Original Research

CCK2-receptor deficiency impairs immune balance by influencing CD4$^+$ T cells development by inhibiting cortical-thymic-epithelial-cells

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Impact Statement

Maintaining immune balance is essential for individual survival, as immunocompromised individuals have high mortality. Neuropeptides, as important mediators of immune balance regulation, have received much attention. The thymus plays an important role in maintaining immune balance. Reconstruction of the thymus microenvironment to promote T cell regeneration has emerged as a new clinical strategy. The immunomodulatory effect of cholecystokinin (CCK) mediated by CCK receptors has been researched for more than 20 years. Our manuscript provides evidence that CCK2 receptor-deficient mice were immunocompromised and were more prone to shock and even death in an endotoxemia/endotoxin shock model. In addition, we found that CCK2 receptor deficiency impairs immune balance by influencing CD4$^+$ T cell development in the thymus by inhibiting major histocompatibility complex class II (MHC II) and CD83 expression on cortical thymic epithelial cells. Our findings reveal that CCK2R is necessary for maintaining immune balance and expand current understanding of neuroimmunomodulation.

Abstract

Immune balance is crucial for an organism's survival and is inseparable from the regulation of the nervous system. Accumulating evidence indicates that cholecystokinin (CCK) plays an important role in mediating the immune response through the activation of cholecystokinin receptors (CCKRs). However, it remains unclear whether CCKRs deficiency may impair immune balance. Here, we showed that CCK2R-deficient adult mice were immunocompromised and had an increased risk of shock and even death in an endotoxemia (ETM)/endotoxin shock (ES) model. In addition, in both adult and juvenile mice, CCK2R deficiency not only influenced the development of CD4 single-positive (SP) thymocytes in thymic positive selection but also decreased the population of CD3$^+$ CD4$^+$ T cells in the spleen. More importantly, CCK2R deficiency inhibited the expression of major histocompatibility complex class II (MHC II) and CD83 on cortical thymic epithelial cells (cTECs) in juvenile and adult mice. Overall, our study suggests that CCK2R is essential for maintaining CD4$^+$ T cell development in the thymus and reveals that CCK2R plays an important role in maintaining immune balance.

Keywords: CCK2 receptor, CD4$^+$ T cells, thymus, cTECs, immunoregulation

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Introduction

"How is immune homeostasis maintained and regulated?" was one of 125 scientific questions posed by *Science* in the new version of its list of critical unanswered questions in cutting-edge. Undoubtedly, a well-balanced immune system is important for survival and for combating diseases, as illustrated by the increased risk of infection in immunocompromised individuals.[1] In recent years, the study of bidirectional neuroimmune communication has grown at an exponential rate. However, our knowledge of how the nervous system affects immunological activity is far more limited. It may seem surprising because we know that emotion can affect our health, for example, we are susceptible to illness after stressful events in our daily lives.[2,3] Therefore, how the nervous system regulates immunity may

reveal novel pathways or mechanisms. Several studies in the last decade have suggested that the nervous system could modulate the immune response. The neuroendocrine pathway is one of the important pathways associated with the regulation of immunity. Neuropeptides have been widely studied as important mediators between the central nervous system and the immune system.[4,5]

Cholecystokinin (CCK), a typical endogenous neuropeptide, is involved in the regulation of various physiological and pathological processes.[6] For example, CCK is involved in the induction and development of acute pancreatitis in experimental animals.[7] In the past 20 years, our research has focused on the immunomodulatory effects of CCK-8, which is the biologically predominant and active form of endogenous CCK. A series of our studies indicated that CCK-8 could regulate inflammatory and immune responses. On one hand, CCK-8 has anti-inflammatory effects. It was able to mitigate lung inflammation induced by lipopolysaccharide (LPS) by inhibiting signaling pathways such as cAMP/PKA and PKC/MAPK/NF-κB, reducing pro-inflammatory cytokines and promoting anti-inflammatory cytokines, which ultimately improved hemodynamics and reduced mortality in rats with endotoxemia (ETM) and endotoxin shock (ES).[8,9] On the other hand, CCK-8 also has immunomodulatory effects. CCK-8 not only inhibits the maturation of the phenotype and function of dendritic cells (DCs), peritoneal macrophages, and B cells and downregulates major histocompatibility complex class II (MHC II), CD80, and CD86 expression[10,11] but also affects the function of $CD4^+$ T cells and modulates the Th1/Th2 cell and Th17/Treg cell balance in mice.[12,13] Importantly, the immunomodulatory effect of CCK is mediated by the activation of cholecystokinin receptors (CCKRs, including CCK1R and CCK2R), especially CCK2R, which is mainly expressed by spleen and immune cells in mice, such as T cells, DCs, and macrophages.[9–11] Overall, these studies indicated that CCKRs might play an important role in the immunomodulatory effect of CCK, especially in maintaining the balance of immune cells such as T cells.

The thymus plays an essential role in maintaining immune balance by mediating the maturation and selection of T cells in mammals. Hypoplasia or removal of the thymus results in T cell deficiency and even being immunocompromised.[14] It is worth noting that the development of T cells in the thymus is variable throughout the life of mammals, reaching maximum size with the most active function (output peaks) at puberty and decreasing gradually due to thymus atrophy with aging after sexual maturity.[15] T cells in the thymus undergo T cell receptor (TCR) rearrangement to express $CD3^+$ co-receptors, $CD4^-$ $CD8^-$ double-negative (DN) thymocytes and $CD4^+$ $CD8^+$ double-positive (DP) thymocytes. Subsequently, DP thymocytes undergo positive selection and negative selection, giving rise to $CD4^+$ or $CD8^+$ single-positive (SP) thymocytes that ultimately output into the periphery as naive T cells. During this process, the thymic microenvironment cooperatively supports T cell differentiation.[16] In particular, cortical thymic epithelial cells (cTECs) are necessary for positive selection to differentiate CD8 SP thymocytes and CD4 SP thymocytes.[17] With the development of mouse and human thymocyte mapping, new immunotherapies to reconstruct the thymic microenvironment to facilitate T cell regeneration are gradually attracting the attention of many scholars.[18,19] A prior study has shown that CCK helped regulate thymus growth by inhibiting thymic lymphocyte chemotaxis.[20] Therefore, whether interfering in the CCK-CCKRs signaling pathway could result in immune imbalance with thymic dysfunction and T cells developmental disorder is unknown.

CRISPR/Cas9 gene-editing technology is widely used in mammals for a deeper understanding of disease mechanisms.[21] CCKR gene knockout mice have also been widely used to study the regulatory mechanism of CCK in diseases; the results suggest that different CCKR deficiencies might affect the maintenance of normal physiological functions.[22,23] However, it remains unclear whether CCKR deficiency influences the development of T cells in the thymus, resulting in immune imbalance.

Here, we assessed the effect of CCKR deficiency on immune balance in adult $CCK1R^{-/-}$, $CCK2R^{-/-}$, and $CCK1/2R^{-/-}$ mice in an LPS-induced ETM/ES model. Second, the effect of different CCKR deficiencies on T cells of the thymus and spleen in juvenile and adult mice was further examined. Finally, the effect of different CCKR deficiencies on the expression of MHC II and CD83 on cTECs during thymic positive selection was analyzed.

Materials and methods

Mice

$CCK1R^{-/-}$, $CCK2R^{-/-}$, and $CCK1R/2R^{-/-}$ mice generated on pure C57BL/6J genetic background were obtained from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). The following primers were used to genotype the mice: CCK1R-forward 5′-GGAGATATAGCAACAGCAAGGC-3′, CCK1R-reverse 5′-CGGCACACAGAAGAACCTTTG-3′, CCK1R-forward 5′-GGAGATATAGCAACAGCAAGGC′, CCK1R-reverse 5′-CAGAATGATAAAAACCTTAGGCCC-3′; CCK2R-forward 5′-GGTGTCCAGGATAGCATTTCC-3′, CCK2R-reverse 5′-CTCAACCCACTTGATGACTCAG-3′, CCK2R-forward 5′-GCAGGGAATTAGTCAGTCACCT′, CCK2R-reverse 5′-GTAGGAGGTTTAGGTCTGTGTGC-3′. All genotyping was performed on tail biopsy samples with Direct PCR® Lysis Reagent (Tail) (Viagen Biotech, U-SA) using real-time PCR to probe for the WT or KO target sequence in each sample as described previously.[24]

All animals were maintained in the animal facility under specific pathogen-free conditions, at 19°C–23°C with a 12/12 h light–dark cycle. All animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals. The experimental protocols were approved by the Local Committee on Animal Care, Use and Protection of Hebei Medical University (20190066).

