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Activation of p38α in T Cells Regulates the Intestinal Host Defense against Attaching and Effacing Bacterial Infections

Eun-Jin Shim,* Bo-Ram Bang,* Seung-Goo Kang,* Jianhui Ma,* Motoyuki Otsuka,† Jiman Kang,* Martin Stahl,‡ Jiahuai Han,§ Changchun Xiao,* Bruce A. Vallance,‡ and Young Jun Kang*†

Intestinal infections by attaching and effacing (A/E) bacterial pathogens cause severe colitis and bloody diarrhea. Although p38α in intestinal epithelial cells (IEC) plays an important role in promoting protection against A/E bacteria by regulating T cell recruitment, its impact on immune responses remains unclear. In this study, we show that activation of p38α in T cells is critical for the clearance of the A/E pathogen Citrobacter rodentium. Mice deficient of p38α in T cells, but not in macrophages or dendritic cells, were impaired in clearing C. rodentium. Expression of inflammatory cytokines such as IFN-γ by p38α-deficient T cells was reduced, which further reduced the expression of inflammatory cytokines, chemokines, and antimicrobial peptide by IECs and led to reduced infiltration of T cells into the infected colon. Administration of IFN-γ activated the mucosal immunity to C. rodentium infection by increasing the expression of inflammatory genes and the recruitment of T cells to the site of infection. Thus, p38α contributes to host defense against A/E pathogen infection by regulating the expression of inflammatory cytokines that activate host defense pathways in IECs. The Journal of Immunology, 2013, 191: 000–000.

Keywords: intestinal epithelial cell, T cell, p38α, IFN-γ, Citrobacter rodentium

Department of Immunology and Microbial Science, The Scripps Research Institute, 10550 North Torrey Pines Road, IMM-16, La Jolla, CA 92037. E-mail address: ykang@scripps.edu

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Address correspondence and reprint requests to Dr. Young Jun Kang, Department of Immunology and Microbial Science, The Scripps Research Institute, 10550 North Torrey Pines Road, IMM-16, La Jolla, CA 92037. E-mail address: ykang@scripps.edu

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Abbreviations used in this article: A/E, attaching and effacing; DC, dendritic cell; Delfb1, β-defensin 1; EHEC, enterohemorrhagic Escherichia coli; EPEC, enteropathogenic Escherichia coli; IEC, intestinal epithelial cell; LP, lamina propria; MLN, mesenteric lymph node.

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Materials and Methods

Mice

p38α−/− mice were described previously (17). To generate macrophage-, dendritic cell (DC)−, or T cell–specific p38α-deficient mice, p38α−/− mice...
were bred with LysM, CD11c, or Lck promoter-driven Cre transgenic mice (The Jackson Laboratory, Bar Harbor, ME). C57BL/6J wild-type mice were obtained from Institutional Breeding Colony at the Scripps Research Institute. Animal studies were performed using sex-matched 8- to 10-wk-old mice and conducted according to the guidelines and approval of the Institutional Animal Care and Use Committee.

Bacterial infection and bacterial Ag preparation

*C. rodentium* strain DBS 100 (American Type Culture Collection, Manassas, VA) in a volume of 200 μl (2 × 10^9 CFU) was orally inoculated in each mouse after fasting for 8 h. IFN-γ (10 μg per mouse; R&D Systems) was injected i.p. at the indicated time points after *C. rodentium* infection. *C. rodentium* lysate was prepared, as previously described (24, 25).

**CFU count, colon tissue collection, and cell isolation**

To assess the level of *C. rodentium* infection, in colonic tissues, a distal piece (~1 cm) of colon was removed, weighed, and homogenized in sterile PBS. Homogenates were serially diluted in PBS and plated on MacConkey agar. The number of colonies was counted after 18 h of incubation at 37°C. To obtain RNA of colon tissues, a piece of colon (~0.5 cm) was collected and kept in RNAlater (Qiagen), according to the manufacturer’s protocol.

Histological analysis

To compare the degree of inflammation, colon tissues were fixed in 10% formalin, and paraffin sections were stained with H&E for histology analysis. The histological scoring was assessed to determine the degree of inflammatory cell infiltration and tissue damage (26). The cell infiltration score was defined as a scale of 0–3 of inflammatory cell infiltration (0, no or occasional inflammatory cells in the LP; 1, slightly increased number of inflammatory cells; 2, moderate infiltration of inflammatory cells; 3, extensive infiltration of inflammatory cells). The histological tissue damage score was determined (0, no damage; 1, mild hyperplasia with superficial epithelial injury; 2, moderate hyperplasia, with focal erosions; 3, severe hyperplasia with multifocal erosions).

