# Supporting Information

## Oertli et al. 10.1073/pnas.1211248110

### SI Materials and Methods

**Induction and Analysis of Allergen-Induced Asthma.** For asthma induction, mice were sensitized by two i.p. injections of 20 μg of ovalbumin (Sigma-Aldrich) emulsified in 2.25 mg of aluminum hydroxide (Alum Imject; Pierce) with a 2-wk interval and challenged with 1% aerosolized ovalbumin using an ultrasonic nebulizer (NE-U17; Omron) for 20 min daily on days 14, 15, and 16 after (second) sensitization. For adoptive Treg transfers, $CD4^+$ $CD25^+$ T cells were immunomagnetically isolated (R&D Systems) from the pooled mesenteric lymph nodes (MLNs) of five donors per group; 200,000 cells were i.v. injected 2 d before the first aerosol challenge. Airway resistance measurements were performed on anesthetized, intubated, and mechanically ventilated mice (FlexiVent; Scireq) in response to increasing doses of inhaled methacholine. Lungs were lavaged via the trachea with 1 mL of PBS. Bronchoalveolar lavage fluid cells were counted using trypan blue dye exclusion. Differential cell counts of lymphocytes, neutrophils, and eosinophils were performed on cytocentrifuged preparations stained with the microscopy Hemacolor set (Merck). Lungs were fixed by inflation and immersion in 10% formalin and embedded in paraffin. Lung tissue sections were stained with H&E and periodic acid-Schiff and were examined in blinded fashion by two independent experimentors on a BX40 Olympus microscope. Peribronchial inflammation was scored on a scale from 0 to 4 on five randomly chosen areas per slide. PAS-positive goblet cells were quantified per 1 mm of basement membrane in the primary bronchus and several medium-sized bronchi using Soft Imaging Systems software.

**Preparation of Murine Gastric and Mesenteric Lymph Node Single-Cell Suspensions and Flow Cytometry.** One-sixth of every stomach (antrum and corpus) and corresponding mesenteric lymph nodes were digested in 1 mg/mL collagenase for 30 min at 37 °C with shaking before mechanical disruption between glass slides and filtering through a cell strainer (40 μm). Single-cell suspensions were either seeded at 100,000 cells/well and assessed for IFN-γ and IL-17 production by ELISA (BD Bioscience) 3 d later or stained straightaway for FACS analysis. The following antibodies were used for surface marker staining: CD11c-biotin, CD4-FITC, CD4-APC (BD Biosciences), CD45-PB, and CD80-APC (BioLegend). IFN-γ-PE-Cy7 (BD Biosciences), IL-17-APC, and FoxP3-APC (both from eBioscience) were used for intracellular staining. Before intracellular cytokine staining, cells were stimulated and blocked in medium containing 2.5 μg/mL Brefeldin A (AppliChem), 0.2 μM ionomycin (Santa Cruz Biotechnology), and 50 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich) for 5 h, stained for extracellular markers, and fixed in 4% paraformaldehyde. Flow cytometry was performed on a Cyan ADP 9 instrument (Beckman Coulter) and analyzed using FlowJo software (Tree Star).

**Western Blotting for GGT and GGT Activity Assay.** The correct deletion of the *ggt* gene in our Δ*ggt* mutants was assessed by Western blotting for γ-glutamyl transpeptidase (GGT) protein and by enzyme activity assay using *p*-nitroanilide as published by Meister et al. (1). Briefly, the release of *p*-nitroanilide was monitored by spectrophotometry at 405 nm. One unit of activity was defined as the quantity of enzyme that released 1 mol of *p*-nitroanilide per minute and per milligram of protein at 37 °C. For Western blotting, an antibody raised in-house against the small GGT subunit was used; 5 μg of protein of *Helicobacter pylori* supernatant was loaded onto SDS polyacrylamide gels.

**Statistics.** GraphPad Prism (GraphPad Software) was used for statistical analyses. The significance of categorical differences in histopathology scores and of unequally distributed experimental animal populations was calculated by the Mann-Whitney test, and the significance of numerical differences and of equally distributed populations with similar variances (e.g., all in vitro-generated results) was calculated by Student *t* test. In all scatter plot graphs, the medians are indicated by horizontal bars. In column bar graphs, the SEM is indicated by vertical bars; n.s., not significant. $P$ values $< 0.05$ were considered statistically significant.

