Anti-Macrophage Migration Inhibitory Factor Antibody Suppresses Chronic Rejection of Heterotopically Transplanted Trachea in Rats

Yorihisa Sumida,[1] Hideki Yamashita,[1] Masao Inoue,[1] Kazutaka Ohsawa,[2] Hiroshi Sato,[2] Atsushi Nanashima,[1] Takeshi Nagayasu[1]

[1] Division of Surgical Oncology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

[2] Biomedical Research Center for Frontier Life Sciences, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

　　Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine essential for delayed hypersensitivity *in vivo,* and is involved in bronchiolitis obliterans and late rejection in lung transplantation. We tested here whether neutralization of MIF using anti-MIF antibody prevents such a response. We examined the MIF mRNA expression level and changes in allograft tracheal epithelium and intraluminal obstruction in a rat allograft model. Lewis rat (RT1^l) underwent heterotopic tracheal transplantation from Brown Norway rats (RT1^n) in the omentum. Anti-MIF antibody was injected in the peritoneum. Rats were divided into three groups (non-treated allograft, allograft treated with normal rabbit IgG and allograft treated with anti-MIF antibody). Implants were harvested on days 7 or 21 for histological analysis. MIF mRNA expression was higher in the allograft at days 7 and 21 than in the isograft. The epithelium in non-treated allograft was almost absent at day 7. The epithelial height in the anti-MIF-treated graft was higher than that in normal IgG-treated grafts. The intraluminal space was mostly replaced by granulation tissue at day 21 in the untreated group. The proportion of obliterans was lowest in the anti-MIF group, the second lowest in the untreated group and the third lowest in the normal IgG-treated grafts, and the difference was significant (*p*<0.001) between the first two groups. Our results indicate that anti-MIF antibody suppresses allogenic tracheal rejection.

ACTA MEDICA NAGASAKIENSIA 51: 51　56, 2006

Keywords: Lung transplantation; Trachea; Macrophage migration inhibitory factor; Allograft; Chronic rejection; Bronchiolitis obliterans

Introduction

　　Orthotopic lung transplantation has become a useful modality for end-stage pulmonary disease and the currently available immunosuppressive regimens have also reduced the rate of acute rejection.[1] However, the long-term outcome in lung transplant recipients is not satisfactory because of postoperative complication of obliterative bronchiolitis (OB).[2] OB is an inflammatory disorder that leads to airway injury and bronchial occlusion by fibrosis.[2-4] Several investigators reported that the frequency and severity of acute rejection correlate with the development of OB.[5,6] Furthermore, patients with OB also show cellular rejection response as evident by perivascular infiltrating lymphocytes.[3] Thus, there is a general agreement that OB is caused by bronchial damage accompanied by graft rejection after lung transplantation. Several studies indicated that chronic allogenic response is the underlying immunological mechanism of OB.[7] T-cell mediated tissue injury or inflammatory cytokines might be associated with OB.[8,9] However, the pathogenesis of OB has not been fully clarified so far.

　　T-cell-dependent macrophage activation and recruitment may be nonspecifically associated with graft injury through the secretion of soluble mediators such as nitric oxide and tumor necrosis factor-α (TNF-α) via the delayed-type hypersensitivity (DTH) reaction, which would be closely related to graft injury by acute and chronic allograft rejection.[8] The macrophage migration inhibitory factor (MIF), a 12.5-kDa protein with multiple pro-inflammatory properties, was first identified in 1966 as a cytokine.[10] Subsequent studies clarified that MIF was an important pro-inflammatory cytokine[11,12] and MIF is associated with DTH reaction in several diseases,[13-15] and is known to play a pivotal role in primary antigenic and mitogenic stimulation of T cell activation and T-cell-dependent antibody production.[16] Furthermore, MIF stimulates the proliferation of fibroblasts[17] and the expression of MIF is increased in rat allograft rejection and human kidney transplants with acute rejection.[18,19] These findings suggest that MIF could play an important role in the process of allograft rejection or it could by itself induce rejection. Makita et

al.[20] reported that anti-MIF antibody suppressed the development of adult respiratory distress syndrome in lipopolysaccharide-induced lung injury.

