CD4 and CD8 T cells require different membrane gangliosides for activation

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Initial events of T-cell activation involve movement of the T-cell receptor into lipid rafts. Gangliosides are major components of lipid rafts. While investigating T-cell activation in ganglioside-deficient mice, we observed that $CD4^+$ and $CD8^+$ T cells required different ganglioside subsets for activation. Activation of $CD4^+$ T cells from GM3 synthase-null mice, deficient in GM3-derived gangliosides, is severely compromised, whereas $CD8^+$ T-cell activation is normal. Conversely, in cells from GM2/GD2 synthase-null mice, expressing only GM3 and GD3, $CD4^+$ T-cell activation is normal, whereas $CD8^+$ T-cell activation is deficient. Supplementing the cells with the corresponding missing gangliosides restores normal activation. GM3 synthase-null mice do not develop experimental asthma. Distinct expression patterns of ganglioside species in $CD4^+$ T and $CD8^+$ T cells, perhaps in uniquely functional lipid rafts, define immune functions in each T-cell subset. Control of ganglioside expression would offer a strategy targeting for specific T-cell subpopulations to treat immune diseases.

glycosphingolipids | repertoire selection

The initial events of T-cell activation involve movement of the T-cell receptor into specialized membrane microdomains known as lipid rafts. The term "lipid rafts" was introduced by Kai Simons and Elina Ikonen, on the basis of the close association of sphingolipids and cholesterol as the detergent resistant complex of signaling molecules present in the membrane microdomains (1). In T lymphocytes, lipid rafts are implicated in signaling from T-cell antigen receptor (TCR) and in localization and function of proteins residing proximal to the receptor, such as coreceptors CD4 and CD8, Src family kinases Lck and Fyn, transmembrane adaptor linker for activation for T cells (LAT), and protein kinase Cθ (2, 3). Gangliosides, sialic acid (SA)-containing glycosphingolipids (GSLs) associated with lipid rafts, are thought to be involved in T-cell activation. For example, following TCR clustering induced by anti-CD3 and anti-CD28 antibodies, polarization of GM1a ganglioside occurs in $CD4^+$ T cells but not in $CD8^+$ T cells (4). In polarized human T cells, asymmetric redistribution results in ganglioside GM3- and GM1a-enriched raft domains segregating to the leading edge and uropod, respectively (5). Additionally, major differences in GM1a expression levels are apparent among cell types or stages of cellular development (6), and at times GM1a expression in certain cell types is much lower than that of other gangliosides (7). Thus, to understand the role of lipid rafts in the differentiation, maturation, and activation of $CD4^+$ T and $CD8^+$ T cells in vivo, it is necessary to understand the ganglioside composition in each respective T-cell subset. To date, however, there has been no report detailing the ganglioside structures in naive or primary T-cell subsets.

In the present study, we analyzed the expression of gangliosides during T-cell differentiation and investigated whether the activation of individual T-cell subsets requires distinct species of gangliosides (Fig. 1*A*). We used two kinds of gene-targeted mice, one carrying disrupted GM3 synthase (GM3S), and so lacking GM3-derived gangliosides (a- and b-series gangliosides) (8), and the other with an altered GM2/GD2 synthase (GM2/GD2S) and expressing only GM3 and GD3 gangliosides while lacking the o-series (9). We found distinct and dramatic changes in ganglioside profiles between primary thymocytes and resting $CD4^+$ T and $CD8^+$ T cells. In particular, $CD4^+$ T cells preferentially express a-series gangliosides, whereas $CD8^+$ T cells express very high levels of o-series gangliosides. Likewise, TCR-dependent activation of $CD4^+$ T cells selectively requires a-series gangliosides, yet for similar activation of $CD8^+$ T cells, o-series gangliosides and not a-series gangliosides are indispensable. Distinct expression patterns of gangliosides in $CD4^+$ T and $CD8^+$ T cells in unique functional lipid rafts may define immune functions in each T-cell subset.

We propose that the repertoire selection from immature thymocytes to mature T-cell subsets is accompanied by selective GSL expression in individual T-cell subsets. This GSL selection process may be indispensable in the formation of distinct and functional lipid rafts in mature T cells.

Results

Distinct Selectivity of Gangliosides Required for $CD4^+$ T- and $CD8^+$ T-Cell Activation. GSLs are built on a ceramide backbone comprising a long-chain amino alcohol, sphingosine, and an amide-linked fatty acid. Gangliosides are SA-containing GSLs (Fig. 1*A*). GM3, the simplest of the "a-series" gangliosides, is synthesized by GM3S, which catalyzes the transfer of SA to the nonreducing terminal galactose (Gal) of lactosylceramide (LacCer). GM3 is altered by GM2/GD2S to form GM2, a downstream a-series ganglioside, or by GD3 synthase to form GD3, the simplest of the "b-series" gangliosides. GM2/GD2S also elongates LacCer to form GA2, the simplest of the "o-series" ganglio-series GSLs. Each branch of GSL biosynthesis is a committed pathway (Fig. 1*A*), so competition between enzymes at a key branch point determines the relative expression levels of o-, a-, and b-series gangliosides.

