# Partial tolerance of autoreactive B and T cells to erythrocyte-specific self-antigens in mice

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## Online Supplementary Appendix

### *Transfusion and immunization*

B6 and B6.HOD mice were immunized subcutaneously with 100 μg of hen egg lysozyme or ovalbumin (Sigma) emulsified in CFA (Difco Labs). Some BALB/c mice were likewise immunized for sera for immunohistology. Serum was collected 14 days later. For peptide immunization experiments, B6 and B6.HOD mice were immunized in the footpad and tail base with 50 μg of OVA323-339 and LCMV GP 61-80 in CFA. Two weeks later, mice were boosted with 25 ug of both peptides in IFA.

### *Immunofluorescence histology*

Tissue was snap frozen in OCT (Sakura Finetek), stored at -80°C, and cut in 8-10 μm sections with a Leica Cryostat (Leica Microsystems). Sections were fixed with 4% paraformaldehyde (Electron Microscopy Science) and endogenous biotin reactivity was quenched by incubation at 37°C for 20 min with 3% hydrogen peroxide (Fisher Scientific). Sections were blocked with PBS + BSA (Sigma) for 1 h at room temperature in a humidified chamber. Sections were stained sequentially with 1-h incubation at room temperature for each antibody: Balb/c anti-HEL, anti-mouse IgG1$^a$ biotin (BD Pharmingen clone 10.9) streptavidin-APC (BD Pharmingen). Secondary lymphoid organ tissues were additionally stained with anti-Thy1.2 (eBioscience clone 53-2.1) and anti-B220 (BD Pharmingen, clone RA3-6B2). Sections were mounted with Prolong gold with DAPI (Molecular Probes) and allowed to dry at room temperature. Slides were stored at 4°C until imaging. Tissues were imaged with a Zeiss LSM 510 Meta (Carl Zeiss Microimaging).

### *ELISA for anti-HEL and anti-OVA humoral response*

Enzyme-linked immunosorbent assays (ELISAs) for anti-HEL IgG and anti-OVA IgG were performed on sera run in triplicate at a 1:50 dilution as previously described.[1] Monoclonal anti-HEL IgG antibody (4B7) was used as an internal standard to allow for comparison between experiments.[2]

### *Staining leukocytes*

A total of 2x10$^6$ leukocytes from bone marrow were stained for flow cytometry in FACS buffer (PBS + 0.2 mg/mL bovine serum albumin [Sigma] + 0.9 mg/mL EDTA [Sigma] + 2% fetal bovine serum [Hyclone]). Duffy-reactive MIMA-29 and HEL-reactive 4B7 monoclonal antibodies were conjugated to Alexa fluor 647 (HEL-AF-647) using Alexa Fluor Monoclonal Antibody Labeling kit (Invitrogen) according to the manufacturer's instructions. Bone marrow leukocytes were stained with anti-CD71 (BD Pharmingen, clone C2), anti-TER119 (BD Pharmingen, clone TER119), and MIMA-29 AF-647 or 4B7-AF-647.

Peripheral blood was collected from mice in ACD. RBCs were stained in FACS buffer with anti-HEL or anti-OVA primary antibodies. Streptavidin-APC (with biotinylated anti-IgG1$^a$) or anti-mouse immunoglobulins were used as secondary antibodies. Peripheral white blood cells were stained with antibodies against CD19 (BD Pharmingen, clone 1D3), CD41 (BD Pharmingen, clone MWReg30) or CD45 (BD Pharmingen, clone 30-F11). Samples were analyzed with a 4-color Accuri Cytometer.

For assessment of BCR specificity for HEL, HEL was biotinylated with EZ Link Sulfo-NHS-Biotinylation kit (Pierce), according to the manufacturer's protocol. HEL was tetramerized with streptavidin-APC (molecular probes). To control for non-specific biotin interactions, mouse albumin (Sigma) was biotinylated and tetramerized with APC-Cy7 (Molecular Probes). Bone marrow, splenocytes, and peritoneal cavity leukocytes were stained with antibodies against BP-1, IgM, CD93, CD43, B220, CD24, and IgD.

### *ELISPOT*

PVDF Enzyme-linked immunosorbent spot (ELISPOT) plates (Millipore, Billerica, MA, USA) were coated with 10 μg/mL of HEL or PBS and incubated overnight at 4°C. The rest of the assay was performed as previously described.[3]

### *Flow cytometric crossmatching*

Crossmatching was performed as previously described.[1]

### *CD4$^+$ T-cell purification and adoptive transfer of cells*

Leukocytes from the spleens of OTIIxThy1.1, SMARTA, TCR75 and OTII/RAG1ko mice were liberated by physical disruption and resuspended in complete RPMI. Mice were adoptively transferred with 10x10$^6$ total splenocytes from OTIIxThy1.1 or enriched CD4$^+$ T cells [CD4$^+$ T-cell isolation kit (Miltenyi Biotec)]. Post purity of CD4$^+$ T-cell enrichment was

70% (OTIIxThy1.1), 85% (SMARTA), 88% (TCR75). In each instance, the percentage of leukocytes $CD19^{+}CD4^{-}$ was less than 1.5% of the total, ungated population. B6 and B6.HOD recipients were injected via lateral tail vein with 1-5x$10^{6}$ enriched $CD4^{+}$ T cells.

