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N-glycan-defective breast cancer cells induce a phenotypic switch in polarization of bone marrow-derived macrophages

Abstract

Purpose: To investigate the effect of *N*-glycan-defective mammary adenocarcinoma cells on the polarization of macrophages.

Methods: *N*-glycan-defective breast cancer cells (MA782 cells) were prepared by swainsonine (SW) treatment and the cytotoxicity of SW to MA782 cells was evaluated using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The *N*-glycan-defective MA782 cells were co-cultured with bone marrow-derived macrophages (BMDMs) for 48 h *in vitro*, and then the BMDMs and the co-cultured supernatant were analyzed for macrophage phenotypic using FQRT-PCR, FCM and ELISA.

Results: SW-treated MA782 cells expressed defective *N*-glycan on the cell surface in a dose-dependent manner ($*p < 0.05$). MTT assays showed that neither the 1 $\mu\text{g/mL}$ nor 5 $\mu\text{g/mL}$ SW treatments showed significant inhibition of MA782 cell growth *in vitro*. The expression of iNOS and agr-1 in the 5 $\mu\text{g/mL}$ SW-treated group were 4.75-fold higher and 3.7-fold lower than that in the untreated group, respectively ($*p < 0.05$). Mean fluorescence intensity of CD16/32 expressed in the cells treated with 5 $\mu\text{g/mL}$ SW was significantly higher in comparison with the untreated group (65 vs. 7, $*p < 0.05$), though the percentage of CD16/32-positive cells were not significantly different. Furthermore, the expression of CD206 and dectin-1 in the 5 $\mu\text{g/mL}$ SW-treated group was significantly decreased ($3.1 \pm 0.3\%$ and $4.1 \pm 1.1\%$, respectively) in comparison with the untreated group ($40 \pm 3\%$ and $8.9 \pm 1.2\%$, respectively, both $p < 0.05$). In addition, the 5 $\mu\text{g/mL}$ SW-treated group secreted more TNF- α (350 ± 25 pg/mL) and less IL-10 (89 ± 7.2 pg/mL) than the untreated group (80 ± 3 pg/mL and 150 ± 10 pg/mL, respectively, both $p < 0.05$).

Conclusion: *N*-glycan-defective MA782 cells can induce the differentiation of BMDM into proinflammatory M1 macrophages *in vitro*.

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Carbohydrates are attached to proteins in two major linkages: *N*-glycans and O-glycans. Carbohydrate chains in mature *N*-glycans include the typical high-mannose, hybrid and complex forms. N-acetylglucosaminyltransferase V (Mgat5 or GnT-V) plays an important regulatory role in the synthesis of complex *N*-glycans and this process is highly conserved between yeast and mammals [1]. Mgat5 and its glycan products, beta-1,6-GlcNAc-branched *N*-glycans, are tumor-associated glycoproteins that are commonly increased in malignancies and their level of expression has been found to correlate with disease progression [2-4]. Furthermore, many studies have demonstrated the association between increased leucoagglutinin (L-PHA) binding and increased tumor cell invasiveness. Likewise, cancers of breast, colon and melanomas show increased levels of beta-1,6-GlcNAc-branched *N*-glycan measured by L-PHA immunohistochemistry [2,5], as L-PHA has been shown to bind specifically to this structure [2]. As reported [5], L-PHA reactivity is also increased in atypical hyperplasia and carcinomas of breast in comparison with normal and benign lesions. Beta-1, 6-branched oligosaccharides are increased in lymph node metastases and are predictors of poor prognosis of breast carcinoma.

