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A Prevalent Cancer Associated Glycan, Globo H Ceramide, Induces Immunosuppression by Reducing Notch1 Signaling

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Abstract

Globo H, a hexasaccharide initially identified as a ceramide-linked glycolipid from human breast cancer cell line MCF-7, is over-expressed on the surface of many common cancers, but its function is unknown. Here we demonstrated the uptake of globo H ceramide (GHCer) by human peripheral blood mononuclear cells (PBMC) upon co-culture with MCF-7 cells. Significantly, the expression of globo H on tumor-infiltrating lymphocytes was observed in 61% of globo H positive breast cancer tissues. Addition of synthetic GHCer to human PBMC, mouse splenocytes or purified CD4+ T cells inhibited their proliferative response to anti-CD3/CD28 to 60 ± 1%, 50 ± 7% and 62 ± 5% of control, respectively, and reduced the secretion of IL-2, IFN-γ and IL-4. GHCer also significantly suppressed the proliferation of splenocytes or purified CD19+ B cells to 45 ± 10% and 26 ± 3% of control in response to LPS or LPS + IL4 +CD40 ligand and their IgM production to 12 ± 5%, 8 ± 3%, and IgG to 34 ± 9%, 35 ± 5%, respectively, with neglible induction of plasma cells. Ceramide displayed no such inhibitory effects. On the other hand, GHCer failed to raise the number of regulatory T cells, or their expression of FOXP3/CTLA4, nor did it increase apoptosis. Notably, GHCer significantly suppressed the Notch1 signaling during activation of human PBMC and murine T and B cells. Furthermore, GHCer upregulated the expression of *id3* and *itch* by 2.1±0.2 and 4.7 ± 0.4 folds, respectively, leading to ID3-dependent downregulation of Notch1 and Itch-mediated Notch1 degradation. These results provide the first evidence for GHCer to facilitate the escape of cancer cells from immune surveillance.

Keywords: Globo H ceramide; Tumor associated glycolipid antigen; Immunosuppression; Notch

Abbreviations: GHCer: Globo H ceramide

Introduction

Globo H, a hexasaccharide (Fucα1→2Galβ1→3GalNAcβ1→3Galα1 →4Galβ1→ 4Glcβ1) was first identified as a ceramide linked glycolipid in human breast cancer cell line MCF-7 [1]. It has been reported to be expressed on a variety of cancers including breast, colon, ovarian, gastric, pancreatic, lung, and prostate cancers [2,3]. It was also found on breast cancer stem cells [4]. Its limited expression in normal tissues made globo H an ideal target of immunotherapy against tumors [5]. So far, all the commercially available cancer immunotherapeutics are directed against protein/glycoprotein antigens. However, tumor associated GD2 has recently been shown to be effective target for immunotherapy [6]. Globo H is being hotly pursued as the next non-protein antigen target for cancer immunotherapy with ongoing multi-national clinical trial, but its function remains unknown.

Cancer cells often displayed altered glycosylation pattern on their surface proteins or glycosphingolipids [7]. Several cancer-associated gangliosides had been shown to be involved in cancer cell migration, invasion, angiogenesis and metastasis [8,9]. These gangliosides could also inhibit immune cell responses, including antigen processing and presentation, T cell proliferation, and cytokine production, such as IFN-γ and IL-4 [10-15]. Purified gangliosides from cancer cells displayed immunosuppressive activities which aided cancers to escape from host immune surveillance [13,16-18]. The mechanisms of gangliosides-induced immunosuppression were mediated by interfering the interaction of IL-2 with its receptor [19], inducing apoptotic cell death [20] and deviation toward Th2 response [21]. The molecular bases for gangliosides-induced T cell dysfunction involved NF-kappa B inhibition through degradation of RelA and p50 proteins [22]. In contrast, there has been no study on the function of globo H to date.

There are multiple mechanisms of cross talk between Notch and NF-kappa B [23].The Notch signaling pathway is an evolutionarily conserved cell signaling system in most organisms, and it can regulate cell proliferation, differentiation, apoptosis, and survival [24]. Notch signaling is an important regulator of leukocyte differentiation and maturation [25]. It is also involved in TCR-mediated T cells activation, proliferation and B cell differentiation into Ig-secreting plasma cells [26,27]. Upon interaction with its ligand, Notch can directly activate transcription of target genes [28]. The regulation of Notch1 is controlled by the E-protein transcription factor E2A and its natural inhibitor ID3 [29]. It is degraded by ubiquitination though E3 ubiquitin ligase, Itch

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[30]. Adler et al. reported that Notch signaling enhanced naïve T cell proliferation by modulating the expression of CD25 [31]. But until now, there is no report on the relationship between glycoshingolipids induced immunosuppresion and Notch signaling.

