

## Supplementary Materials for

### The Tetraspanin CD37 Orchestrates the $\alpha_4\beta_1$ Integrin–Akt Signaling Axis and Supports Long-Lived Plasma Cell Survival

Annemiek B. van Spriel, Sandra de Keijzer, Alie van der Schaaf, Kate H. Gartlan, Mariam Sofi, Amanda Light, Peter C. Linssen, Jan B. Boezeman, Malou Zuidscherwoude, Inge Reinieren-Beeren, Alessandra Cambi, Fabienne Mackay, David M. Tarlinton, Carl G. Figdor,\* Mark D. Wright

\*To whom correspondence should be addressed. E-mail: c.figdor@ncmls.ru.nl

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#### This PDF file includes:

##### Methods

Fig. S1. Production of IgG2b, IgG3, and IgM in *Cd37*<sup>-/-</sup> mice.

Fig. S2. Splenic B cell subsets in *Cd37*<sup>-/-</sup> mice.

Fig. S3. Decreased population of memory B cells in spleens from *Cd37*<sup>-/-</sup> mice.

Fig. S4. Normal proliferation of *Cd37*<sup>-/-</sup> B cells.

Fig. S5. Blocking the  $\alpha_4$  integrin reduces cell rolling and adhesion.

Fig. S6. *Cd37* expression in plasmablasts and plasma cells.

Fig. S7. Impaired Akt phosphorylation in IgG1-secreting *Cd37*<sup>-/-</sup> cells as detected by flow cytometry.

Fig. S8. Average area of  $\alpha_4\beta_1$  integrin fluorescence at the VCAM-1 contact site.

Movies S1 and S2 legends

#### Other Supplementary Material for this manuscript includes the following:

(available at [www.sciencesignaling.org/cgi/content/full/5/250/ra82/DC1](http://www.sciencesignaling.org/cgi/content/full/5/250/ra82/DC1))

Movie S1 (.mp4 format). Rolling and adhesion of wild-type B cells under flow.

Movie S2 (.mp4 format). Rolling and adhesion of *Cd37*<sup>-/-</sup> B cells under flow.

## Methods

### Quantifying B cell subsets in the spleen

Lymphocyte suspensions were obtained by mechanical disruption of spleens into FACS buffer [0.5% (w/v) BSA and 25 mM EDTA in PBS] and stained for 30 min at 4°C with fluorescently labeled anti-mouse antibodies against B220, IgM, CD21, CD23, CD23, and CD1d (BD Biosciences). FACS data were collected on an LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar). B220<sup>+</sup> B cell subsets were gated as follows: Transitional-1 (T1: CD23<sup>low</sup>, IgM<sup>high</sup>, IgD<sup>low</sup>), Transitional-2 (T2: CD23<sup>high</sup>, IgM<sup>int</sup>, IgD<sup>high</sup>), follicular (FO: CD21<sup>int</sup>, CD23<sup>high</sup>), and marginal zone (MZ: CD21<sup>high</sup>, CD23<sup>low</sup>) B cells.

### B cell proliferation assays

Purified B cells were stimulated for various times with F(ab')<sub>2</sub> goat anti-IgM (10 µg/ml, Jackson ImmunoResearch Laboratories) or anti-CD40 (10 µg/ml, FGK45). B cells were pulsed for the last 6 hours with 1 µCi of [<sup>3</sup>H]-thymidine (Amersham) and incorporation was measured with a scintillation counter (Top Count, Packard).

### Colocalization studies

Co-patching experiments were performed as described (25). Briefly, live human Raji B cells ( $2 \times 10^6$ ) were blocked and stained with anti-CD37 (WR17) and anti- $\alpha_4$  integrin (HP 2.1) or with matched isotype controls (10 µg/ml). After washing, cells were incubated with Alexa Fluor 488–conjugated anti-mouse IgG2a and Alexa Fluor 568–conjugated anti-IgG1 (Molecular Probes). The same experiment was performed with anti-CD37 and anti- $\alpha_1\beta_2$  integrin (Ts2/4) as a control. Cells were fixed in 2% PFA, mounted on poly-L-lysine-coated cover slips, and analyzed by confocal laser scanning microscopy (Olympus FV1000). Colocalization was quantified with the Manders' overlap coefficient (Fiji Software).