To investigate functional roles of gangliosides in T-cell development and immunity, we used two types of gene-targeted mice with interrupted ganglioside biosynthesis (Fig. 1*A*). GM3S null mice presumably lack all a- and b-series gangliosides, resulting in LacCer accumulation and compensatory increase of o-series GSLs (8). GM2/GD2S null mice lack all o-series GSLs and all elongated a- and b-series gangliosides, so they accumu-

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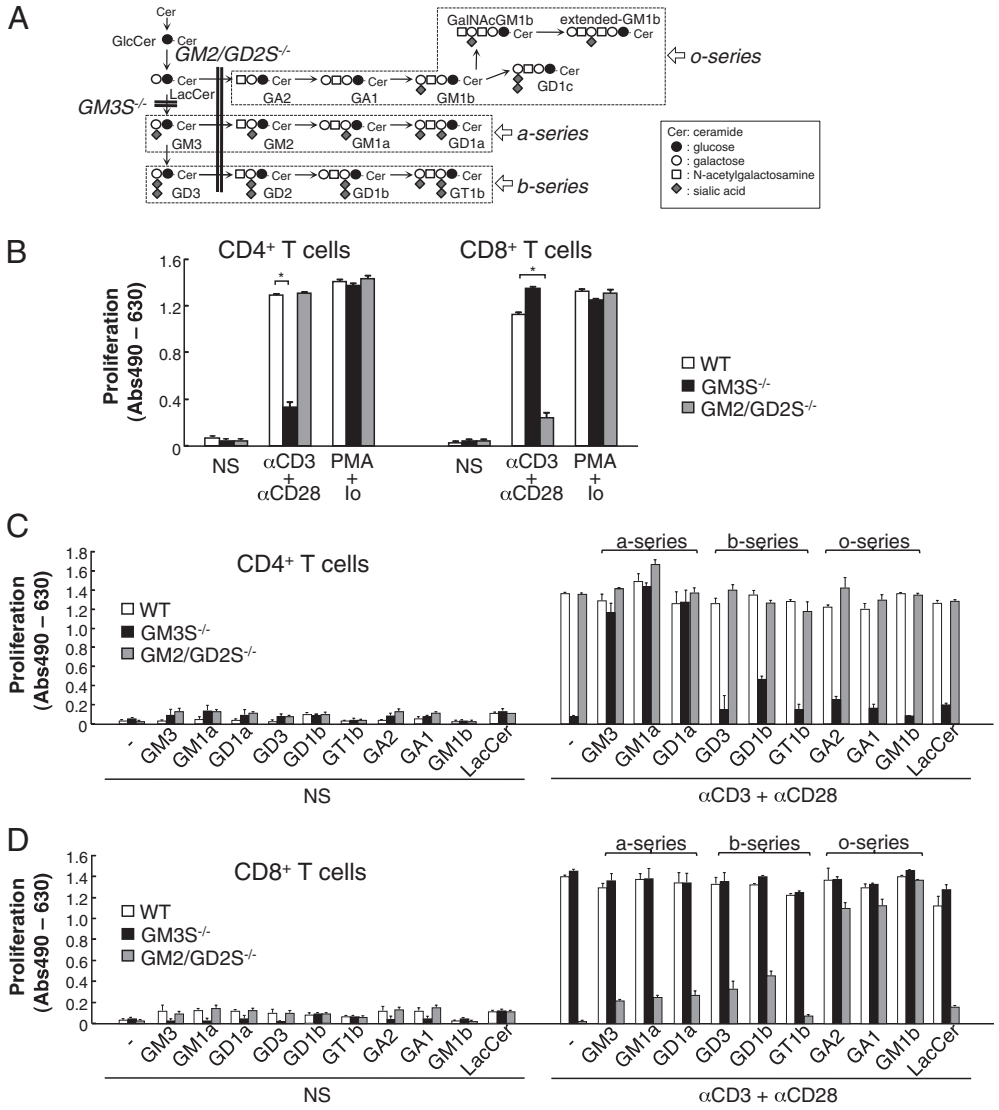

Fig. 1. Distinct selectivity of ganglioside species for CD4$^+$ T and CD8$^+$ T activation. (*A*) Ganglio-series glycosphingolipids are synthesized from ceramide and are divided into o-, a-, and b-series species. (*B*) Activation of CD4$^+$ T and CD8$^+$ T cells upon TCR-mediated stimulation. Purified peripheral CD4$^+$ T and CD8$^+$ T cells from WT, GM3S null, and GM2/GD2S null mice (20–25 mice each) were left unstimulated (NS) or were stimulated for 72 h with anti-CD3 antibody plus anti-CD28 antibody or with PMA plus ionomycin (Io). Proliferative responses were determined after an 8-h pulse with XTT reagent. One representative of three experiments is presented. *$P < 0.01$. (*C* and *D*) Functional rescue experiments by supplementation of gangliosides. Purified peripheral CD4$^+$ T (*C*) and CD8$^+$ T (*D*) cells from WT, GM3S null, and GM2/GD2S null mice were pretreated for 2 h with the indicated GSL (5 μg/mL). The cells were left unstimulated (NS) or were stimulated for 72 h with anti-CD3 antibody plus anti-CD28 antibody. Proliferative responses were determined after an 8-h pulse with XTT reagent. Data are representative of more than three experiments.

late LacCer and express only GM3 and GD3 gangliosides (9), as described later in detail.

