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Cross-Linking of GM1 Ganglioside by Galectin-1 Mediates Regulatory T Cell Activity Involving TRPC5 Channel Activation: Possible Role in Suppressing Experimental Autoimmune Encephalomyelitis

Jianfeng Wang,* Zi-Hua Lu,* Hans-Joachim Gabius,† Christine Rohowsky-Kochan,* Robert W. Ledeen,2* and Gusheng Wu2*

Several animal autoimmune disorders are suppressed by treatment with the GM1 cross-linking units of certain toxins such as B subunit of cholera toxin (CtxB). Due to the recent observation of GM1 being a binding partner for the endogenous lectin galectin-1 (Gal-1), which is known to ameliorate symptoms in certain animal models of autoimmune disorders, we tested the hypothesis that an operative Gal-1/GM1 interplay induces immunosuppression in a manner evidenced by both in vivo and in vitro systems. Our study of murine experimental autoimmune encephalomyelitis (EAE) indicated suppressive effects by bothCtxB and Gal-1 and further highlighted the role of GM1 in demonstrating enhanced susceptibility to EAE in mice lacking this ganglioside. At the in vitro level, polyclonal activation of murine regulatory T (Treg) cells caused up-regulation of Gal-1 that was both cell bound and released to the medium. Similar activation of murine CD4+ and CD8+ effector T (Teff) cells resulted in significant elevation of GM1 and GD1a, the neuraminidase-reactive precursor to GM1. Activation of Teff cells also up-regulated TRPC5 channels which mediated Ca2+ influx upon GM1 cross-linking by Gal-1 or CtxB. This involved co-cross-linking of heterodimeric integrin due to close association of these αβ1, and αβ1, glycoproteins with GM1. Short hairpin RNA (shRNA) knockdown of TRPC5 in Teff cells blocked contact-dependent proliferation inhibition by Treg cells as well as Gal-1/CtxB-triggered Ca2+ influx. Our results thus indicate GM1 in T eff cells to be the primary target of Gal-1 expressed by Treg cells, the resulting co-cross-linking and TRPC5 channel activation contributing importantly to the mechanism of autoimmune suppression.


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Teff cells and may account for some or all of the latter’s elevation. That GM1 cross-linking has a role in Treg cell suppression is strongly suggested in the finding that Teff splenocytes lacking GM1 failed to respond to Treg cell contact with reduced proliferation; such cells were obtained from a knockout (KO) mouse lacking gangliotetraose gangliosides (Ggs), which include GM1. Significantly, those mice showed enhanced susceptibility to MOG-induced EAE. The results of GM1 cross-linking by Gal-1 appear central to the suppressive mechanism and are shown in the present study to involve activation of TRPC5, a cation channel belonging to the transient receptor potential (canonical) family (19). The resulting Ca$^{2+}$ influx is hypothesized to mediate suppression of responder cells. Short hairpin RNA (shRNA) knockdown of this channel activity blocked Gal-1/B subunit of cholera toxin (CtxB)-induced inhibition of Teff cell proliferation as well as Ca$^{2+}$ influx. GM1 cross-linking is shown to involve integrins which, because of their membrane association with GM1, likely undergo simultaneous cross-linking with subsequent signaling and Ca$^{2+}$ influx. Interestingly, CD8$^+$ T cells were found to exhibit parallel properties as CD4$^+$ T cells, in keeping with their now recognized roles as mediators of autoimmune reactivity (20, 21).

Materials and Methods

**Mice**

Female wild-type (WT) C57BL/6 mice, 6–8 wk of age, were purchased from Taconic Farms. Breeding pairs of heterozygotes with disrupted gene for GM2/GD2 synthase (UDP-N-acetyl-galactosamine:GM3/GD3 N-acetyl-galactosaminytransferase, GalNAcT; EC 2.4.1.92; C57BL/6 background), originally produced by Dr. R. Proia and coworkers (22), was provided by Dr. R. Schuurman (Johns Hopkins University School of Medicine, Baltimore, MD). Heterozygous male and KO female offspring were used to produce more breeders. Animals were maintained in the University of Medicine and Dentistry of New Jersey animal facility with 12-h light/dark cycles. They were genotyped by PCR analysis and females were used for EAE studies; heterozygotes and WT showed no differences in this or other studies and were used interchangeably (referred to collectively as WT).

**Gal-1 and related reagents**

Human Gal-1 was isolated after recombinant production, its purity was ascertained by one- and two-dimensional gel electrophoresis and mass spectrometry, and the protein was biotinylated under activity-preserving conditions; activity was checked by solid-phase and cell-binding assays (23, 24). To preclude gradual impairment of lectin binding by oxidation, reactive thiol groups were protected by iodoacetamide treatment during elution in affinity chromatography (25). The Ab was raised in rabbits and activity; such cells were obtained from a knockout (KO) mouse lacking GM1 cross-linking agents were administered every other day, including Gal-1 (40 μg, beginning day 13) and CtxB (20 μg, beginning day 8). Controls were treated with an equal volume of saline. Animals were scored daily for disability. Gal-1 application was terminated at day 23 and scoring continued. Data are means of combined data ± SEM. Mann-Whitney U test was used to assess statistically significant differences between treated mice and controls: a and b, p < 0.0001; c, p < 0.01.

