Members of the galectin family of proteins have been shown to regulate the development and the function of immune cells. We previously identified the increased expression of galectin-1 and galectin-3 mRNA and protein in anergic B cells relative to their naïve counterparts. To investigate the role of these galectins in maintaining B cell tolerance, we crossed mice deficient in galectin-1 or galectin-3 with mice bearing a lupus autoantigen-binding transgenic (Tg) B cell receptor, using a model with a well-characterized B cell tolerance phenotype of deletion, receptor editing and anergy. Here, we present data showing that the global knockout of galectin-1 or galectin-3 yields subtle alterations in B cell fate in autoantibody Tg mice. The absence of galectin-3 leads to a significant increase in the number of Tg spleen B cells, with the recovery of anti-laminin antibodies from a subset of mice. The B cell number increases further in antibody Tg mice with the dual deficiency of both galectin-1 and galectin-3. Isolated galectin-1 deficiency significantly enhances the proliferation of Tg B cells in response to lipopolysaccharide stimulation. These findings add to the growing body of evidence indicating a role for the various galectin family members, and for galectins 1 and 3 in particular, in the regulation of autoimmunity.

Keywords: autoimmunity / CD86 / CD62L / humoral immunity / MHCII

Introduction

Autoimmune diseases such as systemic lupus erythematosus are, at their core, the result of failed immunologic tolerance. The dysregulation of autoreactive B cells and/or T cells leads to aberrantly secreted autoantibodies and myriad clinical manifestations. Recent studies have revealed that galectin family members are involved in the development, functional regulation and apoptosis of immune cells (reviewed in van Kooyk and Rabinovich 2008; Rabinovich and Toscano 2009; Rabinovich et al. 2012). Galectin-1 and galectin-3 are implicated in autoimmune disease regulation or pathogenesis, including lupus, and have been proposed as agents or targets, respectively, for therapeutic intervention (Jiang et al. 2009; Kang et al. 2009; Liu et al. 2011). Some insight into the underlying tolerance defects in B and T cells, and the roles for galectin family members in these processes, is emerging from studies in mouse and man, yet much remains to be discovered to assist the rational design of specific, targeted therapies.

To explore the normal fate of autoreactive B cells and to examine molecular events regulating their development in vivo, we previously generated and characterized an immunoglobulin (Ig) transgenic (Tg) murine model. Developing B cells express a Tg Ig heavy chain, LamH, that in conjunction with diverse endogenous Ig light chains binds to laminin, a lupus nephritis-associated self-antigen (Amital et al. 2007; Hanrotel-Saliou et al. 2011). Our studies revealed that LamH Ig Tg B cells are tightly regulated in vivo by three mechanisms: (i) central deletion, due to the induced apoptosis of developing autoreactive B cells in the bone marrow, (ii) receptor editing, the recombination of additional endogenous heavy or light chain gene loci in attempts to generate a non-autoreactive B cell receptor, and (iii) anergy, the functional inactivation of autoreactive cells. These mechanisms to silence autoreactivity are effective in LamH Ig Tg mice, such that serum anti-laminin autoreactivity is rarely recovered (Rudolph et al. 2002; Brady et al. 2004).

Studies using representational difference analysis, microarray and protein blotting further revealed that LamH Ig Tg anergic B cells express higher levels of mRNA and protein for the galactose-binding lectins galectin-1 and galectin-3 relative to their naïve B cell counterparts (Clark et al. 2007). Galectin-1 was also recently demonstrated to be significantly up-regulated in a subset of human anergic B cells (Charles et al. 2011). This led us to hypothesize that galectin-1 and galectin-3 play a role in the induction and/or the maintenance of B cell tolerance. To investigate this possibility, we generated LamH Ig Tg mice genetically deficient (−/−) in galectin-1 and/or galectin-3 and assessed the in vivo fate of resultant Ig Tg B cells.

Here, we present evidence that the global knockout of galectin-1 and/or galectin-3 does not precipitate overt failure of either central deletion or anergy of autoreactive LamH Ig...
Tg B cells. However, Tg B cells from mice lacking galectin-1 demonstrate enhanced proliferation in response to lipopolysaccharide (LPS) stimulation ex vivo and altered expression of surface CD86. The knockout of galectin-3 prevents the depletion of spleen Tg B cell numbers to the levels observed in galectin-3-sufficient subjects, a finding further exacerbated by the dual deficiency of both galectin-3 and galectin-1, and permits the spontaneous generation of anti-laminin antibodies by Tg B cells in a fraction of subjects. Our results suggest that the genetic loss of galectin-3 and/or galectin-1 in combination with environmental or epigenetic factors can lead to tolerance failure.

