SUPPLEMENTAL MATERIAL

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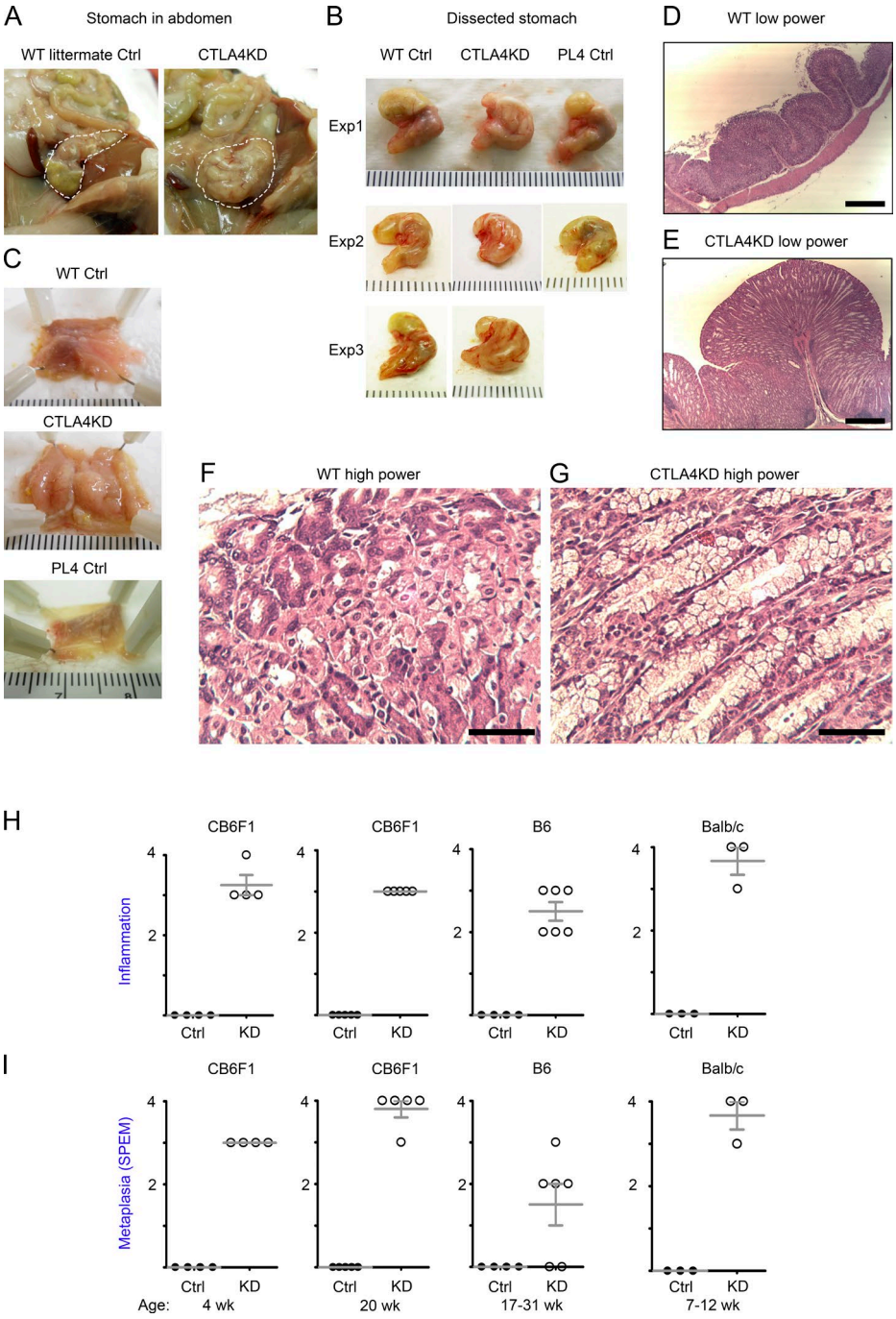

Figure S1. **Characteristic SPEM pathology in the CTLA4KD models varying in extent on different genetic backgrounds. (A–G)** In three experiments, ~20-wk-old CTLA4KD mice, transgene-negative littermates, or PL4 vector transgenic controls on the CB6F1 genetic background were examined for stomach pathology. **(A)** Image of abdominal cavity highlighting stomach before dissection. **(B)** Dissected stomach showing macroscopic difference between CTLA4KD and controls. **(C)** The lumen of the stomach was exposed, washed with PBS, and photographed with a digital camera. **(D–G)** Histological sections of wild-type littermate control and CTLA4KD mice were stained with H&E. The microscopy images taken with a low-power magnification objective were followed with high-power images. There is no histological difference between wild-type and PL4 controls. Data represent two or three animals pooled from three experiments. Bars: (low power) 500 µm; (high power) 50 µm. **(H and I)** Scores of inflammatory and SPEM pathology in the stomach samples from cohorts of CTLA4KD mice on CB6F1, B6, and