1. Meister A, Tate SS, Griffith OW (1981) Gamma-glutamyl transpeptidase. *Methods Enzymol* 77:237–253.

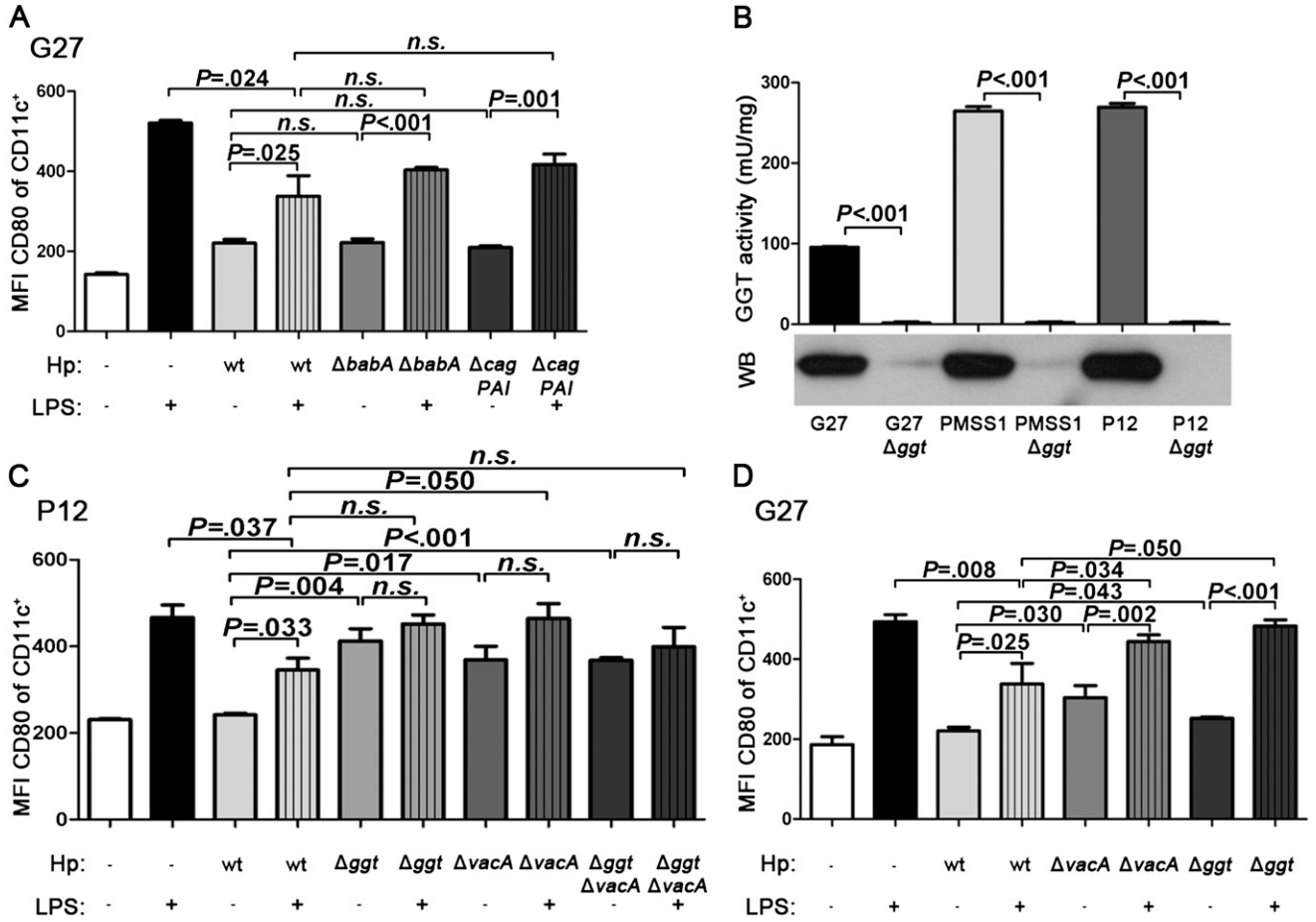


**Fig. S1.** VacA and GGT are required for the inhibition of LPS-induced DC maturation by *H. pylori* strains P12 and G27. (*A*) Bone marrow DCs were infected with *H. pylori* strain G27, G27Δ*babA*, or G27Δ*cag*PAI and/or treated with 0.5 μg/mL *E. coli* LPS for 16 h before the flow cytometric analysis of CD11c and CD80 expression. Data are representative of two independent experiments and are represented as means ± SEM of triplicate cultures. MFI, mean fluorescence intensity. **(***B*) Validation of mutants. All examined Δ*ggt* mutants lack GGT protein as assessed by Western blotting and also lack GGT activity as assessed by *p*-nitroanilide release assay. Δ*vacA* mutants of the toxigenic strains P12 and G27 were routinely assessed for lack of vacuolating cytotoxicity on AGS cells and by genotyping PCR; Δ*vacA* mutants of the nontoxigenic strain PMSS1 were validated by genotyping PCR. (*C*) Bone marrow DCs were infected with *H. pylori* P12, P12Δ*ggt*, P12Δ*vacA*, or P12Δ*ggt*Δ*vacA* (MOI 50) and/or treated with 0.5 μg/mL *E. coli* LPS for 16 h before the flow cytometric analysis of CD11c and CD80 expression. Data are representative of four independent experiments and represented as means ± SEM of triplicate cultures. (*D*) Bone marrow DCs were infected with *H. pylori* strains G27, G27Δ*vacA*, or G27Δ*ggt* and/or treated with 0.5 μg/mL *E. coli* LPS for 16 h before the flow cytometric analysis of CD11c and CD80 expression. Data are representative of two to five independent experiments and are represented as means ± SEM of triplicate cultures. All *P* values were calculated using the Student *t* test.

