Supporting Information

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SI Materials and Methods

Proteins and Reagents. $FVIII_{75–89}$ (VHLFNIAKPRPPWMG), $FVIII_{1723–1737}$ (ERLWDYGMSSSPHVL), and $FVIII_{2210–2229}$ (TASSYFTNMFATWSPSKAR) were synthesized by Anaspec. Ovalbumin (OVA) and HEL (Worthington Biochemical Corporation), $OVA_{323–339}$ peptide (Bachem Americas, Inc.), $PLP_{139–151}$ peptide (US Biological Life Sciences), aluminum hydroxide gel (Alum) (Sigma and Invivogen), and cholera toxin (List Biological Laboratories) were purchased from the suppliers indicated. Anti-CD11c (HL-3, N418), anti-EMR1 (F4/80), anti-CD8α (53-6.7), anti-CD45R (RA3-6B2), anti-CD4 (GK1.5), and anti-CD25 (PC61) antibodies were purchased from BD Biosciences. Anti-Foxp3 (FJK-16s), anti-Ki67 (SolA15), 7AAD, and anti-CD11b (M1/70) antibodies were purchased from eBiosciences. Anti-CD19 (6D5), TCRVa2 (B20.1), TCRb (H57-597), CD38 (90), IgD (11-26c.2a), GL7 (GL7), Ki67 (SolA15, eBio), 7AAD (eBio), Gr1 (RB6-8C5), F4/80 (BM8), and anti-IL10 (JES5-16E3) were purchased from Biolegend.

Generation of pOVA. Chicken OVA protein was dissolved in PBS. The solution pH was raised to 12 by adding 3 M of NaOH, and then the pH was lowered to 2 using trifluoracetic acid to coacervate the protein. This pH cycling was repeated three additional times, and then the solution pH was raised to 7 using 3 M of NaOH. The coacervated protein was then homogenized three times at 20,000 psi using a Microfluidics LV1 microfluidizer with G10Z interaction chamber to reduce the particle size to ∼200 nm as determined by dynamic light scattering (DLS).

Mice and Mouse Cells. C57/BL6, Balb/C, OTII+, and OTII+ $RAG1^{-/-}$ mice were obtained from Jackson Laboratories or Taconic Farms. When indicated, T and B cells were isolated from single-cell suspensions of LNs and/or spleens of young mice and enriched using magnetic sorting (MACS, Miltenyi). In some cases, T cells were labeled with carboxyfluorescein diacetate succinimidyl diester (CFSE, Invitrogen) by incubation for 10 min at 37 °C with CFSE in RPMI medium containing 10% FCS to trace their proliferation in vitro (1 μM) or in vivo (10 μM). FVIII-deficient hemophilia A mice (1) (E16, C57BL/6 background) were maintained in the animal facility of the Uniformed Services University of the Health Sciences.

ELISA. The levels of anti-OVA IgG antibodies were measured by ELISA. Briefly, assay plates (Costar 9017 96-well flat-bottom plates) were coated with 100 μL of OVA protein (5 μg/mL, Worthington Biochemical LS003054) and incubated overnight at 4 °C. Plates were washed with 0.05% Tween-20 in PBS and then blocked with casein in PBS. Assay wells were washed, and serial dilutions of samples were added and incubated at room temperature for 2 h. Anti-OVA antibody was detected by addition of HRP-conjugated goat anti-mouse anti-IgG (AbCam ab98717) followed by addition of substrate (BD Biosciences TMB Substrate Reagent Set, catalog no. 555214). The optical density (OD) of the wells was assessed immediately after adding the stop solution on a plate reader at wavelengths of 450 nm with subtraction at 570 nm. Data analysis was performed using Molecular Device's software SoftMax Pro v6.2.2. A four-parameter logistic curve-fit graph was prepared with the dilution on the *x* axis (log scale) and the OD value on the *y* axis (linear scale), and the half maximum value (EC_{50}) for each sample was determined.

The KLH ELISA followed a similar protocol but used Maxisorp assay plates (NUNC 44-2404-21) and a coating of KLH (Sigma H7127) at 5 μg/mL. The anti-OVA IgE titers were determined using a sandwich ELISA. The Costar plates were coated with anti-mouse IgE (AbD Serotec MCA419) and incubated overnight. Samples were serially diluted and added to blocked wells for 2 h. Samples were detected with biotin-conjugated rabbit anti-OVA IgG (AbCam ab8389) followed by streptavidin–HRP (BD 554066).

mMCP-1 concentrations were determined using Affymetrix Mouse MCPT-1 ELISA Kit (88-7503-88).

