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

3

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

16

17 The authors have no conflicts of interest.

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ABSTRACT

1 **ABSTRACT**

2 *Citrobacter rodentium* (*C. rodentium*), a murine model pathogen for

3 enteropathogenic *Escherichia coli*, colonizes the surface of intestinal epithelial cells

4 and causes mucosal inflammation. This bacterium is an ideal model for

5 investigating pathogen-host immune interactions in the gut. It is well known that

6 gene transcripts for Th1 cytokines are highly induced in colonic tissue from mice

7 infected with *C. rodentium*. However, it remains to be seen whether the Th1 or

8 Th2 cytokines produced by antigen-specific CD4⁺ T cells provide effective

9 regulation of the host immune defense against *C. rodentium* infection. To

10 investigate the antigen-specific immune responses, *C. rodentium* expressing

11 ovalbumin (OVA), a model antigen (OVA-*C. rodentium*), was generated and used to

12 define antigen specific responses under IFN- γ or IL-4 deficient conditions *in vivo*.

13 The activation of antigen-specific CD4⁺ T cells and macrophage phagocytosis were

14 evaluated in the presence of IFN- γ or IL-4 *in vitro*. IFN- γ deficient mice exhibited a

15 loss of body weight and a higher bacterial concentration in feces during OVA-*C.*

16 *rodentium* infection compared to C57BL/6 (wild type) or IL-4 deficient mice. This

17 occurred through the decreased efficiency of macrophage phagocytosis and the

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

6

7 **Keywords**

8 antigen-specific CD4⁺ T cells; *Citrobacter rodentium*; mucosal immune
9 response; IFN- γ ; IL-4

10

1 INTRODUCTION

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

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

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

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

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

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

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

- 1 response to *C. rodentium* infection and consequently eradication of the mucosal
- 2 pathogen.
- 3

1 MATERIALS AND METHODS

2 Animals.

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

14 Antibodies.

15 CD4-PE-Cy5.5 (clone RM4-5) antibody was purchased from eBioscience
16 (San Diego, CA). T-cell Receptor (TCR) V β 5.1-PE (clone MR9-4), TCR
17 V β 5.1-biotin (clone MR9-4), CD69-PE (clone H1.2F3), TCR V α 2-FITC (clone

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

4

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

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

14

15 **T cell proliferation assay.**

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

1 the CD4⁺ T cells was usually over 95% according to examination with a FACS
2 Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). For the T cell
3 proliferation assays, splenic CD4⁺ T cells (1×10^6 cells/ml) purified from OT-II mice
4 were labeled with carboxyfluorescein succinimidyl ester (CFSE) (CellTrace™ CESE
5 Cell Proliferation Kit; Molecular Probes, Eugene, OR). After antigen-presenting
6 cell (APC) incubated with bacterial sonicates from OVA-*C. rodentium* or wild type *C.*
7 *rodentium* (WT-*C. rodentium*), the APC were incubated with CFSE-labeled CD4⁺ T
8 cells for 24 hours. The CFSE-labeled CD4⁺ T cells were stained with anti-CD4 and
9 anti-TCR Vβ5.1 antibodies, and the CFSE intensity of the CD4⁺ T cells gated on
10 TCR Vβ5.1-positive cells was measured by flow cytometry. Splenocytes (1×10^7
11 cells/ml) from C57BL/6 mice were used as APC after irradiation with 70Gy.

12

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

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

1 inoculated with 1×10^9 CFU of OVA-*C. rodentium* using a gavage needle. To
2 ensure maintenance of the plasmid during the *in vivo* infection, 0.1 mg/ml
3 kanamycin was added to the drinking water. Body weight, bacterial concentration
4 in feces, and the histological findings of the colon were assessed for 4 weeks after
5 inoculation. The anti-OVA IgG levels in sera and feces were examined by an
6 enzyme-linked immunosorbent assay (ELISA) as described below. Colonic
7 tissues were stained with hematoxylin and eosin and evaluated as described in
8 'Histological analysis of colon tissue'. Regarding wild type (WT)-*C. rodentium*
9 infection, the mice were not given kanamycin in drinking water.

10

11 **Bacterial counts.**

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

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

5

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

7 C57BL/6 mice were orally infected with 1×10^9 CFU of OVA-*C. rodentium*
8 under the presence of kanamycin in drinking water. Fecal pellets treated as
9 described above were plated on MacConkey agar plates with or without kanamycin,
10 and bacterial colonies were counted. Approximately 150 colonies were selected at
11 random, and the presence of *C. rodentium* in colonies was confirmed by PCR using
12 Tir-specific primers. Regarding PCR-positive colonies, the expression of OVA was
13 confirmed by Immunoblot analysis, and the positive rate of plasmid in feces during
14 OVA-*C. rodentium* infection was evaluated.

15

16 **Histological analysis of colon tissue.**

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

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

8

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

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

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

12

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

15 OT-II mice (4 weeks old) were orally inoculated with 1×10⁹ CFU of *OVA-C.*
16 *rodentium*. Mesenteric lymph node (MLN) cells were collected from OT-II mice at
17 day 0, 7 and 14 after *OVA-C. rodentium* infection. MLN cells were stained with

1 anti-CD69, anti-TCR α 2, and anti-TCR $\nu\beta$ 5.1 antibodies, and the activation of
2 CD4⁺ T cells determined after gating on TCR α 2-positive and TCR $\nu\beta$ 5.1-positive
3 cells as defined by flow cytometry. To evaluate the cytokine production,
4 OVA-specific CD4⁺ T cells were purified from MLN of the infected OT-II mice at day
5 0, 7 and 14 using MACS immunobeads. These cells were then re-stimulated with
6 1 μ M OVA peptide when presented by irradiated splenic APC for 24 hours *in vitro*.
7 The production of IFN- γ and IL-4 from the OVA-specific CD4⁺ T cells was evaluated
8 by intracellular cytokine staining by standard methods as defined by the
9 manufactures (Intracellular Cytokine Staining Starter Kit-Mouse; BD Biosciences,
10 San Jose, CA). C57BL/6 mice (4 weeks old) that received 5×10^6 of CD4⁺ T cells
11 from OT-II mice were orally inoculated with 1×10^9 CFU of OVA-*C. rodentium*, and
12 then the expression of CD69 and the production of IFN- γ and IL-4 on OVA-specific
13 CD4⁺ T cells were determined as described above.

