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Onami, Thandi M.; Zeng, J; Joo, H. M.; Rajini, B.; Wrammert, J. P.; and Sangster, M. Y., "The generation of influenza-specific humoral responses is impaired in ST6Gal I-deficient mice." (2009). *Microbiology Publications and Other Works*.

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This information is current as of February 4, 2011

J Immunol 2009;182:4721-4727

doi:10.4049/jimmunol.0802833

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The Generation of Influenza-Specific Humoral Responses Is Impaired in ST6Gal I-Deficient Mice¹

Junwei Zeng,^{2*} Hye Mee Joo,^{2*} Bheemreddy Rajini,* Jens P. Wrammert,[†] Mark Y. Sangster,* and Thandi M. Onami^{3*}

Posttranslational modification of proteins, such as glycosylation, can impact cell signaling and function. ST6Gal I, a glycosyltransferase expressed by B cells, catalyzes the addition of α -2,6 sialic acid to galactose, a modification found on N-linked glycoproteins such as CD22, a negative regulator of B cell activation. We show that SNA lectin, which binds α -2,6 sialic acid linked to galactose, shows high binding on plasma blasts and germinal center B cells following viral infection, suggesting ST6Gal I expression remains high on activated B cells in vivo. To understand the relevance of this modification on the antiviral B cell immune response, we infected ST6Gal I^{-/-} mice with influenza A/HKx31. We demonstrate that the loss of ST6Gal I expression results in similar influenza infectivity in the lung, but significantly reduced early influenza-specific IgM and IgG levels in the serum, as well as significantly reduced numbers of early viral-specific Ab-secreting cells. At later memory time points, ST6Gal I^{-/-} mice show comparable numbers of IgG influenza-specific memory B cells and long-lived plasma cells, with similarly high antiviral IgG titers, with the exception of IgG2c. Finally, we adoptively transfer purified B cells from wild-type or ST6Gal I^{-/-} mice into B cell-deficient (μ MT^{-/-}) mice. Recipient mice that received ST6Gal I^{-/-} B cells demonstrated reduced influenza-specific IgM levels, but similar levels of influenza-specific IgG, compared with mice that received wild-type B cells. These data suggest that a B cell intrinsic defect partially contributes to the impaired antiviral humoral response. *The Journal of Immunology*, 2009, 182: 4721–4727.

Humoral immunity is characterized by prolonged Ab production, even after resolution of infection, in stark contrast to the T cell response, where effector T cell function is relatively short-lived. Abs, by neutralizing or opsonizing free extracellular pathogens, serve as a critical first-line defense against infection. Humoral immunity is comprised of pre-existing Ab produced by Ab-secreting cells (ASCs)⁴ termed long-lived plasma cells (LLPCs), as well as a population of memory B cells (MBCs) that can differentiate following antigenic stimulation into plasma cells (1–4).

The enzyme ST6Gal I is a glycosyltransferase highly expressed by B and T cells that synthesizes the glycan sequence α -2,6 sialic acid linked to galactose (Sia α 2-6Gal) (5). This structure is typically found on N-linked glycans of glycoproteins, such as the mammalian lectin CD22, a transmembrane glycoprotein expressed by B cells that has high specificity for (Sia α 2-6Gal). CD22 has been shown to be a negative regulator of B cell signaling by recruiting SHP-1 to the BCR, thus attenuating signals via the BCR

(5–7). The plant lectin *Sambucus nigra* agglutinin (SNA) binds the product of ST6Gal I, Sia α 2-6Gal, so SNA lectin binding can be used as an indicator of ST6Gal I expression and/or activity (5).

ST6Gal I null mice were initially described several years ago (5). These mice demonstrate normal T cell activation to anti-CD3 cross-linking in vitro, but defective BCR signaling in vitro, and reduced Ab production following immunization with T-independent and T-dependent Ags (DNP-Ficoll or DNP-keyhole limpet hemocyanin) in vivo (5). However, despite indications that B cell immune function may be compromised in these mice, as well as evidence that addition of cytokines could abrogate in vitro B cell proliferation differences, no studies have documented whether or not protective Ab and memory B cell responses are compromised in these mice when exposed to pathogenic microorganisms such as live viral infection.

In this study, we have compared the outcome of influenza viral infection of wild-type or ST6Gal I-deficient mice. The hemagglutinin (HA) glycoprotein plays a key role in influenza pathogenicity and is involved in host-cell recognition (8). First, we show that effective replication of influenza A/HKx31 (Orthomyxoviridae, influenza A) does occur in the lungs of ST6Gal I^{-/-} mice; however, these mice demonstrate defective early influenza-specific B cell responses. Next, we determine that expansion of CD4 and CD8 influenza-specific T cell numbers in the lung and lymph nodes is normal in ST6Gal I^{-/-} mice. Furthermore, we demonstrate similar overall numbers of influenza specific IgG MBCs and LLPCs, although we note both a reduction in serum levels of influenza specific IgG2c and IgG2c-producing LLPCs in the bone marrow. Finally, we demonstrate an impaired influenza specific IgM response in B cell-deficient mice that receive ST6Gal I^{-/-} B cells, suggesting that a B cell intrinsic defect partially contributes to the impaired humoral response. Overall, our findings suggest that lack of ST6Gal I expression appears to prominently impair the generation of a viral-specific humoral response but plays a lesser role in influenza-specific memory.

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Received for publication August 27, 2008. Accepted for publication February 2, 2009.

