

Absence of kynurenine
3-monooxygenase reduces mortality of
acute viral myocarditis in mice
(キヌレニン3 - モノオキシゲナーゼ
の欠損は急性ウイルス性心筋炎マウス
の死亡率を軽減する)

久保 緋紗子

Original paper

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**Absence of kynurenine 3-monooxygenase reduces mortality of acute viral
myocarditis in mice**

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7 **Footnote**

8 3-HK, 3-hydroxykynurenine; AA, anthranilic acid; EMCV, encephalomyocarditis virus;
9 IDO, indoleamine 2,3-dioxygenase; KA, kynurenic acid; KMO, kynurenine
10 3-monooxygenase; KP, kynurenine pathway; KYN, kynurenine; TRP, tryptophan

Abstract

Infection of the encephalomyocarditis virus (EMCV) in mice is an established model for viral myocarditis. Previously, we have demonstrated that indoleamine 2,3-dioxygenase (IDO), an L-tryptophan - kynurenine pathway (KP) enzyme, affects acute viral myocarditis. However, the roles of KP metabolites in EMCV infection remain unclear. Kynurenine 3-monooxygenase (KMO) is one of the key regulatory enzymes, which metabolizes kynurenine to 3-hydroxykynurenine in the KP. Therefore, we examined the role of KMO in acute viral infection by comparing between $KMO^{-/-}$ mice and $KMO^{+/+}$ mice. KMO deficiency resulted in suppressed mortality after EMCV infection. The number of infiltrating cells and $F4/80^{+}$ cells in $KMO^{-/-}$ mice was suppressed compared with those in $KMO^{+/+}$ mice. $KMO^{-/-}$ mice showed significantly increased levels of serum KP metabolites, and induction of KMO expression upon EMCV infection was involved in its effect on mortality through EMCV suppression. Furthermore, $KMO^{-/-}$ mice showed significantly suppression of CCL2, CCL3 and CCL4 on day 2 and CXCL1 on day 4 after infection. These results suggest that increased KP metabolites reduced chemokine production, resulting in suppressed mortality upon KMO knockdown in EMCV infection. KP metabolites may thus provide an effective strategy for treating acute viral myocarditis.

1

2 **Keywords**

3 Kynurenine 3-monooxygenase, KMO knockout mice, Kynurenine pathway metabolites,

4 Encephalomyocarditis virus, Chemokines, Tryptophan catabolism

5

1. Introduction

Encephalomyocarditis virus (EMCV), a member of the Picornaviridae family that includes the Enterovirus genus, can cause acute myocarditis in various animals including mice. EMCV infection in mice is an established model for viral myocarditis, dilated cardiomyopathy, and congestive heart failure [1]. In the early stages of EMCV infection, inflammatory responses are detected in the heart, where high levels of circulating pro-inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6 via NF- κ B signaling have been measured [2-5]. After EMCV infection, multiple cytokines and chemokines are produced in macrophages, neutrophils, CD4⁺ and CD8⁺ T lymphocytes, and in mast cells [6-8]. The inflammatory response that is induced upon infection plays a critical role in EMCV pathogenesis.

Recently, we demonstrated that the inhibition of indoleamine 2,3-dioxygenase (IDO), which catalyzes the degradation of tryptophan (TRP) to kynurenine (KYN) in the kynurenine pathway (KP), ameliorates EMCV-induced myocarditis [9]. Moreover, it is known that KP regulates inflammation, oxidative stress and immune responses under acute heart attack [10]. Therefore, regulation of KP may provide an effective strategy for treating acute viral myocarditis. However, the roles of KP metabolites after EMCV infection remain unclear.

1 It has recently been reported that kynurenine 3-monooxygenase (KMO)
2 deficiency alters the KP [11, 12]. KMO is a pivotal enzyme in the KP and normally
3 oxidizes KYN to 3-hydroxykynurenine (3-HK) in the presence of reduced nicotinamide
4 adenosine dinucleotide phosphate (NADPH) and molecular oxygen. KMO localizes to
5 the outer mitochondrial membrane and is mainly expressed in peripheral tissues such as
6 the kidney, liver, spleen, and lungs, and in phagocytes such as macrophages [13, 14].
7 Along with others, we have shown that pro-inflammatory cytokines including IFN- γ
8 stimulate KMO activity both in the periphery and in the brain, when the immune system
9 is activated [15-17]. Previous studies have shown that KMO inhibition in blood
10 ameliorates neurodegenerative disorders such as Alzheimer's disease and Huntington's
11 disease [18-21]. Furthermore, a most recent report suggested KMO inhibition as a novel
12 therapeutic strategy in the treatment of acute pancreatitis-induced multi-organ
13 dysfunction syndrome [12]. Therefore, KMO is suggested to play a major role in
14 physiological and pathological events through the KP.

