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Cytomegalovirus infection initiates inflammatory bowel disease by activating a positive MyD88/NF-κB feedback loop in allogeneic skin transplantation mice

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Abstract

Infection with the cytomegalovirus (CMV) is common. Inflammatory bowel disease (IBD) is characterized by chronic inflammation in the gastrointestinal tract. CMV infection is involved in IBD pathogenesis. The abnormal activation of myeloid differentiation factor 88 (MyD88)/nuclear factor- kappa B (NF-κB) signaling, which results in inflammatory cytokine overexpression, is an important factor in IBD pathogenesis. The present study aimed to examine the effect of CMV infection on NF-κB activation and its role in IBD pathogenesis. Since BALB/c rather than C57BL/6 mice belong to the murine CMV (MCMV) susceptible strain, allogeneic skin transplantation was conducted between MyD88 (+/+) or MyD88-knockout Myd88 (–/–) BALB/c (recipient) mice and C57BL/6 (donor) mice. Thereafter, the immune function of the recipient mice was reduced by immunosuppressant cyclosporine, which is meaningful in the pathogenesis of IBD caused by MCMV in immunocompromised mice. MCMV strain K181-eGFP (eGFP K181) or hMIEP-eGFP K181 (knockout MCMV IE1-3 promoter) was used to infect MyD88 (+/+) BALB/c mice while eGFP K181 was also used to infect MyD88 (–/–) BALB/c mice on day 14 post allogeneic skin transplantation. MCMV DNA was detected via nested polymerase chain reaction. Hematoxylin–Eosin staining was used to assess colon necrosis and inflammatory cell infiltration. The serum levels of tumor necrosis factor-alpha, interleukin (IL)-1β, IL-6, IL-8, IL-12, flagellin, lipopolysaccharide, and myeloperoxidase were detected by ELISA and immune reaction. Immunoblots were applied to measure protein levels. eGFP K181 infection significantly induced colon permeability, necrosis, inflammatory cell infiltration, and inflammation in allogeneic skin transplantation mice. hMIEP-eGFP K181 infection significantly inhibited colon permeability, necrosis, inflammatory cell infiltration, and inflammation compared with eGFP K181 infection in allogeneic skin transplantation mice. Moreover, the MyD88-dependent NF-κB signaling pathway was involved in the pathogenesis of eGFP K181-induced colon permeability and inflammation in allogeneic skin transplantation mice. Our findings highlight the importance of CMV infection and the Myd88/NF-κB signaling pathway in IBD and might provide a new direction for the development of drugs for IBD.

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Keywords Cytomegalovirus, Inflammatory bowel disease, Immediate-early protein, Inflammatory cytokines, Myd88/NF-κB

Introduction

Inflammatory bowel disease (IBD) is an inflammatory disease of the gastrointestinal tract [1]. IBD is divided into either ulcerative colitis or Crohn's disease [2]. Its symptoms include abdominal pain, diarrhea, and rectal bleeding, while its causes may include genetic predisposition, gut microbiome alterations, immune defects, etc [3]. Hitherto, IBD has been considered a disease of people in developed countries; however, studies have shown a rapid rise in its prevalence in newly industrialized countries [4]. Human cytomegalovirus (HCMV) is an enveloped, double-stranded DNA virus. According to the International Committee on the Taxonomy of Viruses criteria for virus classification, HCMV belongs to the family *Orthoherpesviridae*, subfamily *Betaherpesvirinae*, and genus *Cytomegalovirus* [5]. Cytomegalovirus (CMV) persists among infected individuals in a latent asymptomatic state across its host's lifetime [6]. It reactivates when the infected host develops an immunosuppressed state which is followed by various breakthrough presentations of CMV-related diseases. CMV, thanks to its extensive tissue affinity, can infect various types of cells [7]. CMV has been detected in the gastrointestinal tract of patients with esophagitis [8] and is associated with IBD [9, 10]. It has been shown that CMV counts in intestinal biopsy specimens were significantly higher in patients with IBD than in controls [11]. Another study also showed a significant difference in steroid resistance between IBD patients with/without CMV infection [7].

Nuclear factor-kappa B (NF-κB) regulates various genes involved in immune and inflammatory responses [12]. This family is composed of five structurally related members—NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel [13]. The most prevalent activated form is a heterodimer consisting of p50 and p65 [14]. Abnormal NF-κB activation results in the elevation of tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-6, IL-8, and IL-12 levels, contributing to IBD pathogenesis [15]. Treatment with the anti-TNF-α antibody has been successful for patients with IBD [16]. CMV infection has also been shown to increase levels of the inhibitor of κB (IκB) kinase (IKK) proteins and decrease IκB levels, culminating in prolonged NF-κB activation [17]. CMV major immediate-early protein IE has multiple functions, and it is important for efficient viral infection [18]. IE transcription is controlled by the major immediate-early promoter/enhancer (MIEP) [19], which is transactivated by NF-κB [17], indicating a positive feedback loop of CMV infection and NF-κB activation. However, the role of CMV infection in NF-κB activation and IBD remains to be elucidated.

Murine CMV (MCMV) infection resembles HCMV, and this resemblance is a useful tool for exploring early immune response after contracting CMV infection [20]. Since BALB/c rather than C57BL/6 mice belong to the MCMV-susceptible strain [21], skin grafting was conducted between BALB/c (recipient) and C57BL/6 (donor) mice. After BALB/c mice received allogeneic skin transplantation, their immune function was reduced by immunosuppressants such as cyclosporine (CsA) [22], which will be meaningful in exploring the pathogenesis of IBD caused by MCMV in immunocompromised mice. Our current study was designed to investigate the combined effects on NF-κB activation and IBD associated with organ transplantation and CsA-mediated immune suppression. We aimed to establish a mouse model of MCMV-associated IBD using mice that are recipients of allogeneic skin transplants.

Materials and methods

Murine cytomegalovirus

MCMV strains K181-eGFP (eGFP K181) and hMIEP-eGFP K181 (Knock out MCMV IE1-3 promoter (sequences up to -146 nucleotides (nt) upstream from the IE1-3 cap site) were generously donated by Dr. Redwood from University of Western Australia, and they were passaged and preserved by Dr. Gan from Anhui Medical University. MCMV was routinely propagated in mouse embryo fibroblasts, maintained in minimum essential medium (MEM; Gibco, Invitrogen Corporation, CA, USA) containing 10% fetal bovine serum (Invitrogen Corporation, Invitrogen Shanghai Office, China), 100 U/mL penicillin, and 10 μg/mL streptomycin, and its titration was determined as previously described [22].

Mice

All the experimental protocols were approved by the Laboratory Animal Ethics Committee of the Zhejiang Academy of Traditional Chinese Medicine [no. (2021) 016]. BALB/c ($n = 138$, female, aged 4–6 weeks), C57BL/6 ($n = 42$, female, aged 4–6 weeks), and myeloid differentiation factor 88 (MyD88)-knockout Myd88 (−/−) BALB/c mice ($n = 30$, female, aged 4–6 weeks) were obtained from the Cyagen (Shanghai, China).