14

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

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

1 cells were also purified from the spleens of C57BL/6 mice. Rag1^{-/-} mice (8-10
2 weeks old) received 1×10⁷ of wild type (C57BL/6) B cells and 5×10⁶ of CD4⁺ T cells
3 from C57BL/6/OT-II, IFN-γ^{-/-}/OT-II, or IL-4^{-/-}/OT-II mice by the intravenous injection.
4 All recipients were orally inoculated with 1×10⁹ CFU of OVA-*C. rodentium* at 5 days
5 after the adoptive transfer. Body weight, bacterial concentration in feces, anti-OVA
6 antibody level, and histological findings of the colon were examined for 4 weeks
7 after the inoculation with OVA-*C. rodentium* as described above. Similarly,
8 IFN-γ^{-/-} recipients that transferred C57BL/6/OT-II or IFN-γ^{-/-}/OT-II cells were infected
9 with 1×10⁹ CFU of OVA-*C. rodentium*, and the body weight, the bacterial
10 concentration in feces, serum anti-OVA IgG levels and the histological findings of
11 colon tissue were evaluated. All the mice were given kanamycin in drinking water
12 during infection.

13

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

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

1 incubated with the bacterial sonicates from OVA-*C. rodentium* for 16 hours with or
2 without 100 U/ml recombinant murine IFN- γ (rIFN- γ) (PEPROTECH, Rocky Hill, NJ)
3 or 30 ng/ml recombinant murine IL-4 (rIL-4) (PEPROTECH), OVA-specific CD4⁺ T
4 cells were incubated with the APC for 24 hours and stained with anti-CD4 and
5 anti-TCR V β 5.1 antibodies. T cell proliferation was determined by measuring the
6 CFSE intensity of CD4⁺ T cells after gating on TCR V β 5.1-positive cells by flow
7 cytometry.

8

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

11 OVA-specific CD4⁺ T cells were purified from the spleens of IFN- γ ^{-/-}/OT-II,
12 C57BL/6/OT-II and IL-4^{-/-}/OT-II mice using MACS immunobeads and were then
13 labeled with CFSE. APC cultured with the bacterial sonicates from OVA-*C.*
14 *rodentium* were incubated with respective CFSE-labeled OVA-specific CD4⁺ T cells
15 for 24 hours and then stained with anti-CD4 and anti-TCR V β 5.1 antibodies.
16 Proliferation of T cells was determined measuring the CFSE intensity of CD4⁺ T
17 cells after gating on TCR V β 5.1-positive cells by flow cytometry.

1

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

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

14

15 **Statistics.**

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

1

2

RESULTS

3

Immunogenicity of *C. rodentium* constitutively expressing OVA

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

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

1 infection was confirmed by PCR from bacterial colonies using Tir-specific primers
2 (26) (Fig. 1D and E). The protein expression of OVA in these bacterial colonies
3 was also confirmed by immunoblot analysis (Fig. 1F). Thus, OVA-*C. rodentium*
4 possesses OVA-specific immunogenicity making of an appropriate model to assess
5 *C. rodentium*-specific immune responses *in vivo*. Furthermore, to determine the
6 frequency of plasmid loss during infection, C57BL/6 mice were orally infected with
7 1×10^9 CFU of OVA-*C. rodentium* under the presence of kanamycin in drinking water.
8 Fecal pellets were plated on MacConkey agar plates with or without kanamycin,
9 and then the number of bacterial colonies, Tir-PCR prevalence, and OVA
10 prevalence were examined. As results, a comparable rate of bacterial colonies
11 (data not shown), the prevalence of Tir-PCR and OVA were observed (table 1, Fig.
12 1E and F). When C57BL/6 mice were orally infected with 1×10^9 CFU of WT-*C.*
13 *rodentium* under the presence of kanamycin in drinking water, there were little
14 bacterial colonies from fecal pellets of the mice for 4 weeks after inoculation, and
15 the colonic tissue was a normal state (data not shown). These results suggest that
16 there is little frequency of plasmid loss during the infection and that the colonic
17 damage is not caused by *C. rodentium* without plasmid even if OVA-*C. rodentium*

1 lost plasmid.

2

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

4 It was reported that T cells and B cells play important roles in eradicating *C.*
5 *rodentium* (19, 22). To evaluate the roles of T cells and B cells in OVA-*C.*
6 *rodentium* infection, C57BL/6 mice and Rag1^{-/-} mice were orally infected with 1×10⁹
7 CFU of OVA-*C. rodentium*. Rag1^{-/-} mice exhibited a loss of body weight (Fig. 2A)
8 and higher bacterial concentrations in their feces at 14 and 21 days after infection
9 (Fig. 2B) compared to C57BL/6 mice. Moreover, *C. rodentium* infection was lethal
10 in Rag1^{-/-} mice by 21 days after OVA-*C. rodentium* infection. This was associated
11 with decreased histological injury in the Rag1^{-/-} mice in comparison to the C57BL/6
12 mice as assessed at day 14 of infection (Fig. 2C). Together these studies in
13 Rag1^{-/-} mice show the importance of adaptive immunity in clearing *C. rodentium*
14 infection.

