Hypoxia Promotes Danger-mediated Inflammation via Receptor for Advanced Glycation End Products in Cystic Fibrosis

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***Rationale*: Hypoxia regulates the inflammatory-antiinflammatory balance by the receptor for advanced glycation end products (RAGE), a versatile sensor of damage-associated molecular patterns. The multiligand nature of RAGE places this receptor in the midst of chronic inflammatory diseases.**

***Objectives*: To characterize the impact of the hypoxia-RAGE pathway on pathogenic airway inflammation preventing effective pathogen clearance in cystic fibrosis (CF) and elucidate the potential role of this danger signal in pathogenesis and therapy of lung inflammation.**

***Methods*: We used *in vivo* and *in vitro* models to study the impact of hypoxia on RAGE expression and activity in human and murine CF, the nature of the RAGE ligand, and the impact of RAGE on lung inflammation and antimicrobial resistance in fungal and bacterial pneumonia.**

***Measurements and Main Results*: Sustained expression of RAGE and its ligand S100B was observed in murine lung and human epithelial cells and exerted a proximal role in promoting inflammation in murine and human CF, as revealed by functional studies and analysis of the genetic variability of *AGER* in patients with CF. Both hypoxia and infections contributed to the sustained activation of the S100B-RAGE pathway, being RAGE up-regulated by hypoxia and S100B by infection by Toll-like receptors. Inhibiting the RAGE pathway *in vivo* with soluble (s) RAGE reduced pathogen load and inflammation in experimental CF, whereas sRAGE production was defective in patients with CF.**

***Conclusions*: A causal link between hyperactivation of RAGE and inflammation in CF has been observed, such that targeting pathogenic inflammation alleviated inflammation in CF and measurement of sRAGE levels could be a useful biomarker for RAGE-dependent inflammation in patients with CF.**

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

An exaggerated and ineffective airway inflammation that fails to eradicate pulmonary pathogens is present in cystic fibrosis (CF). Thus, deciphering the cellular and molecular pathways leading to chronic inflammation could lead to preventive antiinflammatory strategies in CF. Hypoxia affects the inflammatory-antiinflammatory balance by up-regulating the receptor for advanced glycation end products (RAGE), a versatile sensor of damage-associated molecular patterns. The multiligand nature of RAGE places this receptor in the midst of chronic lung inflammatory diseases.

What This Study Adds to the Field

This study causally links the hyperactivation of RAGE by hypoxia and the RAGE ligand S100B by infections to inflammation in murine and human CF, thus providing a unifying conceptual framework within which to accommodate the vicious cycle of airways infection and inflammation in CF. Targeting pathogenic inflammation by administration of soluble RAGE alleviated inflammation in murine CF, whereas measurement of soluble RAGE levels could predict RAGE-dependent inflammation in patients with CF.

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In patients with cystic fibrosis (CF), lung disease is the major cause of morbidity and mortality (1, 2). The progressive decline of pulmonary function is caused by a vicious cycle of airways infection and inflammation. The pulmonary immune response

in CF is characterized by an early and nonresolving activation of the innate immune system, which is dysregulated at several levels (3), does not result in enhanced bacterial clearance (4), and plays a pivotal role in the pathogenesis of lung disease in CF (5). This is supported by several studies that have documented an altered balance of inflammatory and antiinflammatory cytokines in CF (6), such that targeting specific inflammatory and antiinflammatory pathways may represent a valid therapeutic strategy in CF (7, 8).

Development of airways hypoxia is a severe complication in patients with CF (9, 10) in which the CF transmembrane conductance regulator (CFTR) further stabilizes the hypoxia-inducible factor (HIF)-1α (11). Cell adaptation to low oxygen levels occurs by changing the transcription and translation of certain genes (12), mainly the HIFs family genes, which are stabilized by hypoxia (13). HIF-1 exists as a heterodimer, consisting of HIF-1α and HIF-1β subunits. HIF-1β is ubiquitously expressed, whereas HIF-1α is found at very low levels under normoxic conditions because of active proteasomal degradation; however, acute exposure to hypoxia (≤1% oxygen) causes increased HIF-1α protein levels and HIF-1α DNA-binding activity (14). HIF-1α is not only a key mediator of adaptation to hypoxia but is also heavily involved in inflammation (15) and T-cell differentiation (16, 17), through its regulation of the metabolic switch to glycolysis, a switch that is intrinsic to myeloid and T-cell survival and function. Thus, HIF-1α promotes Th17 differentiation (16, 17), a finding consistent with the reduction of indoleamine 2,3-dioxygenase (IDO) and IFN-γ activities under hypoxia (18, 19). However, hypoxia is also an evolutionary mechanism aimed to guarantee tissue homeostasis by innate mechanisms while avoiding autoimmunity (20). Thus, it is not surprising that hypoxia enhanced $FoxP3^+$ regulatory T-cells abundance and function to limit tissue damage in conditions of reduced oxygen availability (21). These data indicate that a better understanding of the molecular pathophysiology of hypoxia in CF might lead to strategies for the prevention and/or treatment of hypoxia-mediated lung complications.

