Increased myeloid cell hypoxia-inducible factor-1 delays obliterative airway disease in the mouse

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BACKGROUND: Obliterative bronchiolitis after lung transplantation is characterized by chronic airway inflammation leading to the obliteration of small airways. Hypoxia-inducible factor-1 (HIF-1) is a master regulator of cellular responses to hypoxia and inflammation. The Von Hippel-Lindau protein (pVHL) drives the degradation of oxygen-sensitive subunit HIF-1α that controls the activity of HIF-1. We investigated the effect of myeloid cell–targeted gene deletion of HIF-1α or its negative regulator pVHL on the development of obliterative airway disease (OAD) in the recipients of tracheal allografts, a mouse model for obliterative bronchiolitis after lung transplantation.

METHODS: Tracheal allografts were heterotopically transplanted from BALB/c donor mice to fully major histocompatibility complex–mismatched recipient mice with HIF-1α or VHL gene deletion in myeloid cells. The recipients were left non-immunosuppressed or received tacrolimus daily. Histologic, immunohistochemical, and real-time reverse transcription polymerase chain reaction analyses were performed at 3, 10, and 30 days.

RESULTS: In the absence of immunosuppression, myeloid cell–specific VHL deficiency of the recipient mice improved epithelial recovery, decreased inflammatory cell infiltration and expression of pro-inflammatory cytokines, increased regulatory forkhead box P3 messenger RNA expression, and reduced OAD development in tracheal allografts. In the presence of tacrolimus immunosuppression, loss of HIF-1α activity in myeloid cells of the recipient by HIF-1α gene deletion accelerated OAD development in mouse tracheal allografts.

CONCLUSIONS: Activity of the HIF-pathway affects the development of allograft rejection, and our results suggest that myeloid cell–specific VHL-deficiency that potentially increases HIF-activity decreases allograft inflammation and the subsequent development of OAD in mouse tracheal allografts.

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A certain degree of both warm and cold lung ischemia is unavoidable in current clinical practice. The cellular responses to hypoxia are regulated by transcription factors called hypoxia inducible factors (HIFs). HIF-1 is a principle regulator of hypoxic adaptation, regulating gene expression involved in glycolysis, erythropoiesis, angiogenesis, proliferation, and stem cell function under hypoxia. HIF-1 is a heterodimer transcription factor constituting of 1 HIF-α sub-unit (HIF-1α, -2α, -3α) and the HIF-1β sub-unit. HIF-1 is the best described of the α sub-units. The HIF-1α and HIF-1β sub-units are both produced at a constant rate. HIF-1β is stable, but HIF-1α is extremely fragile in normal oxygen concentrations and is rapidly degraded via a ubiquitin-mediated pathway. Von Hippel Lindau protein (pVHL) protein is crucial in this process, and its absence leads to stabilization of HIF-1α and activation of HIF-1 even in normoxic conditions.

Cramer et al showed that HIF-1α plays an important role in the regulation of myeloid cell aggregation, invasion, and motility. Furthermore, the overexpression of HIF-1α in macrophages leads to enhanced phagocytosis. On the one hand, HIFs also support the innate immune functions of dendritic cells, mast cells, and epithelial cells. On the other hand, HIF-1 acts as an adaptive and survival factor for ischemic tissue. Therefore, the final effect of HIF-1 in any one setting is likely dependent on the microenvironment.

Ischemia-reperfusion injury initiates an innate immune response leading to neutrophil and monocyte infiltration into the lung allograft. These monocytes differentiate into antigen-presenting dendritic cells and recruit and activate T cells, leading to acute alloimmune activation. Because HIFs have a major role in inflammatory responses during ischemia, we hypothesized that HIF-1 in the allograft infiltrating neutrophils and macrophages may play a major role in innate immune responses and the ensuing alloimmune activation. To test this, we used the heterotopic mouse tracheal allograft model and fully major histocompatibility complex (MHC)-mismatched recipients with myeloid-targeted gene deletion of HIF-1α or VHL that leads to constant HIF-1 inactivation or activation, respectively.

**Methods**

Permission for animal experimentation in this study was obtained from the Provincial State Office of Southern Finland. Mice received 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).

