Title: IgM and IgD B cell receptors differentially respond to endogenous antigens and control B cell fate

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Running Title: Distinct functions of IgM and IgD BCRs \textit{in vivo}
Naive B cells co-express two BCR isotypes, IgM and IgD, with identical antigen-binding domains but distinct constant regions. IgM but not IgD is downregulated on autoreactive B cells. Because these isotypes are presumed to be redundant, it is unknown how this could impose tolerance. We introduced the Nur77-eGFP reporter of BCR signaling into mice that express each BCR isotype alone. Despite signaling strongly \textit{in vitro}, IgD is less sensitive than IgM to endogenous antigen \textit{in vivo} and developmental fate decisions are skewed accordingly. IgD-only $\text{Lyn}^{-/-}$ B cells cannot generate autoantibodies and short-lived plasma cells (SLPCs) \textit{in vivo}, a fate thought to be driven by intense BCR signaling induced by endogenous antigens. Similarly, IgD-only B cells generate normal germinal center, but impaired IgG1$^+$ SLPC responses to T-dependent immunization. We propose a role for IgD in maintaining the quiescence of autoreactive B cells and restricting their differentiation into autoantibody secreting cells.

Keywords: IgM, IgD, B cell receptor, BCR, Lyn, B cell anergy, B cell tolerance, Nur77, Nr4a1, Ets1, autoantibodies, lupus
INTRODUCTION

The pre-immune mature naïve B cell compartment must balance the need for a diverse antibody repertoire with the risk of autoantibody-mediated disease. Early in development, B cells randomly rearrange their immunoglobulin genes through VDJ recombination and encounter a series of tolerance checkpoints that serve to remove autoreactive B cell receptors (BCRs) from the repertoire. Strongly autoreactive immature B cells rearrange additional light chains to “edit” their autoreactivity, and they ultimately undergo deletion if this is unsuccessful (Shlomchik, 2008). Yet, despite stringent counter-selection of autoreactivity, the mature follicular (Fo) B cell compartment retains cells reactive towards endogenous antigens (Wardemann et al., 2003; Zikherman et al., 2012). How these cells are restrained from mounting autoimmune responses is not fully understood.

We previously described a BAC Tg reporter mouse (Nur77-eGFP) in which GFP expression is under the control of the regulatory region of \( Nr4a1 \), an immediate early gene rapidly induced by antigen receptor signaling (Mittelstadt and Defranco, 1993; Winoto and Littman, 2002). We showed that Nur77-eGFP expression in naïve B cells is proportional to strength of antigenic stimulation and consequently to autoreactivity (Zikherman et al., 2012). The most prominent characteristic of GFP\(^{hi}\) reporter B cells is decreased surface IgM BCR relative to GFP\(^{lo}\) cells. Goodnow and colleagues first suggested that IgM downregulation may mark autoreactive B cells in the normal mature repertoire shortly after they reported selective downregulation of IgM in the IgHEL (hen egg lysozyme) BCR Tg / soluble HEL Tg model system of B cell autoreactivity (Goodnow et al., 1988; Goodnow et al., 1989). Indeed, multiple studies in mice and
humans have identified naturally occurring IgD$^+$IgM$^{lo}$ cells as autoreactive and “anergic” or functionally unresponsive (Duty et al., 2009; Kirchenbaum et al., 2014; Quach et al., 2011; Zikherman et al., 2012). These results corroborate observations with several BCR transgenic model systems (Cambier et al., 2007). However, whether or how IgM downregulation might constrain autoreactivity remains unclear because naturally-occurring autoreactive B cells maintain high expression of the IgD BCR isotype (Zikherman et al., 2012).

IgM and IgD are splice isoforms of a common precursor heavy chain mRNA (Moore et al., 1981). While they differ in their Fc domains, both BCR isotypes contain the same antigen-binding domain, as well as identical 3-amino acid cytoplasmic tails, and they pair with Igα/β in order to initiate the canonical BCR signaling cascade (Blum et al., 1992; Radaev et al., 2010). However, the expression pattern of the isotypes differs; IgM expression begins as soon as heavy and light chains recombine early in B cell development, and persists until class switch recombination occurs following B cell activation (Chen and Cerutti, 2010). IgD is uniquely co-expressed with IgM during a narrow developmental window on late transitional and mature naïve Fo B cells as a result of alternate splicing regulated by the zinc-finger protein ZFP318 (Enders et al., 2014; Pioli et al., 2014). This suggests that IgD may play a critical role specifically in mature naïve B cells. Initial characterization of IgM- and IgD-deficient mice revealed only mild phenotypes and substantial redundancy; each isotype could mediate B cell development, initiate antibody responses to T-dependent and -independent immunization, and induce normal levels of steady-state serum IgG (Lutz et al., 1998; Nitschke et al., 1993; Roes and Rajewsky, 1993). This is consistent with prior studies in BCR transgenic model
systems demonstrating that each isotype alone can mediate B cell development, deletion, and activation (Brink et al., 1992).

IgM and IgD differ structurally; IgD has a much longer, flexible hinge region linking its Fab and Fc regions than IgM does (Chen and Cerutti, 2010). Recently, Ubelhart, Jumaa, and colleagues demonstrated that a short and inflexible hinge confers upon IgM the unique capacity to signal in response to monovalent antigens, while both isotypes can respond to multivalent antigens (Ubelhart et al., 2015). However, the Goodnow lab subsequently showed that IgHEL Tg splenic B cells expressing either the IgD or IgM BCR exclusively could mobilize calcium in response to soluble HEL antigen in vitro, and each isotype can mediate a common gene expression program characteristic of anergy in vivo (Sabouri et al., 2016). Therefore, it remains unclear whether IgM and IgD BCRs expressed in an unrestricted B cell repertoire differentially sense bona fide endogenous antigens in vivo, particularly as the identity and nature of such antigens is largely unknown and not restricted to soluble monovalent antigens. For example, 5-10% of circulating naïve B cells in healthy humans express the well-characterized unmutated and autoreactive human heavy chain V-segment IGHV4-34, and these cells exhibit anergy, selective IgM downregulation, and recognize cell-surface antigens on erythrocytes and B cells in vivo (Quach et al., 2011; Reed et al., 2016).

We and others previously hypothesized that downregulation of IgM on Fo B cells might serve to restrain their response to endogenous antigen. Conversely, high expression of IgD on these cells might play a “tolerogenic” role (Zikherman et al., 2012). To determine how IgM and IgD differentially regulate B cell responses to endogenous antigens, we generated Nur77-eGFP reporter mice deficient for either IgM or IgD (Lutz
et al., 1998; Nitschke et al., 1993). Using this reporter of antigen-dependent signaling, we show that IgD is less sensitive than IgM to bona fide endogenous antigens in vivo despite higher surface expression and robust responsiveness to receptor ligation in vitro. Indeed, marginal zone (MZ) and B1a cells that express IgD but lack IgM induce less Nur77-eGFP than WT, and this is not attributable to repertoire differences. To further support these observations, we examine a series of cell fate decisions for which in vivo BCR signaling requirements have been previously defined. We show that IgD drives a pattern of development consistent with reduced endogenous antigen recognition, favoring MZ B cell fate and disfavoring B1a cell fate. Similarly, IgD alone is less efficient than IgM at driving SLPC expansion in response to endogenous antigens, a process that requires robust BCR signaling. However, IgD is sufficient for germinal center B cell differentiation. Our data suggest that reduced endogenous antigen sensing (i.e. signal transduction in response to antigen binding) by the IgD BCR isotype shunts autoreactive IgD$^{hi}$ IgM$^{lo}$ follicular B cells away from differentiation into SLPCs. We propose that predominant IgD expression maintains the quiescence of autoreactive B cells in response to chronic endogenous antigen stimulation, and limits autoantibody secretion in the context of rapid immune responses.

RESULTS

Endogenous antigen is both necessary and sufficient for expression of Nur77-eGFP reporter in B cells in vivo

We previously showed that antigen recognition was both necessary and sufficient for Nur77-eGFP reporter expression by B cells in vivo (Figure 1A)(Zikherman et al.,
Thus, reporter expression reflects endogenous antigen recognition \emph{in vivo}.

Conversely, under steady-state conditions \emph{in vivo}, the Nur77-eGFP reporter does not reflect signaling through other receptors expressed in B cells; indeed, loss of either CD40, or TLR3, 7, and 9 signaling has no effect on reporter expression in B cells \emph{in vivo} (Figure 1-figure supplement 1A). Moreover, neither BAFF, nor IL-4, nor CXCR4-dependent signaling can regulate reporter expression \emph{in vitro} (Figure 1-figure supplement 1B-C) (Zikherman et al., 2012). To further confirm that Nur77 expression in naïve B cells under steady state conditions is not regulated by microbial stimulation of pattern recognition receptors (e.g. TLRs 1, 2, 4, 6, and the TLR-4-like molecule RP105), we studied mice raised under either germ-free conditions or conventional specific-pathogen-free conditions. We observed no induction of endogenous Nur77 protein in splenic B cells or endogenous \textit{Nr4a1} transcript in splenocytes in the presence of commensal flora (Figure 1-figure supplement 1D, E). Moreover, MyD88-deficient and MyD88-sufficient splenocytes and peritoneal B1a cells express comparable amounts of endogenous \textit{Nr4a1} transcript and protein respectively under steady state conditions (Figure 1-figure supplement 1F, G). Taken together, these data demonstrate that Nur77-eGFP expression in B cells under steady-state conditions \emph{in vivo} is a specific readout of antigen-dependent signaling through the BCR (Table 1). We therefore sought to take advantage of the Nur77-eGFP reporter in order to probe the responsiveness of a diverse BCR repertoire of mature B cells expressing either IgM or IgD alone to the vast range of endogenous antigens they may encounter \emph{in vivo}.

\textbf{Dual expression of IgM and IgD BCRs is required to establish a broad dynamic range of BCR responsiveness across the repertoire}
Surface IgM, but not IgD, expression is inversely correlated with endogenous antigen recognition and GFP expression across a diverse repertoire of mature naïve follicular (Fo) B cells, such that B cells reactive to endogenous antigens express high levels of IgD and low levels of IgM on their surface (Figure 1B) (Zikherman et al., 2012). The variation in surface IgM expression across the B cell repertoire is profound, spanning a 100-fold range. However, because IgD is expressed at high and invariant levels in all WT naïve follicular B cells, total surface BCR, unlike IgM, has little dynamic range across the WT repertoire (Figure 1-figure supplement 2A).

To explore how IgM and IgD differentially regulate B cells reactive to endogenous antigens, we generated Nur77-eGFP mice deficient for either IgM or IgD (Lutz et al., 1998; Nitschke et al., 1993). In IgM$^{-/-}$ mice, all B cells express IgD alone prior to class switch recombination (CSR), and in IgD$^{-/-}$ mice, all B cells express IgM alone prior to CSR. We observed that IgM is still downregulated on IgD$^{-/-}$ GFP$^{hi}$ Fo B cells, but the dynamic range of IgM expression is highly restricted relative to the broad range observed on WT cells (Figure 1B).

We previously showed that IgM down-modulation on GFP$^{hi}$ reporter B cells largely accounts for impaired signaling through IgM (Figure 1C) (Zikherman et al., 2012). However, the narrow dynamic range of IgM on IgD-deficient cells renders GFP$^{hi}$ reporter B cells unable to fine-tune signaling through the IgM BCR across the GFP repertoire (Figure 1C). Furthermore, IgD-mediated signaling is not altered across the GFP repertoire in the presence or absence of IgM (Figure 1C). As a result, reduced responsiveness of GFP$^{hi}$ B cells is most profound in B cells that express the IgM isotype in the presence of IgD. This suggests that IgD expression may be necessary to establish
and/or maintain a broad and functionally dynamic range of surface IgM expression across the B cell repertoire. This is consistent with a previously proposed survival function for the IgD BCR (Roes and Rajewsky, 1993; Sabouri et al., 2016).

