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6 **Establishment of a novel mouse model of ulcerative colitis with concomitant**
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9 **cytomegalovirus infection - *in vivo* identification of cytomegalovirus persistent**
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11 **infected cells -**
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23 Abbreviations: MPO: myeloperoxidase, wk: week, wpi: week post infection,
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Abstract**Background**

Human cytomegalovirus (HCMV) infection is considered to be an exacerbating factor in ulcerative colitis (UC) patients. However, the pathogenicity of HCMV in the exacerbation of UC remains unclear. The lack of a model mimicking UC with HCMV infection has posed a challenge for research into pathogenic mechanism of HCMV in flare of UC. Therefore, the aim of our study was to establish a new mouse model of UC with HCMV infection.

Methods

We established latent murine CMV (MCMV) infection in T cell receptor-alpha knockout (TCR α KO) mice at an early age by adjustment of viral dose. Next, we performed immunohistochemical analysis in various organs of infected adult TCR α KO mice to prove the correlation between MCMV infection and development of colitis. We then assessed colitis histologically and cytokine expression in the colon of infected and uninfected TCR α KO mice. Finally, the types of MCMV-infected cells in the inflamed colon were examined by immunohistochemical analysis.

Results

MCMV antigen-positive cells reappeared predominantly in the inflamed colon of TCR α

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6 KO mice. Severe colitis developed in the infected TCR α KO mice compared with
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9 uninfected mice, and Th1/Th17 and Th2 responses were strongly induced.
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12 MCMV-infected cells were mainly perivascular stromal cells including pericytes,
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15 expressing PDGFR- β and CXCL12.
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17 **Conclusions**

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20 Here, we established to our knowledge the first mouse model of UC with HCMV
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23 infection. This model is an excellent tool for clarifying the detailed pathogenicity of
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26 HCMV in the exacerbation of UC and developing new treatment strategy for active UC
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29 with HCMV infection.
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35 **Key words:** ulcerative colitis, cytomegalovirus, perivascular cells
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Introduction

Human cytomegalovirus (HCMV) is a member of the herpes virus family. HCMV usually infects the host asymptotically during childhood, and establishes life-long latency in 50% to 80% of the human population.^{1, 2} The infection is sometimes life threatening for fetuses, infants, and immunocompromised individuals because of direct cytopathic actions that lead to systemic organ injury, but most healthy subjects do not develop any HCMV infection-related disease. Numerous case series have reported the reactivation of HCMV in patients with severe inflammatory bowel disease (IBD) refractory to standard immunosuppressive therapy.³ The prognosis of patients with IBD complicated by HCMV reactivation is poor.⁴ To improve the management of IBD patients, the impact of HCMV infection on IBD must be clarified.

HCMV infection is more frequently detected in patients with ulcerative colitis (UC) than in those with Crohn's disease.^{5, 6} Thus, it is considered that not only the disease activity of UC itself but also immunosuppressive therapies might contribute to HCMV reactivation in the colonic mucosa. Among several cytokines, TNF- α is strongly involved in triggering CMV reactivation.⁷ The detailed mechanisms of UC exacerbation by HCMV infection, however, remain unclear. Because CMV has strict species specificity, and HCMV cannot infect mice, animal models using their unique CMVs are

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6 required for studying the pathologic role of HCMV infection in UC. Additionally, it is
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9 also important to use animals which spontaneously develop chronic colitis resembling
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11 human UC. Currently, animal models suitable for investigating UC with HCMV
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13 infection have not yet been established, although there is only one report that latent
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18 CMV infection exacerbates chemically-induced acute colitis.⁸
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21 The genomes of murine CMV (MCMV) and HCMV are similar at the genetic and
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23 nucleotide composition levels and thus MCMV is a useful tool for understanding
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25 HCMV pathogenesis.⁹ T cell receptor-alpha knockout (TCR α KO) mice spontaneously
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27 develop bowel inflammation, which is considered to be a model of human UC.^{10, 11}
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31 Therefore, we examined the effects of MCMV infection on immune-mediated colitis in
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Therefore, we examined the effects of MCMV infection on immune-mediated colitis in TCR α KO mice and established an experimental UC model with exacerbation by MCMV infection. In addition, we investigated the pathology of this colitis model with MCMV infection, and identified the MCMV-infected cells in the colonic mucosa.

Materials and Methods

Mice and MCMV infection.

C57BL/6 mice and TCR α KO mice (Charles River Japan, Inc., Kanagawa, Japan) were used for the experiments. All mice were housed in specific pathogen-free conditions. MCMV-Smith strain and recombinant MCMV (MCMV-EGFP) were passaged in mouse embryonic fibroblasts. MCMV-EGFP was constructed to express EGFP under the control of the MCMV e1 promoter.¹² Complete viral growth, latency, and pathogenesis are similar between MCMV-EGFP and MCMV-Smith.¹³ Newborn mice 7 days after birth were infected by intraperitoneal injection of various doses (1×10^3 ~ 1×10^6 PFU) of MCMV-Smith or MCMV-EGFP in 50 μ l of DMEM or injected with DMEM as a control.

Detection of infectious virus and viral genomes.

Mice were killed for collection of the liver, spleen, and colon at 7 days post infection. Virus titers in the homogenates of each organ were determined using a standard plaque assay. DNA extraction was performed using ISOGEN (Nippon Gene, Tokyo, Japan). Viral genomes were quantified by real-time PCR using the FastStart Universal SYBR Green Master (Roche, Mannheim, Germany). A fragment of the gB, the major envelope

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6 glycoprotein B of the MCMV, gene was amplified from MCMV genomic DNA by PCR
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9 using oligonucleotides 5'-GAA GAT CCG CAT GTC CTT CAG-3' (forward) and
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12 5'-AAT CCG TCC AAC ATC TTG TCG-3' (reverse).⁷ In this procedure, pSM3fr, made
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15 from the full-length MCMV DNA with a bacterial artificial chromosome system,¹⁴ was
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18 used as the standard. The samples were amplified in a LightCycler® 480 System
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21 (Roche) using the following program: 95°C for 5 min, 1 cycle; and 95°C for 10 s, 60°C
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24 for 10 s, and 72°C for 10 s, 45 cycles. Semi-quantitative PCR was performed with
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27 gene-specific primers for β -actin or gB. The oligonucleotide primers for the β -actin
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30 gene were 5'-GTG GGC CGC CCT AGG CAC CAG-3' (forward) and 5'-CTC TTT
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33 GAT GTC ACG CAC GAT TTC-3' (reverse). The oligonucleotide primer sequences as
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36 mentioned above were used for the gB gene. The samples were amplified in an MJ
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39 Research Dyad Thermal Cycler (Bio-Rad Laboratories AB, Hercules, CA, USA) using
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42 the following program: 94°C for 30 s, the corresponding annealing temperature (57°C
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45 for β -actin and 59°C for gB) for 30 s, and 72°C for 1 min, 36 cycles. The products were
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48 analyzed by agarose gel electrophoresis (2% agarose).

52 **Histologic evaluation.**

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55 The colons of C57BL/6 mice and TCR α KO mice were divided into two segments to
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6 represent the distal and proximal colon. The histology of each colon segment was
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9 evaluated with H&E staining. The severity of inflammation in each section was scored
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12 based on a histologic index ranging from 0 to 19, as previously described.¹⁵ This scoring
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15 system was based on the degree of loss of goblet cells (0-3), crypt abscesses (0-2),
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18 epithelial erosion (0-2), hyperemia (0-3), cellular infiltration in the lamina propria (0-3),
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21 thickness of colonic mucosa (0-3), and increased number of colonic glands (0-3).
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26 **Quantitative analysis of mRNA expression of various cytokines, CXCL12,**
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29 **PDGFR- β , and CXCR4.**
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32 mRNA was assessed in the colonic tissues of MCMV-infected TCR α KO mice and
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34 uninfected TCR α KO mice. Total RNA extraction was performed using ISOGEN
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36 (Nippon Gene) and cDNA was generated as described previously.¹⁶ The protocol for the
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38 real-time PCR was performed as described above. Quantitative gene expression data
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41 were normalized to the expression levels of the 18S ribosomal RNA gene. The
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44 following primer sets were used: TNF- α , 5'-CCA GTG TGG GAA GCT GTC TT-3'
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47 (forward) and 5'-AAG CAA AAG AGG AGG CAA CA-3' (reverse); IFN- γ , 5'-ATG
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50 AAC GCT ACA CAC TGC ATC-3' (forward) and 5'-CCA TCC TTT TGC CAG TTC
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53 CTC-3' (reverse); IL-6, 5'-TCC AGT TGC CTT CTT GGG AC-3' (forward) and
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6 5'-GTG TAA TTA AGC CTC CGA CTT G-3' (reverse); IL-4, 5'-GGT CTC AAC CCC
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9 CAG CTA GT-3' (forward) and 5'-GCC GAT GAT CTC TCT CAA GTG AT-3'
10
11 (reverse); IL-13, 5'-AGA CCA GAC TCC CCT GTG CA-3' (forward) and 5'- TGG
12
13 GTC CTG TAG ATG GCA TTG-3' (reverse); IL-17, 5'-GGC CCT CAG ACT ACC
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15 TCA AC-3' (forward) and 5'- TCT CGA CCC GAA AGT GAA GG-3' (reverse); TGF- β ,
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17 5'-TGA CGT CAC TGG AGT TGT ACG G-3' (forward) and 5'-GGT TCA TGT CAT
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19 GGA TGG TGC-3' (reverse); CXCL12, 5'-CCA GAG CCA ACG TCA AGC AT-3'
20
21 (forward) and 5'-CAG CCG TGC AAC AAT CTG AA-3' (reverse); PDGFR- β , 5'-AGC
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23 CCT TGG TTT GCA GCA CT-3' (forward) and 5'-CGA CTC ACA CCA CCG TAC
24
25 AGT CG-3' (reverse); CXCR4, 5'-TAG GAT CTT CCT GCC CAC CAT-3' (forward)
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27 and 5'-TGA CCA GGA TCA CCA ATC CA-3' (reverse); and 18rs, 5'-GCA CAG TGT
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29 TTG TAG AGC CTG-3' (forward) and 5'-GCC CTG GAA CTT ATT GAT CGG G-3'
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31 (reverse).
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46 **Immunohistochemistry.**

