

# The intramembrane protease Sppl2a is required for B cell and DC development and survival via cleavage of the invariant chain

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**B cell development requires tight regulation to allow for the generation of a diverse repertoire while preventing the development of autoreactive cells. We report, using *N*-ethyl-*N*-nitrosourea (ENU)-induced mutagenesis, the identification of a mutant mouse (chompB) with a block in early B cell development. The blockade occurs after the transitional 1 (T1) stage and leads to a decrease in mature B cell subsets and deficits in T cell-dependent antibody responses. Additionally, chompB mice have decreases in myeloid dendritic cells (DCs). The mutation was mapped to the intramembrane protease signal peptide peptidase-like 2a (Sppl2a), a gene not previously implicated in immune cell development. Proteomic analysis identified the invariant chain (CD74) as a key substrate of Sppl2a and suggests that regulated intramembrane proteolysis of CD74 by Sppl2a contributes to B cell and DC survival. Moreover, these data suggest that modulation of Sppl2a may be a useful therapeutic strategy for treatment of B cell dependent autoimmune disorders.**

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Abbreviations used: DKO, double KO; ENU, *N*-ethyl-*N*-nitrosourea; FO, follicular; mDC, myeloid DC; MZ, marginal zone; NTF, N-terminal fragment; pDC, plasmacytoid DC; SNP, single nucleotide polymorphism; Sppl2a, signal peptide peptidase-like 2a; T1, transitional 1.

B cell development is a tightly controlled process that promotes the survival and expansion of B cells with affinity for invading organisms while eliminating autoreactive B cells and thereby preventing autoimmunity (Browning, 2006; Allman and Pillai, 2008). The recent clinical success of B cell targeted therapies in a multitude of autoimmune disorders has highlighted the potential role of B cells in disease pathology. Work in the last several decades has also revealed that B cells have alternative effector functions in addition to antibody production, and can play a role in the pathology of a multitude of human diseases and provide additional therapeutic intervention points (Browning, 2006; Gürcan et al., 2009).

Although much has been discovered about lymphocyte development and activation through the use of traditional gene targeting approaches, it also has its limitations. These include requirements for a gene in embryonic development, the

development of a particular cell type, or a hypothesis that a gene is involved in the function of a particular cell type or pathway. Forward genetic approaches have classically proven to be valuable tools in many model organisms, and work from the last 10 years has shown that it can also be a useful tool in mice (Beutler et al., 2006; Cook et al., 2006). Random mutagenesis also provides the opportunity to identify mutants with reduced or increased activity as opposed to full ablation, which can provide insight into how a particular molecule is functioning.

To this end, we used a forward genetic screen using *N*-ethyl-*N*-nitrosourea (ENU)-induced mutagenesis in mice to identify novel regulators of immune function. We discovered a

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mouse strain (*chompB*) with a heritable recessive mutation that leads to decreased B cells and T cell–dependent antibody responses and mapped it to the gene encoding signal peptide peptidase-like 2a (*Sppl2a*). The *chompB* mutation revealed an unexpected role for *Sppl2a* in B cell and DC development and that *Sppl2a*-mediated processing of CD74 contributes to the survival of those cells during development and activation.

**RESULTS AND DISCUSSION**

**Identification of a role for *Sppl2a* in B cell and DC function by ENU mutagenesis**

A mutant line with a heritable recessive mutation, named *chompB*, was identified with decreases in peripheral blood B cells and T cell–dependent antibody responses (Fig. 1, A and B). Single nucleotide polymorphism (SNP)-based mapping identified a region of chromosome 2 that was linked to these phenotypes (Fig. S1 A). Candidate gene sequencing identified a T to A conversion in the *Sppl2a* gene, resulting in a methionine to lysine replacement (M252K) in the third transmembrane domain (unpublished data). *Sppl2a* has been shown to cleave TNF and promote inflammatory cytokine production by human DCs but its role *in vivo* has not been established (Fluhrer et al., 2006; Friedmann et al., 2006).

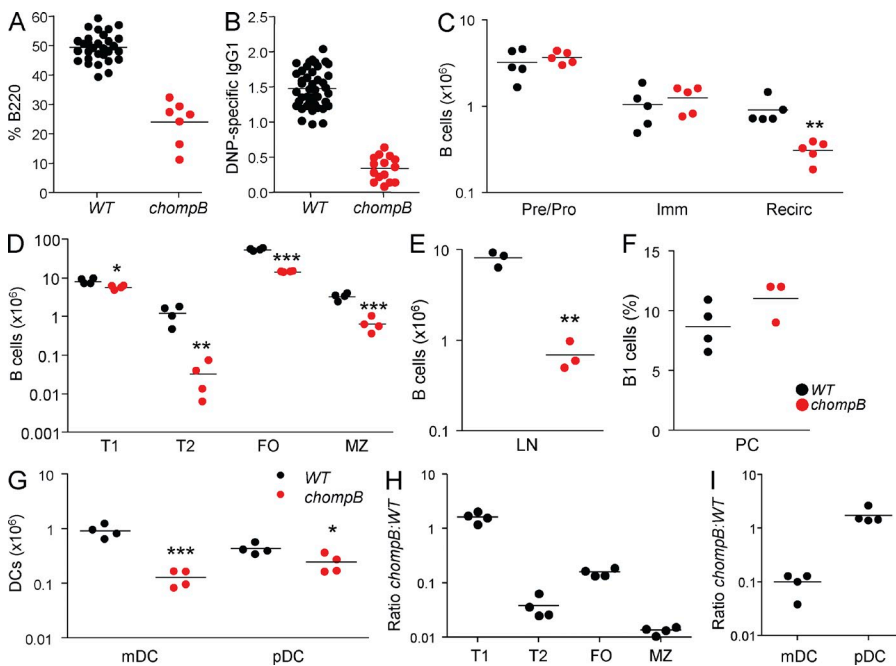
Analysis of B cells from the BM revealed a significant decrease in the number of mature recirculating B cells (Fig. 1 C). Analysis of splenic B cell populations revealed similar numbers of transitional 1 (T1) B cells but significant reductions in the numbers of T2, marginal zone (MZ), and follicular (FO)

