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Microhemorrhage-associated tissue iron enhances the risk for *Aspergillus fumigatus* invasion in a mouse model of airway transplantation

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Competing interests: M.R.N., X.J., and J.R. are co-inventors on a patent for the use of the topical surgical solution for preconditioning transplanted airways: "Iron chelators and the use thereof for reducing transplant failure during rejection episodes" patent # US 9,763,899 B2. The other authors declare that they have no competing interests.

Abstract

Invasive pulmonary disease due to the mold *Aspergillus fumigatus* can be life-threatening in lung transplant recipients, but the risk factors remain poorly understood. To study this process, we used a tracheal allograft mouse model that recapitulates large airway changes observed in patients undergoing lung transplantation. We report that microhemorrhage-related iron content may be a major determinant of *A. fumigatus* invasion and, consequently, its virulence. Invasive growth was increased during progressive alloimmune-mediated graft rejection associated with high concentrations of ferric iron in the graft. The role of iron in *A. fumigatus* invasive growth was further confirmed by showing that this invasive phenotype was increased in tracheal transplants from donor mice lacking the hemochromatosis gene (*Hfe*^{-/-}). The invasive phenotype was also increased in mouse syngrafts treated with topical iron solution and in allograft recipients receiving deferoxamine, a chelator that increases iron bioavailability to the mold. The invasive growth of the iron-intolerant *A. fumigatus* double-knockout mutant (*sreA*/*cccA*) was lower than that of the wild-type mold. Alloimmune-mediated microvascular damage and iron overload did not appear to impair the host's immune response. In human lung transplant recipients, positive staining for iron in lung transplant tissue was more commonly seen in endobronchial biopsy sections from transplanted airways than in biopsies from the patients' own airways. Collectively, these data identify iron as a major determinant of *A. fumigatus* invasive growth and a potential target to treat or prevent *A. fumigatus* infections in lung transplant patients.

INTRODUCTION

Lung transplantation is a lifesaving treatment for persons with end-stage lung diseases (1). However, survival is often limited by infectious complications (1). One in three lung transplant patients suffer from a serious pulmonary disease caused by *Aspergillus fumigatus*, a ubiquitous mold that produces airborne spores (2–4). *Aspergillus* disease after lung transplantation includes airway anastomotic infections, severe asthma, and invasive pulmonary aspergillosis, an infection of the lower respiratory tract (2–4). *A. fumigatus* airway colonization accelerates chronic lung allograft dysfunction, the leading cause of death among lung transplant recipients, and the mortality from invasive pulmonary aspergillosis remains as high as 50% (5–7). An increased burden of *A. fumigatus* disease in lung transplant patients is likely caused by (i) constant graft exposure to the pathogen, (ii) lack of a cough reflex, and (iii) use of immunosuppressive therapy (8, 9). Because these factors are common to all lung transplant patients, there is a great need to increase the field's understanding of modifiable risk factors that contribute to potentially fatal invasive disease.

We developed an airway transplantation mouse model involving an orthotopic tracheal transplant. In this model, visualization of blood vessels is facilitated by their display in two dimensions, unlike the complex three-dimensional vasculature structure in the terminal airways (10). Using this model, we have previously shown that transplanted tracheas undergo a series of distinct microvascular events as a result of acute allograft rejection. These include reanastomosis of the donor with recipient vessels on day 4 posttransplant (11), followed on day 8 posttransplant by progressive ischemia caused by alloreactive CD4⁺ T cells and complement-mediated rejection (11–13). Alloimmune-mediated graft ischemia increased the aggressive growth of *A. fumigatus*, with a disease mimicking the invasive

airway anastomotic infections observed clinically in lung transplant patients (13, 14). However, the mechanisms whereby alloimmunity contributes to this increased virulence remain poorly understood. Following on from previous findings that the iron content in the lungs of transplant recipients increases with the number of acute rejection episodes, the current study was designed to examine whether pathogen invasion is related to the increased quantity of iron in the graft (15–17). Here, we report that alloimmune-related microhemorrhage increases graft iron content, triggering a switch in the *A. fumigatus* growth pattern from one typical of colonization into one more characteristic of invasion; in this manner, the allograft itself can uniquely contribute to the virulence of the pathogen.

RESULTS

Alloimmune-mediated rejection causes microhemorrhage in airway transplants in mice

To examine the effect of acute rejection on microvascular perfusion and leakiness in airway transplants, murine syngeneic and allogeneic airway grafts were studied by Doppler flowmetry and fluorescein isothiocyanate (FITC)–lectin perfusion on days 4 to 10 posttransplant (11–13, 18–20). Doppler flowmetry (measured in blood perfusion units) showed a time-dependent decline in perfusion for allografts, whereas perfusion in syngrafts remained relatively stable (Fig. 1A). Images of FITC-lectin perfusion (Fig. 1, B and C) corroborated the Doppler flowmetry results. To evaluate vascular leakiness, we injected fluorescent microspheres after FITC-lectin infusion. Between days 6 and 8 postallotransplant, there was a time-dependent increase in microsphere extravasation (0.05 μ m diameter) indicative of vascular permeability (Fig. 1, B and D), but such changes were not seen in the concurrently studied syngrafts (Fig. 1, A to D). Allograft microvascular permeability was confirmed by transmission electron microscopy with progressive erythrocyte extravasation seen on days 8 to 10 posttransplant (Fig. 1E), culminating in blood lakes seen in the interstitium of the allograft on day 10 posttransplant (Fig. 1E, right). Tissue oxygenation (PO_2 , mmHg), measured by Oxyprobe, progressively declined in the allografts beginning on day 8 posttransplant but not in the syngrafts studied in parallel (Fig. 1F). Collectively, the data indicated that acute rejection damaged the allograft's microvasculature, resulting in tissue hypoxia and bleeding into the airway transplant.

Tissue hemorrhage and dysregulated iron metabolism increase allograft iron content

Because erythrocyte extravasation could increase iron content in the graft, we used inductively coupled plasma mass spectrometry (ICP-MS) to measure the iron concentration in transplanted tissues. The iron content in the allograft increased as acute rejection progressed, reaching an 18-fold higher concentration than in the nontransplanted control mice (Fig. 2A) or in the syngrafts studied on day 12 posttransplant (fig. S1). To determine the primary form of the iron in the allograft, we stained serial histologic sections with Perl's Prussian blue stain for ferric iron and Turnbull's stain for ferrous iron. As depicted in Fig. 2B for the day 12 posttransplant allografts, ferric iron predominated during acute microvascular rejection; immunofluorescent staining for ferritin, a protein that binds to ferrous iron and stores it in the ferric state, showed both intracellular and extracellular deposition within the allograft transplant compared to nontransplanted mice (Fig. 2C).

