Essential Role for Autophagy in the Maintenance of Immunological Memory Against Influenza Infection

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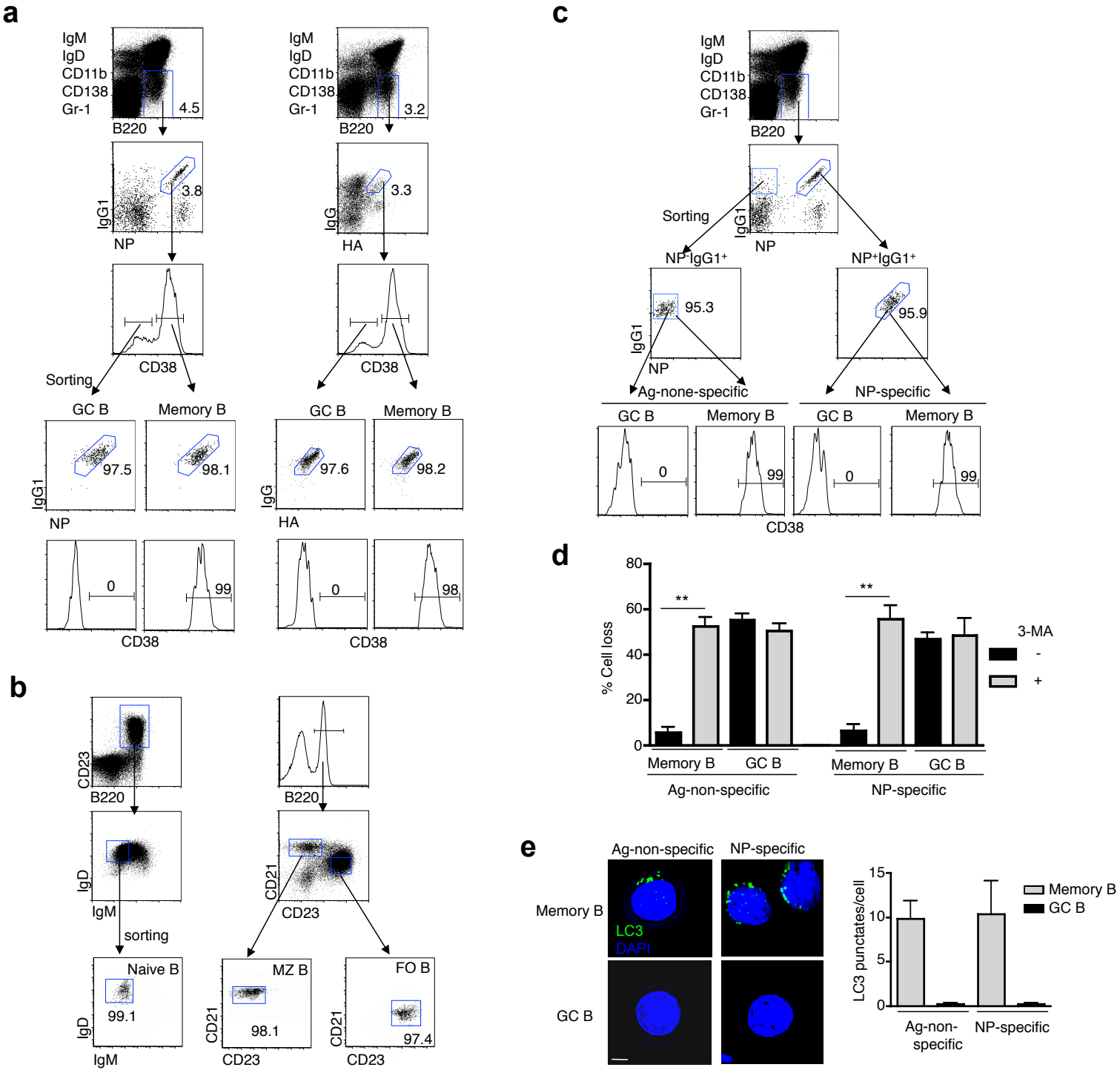
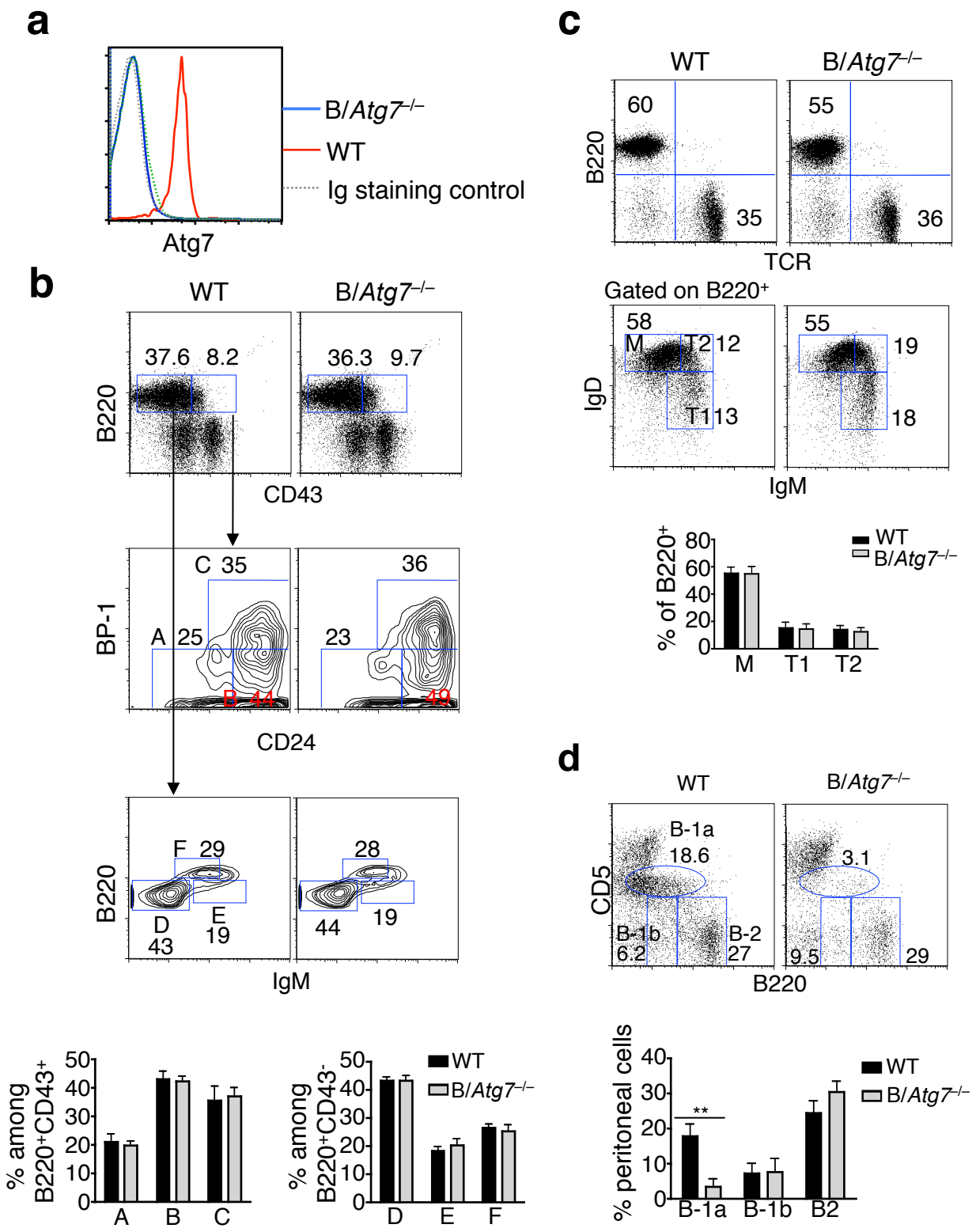
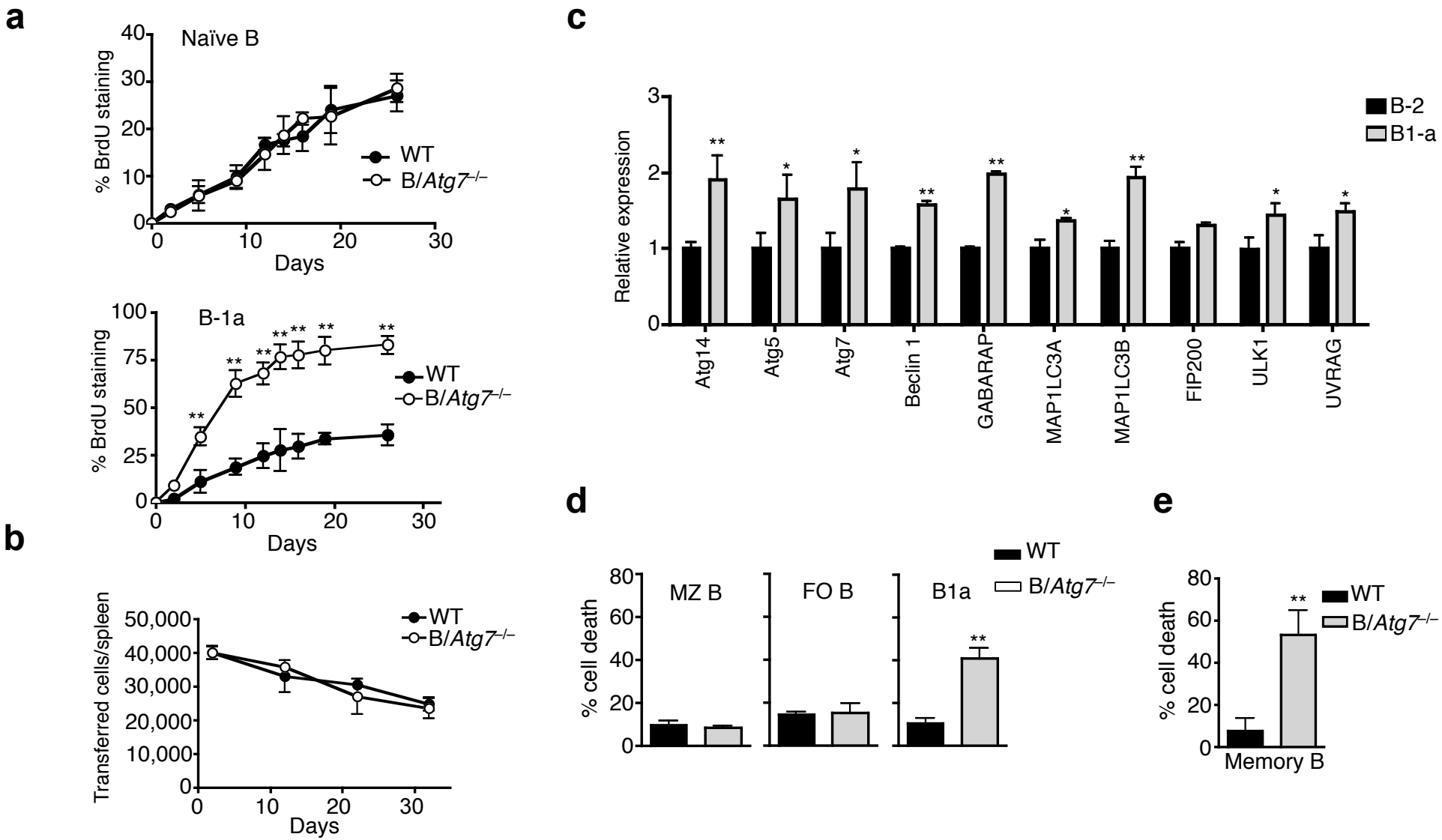


