Su et al. Supplemental Methods

Immunofluorescence staining

Samples of lymph nodes and spleens were embedded in OCT compound (Tissue-Tek) and snap-frozen. Sections (8-mm) were fixed in 4% paraformaldehyde for 10 min, permeabilized with 0.2% Triton X-100 for 10 min, and blocked with 1% IgG-free BSA and 50 mg/ml donkey IgG (Jackson ImmunoResearch) for 1 hour. The sections were incubated with FITC-conjugated anti-B220, PE-conjugated anti-CD3, and Alexa 647-conjugated anti-F480 diluted in 1% BSA and 0.1% Tween-20 at room temperature for 30 min. The sections were washed with PBS and mounted with fluorescence mounting media (Life Technologies). Slides were imaged with a Zeiss LSM 510 laser scanning confocal microscope.

Flow cytometry

Pacific blue conjugated anti-CD3, APC-Cy7 conjugated anti-B220, FITC conjugated anti-TCRβ, PE conjugated anti-IgM, PerCP-Cy5.5 conjugated anti-IgD, Alexa 647 conjugated anti-CXCR4, PerCP-Cy5.5 conjugated anti-CXCR5, PE conjugated anti-CD11a and anti-CD49d (BioLegend) were used for flow cytometry. Isolated lymphocytes were incubated with these antibodies for 30 min at 4°C, washed and immediately taken for analysis with an LSR II flow cytometer (Becton Dickinson) and FlowJo software.

Immunization

T-independent immune responses were measured by immunizing 6- to 8-week-old mice via intraperitoneal injection with 100 μg of trinitrophenol (TNP)-Ficoll (Biosearch Technologies) in PBS. Blood (50 μl) was collected from the retro-orbital venous plexus on days 0 and 7 after immunization. T-dependent immune responses were measured by immunizing 6- to 8-week-old mice via intraperitoneal injection with 100 μg of TNP-keyhole limpet hemocyanin
(KLH) in 200 μl of aluminum hydroxide adjuvant and Imject Alum (Pierce) and boosted with 10 μg of TNP-KLH on day 35. Sera were collected on day 0, 7, and 42. Sera were analyzed by ELISA for isotype-specific anti-TNP Ig (SouthernBiotech).

Platelet Function

Light transmission aggregometry was performed using washed platelets obtained from heparinized whole blood of control or Riam−/− mice and adjusted to 2.5 x 10^8 platelets per ml with modified Tyrode’s buffer (137 mM NaCl, 0.3 mM Na₂HPO₄, 2 mM KCl, 12 mM NaHCO₃, 5 mM HEPES, 5 mM glucose, pH 7.3) containing 0.35% BSA and 2 mM CaCl₂. Par4p or convulxin were added at Low Dose (LD) (50 μM, 75 ng/ml, respectively) or High Dose (HD) (100 μM, 150 ng/ml, respectively) and transmission was recorded over 6 minutes on a Chrono-log 4-channel optical aggregation system (Chrono-log, Havertown, PA). Activation of the αIIbβ3 integrin was measured in washed platelets using JON/A-PE (Emfret Analytics, Germany) as described ¹. Platelets were obtained from heparinized whole blood of control or Riam−/− mice and adjusted to 5 x 10^6 platelets per milliliter with modified Tyrode’s buffer with 2 mM CaCl₂. Baseline fluorescence was quantified in real-time using an Accuri C6 flow cytometer (BD Bioscience) for 10 seconds before the addition of 2 μg/ml of JON/A-PE and agonist (400 μM Par4p or 750 ng/ml of convulxin).