Themis2 lowers the threshold for B cell activation during positive selection

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Abstract

The positive and negative selection of lymphocytes by antigen is central to adaptive immunity and self-tolerance, yet how this is determined by different antigens is incompletely understood. Here we report that Themis2 increased the positive selection of B1 cells and germinal center B cells by self and foreign antigens. We found that Themis2 lowered the threshold for B cell activation by low but not high avidity antigens. Themis2 constitutively bound the adaptor protein Grb2, *src*-kinase Lyn and signal transducer PLCγ2, and increased activation of PLCγ2 and its downstream pathways following B cell receptor stimulation. These findings identify a unique function for Themis2 in differential signaling and provide insight into how B cells discriminate between antigens of different quantity and quality.

During formation of the pre-immune repertoire, highly abundant self-antigens induce the negative selection of immature B cells through deletion, receptor editing or anergy[1], while some less abundant or intracellular self-antigens trigger the positive selection of the polyspecific innate-like B1 B cell subset, at least during early ontogeny[2]. Later, once the repertoire has been formed, some high-avidity and regularly arrayed antigens can generate a sufficiently strong B cell receptor (BCR) signal to induce proliferation and plasma cell differentiation in the absence of T cell help[3]. However, the majority of B cell activation involves interactions with lower avidity and/or low affinity antigens such as soluble proteins, which may be tolerogenic[4], or immunogenic antigens coupled via complement or Fc receptors and displayed on the membranes of macrophages, non-cognate B cells or follicular dendritic cells[5,6]. Little is known about how B cells discriminate between these different antigenic forms, especially when compared to T cell development, partly because of limited tools available to study B cell cognate responses *in vivo* and *in vitro*.

Themis2 (Icb-1), a protein that is primarily expressed in B cells and monocytes[7], is a member of the newly described Themis family of proteins, which is found in animal species from mammals to cnidarians and characterized by the presence of one or two copies of a novel cysteine-containing globular CABIT domain[8]. Themis2 shares 29% identity and 65% homology at the amino acid level with its T cell homologue, Themis1[8]. While not much is known about the function of Themis2, Themis1 has been shown to have a critical function in positive selection of $CD8^+CD4^+$ T cells in the thymus and their transition to the $CD8^{int}CD4^+$ transitional stage[8-12]. Themis1 is constitutively associated with the signal adaptor protein Grb2 via a PxRPxK 'proline-rich' domain[8,9,11,13] and upon T cell activation it is rapidly tyrosine phosphorylated by the protein tyrosine kinases Lck and ZAP70 and recruited by Grb2 to the transmembrane adapter LAT[13-16]. It has been proposed that Themis1 acts as an inhibitory analogue to digital converter in developing T cells, ensuring that low affinity peptide-MHC and T cell antigen receptor (TCR) interactions do not transduce high intensity signals that result in negative selection but instead generate signals appropriate for positive selection[17]. Furthermore, Themis1 and 2 are functionally interchangeable in T

cell development, as transgenic expression of Themis2 can rescue the development and signaling defects in *Themis1*$^{-/-}$ mice[15]. Given this evidence of shared function, we hypothesized that Themis2 might be a regulator of the B cell response to different antigens during development and activation.

Here we investigated the function of Themis2 in B lymphocytes. We have shown that Themis2 regulates the positive selection of B1 B cells by self-antigen and the positive selection of germinal center (GC) B cells in response to foreign antigen. Themis2 lowered the threshold for B cell activation by low avidity antigens, but was not required for activation by high-avidity antigens. Themis2 bound and facilitated the activation of Phospholipase Cγ2 (PLCγ2), downstream Ca2+ mobilization and activation of Erk1/2 signaling pathways. These data provide insights into how B cells discriminate between qualitatively different antigenic signals via the same BCR and highlight how antigens of different quantity and quality may be able to fine-tune the immune response.

Results

The expression and gene-targeting of Themis2

To investigate the function of Themis2 we first used flow cytometry to isolate distinct B and T cell subsets from C57BL/6 (B6) mice and quantified *Themis1* and *Themis2* mRNA expression by quantitative PCR (qPCR). The subsets were defined here (and later) as: pro/pre ($B220^{+}CD43^{+}IgM^{-}IgD^{-}$), immature ($B220^{+}CD43^{-}IgM^{+}IgD^{-}$), B1 ($FSC^{high}B220^{lo}CD19^{+}IgM^{++}IgD^{-}$), follicular ($B220^{++}CD23^{+}CD21^{+}$) and marginal zone (MZ, $B220^{++}CD23^{-}CD21^{++}$) B cells; and double negative (DN, $CD4^{-}CD8^{-}$), double positive (DP, $CD4^{+}CD8^{+}$) and $CD4^{+}$ and $CD8^{+}$ single positive (SP) thymocytes. GC ($B220^{++}GL7^{+}CD95^{+}$) and class-switched ($B220^{++}IgG_1^{+}$) B cells were isolated from B6 mice that had been immunized with sheep red blood cells (SRBCs). *Themis2* mRNA was detected in all B cell populations analyzed, with expression 5-10 fold higher in immature, follicular and B1 B cells than MZ, GC or class-switched B cells **(Fig. 1a)**. There was minimal expression of *Themis2* mRNA in DP thymocytes **(Fig. 1a)**. In contrast, *Themis1* was expressed in DN, DP and $CD4^{+}$ and $CD8^{+}$ SP thymocytes and its absolute expression in the T-lineage was up to ten-fold higher than that of Themis2 in B cells (**Fig. 1b**). *Themis1* mRNA was not detected in B cells (data not shown).

To investigate the function of Themis2 in B cells, we generated Themis2-deficient mice (called $Themis2^{-/-}$ hereafter) by gene targeting and deleting *Themis2* exon 4 in B6 x S129/Sv F_1 ES cells (**Supplementary Fig 1a**). Exon 4 encodes the C terminus of the first CABIT domain and the entire second CABIT domain and PxRPxK motif, and its deletion introduced a frame-shift mutation[8]. *Themis2* mRNA and protein were absent in $Themis2^{-/-}$ splenocytes, as assessed by qPCR and immunoblotting (**Fig. 1c, d**). LC-MS/MS peptide mapping identified twelve high-scoring peptides across Themis2 in wild-type splenocytes, including seven between amino acids 1 and 163 within exons 1-3, but none of these peptides were sequenced by MS/MS in $Themis2^{-/-}$ splenocytes (**Supplementary Fig 1b**). In summary, our findings demonstrated differential expression of *Themis2* and *Themis1* in B cells and T cells respectively, and confirmed the absence of Themis2 protein expression in the $Themis2^{-/-}$ exon 4 knockout mice.

Themis2 is not essential for B cell development or survival

Analysis of B cell development in wild-type and *Themis2*$^{-/-}$ mice showed similar numbers of pro/pre, immature and mature B cells in the bone marrow (BM); B220^{+}CD23^{-}CD21^{-} transitional, follicular and MZ B cells in the spleen and lymph nodes; and B1 B cells in the peritoneal cavity (**Fig 2a and Supplementary Fig. 2a**). Serum IgM antibody titers in unimmunized wild-type and *Themis2*$^{-/-}$ mice were also equivalent (**Fig. 2b**).

Examination of the phenotype of B cells revealed higher expression of IgM on *Themis2*$^{-/-}$ B220^{+}IgD^{+} follicular B cells in the BM and spleen compared to wild-type follicular B cells (**Fig. 2c,d**). High IgM expression has been previously associated with reduced spontaneous BCR signaling[18], suggesting that Themis2 deficiency might affect BCR signaling and the selection of B cells.

To exclude the possibility that alterations in the B cell repertoire in the *Themis2*$^{-/-}$ mice might be masked by compensatory B cell proliferation during development, we generated mixed BM chimeric mice. Equal mixes of CD45.1 wild-type and CD45.2 *Themis2*$^{-/-}$ whole BM cells were injected intravenously into lethally irradiated CD45.1 wild-type recipient mice, and these animals were compared to mice reconstituted with CD45.1 wild-type and CD45.2 wild-type donors. Eight weeks after BM transfer, the reconstitution of pro-pre, immature, transitional, follicular and marginal zone B cells derived from CD45.2 *Themis2*$^{-/-}$ did not differ significantly between subsets and was similar overall to that of CD45.2 wild-type BM in the control mice (**Supplementary Fig. 2b**). These finding confirmed that B cell development and B cell expansion in the absence of Themis2 were normal. In the mixed chimeras, *Themis2*$^{-/-}$ B220^{+}IgD^{+} follicular B cells again showed higher expression of IgM than their wild-type counterparts, indicating that this effect was B cell intrinsic (**Supplementary Fig 2c**).

Although our qPCR analysis failed to detect *Themis1* mRNA in selected B cell subsets, it remained possible that low or stage-specific expression of Themis1 might compensate for loss of Themis2. To test this, we intercrossed *Themis1*$^{-/-}$

and *Themis2*$^{-/-}$ strains to generate *Themis1*$^{-/-}$*Themis2*$^{-/-}$ double-deficient mice. The *Themis1*$^{-/-}$*Themis2*$^{-/-}$ mice showed a block in T cell development at the transition from DP to SP stage, which was the same as that seen in *Themis1*$^{-/-}$ mice, and normal B cell development, as seen in the *Themis2*$^{-/-}$ strain (**Supplementary Fig. 3a-d**). These findings identified separate and non-redundant functions for Themis1 and Themis2 in T and B cells respectively. Whereas Themis2 was not essential for B cell development in naïve mice, its deficiency enhanced IgM expression on follicular B cells, a sign that has been correlated elsewhere with decreased levels of intrinsic BCR signaling[18].