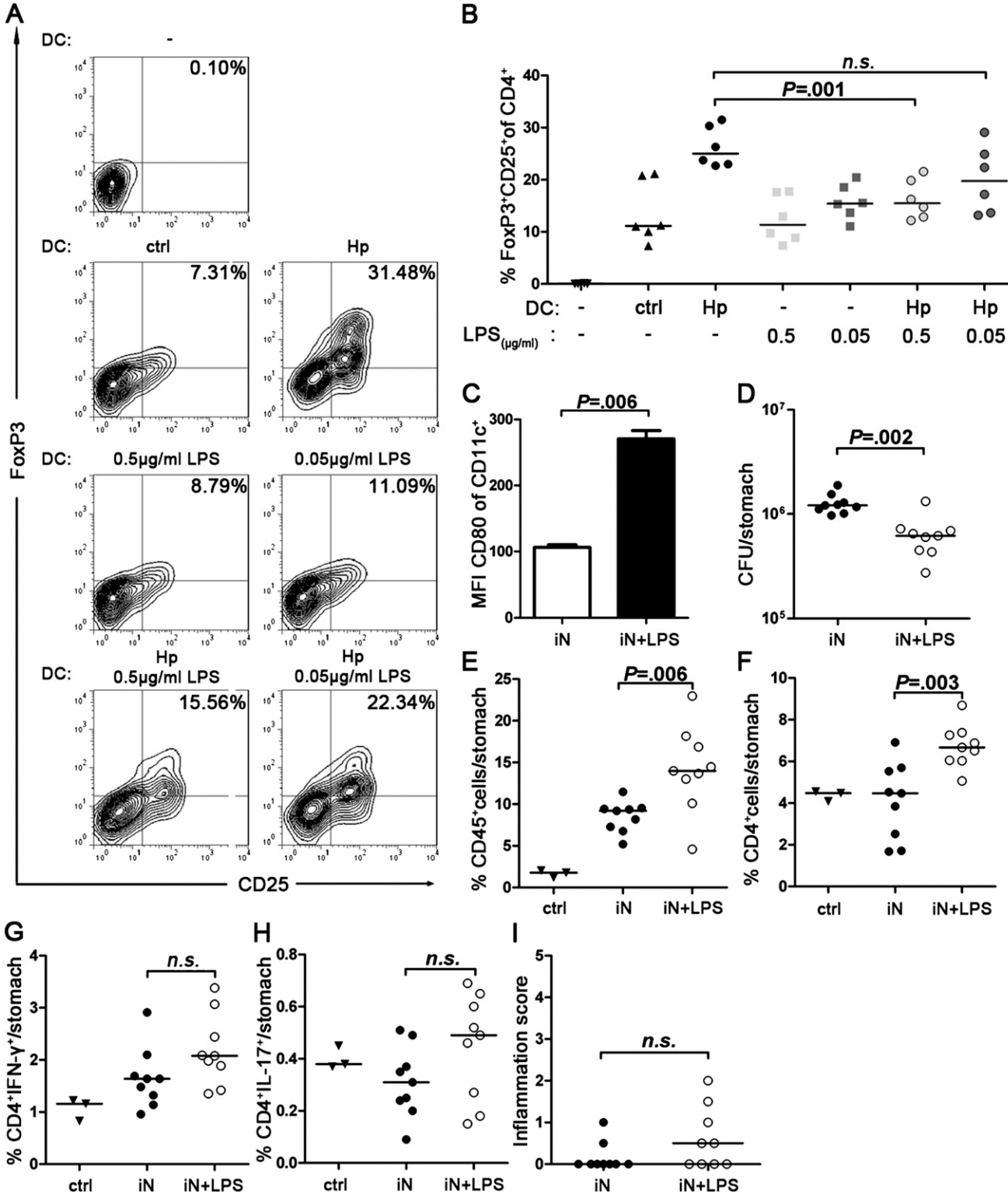


**Fig. S2.** *E. coli* LPS impairs *H. pylori*-induced DC tolerization and breaks neonatally acquired immune tolerance to *H. pylori* infection. (*A* and *B*) Bone marrow DCs were infected or not with *H. pylori* PMSS1 (multiplicity of infection of 50) and/or treated with 0.05 or 0.5 μg/mL *E. coli* LPS. After 16 h, bacteria were killed with antibiotics. DCs were washed thoroughly and cocultured with immunomagnetically isolated, splenic $CD4^+CD25^-$ T cells for 3 d in the presence of rTGF-β, rIL-2, and anti-CD3ε mAb before the flow cytometric analysis of CD4, CD25, and FoxP3 expression. Representative plots of the $CD4^+$ gate are shown in *A* and pooled results from two independent infections are shown in *B*; in *B*, each symbol represents one coculture. (*C*) C57BL/6 mice were infected with *H. pylori* at 7 d of age (infected as neonates, iN) for a total of 2 mo. One group received i.p. doses of 1 μg LPS per gram of body weight every other day for the final 2 wk of infection. MLN single-cell suspensions were stained for CD11c and CD80. The mean fluorescence intensity (MFI) of CD80 expression of CD11c-positive cells is shown for all mice per group, with data represented as means +/− SEM. (*D*) Colony-forming units (CFU) per stomach as determined by plating and colony counting. (*E*) $CD45^+$ leukocyte infiltration into the gastric mucosa of the mice shown in *C* and *D*, as well as several uninfected controls. (*F*) $CD4^+$ T-cell infiltration into the gastric mucosa of the mice shown in *C*–*E*. (*G*) $CD4^+$IFN-$\gamma^+$ T-cell infiltration into the gastric mucosa. (*H*) $CD4^+$IL-$17^+$ T-cell infiltration into the

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gastric mucosa. (*I*) Inflammation scores assigned on a scale from 0 to 6. In *C–I*, each symbol represents one mouse; data are pooled from two experiments. Horizontal lines indicate the medians. *P* values were calculated using the Student *t* test (*B*) and Mann-Whitney test (*C–I*).

