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Supplemental information

B cell extracellular vesicles contain monomeric IgM that binds antigen and enters target cells

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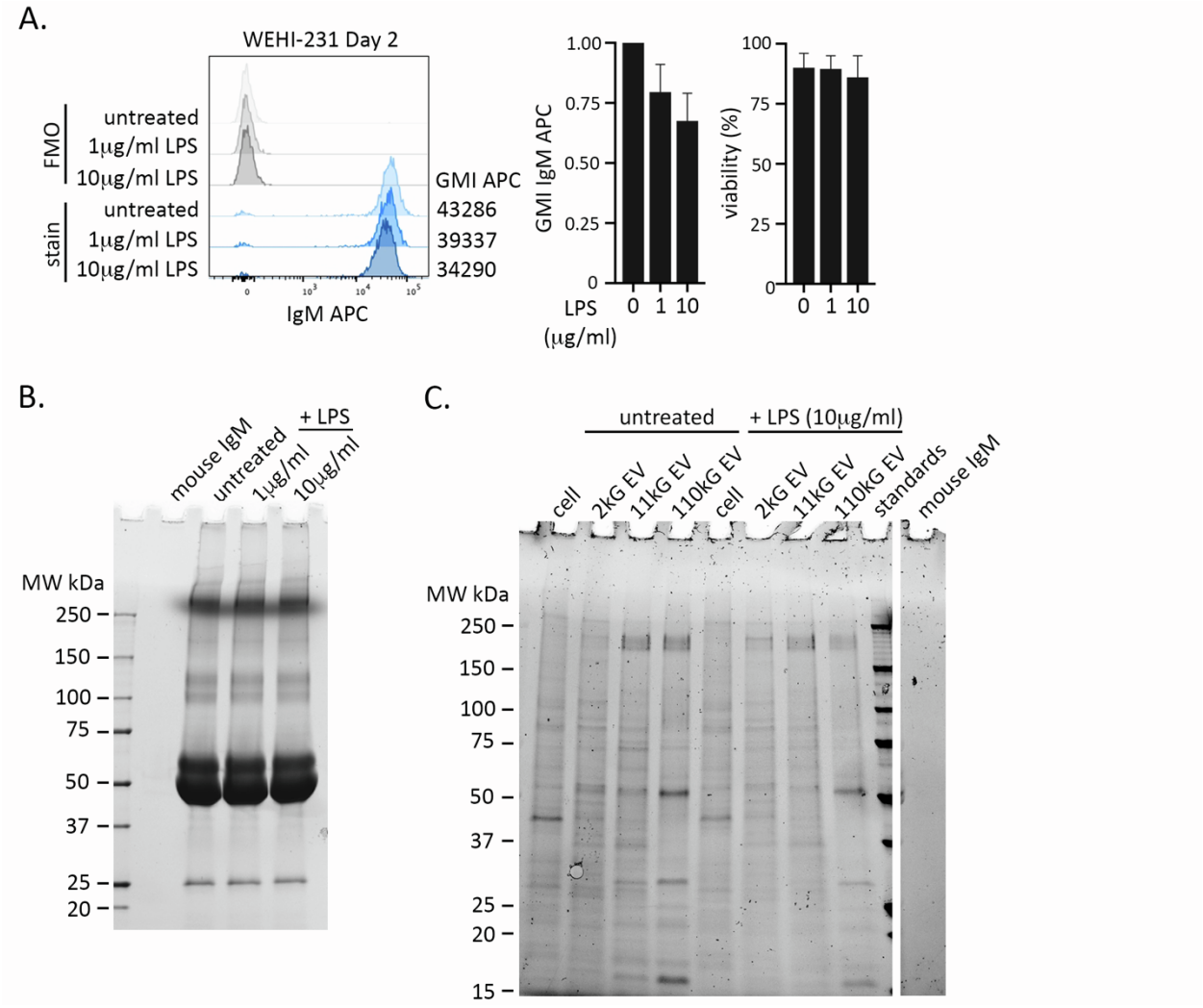

Figure S1. WEHI-231 cell characterization and extracellular vesicle production in response to LPS, related to Figure 1. A. WEHI-231 cells cultured in either complete media alone (untreated) or complete media supplemented with 1μg/ml or 10 μg/ml LPS for two days were evaluated for surface expression of IgM by flow cytometry. Representative histogram overlays of anti-IgM fluorescence intensity of stained cells for each condition compared to FMO control cells are shown, along with the geometric mean intensity (GMI). The GMI IgM APC from two independent experiments are presented (left graph), along with the cell viability at day two (right). **B.** Conditioned media from untreated WEHI-231 cells, or WEHI-231 cells treated with 1 or 10 μg/ml LPS, was centrifuged at 2000 x g and resolved by SDS PAGE (8μl/well). Presented is the stain free gel displaying the total protein for each sample. **C.** Lysate preparations (1μg protein/well) of whole cells, and extracellular vesicle (EV) populations isolated by differential ultracentrifugation (2kG, 11,000 x g (11kG), 110,000 x g (110kG)), from untreated or LPS (10μg/ml) treated WEHI-231 cells were resolved by SDS PAGE. Presented is the stain free gel displaying the total protein for each sample. Purified mouse IgM (3.1ng) was run as a positive control in a non-contiguous lane and is presented at the far right in both images. Data are representative of at least two independent experiments. Data in **A.** are represented as +/- SEM.