**IgM**⁻/⁻ follicular B cells express more surface BCR than IgD⁻/⁻ follicular B cells, but similar Nur77-eGFP

Deletion of either the IgM or the IgD BCR isotype results in compensatory upregulation of the remaining isotype. This may be due in part to a change in competition for pairing with Igα/β heterodimers, which is essential for trafficking of IgM to the cell surface (Hombach et al., 1990; Sabouri et al., 2016). IgM⁻/⁻ B cells express about twice as much surface BCR as IgD⁻/⁻ B cells, as measured by surface anti-light chain staining (Figure 1D and Figure 1-figure supplement 2B-E). Nevertheless, distribution of Nur77-eGFP expression across the naïve B cell repertoire is nearly identical in IgM⁻/⁻ and IgD⁻/⁻ Fo B cells (Figure 1E). Further, at every level of Nur77-eGFP, cells that express IgD alone require more surface BCR to drive an equivalent amount of GFP compared to cells that express IgM alone (Figure 1F). This suggests that on a per-receptor basis, IgD BCR may be less efficient at inducing Nur77-eGFP in response to endogenous antigens. Importantly, this is not attributable to differential requirement dependence upon IgM or IgD expression for signaling via CXCR4 or downstream of canonical TLR ligands (Figure 1-figure supplement 1C, Figure 1-figure supplement 3A).
IgD BCR crosslinking induces robust signaling in vitro

Because IgD drives less Nur77-eGFP per receptor than IgM in vivo, we wanted to determine whether there were defects in signal transduction downstream of IgD. To mimic antigen binding to the membrane-distal end of the BCR and allow for direct comparison between the isotypes, we stimulated IgD-only (IgM−/−) and IgM-only (IgD−/−) cells with anti-Igț and compared downstream signaling. BCR ligation in IgD-only Fo B cells induced equivalent amounts of Erk phosphorylation relative to IgM-only B cells (Figure 2A), and there were no gross differences in pErk kinetics (Figure 2B). Additionally, IgD-only cells induced more robust intracellular calcium increase and S6 phosphorylation than IgM-only cells (Figure 2C and Figure 2-figure supplement 1A). This was not due to differential effects of the Fc portion of the stimulatory antibody on IgM-only and IgD-only B cells as anti-Igț-F(ab’), and anti-Igț stimulation produced identical signaling (Figure 2-figure supplement 1B).

IgD BCRs sense endogenous antigens less efficiently than IgM BCRs

Due in part to increased surface receptor expression, IgD-only B cells induce comparable Erk phosphorylation and enhanced calcium mobilization relative to IgM-only B cells stimulated with anti-Igț. As a result, we observe significantly more CD86 and Nur77-eGFP induction in IgD-only B cells after 18 hours of anti-Igț stimulation in vitro (Figures 2D-2F). However, IgD-only cells induced less Nur77-eGFP and MHC-II upregulation when cultured in the absence of exogenous stimulus (Figures 2G and 2H). This discrepancy suggests that despite increased receptor expression and efficient coupling to downstream signaling machinery, residual ex vivo antigens occupying the
IgD BCR are less efficient at inducing signaling. Consistent with this hypothesis, basal calcium analyzed immediately ex vivo is depressed in cells that express only IgD (Figure 2I). These data suggest that while signal transduction downstream of the IgD BCR is robust in vitro, IgD responds less efficiently than IgM to the relevant antigens it encounters in vivo.

Reduced endogenous antigen sensing by IgD BCR in innate-like B cells

While IgD-only and IgM-only Fo B cells express comparable levels of Nur77-eGFP (Figure 1E), we suspected that competitive pressures for survival might constrain the acceptable range of BCR signaling among mature Fo B cells. We further speculated that compensatory mechanisms such as altered surface receptor and altered BCR repertoire might fine tune how much signaling Fo B cells experience in vivo in order to lie within this range. In contrast to Fo B cells, IgD-only marginal zone (MZ) and B1a cells expressed significantly less Nur77-eGFP than WT cells despite higher levels of surface BCR (Figure 3A, Figure 1-figure supplement 2B-E). To determine whether this difference was due to isotype or altered BCR repertoire, we probed GFP expression in B1a cells specific for phosphatidylcholine (PtC), an endogenous antigen exposed on the surface of dying cells that is thought to select developing B cells into the B1a compartment (Baumgarth, 2011). We found a large difference in GFP even among B1a cells with a common specificity, and this occurs in spite of high surface PtC binding in IgD-only B1a cells (Figures 3B and 3C).

We took an independent and complementary approach to address the same question in MZ B cells; we crossed IgM−/− and IgD−/− reporter mice to a genetic
background that drives over-expression of the B cell survival factor BAFF (Gavin et al., 2005). BAFF overexpression “unrestricts” the BCR repertoire by removing competition for survival, thereby reducing both positive and negative selection pressures and permitting cells with either insufficient or excessive BCR signaling to persist in the repertoire, resulting in a massively expanded MZ compartment (Stadanlick and Cancro, 2008). Importantly, BAFF does not directly regulate reporter GFP expression, and therefore GFP reflects endogenous antigen stimulation on this genetic background (Zikherman et al., 2012). In BAFF Tg mice, IgD-only MZ cells expressed much less GFP than those expressing IgM alone (Figures 3D and 3E). This was not explained by differences in surface receptor levels between these populations as they are comparable on this genetic background (Figure 3-figure supplement 1A). Indeed, IgD-only cells with low GFP and IgM-only cells with high GFP appear to be preferentially rescued in mice with excess BAFF, suggesting that distinct BCR repertoires, far from accounting for GFP differences between IgM-only and IgD-only MZ B cells, actually obscure these differences. Conversely, either “fixing” or unrestriciting the BCR repertoire unmasks impaired GFP upregulation by IgD relative to IgM.

As a result of allelic exclusion of the heavy chain locus during B cell development, IgM+/− heterozygous mice develop two genetically distinct sets of B cells in which half express only IgD and the other half express both IgM and IgD. We exploited this property to confirm that the Nur77-eGFP difference in the MZ and B1a compartments is cell intrinsic and not a consequence of the presence or absence of serum IgM (Figure 3-figure supplement 1B).
Since IgM⁻/⁻ and IgD⁻/⁻ mice were generated on the Balb/c and 129 genetic backgrounds respectively, their germline VDJ loci are different from that of C57/Bl6 mice to which they have been back-crossed. Throughout our study, we have validated our findings in either Balb/c-B6 F1 mice or IgH⁺/⁻ heterozygous mice, which have two sets B cells with identical IgM and IgD isotype expression but distinct germline VDJ loci. We took this approach to confirm that differences in MZ GFP were due to BCR isotype and not VDJ locus (Figure 3-figure supplement 1C).

**Cell-intrinsic skewing of B cell development by the IgM and IgD BCRs**

Generation of the B1a compartment requires endogenous antigen recognition and strong BCR signaling, while the opposite is true for MZ B cells (Figure 4-figure supplement 1A) (Cariappa and Pillai, 2002; Casola et al., 2004). Indeed, B1a cells are modestly reduced in IgM-deficient mice, while MZ B cells are modestly increased (Figure 4-figure supplement 1B-C). Because serum IgM deficiency leads to increased B1a numbers, we assessed B1a and MZ B cell development in a competitive setting to isolate the cell-intrinsic effects of IgM and IgD BCRs (Boes et al., 1998). Analogous to the IgM⁺/⁻ mice described above, IgM⁺/⁻ IgD⁻/⁺ heterozygous mice develop two genetically distinct sets of B cells in which half express only IgD and the other half express only IgM (Figure 4A). Although IgD-only B cells can partially populate the B1a compartment in the absence of competition (Lutz et al., 1998; Ubelhart et al., 2015), IgD-only cells are virtually excluded from this compartment in a competitive setting (Figure 4B). Indeed, this parallels the loss of PtC-binding IgD-only peritoneal B cells observed in competition with wild type cells (Ubelhart et al., 2015). In contrast, IgD-only B cells
preferentially populate the MZ B cell compartment in competition with IgM-only B cells (Figure 4B). These data are consistent with a signal strength model of B1a/MZ B cell development (Figure 4-figure supplement 1A), and this suggests that in vivo signaling, antigen recognition, or both are reduced in IgD-only B cells. These differences in signaling could in turn modulate the generation and/or survival of IgD-only B1a and MZ B cells.

We sought to determine whether skewed development of B cell lineages was driven primarily by properties of IgD, IgM, or both by comparing development of B cell populations in IgM+/−, IgD+/−, IgM+/−, and IgD+/− mice (Figures 4C-4E). We conclude that skewing of MZ and B1a fates is primarily attributable to lack of IgM expression on IgD-only cells rather than lack of IgD expression on IgM-only cells because we observe little skewing in IgD+/− mice (Figure 4E). Further, the competitive advantage of IgD-only cells in the marginal zone niche is reduced in the BAFF Tg setting, consistent with loosening of competitive selection pressures (Figure 4F).

IgM-only B cells exhibit a disadvantage in the mature Fo compartment relative to IgD-only B cells (Figure 4C). We find that absence of IgD rather than excess IgM expression accounts for some of this disadvantage as WT and IgD-only cells compete equally well in this compartment (Figure 4D). This may reflect impaired positive selection or survival in absence of IgD. Indeed, similar to our observations, a disadvantage for Fo B cells lacking IgD was observed in IgD+/− mice previously (Roes and Rajewsky, 1993). As with differences in Nur77-eGFP expression, we confirmed that the developmental fates of IgM-only and IgD-only cells were due to BCR isotype and not VDJ locus (Figure 4-figure supplement 1D).
Either IgD or IgM BCR is sufficient to mediate polyclonal B cell activation and germinal center differentiation in Lyn\(^{-/-}\) mice

Mice deficient for either IgM or IgD can mount T-independent and T-dependent immune responses to model antigens (Lutz et al., 1998; Nitschke et al., 1993; Roes and Rajewsky, 1993). However, endogenous antigens may have unique properties that are absent in model antigen systems. To test whether each BCR isotype was competent to mediate autoimmune responses to endogenous antigens in lupus-prone mice, we generated IgM\(^{+/+}\) and IgD\(^{+/+}\) mice on the Lyn\(^{-/-}\) background (Chan et al., 1997). The Src family kinase Lyn is essential to mediate ITIM-dependent inhibitory signals in B cells and myeloid cells (Scapini et al., 2009; Xu et al., 2005). Lyn\(^{-/-}\) mice consequently develop a spontaneous lupus-like disease characterized by anti-DNA antibodies and nephritis on the C57Bl/6 genetic background (Chan et al., 1997; Hibbs et al., 1995; Nishizumi et al., 1995). It has been shown that both B-cell-specific MyD88 expression and T cells are essential for IgG2a/c anti-dsDNA autoantibody production in Lyn\(^{-/-}\) mice, and conditional deletion of Lyn in B cells is sufficient for autoimmunity (Hua et al., 2014; Lamagna et al., 2014).

Secreted natural IgM is thought to play important homeostatic functions that repress autoimmunity, particularly clearance of dead cell debris (Boes et al., 2000; Manson et al., 2005). By performing our analysis in mice with wild type and IgM-only or IgD-only B cells in a common milieu (IgM\(^{+/+}\) Lyn\(^{-/-}\) and IgD\(^{+/+}\) Lyn\(^{-/-}\) mice), we could control for this and isolate the B cell-intrinsic effects of the IgM and IgD BCRs.

Importantly, precursor Fo B cells are present at roughly equal ratios from WT and IgM-
only or IgD-only loci in these mice, and this is unaffected by VDJ background (Figure 5-figure supplement 1A-C). Moreover, $Lyn^{-/}$ B cells expressing either IgM or IgD alone exhibit enhanced BCR signaling in vitro relative to $Lyn^{+/}$ B cells, suggesting that neither isotype is uniquely coupled to ITIM-containing receptors (Figure 5-figure supplement 2A-B).

Polyclonal B cell activation in $Lyn^{-/}$ mice precedes and is genetically separable from autoantibody production; it is driven by B cell-intrinsic loss of Lyn, is thought to result from enhanced BCR signaling, and is independent of MyD88 expression (Hua et al., 2014; Lamagna et al., 2014). Indeed, $Lyn^{-/}$ reporter B cells have elevated Nur77-eGFP expression consistent with enhanced BCR signal transduction in vivo (Figure 5-figure supplement 3A). We had expected that activation of IgD-only $Lyn^{-/}$ B cells might be impaired due to reduced sensing of endogenous antigens, but neither CD86 nor CD69 upregulation were significantly different on WT and IgD-only B cells in the absence of Lyn (Figure 5A). This implies that both BCRs can provide sufficient signals to drive polyclonal activation by endogenous antigens.