**Immunohistochemistry**

Immunostaining of *C. rodentium* and CD4 T cells was performed as follows. Paraffin section slides were deparaffinized and rehydrated prior to Ag retrieval by boiling in 10 mM sodium citrate buffer (pH 6.0). Sections were blocked in blocking buffer (3% BSA and animal-free blocker [Vector Labs, Burlingame, CA]) and stained with rabbit anti- C. rodentium Ab, followed by Alexa Fluor 488 anti-rabbit IgG (Molecular Probe, Eugene, OR), or stained with FITC-conjugated anti-mouse CD4 (BioLegend, San Diego, CA). Slides were counterstained with VECTASHIELD mounting media with DAPI (Vector Labs, Burlingame, CA) prior to visualization.

**Preparation and stimulation of T cells**

T cells were purified using Pan T Cell Isolation Kit II (Miltenyi Biotech, Auburn, CA) from spleen. To induce T cell activation, cells were seeded on the plates coated with anti-CD3/CD28 Abs. After 3d, culture supernatants were collected to determine IL-2 and IFN-γ levels by ELISA. Cell proliferation was tested using CFSE cell proliferation kit (Invitrogen). Cells were labeled with CFSE and cultured in the plates coated with anti-CD3/CD28 Abs for 3 d. Degree of proliferation was measured using flow cytometer, according to the manufacturer’s protocol.

**Ex vivo colon culture and cytokine measurement**

Colon fragments (~1 cm) were obtained aseptically and weighed. The pieces were washed three times in ice-cold PBS and incubated in DMEM supplemented with 10% FBS and antibiotics for 24 h (24, 25). Culture supernatants were obtained, and cytokine levels were measured by ELISA.

**Preparation of bone marrow–derived DCs**

Bone marrow cells were obtained and cultured in RPMI 1640 supplemented with 10% FBS and GM-CSF (20 ng/ml) for 6 d.

**Preparation of FITC-conjugated *C. rodentium* and analysis of phagocytic activity of macrophages and DCs**

Labeling of *C. rodentium* and phagocytosis analysis was as previously described (27). Cultured *C. rodentium* was labeled with 1 μl/ml FITC solution in PBS for 15 min. Peritoneal macrophages from control or macrophage-specific p38α-deficient mice, or bone marrow–derived DCs from control or DC-specific p38α-deficient mice were cocultured with FITC-conjugated *C. rodentium* for 6 h. Cells were washed and harvested in ice-cold PBS. DCs were stained with anti-CD11c PE Ab. Intracellular level of FITC-labeled *C. rodentium* was measured by flow cytometry.

**Flow cytometry of intracellular cytokine and surface marker expressions**

Isolated cells from LP were treated with brefeldin A (10 μg/ml), PMA (50 ng/ml), and ionomycin (1 μg/ml) for 4 h before intracellular cytokine staining of IL-17 and IFN-γ. Intracellular staining of cytokines was performed using Cytofix/Cytoperm Fixation/Permeabilization Solution kit (BD Biosciences). LP and MLN cells were suspended in FACS buffer and incubated with the indicated Abs for FACS analysis. Fc Block (anti-CD16/CD32), anti-CD4 allophycocyanin, anti-IL-17A FITC, anti–IFN-γ PE, anti-CD11c allophycocyanin, anti–TNF-α FITC, anti–MHCII PE, anti-CD80 PE, and anti-CD86 FITC (eBiosciences) Abs were used, as indicated. Stained cells were analyzed by LSR-II (BD Biosciences) and using FlowJo (version 3.6; Tree Star) software.

**RNA isolation and quantitative PCR analysis**

Total RNA from the colon tissues or LP lymphocytes was isolated using RNeasy kit (Qiagen), and cDNA was synthesized by reverse transcription. The mRNA levels of indicated mouse genes were determined by quantitative PCR analysis using the SYBR Green/ROX qPCR Master Mix (Thermo Scientific). All values were normalized to the housekeeping gene *actin* mRNA, and relative expressions were calculated by the ΔΔ cycle threshold method. Fold induction of genes was compared with the gene expression levels of uninjected mice.

