CD4+ T cells and complement independently mediate graft ischemia in the rejection of mouse orthotopic tracheal transplants

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Abstract

Rationale—While microvascular injury is associated with chronic rejection, the cause of tissue ischemia during alloimmune injury is not yet elucidated.

Objective—We investigated the contribution of T lymphocytes and complement to microvascular injury-associated ischemia during acute rejection of mouse tracheal transplants.

Methods and Results—Using novel techniques to assess microvascular integrity and function, we evaluated how lymphocyte subsets and complement specifically affect microvascular perfusion and tissue oxygenation in MHC-mismatched transplants. To characterize T cell effects on microvessel loss and recovery, we transplanted functional airway grafts in the presence and absence of CD4+ and CD8+ T cells. To establish the contribution of complement-mediated injury to the allograft microcirculation, we transplanted C3-deficient and C3-inhibited recipients. We demonstrated that CD4+ T cells and complement are independently sufficient to cause graft ischemia. CD8+ T cells were required for airway neovascularization to occur following CD4-mediated rejection. Activation of antibody-dependent complement pathways mediated tissue ischemia even in the absence of cellular rejection. Complement inhibition by CR2-Crry attenuated graft hypoxia, complement/antibody deposition on vascular endothelium and promoted vascular perfusion by enhanced angiogenesis. Finally, there was a clear relationship between the burden of tissue hypoxia (ischemia × time duration) and the development of subsequent airway remodeling.
Conclusions—These studies demonstrated that CD4$^+$ T cells and complement operate independently to cause transplant ischemia during acute rejection and that sustained ischemia is a precursor to chronic rejection.

Keywords
T cells; complement; graft ischemia; orthotopic tracheal transplants; microvasculature

INTRODUCTION
Chronic rejection after transplantation is a leading cause of long-term morbidity and mortality in solid organ transplant recipients. Pre-clinical and clinical evidence suggest that the maintenance of a functional microvasculature is required for immunosuppression to reverse acute rejection. Clinical studies of lung, liver and kidney allografts have demonstrated the general principle that chronic rejection of an organ transplant develops in close association with microvascular attrition. In lung transplantation, chronic rejection is manifested by terminal airway fibrosis, also known as BOS. Microvascular loss and the attendant ischemia may be an important cause of BOS. While ischemia-reperfusion injury due to the sudden recirculation of devitalized tissue following transplantation surgery is well recognized, microvascular injury-associated ischemia, which occurs because of acute rejection, was only recently described by our group. Therapeutics targeting critical pathways involved in microvascular injury are expected to improve clinical outcomes in transplantation, but information is lacking about what factors are directly responsible for tissue ischemia during acute rejection.

Mouse orthotopic tracheal transplantation is an ideal model for parsing the role of airway microvasculature repair and remodeling in rejection. This grafted trachea is a functional transplant through which the mouse breathes and, in rejection, the model pathologically replicates lymphocytic bronchiolitis (a large airway precursor of BOS); findings about fibrosis development in large airways from orthotopic tracheal transplant research have been cautiously extrapolated to explain fibrogenesis in terminal bronchioles. We have recently utilized the orthotopic tracheal transplant model because the well-organized planar anatomy of airway microvasculature facilitates the study of relatively long segments of microvessels. Our group has previously demonstrated that acutely rejecting tracheal transplants are relatively hypoxic compared to non-rejecting allograft tissue and exhibit sequential damage characterized, first, by microvascular destruction, followed by airway ischemia and finally, by recipient-derived neovascularization. Recently, the Papworth Autopsy Study demonstrated a marked loss of microvessels in pre-obliterative bronchiolitis (OB) foci of human lung transplants which suggested that a loss of microcirculation and airway ischemia precede the onset of OB. These pre-clinical and clinical studies cumulatively suggest that preserving normal airway circulation is of likely benefit to the overall health (and patency) of the respiratory tree.

Pathologists currently characterize transplant rejection by hematoxylin and eosin (H&E) staining and immunohistochemistry. However, our prior studies have indicated that profound events are occurring in the transplant beyond inflammation: most notably significant tissue hypoxia due to microvascular injury-associated ischemia. The grading of rejection does not typically capture this information, and, subsequently, the dynamic changes of microvascular blood flow and tissue oxygenation remain unknown for solid organ transplants undergoing acute rejection. Endothelial cells are a well-established target for both adaptive and innate immune responses in allograft rejection. Effector CD4$^+$ T cells appear to be directly injurious to allogeneic endothelial cells in vivo. Complement also appears to be a contributor to vascular damage in transplant recipients. The
The current study sought to determine whether T cell subsets and complement were required for the microvascular injury-associated ischemia observed during acute rejection and to observe the impact of this ischemic injury on chronic rejection development in tracheal transplants.

METHODS

Orthotopic tracheal transplantation

The Veterans Affairs Palo Alto Animal Care and Use Committee approved experiments employed in this study. Tracheal transplants were performed according to our established procedure as described previously (3). Surgical and animal details are provided in the online data supplements.

