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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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Lung transplantation is the only effective treatment for many end-stage pulmonary diseases. Chronic lung allograft dysfunction (CLAD) is the leading cause of morbidity, lung allograft loss, and late mortality after lung transplantation.[1] The main known form of CLAD is the classical obliterative bronchiolitis (OB), with irreversible fibroproliferation in respiratory bronchioles.[2] Currently, no effective treatment for CLAD is available. Bronchial epithelial damage and injury of the subepithelial structures seem to be the key initiating event in the development of OB.[3,4] Ischemia-reperfusion injury is one of the most important causes of early allograft injury and may lead to primary graft dysfunction that in turn contributes to the development of OB.[5,6] Therefore, interventions targeting early immune activation could well result in reduced long-term complications.

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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).[7] HIF-1 is a principle regulator of hypoxic adaptation, regulating gene expression involved in glycolysis, erythropoiesis, angiogenesis, proliferation, and stem cell function under hypoxia.[7] 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.[8] 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.[9,10]

Cramer et al[11] 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.[12] On the one hand, HIFs also support the innate immune functions of dendritic cells, mast cells, and epithelial cells.[13] On the other hand, HIF-1 acts as an adaptive and survival factor for ischemic tissue.[14,15] 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.[16] 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.[11,17–20]

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.[20] 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.[11] 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.[21] 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.[21] 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.

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