B cells in *chompB* mutants (Fig. 1 D). A similar reduction in mature B cells was also observed in peripheral lymph nodes of *chompB* mice (Fig. 1 E); however, B1 cells in the peritoneal cavity were unchanged (Fig. 1 F). Analysis of DCs in the spleen revealed a significant decrease in myeloid DCs (mDCs) in *chompB* mice, whereas plasmacytoid DCs (pDCs) were less affected (Fig. 1 G). Other immune cell subsets were unaffected in *chompB* mutants, including T cells, NK cells, and myeloid cells (unpublished results). Despite the relatively ubiquitous expression of *Sppl2a* (Fig. S1 B), the phenotype of *chompB* mice appears to be lymphoid restricted as gross analysis did not reveal any other obvious abnormalities (unpublished data; Lattin et al., 2008).

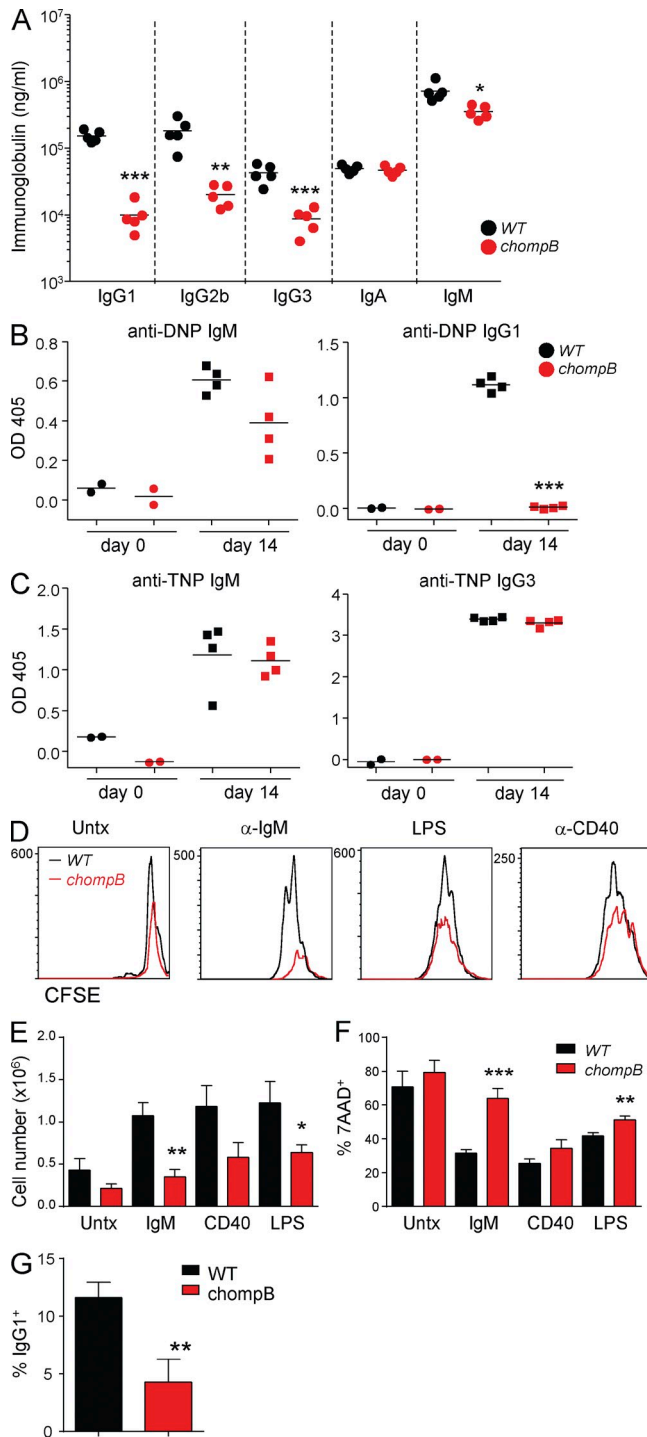
To determine the cellular origin of the *chompB* mutant phenotype, mixed BM chimeras were generated. Analysis of recipient mice revealed similar reductions in T2, MZ, and FO B cells and mDCs from *chompB* mutant BM, consistent with data from intact animals (Fig. 1, H and I). Analyses of single BM chimeras confirmed these observations (unpublished results).