Serial tissue pO2 monitoring

Tissue oxygenation was measured using the OxyLab pO2 monitor (Oxford Optronix Ltd.) as described previously (3) with slight modifications. Details of pO2 monitoring are included in the online data supplement.

Tracheal microvascular perfusion

Whole-mount tracheas were evaluated for microvascular perfusion by fluorescence staining techniques as described previously by (3, 38). Details are provided in the online data supplement.

CD4+ and CD8+ cell depletion

Monoclonal rat anti-mouse CD4 (GK1.5 clone, BioXcell) and rat anti-mouse CD8 (2.43 clone, BioXcell) antibodies were injected (10mg/kg sc) intra-peritoneally at d-1, d0, d1 and d2 to deplete respective T cell populations in WT and C3\(^{-/-}\) allografts recipients. T cell depletion was assessed 4 days after transplantation by flow cytometry. Peripheral blood was stained with FITC-anti mouse CD4 and PE-anti mouse CD8 antibodies and was assessed on a FACS Caliber system (BD Biosciences). Percent depletion was found to be >98%.

Immunoglobulin treatment of RAG1\(^{-/-}\) mice

B6 RAG1\(^{-/-}\) recipients (BALB/c→B6 RAG1\(^{-/-}\)) were given 100\(\mu\)g i.v. of anti-mouse MHCII IgG2b (Southern Biotech), B4-IgM or saline for controls at d0 and every 2nd day thereafter.

Isolation and purification of CD4 and CD8 cells and adoptive transfer

Spleens were harvested from B6 mice, processed, and filtered through a 40\(\mu\)m cell strainer (BD Biosciences) to obtain a cell suspension in cold DMEM with 10% FCS. Details are provided in the online data supplements.

Immunohistochemistry

The tracheal transplants were harvested and embedded in Tissue-Tek O.C.T. compound (Sakura Finetek). A cryostat (HM550; Microm) was used to cut 6\(\mu\)m sections of tracheal graft and the sections were placed on superfrost/plus slides (Fisher Scientific). Details are provided in the online data supplements.

\(\beta\)-galactosidase staining

FVB Tie2-lacZ tracheas were harvested for whole mount analysis as described above. Details are provided in the online data supplement.
CR2-Crry treatment

C57Bl/6 recipient’s (BALB/c→B6 allografts) were given 0.25mg i.p. of CR2-Crry or saline for controls at d0 and every 3rd day thereafter.

Quantitative Real Time RT-PCR

RT-PCR analysis of angiogenic genes were performed by (16). Details are provided in the online data supplement.

Statistics

GraphPad Prism® version 5.0c was used for statistical analysis. Differences between various groups at multiple times were compared using two-way ANOVA with Bonferroni multiple comparisons test for post hoc analyses. For comparisons between multiple experimental groups at a single time point, Kruskall-Wallis test followed by Dunn’s multiple comparisons test for post hoc analyses were used. For all post hoc analyses, all groups were compared to WT-Allo. Student’s unpaired t test was used when comparison was limited to only 2 groups. All data are represented as means ± SEM, and P - value < 0.05 is considered significant.

RESULTS

Loss of perfusion in rejecting tracheal transplants closely correlates with tissue hypoxia

Our group previously reported that with microvascular rejection, late administration of immunosuppression no longer rescues the airway from developing chronic rejection 3. Chronic rejection, which follows untreated acute rejection, is characterized by increased subepithelial fibrosis and a flattened, dysplastic epithelium 3, 15. While our group previously described microvascular injury-associated ischemia 3, we sought to better characterize the relationship between tissue ischemia and tissue hypoxia. To assess microvascular injury-associated ischemia in rejecting allografts, we grafted C57Bl/6 recipients (B6, H-2b) with tracheas from MHC-incompatible BALB/c (H-2d) donors. We also previously reported that syngrafts and allografts undergo a period of non-inflammatory ischemia for ≈4 days until the microcirculation between the recipient and donor fuse at the anastomosis line; this initial ischemic period does not lead to chronic rejection 3. In the current study, tissue oxygenation was assessed by exposing the trachea, making a small hole through the anterior wall and gradually lowering a pO2 probe (Online Figure I). The luminal surfaces of rejecting airway tissue were significantly hypoxic compared to syngrafts (B6→B6) from d10 through d14, but oxygenation progressively increased from d28 until d56 (Fig. 1 A). By contrast, syngrafts consistently maintained a relatively high tissue pO2. (The pO2 of non-transplanted tracheas (i.e. normal airways) was 32-33 mm Hg which was very comparable to syngeneic values of established transplants). To further confirm that the pO2 assessment was a good surrogate for tissue perfusion, we examined blood perfusion using laser doppler flowmetry in allografts and syngrafts and found that rejecting allografts are poorly perfused during the same period that tissue pO2 was low (Fig 1 B). Next, we examined allografts and syngrafts by FITC-lectin perfusion and found that syngrafts showed consistently perfused microvasculature over time while rejecting allografts lost perfusion by d10 but showed re-establishment of a functional airway circulation by d28 (Fig. 1 C, D). Grafts exhibiting tissue pO2s of less than 15-16 mm Hg were ischemic by FITC-lectin assessments in all transplants evaluated throughout this study. Thus, the tracheal tissue pO2 was generally considered to be an accurate surrogate for perfusion status in this study.
**CD4^+ T cells are sufficient to cause persistent graft ischemia while CD8^+ T cells are required for neovascularization of rejected transplants**

