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Increased expression of Tim-3 and its ligand Galectin-9 in rat allografts during acute rejection episodes

Xinwei Qiao, Ke Jiang, Jun Nie, Kai Fan, Zhikun Zheng, Jianjun Wang *, Jinsong Li *

Division of Thoracic Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

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ABSTRACT

The aim of the study is to elucidate the profiles of T-cell immunoglobulin and mucin domain-3 (Tim-3) and its ligand Galecin-9 in acute pulmonary rejection by using a rat model of lung transplantation. Left lung grafts retrieved from Lewis or Fisher 344 rats were orthotopically transplanted into Lewis recipients without any immunosuppressions; the grafts were harvested at day 3, 7 or 10 after transplantation. The grade of acute rejection was histopathologically evaluated. Tim-3, Galectin-9, immune antigen and related cytokines expression were assessed with immunological techniques and real-time polymerase chain reaction (RT-PCR), respectively. Then, our results showed that Tim-3 and its ligand Galectin-9 were markedly up-regulated at protein and mRNA levels in allografts compared with syngrafts. Meanwhile, the decreased CD4/CD8 ratio was associated with acute rejection occurring and Tim-3 expression on $CD4^+$ and $CD8^+$ T cells in allografts was increased. Therefore, our study firstly described that enhanced Tim-3 and its ligand Galectin-9 in allografts might play an important role in the pathogenesis of rat lung transplant rejection, implying new valuable markers for detecting acute allograft rejection.

1. Introduction

Lung transplantation has recently become the ultimate and effective therapeutic option for several end-stage lung diseases. Despite improvements in operative techniques and the advances in immunosuppressants, the success of clinical lung transplantation is poor in comparison to other solid organ transplants. Acute rejection (AR) episodes are the significant cause of morbidity for the transplant patient, and about four percent of deaths are directly caused by AR during the first 30 days after lung transplantation [1]. In addition, the frequency and severity of AR is one of the strongest risk factor for the development of obliterative bronchiolitis (OB), a chronic rejection, which is the leading cause of death in lung allograft recipients [2,3].

AR is a post-transplantation reaction that characterized by varying degrees of mononuclear cell infiltrating into alveolar space and interstitium of the transplanted lung. T lymphocyte infiltration and activation play an important role in acute rejection of lung grafts. Although biopsy has been the preferred standard for monitoring acute rejections in lung transplant recipients [4], it is difficult to determine the rejection at the early stage. Therefore, it is a critical need to establish a method for early and specific diagnosis of the rejection response in clinical lung transplantation.

T-cell immunoglobulin and mucin domain (Tim-3), a type I membrane protein, is not expressed on the surface of naïve T cells, but emerges on the surface of fully differentiated $CD4^+$ Th1 cells [5]. Tim-3 may regulate T cell responses, as shown by the fact that it has multiple functions in animal models of experimental autoimmune encephalomyelitis (EAE) and human multiple sclerosis [5,6]. Recently, Galectin-9 has been identified as a ligand for Tim-3. Binding of Galectin-9 to Tim-3 causes an inhibitory signal that results in apoptosis of Th1 cells and negatively regulates Th1-type immunity [6,7]. Since then it has been shown that Tim-3 is also expressed on Th17 and cytotoxic $CD8^+$ T cells [8,9], implying the suppressive role of Galectin-9 on these two cells as well as Th1 cells.

In autoimmune diseases, previous studies have suggested that important roles of Tim-3 in regulating Th1/Th2 responses [6,10,11]. In alloimmune diseases, Tim-3 also plays an important role in solid organ transplantation since Tim-3 and its ligand have been demonstrated as useful biomarkers for early diagnosis of acute rejection [12–14]. However, the profiles of Tim-3 and its ligand in lung transplantation have not been illustrated before. Therefore, the aim of this study is to assess the expression of Tim-3 and Galectin-9 in pulmonary grafts and to delineate their immunological properties by using a rat model of lung transplantation.

* Corresponding authors. Address: Division of Thoracic Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China.

E-mail addresses: bgjjww@yahoo.com.cn (J. Wang), lijinsong67@hotmail.com (J. Li).

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2. Materials and methods

2.1. Experimental animals and lung transplantation

Specific pathogen-free, male Lewis (LEW; $RT1^{1}$) and Fisher 344 (F344; $RT1^{1v1}$) rats weighing 220–280 g were purchased from Jackson laboratory and kept under conventional conditions. All animals received humane care according to the current version of the Tongji Medical College Animal Care and Use Committee Guidelines.

