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ORIGINAL ARTICLE

Carbon monoxide–releasing molecule attenuates allograft airway rejection

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None declared.

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Summary

Acute rejection after lung transplantation is the main risk factor for the development of bronchiolitis obliterans (BO). Carbon monoxide (CO) can provide anti-inflammatory effects and may serve to limit tissue injury in airway transplant. Here, we tested the ability of carbon monoxide releasing molecule-2 (CORM-2) to prevent airway rejection. Tracheal grafts from BALB/c or C57BL/6 were transplanted to C57BL/6 recipients. Experimental groups were treated with multiple doses of CORM-2. Histopathological evaluation of luminal obliteration was blindly reviewed. Immunohistochemistry and real-time RT-PCR analyses were performed. Allografts treated with CORM-2 revealed a striking reduction of thickening in epithelial and subepithelial airway layers ($P < 0.01$) at day 7 in orthotopic trachea transplantation model compared with allografts treated with vehicle. In heterotopic trachea transplantation model, CORM-2 treated allografts showed a reduction of luminal obliteration ($P < 0.01$) at days 14 and 21. There was also a concordant decrease in $CD3^+$ lymphocytes and macrophages in CORM-2 treated allografts. IFN-γ, IL-2 and IL17A mRNA expressions were reduced dramatically by systemic administration of CORM-2. These data implicate CORM-2-derived CO has an important protective function in experimental BO, and may represent a target for the therapeutic intervention of chronic lung allograft rejection.

Introduction

Bronchiolitis obliterans (BO) manifests in patients as BO syndrome ascertained by spirometry, affects up to 50–60% of patients who survive 5 years after lung transplantation. BO develops as a progressive obliteration of small airways within the lung allograft. Pathologically, there is early lymphocyte infiltration and other signs of inflammation, epithelial cell injury and fibrosis. Aside from infectious complications, BO represents the main factor limiting long-term survival after lung transplantation. Evidence indicates that carbon monoxide (CO) can provide beneficial antiapoptotic and anti-inflammatory effects in several animal models [1,2]. During tissue injury, endogenous CO is generated from heme degradation through heme oxygenase-1 activity. This pathway has been identified as a natural powerful cytoprotective mechanism. More recently, low-dose exogenous CO has been studied in numerous transplant models, including kidney, liver, intestine, lung and heart, with promising results [3–5].

However, exogenous CO administration has been shown to increase carboxyhaemoglobin (COHb), with the theoretical risk of impaired oxygen delivery to organs and tissues. To avoid this problem, novel CO-releasing

molecules (CORMs) have been created to deliver consistent amounts of CO to tissues without a significant impact on COHb levels [6,7]. CORMs have been shown to counteract numerous inflammatory conditions such as sepsis and coronary ischaemia [6,8,9]. However, CORMs have not yet been studied in lung transplant or BO models. We hypothesized that CORMs would prevent inflammation and rejection in mouse trachea transplant models.

Materials and methods

Mice

Male mice aged between 8 and 12 weeks old were used in these experiments. C57BL/6 (H-2b) mice and BALB/c (H-2d) mice were purchased from CLEA Japan, (Tokyo, Japan). Isogeneic tracheal transplants were performed using C57BL/6J mice as recipients and donors. Allogeneic tracheal grafts from donor mice (BALB/c) were transplanted into C57BL/6 recipient mice. All experimental protocols were reviewed by the Committee on the Ethics of Animal Experiments at the School of Medicine, Keio University, and were carried out in accordance with Guidelines for Animal Experiments, issued by the School of Medicine, Keio University Experimental Animal Center.

Tracheal-transplant model

Two well-established tracheal transplant models were used to study the effect of CORM-2 in the pathogenesis of allograft rejection. The first model was the previously described double lumen airway (orthotopic) transplant model for studying chronic airway rejection [10]. Briefly, after anaesthesia, donor mice were exsanguinated, and the whole trachea was harvested under sterile conditions by transecting below the cricoid cartilage distal to the bifurcation of the carina. Recipient mice were subsequently anaesthetized, and the whole trachea was exposed to provide a surgical window for anastomosis. Distal and proximal orifices were created on the recipient trachea to enable anastomosis with both ends of the donor tracheal graft. Running sutures permit airway continuity, enabling airflow over the graft epithelial surface. Histologically, this airway-transplant model mimics lymphocytic bronchiolitis (LB), which was detected as described below. A heterotopic model, characterized by complete epithelial loss and fibroproliferative plug formation by 2 weeks after transplant as previously described, was also used [10,11]. Donor tracheas were harvested as described above. The trachea was inserted in a subcutaneous pouch created after a 3-mm incision and blunt dissection in the back of the neck. The skin pocket was closed with a size 5.0 nylon suture. All surgeries were performed with the use of a surgical microscope at 5–20× magnification (Surgical Scope M680; Leica Microsystems Inc., Bannockburn, IL, USA).

