Cosmc controls B cell homing

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The molecular mechanisms regulating lymphocyte homing into lymph nodes are only partly understood. Here, we report that B cell-specific deletion of the X-linked gene, Cosmc, and the consequent decrease of protein O-glycosylation, induces developmental blocks of mouse B cells. After transfer into wild-type recipient, Cosmc-null B cells fail to home to lymph nodes as well as non-lymphoid organs. Enzymatic desialylation of wild-type B cells blocks their migration into lymph nodes, indicating a requirement of sialylated O-glycans for proper trafficking. Mechanistically, Cosmc-deficient B cells have normal rolling and firm arrest on high endothelium venules (HEV), thereby attributing their inefficient trafficking to alterations in the subsequent transendothelial migration step. Finally, Cosmc-null B cells have defective chemokine signaling responses. Our results thus demonstrate that Cosmc and its effects on O-glycosylation are important for controlling B cell homing.
Naïve lymphocytes continuously patrol the body in search of cognate antigens and readily mount immune responses. Thus, constitutive circulation of lymphocytes between blood and lymphoid systems is essential for immune surveillance. Naïve lymphocytes enter lymph nodes through a complex and partly understood process that begins with a series of molecular interactions requiring glycans on endothelial cells of lymph nodes that are recognized by L-selectin on lymphocytes. This recognition by L-selectin of peripheral node addressins (PNAd) on high endothelial venules (HEV) mediates initial tethering and rolling of lymphocytes. Subsequent chemokine signaling through G protein-coupled receptors, e.g. CCR7 and its ligands CCL19 and CCL21, activate lymphocyte integrins, leading to firm arrest, and finally the diapedesis of the adherent cells into the lymph node. It is not clear, however, whether the glycans on the lymphocytes themselves are important in homing.

The potential importance of lymphocyte glycans in homing was suggested by the seminal studies of Gesner and Ginsberg. Using a radioactivity-based assay, their findings suggested that glycosidase treatment of intact lymphocytes decreased their homing to lymph nodes in recipient rats. The nature and functions of the lymphocyte glycoconjugates implicated in this process remain unknown.

Many studies have demonstrated that mucin-type O-glycans, characterized by extended modifications of the core 1 O-glycan structure Galβ1-3GalNAc1-α-Ser/Thr, which are expressed on granulocytes and activated T cells, are important in many aspects of leukocyte trafficking in inflammation, through interactions with selectins. The formation of active O-glycans in normal cells occur by addition of other sugars, including N-acetylgalactosamine, fucose and sialic acid. There is scant information, however, about the roles of lymphocyte glycans in homing, but some studies suggest the possibility that O-glycosylation might be important.

The presence of galactose residues in core 1 O-glycans requires expression of a single enzyme T-synthase, encoded by T-synthase (C1GalT1), which in the Golgi apparatus converts Tn antigen (CD175) to GalNAcα1-3Galβ1-3GalNAc1-α-Ser/Thr, which are expressed on granulocytes and activated T cells, and are important in many aspects of leukocyte trafficking in inflammation, through interactions with selectins. The formation of active O-glycans in normal cells occur by addition of other sugars, including N-acetylgalactosamine, fucose and sialic acid. There is scant information, however, about the roles of lymphocyte glycans in homing, but some studies suggest the possibility that O-glycosylation might be important.

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Altered B cell development in BC-CosmcKO mice. To investigate the role of Cosmc in B cell development, we analyzed the B cell subsets from the BM and the spleen of both wild-type and BC-CosmcKO mice using flow cytometry (Fig. 2). Notably, we found that the major defects were observed in B cell progenitors after the pro-B cell stage (Hardy fraction B), where Mbl-Cre becomes active, in bone marrow of the BC-CosmcKO mice (Fig. 2a, b). We observed >90% reduction of both small pre-B cell (Hardy fraction D) and recirculating mature B cell (Hardy fraction F), and also on the other side we observed a 3.5-fold increase of immature B cells (Hardy fraction E) in the BC-CosmcKO mice. In the spleen, both the IgM+IgD+ and IgM+IgD− B cells were reduced (Fig. 2c). The marginal zone (MZ) B cells were increased >20-fold, with a concomitant reduction of follicular (FO) B cells in the BC-CosmcKO mice (Fig. 2d). Of note, we observed enhanced expression of CD21 on splenic B cells, as well as IgM and IgD on B cells, complicating the demarcation of the B cell subsets using these markers (Fig. 2a–d). The Cosmc-deficient mature B cells were substantially reduced in the bone marrow, compared to those in the spleen, indicating a possibly impaired recirculation of mature B cells back to the bone marrow. Collectively, these data demonstrate that Cosmc mutation in B cells alters their development in both BM and spleen.