Rat model of orthotopic left lung transplantation was performed using cuff technique as previously described [15]. Briefly, the recipient animals were anesthetized by intraperitoneal pentobarbital. Lung transplantation was performed between LEW to LEW for syngrafts, and F344 to LEW for allografts. All recipient rats did not receive any immunosuppressive agents. Recipients were sacrificed and grafts were harvested at day 3, 7, or 10 after transplantation.

2.2. Histology and immunohistochemistry

In order to confirm the changes in the different state of the transplanted lung with time, histological evaluation of syngraft and allograft lungs was performed. Harvested lungs were fixed in 10% phosphate-buffer formalin and embedded in paraffin. Sections were prepared and stained with hematoxylin and eosin (H&E). Grading for acute cellular rejection was performed according to the ISHLT revision of the working formulation for the classification of pulmonary rejection from 2007 [4].

Parallel sections were analyzed for Tim-3, Galectin-9, CD4 and CD8 by immunohistochemistry which was performed according to a previously established protocol [16]. Briefly, specimens were fixed in cold acetone for 10 min and anti-rat Tim-3 (eBiosciences Inc.), Galectin-9 (Bioworld Inc.), CD4 (Bioss Inc.), CD8 (Bioss Inc.) were employed as primary antibodies at a dilution of 1:200 in immunohistochemical staining. All stained slides were scanned using a microscope (BX51, Olympus) with camera (Axio Cam MRc, Carl Zeiss) and images were analyzed using Image-Pro Plus 6.0 for windows (Media Cybernetics) analysis program.

2.3. Double-labeling immunofluorescence

The procedure was performed as previously described [17]. Briefly, paraffin-embedded sections were deparaffinized, and antigen retrieval was subsequently performed. Antibodies were employed as described in the preceding text. After incubation with the mixed primary antibody (mixture of mouse anti-rat Tim-3 and rabbit anti-rat CD4 or anti-CD8), cyanidin-3 labeled goat anti-mouse secondary antibody and fluorescein isothiocyanate-labeled goat anti-rabbit secondary antibody were used to display Tim-3 and CD4 or CD8 in lung grafts, respectively. All manipulations were kept away from light. The positive signals were analyzed by the Image Pro Plus 6.0 system.

2.4. Quantitative real-time polymerase chain reaction

RNA extracted from grafts with Trizol (Invitrogen) was reverse transcribed to cDNA with reverse transcription kits (Fermentas). Quantitative real-time polymerase chain reaction (PCR) was performed with Quantitect SYBR Green PCR Kit (Fermentas) according to the manufacturer's protocol on Rotor Gene 3000 (Corbett Research, Australia). Each sample was analyzed in triplicate. Cycling conditions consisted of an initial denaturation of 95 °C for 10 min, and followed by 40 cycles of 95 °C for 30 s, followed by 60 °C for 30 s. Results were expressed with the comparative CT method relative to the housekeeping gene β-actin. Oligonucleotides used as primers in this study could be seen in Table 1.

2.5. Statistical analysis

Results were expressed as the mean ± SD. Statistical analysis of continuous variables was performed using the Student's *t*-test or analysis of variance (ANOVA) where appropriate. Spearman's test was used to assess any correlation. A probability level of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Time course of graft histopathology

Our study firstly established the animal model for acute rejection of fully allogeneic F344 donor lungs transplanted to LEW recipients. Lungs from allografts revealed histopathological features typical for acute rejection in this rat strain combination. In allografts at day 3, mononuclear cells began to infiltrate around vessels while peribronchial regions were almost intact (Fig. 1A-b), and a great number of mononuclear cells abruptly infiltrated not only into perivascular region but also into peribronchial region at day 7 after allo-transplantation (Fig. 1A-d). The rejection grade increased sequentially with time, allografts at day 10 were characterized by massive mononuclear cell infiltrating in perivascular and peribronchial regions (Fig. 1A-f). In contrast, syngrafts from day 3 to day 10 post-transplant revealed a sparse influx of mononuclear cells into the alveolar space, infrequently accompanied by marginal basal atelectasis and no evidence of inflammation was present (Fig. 1A-a, c and e).

