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L6H21 prolonged rats survival after limb allotransplantation by inhibiting acute rejection

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Abstract. – OBJECTIVE: Preventing and reducing allograft rejection play a far more important role in limb allotransplantation. We previously found L6H21 could inhibit LPS-induced (lipopolysaccharide LPS) overexpression inflammatory factors in macrophages and specifically targets to MD-2 (myeloid differential protein-2 MD-2) required for TLR4 (Toll-like receptor 4 TLR4) activation and represented an important therapeutic target in inflammatory disorders. Therefore, we evaluated the effect and explored the mechanism of L6H21 in rats' limb allograft model.

MATERIALS AND METHODS: The efficacy of L6H21 was evaluated in limb allograft rats and cyclosporine (CY-A) was used as a positive control agent. T-Lymphocyte in blood was analyzed and dendritic cells (DCs) separated from spleens using flow cytometry. ELISA was used to measure serum cytokine levels. Analysis of protein expressions was performed using Western blotting.

RESULTS: L6H21 reduced the risk of acute rejection and prolonged survival of limb allograft rats. At 3 d and 5 d post-transplant, the ratio of CD4+/CD8+ was decreased in L6H21 group. L6H21 suppressed the content of IL-1α at 7d, IL-5 and IL-10 at both 3 d and 7 d after transplantation. L6H21 decreased the protein expressions of IRF3, p-IRF3, P38, p-P38 and p-IκBα while increased IκBα expression and decreased the ratio of p-IRF3/ IRF3, p-P38/ P38, p-IκBα/IκBα correspondingly.

CONCLUSIONS: L6H21 could reduce the risk of acute rejection and prolong the survival of limb allograft rats through inhibiting the ratio of CD4+/CD8+ in blood and serum cytokine levels and suppressing protein expressions of IRF3, p-IRF3, P38, p-P38 and p-IκBα in DCs. So, it may serve as a potential candidate for the treatment of allograft rejection.

Key Words

Acute rejection, CY-A, Cyclosporine, DCs, Dendritic cells, Limb allotransplantation, L6H21, (E)-2, 3-dimethoxy-4-methoxychalcone.

Introduction

Limb allotransplantation as one of composite tissue allografts that including grafting of heterogeneous tissues consisted of a combination of skin, bone, muscles, fat, subcutaneous tissue, neurovascular tissues, and nerves, all of which with different antigenicities[1,2]. In recent years, as the number of limb allotransplantations is increasing, more attention should be drawn to reduce and prevent acute and chronic rejection[3-5]. Emerging as a solution to limb reconstruction, limb allotransplantation is based on the technical feasibility of replantation and the development of successful immunosuppressive agents[6]. Allograft rejection is a powerful adaptive immune response characterized by substantial T and B cell activation and the generation of long-lasting immunological memory[7]. The toll-like receptor (TLR) family members including the recognition of lipopolysaccharide (LPS) of Gram-negative bacteria (TLR4) recognize microbial cell wall components and microbial nucleic acids[8-10]. TLR4 could be activated by TLR4 polymorphisms were correlated with the incidence of kidney and lung rejection in human[11-13]. As an important subsystem of the overall immune system, the innate immune system comprising the cells and mechanisms that defend the host from infection by other organisms, also known as the non-specific immune system or in-born immunity system, does not confer long-lasting or protective immunity to the host[13]. The innate immune system is an important factor in transplantation immune-biology. During the transplantation procedure, thermal and metabolic stresses are sufficient to trigger the innate immune response and also augment adaptive immunity in the presence of foreign antigen on the donor organ[14]. Although the innate immune mechanisms alone are not sufficient to lead to graft rejection itself, they are responsi-

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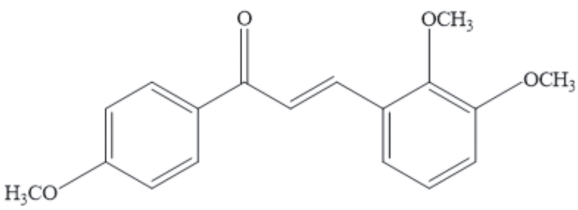
ble for the initial inflammatory events following engraftment and important for optimal adaptive immune responses to the graft, which may play a major role in resistance to tolerance induction[15]. TLR pathways contribute to the pathogenesis of ischemia-reperfusion injury, a form of injury to which all grafts are subjected to the transplantation procedure, which mediated by pattern recognition receptors (PRR) or other components of the innate immune system[7]. Damage-associated molecular patterns (DAMPs), such as LPS could activate TLR, particularly TLR4[16]. As previously reported, we synthesized a series of chalcone derivatives and evaluated their anti-inflammatory efficacy in LPS stimulated macrophages and mouse models[17]. (E)-2, 3-dimethoxy-4-methoxychalcone (L6H21) (Figure 1) involved in the synthetic chalcones showed an excellent anti-inflammatory activity. MD-2 is one of the important therapeutic targets for inflammatory disorders[18-20]. L6H21 could target to MD-2 by inserting to its' hydrophobic region and suppressed mitogen-activated protein kinase (MAPK) phosphorylation, NF-κB activation and cytokine expression in macrophages *in vitro,* subsequently while improved survival, prevented lung injury, decreased serum and hepatic cytokine levels in mice subjected to LPS *in vivo*[20]. Therefore, we hypothesized that the new chalcone derivative L6H21 may serve as an anti-rejection candidate. The objective of our study was to explore effects and mechanism of L6H21 against allograft rejection *in vivo.*

Materials and Methods

Animals and Surgical Procedure

Healthy male Sprague-Dawley rats (SD) weighing 250-280 g as donors and Wistar rats as recipients weighing 250-300 g. Limb replantation model was made according to previous reports[21-23]. Intraperitoneal injection of ketamine was used for anesthesia as a dose of 200 mg kg^{-1}. Care was taken to preserve the skin of the grafted, which was used to monitor the circulation and rejection. The donor limb was orthotopically transplanted into the recipient. With a Kirschner wire (1.2 mm) as an intramedullary rod, osteosynthesis was performed. Using microsurgical technique, the femoral vessels were anastomosed, epineurium was sutured with 9-0 nylon suture and the sciatic nerve was sutured with 11-0 nylon suture. At the end, we checked the permeability of blood vessels. Transplantation from SD to Wistar was across a major histocompatibility barrier[23,24].



