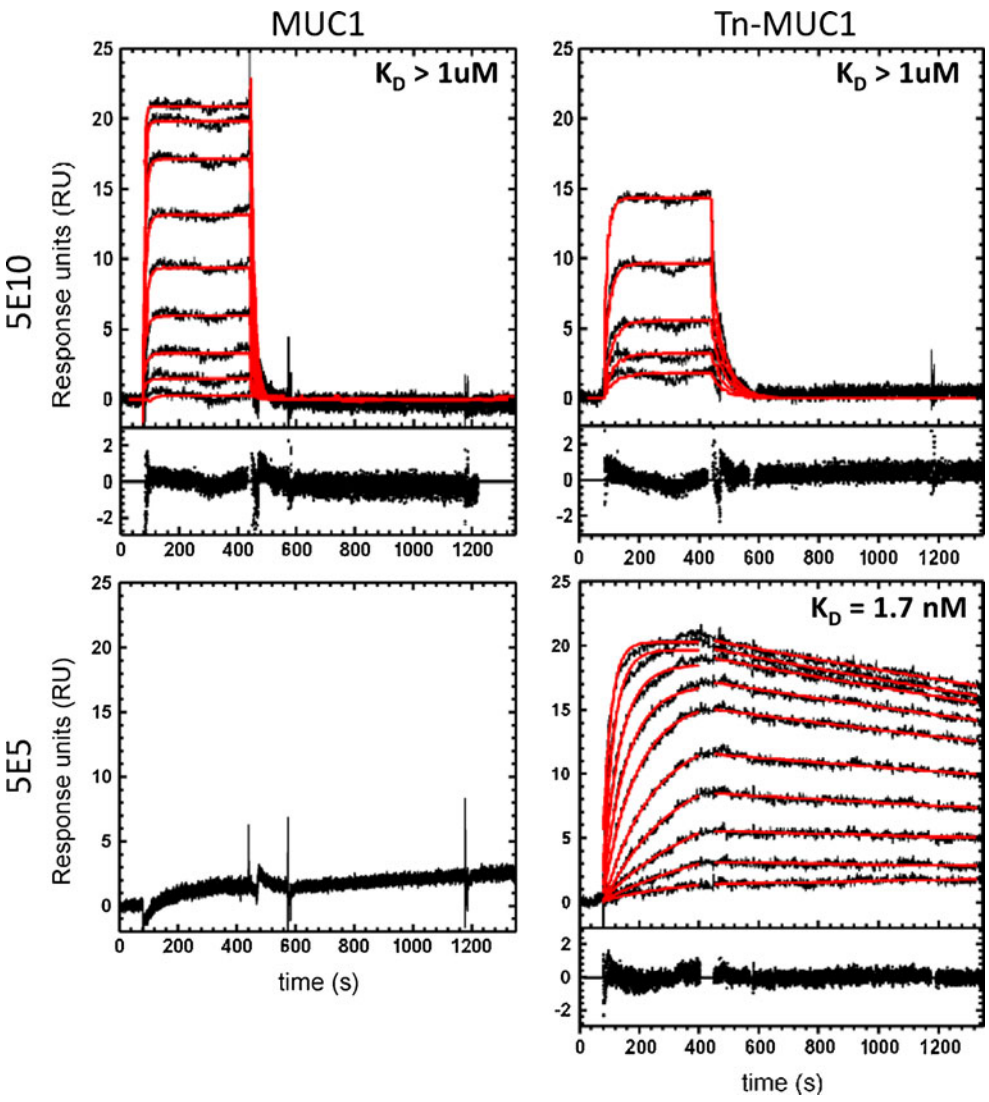

Fig. 3 Binding kinetics of MUC1 antibodies for MUC1 targets. Biacore analysis of the biomolecular interactions between glycopeptide-specific MUC1 antibodies and well-characterized MUC1 glycopeptide targets. Sensorgram overlay plots were generated when serial dilutions of non-glycosylated MUC1 24-mers or chemoenzymatically synthesized MUC1 glycopeptides were injected in flow cells containing immobilized mAb 5E10 (MUC1) or mAb 5E5. The y-axis represents the amount of bound antibody (in resonance units, RU), and the x-axis shows time (in seconds). Residual plots are shown at the bottom of sensorgrams where specific binding is detected. mAb 5E10 bound with low affinity to both non-glycosylated MUC1 (K_{on}=3.5*10^5 M, K_{off}=0.07 M, K_D=2*10^{-7} M), and Tn-MUC1 (K_{on}=2.7*10^4 M, K_{off}= 0.023 M, K_D=8.6*10^{-7} M). In contrast mAb 5E5 bound to Tn-MUC1 with high affinity (K_{on}=9.4*10^4 M, K_{off}= 1.6*10^{-4} M, K_D=1.7*10^{-9} M), while no specific binding to non-glycosylated MUC1 was observed

