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ORIGINAL ARTICLE

Systemic overexpression of matricellular protein CCN1 exacerbates obliterative bronchiolitis in mouse tracheal allografts

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Summary

Obliterative bronchiolitis (OB) involves airway epithelial detachment, fibroproliferation, and inflammation, resulting in chronic rejection and transplant failure. Cysteine-rich 61 (CCN1) is an integrin receptor antagonist with a context-dependent role in inflammatory and fibroproliferative processes. We used a mouse tracheal OB model to investigate the role of CCN1 in the development of lung allograft OB. C57Bl/6 mice received a systemic injection of CCN1-expressing adenoviral vectors 2 days prior to subcutaneous implantation of tracheal allografts from major MHC-mismatched BALB/c mice. We treated another group of tracheal allograft recipients with cyclic arginine–glycine–aspartic acid peptide to dissect the role of αvβ3-integrin signaling in mediating CCN1 effects in tracheal allografts. Allografts were removed 4 weeks after transplantation and analyzed for luminal occlusion, inflammation, and vasculogenesis. CCN1 overexpression induced luminal occlusion ($P < 0.05$), fibroproliferation, and smooth muscle cell proliferation ($P < 0.05$). Selective activation of αvβ3-integrin receptor failed to mimic the actions of CCN1, and blocking failed to inhibit the effects of CCN1 in tracheal allografts. In conclusion, CCN1 exacerbates tracheal OB by enhancing fibroproliferation via an $\alpha_v\beta_3$-integrin-independent pathway. Further experiments are required to uncover its potentially harmful role in the development of OB after lung transplantation.

Introduction

Lung transplantation is the only therapeutic option for many end-stage pulmonary diseases. Despite clinical advances, chronic allograft rejection remains the main factor limiting long-term (>1 year) graft survival and manifests as chronic lung allograft dysfunction (CLAD) [1]. Obliterative bronchiolitis (OB) is part of the pathophysiology of CLAD and involves both pro-inflammatory and pro-proliferative mechanisms that act alone or in concert to cause progressive lung transplant airway obstruction and, ultimately, allograft failure [1–3].

Cysteine-rich 61 (CCN1 or CYR61) is part of the CCN protein family comprising 6 members named CCN1-6 in order of their discovery [4,5]. It is a matricellular protein associated with the extracellular matrix (ECM) upon cellular secretion, and it exerts each of its distinct cellular effects according to the cell type and context through integrins and heparan sulfate proteoglycans [6,7]. CCN1 has a wide repertoire of functions ranging from cardiovascular development to wound healing and inflammatory regulation [8–10]. It acts on a plethora of cells, including fibroblasts and smooth muscle cells (SMC), to induce either cell senescence or proliferation in a context-dependent manner

[11–13]. Ischemia, pressure overload, and a variety of neurohormonal and angiogenic factors induce CCN1 expression [14].

A wide range of synthetic CCN1-mimicking peptides has been produced for specific integrin targeting in cancer studies. One of these products is cyclic arginine–glycine–aspartic Acid peptide (cRGD) – a ligand specific for $\alpha_v\beta_3$ integrin receptor. cRGD acts as a double-edged sword; at low doses, it acts as a partial CCN1 agonist; at high doses, however, as an antagonist [15,16].

Chronic lung allograft dysfunction involves both fibroproliferative and inflammatory processes. So far, no studies have been conducted to discover the role of CCN1 in this setting. Here, we investigated the effects of CCN1 overexpression on the development of experimental pulmonary OB.

Materials and methods

Permission for the animal experiments in this study was obtained from the State Provincial Office of Southern Finland. The animals received humane care in compliance with the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Academy Press (ISBN 0-309-05377, revised 2011)

Experimental tracheal OB model

We used an experimental model of mouse tracheal allograft OB to study the effects of CCN1 overexpression and cRGD peptide treatment on the development of chronic pulmonary rejection.

We performed heterotopic tracheal transplantations from white BALB/c mice to fully MHC-mismatched black C57Bl/6 mice. The tracheal allografts were removed from the recipient and perfused *ex vivo* with a cold phosphate-buffered saline (PBS) containing 10 000 IU/ml penicillin/1000 μg/ml streptomycin solution. Allograft cold ischemia time was standardized to 15 min. All recipients received a daily suboptimal dose of 0.75 mg/kg of tacrolimus (FK506, kindly provided by Astellas) subcutaneously to allow the development of 50–75% tracheal occlusion within 30 days, as described in our previous study [17]. Recipients were sacrificed and tracheal allografts harvested for *ex vivo* analyses 4 weeks after transplantation. All tracheal allografts in both control and treatment groups were cut horizontally in half at the end of the follow-up. One half was suspended in Tissue-tek and snap-frozen for histological analysis and the other in RNALater for RNA extraction and PCR analysis. All cross sections used for histological and immunohistological analyses were cut in the same fashion from the middle part of the tracheal transplants.