The main hypothesis of the present study was that neutralization of MIF using anti-MIF antibody would prevent allograft rejection and the development of OB after lung transplantation. To test our hypothesis, we used a rat model of allograft tracheal transplantation and examined messenger ribonucleic acid (mRNA) of MIF in the allograft. To clarify the effect of anti-MIF antibody in preventing OB, we examined changes in tracheal epithelium and intraluminal obstruction *in vivo*.

Subjects and Methods

Animals

Inbred, unmodified, pathogen-free, major histocompatibility complex (MHC) (RT[1])-incompatible male rats, the Lewis (RT1[l]) and Brown Norway (RT1[n]) rats, were used in the present study. All rats were 8-10-week old and weighed 250-300 g (Charles River Japan Inc., Yokohama, Japan, and SLC Japan Co., Shizuoka, Japan) at the time of the study. They were housed in the Laboratory Animal Resource Center at Nagasaki University Graduate School of Biomedical Sciences in accordance with the institutional guidelines. The study design was approved by the Ethics Committee of Nagasaki University Graduate School of Biomedical Sciences.

Heterotopic tracheal transplantation

Heterotopic tracheal transplantation was performed using the method reported by Reichenspurner et al.[21] After induction of general anesthesia with intraperitoneal injection of pentobarbital (40 mg/kg), the trachea of the donor Brown Norway rat was exposed through a midline cervical incision and upper midline sternotomy. The trachea with the main bronchi were removed by dividing at the pulmonary hilum and immediately submerged in ice-cold 0.9% saline containing 10,000 IU/mL penicillin and 1,000 μg/mL streptomycin solutions (Sankyo Co., Tokyo, Japan). Recipient animals (Lewis rat) were anesthetized with intraperitoneal injection of pentobarbital (40 mg/kg). After upper midline abdominal incision, the trachea of the donor was heterotopically implanted into the greater omentum and was fixed with stitches to avoid migration. The peritoneum and abdominal musculature were closed with 4-0 Dexon and skin was closed with 3-0 nylon suture. In addition to these experimental rats (n=15), we used 5 control recipient Lewis rats, each transplanted with a trachea of a Lewis rat using the above-mentioned procedure (i.e., isograft model).

Preparation of rabbit polyclonal antibody against rat MIF

Polyclonal anti-rat MIF serum was generated by immunizing New Zealand White rabbits with purified recombinant rat MIF. Rat MIF was expressed in *Escherichia coli* and was purified by homogeneity

as described previously.[22] In brief, the rabbits were intradermally inoculated with 100 μg of MIF emulsified in Complete Freunds' adjuvant (CFA), and then immunoglobulin G (IgG) fraction was prepared using the protein A-Sepharose according to the protocol provided by the manufacturer. The anti-rat MIF antibody was kindly provided by Professor Jun Nishihira at the First Department of Medicine and Central Research Institute, Hokkaido University School of Medicine, Sapporo, Japan.

Experimental design and tissue processing

The present experiment was designed to evaluate the effects of anti-MIF antibody (Ab) in suppressing allogenic rejection. To test this effect, we compared (1) 5 rats with allograft and treated with anti-MIF Ab (anti-MIF group), (2) 5 rats with allograft and treated with normal rabbit IgG (Biogenesis, Poole, England) (normal IgG group) and (3) 5 rats with allograft that received no other treatment (non-Tx group). In the normal IgG group, non-immunized rabbit IgG was injected intraperitoneally after tracheal transplantation (IgG fraction was 3 mg/kg/day). In the anti-MIF group, the rats were injected intraperitoneally with anti-MIF antibody at 3 mg/kg/day. All rats were sacrificed at day 7 or 21 after injection and the graft was removed from the recipient and cut into two pieces at the tracheal midsection. One was used for measuring mRNA and the other piece was fixed overnight in 4% paraformaldehyde (pH 7.4) - phosphate-buffered saline (PBS) at 4°C for hematoxylin-eosin (H&E) staining for the histological evaluation and morphometry.