Skin transplantation models

Transplantations were conducted between MyD88(+/+) or MyD88(−/−) BALB/c female (recipient) and C57BL/6 female (donor) mice (aged 4–6 weeks) as previously

described [22]. A square graft (1 cm × 1 cm) was placed on a graft bed prepared on the back of the recipient mouse. The transplantation area was covered with Vaseline gauze and the bandage was removed on day 8 post-transplantation. After surgery, mice received 12 mg/kg CsA (MedChemExpress LLC, Shanghai, China; HY-B0579) daily via intraperitoneal injection throughout the study. For Experiment 1, 80 μL of MEM containing 1×10^5 PFU eGFP K181 or 80 μL of MEM as a control was inoculated intranasally into MyD88(+/+) BALB/c mice on day 14 post allogeneic skin transplantation ($n = 30$ per group). For Experiment 2, 80 μL of MEM containing 1×10^5 PFU eGFP K181 or hMIEP-eGFP K181 were inoculated intranasally into MyD88(+/+) BALB/c mice on day 14 post allogeneic skin transplantation ($n = 24$ per group). For Experiment 3, 80 μL of MEM containing 1×10^5 PFU eGFP K181 was inoculated intranasally into MyD88(+/+) or MyD88(−/−) BALB/c mice on day 14 post allogeneic skin transplantation ($n = 30$ per group). The mice were sacrificed 5, 9, 14, 21, or 28 d postinfection, and serum and colon tissue samples were collected.

Immunofluorescence

MCMV eGFP K181 or hMIEP-eGFP K181 infection in colon tissues of allogeneic skin transplantation mice was confirmed by immunofluorescence at 5, 9, 14, or 21 d postinfection. Colon tissues were dehydrated, paraffin-embedded, sectioned at 4 μm of thickness, and placed into microscope slides. Images were taken with a microscope (Nikon Eclipse C1, Tokyo, Japan).

Polymerase chain reaction (PCR) assessment of viral levels

MCMV DNA levels in colon tissues of allogeneic skin transplantation mice were detected at 5, 9, 14, or 21 d postinfection by real-time quantitative polymerase chain reaction (PCR) [23, 24]. Organ DNA was prepared by using a QIAamp tissue kit (Qiagen, Chatsworth, CA, USA). Real-time quantitative PCR was performed using the Taqman Gene Expression Master Mix and ABI 7500 Fast System. Primers were based on the MCMV IE1 gene sequence as follows: forward primer, 5′-TGCCATACTG CCAGCTGAGA-3′; reverse primer, 5′-GGCTTCATGA TCCACCCTGTT-3′; probe 5′-CTGGCATCCAGGAAA GGCTTGGTG-3′ labeled with the 6-carboxyfluorescein reporter dye and the 6-carboxytetramethylrhodamine quencher dye [23]. The target DNA copy number in each sample was calculated based on the standard curve generated in each individual assay [24].

Hematoxylin–Eosin staining

The colon tissue of allogeneic skin transplantation mice was separated at 5, 9, 14, or 21 d postinfection and preserved as paraffin blocks. Briefly, the tissue sections were coated with hematoxylin for 5 min and then washed with water. Then, the sections were covered with 1% acid ethanol regent for 5 s and washed with water. Next, the blue-promoting solution was added to the sections for 5 s and washed with water. Eosin solution was added for 10 min and then the sections were dehydrated with graded alcohol and cleared in xylene. The image was observed under a microscope.

Measuring the epithelial barrier permeability in mice

The barrier permeability in allogeneic skin transplantation mice was measured at 5, 9, 14, or 21 d postinfection as previously described [25]. After 3 h of starvation, each mouse was intragastrically administered 150 μL fluorescein isothiocyanate (FITC)-dextran (4 kDa, 80 mg/ml; Sigma-Aldrich, Bornem, Belgium; FD500). Blood was collected from the tail vein after 3 h, and serum was extracted. The fluorescence intensity of each serum sample was measured, and FITC-dextran concentrations were obtained from standard curves generated by serial dilution of FITC-dextran.

Enzyme-linked immunosorbent assay (ELISA)

The levels of TNF-α (E-EL-M3063; Elabscience), IL-1β (E-EL-M0037; Elabscience), IL-6 (E-EL-M0044; Elabscience), IL-8 (SEKM-0046; Beijing Solarbio Science & Technology Co., Ltd), and IL-12 (E-EL-M3062; Elabscience) in serum and that of myeloperoxidase (MPO; ab285307; Abcam) in colon tissues of allogeneic skin transplantation mice was measured at 5, 9, 14, 21, or 28 d postinfection using commercial ELISA kits and a microplate reader as previously described [26].

Immunoassays

To measure product-specific antibodies, microtiter plates were coated with flagellin (Sigma-Aldrich, St. Louis, MO, USA; SRP8029; 100 ng/well) or lipopolysaccharide (LPS; Sigma-Aldrich; 297-473-0; 1 μg/well) applied overnight in 0.1 M $NaHCO_3$ (pH 9.6). After overnight coating, serum isolated from allogeneic skin transplantation mice at 28 d postinfection was diluted 1/100 in ELISA wash buffer (HBSS with 0.5% goat serum and 0.1% Tween 20) and applied to coated plates. After 1 h of incubation, product-specific IgG was detected using Goat Anti-Mouse IgG (Abcam, Waltham, MA, USA; ab214879). Peroxidase was then revealed via 3,3′,5,5′-tetramethylbenzidine substrate (Sigma-Aldrich; T8665), and following treatment with H_2SO_4, OD was read at 450 nm [27].

Western blot

Protein was extracted from colon tissues of allogeneic skin transplantation mice at 5, 9, 14, or 21 d postinfection using the radioimmunoprecipitation assay lysis buffer with freshly added protease inhibitor cocktail (Sigma-Aldrich), separated by sodium dodecyl