(E)-2, 3-dimethoxy-4'-methoxychalcone
L6H21
(Compound 30 in Ref JMC, 54, 8110-8123)

Figure 1. Structure of the compound L6H21 (Wu et al)[17].

Experimental Groups and Administration

The animals after transplantation were divided into 3 groups according to the administration immediately after surgery by intraperitoneal injection. Cyclosporine A (CY-A) (Solarbio, Beijing, China) was given[25] in a dose of 10 mg kg^{-1} day^{-1}, L6H21 synthesized and structurally identified as described in previous reports[17,20] was given in a dose of 10 mg kg^{-1} day^{-1} and an equal volume of normal saline (NS) was used as control. All the animals were treated for 7 days.

Evaluation

The general condition of the animals was observed daily. The grafted skin that had erythema for 24 h was taken as onset of rejection of the grafted limbs. Graft warm ischemia time, revascularization surgery, swelling, color, temperature, body hair change, oozing, ulcers, erosions and whether toe was necrotic or not were observed daily.

Detection of T-lymphocyte and Purification of Dendritic Cells

Rat blood of inner canthus was collected at 1, 3, 5, 7 d after treatment. Fluorescence-activated cell sorting of blood Helper T cells (Th, CD4+) and cytotoxic T cells (Tc, CD8+) was performed by flow cytometry. Blood samples were incubated with mouse anti-rat of CD4-PE (Clone W3/25, Bio-Legend, San Diego, CA, USA) and CD8a-FITC (Clone OX-8, Bio-Legend, San Diego, CA, USA). Analyses were performed on 1×10^4 mononuclear cells using FACS Scan (BD, San Diego, CA, USA) and CellQuest software (BD Biosciences, San Diego, CA, USA). DCs were purified from recipient rats' spleens after transplantation with a limb graft. Monocyte suspension derived from spleen was prepared. Then DCs were separated and characterized by lymphocytes DC-associated markers (CD123, MHC Class II and CD11b/c) (Miltenyi Biotechnology, Teterow, Germany) which was performed and analyzed by flow cytometry.

Western Blot Analysis

DCs deprived of spleens of recipient rats were washed with cold phosphate-buffered solution (PBS) and subsequently lysed in 1× sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. The samples of cell lysis were heated to 95-100°C for 10 min followed by cooling on ice and centrifuged at 11.000×g for 1 min at 4°C. The supernatant was run on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred electrophoretically to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After being blocked for 1 h at 25°C with 5% skim milk in Tris-buffered saline containing Tween 20 (TBST), the blots were incubated with primary antibodies against GAPDH (Sungene Biotech, Shanghai, China), IRF3 (Cell Signaling Technology CST, Shanghai, China), p-IRF3 (Cell Signaling Technology CST, Shanghai, China), P38 (Cell Signaling Technology CST, Shanghai, China), p-P38 (Cell Signaling Technology CST, Shanghai, China), IκB (Cell Signaling Technology CST, Shanghai, China) and p-IκB (Cell Signaling Technology CST, Shanghai, China) overnight at 4°C. After being washed with Tris-buffered saline-tween (TBS-T), the membranes were incubated with proper secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA). Blots were then incubated and visualized with enhanced chemiluminescence (ECL, Thermo Scientific, Waltham, MA, USA). The results were normalized to GAPDH to correct for loading.

Measurement of IL-1α, IL-5 and IL-10 by ELISA

Serum content of IL-1α and IL-10 was measured using assay Kits and an assay Kit used to measure IL-5. Experiments were performed exactly according to the kits' instructions of the manufacture and samples were assayed in triplicate. Results were detected using a microplate ELISA reader at 450 nm. The antibodies used in the ELISA were specific for IL-1α, IL-5 and IL-10. The results were expressed as picograms of IL-1α, IL-5 or IL-10 per milliliter (pg ml^{-1}).

Histology

Rats after being treated for 7 d were sacrificed by cervical dislocation and their grafted limbs were collected and frozen at -20°C until analysis. For histological observation, paraffin-embedded rats' grafted limbs sections (5 μm thick) were prepared. Slides were deparaffinized and the sections were stained with Hematoxylin and Eosin (H&E) for histological examination (Nikon ECLIPSE TS2000-0, Tokyo, Japan).

Efficacy Studies

The efficacy of L6H21 was evaluated in recipient rats. Saline and CY-A were used as the negative and positive control respectively. Wistar rats after transplantation surgery were grouped and treated as described above. Animals were weighed and checked for survival every day.

Statistical Analysis

Data are presented as means ± standard deviations (SD) of three to five samples from independent experiments. The differences between two groups were analyzed using Student's *t*-test and more than two groups needing multiple comparisons were evaluated by one-way ANOVA with Bonferroni post-hoc test. Survival curves were built on the basis of a Kaplan-Meier curve, and a log-rank (Mantel-Cox) test was used to determine their statistical differences. SPSS 16.0 (SPSS Inc., Chicago, IL, USA) was used to analyze all the data. When *p*-value was less to 0.05, differences were considered significant.

Results

L6H21 Reduced the Risk of Acute Rejection

1 d after being treated with Saline, rats were not significantly different from that before surgery. At about 3 d post surgery, the grafted limbs began to swell (Figure 2A). Body hair of grafted limbs started to be tarnished at 3 d and turned wet followed by hair falling, a wide range of eschar formation and skin erosion at about 4 d to 5 d after surgery. By days 7, the grafted limbs had turned black and with apparent necrosis. The time for the grafted limbs of rats in CY-A and L6H21 group were noticeably swollen or necrosis was longer than that in Saline group. At 7 d after transplantation, histological examination revealed lymphocytic infiltration around blood vessels with large vacuoles and necrosis in the epidermis of the transplanted limbs in both the traditional and modified models (Figure 2B).

