

Mannan-Binding Lectin Regulates the Th17/Treg Axis Through JAK/STAT and TGF- β /SMAD Signaling Against *Candida albicans* Infection

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Background: Mannan-binding lectin (MBL) is a key molecule in innate immunity and activates the lectin complement pathway, which plays an important role in resisting *Candida albicans* (*C. albicans*) infection. However, the underlying mechanism of this resistance to infection remains unclear.

Methods: In this study, we investigated how MBL regulates the differentiation of CD4⁺ T cells into T helper type 17 (Th17) and T regulatory (Treg) cells against *C. albicans* in mice, as well as the underlying mechanisms. We generated MBL double-knockout (KO) mice and infected them with *C. albicans* by intraperitoneal injection.

Results: Compared with that in wild-type (WT) mice, the percentage of Th17 cells increased in MBL-null mice, whereas Treg cells decreased, indicating that MBL might regulate the Th17/Treg balance. In addition, in MBL-null mice, the expression levels of interleukin (IL)-17A, IL-21, and the master transcription factor of Th17 cells, ROR γ t, significantly increased. Conversely, IL-10, IL-2, and the Treg-specific transcription factor, Foxp3, decreased. Moreover, we found that the levels of TGF- β and IL-6 upregulated in MBL-null mice. Mechanistically, we found that MBL regulated the TGF- β /SMAD pathway through the inhibition of p-SMAD2 and promotion of p-SMAD3, and mediated the JAK/STAT pathway through the inhibition of p-JAK2 and p-STAT3 and promotion of p-JAK3 and p-STAT5. MBL double-KO mice showed a more severe inflammatory response and significantly lower survival rates with *C. albicans* infection.

Conclusion: These results suggest that MBL regulates the Th17/Treg cell balance to inhibit inflammatory responses, possibly via IL-6- and TGF- β -mediated JAK/STAT and TGF- β /SMAD signaling, and play an important role in anti-*C. albicans* infection.

Keywords: mannan-binding lectin, Th17/Treg axis, JAK/STAT, TGF- β /SMAD, *Candida albicans*

Introduction

Mannan-binding lectin (MBL) is the key recognition molecule in the complement activation pathway. In humans, MBL is encoded by a single gene, MBL2, while in rodents, there are two MBL genes, MBL-A and MBL-C.¹ The carbohydrate-recognition domain of MBL senses polysaccharides such as D-mannose, L-fucose, and N-acetylglucosamine on several organisms, including *C. albicans*, which play a key role in innate immunity.²⁻⁴ Recent studies have reported that MBL is also critical for the adaptive immune response.^{5,6} Moreover, Stefan Müller et al found that MBL-deficient mice exhibit increased antibody production and impairment of the adaptive immune response owing to the absence of the first-line defense function of MBL in innate immunity, which can promote the development of adaptive immune responses in the circulatory system.⁷⁻⁹ Crohn's disease is susceptible to a complicated clinical phenotype when accompanied by a defective MBL gene, possibly reflecting the delayed clearance of oligomannan-containing microorganisms by the innate immune system in the absence of MBL.¹⁰

C. albicans is an opportunistic pathogenic fungus that usually colonizes the human gastrointestinal tract, skin, and mucosa.¹¹ MBL levels increase markedly during the infection process in patients with invasive candidiasis, and serum MBL levels exhibit a dynamic decrease during the 2 days preceding positive blood culture sampling, indicating that MBL may play a role during the early stage invasive candidiasis. It has been shown that MBL is expressed in mouse intestinal epithelial cells, and dextran sodium sulfate-induced colitis promotes *C. albicans* dissemination to the kidneys and lungs in MBL-deficient mice after intestinal infection.¹² The gastrointestinal tract of MBL-null mice infected with *C. albicans* will have an excessive inflammatory reaction.^{13,14}

Regulatory T cells (Treg) and T helper type 17 cells (Th17) can eliminate pathogens and reduce tissue damage caused by the inflammatory reaction by inhibiting and promoting inflammatory reactions, respectively.¹³ During an oral *C. albicans* infection in mice, Treg cells induced IL-17 cytokines in responder T cells (Tresp), which markedly enhanced fungal clearance and recovery from infection. The Th17 cells, acting largely through IL-17, confers the dominant response to oral candidiasis through neutrophils and antimicrobial factors.¹⁴ Moreover, Treg cells could promote acute Th17 cell responses to suppress mucosal fungus infections and had a powerful capability to fight infections besides their role in maintaining tolerance or immune homeostasis.¹⁵

Foxp3 is a Treg-specific nuclear transcription factor, and the current understanding of the transcriptional regulation of Foxp3 gene included signaling pathways initiated by TCR, IL-2R/STAT pathway, TGF-beta/Smad pathway, PI3K/Akt/mTOR axis, Notch signal pathway, IFN/IRF and IFN/nitric oxide axis, and epigenetic mechanisms.^{16,17} The retinoid-related orphan receptor gamma t (ROR γ t) is mainly expressed in the Th17 cells of CD4⁺ T cell subsets, which regulate natural immunity and the adaptive immune response by producing inflammatory mediators that can also help remove invading pathogens.^{16,18} Published studies showed that specific JAK/STAT pathways played a critical role in the functional differentiation of distinct Th subsets, and heme oxygenase-1 exerted its inhibitory effect on Th17 cell differentiation by directly associating and blocking STAT3 phosphorylation.¹⁹ In addition, fungal killing by IL-6/23-stimulated human peripheral blood neutrophils was impaired by JAK/STAT inhibitors Ruxolitinib and Stattic and the ROR γ t inhibitor SR1001.²⁰

Until now, the underlying molecular mechanism of MBL against *C. albicans* infection remains unclear. In this study, we established an MBL double-knockout (KO) mouse model and clarified how MBL regulates the balance between Treg cells and Th17 cells through intraperitoneal injection of *C. albicans* in mice.

Materials and Methods

Animals

MBL-null, MBL-A, and MBL-C double-knockout (KO) mice were obtained by CRISPR/Cas9 genome editing on the C57BL/6 background, followed by intercrossing of mice. Laboratory of Immunophenomics performed the genome-editing experiments of mice in Xinxiang Medical University following the protocols as previously described.²¹ WT C57BL/6 mice were used as controls for all experiments and were purchased from Charles River Laboratories. All mice were housed in the Xinxiang Medical University animal care facility, maintained in pathogen-free conditions, and provided standard laboratory chow and water *ad libitum*. All experiments were approved and carried out following the Xinxiang Medical University Animal Care Committee guidelines.

Genotyping

DNA was extracted from the tails of MBL double-KO mice using a commercial DNA extraction kit (GENFINE Company) according to the manufacturer's instructions. Briefly, tails were incubated at 56°C for 6–8 h with lysis buffer solution. Proteinase K. Lysis buffer was then added to the solution. The samples were further incubated at 70 °C for 10 min. After adding ethanol, the samples were applied to the column and centrifuged. DNA was eluted in Buffer TB from the kit and subsequently used for PCR.