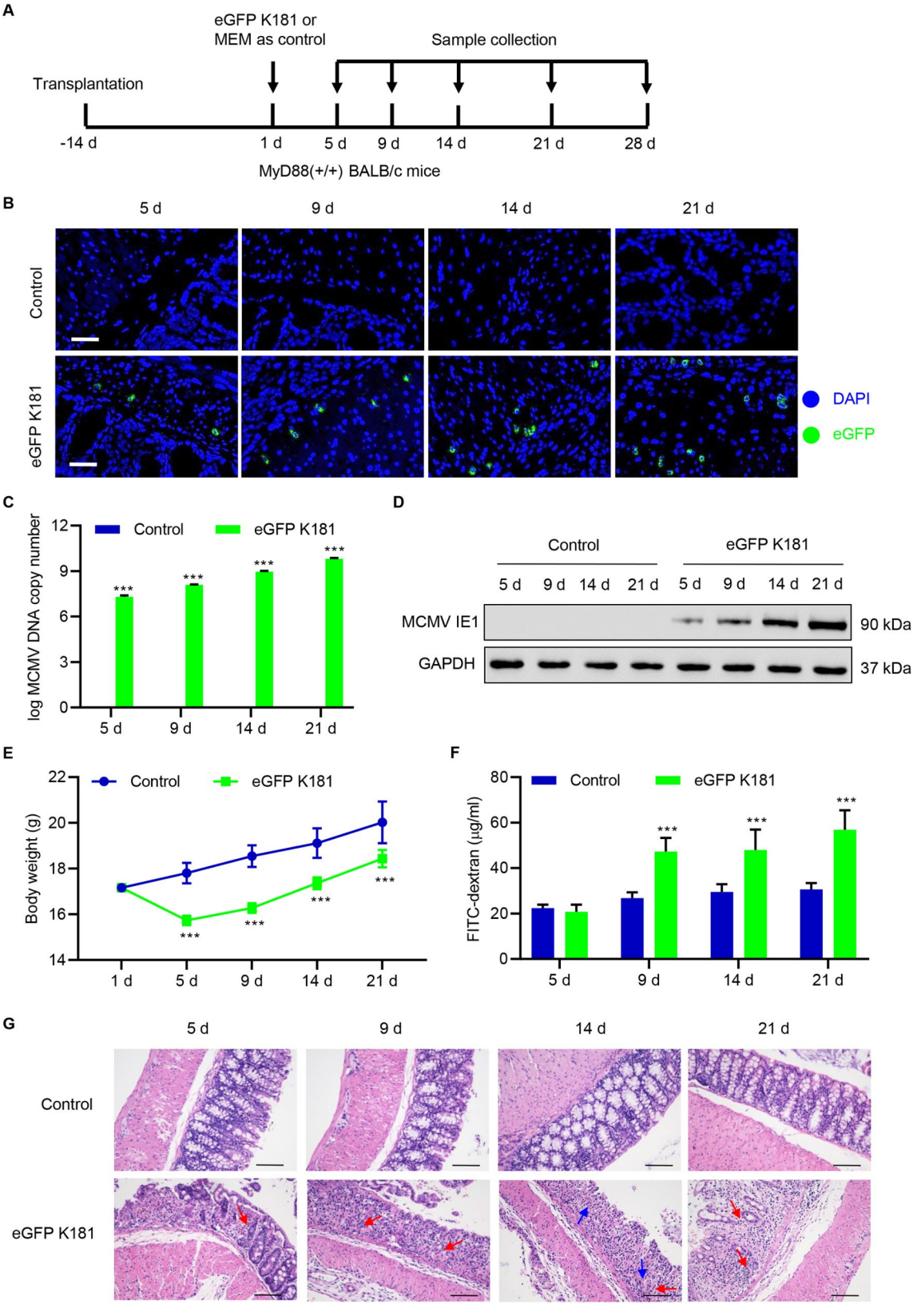

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Fig. 1 Effect of eGFP K181 infection on colon barrier permeability in allogeneic skin transplantation mice. 1×10^5 PFU eGFP K181 were inoculated intranasally into allogeneic skin transplantation BALB/c mice on day 14 posttransplantation. (**A**) A schematic representation of the assay indicating time points of transplantation and eGFP K181 infection. (**B**) MCMV infection in colon tissues was confirmed by immunofluorescence with positive eGFP (Scale bar: 50 μm); (**C**) MCMV DNA and (**D**) IE1 levels in colon tissues were measured by PCR and Western blot, respectively; (**E**) Body weight and (**F**) colon barrier permeability were measured; (**G**) Necrosis (blue arrow) and inflammatory cell infiltration (red arrow) of colon tissues at 5, 9, 14, or 21 d postinfection (Scale bar: 100 μm). Data are shown as the mean ± SD of six mice per group. *** $P < 0.001$ vs. control

sulfate-polyacrylamide gel electrophoresis, immunoblotted to a polyvinylidene fluoride membrane, blocked, and probed with antibodies against MCMV IE1 (KA&M-BIO, Shanghai, China; QM0866R), IKKα (Abcam; ab32041), IKKβ (Abcam; ab32135), p65 (Abcam; ab16502), p52 (Abcam; ab264236), Toll-IL-1 receptor-resistance protein domain-containing adapter-inducing interferon-beta (TRIF; Invitrogen; PA1-20824), MyD88 (Abcam; ab2064), tumor necrosis factor receptor-associated factor 6 (TRAF6; Abcam; ab40675), transforming growth factor-beta activated kinase 1 (TAK1; Abcam; ab109526), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Proteintech Group, Inc., Rosemont, IL, USA; 60004-1-1G), followed by a secondary antibody. Signals were visualized with ECL.

Data analysis

Three independent replicates were performed for all assays. Quantitative data were expressed as the mean ± standard derivation (SD). Comparisons between quantitative data were performed by Student's t-test (two groups) or the analysis of variance (more than two groups) using Prism8.4.2 (GraphPad, La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

Results

eGFP K181 infection increased colon permeability and promoted necrosis and inflammatory cell infiltration in allogeneic skin transplantation mice

To study how MCMV infection affects colon barrier permeability, transplantation was performed between BALB/c (recipient) and C57BL/6 (donor) mice, 80 μL of MEM containing 1×10^5 PFU eGFP K181 was inoculated intranasally into allogeneic skin transplantation mice and serum and colon tissue samples were collected at 5 d, 9 d, 14 d, 21 d, or 28 d postinfection (Fig. 1A). eGFP K181 infection in colon tissues, which was confirmed by immunofluorescence with positive eGFP (Fig. 1B), induced an increase in MCMV DNA copy numbers (Fig. 1C) and the upregulation of MCMV IE1 in colon tissues at 5, 9, 14, or 21 d postinfection (Fig. 1D). eGFP K181 infection also caused decreased body weight (Fig. 1E), enhanced colon barrier permeability (Fig. 1F), and increased necrosis and inflammatory cell infiltration in colon tissues at 5, 9, 14, or 21 d postinfection (Fig. 1G). Together, these data suggest that MCMV infection increased colon permeability and promoted necrosis and inflammatory cell infiltration in mice following skin transplantation.

eGFP K181 infection activated the MyD88/NF-κB signaling pathway in allogeneic skin transplantation mice

To study how MCMV infection increased colon permeability and promoted necrosis and inflammatory cell infiltration in allogeneic skin transplantation mice, we further checked the serum inflammatory cytokine levels. ELISA results showed that eGFP K181 infection time-dependently increased the serum levels of these cytokines (Fig. 2A and E). Western blotting results indicated that eGFP K181 infection time-dependently increased the expression levels of some important factors involved in the MyD88/NF-κB signaling pathway, including MyD88, TRIF, TRAF6, TAK1, IKKα, IKKβ, p65, and p52 in colon tissues (Fig. 2F). The toll-like receptor (TLR)/MyD88/NF-κB pathway plays an important role in the pathogenesis of IBD [28, 29] and thus the levels of TLR ligands—LPS and flagellin—were also measured for the study of MCMV infection-induced changes in inflammation-related intestinal pathologies. Our data showed that eGFP K181 infection significantly increased the serum levels of anti-flagellin IgG, anti-LPS IgG, and MPO (a marker of leukocyte infiltration), in colon tissues at 28 d postinfection (Fig. 2G). These data indicate that MCMV infection activated the NF-κB signaling pathway in allogeneic skin transplantation mice.

hMIEP-eGFP K181 infection inhibited colon permeability, necrosis, and inflammatory cell infiltration in allogeneic skin transplantation mice

The CMV IE1 and IE2 proteinscan activate NF-κB-dependent transcription [30, 31]. Moreover, IE3 gene products are required to activate early-stage gene expression of MCMV [32]. Therefore, we want to elucidate the role of MCMV IE1-3 in colon permeability and inflammation in allogeneic skin transplantation mice. Transplantation was performed as mentioned above, and 80 μL of MEM containing 1×10^5 PFU hMIEP-eGFP K181 (Knock out MCMV IE1-3 promoter) or eGFP K181 was inoculated intranasally into allogeneic skin transplantation mice and the serum and colon tissue samples were collected at 5, 9, 14, or 21 d postinfection (Fig. 3A). MCMV infection in colon tissues was confirmed by immunofluorescence at 5, 9, 14, or 21 d postinfection (Fig. 3B). hMIEP-eGFP K181 infection inhibited MCMV

