Alterations in thymocyte populations under conditions of endotoxin tolerance

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Abstract

Background: Endotoxin tolerance (ET) is a protective phenomenon in which pre-treatment with a tolerance dose of lipopolysaccharide (LPS) leads to dramatically elevated survival. Accumulating evidence has shown that peripheral T cells contribute to the induction of ET. However, what happens to T cell development in the thymus under ET conditions remains unclear. The purpose of this study was to analyze the alterations in thymocyte populations (double-positive [DP] and single-positive [SP] cells) under ET conditions.

Methods: Mice were intraperitoneally injected with LPS at a concentration of 5 mg/kg to establish an LPS tolerance model and were divided into two groups: a group examined 72 h after LPS injection (72-h group) and a group examined 8 days after LPS injection (8-day group). Injection of phosphate-buffered saline was used as a control (control group). Changes in thymus weight, cell counts, and morphology were detected in the three groups. Moreover, surface molecules such as CD4, CD8, CD44, CD69, and CD62L were analyzed using flow cytometry. Furthermore, proliferation, apoptosis, cytokine production, and extracellular signal-regulated kinase (ERK) pathway signaling were analyzed in thymocyte populations. The polymorphism and length of the T-cell receptor (TCR) β chain complementarity-determining region 3 (CDR3) were analyzed using capillary electrophoresis DNA laser scanning analysis (ABI 3730).

Results: Thymus weight and cell counts were decreased in the early stage but recovered by the late stage in a murine model of LPS-induced ET. Moreover, the proportions of DP cells (control: 72.130 ± 4.074, 72-h: 10.600 ± 3.517, 8-day: 84.770 ± 2.228), $CD4^+$ SP cells (control: 15.770 ± 4.419, 72-h: 44.670 ± 3.089, 8-day: 6.367 ± 0.513), and $CD8^+$ SP cells (control: 7.000 ± 1.916, 72-h: 34.030 ± 3.850, 8-day: 5.133 ± 0.647) were obviously different at different stages of ET. The polymorphism and length of TCR β chain CDR3 also changed obviously, indicating the occurrence of TCR rearrangement and thymocyte diversification. Further analysis showed that the expression of surface molecules, including CD44, CD69, and CD62L, on thymocyte populations (DP and SP cells) were changed to different degrees. Finally, the proliferation, apoptosis, cytokine production, and ERK pathway signaling of thymocyte populations were changed significantly.

Conclusion: These data reveal that alterations in thymocyte populations might contribute to the establishment of ET.

Keywords: Immune tolerance; Thymocytes; T-cell receptor; Gene rearrangement; Adaptive immunity

Introduction

Endotoxin tolerance (ET) is an important protective mechanism against infection by Gram-negative bacteria. Pre-treatment with a tolerance dose of lipopolysaccharide (LPS) is important for the induction of ET in response to challenge with high levels of LPS.[1,2] The relevant mechanisms, which involve multi-layer regulatory processes of the immune system, are very complex. Thus far, the innate immune response has been widely implicated in the induction of ET.[3-6] In fact, recent studies have documented that adaptive immune cells, such as T cells, contribute to the induction of ET. T cell depletion mediates decreased sensitivity to LPS in transforming growth factor $\beta1^{-/-}$ mice, which prolongs the survival of mice with several types of autoimmune diseases during the lactation period.[7] In addition, in the absence of functional regulatory T cells (Tregs), $CD4^+$ T cells are pathological cells and contribute to exaggerated immune activation in response to LPS challenge, which impairs the induction of ET.[8] Moreover, the proliferation and inflammatory cytokine secretion of autologous $CD4^+$ T cells induced by macrophages are decreased in the context of ET.[9] These findings demonstrate an important role for T cells in ET induction.

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T cell development is closely related to the formation of central tolerance. In the thymus, T cell development is divided into three stages: double-negative (DN), double-positive (DP), and single-positive (SP). T cells undergo positive selection and negative selection, which require the establishment of central tolerance and migrate into peripheral immune organs and tissues. During the transition from the DP stage to the SP stage, T-cell receptor (*TCR*) gene rearrangement produces some autoreactive TCRs; these TCRs can interact with antigen-presenting cells presenting the high-affinity autoantigen to start the apoptosis program, resulting in clonal deletion of T cells.[10] Interestingly, accumulating evidence has shown that T cell development in the thymus also contributes to the development of various diseases.[11-16] For example, the proportion of thymocytes is abnormal under conditions of *Leishmania* infection combined with malnutrition.[11] In addition, the migration of T cells to peripheral immune organs increases during acute infection with *Trypanosoma cruzi*, which affects thymocyte development.[12] Recently, a study has reported that a reduction in thymic emigrants contributes to the development of coronary heart disease, which may be related to the destruction of immune tolerance caused by T cell confusion and thymus degeneration.[13] These findings raise an interesting question: whether T cell development in the thymus changes under conditions of ET. The biological value of such a change remains largely unknown.

In the present study, we investigated the change in T cell development that occurs during dynamic programming under ET conditions.

Methods

Ethics

All animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals, and were used according to the ethical guidelines of the Zunyi Medical University Laboratory Animal Care and Use Committee (No. 2013016).

Mice and model

Female C57BL/6 mice at 7 to 10 weeks of age were raised in a specific-pathogen-free laboratory at the Biomedical Center of Zunyi Medical University or purchased from Beijing Charles River Experimental Animal Technology Co., Ltd (Beijing, China). The mice were intraperitoneally injected with LPS at a concentration of 5 mg/kg to establish an LPS tolerance model, and two groups were established: a group examined 72 h after LPS injection (72-h group) and a group examined 8 days after LPS injection (8-day group). Phosphate-buffered saline (PBS) was injected for the control group. Thymus tissue was extracted from all mice at the indicated times.

Preparation of single-cell suspensions

The thymus tissues removed from the mice were perfused with PBS, washed, and weighed. Single-cell suspensions were prepared from the tissues via grinding and filtering through a 200-mesh sterile cell strainer, and the whole-cell suspensions were centrifuged at 300 × *g* for 10 min. The supernatant was discarded, and the cells were resuspended in 10 mL of PBS for the next experimental protocol.

Histopathology

Thymus tissues were fixed with 4% formaldehyde, embedded in paraffin, and sectioned into 4-μm-thick slices. The dehydrated slices were dewaxed with xylene, ethanol, and water in sequence. Then, the sections were transferred to hematoxylin for 5 to 10 min and washed under tap water followed by distilled water. Next, the sections were stained with eosin solution for 30 s to 5 min after transfer to ethanol for 5 s. The sections were dehydrated and cleared after washing with 70% ethanol. Finally, the slices were sealed with neutral gum. Images were obtained with an Olympus IX-71 microscope (Beijing, China) at original magnifications of 40×, 100×, 200×, and 400× for each sample.