Given the established importance of T cells in transplant rejection, we next examined how CD4^+ and CD8^+ T cells differentially affect allograft perfusion during acute rejection. First, to evaluate the contribution of CD4^+ cells, B6 recombination activating gene 1 deficient (RAG1^−/−) recipients, which are T and B cell-deficient and complement-replete, were reconstituted with fractionated CD4^+ T cells (Fig. 2 A). Alternatively, wild type (WT) B6 mice were CD8-depleted. In both CD4-reconstituted and CD8-depleted groups, microvascular injury-associated ischemia was first observed on d10, and, in contrast to WT allografts (Fig. 1 A), there was no recipient-derived neovascularization detected as late as 8 weeks following transplantation.

Next, to isolate the contribution of CD8^+ T cells in microvascular injury, RAG1^−/− recipients were reconstituted with CD8^+ T cells, and WT mice were CD4-depleted (Fig. 2 B). While transient hypoxia and ischemia occurred in CD4-depleted animals, CD8-reconstituted RAG1^−/− allografts did not become ischemic. Both CD8^+ replete groups rapidly recovered tissue and pO_2 levels and perfusion by d28. Unexpectedly, while combined anti-CD4/anti-CD8 treatment prevented acute rejection (as manifested by non-significant monocyte/CD3+ T cell infiltration (Figure 3 A/Online Figure II)) in WT recipients, these grafts nevertheless demonstrated pO_2 kinetics similar to WT rejecting allograft controls and transiently lost perfusion at d10 (Fig. 3 B-D). When acute rejection (as manifested by the usual criteria of cellular inflammation rather than pO_2 and perfusion) was prevented with combined CD4/CD8 cell depletion, revascularization occurred despite the absence of CD8^+ cells (i.e. CD8^+ T cells were only required for revascularization following CD4-mediated rejection). Thus, while CD4^+ T cells were sufficient to induce persistent ischemia in allograft rejection, CD8^+ T cells were not isolated as a cause of microvascular injury-associated ischemia. The mild hypoxia noted in CD8^+ reconstituted RAG1^−/− recipients (Fig. 2B) was attributed to CD8^+ T cell inflammation; inflammation is sufficient to induce a modest decline in tissue oxygenation even in the absence of ischemia as previously demonstrated. Surprisingly, the presence of CD8^+ T cells following CD4-mediated acute rejection was required for graft revascularization. Consistent with the previously advanced notion that acute rejection synergizes with ischemia to promote chronic rejection, tracheal pathology in CD4-depleted mice shows less severe airway remodeling at d28 than CD8-depleted recipients (Online Figure III).

**Antibody-dependent complement activation is sufficient to cause microvascular loss in transplants in absence of lymphocytes**

We previously demonstrated that C3 deposits on vascular endothelium prior to microvascular injury-associated ischemia. Because the major role of antibody in complement activation is to focus complement attack to the correct site and increase the effective rate of target deposition, we next investigated whether endothelial deposition of complement and IgG antibody contributed to microvascular injury-associated ischemia, independent of T cell immunity. We performed immunofluorescent staining of lymphocyte subset-depleted allografts for C3d and IgG deposition on vascular endothelial cells (Fig. 4). Confocal imaging showed increased colocalization of C3d and IgG on CD31^+ endothelial cell in CD4^+, CD8^−, and pan-T-cell-depleted allografts. Groups in Figure 2, as well as the combined anti-CD4/anti-CD8 treated animals without overt acute rejection (Fig. 3 A), that all demonstrated microvascular injury-associated ischemia 10 days post-transplant, also have relatively greater endothelial C3d and IgG deposition prior to microvessel loss. Thus, increased vascular C3d and IgG in airway grafts correlated with subsequent microvascular injury-associated ischemia irrespective of the presence of CD4^+ or CD8^+ T cells.
Non-reconstituted RAG1−/− mice do not reject allografts, and maintain perfusion throughout all time points observed (Figure 5 A-C). Of note, although a small amount of endothelial C3d deposition was noted in these antibody-deficient animals (Figure 5 D), this was trivial compared to endothelial C3d deposits in B-cell replete animals (Figure 4). A non-significant drop in oxygenation was transiently evident at d10, but this was not associated with microvascular injury-associated ischemia. As RAG1−/− mice are complement-replete and antibody-deficient, we hypothesized that activation of the complement pathway was antibody-dependent and that the transfer of allospecific antibody would be sufficient to induce microvascular loss in RAG1−/− graft recipients. Adoptive transfer of antibodies specific to I-A^d (donor) Class II MHC (expressed on allograft vascular endothelial cells) was sufficient to induce microvascular injury-associated ischemia in immunodeficient recipients (Figure 5 E). The development of ischemia by d10 coincides with alloimmune antibody responses that are arising in the first two weeks following transplantation. As RAG1−/− mice are complement-replete and antibody-deficient, we hypothesized that activation of the complement pathway was antibody-dependent and that the transfer of allospecific antibody would be sufficient to induce microvascular loss in RAG1−/− graft recipients. Adoptive transfer of antibodies specific to I-A^d (donor) Class II MHC (expressed on allograft vascular endothelial cells) was sufficient to induce microvascular injury-associated ischemia in immunodeficient recipients (Figure 5 E). The development of ischemia by d10 coincides with alloimmune antibody responses that are arising in the first two weeks following transplantation. (Class II MHC-expressing endothelial cells are significantly reduced as acute rejection proceeds (Online Figure IV)). Adoptive transfer of the monoclonal antibody B4 (annexin IV), which binds neoepitopes that become exposed on ischemic tissue and activates complement following intestinal ischemia-reperfusion injury, did not cause microvascular injury-associated ischemia or hypoxia in airway transplants. This latter result is consistent with the fact that, in the current model, antibody/complement injury to vasculature is actually the cause of ischemia whereas naturally occurring antibodies to annexin IV propagate injury in already ischemic tissue. These findings demonstrate for the first time that antibody-mediated complement activation is sufficient to cause microvascular injury-associated ischemia in the absence of lymphocytes.