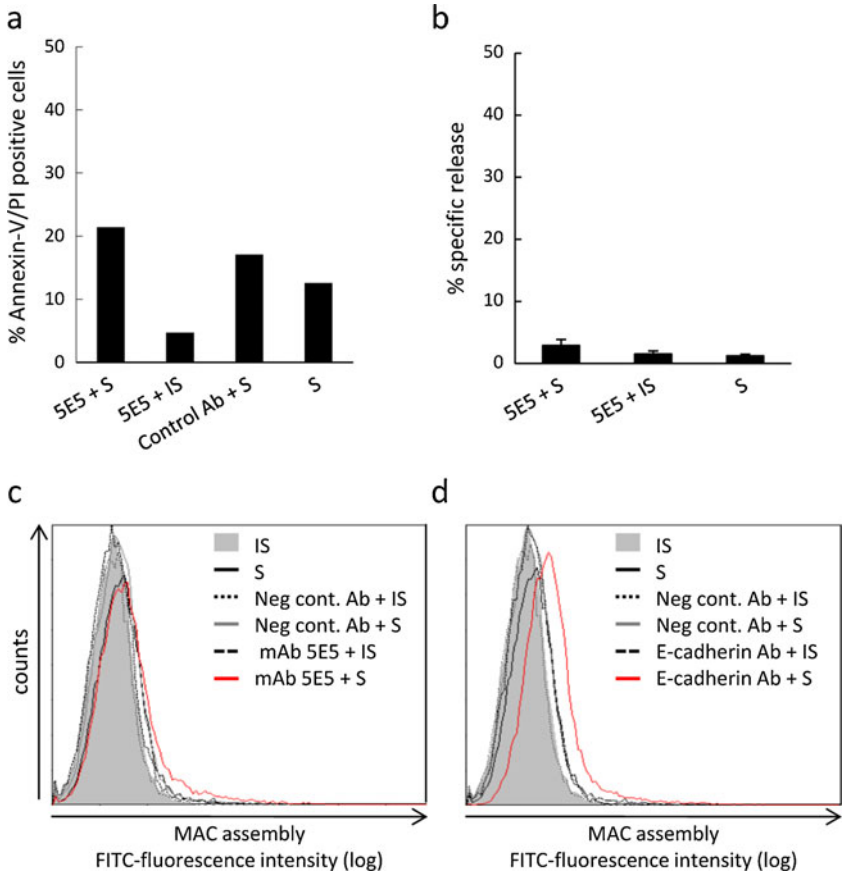

Fig. 4 mAb 5E5-induced CDC in human breast cancer cell lines. Human complement-dependent cytotoxicity (CDC) against the human breast cancer cell line MCF7 induced by mAb 5E5 targeting of Tn-MUC1. **a** Annexin V-FITC-based flow cytometric analysis of mAb 5E5-induced CDC. Cells positive for annexin or PI or both are considered dying or dead. A slight increase in CDC was detected with mAb 5E5 compared to a negative control antibody. **b** 5E5-induced CDC evaluated by standard ^{51}Cr release assay. While the Annexin V-FITC-based assay detects dying cells, the ^{51}Cr release assay only detects lysed cells, explaining the different results obtained by the two methods. **c** Evaluation of membrane attack complex (MAC) assembly by mAb targeting C5b-9. While MAC assembly was seen with E-cadherin antibody, no MAC assembly was seen with mAb 5E5. S = human AB serum, IS = inactivated human AB serum

cells retained the capacity to form the MAC complex (Fig. 4d).

