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CUTTING EDGE

Cutting Edge: Guillain-Barré Syndrome-Associated IgG Responses to Gangliosides Are Generated Independently of CD1 Function in Mice[1]

Yukie Matsumoto, Nobuhiro Yuki,[2]* Luc Van Kaer,† Koichi Furukawa,‡ Koichi Hirata,* and Masahiko Sugita§*

***CD1 molecules present a variety of microbial glycolipids and self-glycolipids to T cells, but their potential role in humoral responses to glycolipid Ags remains to be established. To address this issue directly, we used GM1/GD1a-deficient mice, which, upon immunization with heat-killed* Campylobacter jejuni*, develop Guillain-Barré syndrome-associated IgG Abs against the GM1/GD1a sugar chain epitopes of bacterial lipo-oligosaccharides (LOS). Our results showed that anti-ganglioside Abs of the IgG1, IgG2b, and IgG3 isotypes were produced in the absence of group 2 CD1 (CD1d) expression. Unlike mouse and human group 2 CD1 molecules that specifically bound LOS, none of the group 1 CD1 molecules (CD1a, CD1b, and CD1c in humans) were capable of interacting with LOS. Thus, these results indicate CD1-independent pathways for anti-ganglioside Ab production. The Journal of Immunology,* 2008, 180: 39–43.**

Guillain-Barré syndrome (GBS),[3] characterized by limb weakness and areflexia, is a typical postinfectious autoimmune disease. About one-third of GBS cases develop in response to enteric infection with the Gram-negative bacterium *Campylobacter jejuni* (1). These patients produce IgG autoantibodies that react to self-gangliosides such as GM1 and GD1a expressed in peripheral nerves, resulting in complement-mediated neuronal damage (2–4).

Our previous studies have identified a molecular mechanism to explain the close linkage between the production of these pathogenic autoantibodies with *C. jejuni* infection (5, 6). A clinical isolate of *C. jejuni* (CF90-26) from a GBS patient was found to biosynthesize lipooligosaccharides (LOS) bearing GM1-like and GD1a-like structures (Fig. 1) and, thus, can potentially share antigenic epitopes with self-gangliosides. This "molecular mimicry" theory has been further substantiated by establishing animal models of human GBS. Rabbits sensitized with GM1-like LOS from *C. jejuni* (CF90-26) generate IgG anti-GM1 Abs that induce complement-mediated disruption of sodium channel clusters in the peripheral nerves, leading to the development of flaccid limb weakness (7). It is noteworthy that, whereas most IgG Abs against bacterial polysaccharides are of the IgG2 isotype, the cross-reactive IgG Abs to gangliosides and LOS that develop during *C. jejuni* enteritis-associated GBS comprise primarily IgG1 and IgG3 subclasses (8). This suggests that the induction of autoantibodies may require cognate interactions of B cells with specific T cells that can provide help for class switching into IgG1 and IgG3. Because of the chemical nature of nonprotein Ags, Ag recognition by these T cells would likely be restricted to Ag-presenting molecules that are distinct from the classical MHC class I and class II proteins.

Studies over the past decade have shown that MHC-independent pathways of glycolipid Ag presentation exist and are mediated by CD1 molecules. Human group 1 CD1 molecules (CD1a, CD1b, and CD1c) bind microbial glycolipid Ags, as well as self-glycolipids that include GM1 ganglioside, and present them to T cells bearing diverse TCRs (9, 10). Group 2 CD1 molecules (CD1d) also bind glycolipids of self and microbial origin and present them to NKT cells, including invariant NKT (iNKT) cells that express the invariant Vα14 TCR (11, 12). Although initial functional studies of CD1-restricted T cells have focused on their role in eliminating infected cells and regulating immune responses, their potential role in assisting Ab production has recently been proposed (13, 14).

Considering that B cells express some of the CD1 isotypes and that glycolipid-specific, CD1-restricted T cells with helper functions exist (15), we hypothesized that the generation of pathological IgG autoantibodies against LOS that cross-react with self-gangliosides might occur in a CD1-dependent

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[3] Abbreviations used in this paper: GBS, Guillain-Barré syndrome; α-GC, α-galactosylceramide; h, human (prefix); iNKT, invariant NKT; LOS, lipooligosaccharide; TPBS, Tween-PBS.

