Transplant Immunology 57 (2019) 101244

Contents lists available at ScienceDirect

Transplant Immunology

journal homepage: www.elsevier.com/locate/trim

FK506 combined with GM6001 prevents tracheal obliteration in a mouse model of heterotopic tracheal transplantation

Yiqian Li[a,1], Ping Shu[a,1], Liang Tang[b], Xiaojun Yang[b], Junwei Fan[c,*], Xiaoqing Zhang[a,d,e,f,*]

[a] Department of pharmacy, Shanghai Pulmonary Hospital, Tongji University, Shanghai, China
[b] Department of central Laboratory, Shanghai Pulmonary Hospital, Tongji University, Shanghai, China
[c] Department of general Surgery, Shanghai General Hospital Affiliated to Shanghai Jiao Tong University, Shanghai, China
[d] The International Peace Maternity and Child Health Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China
[e] Shanghai Key Laboratory of Embryo Original Diseases, Shanghai, China
[f] Shanghai Municipal Key Clinical Specialty, Shanghai, China

ARTICLE INFO

Keywords:
Mouse heterotopic tracheal transplantation model
Matrix metalloproteinase inhibitor
Bronchiolitis obliterans
MMP-8
MMP-9

ABSTRACT

Background: Obliterative bronchiolitis (OB) is the major complication limiting the long-term survival of allografts after lung transplantation. In this study, we investigated the effect of tacrolimus (FK506) combined with GM6001 , a matrix metalloproteinase (MMP) inhibitor , on the formation of OB using a mouse heterotopic tracheal transplantation model.

Methods: Syngeneic tracheal grafts were transplanted heterotopically from BALB/c mice to BALB/c mice. Allografts from C57BL/6 mice were transplanted to BALB/c mice. Isograft group, allograft group, allograft + FK506 group, allograft + GM6001 group and allograft + FK506 + GM6001 group was given respectively intraperitoneal injection of saline, saline, FK506, GM6001 and FK506 + GM6001 once a day. At 28 day after transplantation, OB incidence was determined by hematoxylin-eosin staining and the expressions of MMPs and cytokines were assessed using enzyme linked immunosorbent assay, immunohistochemical assays and western blot assay.

Results: The tracheal occlusion rates of isograft group, allograft group, allograft + FK506 group, allograft + GM6001 group and allograft + FK506 + GM6001 group were 0, 74.1 ± 9.79%, 34.4 ± 6.04%, 40.3 ± 8.77% and 26.5 ± 5.73% respectively. There were significant differences between the latter two groups ($P < .001$). The serum MMP-8 and MMP-9 levels of allograft group were significantly higher than those of isograft group ($P < .05$) and had no significant decrease when treated by FK506. The serum MMP-8 and MMP-9 levels of allograft + FK506 + GM6001 group were significantly lower than those of allograft + FK506 group ($P < .05$). MMP-8 and MMP-9 protein expression in the grafts of allograft + FK506 + GM6001 group were lower than those of allograft + FK506 group verified by immunohistochemical staining and western blotting.

Conclusion: FK506 combined with GM6001 could alleviate tracheal obliteration in mouse heterotopic tracheal transplantation model, due to its inhibitory effect on MMPs.

1. Introduction

Lung transplantation is the only effective treatment for end-stage lung disease, which improves lung function and quality of life. Due to the increase of the number of lung transplantation, the improvement of surgical techniques and perioperative management , and the accumulation of experience, the survival rate of patients and grafts have been increased [1]. Obliterative bronchiolitis (OB) is one of the important factors leading to poor prognosis of lung transplantation. The 5-year survival rate of lung transplantation patients with OB was only 30–40%, which is about 30% less than that of lung transplantation patients without OB.

The pathological manifestations of OB are peribronchiolitis and fibrosis of terminal bronchioles. Clinical presentations of OB, which was known as bronchiolitis occlusive syndrome (BOS) include progressive occlusion of the bronchioles, disorder of the small airway function and

* Corresponding authors.
E-mail addresses: fjwnet@163.com (J. Fan), zxqkitten@163.com (X. Zhang).
[1] Li Yiqian and Shu Ping contributed mainly to this work

https://doi.org/10.1016/j.trim.2019.101244
Received 2 May 2019; Received in revised form 8 September 2019; Accepted 13 September 2019
Available online 14 September 2019

breathing block. FK506 anti-rejection therapy is the main treatment for OB at present. However, excessive anti-rejection therapy can easily lead to pulmonary infection and adverse reactions [2–4]. Exploring new therapeutic strategies for OB is the key measure to improve the clinical effect of lung transplantation.

OB following lung transplantation involves chronic inflammatory response injury and abnormal repair process owing to immune and/or non-immune factors. At present, most opinions hold that the primary reason for the occurrence and development of OB is immune factors [5]. OB is the characteristic manifestation of chronic rejection post lung transplantation. Multiple cytokines are involved in the molecular mechanism of OB. A study showed that IL-17 participated in the OB molecular mechanism by regulating the polarization of M1 macrophages in mouse heterotopic tracheal transplantation model [6]. Another study suggested that IL-17a blocking decreased the overall IFN-γ mediated lymphocyte response and thus delayed the occurrence of OB in mouse orthotopic lung transplantation model [7]. IL-18 is a potent pro-inflammatory cytokine that stimulates lymphocytes to produce IFN-γ, regulates macrophage activity, and secretes IL-1, IL -6, CCL4 and other pro-inflammatory cytokines. IL-18 is considered as potential prognostic biomarkers of organ transplant recipients and their donors [8].

Extracellular matrix (ECM) degradation is an important pathological aspect of OB. Matrix metalloproteinases (MMPs) include a group of metal-dependent endopeptidases, which play a key role in the degradation of ECM components [9,10]. Excessive activation of MMPs, involving in the immunopathological damage process through ECM renewal, epithelial injury, fibrosis, tissue remodeling, and inflammation, leads to OB. It has been reported that the occurrence of OB is related to the high expression of MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, tissue inhibitor of matrix metalloproteinase (TIMP -1) and TIMP-2; and the activation of MMP-7 is related to the good prognosis of patients with lung transplantation [11–13].Extracellular matrix metalloproteinase inducer (EMMPRIN) is an internal membrane protein inducting MMPs secretion. EMMPRIN expression is upregulated in the presence of inflammation, immune response, and ischemic injury [14,15]. The activation of MMPs is an important molecular event occurring in OB. The detection of MMPs expression profiling provides a new method to predict the occurrence and development of OB.

MMPs inhibitors are specific inhibitory molecules of MMP, and the mechanism of MMPs inhibitors mainly include preventing the zymogen activation of MMPs and inhibiting the activity of MMPs [16,17]. GM6001, known as Galardin or Ilomastat, is a broad-spectrum metalloproteinase inhibitor and is currently the most potent of the synthetic metalloproteinase inhibitors. The mechanism of action of GM6001 is as follows: by binding to the substrate recognition part of the metalloproteinase molecule and complicating with the required Zn2 + in the active center of the enzyme, the activation of the enzyme is inhibited, thus directly inhibiting the destruction of collagen by MMPs [18,19].

Mouse allogeneic heterotopic tracheal transplantation model is the most commonly used animal model to study the pathogenesis OB after lung transplantation [1]. In this study, the effects of FK506 combined with MMP inhibitor GM6001 on the formation of OB after heterotopic tracheal transplantation in mouse, as well as the expression of various inflammatory factors and MMPs, were investigated to provide experimental basis for the clinical treatment of OB.

2. Materials and methods

2.1. Animals

Specific pathogen-free C57BL/6 and BALB/c male mice weighing 20 ± 2 g were purchased from Shanghai SLAC Laboratory animal Co. Ltd. (Shanghai, China). All experimental procedures were performed in compliance with the Principles of Laboratory animal care (NIH publication Vol 25, No. 28 revised 1996) and the ShangHai Pulmonary Hospital Animal Care and Use Committee Guidelines.