The PCR was run in a thermocycler (Bio-Rad, Hercules, CA, USA) using the 2 \times Taq Plus Master Mix following the manufacturer's instructions. Thermocycling was performed in a final volume of 10 μ L containing 1.2 μ L of cDNA and 10 μ M of each primer. The amplification conditions comprised an initial denaturation at 94°C for 5 min, followed by 35

cycles of 94°C for 30s, 61°C for 30s, 72°C for 40s, and a final extension at 72°C for 20 min. The following primers were used: m-MBL-A-F-PCR, Forward: 5'- GCT TCC ATT ACT CCC TGT CCT T -3' and Reverse: 5'- TCC ATC TCC GAA CAC AGA ACA G -3'; m-MBL-C-F-PCR, Forward: 5'- CCT TCT GCT GTG TGT GGT GA-3' and Reverse: 5'- GCA CAC CTG GTT CTC CCT TT -3'.

C. albicans Strain and Infection

The *C. albicans* strain used in this study was the international standard strain ATCC90028; WT and MBL double-KO mice were matched by gender and age, and more than 15 mice per genotype were used for each experiment. *C. albicans* was cultured in a YPD medium (1% yeast extract, 2% bacto peptone, and 2% dextrose) on a shaker at 28 °C for 48 h.²² Before injection, the fungi were washed twice in phosphate-buffered saline (PBS; Gibco BRL). Mice (6-to-9-weeks-old) were challenged intraperitoneally with 5×10^7 CFUs *C. albicans*.

Flow Cytometry for Detection of Treg and Th17 Cells

On the 7th day after infection, three mice were randomly selected from each group. After the eyeballs of the mice had been removed, blood was collected into the EP tubes containing anticoagulator EDTA. For CD45, CD5, CD4, ROR γ T, and Foxp3 staining, in turn, the cells were washed, fixed, and then stained using a flow cytometry staining kit from BD Biosciences, following the manufacturer's protocol. Data were acquired using Becton Dickinson FACS-Calibur cytometers and analyzed using FlowJo 9.1 software.

Cytokine Measurements

ELISA was used to measure the levels of IL-17A, IL-10, IL-6, TGF- β , IL-2, and IL-21 cytokines in the blood of mice. On day 7 after infection, five mice were randomly selected from each group, euthanized, and the eyeballs removed for blood collection. Blood was collected into tubes containing EDTA as an anticoagulant, and the plasma was collected after centrifugation. According to the manufacturer's instructions, cytokines measurement were performed using ELISA test kits (XinBoSheng Biotechnology Co., Ltd.).

Separation and Purification of CD4⁺ T Cells

After 7 days of infection, five mice were randomly selected from each group and sacrificed by cervical dislocation. The spleen was removed and ground to obtain a cell suspension. Magnetic spleen cell separation was used for sorting. The MS column was placed in a MACS magnetic sorter (Miltenyi Biotech), and the CD4⁺ T cells were sorted and purified using a mouse magnetic bead sorting kit (Miltenyi Biotech). All the procedures were performed according to the kit manufacturer's instructions.

Quantitative RT-PCR

On day 7 after infection, five mice were randomly selected from each group and sacrificed by cervical dislocation. Spleen cells were isolated as described above, and CD4⁺ T cells were sorted using MACS magnetic beads. Total RNA was isolated from CD4⁺ T cells of the four groups using TRIzol reagent (TAKARA) and reverse transcribed into cDNA. The qPCR conditions were one cycle at 95 °C for 30s, 95 °C for 5 s, followed by 34 cycles at 60 °C for 30s. In each sample, the expression levels of the target genes were normalized to that of the β -actin housekeeping gene. Data analysis was performed using the $2^{-\Delta\Delta Ct}$ method. The primers used were synthesized by Wuhan JinkaiRui Bioengineering Co., Ltd. The primers used for the qPCR were as follows: Roryt, sense: 5'-CTG TCC TGG GCT ACC CTA CT-3' and antisense: 5'-GAA GAA GCC CTT GCA CCC C-3'; Foxp3, sense: 5'-GCG AAA GTG GCA GAG AGG TA-3' and antisense: 5'-TGT CAG AGG CAG GCT GGA TA-3'; IL-17A, sense: 5'-CCA GGG AGA GCT TCA TCT GTG T-3' and antisense: 5'-AAG TCC TTG GCC TCA GTG TTT G-3'; IL-10, sense: 5'-GGT TGC CAA GCC TTA TCG GA-3' and antisense: 5'-GAG AAA TCG ATG ACA GCG CC-3'; IL-6, sense: 5'-CCC CAA TTT CCA ATG CTC TCC T-3' and antisense: 5'-CAT AAC GCA CTA GGT TTG CCG-3'; TGF- β , sense: 5'-AGC TGC GCT TGC AGA GAT TA-3' and antisense: 5'-AGC CCT GTA TTC CGT CTC CT-3'; IL-2, sense: 5'-CAA GCA GGC CAC AGA ATT GAA-3' and antisense: 5'-TCA AAT CCA GAA CAT GCC GC-3';

IL-21, sense: 5'-AGC ACA TAG CTA AAT GCC CTT C-3' and antisense: 5'-TGT GGG AAC GAG AGC CTATG-3'; and beta-actin, sense: 5'-AGA TCA AGA TCA TTG CTC CT-3' and antisense: 5'-ACG CAG CTC AGT AAC AGT CC-3'.

Western Blotting

On day 7 after infection, spleen cells were collected as described above. CD4⁺ T cells sorted using MACS magnetic beads were lysed on ice for 15 min, centrifuged at 14,000 rpm for 15 min at 4 °C, and the protein concentration in the supernatant was measured Bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology). The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. Antibodies against STAT3 (ab68153), p-STAT3^{Tyr705} (ab267373), p-STAT5^{Tyr694} (ab278764), SOCS3 (ab280884), ROR γ t (ab207082), and Foxp3 (ab20034) were purchased from Abcam; antibodies against SMAD2 (5339S), p-SMAD2^{Ser465+467} (3108S), SMAD3 (9523S), p-SMAD3^{Ser423+Ser425} (9520S), JAK2 (3230S), JAK3 (8863S), and p-JAK3^{Tyr980/981} (5031S) were purchased from Cell Signaling Technology; the antibody against SMAD7 (MAB2029-SP) was purchased from R&D Systems, and the anti- β -actin antibody (66009-1-Ig) was purchased from Proteintech. Milk was used for blocking, and the primary antibody was incubated overnight with shaking. After washing with 1 \times TBST, a horseradish peroxidase-conjugated secondary antibody was added, incubated for 1 h, and staining was developed using an ECL kit (Millipore Corporation, Billerica, MA, USA). Bands were developed using a TANON exposure apparatus, and the software of ImageJ was used to analyze the intensity of western blot bands.

Histology

After 3 days of infection, mice were randomly selected from 4 groups and were euthanized by cervical dislocation. The liver and kidneys were removed, fixed in 4% paraformaldehyde at 4 °C, dehydrated in a graded ethanol series, cleared in xylene, and embedded in paraffin. The sections were cut into 5- μ m slices for hematoxylin-eosin (H&E) and periodic acid-Schiff (PAS) staining.