Supplementary Figure 1. Sorting of B cell subsets for analyses of cell death and autophagy.
(**a**) Splenocytes of mice immunized as in Figure 1a were used for cell sorting of NP-specific memory and GC B cells. (**b**) Naïve, marginal zone (MZ) and follicular (FO) B cells were sorted from unimmunized mouse spleen and analyzed by flow cytometry. (**c**) NP-specific and non-specific memory B cells and GC B cells were sorted. The purity of sorted cells were analyzed by flow cytometry. (**d**) Antigen-specific and non-specific memory B cells and GC B cells sorted in (c) were cultured in the presence or absence of 3-methyladenine (3-MA) and cell death was quantitated. (**e**) Antigen-specific and non-specific memory and GC B cells were used for immunocytochemistry staining for LC3. LC3 punctates in the cells were also quantified (n=10). Scale bar: 5 μm.

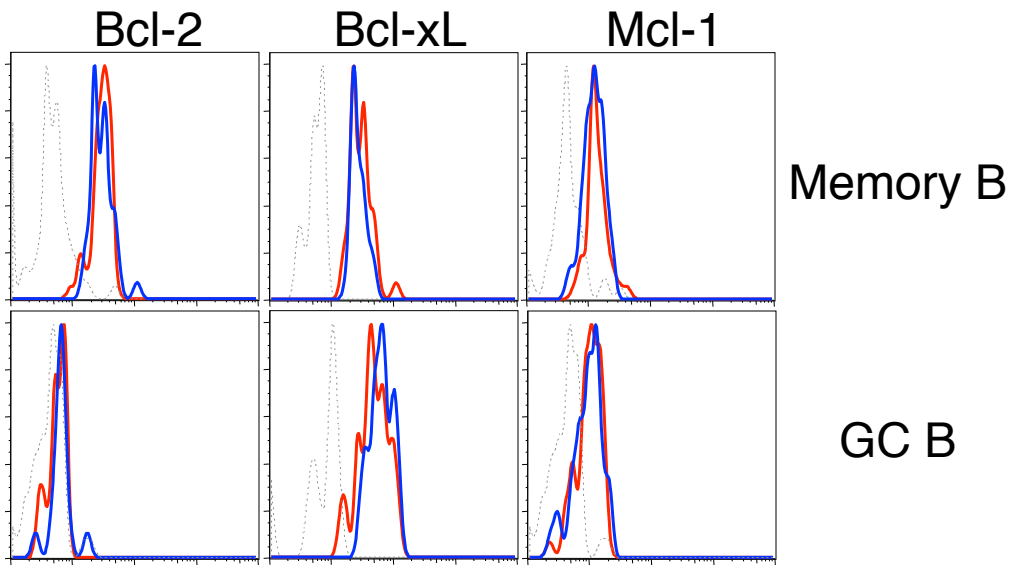


Supplementary Figure 2. B cell development in the absence of Atg7.
(**a**) HA-specific memory B cells from wild type (WT) and B/*Atg7*$^{-/-}$ mice were used for intracellular staining with anti-Atg7 or control IgG, followed by flow cytometry analyses. (**b**) Staining of B cell Hardy fractions in the bone marrow that represent pro- and pre-B cell development stages (Hardy et al., *J. Exp. Med.* 173:1213-1225). (**c**) Staining of T and B cells in the spleen. M: Naïve mature B cells; T1 and T2: Transitional T1 and T2 B cells, respectively. (**d**) Staining of B-1 and B-2 cells in the peritoneal cavity. Data are representative of five mice per group analyzed. $^{**}P<0.01$

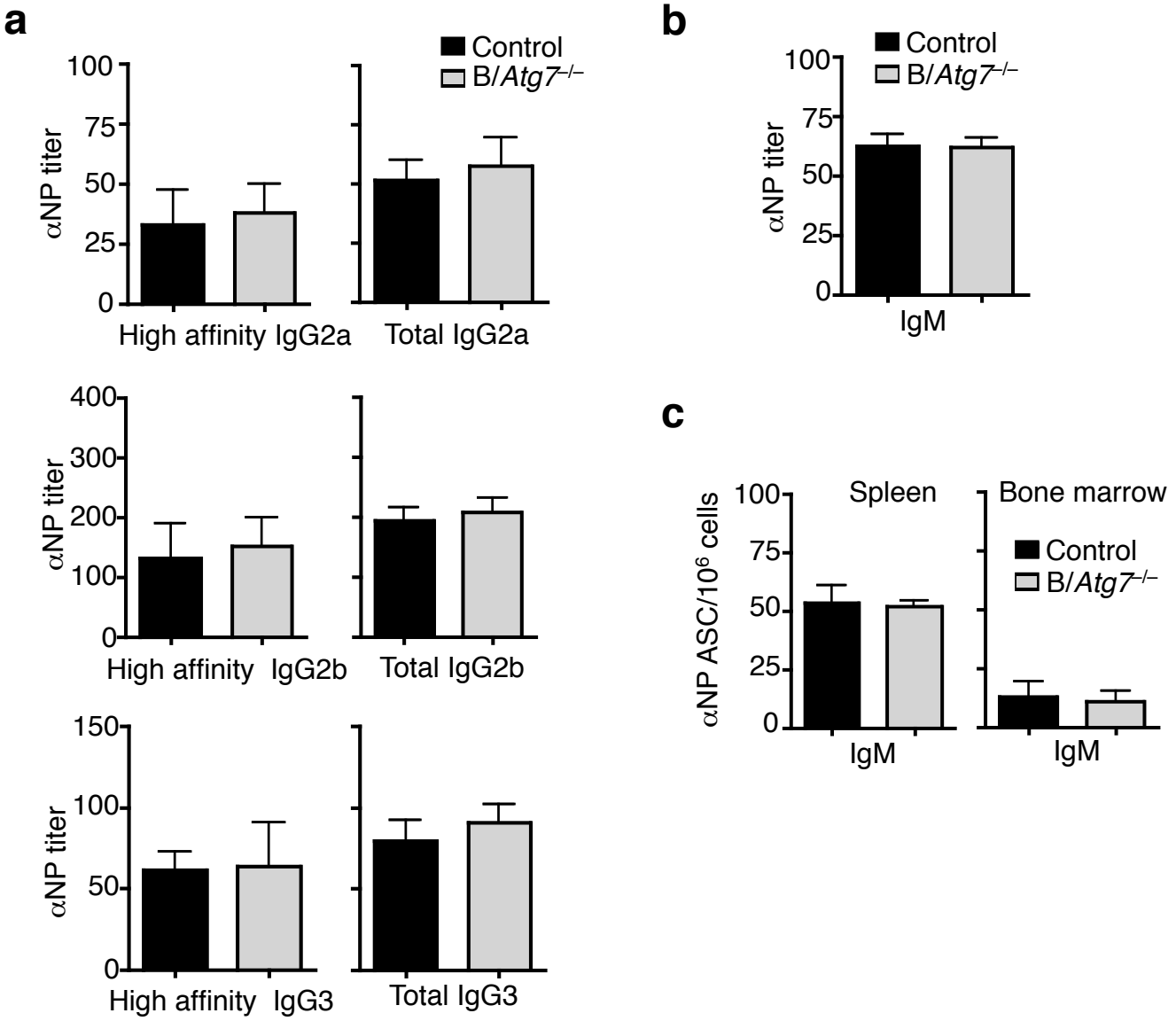


Supplementary Figure 3. Autophagy in B cell subsets.