2.2. Reagents

Mouse IL-10、IL-17、IL-18、TIMP-1、TIMP-2、MMP-2、MMP-3、MMP-7、MMP-8、MMP-9 and EMMPRIN ELISA kits were purchased from American R&D company(Minneapolis, USA). All primary antibodies and second antibody were purchased from Shanghai Abcam Biology limited company (Shanghai, China). All other reagents are domestic analytical pure reagents.

2.3. Model building and group-division of mice

Mouse heterotopic tracheal transplantation was performed to mimic OB pathological process post lung transplantation. In isografts model, tracheal from BALB/c donors were transplanted to BALB/c recipients, while tracheal from C57BL/6 donors were transplanted to BALB/c recipients in allografts model. The donor mice were euthanized by intraperitoneally injecting Ketamine (100 mg/kg). A midline cervical incision was performed to expose the entire trachea. The trachea below the cricoid cartilage distal to the bifurcation was dissected, harvested, and then it was flushed and preserved with cold sterile saline at 4 °C. A small incision was made in the dorsal suprascapular area of the recipient mice. A subcutaneous pouch was made with blunt dissection, and then the donor tracheal segment was placed into it. The skin was closed with interrupted 7–0 Vicryl suture.

All the mice were randomly divided into five groups, and each group contained 12 mice. Isograft group: tracheal of BALB/c donors were transplanted to BALB/c recipients; allograft group: tracheal of C57BL/6 donors were transplanted to BALB/c recipients; allograft + FK506 group: tracheal of C57BL/6 donors were transplanted to BALB/c recipients and the recipients were administered with FK506 by intraperitoneal injection (1 mg/kg/day) [20]; allograft + GM6001 group: tracheal of C57BL/6 donors were transplanted to BALB/c recipients and the recipients were administered with GM6001 by intraperitoneal injection (15 mg/kg/day) [21]; allograft + FK506 + GM6001 group: tracheal of C57BL/6 donors were transplanted to BALB/c recipients and the recipients were administered with FK506 combined with GM6001 by intraperitoneal injection (15 mg/kg/day). Intraperitoneal injection of FK506 and GM6001 were performed from the first to the 27th day after the operation. All groups were dissected to collect serum and peripheral blood for ELISA detection, the grafts were harvested from recipient mice on day 28 after transplantation for histopathology, immunohistochemical analyses, and western blot assay.

2.4. Histological assessment of tracheal obstructive rate

Tracheal grafts were immediately fixed in 4% formalin at room temperature for 24 h. The formalin-fixed tissues were embedded in paraffin, cut into 4-μm sections and then stained with hematoxylin and eosin(H&E). The changes of tracheal structure and inflammatory infiltration were observed under optical microscope. The ratio of occlusion area to cross section of trachea was the luminal obliteration rate. All histologic evaluations were performed by two individual observers in a blinded manner.

2.5. Detection of serum concentration of cytokines and MMPs

The serum concentration of cytokines (IL-10、IL-17、IL-18) and MMPs (TIMP-1、TIMP-2、MMP-2、MMP-3、MMP-7、MMP-8、MMP-9 and EMMPRIN) was detected by enzyme chain immunosorbent assay (ELISA). The operation procedures are strictly in accordance with the kit instructions for testing. The absorbance (A) value of each hole was measured at 450 nm wavelength and zero with a blank hole. The standard curve was made and found out the content of blood sample

from the standard curve.

2.6. Immunohistochemical method

The expression of cytokines and MMPs were determined using immunohistochemical method. The process was in accordance with the instructions for reagents. One slide in each group of sections was stained with PBS solution instead of monoclonal antibody as the negative control. Brown-yellow granule in cytoplasm or nuclei was considered as the positive expression. Stain density and scope of 5 random high-power visual field were scored using Image Pro Plus Image analysis software quantitatively and calculated the integrated optical density value (IOD).

2.7. Western blot assay

Whole protein was extracted using radioimmunoprecipitation assay (RIPA) (Roche, Germany). The lysates were collected for centrifugation (12,000 *g*, 4 °C for 15 min). Pierce BCA Protein Assay Kit (Thermo Scientific, USA) was used to measure protein concentration in supernatants. After that western blotting was carried out according to previous report [28]. Briefly, the proteins separated by SDS-PAGE were electrophoretically transferred onto PDVF membranes (Bio-Rad, USA). Primary antibodies against TIMP-1、TIMP-2、MMP-2、MMP-3、MMP-7、MMP-8、MMP-9、EMMPRIN、IL-10、IL-17、IL-18(1:1000) (Cell Signaling Technology, USA). Actin was used as loading control. Secondary antibody was DyLight 800 goat anti-rabbit IgG (H + L) (KPL, USA). Odyssey infrared imaging system (LI-COR Biosciences, USA) was used for quantitative Western blots.

2.8. Statistical analysis

Statistical analysis was performed using SPSS 19.0 software. All data are presented as means ± SEM. Statistical analysis were performed using a one-way ANOVA followed by a Bonferroni-Holm correction and *t*-test. $P < .05$ was regarded as statistically significant.

3. Results

3.1. Histological assessment of tracheal graft in different groups of mouse heterotopic tracheal transplantation model

In the isograft group, the overall histology of grafts was very similar to the normal trachea: the tracheal structure was clear and complete and epitheliums was normal in the syngeneic tracheal grafts under light microscope. The mucosal epithelium was fully repaired. There was no obstruction and no inflammatory cell infiltration in the lumen (Fig. 1A).

In the allograft group, tracheal epithelial cells undergone complete necrosis and the lumen of tracheal grafts were almost totally occluded by fibrous hyperplasia. The transplanted tracheal cartilage collapsed completely. The lumen was completely blocked by the proliferative fibrous connective tissue, and chronic inflammatory cells were extensively infiltrated (Fig. 1B).

In the allograft + FK506 group, the transplanted tracheal cartilage collapsed partly. The partial lumen was obviously blocked by fibrous tissue hyperplasia. Degree of inflammatory cell infiltrated in allograft + FK506 group was less than that in allograft group (Fig. 1C).

In the allograft + GM6001 group, the transplanted tracheal cartilage collapsed partly. The partial lumen was obviously blocked by fibrous tissue hyperplasia. (Fig. 1D).

In the allograft + FK506 + GM6001 group, the transplanted tracheal cartilage collapsed partly. The lumen was not completely blocked by fibrous tissue hyperplasia. Inflammatory cell infiltrated within tracheal wall in allograft + FK506 + GM6001 group were less than that in allograft group and allograft + FK506 group (Fig. 1E).

3.2. The rate of tracheal occlusion of different groups of mouse heterotopic tracheal transplantation model

No tracheal occlusion occurred in grafts of isograft group. The rates of tracheal occlusion of allograft group, allograft + FK506 group, allograft + GM6001 group and allograft + FK506 + GM6001 group were 74.1 ± 9.79%, 34.4 ± 6.04%, 40.3 ± 8.77% and 26.5 ± 5.73% respectively. There were significant differences between the 4 groups ($P < .001$). FK506 combined with GM6001 significantly reduced the occlusion rate of the transplanted trachea.