Flow cytometry (FCM)

A total of 10^6 to 10^7 single cells in suspension were collected and filtered. Then, 3 mL of red blood cell lysate was added, and the cells were incubated for 10 min. After two washes with PBS, the cells were incubated with peridinin chlorophyll protein-Cyanine 5.5 (PerCP-Cy5.5)-conjugated anti-mouse CD4, allophycocyanin-conjugated anti-mouse CD8, allophycocyanin-conjugated anti-mouse CD44, allophycocyanin-conjugated anti-mouse CD69, and R-phycoerythrin-conjugated anti-mouse CD62L antibodies for 30 min at 4°C in the dark. For intracellular staining, after membrane molecule staining was performed, the cells were fixed with 100 μL of eBioscience intracellular (IC) fixation buffer for 30 min after being washed twice with PBS. Next, the cells were incubated with 300 μL of eBioscience permeabilization buffer and washed once with PBS. Finally, the cells were incubated with allophycocyanin-conjugated anti-mouse IL-4, PerCP-Cy5.5-conjugated anti-mouse interferon-γ (IFN-γ), and allophycocyanin-conjugated anti-mouse Ki-67 antibodies for 30 min at 4°C in the dark. For the apoptosis assay, the cells were subjected to membrane molecule and Annexin V staining, fixation, and lysis. Propidium iodide dye solution was then added. An extracellular signal-regulated kinase (ERK) and phosphorylated ERK (p-ERK) staining were then performed. Briefly, 10^6 to 10^7 single cells in suspension were collected, filtered, fixed with 100 μL of eBioscience IC fixation buffer for 10 min, and then washed with PBS. The cells were permeabilized with 300 μL of eBioscience buffer for 30 min and then incubated with antibodies against ERK and p-ERK for 30 min at 22°C after two washes with PBS. The secondary antibody used was Alexa Fluor® 488-conjugated goat anti-mouse IgG (H^+L) at a 1/500 dilution for 30 min at 22°C. Finally, all of the stained cells were evaluated by FCM.

Capillary electrophoresis DNA laser scanning analysis

According to the international immunogene tics database and GenBank databases, 22 upstream primers for the mouse T cell receptor beta variables (*TRBV)* gene family and one T cell receptor beta chain joint gene 1 common

downstream primer with 5-Carboxy Fluorescein were designed and synthesized by Invitrogen Biotechnology Company (Shanghai, China). The polymerase chain reaction products were subjected to capillary electrophoresis DNA laser scanning analysis (ABI 3730, Thermo Fisher Scientific, USA, MA). The length and polymorphism of the complementarity-determining region 3 (CDR3) were analyzed with GeneMapper 4.1 software (Thermo Fisher Scientific, MA, USA).

Statistical analysis

All values are presented as the mean ± standard deviation from at least three independent experiments. GraphPad Prism 8.0.1 (GraphPad Company, CA, USA) was used to compare and evaluate data among groups. Differences between the two groups were analyzed by Student's *t* test. *P* values < 0.0500 were considered to indicate significance.

Results

The thymus is altered under conditions of LPS tolerance

To examine the alterations in thymic weight, cell counts, and morphology under conditions of ET, we established a murine LPS-induced tolerance model according to the methods in previous work [Figure 1A].[17] A high dose of LPS was used to confirm the successful establishment of the tolerance model [Supplementary Figure 1, http://links.lww.com/CM9/A635]. All mice were monitored for body weight changes. We found that the experimental mice gradually lost weight over 1 to 3 days but that the weights returned to normal levels on the fifth day after LPS injection [Figure 1B]. Then, we examined the thymus weight and cell counts. As shown in Figure 1C and 1D, the weight (control *vs.* 72-h, 34.000 ± 4.899 *vs.* 16.000 ± 3.795, $t = 7.115$, $P < 0.0100$) and total cell numbers (control *vs.* 72-h, 37.220 ± 8.205 *vs.* 6.030 ± 1.514, $t = 9.156$, $P < 0.0100$) of the thymus were significantly lower in the 72-h group than in the control group, consistent with previous reports.[18,19] However, the LPS-induced decreases in weight (control *vs.* 8-day, 34.000 ± 4.899 *vs.* 24.000 ± 3.795, $t = 3.953$, $P < 0.0100$) and total cell numbers (control *vs.* 8-day, 37.220 ± 8.205 *vs.* 22.330 ± 7.941, $t = 3.193$, $P < 0.0100$) of the thymus were attenuated in the 8-day group, although the levels were still lower than those in the control group. Hematoxylin and eosin staining data showed that the boundary of the thymic cortex and medulla was remarkably less clear in the 72-h group than in the control group but that the clarity was recovered in the 8-day group. In addition to a clarification of the boundary of the cortex and medulla, increased numbers of lymphocytes were observed in the 8-day group. In addition, the cortex was scattered in a ring-shaped Hassall body [Figure 1E].

Thymocyte populations are altered under conditions of LPS tolerance

Then, we further analyzed the changes in thymocytes under LPS tolerance conditions. As shown in Figure 2A and 2B, compared with that in the control group, the proportion of DP cells was significantly lower in the 72-h group (control *vs.* 72-h, 72.130 ± 4.074 *vs.* 10.600 ± 3.517, $t = 17.490$, $P < 0.0100$) but higher in the 8-day group (control *vs.* 8-day, 72.130 ± 4.074 *vs.* 84.770 ± 2.228, $t = 4.015$, $P < 0.0500$). The proportions of $CD4^+$ SP cells (control *vs.* 72-h, 15.770 ± 4.419 *vs.* 44.670 ± 3.089, $t = 9.285$, $P < 0.0100$) and $CD8^+$ SP cells (control *vs.* 72-h, 7.000 ± 1.916 *vs.* 34.030 ± 3.850, $t = 10.890$, $P < 0.0100$) were increased in the 72-h group and significantly decreased in the 8-day group. Compared with that in the 72-h group, the proportion of DP cells was significantly higher in the 8-day group (72-h *vs.* 8-day, 10.600 ± 3.517 *vs.* 84.770 ± 2.228, $t = 30.860$, $P < 0.0100$). The proportions of $CD4^+$ SP cells (72-h *vs.* 8-day, 44.670 ± 3.089 *vs.* 6.367 ± 0.513, $t = 21.180$, $P < 0.0100$) and $CD8^+$ SP cells (72-h *vs.* 8-day, 34.030 ± 3.850 *vs.* 5.133 ± 0.643, $t = 12.820$, $P < 0.0100$) were significantly decreased. Moreover, compared with that in the control group, the absolute number of DP cells in the 72-h group was significantly lower (control *vs.* 72-h, 30.030 ± 7.146 *vs.* 0.620 ± 0.200, $t = 7.126$, $P < 0.0100$), while that in the 8-day group was higher (control *vs.* 8-day, 30.030 ± 7.146 *vs.* 18.900 ± 0.497, $t = 2.691$, $P = 0.0546$). The absolute numbers of $CD4^+$ SP cells (control *vs.* 72-h, 5.820 ± 1.606 *vs.* 2.800 ± 0.100, $t = 3.251$, $P < 0.0500$) and $CD8^+$ SP cells (control *vs.* 72-h, 2.600 ± 0.716 *vs.* 2.047 ± 0.245, $t = 1.267$, $P = 0.2740$) were reduced in the 72-h group and reduced further in the 8-day group. Compared with that in the 72-h group, the absolute number of DP cells in the 8-day group was significantly higher (72-h *vs.* 8-day, 0.620 ± 0.200 *vs.* 18.900 ± 0.497, $t = 59.040$, $P < 0.0100$), while the absolute numbers of $CD4^+$ SP cells (72-h *vs.* 8-day, 2.800 ± 0.100 *vs.* 1.423 ± 0.112, $t = 15.850$, $P < 0.0100$) and $CD8^+$ SP cells (72-h *vs.* 8-day, 2.047 ± 0.245 *vs.* 1.150 ± 0.132, $t = 5.578$, $P < 0.0100$) were still lower [Figure 2C].