RT-PCR of rat MIF

Detection of MIF-mRNA was performed as follows. After isolation of the RNA by acid guanidinium thiocyanate-phenol-chloroform extraction, the complementary deoxyribonuclease (cDNA) was prepared from each sample as described previously.[22]

The gene structure of MIF is shown in Figure 1. The gene encoding MIF consists of 3 exons with cDNA of 348 base pairs (bp). The primer was selected between 15 bp from the initiation codon and 15 bp from the termination codon. In all cases, the first primer was the forward PCR primer and the second primer was the reverse PCR primer. The primers were: MIF forward primer: 5'-ATGCCTT AGTTCATC-3'; MIF reverse primer: 3'-GAGTTCGCTTCCACC-5' (size of PCR product was 348 bp) and ß-actin forward primer: 5'-ATGTTTGAGACCTTCAAAAC-3'; ß-actin reverse primer: 5'-A ACGTCACACTTCATGATGGA-3' (size of PCR product was 525
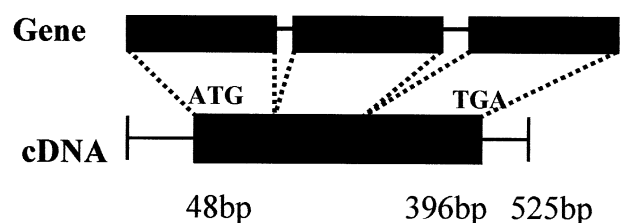

Figure 1. Scheme of gene and cDNA of rat MIF.

bp). The cycle conditions were 95°C for 5 min/95°C for 45 s/60°C for 75s/72°C for 105s for 24 cycles, followed by an extension step of 5 min at 72°C and then stored at 4°C. In a series of preliminary studies, we determined the optimal number of thermal cycles by the exponential portion of the curve for the PCR products and testing several different amounts of initial DNA template. Then, we confirmed that the amount of each PCR product after these PCR cycles correlated with that of the initial DNA template. For electrophoresis, 8 μL of the PCR products were loaded on 2.0% agarose gel in a Trisborate-EDTA buffer. After electrophoresis, the gel images showing ethidium bromide fluorescence were scanned with Image Reader software using a Fluoro-image analyzer and analyzed with Image Gauge software. For the quantitative analysis, the intensity of the PCR products in MIF relative to that in ß-actin was calculated for each sample. To confirm that the correct RCP products had been amplified, the gel bands were excised from the gel and sequenced (ABI733, Applied Biosystems, Foster City, CA).

Histological evaluation and computerized morphometry

The H&E-stained sections of transplanted trachea were examined histologically by light microscopy (magnified ×200). Each image was digitized and analyzed using the software of Scion image Beta 4.02 (Scion Co., Frederick, MD). We quantified the height of the tracheal epithelium and the proportion of obliterated intra-luminal area in the transplanted trachea, using the method reported previously.[23]

Statistical analysis

Results were expressed as mean (or proportion) ± standard deviation. The overall difference among groups in the distribution of the height of epithelium and the frequency of intrluminal obliterans was first analyzed by analysis of variance (ANOVA), and if the overall difference was significant, then Scheffe's multiple comparison method was used for post hoc pairwise comparison. The necessary calculations were performed using StatView 5.0 (SAS Institute Inc., Cary NC).

Results

All procedures were technically successful and the treated animals remained stable. MIF-mRNA was detected in the syngeneic and allogenic implants (Figure 2). The expression level of MIF-mRNA in allograft was higher at days 7 and 21 than in the isograft. Histological examination of the transplanted trachea showed more flattening of the tracheal epithelium at day 7 in the allograft than in the isograft (Figure 3 A and B). However, the epithelium in the anti-MIF Ab-treated allograft was not flattened compared to non-treated rats (Figure 3 C). There was marked intraluminal obliterans at day 21 in the allograft (Figure 4 A), and the intraluminal obliterans was suppressed in anti-MIF Ab-treated allograft (Figure 4 B).
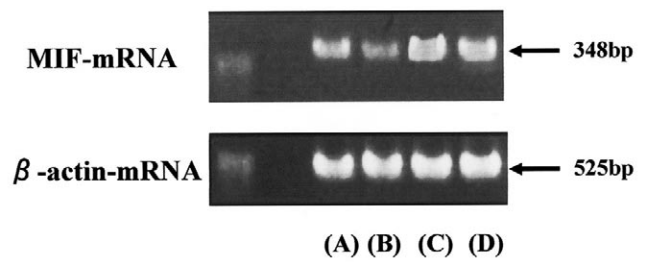