To evaluate the potential contribution of host pathways involved in iron sequestration from pathogens, we used reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) to measure the expression of hepcidin, natural resistance-associated macrophage protein 1 (nRAMP1), ferroportin (FPN), transferrin receptor (TfR), and ferritin (21). Hepcidin is an iron regulatory protein that is expressed during iron excess and inflammation but is down-regulated during hypoxia; nRAMP1 governs hemoglobin iron recycling in macrophages (22). Both nRAMP1 and hepcidin play a key role in the sequestration of iron from intracellular and extracellular pathogens, respectively (21). Hepcidin, ferritin, and nRAMP1 expression in the allograft increased relative to the untransplanted control, peaking on day 8 posttransplant and declining after that (Fig. 2D, $P < 0.05$) (23, 24). Accordingly, the down-regulation of these proteins on day 8 posttransplant may reflect alloimmune-mediated dysregulation of important host iron metabolic pathways (22, 25). Consistent with this interpretation, a direct comparison of day 12 allografts and syngrafts indicated that syngrafts had significantly ($P < 0.05$) higher expression of hepcidin and nRAMP1 than did the allografts (Fig. 2E and fig. S2). Expression of FPN, TfR, and ferritin was similar in allografts and syngrafts (fig. S2). Collectively, the data indicate that acute rejection impairs the ability of the host to sequester and recycle iron, a mechanism potentially responsible for increasing graft iron.

Iron increases *A. fumigatus* metabolism and affects its growth in culture

Having seen that iron was increased in the allotransplants undergoing rejection, we studied the effect of a high iron concentration on *A. fumigatus* metabolism and growth in culture. In the first series of experiments, we examined the effect of iron dextran (0.3 mg/ml) and bovine hemoglobin (0.1 g/ml) on *A. fumigatus* metabolism, using the XTT [2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide] assay. This assay measures the ability of viable fungal cells to cleave the tetrazolium salt, XTT, to a formazan dye, which is quantified colorimetrically (26). The concentration of iron used in these experiments replicates a murine hematocrit of 30%, reflecting tissue microhemorrhage (27). The fungal metabolic activity was significantly ($P < 0.05$) higher in the presence of hemoglobin and iron dextran relative to RPMI culture medium (control) (Fig. 3A), suggesting that free iron released from the breakdown of senescent red blood cells, or hemoglobin itself, supported *A. fumigatus* metabolism.

To better understand the role of fungal iron metabolism genes on *A. fumigatus* growth, the second series of experiments used fungal mutants lacking iron metabolism genes that were derived from the *A. fumigatus* strain AF77, which was the wild-type (WT) strain used. These mutants included the following: *hapX*, which lacks *hapX*, a gene that is required for adaptation to iron starvation through repression of iron-dependent pathways (28); *sreA*, which lacks the *sreA* gene encoding a repressor of iron uptake during high-iron conditions (29); and the double-knockout iron-intolerant mutant strain *sreA/cccA*, which lacks both *sreA* and *cccA*, a gene that is critical for *A. fumigatus* iron detoxification (30). The mutant and WT molds were plated on iron-replete medium (potato dextrose agar) or iron-deficient agar (31), and the growth area (in square millimeters) was measured at 24 hours postseeding. The iron conditions did not affect the growth of the WT strain (Fig. 3B). By contrast, both the *sreA* and the *sreA/cccA* mutants exhibited minimal growth under iron-replete

conditions, whereas growth was similar to that of the WT mold in the iron-deficient agar (Fig. 3B, $P < 0.0001$ and $P = 0.013$, respectively). The opposite was seen for the *hapX* mutant, which grew on potato dextrose agar but exhibited minimal growth on iron-deficient agar (Fig. 3B, $P = 0.001$). Collectively, the data confirm that iron metabolism genes (*sreA*, *hapX*, and *cccA*) are critical for the growth of *A. fumigatus* and its ability to adapt to changing iron conditions.

To better understand the effect of iron on the rate of *A. fumigatus* growth, we measured the hyphal growth area at 24, 40, and 48 hours postseeding of *sreA/cccA*, *hapX*, and WT molds suspended in iron dextran or RPMI culture medium alone (control). In these experiments, the *sreA/cccA* and *hapX* mutants serve as negative and positive controls, respectively. The relative difference in growth rate between the iron dextran-treated and untreated conidia (fungal spores) is shown in Fig. 3C. Iron supplementation did not alter the growth of the WT *A. fumigatus*. However, it inhibited the growth rate of the iron-intolerant mutant *sreA/cccA* and increased the growth rate of the *hapX* mutant, with the most striking differences seen at 24 hours postseeding (Fig. 3C, $P < 0.00001$).

To elucidate the role of iron in *A. fumigatus* growth tropism, we studied the WT strain (CEA10) and the *sreA* mutant (CEA10 background) in a two-well plate assay containing phosphate-buffered saline (PBS) (control) or iron dextran (schematically represented in fig. S3A). Relative to the iron dextran well, the WT strain displayed a convex growth morphology at 24 hours in culture (positive tropism, yellow line) (Fig. 3D, top), whereas the *sreA* mutant showed a concave growth pattern (negative tropism) (Fig. 3D, bottom). To better evaluate the positive iron tropism of the WT *A. fumigatus*, we used glass barriers to direct hyphal growth linearly and placed wells perpendicularly (fig. S3B). Wide-angle ($>45^\circ$) branching, indicative of an aberrant *Aspergillus* hyphal growth pattern (32), was observed in the WT strain, and it was consistently directed toward the iron dextran well (Fig. 3E). This growth pattern was not observed to be directed toward the well containing PBS (fig. S4). The data suggested that iron is a tropism stimulus for *A. fumigatus*.

Tissue iron promotes *A. fumigatus* invasion in the orthotopic tracheal transplant mouse model

Having seen that iron is a potent growth factor and an apparent tropism stimulus for *A. fumigatus* in culture, the next series of experiments sought to determine the role of iron in promoting *A. fumigatus* allograft invasion in the tracheal transplantation mouse model. The experimental approach involved a single intratracheal administration of *A. fumigatus* (strain 10AF) on day 3, day 5, or day 9 posttransplant and evaluation of invasive fungal disease on day 3 postinfection (fig. S5). In the first series of experiments, the depth of fungal invasion and degree of fungal burden were graded histologically over time posttransplant, as previously described (fig. S6) (13). The invasion correlated with increased tissue iron content as quantified by standard morphometric measurements with Prussian blue staining (33). The data summarized in Fig. 4 (A and B) indicate that the depth of *A. fumigatus* invasion increased with time (days 6 to 12) posttransplant and was associated with a time-dependent increase in tissue iron deposition. Given the progressive posttransplant increase in the iron quantity seen histologically and by ICP-MS, we defined three alloimmune-mediated

iron groups as (i) low iron, days 3 to 6 posttransplant; (ii) medium iron, days 5 to 8 posttransplant; and (iii) high iron, days 9 to 12 posttransplant.