**Induction and assessment of EAE**

EAE was induced in groups of 10–11 females by two s.c. immunizations with 100 μl of an emulsion containing 200 μg of MOG35–55 (MEVGW/H9262/L-glutamine, 0.2 mM pyruvate, 0.5 mM nonessential amino acids, 5 mM HEPES, and 50 μM M 2-ME. Treg cells were activated with the above T cell expander along with recombinant human IL-2 (10 ng/ml) as described previously (28, 29). Freshly isolated resting and activated Teff cells were transferred in 100-μl portions to 96-well flat-bottom plates (5 × 10$^5$ cells/well) and the effect of CtxB (10 μg/ml) or Gal-1 (20–100 μg/ml) on proliferation was assessed by [3H]TdR (0.3 μCi) incorporation over an 18-h period. Cells were harvested by filtration and the amount of incorporated radioactivity was measured by scintillation counting. Six wells were used for each experimental condition. Similar assays were conducted with mixed cultures of WT Teff/WT Treg, WT Teff/KO Treg, and KO Treg/WT Treg cells to confirm the nature of Treg cell inhibition. In one experiment, anti-Gal-1 Ab (10 μg/ml) was added to the WT Teff/WT Teff mixed culture

![FIGURE 1.](http://www.jimmunol.org/) EAE suppression by Gal-1 and CtxB and enhancement in ganglioside-deficient mice. WT C57BL/6 female mice, 6–8 wk of age, were used in some trials along with GM1-deficient KO (GalNAcT$^{-/-}$) mice of similar age and background (c). Each animal was immunized twice with 200 μg of MOG35–55 peptide plus pertussis toxin as described. GM1 cross-linking agents were administered every other day, including Gal-1 (40 μg, beginning day 13) and CtxB (20 μg, beginning day 8). Controls were treated with an equal volume of saline. Animals were scored daily for disability. Gal-1 application was terminated at day 23 and scoring continued. Data are means of combined data ± SEM. Mann-Whitney U test was used to assess statistically significant differences between treated mice and controls: a and b, p < 0.0001; c, p < 0.01.

**CD4$^+$ CD25$^+$ Foxp3$^+$ Treg cells and CD4$^+$ and CD8$^+$ Teff cells were isolated from spleens of both WT and KO mice with immunomagnetic bead kits (Miltenyi Biotec) according to the manufacturer’s instructions. For Treg cells this consisted of first isolating total CD4$^+$ T cells by negative selection, followed by positive selection with anti-CD25-coated beads. Purity of Treg cells was verified with the Mouse Regulatory T Cell Staining Kit (eBioscience) and flow cytometry. Teff cells were isolated with anti-CD4- and anti-CD8-coated beads. CD4$^+$ and CD8$^+$ T cells (5 × 10$^5$ cells/ml) were activated by culturing for 3 days at 37°C in 5% CO$_2$ in the presence of anti-CD3/anti-CD28-containing Dynabeads Mouse T Cell Expander (Invitrogen) in RPMI 1640 medium supplemented with heat-inactivated FBS (10%), penicillin (100 U/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, 0.2 mM pyruvate, 0.5 mM nonessential amino acids, 5 mM HEPES, and 50 μM M 2-ME. Teff cells were activated with the above T cell expander along with recombinant human IL-2 (10 ng/ml) as described previously (28, 29). Freshly isolated resting and activated Teff cells were transferred in 100-μl portions to 96-well flat-bottom plates (5 × 10$^5$ cells/well) and the effect of CtxB (10 μg/ml) or Gal-1 (20–100 μg/ml) on proliferation was assessed by [3H]TdR (0.3 μCi) incorporation over an 18-h period. Cells were harvested by filtration and the amount of incorporated radioactivity was measured by scintillation counting. Six wells were used for each experimental condition. Similar assays were conducted with mixed cultures of WT Teff/WT Treg, WT Teff/KO Treg, and KO Treg/WT Treg cells to confirm the nature of Treg cell inhibition. In one experiment, anti-Gal-1 Ab (10 μg/ml) was added to the WT Teff/WT Teff mixed culture.
We used anti-H11005 Incidence of membrane lysates (100 Sigma-Aldrich) treatment and CtxB-HRP overlay as previously described combination with neuraminidase (Clostridium perfringens). Sides were extracted from resting and activated T eff cells with chloroform:alyzed in 0.5% saponin followed by reaction with CtxB-FITC as above.

Aldrich) followed by HPTLC analysis of the coprecipitated ganglioside. 

