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Shiomi, Hideyuki ; Masuda, Atsuhiro ; Nishiumi, Shin ; Nishida, Masayuki ; Takagawa, Tetsuya ; Shiomi, Yuuki ; Kutsumi, Hiromu ;…

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# IFN-γ produced by antigen-specific CD4$^+$ T cells regulates the mucosal immune responses to *Citrobacter rodentium* infection

Hideyuki Shiomi[1)], Atsuhiro Masuda[1)], Shin Nishiumi[1,2)], Masayuki Nishida[1)], Tetsuya Takagawa[1)], Yuuki Shiomi[1)], Hiromu Kutsumi[1)], Richard S. Blumberg[3)], Takeshi Azuma[1)] and Masaru Yoshida[1,2)*]

[1] Division of Gastroenterology, Department of Internal Medicine, Kobe University Graduate School of Medicine, Kobe, Hyogo, Japan

[2] The Integrated Center for Mass Spectrometry, Graduate School of Medicine, Kobe University, Kobe, Hyogo, Japan

[3] Division of Gastroenterology, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts

**Running title**: IFN-γ from CD4$^+$ T cell regulate *C. rodentium* infection

The authors have no conflicts of interest.

***Correspondence**

Masaru Yoshida M.D., Ph.D.

Division of Gastroenterology, Department of Internal Medicine, Kobe University Graduate School of Medicine

7-5-1, Kusunoki-cho, Chu-o-ku, Kobe, 650-0017 Hyogo, Japan

TEL: +81-78-382-6305, Fax: +81-78-382-6309

E-mail: myoshida@med.kobe-u.ac.jp

**ABSTRACT**

*Citrobacter rodentium* (*C. rodentium*), a murine model pathogen for enteropathogenic *Escherichia coli*, colonizes the surface of intestinal epithelial cells and causes mucosal inflammation. This bacterium is an ideal model for investigating pathogen-host immune interactions in the gut. It is well known that gene transcripts for Th1 cytokines are highly induced in colonic tissue from mice infected with *C. rodentium*. However, it remains to be seen whether the Th1 or Th2 cytokines produced by antigen-specific $CD4^+$ T cells provide effective regulation of the host immune defense against *C. rodentium* infection. To investigate the antigen-specific immune responses, *C. rodentium* expressing ovalbumin (OVA), a model antigen (OVA-*C. rodentium*), was generated and used to define antigen specific responses under IFN-$\gamma$ or IL-4 deficient conditions *in vivo*. The activation of antigen-specific $CD4^+$ T cells and macrophage phagocytosis were evaluated in the presence of IFN-$\gamma$ or IL-4 *in vitro*. IFN-$\gamma$ deficient mice exhibited a loss of body weight and a higher bacterial concentration in feces during OVA-*C. rodentium* infection compared to C57BL/6 (wild type) or IL-4 deficient mice. This occurred through the decreased efficiency of macrophage phagocytosis and the

activation of antigen-specific $CD4^+$ T cells. Furthermore, a deficiency in antigen-specific $CD4^+$ T-cell-expressed IFN-γ led to a higher susceptibility to mucosal and gut-derived systemic OVA-*C. rodentium* infection. These results show that the IFN-γ produced by antigen-specific $CD4^+$ T cells plays an important role in the defense against *C. rodentium*. **(231 words)**

**Keywords**

antigen-specific $CD4^+$ T cells; Citrobacter rodentium; mucosal immune response; IFN-γ; IL-4

## INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC) is associated with significant morbidity and mortality in human populations worldwide. EPEC is a leading cause of bacterially induced diarrhea in developing countries and is responsible for approximately 1 million infant deaths per year (4, 8). Enteric bacteria, such as EPEC, evade many systemic host defense mechanisms by restricting their colonization to the luminal surface of the gastrointestinal epithelium (20). The strategies used by these pathogens include intimate adherence to the cecal and colonic epithelium and disruption of cellular structures through formation of attaching and effacing (A/E) lesions (20).

*Citrobacter rodentium* (*C. rodentium*), a murine model pathogen for EPEC infection similarly, colonizes the surface of intestinal epithelial cells by utilization of A/E lesions in association with mucosal inflammation. Colonization of *C. rodentium* peaks 7 days after infection and is usually eradicated by day 28 after the oral administration in immunocompetent mice. Bacterial colonization is limited to the intestinal mucosa with a few bacteria reaching systemic sites. The infected mice exhibit a loss of body weight and diarrhea in association with crypt hyperplasia,

a loss of goblet cells, and mucosal infiltration of the epithelium with lymphocytes, macrophages, neutrophils, and mast cells (10, 11).

The lymphocytic host response to *C. rodentium* is characterized by a large infiltration of $CD4^+$ T cells into the colonic lamina propria, a modest increase in epithelial $CD8^+$ T cells, and a highly polarized Th1 response (5, 6). Additionally, transcripts for Th1 cytokines such as interferon-gamma (IFN-$\gamma$) and interleukin-12 (IL-12) are highly induced in colonic tissue of the infected mice, but those for the Th2 cytokine interleukin-4 (IL-4) are not (6). Recent reports have shown that Th17 cells are also involved in the host defense against *C. rodentium* infection, because antigen-presenting cells from *C. rodentium*-infected mice when stimulated with microbial products, such as lipopolysaccharide, peptidoglycans and zymosan, produce significant amounts of interleukin-23 (IL-23) (9, 23, 24). Furthermore, interleukin-$17RA^{-/-}$ (IL-$17RA^{-/-}$) mice and IL-$23A^{-/-}$ mice are more susceptible to *C. rodentium* infection (7, 12, 29). These studies indicate that Th17 cytokines and possibly Th1 cytokines play an important role in eradicating *C. rodentium* infection.

$CD4^+$ T cell and B cell deficient mice also fail to eradicate *C. rodentium* infection. Among the factors produced by these cells, anti-bacterial IgG is

particularly important. In contrast, mice lacking $CD8^+$ T cells, IgA, secreted IgM, and proteins required for transport of IgA and IgM into the lumen (polymeric Ig receptor and J chain) clear *C. rodentium* normally (2, 11, 19, 27). Thus, it would appear that $CD4^+$ T cells, B cells, and IgG, but not secretory IgA or IgM, play important roles in eradicating this pathogen. Consistent with this, FcRn deficient mice were highly susceptible to *C. rodentium* infection in a pathway that depends upon antibacterial IgG (27).

The specific role of Th1 or Th2 cytokines produced by antigen-specific $CD4^+$ T cells has not yet been examined in the regulation of *C. rodentium* infection. We therefore used *C. rodentium* expressing a model antigen (ovalbumin: OVA) to investigate antigen specific responses to *C. rodentium in vivo* in IFN-$\gamma$ or IL-4 deficient mice. We observed that IFN-$\gamma$ effectively promoted the activation of antigen-specific $CD4^+$ T cells and macrophage phagocytosis. Furthermore, the T cell transfer models supported the functional role for IFN-$\gamma$, but not IL-4, from the antigen-specific $CD4^+$ T cells, the regulation of protective antibodies, the control of the pathogenic burden, and the resolution of infection. Our studies thus show that the IFN-$\gamma$ produced by antigen-specific $CD4^+$ T cells regulates the mucosal immune

response to *C. rodentium* infection and consequently eradication of the mucosal pathogen.

## MATERIALS AND METHODS

### Animals.

Female or male 3- to 4-week-old C57BL/6 mice were purchased from CLEA Japan (Osaka, Japan). OT-II (1), IL-4$^{-/-}$ (25), and RAG1-deficient (RAG1$^{-/-}$) mice (14) (C57BL/6 background) were obtained from The Jackson Laboratory (Bar Harbor, ME), and IFN-$\gamma^{-/-}$ mice (21) (C57BL/6 background) were kindly provided by S. Iwakura (Tokyo University, Japan). OT-II mice were crossed with IL-4$^{-/-}$ and IFN-$\gamma^{-/-}$ mice and those with homozygous mutations in their IL-4 and IFN-$\gamma$ genes were used in this study. C57BL/6/OT-II, IL-4$^{-/-}$/OT-II, and IFN-$\gamma^{-/-}$/OT-II mice were used as donors in T cell transfer studies. All mice were housed and bred in the Animal Unit of the Kobe University School of Medicine in a specific-pathogen-free facility under an approved experimental protocol.

### Antibodies.

CD4-PE-Cy5.5 (clone RM4-5) antibody was purchased from eBioscience (San Diego, CA). T-cell Receptor (TCR) Vβ5.1-PE (clone MR9-4), TCR Vβ5.1-biotin (clone MR9-4), CD69-PE (clone H1.2F3), TCR Vα2-FITC (clone

B20.1), and streptavidin-PerCP-Cy5.5 CD11b-PE (clone M1/70) antibodies were obtained from BD Bioscience (San Jose, CA). Rabbit anti-OVA polyclonal antibody was purchased from Rockland (Gilbertsville, PA).

**Establishment of *C. rodentium* expressing OVA and GFP.**

*C. rodentium* strain DBS100 (catalog no. 51459; ATCC) was kindly provided by Gad Frankel (Division of Cell and Molecular Biology, Imperial College London, United Kingdom). Constitutively OVA-expressing *C. rodentium* (OVA-*C. rodentium*) was created by electroporation of a plasmid vector encoding the chicken OVA construct (28) under the control of the two *gal* operon promoters and a kanamycin-resistance gene into *C. rodentium* strain DBS100 (13). Green fluorescent protein (GFP)-expressing *C. rodentium* (GFP-*C. rodentium*) was kindly provided by C. Sasakawa (Tokyo University, Japan).

**T cell proliferation assay.**

$CD4^+$ T cells were positively selected by magnetic bead sorting with MACS immunobeads (Miltenyi Biotec Inc., Bergisch Gladbach, Germany). The purity of

the $CD4^+$ T cells was usually over 95% according to examination with a FACS Calibur flow cytometer (BectonDickinson, Franklin Lakes, NJ). For the T cell proliferation assays, splenic $CD4^+$ T cells ($1\times10^6$ cells/ml) purified from OT-II mice were labeled with carboxyfluoroscein succinimidyl ester (CFSE) (CellTrace™ CESE Cell Proliferation Kit; Molecular Probes, Eugene, OR). After antigen-presenting cell (APC) incubated with bacterial sonicates from OVA-*C. rodentium* or wild type *C. rodentium* (WT-*C. rodentium*), the APC were incubated with CFSE-labeled $CD4^+$ T cells for 24 hours. The CFSE-labeled $CD4^+$ T cells were stained with anti-CD4 and anti-TCR Vβ5.1 antibodies, and the CFSE intensity of the $CD4^+$ T cells gated on TCR Vβ5.1-positive cells was measured by flow cytometry. Splenocytes ($1\times10^7$ cells/ml) from C57BL/6 mice were used as APC after irradiation with 70Gy.

**Protocol for the induction of colitis by OVA-*C. rodentium* infection.**

OVA-*C. rodentium* was cultured in LB broth medium containing 20 μg/ml kanamycin (Wako, Osaka, Japan) for 6 hour at 37°C with shaking. After 6 hour, the bacterial density was assessed using absorbance at an optical density of 600 nm and confirmed by plating of serial dilutions. Four-week-old mice were orally

inoculated with $1\times10^9$ CFU of OVA-*C. rodentium* using a gavage needle. To ensure maintenance of the plasmid during the *in vivo* infection, 0.1 mg/ml kanamycin was added to the drinking water. Body weight, bacterial concentration in feces, and the histological findings of the colon were assessed for 4 weeks after inoculation. The anti-OVA IgG levels in sera and feces were examined by an enzyme-linked immunosorbent assay (ELISA) as described below. Colonic tissues were stained with hematoxylin and eosin and evaluated as described in 'Histological analysis of colon tissue'. Regarding wild type (WT)-*C. rodentium* infection, the mice were not given kanamycin in drinking water.

**Bacterial counts.**

Fresh fecal pellets were collected from mice at indicated time points after infection, weighed, and dissolved in PBS at 100 mg/ml. The mixture was vortexed, and serial dilutions were plated on MacConkey agar plates containing 20 μg/ml kanamycin. Bacterial colonies were counted 24 hour after culture start. OVA-*C. rodentium* with kanamycin-resistance gene were easily distinguished from commensal flora. The presence of *C. rodentium* in the feces of mice infected with

OVA-*C. rodentium* was confirmed by PCR using Tir-specific primers (forward primer: GCGCGAATTCATGCCTATTGGTAATCTTGGTAATAATAAT and reverse primer: GCGCCCCGGGTTAGACGAAACGTTCAACTCCCGGTGTTGT) (26), after DNA was extracted from bacterial colonies which were selected at random.