Animal preparation

All animals were fasted overnight (with ad libitum access to water) prior to the experiments. To ensure the success of the operation on ETM/ES mice, adult mice (male, 10–24 weeks) were anesthetized using a protocol that preserves spontaneous breathing (pentobarbital sodium, 2%, 50 mg/kg), and the mice were placed on a surgical table. The ETM and ES

models were induced by different doses of LPS (Sigma, 0111: B4, low dose of 11 mg/kg, high dose of 33 mg/kg) administered via the caudal vein. Surgery was performed as previously described.[8] In brief, a polyethylene (PE) catheter was placed in the left carotid artery and connected to pressure transducers to record the mean arterial pressure (MAP). The heart rate (HR) was recorded by electrocardiography. All the data were simultaneously recorded with a physiological data acquisition system (Biopac Systems, Goleta, CA) at different points in time: 0 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, and 8 h.

The organ index and histomorphology of the thymus and spleen

An electronic balance was used to measure the body and organ weights of the mice. The body weight was recorded, followed by the weights of the removed thymus and spleen, in both juvenile (male, 5–6 weeks) and adult mice (male, 10–24 weeks). The immune organ index (%) was calculated according to the following formula: Index = organ weight (mg)/body weight (g) × 100%. The thymus and spleen samples were fixed with 4% paraformaldehyde, embedded in paraffin and cut into 4-μm sections. After deparaffinization in xylene and rehydration in ethanol, the sections were subjected to hematoxylin and eosin (H&E) staining and observed under a light microscope (Olympus, Tokyo, Japan).[25]

Isolation of hematopoietic cells and flow cytometry analysis

The thymus and spleen were harvested when the mice were killed. The thymus and spleen were mechanically disrupted with a 40-μm nylon mesh (BD Falcon) and washed with 1× phosphate-buffered saline (PBS). Red blood cells in the spleen were lysed using 1× red blood cell lysis buffer (BioLegend). Single-cell suspensions were obtained by centrifugation, filtration, and resuspension.[26]

For thymocytes and splenocytes in juvenile mice, these single-cell suspensions were stained with the following fluorescent monoclonal antibodies: anti-mouse CD3 PE/Cy5 (145-2C11, BioLegend), anti-mouse CD4 FITC (GK1.5, BioLegend), and anti-mouse CD8 PE (53-6.7, BioLegend). Flow cytometry was performed on an FC500-type flow cytometer (Beckman Coulter), with data analyzed on EXPO32 ADC v1.2 software (Beckman Coulter).

For thymocytes and splenocytes in adult mice, these single-cell suspensions were stained with the following fluorescent monoclonal antibodies: anti-mouse CD3 FITC (145-2C11, BD Pharmingen), anti-mouse CD4 APC (RM4-5, BD Pharmingen), and anti-mouse CD8 PE (53-6.7, BioLegend). Flow cytometry was performed on a FACSCalibur (BD Bioscience), with data analyzed on FlowJo software (BD Bioscience).

Isolation of cTECs and flow cytometry analysis

The thymuses of juvenile and adult mice were isolated and stromal cell enrichment was performed as previously described.[27] Briefly, thymuses were mechanically disrupted and then digested with DNase I (Sigma–Aldrich) and collagenase type IV (Gibco). After the cells were collected from the digested tissue, anti-CD45 immuno-magnetic beads (Miltenyi Biotec) were used to enrich the TECs in the cell suspension. For cTECs sorting, the single-cell suspensions were stained with anti-mouse CD45 PerCP-Cy5.5 (30-F11, BD Pharmingen), anti-mouse EpCAM PE-Cyanine7 (CD326, G8.8, eBioscience), and anti-mouse Ly-51 PE (6C3, BioLegend).

For analysis of the expression of MHC II and CD83 on cTECs ($CD45^-$ $EpCAM^+$ $LY51^+$), sorted cTECs were prepared by washing and resuspending and then stained with anti-mouse MHC class II APC/Cyanine7 (I-A/I-E, M5/114.15.2, BioLegend) and anti-mouse CD83 APC (Michel-19, BioLegend). Flow cytometry and cell sorting were performed on a FACSAria II (BD Biosciences), and data were analyzed using FACS Diva software.

Statistical analysis

Data were analyzed using SPSS 27.0 software. Data are presented as mean ± standard error of the mean. Analysis of variance (ANOVA), repeated-measures ANOVA, Kruskal–Wallis test, Tukey's test, and a non-parametric test were used as appropriate to determine significance. Results were considered significant at $P < 0.05$.

Results

CCK2R deficiency results in high risk of infection/mortality in ETM/ES adult mice

To rule out a potential effect of gene knockout on basal MAP and HR, adult WT, $CCK1R^{-/-}$, $CCK2R^{-/-}$, and $CCK1R/2R^{-/-}$ mice were injected with normal saline. The results showed that CCKR deficiency had no effect on basal MAP and HR in adult mice without damaging factors (Figure 1(A) to (D)). Thus, ETM and ES mouse models could be established.

In adult mice with ETM treated with a low dose of LPS, the MAP of WT mice decreased over the period from 0 h to 3 h after administration, and the HR decreased with time. These observations suggest that LPS had entered the blood and induced injury and that the ETM model was successfully established. There was no significant difference in MAP and HR in $CCK1R^{-/-}$ mice. In contrast, there were markedly different changes in MAP and HR in both $CCK2R^{-/-}$ and $CCK1R/2R^{-/-}$ mice: there was no significant difference in MAP and HR at 0 h, but the MAP decreased gradually with time and decreased to the shock level (≤60 mmHg) at 8 h. The HR also decreased correspondingly as MAP progressively declined (Figure 2(A) to (D)).

In adult mice with ES induced by a high dose of LPS (three times the low dose), the MAP decreased rapidly from 6 h to 8 h and reached the shock level at 8 h in WT mice. The HR also showed a decrease corresponding to the decline in MAP. The data suggested that the ES model was successfully established. Similarly, the MAP and HR showed the same changes in $CCK1R^{-/-}$ mice. Remarkably, in $CCK2R^{-/-}$ and $CCK1R/2R^{-/-}$ mice, the MAP declined sharply to the shock level at 1 h. All the $CCK2R^{-/-}$ and $CCK1R/2R^{-/-}$ mice were dead at 8 h. The HR decreased at the same time MAP was declining (Figure 3(A) to (D)).