Systemic adenovirus-mediated overexpression of CCN1

The production and purification of the CCN1-expressing and control RR5-expressing adenovectors (a kind gift from Prof. W. Poller, the Department of Cardiology and Pneumology, Universitätsmedizin Berlin, Germany) has been described previously [16]. To study the effect of continuous systemic overexpression of CCN1 on the development of OB, we injected recipient C57Bl/6 mice with 25 μl of adenovirus encoding the CCN1 gene (AdV-CCN1, titer = 1.2 x 10^{11} vg/μl) diluted in 75 μl of sterile PBS through the penile vein ($n = 6$). Recipients were injected 2 days prior to transplantation to secure sufficient hepatic transduction and high serum CCN1 levels at the time of transplant implantation. Recipients injected with 15 μl of AdV-RR5 (titer = 2×10^{10} vg/μl) diluted in 85 μl of PBS served as controls ($n = 6$). All recipients received a daily suboptimal dose of 0.75 mg/kg of tacrolimus (FK506) subcutaneously.

Systemic low-dose cRGD experiment

To study the agonistic effect of cRGD peptide on the development of chronic tracheal allograft OB, we implanted a drug-containing pump underneath the subcutis of allograft recipient C57Bl/6 mice ($n = 7$). A dose of 200 μl of cRGD drug was injected into the pump in sterile conditions and implanted in the subcutis of the recipient during general anesthesia, securing an average dose speed of 3.75 μg/h (3.6 mg/kg/day). Mice with subcutaneous pumps containing sterile PBS solution served as controls ($n = 6$).

Systemic high-dose cRGD experiment

To study the antagonistic effect of cRGD peptide on the development of chronic tracheal allograft OB, we treated allograft recipients ($n = 10$) with a daily high dose of cRGD s.c. (50 mg/kg/day). The drug was administered in two separate daily doses between 12-h intervals (25 mg/kg twice daily) to secure continuous high systemic drug concentration. Mice injected twice daily with PBS s.c. served as controls ($n = 10$). All recipients received a daily suboptimal dose of 0.75 mg/kg of tacrolimus (FK506) subcutaneously.

Immunohistochemistry

Cryostat sections were stained using the peroxidase ABC method (Vectastain Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA, USA), and the reaction was revealed with 3-amino-9-ethylcarbazole (AEC, Vector Laboratories). Counterstaining was performed using Mayer's hemalum. Antibodies and concentrations used were CCN1 (0.5 mg/

ml, MAB4864, R&D Systems, Minneapolis, MN, USA), CD4 (5 μg/ml, 22021D), CD8 (5 μg/ml, 22071D), CD11b macrophages (0.5 mg/ml, 557895 PharMingen, San Diego, CA, USA), CD31 (0.1 mg/ml, ab7388, Abcam, Cambridge, UK), Ki-67 proliferating cells (8.0–12.0 mg/ml, NCL-Ki67p), and α-SMA SMCs (29 mg/ml, A2547, Sigma, St. Louis, MO, USA). The numbers of capillaries, arteries, inflammatory cells, SMC, and proliferating cells were determined by counting all immunohistologically positive blood vessels and cells from the whole trachea cross section with 40× magnification and dividing them by the total surface area of either the whole trachea, the periphery, or the lumen, and are given as a total number of cells per mm^2.

Histology

Tracheal occlusion was determined from cryostat cross sections stained with hematoxylin–eosin using computer-assisted measuring tools (Zeiss Axiovision 4.4, Carl Zeiss International, Oberkochen, Germany). The percentage of luminal occlusion was determined by calculating the ratio of occluded luminal area to the total luminal area. We also used a more widely used semiquantitative method to determine the luminal occlusion of the tracheal sections: 0 for 0%, 1 for <25%, 2 for 26–50%, 3 for 51–75%, and 4 for >75% [18].

RNA isolation and quantitative real-time RT-PCR

Total RNA was isolated from tracheal allograft tissue samples using the RNeasy kit (Qiagen, Hilden, Germany) and reverse transcribed with High-RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed on a RotorGene-6000 (Corbett Research, Doncaster, Australia) using 2× DyNAmo Flash SYBR Green Master min (Finnzymes, Espoo, Finland). Measurement of the PCR product was performed at the end of each extension period.

Statistics

All statistical analyses were performed using SPSS version 22.0 (SPSS, Inc., Chicago, IL, USA). Normality of the variables was determined using the Shapiro–Wilk test and by inspecting histograms. The Mann–Whitney test was used for two-group comparison of nonparametric variables, and Student's *t*-test for parametric variables. *P*-values less than 0.05 were considered statistically significant.

Results

Intravenous injection of AdV-CCN1 results in efficient recipient hepatic transduction

We injected recipient C57Bl/6 mice systemically into the penile vein with 3.0×10^{10} vector genome particles of AdV-CCN1 2 days prior to transplantation and analyzed the hepatic tissue for expression of CCN1 mRNA on the 4th postoperative week.