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49 MCMV-infected cells were detected by rat monoclonal antibody Q3 specific to
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51 MCMV M45 protein, which is an early-late cytoplasmic antigen with a molecular
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53 weight of 150 kDa and a non-functional homolog of ribonucleotide reductase 1
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6 subunit.¹⁷⁻¹⁹ In paraffin-embedded colonic sections prepared as described previously,¹⁶
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9 immunohistochemical staining for the MCMV antigen and MPO was performed using
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12 rat monoclonal antibody Q3 and rabbit polyclonal antibody to MPO (DAKO,
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14 Copenhagen, Denmark), respectively. The sections were then sequentially incubated
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17 with anti-rat or anti-rabbit IgG conjugated with HRP (Nichirei Biosciences, Tokyo,
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19 Japan) and colored with DAB. In frozen colonic sections fixed by isopropyl alcohol,
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22 immunofluorescence double-staining was performed using primary antibodies to M45,
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25 CD80 (Millipore, Billerica, MA, USA), CD163 (Santa Cruz Biotechnology, Santa Cruz,
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28 CA, USA), keratin (Nichirei Biosciences), CD11b-FITC (BD Pharmingen, San Diego,
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31 CA, USA), CD45 (eBioscience, San Diego, CA, USA), α -SMA-FITC (Abcam,
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34 Cambridge, UK), and CD31 (Abcam). The following secondary antibodies were used:
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37 Alexa Fluor 488 anti-rat IgG (for CD80), Alexa Fluor 488 anti-rabbit IgG (for CD45
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40 and CD31), Alexa Fluor 594 anti-rat IgG (for M45), and Alexa Fluor 594 anti-rabbit
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43 IgG (for CD163) (Invitrogen, Carlsbad, CA, USA). For immunofluorescence
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46 triple-staining, we used primary antibodies against α -SMA-FITC (Abcam),
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49 CD146-FITC (Miltenyi Biotec, Auburn, CA, USA), NG2 (Millipore), CXCL12 (Torrey
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52 Pines Biolabs, Secaucus, NJ, USA), and PDGFR- β (Cell Signaling, Danvers, MA,
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55 USA). For secondary antibodies, Alexa Fluor 647 anti-rabbit IgG (for NG2 and
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6 PDGFR- β) and Alexa Fluor 594 anti-rat IgG (for M45; Invitrogen) were used. In
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8 immunofluorescence triple-staining for M45, PDGFR- β and CXCL12, Zenon Alexa
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10 Fluor 488 rabbit IgG labeling kit (Invitrogen) was used. For the secondary antibody
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12 against CXCL12, Zenon Alexa Fluor 488 rabbit IgG labeling reagent was used. The
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14 procedure for formation of the Zenon complex was performed according to
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16 manufacturer's protocol. For immunofluorescence staining of CXCR4, we used
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18 FITC-conjugated antibody against CXCR4 (BD Pharmingen). The stained sections were
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20 examined using a deconvolution fluorescence microscope system (BZ-8000, Keyence,
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22 Osaka, Japan) and the cell count of MPO-, CD80-, and CD163-positive cells was
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24 performed by the Dynamic Cell Count image analysis program (Keyence). The
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26 proportion of cells positive for CD146, NG2, CXCL12, and PDGFR- β among
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28 M45-positive cells was calculated by counting the number of those cells in 10
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30 high-power fields (total magnification: 200X).
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46 ***In situ* hybridization.**

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49 pSM3fr, used as a DNA probe for chromogenic *in situ* hybridization, was kindly
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51 provided by Dr. UH Koszinowski (Max von Pettenkofer-Institute, Ludwig
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53 Maximilians-University, Munich, Germany). It was derived from a BAC labeled with
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6 digoxigenin (DIG)-11-dUTP (Roche) using a nick translation kit (Roche).²⁰
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9 Hybridization and washing procedures were performed as described previously.¹⁷
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12 Sections were subsequently incubated with HRP-conjugated anti-DIG Fab fragments
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14 (1:100, Roche) and colored brown by DAB, followed by hematoxylin counterstain.
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20 **Statistical Analysis**

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23 Analyses were performed using Statview for Windows (SAS Institute, Cary, North
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26 Carolina, United States). All numerical data are expressed as means \pm SEM. Student's
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29 *t*-test and the Mann-Whitney *U*-test were used where appropriate for statistical analysis.
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32 The cumulative survival rate was calculated by the Kaplan-Meier method. A *P* value of
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35 less than 0.05 was considered statistically significant. The statistical test used and the
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38 sample sizes for individual analysis are provided within the figure legends.
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44 **Ethical Considerations**

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47 All animal experiments were performed in accordance with protocols approved by
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50 the Kyoto University Animal Care and Use Committee.
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Results

Establishment of acute and latent MCMV infection in TCR α KO mice.

Because $\alpha\beta$ T cells have an important role in viral clearance, the infectious dose of MCMV in TCR α KO mice was carefully determined by comparing it to the infectious dose in immune-competent C57BL/6 mice so that an adequate survival rate (at least 60%) and latency could be established. Seven days after birth, neonatal mice were intraperitoneally infected with various doses ($1 \times 10^3 \sim 1 \times 10^6$ PFU) of wild-type MCMV-Smith or recombinant MCMV-EGFP, which was genetically modified to express EGFP as a reporter. The two strains are reported to have similar virulence in immune-competent mice.¹³ We found that with infection at 5×10^5 PFU of MCMV-Smith or at 1×10^6 PFU of MCMV-EGFP, approximately 60% of MCMV-infected C57BL/6 mice survived despite severe growth retardation during the acute phase of infection, whereas all MCMV-Smith infected TCR α KO mice died even at doses under 1×10^5 PFU. Similar mortality (approximately 60%) was observed in TCR α KO mice infected with MCMV-EGFP at 2×10^4 PFU. Thus, all further experiments were performed by injection with 5×10^5 PFU of MCMV-Smith for C57BL/6 mice and 2×10^4 PFU of MCMV-EGFP for TCR α KO mice (Supplementary Fig. 1).

To confirm establishment of acute and latent MCMV infection, we measured the

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6 change of body weight and examined the viral titer and copy number of viral genomic
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9 DNA in various organs of infected mice. Growth retardation was seen between 1 to 2
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11 weeks post infection (wpi) in both MCMV-infected C57BL/6 mice and TCR α KO mice,
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13 but the growth rate after 3 wpi was similar to uninfected mice. The growth retardation in
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15 infected C57BL/6 mice and TCR α KO mice was almost the same degree (Fig. 1A). In
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17 infected C57BL/6 mice and TCR α KO mice, both titers and copy numbers in the organs
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19 were high at 1 wpi, and the viral titers decreased to below the detection limit but copy
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21 numbers were scarcely detected in all organs at 4 wpi (Fig. 1B, C).
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23 Immunohistochemical analysis with an antibody against M45, a viral early-late antigen
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25 of MCMV, revealed the presence of M45-positive cells in various organs at 1 wpi and
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27 their disappearance at 4 wpi in both strains of mice (Fig. 1D, E). These findings
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29 suggested that viral replication was not active at 4 wpi or later, and that latency was
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31 established after the acute infection had subsided in both TCR α KO mice and
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33 immune-competent C57BL/6 mice.
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49 **MCMV antigen-positive cells reappeared predominantly in the inflamed colonic**
50 **mucosa of TCR α KO mice.**
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55 We investigated whether MCMV antigen-positive cells reappeared with the
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6 progression of colitis in infected TCR α KO mice. Generally, TCR α KO mice exhibit
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9 signs of chronic colitis beginning at around 16 week (wk) and colitis is established
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12 within 24 wk.²¹ In our animal facility, TCR α KO mice develop colitis at around 12 wk.
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15 Therefore, we assessed colitis histologically in mice at 5, 12, and 24 wk. TCR α KO
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18 mice at both 12 and 24 wk developed colitis, although TCR α KO mice at 5 wk had no
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21 colitis. Furthermore, colitis in TCR α KO mice at 24 wk was severer than at 12 wk, On
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24 the contrary, C57BL/6 mice did not develop colitis even at 24 wk (Supplementary Fig.
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27 2). Immunohistochemistry for the MCMV-M45 antigen indicated the MCMV-infected
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30 cells, which had disappeared once at 4 wpi (Fig. 1E), reappeared predominantly in the
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33 inflamed colonic mucosa of TCR α KO mice at both 12 and 24 wk (11 and 23 wpi). In
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36 contrast, M45-positive cells were scarcely detected at 12 and 24 wk (11 and 23 wpi) in
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39 other organs (spleen and liver) of TCR α KO mice (Fig. 2A, B). Also, in infected
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42 C57BL/6 mice at 24 wk (23 wpi), M45-positive cells were not observed in any organs
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45 (Fig. 2B). These results suggested that the local reappearance of M45-positive cells in
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48 the colon of TCR α KO mice was involved in intestinal inflammation differing from
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51 MCMV systemic infection in acute phase. Among the MCMV-infected TCR α KO mice
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54 older than 12 wk (11 wpi), the proportion of mice manifesting the reappearance of
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57 M45-positive cells in the inflamed colon was increased from 50% to 88% (Fig. 2A).
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6 Furthermore, the number of M45-positive cells per section of colon in infected TCR α
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9 KO mice was significantly higher at 24 wk (23 wpi) than at 12 wk (11 wpi) (Fig. 2C).
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12 These data suggested that the progression of colitis in TCR α KO mice was associated
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15 with the frequent detection of the MCMV-infected cells in the inflamed colonic mucosa.
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18 The copy number of viral genomic DNA in the colonic tissue of most infected TCR α
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20 KO mice was too low to be analyzed by quantitative PCR (data not shown). We then
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22 used semi-quantitative PCR to amplify MCMV DNA in various organs (i.e., colon,
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24 spleen, and liver) obtained from infected TCR α KO mice at 2, 5, and 24 wk (1, 4, and
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26 23 wpi, respectively). MCMV DNA was strongly detected in various organs of TCR α
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28 KO mice at 2 wk (1 wpi), whereas its expression levels were markedly diminished to
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30 near the detection limit at 5 wk (4 wpi). Although MCMV DNA was also barely
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32 detectable in spleen and liver in infected TCR α KO mice at 24 wk (23 wpi), the PCR
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34 signal was detected in the inflamed colon (Fig. 2D).
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44 *In situ* hybridization was used to detect MCMV DNA in a section of inflamed colonic
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46 mucosa in infected TCR α KO mice at 24 wk that was adjacent to the section expressing
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48 M45-positive cells in immunohistochemistry. *In situ* hybridization signals were found in
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50 the cell nuclei that corresponded to the M45-positive cells observed with
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52 immunohistochemistry (Fig. 2E).
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MCMV infection exacerbates colitis in TCR α KO mice.

