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The prevention of tracheal graft occlusion using pioglitazone: A mouse tracheal transplant model study

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ABSTRACT

Bronchiolitis obliterans (BO), which begins with lymphocytic bronchiolitis (LB), is the most serious manifestations of chronic rejection following lung transplantation. We have evaluated the effects of intraperitoneal administration of pioglitazone, a peroxisome proliferator-activated receptor (PPAR) gamma agonist, on LB in mice tracheal transplant model. Tracheal grafts from BALB/c or C57BL/6 were transplanted to C57BL/6 recipients. Animals were divided into three groups (n = 8), isograft (IG) and allograft with (AGP) or without (AGN) pioglitazone. Occlusion rate (OR) of transplanted trachea, CD3, FoxP3 positive cell infiltration, and cytokine levels in the transplanted grafts were analyzed at day 7. In the AGP group, OR was significantly lower than in the AGN group ($p < .01$). An average of 305 ± 56, and 317 ± 84 CD3-positive T-cells/section was shown in the AGN and AGP groups, respectively ($p = .761$). Interestingly, an average of 24 ± 18, and 88 ± 28 FoxP3-positive regulatory T-cells (Tregs)/section was shown in AGN and AGP groups, respectively ($p = .001$). FoxP3 and IL-4 mRNA expression was significantly higher in the AGP group. Pioglitazone can suppress the luminal occlusions of the tracheal graft in an allograft through enhanced Treg infiltration, and may provide new therapies to prevent BO.

1. Introduction

Lung transplantation has become a well-established therapy for patients with severe or terminal pulmonary diseases that cannot be cured completely by medical treatments [1]. Although immunosuppressive therapy efficiently controls the acute rejection, chronic rejection remains the major cause of late mortality following the lung transplantation [2,3]. Bronchiolitis obliterans (BO) and BO-related syndrome (BOS) are recognized as serious complications that can follow lung transplantation, they are associated with increased mortality rate, and affect most lung transplant recipients within five years after the transplantation [4]. BO develops as a progressive obliteration of small airways. This process has been well documented histologically, and represents a series of immunological responses. The process usually begins with the infiltration of lymphocytes into the epithelial and subepithelial tissues, which causes lymphocytic bronchiolitis (LB) and seriously damages normal epithelial cells [5,6]. Consequently, fibroblasts and myofibroblasts are induced to the differentiation, which result in diffuse fibrosis and obstruction of bronchioles [7–9]. Several studies have highlighted a prominent role of infiltrating T-cells in LB [10,11].

Pioglitazone is a peroxisome proliferator-activated receptor (PPAR)-γ agonist, widely used as a type 2 diabetic drug, which inhibits the proliferation of T-cells and the production of inflammatory cytokines [12]. We hypothesized that pioglitazone may improve BO through the inhibition of T-cell infiltration and the downregulation of inflammatory cytokine expressions in the transplanted grafts. To assess this hypothesis, immunohistlogical examination and PCR analysis were performed in mouse orthotopic tracheal transplant model.

Abbreviations: AGN, allograft group without pioglitazone; AGP, allograft group with pioglitazone; BO, bronchiolitis obliterans; BOS, BO-related syndrome; IG, isograft; MAPK, mitogen-activated protein kinase; NF, nuclear factor; OR, occlusion rate; PPAR, peroxisome proliferator-activated receptor; Treg, regulatory T-cell

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2. Materials and methods

2.1. Mice

Male eight to 10-week-old mice were used in this study. C57BL/6 and BALB/c mice were purchased from CLEA Japan (Tokyo, Japan). In order to compare the luminal obstruction of transplanted grafts, three groups of mouse tracheal transplant models were made, including the isograft group (IG) and allograft groups with (AGP) or without pioglitazone administration (AGN). Isogeneic tracheal transplants were performed using C57BL/6 mice as recipients and donors (n = 8). Allogeneic tracheal grafts from BALB/c donor mice were transplanted into C57BL/6 recipient mice (n = 8). All experimental protocols were reviewed and approved by the Committee on the Ethics of Animal Experiments at the Keio University School of Medicine, Japan, and were carried out in accordance with the Guidelines for Animal Experiments, issued by the Keio University School of Medicine Experimental Animal Center.

