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Expression of glycosphingolipids in lymph nodes of mice lacking TNF receptor 1: biochemical and flow cytometry analysis

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Abstract—The expression of gangliosides and neutral glycosphingolipids (GSLs) in the lymph nodes of mice lacking the gene for the tumour necrosis factor-α receptor p55 (TNFR1) has been investigated. GSL expression in the tissues of mice homozygous (TNFR1−/−) or heterozygous (TNFR1+/−) for the gene deletion was analysed by flow cytometry and high-performance thin-layer chromatography (HPTLC) followed by immunostaining with specific antibodies. HPTLC immunostaining revealed that lymph nodes from TNFR1−/− mice had reduced expression of ganglioside GM1b and GalNAc–GM1b, neolacto-series gangliosides, as well as the globo- (Gb3, Gb4 and Gb5) and ganglio-series (Gg3 and Gg4) neutral GSLs. Flow cytometry of freshly isolated lymph node cells showed no significant differences in GSL expression, except for the GalNAc–GM1b ganglioside, which was less abundant on T lymphocytes from TNFR1−/− lymph nodes. In TNFR1−/− mice, GalNAc–GM1b$^+$/CD4$^+$ T cells were twofold less abundant (3.8% vs 7.6% in the control mice), whereas GalNAc–GM1b$^+$/CD8$^+$ T cells were fourfold less abundant (5.0% vs 20.2% in the control mice). This study provides in vivo evidence that TNF signalling via the TNFR1 is important for the activation of GM1b-type ganglioside biosynthetic pathway in CD8 T lymphocytes, suggesting its possible role in the effector T lymphocyte function.

Keywords: Glycosphingolipids; Gangliosides; Lymph nodes; TNF receptor 1; Knockout mice; HPTLC immunostaining; Flow cytometry

1. Introduction

Tumour necrosis factor receptor p55 (TNFR1) has an important role in the morphogenesis of lymphoid organs and generation of immune response to antigens,[1,2] as well as for the development of Peyer's patches and splenic follicular dendritic cells, formation of germinal centres and T cell dependent antibody responses.[3–5] TNFR1 signalling involves different systems, such as phospholipase A_2, phospholipase C and sphingomyelinases.[6–8]

Glycosphingolipids (GSLs) are complex lipids located primarily in the outer leaflet of the plasma membrane. They have a ceramide portion immersed in the lipid layer, and carbohydrate moiety located primarily in the extracellular space.[9,10] They are considered important for cell surface recognition[11] and modulation of the function of a variety of membrane-associated proteins.[12] In the membrane, GSLs are assembled as 'rafts'[13] or

Abbreviations: GSL(s), glycosphingolipid(s); HPTLC, high-performance thin-layer chromatography; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; PBS, phosphate buffered saline; TNF, tumour necrosis factor; TNFR1, TNF receptor p55; TNFR1−/−, mice homozygous for the TNFR1 gene knockout; TNFR1+/−, mice heterozygous for the TNFR1 gene knockout. Glycosphingolipid nomenclature according to the IUPAC-IUB recommendations: lactosylceramide, Lc2 or LacCer, Galβ4Glcβ1Cer; Globotriaosylceramide or Gb3, Galα4Galβ4Glcβ1Cer; Globotetraosylceramide or Gb4, GalNAcβ3Galα4Galβ4Glcβ1Cer; Globopentaosylceramide or Gb5, GalNAcα3GalNAcβ3Galα4Galβ4Glcβ1Cer; Gangliotriaosylceramide or Gg3, GalNAcβ4Galβ4Glcβ1Cer; Gangliotetraosylceramide or Gg4, Galβ3GalNAcβ4Galβ4Glcβ1Cer; Gangliopentaosylceramide or Gg5, GalNAcβ4Galβ3GalNAcβ4Galβ4Glcβ1Cer; Lacto-*N*-*neo*tetraosylceramide or nLc4, Galβ4GlcNAcβ3Galβ4Glcβ1Cer; Lacto-*N*-*neo*hexaosylceramide or nLc6, Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ-4Glcβ1Cer; GM3, II^{3}Neu5Ac-Lc2; GM2,II^{3}Neu5Ac-Gg3; GM1 or GM1a, II^{3}Neu5Ac-Gg4; GM1b, IV^{3}Neu5Ac-Gg4; GalNAc–GM1b, IV^{3}Neu5Ac-Gg5; GD1α, IV^{3}Neu5Ac,III^{6}Neu5Ac-Gg4; GD1a, IV^3-Neu5Ac, II^{3}Neu5Ac-Gg4; GD1c, IV^3(Neu5Ac)$_2$-Gg4 (only Neu5Ac-substituted gangliosides are presented in this list of abbreviations).

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'glycosignalling domains',[14] where they exert their biological activities.[15]

Specific assignment of different GSLs between different lineages of cells involved in immune response have been described (Fig. 1).[16–18] Among the globo-series neutral GSLs, Gb3 seems to be a differentiation marker of human B lymphocytes,[19] Gb4 was described as T lymphocyte maturation marker,[20] whereas Forssman GSL (Gb5) was described as specific for murine splenic macrophages.[21] In the neutral GSL ganglio-series, Gg4 is a foetal thymocyte marker,[22] whereas GM1b-type gangliosides, synthesized from neutral Gg4, are considered important T lineage markers.[23,24] They can be further sialylated to GD1α and GD1c, disialogangliosides found on TH2 lymphocytes[25] and naive TH1-like cells.[26] Disialoganglioside GD1α was detected in murine peritoneal macrophages,[27] and GM1b and its elongation product GalNAc–GM1b were found in murine derived macrophage-like WEHI-3 cells.[28]