We observed a significantly higher amount of CCN1 mRNA in the livers of AdV-CCN1 injected mice ($P < 0.001$; Fig. 1).

Systemic AdV-mediated CCN1 overexpression exacerbates tracheal allograft occlusion but fails to affect the influx of inflammatory cells

To study the effects of chronic systemic CCN1 overexpression on the development of tracheal OB, we injected AdV-CCN1 into the penile vein of allograft recipients 2 days prior to transplant implantation. After 4 weeks, we removed the tracheal transplant and used histological and computer-assisted measurements to evaluate the degree of luminal occlusion and the inflammatory cell influx.

After 4 weeks, the tracheal allografts of recipients with chronic CCN1 overexpression had on average an absolute luminal occlusion of 100%, vs. 70% in the control group ($P < 0.05$; Fig. 2a). The semiquantitative luminal occlusion

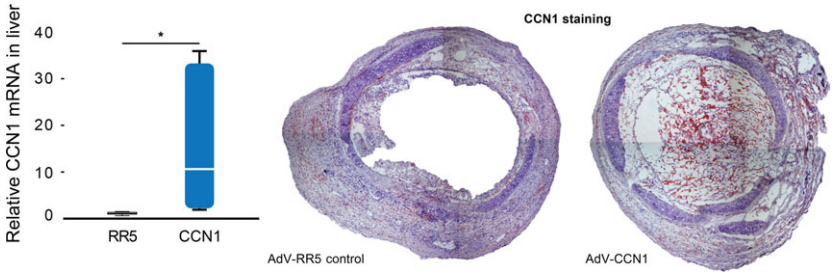

Figure 1 Recipient intravenous AdV-CCN1 administration results in efficient hepatic transduction. Recipients of tracheal allografts were injected with AdV-CCN1 2 days prior to allograft implantation. Real-time RT-PCR was used to analyze recipient hepatic samples for CCN1 mRNA 4 weeks after the transplantation. Representative pictures from tracheal cross sections from each group are also presented. We chose to present tracheal specimens with high degrees of occlusion to fully demonstrate the amount of CCN1 protein in the lumen area. *$P < 0.001$. Data were analyzed with the Mann–Whitney test and are presented as box plots.