### Measuring somatic mutation in memory B cells

Flow cytometric detection of isotype-switched antigen-specific B cells in spleens was performed with FITC-conjugated anti-B220 (RA3-6B2), PE-conjugated anti-IgD (11-26C) and PE-conjugated anti-IgM (331.12), Texas Red–conjugated anti-mouse IgG1 (SBA), and APC-conjugated anti-NP (made in house). Assessment of *V<sub>H</sub>186.2* gene somatic mutation used PCR-amplified cDNA synthesized from single IgG1<sup>+</sup> antigen-specific B cells isolated by flow cytometry from spleens of mice immunized 35 days earlier. Preparation of RNA, cDNA synthesis, *V<sub>H</sub>* gene amplification, DNA sequencing and analysis were performed as described previously (55). Analysis and sorting of antigen-specific B cells used a FACStar<sup>+</sup> cell sorter with an automatic cell deposition unit (BD Biosciences).

### *Cd37* RT-PCR

Murine *Cd37* expression was analyzed at the mRNA level by quantitative RT-PCR as described previously (22). B cells were purified from spleens of immunized WT and *Cd37*<sup>-/-</sup> mice to >97% purity by magnetic cell sorting with B220-MACS beads (Miltenyi Biotec). Cells were co-stained with PE-Cy5–conjugated anti-CD138 and PE-conjugated anti-CD43 (BD-Pharmingen), and Elutra FACS cell sorting was used to purify plasmablasts (CD138<sup>+</sup>, CD43<sup>+</sup>) and plasma cells (CD138<sup>+</sup>, CD43<sup>-</sup>) to 99% purity. Total RNA was extracted from WT and *Cd37*<sup>-/-</sup> plasma cells and plasmablasts with RNazol RT with precipitation carrier PC173 (Molecular Research Center), and was transcribed into cDNA with random hexamer primers (Amersham) and Superscript II RT

(Invitrogen). Real-time RT-PCR was performed in an ABI Prism Sequence Detection system 7000. cDNA was amplified with the SYBR Green PCR mastermix (Applied Biosystems) with mouse *Cd37* primers (5'-GTCCTTTGTGGGTTTGTTCCTT and 5'-GAGACAGCGCAGCTCCTTTAG). The amount of *Cd37* mRNA was normalized to that of the housekeeping gene *PBGD*.

### Electron microscopy analysis of the surface distribution of $\alpha_4$ integrin

For whole-mount transmission electron microscopy, peritoneal macrophages from WT and *Cd37*<sup>-/-</sup> mice were allowed to stretch on glass slides covered by fibronectin-coated Formvar (1 hour 37°C). B cells could not be used because of their poor spreading capacity. After washing with PBS, the cells were fixed in 2% PFA for 15 min at room temperature, washed, blocked with I-buffer (PBS, 1% BSA, 0.25% gelatin, 20mM glycine), and incubated with antibody against  $\alpha_4$  integrin or isotype control antibody (BD Biosciences) in I-buffer for 30 min at 4°C. After washing, cells were fixed in 1% PFA containing 0.1% glutaraldehyde (GTA) to prevent capping induced by secondary antibodies. After washing and blocking in I-buffer, samples were incubated with anti-rat antibody (Cappel Laboratories) with 10-nm gold particles coupled to Protein A, and were fixed in 1% GTA. Subsequently, samples were washed, dehydrated, dried, transferred onto 100 mesh copper grids, and analyzed in a JEOL 1010 transmission electron microscope operating at 60 to 80 kV. Digital images of electron micrographs were processed by custom-written software based on Labview (National Instruments, TX). The distribution pattern of  $\alpha_4$  integrin and inter-particle distance were analyzed as described previously (13).