Polymorphism and length analysis of the TCR β chain CDR3 in the thymus under LPS tolerance conditions

Next, we examined the polymorphism and length of the TCR β chain CDR3. TCRs are T cell surface antigen receptors. Diverse TCRs are involved in the recognition of different antigens by T cells. The TCR CDR3 is the most diverse region, and its length and sequence determine the specificity of the TCR. As shown in Figure 3A, in the control group, the majority of TRBV families of the CDR3 showed Gaussian peaks, while others presented partial-, oligo-, and low-peaks. However, in the 72-h group, there were different numbers of partial-, oligo-, and low-peaks. In the 8-day group, most TRBV families presented multi-peaks and Gaussian peaks. The polymorphism was quantified by the peak area value. Compared with those in the control group, the peak area values of the majority of TRBV families of the CDR3 were lower in the 72-h group but higher in the 8-day group [Figure 3B]. In addition, as shown in Supplementary Table 1, http://links.lww.com/CM9/A635, the length span of the CDR3 became smaller at 72 h after LPS injection but longer at 8 days after LPS injection, reaching 5 to 6 amino acids in length. These data suggested that the polymorphism and length of the TCR β chain CDR3 undergo dynamic programming under conditions of LPS tolerance.

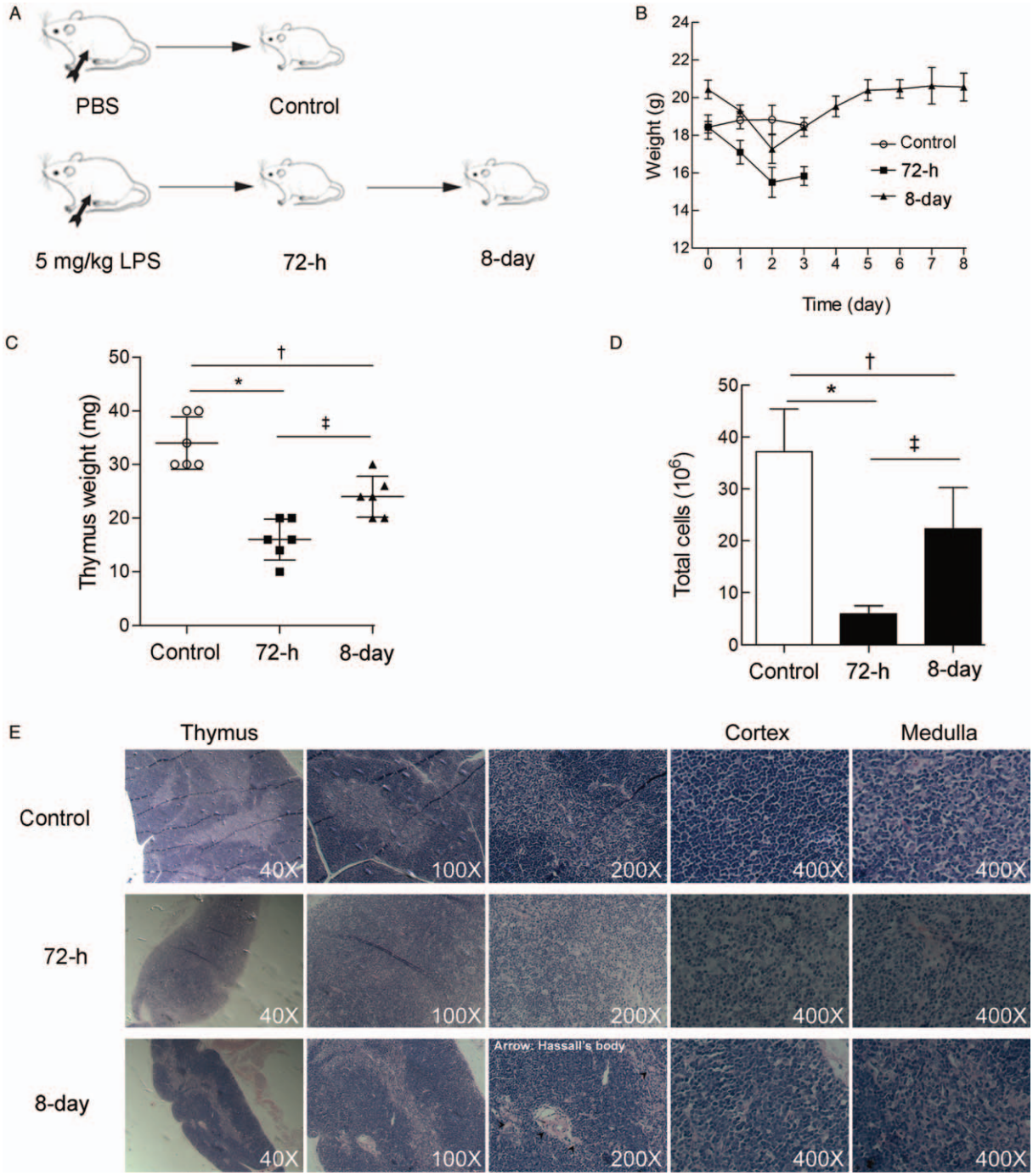

Figure 1: Change in thymic weight, total cell counts, and morphology in a murine LPS-induced tolerance model. C57BL/6 mice (7–10 weeks, $n = 6$) were intraperitoneally injected with 5 mg/kg LPS to establish a tolerance model. (A) Schematic diagram of the establishment of the ET model. (B) Daily changes in body weight in each group. (C) At the indicated time points, thymus tissues were collected. The weight and (D) total cell counts of the thymus were obtained. *Control compared with 72-h, †Control compared with 8-day, ‡72-h compared with 8-day. (E) Thymus sections were stained with hematoxylin-eosin. The data are shown as mean ± SD from three independent experiments. 72-h: Group examined 72 h after LPS injection; 8-day: Group examined 8 days after LPS injection; Control: PBS injection group; ET: Endotoxin tolerance; H&E: Hematoxylin and eosin; LPS: Lipopolysaccharide; PBS: Phosphate-buffered saline; SD: Standard deviation.

Apoptosis, proliferation, and activation of thymocytes under LPS tolerance

Since the proportion and an absolute number of thymocyte populations changed, we further analyzed thymocyte apoptosis and proliferation under LPS tolerance conditions. As shown in Figure 4A and 4B, compared with those in the control group, the proportions of apoptotic $CD4^+$ SP cells (control *vs.* 72-h, 3.000 ± 0.400 *vs.* 1.700 ± 0.100, $t = 5.461$, $P < 0.0100$) and $CD8^+$ SP cells (control *vs.* 72-h, 3.967 ± 0.751 *vs.* 1.067 ± 0.603, $t = 5.218$, $P < 0.0100$) were lower in the 72-h group. The proportion of apoptotic