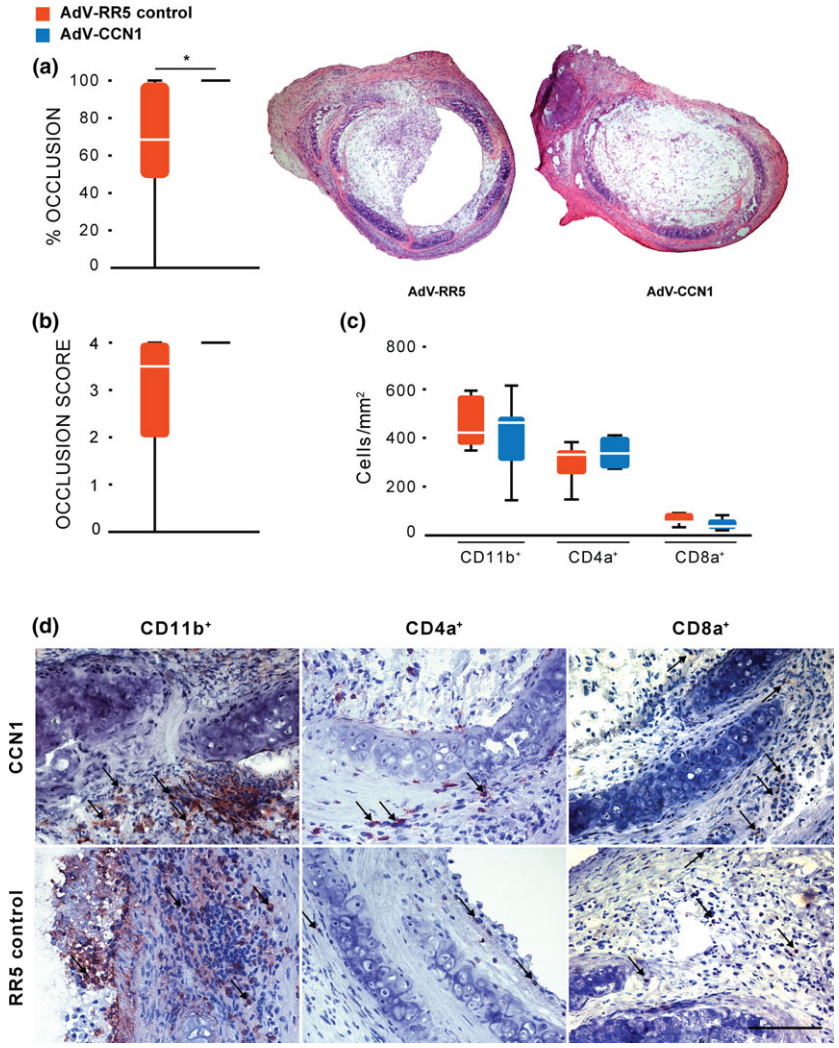

Figure 2 Recipient systemic CCN1 overexpression exacerbates tracheal allograft OB. (a) The degree of tracheal allograft luminal occlusion was determined from tracheal cross sections using computer-assisted imaging techniques to measure the ratio of the occlusion area to the total luminal area. Data are presented as the percentage of occluded luminal area with representative pictures. (b) Semiquantitatively determined tracheal allograft luminal occlusion. (c) The number of intragraft CD11b$^+$ macrophages and CD4a$^+$ and CD8a$^+$ T cells were quantified from immunohistologically stained trachea cross sections. Data are presented as the number of positive cells per mm^2. (d) Representative images of tracheal cross sections stained for CD11b$^+$ macrophages and CD4a$^+$ and CD8a$^+$ T cells. Black arrows indicate positive cells and the black bar represents a distance of 10 μm. *$P < 0.05$. Data were analyzed with the Mann–Whitney statistical test and are presented as box plots.

score was 4 in the CCN1 group and 3.5 in the control group (Fig. 2b). We observed no differences in the intragraft amounts of CD11b$^+$ macrophages, or CD4a$^+$ or CD8a$^+$ T cells between the AdV-CCN1 group and the control group (Fig. 2c,d).

Systemic AdV-mediated CCN1 overexpression induces luminal SMC proliferation in tracheal allografts

We investigated the angiogenic properties of CCN1 and its effects on local cell proliferation by staining tracheal allograft cross sections from CCN1-overexpressing C57Bl/6 recipient mice for α-SMA$^+$ cells, CD31$^+$ capillaries, and Ki-67$^+$ proliferative cells 4 weeks after transplantation.

Although systemic CCN1 overexpression failed to induce local arteriogenesis (Fig. 3a), it significantly increased the number of α-SMA$^+$ cells in the occluded parts of the tracheal allograft lumen ($P < 0.05$; Fig. 3c). Also, CCN1-treated tracheal allografts had a strong trend toward increased mRNA expression of smooth muscle myosin heavy chain (SMMHC) (P=n.s; Fig. 6a). The number of allograft peripheral α-SMA$^+$ blood vessels (Fig. 3b), CD31$^+$ capillaries, and Ki-67$^+$ proliferative cells (Fig. 3d,e) was similar between the groups. We found no differences in the mRNA

