Galectin-9 binds IgM-BCR to regulate B cell signaling

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The galectin family of secreted lectins have emerged as important regulators of immune cell function; however, their role in B-cell responses is poorly understood. Here we identify IgM-BCR as a ligand for galectin-9. Furthermore, we show enhanced BCR microcluster formation and signaling in galectin-9-deficient B cells. Notably, treatment with exogenous recombinant galectin-9 nearly completely abolishes BCR signaling. We investigated the molecular mechanism for galectin-9-mediated inhibition of BCR signaling using super-resolution imaging and single-particle tracking. We show that galectin-9 merges pre-existing nanoclusters of IgM-BCR, immobilizes IgM-BCR, and relocalizes IgM-BCR together with the inhibitory molecules CD45 and CD22. In resting naive cells, we use dual-color super-resolution imaging to demonstrate that galectin-9 mediates the close association of IgM and CD22, and propose that the loss of this association provides a mechanism for enhanced activation of galectin-9-deficient B cells.
cells play a critical role in the immune response and production of protective antibodies. B-cell activation is triggered by binding of antigen to the B-cell receptor (BCR), which initiates a cascade of intracellular signaling through assembly of a multiprotein complex of kinases and adaptors. B-cell activation is accompanied by formation of numerous signaling microclusters. Similar microstructures of antigen receptors have been described in T cells and thus have been proposed to represent the basic unit of lymphocyte signaling. These observations implicate receptor clustering as a mechanism to regulate signaling events, and consequently the cellular outcome of receptor engagement. Indeed, the size and spatial pattern of signaling assemblies significantly contribute to cellular outcomes, with even small variations resulting in altered responses.

Two key parameters influencing the assembly of signaling clusters and regulation of membrane receptor activation are the constitutive nanoscale clustering of membrane proteins referred to as nanoclusters or protein islands, and the cell surface mobility of membrane proteins (or nanoclusters of proteins). These parameters have important implications for receptor triggering and the assembly of signaling complexes as they influence the interaction between protein partners. Several mechanisms have been identified that impact on the organization and mobility of membrane proteins, including the actin cytoskeleton and protein–protein interactions, and membrane microdomains defined by lipid composition.

An often overlooked mechanism controlling membrane protein organization and mobility is the interaction of these cell surface glycoproteins with the family of soluble secreted lectins, known as galectins, which bind and crosslink cell surface proteins, generating glycans-based domains. Indeed, the galectin lattice influences glycoprotein compartmentalization and lateral mobility at the cell surface. These proteins have emerged as important regulators of the immune response. For example, T cells from mice deficient in Mgetc5, which encodes a glycosyltransferase involved in generation of galectin-binding epitopes, have enhanced T-cell receptor (TCR) clustering at the immunological synapse and increased TCR signaling. In comparison to T cells, relatively little is known about galectin–galectin interactions in B cells; however, pre-BCR clustering at the pre-B-cell-stromal cell synapse is dependent on pre-BCR/galectin-1/TCR interactions.

Interestingly, a recent study demonstrated decreased antibody titers upon immunization in mice treated with recombinant galectin-9. Galectin-9 binds to the tandem-repeat subfamily of galectins, which contain two different carbohydrate recognition domains (CRDs) separated by a flexible linker. In T cells, galectin-9 induces cell death of T helper type 1 (Th1) cells through binding to Tim-3, as well as suppresses generation of Th17 cells and promotes induction of Treg. However, the role of galectin-9 in B cells, and the molecular mechanism for decreased antibody production in galectin-9-treated mice has not been investigated.

Here we identify IgM-BCR and CD45 as ligands for galectin-9. Further, we show enhanced BCR microcluster formation and signaling in galectin-9-deficient B cells. Notably, treatment with exogenous recombinant galectin-9 (rGal9) nearly completely abolishes BCR signaling. We investigated the molecular mechanism for galectin-9-mediated inhibition of BCR signaling. We show using super-resolution imaging and single-particle tracking that galectin-9 brings together pre-formed BCR nanoclusters and reduces BCR mobility at the cell surface, and consequently attenuates BCR microcluster formation. We further interrogated the effect of galectin-9 on the organization of cell surface proteins and demonstrate that galectin-9 increases the molecular density of IgM, CD45, CD22, and CD19 within this galectin lattice. Using dual-color direct stochastic optical reconstruction microscopy (dSTORM), we show that galectin-9 mediates close association of IgM and CD22 in resting naive B cells and thus propose a novel role for galectin-9 in regulation of B-cell activation.

**Results**

**Galectin-9 is bound to the surface of primary naive B cells.** To investigate the underlying mechanism for decreased antibody production in galectin-9-treated mice, we initially asked if galectin-9 is bound to the surface of B cells. Primary naive B cells were isolated from C57BL/6 (wild type; WT) and Lgals9−/− (Gal9-KO) mice, stained with a fluorescently labeled antibody specific for galectin-9 and examined by flow cytometry and confocal microscopy. We found that galectin-9 is bound to the surface of WT B cells (Fig. 1a), organized in discrete puncta (Fig. 1b). To investigate the in vivo expression of galectin-9, we immunostained inguinal lymph nodes to identify subcapsular sinus macrophages (CD169), B cells (B220), and galectin-9. We found that galectin-9 was readily detectable within the B-cell follicle (Fig. 1c).

**Galectin-9 regulates BCR microclusters and signaling.** To investigate the effect of galectin-9 deficiency on BCR microcluster formation we employed artificial planar lipid bilayers in which fluorescently labeled anti-BCR antibodies as surrogate antigen are tethered to mimic the in vivo environment of antigen encounter. Naive B cells from WT and Gal9-KO mice were settled on lipid bilayers containing anti-kappa, fixed after 90 s, and visualized by confocal microscopy. WT B cells spread and form multiple BCR-antigen microclusters (Fig. 2a). Galectin-9 KO B cells also spread and form microclusters; however, the area of cell-bilayer contact, and total intensity of antigen is increased (Fig. 2a–c). Moreover, the mean antigen intensity is also increased in Gal9-KO B cells, indicating that the amount of BCR-antigen within individual clusters is increased (Fig. 2d). Given this enhanced microcluster formation, we asked if BCR signaling was altered in Gal9-deficient B cells. Consistent with microcluster data, total tyrosine phosphorylation was increased in Gal9-KO B cells (Fig. 2e). To further investigate this enhanced signaling, we examined phosphorylation of downstream signaling molecules, including CD19, Akt, and extracellular regulated kinase (ERK1/2). Although we did not detect any increase in phospho-CD19 or phospho-Akt, we found that phosphorylation of ERK1/2 was increased in Gal9-KO B cells (Fig. 2f–h). We verified that these differences were not due to altered expression of BCR or CD19 (Supplementary Fig. 1). These data demonstrate that galectin-9 regulates BCR microcluster formation and signaling.