Themis2 increases positive selection to self and foreign antigens

Immunization of *Themis2*$^{-/-}$ mice with the T-independent antigen 4-hydroxy-3-nitrophenylacetyl (NP)-Ficoll or NP coupled to chicken gamma globulin (NP_{19}-CGG) plus adjuvant showed no significant defect in the antibody response compared to wild-type mice (**Supplementary Fig. 4a, b**). However, immunization with intraperitoneal SRBC alone induced fewer GC and switched IgG_1 B cells after 8 days in *Themis2*$^{-/-}$ mice than in wild-type mice (**Fig 3a, b**). Immunization with a lower avidity form of the NP antigen, NP_3-CGG, plus adjuvant, also induced lower antibody titers in *Themis2*$^{-/-}$ mice compared with wild-type mice after 7 and 14 days (**Fig 3c).**

Because subtle effects on the B cell response are difficult to detect in a polyclonal repertoire, we crossed *Themis2*$^{-/-}$ mice with the MD4 transgenic strain (hereafter referred to as Ig^{HEL}), which expresses a high affinity IgM^aIgD^a BCR receptor for hen egg lysozyme (HEL; $K_a=2x10^{10}M^{-1}$) derived from the Balb/c hybridoma HyHEL-10[19]. The development of pro/pre, immature, follicular and MZ B cell subsets was similar in wild-type Ig^{HEL} and *Themis2*$^{-/-}$ Ig^{HEL} mice (**Fig. 4a**). To assess negative selection by self-antigens, wild-type Ig^{HEL} and *Themis2*$^{-/-}$ Ig^{HEL} mice were crossed to transgenic mice expressing either soluble HEL (sHEL) as neo-self antigen under the metallothionein promoter or membrane bound antigen HEL (mHEL) under the class I promoter[20]. Mature $B220^+IgD^+$ B cells from wild-type and *Themis2*$^{-/-}$ Ig^{HEL}/sHEL double transgenic mice showed down-

modulated IgM expression characteristic of B cell anergy[19] (**Supplementary Fig. 5a and b**). Likewise, self-reactive immature Ig^{HEL} cells were deleted from the repertoire by self-antigen with equal efficiency in wild-type and *Themis2*$^{-/-}$ Ig^{HEL}/mHEL double transgenic mice[20] (**Supplementary Fig. 5c**).

To explore the role of Themis2 in B cell positive selection, we crossed *Themis2*$^{-/-}$ Ig^{HEL} mice with transgenic mice expressing the $mHEL^{KK}$ variant of mHEL, which is under the same MHC class 1 promoter but restricted to the endoplasmic reticulum by a C terminal cytoplasmic di-lysine retention motif[21]. The $mHEL^{KK}$ self-antigen induces the positive selection of Ig^{HEL} B1 cells in Ig^{HEL}/$mHEL^{KK}$ double transgenic mice and promotes the generation of large numbers of anti-HEL IgM^a secreting plasma cells[21]. We detected fewer peritoneal B1 cells by flow cytometry and lower serum anti-HEL IgM^a antibody by ELISA in *Themis2*$^{-/-}$ Ig^{HEL}/$mHEL^{KK}$ mice compared to wild-type Ig^{HEL}/$mHEL^{KK}$ controls (**Fig. 4b-d**). Lower serum titers of IgM^a were also seen in naive *Themis2*$^{-/-}$ Ig^{HEL} mice compared to wild-type Ig^{HEL} controls (**Fig. 4d**), suggesting that Themis2 also plays a role in the differentiation of plasma cells in the absence of the cognate antigen.

To further assess the B cell response to a T-dependent antigen, we immunized wild-type mice with monovalent chimeric HEL-ovalbumin (HEL-OVA) in RIBI adjuvant one day after adoptive transfer of equal mixes of wild-type splenic Ig^{HEL} B cells (CD45.1) and wild-type or Themis2$^{-/-}$ splenic Ig^{HEL} B cells (CD45.2) together with splenic $CD4^+$ T cells from OTII mice, which express an MHC Class II I-A^b restricted TCR transgene specific for OVA peptide 323-339. In this experimental system, the naïve adoptively transferred Ig^{HEL} B cells are avidly recruited to the GC, where they bind HEL and receive T cell help from the OTII T cells. The ratio of CD45.1 wild-type:CD45.2 *Themis2*$^{-/-}$ $B220^{++}GL7^+CD95^+HEL^+$ Ig^{HEL} GC B cells is then compared to the ratios of input cells and CD45.1 wild-type:CD45.2 wild-type controls: Eight days after HEL-OVA immunization, the number of *Themis2*$^{-/-}$ Ig^{HEL} GC B cells was significantly reduced compared to wild-type Ig^{HEL} GC B cells (**Fig. 4e, f**). The same experimental protocol using HEL-coupled to SRBC rather than OVA-HEL, also resulted in fewer *Themis2*$^{-/-}$ Ig^{HEL} GC B cells compared to wild-type Ig^{HEL} (**Supplementary Fig. 6a**). Collectively, these

observations indicated that, while Themis2 was not required for B cell negative selection by systemic antigens, it increased the positive selection of B1 cells and plasma cells by intracellular self-antigens and the generation of IgM secreting plasma cells in unimmunized mice. Themis2 also increased the magnitude of the antibody and GC B cell response to foreign antigens, notably when the avidity of these antigens was low.

Themis2 regulates the BCR response to low avidity antigen

To investigate the response of wild-type and *Themis2*$^{-/-}$ Ig^{HEL} B cells to different forms of antigen *in vitro*, we stimulated 50:50 mixtures of CD45.1 wild-type and CD45.2 *Themis2*$^{-/-}$ Ig^{HEL} splenic B cells overnight with either sHEL (1-1,000ng/ml) or anti-IgM F(ab')$_2$ fragments (0.1-10μg/ml) or the TLR4 agonist lipopolysaccharide (LPS) (0.1-1,000ng/ml) and measured the induction of the activation markers CD69 and CD86. We found that the threshold for B cell activation by the low avidity antigen sHEL was significantly higher in *Themis2*$^{-/-}$ Ig^{HEL} B cells than in wild-type Ig^{HEL} B cells (**Fig. 5a**). Similar results were found using soluble duck egg lysozyme, which has a ~1,500 fold lower affinity for Ig^{HEL} (~K_a=1.3x10^{7}M^{-1})[22](**Fig. 5a**). The maximum sHEL-induced CD69 and CD86 expression was also lower in *Themis2*$^{-/-}$ Ig^{HEL} B cells than in wild-type Ig^{HEL} B cells (**Fig 5b**). In contrast, there was no difference in the response of wild-type or *Themis2*$^{-/-}$ Ig^{HEL} B cells to high avidity anti-IgM F(ab')$_2$ or to LPS at any concentration (**Fig 5c**).

We tested next if a similar effect might occur upon encounter of rare membrane bound antigens, of the sort expressed on follicular dendritic or other lymphoid cells *in vivo*. To assess the response to a membrane bound antigen with both low abundance and variable affinity for the Ig^{HEL} receptor, we generated a series of NIH-3T3 cell lines expressing comparably low amounts of mHEL variants with different affinities for the Ig^{HEL} receptor: mHEL-3T3 (K_a=2x10^{10}M^{-1}), mHEL^{2X}-3T3 cells (K_a=8x10^{7}M^{-1}) and mHEL^{3X}-3T3 (K_a=1.5x10^{6}M^{-1})[23]. The mHEL expression was quantified by measuring the fold increase in fluorescence, compared to untransfected NIH-3T3 cells, generated by three tandem copies of

GFP linked to the intracellular C terminus of each mHEL variant, and by extracellular staining with the HEL-specific antibody HyHEL9, whose binding is unaffected by the HEL^{2X} or HEL^{3X} mutations (**Supplementary Fig. 6b**), Overnight incubation of 50:50 mixtures of CD45.1 wild-type and CD45.2 *Themis2*$^{-/-}$ Ig^{HEL} splenic B cells with the mHEL-3T3 cells induced the expression of CD69 and CD86. In this way we found that fewer CD45.2 *Themis2*$^{-/-}$ Ig^{HEL} splenic B cells upregulated CD69 and CD86 in response to the lower affinity antigens ($mHEL^{2X}$ or $mHEL^{3X}$) at low density (fluorescence <3x background) than wild-type Ig^{HEL} B cells (**Fig. 5d**). In contrast, wildtype and *Themis2*$^{-/-}$ Ig^{HEL} B cells were equivalently activated by mHEL at low or high density or $mHEL^{3X}$ at high density (**Fig. 5d**). Similar results were found when the mHEL-3T3, $mHEL^{2X}$-3T3 and $mHEL^{3X}$-3T3 cells co-expressed a constant amount of the integrin receptor ICAM1, showing that failure of the B cells to engage ICAM1 on NIH-3T3 cells was not a limiting factor (**Supplementary Fig. 6c**). These findings demonstrate that Themis2 increases the B cell response to low avidity soluble antigens, regardless of affinity, and to membrane-bound antigens, when they are present at low affinity and low abundance.

Themis2 activates PLCγ2 and downstream pathways

Next we used Ig^{HEL} B cells to investigate the molecular basis of Themis2 function during BCR signaling. To assess calcium mobilization, we loaded equal mixes of CD45.1 Ig^{HEL} splenocytes and CD45.2 wild-type Ig^{HEL} or *Themis2*$^{-/-}$ Ig^{HEL} splenocytes with the calcium indicators Pluronic F-127 and Fura Red, AM and assessed calcium flux induced by sHEL or anti-IgM $F(ab')_2$. *Themis2*$^{-/-}$ Ig^{HEL} B cells showed lower peak calcium flux in response to sHEL compared to wild-type Ig^{HEL} B cells, without a reduction in the time to the peak response (**Fig. 6a, b**). In contrast, the calcium response to anti-IgM $F(ab')_2$ was identical in wild-type and *Themis2*$^{-/-}$ Ig^{HEL} B cells (**Fig. 6a**).