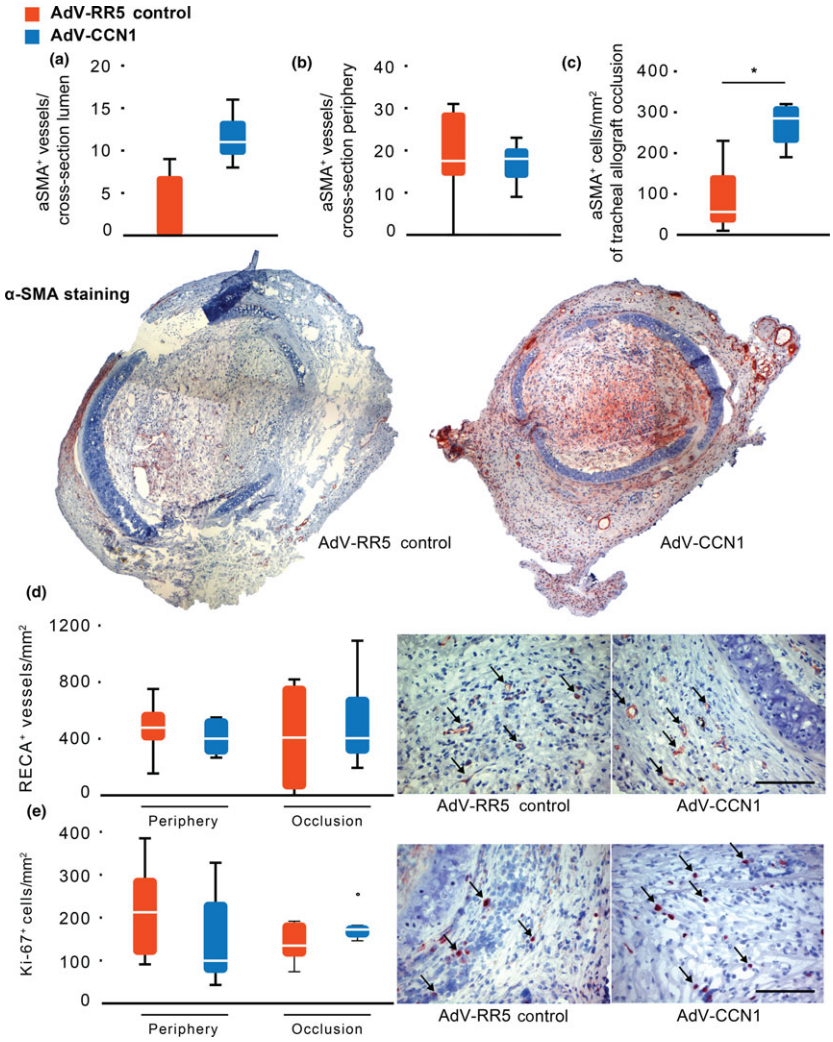

Figure 3 Systemic CCN1 overexpression induces SMC proliferation in tracheal allografts without affecting the number of intragraft proliferating cells. The number of α-SMA$^+$ arteries was quantified separately from the tracheal allograft (a) lumen and (b) periphery at 4 weeks after the transplantation. (c) The total number of α-SMA$^+$ cells in the occlusion of the tracheal allografts was counted at 4 weeks after the transplantation. The data are presented as the number of positive cells per mm^2 of the occluded area and representative images of α-SMA$^+$-stained tracheal cross sections provided. We chose to present maximally occluded tracheal allografts from both groups to illustrate the difference in the cellular composition of the occluded area between the groups. (d) The number of RECA$^+$ capillaries was counted separately from the tracheal allograft periphery and occluded area. The data are presented as the mean number of RECA$^+$ capillaries per mm^2 of the periphery and the occluded area and representative images provided. (e) The number of Ki-67$^+$ cells was determined separately from the tracheal allograft periphery and occluded area. The data are presented as the number of positive cells per mm^2 and representative images provided. The black arrows indicate positive cells, and the black bar represents a distance of 10 μm. *$P < 0.05$. All data were analyzed with the Mann–Whitney statistical test and Student's *t*-test and are presented as box plots.

expression of inflammatory cytokines and chemokines in the tracheal allografts (Fig. 6a).

Recipient treatment with low doses of cRGD peptide fails to mimic the actions of CCN1 in tracheal allografts

Low-dose cRGD acts as an $\alpha_v\beta_3$-integrin receptor agonist. To investigate the role of $\alpha_v\beta_3$ receptor signaling in CCN1-mediated EMT induction, we treated recipients of tracheal allografts with a continuous low dose of cRGD peptide and analyzed tracheal allograft inflammation and the development of OB at 4 weeks after transplantation.

Low-dose treatment with cRGD peptide had no effect on the development of tracheal occlusion (Fig. 4a,b). However, we observed a significant decrease in the number of intragraft CD11b$^+$ macrophages and CD8a$^+$, but not CD4a$^+$ T cells in cRGD-treated tracheas ($P < 0.05$, Fig. 4c, d). The low-dose treatment with cRGD failed to affect the number of α-SMA$^+$ blood vessels in the tracheal allografts (data not presented).

