

In the format provided by the authors and unedited.

Depletion of PD-1-positive cells ameliorates autoimmune disease

Peng Zhao¹, Peng Wang¹, Shuyun Dong¹, Zemin Zhou², Yanguang Cao³, Hideo Yagita⁴, Xiao He², Song Guo Zheng⁵, Simon J. Fisher⁶, Robert S. Fujinami² and Mingnan Chen  ^{1*}

¹Department of Pharmaceutics and Pharmaceutical Chemistry, College of Pharmacy, University of Utah, Salt Lake City, UT, USA. ²Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT, USA. ³Division of Pharmacotherapy and Experimental Therapeutics, The UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA. ⁴Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan. ⁵Division of Rheumatology, Department of Medicine, Penn State Milton S. Hershey Medical Center, Hershey, PA, USA. ⁶Division of Endocrinology, Metabolism, and Diabetes, Department of Internal Medicine, University of Utah, Salt Lake City, UT, USA. *e-mail: mingnan.chen@utah.edu

Table of Contents

Figure S1: Analysis of the coding genes and the purified samples of the proteins used in the study.	2
Figure S2. The selective binding and internalization of α PD-1-ABD-PE by PD-1 ⁺ cells.	3
(a and b) Two representative histograms resulting from flow cytometry analyses of EL4 (a) and B16 cells	3
Figure S3. The selective binding and internalization of α PD-1-ABD-PE to PD-1 ⁺ cells are mediated by PD-1.	5
Figure S4. α PD-1-ABD-PE possesses selective toxicity to primary PD-1 ⁺ lymphocytes <i>in vitro</i>	7
Figure S5. Gating strategies used to quantify PD-1 ⁺ CD4 T cells, Tregs, MOG ₃₈₋₄₉ -specific CD4 T cells.	10
Figure S6. Analysis of immune cells in NOD mice after the α PD-1-ABD-PE treatment.	11
Figure S7. Analysis of immune cells in mice with EAE after the α PD-1-ABD-PE treatment.	14
Figure S8. Analysis of normal adaptive immunity in mice after the α PD-1-ABD-PE treatment.	15

Supplementary Information:

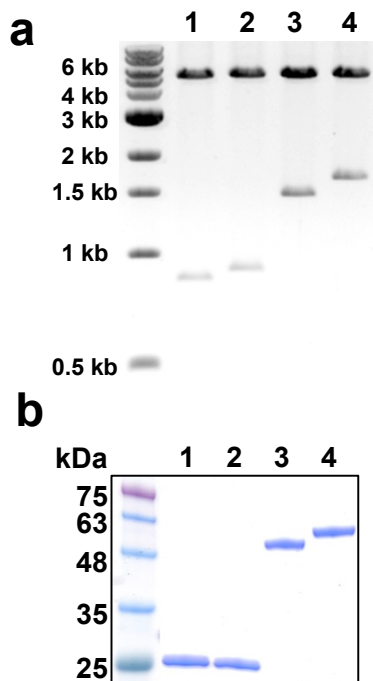


Figure S1: Analysis of the coding genes and the purified samples of the proteins used in the study.

(a) A photo of an agarose gel that contains the coding genes of α PD-1 (lane 1), ABD-PE (lane 2), α PD-1-PE (lane 3), and α PD-1-ABD-PE (lane 4). The lower bands of each lane were the coding genes after they were cleaved from the pET25b(+) vector (upper bands in each lane) by XbaI and BamHI. (b) An SDS-PAGE gel photo of purified α PD-1, ABD-PE, α PD-1-PE, and α PD-1-ABD-PE (5 μ g of each sample). Lane 1: α PD-1, lane 2: ABD-PE, lane 3: α PD-1-PE, lane 4: α PD-1-ABD-PE. Studies described in this figure were repeated at least three times with similar results. Data of one repeat is shown here.