Taken together, these results suggest that CCK2R deficiency results in high risk of infection/mortality in ETM/

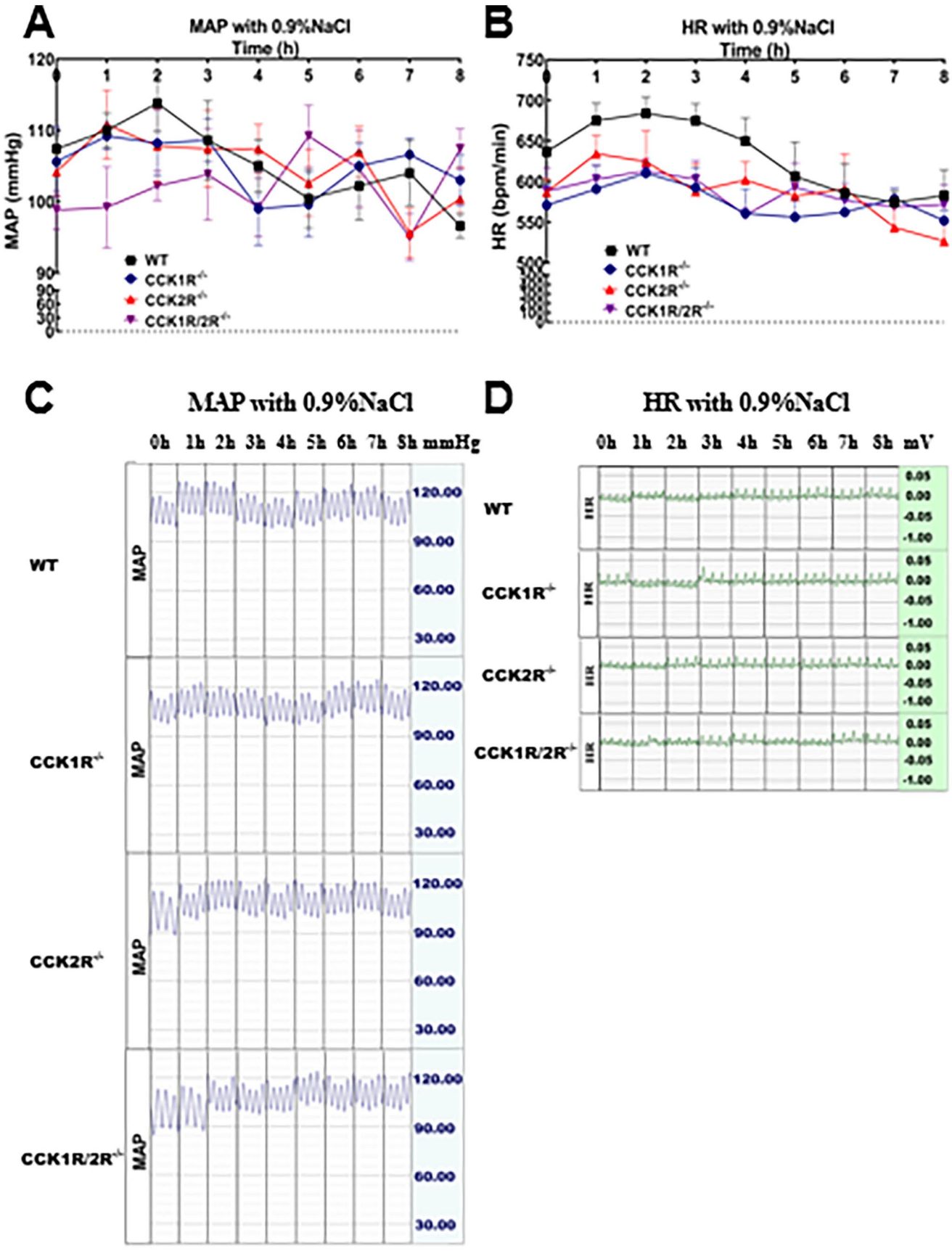

Figure 1. CCK2R deficiency has no effect on basal MAP or HR in mice. The mice (male, 10–24 weeks) were injected with 0.9% NaCl. The MAP and HR were recorded simultaneously using a physiological data-acquisition system at different times (0 h to 8 h). (A and B) The MAP and HR were recorded simultaneously at multiple time points. (C and D) Representative tracings showing alterations in the MAP and HR in different groups. $n = 5$ for each group. Data are expressed as the means ± SEM. $^*P < 0.05$ *vs* WT group.

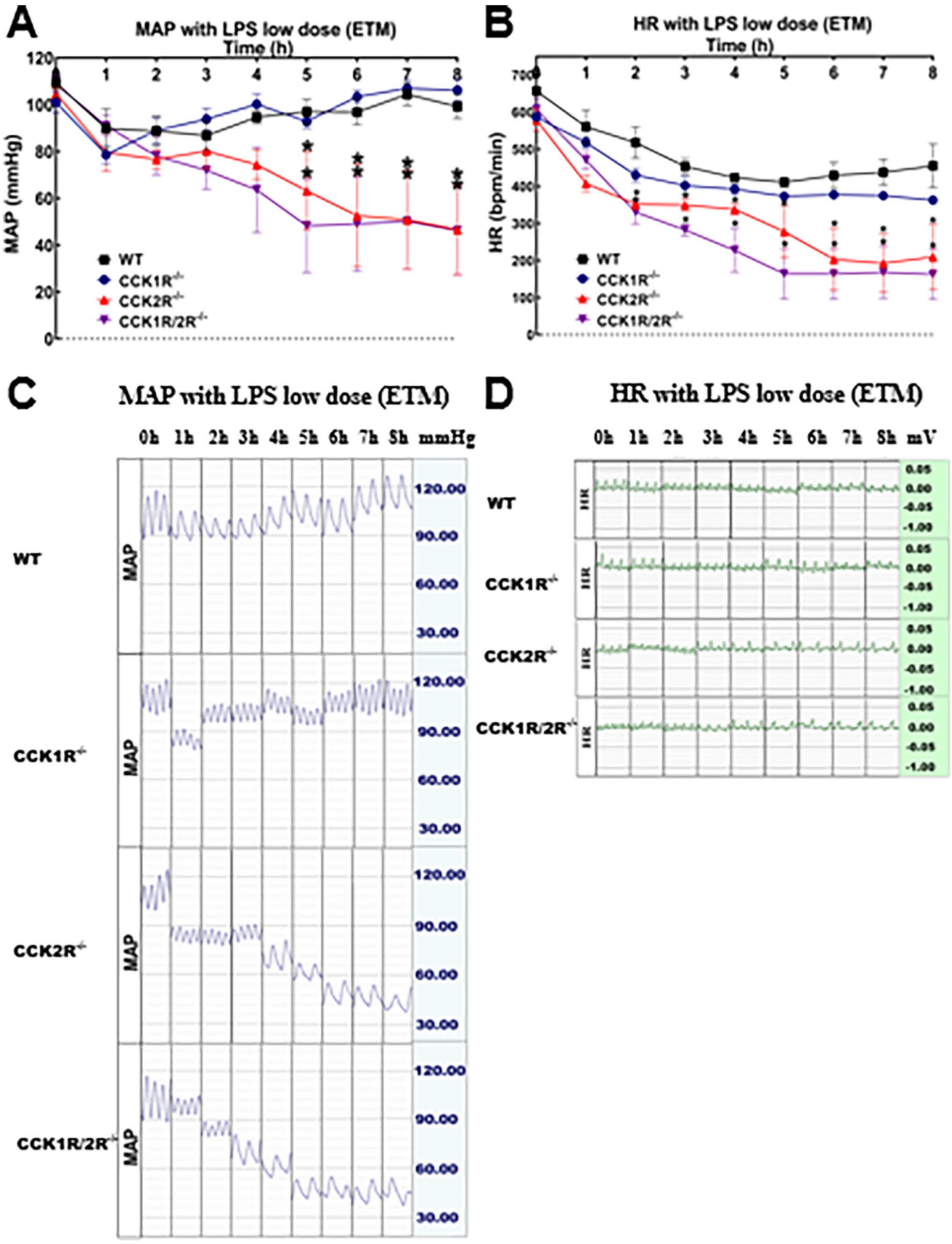

Figure 2. CCK2R deficiency resulted in high risk of infection in ETM mice. The mice (male, 10–24 weeks) were injected with low-dose LPS (11 mg/kg, ETM). The MAP and HR were recorded simultaneously using a physiological data-acquisition system at different times (0 h to 8 h). (A and B) The MAP and HR were recorded simultaneously at multiple timepoints. (C and D) Representative tracings showing alterations in the MAP and HR in different groups. $n = 5$ for each group. Data are expressed as the means ± SEM. $^*P < 0.05$ *vs* WT group.

