Supporting Information

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SI Materials and Methods

Peptide Synthesis. The ERY1 (H_2N-WMVLPWLPGTLDGGSG-CRG-$CONH_2$) and mismatch (MIS; H_2N-PLLTVGMDLWPW-GGSGCRG-$CONH_2$) peptides were synthesized by means of standard solid-phase f-moc chemistry using TGR resin (Nova Biochem) on an automated liquid handler (Chemspeed). The peptide was cleaved from the resin for 3 h in 95% (vol/vol) trifluoroacetic acid, 2.5% (vol/vol) ethanedithiol, and 2.5% (vol/vol) water, and it was precipitated in ice-cold diethyl ether. Purification was conducted on a preparative HPLC-MS (Waters) using a C18 reverse phase column (PerSpective Biosystems). SIINFEKL and p31 (YVRPLWVRME) peptides were purchased from Genscript.

ERY1-Antigen Synthesis. Ten-molar equivalents of sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (Thermo Scientific) dissolved in dimethylformamide were reacted with 5 mg/mL endotoxin-free (<1 EU/mg) ovalbumin (OVA; Hyglos GmbH) in PBS for 1 h at room temperature. Following desalting on a 2-mL Zeba Desalt spin column (Thermo Scientific), 10 equivalents of ERY1 or MIS peptide dissolved in 3 M guanidine-HCl was added and allowed to react for 2 h at room temperature. The conjugate was desalted using 2-mL Zeba Desalt spin columns, 0.2 μm sterile-filtered, dispensed into working aliquots, and stored at −20 °C. Protein concentration was determined via bicinchoninic acid assay (Thermo Scientific). GST was expressed in BL21 *Escherichia coli* and purified using standard glutathione affinity chromatography. On-column endotoxin removal was performed by extensive Triton-X114 (Sigma–Aldrich) washing, and endotoxin removal was confirmed with THP-1 × Blue cells (InvivoGen). The same reaction procedure was used to conjugate ERY1 to GST. Maleimide-activated allophycocyanin (Innova Biosciences) was dissolved in PBS and conjugated with ERY1 or MIS as described above.

Microscopy of Binding to Erythrocytes. A total of 5×10^5 freshly isolated mouse erythrocytes were exposed to 100 nM ERY1-OVA or OVA in PBS containing 10 mg/mL BSA for 1 h at 37 °C. Following centrifugation and washing, cells were labeled with 1:200 diluted goat anti-mouse glycophorin-A (Santa Cruz Biotechnology) and rabbit anti-OVA antibody (AbD Serotec) for 20 min on ice. Following centrifugation and washing, cells were labeled with 1:200 AlexaFluor488 anti-goat IgG (Invitrogen) and AlexaFluor546 anti-rabbit IgG (Invitrogen) for 20 min on ice. Following a final spin/wash cycle, cells were hard set-mounted and imaged on a Zeiss LSM700 inverted confocal microscope with a 63× oil immersion objective. Image analysis was conducted using ImageJ (National Institutes of Health), with identical processing of both images.

In Vivo Binding and Biodistribution. A total of 150 μg of ERY1-OVA or OVA in 0.9% saline (B. Braun Medical) in a volume of 100 μL was injected i.v. into the tail of 8- to 12-wk-old female C57BL/6 mice while under anesthesia with isoflurane. Care was taken to ensure mice were kept at 37 °C with a heating pad during experimentation. At predetermined time points, 5 μL of blood was taken from a small incision on the tail, diluted 100-fold into 10 mM EDTA in PBS, washed three times with PBS with 10 mg/mL BSA, and analyzed for OVA content by flow cytometry and ELISA. OVA was quantified by sandwich ELISA, using a mouse monoclonal anti-OVA antibody (Sigma) for capture, a polyclonal rabbit anti-OVA antibody for detection, and a goat anti–rabbit-IgG-HRP antibody (BioRad) for final detection, followed by TMB substrate (GE Life Sciences). Hematological characterization was performed on an Adviva 2120 Hematology System (Siemens). Erythrocyte-bound ERY1-GST was detected by incubating labeled cells with goat anti-GST (GE Healthcare Life Sciences), followed by incubation with AlexaFluor488 donkey anti-goat (Invitrogen), and was analyzed by flow cytometry. For biodistribution studies, 20 μg of ERY1–antigen-presenting cells (APCs) or MIS-APCs was injected i.v. into the tail vein of 8- to 12-wk-old female C57BL/6 mice as described above. Mice were killed at predetermined time points, and the spleen, blood, and liver were removed. Each organ was digested with collagenase D (Roche) and homogenized to obtain a single-cell suspension for flow cytometry staining.