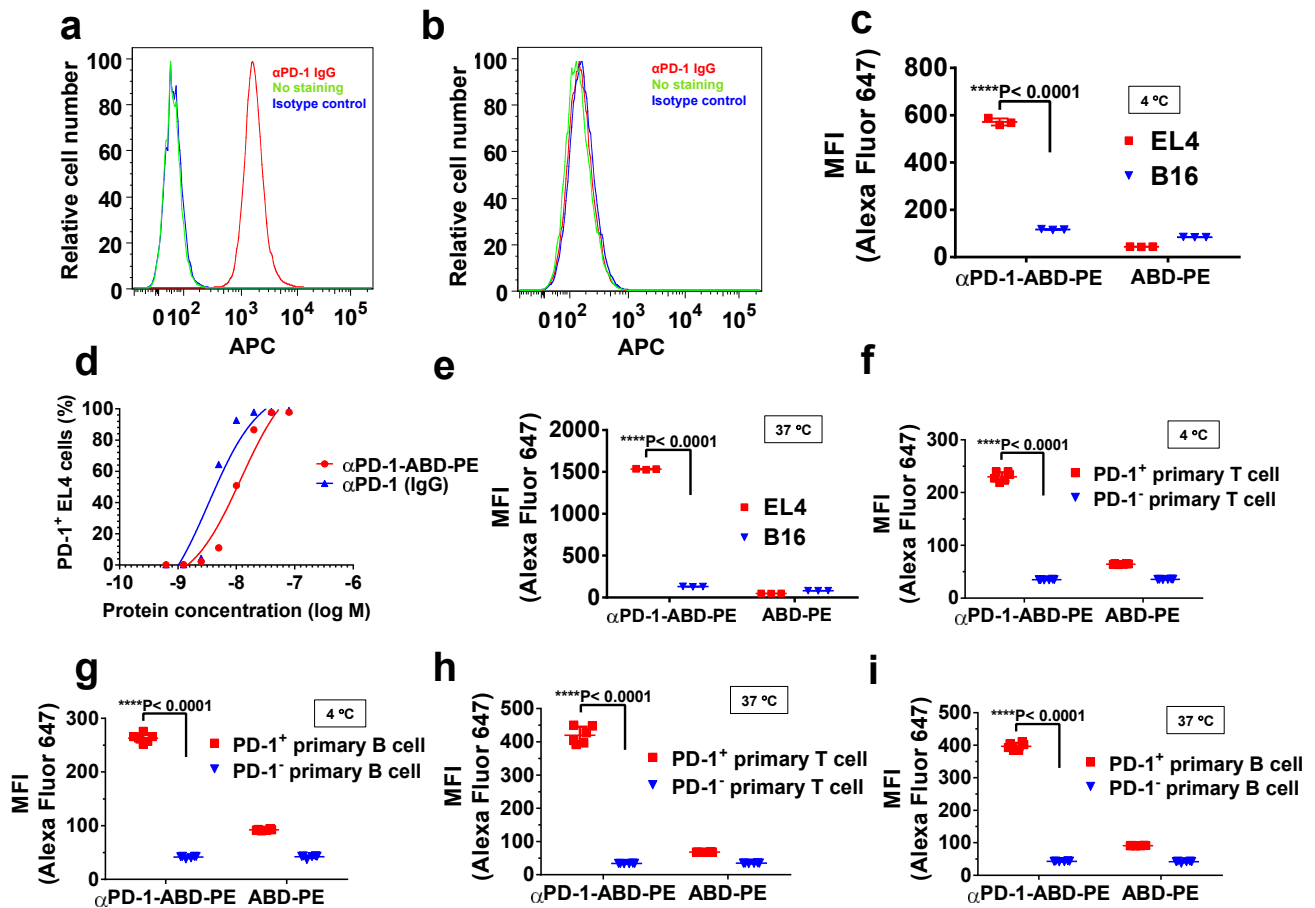


Figure S2. The selective binding and internalization of α PD-1-ABD-PE by PD-1⁺ cells.

(a and b) Two representative histograms resulting from flow cytometry analyses of EL4 (a) and B16 cells (b). These cells were stained with either APC-labeled α PD-1 (full IgG, 100 nM, Red), APC-RatIgG2a isotype control (100 nM, Blue), or nothing (Green). Results reflected from these histograms suggest that the vast majority of EL4 cells express PD-1 on their surface, while almost no B16 cell express PD-1 on their surface. Studies described in the panels a-b were repeated at least three times with similar results. Data of one repeat is shown here. (c) The MFI of EL4 cells (PD-1⁺) and B16 cells (PD-1⁻) after the cells were incubated with Alexa Fluor 647-labeled α PD-1-

ABD-PE or Alexa Fluor 647-labeled ABD-PE at 4°C for 30 minutes. The cells were collected from NOD mice. The MFI means and their SDs are indicated (N=3 biologically independent samples; unpaired two-sided t-test). **(d)** A dose-response binding study of α PD-1-ABD-PE and α PD-1 (full IgG) with EL4 cells. 1 million EL4 cells was used for the assay at 4°C for 30 minutes. The binding affinity (Kd) were derived from sigmoidal dose-response analysis of the curves (N=6). **(e)** The MFI of EL4 and B16 cells after the cells were incubated with the labeled α PD-1-ABD-PE or the labeled ABD-PE at 37°C for 30 minutes. The MFI means and their SDs are indicated (N=3 biologically independent samples; unpaired two-sided t-test). **(f)** The MFI of PD-1⁺ and PD-1⁻ primary T cells after the cells were incubated with the labeled α PD-1-ABD-PE or the labeled ABD-PE at 4°C for 30 minutes. The cells were collected from NOD mice. The MFI means and their SDs are indicated (N=6 biologically independent samples; unpaired two-sided t-test). **(g)** The MFI of PD-1⁺ and PD-1⁻ primary B cells after the cells were incubated with the labeled α PD-1-ABD-PE or the labeled ABD-PE at 4°C for 30 minutes. The cells were collected from NOD mice. The MFI means and their SDs are indicated (N=6 biologically independent samples; unpaired two-sided t-test). **(h)** The MFI of PD-1⁺ and PD-1⁻ primary T cells after the cells were incubated with the labeled α PD-1-ABD-PE or the labeled ABD-PE at 37°C for 30 minutes. The cells were collected from NOD mice. The MFI means and their SDs are indicated (N=6). **(i)** The MFI of PD-1⁺ and PD-1⁻ primary B cells after the cells were incubated with the labeled α PD-1-ABD-PE or the labeled ABD-PE at 37°C for 30 minutes. The cells were collected from NOD mice. The MFI means and their SDs are indicated (N=6 biologically independent samples; unpaired two-sided t-test).

