Supplementary materials for Lu and Cyster report

Materials and Methods

Mice

All mice were on a C57BL/6 background. β2−/− mice were obtained from Eric Brown and Jackson Laboratories. All other mice were obtained either from Jackson Laboratories or Charles River. Animals were used between 7 and 14 weeks of age. Mice were treated with antibodies by intraperitoneal injection in 300 ul of PBS. Bone marrow chimeras were made as previously described (1) and reconstituted for 7 weeks.

Detection of MZ B cells

MZ B cells are distinct from follicular B cells in having lower levels of CD23 and IgD and higher levels of CD21, CD1d and IgM (2, 3). MZ B cells were identified flow cytometrically as B220− CD23lo/− CD21hi or B220− CD23lo/− CD1dhi cells. Findings using these two gating strategies were found to be consistent. Immunohistochemically, MZ B cells were identified by their high expression of IgM and low expression of IgD, or their high expression of CD1d.

Adhesion and Chemotaxis Assays

Fresh splenocytes were isolated into RPMI/5% heat-inactivated FCS, incubated for 30 minutes at 37°C on tissue culture plates to eliminate adherent macrophages, washed with RPMI/0.5% heat-inactivated BSA, and then allowed to adhere to ligand-coated microtiter wells (Immunolon 4, Dynex) for 30 minutes at 37°C at 5% CO2. Nonadherent cells were removed by gentle washing and adherent cells were released by incubating for 15 minutes on ice in RPMI/5mM EDTA. Cells were collected, stained for B220, CD23 and CD21, and analyzed and enumerated by flow cytometry. Adhesion was calculated as a %
of 'input' samples that had been incubated in parallel in BSA-coated wells and collected before washing. Each condition was performed in triplicate wells. Wells were coated by 1 hr incubation at 37°C with ligand in 50µl carbonate buffer and blocked with 1% BSA before use. For chemotaxis assays, cells were prepared similarly, but red blood cells were lysed using Tris-ammonium chloride during the washing procedure. 5 µM Transwell chemotaxis chambers (Corning Costar Corp., Acton, MA) were coated as for adhesion assay using 50 µl of ligand, blocked and washed with BSA and used without drying. Chemotaxis assays were otherwise performed as described (5). Murine ICAM-1-kappa fusion protein (4) was a gift from David Erle (UCSF). Human soluble VCAM-1 was from R&D Systems (Minneapolis, MN).

**Antibodies and antibody treatment**

Anti-αL (clone M17/4, IgG2a) hybridoma was from ATCC. Anti-α4 (clone PS/2, IgG2b) hybridoma was a gift from David Erle. Antibodies were purified on a Sepharose G column (Pharmacia). Anti-VCAM-1 (clone M/K-2, IgG1) was obtained from Southern Biotechnology (Birmingham, AL). Isotype controls (specific for Keyhole Limpet Hemocyanin) were obtained from Pharmingen (San Diego, CA). Macrophages were detected with antibodies ER-TR9 (Accurate Chemical & Scientific Corp. Westbury, NY) specific for marginal zone macrophages, and MOMA1 (gift of G. Kraal), specific for marginal metallophilic macrophages (2). No differences in ER-TR9 and MOMA1 staining were observed between mice treated for three hours with control or integrin blocking antibodies. Antibodies to ICAM-1 (3E2) and VCAM-1 (429) were from Pharmingen. The anti-ICAM-1 and anti-VCAM-1 antibodies interact with integrin-binding sites on the respective molecules, and the integrins compete with the antibodies for ligand binding. Therefore, to maximize the sensitivity of adhesion molecule detection
we stained sections from mice that had been pre-treated with integrin-blocking antibodies. A similar but generally weaker pattern of ICAM-1 and VCAM-1 staining was observed on spleen tissue from untreated mice.

**Figure S1. MZ B cell distribution in β2<sup>-/-</sup>, β7<sup>-/-</sup> and anti-VCAM-1 treated ICAM-1<sup>-/-</sup> mice.** Immunohistochemical analysis of spleen sections from β2<sup>-/-</sup> mice (A) or β7<sup>-/-</sup> mice (B) treated with control IgG, or from ICAM-1<sup>-/-</sup> mice treated with anti-VCAM-1 antibody (C). Sections were stained to detect IgM (blue) and IgD (brown) and are representative of at least 3 mice of each type. Objective magnification, 10x.
Figure S2. Requirement for LTα1β2 in maintenance of marginal zone B cells.

Number of MZ B cells and follicular B cells in mice that had been treated for two weeks with control LFA3-Ig (blue) or with LTβR-Ig (red) (100ug 2x/week). Bars represent mean values obtained from at least 3 mice per group (±sd). *, p<0.05 by Student’s t-test.

Table S1. Elevated integrin expression on MZ B cells. Flow cytometric analysis performed as in figure 1 was used to determine geometric mean fluorescence intensities of the indicated integrin subunits on B220⁺CD23lo⁺CD21hi MZ B cells and B220⁺CD23hi⁺CD21⁺ follicular B cells using CellQuest™ software. Data are shown are mean ±sd and are from at least 4 experiments. Statistical analysis was by the paired Student’s t-test. ns, not significant.

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References