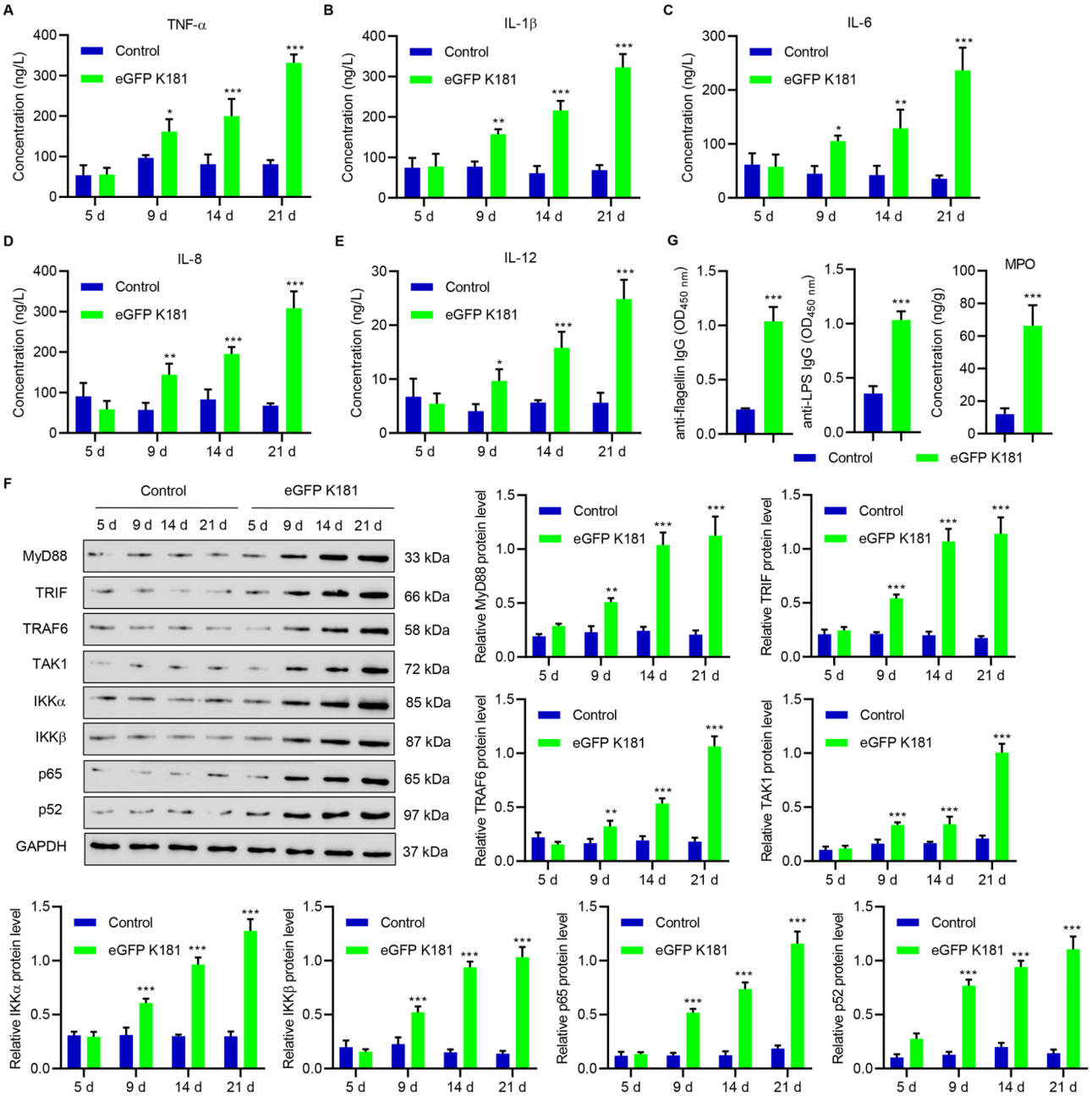

Fig. 2 Effect of eGFP K181 infection on the NF-κB signaling pathway in allogeneic skin transplantation mice. 1×10^5 PFU eGFP K181 were inoculated intranasally into allogeneic skin transplantation BALB/c mice on day 14 posttransplantation. The serum levels of (**A**) TNF-α, (**B**) IL-1β, (**C**) IL-6, (**D**) IL-8, and (**E**) IL-12 were measured by ELISA; (**F**) The expression levels of IKKα, IKKβ, p65, p52, TRIF, MyD88, TRAF6, and TAK1 in colon tissues were measured by Western blot at 5, 9, 14, or 21 d postinfection. (**G**) The levels of anti-flagellin IgG and anti-LPS IgG in serum and MPO in colon tissues were measured at 28 d postinfection. Data are shown as the mean ± SD of six mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control

DNA copy numbers (Fig. 3C) and MCMV IE1 expression in colon tissues compared with eGFP K181 infection (Fig. 3D). hMIEP-eGFP K181 infection also increased body weight and inhibited colon barrier permeability, necrosis, and inflammatory cell infiltration compared with eGFP K181 infection (Fig. 3E and G).

hMIEP-eGFP K181 infection inhibited the NF-κB signaling pathway in allogeneic skin transplantation mice

Next, we checked the effect of hMIEP-eGFP K181 infection on the NF-κB signaling pathway in allogeneic skin transplantation mice. hMIEP-eGFP K181 infection suppressed inflammatory cytokine levels compared with eGFP K181 infection (Fig. 4A and E). Western blotting results indicated that hMIEP-eGFP K181 infection