15 In this study, the role of KP metabolites in immune regulation was examined by
16 acute viral infection of EMCV in KMO^{-/-} mice. We showed that increased levels of serum
17 KP metabolites suppressed the production of various chemokines by preventing the
18 recruitment of immune cells in local infection, resulting in the reduction of EMCV-infected

1 cells and mortality.

2

2. Material and methods

2.1. Mice

Male mice at 6-8 –weeks- of age were used in this study. KMO gene-deficient ($KMO^{-/-}$) mice on a C57BL/6J background were obtained from the Knockout Mouse Project (KOMP) repository. Homozygous $KMO^{-/-}$ and $KMO^{+/+}$ mice were generated by intercrossing heterozygous ($KMO^{+/-}$) mice. All the mouse genotypes were confirmed by standard PCR-based genotyping of genomic DNA extracted from tail snippets. The following primer sequences were used for PCR genotyping: KMO gene sense: 5'-TTCTGACCCCATCTGTGTCTGTTCC-3', antisense: 5'-ATCAGAGCTCCCTAAATATGGTGGC-3', KMO gene deficiency sense: 5'-AACTTCGACCCTTTCCCAC-3', antisense: 5'-GACCACCTCATCAGAGCAG-3'.

The mice were housed in a specific pathogen-free environment within our animal facility prior to use. All experiments were performed in accordance with the Guidelines for Animal Care of the Kyoto University.

2.2. Virus infection

A myocarditic variant of EMCV was generously provided by Dr. Y. Seto (Keio University, Tokyo, Japan). The virus stock was stored at -80°C in HBSS with 0.1%

BSA until use. Virus infection was performed as described previously [9, 22]. Briefly, the mice were injected intraperitoneally with 500 PFU EMCV in 0.2 ml saline and then housed in an isolated room. Mortality after EMCV infection was monitored in $KMO^{-/-}$ mice (n = 27) and in $KMO^{+/+}$ mice (n = 27). To obtain samples, the animals were anesthetized and humanely sacrificed at the indicated times. The day of virus infection was defined as day 0 in the following experiments. All experiments were performed in accordance with the institutional guidelines of the Kyoto University.

2.3. Measurement of serum KP metabolites

For TRP, KYN, kynurenic acid (KA), and anthranilic acid (AA) measurements, serum was diluted (4:1, v/v) in 10% perchloric acid. After thorough mixing, the precipitated proteins were removed by centrifugation ($7,000 \times g$, 10 min, 4°C). Fifty microliters of the resulting supernatant was subjected to high-performance liquid chromatography (HPLC; SHIMADU) analysis. TRP, KYN, KA, and AA were isocratically eluted from a reverse phase column (TSKgel ODS-100V, $3 \mu\text{m}$, $4.6 \text{ mm (ID)} \times 150 \text{ mm (L)}$) (Tosoh, Tokyo, Japan) using a mobile phase containing 10 mM sodium acetate and 1% acetonitrile (pH adjusted to 4.5 with acetic acid) at a flow rate of 0.9 ml/min. TRP and KYN were detected using an ultraviolet and visible

spectrophotometric apparatus (SPD-20A, Shimadzu, Kyoto, Japan) (UV wavelength for TRP: 280 nm, UV wavelength for KYN: 365 nm). AA and KA were detected by a fluorescence detector (RF-20Axs) (Shimadzu, Kyoto, Japan) under the following conditions: for AA, the excitation wavelength was 320 nm, and the emission wavelength was 420 nm; for KA, the excitation wavelength was 334 nm and the emission wavelength was 380 nm.

For 3-HK measurement, serum was diluted (1:4, v/v) in 10% perchloric acid. After thorough mixing, the precipitated proteins were removed by centrifugation (7,000 $\times g$, 10 min, 4°C). Twenty microliters of the supernatant were applied to a 3- μ m HPLC column (HR-80; 80 mm $\times$ 4.6 mm) (ESA, Chelmsford, MA), using a mobile phase consisting of 1.5% acetonitrile, 0.9% triethylamine, 0.59% phosphoric acid, 0.27 mM EDTA, and 8.9 mM sodium heptane sulfonic acid, at a flow rate of 0.5 ml/min. 3-HK was detected electrochemically using an ECD 300 detector (oxidation potential: +0.55 V) (Eicom, Kyoto, Japan) as described previously [17]

2.4. RNA extraction and real-time PCR analysis

Total RNA was extracted from cardiac tissue using Isogen (Nippon Gene, Tokyo, Japan), and RNA concentration was determined spectrophotometrically at 260

nm. RT-PCR was performed by using Revetra Ace Kits (Toyobo, Osaka, Japan).