**Heterotopic mouse tracheal allograft transplantation model**

Allogeneic tracheal transplantations were performed from specific pathogen-free fully MHC-mismatched Balb/C (H-2d; Scanbur, Sollentuna, Sweden) to C57BL/6 (H-2b) recipients with HIF-1α or VHL gene deletion in myeloid cell lineage. HIF-1α and VHL gene deletion confined to neutrophils and macrophages was originally achieved by mating the Sv129/C57BL/6/CB.20 mice containing loxP sequences on either side of the target gene with Sv129/ C57BL/6/CB.20 mice carrying the LysMCre recombinase. The resulting mice were backcrossed to wild-type C57BL/6J mice for 12 generations by Professor Randall Johnson (University of Cambridge, Cambridge, United Kingdom), who provided the mice. The result is a mouse that is deficient in HIF-1α or VHL alleles in all myeloid cells (later defined as mHIF-1α or mVHL). This was controlled by genotyping the animals used in experiments. Littermates (LMs) served as negative controls. All mice were male, weighed 25-30 grams, and were 2 to 3 months old.

Tracheal allografts were transplanted heterotopically into an abdominal subcutaneous pouch from Balb/c donors to gene-deleted or LM recipients. Isoflurane (Baxter, Deerfield, IL) and buprenorphine (Schering-Plough, Kenilworth, NJ) were used for anesthesia and perioperative analgesia. The allografts were removed 3, 10, and 30 days after the transplantation. The number of animals used was 6 per each group.

**Drug regimens**

No immunosuppressive or any other treatment was administered in the first phase. In the absence of immunosuppression, the tracheal allografts develop near total luminal occlusion by 30 days. The degree of airway occlusion may be regulated by tacrolimus immunosuppression in a dose-dependent fashion. In the second phase, the recipients received tacrolimus 0.75 mg/kg daily subcutaneously that reduces OAD development, and tracheal allografts exhibit approximately 30% luminal occlusion at 30 days. The dose was based on our previous dose-response study with this animal model. Tacrolimus was provided by Astellas.

**Histologic evaluation**

The transplanted trachea was excised, embedded in Tissue-Tek (Miles Inc, Elkhart, IN), snap frozen in liquid nitrogen, and stored at −70 °C until used. For histologic evaluation, frozen sections were stained with Mayer’s hematoxylin and eosin. The histologic changes in the respiratory epithelium were evaluated as percentage circumference not covered by epithelium. Luminal occlusion was evaluated as reduction of luminal area using ImageJ 1.59 software (National Institutes of Health, Bethesda, MD). All analyses were done in blinded review by 2 independent observers (J.R. and M.K.).

**Immunohistochemistry**

Cryostat sections were stained using the peroxidase avidin-biotin-peroxidase complex method (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA), and the reaction was revealed by 3-amino-9-ethylcarbazole (Vector Laboratories). The following antibodies and dilutions were used: myeloperoxidase (MPO; 20 μg/ml; catalog number ab9535, Abcam), CD11b (clone M1/70, 5 μg/ml), CD4 (clone RM4-5, 5 μg/ml), CD8 (clone 53-6.7, 5 μg/ml), and CD11c (clone N418, 10 μg/ml). The number of inflammatory cells was recorded by counting positive-staining cells per cross-section using a grid and original magnification ×40 and moving the grid across the tracheal cross-section in 2 perpendicular lines. All analyses were performed in blinded review by 2 independent observers (J.R. and M.K.). The negative controls showed no immunoreactivity.
RNA isolation and quantitative real-time polymerase chain reaction

Total RNA was isolated from a tissue sample dissected from the trachea using RNeasy kit (Qiagen, Hilden, Germany) and reverse transcribed with the High-RNA-to-cDNA kit (Applied Biosystems, Foster City, CA). Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed on a RotorGene-6000 (Corbett Research, Doncaster, VIC, Australia) using 2X DyNAmo Flash SYBR Green Master mix (Finnzymes, Espoo, Finland). Measurement of the PCR product was performed at the end of each extension period. All data were normalized against 18sRNA, which was most stably expressed in the respective experiments.

