SUPPLEMENTAL MATERIAL

Cariappa et al., http://www.jem.org/cgi/content/full/jem.20081399/DC1

Mice

Cmah mutant mice (1) were backcrossed for 10 generations into the C57BL/6 background and then studied. $Siae^{\Delta 2/\Delta 2}$ and $Cmah^{-/-}$ mice, both backcrossed for 10 generations into the C57BL/6 background, were intercrossed to generate $Siae^{\Delta 2/\Delta 2}/Cmah^{-/-}$ double mutant animals.

B lymphocyte purification

The method used by Collins et al. (2) was adhered to.

RT-PCR

Standard methodology was used to prepare total cellular RNA and first-strand complementary DNA from splenocytes. The HPRT primers used in the PCR were as previously published (3). The Siae primers used to detect the deletion of exon 2 were the following: forward, 5′-AGTGCTGCTCATAATCGCGC-GAG-3′; and reverse, 5′-AAGTTTCCTGCGGTGGGCTTG-3′. The reverse primer spanned exons 4 and 5.

CFSE staining and B cell proliferation

Analysis of CFSE-stained cells was performed on nonstimulated and anti-IgM–activated B cells as described earlier (4) with the following modification: CFSE (Invitrogen) was added to a final concentration of 1 μM and no additional surface staining of the B cells was performed.

Lymphocyte proliferation assay

The method described previously (5) was used. Sorted cell fractions were plated at 10^5/well in triplicate in 96-well flat-bottomed microtiter plates with medium alone or with medium and one of the following: goat anti–mouse IgM (Fab')2 (μ chain specific; Jackson ImmunoResearch Laboratories), 1 μg/ml of purified hamster anti–mouse CD40 (BD), and 10 μg/ml LPS from *Escherichia coli* serotype O55:B5, (Sigma-Aldrich). The cells were cultured for 48 h and then pulsed with 2 μCi/well of [^{3}H] thymidine (PerkinElmer) for an additional 18 h, harvested, and read in a beta plate reader.

Annexin V assay for apoptosis

Previously published methodology (6) was used. 5 μl of 10 μg/ml stock of APC-Annexin V (BD) was used and cells were assessed for apoptosis by flowcytometry.

Immunization with T cell–dependent and –independent antigens and ELISA for anti-DNP antibodies

Mice were immunized i.p. with 100 μg DNP-KLH (EMD) or DNP-Ficoll (Biosearch Technologies, Inc.) in PBS on day 0 and boosted on day 28. Serum was collected on designated days and antibodies were quantitated by ELISA as previously described (7).

Stable transfection of U2OS cells, and metabolic labeling-based pulse-chase studies

U2OS cells were stably transfected with a murine C-terminal Flag-tagged Siae cDNA in the pCDNA3.1 vector using the calcium phosphate method. Siae-expressing clones were identified using an anti-Flag Western blot approach. A stable clone expressing Siae was used in all immunofluorescence and metabolic labeling pulse-chase studies.

U2OS-Siae cells were metabolically labeled with 0.5 mCi of ^{35}S-methionine for 10 min and chased with complete medium containing 2 mM methionine for varying time periods. Culture supernatants were immunoprecipitated at various chase time points with anti-Flag antibodies and protein A Sepharose, separated on an SDS-polyacrylamide gel, and visualized by autofluorography.

Immunofluorescence of transfected cells

U20S-Siae cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% NP-40, and stained with labeled anti-Flag antibodies, Wheat germ agglutinin, Lysotracker, or LAMP-1 as indicated in Fig. S2. The method used by Collins et al. (2) was adhered to for the immunofluorescence of purified B lymphocytes seen in Fig. S6. Images were captured using a spinning disc confocal microscope (IX81; Olympus) equipped with a charge-coupled device camera and images were processed with Slidebook 4.2 (Intelligent Imaging Innovations, Inc.).

SUPPLEMENTAL REFERENCES

1. Hedlund, M., P. Tangvoranuntakul, H. Takematsu, J.M. Long, G.D. Housley, Y. Kozutsumi, A. Suzuki, A. Wynshaw-Boris, A.F. Ryan, R.L. Gallo, et al. 2007. N-glycolylneuraminic acid deficiency in mice: implications for human biology and evolution. *Mol. Cell. Biol.* 27:4340–4346.
2. Collins, B.E., B.A. Smith, P. Bengtson, and J.C. Paulson. 2006. Ablation of CD22 in ligand-deficient mice restores B cell receptor signaling. *Nat. Immunol.* 7:199–206.
3. Cariappa, A., I.B. Mazo, C. Chase, H.N. Shi, H. Liu, Q. Li, H. Rose, H. Leung, B.J. Cherayil, P. Russell, et al. 2005. Perisinusoidal B cells in the bone marrow participate in T-independent responses to blood-borne microbes. *Immunity.* 23:397–407.
4. Cariappa, A., L. Chen, K. Haider, M. Tang, E. Nebelitskiy, S.T. Moran, and S. Pillai. 2003. A catalytically inactive form of protein kinase C-associated kinase/receptor interacting protein 4, a protein kinase C beta-associated kinase that mediates NF-kappa B activation, interferes with early B cell development. *J. Immunol.* 171:1875–1880.
5. Cariappa, A., C. Boboila, S.T. Moran, H. Liu, H.N. Shi, and S. Pillai. 2007. The recirculating B cell pool contains two functionally distinct, long-lived, posttransitional, follicular B cell populations. *J. Immunol.* 179:2270–2281.
6. Cariappa, A., T.J. Kim, and S. Pillai. 1999. Accelerated emigration of B lymphocytes in the Xid mouse. *J. Immunol.* 162:4417–4423.
7. Hennet, T., D. Chui, J.C. Paulson, and J.D. Marth. 1998. Immune regulation by the ST6Gal sialyltransferase. *Proc. Natl. Acad. Sci. USA.* 95:4504–4509.