Histopathological evaluation of tracheal transplants

Grafts were harvested at 7, 14 or 21 days after 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 the time of histological analysis. The degree of graft luminal occlusion was ascertained on 5-μm-thick sections, taken from the middle third of the tracheal graft, by histochemical staining, using an elastin stain (Accustain; Sigma-Aldrich, St. Louis, MO, USA), to demarcate the epithelial and subepithelial layers. Immunostaining was performed using serial adjacent sections from each group, using primary Abs directed against a pan-T cell marker (hamster anti-mouse CD3; BD Pharmingen, San Diego, CA, USA), a macrophage marker (rat anti-mouse F4/80; BD Pharmingen) and a neutrophil marker (rat anti-mouse Ly6G; BD Pharmingen). Morphometric measurements of cross-sectional areas were performed by an investigator blinded to section identity, by tracing epithelial and subepithelial areas using a computer-assisted image-analysis system IMAGEJ [12]. Quantitative analysis of recruited T cell, macrophage and neutrophil numbers was performed by manually counting numbers of $CD3^+$ cells in the epithelial and subepithelial layers in each 5-μm section under high-power magnification.

mRNA isolation and real-time PCR analysis

Total RNA was extracted from frozen mouse tracheas using RNeasy Mini Protocol for Tissues kits (Qiagen, Tokyo, Japan) and reverse transcribed using a High Capacity cDNA Archive kit (Applied Biosystems, Tokyo, Japan), using procedures specified by the manufacturer. One microgram of sample RNA was transcribed to cDNA. Real-time PCR was performed on samples of this cDNA using an Applied Biosystems 7000 Real-Time PCR System data-collection system, with data analysis performed using software provided by the manufacturer. Fluorogenic PCR primer sets and probes for all of the specified target genes and endogenous reference housekeeping cDNA (β actin) were purchased as TaqMan Gene Expression Assays (Applied Biosystems).

CORM-2 treatment

The dose of the CORM-2 was chosen based on their previous use in mouse models of kidney and aortic transplantation [13,14]. Experimental groups were treated with multiple doses of CO-releasing molecule, tricarbonyldichlororuthenium(II) dimer (CORM-2; Sigma-Aldrich) at

10 mg/kg intraperitoneally 1 h before transplantation, and 1, 3 and 6 days after transplantation in orthotopic trachea transplant model and grafts were collected on day 7. In heterotopic trachea transplantation model, CORM-2 were given at 10 mg/kg intraperitoneally 1 h before transplantation, and 1, 3, 6, 9, 12, 15 and 18 days after transplantation. The mice were euthanized and the tracheal grafts were collected on days 7, 14 or 21 for histology. Control groups were treated using the same dose schedule except they received vehicle (0.25% DMSO in saline). No immunosuppression was used.

Statistics

A comparison of values between groups was done using the unpaired *t* test. All data were analysed using IBM SPSS Statistics, version 19, software (IBM, Armonk, NY, USA). The data are presented as mean ± standard deviation. Differences were determined to be statistically significant when $P < 0.05$.

Results

Effects of CORM-2 on graft luminal narrowing in heterotopic and orthotopic trachea-transplant model

