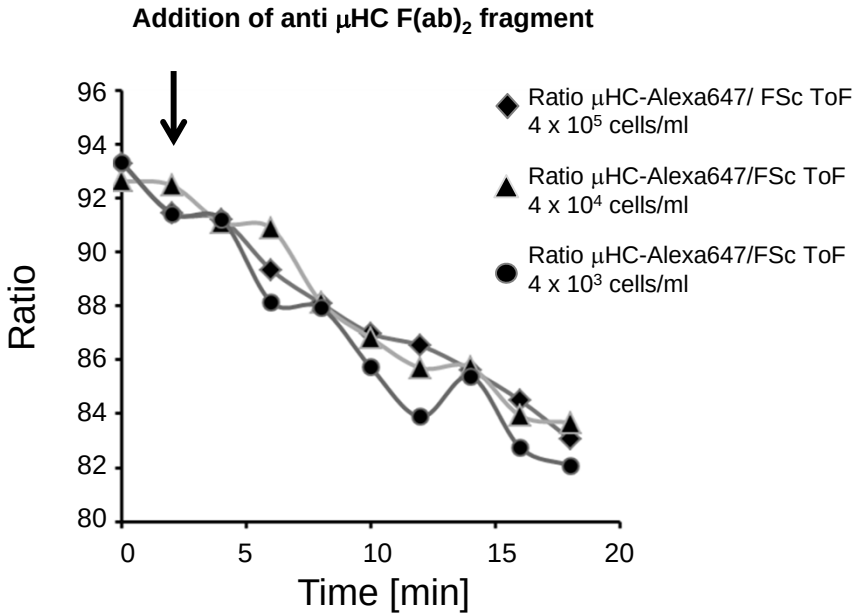
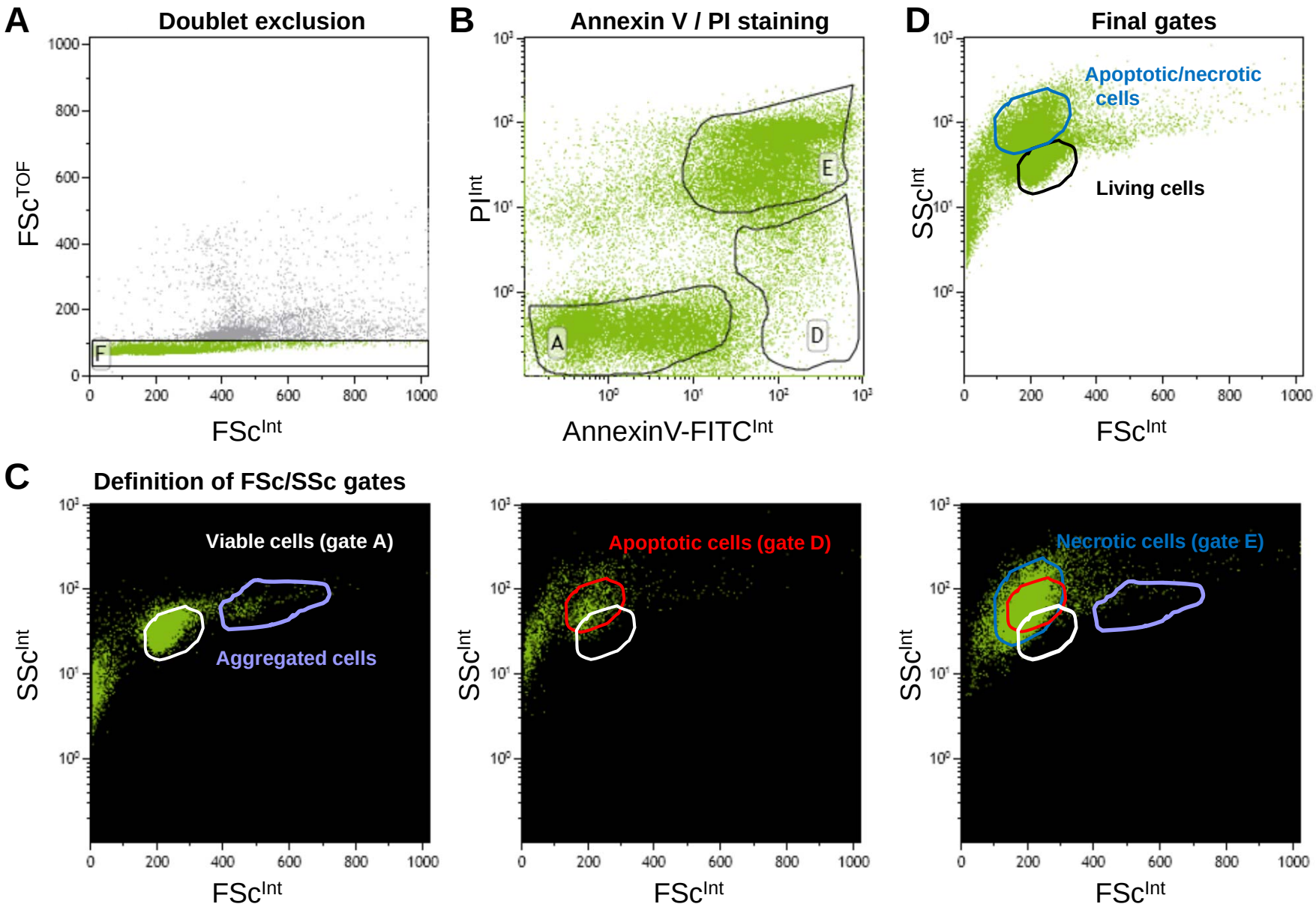
