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研究課題名（和文）Impact of the active lipid-transporter Spinster-homoloque-2 on S1P-mediated immune cell dynamics

研究課題名（英文）Impact of the active lipid-transporter Spinster-homologue-2 on S1P mediated immune cell dynamics

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研究成果の概要（和文）：我々は、S1P輸送体Spns2がS1P分泌を介してリンパ球のリンパ系への移動を制御することを明らかにするために、リンパ内皮細胞特異的Spns2のノックアウトマウスを作製した。当該マウスでは、リンパ液内のS1Pの顕著な減少、パイエル板の肥大化、リンパ節の発育不全、リンパ球の末梢リンパ節への移動の減少、高内皮細静脈の形態と大きさの異常、そして樹状細胞が高内皮細静脈の近傍に局在していないことが観察された。これらの結果はS1Pによるリンパ球の移動を制御するSpns2の重要性を示しており、内皮細胞由来のS1Pがリンパ節内の微小な局所空間での免疫細胞の動きを制御する役割をもつことを明らかにした。

研究成果の概要（英文）：In order to reveal the role of the S1P-specific transporter Spinster-homologue-2 (Spns2) in controlling S1P-mediated lymphocyte egress into the lymphatic system we generated lymphatic endothelial cell-specific Spns2-KO mice. We detected a strong reduction of S1P in the lymph of KO mice, leading to the development of hypertrophic Peyer's patches and hypotrophic lymph nodes (LNs). We could show that lymphocyte immigration into pLNs of KO mice in comparison to WT mice was significantly reduced. We detected severe impairment in morphology and size of high-endothelial venules (HEVs). We observed that DCs were absent in close proximity to the HEVs. Impaired micro-anatomical co-localization of DCs and HEVs in LNs was also observed if mice were treated with S1PR-antagonists. These studies reveal the importance of Spns2 to control S1P-driven lymphocyte egress and uncover new insights in the role of endothelial cell-derived S1P in controlling micro-anatomical migration of immune cells in LNs.

研究分野：医歯薬学

キーワード：Spns2　S1P　HEV　Dendritic cells (DCs)　lymphocyte migration　CCL21

１．研究開始当初の背景

The immune system is in a continuous steady state equilibrium of developing cells and effector cells of the lymphoid and myeloid lineages. This highly orchestrated system is based on a spatiotemporal organization of development, selection and specialization of immune cells in lymphoid organs. Although mature lymphocytes acquire immunocompetence during their differentiation and maturation in primary lymphoid organs, they do not show any immune response. Since interaction with antigens is necessary for naïve lymphocytes to finally differentiate into the effector cells that drive immune responses, committed B- and T-cells have to egress the primary lymphoid organs and migrate to the peripheral secondary lymphoid organs where they undergo antigen-driven selection, activation and maturation.

The driving force that mediates egress of lymphocytes from thymus and secondary lymphoid organs was not understood for a long time until the small lipid mediator sphingosine-1-phosphate (S1P) came to light in experiments that reveal an S1P-gradient between luminal and abluminal compartments. Extensive studies have revealed that S1P is a central factor responsible for lymphocyte distribution in the body[1]. In fact, S1P is able to control the integrity of various effector cell populations within many lymphoid tissues by directing lymphocyte egress. There are five specific cell surface G-protein-coupled receptors for S1P, termed S1PR1–5. These receptors have been implicated in a variety of developmental and disease-related processes. For example, the pro-drug FTY720, approved by the FDA for treatment of the autoimmune disease multiple sclerosis[2], is phosphorylated *in vivo* by sphingosine- kinase-2 (SphK2) and induces downregulation and degradation of S1PR1, but not other S1PRs, on lymphocytes. Loss of S1PR1 prevents lymphocytes from sensing the S1P-gradient from the lymphoid organs into the blood, blocking egress and inducing lymphopenia[3]. Furthermore, S1P/S1PR interactions are involved in the orchestration of inflammatory processes, atherosclerosis, osteoporosis and cancer[4]. With the discovery and description of the bio-functional consequences of S1P/S1P-receptor interactions of multiple cell lineages it became evident that many cell lineages produce and secrete S1P in specific environments. As a consequence, particular attention has been focused on the chemoattraction-inducing S1P-gradient.