C3−/− allograft recipients demonstrate less sustained graft hypoxia and loss of microvascular flow

C3 plays a central role in the activation of both classical and alternative pathways and is a known contributor to pulmonary vascular injury. In the current study, rejecting allografts in C3−/− recipients exhibited declining tissue pO_2 but maintain microvascular flow at the time points assessed (Fig. 6). The d4 C3−/− allografts were noted to exhibit relatively dilated microvessels (Figure 6 B), and further evaluation of these vessel, by microsphere infusion, revealed that them to be relatively leaky (Figure 6 C). Tissue edema due to capillary leak may have accounted for the early relative hypoxia noted in this group. Because the pO_2 was trending down on d8 without detectable ischemia on d10, we assessed d9 allografts and found that perfusion had indeed ceased only on this day (i.e. a brief period of ischemia was detected only on d9; Figure 6 E, F). These findings indicated that recipient C3 was not required for vascular destruction in allograft rejection. While not sufficient to prevent acute rejection, the absence of C3 enhanced the process of vascular repair as evidenced by the rapid reestablishment of tissue perfusion.

Because of the rapid restoration of perfusion to rejected tracheal transplants allografts in C3−/− recipients, we questioned whether this phenomenon occurred because of accelerated recipient neovascularization. To assess for the elimination of donor vessels, we utilized FVB (H-2^q) strain transgenic mice, expressing β-galactosidase on the endothelial cell-specific Tie2 promoter, as tracheal transplants donors and grafted these airways into C3−/− recipients. Specimens were evaluated as whole mounts (Online Figure V). In d6 allografts, β-galactosidase staining is evident in the perfused transplants indicating that the functional microvasculature is of donor origin. Allografts harvested at d10 (not shown) and d12 are β-galactosidase negative but are well perfused; a finding which is consistent with recipient-derived vessels supplying the allograft after rejection on d9. (Importantly for this experiment, the kinetics of ischemia of allografts with the H-2^q haplotype are the same as allografts with the H-2^d haplotype). In summary, although the absence of recipient C3 is not
sufficient to prevent CD4-mediated injury to the microvasculature, recipient C3 deficiency appears to facilitate an enhanced vascular recovery by allowing early neovascularization of the graft via recipient-derived vessels.

**CD4**+ **T cell depletion of C3−/−** **graft recipients prevents airway ischemia**

To assess whether CD4+ and CD8+ cells synergized with C3 complement pathways in allograft rejection with special reference to microvascular function and graft oxygenation, we depleted CD4+ and CD8+ cells in C3−/− recipients. Anti-CD4- or combined anti-CD4/anti-CD8-treated C3−/− recipient groups maintained perfusion (Fig. 7) over all time points assessed. By contrast, CD8 depletion resulted in persistent graft ischemia. This result is similar to WT animals that are CD8-depleted, except that in the case of WT recipients, the drop in tissue pO2 is more rapid; a result consistent with CD4 and C3 both contributing to microvascular injury-associated ischemia. The associated histopathology reveals that both CD4- and combined CD4/CD8-depleted groups exhibit insignificant mononuclear cell infiltrates compared to the more exuberant inflammation noted in CD8-depleted C3−/− recipients (Fig. 7 D). These results demonstrate highly preserved oxygenation and perfusion in the combined absence of both CD4 T+ cells and C3.