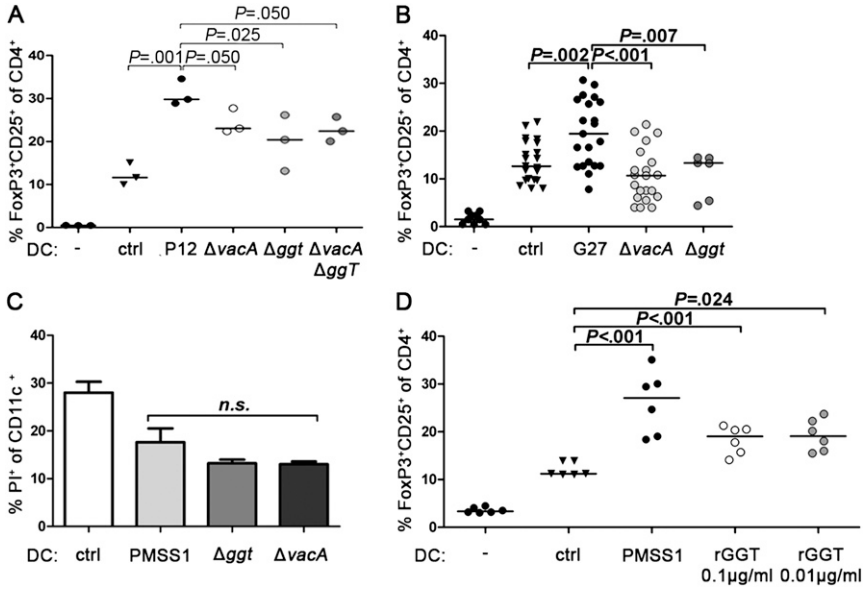


**Fig. S3.** VacA and GGT are required for Treg differentiation induced by *H. pylori*-experienced DCs. (*A*) Bone marrow DCs were infected overnight with *H. pylori* strain P12, P12Δ*ggt*, P12Δ*vacA*, and P12Δ*ggt*Δ*vacA* at a multiplicity of infection (MOI) of 50. After 16 h, bacteria were killed with antibiotics. DCs were cocultured with immunomagnetically isolated, splenic $CD4^+CD25^-$ T cells for 3 d in the presence of rTGF-β, rIL-2, and anti-CD3ε mAb before the flow cytometric analysis of CD4, CD25, and FoxP3 expression. The fraction of $FoxP3^+CD25^+$ cells of the $CD4^+$ gate is shown for triplicate cocultures. (*B*) Bone marrow DCs were infected overnight with *H. pylori* strains G27, G27Δ*ggt*, or G27Δ*vacA* at an MOI of 50 and treated as described in *A*. The fraction of $FoxP3^+CD25^+$ cells of the $CD4^+$ gate are shown for two to six pooled experiments. Uninfected DCs and T cells cultured in the absence of DCs served as controls. (*C*) To assess DC viability upon infection with the various mutants, bone marrow DCs were infected overnight with *H. pylori* strains PMSS1, PMSS1Δ*ggt*, or PMSS1Δ*vacA* at an MOI of 50 and stained with propidium iodide (PI) for flow cytometric analysis. (*D*) To assess the tolerizing capacity of recombinant GGT, DCs were treated for 16 h with the indicated amounts of recombinant protein, washed thoroughly, and cocultured with immunomagnetically isolated, splenic $CD4^+CD25^-$ T cells. The fraction of $FoxP3^+CD25^+$ cells of the $CD4^+$ gate is shown for a representative experiment of two. PMSS1-infected DCs were included for comparison. In *A*, *B*, and *D*, each symbol represents one coculture. Horizontal lines indicate the medians. *P* values were calculated using the Student *t* test.