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

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

3

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

5 To determine whether Th1 and/or Th2 cytokines are important for protection
6 against *C. rodentium* infection, the IFN- $\gamma^{-/-}$, IL-4 $^{-/-}$, and C57BL/6 mice were orally
7 infected with OVA-*C. rodentium*. IFN- $\gamma^{-/-}$ mice exhibited a loss of body weight (Fig.
8 3A) and a higher bacterial concentration in their feces at 14, 21 and 28 days after
9 infection in comparison to IL-4 $^{-/-}$ and C57BL/6 mice (Fig. 3B). Although delayed,
10 the IFN- $\gamma^{-/-}$ mice were able to successfully clear OVA-*C. rodentium* from their feces
11 by 35 days after infection (data not shown). The IFN- $\gamma^{-/-}$ mice also exhibited a
12 significant delay in the development of antigen-specific IgG levels at days 7 and 14
13 after infection in both serum and feces in comparison to the IL-4 $^{-/-}$ and C57BL/6
14 mice (Fig. 3C and D). Macroscopic and microscopic injury was also more severe
15 in the IFN- $\gamma^{-/-}$ mice than that observed in the IL-4 $^{-/-}$ and C57BL/6 mice at days 14 and
16 28. The submucosa and lamina propria of the infected IFN- $\gamma^{-/-}$ mice exhibited a
17 greater number of mononuclear cells and neutrophils in the tissue, and increased

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

8

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

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

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

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

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

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

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

7

8 **IFN- γ effectively induced antigen-specific T-cell activation**

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

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

17

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

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

1 macrophages in response to *C. rodentium*.

1

2

DISCUSSION

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

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

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

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

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

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

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

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

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

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

6

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1

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- 18
- 19

1 **FIGURE LEGENDS**

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

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

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

9

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

11 Susceptibility to infection with 1×10^9 CFU of OVA-*C. rodentium* in Rag1^{-/-}
12 mice. Rag1^{-/-} mice and C57BL/6 mice were infected with OVA-*C. rodentium* or
13 WT-*C. rodentium* at 1×10^9 CFU. Regarding WT-*C. rodentium* infection, the mice
14 were not given kanamycin in drinking water. (A) The body weight changes in Rag^{-/-}
15 mice and C57BL/6 mice. Data are shown as means \pm SD (n=6), and asterisks
16 indicate a significant difference from the values for the uninfected mice at $p < 0.05$
17 according to the Student's t-test. (B) The bacterial loads (CFU/mg) of OVA-*C.*

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

8

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

10 Susceptibility to infection with 1×10^9 CFU of OVA-*C. rodentium* in IFN- γ ^{-/-}
11 mice, IL-4^{-/-} mice and C57BL/6 mice. (A) The body weight changes in IFN- γ ^{-/-} mice,
12 IL-4^{-/-} mice, and C57BL/6 mice during OVA-*C. rodentium* infection. Data are
13 shown as means \pm SD (n=8), and asterisks indicate a significant difference from the
14 values for the C57BL/6 mice at $p < 0.05$ according to the Student's t-test. (B) The
15 bacterial loads (CFU/mg) of OVA-*C. rodentium* in feces 7, 14, 21 and 28 days after
16 infection. Data are shown as means \pm SD (n=8), and two asterisks indicate a
17 significant difference. No colonies were detected in feces of the respective

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

11

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

14 OT-II mice were orally inoculated with 1×10^9 CFU of OVA-*C. rodentium*.
15 (A) Evaluation of CD69 expression on OVA-specific CD4⁺ T cells after *in vivo*
16 stimulation with OVA-*C. rodentium*. (B) After *in vivo* stimulation with OVA-*C.*
17 *rodentium*, the OVA-specific CD4⁺ T cells were treated with OVA peptide for 24

1 hours *in vitro*. The production of IFN- γ and IL-4 was examined by an intracellular
2 cytokine staining method using flow cytometry. (C, D) C57BL/6 that received
3 5×10^6 of CD4⁺ T cells from OT-II mice were orally infected with 1×10^9 CFU of OVA-*C.*
4 *rodentium*, and the expression of CD69 (C) and the production of IFN- γ and IL-4 (D)
5 on OVA-specific CD4⁺ T cells were evaluated. Typical images are shown from at
6 least triplicate determinations.

7

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

10 Susceptibility to infection with 1×10^9 CFU of OVA-*C. rodentium* in Rag1^{-/-}
11 mice receiving IFN- γ ^{-/-}/OT-II, IL-4^{-/-}/OT-II and C57BL/6/OT-II cells. IFN- γ ^{-/-}/OT-II,
12 IL-4^{-/-}/OT-II and C57BL/6/OT-II cells were transferred into Rag1^{-/-} mice, which had
13 previously been injected with C57BL/6-B cells, and then these mice were infected
14 with 1×10^9 CFU of OVA-*C. rodentium*. (A) The body weights of the mice were
15 measured. Data are shown as means \pm SD (n=6), and asterisks indicate a
16 significant difference from the values for the Rag1^{-/-} mice receiving C57BL/6/OT-II
17 cells at p<0.05 according to the Student's t-test. (B) The bacterial loads (CFU/mg)

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

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

9

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

11 (A) APC were cultured with bacterial sonicates from OVA-*C. rodentium* for
12 16 hours with or without IFN- γ or IL-4, and then CFSE-labeled OVA-specific OT-II
13 CD4⁺ T cells from OT-II mice were incubated with the APC for 24 hours. The
14 number of OVA-specific CD4⁺ T cells in the presence of IFN- γ or IL-4 was examined
15 by flow cytometry. (B) APC cultured with bacterial sonicates from OVA-*C.*
16 *rodentium* were incubated with CFSE-labeled OVA-specific CD4⁺ T cells from
17 IFN- $\gamma^{-/-}$ /OT-II, IL4^{-/-}/OT-II or C57BL/6/OT-II mice for 24 hours, and the T cells

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

5

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

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

Figure 2.