Results

Expression of the LamH transgene

The LamH Ig transgene is distinguished from endogenous IgM by IgM-a allotype reagents; endogenous B6 mice are IgM-b allotype. Expression of the transgene was detected on the surface of the majority of B cells from LamH Ig Tg+ mice regardless of galectin status (Table I and Figure 1). Tg IgM-a was also detected in the serum of all Tg+ mice, though at low levels relative to total circulating IgM, similar to the phenotype previously reported for LamH Ig Tg+ B6 mice (Brady et al. 2004). Serum Tg IgM-a levels did not differ significantly with the knockout of either galectin-1 or galectin-3 (data not shown).

Markers of B cell receptor editing in LamH Ig Tg+ mice did not differ with galectin status. The frequency of coexpression of endogenous heavy chain (b-allotype IgM), indicating the secondary rearrangement at the endogenous loci despite the presence of the rearranged Tg H chain, did not differ in LamH Ig Tg+ subjects with and without galectin deficiency (Figure 1 and Table I). Similarly, the frequency of lambda light chain expression, which suggests multiple attempts at light chain rearrangement to replace an autoreactive receptor, did not differ between Tg+ groups (Table I). LamH Ig Tg+ galectin-3−/− mice had a significantly larger proportion of lambda+ B cells when compared with their non-Tg galectin-3−/− counterparts, similar to the previously reported phenotype in galectin-sufficient LamH Ig Tg+ B6 mice (shown in Table I and in Brady et al. 2004), suggesting enhanced editing of LamH Ig Tg+ cells in these strains.

Deletion of Tg B cells

A significant reduction in the number of splenic B cells was seen in all LamH Ig Tg+ groups compared with their respective non-Tg controls, regardless of the presence or the knock-out of either galectin-1 or galectin-3 (Figure 1C).

The B cell count in Ig Tg+ galectin-3−/− mice was significantly higher (71% increase) than in Tg+ galectin+ subjects (Figure 1C and Table I). A significant increase in spleen weight was also noted in Tg+ galectin-3−/− vs Tg+ galectin+ mice (Table I). No significant differences were observed between non-Tg-galectin+ and galectin−/− mice, although there was a trend in the increased B cell count in non-Tg galectin-3−/− mice vs the non-Tg galectin+ group (P = 0.0523).

The overall decrease in B cells in LamH Ig Tg+ mice is also evident in the central compartment as a reduction in the percentage of bone marrow B220+ lymphocytes and of IgM+ bone marrow B cells in LamH Ig Tg+ mice relative to non-Tg mice of the same galectin status (Table I and Figure 1B).

To investigate whether galectin-3 impacts the survival of LamH Ig Tg B cells, which could alter the total B cell number as seen in LamH Ig Tg+ galectin-3−/− subjects, we examined whether addition of exogenous galectin-3 to overnight cultures of LamH Ig Tg+ galectin3−/− B cells impacted cell survival. Figure 2A and B shows that addition of recombinant mouse galectin-3 did not consistently increase the percentage of live B cells in culture, either in the presence or in the absence of LPS.

Another potential mechanism by which galectin-3 could impact B cell number is via interaction in vivo with either laminin or laminin-specific B cell receptors. Galectin-3 has been shown to bind to laminin (our data not shown, and Barboni et al. 1999). To investigate whether galectin-3 and lupus anti-laminin autoantibodies bind the same epitope(s) on laminin, two laminin-reactive antibodies, IgG H50-9 (Foster et al. 1993) and IgM A10C (Fitzsimons et al. 2000), were incubated with various concentrations of galectin-3 prior to assay for laminin binding. Addition of exogenous galectin-3 did not inhibit the binding of either antibody to laminin (Figure 2C). Rather, increasing amounts of galectin-3 enhanced Ig binding to laminin, suggesting that galectin-3/ laminin interactions facilitate the exposure of distinct laminin epitopes recognized by anti-laminin Ig. Anti-Ig detection reagent did not identify deposited Ig in control wells incubated with galectin-3 alone (not shown), ruling out physical interaction between the anti-laminin Ig and the galectin-3.

Serum anti-laminin autoreactivity

We previously reported strict regulation of the LamH Ig Tg B cells in the B6 background, such that spontaneous serum anti-laminin autoreactivity is rarely recovered (Rudolph et al. 2002; Brady et al. 2004). Superimposition of galectin-1−/− or galectin-3−/− mutations onto LamH Ig Tg+ B6 mice did not alter this phenotype in the majority of mice studied. However, one of 13 LamH Ig Tg+ galectin-3−/− mice had high serum levels of anti-laminin autoreactivity (Figure 3A).