In this study, we first demonstrated the uptake of GHCer by immune cells *in vitro* and by tumor infiltrating-lymphocytes in breast cancer *in vivo*. Addition of GHCer to immune cells could inhibit their proliferation and cytokine or immunoglobulin secretion. We also elucidated the molecular mechanism of GHCer induced immunosuppression which involved inhibition of Notch1 signaling through the induction of ID3 and Egr2/3 accompanied *itch* expression. The key immunosuppressive effects and downregulation of Notch1 by GHCer were demonstrated in both human and murine immune cells, with further detailed analysis in murine system, given the readily accessible immune cells from inbred strain of mice. Our findings illustrated for the first time the role of GHCer in the escape of cancer cells from immune surveillance.

Material and Methods

Materials

Female BALB/c mice (8-12 weeks old) were obtained from National Laboratory Animal Center (Taipei, Taiwan) and housed under specific pathogen free condition. All animal studies were approved by the Institutional Animal Care and Use Committee of the Academia Sinica. Normal human blood was obtained from Taipei Blood Center with the approval of the Human Subject Research Ethics committee of both Academia Sinica and the Taiwan Blood Services Foundation. Tissue sections of human breast cancer were obtained from Chunghua Christian Hospital (Chunghua, Taiwan) and were fully encoded to protect patient confidentiality. This study was approved by the Institutional Review Board of Human Subjects Research Ethics Committees of Academia Sinica (Taipei, Taiwan) and Chunghua Christian Hospital. The MCF-7 cells were obtained from ATCC and maintained in MEM medium supplemented with 10% heat-inactivated FCS, 100 ug/ml streptomycin, and 100 U/ml penicillin (Invitrogen, Frederick, MD).

Reagents and Flow cytometry

GHCer with fatty acid tail 16:0 was obtained from OBI Pharma Inc. and dissolved with PBS at a concentration of 2 mg/ml. LPS was purchased from Sigma-Aldrich. IL-4 and CD40L were purchased from PeproTech. Antibodies against CD3-LEAF (145-2C11 and OKT3), CD28-LEAF (37.51 and CD28.2), CD4-PE (RM4-5 and OKT4), CD19-PE (6D5), CD25-FITC (3C7), CTLA4-PE (UC10-4B9), FOX-P3-PE (150D) and Notch1-PE (mN1A) were purchased from Biolegend. Monoclonal antibody against globo H was prepared from VK9 hybridoma provided by Dr. Ragupathi (52). Single cell suspensions of lymphoid cells were stained with fluorescence labeled mass following the manufacturer's instructions. Flow cytometry was performed with FACSCanto (BD, Franklin Lakes, NJ) and the data were analyzed by FCS Expresss V3 software (De Novo Software, Canada).

Isolation of T and B cells from mouse splenocytes

CD4+ T cells were purified by anti-CD4 magnetic microbead following the manufacturer's instructions (Miltenyi Biotec, Gladbach, Germany). The CD19+ B cells were positive selected by anti-CD19-PE Ab followed by anti-PE magnetic microbead. The purity of isolated cells was greater than 95% by FACS analysis.

Cell proliferation and cytokine production assays

Mouse splenocytes, purified CD4+ T cells and human PBMCs (5, 2 and 2 X 10^5/well, respectively) were stimulated with/without anti-CD3/28 mAbs (1 and 3μg/ml respectively) coated wells at 37°C for 3 days. Purified CD19+ B cells were activated by LPS (2 μg/ml) with/without IL-4 (10 ng/ml) + CD40L (1 ng/ml). [^{3}H] thymidine (1 μCi/well) was added to the cells at the last 16 hrs before harvest. The [^{3}H] thymidine incorporation was determined by TopCount NXT gamma counter (Packard, Meriden, CT). Cell culture supernatants were determined for IL-2, IL-4, IFN-γ, IL-10 and TGF-β by DuoSet ELISA Development System according to the manufacturer's procedures (R&D System, USA).

Apoptosis assay

Mouse splenocytes (5X10^5 cells/well) were stimulated with anti-CD3/28 antibody-coated wells and incubated at 37°C for 24, 48 and 72 h in 96 well flat bottom plates. Cells were stained with annexin V-FITC and propidium iodide provided by the Apoptosis Detection kit (BD Biosciences). Data was performed by flow cytometry analysis.

Quantitative real-time PCR

Total RNA was extracted from purified mouse CD4+ T cells with TRIzol reagent (Invitrogen Life Technologies), and 1μg of RNA was reversely transcribed into cDNA using Omiscript RT (Qiagen,Hilder, Germany). cDNA (20 ng) mixed with 1X SYBR Green Master Mix (Applied Biosystems) was used to perform real-time quantitative PCR on an Applied Biosystems PRISM 7300-HT. The values of cycle threshold were used to calculate the fold differences of gene expression relative to the expression of the housekeeping gene, β-actin. The primers used in this assay were listed as following: *id3*; forward, 5'- TCCTGGCACCTCCCGAAC-3'; reverse, 5'- TAAGTGAAGAGGGCTGGGTTAAG-3', *itch*; forward, 5'- GTGTGGAGTCACCAGACCCT-3'; reverse, 5'- GCTTCTACTTGCAGCCCATC-3', *egr2*; forward, 5'-GTGCCAGCTGCTATCCAGAAG -3'; reverse, 5'- GGCTGTGGTTGAAGCTGGAG -3', *egr3*; forward, 5'- CAACGACATGGGCTCCATTC -3'; reverse, 5'- GGCCTTGATGGTCTCCAGTG -3', *cbl-b*; forward, 5'- CTTAAATGGGAGGCACAGTAGAAT-3'; reverse, 5'-CAGTACACTTTATGCTTGGGAGAA-3'.