Although GC fate is disfavored relative to SLPC fate in $Lyn^{-/}$ mice, GCs do arise spontaneously with time (Hibbs et al., 1995; Hua et al., 2014). We found that both IgD-only and IgM-only cells in Lyn-deficient mice made comparable contributions to the GC compartment in competition with wild type B cells, independent of VDJ background (Figure 5B and Figure 5-figure supplement 3B-C). We propose that endogenous antigen sensing by IgD, while normally dampened, is sufficient to drive both polyclonal activation and GC differentiation on the $Lyn^{-/}$ background. This further implies that
antigen capture by IgD and presentation on MHC-II is robust enough to recruit adequate T cell help to support these cell fates.

IgM BCR is required to drive dsDNA antibody production in Lyn⁻/⁻ mice

IgM- and IgD-deficient B cells express BCR heavy chain allotype [a] (IgHᵃ), and their secreted antibodies can be differentiated from WT B6 antibodies (IgHᵇ) even after isotype switching (e.g. IgG2a[a] vs. IgG2a[b] – also referred to as IgG2c). Allotype-specific antibodies have been previously used in the context of lupus mouse models to track autoantibodies generated by B cells of distinct genetic origin (Mills et al., 2015; Pisitkun et al., 2006). We took an analogous approach to assess IgG2a/c anti-dsDNA autoantibodies emanating from each genetic locus in IgM⁺/⁺, IgD⁺/⁺, and IgHᵃ/b control mice deficient for Lyn. In order to compensate for reduced penetrance of autoantibody production in IgM⁺/⁺ Lyn⁻/⁻ mice, we collected serum from this genotype at time points beyond 24 weeks to increase the number of samples in which tolerance had been broken. While B cells expressing IgM (of either IgH locus) generated IgG2a/c anti-dsDNA, IgD-only B cells were relatively protected from generating anti-dsDNA antibodies, even at late time points (Figures 5C-5G, Figure 5-figure supplement 3D-E). Importantly, this is attributable to BCR isotype and not VDJ locus (Figure 5-figure supplement 3D-E).

We noted that anti-dsDNA antibody titers fluctuated substantially over time in Lyn⁻/⁻ mice, in some cases dropping markedly over a four-week period (Figures 5C and 5D). Anti-dsDNA IgG autoantibodies in patients with systemic lupus erythematosus (SLE) similarly fluctuate over time – in contrast to other anti-nuclear specificities - and correlate with disease flares (Liu et al., 2011). Indeed, unmutated germline BCRs from
Naïve B cells are recruited directly into the circulating plasmablast compartment during SLE flares (Tipton et al., 2015). Taken together, this suggests that anti-dsDNA antibodies in mice and humans are secreted at least in part by SLPCs emerging from an extra-follicular immune response, rather than long-lived plasma cells (LLPCs) that originate in GCs. Our data suggests that, in the absence of Lyn, the IgD BCR is sufficient to mediate B cell activation and GC entry, but the IgM BCR is essential to produce dsDNA-specific SLPCs.

**IgM BCR is required for expansion of unswitched plasma cells in Lyn−/− mice**

In addition to stochastic generation of autoantibodies over time, Lyn−/− mice exhibit a massive expansion of the splenic IgM plasma cell compartment that corresponds to a roughly 10-fold increase in serum IgM (Hibbs et al., 1995). While natural IgM is thought to be secreted by B1a-derived plasma cells (Baumgarth, 2011), elevated serum IgM in Lyn−/− mice arises from the follicular B2 compartment since Lyn−/− mice lack MZ B cells, and bone marrow chimeras lacking the B1a compartment reconstitute the hyper-IgM secretion phenotype (Luo et al., 2014). Moreover, while BCR signaling is prominently enhanced in Lyn−/− B2 B cells, it is markedly dampened in Lyn−/− B1a cells (Figure 6-figure supplement 1A) (Skrzypczynska et al., 2016).

IgM plasma cell expansion is temporally and genetically separable from autoimmunity in Lyn−/− mice; this phenotype develops in mice as young as 7-10 weeks of age with 100% penetrance, is B-cell intrinsic, and is not dependent upon MyD88 expression (Hua et al., 2014; Infantino et al., 2014; Lamagna et al., 2014). A pathway involving Btk, Ets1, and Blimp-1 is thought to drive this phenotype. Ets1 inhibits
differentiation into plasma cells in part by antagonizing the key transcriptional regulator of plasma cell fate, Blimp-1 (John et al., 2008). Mice deficient for Ets1 display IgM plasma cell expansion, Ets1 levels are reduced in Lyn−/− mice, and restoration of Ets1 expression normalizes IgM plasma cell numbers (Luo et al., 2014). Furthermore, reduced levels of Btk suppress IgM plasma cell expansion in Lyn−/− mice, and Ets1 levels are concomitantly restored to normal levels in BtkLoLyn−/− mice (Gutierrez et al., 2010; Luo et al., 2014; Mayeux et al., 2015). Therefore, exaggerated BCR stimulation of B2 B cells by endogenous antigens promotes IgM plasma cell expansion in Lyn−/− mice (Figure 6-figure supplement 2A). Indeed, enhanced BCR signaling has been shown to favor SLPC fate over GC differentiation in Lyn-sufficient mice as well (Chan et al., 2009; Nutt et al., 2015; Paus et al., 2006). We therefore wondered whether impaired endogenous antigen sensing by IgD influenced Ets1 expression and unswitched PC expansion in Lyn−/− mice.

As previously shown, Lyn−/− B cells downregulate Ets1 protein expression (Figure 6A) (Luo et al., 2014). However, Lyn-deficient B cells expressing only IgD did not do so, consistent with a reduced ability of IgD to transmit antigen-dependent signals in vivo, (Figure 6A). As previously reported, we found that the frequency of plasma cells in the spleens of Lyn−/− mice was increased fourfold relative to Lyn+/+ mice, but the bone marrow plasma cell compartment size was relatively unaffected (Figures 6B-C) (Infantino et al., 2014). The increase in splenic plasma cells was attributable entirely to unswitched (IgM+) plasma cells (Figure 6C). However, unswitched plasma cell expansion was completely absent in IgM−/− Lyn−/− spleens (Figure 6C). This was reflected in steady-state serum antibody levels; while Lyn−/− mice have elevated serum IgM relative to WT
mice, \( I_{gM}^{-/-} \text{Lyn}^{-/-} \) mice did not have elevated serum IgD relative to \( I_{gM}^{-/-} \) mice (Figures 6D-E).

To confirm that resistance to unswitched plasma cell expansion was a cell-intrinsic feature of IgD-only \( \text{Lyn}^{-/-} \) B cells, we assessed this phenotype in \( I_{gM}^{+/+} \text{Lyn}^{-/-} \) mice. Similar to the non-competitive setting, IgD-only \( \text{Lyn}^{-/-} \) cells did not contribute to an expansion in the unswitched plasma cell compartment (Figures 6F-G). This effect was due to isotype and not VDJ allele usage because both WT \( I_{gH}^{a} \) and WT \( I_{gH}^{b} \text{Lyn}^{-/-} \) B cells generated unswitched plasma cells in a competitive setting (Figure 5-figure supplement 3C).

\textbf{IgM BCR is required for efficient generation of short-lived IgG1\(^+\) plasma cells but is dispensable for GC B cell fate}

IgD-only \( \text{Lyn}^{-/-} \) Fo B cells are completely prevented from generating an expanded unswitched SLPC compartment in response to chronic endogenous antigen stimulation, but they are competent to enter the GC. To determine how this defect relates to responses towards exogenous model antigens, we sought to determine whether IgD-only \( \text{Lyn}^{+/+} \) B cells exhibit skewing of cell fate in the context of a T-dependent response to a model antigen. To generate a robust polyclonal B cell response in which T cell help should not be limiting, we used the classic immunogen sheep RBCs. During this immune response, Fo B cells can either enter the germinal center or differentiate into IgG1\(^+\) extrafollicular plasma cells (Chan et al., 2009; Paus et al., 2006). We found that wild type, \( I_{gM}^{-/-} \), and \( I_{gD}^{-/-} \) mice all produced extremely robust GC responses 5 days after SRBC injection, but
B cells expressing IgD alone were unable to produce IgG1+ SLPCs at this time point (Figures 7A-D).

Since serum IgM is absent in IgM−/− mice and is known to enhance PC generation (Boes et al., 1998), we wanted to assess the cell-intrinsic effect of IgD and IgM BCRs on the immune response to SRBCs. To do so, we immunized IgM−/− mice in which half of Fo B cells express IgD alone and half express both IgM and IgD. We could track the relative contribution of these two cell populations to the GC response because the bulk of GC B cells have not yet isotype-switched and express surface IgM or IgD. Both cell types made a robust contribution to the germinal center response (Figure 7E). Next we tracked the generation of IgG1+ PCs by detecting allotype [a] or allotype [b] IgG1. In contrast to the GC response, IgD-only B cells were significantly disfavored in this compartment, although the defect was less severe than that observed in IgM−/− mice, which lack serum IgM (Figure 7F). To control for differences in the VDJ locus, we performed this experiment using IgHα/β control mice and observed no significant differences between allotypes, implying that BCR isotypes rather than VDJ locus accounted for our observations (Figure 7-figure supplement 1A-B).

In contrast to impaired IgG1 SLPC responses by IgD-only B cells, we observed that IgD-only B cells were able to generate unswitched plasma cells in response to SRBC immunization in both non-competitive and competitive settings (Figure 7-figure supplement 1C-D). Unswitched PC numbers correlate well with MZ B cell numbers in these mice (Figure 4-figure supplement 1B and 4D). Moreover, MZ B cells efficiently generate short-lived unswitched plasma cells even in response to TD-antigens (Phan et al., 2005; Song and Cerny, 2003). We therefore suspect that IgG1 PCs emanate from the
Fo B cell compartment, while the unswitched PC response may originate in the MZ B cell compartment. Normal SLPC generation by IgM-deficient MZ B cells in response to immunization would be consistent with previously reported normal T-independent II responses in IgM-deficient mice (Lutz et al., 1998). However, we cannot exclude a contribution of follicular-derived PCs that have failed to class switch to IgG1. We sought to confirm that the IgG1+ PC defect was generalizable to other T-dependent immunogens. The B cell response to NP hapten is stereotyped and makes predominant use of the VH186-2 heavy chain (Loh et al., 1983). We therefore immunized both \( \text{IgM}^{+/-} \) and \( \text{IgH}^{\text{a/b}} \) mice with NP hapten conjugated to rabbit serum albumin (NP-RSA) in order to control for differences in VDJ locus. Although allotype [a] makes a less robust response to NP than allotype [b], we normalized responses of IgD-only cells to wild type allotype [a] cells and found that GC responses at early time points were intact, but IgG1+ PCs and NP-specific IgG1 titers were significantly reduced 7-8 days after immunization (Figures 7G-I). These data suggest that IgD-only follicular B cells are competent to enter the GC, but they have defects in adopting the SLPC fate (or class-switching) in response to T-dependent immunization.

**DISCUSSION**

We and others previously hypothesized that a major tolerance strategy employed by autoreactive Fo B cells is selective downregulation of IgM. However, since all Fo B cells express a high and invariant amount of surface IgD, it was unclear whether and how loss of IgM expression could affect B cell responses to antigenic stimulation. It was recently reported that IgM, but not IgD, is uniquely responsive to monomeric antigens
due to a short and inflexible linker region coupling the Fab and Fc portions of IgM (Ubelhart et al., 2015). This could account for selective quiescence of mildly autoreactive B cells to monovalent endogenous antigens. However, the identity and structural characteristics of endogenous antigens are not well understood, and include cell-surface antigens on the surface of red cells and B cells (Quach et al., 2011; Reed et al., 2016). Moreover, recent work from Goodnow and colleagues showed that HEL-specific IgD BCR, when expressed on primary mouse splenocytes, can indeed mobilize calcium \textit{in vitro} and can mediate gene expression changes \textit{in vivo} in response to monovalent cognate antigen (Sabouri et al., 2016).

How do our observations help move beyond and reconcile the seemingly contradictory published work on antigen sensing by IgM and IgD? \textit{In vitro} signaling studies of HEL-specific BCRs by the Jumaa and Goodnow labs differ in several important ways; the former work assesses calcium signaling in pre-B cells that lack Slp-65 expression, while the latter studies splenic B cells with a considerably more mature phenotype. In addition, differences in reagents or strength of stimulus may also contribute to opposing conclusions. Our data suggests that the reduced sensitivity of IgD to bona fide endogenous antigen may lie somewhere between these two extremes; we find that IgD can sense endogenous antigens, just less efficiently than IgM. Consistent with this conclusion, both HEL-specific IgM and IgD BCRs can mediate B cell anergy and drive a common gene expression program in response to chronic high affinity soluble antigen exposure \textit{in vivo} (Brink et al., 1992; Sabouri et al., 2016).