2.2. Tracheal transplant mouse model

A well-established mouse tracheal transplant model was used to examine the effects of pioglitazone in the pathogenesis of allograft rejection [13,14]. Following the application of anesthesia, donor mice were exsanguinated, and the whole trachea was harvested by transection from the distal of cricoid cartilage to the bifurcation of the carina, under sterile conditions. Subsequently, recipient mice were anesthetized and the whole trachea was exposed to provide a surgical window for anastomosis. Proximal and distal orifices were created on the recipient trachea, in order to connect it to the both ends of the donor tracheal graft, and running sutures were performed with a size 10–0 nylon. This anastomosis permits airway continuity and enables airflow over the epithelial surface of transplanted graft. All surgeries were performed using a microscope at 5–20× magnification (Surgical Scope M680; Leica Microsystems Inc., Bannockburn, IL, USA).

2.3. Pioglitazone treatment

Pioglitazone dosage was chosen based on the results of a previous experiments on KK-Ay mice, a type 2 diabetes model [15]. The dose of 5 mg/kg/day for mice is clinically equivalent to the therapeutic dose of pioglitazone used in humans [15]. Pioglitazone (Takeda Pharmaceutical, Tokyo, Japan) was dissolved in 0.5% methylcellulose and administered by intraperitoneal injection. The experimental group was administered pioglitazone twice a day from the day 2 before surgery until the day 7 after the transplantation.

2.4. Histopathological evaluation of tracheal transplants

Transplanted grafts were harvested at day 7 following the transplantation, embedded en bloc in tissue freezing medium (Tissue-TEK, Sakura, Tokyo, Japan), placed in embedding molds (Sakura Finetek, Tokyo, Japan), snap-frozen in liquid nitrogen, and stored at −80 °C until further histological analysis. The degree of luminal occlusion of the tracheal graft was determined using hematoxylin and eosin staining of 6-μm thick sections taken from the middle part of each transplanted graft. Histologically, this airway-transplant model mimics lymphocytic bronchiolitis, which allows studies focusing on immunemediated inflammation. One section of each harvested graft was used for quantitative and morphometric analysis as we previously described [14,16]. To calculate the occlusion rate (OR) of the transplanted grafts, the morphometric measurements of the cross-sections of transplanted grafts were performed using the image analysis software ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA) [17]. Immunostaining of the serial adjacent sections from each transplanted graft was performed using primary antibodies against a mature T-cell marker (hamster anti-mouse CD3, BD Pharmingen, San Diego, CA, USA) and a regulatory T-cell (Treg) marker (rat anti-mouse FoxP3, BD Pharmingen, San Diego, CA, USA). Mature T-cell and Treg recruitment was assessed by manually counting the numbers of CD3-positive and FoxP3-positive cells in the epithelial and subepithelial area in each 6-μm section using high-power magnification.

2.5. mRNA isolation and real-time PCR analysis

Total RNA was extracted from frozen transplanted grafts using RNeasy Mini Protocol for Tissues kits (Qiagen, Tokyo, Japan). A 1-μg sample of RNA was transcribed into cDNA using a High Capacity cDNA Archive kit (Applied Biosystems, Tokyo, Japan), according to the manufacturer's instructions. Real-time PCR was performed with each cDNA sample using an Applied Biosystems 7500 Real-Time PCR data-collection system, and data analysis was performed using software provided by the same manufacturer. Fluorogenic PCR primer sets and probes for all specific target genes and endogenous reference housekeeping cDNA (β-actin) were used for gene expression analysis with Taqman Gene Expression Assays (Applied Biosystems, Tokyo, Japan).

2.6. The expression of cytokines in the grafts

The levels of cytokines were determined in the grafted tissue. We have determined the levels of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and different interleukins (IL-2, -4, -6, -10).

2.7. Statistical Analysis

ANOVA with a post hoc Bonferroni test was used to compare conditions between groups. All data were analyzed using SPSS Statistics Desktop 24.0 software (IBM, Armonk, NY, USA). The results are presented as mean ± standard deviation (SD). A p-value of < 0.05 was considered significant.