To determine if enhanced microcluster formation and signaling is specifically due to loss of galectin-9 at the cell surface, we titrated the amount of recombinant galectin-9 (rGal9) added to Gal9-KO B cells from 0.1 to 1 μM and then stained cells with anti-galectin-9 antibody and compared this to untreated WT cells. We found that 0.1 μM rGal9 added to Gal9-KO cells was roughly equivalent to the level of endogenous galectin-9 on WT cells (Fig. 3a). We treated Gal9-KO B cells with 0.1 μM rGal9 and then stimulated with anti-IgM and examined ERK phosphorylation as we found this pathway was enhanced in galectin-9-deficient cells. We found that this treatment was sufficient to restore BCR signaling to that observed in WT B cells (Fig. 3b), demonstrating that the effect of deficiency of galectin-9 is indeed due to lack of galectin-9 at the cell surface. Given that galectin family members share the basic glycan ligand, N-acetyllactosamine, we examined an alternative galectin-deficient mouse model. In contrast to Gal9-KO B cells, we found no difference in BCR
microcluster formation or downstream signaling in galectin-1-deficient B cells (Supplementary Fig. 2). These data, while not exhaustive of the galectin family, provide strong evidence for specificity of galectin-9 in regulation of these early events of B-cell activation, consistent with studies demonstrating that structural differences in the carbohydrate recognition domain (CRD) of galectin-1 and galectin-9 define specific interactions with cell surface glycoproteins.

We noted that ligands for galectin-9 at the cell surface were not saturated in primary naive B cells and we could add 10-fold more rGal9 to both WT and Gal9-KO B cells (Fig. 3a). Thus, we asked what happens to BCR signaling if we increase the amount of galectin-9 bound to the surface. We observed a substantial reduction in total tyrosine phosphorylation, as well as phosphorylation of CD19, Akt, and ERK upon pre-treatment of WT cells with rGal9 (Fig. 3c–f). These differences were not due to altered expression of BCR, CD19, or CD45 by rGal9 (Supplementary Fig. 3), nor galectin-9-induced cell death (Supplementary Fig. 4), as has been reported for thymocytes and T cells.

**Galectin-9 binds IgM-BCR and CD45.** Our data demonstrate an important role for galectin-9 in regulating BCR signaling; however, the cell surface glycoproteins that bind galectin-9 in B cells have not been identified. To identify potential ligands we performed a pull-down assay using rGal9 under stringent cell lysis conditions to disrupt protein–protein interactions followed by western blotting (Fig. 4b). Consistent with our mass spectrometry results, we identified CD45 and IgM-BCR as potential ligands of galectin-9. To verify that these were indeed direct ligands of galectin-9, we performed far-western analysis using FLAG-tagged rGal9 as bait protein. We detected galectin-9-binding proteins corresponding to the size of CD45 and IgM in reference blots (Fig. 4c) in primary murine B cells. Importantly, a band corresponding to the size of IgM was not detected in far-western using cell lysates from the A20 B-cell line that does not express endogenous IgM. Conversely, a band corresponding to the size of IgM was detected in lysates of A20 B cells expressing hen egg lysozyme (Hel)-specific IgM (D1.3). Neither CD45 (B220) nor IgM bands were detected when Jurkat T cells were used. These data provide strong evidence that galectin-9 binds IgM-BCR and CD45.

**Galectin-9 alters IgM-BCR nanoclusters.** Identification of IgM-BCR as a ligand of galectin-9, and the potential of galectins to act as cell surface scaffolding proteins led us to ask whether galectin-9 has a role in organizing IgM-BCR nanoclusters. To investigate this, we employed dSTORM, which permits a lateral resolution of approximately 20 nm, and has been used to study the organization of IgM, IgD, and IgG BCR. Primary WT and Gal9-KO B cells were labeled with Alexa Fluor 647-conjugated anti-IgM Fab fragment, settled on non-BCR-stimulating anti-major histocompatibility complex (MHC) II-coated coverslips to adhere cells, then fixed and imaged by total internal reflection fluorescence microscopy (TIRFM). IgM-BCR are organized into nanoscale clusters in WT B cells. To quantify clustering tendency of IgM-BCR, we used two methods commonly used to assess distribution of proteins from super-resolution images. The
Galectin-9 regulates BCR microcluster formation and signaling. a Representative images of primary naive WT (top) and Gal9-KO B cells (bottom) fixed on bilayers containing anti-kappa as surrogate antigen (Ag) after 90 s of spreading and imaged by confocal microscopy. Brightfield (left) and confocal (right) visualizing antigen mapped to an 8-bit fire color scale (ImageJ). Scale bar 2 μm. Quantification of b area of spreading, c total antigen fluorescence intensity at the cell-bilayer contact, and d mean intensity of antigen for WT (black circles) and Gal9-KO (blue diamonds) cells (each dot represents 1 cell, 200 cells measured per condition), with the mean ± SEM indicated by the red bar. ***p < 0.0001, Mann-Whitney test. Data representative of at least three independent experiments. e-h Primary naive B cells from WT and Gal9-KO mice were settled onto anti-IgM-coated plates for the indicated time. Cells were lysed and subjected to SDS-PAGE followed by immunoblotting with e anti-phosphotyrosine and anti-ERK1/2, f anti-phospho-CD19, g anti-phospho-Akt, and h anti-phospho-ERK1/2 (pERK) and anti-β-tubulin. Data representative of at least three independent experiments. f Quantification of the fold increase in pCD19, pAkt, and pERK, with the mean ± SEM indicated by bar. Data were analyzed by two-way ANOVA, followed by Sidak’s multiple comparisons test; *p < 0.05, **p < 0.01.
Galectin-9 immobilizes IgM-BCR. The mobility of IgM-BCR is a critical factor regulating BCR signaling. Given dSTORM analysis showed galectin-9 increased cluster size, we wondered whether altered BCR mobility may account for suppressed BCR signaling upon treatment with rGal9. To investigate the effect of galectin-9 on IgM mobility in the steady state, we labeled single particles of IgM on WT and Gal9-KO B cells with Atto-633-conjugated anti-IgM Fab fragments. Cells were settled on anti-MHC II-coated coverslips and single molecules of IgM were visualized using TIRFM. The median diffusion coefficient of IgM-BCR on Gal9-KO B cells was approximately 30% higher than that on WT B cells (0.037 μm²/s compared to 0.028 μm²/s) (Fig. 6a), consistent with a decrease in the relative frequency of slow-moving IgM-BCR in Gal9-KO B cells (Fig. 6b). To examine the mobility of IgM-BCR inside the galectin-9 lattice, we treated Gal9-KO B cells with fluorescently labeled rGal9 and visualized and tracked IgM-BCR. We defined regions of high and low galectin-9 using the fluorescence intensity of galectin-9 and then calculated diffusion coefficient in these regions (Fig. 6c). The median diffusion coefficient of IgM in galectin-9-high regions was reduced twofold compared to galectin-9-low regions (0.012 μm²/s compared to 0.028 μm²/s) (Fig. 6d). Approximately 65% of tracked IgM molecules are largely immobilized inside the mask compared to 45% of tracked IgM molecules outside the mask (Fig. 6e). This finding provides direct evidence for galectin-9 to restrict the mobility of IgM molecules on the B-cell membrane.

