

# Integrin $\alpha 9$ on lymphatic endothelial cells regulates lymphocyte egress

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**Sphingosine 1-phosphate (S1P) plays a role in lymphocyte egress from lymphoid organs. However, it remains unclear how S1P production and secretion are regulated. We show that under inflammatory conditions,  $\alpha 9$  integrin, which is closely associated with activated  $\beta 1$  integrin, and its ligand, tenascin-C, colocalize on medullary and cortical sinuses of draining lymph nodes (dLNs), which is a gate for lymphocyte exit, and that inhibition of lymphocyte egress is evident by blockade of  $\alpha 9$  integrin-mediated signaling at dLNs. Based on in vitro analysis using lymphatic endothelial cells obtained from mice embryos, we suggested the possibility that stimulation of lymphatic endothelial cells by tenascin-C enhances S1P secretion in an  $\alpha 9$  integrin-dependent manner without affecting S1P synthesis and/or degradation. Blockade of  $\alpha 9$  integrin-mediated signaling reduced lymphocyte egress from dLNs in several models, including experimental autoimmune encephalomyelitis, where it improved clinical scores and pathology. Therefore, manipulating  $\alpha 9$  integrin function may offer a therapeutic strategy for treating various inflammatory disorders.**

matricellular proteins | lymphocyte trafficking | autoimmune disease

The lymphatic system plays a role in lymphocyte migration and immune surveillance and contributes to various physiological and pathological conditions (1). Mechanisms of T-cell recruitment from peripheral blood into lymphoid organs have been well-characterized (2–4). Naïve T cells migrate from peripheral blood into draining lymph nodes (dLNs) via postcapillary high endothelial venules (HEVs), where T-cell entry to dLNs is dependent on the interaction of integrins  $\alpha 4\beta 1$  and  $\alpha L\beta 2$  on T cells with vascular addressins on HEVs. In the dLN, T cells are activated, differentiate into effector cells, migrate to lymphatic sinuses, and exit the LN. This egress is dependent on a sphingosine 1-phosphate (S1P) gradient (5). S1P is present at high concentrations in plasma and lymph but at low concentrations in LNs (6). Most cells can synthesize S1P; however, erythrocytes lack S1P-degrading enzymes and are the main cellular sources of plasma S1P (6), whereas lymphatic endothelial cells (LECs) are cellular sources of lymph S1P (7, 8). The finding that mice with an LEC-specific deficiency of sphingosine kinases (sphks), which convert sphingosine to S1P, have significantly fewer lymphocytes in lymph and blood (7), suggests that LECs are an important source of lymph S1P. However, the mechanisms regulating S1P production and secretion in LECs during inflammation remain to be elucidated.

Expansion of lymphatic networks under inflammation significantly affects the migration and egress of lymphocytes. Factors, including vascular endothelial growth factor receptor 3 (VEGFR3), lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), transcription factor prox-1, and integrins, regulate lymphatic vessel growth and development (9–13). Among the integrins,  $\alpha 9$  integrin ( $\alpha 9$  hereafter) is critical for the development of lymphatic valves (14, 15). Furthermore, prox-1-induced lymphatic endothelial differentiation is mediated at least in part through  $\alpha 9$  (10). The  $\alpha 9\beta 1$  integrin is also a receptor for the lymphangiogenic factors

VEGF-C and -D (16). However, the role of  $\alpha 9$  in lymphocyte trafficking remains unclear. Therefore, we sought to determine the role of  $\alpha 9$  and its ligands in lymphatic development and lymphocyte trafficking during tissue inflammation. Interestingly, tenascin-C (TN-C) colocalized with  $\alpha 9$  on medullary and cortical sinus-lining LECs of dLNs. Of note, the activated  $\beta 1$  integrin subunit, which forms a heterodimeric receptor with  $\alpha 9$ , was increased in inflamed dLNs. By using embryo-derived LECs, we suggested the possibility that stimulation of  $\alpha 9^+$  LECs with TN-C induced secretion of S1P. Under inflammatory conditions, the involvement of  $\alpha 9$ -mediated signaling in lymphocyte egress from dLNs was evident, whereas that from non-dLNs or noninflamed LNs was not detected. We also found that inhibition of  $\alpha 9$ -mediated signaling in experimental autoimmune encephalomyelitis (EAE) reduced lymphocyte egress from dLNs and attenuated clinical scores and pathology. Thus, inhibition of lymphocyte egress by targeting  $\alpha 9$ -mediated signaling has promise as a potential therapeutic intervention in inflammatory diseases.

## Results

**Expression of  $\alpha 9$  Integrin and Its Ligand, TN-C, During Complete Freund Adjuvant-Induced Lymphangiogenesis.** We first determined the effects of inflammation on expression of  $\alpha 9$ , its ligands, and the characteristics of relevant LNs. Immunohistochemical staining of sections of dLNs revealed the presence and distribution of LECs and  $\alpha 9$ . Medullary and cortical sinuses (MSs and CSs, respectively) were defined by the anatomical location of LYVE-1<sup>+</sup> cells in relation to the CD3<sup>+</sup> T-cell zone and peripheral node

## Significance

The lymphatic system plays critical roles in immune surveillance by providing a route for circulating immune cells.  $\alpha 9$  integrin is a signature molecule of lymphatic endothelial cells (LECs), and has been suggested to have a significant role in lymphatic functions. Here we found that in draining lymph nodes (LNs) of mice treated with complete Freund adjuvant, blockade of  $\alpha 9$  integrin induced deficiency of lymphocytes in medullary and cortical sinuses, which is the characteristic feature of impaired lymphocyte egress from LNs. In addition, we found that stimulation of embryo-derived LECs with the  $\alpha 9$  ligand tenascin-C induced secretion of sphingosine 1-phosphate, which is the critical factor for lymphocyte egress. Thus, we suggest a possible role of  $\alpha 9$  integrin-dependent lymphocyte egress from inflamed LNs.