BC-CosmcKO mice exhibited reduced B cell numbers in bone marrow and peripheral lymphoid tissues. Paradoxically, BC-CosmcKO mice exhibited increased levels of total IgM in serum, which may reflect the increased numbers of MZ B cells (Fig. 2e). Meanwhile, BC-CosmcKO mice demonstrated reduced serum IgA level, an immunoglobulin isotype that is primarily produced by intestinal B cells. BC-CosmcKO mice had scarce B cells in Peyer’s patches, which likely contributes to the decreased amount of serum IgA. In addition, dynamic changes in IgG isotypes level...
were observed, with increased levels of IgG2b, IgG2c, and a surprisingly marked reduction of IgG3 (Fig. 2e).

Cosmc controls B cell homing to LNs and non-lymphoid organs. We were intrigued by the disproportionate reduction of resident B cells number in the spleen, PLNs, and PPs of the BC-CosmcKO mice as compared to WT. To determine whether homing of B cells into those tissues is affected, we conducted adoptive transfer experiments. We isolated splenic cells from either BC-CosmcKO mice or littermate controls, labeled them with CellTrace violet, and co-injected into WT recipient mice with CFSE-labeled WT splenocytes as an internal control. Mice were sacrificed at 2 or 20 h post-injection, and flow cytometry was used to determine the frequencies of their appearance in PBL, spleen, MLNs, PPs, and PLNs. Remarkably, at 2 h, there were few Cosmc-deficient B cells in LNs and PPs (Fig. 3a), as we observed less than 2% of Cosmc-deficient B cells migrated to PLNs, MLNs, and PPs, as compared to control B cells. After 20 h, the accumulation of Cosmc-deficient B cells remained significantly reduced, with only 3% in PLNs, and less than 1% in both MLNs, and PPs (Fig. 3b), compared to control B cells. We also observed that a negligible number of the Cosmc-deficient B cells had migrated into the lung and liver (Fig. 3c). We obtained similar results when we used BC-CosmcKO mice as recipients (Supplementary Fig. 2A, B). Collectively, these data demonstrate that Cosmc is essential for normal B cell migration to both lymphoid and non-lymphoid organs, in a cell-intrinsic manner.

Glycan profiling of B cells. To gain insights into the nature of glycans on Cosmc-deficient B cells, we first analyzed the released Asn-linked oligosaccharides (N-glycans) from purified WT and Cosmc-deficient B cells using mass spectrometry. Our analysis of the N-glycan profile showed no significant differences compared to WT (Supplementary Fig. 3A, B), indicating that the deletion of Cosmc in B cells does not affect N-glycosylation pathways. In
parallel studies, we also analyzed glycosylation of mouse IgG. IgG N-glycopeptide analysis revealed very similar glycan profiles among all IgG subtypes with minor differences in IgG sialylation (Supplementary Fig. 4A–D). Importantly, we observed that B cells derived from the BC-Cosmc KO mice lacked extended O-glycans in their glycoproteins as compared to WT (Fig. 4a, b). This was confirmed on a protein-specific level in our analysis of the hinge-region of IgG2b. Thr104 (UniProt annotation) was identified to be partially O-glycosylated (Supplementary Fig. 5A, B), which is consistent with a previous report. While IgG2b from WT mice sera expresses mono- and disialylated core 1 O-glycans, the IgG2b from BC-Cosmc KO mice shows exclusively the Tn antigen (Fig. 4c). These results demonstrate that Cosmc deletion does not affect N-glycan structures, but causes the loss of extended O-glycans, resulting in the expression of the Tn antigen on B cells. Also consistent with a previous study, N-glycans from B cells include biantennary complex-type N-glycans capped with the sialic acid Neu5Gc, as well as Neu5Ac (Supplementary Fig. 3A). Notably, after neuraminidase (sialidase) treatment, the binding of PNA, which binds to the core 1 disaccharide Galβ1–3GalNAcβ1–4GaIC, was enhanced on both WT B and T cells, as expected (Supplementary Fig. 6A, B). By contrast, the binding of Malattia amurensis lectin-II (MAL-II), which is specific for α2–3-linked sialic acid on the core 1 disaccharide, as well as the binding of Sambucus nigra agglutinin (SNA), specific for α2–6-linked sialic acids, were decreased in both WT B and T cells (Supplementary Fig. 6A, B). Together, these results demonstrate that glycoproteins of WT murine B cells express extended and sialylated O-glycans, which are lacking on BC-Cosmc-deficient B cells.