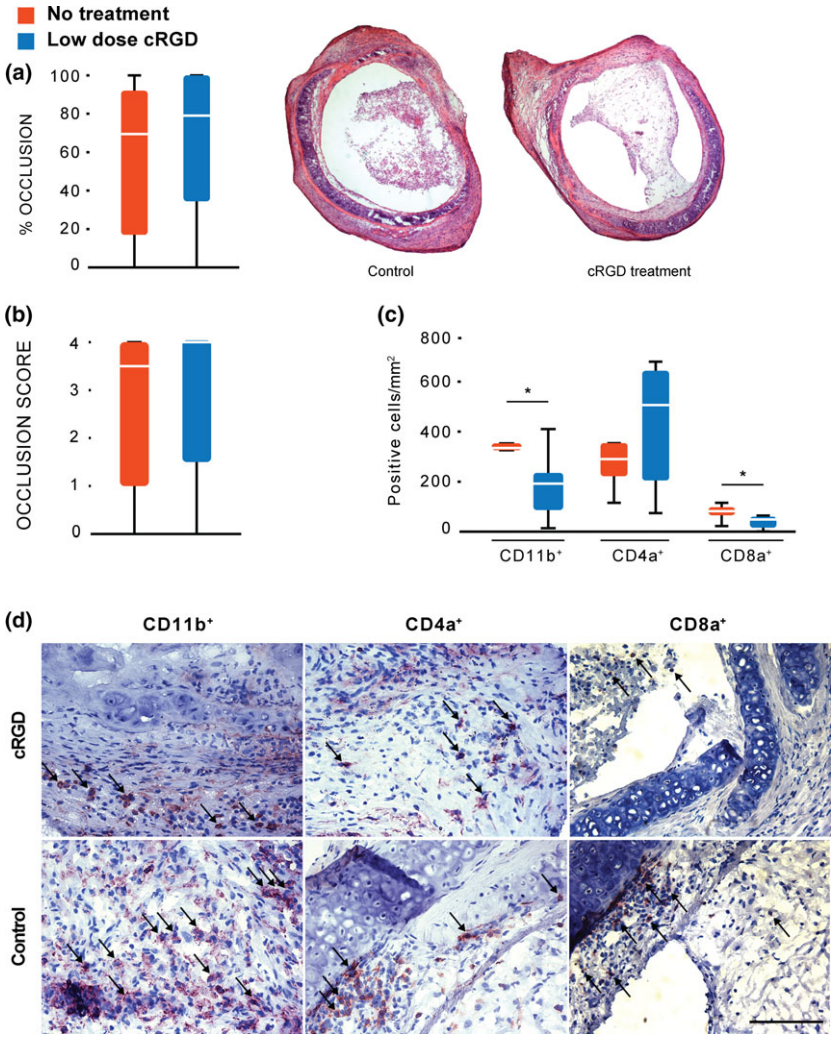

Figure 4 Low-dose systemic cRGD peptide treatment reduces intragraft inflammation but fails to affect tracheal allograft OB. (a) The degree of tracheal allograft luminal occlusion was determined from tracheal cross sections using computer-assisted imaging techniques to measure the ratio of the occlusion area to the total luminal area at 4 weeks after transplantation. Data are presented as the percentage of occluded luminal area with representative pictures. (b) Semiquantitatively determined tracheal allograft luminal occlusion. (c) The number of intragraft $CD11b^+$ macrophages and $CD4a^+$ and $CD8a^+$ T cells were quantified from immunohistologically stained trachea cross sections at 4 weeks after transplantation. Data are presented as the number of positive cells per mm^2. (d) Representative images of tracheal cross sections stained for $CD11b^+$ macrophages and $CD4a^+$ and $CD8a^+$ T cells. Black arrows indicate positive cells, and the black bar represents a distance of 10 μm. $*P < 0.05$. Data were analyzed with the Mann–Whitney statistical test and Student's *t*-test and are presented as box plots.

Recipient treatment with high doses of cRGD peptide treatment fails to attenuate the development of chronic OB

To investigate the possible effect of high-dose cRGD peptide on tracheal allograft inflammation and the development of OB, we treated tracheal allograft recipients with a high dose of cRGD peptide s.c. twice daily. We removed the allograft after 4 weeks and analyzed it for inflammatory cell influx and the development of tracheal occlusion.

High-dose cRGD peptide did not affect the development of tracheal occlusion and the development of OB (Fig. 5a, b). There was no significant change in the amount of intragraft $CD11b^+$ macrophages, $CD4a^+$ and $CD8a^+$ T cells (Fig. 5c,d), or inflammatory cytokines and chemokines (Fig. 6b). However, the mRNA levels of IL-10 were significantly reduced in tracheal allografts in the high-dose cRGD group ($P < 0.05$; Fig. 6b).

Discussion

Here, for the first time, we investigated the role of matricellular protein CCN1 in the alloimmune setting of tracheal transplant OB. First, we showed that systemic CCN1