The following PCR primers were used: EMCV, sense,
5'-GTCGTGAAGGAAGCAGTTCC-3', and antisense,
5'-CACGTGGCTTTTGGCCGCAGAGGC-3'. KMO, sense,
5'-GTTATTGGCGGTGGTTTGGTTG-3', and antisense,
5'-GGGCCAAGTTAATGCTCCTTC-3'. 18s, sense,
5'-GGATTGACAGATTGATAGC-3', and antisense,
5'-TATCGGAATTAACCAGACAA-3'. EMCV, KMO, and 18s were quantified by using
real-time PCR in a 7900HT Fast Real-Time system (Applied Biosystems, Foster City,
CA). The EMCV, KMO, and 18s reactions were performed using Ssoadvanced SYBR
Green Supermix (Bio Rad, Hercules, CA). The data were analyzed with the 7900HT
software (version 2.3; Applied Biosystems). Heart homogenate from EMCV-infected
WT mice on day 2 was used as a standard for expression of EMCV. Liver homogenate
from WT mice was used as a standard for expression of KMO and 18s. The expression
values of EMCV, KMO and 18s were interpolated from the standard curve. RT-PCR
data were calculated as the ratio of gene to 18s expression by the relative quantification
method. Data of KMO mRNA are presented as normalized transcript expression in the
samples relative to normalized transcript expression in control mice (uninfected).

2.5. Measurement of serum cytokines and chemokines

The cytokines and chemokines in the serum samples were measured using a 23-plex kit, which is a multiplex detection kit (Bio-Rad, Hercules, CA). This kit detects the levels of CCL2, CCL3, CCL4, CCL5, CXCL1, TNF- α , IL-1 β , IL-6, IL-10, IL-1 α , IL-2, IL-3, IL-4, IL-5, IL-9, IL-12(p40), IL-12(p70), IL-13, IL-17A, G-CSF, GM-CSF, IFN- γ , and Eotaxin. The assay was performed according to the manufacturer's protocol. Fifty microliters of each prepared sample was diluted at a ratio of 1:3 in the sample diluent. All samples were assayed in duplicate and analyzed on a Luminex 200 Labmap system (Luminex, Austin, TX). Data analyses were performed using the Bio-Plex Manager software (version 4.1.1; Bio-Rad). Cytokine concentrations were interpolated from an appropriate standard curve.

2.6. Histological and immunohistochemical analyses

Hearts were fixed in 10% formalin in PBS overnight and then embedded in paraffin. Sections (thickness, 1 μ m) were used for hematoxylin and eosin (HE) staining and F4/80 immunostaining, as described previously [23]. The primary antibody was rabbit polyclonal anti-F4/80 antibody (ab100790, Abcam, Cambridge, U.K.). For

secondary antibody, the VECTASTAIN anti-rabbit IgG antibody kit (Vector Laboratories, Burlingame, CA) was used. Sections were visualized under a light microscope (Olympus, Tokyo, Japan).

2.7 Statistical analyses

All data are expressed as the mean $\pm$ SEM. The survival rates of mice were analyzed by the Kaplan-Meier method. Statistical differences among more than three groups were determined by one-way ANOVA followed by a post-hoc Tukey's -test. For the values obtained in time course experiments, statistical analysis was performed using two-way ANOVA followed by Bonferroni's multiple-comparison test. A p value of less than 0.05 was considered statistically significant.

3. Results

3.1. KMO deficiency reduces mortality after EMCV infection

To investigate the role of KMO in EMCV infection, the effects of KMO deficiency were examined. On day 14 after EMCV infection, the survival rate of KMO^{-/-} mice was 37 %, whereas the survival rate of KMO^{+/+} mice was 11 % (Fig.1A). Moreover, histological changes in the hearts from KMO^{+/+} and KMO^{-/-} mice were compared on day 4 after EMCV infection. The cellular infiltration in KMO^{-/-} mice was suppressed compared with that in KMO^{+/+} mice (Fig. 1B). The number of F4/80⁺ cells, which are known as EMCV-infected cells [24], in KMO^{-/-} mice was lower than that in KMO^{+/+} mice on day 4 after EMCV infection (Fig. 1C). On day 2, the levels of EMCV genomic RNA in the hearts of KMO^{-/-} mice were significantly less than those in the KMO^{+/+} mice (Fig.1D). Furthermore, in KMO^{+/+} mice, the KMO mRNA levels in the hearts increased significantly on day 2 after EMCV infection (Fig.1E). These results indicate that induced expression of KMO upon EMCV infection is involved in mortality.

3.2. KMO gene deficiency markedly changes the levels of serum KP metabolites

The effects of KP metabolites were investigated in mice (KMO^{+/+} and KMO^{-/-}

groups) infected with EMCV, because KMO deficiency has been associated with significantly reduced mortality. In KMO^{+/+} mice, serum KYN and 3-HK levels were increased on day 2 after EMCV infection (Fig.2). On the other hand, the concentrations of serum TRP, AA, and KA in KMO^{-/-} mice were not significantly changed upon EMCV infection. Moreover, the concentrations of serum KYN, KA, and AA were dramatically elevated in KMO^{-/-} mice than in KMO^{+/+} mice. In addition, 3-HK levels in the sera of KMO^{-/-} mice were not detected. These results suggest that increased KP metabolites, excluding TRP and 3-HK, upon EMCV infection promoted immune regulation via KMO activation.