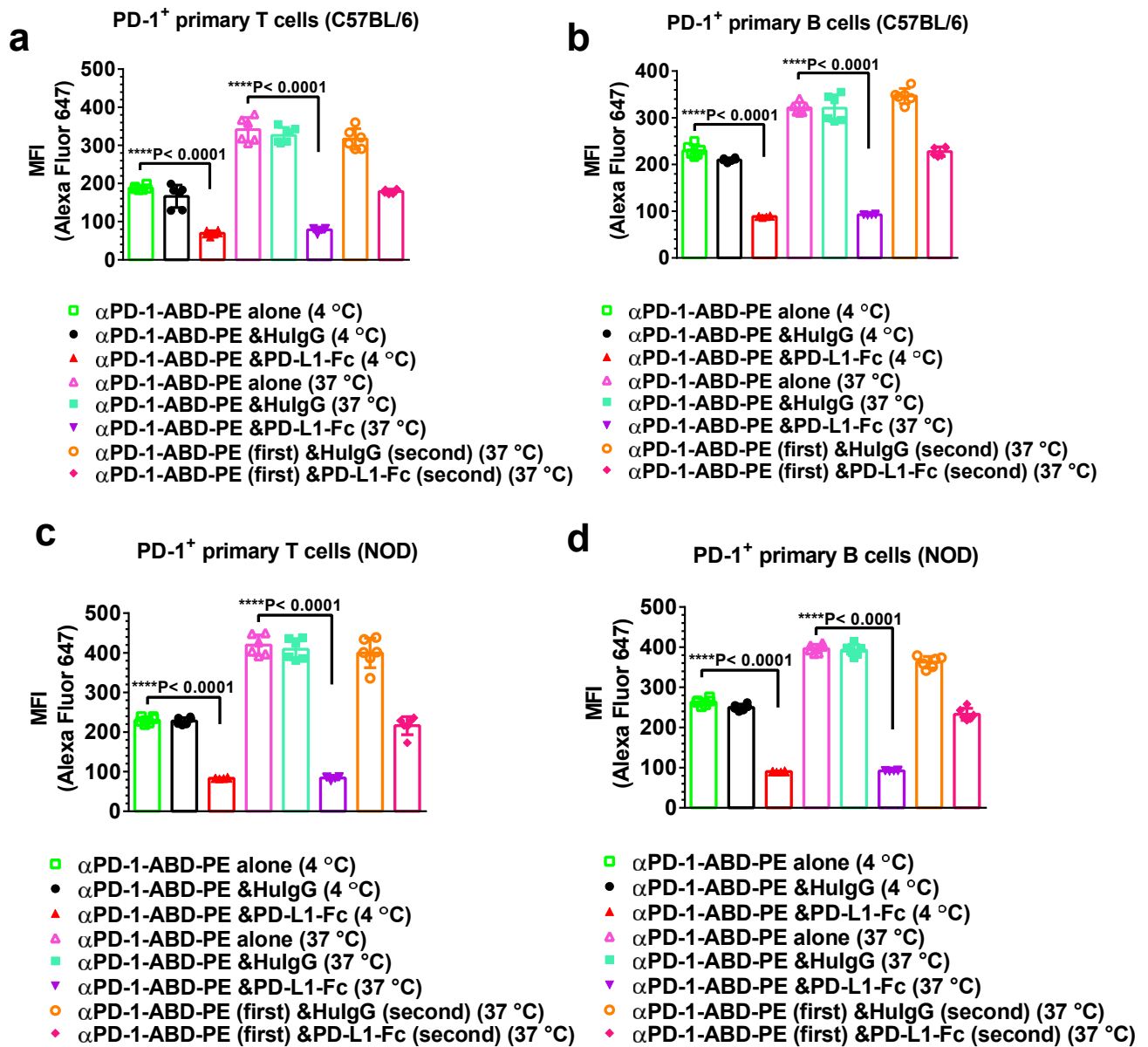


Figure S3. The selective binding and internalization of α PD-1-ABD-PE to PD-1⁺ cells are mediated by PD-1.

(a-d) The MFI of PD-1⁺ primary T cells (**a**, **c**) and PD-1⁺ primary B cells (**b**, **d**) after the cells were incubated with Alexa Fluor 647-labeled α PD-1-ABD-PE under the conditions noted in the figure. The MFI means and their SDs are indicated (N=6 biologically independent samples; unpaired two-sided t-

test). Cells of “a” and “b” were collected from C57BL/6 mice; cells of “c” and “d” were collected from NOD mice.