3.2. Decreased CD4/CD8 ratio in filtrating lymphocytes during acute rejection

To evaluate the adaptive T-cell response during acute rejection of rat lung allografts, we compared graft T-cell infiltration by immunohistochemical staining in isografts and allografts. The expression of $CD4^+$ and $CD8^+$ T cells in isografts was relatively stable from day 3 to day 10 (Fig. 1C–G). In allograft lungs, while $CD4^+$ T cells decreased from 22.56 ± 1.17 to 13.01 ± 1.02 from day 3 to day 10 (Fig. 1C and E), $CD8^+$ T cells increased from 7.38 ± 1.02 to 18.67 ± 1.45 (Fig. 1D and F), and the CD4/CD8 ratio decreased from 3.08 ± 0.27 to 0.70 ± 0.08 (Fig. 1G). In addition, a lower CD4/CD8 ratio in allografts was associated with the stronger acute rejection occurring (Fig. 1G).

3.3. Expression of Tim-3 and Galectin-9 protein

Immunohistochemical analysis was used to detect the expression of Tim-3 and Galectin-9 protein in the pulmonary grafts.

Table 1
Sequences of primers used for real-time polymerase chain reaction.

Genes	Primers (5′ → 3′)	Products (bp)
β-Actin	Forward: CACGATGGAGGGGCCGGACTCATC Reverse: TAAAGACCTCTATGCCAACACAGT	240
Tim-3	Forward: ATCTGCCCTGCAGCTACACT Reverse: TCAGCGACATGTCTCCTTTG	186
Galectin-9	Forward: ATCCCTCCTATGGCATACCC Reverse: AGGTGGAAAGCAATGTCACC	167
IL-17	Forward: ACAGTGAAGGCAGCGGTACT Reverse: GCTCAGAGTCCAGGGTGAAG	177
IFN-γ	Forward: AACAACCCACAGATCCAGCA Reverse: CTTATTGGCACACTCTCTACC	132