Microscopy of Tissue Samples. The following antibodies were used for tissue sample microscopy: CD45, F4/80, H-2kb-SIINFEKL 25-d1.16, biotin-CD3e (eBioscience), rabbit anti-OVA, anti-insulin (Abcam), and streptavidin-AlexFluor488 (Invitrogen). Organs were freshly isolated from euthanized mice and frozen in optimal cutting temperature (OCT) embedding matrix with liquid nitrogen. Slices were blocked for 1 h at room temperature with 10% (vol/vol) horse serum (Invitrogen Gibco) in PBS + 20 mg/mL BSA, washed with PBS, incubated with primary antibodies for 2 h at room temperature, washed with PBS, and incubated with the appropriate secondary antibody and DAPI for 1 h at room temperature. Following a final wash with PBS, samples were hard set-mounted and visualized on an LSM700 inverted confocal microscope. Image analysis was conducted using Fiji (National Institutes of Health), with identical processing of both images. A local normalization filter was applied to the nuclear DAPI signal to avoid nonhomogeneous nuclei throughout the entire field of view.

Flow Cytometry. The following anti-mouse antibodies were used for flow cytometry: CD1d Pacific Blue, CD3ε peridinin chlorophyll protein (PerCP)-Cy5.5, CD8α phycoerythrin (PE)-Cy7, CD11b PE-Cy7, CD11c Pacific Blue, biotinylated CD45, CD45.2 Pacific Blue, CD45 Pacific Blue, IFN-γ–APC, CD8α APC-eF780, CD44 PE-Cy5.5, CD62L PE, CD205 PE-Cy7, F4/80 PE, I-A/I-E MHC-II FITC, biotin–programmed death-1, and CD4 Pacific Blue (all from eBioscience), in addition to fixable live/dead dye (Invitrogen), an annexin-V–Cy5 labeling kit (BioVision), streptavidin Pacific Orange (Invitrogen), streptavidin APC-eF780 (eBioscience), anti–OVA-FITC, and anti-6 × His PE (Abcam). Samples were analyzed on a CyAn ADP flow cytometer (Beckman Coulter). Cells were washed first with PBS, stained for 20 min on ice with live/dead dye, blocked for 20 min on ice with 24G2 hybridoma medium, surface-stained for 20 min on ice, fixed in 2% (vol/vol) paraformaldehyde for 20 min on ice, and intracellularly stained in the presence of 0.5% saponin for 45 min on ice, followed by a final wash before analysis. For apoptosis staining, annexin-V–Cy5 was added 5 min before analysis. For biotinylated antibodies, cells were stained with streptavidin conjugates for 20 min on ice, washed, and analyzed. The dissociation constant of ERY1-OVA was determined by equilibrium binding measurements via flow cytometry, as described elsewhere (1).

1. Feldhaus M, Siegel R (2004) Flow cytometric screening of yeast surface display libraries. *Methods Mol Biol* 263:311–332.