Galectin-9 increases the density of IgM-BCR and co-receptors. We hypothesized that galectin-9-mediated immobilization of IgM-BCR would reduce BCR localization to the contact with an antigen-containing membrane. To test this, we settled WT B cells or cells pre-treated with rGal9 on planar lipid bilayers containing anti-kappa as surrogate antigen for 90 s and then fixed and visualized by confocal microscopy. We found that BCR-antigen aggregation is reduced in cells treated with rGal9 (Fig. 6f, g). These findings suggest that galectin-9 binds IgM-BCR inducing aggregation and immobilization of IgM nanoclusters, which consequently affects the formation of signaling microclusters upon BCR stimulation.

**Fig. 3** Treatment with exogenous galectin-9 suppresses BCR signaling. a Representative flow cytometric histograms of WT (left) and Gal9-KO (middle) B cells either untreated or treated with various concentrations of recombinant galectin-9 (rGal9; 0.1, 0.2, 0.5, and 1 μM) followed by surface staining for galectin-9 and analyzed using flow cytometry. Overlay of endogenous galectin-9 surface expression in WT cells, and Gal9-KO cells treated with 0.1 μM rGal9 (right). b Naïve B cells from WT and Gal9-KO mice treated with 0.1 μM rGal9 were settled onto anti-IgM-coated plates for the indicated time. Cells were lysed and subjected to SDS-PAGE followed by immunoblotting with anti-phospho ERK1/2 and anti-β-tubulin (left panel). Quantification of the fold change in pERK over time, averaged over two independent experiments with the mean ± SEM indicated by the bar (right panel). c-f Naïve B cells from WT mice were treated with 1 μM rGal9 and settled onto anti-IgM-coated plates for the indicated time. Cells were lysed and subjected to SDS-PAGE followed by immunoblotting with c anti-phosphotyrosine and ERK1/2, d anti-phospho-CD19, e anti-phospho-Akt, and f anti-phospho ERK1/2 and anti-β-tubulin. Quantification of the fold change in pCD19, pAkt, and pERK over time, averaged over three independent experiments, with the mean ± SEM indicated by the bar is shown in the right panel. Statistical significance measure by two-way ANOVA followed by Sidak’s multiple comparisons test; **p < 0.0001, *p < 0.01, *p < 0.05.
calcium upon addition of rGal9 (Supplementary Fig. 5). This finding raises the question of why treatment with rGal9, which clusters BCR, does not itself induce B-cell activation. Our data also identified CD45, the most abundant phosphatase on the B-cell surface, as a ligand of galectin-9 (Fig. 4). CD45 is a member of the protein tyrosine phosphatase family and plays an important role in both positive and negative regulation of B-cell activation through dephosphorylation of the activating and inhibitory sites of the Src family kinase Lyn. This dual regulation of BCR signaling by CD45 is dependent on the localization of CD45, as CD45 constitutively dephosphorylates and inactivates Lyn within glycosphingolipid-enriched domains (GEMs), and BCR ligation induces sequestration of CD45 from GEMs. We predicted that galectin-9 modifies the spatial organization of IgM and CD45 to attenuate signaling. To visualize the effect of galectin-9 on organization of CD45 relative to IgM-BCR, we treated primary WT B cells with 1 μM rGal9 and then fixed and immunostained using anti-CD45, anti-IgM, and anti-galectin-9 antibodies, and imaged by confocal microscopy. Interestingly, rGal9 often forms a cap at one side of the cell, consistent with the ability of galectin to form a lattice-like structure through galectin-glycoprotein interactions (Fig. 7a). We also observed that within the galectin-9 cap, the fluorescence intensity of CD45 and IgM is increased (Fig. 7a, b). To quantify this observation, we developed a protocol to define galectin-9-high regions (Gal9high) and galectin-9-low regions (Gal9low) (Fig. 7c) and found that the mean fluorescence intensity of both CD45 and IgM is significantly higher in Gal9high regions compared to Gal9low regions (Fig. 7d).

Reorganization of IgM and CD45 is not due to a global change in membrane structure, as the intensity of IgD, an isotype of BCR that is expressed 10-fold higher than IgM, was not increased within the galectin-9 lattice (Supplementary Fig. 6a–c).

One of the best characterized inhibitory co-receptors expressed on B cells is CD22. The extracellular domain of CD22 is also glycosylated and contains a sialic acid-binding domain, which binds α,β-linked sialic acids and mediates homotypic interactions between CD22 molecules and heterotypic interactions with other glycosylated proteins, including CD45 and IgM. Thus, we investigated if rGal9 also alters organization of CD22. We observed clusters of CD22 with higher fluorescence intensity inside the galectin-9 cap, which colocalized with IgM clusters (Fig. 7e, f). Consistent with this, the mean fluorescence intensity of CD22 is increased inside Gal9high regions (Fig. 7g). These findings suggest that rGal9 increases the density of CD22, coincident with the enrichment of IgM, within the galectin-9 lattice. Again, to confirm the specificity of CD22 enrichment within the galectin-9 lattice, we examined localization of inhibitory receptor FcγRIB, upon treatment with rGal9, and found no enrichment within Gal9high regions (Supplementary Fig. 6d–f).

Our findings suggest that galectin-9 enhances the interaction between IgM and CD22 and may provide a mechanistic basis for...
the inhibitory effect of galectin-9 on B-cell activation. The intracellular domain of CD23 contains three immunoreceptor tyrosine-based inhibitory motifs that are phosphorylated upon BCR ligation and recruit other phosphatases such as Src homology region 2 domain-containing phosphatase-1 (SHP-1), which dephosphorylates Syk, PLCγ2, and CD19 to dampen BCR signaling. Given our finding of reduced phosphorylation of CD19 in BCR stimulated cells pre-treated with rGal9 (Fig. 3d), we examined if CD19 is also localized within the galectin-9 lattice, and found that CD19 is indeed enriched within Gal9-rich regions (Fig. 7h–j). These findings suggest that galectin-9 reorganizes IgM-BCR and its co-receptor CD19 together with inhibitory co-receptors CD45 and CD22 to suppress BCR signaling.

GEMs (or lipid rafts) play an important role in initiation of BCR signaling, and the localization of proteins within these domains have important consequences for activation of signaling. We found that when B cells are treated with rGal9, galectin-9 often forms a cap on one side of the cell, reminiscent of the polarization of lipid rafts upon lymphocyte activation. Thus, we asked whether galectin-9 alters the distribution of lipid rafts and association of CD45 and CD22 with these domains. We treated primary B cells with rGal9 and then labeled lipid rafts and association of CD45 and CD22 with these domains. Thus, we asked whether galectin-9 alters the distribution of lipid rafts and association of CD45 and CD22 with these domains. We observed a similar distribution of correlation coefficients and nearest-neighbor distance in Gal9-KO B cells (Fig. 9f, g), suggesting that galectin-9 does not influence the association of IgM and CD45 to a significant degree in the steady state.

