Methods

Mice. SAP-deficient mice were previously described[14] and backcrossed to B6 for at least 10 generations. B6 (Jax 664), CD45.1 congenic (Jax 2014), HEL-specific Ig-transgenic MD4 (Jax 2595), μMT (Jax 2288), GFP-expressing (Jax 4353), CFP-expressing (Jax 4218), $OVA_{323\text{-}339}$-specific TCR transgenic OT-2 (Jax 4194) mice were purchased from the Jackson Laboratory. I-Aβ$^{-/-}$ mice were purchased from Taconic Farms. Relevant mice were interbred to obtain CFP-MD4, *sap*$^{+/+}$ CFP-OT-2, and *sap*$^{+/+}$ or *sap*$^{-/-}$ GFP-OT-2 mice. All mice were maintained under specific pathogen-free conditions, and used in accordance of NIH institutional guidelines for animal welfare.

Cell preparation, antigen, and immunization. To isolate DCs, mouse spleens were digested with 400 μg/ml Liberase CI and 20 μg/ml DNase I for 30 minutes (Roche) before subjected to CD11c Microbeads-based isolation protocol (Miltenyi Biotec). DCs were pulsed with indicated doses of OVA_{323} peptide antigen in the presence of LPS for 2 hours, washed, and used for *in vitro* cell conjugation assay or for subcutaneous injection into mice after being labelled with 75 μM CMF_2HC. OT-2 T cells and B cells of either a MD4 or polyclonal repertoire were isolated by CD4 T cell isolation kit and naïve B cell isolation kit (Miltenyi Biotec), respectively. For intravital imaging experiments involving dye-labelled cells, the MD4 B cells were always labelled with 75 μM CMF_2HC, while *sap*$^{+/+}$ and *sap*$^{-/-}$ OT-2 T cells were interchangeably labelled with either 2 μM CFSE or 4 μM CMTPX. All dyes were purchased from Invitrogen. Model antigens used for subcutaneous immunization included OVA protein and chemical conjugates of OVA and HEL or BSA and HEL. The purified proteins were purchased form Sigma-Aldrich, and conjugates were made with the HydraLink heterobifunctional conjugation kit (SoluLink). Mice were immunized with the indicated antigen mixed with alum and 0.2 μg LPS.

Transient transfection of T cells. For certain experiments, blasting OT-2 T cells were transfected with 4 μg of DNA constructs expressing GFP-tagged SAP or GFP alone by Amaxa nucleofector (program X-001) before being analyzed for conjugation formation

with B cells. Reconstituted expression of SAP proteins in *sap*$^{-/-}$ T cells were verified by Western blotting.

Construction of mixed bone marrow chimeras. B6 or CD45.1 congenic mice were lethally irradiated by γ ray from a cesium source (900 rad) and then received intravenous transfer of a total of 1.25×10^5 bone marrow lineage-negative cells, which were isolated using the mouse lineage depletion kit (Miltenyi Biotec). To make chimeric mice that lack class II MHC, a mixture of 80% μMT and 20% I-Aβ$^{-/-}$ donor BM cells were used. For certain control experiments, I-Aβ$^{-/-}$ donor cells were replaced with regular B6 cells.

Flow cytometry and immunohistochemistry. All reagents were purchased from BD PharMingen unless otherwise indicated. To examine surface phenotypes of OT-2 T cells or MD4 B cells, draining LN cells were washed, incubated with 50 μg/ml rat and hamster IgG whole molecules (Pierce) and 10% 2.4G2 hybridoma supernatants, and then stained with indicated Abs in PBS supplemented with 2 mM EDTA and 0.5% foetal calf serum. Staining reagents included AlexaFluor 700 anti-CD4 (eBioscience), AlexaFluor 700 CD38 (eBioscience), APC-Cy7 anti-CD19, PE anti-Fas, PE anti-ICOS, PE anti-OX40, PE anti-I-Aβ, APC anti-CD11c, biotinylated anti-CD40L, biotinylated anti-CXCR5, biotinylated anti-IgM^a, 7-AAD, streptavidin APC, and isotype-matched non-specific antibodies. Cells were stained on ice with primary reagents for 1 hour and with the secondary reagent for 20 minutes. Cytometric data were collected on LSR II cytometer (BD) and analyzed with FlowJo software (TreeStar). Dying cells and cell multiplets were excluded from analysis based on 7-AAD fluorescence and FSC-H/FSC-A. Quantitative analysis of cell surface phenotypes involved determining percentages of positive cells based on a cut-off established by the isotype control (for CXCR5 and CD40L) or calculating the normalized expression level based on geometric mean fluorescence intensities ($Log_{10}(MFI^{sample})/ Log_{10}(MFI^{isotype})$ for ICOS and OX40). For immunohistochemical staining of LN sections, the protocol was as previously

described[44]. Staining reagents included AlexaFluor 647 anti-B220, biotinylated anti-IgD, purified GL7 (eBioscience), AlexaFluor 647 anti-rat IgM (Invitrogen), and streptavidin AlexaFluor 568 (Invitrogen). All stained slides were mounted with ProlongGold antifade reagents (Invitrogen) and examined with a Leica TCS SP5 confocal system.

Intravital imaging and quantitative data analysis. To maintain consistency of visual presentation in all figures and movies, the *sap*$^{+/+}$ and *sap*$^{-/-}$ T cells were pseudo-coloured in red and green, respectively. An excitation wavelength between 783 and 800 nm was used for dye-labelled cells. For imaging experiments involving GFP- or CFP-expressing transgenic T or B cells, with or without additional CMTPX-labelled naïve B cells, an excitation wavelength between 840 and 850 nm was used, a compromise wavelength for simultaneous visualization of CFP and GFP or all of the three fluorochromes. While the overlap between normalized emission curves of CFP and GFP is substantial, the GFP transgenic line used here (Jax stock 4353) is ~100 times "brighter" than the CFP transgenic line (Jax stock 4218), allowing real GFP signals be readily distinguished from bleed-through signals from CFP. Post data acquisition, 4-D image datasets were analyzed using Bitplane Imaris software package. Cell-cell contacts were scored manually in blind analyses. Cell migration was analyzed through automatic cell tracking aided by manual supervision. Only cell tracks that lasted longer than 5 minutes were included in quantitative analysis. For certain experiments, it was necessary to quantitatively analyze cell positioning over time in relationship to the GC in space. To achieve this, the boundary of GC was manually traced for each optical slice in a time-averaged series and then mathematically defined as a triangulated surface in 3-D space by an optimized Dirichlet tessellation algorithm implemented in C++ (Klauschen *et al.*, manuscript in preparation). The distance of a cell at any give time to the GC surface can then be calculated (see Supplementary Figure 6 for a flowchart of the relevant data processing).

For all imaging experiments, final presentations of time-lapse image sequences as stills or movies were created using Photoshop, AfterEffect, and Illustrator (Adobe).