Here we show that B cells expressing IgD BCR alone inefficiently sense endogenous antigens, and exhibit impaired \textit{in vivo} cell fate decisions that are dependent
upon BCR signal strength. This is epitomized by dramatic skewing of IgD-only B cells away from the B1a and towards the MZ fate in a competitive setting. Importantly, signal transduction mediated by the IgD BCR in response to anti-Igκ stimulation in vitro is intact for all assayed parameters, suggesting that antigen sensing and signal initiation rather than signal transduction downstream of IgD is impaired. We propose that this impaired sensing is conferred by structural properties of IgD, either through direct binding of antigen and signal transduction through the BCR or through differential pairing with co-receptors that directly modulate the BCR signaling pathway.

Characterization of bona fide endogenous antigens that drive Nur77-eGFP expression in the polyclonal repertoire is necessary in order to further dissect the biophysical basis for reduced antigen sensing by IgD in vivo. These antigens may not be exclusively restricted to those that are germline-encoded or generated by host enzymes; rather they may include non-inflammatory antigens taken up through the gastrointestinal tract and recognized by specific BCRs. At least some relevant endogenous antigens may be membrane-bound (Quach et al., 2011; Reed et al., 2016), and recent studies have illustrated the importance of membrane spreading, contraction, and stiffness discrimination in BCR signaling (Fleire et al., 2006; Shaheen et al., 2017). We propose that relevant endogenous antigens are presented in contexts that do not efficiently trigger signaling through the flexible structure of IgD. This might explain the discrepancy between weak responsiveness of IgD to endogenous antigens in vivo and strong signaling in response to crosslinking antibodies in vitro.

An alternative mechanism is that differential clustering of co-receptors with IgD and IgM might influence how BCR signals are integrated in vivo. It has long been
proposed that there are not only quantitative, but also qualitative differences between IgM and IgD signaling. Early studies of IgM and IgD signaling in cell lines suggested that kinetics but not quality of signaling triggered by each isotype differed (Kim and Reth, 1995). However, we have been unable to identify a difference in kinetics of BCR signaling in primary IgM- or IgD-deficient B cells in vitro. More recently, studies using TIRF, dSTORM, and PLA (proximity ligation assay) showed that IgD is more densely clustered on the cell surface than IgM, and these distinct IgM- and IgD-containing “islands” are differentially associated with co-receptors such as CD19 in resting and activated B cells (Klasener et al., 2014; Maity et al., 2015; Mattila et al., 2013). Since function of the ITIM-containing inhibitory co-receptor CD22 depends on its ability to efficiently access the BCR, differences in cluster density of IgM and IgD might render them differentially sensitive to such inhibitory tone (Gasparrini et al., 2016). It is possible that differential association with such co-receptors contributes to differences in the function of the IgM and IgD BCRs in vivo by modulating BCR signal strength, or by selectively perturbing specific downstream signaling events.

In addition to B cell co-receptors that have long been known to directly modulate canonical BCR signaling, a growing list of immunoreceptors expressed on B cells have more recently been shown to require expression of the BCR for optimal function; Becker et al. have shown that expression of the IgD BCR is critical for CXCR4-dependent signaling (Becker et al., 2017). This may influence the biology of B cells expressing only IgM; indeed, CXCR4-deficient B cells exhibit reduced plasma cell migration from spleen to bone marrow (but intact splenic SLPC responses) (Nie et al., 2004). Importantly, defective CXCR4 signaling in IgM-only B cells could not account for reduced Nur77-
eGFP expression in IgD-only B cells because reporter expression is insensitive to CXCR4 signaling (Figure 1-figure supplement1C). Nor would it account for skewed cell fate decisions by IgD-only B cells in competition with wild type B cells, both of which retain intact CXCR4 signaling. B cell responses to TLR4 ligands also require expression of the BCR, but importantly canonical TLR ligands do not exhibit selective dependence on either the IgM or the IgD isotypes (Figure 1-figure supplement3A). Finally, recent work establishes a role for the Fc-receptor for IgM in modulating B cell responses, and could therefore play a role downstream of serum IgM that is absent in IgM−/− mice (Nguyen et al., 2017a; Nguyen et al., 2017b). We control for this effect by confirming that all phenotypes in this study are cell-intrinsic and independent of secreted IgM.

Accumulating evidence suggests that strong BCR signals favor SLPC over GC fate (Chan et al., 2009; Nutt et al., 2015; Paus et al., 2006). This is thought to be transcriptionally mediated at least in part by loss of Ets1 expression and induction of Irf4 in a BCR signal-strength dependent manner (Nutt et al., 2015). Here we show that IgM and IgD are each sufficient to drive GC responses, but IgD is less efficient at promoting SLPC fate in response to endogenous antigens, implying that IgD hi IgM lo B cells may similarly be shunted away from SLPC responses. This discrepancy is unlikely to be attributable to differences in antigen capture and presentation by the IgM and IgD BCRs, as GC entry is highly T cell-help dependent. Instead, we provide evidence to suggest that reduced antigen-dependent BCR signals are transduced in vivo by IgD. We show that, in the absence of Lyn, Ets1 downregulation and unswitched PC expansion require the IgM BCR. IgM−/− Lyn−/− mice lacking IgD expression phenocopy Btk−/− Lyn−/− mice; both strains are protected from Ets1-downregulation, PC expansion, and anti-dsDNA autoantibody
production in vivo (Mayeux et al., 2015; Whyburn et al., 2003). This suggests that robust
Btk-dependent Ets-1 downregulation in response to endogenous antigens relies upon
efficient antigen sensing by the IgM BCR and is important to drive unswitched PC
expansion in the absence of Lyn. It will be important to explore whether a similar
mechanism accounts for impaired IgG1+ SLPC responses by Lyn-sufficient B cells with
low or absent IgM expression.

We propose that the purpose (and consequence) of IgM downregulation on
autoreactive B cells is to limit their direct differentiation into SLPCs in response to
chronic endogenous antigen stimulation, and prevent secretion of auto-antibodies in the
context of an acute humoral immune response (Figure 7-figure supplement 2A).
However, IgD is sufficient to drive germinal center differentiation without the
contribution of IgM. This has been well-demonstrated both in the present study as well as
prior work (Lutz et al., 1998). Indeed, not only are autoreactive IgD^{hi} IgM^{lo} B cells
competent to enter the germinal center, they appear to do so with greater efficiency,
perhaps due in part to improved survival (Sabouri et al., 2016; Sabouri et al., 2014). It is
worth emphasizing that selection pressures and tolerance mechanisms operating within
the germinal center must independently ensure that somatic hypermutation both abolishes
germ-line-encoded autoreactivity and prevents de novo acquisition of autoreactivity.
Goodnow and colleagues recently showed that autoreactive BCRs can indeed be
“redeemed” by somatic hypermutation, providing proof-of-principle that entry of
autoreactive B cells into the GC does not necessarily pose a risk to the organism (Sabouri
et al., 2014).
Why, though, are autoreactive B cells preserved in the periphery and not deleted? Why is IgD necessary at all? One suggestion is that such BCRs are retained in the pre-immune B cell compartment to fill holes in the repertoire, and that IgD is essential for their survival; indeed, it has been shown that loss of IgD expression, with or without compensatory upregulation of IgM, leads to loss of mature naïve B cells in competition (Roes and Rajewsky, 1993; Sabouri et al., 2016). We similarly find that IgD-only B cells have a profound competitive advantage in the follicular B cell compartment relative to IgM-only (but not WT) B cells, implying an obligate survival function for the IgD isotype BCR. This is consistent with a well-appreciated pro-survival function of the BCR as demonstrated by Rajewsky and colleagues (Kraus et al., 2004; Lam et al., 1997). Since IgD expression is needed to keep IgM<sup>lo</sup> B cells alive, this may explain why IgD expression facilitates a broad dynamic range of IgM expression across the B cell repertoire (Figure 1B, C), allowing B cells to fine-tune the intensity of their SLPC responses according to their autoreactivity. IgD expression on IgM<sup>lo</sup> cells could serve to mediate antigen capture and participation in T-dependent immune responses, while simultaneously limiting SLPC responses as we show here.

We demonstrate here for the first time that IgD BCRs sense endogenous antigen more weakly than IgM BCRs in vivo, are inefficient at driving SLPC responses to endogenous antigens, and thereby may function to divert autoreactive follicular B cells from direct PC differentiation. We propose that this property of the IgD BCR limits generation of auto-reactive antibodies in the context of immediate humoral immune responses. Taken together with recent work from the Jumaa and Goodnow groups, we propose a unified model of how IgM downregulation in the face of high IgD expression
regulates the fate of naïve autoreactive B cells in vivo while retaining their contribution to
the mature BCR repertoire.

**MATERIALS AND METHODS**

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Mice. Nur77-eGFP mice and Lyn−/− mice have been previously described (Chan et al., 1997; Zikherman et al., 2012). BAFF Tg mice were originally generated in the Nemazee lab, in which BAFF is under the control of a myeloid promoter, human CD68 (Gavin et al., 2005) (source: MMRRC UC Davis). IgD−/− and IgM−/− mice were previously described, and the former were generously shared by Dr. Hasan Jumaa (Lutz et al., 1998; Nitschke et al., 1993). TLR7−/−, CD40L−/−, Unc93b13d/3d, Nr4a1−/−, and MB1-Cre MyD88fl/fl mice were previously described (Hobeika et al., 2006; Hou et al., 2008; Lee et al., 1995; Lund et al., 2004; Renshaw et al., 1994; Tabeta et al., 2006). All strains were backcrossed to the C57BL/6 genetic background for at least six generations. Mice were used at 5-12 weeks of age for all functional and biochemical experiments unless otherwise noted. Germ-free (GF) and specific pathogen free (SPF) C57BL/6 mice used for direct comparison were purchased from Taconic and Jackson Laboratory and kept in microisolators under GF or SPF (ISOcage P - Bioexclusion System, Tecniplast) conditions. All GF mice were housed in closed caging systems and provided with standard irradiated chow diet, acidified water and housed under a 12-hour light cycle; 7-week-old males mice were used. GF and control mice were generously provided by Drs.
Sergio Baranzini and Anne-Katrin Proebstel at UCSF. All other mice used in our studies were housed in a specific pathogen free facility at UCSF according to the University Animal Care Committee and National Institutes of Health (NIH) guidelines.

**Antibodies and Reagents.** Fluorescently-conjugated or biotin-conjugated antibodies to B220, CD5, CD19, CD21, CD23, CD69, CD86, CD93 (AA4.1), CD95 (Fas), CD138, CXCR4, IgA, IgM, IgM[a], IgM[b], IgD, IgD[a], IgGl[a], IgGl[b], Igκ, Igλ, GL-7, MHC Class II, and fluorescently-conjugated streptavidin were from Biolegend, eBiosciences, BD Biosciences, Tonbo, or Life Technologies. NP-PE was from Biosearch Technologies. 100 nm Rhodamine PtC liposomes were from FormuMax. Antibodies for intra-cellular staining, pErk Ab (clone 194g2) and pS6 Ab (2F9), were from Cell Signaling Technologies, and Nur77 Ab (clone 12.14) conjugated to PE was from eBioscience. Goat anti-mouse IgM F(ab’)2 was from Jackson ImmunoResearch. Goat anti-mouse Igκ and goat F(ab’)2 anti-mouse Igγ were from Southern Biotech. Anti-IgD was from MD Biosciences. CXCR4 ligand (CXCL12/hSDF-1α) was from Peprotech. LPS (Cat. L8274) was from Sigma. CpG DNA (ODN 1826 Biotin; Cat. tlrl-1826b) and Pam3CSK4 (Cat. tlrl-pms) were from InvivoGen.

**Flow Cytometry and Data Analysis.** Cells were stained with indicated antibodies and analyzed on a Fortessa (Becton Dickson) as previously described (Hermiston et al., 2005). Data analysis was performed using FlowJo (v9.7.6) software (Treestar Incorporated, Ashland, OR). Statistical analysis and graphs were generated using Prism v6 (GraphPad Software, Inc). Figures were prepared using Illustrator CS6 v16.0.0.
Median Igk levels (Figure 1F) were calculated for 200 equally sized “bins” spanning the Nur77-eGFP spectrum using Canopy v1.4.1 (Enthought); source code is provided, and parameters can be modified to condense any 2D FACS plot for comparisons of multiple samples.