In the second series of experiments to evaluate the role of iron in promoting *A. fumigatus* invasive growth, allotransplant murine recipients (day 8, medium iron group) were given intraperitoneal injections of PBS (control), or the iron chelators deferoxamine (DFO) or deferriox (DFX). These chelators were selected because DFO is an *A. fumigatus* xenosiderophore, which means that the mold is capable of acquiring iron from the DFO-iron complex, whereas DFX is a non-xenosiderophore iron chelator, which means that the mold cannot access iron from the DFX-iron complex (31, 34–36). *Aspergillus* invasion was significantly ($P < 0.05$) increased in DFO-treated compared to DFX-treated transplant recipients, where the mold displayed a less invasive phenotype (Fig. 4C) (37–39).

The third series of experiments examined the ability of iron to increase the depth of *A. fumigatus* infection when using hemochromatosis gene knockout mice (*Hfe*^{-/-}) as transplant donors. These mice were selected because they are characterized by iron overload in multiple tissues, including the airways, due to an increased uptake of iron from their diet (40). *Aspergillus* was significantly ($P < 0.05$) more invasive in day 8 syngrafts [B6(*Hfe*^{-/-}) → B6(^{WT}), Fig. 4D] and allografts [B6(*Hfe*^{-/-}) → BALB/c, Fig. 4E] from *Hfe*^{-/-} donors than in syngrafts [B6(^{WT}) → B6(^{WT})] and day 8 allografts [B6(^{WT}) → BALB/c] from a WT donor. Collectively, the data indicate that (i) graft iron overload is a potent risk factor for *A. fumigatus* invasion in the orthotopic tracheal transplantation mouse model and (ii) targeting fungal iron acquisition through non-xenosiderophore iron chelation could lessen pathogen virulence.

Having seen that fungal iron metabolism genes were critical for *A. fumigatus* growth in culture, we hypothesized that mutants with severe iron intolerance would be less invasive in the orthotopic tracheal transplant model during high allograft iron conditions. The depth of fungal invasion was examined for day 12 allotransplants infected with the WT strain (AF77) or the following mutant strains: *hapX*, *sreA*, or *sreA/cccA*. The data summarized in Fig. 4F show that under iron-overload conditions, the iron-intolerant mutant (*sreA/cccA*) was significantly ($P < 0.05$) less invasive than both the WT and the *sreA* molds. Invasion for the *hapX* mutant was similar to that for the WT strain. The data indicate that knockout of both *sreA* and *cccA* in *A. fumigatus* was required to lessen fungal invasion.

Because of the small diameter of the murine trachea, iron-induced fungal overgrowth in the orthotopic tracheal transplant model could potentially have promoted invasion through hyphal compression into the transplanted tissue by an increased mycelial mass. Having observed that iron increased fungal metabolism and growth in culture, we also quantified the fungal burden, because it may be a confounding factor in this mouse model. Fungal burden, defined as the luminal area occluded by fungal elements, was graded histologically for each experimental group shown in Fig. 4 (A to F) (13). The data summarized in fig. S7 indicate that fungal burden correlated with invasion for only two comparison groups: (i) day 12 compared to day 6 allotransplants and (ii) *sreA*-compared to *sreA/cccA*-infected allotransplants. In all other groups, fungal burden and invasion were poorly correlated,

suggesting that fungal overgrowth was not a confounding variable to explain *A. fumigatus* invasion in the tracheal transplantation model.

Exogenous iron stimulates *A. fumigatus* invasion in syngrafts

To confirm that iron plays a significant role in fungal invasion, we examined the depth of *A. fumigatus* invasion in syngrafts topically treated with iron sulfate (FeSO_4) or vehicle solutions, because it related to Doppler flowmetry perfusion and tissue PO_2 . *A. fumigatus* was deeply invasive in syngrafts treated with an FeSO_4 solution. By contrast, in syngrafts treated with vehicle solution, the mold displayed a colonization phenotype with a large fungal burden in the airway and no histologic evidence of invasion in serial tracheal sections (Fig. 5, A and B). Iron deposits occurred in the mucosal, sub-mucosal, and cartilaginous layers of the tracheal wall of the FeSO_4 -treated syngrafts (Fig. 5C, top), whereas, in syngrafts treated with vehicle, iron deposits were rarely found, and they were primarily located at the airway anastomosis (Fig. 5C, bottom). Moreover, differences were not seen between the syngeneic treatment groups regarding Doppler flowmetry perfusion (Fig. 5D) or tissue PO_2 (Fig. 5E). *Aspergillus* was also consistently more invasive in the FeSO_4 -treated allotransplants (positive control) (fig. S8). Collectively, the data suggest that the differences in fungal invasion between FeSO_4 -treated and control transplants were attributable to tissue iron content rather than differences in graft perfusion or tissue hypoxia.

Acute rejection is characterized by a proinflammatory macrophage phenotype

The immune system is critical for host control of invasive mold infections, as evidenced by the high rate of invasive pulmonary aspergillosis in patients with prolonged neutropenia (41), and both iron and microvascular ischemia may impair the immune response (42, 43). To examine the effect of iron on the immune system, we stained day 12 allotransplants treated with a topical solution applied at the time of transplantation, containing FeSO_4 or vehicle (control), with antibodies to proteins that specifically identify immune effector cells including neutrophils (Ly6G^+), dendritic cells (DCs) (CD11c^+), B cells (CD45R^+), and CD4 and CD8 T cells. For each trachea, the number of stained cells was counted in 10 high-power fields, and the results were expressed as mean positive staining cells per trachea. Syngrafts were studied in parallel and served as controls. The numbers of all of these immune effector cells were higher in the poorly perfused allografts than in the well-perfused syngrafts (fig. S9, A to F). The number of cells staining with F4/80 antibody (macrophages) was significantly ($P < 0.05$) higher in the FeSO_4 solution-treated compared to the control solution-treated syngrafts (Fig. 6, A and B). The data suggested that (i) alloimmune-mediated microvascular damage and iron overload did not impair immune effector cell accumulation in the mouse tracheal transplant and (ii) iron and alloimmunity increased the number of macrophages in the tracheal transplant.

To further characterize the innate immune response within the allograft, we used flow cytometry to identify the number of neutrophils, DCs, and macrophages in tracheal samples from day 12 allotransplants compared with syngrafts. The common leukocyte antigen (CD45) was used to define granulocyte and monocyte populations. Confirming the results of the immunohistochemistry studies, a large number of neutrophils were identified in allotransplants as defined by the expression of CD11b and Ly6G but not F4/80 (fig. S9G).