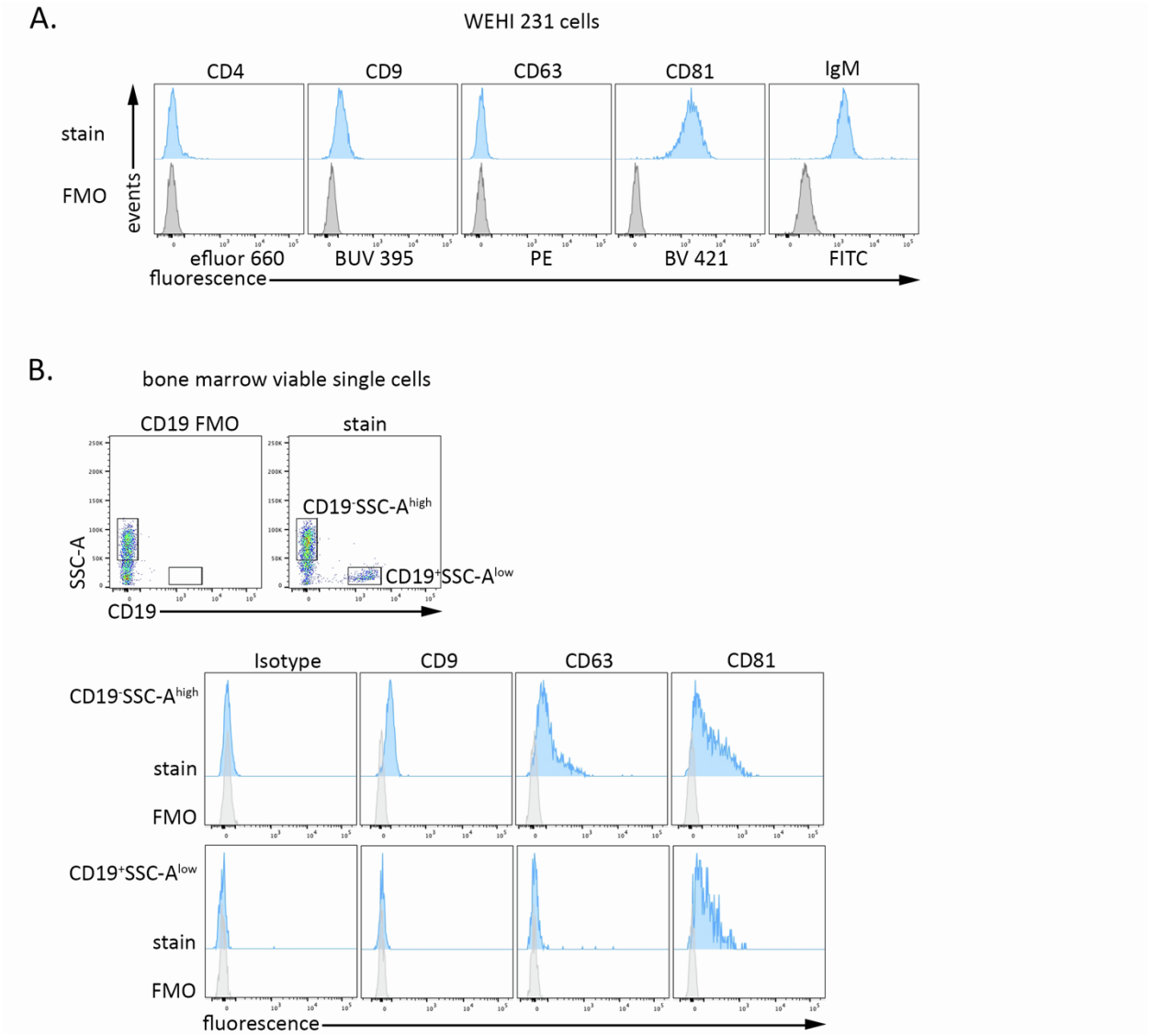

Figure S2. CD9, CD81, and IgM, but not CD63, are expressed on the cell surface of WEHI 231, related to Figure 2. WEHI 231 cells were stained for the indicated antigens and analyzed by flow cytometry. **A.** Histograms display the fluorescence of singlet, viable stained cells (upper) compared to FMO stained cells (lower). **B.** Whole bone marrow from WT C57BL/6 mice was stained for the tetraspanins CD9, CD63, and CD81, along with CD19. Isotype control antibody was included in the panel. Population gates were first set on singlet, viable cells for $CD19^{-}SSC\text{-}A^{high}$ cells and $CD19^{+}SSC\text{-}A^{low}$ cells (upper row). The expression of CD9, CD63, CD81, and isotype control fluorescence in each population, as compared to FMO controls, is presented (center and lower rows).