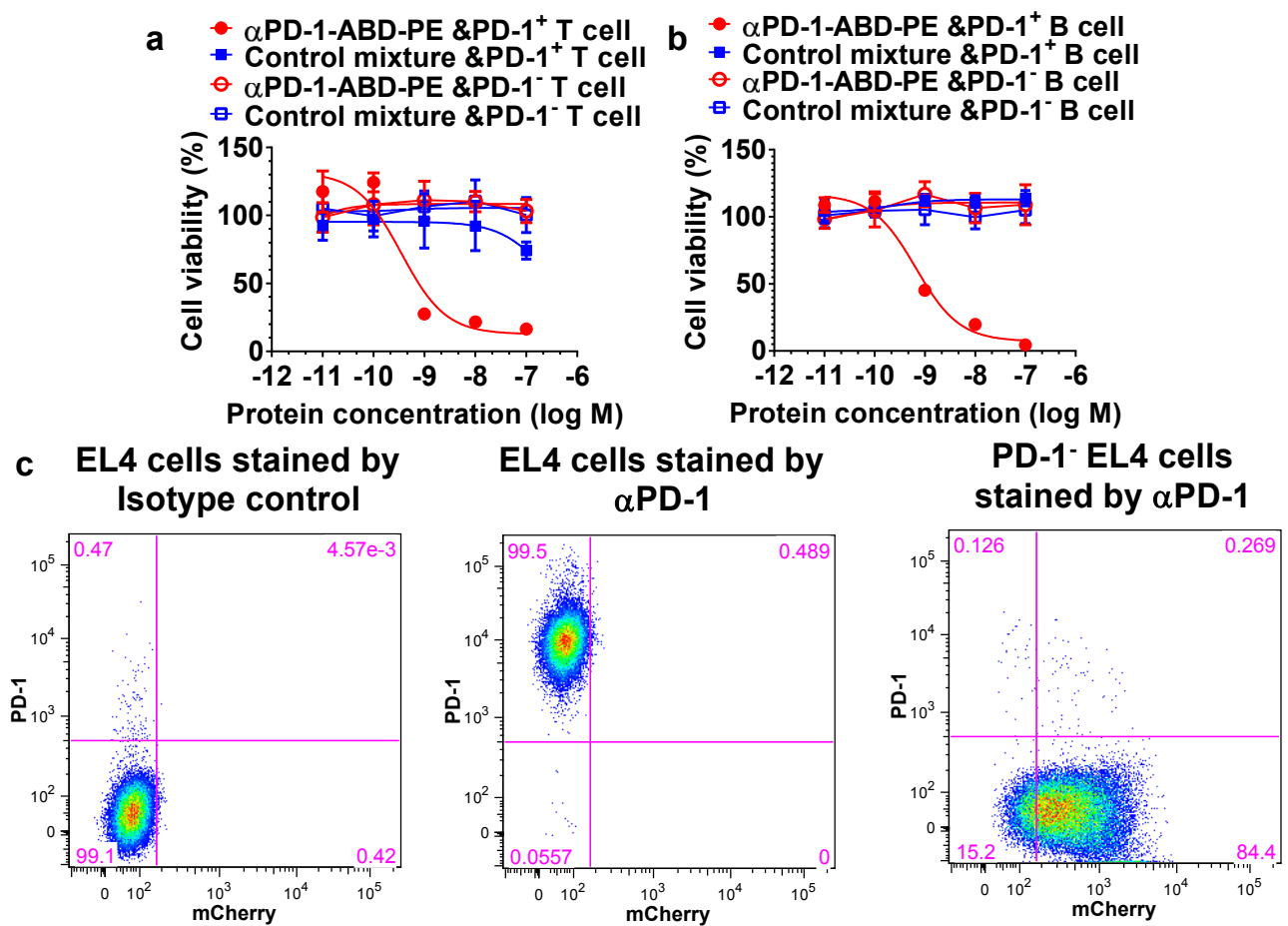
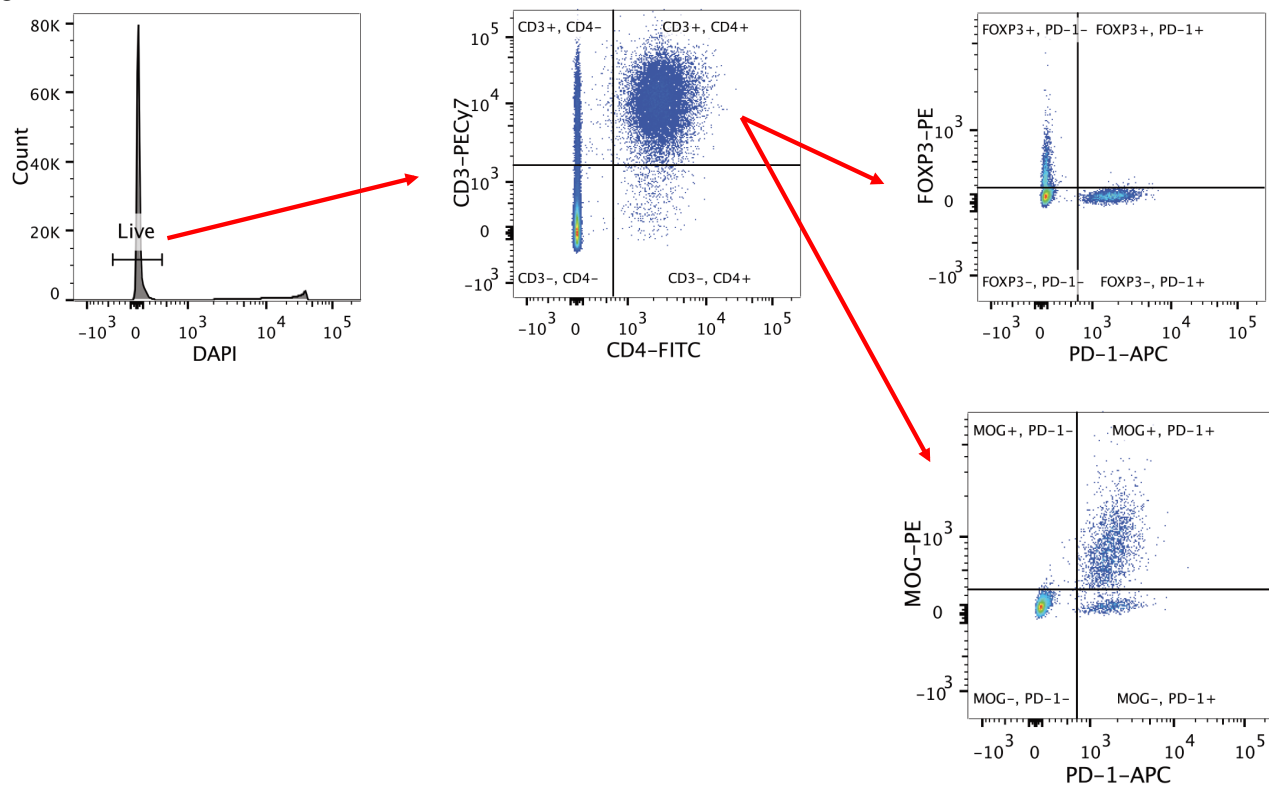


Figure S4. α PD-1-ABD-PE possesses selective toxicity to primary PD-1⁺ lymphocytes *in vitro*.