**Evaluation for the frequency of plasmid loss during infection.**

C57BL/6 mice were orally infected with $1x10^9$ CFU of OVA-*C. rodentium* under the presence of kanamycin in drinking water. Fecal pellets treated as described above were plated on MacConkey agar plates with or without kanamycin, and bacterial colonies were counted. Approximately 150 colonies were selected at random, and the presence of *C. rodentium* in colonies was confirmed by PCR using Tir-specific primers. Regarding PCR-positive colonies, the expression of OVA was confirmed by Immunoblot analysis, and the positive rate of plasmid in feces during OVA-*C. rodentium* infection was evaluated.

**Histological analysis of colon tissue.**

For the histological analysis, the terminal 0.5 cm of colon tissue was

removed and soaked into 10% formalin in PBS. Next, paraffin-embedded sections were prepared and stained with hematoxylin and eosin. The histological analysis was performed according to a previous report (16). For histopathological grading of colitis, five criteria, i.e., hypervascularization, presence of mononuclear cells, epithelial hyperplasia, epithelial injury and presence of granulocytes, were scored from 0 to 3 yielding an additive score between 0 (no colitis) and 15 (maximal colitis activity).

**Determination of anti-OVA antibody in serum or fecal samples.**

Whole blood and fecal pellets were collected from mice infected with OVA-*C. rodentium*. The serum was separated by centrifugation at 16,000 x g for 10 min, and stored at -20$^{o}$C until the following experiments. Fecal pellets were weighed and then added to sterile PBS containing 0.1% sodium azide at 100 mg/ml. The mixture was homogenized by continuous shaking for 10 min on a vortex mixer, and centrifuged at 15,000 x g for 5 min. The supernatants obtained were collected and stored at -80$^{o}$C. For analysis of the antigen-specific antibody response, 96-well plates were coated overnight at 4$^{o}$C with 100 μl of a bicarbonate solution (pH 9.6)

containing 100 μg/ml OVA. After washed with PBS containing 0.05% Tween 20 (PBST), the plates were blocked by addition of 1.5% (wt/vol) bovine serum albumin (BSA) in PBS for 1 hour at 37°C. The plates were then washed twice with PBST before the sera or fecal lysates from individual mice were added and serially diluted in PBST containing 0.2% (wt/vol) BSA, and incubated for 2 hours at 37°C. For the determination of specific IgG titers, the plates were washed with PBST, followed by addition of 100 μl of an anti-mouse IgG-specific horseradish peroxidase (HRP) conjugate (DakoCytomation; Denmark A/S) diluted 1:1000 in PBST containing 0.2% (wt/vol) BSA. After the incubation for 2 hours at 37°C, the plates were washed with PBST, and then the bound antibody was detected by addition of *o*-phenylenediamine substrate and measurement of absorbance at 490 nm.

**Antigenic challenge with OVA-*C. rodentium* and evaluation of early activation and cytokine production in OVA-specific OT-II CD4$^+$ T cells.**

OT-II mice (4 weeks old) were orally inoculated with $1\times10^9$ CFU of OVA-*C. rodentium*. Mesenteric lymph node (MLN) cells were collected from OT-II mice at day 0, 7 and 14 after OVA-*C*. *rodentium* infection. MLN cells were stained with

anti-CD69, anti-TCR vα2, and anti-TCR vβ5.1 antibodies, and the activation of $CD4^+$ T cells determined after gating on TCR vα2-positive and TCR vβ5.1-positive cells as defined by flow cytometry. To evaluate the cytokine production, OVA-specific $CD4^+$ T cells were purified from MLN of the infected OT-II mice at day 0, 7 and 14 using MACS immunobeads. These cells were then re-stimulated with 1 μM OVA peptide when presented by irradiated splenic APC for 24 hours *in vitro*. The production of IFN-γ and IL-4 from the OVA-specific $CD4^+$ T cells was evaluated by intracellular cytokine staining by standard methods as defined by the manufactures (Intracellular Cytokine Staining Starter Kit-Mouse; BD Biosciences, San Jose, CA). C57BL/6 mice (4 weeks old) that received $5\times10^6$ of $CD4^+$ T cells from OT-II mice were orally inoculated with $1\times10^9$ CFU of OVA-*C. rodentium*, and then the expression of CD69 and the production of IFN-γ and IL-4 on OVA-specific $CD4^+$ T cells were determined as described above.

**Adoptive transfer and induction of OVA-*C. rodentium* infection.**

$CD4^+$ T cells were purified from the spleens of C57BL/6/OT-II, IFN-$\gamma^{-/-}$/OT-II, and IL-$4^{-/-}$/OT-II mice using MACS immunobeads as described above. Similarly, B

cells were also purified from the spleens of C57BL/6 mice. Rag1$^{-/-}$ mice (8-10 weeks old) received 1×10$^{7}$ of wild type (C57BL/6) B cells and 5×10$^{6}$ of CD4$^{+}$ T cells from C57BL/6/OT-II, IFN-γ$^{-/-}$/OT-II, or IL-4$^{-/-}$/OT-II mice by the intravenous injection. All recipients were orally inoculated with 1×10$^{9}$ CFU of OVA-*C. rodentium* at 5 days after the adoptive transfer. Body weight, bacterial concentration in feces, anti-OVA antibody level, and histological findings of the colon were examined for 4 weeks after the inoculation with OVA-*C. rodentium* as described above. Similarly, IFN-γ$^{-/-}$ recipients that transferred C57BL/6/OT-II or IFN-γ$^{-/-}$/OT-II cells were infected with 1×10$^{9}$ CFU of OVA-*C. rodentium*, and the body weight, the bacterial concentration in feces, serum anti-OVA IgG levels and the histological findings of colon tissue were evaluated. All the mice were given kanamycin in drinking water during infection.

**Measurement of antigen-induced antigen-specific T cell proliferation in the presence of IFN-γ or IL-4.**

OVA-specific CD4$^{+}$ T cells were purified from the spleens of OT-II mice using MACS immunobeads and were then labeled with CFSE. After APC were

incubated with the bacterial sonicates from OVA-*C. rodentium* for 16 hours with or without 100 U/ml recombinant murine IFN-γ (rIFN-γ) (PEPROTECH, Rocky Hill, NJ) or 30 ng/ml recombinant murine IL-4 (rIL-4) (PEPROTECH), OVA-specific $CD4^+$ T cells were incubated with the APC for 24 hours and stained with anti-CD4 and anti-TCR Vβ5.1 antibodies. T cell proliferation was determined by measuring the CFSE intensity of $CD4^+$ T cells after gating on TCR Vβ5.1-positive cells by flow cytometry.

**Evaluation of proliferation in IFN-γ or IL-4 deficient antigen-specific $CD4^+$ T cells.**

OVA-specific $CD4^+$ T cells were purified from the spleens of IFN-$\gamma^{-/-}$/OT-II, C57BL/6/OT-II and IL-$4^{-/-}$/OT-II mice using MACS immunobeads and were then labeled with CFSE. APC cultured with the bacterial sonicates from OVA-*C. rodentium* were incubated with respective CFSE-labeled OVA-specific $CD4^+$ T cells for 24 hours and then stained with anti-CD4 and anti-TCR Vβ5.1 antibodies. Proliferation of T cells was determined measuring the CFSE intensity of $CD4^+$ T cells after gating on TCR Vβ5.1-positive cells by flow cytometry.

**Preparation of peritoneal macrophages and evaluation of phagocytic activity.**

Macrophages were prepared as described previously (13). Briefly, C57BL/6 mice were injected with 1 ml of 3% thioglycolate into the peritoneal cavity. The peritoneal cells were harvested by peritoneal lavage with 10 ml cold sterile PBS twice. Peritoneal macrophages were treated with 100 U/ml rIFN-γ or 30 ng/ml rIL-4 for 16 hours and incubated with GFP-*C. rodentium* for 60 min. The cells were then stained with TOPRO-3 and Alexa Fluor 546-conjugated phalloidin and examined by confocal microscopy (LSM5Pascal; Carl Zeiss, Germany). The cells were also stained with anti-CD11b-PE antibody, and phagocytic activity was determined as green fluorescence intensity per CD11b-positive cell by flow cytometry.

**Statistics.**

Statistical significance was determined by the two-tailed Student's *t* test. *P* values less than 0.05 were considered significant.

## RESULTS

### Immunogenicity of *C. rodentium* constitutively expressing OVA

To assess the host antigen-specific T cell responses during *C. rodentium* infection, *C. rodentium* constitutively expressing OVA was generated as previously described (13, 27). As shown in Fig. 1A, the OVA protein expression could be detected in OVA-*C. rodentium*, but not in WT-*C. rodentium*. The bacterial sonicates from OVA-*C. rodentium* when presented by APC were also able to stimulate OVA-specific $CD4^+$ T cells from OT-II mice *in vitro* as defined by division of the CFSE signal consistent with proliferation (Fig. 1B). These results indicate that the OVA expressed by *C. rodentium* is recognized by OVA-specific $CD4^+$ T cells.

Given these results, the antigenicity of OVA-*C. rodentium in vivo* was also investigated by measuring the serum anti-OVA IgG level in C57BL/6 mice after infection with OVA-*C. rodentium* or WT-*C. rodentium*. As shown in Fig. 1C, the serum anti-OVA IgG levels were significantly elevated in mice infected with OVA-*C. rodentium* at 7, 14 and 28 days after infection. The presence of *C. rodentium* in the feces of mice infected with OVA-*C. rodentium* at 7, 14 and 28 days after

infection was confirmed by PCR from bacterial colonies using Tir-specific primers (26) (Fig. 1D and E). The protein expression of OVA in these bacterial colonies was also confirmed by immunoblot analysis (Fig. 1F). Thus, OVA-*C. rodentium* possesses OVA-specific immunogenicity making of an appropriate model to assess *C. rodentium*-specific immune responses *in vivo*. Furthermore, to determine the frequency of plasmid loss during infection, C57BL/6 mice were orally infected with $1x10^9$ CFU of OVA-*C. rodentium* under the presence of kanamycin in drinking water. Fecal pellets were plated on MacConkey agar plates with or without kanamycin, and then the number of bacterial colonies, Tir-PCR prevalence, and OVA prevalence were examined. As results, a comparable rate of bacterial colonies (data not shown), the prevalence of Tir-PCR and OVA were observed (table 1, Fig. 1E and F). When C57BL/6 mice were orally infected with $1x10^9$ CFU of WT-*C. rodentium* under the presence of kanamycin in drinking water, there were little bacterial colonies from fecal pellets of the mice for 4 weeks after inoculation, and the colonic tissue was a normal state (data not shown). These results suggest that there is little frequency of plasmid loss during the infection and that the colonic damage is not caused by *C. rodentium* without plasmid even if OVA-*C. rodentium*

lost plasmid.

**OVA-*C. rodentium* can induce colitis similar to WT-*C. rodentium***

It was reported that T cells and B cells play important roles in eradicating *C. rodentium* (19, 22). To evaluate the roles of T cells and B cells in OVA-*C. rodentium* infection, C57BL/6 mice and Rag1$^{-/-}$ mice were orally infected with $1\times10^{9}$ CFU of OVA-*C. rodentium*. Rag1$^{-/-}$ mice exhibited a loss of body weight (Fig. 2A) and higher bacterial concentrations in their feces at 14 and 21 days after infection (Fig. 2B) compared to C57BL/6 mice. Moreover, *C. rodentium* infection was lethal in Rag1$^{-/-}$ mice by 21 days after OVA-*C. rodentium* infection. This was associated with decreased histological injury in the Rag1$^{-/-}$ mice in comparison to the C57BL/6 mice as assessed at day 14 of infection (Fig. 2C). Together these studies in Rag1$^{-/-}$ mice show the importance of adaptive immunity in clearing *C. rodentium* infection.

When C57BL/6 mice were examined for responses to WT-*C. rodentium* or OVA-*C. rodentium*, a comparable degree of body weight loss (Fig. 2A), burden of bacteria (Fig. 2B) and pathology (Fig. 2C and data not shown) were observed.

These results were consistent with a previous report by Vallance et al. (22) and indicate that OVA-*C. rodentium* has the same pathogenicity as WT-*C. rodentium*.