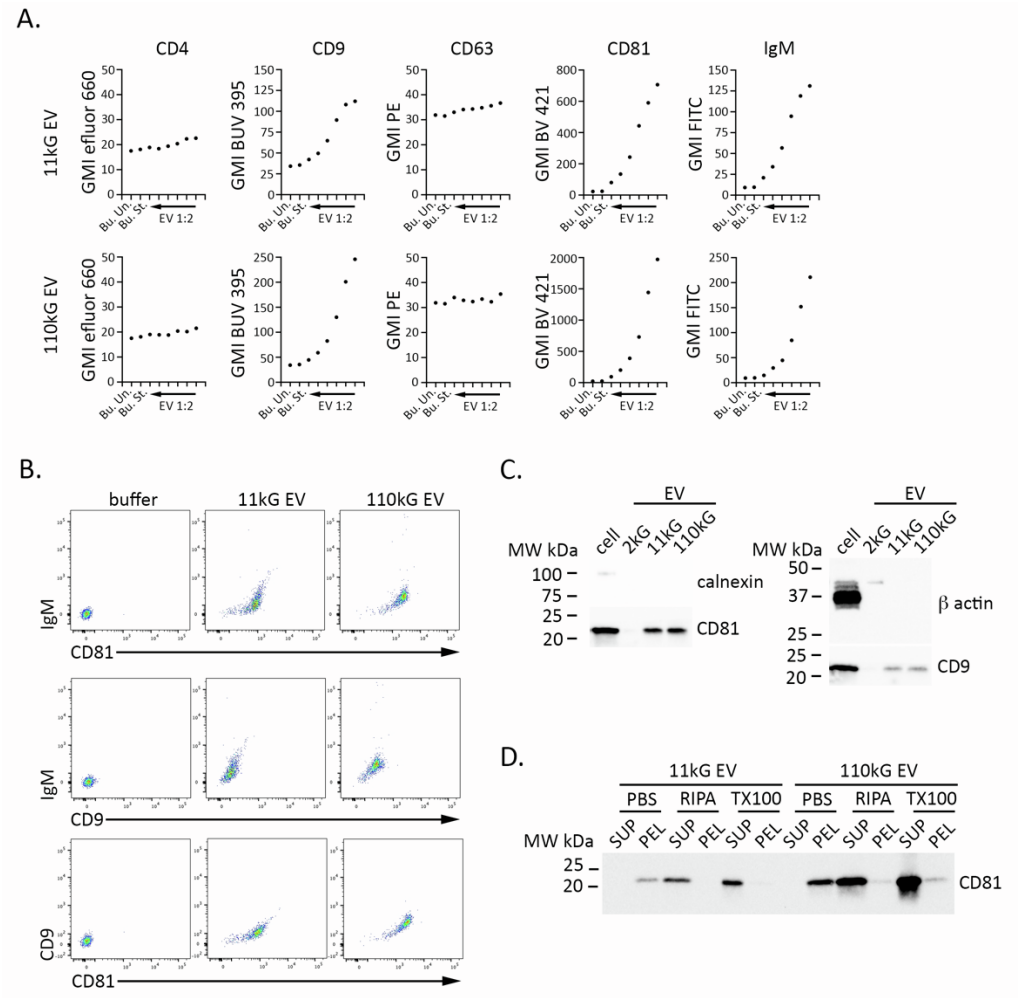

Figure S3. Application of differential ultracentrifugation (DUC) to WEHI 231 conditioned media results in isolated EV populations that display hallmark EV characteristics, related to Figure 2. Equivalent amounts of WEHI 231 11kG and 110kG EVs isolated by DUC were titrated 1:2 and adsorbed onto aldehyde sulfate microbeads, stained for surface expression of IgM, canonical EV marker proteins, and negative control protein (CD4) and analyzed by flow cytometry. Beads incubated with buffer alone were included as a control. **A.** Presented is the bead GMI for each antigen across all 11kG and 110kG titrations (far right data point most concentrated) compared to beads incubated in buffer alone. Buffer only unstained (Bu. Un.) and stained (Bu. St.) are indicated at the far left of each graph. **B.** Bi-parametric analysis of IgM/CD81 (top row), IgM/CD9 (center), and CD9/CD81 (bottom) for buffer, 11kG EV, and 110kG EV samples is presented. **C.** WEHI 231 cell lysate (10μg**)** and EV preparations (2kG, 11kG, 110kG) were prepared in non-reducing sample buffer and analyzed by SDS/PAGE for expression of calnexin and CD81 (left image) and actin and CD9 (right). **D.