TRPC5 channel determination and intracellular Ca\(^ {2+}\) concentration ([Ca\(^ {2+}\)]\(_i\)) measurement

Gene expression of TRPC 1, 4, and 5, and GAPDH in resting and activated T eff cells was analyzed with RT-PCR as described elsewhere (32). Mouse brain mRNA was used as positive control. The reactions employed 40 cycles, and products were visualized on a 1% agarose gel with ethidium bromide. To verify, the DNA bands were extracted from the gel with the QIAquick gel extract kit (Qiagen) and sequenced in the Molecular Resource Facility of this institution; resulting sequences were identified by the BLAST protocol on the National Institutes of Health-National Center for Biotechnology Information web site. Protein expression of TRPC5 in these cells was determined by immunocytochemical staining using rabbit anti-TRPC5 Ab in fixed and permeabilized cells. TRPC5 expression was suppressed (knocked down) by infecting T eff cells with retrovirus with incorporated shRNA for TRPC5. Such retrovirus was prepared by transfecting HuSH-29 shRNA plasmids (from OriGene) into Phoenix eco-packaging retrovirus source Facility of this institution; resulting sequences were identified by the BLAST protocol on the National Institutes of Health-National Center for Biotechnology Information web site. Protein expression of TRPC5 in these cells was determined by immunocytochemical staining using rabbit anti-TRPC5 Ab in fixed and permeabilized cells. TRPC5 expression was suppressed (knocked down) by infecting T eff cells with retrovirus with incorporated shRNA for TRPC5. Such retrovirus was prepared by transfecting HuSH-29 shRNA plasmids (from OriGene) into Phoenix eco-packaging retrovirus source Facility of this institution; resulting sequences were identified by the BLAST protocol on the National Institutes of Health-National Center for Biotechnology Information web site. Protein expression of TRPC5 in these cells was determined by immunocytochemical staining using rabbit anti-

\[\text{TRPC5 channel determination and intracellular Ca}^{2+}\text{concentration ([Ca}^{2+}\text{]}_i\text{) measurement}\]

![FIGURE 2. Histopathology of WT and GM1-deficient KO spinal cord sections stained with Luxol Fast blue. Arrows indicate areas of demyelination. Sections from EAE animals (cf. Fig. 1) treated with Gal-1 (b) and CtxB (c) showed less demyelination than those from WT mice (a). CtxB being especially effective. GalNAC\(^{-}\) KO mice showed significantly more demyelination than WT mice (d). Boxed areas of initial sections (bar scale, 500 μm) are expanded (bar scale, 150 μm) to show more detailed histopathology, especially in the KO sample. In addition to revealing demyelination, Luxol Fast blue also indicated massive inflammation in KO and somewhat less in samples from Gal-1/CtxB-treated animals compared with untreated animals. The examples shown are typical of many similar sections.](http://www.jimmunol.org/)

### Table I. Clinical parameters of MOG-induced EAE

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidence</th>
<th>Day of Onset (mean ± SD)</th>
<th>Maximum Score (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT control</td>
<td>30/32</td>
<td>15.0 ± 0.53</td>
<td>2.02 ± 0.27</td>
</tr>
<tr>
<td>GM1-null</td>
<td>21/21</td>
<td>12.6 ± 0.45^*</td>
<td>2.88 ± 0.31*</td>
</tr>
<tr>
<td>WT + CtxB</td>
<td>12/21</td>
<td>16.5 ± 0.75</td>
<td>0.21 ± 0.06*</td>
</tr>
<tr>
<td>WT + Gal-1</td>
<td>22/30</td>
<td>15.8 ± 0.47</td>
<td>0.89 ± 0.22**</td>
</tr>
<tr>
<td>Days 24–31 after first immunization</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>10/10</td>
<td>NA</td>
<td>2.10 ± 0.39</td>
</tr>
<tr>
<td>WT + Gal-1</td>
<td>26/30</td>
<td>NA</td>
<td>1.73 ± 0.31***</td>
</tr>
</tbody>
</table>

\(^{a}\) EAE was induced by two immunizations of WT and Gg-null KO mice with MOG\(_{35–55}\). They were treated with CtxB, Gal-1, or saline (WT control) as described. Incidence = mice with EAE/total; Day of onset, period from first of two immunizations. Maximum score included all mice.

\(^{*}\), \(p < 0.001; \#\), \(p < 0.05\) vs WT control. \(^{**}\), \(p < 0.001\), WT + Gal-1 up to day 23 vs WT control for same period; \(^{***}\), \(p < 0.05\), WT + Gal-1 days 24–31 vs days 1–23 for the same group. NA, not applicable.
cells using Lipotransfectamine 2000 (Invitrogen). The transfected cells were transferred to the above culture medium for Teff cells when 80–90% confluent; conditioning medium containing secreted retrovirus was collected 24 h later. For infection, this virus-containing medium, supplemented with 4 μg/ml polybrene (Sigma-Aldrich), was applied in equal volume to Teff cells on the second day of culture. Control infection was conducted with virus containing nonsense shRNA. Cells were harvested after 48 h and suppression of TRPC5 expression was tested by RT-PCR and immunocytostaining as above.