BALB/c genetic backgrounds. For CB6F1 mice, the 4-wk and 20-wk inflammatory and SPEM pathology data are from Fig. 1 and shown here again for comparison. For B6 mice, data represent four to six animals per group pooled from three experiments. For BALB/c mice, data represent three animals per group pooled from two experiments. Each data point represents one animal (mean ± SEM). The incidences of inflammation and SPEM in CTLA4KD versus control mice were significantly different in all genetic background and age groups ($P < 0.05$, χ^2 test).

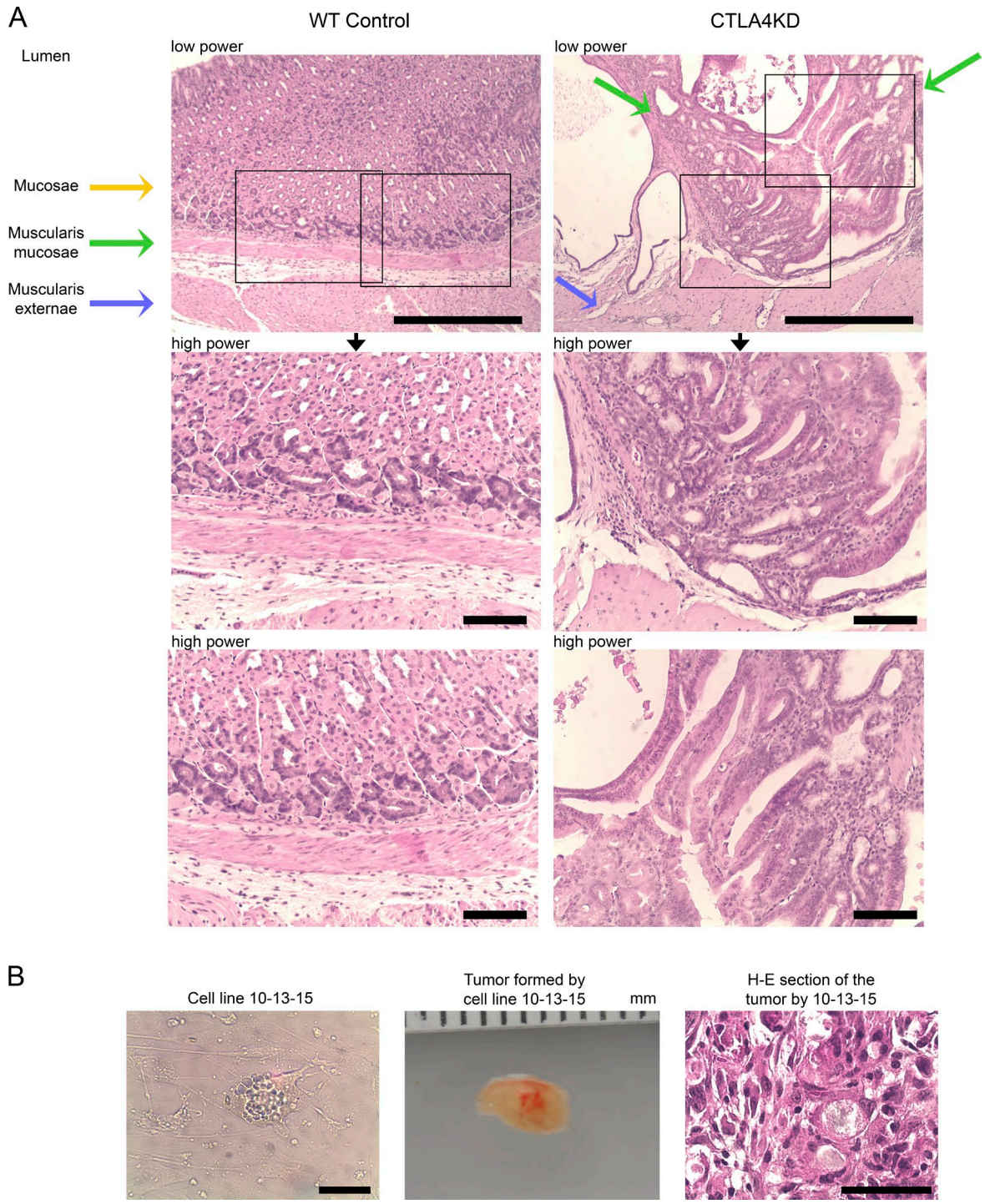

Figure S2. **Evidence for malignant transformation of the gastric mucosal cells in aging CTLA4KD mice. (A)** Destructive cancerous growth in the stomach of old CTLA4KD mice. The histopathology of the stomach of a 78-wk-old CTLA4KD and a control mouse (different animals from the ones shown in Fig. 1, group 5). The low magnification power images (bars, 500 µm) were followed with a higher power "zoomed in" images (bars, 100 µm) of the highlighted two areas for each of the low-power images. Of note, the tumor growth breaching through the muscularis mucosae was shown to indicate the invasive nature of the adenocarcinoma. All