**Statistical analysis**

Differences were tested using the Student t test. The p values are shown, and a p value <0.05 was considered statistically significant.

**Results**

p38α in T cells, not in macrophages or DCs, plays an essential role in protection against *C. rodentium infection*

We previously reported that p38α in IECs promotes T cell recruitment to provide protection against *C. rodentium* infection (24). To test the role of p38α in immune cells against *C. rodentium* infection, we generated macrophage-specific (p38αADΔC) (17), DC-specific (p38αADΔC), or T cell–specific (p38αAT) p38α-deficient mouse strains. Expression of p38α was defective in thymocytes, purified splenic T cells, and LP T cells, but was intact in other tissues and cells, including B cells and macrophages in p38αAT mice (Supplemental Fig. 1A, 1B). Flow cytometry analysis also confirmed the deletion of p38α in DCs of p38αADΔC mouse (Supplemental Fig. 1C). The ratio of CD4+ and CD8+ cells among CD3+ T cells in the thymus, spleen, and LP was comparable between control (p38αADΔC) and p38αAT mice (Supplemental Fig. 1D), indicating that deletion of p38α does not affect the development of T cells. Also, p38α-deficient T cells did not show any significant differences in TCR-mediated responses such as proliferation and IL-4 production compared with the control cells, whereas production of IFN-γ was significantly reduced in p38α-deficient T cells (Supplemental Fig. 2). This observation is con-
sistent with other reports using inhibitors or kinase-dead knock-in mutant mice (21, 28).

To test the role of p38α in immune cells against *C. rodentium* infection, p38αΔfl/fl, p38αΔMAC, p38αΔDC, or p38αΔT mice were orally inoculated with *C. rodentium*. Colon tissues were collected to measure *C. rodentium* CFU after 1, 2, 3, or 4 wk postinfection. *p* < 0.05, **p < 0.01. Error bars indicate SD. The results shown are representative of two to three experiments.

*p38α regulates the production of inflammatory cytokines and the recruitment of T cells*

We further investigated the mechanism of p38α-mediated T cell responses against *C. rodentium* infection. Because DCs are on the front line of host defense against the bacterial infection, we tested whether the activation of DCs was affected in p38αΔT mice. DCs were obtained from p38αΔm and p38αΔT mice after 1 wk of *C. rodentium* infection. No significant differences in the expression of surface markers like CD80, CD86, or MHC II, and production of the inflammatory cytokine TNF-α were observed in CD11c+ DCs in MLNs of p38αΔm and p38αΔT mice (Fig. 3A). Also, activation of DCs in LP was comparable between p38αΔm and p38αΔT mice (data not shown). These results indicated deletion of p38α is not a significant player in inflammatory cells and tissue damage was assessed (n = 6). *p < 0.05, **p < 0.01. Error bars indicate SD. (C) Detection of *C. rodentium* in the colon tissues. Colon tissue specimens of infected mice were stained with anti-*C. rodentium* Ab (green), and nuclei were counterstained with DAPI (blue). Inflammation and bacterial staining of colon tissue of uninfected mouse are shown. Original magnification ×100.

FIGURE 1. p38α in T lymphocytes, not in macrophages or DCs, plays an essential role in host defense against *C. rodentium*. Bacterial loads in the colon tissues of tissue-specific p38α-deficient mice. Control (p38αΔfl/fl), or (A) T cell (p38αΔT, n = 6–7), (B) macrophage (p38αΔMAC, n = 6–7), or (C) DC (p38αΔDC, n = 5–6)-specific mice were orally inoculated with *C. rodentium*. Colon tissues were collected to measure *C. rodentium* CFU after 1, 2, 3, or 4 wk postinfection.
p38α in T cells did not overtly affect the function of DCs during *C. rodentium* infection.