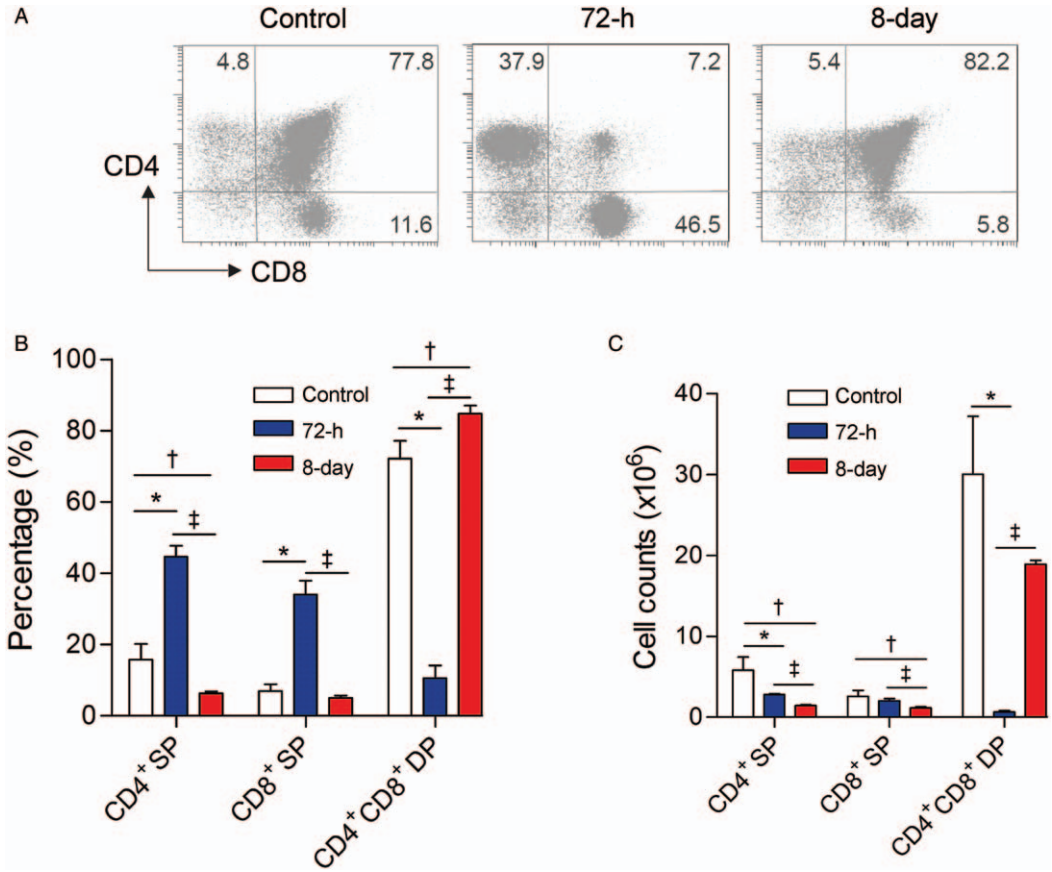

Figure 2: Changes in the proportions and cell counts of $CD4^+$ SP, $CD8^+$ SP, and $CD4^+CD8^+$ DP cells in a murine LPS-induced tolerance model. C57BL/6 mice (7–10 weeks, $n = 6$) were intraperitoneally injected with 5 mg/kg LPS to establish a tolerance model. (A) The proportions of $CD4^+$ SP, $CD8^+$ SP, and $CD4^+CD8^+$ DP cells were analyzed by FCM. (B) The proportions and (C) cell counts of $CD4^+$ SP, $CD8^+$ SP, and $CD4^+CD8^+$ DP cells were calculated. *Control compared with 72-h, †Control compared with 8-day, ‡72-h compared with 8-day. The data are shown as the mean ± SD from three independent experiments. 72-h: Group examined 72 h after LPS injection; 8-day: Group examined 8 days after LPS injection; Control: PBS injection group; DP: Double-positive; FCM: Flow cytometry; LPS: Lipopolysaccharide; SD: Standard deviation; SP: Single-positive.

$CD4^+$ SP cells showed no significant change in the 8-day group, but the proportion of apoptotic $CD8^+$ SP cells was increased (control *vs.* 8-day group, 3.967 ± 0.751 *vs.* 8.900 ± 0.700, $t = 8.326$, $P < 0.0100$), and the proportion of apoptotic $CD4^+CD8^+$ DP cells was decreased (control *vs.* 8-day, 1.800 ± 0.200 *vs.* 0.500 ± 0.200, $t = 7.961$, $P < 0.0100$). Compared with those in the 72-h group, the proportion of apoptotic DP cells in the 8-day group was lower (72-h *vs.* 8-day, 7.200 ± 3.600 *vs.* 0.500 ± 0.200, $t = 3.219$, $P < 0.0500$), while the proportions of apoptotic $CD4^+$ SP cells (72-h *vs.* 8-day, 1.700 ± 0.100 *vs.* 3.833 ± 0.503, $t = 7.201$, $P < 0.0100$) and $CD8^+$ SP cells (72-h *vs.* 8-day, 1.067 ± 0.603 *vs.* 8.900 ± 0.700, $t = 14.690$, $P < 0.0100$) were higher. In addition, compared with those in the control group, the proportions of proliferating $CD4^+$ SP cells (control *vs.* 72-h, 7.000 ± 1.900 *vs.* 2.333 ± 1.721, $t = 3.220$, $P < 0.0500$), $CD8^+$ SP cells (control *vs.* 72-h, 6.700 ± 0.100 *vs.* 2.600 ± 1.400, $t = 5.060$, $P < 0.0100$), and $CD4^+CD8^+$ DP cells (control *vs.* 72-h, 4.620 ± 0.951 *vs.* 2.283 ± 1.151, $t = 2.710$, $P = 0.0536$) were lower in the 72-h group and still lower in the 8-day group. Compared with the 72-h group, the 8-day group exhibited an unchanged proportion of proliferating SP cells but a lower proportion of proliferating DP cells (72-h *vs.* 8-day, 2.283 ± 1.151 *vs.* 0.233 ± 0.153, $t = 3.057$, $P < 0.0500$) [Figure 4C–E].

Next, we evaluated the proportions of cells expressing CD44, CD62L, and CD69 in the thymocyte subsets. Compared with the control group, the 72-h group exhibited higher proportions of $CD44^-$ (control *vs.* 72-h, 2.030 ± 0.851 *vs.* 13.000 ± 3.600, $t = 5.135$, $P < 0.0100$) and $CD69^-$ (control *vs.* 72-h, 6.300 ± 1.609 *vs.* 19.170 ± 5.450, $t = 3.922$, $P < 0.0500$) expressing DP cells, a lower proportion of CD69-expressing $CD4^+$ SP cells (control *vs.* 72-h, 48.670 ± 9.163 *vs.* 10.530 ± 0.153, $t = 7.207$, $P < 0.0100$) and a higher proportion of CD62L-expressing $CD4^+$ SP cells (control *vs.* 72-h, 52.300 ± 0.300 *vs.* 81.470 ± 1.361, $t = 36.240$, $P < 0.0100$). The proportions of CD69-expressing $CD4^+$ SP cells (control *vs.* 8-day, 48.670 ± 9.163 *vs.* 7.900 ± 2.524, $t = 7.429$, $P < 0.0100$) and CD69-expressing $CD8^+$ SP cells (control *vs.* 8-day, 13.100 ± 5.803 *vs.* 3.400 ± 0.656, $t = 2.877$, $P < 0.0500$) were decreased in the 8-day group. Furthermore, compared with the 72-h group, the 8-day group exhibited much lower proportions of CD44-expressing DP cells (72-h *vs.* 8-day, 13.000 ± 3.600 *vs.* 0.267 ± 0.058, $t = 6.126$, $P < 0.0100$), CD69-expressing DP cells (72-h *vs.* 8-day, 19.170 ± 5.450 *vs.* 0.500 ± 0.265, $t = 5.925$, $P < 0.0100$), and CD62L-expressing $CD4^+$ SP cells (72-h *vs.* 8-day, 81.470 ± 1.361 *vs.* 56.000 ± 2.800, $t = 14.170$, $P < 0.0100$). These results suggest that stimulation with a tolerance dose of LPS may