To dissect the differential effects of high and low avidity antigen stimulation on the BCR signaling pathways, we sorted splenic follicular Ig^{HEL} B cells from CD45.1 wild-type Ig^{HEL} and CD45.2 wild-type Ig^{HEL} or *Themis2*$^{-/-}$$Ig^{HEL}$ mice by negative

selection and stimulated equal mixes of B cells with sHEL or anti-IgM F(ab')$_2$. Intracellular staining of the splenic follicular Ig^{HEL} B cells showed similar activation of Syk, BLNK and downstream p38 and AKT in *Themis2*$^{-/-}$ Ig^{HEL} and wild-type Ig^{HEL} B cells after stimulation with sHEL **(Fig. 6c and Supplementary Fig. 7a)**. However, phosphorylation of both PLCγ2 and ERK1/2 was reduced in *Themis2*$^{-/-}$ Ig^{HEL} B cells compared to the co-cultured wild-type Ig^{HEL} B cells following stimulation with sHEL **(Fig. 6c, d and Supplementary Fig. 7c)**. These results are consistent with data indicating that up-regulation of CD69 and CD86 is primarily mediated by the ERK signaling pathway[24]. In contrast to the findings with sHEL, there was no difference in the phosphorylation of PLCγ2 and ERK1/2 in wild-type and *Themis2*$^{-/-}$ Ig^{HEL} B cells stimulated with anti-IgM F(ab')$_2$ (**Supplementary Fig. 6b, c**).

Intracellular staining showed equivalent recruitment of the Ras guanine nucleotide exchange factor SOS1 to the cell membrane in sHEL-stimulated wild-type Ig^{HEL} and *Themis2*$^{-/-}$ Ig^{HEL} B cells **(Fig. 6e**), suggesting that Themis2 does not directly affect Ras-ERK activation via the alternative pathway of SOS1-dependent activation. Consistent with the lack of a discernable defect in AKT activation, CD19, which can augment calcium and ERK dependent signaling, was normally phosphorylated in Themis2$^{-/-}$ Ig^{HEL} B cells in response to sHEL (**Fig. 6d**). In contrast to the observations reported in *Themis1*$^{-/-}$ T cells[25,26], there was no observable defect in SHP1 phosphorylation in *Themis2*$^{-/-}$ Ig^{HEL} B cells compared to wild-type Ig^{HEL} B cells or in the phosphorylation of SHP2, which is widely reported to be a positive regulator of ERK[17,27] (**Fig, 6f)**.

To gain further insight into the effect of Themis2 on ERK and Ca^{2+} signaling, we tested if Themis2 associated with PLCγ2. Immunoprecipitation of Themis2 from wild-type Ig^{HEL} B cells before and after stimulation with sHEL and anti-IgM F(ab')$_2$ demonstrated that Themis2 is constitutively associated with PLCγ2 and the *src*-kinase Lyn (**Fig. 7a**). Consistent with previous reports[7,15], Themis2 also co-immunoprecipitated Grb2. To explore the significance of these associations in more detail, we expressed Flag-tagged Themis2, Lyn, Syk and PLCγ2 in HEK 293 cells. Confirming the results in primary B cells, Themis2 associated with both PLCγ2 (**Fig. 7b and c**) and Lyn (**Fig. 7d and e**) in transfected HEK293 cells. A

mutant Themis2 protein lacking the PxRPxK Grb2 binding motif (Themis2-dPRR) also immunoprecipitated Lyn, and PLCγ2 (**Fig. 7f and Supplementary Fig. 7d, e**) indicating that the association between Themis2, PLCγ2 and Lyn was not mediated by Grb2. The association between Themis2 and PLCγ2 in HEK 293 cells was only slightly enhanced by PLCγ2 phosphorylation (**Fig. 7c**); however, Lyn-mediated phosphorylation of Tyr759 on PLCγ2, which has been shown to augment PLCγ2 enzymatic activity[28,29] and Lyn-mediated phosphorylation of Tyr1217 on PLCγ2 were enhanced in the presence of Themis2 (**Fig. 7e, g**). These results demonstrate that Themis2 interacts constitutively with Grb2, Lyn and PLCγ2 and increases the activation of PLCγ2 and downstream pathways in response to low avidity antigens.

Discussion

In this study we have shown that Themis2 sets the threshold for B cell activation by low avidity antigens, increasing the activation of PLCγ2 and downstream pathways. Themis2 increases the positive selection of B cells by both self and foreign antigens, but is not required for negative selection. Our findings also suggest that Themis2 may increase the constitutive activation by the BCR in the absence of self or foreign antigen, leading to IgM downregulation and increased rate of spontaneous plasma cell differentiation.

Loss of Themis2 raises the threshold for the antigen-induced up-regulation of CD69, which is required for localization of activated B cells in the lymph node[30], and reduces the expression of CD86, which is required for receiving T cell help. Themis2 may therefore be required to increase the sensitivity of naïve B cells to antigens of low abundance. Because Themis2 is a putative adaptor protein and lacks any known enzymatic activity, its binding to both Lyn and PLCγ2 may facilitate activation of PLCγ2 in response to BCR stimulation by stabilizing the interaction of Lyn and PLCγ2. The precise nature of this mechanism of activation and whether the unique CABIT domains in Themis2 have a role in this process remain to be determined.

B cell responses to antigens of relatively low avidity are likely to be important in physiological settings especially during the initial phase of T-dependent antibody responses; yet *in vitro* and biochemical studies of B cell activation have almost exclusively used high avidity analogues of T-independent antigens, typically anti-IgM $F(ab')_2$. Our data suggest that these forms of antigenic stimulation are likely to miss important aspects of differential BCR signaling. Antigens of different affinity are routinely used to assess T cell responses, and our findings indicate that similar approaches need to be implemented in the study of B cell selection.

It was recently reported that Themis2-deficiency had no effect on B cell development or antibody titres following immunization with NP-Ficoll and NP_{21}-CGG or B cell activation by anti-IgM in vitro[31]. However, that study did not include an analysis of lower valency forms of antigen such as NP_3-CGG or sHEL. Another recent study implicated Themis1 in transmitting T cell receptor (TCR) signals from low affinity, but not high affinity antigens and in attenuating rather than increasing signal strength[17]. The authors compared developmentally matched OVA-specific T cells and found an increase in TCR-dependent calcium flux and ERK signaling in the absence of Themis1. From these results, they proposed that in the absence of Themis1, signals generated from weak TCR-ligand interactions are enhanced, converting positive to negative selection and attributed this in part to a failure to activate the inhibitory phosphatase SHP1 and/or recruit SHP1 to LAT[17,25]. These results seem inconsistent with our finding that Themis2 is a positive regulator of signaling in B cells. Our data has shown that loss of Themis2 does not diminish sHEL or anti-IgM induced phosphorylation of SHP1 in B cells. Moreover, the effects of Themis2 and SHP1 deficiency on Ig^{HEL} B cells are very different, the latter characterized by increased spontaneous and antigen-induced B cell signaling to sHEL and anti-IgM $F(ab')_2$[32].

The fact that Themis2 can substitute for Themis1 in T cell development implies that, at least in thymocytes, the two proteins may have a similar mechanism of action. However, cell-specific dissimilarities, including redundancy, and a notable difference in the level of cellular expression of Themis2 in T cells and Themis1 in B cells may mean this interpretation is too simplistic. For example, the phosphatase SHP2 binds to the same Themis1-Grb2 complex as SHP1[17] but is

a positive regulator of ERK downstream of a variety of receptors[33]. SHP2 activation appears unimpaired in Themis2-deficient B cells; however, in principle, differential signaling in B and T cells might be due to competition between positive and negative regulators linked to Themis-family proteins. The amount of Themis-family proteins and other binding partners, including inhibitory coreceptors, could result in different outcomes in different cells.

A further layer of complexity and control is likely to be added by the cellular compartmentalization of signaling[34]. Most cellular ERK activation occurs on internal membranes, in a RASGRP dependent manner, and calcium dependent RasGAPs like CAPRIL and RASAL are also regulated through intracellular compartmentalization[35]. One study has linked the pattern of intracellular Ras-MAPK signaling to qualitative differences in positive and negative signaling by different antigens in the thymus[36]. Speculatively, Themis2 could additionally be affecting positive selection by recruiting PLCγ2 locally and having specific effects on these pathways. Untangling these processes and how they are dependent on different environmental cues and developmental stages, as well as on the nature and dose of antigens, will give us a better understanding of how B cells integrate upstream signals and switch between different outcomes.

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Author Contributions

D.C, M. D-L, S.C. E.Z., C.A., P.E.L and R.J.C. designed the research; D.C., M. D-L, S.C. E.Z., R.L., S.U., D.B., S.C.B., K.R.B., T.L.C., H.F., C.W., M.M., A.G.d.P. and C.A. performed and analyzed experiments; P.E.L and R.J.C. supervised the research; M. D-L, D.C., P.E.L and R.J.C. wrote the manuscript.