Figure 2. MIF-mRNA expression by RT-PCR in the transplants. (A) Isograft at day 7. (B) Isograft at day 21. (C) Allograft at day 7. (D) Allograft at day 21. The ß-actin-mRNA was used for control.
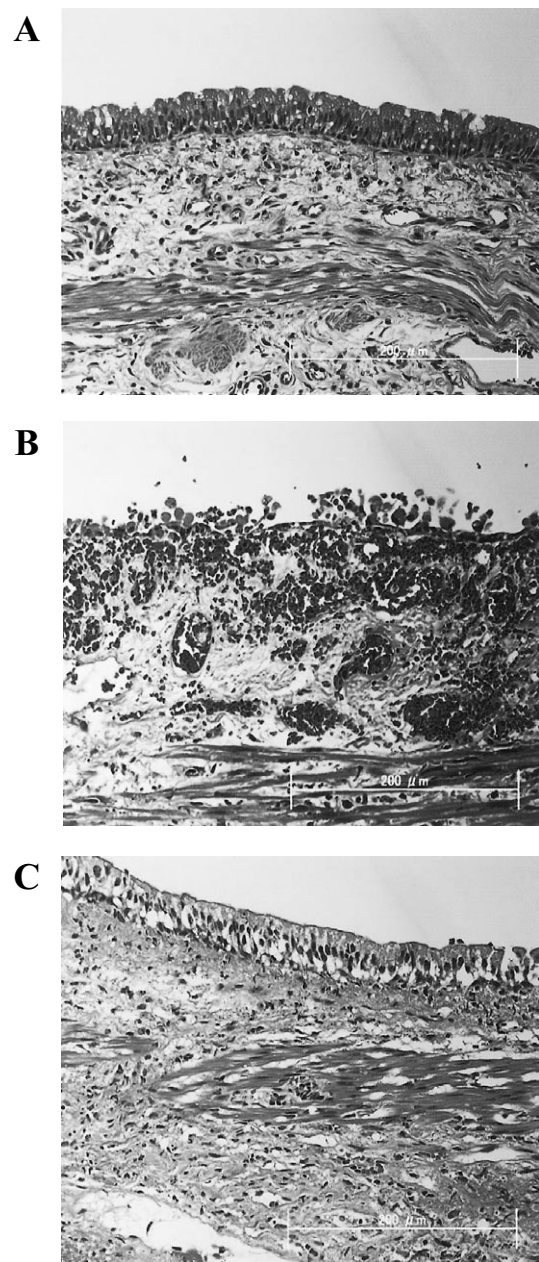