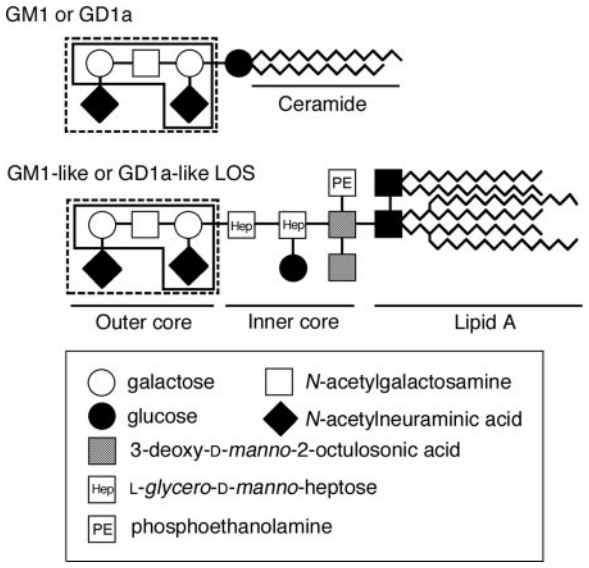

FIGURE 1. Carbohydrate mimicry between gangliosides and *C. jejuni* LOS. The terminal tetrasaccharide of GM1-like LOS is identical with that of GM1 (shown by solid lines). The terminal pentasaccharide structure of GD1a-like LOS is identical with that of GD1a (shown by dashed lines).

manner. Thus, the present study has been designed to directly address this hypothesis. First, we examined the capacity of each CD1 isoform to bind the *C. jejuni* LOS in a cell-free ELISA system. Next, we set up an in vivo system in which high titers of anti-GM1/GD1a Abs were produced by sensitization of GM1/GD1a-deficient mice with *C. jejuni* bacteria. We then generated GalNAcT$^{-/-}$CD1d$^{-/-}$ mice to explicitly analyze the impact of CD1d function on the production of autoantibodies. We found that, unlike other CD1 isoforms, CD1d strongly bound with *C. jejuni* LOS, but autoantibodies were produced independently of CD1d function.

Materials and Methods

Mice and immunization protocols

GM1/GD1a-deficient mice lacking the functional gene for (*N*-acetylneuraminyl)-galactosylglucosylceramide *N*-acetylgalactosaminyltransferase (Enzyme Commission no. EC 2.4.1.92) (GalNAcT$^{-/-}$ mice (B6.129-*B4galnt1*^{tm1Sia})) (16) and CD1d-deficient mice (CD1d$^{-/-}$ mice (B6.129S6-*Cd1d1*^{tm1Luc})) (17) were generated previously and backcrossed over eight generations with C57BL/6. GalNAcT$^{-/-}$CD1d$^{-/-}$ mice were obtained by intercrossing GalNAcT$^{-/-}$ mice with CD1d$^{-/-}$ mice. Seven- to 10-wk-old GalNAcT$^{-/-}$ and GalNAcT$^{-/-}$CD1d$^{-/-}$ mice were immunized i.p. with 1 mg (dry weight) of proteinase K-treated, heat-killed *C. jejuni* (CF90-26) (18) emulsified in IFA (Sigma-Aldrich), followed by two additional immunizations with the same Ag preparation at 2-wk intervals. Serum anti-GM1/GD1a Ab titers were measured by ELISA as described (5). Animals were treated according to the Guidelines for the Care and Use of Laboratory Animals of Dokkyo Medical University School of Medicine, and the experimental protocols were approved by the Animal Care and Use Committee, Dokkyo Medical University School of Medicine (Tochigi, Japan).

CD1 and LOS binding assays

LOS bearing GM1-like and GD1a-like structures were prepared from *C. jejuni* (CF90-26) as described previously (18). The CD1-IgG2a fusion proteins were purified as described (19, 20). To assess the ability of CD1 to bind GM1-like LOS, CD1-IgG2aFc fusion proteins and the isotype control UPC10 mAb (Sigma-Aldrich) were diluted at 10 μg/ml in PBS and mixed with LOS at the indicated concentrations. The mixture (100 μl/well) was added to protein A-coated 96-well plates (MaxiSorp; Nunc) and incubated at 37°C overnight, followed by blocking with 0.5% casein in PBS. To detect LOS-bound CD1, the plates were incubated with biotin-labeled anti-GM1 mAb (GB2) (5) and HRP-labeled streptavidin (Sigma-Aldrich). Development was done with *o*-phenylenediamine substrate.