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¹ This work was supported by National Institutes of Health Grant AI05771901 and University of Tennessee startup funds (to T.M.O.) and in part by National Institute of General Medical Sciences-Consortium for Functional Glycomics Grant GM62116.

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⁴ Abbreviations used in this paper: ASC, Ab-secreting cell; CLN, cervical lymph node; HA, hemagglutinin; LLPC, long-lived plasma cell; MBC, memory B cell; MedLN, mediastinal lymph node; p.i., postinfection; PNA, peanut lectin (agglutinin); SNA, *Sambucus nigra* agglutinin; Sia α 2-6Gal, α -2,6 sialic acid linked to galactose.

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Materials and Methods

Mice and immunizations

ST6Gal I-deficient mice were generated by J. Marth (University of California, San Diego, La Jolla, CA) and obtained from the Consortium for Functional Glycomics and bred in-house (5). C57BL/6 female mice and B6.129S2-Igh-6^{tm1Cgn}/J (μ MT^{-/-} mice) were purchased from The Jackson Laboratory. Mice were maintained under specific pathogen-free conditions at the University of Tennessee in accordance with university IACUC guidelines and used at 6–12 wk of age. Anesthetized mice were intranasally infected with 10^{6.8} EID₅₀ influenza A/HKx31 in 30 μ l of PBS as described previously (9, 10). μ MT^{-/-} mice were primed with 10^{7.4} EID₅₀ influenza A/HKx31 i.p.

Tissue harvest and flow cytometry

At specified time points (days 7, 10, >40 postinfection (p.i.)), animals were sacrificed, and tissues were harvested for viral titer (lungs only), immunofluorescence, or isolation of lymphocytes. For isolation of lymphocytes from lungs, lungs were perfused with 5 ml of cold PBS before removal and processed as described previously (11). Single-cell suspensions were stained with mAbs purchased from BD Pharmingen (CD4, CD8, CD44, B220, CD138, FAS, GL7, IFN- γ , and TNF- α), Vector Laboratories (SNA lectin and peanut agglutinin (PNA)), or Southern Biotechnology Associates (IgD). Intracellular cytokine staining was assayed on cells from spleen, lung, and MedLN. All samples were run on a FACSCalibur (BD Biosciences). All data were analyzed with FlowJo software (Tree Star). Unpaired Student's *t* tests were performed to determine statistical significance with * denoting *p* < 0.05, ** denoting *p* < 0.01, and *** denoting *p* < 0.001.

Immunofluorescence

MedLNs were removed from mice on day 7 p.i. and frozen in OCT at -80°C. The frozen tissues were cut at 8- μ m thickness, thaw-mounted onto slides, air-dried, and fixed in cold acetone for 10 min. The sections were then blocked with 3% BSA and stained for germinal centers with PNA-FITC (Vector Laboratories), anti-B220-PE, and/or GL7-FITC (BD Pharmingen) as indicated. After staining, sections were washed with PBS, mounted using Vectashield mounting medium (Vector Laboratories), and analyzed using a laser scanning confocal microscope (Leica SP2).

Influenza viral titer determination

Lungs were collected on indicated days p.i. and homogenized in Hanks' media with 0.1% BSA (Life Technologies). Eight replicates of the homogenized lung were serially diluted 10-fold across 96-well U-bottom plates in 1 \times MEM with 0.3% BSA, 1 μ g/ml *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (Worthington), penicillin (100 IU/ml), streptomycin (100 μ g/ml), and 5% FBS. The titrated lung samples were added to serum-free MEM on a Madin-Darby canine kidney cell monolayer prepared in 96-well tissue culture plates and incubated for 48 h at 37°C and 5% CO₂. Wells that were positive for viral growth were identified by testing supernatants for hemagglutinating activity using 0.5% chicken RBCs.

ELISPOT assay to detect influenza specific ASCs

A preparation of concentrated viral particles was disrupted for 10 min at room temperature in a 1/10 dilution of disruption buffer (0.5% Triton X-100, 0.6 M KCl, and 0.05 M Tris-HCl (pH 7.5)) in PBS, further diluted in PBS, and plated at 1 μ g/well in nitrocellulose-bottomed 96-well Multiscreen HA filtration plates (Millipore). After overnight incubation at 4°C, plates were washed with PBS, and blocked with cell culture medium containing 10% FBS. Plates were emptied by flicking, and cell suspensions (including resuspended cells from 96-well MBC assay plates) were added in volumes of ~100 μ l/well. After incubation for 3–4 h at 37°C in a humidified atmosphere containing 5% CO₂, plates were thoroughly washed with PBS alone and PBS containing 0.1% Tween 20. Alkaline phosphatase-conjugated goat anti-mouse IgG, IgM, or IgA (Southern Biotechnology Associates) diluted to 2 μ g/ml in PBS containing 5% BSA was added (100 μ l/well), and the plates were incubated overnight at 4°C. The plates were then washed extensively with PBS alone and PBS containing 0.1% Tween 20, including washing the underside of the nitrocellulose filters. Spots were developed at room temperature by the addition of 1 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich) in diethanolamine buffer (10% diethanolamine, 0.1 M NaCl, 5 mM MgCl₂, and 0.1 M Tris HCl (pH 9.5)) in 100 μ l/well. After spot development, plates were washed with PBS, dried, and spots representing individual ASCs were counted using an Olympus SZX9 stereozoom microscope.