We were able to characterize the active and specific S1P-transporter spinster-homologue2 (Spns2) in mice that controls S1P-induced lymphocyte egress from primary to secondary lymphoid organs[5]. In fact, we could detect accumulation of egress-competent mature T-cells in the thymus and a strong reduction of recirculating lymphocytes (B and T-cells) in the peripheral lymphoid organs in Spns2-deficient mice. Hence, Spns2 is responsible for S1P-secretion as Spns2-deficient mice show impaired trafficking of mature lymphocyte populations that would normally be controlled by S1P[5], a phenotype comparable to S1PR1-deficient mice[6].

This is further confirmed by the fact that total wildtype bone marrow reconstitution cannot rescue the Spns2-knockout-phenotype, whereas total blood cells from Spns2-deficient animals do not show impaired S1P-secretion *in vitro*. In addition, conditional deletion of Spns2 in endothelial cells (ECs) resulted in a comparable phenotype identifying ECs as important Spns2-dependent S1P-provider[5].

２．研究の目的

In this project we investigate the role of Spns2 on lymphocyte migration in lymph nodes by conditional deletion of Spns2 in lymphatic ECs (Lyve1+) and high endothelial venules (HEVs) by taking advantage of our Spns2-flox mice and Lyve1-CRE mice. With this approach we plan to characterize the unknown function of Spns2 in the regulation of lymphocyte homeostasis in secondary lymphoid organs and the lymphatic system. We intend to get new insights in the regulation of the cellular homeostasis in the lymphatic system in the steady state. With this project we hope to significantly contribute to the characterization of the S1P-transporter Spns2 in mammals. Since S1P is involved in many pathological processes that lead to inflammation and the development of e.g. autoimmune diseases or cancer, we will provide, with our experimental systems, a platform to initiate the search for therapeutic strategies that use Spns2 as a potential target.

３．研究の方法

The following experimental approaches take advantage of Lyve1-CRE/Spns2Δ/Δ mice that specifically lack Spns2 in lymphatic ECs, and, which we were recently able to generate and establish in our laboratory. We performed Immunohistochemistry of lymphocyte pop-ulations and immigration sites, e.g. high endothelial venules (HEVs), of Lyve1-Spns2Δ/Δ and control mice in order to check the integrity of the HEV architecture. Furthermore, we investigated in short-term homing experiments

with wildtype congenic splenocytes, if lymphocyte immigration and egress into and from secondary lymphoid organs was unaffected. Furthermore, we checked if dendritic cell immigration via afferent lymphatics into peripheral lymph nodes (pLNs) is normal in Lyve1-Spns2Δ/Δ by subcutaneous footpad injection of mature in vitro differentiated bone marrow-derived DCs (BMDCs). In addition, we take advantage of the S1PR-antagonists FTY720, W146 and TY52156 in order to monitor the influence the inhibition of S1PR-Gi signaling on DC localization in the LN. We also monitored S1P concentration in blood and lymph by LC-MS/MS and the composition of the commensal bacterial populations by 16s-pyrosequencing of the feces of Lyve1-Spns2Δ/Δ.

４．研究成果

In order to investigate the implications of Spns2-deficiency in Lyve-1 expressing endothelial cells we generated Spns2-deficient Lyve1-Spns2$^{\Delta/\Delta}$ mice by deleting loxP-flanked Spns2 of Spns2$^{f/f}$ mice through intercrossing with Lyve-1 CRE mice. We found normal organ morphology and size of spleen, thymus and mesenteric lymph nodes (mLNs) of Lyve1-Spns2$^{\Delta/\Delta}$ mice, but in comparison to wildtype Spns2$^{f/f}$ mice peripheral lymph nodes (pLNs) are significantly hypotrophic whereas Peyer's patches (PPs) are markedly hypertrophic. Lyve1-specific deletion of Spns2 strongly reduced S1P levels in lymph fluid, but not blood, to 85.3% of those in Spns2$^{f/f}$ mice. Hypotrophic pLNs of Lyve1-Spns2$^{\Delta/\Delta}$ mice show strongly reduced CD4^{+} and CD8^{+} single-positive T-cell and mature recirculating B-cell (CD19^{+}/CD23^{+}/IgD^{+}) populations. In contrast, hypertrophic PPs of Lyve1-Spns2$^{\Delta/\Delta}$ mice have dramatically enlarged CD4^{+} and CD8^{+} single-positive T-cell and mature recirculating B-cell populations. Furthermore, impairment of recirculating lymphocyte populations is detectable throughout varying lymphatic organs in Lyve1-Spns2$^{\Delta/\Delta}$ mice. These experiments suggest that LYVE-1^{+} LECs make a significant contribution to lymph S1P-levels by secreting S1P by Spns2 into the lymphatics and thereby control lymphocyte egress from secondary lymphoid organs into the lymphatic system.

