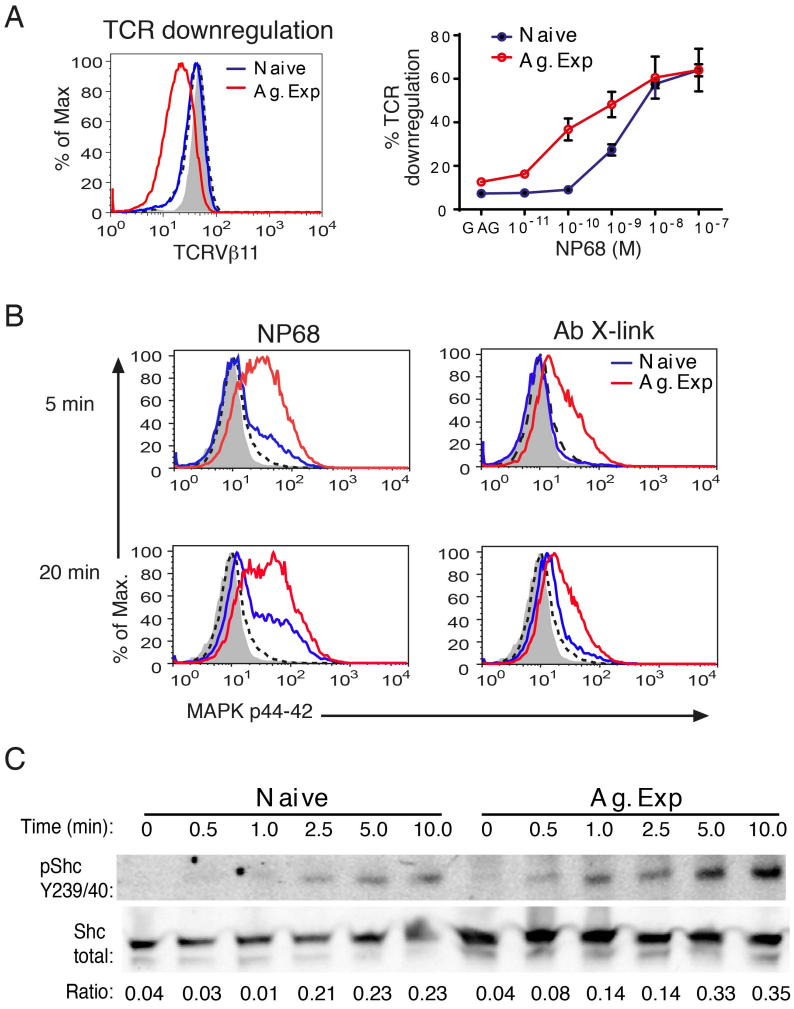
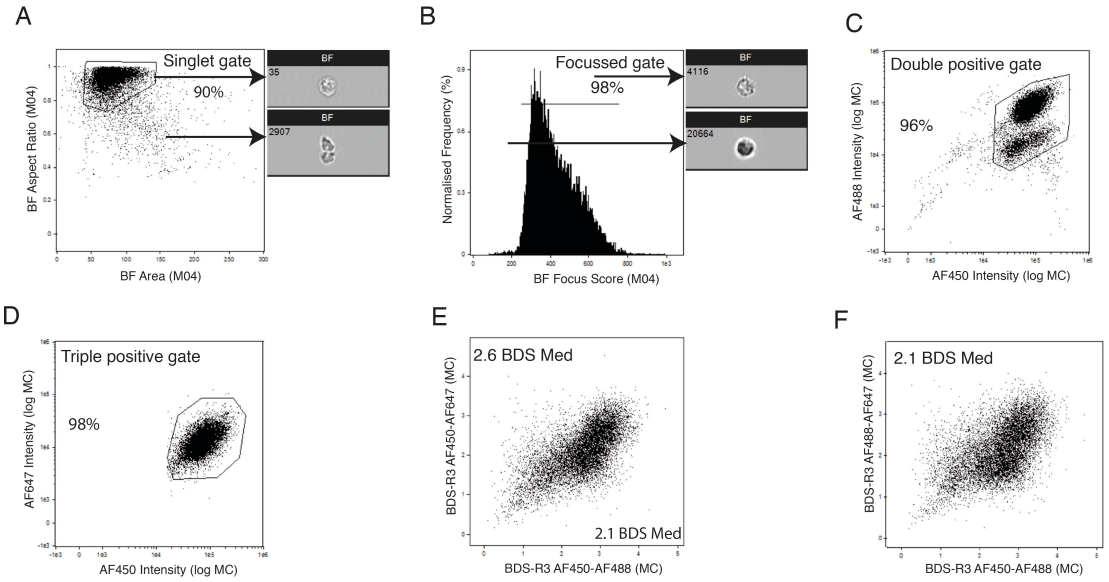