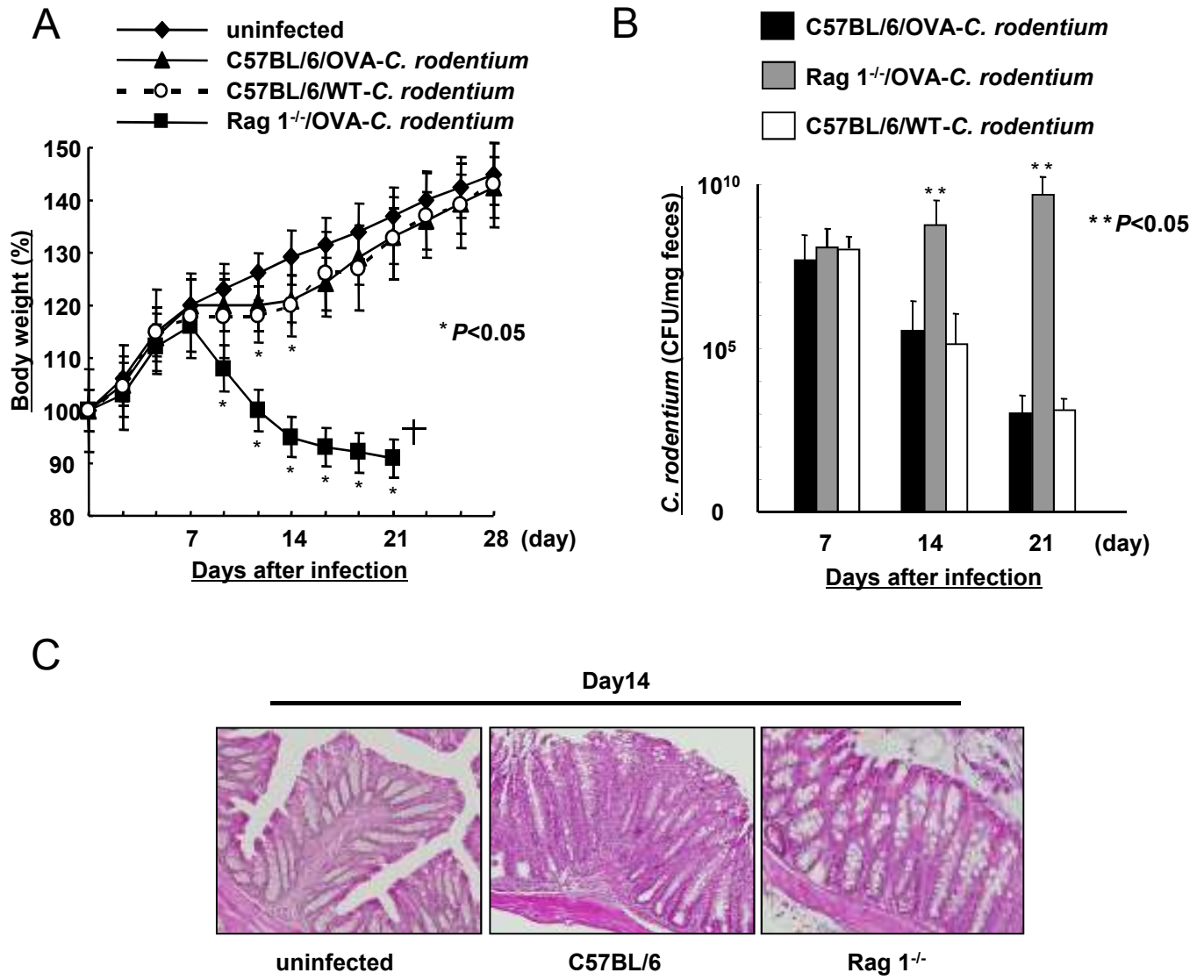


Figure 3.