Determination of Survival Rate and Fungal Burden

WT and MBL double-KO mice were simultaneously intraperitoneally infected with 200 μ L of a *C. albicans* suspension (1×10^8 CFUs) according to a previous study,²³ and the survival rates were monitored for 14 days. In addition, to compare differences in tissue load, WT and MBL double-KO mice were infected intraperitoneally with a sublethal dose of *C. albicans* (5×10^7 CFUs) after 3 days of infection. The liver, kidneys, and spleen were sterilely dissected from each mouse, weighed, and homogenized in sterile PBS. Serial dilutions of the organ homogenate were spread onto agar plates. After 48 h of incubation at 37 °C, the CFUs were counted and determined as CFUs/g of tissue.

Statistical Analysis

All data were processed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). The SPSS statistical package (SPSS for Windows 16; SPSS Inc., Chicago, IL, United States) was utilized to apply Duncan's multiple range test and one-way analysis of variance (ANOVA) to determine the statistical significance of biological research data, such as the change in the quantity of Treg and Th17 and the expression of cytokines. The survival periods were compared using the Kaplan–Meier method. Tests of the differences among groups were conducted, and the threshold value of $P < 0.05$ indicated that the difference was statistically significant.

Results

Generation of MBL Double-Knockout Mice

As described in published articles,²⁴ agarose electrophoresis, genotyping, and sequencing analyses were used to confirm that mice homozygous for MBL-*A* and MBL-*C* gene double-KO were successfully obtained.

Establishment of a Mouse Model of *C. albicans* Infection

The mouse abdominal cavity was opened 3 days after infection, and the infection foci were identified in the liver and kidneys (S1 Fig A, B). When the liver and kidney suspensions were coated on Sabouraud's medium, white colonies with a smooth surface were observed (S1 Fig C, D). Single colonies were transferred to new plates for green-color colony *Candida* selection, and green colonies could be observed (S1 Fig E, F). Moreover, periodic acid-Schiff (PAS) staining showed that both types of *C. albicans* cells (yeast and branched hyphae) were present in the livers and kidneys of both WT (WT) and MBL double-KO mice (S1 Fig G, H). These results showed that the mouse model of systemic *C. albicans* infection had been successfully established.

The Change of Treg, Th17 Cells and the Expression of Foxp3 and ROR γ t in MBL Double-KO Mice

To explore the roles of MBL in Treg and Th17 cell differentiation, the proportion of Treg and Th17 cells in peripheral blood and the expression levels of the nuclear transcription factors Foxp3 and ROR γ t were compared between WT and MBL double-KO mice by flow cytometry (S2 Fig) and western blotting. The results showed that the proportion of Treg cells decreased significantly ($P < 0.01$) in MBL double-KO mice, and the percentage of Treg cells in *C. albicans* infected mice was significantly higher ($P < 0.05$) than that in uninfected mice while MBL double-KO could inhibit the increase of Treg cells (Figure 1A and B), indicating that MBL can induce the differentiation of CD4⁺ T cells into Treg cell subsets, especially, during *C. albicans* infection. Simultaneously, the proportion of Th17 cells in the MBL double-KO mice was significantly higher ($P < 0.05$) than that in WT mice, and the number of Th17 cells in *C. albicans* infected mice was significantly higher ($P < 0.05$) than that in uninfected mice and MBL double-KO could promote the increase of Th17 cells (Figure 1C and D), suggesting that MBL can inhibit the differentiation of CD4⁺ T cells into Th17 cell subsets in both normal and pathogen-infected conditions. Foxp3 and ROR γ t are important transcriptional Treg and Th17 cell differentiation and function regulators. Therefore, we also compared the protein and mRNA levels of Foxp3 and ROR γ t between WT and MBL double-KO mice. We found that Foxp3 expression decreased, while that of ROR γ t was increased, in MBL double-KO mice compared with that of WT mice in both normal and pathogen-infected conditions (Figure 1F–H). These results revealed that MBL positively regulates Treg differentiation and Foxp3 expression, negatively regulating Th17 differentiation and ROR γ t expression.

The Change of the Serum Levels of IL-17A, IL-10, IL-6, TGF- β , IL-2, and IL-21 in MBL Double-KO Mice

Th17 and Treg cells are known to be involved in fungal infection-associated immunoreactions. Furthermore, IL-17 and IL-21 are hallmarks of Th17 cells,²⁵ while IL-10 and IL-2 are hallmarks of Treg cells.^{26,27} TGF- β induces the differentiation of Tregs. However, when combined with IL-6, TGF- β can differentiate CD4⁺ T cells into Th17 cells.²⁸ As previously described, we observed dynamic changes in the Treg/Th17 cell balance in mouse peripheral blood. Next, we measured the serum levels of the related cytokines by ELISA. The measured cytokines included IL-17A, IL-10, IL-6, TGF- β , IL-2, and IL-21. The results of ELISA showed that, following *C. albicans* infection, the secretion of cytokines related to Th17 and Treg cells in the WT group was significantly different from that of the MBL-null group. The levels of IL-17A (Figure 2A), IL-6 (Figure 2C), TGF- β (Figure 2D), and IL-21 (Figure 2F), in the MBL double-KO mice, was significantly higher ($P < 0.01$) than that in the WT with both normal and *C. albicans* infected conditions, respectively, whereas the levels of IL-10 (Figure 2B) and IL-2 (Figure 2E) showed a significant decrease ($P < 0.01$). Collectively, these findings indicate that the changes in serum cytokine levels were consistent with the changes in Treg and Th17 cell proportions.

The Levels of Cytokine mRNA Expression in Mouse Splenocytes

We carried out qRT-PCR to compare the mRNA expression levels of IL-17A, IL-10, IL-6, TGF- β , IL-2, and IL-21 among WT mice, WT mice with infection *C. albicans*, MBL double-KO mice, and MBL double-KO mice with infection *C. albicans*. The results showed that, with *C. albicans* infection, the mRNA expression of Th17- and Treg-related factors