** Isolated 11kG and 110kG EV were divided evenly three ways and pelleted. Each pellet was subsequently resuspended in either PBS, RIPA buffer, or 0.25% TX100 and incubated on ice for 20min. The RIPA and TX100 samples were vortexed briefly throughout the incubation. The samples were then centrifuged at the appropriate pelleting speed (11,000 x g or 110,000 x g). The supernatant was removed with care to avoid disturbing the pellet, and then combined with sample buffer. The pelleted material was washed in PBS and then centrifuged at the appropriate speed. The supernatant was then removed with care and discarded, and the pelleted material was resuspended in sample buffer. Following the initial buffer incubation, sample processing was identical for each 11kG and 110kG cohort. The supernatant (SUP) and pelleted material (PEL) were then resolved by SDS/PAGE and evaluated for CD81 expression by immunoblot. Data are representative of two independent experiments.

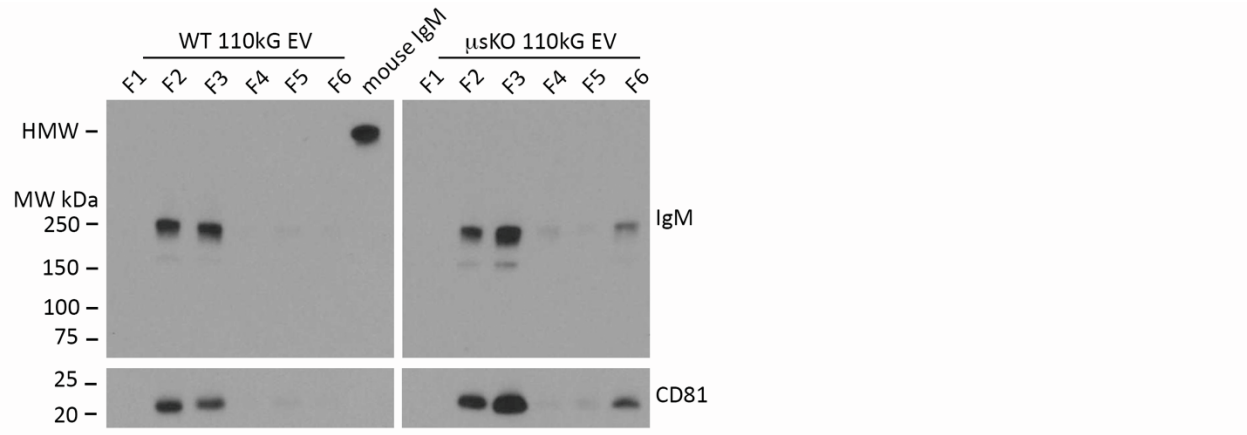

Figure S4. EV isolated by differential ultracentrifugation (DUC) and sucrose gradient fractionation from WT and μsKO splenic B cells culture media contain low molecular weight IgM, related to Figure 4. DUC isolated 110kG EV samples were fractionated by sucrose density centrifugation, washed, and resuspended in an equivalent volume of buffer. The samples were then resolved by SDS/PAGE, followed by immunoblot detection of IgM and CD81. Mouse IgM was run as a positive control. Shown are WT (left) and μsKO (right) samples run on non-contiguous lanes. Data are representative of two independent experiments.