Assays for Anti-FVIII Antibody Levels and Inhibitor Titers. Anti-FVIII antibody levels were determined by ELISA. ELISA plates were coated with 1 μg/mL of recombinant FVIII (rFVIII) overnight at 4 °C. For quantification, a standard curve was generated with a mixture of monoclonal anti-FVIII A2 and C2 antibodies.

The FVIII inhibitor titer was determined by Bethesda assay using a previously published protocol with minor modification (2). The inhibitor titer was established from a semilogarithmic plot of residual FVIII activity (logarithmic) and units of inhibitor activity (linear) (2). The dilution of test plasma giving a residual FVIII activity of 50% is said to contain one Bethesda unit FVIII inhibitor activity per milliliter.

Immunizations. Three major modalities of immunizations were used: OVA(25 μg)+CpG (20 μg) s.c. in the hind limbs or front limbs, pOVA (10 μg) intravenously, KLH (50 μg) s.c. in the base tail (50 μg) or the front limbs (50 μg), OVA (5 mg) with cholera toxin (CTx, 10μg) weekly, and the aforementioned OVA+Alum.

BALB/c female mice aged 8–10 wk were administered 5 mg of OVA with 10 μg of CTx (List Biologicals) weekly. In animals immunized with OVA+CpG on days 0, 14, and 28, a delayed-type hypersensitivity response was evaluated by injecting one hind limb with PBS and the other with 10 μg of OVA. The following day, the two limbs were measured with a caliper to determine the degree of inflammation. In other instances, repetitive infusions with pOVA i.v. led to anaphylaxic reactions that were scored by three blinded scorers as previously described (3): 0, no symptom; 1, lethargy; 2, lethargy and inability to right; 3, moribund.

1. Bi L, et al. (1995) Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. *Nat Genet* 10(1):119–121.
2. Kasper CK, et al. (1975) Proceedings: A more uniform measurement of factor VIII inhibitors. *Thromb Diath Haemorrh* 34(2):612.
3. Liu E, et al. (2002) Anti-peptide autoantibodies and fatal anaphylaxis in NOD mice in response to insulin self-peptides B:9-23 and B:13-23. *J Clin Invest* 110(7):1021–1027.