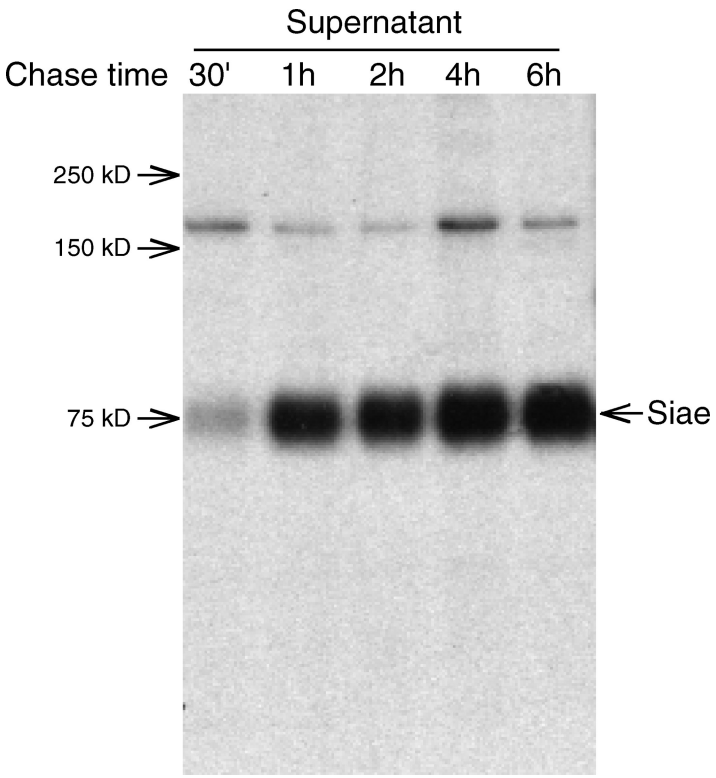

Figure S1. Siae is secreted from stably transfected U2OS cells. U2OS-Siae cells were pulsed with 0.5 mCi of ^{35}[S] Methionine for 10 min and supernatants were immunoprecipitated immediately after labeling (0') and at the indicated chase time periods. Flag-tagged Siae was identified in cell lysates and in supernatants by immunoprecipitation with anti-Flag antibodies followed by separation on SDS/PAGE and autofluorography.