To determine the influence of CORM-2 on graft luminal narrowing, morphometric analyses were performed. Allografted heterotopic tracheal transplants (BALB/c into C57Bl/6) demonstrated concentric graft luminal occlusion at day 14 and 21 ($P < 0.01$; Fig. 1a). To study the role of CORM-2, BALB/c tracheas were transplanted into C57Bl/6 mice treated with CORM-2. Allografts treated with CORM-2 showed a reduction of luminal obliteration at day 14 (6.7 ± 10.0% compared to 81.0 ± 15.1%, allografts treated with vehicle, $P < 0.01$, Fig. 1a and b). At day 21, the lumens in the allografts were obviously obliterated, but the allografts treated with CORM-2 were less obliterated. The difference in these two groups was significant (84.0 ± 13.4% compared to 15.8 ± 12.8%, $P < 0.01$, Fig. 1a). In orthotopic trachea transplantation model, allografts treated with CORM-2 demonstrated a significant decrease in graft luminal narrowing (34.3 ± 13.3% compared to 58.4 ± 13.9%, allografts treated with vehicle, $P < 0.01$, Fig. 2). These data suggest an important protective role for recipient CORM-2, because its addition ameliorates the histological equivalent of BO in this mouse model.

Quantification of graft T cell infiltration in orthotopic trachea-transplant model

To study the effect of CORM-2 in T cell infiltration into airway grafts, pan-T cell–labelled $CD3^+$ cells were quantified using immunohistochemically stained frozen sections. Comparisons were made between allografts treated with CORM-2 or vehicle and isografts. There were significant differences in the number of infiltrating T cells between allografts treated with vehicle and allografts treated with CORM-2 (number of cells per slice, 259 ± 83 vs. 85 ± 44;

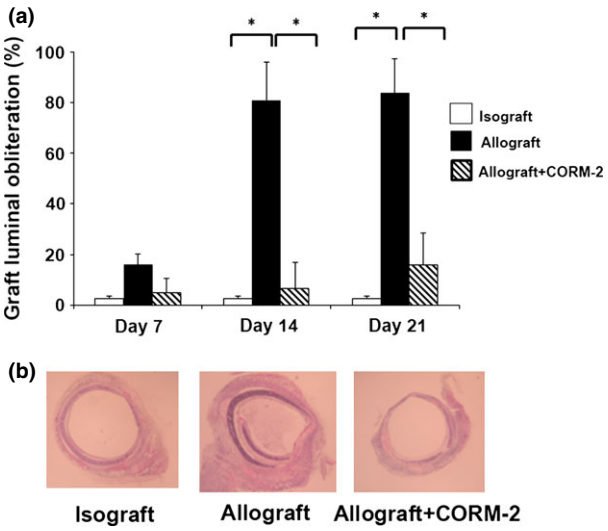

Figure 1 (a) Effect of CORM-2 on graft luminal narrowing from day 7 to day 21 after surgery in heterotopic trachea transplant model ($n = 6$ each). Allografted heterotopic tracheal transplants demonstrated concentric graft luminal occlusion at day 14 and 21 (*$P < 0.01$). Each bar represents mean ± SD. Allografts treated with CORM-2 showed a reduction of luminal obliteration (*$P < 0.01$) at day 14 and 21. No significant differences were seen at day 7. (b) Representative histology sections of airway grafts at day 14 are shown (original magnification ×400).

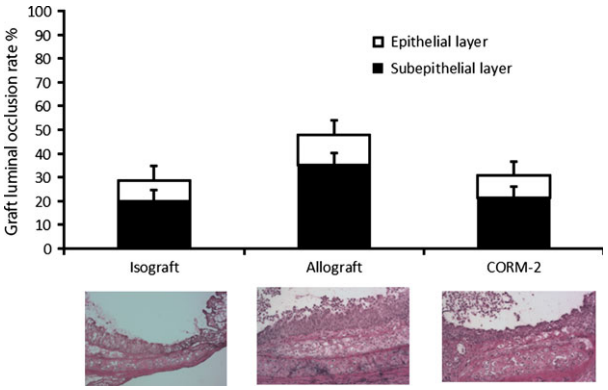

Figure 2 Effect of CORM-2 on graft luminal narrowing 1 week after surgery in orthotopic trachea transplant model ($n = 6$ each). Representative EVG stainings of airway grafts are shown (original magnification ×400). Allografts treated with CORM-2 demonstrated a significant decrease in graft luminal narrowing (34.3 ± 13.3% compared to 58.4 ± 13.9%, allografts treated with vehicle, $P < 0.01$).

$P = 0.0032$; Fig. 3). These quantitative data demonstrate that numbers of CD3-positive cells directly correlate with the exacerbation of airway luminal narrowing.

