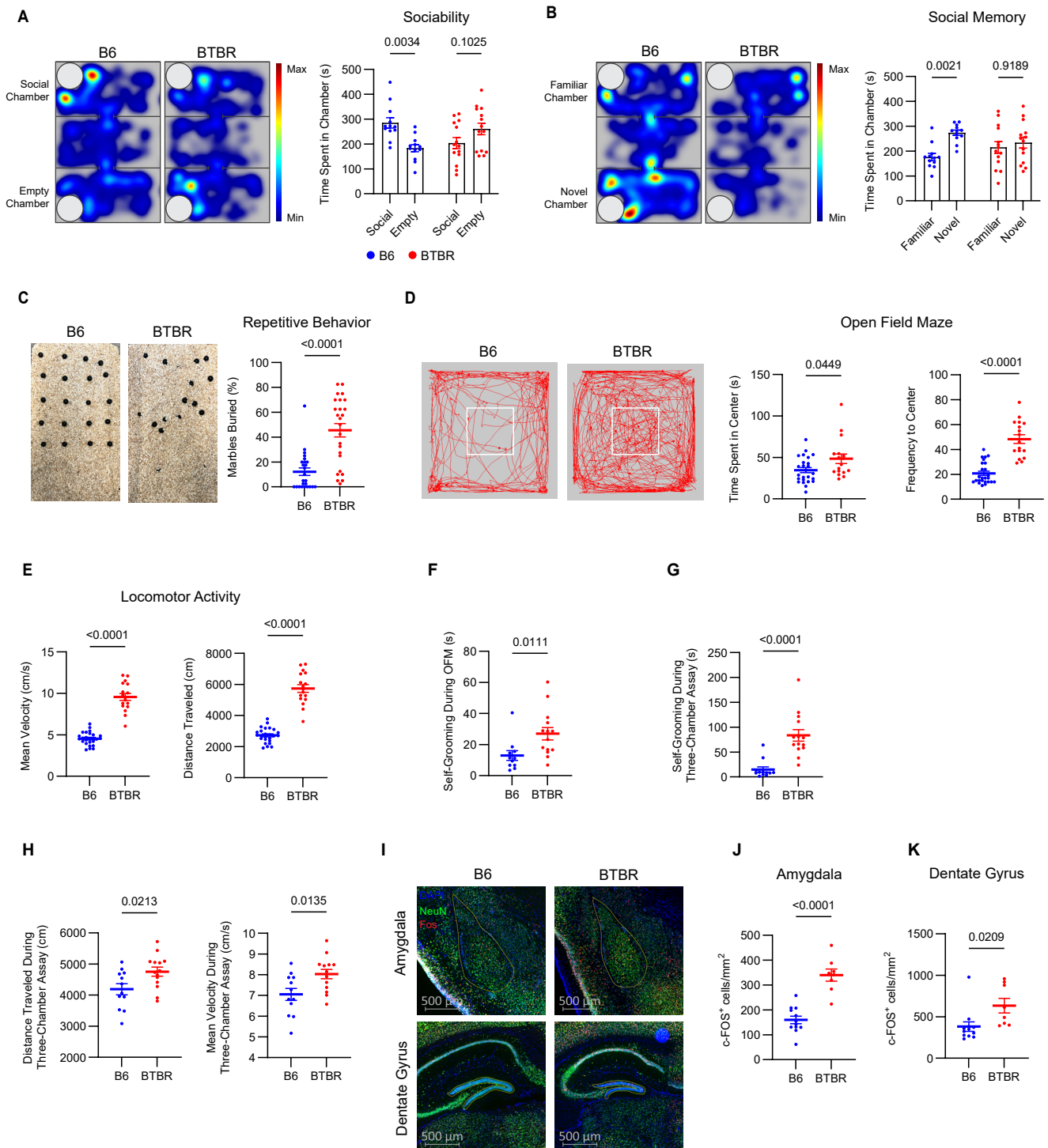
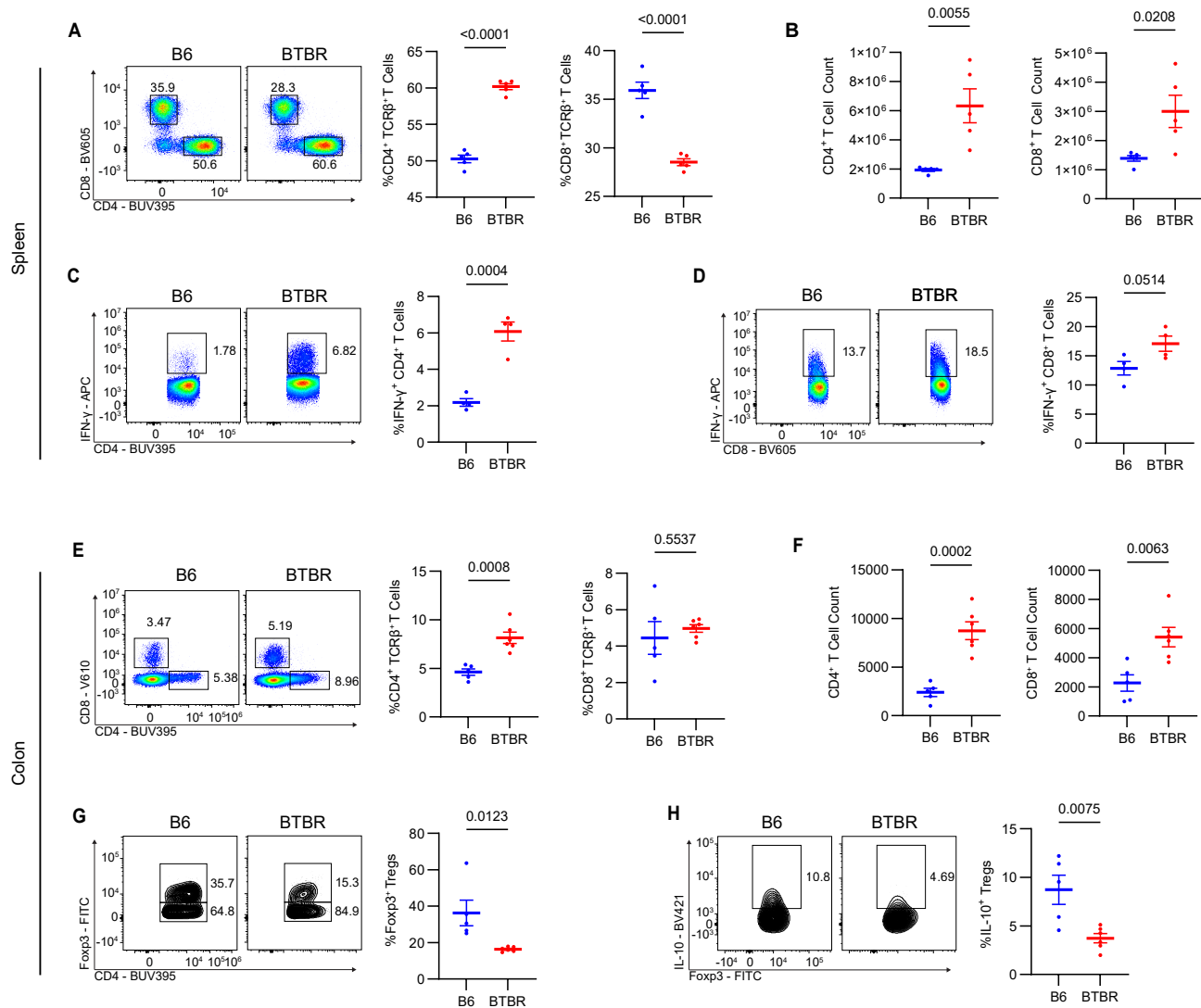