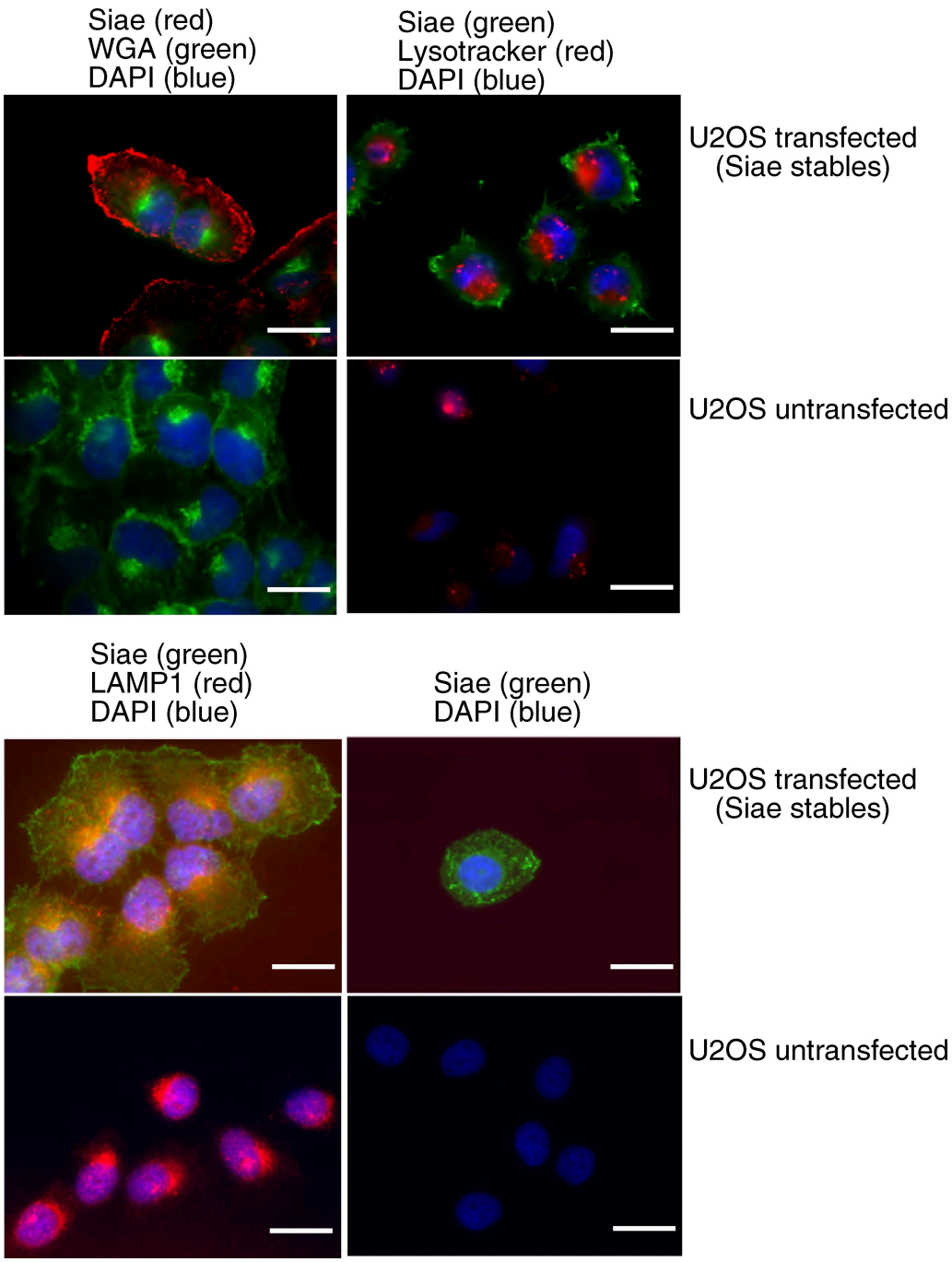

Figure S2. Siae is expressed on the surface of transfected U2OS cells and also partially in lysosomes. U2OS-Siae cells were permeabilized and stained with anti-Flag antibodies (red, top left; and green, top right panels and all bottom), wheat germ agglutinin (WGA; green), Lysotracker (red), or antibodies to LAMP-1 (red). Bars, 20 μm.

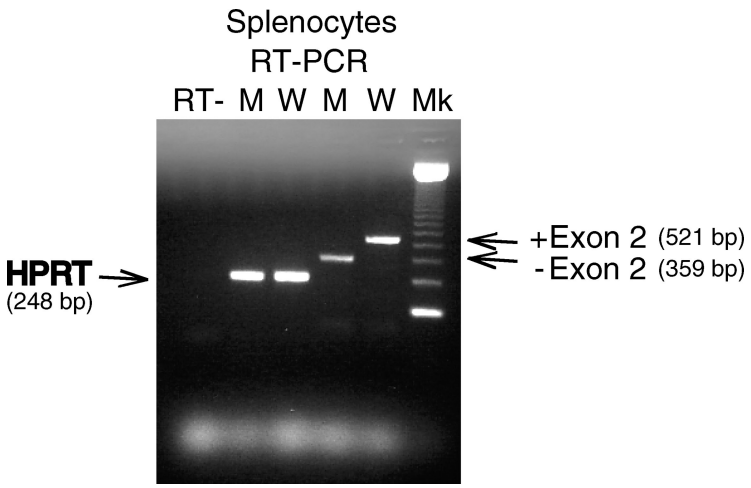

Figure S3. Truncated *Siae* messenger RNA in mutant mice as revealed by RT–PCR. M, mutant; W, WT; Mk, 123-bp marker; RT−, no reverse transcriptase; and HPRT, hypoxanthine phosphoribosyltransferase.