Statistics and Replicates. We define biological replicates as independent analyses of cells isolated from different mice of the same genotype, and we define technical replicates as analyses of cells isolated from the same mouse and used in the same experiment. When technical replicates were used, we averaged them to calculate a value for the biological replicate. All reported values and statistics correspond to biological replicates only, and all “n” values reported reflect the number of biological replicates. Wherever MFI s are directly compared, we collected all samples in a single experiment to avoid potential error that could arise from fluctuations in our flow cytometers. In some instances, MFI ratios were compiled from different experiments for statistical purposes, but qualitative findings were consistent experiment to experiment. As our panels reproducibly generated flow plots with well-defined populations, population sizes were calculated from data compiled from different experiments. When comparing two groups, we employed Welch’s t test, which is more stringent than Student’s t test and does not assume that the groups have the same standard deviation. We used paired difference t tests when studying parameters in allotype-heterozygous mice that are sensitive to cell-extrinsic factors (e.g. dose of immunogen or severity of autoimmune disease). When comparing three genotypes (e.g. receptor levels on WT vs. IgM^- vs. IgD^-), we used one-way ANOVA and Tukey’s multiple comparison test to calculate p values.
Intracellular Nur77, pErk and pS6 Staining. Ex vivo or following stimulation, cells were fixed in 2% paraformaldehyde, permeabilized with 100% methanol, and stained with anti-Nur77, anti-pErk or anti-pS6 followed by lineage markers and secondary antibodies if needed.

Intracellular Plasma Cell Staining. Cells were fixed in 2% paraformaldehyde and permeabilized in BD Perm/Wash. Stains for antibody isotypes and allotypes were prepared in BD Perm/Wash.

Intracellular Calcium Flux. Cells were loaded with 5 μg/mL Indo-1 AM (Life Technologies) and stained with lineage markers. Cells were rested at 37 C for 2 minutes, and Indo-1 fluorescence was measured immediately prior to stimulation to calculate basal calcium.

In vitro B Cell Stimulation. Splenocytes or lymphocytes were harvested into single cell suspension, subjected to red cell lysis using ACK buffer, and plated at a concentration of 7.5 x 10^5 cells/200 μL in round bottom 96 well plates in complete RPMI media with stimuli for 18 hours prior to analysis. In vitro cultured cells were stained using fixable near IR live/dead stain (Life technologies) per manufacturer’s instructions.

ELISA. Serum antibody titers for total IgM, total IgD, anti-dsDNA IgG2a, and NP-specific IgG1 were measured by ELISA. For total IgM and total IgD, 96-well plates
were coated with 1 μg/mL anti-IgM F(ab’)2 (Jackson) or 1 μg/mL anti-IgD (BD 53448), respectively. Sera were diluted serially, and total IgM and total IgD were detected with anti-IgM-biotin (eBioscience) and SA-HRP (Southern Biotech) or anti-IgD-HRP (American Research Products). dsDNA plates were generated by serially coating plates with 100 μg/mL poly-L-lysine (Sigma-Aldrich) and 0.2 U/mL poly dA-dT (Sigma-Aldrich) in 0.1 M Tris-HCl pH 7.6. Sera were diluted serially on dsDNA plates, and autoantibodies were detected with IgG2[a]-biotin, IgG2a[b]-biotin, and SA-HRP. NP plates were generated by coating 96-well plates with 1 μg/mL NP-BSA (Biosearch, conjugation ratio 23) in PBS. Sera from NP-RSA immunized and unimmunized mice were diluted serially, and NP-specific IgG1 was detected with IgG1[a]-biotin, IgG1[b]-biotin, and SA-HRP. All ELISA plates were developed with TMB (Sigma) and stopped with 2N sulfuric acid. Absorbance was measured at 450 nm. For total IgM, total IgD, and dsDNA IgG2a, OD values or OD ratios were calculated and displayed. For NP-specific IgG1, titers were calculated at OD = 0.2 and normalized such that the average IgG1[a]/IgG1[b] ratio in immunized Balb/c-B6 F1 mice equals 1.0.

B Cell Purification and Western Blots. Splenic B cells were purified by negative selection with a MACS kit according to manufacturer’s protocol (Miltenyi Biotech, cat# 130-090-862). Purified B cells were lysed in 2N 4X SDS and boiled at 95C for 5 minutes. 500,000 cells per lane were loaded onto NuPAGE 4-12% Bis-Tris gels (Invitrogen NP0335), run for 50 minutes at 200V, and transferred onto PVDF membranes using XCell II Blot Module (Invitrogen). Membranes were blocked with 3% BSA. Proteins were detected with rabbit monoclonal anti-Ets1 (Epitomics, custom lot provided by Lee Ann Garrett-
Sinha), mouse anti-mouse GAPDH (Santa Cruz Biotechnology 32233), and HRP-
conjugated secondary antibodies goat anti-rabbit IgG (H+L) and goat anti-mouse IgG
(H+L) (SouthernBiotech). Membranes were developed with Western Lightning Plus ECL
(Perkin Elmer 0RT2651 and 0RT2751) and imaged using a ChemiDoc Touch Imaging
System (Bio-Rad). Quantifications were performed using Image Lab v. 5.2.14 (Bio-Rad).

**Immunizations.** Mice were immunized i.p. with 200 uL of 10% sheep red blood cells
(Rockland R406-0050) diluted in PBS. Mice were sacrificed 5 days after immunization, and serum and spleens were harvested. Mice were immunized i.p. with 100 ug of NP-
conjugated rabbit serum album (NP-RSA, Biosearch N-5054-10, conjugation ratio 10)
prepared in PBS with Alhydrogel 1% adjuvant (Accurate Chemical and Scientific Corp.
21645-51-2). NP-RSA immunized mice were sacrificed 7-8 days following
immunization, and serum and spleens were harvested. To ensure that our calculated
values accurately reflect the magnitude and variability of immune response induced by
immunization, we analyzed all mice that had larger plasma cell and germinal center
compartments than unimmunized mice. One IgM+/− mouse immunized with SRBCs
(Figure 7E-F) was excluded because it did not display an expanded plasma cell
compartment; no other mice were excluded from analysis.

**Quantitative PCR.** Splenocytes were lysed in TRIzol (Invitrogen) and stored at -80 C.
cDNA was prepared using a SuperScriptIII kit (Invitrogen). Quantitative PCR reactions
were run on a QuantStudio 12K Flex thermal cycler (Applied Biosystems) with FastStart
Universal SYBR Green Master Mix (Roche). Nr4a1 (forward: gcctagcactgccaaattg;
reverse: ggaaccagagagcaagtcat) and GAPDH (forward: aggtcgggtgaacggatttg; reverse: ttagaccatgttaggtgaggtca) primers were used at 250 nM each.

**FIGURE LEGENDS**

**Figure 1. IgD expression enables a dynamic range of IgM responsiveness.**

(A) GFP expression in mature follicular splenic B cells (CD19+CD23+CD93−) from Nur77-eGFP BAC Tg reporter mice with either a wild-type BCR repertoire (left), or harboring IgHEL Tg specific for the cognate antigen HEL (hen egg lysozyme) in the absence (middle), or presence (right) of endogenous cognate antigen driven by soluble HEL Tg. WT mature follicular B cells lacking GFP reporter are included for reference (gray shaded histograms).

(B) Surface IgM and IgD expression in splenic Fo B cells from WT, \(IgM^{−/−}\), and \(IgD^{−/−}\) mice expressing the Nur77-eGFP reporter.

(C) Splenocytes from WT, \(IgM^{−/−}\), and \(IgD^{−/−}\) mice were loaded with Indo-1 and stimulated with 2.5 μg/mL of F(ab’)2 anti-IgM or 1:400 anti-IgD. Fo B cells with the highest 20% and lowest 20% Nur77-eGFP expression are compared.

(D) Representative histograms and quantification of surface Igκ expression in \(IgM^{−/−}\) and \(IgD^{−/−}\) Fo B cells normalized to WT.

(E) Representative histograms and quantification of Nur77-eGFP expression in \(IgM^{−/−}\) and \(IgD^{−/−}\) Fo B cells normalized to WT.

(F) Median surface Igκ expression of WT, \(IgM^{−/−}\), and \(IgD^{−/−}\) Fo B cells was calculated for 200 bins of equal width across the Nur77-eGFP spectrum.

For (A), (B) and (F), data are representative of at least \(n = 4\) independent experiments.
For (C), n = 3 independent experiments for anti-IgM and n = 2 independent experiments for anti-IgD. For (D) and (E), n = 7 and n = 4, respectively, WT, IgM\(^{-/-}\), and IgD\(^{-/-}\) mice. Welch’s t test was used to calculate p values, and mean + SEM is displayed. ***p < 0.001.

Figure 1-figure supplement 1. Regulation of Nur77 and Nur77-eGFP reporter expression.

(A) Nur77-eGFP expression in mature Fo (B220+CD93-CD23+) B cells from mice with mutations in various signaling pathways (CD40L\(^{-/-}\), Unc93b1\(^{3d/3d}\), TLR7\(^{-/-}\)). This Unc93b1 mutation abolishes signaling through TLR3, TLR7, and TLR9.

(B) Nur77-eGFP and CD69 induction in B220+ splenocytes from Nur77-eGFP reporter mice stimulated with either anti-IgM or IL-4 \textit{in vitro} for 24 hours.

(C) Splenic B cells were stimulated with CXCR4 ligand (SDF-1) \textit{in vitro} for 18 hours and Nur77-eGFP expression and CXCR4 downregulation were assessed.

(D-E) Splenocytes from N=5 Germ-free and SPF mice were taken directly \textit{ex vivo} and either permeabilized and stained to detect B220+ cells and endogenous Nur77 by intracellular staining (D), or total Nr4a1 transcript by qPCR (E).

(F) PerC (peritoneal cavity) cells from n = 4 MB1 Cre\(^{+}\) MyD88\(^{0/0}\) and controls were permeabilized and stained immediately \textit{ex vivo} to detect B1a cells and endogenous Nur77 by intracellular staining. In D-E, Nr4a1\(^{-/-}\) splenocytes serve as a control both for Ab and primer specificity.

(G) Splenocytes from mice analyzed in (F) were harvested directly \textit{ex vivo} to detect total Nr4a1 transcript by qPCR.
Data in (A) and (C) are representative of n = 2 independent experiments. Data in (B) are representative of n = 3 independent experiments.

**Figure 1-figure supplement 2. Quantification of surface BCR expression on B cell subsets.**

(A) Surface Igκ expression relative to Nur77-eGFP reporter in Fo B cells from WT, IgM−/−, and IgD−/− mice.

(B) Mean surface Igκ was calculated for splenic B cell (B220+) subsets in WT, IgM−/−, and IgD−/− mice. T1 (CD93+CD23−); T2 (CD93+CD23+); Fo (CD93-CD23+); MZ (CD21hiCD23lo).

(C) Surface Igλ on B cell subsets described in (B)

(D) Surface Igκ MFI of Igκ+ cells in peritoneal B cell (CD19+) subsets. B1a (CD5+CD23−); B1b (CD5-CD23-); B2 (CD5-CD23+).

(E) Surface Igλ MFI of Igλ+ cells in peritoneal B cell subsets described in (D).

Data in (A) are representative of n = 3 independent experiments. Values in (B-E) were calculated from n = 3 mice of each genotype. One-way ANOVA with Tukey’s multiple comparisons test (B-E) was used to calculate p values, and mean + SEM is displayed. *p < 0.05, **p < 0.01, ***p < 0.001.

**Figure 1-figure supplement 3. Induction of Nur77-eGFP and CD69 in TLR-stimulated IgM−/− and IgD−/− B cells.**

(A) Nur77-eGFP and CD69 upregulation in IgM−/− and IgD−/− splenic B cells stimulated with indicated doses of LPS, CpG DNA, and Pam3CSK4. Histograms compare
unstimulated cells with cells incubated with indicated stimuli for 18 hours at 37°C.

Histograms are representative of two independent experiments.

