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A novel MyD88 inhibitor attenuates allograft rejection after heterotopic tracheal transplantation in mice

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ABSTRACT

Background: After lung transplantation, the major complication limiting the long-term survival of allografts is obliterative bronchiolitis (OB), characterized by chronic rejection. Innate immune responses contribute to the development of OB. In this study, we used a murine heterotopic tracheal transplantation mouse model to examine the effects of a newtype of innate immune inhibitor, TJ-M2010–5.

Methods: Syngeneic tracheal grafts were transplanted heterotopically from C57BL/6 mice to C57BL/6 mice. Allografts from BALB/c mice were transplanted to C57BL/6 mice. The allograft recipients were treated with TJ-M2010–5, and anti-mouse CD154 (MR-1). The grafts were harvested at 7, 14, and 28 days and evaluated by histological and real-time RT-PCR analyses.

Results: In untreated allografts, almost all epithelial cells fell off at 7 days and tracheal occlusion reached a peak at 28 days. However, the loss of the epithelium and airway obstruction were significantly improved in mice treated with TJ-M2010–5 combined with MR-1. The relative mRNA expression levels of pro-inflammatory cytokines were upregulated in allogeneic tracheal grafts, and treatment with the two drugs reduced the production of pro-inflammatory cytokines and infiltration of inflammatory cells.

Conclusions: In heterotopic tracheal transplantation models, TJ-M2010–5 combined with MR-1 could ameliorate the development of OB.

1. Introduction

Lung transplantation is an effective therapeutic option for many end-stage pulmonary diseases. However, despite the joint application of immunosuppressive therapy, obliterative bronchiolitis (OB), characterized by gradual fibroproliferation and the occlusion of respiratory bronchioles, still limits the long-term survival of allografts and patients after lung transplantation [1–4]. The precise pathophysiological mechanisms of OB and effective therapeutic strategies have not been established [5]. Injury to the bronchial epithelium and subepithelial constructions are critical factors leading to the occurrence and development of OB [3,6]. After lung transplantation, recipients experience acute and chronic rejection. During these two phases, both innate and adaptive immune responses play key roles in the pathogenesis of OB [7].The pathogenesis of OB is highly complex, including ischemia-reperfusion injury, rejection, and infection, resulting in repeated inflammatory injury and repair and eventually in tracheal tissue remodeling. As one of the main immune systems of cavity organs, innate immunity has received increasing attention in lung transplant studies. Innate immunity is the first-line immune response against invading pathogens and tissue damage and plays a vital role in adaptive immunity [8–11]. Toll-like receptors (TLRs) are important for innate immunity; they can be pathologically activated by a set of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular

Abbreviations page: DAMPs), damage-associated molecular patterns; DCs, dendritic cells; MR-1, anti-mouse CD154; MyD88, myeloid differentiation factor 88; OB, obliterative bronchiolitis; PAMPs, molecular patterns; PRRs, pattern recognition receptors; TLRs, Toll-like receptors

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patterns (DAMPs) during tissue damage [12,13], and subsequently regulate the maturation and differentiation of antigen-presenting cells, such as macrophages and dendritic cells (DCs), connecting innate and adaptive immunity. MyD88 (myeloid differentiation factor 88) is an adaptor protein common to all TLR signaling networks, except TLR3 [10]. It is vital for TLR signaling and plays an important role in the pathogenesis of OB.

We developed the novel MyD88 molecular inhibitor TJ-M2010–5 based on the MyD88 molecular structure. It was designed to bind to the TIR domain of MyD88 and can inhibit the dimerization of the MyD88. In this study, we used the mouse heterotopic tracheal transplantation model, which is used extensively in studies of the pathogenesis of OB after lung transplantation, to test whether our original MyD88 inhibitor TJ-M2010–5 is able to attenuate OB.

2. Materials and methods

2.1. Mice

Specific pathogen-free BALB/c and C57BL/6 6- to 8-week-old inbred male mice were purchased from Hunan SJA Laboratory Animal Company (Changsha, China). All experimental procedures were in accordance with the regulations of our Institutional Animal Care and Use Committee.