Intracellular free Ca^{2+} measurements ([Ca^{2+}]_i) were performed as described previously (32, 33). Activated CD4^+ or CD8^+ Teff cells, with or without TRPC5 shRNA treatment, were reacted or not with neuraminidase (0.5 U/ml) at 37°C for 2 h, then exposed to 5 μM fura 2-AM and 0.25 mM sulfinpyrazone for 30 min. Aliquots of 1–1.5 × 10^5 cells were suspended in 4-morpholinepropanesulfonic acid (20 mM, pH 7.2)-buffered physiological saline solution consisting of 140 mM NaCl, 5 mM KCl, 1 mM MgCl_2, 10 mM glucose, 0.25 mM sulfinpyrazone, 1% BSA, and 0 or 5 mM Ca^{2+}. Measurements were run 600–800 s and CtxB (5 μg/ml) or Gal-1 (20 μg/ml) was applied at the times indicated. [Ca^{2+}]_i levels are expressed as the fluorescent ratio between the two excitation wavelengths (R_340/380). Fluorescence intensity was recorded at 510-nm emission. For inhibition studies, cells in suspension were preincubated for 15–30 min with SK&F96365. Measurements were conducted with a RF-M 2001 fluorometer (Photon Technology) equipped with magnetic stirrer.

Results

EAE suppression by Gal-1 and CtxB: enhancement in GM1-deficient mice

Using MOG35–55 peptide-immunized mice obtained as above, disease severity of Gal-1- and CtxB-treated animals was significantly reduced compared with controls over the observed periods (Fig. 1, a and b). Discontinuance of Gal-1 injections at day 23 resulted in a progressive rise of clinical symptoms to the point of no significant difference compared with controls at day 31. Comparison of the curve segment for Gal-1 (days 1–23) vs the segment Gal-1 (days 24–31) following cessation of treatment gave statistical difference (p < 0.05). Whereas >90% of control mice developed symptoms, the incidence was less for the mice given CtxB (57%) or Gal-1 (73%); day of onset did not differ (Table I). Genetically altered mice (GalNAcT^−/−/−) lacking Ggs (including GM1) showed enhanced susceptibility to MOG35–55 immunization (Fig. 1c), with an incidence of 100% and significantly shorter time of onset (Table I).

Histopathological examination of spinal cord sections stained with Luxol Fast blue revealed demyelination in untreated WT mice (Fig. 2a) and less in WT mice treated with Gal-1 (Fig. 2b) and...
CxtB (Fig. 2c). The KO mutant, on the other hand, showed significantly enhanced demyelination in association with abundant lymphocyte invasion (Fig. 2d).

**Up-regulation of gangliosides GM1 and GD1α in activated T cells: interaction with Gal-1**

CD4⁺ (−10⁷) and CD8⁺ (~8 × 10⁶) T cells were isolated from spleens of both WT- and KO mice. Analysis of the purified gangliosides by HPTLC revealed initially low levels of GM1 and pronounced elevation of that ganglioside in whole cells following activation of both CD4⁺ and CD8⁺ cells (Fig. 3a); GD1α was similarly increased, principally in CD4⁺ T cells; resting CD8⁺ T cells contained substantially more GD1α than CD4⁺ T cells, which was somewhat elevated after activation. CD4⁺ T cells also showed elevation of a slower migrating ganglioside of unknown identity (possibly GD1b). To determine the change in cell surface GM1, resting and activated cells were treated with CxtB-FITC and analyzed by flow cytometry, which indicated a significant increase: 7.3-fold for CD4⁺ and 3.3-fold for CD8⁺ T cells in mean fluorescence intensity of GM1 (Fig. 3b). To determine intracellular GM1, activated cells were reacted with nonfluorescent CxtB (to block surface GM1) and then permeabilized with saponin followed by CxtB-FITC treatment and flow cytometric analysis. This revealed a sizeable intracellular pool in both cell types that increased during activation, although less dramatically than surface GM1 (Fig. 3b). To determine whether the up-regulated GM1 was able to bind Gal-1, activated cells from spleens of WT and KO mice were treated with Gal-1-biotin plus streptavidin-FITC and examined with fluorescence microscopy. Considerable Gal-1 binding was apparent with the activated WT CD4⁺ and CD8⁺ T cells, in contrast to marked elevation of Gal-1 in Treg cells. Band at ~28 kDa is Gal-1 homodimer. e, Activated (Act.) Treg cells, with or without saponin permeabilization, were stained with anti-Gal-1 Ab followed by goat anti-rabbit Ab linked to FITC. Following activation, Gal-1 is expressed in cytosol/nucleus and on the cell surface. Res., Resting. Scale bar, 20 μm.

**FIGURE 4.** Up-regulation of Gal-1 in activated Treg cells. a, Isolation of Treg cells from WT mouse spleen with immunomagnetic beads gave a population of cells that stained positively with anti-Foxp3. b, Flow cytometric analysis (eBioscience mouse Treg staining kit) indicated that 87% of the purified cells were CD4⁺ CD25⁺ Foxp3⁺ T cells. c, Activation of Treg cells with anti-CD3/anti-CD28 followed by immunoblot analysis indicated significant elevation of Gal-1 (14–15 kDa), both cell associated and released into the medium (Med.). d, Activation of T cells followed by immunoblot analysis indicated little if any Gal-1 expression by CD4⁺ or CD8⁺ Teff cells, in contrast to marked elevation of Gal-1 in Treg cells. Band at ~28 kDa is Gal-1 homodimer. e, Activated (Act.) Treg cells, with or without saponin permeabilization, were stained with anti-Gal-1 Ab followed by goat anti-rabbit Ab linked to FITC. Following activation, Gal-1 is expressed in cytosol/nucleus and on the cell surface. Res., Resting. Scale bar, 20 μm.
indicates that Gal-1 and CtxB react with the same receptor and those results along with those of Fig. 3c suggest GM1 as the primary binding site of Gal-1 on CD4+H11001 and CD8+H11001 T cells.