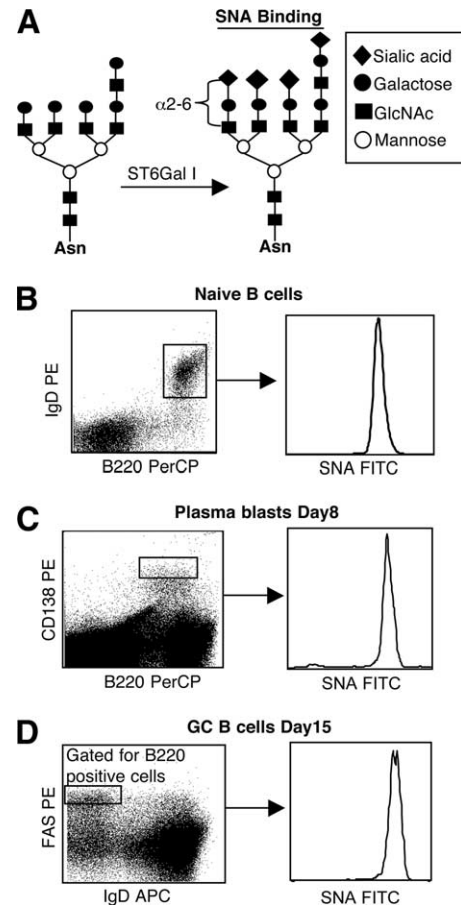


FIGURE 1. Binding of SNA on B cell populations. Splenocytes from uninfected C57BL/6 mice or mice infected with 2×10^5 PFU of LCMV Armstrong i.p. either 8 or 15 days earlier were used for the analysis. The left panel in B–D shows the gating, and the right panel shows SNA binding as histograms of the gated population. A, Cartoon depicting the glycosyltransferase ST6Gal I, which catalyzes addition of terminal α 2,6 sialic acids to galactose. SNA lectin binds Sia α 2-6Gal β 1-4GlcNAc. B, Naive B cells from uninfected mice gated on B220⁺/IgD⁺ cells, or CD138⁺/B220^{low} plasma blasts on day 8 (C), or B220⁺/FAS⁺/IgD⁺ germinal center cells (D) all show high SNA binding.

Memory B cell assay

Influenza-specific MBC frequencies were determined by a previously described LDA based on *in vitro* stimulation of MBCs to differentiate into ASCs (10, 12). Briefly, 2-fold dilutions of cells were incubated in 96-well tissue culture plates (routinely 12 wells per dilution), together with 10⁶ irradiated (3000 rad) syngeneic naive spleen cell feeders plus β -propiolactone-inactivated HKx31 (Charles River Laboratories). After incubation, cells in each well were transferred to ELISPOT plates for the enumeration of influenza-specific IgG ASCs. Pre-existing virus-specific ASC numbers at the time of sampling were determined by direct *ex vivo* ELISPOT assay. After *in vitro* MBC activation and ELISPOT analysis, individual wells were scored positive for virus-specific MBC if progeny ASC numbers were greater than twice the mean pre-existing ASCs. The virus-specific MBC frequency was calculated from the number of negative wells per cell dilution by extrapolation to the dilution that gave 37% negative wells (12). Linearity between the proportion of negative cultures and the input cell dose indicated direct measurement of MBC. No influenza-specific IgG ASCs were detected after *in vitro* stimulation of lymphocytes from naive mice or from mice infected intranasally 8 wk previously with an unrelated virus.

B cell transfer and ELISA

MACS B220 beads (Miltenyi Biotec) were used for purification of B cells according to the manufacturer's instructions. Purity of B220⁺ B cells was 95% determined by FACS. B220⁺ spleen cells (2×10^7) were adoptively