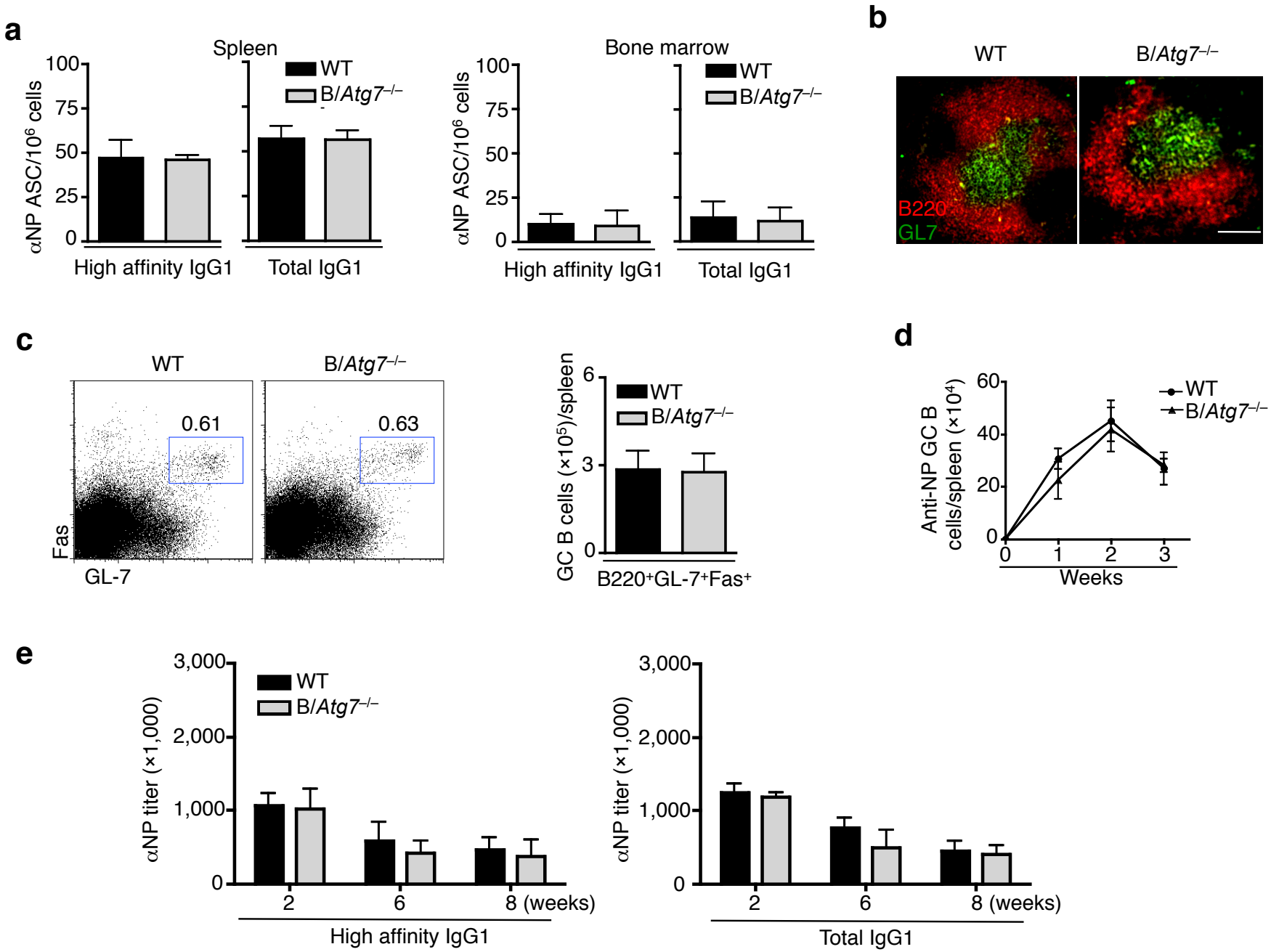
(**a**) Wild type (WT) and B/*Atg7*$^{-/-}$ mice were fed with BrdU in drinking water. BrdU^{+} cells among naïve B cells in the spleen or B-1a cells in the peritoneal cavity were quantitated at indicated time (3 mice for each time point per group). $^{**}P<0.01$. (**b**) B220^{+}IgM^{low}IgD^{+}CD23^{+}IgG^{-} naïve B cells sorted from the spleen of WT or B/*Atg7*$^{-/-}$ mice were adoptive transferred into congenic CD45.1 mice. Total transferred cells in the spleen of recipients were quantitated by flow cytometry between day 2 and day 32 after the transfer (3 mice for each time point per group). (**c**) Real-time PCR for autophagy genes in conventional B-2 cells and in B-1a cells. $^{*}P<0.05$, $^{**}P<0.01$. (**d**) Increased cell death in *Atg7*-deficient B-1a cells. B220^{+}CD23$^{low/-}$CD21^{high} MZ B cells, B220^{+}CD23^{+}CD21^{-} FO B cells from the spleen and CD5^{+}B220^{low}CD11b^{+}CD23^{-} B-1a cells from the peritoneal cavity were sorted. Percentage of cell death after *in vitro* culture was determined. $^{**}P<0.01$ (n=3). (**e**) IgG1 NP-specific memory B cells were sorted from mice two weeks after immunization with NP-KLH. Cell death was analyzed as in Figure 1. $^{**}P<0.01$ (n=3).



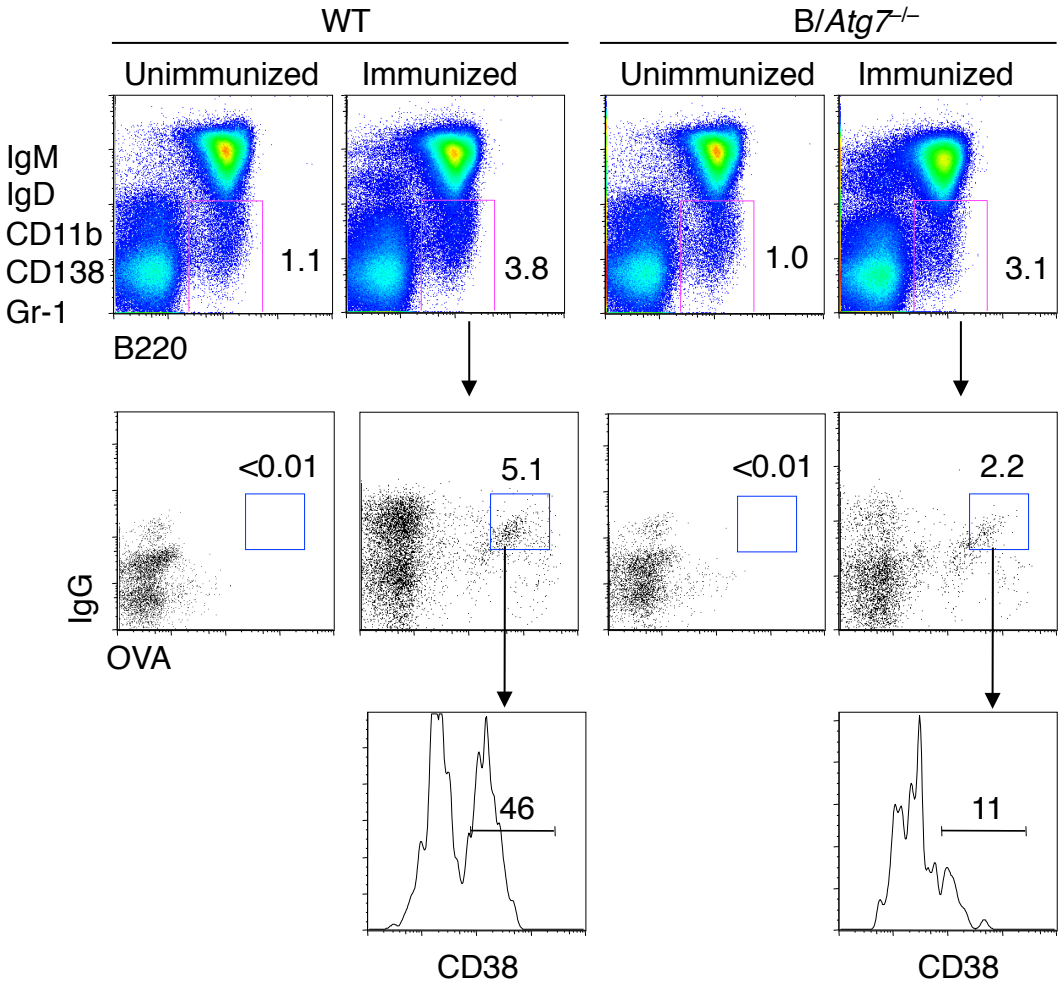
Supplementary Figure 4. Expression of anti-apoptotic Bcl-2 family proteins in memory B cells. $B220^{+}IgG1^{+}NP^{+}CD38^{+}$ memory B cells and $B220^{+}IgG1^{+}NP^{+}CD38^{-}$ GC B cells from wild type (red line) or B/*Atg7*$^{-/-}$ (blue line) mice were stained for surface markers, followed by intracellular staining with FITC-anti-Bcl-2, Alexa fluor 488-anti-Bcl-xL, FITC-anti-Mcl-1 (solid lines) or isotype controls (dotted lines) and analyzed by flow cytometry.