**CR2-Cry treatment restores tissue oxygenation and microvascular flow in tracheal transplants and limits airway remodeling**

Given that the absence of recipient C3 attenuated graft hypoxia and promoted perfusion during acute rejection, we next tested if the complement C3 inhibitor CR2-Cry could similarly benefit allograft tissue pO2 and microvascular flow. Similar to C3−/− recipients, perfusion was transiently only lost (≈24 hours) on d9 (Fig. 8). Thus, neither recipient C3 deficiency nor CR2-Cry therapy prevents microvascular rejection when CD4+ T cells are present. However, both groups retain columnar epithelium and exhibit less subepithelial fibrosis compared to WT allografts at d28 (Online Figure III). PicroSirius staining for fibrosis indicated that all experimental groups associated with airway ischemia have varying degrees of subepithelial fibrosis (Online Figure VI A). Groups undergoing rejection with only limited airway ischemia (i.e. CR2-Cry, C3−/−, CD4-depleted, CD4/CD8-depleted) preserved overlying columnar epithelium whereas groups undergoing rejection characterized by prolonged ischemia (i.e. WT-Allo, CD8-depleted) display a thin dysplastic epithelium. Of interest, the group with the most prolonged ischemia (CD-8 depleted) exhibited relatively low Collagen I, II and III mRNA transcripts (Online Figure VI B).

The brief period of poor oxygenation and perfusion in CR2-Cry-treated mice is followed by a rapid rise in tissue pO2 (Fig. 8 A) and accelerated neovascularization evident beginning d10 post-transplantation (Fig. 8 B); this rapid recovery of graft oxygenation and perfusion had the same kinetics as that observed in C3−/− recipients (Fig. 6). The rapid reinvestment of perfusing vessels into rejecting allografts in C3−/− and CR2-Cry-treated recipients suggested that enhanced angiogenesis was occurring. Complement activation has been previously associated with dysregulated angiogenesis 27, 28. To investigate if complement inhibition promoted proangiogenic factors, we compared the tracheal expression of proangiogenic factors in CR2-Cry-treated recipients to rejecting C3−/− and WT recipients after 10 days of acute rejection. CR2-Cry-treated recipients expressed significantly higher transcript copies of VEGF, VEGF-R2, SDF1, and PLGF (Online Figure VII). Rejecting tracheal transplants in C3−/− recipients similarly demonstrated relatively high levels of proangiogenic factors ANGPT1 and Tie2. These findings indicate that enhanced angiogenesis in allograft recipients with inhibited or absent C3 activity may occur because of augmented graft-intrinsic signaling that promotes microvascular invasion.
CR2-Crry treatment reduces C3d and IgG deposition on graft vascular endothelium

CR2-Crry-treated recipients demonstrate decreased C3d and IgG compared to untreated WT allografts (Fig. 8 D-G). With activation of complement pathways, the terminal components of the complement cascade (C5b-C9) form a membrane attack complex (MAC) which leads to the rapid activation and destruction of target vascular endothelial cells in lung transplant rejection. CR2-Crry treatment effectively prevented endothelial MAC formation in these airway microvessels (Online Figure VIII).

Discussion

While microvascular loss is strongly associated with chronic rejection, little is known about how inflammatory mediators specifically affect vascular flow during this process. The ability to attribute physiologic derangements to individual immune components requires an experimental model that can be interrogated for microvascular injury-associated ischemia. By focusing on the functionality of graft microvasculature, the definition of allograft health broadens within this study. (Online Table 1 summarizes experimental results, and Online Table 2 interprets these findings.) The cumulative data demonstrate that CD4+ T cells and complement independently mediate graft ischemia during acute rejection.

In the current study, CD4+ T cells were found sufficient, independent of antibody and complement, to induce microvascular injury-associated ischemia whereas CD8+ T cells, in isolation, did not induce microvascular injury-associated ischemia. Surprisingly, CD4 T cell-mediated injury did not recover in the absence of CD8+ T cells; CD8+ T cells were required for airway neovascularization to occur following CD4-mediated rejection. T cells have previously been implicated as important pro-angiogenic cells. CD8+ T cells contribute to the early phase of collateral vessel development in hind limb ischemia by promoting the recruitment of angiogenic CD4+ T cells in an IL-16-dependent manner. CD8-recruitment of other angiogenic monocytes and macrophages, that are also key to vascular collateralization following ischemic injury, may also explain the requirement of CD8+ T cells to revascularize rejected airway transplants.

Airway ischemia during acute rejection was closely associated with the presence of subepithelial fibrosis. Of note, in the absence of CD8+ T cells following acute rejection, the microvascular injury-associated ischemia is most prolonged, but subepithelial fibrosis is surprisingly limited, and collagen transcription, minimal. It is possible that the complete absence of perfusion to the transplant for many days also decreased the delivery of fibrosis-causing elements in the blood such as cytokines and fibrocytes. The duration of airway ischemia closely correlated with the appearance of the overlying epithelium with a brief cessation of airway perfusion during rejection still allowing the preservation of an intact overlying columnar lining but prolonged ischemia being tightly associated with a flattened dysplastic-appearing overlying epithelium. Thus, as our other studies have recently demonstrated, microvascular injury-associated ischemia in airways, during acute rejection episodes, appear to be causally-linked to the airway remodeling observed in chronic rejection.