***Sppl2a* is required for immunoglobulin production and T cell–dependent antibody responses**

We next assessed B cell function by measuring serum immunoglobulin levels and the antigen-specific response after immunization. Significant decreases in IgG1, IgG2b, and IgG3 levels were observed in *chompB* mice compared with controls, whereas levels of IgA and IgM were less affected (Fig. 2 A). Consistent with the initial screening results, *chompB* mutant



**Figure 1. *chompB* mice have a block in B cell and DC development.** (A and B) Mutagenized mice were screened for blood B cells by B220 staining (A) and DNP-KLH-specific IgG by ELISA (B). (C) BM B cells were enumerated by flow cytometry using the following criteria: pre/pro, B220<sup>+</sup>IgM<sup>-</sup>; immature, B220<sup>+</sup>IgM<sup>+</sup>CD24<sup>+</sup>; mature/recirculating, B220<sup>+</sup>IgM<sup>+</sup>CD24<sup>-</sup>. (D) Splenic B cell subsets were enumerated using the following criteria: T1, IgM<sup>hi</sup>CD21<sup>-</sup>; T2, IgM<sup>hi</sup>CD21<sup>hi</sup>CD23<sup>+</sup>; FO, IgM<sup>+</sup>CD21<sup>lo</sup>; MZ, IgM<sup>hi</sup>CD21<sup>hi</sup>CD23<sup>-</sup>. (E) B cell numbers in the lymph nodes were determined by gating on B220<sup>+</sup> cells. (F) B1 cell numbers from the peritoneal cavity were enumerated using CD19<sup>+</sup>B220<sup>int</sup>CD5<sup>int</sup>. (G) DC numbers were determined using the following criteria: mDC, CD19<sup>-</sup>CD3<sup>-</sup>NK1.1<sup>-</sup>CD11c<sup>hi</sup>; pDC, CD19<sup>-</sup>CD3<sup>-</sup>NK1.1<sup>-</sup>CD11c<sup>+</sup>PDCA1<sup>+</sup>. (H and I) 50/50 Mixed BM chimeras were analyzed by flow cytometry 8–12 wk after reconstitution. Data are expressed as the ratio of *chompB* to *WT* cells. Analysis of the B cells (H) and DCs (I) was performed using the gating schemes as in D and E after gating on CD45 variants to determine donor. \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001 (mean, n = 3–5 in at least two independent experiments).



**Figure 2. Defective T cell-dependent antibody responses and B cell activation in *chompB* mutants.** (A) Ig levels were analyzed in 6–12-wk-old WT and *chompB* mutant mice. (B) 6–12-wk-old WT and *chompB* mice were immunized with DNP-KLH and, after 14 d, DNP-specific antibody levels were determined by ELISA. (C) 6–12-wk-old WT and *chompB* mice were immunized with TNP-Ficoll and, after 14 d, TNP-specific antibody levels were determined by ELISA. (D–F) Purified B cells from WT and *chompB* mice were labeled with CFSE and cultured with the indicated stimuli along with 10 ng/ml rIL-4 for 72 h. CFSE dilution and cell death were then assessed by flow cytometry, and total cell numbers were determined

mice had significant decreases in DNP-specific IgG1 after immunization; however, DNP-specific IgM levels were unaffected (Fig. 2 B). The dramatic decrease in DNP-specific IgG1 cannot be explained solely by the threefold decrease in FO B cells, although it could be a contributing factor. Additionally, assessment of the ability of *chompB* B cells to respond to T cell help via class switching revealed a reduced ability to differentiate into IgG1<sup>+</sup> cells (Fig. 2 G). The response to the T cell-independent antigen (TNP-Ficoll) was unchanged in *chompB* mutants, consistent with a lack of effect on the number of B1 B cells, which are known to contribute to this response (Fig. 2 C; Martin et al., 2001; Defrance et al., 2011).

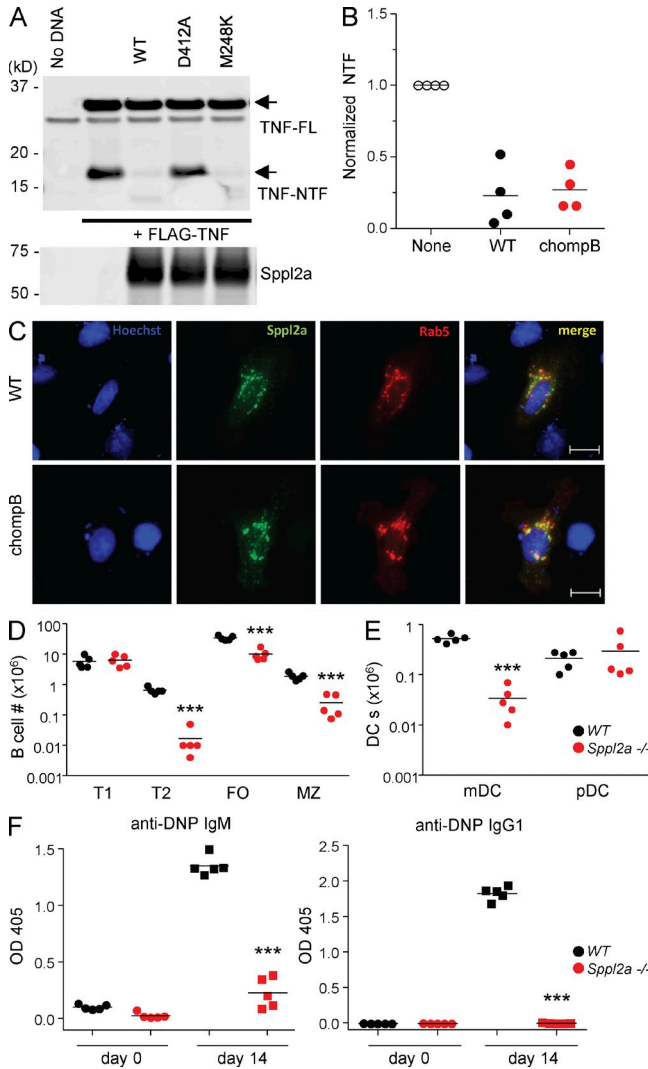
We next assessed the ability of *chompB* mutant B cells to respond to stimuli in vitro. *chompB* B cells failed to proliferate in response to anti-IgM and exhibited reduced responses to LPS and anti-CD40 stimulation as indicated by dilution of CFSE (Fig. 2 D). Additional analyses revealed significantly decreased cell expansion as well as increased cell death in response to anti-IgM stimulation and, to a lesser extent, LPS (Fig. 2, E and F). Because FO B cells comprise 60% of B cells in the spleen of *chompB* mice as compared with 75% in control mice (unpublished results), the inability to proliferate in response to anti-IgM cannot be explained solely by alterations in B cell subsets, although this may be a contributing factor.