### Confocal microscopy and FRAP

*Cd37*<sup>-/-</sup> and WT B cells were purified (to >98%) from spleens by B220-MACS sorting. B220-positive cells were labeled for  $\alpha_4\beta_1$  integrin with a non-blocking antibody, Alexa Fluor 488-conjugated 9C10 (Serotec), in phenol red-free RPMI containing 1% goat serum.  $1 \times 10^6$  cells were adhered to 0.15 mm WillCo-dishes (Willco Wells, the Netherlands) coated with VCAM-1 (1  $\mu$ g/ml) at 37°C. Confocal microscopy was performed on an LSM510 meta confocal laser scanning microscope (Zeiss). The area of fluorescence and the average fluorescence intensity at the contact site were measured for each cell in ImageJ. FRAP experiments were performed with a 2.1- $\mu$ m square region of interest in the basal plane of the plasma membrane. Photobleaching was performed operating at 100% of laser power by scanning the bleached region of interest (ROI) for 3 iterations, which yielded a total bleach time of 0.150 s. Recoveries were collected with time intervals of 100 ms. Fluorescence intensity data for the bleached ROI were calculated with Zeiss software. After background correction and normalization to prebleach with double normalization, the single data files (approximately 20 curves for each condition) were averaged to create a single curve. The curves were fitted with a single exponential:

$$I(t) = A(1 - e^{-t/\tau}) \quad [\text{eq. 1}]$$

following calculation of the half maximum of the recovery:

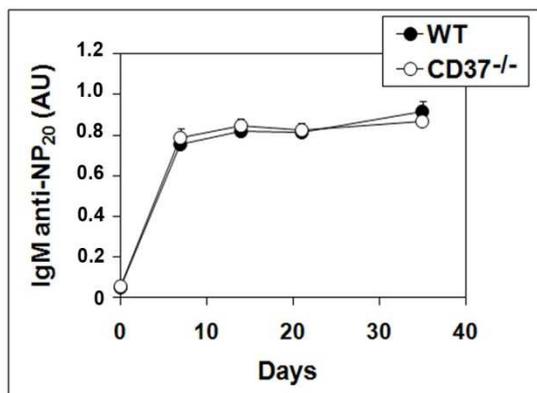
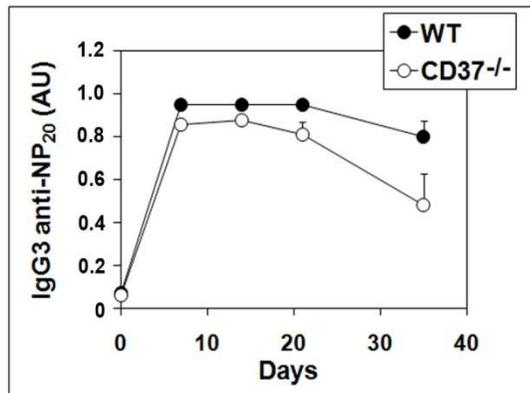
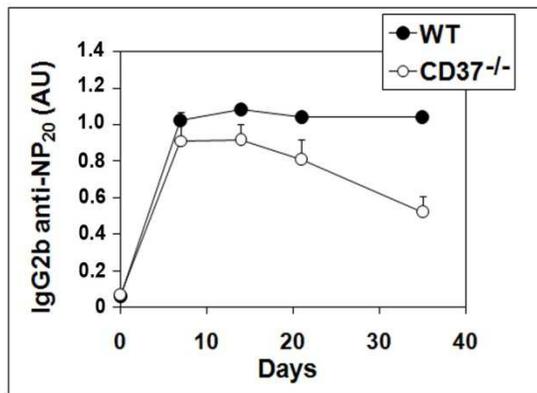
$$\tau_{1/2} = \frac{\ln 0.5}{-\tau} \quad [\text{eq. 2}]$$

and the mobile fraction calculation with:

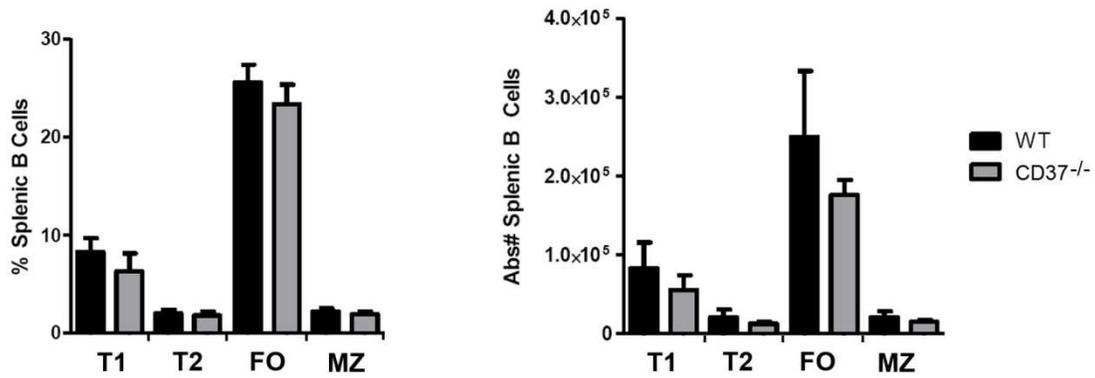
$$Mob = \frac{-A}{1 - (\gamma_0 + A)}$$

[eq. 3]

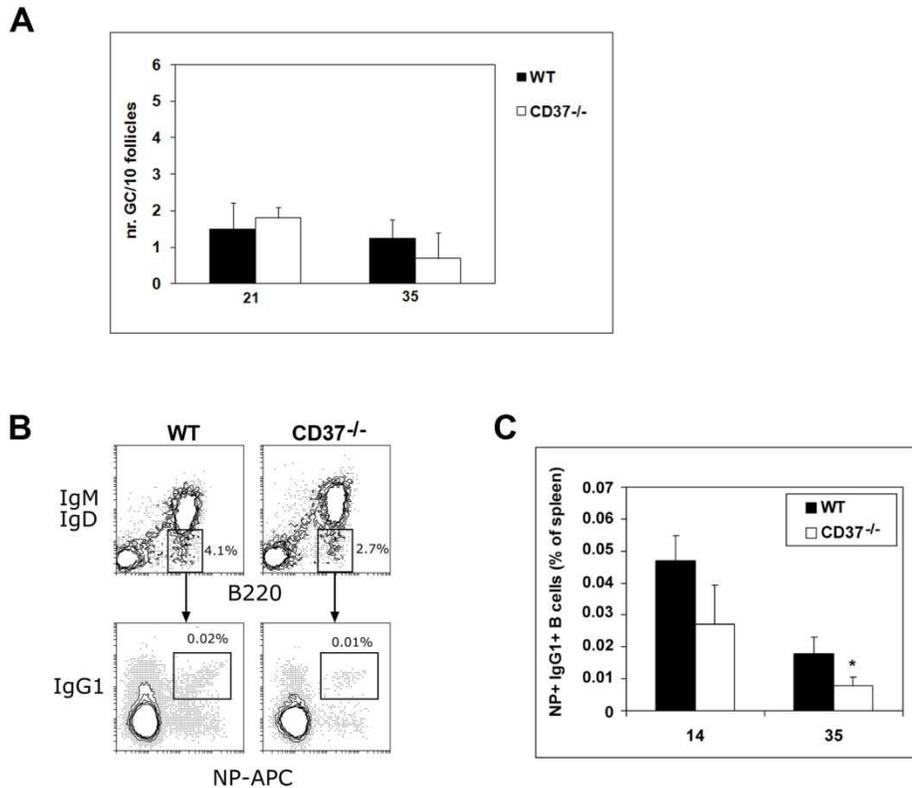
The data were compared with the two sample Kolmogorov-Smirnov test function in Matlab (The Mathworks).