We next examined the function of T cells in *C. rodentium*-infected p38α<sup>fl/fl</sup> and p38α<sup>AT</sup> mice. Because the expression of IFN-γ and IL-17 is critical for the mucosal immunity against *C. rodentium* infection (6, 11), the intracellular levels of IFN-γ and IL-17 in the intestine-associated LP T lymphocytes were examined by flow cytometry. Expression of IFN-γ was significantly reduced in CD4<sup>+</sup> T cells of p38α<sup>AT</sup> mice postinfection compared with p38α<sup>fl/fl</sup> mice. However, abrogation of p38α in T cells did not affect the expression of IL-17, indicating that p38α regulates Th1 responses in bacterial infection (Fig. 3B). We further analyzed the expression of proinflammatory cytokines in LP lymphocytes in *C. rodentium*-infected p38α<sup>fl/fl</sup> and p38α<sup>AT</sup> mice by quantitative PCR method. Consistent with the flow cytometry results, induction of IFN-γ was reduced in T cells from p38α<sup>AT</sup> mice, whereas that of IL-17 was similar between p38α<sup>fl/fl</sup> and p38α<sup>AT</sup> mice (Fig. 3C). Inflammatory cytokines such as IL-2, IL-12, and IL-22 are important components of T cell–mediated host defense against the enteric bacterial infections. Expression levels of IL-2, IL-12, and IL-22 were significantly reduced, whereas IL-10 level was not affected in p38α<sup>AT</sup> mice, indicating that p38α regulated the production of inflammatory cytokines by T cells that are known to provide host protection against *C. rodentium* infection. Because the infiltration of inflammatory cells was reduced in the colon tissues of p38α<sup>AT</sup> mice (Fig. 2A, Supp. Fig. 3), the recruitment of immune cells into the colonic mucosa was further tested. Flow cytometry analysis of CD4<sup>+</sup> T cells in isolated LP lymphocytes showed that recruitment of CD4<sup>+</sup> T cells into the colonic mucosa in p38α<sup>AT</sup> mice was decreased (Fig. 3D), which indicated that p38α in T cells also affected the infiltration of CD4<sup>+</sup> T cells to the site of *C. rodentium* infection. These results suggested that p38α in T cells regulated the activation and infiltration of T cells to protect the host from infection by A/E bacterial pathogens.

**IEC function was affected by reduced T cell activation in *C. rodentium*-infected p38α<sup>AT</sup> mice**

One of the functions of IECs is to maintain a protective barrier against luminal pathogens. The tight junctions between epithelial cells are known to play an important role in protecting against the translocation and escape of the enteric bacteria from the intestinal lumen (29). We tested the integrity of epithelial cells in p38α<sup>fl/fl</sup> and p38α<sup>AT</sup> mice by measuring *C. rodentium* CFUs in liver tissues following infection (30). Bacterial counts in liver tissues were comparable between p38α<sup>fl/fl</sup> and p38α<sup>AT</sup> mice after 1 or 2 wk of infection (Fig. 4A), suggesting that IEC barrier function was not affected by reduced activation of T cells in p38α<sup>AT</sup> mice.

Next, we tested whether reduced T cell activation resulted in changes of mucosal defense function in p38α<sup>AT</sup> mice by comparing the expression levels of proinflammatory cytokines, chemokines, and antimicrobial peptides in colon tissues. Ex vivo production of cytokines was measured by incubating colon tissue fragments from *C. rodentium*-infected mice, and we found that the levels of IFN-γ, IL-22, and IL-6 were lower in the colon of p38α<sup>AT</sup> mice compared with control mice, whereas IL-17 and TNF levels did not differ (Fig. 4B, 4C). Expression of proinflammatory cytokines, chemokines, and antimicrobial peptides from colonic tissues of *C. rodentium*-infected p38α<sup>fl/fl</sup> and p38α<sup>AT</sup> mice was further examined by quantitative PCR analysis (Fig. 4D). The mRNA levels of inflammatory cytokines in the colonic tissues were similar; IFN-γ, IL-22, and IL-6 levels were lower in the colon of p38α<sup>AT</sup> mice, whereas IL-17 and TNF levels were comparable. We previously reported that p38α in IECs promotes the expression of chemokines such as CXCL10 and CCL25 that recruit T cells (24). In the colon tissue of p38α<sup>AT</sup> mice, expression...
of CXCL10 and CCL25 was significantly reduced, whereas CXCL2 levels were comparable (Fig. 4D). The expression of intestinal antimicrobial peptides did not differ between C. rodentium–infected p38αβfl/fl and p38αβAT mice, except that expression of β-defensin 1 (Defb1) was significantly reduced in p38αβAT mice, indicating that Defb1 expression was regulated by inflammatory cytokines produced by T cells (Fig. 4D).