B cell response to stimulus

When purified B cells were stimulated in culture with LPS, anti-laminin IgM antibodies were occasionally generated. Shown in Figure 3B, anti-laminin autoreactivity was detected in supernatants of mitogen-stimulated B cells from 2 of 11 (18%) LamH Ig Tg+ galectin+ mice. Three of six (50%) LamH Ig Tg+ galectin 3−/− B cell culture supernatants also contained laminin autoreactivity, whereas none of the LamH Ig Tg+ galectin-1−/− cultures generated substantial levels of anti-laminin reactivity. Similar results were observed when whole splenocytes were cultured with mitogen (not shown).

To assess proliferation, purified B cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and cultured in the presence of either LPS or B cell receptor cross-linker anti-IgM F(ab′)2. Following 2.5 days of stimulation, proliferation was assessed by flow cytometry. In response to LPS stimulation, a significantly higher percentage of B cells from LamH Ig Tg+ galectin-1−/− mice proliferated compared
B cells from two of three immunized Tg+ galectin-1 deficient mice had little to no Tg anti-laminin autoreactivity was recovered from serum- or mitogen-stimulated B cells of any Laminin immunization of galectin-1 deficient Tg−/− mice, three Tg−/− mice with galectin-1 deficiency were immunized with laminin in complete Freund’s adjuvant in an attempt to induce an anti-laminin response. Seventeen days following immunization, little to no Tg anti-laminin autoreactivity was recovered from the serum of any of the three subjects. However, in contrast to results using B cells from unimmunized mice anti-laminin Tg Ig were detected in supernatants of mitogen-stimulated B cells from two of three immunized Tg+ galectin-1−/− mice (Table II).

### Table I. B cell profile and receptor expression

<table>
<thead>
<tr>
<th></th>
<th>Non-Tg</th>
<th>LamH Ig Tg+</th>
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<tbody>
<tr>
<td></td>
<td>Galectin+</td>
<td>Galectin-1−/−</td>
</tr>
<tr>
<td></td>
<td>Galectin-1−/−</td>
<td>Galectin-3−/−</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen weight (mg)</td>
<td>0.068 ± 0.011 (16)</td>
<td>0.075 ± 0.008 (5)</td>
</tr>
<tr>
<td>Spleenocyte count (millions)</td>
<td>94.0 ± 22.3 (13)</td>
<td>102.0 ± 14.2 (4)</td>
</tr>
<tr>
<td>% B220+ of lymphocytes</td>
<td>48.8 ± 11.9 (15)</td>
<td>49.3 ± 13.3 (5)</td>
</tr>
<tr>
<td>B cell count (millions)</td>
<td>27.0 ± 11.5 (12)</td>
<td>36.6 ± 20.7 (4)</td>
</tr>
<tr>
<td>%IgMa</td>
<td>2.1 ± 1.8 (14)</td>
<td>1.9 ± 1.2 (4)</td>
</tr>
<tr>
<td>%IgMb</td>
<td>87.9 ± 7.6 (14)</td>
<td>92.3 ± 3.4 (4)</td>
</tr>
<tr>
<td>% lambda</td>
<td>5.7 ± 1.0 (11)</td>
<td>6.5 ± 1.3 (5)</td>
</tr>
<tr>
<td>Bone marrow</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% B220</td>
<td>40.4 ± 11.1 (15)</td>
<td>35.1 ± 4.1 (5)</td>
</tr>
<tr>
<td>% IgM</td>
<td>50.9 ± 10.4 (14)</td>
<td>54.0 ± 10.9 (5)</td>
</tr>
<tr>
<td>B cell markers of antigen contact</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surface Ig MFI</td>
<td>1.00 ± 0.12 (11)</td>
<td>0.97 ± 0.29 (3)</td>
</tr>
<tr>
<td>CD62L MFI</td>
<td>1.00 ± 0.03 (14)</td>
<td>1.13 ± 0.08* (4)</td>
</tr>
<tr>
<td>%CD62L of B220+</td>
<td>44.3 ± 28.1 (12)</td>
<td>45.8 ± 37.2 (2)</td>
</tr>
<tr>
<td>MHCI MFI</td>
<td>1.00 ± 0.04 (14)</td>
<td>1.12 ± 0.10* (4)</td>
</tr>
<tr>
<td>%MHCI of B220+</td>
<td>94.5 ± 2.6 (14)</td>
<td>95.1 ± 1.7 (4)</td>
</tr>
</tbody>
</table>

with B cells from either non-Tg or Tg+ galectin+ mice (Figure 4A and B). There was a trend to higher LPS-induced proliferation of LamH Ig Tg+ galectin-1−/− when compared with LamH Ig Tg+ galectin-3−/− B cells (P = 0.0588). No difference in response to receptor cross-linking was detected.