Up-regulation of Gal-1 in activated Treg cells: intracellular and cell surface expression

CD4+CD25+ Treg cells were isolated from WT mouse spleens with immunomagnetic beads based on negative selection of CD4+ cells followed by positive selection with anti-CD25-coated beads. These Treg cells, representing ~10% of both WT and KO CD4+ T cells, were shown by immunofluorescence to express Foxp3 (Fig. 4a). Flow cytometric analysis using a rat anti-Foxp3 mAb linked with PE indicated that 87% of CD4+CD25+ cells were Foxp3+ (Fig. 4b). These cells were activated or not with anti-CD3/anti-CD28 and IL-2 and proteins in the resulting supernatants precipitated with trichloroacetic acid. Immunoblot analysis revealed only a trace of Gal-1 in the resting cells and strong up-regulation of a band at ~14–15 kDa in both cells and medium following activation that corresponded to Gal-1 (Fig. 4c). In contrast, little if any Gal-1 expression was seen in CD4+ or CD8+ T cells, whether resting or activated (Fig. 4d). T cells from KO and WT spleen had a similar Gal-1 phenotype. Activated Treg cells were immunostained with anti-Gal-1 Ab with and without saponin permeabilization, revealing Gal-1 expression in cytosol/nucleus (with) and on the cell surface (without) detergent (Fig. 4e).

Involvement of Gal-1 and GM1 in immunosuppression

To determine whether GM1-Gal-1 interaction is involved in Treg cell function, activated CD4+ or CD8+ Teff cells from spleen were cocultured with Treg cells or GM1 cross-linking agents and [3H]TdR incorporation determined in the final 18 h. Increasing ratios of Treg:Teff cells resulted in progressively greater suppression (Fig. 5a), as did increasing concentration of [3H]TdR incorporation determined in the final 18 h. Thymidine incorporation was significantly suppressed by both agents relative to control. Resting (Res.) cells showed significantly less [3H]TdR uptake, which was nevertheless inhibited by CtxB and Gal-1. Anti-Gal-1, but not rabbit control IgG, added during the final 18 h blocked inhibition. Analysis was by two-tailed Student’s t test, compared with controls (a–c) or WT Teff (d), *p < 0.01; **p < 0.001; and ***p < 0.0001. Each panel represents one of two similar experiments.

FIGURE 5. Involvement of GM1 and Gal-1 in immunosuppression. a. Teff and Treg cells were isolated and activated (Act.) for 3 days as described, followed by coculturing another 18 h with [3H]TdR incorporation. Increasing ratios of Treg:Teff cells resulted in progressively greater suppression. b. CD4+ and CD8+ Teff cells from WT spleen were activated for 3 days and cultured for an additional 18-h period in the presence of [3H]TdR and CtxB or Gal-1. Thymidine incorporation was significantly suppressed by both agents relative to control. Resting (Res.) cells showed significantly less [3H]TdR uptake, which was nevertheless inhibited by CtxB and Gal-1. c. Anti-Gal-1, but not rabbit control IgG, added during the final 18 h blocked inhibition. d. Spleen Treg cells from WT mice did not suppress proliferation of Teff cells from GM1-deficient KO mice, whereas Treg cells from KO mice did suppress Teff cells from WT mice. Analysis was by two-tailed Student’s t test, compared with controls (a–c) or WT Teff (d). *p < 0.01; **p < 0.001; and ***p < 0.0001. Each panel represents one of two similar experiments.

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CoxB in the absence of Treg cells (Fig. 5b). Gal-1 alone also produced significant suppression of activated cells. Resting CD4+ and CD8+ T cells showed considerably less proliferation and this too was suppressed by CtxB and Gal-1. Treg cell-induced inhibition of [3H]TdR incorporation was blocked by anti-Gal-1 Ab (Fig. 5c), indicating suppression by Gal-1 expressed on the surface of (or released from) Treg cells. Significantly, Teff cells from KO mice lacking GM1 did not experience reduced [3H]TdR incorporation when cocultured with WT Treg cells; in contrast, WT Teff cells cocultured with KO Tregs showed significant inhibition (Fig. 5d), consistent with the normal Gal-1 expression and up-regulation by KO Treg cells (Fig. 4d). CD4+ and CD8+ Teff cells behaved similarly in these tests.