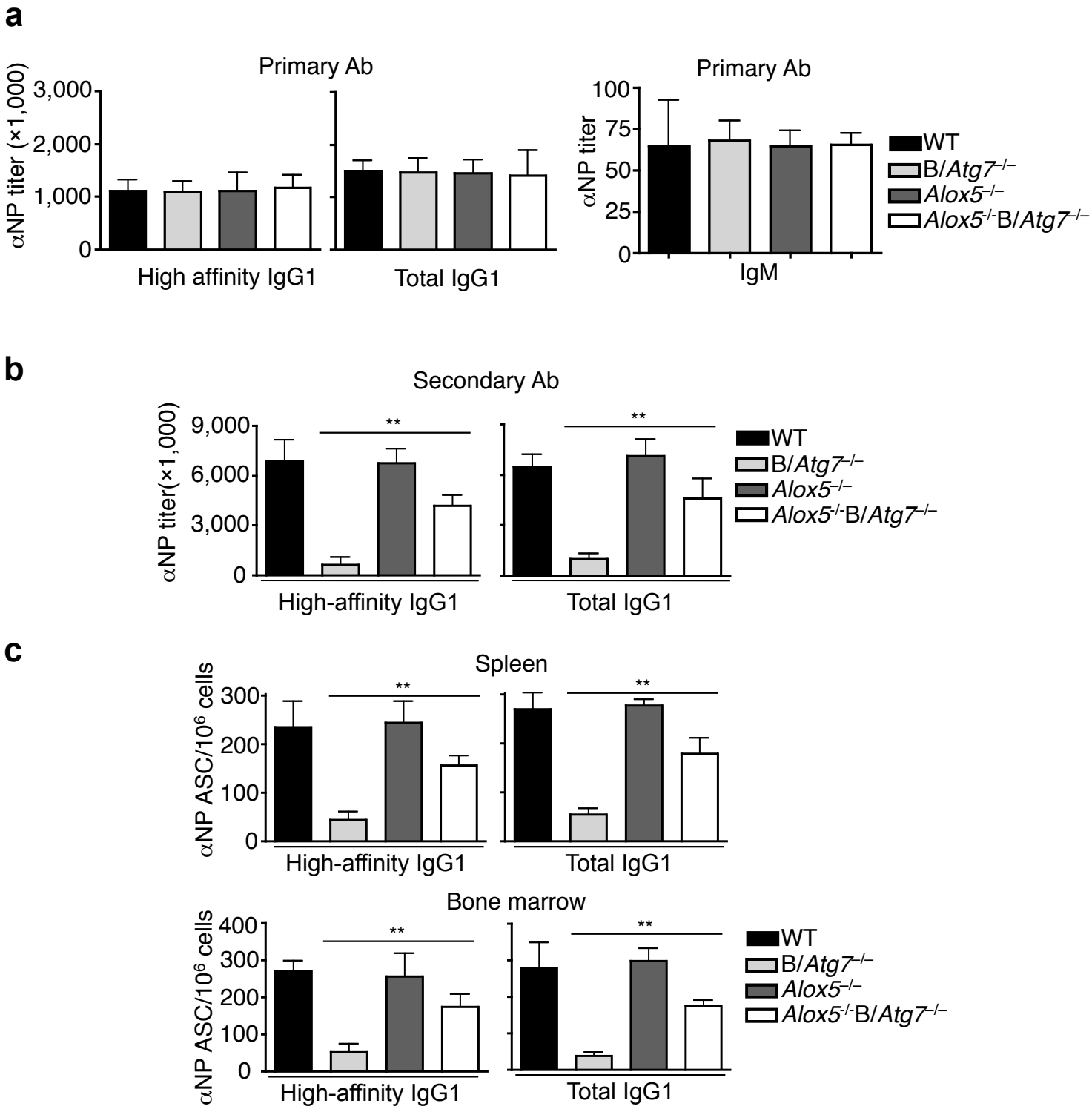
Supplementary Figure 5. Primary antibody responses in B/*Atg7*$^{-/-}$ mice. (**a**) Mice were immunized with NP-KLH precipitated in Alum. Two weeks later, ELISA for different subclasses of NP-specific antibodies was performed. Relative titers are shown as means ± SEM (*n*=10). (**b**) Mice were immunized with NP-KLH precipitated in Alum. Two weeks later, ELISA for IgM anti-NP in the sera was performed. Data are presented as mean ± SEM (*n*=10). (**c**) ELISPOT for IgM anti-NP ASC in the spleen and bone marrow from mice in (A) was performed. Data are presented as mean ± SEM (*n*=10).