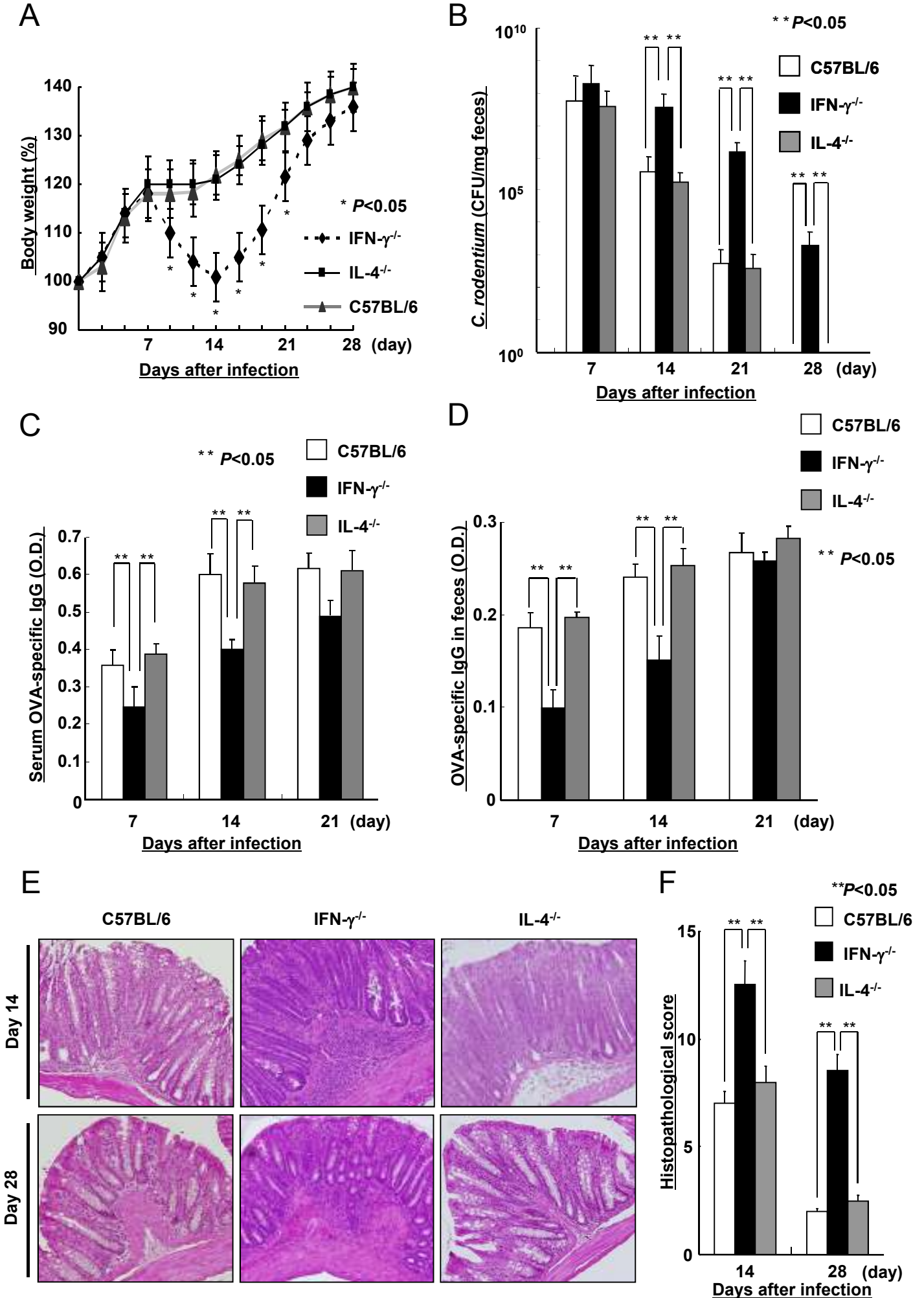
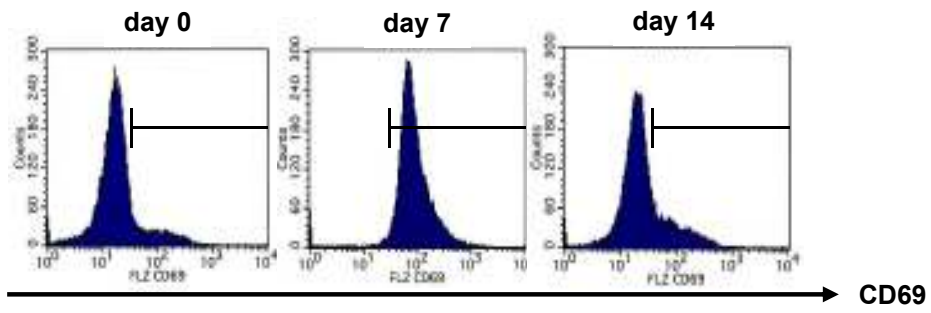


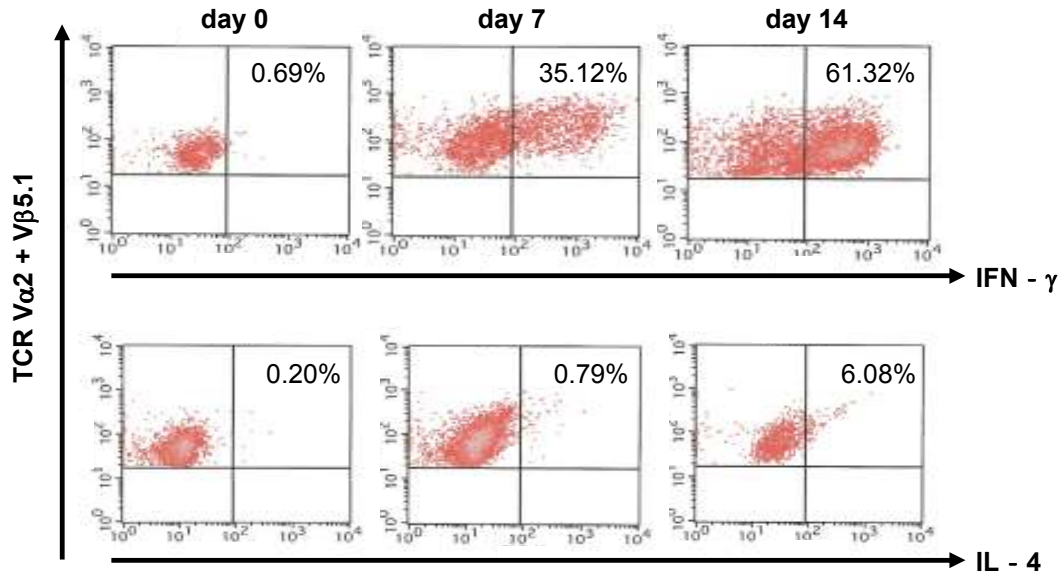
Figure 4.