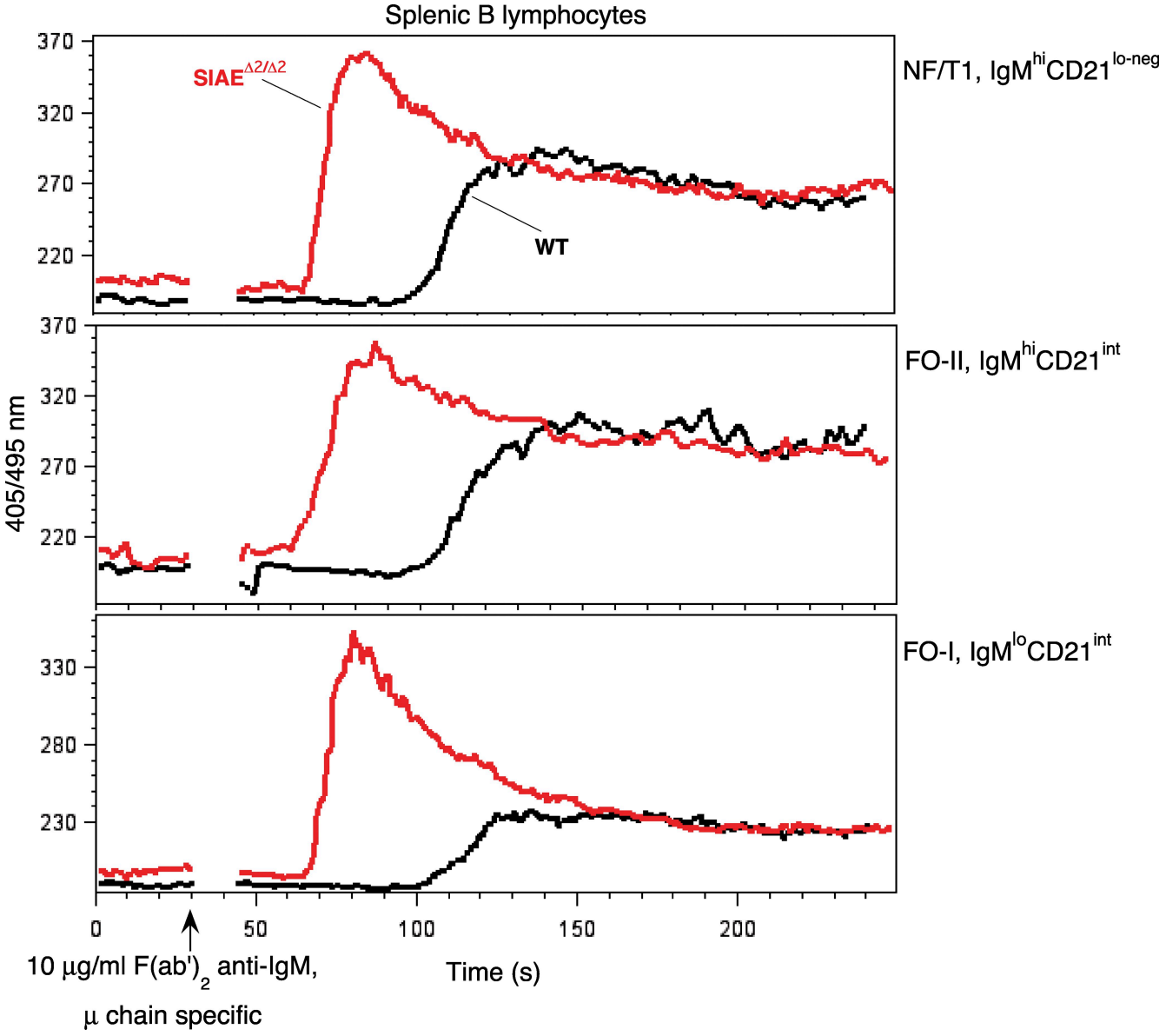

Figure S4. Purified B lymphocytes show accelerated and enhanced BCR signaling in *Siae* mutant mice. B cells were purified from WT and *Siae* mutant mice and intracellular calcium was assessed after BCR cross-linking.

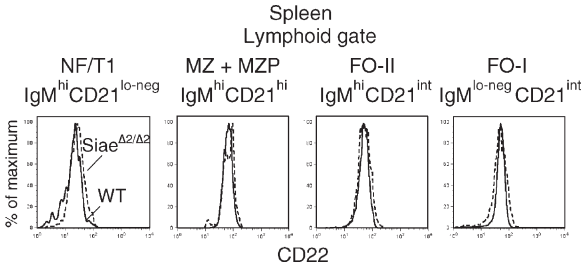

Figure S5. Surface expression of CD22 on splenocytes in *Siae*$^{\Delta 2/\Delta 2}$ mice is unaltered compared to WT mice. Flow cytometric analysis of CD22 expression on WT and mutant B cells was perfomed. Results are representative of three mice per group.

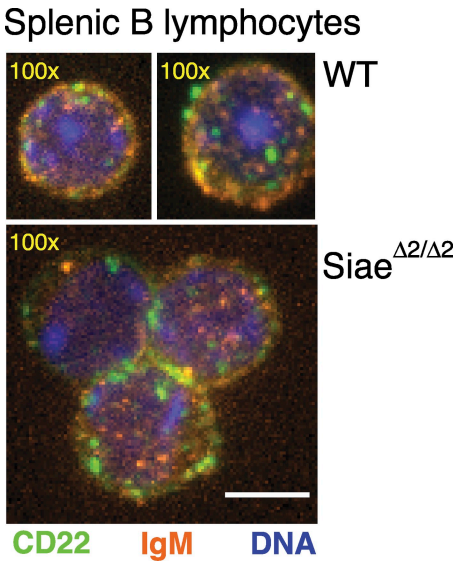

Figure S6. Distribution of CD22 on the surface of unstimulated splenic B lymphocytes is unaltered in *Siae* mutant mice compared to their WT counterparts. B cells were examined using spinning disk confocal microscopy. Bar, 5 µm. Representative fields are shown.