**Susceptibility to *C. rodentium* is enhanced in the absence of IFN-γ**

To determine whether Th1 and/or Th2 cytokines are important for protection against *C. rodentium* infection, the IFN-$\gamma^{-/-}$, IL-$4^{-/-}$, and C57BL/6 mice were orally infected with OVA-*C. rodentium*. IFN-$\gamma^{-/-}$ mice exhibited a loss of body weight (Fig. 3A) and a higher bacterial concentration in their feces at 14, 21 and 28 days after infection in comparison to IL-$4^{-/-}$ and C57BL/6 mice (Fig. 3B). Although delayed, the IFN-$\gamma^{-/-}$ mice were able to successfully clear OVA-*C. rodentium* from their feces by 35 days after infection (data not shown). The IFN-$\gamma^{-/-}$ mice also exhibited a significant delay in the development of antigen-specific IgG levels at days 7 and 14 after infection in both serum and feces in comparison to the IL-$4^{-/-}$ and C57BL/6 mice (Fig. 3C and D). Macroscopic and microscopic injury was also more severe in the IFN-$\gamma^{-/-}$ mice than that observed in the IL-$4^{-/-}$ and C57BL/6 mice at days 14 and 28. The submucosa and lamina propria of the infected IFN-$\gamma^{-/-}$ mice exhibited a greater number of mononuclear cells and neutrophils in the tissue, and increased

crypt hyperplasia (Fig. 3E). This was supported by a quantitative evaluation of the histological findings as shown in Fig. 3F. Consistent with this, the colons of the IFN-$\gamma^{-/-}$ mice were shorter and more edematous than that of the IL-$4^{-/-}$ and C57BL/6 mice (data not shown). In contrast, there was no inflammatory response observed in the IL-$4^{-/-}$ or C57BL/6 mice on day 28 after infection. These results indicate that IFN-$\gamma^{-/-}$ mice are more susceptible to *C. rodentium* infection than either IL-$4^{-/-}$ or C57BL/6 mice.

**Th1 or Th2 Cytokine production from antigen specific $CD4^+$ T cells during *C. rodentium* infection**

To assess the cytokine profile from antigen-specific $CD4^+$ T cells during native *C. rodentium* infection, OT-II mice were orally infected with OVA-*C. rodentium*. First, to determine the early activation of $CD4^+$ T cells, the expression of CD69 on OVA-specific OT-II $CD4^+$ T cells purified from MLN during OVA-*C. rodentium* infection was investigated. At day 7 after infection, the expression levels of CD69 were elevated but observed to decrease at day 14 after infection (Fig. 4A). Next, OVA-specific OT-II $CD4^+$ T cells were purified from MLN at day 7

and 14 after OVA-*C*. *rodentium* infection. These cells were re-stimulated with APC that were treated with OVA peptide for 24 hours *in vitro*, before the measurement of IFN-γ and IL-4 by an intracellular cytokine staining method. At days 7 and 14, the IFN-γ levels produced by OVA-specific OT-II $CD4^+$ T cells were observed to be progressive in elevated (Fig. 4B). In contrast, there was no evidence of IL-4 production of OVA-specific OT-II $CD4^+$ T cells during OVA-*C. rodentium* infection (Fig. 4B). However, the exaggerated responses might be induced in OT-II mice infected with OVA-*C. rodentium*, because the precursor frequency of OVA-specific $CD4^+$ T cells in these T cell receptor transgenic mice is too high. Therefore, OVA-specific OT-II $CD4^+$ T cells were transferred into C57BL/6 mice, and then these C57BL/6 recipients were infected with OVA-*C. rodentium* followed by measurement of the expression levels of CD69, IFN-γ and IL-4 on OVA-specific OT-II $CD4^+$ T cells. The expressions of CD69, IFN-γ and IL-4 were consistent with results of OT-II mice directly infected with OVA-*C. rodentium*. (Fig. 4C and D). These results indicate that IFN-γ is a dominant cytokine produced by antigen-specific $CD4^+$ T cells in response to *C. rodentium* infection.

**IFN-γ produced by antigen specific CD4$^+$ T cells plays an important role in the prevention of *C. rodentium* infection**

CD4$^+$ T cells and B cells play important roles in clearance of *C. rodentium*, because CD4$^+$ T cell and B cell deficient mice failed to eradicate *C. rodentium* infection (2, 11, 19). To determine the contribution of antigen-specific CD4$^+$ T cell expressed IFN-γ and IL-4, IFN-γ$^{-/-}$/OT-II, IL-4$^{-/-}$/OT-II, and C57BL/6/OT-II cells were adoptively transferred into Rag1$^{-/-}$ recipients that received wild type B cells. Subsequently, these Rag1$^{-/-}$ recipients were infected with OVA-*C. rodentium*. The mice that received the IFN-γ$^{-/-}$/OT-II cells, despite the presence of B cells, exhibited the significant loss of body weight (Fig. 5A) and greater concentration of *C. rodentium* in their feces at 21 and 28 days after infection (Fig. 5B) in comparison to either Rag1$^{-/-}$ mice that received IL-4$^{-/-}$/OT-II or C57BL/6/OT-II cells. Importantly, Rag1$^{-/-}$ mice that received IL-4$^{-/-}$/OT-II or C57BL/6/OT-II cells exhibited similar to body weight loss and bacterial burden. Consistent with these observations, the Rag1$^{-/-}$ mice that received IFN-γ$^{-/-}$/OT-II cells exhibited decease of OVA-specific IgG levels on days 7 and 14 in sera and on day 14 in feces in comparison to the Rag1$^{-/-}$ mice that received C57BL/6/OT-II or IL-4$^{-/-}$/OT-II cells (Fig. 5C and 5D). Consistent

with the increased pathogen burden, the mice that received IFN-$\gamma^{-/-}$/OT-II cells demonstrated more severe histological injury than the mice that received C57BL/6/OT-II or IL-$4^{-/-}$/OT-II cells on days 14 and 28 after infection (Fig. 5E and 5F). By 35 days after infection, the $Rag1^{-/-}$ mice that received IFN-$\gamma^{-/-}$/OT-II cells were able to clear infection. However, in these models, IFN-$\gamma$ produced by cells except $CD4^+$ T cells, for example, NK cells, neutrophils, macrophages and dendritic cells, might also promote key functions against *C. rodentium* infection. To remove the possible effects of IFN-$\gamma$ produced from cells except $CD4^+$ T cells, IFN-$\gamma^{-/-}$ recipients that transferred IFN-$\gamma^{-/-}$/OT-II cells or C57BL/6/OT-II cells were infected with OVA-*C. rodentium*, and the body weight, the bacterial concentration in feces, the serum anti-OVA IgG levels and the histological findings of colon tissue were assessed for 4 weeks after inoculation. The IFN-$\gamma^{-/-}$ mice that received C57BL/6/OT-II cells exhibited the significant increase in the body weight (Fig. 5G) and the lesser concentration of *C. rodentium* in feces at 14, 21 and 28 days after infection (Fig. 5H) compared with the IFN-$\gamma^{-/-}$ mice that received IFN-$\gamma^{-/-}$/OT-II cells. Consistent with these observations, the IFN-$\gamma^{-/-}$ mice that received C57BL/6/OT-II cells revealed the increase in the serum OVA-specific IgG levels on days 7 and 14

in comparison to the IFN-$\gamma^{-/-}$ mice that received IFN-$\gamma^{-/-}$/OT-II cells (Fig. 5I). In addition, the IFN-$\gamma^{-/-}$ mice that received C57BL/6/OT-II cells exhibited the milder histological injury than the IFN-$\gamma^{-/-}$ mice that received IFN-$\gamma^{-/-}$/OT-II cells (Fig. 5J and K). These results indicate that the IFN-$\gamma$ produced by antigen-specific $CD4^+$ T cells plays an important role in the susceptibility to *C. rodentium* at a critical period after infection.

**IFN-$\gamma$ effectively induced antigen-specific T-cell activation**

These studies show that susceptibility to *C. rodentium* is dependent upon IFN-$\gamma$. Therefore, antigen specific T cell responses during *C. rodentium* infection in the presence of IFN-$\gamma$ were evaluated. APC were cultured with bacterial sonicates from OVA-*C. rodentium* for 16 hours with or without IFN-$\gamma$ or IL-4. Subsequently, the antigen loaded APC were incubated with CFSE-labeled OVA-specific $CD4^+$ T cells from OT-II mice for 24 hours. T cell responses were then examined by flow cytometry. The presence of IFN-$\gamma$ led to proliferation of OVA-specific $CD4^+$ T cells as demonstrated by increased cell divisions compared to APC that were loaded with OVA-*C. rodentium* without IFN-$\gamma$ (Fig. 6A). In contrast, the presence of IL-4

decreased induction of OVA-specific $CD4^+$ T cells proliferation by OVA-*C. rodentium* loaded APC (Fig. 6A). APC that were loaded with WT-*C. rodentium* did not enhance the proliferation of OVA-specific $CD4^+$ T cells (data not shown). Furthermore, to examine whether IFN-γ produced by antigen-specific $CD4^+$ T cells is involved in infection-induced acquired immune responses, the proliferation of OVA-specific $CD4^+$ T cells from C57BL/6/OT-II, IFN-$\gamma^{-/-}$/OT-II and IL-$4^{-/-}$/OT-II mice was evaluated *in vitro*. APC cultured with bacterial sonicates from OVA-*C. rodentium* were incubated with CFSE-labeled OVA-specific $CD4^+$ T cells from IFN-$\gamma^{-/-}$/OT-II, C57BL/6/OT-II or IL-$4^{-/-}$/OT-II mice for 24 hours. The proliferation of OVA-specific $CD4^+$ T cells was upregulated in C57BL/6/OT-II mice compared with IFN-$\gamma^{-/-}$/OT-II mice as shown by an enhancement of cell divisions (Fig. 6B). On the other hand, $IL4^{-/-}$/OT-II cells led to the similar degree of cell proliferation to C57BL/6/OT-II cells (data not shown). These results indicate that IFN-γ produced by antigen-specific $CD4^+$ T cells can upregulate the ability of APC to stimulate the proliferation of antigen-specific T cells and effectively induce acquired immune responses during *C. rodentium* infection.

**IFN-γ enhances the macrophage phagocytic activity that is directed against *C. rodentium* infection**

Finally, the phagocytic activity of macrophages in response to *C. rodentium* in the presence of IFN-γ or IL-4 was investigated. A genetically engineered *C. rodentium* strain that constitutively expresses GFP (GFP-*C. rodentium*) was used. Flow cytometry analysis and confocal microscopy confirmed the expression of GFP in GFP-*C. rodentium* but not in control *C. rodentium* (Fig. 7A). Peritoneal macrophages from C57BL/6 mice were collected after peritoneal injection of thioglycolate and cultured with GFP-*C. rodentium* for 60 min with or without IFN-γ or IL-4. The cells were stained with TOPRO-3 and phalloidin, and the number of GFP signals per macrophage was examined by confocal microscopy. IFN-γ increased the number of GFP-*C. rodentium* incorporated into macrophages compared to the vehicle control (Fig. 7B). On the other hand, a marked decrease in the GFP-*C. rodentium* uptake by macrophages after treatment with IL-4 was observed. In addition, similar results were obtained by measurement of the phagocytic activity as defined by flow cytometry which detected the GFP signal in CD11b positive cells (Fig. 7C). These results indicate that IFN-γ enhances the phagocytic activity of

macrophages in response to *C. rodentium*.

## DISCUSSION

In this work, we demonstrated that IFN-γ produced by the antigen-specific $CD4^+$ T cells regulates the mucosal immune response to *C. rodentium* infection. From the experiments using immune cell-deficient mice, $CD4^+$ T cells, B cells and IgG, but not secretory IgA or IgM were reported to play critical roles in the eradication of *C. rodentium* (15). This pathogen is therefore appropriate for evaluating the roles of $CD4^+$ T cells and moreover the Th1 and Th2 cytokines produced by $CD4^+$ T cells in defense of the mucosal tissue against bacterial infection. In our studies, *C. rodentium* expressing OVA, a model antigen, was used to investigate antigen specific immune responses. The pathogenesis exerted by OVA-*C. rodentium* was similar to that of *C. rodentium*. This experimental model has at least three benefits. First, adaptive immunity to *C. rodentium* requires the development of systemic and $CD4^+$ T cell-dependent antibody responses (3). The OVA tag expressed by OVA-*C. rodentium* is non-cross-reactive with antigens in the colonic fluid, and so antigen-specific antibody responses can be assessed without affecting other factors. Second, our

model can be used to evaluate various aspects of antigen-specific $CD4^+$ T cell functions relevant to antimicrobial immunity with transgenic mice such as OT-II mice. Finally, OVA-*C*. *rodentium* possesses a kanamycin-resistance gene. Therefore, OVA-*C*. *rodentium* is able to easily distinguish from commensal flora. Furthermore, the antibiotic treatment diminishes the load of bacteria in the colon, which also may have abolished the secondary actions that are caused by other bacteria in the colon with a disrupted epithelial barrier. Thus, our experimental model is suitable for examining the antigen specific immune responses against *C. rodentium* infection.