**Figure 2. IgD signals strongly in vitro but weakly in vivo.**

(A) Median intracellular pErk in splenic Fo (B220+CD23+) B cells stimulated anti-Igκ for 15 minutes.
(B) Erk phosphorylation kinetics in Fo B cells stimulated with 15 μg/mL anti-Igκ.
(C) Splenocytes from IgM−/− and IgD−/− mice were loaded with Indo-1 and stimulated with 2.5 or 5 μg/mL anti-Igκ. (D) CD86 induction in CD23+ IgM−/− and IgD−/− splenocytes stimulated with anti-Igκ for 18 hours.
(E) Summary data for CD86 MFI in (D).
(F) Nur77-eGFP induction in cells from (D).
(G) Nur77-eGFP in cells from (D) incubated with medium alone (0 μg/mL anti-Igκ).
(H) Representative histograms and summary data for MHC-II induction in unstimulated cells from (D).
(I) Basal calcium in unstimulated IgM−/− and IgD−/− Fo (B220+CD23+CD93−) B cells was calculated by normalizing the geometric mean of [Indo-1(violet)/Indo-1(blue)] to WT B cells in the same experiment.

For (A), signaling in cells from n = 2 IgM−/− and IgD−/− mice is displayed, and results for 1.5, 3, and 15 μg/mL of anti-Igκ were replicated in n = 3 independent experiments. Data in (B) was compiled from n = 3 independent experiments with n = 3 mice of each genotype in each experiment. Data in (C) are representative of n = 4 independent experiments for 5 μg/mL and n = 2 independent experiments for 2.5 μg/mL.
values were calculated for splenocytes from $n = 3$ mice of each genotype. For (I), basal calcium ratios from $n = 6$ independent experiments are compiled. Welch’s t test was used to calculate p values, and mean ± SEM is displayed. *p < 0.05, **p < 0.01, ***p < 0.001.

**Figure 2-figure supplement 1. S6 and calcium signaling in $IgM^{+/−}$ and $IgD^{+/−}$ B cells.**

(A) S6 phosphorylation kinetics in splenic CD23+ B cells following stimulation with 15 $\mu$g/mL anti-Igκ.

(B) Splenocytes from $IgM^{−/−}$ and $IgD^{−/−}$ mice were loaded with Indo-1 and stimulated with 10 or 5 $\mu$g/mL anti-Igκ or anti-Igκ F(ab’)$_2$.

Data (A) and (B) are representative of $n = 3$ and $n = 2$ independent experiments, respectively. Welch’s t test was used to calculate p values, and mean ± SEM is displayed.

* $p < 0.05.$

**Figure 3. Reduced in vivo antigen sensing by IgD in innate-like B cells.**

(A) Nur77-eGFP in peritoneal B1a (CD19+CD5+CD23−) and splenic MZ (B220+CD21hiCD23lo) B cells from WT and $IgM^{−/−}$ mice.

(B) Nur77-eGFP in PtC-binding peritoneal B1a cells from WT, $IgM^{−/−}$, and $IgD^{−/−}$ mice.

(C) Nur77-eGFP and PtC MFIs were calculated for total B1a and PtC-binding B1a cells.

The ratio of the $IgM^{+/−}$ (IgD-only) MFI to the $IgD^{+/−}$ (IgM-only) MFI is displayed with a 95% confidence interval.

(D) Representative histograms of Nur77-eGFP in $IgM^{−/−}$ and $IgD^{−/−}$ splenic MZ B cells from mice without (left) and with (right) a BAFF overexpression transgene.

(E) Quantification of Nur77-eGFP in MZ B cells from (D).
For (A) and (E), \( n = 3 \) mice of each genotype were analyzed. For (B), histograms are representative of \( n = 4 \) mice of each genotype. For (C), ratios are pooled for \( n = 4 \) mice of each genotype from three independent experiments. For (D), histograms are representative of \( n = 3 \) mice of each genotype. Welch’s t test (A and E) was used to calculate p values, and mean + SEM is displayed (except in C). *p < 0.05.

Figure 3-figure supplement 1. The effect of BAFF, competition, and allotype on Igκ and Nur77-eGFP expression.

(A) Surface Igκ expression on IgM\(^{-/-}\) and IgD\(^{-/-}\) splenic MZ B cells from mice without (left) and with (right) a BAFF overexpression transgene.

(B) Nur77-eGFP expression in peritoneal B1a and splenic MZ B cells from IgM-null (IgD\([a]+\)) and WT (IgM\([b]+\)) loci in IgM\(^{-/-}\) mice.

(C) Nur77-eGFP expression in IgM\([a]+\) and IgM\([b]+\) splenic MZ B cells in 6-month-old IgH\(^{a/b}\) mice with a BAFF overexpression transgene.

Values (A) were calculated from \( n = 3 \) mice of each genotype. Values in (B) and (C) were calculated in \( n = 6 \) and \( n = 4 \) mice, respectively. Welch’s t test (A) and a paired t test (B-C) were used to calculate p values, and mean + SEM is displayed. **p < 0.01, ***p < 0.001.

Figure 4. Cell-intrinsic skewing of B cell developmental decisions by IgM and IgD BCRs.

(A) Allelic exclusion leads to a 1:1 mixture of IgM-only and IgD-only B cells in IgM\(^{+/+}\) IgD\(^{+/+}\) mice.
(B) Proportion of peritoneal B1a (CD19+CD5+CD23-) and splenic MZ (B220+CD21^{hi}CD23^{lo}) B cells originating from each Ig locus in IgM^{+/-} IgD^{+/-} mice.

(C) Relative competition between IgM+ and IgD+ B cells in IgM^{+/-} IgD^{+/-} mice was calculated for bone marrow, splenic, and peritoneal B cell compartments. Results include data from (B) for reference. Immature (CD23-CD93+); T2-like (CD23+CD93+); mature recirculating (CD23+CD93-); T1 (CD93+CD23-); T2 (CD93+CD23+); Fo (CD93-CD23+); MZ (CD21^{hi}CD23^{lo}); B1a (CD5+CD23-); B1b (CD5-CD23-); B2 (CD5-CD23+).

(D) Relative competition between WT (IgM[b]+) and IgM-null (IgD[a]+) B cells in IgM^{+/-} mice was determined as in (C).

(E) Relative competition between WT (IgM[b]+) and IgD-null (IgM[a]+) B cells in IgD^{+/-} mice was determined for splenic and peritoneal compartments as described in (C).

(F) Competition in peritoneal B1a and splenic MZ compartments in IgM^{+/-} mice with or without a BAFF overexpression transgene. Results include data from (D) for reference. For (B) and (C), n = 3-5 mice were analyzed. For (D), n = 3-8 mice were analyzed. For (E), n = 5 mice were analyzed. For (F), n = 4-5 mice of each genotype were analyzed.

Welch’s t test was used to calculate p values, and mean ± SEM is displayed. **p < 0.01, ***p < 0.001.

Figure 4-figure supplement 1. B cell subset development in IgM^{+/-}, IgD^{+/-}, WT, and IgH^{a/b} mice.

(A) Signal strength model of B1a and MZ B cell development. While B1a and MZ cells originate from different precursor populations, their development is thought to be signal
strength-dependent. (B) B cell compartments as a percentage of total B220+ splenic B cells. T1 (CD93+CD23-); T2 (CD93+CD23+); Fo (CD93-CD23+); MZ (CD21<sup>hi</sup>CD23<sup>lo</sup>). (C) B cell compartments as a percentage of total CD19+ peritoneal B cells. B1a (CD5+CD23-); B1b (CD5-CD23-); B2 (CD5-CD23+). (D) Relative competition between IgM[a]+ and IgM[b]+ B cells in Balb/c-B6 F1 mice was calculated for splenic and peritoneal compartments described in (B-C). For (B), n = 10-12 mice of each genotype were analyzed. For (C), n = 6 mice of each genotype were analyzed. For (D), n = 3 mice were analyzed. Mice in (D) were 6-14 weeks old. One-way ANOVA with Tukey’s multiple comparisons test (B-C) was used to calculate p values, and mean ± SEM is displayed. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 5. IgD can drive polyclonal activation and germinal center entry, but not anti-dsDNA IgG2a production, in Lyn<sup>−/−</sup> mice. (A) Surface CD69 and CD86 expression on CD23+ splenic B cells from each Ig locus in IgM<sup>+/−</sup> mice on Lyn<sup>+/+</sup> and Lyn<sup>−/−</sup> backgrounds. (B) Percentage of unswitched germinal center (CD19+ Fas<sup>hi</sup> GL-7<sup>hi</sup> IgM/IgD+) B cells from each Ig locus in IgM<sup>+/−</sup> and IgD<sup>+/−</sup> mice on the Lyn<sup>−/−</sup> background. (C) Anti-dsDNA IgG2a titers from each Ig locus in IgM<sup>+/−</sup> Lyn<sup>−/−</sup> mice were calculated by ELISA using pooled IgH<sub>αβ</sub> autoimmune serum with high-titer autoantibodies from each locus as a reference (titer set at 1000). (D) Anti-dsDNA IgG2a titers in IgD<sup>+/−</sup> Lyn<sup>−/−</sup> mice were calculated as in (C).
(E) Paired anti-dsDNA titers from each locus in $\text{IgM}^{+/\pm}$ $\text{Lyn}^{\pm/-}$ mice from (C) with additional mice from 24 weeks to 12 months.

(F) Paired anti-dsDNA titers from each locus in $\text{IgD}^{+/\pm}$ $\text{Lyn}^{\pm/-}$ mice from (D).

(G) Ratio of anti-dsDNA IgG2a[a] to IgG2a[b] from all samples in (E) and (F) with an anti-dsDNA IgG2a titer > 250 from either locus; cutoff defined by titers in young WT mice.

For (A), n = 4 $\text{IgM}^{+/\pm}$ $\text{Lyn}^{\pm/-}$ mice are compared to a reference $\text{IgM}^{+/\pm}$ $\text{Lyn}^{\pm/+}$ mouse. Qualitatively similar results were obtained in two independent experiments. For (B) n = 5-6 mice of each genotype were analyzed. For (C) and (D), n = 9 and n = 12 mice were tracked. For (G), n = 36 $\text{IgM}^{+/\pm}$ $\text{Lyn}^{\pm/-}$ and n = 39 $\text{IgD}^{+/\pm}$ $\text{Lyn}^{\pm/-}$ anti-dsDNA IgG2a+ samples were compared. Welch’s t test was used to calculate p values, and mean ± SEM is displayed. **p < 0.01.

Figure 5-figure supplement 1. Splenic B cell subsets in $\text{IgH}^{a/b}$, $\text{IgM}^{+/\pm}$, and $\text{IgD}^{+/\pm}$ $\text{Lyn}^{\pm/-}$ mice.

(A) Gating scheme for determining Ig locus of origin for splenic B cell subsets in 6-month-old $\text{IgH}^{a/b}$ $\text{Lyn}^{\pm/-}$ mice. CD21$^\text{hi}$ cells did not express surface BCR and were not considered bona fide MZ B cells.

(B) Quantification of compartments in (A). T1 (CD93$^+$CD23$^-$); T2 (CD93$^+$CD23$^+$); Fo (CD93$^-$CD23$^+$).

(C) Percentage of Fo B cells originating from each Ig locus in $\text{IgM}^{+/\pm}$ and $\text{IgD}^{+/\pm}$ mice on the $\text{Lyn}^{\pm/-}$ background.
Percentages in (A-C) were calculated using \( n = 5-6 \) mice of each genotype, and mean + SEM is displayed.

**Figure 5-figure supplement 2. Signaling in \( \text{IgM}^{+/} \) and \( \text{IgD}^{+/} \) \( \text{Lyn}^{-/-} \) B cells.**

(A) \( \text{IgM}^{+/} \) and \( \text{IgD}^{+/} \) mice on either \( \text{Lyn}^{+/+} \) or \( \text{Lyn}^{-/-} \) backgrounds were loaded with Indo-1 and stimulated with anti-Igκ. WT (\( \text{IgM}[b]+ \)) B cells were gated out to isolate IgM-null (IgD-only) and IgD-null (IgM-only) cells. Mature (CD19+CD23+) B cells are displayed. (B) Intracellular Erk phosphorylation in mature (B220+CD23+) B cells from mice in (A) stimulated with anti-Igκ for 5 minutes. Shaded histogram corresponds to IgM[b]+ cells from \( \text{IgD}^{+/} \) \( \text{Lyn}^{-/-} \) mice incubated with medium alone. (A) and (B) are representative of two independent experiments.