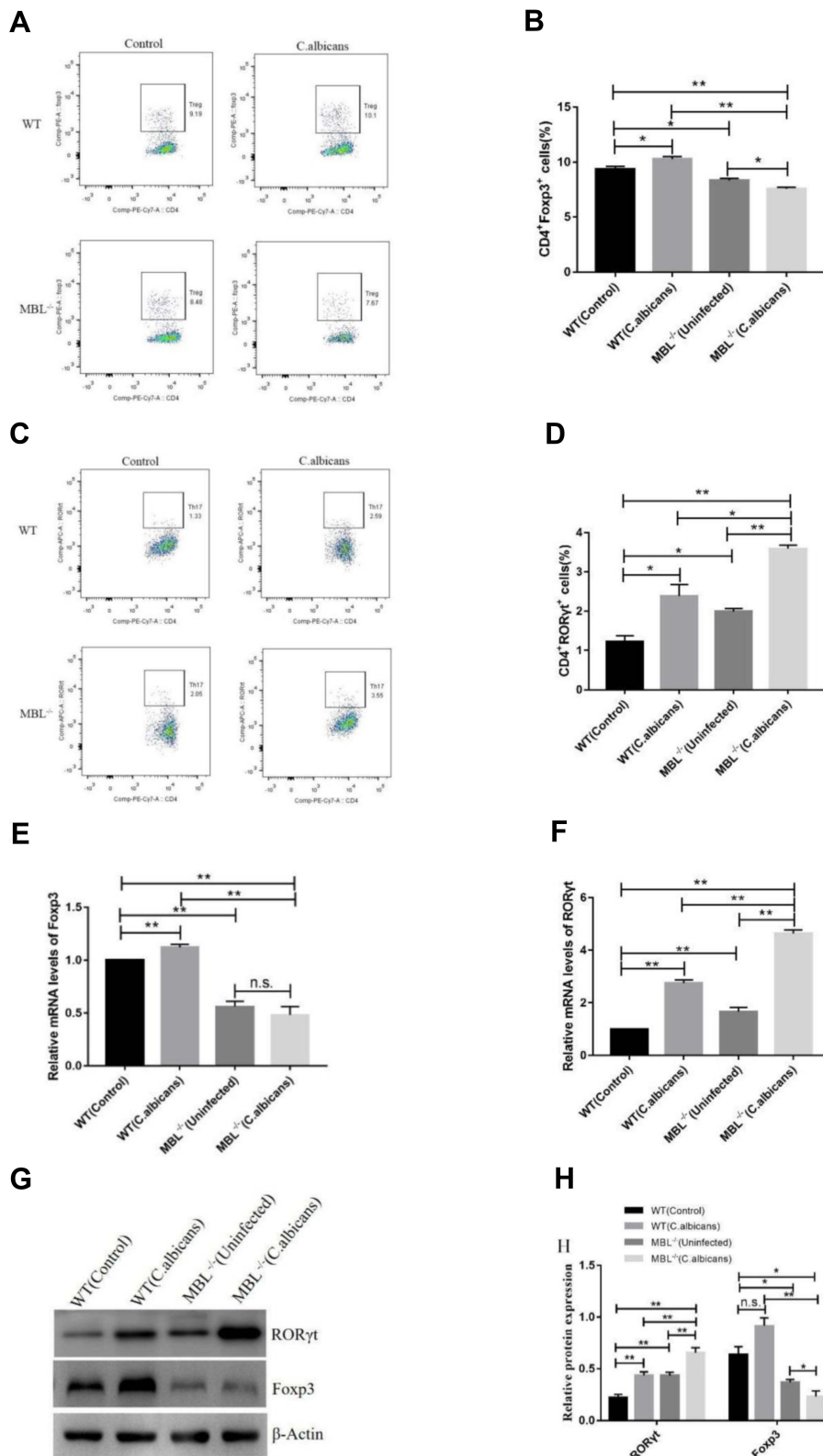


Figure 1 MBL regulates Th17 and Treg cell differentiation as well as RORγt and Foxp3 expression. After 3 days of infection, blood was collected from WT and MBL double-KO mice to analyze the proportion of Treg and Th17 cells and the expression levels of the lineage-specific nuclear transcription factors Foxp3 and RORγt. **(A)** Representative flow cytometry and **(B)** composite percentages of CD4⁺Foxp3⁺Treg cells in each group. **(C)** Representative flow cytometry and **(D)** composite percentages of CD4⁺RORγt⁺Th17 cells in each group. **(E)** The mRNA levels of Foxp3. **(F)** The mRNA levels of RORγt. **(G)** The protein levels of RORγt and Foxp3. **(H)** Histogram showing the relative expression levels of RORγt and Foxp3 as measured in three independent experiments. One-way analysis of variance (ANOVA) was used for statistical difference analysis (**P* < 0.05, ***P* < 0.01).

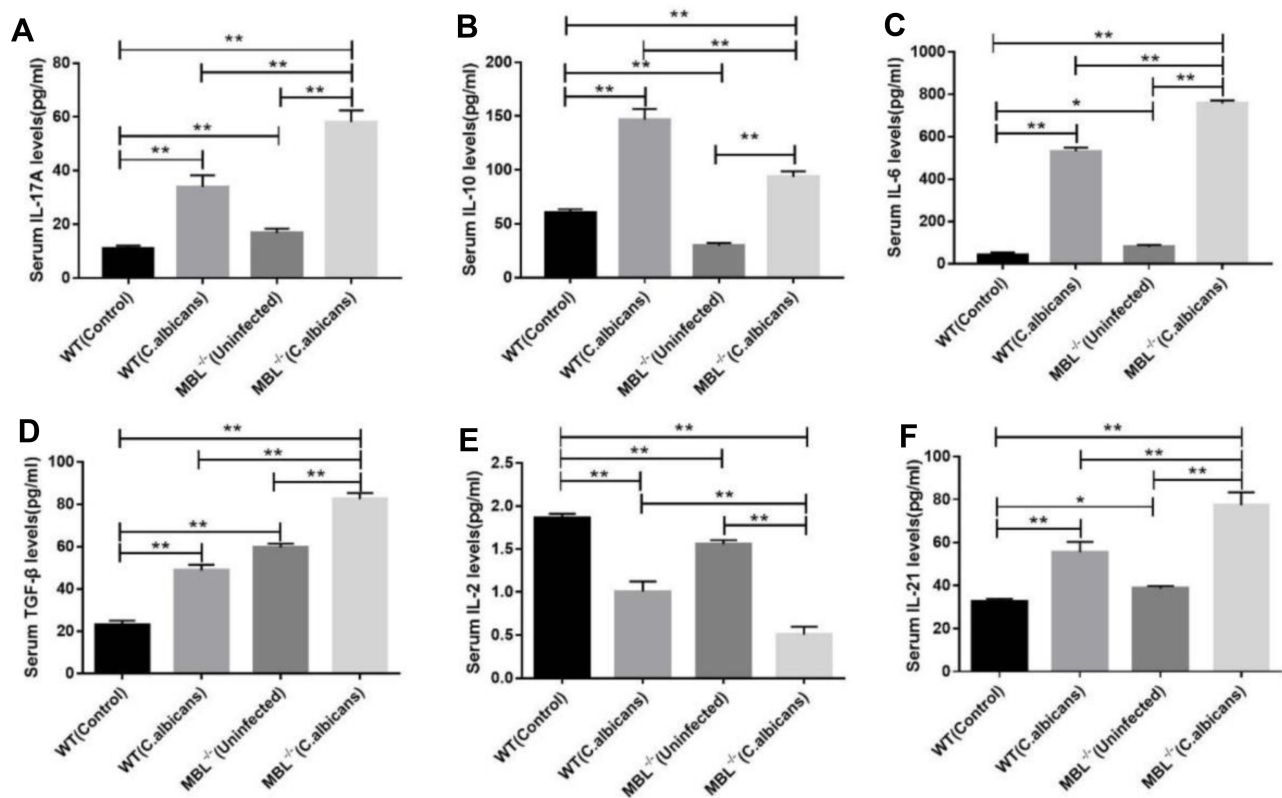


Figure 2 Changes of the relative levels of Th17- and Treg-related cytokines in the different groups. The concentrations (Mean \pm SD) of (A) IL-17A, (B) IL-10, (C) IL-6, (D) TGF- β , (E) IL-2, and (F) IL-21 in each group. One-way analysis of variance (ANOVA) was used for statistical difference analysis (* $P < 0.05$, ** $P < 0.01$). These data are representative of three independent experiments.