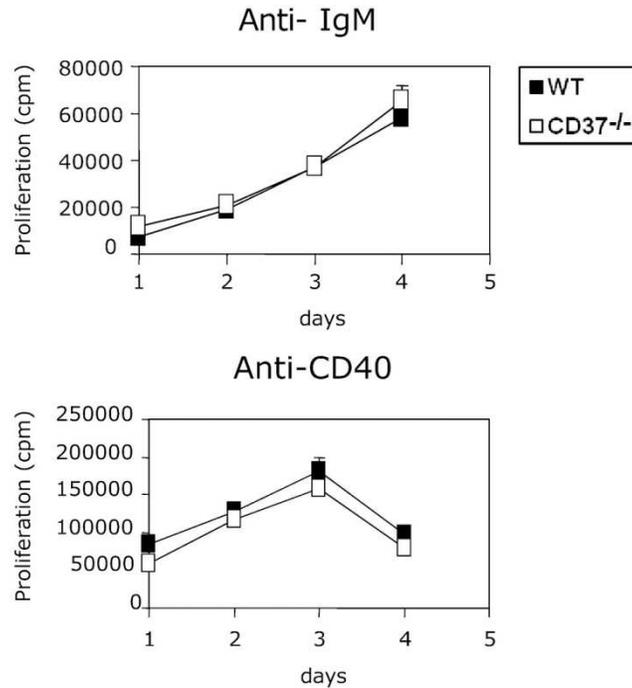
**Fig S1.** Production of IgG2b, IgG3, and IgM in *Cd37<sup>-/-</sup>* mice. WT (filled circles) and *Cd37<sup>-/-</sup>* (open circles) mice were immunized i.p. with NP-KLH in alum, and serum was assayed by ELISA against NP<sub>20</sub>-BSA to measure total NP-specific IgG2b, IgG3, and IgM. Antibody titers are expressed in arbitrary units and are presented as means  $\pm$  SEM ( $n = 6$  mice of each genotype). Experiments were performed three times yielding similar results.



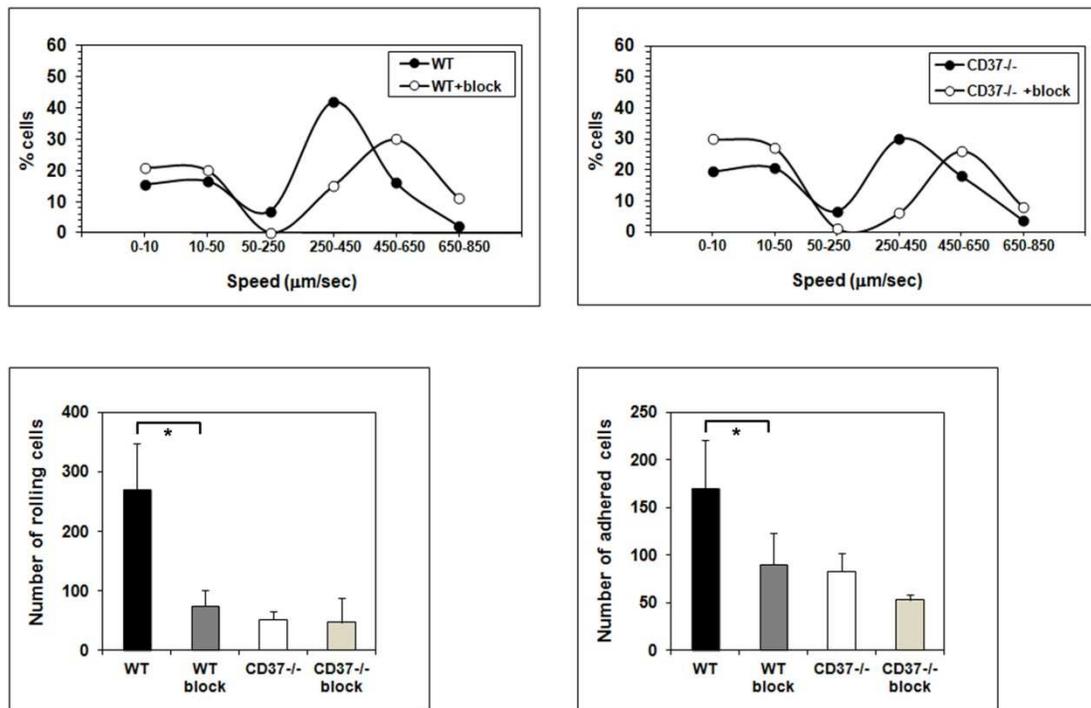
**Fig. S2.** Splenic B cell subsets in *Cd37<sup>-/-</sup>* mice. Splenic B cell subsets (T1: CD23<sup>low</sup>, IgM<sup>high</sup>, IgD<sup>low</sup>; T2: CD23<sup>high</sup>, IgM<sup>int</sup>, IgD<sup>high</sup>; follicular CD21<sup>int</sup>, CD23<sup>high</sup>; and MZ: CD21<sup>high</sup>, CD23<sup>low</sup> B cells) from naïve WT and *Cd37<sup>-/-</sup>* mice were quantified by flow cytometry and expressed as percentages (left panel) and as absolute numbers (right panel).  $n = 6$  mice of each genotype. Data are presented as means  $\pm$  SEM. Experiments were performed twice yielding similar results.