3. Results

3.1. Pioglitazone suppressed graft luminal occlusion in the orthotopic tracheal transplant model

Occlusion rates of Isografts (IG), allografts (AGN), and allografts with pioglitazone (AGP) at day 7 were 45.0 ± 9.1%, 63.7 ± 8.0%, and 53.6 ± 6.8%, respectively (Fig. 1A). AGN group developed concentric graft luminal occlusions (Fig. 1B) and had a significantly higher OR than the IG group (p = .001), while the AGP group had a significantly lower OR compared with the AGN group (p = .045). In the IG group, mature T-cell infiltration did not increase (35 ± 17 cells). There was no significant difference in the number of infiltrating mature T-cells between the AGN and AGP groups (305 ± 56 cells and 317 ± 84 cells, respectively; p = .943) (Fig. 2).

3.2. Pioglitazone enhanced Treg infiltration and intragraft expression of FoxP3 mRNA in the orthotopic trachea transplant model

In the IG group, Treg infiltration was not accelerated (8 ± 8 cells). A significantly higher number of infiltrating Tregs was observed in the AGP group than in the AGN group (88 ± 23 and 24 ± 18, respectively; p < .01) (Fig. 3). Furthermore, FoxP3 mRNA expression level was 1.6 times higher in the AGP group than in the AGN group (p = .037) (Fig. 4).

3.3. Intra-graft cytokine expression

IFN-γ mRNA expression level was 96.2 times higher in the AGN group than in the IG group (p = .005), and it was 0.27 times lower in the AGP group than in the AGN group (p = .032) (Fig. 5A). The

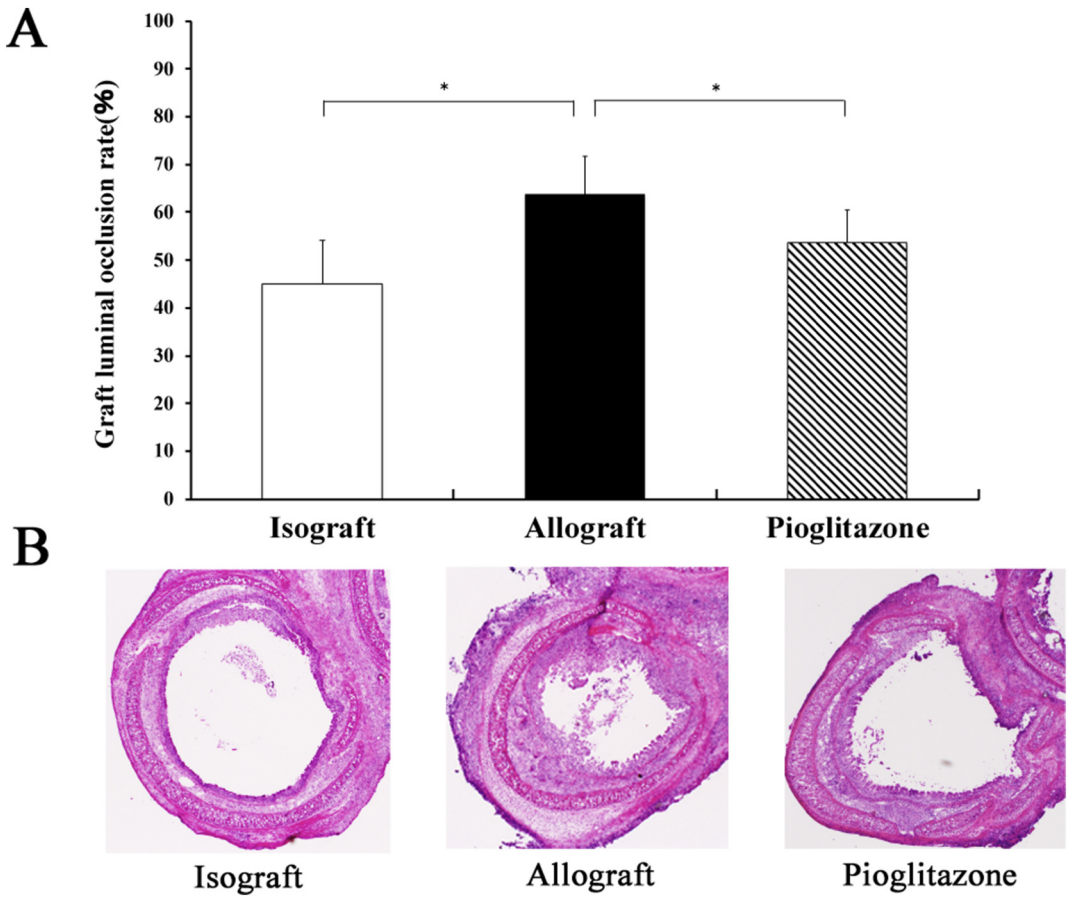

Fig. 1. (A) Effect of pioglitazone on graft luminal narrowing at day 7 following the transplantation, in an orthotopic tracheal transplant model (n = 8, each group) (*p < .05). Allografts treated with pioglitazone showed a significant decrease in graft luminal narrowing over that in the untreated controls (53.3 ± 6.8% as against 63.7 ± 8.0%, p < .05). (B) Representative hematoxylin and eosin staining of airways in each group are shown (original magnification 400×).