Consistent with the strong reduction of lymph S1P-levels flow cytometric analyses of the lymph collected from the cysterna chyli of Lyve1-Spns2Δ/Δ mice confirmed complete absence of recirculating lymphocytes, indicating severe impairment of lymphocyte-egress into lymphatics from secondary lymphoid organs. These data indicate that S1P-dependent recruitment of lymphocytes from the follicle into the lymphatics of Lyve1-Spns2Δ/Δ mice is impaired and egress of recirculating B- and T-cells from pLNs and PPs into the lymphatic system is strongly reduced. However, the drastic difference in organ volume and lymphocyte homeostasis between pLNs and PPs of Lyve1-Spns2Δ/Δ mice suggests that lymphocyte immigration from the blood into pLNs is already affected. Therefore, to affirm a discrepancy in lymphocyte immigration into secondary lymphoid organs between control and lymphatic Spns2-deficient mice, we tested short-term lymphocyte trafficking to lymphoid tissues by transplantation of wildtype congenic CD45.1+ splenocytes i.v. into Spns2f/f and Lyve1-Spns2Δ/Δ mice. Flow cytometric analyses 2h after injection of cells show that cell immigration into pLNs of Spns2f/f mice is 5.2 fold more efficient than into pLNs of Lyve1-Spns2Δ/Δ mice. Furthermore, appearance of transplanted cells in mLNs of Spns2f/f mice is 1.68 fold higher than in mLNs of Lyve1-Spns2Δ/Δ mice. Because of the established function of specialized high endothelial venules (HEVs) to facilitate chemotactically controlled extravasation of lymphocytes from the blood system into LNs as well as PPs and our observation of reduced cellular immigration into LNs, we sought to determine if Lyve-1 specific ablation of Spns2 had an effect on HEVs. In fact, we observed a strong malformation of PNAd+ HEVs in pLNs of Lyve1-Spns2Δ/Δ mice. Furthermore, MAdCAM-1+ HEVs in mLNs and PPs of Lyve1-Spns2Δ/Δ mice are reduced in size and appeared collapsed. However, the strong hypertrophy and accumulation of recirculating B- and T-cells as well as the equal immigration rates of i.v. transplanted wildtype congenic splenocytes in PPs of Lyve1-Spns2Δ/Δ mice suggest that lymphocyte immigration is not affected but the egress into the intestinal lymphatic sinuses is impaired. In order to functionally address the efficacy of lymphocytes egress from secondary lymphoid organs, splenocytes of congenic eGFP+ splenocytes were adoptively transferred into Spns2f/f and Lyve1-Spns2Δ/Δ recipients. Upon an equilibration period of 48h surface integrins on circulating lymphocytes were saturated with anti-αL and anti-α4 antibodies. Therefore, lymphocyte rolling and lymphocyte immigration was blocked and lymphocyte egress rates could be quantified 20h later. Strikingly, in PPs of Lyve1-Spns2Δ/Δ mice the number of transplanted eGFP+ cells 20h after integrin

blocking in comparison to those in PPs of Spns2f/f was 12.8 fold increased. Furthermore, total mature rec. B-cells were 79 fold elevated in PPs of Lyve1-Spns2Δ/Δ mice in comparison to control recipient mice. In addition, CD4+ and CD8+ single-positive T-cells had left PPs of Spns2f/f mice completely 20h after integrin blocking, whereas a significant amount of cells was still trapped in PPs of Lyve1-Spns2Δ/Δ mice. However, recirculating lymphocyte populations in LNs of Lyve1-Spns2Δ/Δ mice didn't accumulated (mLNs) or were rather decreased (pLNs) possibly because of the impaired integrity of PNAd+ HEVs and the resulting block of lymphocyte immigration. Taken together our data show that the Spns2-dependent contribution of LEC to lymph S1P-levels controls S1P-dependent lymphocyte egress from LNs and PPs into the lymphatic system. Unexpectedly, Spns2-deficiency in Lyve1+ endothelial cells led to severe impairment in function, morphology and size of PNAd+ HEVs. Therefore, reduced immigration of lymphocytes results in the development of hypotrophic LNs, whereas PNAd-/ MAdCAM-1+ HEVs of PPs of Lyve1-Spns2Δ/Δ mice facilitate lymphocyte immigration normally.