Quantification of graft macrophage and neutrophil infiltration in orthotopic trachea-transplant model

To study the effect of CORM-2 in macrophage and neutrophil into airway grafts, macrophage or neutrophil cells were quantified using immunohistochemically stained frozen sections. Comparisons were made between allografts treated with CORM-2 or vehicle and isografts. There were significant differences in the number of infiltrating macrophages between allografts treated with CORM-2 and allografts treated with vehicle (number of cells per slice, 103 ± 53 vs. 301 ± 128; $P < 0.01$; Fig. 4). However, there were no significant differences in the number of infiltrating neutrophils between allografts treated with CORM-2 and allografts treated with vehicle (number of cells per slice, 151 ± 144 vs 173 ± 107; $P = 0.79$; Fig. 5).

Intragraft expression of Th cytokines

Studies were performed to investigate the relationship between CORM-2 and expression of Th1 cytokines, which are known to upregulate intragraft inflammatory-immune events. We examined the expression of IFN-γ and IL-2 mRNA in orthotopic tracheal-transplant tissue of isografts and allografts, as well as allografts treated with CORM-2. IFN-γ mRNA was markedly increased (62.0-fold; $P < 0.01$) in allografts compared with isografts, and it decreased in al-

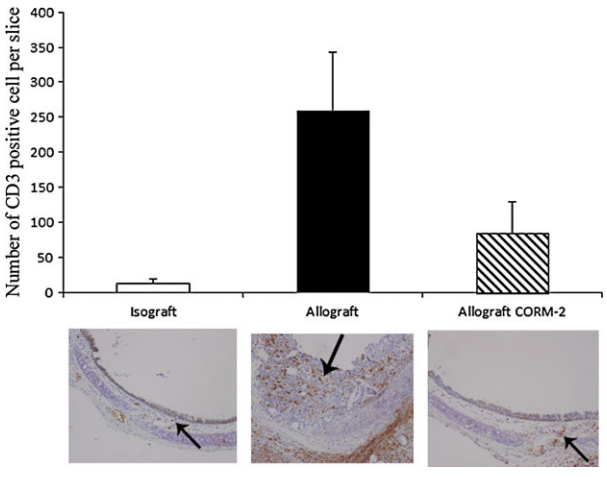

Figure 3 Quantitative analysis of T cell infiltration by counting the number of $CD3^+$ cells per slice under high-power magnification. Total $CD3^+$ cell counts were obtained for an entire section taken from the middle third of the orthotopically transplanted tracheal graft at day 7. Each bar represents mean ± SD (n = 5–6). Representative immunohistochemical stainings for the pan-T cell marker CD3 (brown; T cell demonstrated by arrow) in sections of airway grafts are shown. The bar graph shows the analysis of positive immunostaining of migratory CD4 positive cells in the allograft at day 7.

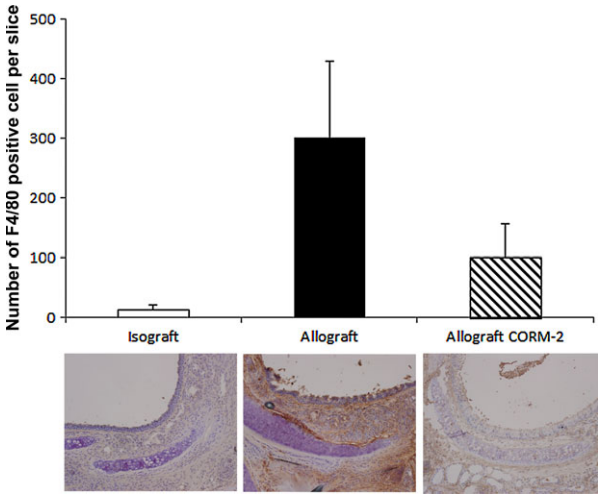

Figure 4 Quantitative analysis of macrophage infiltration in the orthotopic trachea transplant model. Each bar represents mean ± SD (n = 5–6). Representative immunohistochemical stainings for the macrophage in sections of airway grafts are shown. The bar graph shows the analysis of positive immunostaining of migratory macrophages in the allograft at day 7. There were significant differences in the number of infiltrating macrophages between allografts treated with CORM-2 and allografts treated with vehicle ($P < 0.01$).