(a-b) The relative viability of PD-1⁺ and PD-1⁻ primary T cells **(a)** and B cells **(b)** after they were incubated with α PD-1-ABD-PE or a control mixture of α PD-1 and ABD-PE for 72 hours. The mean viabilities and their SDs at different concentrations of α PD-1-ABD-PE and the control mixture were shown. The viability data of PD-1⁺ primary cells after the α PD-1-ABD-PE treatment were fitted to a sigmoidal dose-response model (N=6 biologically independent samples; unpaired two-sided t-test) and the IC₅₀ was obtained through the fitting. The cells were collected from NOD mice. **(c)** Representative scatterplots showing the PD-1 expression on EL4 and PD-1⁻ EL4 cells. mCherry is the transfection marker. Left: EL4 cells stained with isotype control; middle: EL4 cells stained with α PD-1 (Clone:

RMP1-30); right: PD-1- EL4 cell stained with α PD-1. Studies described in panel were repeated at least three times with similar results.

a



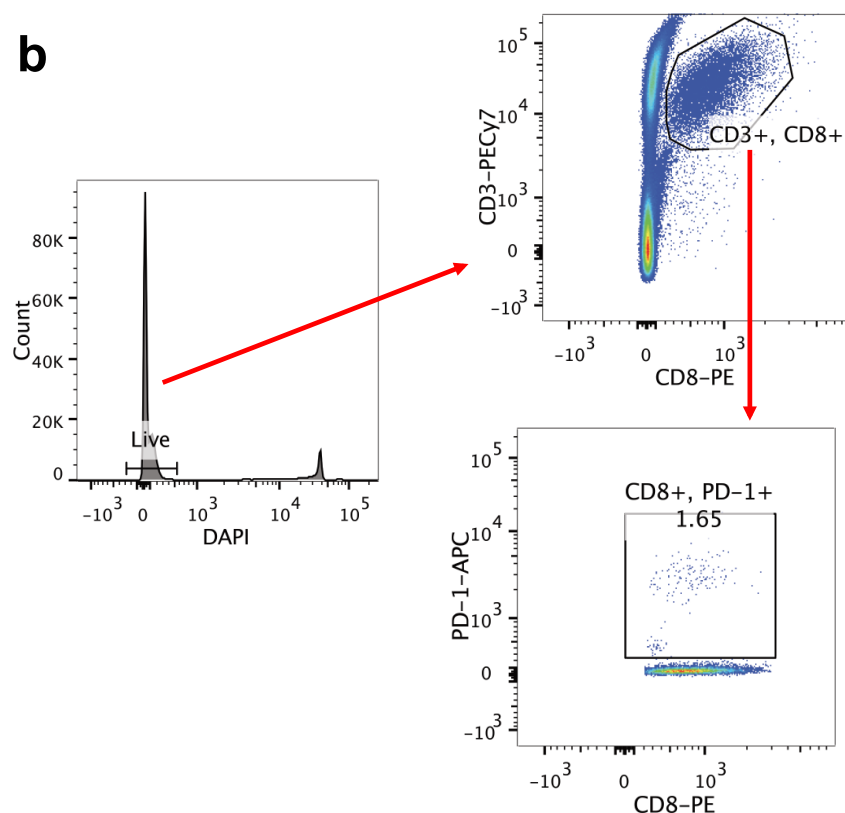
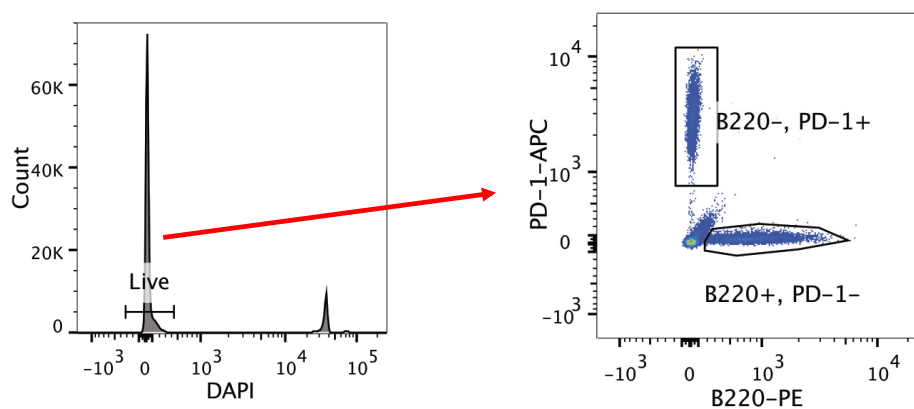
b**c**

Figure S5. Gating strategies used to quantify PD-1+ CD4 T cells, Tregs, MOG₃₈₋₄₉-specific CD4 T cells.

(a), PD-1+ CD8 T cells (b), and PD-1+ B220 cells (c) by flowcytometry. Dead cells were first stained by 3 μ M DAPI for 10 min, and then washed 3 times by a centrifugation (300 g for 5 minutes) to remove free DAPI. Then, cells were stained for markers indicated in the figures by antibodies.