The following sequence of key results subsequently led to the discovery of two independent effector pathways for microvascular injury-associated ischemia: 1) Allografts in unreconstituted complement-replete RAG1−/− mice do not become ischemic. 2) CD4-reconstitution of RAG1−/− recipients results in transplant ischemia. 3) CD4-replete/C3-inhibited recipients demonstrate graft ischemia. 4) Adoptive transfer of donor-specific MHC Class II antibodies restores graft ischemia in B and T cell-deficient/complement-replete RAG1−/− recipients. 5) C3-deficient/antibody-replete/CD4-depleted WT recipients do not develop ischemia. The latter two results indicate that in the absence of CD4 cells, C3 and
antibody must both be present for graft ischemia to occur. The cumulative results indicate that CD4$^+$ T cells and antibody-dependent complement activation independently mediate microvascular injury-associated ischemia. Because C3-deficient/CD4-depleted WT recipients do not become ischemic, additional effector pathways are not required to explain the loss of perfusion in airway transplants.

Complement activation causes dysregulation of angiogenic factors required for normal fetal development, and C3 inhibition with Crry-Ig blocks the pathological increase in soluble VEGFR-1, a potent inhibitor of VEGF activity, and rescues pregnancies in mice. CR2-Crry treatment up-regulated proangiogenic factors within the transplant, which may have caused the rapid reinvestment of microvessels into these rejected airways. C3$^{-/-}$ recipients had a similar (although not identical) profile of upregulated proangiogenic factors associated with a faster revascularization period. A recent study in an animal model of retinopathy of prematurity mirrors these findings. The loss of donor-derived microvasculature was evident in C3$^{-/-}$ recipients; their rapid replacement with non-transgenic vessels is consistent with recipient vessels growing into the transplant as an accelerated response to increased graft-derived proangiogenic factors. C3$^{-/-}$ recipients exhibited leakier and more dilated allograft vessels earlier in rejection for unknown reasons. In C3$^{-/-}$ mice, thrombin substitutes for C3-dependent C5 convertase leading to the generation of C5a, the latter complement component being an anaphylatoxin that can trigger vasodilation and increase capillary permeability. Given that the generation of thrombin is a feature of allograft rejection, it is possible that excessive C5a activation could account for increased microvascular dilation and permeability in low/inhibited C3 conditions. Regardless of this finding, the ischemic burden of C3$^{-/-}$ and CR2-Crry-treated animals was substantially less than in WT-rejecting animals. We have recently reported that manipulating airway allografts by selectively increasing proangiogenic factors by HIF-1α modulation similarly accelerates recipient-derived angiogenesis and limits fibrotic graft remodeling.

This study strongly suggests that the duration of hypoxia and ischemia in allograft rejection is relevant to the subsequent development of significant airway remodeling as manifested by a loss of normal epithelium and increased subepithelial fibrosis. Lung transplant recipients are particularly vulnerable to the deleterious effects of airway hypoxia and ischemia. The lung is unique among solid organ transplants because of the lack of the surgical restoration of a vascular connection to the systemic circulation. Blood supply to the airways in lung transplant recipients, in contrast to the normal dual circulation, presumably comes from the deoxygenated pulmonary artery circulation. Therefore, from the outset, lung transplant airways have an impaired microcirculation due to the lack of bronchial artery restoration, which, we have recently demonstrated, results in relative airway tissue hypoxia in lung transplant patients. We have hypothesized that this baseline airway hypoxia may be a diathesis for chronic rejection in lung transplant recipients, and therapies which preserve microvascular integrity may be especially relevant.

Profound tissue ischemia, undetectable by histology, may ‘silently’ occur during acute rejection in transplant recipients. Knowledge of the immune factors which cause microvascular-injury associated ischemia should help to more logically target therapeutics designed to preserve microvessel integrity. Captured perfusion and oxygenation data from this study reveal, paradoxically, that CD8$^+$ T cell depletion interferes with angiogenesis in ischemic tissue and may harm graft recovery in patients. Further, this new physiologic information suggests that targeted complement inhibitors could safely synergize with conventional therapy during rejection episodes to prevent microvascular injury-associated ischemia and potentially limit the development of chronic rejection.
Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-Standard Abbreviations and Acronyms

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<tr>
<td>Allo</td>
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<td>B6</td>
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<td>BOS</td>
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<td>CR2-Crry</td>
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REFERENCES


Novelty and Significance

What is Known?

- Although lung transplantation is the last option for patients with serious lung disease, life expectancy with this procedure is significantly limited by the high incidence of chronic rejection, known as the bronchiolitis obliterans syndrome (BOS).
- Microvascular injury is strongly associated with chronic rejection in solid organ transplantation, and promoting vascular health during acute rejection could prevent chronic rejection.
- The immune factors responsible for the tissue ischemia associated with microvascular injury have not been previously elucidated.