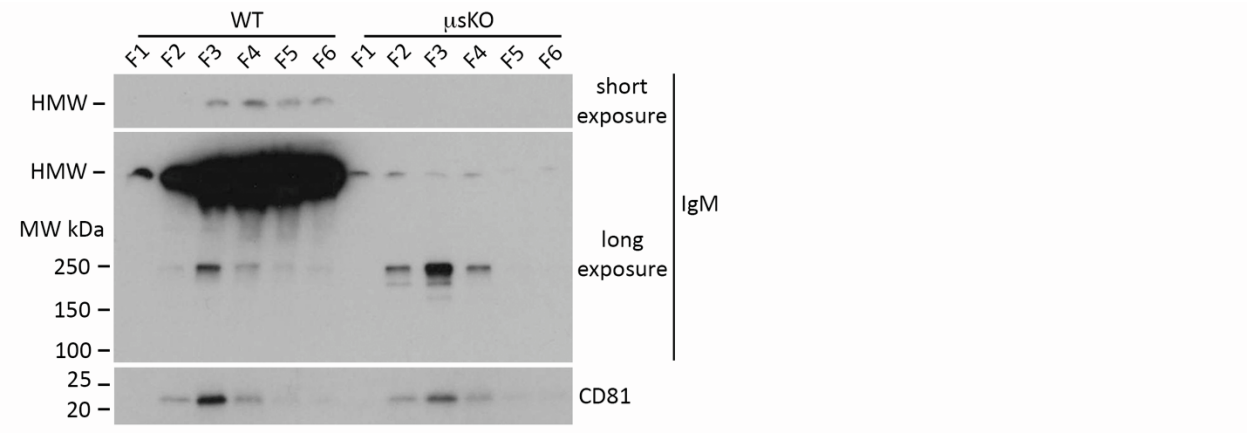

Figure S5. EV isolated from WT and μsKO mouse plasma contain low molecular weight IgM, related to Figure 5. Mouse plasma from WT and μsKO mice was harvested and first subjected to differential ultracentrifugation (DUC) to isolate the 110kG EV population. Following subsequent sucrose gradient fractionation, each resultant fraction was resuspended in an equivalent volume of buffer, resolved by SDS/PAGE under unreduced conditions, and immunoblotted for IgM and CD81.

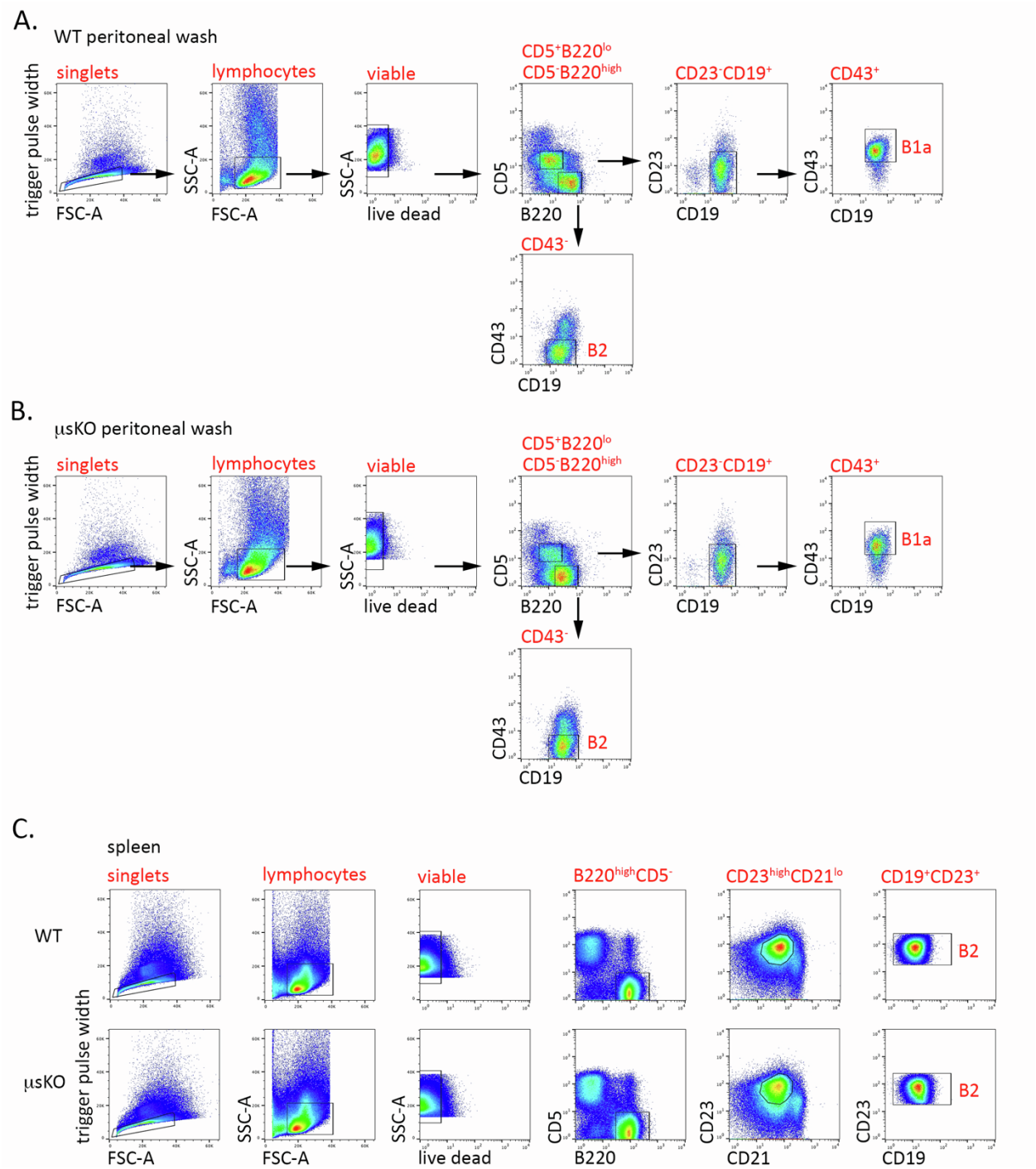

Figure S6. Gating scheme for sorted peritoneal B1a, B2 and splenic B2 cell populations from WT and μsKO mice, related to Figure 5. A. Dot plots display the gating scheme for WT peritoneal B1a and B2 cells. **B.** Dot plots display the gating scheme for μsKO peritoneal B1a and B2 cells. **C.** Dot plots display the gating scheme for WT (upper) and μsKO (lower) splenic B2 cells. Data are representative of at least two independent experiments.