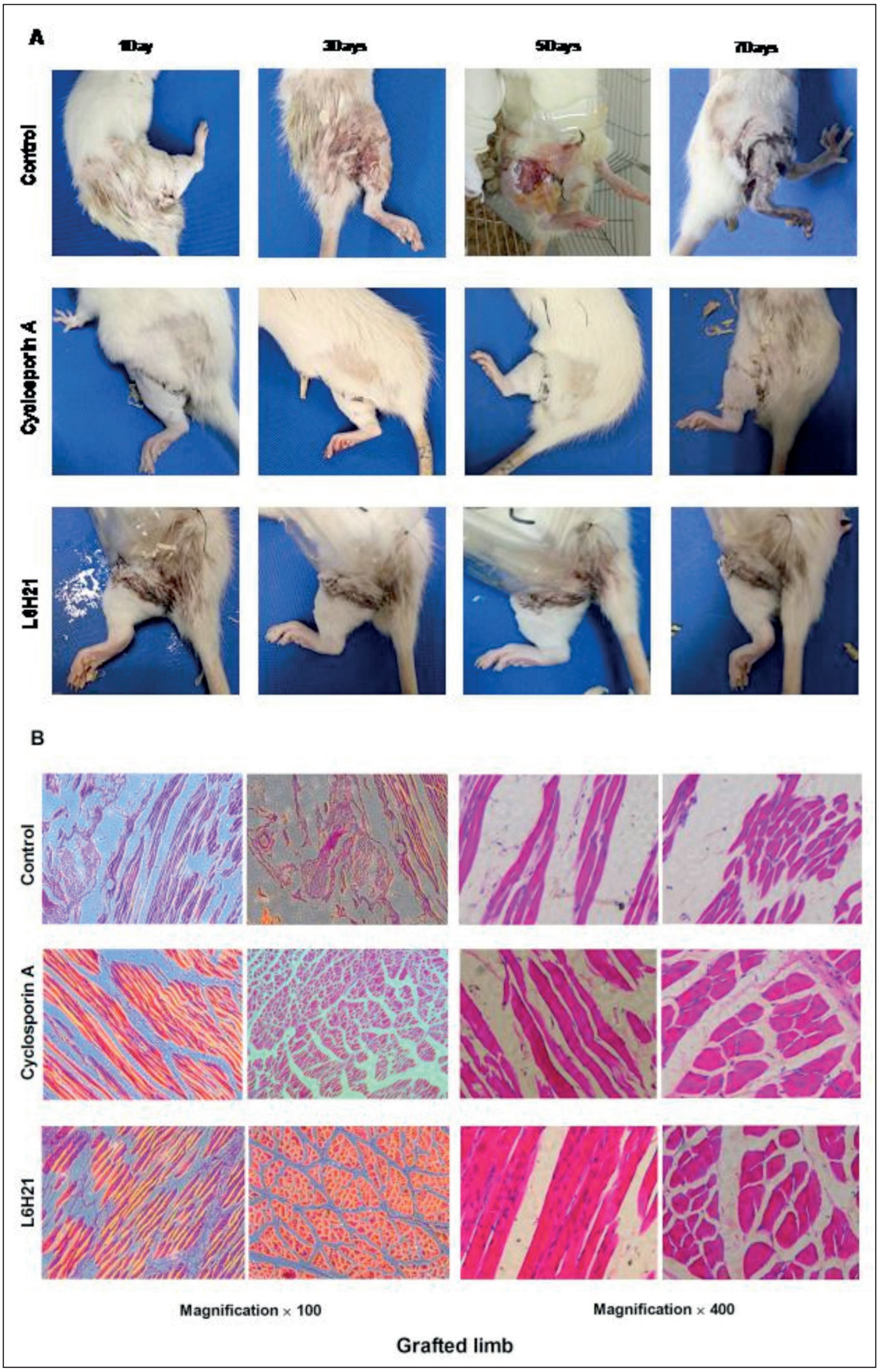


Figure 2. L6H21 attenuated the risk of acute rejection. (***A*** and **B**) SD rats were used as donors and Wistar rats as recipients to made limb replantation model. The animals after transplantation were treated with Cyclosporine A (CY-A) and L6H21 at a dose of 10 mg kg^{-1} day^{-1} and an equal volume of normal saline (NS) (Control) by intraperitoneal injection (n=5) for 7 days. ***A***, Allografted limb of rats at 1, 3, 5, 7 d after transplantation and treatment. **B**, Rats were sacrificed by cervical dislocation at 7 d after treatment. Allografted limb histopathological analysis was performed using Hematoxylin and eosin (H&E) staining as described in the Methods (n=5).

L6H21 Decreased the Ratio of CD4+/CD8+

As shown in Figure 3, at 1 d after surgery, no CD4+/CD8+ ratio difference was found among all the groups. At 3 d post surgery, CD4+/CD8+ T cell ratio was obviously increased in saline group. It was similar to CY-A that L6H21 significantly reduced the ratio of CD4+/CD8+ T. At 5 d after surgery, although the ratio of CD4+/CD8+ T cell was decreased in all groups, CY-A or L6H21 significantly reduced CD4+/CD8+ T cell ratio. CD4+/CD8+ T cell ratio in all groups was no difference at either 7 d or 1 d.

L6H21 Affected the Content of IL-1α, IL-5 and IL-10

It was shown in Figure 4A that CY-A suppressed the content of IL-1α in peripheral blood compared with control at 3 d after transplantation, while L6H21 had no significant effect on IL-1α content. At 7 d after transplantation, it was increased which was significantly decreased by either CY-A or L6H21. Change in concentration of IL-5 was similar to that of IL-10 (Figure 4B-4C). At 3 d after transplantation, the content of IL-5 and IL-10 increased which was significantly inhibited by CY-A or L6H21. Despite both IL-5 and IL-10 content being significantly decreased at 7 d compared with 3 d in saline group, CY-A and L6H21 significantly suppressed IL-5 and IL-10 content at 7 d. IL-5 content in L6H21 group at 3 d and 7 d as well as IL-10 content at 3 d was significantly decreased compared with that in CY-A group, which was worthy of our attention.