We have recently shown that mice with the knockout for the TNFR1 gene have reduced expression of various gangliosides and neutral GSLs, especially GM1b-type gangliosides (GM1b and GalNAc–GM1b) in the lungs, spleen and thymus.[29] Lymph nodes were not analysed because of the methodological limitations of the lipid extraction protocol. Recently, we developed a method suitable for analysis of small samples,[30] and used this method to analyse GSL expression in lymph nodes, using specific polyclonal antibodies and high-performance thin-layer chromatograms.[31] We used the same

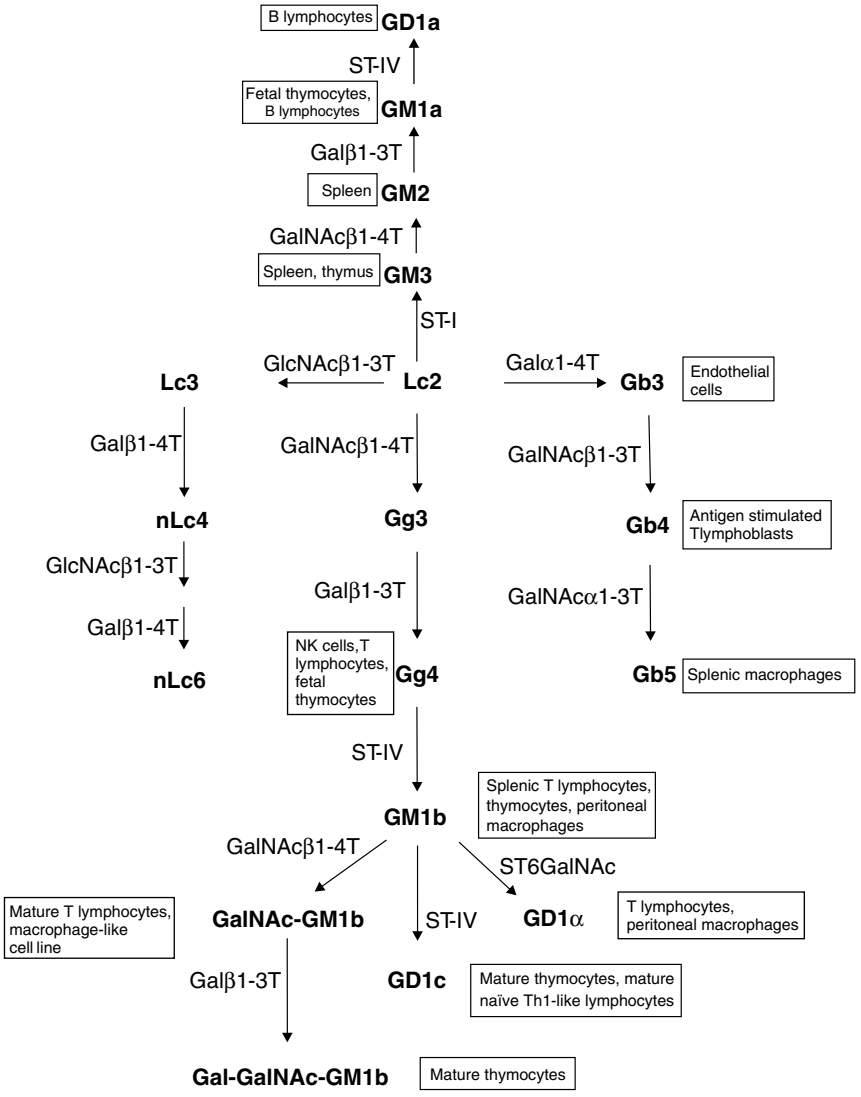

Figure 1. A schematic overview of GSL biosynthesis and expression in murine lymphoid cells and tissues. Galα1-4T, galactosyl-(α1-4)-transferase; Galβ1-3T, galactosyl-(β1-3)-transferase; Galβ1-4T, galactosyl-(β1-4)-transferase; GalNAcα1-3T, *N*-acetyl-galactosaminyl-(α1-3)-transferase; GalNAcβ1-3T, *N*-acetyl-galactosaminyl-(β1-3)-transferase; GalNAcβ1-4T, *N*-acetyl-galactosaminyl-(β1-4)-transferase; GlcNAcβ1-3T, *N*-acetyl-glucosaminyl-(β1-3)-transferase; ST-I and ST-IV, sialyl-transferases I and IV and V; ST6GalNAc, sialyltransferase that transfers sialic acid to position 6 of *N*-acetylgalactosamine residue.

antibodies for the characterization of GSL expression on lymph node cell populations by flow cytometry.

2. Results

2.1. HPTLC analysis of GSL expression

Resorcinol staining of gangliosides (Figs. 2A and 4A) and orcinol staining of neutral GSLs (Figs. 3A and 5A) indicated lower expression of a number of GSLs in TNFR1−/− lymph nodes compared with TNFR1+/− controls. As the staining was weak, we used immunooverlay of lipid chromatograms with specific antibodies to discern the structural differences between the two groups. Control heterozygous mice expressed traces of two bands of GM1b-type gangliosides, which were absent in the lymph nodes of TNFR1−/− mice (Fig. 2B). The upper band (marked I in the figure) represented GM1b(Neu5Ac) with C24-fatty acid and the lower one (marked II in the figure) could contain ganglioside GM1b(Neu5Ac,C16) and/or GM1b(Neu5Gc,C24). YAC-1 and MDAY-D2 reference gangliosides were marked with roman numerals from −II to VIII and their structures are given in Table 1.