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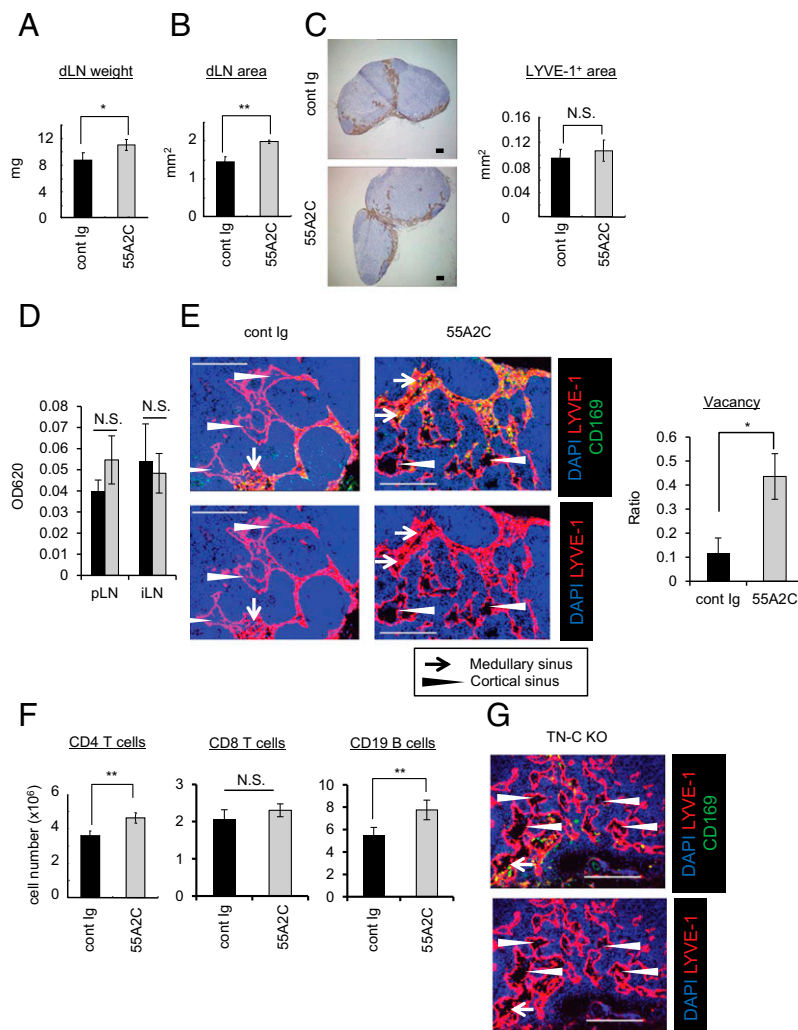
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addressin<sup>+</sup> HEVs (17). In addition, a recent study demonstrated that LYVE-1<sup>+</sup> CSs were connected to the CD169<sup>+</sup> macrophage-rich medulla (17). Therefore, we could distinguish both sinuses by their anatomical location and relation to CD169<sup>+</sup> macrophage (Fig. S1). We analyzed dLNs from mice injected or not with emulsified complete Freund adjuvant (CFA), a protocol that triggers LN expansion and lymphangiogenesis (18, 19). The weight and area of dLNs increased after CFA injection (Fig. S2 A and B, respectively). Areas positive for LYVE-1 (a marker for LECs) as well as those positive for  $\alpha 9$  increased in a time-dependent manner (Fig. S2C, Right). Both LYVE-1<sup>+</sup> MS and CS LECs of naïve and inflamed LNs expressed  $\alpha 9$  (Fig. S2C, Left). Only MSs were associated with CD169<sup>+</sup> macrophage (Fig. S2C, Center). However, the expression level of  $\alpha 9$  on CS LECs was weaker than that of MS LECs. We then examined the expression of two representative ligands of  $\alpha 9$ , TN-C and FN-EDA. These proteins are known to be induced during inflammation (20, 21). TN-C, but not FN-EDA, colocalized with LYVE-1<sup>+</sup> cells in both MSs and CSs of naïve and inflamed dLNs (Fig. S2D). Collectively,  $\alpha 9\beta 1$  integrin and its ligand TN-C were expressed on both MS and CS LECs of naïve and inflamed LNs and suggested that LEC function might be regulated by the interaction between  $\alpha 9\beta 1$  and TN-C.

**Blockade of  $\alpha 9$  Integrin-Mediated Signaling in Inflamed LNs Induces both Vacant Cortical and Medullary Sinuses.** To examine whether  $\alpha 9$  plays a role in LEC function, we treated mice with an  $\alpha 9$ -blocking antibody (55A2C) on the day before CFA administration. Six days

after CFA injection, inguinal LNs, which were dLNs, were removed and analyzed. The weight and area of dLNs were significantly increased in 55A2C-treated mice compared with control (cont) Ig-treated mice (Fig. 1 A and B). As shown in Fig. 1C, the LYVE-1-positive area was similar between 55A2C- and cont Ig-treated mice. In addition, we examined whether 55A2C affected lymphatic flow by injection of Evans blue into a CFA-injected footpad. As shown in Fig. 1D, the accessibility of dye to popliteal and inguinal LNs of 55A2C-treated mice was comparable to that of cont Ig-treated mice, collectively suggesting that blockade of  $\alpha 9$  did not affect CFA-induced lymphangiogenesis and lymphatic function. To determine how 55A2C enhanced CFA-induced dLN enlargement, we analyzed morphologies of LN sections stained for LYVE-1 and CD169. Although MSs and CSs in cont Ig-treated mice contained abundant lymphocytes, those in 55A2C-treated mice were nearly completely devoid of lymphocytes (we call these sinuses “vacant”; compare sinuses indicated by arrows and arrowheads in Fig. 1E). CD4<sup>+</sup> T cells and CD19<sup>+</sup> B cells in dLNs were increased in 55A2C-treated mice compared with cont Ig-treated mice (Fig. 1F). Notably, we found that in mesenteric LNs, which were non-draining lymph nodes, there was no difference in lymphangiogenesis (Fig. S3 A–C), vacancy (Fig. S3D), and cellularity (Fig. S3E) between cont Ig- and 55A2C-treated mice. To test whether the absence of lymphocytes in MSs and CSs involves the  $\alpha 9$  ligand TN-C, we examined the presence of lymphocytes in the MSs and CSs of TN-C-deficient (TN-C KO) mice treated with CFA. Both MSs and CSs were vacant in TN-C KO mice (Fig. 1G).