#### The *chompB* allele is a loss of function mutation in *Sspl2a*

We next investigated the nature of the defect in the *chompB* mutant *Sspl2a*. Epitope-tagged WT, *chompB*, and protease dead *Sspl2a* were expressed at similar levels (Fig. 3 A). Both WT and *chompB* versions of *Sspl2a* colocalized with the endosome marker Rab5, ruling out misfolding or altered localization (Fig. 3 C). WT and *chompB* mutant *Sspl2a* were also capable of cleaving the previously described substrate TNF to a similar extent (Fluhrer et al., 2006; Friedmann et al., 2006; Fig. 3, A and B). These results demonstrate the localization and function of the *chompB* mutant *Sspl2a*, at least when overexpressed, is largely comparable to WT. We speculate that the function and/or localization of the mutant *Sspl2a* at endogenous expression levels are altered. However, because of a lack of commercially available anti-*Sspl2a*-specific antibodies, the endogenous expression of the *chompB* mutant was unable to be determined. Preliminary results confirmed that the mutation in *Sspl2a* was responsible for the phenotype of *chompB* mice, as transduction and transplant of *chompB* bone marrow with WT *Sspl2a* rescued the B cell developmental blockade (unpublished data).

To confirm that the phenotypes observed in the *chompB* mutants were a result of loss of function of *Sspl2a*, we obtained

by cell counting. (G) Expression of surface IgG1 in response to anti-CD40 and IL-4 was assessed by flow cytometry after 96 h of culture (mean,  $n = 4$ –5 from two independent experiments, A–C; results are representative of at least three replicates from two independent experiments, D; mean and SEM of four independent experiments, E and F; and mean and SEM,  $n = 4$  from two independent experiments, G). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

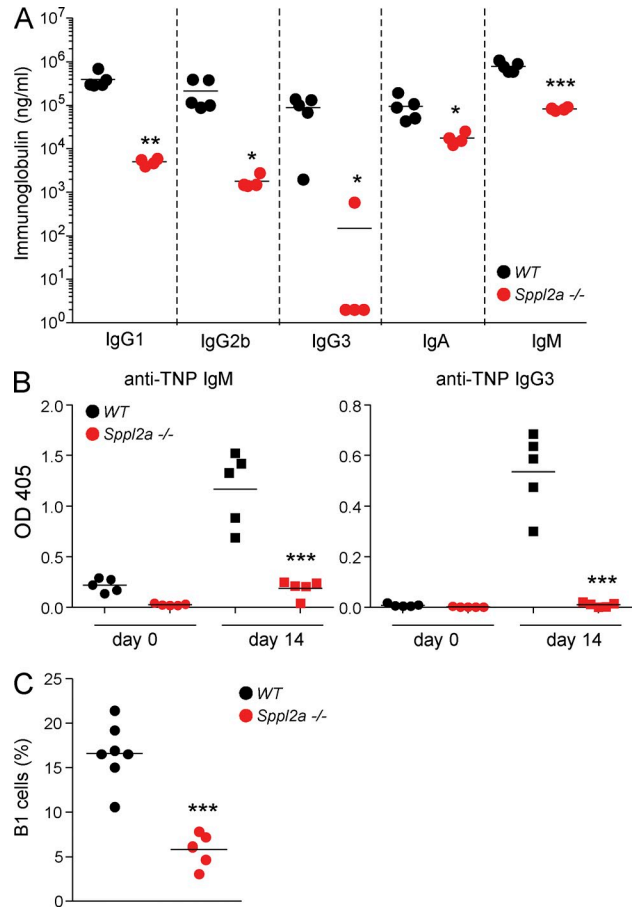


**Figure 3. Mutation in *Spp12a* is responsible for the observed defects in *chompB* mice.** (A and B) HEK293T cells were cotransfected with the indicated plasmids, and TNF and *Spp12a* levels were determined by Western blot. TNF-NTF (N-terminal fragment) was first normalized to full length TNF, followed by normalization to samples without *Spp12a* for quantification (representative of four independent experiments for A and mean,  $n = 4$  from four independent experiments for B). (C) U2OS cells were cotransfected with V5-m*Spp12a* together with Rab5-RFP. Cells were then stained with an anti-V5 antibody and Hoechst dye and analyzed by microscopy (representative of two independent experiments). Bars, 20  $\mu\text{m}$ . (D and E) Splenocytes from *WT* and *Spp12a*<sup>-/-</sup> mice were analyzed by flow cytometry as described in Fig. 1 (mean,  $n = 5$  from three independent experiments). (F) 6–12-wk-old *WT* and *Spp12a*<sup>-/-</sup> mice were immunized with DNP-KLH. DNP-specific antibody levels were determined by ELISA after 14 d (mean,  $n = 4$ –5 from two independent experiments). \*\*\*,  $P < 0.001$ .