In vitro stimulation of NKT hybridoma cells

The Vα14$^+$ NKT cell hybridoma DN32.D3 was obtained from Dr. A. Bendelac (University of Chicago, IL), and the Vα14$^+$ NKT cell hybridomas N38-2C12, N38-3C3, N57-2C12, and N57-2B6 were obtained from Dr. K. Hayakawa (Fox Chase Cancer Center, Philadelphia, PA). Plate-bound mCD1d was loaded with lipids as described above. After removing the free lipids, 5×10^4 NKT hybridoma cells were added to each well and cultured for 20 h. IL-2 released into the culture medium was measured by ELISA according to the manufacturer's instructions (BD Biosciences).

In vivo activation of NKT cells

Mice were injected i.p. with α-galactosylceramide (α-GC) (5 μg in 0.025% Tween-PBS (TPBS)), *C. jejuni* LOS (50 μg in IFA or TPBS), or *Escherichia coli* LPS (50 μg in TPBS), and the sera as well as splenocytes were obtained at the time points indicated in the figure legends. The serum IL-4 and IFN-γ levels were determined by ELISA (eBioscience). For flow cytometric analysis, splenocytes were labeled with anti-TCRβ, anti-CD69, anti-NK1.1, and anti-B220 Abs, followed by staining with an α-GC-loaded tetramer as described (19). Intracellular cytokine staining was performed after fixation and permeabilization with BD FACS Permeabilizing Solution 2 according to the manufacturer's instructions (BD Biosciences).

Results

Differential binding of LOS to CD1 isoforms

LOS-specific IgG Abs that cross-react with self-gangliosides represent a hallmark of *C. jejuni*-associated GBS, but a molecular basis for the induction of these autoantibodies remains to be elucidated. Considering their role in glycolipid-specific immune responses, CD1 molecules may play a critical role in mounting the humoral immune response to LOS. To address this possibility, we first assessed the capacity of each CD1 isoform to bind LOS by using a cell-free ELISA system. Recombinant soluble CD1-Fc fusion proteins and LOS bearing GM1-like structures were incubated in protein A-coated plates. After extensive washing, LOS loaded onto CD1-Fc fusion protein was detected by anti-GM1 mAb (GB2) recognizing the GM1 epitope on LOS. As shown in Fig. 2*A*, no significant interactions of the GM1-like LOS were observed with any of the human (h) group 1 CD1 molecules (hCD1a, hCD1b, and hCD1c). In sharp contrast, a dose-dependent binding of the GM1-like LOS was readily detected for the hCD1d molecule. Pretreatment of hCD1d-Fc with anti-hCD1d Ab, but not with an isotype-matched control Ab, totally abrogated LOS binding (Fig. 2*B*), confirming the specific interaction between hCD1d and LOS. In agreement with the generally accepted notion that the repertoire of CD1d-bound glycolipid Ags is overlapping if not identical between humans and mice, significant LOS binding was also demonstrated for mouse CD1d (mCD1d) (Fig. 2*C*). Thus, these results demonstrate that only group 2 CD1 (CD1d) can interact specifically with bacterial LOS.

Failure of LOS to activate NKT cells

Given the ability of CD1d to bind GM1-like LOS, we then examined whether LOS could activate NKT cells in two different settings. First, Vα14$^+$ iNKT hybridoma cells were stimulated in vitro by plate-bound CD1d molecules that were incubated with α-GC, LOS, GM1, *E. coli* LPS, or *Salmonella* LPS, and IL-2 release into the culture medium was measured. As shown in Fig. 3*A*, only α-GC but none of the other stimuli, including *C. jejuni* LOS, was able to induce IL-2 production by the hybridoma cells. Secondly, in vivo activation of NKT cells was assessed in α-GC-, LOS-, or LPS-injected mice. When mice were injected with 50 μg of LOS emulsified in IFA (the