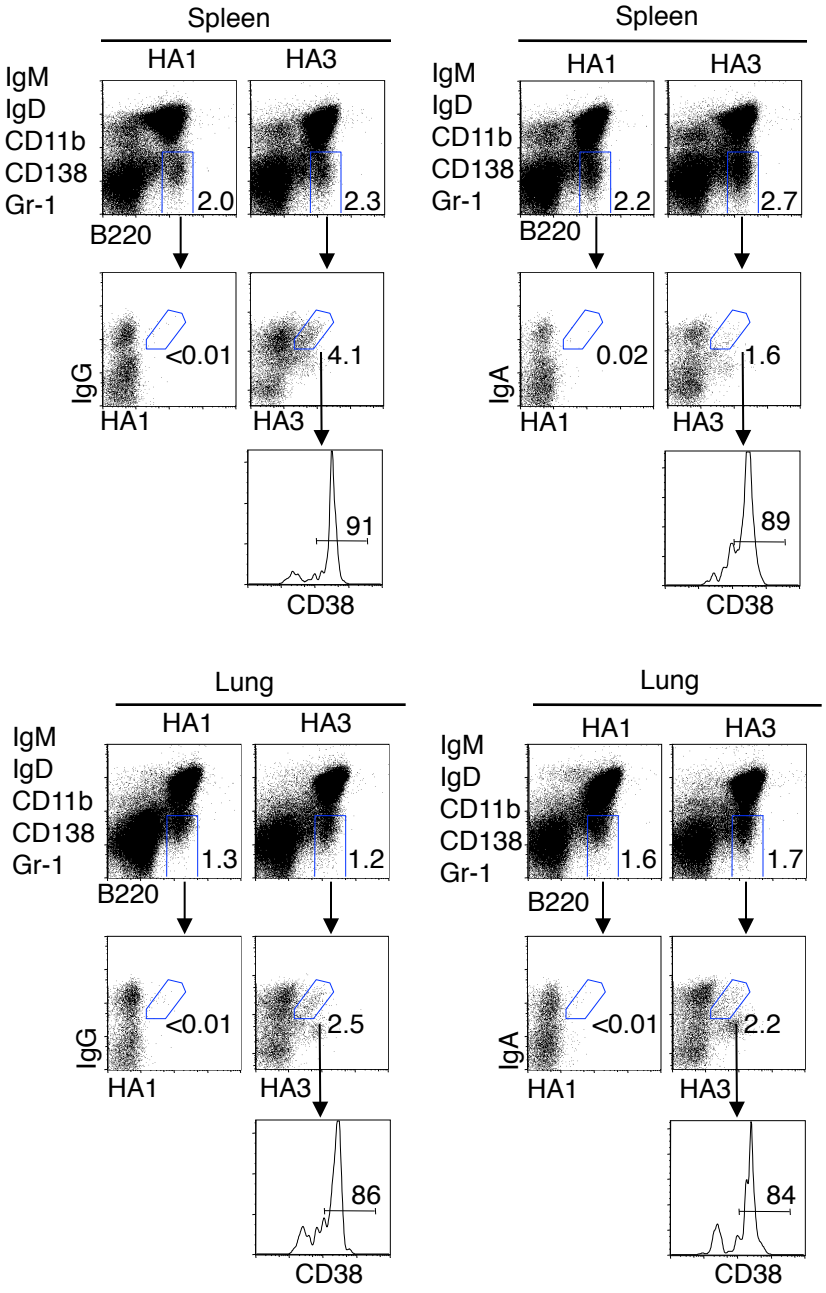
Supplementary Figure 6. Primary antibody and GC B cell responses in B/Atg7$^{-/-}$ mice. (**a**) B/*Atg7*$^{-/-}$ mice or *CD19-cre* mice as wild type (WT) controls were immunized with Alum-precipitated NP-KLH. Two weeks later, ELISPOT for NP-specific IgG1 ASC in the spleen and bone marrow were determined by ELISPOT. (**b**) Frozen spleen sections were stained with PE-anti-B220 and FITC-anti-GL7, and analyzed under a fluorescent microscope. Scale bar: 20 μm. (**c**) B220^{+}GL-7^{+}Fas^{+} GC B cells in the spleen of mice immunized as in (a) were analyzed by flow cytometry. Total GC B cell numbers per spleen were calculated. (**d**) Total anti-NP GC B cells in the spleen after immunization as in (a). (**e**) ELISA for anti-NP IgG1 in B/*Atg7*$^{-/-}$ mice or WT controls at different weeks after immunization.



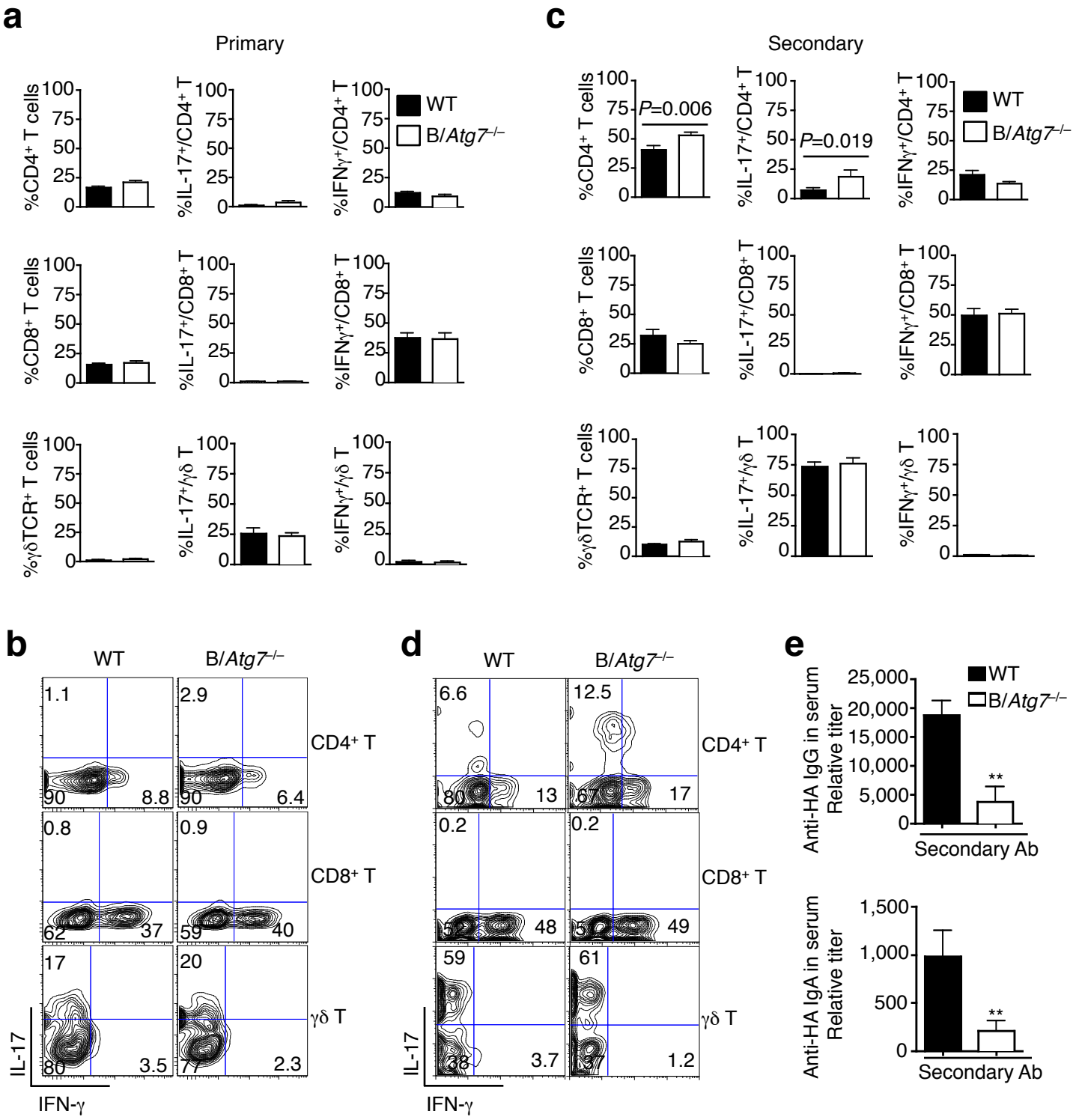
Supplementary Figure 7. Loss of memory B cells due to autophagy deficiency. Mice immunized with Alum-precipitated ovalbumin (OVA) were re-challenged with soluble OVA two months later. An example of flow cytometry of OVA-specific memory B cells in the spleen of immunized mice or unimmunized control is shown.