expression level of IL-2 mRNA was 9.1 times higher in the AGN group compared with the IG group (p < .001), but there was no significant difference between the AGP and AGN groups (Fig. 5B). The expression level of IL-10 mRNA was 3.7 times higher in the AGN group compared with the IG (p < .01), but there was no significant difference between the AGP and AGN groups (Fig. 5C). The expression level of IL-4 was 2.9 times higher in the AGP group than in the AGN group (p = .048) (Fig. 5D). TNF-α expression level was shown to be 6.4 times higher in the AGN group than in the IG group (p = .003), and it was 0.64 times lower in the AGP group compared with the AGN group (p = .023) (Fig. 5E). IL-6 expression level was 11.5 times higher in the AGN compared with the IG group (p < .01) and it was 0.58 times lower in the AGP than in the AGN group (p = .023) (Fig. 5F).

4. Discussion

Primary graft dysfunction and BO are the main causes of death after lung transplantation. Despite the considerable advances in immunosuppressive therapies, as well as the organ preservation and surgical techniques, the overall five-year survival rate remains at 50% [18]. Although the cause of BO is multifactorial, clinical and experimental studies demonstrated that the acute rejection and immunemediated inflammation, which begins with T-cell infiltration, is one of the major factors that lead to the development of BO [3,10,16]. This study have examined the effect of an oral type 2 diabetes medication, pioglitazone, which is a PPAR-γ ligand, on the immune response that leads to the development of BO in mouse orthotopic trachea transplant model. Previous studies have used orthotopic tracheal transplant model to study BO and the related conditions. Whether proximal, central airways, such as trachea, can be used as the experimental model of bronchiole is still controversial. However, various studies have been performed using the trachea-orthotopic tracheal transplantation and heterotopic tracheal transplantation in subcutaneous, intraomental, or intrapulmonary sites. Advantages and limitations of these models have been extensively discussed previously [19]. Immune response and epithelial damage/regeneration have been shown to be similar in the trachea and bronchiole, despite the size differences [13,19]. Thus, we have used the orthotopic tracheal transplantation in this study, because the pathological changes can be observed early, the results are consistent, and the procedure is relatively simple.

We have shown that the administration of the PPAR-γ agonist, pioglitazone, inhibits the obliteration of the transplanted graft lumen and prevents the elevation of inflammatory cytokines. T cell infiltration into the grafts was quantified by counting the number of CD3 positive cells, as both CD4 and CD8 positive T cells have been demonstrated to play a critical role in the development of airway rejection [20]. The accumulation of T-cells has been regarded as an important factor in the development of BO, since T-cell infiltration typically precedes and accompanies fibrosis in the bronchioles [13,16]. Our results showed that the thickening of tracheal occlusions was prevented by pioglitazone administration, without the inhibition of T-cell infiltration into the transplanted tracheal graft. Furthermore, Treg infiltration was shown to be increased in the grafts treated with pioglitazone. We did not evaluate CD4 and CD8 positive cell infiltrations in the tracheal graft in this study. Further study is needed to specify the histological effect of relationships between of Th1/ Th2 cytokines and CD4/ CD8 positive cells. Huang et al. reported that the activators of PPAR-γ promote Treg