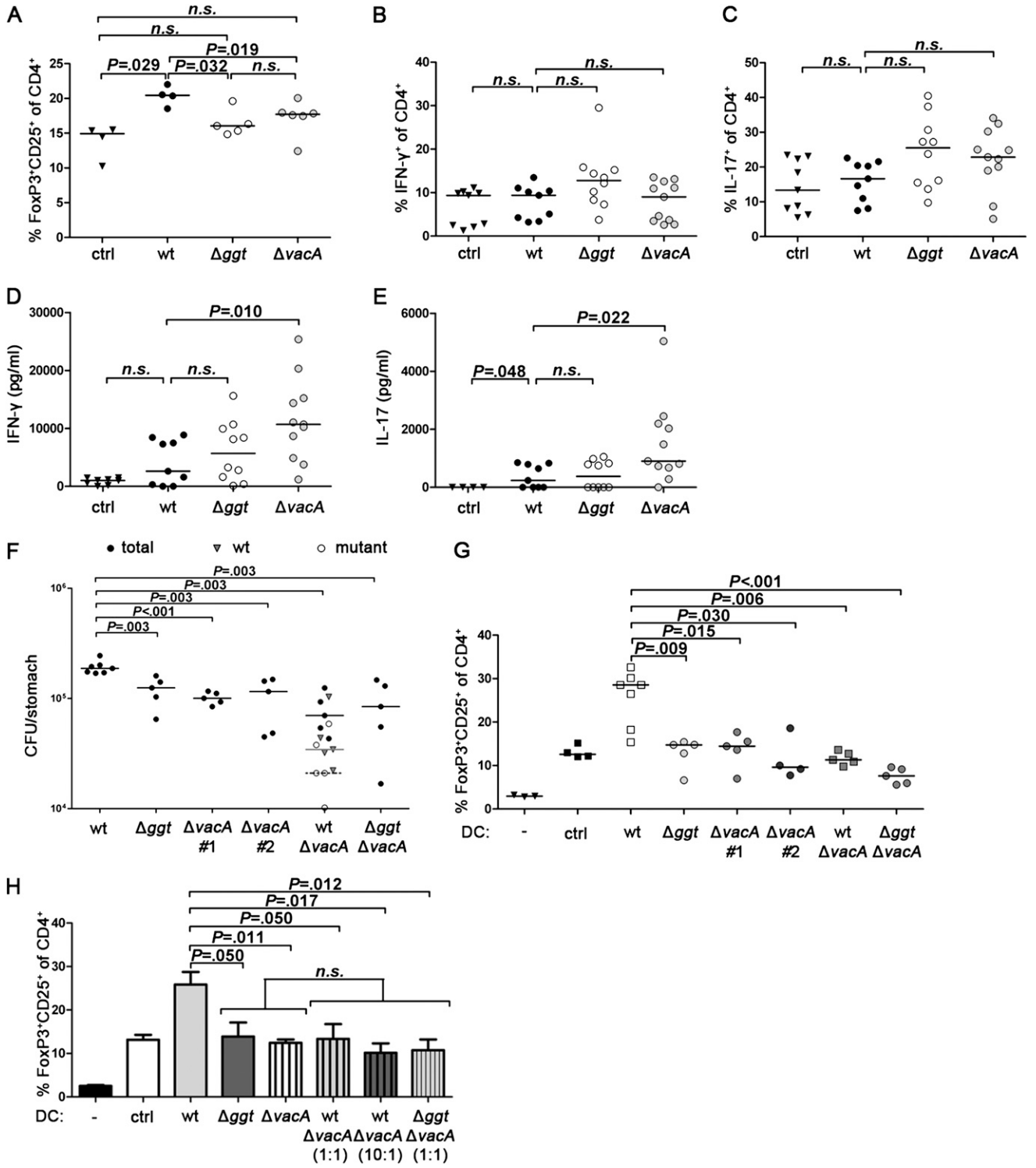


**Fig. S4.** VacA and GGT induce *H. pylori*-specific Treg responses and suppress Th1 and Th17 responses. C57BL/6 mice were infected with *H. pylori* PMSS1, PMSS1Δ*ggt*, or PMSS1Δ*vacA* at 6 wk of age for 1 mo. After mice were killed, MLN single-cell preparations of each mouse were stained with antibodies for CD4, CD25, FoxP3, IL-17, and IFN-γ for FACS analysis and seeded for cytokine quantification by ELISA. (*A*) Proportion of FoxP3$^+$CD25$^+$ cells in % of all CD4$^+$ cells. (*B*) Proportion of IFN-γ$^+$ cells in % of all CD4$^+$ cells. (*C*) Proportion of IL-17$^+$ cells in % of all CD4$^+$ cells. Note that in *A*–*C* the fraction of CD4$^+$ cells in % of all MLN cells remains relatively constant. (*D*) MLN single-cell preparations were seeded at 100,000 cells per well and assessed after 3 d for IFN-γ production by ELISA. (*E*) The same supernatants were also subjected to IL-17A ELISA. Each symbol represents one mouse; horizontal lines indicate medians. Note that of the three independent studies pooled for Fig. 2 *A*–*C*, only one study was analyzed with respect to FoxP3/CD25 expression (shown in *A*), whereas two studies were analyzed for cytokine expression by FACS and