Competing Financial Interests

The authors declare no competing financial interests

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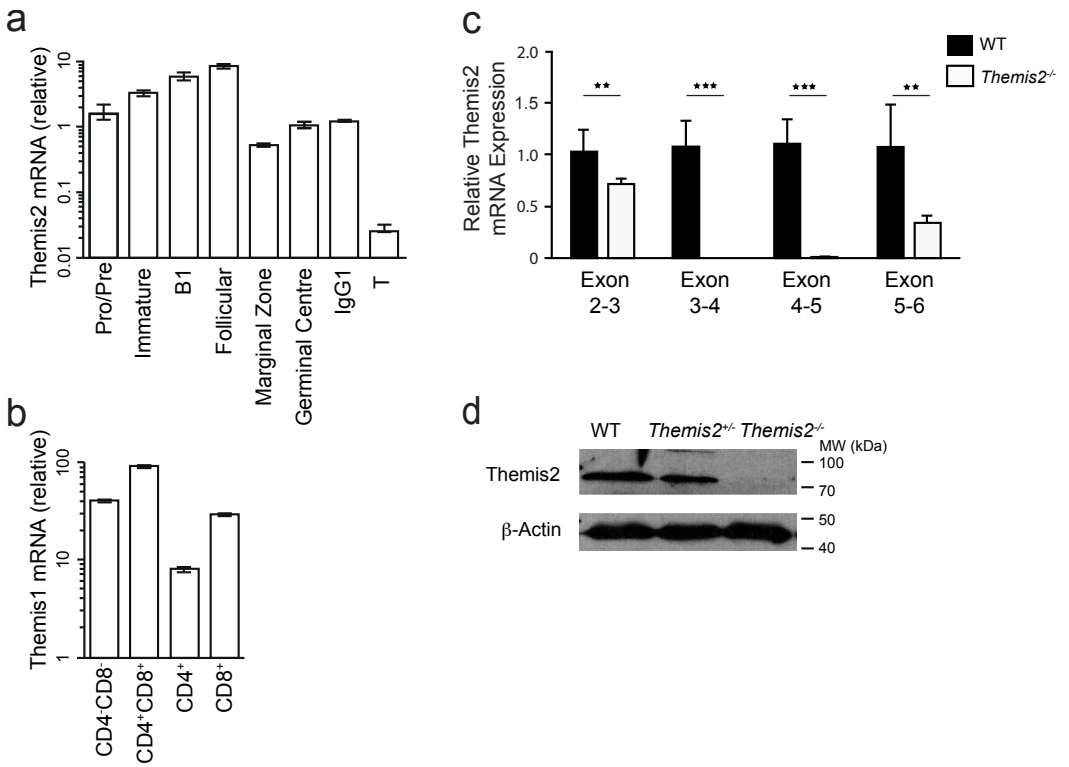

Figure 1. The expression and gene targeting of Themis2. (a, **b**) mRNA expression of *Themis2* (**a**) and *Themis1* (**b**) in flow-sorted cells from wild-type (WT) mice. (**a**) *Themis2* expression in BM pro/pre ($B220^{+}CD43^{+}IgM^{-}IgD^{-}$) and immature ($B220^{+}CD43^{-}IgM^{+}IgD^{-}$) B cells; peritoneal B1 ($B220^{lo}CD19^{+}IgM^{+}IgD^{-}$) B cells; splenic follicular ($B220^{++}CD23^{+}CD21^{+}$) and marginal ($B220^{++}CD23^{-}CD21^{++}$) B cells and germinal center ($B220^{+}GL7^{+}CD95^{+}$) and IgG1 ($B220^{+}IgG1^{+}$) B cells following immunization with SRBC. (**b**) Themis1 expression in thymic cell populations. Columns show means from triplicate sorts and qPCR with maximum and minimum measurements. (**c**) Relative expression of *Themis2* splenic mRNA across indicated exon junctions. Bars show means with 95% confidence limits and comparison by unpaired t test, **=p<0.01 and ***=p<0.001. (**d**) Representative western blot analysis of Themis2 and β-actin protein expression in splenocytes from mice carrying wild-type, heterozygote (*Themis2*$^{+/-}$) and homozygote (*Themis2*$^{-/-}$) targeted alleles of *Themis2* stained with a rabbit anti-Themis2 antibody[14].

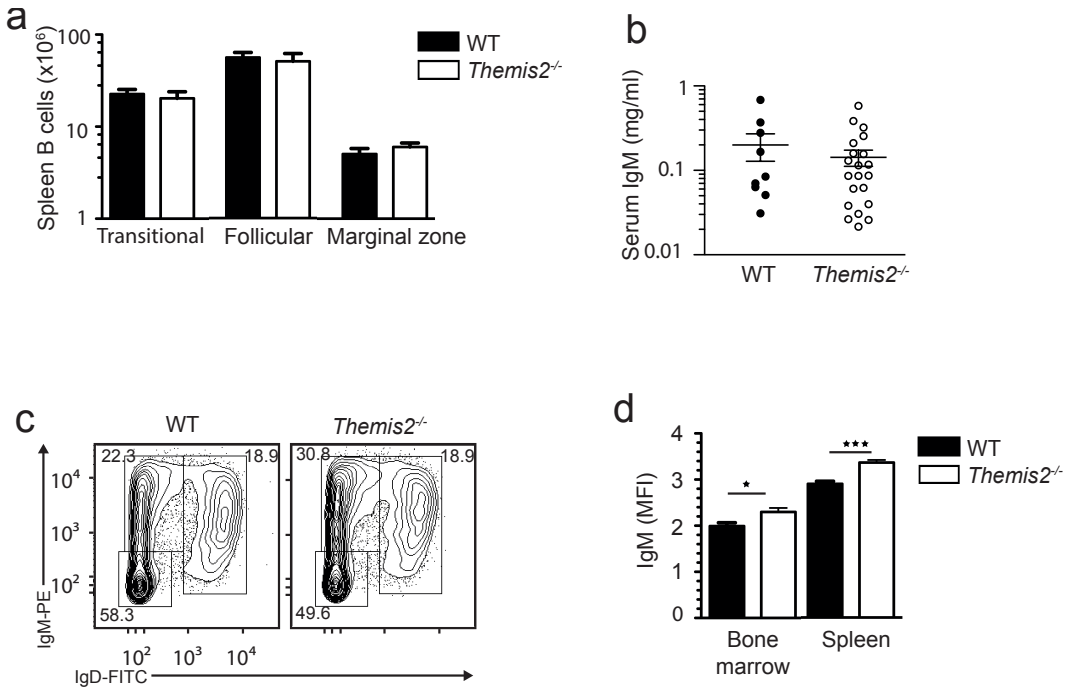

Figure 2. Themis2 is not required for generation of a pre-immune B cell repertoire but reduces BCR expression on mature B cells. (a) Number of B cells in 6 wild-type and 5 *Themis2*$^{-/-}$ mice, gated on transitional (B220^{++}CD23^{-} CD21^{lo}), follicular (B220^{++}CD23^{+}CD21^{+}) and MZ (B220^{++} CD23^{-}CD21^{++}) B cells in the spleen. Columns show means and SEM. **(b)** Serum IgM antibody titers in 9 wild-type and 21 *Themis2*$^{-/-}$ adult mice, aged 8-14 weeks. Bars show means and 95% confidence limits. **(c)** Flow cytometry of wild-type and *Themis2*$^{-/-}$ B220^{+} BM B cells showing the frequency and gating of pre/pro (IgM^{-}IgD^{-}), immature (IgM^{+}IgD^{-}) and mature (IgM^{+}IgD^{+}). Histograms are representative of more than 10 mice. (**d**) Mean cell surface IgM expression on B220^{+}IgD^{+} wild-type and *Themis2*$^{-/-}$ B cells in BM and spleen, expressed as mean fluorescent intensity (MFI) by flow cytometry and gated as in (**c**). Bars show means and 95% confidence limits and comparison by unpaired t test, *=p<0.05 and ***=p<0.001. Data are representative of results from 3 independent experiments.