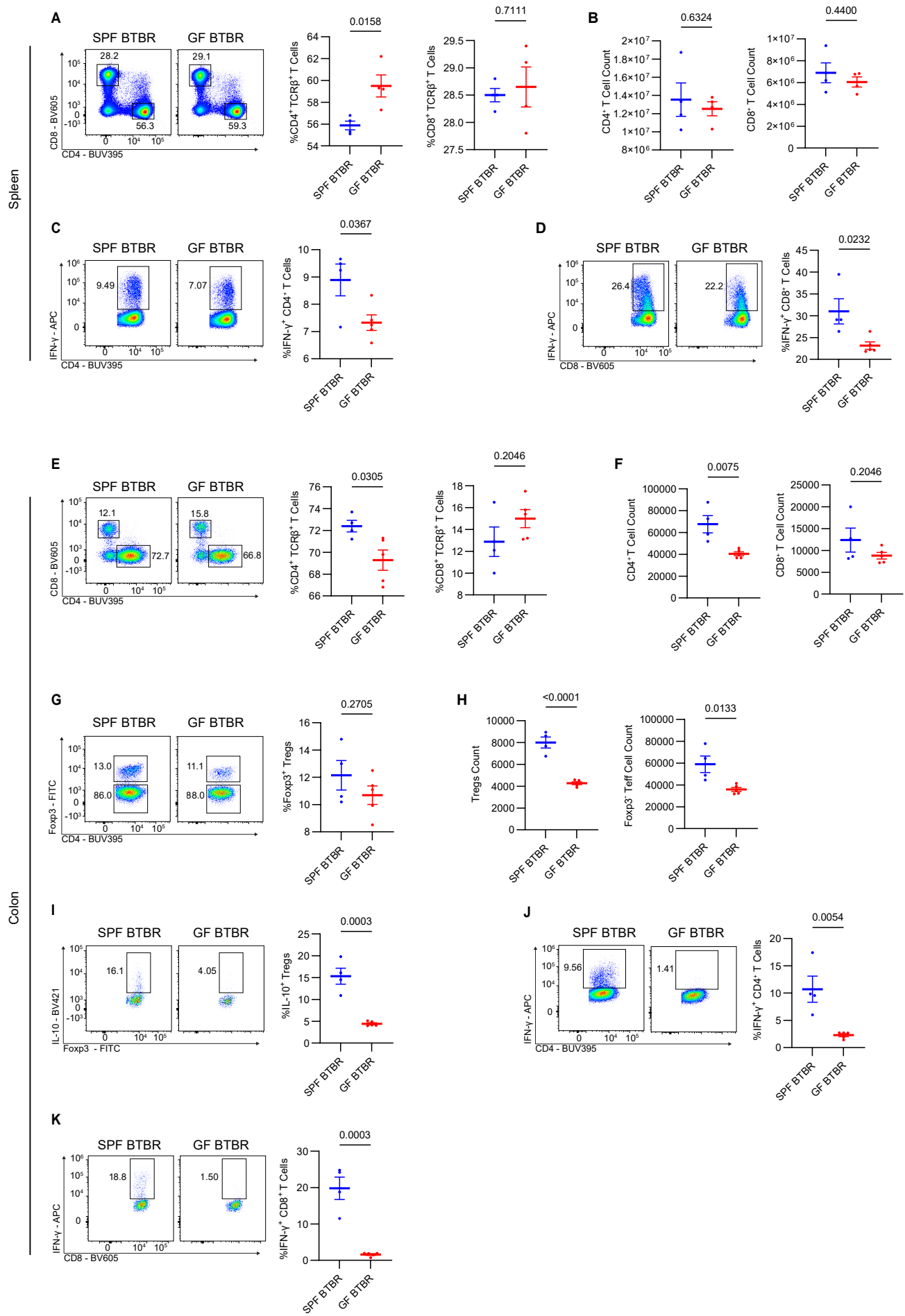