Immunohistochemical staining of clinical specimens of breast cancer

For globo H staining, tissue sections were deparaffinized followed by antigen retrieval by autoclave for 121°C, 5 mins in AR-10 solution (Biogenex). Slides were incubated with VK9 antibody (1:100 dilutions in antibody dilution buffer, Ventana Medical Systems, Inc.) overnight at 4°C followed by polymer-HRP IHC detection system (Biogenex). The slides were counter stained with hematoxylin and mounted. CD3 immunohistochemical staining was performed automatically with Ventanas Benchmark® XT by using the rabbit anti-human CD3 (DAKO Corporation, Carpinteria, CA). Digital images were captured by Aperio ScanScope XT Slide Scanner (Aperio Technologies, Vista, CA, USA) under 20 × magnification.

Statistical analysis

The statistical significance between each group was analyzed by unpaired two-tailed Student's t test. P value less than 0.05 was considered significant.

Results

Uptake of globo H by lymphocytes *in vitro* and *in vivo*

It has been reported that tumor cells could shed gangliosides into microenvironment to help its growth (32-34). To test whether globo

H could be transferred from cancer cells to immune effector cells, we cultured the human PBMC in the presence/absence of breast cancer cell line MCF-7 which expressed high level of globo H on its surface. Three days after incubation with MCF-7, globo H was detected on the surface of PBMC(Figure 1A) as well as CD4+ cells (Figure 1B) while it was undetectable on PBMC cultured without MCF-7 cells. Similarly, globo H could be detected on BALB/c mouse splenocytes upon incubation with chemically synthesized GHCer for 3 days but not with the ceramide (Figure 1C). Next, we explored the possibility of globo H uptake by tumor infiltrating-lymphocytes. A total of 98 tumor tissues of breast cancer were examined by Immunohistochemical staining with anti-globo H. Figure1D showed representative tissue sections. In the left upper panel, globo H was easily discernible on the infiltrating lymphocytes surrounding the tumor which was strongly positive for globo H. On the other hand, globo H was undetectable on the infiltrating lymphocytes in tumors which were slightly positive or negative for globo H, as shown in upper middle and upper right panel, respectively, of Figure 1D. The sections were stained with anti-CD3 antibody which revealed that most tumor infiltrating lymphocytes were T cells (Figure 1D, lower panel). All together, 61 of 98 breast cancer samples (62.2%) showed positive staining of tumors for globo H and 37 of 98 samples (37.7%) showed positive staining of both tumors and lymphocytes for globo H (Figure 1E). These results indicated that globo H could be shed or transferred from cancer cells to the immune cells *in vitro* and *in vivo*.

GHCer inhibited the proliferation of mouse and human immune cells

To determine whether the incorporation of GHCer could influence the proliferation of immune cells, splenocytes or purified CD4+ T cells from BALB/c mouse were pretreated with GHCer or ceramide for 24 hours before stimulation with plate bound antibodies against mouse CD3 and CD28 for 3 days. As determined by [^{3}H]-thymidine incorporation, GHCer but not ceramide inhibited anti-CD3/CD28 induced proliferation of splenocytes and CD4+ T cells to 50 ± 7% and 62 ± 5% of the control, respectively (Figures 2A and 2B). GHcer or ceramide had no effect on proliferation without anti-CD3/CD28 stimulation. To asses whether GHCer also affect B cell proliferation, murine splenocytes or purified CD19+ B cells were stimulated with LPS or LPS+IL-4+CD40L in the presence or absence of GHCer or ceramide for 4 days and cell proliferation was determined by [^{3}H]-thymidine incorporation. As shown in Figure 2C, upon stimulation with LPS, GHCer but not ceramide inhibited the proliferation of mouse splenocytes to 15 ± 2% of the control. The proliferation of CD19+ B cells stimulated with LPS or LPS+IL-4+CD40L was reduce by GHCer to 45 ± 10% and 26 ± 3% of control, respectively (Figure 2D). Similar effects of GHCer on human PBMC were observed. As shown in Figure 2E, GHcer but not ceramide-decreased anti-human CD3/ CD28 induced proliferation of human PBMC to 60 ± 1% of control. These results suggested that GHCer could inhibit the proliferation of mouse and human immune cells.