3.3. KMO is involved in chemokine production after EMCV infection

To assess the mechanisms of KMO activity against EMCV proliferation, the changes in serum chemokine and cytokine levels were compared between KMO^{+/+} and KMO^{-/-} mice after EMCV infection (Fig.3). The serum levels of CCL2, CCL3, and CCL4 after day 2 of infection were significantly lower in KMO^{-/-} mice than in KMO^{+/+} mice. The serum level of CXCL1 after day 4 of infection was significantly lower in KMO^{-/-} mice than in KMO^{+/+} mice. The CCL5 levels tended to be lower in KMO^{-/-} mice than in KMO^{+/+} mice.

4. Discussion

In this study, the role of KMO in immune regulation was examined in the context of acute viral infection of EMCV by using KMO^{-/-} mice. We demonstrated that induction of chemokines and recruitment of F4/80⁺ cells was reduced in KMO^{-/-} mice, resulting in increased KP metabolites upon local infection, and significantly reduced mortality in KMO^{-/-} mice.

A recent study has suggested that KMO inhibition prevents multiple organ failure in rodent models of acute pancreatitis and that KA elevation during KMO blockade may exert an anti-inflammatory effect on innate immune cells [12]. Similarly, our results showed that KMO deficiency suppressed mortality in EMCV infected mice. On the contrary, blockade of KMO exacerbated the disease in a Th17-driven model of autoimmune gastritis [25]. Thus, KMO shows both beneficial as well as disadvantageous effects in various diseases.

In this study, our results demonstrated that KP metabolites, especially KYN and KA, were significantly increased in KMO^{-/-} mice. As a considerable number of reports have given evidence of the interaction between the KP, cytokines, and the immune system, there is increasing interest in the role of KP metabolites in inflammatory diseases [26, 27]. KYN and KA are reported to have immunosuppressive effects. KYN

1 induces apoptosis in natural killer (NK) cells through the generation of reactive oxygen
2 species, and inhibits T cell and NK cell proliferation [28-30]. KA activates the G
3 protein-coupled receptor GPR35, and in doing so, inhibits TNF release from
4 macrophages [31]. Accordingly, KYN and KA elevation suppress the recruitment and
5 activation of immune cells. Indeed, our previous report has demonstrated that the
6 number of macrophages in the spleen of IDO^{-/-} mice (low levels of KP metabolites
7 model) was significantly higher than that in the IDO^{+/+} mice after EMCV infection.
8 Furthermore, these effects were canceled in KP metabolites-treated IDO^{-/-} mice [9]. In
9 addition, this study demonstrated that the infiltrated cells, mainly F4/80⁺ macrophages and
10 neutrophils, were suppressed in KMO^{-/-} mice compared with that in KMO^{+/+} mice on day 4
11 after EMCV infection. These results suggested that increased KP metabolite levels may
12 result in the suppression of F4/80⁺ macrophage and neutrophil recruitment.

13 When pathogens invade host cells, they activate the innate immune system
14 and stimulate the production of chemokines and cytokines, which recruit immune cells
15 such as macrophages. KMO was activated by the proinflammatory signaling pathway in
16 mainly macrophages [15]. Our result demonstrated that the levels of KMO mRNA,
17 serum 3-HK, and various chemokines peaked on day 2 after EMCV infection and
18 tended to reduce on day 4 and day 7 parallel to viral load. Induction of KMO expression

1 by EMCV promoted viral replication and tissue damage, suggesting that KMO has a
2 deleterious function in acute EMCV infection.

3 We found that accumulation of KP metabolites decreased the production of
4 chemokines. Although levels of serum CCL2, CCL3, and CCL4, which are produced by
5 mainly macrophages [32, 33], in KMO^{-/-} mice on day 2 after EMCV infection were
6 significantly reduced, compared with in KMO^{+/+} mice, CCL5 levels were not
7 significantly different. The level of serum CCL5 has been reported to mirror the activity
8 of CD8⁺ T cells [34]. CXCL1 is secreted from mainly neutrophils and macrophages in
9 response to various proinflammatory cytokines [35, 36]. Thus, we speculated that
10 CXCL1 level was increased on day 4 after EMCV infection through recruitment of
11 neutrophils into the infection focus. It is possible that the difference in cells producing
12 the chemokine may affect the chemokine reactivity against EMCV infection. It is
13 known that production of chemokines during virus infection is regulated by NF- κ B
14 signaling [4, 5]. Inhibition of NF- κ B upon EMCV infection in mice reduced mortality
15 in the animals, attenuated necrosis and cellular infiltration, and decreased the production
16 of IL-1 β , IL-6, TNF- α , and NO in the mouse heart [2, 4]. KP metabolites are known to
17 inhibit NF- κ B activation [37]. Thus, we speculate that KP metabolites may suppress the
18 production of CCL2, CCL3, CCL4, and CXCL1 by inhibiting NF- κ B signaling,