2.2. Reagents

TJ-M2010–5 (3-(4-(4-benzylpiperazin-1-yl)-N-(4-phenylthiazol-2-yl)) propanamide) was synthesized at the Academy of Pharmacy, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China (WIPO Patent Application Number: PCT/CN2012/070811). Chemical structure of TJ-M2010–5(Fig. 1), general synthesis pathways and docking molecular interaction of TJ-M2010–5 with MyD88 are illustrated in our previous studies [14].

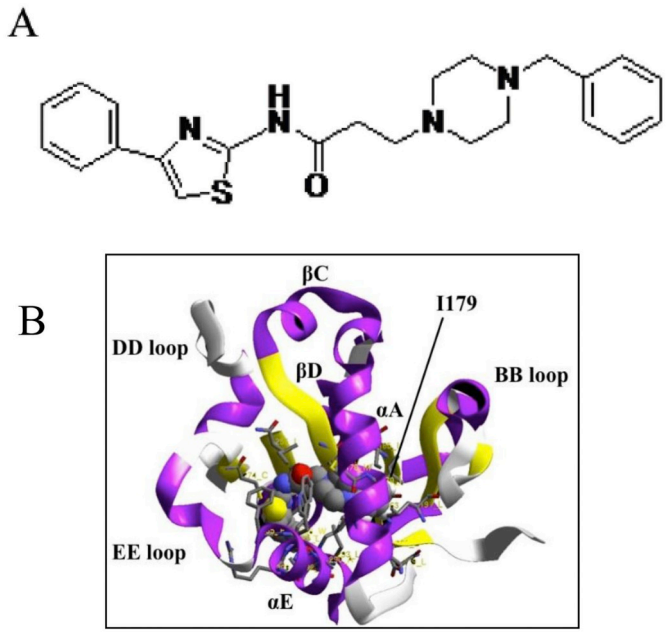

Fig. 1. (A) Chemical structure of TJ-M2010-5 (3-(4-(4-benzylpiperazin-1-yl)-N-(4-phenylthiazol-2-yl)) propanamide). (B) Predicted configuration of TJ-M2010-5 binding to the MyD88 in box3. TJ-M2010-5 may bind to I179 of the BB loop and act on amino-acid residues of αE, βD, βC, αA, DD loop, and EE loop of MyD88.

2.3. Heterotopic tracheal transplantation and treatment protocol

In this heterotopic tracheal transplantationmodel, transplanted tracheal grafts were subcutaneously transplanted into recipients [15]. Isografts were transplanted heterotopically from BALB/c donors to BALB/c recipients, while allografts were transplanted from BALB/c donors to C57BL/6 mice. The grafts were harvested at 7, 14, and 28 days after transplantation. Recipient mice were randomly divided into five groups: group 1, syngraft; group 2, allograft; group 3, TJ-M2010–5 injected intraperitoneally (50 mg/kg/day); group 4, MR-1 injected intraperitoneally (10 mg/kg); group 5, TJ-M2010–5 combined with MR-1. These grafts were harvested for histological and quantitative real-time polymerase chain reaction analyses.

2.4. Histopathologic evaluation

The harvested grafts were immediately fixed in 4% paraformaldehyde. After 24 h, fixed grafts were embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E). In our analysis, luminal obliteration was evaluated as the area between the epithelium and inner trachea divided by the total area of the inner trachea. The pathological changes of the tracheal epithelium were evaluated as the length of the lumen not covered by the epithelium divided by the total luminal circumference of the trachea [16]. All histologic evaluations were performed by two individual observers in a blinded manner.

2.5. Quantitative reverse-transcriptase polymerase chain reaction

Total RNAs were extracted from tracheal grafts using the Trizol method (Invitrogen). Quantitative real-time polymerase chain reaction (PCR) was performed using a StepOne System (Life Technologies) and it was performed using the following forward and reverse primer pairs: *β-actin*, AGG CCA ACC GTG AAA AGA TG and TGG CGT GAG GGA GAG CAT AG; *IL-1β*, GCA CTA CAG GCT CCG AGA TGA A and GTC GTT GCT TGG TTC TCC TTG T; *IFN-γ*, AGC GGC TGA CTG AAC TCA GAT TGT AG and GTC ACA GTT TTC AGC TGT ATA GGG; *TNF-α*, CAC GCT CTT CTG TCT ACT GAA CTT C and ATG ATC TGA GTG TGA GGG TCT GG; *IL-17A*, ATG CTG TTG CTG CTG CTG AG and TGG AAC GGT TGA GGT AGT CTG AG; *IL-6*, AGT GGC TAA GGA CCA AGA C and ATA ACG CAC TAG GTT TGC CGA; *IL-10*, GCC TTA TCG GAA ATG ATC CA and AGG GTC TTC AGC TTC TCA CC.