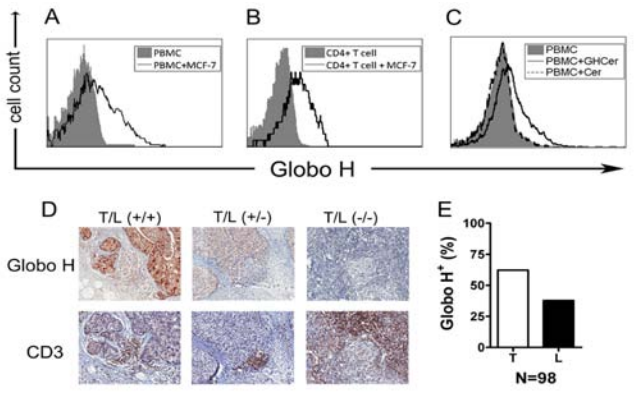

Figure 1: Uptake of globo H by lymphocytes in vitro and in vivo. Human PBMCs were cultured with/without human breast cancer cell, MCF-7. Three days later, PBMCs were harvested from culture medium and the expression of Globo H on PBMC (A) and CD4+ T cells (B) was determined by flow cytometry with mAb VK9. Splenocytes from BALB/c mouse were incubated with synthetic Globo-H-ceramide or ceramide for 3 days, and stained with mAb VK9 to detect the expression of Globo H by flow cytometry (C). (D) Tissue sections of breast cancer with Immunohistochemical staining of VK9 or anti-human CD3 antibody. T: tumor, L: lymphocyte, +: globo H positive, -: globo H negative. (E) The percent of globo H expression in the tumors and infiltrating lymphocytes. T: the percent of globo H positive tumors in 98 clinical breast cancer samples. L: the percent of globo H positive tumors containing globo H positive lymphocytes in 98 samples.

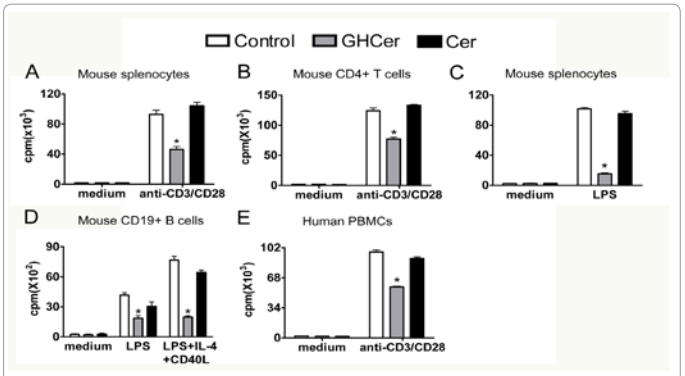

Figure 2: Inhibition of proliferation of immune cell by GHCer. Indicated immune cells were incubated with GHCer, ceramide or medium control for 24 hrs and then activated by anti-mouse CD3 and CD28 mAbs (A and B), LPS (C), LPS in the presence of IL4 and mouse CD40 ligand (D), or anti-human CD3 and CD28 mAbs (E). Cell proliferation was determined by [^{3}H]-thymidine incorporation assay. The data are presented as mean ± SD of triplicate. *, p<0.05, compared with control.

Effects of GHCer on the production of cytokines and immunoglobulin by stimulated mouse cells

To investigate the effects of GHCer on the cytokine production of activated T cells, murine splenocytes or purified CD4+ T cells were pretreated with GHCer or ceramide for 24 hrs before activation by anti-CD3 and anti-CD28 antibodies. Three days later, culture supernatant was collected for determination of IL-2, IFN-γ and IL-4 by ELISA assay. As shown in Figure 3A, the secretion of IL-2 from activated splenocytes or CD4+ T cells was suppressed by GHCer but not ceramide to 19 ± 1% and 72 ± 4% of control, respectively. GHCer also inhibited IFN-γ secretion to 40 ± 3% and 37 ± 2% (Figure 3B) and IL-4 secretion to 12 ± 2% and 31 ± 8% of control, respectively, (Figure 3C). We next examined whether GHCer treatment could affect the secretion of immunoglobulins and the generation of CD138+ plasma cell from LPS-stimulated B cell. Purified mouse CD19+ B cells were treated with GHCer or ceramide before activation by LPS or LPS in the presence of IL-4 and CD40 ligand. Four days later, supernatant was collected for measurement of IgM and IgG by ELISA assay. As shown in Figures 3D and 3E, after stimulation by LPS or LPS in the presence of IL-4 and CD40 ligand, the secretion of IgM was decreased by GHCer to 12 ± 5%, 8 ± 3% of control, and for IgG, to 34 ± 9%, 35 ± 5%, respectively. The percentage of CD138+ plasma cells was reduced to negligible levels in GHCer treated CD19+ B cells (Figure 3F). Ceramide neither alter

the secretion of cytokines or immunoglobulins, nor the generation of plasma cells. These results showed that GHCer had significant impact on T or B cell activation by anti-CD3/CD28 or LPS.