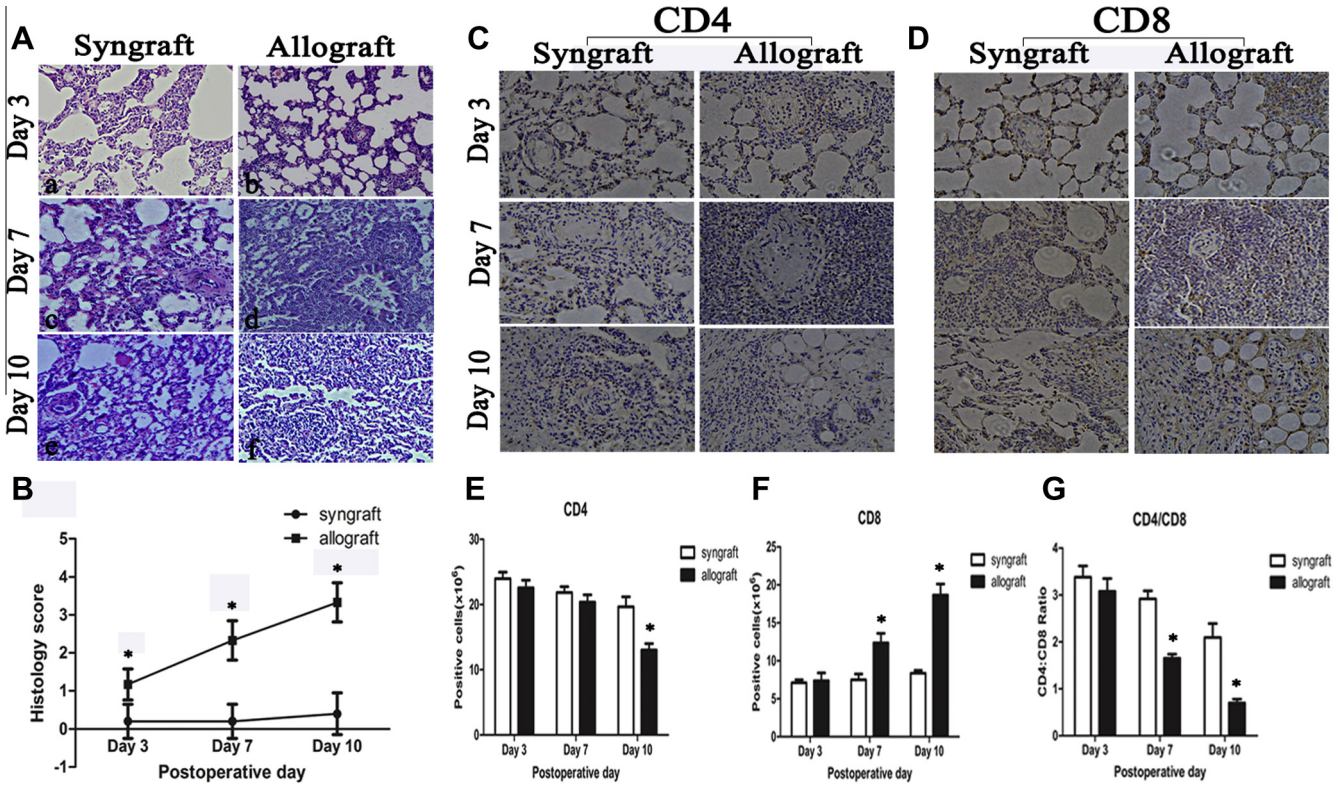

Fig. 1. Representative histopathological findings and T lymphocytes infiltration in syngrafts and allografts after transplantation. (A) Histological features of syngrafts and allografts at day 3, 7 and 10; (B) Acute rejection pathology score means; (C and D) Immunohistochemical staining for $CD4^+$ and $CD8^+$ T lymphocytes infiltration in syngrafts and allografts; (E and F) Quantitive analysis of $CD4^+$ and $CD8^+$ T lymphocytes infiltration; (G) Acute rejection following lung transplant associated with a decreased graft CD4/CD8 ratio. (Original magnification, 200×; $^*P < 0.05$; $n = 6–8$ rats per group).

The protein expression of them in isografts changed slightly after transplantation. In contrast, their protein levels in allografts were significantly elevated in a similar manner, which rose at day 3, then decreased to the minimal levels at day 7, and increased to the maximum at day 10 (Fig. 2C and D; $P < 0.05$). Moreover, Tim-3 in lung grafts was expressed mainly in infiltrating mononuclear cells (Fig. 2A), and Galectin-9 expression was mainly localized in the vascular endothelial and bronchial epithelial cells (Fig. 2B).

3.4. Tim-3 expression on $CD4^+$ T cells and $CD8^+$ T cells

In order to understand the regulatory systems of Tim-3 in lung transplantation, surface expression of Tim-3 on T lymphocytes infiltrating in rat lung grafts was examined by double staining Immunofluorescence. Both $CD4^+$ and $CD8^+$ T cells in lung grafts exhibited the expression of surface Tim-3 (Fig. 3A and B), and the frequencies of Tim-3^+CD4^+ cells and Tim-3^+CD8^+ cells were significantly higher in allotransplant recipients than in syngeneic recipients (Fig. 4C and D). Furthermore, a trend toward decreased frequency of Tim-3^+ cells in $CD4^+$ T cells in allografts was observed after transplantation. In contrast to Tim-3^+CD4^+ cells, an increase of Tim-3^+ cells on $CD8^+$ T cells was detected with time (data not show).

3.5. Tim-3, Galectin-9 and related cytokines mRNA expression by RT-PCR

The expression of related cytokines as well as Tim-3 and Galectin-9 mRNA in lung grafts from allogeneic and syngeneic recipients was investigated by RT-PCR. While mRNA expression was relatively stable in syngrafts with time, gene expression of Tim-3, IFN-γ and IL-17 in allografts significantly up-regulated in a time-dependent manner, which elevated at day 3, then decreased to the minimum at day 7, and increased to the maximal levels at day 10 (Fig. 3E, G and H). Somewhat differently, the intragraft gene expression of Galectin-9 reached maximal levels at day 3 after allotransplantation (Fig. 3F). In agreement with the cytotoxic response, relative mRNA expression of IFN-γ and IL-17 was higher in samples with acute rejection ($P < 0.05$).

3.6. Correlation analysis

Considering Tim-3 as a surface molecule preferentially expressed on Th1 and Th17 cells, we speculated that Tim-3 was involved in Th1 and Th17 immune response. The correlation of Tim-3 with Th1-related cytokine (IFN-γ), and with Th17-related cytokine (IL-17) was detected in rejection group. Indeed, higher levels of Tim-3 mRNA were associated with high levels of IFN-γ mRNA (Fig. 4A) and IL-17 mRNA (Fig. 4B), respectively.

Since Galectin-9 was identified as the Tim-3 ligand, we addressed to determine the correlation of these two molecules. A positive correlation between their protein levels was shown in Fig. 4E, and a correlation between their mRNA levels was also found when rejection grade was A2 or greater (Fig. 4C).

It was demonstrated that the pro-inflammatory cytokine IFN-γ could induce Galectin-9 expression, in the current study we observed a correlation between them when rejection grade was greater than A2 (Fig. 4D). Meanwhile, Galectin-9 mRNA expression was associated with the severity of allograft rejection (Fig. 4F).