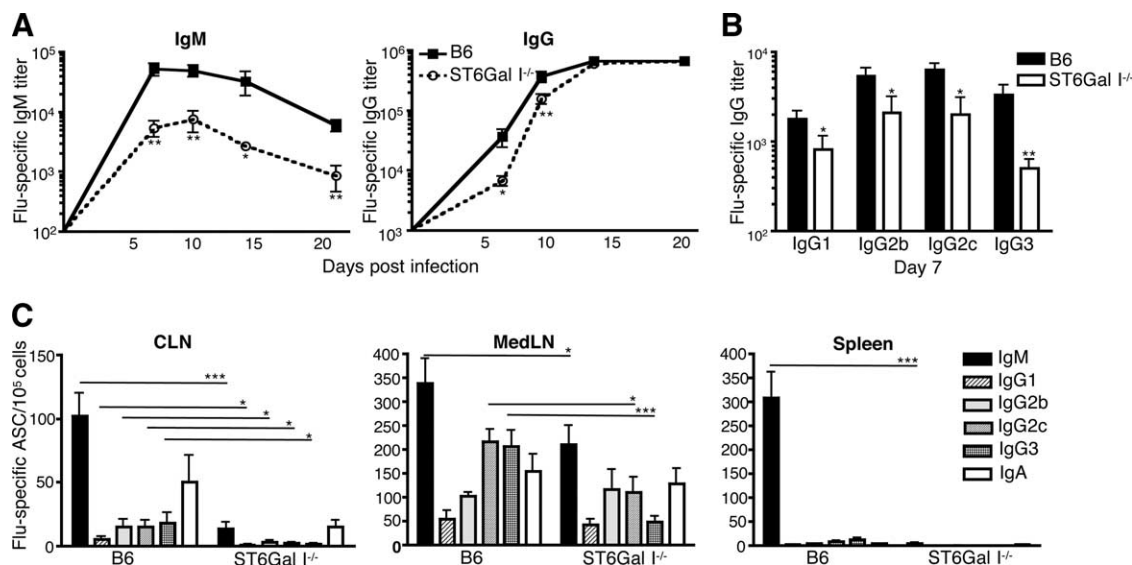


FIGURE 2. Influenza-specific B cell responses in ST6Gal I^{-/-} mice during acute infection. B6 or ST6Gal I^{-/-} mice were infected with influenza A/HKx31 i.n. and bled on indicated days. Serum virus-specific IgM (left) and IgG (right) (A) and IgG (B) isotypes on day 7 were measured by ELISA. Data are expressed as the reciprocal endpoint Ab titer on serial titration of influenza Ab. C, Cell suspensions of CLN, MedLN, and spleen were prepared from individual mice on day 7 p.i., and the ELISPOT assay was used to measure the number of cells producing virus-specific IgM, IgG1, IgG2b, IgG2c, IgG3 or IgA as indicated, with $n = 6-8$ mice/group. Results are expressed as the number of ASC/10⁵ nucleated cells. (* indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$.)

transferred into individual primed μ MT-deficient mice i.v. followed by infection with influenza i.n. 24 h later. For serum Ab determination by ELISA, plates were coated with purified, detergent disrupted influenza A/HKx31 virus (0.5 μ g/well) and incubated at 4°C overnight. Plates were washed with PBS-Tween 20 (0.05%), blocked with PBS/FBS (3%), and washed again. Serial 3-fold serum dilutions were prepared in PBS-Tween

20 (0.05%)-BSA (0.5%). The plates were incubated at room temperature 4 h or overnight at 4°C, then washed thoroughly. Plate-bound secreted Abs were detected using alkaline phosphatase-conjugated goat anti-mouse Abs with specificity for IgM, IgG, IgG1, IgG2b, IgG2c, or IgG3 (Southern Biotechnology Associates) diluted in PBS-BSA (1%). After 4 h of incubation at room temperature, plates were washed extensively, and color

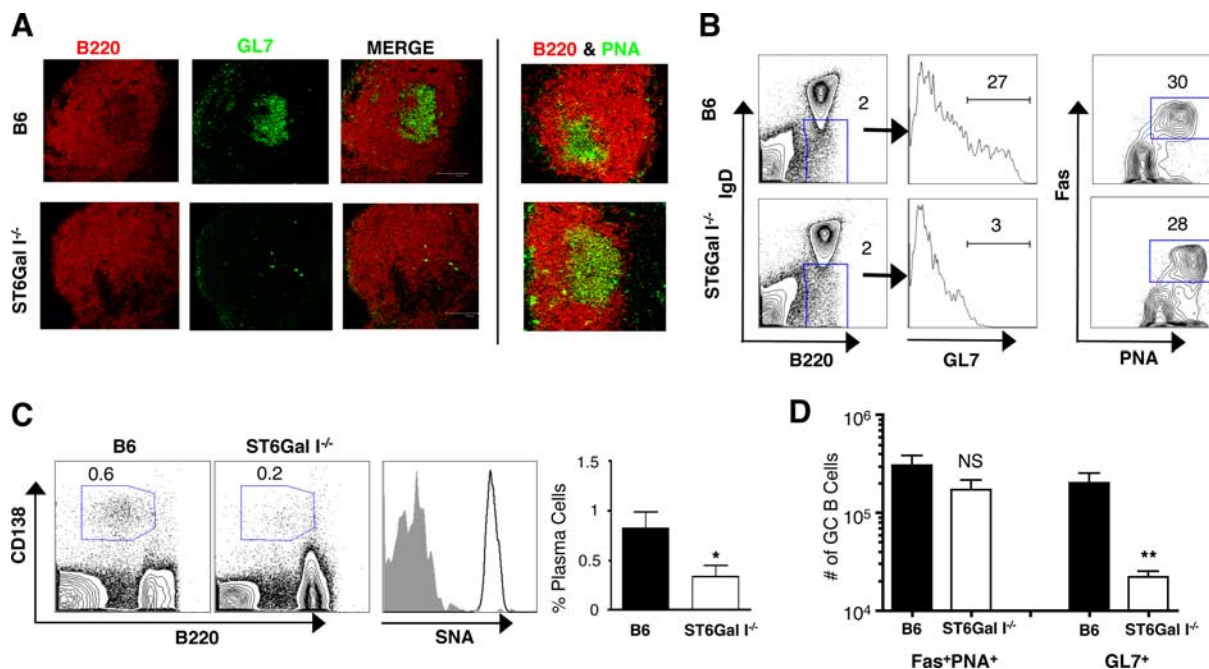


FIGURE 3. Germinal center B cells of ST6Gal I^{-/-} mice do not bind GL7. Mice were infected with influenza x31 intranasally, and on day 7 p.i., lymph nodes were isolated and either frozen for immunofluorescence or stained for FACS analysis using germinal center or plasma cell markers. Numbers show percentages of gated population. A, Immunofluorescence staining of MedLN using B220 and GL7 or B220 and PNA as indicated. B220 in red and GL7 or PNA staining in green. B, FACS analysis of germinal center B cells in CLN of same mice, gating on IgD^{low}B220⁺ cells. Histogram shows GL7 binding on gated IgD^{low}B220⁺ cells. Dot plot shows PNA^{high}Fas⁺ cell population of gated IgD^{low}B220⁺ cells. C, FACS analysis of plasma cells in CLN gating on CD138⁺B220⁺ cells. Histogram overlay of SNA binding of gated population. Filled histogram shows ST6Gal I^{-/-}, and open histogram shows B6. Bar graph shows frequencies of total plasma cells in indicated mice. D, Total number of germinal center B cells in CLN gating on IgD^{low}B220⁺PNA^{high}Fas⁺ or IgD^{low}B220⁺GL7⁺ cells. Bar graphs show data from $n = 5$ mice. (*, $p < 0.05$; and **, $p < 0.01$.)