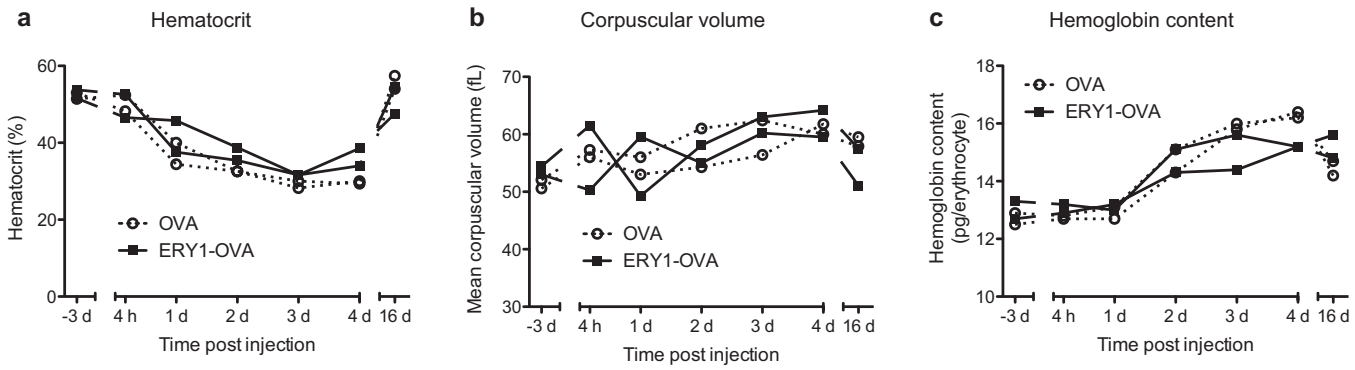

Fig. S1. Erythrocyte binding does not alter hematological behavior. Hematocrit (*A*), mean corpuscular volume (*B*), and erythrocyte hemoglobin content (*C*) measured at varying time points following administration of either 10 μg of OVA (○) or ERY1-OVA (■).

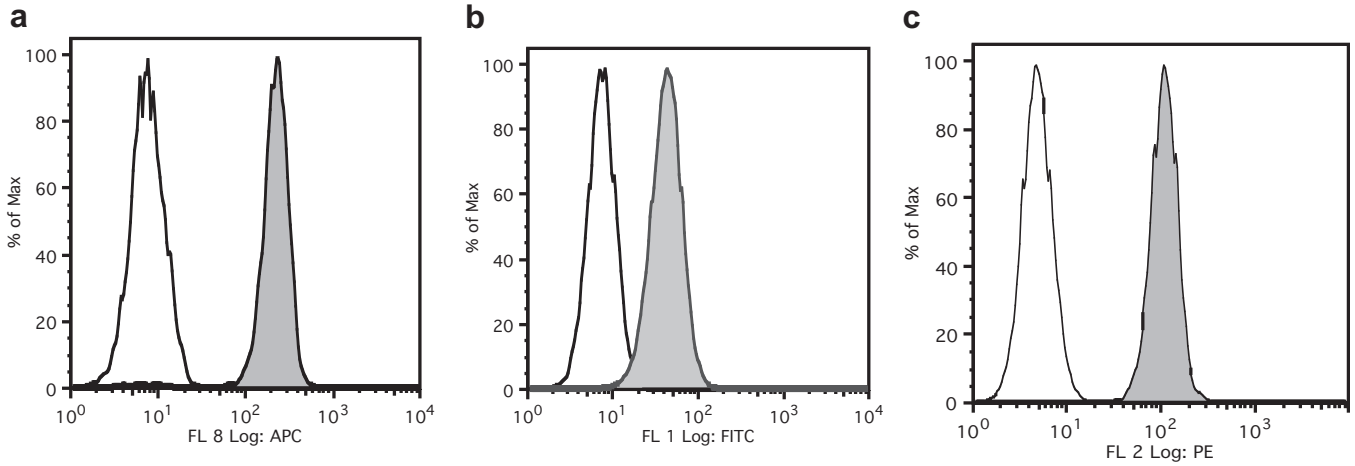

Fig. S2. Flow cytometric confirmation of erythrocyte binding by various antigen constructs. (*A*) Binding histogram of ERY1-allophycocyanin (gray-filled histogram) and MIS-allophycocyanin (empty histogram). (*B*) Binding histogram of ERY1-GST (gray-filled histogram) and GST (empty histogram). (*C*) Binding histogram of TER119-SINIFEKL (gray-filled histogram) and isotype anti-6 × HIS mAb control (empty histogram). Max, maximum; PE, phycoerythrin.