Cross-linking of GM1 in Teff cells activates TRPC5 Ca2+ channels

The underlying mechanism(s) of Teff suppression by GM1 cross-linking is undoubtedly complex, but a key element was revealed in this study to be stimulation of Ca2+ influx through activation of TRPC5 channels. Monitoring [Ca2+]i with fura-2 in WT CD4+ Teff cells showed Gal-1 and CtxB to be similarly effective in inducing Ca2+ influx that was blocked by SK&F96365, a TRP channel blocker (34) (Fig. 6a). Influx was augmented by pretreatment of the cultured cells with neuraminidase, which increased GM1 on the cell surface (Fig. 6b). Small rises in intracellular Ca2+ were observed in the absence of extracellular Ca2+, suggesting the possibility of release from intracellular stores. Significantly, no rise in intracellular Ca2+ was seen in Gal-1-treated Teff cells from KO mice (Fig. 6c and d). Since SK&F96365 is a general inhibitor for TRP channels, more direct evidence for TRPC5 was sought by RT-PCR product sequencing and BLAST identification. TRPC5 mRNA (BLAST identification no. NM_009428.1) expression was very low in the resting state and up-regulated after activation in both CD4+ and CD8+ T cells (Fig. 7a). TRPC1 mRNA (BLAST identification no. NM_016984) was strongly expressed in resting cells and remained unchanged following activation. TRPC4 mRNA (BLAST identification no. NM_011643.1), the other member of this TRPC subfamily, was undetectable in either the resting or activated state. Immunostaining with anti-TRPC5 Ab also revealed up-regulation of this protein in activated CD4+ T cells (Fig. 7b). Infection of CD4+ and CD8+ T cells with retrovirus incorporated with TRPC5 shRNA-containing HuSH-29 plasmid effectively suppressed TRPC5 expression as determined by RT-PCR (Fig. 7c) and immunostaining (Fig. 7d). This knockdown of TRPC5 impacted Teff cell proliferation by Gal-1 and CtxB. Experiments a–c and f were each done twice with similar outcome. Res., Resting; ctl, control. Scale bars, 20 μm.

FIGURE 7. Up-regulation of TRPC5 protein in activated (Act.) Teff cells and effect of TRPC5 knockdown. a, RT-PCR analysis revealed up-regulation of TRPC5 mRNA in activated CD4+ and CD8+ cells and only trace detection of these before activation. b, Immunostaining with anti-TRPC5 Ab also indicated up-regulation of TRPC5 protein in activated CD4+ and CD8+ cells. Knockdown of TRPC5 with shRNA was indicated by RT-PCR (c) and immunostaining (d) with anti-TRPC5 Ab. e, Knockdown of TRPC5 blocked CtxB-induced influx of Ca2+ in Teff cells as monitored by fura 2. f, Knockdown of TRPC5 in CD4+ and CD8+ cells blocked inhibition of Teff proliferation by Gal-1 and CtxB. Experiments a–c and f were each done twice with similar outcome. Res., Resting; ctl, control. Scale bars, 20 μm.

Cross-linking of GM1 in Teff cells activates TRPC5 Ca2+ channels

The underlying mechanism(s) of Teff suppression by GM1 cross-linking is undoubtedly complex, but a key element was revealed in this study to be stimulation of Ca2+ influx through activation of TRPC5 channels. Monitoring [Ca2+]i with fura-2 in WT CD4+ Teff cells showed Gal-1 and CtxB to be similarly effective in inducing Ca2+ influx that was blocked by SK&F96365, a TRP channel blocker (34) (Fig. 6a). Influx was augmented by pretreatment of the cultured cells with neuraminidase, which increased GM1 on the cell surface (Fig. 6b). Small rises in intracellular Ca2+ were observed in the absence of extracellular Ca2+, suggesting the possibility of release from intracellular stores. Significantly, no rise in intracellular Ca2+ was seen in Gal-1-treated Teff cells from KO mice (Fig. 6c and d). Since SK&F96365 is a general inhibitor for TRP channels, more direct evidence for TRPC5 was sought by RT-PCR product sequencing and BLAST identification. TRPC5 mRNA (BLAST identification no. NM_009428.1) expression was very low in the resting state and up-regulated after activation in both CD4+ and CD8+ T cells (Fig. 7a). TRPC1 mRNA (BLAST identification no. NM_016984) was strongly expressed in resting cells and remained unchanged following activation. TRPC4 mRNA (BLAST identification no. NM_011643.1), the other member of this TRPC subfamily, was undetectable in either the resting or activated state. Immunostaining with anti-TRPC5 Ab also revealed up-regulation of this protein in activated CD4+ T cells (Fig. 7b). Infection of CD4+ and CD8+ T cells with retrovirus incorporated with TRPC5 shRNA-containing HuSH-29 plasmid effectively suppressed TRPC5 expression as determined by RT-PCR (Fig. 7c) and immunostaining (Fig. 7d). This knockdown of TRPC5

FIGURE 8. Association of GM1 with integrins in Teff cells. a, IP of α4-integrin and β1-integrin followed by immunoblot analysis with anti-β1 Ab indicated β1 association with both α4 and α5 integrin in CD4+ and CD8+ T cells. h, Similar IP followed by chloroform-methanol extraction of the immunoprecipitates and HPTLC analysis revealed GM1 association with both integrins in both CD4+ and CD8+ T cells. Appearance of two GM1 bands is likely the result of two classes of fatty acids of varying chain length within ceramide. The CD4 “none” lane was a control to rule out nonspecific absorption of GM1 to protein A beads. BBG, Bovine brain gangliosides (standards).
also blocked CtxB-mediated Ca\textsuperscript{2+} influx (Fig. 7e) as well as Gal-1- or CtxB-induced suppression of proliferation (Fig. 7f).