GHCer has no effect of on the induction of cell apoptosis or Treg cells

Regulatory T cells (Treg) had been shown to exert negative immunoregulatory effects directly through CTLA4 or indirectly by the secretion of IL-10 and TGF-β. To determine whether the inhibitory effects of GHCer on T cell activation was mediated by Treg, we analyzed the production of IL-10, TGF-β and the population of Treg after anti-CD3/CD28 mAbs activation. As shown in Figure 4A and 4B, IL-10 and TGF-β production did not change significantly in the GHCer treated cells when compared to the control cells after stimulation for 3 days. Similarly, GHCer treatment did not alter the percentage and absolute number of regulatory T cells (Figure 4C and 4D) nor their expression of foxp3 and CTLA4 (Figures 4E and 4F) after activation for 2 days. To explore the possibility of apoptosis induced by GHCer, murine splenocytes were activated by anti-CD3/CD28 mAbs for three days in the presence/absence of GHCer and apoptotic cells were determined by staining with Annexin V and PI. As shown in Figure 4G, the percentage of apoptotic cells (Annexin V+) was lower in GHCer treated cells (46 ± 1.5% and 60 ± 1.1%) than control cells (55 ± 2.1% and 67 ± 1.8%) at 24 and 48 hrs after stimulation, respectively. These results indicated that the inhibition of cell proliferation by GHCer was neither mediated by expansion of Tregs nor the induction of cell apoptosis.

GHCer decreased the expression of CD25 and Notch1 during cell activation

CD25, the IL-2 receptor, which was up-regulated upon T cell activation, was crucial for T cell proliferation. To determine whether GHCer affected CD25 expression, murine splenocytes or purified CD4+ T cells were incubated with GHCer or ceramide for 24 hrs before activation by anti-mouse CD3/CD28 mAbs. Two days later, cells were collected and the expression of CD25 on CD4+ T cells was analyzed by flow cytometry. As shown in Figures 5A and 5B, expression of CD25 on CD4+ T cells was decreased by GHCer. Since Notch1 signaling has been shown to modulate CD25 expression on T cells, we further analyzed the GHCer influence on Notch1 expression. As shown in Figures 5C and 5D, Notch1 expression in both mouse splenocytes and CD4+ T cells was inhibited by GHCer 2 days after stimulation by anti-CD3/CD28 mAbs. Notch signaling was also essential for B cell differentiation into Ig-secreting plasma cells [27]. Hence we examined the influence of GHCer on Notch1 expression during B cell activation. Purified mouse CD19+ B cells were incubated with GHCer or ceramide before activation by LPS combined with IL-4 and mouse CD40 ligand. On day2, Notch1 expression on CD19+ B cells was completely abolished by GHCer treatment when compared to the control or ceramide treated cells (Figure 5E). Similarly, GHcer also inhibited the upregulation of Notch1 expression on human CD4+ T cells during anti-CD3/CD28 mAbs (Figure 5F). These findings suggested that inhibition of immune cell proliferation and differentiation by GHCer might be attributed to the suppression of the Notch1 signaling.

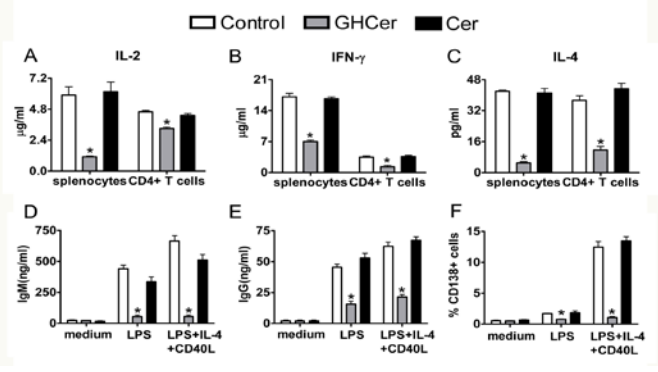

Figure 3: Effects of GHCer on the production of cytokine and immunoglobulin. Mouse splenocytes or purified CD4+ T cells were incubated with GHCer, ceramide or medium control for 24 hrs, and then activated by anti-mouse CD3 and CD28 mAbs for 3 day. Supernatants were collected for determination of IL-2 (A), IFN-γ (B) and IL-4 (C) by ELISA assay. Mouse splenocytes or purified CD19+ B cells were incubated with GHCer or ceramide for 24 hrs and activated by LPS or LPS combined with IL-4 and mouse CD40 ligand for 4 days. Supernatants were collected to determine the production of IgM (D) and IgG (E) by ELISA assay and cells were harvested to examine the population of CD138+ plasma cells (F) by flow cytometry. The data are presented as mean ± SD of triplicate. *, p<0.05, compared with control.