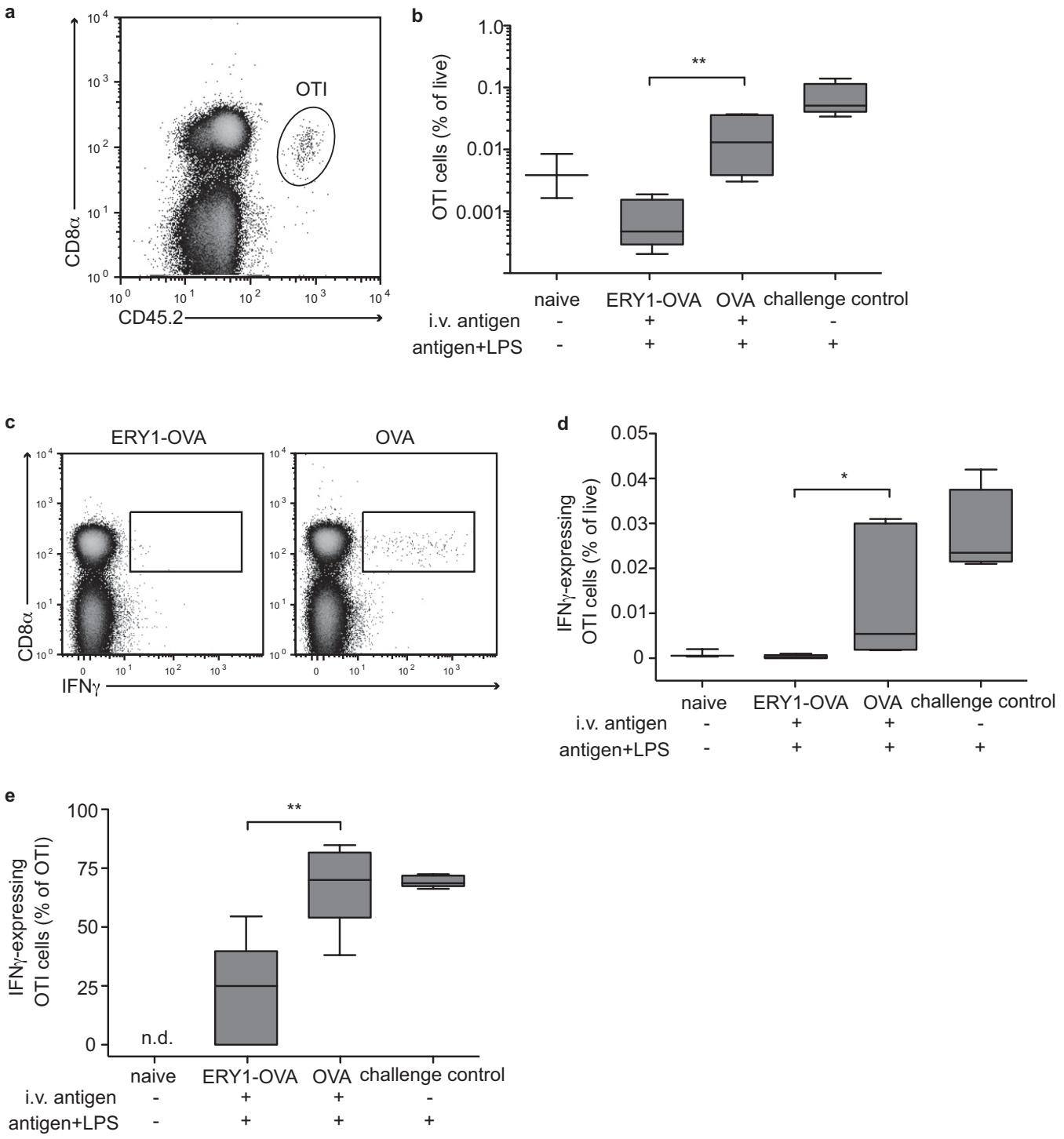

Fig. S3. Erythrocyte binding induces tolerance to antigen challenge. (*A*) Flow cytometric detection of OTI CD8$^+$ T-cell populations in the spleen (CD3ε^+ CD8α^+ CD45.2$^+$). (*B*) OTI CD8$^+$ T-cell population quantification in the spleen 4 d following antigen challenge in CD45.1$^+$ mice. $^{**}P < 0.01$. (*C*) Flow cytometric detection of IFN-γ–expressing OTI CD8$^+$ T cells. (*D*) IFN-γ–expressing OTI CD8$^+$ T cells in the spleen 4 d following antigen challenge and in vitro restimulation with SIINFEKL peptide. $^{*}P < 0.05$. (*E*) IFN-γ–expressing OTI CD8$^+$ T-cell population quantification in the spleen 4 d following antigen challenge and in vitro restimulation with SIINFEKL peptide, represented as a percentage of the remaining splenic OTI CD8$^+$ T-cell population. $^{**}P < 0.01$. Data were obtained by multiparameter flow cytometry, represented as mean ± minimum to maximum (n = 5).