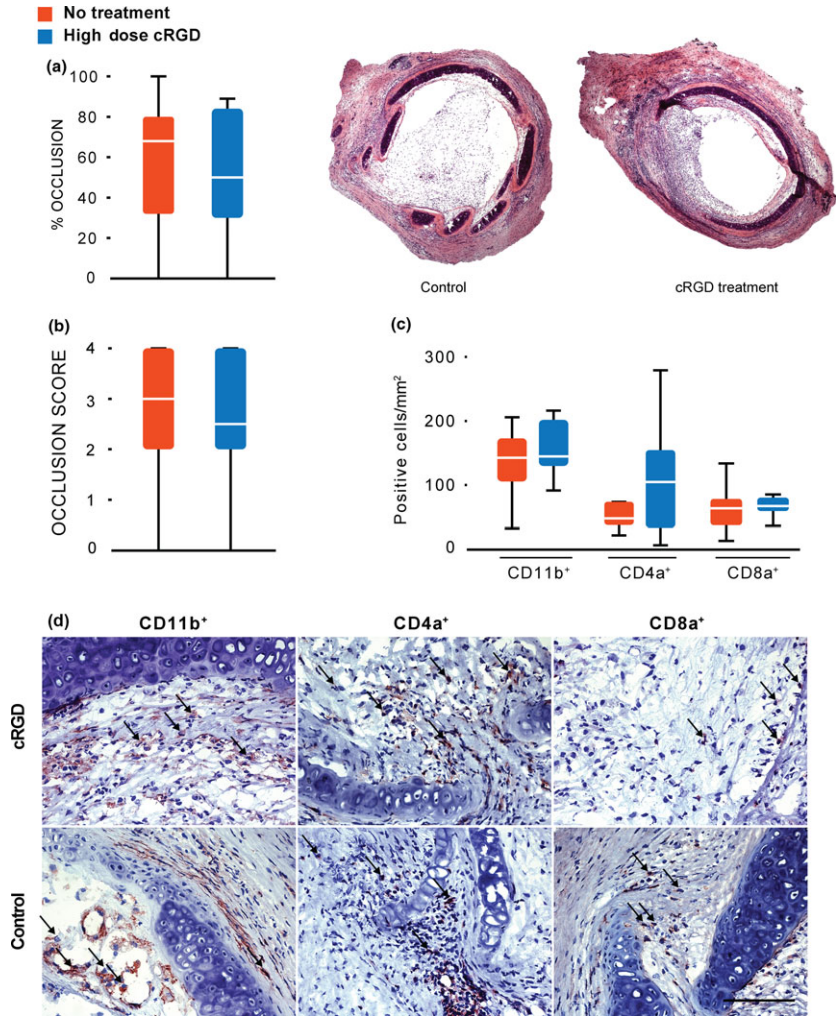

Figure 5 High-dose systemic cRGD peptide treatment fails to affect tracheal allograft OB. (a) The degree of tracheal allograft luminal occlusion was determined from tracheal cross sections using computer-assisted imaging techniques to measure the ratio of the occlusion area to the total luminal area at 4 weeks after transplantation. Data are presented as the percentage of occluded luminal area with representative pictures. (b) Semiquantitatively determined tracheal allograft luminal occlusion. (c) The number of intragraft $CD11b^+$ macrophages and $CD4a^+$ and $CD8a^+$ T cells were quantified from immunohistologically stained trachea cross sections at 4 weeks after transplantation. Data are presented as the number of positive cells per mm^2. (d) Representative images of tracheal cross sections stained for $CD11b^+$ macrophages and $CD4a^+$ and $CD8a^+$ T cells. Black arrows indicate positive cells, and the black bar represents a distance of 10 μm. Data were analyzed with the Mann–Whitney statistical test and Student's *t*-test and are presented as box plots.

overexpression exacerbated OB. Next, we used a cRGD peptide to dissect the role of $\alpha_v\beta_3$ integrin receptor signaling in the actions of CCN1 and, subsequently, the pathogenesis of tracheal OB.

Systemic CCN1 overexpression increases the development of luminal occlusion and EMT in tracheal allografts

Overexpression of CCN1 had no effects on inflammatory cell influx, or expression levels of cytokines and chemokines, suggesting that its main pathological effect was through exacerbation of noninflammatory mechanisms related to excessive fibroproliferation. Tracheal OB is characterized by early airway epithelial damage and an ensuing excessive fibroproliferation leading to obstruction of the airway lumen by granulation- and scar tissue [3]. CCN1 stimulates SMCs to migrate both *in vitro* and *in vivo* and induces vascular SMC (VSMC) proliferation and neointimal thickening after balloon injury in rat carotid arteries [13,19]. Accordingly, it may expedite the aberrant tissue repair that follows the airway epithelial damage in tracheal allografts, thus augmenting the

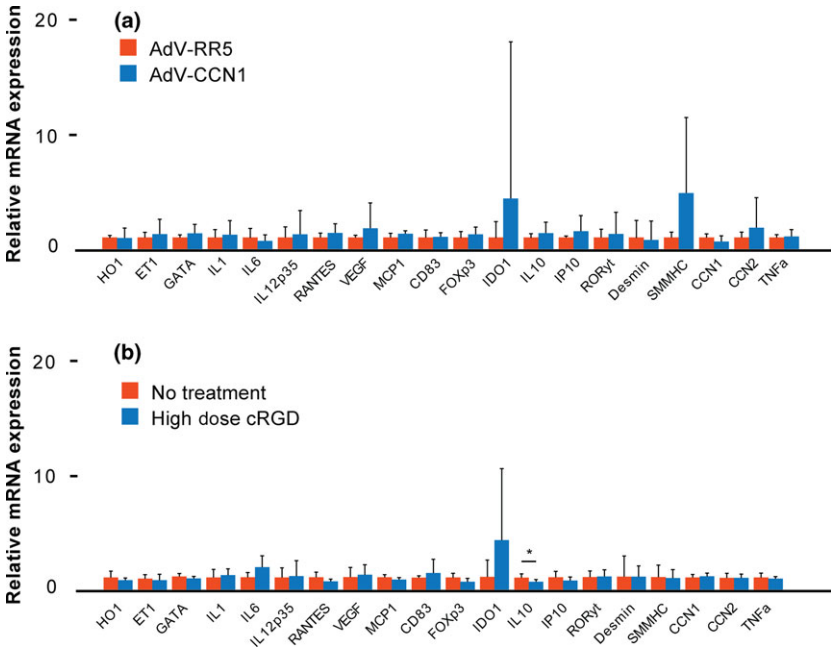

Figure 6 Cytokine, chemokine, and growth factor mRNA expression in (a) CCN1- and (b) high-dose cRGD-treated mice tracheal allografts at 30 days after transplantation. *$P < 0.05$. Data were analyzed with the Mann–Whitney statistical test.

fibroproliferation and the obstruction of the tracheal lumen – as witnessed in our model. Furthermore, epithelial-to-mesenchymal transition (EMT) has a pivotal role in the pathogenesis of pulmonary OB, and CCN1 has been shown to play an important part in EMT occurring in both gastric stomach ulcers and pancreatic carcinogenesis [20–22]. According to our results, CCN1 overexpression increased the number of intragraft α-SMA$^+$ cells and SMMHC mRNA expression – indicating increased fibroproliferation, and potentially also EMT.