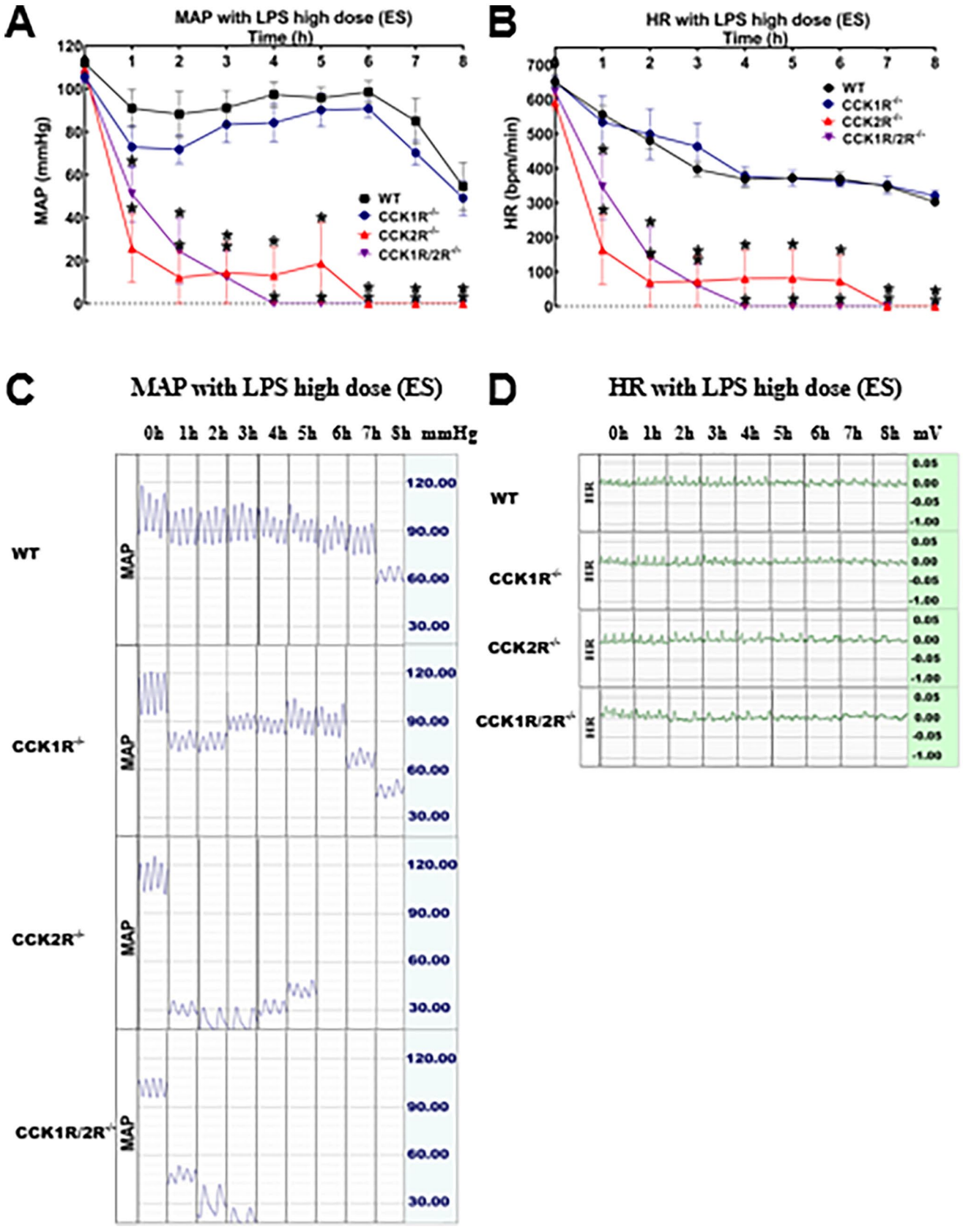

Figure 3. CCK2R deficiency resulted in high risk of mortality in ES mice. The mice (male, 10–24 weeks) were injected with high-dose LPS (33 mg/kg, ES). The MAP and HR were recorded simultaneously using a physiological data acquisition system at different times (0 h to 8 h). (A and B) The MAP and HR were recorded simultaneously at different times. (C and D) Representative tracings showing alterations in the MAP and HR in different groups. $n = 5$ for each group. Data are expressed as the means ± SEM. $^{*}P < 0.05$ *vs* WT group.

ES models, which may be related to immunocompromised in adult mice.

CCK2R deficiency resulted in thymus atrophy and spleen hyperplasia in juvenile and adult mice

Because the thymus reaches its maximum size and activity at puberty (approximately 5–6 weeks), we added juvenile mice (5–6 weeks) to assess the effect of CCKR deficiency on thymus and spleen.[15]

Compared with WT mice, juvenile and adult mice with CCK2R and CCK1R/2R deficiency showed decreased in thymus indices but increased in spleen indices. There was no significant difference in thymus and spleen indices of the CCK1R$^{-/-}$ mice (Figure 4(A) to (F)).

Regarding histomorphology, the juvenile and adult mice with CCK2R and CCK1R/2R deficiency showed a thinner thymus cortex than WT mice. Compared with WT mice, the mutant mice had irregular splenic corpuscles, reduced lymphocytes, and intercellular enlargement in the central area of the spleen. There was no significant difference in either the thymus or the spleen in CCK1R$^{-/-}$ mice (Figure 4(G) and (H)).

Thus, these results suggest that CCK2R deficiency resulted in thymus atrophy and spleen hyperplasia with immunocompromised in both juvenile and adult mice.

CCK2R deficiency influenced CD4$^+$ T cell development in the thymus of juvenile and adult mice

We examined the proportions of T cell subsets in the thymus. In juvenile mice, compared with WT mice, although the proportions of DP thymocytes and CD8 SP thymocytes were normal (Figure 5(C), (D), (F)), the proportions of CD3$^+$ thymocytes and CD4 SP thymocytes were notably reduced in CCK2R$^{-/-}$ and CCK1R/2R$^{-/-}$ mice (Figure 5(A) to (C) and (E)). The reduction in CD3$^+$ thymocytes might be associated with the reduction in CD4 SP thymocytes. Moreover, there was no significant difference in CCK1R$^{-/-}$ mice (Figure 5(A) to (F)). In addition, the same phenomenon was observed in adult mice with CCK1R, CCK2R, and CCK1R/2R deficiencies (Figure 5(G) to (L)). On this basis, we can infer that CCK2R deficiency influenced CD4$^+$ T cell development by blocking CD4 SP thymocytes maturation in both juvenile and adult mice.

CCK2R deficiency reduced the population of CD3$^+$ CD4$^+$ T cells in the spleen of juvenile and adult mice

The effect of CCKR deficiency on T cell subsets in the spleen was also assessed. Among juvenile mice, the results showed that the proportions of CD3$^+$ T cells and CD3$^+$ CD4$^+$ T cells (Figure 6(A) to (D)) but not CD3$^+$ CD8$^+$ T cells (Figure 6(E) and (F)) in the spleen were significantly reduced in mice with CCK2R and CCK1R/2R deficiency compared with WT mice. There was no significant difference in the CCK1R$^{-/-}$ mice (Figure 6(A) to (F)). Similarly, such a phenomenon was observed in adult mice with CCK1R, CCK2R, and CCK1R/2R deficiency (Figure 6(G) to (L)). Therefore, we inferred that CCK2R deficiency reduced the populations of CD3$^+$ T cells and CD3$^+$ CD4$^+$ T cells in the spleen, which might be associated with decreased in thymus output in both juvenile and adult mice.