In previously studies, blocking expression of Mgat5-modified glycans in murine mammary adenocarcinoma MA782 cells using short hairpin RNA (shRNA) has been shown to prevent Mgat5-suppressed tumor progression both *in vivo* and *in vitro* and to activate CD4⁺ T cells and macrophages [6], but the incipient mechanism for this phenomenon is still ill-defined. Tumor cells use multiple mechanisms to invade extracellular matrix and metastasize to distant organs. The interaction between the tumor cells and stromal cells in the tumor microenvironment plays an important role in tumor growth and metastasis and macrophages are prominent stromal cells in this interaction. Macrophages secrete a variety of growth factors, cytokines, chemokines and enzymes that regulate tumor growth, angiogenesis, invasion and metastasis [7]. Macrophages are highly versatile, as local environmental factors shape their phenotypic and functional properties [8]. Operationally, two distinct activation states have been defined for macrophages: the M1 (or classically activated) and M2 (or alternatively activated) macrophages, each associated with specific phenotypes and functions [8-10]. M1 macrophages typically produce copious amounts of proinflammatory cytokines (tumor necrosis factor alpha, interleukin-12) and generate reactive oxygen species. M1 macrophages participate as inducers and effectors in T helper-1 immunity. M2 macrophages secrete high levels of anti-inflammatory cytokines (IL-10) and are characterized by increased arginase-1 (Arg-1) activity and sur-

face expression of macrophage mannose receptor (MMR, CD206) and dectin-1. Functionally, M2 macrophages are considered to tune inflammatory responses and promote tissue repair [11,12].

In this study, beta-1,6-GlcNAc-branched *N*-glycan-defective MA782 cells were prepared by Swainsonine (SW) treatment, and the effect of beta-1,6-GlcNAc-branched *N*-glycan on the polarization of macrophages was investigated.

Materials and Methods

Macrophage isolation and culture.

Bone marrow-derived macrophages (BMDMs) were obtained using the technique described previously [13]: DMEM (Invitrogen Life Technologies, Carlsbad, CA), containing 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.37% (wt/vol) NaHCO₃, 10% (vol/vol) heat-inactivated FBS, and 10% (vol/vol) L929 cell-conditioned medium, as a source of M-CSF, was used for the isolation, culture, and stimulation of the macrophages. Bone marrow cells were aseptically flushed from the dissected pelvises, femurs and tibias of BALB/c mice with a jet of complete medium directed through a 25-gauge needle. The bone marrow cells were adjusted to a density of $\sim 3 \times 10^5$ cells/mL of medium and distributed in 2 mL aliquots into 22 mm diameter wells of 12-well tissue culture plates or 13 mL aliquots into 100 mm diameter culture dishes. The cells were maintained at 37°C under a 10% (vol/vol) CO₂ atmosphere for 5 d and thereafter the medium was changed on alternate days until the cells were used between days 5 and 7.

Swainsonine treatment

MA782 cells derived from mouse mammary adenocarcinoma¹⁴ were grown in DMEM that was supplemented with 10% FBS. For SW treatment, MA782 cells were diluted to 1×10^6 cells/mL, and seeded into 6-well plate at 2 mL per well. SW (Sigma, St. Louis, MO, USA) was added at 1 µg/mL or 5 µg/mL to the medium with MA782 cells. After 24 h incubation, MA782 cells were harvested and washed several times with fresh medium.

Transfection

The cDNA of Mgat5 was cloned into eukaryotic expression vector pcDNA3.1A to yield plasmid pcDNA3.1-Mgat5 as described previously [6]. Plasmids were purified using endotoxin-free DNA extraction kit (QIAGEN, Valencia, CA). A total of 5×10^5 cells/mL were plated in 60 mm plates at a density ap-

appropriate to achieve 60% confluency 24 h before transfection. Cells were transfected with 4 µg of each plasmid using Lipofectin2000 (Invitrogen Life Technologies). Cells were harvested 72 h post transfection and suspended in PBS.

L-PHA binding assay

After being treated with SW for 24 h, MA782 cells were collected. FITC-conjugated leucoagglutinin (L-PHA, Vector Laboratories, Peterborough, UK) was added to a final concentration of 2.5 µg/mL in PBS, and cells were incubated for 30 min at 4°C. The cells were collected by centrifugation and then suspended in 1 mL PBS-BSA for flow cytometry (FCM) analysis.