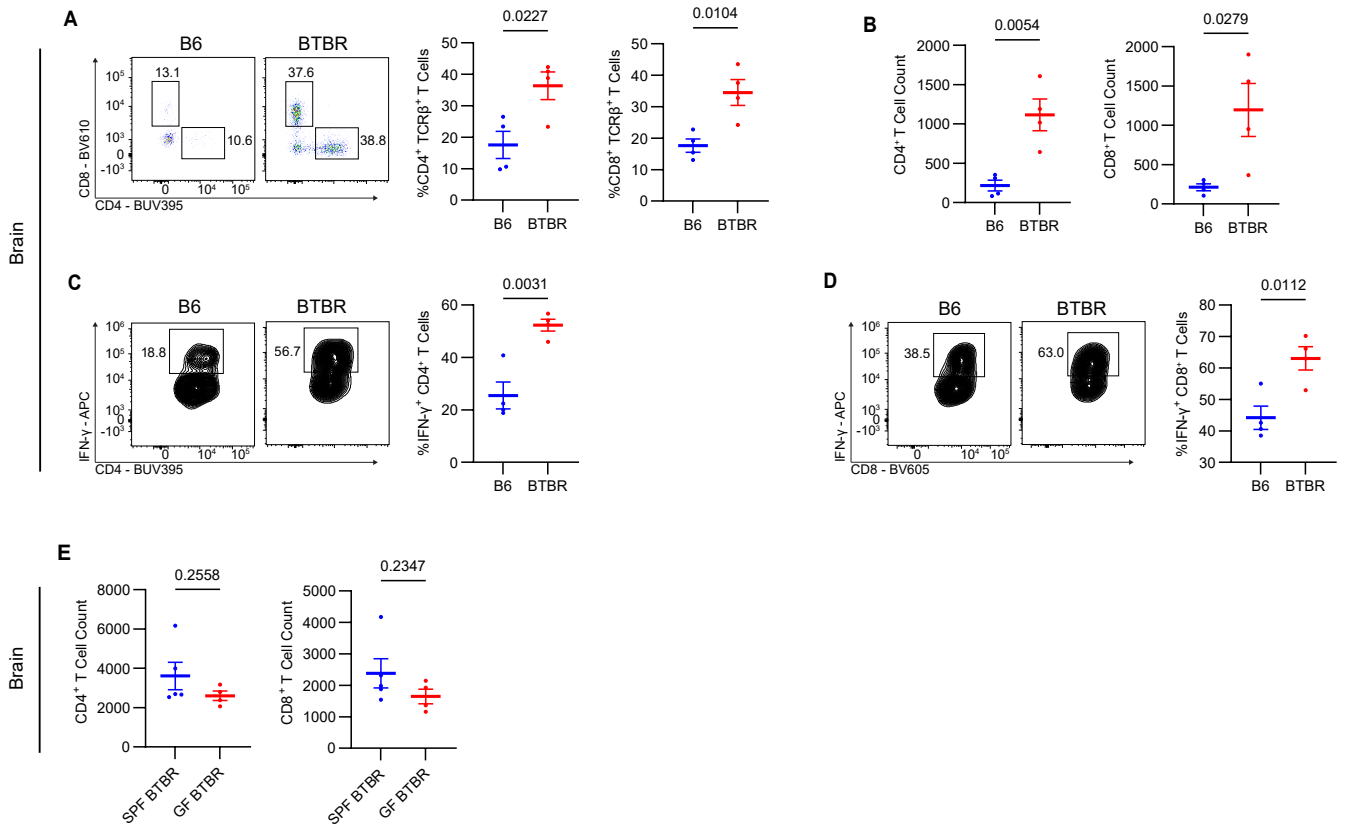


Supplementary Fig. 1 | BTBR mice display ASD-associated behaviors. **A** Representative heat maps and time spent in either the social or empty chambers during the three-chamber sociability test. B6 $n = 12$, BTBR $n = 14$. P value from two-way ANOVA, $F_{1,48} = 14.41$. **B** Representative heat maps and time spent in either the familiar or novel chambers during the three-chamber social novelty test. B6 $n = 12$, BTBR $n = 14$. P value from two-way ANOVA, $F_{1,48} = 4.197$. **C** Representative images and percent of marbles buried during the marble burying test. $n = 25$. $P = 0.000003$. P value from two-tailed Welch's t -test, $t(35.10) = 3.619$. **D** Representative traces, time spent in the center zone, and frequency to the center zone during the open field maze. B6 $n = 25$, BTBR $n = 17$. Frequency to center $P = 0.0000003$. P value from two-tailed Welch's t -test, $t(25.30) = 2.110$, $t(23.75) = 6.983$, respectively. **E** Mean velocity and distance traveled of mice during the OFM. B6 $n = 25$, BTBR $n = 17$. Mean velocity $P = 0.0000000003$, Distance traveled $P = 0.0000000003$. P value from two-tailed Welch's t -test, $t(20.67) = 11.31$, $t(17.08) = 5.360$. **F** Time spent self-grooming during OFM. B6 $n = 11$, BTBR $n = 14$. P value from two-tailed Welch's t -test, $t(20.68) = 11.32$. **G** Time spent self-grooming during the three-chamber assay. B6 $n = 12$, BTBR $n = 14$. $P = 0.000004$. P value from two-tailed Welch's t -test, $t(17.89) = 5.370$. **H** Distance traveled and mean velocity during the three-chamber assay, respectively. B6 $n = 12$, BTBR $n = 14$. P value from two-tailed Welch's t -test, $t(22.19) = 2.477$, $t(21.48) = 2.690$, respectively. **I, J, K** Representative immunofluorescence staining and number of c-FOS⁺ neurons per area in the amygdala ($t(18) = 6.605$. B6 $n = 12$ sections, 5 mice, BTBR $n = 8$ sections, 3 mice) and dentate gyrus of the hippocampus (two-tailed Student's t -test, $t(18) = 2.531$. B6 $n = 12$ sections, 5 mice, BTBR $n = 8$ sections, 3 mice). $P = 0.000003$ (**J**). P values from two-tailed Student's t -test. All data represented as mean $\pm$ SEM. Source data are provided as Source data file.