What New Information Does This Article Contribute?

- CD4+ T cells and antibody-dependent complement activity are independently sufficient to induce airway tissue ischemia during rejection of functional airway transplants.
- Paradoxically, CD8+ T cells are required for airway neovascularization following CD4-mediated vascular rejection.
- Complement antagonism synergizes with CD4-depletion to prevent transplant ischemia and chronic rejection.

Microvascular loss and the resulting ischemia may be an important cause of BOS. While ischemia-reperfusion injury due to the sudden vascular investment of devitalized tissue is a well-recognized cause of graft damage, microvascular injury-associated ischemia, which occurs because of acute transplant rejection, has only recently been described by our group. Using a mouse model of orthotopic tracheal transplantation, we performed a series of experiments that isolated the role of the four immune components most likely responsible for microvascular injury: CD4+ T cells, CD8+ T cells, complement and immunoglobulin. Using lymphocyte depletion, adoptive transfer, and knockout mouse recipients, we show that CD4+ T cells and antibody-dependent complement pathways were independently capable of inducing airway transplant ischemia. Although, CD8+ T cells are known to participate in allograft rejection, we found that this cell subset did not induce tissue ischemia but instead promoted graft revascularization. Assessing graft perfusion during rejection episodes could provide insight into transplant health and can guide new therapeutic approaches. These findings suggest that treating acute rejection with complement inhibition, while avoiding CD8+ T cell depletion, could prevent the subsequent development of chronic rejection.
Figure 1. The progressive hypoxia of acutely rejecting allografts is reversed as perfusion is restored during chronic rejection

(A) Tissue pO\(_2\) (Mean±SE, mmHg) was plotted over different time points (d4 - d56), (n=4-6 animals/time point). Rejecting allografts become progressively hypoxic over time beginning with early acute rejection responses observed in the first week following transplantation. Airway pO\(_2\) nadirs on d14 and increases during chronic rejection. B) Blood perfusion (Mean ±SE, units) was plotted over different time points (d4-d28), (n=4-6 animals/time points). Rejecting allografts demonstrate less blood perfusion as compare to syngrafts during acute rejection. (C) FITC-lectin perfusion profile of whole mounts tracheal grafts from d4 to d28 illustrates that falling airway pO\(_2\)s correlate with the loss and restoration of perfusion during allograft rejection. (D) Morphometric assessments of perfused vasculature (FITC-lectin perfusing vessels/unit area) in tracheal grafts at different time points following
transplantation demonstrate neovascularization after 28 days of rejection. Data are shown as means with SEM for five independent experiments. *, p<0.05. Original magnification, X10.
Figure 2. Effects of CD4$^+$ and CD8$^+$ T cells on kinetics of tissue pO2 and perfusion

(A) Tissue pO2 kinetics, in rejecting CD4-reconstituted/CD8-deficient RAG1$^{-/-}$ or CD4-replete/CD8-depleted WT recipients, were serially evaluated between d4 and d56 (n=4-6 animals/time point). CD4$^+$ cells are sufficient to induce sustained graft hypoxia. Absence of CD8$^+$ cells is correlated with persistent graft hypoxia relative to CD8-replete control rejection responses (See Fig. 1a). (B) Tissue pO2 kinetics, in rejecting CD8-reconstituted/CD4-deficient RAG1$^{-/-}$ or CD8-replete/CD4-depleted WT recipients, were serially evaluated between d4 and d56 (n=4-6 animals/time point). CD8$^+$ reconstitution does not result in significant hypoxia in RAG1$^{-/-}$ recipients. (C) FITC-lectin perfusion profiles were serially assessed in whole mounts from d8 to d28 and demonstrated that perfusion was lost.
in CD4-reconstituted/CD8-deficient RAG1−/− and CD4-replete/CD8-depleted WT recipients. By contrast, CD8-reconstituted/CD4-deficient RAG1−/− recipients were not ischemic and CD8-replete/CD4-depleted WT recipients demonstrated an accelerated restoration of blood flow by d12, which was earlier than the WT response illustrated in Fig. 1c. (D) Morphometric assessment of perfused vasculature in tracheal graft at different time points. Data are shown as means with SEM of four independent experiments. *, p<0.05. Original magnification, X10.
Figure 3. Combined CD4+ and CD8+ T cell depletion prevents acute rejection, but grafts become ischemic

(A) H&E of BALB/c →B6 tracheal transplants 10 days after transplantation shows no acute mononuclear cell infiltration (H&E, Original magnification, X40). (B) CD4/CD8-depleted recipients exhibit significant hypoxia similar to rejecting non-depleted WT recipients (n=4-6 animals/time point). (C) Reperfusing vessels in pan T-depleted recipients are detected on d12. (D) % perfused vasculature is significantly decreased on d10 and d12 relative to syngrafts. Data are shown as means with SEM. *, p<0.05. (FITC-lectin, Original magnification, X10).
Figure 4. Increased microvascular deposition of C3d and IgG identifies grafts that will lose perfusion