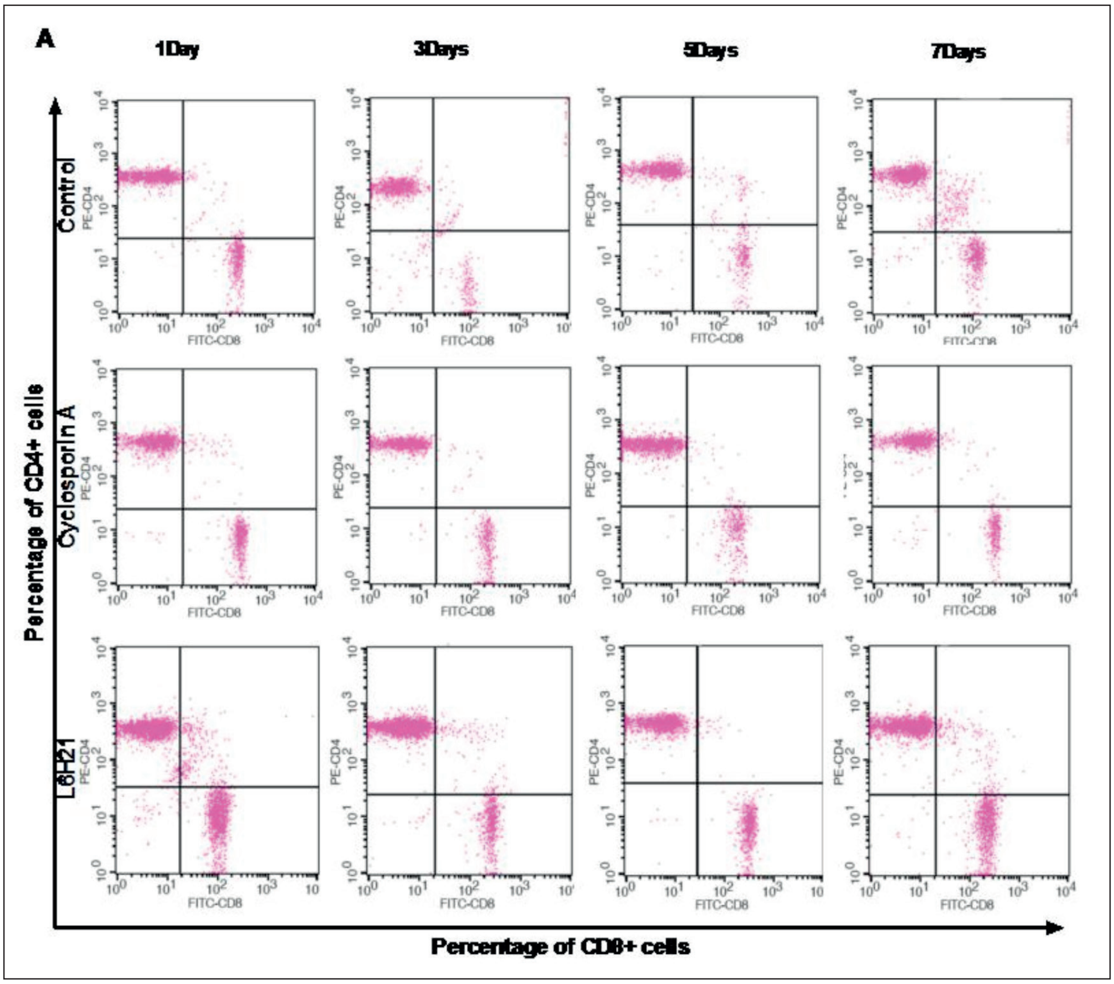


Figure 3. L6H21 suppressed the ratio of CD4+/CD8+. (A-C) CD4+ and CD8+ T cells were detected by flow cytometry. Percentages of CD4+ and CD8+T cells were obtained and the ratio of CD4+/CD8+ was calculated by percentages of CD4+ and CD8+ T cells. The ratio of CD4+/CD8+ in peripheral blood of rats was analyzed by flow cytometry at 1, 3, 5, 7 d after transplantation and treatment with Cyclosporine A (CY-A) and L6H21 at a dose of 10 mg kg^{-1} day^{-1} and an equal volume of normal saline (NS) (Control) by intraperitoneal injection. All data are presented as mean±SD, n=3. *$p<0.05$, **$p<0.01$ *vs.* control.