In contrast to CD45, dual-color dSTORM of IgM and CD22 showed a larger degree of colocalization based on visual inspection of dSTORM images (Fig. 9h). Consistent with previous reports, CD22 is highly clustered on the surface of resting primary naive B cells. We noted a small but statistically insignificant difference in both the mean Hopkins index and the H function curve, with CD22 appearing slightly less clustered in Gal9-KO B cells compared to WT cells (Fig. 9i, j). However, we noted no difference in mean cluster diameter or cluster area of CD22 between WT and Gal9-KO B cells (Fig. 9k, l). CBC analysis of WT cells revealed a distribution histogram with two peaks, one approaching −1 and one approaching +1, indicating some degree of colocalization between IgM and CD45 in the resting state; however, the median CBC value for IgM and CD45 was 0.018 indicating that across the population of molecules the distribution is largely uncorrelated (Fig. 9f). We observed a similar distribution of correlation coefficients and nearest-neighbor distance in Gal9-KO B cells (Fig. 9f, g), suggesting that galectin-9 does not influence the association of IgM and CD45 to a significant degree in the steady state.
Galectin-9 alters IgM-BCR nanoclusters. **Fig. 5**

**a** TIRFM image of surface IgM and fluorescently labeled rGal9 before bleaching for image acquisition (two left panels respectively). dSTORM images reconstructed from single-molecule localization processed by Thunderstorm software mapped to a fire color scale as indicated; the magnified region (3 × 3 µm) from ROI (white box) is shown as 2D image (middle) and 3D surface plot (right) in the order of WT (top), Gal9-KO (middle), and Gal9-KO + 1 µM rGal9 (bottom). Scale bar represents 2 µm.

**b** Quantification of the distribution of IgM by $H$ function and **c** Hopkins index of localizations inside ROIs. **d** Number of clusters (one point per ROI). **e** Cluster radii (one point per cluster). **f** Number of molecules (one point per cluster). **g** Percentage of localization in clusters (one point per ROI). Each category contains at least 15 ROIs from three independent experiments (at least four cells per experiment). Statistical analysis was performed using Kruskal-Wallis test with Dunn’s multiple comparison test (d, e, f) and one-way ANOVA with Tukey’s multiple comparison test (b, g). Red bars indicate mean ± SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$
These findings suggest that galectin-9 mediates close association between IgM and CD22, at least to some extent, in the steady state.

Discussion

Here we identify galectin-9 as a negative regulator of B-cell activation. We found that BCR microcluster formation and signaling are enhanced in galectin-9-deficient B cells. Importantly, we show that the ligands of galectin-9 at the cell surface are not saturated in freshly isolated naive B cells and treatment with rGal9 nearly abolishes BCR signaling. Using a pull-down assay and mass spectrometry we identified IgM and CD45 as ligands of galectin-9 in primary B cells. We further show that galectin-9 plays a role in regulating the spatial organization and dynamics of IgM-BCR and its localization with regulatory surface proteins. We propose that galectin-9 organizes IgM-BCR into larger clusters that restrict the mobility of IgM and relocates inhibitory molecules, including CD45 and CD22 to directly inhibit BCR signaling (Fig. 10).

The spatial organization of cell surface proteins in constitutive nanoscale clusters has emerged as a general principle of cell surface proteins. However, the molecular mechanism regulating the size, composition, and stability of these assemblies is an open question. The formation and stability of IgM-BCR nanoclusters is not dependent on the actin cortex, as depolymerization of actin does not alter the size or density of IgM-BCR nanoclusters. Thus, we hypothesized that the galectin-9 lattice may play a role in the nanoscale organization of IgM-BCR. In contrast, super-resolution dSTORM revealed no difference in the size or density of IgM-BCR nanoclusters in galectin-9-deficient B cells. This is consistent with a recent study demonstrating that glycan-based interactions do not mediate the nanoclustering of dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin. It may be that formation of BCR nanoclusters (or oligomers) is largely dependent on the direct interaction of BCR components, as suggested by Reth and colleagues. However, we do find that addition of rGal9 induces significant changes in IgM-BCR organization. Inside the galectin-9 lattice, the number of IgM-BCR nanoclusters decreased while the radius of clusters, and the number of molecules in each cluster, increased. Thus, we propose that galectin-9 provides a second layer of organization to IgM-BCR by merging pre-existing nanoclusters.

But how does this observation square with our finding that rGal9 suppresses BCR signaling? Indeed, these observations are somewhat counter-intuitive given that BCR stimulation induces the coalescence of BCR nanoclusters to form larger-scale signaling microclusters. So, how does the larger cluster of IgM induced by galectin-9 not activate B cells, but instead inhibit BCR signaling? We propose two potential mechanisms for galectin-9-mediated control of BCR signaling. First, we propose that galectin-9 induced merging of pre-existing nanoclusters decreases...
**Fig. 7** The galectin-9 lattice increases the molecular density of IgM-BCR and co-receptors. 

a Representative confocal images of primary WT B cells treated with 1 µM rGal9 and immuno-stained for CD45 (cyan), IgM (magenta), and galectin-9 (Gal9; yellow). 

b Fluorescence intensity profile of CD45, IgM, and Gal9 along the cell membrane. 

c Representative example of masking output of algorithm to detect regions of high galectin-9 (Gal9<sup>high</sup>) and low galectin-9 (Gal9<sup>low</sup>). 

d Mean fluorescence intensity of CD45 (left) and IgM (right) in Gal9<sup>high</sup> and Gal9<sup>low</sup> regions. 

e Representative confocal images of WT B cells treated with 1 µM rGal9 and immunostained for CD22 (cyan), IgM (magenta), and Gal9 (yellow). 

f Fluorescence intensity profile of CD22, IgM, and Gal9 along the cell membrane. 

g Mean fluorescence intensity of CD22 (left) and IgM (right) in Gal9<sup>high</sup> and Gal9<sup>low</sup> regions. 

h Representative confocal images of WT B cells treated with 1 µM rGal9 and immunostained for CD19, IgM, and Gal9. 

i Fluorescence intensity profile of CD19, IgM, and Gal9 along the cell membrane. 

j Mean fluorescence intensity of CD19 (left) and IgM (right) in Gal9<sup>low</sup> and Gal9<sup>high</sup> regions. 