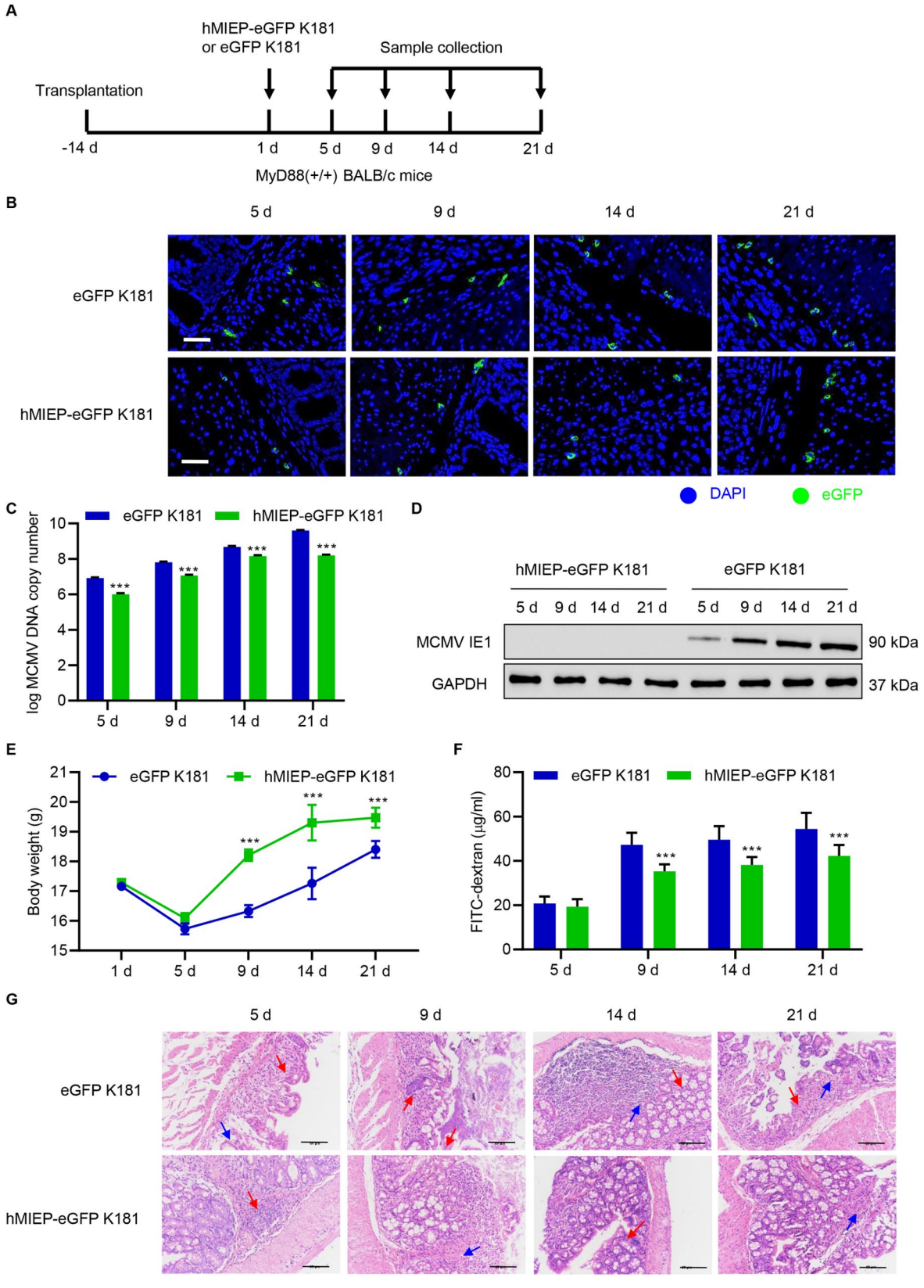

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Fig. 3 Effect of hMIEP-eGFP K181 infection on colon barrier permeability in allogeneic skin transplantation mice. 1×10^5 PFU hMIEP-eGFP K181 or eGFP K181 were inoculated intranasally into allogeneic skin transplantation BALB/c mice on day 14 posttransplantation. (**A**) A schematic representation of the assay indicating time points of transplantation and MCMV infections. (**B**) MCMV infection in colon tissues was confirmed by immunofluorescence with positive eGFP (Scale bar: 50 μm); (**C**) MCMV DNA and (**D**) IE1 levels in colon tissues were measured by PCR and Western blot, respectively; (**E**) Body weight and (**F**) colon barrier permeability were measured; (**G**) Necrosis (blue arrow) and inflammatory cell infiltration (red arrow) of colon tissues at 5, 9, 14, or 21 d postinfection (Scale bar: 100 μm). Data are presented as the mean ± SD of six mice per group. *** $P < 0.001$ vs. eGFP K181

inhibited the expression levels of some important factors involved in the MyD88/NF-κB signaling pathway including MyD88, TRIF, TRAF6, TAK1, IKKα, IKKβ, p65, and p52 in colon tissues compared with eGFP K181 infection (Fig. 4F). All the findings indicate that the MCMV IE-induced activation of the NF-κB signaling pathway contributed to colon inflammation in allogeneic skin transplantation mice.

eGFP K181 infection promotes colon permeability, necrosis, and inflammatory cell infiltration in allogeneic skin transplantation mice via MyD88-dependent NF-κB activation

To further investigate the regulation of NF-κB in MCMV infection, transplantation was performed as mentioned above, and 80 μL of MEM containing 1×10^5 PFU eGFP K181 was inoculated intranasally into MyD88(+/+) or MyD88(−/−) allogeneic skin transplantation mice and serum and colon tissue samples were collected at 5, 9, 14, 21, or 28 d postinfection (Fig. 5A). eGFP K181 infection in colon tissues was confirmed by immunofluorescence both in MyD88(+/+) and MyD88(−/−) mice (Fig. 5B), and the MCMV DNA copy numbers (Fig. 5C) and MCMV IE1 expression (Fig. 5D) in colon tissues were decreased in Myd88(−/−) mice after eGFP K181 infection compared with Myd88(+/+) mice at 5, 9, 14, or 21 d postinfection. Moreover, the body weight was increased and colon barrier permeability, necrosis, and inflammatory cell infiltration were decreased in MyD88(−/−) mice after eGFP K181 infection compared with MyD88(+/+) mice (Fig. 5E and G). This finding indicates that MCMV infection increased colon permeability and promoted necrosis and inflammatory cell infiltration via MyD88-dependent NF-κB activation in mice following skin transplantation.

eGFP K181 infection activated the NF-κB signaling pathway in allogeneic skin transplantation mice via MyD88

We also further checked MyD88's role in NF-κB signaling pathway activation. Our results showed that inflammatory cytokine levels were decreased in MyD88(−/−) mice after eGFP K181 infection (Fig. 6A and E). Levels of some important factors involved in the MyD88/NF-κB signaling pathway, including TRIF, TRAF6, TAK1, IKKα, IKKβ, p65, and p52 in colon tissues, were decreased in MyD88(−/−) mice after eGFP K181 infection (Fig. 6F). The levels of anti-flagellin IgG, anti-LPS IgG, and MPO were also decreased in MyD88(−/−) mice after eGFP K181 infection (Fig. 6G). Together, the findings indicate that MCMV infection increased colon inflammation via MyD88-dependent NF-κB activation in allogeneic skin transplantation mice.

Discussion

Herein, we reported that CMV infection increased colon permeability, promoted necrosis and inflammatory cell infiltration, and enhanced colon inflammation in allogeneic skin transplantation mice. We also proved that MCMV infection increased colon inflammation by activating NF-κB signaling. Mechanism studies showed that MCMV's activation of NF-κB signaling was MyD88-dependent in allogeneic skin transplantation mice. For the first time, our study indicated that MCMV infection initiates IBD by activating a positive MyD88/NF-κB feedback loop in allogeneic skin transplantation mice (Fig. 7).

CMV causes severe disease in immunocompromised individuals. CMV infection also exacerbated ulcerative colitis [10]. CMV infection in IBD was associated with an increased risk of mortality [33]. Traditionally, research systems studying the molecular biology and pathogenic mechanisms of CMV use either HCMV in cultured human cells or MCMV in its natural host species. Compared with those in vitro studies, in vivo approaches are advantageous because they better reflect the complexity of human conditions. Ni et al. have described a mouse model of MCMV-induced interstitial pneumonitis after skin transplantation and CsA immunosuppression, suggesting the roles of HCMV infection in the pathogenesis of interstitial pneumonia in transplant recipients [22]. To study the role of MCMV infection in IBD, we develop a progressive mouse model of MCMV colon inflammation following allogeneic skin transplantation between BALB/c and C57BL/6 mice and administered CsA to suppress allograft rejection. Our findings demonstrate that a dose (1×10^5 PFU) of MCMV significantly induced weight loss, colon permeability, necrosis, and inflammatory cell infiltration, suggesting the progressive course of colon inflammation occurred after the MCMV infection of skin-transplanted mice, and viral replication in colon tissues resulted in pathological abnormalities. However, the discrepancy in weight loss, the MCMV copy number, and the infection level can be attributed to several factors. First, the detection method for eGFP fluorescence might not be as sensitive as the method for quantifying the MCMV copy number. The assay for the infection