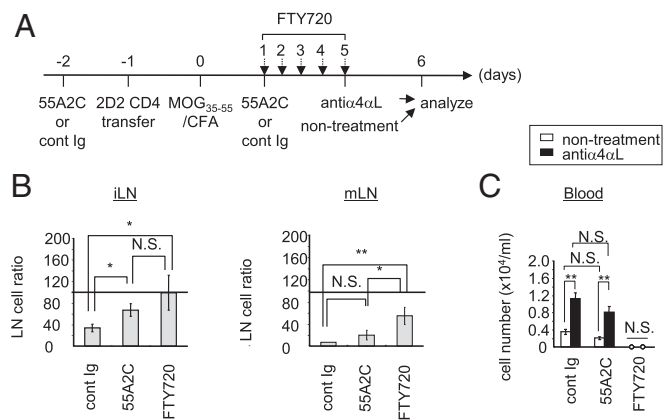
**Fig. 1.** Blockade of  $\alpha 9$  integrin-mediated signaling induced vacant sinuses. (A–C, E, and F) Mice were treated with anti- $\alpha 9$  (55A2C) or control (cont Ig) antibody 1 d before CFA injection. Six days after CFA injection, inguinal LNs (dLNs for the present studies) were analyzed. (A and B) The mean weight (A) and area (B) of dLNs ( $n = 4$  per group) are shown. (C) Draining LN sections stained for LYVE-1. (Scale bars, 100  $\mu\text{m}$ .) Areas of LYVE-1-positive regions were calculated by Scion Image software ( $n = 4$  per group). (D) Effect of 55A2C on lymphatic flow. The graph shows the OD<sub>620</sub> value ( $n = 3$  per group). pLN, popliteal LN. (E) Representative three-color staining of dLN sections with LYVE-1 (red), CD169 (green), and DAPI (blue) ( $n = 4$  per group). (Scale bars, 200  $\mu\text{m}$ .) Vacancy was calculated according to the formula vacant sinus number/total sinus number. (F) Total number of dLN CD4<sup>+</sup>, CD8<sup>+</sup>, and CD19<sup>+</sup> cells per mouse for each group ( $n = 10$ ), determined by FACS. Bars indicate means ( $\pm$ SEM). \* $P < 0.05$  and \*\* $P < 0.01$  denote values significantly different between the indicated pairs. N.S., no significant difference. (G) Representative three-color staining of iLN sections from TN-C KO mice stained with LYVE-1 (red), CD169 (green), and DAPI (blue) ( $n = 4$  per group). TN-C KO mice were injected with emulsified CFA and, at day 6, iLNs were removed and stained with LYVE-1. (Scale bars, 200  $\mu\text{m}$ .) (E and G) Arrows indicate CD169<sup>+</sup> macrophage-associated MSs. Arrowheads indicate CD169<sup>+</sup> macrophage-free CSs. Note that LNs obtained from 55A2C-treated and TN-C KO mice showed vacant MSs and CSs. To clearly demonstrate the presence of vacant sinuses in 55A2C-treated LNs and in LNs from TN-C KO mice, two-color staining pictures with DAPI and LYVE-1 are depicted (Lower).

In combination, these studies demonstrate that the interaction between  $\alpha 9$  and TN-C affects the number of lymphocytes within lymphatic sinuses in the dLN.

**$\alpha 9$  Integrin-Mediated Signaling Is Involved in CD4<sup>+</sup> T-Cell Egress from dLNs.** FTY720, an S1P receptor agonist, inhibited lymphocyte egress from LNs and induced vacant lymphatic sinuses (22). Mice with LEC-specific ablation of sphks have a virtual absence of lymphocytes within lymphatic sinuses (7). We therefore suspected that  $\alpha 9$  on LECs, like S1P, might be important in lymphocyte egress from dLNs. The enhanced accumulation of lymphocytes in dLNs of CFA-treated mice by 55A2C treatment (Fig. 1F) could be due to increased lymphocyte entry and/or impaired lymphocyte egress. To determine whether blockade of  $\alpha 9$  affected lymphocyte entry to LNs, we transferred myelin oligodendrocyte glycoprotein (MOG)<sub>35-55</sub>-specific T-cell receptor (TCR) transgenic (2D2) CD4<sup>+</sup> T cells into recipient mice treated with 55A2C or cont Ig a day before cell transfer. Twenty-four hours later, we determined the number of 2D2 cells, defined as TCR V $\alpha$ 3.2<sup>+</sup> V $\beta$ 11<sup>+</sup>, in LNs. The number of inguinal and mesenteric LNs (iLNs and mLNs, respectively) was similar between the two groups (Fig. S4A), indicating that  $\alpha 9$  was dispensable for lymphocyte entry. We then tested whether blockade of  $\alpha 9$  by 55A2C affects T-cell proliferation in the dLN. Carboxy-fluorescein succinimidyl ester (CFSE)-labeled 2D2 cells were transferred into 55A2C- or cont Ig-treated mice, and mice were immunized with MOG<sub>35-55</sub>/CFA (Fig. S4B). Cell division in dLNs, as indicated by CFSE dilution, was similar in both 55A2C- and cont Ig-treated mice. Thus,  $\alpha 9$  is not involved in antigen-induced T-cell proliferation.