TCR V α 2 and V β 5.1 gated

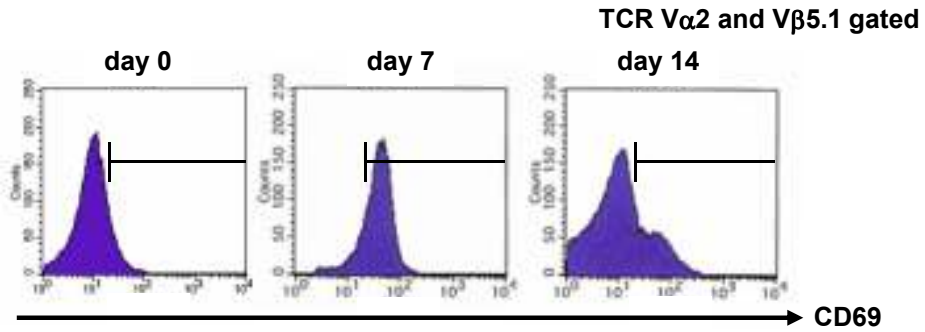
A



B



C



D

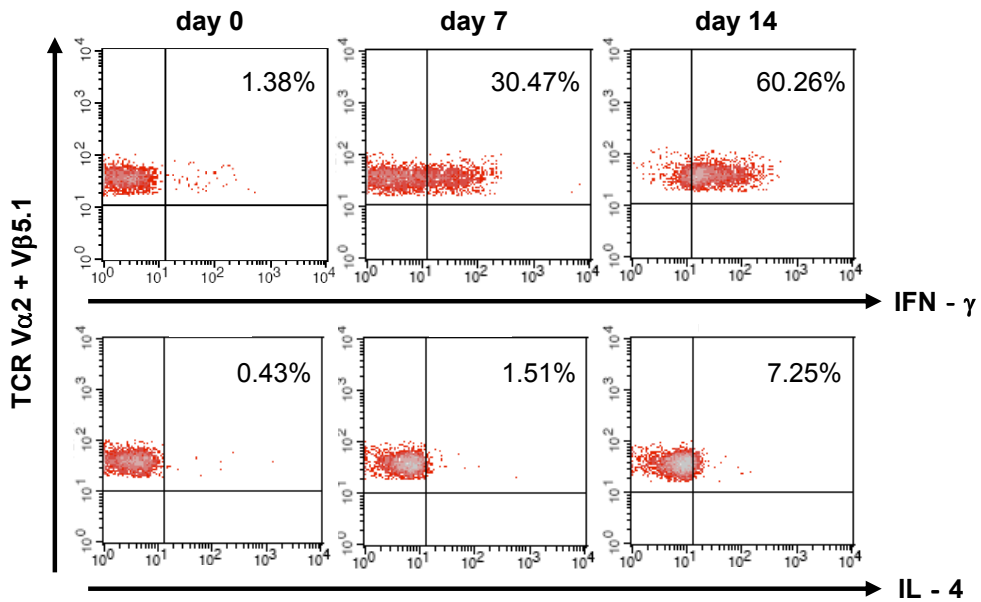


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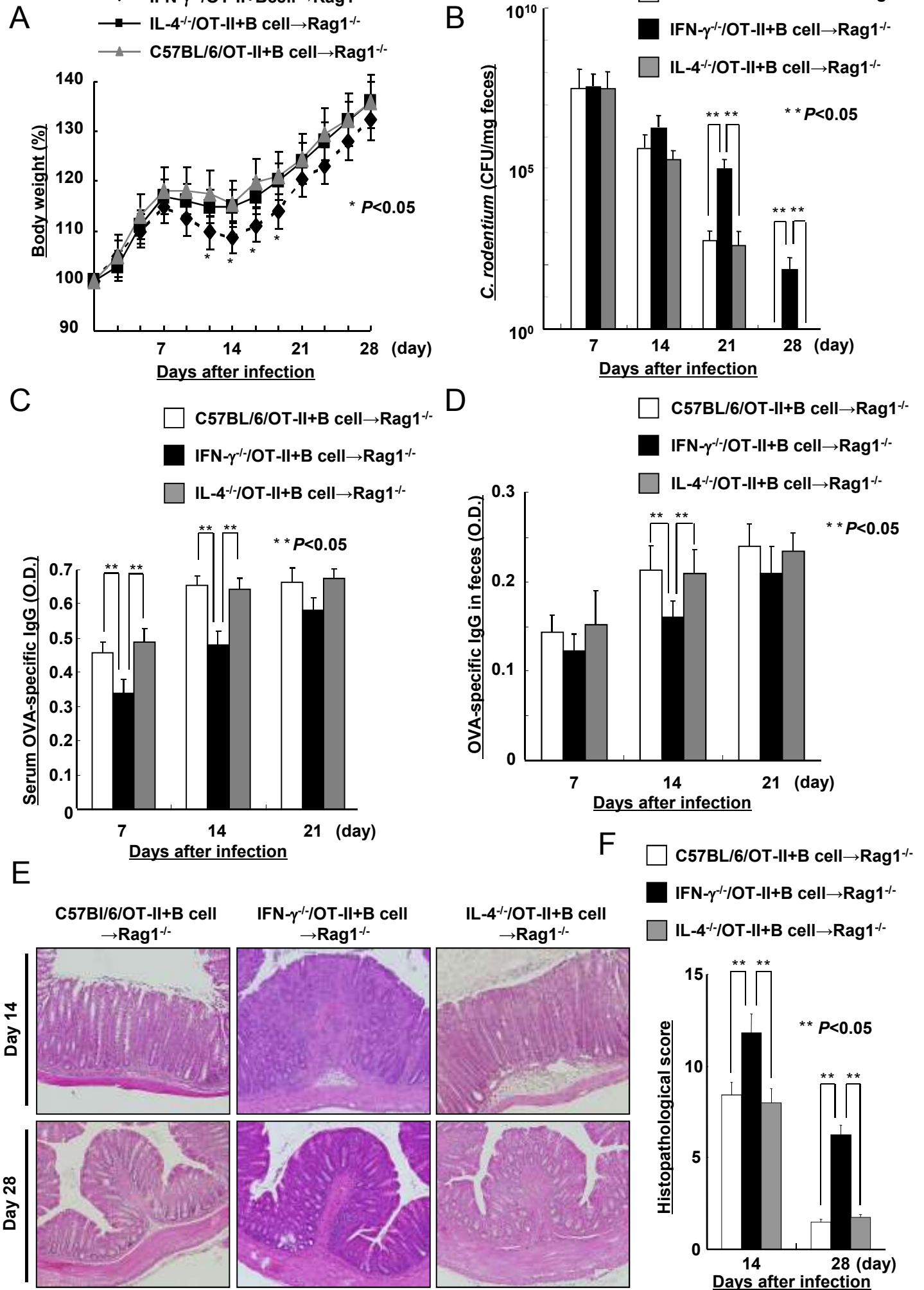


Figure 5.