A single positive GalNAc–GM1b band was detected in the lymph nodes of control mice at the position, which could contain mixed fractions GalNAc–GM1b(Neu5Ac) with C16-fatty acid and GalNAc–GM1b(Neu5Gc) with C24-fatty acid.[29] This band was absent in the lymph node of TNFR1−/− mice (Fig. 2C). Lymph nodes from control heterozygous mice expressed

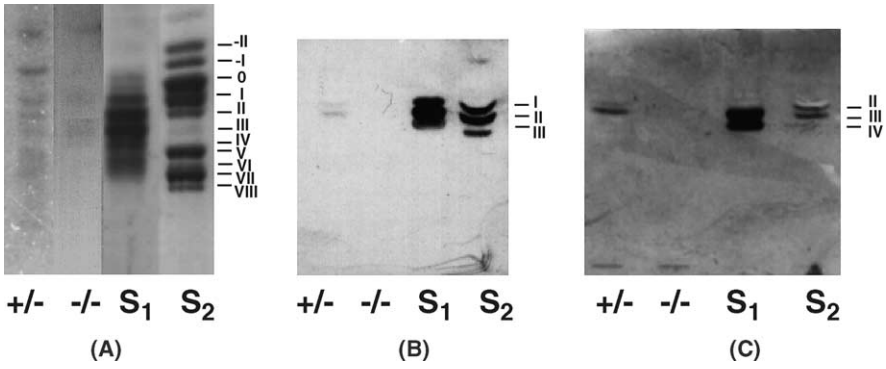

Figure 2. Detection of GM1b-type gangliosides in the lymph nodes of TNFR1−/− and TNFR1+/− mice: resorcinol stain (A), immunodetection of GM1b with anti-Gg4 antibody after neuraminidase treatment (B) and immunodetection with anti-GalNAc–GM1b antibody (C). Gangliosides amounts corresponding to 40 mg wet weight for resorcinol staining (A) and to 13.3 mg wet weight for HPTLC immunostaining (B, C) were chromatographed together with 10 μg of standard gangliosides from YAC-1 (lane S_1) and 10 μg of gangliosides from MDAY-D2 (lane S_2). YAC-1 and MDAY-D2 gangliosides are marked with roman numerals from −II to VIII and their structures are given in Table 1.

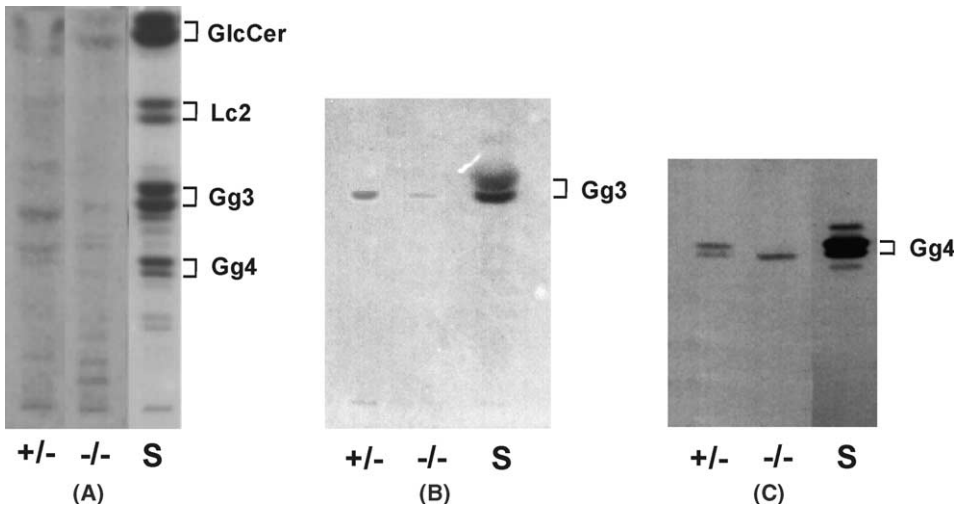

Figure 3. Detection of ganglio-series neutral GSLs in the lymph nodes of TNFR1−/− and TNFR1+/− mice: orcinol stain (A), immunostain with anti-Gg3 (B) and immunostain with anti-Gg4 antibody (C). GSLs amounts corresponding to 40 mg wet weight for orcinol staining (A) and to 13.3 mg wet weight for HPTLC immunostaining (B, C) were chromatographed together with 8 μg of neutral GSLs from YAC-1 (lane S). Upper and lower bands of pairs of YAC-1 neutral GSLs correspond to substitution with C24 and C16 fatty acids, respectively.

Table 1. Major gangliosides from murine lymphoma YAC-1 and lymphoreticular MDAY-D2 cells

Ganglioside fraction	Fatty acid	Symbol[a]	Sialic acid	YAC-1	MDAY-D2
–II	24:0, 24:1	G_{M2}	Neu5Ac	–	+
–I	16:0	G_{M2}	Neu5Ac	–	+
0	24:0, 24:1	G_{M1a}	Neu5Ac	–	+
I	16:0	G_{M1a}	Neu5Ac	–	+
	24:0, 24:1	$^{*}G_{M1b}$	Neu5Ac	+	+
II	16:0	$^{*}G_{M1b}$	Neu5Ac	+	+
	24:0, 24:1	GalNAc-G_{M1b}	Neu5Ac	+	+
	24:0, 24:1	$^{*}G_{M1b}$	Neu5Gc	+	–
III	16:0	$^{*}G_{M1b}$	Neu5Gc	+	–
	16:0	GalNAc-G_{M1b}	Neu5Ac	+	+
	24:0, 24:1	GalNAc-G_{M1b}	Neu5Gc	+	–
IV	16:0	GalNAc-G_{M1b}	Neu5Gc	+	–
V	24:0, 24:1	G_{D1a}	Neu5Ac	–	+
VI	16:0	G_{D1a}	Neu5Ac	–	+
VII	24:0, 24:1	$^{*}G_{D1\alpha}$	Neu5Ac	–	+
VIII	16:0	$^{*}G_{D1\alpha}$	Neu5Ac	–	+

[a] G_{M1b}- and $G_{D1\alpha}$-gangliosides detectable by immunostaining with anti-$GgOse_4Cer$ antibody after *V. cholerae* neuraminidase treatment are marked with asterisks; structural data drawn from Müthing et al.[32,33]

Gg3, whereas only traces were found in the lymph nodes of TNFR−/− mice (Fig. 3B). Rabbit anti-Gg4 antibody labelled two bands in the control animals, in contrast to a single band detected in the TNFR−/− lymph nodes (Fig. 3C).