We compared the manifestations of colitis between infected and uninfected mice. In addition, we compared the severity of colitis in both the proximal and distal colon. Colon length was significantly shorter in infected TCR α KO mice than in uninfected KO mice at 24 wk (Fig. 3A). As compared to uninfected KO mice, histologic examination revealed severe hyperplasia of the epithelial cells, infiltration of inflammatory cells, and crypt loss in infected TCR α KO mice at around 12 wk. Furthermore, colitis in the infected TCR α KO mice at 24 wk was associated with crypt abscesses, while it was hardly detected in the colon of uninfected TCR α KO mice. In contrast, colitis was not observed in either uninfected or infected C57BL/6 mice at 24 wk (Fig. 3B). The total colitis score in the colon was higher in infected TCR α KO mice than in uninfected TCR α KO mice at both 12 and 24 wk (Fig. 3C). These findings suggested that infected TCR α KO mice develop more severe colitis than uninfected TCR α KO mice.

Reaction of proinflammatory cells during exacerbation of colitis induced by MCMV infection in TCR α KO mice.

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6 To investigate the effect of MCMV infection on the number and phenotype of
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8 infiltrating neutrophils and macrophages, we performed immunohistochemical analysis
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10 for myeloperoxidase (MPO), CD80 (for M1 macrophages), and CD163 (for M2
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12 macrophages) on colonic sections in TCR α KO mice at 24 wk. The M1/M2 ratio was
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14 significantly higher in infected TCR α KO mice than in uninfected TCR α KO mice (Fig.
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16 4A). Immunohistochemistry for MPO revealed that the number of infiltrating
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18 neutrophils was significantly greater in the proximal colon of infected TCR α KO mice
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20 compared with uninfected TCR α KO mice (Fig. 4B).
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29 We then investigated the gene expression of several cytokines in the colonic tissue of
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31 both infected and uninfected TCR α KO mice by real-time PCR. We first compared the
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33 results between the two age groups. In both uninfected and infected TCR α KO mice, the
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35 gene expression of each cytokine in the colon was higher at 24 wk than at 12 wk, except
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37 for *IL-6* in uninfected mice, indicating that the severity of colitis increased with age.
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39 Next, we analyzed the contribution of MCMV infection to cytokine expression in the
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41 colonic tissues. Expression levels of *TNF- α* , *IFN- γ* , and *IL-6*, which are known to be
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43 enhanced under CMV infectious conditions,^{17, 22, 23} were higher in infected TCR α KO
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45 mice than in uninfected TCR α KO mice. Gene expression of *IL-6* at 24 wk differed
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47 significantly between uninfected and infected TCR α KO mice. Expression levels of Th2
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6 cytokines *IL-4* and *IL-13*, which are strongly involved in the development of colitis in
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9 TCR α KO mice, were also higher in infected TCR α KO mice than in uninfected TCR α
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12 KO mice. Furthermore, gene expression of *IL-17* and *TGF- β* was also increased in
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15 infected TCR α KO mice (Fig. 4C). Thus, Th1/Th17 and Th2 immune responses were
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18 enhanced in the colonic mucosa of infected TCR α KO mice.
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20 21 22 23 **MCMV exists in perivascular stromal cells, including pericytes.** 24

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26 We examined the types of MCMV-infected cells in this mouse model to elucidate
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28 how MCMV infection affects intestinal inflammation (Fig. 5). We first investigated
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30 markers corresponding to known MCMV-permissive cells, that is, keratin for epithelial
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32 cells, CD11b for macrophages, CD45 for leukocytes, α -SMA for smooth muscle cells,
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35 and CD31 for endothelial cells. Immunohistochemical analysis indicated that
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38 M45-positive cells did not correspond to the above-mentioned cells. Infected cells were
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41 located in the vicinity of endothelial cells, however, as indicated by the white arrows in
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44 Figure 5 (lower right two panels).
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50 Therefore, we investigated whether the infected cells corresponded to perivascular
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52 cells (e.g., vascular smooth muscle cells or pericytes) surrounding endothelial cells.
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55 Generally, vascular smooth muscle cells are always positive for α -SMA but not always
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6 for NG2, whereas pericytes on capillaries are positive for both NG2 and CD146, but
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8 negative for α -SMA.^{24,25} Results of triple-staining for M45, α -SMA, and NG2 showed
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10 that some infected cells were positive for NG2, but negative for α -SMA (Fig. 6A-1).
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12 These infected cells expressing NG2 were also positive for CD146 (Fig. 6A-2). These
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14 findings indicated that the infected cells were pericytes on capillaries, but not vascular
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16 smooth muscle cells. The proportion of NG2- and CD146-double-positive pericytes was
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18 15% among the M45-positive cells. For further identification of the infected cells,
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20 triple-staining was performed for M45, CXCL12, and PDGFR- β . CXCL12 and
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22 PDGFR- β were expressed on perivascular cells, including pericytes.²⁶⁻²⁹ Among
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24 M45-positive cells, the proportion of CXCL12-positive cells and PDGFR- β -positive
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26 cells was 53% and 79%, respectively, and all the infected cells positive for CXCL12
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28 expressed PDGFR- β . Taken together, the data showed that the infected cells were
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30 mainly perivascular stromal cells, including pericytes, that were positive for CXCL12
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32 and PDGFR- β (Fig. 6A-3).
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46 We also found that gene expression of *CXCL12* and *PDGFR- β* was higher in infected
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48 TCR α KO mice than in uninfected TCR α KO mice at 12 and 24 wk (11 and 23 wpi,
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50 respectively) ; the increase in *CXCL12* was significant at 12 wk (Fig. 6B).
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55 In general, CXCL12 is a member of a large family of structurally related
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6 chemoattractive cytokines and its primary physiologic receptor is CXCR4. Also,
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9 CXCL12-CXCR4 axis plays an important in the pathophysiology of inflammatory
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12 bowel diseases.²⁹ Therefore, we investigated the expression of CXCR4 in the colonic
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15 tissue of both infected and uninfected TCR α KO mice by real-time PCR and
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18 immunohistochemistry. In both uninfected and infected TCR α KO mice, the gene
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21 expression of *CXCR4* in the colon was higher at 24 wk than at 12 wk, indicating that the
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24 severity of colitis correlated with CXCR4 expression. And, expression level of *CXCR4*
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27 at 24 wk was higher in infected TCR α KO mice than in uninfected TCR α KO mice (Fig.
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30 7A). The number of CXCR4-positive cells was higher in the colonic mucosa of infected
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33 TCR α KO mice than in uninfected TCR α KO mice at 24wk (Fig. 7B). These data
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36 strongly suggest that the increased expression of CXCL12 in inflamed colonic tissue of
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39 MCMV-infected TCR α KO mice contributed to the enhancing migration of
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42 CXCR4-positive cells.
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Discussion

Several clinical studies have investigated the role of HCMV infection in the exacerbation of IBD, especially UC. Current data are conflicting, however, as to whether HCMV infection worsens intestinal inflammation or is only a surrogate marker for severe disease.^{30,31} One of the reasons for the uncertainty of the pathogenic role of HCMV infection in IBD is that there has been no animal IBD model suitable for investigating the role of HCMV infection in the pathophysiology of IBD. Here, by infecting TCR α KO mice with MCMV, we established a remarkable mouse model for investigating the pathogenicity of HCMV in IBD. First, we successfully established a latent MCMV-infected condition in both C57BL/6 mice and TCR α KO mice, in which we confirmed the disappearance of viral particles and MCMV-antigen positive cells but the scarce detection of MCMV-DNA copies in several organs. Interestingly, the reappearance of MCMV-antigen positive cells was observed predominantly in the colon, and not in the spleen or liver, of infected TCR α KO mice with the development of colitis. Of note, this reappearance of MCMV-antigen positive cells was not observed in the colon of infected C57BL/6 mice which had no colonic inflammation. Similar to cases of UC patients with HCMV infection, MCMV infection had a deteriorating effect on colitis in TCR α KO mice. We demonstrated for the first time the involvement of

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6 MCMV infection in the progression of spontaneous colitis. Finally, we identified
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9 MCMV-infected cells in the colon of this mouse model. MCMV established persistent
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12 infection in perivascular stromal cells, including pericytes expressing CXCL12 and
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15 PDGFR- β . Thus, our mouse model provides a unique tool for understanding the
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18 association between HCMV infection and exacerbation of colitis in patients with UC.