### *MHCII tetramer-based enrichment of antigen-specific T cells*

Spleen and lymph nodes were harvested, stained with tetramer, and positively enriched as previously described,[4] using $OVA_{326-334}$, $OVA_{329-337}$, and LCMV $GP_{66-77}$.

## References


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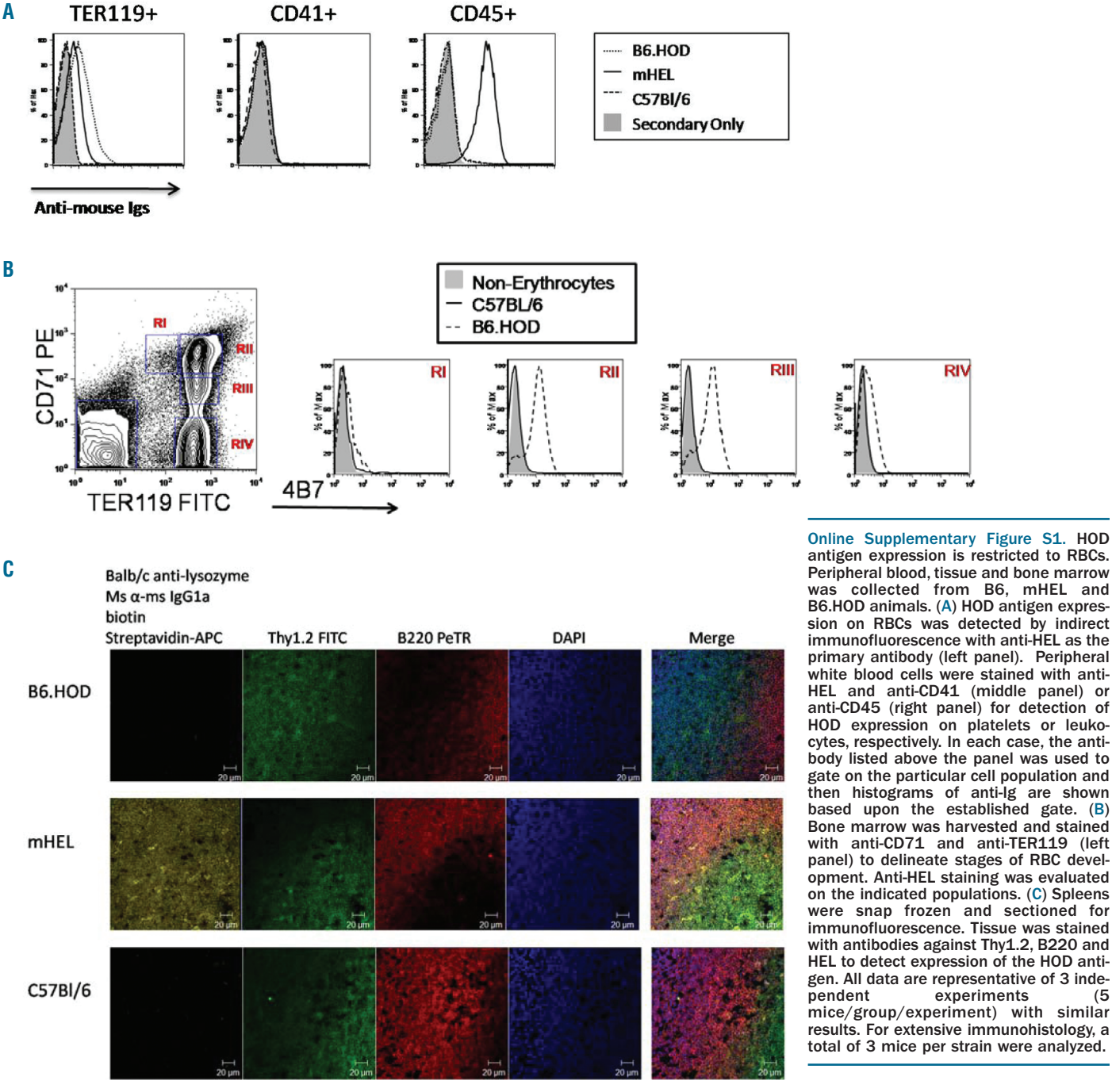
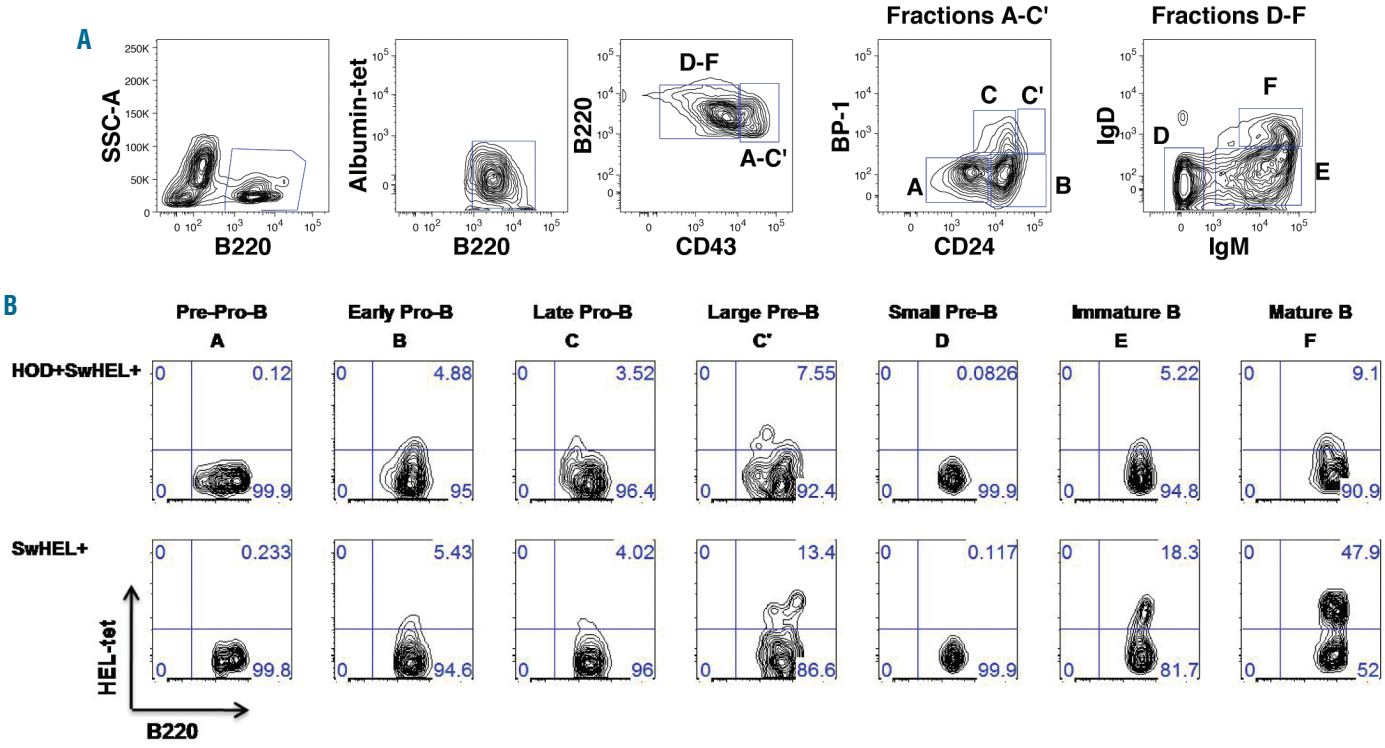