We next examined the effect of  $\alpha 9$  blockade on lymphocyte egress using methods described previously (23). The protocol is outlined in Fig. 2A. 2D2 CD4<sup>+</sup> T cells were transferred into recipient mice treated with either 55A2C or cont Ig on the day before cell transfer. One day later, mice were immunized with MOG<sub>35-55</sub>/CFA. To control for the effect of lymphocyte entry into LNs, for each treatment group we treated a subgroup of mice with anti- $\alpha 4$  and - $\alpha L$  integrin antibodies (anti $\alpha 4\alpha L$  hereafter) on day 6 after cell transfer (23). We also treated a group of mice with FTY720 to serve as a positive control for inhibition of lymphocyte egress from LNs (22, 24). Seven days after cell transfer, we counted 2D2 CD4<sup>+</sup> T cells in LNs from each group. To demonstrate lymphocyte egress, we calculated the ratio using the formula (2D2 CD4<sup>+</sup> cell number in anti $\alpha 4\alpha L$ -treated group/2D2 CD4<sup>+</sup> cell number in nontreated group)  $\times$  100. For the FTY720-treated mice, the ratio was  $98.5 \pm 32.2$  in iLNs (Fig. 2B, Left). In contrast, for cont Ig-treated mice, the ratio was significantly lower ( $33.4 \pm 7.1$ ) (Fig. 2B, Left), demonstrating that blocked lymphocyte entry without inhibition of lymphocyte egress leads to a reduced LN cell number, as shown in Fig. S5A. The ratio was significantly higher in the 55A2C-treated group ( $66.7 \pm 11.7$ ) compared with the cont Ig-treated group but not significantly lower than for the FTY720-treated group (Fig. 2B, Left). Consistent with this analysis, there was no difference between the presence and absence of anti $\alpha 4\alpha L$  in 55A2C- and FTY720-treated mice (Fig. S5A). Thus,  $\alpha 9$  blockade appears to inhibit lymphocyte egress.

Of note, in non-dLNs (mLNs), the cell ratios were low and not significantly different between the cont Ig-treated ( $6.5 \pm 2.0$ ) and 55A2C-treated ( $19.5 \pm 8.2$ ) groups (Fig. 2B, Right), indicating that the reduced LN cell ratio in both groups was mainly due to a low rate of lymphocyte entry into non-dLNs. However, for the FTY720-treated group, the cell ratio in mLNs was significantly higher ( $53.9 \pm 15.2$ ), indicating again that lymphocyte entry, blocked by anti $\alpha 4\alpha L$  treatment, is compensated by blocking lymphocyte egress. Thus, involvement of  $\alpha 9$  in lymphocyte egress is evident in dLNs but not in non-dLNs, whereas the more dramatic effect of FTY720 is apparent in both dLNs and non-dLNs. Nevertheless, absolute cell numbers and/or 2D2:CD4 ratios were higher in dLNs compared with those in mLNs, indicating that lymphocyte entry is higher or expansion of 2D2 cells is more effective in dLNs (Fig. S5 A and B, respectively).

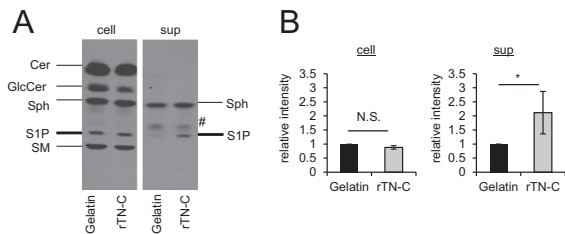


**Fig. 2.** CD4<sup>+</sup> T-cell egress from dLNs was dependent on  $\alpha 9$  integrin. (A) Protocol for analysis of lymphocyte egress. 2D2 CD4<sup>+</sup> T cells were transferred into mice treated, as indicated, with 55A2C or cont Ig and immunized with MOG<sub>35-55</sub>/CFA, as shown. As a control, some mice were treated intraperitoneally with FTY720. Five days after immunization, mice were treated with anti $\alpha 4\alpha L$ . On the following day, iLNs, mLNs, and blood were analyzed. (B) Number of transferred 2D2 CD4<sup>+</sup> T cells in iLNs and mLNs, defined as TCR V $\alpha$ 3.2<sup>+</sup>V $\beta$ 11<sup>+</sup>. The graphs show ratios using the formula (2D2 CD4<sup>+</sup> T-cell number in anti $\alpha 4\alpha L$ -treated group/2D2 CD4<sup>+</sup> T-cell number in nontreated group)  $\times$  100. Bars indicate means ( $\pm$ SEM) [ $n = 11$  per group (cont Ig and 55A2C),  $n = 4$  (FTY720)]. (C) Absolute number of 2D2 cells in the blood. White and black bars represent the absence or presence of anti- $\alpha 4\alpha L$  antibodies, respectively. \* $P < 0.05$  and \*\* $P < 0.01$  denote values significantly different between the indicated groups.

To further analyze the functional difference between  $\alpha 9$ -mediated signaling and FTY720, circulating 2D2 cells were counted in peripheral blood of mice treated with FTY720 or 55A2C and with or without anti $\alpha 4\alpha L$  as described above (Fig. 2C). FTY720 induced almost complete disappearance of blood 2D2 cells due to sequestration within lymphoid organs. In contrast, blood 2D2 T cells in cont Ig-treated mice increased with anti $\alpha 4\alpha L$  treatment (Fig. 2C), demonstrating that lymphocyte egress from LNs was intact. In 55A2C-treated mice, blood 2D2 T cells also increased with anti $\alpha 4\alpha L$  treatment, demonstrating that overall lymphocyte egress was not impaired by blocking  $\alpha 9$ -mediated signaling. These data are consistent with the interpretation that  $\alpha 9$ -mediated lymphocyte egress is evident in dLNs of inflamed tissues.