*Spp12a*<sup>-/-</sup> mice. Analysis of *Spp12a*<sup>-/-</sup> mice revealed similar decreases in splenic B cells and mDCs compared with *chompB* mutants (Fig. 3, D and E). Also consistent with the *chompB* mice, DNP-specific IgG1 was decreased after immunization in *Spp12a*<sup>-/-</sup> mice (Fig. 3 F).

**The *chompB* mutation is a hypomorphic allele of *Spp12a***

Despite the similarities, there were also differences between *chompB* mutant and *Spp12a*<sup>-/-</sup> mice. Unlike the *chompB* mice, the levels of DNP-specific IgM were decreased in *Spp12a*<sup>-/-</sup> mice after immunization (Fig. 3 F). Analysis of steady-state Ig levels also revealed decreases in all isotypes tested in the *Spp12a*<sup>-/-</sup> mice (Fig. 4 A). Additionally, the response to TNP-Ficoll revealed a decrease in TNP-specific IgM and IgG3 in the *Spp12a*<sup>-/-</sup> mice (Fig. 4 B). Unlike the *chompB* mutants, there was a significant decrease in B1 B cells in the *Spp12a*<sup>-/-</sup> mice and this likely explains some of the observed differences between the two strains (Fig. 4 C). The similarities between the *chompB* and *Spp12a*<sup>-/-</sup> mice suggest that the *chompB* mutation in *Spp12a* is a loss of function allele. However, the intact T cell-independent responses, B1 cell numbers, intact IgA and IgM levels, and assessment of activity when over-expressed together suggest that the *chompB* mutation is a hypomorphic allele that maintains some level of activity in vivo.



**Figure 4. The *chompB* mutation in *Spp12a* is a hypomorphic allele.**

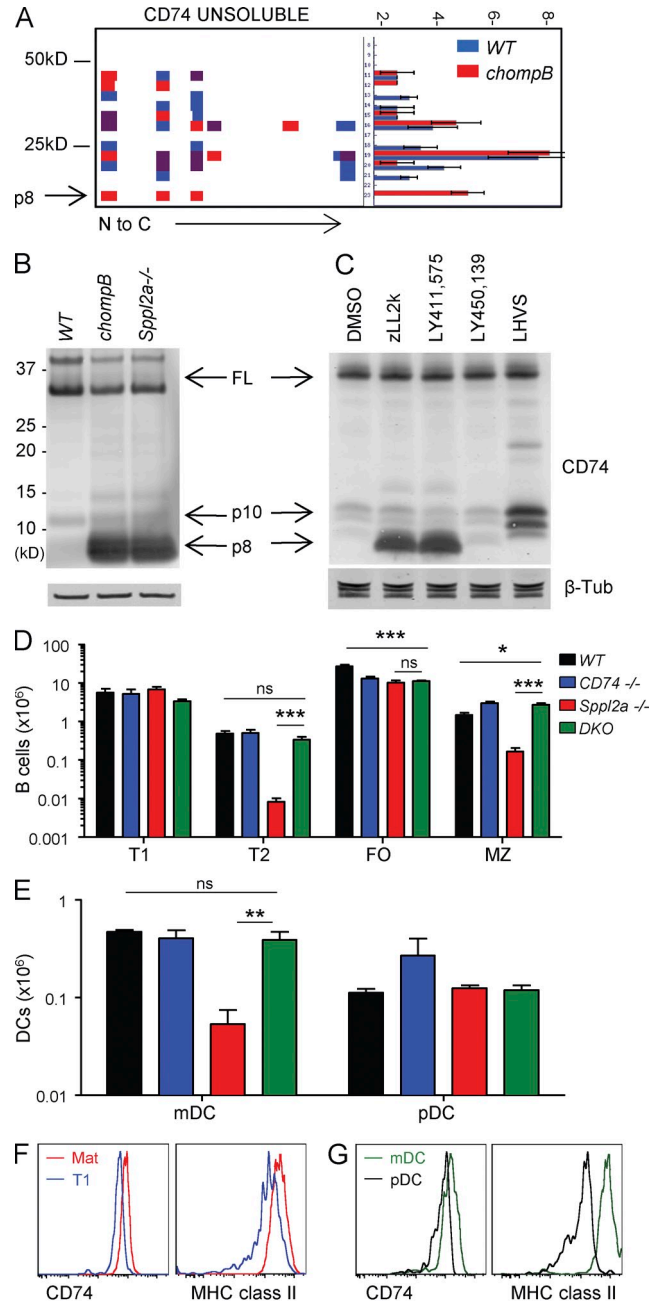
(A) Plasma Ig levels were determined from *WT* and *Spp12a*<sup>-/-</sup> mice (mean,  $n = 4$ –5 from two independent experiments). (B) 6–12-wk-old *WT* and *Spp12a*<sup>-/-</sup> mice were immunized with TNP-Ficoll and TNP-specific antibody levels were determined by ELISA 14 d later (mean,  $n = 4$ –5, two independent experiments). (C) B1 cell numbers from the peritoneal cavity were defined as in Fig. 1 (mean,  $n = 5$ –7 from three independent experiments). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