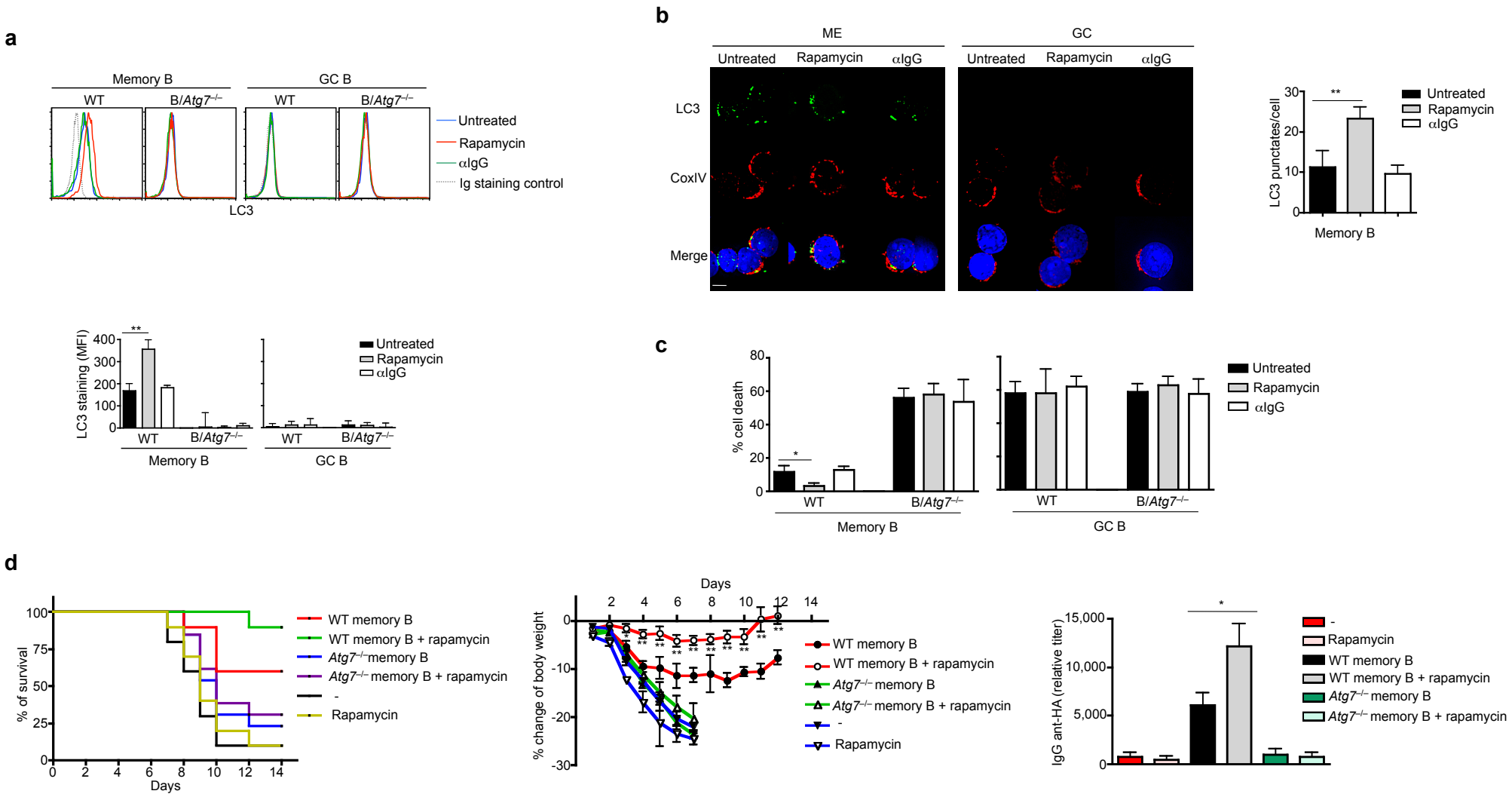
Supplementary Figure 8. Rescue of secondary antibody responses in B/*Atg7*$^{-/-}$ mice by deletion of Alox5. (**a**) Two-month-old *Alox5*$^{-/-}$B/*Atg7*$^{-/-}$, *Alox5*$^{-/-}$, B/*Atg7*$^{-/-}$ and wild type (WT) mice were immunized with NP-KLH. Primary antibody responses were determined as in Figure 3. (**b**, **c**) Mice immunized as in (a) were challenged with the antigen 2 months later. Anti-NP antibodies in the sera were determined by ELISA 5 days later (b). NP-specific ASC were determined by ELISPOT (c). ***P*<0.01. Five mice of each group were used in the experiments.