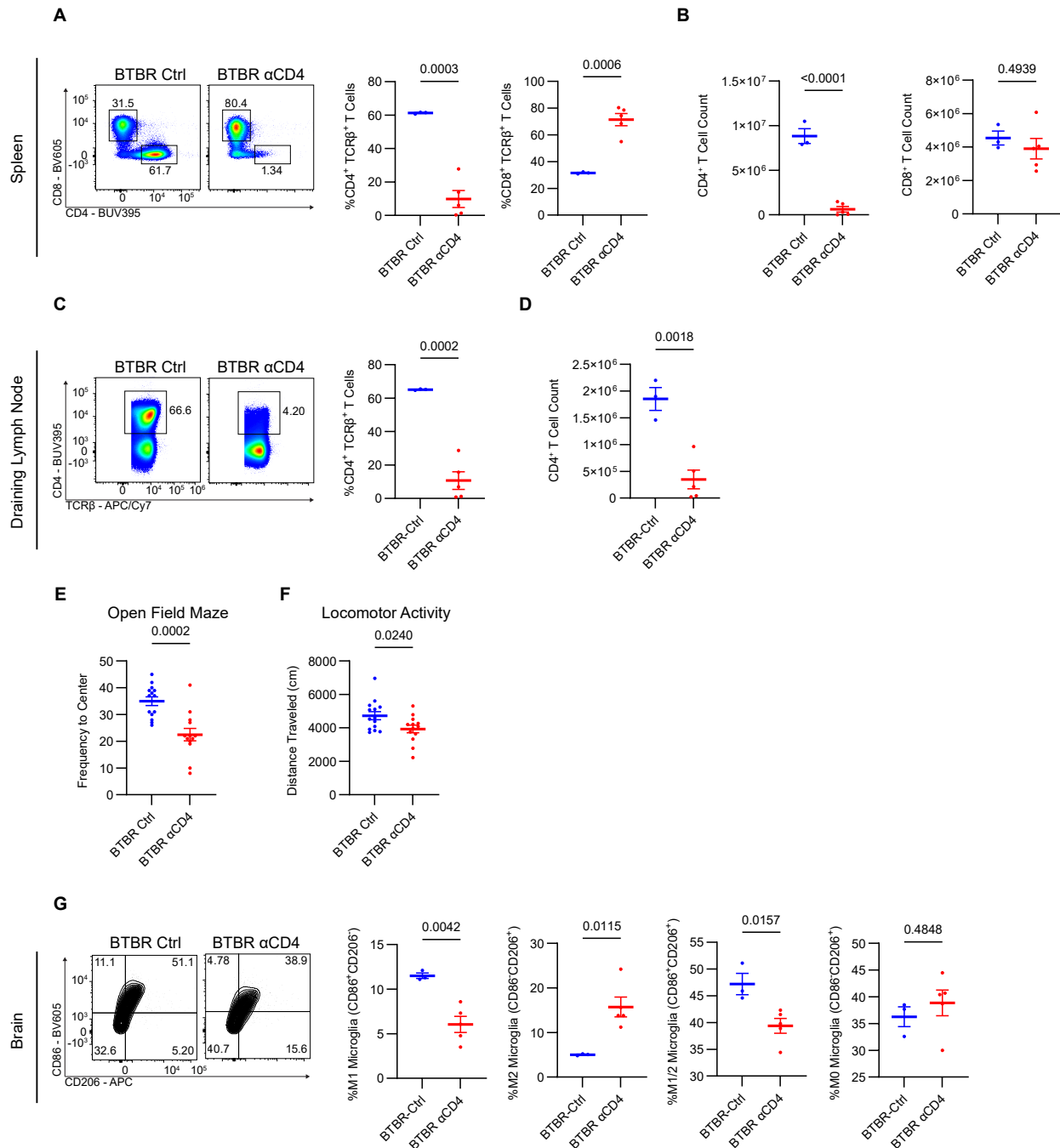




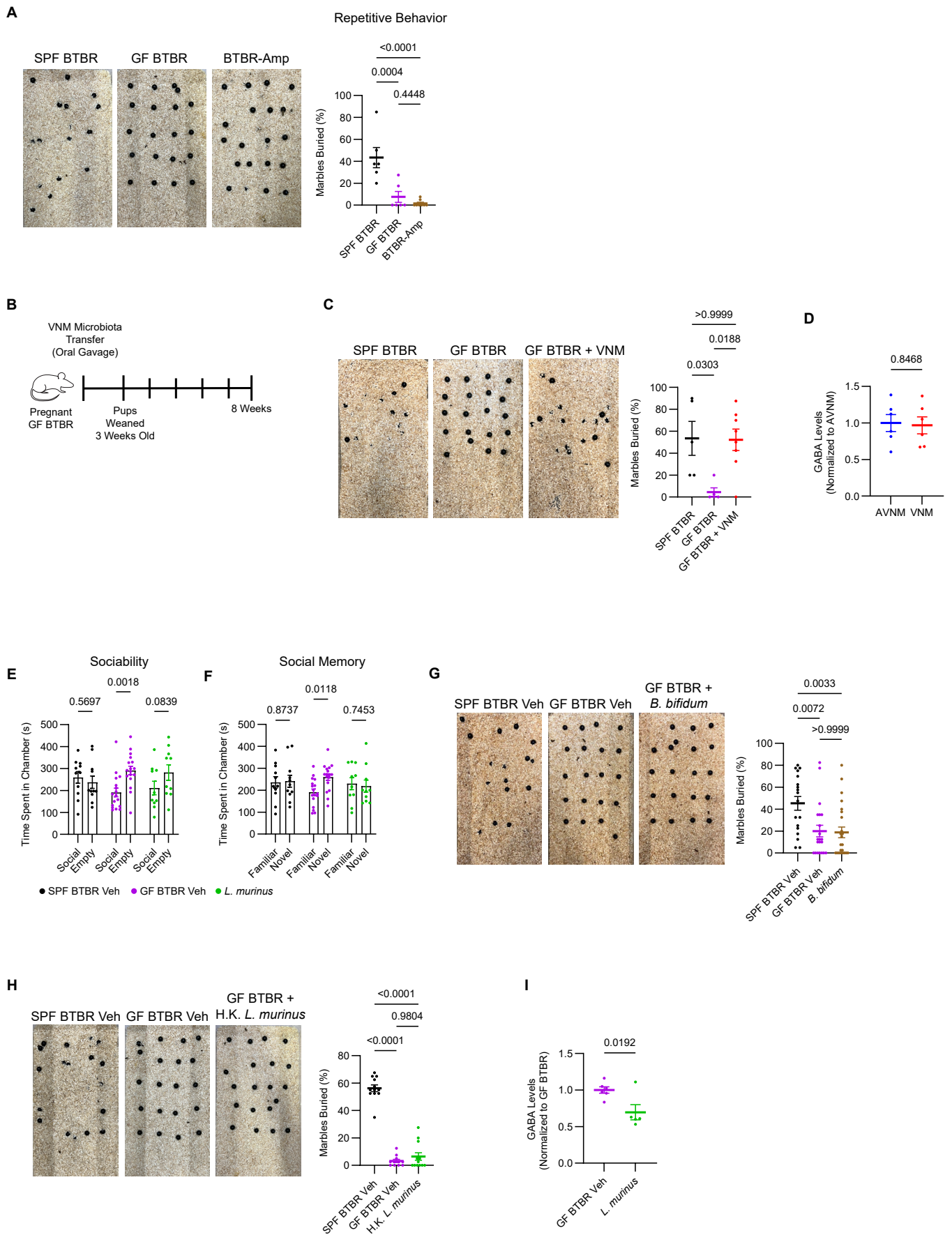
Supplementary Fig. 3 | Microbiota controls adaptive immune activation in BTBR mice. **A** Representative flow cytometry plots and frequencies of CD4⁺ or CD8⁺ T cells gated from TCRβ⁺ cells in the spleen. *n* = 4 mice, *P* values from two-tailed Student's *t*-test, *t*(6) = 3.329, *t*(6) = 0.3884, respectively. **B** Cell number of CD4⁺ or CD8⁺ T cells in the spleen from flow cytometry analysis. *n* = 4 mice, *P* values from two-tailed Student's *t*-test, *t*(6) = 0.5038, *t*(6) = 0.8267. **C, D** Representative flow cytometry plots and frequencies of IFN-γ⁺ Foxp3⁻ CD4⁺ (**C**) or CD8⁺ (**D**) T cells in the spleen. SPF BTBR *n* = 4 mice, GF BTBR *n* = 5 mice, *P* values from two-tailed Student's *t*-test, *t*(7) = 2.576, *t*(7) = 2.892, respectively. **E** Representative flow cytometry plots and frequencies of CD4⁺ or CD8⁺ T cells gated from TCRβ⁺ cells in the colonic lamina propria (cLP). SPF BTBR *n* = 4 mice, GF BTBR *n* = 5 mice. *P* values from two-tailed Student's *t*-test, *t*(7) = 2.703, *t*(7) = 1.399. **F** Cell number of CD4⁺ or CD8⁺ T cells in the cLP from flow cytometry analysis. SPF BTBR *n* = 4 mice, GF BTBR *n* = 5 mice. *P* values from two-tailed Student's *t*-test, *t*(7) = 3.720, *t*(7) = 1.399. **G** Representative flow cytometry plots and frequency of Foxp3⁺ Tregs in the cLP. SPF BTBR *n* = 4 mice, GF BTBR *n* = 5 mice. *P* value from two-tailed Student's *t*-test, *t*(7) = 1.196. **H** Cell number of Foxp3⁺ Tregs or Foxp3⁻ CD4⁺ Teffs in the cLP from flow cytometry analysis. SPF BTBR *n* = 4 mice, GF BTBR *n* = 5 mice. *P* = 0.00008, from two-tailed Student's *t*-test, *t*(7) = 8.035, *t*(7) = 