Lymph nodes of control TNFR1+/− mice gave four bands of neolacto-series gangliosides, referring to double bands of (α2-3)-sialylated IV^3nLc4 and (α2-6)-sialylated IV^6nLc4 neolacto-core gangliosides.[29] These bands were completely absent in the lymph nodes from TNFR1−/− mice (Fig. 4B).

Globo-series neutral GSLs (Gb3, Gb4 and Gb5) were detected by specific antibodies in the lymph nodes from the control heterozygous mice (Fig. 5B–D). The expression of Gb4 was decreased in the lymph nodes of TNFR1−/− mice (Fig. 5C), whereas Gb3 and Gb5 were completely absent (Fig. 5B and D).

Collectively, these results showed that the expression of globo- and ganglio-series neutral GSLs, GalNAc–GM1b and GM1b gangliosides and neolacto-series gangliosides was down-regulated in the lymph nodes of TNFR1−/− mice.

2.2. Flow cytometry analysis of GSL expression

For the flow cytometry, we used the same set of antibodies used in the HPTLC detection of GSLs. Due to the nature of antibody recognition, we could not detect ganglioside GM1b and neolacto-series gangliosides because of the interference of neutral GSLs. In thin layer chromatography, ganglioside GM1b is recognized by antibody against neutral GSL Gg4 after *V. cholerae* neuraminidase treatment, which cleaves off the sialic acid.[34] The analogous treatment is used for the biochemical detection of neolacto-series gangliosides, which can be detected by anti-nLc4 antibody after neuraminidase treatment. Also, Gb5 (Forssman antigen)

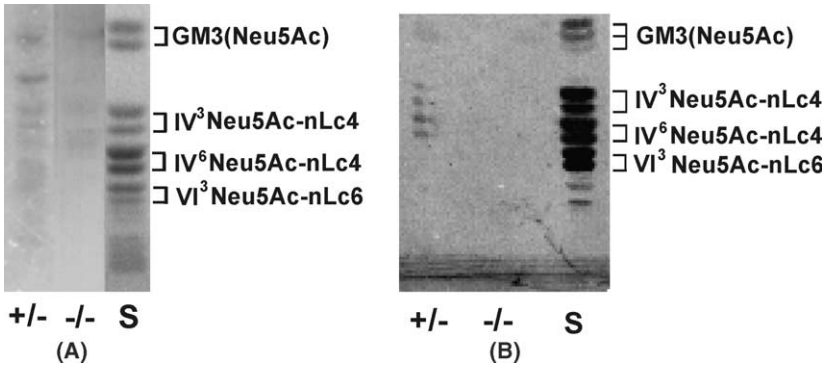

Figure 4. Detection of neolacto-series gangliosides in the lymph nodes of TNFR1−/− and TNFR1+/− mice: resorcinol stain (A) and immunostain (B). Ganglioside amounts corresponding to 40 mg wet weight for resorcinol staining (A) and to 13.3 mg wet weight for HPTLC immunostaining (B) were chromatographed together with 2.5 μg human granulocyte gangliosides (lane S). The HPTLC immunostain was performed with anti-nLc4 antibody after *V. cholerae* neuraminidase treatment. Upper and lower bands of pairs of granulocyte gangliosides correspond to substitution with C24 and C16 fatty acids, respectively.

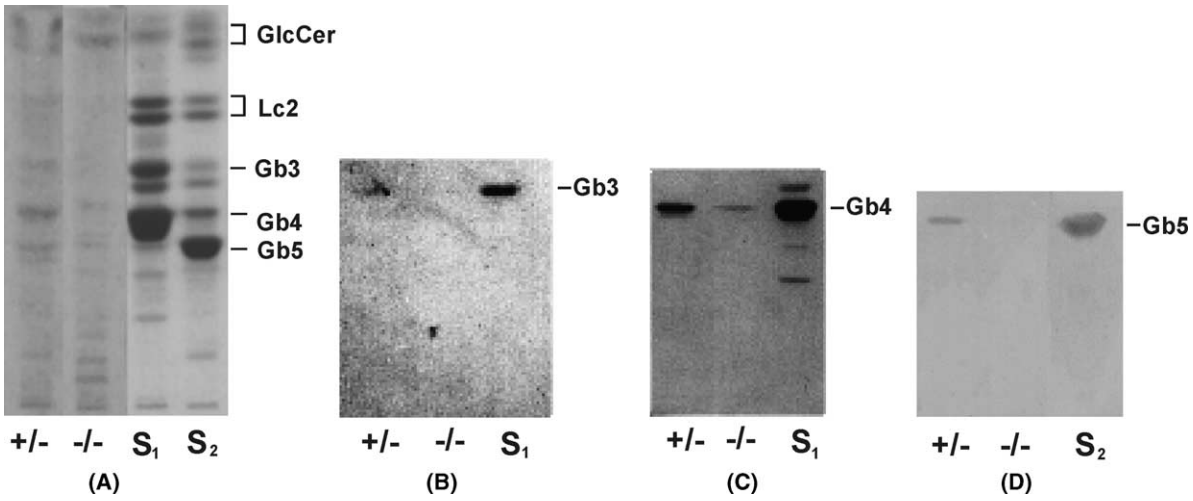

Figure 5. Detection of globo-series neutral GSLs in the lymph nodes of TNFR1−/− and TNFR1+/− mice: orcinol stain (A); and immunostain with anti-Gb3 (B), anti-Gb4 (C) and anti-Gb5 antibody (D). GSLs amounts corresponding to 40 mg wet weight for resorcinol staining (A) and to 13.3 mg wet weight for HPTLC immunostaining (B–D) were chromatographed together with 5 (lanes S_1, panels A and B) or 2.5 μg (lane S_1, panel C) neutral GSLs from human erithrocytes or 2.5 μg neutral GSLs from sheep blood cells (lane S_2, panels A and D).

could not be detected on cells in flow cytometry, most probably because of weak expression of this antigen on lymphocytes.[21]

The only notable difference between TNFR1−/− and the control mice was in the expression of GalNAc–GM1b ganglioside on lymph node cells (Fig. 6). In the control mice, 45% of lymph node lymphocytes expressed this ganglioside, compared to 35% in the TNFR1−/− mice. The expression of ganglio-series neutral GSLs, Gg3 and Gg4, as well as the expression of globo-series neutral GSLs, Gb3 and Gb4, did not differ significantly between the control and TNFR1−/− mice (Fig. 6).