To investigate whether the enhanced LPS-induced proliferation of LamH Ig Tg+ galectin-1−/− B cells could be reversed by addition of exogenous galectin-1, proliferation assays were performed with LPS +/− 10 µg/mL recombinant galectin-1. Addition of galectin-1 had only a minor and non-uniform effect on the percent of divided cells (Figure 4C).

Conversely, to investigate whether the level of LPS-induced proliferation of LamH Ig Tg+ galectin+ B cells could be increased by blocking exogenous galectin binding, proliferation assays were performed with LPS +/− 10 µM lactose. Addition of lactose failed to enhance B cell proliferation (Figure 4D).

### Laminin immunization of galectin-1-deficient Tg+ mice

Because spontaneous anti-laminin autoreactivity was not recovered from serum- or mitogen-stimulated B cells of any LamH Ig Tg+ galectin-1−/− mice, these Tg+ mice with galectin-1 deficiency were immunized with laminin in complete Freund’s adjuvant in an attempt to induce an anti-laminin response. Seventeen days following immunization, little to no Tg anti-laminin autoreactivity was recovered from the serum of any of the three subjects. However, in contrast to results using B cells from unimmunized mice anti-laminin Tg Ig were detected in supernatants of mitogen-stimulated B cells from two of three immunized Tg+ galectin-1−/− mice (Table II).

### Surface marker expression

A number of B cell surface markers that were previously determined to have altered expression on LamH Ig Tg+ anergic B cells relative to their naïve B cells counterparts were examined. These differences included increased surface expression of CD86, decreased percentages of CD62L- and MHCI-positive cells and decreased total surface Ig density on LamH Ig Tg+ B cells (Brady et al. 2004). In this study, the expression of multiple markers was altered on B cells of LamH Ig Tg+ mice with deficiency of either galectin-1 or galectin-3 in a pattern similar to that reported previously (Table I), suggesting that the anergic phenotype was intact. This includes a trend toward decreased total surface Ig in LamH Ig Tg+ galectin-1−/− relative to non-Tg galectin-1−/− mice (P = 0.053).

To further assess the impact of galectin deficiency on surface markers, LamH Ig Tg+ B cells from galectin-deficient and galectin+ mice were compared. For the most part, these groups were similar in surface marker expression (Table I). However, two differences were noted: LamH Ig Tg+ galectin-1−/− B cells are unique in that they do not show the increase in the surface density of CD86 that is observed on anergic LamH Ig Tg+ B cells from mice with an intact galectin-1 gene (Figure 5). Additionally, LamH Ig Tg+ galectin-3−/− mice have a higher percentage of CD62L+ B cells than LamH Ig Tg+ galectin+ controls (Table I).

### Effect of galectin-1 or galectin-3 knockout on B cells from non-Tg B6 mice

Overall, the examination of non-Tg mice with a broad polyclonal B cell repertoire revealed that relatively few variables measured in this study were altered in mice with the knockout of galectin-1 or galectin-3 compared with galectin-sufficient
mice (Table I). There were no significant differences in the total splenocyte count, B cell surface Ig expression or serum total IgM levels (data not shown). Table I shows the few surface markers for which differential expression was noted: spleen B cell CD62L and MHCII surface density were significantly increased with the knockout of galectin-1 in non-Tg mice. Additionally, the surface density of CD86 was significantly decreased in non-Tg galectin-3−/− mice relative to non-Tg galectin-3+ (mean normalized mean fluorescence intensity (MFI) of 0.73 ± 0.24, n = 3, vs 0.99 ± 0.14, n = 13, respectively, P < 0.05).

**Phenotype of LamH Ig Tg+ galectin-1−/− galectin-3−/− double-knockout mice**

Functional redundancy among galectin family members is common. To elucidate phenotypic changes that might be exacerbated by loss of both galectin-1 and galectin-3, the effect of galectin-1−/− galectin-3−/− double knockout on the in vivo fate of LamH Ig Tg+ B cells was examined. The total splenic B cell count of LamH Ig Tg+ galectin-3−/− double-knockout subjects was significantly higher than concurrently assayed Tg+ galectin-3−/− single-knockout subjects (Table III). Normalized CD86 MFI levels did not differ

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**Fig. 1.** B cell profiles. Representative flow cytometry plots of (A) splenocytes and (B) bone marrow from anti-laminin Ig transgene (LamH Tg+) galectin knockout (−/−) mice and non-Tg or galectin sufficient (+) controls. Cells were gated on lymphocytes based on FSC and SSC properties, with further gating on B220+ B cells when indicated. IgM-a, transgene-encoded IgM; IgM-b, endogenous IgM; IgM, total IgM (IgM-a + IgM-b). (C) Total splenic B cell count, in millions, by genotype for galectin knockout mice (−), with (+) or without (−) the LamH Ig Tg, and galectin-sufficient controls (+). *P < 0.05 for pairwise comparison as noted.
significantly between groups. The Tg+ galectin-1−/− galectin-3−/− double-knockout mice also had no detectable spontaneous serum anti-laminin autoantibodies. No other significant differences were noted between these groups in the measured B cell parameters, though it is interesting that double-knockout LamH Ig Tg+ B cells appear to hyperproliferate to B cell receptor cross-linking (mean % divided, 70.4 ± 6.9 vs 42.6 ± 8.8 in LamH Ig Tg+ galectin+ mice, n = 2 per group).