To further define the nature of the cross-linking phenomenon, we sought to identify the protein with which GM1 associates in Teff cells and which would undergo co-cross-linking. Membranes from CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells were subjected to IP with anti-α\textsubscript{4} integrin mAb and anti-α\textsubscript{5} integrin mAb followed by HPTLC analysis of the coprecipitated ganglioside. GM1 was indicated as the principle Gg-type ganglioside associated with both α\textsubscript{4} and α\textsubscript{5} integrin (Fig. 8b). The appearance of two GM1 bands seen here often results from the presence of two groups of fatty acids varying in chain length within the ceramide moiety (cf Fig. 3e). Similar IP of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell membranes with the above two Abs followed by immunoblot analysis with anti-β\textsubscript{1} integrin Ab revealed β\textsubscript{1} integrin association with both α\textsubscript{4} and α\textsubscript{5} integrin (Fig. 8a). Hence the membranes of both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells appear to contain GM1 closely linked with both α\textsubscript{4}β\textsubscript{1} and α\textsubscript{5}β\textsubscript{1} heterodimers.

Discussion

This study has shown cross-linking of GM1 ganglioside in Teff cells by Gal-1 expressed by Treg cells to be a crucial element in responder cells. Integrin associated with GM1 was seen to undergo co-cross-linking, resulting in TRPC5-mediated Ca\textsuperscript{2+} influx. We demonstrated significant elevation of GM1 in Teff cells subjected to polyclonal activation while similar activation of Treg cells caused up-regulation of Gal-1 that was both cell associated and released to the medium. Mixed culture experiments showed suppressed proliferation of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells by both Gal-1 and CtxB, analogous to that produced by activated Treg cells; moreover, Treg cell-induced inhibition of Teff cell proliferation was blocked by anti-Gal-1 Ab (Fig. 5). Toscano et al. (14) also demonstrated such inhibition by Gal-1 and further showed that genetically induced Gal-1 deficiency resulted in enhanced susceptibility to EAE. Fittingly, our analogous results with genetically induced GM1 deficiency (Figs. 1 and 2) provides a salient clue for defining galectin surface receptor pairing. Whereas Gal-1 binds to a variety of protein and lipid galactosides (11, 14), CtxB is relatively specific for GM1 (35, 36). Hence, our finding of parallel effects of Gal-1 and CtxB in suppressing Tcell proliferation as well as EAE point to GM1 as the primary target of Gal-1 expressed by Treg cells. The B subunit of Escherichia coli heat-labile enterotoxin shows considerable structural and functional similarity to CtxB and these two GM1 cross-linking agents have been shown to suppress a variety of autoimmune disorders (37–39). Homodimeric Gal-1, also an effective GM1 cross-linker, similarly suppresses several animal models of autoimmunity (see below).

This demonstration of GM1 involvement in autoimmune suppression appears contrary to a recent report which found no difference in EAE clinical symptoms between similar ganglioside-null KO and WT mice (40). A likely explanation of the discrepancy was the methodology for inducing EAE: we immunized twice with 200 μg of MOG\textsubscript{35–55}, 1 wk apart, as opposed to once with an apparently lesser amount of the same peptide. A stronger challenge was apparently needed to demonstrate the difference. This was further indicated by the earlier time of onset (Table I) and most strikingly by enhanced histopathology (Fig. 2c), an aspect not considered in the previous study (40). That the mutants did not show even greater pathology would suggest the existence of a parallel pathway(s) that mediates autoimmune suppression. In that regard, differential sialylation of cell surface glycoproteins was invoked to explain the greater reactivity of Gal-1 toward T\textsubscript{H}1- and T\textsubscript{H}17-differentiated T cells compared with T\textsubscript{H}2 cells (14). The ability of homodimeric Gal-1 to bind, albeit sparsely, to ganglioside-deficient T cells supports some Gal-1 interaction with cell surface glycoproteins, although the more pronounced binding to WT cells (Fig. 3c) suggests primary binding of Gal-1 to GM1 in this system. This was supported by evidence that Gal-1 and CtxB compete for the same binding site in activated CD4\textsuperscript{+} T cells (Fig. 3e). This finding of GM1 as the primary glycoconjugate target of Gal-1 in Teff cells is similar to the previously described interaction of these two molecules in neuroblastoma cells (16). Absence of GM1 in the KO mice may account, at least in part, for the progressive debilitation these animals experience (41), although it is not known to what extent this represents an autoimmune phenotype.