IFN-γ is an immunoregulatory cytokine crucially involved in a wide range of infectious diseases. It has been reported that IFN-γ promotes immune responses at the initiation of several bacterial infections; i.e., macrophage activation, neutrophil recruitment, Th1-cell development, Th2 response inhibition and epithelial defense including the induction of antimicrobial defenses (17, 20). The colonic tissue from mice infected with *C. rodentium* exhibited the increased expression of IFN-γ and more mucosal damage compared to that of uninfected mice (6). The importance of IFN-γ expression was confirmed by the marked increase in the susceptibility of IFN-$\gamma^{-/-}$ mice to OVA-*C. rodentium* infection. Moreover, IFN-γ promoted the efficacy

of antigen-specific T cell activation and macrophage phagocytosis during *C. rodentium* infection as well as the production of anti-bacterial IgG. The deficiency of IFN-γ may lead to the decrease in efficiency of various aspects of antigen-presenting cell functions relevant to antimicrobial immunity, including endocytosis, phagocytosis, and antigen-presenting cell activation, resulting in a less mature phenotype of the latter types of cells. Consistent with these phenomena, in the absence of IFN-γ produced by antigen-specific $CD4^+$ T cells, there is less stimulation of antigen-specific T cells and thus adaptive immunity. This stimulation of T cells is associated with the increased B-cell production of bacterium-specific IgGs. The increased susceptibility to *C. rodentium* infection in the context of IFN-γ deficiency can thus be explained by a broad decline in the efficacy of the immune response against this mucosal pathogen.

The lymphocytic host responses to *C. rodentium* are characterized by a large infiltration of $CD4^+$ T cells into the colonic lamina propria and highly polarized Th1 responses (5, 6). $CD4^+$ T cell deficient mice failed to clear *C. rodentium* infection, and their titers of serum anti-*C. rodentium* antibody were significantly reduced compared to those of the control mice. $CD4^+$ T cells thus play an

important role in the mucosal and systemic immune response to this bacterium. In addition, our studies demonstrated that the level of IFN-γ was higher than other cytokines produced by antigen-specific $CD4^+$ T cells during *C. rodentium* infection. Moreover, our adaptive transfer model exhibited that IFN-γ deficiency from antigen-specific $CD4^+$ T cells was more susceptible to this bacterial infection and a significant delay in the development of antigen-specific IgG levels. In contrast to our results, a previous report showed that IFN-γ deficiency did not affect the bacterial burden or the development of protective antibodies in IFN-$\gamma^{-/-}$ mice and adoptively transferred $CD4^{-/-}$ recipients during *C. rodentium* infection (3). We speculate some possibilities in these distinct results, although the reason for this discrepancy is unclear. First, more than double the amount of *C. rodentium* were administered in our experiments compared with the experiments by *et al* (ref), possibly resulting in the difference of bacterial burden. Second, other T cells such as $CD8^+$ T cells and NKT cells, of which the involvement was not eliminated in our experimental conditions (Fig. 5A-F), might be involved in development of protective antibodies, although it was reported that CD8+ T cells and NKT cells could not be associated with elimination of *C. rodentium*.(ref) Finally, most importantly, our

unique experimental system using *C. rodentium* expressing OVA might have allowed for an enhanced activation of antigen-specific $CD4^+$ T cells, leading to strong immune responses for the eradication of *C. rodentium*. Furthermore, adoptive transfer of OVA-specific $CD4^+$ T cells from C57BL/6/OT-II mice into IFN-γ recipients were decreased in the susceptibility to OVA-*C. rodentium* (Fig. 5G-K). Therefore, it is suggested that IFN-γ from antigen-specific $CD4^+$ T cells promote the key function against *C. rodentium* infection.

A unique aspect of our study is the regulation of anti-bacterial IgG by IFN-γ from antigen specific $CD4^+$ T cells. The protective effects of IgG might contribute to the complement-fixing activity of high affinity binding to activated FcγRs, such as FcγRI receptors and FcγRII/III receptors (13, 18). Bacterium-specific IgG plays a critical role in *C. rodentium* eradication. Therefore, the decreased production of IgG may be associated with susceptibility to *C. rodentium* infection. The results from these studies support an important role for the IFN-γ produced by antigen-specific $CD4^+$ T cells in coordinating the innate and adaptive immune responses to this pathogen.

In conclusion, IFN-γ produced by antigen-specific $CD4^+$ T cells regulates a

variety of important processes in the protection against *C. rodentium* infection. This indicates the development of cellular and humoral (IgG-) immunity, the enhancement of microbiocidal activity by immune cells. Thus IFN-γ produced by antigen-specific $CD4^+$ T cells in response to *C. rodentium* regulates innate and adaptive immune pathways in response to primary infection at the mucosal surface.

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## REFERENCES


1. **Barnden, M. J., J. Allison, W. R. Heath, and F. R. Carbone.** 1998. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. Immunol Cell Biol **76:**34-40.
2. **Bry, L., and M. B. Brenner.** 2004. Critical role of T cell-dependent serum antibody, but not the gut-associated lymphoid tissue, for surviving acute mucosal infection with Citrobacter rodentium, an attaching and effacing pathogen. J Immunol **172:**433-441.
3. **Bry, L., M. Brigl, and M. B. Brenner.** 2006. CD4+-T-cell effector functions and costimulatory requirements essential for surviving mucosal infection with Citrobacter rodentium. Infect Immun **74:**673-681.
4. **Frankel, G., A. D. Phillips, M. Novakova, H. Field, D. C. Candy, D. B. Schauer, G. Douce, and G. Dougan.** 1996. Intimin from enteropathogenic Escherichia coli restores murine virulence to a Citrobacter rodentium eaeA mutant: induction of an immunoglobulin A response to intimin and EspB. Infect Immun **64:**5315-5325.
5. **Higgins, L. M., G. Frankel, I. Connerton, N. S. Goncalves, G. Dougan, and T. T. MacDonald.** 1999. Role of bacterial intimin in colonic hyperplasia and inflammation. Science **285:**588-591.
6. **Higgins, L. M., G. Frankel, G. Douce, G. Dougan, and T. T. MacDonald.** 1999. Citrobacter rodentium infection in mice elicits a mucosal Th1 cytokine response and lesions similar to those in murine inflammatory bowel disease. Infect Immun **67:**3031-3039.
7. **Ishigame, H., S. Kakuta, T. Nagai, M. Kadoki, A. Nambu, Y. Komiyama, N. Fujikado, Y. Tanahashi, A. Akitsu, H. Kotaki, K. Sudo, S. Nakae, C. Sasakawa, and Y. Iwakura.** 2009. Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses. Immunity **30:**108-119.
8. **Kaper, J. B., J. P. Nataro, and H. L. Mobley.** 2004. Pathogenic Escherichia

coli. Nat Rev Microbiol **2:**123-140.

9. **LeibundGut-Landmann, S., O. Gross, M. J. Robinson, F. Osorio, E. C. Slack, S. V. Tsoni, E. Schweighoffer, V. Tybulewicz, G. D. Brown, J. Ruland, and C. Reis e Sousa.** 2007. Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. Nat Immunol **8:**630-638.
10. **Luperchio, S. A., and D. B. Schauer.** 2001. Molecular pathogenesis of Citrobacter rodentium and transmissible murine colonic hyperplasia. Microbes Infect **3:**333-340.
11. **Maaser, C., M. P. Housley, M. Iimura, J. R. Smith, B. A. Vallance, B. B. Finlay, J. R. Schreiber, N. M. Varki, M. F. Kagnoff, and L. Eckmann.** 2004. Clearance of Citrobacter rodentium requires B cells but not secretory immunoglobulin A (IgA) or IgM antibodies. Infect Immun **72:**3315-3324.
12. **Mangan, P. R., L. E. Harrington, D. B. O'Quinn, W. S. Helms, D. C. Bullard, C. O. Elson, R. D. Hatton, S. M. Wahl, T. R. Schoeb, and C. T. Weaver.** 2006. Transforming growth factor-beta induces development of the T(H)17 lineage. Nature **441:**231-234.
13. **Masuda, A., M. Yoshida, H. Shiomi, S. Ikezawa, T. Takagawa, H. Tanaka, R. Chinzei, T. Ishida, Y. Morita, H. Kutsumi, H. Inokuchi, S. Wang, K. Kobayashi, S. Mizuno, A. Nakamura, T. Takai, R. S. Blumberg, and T. Azuma.** 2008. Fcgamma receptor regulation of Citrobacter rodentium infection. Infect Immun **76:**1728-1737.
14. **Mombaerts, P.** 1995. Lymphocyte development and function in T-cell receptor and RAG-1 mutant mice. Int Rev Immunol **13:**43-63.
15. **Mundy, R., T. T. MacDonald, G. Dougan, G. Frankel, and S. Wiles.** 2005. Citrobacter rodentium of mice and man. Cell Microbiol **7:**1697-1706.
16. **Neurath, M. F., B. Weigmann, S. Finotto, J. Glickman, E. Nieuwenhuis, H. Iijima, A. Mizoguchi, E. Mizoguchi, J. Mudter, P. R. Galle, A. Bhan, F. Autschbach, B. M. Sullivan, S. J. Szabo, L. H. Glimcher, and R. S. Blumberg.** 2002. The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease. J Exp Med **195:**1129-1143.
17. **Ohteki, T., T. Fukao, K. Suzue, C. Maki, M. Ito, M. Nakamura, and S.**

**Koyasu.** 1999. Interleukin 12-dependent interferon gamma production by CD8alpha+ lymphoid dendritic cells. J Exp Med **189:**1981-1986.

18. **Ravetch, J. V., and J. P. Kinet.** 1991. Fc receptors. Annu Rev Immunol **9:**457-492.
19. **Simmons, C. P., S. Clare, M. Ghaem-Maghami, T. K. Uren, J. Rankin, A. Huett, R. Goldin, D. J. Lewis, T. T. MacDonald, R. A. Strugnell, G. Frankel, and G. Dougan.** 2003. Central role for B lymphocytes and CD4+ T cells in immunity to infection by the attaching and effacing pathogen Citrobacter rodentium. Infect Immun **71:**5077-5086.
20. **Simmons, C. P., N. S. Goncalves, M. Ghaem-Maghami, M. Bajaj-Elliott, S. Clare, B. Neves, G. Frankel, G. Dougan, and T. T. MacDonald.** 2002. Impaired resistance and enhanced pathology during infection with a noninvasive, attaching-effacing enteric bacterial pathogen, Citrobacter rodentium, in mice lacking IL-12 or IFN-gamma. J Immunol **168:**1804-1812.
21. **Tagawa, Y., K. Sekikawa, and Y. Iwakura.** 1997. Suppression of concanavalin A-induced hepatitis in IFN-gamma(-/-) mice, but not in TNF-alpha(-/-) mice: role for IFN-gamma in activating apoptosis of hepatocytes. J Immunol **159:**1418-1428.
22. **Vallance, B. A., W. Deng, L. A. Knodler, and B. B. Finlay.** 2002. Mice lacking T and B lymphocytes develop transient colitis and crypt hyperplasia yet suffer impaired bacterial clearance during Citrobacter rodentium infection. Infect Immun **70:**2070-2081.
23. **van Beelen, A. J., Z. Zelinkova, E. W. Taanman-Kueter, F. J. Muller, D. W. Hommes, S. A. Zaat, M. L. Kapsenberg, and E. C. de Jong.** 2007. Stimulation of the intracellular bacterial sensor NOD2 programs dendritic cells to promote interleukin-17 production in human memory T cells. Immunity **27:**660-669.
24. **Veldhoen, M., R. J. Hocking, R. A. Flavell, and B. Stockinger.** 2006. Signals mediated by transforming growth factor-beta initiate autoimmune encephalomyelitis, but chronic inflammation is needed to sustain disease. Nat Immunol **7:**1151-1156.
25. **von der Weid, T., M. Kopf, G. Kohler, and J. Langhorne.** 1994. The immune response to Plasmodium chabaudi malaria in interleukin-4-deficient

mice. Eur J Immunol **24:**2285-2293.

26. **Wei, O. L., A. Hilliard, D. Kalman, and M. Sherman.** 2005. Mast cells limit systemic bacterial dissemination but not colitis in response to Citrobacter rodentium. Infect Immun **73:**1978-1985.

27. **Yoshida, M., K. Kobayashi, T. T. Kuo, L. Bry, J. N. Glickman, S. M. Claypool, A. Kaser, T. Nagaishi, D. E. Higgins, E. Mizoguchi, Y. Wakatsuki, D. C. Roopenian, A. Mizoguchi, W. I. Lencer, and R. S. Blumberg.** 2006. Neonatal Fc receptor for IgG regulates mucosal immune responses to luminal bacteria. J Clin Invest **116:**2142-2151.

28. **Yoshida, M., Y. Shirai, T. Watanabe, M. Yamori, Y. Iwakura, T. Chiba, T. Kita, and Y. Wakatsuki.** 2002. Differential localization of colitogenic Th1 and Th2 cells monospecific to a microflora-associated antigen in mice. Gastroenterology **123:**1949-1961.

29. **Zheng, Y., P. A. Valdez, D. M. Danilenko, Y. Hu, S. M. Sa, Q. Gong, A. R. Abbas, Z. Modrusan, N. Ghilardi, F. J. de Sauvage, and W. Ouyang.** 2008. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. Nat Med **14:**282-289.