Initial analysis of GM3S null and GM2/GD2S null mice did not reveal any obvious alteration in the cellularity of the lymphoid organs (Fig. S1*A*). To assess T-cell development, we performed flow cytometry for CD4 and CD8 on thymocytes and found similar distribution patterns in the thymocyte populations of the null and wild-type (WT) mice (Fig. S1*B*). In the spleen and lymph nodes, there was no obvious alteration in relative distribution within the T-cell and B-cell populations (Fig. S1*C*) and the CD4$^+$ T- and CD8$^+$ T-cell populations (Fig. S1*D*). These data indicate no gross defects in T-cell development despite the altered ganglioside expression. Then we examined the T-cell activation in purified peripheral CD4$^+$ T and CD8$^+$ T cells of the two null mice (Fig. 1*B*). Remarkably, CD4$^+$ T cells (but not CD8$^+$ T cells) from GM3S null mice exhibited severe defects in their proliferative response to the anti-CD3/anti-CD28 antibody mixture, but maintained a normal response to phorbol myristate acetate (PMA) plus ionomycin (Io). Conversely, CD8$^+$ T cells (but not CD4$^+$ T cells) from GM2/GD2S null mice exhibited similar severe defects in TCR-mediated activation when stimulated with the antibody mixture. Following TCR stimulation with the antibody mixture, both IL-2 production and IFN-γ production were severely reduced in both GM3S null CD4$^+$ T cells and GM2/GD2S null CD8$^+$ T cells (Fig. S2). Responses to an alloantigen, a superantigen, and a specific antigen peptide were similar to those observed with the anti-CD3/anti-CD28 mixture (Fig. 2). In contrast to T cells, splenic B cells isolated from GM3S null and GM2/GD2S null mice exhibited no obvious alterations in proliferative responses to various concentrations of anti-IgM antibody and lipopolysaccharide (Fig. S3). In experiments to determine which gangliosides have an essential role in

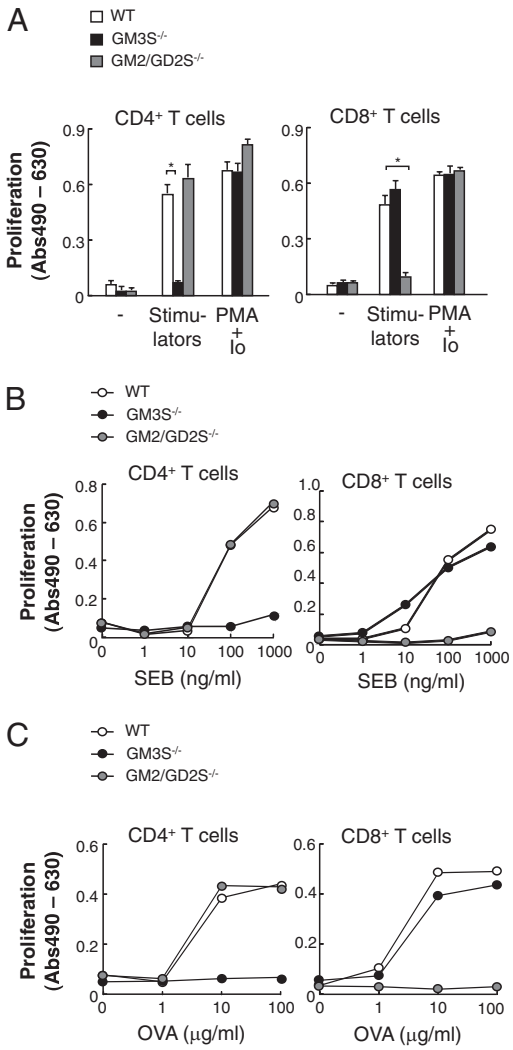

Fig. 2. Antigen-specific responses of $CD4^+$ T and $CD8^+$ T cells of GM3S null and GM2/GD2S null mice. (*A* and *B*) Peripheral $CD4^+$ T and $CD8^+$ T cells were purified from WT, GM3S null, and GM2/GD2S null mice. (*A*) Mixed lymphocyte reaction. The cells were left untreated (–), mixed with MMC-treated MHC-mismatched splenocytes from BALB/c as stimulators, or treated with PMA plus ionomycin (Io) for 72 h. (*B*) Superantigen-induced activation. The indicated cells were stimulated with indicated amounts of *Staphylococcal* enterotoxin B (SEB) and MMC-treated WT splenocytes as APCs. (*C*) Antigen-specific T-cell responses. Purified $CD4^+$T and $CD8^+$T cells from WT, GM3S null and GM2/GD2S null mice were obtained 7 d after immunization with TNP-OVA. The resulting cells were cocultured with MMC-treated WT splenocytes as APCs, together with the indicated amounts of OVA peptide. Proliferative responses were determined after an 8-h pulse with XTT reagent. Data are representative of three experiments. $*P < 0.01$ vs. WT.

TCR-mediated activation of T-cell subsets, $CD4^+$ T cells and $CD8^+$ T cells from WT, GM3S null, or GM2/GD2S null mice were pretreated with gangliosides followed by anti-CD3/anti-CD28 stimulation. Proliferative responses of GM3S null $CD4^+$ T cells were restored following pretreatment with any of the a-series species (GM3, GM1, and GD1a), but were not restored with any o- or b-series species (Fig. 1*C*). Conversely, the proliferative responses of GM2/GD2S null $CD8^+$ T cells were restored following pretreatment with any of the o-series species (GA2, GA1, and GM1b), but not with any of the a- or b-series species (Fig. 1*D*). Importantly, the gangliosides themselves had no effect in the absence of stimulation. These results indicate that o-series ganglioside expression is essential for TCR-mediated activation of $CD8^+$ T cells and a-series ganglioside expression is indispensable for that of $CD4^+$ T cells. Data from these lipid-restoring experiments demonstrate that a different series of gangliosides is necessary in each T-cell subset for proper T-cell activation following TCR stimulation. These data suggest that different species of gangliosides are essential in TCR-mediated activation of $CD4^+$ T cells vs. $CD8^+$ T cells.