3.291. **I** Representative flow cytometry plots and frequency of IL-10⁺ Tregs in the cLP. SPF BTBR *n* = 4 mice, GF BTBR *n* = 5 mice. *P* value from two-tailed Student's *t*-test, *t*(7) = 6.628. **J, K** Representative flow cytometry plots and frequencies of IFN-γ⁺ Foxp3⁻ CD4⁺ (**J**) or CD8⁺ (**K**) T cells in the cLP. *P* values from two-tailed Student's *t*-test, *t*(7) = 3.963, *t*(7) = 6.695. SPF BTBR *n* = 4 mice, GF BTBR *n* = 5 mice. Flow cytometry data from 2 independent experiments with minimum 3 mice. All data represented as mean ± SEM. Source data are provided as Source data file.



Supplementary Fig. 4 | BTBR mice have greater populations and activated brain-resident T cells. **A** Representative flow cytometry plots and percentages of CD4⁺ or CD8⁺ T cells gated from TCRβ⁺ CD45⁺ live cells within the whole brain parenchyma. B6 and BTBR $n = 4$ mice. P values from two-tailed Student's t -test, $t(6) = 3.045$ and $t(6) = 3.671$, respectively. **B** Absolute cell numbers of CD4⁺ or CD8⁺ T cells assessed by flow cytometry. $n = 4$ mice. P values from two-tailed Student's t -test, $t(6) = 4.253$ and $t(6) = 2.883$, respectively. **C**, **D** Representative flow cytometry plots and frequency of IFN-γ⁺ Foxp3⁻ CD4⁺ or IFN-γ⁺ CD8⁺ T cells in the brain parenchyma. $n = 4$ mice, P values from two-tailed Student's t -test, $t(6) = 4.760$ and $t(6) = 3.610$, respectively. **E** Absolute cell numbers of CD4⁺ or CD8⁺ T cells assessed by flow cytometry. SPF BTBR $n = 5$ mice, GF BTBR $n = 4$ mice. P values from two-tailed Student's t -test, $t(7) = 1.238$, $t(7) = 1.300$. Flow cytometry data from 2 independent experiments with minimum 3 mice. All data represented as mean ± SEM. Source data are provided as Source data file.