We next tested the ability of 5E5 to induce antibody-dependent cellular cytotoxicity (ADCC). MAb 5E5 induced killing of a significant proportion of breast cancer cells by ADCC. We first established the assay using MCF7 as the target cells. Importantly, substantial ADCC was observed only after incubation of human PBMCs overnight with a cytokine cocktail consisting of IL-2, IL-15, IL-21, and IFN-α (Fig. 5). Pre-incubation of PBMCs with IFN-α alone had limited effects, while the addition of the cytokine cocktail significantly improved specific 5E5-induced ADCC compared to a negative control antibody (Fig. 5). Next, both MCF7 and T47D cells were tested in the ADCC assay. While 5E5 induced significant cytotoxicity of both cells, no killing was observed in the MUC1 negative-control cell line HEK293 (Fig. 6). The antibody HMFG2, which targets a peptide epitope in the MUC1 tandem repeat [32], was also tested and induced substantial killing (Fig. 6). In contrast the two other glyco-peptide MUC1 antibodies 2D9 (Tn-MUC1) and 1B9 (T-MUC1) did not induce ADCC (not shown), regardless of the surface expression of these antigens. This is consistent with the relatively low affinity of these antibodies for Tn-MUC1 and T-MUC1 [3, 5].

Discussion

Aberrant glycopeptide epitopes on MUC1 are emerging as promising targets for breast cancer immunotherapy, because

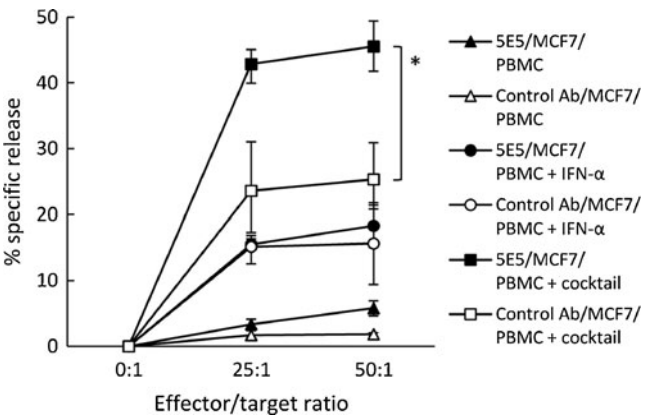

Fig. 5 Optimization of ADCC assay. ^{51}Cr-release ADCC assay with mAb 5E5 and breast cancer cell line MCF7. Human PBMCs was used as effector cells with and without preincubation with IFN-α alone or with a cytokine cocktail (IFN-α, IL-2, IL-15, and IL-21) (*$p<0.001$). The Y-axis indicates the percentage of specific release calculated according to the following equation: (experimental release − spontaneous release ×100)/(maximal release − spontaneous release)

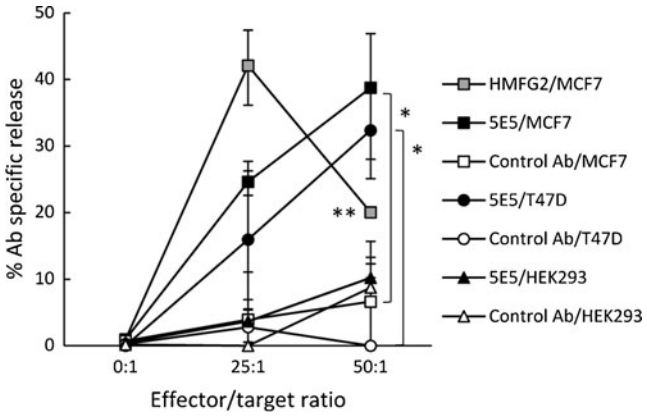

Fig. 6 mAb 5E5 induced ADCC in human breast cancer cell lines. mAb 5E5 (Tn-MUC1) induced ADCC in two human breast cancer cell lines, T47D and MCF7, when human PBMCs were preincubated with the cytokine cocktail (*$p<0.003$). mAb HMFG2 (MUC1) was used as a positive control antibody (**sd=+11.1/−9.6), and the MUC1-negative cell line HEK293 was used as a negative control cell line. The y-axis indicates Ab-specific release calculated using the equation: Ab − specific release = (specific release obtained in the presence of mAb 5E5) − (specific release obtained in the absence of antibody)