Our proposed mechanism involving cross-linking of GM1 as a key event in immunosuppression is consistent with the dramatic elevation of GM1 in Teff cells during activation (Fig. 3, a and b). This accords with the recent observation of “GM ganglioside” elevation in activated CD4\textsuperscript{+} T cells (42). The present study demonstrated GM1 elevation in CD8\textsuperscript{+} T cells as well, while the increase of GD1a, a neuraminidase-reactive substrate that yields GM1, would correlate with the reported up-regulation of neuraminidase in T cells during activation (43). Such reaction might account for all or part of the GM1 rise during T cell activation. The proposed role for GM1 could provide a rationale for the several animal model examples of autoimmune suppression by administered GM1 (and Gg mixtures containing GM1) (44–49).

A more detailed understanding of Treg cell suppression requires elucidation of events subsequent to cross-linking of GM1 in the Teff cell. Earlier studies using CtxB application to rat lymphocytes (50) and Jurkat T cells (51) revealed Ca\textsuperscript{2+} influx through an undefined channel while similar studies with neural cells showed CtxB-induced neurite outgrowth as a consequence of the Ca\textsuperscript{2+} increase (33, 52, 53). The Ca\textsuperscript{2+} channel was revealed to be voltage independent (33) and a recent report specified the channel type as TRPC5 (32), a member of the canonical subfamily belonging to the transient receptor potential superfamily of signal transduction-gated ion channels (19). The present study revealed TRPC5 as the channel type that is activated by Gal-1 and CtxB cross-linking of GM1 in Teff cells. That such cross-linking, as opposed to mere binding, of GM1 is necessary was suggested in the observation that IgM but not IgG anti-GM1 Ab induced similar Ca\textsuperscript{2+} influx as CtxB (54). The fact that GM1 is associated with integrins (Fig. 8) suggests integrin-activated signaling, analogous to that in neural cells (32).

Our finding of TRPC5 expression may seem at odds with a recent report claiming absence of TRPC5 in mouse spleen and T lymphocytes (55). We were able to verify the virtual absence of TRPC5 in resting murine T cells, but observed significant up-regulation of this protein following T cell activation based on RT-PCR and immunostaining (Fig. 7, a and b). Knockdown of TRPC5 mRNA with shRNA blocked Ca\textsuperscript{2+} influx and in addition eliminated the inhibitory effect of CtxB/Gal-1 on Tcell proliferation (Fig. 7, c–f). This form of signaling can be distinguished from other Ca\textsuperscript{2+} effects in immune cells, such as that occurring during formation of the immunological synapse that results in modest but sustained intracellular Ca\textsuperscript{2+} elevation (56, 57). We previously showed that elevation of cell surface GM1 with neuraminidase increased ion flow, permitting channel recordings in neural cells (32), analogous to the current finding that neuraminidase pretreatment increased CtxB-induced Ca\textsuperscript{2+} influx in Teff cells (Fig. 6, a and b). Gal-1 was previously shown to induce Ca\textsuperscript{2+} influx in Jurkat cells in a nonvoltage regulated manner (58).

Further elucidation of the cross-linking phenomenon revealed GM1 association with α\textsubscript{4} and α\textsubscript{5} integrin, both of which were associated with β\textsubscript{1} integrin. This is similar in one respect to the
finding in a previous study of neural cells (32) in which the pivotal role of the αβ heterodimer, which experiences cross-linking with GM1, was revealed as initiating a signaling cascade that culminates in TRPC5 activation. Integrin clustering, its presence indicated in a model of tumor cell aneiksis (15), is necessary and often sufficient for activation of the crucial enzyme focal adhesion kinase through tyrosine phosphorylation, which in turn results in assembly of a multicomponent signaling complex (59).

The above-mentioned study of neural cells (32) suggests it is the αβ heterodimer that triggers TRPC5 channel activation. This study has thus revealed one likely pathway by which Treg cells activate the above mechanism to render tight control of autoggressive T cells. This may account for the proximity requirement of Treg suppression, involving either cell-cell contact or a high concentration of the lectin released in spatial proximity. Due to its nature with intimate contacts in the interface, Gal-1 can potently cross-link glycans, and its reactivity to GM1 has been shown with free pentasaccharide (even revealing conformer selection), neoganglioprotein, and in a cell system of neuroblastoma (16, 25, 60–63). Gal-1 was previously proposed as a negative regulator of the immune response in keeping with its ability to suppress animal models of autoimmune uinmunity such as EAE (64), collagen-induced arthritis (65), experimental colitis (66), autoimmune uveitis (14). Con A-induced hepatitis (67), and autoimmune diabetes (68). As mentioned, GM1 has also proved an effective modulator of autoimmune. It is intriguing to speculate that some naturally occurring autoimmune diseases may result from malfunction of one or another reaction in the proposed Treg cell sequence, analogous to aberrations revealed in Treg cells of autoimmune patients (4–10). The ability of both Gal-1 and GM1 to restore autoimmune regulation in animal models where natural suppression has been compromised promises them as appealing candidates for therapeutic design in human disorders.

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Disclosures

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References