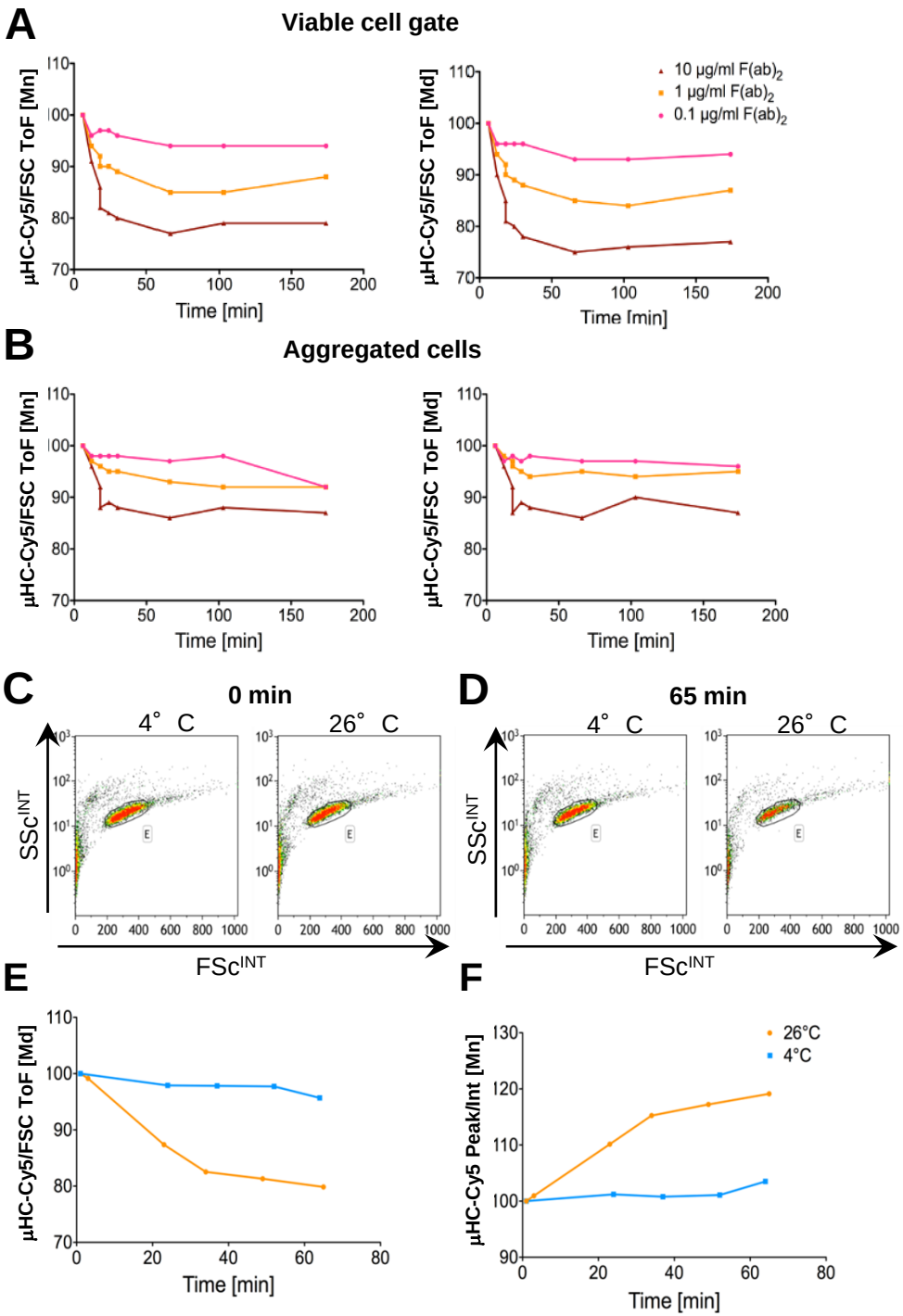