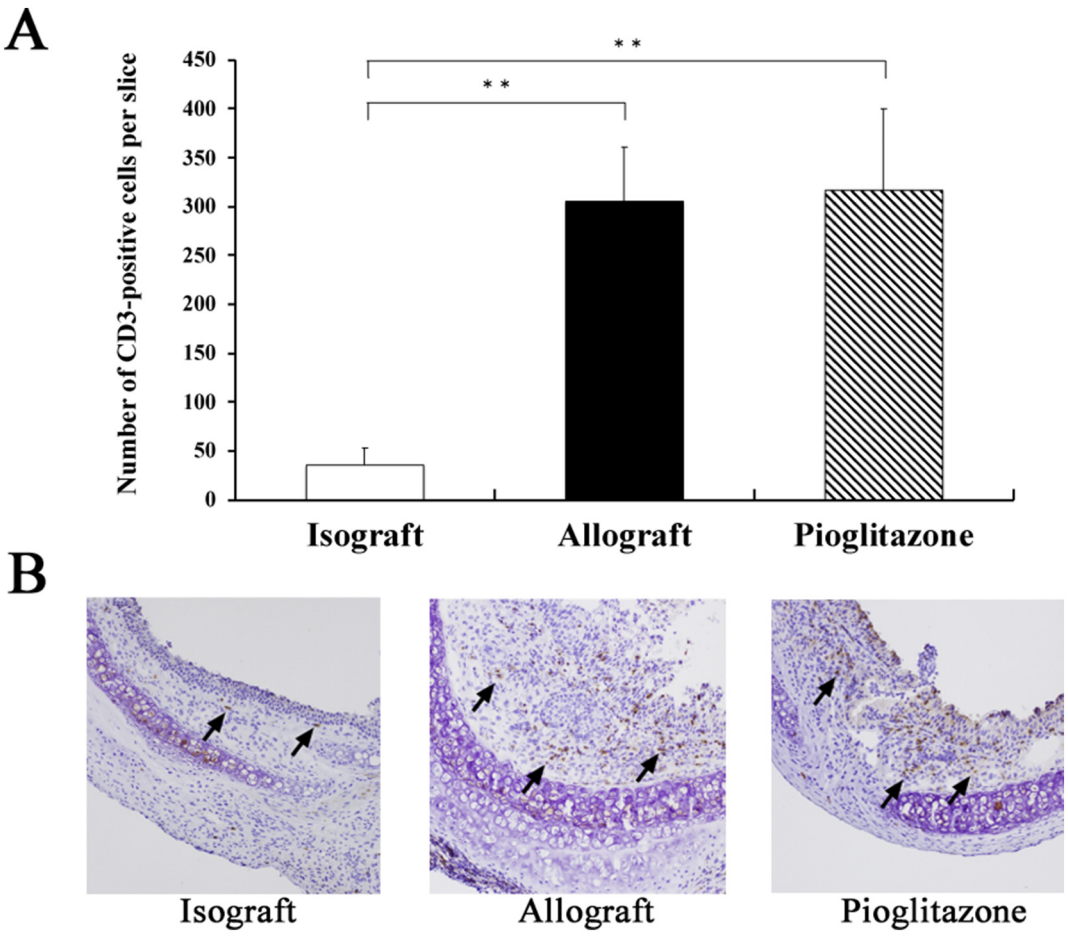

Fig. 2. (A) Quantitative analysis of T-cell infiltration by counting the number of CD3-positive cells per section under high-power magnification. Total CD3+ cell counts were obtained for the entire section taken from orthotopically transplanted tracheal grafts at day 7. Each bar represents mean ± SD (n = 8, each group) (**p < .01). (B) Representative immunohistochemical staining for the mature T-cell marker, CD3, in tracheal graft sections are shown (T-cells indicated by black arrows).

development and reduced chronic rejection in mouse cardiac transplant model, and also showed that PPAR-γ deficient recipients displayed reduced cardiac allograft survival and decreased proportion of Tregs, although the mechanisms of Treg accumulation following the pioglitazone administration are not clear [21]. An increasing amount of clinical evidence shows that Tregs play an important role in the induction and maintenance of transplantation tolerance [22,23]. Among the human lung transplant recipients, stable patients had a significantly higher percentage of Tregs in bronchoalveolar lavage than did the patients who subsequently developed BOS [24]. Additionally, the acceptance rate of allograft was shown to be associated with an increased intra-graft expression levels of Treg-attracting chemokines, such as CCL17 and CCL22 [25,26]. A recent animal study showed that the administration of anti-MHC class I antibodies to a Treg-depleted mouse increases bronchial luminal occlusion and fibrous deposition, in comparison with the mice containing Tregs, in a mouse obliterative airway disease model [27]. In our results, increase of percentages of Tregs in T cells by the administration of pioglitazone have ameliorated LB, without decrease of total number of T cells.