## FIGURE LEGENDS

**Figure 1. Immunogenicity of *C. rodentium* constitutively expressing OVA.**

(A) Establishment of a genetically engineered *C. rodentium* strain that constitutively produces OVA. The expression of OVA in *C. rodentium* was confirmed by immunoblot analysis. As a positive control, OVA proteins were used. The band was not detected by immunoblotting with secondary antibody only (data not shown). (B) APC were cultured with bacterial sonicates from OVA-*C. rodentium* or WT-*C. rodentium*, and then the CFSE-labeled OVA-specific $CD4^+$ T cells purified from OT-II mice were incubated with APC for 24 hours. The number of OVA-specific $CD4^+$ T cells was examined by flow cytometry. The arrows indicate increasing rounds of cell division. (C) The serum OVA-specific IgG level in C57BL/6 mice 7, 14 and 28 days after infection with OVA-*C. rodentium* was evaluated by ELISA. Data are shown as means ± SD (n=6), and two asterisks indicate a significant difference from the values for the mice infected with WT-*C. rodentium* at $p<0.05$ according to the Student's t-test. O.D., optical density. The OVA-specific IgG in serum from the uninfected mice were not detected by ELISA

(data not shown). (D) DNA was extracted from colonies isolated from the feces of mice infected with OVA-*C. rodentium* or WT-*C. rodentium*, and PCR was performed with Tir-specific primers. (E) Fecal pellets from mice infected with OVA-*C. rodentium* were plated on MacConkey agar plates with or without kanamycin, and then the presence of *C. rodentium* in bacterial colonies was confirmed by PCR using Tir-specific primers. (F) The expression of OVA in PCR-positive colonies at plates with kanamycin or without kanamycin was confirmed by immunoblot analysis.

**Figure 2. OVA-*C. rodentium* can induce colitis.**

Susceptibility to infection with $1\times10^9$ CFU of *OVA-C. rodentium* in $Rag1^{-/-}$ mice. $Rag1^{-/-}$ mice and C57BL/6 mice were infected with OVA-*C. rodentium* or WT-*C. rodentium* at $1\times10^9$ CFU. Regarding WT-*C. rodentium* infection, the mice were not given kanamycin in drinking water. (A) The body weight changes in $Rag^{-/-}$ mice and C57BL/6 mice. Data are shown as means ± SD (n=6), and asterisks indicate a significant difference from the values for the uninfected mice at $p<0.05$ according to the Student's t-test. (B) The bacterial loads (CFU/mg) of OVA-*C.*

*rodentium* or WT-*C. rodentium* in feces 7, 14 and 21 days after infection. Data are shown as means ± SD (n=6), and two asterisks indicate a significant difference from the values for the C57BL/6 mice infected with WT-*C. rodentium* at $p<0.05$ according to the Student's t-test. No colonies were detected in feces of the respective uninfected mice (data not shown). (C) Histological findings of the colons of Rag1$^{-/-}$ mice (right) and C57BL/6 mice (middle) at 14 days after OVA-*C. rodentium* infection. Magnification: ×100.

**Figure 3. Susceptibility to OVA-*C. rodentium* in the absence of IFN-γ.**

Susceptibility to infection with $1\times10^9$ CFU of OVA-*C. rodentium* in IFN-$\gamma^{-/-}$ mice, IL-4$^{-/-}$ mice and C57BL/6 mice. (A) The body weight changes in IFN-$\gamma^{-/-}$ mice, IL-4$^{-/-}$ mice, and C57BL/6 mice during OVA-*C. rodentium* infection. Data are shown as means ± SD (n=8), and asterisks indicate a significant difference from the values for the C57BL/6 mice at $p<0.05$ according to the Student's t-test. (B) The bacterial loads (CFU/mg) of OVA-*C*. *rodentium* in feces 7, 14, 21 and 28 days after infection. Data are shown as means ± SD (n=8), and two asterisks indicate a significant difference. No colonies were detected in feces of the respective

uninfected mice (data not shown). (C, D) The OVA-specific IgG levels in serum (C) and feces (D) 7, 14 and 21 days after infection were mesured by ELISA. Data are shown as means ± SD (n=8), and two asterisks indicate a significant difference. The OVA-specific IgG in serum and feces from the respective uninfected mice were not detected by ELISA (data not shown). (E, F) Histological findings (E) and the histological score (F) of the colonic tissue from IFN-$\gamma^{-/-}$, IL-$4^{-/-}$ and C57BL/6 mice were evaluated at 14 and 28 days after infection. Magnification: ×100. Data are shown as means ± SD (n=8), and two asterisks indicate a significant difference. The histological score in the colonic tissue from the uninfected mice was 0 point; i.e., no colitis (data not shown).

**Figure 4. cytokine production in antigen specific $CD4^+$T cells during *C. rodentium* infection.**

OT-II mice were orally inoculated with $1\times10^9$ CFU of OVA-*C. rodentium*. (A) Evaluation of CD69 expression on OVA-specific $CD4^+$ T cells after *in vivo* stimulation with OVA-*C*. *rodentium*. (B) After *in vivo* stimulation with OVA-*C*. *rodentium*, the OVA-specific $CD4^+$ T cells were treated with OVA peptide for 24

hours *in vitro*. The production of IFN-γ and IL-4 was examined by an intracellular cytokine staining method using flow cytometry. (C, D) C57BL/6 that received $5\times10^6$ of $CD4^+$ T cells from OT-II mice were orally infected with $1\times10^9$ CFU of OVA-*C. rodentium*, and the expression of CD69 (C) and the production of IFN-γ and IL-4 (D) on OVA-specific $CD4^+$ T cells were evaluated. Typical images are shown from at least triplicate determinations.

**Figure 5. Cytokines produced from antigen-specific $CD4^+$ T cells regulate *C. rodentium* infection.**

Susceptibility to infection with $1\times10^9$ CFU of OVA-*C. rodentium* in Rag1-/- mice receiving IFN-$\gamma^{-/-}$/OT-II, IL-$4^{-/-}$/OT-II and C57BL/6/OT-II cells. IFN-$\gamma^{-/-}$/OT-II, IL-$4^{-/-}$/OT-II and C57BL/6/OT-II cells were transferred into Rag$1^{-/-}$ mice, which had previously been injected with C57BL/6-B cells, and then these mice were infected with $1\times10^9$ CFU of OVA-*C. rodentium*. (A) The body weights of the mice were measured. Data are shown as means ± SD (n=6), and asterisks indicate a significant difference from the values for the Rag$1^{-/-}$ mice receiving C57BL/6/OT-II cells at $p<0.05$ according to the Student's t-test. (B) The bacterial loads (CFU/mg)

of OVA-*C. rodentium* in feces 7, 14, 21 and 28 days after infection. Data are shown as means ± SD (n=6), and two asterisks indicate a significant difference. No colonies were detected in feces of the respective uninfected mice (data not shown). (C, D) The OVA-specific IgG levels in serum (C) and feces (D) at 7, 14 and 21 days after infection were evaluated by ELISA. Data are shown as means ± SD (n=6), and two asterisks indicate a significant difference. The OVA-specific IgG in serum and feces from the respective uninfected mice were not detected by ELISA (data not shown). (E, F) Histological findings (E) and the histological score (F) of colonic tissue from the respective Rag1$^{-/-}$ recipients were evaluated 14 and 28 days after infection. Magnification: ×100. Data are shown as means ± SD (n=6), and two asterisks indicate a significant difference. The histological score in the colonic tissue from the uninfected mice was 0 point; i.e., no colitis (data not shown). Susceptibility to infection with 1×10$^9$ CFU of *OVA-C. rodentium* in the IFN-$\gamma^{-/-}$ mice receiving IFN-$\gamma^{-/-}$/OT-II or C57BL/6/OT-II cells. (G) The body weights of the mice were measured. Data are shown as means ± SD (n=6), and asterisks indicate a significant difference from the values for the IFN-$\gamma^{-/-}$ mice receiving IFN-$\gamma^{-/-}$/OT-II cells at $p<0.05$ according to the Student's t-test. (H) The bacterial loads (CFU/mg)

of OVA-*C. rodentium* in feces 7, 14, 21 and 28 days after infection. Data are shown as means ± SD (n=6), and two asterisks indicate a significant difference. (I) The OVA-specific IgG levels in serum at 7, 14 and 21 days after infection were evaluated by ELISA. Data are shown as means ± SD (n=6), and two asterisks indicate a significant difference. (J and K) Histological findings (J) and the histological score (K) of colonic tissue from the respective IFN-$\gamma^{-/-}$ recipients were evaluated 14 and 28 days after infection. Magnification: ×100. Data are shown as means ± SD (n=6), and two asterisks indicate a significant difference.

**Figure 6. IFN-γ effectively induces antigen-specific T-cell activation.**

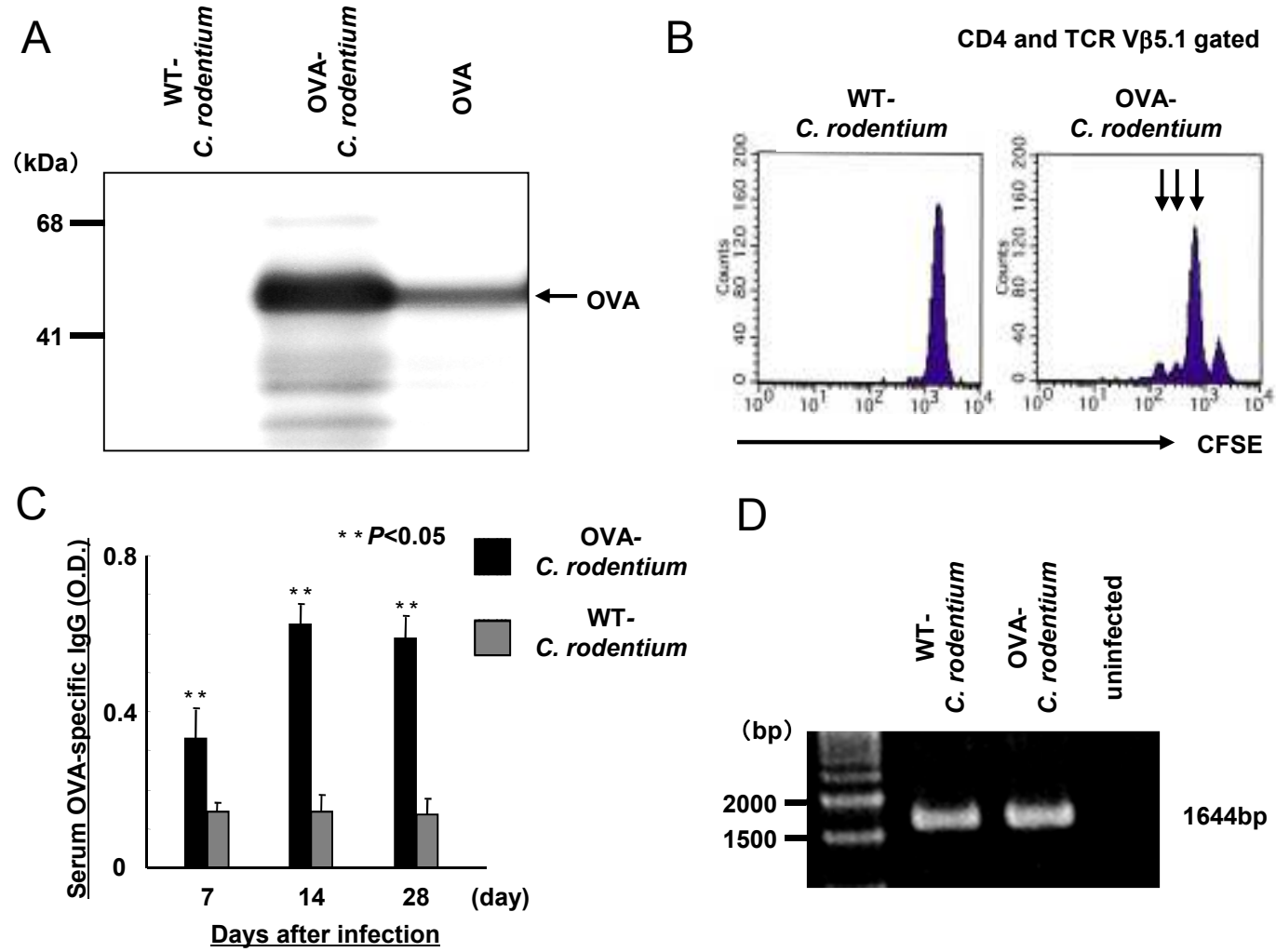
(A) APC were cultured with bacterial sonicates from OVA-*C. rodentium* for 16 hours with or without IFN-γ or IL-4, and then CFSE-labeled OVA-specific OT-II $CD4^+$ T cells from OT-II mice were incubated with the APC for 24 hours. The number of OVA-specific $CD4^+$ T cells in the presence of IFN-γ or IL-4 was examined by flow cytometry. (B) APC cultured with bacterial sonicates from OVA-*C. rodentium* were incubated with CFSE-labeled OVA-specific $CD4^+$ T cells from IFN-$\gamma^{-/-}$/OT-II, $IL4^{-/-}$/OT-II or C57BL/6/OT-II mice for 24 hours, and the T cells

proliferation was evaluated by flow cytometry. IL4$^{-/-}$/OT-II cells led to the similar degree of cell proliferation to C57BL/6/OT-II cells (data not shown). Arrows indicate increasing rounds of cell division. Typical images are shown from at least triplicate determinations.