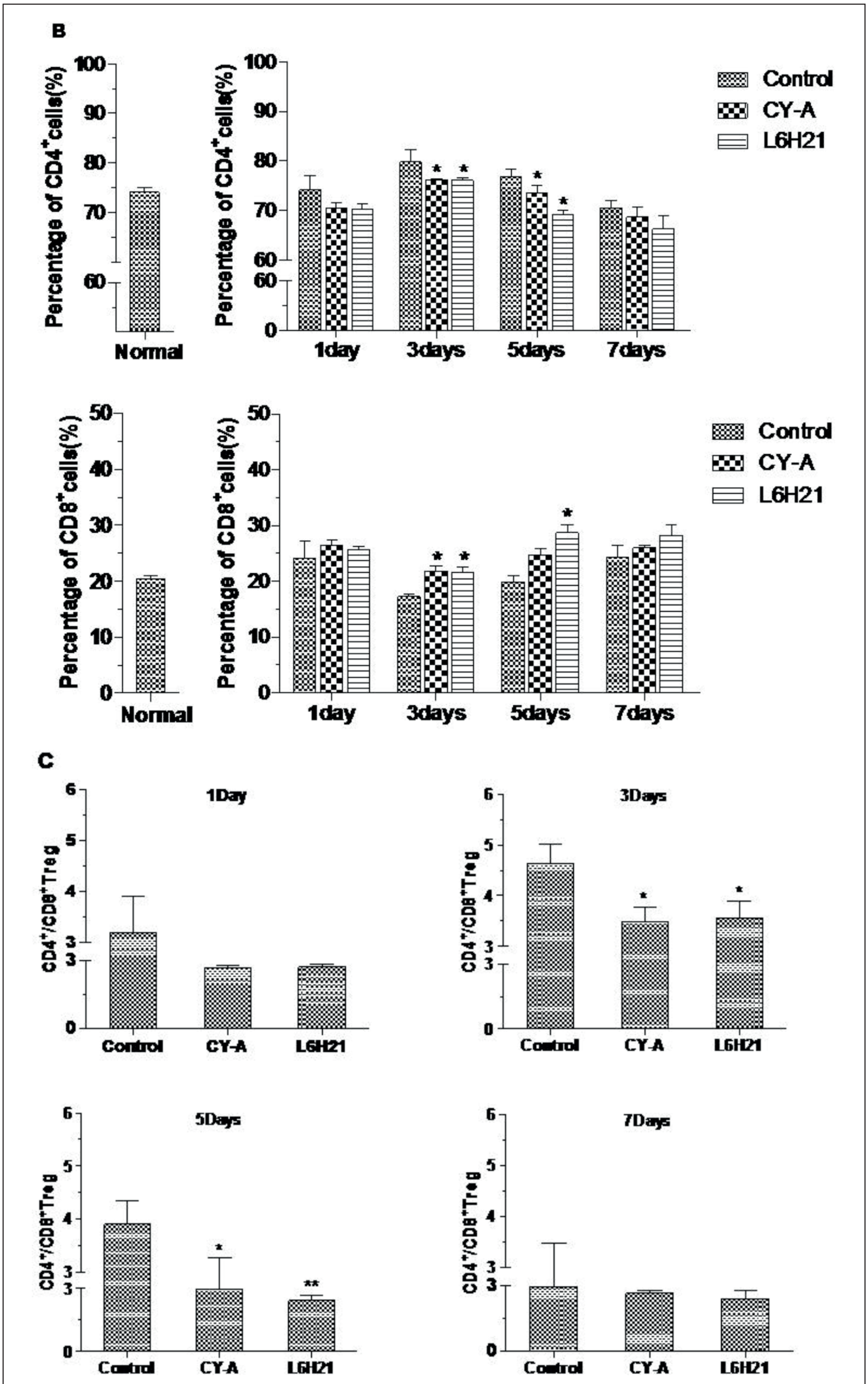


Figure 3. (Continued).

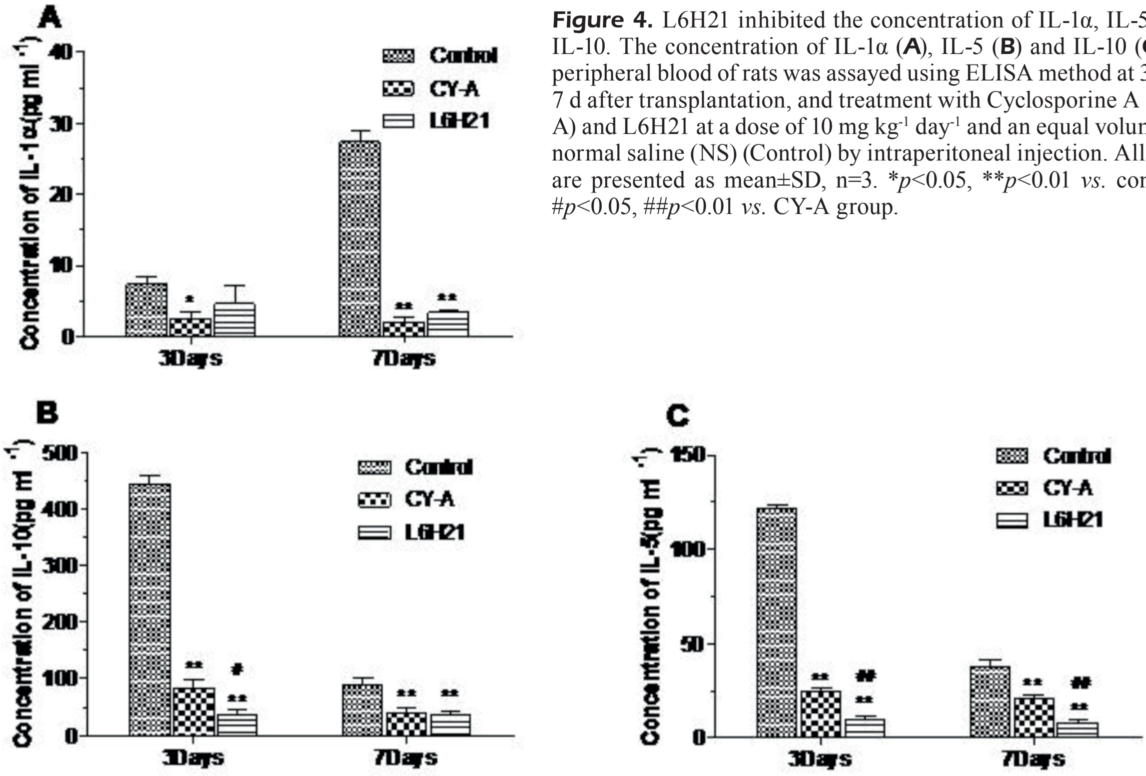


Figure 4. L6H21 inhibited the concentration of IL-1α, IL-5 and IL-10. The concentration of IL-1α (**A**), IL-5 (**B**) and IL-10 (**C**) in peripheral blood of rats was assayed using ELISA method at 3 and 7 d after transplantation, and treatment with Cyclosporine A (CY-A) and L6H21 at a dose of 10 mg kg^{-1} day^{-1} and an equal volume of normal saline (NS) (Control) by intraperitoneal injection. All data are presented as mean±SD, n=3. *$p<0.05$, **$p<0.01$ *vs.* control; #$p<0.05$, ##$p<0.01$ *vs.* CY-A group.

Protein Expression of IRF3, p-IRF3, P38, p-P38, IκBα, p-IκBα in DCs of Rats Treated With L6H21

As shown in Figure 5, the protein expressions of IRF3, p-IRF3, P38, p-P38 and P-IκBα in both CY-A and L6H21 groups were obviously decreased compared with saline group. However the IκBα was remarkably increased in CY-A and L6H21 groups. The p-IRF3, p-P38 and p-IκBα were especially low in CY-A and L6H21 groups. The ratio of p-IRF3/IRF3, p-P38/P38, p-IκBα/IκBα was correspondingly lower in CY-A and L6H21 groups. As compared with CY-A, the p-P38 and p-IκBα as well as the ratio of p-P38/P38, and p-IκBα/IκBα was significantly decreased in L6H21 group. Furthermore, there was no significant difference in the protein expressions of IRF3, p-IRF3, P38 and IκBα between L6H21 and CY-A group.