Discussion

We have shown that the global knockout of galectin-1 or galectin-3 yields subtle alterations in the B cell fate in an anti-laminin autoantibody Tg model. In LamH Ig Tg+ mice, galectin-3 deficiency significantly increases the total number of spleen Tg B cells and supports the secretion of anti-laminin Ig, either spontaneously or after endotoxin stimulation, whereas galectin-1 deficiency enhances B cell proliferation in response to a Toll-like receptor (TLR)4 ligand. These findings add to the growing body of evidence, indicating a role for the various galectin family members, and for galectin-1 and galectin-3 in particular, in the regulation of autoimmunity (reviewed in van Kooyk and Rabinovich 2008; Rabinovich and Toscano 2009; Rabinovich et al. 2012).

Systemic galectin-3 deficiency was previously shown to lessen clinical manifestations of experimental autoimmune encephalomyelitis, antigen-induced arthritis and concanavalin A-induced hepatitis relative to disease in galectin-3 sufficient controls (Jiang et al. 2009; Forsman et al. 2011; Volarevic et al. 2012). In these models, galectin-3 promotes the
activation of T cells and the maturation of dendritic cells and alters the inflammatory cytokine profile. Conversely, exogenous galectin-1 administered parenterally or via genetically engineered fibroblasts ameliorates disease in several models of induced autoimmunity and in spontaneous BWF1 murine lupus (Rabinovich et al. 1999; Santucci et al. 2000, 2003; Baum et al. 2003; Liu et al. 2011). The therapeutic efficacy of galectin-1 has been attributed at least in part to the induction of apoptosis in activated T cells and altered cytokine secretion. In addition to their effects on cellular immunity, galectin-1 and galectin-3 have been shown to modulate development, localization and differentiation of B cells (Acosta-Rodriguez et al. 2004; Clark et al. 2007; Oliveira et al. 2009, 2011; Rabinovich and Toscano 2009; Tabrizi et al. 2009; Mourcin et al. 2011; Tsai et al. 2011); however, their involvement in the regulation of humoral autoimmunity and the maintenance of B cell tolerance has not been previously well characterized.

Our results indicate that galectin-3 and galectin-1 both play a role in promoting B cell tolerance, a finding previously suggested by the discovery of their up-regulation in anergic B cells (Clark et al. 2007; Charles et al. 2011). In our model system, galectin-3 contributes to the control of autoreactive B cell numbers and functional inactivation, such that its deficiency can lead to overt loss of tolerance to laminin. Knockout of galectin-3 in LamH Ig Tg+ mice was associated with the presence of anti-laminin autoantibodies in the serum of one mouse and in the supernatants of mitogen-stimulated B cells from 50% of mice. In contrast, anti-laminin Ig rarely appear in the serum or B cell culture supernatants in galectin-3-sufficient B6 LamH Ig Tg+ mice (herein and Rudolph et al. 2002). In the context of the concurrent significantly increased number of peripheral B cells observed in

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**Table II. Anti-laminin Ig from LamH Ig Tg+ galectin-1−/− subjects with or without immunization**

<table>
<thead>
<tr>
<th>Subject(s)</th>
<th>Serum (OD405)</th>
<th>B cell supernatant (OD405)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminin immunization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse 1</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Mouse 2</td>
<td>0.002</td>
<td>0.249</td>
</tr>
<tr>
<td>Mouse 3</td>
<td>0.008</td>
<td>0.048</td>
</tr>
<tr>
<td>Unimmunized</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean OD ± SD (n = 4–6)</td>
<td>0.016 ± 0.019</td>
<td>0.019 ± 0.011</td>
</tr>
</tbody>
</table>