these, in contrast to peptide epitopes, are selectively expressed by cancer cells and MUC1 is overexpressed in 90 % of breast cancers as well as in many other types of cancers [33]. Glycans on breast cancer cells are characteristically altered from normal elongated core 2 type O-glycans to shorter immature core 1-type O-glycans such as T, sialyl-T, and Tn [4, 7, 34], which are associated with an unfavorable prognosis [35]. In this study, we demonstrated that the murine mAb 5E5 targeting one immunodominant aberrant MUC1 glycopeptide epitope reacted with the cell membrane of breast cancer cells and mediated ADCC tumor killing *in vitro*. The cancer specificity of mAb 5E5 has been demonstrated previously by immunohistochemistry of breast and ovarian cancer tissues [3, 36]. It has, however, been unclear if the epitope was available for recognition by immune effector mechanisms such as CDC and ADCC on the cell surface, or if the cancer-selectivity of 5E5 primarily reflected accumulation of the truncated glycoform of MUC1 inside cancer cells. Here we confirmed cell membrane staining of the 5E5 epitope (Tn-MUC1) in breast cancer tissues and cell lines and found that mAb 5E5 was able to induce potent ADCC activity. This may be due to the high affinity (K_D= 7 nM) of 5E5, since other MUC1 MAbs with considerable lower affinity (2D9 (Tn-MUC1) and 1B9 (T-MUC1) failed to induce ADCC despite ability to label the cell surface [3, 25].

In this study, we activated PBMCs from healthy donors with a combination of IL-2, IL-15, IL-21, and IFN-α, which increases expression of Fc-γ-receptors (FcγR) [37]. This was found to be essential for induction of significant ADCC and is in accordance with the importance of activation of natural killer (NK) in ADCC and the suggestion that co-treatment with cytokines can enhance effector cell activation in passive immunotherapy [38–40]. We did not detect induction of CDC by 5E5 or the other MUC1 antibodies in agreement with lack of MAC formation by mAb 5E5 (Fig. 4). This might be explained by the low level of cell surface expression of Tn-MUC1 as well as the biochemical and biophysical nature of the target antigen. Earlier attempts to mediate CDC through mucin targets have failed and mucins are therefore considered unsuitable for effective complement activation [41]. However, inhibitory complement regulators are often over expressed by tumor cells [42, 43], which could compromise CDC induction as well [44].

A number of mAbs with reactivity to all glycoforms of MUC1 have been tested for their ability to induce ADCC. Most of these antibodies target non-glycosylated epitopes and therefore do not discriminate between MUC1 derived from cancer cells *versus* healthy cells [45]. One MUC1 antibody (HMFG1) have been tested in its humanized form in a small Phase I trial and was well tolerated but it was not clear if there was any effect on survival [46]. A major advantage with targeting Tn-MUC1 with MAb immunotherapies is that there is little or no circulating Tn-MUC1 in patients to inhibit efficient tumor cell targeting of MAbs. This is in contrast to non-selective MUC1 antibodies like HMFG1 that bind circulating MUC1 shed from healthy cells that escape the hepatic clearance mechanisms [23, 47]. An exception is the antibody PankoMab, which targets a Tn-glycopeptide in the -PDTR- region of the MUC1 tandem repeat [48]. Like 5E5, PankoMab is tumor-specific and has ADCC activity [49].

We previously showed that vaccination of disease-free breast cancer patients with varying amounts (3–30 μg injected three times) of Tn-106-mer-MUC1[22] induced a glycopeptide-specific immune response similar to mAb 5E5 [23]. Using a MUC1 glycopeptide array that presented all possible variants of the GalNAc-MUC1 epitopes we have previously investigated MUC1 reactivity in Tn-MUC1 vaccinated patients. Analysis of the epitopes recognized by the patients demonstrated that the majority of vaccinated patients reacted with both the bis-glycosylated-GST-epitope similar to mAb 5E5. Many other factors are important for the induction of effective ADCC responses in vaccinated patients. For example variable expression of NK receptors such as CD94, NKG2D, NKp65, NKp80 may affect the activation threshold through CD16 [50]. Furthermore, FcγR polymorphism are known to correlate with variation in clinical responses to tumor targeting mAbs in certain malignancies [51]. It will therefore be interesting in the future to investigate serum samples from patients vaccinated with Tn-MUC1 vaccines for their ability to induce ADCC, and to test if this correlates with disease development.

In conclusion, mAb 5E5 shows promise as an immunotherapeutic antibody in its humanized form for use in

passive immunization strategies due to its affinity, specificity, and its ability to induce ADCC.

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