Fig. 2 Cosmc is required for B cell development. Single cell suspensions were prepared from bone marrow and spleen of WT and BC-Cosmc KO mice and stained with indicated antibodies. Each symbol (black square and open circle for WT and BC-Cosmc KO, respectively) represents an individual mouse. Representative flow cytometric contour plots and numbers of B cell subsets were shown in (a, b) bone marrow (n = 16 for WT and n = 15 for BC-Cosmc KO mice), in %B cells bar graphs: p values of fraction (a) 0.0003, (b) 0.0032, (c) 0.0177, (d) < 0.0001, (e) < 0.0001, (f) 0.7302, (g) < 0.0001, in #B cells bar graphs: p values of fraction (a) 0.2217, (b) 0.0167, (c) 0.0148, (d) < 0.0001, (e) < 0.0001, (f) < 0.0001, and (c, d) spleen (n = 13 for WT and n = 14 for BC-Cosmc KO mice for c, and n = 11 for WT and n = 10 BC-Cosmc KO mice for d). In %B cells bar graphs of c, p values of IgM+ IgD− = 0.0003, of IgM− IgD+ = 0.5633. In %B cells bar graphs of e, p values of IgM− IgD− < 0.0001, of IgM+ IgD+ = 0.0001. In %B cells bar graphs of d, p values of MZB < 0.0001, of FO < 0.0001. Hardy’s gating schemes were used to measure B cells at different developmental stages (a), with top row gated on B220+ CD43+ cells, and bottom row gated on B220− CD43− cells. Serum from naïve BC-Cosmc KO mice and WT littermate control (for IgM, IgA, and IgG2b, n = 15 for WT and n = 16 for BC-Cosmc KO; for IgG1 and IgG3, n = 15 for both groups; for IgG2c, n = 12 for WT and n = 16 for BC-Cosmc KO) were assessed for indicated immunoglobulin isotypes levels by sandwich ELISA with appropriate immunoglobulin standards. Each symbol represents the datum from an individual mouse. For IgM, p value < 0.0001, for IgA, p value = 0.0003, for IgG1, p value = 0.4629, for IgG2b, p value < 0.0001, for IgG2c, p value < 0.0001, for IgG3, p value < 0.0001. Data are presented as average ± SD of each genotype. Unpaired two-tailed Student’s t tests were performed to determine statistical significance with *** denoting p < 0.001, ** p < 0.01. Source data are provided as a Source Data file.
analyzed by flow cytometry. The homing index was calculated as the [percentage of dye^+ Tn^− or Tn^+ B220^+ (or CD19^+) B cells] / tissue ratio to the input ratio. Data are presented as average ± SD of each genotype with each individual value plotted. Source data are provided as a Source Data file.

Removing sialic acid on lymphocytes blocks migration to LNs. The above results demonstrate that extended O-glycans are required for proper B cell homing to lymph nodes. As O-glycans are frequently capped with sialic acid, we treated lymphocytes with neuraminidase in order to identify whether sialic acids on lymphocytes play a role their homing to lymph nodes (Fig. 4a, c). This treatment, however, is not specific for O-glycans, as it releases sialic acids from both O- and N-glycans, and there is no available neuraminidase that specifically can desialylate O-glycans (Supplementary Fig. 6A, B). We adoptively transferred a preparation of neuraminidase-treated and dye-labeled bulk WT splenocytes into WT recipient mice and examined their distribution within diverse organs using flow cytometry. We observed that in WT recipients, PBS-treated WT cells were distributed as expected into lymph nodes and other organs. However, neuraminidase-treated WT splenocytes accumulated in the liver; strikingly, the majority of these cells were Thy1.2^+ T cells, which were 5-fold increased, compared to untreated T cells (Fig. 4d, Supplementary Fig. 6C). Remarkably, neither neuraminidase-treated B cells nor control B cells were targeted to the liver, as the neuraminidase-treated B cells showed substantially impaired migration to the liver, compared to control B cells (Fig. 4d). Both neuraminidase-treated T and B cells showed significantly reduced accumulation in PLNs, PPs, and lung (Fig. 4e, f, Supplementary Fig. 6D). These results demonstrate that surface sialic acid on lymphocytes is crucial for T and B cell maintenance in the periphery. More importantly, based on this result and those above with BC-CosmcKO mice, we conclude that sialylated O-glycans are required for B cell homing.