One interesting mechanism by which hypoxia may regulate the inflammatory-antiinflammatory balance is the induction of expression of the receptor for advanced glycation end products (RAGE), a versatile sensor of damage-associated molecular patterns (22), that is expressed at high levels in the lung (23) where it plays a major role in both homeostasis and pathology (24). Genome-wide association studies have shown that variants of *AGER*, the gene encoding RAGE, are correlated with lung disease in patients with (25, 26) and without (27, 28) CF. RAGE is a membrane receptor capable of activating several proinflammatory signaling pathways on binding to different ligands (29). It also has an inhibitory decoy receptor, the soluble (s) RAGE, the deficiency of which is linked to heightened inflammation in various chronic pulmonary diseases (30), whereas its administration showed therapeutic potential in lung injury (31). Thus, RAGE contributes to lung diseases such that measurement of sRAGE levels could be a useful biomarker for RAGE-dependent pathology in the lung (32–34), whereas targeting RAGE signaling is likely to be important in the therapeutic alleviation of lung injury and associated persistent inflammation.

In this study, we tested the hypothesis that hypoxia may contribute to RAGE-dependent lung pathology in CF. Specifically, we assessed whether hypoxia is an up-stream regulator of RAGE in experimental and clinical CF and whether targeting hypoxia and/or RAGE is a drugable strategy in CF. We resorted to *Cftr tm1 Unc* ($Cftr^{-/-}$) mice, reported to mimic, to some extent, human CF (7) and patients with CF to verify that hypoxia may lead to unopposed RAGE activation in response to *Aspergillus fumigatus*, a common pathogen in CF (35), to the pathogenesis of which adaptation to hypoxia (36) and hyperfunction of RAGE contribute both in mice (37) and humans (38). We also evaluated RAGE activation in infection by *Pseudomonas aeruginosa* that, of interest, is known to induce HIF-1α stabilization (39). We demonstrated that RAGE is up-regulated in murine and human CF by hypoxia and is associated with lung disease severity in infections. The hypoxic pathway does not operate in isolation, but rather in concert with the Toll-like receptor (TLR) promoting the production of S100B, an EF-hand (i.e., two helix–loop–helix motifs) calcium-binding protein that acts through RAGE (40). We also obtained a proof-of-concept demonstration that targeting the hypoxia-RAGE pathway *in vivo* restored effective inflammation and pathogen clearance in the lung.

METHODS

Animals

Six- to eight-week C57BL/6 mice were purchased from Charles River (Calco, Italy). Genetically engineered homozygote $Cftr^{-/-}$ mice (7) were bred at the CF core animal facility at San Raffaele Hospital, Milan. Experiments were performed according to the Italian Approved Animal Welfare Assurance 245–2011-B.

Aspergillus fumigatus Infection

Mice were anesthetized by intraperitoneal injection of 2.5% avertin (Sigma Chemical, St. Louis, MO) before intranasal instillation of 2×10^7 resting conidia per 20 μl saline of *A. fumigatus* (AF 293), *Aspergillus terreus*, *Aspergillus nidulans*, or *A. fumigatus* (AF 300) and its mutant (AF 300niaD7, hereafter referred as AF 300 mutant) unable to grow in hypoxic conditions (41). For histology, paraffin-embedded tissue was stained with periodic acid–Schiff and bronchoalveolar lavage fluid collection was done as described (7).