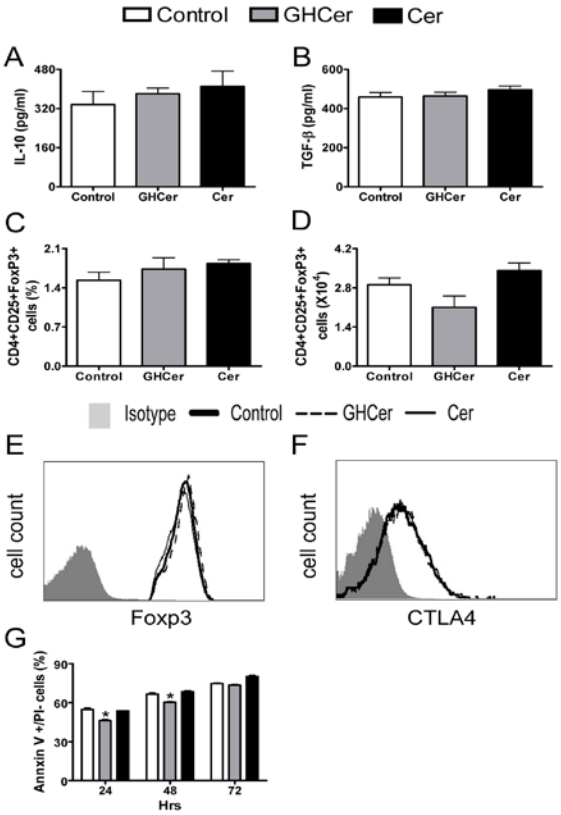

Figure 4: GHCer could neither expand Treg population nor induce apoptosis of splenocytes. Mouse splenocytes were incubated with GHCer, ceramide or medium control for 24 hrs, before activation by anti-mouse CD3/CD28 for 3 days. Supernatants were collected for determination of IL-10 (A) and TGF-β (B) by ELISA assay. Cells were stained with antibodies against CD4, CD25, Foxp3 and CTLA4 at day2 and the percentage (C), absolute number (D), expression of Foxp3 (E) and CTLA4 (F) of Treg cells were determined. Splenocytes were harvested at the indicated time points for determination of apoptotic cells by staining with annexin V and PI, followed by flow cytometric analysis. The percentage of apoptotic (annexin V+/PI-) cells was shown (G) and data are presented as mean ± SD of triplicate. *, p<0.05, compared with control.

ID3 and Egr2/3 regulated E3 ubiquintin ligase may contribute to Notch1 suppression by GHCer

It was reported that Notch1 was negatively regulated at transcriptional level by ID3, the E2A antagonist [29] and at the protein

level via ubiquintination by Itch [30]. Thus, we assessed the influence of GHCer on the expression of *id3* and *itch* during T cell activation. Mouse splenic CD4+ T cells were incubated for 24 hrs with GHCer or ceramide, before activation by anti-mouse CD3/CD28 mAbs. RNA was collected at 2 and 12 hrs to analyze the gene expression by real-time quantitative PCR. As shown in Figures 6A and 6B, the expression of *id3* and *itch* at 2 hrs was not affected by GHCer but raised to 2.1 ± 0.2 and 4.7±0.4 folds higher, respectively, than in control cells at 12 hrs. As *itch* was regulated by Egr2/3 [35,36], we investigated the expression of *egr2/3* after GHCer treatment. Treatment of CD4+ T cells with GHCer increased the expression of *egr2* and *egr3* to 1.6 ± 0.1 and 1.2 ± 0.03 folds respectively, of control at 2 hrs after activation which preceded *itch* upregulation (Figures 6C and 6D). Since overexpression of Egr2 and Egr3 also increased the expression of *cbl-b*, an E3 ubiquintin ligase [35], we determined *cbl-b* expression in GHcer treated CD4+ T cells. As shown in Figure 6E, GHCer promoted the expression of *cbl-b* to 2.1 ± 0.3 folds of control at 12 hrs. In conclusion, these data suggested that GHCer could downregulate Notch1 expression through two pathways, one at transcriptional level by ID3, and the other at protein level through Egr2/3 controlled *itch* expression.

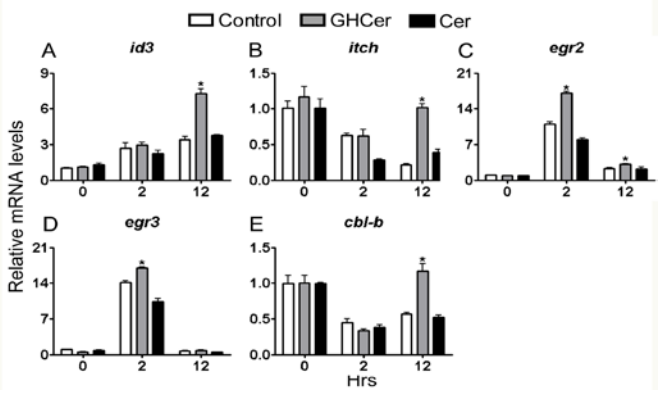

Figure 6: Effect of GHCer on the upregulation of *id3* and Egr2/3-mediated increase of E3-ubiquintin ligases during T cell activation. CD4+ T cells isolated from mouse spleen were incubated with GHCer, ceramide or medium control for 24 hrs, and then activated by anti-CD3- and CD28 mAbs. At 2 and 12 hrs, total RNA was extracted for quantitation of *id3* (A), *itch* (B), *egr2* (C), *egr3* (D), *cbl-b* (E) by real-time quantitative PCR. The mRNA levels were normalized to the level of *β-actin* and compared to resting cells. The data are presented as mean ± SD of triplicate determinations. *, p<0.05, compared with medium control.