development with *p*-nitrophenyl phosphate (Sigma-Aldrich) in diethanolamine buffer was read at 405 nm using a Synergy2 MultiDetection Microplate Reader (Bio-Tek Instruments). The virus-specific serum Ab titer is expressed as the reciprocal of the highest dilution giving an absorbance value more than twice that for simultaneously titrated samples from naive mice.

Results

SNA binding is high on naive and in vivo activated B cells

The plant lectin SNA preferentially binds α 2-6-linked sialic acids and previous reports have demonstrated high SNA binding on naive lymphocytes that is abrogated in lymphocytes lacking ST6Gal I expression (5). Gene expression studies comparing naive B cells to in vitro activated B cells revealed that ST6Gal I expression remains high on activated B cells (13).

We examined SNA lectin binding by flow cytometry on B cells following in vivo B cell activation (Fig. 1). We observed that on day 8 following viral infection, B220⁺CD138⁺ plasma blasts remain SNA high similar to B220⁺IgD⁺ naive B cells in uninfected mice. Additionally, on day 15 p.i., B220⁺Fas⁺gD^{low} germinal center B cells also show high SNA lectin binding (Fig. 1).

Impaired generation of influenza-specific humoral responses in ST6Gal I-deficient mice

To examine the functional relevance of this modification for antiviral humoral responses, we obtained ST6Gal I-null mice. ST6Gal I^{-/-} mice develop normally and show a normal lymphoid compartment and architecture (5, 14). However, B cells from ST6Gal I^{-/-} mice show impaired proliferation in vitro to anti-IgM, anti-CD40, and LPS stimulation that can be rescued by the addition of IL-4 or gene ablation of CD22 (5, 6, 15). These mice also show defective T-dependent and T-independent humoral responses to hapten-protein immunization. Previous studies examining Ab responses following viral infection have sometimes contrasted with results using hapten-protein immunizations (16, 17). We infected ST6Gal I^{-/-} mice with influenza A/HKx31 and serially bled them to determine influenza-specific IgM and IgG by serum ELISA (Fig. 2A). We observed ~10-fold reductions and ~5-fold reductions in the levels of influenza-specific IgM and IgG, respectively, on day 7 p.i. (Fig. 2A). Analysis of IgG isotypes on day 7 p.i. revealed that all isotypes were significantly reduced compared with wild-type mice, with IgG3 and IgG2c showing the most impairment of the IgG isotypes (3- and 7-fold, respectively; Fig. 2B). By day 14 p.i., influenza-specific IgG was similar comparing wild-type and ST6Gal I^{-/-} mice, while influenza-specific IgM still remained impaired.

One possible explanation for the differences observed could be aberrantly glycosylated Abs are preferentially scavenged in vivo by lectin-bearing macrophages (18). We determined whether ST6Gal I^{-/-} mice are defective in the generation of viral-specific plasma cells (Fig. 2C). Examination of influenza-specific ASCs by ELISPOT in the CLN, the MedLN, and spleen show significantly reduced numbers at day 7. Influenza-specific IgM producing ASCs showed the most impairment, especially in the spleen. Influenza-specific IgA producing ASCs were reduced, but the reduction was not statistically significant (Fig. 2C). These results show that the reduced serum levels of antiviral Abs are likely not the effect of preferential scavenging by macrophages, but rather a defect in the generation of the humoral immune response.

Germinal centers of ST6Gal I^{-/-} mice do not bind GL7

To determine whether any differences in germinal center formation are present in ST6Gal I^{-/-} mice, we performed immunostaining in the lymph nodes. Our analysis of the MedLN by immunofluorescence on day 7 p.i. revealed that germinal center B cells in ST6Gal I^{-/-} mice show no expression of the germinal center marker GL7,

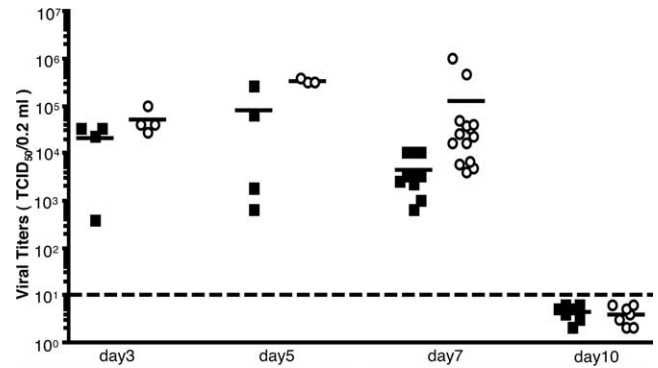


FIGURE 4. Influenza viral infection and clearance in lungs. B6 and ST6Gal I^{-/-} mice were infected intranasally, and on indicated days postinfection, lungs were collected and assayed for influenza viral titer. Filled symbols are B6 mice, and open symbols are ST6Gal I^{-/-} mice. Each symbol represents one mouse tested. The limit of detection is shown by the dotted line.