Pseudomonas aeruginosa Chronic Infection

A mucoid strain isolated from a patient with CF (42) was embedded in agar beads, as described (43). Twenty microliters of the agarose bead suspension (containing 2×10^6 *P. aeruginosa* cfu) was injected into the trachea with a 26-gauge needle and a small syringe into anesthetized mice.

Treatments

Mice were treated intranasally with 20 μg of sRAGE every other day, starting the day before and up to 7 (*A. fumigatus* infection) or 13 (*P. aeruginosa* infection) days after the infection. Additional details are reported in the online supplement.

Hypoxyprobe Treatment

Mice were intravenously injected with hypoxyprobe at a dose of 60 mg/kg weight of the mouse (Hypoxyprobe Inc., Burlington, MA). After 60 minutes, mice were killed and the lung of each mouse was filled with an embedding medium used for frozen tissue to ensure optimal cutting temperature (OCT) and after embedding in OCT immediately frozen in liquid nitrogen. Additional details are reported in the online supplement.

Western Blotting

Blots of lung lysates were incubated with primary antibodies followed by appropriate antimouse or antirabbit secondary horseradish peroxidase–linked antibody (Cell Signaling). Blots were developed with the LiteA-Blot Plus Chemiluminescence detection kit (Euroclone, Milan, Italy). Further details are in the online supplement.

ELISA Assays

ELISA for sRAGE was performed using the Quantikine Human RAGE Immunoassay kit (R&D Systems, Minneapolis, MN). For S100B ELISA, human S100B ELISA KIT (Millipore, Billerica, MA) was used following manufacturer's instructions.

Immunohistochemistry

Lung sections were incubated overnight with polyclonal anti-S100B antibody (1:100) or polyclonal anti-RAGE antibody followed by the secondary antibodies (i.e., horseradish peroxidase–conjugated goat antirabbit IgG

[Sigma-Aldrich, St. Louis, MO] for S100B, and AlexaFluor 594 donkey antigoat IgG [Invitrogen, Monza MB, Italy] for RAGE). Cells were counterstained with hematoxylin or DAPI (4',6- diamidino-2-phenylindole). Endogenous peroxidase activity was quenched using 3% H_2O_2 in phosphate-buffered saline. Fluorescence and immunofluorescence microscopy was performed on an Olympus (Segrate, Italy) microscope (BX51) and analySIS image processing software.

Human Bronchial Epithelial Cells

Human bronchial epithelial (HBE) cells homozygous for the δF508 mutation were obtained from lung transplants (patients with CF) or lung resections (patients without CF) and cultured as described (44). For growth conditions and treatment, *see* the METHODS section in the online supplement.

Statistical Analysis

Results are expressed as means ± SD. Statistical analysis was performed with Student *t* test or one-way analysis of variance Bonferroni post-test and analyzed by GraphPad Prism 4.03 program (GraphPad Software, San Diego, CA). Values of *P* less than or equal to 0.05 were considered significant.

RESULTS

RAGE and S100B Expressions Are Up-regulated in CF Mice with Aspergillosis

RAGE is expressed on epithelial and myeloid lung cells of C57BL/6 (wild-type [WT]) mice with aspergillosis (37). We assessed the expression of RAGE gene and protein in the lungs of *A. fumigatus*–infected CF mice by immunofluorescence staining, Western blotting, and reverse transcriptase polymerase chain reaction. RAGE expression was observed at protein (Figure 1A) and mRNA (Figure 1B) levels in uninfected WT and, more, CF mice; it maximally occurred, and remained elevated thereafter, at 7 days postinfection in CF mice, a time at which it returned to baseline levels in WT mice (Figures 1A and 1B). Multiple RAGE isoforms arise through alternative splicing and/or proteolysis in the different mouse organs (45). Mouse lung expresses three major RAGE isoforms of 57.4, 52.6, and 45.1 kD recognized by N-16 antibody: (*1*) the xRAGE, likely an additional form of membrane RAGE; (*2*) the "full-length" transmembrane isoform (mRAGE), and (*3*) a soluble (sRAGE) isoform (45, 46). All three isoforms were present in the lung of WT and CF mice (Figure 1C). Interestingly, sRAGE increased during infection in WT mice but apparently failed to do so in CF mice, particularly at 7 days postinfection when sRAGE could not be detected. RAGE expression in CF mice was associated with signs of RAGE activation, as indicated by the sustained levels of ERK p42/p44 phosphorylation (Figure 1D) and canonical nuclear factor (NF)-κB (p-IKKβ) activation (Figure 1E), both pathways known to occur downstream RAGE (47). Both pathways were apparently down-regulated, as a result of RAGE inhibition (37), in WT mice in which noncanonical NF-κB (p-IKKα) activation (Figure 1E) and p38 phosphorylation (Figure 1F) were instead progressively increasing.