Induction of chemokine expression by IECs contributes to host defense against C. rodentium infection (4), and expression of chemokines in the intestinal mucosa regulates the recruitment of effector lymphocytes to the intestine (31, 32). Therefore, we examined the expression of cytokines and chemokines by IECs of C. rodentium–infected p38αβAT mice. Similar to the colon tissues, expression level of IFN-γ and IL-22 was significantly lower in the IECs of p38αβAT mice. Also, expression of CXCL10 was significantly reduced in IECs of p38αβAT mice (Fig. 4E), indicating that p38αβ-mediated T cell activation regulated the expression of inflammatory cytokines and chemokines in the IECs of C. rodentium–infected mice, which further recruited T cells to the site of bacterial infection. These results suggested that p38αβ-mediated T cell activation limited the A/E bacterial burden by promoting defense mechanisms within the intestinal mucosa.

Treatment of IFN-γ activates the host defense against C. rodentium infection in vivo

Given the results that reduced expression of inflammatory cytokines such as IFN-γ by p38αβ-deficient T cells resulted in the impaired host defense against C. rodentium infection, we tested the recovery/promotion of host defense by IFN-γ treatment in C. rodentium infection. First, we examined whether IFN-γ administration recovered the reduced host defense activity of p38αβAT mice. Bacterial counts in colon tissues were lower, and the development of transmissible colonic hyperplasia was less significant in IFN-γ–treated p38αβAT mice compared with the mice treated with PBS (Fig. 5A, and data not shown), indicating that IFN-γ recovered the reduced host defense against the enteric bacterial infection that was impaired by p38αβ deletion in T cells. We further evaluated whether administration of IFN-γ enhanced the immune response of C. rodentium–infected wild-type mice. The bacterial CFU was significantly lower in the colon tissues by IFN-γ administration (Fig. 5B). Also, treatment of IFN-γ in-
creased the recruitment of inflammatory cells and ameliorated the colonic tissue damage (Fig. 5C, 5D). Recruitment of T cells to the site of bacterial infection and the expression of some essential genes for host defense were significantly upregulated in the C. rodentium-infected wild-type mice treated with IFN-γ (Fig. 5E, 5F), suggesting that administration of IFN-γ activated the mucosal immune response against C. rodentium infection.

In this study, we showed that p38α was essential for the T cell–mediated immune response against C. rodentium infection. Although p38α has been previously reported to play an important role in innate immune responses against many different types of microbial infections, we found that activation of p38α did not limit C. rodentium infection in macrophages and DCs. In line with the role of p38α in IECs regulating the recruitment of T cells, our current study suggests that p38α regulates T cell– and IEC-mediated host defense against A/E pathogen infection.

Discussion

NF-κB and p38 MAPK signaling pathways are critical in the development of host defense against pathogenic enteric bacterial infections. The NF-κB pathway is essential for maintaining immune homeostasis in IECs, as abrogation of NF-κB signaling within IECs dramatically impaired mucosal immune responses, dysregulated IEC integrity, and led to the subsequent failure to clear bacterial pathogen burdens (12, 33, 34). Using IEC-specific p38α-deficient mice, we previously showed that p38α in IECs plays a protective role in host defense against C. rodentium infection by recruiting T cells to the site of infection, whereas the immune functions were not affected (24). In this study, we demonstrated that mice lacking p38α in T cells, but not in macrophages or DCs, failed to clear C. rodentium infection, indicating that p38α regulates the adaptive immunity to limit the degree of A/E pathogen infection. In TLR-mediated innate immune responses, p38α regulates the activation of inflammatory signaling pathways (17). TLR2 is required in maintaining mucosal integrity, and MyD88-mediated signaling pathway is essential for a protective innate immunity by neutrophils in C. rodentium infection (4, 7). However, TLR4 deficiency showed a delayed spread and colonization of C. rodentium, indicating that TLR4-mediated responses against this A/E pathogen are not host protective despite that the role of TLR4 in mucosal immunity against the enteric bacterial infection. Also, administration of IFN-γ increased the expression of CXCL10 to enhance the host defense mechanism of C. rodentium–infected wild-type mice by recruiting T cells to the site of bacterial infection. Expression of IFN-γ and Defb1 was also increased, indicating that IFN-γ activated the mucosal immune response to protect a host from the enteric bacterial infection. Our data have suggested that a strategy that activates the host mucosal immunity can be used as a treatment of A/E pathogen infection. Treatment of the A/E pathogen infection such as E. coli O157:H7 is limited to the replacement of fluids and electrolytes to prevent dehydration because antibiotics may increase the chance of developing hemolytic uremic syndrome, a potentially fatal complication caused by Shiga toxin–mediated kidney failure (47). Therefore, activation of host mucosal immune response such as targeting the p38α signaling can be used as a potential future method for the treatment of A/E infection.