2.6. Statistics

All data are reported as means ± SEM. Statistical differences in mean values between groups were determined using the unpaired *t*-test. Differences were considered statistically significant when $P < .05$.

3. Results

3.1. Effect of TJ-M2010–5 combined with MR-1treatment on histology

In syngeneic tracheal grafts, no luminal occlusion was observed and the grafts were completely lined with normal epithelium. The overall histology was very similar with a normal trachea. In the allografts, the ischemic injury was combined with an acute alloimmune response leading to progressive epithelial destruction 7 days after transplantation. At 28 days, the allografts had undergone complete epithelial necrosis and were almost totally occluded by the fibroproliferative lesion. The allografts were significantly rejected. However, TJ-M2010–5 combined with MR-1 significantly delayed tracheal allograft epithelial necrosis and significantly attenuated tracheal occlusion (Fig. 2).

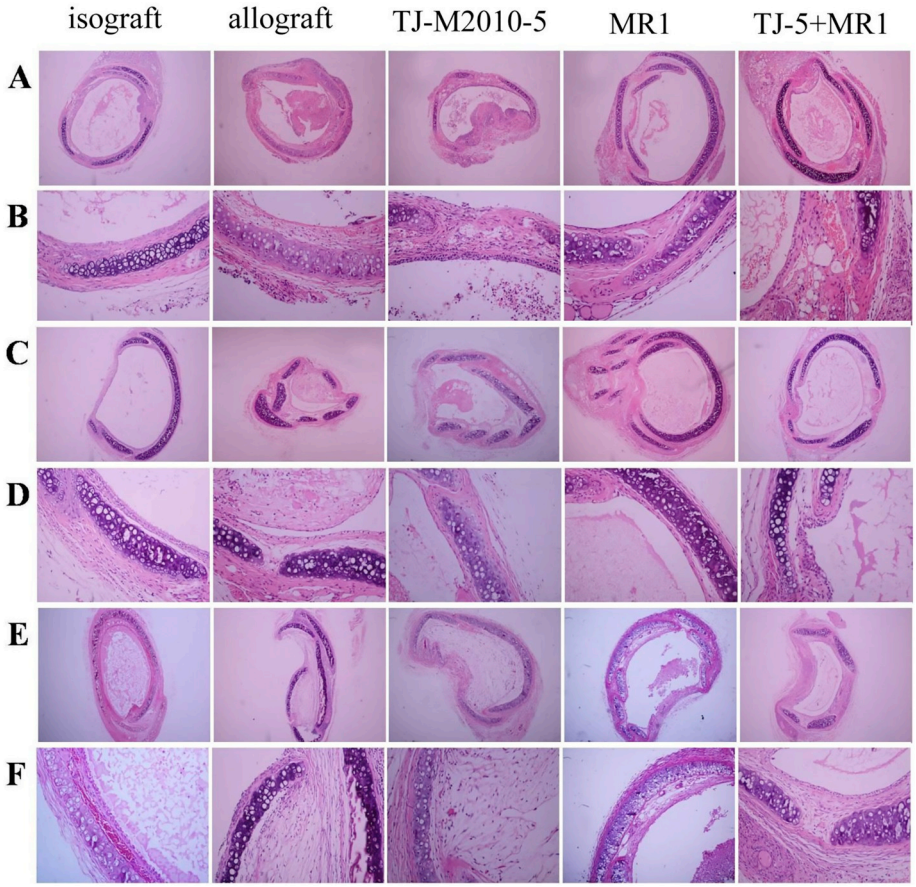

Fig. 2. Histologic analysis of tracheal grafts. Histologic findings of harvested tracheal grafts on day 7 (A, B), 14 (C, D), 28 (E, F) after heterotopic tracheal transplantation (magnification: A, C, E 100×; B,D,F 400×).