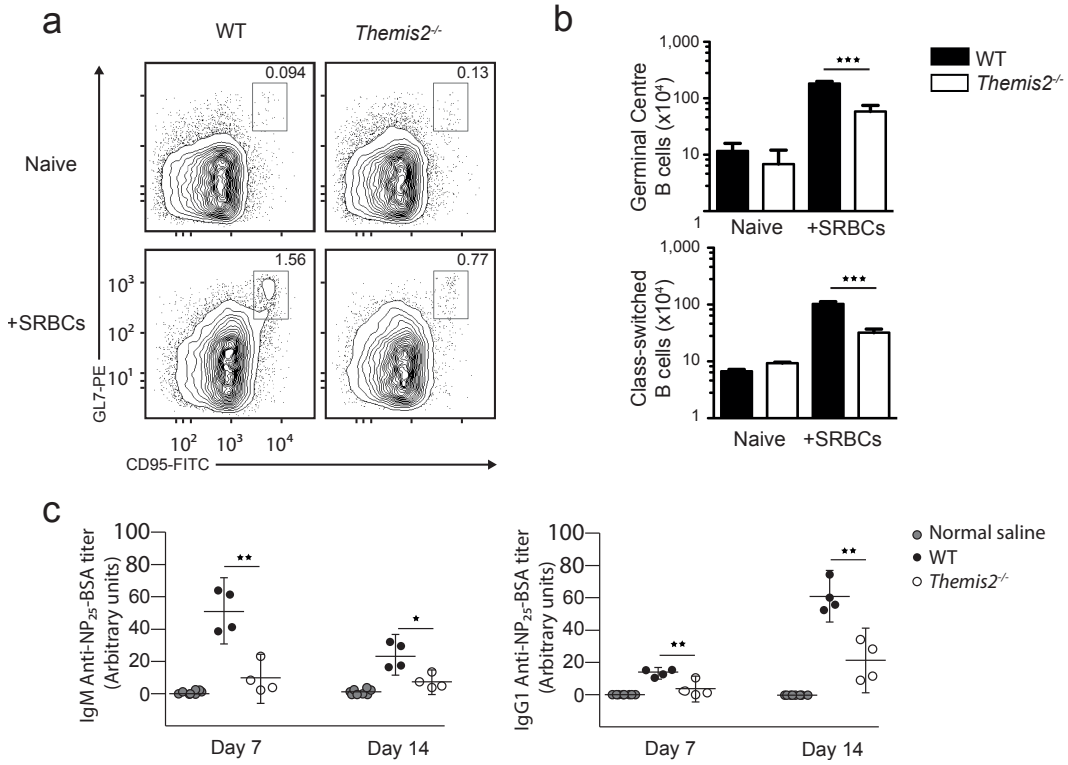

Figure 3. Reduced germinal center and switched B cells in the absence of Themis2 (a) Flow cytometry of wild-type and *Themis2*$^{-/-}$ splenic B220^{+} B cells in naïve mice and in mice 8 days after intraperitoneal (IP) immunization with SRBCs, showing the gating and typical frequency of GC (B220^{+}GL7^{+}CD95^{+}) B cell numbers. Data are representative of more than 12 mice in 3 independent experiments. (**b**) Number of GC (B220^{+}GL7^{+}CD95^{+}) B cells (upper panel) and class-switched (B220^{+}IgG1^{+}) B cells (lower panel) in wild-type and *Themis2*$^{-/-}$ mice, 8 days after immunization with SRBCs, with bars showing means and SEM. Data are representative of results from 3 independent experiments. **(c)** IgM and IgG_1 anti-NP_{25} antibody titers in WT and *Themis2*$^{-/-}$ mice 7 and 14 days after primary immunization with NP_3-CGG. Symbols represent individual mice, bars means and 95% confidence limits and comparison by unpaired t test, *=p<0.05, **=p<0.01 and ***=p<0.001

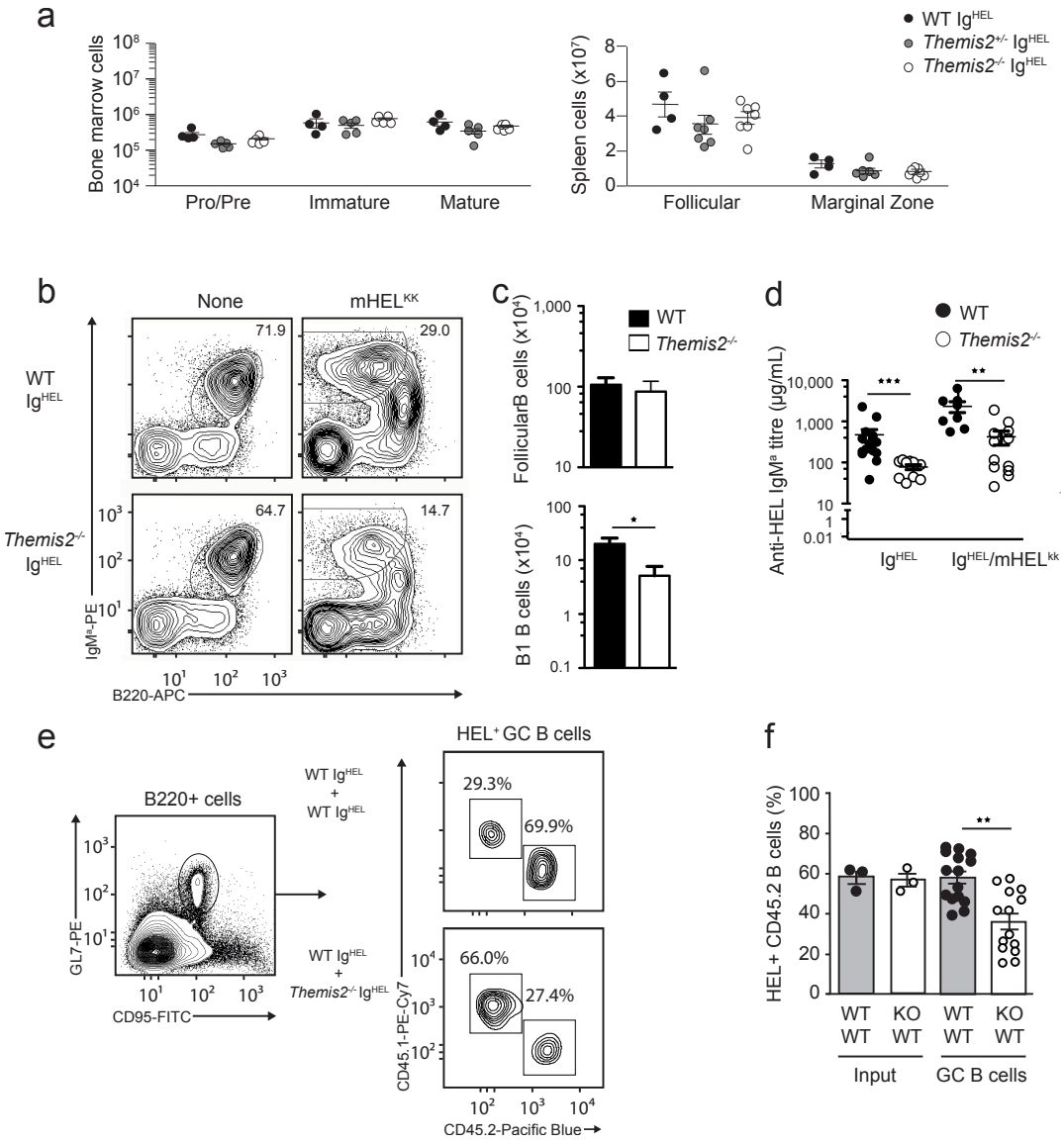

Figure 4. Themis2 is required for B cell selection by self and foreign antigen. (a) Numbers of B cell subsets in the BM and spleens of wild-type, *Themis2*$^{+/-}$ and *Themis2*$^{-/-}$ Ig^{HEL} transgenic mice (gated as in Fig 2). Symbols represent individual mice, bars means and SEM. **(b-c)** Phenotype and enumeration of peritoneal B cells from wild-type and *Themis2*$^{-/-}$ Ig^{HEL} and Ig^{HEL}/mHEL^{KK} mice, showing the flow cytometry gating **(b)** and quantification **(c)** of HEL-binding B220^{++}IgM^{a+} follicular B cells in Ig^{HEL} mice (left histograms and upper graphs) and B220^{lo}IgM^{a++} B1 B cells in Ig^{HEL}/mHEL^{KK} mice (right histograms and lower graphs). Columns show means and SEMs and data are representative of 3 experiments, n>10. (**d**) Serum anti-HEL IgM^{a} in adult wild-type and *Themis2*$^{-/-}$ Ig^{HEL} and Ig^{HEL}/mHEL^{KK} mice. Symbols represent individual

animals and bars show means and SEM. **(e)** Wild-type B6 mice were injected iv with 50:50 mixtures of CD45 allotype-marked WT:WT or WT:*Themis2*$^{-/-}$ Ig^{HEL} B cells and OTII T cells and immunized with OVA-HEL/RIBI 1 day later. Flow cytometry shows typical gating of GC B cells at 8 days and their resolution into HEL^{+}CD45.1 (wild-type) and HEL^{+}CD45.2 (wild-type or *Themis2*$^{-/-}$) cells. **(f)** Percentage of wild-type or *Themis2*$^{-/-}$ HEL^{+}CD45.2 GC B cells at input and in the GC 8 days after immunization. Columns show means and SEM and symbols represent individual mice at 8 days or separate input samples. Data combined from 3 experiments and comparison by unpaired t test, *=p<0.05, **=p<0.01 and ***=p<0.001.