Intravital microscopy of Cosmc-deficient B cell homing. To directly assess whether other aspects of B cell homing, such as cell rolling or adhesion to endothelium, which largely is known to depend on L-selectin, might be defective in the Cosmc-deficient B cells, we performed intravital microscopy (IVM) in inguinal lymph nodes. For the five discrete venular orders examined, the rolling and sticking fraction of B cells in HEVs was not affected in BC-CosmcKO mice (Fig. 5a, b, representative videos are shown in Supplementary Movies 1 and 2). Interestingly, when we measured the rolling velocity (V_roll), which reflects cell movement while adhesively contacting the HEV surface, we found that the median V_roll of Cosmc-deficient B cells was increased to 122.4 µm/s in order III HEVs, 68% higher than it was in WT control (Fig. 5c). V_roll has been reported to be sensitive to L-selectin expression level, with higher expression of CD62L resulting in lowered V_roll^12. These data suggest that Cosmc contributes to some extent to the rolling interaction after tethering, but these relatively modest effects are unlikely to contribute to the major defects in homing of Cosmc-deficient B cells.

Based on the above results that Cosmc-deficient B cells lack major defects in rolling and arrest, we considered the possibility that the cells may be defective in terminal steps of transmigration and diapedesis. Lymphocytes enter the lymph nodes through HEVs, which involves a cascade of molecular events between lymphocytes and HEVs^1–3^7. To dissect the molecular mechanisms of the defective migration of Cosmc-deficient B cells, we examined specific factors that might be affected in the cells. Using flow cytometry, we analyzed the expression levels of several known migration-related molecules, such as L-selectin (CD62L), integrin α4β7,28,29 and integrin α4β7 on Cosmc-deficient B cells as compared to WT littermate control (Fig. 5d). We also cytometrically examined the surface expression of several chemokine receptors that are known to contribute to lymphocyte migration^30–32. Notably, the exact chemokine receptor(s) required for B cell homing to lymph nodes at the genetic level are not well understood, and there may be functional redundancy. We observed higher expression levels of CXCR4, CXCR5, CCR7 on Cosmc-deficient B cells as compared to WT (Fig. 5e). It is known that CCR7, a receptor for both CCL19 and CCL21, has several sites of O-glycosylation in its N-terminal domain^33,34. Additionally, a previous study demonstrated that sialylation and O-glycosylation of CCR5 in its extracellular domain is important for normal chemokine binding^35. To explore this further, we immunoprecipitated mouse CCR7 and then blotted with PNA lectin, which binds to core 1 O-glycans. The binding of PNA is consistent with the presence of O-glycans on murine CCR7 (Supplementary Fig. 7).

Thus, we reasoned that chemokine receptors in Cosmc-deficient B cells, lacking sialylated extended O-glycans, might have altered chemotactic responsiveness. To test this possibility, we explored whether Cosmc-deficient B cells exhibited defective chemotaxis in a transwell migration assay. Compared to their WT counterpart, the response of Cosmc-deficient B cells to CXCL13 was normal, whereas it was significantly reduced for CCL21 and
CXCL12 (Fig. 5f). These results demonstrate that Cosmc-deficient B cells have impaired chemokine responsiveness.

Discussion
Multiple factors have been reported in mediating lymphocyte homing to lymphoid organs1–3, yet little is known about the role of O-glycans in B cell homing. Here we present our discovery that the deletion of Cosmc in murine B cells causes a loss of core-1 O-glycans, associated with a marked reduction of B cells in PLNs, PPs, and non-lymphoid organs. We found that the loss of Cosmc in B cells disturbs B cell development, and ablates their homing to PLNs, GALTs, and non-lymphoid organs. Our results demonstrate that the rolling and firm attachment of Cosmc-deficient B cells in the blood venules were comparable to those of WT B cells, suggesting that extended O-glycans on B cells are not required for those steps. However, Cosmc-deficient B cells exhibit defective responses to chemokines in vitro. Together, these data demonstrate that the transmigration of B cells into lymph nodes requires a functional Cosmc and extended O-glycans. The trafficking pattern of Cosmc-deficient B cells phenocopied that of desialylated B cells supports a role for sialylated O-glycans on B cells in normal B cell homing.

We also demonstrated that sialidase treatment of lymphocytes impairs their migration to lymph nodes and PPs, and causes them...
to accumulate in the liver, consistent with earlier studies on the effects of glycosidase treatments of lymphocytes. Strikingly, we found that most of the enriched desialylated lymphocytes in the liver are T cells, with only trace numbers of B cells (Fig. 4). Thus, the loss of sialic acid differentially affects the trafficking of B and T cells to liver, but not other organs.