3.2. TJ-M2010–5 combined with MR-1 markedly reduced the extent of airway obstruction

TJ-M2010–5 combined with MR-1 significantly reduced luminal occlusion. Compared with positive allografts, intraperitoneal injection of TJ-M2010–5 and MR-1 markedly reduced luminal obliteration at 7 days ($P < .01$, Fig. 3A). In addition, the ratio of airway occlusion was also significantly decreased at 14 days ($P < .05$, Fig. 3B). Similar to these results, the combination of the two drugs significantly lowered luminal obliteration at 28 days after tracheal transplantation ($P < .05$, Fig. 3C).

3.3. TJ-M2010–5 combined with MR-1 reduced the loss of the epithelium

Allografts exhibited substantial epithelial loss on day 7. However, significant changes were observed in the loss of epithelial cells when we compared the group treated with the intraperitoneal injection of TJ-M2010–5 and MR1 with the positive allograft group ($P < .05$, Fig. 4A). The allografts treated with both TJ-M2010–5 and MR-1 were protected against the loss of the luminal epithelium on day 14 compared with the positive controls ($P < .05$, Fig. 4B). On day 28, both TJ-M2010–5- and MR-1-treated tracheas were significantly protected against the loss of the luminal epithelium ($P < .05$, Fig. 4C).

3.4. TJ-M2010–5 plus MR-1 alleviated OB by reducing the relative mRNA expression levels of multiple cytokines

We detected significantly higher relative mRNA levels of pro-inflammatory cytokines in allografts than in isograft controls. However, TJ-M2010–5 significantly reduced the mRNA levels of these cytokines. At 7 days, TJ-M2010–5 plus MR-1 significantly reduced the mRNA level of *IFN*-γ. The difference between the TJ-M2010–5 plus MR-1 and MR-1 groups was statistically significant ($P < .05$, Fig. 5A). Treatment with only TJ-M2010–5 more clearly reduced the mRNA level of *IFN*-γ compared with the reductions in the other groups. However, the mRNA level of *IFN*-γ was not affected by treatment with MR-1. TJ-M2010–5

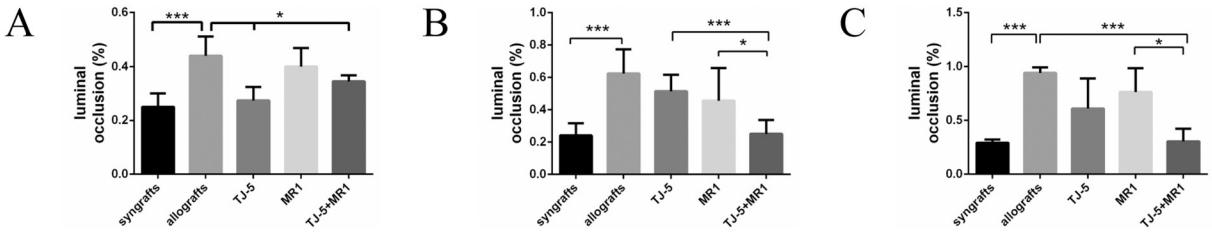

Fig. 3. The ratio of luminal occlusion in tracheal grafts. Intraperitoneal injection of TJ-M2010-5 and MR-1 significantly lowered luminal obliteration at 7 days (A), 14 days (B), 28 days (C) respectively (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