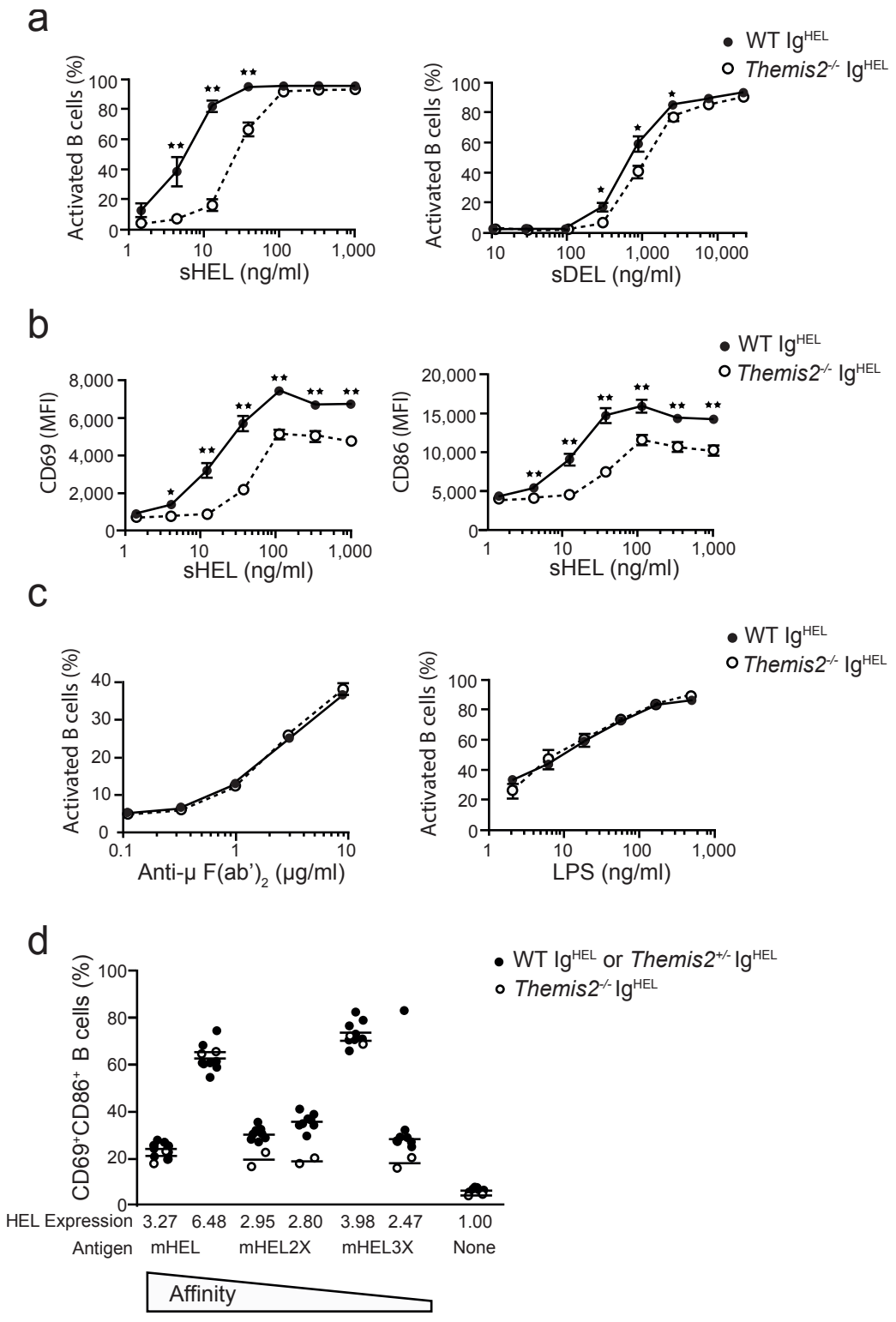

Figure 5. Themis2 sets the threshold for B cell activation by soluble and low abundance membrane bound antigen. (a) Percent of $CD69^{+}CD86^{+}$ activated wild-type and *Themis2*$^{-/-}$ Ig^{HEL} B cells following 16hr stimulation with sHEL (left) or sDEL (right). Data are representative of 6 (sHEL) and 3 (sDEL)

independent experiments and comparison by unpaired t test, *=p<0.05 and **=p<0.01. **(b)** The expression of CD69 (left) and CD86 (right) on wild-type and *Themis2*$^{-/-}$ Ig^{HEL} B cells following 16hr activation with sHEL. Comparison by unpaired t test, **=p<0.01. **(c)** Percentage of wild-type and *Themis2*$^{-/-}$ Ig^{HEL} B cells activated (CD69^{+}CD86^{+}) following 16hr stimulation with anti-IgM F(ab')$_2$ (left) or LPS (right). Data are representative of 6 experiments. **(d)** pBMN retrovirus expressing mHEL variants linked to 3 tandem intracellular GFP molecules was stably transfected into NIH3T3 cells, which were incubated overnight with CD45.2 and CD45.1 allotype-marked WT:WT or WT:*Themis2*$^{-/-}$ Ig^{HEL} B cells. The percentage of activated Ig^{HEL} B cells (CD69^{+}CD86^{+}) following overnight incubation with NIH3T3 cell clones expressing different low levels of mHEL (2 clones), and variants mHEL^{2X} and mHEL^{3X} (2 clones each). Data are representative of three separate experiments with different clones. Symbols represent separate cultures and bars show means from triplicate assays. The mean levels of mHEL expression on each clone relative to untransfected cells is displayed below the X axis and here calculated from flow cytometric staining with the anti-HEL antibody (HyHEL9).

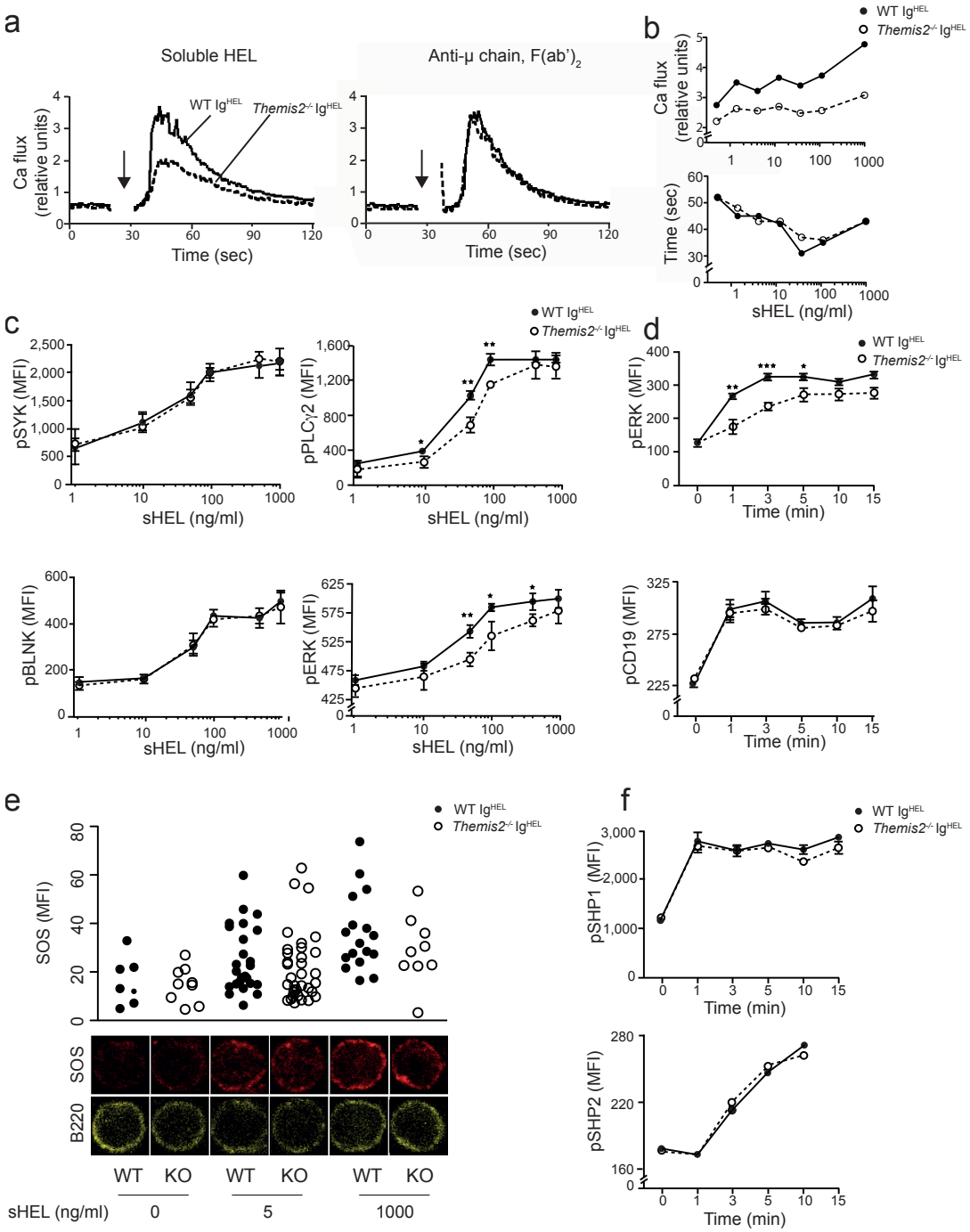

Figure 6. Themis2 sets the calcium, PLCγ2 and ERK response to low but not high avidity antigen. (a) Median calcium flux in mixed naive CD45.2 *Themis2*$^{-/-}$ and CD45.1 wild-type Ig^{HEL} B cells in response to stimulation with sHEL or anti-IgM F(ab')$_2$. Data are representative of 4 experiments. **(b)** Peak median height and time to peak height of calcium flux in response to varying concentrations of sHEL. Symbols represent individual samples. **(c)** Mean phospho-specific antibody binding to intracellular signaling molecules downstream of the BCR, 5

min after stimulation of mixed MACS sorted Ig^{HEL} CD45.2 *Themis2*$^{-/-}$ (open circles) and CD45.1 wild-type (closed circles) B cells with sHEL. **(d)** Time course of activation of pERK and pCD19 in CD45.2 *Themis2*$^{-/-}$ and CD45.1 wild-type Ig^{HEL} B cells following stimulation with 100ng/ml sHEL. Results derived from triplicate samples and bars indicate 95% confidence limits with unpaired t tests *=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$. Graphs are representative of at least 3 separate experiments in each case. **(e)** Mean SOS fluorescence intensity in confocal images from mixed wild-type and *Themis2*$^{-/-}$ Ig^{HEL} B cells stimulated for 10 min with 5ng/ml or 1μg/ml HEL, and identified as wild-type or *Themis2*$^{-/-}$ by counterstaining with B220 alone (*Themis2*$^{-/-}$*)* or B220 and CD45.1 (WT). **(f)** Mean anti-SHP1 and SHP2 phospho-specific antibody binding following stimulation of 50:50 mixtures of CD45.1 wild-type and CD45.2 *Themis2*$^{-/-}$ Ig^{HEL} B cells with sHEL. Data show means from triplicate samples and graphs are representative of 3 or more experiments.