Here we did not explore in great detail the fate of enzymatically desialylated lymphocytes, as such treatments can affect many types of sialylated glycans beyond O-glycans, and even glycolipid glycans. Others have reported that neuraminidase treatment of platelets led to their clearance by hepatic Kupffer cells, which was promoted by the Ashwell–Moll receptor (AMR) or asialoglycoprotein receptor (36, 37). Thus, it is possible that AMR and/or other unidentified related hepatic molecules may be involved in the active enrichment of desialylated T cells, but not B cells, in the liver. Interestingly, adhesion and/or migration of naïve subset-specific lymphocytes have been reportedly regulated by the expression level of surface L-selectin, distinctive usage of chemokine receptors, and possibly other molecules. Our results indicate that sialylation, as a novel mechanism, contributes to this process. Of note, intravascular staining has established that the vast majority of lymphocytes detected in liver and lung are located in the vasculature, but not in the interstitial tissues. In our study, the livers and lungs were perfused with cold PBS, which will likely flush away most of the transferred cells in the precapillary vessels. The reduced B cells recovered from transfer experiments thus suggest that, in addition to its pivotal role in mediating lymphocyte to lymphoid tissue, sialic acid on B cells may be important for the retention of B cells in the capillary vessels of non-lymphoid organs.

Our results provide novel insights into the roles of B cell O-glycans, which have not been extensively studied previously. The
O-GalNAc O-glycans are critical in mediating neutrophil and activated T cell trafficking4,5, as well as establishment and maintenance of T cell populations in the periphery14. Both Tsyn−/− and C2GnT1−/− neutrophils showed severely impaired recruitment into inflamed peritoneum, likely due to a defect in E-selectin binding2,48. By contrast, upon activation T cells quickly express enzymes generating O-glycans that terminate with the Sialyl Lewis X moiety, which allows the activated T cells to bind to selectins on endothelial cells and eventually promotes T cell extravasation into inflamed tissues15,16. However, there have been only a few studies on the potential roles of lymphocyte-expressed O-glycans in B cell biology. For example, similar percentage and numbers of resident B cells found in the lymphoid tissues of C2GnT1−/− and WT mice indicated that the absence of core-2 O-glycans on naïve lymphocytes does not affect B lymphocyte migration15. Decreased resident lymphocytes in certain PLNs of PPGALNacT1-deficient mice were identified, but that seems attributable to reduced L-selectin ligand levels in the lymph node15.

As global deletion of extended O-glycans causes embryonic death in mice, to achieve B cell-specific deletion of core-1 O-glycans, we crossed Cosmcfl/fl mice with mice expressing transgenic Cre-recombinase under the promoter of the Mbl gene41. This approach efficiently generates BC-CosmcKO mice with a high rate of Cosmc deletion in B cells in the peripheral tissues (Supplementary Fig. 1). We observed a clear trafficking defect as was manifested in the substantially reduced percentage and numbers of resident B cells in lymph nodes and PPs, whereas B cells were mildly reduced in the spleen and bone marrow (Fig. 1). A disruption to homing via Cosmc deletion was confirmed by both short- and long-term adoptive transfer models. To our knowledge, in regard to single gene knockout models, Cosmc represents the only single gene identified to date that fully controls B cell trafficking to PLNs, GALTs, and non-lymphoid organs.

Previous studies have established the critical roles of lymphocyte-expressed L-selectin in initiating rolling26,27, and β7 in forming firm attachment of lymphocytes in HEVs28,29. Lack of either molecule, or blockade of their interaction with receptors on endothelium, led to defective lymphocyte trafficking48−50. In addition, chemokine receptors have also been shown to be important to activate integrin33,34. When multiple chemokine receptors were desensitized, lymphocytes demonstrated impaired arrest and subsequently reduced homing30. We initially speculated that lack of extended O-glycans might lead to downregulation of migration-related molecules. We observed, however, that Cosmc-deficient B cells, possibly through a compensatory mechanism, upregulated their surface expression of L-selectin, integrins, and chemokine receptors (Fig. 5d). This upregulation, however, does not functionally compensate for the deficiency caused by the loss of Cosmc during transmigration. It is interesting to note that as a consequence of Cosmc deletion, the Cosmc-deficient B cells may reprogram in a sense to overcome the extravasation blockade by upregulating L-selectin, which led to the increase of velocity of B cell during initial rolling. It is clear, however, that none of these potentially compensatory effects can salvage the B cell migration defect resulting from Cosmc deletion. In terms of the mechanism for B cell trafficking into lymph nodes, our results demonstrate a key role for sialylated O-glycans in chemokine receptor activity. We recently noted that most chemokine receptors, and especially the subfamily of CC chemokine receptors (CCR), have potential O-glycosylation sites in their extracellular N-terminus, and thus could be functionally impacted by the loss of extended O-glycans51. For CCR7, it was reported that sialylation is important for its functions in promoting CCL19 induced breast cancer cell growth52. In addition, the presence of N-glycans and sialic acid on T cell-expressed CCR7 can modulate its receptor functions53. Our results suggest that sialylated O-glycans of CCR7 are required for its functional activity. It is known that CCR7 is O-glycosylated at several sites in its N-terminal surface domain53,54. The detailed structures of the O-glycans in B cell CCR7 of mouse or human origin remain unknown, but our results suggest that core-1 type O-glycans occur on murine CCR7 (Supplementary Fig. 7). However, we are cautious to interpret the contribution of potential Cosmc-dependent CCR7-ligand interaction to B cell homing because the presence of CCR7 appears dispensable for B cell migration55. But in addition, Cosmc-deficient B cells exhibit impaired response to CXCL12 (CXCR4 ligand), but unaltered response to CXCL13 (CXCR5 ligand). Thus, aspects of known chemokine signaling are clearly impaired in Cosmc-deficient B cells, but the overall functional chemokine signaling pathways required to mediate the activation of sequential integrins is poorly understood and remains to be explored in more detail.