3.3. Serum cytokines and MMPs of different groups of mouse heterotopic tracheal transplantation model determined by ELISA

On the whole, the serum MMPs and cytokines of allograft group was higher than those of isograft group, while serum MMPs and cytokines of allograft + FK506 group and allograft + FK506 + GM6001 group were lower than those in allograft group. Serum protein expression of MMP-8 ($P = .0005$), MMP-9 ($P = .0013$), IL-17 ($P = .0054$), and IL-18 ($P = .0087$) had significantly difference in total four groups. Serum protein expression of MMP-3 ($P = .0408$), MMP-8 ($P = .0175$), MMP-9 ($P = .0127$), IL-17 ($P = .0334$) and IL-18 ($P = .0414$) were significantly increased in allograft group compared to isograft group. Serum protein expression of MMP-7 ($P = .0426$), MMP-8 ($P = .0026$), MMP-9 ($P = .0021$) were significantly decreased in allograft + GM6001 group compared to allograft group. Serum protein expression of IL-17 ($P = .0206$) and IL-18 ($P = .0247$) were significantly reduced in allograft + FK506 group compared to the isograft group and allograft group. Serum protein expression of MMP-8 ($P < .0001$), MMP-9 ($P = .0016$), IL-17 ($P = .0105$) and IL-18 ($P = .0056$) were significantly reduced in the allograft + FK506 + GM6001 group compared to isograft group and allograft group. Serum protein expression of MMP-8 ($P = .0331$) and MMP-9 ($P = .0161$) were significantly lower in the FK506 and GM6001 combined treatment group than in the FK506 alone treatment group. (Table 1).

3.4. The protein expression of cytokines and matrix metalloproteinases in tracheal graft of different groups of mouse heterotopic tracheal transplantation model determined by immunohistochemistry

The protein of MMP -8 and MMP-9 were expressed in fibroblasts, epithelial cells, airway smooth muscle cells and many inflammatory cells, such as macrophages and neutrophils. Compared to isograft group (Fig. 2A and F), the protein of MMP-8 and MMP-9 were up-regulated expressed in tracheal grafts after transplantation in allograft group (Fig. 2B and G). Compared to allograft group, the expressions of MMP-8 and MMP-9 were reduced in tracheal grafts of allograft + FK506 group (Fig. 2C, H), allograft + GM6001 group (Fig. 2D, I) and allograft + FK506 + GM6001 group (Fig. 2E, J). At 28 day post-operation, the expression IOD values of MMP-8 in isograft group, allograft group, allograft + FK506 group , allograft + GM6001 group and allograft + FK506 + GM6001 group were 43.1 ± 19.4、183.6 ± 25.3、110.2 ± 20.5、85.1 ± 11.2 and 79.5 ± 12.1, respectively. The expression IOD values of MMP-9 were 36.3 ± 9.2、130.9 ± 21.4、70.3 ± 18.0 , 43.0 ± 19.3 and 37.4 ± 18.3, respectively. MMP-8 and MMP-9 expression were significantly different among the 5 groups ($P < .001$), with the lowest expression in isograft group and the highest expression in allograft group, and the expression of MMP-8 and MMP-9 was significantly inhibited in allograft + FK506 + GM6001 group ($P < .001$). There was no significant difference in the levels of other cytokines and MMPs between the four groups (all $P > .05$).

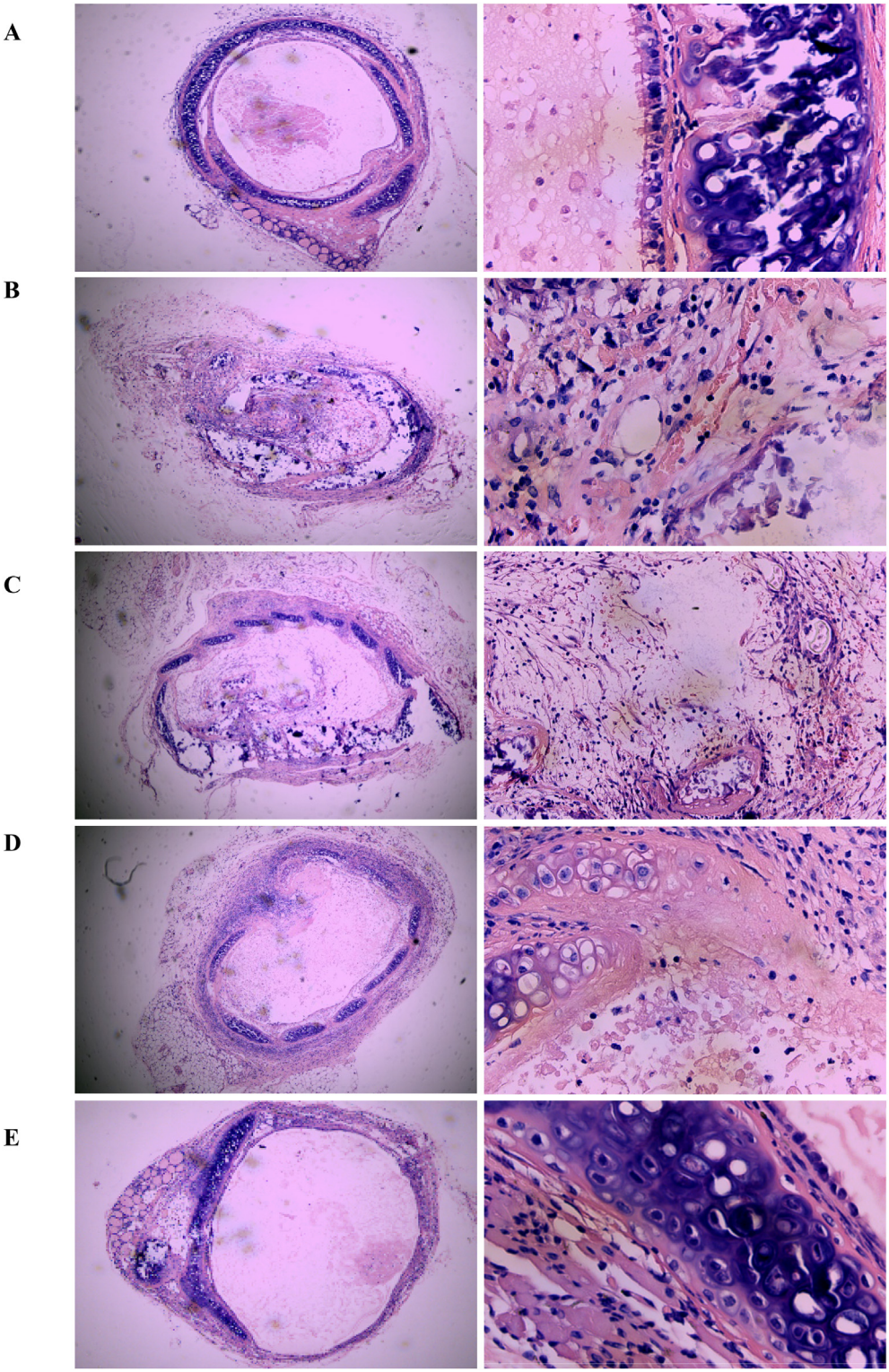

Fig. 1. Pathologic histological changes of tracheal graft in different groups of mouse heterotopic tracheal transplantation model (hematoxylin-eosin staining, the magnification of the left side of Figure was 40, the magnification of the right side of figure was 400).
A: isograft group, B: allograft group, C: allograft + FK506 group, D: allograft + GM6001 group, E: allograft + FK506 + GM6001 group.

3.5. The protein expression of matrix metalloproteinases in tracheal graft of different groups of mouse heterotopic tracheal transplantation model determined by western blot analysis

The protein expression of MMP-8 and MMP-9 in the transplanted trachea of allograft group was higher than those of isograft group, and the protein content of MMP -8 and MMP -9 gradually decreased in the treated trachea of allograft + FK506 group and allograft + FK506 + GM6001 group. The optical density ratio of the target protein and internal reference protein was used as the relative protein expression content. The expression levels of MMP -8 protein in four groups were: isograft group (0.0053), allograft group (0.11), allograft

Table 1
Serum cytokines and MMPs of different groups of mouse heterotopic tracheal transplantation model.