It has been accepted that during the induction of transplant tolerance, decrease of Th1 associated cytokines is observed, which may associate with sustained or increased expression of Th2 cytokines. Th1 cells secrete IFN-γ, IL-2, and TNFs, and they play important roles in allograft rejection and transplant tolerance [28]. PPAR-γ agonists have been reported to decrease Th1 cytokines, such as IFN-γ and TNF-α production in activated T-cells and enhance apoptosis [29]. In our investigations, we observed significant differences in the expression of Th1 cytokine IFN-γ and TNF-α between the untreated and pioglitazone-treated allografts. In a rat model, treatment with pioglitazone was demonstrated to alter the helper T-cell balance from Th1 to Th2 in the myocardium of animals with autoimmune myocarditis, through the upregulation of Th2 cytokine IL-4 mRNA expression, and through the reduction of Th1 cytokine IFN-γ mRNA levels [30]. Our findings are consistent with the results of this study. We found that the levels of IL-4 were significantly upregulated in grafts treated with pioglitazone. The role of Th2 cytokines in the development of BO/LB has not been extensively investigated. Kastelijn et al. showed that serum concentrations of IL-4 were lower in the transplant patients who developed BOS than in the stable patients. They suggested that the decrease in IL-4 in the transplant recipient may reduce the induction of allograft tolerance, leading to BOS [31]. We have shown that IL-6 was overexpressed in the allograft, and pioglitazone administration reduced intragraft IL-6 mRNA expression. IL-6 have been shown to have the counter-regulatory effects on development of Tregs. IL-6 have shown to inhibit Tregs and prevent conversion of Th17 cells into Treg cells [32]. In our experiment, decrease of IL-6 by pioglitazone may have promoted Treg infiltrations in the graft, and then resulted in amelioration of graft occlusion. Pathways of IL-6 reduction with pioglitazone have not been elucidated. One explanation of the mechanism is that PPAR-γ ligands regulate MAPK and NF-κB in dendritic cells, leading to a decrease in IL-6 [33]. In our experimental model, pioglitazone may cause the transplant tolerance in tracheal grafts through suppression of MAPK and NF-κB pathways.