**Figure 5-figure supplement 3. Nur77-eGFP in \( \text{Lyn}^{-/-} \) B cells and the role of BCR allotype in \( \text{Lyn}^{-/-} \) phenotypes.**

(A) Nur77-eGFP expression in splenic mature follicular B cells from \( \text{Lyn}^{+/+} \) and \( \text{Lyn}^{-/-} \) mice. (B) Gating scheme for determining the Ig locus of origin for germinal center B cells in 6-month-old \( \text{IgM}^{+/} \) \( \text{Lyn}^{-/-} \) mice. (C) Relative contribution of IgH[a]+ and IgH[b]+ B cells to the splenic unswitched GC (CD19+ Fas+ GL-7+ IgM+) and unswitched PC (CD138+ IgM+) compartments of 6-month-old \( \text{IgH}^{a/b} \) \( \text{Lyn}^{-/-} \) mice. (D) Anti-dsDNA IgG2a from each Ig locus in 5-6 month old \( \text{IgM}^{+/}, \text{IgD}^{+/}, \) and \( \text{IgH}^{a/b} \) mice on the \( \text{Lyn}^{-/-} \) background was quantified by ELISA.
The OD ratio of knockout (allotype a) to WT (allotype b) anti-dsDNA IgG2a was calculated for all mice in (D) in which either locus produced an OD > 0.3. Histograms in (A) are representative of at least n = 3 mice of each genotype. Percentages in (B-C) were calculated using n = 5-6 mice of each genotype, and mean ± SEM is displayed. For (D), n = 10-13 mice of each genotype were analyzed. For (E), n = 5-9 autoantibody-positive mice were analyzed. Welch’s t test (E) was used to calculate p values, and mean ± SEM is displayed. *p < 0.05.

Figure 6. Cell-intrinsic IgM expression is required for unswitched plasma cell expansion in Lyn−/− mice.

(A) Representative blot and quantification of Ets1 and GAPDH protein in purified splenic B cells from WT, Lyn−/−, and IgM−/− Lyn−/− mice.

(B) Composition of the CD138+ plasma cell compartments in the spleens and bone marrow of WT, Lyn−/−, and IgM−/− Lyn−/− mice was determined by intracellular staining of IgM, IgD, and IgA.

(C) Percentages in (B) multiplied by the fraction of live cells positive for CD138 in each tissue. Unswitched cells are positive for either IgM or IgD. Statistics correspond to unswitched plasma cell percentages; differences in IgA+ cells were not significant.

(D) Serum IgM in 16-week-old mice was quantified for B6.IgHa (WT) and Lyn−/− mice by ELISA. A sample from an IgM−/− mouse is shown for reference.

(E) Serum IgD in 16-week-old mice was quantified for IgM−/− and IgM−/− Lyn−/− mice by ELISA. A sample from a WT mouse is shown for reference.

(F) Gating scheme for quantifying the unswitched splenic plasma cell composition of
IgM+/− Lyn−/− mice. (G) Percentage of unswitched splenic plasma cells (CD138+B220loIgM/IgD+) from each locus in IgM+/− and IgD+/− mice on the Lyn−/− background. For (B) and (C), figures are representative of n = 4-5 mice of each genotype. For (D), values from n = 3 WT and n = 4 Lyn−/− mice are averaged. For (E), values from n = 3 IgM−/− and n = 4 IgM−/− Lyn−/− mice are averaged. For (F) and (G), n = 4-5 mice of each genotype were used. Welch’s t test was used to calculate p values, and mean ± SEM is displayed. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 6-figure supplement 1. BCR signaling in Lyn−/− peritoneal B cell subsets.
(A) Intracellular Erk phosphorylation in peritoneal B220+ B cells stimulated with anti- Igκ for 5 minutes. B1a (CD5+CD23−); B2 (CD5−CD23+). Histograms in (A) are representative of cells from n = 2 WT and Lyn−/− mice.

Figure 6-figure supplement 2. Model of how Lyn restrains follicular B cells.
(A) Lyn restrains BCR signaling in Fo B cells. Loss of Lyn leads to Btk-dependent repression of Ets1 and expansion of unswitched (IgM+) plasma cells.

Figure 7. IgD-only cells have intact germinal center responses but impaired IgG1+ plasma cell responses.
(A) Splenic B (CD19+) cells from unimmunized and WT, IgM−/−, and IgD−/− mice 5 days after i.p. immunization with 200 µL of 10% SRBCs. (B) Quantification of germinal center (Fashi GL-7hi) cells in (A).
(C) Splenocytes from unimmunized and WT, IgM\textsuperscript{+/−}, and IgD\textsuperscript{+/−} mice 5 days after i.p. immunization with 200 μL of 10% SRBCs.

(D) Quantification of CD138\textsuperscript{+} IgG1\textsuperscript{+} plasma cells in (C).

(E) WT (IgM\textsuperscript{[b]+}) and IgM-null (IgD\textsuperscript{[a]+}) germinal center B cells as a percentage of live splenocytes in unimmunized and IgM\textsuperscript{+/−} mice 5 days after i.p. immunization with 200 μL of 10% SRBCs.

(F) WT (IgG1\textsuperscript{[b]+}) and IgM-null (IgG1\textsuperscript{[a]+}) switched plasma cells (CD138\textsuperscript{+}IgG1\textsuperscript{+}) as a percentage of live splenocytes in unimmunized and IgM\textsuperscript{+/−} mice 5 days after i.p. immunization with 200 μL of 10% SRBCs.

(G) Fraction of unswitched NP-specific germinal center cells (CD19\textsuperscript{+} Fas\textsuperscript{hi} GL-7\textsuperscript{hi} IgM/IgD\textsuperscript{+}) from the IgH\textsuperscript{[a]} locus in the spleens of Balb/c-B6 F1 and IgM\textsuperscript{+/−} mice 7-8 days after i.p. immunization with 100 μg NP-RSA.

(H) Fraction of IgG1\textsuperscript{+} CD138\textsuperscript{+} plasma cells from the IgH\textsuperscript{[a]} locus in Balb/c-B6 F1 and IgM\textsuperscript{+/−} mice 7-8 days after i.p. immunization with 100 μg NP-RSA.

(I) NP-specific IgG1\textsuperscript{[a]} and IgG1\textsuperscript{[b]} titers at OD = 0.2 were calculated for the mice in (G-H) by ELISA. The IgG1\textsuperscript{[a]} to IgG1\textsuperscript{[b]} titer ratio was calculated for each mouse, and all ratios were normalized such that the average IgG1\textsuperscript{[a]}/IgG1\textsuperscript{[b]} ratio in Balb/c-B6 F1 samples = 1.0.

For (A-D), statistics from n = 4 unimmunized mice of each genotype and n = 3 WT, n = 6 IgM\textsuperscript{+/−}, and n = 7 IgD\textsuperscript{+/−} immunized mice were pooled. For (E-F), n = 5 unimmunized and n = 5 immunized mice are shown. For (G-I), n = 5 Balb/c-B6 F1 mice and n = 3 IgM\textsuperscript{+/−} mice are shown. One-way ANOVA with Tukey’s multiple comparisons test (B...
and D), a paired t test (E-F), and Welch’s t test (G-I) were used to calculate p values, and mean + SEM is displayed. *p < 0.05, **p < 0.01, ***p < 0.001.

**Figure 7-figure supplement 1. Role of BCR allotype and generation of unswitched plasma cells in SRBC-immunized mice.**

(A) Quantification of IgM[a]+ and IgM[b]+ germinal center B cells (CD19+ Fas$^{hi}$ GL-7$^{hi}$) as a percentage of live splenocytes in $IgH^{a/b}$ mice 5 days after i.p. immunization with 200 $\mu$L of 10% SRBCs.

(B) Quantification of IgM[a]+ and IgM[b]+ plasma cells (CD138+) as a percentage of live splenocytes in $IgH^{a/b}$ mice 5 days after i.p. immunization with 200 $\mu$L of 10% SRBCs.

(C) Quantification of splenic unswitched (IgM+ or IgD+) CD138+ plasma cells from unimmunized and WT, $IgM^{+/ -}$, and $IgD^{+/ -}$ mice 5 days after i.p. immunization with 200 $\mu$L of 10% SRBCs.

(D) WT (IgM[b]+) and IgM-null (IgD[a]+) unswitched plasma cells (CD138+) as a percentage of live splenocytes in unimmunized and $IgM^{+/ -}$ mice 5 days after i.p. immunization with 200 $\mu$L of 10% SRBCs.

For (A) and (B), n = 3 mice were analyzed. Paired t tests (A-B and D) and one-way ANOVA with Tukey’s multiple comparisons test were used to calculate p values, and mean + SEM is displayed. For (C), cell numbers from n = 4 unimmunized mice of each genotype and n = 3 WT, n = 6 $IgM^{+/ -}$, and n = 7 $IgD^{+/ -}$ immunized mice were analyzed. For (D), n = 5 unimmunized and n = 5 immunized were analyzed. **p < 0.01, ***p < 0.001.
Figure 7-figure supplement 2. Role of IgM and IgD in regulating rapid antibody responses.

(A) Model: Control of peripheral B cell tolerance by IgM and IgD. Selective downregulation of IgM is a well-described feature of autoreactive B cells, and this study demonstrates how loss of IgM could desensitize B cells towards self-antigens. High and invariant IgD expression provides survival signals to allow these autoreactive cells to be maintained in the repertoire. Upon activation by foreign antigen, the least autoreactive cells receive strong signals through IgM and can quickly differentiate into plasma cells with little danger of autoimmunity. In contrast, the most autoreactive B cells are activated through IgD, which is less efficient at transducing signals in vivo. This weak signaling forces IgD^{hi} IgM^{lo} cells into the germinal center where they can be “redeemed” via somatic hypermutation, as proposed by Goodnow and colleagues. In this way, dual expression of IgM and IgD expression facilitates repertoire diversity and balances immune responsiveness with autoimmune potential.

SOURCE DATA FIGURE LEGENDS

Figure 1-source data 1.
- Numerical data corresponding to receptor and Nur77-eGFP levels in Figure 1D-F.
- Source code used to calculate median Igx for given levels of Nur77-eGFP in Figure 1F.
- Numerical data corresponding to endogenous Nur77 in germ-free mice depicted in Figure 1-figure supplement 1D.
- Numerical data corresponding to *Nr4a1* transcript in germ-free mice depicted in Figure 1-figure supplement 1E.

- Numerical data corresponding to endogenous Nur77 in MyD88-conditional-knockout peritoneal B1a cells in Figure 1-figure supplement 1F.

- Numerical data corresponding to *Nr4a1* transcript in MyD88-conditional-knockout splenocytes in Figure 1-figure supplement 1G.

- Numerical data corresponding to surface BCR levels on splenic B cells subsets in Figure 1-figure supplement 2B-C.

- Numerical data corresponding to surface BCR levels on peritoneal B cell subsets in Figure 1-figure supplement 2D-E.

**Figure 1-source data 2.** Numerical data corresponding to receptor levels in Figure 1D.

**Figure 1-source data 3.** Numerical data corresponding to Nur77-eGFP levels in Figure 1E.

**Figure 2-source data 1.**

- Numerical data corresponding to ERK phosphorylation in Figure 2A and B

- Numerical data corresponding to activation marker upregulation in Figure 2E-H.

- Numerical data corresponding to basal calcium in Figure 2I.

- Numerical data corresponding to S6 phosphorylation in Figure 2-figure supplement 1A.

**Figure 2-source data 2.** Numerical data corresponding to basal calcium in Figure 2I.

**Figure 3-source data 1.**
- Numerical data corresponding to Nur77-eGFP expression in innate-like B cells in Figure 3A.

- Numerical data corresponding to Nur77-eGFP expression and PtC binding in B1a cells in Figure 3C.

- Numerical data corresponding to Nur77-eGFP and surface receptor levels in BaffTg+ MZ B cells in Figure 3E and Figure 3-figure supplement 1A.

- Numerical data corresponding to Nur77-eGFP expression in innate-like B cells in Figure 3-figure supplement 1B.

- Numerical data corresponding to Nur77-eGFP expression in MZ B cells in Figure 3-figure supplement 1C.

**Figure 3-source data 2.** Numerical data corresponding to Nur77-eGFP expression and PtC binding in B1a cells in Figure 3C.