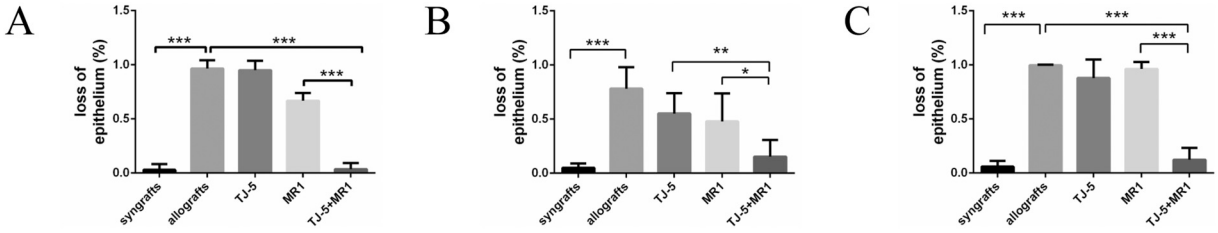

Fig. 4. The loss of the luminal epithelium in tracheal grafts. The loss of epithelial cells was markedly reduced by treatment with TJ-M2010-5 and MR-1 (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (A-C): 7, 14, 28 days after tracheal transplantation.

and TJ-M2010–5 plus MR-1 both inhibited the production of IL-1β ($P < .01$, Fig. 5B). TJ-M2010–5 plus MR-1 decreased the mRNA level of *IL-6* ($P < .05$, Fig. 5C). There was no significant difference between the MR-1 group and the TJ-M2010–5 plus MR-1 group ($P = .0832$). In addition, the allografts treated with both TJ-M2010–5 and MR-1 exhibited significantly decreased cytokine mRNA levels at 14 days. IFN-γ, IL-1β, IL-6, and TNF-α levels decreased, while IL-10 levels clearly increased by treatment with TJ-M2010–5 plus MR-1 (Fig. 6). Similar to the above results, combining TJ-M2010–5 with MR-1 markedly lowered *IFN-γ*, *IL-1β*, and *TNF-α* mRNA levels at 28 days (Fig. 7).

4. Discussion

Despite triple or quadruple immunosuppressive therapy, acute and chronic rejection continues to be the major limitation to long-term survival after lung transplantation [17]. The lung is a non-solid organ that possesses an extensive system of innate immune responses [9], explaining why the curative effect of clinical immunosuppressors is consistently unsatisfactory. OB is the end result of innate and adaptive immune responses. Airway inflammation and epithelial injury may be important factors leading to airway chronic remodeling and luminal obstruction [2]. We hypothesized that the inhibition of the innate and adaptive immune responses by TJ-M2010–5 plus MR-1 could markedly alleviate OB and slow its development.

In this study, we investigated the immune responses caused by ischemia and alloantigens using a heterotopic mouse tracheal transplantation model. In syngeneic tracheal grafts, ischemia causes self-limiting transient pathological changes and inflammatory responses. However, allografts exhibit a loss of epithelial cells and luminal obstruction due to the alloantigens. In this model of OB, allografts exhibit obliterative changes, similar to those observed after lung transplantation [7,18,19]. We observed that TJ-M2010–5 plus MR-1 could significantly reduce the loss of the epithelial cells comparedwith positive allografts. Airway obstruction was also markedly ameliorated by this treatment. These results revealed that the combination of an MyD88 inhibitor and MR-1 could significantly prevent the progression of OB. Our results are also in agreement with those of other studies showing the importance of epithelial cells in OB. It was previously reported that the respiratory airway epithelium is the major target of immunologic injuries [20]. Airway epithelial damage and injury to sub-epithelial structures probably play important roles in the development of OB [20]. The epithelium is the first line of immune defense and is initially damaged by ischemic injury [20,21]. Acute ischemia could induce the inflammatory response primarily involved in innate immunity. Adaptive immune responses also participate in this process [22,23]. Ischemic injury leads to the release of endogenous molecules capable of activating pattern recognition receptors (PRRs), which are critical for the activation ofinnate immune responses [10]. TLRs are among the most important families of PRRs, and play a key role in activating innate immune responses and promoting adaptive immunity [24]. TJ-M2010–5, a novel MyD88 inhibitor, can alleviate ischemic injury by blocking most TLR/MyD88 signalingpathways.

TLR/MyD88 signaling pathways are present on mononuclear phagocytes, DCs, polymorphonuclear leukocytes, and epithelial cells [17]. Activation of this pathway can stimulate the production of a variety of inflammatory cytokines and chemokines and induce an adaptive response to the allograft [25].TLR signaling results in the rapid production of proinflammatory cytokines and chemokines, enhanced antigen presentation, and the up-regulation of MHC expression by interactions with pathogens [17,26].The delicate balance between proinflammatory and anti-inflammatory cytokines is critical to the repair of tracheal injury, and changes in this balance influence allogeneic airway remodeling. Interactions between Th1, Th2, and Th17 cells or immune responses may lead to the fibro-obliteration of allogeneic tracheas during the process of OB.