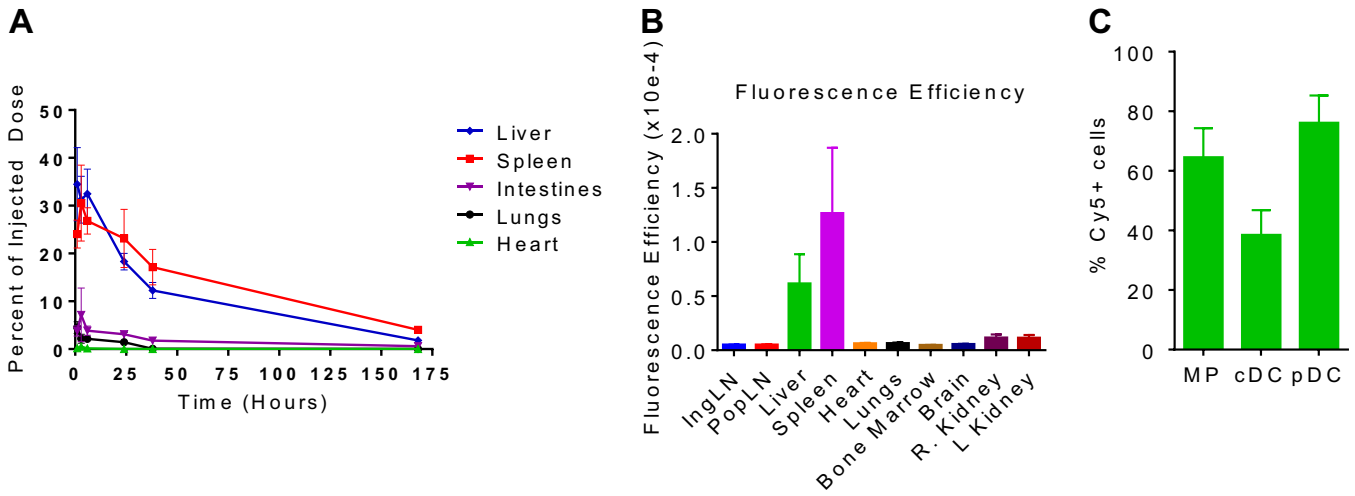

Fig. S1. Biodistribution of fluorescently labeled NPs. (*A*) Balb/C animals were immunized with pOVA on days –27 and –13 before the injection of NPs. On day 0, fluorescently tagged Cy7–tNPs were injected i.v. in in vivo whole animal with imaging by FMT. Mice were subjected to imaging on a FMT instrument. Fluorescence signal was quantified at 1, 3, 6, 24, and 48 h ($n = 3$) and at 168 h ($n = 1$) after injection for selected organs. (*B*) Ex vivo analysis of tNP biodistribution. Animals were killed at 48 h ($n = 2$), and selected organs were harvested and analyzed for fluorescence signal. (*C*) Cellular localization of NP in the spleen. Animals were injected i.v. with tNP (containing rapamycin+OVA) labeled with Cy5. Spleen cells were harvested the next day and macrophages (MPs; F4/80+ CD11b+Gr1–), conventional DC (cDC; MHCCLII+CD11c+CD11b+), and plasmacytoid DC (pDC; MHCCLII+CD11c+B220+PDCA1+) were analyzed by flow cytometry ($n = 3$ per group).

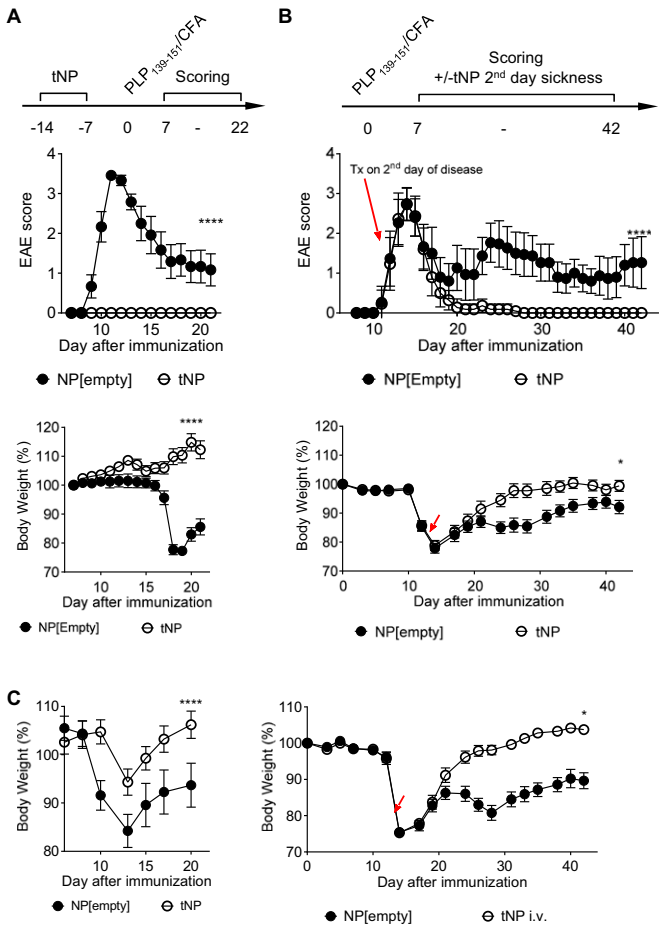

Fig. S2. Induction of tolerance in R-EAE using tNP. (*A*) Prophylactic treatment. SJL mice ($n = 12$) were treated prophylactically with two injections of tNP loaded with $PLP_{139–151}$ and rapamycin or with placebo NPs (empty NP) on days –14 and –7. R-EAE was induced on day 0 by injection of $PLP_{139–151}$ emulsfied in CFA followed by pertussis toxin (PTx). Disease was scored by blinded observers. (*B*) Therapeutic treatment. R-EAE in SJL mice ($n = 15$) was induced on day 0 by s.c. administration of $PLP_{139–151}$/CFA. Animals were individually monitored for onset of disease and treated on the second day of detectable disease (score ≥ 1, around day 12) with either empty NP or tNP. Body weight of animals was measured three times per week. The results are represented as the mean ± 95% confidence interval. For statistical analysis, Student *t* test with two-tailed distribution was used (****$P < 0.0001$). (*C*) Body weight measurements in EAE. Mice described in Fig. 6 *A* and *B* were monitored for body weight three times per week (*Left* and *Right* panels, respectively). The results are represented as the mean ± 95% confidence interval. For statistical analysis, Student *t* test with two-tailed distribution was used (****$P < 0.0001$).