The abnormal HEV morphology and function and the concomitant difference in lymphocyte immigration to pLNs and PPs of Lyve1-Spns2$^{\Delta/\Delta}$ mice caught our attention. In order to monitor Lyve1 CRE-mediated gene deletion in HEVs we intercrossed Lyve1 CRE-mice to mice carrying tdTOMATO preceded by a LoxP-flanked transcriptional stop in the Rosa26 locus. Immunofluorescent staining with an anti-PNAd antibody (MECA-79) revealed that PNAd$^+$ HEVs of pLNs simultaneously express tdTOMATO. However, flow cytometric analyses of endothelial cells isolated from pLNs of adult Spns2$^{f/f}$ and Lyve1-Spns2$^{\Delta/\Delta}$ mice didn't show any CD45$^-$/PNAd$^+$/Lyve1$^+$ double-positive endothelial cells. Furthermore, qRT-PCR analyses confirmed the deletion of Spns2 in purified CD45$^-$/PNAd$^+$ single-positive high-endothelial cells and CD45$^-$/Lyve1$^+$ single-positive LECs.

The stimulation of the lymphotoxin-β receptor (LTβr) on HEVs beside the provision of other angiogenic factors by dendritic cells (DCs) has been accounted as determinant responsible for the maturation of adult PNAd$^+$ HEVs. To address the potential role of DCs in the maintenance of HEV architecture and function in pLNs of Lyve1-Spns2$^{\Delta/\Delta}$ mice, we analysed DC homeostasis. We observed that endogenous DCs were in close vicinity to PNAd$^+$ HEVs in pLNs of Spns2$^{f/f}$ mice. In contrast, endogenous DCs were absent in areas of close proximity to PNAd$^+$ HEVs in pLNs of Lyve1-Spns2$^{\Delta/\Delta}$ mice and thus unable to provide cell-bound LTβ to high-endothelial cells. Transmission electron micrographs (TEM) confirmed the drastic morphological changes in atrophic HEVs in pLNs of Lyve1-Spns2$^{\Delta/\Delta}$ mice. The number of high-endothelial cells of a HEV appears to be reduced and the vessel lumen is collapsed in pLNs of Lyve1-Spns2$^{\Delta/\Delta}$ mice when compared to those in pLNs of Spns2$^{f/f}$ mice. Given the specific reduced DC-HEV interactions in pLNs but not mLNs and PPs of Lyve1-Spns2$^{\Delta/\Delta}$ mice as well as the impaired integrity of HEVs, we wondered if the immigration of activated DCs by afferent lymphatics and chemotactic micro-localization around HEVs is affected by LYVE1-specific ablation of Spns2 in HEVs and LECs. In order to characterize the chemotactic micro-localization of DCs around HEVs in pLNs we injected fluorescently labelled mature bone-marrow derived DCs (BMDCs) into the footpad of Spns2$^{fl/fl}$ and Lyve1-Spns2$^{\Delta/\Delta}$ mice and investigated BMDC-migration into pLNs 24h later. BMDCs immigrated to pLNs by afferent lymphatics in frequencies comparable to the controls. Strikingly, total numbers of homed BMDCs were strongly reduced in a restricted area around HEVs within a distance of 40µm from the basal lamina of HEVs in pLNs of Lyve1-Spns2$^{\Delta/\Delta}$ mice when compared to the number of migrated BMDCs in pLNs of Spns2$^{f/f}$ mice. The same effect could be observed when BMDCs were transplanted into WT mice treated with the S1PR antagonist FTY720, W146 but not with TY52156. These data indicate that Spns2-dependent release of S1P from HEVs in pLNs of Lyve1-Spns2$^{\Delta/\Delta}$ mice causes impaired chemotactic micro-localization of activated DCs immigrated by afferent lymphatics around HEVs in contrast to mLNs and PPs, and, in a so far undescribed mechanism. Hence, DCs are unable to provide LTβ to HEVs for proper development and function and therefore lymphocytes are restricted in their immigration to pLNs which causes the hypotrophy. This hypothesis is supported by the partial rescue of HEV morphology in pLNs of Lyve1-Spns2$^{\Delta/\Delta}$ mice observed upon treatment with agonistic anti-LTβr antibody.