Reflective of that, Cosmc-deficient B cells showed normal attachment to the HEV when measured by IVM. Thus, our results indicate that future studies should explore overall signaling responsiveness in terms of potential O-glycosylation on many migration-related molecules. One such case is human CD99, which is heavily O-glycosylated and has been shown to be pivotal in mediating the diapedesis of monocytes through endothelial junctions53,54. At present, however, the homolog of CD99 in mice has not been identified, but for human B cells it would be informative and worthwhile to investigate the functional contribution of O-glycans to CD99-mediated leukocyte transmigration. Moreover, PNA binding to immunoprecipitated CCR7 (Supplementary Fig. 7) also suggests a potentially important role of O-glycosylation in chemotactic responsiveness of lymphocytes, which contributes to the homing defects in Cosmc-deficient T cells that we observed in our recent study14. So far, no known glycan-binding protein (GBP) or lectin other than L-selectin expressed by lymphocytes has been reported to be important in lymphocyte homing to lymph nodes. Thus, future studies should consider the possibility that loss of sialylated O-glycans in Cosmc-deficient B cells might impair B cell interactions with an as yet unidentified adhesive or signaling GBP that recognizes normal O-glycans. In addition, recent notable technical advances have been made in visualizing how normal lymphocytes undergo transendothelial migration55. Such novel methodology may reveal more details about the behavioral changes of Cosmc-deficient B cells during their migration through HEVs.

In addition, our results indicate a complex regulation of B cell development by Cosmc. BC-CosmcKO mice showed dynamic changes in frequencies and absolute numbers of B lineage progenitors, which suggest that Cosmc is required for the normally progressive development of B cell in the bone marrow. The basis for the upregulation of surface expression of certain B cell subset markers is unclear and further studies are warranted to better understand the roles of Cosmc in regulating B cell development, localization, germinal center B cell response, and other functions. The increase of T cell subsets in the spleen of BC-CosmcKO mice is also interesting, which could be due to unidentified lymphocyte homeostasis mechanisms. Considering B cells play a role in T cell priming22,56, it would be intriguing to examine how the Cosmc-deficient B cells interact with T cells under certain disease settings. Our studies conclusively demonstrate that deletion of Cosmc in B cells alters their development and ablates their ability to migrate to lymph nodes. Particularly, Cosmc-deficient B cells are functional to roll and firmly attach to endothelium, but defective in their transmigration across the HEV barrier into lymph nodes. Future work is warranted to uncover the O-glycan-bearing molecules on B cells and their potential recognition partners within the endothelium. Here we reveal a novel glycosylation-based mechanism for
lymphocytes to access lymph nodes and provide new perspective for understanding lymphocyte trafficking in human health and disease.

Methods

**Mice.** *Cosmc*−/− females were created from our previous work, and crossed with *Mbi-Cre* transgenic male mice (kindly provided by Dr. Michael Reth) to generate B cell-specific *Cosmc* knockout line. B cell-specific *Cosmc* knockout line was co-housed with WT littermate. 

**Total RNA isolation.** Total RNA was isolated from WT and BC-Cosmc KO mice cells using RNAeasy Mini Kit (QIAGEN Ref#74104) was dissolved in RNase-free water. One microgram of total RNA from both groups was used for the synthesis of first strand cDNA using reverse transcriptase (SuperScript III, Invitrogen Ref#18080-044).