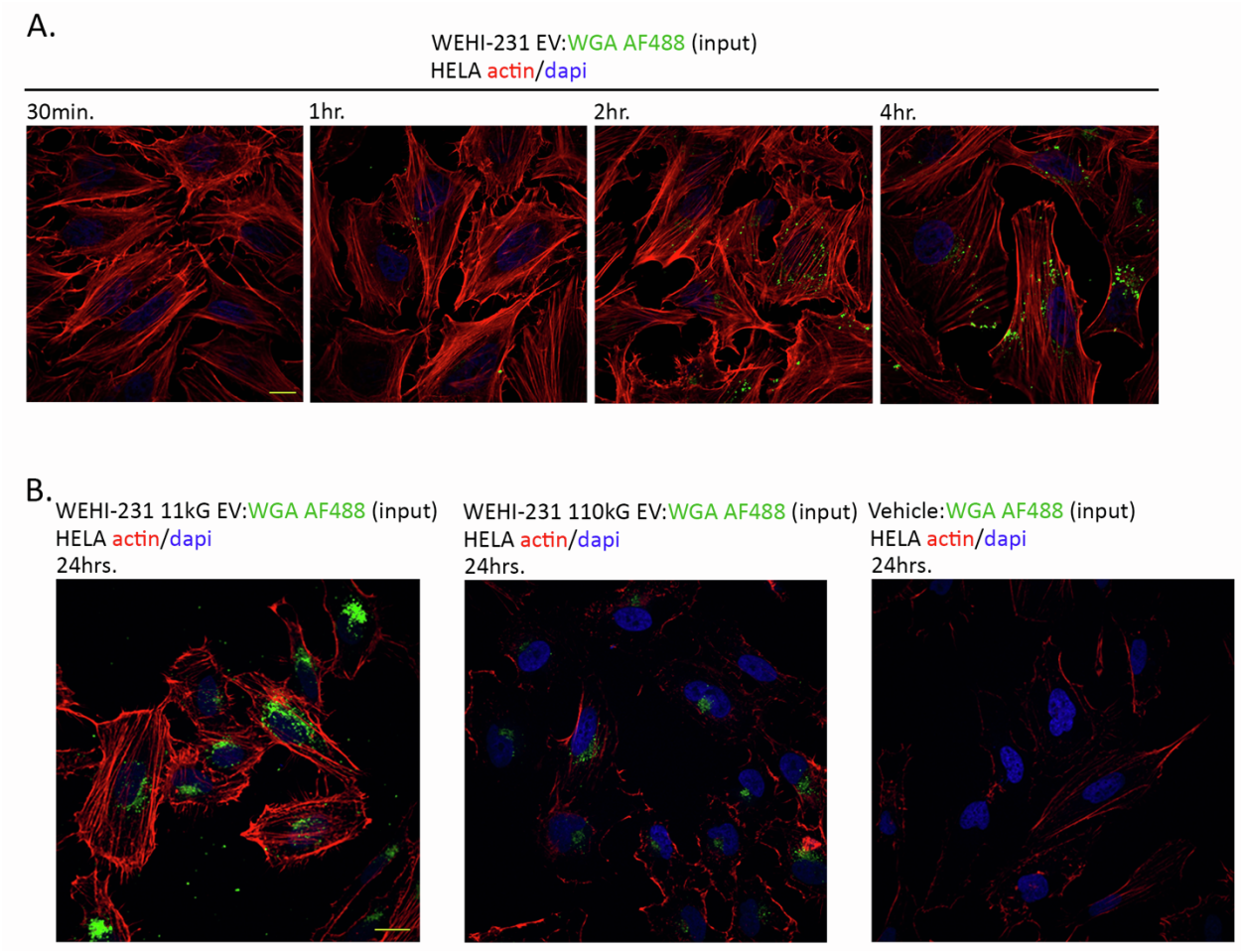

Figure S7. EV produced by WEHI-231 accumulate in secondary cells, related to Figure 7. Following isolation by differential ultracentrifugation, WEHI-231 EV were labeled with wheat germ agglutinin AF488 (WGA AF488) and subsequently applied to HELA monolayers. After the indicated times, the cells were stained with phalloidin rhodamine and dapi, and then imaged by confocal microscopy. **A.** Representative images show HELA monolayers at 30min., 1hr., 2hr., and 4hr. post-application of WEHI-231 EV:WGA AF488, with pseudocolored WGA AF488 (green), actin (red), and dapi (blue). **B.** Representative images show HELA monolayers 24hrs. post-application of 11kG EV (left), 110kG EV (center), and buffer only (right). Scale bars indicate 20μm.