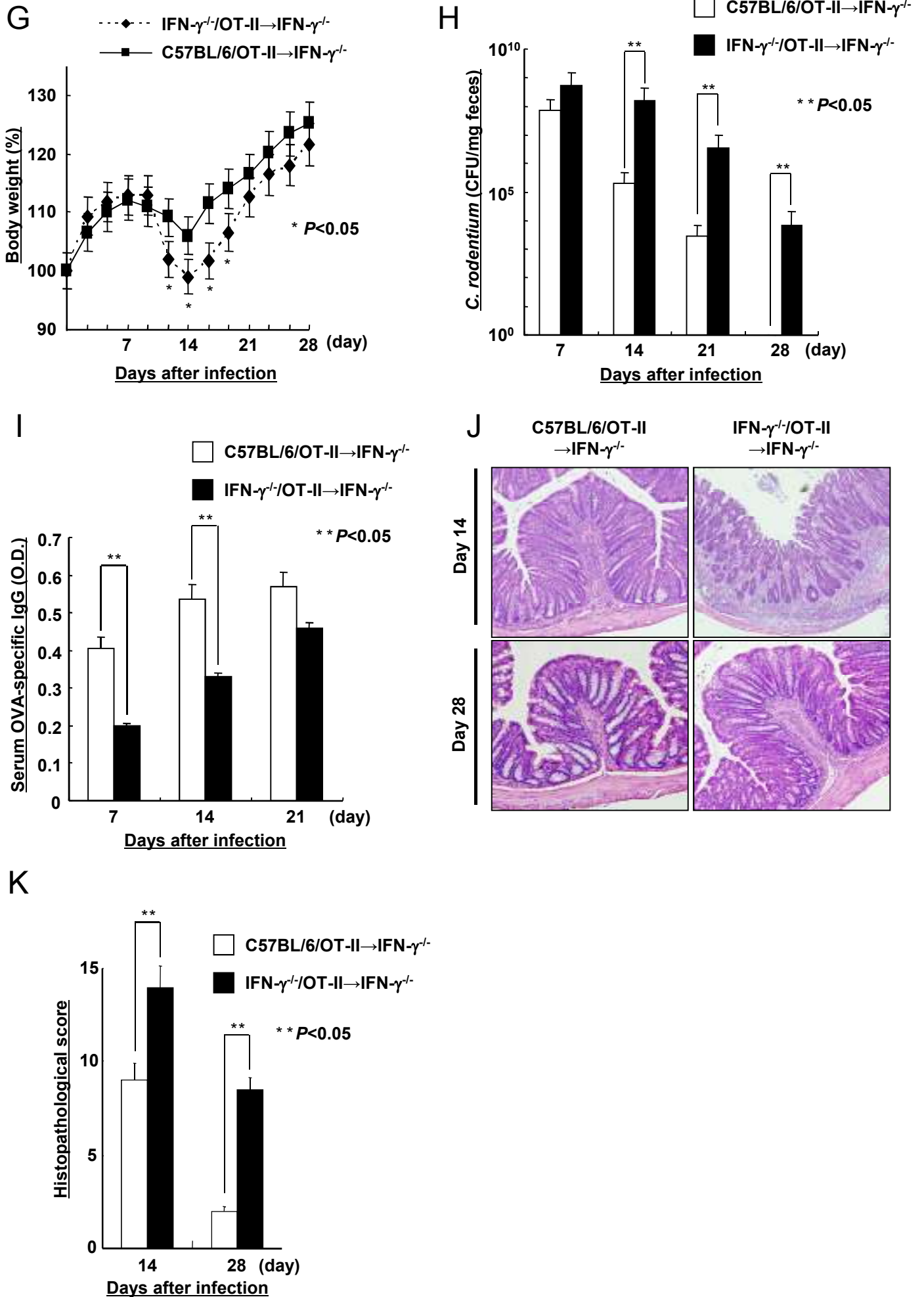


Figure 6.