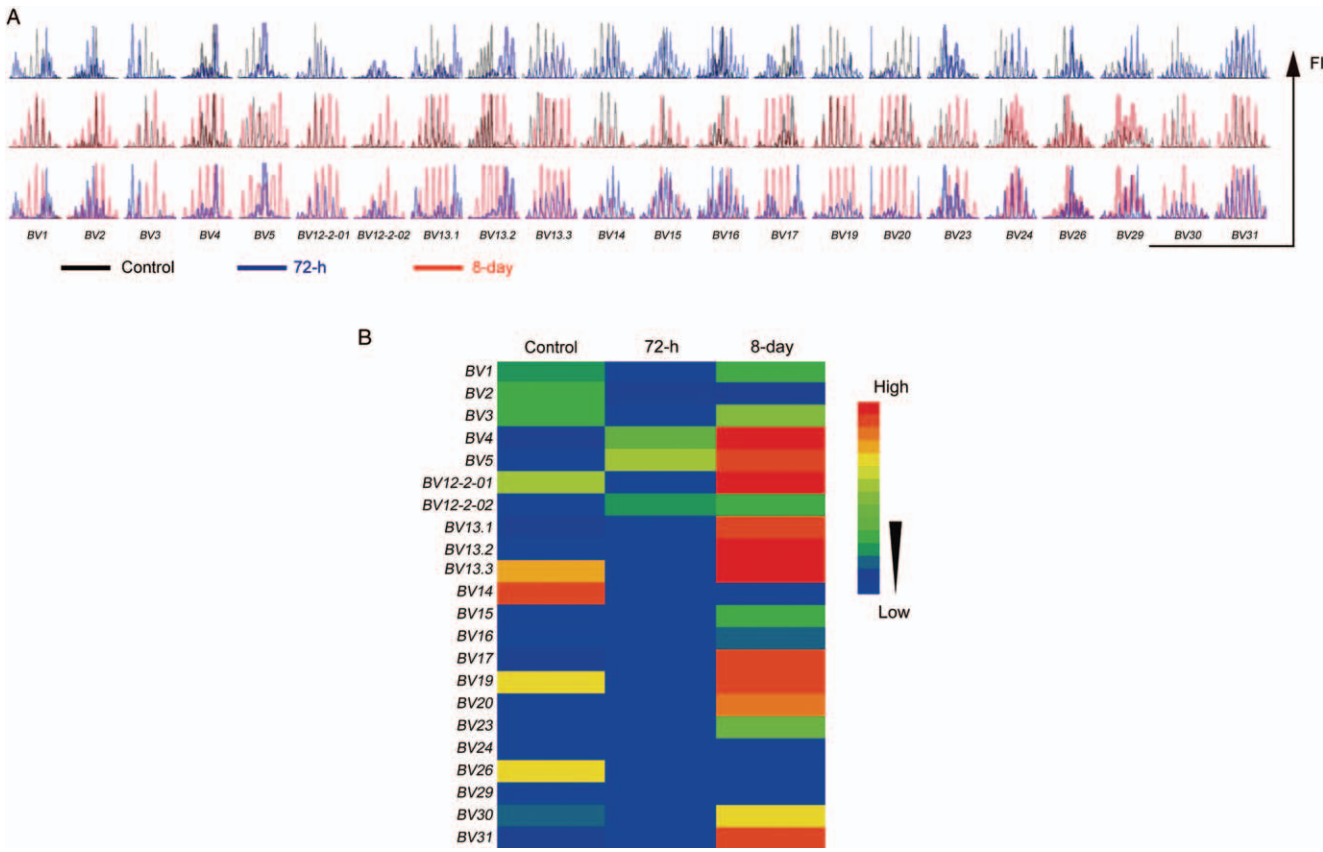

Figure 3: Changes in TCR β chain CDR3 polymorphism and length in a murine LPS-induced tolerance model. C57BL/6 mice (7–10 weeks, $n = 6$) were intraperitoneally injected with 5 mg/kg LPS to establish a tolerance model. (A) Polymorphism of the TCR β chain CDR3 was determined by capillary electrophoresis. (B) Heat map clustering. 72-h: Group examined 72 h after LPS injection; 8-day: Grouped examined 8 days after LPS injection; CDR3: Complementarity-determining region 3; Control: PBS injection group; FI: Fluorescence intensity; LPS: Lipopolysaccharide; SD: Standard deviation; TCR: T-cell receptor.

alter the development of DP cells, thereby indirectly regulating SP cell maturation status [Figure 4C and 4D, 4F–H].

Secretion of related cytokines and ERK pathway signaling are altered in thymocyte populations under LPS tolerance

We also observed the secretion of interleukin-4 (IL-4) and IFN-γ by three thymocyte populations. As shown in Figure 5A to 5C, compared with the control group, the 72-h group exhibited higher expression levels of IL-4 in DP cells (control *vs.* 72-h, 0.333 ± 0.153 *vs.* 1.967 ± 0.058, $t = 17.320$, $P < 0.0100$), $CD4^+$ SP cells (control *vs.* 72-h, 0.667 ± 0.208 *vs.* 1.650 ± 0.250, $t = 5.235$, $P < 0.0100$), and $CD8^+$ SP cells (control *vs.* 8-day, 0.733 ± 0.252 *vs.* 1.533 ± 0.351, $t = 3.207$, $P < 0.0500$). The expression of IL-4 in $CD4^+$ SP cells (control *vs.* 8-day, 0.667 ± 0.208 *vs.* 19.370 ± 2.967, $t = 10.890$, $P < 0.0100$), $CD8^+$ SP cells (control *vs.* 8-day, 0.733 ± 0.252 *vs.* 16.870 ± 0.451, $t = 54.110$, $P < 0.0100$), and $CD4^+CD8^+$ DP cells (control *vs.* 8-day, 0.333 ± 0.153 *vs.* 3.700 ± 0.819, $t = 7.003$, $P < 0.0100$) was significantly elevated in the 8-day group. Moreover, compared with the 72-h group, the 8-day group exhibited higher expression of IL-4 in DP cells (72-h *vs.* 8-day, 1.967 ± 0.058 *vs.* 3.700 ± 0.819, $t = 3.659$, $P < 0.0500$) and remarkably higher expression of IL-4 in $CD4^+$ SP cells (72-h *vs.* 8-day, 1.650 ± 0.250 *vs.* 19.370 ± 2.967, $t = 10.310$, $P < 0.0100$) and $CD8^+$ SP cells (72-h *vs.* 8-day, 1.533 ± 0.351 *vs.* 16.870 ± 0.451, $t = 46.470$, $P < 0.0100$). Compared with the control group, the 72-h group exhibited lower expression levels of IFN-γ in $CD4^+$ SP cells (control *vs.* 72-h, 1.700 ± 0.264 *vs.* 0.107 ± 0.011, $t = 10.420$, $P < 0.0100$). Compared with the 72-h group, the 8-day group exhibited lower expression of IFN-γ in DP cells (72-h *vs.* 8-day, 0.433 ± 0.058 *vs.* 0.267 ± 0.058, $t = 3.536$, $P < 0.0500$) but higher expression of IFN-γ in $CD4^+$ SP cells (72-h *vs.* 8-day, 0.107 ± 0.011 *vs.* 1.100 ± 0.400, $t = 4.299$, $P < 0.0500$) and $CD8^+$ SP cells (72-h *vs.* 8-day, 0.350 ± 0.150 *vs.* 1.867 ± 0.058, $t = 16.340$, $P < 0.0100$) [Figure 5A, 5B, and 5D].