**Methods**

**Cell isolation, RT-PCR, and enzyme assays.** B cells from WT and BC-Cosmc KO mice were isolated using B cell isolation kit (Miltenyi Biotec, Cat#130-090-862) with purity over 92% as measured by CD19 positivity by flow cytometry. *Cosmc* gene expression was measured by semi-quantitative RT-PCR. Briefly, total RNA isolated from WT and BC-Cosmc KO B cells using RNAeasy Mini Kit (QIAGEN Ref#74104) was dissolved in RNase-free water. One microgram of total RNA from both groups was used for the synthesis of first strand cDNA using reverse transcriptase (SuperScript III, Invitrogen Ref#18080-044). PCR was performed with Phusion High-Fidelity PCR kit (New England Biolabs) in a 25 μl reaction system with primers (Forward primer 5′-ATGAACTATGTCTGGGACACACA 3′ and Reverse primer 5′-TGTGCTTGTAGGGGCTTGCA-3′). Products were analyzed by electrophoresis on a 1% Tris-acetate EDTA agarose gel.

**For T-synthase and α-Mannosidase activity assays, isolated splenic B cells from both WT and BC-Cosmc KO mice were lysed in Tris-Buffered Saline containing 0.5% Triton X-100 and complete-Mini protease inhibitor (Roche, Ref#1183617001) cocktail on ice. 10 μl cell extract supernatants were added to a final 50 μl reaction system, for T-synthase activity, containing 1 mM GalNAc-α-4-MU, 500 mM UDP-Gal, 20 mM MgCl2, 0.2% Triton X-100, 800 units of *O*-glycosidase, in 50 mM MES-NaOH buffer (pH 6.8), or a 50 μl reaction system, for α-Mannosidase activity, containing 100 mM Man-α-4-MU (0.2% Triton X-100, 20 mM Tris-HCl (pH 7.8), for 45 min at 37 °C in a 96-well black plate. Reactions were stopped by adding 100 μL of 1.0 M glycine-NaOH (pH 10.0) and the relative fluorescence intensity were measured on a Victor Multi-Label Counter (PerkinElmer) using umbelliforene more.

**Antibodies and flow cytometry.** Antibodies were purchased from BD, Biolegend, eBioscience, and listed as follows: CD19, B20, CD26L2, β7, α4β7, CCRX5, CCR7, CCR4, CD4, Ly51 CD24, CD23, IgM, IgG, Thy-1.2-PE, or PerCp, or FITC, or PE-Cy7, or APC-Cy7, or Brilliant Violet 510, or Alexa Fluor-700, or PerCP, legend, eBioscience, and listed as follows: CD19, B220, CD62L, CCR7, CXCR4, CD43, Ly51 CD24, CD23, IgM, IgD, Thy1.2-PE, or PerCp, eBioscience, and listed: IgM, IgG1, IgG2a, IgG2b, IgG3, IgA (Southern Biotech).

**Characterization of B cell glycan.** Approximately 5 million splenic B cells were purified, homogenized and extracted. The cells were next lysed and homogenized prior to incubation with DT4 (1,4-dithiothreitol) and IAA (iodoacetoamide) to denature the proteins. After dialysis to remove the DT and IAA, the proteins were trypsinized (TPCK-trypsinized) and the peptides were purified. The peptides were treated with PNGaseF to remove the N-linked N-glycans and PNGaseF-treated peptides were recovered and purified on C18 column. The N-glycans were then permethylated and analyzed by MALDI-TOF spectrometry.

**Characterization of IgG glycan composition.** IgG was purified from free WT and BC-Cosmc KO mice sera using protein G agarose beads (Roche). Briefly, 50 μl protein G agarose beads were equilibrated with 2 x 500 μl PBS, followed by centrifugation at 2000 g for 30 s and removal of the supernatant. 80 μl of PBS and 10 μl mouse serum were added to the beads and incubated on a shaker for 1 h at RT. The bead solution was transferred to empty tips (Glycen, followed by glycerol solution (50 μl), PBS washing steps and a final elution with 40 μl 0.1 M glycine- HCl pH 2.7. The elution fraction was neutralized with 6.1 μl 1 M Tris-HCl pH 8.6.

**For subsequent SDS-PAGE:** 22 μl 4x non-reducing Laemmli buffer were added for denaturation at 95 °C for 5 min. Each sample was loaded in two lanes (duplicate MS analysis) and the cut bands were used for in-gel tryptic digestion as described elsewhere. The peptide fraction was removed from 50 μl of elution and 50% acetonitrile were added and incubated on the shaker for 10 min at RT. Both supernatants were combined and dried in a speed vac concentrator. The samples were taken into 20 μl water and diluted 3x in 0.1% formic acid.