By contrast, there was a relative lack of DCs in both allotransplants and syngeneic transplants, as determined by the expression of CD11b, CD11c, and MHC II (major histocompatibility class II) and the absence of Ly6G (fig. S9H). Macrophages were identified on the basis of the expression of CD11b, CD11c, and F4/80 (Fig. 6C), and they were then re-gated using CD206 antibody to differentiate between M1 (classically activated) and M2 (alternatively activated) macrophage subpopulations (Fig. 6D). Two distinct macrophage populations were identified in allotransplants relative to syngeneic transplants with an increase in classically activated macrophages (M1 phenotype: 22% versus 4%, respectively) (Fig. 6D). Collectively, the data suggested that, during acute rejection, (i) the innate immune system was intact in allotransplants and (ii) macrophages were activated, displaying a proinflammatory phenotype.

Airway tissue iron is increased in lung transplant patients

To confirm the applicability of our findings from the mouse tracheal transplant model to human lung transplant recipients, we collected endobronchial biopsies superior (recipient side) and inferior (lung transplant side) to the transplanted lung airway anastomosis in persons >6 weeks posttransplant ($n = 5$; schematically represented in Fig. 7A). Endobronchial biopsy specimens were serially sectioned and examined, using Prussian blue iron stain (Fig. 7, B and C). For each biopsy, the number of stained cells was counted in 10 histologic sections, and the results were expressed as mean Prussian blue–positive cells per biopsy. Biopsies from transplanted lungs showed a higher number of cells staining positive for iron compared to paired biopsies from the patient's nontransplanted native airway (Fig. 7, B to D, and Table 1). Recapitulating findings from previous studies, iron was also present in the bronchoalveolar lavage fluid from these patients, as determined by ICP-MS (Table 1). These data support the hypothesis that higher iron in the airway graft promotes *A. fumigatus* infection in patients after lung transplantation.

DISCUSSION

The salient feature of the data presented in this report is the finding that acute rejection–mediated microhemorrhage leads to tissue iron overload, triggering a switch in *A. fumigatus* growth from a colonization to an invasion phenotype (schematically modeled in fig. S10). Lung transplantation is increasingly used to treat end-stage lung diseases. The annual number of lung transplants markedly increased from 32 in 1987 to more than 3800 in 2013 (1). Unfortunately, the current median survival is only 5.7 years posttransplant, and death is often due to infectious disease complications (1). *Aspergillus*-related pulmonary diseases are estimated to occur in up to 30% of lung transplant recipients (44–46). The majority of disease is caused by *A. fumigatus* (60 to 73%), followed by *Aspergillus flavus* (7 to 10%) and *Aspergillus niger* (6 to 9%) (47). *Aspergillus*-related pulmonary disease in lung transplant patients is caused by fungal colonization (68 to 79%) or tissue invasion (20 to 42%) (7, 46). Colonization, although not immediately life-threatening, results in severe asthma or the development of aspergillomas (a contained fungal ball), or it accelerates chronic lung allograft dysfunction (48). Tissue invasion is a more virulent presentation that often results in fatal airway anastomotic infections and invasive pulmonary aspergillosis in the lower respiratory tract (9, 14, 46, 49). We have previously shown that *A. fumigatus*

growth is mostly invasive in progressive allograft rejection, whereas the infection results in airway colonization in syngrafts (13). Because all lung transplant recipients receive immunosuppressive therapy, however, immune suppression cannot explain on its own the switch from fungal colonization to invasive growth, and the responsible factors remain poorly understood.

Iron is a vital nutrient for nearly all living organisms. Iron, in excess, has been associated with increased virulence of *Yersinia enterocolitica*, *Vibrio vulnificus*, *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Salmonella typhi*, and the invasive mold *Rhizopus oryzae* (50–54). Our interest in the role of iron overload in *A. fumigatus* virulence was stimulated by previous reports of an increased incidence of invasive mold infections in patients with liver cirrhosis and in bone marrow and liver transplant recipients with iron overload (55, 56). In addition, patients with chronic lung allograft dysfunction have increased ferritin concentrations in their bronchoalveolar lavage fluid (15, 17). Here, we found that (i) rejection-mediated microhemorrhage resulted in a time-dependent increase in the iron concentration in the graft, (ii) iron was a potent stimulus for *A. fumigatus* growth both in culture and in vivo, and (iii) hemoglobin was also a stimulus for *A. fumigatus* metabolism. Whereas previous studies had shown that both *A. nidulans* and *A. fumigatus* are unable to use hemoglobin or heme as an iron source (52, 57), we used a 100-fold higher concentration of hemoglobin (2 mM versus 10 μ M) to replicate the amount of iron seen during tissue bleeding (52). However, we do not exclude the possibility that *A. fumigatus* may be able to use heme as an alternative nutrient source, as hemin supplementation was shown to rescue defective heme biosynthesis in *A. niger* (58).

Iron contributes to *A. fumigatus* growth both in culture and in the orthotopic tracheal transplantation mouse model. We term this response “ferrotropism,” in contrast to the behavior of *sreA* mutants, which grow away from iron (or negative tropism). We hypothesized that the *sreA* mutant was exhibiting negative tropism to avoid iron toxicity (30). Here, iron played a determinant role in the switch from colonization to invasive growth of *A. fumigatus*, a pattern characteristically seen in patients with lung transplant rejection. In syngrafts, the mold displayed a colonization growth phenotype associated with rare tissue iron deposits. By contrast, the concentration of iron was increased in the transplanted trachea of recipient mice as a result of alloimmune-mediated microhemorrhage, and it contributed to the invasive growth as documented by gain- and loss-of-function experiments. Specifically, the mean depth of invasion progressively increased in (i) allografts beginning at day 8 posttransplantation, (ii) iron-rich *Hfe*^{-/-} donor tracheal grafts, (iii) allograft recipients treated with the xenosiderophore chelator DFO, and (iv) syngrafts treated with exogenous FeSO₄. However, the depth of invasion decreased in allograft recipients treated with the non-xenosiderophore iron chelator DFX and when animals were infected with the severely iron-intolerant *A. fumigatus sreA/cccA* null mutant.