ERK is a member of the mitogen-activated protein kinase family, and the related signaling pathway is key to the normal development, growth, and differentiation of cells.[20,21] A very large number of studies have shown that the ERK signaling pathway is heavily involved in the development and apoptosis of immune cells, including T cells.[22,23] It has been reported that knockout of B-Raf strongly disrupts differentiation of DP cells into SP cells and that this effect is associated with significant down-regulation of ERK signaling pathway activity. Thus, we preliminarily tested the expression of ERK and p-ERK in thymocyte populations by FCM. As shown in Figure 5E and 5F, compared with the control group, the 72-h group exhibited much higher proportions of ERK-expressing $CD4^+$ SP cells (control *vs.* 72-h, 0.700 ± 0.300 *vs.* 2.750 ± 0.150, $t = 10.590$, $P < 0.0100$) and $CD4^+$ $CD8^+$ DP cells (control *vs.* 72-h, 0.400 ± 0.100 *vs.*

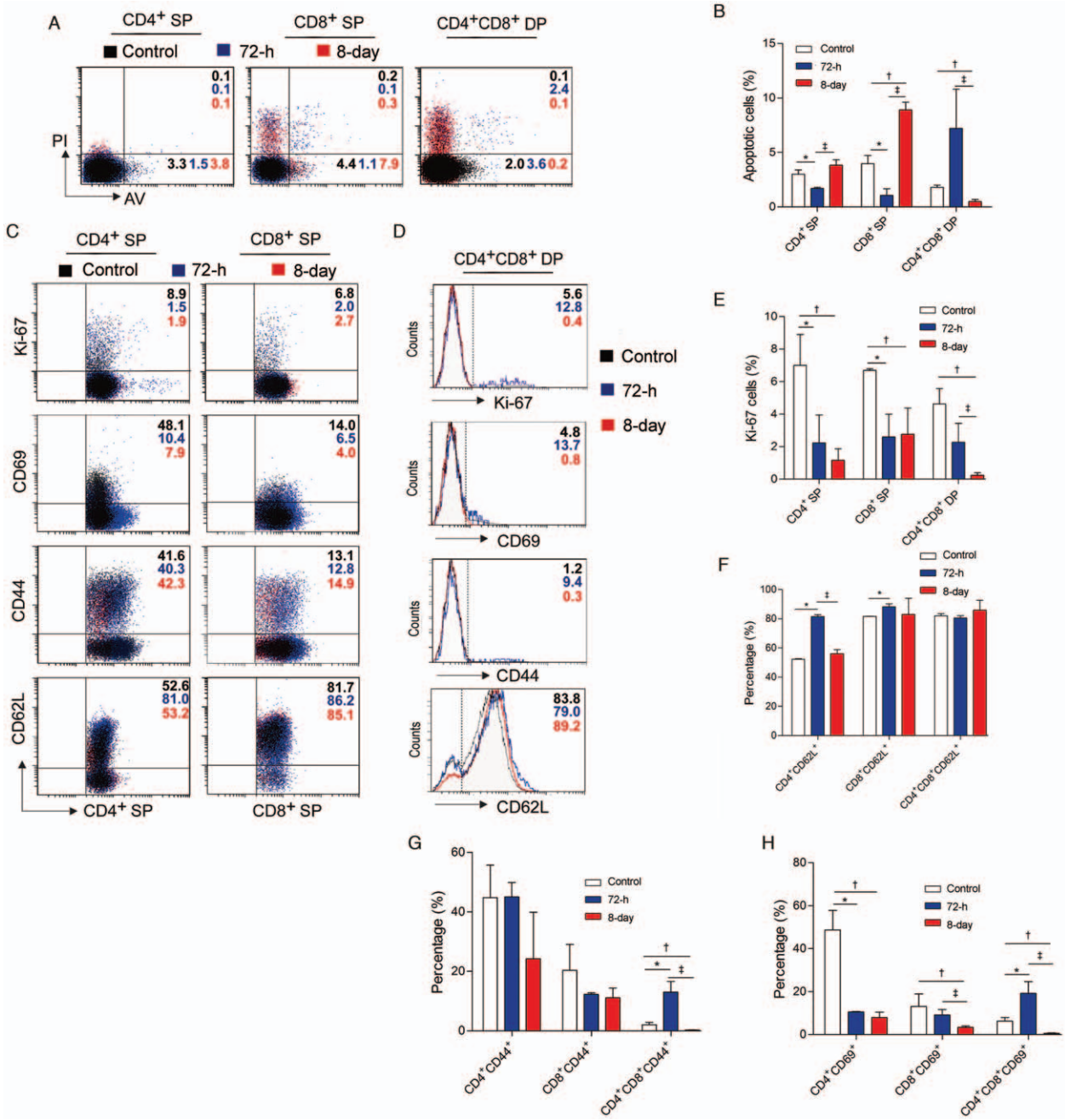

Figure 4: Changes in the proportions of $CD4^+$ SP, $CD8^+$ SP, and $CD4^+CD8^+$ DP cells expressing proliferation-, apoptosis-, and activation-related molecules in a murine LPS-induced tolerance model. C57BL/6 mice (7–10 weeks, $n = 6$) were intraperitoneally injected with 5 mg/kg LPS to establish a tolerance model. The proportions of apoptotic (AV, PI) $CD4^+$ SP, $CD8^+$ SP, and $CD4^+CD8^+$ DP cells were detected by FCM (A) and calculated (B). The proportions of $CD4^+$ SP, $CD8^+$ SP, and $CD4^+CD8^+$ DP cells expressing proliferation-(Ki-67) and activation-related molecules (CD69, CD44, and CD62L) were detected by FCM (C and D) and calculated (E–H). *Control compared with 72-h, †Control compared with 8-day, ‡72-h compared with 8-day. The data are shown as the mean ± SD from three independent experiments. 72-h: Group examined 72 h after LPS injection; 8-day: Group examined 8 days after LPS injection; AV: Annexin V; Control: PBS injection group; DP: Double-positive; FCM: Flow cytometry; LPS: Lipopolysaccharide; PI: Propidium iodide; SD: Standard deviation; SP: Single-positive.

14.670 ± 5.508, $t = 4.486$, $P < 0.0500$). Most importantly, the proportions of p-ERK-expressing $CD4^+$ SP cells (control *vs.* 72-h, 0.850 ± 0.150 *vs.* 3.300 ± 0.755, $t = 5.513$, $P < 0.0100$), $CD8^+$ SP cells (control *vs.* 72-h, 0.500 ± 0.100 *vs.* 2.000 ± 0.608, $t = 4.215$, $P < 0.0500$), and $CD4^+CD8^+$ DP cells (control *vs.* 72-h, 0.600 ± 0.100 *vs.* 12.800 ± 3.205, $t = 6.591$, $P < 0.0100$) were increased significantly [Figure 5E and 5G]. Notably, the proportions of ERK- and p-ERK-expressing $CD8^+$ SP and $CD4^+CD8^+$ DP cells were not obviously changed at 8 days after LPS injection, while the proportion of p-ERK-expressing $CD4^+$ SP cells was still increased (control *vs.* 8-day, 0.850 ± 0.150 *vs.* 3.500 ± 0.436, $t = 9.957$, $P < 0.0100$). These data indicate that the changes in thymocytes under LPS-induced tolerance conditions might be related to altered transduction of relevant signaling pathways.

Discussion

Continuous improvements have been made in intensive care support; however, surgical treatments and the use of