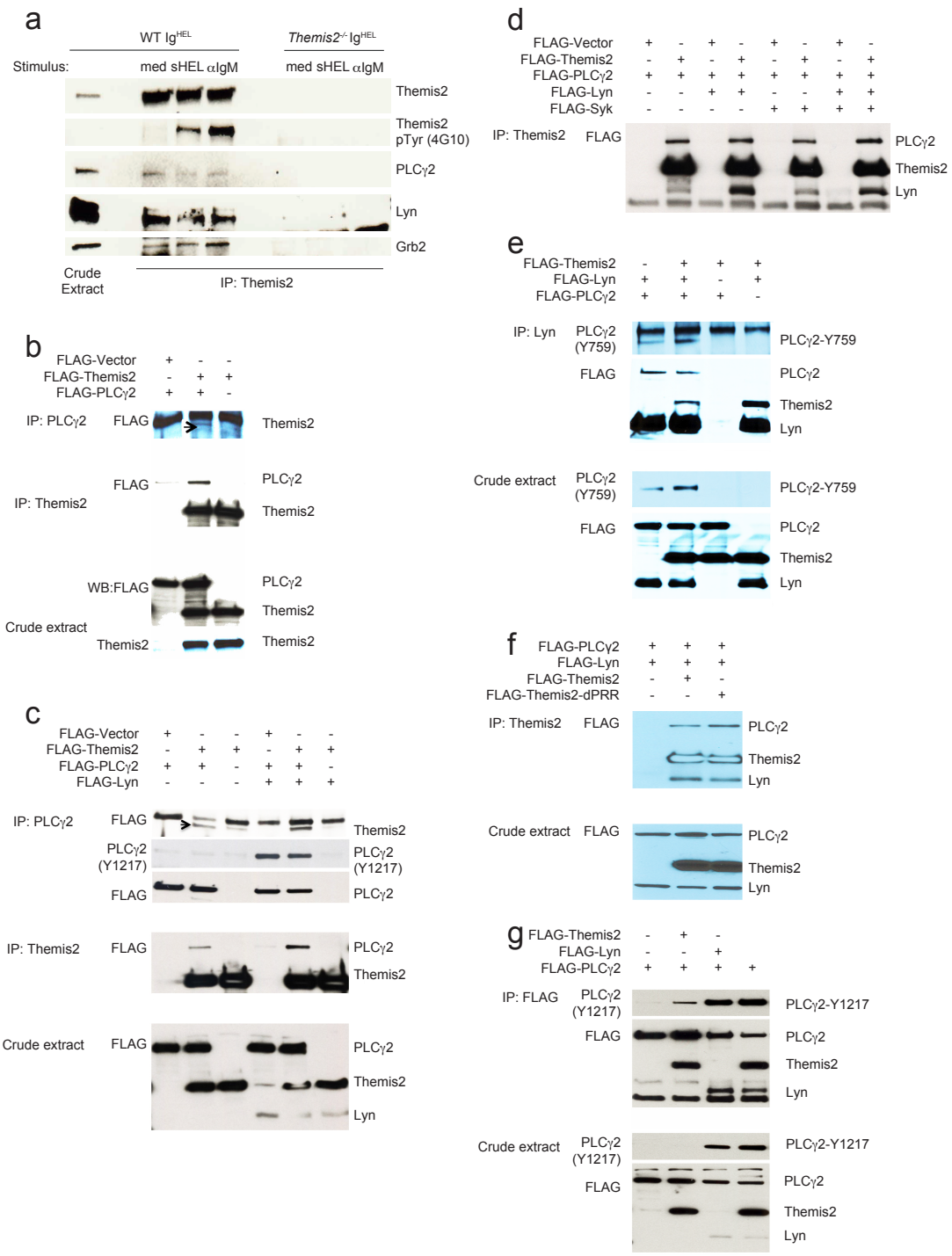

Figure 7. Themis2 binds and facilitates the activation of PLCγ2 (**a**) Western blot (WB) of crude lysate and Themis2 immunoprecipitates (IPs) from wild-type and *Themis2*$^{-/-}$ Ig^{HEL} B cells, stimulated for 5 min with medium, 100ng/ml sHEL or 10μg/ml anti-IgM F(ab')$_2$. Data representative of 3 independent experiments using 4 wild-type and 4 *Themis2*$^{-/-}$ mice each. (**b**) WB of anti-Themis2 and anti-PLCγ2 IP from HEK 293 cells expressing combinations of Flag-tagged Themis2

and PLCγ2 (**c**). WB of anti-Themis2 and anti-PLCγ2 IP from HEK 293 cells expressing combinations of Flag-tagged Themis2, Lyn and PLCγ2. Y1217 phosphorylation denotes PLCγ2 activation. (**d**). WB of anti-Themis2 IP from HEK 293 cells expressing combinations of Flag-tagged Themis2, Lyn, Syk and PLCγ2 (**e**). WB of anti-Lyn IP from HEK 293 cells expressing combinations of Flag-tagged Themis2, Lyn and PLCγ2. Y759 phosphorylation denotes PLCγ2 activation (**f**). WB of anti-FLAG IP from HEK 293 cells expressing combinations of Flag-tagged Themis2, Lyn and PLCγ2. **(g)** WB of anti-Themis2 IP from HEK 293 cells expressing combinations of Flag-tagged PLCγ2, Lyn, Themis2, and tagged Themis2-dPRR. Data are representative of 3 or more independent experiments.

Methods

Mice

Themis2-null mice were generated by homologous recombination and deletion of Themis2 exon 4 in C57BL/6 (B6) X S129/Sv F_1 ES cells. The targeted allele was backcrossed onto the B6 background for at least 10 generations (genotyping assay available on request) before intercrossing to create homozygous Themis2 deficient animals. Ig^{HEL} (C57BL/6-Tg(IghelMD4)4Ccg/J), sHEL (C57BL/6-Tg(ML5sHEL)5Ccg/J), mHEL (C57BL/6-Tg(KLK4mHEL)6Ccg/J), $mHEL^{KK}$ and OTII (C57BL/6-Tg(TcraTcrb)425Cbn/Crl) mice were as described previously[21] and maintained on a B6 background. For BM chimeras, B6 mice were irradiated with two doses of 4.5 Gy spaced by 3 hours and were injected with at least $5x10^6$ bone marrow cells (single samples or 50:50 mixture of WT B6.SJL $CD45.1^+$ BM and either $Themis2^{-/-}$ or wild-type B6 ($CD45.2^+$) BM. They were allowed to reconstitute for 8–10 weeks before immunization or analysis. All experiments included age and sex-matched littermate control animals. All experiments were approved by the NIHR or the Oxford University Ethical Review Committee and done under UK Home Office License.

Flow Cytometry

Cell suspensions from BM (one femur and tibia), spleen, thymus, mesenteric lymph nodes and peritoneal cavity were counted on a hemocytometer and stained, as described previously[21] with mAbs against the following antigens (from eBioscience, unless otherwise stated): B220, RA3-6B2-allophycocyanin (APC), phycoerythrin (PE) or fluorescein isothiocyanate (FITC); CD19, eBio1D3-PE–indotricarbocyanine (Cy7); CD21/35, 7G8-FITC (BD Pharmingen); CD24, M1/69-FITC; CD4, GK1.5-FITC or PE; CD43, eBioR2/60-PE; CD45.1, A20-APC or PE-Cy7 (BD Pharmingen); CD45.2, PE-104, Pacific blue-104 (Biolegend); CD5, 53-7.3-peridinin chlorophyll protein (PerCP; BD Pharmingen); CD69, H1.2F3-FITC; CD8, 53-6.7-PE; CD86, GL1-PE; CD95, 15A7-FITC, GL-7, G7-PE; IgM^a, DS-1–PE (BD Pharmingen); IgD^a, AMS9.1-FITC (BD Pharmingen); IgD, 11-26-FITC (BD Pharmingen); IgM, 11/41-PE; IgG1, P3-PE; Live/Dead Indicator for 488nm laser and 633nm laser (Invitrogen). HEL-binding cells were detected by incubating

cells with 200 ng/ml of unlabeled HEL and counterstaining with HyHEL9 Tricolor (Tc). Intracellular staining was performed using the Cytofix/Cytoperm buffer (BD Bioscience) and the following antibodies against phosphorylated epitopes, which were from Cell Signaling Technology, unless otherwise stated: p-p38, Rabbit IgG anti-pThr180/pTyr182, clone 3D7; p-Akt, Rabbit IgG anti-pSer473, clone 193H12; p-Akt, Rabbit IgG anti-pThr308, clone C31E5E; p-BLNK, Mouse IgG anti-pTyr84, clone J117-1278 (BD Pharmingen); p-Erk1/2, Rabbit IgG anti-pThr202/pTyr204, (Erk1) and anti-pThr185/pTyr187 (Erk2), clone D13.14.4E; p-PLCγ2, Mouse IgG anti-pTyr759, clone K86-689.37.73; pSyk, Mouse IgG anti-pTyr319/pTyr352, clone 17A/P-ZAP70 (BD Pharmingen); pCD19, Rabbit anti-pTyr531; pSHP1, Rabbit anti-pTyr564; pSHP2, Rabbit anti-pTyr580. Data were acquired on a FACSCanto, FACSCalibur, FACSort or LSR II (BD) and were analyzed with FlowJo Software (Tree Star).

Quantitative RT-PCR

Total RNA was extracted from splenocytes or flow cytometry sorted populations with RNEasy kits (Qiagen) followed by cDNA synthesis using the Superscript III First Strand Synthesis SuperMix for quantitative RT-PCR (qRT-PCR) kit (Life Technologies). Samples were analyzed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems), using multiple wavelength detection for control and target (*Themis1*, *Themis2* and β-actin mRNA), which were probed using the TaqMan Gene Expression Assays and Power SYBR Green PCR Master Mix (Life Technologies). Data from *Themis2* expression across cells were normalized to β-actin and analyzed using the comparative threshold cycle method. *Themis2* mRNA expression in wild-type and *Themis2*$^{-/-}$ (5 mice of each genotype) was assayed using primers spanning adjacent exons (available on request) and normalized against CD19, for example DC_T WT1 (Exon 2-3) = C_T WT1 (Exon 2-3) - C_T WT1 (CD19).