Cell proliferation assay.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma, St.Louis, MO, USA) proliferation assay was used to determine the effects of SW on MA782 cell growth. The cells were resuspended in fresh medium, diluted to 1×10^5 cells/mL, and seeded into four 96-well plates at 100 µL per well. Cells were treated with SW at doses of 1, 3 or 5 µg/mL, and grown for up to 4 days. One plate was used for MTT assay each day for a total of 4 days. Twenty microliters of MTT (5 mg/mL) was added to each well, and the cells were incubated at 37°C for another 4 h. The culture medium was then replaced with 150 µL dimethyl sulfoxide (Sigma). After shaking for 10 min, the plate was read for the absorbance at 490 nm on a plate reader. The proliferation rate was determined as follow: absorbance of treated group/absorbance of untreated group.

Flow cytometry assay

BMDMs were stained with the following monoclonal antibodies diluted in 1% FBS in PBS: PE anti-F4/80 (eBioscience, San Diego, CA), PE anti-CD16/32 (BD Biosciences, San Diego, CA), PE anti-CD206 (BD Biosciences), PE anti-Dectin-1 (eBioscience). The surface molecule F4/80, CD16/32, CD206 and Dectin-1 were analysed according to the protocols of the manufacturer in a Beckman Coulter EPICS ALTRA II flow cytometer.

Co-culture of N-glycan-defective MA782 cells and bone marrow-derived macrophages

Bone marrow-derived macrophages and SW-treated or -untreated MA782 cells were co-cultured in 6-well plate in a ratio of 1:10 at 37°C. There were 2×10^5 BMDMs and 2×10^6 N-

glycan-defective MA782 cells in each well of a 6-well plate with a total culture medium volume of 2 mL. After 48 h incubation, the supernatants of each well were collected separately via centrifugation for the ELISA assay and the BMDMs were collected for the phenotypic analysis.

Enzyme-Linked Immunosorbent Assay (ELISA)

After co-cultured for 48 h, the medium of each well were collected separately with centrifugation, and IL-10 and TNF-α contents were then determined with ELISA kit (eBioscience) as described by the manufacturer.

RNA Isolation and Real Time Fluorescence Quantitative RT-PCR (FQ RT-PCR)

Total RNA was extracted from the cell lysates using TRIZOL (Gibco-Invitrogen). RNA was measured by the real-time reverse transcription polymerase chain reaction using the QuantiTect™ SYBR Green PCR Handbook Kit (QIAGEN). The samples were heated to 95°C for 5 min, followed by 30 cycles of 95°C for 10 s, 59°C for 15 s, and 72°C for 20 s. The oligonucleotide primers used were as follows: iNOS forward: 5'-CTG CAGCACTTGGATCAGGAACCTG -3', reverse: 5'-GGA GTAGCCTGTGTGCACCTGGAA-3'; Arg-1 forward: 5'-CAGAAGAATGGAAGAGTCAG-3', reverse: 5'-CAGATAT GCAGGGAGTCACC-3'; GAPDH forward: 5'-ACCACA GTCCATGCCATCAC-3', reverse: 5'-TCCACCACCCTG TTGCTGTA-3'. Primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. The relative expression quantity of target gene in each sample was normalized by comparing with that of GAPDH.

Measurement of nitrite production using the Griess reaction

Nitrite in culture supernatants was measured by adding 100 µL of Griess reagent (1% sulfanilamide and 0.1% N-[1-naphthyl]ethylenediamine dihydrochloride in 5% phosphoric acid) to 100 µL samples of medium. All measurements were performed in triplicate. The concentration of NO₂⁻ was determined using a standard curve generated with NaNO₂.

Arginase assay.

Cells were lysed in 0.1% Triton X-100. Tris-HCl was added to yield final concentrations of 12.5 mM and MnCl₂ added to final concentrations of 1 mM. Arginase was activated by heating for 10 minutes at 56°C, and L-arginine substrate was added to final concentrations of 250 mM. Reactions were incubated at 37°C for 30 minutes and