Taken together, the data presented in this study indicate a

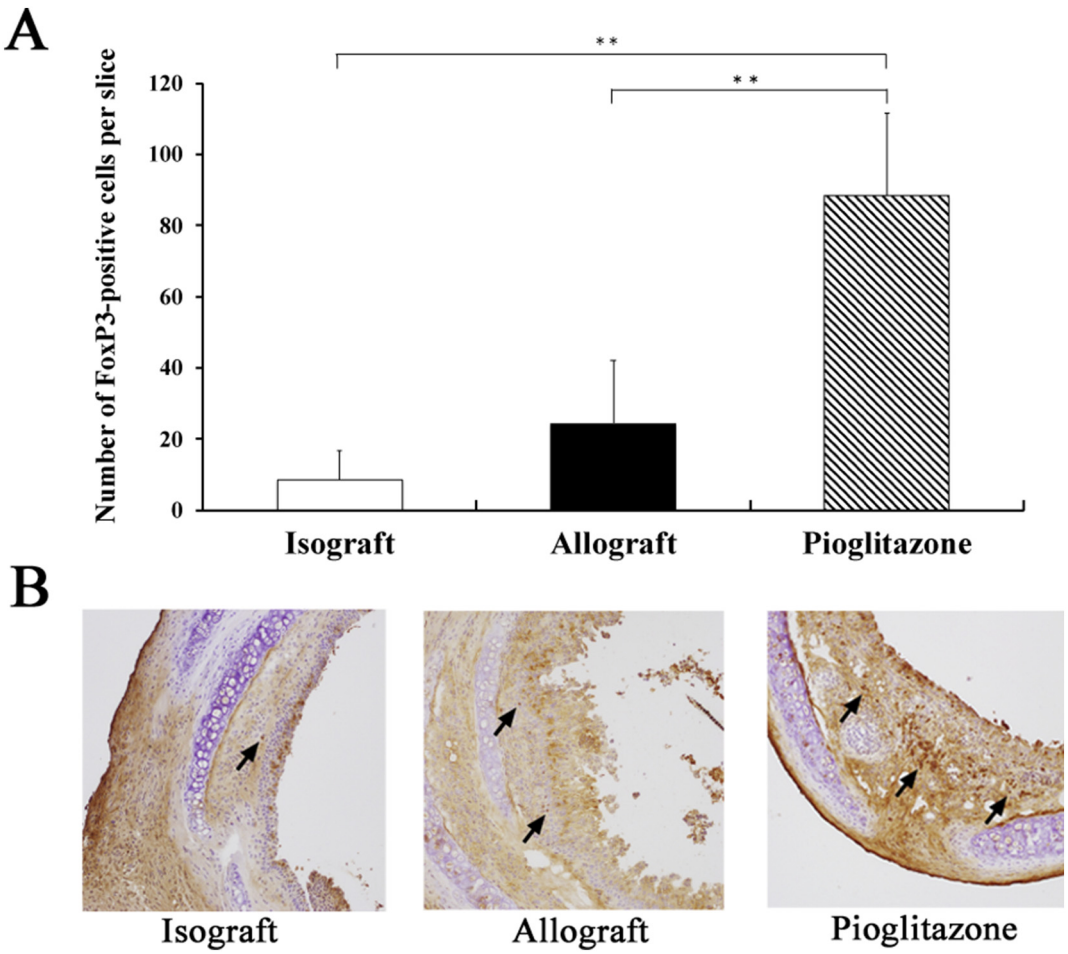

Fig. 3. (A) Quantitative analysis of the regulatory T-cell infiltration in the orthotopic tracheal transplant model at day 7 after the transplantation. Each bar represents mean ± SD (n = 8, each group) (*p < .05, **p < .01). (B) Representative immunohistochemical staining for the regulatory T-cell marker FoxP3 in the sections of tracheal grafts are shown (regulatory T cells indicated by black arrows).

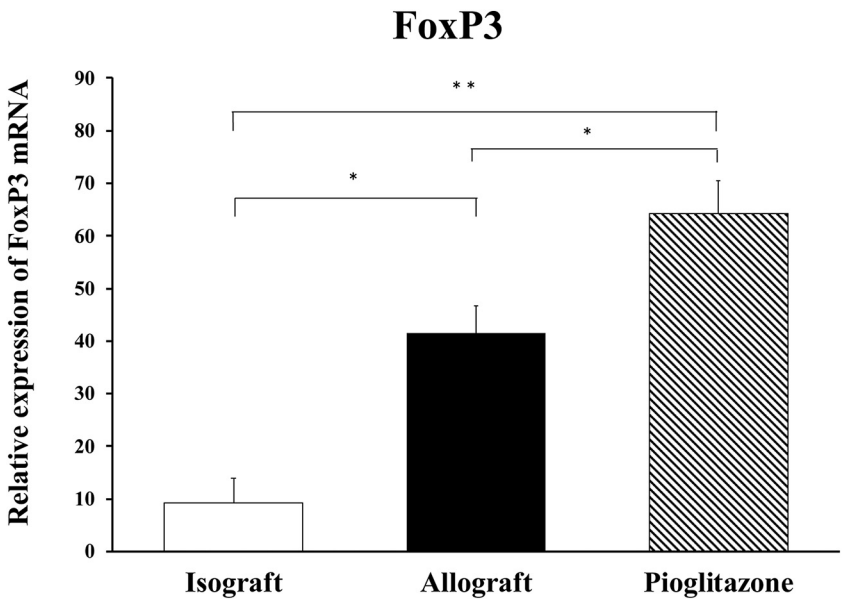

Fig. 4. Quantitative analysis of FoxP3 mRNA. FoxP3 extracted from orthotopically transplanted tracheal isografts, allografts, and allografts treated with pioglitazone. (n = 8, mean ± SD) (*p < .05, **p < .01).