Supplemental FIGURE 1. Titration of cell numbers.
Murine spleen cells (4 x 10^5 - 4 x 10^3 /ml) that had been rested overnight were stained with anti-μHCFab-Cy5. Cells were measured in the flow cytometer for 2 min. Cross-linking of Cy5-labeled anti μHCFab was carried by the addition of goat anti-μHC $F(ab)_2$ fragments (10μg/ml; see arrows) at ~25°C and then cells were analyzed for another 18 min at ~25°C. At various time-points the fluorescence time-of-flight (FL-ToF) and forward scatter time-of-flight (FSc-ToF) signals were calculated.

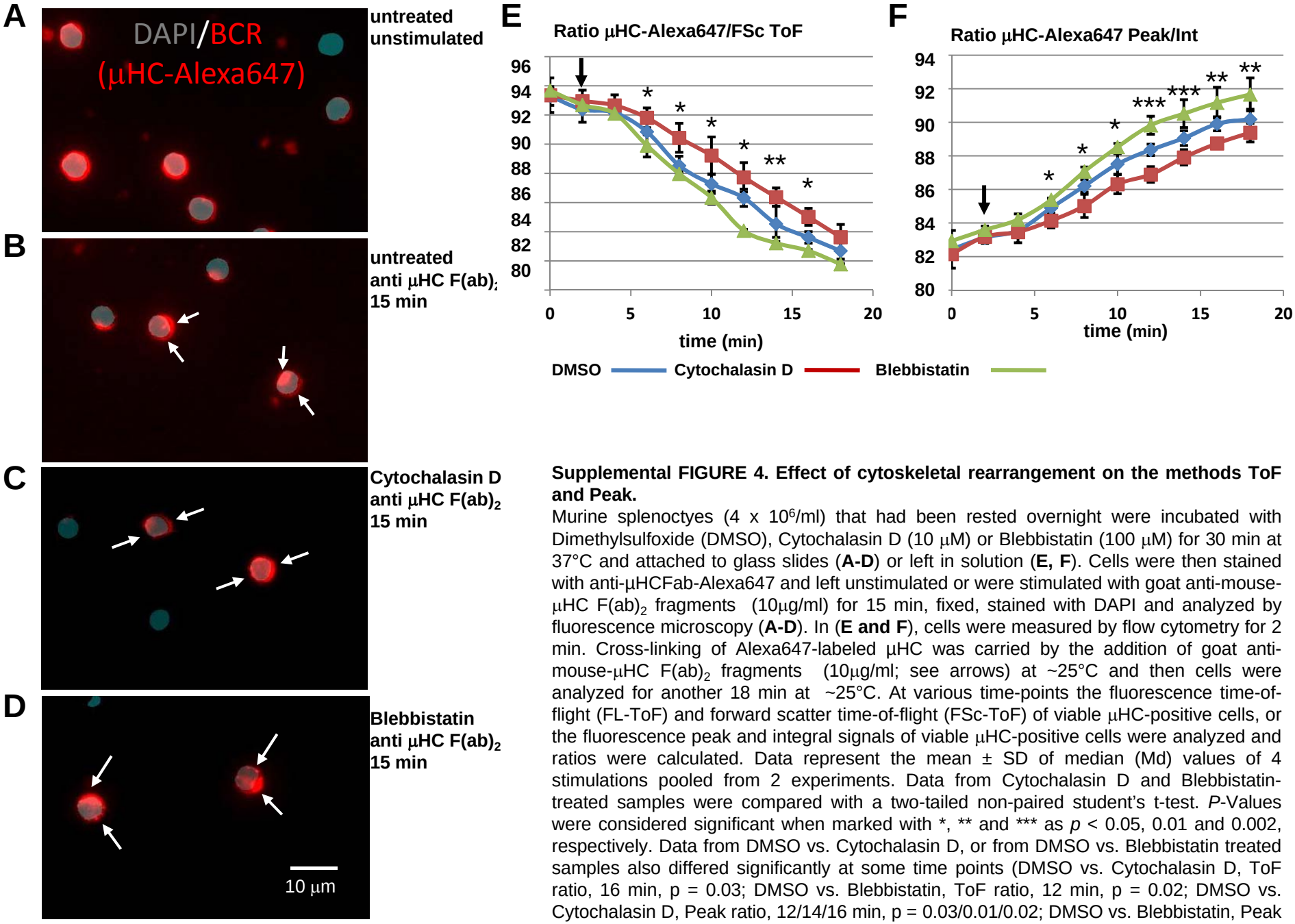


Supplemental FIGURE 2. Determination of the viable and apoptotic/necrotic cell gates of primary splenocytes by Annexin V / propidium iodide staining.

Murine spleen cells that had been rested overnight were stained with Annxin V-FITC and propidium iodide (PI). Single cells **(A)** were classified as **(B)** viable cells (Annexin V negative, PI negative; gate A), apoptotic cells (Annexin V positive, PI negative; gate D) and necrotic cells (Annexin V positive, PI positive; gate E). These gates were then used to define the FSCc/SSc parameters of the different populations **(C)**. **(D)** reveals the final gates used to distinguish cell populations that are mostly viable (black gate) or mostly apoptotic or necrotic (blue gate).