but show high binding by the PNA lectin (Fig. 3A). These data are consistent with results demonstrating that the mAb GL7 shows specificity for a sialylated glycan epitope expressed on germinal center B cells (19). FACS analysis of lymphocytes from the CLN of these mice gating on IgD^{low}B220⁺ cells showed similar percentages and numbers of GC cells as measured by expression of germinal center markers Fas and PNA, and confirmed loss of GL7 binding (Fig. 3, B and D). We also noted that the mean fluorescence intensity of PNA binding was significantly higher in ST6Gal I^{-/-} GC cells compared with wild-type (mean fluorescence intensity = 1178 vs 549). The frequencies of total plasma cells (CD138⁺B220⁺) were reduced in ST6Gal I^{-/-} mice, consistent

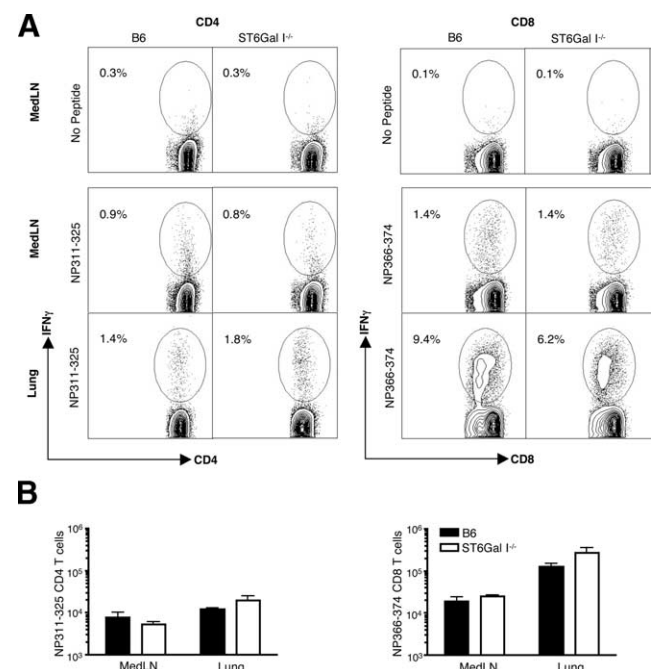


FIGURE 5. Influenza-specific T cell responses. At day 10 p.i., lymphocytes from MedLN and lung were stimulated with indicated peptides for 5 h and stained for intracellular cytokine production of IFN- γ . **A**, Representative staining for each tissue gating on CD4 or CD8 T cells. Numbers indicate the percentage of CD4 or CD8 T cells specific for each peptide. **B**, Total NP311-325-specific CD4 or NP366-374-specific CD8 T cells were enumerated in MedLN and lung at day 10; $n = 4$ mice in each group.

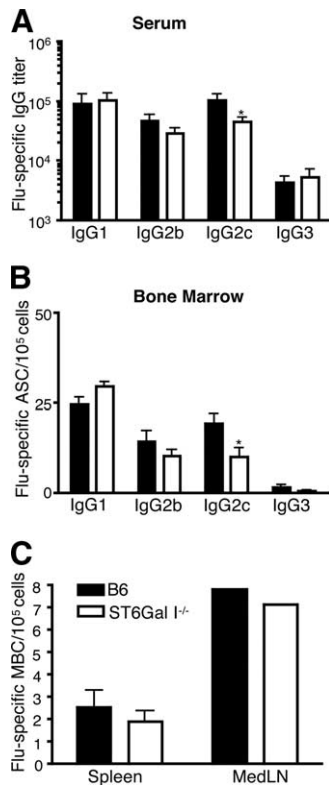


FIGURE 6. Immune influenza-specific IgG responses. *A*, Influenza-specific IgG isotypes from day 180 sera samples of B6 or ST6Gal I^{-/-} mice. *B*, ELISPOT assay was used to determine the virus-specific IgG1, IgG2b, IgG2c, and IgG3 ASCs in bone marrow of mice (LLPCs). *C*, Memory B cell numbers from spleen and MedLN; cells are pooled from four mice for MedLN; *n* = 4–8 mice/group.

with the reduction in viral specific ASCs and as expected, showed no binding of the SNA lectin (Figs. 2*C* and 3*C*).

Productive infection and replication of influenza in ST6Gal I^{-/-} mice

Entry of influenza virus, as well as the productive infection and replication in lung epithelial cells is dependent on sialic acid modification of glycoproteins. Productive influenza infection in humans depends specifically on sialyloligosaccharides containing terminal *N*-acetyl sialic acid linked to galactose by an α 2,6-linkage, whereas in mice the data suggest α 2,3-linked sialic acids are important (20, 21). Influenza viral titers were determined in the lungs of mice infected with A/HKx31 (Fig. 4). We confirm that similar to wild-type mice, lungs from ST6Gal I^{-/-} mice become productively infected with influenza A/HKx31 and show comparable viral titers at days 3 and 5 p.i. By day 7 p.i., we see a trend toward higher viral titers in the lungs of ST6Gal I^{-/-} mice, which coincides with the early impairment of viral-specific Ab responses observed in these mice. However, this difference is not statistically significant (*p* = 0.07), and by day 10 virus was cleared by both wild-type and ST6Gal I^{-/-} mice. These results extend the findings in a recently published report showing several human influenza A viruses are able to productively infect the respiratory tract of mice lacking ST6Gal I expression (22).