	Isograft group (n = 12)	Allograft group (n = 12)	Allograft + FK506 group (n = 12)	GM6001 (n = 12)	Allograft + FK506 + GM6001 group (n = 12)	P^a	P^b	P^c	P^d	P^e	P^f
TIMP-1(μg/L)	42.78 ± 6.115	44.02 ± 6.578	43.84 ± 7.359	42.88 ± 5.088	40.75 ± 6.727	0.5676	0.4996	0.9322	0.6044	0.2391	0.2993
TIMP-2(μg/L)	8991 ± 1227	9271 ± 1468	8880 ± 1394	8.982 ± 0.8714	8871 ± 1196	0.9251	0.6167	0.5731	0.5782	0.502	0.9869
MMP-2(μg/L)	276.4 ± 42.62	320.8 ± 48.14	305.5 ± 53.59	310.8 ± 50.25	301.5 ± 38.76	0.1377	0.0641	0.4804	0.5476	0.2138	0.931
MMP-3(μg/L)	211.6 ± 35.6	242.4 ± 31.17	232.5 ± 38.27	230.1 ± 28.23	225.4 ± 36.08	0.1887	0.0408	0.5115	0.1114	0.0909	0.6794
MMP-7(μg/L)	5.534 ± 0.9877	5.663 ± 1.02	5.452 ± 0.9801	4.803 ± 0.6743	5.416 ± 0.9595	0.9262	0.7903	0.6485	0.0426	0.6159	0.9267
MMP-8(μg/L)	168.4 ± 29.34	193.8 ± 19.75	174.1 ± 30.46	168.6 ± 19.04	149.4 ± 9.68	0.0003	0.0175	0.05	0.0026	< 0.0001	0.0331
MMP-9(μg/L)	45.12 ± 10.14	58.58 ± 9.485	51.04 ± 8.002	49.63 ± 8.23	42.15 ± 7.127	0.0009	0.0127	0.0781	0.0021	0.0016	0.0161
EMMPRIN(μg/L)	71.39 ± 9.451	76.95 ± 12.77	72.94 ± 7.306	0.06998 ± 0.009854	67.68 ± 11.58	0.2704	0.259	0.3219	0.1216	0.1195	0.2144
IL-10(μg/L)	397 ± 32.53	427.1 ± 39.43	411.1 ± 45.09	0.4084 ± 0.02367	402.7 ± 38.79	0.3733	0.1251	0.4346	0.1458	0.1134	0.6628
IL-17(μg/L)	33.67 ± 1.815	36.69 ± 4.112	33.05 ± 3.33	0.03589 ± 0.004218	32.06 ± 3.81	0.0022	0.0334	0.0206	0.4885	0.0105	0.3481
IL-18(μg/L)	94.83 ± 10.68	106.1 ± 13.26	94.17 ± 9.449	0.1034 ± 0.01171	90.94 ± 7.087	0.0024	0.0414	0.0247	0.1791	0.0056	0.2421

P^a: Statistical differences among the five groups, P^b: Statistical differences between isograft group and allograft group, P^c: Statistical differences between allograft group and allograft + FK506 group, P^d: Statistical differences between allograft group and allograft + GM6001 group , P^e: Statistical differences between allograft group and allograft + FK506 + GM6001 group, P^f: Statistical differences between allograft + FK506 group and allograft + FK506 + GM6001 group.

+ FK506 group (0.090), allograft + GM6001 group (0.015) and allograft + FK506 + GM6001 group (0.010). The four groups of MMP -9 protein expression levels were: isograft group (0.036), allograft group (0.80), allograft + FK506 group (0.30), allograft + GM6001 group (0.040) and allograft + FK506 + GM6001 group (0.020) (Fig. 3A and B).

4. Discussion

OB is a serious complication to affect the prognosis of lung transplant patients. The main clinical features of OB are progressive dyspnea, dry cough, and progressive decline in lung function. Immunosuppressive therapy can only delay the progression of OB [22]. Exploring the molecular mechanism of OB and formulating reasonable treatment strategies are the key issues to improve the prognosis of patients with lung transplantation.

The occurrence of OB after lung transplantation can be divided into three steps: ① the initial step is immune and/or non-immune factors including rejection, lymphocytic bronchiolitis, cytomegalovirus infection, gastroesophageal reflux and other damage to lung epithelial cells; ② then the immune response appeared: multiple cytokines and complement are upregulated and an inflammatory cascade initiates; ③ the last step is tissue remodeling: repeated damage and repair of the bronchioles, resulting in airway fibrosis and occlusion. Multiple cytokines have been found to be involved in the OB molecular mechanism after lung transplantation [23–25]. These results suggest that cytokines play a key role in OB molecular mechanism after lung transplantation. The final histopathological features of OB are bronchiolar lymphocyte and neutrophil infiltration, airway epithelial injury, extracellular matrix (ECM) degradation, tissue remodeling and fibrosis.

The activation of MMPs is involved in the immunopathological damage process of OB through ECM renewal, fibrosis, tissue remodeling, inflammation, etc., which is an important molecular event of OB after lung transplantation [26,27]. Therefore, cytokines and MMPs play a key role in the pathophysiology and mechanism of OB. We speculated that OB progression could be inhibited by changing the expression of cytokines and reducing the activity of MMPs.

The development of animal models is indispensable to the study of OB pathogenesis and prevention measures. The researchers explored and established a series of animal models for OB study after lung transplantation, including orthotopic lung transplantation, orthotopic tracheal transplantation, and heterotopic tracheal transplantation in different locations in small rodents and large mammals. However, each model has its own advantages and disadvantages. At present, there is no perfect animal model, But the most important question for researchers is which model is the best fit for their research. Mouse heterotopic tracheal transplantation model is the most commonly used animal model for OB, which can be used to explore the molecular mechanism of immune rejection and fiber tissue reconstruction after lung transplantation. After heterotopic tracheal transplantation, airway epithelial cells were completely shed in a short time and the lumen was finally completely occluded, which can be used as a model for the study of advanced OB lesions [28–30]. In this study, no tracheal occlusion occurred in the isograft group. In the allograft group, the tracheal occlusion rate was 74.1 ± 9.79%. The transplanted tracheal cartilage was completely collapsed and destroyed, and the lumen was completely blocked by proliferative fibrous connective tissue, and chronic inflammatory cells were extensively infiltrated.

Cytokines are one of the therapeutic targets for OB. In this study, serum levels of IL-10, IL-17 and IL-18 were detected and analyzed in the isograft group, allograft group, allograft + FK506 group and allograft + FK506 + GM6001 group. The results indicated that there was no difference in IL-10 serum levels among the groups. However, serum IL-17 and IL-18 levels were significantly higher in allograft group than in isograft group, and serum IL-17 and IL-18 levels of allograft + FK506 group were significantly lower than those of allograft group. It was suggested that IL −17 and IL −18 might be related to graft rejection,