**Online Supplementary Figure S1.** HOD antigen expression is restricted to RBCs. Peripheral blood, tissue and bone marrow was collected from B6, mHEL and B6.HOD animals. (A) HOD antigen expression on RBCs was detected by indirect immunofluorescence with anti-HEL as the primary antibody (left panel). Peripheral white blood cells were stained with anti-HEL and anti-CD41 (middle panel) or anti-CD45 (right panel) for detection of HOD expression on platelets or leukocytes, respectively. In each case, the antibody listed above the panel was used to gate on the particular cell population and then histograms of anti-Ig are shown based upon the established gate. (B) Bone marrow was harvested and stained with anti-CD71 and anti-TER119 (left panel) to delineate stages of RBC development. Anti-HEL staining was evaluated on the indicated populations. (C) Spleens were snap frozen and sectioned for immunofluorescence. Tissue was stained with antibodies against Thy1.2, B220 and HEL to detect expression of the HOD antigen. All data are representative of 3 independent experiments (5 mice/group/experiment) with similar results. For extensive immunohistology, a total of 3 mice per strain were analyzed.




**Online Supplementary Figure S2.** RBC expression of HOD antigen induces central B-cell tolerance. Femoral marrow and splenocytes were harvested from SwHEL and B6.HODxSwHEL F1 mice and stained with tetramerized HEL and the indicated surface antibodies to determine BCR specificity and delineate the stages of B-cell development. Flow cytometry was used to establish (A) bone marrow gating strategy, (B) the percentage of HEL-reactive B cells in different stages of development, and (C) total $IgM^{+}IgD^{+}$ (Fraction F). All data are representative of 3 independent experiments (2-5 mice/group/experiment) with similar results. No fewer than 8 mice were analyzed in any given group.