Statistical analyses

All data are expressed as mean ± standard error of the mean. The Mann-Whitney U test was used for 2-group comparisons, and the analysis of variance test with Dunnett correction was used for parametric multiple group comparison. Analyses were done with SPSS 15.0 software (IBM Corp, Armonk, NY). Statistical significance was set at \( p < 0.05 \). The mHIF-1α−/− and mVHL−/− groups were not compared with each other.

Results

The development of OAD was delayed in mVHL−/− recipients of tracheal allografts. In the first phase of our study, we investigated whether the development of OAD in tracheal allografts is altered in mHIF-1α−/− or mVHL−/− recipient mice in the absence of immunosuppression. At Day 3, during the ischemic phase, all tracheal allografts showed equally prominent respiratory epithelial cell injury (Figure 1A). The epithelium in mVHL−/− recipients was fully recovered at 10 days and remained partly preserved at 30 days (Figure 1A, C), whereas total loss of epithelium was
seen in mHIF-1α−/− recipients and in LMs at 30 days (p = 0.016, Figure 1A). No tracheal occlusion was observed in any of the groups at 3 or 10 days (Figure 1B). In mVHL−/− recipients, the airway occlusion was significantly decreased at 30 days compared with tracheal allografts in LMs (p = 0.001; Figure 1B, D–F).

The development of OAD in tracheal allografts was increased in mHIF-1α−/− recipients in the presence of T-cell immunosuppression. In the second phase of our study, we investigated how changes in HIF-1 activity affect the development of OAD in tracheal allografts in the presence of low-dose tacrolimus. In all allografts, almost total loss of epithelium was seen at 30 days despite immunosuppression (Figure 2A). However, OAD in tracheal allografts was significantly increased in mHIF-1α−/− recipients at 30 days (p = 0.02, Figure 2B–E). There was no difference in the degree of preserved epithelium or occlusion between mVHL−/− and LMs (Figure 2A–D).

The infiltration of inflammatory cells in tracheal allografts was reduced in mVHL−/− recipients. The number of CD11b+ macrophages and CD4+ T cells was significantly reduced in tracheal allografts transplanted to mVHL−/− recipients at 10 days, (p = 0.024 and p = 0.014; Figure 3B, C, and F) compared with LMs. No difference was observed in the number of allograft-infiltrating inflammatory cells between the groups at 3 days (Figure 3A–E). In the recipient mice treated with tacrolimus immunosuppression, no difference was observed in the number of allograft-infiltrating inflammatory cells between the groups at 3 or 10 days (data not shown).

On the basis of the histologic and immunohistochemical results, we decided to concentrate on mVHL−/− recipients to define the beneficial effect on the mechanisms of the delayed OAD development.

The messenger (m)RNA expression of proinflammatory cytokines was reduced and T-regulatory cell (Treg) transcription factor was increased in tracheal allografts in mVHL−/− recipients. On the basis of our previous studies with the tracheal allograft model,22 we chose to investigate the mRNA expression of allografts at 10 days, at the time of peak of alloimmune activation. The mRNA expression of Treg transcription factor forkhead box P3 (FoxP3) was significantly increased (p = 0.002), meanwhile the mRNA expression of Th17-related cytokine interleukin (IL)-6, monocyte chemotactic protein (MCP)-1, and heme oxygenase-1 (HO-1) was significantly reduced in mVHL−/− recipients. There was no significant difference between the groups in the mRNA expression of inflammatory cytokine IL1β and Th1-related transcription factor T-Bet, cytokines IL-2, IL-12p35, Th2-related IL-4, Th17-related transcription factor retinoic acid receptor-related orphan receptor c, Treg-related cytokine IL-10, endothelin-1, and growth factors transforming growth factor-β or connective tissue growth factor (Figure 4A–C).

The mRNA expression of Treg transcription factor was increased in the spleens of mVHL−/− recipients of tracheal allografts. We also investigated the mRNA expression in the...
recipients’ secondary lymphoid organ spleen at 10 days. The mRNA expression of MCP-1, IL-1β, and FoxP3 was significantly increased in the spleens of the mVHL<sup>−/−</sup> mice ($p = 0.035$, $p = 0.022$, and $p = 0.035$; Figure 4D). There was no significant difference in the mRNA expression of HO-1 or IL-6 (Figure 4D).