six CTLA4 KD mice examined in the old age group exhibited destructive cancerous growth, evident with tumor growth breaching through the muscularis mucosae layer (n = 6, 100% penetrance, $P < 0.001$, χ^2 test). The lesion was mainly adenocarcinoma but with heterogeneous tissue types. **(B)** A cell line was established from the gastric mucosa of an aging CTLA4KD mouse but not the control mouse. The cell line, 10-13-15, formed tumors in wild-type CB6F1 or Rag1°/CB6F1 mice (n = 4) 10–13 d after subcutaneous injection. The tumors were analyzed by H&E staining of sections. Bars, 50 µm. Data represent the tumors from four mice pooled from two experiments.

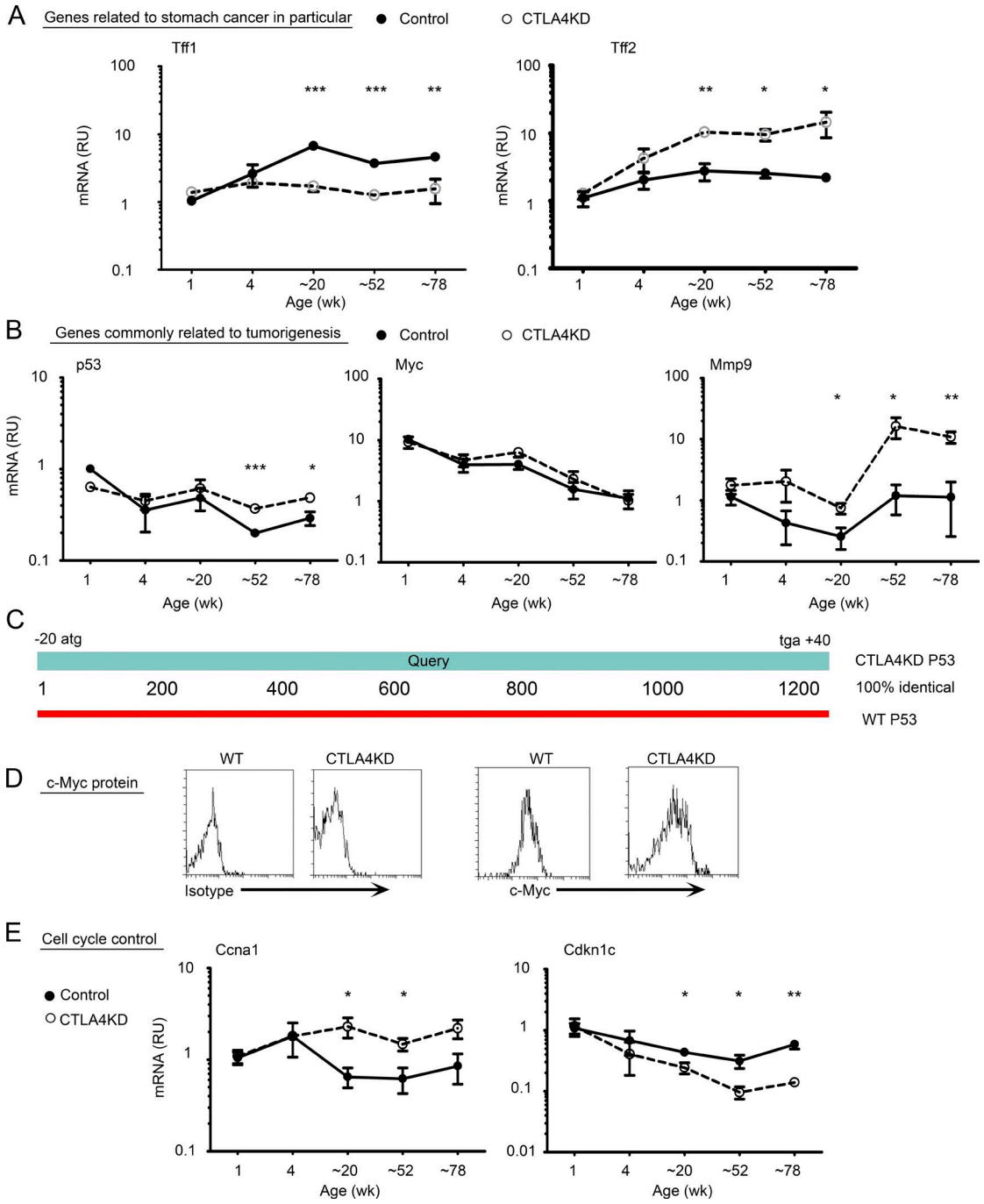

Figure S3. **Tumorigenic gene expression in gastric mucosae of CTLA4KD mice. (A)** mRNA expression for Tff1 and Tff2 in the mouse stomach. Data represent four to six mice per group pooled from two experiments (mean ± SEM; ANOVA). **(B)** A few genes commonly associated with tumorigenesis were arbitrarily selected and examined for mRNA expression, including the *p53* tumor suppressor and c-*Myc* proto-oncogene. *Mmp9* was selected because it is associated with tumor growth and/or metastasis in many studies (n = 4–6 mice). Data represent four to six mice per group pooled from two experiments (mean ± SEM; ANOVA). **(C)** Sequencing alignment of p53 transcripts from the gastric tumor tissue in CTLA4KD mice versus normal mucosae in control mice. No mutation was detected in the entire transcript from the −20 to +40 position (n = 3 pairs of CTLA4KD vs. control mice). **(D)** c-Myc protein expression in gastric tumors from CTLA4KD mice versus normal mucosae from controls (n = 4 pairs of mice), analyzed by flow cytometry intracellular staining. The cells from the tumor samples exhibited a more heterogeneous profile but did not show overall overexpression versus controls. **(E)** Two cell cycle control genes, cyclin A1 (*Ccna1*) and cyclin-dependent kinase inhibitor 1c (*Cdkn1c*), which had altered methylation in human gastric cancer (Sapari et al., 2012; Loh et al., 2014), were analyzed for mRNA expression. Data represent four to six mice per group pooled from two experiments (mean ± SEM; ANOVA). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

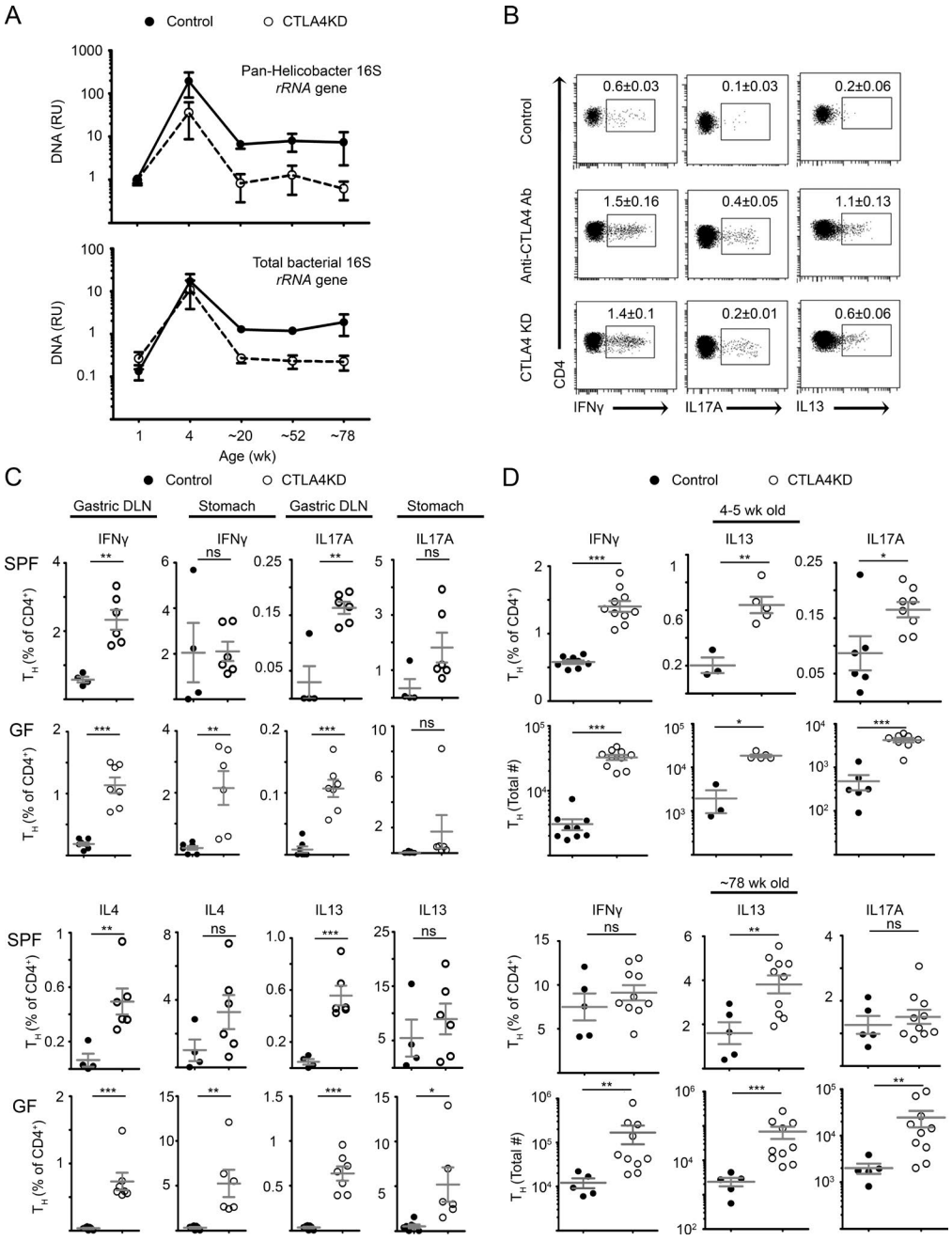