**Figure 7. Phagocytosis of *C. rodentium* by macrophages is regulated by IFN-γ and IL-4.**

(A) The expression of GFP in *C. rodentium* was confirmed by flow cytometry and confocal microscopy. (B) Peritoneal macrophages were incubated with GFP-*C. rodentium* for 60 min with IFN-γ or IL-4 and then were subjected to confocal microscopy with TOPRO-3 and phalloidin (Green: GFP; Blue: nuclei; Red: actin). Magnification: ×630. (C) Peritoneal macrophages were incubated with GFP-*C. rodentium* for 60 min with IFN-γ or IL-4 and then were subjected to flow cytometry to evaluate the phagocytic activity of macrophages. Typical images are shown from at least triplicate determinations.

Figure 1.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C. rodentium
OVA-
C. rodentium
Counts
CFSE
C
** P<0.05
OVA-
C. rodentium
WT-
C. rodentium
Serum OVA-specific IgG (O.D.)
0.8
0.4
0
7
14
28
(day)
Days after infection
D
WT-
C. rodentium
OVA-
C. rodentium
uninfected
(bp)
2000
1500
1644bp

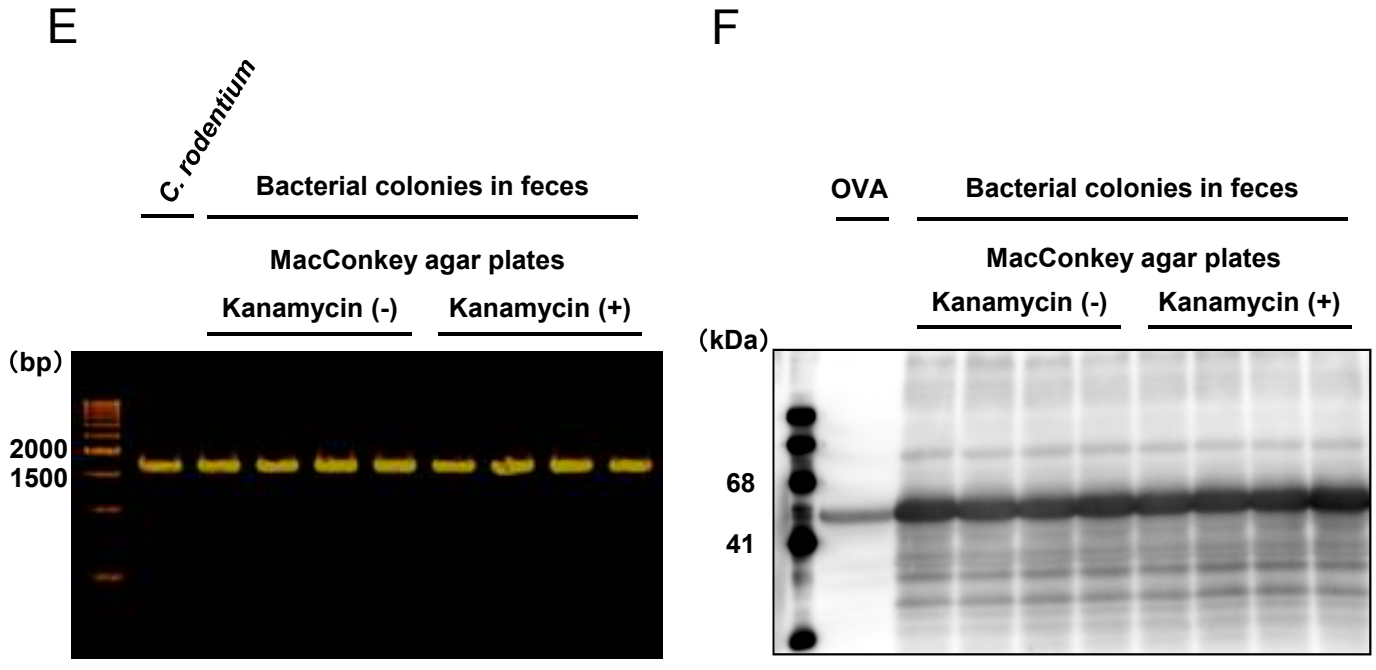
E
C. rodentium
Bacterial colonies in feces
MacConkey agar plates
Kanamycin (-)
Kanamycin (+)
(bp)
2000
1500
F
OVA
Bacterial colonies in feces
MacConkey agar plates
Kanamycin (-)
Kanamycin (+)
(kDa)
68
41

**Figure 2.**

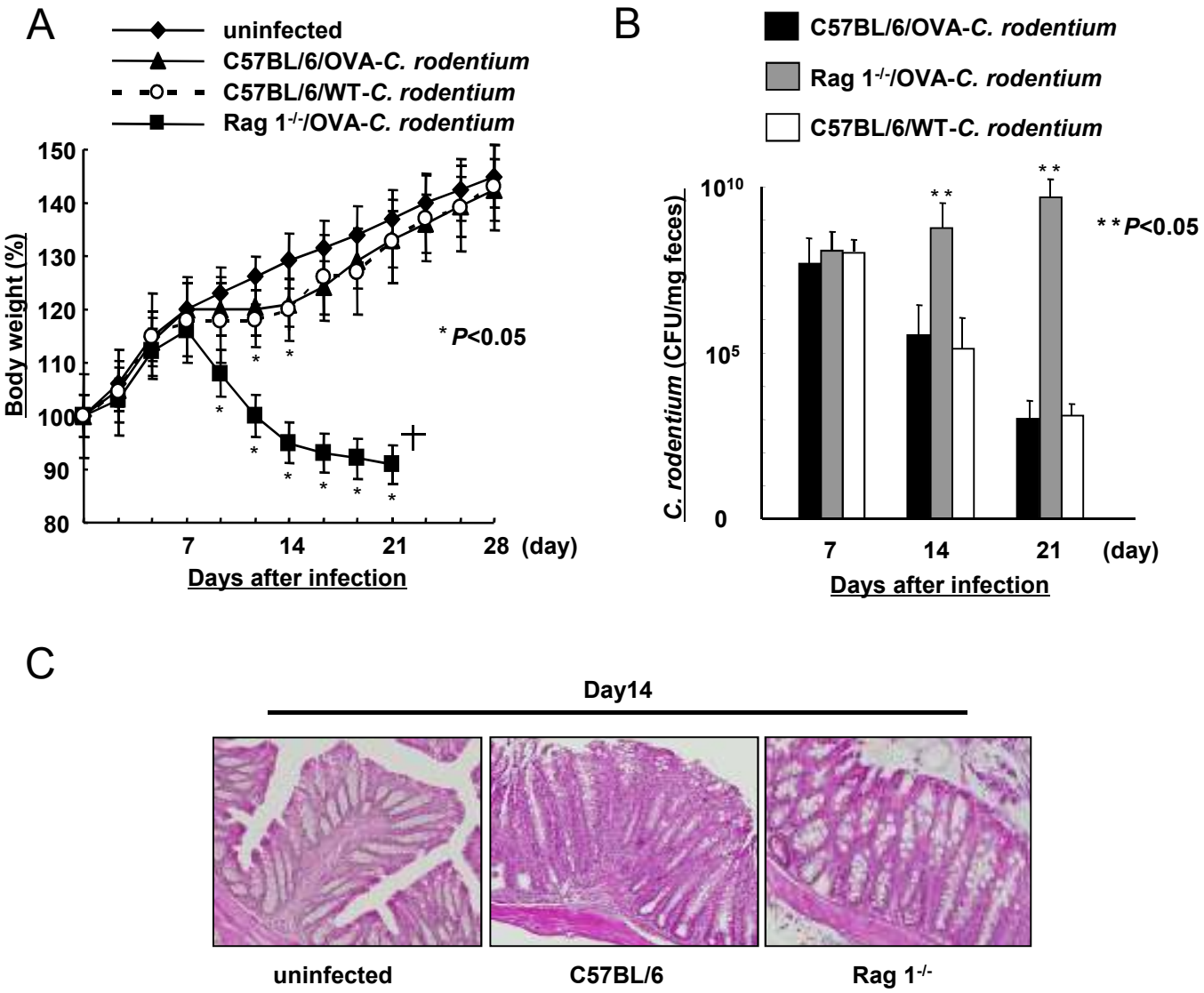
A
uninfected
C57BL/6/OVA-C. rodentium
C57BL/6/WT-C. rodentium
Rag 1-/-/OVA-C. rodentium
Body weight (%)
150
140
130
120
110
100
90
80
* P<0.05
7
14
21
28 (day)
Days after infection
B
C57BL/6/OVA-C. rodentium
Rag 1-/-/OVA-C. rodentium
C57BL/6/WT-C. rodentium
C. rodentium (CFU/mg feces)
10^10
10^5
0
** P<0.05
7
14
21
(day)
Days after infection
C
Day14
uninfected
C57BL/6
Rag 1-/-

Figure 3.

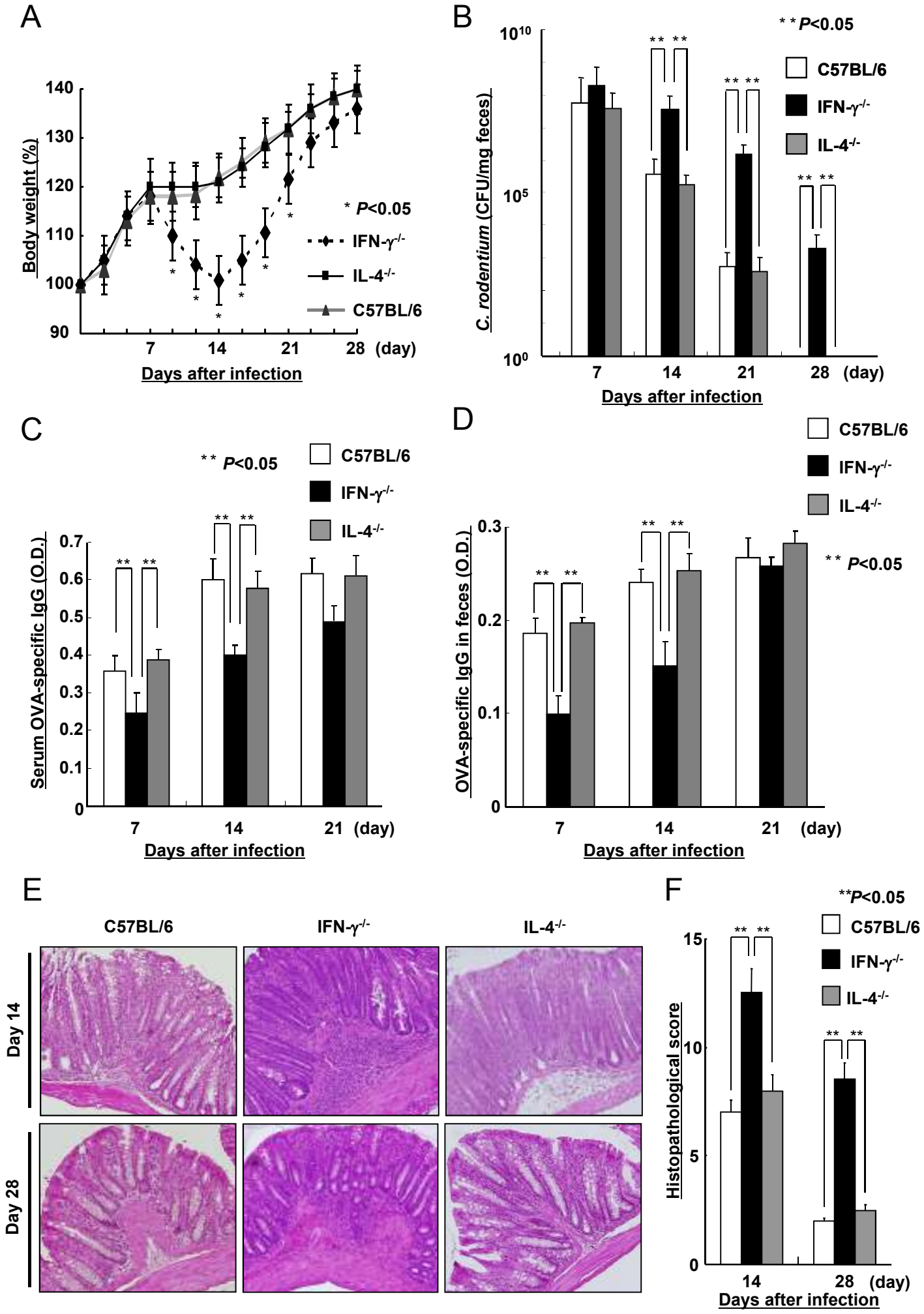
A
Body weight (%)
Days after infection
(day)
* P<0.05
IFN-γ-/-
IL-4-/-
C57BL/6
B
C. rodentium (CFU/mg feces)
** P<0.05
C57BL/6
IFN-γ-/-
IL-4-/-
Days after infection
(day)
C
Serum OVA-specific IgG (O.D.)
** P<0.05
C57BL/6
IFN-γ-/-
IL-4-/-
Days after infection
(day)
D
OVA-specific IgG in feces (O.D.)
** P<0.05
C57BL/6
IFN-γ-/-
IL-4-/-
Days after infection
(day)
E
C57BL/6
IFN-γ-/-
IL-4-/-
Day 14
Day 28
F
Histopathological score
**P<0.05
C57BL/6
IFN-γ-/-
IL-4-/-
Days after infection
(day)

Figure 4.