4. Discussion

In lung transplantation, the frequency and severity of acute rejection have been shown to promote the development of OB,

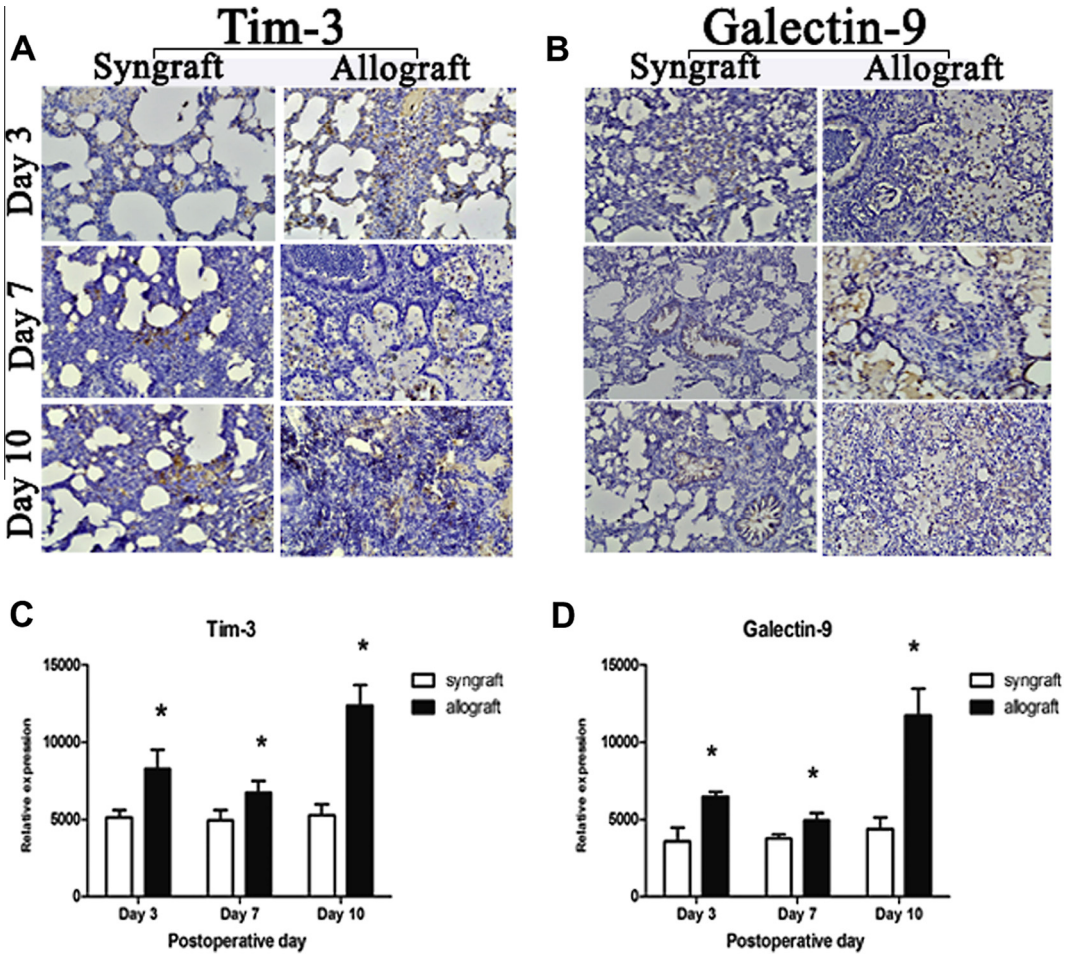

Fig. 2. Immunohistochemical analysis of Tim-3 and Galectin-9 protein expression in lung grafts after transplantation. Immunohistochemical staining for Tim-3 (A) and Galectin-9 (B) protein expression; Quantitive analysis of Tim-3 (C) and Galectin-9 (D) protein expression. (Original magnification, 200×; $^{*}P < 0.01$; $n = 6–8$ rats per group).

which ultimately causes graft dysfunction [2,3]. However, the mechanisms that lead to both acute and chronic lung allograft rejection have still not been clarified. The aim of this study was to investigate whether Tim-3 and its ligand Galetin-9 were involved in the immune reactions of acute allograft rejection using the rat lung allo-transplantation model and to explore underlying mechanisms.

Rat models of orthotopic lung allografts have been generally used to study early postoperative problems, such as ischemia-reperfusion, airway dehiscence, and acute rejection. In this article, we firstly established the F344 to LEW rat strain combination in lung transplantation, to decipher potential mechanisms of pulmonary acute rejection. In contrast to lung transplantation in the rat Dark Agouti (DA) to Lewis (LEW) combination [18], lung allografts transplanted in this model were less vigorously rejected.