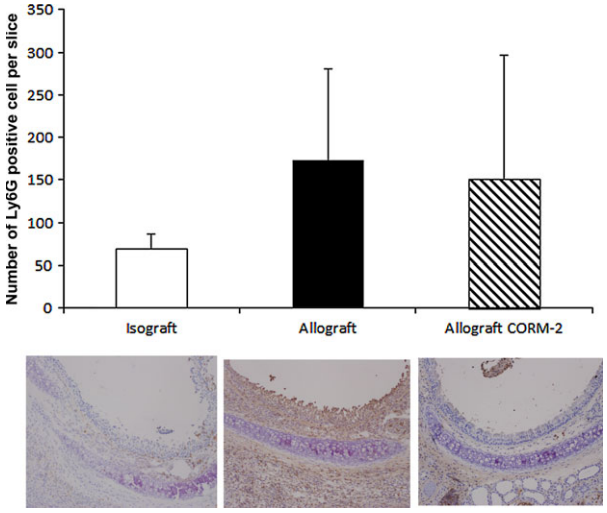

Figure 5 Quantitative analysis of neutrophil infiltration in the orthotopic trachea transplant model. Each bar represents mean ± SD (n = 5–6). Representative immunohistochemical stainings for the neutrophil in sections of airway grafts are shown. The bar graph shows the analysis of positive immunostaining of migratory neutrophils in the allograft at day 7. There were no significant differences in the number of infiltrating neutrophils between allografts treated with CORM-2 and allografts treated with vehicle ($P = 0.79$).

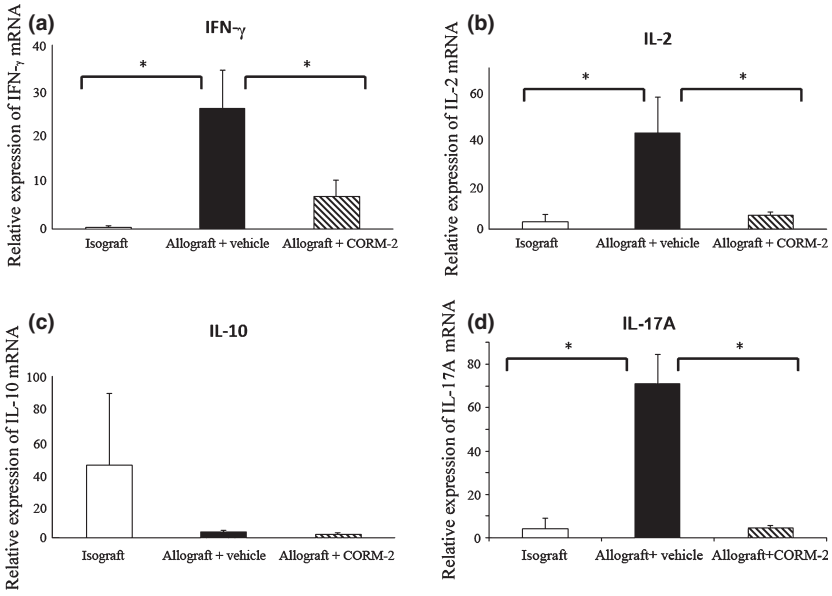

Figure 6 Quantitative analysis of Th1, Th2 and Th17 type cytokine mRNA. IFN-γ (a), IL-2 (b), IL-10 (c) and IL-17A (d) extracted from orthotopically transplanted trachea of isografts, allografts and allografts treated with CORM-2. (n = 6 transplants for each group, with mean ± SD shown) (*$P < 0.01$).

lografts treated with CORM-2 compared with allografts by 73% ($P < 0.01$) (Fig. 6a). Similarly, the expression of IL-2 mRNA was strongly upregulated in allografts compared with isografts (4.8-fold; $P < 0.01$), and it decreased in allografts treated with CORM-2 compared with allografts by 75% ($P < 0.01$) (Fig. 6b). When a prototypical Th2 cytokine (IL-10) was examined, no difference was found between allograft treated with vehicle and CORM-2 (Fig. 6c). We also analysed pro-inflammatory Th17 cytokine IL-17A mRNA. IL-17A mRNA was markedly increased (16.2-fold; $P < 0.01$) in allografts compared with isografts, and it decreased in allografts treated with CORM-2 compared with allografts by 93% ($P < 0.01$) (Fig. 6d).