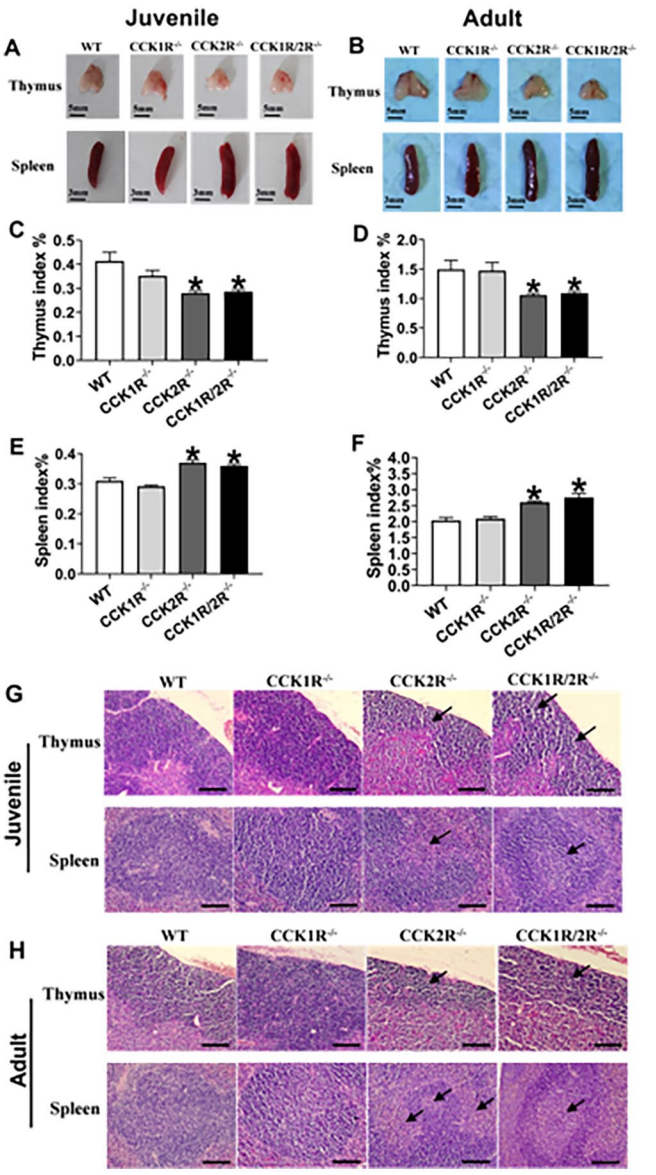

Figure 4. CCK2R deficiency resulted in thymus atrophy and spleen hyperplasia in juvenile and adult mice (male; juvenile, 5–6 weeks, when the thymus is at its maximum size during puberty; adult, 10–24 weeks). All samples were collected from different groups of mice. (A and B) Representative images of the thymus and spleen. (C to F) The organ indices of the thymus and spleen were calculated as follows: Index = organ weight (mg)/body weight (g) × 100%. (G and H) Representative images of the histomorphology of the thymus and spleen. H&E staining, bar = 200 μm (thymus) and 100 μm (spleen). $n = 5$ for each group. Data are expressed as the means ± SEM. *$P < 0.05$ *vs* WT group.

CCK2R deficiency influenced CD4$^+$ T cell development by inhibiting MHC II and CD83 expression on cTECs in juvenile and adult mice

cTECs as a part of the thymic microenvironment are important for positive selection of T cells. In particular, the MHC II on cTECs is crucial for the differentiation of CD4 SP thymocytes. Meanwhile, the stability of MHC II depends on the

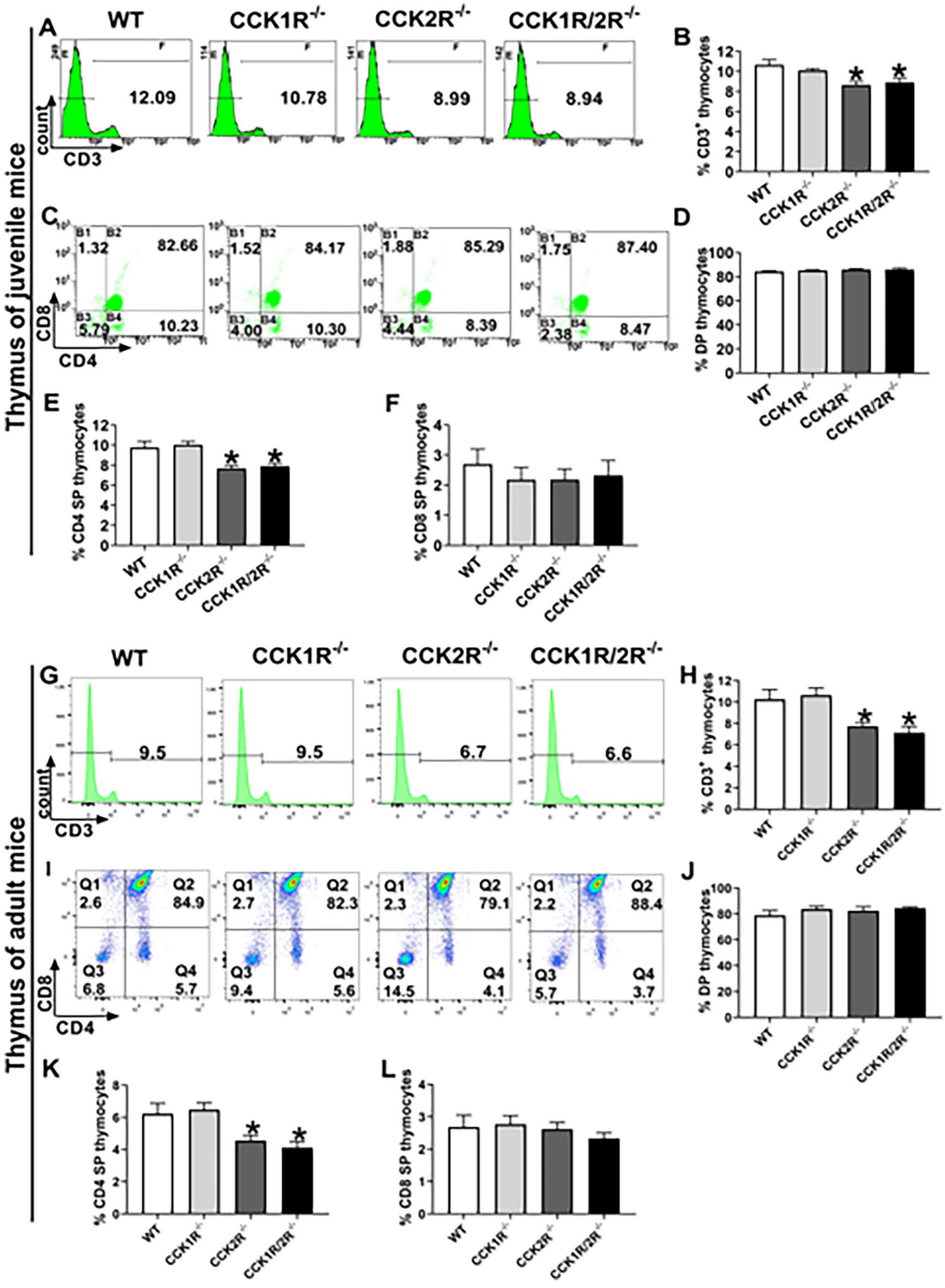

Figure 5. CCK2R deficiency influenced CD4$^+$ T cells development in the thymus in juvenile and adult mice (male; juvenile, 5–6 weeks, when the thymus is at its maximum size during puberty; adult, 10–24 weeks). The thymuses were collected and mechanically disrupted to obtain the cell suspensions for FCM. (A to F) Representative profiles and the proportion of CD3$^+$ thymocytes, DP thymocytes, CD4 SP thymocytes, and CD8 SP thymocytes in different groups of juvenile mice. (G to L) Representative profiles and proportions of CD3$^+$ thymocytes, DP thymocytes, CD4 SP thymocytes, and CD8 SP thymocytes in different groups of adult mice. $n = 5$ for each group. Data are expressed as the means ± SEM. *$P < 0.05$ *vs* WT group.