FIGURE 1. Swainsonine inhibited beta-1,6-GlcNAc-branched N-glycan biosynthesis in MA782 cells without cytotoxicity. (A) MA782 cells, treated with SW, at indicated concentration. At 24 h post-incubation, cells were harvested, stained with FITC-labeled L-PHA, and analyzed by FCM. The illustrated data were from a representative of three separate experiments. The percentages of PHA-positive cells are shown. The gray shadow represented isotype control. (B) The statistical data of the percentages of PHA⁺ MA782 cells from each group after SW treatment. (C) The statistical data of the percentages of PHA⁺ MA782 cells 24 h and 48 h after SW treatment. (D) Measurements of the SW-treated MA782 cells' growth and proliferation in vitro by MTT assay as described in the Methods. Data represent mean $\pm$ SEM of 3 independent experiments.

stopped by the addition of $\text{H}_2\text{SO}_4/\text{H}_3\text{PO}_4$. After addition of α -isonitrosopropiophenone and heating for 30 minutes at 95°C , urea production was measured by absorbance at 540 nm and normalized to total protein content. All measurements were performed in triplicate.

Statistical Analysis

Data were expressed as the mean $\pm$ standard error of the mean (SEM) of at least three independent experiments each performed in triplicate. All statistical analyses were performed in the SPSS 11.0 statistical software package. Experimental data were analyzed by ANOVA, with the Student-Newman-Keuls

method for multiple comparisons among means. Values of $p < 0.05$ were considered statistically significant.

Results

Swainsonine inhibited N-glycan biosynthesis in MA782 cells without cytotoxicity

The plant lectin leucoagglutinin (L-PHA) binds specifically to mature Mgat5 products [15] and, therefore, has been used as a probe for Mgat5-modified glycans. Inhibition of Mgat5-modified N-glycan product expression by SW was examined by L-PHA binding assay. The results showed that L-PHA binding

FIGURE 2. Beta-1,6-GlcNAc-branched *N*-glycan-defective MA782 cells induced iNOS expression in BMDMs. (A) After cultured *in vitro* for 7 days as described in the methods, the expression of F4/80 on BMDMs was detected by FCM. The gray shadow represented isotype control and the data were from a representative of three separate experiments. BMDMs co-cultured with SW-treated or parental MA782 cells for 48h. (B) FQRT-PCR showed that iNOS expression increased, whereas arg-1 expression decreased in SW-treated groups in comparison with untreated group (**p*<0.05). The BMDMs cultured alone was set as control group. Data are mean $\pm$ SEM, *n*=3 independent experiments. (C) The activity of iNOS and Arg-1 analyzed by Griess reaction and arginase assay as described in the methods. The BMDMs cultured alone were set as control. Data represent mean $\pm$ SEM of 3 independent experiments.

to Mgat5-modified glycans on MA782 cells was significantly decreased in SW-treated cells compared with parental cells in a dose-dependent manner (Fig. 1A, 1B, **p*<0.05). Furthermore, the *N*-glycan were still defective on MA782 cells 48 h after SW treatment as measured by L-PHA binding assay (Fig. 1C, **p*<0.05). Cell proliferation assay showed that SW had no influence on the MA782 cell growth at either 1 µg/mL or 5 µg/mL, in comparison with parental cells (Fig. 1D). These data indicate that SW significantly inhibits *N*-glycan biosynthesis in MA782 cells *in vitro* for at least 48 h, without cytotoxicity.

N-glycan-defective MA782 cells induced iNOS expression in BMDMs

After been cultured *in vitro* for 7 consecutive days, bone marrow-derived macrophages were harvested for phenotypic analysis. The flow cytometry data showed that more than 90% of the BMDMs expressed the macrophage-specific marker, F4/80 (Fig. 2A). To investigate the effect of *N*-glycan-defective MA782 cells on the BMDMs, the BMDMs were co-cultured