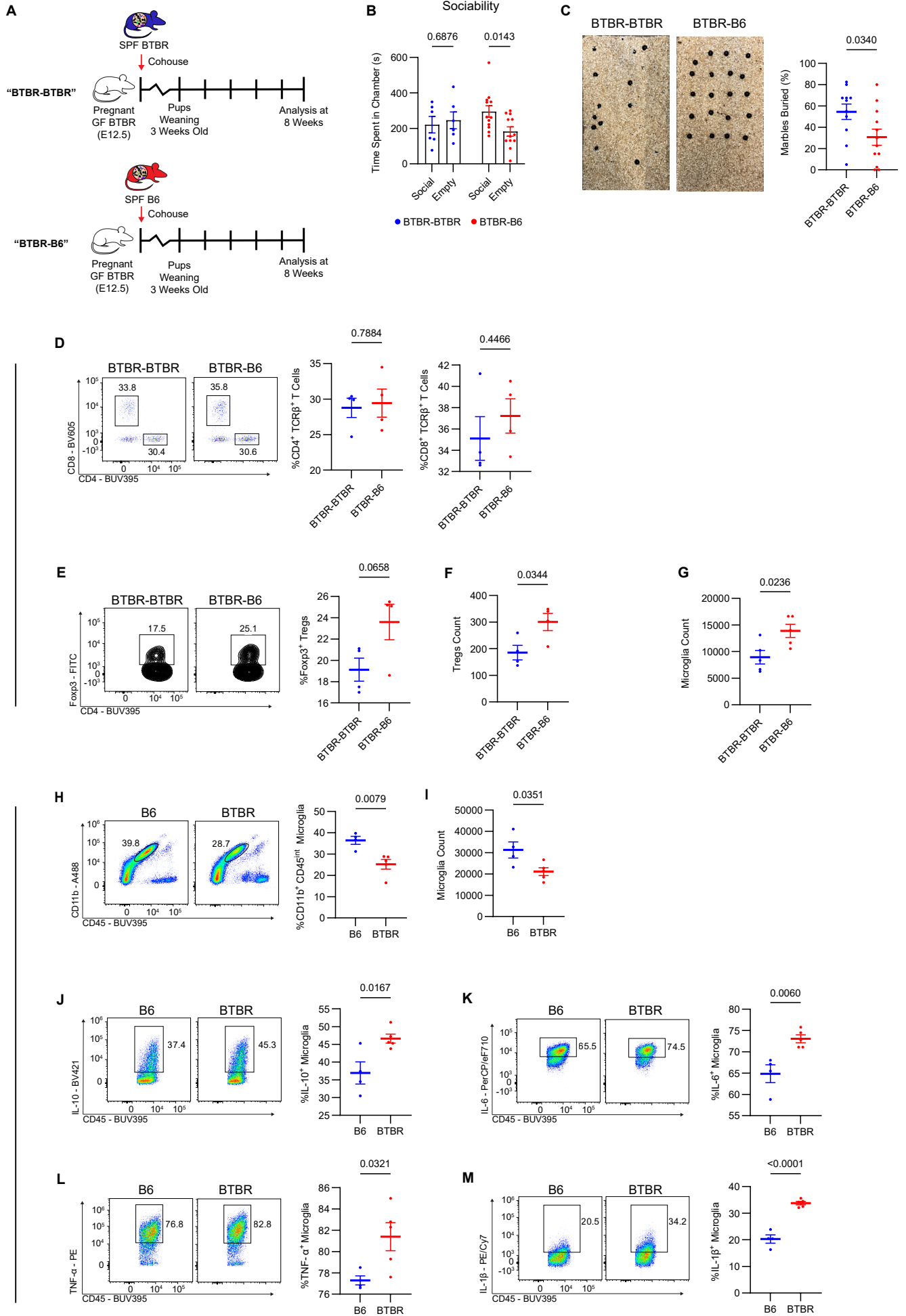


Supplementary Fig. 5 | CD4⁺ T cell depletion in BTBR mice. **A** Representative flow cytometry plots and frequencies of CD4⁺ or CD8⁺ T cells gated from TCRβ⁺ CD45⁺ live cells from the spleen. BTBR Ctrl *n* = 3 mice, BTBR αCD4 *n* = 5 mice. *P* values from two-tailed Student's *t*-test, *t*(6) = 7.58, *t*(6) = 6.514. **B** Absolute cell number of CD4⁺ or CD8⁺ T cells in the spleen from flow cytometry analysis. BTBR Ctrl *n* = 3 mice, BTBR αCD4 *n* = 5 mice. CD4⁺ T cell *P* = 0.00003. *P* values from two-tailed Student's *t*-test, *t*(6) = 11.15, *t*(6) = 0.7282. **C, D** Representative flow cytometry plots, frequency (**C**) and cell count (**D**) of CD4⁺ T cells in pooled draining lymph nodes. BTBR Ctrl *n* = 3 mice, BTBR αCD4 *n* = 5 mice. *P* values from two-tailed Student's *t*-test, *t*(6) = 7.92 (**C**), *t*(6) = 5.331 (**D**). **E, F** Frequency to the center zone and the distance traveled during the open field maze. BTBR Ctrl *n* = 14, BTBR αCD4 *n* = 13. *P* value from two-tailed Welch's *t*-test, *t*(21.61) = 4.409 and *t*(24.98) = 2.402, respectively. **G** Representative flow cytometry plots and frequencies of M1 (CD86⁺CD206⁻), M2 (CD86⁺CD206⁺), M1/2 (CD86⁺CD206⁺), or M0 (CD86⁻CD206⁻) microglia in the brain. BTBR Ctrl *n* = 3 mice, BTBR αCD4 *n* = 5 mice. *P* values from two-tailed Student's *t*-test, *t*(6) = 4.481, *t*(6) = 3.590, *t*(6) = 3.334, *t*(6) = 0.7443, respectively. Flow cytometry data are representative of at least 2 independent experiments with minimum 3 mice. All data represented as mean ± SEM. Source data are provided as Source data file.