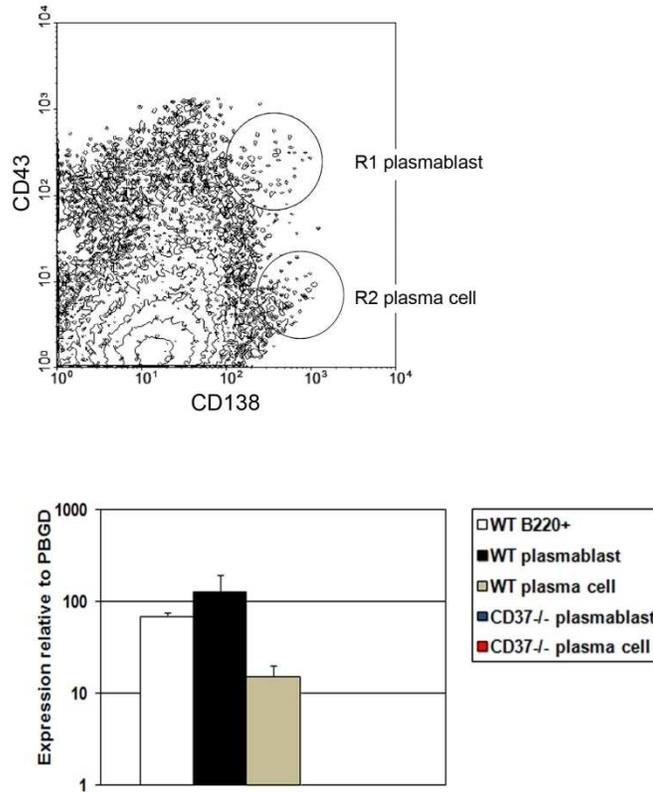
**Fig. S3.** Decreased population of memory B cells in spleens from *Cd37*<sup>-/-</sup> mice. **(A)** Quantification of the number (nr.) of GCs (GL7<sup>+</sup>) per B cell follicle in spleens from WT and *Cd37*<sup>-/-</sup> mice on days 21 and 35 after immunization with NP-KLH. Data are presented as means  $\pm$  SEM ( $n = 3$  mice of each genotype for day 21, and  $n = 6$  mice for day 35). Experiments were performed three times yielding similar results. **(B)** Analysis of B220-positive B cells (negative for both IgM and IgD) that were NP-positive and switched to IgG1 (boxed) 35 days after immunization as identified by flow cytometry. Population sizes are given as percentages of the total number of splenocytes. **(C)** Quantification of IgG1<sup>+</sup> memory B cells in the spleens of WT and *Cd37*<sup>-/-</sup> mice by flow cytometry after immunization. Graph depicts the percentages of splenic B cells expressing IgG1 and specific for NP in WT (filled bars) and *Cd37*<sup>-/-</sup> (open bars) mice ( $n = 3$  mice of each genotype on day 14;  $n = 6$  mice of each genotype on day 35). Asterisk indicates significant difference ( $*P < 0.02$ ). Experiments were performed three times yielding similar results.