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21 First, we assessed the optimal dose of MCMV that effectively induced latent infection
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24 without complete elimination by host immunity. In TCR α KO mice, MCMV-EGFP was
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27 less toxic than MCMV-Smith, although the overall pathogenicity of MCMV-EGFP is
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30 reported to be similar to the Smith strain in immune-competent mice.¹³ The region of
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33 the MCMV m128 gene, which is lost in the construction of MCMV-EGFP, has no effect
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36 on viral proliferation and pathogenicity.³² In TCR α KO mice, however, which have an
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39 aberrant immune system compared with C57BL/6 mice (wild-type mice), the lack of the
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42 m128 gene might have resulted in an unexpected immune response and thus in different
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45 susceptibility between the two MCMV strains in our study.

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48 There is only one report that MCMV infection exacerbates chemically induced
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51 colitis, but it was not shown whether MCMV infection directly affected the
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54 development of colitis.⁸ In this study, the correlation between the progression of colitis
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57 and MCMV infection was indicated by immunohistochemical analysis and PCR
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6 analysis. Notably, the reappearance of MCMV-positive cells was predominantly
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9 observed in the inflamed colonic mucosa of TCR α KO mice. Furthermore,
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12 semi-quantitative PCR analysis revealed that the intensity of the PCR signal in the
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15 colonic tissue was higher than that in other organs. These results are consistent with
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18 previously reported clinical findings that HCMV reactivation is detected mainly in the
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21 inflamed colon in UC patients, even when peripheral markers of CMV infection are all
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24 negative.³³ These data suggest that preceding inflammation might promote MCMV
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27 replication in the colonic mucosa. In contrast to MCMV-infected TCR α KO mice,
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30 neither colitis nor M45-positive cells were observed in the colonic mucosa of any
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33 MCMV-inoculated C57BL/6 mice at 24 wk, despite the detection of very weak signals
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36 for MCMV-DNA by PCR (data not shown). This suggests that MCMV was maintained
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39 in a state of latency in the non-inflamed mucosa of immune-competent mice. CMV
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42 reactivation is mainly triggered by TNF- α .⁷ Thus, elevated cytokine levels, including
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45 TNF α , in the inflamed colonic mucosa of MCMV-infected TCR α KO mice might
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48 contribute to the reactivation of MCMV.

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50 To investigate cytokine response of immune cells in both C57BL/6 and TCR α KO
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53 mice after initial infection, we evaluated *in vitro* cytokine production from splenocytes
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56 in C57BL/6 and TCR α KO mice immunized with MCMV. Cytokine production in both
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6 MCMV-inoculated C57BL/6 and TCR α KO mice increased in comparison with
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8 uninoculated mice (Supplementary Fig. 3). These results suggest that MCMV-sensitized
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10 immune cells are ready to suppress virus replication in even latent infection by
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12 producing cytokines, while it might be that these sensitized lymphocytes are easily
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14 activated by any type of stimulation, including local inflammation and virus.
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17 Considering no significant difference of cytokine production from splenocytes between
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19 MCMV-immunized C57BL/6 and TCR α KO mice, the mechanism of viral reemergence
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21 in colonic mucosa of infected TCR α KO mice might be mainly associated with local
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23 intestinal inflammation and increased viral load in the colonic mucosa could contribute
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25 to exacerbation of colitis by enhancing cytokine production.
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35 In the colon of MCMV-infected TCR α KO mice at 24 wk, the viral titer was below
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37 the detection limit of the plaque assay and expression of IE1, E1 and EGFP, which are
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39 essential markers for viral replication, was hardly detected in immunostained or frozen
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41 sections (data not shown). These data together indicate a low level of viral replication.
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44 On the other hand, M45 antigen-positive cells were frequently observed in the colonic
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46 mucosa of these mice. The detailed reason for this phenomenon is unknown, but could
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48 be related to the unique immune status of TCR α KO mice. The aberrant T cell immunity
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50 in TCR α KO mice might result in an attenuated T cell response to the M45 antigen,
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6 which essentially plays an important role in viral clearance during both acute and
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9 chronic phase of MCMV infection in immune-competent mice.³⁴ Thus, it seems that the
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12 reappearance of M45-positive cells in the colon of infected TCR α KO mice reflects
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15 preferential escape of M45 expressing MCMV-infected cells from host
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18 immunosurveillance. The atypical reappearance of MCMV antigen-positive cells in this
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21 study may be interpreted as a kind of incomplete reactivation induced locally by
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24 inflammation in the colonic mucosa of TCR α KO mice.

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26 Histologic findings revealed that MCMV-infected TCR α KO mice manifesting the
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29 reappearance of M45-positive cells developed more severe colitis than uninfected mice.
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32 We then evaluated the mechanism of the progression of colitis by MCMV infection.
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35 CMV infection is known to alter the pattern of cytokine production by modulating the
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38 immune responses.^{17, 22, 23} In the present study, in addition to Th2 cytokines, Th1
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41 cytokines such as TNF- α and IFN- γ were strongly induced in MCMV-infected TCR α
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44 KO mice. Similar persistent expression of Th1 cytokines occurs in MCMV infection.^{17,}
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47 ³⁵ In MCMV-infected foci, antigen-specific CD8⁺ memory T-cells induce long-term
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50 macrophage activation through IFN- γ production.³⁵ We also found in this study that the
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53 expression of both the *IL-6* and *TGF- β* genes was elevated in the colon of the infected
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56 TCR α KO mice compared with uninfected TCR α KO mice. Persistent CMV infection in
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6 human induces an increase in TGF- β production.³⁶ Further, CMV-infected stromal cells
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9 increase the production of IL-6 in humans.³⁷ The increased *IL-6* gene expression
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12 observed in the present study is consistent with clinical data on human UC patients with
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15 concomitant HCMV infection.³⁸ Considering the increased gene expression of *IL-6* and
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18 *TGF- β* in the colonic tissue of infected TCR α KO mice, it seems reasonable that *IL-17*
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21 gene expression was enhanced in infected TCR α KO mice. Together, these findings
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24 indicate that the induction of Th1/Th17 responses was associated with the exacerbation
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27 of colonic inflammation in infected TCR α KO mice.

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29 Our immunohistochemical analysis demonstrated an increased number of neutrophils
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32 and predominant infiltration of M1 macrophages in the inflamed mucosa of infected
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35 TCR α KO mice. Both IL-17 and TGF- β induce the migration of neutrophils, and the
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38 combination of IFN- γ and TNF- α induces the differentiation of macrophages toward the
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41 M1 subtype.^{39,40} CMV infection has been shown to induce the migration of neutrophils
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44 by the production of several chemotactic factors.⁴¹ Thus, the increased cytokine
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47 expression observed in infected TCR α KO mice could contribute to the infiltration of
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50 neutrophils and M1 macrophages in the colonic mucosa.

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52 Finally, we tried to identify the MCMV-infected cells in the inflamed colonic mucosa
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55 of TCR α KO mice. Many reports on HCMV-infected cells in the colonic tissue of
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6 immunocompromised patients (e.g., AIDS patients and organ transplant recipients) or
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9 severe UC patients revealed that the HCMV-infected cells are endothelial cells,
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12 epithelial cells, other stromal cells, or macrophages.^{5, 42-45} Foucar et al. proposed that
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15 HCMV infection in epithelial cells or endothelial cells is a feature of a lethal disease
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18 condition of HCMV infection.⁴² Kuwabara et al. showed that the HCMV positivity in
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21 endothelial cells tends to correlate with the severity of HCMV infection in patients with
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24 UC.⁵ The results of the present study clearly demonstrated that most of the
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27 MCMV-infected cells in the colonic mucosa of TCR α KO mice were stromal cells near
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30 the endothelial cells. Triple immunofluorescence staining further demonstrated that
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33 MCMV-infected cells were perivascular stromal cells, including pericytes, but not
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36 endothelial cells. It remains unclear why the infected cells in the colonic mucosa
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39 differed between our experimental data and previous clinical reports. It might be due to
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42 the difference in species. Alternatively, it may reflect the difference of the disease stages.
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45 Indeed, most of the previous human reports examined autopsy samples from
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48 immunocompromised patients or surgical specimens from UC patients who underwent
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51 colectomy for severe condition, including perforation and toxic megacolon. In contrast
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54 to such severe conditions, TCR α KO mice at 24 wk might reflect an early stage of
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57 MCMV reactivation. Consistent with our data, pericytes and perivascular cells including
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6 pericytes are reported to be the sites of CMV infection in humans.^{42, 46} Together, our
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9 animal data and previous clinical observations suggest that HCMV infection in the
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12 colonic mucosa of patients with IBD may spread from perivascular stromal cells to
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15 endothelial cells and epithelial cells with the progression of colitis, which may lead to
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18 the formation of ulcers and systemic dissemination.