Immunohistochemistry revealed S1PR1 expression on PNAd$^+$ HEVs in pLNs of Spns2$^{f/f}$ and Lyve1-Spns2$^{\Delta/\Delta}$ mice. Furthermore, we assayed PNAd$^+$ HEVs in pLN sections of Spns2$^{f/f}$ and Lyve1-Spns2$^{\Delta/\Delta}$ mice for S1P activated S1PR1-G_i signaling represented by its

downstream target phosphorylated Akt (pAkt). $PNAd^{+}$ HEVs in pLNs of $Spns2^{f/f}$ show distinct phosphorylation of Akt contrasted by the reduced levels of pAkt in high-endothelial cells in pLNs of Lyve1-$Spns2^{\Delta/\Delta}$ mice. Hence, a S1PR1-G_i directed signaling pathway on HEVs in pLNs of Lyve1-$Spns2^{\Delta/\Delta}$ mice appears to be defective although the proportion of S1PR1 surface expression is comparable to those on HEVs in pLNs of $Spns2^{f/f}$.

Taken together all these studies reveal the importance of Spns2 to control S1P-driven lymphocyte egress from primary and secondary lymphoid organs. Our findings uncovered and describe a Spns2/S1P-mediated multicellular network that regulates lymphocyte recirculation during immune surveillance with significant implications on HEV integrity and immunity. Since S1P is involved in many pathological processes that lead to inflammation and the development of e.g. autoimmune diseases, we provided with our experimental systems a platform to initiate the search for therapeutic strategies that use Spns2 as a potential target.

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５．主な発表論文等

（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計１件）

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〔学会発表〕（計９件）

08/2016 16th International Congress of Immunology, Melbourne, Australia, Presenter: Simmons S., Title: Implications of Spns2-deficiency on S1P-driven lymphocytes egress, HEV-integrity and immunity. oral presentation

01/2016 Riken Immunology Workshop "Environment Controlling normal and diseased hematopoietic and immune systems", Yokohama, Japan, Presenter: Simmons S., Title: Dendritic cell dynamics and HEV-integrity controlled by lymphatic endothelial cell-derived S1P. Poster presentation

11/2015 The 44th Annual Meeting of the Japanese Society for Immunology, Sapporo, Japan, Presenter: Simmons S., Title: Spinster-homologue-2 (Spns2) controls S1P- driven lymphocyte egress from lymphoid organs. Oral and poster presentation

09/2015 The 3rd European Congress of Immunology, Glasgow, UK, Presenter: Simmons S., Title: Spinster-homologue-2 (Spns2) controls S1P- driven lymphocyte egress from lymphoid organs. Poster presentation

07/2015 Seminar series of the Max Planck Institute for Infection Biology, Berlin, Germany, Presenter: Simmons S., Title: Spinster-homologue-2 (Spns2) controls S1P-driven lymphocyte egress from lymphoid organs. Invited talk

11/2014 International Symposium “Imaging the inner life of cells”, Osaka University, Osaka, Japan, Presenter: Simmons S., Title: Sphingosine-1-Phosphate: a Master Regulator of Osteoclast Precursor Migration, Lymphocyte Egress and Immunity. Invited talk

12/2014 The 43rd Annual Meeting of the Japanese Society for Immunology, Kyoto, Japan, Presenter: Simmons S., Title: Lymphocytes caught before the act–how Spinster-homologue-2 (Spns2) controls S1P-driven lymphocyte egress from lymphoid organs. Oral and poster presentation

09/2014 The 44th Annual Meeting of the German Society for Immunology, Bonn, Germany, Presenter: Simmons S., Title: Lymphocytes caught before the act–how

Spinster-homologue-2 (Spns2) controls S1P-driven lymphocyte egress from lymphoid organs. Oral and poster presentation
01/2014 Gordon Research Conference on Glycolipid & Sphingolipid Biology, Ventura CA United States. Presenter: Simmons S., Title: Spns2 controls S1P-mediated B- and T-lymphoid cell migration from primary to secondary lymphoid tissues and back again. Poster presentation
〔図書〕（計 0 件）
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