It is well established that T lymphocytes play a central role in mediating transplant rejection, orchestrating the generation of inflammatory events that ultimately lead to graft destruction. Investigation in a rat lung transplant model found a decreased CD4/CD8 ratio in allografts mainly due to an increase of $CD8^+$ T cells [19]. The similar result was detected in bronchoalveolar lavage (BAL) fluid of lung-transplanted patients [20]. In the present paper, analysis of T-lymphocyte subpopulations by immunohistochemistry pointed out a decrease of the CD4/CD8 ratio during acute rejection that was due to an elevation of CD8 positive cells and a decline of CD4 positive cells. Our results were in agreement with previous studies showing the decrease of CD4/CD8 ratio was associated with alloimmune response, and the lower CD4/CD8 ratio correlated with increasing frequency of rejection.

Tim-3, a novel member of the Tim family of molecules, is the first molecule identified to be specifically expressed on terminally differentiated $CD4^+$ Th1 but not on Th2 cells [5], which has also been found on cytotoxic $CD8^+$ T cells and Th17 [8,9]. By using Immunological techniques, we detected that Tim-3 expressed mainly on infiltrating mononuclear cells, then indicated that it was expressed on $CD4^+$ and $CD8^+$ T lymphocytes in lung grafts, and the frequencies of Tim-3 expressed on T cell subsets in allografts were higher than in syngrafts. Therefore, the increase of Tim-3^+ cells in T lymphocytes was the reason for enhancement of Tim-3 expression in allografts, and the changes of T-lymphocyte subpopulations had an impact on Tim-3 expression during acute rejection episodes. Although the actual regulatory mechanisms for the control of cell surface Tim-3 were unknown, these data indicated that $CD4^+$ and $CD8^+$ T cells might have different regulatory systems with regard to Tim-3.

Galectin-9, as a ligand for Tim-3, was detected on the surface of many cell types, such as endothelial cells and fibroblasts [7], which was in agreement with our observations that Galectin-9 was localized in bronchial epithelium and endothelial cells in lung tissues and increased in allografts with acute rejection. Interestingly, Galectin-9 mRNA expression was inconsistent with its protein levels in the article, and this inconsistency might be due to Galectin-9 in the translation process affected by certain cytokines. Inflammatory cytokines such as IFN-γ and IL-1β were able to induce its