in the WT mice was significantly different from that in MBL double-KO mice (Figure 3). Compared with the infected WT mice, the mRNA expression of IL-10 (Figure 3B) and IL-2 (Figure 3E) of the mice in the infected MBL double-KO group were decreased, whereas that of IL-17A (Figure 3A), IL-6 (Figure 3C), TGF- β (Figure 3D), and IL-21 (Figure 3F) was increased, and the difference was significant ($P < 0.01$). These results suggested that MBL has anti-inflammatory activity by regulating the expression of proinflammatory and anti-inflammatory cytokines during CD4⁺ T cell differentiation.

MBL Mediates Treg and Th17 Cell Differentiation Through the JAK/STAT and TGF- β /SMAD Signaling Pathways

The JAK/STAT and TGF- β /SMAD signaling pathways contribute to the differentiation of T helper cells.^{29,30} To assess the potential mechanism involved in how MBL modulates the differentiation of CD4⁺ T cells in *C. albicans*-infected mice, we measured the protein expression levels of key factors in the two signaling pathways using western blot analysis. Compared with WT mice infected with *C. albicans*, the expression of Foxp3, p-JAK2, p-JAK3, p-STAT5, and p-SMAD3 was decreased, whereas that of ROR γ t, p-STAT3, and p-SMAD2 was increased, in MBL double-KO mice with *C. albicans* infected conditions, respectively. Moreover, compared with infected WT mice, JAK2, JAK3, and STAT3 were decreased, and SMAD2 was increased in MBL double-KO mice infected with *C. albicans*. However, no significant changes were found in the levels of β -actin and total STAT5 and SMAD3 proteins. The levels of SOCS3³¹ and SMAD7,³² regulators of the JAK/STAT and TGF- β /SMAD signaling pathways, were also decreased (Figure 4). Therefore, the results revealed that MBL regulates the phosphorylation status of the STAT and SMAD protein families during Treg and Th17 cell differentiation.

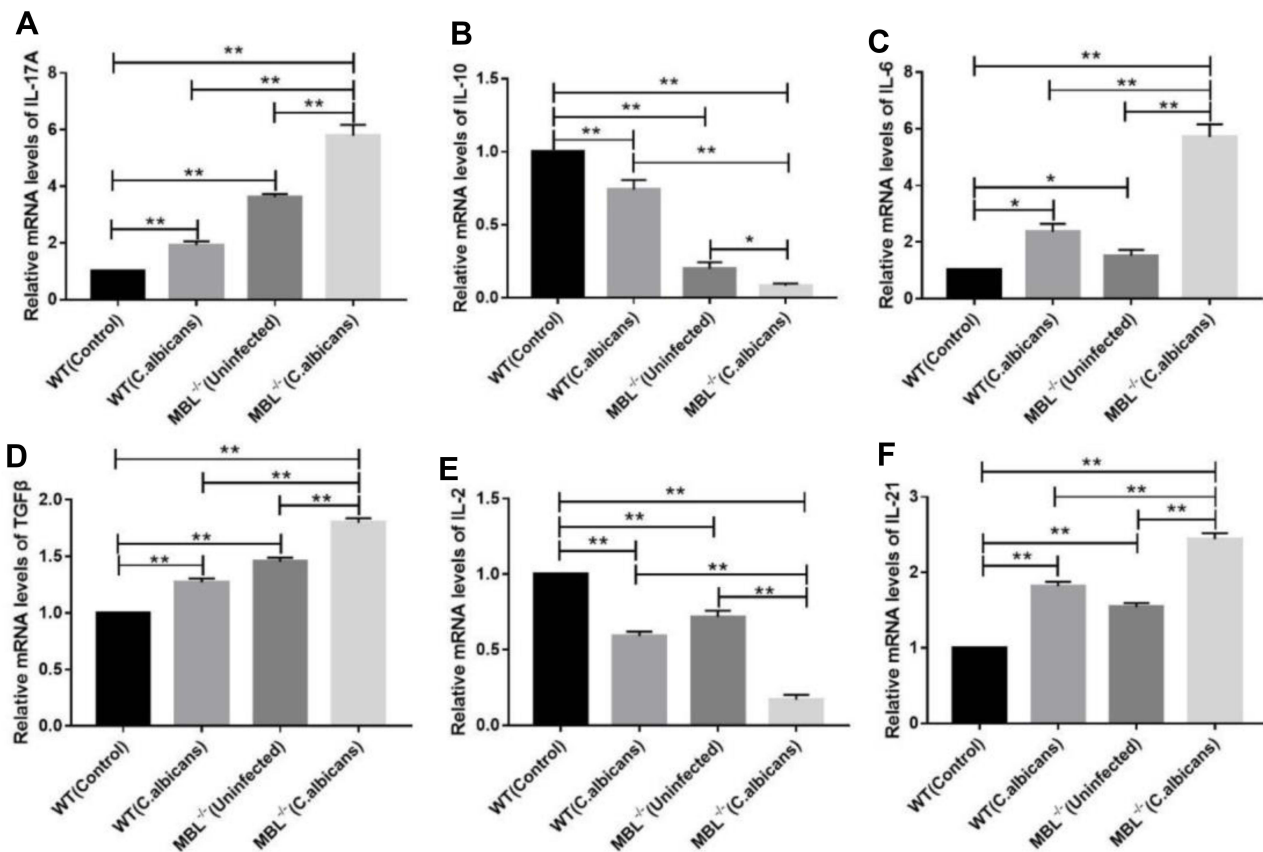


Figure 3 mRNA expression of Th17- and Treg-related factors in the different groups. Total RNA was isolated from the CD4⁺ T cells, and quantitative real-time PCR was performed to measure the mRNA levels of Il17a (A), Il10 (B), Il6 (C), Tgfb (D), Il2 (E), and Il21 (F) in each group. The results represent the means of three independent experiments. One-way analysis of variance (ANOVA) was used for statistical difference analysis (*P < 0.05, **P < 0.01).

MBL Ameliorated the Inflammatory Response of the Liver and Kidney in Mice with *C. albicans* Infection

To evaluate the effects of MBL on immune inflammation *in vivo*, we generated a murine model of *C. albicans* infection and compared tissue inflammatory responses between *C. albicans*-infected WT and MBL-null mice. The results of hematoxylin and eosin (H&E) staining showed that, compared with that of infected WT mice, the liver and kidney of infected MBL-null mice presented a greater inflammatory response, wherein most renal units disappeared in the kidney, and multifocal necrotic tissue appeared in the renal medulla and cortex. In addition, *C. albicans* spores were observed and were surrounded by a large number of inflammatory cells. Moreover, liver tissue infection and peripheral hepatocyte water-like degeneration and fibrosis were more apparent in infected MBL-null mice. In contrast, the inflammation status of infected WT mice was relatively light, showing the disappearance of some of the nephrons, the appearance of a few inflammatory cells, and the ballooning of a small number of hepatocytes (Figure 5A). The results of the PAS staining showed that the liver and kidney of infected MBL double-KO mice were infiltrated with more hyphae and thick-film spores compared with the infected WT mice (Figure 5B).