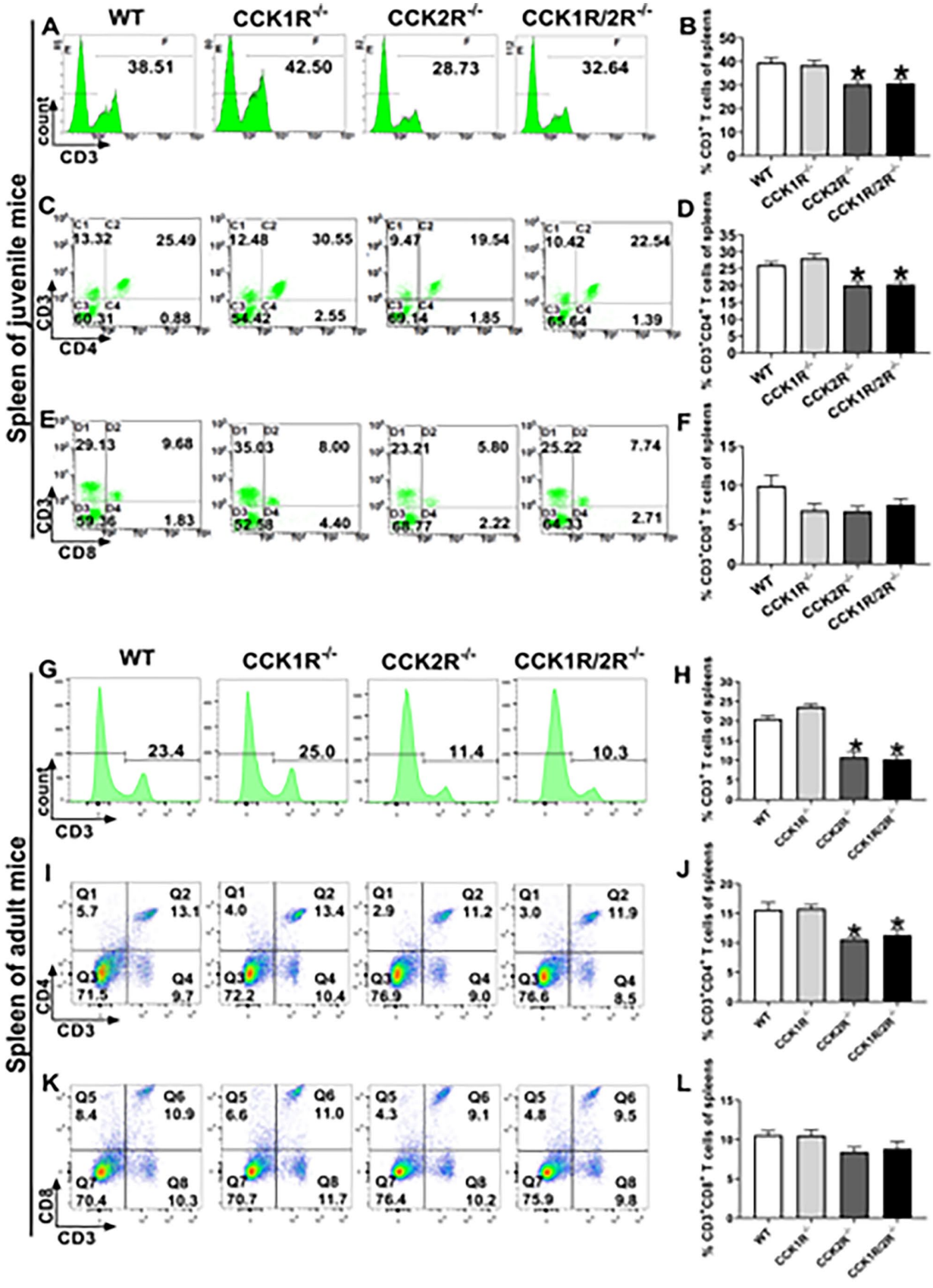

Figure 6. CCK2R deficiency reduced the population of $CD3^+$ $CD4^+$ T cells in the spleen in juvenile and adult mice (male; juvenile, 5–6 weeks, when the thymus is at its maximum size during puberty; adult, 10–24 weeks). The spleen, as an important peripheral immune organ, was collected and mechanically disrupted to obtain the cell suspensions for FCM. (A to F) Representative profiles and the proportion of $CD3^+$ T cells, $CD3^+$ $CD4^+$ T cells, and $CD3^+$ $CD8^+$ T cells of spleen in different groups of juvenile mice. (G to L) Representative profiles and proportions of $CD3^+$ T cells, $CD3^+$ $CD4^+$ T cells, and $CD3^+$ $CD8^+$ T cells in the spleen in different groups of adult mice. $n = 5$ for each group. Data are expressed as the means ± SEM. $^*P < 0.05$ *vs* WT group.

expression of CD83 on cTECs. To further clarify the factor by which CCK2R deficiency impaired $CD4^+$ T cell development, the proportions of cTECs and the expression levels of MHC II and CD83 on cTECs were evaluated. In juvenile mice, the proportions of cTECs and the expression levels of MHC II and CD83 on cTECs were all reduced in mice with CCK2R and CCK1R/2R deficiency compared with WT mice. There was no significant difference in $CCK1R^{-/-}$ mice (Figure 7(A) to (F)). As expected, the same phenomenon was observed in adult mice with CCK1R, CCK2R, and CCK1R/2R deficiency (Figure 7(G) to (L)). Collectively, these findings demonstrate that CCK2R deficiency impaired the microenvironment associated with $CD4^+$ T cell development, especially in the positive selection of CD4 SP thymocytes (Figure 8).

Discussion

The endocrine system is one of the most potent tools available to the brain, allowing it to regulate a myriad of physiological processes, also including the immune system. In this study, we first assessed the effect of CCKR deficiency on immune balance in $CCK1R^{-/-}$, $CCK2R^{-/-}$, and $CCK1/2R^{-/-}$ adult mice by LPS-induced ETM/ES model. Deficiencies of different CCKR had no effect on MAP or HR in the basal state with no damage. Then, the ES/ETM mouse model was established. We found that in both the ETM and ES models, mice with CCK2R deficiency but not CCK1R deficiency had an increased risk of shock and even death. These data were consistent with the previous results of intravenous CCKR blockers in rats with LPS-induced ETM/ES.[8] ETM and ES is a common complication of combat injuries and trauma and is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection. It is also one of the significant causes of death and increased health care costs in modern intensive care units. Disruption of immune homeostasis is now recognized as one of the major causes of death, which particularly in older patients and those who are immunocompromised.[28,29] Evidence of immunocompromise includes the failure of immune cells, particularly impaired T cells.[30] Our previous research indicated that CCK-8 has anti-inflammatory and immunomodulatory effects, which are mainly mediated by CCK2R.[8–13] Especially in regulating immune cell balance, our findings showed that selective antagonists of CCK2R could suppress the effects of CCK-8 on $CD4^+$ T cell subset-specific transcription factors so as to disrupt the Th1/Th2 cell and Th17/Treg cell balance from mice spleens.[12,13] Therefore, we inferred that CCK2R deficiency impaired immune balance and resulted in immunocompromised in mice.

The thymus is the main site for T cell development and maturation. Reduced T cell generation in the thymus, due to normal aging or pathological factors (such as chronic inflammation), could lead to imbalance in peripheral T cells and increased susceptibility to infection.[31] Research has shown that the organ index of the spleen is increased in CCK2R-deficient mice, while that of the thymus was decreased.[32] Given this information, is the immunocompromised state of CCK2R-deficient mice associated with thymus atrophy and spleen hyperplasia? Is their immunocompromised state associated with disordered T cell development? Considering that the thymus begins to atrophy with aging after sexual maturity, most researchers have selected juvenile mice (5–6 weeks), in which the thymus is at its largest size and highest activity, for studies of the thymus.[33] However, in our study, to ensure the success of the operation on ETM/ES mice, adult mice (10–24 weeks) were selected in the first part of the experiment. Therefore, in subsequent experiments, we chose to use both juvenile and adult mice with CCKR deficiency for further research without any external intervention.