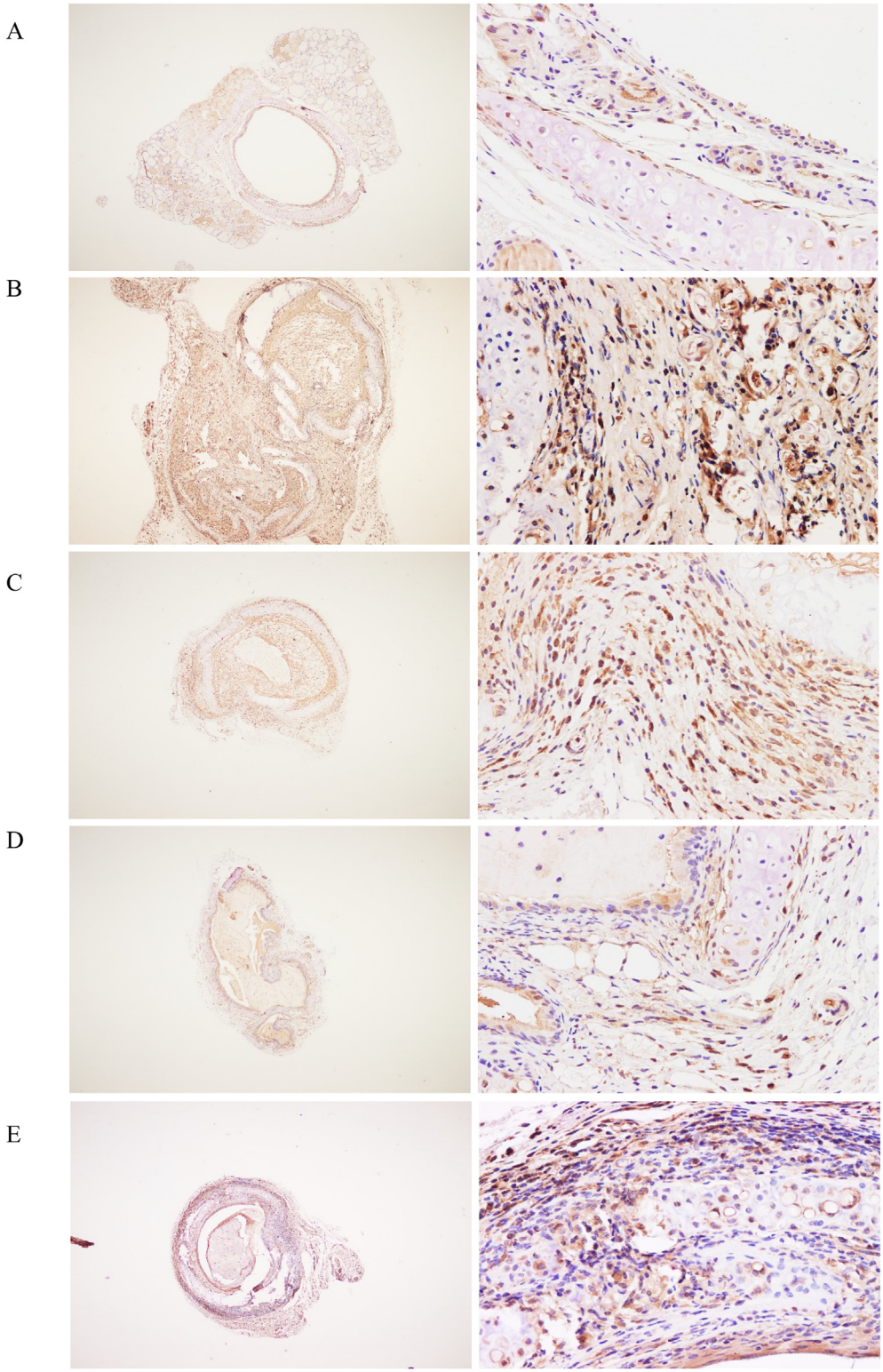

Fig. 2. The protein expression of MMP-8 and MMP-9 in tracheal graft of different groups of mouse heterotopic tracheal transplantation model(immunohistochemical staining, the magnification of the left side of Figure was 40, the magnification of the right side of figure was 400).
A: MMP-8 protein expression in tracheal graft of isograft group,
B: MMP-8 protein expression in tracheal graft of allograft group,
C: MMP-8 protein expression in tracheal graft of allograft + FK506 group,
D: MMP-8 protein expression in tracheal graft of allograft + GM6001 group,
E: MMP-8 protein expression in tracheal graft of allograft + FK506 + GM6001 group,
F: MMP-9 protein expression in tracheal graft of isograft group,
G: MMP-9 protein expression in tracheal graft of allograft group,
H: MMP-9 protein expression in tracheal graft of allograft + FK506 group,
I: MMP-9 protein expression in tracheal graft of allograft + GM6001 group,
J: MMP-9 protein expression in tracheal graft of allograft + FK506 + GM6001 group.

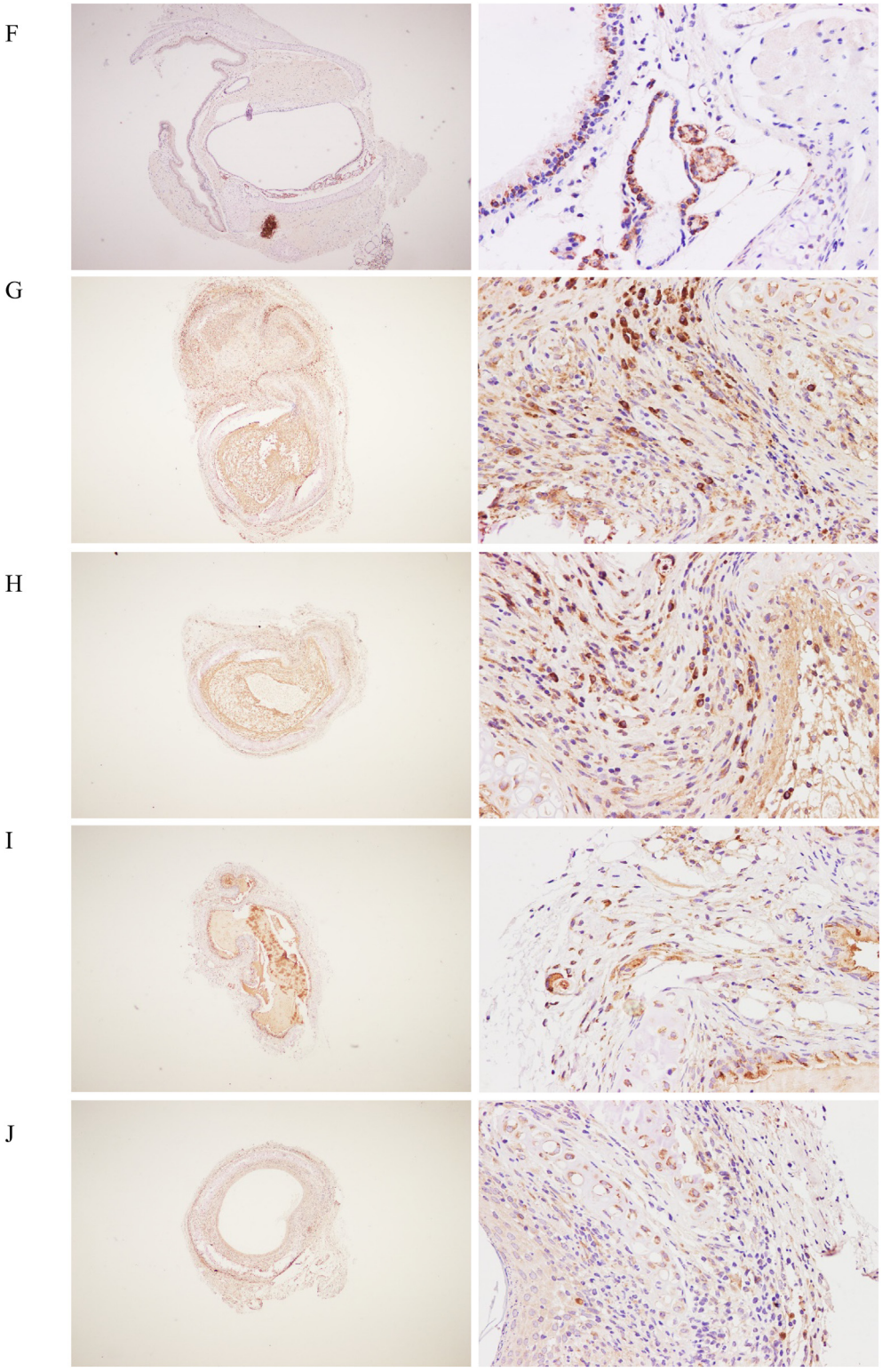

Fig. 2. (*continued*)

while FK506 anti-rejection reduced serum IL −17 and IL −18 levels. Haouami Y [31] et al. reported that IL −17 was involved in the rejection of kidney transplantation, and the levels of IL −17 mRNA in the transplanted kidney and serum IL −17 in the rejection group were significantly higher than those in the control group. Serum IL −17 level in renal transplant patients was good in predicting the sensitivity and specificity of rejection, and was a biomarker with promising application in rejection. Sun B [32] et al. found that IL-17 G-197A gene polymorphism was associated with rejection of liver transplantation recipients. IL-17 levels were significantly decreased after FK506 treatment in both cell culture and mouse experiments [33,34]. Pawlus J [35] et al. found that the polymorphism of IL-18 rs1946518 locus was associated with chronic rejection of renal transplant recipients. Another study showed that IL-18 mRNA increased glomerular filtration rate of renal transplant recipients, which was related to renal transplant function. Cell culture experiments also showed that FK506 significantly