LC-MS/MS

Splenocytes from wild-type and *Themis2*$^{-/-}$ mice (2 of each genotypes) were lysed and sonicated in 50mM Tris pH 8.0, 2%SDS. Protein extracts were submitted to cysteine reduction and alkylation with iodoacetamide, and loaded onto a 1D SDS-

PAGE gel, to isolate the whole protein sample in a single band after brief electrophoretic migration. Proteins were in-gel digested overnight with sequencing grade trypsin (Promega). Resulting peptides were extracted and analyzed by nanoLC-MS/MS with an UltiMate 3000 RSLCnano system (Dionex) coupled to a Q-ExactivePlus mass spectrometer (ThermoScientific), using a 300min acetonitrile gradient separation on an in-house packed reverse phase C-18 analytical column (75µm inner diameter × 50cm), to optimize proteomic coverage. Raw MS files were analyzed with the MaxQuant software (version 1.5.2.8). MS/MS sequencing data was searched with the Andromeda search engine against "mouse" entries of the UniProtKB/Swiss-Prot protein database (release 2015_05). Validation of the identifications was performed with a false discovery rate set to 1% at protein and peptide sequence match level. For label-free relative quantification of the wild-type and *Themis2*$^{-/-}$ samples, the "match between runs" option of MaxQuant was enabled to allow cross-assignment of MS features detected in the different runs, in order to confirm the absence of Themis2 peptide MS signal in the *null* allele samples.

Immunizations and enzyme-linked immunosorbent assays

Mice were injected IP with two 150µl injections of 0.9% (vol/vol) sterile saline containing a total of 50µg NP_{25}-aminoethyl carboxymethyl–Ficoll (Biosearch Technologies), or 50µg alum–precipitated NP_{19}-CGG or NP_3-CGG (Biosearch Technologies). ELISA plates were coated overnight at 4°C with NP_{25}-BSA or NP_5-BSA (5 µg/ml; Biosearch Technologies), blocked with 5% (wt/vol) milk, and serum samples were serially diluted in 1% (wt/vol) milk. Plates were incubated with horseradish peroxidase–conjugated goat antibody to mouse IgM, IgG_3 or IgG1 (Southern Biotechnology) and developed with SureBlue TMB Microwell Peroxidase Substrate and TMB Stop Solution (KPL). Absorbance was measured at 450nm on a BioTek ELx808 ELISA plate reader. Background for assays was determined by incubation of serum from naïve animals. Total IgM, and IgG ELISAs and the anti-HEL capture ELISA were performed as described previously[21]. For SRBC immunization, mice were given IP injection into both flanks of a total of 200µl SRBCs (Patricell) diluted 1.5 times in PBS. Conjugation of SRBC to HEL was as described previously[21].

HEL-OVA antigen was generated as a His-tagged monomeric chimeric molecule transiently expressed in HEK293T cells and purified by affinity chromatography, followed by FPLC. 500μg purified HEL-OVA was mixed with RIBI Sigma Adjuvant System (S6322) in 3 mL PBS and mice were injected IP with two 150μl injections containing a total of 50μg HEL-OVA.

Ex vivo culture and stimulation

B cells were sorted from RBC-lysed single-cell suspensions by magnetic negative depletion using biotinylated Abs (from eBioscience) against CD43 (eBioR2/60-Bi), CD11c (N418-Bi), CD11b (M1/70-Bi) and Ly76 (TER119-Bi) and streptavidin-Dynabeads. B cells were cultured in DMEM, 100 mM nonessential amino acids, 20 mM HEPES buffer, 10% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, 100 mM 2-βmercaptoethanol. Ig^{HEL} B cells were stimulated with anti-IgM $F(ab)_2$ (Jackson Immunoresearch), LPS (Alexis), or sHEL (Sigma-Aldrich). CD45.1/2 allotype marked mixtures of wild-type and *Themis2*$^{-/-}$ Ig^{HEL} B cells (total $2x10^6$ cells) were incubated at 37°C in 5% CO2 in 0.25 ml of complete medium. After 17 h, cells were analyzed by flow cytometry for surface expression of CD69 and CD86.

Constructs expressing mHEL were generated in the retroviral vector pBMN using cDNA constructs for wild-type HEL and HEL^{2X} and HEL^{3X} variants, which were generated by mutagenesis. Retroviruses were stably transfected into NIH-3T3 cells, with and without co-expression of ICAM-1. Chimeric proteins contained the MHC Class I transmembrane domain, as used previously in mHEL[22] and three C terminal tandem copies of eGFP on the intracellular surface. mHEL expression was quantified by measuring eGFP fluorescence and binding to the HEL-specific monoclonal antibody HyHEL9-Tc, whose epitope is not affected by the 2X and 3X mutations. Cell lines expressing low levels of mHEL were selected by flow cytometry and then cultured overnight with 50:50 mixtures of CD45.1 wild-type Ig^{HEL} and CD45.2 *Themis2*$^{-/-}$ Ig^{HEL} B cells.

Constructs expressing Themis2 were subcloned into pFLAG-CMV2 vector by PCR with mouse cDNA for Icb1 from Imagenes. Constructs expressing Lyn, Syk and

PLCγ2 were purchased from Origene. The Themis2-dPRR construct was generated by sequentially inserting two PCR fragments into the BglII, KpnI, and SalI sites of pFLAG-CMV2 vector using Quick-Fusion (Bimake). The first PCR fragment was amplified using primers 5'-ATTCATCGATAGATCTAGAGCCGGTGCCGCTGCAGGAC-3' and 5'-CTCTAGAGTCGACTGGTACCGGCTTGGCTCTCAGAGGCTG-3'. The second PCR fragment was amplified using primers 5'-CTGAGAGCCAAGCCGGTATGAATAAGAAACAGCAGAACATAC-3' and 5'-ATCCTCTAGAGTCGACTCAAATTTCTTCATAGTCATG-3'.

Calcium flux analysis

Wild-type and *Themis2*$^{-/-}$ Ig^{HEL} B cells were first stained separately for B220 using different fluorochromes and a live/dead indicator. Cells were mixed in equal parts and resuspended in HBSS Buffer containing 0.02% Pluronic F-127 and 1μg/ml Fura-Red, AM (Invitrogen), already warmed to 37°C. The cells were incubated at 37°C for 30 min before washing and resuspending at $1x10^7$ cells/ml in HEPES saline solution. Analysis was performed using an LSRII equipped with the following lasers: blue (488 nm, 80mW), green (532 nm, 150 mW), red (642 nm, 40 mW) and violet (406 nm, 25 mW). Calibration was performed using CS&T Beads (BD Biosciences). Before analysis by flow cytometry, cells and 4X stimulant were warmed for 5 minutes in a 37°C water bath. Increases in FuraRed emission were monitored off the Violet laser (630LP and 660/20 BP), while a decrease in emission was detected off the Green laser (685LP and 710/50 BP). The ratiometric, 'FuraRed Ratio' was calculated as the increasing/decreasing signal using the Kinetics tool in FlowJo software version 9.3.3 (Tree Star Inc., OR, USA).

Immunoprecipitations and Western blotting

Cells were lysed with lysis buffer (10mMTris pH7.5, 150mM NaCl, 2mM EGTA 50mM β-glycerophosphate, 1% Nonidet P-40, 2mM Na_3VO_4, 10mM NaF, protease inhibitor cocktail (Roche)). Cell lysates were cleared by centrifugation at 14,000 x g for 10 min. Cell lysates were pre-cleared with Protein G-Sepharose

beads (GE Healthcare) or Protein A-Dynabeads (Life Technologies) for 1h and immunoprecipitated with antibodies conjugated to Protein G-sepharose beads or Protein A-Dynabeads for 4h to overnight, followed by three washes in ice-cold lysis buffer. Antibodies used for immunoprecipitation were anti-PLCγ2 (SC407, SantaCruz), anti-Lyn (SC15, SantaCruz), anti-FLAG (F1804, Sigma Aldrich), and anti-Themis2 (affinity purified rabbit polyclonal raised against the C-terminal peptide residues CKISVHKKDRKPNPQTQN of mouse Themis2)[14].

Western blots were performed as previously described[21] and probed with anti-PLCγ2 (SC407, SantaCruz), anti-Grb2 (3972, Cell Signaling), anti-Lyn (628102, BioLegend), anti-Phosphotyrosine, 4G10 Platinum (16-452, Millipore), anti-FLAG (F1804, Sigma Aldrich), anti-Themis2[14], anti-Myc (M047-3, MBL International), anti-pTyr759-PLCγ2 (3874, Cell Signaling), and anti-pTyr1217-PLCγ2 (3871, Cell Signaling). Blots were developed with HRP conjugated goat anti-rabbit or anti-mouse antibodies followed by enhanced chemiluminiscence (SuperSignal™ West Pico Chemiluminescent Substrate, ThermoScientific).

Confocal Microscopy

Equal mixtures of purified CD45.1 wild-type Ig^{HEL} and CD45.2 *Themis2*$^{-/-}$ Ig^{HEL} B cells in complete medium, were warmed at 37^{o}C and stimulated with sHEL at varying concentrations for 10min before adding an equal volume of 4% w/v of paraformaldehyde in PBS and cyto-spinning onto poly-L-Lysine slides. Slides were stained with CD45.1-FITC, B220-APC and anti-SOS (rabbit anti-SOS1/2 Santa Cruz), followed by Goat anti-Rabbit IgG-AlexaFluor-594 before imaging on a Zeiss 510 Metahead Confocal microscope. Membrane associated SOS was quantified by measuring its mean fluorescence density.

Statistics

GraphPad Prism Software was used for statistical analyses, and unpaired, two tailed Student's t tests were used for statistical comparison between groups, unless otherwise specifically mentioned.