L6H21 Prolonged Survival and Maintained Rate of Weight-Gain in Rats Underwent Transplant Surgery

Survival of recipient rats after different treatments were presented in a Kaplan-Meier plot as indicated in Figure 6A. All the animals in saline group died and the median survival time was only 3.5 day. Animals treated with CY-A had a median survival time of 14 days. CY-A and L6H21 were found to be significantly more effective in prolonging recipient rats survival than Saline ($p<0.05$). As shown in Figure 6B, weight-gain rate curve of animals treated with L6H21 started to be steadily elevated at 5 d and later post transplantation.

Discussion

Transplant rejection is a frequent and severe complication after limb allotransplantation which was one of composite tissue allografts, particularly acute rejection occurring within the first period post transplantation involving skin, lung, gut and liver organs regularly[26-28]. Infiltrations of monocytes/macrophages are recognized as a hallmark of acute allograft rejection[29]. It was reported that the absence of immunosuppressive macrophages may increase the susceptibility of human lung allografts to the rejection process[29,30].

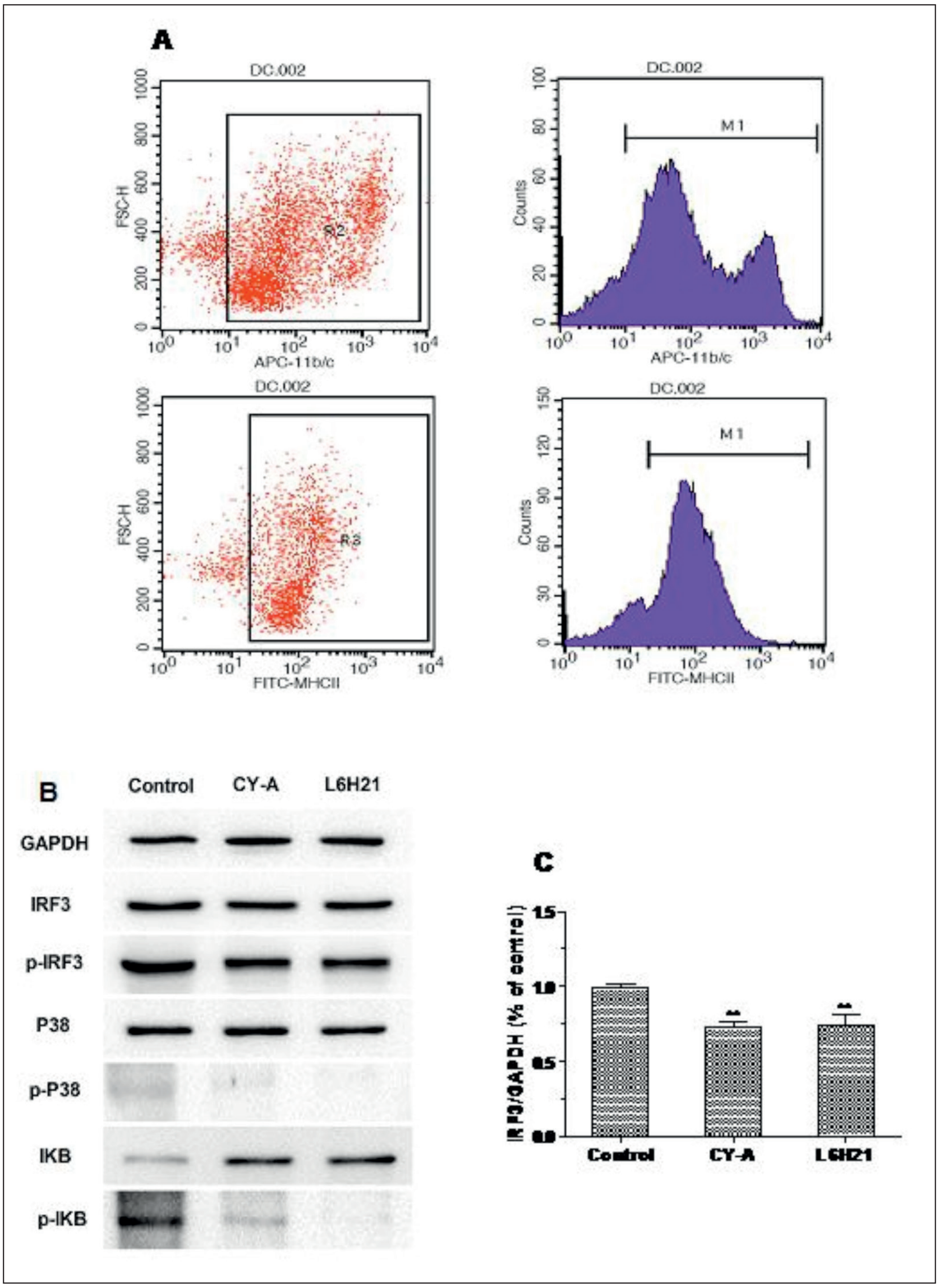
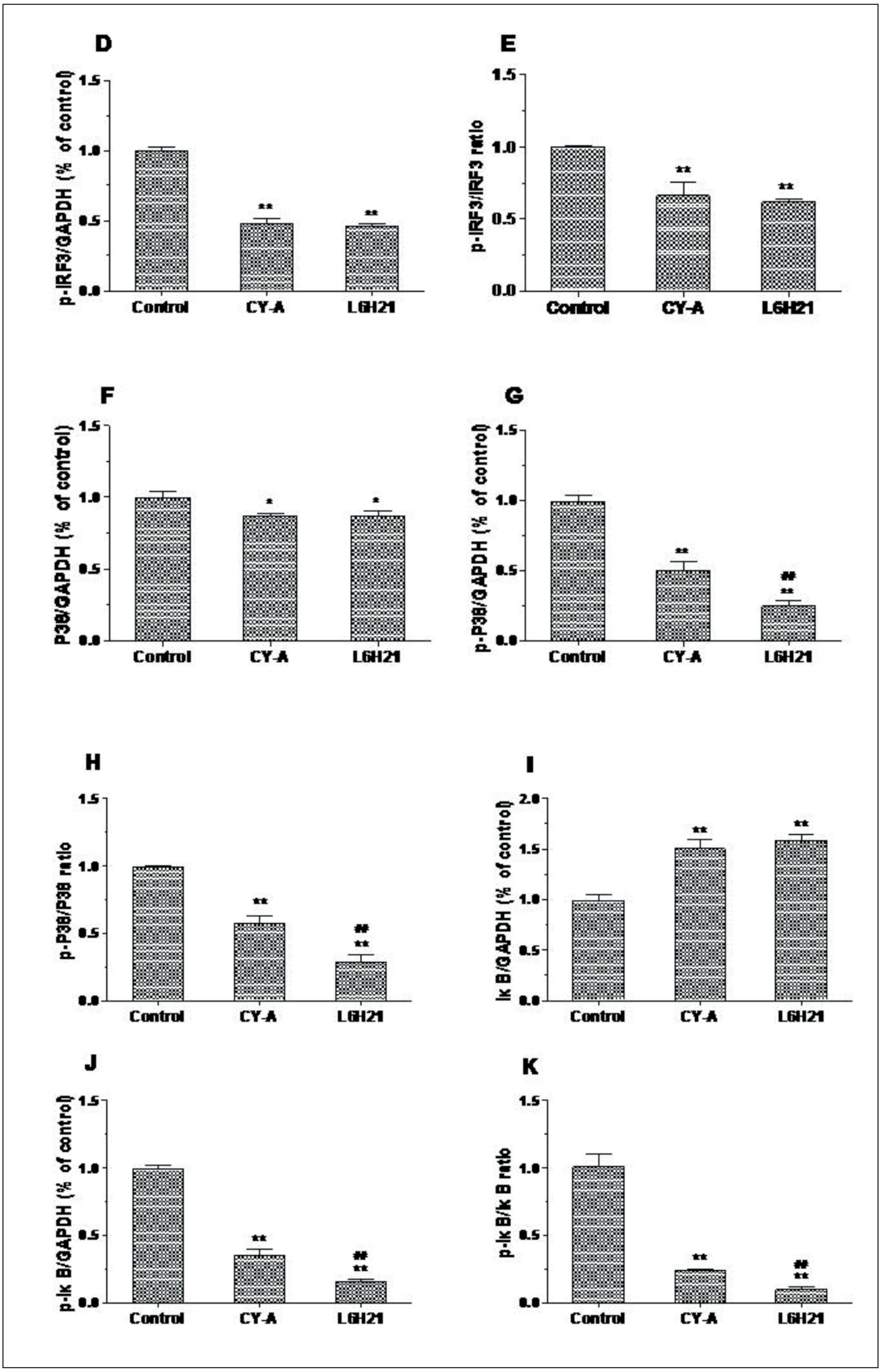