We compared ganglioside profiles among thymocytes and peripheral $CD4^+$ T and $CD8^+$ T cells from WT, GM3S null, and GM2/GD2S null mice using TLC (Fig. 3). Acidic lipid fractions from thymocytes and $CD4^+$ T and $CD8^+$ T cells were separated by TLC, and lipids were stained with HRP-conjugated cholera toxin B subunit (CTx-B) (Fig. 3*A*). CTx-B recognizes a monosialo-ganglio-triose moiety, Galβ1-3GalNAcβ1-4 (SAα2-3)Galβ1-, which is found in GM1a, fucosylated GM1a, and GalGalNAcGM1b (extended-GM1b) (6). In WT $CD4^+$ T cells, GM1a was dominantly expressed, but extended-GM1b was expressed much more than GM1a in WT $CD8^+$ T cells. The thymocytes and peripheral $CD4^+$ T and $CD8^+$ T cells of GM3S null mice lacked a-series GM1a and expressed o-series extended GM1b, whereas the cells from GM2/GD2S null mice did not express either one (Fig. 3*A*). GM2/GD2S null cells also did not express complex gangliosides but did express GM3 and GD3 (Fig. 3*B*). None of the cells from GM3S null mice exhibited any accumulation of LacCer (Fig. 3*C*). In contrast, the cells from GM2/GD2S null mice exhibited an absence of GA1 and an accumulation of LacCer (Fig. 3*C*). Taken together, these results indicate that the ganglioside expression patterns of GM3S null mice, compared with those of WT cells, switch from an a-series–rich pattern to an o-series–rich pattern. GM2/GD2S null mice express LacCer, GM3, and GD3 in thymocytes and peripheral $CD4^+$ T cells, but only LacCer in $CD8^+$ T cells.

Differentiation from Thymocytes to Mature T-Cell Subsets Is Accompanied by Selective GSL Expression. We examined the expression of *GM3S* and *GM2/GD2S* genes in thymocytes and peripheral $CD4^+$ T and $CD8^+$ T cells from WT mice. *GM3S* expression was increased to 180% in $CD4^+$ T cells and was decreased to 30% in $CD8^+$ T cells compared with expression in thymocytes (Fig. 4*A*, *Left*). *GM2/GD2S* expression was markedly increased in both $CD4^+$ T and $CD8^+$ T cells compared with thymocytes (Fig. 4*A*, *Right*). These gene expression patterns suggest that $CD4^+$ T cells dominantly express a-series gangliosides due to up-regulation of *GM3S* and $CD8^+$ T cells would carry o-series GSLs due to down-regulation of *GM3S* and up-regulation of *GM2/GD2S* expression.

Liquid chromatography (LC)-MS analysis demonstrated that thymocytes and peripheral $CD4^+$ T and $CD8^+$ T cells predominantly express GM1, GD1, GalNAcGM1b, and extended GM1b. Whereas GM1 and GD1 levels were comparable in all three cell types, GalNAcGM1b and extended-GM1b levels were dramatically increased in the $CD8^+$ T-cell subset (Fig. 4*B*, and Fig. S4 *A* and *B*). MS^2 analysis distinguished positional isomers of GM1 (GM1a and GM1b) and GD1 (GD1b and GD1c) and their ratios (Fig. S4 *C–E*, and Fig. S5). GD1a was not detectable in any of the three cell types. Consistent with CTx-B TLC staining (Fig. 3*A*), LC-MS indicates that the expression pattern of ganglioside species is altered during T-cell development, and o-series and a-series gangliosides are dominantly expressed in $CD8^+$ T and $CD4^+$ T cells, respectively (Fig. 4*B*).

Airway Inflammatory Responses in GM3S Null Mice. Allergic airway inflammation is tightly regulated by adaptive immunity, in which $CD4^+$ T cells play a crucial role via Th2 cytokine production. We examined allergic airway responses induced by inhalation of ovalbumin (OVA) in OVA-sensitized mice (experimental asthma). OVA challenge induced extensive mucus hypersecretion, a cardinal feature of asthma, in WT but not in the GM3S null