Shown is OD405 for Ig binding on laminin-coated wells minus binding on diluent-coated wells. Sera were assayed at 1:20 dilution, supernatants from LPS-stimulated B cells were assayed undiluted for n = 3 LamH Ig Tg+ galectin-1−/− galectin-3+/− mice. Summary values for unimmunized LamH Ig Tg+ Gal-1−/− mice are shown for reference.
gallactin-3-deficient Tg mice, one possible explanation is defective deletion of laminin-reactive B cells. Such a defect could occur due to a requirement for galectin-3 for the induction of apoptosis in developing autoreactive B cells in the bone marrow, a compartment in which galectin-3 is known to be expressed (Oliveira et al. 2007). While galectin-3 is generally considered to be anti-apoptotic (Hernandez and Baum 2002; Hoyer et al. 2004; Liu and Rabinovich 2005), it has been shown to induce apoptosis in activated T cells (Stillman et al. 2006; Stowell et al. 2008); its expression and role in apoptosis in developing B cells has not been well characterized. Alternatively, galectin-3 could facilitate B cell receptor signaling after the engagement of self-antigen by developing B cells, such that a signal threshold that triggers deletion is surpassed. Support for this mechanism is garnered by our finding that addition of galectin-3 increased, rather than inhibited, binding of anti-laminin autoantibodies to self-antigen. In this scenario, galectin-3 deficiency would permit the survival of autoreactive cells that would otherwise not escape the bone marrow. If fewer autoreactive B cells are deleted during development, a heavier burden is placed on editing or maintenance of anergy to prevent autoimmunity. Anergy can be circumvented in some conditions of B cell culture (Hartley et al. 1993), which could explain the increased rate of recovery of laminin autoantibodies in gallactin-3/−/− B cell supernatants.

It is possible that the effect of gallactin-3 deficiency to boost Tg B cell numbers is manifest primarily in the periphery. Potential checkpoints for gallactin-3 control of autoreactive B cells include shortening the life span of anergic B cells, excluding autoreactive cells from follicles or limiting homeostatic proliferation or expansion of activated B cells, such that galectin-3 deficiency permits autoreactive cells to accumulate. These possibilities seem at odds with reports of a role of gallactin-3 in mediating IL-4 induced B cell survival and differentiation (Acosta-Rodriguez et al. 2004), though gallactin-3 functions likely vary with cell subset or differentiation state as well as gallactin cellular localization. We found that exogenous gallactin-3 did not alter survival of Tg+ gallactin-3-deficient B cells in culture. Other potential mechanisms for the gallactin-3 modulation of B cell numbers are suggested by observations in T cells. Gallactin-3:glycan lattices limit T cell receptor (TCR) clustering, decrease lateral movement across the cell membrane and increase agonist thresholds for TCR signaling (Demetriou et al. 2001). One predicts that gallactin-3 deficiency could thus lower activation thresholds in differentiated cells and permit cell expansion. Gallactin-3 lattices are also implicated in maintaining murine cytolytic T cell anergy; lattices trap T cell receptors and prevent their colocalization with CD8, whereas gallactin removal by competitive binding restores T cell function (Demetriou et al. 2008). Future studies can dissect if analogous mechanisms are operative in B cells.

Tg B cells from gallactin-1-deficient mice had a distinct tolerance phenotype. Neither serum nor LPS-stimulated B cells of unimmunized LamH Ig Tg+ gallactin-1/−/− mice yielded anti-laminin Ig, similar to previous results in LamH Ig Tg+ B6 gallactin-sufficient mice (Rudolph et al. 2002). However, residual B cells from LamH Ig Tg+ gallactin-1/−/− mice hyperproliferate in response to LPS, suggesting a role for gallactin-1 in dampening B cell TLR4 signaling or responses. This would expand the role for gallactin-1 in downmodulating LPS-induced effects in immune cells, as gallactin-1 has also been shown to...
reduce macrophage and dendritic cell responses to endotoxin (Rabinovich et al. 2000; Santucci et al. 2000; Kuo et al. 2011). Galectin-1 control of TLR-induced proliferative responses in B cells may require intracellular actions of galectin-1, as in our system supplementation of exogenous galectin-1 did not alter the proliferation of galectin-deficient cells in culture. B cell receptor (BCR)-stimulated proliferation was not altered in our galectin-1-deficient Tg B cells, suggesting a complex role in antigen receptor signaling, as exogenous galectin-1 was previously shown to decrease mouse primary B cell proliferation in response to anti-IgM-mediated BCR cross-linking (Yu et al. 2006).

It is of note that Tg+ anti-laminin autoantibodies were recovered in culture supernatants of LPS-stimulated B cells derived from LamH Ig Tg+ galectin-1−/− mice that had previously been immunized with laminin in Complete Freund’s adjuvant. This finding suggests that some anti-laminin B cells do escape deletion in vivo and can be activated in galectin-1−/− mice under certain conditions, possibly from environmental toxins or microbes. A role for key mechanisms under the redundant control of galectin-1 and galectin-3 cannot be excluded by our data. B cell hyperproliferation to endotoxin in the setting of galectin-1 deficiency could exacerbate autoreactivity in this setting.