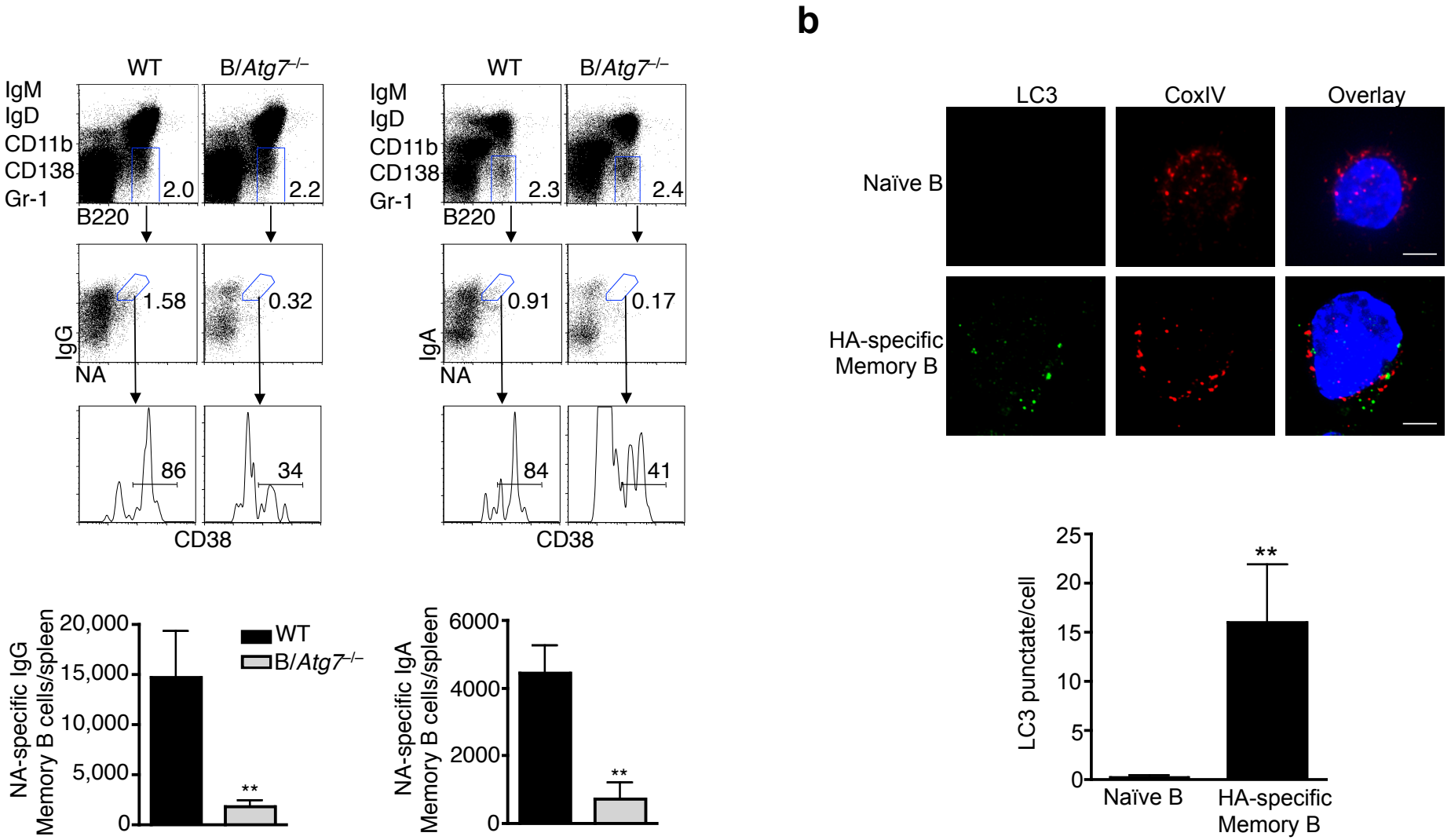
Supplementary Figure 9. Staining of influenza A HA-specific memory B cells. Wild type mice were immunized with influenza A as in Figure 5. The cells from the spleen and lung were stained as in Figure 5 except that HA1 instead of HA3 was used for staining in some groups as negative controls as previously described (Onodera et al., *Proc. Natl. Acad. Sci. U.S.A*. 109: 2485–2490). The cells were analyzed by flow cytometry.



Supplementary Figure 10. T cell responses in B/*Atg7*$^{-/-}$ mice after immunization with the influenza A virus. (**a**,**b**) Primary T cell responses in the spleen were examined by intracellular staining of cytokines 3 weeks after immunization with influenza A viruses (*n*=6). The examples of one set of mice are shown in (b). (**c**,**d**) To determine secondary T cell responses, immunized mice were re-challenged with influenza A viruses 8 weeks after immunization. Secondary T cell responses were examined 14 days later (*n*=7). Increased CD4$^+$ T cell expansion and IL-17 production may reflect prolonged T cell stimulation due to the higher viral load in B/Atg7$^{-/-}$ mice. The examples of one set of mice are shown in (d). (**e**) Anti-HA antibodies in the sera of mice 14 days after secondary influenza infection. $^{**}P<0.01$, *n*=7.



Supplementary Figure 11. Autophagy in memory B cells.
(**a**) HA-specific memory B cells with or without treatment with rapamycin or anti-IgG were used for intracellular staining with anti-LC3 or control IgG, followed by flow cytometry analyses. ***P*<0.01. (**b**) Cells as in (a) were used for immunocytochemistry staining for LC3 and CoxIV. Scale bar: 5 μm. ***P*<0.01. (**c**) Cells as in (a) were cultured as in Figure 2 for cell death analyses. **P*<0.05. (**d**) HA-specific WT or *Atg7*$^{-/-}$ memory B cells were sorted from pooled spleens and lungs from influenza infected mice and adoptively transferred i.v. (10^4/mice) into *Atg7*$^{-/-}$ mice which had received a sublethal dose (7.5 $TCID_{50}$) of H3N2 influenza A virus 3 months ago. One day after transfer, *Atg7*$^{-/-}$ recipients were re-infected with a lethal dose of influenza (2,500 $TCID_{50}$). Weight loss and survival of B/*Atg7*$^{-/-}$ mice were monitored for 14 days. Some groups were treated with rapamycin i.p. daily from day -1 to day 13 during infection. Sorted naïve cells (2×10^5) were co-injected as filler cells to minimize cell loss during injection (12 mice/group). Rapamycin treatment versus no rapamycin in groups with transfers of WT memory B cells: *P*<0.05; Weight loss and anti-HA titers, **P*<0.05, ***P*<0.01.



Supplementary Figure 12. Autophagy in mouse and human influenza-specific memory B cells.
(**a**) Wild type (WT) and B/Atg7$^{-/-}$ mice were immunized with influenza virus at a sublethal dose of 7.5 $TCID_{50}$ intranasally. Two months later, neuraminidase (NA)-specific memory B cells in the spleen were examined (*n*=6 mice/group). ***P*<0.01. (**b**) Human $CD19^{+}CD27^{-}IgG^{-}IgA^{-}$ naïve B cells and H3N2 Influenza A HA-specific $CD19^{+}CD27^{+}IgG^{+}HA^{+}$ memory B cells were sorted and used for immunocytochemistry staining of LC3 and CoxIV. The nuclei were counterstained with DAPI. LC3 punctates per cell were quantitated. Data are representative of five donors. ***P*<0.01. Scale bar: 5 μm.