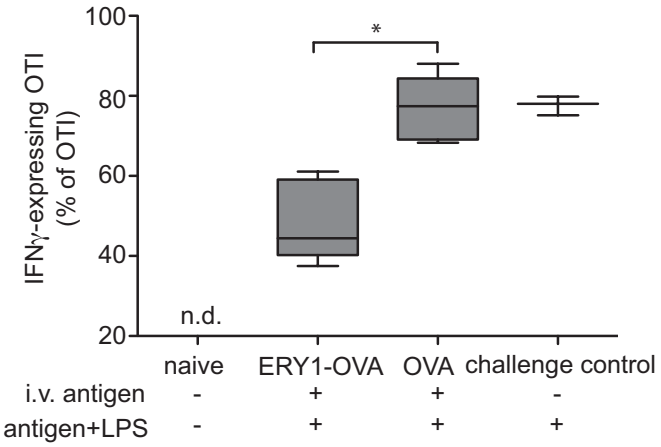

Fig. S4. ERY1-OVA induces functional nonresponsiveness of remaining OTI CD8$^+$ T cells. OTI CD8$^+$ T-cell population quantification in the draining lymph nodes 4 d following antigen challenge in CD45.1$^+$ mice, represented as a percentage of the remaining splenic OTI CD8$^+$ T-cell population. *$P < 0.05$. Data were obtained by multiparameter flow cytometry, represented as median ± minimum to maximum ($n = 5$).

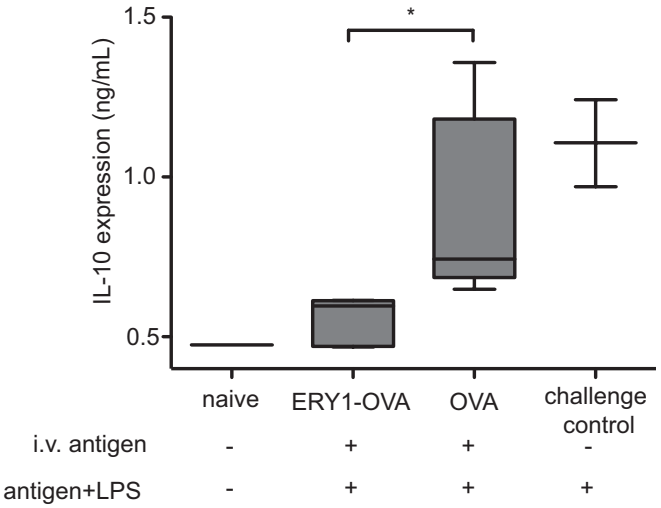

Fig. S5. ERY1-OVA attenuates IL-10 expression following restimulation of draining lymph node cells with OVA antigen in the OTI challenge-to-tolerance model. *$P < 0.05$. Data were obtained by ELISA, represented as median ± minimum to maximum ($n = 5$).