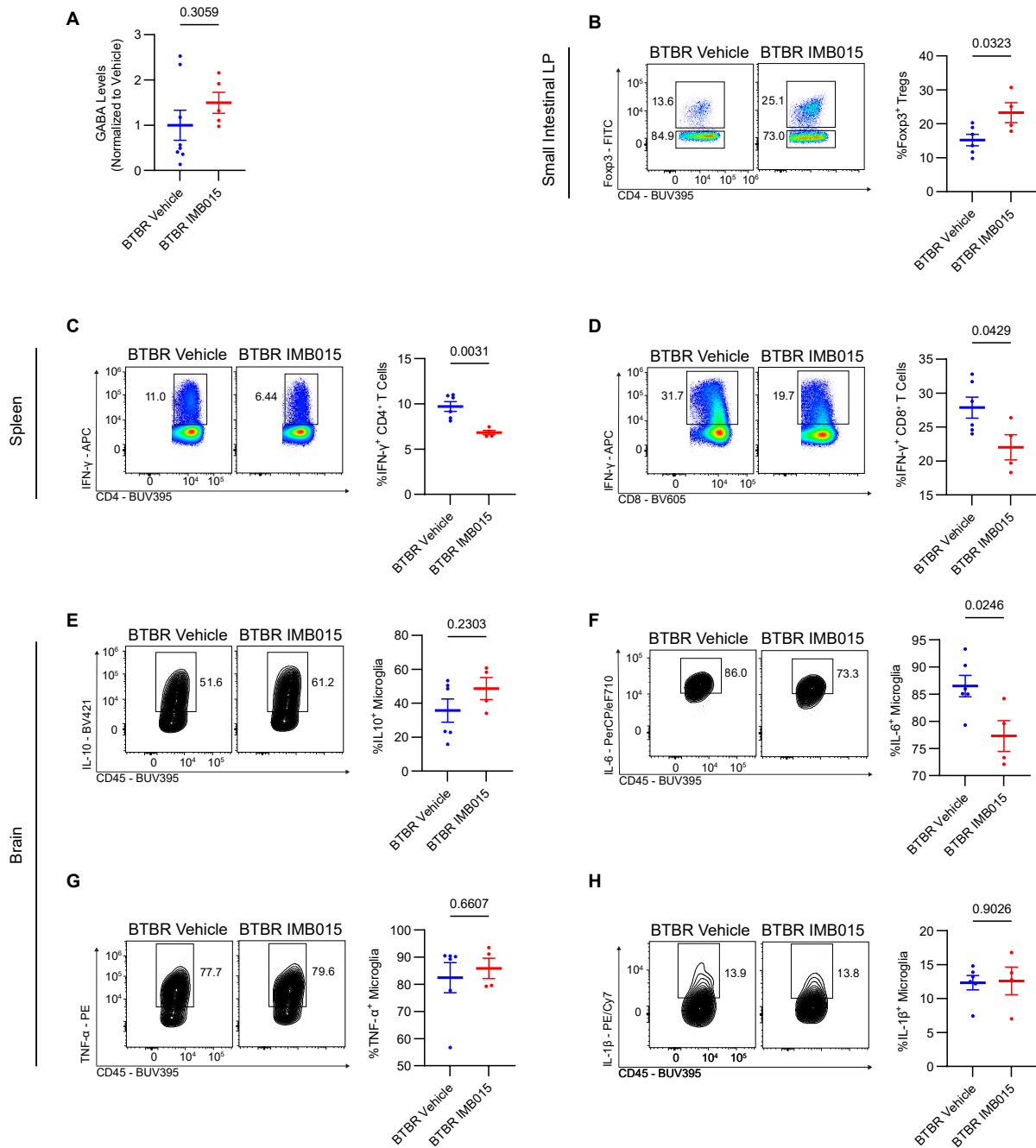


Supplementary Fig. 6 | ASD-associated microbiota recapitulates repetitive behavior in GF BTBR mice. A

Representative images and percent of marbles buried during the marble burying test. SPF BTBR $n = 6$, GF BTBR $n = 6$, Ampicillin-treated BTBR (BTBR-Amp) $n = 8$. SPF BTBR vs BTBR-Amp $P = 0.00004$. P value from one-way ANOVA, $F_{2,17} = 1.968$. **B** Feces from VNM mice were transplanted into pregnant GF BTBR mice by oral gavage. Offspring were analyzed at 8 weeks of age. **C** Representative images and percent of marbles buried during the marble burying test. SPF BTBR $n = 5$, GF BTBR $n = 5$, GF BTBR + VNM $n = 8$. P value from one-way ANOVA, $F_{2,15} = 6.049$. **D** Relative fecal levels of GABA as normalized to AVNM group. $n = 6$. P value from two-tailed Student's t -test, $t(10) = 0.1982$. **E** Time spent in the social or empty chamber during the three-chambers sociability assay. SPF BTBR Vehicle (Veh) $n = 11$, GF BTBR Vehicle (Veh) $n = 17$, *L. murinus* $n = 10$. P value from two-way ANOVA, $F_{2,70} = 3.148$. **F** Time spent in the familiar or novel chamber during the three-chambers social novelty assay. SPF BTBR Veh $n = 11$, GF BTBR Veh $n = 17$, *L. murinus* $n = 10$. P value from two-way ANOVA, $F_{2,70} = 2.050$. **G** Representative images and percent of marbles buried during the marble burying test. SPF BTBR Veh $n = 18$, GF BTBR Veh $n = 21$, GF BTBR + *B. bifidum* $n = 24$. P value from one-way ANOVA, $F_{2,60} = 7.046$. **H** Representative images and percent of marbles buried during the marble burying test. SPF BTBR Veh $n = 12$, GF BTBR Veh $n = 11$, Heat-killed (H.K). *L. murinus* $n = 12$. SPF BTBR vs GF BTBR $P = 0.0000000000000001$, SPF BTBR vs H.K. *L. murinus* $P = 0.0000000000000001$. P value from one-way ANOVA, $F_{2,32} = 167.9$. **I** Relative fecal levels of GABA as normalized to GF BTBR. GF BTBR Veh $n = 6$, *L. murinus* $n = 5$. P value from two-tailed Student's t -test, $t(9) = 2.846$. All data represented as mean $\pm$ SEM. Source data are provided as Source data file.