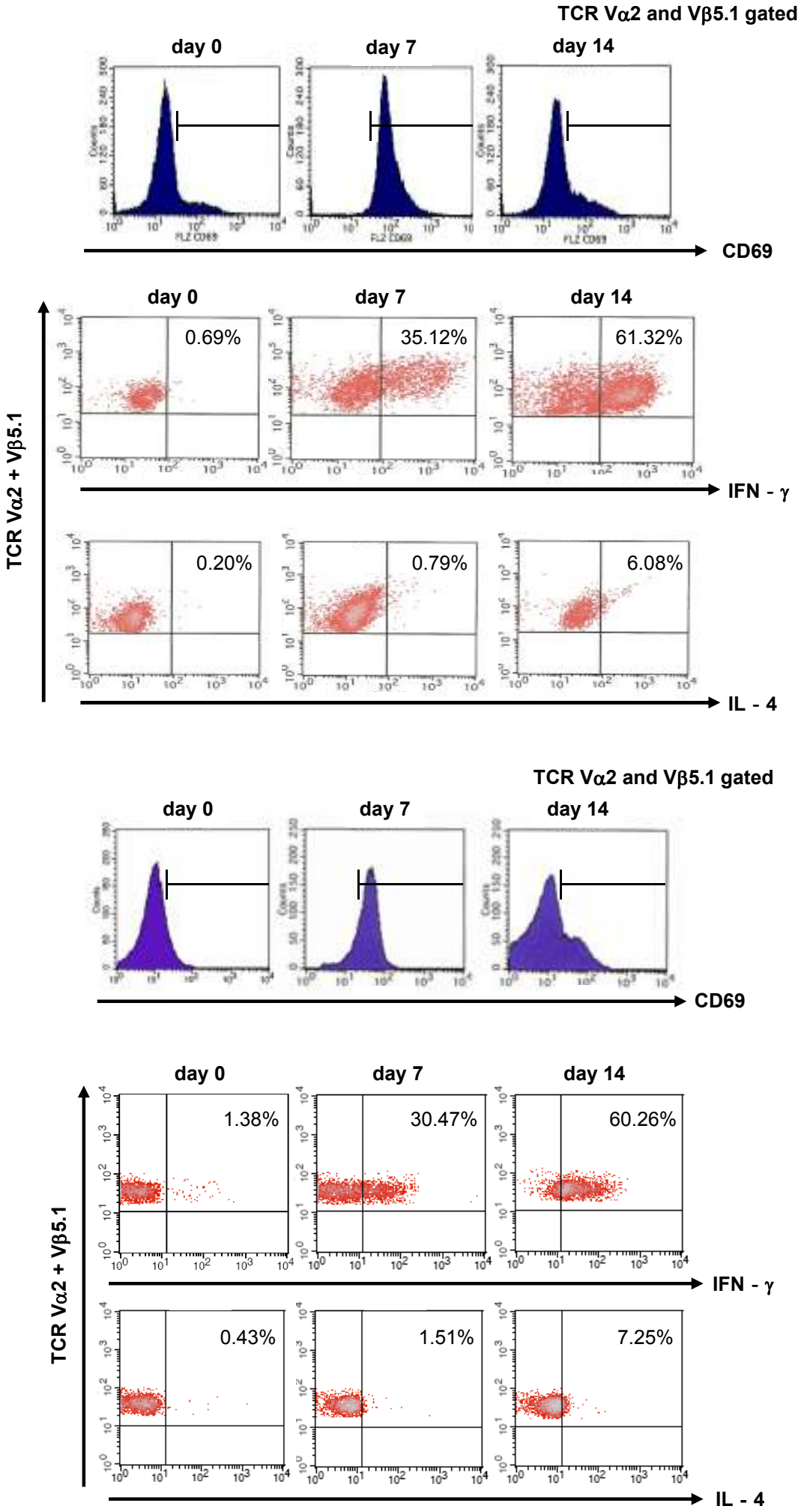
A
TCR Vα2 and Vβ5.1 gated
day 0
day 7
day 14
CD69
B
day 0
day 7
day 14
0.69%
35.12%
61.32%
IFN - γ
0.20%
0.79%
6.08%
IL - 4
TCR Vα2 + Vβ5.1
C
TCR Vα2 and Vβ5.1 gated
day 0
day 7
day 14
CD69
D
day 0
day 7
day 14
1.38%
30.47%
60.26%
IFN - γ
0.43%
1.51%
7.25%
IL - 4
TCR Vα2 + Vβ5.1

Figure 5.

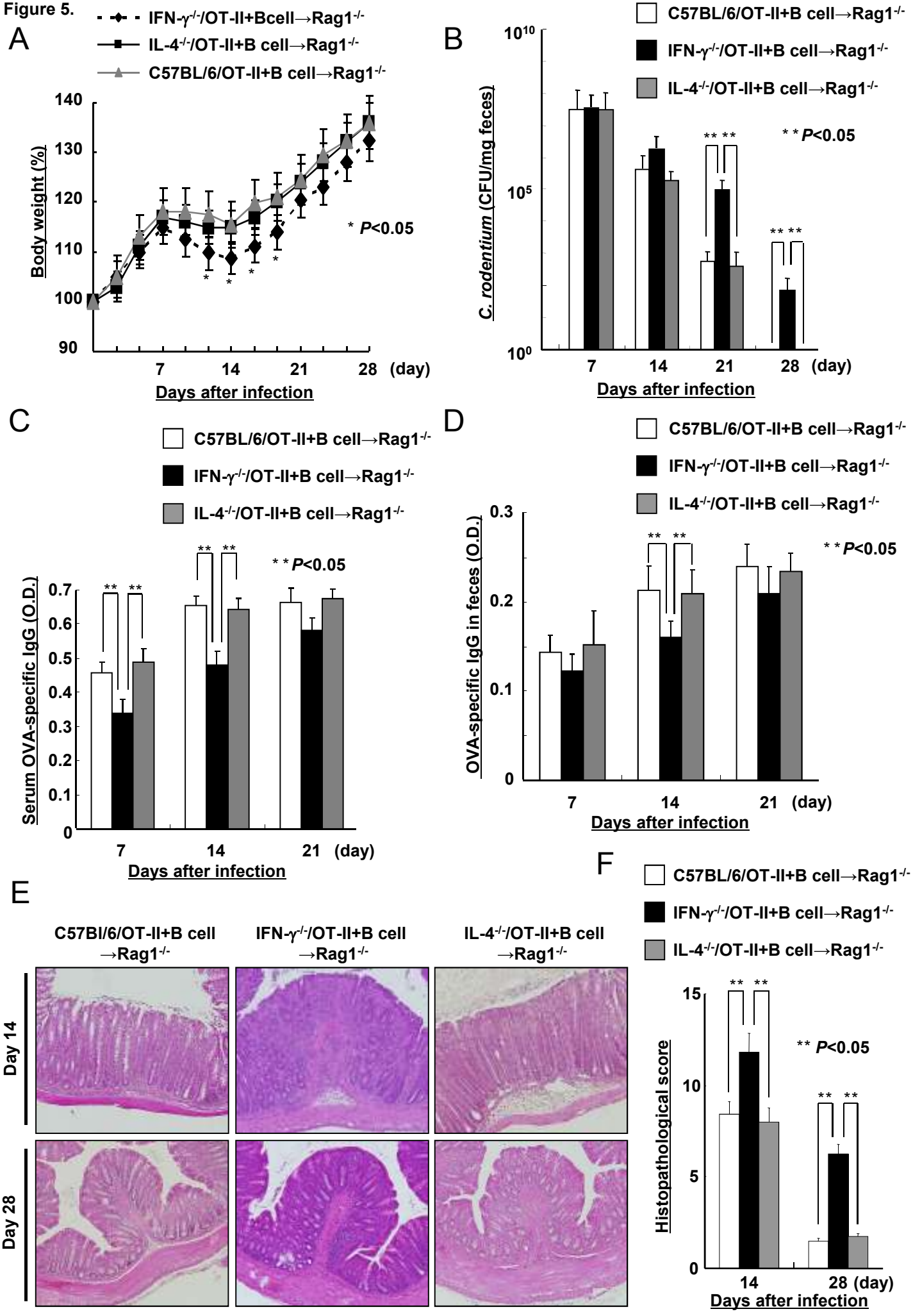
A
IFN-γ-/-/OT-II+Bcell→Rag1-/-
IL-4-/-/OT-II+B cell→Rag1-/-
C57BL/6/OT-II+B cell→Rag1-/-
Body weight (%)
140
130
120
110
100
90
7
14
21
28 (day)
Days after infection
* P<0.05
B
C57BL/6/OT-II+B cell→Rag1-/-
IFN-γ-/-/OT-II+B cell→Rag1-/-
IL-4-/-/OT-II+B cell→Rag1-/-
C. rodentium (CFU/mg feces)
10^10
10^5
10^0
** **
** **
* * P<0.05
7
14
21
28 (day)
Days after infection
C
C57BL/6/OT-II+B cell→Rag1-/-
IFN-γ-/-/OT-II+B cell→Rag1-/-
IL-4-/-/OT-II+B cell→Rag1-/-
Serum OVA-specific IgG (O.D.)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
** **
** **
* * P<0.05
7
14
21 (day)
Days after infection
D
C57BL/6/OT-II+B cell→Rag1-/-
IFN-γ-/-/OT-II+B cell→Rag1-/-
IL-4-/-/OT-II+B cell→Rag1-/-
OVA-specific IgG in feces (O.D.)
0.3
0.2
0.1
0
** **
* * P<0.05
7
14
21 (day)
Days after infection
E
C57Bl/6/OT-II+B cell →Rag1-/-
IFN-γ-/-/OT-II+B cell →Rag1-/-
IL-4-/-/OT-II+B cell →Rag1-/-
Day 14
Day 28
F
C57BL/6/OT-II+B cell→Rag1-/-
IFN-γ-/-/OT-II+B cell→Rag1-/-
IL-4-/-/OT-II+B cell→Rag1-/-
Histopathological score
15
10
5
0
** **
** **
** P<0.05
14
28 (day)
Days after infection

Figure 5.