To confirm this hypothesis, we compared the effects of 55A2C and FTY720 under noninflammatory conditions by monitoring 2D2 T cells transferred to recipient mice, as above, but without subsequent MOG<sub>35-55</sub> immunization (Fig. S6A). Under these conditions, both iLNs and mLNs are noninflamed. Note that absolute cell numbers and 2D2:CD4 ratios were significantly low (Fig. S6B, Top and Bottom, respectively) compared with those in inflamed iLNs (Fig. S5). In both the cont Ig- and 55A2C-treated groups, the cell ratio in iLNs and mLNs was reduced significantly relative to the FTY720-treated group (Fig. S6B, Middle), demonstrating that inhibition of lymphocyte entry in both groups caused the reduced LN cell ratio. Thus, lymphocyte egress from noninflamed LNs is not effectively blocked by 55A2C.

To further understand how 55A2C affects lymphocyte egress, we performed an additional two experimental conditions using mice immunized with either emulsified CFA or MOG peptide. As shown in Fig. S7 A and B, in mice treated with MOG peptide alone, it seemed that MOG immunization did not induce inflammation in LNs because absolute cell number and 2D2:CD4 ratios were significantly low compared with those in Fig. S5 A and B. Inhibition of lymphocyte egress by 55A2C was not evident under this condition, as expected. In mice treated with CFA, unexpectedly, lymphocyte egress was not inhibited by 55A2C treatment (Fig. S7 C and D). However, note again that absolute 2D2 cell numbers and 2D2:CD4 ratios were significantly low compared with those in Fig. S5 A and B. In contrast,



**Fig. 3.** Stimulation of  $\alpha 9$  integrin on LECs induced S1P secretion. S1P release assay. Isolated ED-LECs were stimulated with gelatin or rTN-C (20  $\mu\text{g}/\text{mL}$ ) for 22 h and labeled for 1 h with [ $^3\text{H}$ ]sphingosine. (A) Lipids were extracted from cells and supernatants (sup) and analyzed by TLC. Cer, ceramide; GlcCer, glucosylceramide; SM, sphingomyelin. #, an unidentified lipid. Representative data of three independent chromatographic experiments are shown. Sph detected in the supernatant is an exogenously added Sph. (B) The graphs show the mean ( $\pm\text{SD}$ ) of three independent experiments. Values represent fold changes relative to the gelatin control. \* $P < 0.05$  denotes values significantly different between the indicated groups.

FTY720 inhibits egress of 2D2 cells under the above two different conditions.

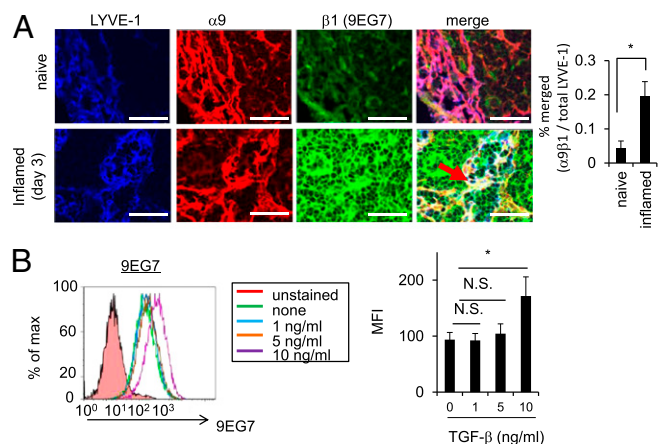
**$\alpha 9$  Integrin-Mediated Signaling Induces S1P Secretion.** To clarify further the basis for regulation of lymphocyte egress by  $\alpha 9$ -mediated signaling, we isolated LECs from embryos to perform in vitro analysis. Embryo-derived LECs (ED-LECs) expressed  $\alpha 9$  integrin and VEGFR3, a signature marker for LECs (Fig. S8A). For these studies, we prepared mutated proteins that specifically interact with  $\alpha 9\beta 1$ . TN-C has several domains, which bind to  $\alpha 9\beta 1$ ,  $\alpha 9\beta 3$ ,  $\alpha 9\beta 6$ , and Toll-like receptor 4 (26). We used the third fibronectin type III repeat domain. This domain includes not only the  $\alpha 9\beta 1$  integrin-binding sequence (AEIDGIEL) but also the  $\alpha 9\beta 3$  and  $\alpha 9\beta 6$  integrin-binding sequence (RGD). Therefore, to avoid RGD-recognizing integrin-mediated binding, we used a sequence in which RGD was mutated to RAA (rTN-C). We confirmed that ED-LECs bound to rTN-C (Fig. S8B). Treatment with 55A2C blocked ED-LEC binding to rTN-C (Fig. S8C), indicating that ED-LECs express functional  $\alpha 9$ . Although LECs expressed  $\alpha 9$  in the LN (Fig. S2C), 55A2C did not affect CFA-induced lymphangiogenesis (Fig. 1C). Consistent with this finding, rTN-C did not induce ED-LEC proliferation in vitro (Fig. S8D), suggesting that the reduced lymphocyte egress with 55A2C treatment was not due to inhibition of lymphangiogenesis. Because S1P is known to regulate lymphocyte egress (27), we tested whether  $\alpha 9$  stimulation induced S1P production. ED-LECs stimulated with  $\alpha 9$  ligands were labeled with [ $^3\text{H}$ ]sphingosine. Exogenously added sphingosine enters the cells and is metabolized to S1P or other sphingolipids. Most of the S1P is rapidly degraded, whereas a portion of the S1P is transported to the extracellular space (28). Lipids extracted from both LEC and culture supernatants were analyzed by TLC. ED-LECs produced cell-associated S1P regardless of  $\alpha 9$  ligand stimulation (Fig. 3A and B). In contrast, only minimal S1P was present in culture supernatants derived from gelatin-stimulated ED-LECs, indicating that S1P was barely secreted without  $\alpha 9$  ligand stimulation (Fig. 3A and B). However, stimulation by rTN-C increased S1P in culture supernatants (Fig. 3A and B). Based on data from in vitro experiments using ED-LECs, there is the possibility that ligation of  $\alpha 9\beta 1$  induces S1P secretion without affecting S1P synthesis and/or degradation.