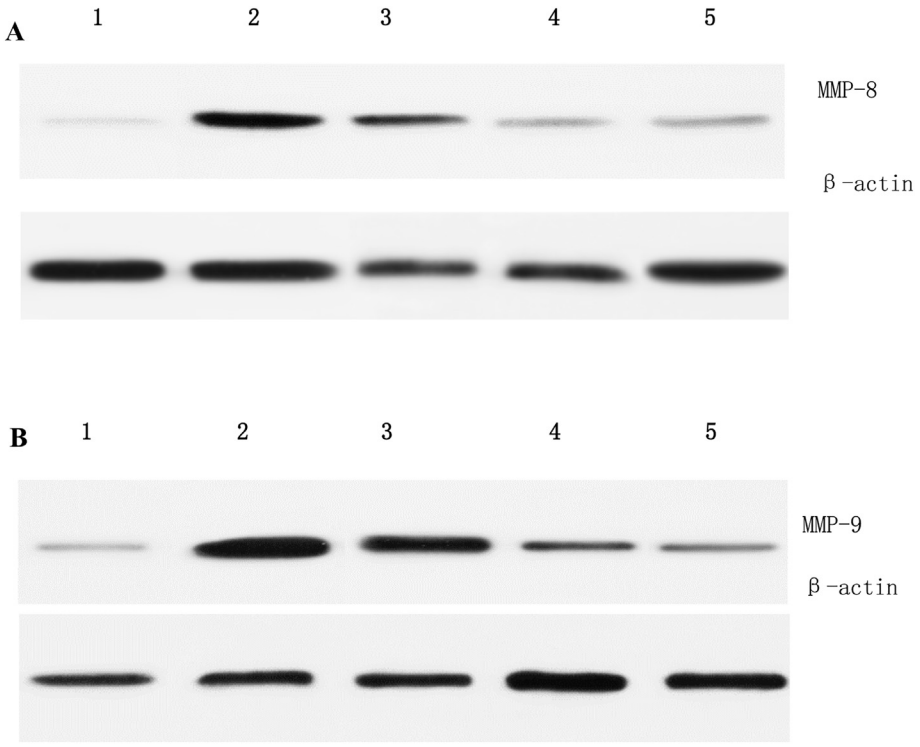

Fig. 3. The protein expression of MMP-8 and MMP-9 in tracheal graft of different groups of mouse heterotopic tracheal transplantation model determined by western blot analysis.
A: isograft group, B: allograft group, C: allograft + FK506 group, D: allograft + GM6001 group, E: allograft + FK506 + GM6001 group.

reduced IL-18 levels [36]. The above studies combined with our data suggested that IL-17 and IL-18 involved in the molecular mechanism of organ transplantation rejection. FK506 can effectively reduce IL-17 and IL-18 levels, thus alleviating rejection.

In this study, the serum levels of MMP-2, MMP-3, MMP7, MMP-8, MMP-9, TIMP-1, TIMP-2 and EMMPRIN in recipient mice of isograft group, allograft group, allograft + FK506 group, allograft + GM6001 group and allograft + FK506 + GM6001 group were detected to explore the role of matrix metalloproteinases and related molecules in the occurrence and treatment of OB. The data indicated that serum levels of MMP-2, MMP-3, MMP-7, TIMP-1, TIMP-2 and EMMPRIN were not significantly different in the 5 groups, while serum levels of MMP-8 and MMP-9 were significantly different in the 5 groups. Serum levels of MMP-8 and MMP-9 was significantly higher in the allograft group than in the isograft group; and serum levels of MMP-8 and MMP-9 of allograft + FK506 + GM6001 group was significantly lower than those of allograft + FK506 group.

Both immunohistochemistry and Western blot results showed: compared with isograft group, MMP-8 and MMP-9 expression were increased in tracheal grafts of allograft group after transplantation. Compared with allograft group, MMP -8 and MMP -9 expressions in tracheal grafts after transplantation in allograft + FK506 group and allograft + FK506 + GM6001 group were significantly reduced. Heijink IH [11] et al. confirmed that MMP-8 and MMP-9 were significantly increased in alveolar lavage fluid of patients with BOS after lung transplantation. Current studies showed that MMP-9 is involved in the pathogenesis of chronic rejection after lung transplantation in adults, and its expression has a certain prediction effect on adult BOS [37–39]. The experimental data and literature reports suggested that matrix metalloproteinase was involved in the OB molecular mechanism after lung transplantation. After the treatment with MMP inhibitor GM6001, it was observed that the occlusion rate of the mouse model of heterotopic tracheal transplantation was significantly reduced, and the level of serum MMPs and the expression of graft MMPs protein were reduced. Therefore, it can be speculated that GM6001 inhibits the formation of obliterative bronchiolitis after heterotopic tracheal transplantation in mouse by reducing the expression of MMP-8 and MMP-9.

The results of this study showed that FK506 could reduce OB by reducing cytokines IL-17, IL-18 and GM6001 reduces OB by reducing MMPs. Combined application of FK506 and GM6001 in a mouse model of heterotopic tracheal transplantation showed good control effect on OB. The pathological changes of the transplanted trachea were significantly reduced.

In summary, the mouse heterotopic tracheal transplantation model used in this study successfully depicted OB pathophysiological process in lung tissue protein expression, including increased MMP-8, MMP-8, MMP-9, IL-17 and IL-18. Furthermore, we showed that anti-rejection therapy with FK506 as the main drug combined with MMP inhibitor was an effective strategy for the treatment of OB after lung transplantation. Further studies are needed in orthotopic lung transplantation models and the selection of suitable MMP inhibitors for clinical application to provide more reliable basic research data for clinical application.

5. Limitations

Although the present study initially proved that GM6001 could alleviate tracheal obliteration in mouse heterotopic tracheal transplantation model, toxic side effects of GM6001 should been observed in further study.

Author contributions

Xiaoqing Zhang and Junwei Fan designed and performed experiments. Yiqian Li, Ping Shu made animal experiments. Liang Tang and Xiaojun Yang detect the genes expression. All the authors wrote the paper.

Fund program

Shanghai Municipal Natural Science Foundation(17ZR1423300, to Xiaoqing Zhang); Youth Medical Talents-Clinical Pharmacist Program, Shanghai "Rising Stars of Medical Talent" Youth Development Program (2017, to Xiaoqing Zhang); The hospital supports the discipline construction plan.

Declaration of Competing Interest

The authors declare no competing financial interests.