MBL Double-KO Mice are Susceptible to *C. albicans* Infection

To investigate the role of MBL in *C. albicans* infection, we challenged C57BL/6 WT and MBL double-KO mice with *C. albicans* and monitored the survival rates and tissue burden of the two groups. The survival rates of the *C. albicans*-infected MBL-deficient mice were decreased compared with those of the WT controls (Figure 6A). On the 14th day of infection, the mortality rate of MBL double-KO mice was 100%, whereas that of WT mice was 80%. In addition, after 3

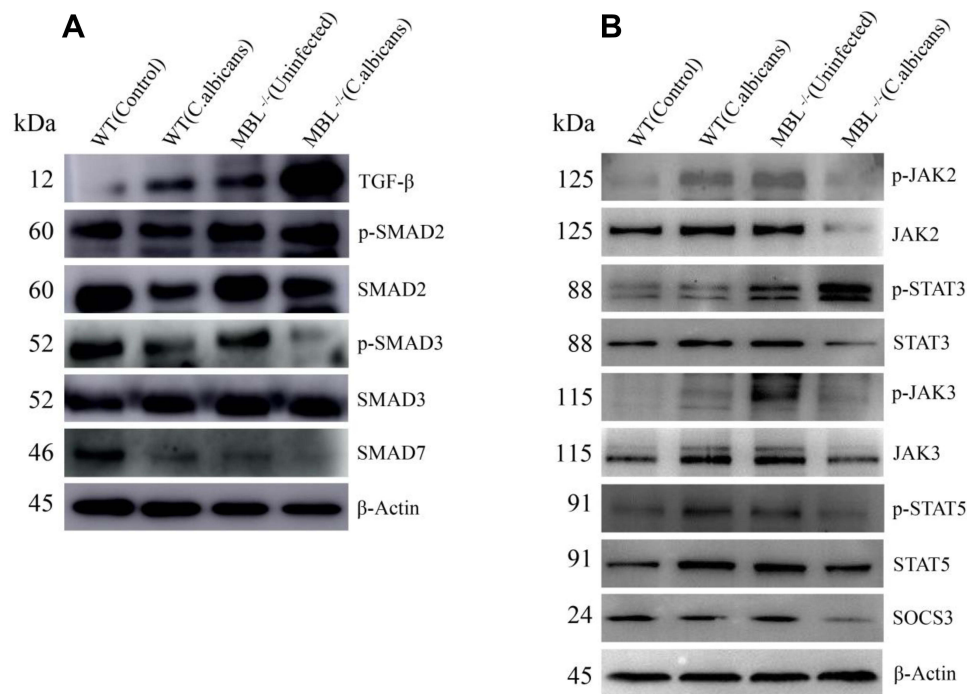


Figure 4 MBL acts through the JAK/STAT and TGF- β /SMAD signaling pathway in *C. albicans*-infected mice. The protein expression of **(A)** TGF- β , p-SMAD2/3, SMAD2/3, SMAD7, **(B)** p-JAK2/3, p-STAT3/5, JAK2/3, STAT3/5, and SOCS3 in splenocytes was determined by western blot. Representative images from one of three independent experiments are shown.

days of infection, the fungal burden of the liver, kidney, and spleen was higher in MBL double-KO mice than in WT mice (Figure 6B–D), which indicates that MBL is critically important for host defense against *C. albicans* infection.

Discussion

Plasma MBL, a secreted pattern recognition molecule, is an intrinsic component of the complement system. MBL deficiency is related to a variety of infectious conditions, including fungal infections. Although the role of MBL in the innate immune system has been widely studied, its role in adaptive immune responses remains poorly understood. Our current research aimed to increase our understanding of how MBL affects CD4⁺ T cell differentiation in the adaptive immune system under the condition of infection.

Noha M. Hammad showed that MBL could be used as a candidate antifungal agent against *Candida* infection.³³ In our previous cytological studies in vitro, we found that a high concentration of MBL inhibited LPS-stimulated TNF- α and IL-12 production in THP1/CD14 cells, peptidoglycan-stimulated TNF- α and IL-6 production in THP1/CD14 cells, and *C. albicans*-stimulated TNF- α and IL-8 production in THP1/CD14 cells.^{2–4} It has been reported that two-phase states of *C. albicans* can stimulate dendritic cells and monocytes to produce cytokines such as IL-8, IL-6, MCP-1, and TNF- α .³⁴ In this study, our results demonstrated that, under the conditions of this mouse model, MBL has a role in limiting excessive pro-inflammatory responses in tissues and resisting *C. albicans* infection.

Protection against *C. albicans* is determined not only by host immune resistance but also by the ability to control *Candida*-induced immunopathology appropriately. Excessive inflammatory responses can be damaging, and the overproduction of proinflammatory cytokines (eg, TNF- α , IL-12, IFN- γ , and IL-18) results in sustained sepsis, shock, and even death. Recent studies^{35–39} have pointed towards a surprisingly complex relationship between Th17 and Treg responses during *C. albicans* infections, wherein Th17-mediated immunity is crucial for protection against *C. albicans* infections, especially mucocutaneous infections, while Treg mainly restrains immunopathology. Our study found that the proportion of Th17 and Treg cells in WT mice infected with *C. albicans* was significantly higher than that in control mice, which indicated that Th17 and Treg cells played an important role in mice anti-*C. albicans*. However, excessive Th17 cells or a decrease of Treg cells could reduce the ability against *C. albicans* infection in mice. So, it is important to