### Sppl2a-mediated cleavage of the N terminus of CD74 is required for B cell survival

In addition to TNF, Sppl2a has been shown to cleave several substrates *in vitro*; however, none of these have been implicated in B cell development (Kirkin et al., 2007; Martin et al., 2008). To identify additional substrates, an unbiased proteomics approach was used to compare the proteomes from WT and *chompB* B cells (Dix et al., 2008). This analysis identified accumulation of a fragment of CD74 in membrane-enriched fractions from *chompB* but not WT B cells (Fig. 5 A). Although CD74 has been well studied for its role in MHC class II antigen presentation, it has also been implicated in B cell development (Kenty and Bikoff, 1999; Matza et al., 2002b; Stumptner-Cuvelette and Benaroch, 2002). Analysis of CD74 by Western blot revealed accumulation of an 8-kD fragment (p8) in both *Sppl2a*<sup>-/-</sup> and *chompB* cells consistent with the observations in the proteomics approach (Fig. 5 B). Cleavage of the p8 fragment by Sppl2a should generate a 4-kD fragment (p4) but it is not detectable by Western blot. CD74 processing was further analyzed in A20 mouse B cells using protease inhibitors (Fig. 5 C). Cathepsin inhibition (LHVS) led to accumulation of the CLIP-containing 10-kD fragment consistent with previous results (Riese et al., 1996). Treatment of B cells with Spp family active inhibitors (zLL2k and LY411,575) also led to accumulation of CD74 p8 (Nyborg et al., 2004). Because LY411,575 also has  $\gamma$ -secretase activity, we tested a  $\gamma$ -secretase selective inhibitor (LY450,139) which failed to block the processing of CD74 p8. These data are in contrast with a previous study demonstrating a role for  $\gamma$ -secretase, but not the Spp family, in CD74 processing (Becker-Herman et al., 2005). The reasons for the discrepancies are unclear but could be a result of the differences in the systems used.

### Deletion of CD74 prevents B cell and mDC loss in *Sppl2a*<sup>-/-</sup> mice

The accumulation of CD74 p8 in the absence of Sppl2a could affect B cell and DC development/survival in several ways. Two possibilities are either that Sppl2a cleavage of p8 releases p4 which mediates cell signaling, or that accumulation of p8 in the endosome is toxic. To differentiate between these possibilities, *Sppl2a:CD74* double KO mice (DKO) were generated. If signaling via p4 were the predominant function of Sppl2a, DKO mice should have a similar phenotype as the *Sppl2a*<sup>-/-</sup> mice. Alternatively, if the primary function of Sppl2a is to clear p8, the *Sppl2a* phenotype should be rescued in DKO mice. Analysis of B cell populations in the spleen revealed a restoration of T2 and MZ B cells to WT levels in DKO mice (Fig. 5 D). Analysis of DCs also revealed a restoration in the mDC population to WT levels in DKO mice (Fig. 5 E). FO B cells were not restored in DKO mice, as *CD74*<sup>-/-</sup> mice have decreased FO B cell numbers. Collectively, these data demonstrate that removal of CD74 p8 from endosomal membranes is the primary function of Sppl2a and that CD74 is the key substrate of Sppl2a with respect to B cell and DC development/survival.



**Figure 5. Sppl2a-mediated cleavage of CD74 is responsible for the B cell developmental blockade in *chompB* mice.** (A) Peptogram of CD74 from WT and *chompB* membrane-enriched fractions. The left side depicts the weight in kD and the right side depicts the frequency. (B) Western blot analysis of sorted mature B cells (CD24<sup>lo</sup>CD21<sup>int</sup>) using antibodies to CD74 and  $\beta$ -tubulin (representative of two independent experiments). (C) CD74 cleavage in A20 cells assessed by Western blot (results are representative of at least three independent experiments). (D and E) B cell and DC numbers were determined using the same parameters in Fig. 1 (mean,  $n = 4$  from three independent experiments). (F and G) Intracellular CD74 and MHC class II levels were determined using the gating schemes in Fig. 1 for the indicated B cell (F) and DC (G) populations ( $n = 3$ , from two independent experiments). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

One open question relates to why post-T1 B cells and mDCs are particularly sensitive to loss of Sppl2a. One hypothesis is that the sensitivity could be driven by increased expression of Sppl2a or CD74 in these cell types. Analysis of Sppl2a mRNA expression revealed similar levels in T1 and mature B cells (Fig. S1 C). In contrast, analysis of intracellular levels of CD74 and MHC class II on WT B cell subsets revealed an increase between T1 and mature B cells, consistent with timing of the B cell developmental blockade in *Sppl2a*<sup>-/-</sup> mice (Fig. 5 F). Analysis of DCs also revealed elevated levels of both proteins in mDCs compared with pDCs (Fig. 5 G). Collectively, these data suggest that Sppl2a is especially important in cells that contain high levels of MHC II and CD74 and that Sppl2a-mediated processing of CD74 p8 is important for B cell and DC survival.