ELISA (*B*–*E*). (*F* and *G*) C57BL/6 mice were infected with *H. pylori* PMSS1, PMSS1Δ*ggt*, or PMSS1Δ*vacA* clones 1 or 2, or with equal amounts of PMSS1 and PMSS1Δ*vacA* (clone 1), or with equal amounts of PMSS1Δ*ggt* and PMSS1Δ*vacA* (clone 1), at 6 wk of age for 1 mo. After mice were killed, colony-forming units (CFU) were determined (separately for wild-type and mutant bacteria, where necessary, *F*) and MLN DCs were prepared for each mouse and cocultured with CD4$^+$CD25$^-$ T cells; cocultures were stained with antibodies for CD4, CD25, and FoxP3. The proportion of FoxP3$^+$CD25$^+$ cells

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in % of all $CD4^+$ cells is shown *G*. In *F*, *P* values refer to comparisons between groups in terms of total cfu. (*H*) Bone marrow DCs were infected overnight with *H. pylori* PMSS1, PMSS1Δ*ggt*, or PMSS1Δ*vacA* or the specified strain combinations (ratios as indicated in the figure) at an MOI of 50. After 16 h, bacteria were killed with antibiotics. DCs were cocultured with $CD4^+CD25^-$ T cells for 3 d before the flow cytometric analysis of CD4, CD25, and FoxP3 expression. The fraction of $FoxP3^+CD25^+$ cells of the $CD4^+$ gate are shown for triplicate cocultures.