Data representative of at least three independent experiments. Each dot represents 1 cell, at least 30 cells measured per condition per experiment. Mean ± SEM indicated by the red bar. Statistical significance assessed by Mann-Whitney; ****p < 0.0001 ***p < 0.001, *p < 0.05. Scale bar 2 µm.
the lateral diffusion of IgM-BCR and consequently suppresses BCR signaling. The mobility of IgM-BCR on the cell membrane can be described as Brownian motion restricted by the underlying actin cytoskeleton. The theory of Brownian motion postulates that the larger the particle the slower the movement of the particle. Thus, we would predict that larger clusters of IgM-BCR inside the galectin-9 lattice have a lower diffusion coefficient compared to IgM-BCR. Thus, we propose that galectin-9 restricts IgM-BCR mobility by organizing IgM-BCR into larger clusters. The mobility of BCR on the cell surface is correlated with BCR signaling; simply depolymerizing actin increases BCR mobility and triggers spontaneous signaling. Consistent with this, treating B cells with lipopolysaccharide increases actin severing and BCR mobility, which lowers the threshold for B-cell activation. Increased diffusion of the BCR (or BCR nanoclusters) may be important for BCRs to join newly forming microclusters. In agreement with this, treatment with rGal9 decreased BCR microcluster formation upon stimulation by membrane-bound antigen. Conversely, increased BCR diffusion would be associated with more microclusters, in line with our observations.
observation of increased BCR mobility in galectin-9-deficient B cells and enhanced B-cell activation.

Additionally, we propose that galectin-9 induced merging of BCR nanoclusters regulates the interaction between IgM and the co-receptors CD45 and CD22 to suppress BCR signaling. Our finding of CD45 enrichment in the galectin-9 lattice is consistent with our identification of CD45 as a ligand of galectin-9. The N-terminal of CD45 has multiple N-glycosylation sites, providing potential binding sites for galectin family members. Indeed, in T cells, CD45 was reported to bind to galectin-136–38 and galectin-933. In addition, galectin-3 has been reported to bind to CD45 on diffuse large B-cell lymphoma59. These findings suggest that CD45 may be a promiscuous ligand of the galectin family, although differential glycosylation of CD45 in different immune
cell subsets may provide specificity for distinct galectin binding. Nonetheless, the enrichment of CD45 together with IgM inside the galectin-9 lattice is consistent with the inhibitory effect of galectin-9 on BCR signaling. Previous studies suggest that BCR activation is accompanied by the segregation of BCR and CD45 in order to promote Igα–Igβ phosphorylation\(^{41,60}\), consistent with the finding that CD45 is excluded from BCR microclusters\(^2\). Our finding that addition of rGal9 induces co-patching of CD45 and IgM and concomitant abrogation of BCR signaling is consistent with this model.

We also observed the enrichment of CD22 inside the galectin-9 lattice. CD22 is a known negative regulator of BCR signaling, which recruits the phosphatase SHP-1\(^{45}\), which dephosphorylates CD19\(^{61}\) terminating the amplification of BCR signaling. In addition, SHP-1 dephosphorylates PLC\(\gamma\)2 and attenuates MAPK activation\(^{42}\). Thus, the localization of CD22 together with IgM-BCR upon rGal9 treatment is consistent with our observation of reduced phosphorylation of CD19 and ERK1/2 and likely contributes to the inhibitory effect of galectin-9 on B-cell activation. Moreover, in resting naive B cells, dual-color dSTORM
demonstrated that the colocalization of IgM and CD22 is reduced in galectin-9-deficient B cells, providing a plausible mechanism for enhanced BCR signaling in Gal9-KO B cells.

Interestingly, we found that the ligands for galectin-9 are not saturated in naïve B cells and raises the possibility that BCR signaling could be modulated under conditions that affect the secretion of galectin-9. It is currently not known if B cells secrete galectin-9, which binds to the cell surface, or if other cells secrete galectin-9, which then binds to the surface of B cells. Indeed, galectin-9 is expressed in multiple cell types, including T cells, natural killer cells, and monocytes. Alterations in galectin-9 secretion and its association with cell surface proteins would provide a mechanism to adjust the threshold of B-cell activation and thus tune B-cell responses. Moreover, recent reports indicate that galectin-9 is induced under inflammatory conditions in peripheral blood mononuclear cells and mesenchymal stromal cells, and elevated in the serum of patients in a variety of infections, including HIV, cytomegalovirus, and dengue infection. How this affects the B-cell response in these infections is an important question to address.

Methods

Mice. C57BL/6 WT mice were obtained from Charles River, Lgals9−/− (Gal9-KO) mice were obtained from Steven Beverley (Washington University) on behalf of The Scripps Research Institute, and Lgals1−/− (Gal1-KO) were obtained from The Jackson Laboratory. Mice were used at 2–6 months of age for all functional and biochemical experiments and were age- and sex-matched within each experiment. Mice were housed in specific pathogen-free animal facility at University of Toronto Scarborough, Toronto, Canada. All procedures were approved by the Local Animal Care Committee at the University of Toronto Scarborough.

Cell lines and culturing. A20 B cells and A20 expressing Hel-specific IgM (D1.3) were obtained from F. Batista (Ragon Institute), and Jurkat T cells were obtained from T. Watta (University of Toronto). Cell lines were not tested for mycoplasma contamination. Cells were maintained at 37 °C in 5% CO2 in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and streptomycin (all from Gibco), and 50 µM 2-mercaptoethanol (Amresco).

Cell isolation. Splenocytes were isolated from C57BL/6 WT, Gal9-KO, and Gal1-KO mice using 70 µm cell strainer and phosphate-buffered saline (PBS, pH 7.4, Life Technologies) to collect a single-cell suspension. B cells were purified using negative isolation kit (Miltenyi Biotec Inc. or Stem Cell technologies) according to the manufacturer’s protocol.

Immunohistochemistry. Inguinal lymph nodes from C57BL/6 mice were isolated and immersed in Optimal Cutting Temperature compound (TissueTek) followed by 4% paraformaldehyde (PFA), permeabilized with 0.3% Tween-20 in PBS for 5 min, then blocked with 5% goat serum in PBS for 30 min. Sections were incubated with CD16/FITC (clone 3D6,112, Biologend), B220-allophycocyanin (APC) (clone RA3-682, Invitrogen), and goat anti-mouse galectin-9 (R&D Systems, Cat. No. AF5335) at 1:200 v/v in blocking buffer for 1 h at room temperature. Sections were washed five times with 0.5% Tween-20 in PBS, and then incubated with secondary antibody Cy3-conjugated bovine anti-goat (Jackson Immunoresearch, Cat. No. 105-095-025) at 1:200 v/v for 1 h at room temperature. Slides were washed five times and then mounted in Fluoro-Gel with DABCO® Anti-fading Mounting Medium. Slides were imaged by laser scanning confocal microscopy with Zeiss LSM510 Meta using a ×40/0.6 numerical aperture (NA) 1.40 oil-immersion objective.