The difference in GalNAc–GM1b expression, observed by single staining, was further analysed by double staining using GalNAc–GM1b antibody and PE-conjugated primary antibodies to CD19, a mature B-cell marker, or CD3, expressed on all mature T-cells (Fig. 7). In both groups of mice, there were 9.9% of GalNAc–GM1b$^+$/CD19$^+$ lymph node cells. GalNAc–GM1b$^+$/CD3ε$^+$ cells constituted 31.8% of CD3ε$^+$ lymph node cells in the control animals, compared to 9.6% of those cells in TNFR1−/− mice. This result indicated that the difference in the expression of GalNAc–GM1b observed by HPTLC immunostaining and flow cytometry anti-GalNAc–GM1b single staining was due to lower expression of GalNAc–GM1b on T lymphocytes.

To analyse further GalNAc–GM1b expression on T lymphocyte subpopulations, we performed three-colour

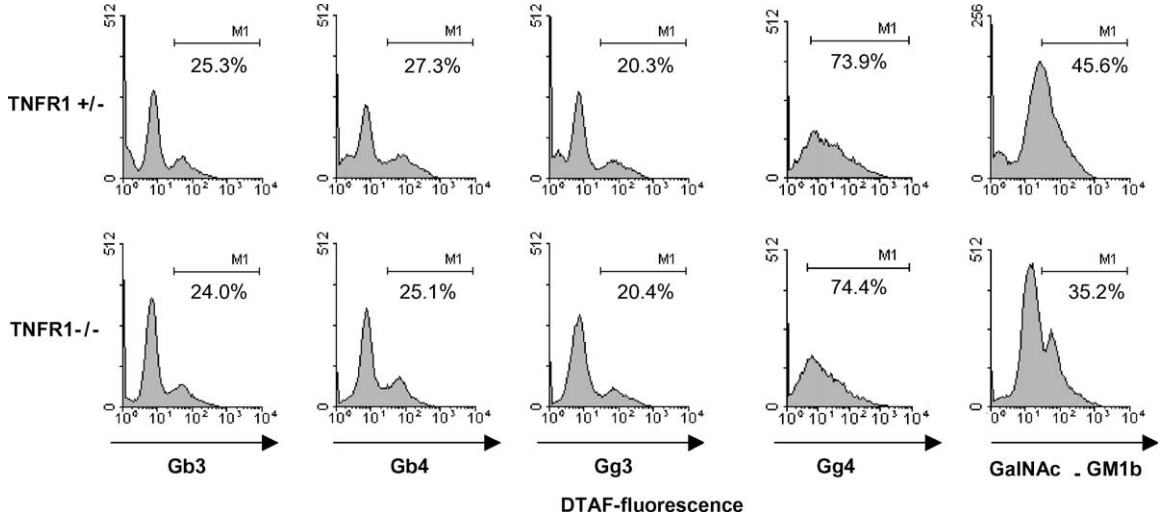

Figure 6. Flow cytometric analysis of GSL expression in the lymph node lymphocytes from control and TNFR1−/− mice. Single-colour histograms represent lymphocyte staining with different anti-GSL antibodies; *x*-axis, DTAF fluorescence intensity; *y*-axis, frequency of cells displaying certain fluorescence intensity.

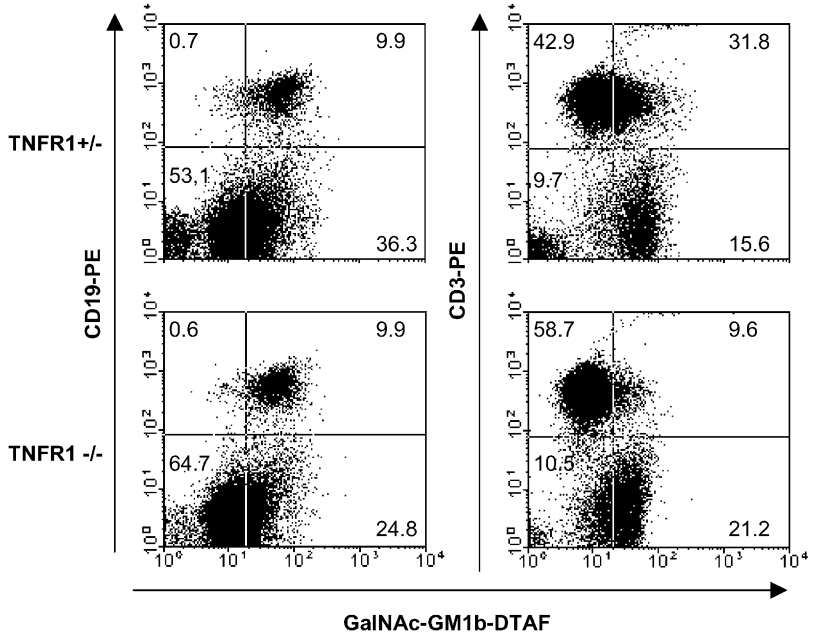

Figure 7. Two colour dot plots showing distribution of GalNAc–GM1b positive cells (GalNAc–GM1b-DTAF, *x*-axis) among B lymphocytes and T lymphocytes, in control (TNFR1+/−) and TNFR1 knockout mice (TNFR1−/−). B lymphocytes were stained with anti-CD19 antibody (CD19-PE, *y*-axis), and T lymphocytes were stained with anti-CD3ε antibody (CD3-PE, *y*-axis).