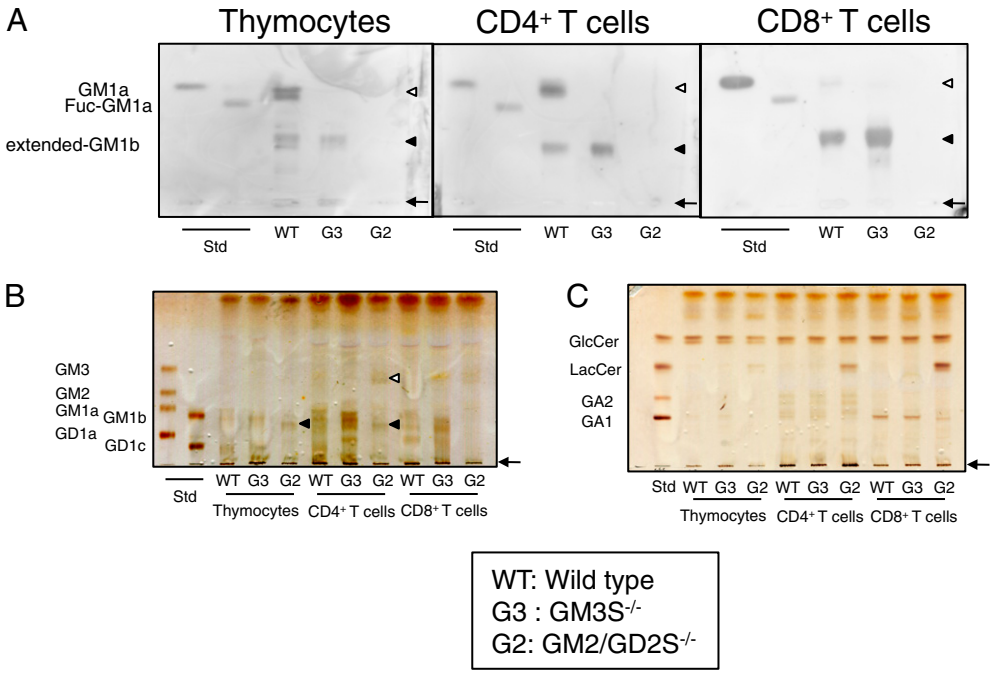

Fig. 3. Glycosphinogolipid expression patterns in thymocytes and peripheral primary CD4$^+$ T and CD8$^+$ T cells in WT, GM3S$^{-/-}$, and GM2/GD2S$^{-/-}$ mice. Neutral and acidic lipids were obtained from thymocytes, CD4$^+$ T cells, and CD8$^+$ T cells of WT, GM3S$^{-/-}$ (G3), and GM2/GD2S$^{-/-}$ (G2) mice. (*A*) The acidic lipids were separated on HPTLC plates and were stained with HRP-conjugated choleragenoid (CTx-B). An arrow indicates the origin for TLC. White and black arrowheads indicate GM1a and extended-GM1b bands, respectively. (*B* and *C*) Acidic (*B*) and neutral (*C*) lipids were separated on HPTLC plates and visualized with orcinol-sulfuric acid. White and black arrowheads in (*B*) indicate GM3 and GD3 bands, respectively. Fuc-GM1a, fucosylated GM1a; std, standard lipids.

mice (Fig. 5*A*). Although both types of mice challenged with OVA exhibited airway infiltration of inflammatory cells including eosinophils and lymphocytes, the numbers of infiltrating cells were significantly lower in GM3 null mice (Fig. 5*B*). Serum OVA-specific IgE levels were greatly increased on days 3 and 5 after OVA challenge in WT mice, but not in GM3S null mice (Fig. 5*C*). Decreased levels of Th2 cytokines in the bronchoalveolar lavage (BAL) fluids were also observed in GM3S null mice (Fig. 5*D*). In adoptive transfer experiments, CD4$^+$ T cells isolated from OVA-sensitized WT and GM3S null mice were transferred into naive WT mice, and the recipient mice were challenged with OVA. Infiltration of inflammatory cells, particularly eosinophils and lymphocytes, was suppressed in the recipient mice of CD4$^+$ T cells from GM3S null mice (Fig. 5*E*). In the recipient mice of CD4$^+$ T cells from GM2/GD2 synthase null mice, there was no difference in the numbers of infiltrated cells compared with those of recipient mice of CD4$^+$ T cells from WT mice (Fig. 5*F*). These results indicate that the immune function of CD4$^+$ T cells in vivo is selectively deficient in GM3S null mice.

Discussion

In the present study, we determined in great detail the ganglioside expression patterns of primary T cells, specifically those of immature thymocytes and mature peripheral CD4$^+$ T cells and CD8$^+$ T cells. Our most important finding was that distinct expression of gangliosides exists in CD4$^+$ T cells and CD8$^+$ T cells, and defines the immune function of each T-cell subset. CD4$^+$ T cells preferentially express a-series gangliosides, and cells isolated from GM3S null mice exhibit severe impairments in TCR-mediated cytokine production and clonal expansion, but can be rescued by reintroducing a-series gangliosides. Similarly, CD8$^+$ T cells preferentially express o-series GSLs, and cells isolated from GM2/GD2S null mice exhibit severe impairments in TCR-mediated cytokine production and clonal expansion. They can be rescued by reintroducing o-series gangliosides. These results suggest that CD4$^+$ T-cell and CD8$^+$ T-cell subsets require a-series and o-series ganglioside expression, respectively, to undergo activation upon TCR ligation.

Why are CD4$^+$ T cells and CD8$^+$ T cells dependent on expression of distinct gangliosides for proper immune function?

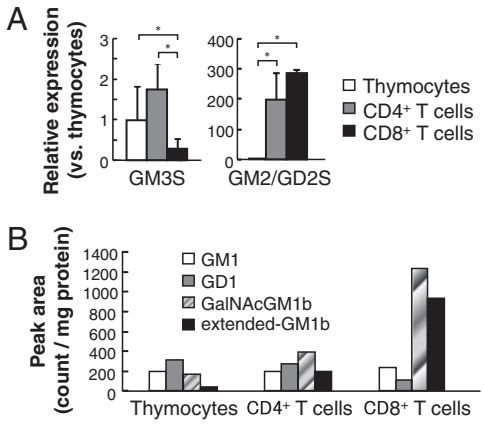

Fig. 4. Distinct expression of ganglioside species during T-cell repertoire selection. (*A*) Gene expression of GM3S and GM2/GD2S was determined by quantitative real-time PCR analysis. Data represent the relative gene expression in peripheral T-cell subsets compared with that in thymocytes (set to 1.0 separately for each target gene). *$P < 0.05$. (*B*) Gangliosides of mouse thymocytes and CD4$^+$ and CD8$^+$ T cells analyzed by LC-MS. Peak areas of GM1, GD1, GalNAcGM1b, and extended-GM1b were determined in the mass chromatograms obtained by C30 column chromatography-MS with GM3 (NeuAc, d18:1–14:0) as an internal standard. GM1 includes GM1a [*N*-glycolylneuraminic acid (NeuGc)] and GM1b (NeuGc), each carrying d18:1–16:0, –18:0, –20:0, –22:0, –24:1, and –24:0. GD1 includes GD1b (NeuGc) and GD1c (NeuGc) each carrying d18:1–16:0, –18:0, –20:0, –22:0, –24:1, and –24:0. GD1a was not detectable in any of the three cell types.