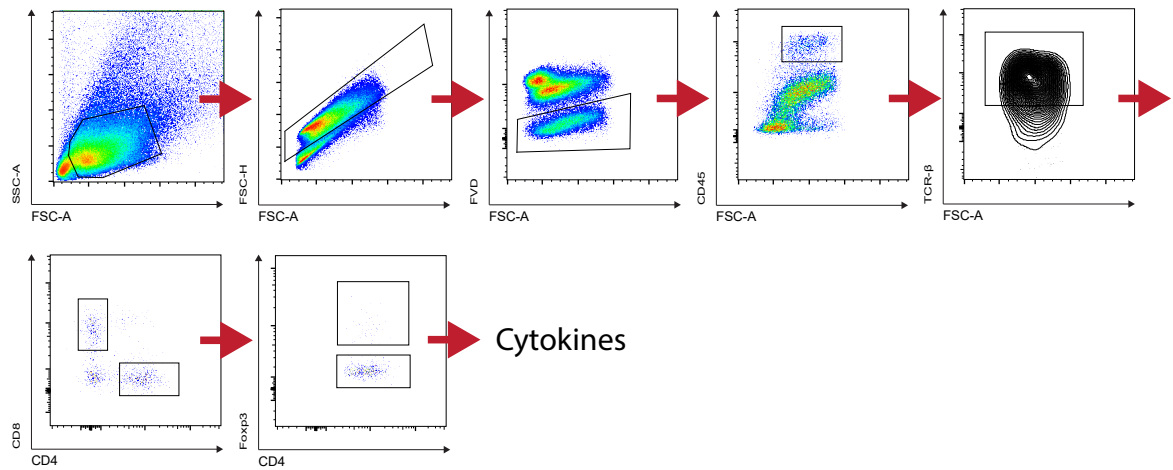


Supplementary Fig. 7 | B6 microbiota regulates ASD-associated behaviors and brain-resident immune activation. A Prenatal microbiota transfer model: Pregnant GF BTBR mice at E12.5 were cohoused with either SPF B6 (BTBR-B6) or SPF BTBR (BTBR-BTBR) female mice. Pups were tested at 8 weeks of age. **B** Time spent in the social or empty chambers during the three-chamber sociability test for prenatal model. BTBR-BTBR $n = 6$, BTBR-B6 $n = 12$. P values from two-way ANOVA, $F_{1,32} = 3.341$. **C** Representative images and percent of marbles buried during the marble burying test in prenatal model. BTBR-BTBR $n = 11$, BTBR-B6 $n = 12$. P values from two-tailed Welch's t -test, $t(21) = 2.267$. **D** Representative flow cytometry plots and frequencies of CD4⁺ or CD8⁺ T cells in the brain following postnatal (at 3 weeks of age) microbiota transfer. Gated from TCR β ⁺ CD45⁺ live cells in the brain. $n = 4$ mice. P values from two-tailed Student's t -test, $t(6) = 0.2806$, $t(6) = 0.8143$. **E** Representative flow cytometry plots and frequencies of Foxp3⁺ CD4⁺ Tregs in the brain following postnatal microbiota transfer. $n = 4$ mice. P value from two-tailed Student's t -test, $t(6) = 2.246$. **F** Absolute cell number of Tregs in the brain from flow cytometry. BTBR-BTBR and BTBR-B6 $n = 4$ mice. P value from two-tailed Student's t -test, $t(6) = 2.726$. **G** Absolute cell number of CD11b⁺ CD45^{int} microglia in the brain. $n = 4$ mice. P value from Student's t -test, $t(8) = 2.790$. **H** Representative flow cytometry plots and frequencies of microglia in the brain of B6 or BTBR mice. B6 $n = 4$ mice, BTBR $n = 5$ mice. P values from two-tailed Student's t -test, $t(7) = 3.678$. **I** Microglia cell count in the brain from flow cytometry. B6 $n = 4$ mice, BTBR $n = 5$ mice. P values from two-tailed Student's t -test, $t(7) = 2.607$. **J-M** Representative flow cytometry plots and frequencies of IL-10⁺ (**J**), IL-6⁺ (**K**), TNF- α ⁺ (**L**), and IL-1 β ⁺ (**M**) microglia in the brain. B6 $n = 4$ mice, BTBR $n = 5$ mice. $P = 0.00005$ (**L**). P values from two-tailed Student's t -test, $t(7) = 3.127$ (**J**), $t(7) = 3.889$ (**K**), $t(7) = 2.667$ (**L**), $t(7) = 8.701$ (**M**). All data represented as mean $\pm$ SEM. Source data are provided as Source data file.



Supplementary Fig. 8 | Probiotic *L. reuteri* IM015 treatment suppresses inflammation in SPF BTBR mice. **A** Relative GABA levels in the feces, as normalized to BTBR vehicle mice. BTBR Vehicle $n = 8$, BTBR IMB015 $n = 5$. P value from two-tailed Student's t -test, $t(11) = 1.074$. **B** Representative flow cytometry plot and frequency of Fopx3⁺ Tregs gated from CD4⁺ T cells in the small intestinal lamina propria (LP). BTBR Vehicle $n = 6$ mice, BTBR IMB015 $n = 4$ mice. P value from Student's t -test, $t(8) = 2.586$. **C**, **D** Representative flow cytometry plots and frequencies of IFN- γ ⁺ Fopx3⁺ CD4⁺ (**C**) or CD8⁺ (**D**) T cells gated from TCR β ⁺ cells of the spleen. BTBR Vehicle $n = 6$ mice, BTBR IMB015 $n = 4$ mice. P values from Student's t -test, $t(8) = 4.181$ (**C**), $t(8) = 2.404$ (**D**). **E-H** Representative flow cytometry plots and frequencies of IL-10⁺ (**E**), IL-6⁺ (**F**), TNF- α ⁺ (**G**), and IL-1 β ⁺ (**H**) microglia in the brain. BTBR Vehicle $n = 6$ mice, BTBR IMB015 $n = 4$ mice. P values from two-tailed Student's t -test, $t(8) = 1.298$ (**E**), $t(8) = 2.763$ (**F**), $t(8) = 0.4557$ (**G**), $t(8) = 0.1263$ (**H**). Flow cytometry data are representative of at least 2 independent experiments with minimum 3 mice. All data represented as mean $\pm$ SEM. Source data are provided as Source data file.

Gating strategy for T cells within the brain parenchyma



Gating strategy for microglia within the brain parenchyma