Discussion

Lung transplantation remains the only effective therapy for a large number of patients with end-stage lung disease. Despite significant advances in immunosuppressive therapies, as well as organ preservation and surgical techniques, the overall 5-year survival rate remains at 50% [15]. Aside from infections, mortality after lung transplantation results mainly from the development of chronic graft dysfunction (BO), which develops in >60% of lung transplant recipients [16]. Although the pathogenesis of BO is multifactorial, clinical studies implicated acute rejection as one of the major causative factors leading to the development of BO [17] with a prominent role for T lymphocytes in its pathogenesis [18].

Recently, CO has been shown to have anti-inflammatory effects, and administration of inhaled CO protected rat or mouse organs from transplant related injury [14]. Unfortunately, the prospect of using inhaled CO in clinical transplantation is limited due to difficulty in CO storage and delivery in a controlled manner. Moreover, CO binds to haemoglobin and forms COHb with an affinity 240 times higher than that of oxygen, interfering with oxygen transport and delivery to the organs with potentially life-threatening consequences [19]. The availability of CORM capable of releasing CO in biological systems in a controlled manner has a better future over inhaled CO and provides the opportunity to investigate CO-mediated biological effects in more detail [20,21], and we evaluated the effects of CORM-2 in mice trachea transplantation model.

Leucocyte infiltration and transmigration is a hallmark inflammatory event in the early and intermediate stages of BO development. This process is regulated at multiple levels by the expression and activation of different molecules. In the present study, CORM-2 treated allografts showed marked decrease of T cell and macrophage infiltration in the tissue compared with allografts treated with vehicle. Several mechanisms are implicated for the effect of CORM-2. CO released by CORM-2 exerts potent antiproliferative effects through the activation of p38 mitogen activated protein kinase (MAPK) signalling and induction of HO-1 protein [22]. Another possible explanation is CO liberated from CORM-2 inhibits the NF-κB and subsequent ICAM-1 expression and provides anti-inflammatory effect [23].

Minamoto *et al.* reported that HO-1 expressed by the graft was more important than that by the recipient in ameliorating airway rejection in a mouse trachea transplantation model and also showed administration of exogenous CO, which acted immediately on epithelial cells, elicited beneficial effects even in the HO-1 deficient graft [24].

Previous findings indicate that the cellular immune response during rejection of allografts of lungs and other organs is associated with local production of the Th1 cytokines [25]. IFN-γ is one of the Th1 cytokines and believed to promote the rejection response by increasing expression of major histocompatibility complex class II antigens, stimulating cytotoxic T lymphocyte development, and inducing alloantibody production. In the present study, allografts treated with CORM-2 had significantly lower levels of IFN-γ, IL-2 and IL-17A production, whereas their levels of IL-10 did not change intensely. Hegazi *et al.* [26] showed CO alters IFN-γ signalling by inhibiting a member of the IFN regulatory factor-8, and resulting in protection of intestinal inflammation in Th-1 mediated murine model. All these present results are consistent with previous reports of a direct effect of CO on T cells, including a reduction of cytokine production [24,27]. Also our results are compatible with the study that has demonstrated CORM-2 can reduce lymphocyte infiltration in the transplanted kidney [13]. Elevation of IL-17A, pro-inflammatory cytokine, in the bronchoalveolar lavage in lung transplant patients who experience primary graft dysfunction or chronic rejection suggesting its involvement on BO [28]. Ning *et al.* [29] reported that CO inhibited the production of IL-17 in human pulmonary epithelial cells. Our study here showed that the administration of CORM-2 can also inhibit the IL-17A mRNA level in trachea transplant model, and added further insight to the anti-inflammatory effect of CORM-2. In conclusion, the data presented in this study suggest a protective role of CORM-2, one of the novel CORMs, in mice trachea transplantation model. CORM-2 leads to decreased airway graft luminal occlusion, T cell and macrophage infiltration. These data indicated a strong protective role for external CO against BO. The potential mechanism of this beneficial effect of CORM-2 appears to suppress leucocyte infiltration, and decrease the production of IL-2, IFN-γ and IL-17A. However, the therapeutic potential of anti-inflammation or anti-oxidative stress strategies in this setting should be further validated by future studies.

Authorship

TO, KK and TS: participated in the performance of the study. TO and KK: participated in the writing of the article. TO, KK and TS: participated in the research design. TO and KK: participated in the data analysis. TO and MS: performed histological analysis. TS, TH, IK, TG and MK: contributed to the revision of the article.

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