**Discussion**

In this study, we show that myeloid cell-specific deletion of HIF-1 regulating VHL of the recipient mice led to a significant reduction in the OAD development in heterotopic tracheal allografts. In contrast, myeloid cell–specific HIF-1α gene deletion accelerated OAD development even in the presence of T-cell inhibition with tacrolimus. These results suggest a beneficial role for myeloid cell–specific VHL-deficiency in this model.

In this heterotopic non-vascularized model, tracheal neovascularization is preceded by an ischemic phase for the first 2 to 3 days after transplantation, resulting in ischemic injury to the respiratory epithelium. In syngrafts, ischemia induces a self-limiting, alloantigen-independent innate immune response, the epithelium recovers completely, and no OAD develops. In the allografts without immunosuppression, the early ischemic injury is followed by a predominantly pro-inflammatory milieu. Alloantigen-dependent Th1 and Th17 responses peak at 7 to 10 days, followed by progressive epithelial damage and a nearly total airway occlusion 30 days after transplantation.
Inflammatory cells play a major role in the development of immune responses, and because of this we chose to use mice with HIF-1α and VHL gene deletion in myeloid cells only in this study. The development of OAD was seen all groups, which was not a surprise considering the robust nature of the model where the trachea is transplanted over a major MHC mismatch. Without any immunosuppression we were able to investigate the beneficial effects of VHL deficiency. HIF-1α expression was previously reported to have an inhibitory role in T-cell functioning. In our study, myeloid cell–specific VHL deficiency in the recipients reduced accumulation of CD11b+ macrophages and CD4+ T cells in mouse tracheal allografts during the peak of the alloimmune response at 10 days. In addition, the mRNA expression of pro-inflammatory cytokines MCP-1, IL-6, and HO-1 was also significantly reduced in mVHL−/− recipients. However, even low-dose tacrolimus preventing T-cell signal transduction and IL-2 production did not delay the development of OAD in the mHIF-1α−/− recipients.

There are a few plausible mechanisms of action by which myeloid cell–specific VHL deficiency reduced OAD in our study. VHL deficiency likely leads to increased HIF-1 activity and thus was associated with a lesser degree of epithelial loss at 10 days. Because the rate of epithelial loss was similar in all groups at 3 days, it is unlikely that early ischemia-related epithelial injury was affected by myeloid cell HIF-1 expression. In this model, even the epithelium in syngrafts suffers extensive early damage and then recovers in the absence of alloimmune activation. Earlier, the increased vascular endothelial growth factor-A expression was shown to improve epithelial recovery in this model. Furthermore, allograft overexpression of HIF-1α promotes repair of airway microvasculature through the induced expression of pro-angiogenic factors in mice. However, we could not demonstrate pro-angiogenic effects in mVHL−/− mice in our study (data not shown). It is more likely that the markedly reduced acute inflammatory response allowed epithelial regeneration in tracheal allografts. As a result, the complete loss of the tracheal epithelium was delayed in mVHL−/− mice. The importance of epithelial injury in OAD development is well established, and reduced injury may explain the delay in OAD development in our study. However, epithelial regeneration does not explain the reason for initially reduced inflammatory response.

The cytokine IL-6 is a crucial mediator in priming naïve T cells toward the Th17 phenotype that is important in adaptive immune responses on epithelial surfaces. Recent studies show that IL-6 is also a potent inhibitor of transforming growth factor-β–driven induction of CD4+ FoxP3+ Tregs. Interestingly, we observed reduced mRNA expression of IL-6 and simultaneously increased mRNA expression of FoxP3 in mVHL−/− allografts in this study. Furthermore, we also observed reduced mRNA expression of MCP-1 and reduced allograft infiltration of inflammatory cells into the allograft. Altogether, these results suggest a decreased adaptive immune response in mVHL−/− and Treg