(A) Immunofluorescent staining demonstrates increased vascular C3d on post-operative day 6 in the T-cell depleted allografts relative to syngrafts; these allograft groups lose perfusion on d10. Increased C3d/IgG endothelial deposition is noted even the combined CD4/CD8 T cell depleted graft recipients that do not exhibit acute rejection. Arrows highlighted overlaid C3d on CD31+ cells. A small amount of endothelial C3d expression is noted in the adventitia of the syngrafts (n=8/group). (B) A similar pattern of vascular IgG deposition was noted, with greater deposition in the allograft groups than the syngraft group. (C) Morphometric assessments of C3d/CD31 colocalization revealed that all allograft groups demonstrated significantly more endothelial complement deposition. (D) Morphometric
assessments for IgG/CD31 colocalization similarly showed increased immunoglobulin deposition in allografts. Data are shown as means with SEM and representative images of at least four different experiments. *, p<0.05. Original magnification, X40. SE symbolizes sub epithelial area in grafts.
Figure 5. Effects of absence of T and B-lymphocytes on kinetics of tissue pO2 and loss of microvascular flow

(A) Tissue pO2 values of Balb/c allografts in non-reconstituted and CD4+/CD8+ reconstituted RAG1−/− recipients were plotted over different time points (d10-d58), (n=4-6 animals/time point). B, C) FITC-lectin perfusion profile and % perfused vasculature show that perfusion is maintained over time in RAG1−/− recipients and lost in reconstituted animals. (FITC-lectin, Original magnification, X10). D) Immunofluorescence colocalization of C3d in CD31+ vascular endothelial cells in RAG1−/− mice at day 6 of transplantation. E) Tissue pO2 and FITC-lectin perfusion in donor-specific MHC-II IgG2 and B4-IgM reconstituted RAG1−/− allografts at d10 post transplantation. Adoptive transfer of donor-specific antibodies causes ischemia whereas administration of B4-IgM antibodies (specific
to annexin IV) did not cause graft hypoxia or ischemia. Original magnification, X40. Data are shown as means with SEM. *, p<0.05. Data are shown as means with SEM.
Figure 6. Absence of complement in the allograft recipient limits graft hypoxia and ischemia during rejection

(A) BALB/c→B6 C3−/− allografts were assessed for tissue pO2 over different time points (d4-d28), (n=4-6 animals/time point) and demonstrated a brief period of hypoxia on d9. (B) FITC-lectin perfusion profile of whole mounts tracheal grafts from d4 to d28 illustrate rapid recovery of microvascular flow from d10 of perfusion during allograft rejection. D4 microvessels appear dilated in C3−/− recipients. (C) Significant microvascular permeability was noted in d4 tracheal C3−/− recipients by using FITC-lectin in conjunction with R50 Fluromax red microsphere infusion. (D) Morphometric assessments of perfused vasculature (FITC-lectin perfusing vessels/unit area) in tracheal grafts at different time points. (E) FITC-
lectin perfusion studies in C3−/− recipients demonstrate a brief period of ischemia on d9. (F) Morphometric assessment of perfused vessels on d9. Data are shown as means with SEM of four different experiments. *, p<0.05. Original magnification, X10.
Figure 7. CD4+ T cell depletion synergizes with C3 deficiency to prevent airway hypoxia and maintain perfusion in rejection

(A) Lymphocyte subset-depleted Balb/c→B6 C3−/− recipients were serially assessed for graft pO2 between d4-d28 (n=4-6 animals/time points). While CD4-depleted C3−/− recipients exhibited a pO2 profile similar to syngrafts, CD8-depleted C3−/− recipients reveal prolonged and unremitting graft hypoxia. (B, C) FITC-lectin perfusion profiles and morphometric assessments of whole mount tracheal grafts from d4 to d28 demonstrate preserved perfusion in CD4-depleted and CD4/CD8 depleted allografts over time in C3−/− recipients (FITC-lectin, original magnification, X10). (D) Tracheal transplants harvested on d10 post-transplant in C3−/− recipients subjected to CD4+ and/or CD8+ cell depletion (H&E,
original magnification, X40). Data are shown as means with SEM of four different experiments, *, p<0.05. SE symbolizes sub epithelial area in grafts.
Figure 8. Effects of CR2-Crry treatment on kinetics of tissue hypoxia and loss of microvascular flow in rejecting allografts

(A) Allograft tissue pO2 in CR2-Crry treated recipients (n=4-6 animals/time point). (B, C) FITC-lectin perfusion profile and % perfused vasculature (per unit area) during course of treatment from d10 - d28 in WT-allografts (FITC-lectin, original magnification, X10. (D-G) Immunofluorescent staining for C3d, IgG and colocalization of C3d and IgG on vascular endothelial cells. Arrows indicate colocalization of C3d and IgG on CD31+ cells. (Image representative of n= 6). Data are shown as means with SEM. *, p<0.05. Original magnification, X40.