Without immunosuppressors, allografts are ultimately rejected via adaptive immune responses, which develop by the activation of antigen-presenting cells (DCs) and the generation of allograft-specific Th1 cells or Th17 cells [27–29].The presence of alloantigens in allografts subsequently leads to increased mRNA expression of proinflammatory cytokines as well as an increased influx of inflammatory cells. *IFN-γ* is a typical Th1 cytokine associated with immune rejection. The mRNA expression of *IFN-γ* was markedly reduced by treatment with the two agents. *IL-1β* and *TNF-α* are pro-inflammatory cytokines produced by monocytes, which rapidly migrate to inflammatory sites and differentiate into macrophages or DCs. They are the most important cells in innate immunity. Our results showed that the combination of TJ-M2010–5 with MR-1 significantly reduced the production of IL-1β and TNF-α. IL-1β can promote the production of TNF-α and IL-6, which results in neutrophil migration, fibroblast proliferation, and chronic inflammation [30]. Our previous studies have demonstrated that MyD88 is closely related to the maturation of DCs [31]. The absence of

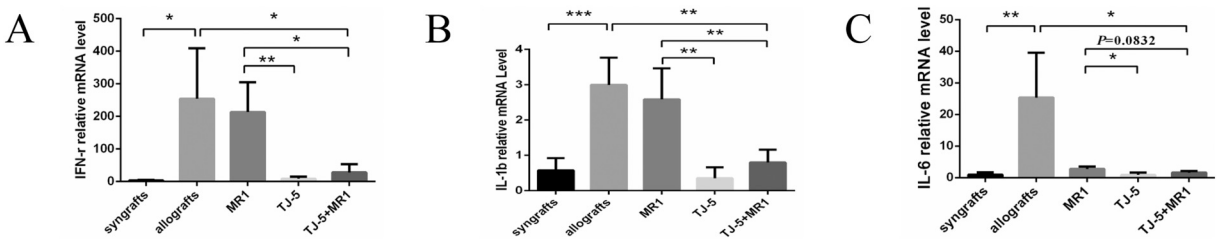

Fig. 5. The relative mRNA expression of cytokines in allografts at day 7. Relative levels of IFN-γ (A), IL-1β (B), IL-6 (C) in tracheal grafts were quantified by real-time PCR. The level of each gene was expressed as relative folds compared to house keeping gene GAPDH. TJ-M2010-5 plus MR-1 significantly reduced relative levels of these cytokines.