**Figure 4-source data 1.**

- Numerical data corresponding to competition between IgM-only, IgD-only and WT B cells in Figure 4B-E.

- Numerical data corresponding to competition between IgD-only and WT B cells in Figure 4F.

- Numerical data corresponding to splenic and peritoneal B cell compartment sizes in Figure 4-figure supplement 1B-C.

- Numerical data corresponding to competition between IgH[a] and IgH[b] B cells in Figure 4-figure supplement 1D.
Figure 4-source data 2. Numerical data corresponding to competition between IgM-
only, IgD-only and WT B cells in Figure 4B-E.

Figure 4-source data 3. Numerical data corresponding to competition between IgD-only
and WT B cells in Figure 4F.

Figure 5-source data 1.

- Numerical data corresponding to activation marker upregulation in Figure 5A.
- Numerical data corresponding to germinal center composition in Figure 5B.
- Numerical data corresponding to autoantibody production in Figure 5C-G.
- Numerical data corresponding to cellular phenotypes of $\text{IgH}^{\text{a/b}} \text{Lyn}^{-/-}$ mice in Figure 5-
figure supplement 1A-B and Figure 5-figure supplement 3C.
- Numerical data corresponding to follicular B cell competition in Lyn-deficient $\text{IgM}^{+/+}$
and $\text{IgD}^{+/+}$ mice in Figure 5-figure supplement 1C.
- Numerical data corresponding to germinal center composition in Figure 5-figure
supplement 3B.
- Numerical data corresponding to autoantibody production in Figure 5-figure supplement
3D-E.

Figure 6-source data 1.

- Numerical data corresponding to Ets1 expression in splenic B cells in Figure 6A.
- Numerical data corresponding to plasma cell compartments in Figure 6B-C.
- Numerical data corresponding to serum IgM titers in Figure 6D.
- Numerical data corresponding to serum IgD titers in Figure 6E.
- Numerical data corresponding to plasma cell competition in Figure 6F and G.

**Figure 6-source data 2.** Numerical data corresponding to Ets1 expression in splenic B cells in Figure 6A.

**Figure 6-source data 3** Numerical data corresponding to plasma cell compartments in Figure 6B-C.

**Figure 7-source data 1.** Numerical data corresponding to germinal center and plasma cell responses in Figure 7A-F.

- Numerical data corresponding to NP-RSA responses in Figure 7G-I
- Numerical data corresponding to germinal center and plasma cell responses in Figure 7-figure supplement 1A-B.
- Numerical data corresponding to unswitched plasma cell responses in Figure 7-figure supplement 1C-D.

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<td>TLR7</td>
<td>Nur77-eGFP</td>
<td>B cells</td>
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<td>Un93b1 3d/3d</td>
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<td>MyD88 fl/fl MB1-Cre</td>
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<td>End. Nur77 protein / transcript</td>
<td>PerC B1a cells / spleen</td>
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<td>Const. act. STAT5</td>
<td>Jak/Stat</td>
<td>Nur77-eGFP</td>
<td>Thymocytes</td>
<td>Moran et al. JEM 2011, Figure 8.</td>
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<td>Does not induce Nur77 in vitro</td>
<td>BAFF</td>
<td>BAAFR</td>
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<td>Zikherman et al. Nature 2012: Figure S1G</td>
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<td>IL-4</td>
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<td>IL-2, IL-15</td>
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<td>Nur77-eGFP</td>
<td>CD8 (IL-2, 15), CD4 (IL-2)</td>
<td>Au-Yeung et al. JI 2017, Figures S1A, 3C, 4A</td>
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<td>CXCL12/ SDF-1</td>
<td>CXCR4</td>
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<td>Induces Nur77 in vitro but does not require IgM or IgD specifically</td>
<td>LPS</td>
<td>TLR4, Rp150</td>
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<td>Modulates pathway at steady state in vivo</td>
<td>IgHEL Tg</td>
<td>Antigen/BCR</td>
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Figure 1. IgD expression enables a dynamic range of IgM responsiveness

(A) Representative histograms and quantification of surface Igκ expression in IgM-/- and IgD-/- Fo B cells normal and transgenic for Nur77-eGFP. 

(B) Indirect immunofluorescence for surface Igκ expression in WT (IgM+IgD) and IgM-/- (IgD-only) B cells, with or without Nur77-eGFP transgene.

(C) Intracellular staining for IgM and IgD in WT and IgM-/- (IgD-only) B cells, with or without Nur77-eGFP transgene.

(D) Median surface Igκ expression of WT, IgM-/-, and IgD-/- Fo B cells was calculated for 200 bins of equal width.

(E) Surface Igκ expression (fraction of WT) in IgM-/- and IgD-/- B cells, with or without Nur77-eGFP transgene.

(F) Median Igκ expression in WT, IgM-/- (IgD-only), IgD-/- (IgM-only), and IgM-/- IgD-/- B cells, with or without Nur77-eGFP transgene.
**A**

- **WT** Nur77<sup>eGFP</sup> Tg
- **Mutant** Nur77<sup>eGFP</sup> Tg

- **CD40L**<sup>−/−</sup>
- **Unc93b1<sup>−/−</sup>
- **TLR7**<sup>−/−</sup>

GFP % of Max

**B**

- **No Stim.**
- **Anti-IgM 6.4 μg/ml**

GFP % of Max

**C**

- **SDF1 (CXCL12)**
  - 100 ng/ml
  - 33.3 ng/ml
  - 11.1 ng/ml
  - 3.7 ng/ml
  - Media

CXCR4 % of Max

**D**

- **Spleenic B Cells**

endogenous Nur77 (MFI)

**E**

- **Total splenocytes**

Relative Expression (MFI)

**F**

- **Peritoneal B1a**

endogenous Nur77 (MFI)

**G**

- **Total splenocytes**

Relative Expression (MFI)
**FIGURE 1**

(A) Surface Igk expression relative to Nur77-eGFP in reporter Fo B cells from WT, IgM-/-, and IgD-/- mice.

(B) Mean surface Igk was calculated for splenic B cell (B220+) subsets in WT, IgM-/-, and IgD-/- mice.

(C) Surface IgM on B cell subsets described in (B).

(D) Surface Igk MFI of Igk+ cells in peritoneal B cell (CD1λ+) subsets. B1a (CD5+CD23-); B1b (CD5-CD23-); B2

(E) Surface IgM MFI of IgM+ cells in peritoneal B cell (CD1λ+) subsets described in (D).

Data in (A) are representative of n = 3 independent experiments. Values in (B-E) were calculated from n = 3 mice of each genotype. One-way ANOVA with Tukey's multiple comparisons test (B-E) was used to calculate p values, and mean + SEM is displayed. *p < 0.05, **p < 0.01, ***p < 0.001.
(A) Median intracellular pErk in splenic Fo (B220+CD23+) B cells stimulated with anti-Igκ for 15 minutes.

(B) Erk phosphorylation kinetics in Fo B cells stimulated with 15 μg/m/ anti-Igκ.

(C) Splenocytes from IgM-/- and IgD-/- mice were loaded with Indo-1 and stimulated with 2.5 or 5 μg/m/ anti-Igκ.

(D) CD86 induction in CD23+ IgM-/- and IgD-/- splenocytes stimulated with anti-Igκ for 1 hour.

(E) Summary data for CD86 MFI in (D).

(F) Nur77-eGFP induction in cells from (D).

(G) Nur77-eGFP in cells from (D) incubated with medium alone (0 μg/m/ anti-Igκ).

(+ and *) Representative histograms and summary data for MHC-II induction in unstimulated cells from (D).

The geometric mean of Indo-1 (violet)/ Indo-1 (blue) relative to WT B cells in the same experiment.

For (A), signaling in cells from n = 2 IgM-/- and IgD-/- mice is displayed, and results for 1.5, 3, and 15 μg/m/ of anti-Igκ were replicated in n = 3 independent experiments. Data in (B) was compiled from n = 3 independent experiments with n = 3 mice of each genotype in each experiment. Data in (C) are representative of n = independent experiments for 5 μg/m/ and n = 2 independent experiments for 2.5 μg/m/.

For (D-+), values were calculated for splenocytes from n = 3 mice of each genotype. For (I), basal calcium ratios from n = independent experiments are compiled. Welch's t test was used to calculate p values, and mean ± SEM is displayed. *p < 0.05, **p < 0.01, ***p < 0.001.
(A) S phosphorylation kinetics in splenic CD23+ B cells following stimulation with 15 µg/mL anti-Igκ.

(B) Splenocytes from IgM-/- and IgD-/- mice were loaded with Indo-1 and stimulated with 10 or 5 µg/mL anti-Igκ or anti-Igκ F(ab')2.

Data (A) and (B) are representative of n = 3 and n = 2 independent experiments, respectively. Welch's t test was used to calculate p values, and mean ± SEM is displayed. *p < 0.05.
(A) Surface Igκ expression on IgM-/- and IgD-/- splenic MZ B cells from mice without (left) and with (right) a BAFF overexpression transgene.

(B) Nur77-eGFP expression in peritoneal B1a and splenic MZ B cells from IgM-null (IgD-only) and WT (IgM+/IgD+) loci in IgM+/IgD+ mice.

(C) Nur77-eGFP expression in IgM+/IgD+ and IgM+/-IgD-/- splenic MZ B cells in -month-old IgM+/IgD+ mice with a BAFF overexpression transgene.

Values (A) were calculated from n = 3 mice of each genotype. Values in (B) and (C) were calculated in n =  and n =  mice, respectively. Welch's t test (A) and a paired t test (B-C) were used to calculate p values, and mean ± SEM is displayed. **p < 0.01, ***p < 0.001.
A

B

C

D

**T1 T2 Fo MZ**

0 20 40 60

% of Splenic B Cells

IgM-/- (IgD-only)

IgD-/- (IgM-only)

WT (IgM+IgD)

0 20 40 60

% of Peritoneal B Cells

IgM-/- (IgD-only)

IgD-/- (IgM-only)

WT (IgM+IgD)

A

B1a MZ

Precursor Cells

Strong BCR Signal

Weak BCR Signal

B1a

MZ

Balb/c (IgH[a]) Locus

B6 (IgH[b]) Locus

Spleen PerC

T1 T2 Fo MZ B1a B2

0.0 0.5 1.0

Fraction of Compartment

T1 T2 Fo MZ B1a B2

spleen PerC
A

B

C

D

E

F

G
A

B220+ CD23

CD21 lo CD23

CD21 b CD93 (AA4.1)

IgM[b]

23.9% 31.2%

54.1% 45.9%

T1 T2 Fo

B

Fraction of Compartment

C

Fraction of Fo B Cells

WT (IgM+IgD) IgM-null (IgD-only) IgD-null (IgM-only)

IgM+/- IgD+/-
WT Unstimulated anti-Igκ. WT (IgM>b@+) B cells were gated out to isolate IgM-null (IgD-only) and IgD-null (IgM-only) cells. Mature

(B) Intracellular Erk phosphorylation in mature (B220+CD23+) B cells from mice in (A) stimulated with anti-Igκ for 5 minutes. Shaded histogram corresponds to IgM>b@+ cells from IgD+/-/yn-/- mice incubated with medium alone.
Perk
B2
B1a
Media 5 ug/mL anti-Igκ 10 ug/mL anti-Igκ Lyn+/+ Lyn−/−

(A) Intracellular Erk phosphorylation in peritoneal B220+ B cells stimulated with anti-Igκ for 5 minutes. B1a histograms in (A) are representative of cells from n = 2 WT and Lyn−/− mice.
WT (IgM+IgD)

IgM-/-(IgD only)

IgD-/-(IgM only)

- + - + - + SRBC

0.0

0.5

1.0

1.5

2.0

2.5

3.0

3.5

4.0

4.5

5.0

G (GC (% of Splenic B Cells))

Fas

GL-7

Intracellular IgG1

IgG1+ PC (% of Splenocytes)

Balb-B6 F1

IgM

+/-

0

10

20

30

40

50

**

IgG1+ PC (% IgH[a])

Balb-B6 F1

IgM+/-

0

5

10

15

20

25

30

35

40

45

50

**

NP-Specific IgG1

IgG1[a]/IgG1[b] (AU)

**

UNSW  NP+ GC (%IgH[a])

IgG1+ PC (% of Splenocytes)

F

IgM+/- IgM+/

-IgD+/-

SRBC

+SRBC

**

IgM-null (IgD-only)

WT (IgM+IgD)