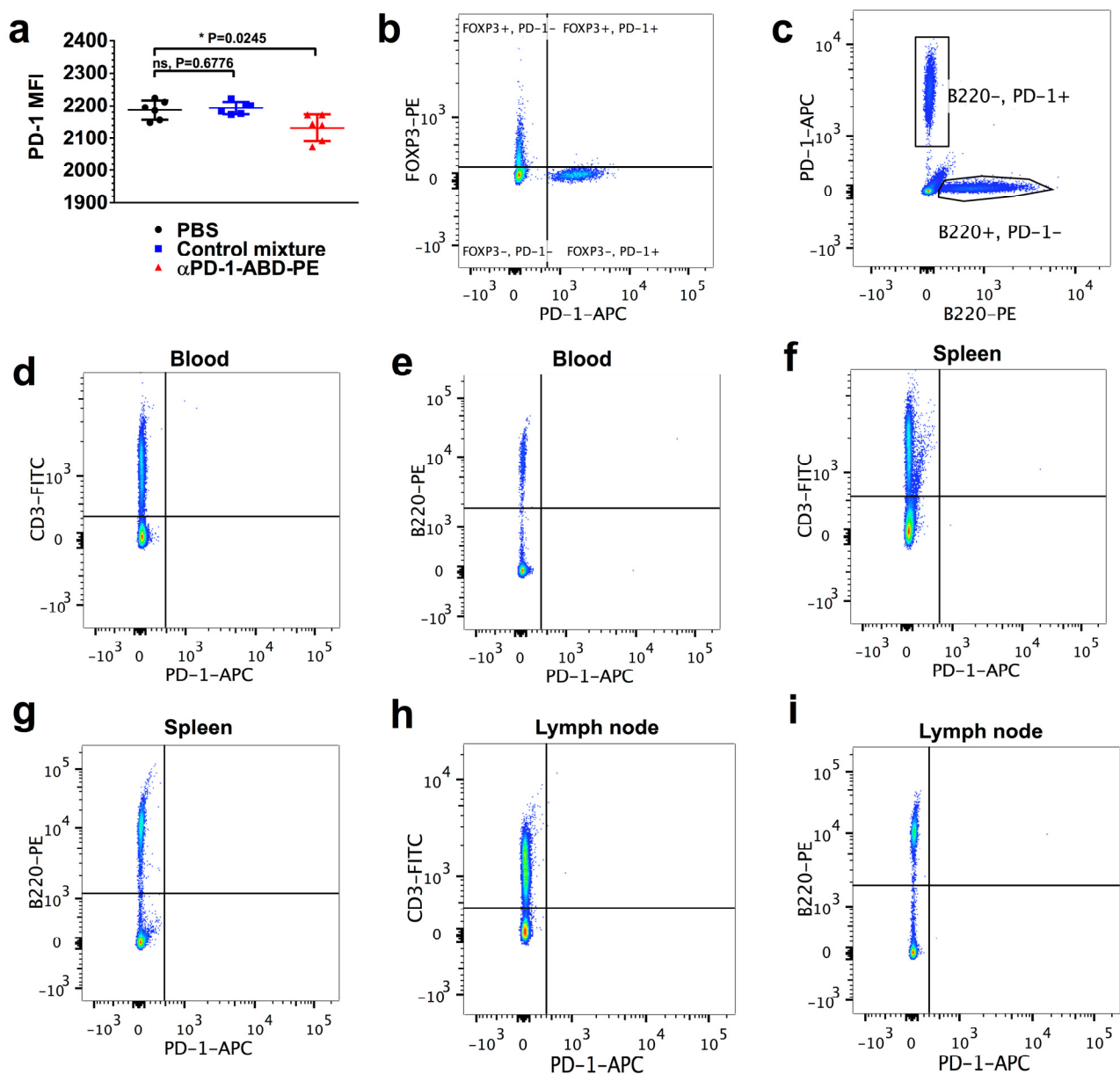


Figure S6. Analysis of immune cells in NOD mice after the α PD-1-ABD-PE treatment.

(a) The MFI (PD-1 expression) of PD-1⁺ cells in pancreases of 18-week old NOD mice after these mice were treated with one dose of α PD-1-ABD-PE, a control mixture of α PD-1 and ABD-PE, or PBS. Each dot represents the MFI of the cells from a single mouse. The MFI means and their SDs are indicated. (N=6 mice; unpaired two-sided t-test). **(b-c)** Representative scatter plots for Tregs **(b)** and B cells **(c)** in

a pancreas that was collected from the NOD mice treated with PBS. The cells were stained with α PD-1 to show PD-1⁺ populations. There was no PD-1⁺ Treg or PD-1⁺ B cell population in the pancreases. The plots represent 6 mice that were treated with PBS. Results for the mice treated with α PD-1-ABD-PE and the control mixture were the same. **(d-i)**. Representative scatter plots for T cells **(d, f, h)** and B cells **(e, g, i)** in blood **(d, e)**, spleens **(f, g)**, and lymph nodes **(h, i)** that was collected from NOD mice treated with PBS. The cells were stained with α PD-1 to reveal PD-1⁺ populations. There was no PD-1⁺ T or PD-1⁺ B cell population in these samples. The plots represent 6 mice that were treated with PBS. Results for the mice treated with α PD-1-ABD-PE and the control mixture were the same.