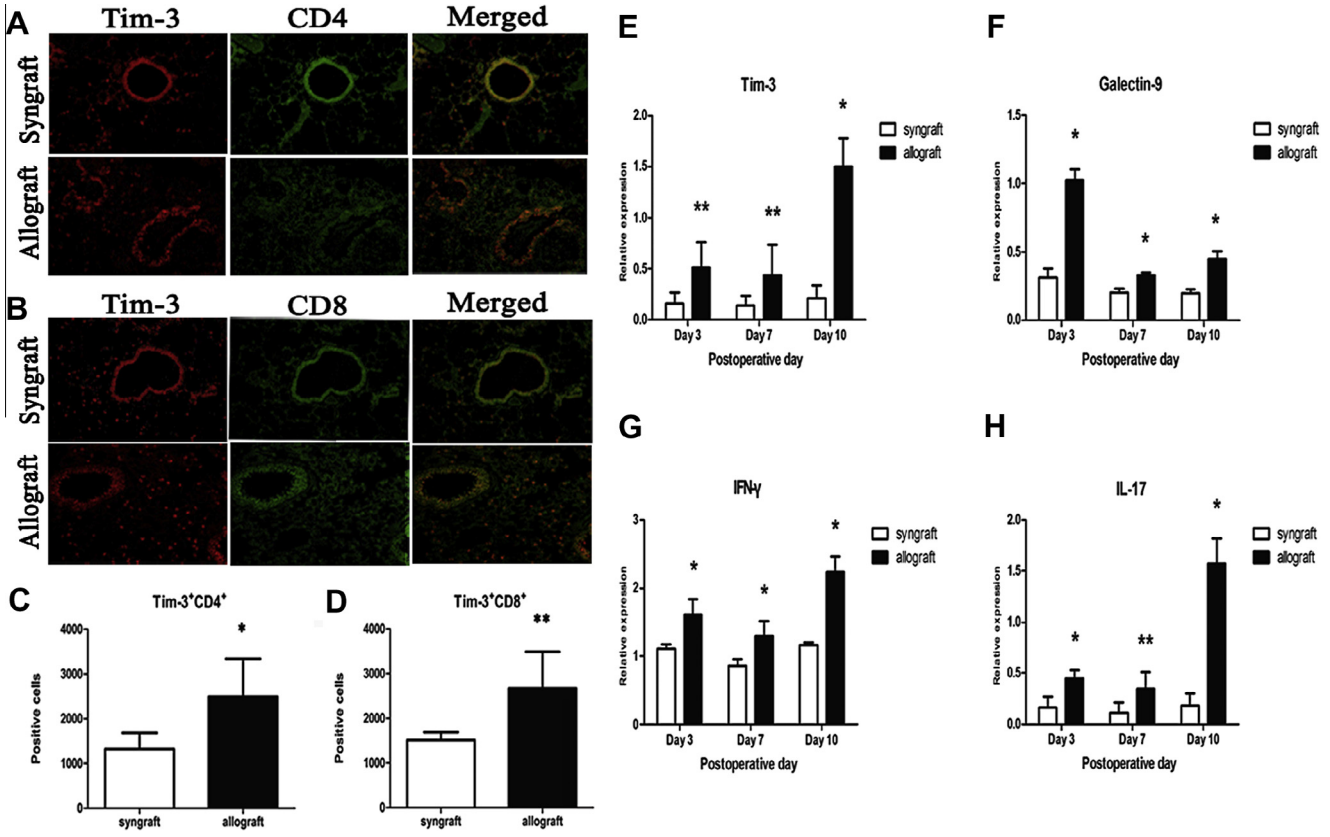

Fig. 3. Detection of Tim-3 and related molecules in lung grafts after transpaltation. (A–D) Double-labeling immunofluorescence for the expression of Tim-3 on $CD4^+$ and $CD8^+$ T lymphocytes in lung grafts at day 7. Tim-3 antibody was labeled with cyanidin-3 (red), and CD4 or CD8 antibody was labeled with fluorescein isothiocyanate (green). (A and B) Tim-3 expression on $CD4^+$ and $CD8^+$ T cells; (C and D) Quantitive analysis of Tim-3^+CD4^+ and Tim-3^+CD8^+ T cells in allografts and syngrafts; (E–H) Time course of Tim-3, Galectin-9, IFN-γ and IL-17 mRNA expression by real-time PCR, respectively. (Original magnification, 200×; $^{**}P < 0.05$, $^{*}P < 0.01$; $n = 6–8$ rats per group). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