Viral-specific CD4 and CD8 T cells in the lungs and MedLN

Since T cells also express ST6Gal I, we enumerated influenza-specific T cells isolated from the lungs or draining nodes (MedLN) of wild-type or ST6Gal I^{-/-} mice on day 10 using CD4-specific

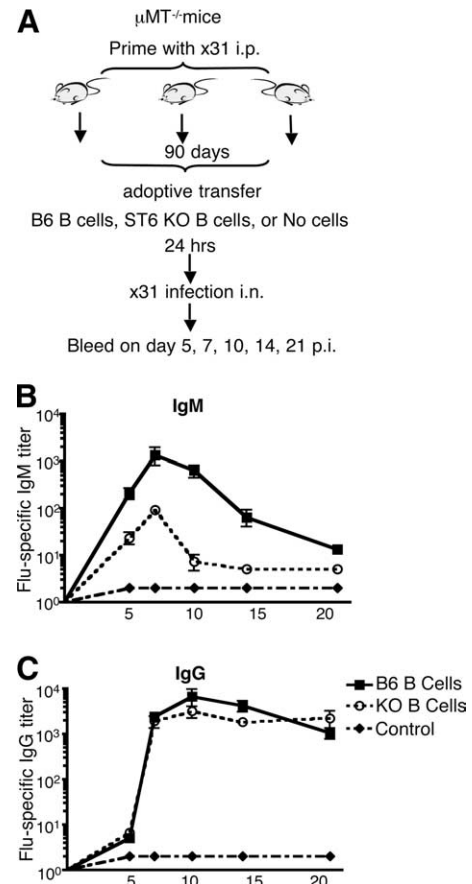


FIGURE 7. Loss of expression of ST6Gal I by B cells results in defective influenza specific IgM. *A*, Cartoon depicting experimental protocol where μ MT^{-/-} recipient mice were primed with 10^{7.4} EID₅₀ i.p.; 90 days later, 2 × 10⁷ purified naive B220⁺ B cells from B6 or ST6Gal I^{-/-} mice were adoptively transferred i.v. into mice. Control mice received no transferred cells. Mice were infected with 10^{6.8} EID₅₀ influenza A/HKx31 intranasally 1 day later and bled on indicated days. Serum virus-specific Abs for IgM (*B*) or IgG (*C*) levels were detected by ELISA, *n* = 3 mice in each group.

(NP311–325) or CD8-specific (NP366–374) peptides performing intracellular cytokine staining to detect IFN- γ (23). ST6Gal I^{-/-} mice generate similar numbers of viral-specific CD4 and CD8 T cells in the lung and MedLN, show similar coproduction of TNF- α and IL-2, and show similar numbers of CD44^{high} CD4 and CD8 T cells in these tissues (Fig. 5 and data not shown). However, we did note reduced numbers of viral specific CD8 T cells in the spleen on day 10 consistent with our observations of reduced viral specific T cells following LCMV infection in these mice (T. Onami, manuscript in preparation).

Viral-specific memory B cell responses

ST6Gal I^{-/-} mice control influenza infection by day 10, despite impaired influenza-specific humoral responses (Fig. 4). The strong antiviral T cell responses in the lungs of these mice likely contribute to this effective viral clearance. Immune ST6Gal I^{-/-} mice show high levels of class-switched IgG Ab; however, a significant reduction in serum levels of IgG2c was observed, supported by a significant reduction in frequencies of influenza-specific IgG2c-producing LLPCs in the bone marrow (Fig. 6, *A* and *B*). Examination of MBCs in the spleen and MedLN reveal comparable frequencies of total influenza-specific IgG MBCs (Fig. 6*C*). These results clearly indicate that, with the exception of the reduced

IgG2c levels, immune ST6Gal I^{-/-} mice generated similar high titers of antiviral-specific Ab.

B cell expression of ST6Gal I is required to generate optimal levels of viral-specific IgM

Our data indicate that ST6Gal I^{-/-} mice are able to generate strong antiviral humoral memory despite early impairments in the generation of the viral-specific humoral response (Figs. 2 and 6). To determine whether it is the expression of ST6Gal I by B cells alone that results in impairment of the early antiviral IgM and IgG response, we transferred purified naive B cells from either wild-type or ST6Gal I^{-/-} mice into primed B cell-deficient μ MT^{-/-} mice. Recipient mice were intranasally infected with influenza and serially bled to determine influenza-specific Ab levels. Mice that received ST6Gal I^{-/-} B cells show impaired influenza-specific IgM levels but similar viral-specific IgG (Fig. 7). These data argue that the observed impairment in production of viral-specific IgM in ST6Gal I^{-/-} mice is likely B cell intrinsic; however, the impairment in early production of viral-specific IgG is not B cell intrinsic. Thus, expression of ST6Gal I by B cells, at least in part, is required for normal antiviral humoral immunity.