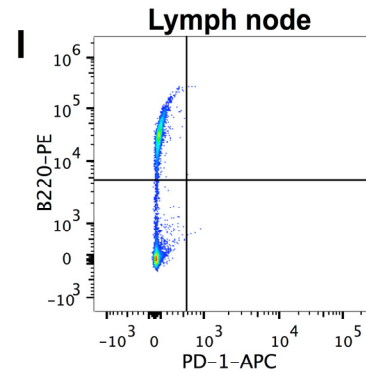
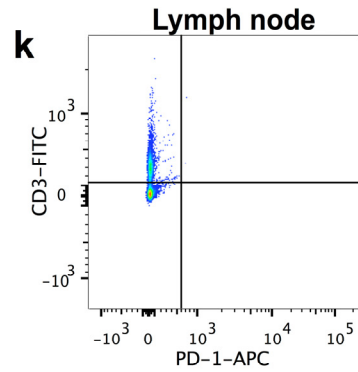
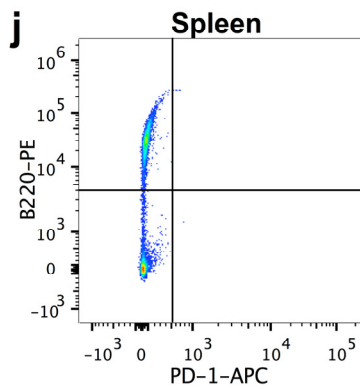
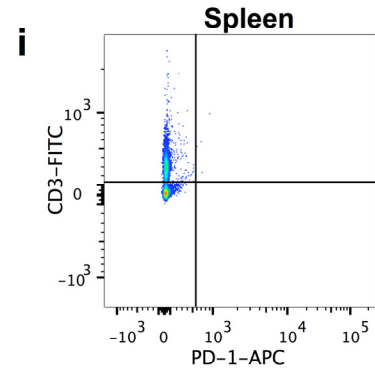
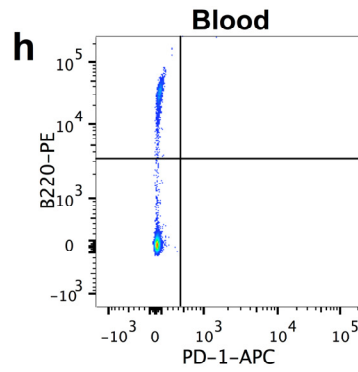
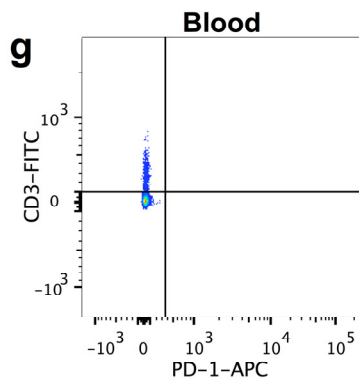
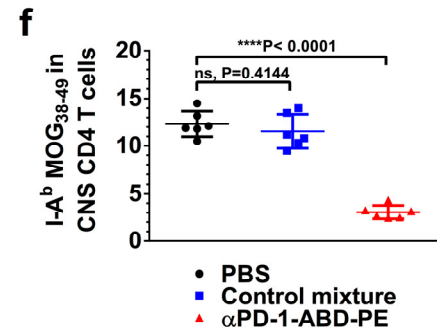
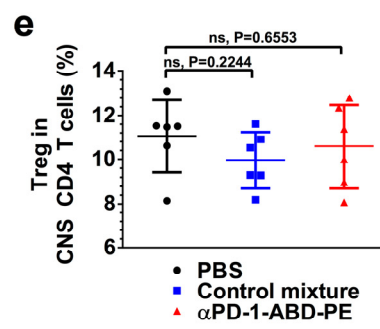
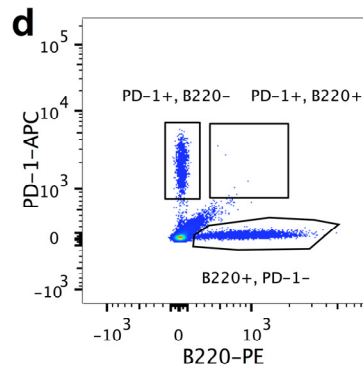
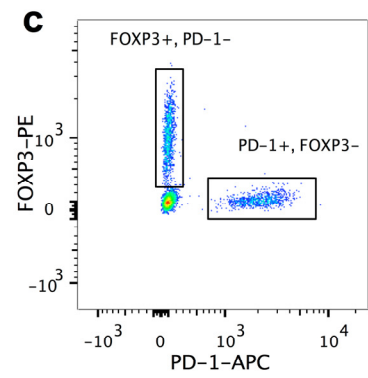
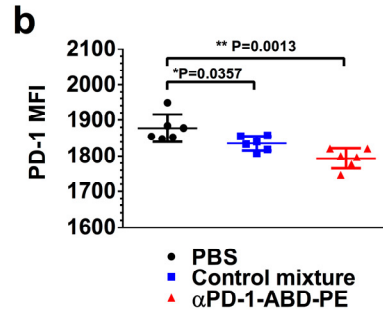
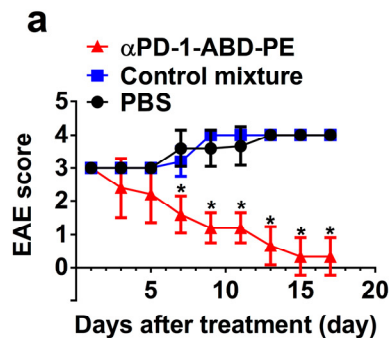


Figure S7. Analysis of immune cells in mice with EAE after the α PD-1-ABD-PE treatment.

(a) The EAE score changes of mice that were treated with one dose of α PD-1-ABD-PE, a control mixture of α PD-1 and ABD-PE, or PBS. The shown data are mean EAE clinical scores and their standard deviations at each observation time point after the induction of EAE. (N=5 mice; *P $\leq$ 0.01; unpaired two-sided t-test). The X-axis represents the number of days after treatment started for an individual mouse. **(b)** The MFI (PD-1 expression) of PD-1⁺ cells in the CNS of the mice that were treated with one dose of α PD-1-ABD-PE, a control mixture of α PD-1 and ABD-PE, or PBS. Each dot represents the MFI of the cells from a single mouse. The MFI means and their SDs are indicated. (N=6 mice; unpaired two-sided t-test). **(c-d)**. Representative scatter plots for Tregs **(c)** and B cells **(d)** in the CNS of mice with EAE and treated with PBS. The cells were stained with α PD-1 to show PD-1⁺ populations. There was no PD-1⁺ Treg or PD-1⁺ B cell population in the CNS. The plots represent 6 mice that were treated with PBS. Results for the mice treated with α PD-1-ABD-PE and the control mixture were the same. **(e-f)** The fraction of Tregs cells **(d)** and MOG₃₈₋₄₉-specific CD4 T cells **(e)** in the collected mononuclear cells from the CNS of the mice that were treated with one dose of α PD-1-ABD-PE, a control mixture of α PD-1 and ABD-PE, or PBS. Each dot represents the fraction result of a single mouse. The fraction means and their SDs are indicated. (N=6 mice; unpaired two-sided t-test). **(g-l)** Representative scatter plots for T cells **(g, i, k)** and B cells **(h, j, l)** in blood **(g,h)**, spleens **(i, j)**, and lymph nodes **(k,l)** that was collected from the mice with EAE and treated with PBS. The cells were stained with α PD-1 to reveal PD-1⁺ populations. There was no PD-1⁺ T or PD-1⁺ B cell population in these samples. The plots represent 6 mice that were treated with PBS. Results for the mice treated with α PD-1-ABD-PE and the control mixture were the same.