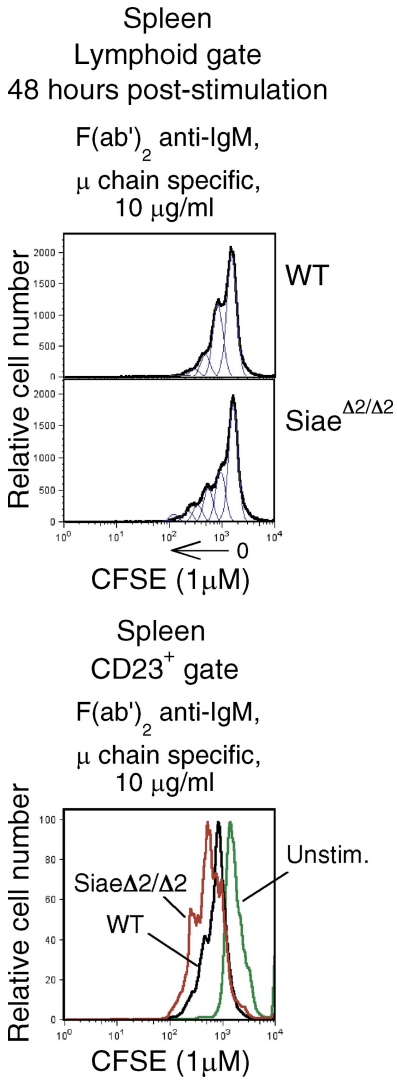

Figure S7. Siae mutant splenocytes proliferate more than WT B cells. Generations are marked in blue (top). CD23$^+$ cells were gated on (bottom). Results are representative of three mice per group.

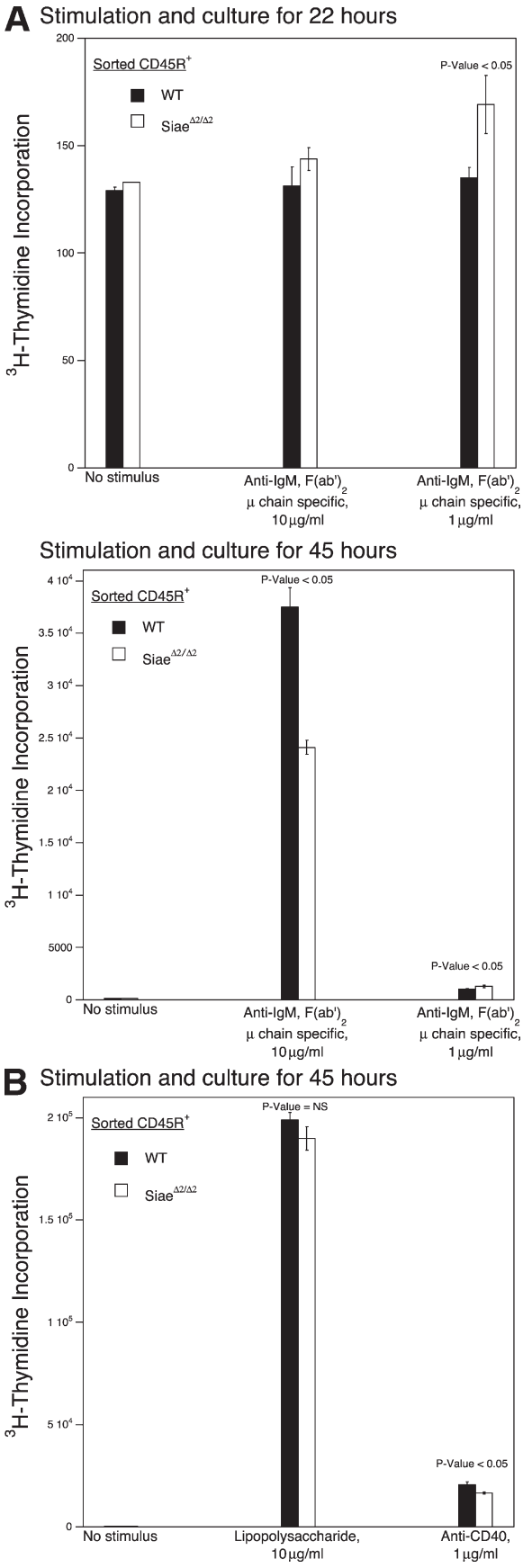

Figure S8. Stimulus strength–dependent inhibition of proliferation in Siae mutant B cells. (Aand B) Proliferation after 22 and 45 h (A), and after 45 h (B) . *n* = 3 mice per group. Error bars represent SEM.