References

[1] S.D. Nathan, The future of lung transplantation, Chest. 147 (2) (2015 Feb) 309–316.
[2] A.F. Barker, A. Bergeron, W.N. Rom, M.I. Hertz, Obliterative bronchiolitis, N. Engl. J. Med. 370 (19) (2014 May 8) 1820–1828.
[3] P.R. Aguilar, A.P. Michelson, W. Isakow, Obliterative bronchiolitis, Transplantation. 100 (2) (2016 Feb) 272–283.
[4] H. Treede, A.R. Glanville, W. Klepetko, C. Aboyoun, E. Vettorazzi, R. Lama, C. Bravo, C. Knoop, J.D. Aubert, H. Reichenspurner, European and Australian Investigators in Lung Transplantation. Tacrolimus and cyclosporine have differential effects on the risk of development of bronchiolitis obliterans syndrome: results of a prospective, randomized international trial in lung transplantation, J. Heart Lung Transplant. 31 (8) (2012 Aug) 797–804, https://doi.org/10.1016/j.healun.2012.03.008 Epub 2012 May 2. PubMed PMID: 22554673.
[5] G.M. Verleden, R. Vos, B. Vanaudenaerde, L. Dupont, J. Yserbyt, D. Van Raemdonck, S. Verleden, Current views on chronic rejection after lung transplantation, Transpl. Int. 28 (10) (2015 Oct) 1131–1139.
[6] Q. Meng, J. Liu, F. Lin, L. Bao, Y. Jiang, L. Zheng, J. Tie, L. Zhang, X. Liang, L. Wei, Y. Li, H. Fan, X. Zhou, IL-17 contributes to the pathogenesis of obliterative bronchiolitis via regulation of M1 macrophages polarization in murine heterotopic trachea transplantation models, Int. Immunopharmacol. 52 (2017 Nov) 51–60, https://doi.org/10.1016/j.intimp.2017.08.022 Epub 2017 Aug 31. PubMed PMID: 28863322.
[7] P.K. Gupta, S.R. Wagner, Q. Wu, R.A. Shilling, IL-17A blockade attenuates Obliterative bronchiolitis and IFN-γ cellular immune response in lung allografts, Am. J. Respir. Cell Mol. Biol. 56 (6) (2017 Jun) 708–715, https://doi.org/10.1165/rcmb.2016-0154OC PubMed PMID: 28118023; PubMed Central PMCID: PMC5516289.
[8] C. Liu, J. Chen, B. Liu, S. Yuan, D. Shou, L. Wen, X. Wu, W. Gong, Role of IL-18 in transplant biology, Eur. Cytokine Netw. 29 (2) (2018 Jun 1) 48–51, https://doi.org/10.1684/ecn.2018.0410. Review PubMed PMID: 30078783.
[9] N. Cui, M. Hu, R.A. Khalil, Biochemical and biological attributes of matrix metalloproteinases, Prog. Mol. Biol. Transl. Sci. 147 (2017) 1–73, https://doi.org/10.1016/bs.pmbts.2017.02.005 Epub 2017 Mar 22. Review. PubMed PMID: 28413025;PubMed Central PMCID: PMC5430303.
[10] J. Liu, R.A. Khalil, Matrix metalloproteinase inhibitors as investigational and therapeutic tools in unrestrained tissue remodeling and pathological disorders, Prog. Mol. Biol. Transl. Sci. 148 (2017) 355–420, https://doi.org/10.1016/bs.pmbts.2017.04.003 Epub 2017 May 10. Review. PubMed PMID: 28662828; PubMed Central PMCID: (PMC5548434).
[11] I.H. Heijink, D. Rozeveld, S. van der Heide, W. van der Bij, R. Bischoff, A.J. van Oosterhout, M. van der Toorn, Metalloproteinase profiling in lung transplant recipients with good outcome and bronchiolitis obliterans syndrome, Transplantation. 99 (9) (2015 Sep) 1946–1952, https://doi.org/10.1097/TP.0000000000000602 PubMed PMID: 25651312.
[12] L.M. Eerola, H.S. Alho, P.K. Maasilta, K.A. Inkinen, A.L. Harjula, S.H. Litmanen, U.S. Salminen, Matrix metalloproteinase induction in post-transplant obliterative bronchiolitis, J. Heart Lung Transplant. 24 (4) (2005 Apr) 426–432 PubMed PMID:15797744..
[13] K. Krenn, M. Gmeiner, P. Paulus, N. Sela, L. Torres, K. Zins, G. Dekan, S. Aharinejad, Effects of azithromycin and tanomastat on experimental bronchiolitis obliterans, J. Thorac. Cardiovasc. Surg. 149 (4) (2015 Apr) 1194–1202, https://doi.org/10.1016/j.jtcvs.2014.11.088 Epub 2014 Dec 4. PubMed PMID: 25595376.
[14] D. Guindolet, E.E. Gabison, Role of CD147 (EMMPRIN/basigin) in tissue remodeling, Anat Rec (Hoboken) (2019 Feb 15), https://doi.org/10.1002/ar.24089 [Epub ahead of print] PubMed PMID: 30768865.
[15] S.N.I. von Ungern-Sternberg, A. Zernecke, P. Seizer, Extracellular matrix metalloproteinase inducer EMMPRIN (CD147) in Cardiovascular disease, Int. J. Mol. Sci. 19 (2) (2018 Feb 8), https://doi.org/10.3390/ijms19020507 pii: E507. (Review. PubMed PMID: 29419744; PubMed Central PMCID: PMC5855729).
[16] U. Schlomann, K. Dorzweiler, E. Nuti, T. Tuccinardi, A. Rossello, J.W. Bartsch, Metalloprotease inhibitor profiles of human ADAM8 in vitro and in cell-based assays, Biol. Chem. (2019 Mar 4), https://doi.org/10.1515/hsz-2018-0396 pii:/j/bchm.ahead-of-print/hsz-2018-0396/hsz-2018-0396.xml. [Epub ahead of print] PubMed PMID: 30738011.
[17] A. Almahdy, G. Koller, F. Festy, J.W. Bartsch, T.F. Watson, A. Banerjee, An MMP-inhibitor modified adhesive primer enhances bond durability to carious dentin, Dent. Mater. 31 (5) (2015 May) 594–602, https://doi.org/10.1016/j.dental.2015.03.003 Epub 2015 Mar 21. PubMed PMID: (25804191).
[18] X.M. Li, Y. Tan, C.Q. Huang, M.C. Xu, Q. Li, D. Pan, B.Q. Zhao, B.R. Hu, MMP inhibitor Ilomastat improves survival of mice exposed to γ-irradiation, Biomed. Environ. Sci. 31 (6) (2018 Jun) 467–472, https://doi.org/10.3967/bes2018.062 PubMed PMID: 30025561.
[19] J. Song, P. Peng, J. Chang, M.M. Liu, J.M. Yu, L. Zhou, X. Sun, Selective non-zinc binding MMP-2 inhibitors: novel benzamide Ilomastat analogs with anti-tumor metastasis, Bioorg. Med. Chem. Lett. 26 (9) (2016 May 1) 2174–2178, https://doi.org/10.1016/j.bmcl.2016.03.064 Epub 2016 Mar 16. PubMed PMID: (27038494).
[20] S. Herbst, A. Shah, M. Carby, G. Chusney, N. Kikkeri, A. Dorling, E. Bignell, S. Shaunak, D. Armstrong-James, A new and clinically relevant murine model of solid-organ transplant aspergillosis, Dis. Model. Mech. 6 (3) (2013 May) 643–651, https://doi.org/10.1242/dmm.010330 Epub 2012 Dec 20. PubMed PMID: 23264562; PubMed Central PMCID: (PMC3634648).
[21] W. Wang, The Protective Role of GM6001 on TNBs-Induced Ulcerative Colitis in Rats, Master thesis Dalian,Dalian medical university, 2007.
[22] C.R. Ensor, C.J. Iasella, K.M. Harrigan, M.R. Morrell, C.A. Moore, N. Shigemura, A. Zeevi, J.F. McDyer, R. Venkataramanan, Increasing tacrolimus time-in-therapeutic range is associated with superior one-year outcomes in lung transplant recipients, Am. J. Transplant. 18 (6) (2018 Jun) 1527–1533, https://doi.org/10.1111/ajt.14723 Epub 2018 Apr 6. PubMed PMID: (29513387).
[23] E.A. Kastelijn, C.H. van Moorsel, H.J. Ruven, J.W. Lammers, J.C. Grutters, Genetic polymorphisms and bronchiolitis obliterans syndrome after lung transplantation: promising results and recommendations for the future, Transplantation. 93 (2) (2012 Jan 27) 127–135.
[24] K. Budding, E.A. van de Graaf, T. Kardol-Hoefnagel, J.C. Broen, J.M. Kwakkel-van Erp, E.D. Oudijk, D.A. van Kessel, C.E. Hack, H.G. Otten, A promoter polymorphism in the CD59 complement regulatory protein gene in donor lungs correlates with a higher risk for chronic rejection after lung transplantation, Am. J. Transplant. 16 (3) (2016 Mar) 987–998.
[25] E.A. Kastelijn, C.H. van Moorsel, G.T. Rijkers, H.J. Ruven, V. Karthaus, J.M. Kwakkel-van Erp, E.A. van de Graaf, P. Zanen, D.A. van Kessel, J.C. Grutters, J.M. van den Bosch, Polymorphisms in innate immunity genes associated with development of bronchiolitis obliterans after lung transplantation, J. Heart Lung Transplant. 29 (6) (2010 Jun) 665–671.
[26] A. Koutsokera, P.J. Royer, J.P. Antonietti, A. Fritz, C. Benden, J.D. Aubert, A. Tissot, K. Botturi, A. Roux, M.L. Reynaud-Gaubert, R. Kessler, C. Dromer, S. Mussot, H. Mal, J.F. Mornex, R. Guillemain, C. Knoop, M. Dahan, P.M. Soccal, J. Claustre, E. Sage, C. Gomez, A. Magnan, C. Pison, L.P. Nicod, SysCLAD Consortium. Development of a multivariate prediction model for early-onset bronchiolitis obliterans syndrome and restrictive allograft syndrome in lung transplantation, Front Med (Lausanne). 4 (2017 Jul 17) 109, https://doi.org/10.3389/fmed.2017.00109. eCollection 2017 PubMed PMID:28770204; PubMed Central PMCID: PMC5511826.
[27] C. Müller, A. Andersson-Sjöland, H.H. Schultz, L.T. Eriksson, C.B. Andersen, M. Iversen, G. Westergren-Thorsson, Early extracellular matrix changes are associated with later development of bronchiolitis obliterans syndrome after lung transplantation, BMJ. Open. Respir. Res. 4 (1) (2017 Apr 12) e000177, , https://doi.org/10.1136/bmjresp-2016-000177. eCollection 2017 PubMed PMID: 28469930; PubMed Central PMCID: PMC5411729.
[28] L. Keil, A.L. Schaub, S.W. Hirt, C. Schmid, K. Lehle, M. von Suesskind-Schwendi, Synergism of imatinib, vatalanib and everolimus in the prevention of chronic lung allograft rejection after lung transplantation (LTx) in rats, Histol. Histopathol. 18088 (2019 Feb 1) 30706940, , https://doi.org/10.14670/HH-18-088 [Epub ahead of print] PubMed PMID.
[29] J. Guihaire, R. Itagaki, M. Stubbendorff, X. Hua, T. Deuse, S. Ullrich, E. Fadel, P. Dorfmüller, R.C. Robbins, H. Reichenspurner, U. Schumacher, S. Schrepfer, Orthotopic tracheal transplantation using human bronchus: an original xenotransplant model of obliterative airway disorder, Transpl. Int. 29 (12) (2016 Dec) 1337–1348, https://doi.org/10.1111/tri.12854 Epub 2016 Oct 4. PubMed PMID: (27614085).
[30] K. Fan, X.W. Qiao, J. Nie, L. Yuan, H.Z. Guo, Z.K. Zheng, J.S. Li, J.J. Wang, K. Jiang, Orthotopic and heterotopic tracheal transplantation model in studying obliterative bronchiolitis, Transpl. Immunol. 28 (4) (2013 Jun) 170–175, https://doi.org/10.1016/j.trim.2013.04.006 Epub 2013 Apr 22. PubMed PMID: (23619376).
[31] Y. Haouami, T. Dhaouadi, I. Sfar, M. Bacha, T. Gargah, R. Bardi, E. Abderrahim, R. Goucha, T. Ben Abdallah, Y. Gorgi, The role of IL-23/IL-17 axis in human kidney allograft rejection, J. Leukoc. Biol. 104 (6) (2018 Dec) 1229–1239, https://doi.org/10.1002/JLB.5AB0318-148R Epub 2018 Jul 19. PubMed PMID: (30024651).
[32] B. Sun, J. Gao, W. Shi, Y. Guo, J. Fan, J. Zhang, X. Li, G. Liu, The interleukin-17 G-197A polymorphism is associated with cyclosporine metabolism and transplant rejection in liver transplant recipients, Pharmacogenomics. (2019 Feb 25), https://doi.org/10.2217/pgs-2018-0198 [Epub ahead of print] PubMed PMID: 30799725.
[33] T. Yago, Y. Nanke, M. Kawamoto, H. Yamanaka, S. Kotake, Tacrolimus potently inhibits human osteoclastogenesis induced by IL-17 from human monocytes alone and suppresses human Th17 differentiation, Cytokine. 59 (2) (2012 Aug) 252–257, https://doi.org/10.1016/j.cyto.2012.04.012 Epub 2012 May 11. PubMed PMID: 22579702.
[34] X. Tian, F. He, J. Jia, H. Zhang, Tacrolimus down-regulates the mRNA levels of IL-17 and IL-23 in the muscle tissues of mice with experimental autoimmune myositis, Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 33 (2) (2017 Feb) 206–209 Chinese. PubMed PMID: 29762990.
[35] J. Pawlus, A. Sierocka, K. Tejchman, Z. Ziętek, M. Romanowski, A. Pawlik, J. Sieńko, M. Żukowski, K. Ciechanowski, M. Ostrowski, T. Sulikowski, The impact of interleukin 12B (1188A > C), interleukin 16 (−295T > C), and interleukin 18 (607C > A, 137G > C) gen polymorphisms on long-term renal transplant function and recipient outcomes, Transplant. Proc. 46 (6) (2014 Jul-Aug) 2079–2082, https://doi.org/10.1016/j.transproceed.2014.06.019 PubMed PMID: 25131111.