1 resulting in the improvement of survival rates upon EMCV infection. Indeed, previous
2 research has shown that CCL2-knockout or CCL3-knockout mice survived longer than
3 WT mice upon viral infection (retrovirus, MCV infection) [38, 39]. These reports
4 suggest that chemokines contribute to disease severity through migrating infected cells.
5 In contrast, CCL2-knockout or CCL3-knockout mice survived for shorter periods
6 compared to WT mice after infection with other viruses, such as paramyxovirus or
7 Rocio virus [40, 41]. Therefore, the chemokine-mediated acute inflammatory response
8 protects mice by delaying the lethal sequelae of infection. In this study, the induction of
9 chemokines production due to the increase in levels of KP metabolites following EMCV
10 infection may have contributed to the migration of infected-cells and affected
11 pathogenesis through regulation of immune cell recruitment and/or NF-kB signaling.
12 However, further studies are still needed to clarify these mechanisms.

13 In conclusion, we demonstrated that accumulation of KP metabolites decreased
14 the production of chemokines, resulting in suppressed mortality upon KMO knockdown
15 after EMCV infection. Consequently, KP metabolites may provide an effective strategy
16 for treating acute viral myocarditis.

1 **Conflicts of interest**

2 The authors declare no conflicts of interest.

3

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6

7

Figure legend

FIGURE 1.

KMO gene deficiency reduced mortality through EMCV suppression

(A) The survival rate of $KMO^{-/-}$ mice ($n = 27$) was significantly higher than that of $KMO^{+/+}$ mice ($n = 27$). Statistically significant differences between groups were determined using Kaplan-Meier analyses. (B, C) Histopathological examination of the heart on day 4 after EMCV infection was performed. Heart sections were used for hematoxylin and eosin (HE) staining and F4/80 immunostaining, and examined by light microscopy. Scale bars indicate 100 μm (B upper panel), 10 μm (B lower panel), and 25 μm (C). Lung sections were used as control (C lower left panel: negative control, lower right panel: positive control). Arrows indicate F4/80⁺ cells. These experiments were performed with four mice in each group and produced the same results. (D, E) $KMO^{+/+}$ mice and $KMO^{-/-}$ mice were infected with EMCV, and the animals were humanely sacrificed at the indicated times. The cardiac levels of EMCV RNA and KMO mRNA from each group of mice were determined using real-time quantitative PCR. Data are expressed as the mean $\pm$ SEM of five mice (control) or eight mice (day 2, 4, 7) per group. Statistically significant differences between groups were determined using ANOVA; * $p < 0.05$, ** $p < 0.01$. nd: not detected.

FIGURE 2.

Time course of serum KP metabolites in KMO^{+/+} and KMO^{-/-} mice after EMCV infection

Serum KP metabolite levels were measured using HPLC. Data are expressed as the mean $\pm$ SEM of five mice per time point per group. Statistically significant differences between groups were determined using ANOVA; *p < 0.001 compared with KMO^{+/+} mice. #p < 0.05, ##p < 0.01, ###p < 0.001 compared with uninfected KMO^{+/+} mice (control). nd: not detected.