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21 Interestingly, most of the MCMV-infected cells in the colonic mucosa expressed
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24 CXCL12 and PDGFR- β in our TCR α KO mice. Moreover, gene expression of both
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27 *CXCL12* and *PDGFR- β* was elevated in the colon of those mice. CMV infection
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30 enhances PDGFR- β expression in infected cells.⁴⁷ Importantly, both PDGFR- β and
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33 CXCL12 have crucial roles in chemotaxis as well as angiogenesis. PDGFR- β activation
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36 stimulates chemotaxis of monocytes and granulocytes.^{48, 49} CXCL12 also induces the
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39 migration of T cells, B cells, and neutrophils in inflammatory conditions.^{29, 50} In fact, the
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42 number of CXCR4-positive cells was increased in the inflamed colon of infected TCR α
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45 KO mice. Thus, the data suggest that MCMV infection increased PDGFR- β and
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48 CXCL12 expression, which resulted in the migration of various immune cells, leading
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51 to the exacerbation of colitis in TCR α KO mice.

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54 In conclusion, we developed the first mouse model of human UC with concomitant
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57 HCMV infection. We demonstrated that MCMV infection exacerbated colitis in TCR α
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6 KO mice. We also showed that most of the infected cells in the colonic mucosa were
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9 perivascular stromal cells, including pericytes, expressing PDGFR- β and CXCL12.
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12 Further studies using this mouse model will contribute to a better understanding of the
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14 detailed pathogenicity of HCMV infection in the exacerbation of IBD, which might lead
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17 to the development of therapeutic strategies for IBD patients with HCMV infection.
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20 21 22 **Acknowledgements** 23

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Figure legends

Figure 1. Establishment of acute and latent MCMV infection in C57BL/6 mice and TCR α KO mice. (A) Serial change in body weight in uninfected and infected mice. The data are expressed as the percentage change from the starting body weight. C57BL/6 mice were infected with 5×10^5 PFU of MCMV-Smith and TCR α KO mice were infected with 2×10^4 PFU of MCMV-EGFP. (B) and (C) MCMV titers (B) and copy numbers (C) at 1 and 4 wpi in colon, spleen, and liver of C57BL/6 and TCR α KO mice. MCMV titer was examined by plaque assay. MCMV-DNA was estimated by real-time PCR. (D) and (E) Immunohistochemistry for M45, an early-late antigen of MCMV, and the fluorescence view of EGFP in the colon, spleen, and liver of C57BL/6 and TCR α KO mice at 1 wpi (D) and 4 wpi (E). At 1 wpi, M45-positive cells were observed in various organs of both C57BL/6 and TCR α KO mice. EGFP fluorescence was also detected in various organs of TCR α KO mice at 1 wpi (D). At 4 wpi, M45-positive cells disappeared in all examined organs of both C57BL/6 and TCR α KO mice (E). Scale bars, 30 μ m (D), 100 μ m (E). Data are expressed as the mean \pm SEM, n = 8 mice/group.

Figure 2. Viral antigen-positive cells reappeared in the inflamed colonic mucosa of TCR α KO mice. (A) The percentages of mice manifesting the reappearance of

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6 M45-positive cells in the colon, spleen, and liver of MCMV-infected TCR α KO mice (n
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8 = 11). (B) Immunohistochemistry for M45 in colon, spleen, and liver of
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10 MCMV-infected C57BL/6 mice and TCR α KO mice at 24 wk (23 wpi). A high-power
11
12 view of the colon in infected TCR α KO mice is shown in the lower left panel. Scale
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14 bars, 100 μ m. (C) The number of M45 positive cells was examined per cross section of
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16 proximal and distal colon in infected TCR α KO mice at 12 and 24 wk (11 and 23 wpi).
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18 Results are presented as means \pm SEM, n = 10-11 mice/group, * $P < 0.05$ between
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20 infected TCR α KO mice at 11 wpi and 23 wpi by Student's t -test. (D) Detection of
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22 MCMV DNA in various organs (colon, spleen, and liver) of infected TCR α KO mice at
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24 2, 5, and 24 wk (1, 4, and 23 wpi, respectively) by semi-quantitative PCR. Primers sets
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26 gene-specific for gB, the major envelope glycoprotein B of MCMV, or β -actin were
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28 used. (E) Immunohistochemistry for M45 in colon of infected TCR α KO mice at 24 wk
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30 (23 wpi) (left panel) and its high-power view (upper right panel). *In situ* hybridization to
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32 MCMV-DNA in serial sections is shown in the lower right panel. Scale bars, 30 μ m.
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49 **Figure 3.** MCMV infection exacerbates colitis in TCR α KO mice. C57BL/6 mice and
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51 TCR α KO mice were infected by intraperitoneal injection of MCMV at 1 wk of age.
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53 Macro- and microscopic findings of MCMV-infected mice at 12 and 24 wk (11 and 23
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6 wpi) were compared to those of uninfected mice of the same age. (A) Colon length in
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9 C57BL/6 mice with and without MCMV infection at 24 wk and that in TCR α KO mice
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12 with and without MCMV infection at 12 and 24 wk. Results are presented as means \pm
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15 SEM, n = 10–13 mice/group. * $P < 0.05$ between uninfected and infected mice by
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18 Student's *t*-test. (B) Histologic findings of the distal colon in C57BL/6 mice with and
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21 without MCMV infection at 24 wk and TCR α KO mice with and without MCMV
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24 infection at 12 and 24 wk. A high-power view of the colon in infected TCR α KO mice
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26 at 24 wk in the lower right panel shows a crypt abscess. Scale bars, 100 μ m. (C)
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29 Histologic colitis score of C57BL/6 mice with and without MCMV infection at 24 wk
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32 and TCR α KO mice with and without MCMV infection at 12 and 24 wk. Results are
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35 presented as means \pm SEM, n = 10–13 mice/group. * $P < 0.05$ between uninfected and
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38 infected TCR α KO mice by Mann-Whitney *U*-test.
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Figure 4. MCMV infection affects immune responses in the inflamed colonic mucosa in TCR α KO mice. (A) Immunohistochemistry for CD80 and CD163 was performed using the colonic tissue of uninfected and infected TCR α KO mice at 24 wk (23 wpi). M1 (CD80) / M2 (CD163) macrophage ratio per high-power field was calculated using a dynamic cell count image analysis program (Keyence), n = 8 mice/group. Scale bars, 50

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6 μm . (B) Immunohistochemistry for MPO in colonic tissue of uninfected and infected
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9 TCR α KO mice at 24 wk. The number of MPO-positive cells per cross-section of the
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12 distal and proximal colon was counted. $n = 8$ mice/group. Scale bars, 50 μm . (C) The
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15 gene expression of several cytokines in the colonic tissue of uninfected and infected
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18 TCR α KO mice at 12 and 24 wk (11 and 23 wpi) was determined by quantitative
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21 real-time PCR and was normalized to the expression levels of the 18S ribosomal RNA
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24 gene. Data are expressed as the mean \pm SEM, $n = 10\text{--}13$ mice/group. * $P < 0.05$
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27 between uninfected and infected TCR α KO mice by Student's *t*-test.
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32 **Figure 5.** Characterization of MCMV-infected cells in the colon of TCR α KO mice.
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35 Immunofluorescence double-staining was performed in the colon of the infected TCR α
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38 KO mice at 24 wk (23 wpi). Colonic tissue sections from the infected TCR α KO mice
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41 were stained with antibodies against M45 (red), an early-late antigen of MCMV, and
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44 various cell markers (green), including keratin, CD11b, CD45, α -SMA, and CD31,
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47 followed by DAPI (blue). None of the markers tested were co-stained with M45.
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50 Arrows indicate the presence of M45-positive cells in the vicinity of CD31-positive
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53 endothelial cells. Scale bars, 50 μm .
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6 **Figure 6.** Characterization of MCMV-infected cells in the colon of TCR α KO mice.
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9 Immunofluorescence triple-staining for M45, an early-late antigen of MCMV, and
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11 perivascular cell markers. Colonic tissue sections from infected TCR α KO mice at 24
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13 wk (23 wpi) were stained with antibodies against M45 (red), markers for perivascular
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15 cells (green or blue), and DAPI (cyan). (A-1) M45, α -SMA, NG2, and DAPI were
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17 expressed as red, green, blue, and cyan fluorescence, respectively. (A-2) M45, CD146,
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19 NG2, and DAPI were expressed as red, green, blue, and cyan fluorescence, respectively.
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21 (A-3) M45, CXCL12, PDGFR- β , and DAPI were expressed as red, green, blue, and
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23 cyan fluorescence, respectively. M45 (red) were co-localized with NG2 (blue) and
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25 CD146 (green) in some cells but not with α -SMA (green) (A-1) and (A-2).
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27 M45-positive cells (red) expressed CXCL12 (green) and PDGFR- β (blue) (A-3). (B)
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29 mRNA expression of *CXCL12* and *PDGFR- β* in the colonic tissue of uninfected and
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31 infected TCR α KO mice at 12 and 24 wk (11 and 23 wpi, respectively) was determined
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33 by quantitative real-time PCR and was normalized to the expression levels of the 18S
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35 ribosomal RNA gene. Data are expressed as the mean \pm SEM, n = 10 mice/group. * P <
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37 0.05 between uninfected and infected TCR α KO mice by Student's t -test. Scale bars, 30
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6 **Figure 7.** Expression of CXCR4 in the colonic tissue. (A) mRNA expression of
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8 *CXCR4* in the colonic tissue of uninfected and infected TCR α KO mice at 12 and 24 wk
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10 (11 and 23 wpi, respectively) was determined by quantitative real-time PCR and was
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12 normalized to the expression levels of the 18S ribosomal RNA gene. Data are expressed
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14 as the mean \pm SEM, n = 10 mice/group. (B) Immunohistochemistry for CXCR4 in
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16 colonic tissue of uninfected and infected TCR α KO mice at 24 wk. The number of
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18 CXCR4-positive cells per high-power field was calculated by counting ten high-power
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20 fields. n = 8 mice/group. Scale bars, 50 μ m.
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List of Supplemental Digital Content