with *N*-glycan-defective MA782 cells for 48 h, then the expression of iNOS and Arg-1 in macrophages were detected using FQRT-PCR and enzyme activity tests, as described above. The results of FQRT-PCR analysis showed that the expression of iNOS mRNA in macrophages co-cultured with *N*-glycan-defective MA782 cells was significantly increased in comparison with macrophages co-cultured with parental MA782 cells, whereas the expression of Arg-1 was significantly decreased (Fig. 2B, $*p < 0.05$). The results of the Griess reaction showed that there was more accumulated nitrite in the culture medium of macrophages co-cultured with *N*-glycan-defective MA782 cells than in the medium of macrophages co-cultured with parental MA782 cells (Fig. 2C, $*p < 0.05$). Moreover, higher arginase activity was observed in macrophages co-cultured with parental MA782 cells detected by arginase assay as described (Fig. 2C, $*p < 0.05$). These data indicate that *N*-glycan-defective MA782 cells induce iNOS expression in BMDMs, but not arg-1 expression. It may be that the *N*-glycan-defective MA782 cells induce BMDMs to differentiate mainly into proinflammatory M1 macrophages.

N-glycan-defective MA782 cell-induced CD16/32 expression in BMDMs

The surface molecule expression is different for M1 and M2 macrophages. To evaluate the effect of *N*-glycan-defective MA782 cells on the surface molecules expressed in the BMDMs, the expression of CD16/32, CD206 and dectin-1 in the BMDMs co-cultured with *N*-glycan-defective MA782 cells was analyzed by flow cytometry. The results of FCM showed that BMDMs co-cultured with *N*-glycan-defective MA782 cell expressed more CD16/32 with less CD206 and dectin-1 expression than BMDMs co-cultured with MA782 cells (Fig. 3A, 3B, 3C, $*p < 0.05$). Taken together, phenotypic differences were apparent between the macrophages co-cultured with *N*-glycan-defective MA782 cells and those co-cultured with parental MA782 cells, and BMDMs co-cultured with *N*-glycan-defective MA782 cell were probably polarized to the M1 phenotype.

N-glycan-defective MA782 cells induced TNF- α expression in BMDMs

To further verify the ability of *N*-glycan-defective MA782 cells to induce a phenotypic switch in BMDM polarization, the cytokines secreted into the culture medium after co-cultured for 48 h were detected by ELISA. The results of ELISA imply that macrophages co-cultured with *N*-glycan-defective MA782 cell secreted more TNF- α and less IL-10 into supernatant

than macrophages co-cultured with parental MA782 cells (Fig. 4A, 4B, $*p < 0.05$). Taken together, these data support the hypothesis that *N*-glycan-defective MA782 cells induce BMDMs to differentiate into proinflammatory M1 macrophages.

Mgat5 overexpressed MA782 cells induced Arg-1 expression in BMDMs

Although SW can inhibit *N*-glycan synthesis, this chemical is also well-known for other functions. To confirm that the polarization of BMDMs is caused by the effect of *N*-glycan on MA782 cells, but not other factors, MA782 cells were transfected with the plasmid pcDNA3.1-Mgat5 and the vector plasmid pcDNA3.1A. The enhanced expression of *N*-glycan on MA782 cells 72 h after transfection with pcDNA3.1-Mgat5 was observed as measured by L-PHA binding assay (Fig. 5A, $*p < 0.05$). *N*-glycan-enhanced MA782 cells were co-cultured with BMDMs for 48 h, then the expression of iNOS and Arg-1 in macrophages was detected. The results of FQRT-PCR showed that the expression of Arg-1 mRNA in macrophages co-cultured with *N*-glycan-enhanced MA782 cells was significantly increased in comparison with those co-cultured with parental MA782 cells or MA782 cells transfected with vector plasmid, whereas the expression of iNOS was significantly decreased (Fig. 5B, $*p < 0.05$). These data indicate that the overexpression of Mgat5 in MA782 cells, which leads to the enhanced *N*-glycan expressed on the cells surface, could induced Arg-1 expression in BMDMs.