Surface staining of galectin-9 for flow cytometry and imaging. C57BL/6, Gal9-KO, or rGal9-treated B cells were first blocked with purified rat anti-mouse CD16/32 (Fc-block, 1:250 v/v, clone 3C9-4G8, Invitrogen) in fluorescence-activated cell sorting (FACS) buffer (1% bovine serum albumin (BSA), 4 mM EDTA, and 0.01% NaN3 in PBS) for 30 min at 4 °C. Cells were washed twice with FACS buffer and then immunostained with phycoerythrin-conjugated rat anti-mouse galectin-9 antibody (clone 108A2, Biologend) at 1:200 v/v, or stained with goat anti-galectin-9 antibody (R&D Systems, Cat. No. AF5335) at 1:100 v/v in FACS buffer for 30 min at 4 °C, followed by Cy3-conjugated AffiniPure bovine anti-goat antibody (Jackson ImmunoResearch, Cat. No. 105-005-154), or stained with Alexa Fluor 488 or Alexa Fluor 644 AffiniPure Fab fragment rat anti-mouse IgM, µ-chain-specific (Jackson Immunoresearch Laboratories, Cat. No 115-547-020 or Cat. No 115-607-020) for 1 h at 4 °C. Cells were then resuspended in FACS buffer for flow analysis or imaged by spinning disc confocal microscopy. Cells were analyzed by flow cytometry (BD Fortessa) and plotted using Flowjo software (TreeStar). Cells were imaged by spinning disc confocal microscopy (Quorum Technologies) consisting of an inverted fluorescence microscope (DMi6000B, Leica) equipped with a 63×/1.4 NA oil-immersion objective and an electron-multiplying charge-coupled device (EMCCD) camera (ImagEM; Hamamatsu). Fluorescence intensity images of galectin-9 were pseudo-colored using the fire look up table in ImageJ. Quantification of number of puncta for all z-sections was performed in ImageJ using a threshold of 2× background intensity followed by “Analyze Particles” algorithm with the following parameters: size 5–100; circularity 0.00–1.00.

Planar lipid bilayer. Artificial planar lipid bilayers were prepared by liposome spreading in FCS2 chambers (Biotechnics). 1, 2-dioleoyl-sn-glycerol-3-phospho-coline liposomes (Avanti Polar Lipids, Inc.) were mixed with 2.5 × 10−6 % biotinylated (N-Biotinyl Cap PE; Avanti Polar Lipids, Inc.) liposomes. Alexa Fluor 633 streptavidin (1:1000 v/v Life Technologies, Cat. No S21375) was used to tether 50 mM ammonium bicarbonate (pH 8.0). Both eluates were then subjected to in-solution tryptic digestion followed by peptide desalting and concentration using OXMIX C18 tips (Agilent) according to the manufacturer’s instructions. Eluted peptides were dried by vacuum centrifugation and stored at −20 °C prior to analysis. Peptide separations were performed with an increasing acetone/10% 2-propanol gradient. After washing, the 10% 2-propanol liquid was removed. All fractions were then dried by vacuum centrifugation and stored at −80 °C.

Pull-down assay and mass spectrometry. The protocol was adapted from ref. 31. In all, 5 × 107 purified B cells from Gal9-KO mice were lysed in NP40 lysis buffer (50 mM HEPES (pH 8.0), 150 mM NaCl, 1% TritonX-100 (DTT), 5 mM EDTA, 0.1% Nonidet-P40, and Roche Complete mini EDTA-free protease inhibitor) with three cycles of freeze/thaw using liquid nitrogen. Lysates were cleared by centrifugation at 21 000 × g for 10 min at 4 °C. The cell lysate was incubated with anti-FLAG beads (Sigma-Aldrich) conjugated with 10 µg FLAG-tagged rGal9 (kindly provided by M. Ostrowski, University of Toronto) for 3 h at 4 °C. Beads were subjected to magnetic separation and washing in lysis buffer. Bound proteins were first eluted by 200 mM lactose solution (in distilled water), followed by denaturing elution (125 mM Na2HPO4). Denatured eluents were evaporated to dryness to remove NH4OH using a vacuum centrifuge, followed by resuspension in 50 mM ammonium bicarbonate (pH 8.0). Both eluates were then subjected to in-solution digestion following by peptide desalting and concentration using OXMIX C18 tips (Agilent) according to the manufacturer’s instructions. Eluted peptides were dried by vacuum centrifugation and stored at −20 °C in a cold spot. Peptide separations were performed with an increasing acetone/10% 2-propanol gradient. After washing, the 10% 2-propanol liquid was removed. All fractions were then dried by vacuum centrifugation and stored at −80 °C.

Oxidation of methionine and tryptophan, as well as deamidation of asparagine and glutamine was permitted as potential modifications during re-analysis. Results were further compared using ProHitsVM.

Pull-down assay for western blotting. A total of 1–3 × 107 purified B cells from Gal9-KO mice were lysed in RIPA buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride (PMSF)). Lysates were cleared by centrifugation at 21 000 × g for 10 min at 4 °C. Cell lysates were incubated with anti-FLAG beads (Sigma-Aldrich) conjugated with 10 µg FLAG-tagged rGal9 (kindly provided by M. Ostrowski, University of Toronto) for 3 h at 4 °C. Beads were subjected to anti-FLAG conjugated goat anti-mouse IgM (Jackson Immunoresearch Laboratories, Cat. No. 115-005-020) were incubated at 1:1000 overnight at 4 °C with gentle shaking. Secondary antibody was 1:1000 HPLC equipped with a 10.5 cm C18 column coupled online to a LTQ XL mass spectrometer (Thermo). MS1 scans were acquired between 400 and 1800 m/z, and up to six of the most intense parent ions were selected for collision dissociation ionization with an isolation width of 2 Da and normalized collision energy of 35 eV. Parent ions selected for analysis more than three times in 30 s were dynamically excluded from analysis for 45 s. Raw spectra were converted to mzXML format using msConvert (PMID: 23051804) and searched against the mouse and Escherichia coli proteomes as well as the sequence of Lgals9 and a database of commonly observed contaminants using X! Tandem (PMID: 14976030). Parent ion tolerances were set to ±3 and −2 Da and fragment ion mass tolerance was set to ±0.4 Da. Oxidation of methionine and tryptophan, as well as deamidation of asparagine and glutamine was permitted as potential modifications during refinement. Results were further compared using ProHitsVM.
antibodies horseradish peroxidase (HRP)-conjugated donkey anti-rabbit and donkey anti-goat (Abcam, Cat. No ab102182 and ab71710) were incubated at 1:10 000 in 5% non-fat milk in TBST for 1 h at room temperature with gentle shaking. Membranes were washed with TBST and developed with chemiluminescent substrate (West Pico, Thermo Fisher), and imaged by ChemiDoc (Bio-Rad).

Far-western blotting. In all, 1–5 x 10^7 purified primary murine B cells, D13 B cells, A20 B cells, or Jurkat T cells were lysed in RIPA buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% Triton-X 100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, and 1 mM PMSF). Lysates were cleared by centrifugation at 21 000 x g for 10 min at 4 °C. A unit of 25 µg of whole-cell lysate was resolved by 10% SDS-PAGE and transferred to PVDF membrane. Proteins were re-natured in decreasing concentrations of guanidine-HCl (6–0 M) according to ref. 34. Membranes were then blocked with 5% non-fat milk in TBST for 30 min at room temperature with gentle shaking. A unit of 5 µg/ml FLAG-AHF recombinant galega-maltose-binding protein was incubated in 5% non-fat milk in TBST used as a bait protein, blots were incubated with bait protein overnight at 4 °C with gentle shaking. Blots were washed and incubated with HRP-conjugated anti-goat antibody (Jackson Immunoresearch, Cat. No. 115-035-003) at 1:10 000 in 5% non-fat milk in TBST for 1 h at room temperature with gentle shaking. Membranes developed with chemiluminescent substrate (West Pico, Thermo Fisher) and imaged by ChemiDoc (Bio-Rad).