Figure 3. Histological findings of epithelium at the membranous part of the trachea in transplanted rat. **A.** Isograft. **B.** Non-treated allograft. **C.** Anti-MIF Ab treated allograft.
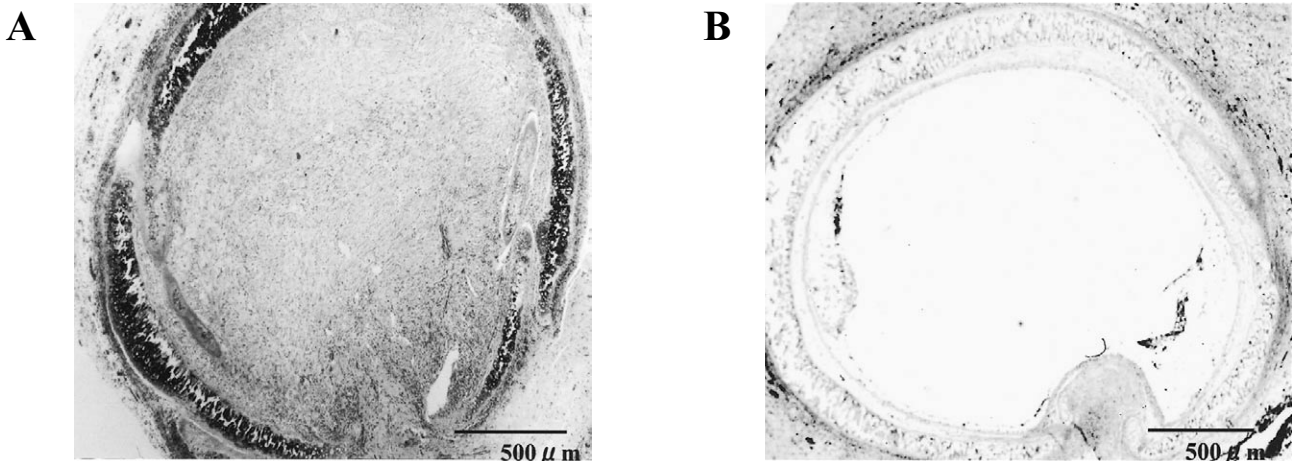
A

B

Figure 4. Histological findings of intraluminal granulomatous obliterans of trachea in transplanted rat. **A.** Non-treated allograft. **B.** Anti-MIF Ab treated allograft.
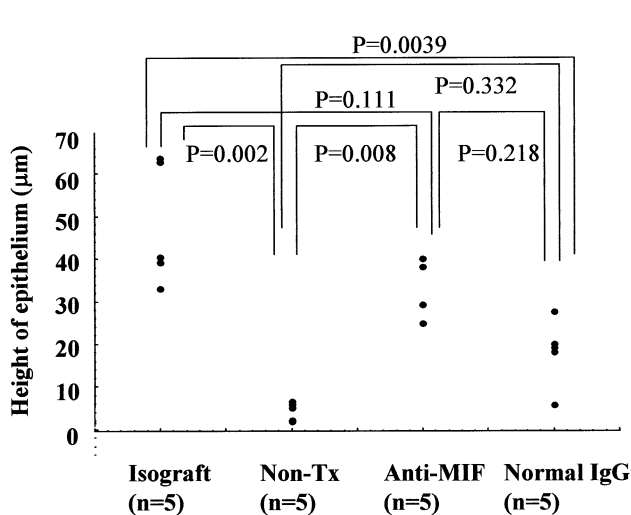

Figure 5. The distribution of the height of epithelium in each group. The post hoc pairwise comparison was based on Scheffe's multiple comparison method.
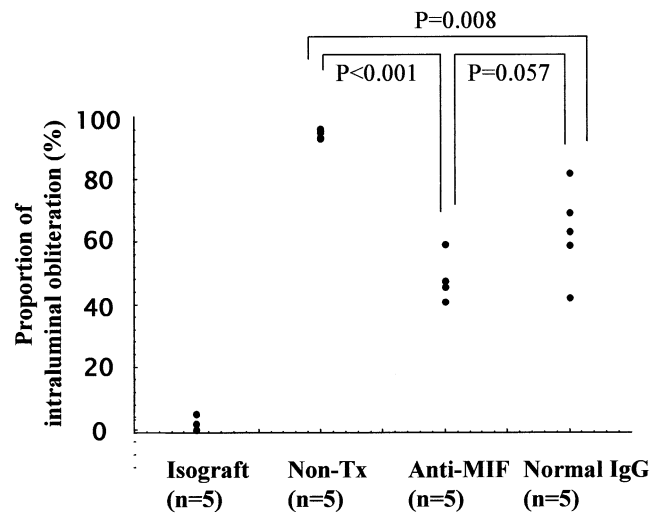

Figure 6. The proportion of intraluminal obliterans in each group. The post hoc pairwise comparison was based on Scheffe's multiple comparison method.