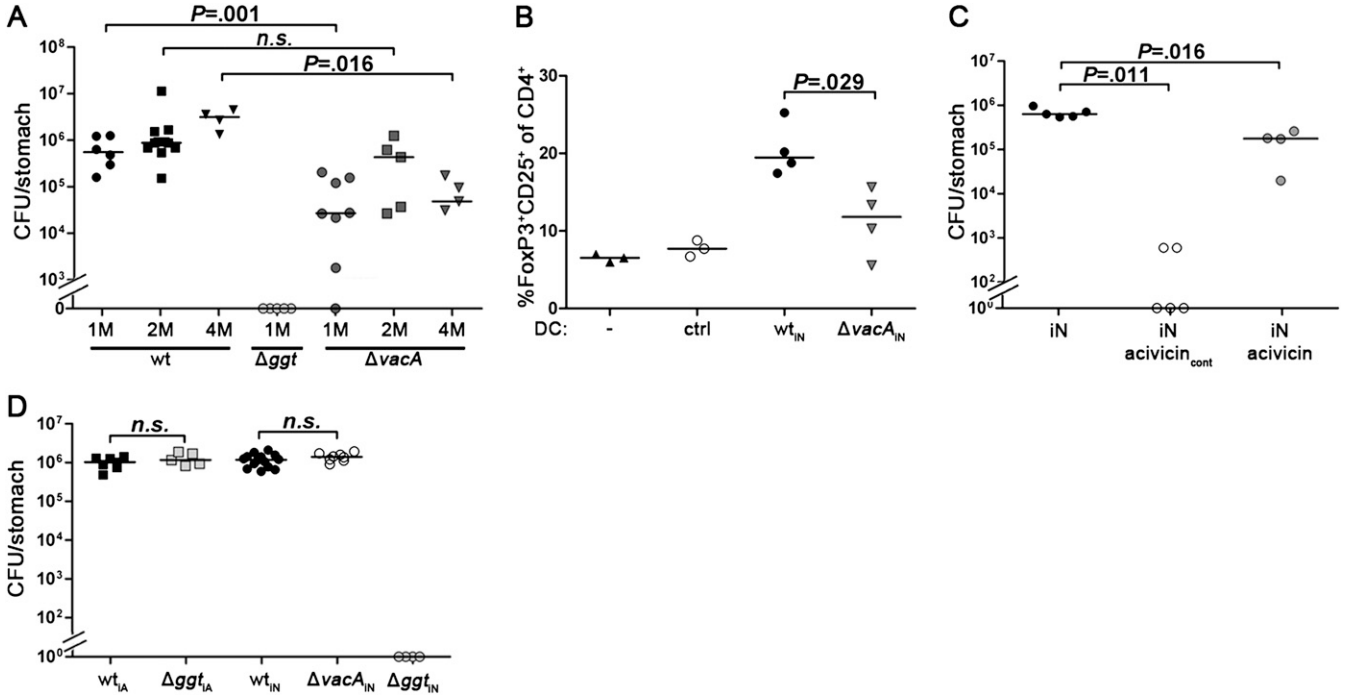


**Fig. S5.** VacA and the enzymatic activity of GGT contribute to neonatally acquired immune tolerance independent of T cells. (*A*) C57BL/6 mice were infected with *H. pylori* PMSS1, PMSS1Δ*ggt*, or PMSS1Δ*vacA* at 7 d of age (iN, infected as neonates) for 1, 2, or 4 mo. Colony-forming units (CFU) per stomach are shown. (*B*) $CD11c^+$ MLN DCs were immunomagnetically isolated from all mice killed at 4 mo p.i., cocultured with $CD4^+CD25^-$ T cells, and subjected to flow cytometric analysis of CD4, CD25, and FoxP3 expression. $CD25^+FoxP3^+$ cells in the $CD4^+$ gate are shown. (*C*) C57BL/6 mice were infected with *H. pylori* PMSS1 at 7 d of age (iN) for 6 wk. One group received acivicin continuously i.p. every other day at 2 mg/kg body weight, starting from the day of infection ($acivicin_{cont}$). Another group received acivicin at the same dosage, but only during the last 2 wk of the infection. Colony-forming units per stomach are shown. (*D*) TCR-$\beta^{-/-}$ mice were infected with *H. pylori* PMSS1, PMSS1Δ*ggt*, or PMSS1Δ*vacA* at 7 d (iN, infected as neonates) or 6 wk of age (iA, infected as adults) for 1 mo. Colony-forming units per stomach are shown. Horizontal lines in *A*–*D* indicate the medians. *P* values were calculated using the Mann-Whitney test.

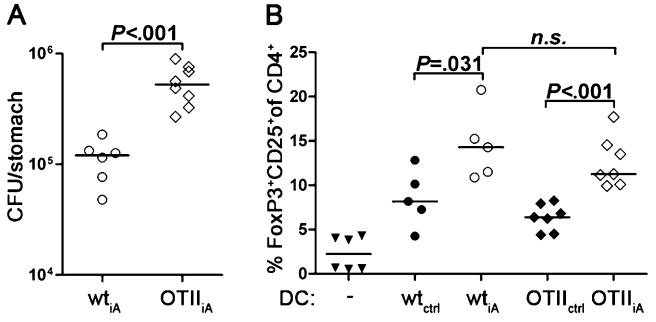


**Fig. S6.** A normal T-cell repertoire is not required for the tolerizing effects of VacA- and GGT-proficient *H. pylori* on DCs. (*A* and *B*) C57BL/6 or OT II TCR-transgenic mice were infected with *H. pylori* PMSS1 at 6 wk of age for 1 mo. $CD11c^+$ MLN-DCs were immunomagnetically isolated from all infected mice and from uninfected controls, cocultured for 3 d with splenic $CD4^+CD25^-$ T cells, rTGF-β, rIL-2, and anti-CD3ε mAb, and subjected to flow cytometric analysis of CD4, CD25, and FoxP3 expression. Colony-forming units (CFU) per stomach are shown in *A*. The fraction of $FoxP3^+CD25^+$ cells of the $CD4^+$ gate is shown in *B* for all donors. T cells cultured in the absence of DCs served as additional controls. Data in *A* and *B* are from one study. Horizontal lines indicate the medians. *P* values were calculated using the Mann-Whitney test.