The expression of several iron metabolism genes including hepcidin and nRAMP1 was markedly up-regulated in the day 8 allografts and declined thereafter, suggesting that a factor related to acute rejection down-regulates their expression. Although the genes that are potentially involved in the iron response and their respective contributions to *A. fumigatus* invasion are still unknown, our findings highlight the complexity of iron-associated

metabolic pathways in alloimmune-mediated graft rejection. Consistent with previous findings that hepcidin expression is up-regulated by iron excess and inflammation but is down-regulated during hypoxia (51, 59), we found that relative to syngrafts, hepcidin expression in the allograft peaked in the moderately perfused day 8 transplants but decreased during progressive hypoxia at day 12 posttransplantation. Because hepcidin functions to sequester iron from invading pathogens and has potent antimicrobial activity (59), the data suggest that hepcidin down-regulation may contribute to *A. fumigatus* invasive growth. Also congruent with a previous finding that decreased nRAMP1 activity results in inefficient iron recycling leading to a buildup of ferritin within macrophages (22), we found that nRAMP1 was down-regulated in allografts compared to syngrafts and that high amounts of ferritin were seen in allotransplants likely resulting from the inefficient processing of extravasated erythrocytes.

Furthermore, our data suggest that macrophages, which are essential for erythrophagocytosis and are known to kill *Aspergillus* conidia (41), were dysfunctional in iron overload and potentially contributed to tissue damage through oxidative stress (43). Consistent with this, flow cytometry analysis indicated that the macrophages in day 12 allotransplants displayed a proinflammatory phenotype. Similar changes in macrophage phenotype have been previously described in association with both acute transplant rejection and tissue iron overload (43, 60). Thus, the alloimmune-mediated dysregulation of iron metabolic pathways likely contributes to transplant iron overload, macrophage induced-proinflammatory stress, and an increased risk of *A. fumigatus* invasion.

SreA is an *A. fumigatus* transcriptional regulator that represses iron uptake under iron-overload conditions and promotes iron-consuming pathways (29). Supporting the conclusion that iron acts directly on *A. fumigatus*, triggering a switch to an invasive phenotype in the mouse tracheal transplant model, we found that the *A. fumigatus* mutant deleted in the *sreA* gene exhibited an invasive phenotype. Accordingly, a mutation that increased fungal iron uptake was associated with increased virulence. Consistent with previous findings that the *sreA* mutant adapts to increased iron uptake by up-regulating the iron detoxification gene *cccA* (30), we found that the severely iron-intolerant double-knockout *sreA/cccA* mutant was less invasive than the WT *A. fumigatus* strain in the day 12 allograft. The growth of the *hapX* mutant was similar to that of the WT, lending further support to the conclusion that, in acute rejection, the environmental niche is iron-sufficient.

Immune dysregulation is an established risk factor for *Aspergillus* infection with an increased risk of invasive disease seen in individuals with chronic granulomatous disease (a genetic immunodeficiency), persons with hematologic malignancies, and solid organ and hematopoietic cell transplant patients (41). Following on from previous observations that both iron overload and microvascular damage contribute to immune dysregulation (42, 43), we used immunohistochemistry and flow cytometry to understand the impact of alloimmune-mediated microvascular damage on fungal invasion in the orthotopic tracheal transplantation mouse model. The data indicated that the immune effector cell numbers were not affected in the transplant due to microvascular ischemia or iron overload, suggesting that at least within the examined parameters, alloimmune-mediated immune dysregulation does not appear to be involved in increased fungal invasion.

Limitations of the current study include an incomplete in vivo characterization of ferrotropism, resulting from technical difficulties that prevented tissue iron colocalization with fungal invasion. Specifically, to evaluate the highest grade of fungal invasion, histologic sections were cut throughout the tracheal graft and stained for Grocott's methenamine silver stain; the tissue sections were not amenable to concomitant staining for iron and fungal elements. Another limitation is that *A. fumigatus* infections often occur in the human lower respiratory tract, whereas the orthotopic tracheal transplantation mouse model only evaluates the trachea. However, this model does replicate *A. fumigatus* invasive airway anastomotic infections observed in lung transplant recipients. The translational relevance of the pre-clinical findings was confirmed by an increased iron content in human allograft endobronchial tissue and transplant bronchoalveolar lavage fluid. The orthotopic tracheal transplantation model is particularly useful to address the role of iron in allograft invasion because the iron concentration of the transplant can be modulated at the time of surgery and because the planar anatomy of the trachea favors an accurate quantitation of fungal invasion (12, 13, 18, 61, 62).

Implicit in the interpretation that high iron contributes to *A. fumigatus* virulence in alloimmune-mediated rejection is the conclusion that new treatments to lower allograft iron or to prevent microvascular damage during acute rejection could be clinically impactful, reducing the burden of these anastomotic infections in lung transplantation. Overall, our studies show that the availability of iron in the tracheal graft tissues influences the invasiveness of *A. fumigatus* and that this invasiveness could potentially be modulated or subverted by treatment with DFX. In this context, it should be mentioned that systemically delivered iron chelators previously showed promise in the treatment of mucormycosis and aspergillosis in animal models but unfortunately increased mortality in a small clinical trial (31, 34, 35). Systemic chelation carries the risk of hepatotoxicity, acute renal failure, and agranulocytosis (63). Delivery of a non-xenosiderophore iron chelator, such as DFX, directly to the lungs through inhaled medications or topically at the time of transplantation could provide a safer therapeutic approach (12).

MATERIALS AND METHODS

Study design

The objective of this study was to define new risk factors in lung transplantation that determine the invasive growth of *A. fumigatus*. Using a murine orthotopic tracheal transplantation model, we studied the microvascular damage that occurs during acute rejection and found that allograft iron increased as a result of tissue hemorrhage. Using *A. fumigatus* iron metabolism mutants and WT strains in culture, we found that iron increased fungal metabolism and determined the direction of hyphal growth. In the tracheal transplantation model, iron overload promoted an invasion phenotype. Iron was manipulated in this mouse model through transplants using (i) tissue iron-rich *Hfe*^{-/-} donors, (ii) allografts treated with the xenosiderophore chelator DFO or the non-xenosiderophore chelator DFX, and (iii) transplants treated with exogenous FeSO₄ solution. The experimental design of the animal models used in this study is depicted in fig. S5. The sample size of the orthotopic tracheal transplantation model experiments was based on our previous experience

with this animal model. In animal studies, researchers were blinded to the experimental group in evaluating histology sections. We assessed the clinical relevance of our hypothesis in human lung transplant recipients and found consistently positive tissue iron staining in transplanted airway biopsies compared to the patient's native airway. The size of the clinical study was based on a convenience sample of patients ($n = 5$) receiving a bronchoscopy as part of their routine clinical care. Figure legends include details of replicate experiments. No data were excluded from this study.

Study approval

All animal studies were performed with the approval of the Veterans' Affairs Palo Alto Health Care System's Institutional Animal Care and Use Committee (protocol number NIM1483). The Stanford University Applied Panel on Biosafety approved all microbiological procedures (protocol number 1007-MN0312). The Institutional Review Board (IRB protocol number 17020) at Stanford University approved the human study, and lung transplant recipients gave written consent to participate in this study.