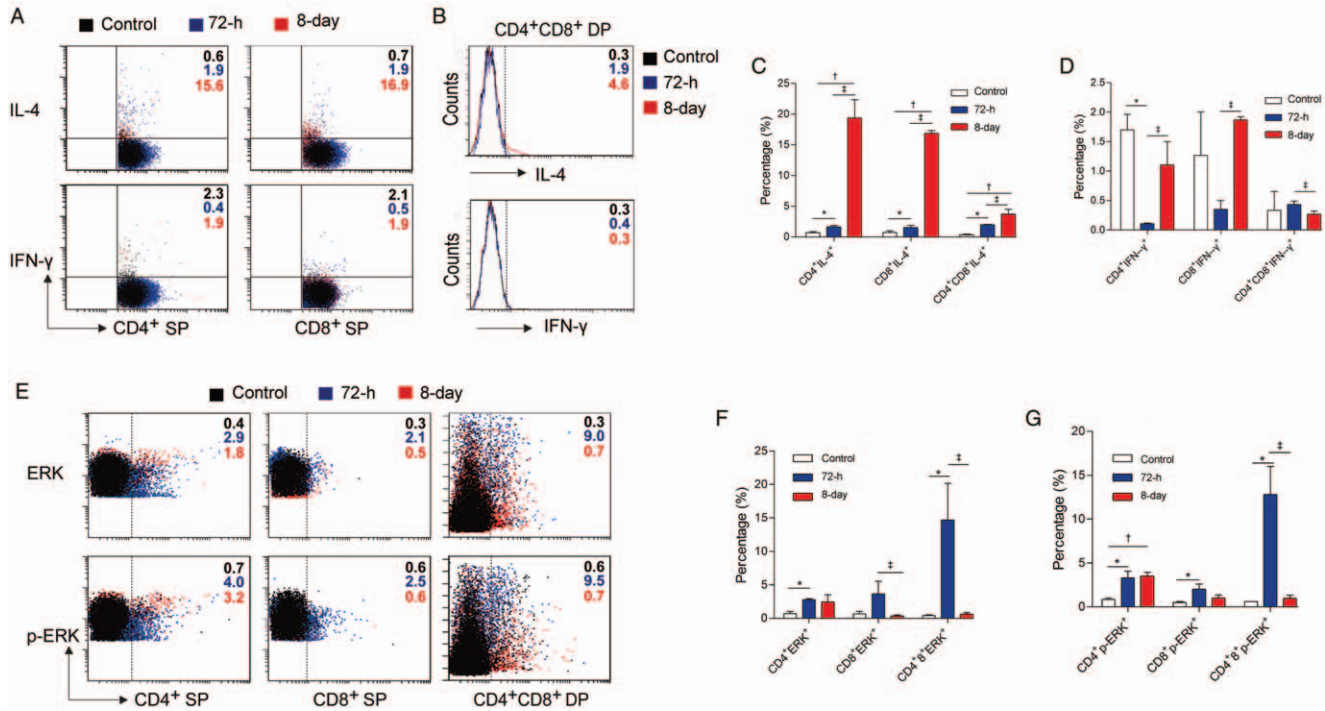

Figure 5: Changes in the expression levels of IL-4, IFN-γ, ERK, and p-ERK in $CD4^+$ SP, $CD8^+$ SP, and $CD4^+CD8^+$ DP cells in a murine LPS-induced tolerance model. C57BL/6 mice (7–10 weeks, $n = 6$) were intraperitoneally injected with 5 mg/kg LPS to establish a tolerance model. The expression levels of IL-4 and IFN-γ in $CD4^+$ SP, $CD8^+$ SP, and $CD4^+CD8^+$ DP cells were measured by FCM (A and B) and calculated (C and D). The expression levels of ERK and p-ERK in $CD4^+$ SP, $CD8^+$ SP, and $CD4^+CD8^+$ DP cells were measured by FCM (E) and calculated (F and G). *Control compared with 72-h, †Control compared with 8-day, ‡72-h compared with 8-day. The data are shown as the mean ± SD from three independent experiments. 72-h: Group examined 72 h after LPS injection; 8-day: Group examined 8 days after LPS injection; Control: PBS injection group; DP: Double-positive; ERK: Extracellular signal-regulated kinase; FCM: Flow cytometry; LPS: Lipopolysaccharide; p-ERK: Phosphorylated extracellular signal-regulated kinase; IL-4: interleukin-4; IFN-γ: interferon-γ; SD: Standard deviation; SP: Single-positive.

antibiotics are increasing with the aging of the population, so mortality during the acute phase of endotoxemia remains high.[24-26] Importantly, accumulating evidence has shown that the proportion and function of T cells and their subpopulations play decisive roles in the development of ET.[17,28,29] For instance, Vogel *et al*[27] found that the T cell activation inhibitor, mitochondrial reduces LPS-mediated calcium influx and IL-2 production, thereby attenuating T cell activation capacity and participating in the establishment of ET. Our recent work has shown that DC tolerance-derived induced Tregs also contribute to the formation of ET.[17] Interestingly, the development of thymocytes is closely related to the occurrence of various diseases, including infectious diseases and rheumatoid arthritis (RA).[30-32] In patients with RA, due to abnormal development of thymocytes, the proportion of recent thymic emigrants is reduced, and the turn-over of peripheral $CD4^+$ T cells is high to increase stability.[33] Moreover, in an RA model, combined methotrexate and etanercept treatment has been found to decrease the proportion of thymic $CD4^+/CD8^+$ T cells with severe disorder and to alleviate pathological outcomes in RA.[34] The above findings show that in-depth research on thymocytes under specific disease conditions is beneficial. However, whether there are changes in T cell development in the thymus and the possible value of such changes in the context of ET have not been reported.

In the present study, we found that the thymus weight and the total number of cells were significantly reduced and that the thymus structure was obviously changed under ET conditions compared with the control condition, consistent with previous reports.[12,13] Importantly, we found that the proportion and an absolute number of thymocytes were changed significantly under ET conditions. Further analysis showed that the proportion and the absolute number of $CD4^+CD8^+$ DP cells were markedly lower in the 72-h group than in the control group. Moreover, the absolute numbers of $CD4^+$ SP and $CD8^+$ SP cells were decreased in the 72-h group, although the proportions of $CD4^+$ SP and $CD8^+$ SP cells were significantly elevated. Similarly, a previous study has demonstrated that in mouse models of inflammatory enteritis induced by dextran sulfate sodium, the subset of $CD4^+/CD8^+CD62L^+$ T cells is enriched in the thymus, whereas the numbers of immature DP thymocytes are significantly reduced.[35] Interestingly, we found that the proportion and the total number of DP cells had returned to normal levels by 8 days post-injection. However, the absolute number and a total number of $CD4^+$ SP and $CD8^+$ SP cells had not yet recovered by 8 days; they were still lower than the numbers in the control group. These data suggest that under ET conditions, the production of DP cells is severely impaired such that abnormal SP cell development subsequently occurs. Moreover, the impact of challenge with a tolerance dose of LPS on thymocyte development may last for a long time. This possibility needs to be verified in further research.

Different T cell clones have TCR CDR3s of different lengths and sequences, forming a diverse *CDR3* gene

profile. The length and sequence of CDR3 determine the TCR structure and specificity. Consistent with the findings of previous studies,[36] we found that the majority of TRBV families of the CDR3 showed standard bell-shaped Gaussian distributions of polyclonal proliferation with CDR3 lengths spanning four amino acid discrepancies. However, in the 72-h group, different numbers of families showed non-normally distributed oligoclonal T cell populations with CDR3 lengths spanning 2 to 3 amino acid discrepancies. The above data suggest that T cell polyclonality decreases under ET conditions. This effect may be caused by impairment of rearrangement and selection programming, resulting in thymic TCR repertoire changes. Similarly, others have reported that circulating T cell subsets in type 1 diabetes patients have shorter TCR CDR3s because of increased deletions/reduced insertions during rearrangement.[37] Moreover, the CDR3 lengths of circulating TCRs in immunoglobulin A nephropathy patients are significantly shorter than those in healthy controls as a result of both rearrangement and selection.[38] However, in the 8-day group, the TCR CDR3 profiles of most TRBV families presented multi-peak and normal distributions, and the CDR3 lengths increased to span 5 to 6 amino acid discrepancies. Considering these data together, we hypothesized that there may be an unknown rearrangement and selection program in ET that alters the TRBV–CDR3 lineage. Moreover, the increase in T cell diversity might be an indicator of the recovery of thymocyte development. Although we concluded that new clones of T cells were produced and that diversity increased, the specificity of the clones has not been confirmed. Therefore, given the important role of T cell clone diversity in thymocyte development, further analyses, such as high-throughput sequencing analysis, of the clone diversity among distinct thymocyte populations in ET will be valuable for elucidating the role of T cell clone diversity in T cell development in the context of ET.