We have provided evidence that *chompB* mutant B cells have survival defects as a result of accumulation of CD74 p8. In contrast, previous work has suggested a role for CD74 in B cell development via p4 signaling (Matza et al., 2002a,b). However, the observation that mature B cell numbers are restored in *MHC class II:CD74* double-deficient mice suggests that the loss of FO B cells in *CD74*<sup>-/-</sup> mice results from altered MHC class II trafficking rather than lack of p4 signaling (Maehr et al., 2004). These data, together with the current work, provide strong support for the hypothesis that p8 accumulation, rather than p4 loss, leads to B cell and mDC defects in *Sppl2a*<sup>-/-</sup> mice. Two accompanying manuscripts in this issue by Schneppenheim et al. and Bergmann et al. support the general conclusions we have reached.

We speculate that Sppl2a permits higher levels of CD74 and MHC class II expression and thereby facilitates effective sampling of the immune environment. Rapid turnover of MHC-peptide complexes ensures that antigen-presenting cells are able to present the most current range of foreign antigens to ensure a rapid response to invading pathogens. However, a byproduct of this rapid turnover is high levels of membrane-bound p8 which are toxic to the cell. By degrading p8, Sppl2a prevents accumulation of this otherwise toxic byproduct of MHC Class II antigen processing and permits higher levels of MHC Class II and CD74 needed for efficient antigen sampling in specialized antigen-presenting cells.

The past decade of clinical experience with B cell depleting antibodies, such as Rituximab, has revealed a critical role for B cells in the pathogenesis of several autoimmune diseases. Our results raise the possibility that targeting Sppl2a may prove useful for the treatment of autoimmune disease. However, given the essential role of Sppl2a in mediating B and DC development, a thorough assessment of the therapeutic potential of Sppl2a will require the generation of appropriate inducible models that allow elimination of Sppl2a in mature cells.

## MATERIALS AND METHODS

**Mice.** All mice used were 6–12 wk of age and were maintained in the specific pathogen-free facility of the Genomics Institute of the Novartis Research Foundation. C57BL/6 ENU-mutagenized mice were generated as described elsewhere (Miosge et al., 2002). B cell percentages in whole blood were measured using antibodies to B220 (eBioscience) and cells were analyzed by

flow cytometry. SNP mapping of the affected mice was performed as described elsewhere (Wilshire et al., 2003). *Sppl2a*<sup>-/-</sup> mice were purchased from Delta-gen and backcrossed to C57BL/6 for five generations to >99% pure background using speed congenics. *CD74*<sup>-/-</sup> mice (B6.129S6-Cd74tm1Liz/J; The Jackson Laboratory) have been described previously (Bikoff et al., 1993). All procedures were approved by the Genomics Institute of the Novartis Research Foundation Institutional Animal Care and Use Committee. Littermates were used as controls in the majority of experiments, but in some instances, C57BL/6 mice from our internal colony were used as well.

**Bone marrow transplant.** Traditional mixed and single bone marrow chimeras were set up as previously described except the cells were transferred into the retro-orbital sinus region. (Miller et al., 2007).

**Immunoglobulin levels.** Immunoglobulin levels were measured from mouse plasma using an isotyping kit (Millipore) according to the manufacturer's instructions.

**Plasmids and cell transfection.** Cell transfections in 293T cells were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. In brief,  $2 \times 10^5$  293T cells were cultured in 24-well plates (Costar), followed by additions of 1  $\mu$ g of the indicated plasmids together with Lipofectamine in Opti-MEM (Invitrogen). 24 h after transfection, cells were released from the plate using 0.05% Trypsin (Invitrogen) and whole cell lysates, and Western blotting procedures were performed as described below. Plasmids for human Sppl2a (pCI-hSPPL2a) were described previously (Friedmann et al., 2006), and the *chompB* equivalent mutant (pCI-hSP-PL2aM248K) and protease inactive mutant (pCI-hSPPL2aD412A) were generated by site-directed mutagenesis of the appropriate base pairs. Plasmids for mouse Sppl2a were generated by inserting the full length cDNA into the pCDNA3.1/V5-His-TOPO vector (Invitrogen) by PCR. The *chompB* version was generated using site-directed mutagenesis of the appropriate base pairs. N-terminally tagged human TNF (pBICEP-CMV-2-TNF) was generated by introducing the full length cDNA into the pBICEP-CMV-2 vector (Sigma-Aldrich) using *Sall* and *NotI* sites. pCMV6-AC-RFP\_Rab5 (expressing RFP-labeled endosome marker Rab5) was purchased from Origene.

**Immunofluorescence.** For immunofluorescence studies of transiently expressed proteins in U2OS cells, DNA (0.06  $\mu$ g/well; 96-well plate) was diluted in Opti-MEM (Invitrogen) and incubated at room temperature for 5 min before addition of PEI (Polysciences) solution and 10-min incubation at room temperature.  $10^4$  cells/well were added to the preformed complexes and incubated for 20 min at room temperature before the mixture was transferred into a clear-bottom 96-well plate. 24 h after transfection, cells were fixed with 4% paraformaldehyde for 15 min at room temperature, followed by 1 h of treatment with blocking solution (4% BSA in PBS/0.1% Tween-20). Primary antibody directed against the V5 tag (Sigma-Aldrich) was added in PBS/0.1% Tween-20/1% BSA for 1 h and washed three times with PBS/0.1% Tween-20. The secondary antibody goat anti-mouse IgG Alexa Fluor 488 (Invitrogen) and Hoechst dye (1:10,000) were added in PBS/0.1% Tween-20/1% BSA for 1 h, followed by three washes with PBS/0.1% Tween-20. Fluorescence images were taken with an Axio fluorescence microscope (Carl Zeiss).