Orthotopic tracheal transplantation model

Five-week-old male C57BL/6, BALB/c, C57BL/6^{Hfetm1Gln} mice were purchased from the Jackson Laboratory. Mice were randomly assigned to groups that consisted of 3 transplanted mice in all experiments. The orthotopic tracheal transplantation model was used, as previously described (12–13, 18, 19). For iron chelator experiments, animals were injected intraperitoneally with DFX (150 mg/kg), DFO (150 mg/kg), or 1× PBS on days 1, 3, 5, and 7 posttransplant. A topical solution containing FeSO₄ or vehicle control was applied before transplantation by immersing the excised tracheal graft in the solution (30 s). Animals were inoculated intratracheally with *A. fumigatus* on day 3, day 5, or day 9 posttransplant, after which all infected animals received a single dose of corticosteroid (1 mg of triamcinolone acetonide) subcutaneously (12, 13, 18, 20). This dose replicates the induction dose for a glucocorticoid suppression model (64). Hemorrhage and iron overload were observed in both corticosteroid-treated and untreated animals. Empirically, we have shown that triamcinolone administration at the time of inoculation with *A. fumigatus* increases the probability of infection occurring in transplanted animals. We chose to limit the amount of immune suppression in these animals, as immune suppression itself is a potent driver for *Aspergillus* invasion. This method facilitated the study of additional risk factors for invasion. At day 3 postinfection, all surviving mice were euthanatized using CO₂ asphyxia. The design of animal experiments is schematically represented in fig. S5.

Preparation of *A. fumigatus* conidia

D. Stevens provided the *A. fumigatus* strain 10AF (American Type Culture Collection 90240) used in the tracheal transplant mouse model. H. Haas provided the mutant strains *hapX*, *sreA*, and *sreA* *cccA* (all on an AF77 background) and the WT strain (AF77) used in in vivo and in vitro studies. R. Cramer provided the WT (CEA10) and *sreA* mutant (on a CEA10 background) used in the tropism experiments. Cultures were revived from long-term storage at −80°C and grown on PDA with conidia harvested and suspensions made, as previously described (13). In vivo experiments used 10⁸ conidia/ml for each isolate (13). For in vitro experiments, conidial iron stores were depleted by growth at 37°C on PDA

plates containing 1 mM ascorbate (Sigma-Aldrich) and 1 mM ferrozine (Sigma-Aldrich) for 4 days (31). For the XTT experiments, a conidial inoculum (10^5 /ml) was prepared using a spectrophotometer. For culture plate assays, conidial suspensions were diluted to 10^4 to 10^8 conidia/ml.

Measurement of microvascular perfusion and vascular leakiness

Microvascular perfusion, tissue PO_2 , and FITC-lectin perfusion were determined, as previously described (12, 13, 18–20). In brief, microvascular perfusion was determined using a laser Doppler flowmetry probe on the anterior membranous portion between tracheal rings (12, 13, 18–20). Tissue PO_2 was measured by Oxyprobe, as described elsewhere (13, 18–20). FITC-lectin perfusion studies were performed to confirm whether vessels were functional and continuous with the systemic circulation (11, 13, 18–20). For microvascular permeability, we instilled 100 μ l of fluorescent microbeads (0.05 μ m diameter, Thermo Fisher Scientific) into the inferior vena cava after FITC-lectin infusion. Microbead extravasation into the surrounding tissue was used to measure the degree of vascular permeability, whereas FITC-lectin staining represents the vascular architecture.

XTT assay

Comparative metabolism of *A. fumigatus* in RPMI, iron dextran (0.3 mg/ml, Sigma-Aldrich), or bovine hemoglobin (0.1 g/ml, Sigma-Aldrich) was examined by the XTT assay, as previously described (26). For additional details, please see the Supplementary Materials.

Fungal tropism

Tropism studies were performed using plates schematically represented in fig. S3. Cultures were grown at 37°C on PDA plates containing 1 mM ascorbate (Sigma-Aldrich) and 1 mM ferrozine (Sigma-Aldrich) (31). Conidial suspensions (10^6 conidia/ml: WT or *sreA* null mutant *A. fumigatus* strains) were spotted on a 2-mm filter paper and grown (48 hours) (fig. S3A). Iron dextran (40 μ l, at a concentration of 100 mg/ml, Sigma-Aldrich) was used in the treatment well. We used 1 $\times$ PBS (40 μ l) in the control wells. For positive tropism studies, conidial suspensions were spotted on filter paper, and glass slide coverslips were placed to the right and left of the filter paper (fig. S3B). The area of interest was defined by examination with a surgical microscope before slide preparation (65). Slides were prepared at 2-hour intervals (16 to 24 hours postinoculation) fixed with 4% paraformaldehyde in 1 $\times$ PBS, dried (48 hours) at room temperature, and imaged by light microscopy (magnification, $\times 5$ to $\times 40$). Positive tropism was defined as growth with wide-angle branching ($>45^\circ$) with hyphae deviating toward the treatment or control well. *A. fumigatus* is classically defined as having narrow-angle branching (which differentiates *A. fumigatus* from zygomycetes). Negative tropism was defined as hyphal tip growth away from the treatment well. Still images were photographed with an Olympus 12–40mm f2.8 lens. Images were transformed using ImageJ software, and hyphal tips were distinguished from the background by measuring a pixel intensity equal to or greater than a threshold value of 50 (range, 0 to 255). Three biologic replicates and three technical replicates were performed for each experiment.

Tissue preparation, grading of fungal invasion, and fungal burden

The extent of fungal invasion was semiquantitatively graded, as previously described (fig. S6) (13, 18). All tracheal samples were cut longitudinally in 5- μ m sections through the entire tracheal segment and stained with Grocott's methenamine silver (Histo-Tec Laboratories). A board-certified anatomic pathologist (R.A.S.), blinded to the experimental group, independently graded representative tracheal sections for depth of fungal invasion. The degree of fungal burden was determined using the following scoring system: 0, no fungal elements; 1, fungal hyphae in less than 25% of the luminal area; 2, hyphae occluding 25 to 49% of the tracheal area; 3, hyphae occluding 50 to 75% of the tracheal area; and 4, greater than 75% occlusion of the tracheal lumen (13).