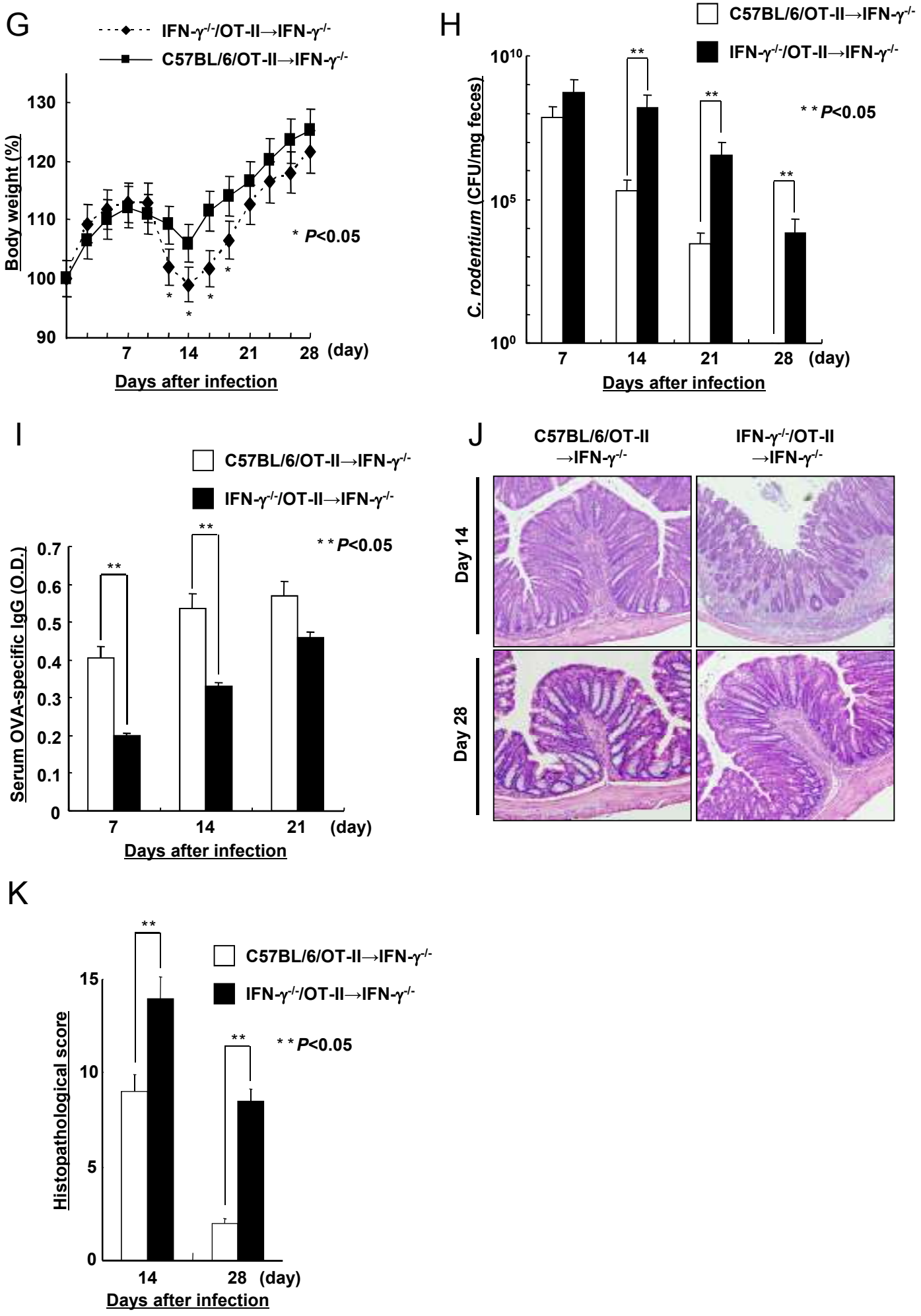
G
IFN-γ-/-/OT-II→IFN-γ-/-
C57BL/6/OT-II→IFN-γ-/-
Body weight (%)
130
120
110
100
90
* P<0.05
7
14
21
28
(day)
Days after infection
H
C57BL/6/OT-II→IFN-γ-/-
IFN-γ-/-/OT-II→IFN-γ-/-
C. rodentium (CFU/mg feces)
10^10
10^5
10^0
** P<0.05
7
14
21
28
(day)
Days after infection
I
C57BL/6/OT-II→IFN-γ-/-
IFN-γ-/-/OT-II→IFN-γ-/-
Serum OVA-specific IgG (O.D.)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
** P<0.05
7
14
21
(day)
Days after infection
J
C57BL/6/OT-II →IFN-γ-/-
IFN-γ-/-/OT-II →IFN-γ-/-
Day 14
Day 28
K
C57BL/6/OT-II→IFN-γ-/-
IFN-γ-/-/OT-II→IFN-γ-/-
Histopathological score
15
10
5
0
** P<0.05
14
28
(day)
Days after infection

**Figure 6.**

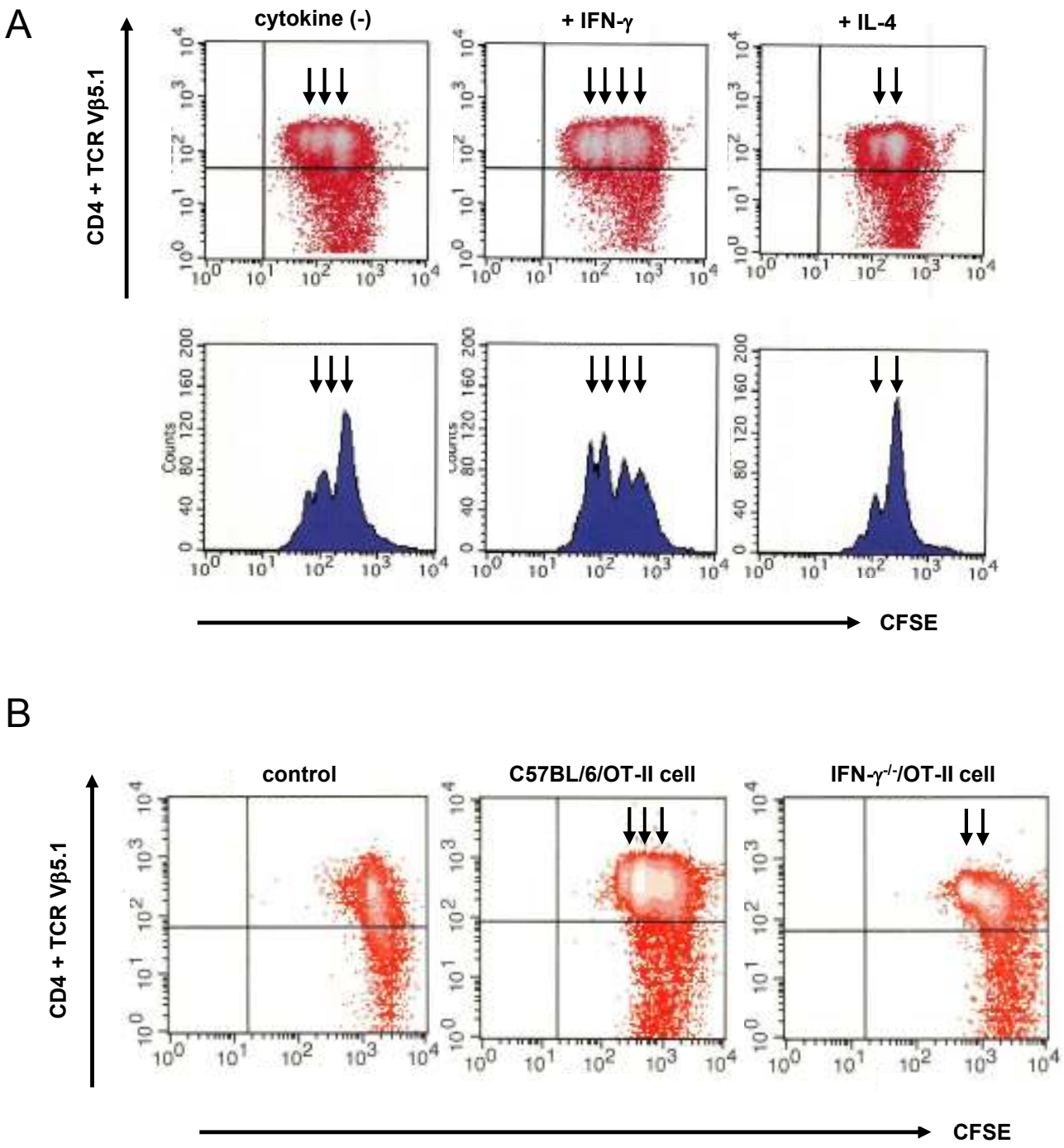
A
cytokine (-)
+ IFN-γ
+ IL-4
CD4 + TCR Vβ5.1
Counts
CFSE
B
control
C57BL/6/OT-II cell
IFN-γ-/-/OT-II cell
CD4 + TCR Vβ5.1
CFSE

**Figure 7.**

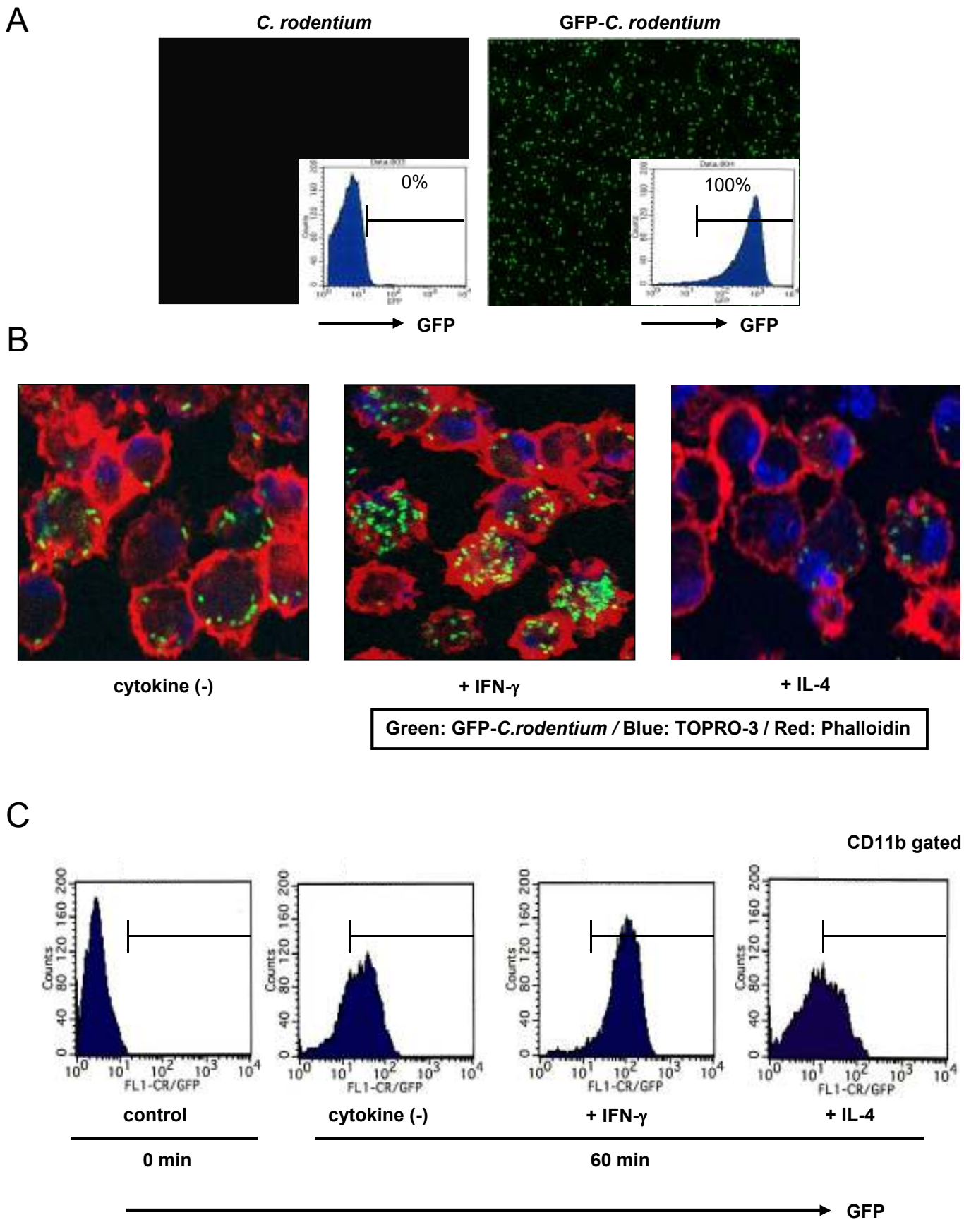
A
C. rodentium
GFP-C. rodentium
0%
100%
GFP
GFP
B
cytokine (-)
+ IFN-γ
+ IL-4
Green: GFP-C.rodentium / Blue: TOPRO-3 / Red: Phalloidin
C
CD11b gated
Counts
FL1-CR/GFP
control
cytokine (-)
+ IFN-γ
+ IL-4
0 min
60 min
GFP

Table 1. The prevalance of Tir and OVA in bacterial colonies in feces

| | day 7 | |
|---|---|---|
| | MacConkey agar plates | |
| | kanamycin (+) (%) | kanamycin (-) (%) |
| Tir-positive[a] | 100 | 98.0 |
| OVA-positive[b] | 98.6 | 96.6 |
| | day 14 | |
| | MacConkey agar plates | |
| | kanamycin (+) (%) | kanamycin (-) (%) |
| Tir-positive[a] | 100 | 97.3 |
| OVA-positive[b] | 99.3 | 97.2 |

All mice were given kanamycin in drinking water during OVA-*C. rodentium* infection.

[a] The values (%) represent [(the number of Tir-positive colonies)/(the number of colonies)] x 100.

[b] The values (%) represent [(the number of OVA-positive colonies)/(the number of Tir-positive colonies)] x 100.