Figure 5 shows the distribution of height of the epithelium at day 7 in each group. The epithelium in non-Tx group (4.8±2.1 μm) was little observed compared to the isograft group (49.7±15.9 μm) at day 7. The epithelial height in the anti-MIF group (32.3±6.5 μm) was significantly greater (p=0.008) than that in the non-Tx group, while it did not differ much from that in the normal IgG group (17.9±9.1 μm) or in the isograft group. Figure 6 shows the distribution of the proportion of intraluminal obliteration at day 21 in each group. The intraluminal space was mostly replaced by fibrous granulation tissue in the non-Tx group (94.6±1.4%). The proportion of obliterans in the anti-MIF group (46.8±7.6%) was significantly lower (p<0.001) than that in the non-Tx group, which was in turn lower than that in the normal IgG group (63.1±14.6%).

Discussion

As described above, OB is the major cause of morbidity and mortality in patients who undergo heart-lung and lung transplantation. The long-term survival is poor in approximately 50% of the patients who develop OB.[23] The pathogenesis of OB is not fully understood at this stage; however, OB is considered the result of chronic rejection.[7,24,25] Hertz et al.[26] used a murine model of heterotopic lung transplantation and showed that the allograft was infiltrated by immune cells, which was then occluded with fibroblasts or collagen scar resembling OB within 4 weeks after transplantation. In the present study, the transplanted trachea in non-treated allograft showed marked disappearance of the epithelium at day 7 and complete obstruction of the intraluminal space at day 21. The results indicate that the fibrous granulation was induced by the loss of tracheal epithelium. Therefore, changes in tracheal or bronchial epithelium seem to be

an important sign for predicting the development to OB. In this regard, it is necessary to suppress the flattening of tracheal or bronchial epithelium in order to prevent OB.

The immunological mechanism of OB is considered as graft rejection caused by infiltrating T-cells or activated macrophages in the graft.[27] T-lymphocytes are the central mediators of allograft rejection, and directly cause tissue damage through their cytotoxicity against donor cells.[27,28] In this regard, David et al.[27,28] showed that the allo-reactive CD8^{+} T-cells and CD4^{+} T-cells caused OB. T-cell-dependent macrophage activation and recruitment may contribute to graft injury nonspecifically through DTH reactions. Suppressing this immunological adverse response may reduce the rejection in lung transplantation. In the present study, we hypothesized that neutralization or prevention of production of harmful cytokines such as a TNF-α, infiltration of macrophages and T-lymphocytes may reduce allograft rejection.

Figure 7 shows the scheme of interactive responses by MIF.[20] MIF is a pro-inflammatory cytokine, and is a central mediator of DTH reaction *in vivo*.[16] MIF is secreted by macrophages or T-cells in various organs in response to harmful stimulants such as endotoxin oxygen radicals. Subsequently, MIF plays a pivotal role in the primary antigenic and mitogenic stimulation of T-cells or T-cell-dependent antibody production, which should adversely affect inflammatory changes.[17] Furthermore, MIF stimulates the proliferation of fibroblasts[17] and is expressed in the tissue of rat showing allograft rejection.[18] These findings suggest that MIF is a potential player in the process of allograft rejection. Therefore, we examined the effect of blocking this protein by neutralization using antibody. Our results showed that animals treated with anti-MIF antibody prevented flattening or disappearance of epithelium, and reduced the granulomatous obliterans in the transplanted trachea. Abe et al.[29] also reported that downregulation and trafficking of anti-tumor T-lymphocytes and neutralization of T-cell cytotoxicity by MIF might enhance survival of transplants. Alho et al.[30] reported that blocking TNF-α provided benefit in the clinical setting. TNF-α regulates the gene expression of MIF through tyrosine kinase-dependent pathway.[31] In the next step, we plan to clarify the association of CD8^{+} lymphocytes and macrophages in the allograft, and examine the effect of suppression of these inflammatory cells by using anti-MIF antibody. Based on the present results, we believe that anti-MIF antibody is a potentially useful therapy for the prevention of allograft rejection through MIF-mediated DTH reaction in patients who undergo lung transplantation.