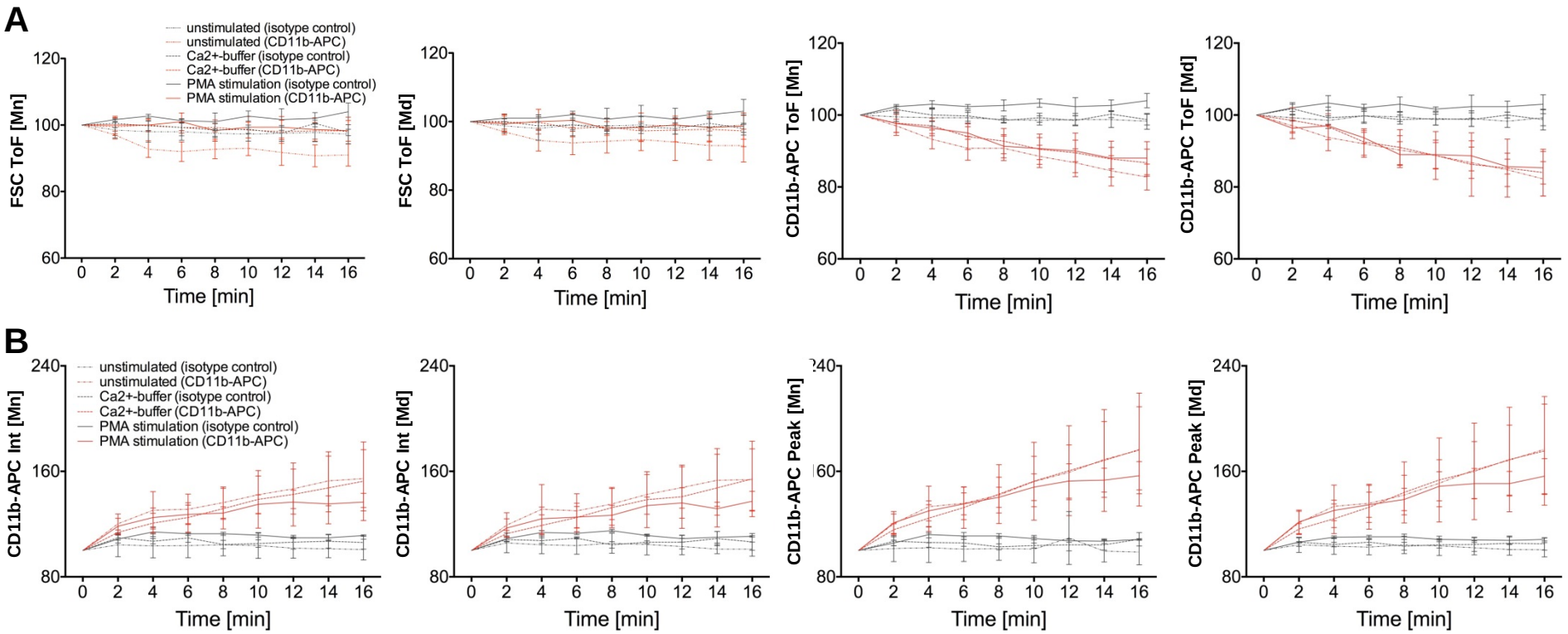


Supplemental FIGURE 3 Dependence of BCR clustering on antibody concentration and temperature. WEHI231 B cells were stained with anti-µHCFab-Cy5 (FL6) on ice for 10 min. To induce clustering of the BCR the cells were subsequently incubated with various concentrations of goat anti-µHCF(ab)$_2$ antibodies ranging from 0.1–10 µg/ml for the time-points indicated. The ratio of FL6-/FSc-ToF was calculated for (**A**) viable single cells and (**B**) aggregated cells. After staining of viable WEHI231 B cells with anti-µHCFab-Cy5 at room temperature, cells were incubated with goat anti-µHCF(ab)$_2$ (10µg/ml) either at (**C**) 4° C or at (**D**) 26° C. Comparison of BCR clustering at 4 and 26° C by method (**E**) ToF and (**F**) Peak.



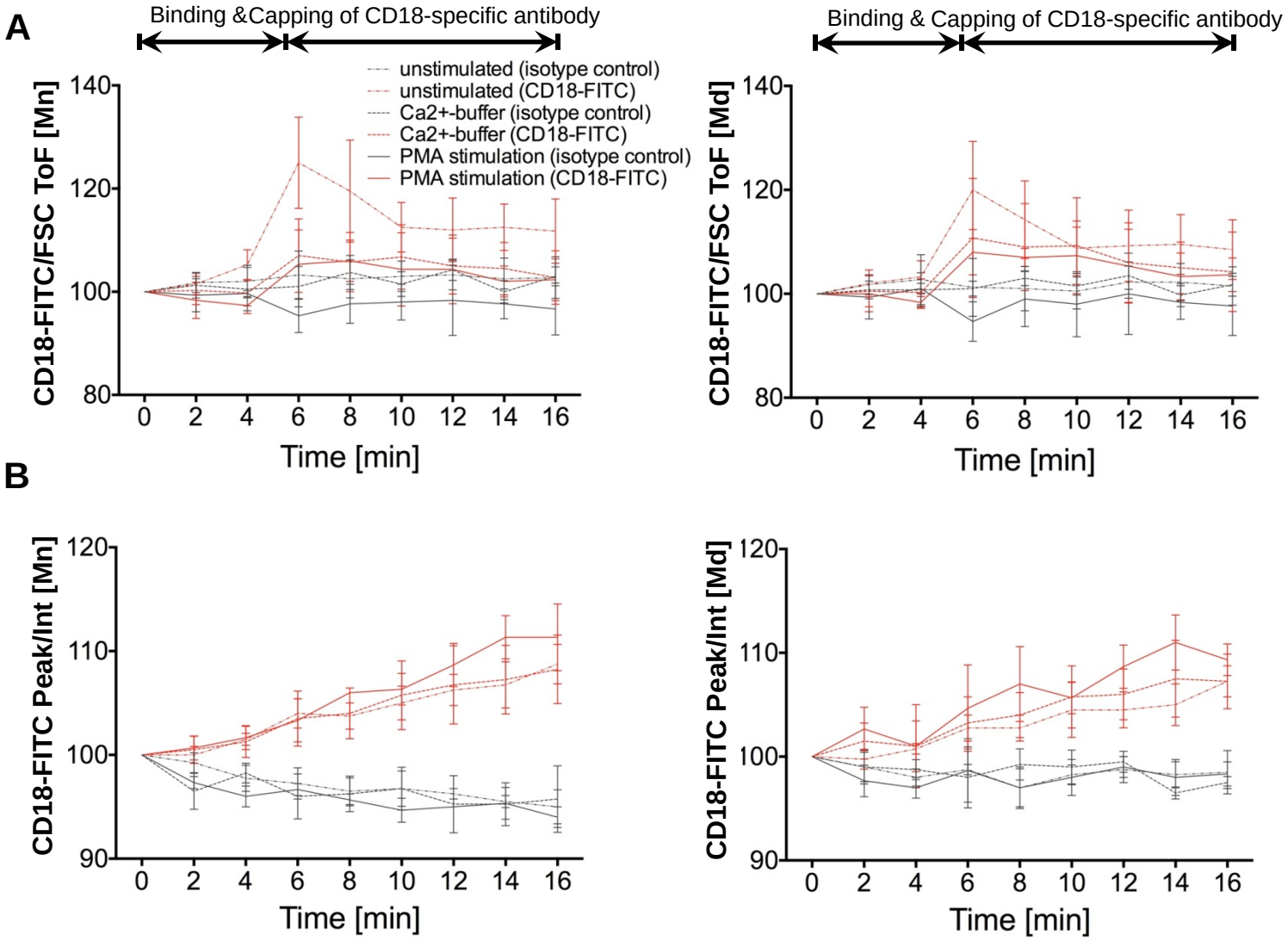
Supplemental FIGURE 4. Effect of cytoskeletal rearrangement on the methods ToF and Peak.