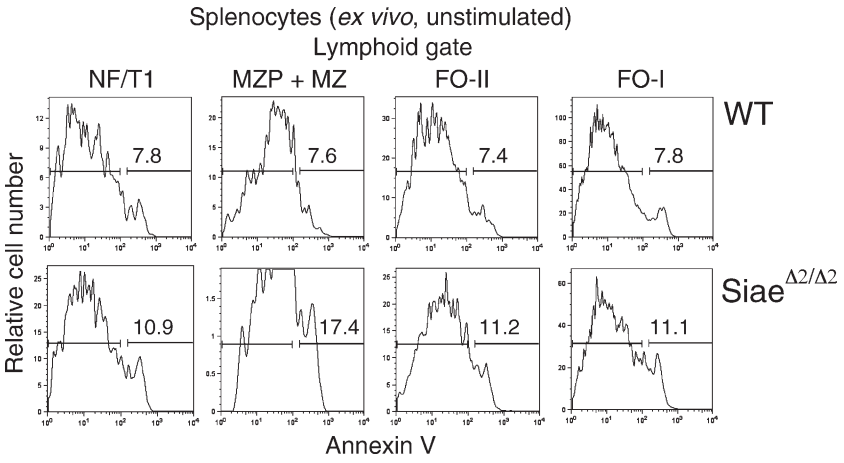

Figure S9. Mutant resting B splenocytes show a mild increase in apoptosis. Annexin V staining was performed on resting B cells from WT and *Siae* mutant mice with three mice per group.