Discussion

In a previous study, Mgat5 specific-short hairpin RNA (shRNA) not only blocks expression of Mgat5 and Mgat5-modified glycan products, but also inhibits MA782 cell growth *in vitro* [6]. To avoid the influence of the viability of MA782 cells on the results of this study, when co-cultured with BMDMs, Mgat5 specific shRNA was replaced by SW, an *N*-glycan inhibitor that inhibits Golgi alpha-mannosidase II and ultimately causes the inhibition of N-linked beta-1,6-oligosaccharide expression upstream of the action of Mgat5 [16]. The L-PHA binding assay showed that L-PHA binding to Mgat5-modified glycans on MA782 cells was significantly decreased in SW-treated cells in comparison with parental cells and that this decrease was dose-dependent (Fig. 1A, 1B, $*p < 0.05$). Furthermore, the MTT assay showed that SW had no influence on the MA782 cell growth (Fig. 1D). Jun Hama-guchi *et al.* also reported that SW, at the concentration of 5 $\mu\text{g}/\text{mL}$, showed no cytotoxicity on murine colorectal cancer cells [17]. These data indicated that SW could effectively inhibit *N*-

FIGURE 3. Beta-1,6-GlcNAc-branched *N*-glycan-defective MA782 cells induced CD16/32 expression in BMDMs. BMDMs were co-cultured with SW-treated or parental MA782 cells for 48 h. (A) The expression of CD16/32, CD206, dectin-1 on BMDMs was analyzed by FCM. The gray shadow represented isotype control and the BMDMs cultured alone were set as control. Statistical analysis of the mean fluorescence intensity (MFI) (B) and percentage of positive cells (C) are shown. Data represent mean $\pm$ SEM of 3 independent experiments.

FIGURE 4. Beta-1,6-GlcNAc-branched *N*-glycan-defective MA782 cells induced TNF- α expression in BMDMs. BMDMs were co-cultured with SW-treated or parental MA782 cells for 48h and the supernatants were collected. The content of TNF- α (A) and IL-10 (B) in the supernatant, secreted from BMDMs, was analyzed by ELISA. The supernatant from BMDMs cultured alone was set as control. Data represent mean $\pm$ SEM of 3 independent experiments.

FIGURE 5. *N*-glycan-enhanced MA782 cells induced Arg-1 expression in BMDMs. (A) After transfection with indicated plasmid for 72h, The *N*-glycan expressed on MA782 cells was detected by L-PHA binding assay. Data are mean $\pm$ SEM, $n=3$ independent experiments. (B) 2×10^5 BMDMs co-cultured with 2×10^6 *N*-glycan-enhanced MA782 cells FQRT-PCR showed that iNOS expression decreased, whereas arg-1 expression increased in pcDNA3.1-Mgat5 groups in comparison with vector or untransfected group (* $p < 0.05$). Data represent mean $\pm$ SEM of 3 independent experiments.

glycan biosynthesis in MA782 cells without cytotoxicity *in vitro*.

Macrophages are a major component of inflammatory infiltrate of tumors [18,19]. Tumor-associated macrophages (TAMs) have complex dual functions in terms of their anti-tumor or pro-tumor effects. This functional complexity is related to the heterogeneity of macrophage population, which holds a continuum of diverse functional states. At one end of the continuum are the classically-activated M1 macrophages that produce effector molecules, such as reactive oxygen intermediates, reactive nitrogen intermediates and TNF- α , to limit tumor growth. Ohri *et al.* recently reported that the M1 macrophage density in the tumor islets is positively associated with extended survival of NSCLC patients [20]. At the other end are the alternatively-activated M2 macrophages that promote tumor growth and metastasis by secretion of matrix-degrading enzymes, angiogenic factors and immunosuppressive cytokines/chemokines [10]. The balance of these macrophage forms determines the anti- or pro-tumor effects of the macrophage population [18]. In this study, parental MA782 cell-stimulated BMDMs up-regulate the expression of both M1 macrophage markers (iNOS, TNF- α) and M2 macrophage markers (arg-1, CD206, dectin-1, IL-10) (Fig. 2, Fig. 3, Fig. 4), in comparison with BMDMs cultured alone, although for most markers, these differences were not statistically significant. These data indicate that MA782 cells induce polarization of BMDMs to both M1 macrophages and M2 macrophages. Interestingly, this balance could be switched when BMDMs co-cultured with *N*-glycan-defective MA782 cells: BMDMs stimulated by *N*-glycan-defective MA782 cells expressed more M1 macrophage markers, such as iNOS, CD16/32 and TNF- α (Fig. 2, Fig. 3, Fig. 4), and less M2 macrophages markers, such as arg-1, CD206, dectin-1 and IL-10 (Fig. 2, Fig. 3, Fig. 4), than those co-cultured with parental MA782 cells. Then, to further confirm the role of *N*-glycan in inducing a phenotypic switch in BMDMs polarization, the *N*-glycan expression on MA782 cells was upregulated following transfection with the plasmid pcDNA3.1-Mgat5, which encoded the cDNA of mouse Mgat5 (Fig. 5A). Interestingly, BMDMs co-cultured with *N*-glycan-enhanced MA782 cells expressed more Arg-1 and less iNOS than those co-cultured with MA782 cells transfected with the vector plasmid (Fig. 5B, $p < 0.05$). These findings suggest that the polarization of BMDMs co-cultured with MA782 cells was closely related to the changes in *N*-glycan on the MA782 cells. All of these data indicate that *N*-glycan-defective MA782 cell induce a phenotypic switch in BMDM polarization, where BMDMs differentiated mainly into proinflammatory M1 macrophages.