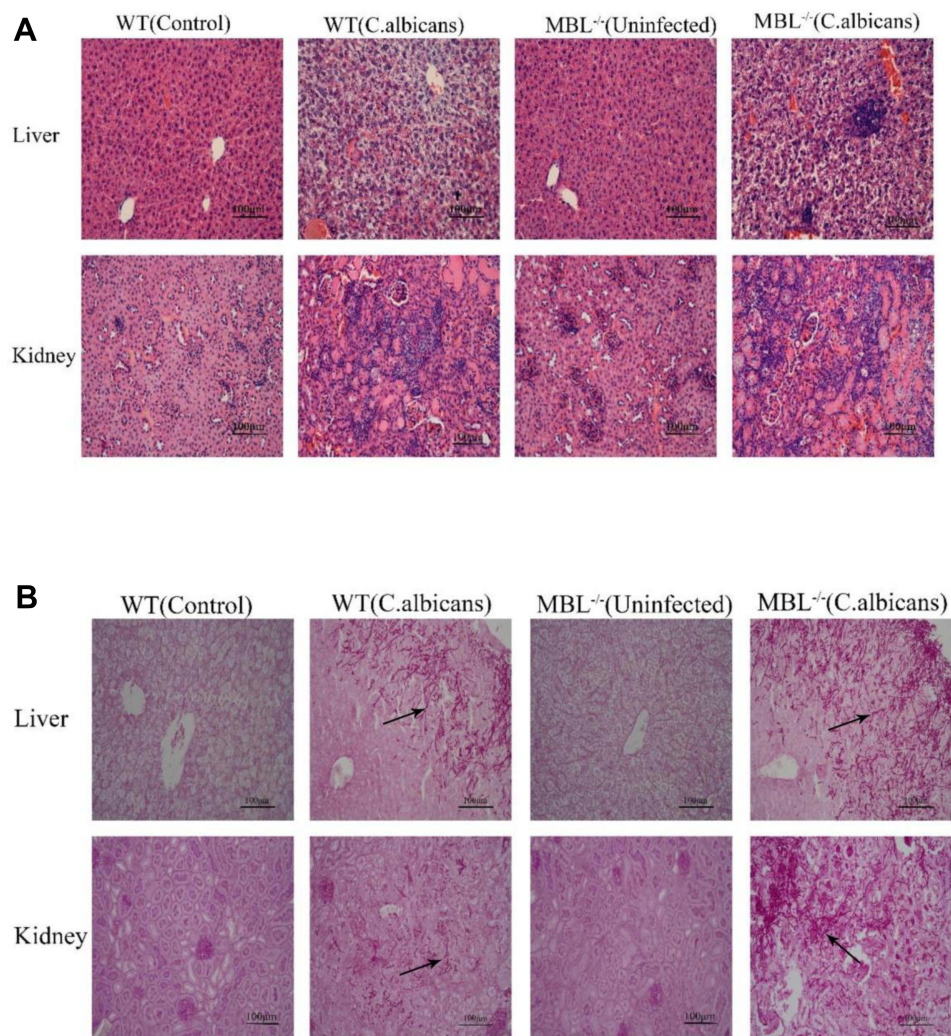


Figure 5 Histopathological changes in WT and $MBL^{-/-}$ mouse tissues after invasive *Candida albicans* infection. **(A)** Representative images of Hematoxylin and eosin (H&E)-stained liver and kidney. **(B)** Representative images of PAS-stained liver and kidney. ($\times 400$ magnification, Scale bar = $100\mu\text{m}$). The arrows indicate representative *C. albicans* hyphae.

maintain the balance between Th17 and Treg cells in the process of anti-pathogen microbial infection, but the mechanism needs further study.

There is some evidence indicating that pattern recognition receptors on antigen-presenting cells recognize *C. albicans*, leading to the secretion of IL-1 β , IL-23, and IL-6, as well as other specific cytokines.^{40,41} The release of these cytokines facilitates the differentiation of $CD4^{+}$ T cells into Th17 cells, which expresses IL-17 (also known as IL-17A), IL-17F, and IL-22. In addition to Th17 cells, Tregs are also an important subpopulation of $CD4^{+}$ T cells. In a stable state, Tregs suppress immunity by consuming the cytokine IL-2. Under inflammatory conditions, however, Treg cells show more potent immunosuppressive effects. This inducible iTreg may be mediated by IL-10 or TGF- β in an immunosuppressive environment, leading to antigen-specific or bypassing immunosuppression.^{42,43}

Accumulating evidence has indicated that the IL-6-mediated JAK/STAT and TGF- β -mediated TGF- β /SMAD signaling pathways play important roles in naive $CD4^{+}$ T cell differentiation.^{17,44–46} Moreover, the IL-6/JAK/STAT and TGF β /SMAD pathways may influence the Th17/Treg balance. Consequently, we investigated the involvement of JAK/STAT and TGF- β /SMAD signaling in mice infected with *C. albicans*. The cytokine environment is a major determinant of the Th17/Treg balance. IL-6 is an important inflammatory cytokine that activates the tyrosine/serine phosphorylation of STAT3 via the cytokine receptor pathway, and the phosphorylation of STAT3 promotes the synthesis of ROR γ t. The