First, the organ indices and histomorphology of the thymus and spleen were observed and recorded. We found that in both juvenile and adult mice with CCK2R deficiency but not CCK1R deficiency, the organ index of thymus was significantly decreased, while that of the spleen was increased, which was consistent with the literature.[32] Moreover, H&E staining showed thinning of the thymus cortex, irregular morphology of the white pulp, loss of lymphocytes, and intercellular enlargement in the central area of the spleen. These results suggested that CCK2R deficiency could cause typical atrophy in the thymus and compensatory hyperplasia in the spleen, which was considered as inhibited the immune system. In addition, this may be related to inhibited thymus T cell output to the spleen. However, the effect of CCK2R deficiency on T cells in the thymus is unknown.

Subsequently, we evaluated the T cells proportions of the thymus in both juvenile and adult mice. The results showed that the proportions of $CD3^+$ thymocytes and CD4 SP thymocytes but not the proportions of DP thymocytes or CD8 SP thymocytes were reduced in mice with CCK2R and CCK1R/2R deficiency. The reduction in $CD3^+$ thymocytes might be associated with the reduction in CD4 SP thymocytes. During the positive selection process in the thymus cortex, DP thymocytes differentiate into SP thymocytes: CD8 expression increased in DP thymocytes bound to MHC I, and these cells differentiated into CD8 SP thymocytes; CD4 expression increased in DP thymocytes bound to MHC II increased, and these cells and differentiated into CD4 SP thymocytes. Our results suggested that CCK2R deficiency might interfere with the positive selection process in the thymus cortex (consistent with cortical thinning shown by H&E staining), especially impairing CD4 SP thymocytes. However, the effect of CCK2R deficiency on T cells in the spleen is unknown.

The spleen, which is the largest peripheral immune organ, plays an important role in the pathophysiology of ETM/ES.[34] In our study, mice of both age groups showed the same trend: the proportions of $CD3^+$ T cells and $CD3^+$ $CD4^+$ T cells but not $CD3^+$ $CD8^+$ T cells in the spleen were decreased in mice with CCK2R and CCK1R/2R deficiency. These results were consistent with the changes in the thymus and the histomorphology observed in the spleen by H&E-staining. We inferred that in both juvenile and adult mice, the decrease in $CD3^+$ T cells and $CD3^+$ $CD4^+$ T cells in the spleen was related to the decrease in $CD3^+$ thymocytes and CD4 SP thymocytes from thymus output, which was immune imbalance.

Normal function of the thymus is essential for reducing mortality caused by a variety of immune-related clinical diseases, such as infection.[35] Therefore, CCK2R deficiency adult mice with immunocompromised in the ETM/ES model

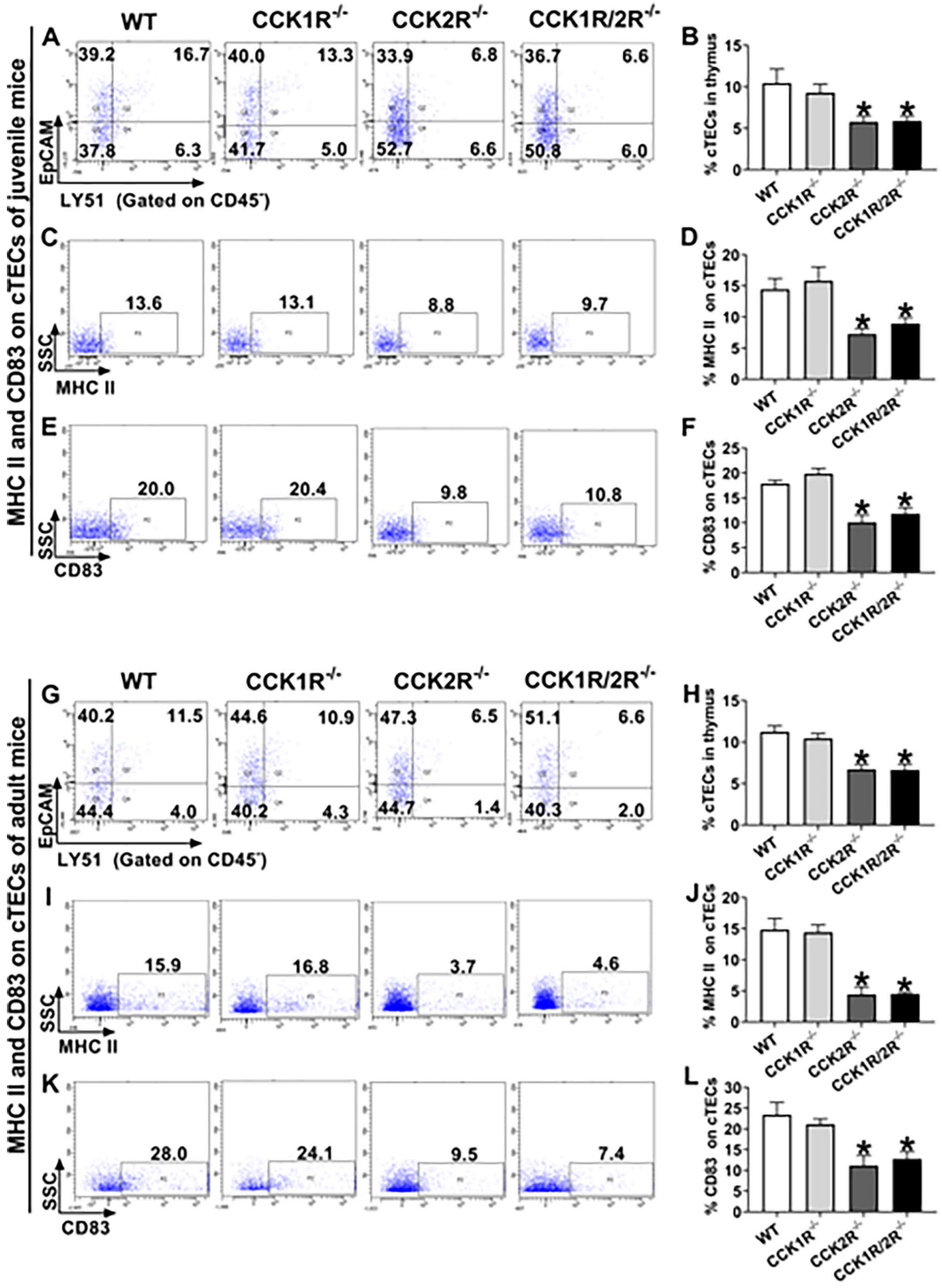

Figure 7. CCK2R deficiency impaired cTECs and inhibited the expression of MHC II and CD83 on cTECs in juvenile and adult mice (male; juvenile, 5–6 weeks, when the thymus is at its maximum size during puberty; adult, 10–24 weeks). The thymuses were mechanically disrupted and digested with DNase I and collagenase type IV. Anti-CD45 immuno-magnetic beads were used to enrich TECs in the cell suspension. cTECs ($CD45^-$ $EpCAM^+$ $LY51^+$) were sorted by FCM, and the expression of MHC II and CD83 was also analyed by FCM. (A to F) Representative profiles and proportion of cTECs, the expression of MHC II and CD83 on cTECs in different groups of juvenile mice. (G to L) Representative profiles and proportions of cTECs, the expression of MHC II and CD83 on cTECs in different groups of adult mice. $n = 5$ for each group. Data were expressed as the means ± SEM. $^*P < 0.05$ *vs* WT group.