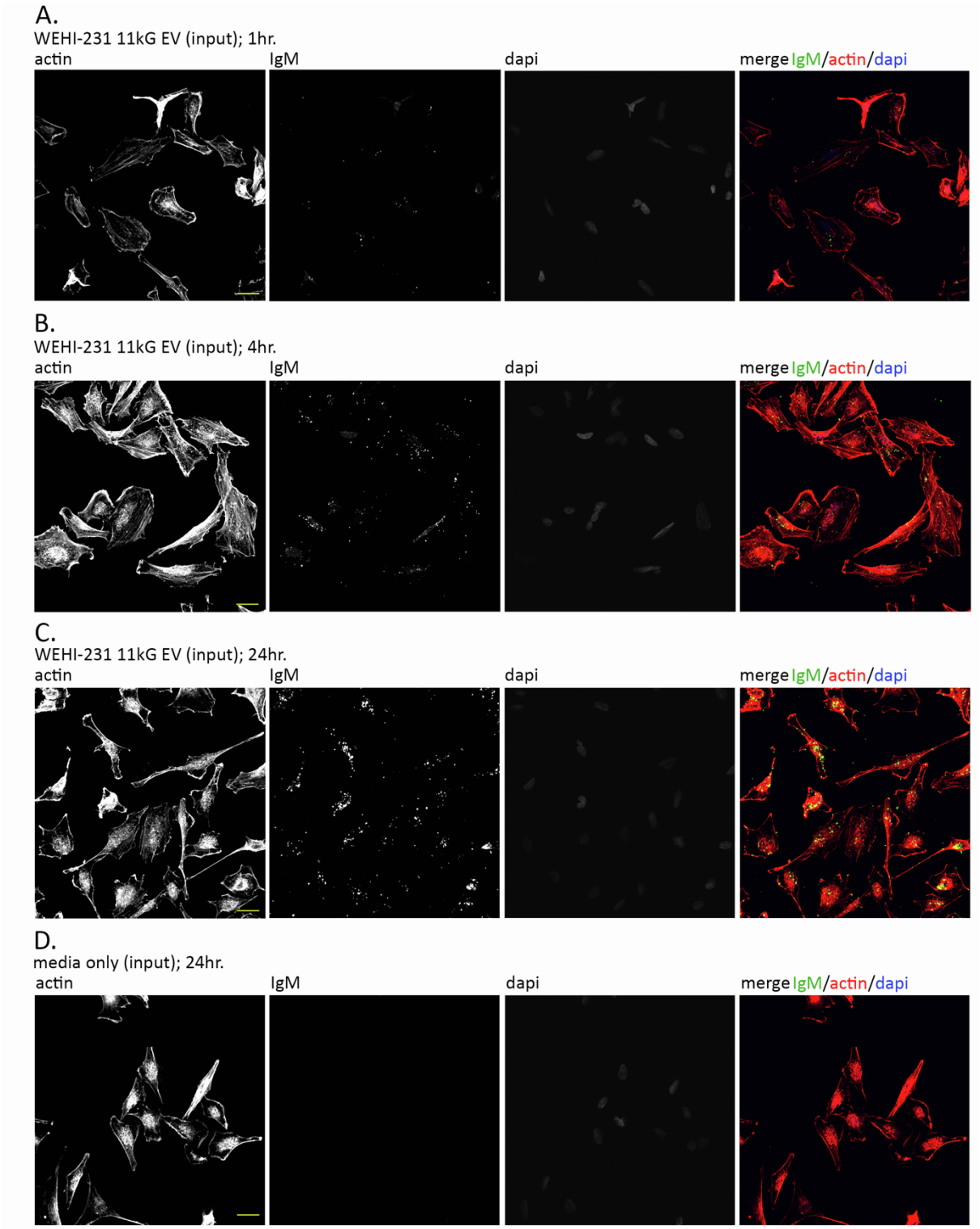

Figure S8. IgM contained in B cell-derived EV can accumulate intracellularly in secondary cells, related to Figure 7. HELA monolayers treated with isolated WEHI-231 11kG EV were subjected to immunofluorescent staining for mouse IgM, phalloidin (actin), and dapi and then imaged by confocal microscopy. Displayed are representative images for treatment periods of 1hr. (**A.**), 4hr. (**B.**), or 24hr. (**C.**). Stained HELA monolayers treated with media alone for 24hr. are shown in **D.**. Scale bars indicate 20μm.