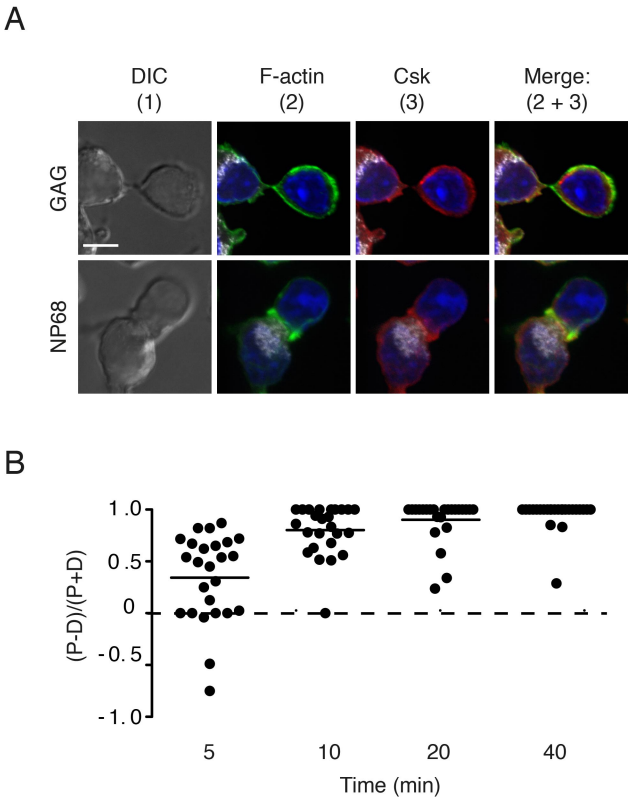
Supplementary Figure 1: Enhanced responses of Ag.experienced CD8$^+$ T cells upon TCR stimulation

LN cells from Rag-1$^{-/-}$F5 mice (naïve) and Ag.experienced CD8$^+$ T cells generated by stimulation with 10^{-7}M NP68 for 72 h then rested in IL-2 and IL-15 for 96 h, were activated with peptide (NP68) and their responses were assessed. (A) The percentage of CD8$^+$cells that downregulate Vβ11 TCR in response to a titration of peptide at 3 h is shown. Histogram shows expression of surface Vβ11 TCR at 10^{-9}M NP68 in naïve unstimulated T cells (filled); Ag-experienced unstimulated level (dashed line); naïve peptide stimulated (blue); and Ag-experienced peptide stimulated (red). Data are representative of at least 6 mice of each phenotype from two independent experiments. Values are the mean of 6 data points ± SD. (B) Histograms show the levels of pERK following 5 and 20 min 10^{-9}M NP68 or TCR/CD8 crosslinking (Ab X-link) in naïve unstimulated T cells (filled); Ag.experienced unstimulated (dashed line); naïve peptide stimulated (blue); and Ag-experienced peptide stimulated (red). Data are representative of at least 6 mice of each phenotype from 2 independent experiments. Values are the mean of 6 data points ± SD. (C) T cells were stimulated for indicated times with 10^{-7}M NP68 and whole cell lysates run on Tris-Bis gels and immunoblotted for pSHC Y239/40 and total SHC indicates loading. Relative pSHC levels were determined by normalizing data of phospho-SHC to total SHC, with densitometry quantitation by infrared imaging using the LI-COR Odyssey system.



Supplementary Figure 2: Imaging Flow Cytometry gating and analysis strategy

Gating and analysis strategy for measuring pair-wise co-localisation of TCR/CD8 (AF450), SFK (AF488) and Csk (AF647) signals on live, single, in focus, triple stained cells. (A) Single, live cells were defined using the area (x-axis) and aspect ratio (y axis) of the bright field (BF) channel default mask (M04). Single cells have a high aspect ratio and intermediate area whereas doublets have lower aspect ratio and double area values (see example images). (B) Poorly focused events were eliminated with "gradient RMS' calculated from the pixels identified by the M04 mask. Values above 200 units were considered to be in focus. Representative images are shown of events within and without the focused gate. (C) TCR/CD8-AF450 and SFK-AF488 double positive cells were gated using the "total intensity' feature which sums the pixel values in a given channel mask AF450 (M01), x axis and AF488 (M02), y axis). Values are plotted on a log scale. (D) Identification of triple positive cells using the sum pixel value of the AF647 (M05) signal on a log scale (y-axis). (E) The BDS score was calculated using the complete mask (MC) and the AF450 versus AF488 channel pixel values (X-axis), the AF488 versus AF647 channel pixel values (y-axis) or the (F) AF488 versus AF647 channel pixel values (y-axis). In all cases, median values were reported for the triple positive populations. A dissimilar cut off value of <1.5 units was determined using the MRB control of TCR/CD8-AF450 versus SFK-AF488 signals as a highly correlated distribution. The BDS score functions by first subtracting away all light from sub-optimal focal planes using a 3 pixel-structuring element (R3) so that only bright-detail pixels within the plane of best focus are used for Pearson's correlation. This minimises co-localisation on the x and y-axis of a 2D slice being confounded by a lack of z resolution. In all cases gated percentages (%) are shown and median values (SEM) included for BDS scores. Compensation values generated from single stained controls post-acquisition for Ch1, 2, 3, 4 , 5 and 6 respectively were AF450:100, 16.6, 4.1, 2.7, 1.1, 0.6; AF488 11.3, 100, 20, 7.5, 1, 0.25; AF647: 6, 8, 6, 1, 100, 20.



Supplementary Figure 3: Immunological synapse formation is required for Csk redistribution in Ag.experienced CD8$^+$ T cells

Ag.experienced CD8$^+$ T cells were conjugated to NP68- or GAG-pulsed RMA-S cells for 5-40 min, and conjugates were fixed and fluorescently labeled with Abs to Csk and BODIPYFL-phalloidin to label F-actin accumulation at the SMAC. (A) Representative conjugates containing GAG-pulsed RMA-S (top panel) or NP68-pulsed RMA-S (bottom panel) each intracellularly labelled with Mitotraker (white) and a single Ag.experienced CD8$^+$ T cell. Scale bar represents 6 μM. (B) Using Volocity software, the sum of fluorescence above background of each time point was calculated in both the proximal and distal half of the cell. The amount of Csk fluorescent units were entered into the formulae (P-D)/(P+D). The data set of 1 experiment, comprising 25 images for each condition. The data are representative of at least 2 independent experiments.