**The Mechanism of  $\alpha 9$  Integrin-Dependent T-Cell Egress from Draining Lymph Nodes.** We found that blockade of  $\alpha 9$ -inhibited lymphocyte egress is evident in dLNs but not in non-dLNs or LNs of naive mice (Fig. 2 and Fig. S6). Therefore, we examined the mechanism of inflammation-induced and  $\alpha 9$ -dependent lymphocyte egress. At first, we examined the expression level of  $\alpha 9$  on LECs before and after treatment with CFA. As shown in Fig. S9A, LECs were defined as  $\text{CD}45^- \text{CD}31^+ \text{podoplanin}^+$  cells, and blood vascular endothelial cells (BECs) were defined as

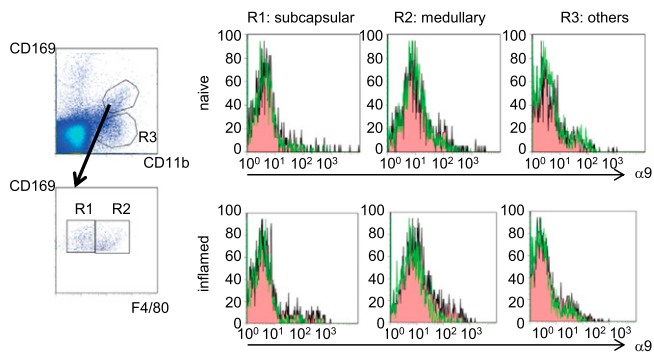
$\text{CD}45^- \text{CD}31^+ \text{podoplanin}^-$  cells. We found that LECs from LNs expressed  $\alpha 9$ , whereas BECs barely expressed  $\alpha 9$  (Fig. S9A). Of note,  $\alpha 9$  expression on LECs did not differ between naive and inflamed conditions (Fig. S9A). Next, we examined expression of TN-C and FN-EDA in the dLN and found that two  $\alpha 9$  integrin ligands were increased in response to CFA (Fig. S9B). However, as shown in Fig. S2D, TN-C was colocalized with LECs even under the naive condition. Therefore, we focused on the activation status of  $\alpha 9$  on LECs during inflammation. The avidity of binding of integrins to their ligand is regulated, at least in part, by the integrin activation state (29). We noted an increased activation state of the  $\beta 1$  integrin subunit, the partner of  $\alpha 9$ , as defined by staining with the activation-specific antibody 9EG7 (30) in lymphatic sinuses of inflamed LNs (Fig. 4A). Cytokines including IL-17 and TNF- $\alpha$  can stimulate endothelial cells to secrete chemokines (31) that can, in turn, induce integrin activation. We performed experiments using ED-LECs and found that among the cytokines we examined, only TGF- $\beta$  induced expression of the 9EG7 activation epitope without affecting the overall expression of  $\alpha 9$  and  $\beta 1$  integrins (Fig. 4B and Fig. S9C and D). Indeed, TGF- $\beta$  expression was up-regulated in the inflamed LN (Fig. S9E).

**Medullary Sinus Macrophages Were Not Involved in  $\alpha 9$ -Mediated Lymphocyte Egress.** LYVE-1 staining in the medullary area was not restricted to LECs but was also detected in MS-associated  $\text{CD}169^+$  macrophages (17). To determine whether medullary macrophages play a role in  $\alpha 9$ -mediated lymphocyte egress, we examine the expression of  $\alpha 9$  on  $\text{CD}169^+$  macrophages. As shown in Fig. 5,  $\text{CD}169^+$  macrophages can be divided by expression of F4/80 (32).  $\text{CD}11b^+ \text{F}4/80^+ \text{CD}169^+$  (R2), which is defined as medullary sinus macrophages, did not express  $\alpha 9$ . In addition, subcapsular sinus macrophages defined as  $\text{CD}11b^+ \text{F}4/80^- \text{CD}169^+$  (R1) barely express  $\alpha 9$ . Other  $\text{CD}11b^+$  cells ( $\text{CD}11b^+ \text{CD}169^-$ ; R3) also do not express  $\alpha 9$ . Furthermore, expression of  $\alpha 9$  did not change during inflammation (Fig. 5, Lower). Thus, it is unlikely that medullary sinus macrophages are involved in  $\alpha 9$ -mediated lymphocyte egress.

**Inhibition of  $\alpha 9$  Integrin-Mediated Signaling Ameliorated EAE.** We asked whether  $\alpha 9$ -mediated regulation of lymphocyte egress plays a role in the pathogenesis of EAE. EAE is induced by infiltration of Th1 and Th17 cells into the central nervous system



**Fig. 4.** Activated  $\beta 1$  integrin was increased on LECs within dLNs. (A) Representative pictures of naive and inflamed LNs stained with LYVE-1 (blue),  $\alpha 9$  (red), and 9EG7 (green), a marker of activated  $\beta 1$  integrin. White indicates the area of colocalization of  $\alpha 9$ , activated  $\beta 1$  integrin, and LYVE-1 (some of a representative area is indicated by an arrow). The graph shows % area of activated  $\beta 1$  integrin associated with  $\alpha 9$  within the total LYVE-1 $^+$  area. (Scale bars, 50  $\mu\text{m}$ .) (B) The induction of activated  $\beta 1$  integrin by TGF- $\beta$  stimulation as described in *S1 Materials and Methods*. The graph shows the mean fluorescence intensity (MFI) of 9EG7 ( $n = 3$  per group).



**Fig. 5.** Expression of  $\alpha 9$  integrin on medullary sinus macrophages. LN cells from naive and inflamed conditions (3 d after CFA immunization) were stained with CD11b, F4/80, CD169, and  $\alpha 9$  integrin. R1 and R2 denote CD169<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>-</sup> subcapsular macrophages and CD169<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> medullary sinus macrophages, respectively. R3 denotes CD11b<sup>+</sup>CD169<sup>-</sup> cells. Fluorescence intensity of  $\alpha 9$  integrin is indicated by green histograms, whereas the isotype control is indicated by pink histograms. Data are representative of six mice.