Residual spleen B cells in LamH Ig Tg+ galectin-1−/− mice are unique compared with their Tg+ counterparts in galectin-sufficient and galectin-3-deficient mice in that they do not express increased surface CD86 (B7.2). Up-regulation of this costimulatory molecule is typically observed in antigen experienced B cells, as a consequence of B cell receptor cross-linking during either cell activation (Lenschow et al. 1994) or anergy induction in some settings (Brady et al. 2004; Terrier et al. 2011). Its normalization in the galectin-1-deficient LamH Ig Tg+ B cells suggests either lack of prior antigen contact, an unlikely circumstance given the evidence of concurrent marked cell deletion, a shift in the residual B cell repertoire to non-autoreactivity, presumably via receptor editing or dependence on galectin-1 in the signaling networks responsible for CD86 up-regulation. Some support for the latter possibility comes from studies of galectin-1-induced maturation of, and CD86 up-regulation on, human monocyte-derived dendritic cells (Fulcher et al. 2006). CD86 on activated B cells promotes T cell activation via interactions with CD80/CD86, B7.1/2, in a manner similar to CD28/CD152 (CTLA-4) (Damle et al. 1992; Freeman et al. 2004), and both galectin-1 and galectin-3 are capable of inducing T cell apoptosis (Stillman et al. 2006). Study of B cells from autoantibody Tg mice genetically lacking both galectin-1 and galectin-3 revealed a synergistic effect on the control of spleen autoreactive B cell numbers. Whether this reflects redundant effects on the control of B cell deletion or apoptosis revealed only in the dual knockout or reflects additive independent effects on deletion and proliferation will require further investigation.

We also observed an increase in the B cell surface expression of MHCIId and CD62L (L-selectin) in non-Tg galectin-1 knockout mice, compared with their non-Tg galectin-sufficient counterparts. MHCIId and CD62L are constitutively expressed on naïve B cells; following antigen activation, surface MHCIId is normally up-regulated to facilitate antigen presentation to T cells, whereas surface CD62L decreases to modulate B cell adhesion and trafficking (Reichert et al. 1983). Their increased constitutive expression on resting B cells could modify initiation of adaptive immune responses. There is precedence for an influence of galectin-1 on the biology of both molecules. Exogenous galectin-1 selectively inhibits constitutive and interferon-induced MHCIId expression on human monocytes and mouse inflammatory macrophages, resulting in decreased ability to stimulate T cells (Barrionuevo et al. 2007). Conversely, galectin-1 exposure up-regulates Human leukocyte antigen DR on immature human monocyte-derived dendritic cells (Fulcher et al. 2006), suggesting that MHCIId modulation by galectin-1 is developmental or differentiation stage-dependent. Inflammatory macrophages from galectin-1−/− mice express increased surface MHCIId, an effect that has functional consequences and is largely reversible with addition of recombinant galectin-1 (Barrionuevo et al. 2007). Galectin-1 colocalizes with CD62L in mouse inflammatory neutrophils, and galectin-1 exposure modulates...
Rudolph et al. (2002) and Brady et al. (2004). Galectin-1 types are pooled to generate the control (galectin-sufficient) group onto the C57BL/6J background (Jackson Labs, Bar Harbor, ME). A detailed description of the knockouts and initial phenotyping are provided via the Consortium for Functional Glycomics. Animal Care and use Committees. Cloning of the anti-laminin antibody (LamH) was performed previously by 14 generations (Clark et al. 2011). To assay for laminin-binding activity, Immulon II plates were coated overnight at 4°C with 10 µg/mL of laminin Engelbreth-Holm-Swarm (Sigma, St. Louis, MO) in phosphate buffered saline (PBS). Plates were blocked, rinsed, incubated with mouse sera (diluted 1:20) or B cell supernatants (undiluted) and detected using the alkaline phosphatase-conjugated anti-IgM antibody (Southern Biotech). Control Ig is anti-laminin mAb A10C supernatant (Foster and Fitzsimons 1998) and H50-9 antibody (Foster et al. 1993), which is detected with a goat-anti-mouse IgG alkaline phosphatase conjugated antibody. Results were recorded as the mean sample binding OD to laminin after the subtraction of mean OD on diluent (PBS)-coated plates. In cases where binding to laminin was lower than binding to diluent-coated plates, the reported laminin-binding value was zero.

For laminin-binding assays in the presence of exogenous galectin-3, the concentration of H50-9 and A10C control antibodies that generated half-maximal binding (300 ng/mL for H50-9, 1:2 dilution for A10C supernatant) was assayed for laminin binding in the presence of 0, 222 and 667 ng/mL of recombinant mouse galectin-3 (R&D Systems, Minneapolis, MN).