Preparation of iron sulfate surgical topical solution

Nanoparticle surgical solution was prepared, as previously described (12). To prepare the treatment solution, the active ingredient [FeSO_4 , 38.3 mg in 100 mg of Slow Fe powder (Novartis)] was prepared as a dry powder, and equal amounts of active ingredient and lecithin (48.49% each, by weight) were mixed with a 0.5% aqueous solution of Probumin (3.02% by weight). The control (vehicle only) formulation containing lecithin was prepared by making a suspension of lecithin (94.14% by weight) in a 0.5% aqueous solution of Probumin (5.86% by weight). The liquid suspensions were then lyophilized. The final nanoparticle solution was prepared by mixing the dry powders with a 1:9 (w/v) ratio of 40% propylene glycol in deionized water.

Flow cytometry

Tracheal tissue was harvested from the allotransplants or syngeneic transplants under surgical microscopy and digested with 0.2% collagenase/g of tissue at 37°C for 30 min. Single cells were obtained by pressing the sample through a 100- μ m cell strainer and subjected to flow cytometry with BD LSRFortessa (BD Biosciences). Compensation and data collection were done with BD FACSDiva software (BD Biosciences). After the exclusion of doublets and debris, initial gating was done using the forward-scatter and side-scatter dot plot in combination with CD45 fluorescence to define granulocyte, monocyte, and lymphocyte populations. Macrophages were identified on the basis of the expression of CD11b, CD11c, and F4/80. M1 versus M2 macrophages were identified on the basis of the expression of CD206. DCs were enumerated on the basis of the expression of CD11b, CD11c, and MHC II, and the absence of Ly6G. Neutrophils were identified on the basis of the expression of CD11b and Ly6G but not F4/80. Analyses were performed with FlowJo.

Study of lung transplant recipients and sample preparation

We prospectively identified five lung transplant patients for inclusion in this study. The status of the subjects was at least 6 weeks posttransplantation, and subjects were included on the basis of the need for a bronchoscopy as part of their routine clinical care. Bronchoalveolar lavage fluid was collected before performing endobronchial biopsies by instilling 30 ml of sterile saline as the third or final lavage. A minimum of 10 ml of bronchoalveolar fluid (BAL) was aliquoted for iron measurements, and the remaining fluid was sent for routine microbiologic culture. The BAL was filtered through sterile gauze and

stored at -80°C until it was assessed for iron content by ICP-MS (as described above). We also obtained 2 endobronchial biopsy samples from the airway superior (native) and inferior (transplanted) to the lung transplant airway anastomosis. These samples were fixed in formalin, and the whole sample was sectioned ($5\text{ }\mu\text{m}$) for staining with Prussian blue to detect the presence of iron. Ten histologic sections were evaluated in 4 slide intervals to ensure that the same cell was not counted twice. Positively stained cells were manually counted, and results expressed the mean number of Prussian blue positively stained cells per biopsy. Patient characteristics are shown in Table 1.

Statistics

GraphPad Prism version 7.0c was used for statistical analysis. Differences in blood perfusion units, percent area perfused, and microbead density area between syngrafts and allografts were evaluated using Student's t test. All t tests were two-tailed. Histologic differences in depth of fungal invasion, fungal burden, and Prussian blue staining for iron content in the graft were evaluated by a nonparametric Mann-Whitney U test. For RT-qPCR data, multiple t tests were performed and corrected for multiple comparisons using the Holm-Sidak method for determining significance ($\alpha = 0.05$). Results from the XTT assay were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. Iron mutant growth studies were evaluated by Student's t test. Immunohistochemistry studies were analyzed using two-way ANOVA with Tukey's post hoc test for multiple comparisons. Differences in the number of cells from recipient and donor airways that stained positive for iron were evaluated using a one-tailed Wilcoxon matched-pairs signed rank test. As previous studies have shown that biopsies from transplanted airways have high iron content, a one-tailed test was chosen to maximize power to study the hypothesis that transplanted airways have a higher iron content than native airways (11, 16, 17, 66). Significance values were set at $P < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Alloimmune-mediated rejection causes microhemorrhage in airway transplants
Comparison of allotransplants and syngeneic transplants ($n = 3$ to 6 per group) by day posttransplant for (A) mean blood perfusion units measured by Doppler flowmetry and (B) fluorescein isothiocyanate (FITC)-conjugated lectin perfusion. a.u., arbitrary units. (B) Microbeads (yellow arrows) are extravasated (red) or colocalized (yellow) with microvascular FITC-lectin staining (green). Magnification, $\times 10$. Scale bar, 100 μ m. (C) Mean percent area perfused in the tracheal transplant model. (D) Microbead density area, measured by the percentage of the tracheal transplant with extravasated fluorescent microbeads. Data are means $\pm$ SEM and analyzed by Student's t test. (E) Transmission electron micrograph of transplanted mouse trachea depicts vascular intimal layer (dashed red ellipse) with erythrocyte collections (yellow asterisks). The white arrow indicates an extravasating erythrocyte. Magnification, $\times 4500$. (F) Tissue oxygen tension (PO_2 , mmHg) by day posttransplant in allotransplants and syngeneic transplants ($n = 3$ to 6 per group) measured by Oxyprobe. Data are means $\pm$ SEM and analyzed by Student's t test.

Fig. 2. Acute rejection induces tissue hemorrhage and dysregulates iron metabolism, increasing allograft iron content

(A) Iron concentration measured by inductively coupled plasma mass spectroscopy. Data are means $\pm$ SEM of graft iron content in parts per billion (ppb) per milligram of dry weight of trachea ($n = 9$) shown as a function of day posttransplant and analyzed by Student's t test.

(B) Representative serial sections of day 12 allotransplant mouse trachea stained with Perl's Prussian blue stain for ferric iron and Turnbull's stain for ferrous iron. Turnbull stain positive control is an untransplanted graft treated with ferrous sulfate topical solution. Magnification, $\times 20$. Scale bar, 100 μm .

(C) Representative immunofluorescent staining of day 12 mouse tracheal allotransplant ($n = 3$) with anti-light-chain ferritin antibody (red) compared to untransplanted graft (control). Nuclei are counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue). Magnification, $\times 20$. Scale bar, 100 μm .

(D) Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) for host iron metabolism genes in allotransplants ($n = 3$ to 9) on days 6 to 12 posttransplant compared to untransplanted grafts (control). Data are means $\pm$ SEM and analyzed by multiple t tests with the Holm-Sidak method to correct for multiple comparisons.

(E) RT-qPCR fold change differences for hepcidin and natural resistance-associated macrophage protein 1 (nRAMP1) in day 12 mouse tracheal allotransplants and syntransplants ($n = 5$) relative to untransplanted trachea * $P = 0.0079$, ** $P = 0.04$ analyzed by nonparametric Mann-Whitney U test.