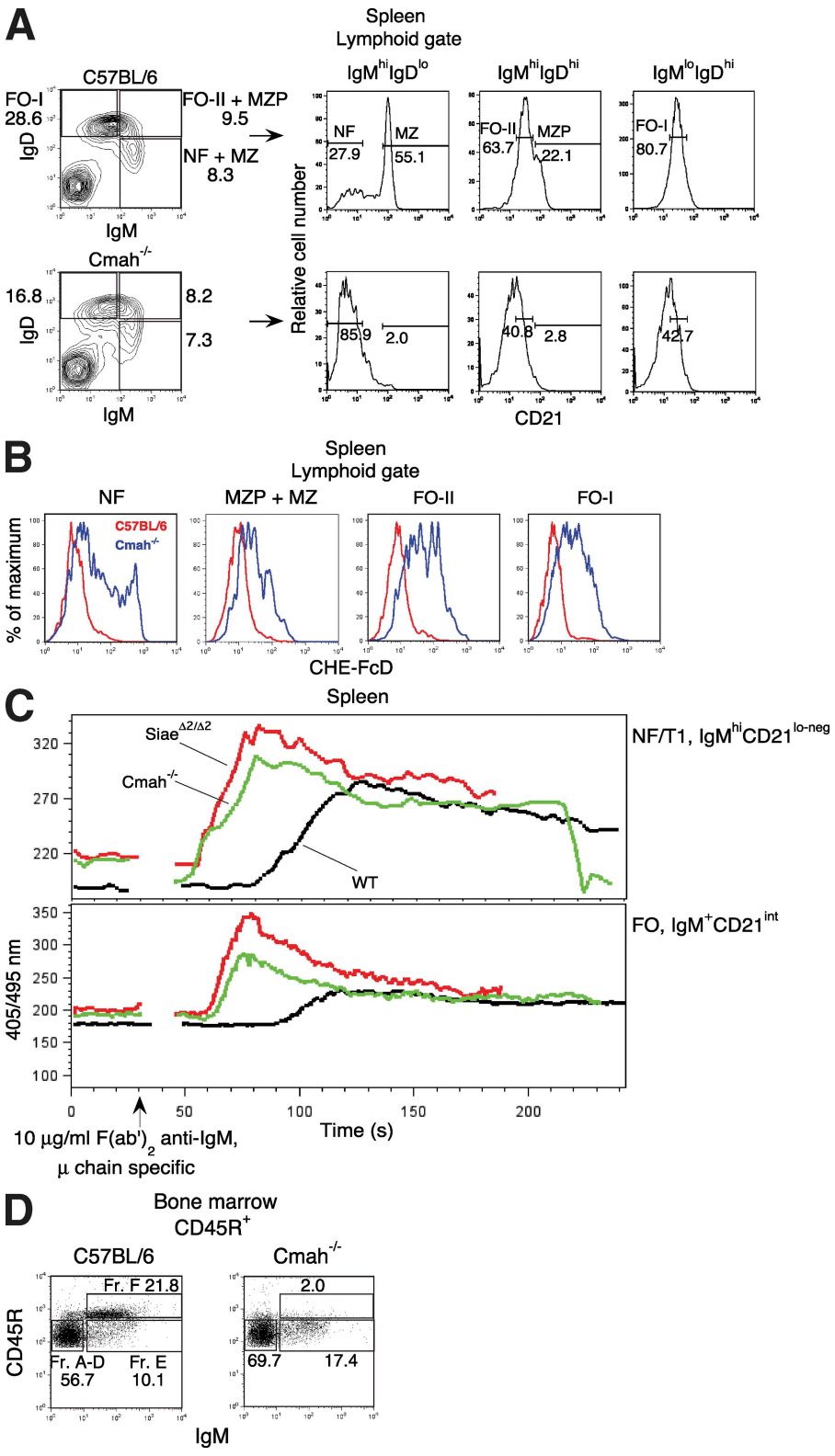

Figure S10. Analysis of *Cmah* mutant mice. (A) Flow cytometric analyses reveal a reduction in MZ B cells in *Cmah*$^{-/-}$ mice. Please refer to Fig. 5B for expansion of abbreviations. Results are representative of more than five mice per group. (B) Increased 9-*O*-acetylation of α2–6-linked sialic acid in *Cmah* mutant B cells. Results are representative of three mice per group. (C) Accelerated and enhanced BCR signaling in *Cmah* mutant mice as assessed by intracellular accumulation of calcium. Results are representative of three mice per group. (D) Perisinusoidal BM B cells are reduced in *Cmah*$^{-/-}$ mice. Results are representative of three mice per group. Please refer to Fig. 5C for expansion of abbreviations.