staining with primary antibody to GalNAc–GM1b, APC-conjugated primary antibody to CD3 and PE-conjugated primary antibodies to CD4 or CD8 (Fig. 8). In control mice, there were 7.6% $CD4^+$ T lymphocytes expressing GalNAc–GM1b, compared with 3.8% in the lymph nodes of TNFR1−/− mouse. Decreased GalNAc–GM1b expression was even more pronounced on $CD8^+$ T lymphocytes: in control mice, 20.2% of $CD8^+$ T lymphocytes expressed GalNAc–GM1b, but only 5.0% of $CD8^+$ T lymphocytes from TNFR1−/− lymph nodes. These results indicated that GalNAc–$GM1b^+$ T lymphocytes belonged mostly to the $CD8^+$ T cell subpopulation.

3. Discussion

This study provides in vivo evidence that TNFR1 signalling is necessary for the acquisition of specific pattern of GSL expression in the lymph nodes.

HPTLC immunostaining revealed decreased expression of ganglio-series neutral GSLs and their elongation products, GM1b-type gangliosides, in TNFR1−/− mouse lymph nodes. These GSLs belong to the asialo biosynthetic pathway, which starts from LacCer and elongates with *N*-acetylgalatosamine to Gg3.[10] Gg3 is further elongated to Gg4 by the addition of galactose residue. Sialylation of Gg4 by sialyltransferase-IV forms ganglioside GM1b, characteristic for splenic T lymphocytes[35] and thymocytes,[24] which can be elongated to GalNAc–GM1b by the addition of *N*-acetylgalactosamine to the terminal galactose. The formation of GalNAc–GM1b is related to T lymphocyte activation.[24,35,36] Our findings imply that TNFR1 signalling is necessary for the activation of the asialo pathway of ganglioside biosynthesis in the lymph nodes. Flow cytometric analysis of the lymph node lymphocytes confirmed the importance of TNFR1 signalling for the expression of ganglioside GalNAc–GM1b on lymph node T lymphocytes, especially the CD8 subpopulation.

Flow cytometry did not detect differences in the expression of neutral GSL precursors of the asialo pathway, Gg3 and Gg4. This may be related to the cross-reactivity of antibodies to carbohydrate epitopes on glycolipids and glycoproteins. Such epitopes on glycoproteins are especially abundantly expressed on B lymphocytes and give a strong signal in flow cytometry analysis,[37] possibly masking weakly expressed glycosphingolipids.

Predominant expression of GalNAc–GM1b on CD8 T lymphocytes and the role of TNF signalling in the acquisition of this ganglioside on CD8 subpopulation is not clear. Recent studies indicated that glycosyl residues corresponding to GM1b might have an important role in the regulation of T lymphocyte function and survival. Immature CD4/CD8 double-positive thymocytes bind MHC-I tetramers more avidly than mature CD8 single-positive thymocytes. This is influenced by *O*-glycan modification controlled by a sialyltransferase that adds sialic acid to CD8ß on mature thymocytes, thus decreasing CD8αβ-MHC-I avidity.[38] Enzyme ST-IV catalyses the transfer of (α2-3) linked sialic acid to

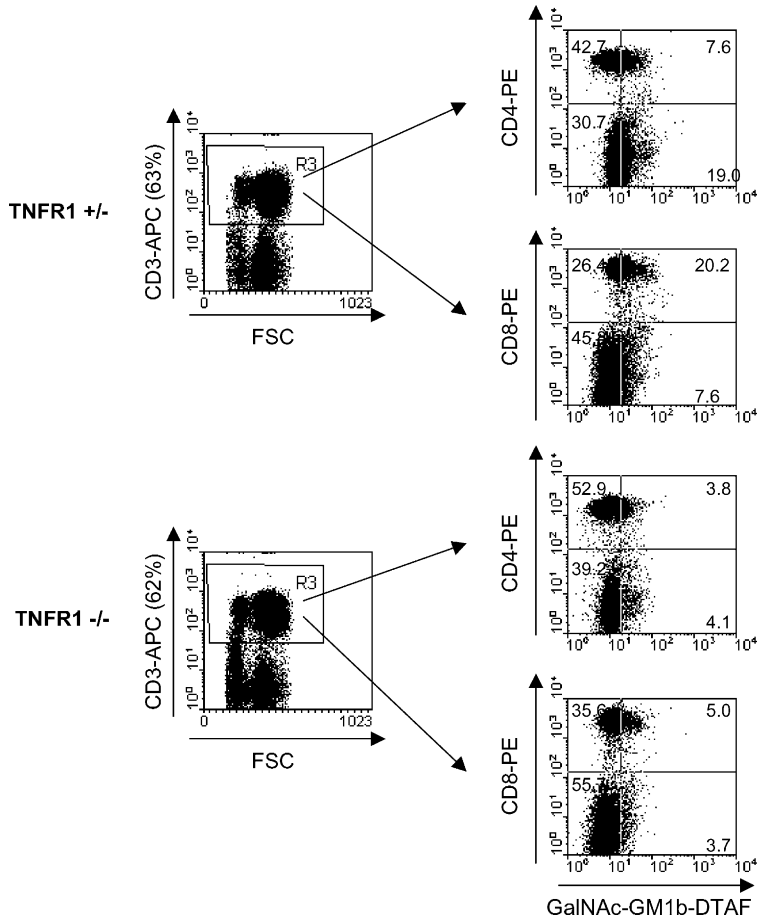

Figure 8. Phenotypic analysis of the GalNAc–GM1b distribution on CD4 and CD8 T lymphocytes in control (TNFR1+/–) and TNFR1 knockout mice (TNFR1–/–). T lymphocytes were gated according to CD3ε expression (CD3-APC) in FSC (forward scatter, *x*-axis) versus CD3-APC dot plots. Further analysis included only T lymphocytes. T lymphocytes were further analysed according to the expression of GalNAc–GM1b (GalNAc–GM1b-DTAF, *x*-axis) versus the expression of CD4 or CD8.