Disclosures

The authors have no financial conflicts of interest.

References


**Supplemental Figure S1**

(A & B) T cell-specific deletion of p38α. Lysates of tissues and immune cells from control (p38α<sup>fl/fl</sup> or C) and T cell-specific p38α-deficient (p38α<sup>KO</sup> or KO) mice were analyzed by immunoblotting using anti-p38α antibodies. Equal loading of proteins was assessed by detecting GAPDH levels (A). (B & C) LP lymphocytes were stained with anti-CD3-APC and anti-CD4-FITC (B) or mesenteric lymph node (LN) cells were stained with anti-CD11c-FITC (C) Abs. Cells were further permeabilized and stained with rabbit anti-p38α<sup>g68</sup>/g39 followed by anti-rabbit-PE Abs. Intracellular p38α was detected in CD3+CD4+ or CD11c+ cells. 1, isotype Ab control; 2, p38α<sup>fl/fl</sup> LP cells; 3, p38α<sup>KO</sup> LP (in B) or p38α<sup>KO</sup> DC LN cells (D) Subsets of T cells.

(D) Subsets of T cells. Cells from the thymus, spleen, or lamina propria were obtained from p38α<sup>fl/fl</sup> or p38α<sup>KO</sup> mice, and stained with fluorescein-conjugated antibodies. The proportion of CD4 and CD8 T cells in the CD3<sup>+</sup> cell population were analyzed by flow cytometry. The percentage of each subset of T cells is shown as mean ± s.d. in the table.
Supplemental Figure S2. Role of p38α in T cell activation in vitro. T cells from the spleens of p38α<sup>fl/fl</sup> or p38α<sup>ΔT</sup> mice were obtained. (A) Purified T cells were stained with CFSE and stimulated with medium or anti-CD3/CD28 antibodies for 3 days. Proliferation of T cells was analyzed by flow cytometry. (B) T cells were stimulated with medium or anti-CD3/CD28 antibodies, and culture supernatants were obtained. IFN-γ and IL-4 levels in culture supernatants were measured by ELISA. *, p<0.05, and error bars indicate s.d.
Supplemental Figure S3. Innate immunity is not regulated by p38α in *C. rodentium* infection. (A & B) Inflammation of colon tissues in the *C. rodentium*-infected mice. Colon tissues of *C. rodentium*-infected p38α<sup>fl/fl</sup> or p38α<sup>ΔMAC</sup> (A), or p38α<sup>fl/fl</sup> or p38α<sup>ΔDC</sup> (B) mice were obtained after 2 weeks of infection and stained with hematoxylin and eosin. Original magnification is shown. Scale bar = 20 μm. (C & D) Role of p38α in phagocytosis of *C. rodentium*. Peritoneal macrophages from p38α<sup>fl/fl</sup> or p38α<sup>ΔMAC</sup> mice (C), or BM-derived DCs from p38α<sup>fl/fl</sup> or p38α<sup>ΔDC</sup> mice (D) were incubated with FITC-labeled *C. rodentium* (1 mg/ml of FITC in PBS for 15 min). Cell:bacteria=1:100. After 6 hours, cells were washed and harvested in ice-cold PBS to measure the intracellular phagocytosis of *C. rodentium*. DCs were stained with anti-CD11c-PE antibodies. (E) p38α in macrophages is not involved in the host response to *C. rodentium*. Peritoneal macrophages from p38α<sup>fl/fl</sup> or p38α<sup>ΔMAC</sup> mice were obtained and stimulated with medium, LPS (100 ng/ml), live *C. rodentium* (10<sup>9</sup> CFU), or *C. rodentium* lysates (100 μg/ml) for 24 hours. TNF-α levels in culture supernatants were measured by ELISA.
Supplemental Figure S4. Inflammation in \textit{C. rodentium}-infected mice. H & E staining of colon tissues of \textit{C. rodentium}-infected p38$\alpha^{fl/fl}$ or p38$\alpha^{AT}$ mice. Colon segments were obtained after 2 weeks of infection. Boxed area is x200 of the original x100 magnification. Arrows in the figure indicate the infiltration of inflammatory cells. Scale bar = 20 $\mu$m.