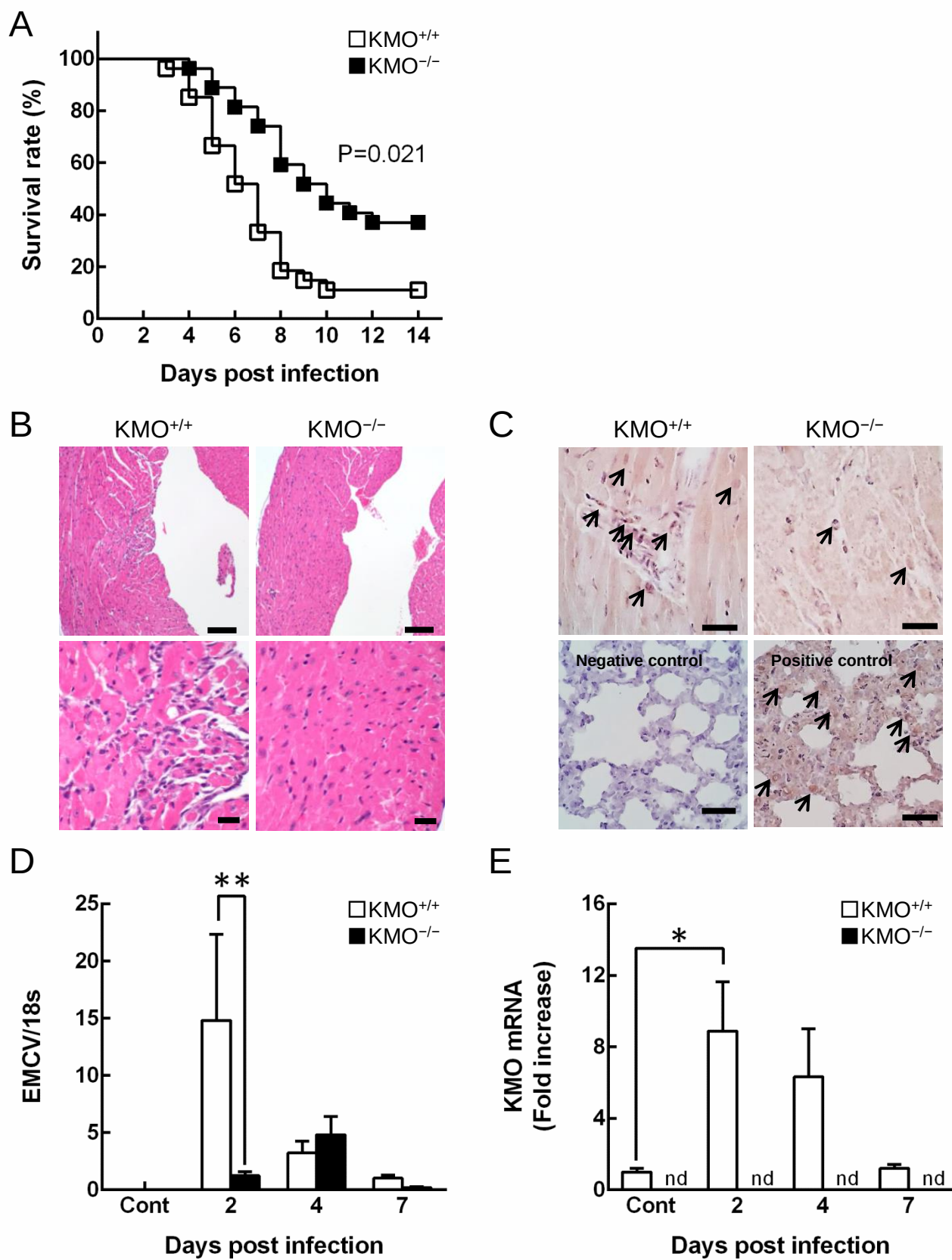
FIGURE 3.

KMO deficiency suppressed the production of various chemokines in EMCV infected mice

The animals were humanely sacrificed at the indicated times. Serum chemokine and cytokine levels in KMO^{+/+} or KMO^{-/-} mice after EMCV infection were quantified using a multiplex detection kit. Data are expressed as the mean $\pm$ SEM of five mice per time point per group. Statistically significant differences between groups were determined using ANOVA; *p < 0.05, **p < 0.01, ***p < 0.001. Control is uninfected state. There was no significant difference in the serum levels of the other

1 cytokines measured by the kit (data not shown).
2
3
4 Graphical abstract
5 Schematic over view of the role of KMO regulation upon EMCV infection
6 EMCV infection induced IDO activation and increased the KP metabolites.
7 KMO deficiency increased KP metabolites (KYN, KA, and AA), and KYN decreased
8 the chemokine expression via NF- κ B signaling. The increased KP metabolites
9 suppressed the immune cells. The KP metabolites might contribute to suppress infected
10 cells and ameliorate the survival rate.