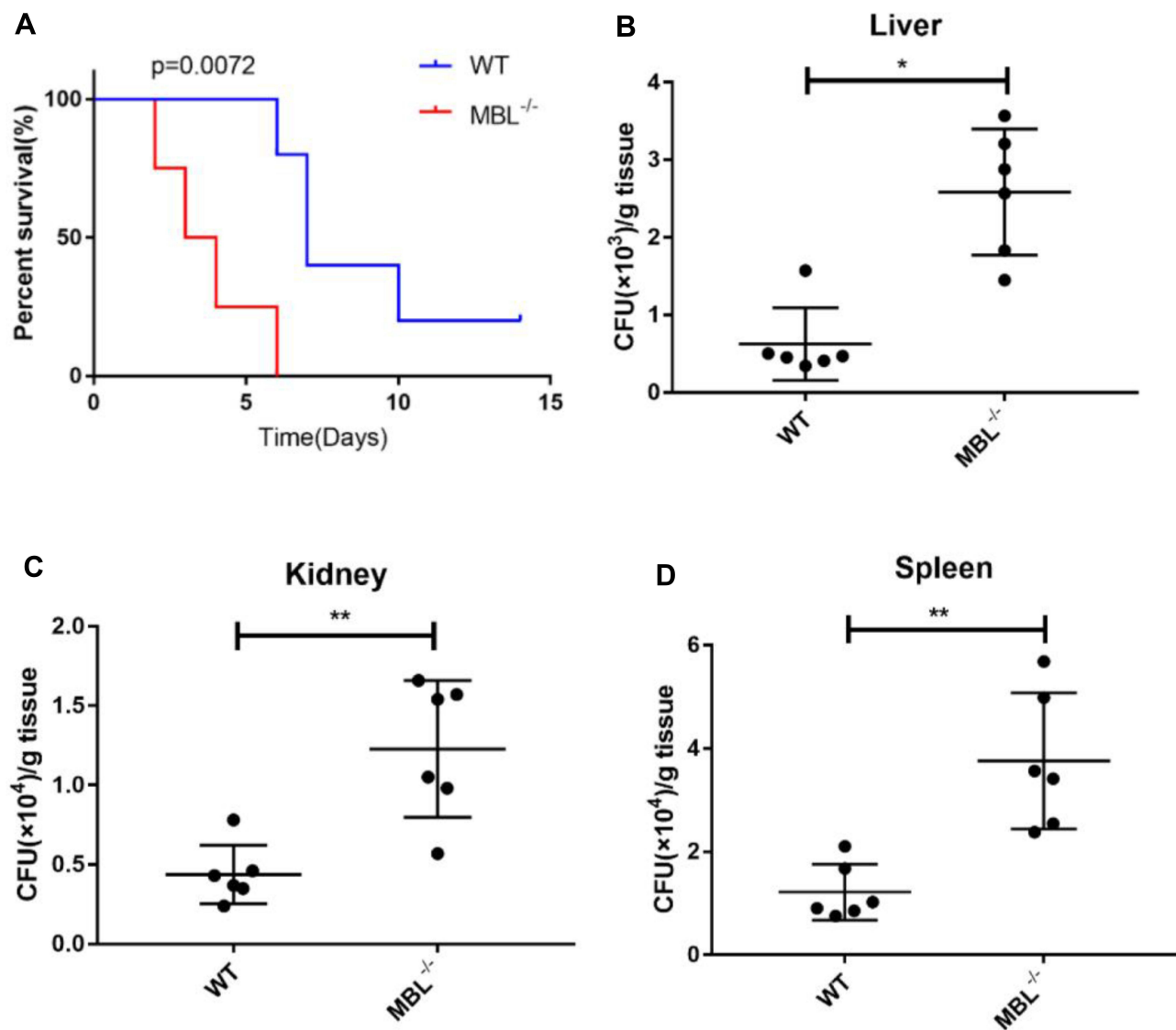


Figure 6 MBL deficiency influenced survival rates and fungal tissue burden. **(A)** Survival curves comparing the control and MBL-deficiency groups. **(B)** The number of colony-forming units (CFUs) in the livers of WT and MBL-deficient mice was determined on day 3 after intraperitoneal infection with 5×10^7 CFUs *C. albicans*. **(C)** The number of CFUs in the kidneys of WT and MBL-deficient mice was determined on day 3 after intraperitoneal infection with 5×10^7 CFUs *C. albicans*. **(D)** The number of CFUs in the spleens of WT and MBL-deficient mice was determined on day 3 after intraperitoneal infection with 5×10^7 CFUs *C. albicans*. One-way analysis of variance (ANOVA) was used for statistical difference analysis (* $P < 0.05$, ** $P < 0.01$).

combined action of IL-2 and TGF- β can promote the tyrosine phosphorylation of STAT5, while the phosphorylation of STAT5 promotes the synthesis of Foxp3.

Moreover, TGF- β has a joint function in differentiating both Th17 and Treg. TGF- β first binds to the TGF- β receptor (TGF- β R), mainly activating the SMAD transcription factors SMAD2 and SMAD3, and the common SMAD mediator, SMAD4. The activated SMAD complexes translocate into the nucleus and regulate the transcription of ROR γ t and Foxp3, critical transcriptional factors of Th17 and Treg. The increase in the expression of ROR γ t promotes the differentiation of CD4⁺ T cells into the Th17 subgroup, while the increase in Foxp3 expression promotes the differentiation of CD4⁺ T cells into the Treg subgroup.

Therefore, we investigated whether MBL regulates the Th17/Treg balance through the JAK/STAT and TGF- β /SMAD cytokine receptor signaling pathways. The results showed that, compared with infected WT mice, the levels of p-STAT3^{Y705}, p-SMAD2^{S465+467}, and ROR γ t increased significantly in infected MBL-deficient mice, while the protein levels of SOCS3 and SMAD7 decreased. Furthermore, in MBL double-KO mice infected with *C. albicans*, the levels of

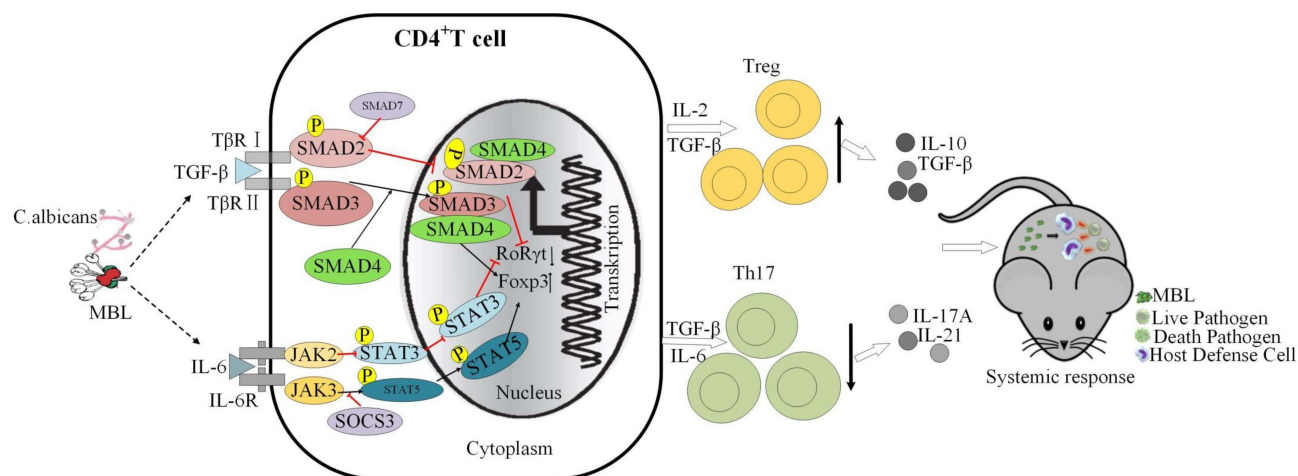


Figure 7 Model of MBL regulating the Th17/Treg balance in *C. albicans* infection. (1) MBL efficiently inhibits *C. albicans* infection; (2) MBL inhibits the expression of TGF- β and IL-6 after infection; (3) TGF- β interacts with its receptor, TGF- β R, to activate Foxp3 through the SMAD3/4 signaling pathway; in addition, IL-6 interacts with IL-6R to activate Foxp3 through the JAK3/STAT5 signaling pathway; (4) TGF- β interacts with TGF- β R to activate SMAD2/4 signaling pathway and stimulate ROR γ t; (5) Foxp3 promotes the differentiation of CD4⁺ T cells into the Treg subgroup, whereas ROR γ t promotes the differentiation of CD4⁺ T cells into the Th17 subgroup; (6) MBL regulates inflammatory responses by promoting changes in the Th17/Treg balance.

p-STAT5^{Y694}, p-JAK2^{Y1007+Y1008}, p-JAK3^{Tyr980/981}, p-SMAD3^{S423+S425}, and Foxp3 were lower than those in infected, WT mice, indicating that MBL can regulate the Th17/Treg balance through the JAK/STAT and TGF- β /SMAD cytokine receptor pathways.

Conclusion

We determined the MBL-related effects on adaptive immune responses to *C. albicans* infection, as well as the associated mechanisms, focusing on the differentiation of naive CD4⁺ T cells. Our results suggested that MBL could regulate the Th17/Treg cell balance by IL-6- and TGF- β -mediated JAK/STAT and TGF- β /SMAD signaling, and Th17/Treg cell balance might maintain an appropriate inflammatory response against pathogen infection (Figure 7). This study extends our knowledge of how innate immunity-related molecules regulate the inflammatory response and provide a potential way to prevent and treat fungal diseases by regulating the level of MBL.

Ethics Approval and Consent to Participate

All experiments were approved and carried out following the Xinxiang Medical University Animal Care Committee guidelines (Reference No. 2015016).

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Disclosure

The authors declare that they have no competing interests in this work.

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