**Proteomics approach for substrate identification.** Purified WT and *chompB* mutant B cells were resuspended in 50 mM Tris, pH 8.0, with appropriate amount of protease inhibitors (lysis solution) and probe was sonicated  $3 \times 10$  bursts on ice. Protein samples were centrifuged at 100,000 *g* for 45 min at 4°C. The supernatant, the soluble proteome, was moved to a new tube and the pellet, the insoluble proteome, was washed three times with lysis solution and resuspended back into lysis solution by probe sonication. Proteins were quantified and 100  $\mu$ g was mixed with protein loading dye for separation by SDS-PAGE. The rest of the procedure was performed as described previously (Dix et al., 2008).

**B cell isolation and cell culture.** Cell culture and isolation was performed as described elsewhere (Beisner et al., 2003). Resting splenic B cells

were purified magnetically by depletion with CD43, Thy1.2, and CD11b MACS beads (Miltenyi Biotec). The purity of these negatively selected cells was determined to be >95% B220<sup>+</sup> by flow cytometry. For assessment of proliferation, purified B cells were labeled with 5  $\mu$ M CFSE (Life Sciences) and cultured for 72 h in the presence of the indicated stimuli, together with 10 ng/ml rIL-4 (eBioscience). After 72 h, cells were stained with B220 APC and 7AAD, and proliferation and cell death was assessed by flow cytometry. For assessment of cell numbers from proliferation experiments, cell counts were determined using a Vicell (Beckman Coulter). For analysis of class switching, purified B cells were stimulated for 96 h with anti-CD40 and 10 ng/ml rIL-4 (eBioscience). IgG1 surface staining on B220<sup>+</sup> cells was assessed by flow cytometry.

**Flow cytometry.** Cells were stained for 20 min at 4°C using FITC-, PE-, PerCP-, allophycocyanin-, Pacific blue-, APC-Cy7-, APC-Cy5-, and biotin-conjugated Abs against B220, CD21, CD23, IgM, IgD, CD4, CD8, CD11c, CD19, CD3, NK1.1, PDCA1, CD95, GL7, CD45.1, CD45.2, Gr1, CD11b, MHC class II, and Ter119 (BD, eBioscience, and BioLegend). Cells were collected on either an LSR-II or Fortessa (BD) flow cytometer and data were analyzed using FlowJo (Tree Star). Cell sorting was performed on a FACSAria. For intracellular staining of CD74 and MHC class II, surfaced-stained cells were fixed, permeabilized, and stained for each marker using a kit from eBioscience according to the manufacturer's instructions.

**Western blotting.** Cells were harvested in 1 $\times$  lysis buffer (Cell Signaling Technology) containing protease inhibitors, phosphatase inhibitors (Sigma-Aldrich), and 1 mM PMSF. Samples were electrophoresed through NuPAGE Novex 10% Bis-Tris precast polyacrylamide gels in MOPS buffer (Invitrogen) and transferred to nitrocellulose membranes using the iBlot Dry Blotting System (Invitrogen). Membranes were probed with antibodies against mouse CD74 (BD),  $\beta$ -tubulin (Millipore), FLAG (Sigma-Aldrich), and HA (Invitrogen), followed by IRDye 800- (Rockland Immunochemical) or Alexa Fluor 680 (Invitrogen)-conjugated secondary antibodies. Membranes were scanned and analyzed with the Odyssey system (LI-COR Biosciences).

**Immunizations and antigen-specific antibody determination.** Mice were immunized intraperitoneally with 25  $\mu$ g TNP-Ficoll or 100  $\mu$ g DNP-KLH (Biosearch Technologies) in ALUM (Thermo Fisher Scientific). Levels of DNP or TNP-specific Ig were assayed by ELISA as described previously (Schopf et al., 1999). HRP-conjugated rat anti-mouse IgM, IgG1, and IgG3 were purchased from BD. Plates were developed using the Super AquaBlue ELISA substrate system (eBioscience).

**Quantitative mRNA expression analysis.** RNA was isolated from mature and T1 B cells using the RNeasy kit (QIAGEN). cDNA was generated with the QuantiTect Rev. Transcription kit (QIAGEN). mGAPDH and mSppl2a were amplified using SYBR Green (Life Technologies) on the ABI 7900HT. Primer sequences are as follows: mGAPDH forward: 5'-CATGGCCTTC-CGTGTTCCCTA-3', mGAPDH reverse: 5'-CCTGCTTACCACCTTC-TTGAT-3'; mSppl2a forward: 5'-CCATCCCTGGCCTAACTTTGA-3', and mSppl2a reverse: 5'-TCTTCTGCGTCTTCCACTGA-3'. The following program was used: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. mSppl2a mRNA levels shown in the graph are normalized to GAPDH levels.

**Statistics.** All statistical analysis was performed with Prism software using an unpaired two-tailed Student's *t* test.

**Online supplemental material.** Fig. S1 shows identification of Sppl2a by ENU mutagenesis. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20121072/DC1>.

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