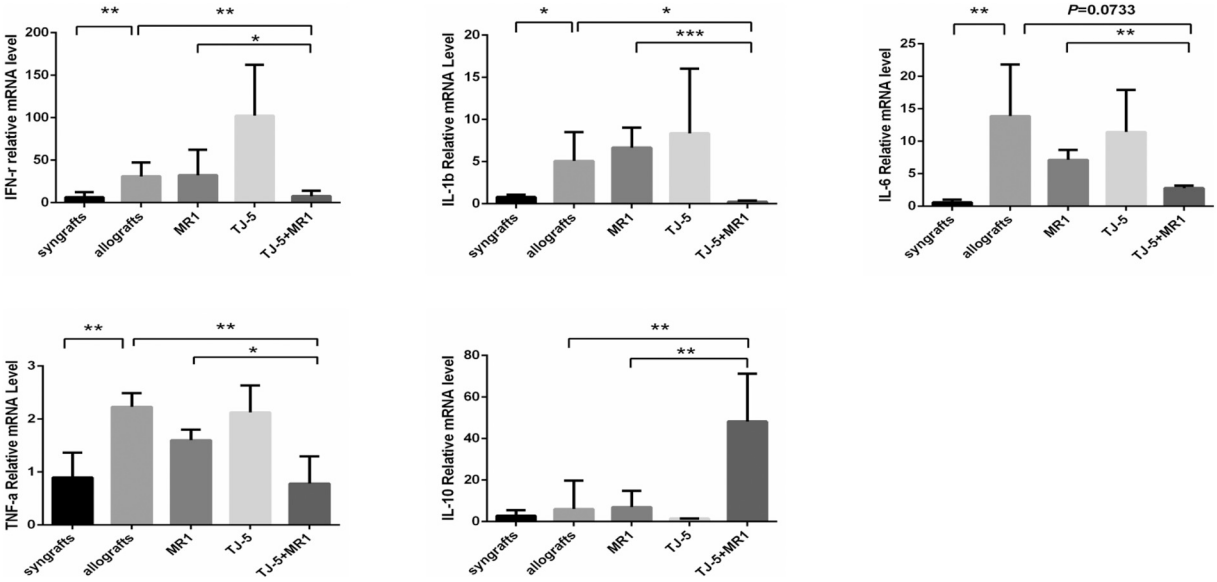

Fig. 6. The relative mRNA expression of cytokines in allografts at 14 days. IFN-γ, IL-1β, IL-6, and TNF-α levels were decreased by treatment with TJ-M2010-5 plus MR-1, while IL-10 levels clearly increased.

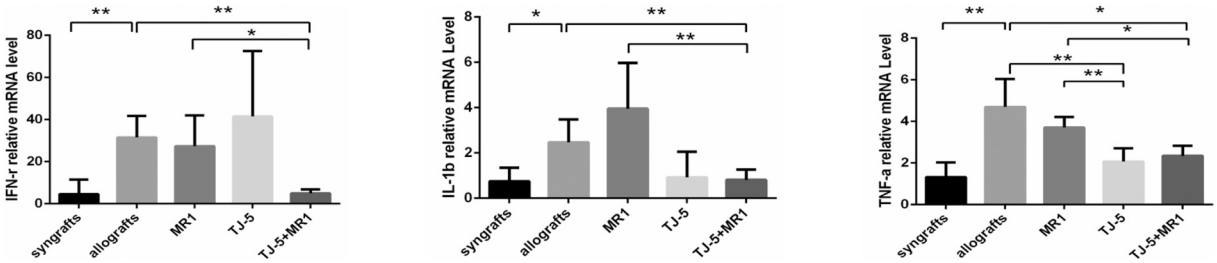

Fig. 7. The relative mRNA expression of cytokines in allografts at day 28. TJ-M2010-5 with MR1 markedly lowered *IFN-γ*, *IL-1β*, and *TNF-α* mRNA levels at 28 days.

MyD88 resulted in defects in the generation of mature DCs, which ultimately prolonged the survival of grafts. Walker et al. [32] indicated that DC production of IL-6 is reduced when MyD88 is absent, which leads to increased T cell suppression by Tregs. IL-6 is a key regulator of the induction of naive T cells toward the Th17 phenotype, which is important in adaptive immune responses on epithelial surfaces [33]. Many studies have indicated that these cytokines and immune responses are related and interact with each other.

TJ-M2010–5 is a new inhibitor produced by our team that can block the majority of MyD88 signaling. MR-1 is an anti-mouse CD154 monoclonal antibody that inhibits the CD40/CD154 costimulatory pathway. TJ-M2010–5 or MR-1 alone had a weak effect on allografts. However, combined treatment with TJ-M2010–5 and MR-1 significantly alleviated the pathological changes in OB. In conclusion, TJ-M2010–5 plus MR-1 maybe an effective treatment for OB after lung transplantation.

5. Limitations

We focused on mRNA expression levels in tissue samples. mRNA analyses provide insight into cellular activity, but do not necessarily reflect protein expression. mRNA analyses also provide little information regarding readily synthesized intracellular proteins and their deposition and release.

Authorship page

The authors declare financial interests.

This work is attributed to the Institute of Organ Transplantation, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Key Laboratory of Ministry of Health, and Key Laboratory of Ministry of Education.

Ping Zhou and Sheng Chang participated in research design. Min Yang participated in the performance of the study. Min Yang and Gen Chen participated in the writing of the paper. Gen Chen and Xue Zhang contributed to the revision of the paper. Zuochuan Ding, Yan Miao, Yang Yang participated in data analysis.

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