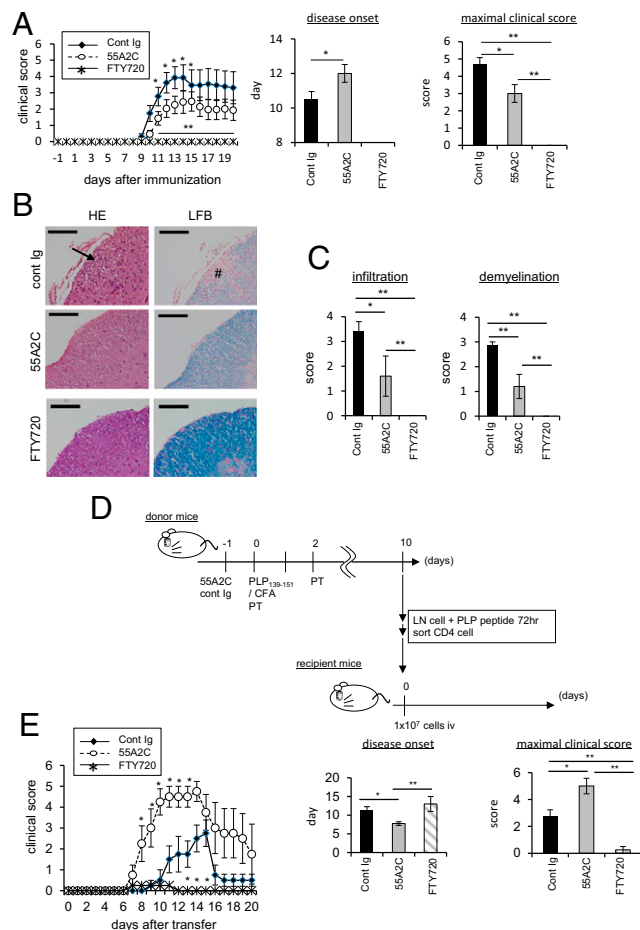
(CNS) (33, 34), and these cells expand in dLNs before being recruited into the CNS. Given the role of  $\alpha 9$  in lymphocyte egress from dLNs, we tested whether blockade of  $\alpha 9$ -mediated signaling in vivo has any therapeutic effect in EAE. Prophylactic treatment with a single injection of 55A2C ameliorated EAE symptoms (Fig. 6A) and significantly reduced cellular infiltration and demyelination (Fig. 6B and C). As reported previously, FTY720 has strong therapeutic effects (Fig. 6A–C). To confirm that 55A2C blockade of  $\alpha 9$ -mediated signaling affected lymphocyte egress in EAE, CD4<sup>+</sup> T cells obtained at day 10 from iLNs of cont Ig-, 55A2C-, or FTY720-treated mice were adoptively transferred into recipient mice (Fig. 6D). Mice that received CD4<sup>+</sup> T cells from 55A2C-treated mice showed earlier EAE onset and more severe clinical symptoms than mice given CD4<sup>+</sup> T cells from cont Ig-treated mice (Fig. 6E). These results are consistent with the notion that there is retention of effector T cells in the iLNs of 55A2C-treated mice. Interestingly, CD4<sup>+</sup> T cells obtained from FTY720-treated mice failed to induce EAE (Fig. 6E), indicating that 55A2C and FTY720 show distinct roles in the pathogenesis of transferred EAE.

## Discussion

In response to inflammation, the lymphatic system undergoes remodeling, namely lymphangiogenesis (Fig. S2). This process is regulated by several factors including integrins (9–13). In this study, we found that  $\alpha 9$ -mediated signaling is dispensable for CFA-induced lymphangiogenesis (Fig. 1C). It was shown that the interaction of FN-EDA with its receptor  $\alpha 9\beta 1$  plays a role in the development of lymphatic valves, although both FN-EDA and TN-C were colocalized in developing lymphatic valves (15). We found that blockade of  $\alpha 9$  did not cause defects of lymphatic flow (Fig. 1D). Thus, in adult mice,  $\alpha 9$ -mediated signaling is also dispensable for lymphatic flow. However, we could not exclude the possibility that 55A2C affected lymphatic microanatomy. We also found that TN-C and  $\alpha 9$  were colocalized in dLNs in the inflammation model of CFA-induced lymphangiogenesis (Fig. S2). Importantly, TN-C was found adjacent to the LECs of MS and CSs of dLNs (Fig. S2D), and both sinuses were involved in lymphocyte egress (23). Of note, the activated  $\beta 1$  integrin subunit, which is the heterodimeric partner of  $\alpha 9$  that forms the  $\alpha 9\beta 1$  integrin, was significantly induced in inflamed LNs (Fig. 4A), suggesting that any interaction of TN-C and  $\alpha 9\beta 1$  occurs in the dLN. In this regard, activation of integrin is critical for its function, and we found that activation of  $\beta 1$  integrin on LECs was induced by TGF- $\beta$  (Fig. 4B), which was up-regulated in inflamed LNs (Fig. S9E). Consistent with this hypothesis, inhibition of  $\alpha 9$  signaling induced vacant sinuses (Fig. 1E) and led to increased CD4<sup>+</sup> T cells and CD19<sup>+</sup> B cells in dLNs in CFA-

induced lymphangiogenesis (Fig. 1F). In addition, we demonstrated that the importance of TN-C is further supported by the finding that sinuses were vacant in dLNs of CFA-injected TN-C KO mice (Fig. 1G). In this regard, the cellular source(s) of TN-C in LNs should be clarified in future studies.