**Confocal microscopy.** Spleen, lymph nodes, and Peyer’s patches were harvested from mice and frozen in OCT at −80 °C. The frozen tissues were cut at 6-μm thickness. Sections were air-dried, fixed with cold 1:1 methanol/acetic acid (1:1, v/v) for 10 min at −20 °C. After being rinsed 3 times with PBS containing 0.05% Tween 20, tissues were blocked with 10% goat serum for 2 h and then incubated with...
anti-mouse CD19-PE and anti-CD12-FITC overnight. The sections were then counterstained with Hoechst 33342 and mounted using ProLong gold reagent. The slides were then processed with a Zeiss LSM880 confocal microscope and analyzed by ImageJ (Fiji).

Homing assays. The in vivo homing assay was performed as described with modifications. Single cell suspensions were prepared from the spleens of donor mice and labeled with CellTrace Violet according to manufacturer’s protocol. Internal control wild-type spleenocytes were labeled with CFSE. Donor splenocytes (2 × 10^7) and equal numbers of internal control (1–1.2 × 10^7) were intravenously co-injected into recipient mice in a volume of 300 μl of PBS. For some experiments, donor WT and BC-CosmcKO spleenocytes and internal control were co-injected into recipients. An aliquot of the injection mixture was analyzed by flow cytometry for the injected ratio of Violet+ B220+ (or CD19+) Tn+, or Tn+/CFSE+B220+ (or CD19+) cells (Ri). After either 2 or 20 h of migration, single cell suspensions of blood and tissues were prepared and stained with antibodies, and the percentage of CellTrace-Violet and CFSE was determined by flow cytometry. The ratio of Violet+ B220+ (or CD19+) Tn+, or Tn+/CFSE+B220+ (or CD19+) cells within individual organs or blood (Ri) was measured, and the results were presented as the ratio of Ro/Ei in each tissue.

Chemotaxis assay. The responsiveness of splenic B cells to chemokines was examined using 6.5-mm Transwell inserts with a 5-µm pore size (Corning). Fresh single cell suspension from spleen was prepared in complete RPMI1640 and incubated for 30 min at 37°C, and resuspended in RPMI with 0.5 % BSA (10^7 cells/ml). 100 µl suspension was placed to each insert in a well containing 580 µl B220+ or B220− cells/ml). 100 µl suspension was placed to each insert in a well containing 580 µl B220+ or B220− cells/ml). 100 µl suspension was placed to each insert in a well containing 580 µl B220+ or B220− cells/ml). 100 µl suspension was placed to each insert in a well containing 580 µl.

Intravital microscopy. Intravital microscopy of the inguinal lymph nodes was done based on previous studies. Single cell suspensions from spleen were prepared in complete RPMI1640 and incubated for 30 min at 37°C, and resuspended in RPMI with 0.5% BSA (10^7 cells/ml). 100 µl suspension was placed to each insert in a well containing 580 µl of solution of chemokines (R&D Systems) with indicated concentration. Migration was allowed for 4 h at 37°C. Cells migrated to the lower chamber were collected, counted, and analyzed by flow cytometry.

Immunoprecipitation of CCR7 complex and western blotting. CCR7, Abcam (Catalog number: ab32527), Protein A/G Magnetic beads, Thermo Scientific (Product Number: 88802), ECL Anti-mouse IgG HRP linked whole antibody from Sheep (GE Healthcare Cat#NA9131V), biotinylated PNA (Vector Laboratories), and streptavidin (Vector Laboratories) were used in the experiment. The following dilutions of antibodies and lectins were used: CCR7 (1:5000), Biotinylated PNA (diluted to 1 µg/ml), streptavidin at 1:10,000 dilution in TTBS. Lysates were centrifuged at 13,000 × g for 5 min each time. 125I-labeled biotin (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) for 1 h at RT and further incubated with diluted primary antibody overnight with 1x TBS 5% non-fat milk. The membranes were washed 2x with 1x TBS for 5 min each followed by incubation with HRP-conjugated secondary antibody prepared in 1x TBS 5% non-fat milk and the membranes were washed 5x with 1x TBS and developed. Signals were detected by using ECL Prime Western Blotting Detection Reagent. For PNA, PVDF membrane was blocked with 5% BSA for 1 h. Biotinylated PNA was prepared in 1% BSA and secondary streptavidin reagents were prepared in 0.5% BSA.

Statistics. Unless stated otherwise, Group comparisons were analyzed using an unpaired two-tailed unpaired Student’s t test with Prism software.

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