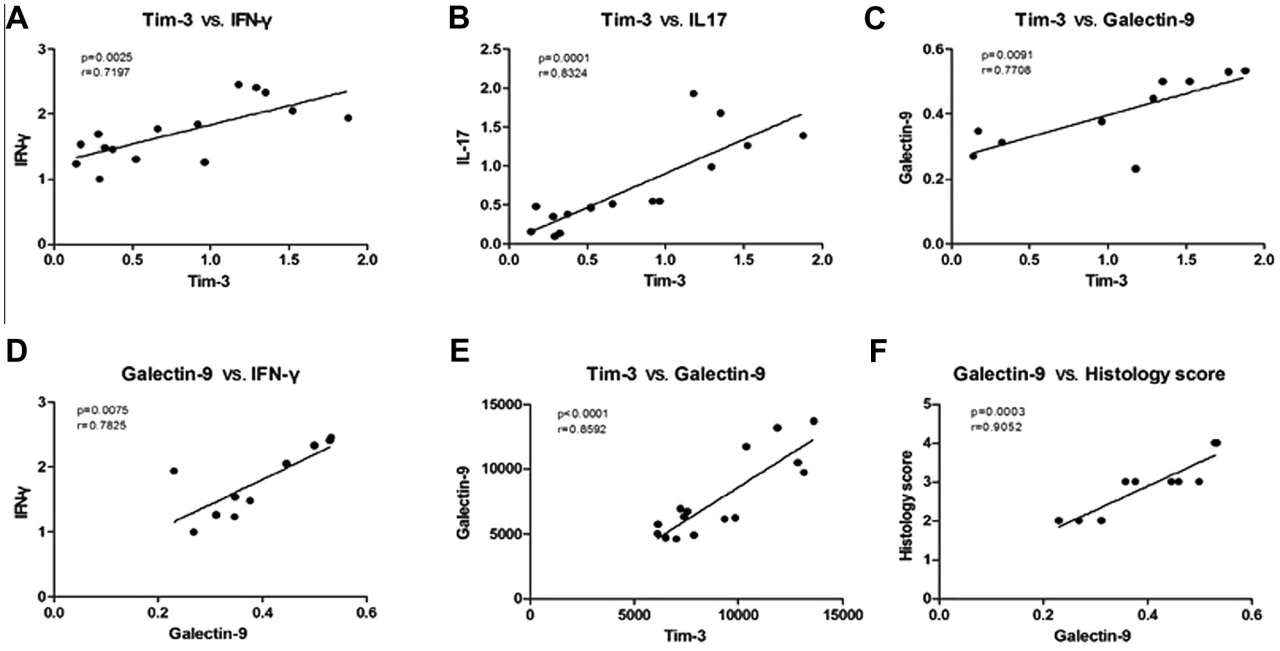

Fig. 4. Correlation analysis in allografts during acute rejection episodes. (A and B) Correlation between Tim-3 and the inflammatory cytokines IFN-γ and IL-17 mRNA levels; (C and D) The Correlation of Galectin-9 with Tim-3 mRNA levels as well as with IFN-γ mRNA levels when rejection grade was greater than A2; (E) Correlation between Tim-3 and Galectin-9 protein levels; (F) Correlation between Galectin-9 mRNA levels and the severity of allograft rejection when rejection grade was greater than A2. ($n = 10–15$ rats per group).

expression in endothelial cells and fibroblasts [21], and our study displayed that there was a correlation between IFN-γ and Galectin-9 mRNA expression.

Initial studies demonstrated that Tim-3 played functional roles in EAE [5]. Tim-3 was proposed as a negative regulator of tissue-destructive immune responses by soluble Tim-3 interfering with Tim-3–Tim-3 ligand (Tim-3L) interaction that normally served to control Th1 responses [22]. A subsequent study suggested that Tim-3 blockage resulted in abrogation of tolerance induction by costimulation blockage in an experimental model [11]. Furthermore, in human renal transplant, Tim-3 was detected increased in urine, blood and kidney allograft nephrectomy samples from recipients during an acute rejection episode [13,14,23]. In addition, a higher mRNA expression of Galectin-9 was found in allograft nephrectomy samples of more sever rejection [24]. In consistent with previous studies, our results displayed that Tim-3 and its ligand Galectin-9 in lung allografts were up-regulated and positively correlated with each other during acute rejection episodes, and the higher the expression, the more acute rejection episodes occurred. Our data suggested that these two molecules implicated in the pathogenesis of murine experimental lung transplantation and could be served as a potential marker of AR. Furthermore, both of them were highly elevated in the early stage of rejection, then the expression might be influenced by some cytokines and began to decline. Thus, it was more sensitive by using them to monitor pulmonary acute rejection responses in the early stage.

As it is demonstrated that Th1 immune responses are important in cell-mediated immunity, and there is a strong support for the hypothesis that Th1 cells are critical for the allograft rejection. In this sense, we investigated the pro-inflammatory Th1-restrict IFN-γ related to the AR response in rats undergoing allograft rejection, and found that IFN-γ mRNA was markedly enhanced in allotransplant recipients with acute rejection, and correlated well with Tim-3. Several studies suggested that IL-17 and Th17-associated cytokines have been linked to the development of allograft rejection after lung transplantation in humans and animal models [25,26]. In this study, we also showed that IL-17 transcripts were significantly increased in allografts during acute rejection episodes, which were in consistent with studies suggesting a pathogenic role for IL-17 in rejection. Furthermore, a correlation between IL-17 and Tim-3 mRNA expression in allografts was found. These findings raised the possibility that Tim-3 engaged in the regulation of Th1 and Th17 responses in lung transplantation.

Galectin-9, a physiological ligand of Tim-3, bounding to Tim-3 on Th1 and Th17 cells induces cell apoptosis and/or suppresses cell differentiation, and activation of the Tim-3–Galectin-9 pathway attenuates cytotoxicity and prolongs the survival of skin or cardiac allografts in mice [27–29], while *in vitro* Galectin-9 suppresses Th17 development independently of Tim-3 [30]. This pathway may become a new therapeutic strategy to prevent acute pulmonary rejection, and further studies will be required to verify the hypotheses.

Our data provided new mechanistic insights into the acute rejection of lung allografts and revealed the prominent role of Tim-3 and its ligand Galectin-9 in the severity of acute rejection, which could be served as surrogate markers of AR in lung transplantation. Indeed there were some limitations in this study, and more studies should be performed to validate these findings in human lung transplantation.

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