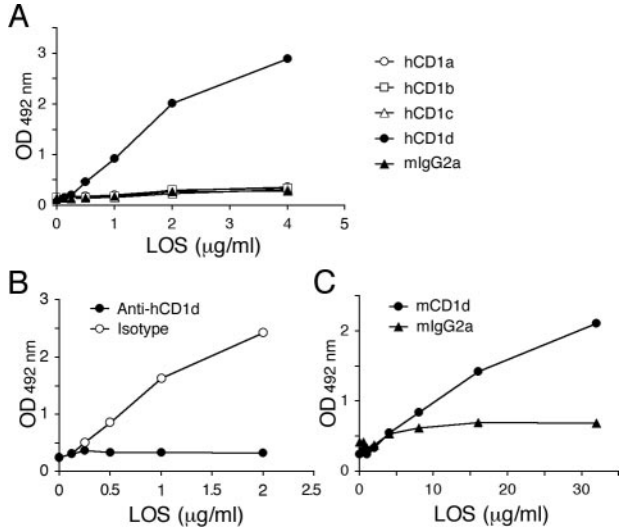

FIGURE 2. Specific binding of the GM1-like LOS to CD1d but not to other CD1 isoforms. *A*, Serially diluted (0.125–4 μg/ml) GM1-like LOS and hCD1a-Fc, hCD1b-Fc, hCD1c-Fc, hCD1d-Fc, or the negative control mouse IgG2a (mIgG2a) were incubated in protein A-coated plates, and GM1-like LOS loaded onto plate-bound hCD1 isoforms were detected by ELISA. *B*, A binding assay with GM1-like LOS and hCD1d was performed in the presence of either anti-hCD1d mAb (d55) or isotype control mAb. *C*, Binding of the serially diluted (0.5–32 μg/ml) GM1-like LOS to plate-bound mCD1d and to mouse IgG2a was examined by ELISA as conducted in *A*.

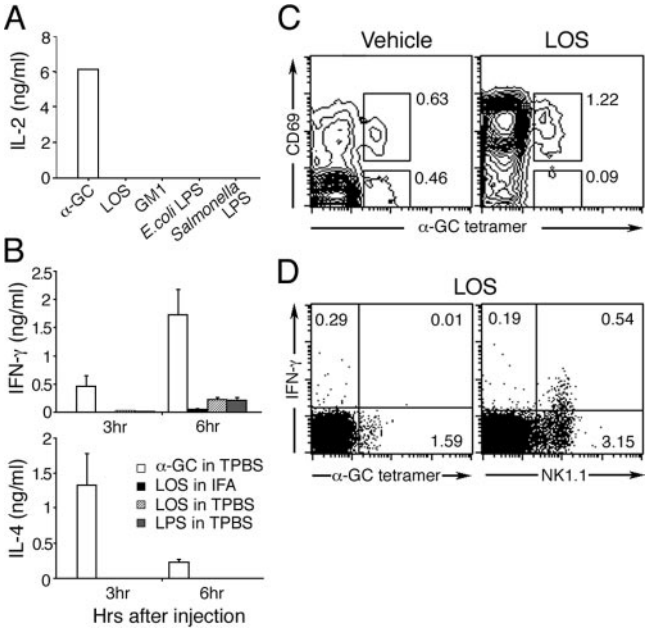

FIGURE 3. Failure of LOS to activate NKT cells. *A*, Vα14$^+$ NKT hybridoma cells (DN32.D3) were cultured with plate-bound CD1d in the presence of 0.1 μM α-GC, 1 μM *C. jejuni* LOS, 1 μM GM1, 1 μM *E. coli* LPS, or 1 μM *Salmonella* LPS. IL-2 production by the hybridoma cells was assessed. Similar results were obtained with the Vα14$^+$ N38-2C12, N38-3C3, N57-2C12, and N57-2B6 hybridoma cell lines (data not shown). *B*, GalNAcT$^{-/-}$ mice were injected i.p. with α-GC (5 μg in TPBS), *C. jejuni* LOS (50 μg in IFA or 50 μg in TPBS), or *E. coli* LPS (50 μg in TPBS). The serum IL-4 and IFN-γ levels were determined at 3 and 6 h after injection. *C*, Splenocytes were obtained at 6 h after injection with LOS (50 μg in TPBS) or vehicle only, and flow cytometric analysis was performed. Numbers indicate the percentages of CD69$^+$tetramer$^+$ or CD69$^-$tetramer$^+$ cells among TCRβ^+ cells. *D*, IFN-γ production in the spleen at 6 h after injection with LOS (50 μg in TPBS). Numbers indicate the percentages of IFN-γ^+tetramer$^+$ or IFN-γ^-tetramer$^+$ cells among TCRβ^+B220$^-$ cells (*left panel*) and IFN-γ^+NK1.1$^+$ or IFN-γ^-NK1.1$^+$ cells (*right panel*) among TCRβ^-B220$^-$ cells.