In conclusion, we have demonstrated in the present study that anti-MIF antibody significantly suppressed the disappearance or flattening of tracheal epithelium and intraluminal obliterans by fibrous granulation, representing a block of the adverse immunological responses caused by MIF and associated with allograft rejection. Clinical application of anti-MIF antibody could potentially prevent bronchitis obliterans and promote allograft tolerance.

Acknowledgments

Figure 7. Schematic illustration of interactions between MIF and inflammatory responses.[20]

References

1. Hartwig MG, Appel JZ 3rd, Cantu E 3rd et al. Improved results treating lung allograft failure with veno-venous extracorporeal membrane oxygenation. *Ann Thorac Surg* 80: 1872-1879, 2005
2. Sandmeier P, Speich R, Grebski E et al. Iron accumulation in lung allografts is associated with acute rejection but not with adverse outcome. *Chest* 128: 1379-1384, 2005
3. Yousen SA, Ray L, Paradis IL, Dauber JA, Griffith BP. Potential role of dendritic cells in bronchiolitis obliterans in heart-lung transplantation. *Ann Thorac* 49: 424-428, 1990
4. Cooper JD, Billingham M, Egan T et al. A working formulation for the standardization of nomenclature and for clinical staging of chronic dysfunction in lung allografts. International Society for Heart and Lung Transplantation. *J Heart Lung Transplant* 12: 713-716, 1993
5. Bando K, Paradis IL, Similo S et al. Obliterative bronchiolitis after lung and a heart-lung transplantation: an analysis of risk factors and management. *J Thorac Cardiovasc Surg* 110: 4-13, 1995
6. Scott JP, Higenbottam TW, Sharples L et al. Risk factors for obliterative bronchiolitis in heart-lung transplant recipients. *Transplantation* 51: 813-817, 1991
7. Zheng L, Orsida B, Whitford H et al. Longitudinal comparisons of lymphocytes and subtypes between airway wall and bronchoalveolar lavage after human lung transplantation. *Transplantation* 80: 185-192, 2005
8. Sirak J, Orosz CG, Wakely E et al. Alloreactive delayed-type hypersensitivity in graft recipients: Complexity of response and divergence from acute rejection. *Transplantation* 63: 1300-1307, 1997
9. Majeski EI, Harley RA, Bellum SC, London SD, London L. Differential role for T cells in the development of fibrotic lesions associated with reovirus 1/L-induced bronchiolitis obliterans organizing pneumonia versus acute respiratory distress syndrome. *Am J Respir Cell Mol Biol* 28: 208-217, 2003
10. Bloom BR, Benett B. Mechanism of a reaction in vitro associated with delayed-type hypersensitivity. *Science* 153: 80-82, 1966
11. Bernhagen J, Calandra T, Mitchell RA, Martin SB, Bucala R. MIF is a pituitary-derived cytokine that potentiates lethal endotoxemia. *Nature* 365: 756-759, 1993
12. Bucala R. MIF rediscovered: cytokine, pituitary hormone, and glucocorticoid-induced regulator of the immune response. *FASEB J* 10: 1607-1613, 1996
13. Bernhagen J, Bacher M, Calandra T et al. An essential role for macrophage migration inhibitory factor in the tuberculin delayed-type hypersensitivity reaction. *J Exp Med* 183: 277-282, 1996
14. Mikulowska A, Metz NC, Bucala R, Holmdahl R. Macrophage migration inhibitory factor is involved in the pathogenesis of collagen type a-induced arthritis in mice. *J Immunol* 158: 5514-5517, 1997
15. Bernhagen J, Calandra T, Mitchell RA et al. MIF is a pituitary-derived cytokine that potentiates lethal endotoxaemia. *Nature* 365: 756, 1993
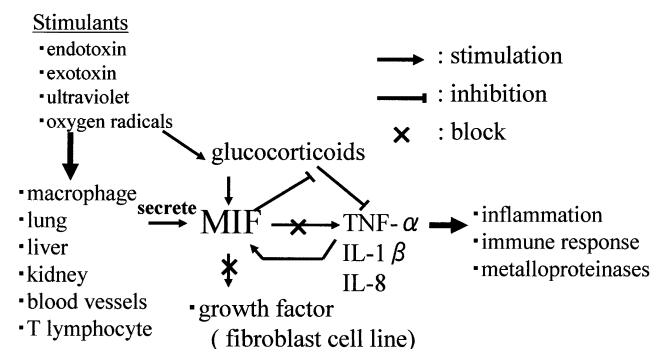
16. Bacher M, Metz CN, Calandra T et al. An essential regulatory role for macrophage migration inhibitory factor in T-cell activation. *Proc Natl Acad Sci USA* 93: 7849-7854, 1996