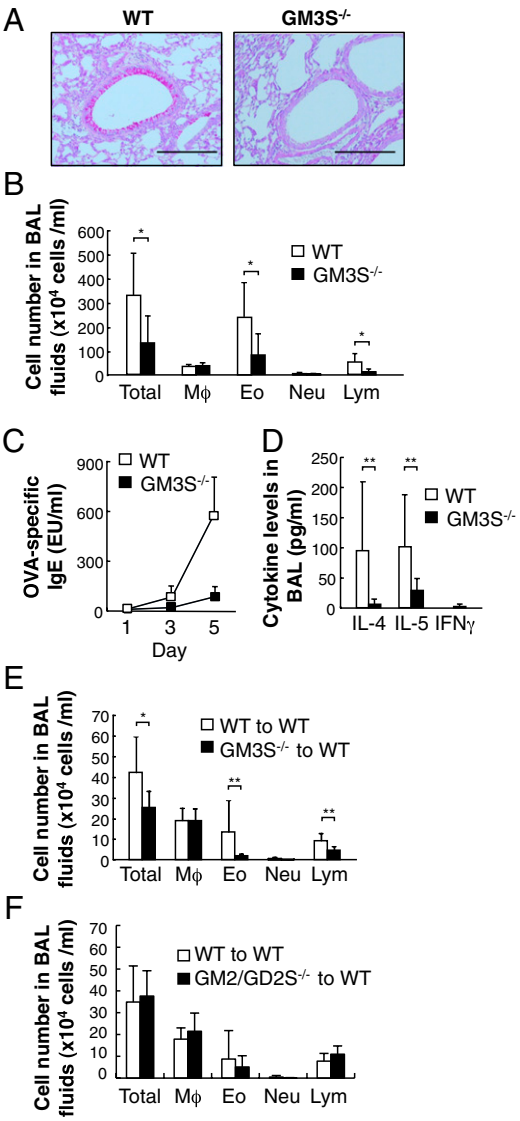

Fig. 5. Allergic airway inflammation in GM3S null mice. (*A* and *B*) Sensitized WT and GM3S null mice were challenged with OVA. Five days after the challenge, BAL fluid and lung tissues were collected. The representative lung sections stained with PAS (*A*) and the numbers of total cells (Total), macrophages (Mϕ), eosinophils (Eo), neutrophils (Neu), and lymphocytes (Lym) in BAL fluids (*B*) are shown. (WT, $n = 7$; GM3S null, $n = 9$). (Scale bar in *A*, 200 μm.) (*C*) Sera from sensitized WT and GM3S null mice were obtained at 1 (WT, $n = 6$; GM3S null, $n = 6$), 3 (WT, $n = 6$; GM3S null, $n = 6$) and 5 d (WT, $n = 6$; GM3S null, $n = 6$) after OVA challenge. OVA-specific IgE in the sera was measured. (*D*) Cytokine levels in BAL fluid (WT, $n = 6$; GM3S null, $n = 6$) were measured 1 d after challenge. (*E* and *F*) Cell numbers in BAL fluid of mice adoptively transferred with $CD4^+$ T cells. $CD4^+$ T cells from sensitized WT and GM3S null mice (*E*) or GM2/GD2S null mice (*F*) were transferred to naive WT mice. BAL fluid was obtained from the recipient mice 5 d after OVA challenge [(*E*) WT recipient, $n = 6$; GM3S null recipient, $n = 7$; (*F*) WT recipient, $n = 10$; GM2/GD2S null recipient, $n = 6$]. The numbers of total cells (Total), macrophages (Mϕ), eosinophils (Eo), neutrophils (Neu), and lymphocytes (Lym) in BAL fluid were counted. $*P < 0.05$, $**P < 0.01$.

Although there are some commonalities between the mechanisms of TCR-mediated signaling in $CD4^+$ T cells and $CD8^+$ T cells, the two subsets do have different cellular and molecular modifications. CD4 and CD8 can localize to lipid rafts by palmitoylation in their juxtamembrane region (10, 11), yet raft localization seems not to be determined by lipid modification alone (11, 12). To ensure that CD4 and CD8 undergo proper intracellular trafficking and successful localization on the plasma membrane, it might be vital for CD4/CD8 to interact with rafts carrying a specific ganglioside. CD28, a B7-type costimulatory molecule, provides functional differences between $CD4^+$ T cells and $CD8^+$ T cells (13, 14). In human and mouse $CD4^+$ T cells, CD28 promotes the clustering of CTx-B–detectable rafts at the immunological synapse through its downstream signaling molecule protein kinase Cθ (15). However, $CD8^+$ T cells do not reorient CTx-B–detectable rafts to the T-cell/antigen-presenting cell (APC) interface during activation (4, 16). Considering the combined information, we speculate that ganglioside compositions in rafts necessary for signaling events mediated by TCR and costimulatory molecules differ between $CD4^+$ T cells and $CD8^+$ T cells.