Cell labeling for single-particle tracking. Mouse rGal9 (R&D Systems) was reconstituted at 0.5 µg/ml in PBS containing 2% FBS for 15 min at 4 °C, then washed twice with PBS. Cells were then incubated with 0.5 µg/ml Alexa Fluor® 555 NHS Ester for 1 h at room temperature with gentle mixing. Following labeling, the mixture was dialyzed against 20 mM MOPS, 500 mM sodium chloride, 0.5 mM EDTA, and 1 mM DTT using 10 000 molecular weight cutoff Slide-A-Lyzer® Dialysis Cassette (Thermo Scientific). A volume of 20 µl of Alexa Fluor® 555 NHS Ester was added to 200 µl of primary murine B cells, D1.3 B cells, or A20 B cells (Cat. No. ab118416). The mixture was then incubated with 0.5 µg/ml Alexa Fluor® 488-conjugated Fab fragment goat anti-mouse IgM, µ-chain-specific (Jackson Immunoresearch, Cat. No. 115-007-020) and incubated with 40 µg/ml Attotect® 633 NHS Ester for 1 h at room temperature with gentle mixing. Following labeling, the mixture was dialyzed against PBS.

Glass coverslip coating for single-particle tracking. Glass coverslips were cleaned in a chromic acid (Fisher) bath for 20 min followed by rinsing with water and acetone. Coverslips were air-dried and then incubated with 1 µg/ml anti-MHC class II (clone M5/114, Sunnybrook Research Institute) for 2 h at room temperature and then washed with PBS.

Cell labeling for single-particle tracking. Primary murine B cells from WT and Gal9-KO mice were labeled with 4 ng/ml Attotect® 633-labeled goat anti-mouse IgM Fab fragment (Jackson Immunoresearch, Cat. No. 115-007-020) in 0.5% FBS in PBS for 15 min at 4 °C. For rGal9 treatment, 5 x 10^6 B cells from Gal9-KO mice were incubated with 0.5 µM labeled rGal9 mixed with 0.5 µM non-labeled rGal9 in complete media for 30 min at 37 °C. Labeled cells were washed twice with PBS and resuspended in chamber buffer. Labeled cells were stored on ice prior to imaging. Just before imaging, cells were incubated at 37 °C for 5 min.

Instrument for single-particle tracking and dSTORM. Single-molecule fluorescence microscopy was performed with a TIRF microscope (Quantum Technologies) based on an inverted microscope (DMi6000B; Leica), HCX PL APO ×100/1.47 oil-immersion objective, and Evolve Delta EMCCD camera (Photometrics). Images were acquired continuously at 20 frames/s for 10 s with an EM gain of 200 and the immersion objective, and Evolve Delta EMCCD camera (Photometrics). Images were acquired at 20 frames/s for 10 ms with an EM gain of 200 and the

Sample preparation for dSTORM. Primary B cells from WT and Gal9-KO mice were stained with Alexa Fluor 647-conjugated Fab fragment goat anti-mouse IgM, µ-chain-specific (Jackson Immunoresearch, Cat. No. 115-607-020) at 1.5 µg/ml in PBS containing 2% FBS for 15 min at 4 °C, then washed twice with PBS. Cells were then incubated with 0.5 µg/ml Alexa Fluor® 647-conjugated Fab fragment goat anti-mouse IgM, µ-chain-specific (Jackson Immunoresearch, Cat. No. 115-547-020) at 2.8 µg/ml and either Alexa Fluor 647 anti-mouse CD22 (OX-97, Biologend) at 2.5 µg/ml or Alexa Fluor 647 anti-mouse-human B220 (RA3-6B2, Biologend) at 2.5 µg/ml in PBS containing 2% FBS for 20 min at 4 °C then washed twice with PBS. Cells were then incubated with 2% FBS and 0.5% Triton X-100 in PBS containing 2% FBS for 10 min at room temperature, washed twice with PBS, and resuspended in 0.1% SDS, 100 mM NaCl, 1 mM PMSF, 0.2% Triton, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, and 1 mM PMSF. Lysates were cleared by centrifugation at 21 000 x g for 10 min at 4 °C. A unit of 25 µg of whole-cell lysate was resolved by 10% SDS-PAGE and transferred to PVDF membrane. Proteins were re-natured in decreasing concentrations of guanidine-HCl (6–0 M) according to ref. 34. Membranes were then blocked with 5% non-fat milk in TBST for 30 min at room temperature with gentle shaking. A unit of 5 µg/ml FLAG-AHF recombinant galega-maltose-binding protein was incubated in 5% non-fat milk in TBST used as a bait protein, blots were incubated with bait protein overnight at 4 °C with gentle shaking. Blots were washed and incubated with HRP-conjugated anti-goat antibody (Jackson Immunoresearch, Cat. No. 115-035-003) at 1:10 000 in 5% non-fat milk in TBST for 1 h at room temperature with gentle shaking. Membranes developed with chemiluminescent substrate (West Pico, Thermo Fisher) and imaged by ChemiDoc (Bio-Rad).

dSTORM acquisition and image reconstruction. dSTORM images were acquired on a TIRF microscope as described above. For Alexa Fluor® 647, photoconversion was achieved with 633-nm laser (intensity ranged from 80 to 100 mW/cm^2) illumination and conversion from the dark state with 488-nm laser illumination (intensity range from 5 to 20 mW/cm^2). Ten thousand images were acquired at a frame rate of 20 frames/s. Image reconstruction was performed on a TIRF microscope, first image Alexa 647 followed by Alexa 488 to prevent photobleaching of Alexa 647. For Alexa Fluor® 647, photoconversion was achieved with 633-nm laser (intensity ranged from 80 to 100 mW/cm^2) illumination and conversion from the dark state with 402-nm laser illumination (intensity range from 5 to 20 mW/cm^2). For Alexa Fluor® 648, photoconversion was achieved with 488-nm laser (intensity ranged from 80 to 100 mW/cm^2). Eight thousand images were acquired at a frame rate of 30 frames/s. Fiducial markers, which were visible in both the 488- and 647-nm channels, were used to align the two channels. The images of the beads in both channels were used to calculate a polynomial transformation that matched the position of fiducial marker (5-nm channel) with the position of Alexa Fluor® 488 (6-nm channel), using the MultiStackReg plugin of ImageJ. The transformation matrix was applied to each frame of the 488-nm channel stack. Reconstructed images were acquired using ThunderSTORM plugin for ImageJ according to the parameters shown in Supplementary Fig. 8A. Briefly, camera setup was as follows: pixel size (101.5 nm); 488- and 647-nm channels, and a 3 x 3 µm region, were used to align the two channels. The 3 x 3 µm region was used to reduce processing time, 4000 frames were processed for cluster analysis. For each reconstructed image of WT and Gal9-KO B cells, a 3 x 3 µm region was manually selected but excluded from the cell boundary. For each reconstructed image of rGal9-treated Gal9-KO B cells, a 3 x 3 µm region that contained at least two cells was required for analysis. Track selection was performed using ThunderSTORM plugin for ImageJ. Diffraction index and Ripley's H function analysis were performed by SuperCluster, an analysis tool kindly provided by the University of New Mexico's Spatio Temporal Modeling Center (http://sttc.unm.edu/). Cluster analysis was performed by a Bayesian, a model-based approach. In brief, uncertainty, x and y coordinates of each localization in post-processed reconstructed images were exported. The selected 3 x 3 µm regions were analyzed by the published R code of Bayesian cluster analysis with an alpha value of 20, background of 0.5, resq of (5, 200, 10), and thresh of (0, 50, 5). The analyzed data were post-processed to extract data about percentage of molecules localizing in clusters, cluster radius, number of clusters, and number of molecules per clusters.