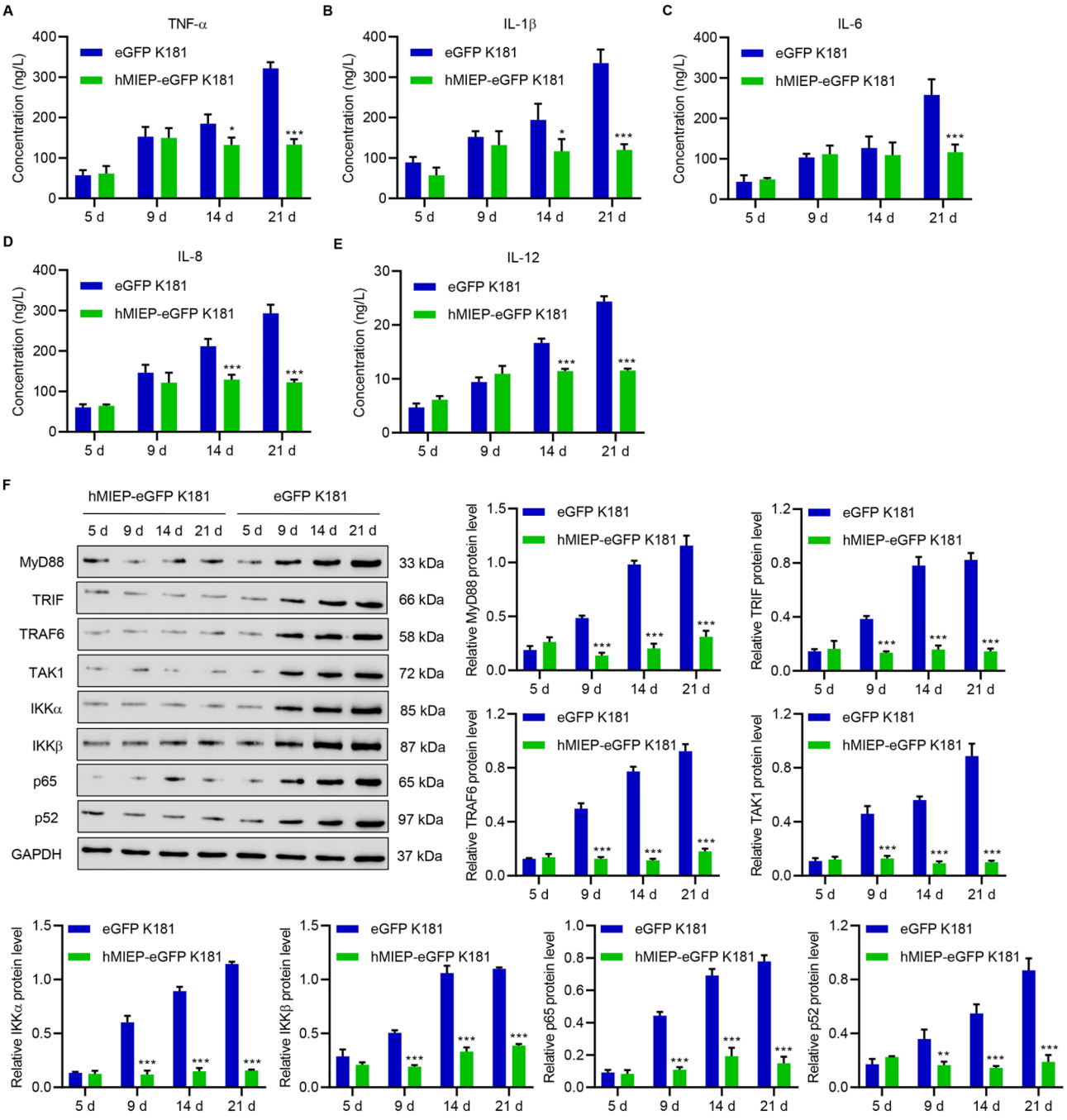

Fig. 4 Effect of hMIEP-eGFP K181 infection on the NF-κB signaling pathway in allogeneic skin transplantation mice. 1×10^5 PFU hMIEP-eGFP K181 or eGFP K181 were inoculated intranasally into allogeneic skin transplantation BALB/c mice on day 14 posttransplantation. The serum levels of (**A**) TNF-α, (**B**) IL-1β, (**C**) IL-6, (**D**) IL-8, and (**E**) IL-12 were measured by ELISA; (**F**) The expression levels of IKKα, IKKβ, p65, p52, TRIF, MyD88, TRAF6, and TAK1 in colon tissues were measured by Western blot at 5, 9, 14, or 21 d postinfection. Data are shown as the mean ± SD of six mice per group. * $P < 0.05$, *** $P < 0.001$ vs. eGFP K181

level could be measuring a downstream effect of the viral presence, and there may be a saturation or a delay in the manifestation of this effect [34]. Second, the host immune response may play a role. As the virus replicates and the copy number increases, the immune system is also mounting a response [35]. In the MCMV infection group, it is possible that the immune response is able to contain the spread of the virus to new cells, preventing a proportional increase in the overall infection level. The virus may be replicating within a limited set of cells, and the immune system is controlling its dissemination, thus maintaining a relatively stable infection level. More importantly, MCMV infection will eventually enter the latent infection phase, during which MCMV-infected