Ganglioside expression in primary T cells has previously been studied using biochemical analyses (TLC and HPLC) of whole T-cell populations. However, whole T-cell populations are truly a "mixed population," so any such results would be of limited value to study specific T-cell subsets. In contrast, we separated $CD4^+$ T cells and $CD8^+$ T cells from cell mixtures isolated from lymph nodes and spleen and then performed TLC and LC-MS analyses. Previously, FACS analyses with monoclonal antibodies (mAbs) against several ganglioside species determined that mature peripheral $CD4^+$ T cells and $CD8^+$ T cells express differential species of gangliosides (17, 18). Our LC-MS analysis (Fig. 4*B* and Figs. S4 and S5) demonstrated that expression of GalNAcGM1b and extended-GM1b is much higher in $CD8^+$ T cells than in $CD4^+$ T cells. $CD4^+$ T cells express a larger amount of GD1c than $CD8^+$ T cells do.

Staining with CTx-B, commonly used to detect GM1a and visualize rafts, has demonstrated that $CD8^+$ T cells express higher levels of GM1a in the rafts than $CD4^+$ T cells (19). However, this toxin also reacts to other gangliosides including fucosylated-GM1a and extended-GM1b, both of which have a monosialo-ganglio-triose structure, Galβ1-3GalNAcβ1-4(SAα2-3)Galβ1- (6). In fact, we were able to detect two CTx-B–reactive gangliosides, GM1a and extended GM1b in T cells with different quantities in individual T-cell subsets (Fig. 3*A*). The presence in a single cell of a variety of rafts with different gangliosides has been suggested (4, 5). Cross-linking gangliosides using CTx-B or a homolog, the heat-labile enterotoxin of *Escherichia coli*, can induce apoptosis in $CD8^+$ T cells but not in $CD4^+$ T cells (20), which results from the induction of caspase-dependent signaling caused by activation of NF-κB and c-Myc (20). Although this process is known to proceed in a Fas- and p55 tumor necrosis factor receptor-independent pathway (21), the events in rafts following the ganglioside cross-linking remain undetermined. Considering the differences in the expression of CTx-B–binding ganglioside species between $CD4^+$ T cells and $CD8^+$ T cells (Fig. 3*A*), it seems possible that apoptosis by CTx-B cross-linking may involve extended-GM1b rafts and not GM1a rafts. This result strongly suggests that each T-cell subset has unique rafts in the plasma membrane and that these rafts provide distinct functions in different intracellular events following receptor-mediated stimulation.

FACS analyses have revealed that $CD4^+$ T cells and $CD8^+$ T cells each include subpopulations expressing a particular GSL not observed in the rest of the population. GA1-positive $CD8^+$ T cells exhibit more robust activation via the TCR and play a critical role in CD40/CD28 costimulated and blockade-resistant allograft rejection, compared with GA1-negative $CD8^+$ T cells (22, 23). These findings imply that each subpopulation of $CD4^+$ T cells and $CD8^+$ T cells has a unique ganglioside expression pattern in its rafts, which may be responsible for exerting specific functions of Th and Tc effector cells. GA1-positive mouse $CD8^+$ T cells produce higher levels of IFN-γ in vitro upon TCR stimulation than GA1-negative $CD8^+$ T cells, and both clonal expansion of $CD8^+$ T cells and cytotoxic T-cell–

dependent allograft rejection are suppressed by administration of anti-GA1 mAb in vivo (22). It remains unclear whether, in addition to GA1, the other o-series species expressed in primary $CD8^+$ T cells are essential for TCR-mediated activation. In mouse $CD4^+$ T cells, few studies have addressed whether expression of a-series gangliosides is indispensable for TCR-mediated activation. Human $CD4^+$ T cells isolated from peripheral blood mainly express GM3 (24), which would form GM3-containing rafts available as a platform for TCR signal transduction. The importance of GM3-containing rafts may be corroborated by the observation that GM3 forms a complex with CD4 and Lck on plasma membranes and is coimmunoprecipitated with ZAP-70 after cross-linking with anti-CD3 plus anti-CD28 mAb (24, 25). Consequently, the future challenge will be to examine immune responses in primary T-cell subpopulations separated on the basis of ganglioside species.

Mouse $CD4^+$ T cells and $CD8^+$ T cells activated by anti-CD3 antibody exhibit different profiles for *N*-linked glycans (26). In addition, differentially *N*-glycosylated TCR complexes have been observed in $CD4^+$ T cells compared with $CD8^+$ T cells, which might affect the functional avidity of TCR-transduced T cells (27, 28). The CD4 and CD8 coreceptors, which enhance the TCR–MHC interaction upon binding, have also been shown to be glycosylated (29). These findings, combined with multiple lines of evidence that gangliosides interact with *N*-linked glycans of glycoproteins (30), suggest that a specific ganglioside species expressed on the plasma membrane of $CD4^+$ T and $CD8^+$ T cells might be responsible for the interaction between the TCR and CD4/CD8, to ensure the induction of proper TCR signaling.

Roles of GSLs in TCR signaling and T-cell activation have also been studied using inhibitors of GSL biosynthesis (31). We previously demonstrated that treatment of Jurkat cells with D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), an inhibitor of GlcCerS (32), preferentially reduces the levels of GSLs in rafts without affecting SM or cholesterol levels. The alteration of raft lipid content results in the up-regulation of TCR signaling via GPI-anchored protein (31). In contrast, treatment of human peripheral blood lymphocytes with this inhibitor attenuated TCR signaling and T-cell activation, indicated by reduction in the expression of markers for T-cell activation and IFN-γ secretion (33).