17. Mitchell RA, Metz CN, Peng T et al. Sustained mitogen-activated protein kinase (MAPK) and cytoplasmic phospholipase A2 activation by macrophage migration inhibitory factor (MIF). *J Biol Chem* 274: 18100-18106, 1999

18. Lan HY, Yang N, Brown FG et al. Macrophage migration inhibitory factor expression in human renal allograft rejection. *Transplantation* 66: 1465-1471, 1998

19. Brown FG, Nikolic-Paterson DJ, Metz C et al. Up-regulation of macrophage migration inhibitory factor in acute renal allograft rejection in the rat. *Clin Exp Immunol* 118: 329-336, 1999

20. Makita H, Nishimura M, Miyamoto K et al. Effect of anti-macrophage migration inhibitory factor antibody on lipopolysaccharide-induced pulmonary neutrophil accumulation. *Am J Respir Crit Care Med* 158: 573-579, 1998

21. Reishenspruner H, Soni V, Nitschke M et al. Enhancement of obliterative airway disease in rat tracheal allografts infected with recombinant rat cytomegalovirus. *J Heart Lung Transplant* 17: 439-451, 1998

22. Nishihira J, Kuriyama T, Sakai M et al. The structure and physicochemical properties of rat liver macrophage migration inhibitory factor. *Biocehem Biophys Acta* 1247: 159-162, 1995

23. Reichenspurner H, Girgis R, Robbins R et al. Obliterative bronchiolitis after lung and heart-lung transplantation. *Ann Thorac Surg* 60: 1845-1853, 1995

24. Martinez FJ, Chang A. Surgical therapy for chronic obstructive pulmonary disease. *Semin Respir Crit Care Med* 26: 167-191, 2005

25. Verleden GM, Dupont LJ, Van Raemdonck DE. Is it bronchiolitis obliterans syndrome or is it chronic rejection: a reappraisal? *Eur Respir J* 25: 221-224, 2005

26. Hertz MI, Jessurun J, King MB et al. Reproduction of the obliterative bronchiolitis lesion after heterotopic transplantation of mouse airway. *Am J Pathol* 142: 1945-1951, 1993

27. David MR, Stacy LD, Marshall IH et al. Trachea allograft class I molecules directly activate and retain CD8^{+} T cells that cause obliterative airways disease. *J Immunol* 171: 6919-6928, 2003

28. David MR, Stacy LD, Benjamin DE et al. Indirect minor histocompatibility antigen presentation by allograft recipient cells in the draining lymph node leads to the activation and clonal expansion for CD4^{+} T cells that cause obliterative airways disease. *J Immunol* 172: 3469-3479, 2004

29. Abe R, Peng T, Sailors J et al. Regulation of the CTL response by macrophage migration inhibitory factor. *J Immunol* 166: 747-753, 2001

30. Alho HS, Maasilta PK, Harjula ALJ et al. Tumor necrosis factor-alpha in a porcine bronchial model of obliterative bronchiolitis. *Transplant* 76: 516-523, 2003

31. Horikawa J, Sakaue S, Furuya Y et al. Tumor necrosis factor-alpha regulates the gene expression of macrophage migration inhibitory factor through tyrosine kinase-dependent pathway in 3T3-L1 adipocytes. *J Biochem* 123: 733-739, 1998