FTY720 induces down-modulation of S1P receptors (22), and ablation of sphks in LECs eliminates S1P synthesis and also impairs lymphocyte egress (7). The data presented in this paper provide evidence that  $\alpha 9$ -mediated signaling also affects lymphocyte egress, albeit in a more localized fashion, by regulating S1P secretion from LECs. The level of S1P production is regulated in part by the balance between synthesis and degradation,



**Fig. 6.** Blockade of  $\alpha 9$  integrin ameliorated the clinical score of EAE and reduced cellular infiltration and demyelination of the spinal cord. (A) Clinical score, disease onset, and maximal clinical score of EAE mice treated with 55A2C, cont Ig, or FTY720. Error bars indicate means ( $\pm$ SEM) [ $n > 13$  per group (cont Ig and 55A2C),  $n = 5$  (FTY720)]. (B) Representative histologies of the spinal cord. On days 11–12 after immunization, spinal cords were collected from cont Ig-, 55A2C-, or FTY720-treated mice and sections were stained with hematoxylin/eosin (HE) (Left) and Luxol fast blue (LFB) (Right). (Scale bars, 100  $\mu$ m.) An arrow indicates inflammatory cell infiltration. #, demyelination of the spinal cord. (C) Statistical evaluation of histology in terms of inflammatory cell infiltration and demyelination in the spinal cord ( $n > 5$  per group). (D) Protocol for the adoptive transfer experiment. Donor mice were treated with 55A2C, cont Ig, or FTY720. On the following day, mice were immunized with proteolipid protein (PLP)<sub>139–151</sub>/CFA. Ten days after immunization, LN cells collected from each group were restimulated with PLP<sub>139–151</sub>. After stimulation, CD4<sup>+</sup> T cells were purified by the MACS procedure (Miltenyi Biotec) and  $1 \times 10^7$  cells were transferred into recipient mice. PT, pertussis toxin. (E) Clinical score, day of disease onset, and maximal clinical score of EAE in recipient mice ( $n = 4$  per group). \* $P < 0.05$  and \*\* $P < 0.01$  denote values significantly different versus cont Ig-treated mice.

mediated by sphks and lyase/phosphatases (35). Our data indicate that interaction of TN-C with  $\alpha 9$  in LECs did not affect S1P synthesis or degradation but induced S1P secretion (Fig. 3), possibly by up-regulation of the S1P transporter. Recent studies demonstrated that spinster homolog 2 (spns2) is critical for S1P secretion from LECs (8, 36). spns2 plays a role in S1P secretion under at least the naïve (nonimmunized) condition (8). Thus, future detailed study is needed for elucidating which S1P transporter is responsible for  $\alpha 9$ -mediated S1P secretion.

Under noninflamed and inflamed conditions, S1P is present at higher concentrations in plasma and lymph than in LNs, and lymphocyte egress from LNs is regulated by S1P under both conditions. As expected, FTY720 inhibited lymphocyte egress under both inflamed and noninflamed conditions (Fig. 2 and Fig. S6). In contrast, in naïve mice, blockade of lymphocyte egress from LNs by 55A2C was not evident (Fig. S6). However, under inflamed conditions, inhibition of  $\alpha 9$ -mediated signaling by 55A2C induced vacant sinus and increased lymphocyte numbers in dLNs (Fig. 1 E and F). These results suggest that under naïve conditions, lymphocyte egress and presumably S1P secretion occur by pathways that are independent of  $\alpha 9$ -mediated signaling. In contrast, under inflamed conditions, lymphocyte egress from dLNs seems to be dependent on  $\alpha 9$ -mediated signaling. TN-C, a ligand for  $\alpha 9$ , is distributed broadly within dLNs and non-dLNs and overlaps with  $\alpha 9$  expression sites (Fig. S2 C and D). Restriction of the role of  $\alpha 9$  on LECs of inflamed LNs might be explained by the presence of the activated form of  $\alpha 9\beta 1$  integrin on LECs under inflamed conditions (Fig. 4). However, unexpectedly, in egress experiments using transferred 2D2 cells, we could not observe any difference between cont Ig- and 55A2C-treated mice under CFA-immunized conditions (Fig. S7D). It should be noted that absolute numbers and 2D2:CD4 ratios were significantly low under the CFA-alone-immunizing condition compared with those under the MOG/CFA-immunized condition (Fig. S5). Thus, these unexpected results may

be due to the limited capacity of LNs that can handle the number of lymphocytes for egress, and thus egress of endogenous lymphocytes seems to be dominant whereas the small number of 2D2 cells does not have the chance for egress. Alternatively,  $\alpha 9$ -dependent 2D2 cell egress might be masked by expanded endogenous CD4 T cells under the CFA-alone condition. It is also possible that another unidentified mechanism operates in  $\alpha 9$ -mediated regulation of lymphocyte egress.

A model for the possible role of  $\alpha 9$  and its ligand TN-C in regulating lymphocyte egress is illustrated in Fig. S10. Our data supporting this model are the following:  $\alpha 9$ , which closely associates with activated  $\beta 1$  integrin, was strongly expressed by sinus-lining LECs of dLNs. Based on in vitro analysis, the possibility that secretion of S1P was induced by the interaction of  $\alpha 9$  with TN-C was suggested. Blockade of  $\alpha 9$ -mediated signaling by 55A2C resulted in inhibition of lymphocyte egress and attenuation of severity of EAE. However, unlike FTY720, blockade of lymphocyte egress by 55A2C was not evident under noninflamed conditions. These data suggest that  $\alpha 9$  might be a target for the treatment of immune-mediated diseases, through regulation of lymphocyte egress from inflamed LNs.

## Materials and Methods

SJL/J and C57BL/6 mice were purchased from Charles River Laboratories and Japan SLC, respectively. MOG-specific TCR transgenic (2D2) mice (37) were purchased from The Jackson Laboratory. TN-C knockout mice were provided by K. Matsumoto (RIKEN BioResource Center, Tsukuba, Ibaraki, Japan). Mice were maintained under pathogen-free conditions in the Laboratory of Animal Experiment for Disease Model (Institute for Genetic Medicine). These animal experiments were performed within a protocol approved by the Hokkaido University Committee for Animal Care and Use. Details of other materials and methods are given in *SI Materials and Methods*.

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