Supplemental Digital Content 1.doc (Supplementary Materials and Methods)

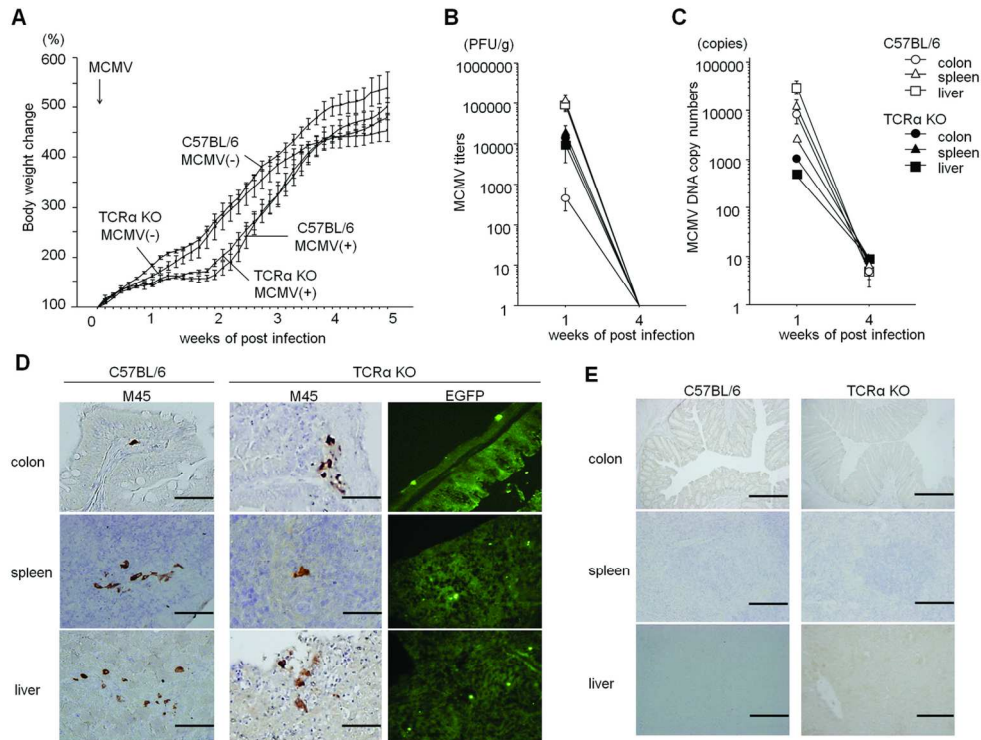
Supplemental Digital Content 1.doc (Supplementary Figure 1)

Supplemental Digital Content 1.doc (Supplementary Figure 2)

Supplemental Digital Content 1.doc (Supplementary Figure 3)