Discussion

In this article, we have evaluated the influence of posttranslational modification of glycoproteins on the immune response to influenza virus infection. Changes in the posttranslational modification of glycoproteins during immune responses have been reported for decades, but their functional importance remains obscure (24–32). We demonstrate that in contrast to observations with PNA lectin binding, which changes during activation/differentiation of B cells, naive and activated B cells in vivo show similar high binding of SNA (Fig. 1). This supports recently published data showing that ST6Gal I gene expression does not change following B cell activation in vitro (13). Using ST6Gal I^{-/-} mice, which lack the ability to catalyze the addition of α 2,6 sialic acids to N-linked glycoproteins, we investigated the role of this modification on the generation of an influenza-specific humoral response. Serum levels of influenza-specific Abs in these mice are reduced early in the immune response, with influenza-specific IgM responses showing the greatest impairment (Fig. 2A). These data are supported by reductions in the frequencies of viral-specific plasma cells, suggesting that the impaired viral-specific Ab levels are likely a result of altered generation of short-lived viral-specific plasma cells and not a result of preferential scavenging of Abs lacking α 2,6 sialic acids (Figs. 2C and 3C). Additionally, ST6Gal I^{-/-} mice show no differences in influenza viral replication in the lungs, so the reduced Ab levels are not due to reduced viral load or Ag availability in these mice (Fig. 4). We did observe comparable influenza-specific CD4 and CD8 T cell responses in the lung and draining lymph nodes of these mice, and this most likely explains why ST6Gal I^{-/-} mice are able to control and clear influenza viral infection in the lungs by day 10, similar to wild-type mice (Fig. 5). Despite significant impairments in the generation of an early antiviral humoral response, ST6Gal I^{-/-} mice are able to generate potent antiviral humoral immunity, with high levels of class-switched IgG Abs by day 14 and memory time points (Figs. 2A and 6). This is the first reported analysis of antiviral immune responses in ST6Gal I^{-/-} mice.

To determine whether the observed impairment of antiviral humoral responses were B cell intrinsic, we adoptively transferred ST6Gal I^{+/+} or ST6Gal I^{-/-} B cells into primed B cell-deficient μ MT^{-/-} mice (Fig. 7). Primed mice were used to ensure adequate CD4 T help in these mice. The antiviral IgM response to infection was substantially impaired in mice that received ST6Gal I^{-/-} B

cells. However, the antiviral IgG response was similar compared with mice that received wild-type B cells. These results suggest that the defective antiviral IgM response observed in ST6Gal I^{-/-} mice is likely B cell intrinsic. While we must be cautious with our interpretations of antiviral responses in B cell-deficient mice due to known alterations in lymphoid architecture, the results raise the possibility that subtle defects in an additional cell compartment in ST6Gal I^{-/-} mice may contribute to the delayed antiviral IgG response. Future studies will investigate how ST6Gal I deficiency affects other lymphoid cells and how this loss contributes to the defective antiviral response.

Recent reports have demonstrated that loss of expression of ST6Gal I by B cells results in preferential colocalization of the BCR with CD22, a negative regulator of BCR signaling, in clathrin-rich membrane microdomains (6, 15). Aged CD22-deficient mice have been reported to develop IgM autoantibodies (33). Ablation of CD22 expression in ST6Gal I^{-/-} mice rescues BCR signaling defects (6). In naive B cells, modification of CD22 by ST6Gal I normally results in segregation of CD22 molecules, due to homotypic interactions with the α 2,6 sialic acid *cis*-ligands of other CD22 molecules (6). Following Ag receptor engagement, CD22 is tyrosine phosphorylated, and phosphorylated CD22 recruits SHP-1 (Src homology domain2-containing tyrosine phosphatase), mediating signal inhibition via the BCR (33, 34). Due to the absence of these *cis*-CD22 ligands in ST6Gal I^{-/-} mice, there is increased association of sIgM with the inhibitory receptor CD22 on naive B cells; following Ag receptor engagement in vivo, this could result in immediate attenuated BCR signaling. We speculate that a possible in vivo outcome of this could be significantly impaired differentiation of naive B cells into extrafollicular short-lived plasma cells following viral infection of ST6Gal I^{-/-} mice (33, 35–38). Viral-specific B cells in the ST6Gal I^{-/-} mice, with “weak or moderate BCR” signaling could enter germinal centers and undergo affinity maturation and differentiation into plasma cells. This could explain why we observe that after day 14 p.i., influenza-specific IgG responses are quite comparable to wild-type mice. Thus, in a normal immune response, ST6Gal I modification of glycoproteins on naive B cells may influence the signaling threshold required following Ag receptor signaling, and the biological outcome in vivo would favor differentiation of a greater proportion of Ag-specific B cells into extrafollicular short-lived plasma cells, important in the early control of pathogens. Alternatively, the repertoire of B cells available in ST6Gal I^{-/-} mice could be different from wild-type mice due to differences in signaling strength during development (39, 40).

In conclusion, our data demonstrate that despite early defects in the generation of an antiviral humoral response, ST6Gal I^{-/-} mice are able to clear virus and generate potent humoral memory. Furthermore, our data suggest that loss of ST6Gal I expression by B cells may be responsible for the impaired antiviral IgM response. Finally, loss of expression of ST6Gal I in non-B cells likely contributes to the impaired IgG humoral response. Future experiments will elucidate how the expression of this enzyme, by different lymphocyte populations, influences the generation of an antiviral immune response.

Acknowledgments

We thank Dr. Rafi Ahmed at the Emory Vaccine Center and Dr. Linda Baum at University of California Los Angeles for helpful discussions and suggestions, Dr. Kendall Smith at Cornell University’s Weill Medical College for the NP311-325 CD4 peptide, Dr. Barry Rouse for critical review of the manuscript, and members of the Onami lab for technical assistance.

Disclosures

The authors have no financial conflict of interest.

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