Figure 5. L6H21 suppressed the proteins expression of IRF3, p-IRF3, P38, p-P38, and p-IκBα while promoted IκBα expression in dendritic cells (DCs). DCs were separated from recipient rats' spleens after transplantation and treatment with Cyclosporine A (CY-A) and L6H21 at a dose of 10 mg kg^{-1} day^{-1} and an equal volume of normal saline (NS) (Control) by intraperitoneal injection which was described in method section. **A**, DCs were characterized by MHC Class II and CD11b/c performed by flow cytometry. The proteins expression of IRF3 (**C**), p-IRF3 (**D**), P38 (**F**), p-P38 (**G**), IκBα (**I**) and p-IκBα (**J**) were measured by Western blotting and GAPDH was used as positive control (**B**). **E**, **H**, and **K** show the ratio of p-IRF3/ IRF3, p-P38/P38, p-IκBα/IκBα, respectively. All data are presented as mean±SD, n=3. *p<0.05, **p<0.01 *vs.* control; #p<0.05, ##p<0.01 *vs.* CY-A group.



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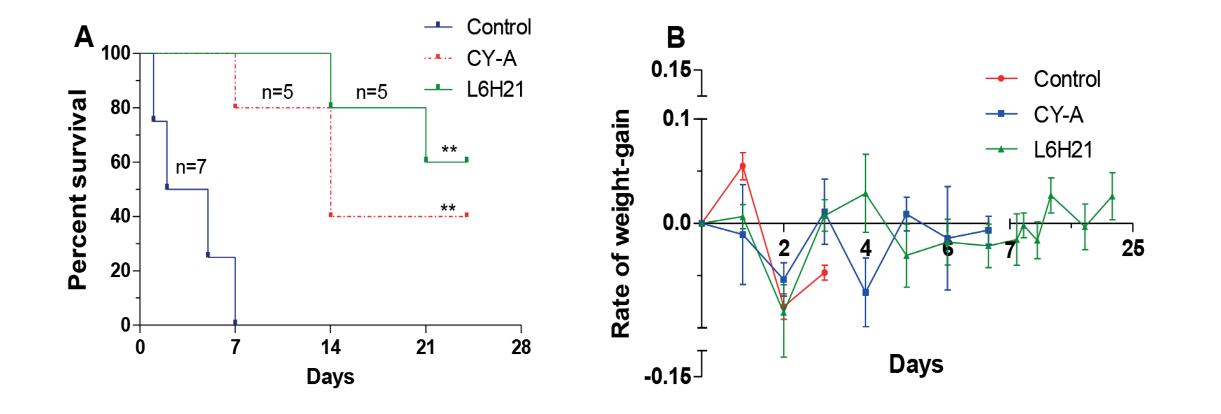

Figure 6. L6H21 prolonged survival in rats underwent transplant surgery. Rats after allogeneic transplantation were treated with Cyclosporine A (CY-A) (n=5) and L6H21 (n=5) at a dose of 10 mg kg^{-1} day^{-1} and an equal volume of normal saline (NS) (Control) (n=7) by intraperitoneal injection for 7 days. Considering the animals after transplantation treated with NS were likely to die, there were 7 rats in control group, which was more than the other two groups. **A**, Showed survival rates and body weight was showed in **B**,. ***p*<0.01 *vs.* control.