Activated human T cells are increased in GM1a levels (34, 35). Studies in cells from patients with systemic lupus erythematosus, an autoimmune rheumatic disease characterized by abnormalities in T-cell activation, have determined that the expression of a raft-associated ganglioside, GM1a, is increased in $CD4^+$ T cells, but not in $CD8^+$ T cells, compared with controls (36, 37). It is thought that GM1a expression is enhanced in self-reactive $CD4^+$ T cells, which causes persistence of abnormal cell activation (37, 38). We demonstrated that allergic airway responses caused by OVA inhalation were improved in GM3S null mice, which lack GM1a, resulting in dysfunction of $CD4^+$ T cells. This finding, as well as a recent report in which airway inflammation is suppressed by administration of antisense oligonucleotides against GM3S (39), suggests that inhibition of a-series ganglioside synthesis would be a powerful choice for a treatment of allergic airway diseases.

Recently, novel $CD4^+$ T-cell subsets, in addition to Th1 and Th2 cells, Th17 and regulatory T (Treg), have been described. In allergic airway inflammation, the balance between effector Th2 cells and suppressive Treg cells is skewed toward Th2 predominance (40). Th17 cells have been suggested to contribute to neutrophilic, steroid-resistant severe asthma and to enhance Th2-mediated airway inflammation, although a role for the cells in asthma remains to be determined (41). Reportedly, GM3S null mice exhibit a decreased number of Th17 cells skewed by in vitro culture (42). Because the adoptive transfer experiments confirmed the marked reduction of allergic responses in WT mice that received a whole $CD4^+$ T-cell population from sensitized GM3S null mice (Fig. 5*E*), we are underway to identify which $CD4^+$ T-cell subset is affected in GM3S null mice in vivo.

The present study demonstrates that the functional repertoire selection from double-positive ($CD4^+CD8^+$) to single-positive cells is accompanied by selective GSL expression in individual T-cell subsets and this GSL selection process would be critical for the formation of a functional microcluster and/or lipid rafts in mature T cells. These findings may open up a strategy for targeting specific T-cell subpopulations to treat immune diseases by controlling ganglioside expression in lipid rafts.

Materials and Methods

Experimental Animals. All animals were maintained in accordance with the guidelines of the Tohoku Pharmaceutical University for the care and use of laboratory animals. C57BL/6 mice were obtained from Japan SLC. The GM3S null and GM2/GD2S null mice were generated as previously described (8, 43). Both types of null mice were backcrossed with C57BL/6 mice over >11 generations. Mice were analyzed for the *GM3S* and *GM2/GD2S* genotypes by PCR as described previously (8, 43).

T-Cell Purification. Thymus, spleen, and lymph nodes were collected from WT, GM3S null, and GM2/GD2S null mice. Detailed methods can be found in *SI Materials and Methods*.

T-Cell Activation. For antibody-mediated activation, T-cell subsets purified by negative selection were incubated with 0.1 μg/mL of anti-CD3ε antibody (145-2C11) in the presence of 0.2 μg/mL of anti-CD28 (PV-1) antibody for 72 h. For GSL supplementation experiments the cells were pretreated with the indicated GSLs for 2 h before TCR-mediated stimulation. For the antigen-specific proliferative response assays, $CD4^+$ T cells and $CD8^+$ T cells were prepared from the spleen 7 d after immunization with OVA conjugated with trinitrophenyl (TNP) (Biosearch Technologies). The cells were cocultured with mitomycin C (MMC)-treated WT splenocytes for 72 h in the presence or absence of TNP-OVA antigen. For mixed lymphocyte reactions (MLR), $CD4^+$ T and $CD8^+$ T cells were cocultured for 72 h with MMC-treated splenocytes from BALB/c mice. Cell proliferation was measured by 2,3-bis-(2-methoxy-4- nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay with a cell proliferation kit (MD Biosciences), according to the manufacturer's instructions.

Allergen-Induced Airway Inflammation. Airway inflammation was generated as previously described (44). Detailed methods can be found in *SI Materials and Methods*.

Preparation of $CD4^+$ T Cells for Adoptive Transfer. On day 17, spleens collected from sensitized WT, GM3S null, and GM2/GD2S null mice were excised, and the splenocytes were disaggregated. The washed splenocytes were resuspended and cultured in the presence of 200 μg/mL OVA. Three days after the culture, $CD4^+$ T cells were purified by positive selection on an autoMACS separator (Miltenyi Biotec), using anti-mouse CD4 (L3T4) MicroBeads (Miltenyi Biotec) according to the manufacturer's protocol. The $CD4^+$ T cells (2×10^6 cells/ 200 μL in saline) were transferred to naive WT mice by tail vein injection. Three days after the injection, the recipient mice were aerosol challenged; BAL fluids were collected 5 d after the challenge as described above.

ELISA. Concentrations of IL-2 and IFN-γ in culture supernatants were measured by ELISA as described elsewhere (45), using two types of anti-mouse IL-2 mAbs and anti-mouse IFN-γ mAb. Serum levels of OVA-specific IgE were measured with ELISA according to published methods (42). IL-4 and IL-5 contents in BAL fluid were measured using an ELISA kit (R&D Systems) according to the manufacturer's protocol.

Lipid Analysis. Lipid analysis was performed as described previously (31). Detailed methods can be found in *SI Materials and Methods*.

Liquid Chromatography–Mass Spectrometry (LC-MS). LC-MS analyses of ganglioside species were performed as described previously (46) with modifications as follows. Detailed methods can be found in *SI Materials and Methods*.

Quantitative Real-Time PCR. Total RNA was isolated using TRI Reagent (Molecular Research Center). Detailed information can be found in *SI Materials and Methods*.

Statistics. Values in the text are the means ± SD. Data were compared using Student's *t* test for two-group comparison or ANOVA for multigroup comparison. Significant differences were post hoc analyzed using Scheffé's test. Differences were considered significant at $P < 0.05$.

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