It is well known that activation, proliferation, and apoptosis are involved in the development of thymocytes in the thymus. We further found that the proportion of apoptotic DP cells was increased in the 72-h group, which was consistent with the above findings. Perhaps due to the reduced production of SP clones, the proportion of apoptotic SP cells was reduced. Furthermore, the opposite trend occurred in the 8-day group. However, we noticed that the proportions of proliferating $CD4^+$ SP, $CD8^+$ SP, and $CD4^+CD8^+$ DP cells in the experimental group were continuously reduced. Furthermore, we found that the expression of CD44 and CD69, important activation indicators, in DP cells was increased in the 72-h group. In SP cells, CD62L expression was increased, while CD69 and CD44 expression were decreased; the opposite trends were observed in DP cells. Taken together, these data suggest that different populations of thymocytes are altered in different ways in ET, indicating the complexity of thymocyte development in the context of ET.

Experiments have confirmed that some factors are useful as indicators for T cell functional maturation during thymocyte development, such as secreted IL-4 and IFN-γ.[39] Correspondingly, Marino *et al*[40] reported that compared with DN and DP cells, SP cells have a milder response to IL-4 and IFN-γ and respond to a broader range of cytokines during T cell development. Herein, we found that the IL-4 secretion of DP cells was increased significantly in the context of ET. Importantly, the IL-4 secretion of SP cells showed a trend of elevation and was especially sharply elevated at 8 days. In contrast, the IFN-γ secretion of SP was decreased 72 h after LPS injection and recovered by 8 days after LPS injection. These data suggest that functional maturation of SP cells recovered in the late stage of ET, consistent with our above findings regarding the generation of new T cell clones and the increased diversity of the TCR CDR3 in the late stage of ET.

Numerous studies have indicated that the ERK signaling pathway is closely related to thymocyte development, and its activation also plays key roles in the development of diverse diseases.[41,42] For example, thyroid-stimulating hormone promotes T cell development and enhances recent thymic output in mice with subclinical hypothyroidism, possibly by protecting thymocytes from apoptosis, which is related to ERK pathway activation.[41] The ERK signaling pathway is closely related to the formation of ET. Dual-specificity phosphatase 3 (DUSP3) is a regulator of the innate immune response, and sepsis in DUSP3-deficient mice decreases TNF production and impairs ERK1/2 activation, which enhances the tolerance of the mice to LPS-induced multi-bacterial infection.[43] Interestingly, a recent study has shown that macrophage colony-stimulating factor (M-CSF) secreted by neurons and astrocytes regulates the formation of ET in microglia. Activation of the microglial M-CSF receptor (CSF1R) and its downstream ERK1/2 signaling are involved in M-CSF-mediated microglial ET formation.[44] Consistent with these findings, in the present study, we found that the proportions of $CD4^+$ SP, $CD8^+$ SP, and $CD4^+CD8^+$ DP cells expressing total ERK and p-ERK were elevated at 72 h and decreased at 8 days. Given that the development of thymocytes recovered at the late stage of ET, we propose that optimal transduction of ERK signaling might be an important molecular mechanism of thymocyte development in ET. Other signaling pathways, including the protein kinase B and c-Jun N-terminal kinase pathways, are also documented to be involved in thymocyte development.[45] The exact roles of these pathways and especially the crosstalk among these signaling pathways in the reprogramming of thymocytes in ET remain to be elucidated in the future. This study provides a rare description of thymocyte development in ET; nevertheless, there are some limitations. For example, the microenvironment, in which there are complex interactions among thymocytes and stromal cells, is crucial for thymocyte development and transduction of related signaling pathways and was not investigated in the current study. Moreover, the exact biological value of altered thymocyte development during ET establishment and the potential clinical applications have yet to be determined. Investigation of these core issues might be crucial for the elucidation of the mechanisms of ET and for the development of new strategies to treat clinically important diseases.

To conclude, this study explored the development of thymocytes in the context of LPS-induced tolerance. We reveal that thymocyte development is significantly altered

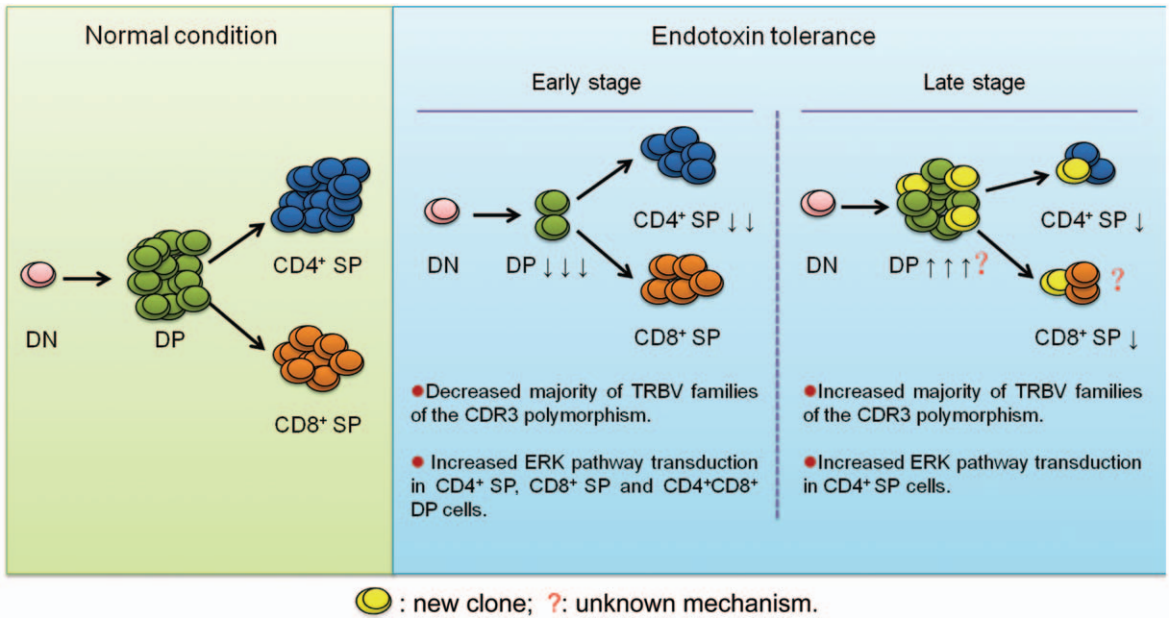

Figure 6: A schematic diagram on the alterations in thymocyte populations under conditions of ET. In the early stage of ET, the cell counts of DP and $CD4^+$ SP cells were significantly decreased. Meanwhile, the majority of TRBV families of the CDR3 polymorphism were decreased. The transduction of the ERK pathway in SP and DP cells was increased significantly. In the late stage of ET, the cell counts of DP cells were significantly increased, while that in SP cells were decreased. The majority of TRBV families of the CDR3 polymorphism were increased. The transduction of the ERK pathway in $CD8^+$ SP and DP cells was not obviously changed, while that in $CD4^+$ SP cells was still increased. CDR3: Complementarity-determining region 3; DN: Double-negative; DP: Double-positive; ERK: Extracellular signal-regulated kinase; ET: Endotoxin tolerance; SP: Single-positive.

under conditions of ET. This developmental alteration is accompanied by altered activation, proliferation, and cytokine secretion of thymocyte subpopulations and by a generation of new T cell clones, which might be related to altered transduction of the ERK pathway [Figure 6]. These findings provide potentially valuable fundamental experimental evidence supporting the subsequent investigation of the mechanism of ET and the pathogeneses of related clinical diseases as well as the development of prevention strategies.

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Conflicts of interest

None.

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