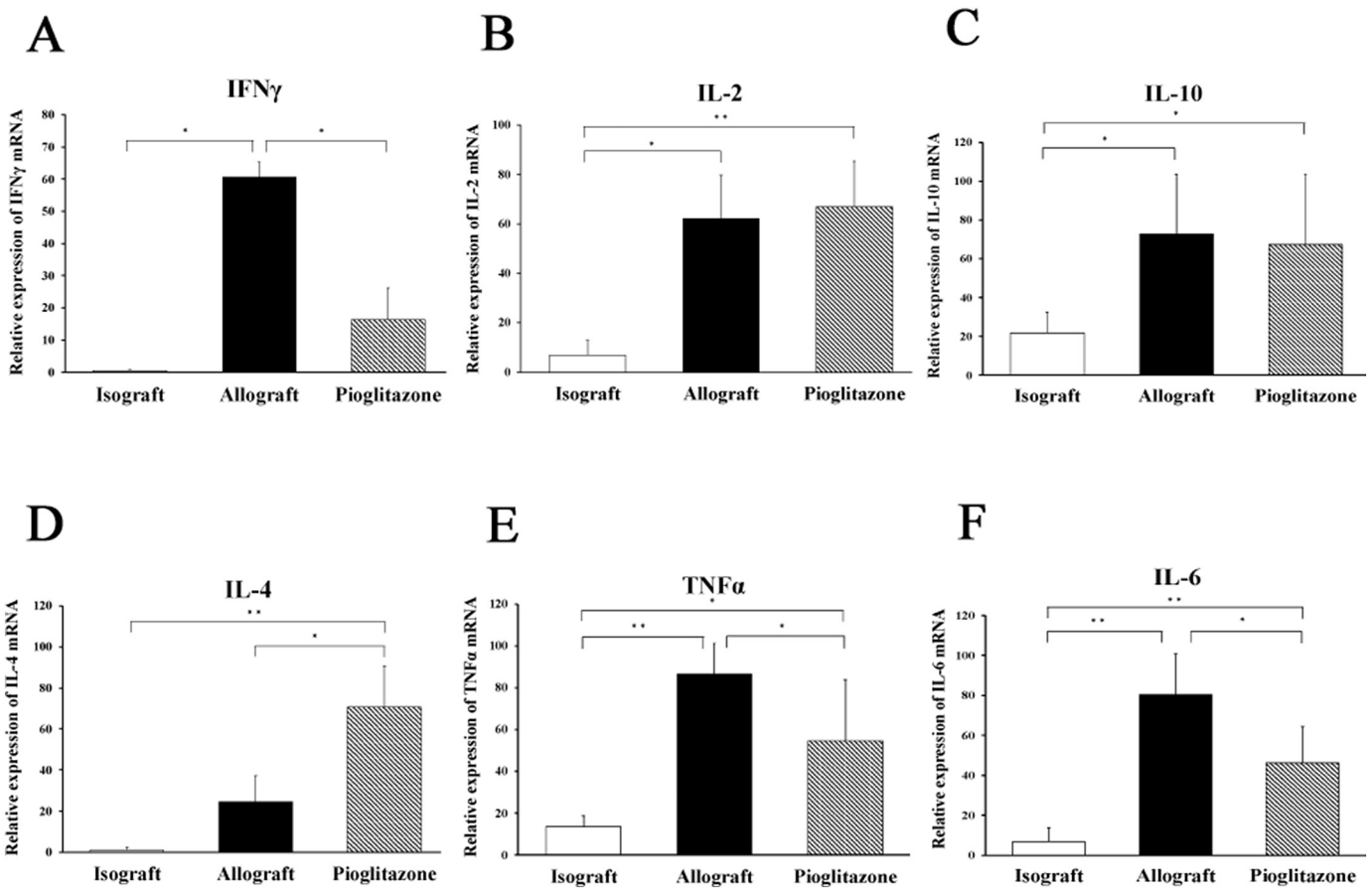

Fig. 5. Quantitative analysis of T-cell and macrophage cytokine mRNA levels. IFN-γ (A), IL-2 (B), IL-10 (C), IL-4 (D), TNFα (E), and IL-6 (F) extracted from orthotopically transplanted tracheal isografts, allografts, and allografts treated with pioglitazone. (n = 8, mean ± SD,) (*p < .05, **p < .01).

protective role of pioglitazone, a PPAR-γ agonist, in a mouse tracheal transplantation model. The administration of pioglitazone leads to a decrease in tracheal graft luminal occlusions and the induction of Treg accumulation in the graft. This indicates a protective role of pioglitazone against BO. The potential mechanism of these effects includes an increase in IL-4 production and a decrease in IFN-γ, TNF-α, and IL-6 production. However, the anti-inflammatory potential, and efficacy in the maintenance of transplant tolerance needs to be validated in the future.

Conflict of interest statement

The authors declare no conflicts of interest.

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