Fig.1



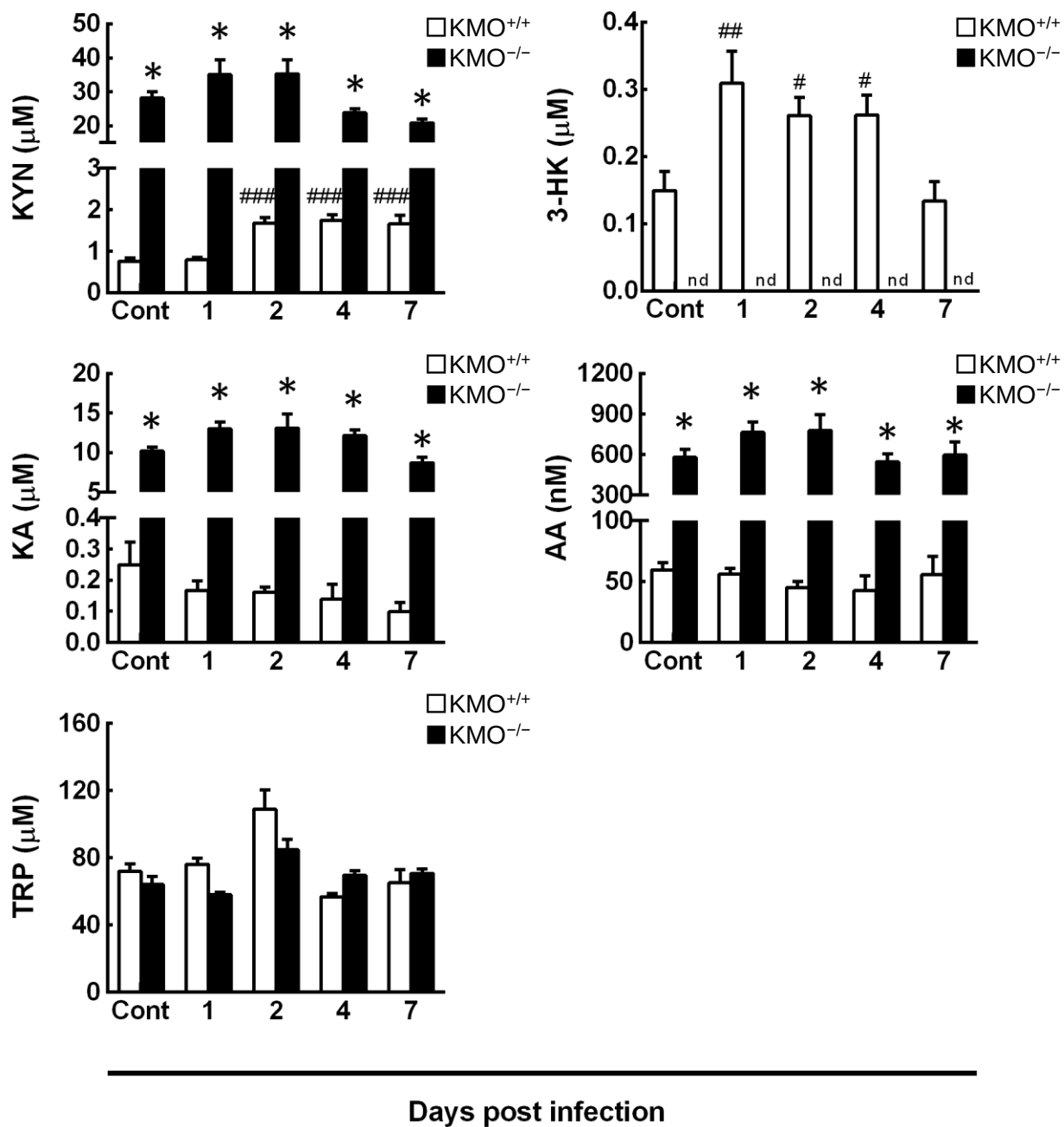
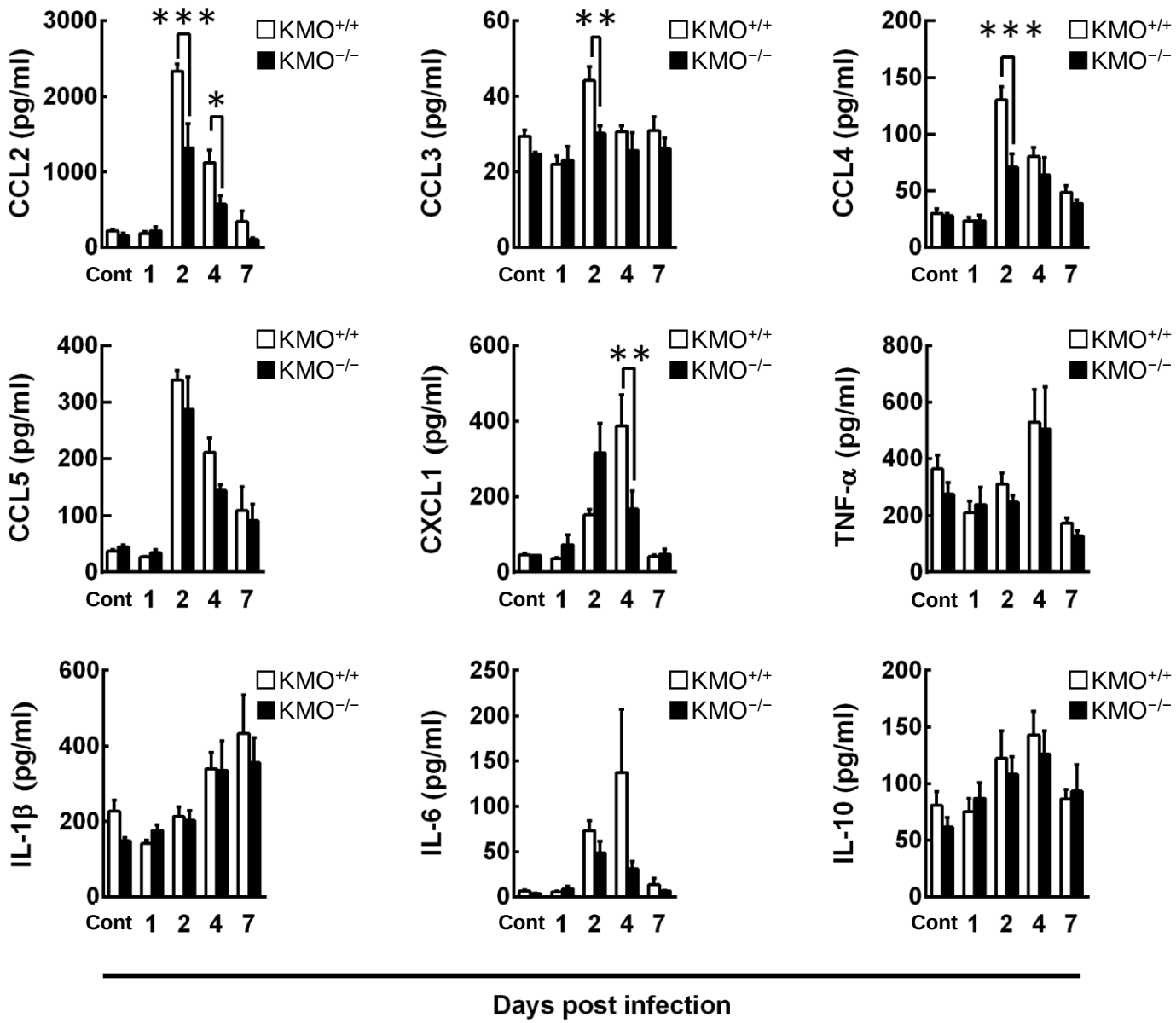