In vitro proliferation and differentiation assays
B cells were purified from single-cell RBC-lysed splenocyte suspensions using CD43 micro bead depletion of non-B cells (Miltenyi Biotec, Auburn, CA). Purified B cells were plated in proliferation media (Roswell Park Memorial Institute supplemented with 10% fetal bovine serum, 2 mM l-glutamine, beta-mercaptoethanol and 100 U/mL penicillin–streptomycin), with and without 50 µg/mL of LPS stimulation (#L6386, Sigma) at 1 × 10⁶ cells/mL in 1 mL/well of a 24-well plate. Mature supernatants harvested after 8–10 days of culture were assayed, undiluted, for laminin binding by ELISA.

For cell proliferation assays, purified B cells were labeled with 2.5–5.0 µM CFSE (Molecular Probes, Eugene, OR) as per the manufacturer’s protocol. Labeled B cells were cultured at 1.25 × 10⁶ cells/200 µL per well, in proliferation media as defined above, in a 96-well plate ±50 µg/mL of LPS (#L6386, Sigma), 80 µg/mL of goat-anti-mouse IgM F(ab')2 (Pierce/Thermo Scientific, Rockford, IL) or LPS as above plus 10 mM lactose (Sigma) or 10 µg/mL recombinant mouse galectin-1 (R&D Systems). Following 2.5 days in culture, cells were collected and stained with B220-PE and assessed by flow cytometry as detailed above. The percent of divided cells were determined as %B220⁺ x % lymphocytes x number of splenocytes.

Ig concentration and laminin-binding enzyme-linked immunosorbent assay (ELISA)
Quantitation of LamH Tg IgM in serum was as described (Clark et al. 2011). To assay for laminin-binding activity, Immulon II plates were coated overnight at 4°C with 10 µg/mL of laminin Engelbreth-Holm-Swarm (Sigma, St. Louis, MO) in phosphate buffered saline (PBS). Plates were blocked, rinsed, incubated with mouse sera (diluted 1:20) or B cell supernatants (undiluted) and detected using the alkaline phosphatase-conjugated anti-IgM antibody (Southern Biotech). Control Ig is anti-laminin mAb A10C supernatant (Foster and Fitzsimons 1998) and H50-9 antibody (Foster et al. 1993), which is detected with a goat-anti-mouse IgG alkaline phosphatase conjugated antibody. Results were recorded as the mean sample binding OD to laminin after the subtraction of mean OD on diluent (PBS)-coated plates. In cases where binding to laminin was lower than binding to diluent-coated plates, the reported laminin-binding value was zero.

Materials and methods

Animals
All studies and procedures were approved by the Duke University and the Durham Veterans Affairs Medical Center Animal Care and use Committees. Cloning of the anti-laminin antibody (LamH) was performed previously by 14 generations (Clark et al. 2011).

In vitro proliferation and differentiation assays
B cells were purified from single-cell RBC-lysed splenocyte suspensions using CD43 micro bead depletion of non-B cells (Miltenyi Biotec, Auburn, CA). Purified B cells were plated in proliferation media (Roswell Park Memorial Institute supplemented with 10% fetal bovine serum, 2 mM l-glutamine, beta-mercaptoethanol and 100 U/mL penicillin–streptomycin), with and without 50 µg/mL of LPS stimulation (#L6386, Sigma) at 1 × 10⁶ cells/mL in 1 mL/well of a 24-well plate. Mature supernatants harvested after 8–10 days of culture were assayed, undiluted, for laminin binding by ELISA.

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cells was calculated for the B220+ population using the FlowJo proliferation platform.

Viability assay
B cells were purified by negative selection and cultured overnight in proliferation medium with and without 50 µg/mL of LPS and/or 10 µg/mL of recombinant mouse galectin-3 (R&D Systems), at a concentration of 1 × 10^5 cells/mL in 200 µL/well of a 96-well plate. Following 24 h incubation, cells were assayed for apoptosis/death using Annexin V-FITC and 7-AAD. live cells is determined as cells negative for both Annexin 7-Aminoactinomycin D (AAD) (BD Pharmingen, San Jose, CA) as per the manufacturer’s protocol. The percentage of live cells is determined as cells negative for both Annexin V-FITC and 7-AAD.

Immunization
Three LamH Ig Tg+ galectin-1−/− galectin-3+/− mice were immunized subcutaneously with 50 µg laminin EHS (Sigma) in Complete Freund’s Adjuvant.

Statistical analysis
Statistical analysis was performed using JMP software (SAS Institute, Cary, NC). Data are shown as mean values ± SD. Comparisons between two groups were performed using the non-parametric Wilcoxon test. A P-value of <0.05 is considered significant.

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Conflict of interest
None declared.

Abbreviations
B6, C57BL/6J mouse strain; CFSE, carboxyfluorescein diacetate succinimidyld ester; Ig, immunoglobulin; LPS, lipopolysaccharide; OD, optical density at 405 nm; Tg, transgenic.