Classically activated macrophages (M1) as one of populations with distinct functions based on their responses to environmental stimuli, stimulated by LPS, can secrete high levels of pro-inflammatory cytokines and mediators, such as, TNF-α, IL-1β and IL-6[31]. We previously found that chalcone derivative L6H21 could inhibit LPS-induced overexpression of TNF-α and IL-6 in macrophages. *In vivo*, L6H21 pretreatment decreased serum and hepatic cytokine levels in mice subjected to LPS. Moreover, mice with MD-2 gene knockout were universally protected from the effects of LPS-induced septic shock. MD-2 is an assistant protein of TLR4. Treatment with L6H21 in mouse RAW 264.7 macrophages significantly inhibited the LPS-induced TLR4/MD-2 complex in a dose-dependent manner. Compound L6H21 inserted into the hydrophobic region of the MD-2 pocket, forming hydrogen bonds with Arg^{90} and Tyr^{102} in the MD-2 pocket. L6H21 binds directly to MD-2, and specifically antagonizes the LPS-TLR4/MD-2 interactions[20]. In present study, L6H21 may play a similar role in immunosuppressive macrophages through binding with MD-2. Transplant rejection impairs immune reconstitution after transplantation and effective therapies aimed at restoring T cell counts in transplant rejection patients[32]. Transplant rejection insult to the peripheral lymphoid niche is responsible for affecting $CD4^+$ T cell reconstitution particularly during transplant rejection[32]. As previous reports, CD8+ T cell was associated with skin[33], heart[34], pancreatic islet[35] and kidney allograft[36]. CD4+ T cells as T helper 1 (Th1) cells are supposed to have a major role in the early acute rejection phase[37]. Both CD4+ cytotoxic T cells (CTLs) and CD8+ CTLs and NK cells together with soluble inflammatory agents mediate target tissue inflammation and destruction in the effector phase[38]. CD4+ and CD8+ T cell reconstitution was correlated with better survival in patients after allogeneic hematopoietic stem cell transplantation[37]. The increased early CD4+/CD8+ T cell ratio has been also reported as a promising cellular biomarker for acute rejection[37] and a higher CD4+/CD8+ was associated with a significantly increased risk of acute rejection grades[39]. At 1, 3, 5, 7 d after treatment with L6H21, Saline (negative control) and CY-A (positive control), animals' blood CD4+/CD8+ T cell was analyzed by flow cytometry. CD4+/CD8+ T cell ratio in CY-A or L6H21 group decreased most significantly at 3 d post transplantation and there was no difference at 7 d among all the groups. IL-1α is responsible for the production of inflammation and plays one of the central roles in the regulation of the immune responses. Inflammation is a key instigator of the immune responses that drive allograft rejection and IL-1α released from endothelial cells (ECs) damaged during transplant drives allograft rejection[40]. IL-5 is a key mediator in eosinophil activation, as well as stimulates B cell growth and increases immunoglobulin secretion. IL-10 is generally considered as an anti-inflammatory cytokine with multiple, pleiotropic, effects in immunoregulation and inflammation, which can block NF-κB activity. According to the results of T cell assay experiment, we measured animals' blood content of IL-1α, IL-5 and IL-10 after surgery. L6H21 reduced IL-1α and IL-5 content in blood. IL-10 blood content in negative control group at 3 d after surgery was significantly increased compared with the other two groups which remain decreased by L6H21 or CY-A at both 3 d and 7 d. Barbara et al[41] reported that Th1 cells secrete IL-10 could trigger islet rejection as efficiently as Th1 cells, which ensure that Th2-type immune activation was occurring within the local environment of the islet graft. Another study[42] showed that IL-10 elevated during rejection episodes in human liver allograft. Maturation/activation state of DCs is fundamental in the induction of tolerance[43]. Immature DCs can induce and maintain peripheral T-cell tolerance, while mature DCs induce T-cells immunity[43-45]. As reported, DC counts can be diminished during transplant rejection and transplant rejection-causing T cells can eliminate mature DCs and induce damage to the blood and marrow (BM) microenvironment, resulting in impaired DC production from donor hematopoietic stem cells[32]. MHC-allopeptide complexes expressed by DCs represent targets for transplant rejection-causing T cells and their elimination could limit overwhelming T cell activation[46]. IRF3 (IFN regulatory factor-3) could impact on the incidence and intensity of transplant rejection by modulating innate immune reactions and has an important role in the inflammatory response, particular in the activation of inflammatory responses initiated by DCs[47]. As reported, DCs express TLR4[48-50], and we previously found L6H21 binding to MD-2 protein that recognizes LPS, which is required for TLR4 activation, and represents an attractive therapeutic target for severe inflammatory disorders[20]. P38 involved in MAPK signaling pathway activated by a variety of cellular stresses including inflammatory cytokines, LPS and osmotic shock, was associated with differentiation, apoptosis and autophagy. Inhibitor of NF-κB (IκBα) involved in regulation of transcriptional responses to NF-κB, was associated with cells immune, pro-inflammatory responses, apoptosis, differentiation and growth. We purified DCs from recipient rats' spleens and assayed protein expression of IRF3, p-IRF3, P38, p-P38, IκBα, P-IκBα. It was demonstrated that L6H21 inhibited IRF3, p-IRF3, P38, p-P38 and P-IκBα expression, as well as upregulated IκBα significantly compared with control. Protein expressions of p-P38 and p-IκBα, as well as the ratio of p-P38/ P38 and p-IκBα/IκBα, were significantly decreased in L6H21 group compared with that in CY-A group. CY-A is one of the widely used immunosuppressive drugs in clinic. Although CY-A suppresses acute allograft rejection, it hinders transplant tolerance induction[51,52]. Furthermore, CY-A has side effects like hypertrichosis, gingival hypertrophy, tremor and increased blood pressure, especially hepatic and nephrotoxicity were its' main secondary effects. It is necessary to find drug candidate for allograft rejection. Our results indicated that L6H21 could decrease blood content of IL-5 at both 3 d and 7 d and IL-10 at 3 d after transplantation, along with reduce protein expressions of p-P38 and p-IκBα in DCs as well as the ratio of p-P38/P38, and p-IκBα/IκBα correspondingly.

Conclusions

L6H21 could reduce the risk of acute rejection and prolong the survival of limb allograft rats through inhibiting the ratio of CD4+/CD8+ in blood and serum cytokine levels and suppressing protein expressions of IRF3, p-IRF3, P38, p-P38 and p-IκBα in DCs. So, it may serve as a potential candidate for the treatment of allograft rejection. In summary, we evaluated the effect of L6H21, a chalcone derivative, on allograft rejection in limb allograft model *in vivo* together with explored the mechanism. Therefore, L6H21 may serve as a potential candidate for the treatment of allograft rejection.

Acknowledgements
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Conflict of Interests
All authors declare that they have no conflict of interests related to this study.

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