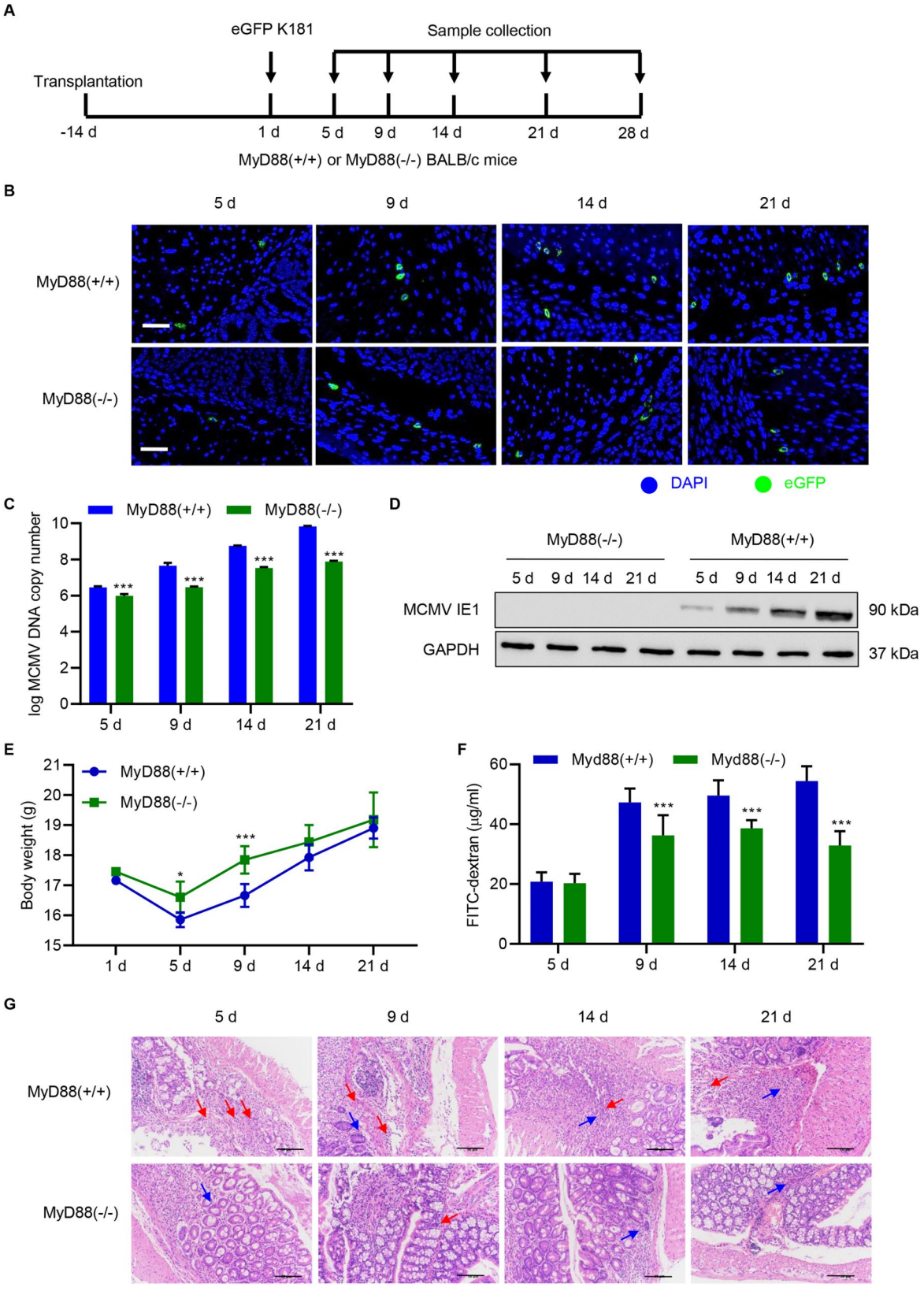

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Fig. 5 Effect of eGFP K181 infection on colon barrier permeability in allogeneic skin transplantation mice. 1×10^5 PFU eGFP K181 were inoculated intranasally into allogeneic skin transplantation MyD88(−/−) and MyD88(+/+) BALB/c mice on day 14 posttransplantation. (**A**) A schematic representation of the assay indicating time points of transplantation and eGFP K181 infection. (**B**) MCMV infection in colon tissues was confirmed by immunofluorescence with positive eGFP (Scale bar: 50 μm); (**C**) MCMV DNA and (**D**) IE1 levels in colon tissues were measured by PCR and Western blot, respectively; (**E**) Body weight and (**F**) colon barrier permeability were measured; (**G**) Necrosis (blue arrow) and inflammatory cell infiltration (red arrow) of colon tissues at 5, 9, 14, or 21 d postinfection (Scale bar: 100 μm). Data are shown as the mean ± SD of six mice per group. * $P < 0.05$, *** $P < 0.001$ vs. Myd88(+/+)

cells will express IE1 but not release mature virus particles. Regarding the unexpected pattern of body weight in the MCMV infection group, it is also possible that early MCMV infection can cause a variety of physiological disruptions, such as anorexia, inflammation, and metabolic dysregulation, all of which contribute to weight loss [36]. However, the subsequent gradual increase in body weight is likely a result of the host's recovery mechanisms. As the immune response begins to gain the upper hand against the virus, the acute symptoms start subsiding. The host may begin to regain its appetite, and the inflammation-related metabolic costs decrease. These findings indicate a critical role of MCMV infection in the regulation of colon inflammation and improve our understanding of IBD pathogenesis.

NF-κB plays an essential role in multiple biological processes [37]. NF-κB activation promotes the proliferation and invasion of tumor cells [38]. NF-κB activation also regulates a proapoptotic program in retinal pericytes [39]. Atreya et al. indicated that activating NF-κB contributed to chronic mucosal inflammation in IBD patients [40]. Wang et al. reported that IL-6 induces NF-κB activation to enhance the expression of intercellular adhesion molecule 1, which is involved in the neutrophil-epithelial interactions in IBD [41]. CMV infection has been shown to promote the LPS or flagellin stimulation of macrophages [42]. Furthermore, LPS and flagellin can subsequently activate NF-κB signaling through either TLR/TRIF or TLR/MyD88 pathways [43]. In this study, we demonstrated that MCMV infection increased the levels of flagellin and LPS, which could result in NF-κB activation. This finding offers a more comprehensive explanation for why MCMV infection promotes colon permeability and inflammation in allogeneic skin transplantation mice.

The role of NF-κB in the life cycle of CMV is complicated, and CMV-mediated NF-κB activation plays a key role in MIEP activation, IE expression, and CMV gene cascade initiation [17]. Subsequently, the *de novo* synthesis of p50 and p65 occurs [44]. The CMV IE1 and IE2 proteins can activate NF-κB-dependent transcription [30, 31], and IE3 gene products are required to activate the early-stage gene expression of MCMV [32]. Moreover, MIEP, which is essential for CMV growth, controls IE gene expression to affect viral replication [45, 46]. We discovered that hMIEP-eGFP K181 (Knockout MCMV IE1-3 promoter) slowed down viral replication and decreased colon permeability, necrosis, inflammatory cell infiltration, inflammation, and NF-κB activation. These findings provide more information regarding the regulatory mechanism of MCMV in the NF-κB signaling pathway in colon permeability and inflammation in allogeneic skin transplantation mice.

MyD88 mediates the induction of inflammatory cytokines through NF-κB [42]. Diomede et al. have demonstrated that LPS leads MyD88 activation, triggering proinflammatory cytokine secretion [13]. MyD88 can induce the phosphorylation of IκBα, which will be degraded by proteasome. Once IκBα is degraded, NF-κB translocates to the nucleus and binds to the promoter region of inflammatory genes to boost their transcription [42]. Our results showed that MyD88 knockout reduced MCMV infection induced an increase in colon permeability and colon inflammation and also inhibited the protein expression of MCMV IE1, which has multiple functions and is important for efficient viral infection [18]. The infection level was decreased in MyD88(−/−) mice, perhaps due to the limited expression of MCMV IE1 caused by the absence of MyD88, preventing viral replication and the confinement of the virus to infected cells. These data confirmed that there is positive feedback between CMV infection and MyD88/NF-κB activation. We mainly dealt with animals, a situation that might not be representative of the experience with patients. Future studies with patients' specimens will provide more relevant data.

In conclusion, our findings demonstrate the significance of MyD88-dependent NF-κB activation in MCMV infection and colon inflammation, which may benefit the treatment of IBD.