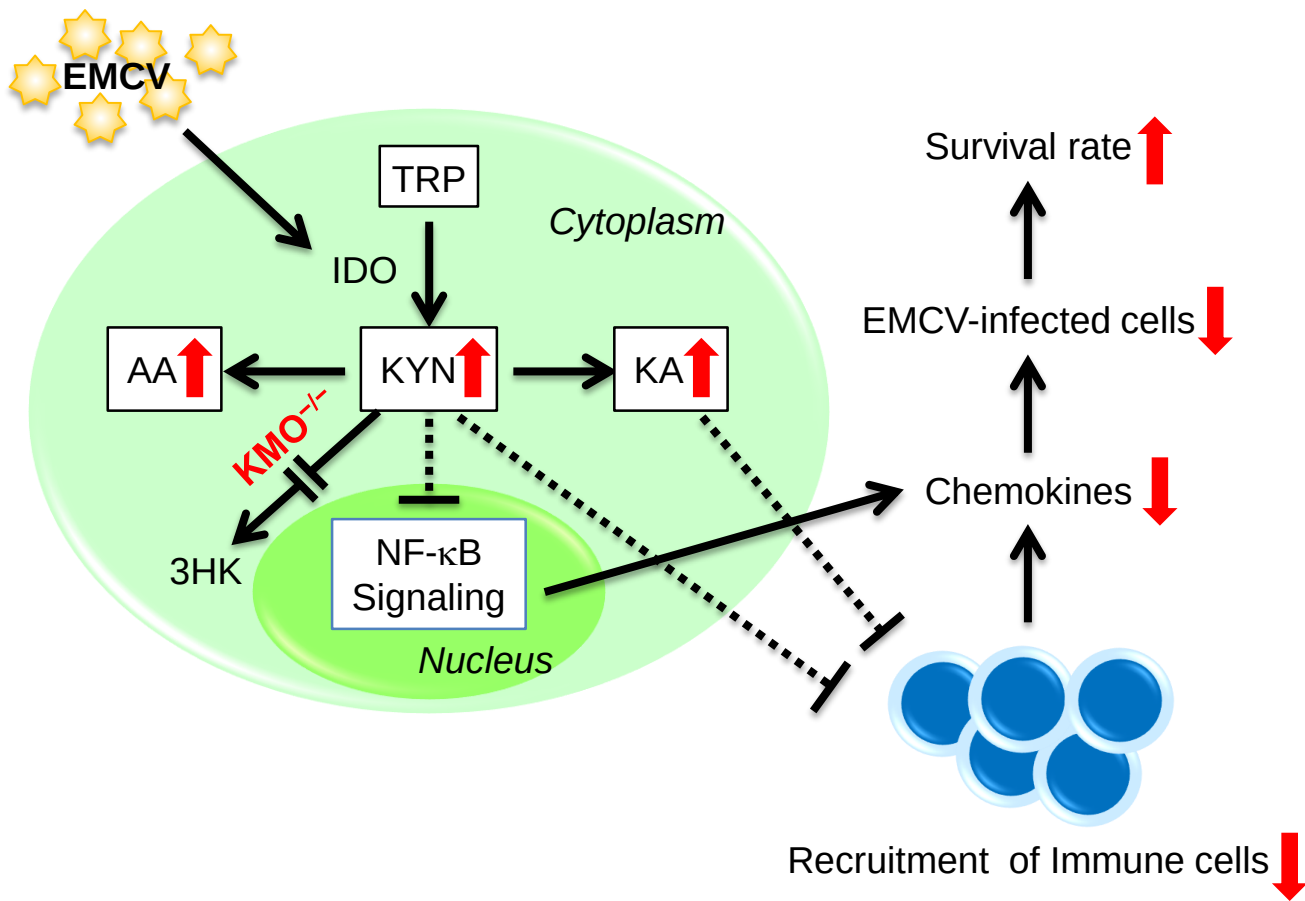


Fig.2

Fig.3





Graphical abstract