Murine splenoctyes (4 x 10^6/ml) that had been rested overnight were incubated with Dimethylsulfoxide (DMSO), Cytochalasin D (10 μM) or Blebbistatin (100 μM) for 30 min at 37°C and attached to glass slides (**A-D**) or left in solution (**E, F**). Cells were then stained with anti-μHCFab-Alexa647 and left unstimulated or were stimulated with goat anti-mouse-μHC $F(ab)_2$ fragments (10μg/ml) for 15 min, fixed, stained with DAPI and analyzed by fluorescence microscopy (**A-D**). In (**E and F**), cells were measured by flow cytometry for 2 min. Cross-linking of Alexa647-labeled μHC was carried by the addition of goat anti-mouse-μHC $F(ab)_2$ fragments (10μg/ml; see arrows) at ~25°C and then cells were analyzed for another 18 min at ~25°C. At various time-points the fluorescence time-of-flight (FL-ToF) and forward scatter time-of-flight (FSc-ToF) of viable μHC-positive cells, or the fluorescence peak and integral signals of viable μHC-positive cells were analyzed and ratios were calculated. Data represent the mean ± SD of median (Md) values of 4 stimulations pooled from 2 experiments. Data from Cytochalasin D and Blebbistatin-treated samples were compared with a two-tailed non-paired student's t-test. *P*-Values were considered significant when marked with *, ** and *** as $p < 0.05$, 0.01 and 0.002, respectively. Data from DMSO vs. Cytochalasin D, or from DMSO vs. Blebbistatin treated samples also differed significantly at some time points (DMSO vs. Cytochalasin D, ToF ratio, 16 min, p = 0.03; DMSO vs. Blebbistatin, ToF ratio, 12 min, p = 0.02; DMSO vs. Cytochalasin D, Peak ratio, 12/14/16 min, p = 0.03/0.01/0.02; DMSO vs. Blebbistatin, Peak ratio, 8/12/14/16/18 min, p = 0.04/0.002/0.006/0.02/0.01).



Supplemental FIGURE 5. FSc ToF, CD11b-APC ToF, CD11b-APC Peak and CD11b-APC Integral

Neutrophils from four healthy volunteers were isolated. Cells were resuspended in buffer containing Ca^{2+} or PMA in the presence of the APC-conjugated antibody to CD11b. APC-labelled IgG1k served as control. (A) FSc- and CD11b-APC ToF as well as (B) CD11b-APC ToF peak and integral were recorded over 1000 s and are displayed as Mean (Mn) or Median (Md) signals ± SD.



Supplemental FIGURE 6. CD18-FITC/FSc ToF and CD18-FITC Peak/Integral.
Neutrophils from four healthy volunteers were isolated. Cells were resuspended in buffer containing Ca^{2+} or PMA in the presence of freshly added FITC-labelled monoclonal antibody to CD18 or a FITC-conjugated isotype matched control antibody. (A) CD18-FITC and FSc ToF were recorded and are displayed as ratios of the Mean (Mn) or Median (Md) ± SD. (B) CD18-FITC peak and integral were recorded and are displayed as ratios of the Mean (Mn) or Median (Md) ± SD.