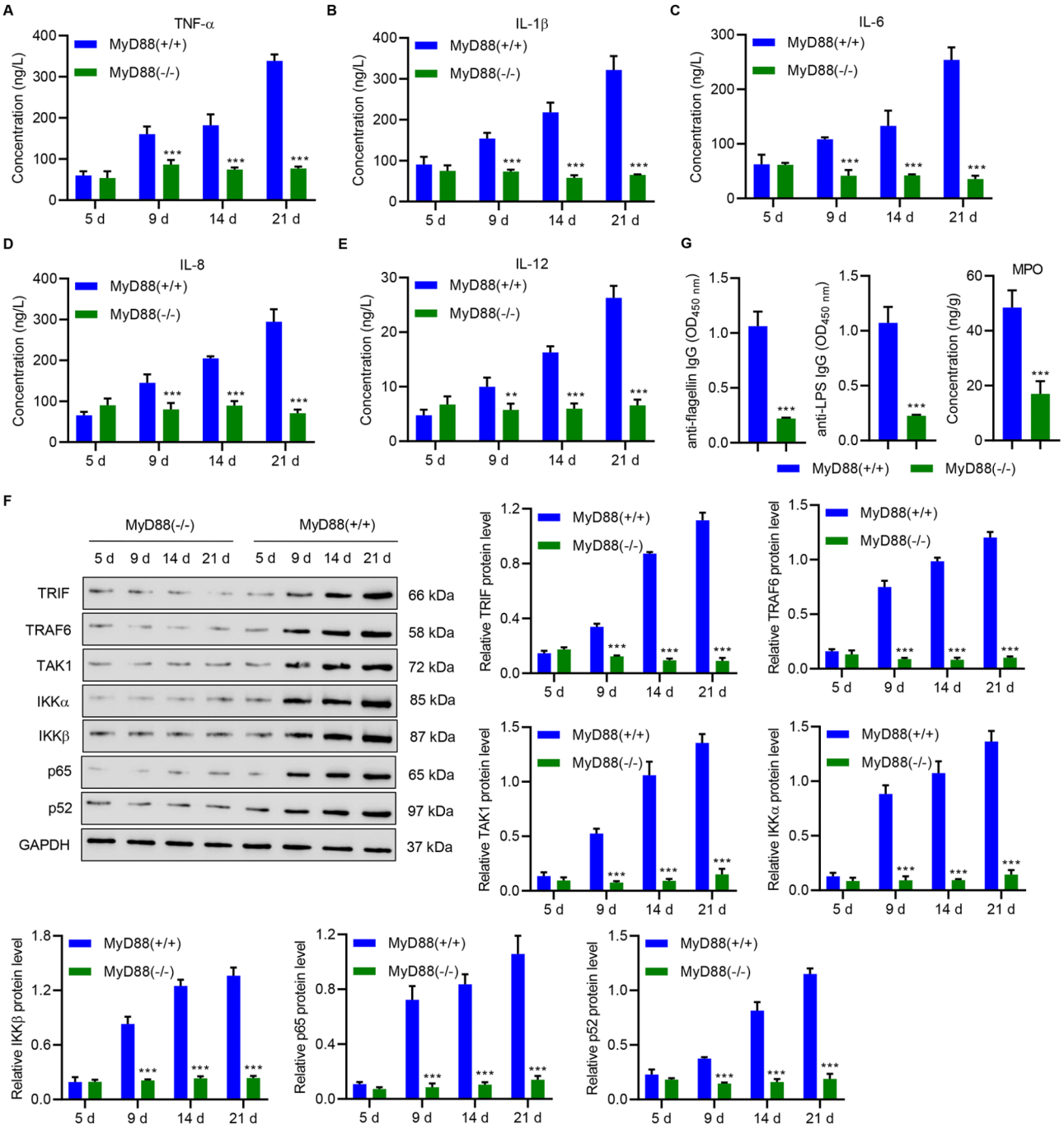

Fig. 6 Effect of eGFP K181 infection on colon inflammation and the NF-κB signaling pathway in allogeneic skin transplantation mice. 1×10^5 PFU eGFP K181 were inoculated intranasally into allogeneic skin transplantation MyD88(−/−) and MyD88(+/+) BALB/c mice on day 14 posttransplantation. The serum levels of (**A**) TNF-α, (**B**) IL-1β, (**C**) IL-6, (**D**) IL-8, and (**E**) IL-12 were measured by ELISA; (**F**) The expression levels of IKKα, IKKβ, p65, p52, TRIF, MyD88, TRAF6, and TAK1 in colon tissues were measured by Western blot at 5, 9, 14, or 21 d postinfection. (**G**) The levels of anti-flagellin IgG and anti-LPS IgG in serum and MPO in colon tissues were measured at 28 d postinfection. Data are shown as the mean ± SD of six mice per group. ** $P < 0.01$, *** $P < 0.001$ vs. Myd88(+/+)

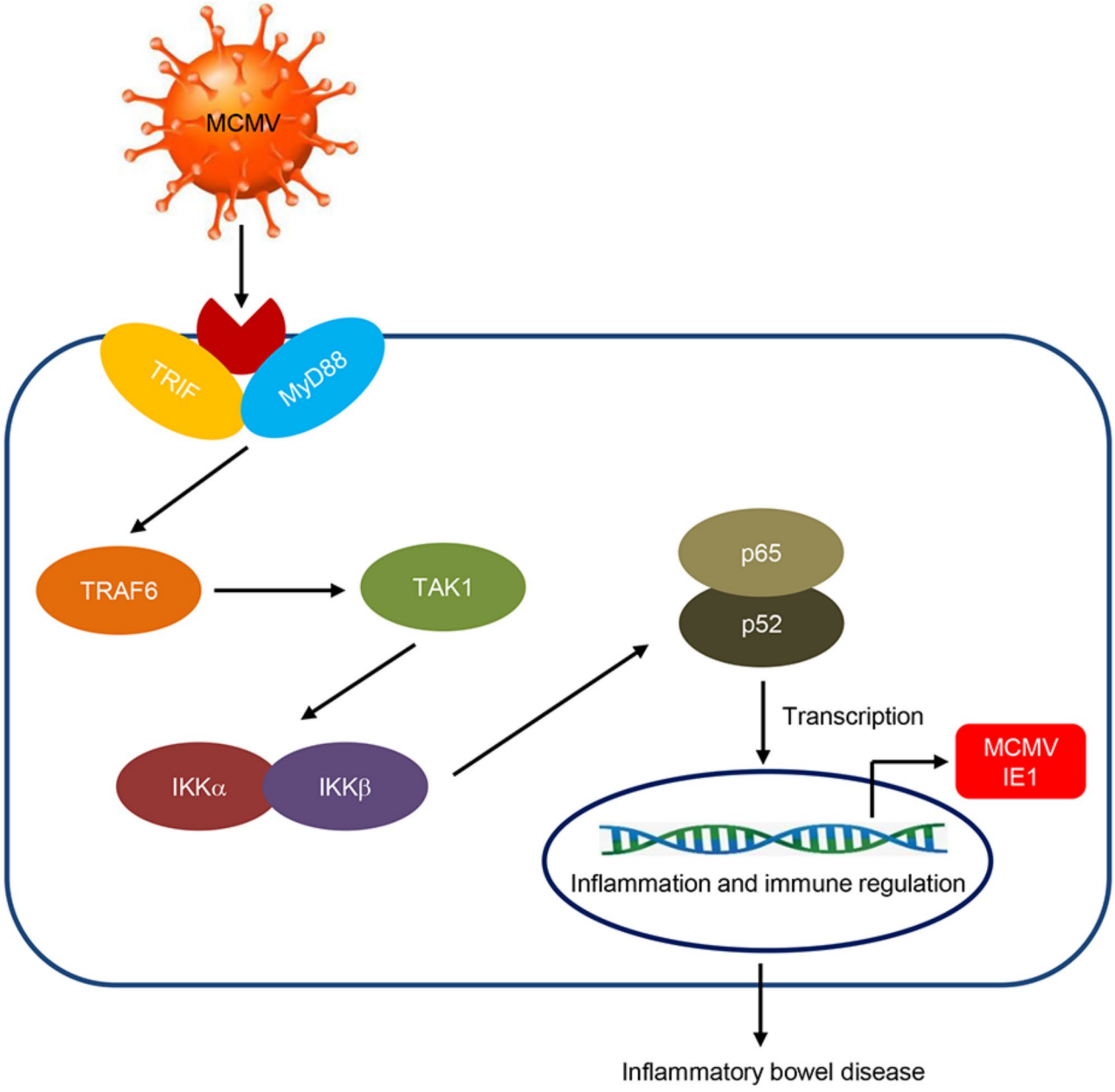

Fig. 7 A graphical summary of the study. MCMV infection initiates IBD by activating a positive MyD88/NF-κB feedback loop in allogeneic skin transplantation mice

Supplementary Information

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Supplementary Material 1

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Author contributions
MXC, SYL and TBS designed the study, analyzed the data and wrote the manuscript. YC, RF and JYW conceived the study, performed the experiments and analyzed the data. All authors contributed to the article and approved the submitted version.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

All the experimental protocols were approved by the Laboratory Animal Ethics Committee of the Zhejiang Acedemy of Traditional Chinese Medicine [no. (2021) 016].

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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