Gal(β1-3)GalNAc, which is the final step in GM1b synthesis.[39] ST-IV is also crucial for the homeostasis of CD8 T lymphocyte. Mice with a gene knockout for ST-IV have significantly less CD8 T lymphocytes.[40] This has been related to disturbed membrane carbohydrates, which seem to be crucial for the provision of anti-apoptotic signal to T lymphocytes during their development.[41] The difference in GalNAc–GM1b expression between CD4 and CD8 population may be related to the different role of TNF in auto-regulatory apoptosis, which is regulated by TNF in CD8 T lymphocytes, and by FasL in CD4 T lymphocytes.[41] In addition, our result is in accordance with earlier findings of Nakamura et al.,[42] who described that elongation product of GM1b, GD1c disialoganglioside, was strongly expressed on CD4 T cells but not on CD8 T cells in mouse lymph nodes. This indicated that less GM1b as a precursor is available for GalNAc–GM1b synthesis in CD4 T cells than in CD8 population.

Double bands of terminally sialylated nLc4 were detected in the control TNFR1+/– but not in the lymph nodes of TNFR1–/– mice, suggesting that signalling via TNFR1 is necessary for the expression of neolacto-core gangliosides in the lymph node. This is also the first evidence of the presence of neolacto-series gangliosides in mouse lymph nodes. Only scarce data were available about neolacto-type and related gangliosides in the lymphoid and non-lymphoid mouse tissues.[29,43] Recently, mouse β1,3 *N*-acetylglucosaminyltransferase GlcNAc(β1,3)Gal(β1,4)Glc-ceramide (Lc3) synthase gene has been cloned.[43] This enzyme is the key regulator of lacto-series glycosphingolipid biosynthesis. It is broadly expressed during embryonic development, whereas its expression is postnatally restricted to splenic B lymphocytes, placenta and cerebellar Purkinje cells. Lc3 synthase activity has never been detected in lymphoid cell lines,[44,45] which may reflect the differences in the activation of different GSL biosynthetic pathways or substrate availability between cells in vivo and transformed cell lines in vitro.[46]

Our study also showed that the signalling via TNFR1 influenced globo-series neutral GSL expression in the lymph nodes at the biochemical level. Neutral glycosphingolipid Gb3 is CD77, a marker of follicular centre

B lymphocytes.[19,47] It is also expressed on the human umbilical vein endothelial cells, endothelium of capillaries, venules and veins of the muscle tissue.[48,49] In the lymph nodes from C57BL/6 mice, Gb3 is strongly expressed on cortical vessels and nodular area, and weakly in the cortical area.[37] Binding of CD40 to its ligand induces CD77 expression on B lymphocytes.[50] CD77 is expressed only on the cells sensitive to verotoxin-1, derived from *E. coli*, which specifically recognizes Gb3.[49] In vitro analysis showed that TNF-α increases expression of Gb3 on the human cerebral endothelial cells.[51] Conversion of LacCer to Gb3 is essential for the subsequent production of Gb4 and Gb5,[52] so that reduced expression of Gb4 and Gb5 in TNFR1−/− lymph nodes could be the consequence of low substrate availability for the downstream enzymes. We were not able to demonstrate these differences in flow cytometry, which is most probably related to the methodological differences of detecting GSLs from the whole organ in lipid chromatography and on live cells in flow cytometry.[37] Possible explanations include cross-reactivity with epitopes on glycoproteins and contribution of stromal elements to the GSLs detected in whole organs,[37] as well as predominant localization of certain GSLs in intracellular structures.[53]

TNF can influence the activity of glycosyltransferases in vitro, which subsequently alters cell attachment and migration.[54,55] GSLs have been implicated in the immune cell circulation and/or localization of cells in lymphoid organs.[56] Lymph node microenvironment can also influence cell trapping, such as TNF-dependent monocyte recruitment in inflamed lymph nodes.[57] We can postulate that altered GSL expression due to lack of TNFR1 signalling may influence the morphological compartmentalization of lymphoid organs, resulting in the specific phenotype of TNFR1-knockout mice.[1]

Molecular mechanism responsible for the effects of TNFR1 on GSL expression remains unclear. It may involve the expression of genes encoding the enzymes that catalyse GSL synthesis or the activity of those enzymes by the induction of transferase-modifying proteins. Further analysis of those enzymes on RNA and protein level are needed to elucidate the potential mechanism. Considering the advances in understanding the biosynthesis of GSLs,[9,46] in vivo approach, such as the one used in our study, may be a suitable model for studying the role of GSLs in immune cell functions.

4. Experimental

4.1. Animals

Mice lacking the gene for the tumour necrosis factor receptor p55,[1] were bred onto a C57BL/6 background and kept under standard housing conditions (laboratory rodent chow and water ad libitum and 12 h light–dark cycle) at the Animal Facility of the Rijeka University School of Medicine (Rijeka, Croatia). For the study, 10-week old males were sacrificed by cervical dislocation under CO_2 anaesthesia. Inguinal, axillary and mesenterial lymph nodes were dissected out and stored at −20 °C until GSL extraction.

4.2. Isolation of GSLs from tissues

Lymph nodes were pooled, suspended in distilled water in a 1/2 ratio (w/v), homogenized for 10 min with a dispersing tool (Polytron PT1200C, Kinematica AG, Littau/Luzern, Switzerland), and isolated according to standard procedures.[58] Gangliosides were separated from neutral GSLs by anion exchange chromatography on DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals, Freiburg, Germany) as described previously.[29] After final column chromatography purifications (Iatrobeads column 6RS-8060 for gangliosides, Macherey-Nagel, Düren, Germany; silica gel 60 for neutral GSLs, Merck, Darmstadt, Germany) GSL fractions were adjusted to defined volumes of chloroform/methanol (2:1) corresponding to 2 mg wet weight per microlitre.