optimum condition to induce specific IgG Abs to GM1/GD1a in GalNAcT$^{-/-}$ mice), only a marginal elevation of serum IFN-γ levels was detected at 6 h and IL-4 was not detectable at any time point, which contrasted sharply with the robust IFN-γ and IL-4 response induced by α-GC injection (Fig. 3*B*). Injection of LOS as a form that was dissolved in TPBS rather than in IFA resulted in augmented IFN-γ responses, but still no IL-4 response was detected (Fig. 3*B*). In these mice many cell types, including α-GC-loaded CD1d tetramer$^+$ cells, appeared to express the early activation marker CD69 (Fig. 3*C*), but the IFN-γ-producing cells were identified as tetramer$^-$NK1.1$^+$TCRβ^- cells, presumably representing NK cells (Fig. 3*D*). Taken together, LOS failed to activate iNKT cells in a manner that was comparable to α-GC.

Production of IgG anti-ganglioside Abs in GalNAcT$^{-/-}$CD1d$^{-/-}$ mice

To address directly whether Ab responses to LOS bearing the GM1-like and GD1a-like structures might depend on CD1d function, GalNAcT$^{-/-}$CD1d$^{-/-}$ mice and GalNAcT$^{-/-}$CD1d$^{+/+}$ mice were immunized with *C. jejuni* bacteria and serum titers of specific IgG Abs to GM1 and GD1a were compared. As shown in Fig. 4*A*, both groups of animals elicited similar levels of IgG responses to GM1 (*left panel*), and titers of anti-GD1a Abs were even higher in GalNAcT$^{-/-}$CD1d$^{-/-}$ mice than those in GalNAcT$^{-/-}$CD1d$^{+/+}$ mice (*right panel*).

We further determined the IgG subclasses of the anti-GM1 and anti-GD1a Abs detected in the sera of GalNAcT$^{-/-}$CD1d$^{-/-}$ and GalNAcT$^{-/-}$CD1d$^{+/+}$ mice after the third immunization. We found that the majority of the specific Abs in both groups of animals were of the IgG3 and IgG2b subclasses, which are the IgG subclasses typically seen in T-independent Ab responses. Class switching to IgG1, indicative of T-dependent responses, was also observed in both GalNAcT$^{-/-}$CD1d$^{-/-}$ and GalNAcT$^{-/-}$CD1d$^{+/+}$ mice, and no statistically significant differences were observed between the groups (Fig. 4*B*). Class switching to IgG2a was detected in none of the GalNAcT$^{-/-}$CD1d$^{-/-}$ and GalNAcT$^{-/-}$CD1d$^{+/+}$ mice. Thus, these results indicate that development of IgG anti-ganglioside Abs in the murine model occurs independently of CD1d function.

Discussion

Apart from their unique capacity to activate innate immune cells such as NK cells and dendritic cells during the early phases of a variety of immune responses, it has recently been noted that CD1d-restricted NKT cells influence Ab production. NKT cells activated with α-GC are able to enhance humoral immunity to both T-dependent and T-independent Ags (21, 22). In murine infection models, such as those with *Borrelia hermsii* (13) and *Plasmodium berghei* (23), CD1d-dependent Ab production and its role in host defense have also been reported. In addition, CD1d- and CD8$^+$ T cell-dependent pathways for IgG responses to pneumococcal polysaccharides may exist, although the identity of the CD8$^+$ T cells involved in these pathways has not been determined (14). Despite these examples of CD1d-dependent Ab production, it remains to be determined whether the generation of Ab responses to glycolipids, such as