Discussion

GHCer is a glycosphingolipid of globo series, which is expressed on a variety of cancers [2,3,37], but its function remains an enigma. In this study, we first demonstrated that human PBMC acquired globo H when co-cultured with globo H expressing cancer cells. GHCer could suppress proliferation and activation of human and murine immune cells which might contribute to the escape of cancer cells from immune surveillance. Previously, several cancer associated gangliosides were shown to induce immunosuppression [16]. To date, this is the first study to show that globoside can also inhibit immune activation. The length of the ceramide chain also played a role in the ganglioside induced immunosuppression. Gangliosides with shorter fatty acyl chain (C16:0, C18:0) was more immunosuppressive and easily shed than those with a longer fatty acyl chain (C22:0/C24:1, C24:0) [38]. In MCF-7 cells, the most abundant fatty acyl chain linked to the globo H is C16:0 [39], and we showed that the synthetic GHCer with C16:0 fatty acyl chain indeed inhibited the activation of lymphocytes. Ceramide (C16:0) alone did not have any effect, suggesting that globo H residue was necessary for the immunosuppression.

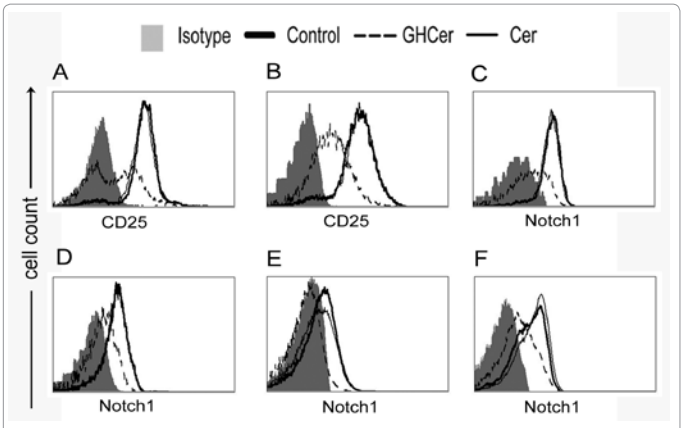

Figure 5: GHcer dampened the upregulation of CD25 and Notch1 during lymphocyte activation. Mouse splenocytes (A and C) and purified CD4+ T cells (B and D) were incubated with GHCer, ceramide or medium control for 24 hrs, and then activated by anti-mouse CD3 and CD28 mAb. Two days after activation, CD4+ T cells were gated to determine the expression of CD25 (A and B) and Notch1 (B and D) by flow cytometry. Purified mouse splenic CD19+ B cells were incubated with GHCer, ceramide or medium control for 24 hrs, and then activated by LPS combined with IL-4 and mouse CD40 ligand. Three days after activation, Notch1 expression was determined by flow cytometry (E). Human PBMC were incubate with GHCer or Cer for 24 hrs, and then activated by anti-human CD3 and CD28 mAb. Two days after activation, Notch1 expression on CD4+ T cells was determined by flow cytometry (F).

Cancer cells can evade the host immune system by several mechanisms, such as shifting the microenvironment from Th1 to Th2 polarity [40], inducing the immunosuppressive cytokine IL-10 and TGF-β [41], and regulatory T cells [42]. It had been shown that tumor derived gangliosides can shift theTh1/Th2 balance to promote the cancer progression. However, there were conflicting reports about the effects of various gangliosides on Th1 and Th2 responses. For instance, Rayman et al. reported that GD1a derived from renal cell carcinoma inhibited the development of Th1 cell response by inducing the apoptosis of Th1 cytokine producing T cell [43]. Similarly, Crespo et al. indicated that GT1b, GD1a and GM1 shifted the immune response toward Th2 by inhibiting the IFN-γ production and enhancing IL-4 dependent differentiation of $CD4^+$ T cells [21]. On the other hand, purified GT1b, GD1b and GQ1b had been reported to enhance the production of IL-2 and IFN-γ but suppress IL-4 [44]. In addition, Kaushik et al. showed that gangliosides from some renal cell carcinoma patients suppressed both Th1 and Th2 cytokine responses [10]. So far, the effect of globo series glycosphingolipid on Th1 and Th2 cytokine has not been investigated. In this study, we demonstrated that acquisition of GHCer by immune cells decreased the production of IFN-γ, IL-2 and IL-4 during T cell activation. Such suppressed Th1 and Th2 responses might create a microenvironment conducive to tumor growth. In short, the composition of carbohydrate moiety of glycosphingolipids on different tumors likely dictated the balance of Th1/Th2 activities, leading to tumor progression.