4.3. Antibodies

Chicken polyclonal antibodies were used to detect Gg3, Gb3, Gb4, nLc4, and GalNAc–GM1b and a rabbit polyclonal antibody to detect Gg4.[29,30] Chicken antibodies were of the IgY isotype, the equivalent of IgG in mammals. All antibodies were produced and characterized by the laboratory of Dr. J. Müthing.[29–35,37,59–61] Forssman GSL (Gb5) was detected with a supernatant from monoclonal antibody-producing rat–mouse hybridoma.[41] Preimmune chicken or rabbit sera were used as negative controls in all experiments. Secondary alkaline phosphatase-conjugated affinity chromatography-purified rabbit anti-chicken and goat anti-rabbit antibodies were used for HPTLC immunostaining and dichlorotriazinyl-amino fluorescein (DTAF) conjugated antibodies (Dianova, Hamburg, Germany) for flow cytometry. Monoclonal anti-mouse CD19 antibody and monoclonal anti-mouse CD3 antibody, both conjugated with phycoerythrin (PE), were used for double flow cytometry staining (Pharmingen, San Diego, CA). Monoclonal anti-mouse CD4 and anti-mouse CD8 antibodies conjugated with PE, and anti-mouse CD3ε antibody conjugated with allophycocyanin (APC), were used for three-colour flow cytometry staining (Pharmingen).

4.4. HPTLC immunostaining

Glycosphingolipids were separated on silica gel 60 precoated HPTLC plates (size 10 cm × 10 cm, thickness 0.2 mm, Merck, Darmstadt, Germany; Art. No. 5633).

Neutral GSLs were separated in chloroform/methanol/water (120:70:12) and gangliosides in chloroform/methanol/water (120:85:20) containing 2 mM $CaCl_2$ (all ratios are v/v).[59] The overlay technique was performed as described previously.[34,35,61,62] All polyclonal GSL-specific antibodies were diluted 1:1000, except anti-Forssman antibody, which was diluted 1:40. Secondary affinity chromatography-purified alkaline phosphatase labelled rabbit anti-chicken IgG, goat anti-rabbit IgG, rabbit anti-rat IgG antisera (Dianova, Hamburg, Germany) were diluted 1:2000. Bound antibodies were visualized with 0.05% (w/v) 5-bromo-4-chloro-3-indolylphosphate (Biomol, Hamburg, Germany) in glycine buffer. Each antibody analysis was performed twice, with identical results. Before immunooverlay, neutral GSLs were visualized with orcinol and gangliosides by resorcinol staining.[29]

Terminally sialylated ganglioside GM1b with Gg4 backbone and neolacto-series gangliosides with nLc4 backbone were detected after pretreatment with *V. cholerae* neuraminidase, as described previously.[29] Briefly, after separation and fixation of gangliosides, the plate was incubated with 2.5 mU/mL *V. cholerae* neuraminidase (Behring Werke AG, Marburg, Germany) for 2 h at 37 °C in 0.05 M sodium acetate, 9 mM $CaCl_2$ (pH 5.5) and overlayed with anti-Gg4 and anti-nLc4 antibody, for the labelling of GM1b-type and neolacto-core gangliosides, respectively.

We used the same reference GSLs as described previously.[29,63] Ganglioside mixtures from human granulocytes, composed of GM3 (Neu5Ac), IV^3Neu5Ac-nLc4 (IV^3nLc4), IV^6Neu5Ac-nLc4 (IV^6nLc4) and VI^3Neu5Ac-nLc6 (VI^3nLc6), and from murine T lymphoma YAC-1 and lymphoreticular tumour cell line MDAY-D2 composed of GM1b-type gangliosides were used as references. Neutral GSL fractions were prepared from human erythrocytes, sheep red blood cells, and murine YAC-1 cells, containing as major compounds Gb3 and Gb4, Gb5 (Forssman GSL) and Gg3 and Gg4, respectively.

4.5. Flow cytometry

Single cell suspensions of lymph nodes were prepared in ice-cold 0.1 M PBS with 0.1% sodium azide. 10^6 cells were incubated with primary anti-GSL antibodies and/or 1 μg of PE-conjugated antibodies reactive to mouse CD19, CD4 and CD8 and/or PE or APC conjugated antibodies reactive to mouse CD3ε, (Pharmingen) for 30 min on ice. Antibodies were diluted in 0.1 M PBS with 0.1% NaN_3, according to our standard protocol.[36] After two washes in 0.1 M PBS with 0.1% sodium azide, 0.5 μg of secondary DTAF-conjugated, affinity chromatography-purified rabbit anti-chicken IgY and goat anti-rabbit IgG antibodies (Dianova) were added and incubated on ice for 30 min. Finally, cells were resuspended in 1 mL of 0.1 M PBS with 0.1% sodium azide.

Two-colour fluorescence was measured at the excitation wavelength of 496 nm, and three-colour fluorescence at the excitation wavelengths of 496 and 595 nm using a FACSCalibur (Becton-Dickinson, San Jose, CA). Fluorescence was further quantified on the forward scatter/side scatter (FSC/SSC) dot plots. Three-colour fluorescence was quantified on CD3ε positive T lymphocytes, gated according to APC fluorescence. A total of 5×10^5 cells was analysed. Negative control for anti-CD19, anti-CD3, anti-CD4 and anti-CD8 antibodies were non-immune species-matched PE-labelled immunoglobulins. They were used in the same dilution as the primary antibody, followed by the incubation with DTAF-labelled secondary antibody. Nonspecific binding of secondary antibody was excluded by incubating the cells only with the DTAF-labelled secondary antibody.

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