Figure S4. **Chronic dysregulation of both type 1 and type 2 cytokine responses in CTLA4KD mice without evidence of pathogenic *Helicobacter* infection. (A)** Gastric metaplasia in CTLA4KD mice was not associated with pathogenic *Helicobacter* infection. Quantitative PCR for all *Helicobacter* species using primers specific to the 16S *rRNA* gene of *Helicobacter* species, or quantitative PCR for all bacteria using primers that are homologous to the *16S rRNA* gene of all bacterial species, relative to eukaryotic genomic DNA of stomach mucosal samples assayed by primers specific to the β-casein gene. Data at each age-point represent three to four mice per group pooled from two cohorts of animals (mean ± SEM). **(B)** Representative flow cytometry of the T_H subset differentiation in the gastric draining lymph nodes in mice treated with anti-CTLA4 antibody or in CTLA4KD mice, by intracellular cytokine staining for IFN-γ (T_H1), IL-13 (T_H2), and IL-17A (T_H17). Data represent 3–10 mice pooled from two to three experiments (mean ± SEM; $P < 0.05$, Student's *t* test). **(C)** Summary of the percentages of T_H cells in the draining lymph nodes and stomach tissue in CTLA4KD mice maintained in SPF or GF facilities and analyzed at 9–10 wk of age. The cells were stimulated for ~6 h by phorbol dibutyrate (PDBU) plus