Cysteine-rich 61 has a crucial role in maintaining the balance between cell senescence and proliferation in the process of fibrosis. Under physiologic conditions, accumulating CCN1 produced by myofibroblasts induces local cell senescence in granulation tissue once sufficient tissue concentrations have been reached [11]. In our model, however, CCN1 was continuously expressed from the liver into the blood circulation and not locally into the tracheal allograft, whereby its cell senescence-inducing effects were absent. In addition, circulating CCN1 acts on circulating CD34$^+$ progenitor cells to promote tissue regeneration and vasculogenesis in sites of tissue damage [23]. Although CCN1 overexpression increased luminal occlusion in our model, the amount of intragraft proliferating cells was not increased. This suggests that the proliferating cells may have originated from a separate source. In our model, CCN1 was overexpressed into the blood circulation from the liver of the recipients transduced with AdV-CCN1 and may have influenced circulating progenitor cells – for instance CD34$^+$ cells – to localize to the allograft to initiate wound healing.

Selective $\alpha_v\beta_3$-integrin receptor activation fails to mimic, and blocking fails to reduce CCN1-mediated effects in tracheal allografts

Systemic CCN1 overexpression attenuates inflammatory cell influx in experimental autoimmune myocarditis by rendering inflammatory cells unresponsive to chemotactic stimuli – an action mimicked by $\alpha_v\beta_3$ integrin receptor-binding cRGD peptides [16]. On the other hand, CCN1 instigates a pro-inflammatory program in mouse macrophages via the $\alpha_m\beta_2$-integrin receptor and induces macrophage influx into hepatic tissue of mice with nonalcoholic fatty liver disease [24,25]. In our allogenic tracheal OB model, however, CCN1 did not affect the numbers of intragraft inflammatory cells. Recipient treatment with either low-dose or high-dose cRGD peptide failed to mimic or block, respectively, the increased luminal occlusion and accelerated fibroproliferation that CCN1 overexpression induced in tracheal allografts. CCN1 exerts its effect on fibroblasts and SMCs via many receptors, including $\alpha_v\beta_5$ and syndecan-4 receptors [26]. In particular, $\alpha_6\beta_1$ has previously been shown to mediate VSMC migration and proliferation by CCN1 [13,19]. Thus, selective activation or blocking of the $\alpha_v\beta_3$-integrin receptor with cRGD peptides may not have been sufficient to replicate or diminish, respectively, the fibroproliferative effects of CCN1.

Study limitations

Although the mouse tracheal allograft model is commonly used for investigating lung transplant chronic rejection, it has certain limitations. Tracheal tissue histology differs from the bronchioles, and – contrary to human lung transplants – the tracheal allograft is completely devoid of blood flow for the first day after implantation. Also, the tracheal allograft implantation site is mostly pathogen free and isolated from the external environment and microbes. However, although this model is not directly comparable to clinical lung transplantation, it serves an important purpose in dissecting pathway-specific mechanisms of OB in lung transplantation and, as such, has allowed us to obtain new insight on the potential role of CCN1 and its integrin receptors in this pathological process.

Conclusions

Cysteine-rich 61 is an extracellular matrix protein with an ambiguous role within a variety of inflammatory and fibroproliferative contexts. Here, we show that actions of CCN1 in wound healing and EMT induction outweigh its immunomodulatory ones to exacerbate experimental OB. However, our results only offer a fraction of all the possible effects that CCN1 may have in an alloimmune setting and warrants further research to uncover its true potential as both an immunomodulator and remodeling agent. The next logical step would be to investigate the difference between local and systemic overexpression of CCN1 and the potential of other selective integrin receptor blocking agents on preventing the excessive fibroproliferation and EMT occurring in rejecting tracheal allografts.

Authorship

AR: codesigned the study, performed the animal experiments, collected and analyzed the data, and wrote the manuscript. AIN: codesigned the study, analyzed the data, and advised on the writing of the manuscript. RT: collected data and participated in performing the animal experiments. SOS: participated in performing the animal experiments. RK: designed and tested the primers used in PCR and advised on the writing of the manuscript. RA and ER: assisted with data collection and laboratory procedures and methods. XW: designed the viral vectors and collected data. WP: codesigned the study and contributed new tools, vectors, and reagents to perform the study. KBL: codesigned the study, contributed the means to perform the study, supervised the research, and advised on the writing of the manuscript. All of the authors concur with submitting the manuscript for consideration for publication.

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