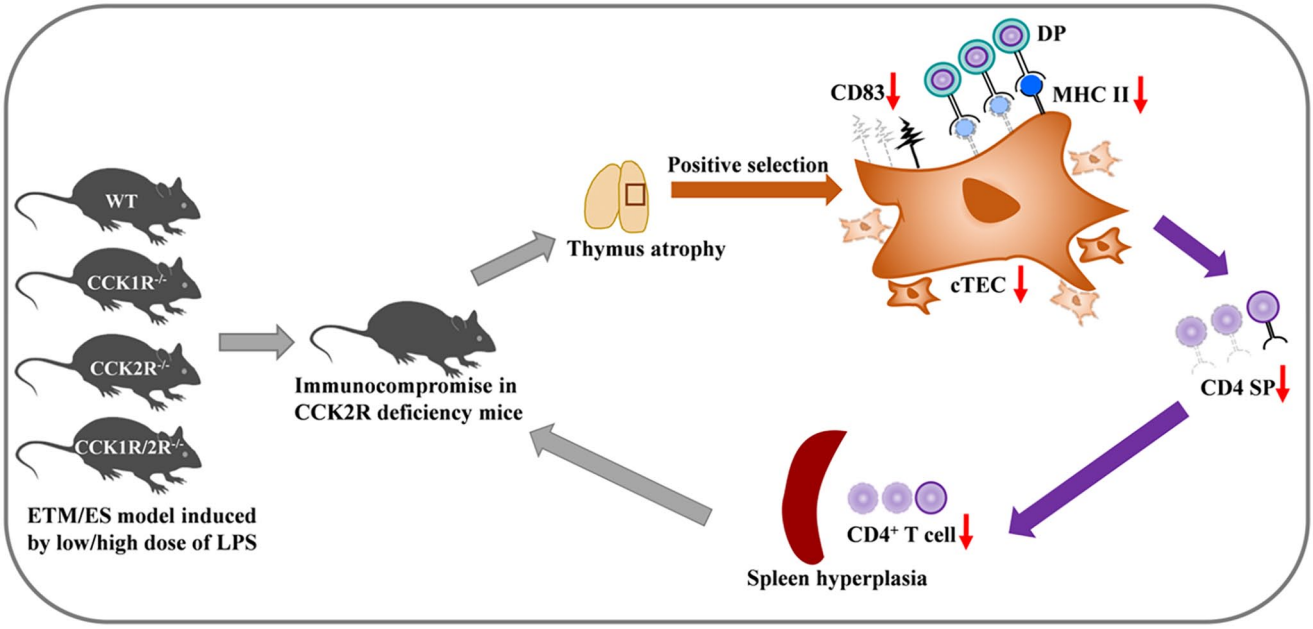

Figure 8. Graphical abstract. CCK2R deficiency not only leads to disordered development of CD4 SP thymocytes by inhibiting MHC II and CD83 expression on cTECs but also causes the population of $CD4^+$ T cells to decrease in the spleen, resulting in immunocompromised mice. This finding indicated that CCK2R plays an irreplaceable role in maintaining immune balance.

might be related to disordered T cell development in the thymus. Our previous studies showed that CCK has various immunomodulatory effects mostly mediated by CCK2R,[12,13] which suggested that CCK2R plays an important role in maintaining the development of T cells, as well as immune balance. The effect of CCK2R deficiency on $CD4^+$ T cells in the thymus and spleen may be one of the mechanisms related to more prone to shock or death in CCK2R-deficient mice with ETM/ES. However, what causes CD4 SP thymocytes development disorder in CCK2R-deficient mice? Is it related to the cTECs in CCK2R-deficient mice during thymic positive selection?

Thymic epithelial cells (TECs) play an important role in T cells maturation. In particular, positive selection in the thymus is associated with cTECs. During positive selection, MHC II expressed on cTECs plays a decisive role in the differentiation of CD4 SP thymocytes into $CD4^+$ T cells.[36] Moreover, CD83, as one of the factors maintaining the stability of MHC II, plays an important role in the positive selection of CD4 SP thymocytes.[37] Research has shown that CD4 SP thymocytes but not DP thymocytes and $CD8^+$ SP thymocytes are inhibited and peripheral $CD3^+$ $CD4^+$ T cells are decreased in $CD83^{-/-}$ mice.[38] Therefore, we detected these indices to assess the effect of CCKR deficiency on CD4 SP thymocytes during positive selection. We found that CCK2R deficiency resulted in reduced the proportions of cTECs and inhibited the expression of MHC II and CD83 on cTECs in both juvenile and adult mice. The changes in the proportions of cTECs were consistent with the histomorphology of the thymus in $CCK2R^{-/-}$ and $CCK1R/2R^{-/-}$ mice. Our previous studies have shown that CCK2R knockdown can regulate the expression of MHC II on DCs and affect the proliferation of $CD4^+$ T cells.[10,11,13] The effect of CCK2R deficiency on cTECs might be one of the mechanisms leading to disordered development of CD4 SP thymocytes. Thus, we inferred that CCK2R was essential for the development of the thymus in mice, especially for CD4 SP thymocytes in positive selection.

Specifically, according to the results from mice at two age stages, we also deduced that the effect of CCK2R deficiency on immune imbalance, especially the disordered development of $CD4^+$ T cells, was sustained from adolescence to adulthood. More importantly, there was no effective compensatory mechanism in mice. Clinical research shows evidence that the clinical features of immunocompromised patients include the failure of immune cells, particularly T cells.[30] Reconstruction of the thymus microenvironment for T cells regeneration as a new clinical strategy has been gradually studied.[18]

The nervous system and the immune system are both crucial for survival. Maintaining immune balance is inseparable from the regulation of the nervous system. The endocrine system is one of the most potent tools available to the brain, allowing it to regulate a myriad of physiological processes, including the immune system. Therefore, the nervous system, endocrine system, and immune system interact with each other to regulate the delicate balance of immune homeostasis within the body, their interaction could provide targets for future clinical therapies. The typical example of this interplay is that glucocorticoids inhibit the immune response during stress and are administered routinely as potent immunosuppressive drugs.[39] Indeed, studies have shown that the CCK-CCKR signaling system has various regulatory effects on the neuroendocrine system. For example, CCK can enhance glucocorticoid secretion through the CCK2-R-mediated stimulation of corticotropin-releasing hormone-dependent ACTH release.[40] In addition, as with ETM/ES, the mortality associated with COVID-19 is mainly the result of dysregulated immunopathology in response to

the virus, including the critical impact of polyfunctional $CD4^+$ and $CD8^+$ T cell responses, rather than organ injury due to viral replication itself.[41] There is an urgent need for drugs or combination regimens that on the race between viral replication and the elicitation of a productive and coordinated immune response, to maintain immune balance inside the body.[41] Therefore, given the expression of CCK2R in human lymphocytes,[42] interfering with the CCK-CCKR signaling pathway may provide new ideas for the treatment of immune imbalance diseases related to $CD4^+$ T cells in humans. Moreover, CCK2R is widely distributed in the central nervous system, and so it is unknown whether CCK2R deficiency causes changes in the secretion of other neuropeptides or hormones. Meanwhile, the thymus is an immune organ sensitive to a variety of neuropeptides and hormones, such as glucocorticoids and sex hormones. It is not clear whether the developmental disorder of the thymus T cells caused by CCK2R deficiency is related to changes in other neuropeptides or hormones. All these scientific questions will be among our future directions to explore the effects of the neuropeptide CCK on immune balance.

In summary, our research demonstrated that CCK2R deficiency not only leads to disordered development of CD4 SP thymocytes by inhibiting MHC II and CD83 expression on cTECs but also reduces the population of CD4+ T cells in the spleen, which results in immunocompromised mice. This finding indicates that CCK2R plays an irreplaceable role in maintaining immune balance.

AUTHORS' CONTRIBUTIONS

All authors participated in the design of the study, interpretation of the results, analysis of the data and review of the manuscript. BC conceived the study design. XJZ conducted the experiments and revised the manuscript. DZ performed most of the experiments and wrote the manuscript. MMJ analyzed data. CW, RFM, and GMX collected the samples. YML and CLM supervised the experiments. GYZ, DW, and XXJ provided technical support. All authors read and approved the final manuscript.

DECLARATION OF CONFLICTING INTERESTS

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SUPPLEMENTAL MATERIAL

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