Several lines of evidence indicate that the inflammatory cells and cytokines found in tumors are more likely to contribute to tumor growth, progression and immunosuppression than they are to mount an effective host antitumor response¹⁹. Macrophages play an important role in this scenario as these cells produce large quantities of pro- and anti-inflammatory cytokines, which can promote cancer dissemination and metastasis. Blot *et al.* showed that the deleterious role of macrophages in cancer progression is due to the cooperation of monocytes with cancer cells [21], and the modifications of macrophages functions in tumours could be explained by a switch of M1 macrophages to M2 macrophages, which have protumoral properties [22,23]. Our data indicate that *N*-glycan-defective breast cancer cells can induce BMDMs to differentiate into M1 macrophages, which suggests that down-regulated the expression of *N*-glycan on breast cancer cells could enhance the antitumor effects of TAMs. Further, it has been accepted that fully activated macrophages have the potential to inhibit tumour development. TAMs that express an M1 phenotype are capable of killing tumour cells mainly by secreting inflammatory cytokines such as Tumour Necrosis Factor α [24]. Bolpetti *et al.* found IL-10 can facilitate tumor growth and decrease anti-tumor antigen T cell response [25]. Our data showed that *N*-glycan-defective MA782 cells can induce BMDMs to express more TNF α and less IL-10 (Fig. 4A, 4B), which should be beneficial to the antitumor immune response.

Knockdown of Mgat5-inhibited MA782 cell growth *in vivo* has been reported following activation of CD4⁺T cells and macrophages. This phenomenon might be due to the reduction of beta-1,6-GlcNAc-branched *N*-glycan on tumor cells that results from the knockdown of Mgat5 and/or the alteration or exposure of some Ag epitopes, which in turn activates APC in subcutaneous tissue and eventually up-regulates CD4⁺T cell proliferation and TNF- α and IFN- γ production [6]. In the present study, *N*-glycan-defective MA782 cells have been found to induce the differentiation of BMDMs to proinflammatory M1 macrophages, which could produce effector molecules to limit tumor growth. Thus, *N*-glycandefective tumor cells may induce the differentiation of tumor-associated macrophages into M1 macrophages, which have powerful antigen present capability, therefore enhance Th1 immunity response, and eventually inhibit tumor cells growth *in vivo*.

In conclusion, the beta-1,6-GlcNAc-branched *N*-glycan on MA782 cells has been found to be critical in tumor development and host immune response. More importantly, *N*-glycan-defective tumor cells can induces a phenotypic switch in

macrophages polarization and this maybe the incipient mechanism for the inhibition of *N*-glycan-defective tumor cell growth *in vivo*.

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List of Abbreviations

BMDMS	bone marrow-derived macrophages
SW	swainsonine
GlcNAc	N-acetylglucosamine
L-PHA	leukoagglutinin
Mgat5	N-acetylglucosaminyltransferase V
APC	antigen present cell
FQRT-PCR	real time fluorescence quantitative RT-PCR
FCM	flow cytometry
TAMs	Tumor-associated macrophages

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