Fig. 3. Iron increases *A. fumigatus* metabolism and affects its growth direction in culture (A) Comparative *A. fumigatus* metabolism [2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide assay] in RPMI culture medium (control) or in the presence of hemoglobin (HgB) or iron dextran ($n = 32$ wells) analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. (B) Growth area (in square millimeters) of *A. fumigatus* WT and *sreA*, *hapX*, and *sreA/cccA* mutants on iron-sufficient (Fe⁺) and iron-deficient (Fe⁻) agar plates ($n = 3$) analyzed by Student's *t* test. (C) Relative difference in growth area of *A. fumigatus* WT and mutant strains in wells containing iron dextran supplemented or unsupplemented RPMI culture medium ($n = 3$) analyzed by Student's *t* test. (D) Representative *A. fumigatus* cultures comparing hyphal growth morphology in WT and *sreA* mutant exposed to iron dextran and phosphate-buffered saline (PBS). Growth morphology (yellow line) is shown relative to the iron dextran well (24 hours). The convex pattern denotes positive tropism for iron; the concave pattern denotes negative tropism. (E) Representative light microscopy image of WT *A. fumigatus* hyphal growth (18 hours). Magnification, $\times 5$. Scale bar, 50 μ m. The inset depicts hyphal tips with wide-angle ($>45^\circ$) branching toward the iron dextran-containing well ($n = 3$).

Fig. 4. Tissue iron promotes *A. fumigatus* invasion in the orthotopic tracheal transplant mouse model

(A) *A. fumigatus* invasion in mouse tracheal allotransplants ($n = 4$ to 9 per group) on days 6 to 12 posttransplant graded using a 0-to-4 histologic scale. Data are means $\pm$ SEM and analyzed by nonparametric Mann-Whitney *U* test. (B) Perl's Prussian blue staining of the iron area (in square micrometers) in mouse tracheal allotransplants ($n = 4$ to 9 per group) on days 6 to 12 posttransplant. Data are means $\pm$ SEM analyzed by nonparametric Mann-Whitney *U* test. (C) Invasion by *A. fumigatus* of day 8 allotransplants ($n = 8$ to 9 per group) after treatment by intraperitoneal injections of PBS (control), DFO (deferoxamine), or DFX (deferrioxamine), analyzed by nonparametric Mann-Whitney *U* test. (D) Invasion by *A. fumigatus* of day 8 syngeneic transplants ($n = 5$ to 9 per group) from hemochromatosis knockout ($Hfe^{-/-}$) donor mice and WT donor mice (control). (E) Invasion by *A. fumigatus* of day 8 allotransplants ($n = 5$ to 9 per group) from $Hfe^{-/-}$ and WT donor mice, analyzed by nonparametric Mann-Whitney *U* test. (F) Invasion of day 12 allotransplants ($n = 5$ to 6 per group) by *A. fumigatus* *hapX*, *sreA*, and *sreA/cccA* mutants or WT *A. fumigatus*, analyzed by nonparametric Mann-Whitney *U* test.

Fig. 5. Exogenous iron stimulates *A. fumigatus* invasion in syngrafts

(A) Representative Grocott's methenamine silver staining of mouse tracheal syngrafts treated with vehicle (control, left) or FeSO_4 topical solution reveals deep invasion by *A. fumigatus* (red arrows) in the FeSO_4 solution-treated graft. Magnification, $\times 20$. Scale bar, 100 μm . (B) Invasion by *A. fumigatus* of day 8 syngrafts ($n = 6$ to 11 per group) treated with topical FeSO_4 solution or vehicle control. Data are means $\pm$ SEM analyzed by nonparametric Mann-Whitney U test. (C) Representative Prussian blue staining of syngrafts ($n = 4$ per group) treated with FeSO_4 topical solution (top) or vehicle control (bottom). Black arrowheads depict tissue iron deposits (blue). Magnification, $\times 20$. Scale bar, 100 μm . (D) Doppler flowmetry perfusion studies comparing blood perfusion units between syngrafts ($n = 4$ per group) treated with FeSO_4 solution or vehicle control, analyzed by Student's t test. (E) Graft tissue oxygen tension (PO_2 , mmHg) comparing vehicle control or FeSO_4 solution-treated syngrafts ($n = 4$ per group). Analyzed by Student's t test. TL, tracheal lumen; C, cartilage ring.

Fig. 6. Acute rejection is characterized by a proinflammatory macrophage phenotype
(A) Mean number of macrophages (F4/80⁺ cells per trachea) in day 12 mouse tracheal allotransplants and syngeneic transplants ($n = 3$ per group) treated with FeSO₄ solution or vehicle control. Data are means $\pm$ SEM analyzed by two-way ANOVA with Tukey's multiple comparison test. **(B)** Representative immunohistochemistry with F4/80 antibody staining of mouse tracheal sections from day 12 allotransplants and syngeneic transplants ($n = 3$ per group) treated with FeSO₄ solution or vehicle control. Magnification, $\times 40$. Scale bar, 50 μ m. **(C)** Representative flow cytometry analysis of mouse tracheal transplants comparing day 12 syngeneic transplants and allotransplants gated on cell surface CD11b and F4/80 expression. **(D)** Representative flow cytometry analysis of mouse tracheal transplants comparing day 12 syngeneic transplants and allotransplants re-gated on the expression of CD206. M1 denotes classically activated macrophages, and M2 denotes alternatively activated macrophages.

Fig. 7. Iron is increased in allograft airways from lung transplant patients

(A) Schematic diagram depicting biopsy strategy for human lung transplant patients, comparing paired samples from nontransplanted (recipient, black) and transplanted (donor, red) airways. (B) Proportion of cells staining positive for iron in biopsy samples from lung transplant patients ($n = 5$) comparing recipient and transplanted airways. Data are means $\pm$ SEM analyzed by Wilcoxon matched-pairs signed rank test. (C) Representative Prussian blue staining of a recipient airway biopsy and (D) a transplant airway biopsy, depicting iron-laden (blue) macrophages. Magnification, $\times 40$. Scale bar, 50 μ m.

Table 1

Iron studies in human lung transplant patients

CF, cystic fibrosis; IPF, idiopathic pulmonary fibrosis; ND, not detected; BAL, bronchoalveolar fluid.

Age (years)	Diagnosis	Time posttransplant (weeks)	# Cells iron stain positive/biopsy		BAL iron content (µg/liter)	Infection	Rejection grade
			Transplant	Recipient			
27	CF	58	22	1	431.4	<i>Pseudomonas aeruginosa</i>	A0
53	IPF	118	149	29	353.4	ND	A0
35	CF	106	54	34	430.5	ND	A1
67	IPF	23	347	40	279.3	<i>P. aeruginosa</i>	A2
21	CF	8	127	23	550.9	<i>Enterobacter cloacae</i>	A1