[36] Z.X. Pan, H.F. Liu, D.S. Tang, D. Liang, X.W. Chen, IL-18 expression in whole blood cell cultures from active lupus nephritis and the inhibitory effects of FK506, cyclosporin A and dexamethasone, Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 20 (6) (2004 Nov) 741–743 Chinese. PubMed PMID: 15555450.

[37] E. Vandermeulen, S.E. Verleden, H. Bellon, D. Ruttens, E. Lammertyn, S. Claes, J. Vandooren, E. Ugarte-Berzal, D. Schols, M.P. Emonds, D.E. Van Raemdonck, G. Opdenakker, G.M. Verleden, R. Vos, B.M. Vanaudenaerde, Humoral immunity in phenotypes of chronic lung allograft dysfunction: a broncho-alveolar lavage fluid analysis, Transpl. Immunol. 38 (2016 Sep) 27–32, https://doi.org/10.1016/j.trim.2016.08.004 Epub 2016 Aug 22. PubMed PMID: (27561239).

[38] V.E. Kennedy, J.L. Todd, S.M. Palmer, Bronchoalveolar lavage as a tool to predict, diagnose and understand bronchiolitis obliterans syndrome, Am. J. Transplant. 13 (3) (2013 Mar) 552–561, https://doi.org/10.1111/ajt.12091 Epub 2013 Jan 28. Review. PubMed PMID: 23356456; PubMed Central PMCID: (PMC3582805).

[39] M. Pain, P.J. Royer, J. Loy, A. Girardeau, A. Tissot, P. Lacoste, A. Roux, M. Reynaud-Gaubert, R. Kessler, S. Mussot, C. Dromer, O. Brugière, J.F. Mornex, R. Guillemain, M. Dahan, C. Knoop, K. Botturi, C. Pison, R. Danger, S. Brouard, A. Magnan, COLT Consortium, T cells promote bronchial epithelial cell secretion of matrix Metalloproteinase-9 via a C-C Chemokine receptor Type 2 Pathway: implications for chronic lung allograft dysfunction, Am. J. Transplant. 17 (6) (2017 Jun) 1502–1514, https://doi.org/10.1111/ajt.14166 Epub 2017 Jan 23. PubMed PMID: 27982503.