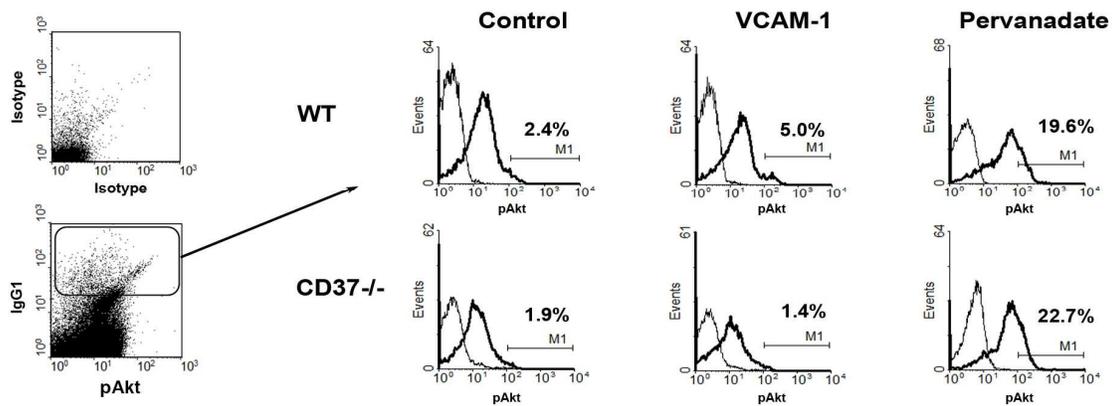
**Fig. S4.** Normal proliferation of *Cd37*<sup>-/-</sup> B cells. Proliferation of purified splenic B cells from WT and *Cd37*<sup>-/-</sup> mice. B cells from WT (filled squares) and *Cd37*<sup>-/-</sup> (open squares) mice were stimulated with F(ab')<sub>2</sub> fragments of anti-IgM (top panel) and anti-CD40 (bottom panel). Proliferation was assessed on the indicated days by measurement of the amount of [<sup>3</sup>H]-thymidine incorporation. Non-stimulated cells did not proliferate at any stage. Data are presented as means ± SEM (*n* = 4 mice of each genotype). Experiments were performed four times yielding similar results.



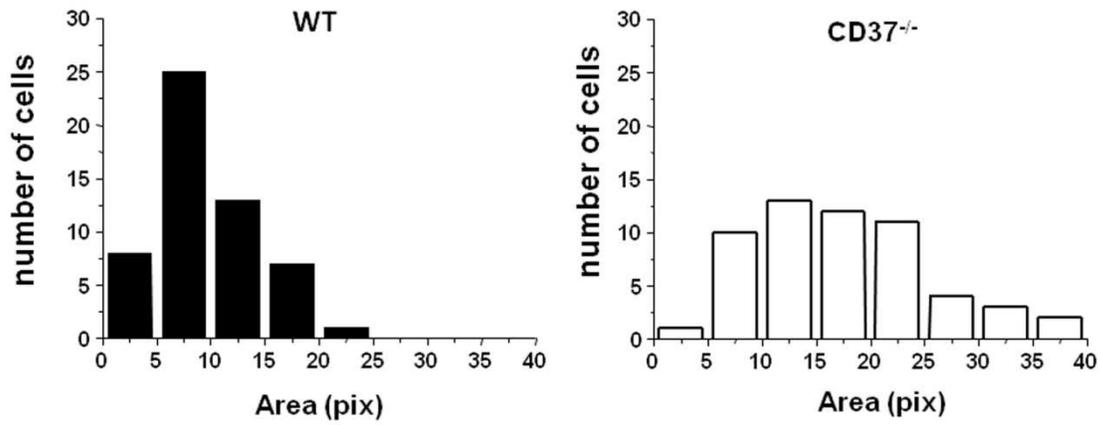
**Fig. S5.** Blocking the  $\alpha_4$  integrin reduces cell rolling and adhesion. Purified (B220-MACS sorted) splenic WT and  $Cd37^{-/-}$  B cells were perfused at physiological shear flow ( $2 \text{ dyne/cm}^2$ ) through a fibronectin-coated chamber. In blocking experiments, B cells were incubated with blocking antibodies against the  $\alpha_4$  integrin (Ps/2 at  $10 \mu\text{g/ml}$ ). Video recordings of individual cells were analyzed and the rolling velocities and percentages of rolling cells (defined as those moving at 10 to  $650 \mu\text{m/s}$ ) were quantified (top panels). The numbers of rolling and adhering WT and  $Cd37^{-/-}$  B cells on  $\alpha_4$  integrin ligand were determined in the absence and presence of blocking antibodies against  $\alpha_4$  integrin (bottom panels). Data are presented as means  $\pm$  SEM ( $n = 3$  mice of each genotype).  $*P < 0.05$ . Experiments were performed three times yielding similar results.