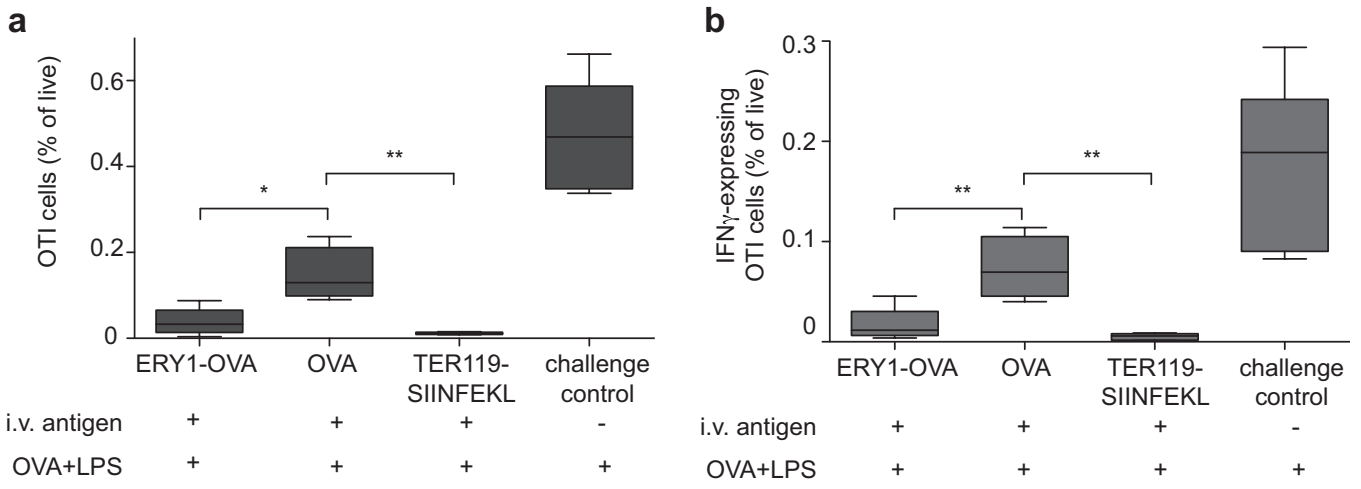

Fig. S6. TER119-SIINFEKL induces deletional tolerance to antigen challenge. (*A*) OTI CD8$^+$ T-cell population quantification in draining lymph nodes 4 d following antigen challenge in CD45.1$^+$ mice. (*B*) Flow cytometric detection of IFN-γ–expressing OTI CD8$^+$ T cells. Data were obtained by multiparameter flow cytometry, represented as mean ± minimum to maximum (n = 5). *P < 0.05; **P < 0.01.

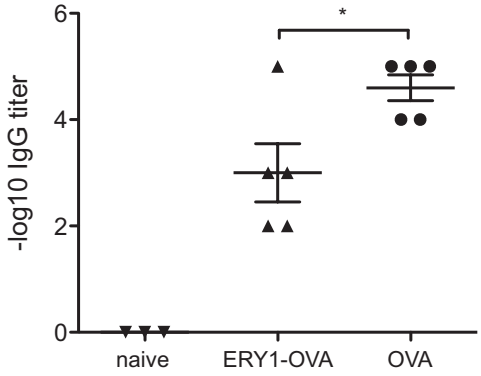

Fig. S7. Erythrocyte binding attenuates antigen-specific humoral responses in C57BL/6 mice. OVA-specific IgG detection in serum 19 d following two injections of 1 μg of OVA (●) or 1 μg of ERY1-OVA (▲) 6 d apart in C57BL/6 mice. *$P \leq 0.05$.