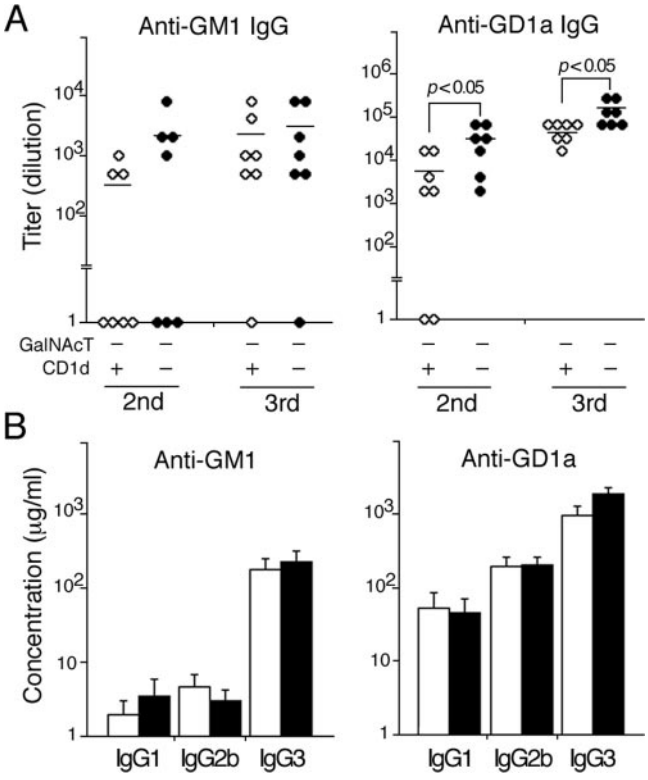

FIGURE 4. Production of IgG anti-GM1 and anti-GD1a Abs after immunization with *C. jejuni* LOS. *A*, Serum IgG Abs specific for GM1- and GD1a-oligosaccharides in GalNAcT$^{-/-}$ mice (open circles) and GalNAcT$^{-/-}$ CD1d$^{-/-}$ mice (filled circles) were measured by ELISA after the second and third immunizations. Each symbol represents a single animal. Mann-Whitney *U* test was used to determine the level of statistical significance. *B*, Subclass analysis of IgG anti-GM1 and anti-GD1a Abs in GalNAcT$^{-/-}$ mice (open bars) and GalNAcT$^{-/-}$CD1d$^{-/-}$ mice (filled bars). Statistical significance between GalNAcT$^{-/-}$ and GalNAcT$^{-/-}$CD1d$^{-/-}$ groups was examined by repeated measure ANOVA.

those derived from pathogens, requires expression of CD1d molecules.

In the present study, we have demonstrated that IgG responses directed against the sugar chain epitopes on LOS can occur even in the absence of CD1d function. Thus, this study and the work described above (14) highlight distinct pathways of Ab production to polysaccharides and LOS. Polysaccharide Ags contain repetitive carbohydrate epitopes that allow physical cross-linking of specific BCRs in lipid rafts (24). Further, surface CD1d molecules expressed on B cells, especially on CD1d^{high} marginal zone B cells, may comigrate in rafts and transmit an activation signal via their cytoplasmic tail tyrosine-containing motif, as suggested for CD1d^{+} intestinal epithelial cells (25, 26). In contrast, LOS, which is devoid of repetitive carbohydrate epitopes, may not be efficient in stabilizing lipid rafts (24), but their lipid A structure can transmit supporting signals for IgG responses directed against the sugar chain epitopes of LOS.

Without direct TCR interaction with the CD1d-ligand complex, NKT cell activation can potentially occur in response to cytokines released from APCs that are stimulated with microbial components such as LPS (27). However, we could not detect any significant cytokine production by iNKT cells after i.p. injection of *C. jejuni* LOS (Fig. 3, and data not shown), making it unlikely that the IgG anti-LOS response might be mediated by indirect activation of iNKT cells.

Pathogenic anti-ganglioside Abs that develop in *C. jejuni* enteritis-associated GBS belong to the IgG1 and IgG3 subclasses, suggestive of T cell-dependent responses (8). Because bacterial LPS and LOS cannot directly activate class switch recombination in human B cells, T cell-dependent pathways should exist for the production of anti-ganglioside Abs. We observed that splenic T cells from both GalNAcT$^{-/-}$ and GalNAcT$^{-/-}$ CD1d$^{-/-}$ mice were capable of producing large amounts of IL-10 and IL-13 at similar levels in response to GM1-like LOS (data not shown) and, thus, it is possible that T cell-derived, cytokine-dependent pathways for B cell activation and differentiation, rather than cognate B cell-T cell interactions, may play a substantial role in the production of anti-ganglioside Abs.

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Disclosures

The authors have no financial conflict of interest.

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