**Fig. S6.** *Cd37* expression in plasmablasts and plasma cells. B cells were purified from the spleens of immunized WT and *Cd37*<sup>-/-</sup> mice by B220 magnetic bead sorting (to >97% purity), labeled with PE-Cy5-conjugated anti-CD138 and PE-conjugated anti-CD43 antibodies (BD-Pharmingen) followed by Elutra FACS cell sorting to purify mature B cells (B220<sup>+</sup>, CD138<sup>-</sup>), plasmablasts (CD138<sup>+</sup>, CD43<sup>+</sup>), and plasma cells (CD138<sup>+</sup>, CD43<sup>-</sup>) (to 99% purity). *Cd37* mRNA abundance [as determined by the  $\Delta C(t)$  method] in purified B cells, plasmablasts, and plasma cells was determined by quantitative RT-PCR, because a commercial antibody against murine *Cd37* is not available). The abundance of *Cd37* mRNA was normalized to that of the housekeeping gene *PBGD*. B cells from *Cd37*<sup>-/-</sup> mice were used as the negative control. Data are presented as means  $\pm$  SD ( $n = 3$  mice of each genotype). Experiments were performed twice yielding similar results.



**Fig. S7.** Impaired Akt phosphorylation in IgG1-secreting *Cd37<sup>-/-</sup>* cells as detected by flow cytometry. Fourteen days after immunization, WT and *Cd37<sup>-/-</sup>* splenocytes were stimulated ex vivo with VCAM-1 (2  $\mu$ g/ml) for 10 min at 37°C, followed by intracellular staining for pAkt and murine IgG1. Experiments were repeated 2 times with 6 mice of each genotype per experiment. Quantification of the percentages of cells containing pAkt after gating on IgG1-secreting cells by flow cytometry. Thick line: pAkt; thin line: isotype control. Pervanadate was used as a positive control.



**Fig. S8.** Average area of  $\alpha_4\beta_1$  integrin fluorescence at the VCAM-1 contact site. WT and *Cd37*<sup>-/-</sup> B cells were labeled with antibody against  $\alpha_4\beta_1$  Ab (Alexa Fluor 488-conjugated 9C10), adhered to VCAM-1 (1  $\mu$ g/ml), and analyzed at the contact site at 37°C with confocal laser scanning microscopy. The frequency of area of fluorescence of  $\alpha_4\beta_1$  integrin labeling was measured in WT ( $n = 54$ ) and *Cd37*<sup>-/-</sup> ( $n = 56$ ) cells. Experiments were performed twice yielding similar results.

**Movie S1.** Rolling and adhesion of wild-type B cells under flow. WT B cells were perfused on fibronectin at a shear stress rate of 2 dyne/cm<sup>2</sup> for 60 s, and video recordings of one microscopic field (0.14 mm<sup>2</sup>) were analyzed continuously at 10 images/s.

**Movie S2.** Rolling and adhesion of *Cd37*<sup>-/-</sup> B cells under flow. *Cd37*<sup>-/-</sup> B cells were perfused on fibronectin at a shear stress rate of 2 dyne/cm<sup>2</sup> for 60 s and video recordings of one microscopic field (0.14 mm<sup>2</sup>) were analyzed continuously at 10 images/s. Note the decreased number of rolling and adherent *Cd37*<sup>-/-</sup> B cells compared to WT B cells.