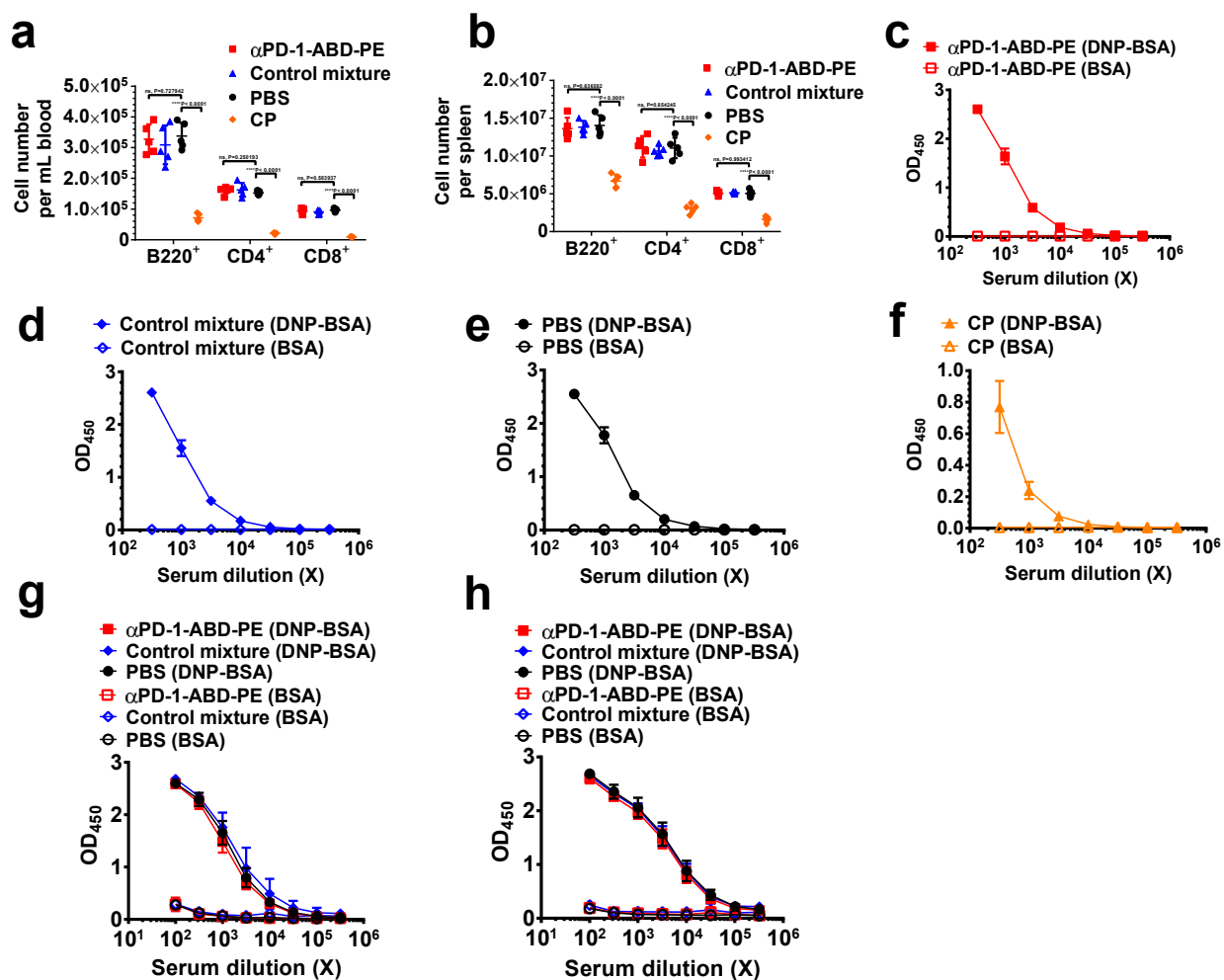


Figure S8. Analysis of normal adaptive immunity in mice after the α PD-1-ABD-PE treatment.

(a-b) B220⁺, CD4⁺, CD8⁺ cell numbers in blood (a) and spleens (b) in the NOD mice that were treated with one dose of α PD-1-ABD-PE, a control mixture of α PD-1 and ABD-PE, PBS, or CP. The number means and their SDs are indicated (N=6 mice; unpaired two-sided t-test). (c-f) The data curves in Figure 6c are separated into four subfigures based on the treatments. The subfigures are prepared to show curve details that are hidden due the overlap of these curves (N=6 mice). (g-h) ELISA results of the anti-DNP humoral responses in the C57BL/6 mice (g) and the NOD mice (h) that that were pre-treated with five doses of α PD-1-ABD-PE, a control mixture of α PD-1 and ABD-PE, or PBS. The results were measured by OD₄₅₀ after a background OD₅₇₀ subtraction. The mean $\pm$ SD of OD₄₅₀ for the serum

samples at indicated dilutions were shown. The same samples were loaded into both DNP-BSA-coated and BSA-coated (control) ELISA plates, separately. The materials used to coat the plates are written in the parentheses (N=5 mice; unpaired two-sided t-test).