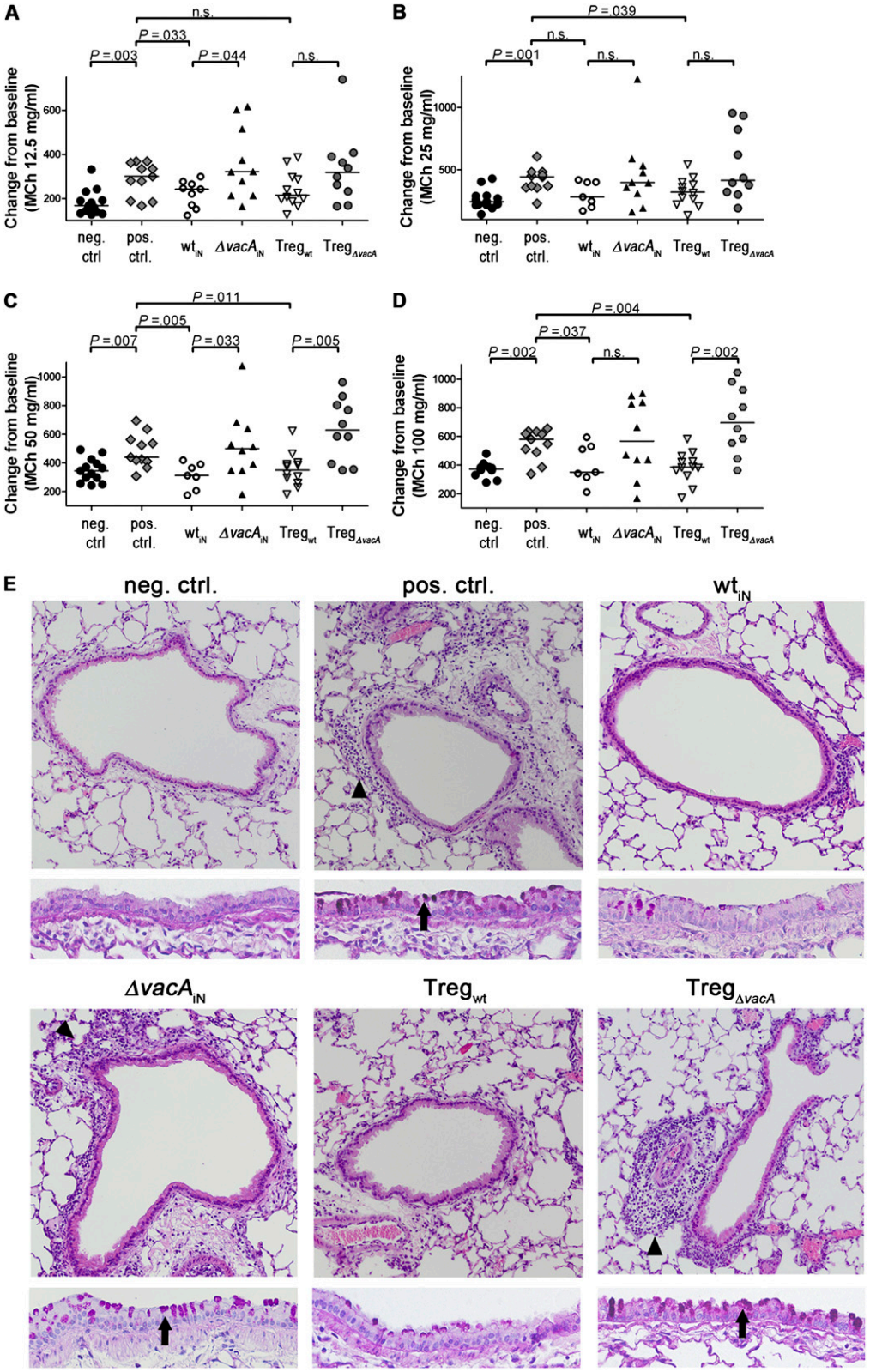


**Fig. S7.** The *H. pylori*-mediated protection against asthma and generation of Tregs with protective activity depends on VacA. Mice were treated as described in Fig. 4. (*A–D*) Airway hyper-responsiveness as assessed upon exposure to increasing doses of methacholine (12.5, 25, 50, and 100 mg/mL). Horizontal lines indicate the medians. *P* values were calculated using the Mann-Whitney test. (*E*) Tissue inflammation and goblet cell metaplasia as assessed on H&E- and PAS-stained tissue sections. Representative micrographs taken at 100× (H&E, *Upper*) and 400× (PAS, *Lower*) original magnification are shown for representative mice of the groups shown in Fig. 4; arrowheads mark clusters of inflammatory cells in H&E-stained sections, and arrows point to PAS-positive goblet cells.