Regulatory T cells, formerly known as suppressor T cells, can potently inhibit the T cell proliferation. Our investigation showed that GHCer neither increased the regulatory T cell population, nor enhanced their expression of foxp3 and inhibitory molecular CTLA4. GHCer also failed to induce the suppressive cytokine IL-10 and TGF-β. These findings implied that GHCer-induced immunosuppression was

not mediated by the Tregs. This is consistent with the previous finding that NGcGM3- induced immune suppression was mainly by reducing the functions of dendritic cells and CD4+CD25- T lymphocyte, rather than enhancing the regulatory T cell activity [45].

Although the cancer associated gangliosides-induced immune suppression had been reported by many studies, the molecular mechanisms were addressed by only a few studies which focused mainly on NF-kappa B. Irani et al. reported that brain derived gangliosides could inhibit the NF-kappa B activation in mitogen stimulated T cells [11]. Thornton et al. showed that gangliosides isolated from renal carcinoma suppressed the activation of NF-kappa B by degrading RelA and p50 proteins to impair nuclear localization of the p68/p50 NF-kappa B heterodimer in T cells [22]. Treatment of hepatocytes with GD3 blocked the activation of NF-kappa B and subsequent gene expression [46], implying that NF-kappa B played an important role in the gangliosides induced immunosuppression. However, the basis for the inhibition of NF-kappa B activation by gangliosides remains to be elucidated. NF-kappa B activity was reported to be modulated by several pathways, including Notch signaling. Shin et al. described that intracellular domain of Notch1 could directly interact with NF-kappa B, leading to retention of NF-kappa B in the nucleus [47]. Cheng et al. also demonstrated that NF-kappa B activity was regulated by Notch1 via transcriptional control of NF-kappa B during B cell activation [48]. Notch signaling is crucial in determining T and B lymphoid lineage decision. Inhibition of Notch1 cleavage in activated T cells resulted in decreased cell proliferation, and reduced production of IL-2 and IFN-γ [26]. Furthermore, the intracellular domain of Notch1 was shown to directly regulate IFN-γ expression through complexes formed on the IFN-γ promoter [47]. Our study provided the first evidence that cancer associated globo series glycosphingolipid, GHCer, could inhibit Notch1 expression during T and B cell activation. It will be of interest to investigate if gangliosides induced suppression of NF-kappa B is also mediated by reducing Notch 1 signaling. Such diminished Notch1 signaling may contribute to the GHCer-induced decreases in the cell proliferation, IL-2 and IFN-γ production and CD25 upregulation. Furthermore, we showed that treatment of T cells with GHCer boosted their expression of *egr2* and *egr3*, which in turns upregulated *itch* to degrade the Notch1 protein. The antagonist of E2A-mediated Notchl transcription, *id3* was also enhanced by GHCer. Henceforth, our findings indicated that GHCer inhibited T cell activation by enhancing the expression of *id3* and *itch*, leading to diminished Notch1 signaling. In addition to *itch*, Egr2/3 also regulated *cbl-b* expression, which was found to be increased by GHCer treatment. In light of the report that ablation of *cbl-b* in mice provided protection against engraftment of a variety of solid and hematopoietic tumors [49], the upregulation *cbl-b* in T cells by GHCer might further dampen host immunity and favor tumor growth.

Egr2 and Egr3 are zinc-finger transcription factors of the early growth response gene family which negatively regulate T cell activation and induce T and B cell tolerance. Absence of full costimulation signal in TCR ligation would lead to induction of *egr2/3* expression causing cell unresponsiveness [35,50,51]. The basis for our observed upregulation of *egr2/3* by GHcer was not clear. It was possible that incorporation of GHCer in the immune cells might diminish costimulatory signaling or activate signaling molecules such as NFAT to induce egr2/3 expression [52]. Further investigation will be necessary to elucidate the underlying mechanisms.

Our findings provide a new insight that globo H released from cancer cells might alter the microenvironment in favor of tumor progression by suppressing the activation of immune cells. This was corroborated by our demonstration of globo H expression on tumor-infiltrating lymphocytes in a majority of globo H positive breast cancer tissues Thus, depletion of GHCer might be effective in overcoming immunosuppression with ensuing tumor regression. In view of the present study and our previous report that globo H was expressed on the breast cancer stem cells, globo H is an ideal target for cancer immunotherapy with globo H vaccine or anti-globo H monoclonal antibody. Our data further strengthened the scientific rationales for the ongoing phase II/III clinical trial of globo H vaccine in breast cancer.

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