CBC analysis. Colocalization of dual dSTORM data was conducted using CBC analysis using ThunderSTORM. Briefly, this method uses coordinate information of each molecule instead of an intensity-based approach, which would depend on the reconstruction and post-processing parameters chosen. Furthermore, this...
define a mask delineating the membrane region. Gaβ9β10β regions were determined by the fluorescence signal of Gaβ9. Gaβ9β10β regions were determined by subtracting the Gaβ9β10β regions from the membrane region. The mean fluorescence intensity of CD45, CD22, CD19, IgD, FcγRIIb, and IgM was calculated in Velocity.

### Lipid raft staining
Primary murine WT B cells and 1 µM rGal9-treated B cells were labeled with 1 µg/mL Alexa Fluor 555-conjugated Cholera toxin subunit B (Vybrant® Alexa Fluor® 555 Lipid Raft Labeling Kit, Thermo Fisher Scientific) for 10 min, followed by staining with Fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide according to the manufacturer’s protocol (Annexin V Apoptosis Detection Kit, ebioscience), and analyzed by flow cytometry (BD Fortessa). Data were analyzed using FlowJo software.

### Statistical analysis
Statistical analysis was performed using GraphPad Prism. The distribution of data was tested using D’Agostino-Pearson omnibus normality test. Comparisons between two groups were performed using Mann-Whitney for data with non-normal distribution. Comparisons between multiple groups were performed by ordinary one-way analysis of variance (ANOVA) for data with normal distribution and Kruskal-Wallis test for data with non-normal distribution. For post hoc analysis, Tukey’s multiple comparison was used for normally distributed data and Dunnett’s or Alexa Fluor-conjugated secondary antibodies were used at 1:10 000 v/v in 5% non-fat skimmed milk in TBST or 1% BSA in TBST and incubated for 2 h with shaking at room temperature. Membranes were incubated with SuperSignal (Thermo Fisher Scientific) chemiluminescent substrate and then imaged by ChemiDoc (Bio-Rad). Densitometric analysis of western blots was performed using ImageJ (NIH). The amount of phosphorylated protein was normalized to loading control and then multiplied by the ratio of signal treated to signal untreated. Eight-well Lab-Tek chambers were imaged by spinning disk confocal microscopy (Quorum Technologies) consisting of an inverted fluorescence microscope (DMi6000B; Leica) equipped with a ×63/1.4 NA oil-immersion objective and an EMCCD camera (Immunity). Individual fluorescence images were acquired using Metamorph software (Molecular Devices). Images were analyzed using Velocity software (Perkin Elmer). The fluorescence signal of CD45, CD22, CD19, IgM, IgD, or FcγRIIb was used to

### Cell stimulation for western blot
Forty-two-well plates were coated with 5 µg/mL AflimPure Fab(g)2 fragment anti-mouse IgM, IgM-specific (Jackson ImmunoResearch Laboratories). Plates, Cat. No. 115-006-020 in PBS over night at 4 °C. Primary naive splenic B cells from WT and Gaβ9-KO mice were stimulated for the indicated time, then lysed with Laemmli buffer containing 100 µM DTT followed by SDS-PAGE. For rGal9 treatment, Gaβ9-KO or WT B cells were pre-treated with 0.1 or 1 µM, respectively, with recombinant mouse galectin-9 (rGaβ9, E. coli-derived, R&D Systems) in RPMI containing 1% FBS (both Gibco) and incubated for 30 min at 37 °C. Cells were then washed once with RPMI and then stimulated as described above. Proteins were transferred to PVDF and blocked with 5% non-fat skimmed milk or 5% BSA in TBST for 1 h at room temperature or overnight (4°C) at 4 °C with shaking. Membranes were immunoblotted with the following antibodies or for 1 h for 4°C: mouse anti-β2-microglobulin (Sigma, 1:5000 in 1% BSA/TBST); rabbit anti-phospho-p44/42 MAPK (ERK1/2 Cell Signaling, Cat. No. 9101, 1:1000 in 1% BSA/TBST); rabbit anti-ERK p44/42 MAPK (ERK2/Cell Signaling, Cat. No. 9102); 4G10 (Sunnybrook Research Institute, 1:1000 in 1% BSA/1% NaN3/TBST); phospho-CD19 (Tyr531 Cell Signaling, Cat. No. 3571, 1:1000 in 1% BSA/1% NaN3/TBST); and phospho-Akt (Tyr308 Cell Signaling, Cat. No. 4056, 1:1000 in 1% BSA/1% NaN3/TBST). HRP-conjugated secondary antibodies were used at 1:10 000 v/v in 5% non-fat skimmed milk in TBST or 1% BSA in TBST and incubated for 2 h with shaking at room temperature. Membranes were incubated with SuperSignal (Thermo Fisher Scientific) chemiluminescent substrate and then imaged by ChemiDoc (Bio-Rad). The data that support the findings of this study are available from the corresponding author upon reasonable request.

### Code availability
ThunderStorm plugin for ImageJ is available at http://itzmen.github.io/thunderstorm/. SuperCluster is available from the University of New Mexico’s Spatial Temporal Modeling Center (http://stmc.unm.edu/). Bayesian cluster analysis code is available in supplementary information in ref 38. Single-particle tracking algorithms implemented in Matlab (The MathWorks) is available at http://physics.georgetown.edu/matlab/.

### Data availability
Lgal9–/– mice require an MTA from The Scripps Research Institute (TSRI). The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

A.C., N.A. and B.T. designed the research study. A.C., N.A., F.H.M.B., L.W., L.K.S., A.T.Q., Z.H., H.F. and R.T. conducted the experiments and data analysis. M.S. and D.M.O. provided input and assistance on dSTORM analysis. A.T.Q. and A.S. performed mass spectrometry analysis. A.C., N.A., F.H.M.B., L.W., L.K.S. and B.T. wrote the manuscript.

Additional information

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