**Figure 4** The messenger RNA (mRNA) expression of T-regulatory cell transcription factor was increased in tracheal allografts and spleens of Von Hippel-Lindau knockout (VHL-ko) recipients. Tracheal allografts were heterotopically transplanted from Balb/C donors to major histocompatibility complex–mismatched recipients with VHL (mVHL−/−) deficiency in myeloid cells. Quantitative reverse-transcription polymerase chain reaction analysis was performed from the (A-C) tracheal samples and from the (D) spleens 10 days after transplantation. mRNA data were normalized against 18sRNA and represented as a fold-increase compared with tracheal allografts in littermate control mice. Data are expressed as mean ± standard error of the mean (n = 6 per group). Statistical analyses with Mann-Whitney U test. CTGF, connective tissue growth factor; ET, endothelin; FoxP3, forkhead box P3; HO, heme oxygenase; IL, interleukin; MCP, monocyte chemotactic protein; RORc, retinoic acid receptor–related orphan receptor; TGF, transforming growth factor. *p < 0.05, **p < 0.01, ***p < 0.005.
pathway activation in these allografts at 10 days. Although concomitantly increased mRNA expression of pro-inflammatory cytokines was seen in the spleens of mVHL−/−, increased FoxP3 expression was also observed, suggesting increased Treg activity also in the secondary lymphoid organ. Ben-Shoshan et al.29 recently reported that hypoxia controls Treg homeostasis via HIF-1. The number as well as the potency of Tregs are also enhanced in hypoxia.29 In our study, VHL-deficiency in the myeloid cells may have resulted in increased Treg activity leading to reduced inflammatory and immune responses. However, we also observed decreased HO-1 expression in mVHL−/− during the peak alloimmune response at 10 days. On the one hand, this is somewhat at odds with reports suggesting HO-1 is protective in the setting of transplant rejection where HO-1 has been reported to have anti-apoptotic and protective effects.30 On the other hand, increased HO-1 expression is also linked to the development of chronic rejection in heart transplantation.31 In our study, reduced HO-1 expression at the time of peak inflammation and acute rejection is likely to be secondary to the reduction of the number of T cells as HO-1 expression is increased during acute T-cell-mediated rejection in lung allografts.32,33

We acknowledge that mVHL−/− leads to stabilization of both HIF isoforms, HIF-1α and HIF-2α.10 However, we did not dissect which of the isoforms is responsible for the beneficial effect, and furthermore, we did not compare mVHL−/− directly to HIF-1α−/− but separately to the control. Previous studies showed that macrophages and neutrophils are able to express both HIF-1α and HIF-2α.34,35 However, because the OAD development was significantly accelerated in mHIF-1α−/− recipients, one may assume that HIF-1α activity was the major contributor to the decreased OAD development in mVHL−/− recipients. In addition, Thompson et al.35 reported that HIF-2α regulates mainly key neutrophil functions, but we did not detect any significant difference in the number of MPO+ neutrophils between the groups at 3 or 10 days in our study.

The tracheal allograft model used in our study has several limitations that should be kept in mind when interpreting the results. Firstly, the trachea differs anatomically from the bronchiol and is not in contact with the environment.17 Also, this is a non-vascularized model, and therefore, the early ischemic injury is very severe.36 Owing to the robust nature of this model, the loss of epithelium and OAD development are much more accelerated compared with the clinical setting. In addition, the acute and rapid development of the obliterative lesion compared with the slower and more chronic process seen in clinical BOS may limit extrapolation of treatment effects into the clinic. However, despite the model’s shortcomings, it has the advantage of being easily reproduced, and the obliterative lesion resembles that of the fibroproliferative lesions seen in human OB.11 In addition, we used knockout animals with HIF-1α or VHL deficiency in myeloid cells. However, one should keep in mind, that although most of the myeloid cells have a targeted deletion, the deletion efficiency of HIF-1α or VHL is not 100%.11

In conclusion, VHL-deficiency in myeloid cells improves epithelial recovery, reduces allograft inflammation, and increases regulatory FoxP3 mRNA expression in mouse tracheal allografts. Importantly, this results in better-preserved tracheal epithelium and a decrease in OAD development. In contrast, knocking out myeloid cell expression of HIF-1 accelerates OAD development in mice receiving low-dose tacrolimus therapy. Our results suggest that manipulation of the HIF-1 pathway may have a protective role in preventing OAD in an experimental model for lung transplantation.

Disclosure statement
None of the authors has a financial relationship with a commercial entity that has an interest in the subject of the presented manuscript or other conflicts of interest to disclose.

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