ionomycin and analyzed by intracellular cytokine staining of T cells. Data represent three to seven mice pooled from two analysis experiments from one GF derivation (mean ± SEM; Student's *t* test). **(D)** Summary of the total numbers of T_H cells in the draining lymph nodes of young and aging adult CTLA4KD mice (at age of 4–5 wk or ~78 wk) in SPF facilities. Data represent 3–10 mice for each of the 4–5-wk groups and 5–10 mice for each of the ~78-wk groups pooled from two to three experiments (mean ± SEM; Student's *t* test and Mann–Whitney test). Each data point represents one animal (mean ± SEM). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

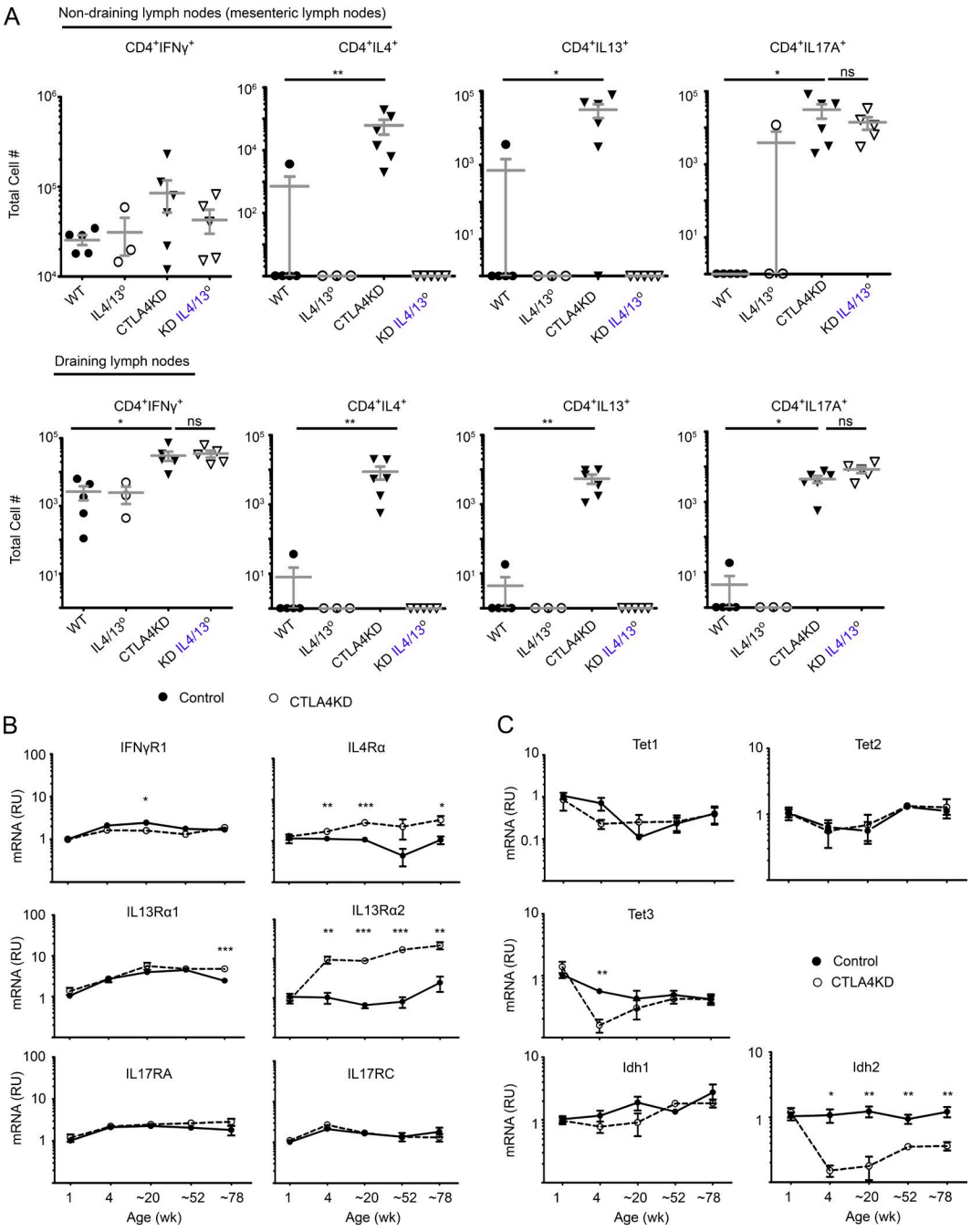

Figure S5. **The role of type 2 cytokine signaling and epigenetic alteration in the gastric mucosal cells for the tumorigenic effect of immune dysregulation caused by CTLA4 insufficiency. (A)** IL-4/IL-13 deficiencies did not alter the population size of T_H1 and T_H17 subsets in the CTLA4KD model. Lymphocytes were isolated from nondraining lymph nodes (mesenteric lymph nodes) and gastric draining lymph nodes. Intracellular cytokine staining was performed for IFN-γ (T_H1), IL-4 and IL-13 (T_H2), and IL-17A (T_H17). Summary of the total numbers of T_H cells in 8–10-wk-old CTLA4KD IL4/IL13° mice in comparison to three types of littermate controls: wild-type, IL4/IL13°, and CTLA4KD(IL4/IL13$^+$). Data represent three to six mice per group pooled from two experiments. Each data point represents one animal (mean ± SEM; Kruskal–Wallis test). **(B)** Expression of cytokine receptors in gastric mucosal tissue samples of CTLA4KD mice versus controls from the SPEM (4–52 wk) to GA (52–78 wk) stages, assessed with quantitative RT-PCR for mRNA expression levels. The data represent four to eight mice in each group pooled from two to three experiments (mean ± SEM; ANOVA). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. **(C)** Assessment of methylation regulatory gene expression in gastric mucosae of CTLA4KD versus control mice. Differential mRNA expression of epigenetic regulatory genes in gastric tissues from CTLA4KD mice or transgene-negative controls on the CB6F1 background was assessed with quantitative RT-PCR. The data represent four to five mice pooled from two to three cohorts of animals for each group at each age point (mean ± SEM; ANOVA). *, $P < 0.05$; **, $P < 0.01$. RU, relative units.