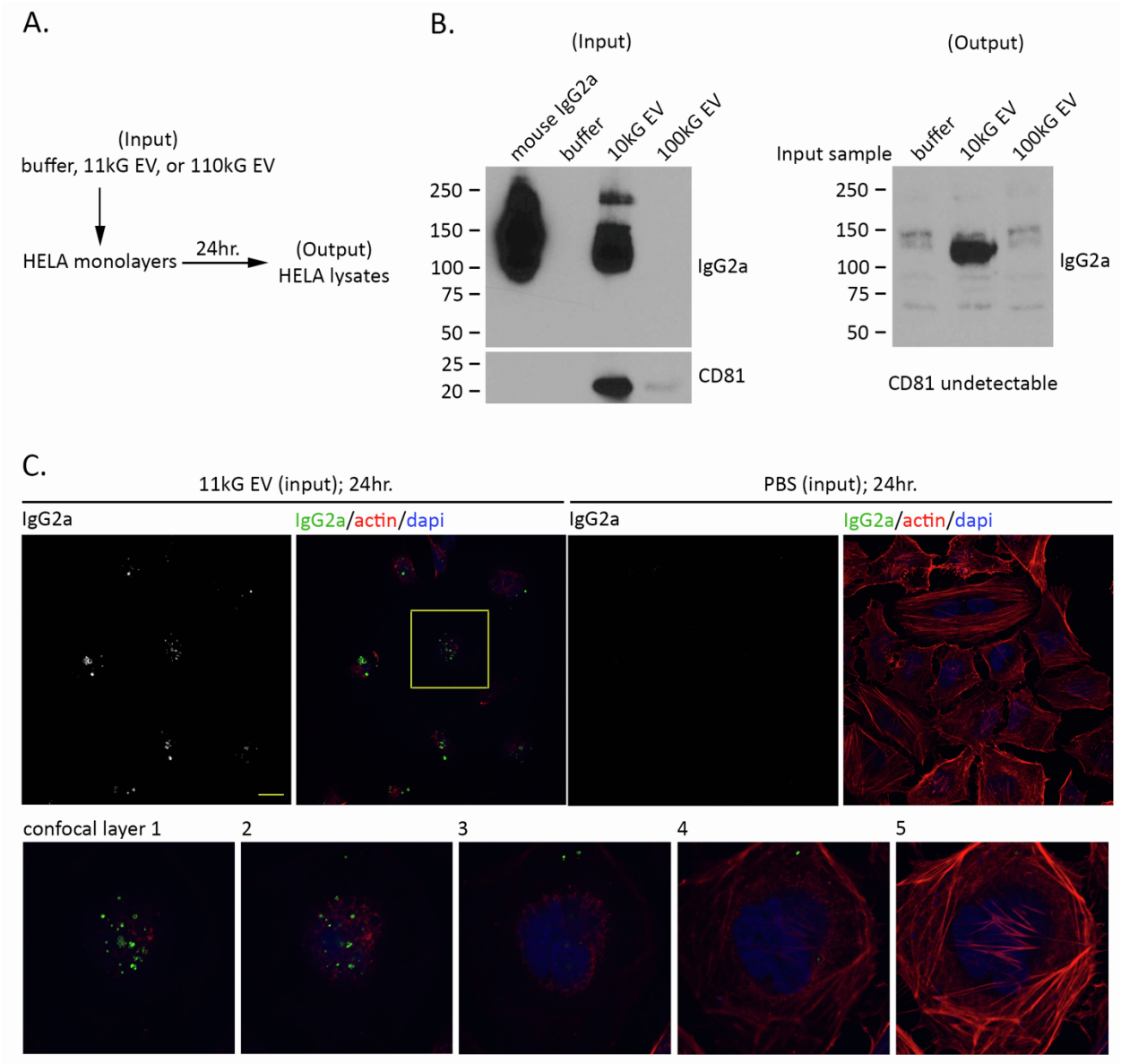

Figure S9. IgG2a contained in S9.6 hybridoma EV can accumulate intracellularly in secondary cells, related to Figure 7. EV (11kG and 110kG) were isolated from S9.6 hybridoma cell culture supernatant and subsequently applied to HELA monolayers, followed by HELA cell lysate preparation and immunoblot analysis or immunofluorescent confocal microscopy. **A.** Schematic showing the experimental procedure, and designation of buffer, 11kG EV, or 110kG (EV) as (input) and HELA lysates as (output). **B.** Immunoblot for IgG2a and CD81 in isolated EV prior to application (left, (input)) and in HELA lysate preparations generated 24hrs. post-EV application (right, (output)). Mouse IgG2a was run as a positive control. **C.** Representative microscopy images of a single confocal layer (0.75μm) 24hr. post-application of 11kG EV (left set) or PBS (right set) that were subjected to the identical staining reagents utilized above. A region of interest (yellow box) from five sequential layers was magnified and is presented in the lower panel (1-5). Scale bar indicates 20μm.