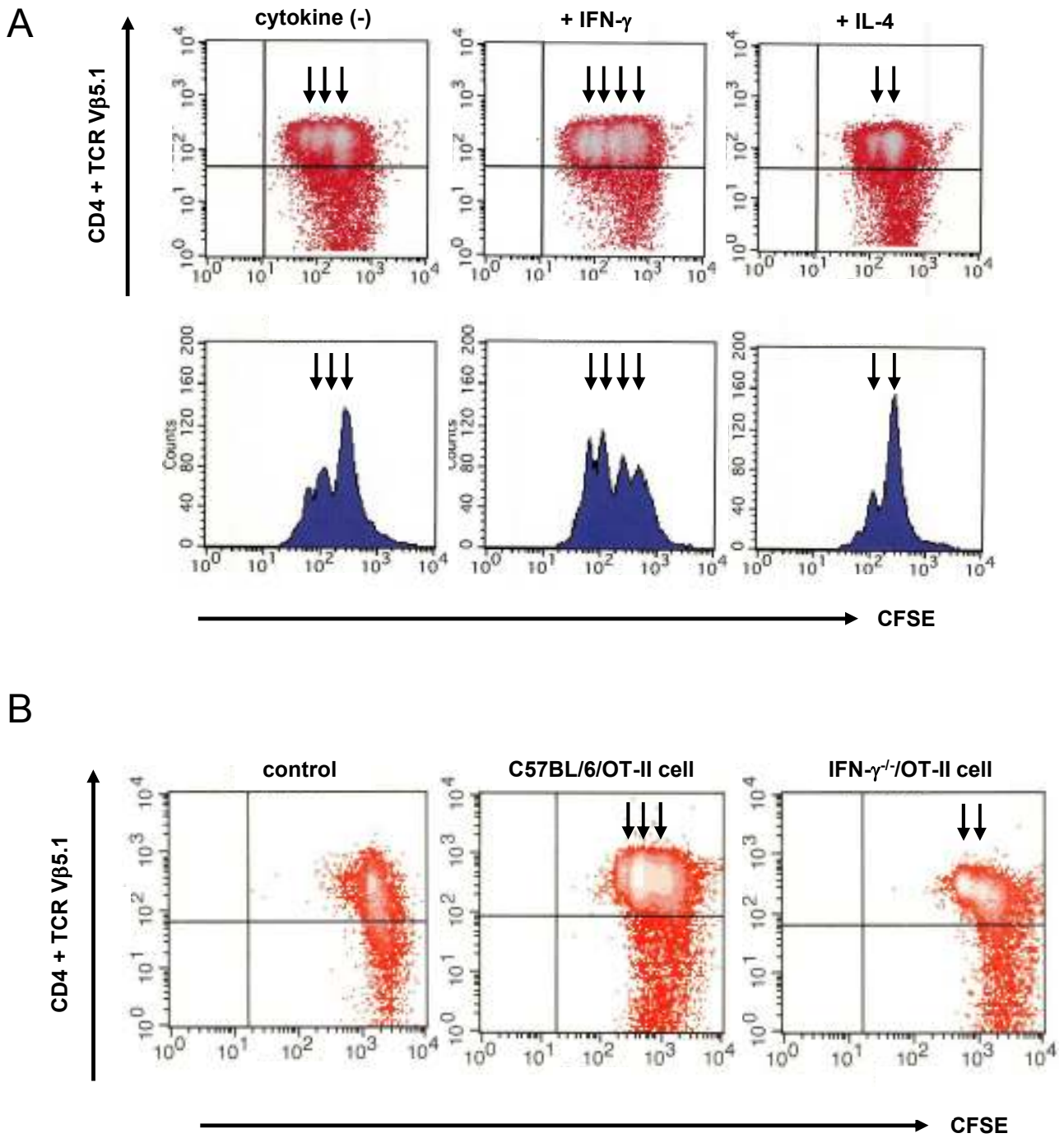


Figure 7.

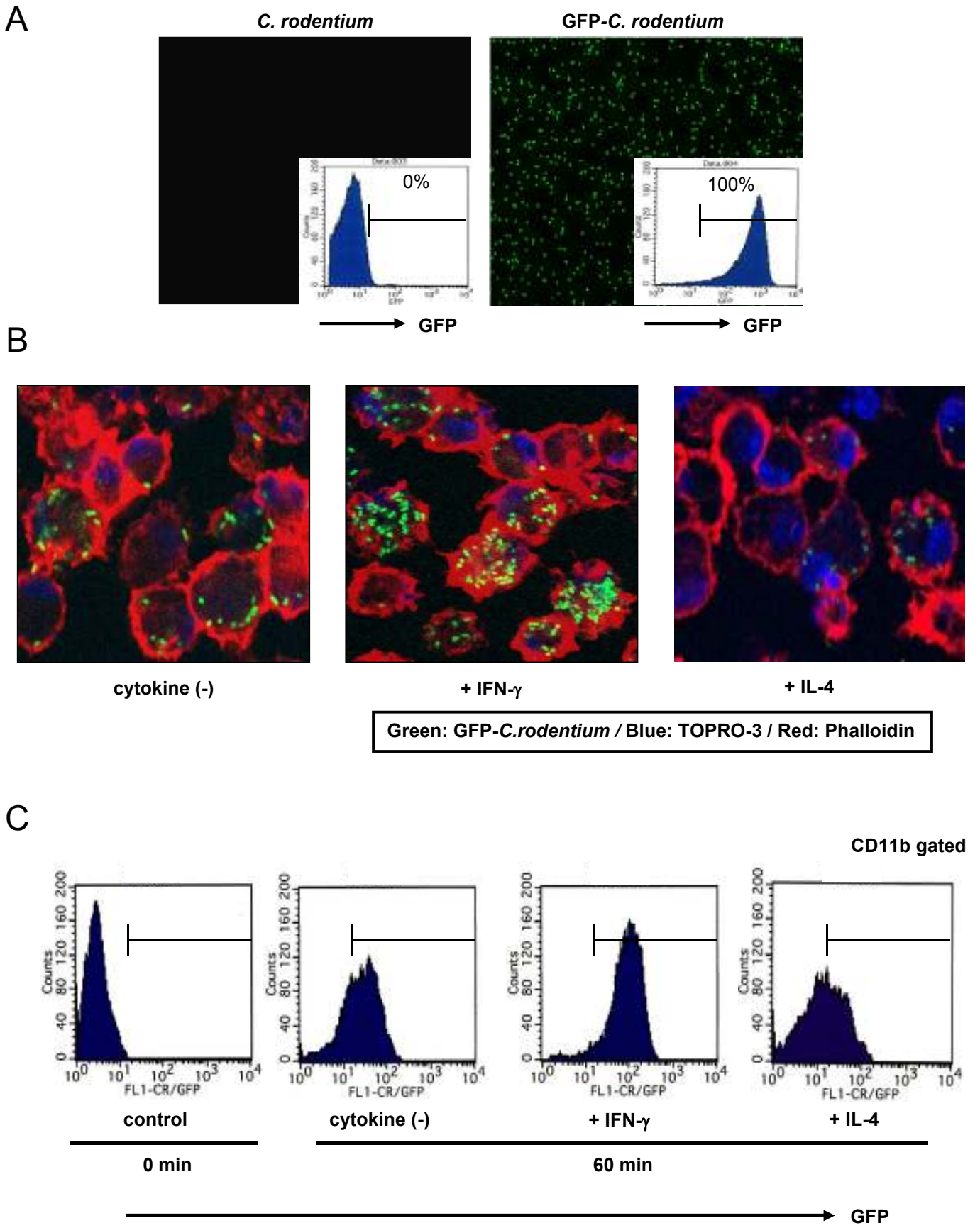


Table 1. The prevalence of Tir and OVA in bacterial colonies in feces

	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.