For Peer Review

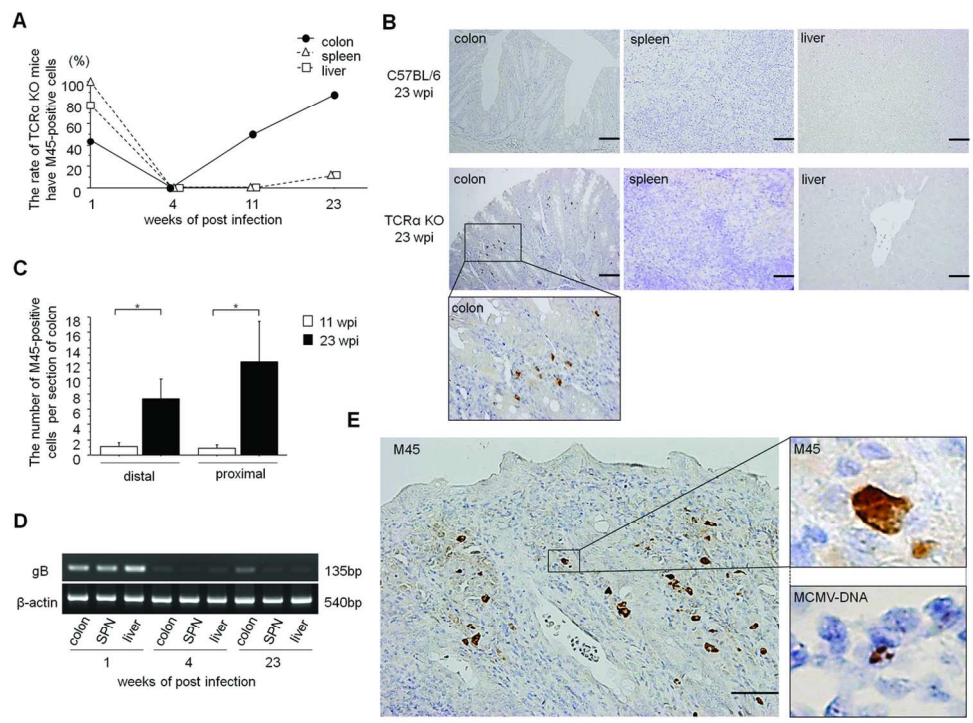
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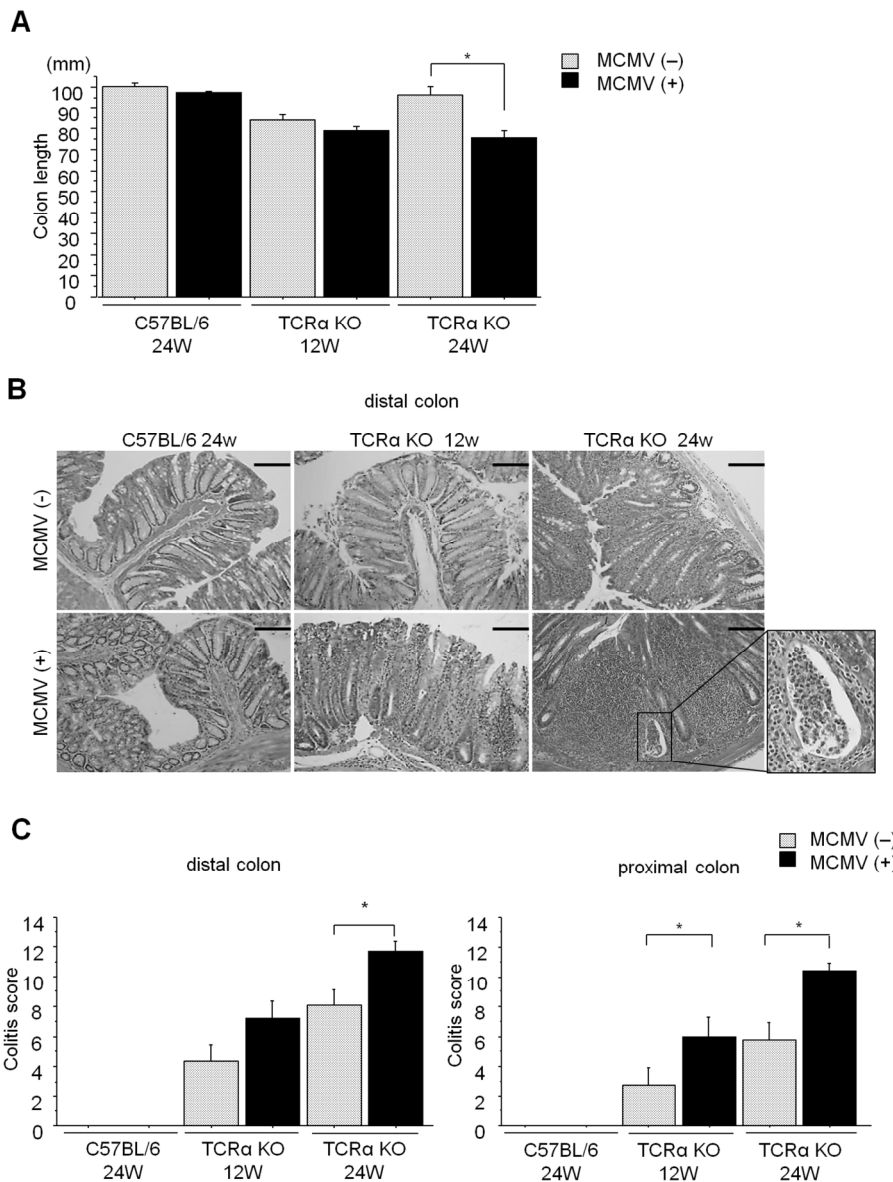
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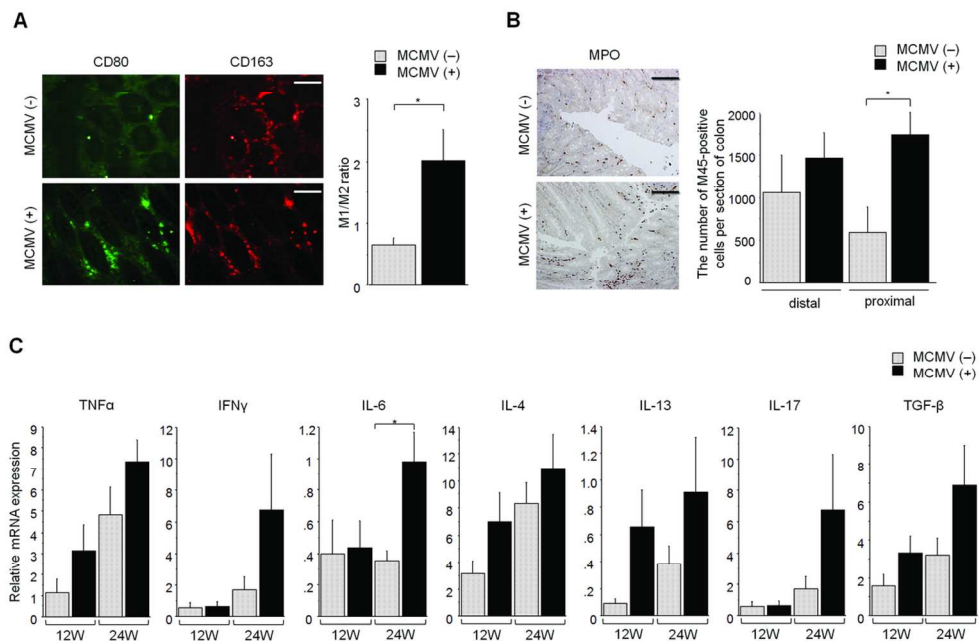
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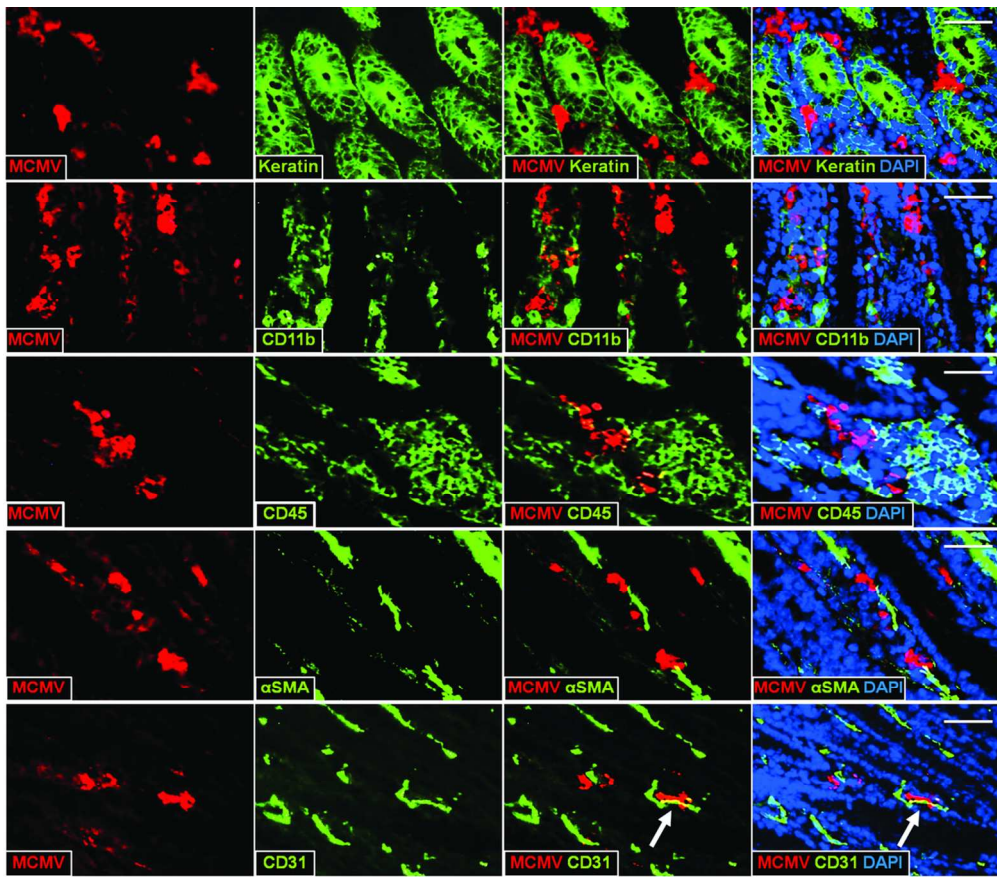
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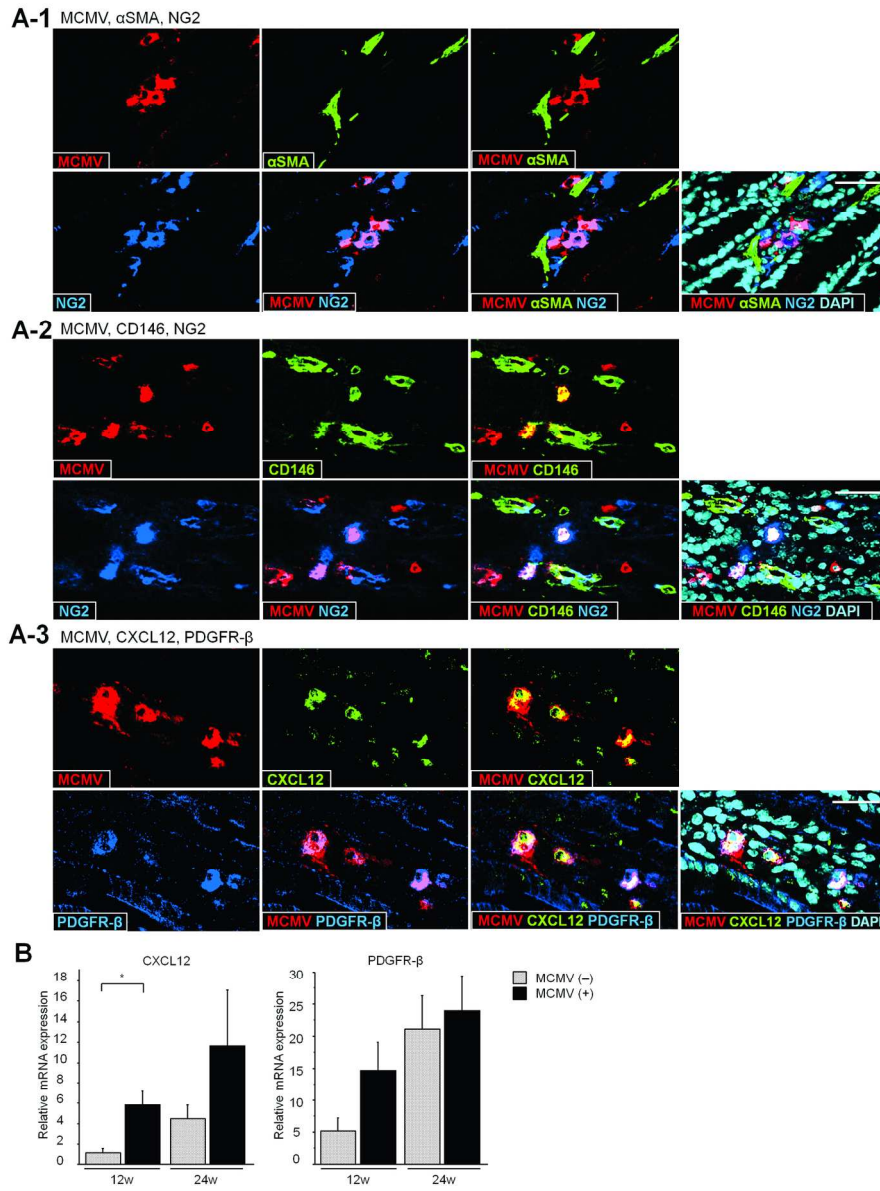
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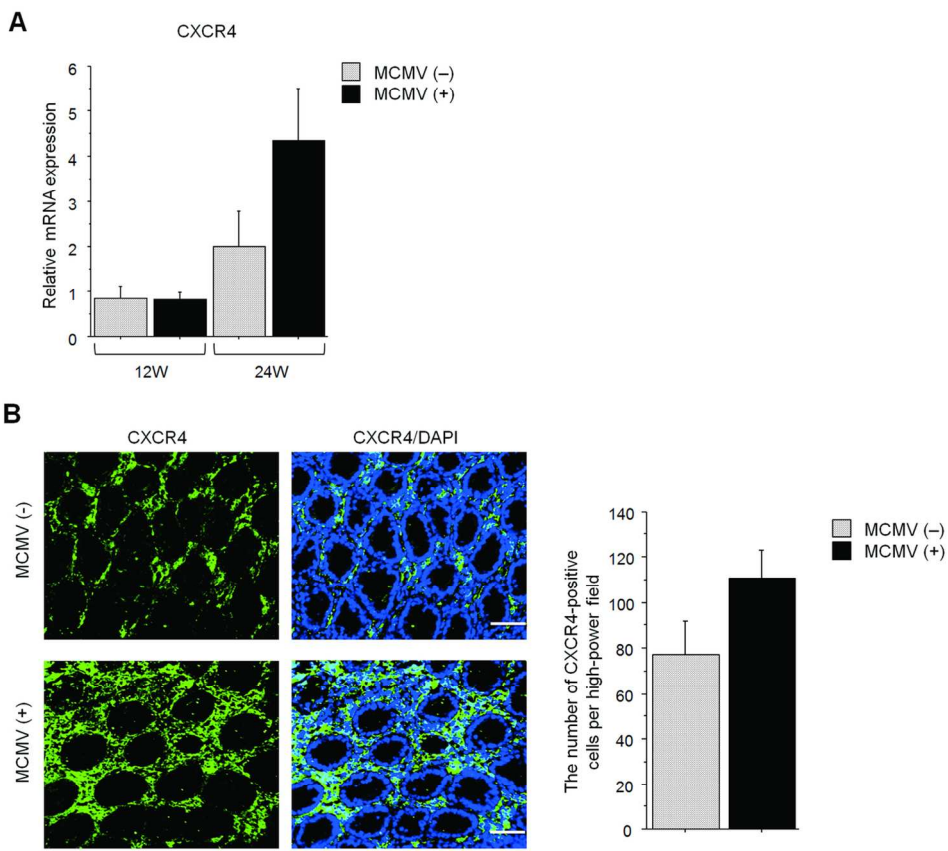
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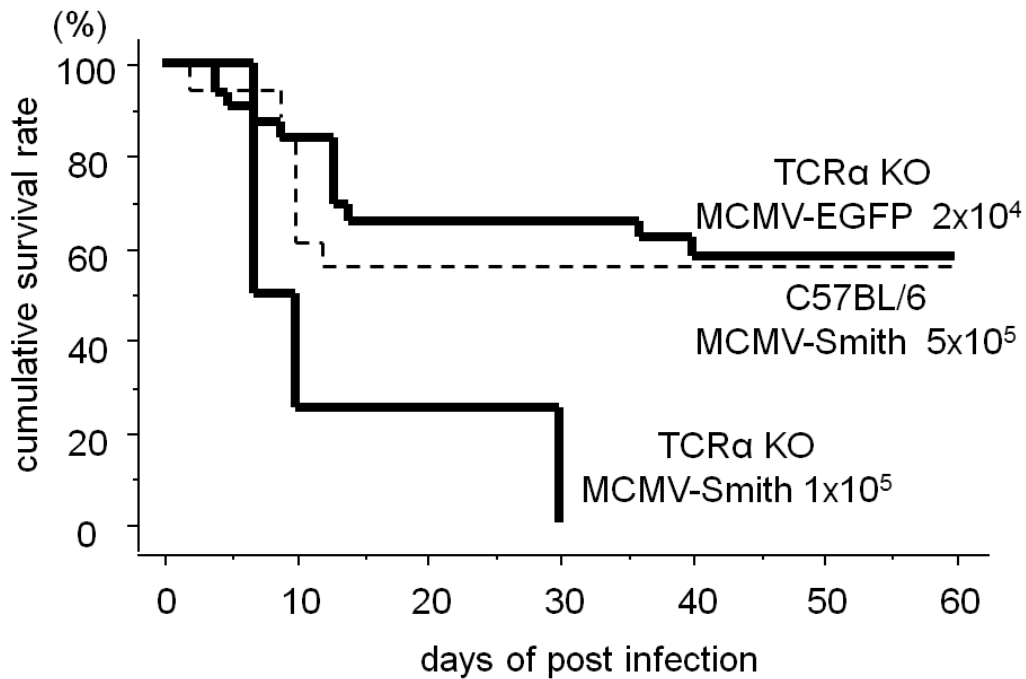
Expression of CXCR4 in the colonic tissue.
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Supplementary Materials and Methods