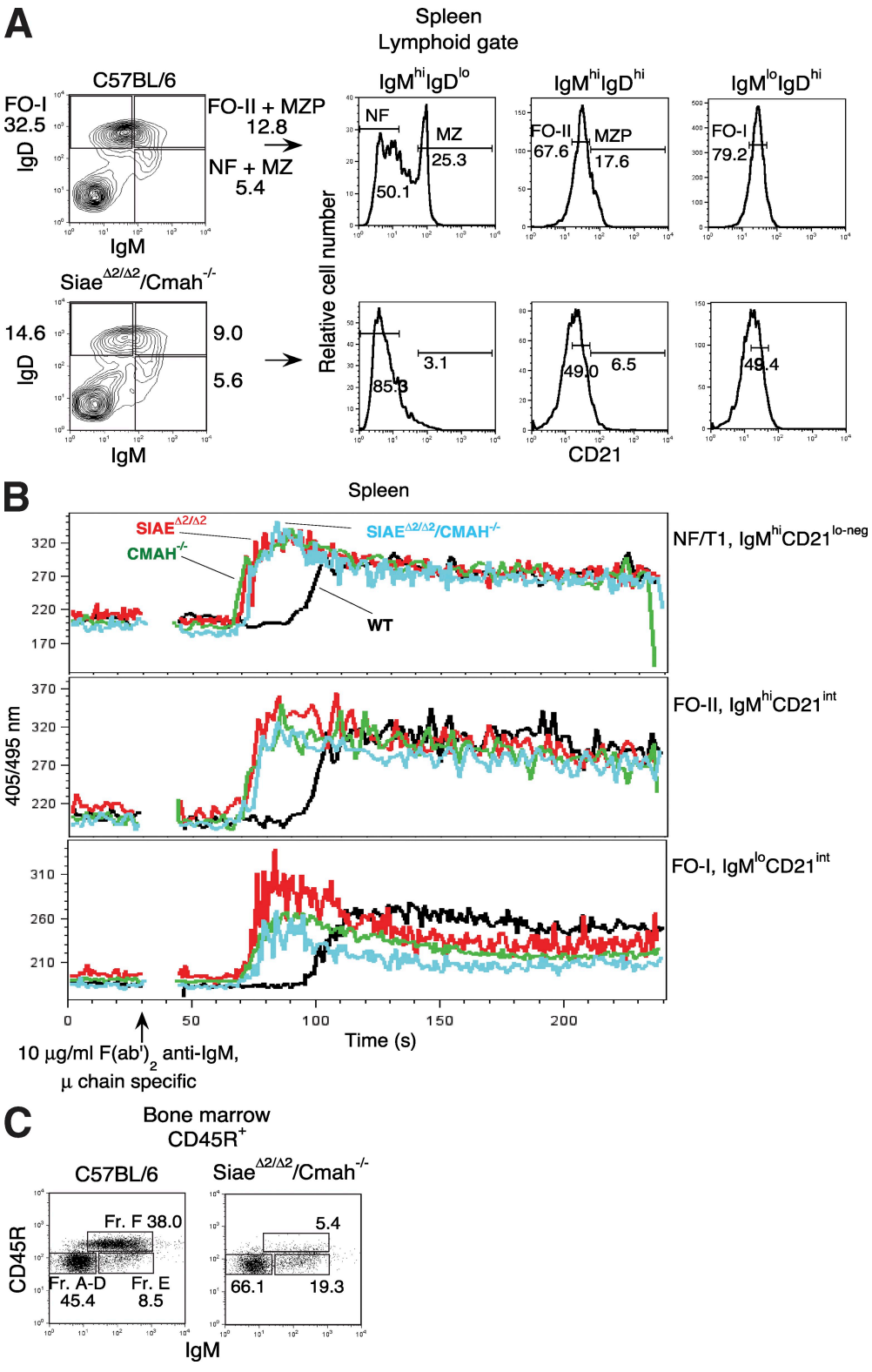

Figure S11. Analysis of *Siae/Cmah* double mutant mice. (A) Flow cytometric analyses reveal a reduction in MZ B cells in *Siae/Cmah* double mutant mice. Please refer to Fig. 5B for expansion of abbreviations. Results are representative of five mice per group. (B) Accelerated and enhanced BCR signaling in *Siae/Cmah* double mutant mice as assessed by intracellular accumulation of calcium. Results are representative of three mice per group. (C) Perisinusoidal BM B cells are reduced in *Siae/Cmah* mice double mutant mice. Results are representative of three mice per group. Please refer to Fig. 5C for expansion of abbreviations.

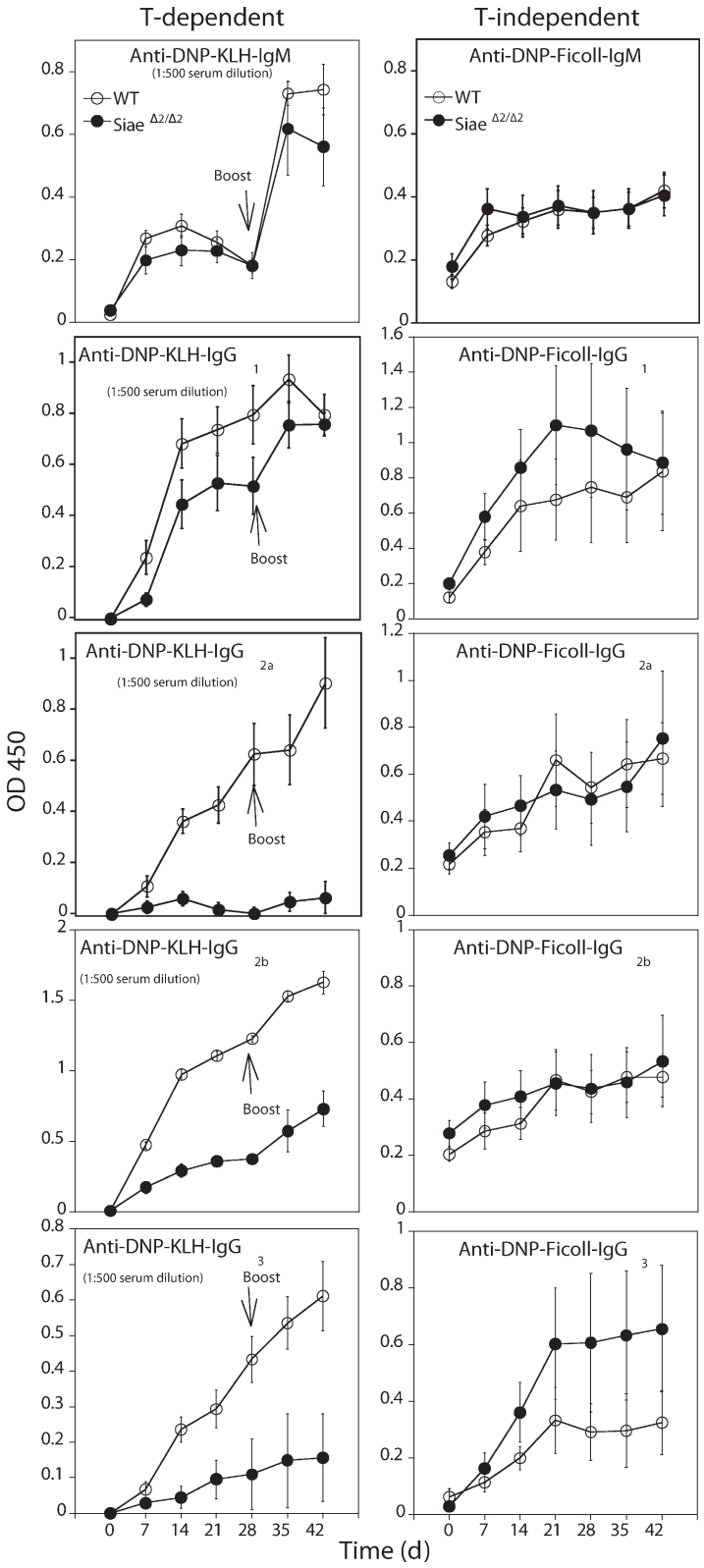

Figure S12. Antibody responses to synthetic T-dependent and T-independent antigens in Siae mutant and WT mice. Five mice were analyzed in each group. Error bars represent SEM.