Isolation and stimulation of MCMV-sensitized splenocytes

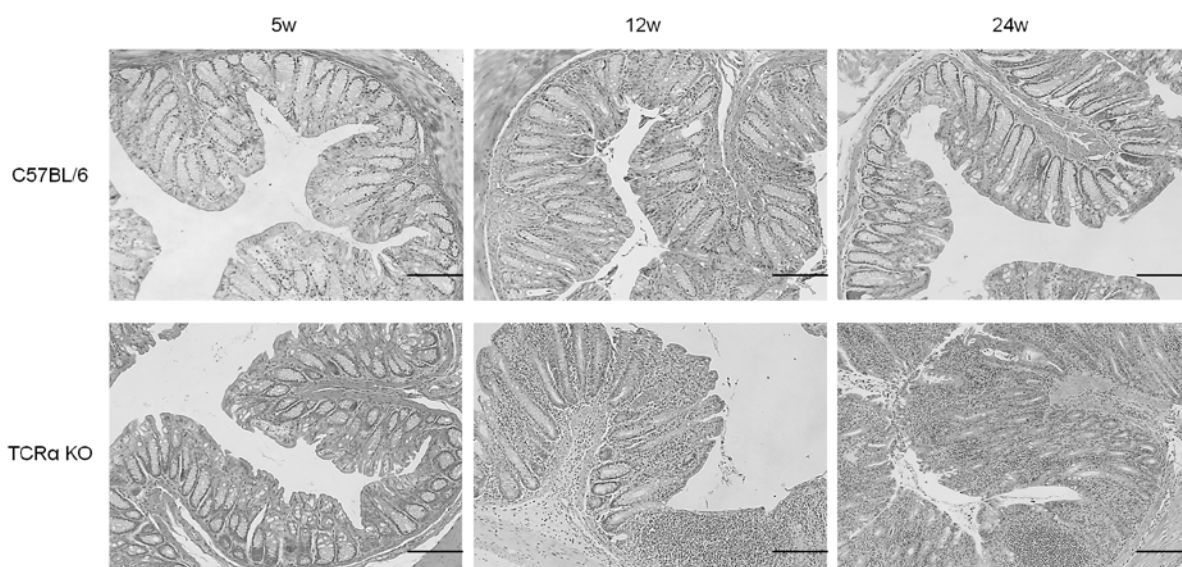
For immunization with MCMV, C57BL/6 mice and TCR α KO mice at 4 wk were inoculated with MCMV-EGFP at 2×10^4 PFU intraperitoneally. Spleens of uninoculated control mice and inoculated mice at 7 wk (3 wk after inoculation) were mechanically disrupted and filtered through a cell strainer. 2×10^5 cells/well were plated in a 96-well plate and incubated with MCMV-EGFP at 1, 3, or 10 MOI (2×10^5 , 6×10^5 , 2×10^6 PFU) for 72 hours in 5% CO₂ at 37°C. Cytokine levels in the supernatant of the culture medium were measured by enzyme-linked immunosorbent assay kit (eBioscience).

Supplementary Figure 1



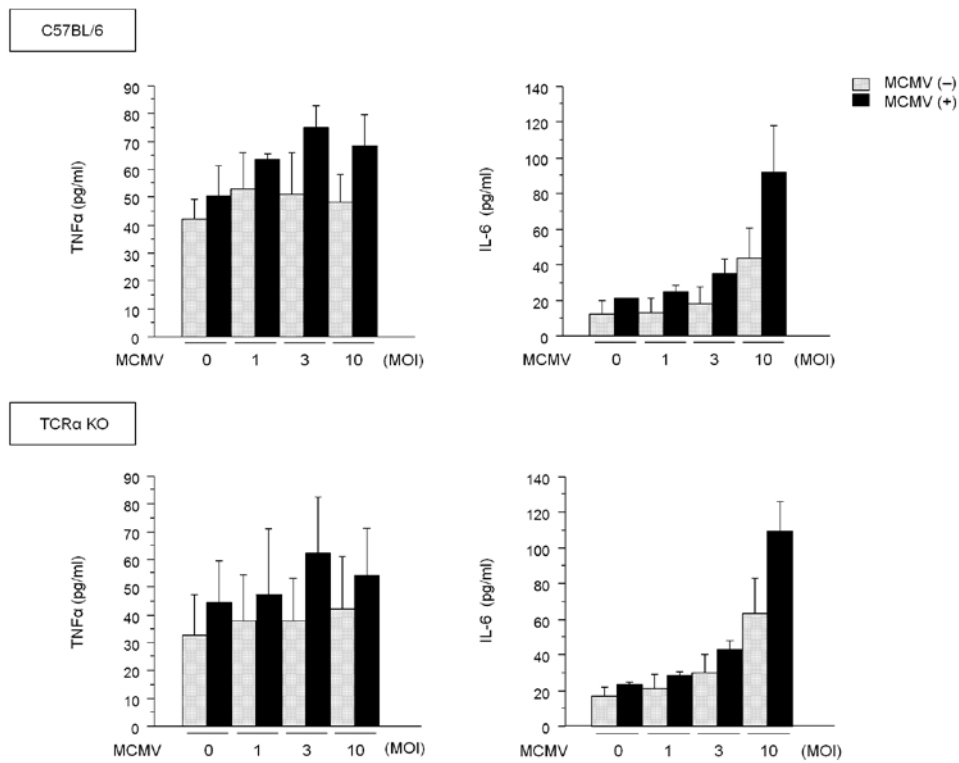
Supplementary Figure 1. Cumulative survival rate of mice inoculated with MCMV-Smith (1 or 5×10^5 PFU) or MCMV-EGFP (2×10^4 PFU) calculated by the Kaplan-Meier method. Newborn mice 7 days after birth were infected by intraperitoneal injection of various doses ($1 \times 10^3 \sim 1 \times 10^6$ PFU) of MCMV-Smith or MCMV-EGFP and viral dose was adjusted to induce a state of latency. Similar mortality (approximately 60%) was observed in C57BL/6 mice infected with MCMV-Smith at 5×10^5 PFU and TCR α KO mice infected with MCMV-EGFP at 2×10^4 PFU. All MCMV-Smith infected TCR α KO mice died even at the minimum dose under 1×10^5 PFU. The dotted line and thick line represent the survival rate of C57BL/6 mice and of TCR α KO mice, respectively.

Supplementary Figure 2



Supplementary Figure 2. Representative histological findings of the distal colon in C57BL/6 mice TCR α KO mice at 5, 12, and 24 wk. Colitis was observed in TCR α KO mice at both 12 and 24 wk but not at 5 wk. TCR α KO mice at 12 and 24 wk showed hyperplasia of epithelial cells, infiltration of mononuclear cells in the colonic lamina propria, and loss of Goblet cells. In contrast, C57BL/6 mice did not develop colitis even at 24 wk. Scale bars, 100 μ m.

Supplementary Figure 3



Supplementary Figure 3. The effect of MCMV on proinflammatory cytokine production from splenocytes of MCMV-immunized C57BL/6 mice and TCR α KO mice. Splenocytes of both naïve mice (dotted bars) and immunized mice (filled bars) were stimulated with medium alone or MCMV-EGFP (at 1, 3, or 10 MOI) for 72 hours, and proinflammatory cytokine secretion in the supernatant was measured by ELISA. Data are expressed as the mean \pm SEM. n = 4 mice/group.