On assessing which putative ligands of RAGE were concomitantly expressed in CF, we looked for high-mobility group box 1 (HMGB1) and S100B, a member of the S100-calgranulin family, expression in the lung during infection. We found that the kinetics of HMGB1 gene (Figure 1G) and protein (Figure 1H) expression was similar in CF and WT mice. In contrast, S100B gene (Figure 1G) and protein (Figure 1I) expression was not only higher in CF than WT mice at the basal level but continued to be elevated in CF mice throughout the infection as opposed to WT mice. Indeed, immunohistochemistry showed that, in CF mice, S100B immunereactivity was high and sustained in bronchiolar epithelial cells, known to be major sources of S100B in infection (37), but also in infiltrating inflammatory cells (Figure 1I). These data suggest that although HMGB1 and S100B pairs with RAGE very early in infection in either type of mice, a sustained S100B-RAGE expression only occurs in CF mice.

Hypoxia Regulates RAGE Expression in CF Mice

The ability of HIF-1α to bind to the RAGE promoter and transcriptionally activate it (22) is responsible for the increased expression of RAGE in ischemic hypoxia (48). To determine whether the HIF-1α–RAGE pathway is active in CF, we first visualized hypoxia *in vivo* by immunofluorescence with the hypoxia marker, pimonidazole hydrochloride (22, 49), and measured HIF-1 gene and protein expression levels in CF mice with aspergillosis. The amount and extent of hypoxia was significantly increased in CF mice because it could be detected in the lungs of uninfected mice and in lesions (*red*) throughout the course of the infection (Figure 2A). In terms of HIF-1 gene expression, HIF-1α, but not HIF-1β, gene was up-regulated early in infection in either type of mice to return to baseline levels a week after the infection in WT but not CF mice in which HIF-1α mRNA (Figure 2B) and protein (Figure 2C) maintained elevation. This up-regulation of *Hif1a* mRNA was mirrored by similar changes in the mRNA levels of the HIF-1α–dependent glycolytic genes (50), such as the transporters glucose transport 1 (*Glut1*), pyruvate kinase (*PK*), and lactate dehydrogenase (*Ldh-α*) (Figure 2D).

We next assessed whether blocking HIF-1α affects RAGE expression, infection, and inflammation. To this purpose, WT and CF mice were infected and concomitantly subjected to HIF-1α blocking by means of delivery of specific siRNA into the lung. Treatment with HIF-1α siRNA, which *per se* reduced HIF-1α gene and protein expression (*see* Figures E1A and E1B in the online supplement), reduced RAGE protein expression in the lungs of infected mice (Figure 3A). Of interest, all the isoforms decreased in WT mice but not in CF mice in which sRAGE actually increased (Figure 3B), a finding suggesting that HIF-1α differently affects the expression of the RAGE isoforms in infection. Treatment was associated with a divergent effect in WT versus CF mice. It decreased the ability to control infection and inflammation in WT mice, as evidenced by the increased fungal burden (Figure 3C), tissue inflammation (Figure 3D), inflammatory cytokine (tumor necrosis factor-α and IL-17A) production, and decreased IL-10 (Figure 3E). Opposite results were obtained in CF mice in which fungal growth restriction (Figure 3C), amelioration of tissue inflammatory pathology (Figure 3D), and increased IL-10 over proinflammatory cytokine production (Figure 3E) were observed on HIF-1α inhibition. Confirming the Western blotting data, sRAGE production in CF mice was increased by the treatment (Figure 3E). Finally, consistent with the asthma exacerbation by hypoxia (51), blocking HIF-1α decreased IL-4 and IL-13 production in CF mice (Figure 3E). These results indicate that the sustained activation of the HIF-1α–RAGE pathway in CF in response to hypoxic stress results in pathogenic inflammatory and allergic responses. In contrast, consistent with ability of the RAGE axis to restrain pathogen-induced inflammation in WT mice (37), blockade of the HIF-1α–RAGE pathway may impair the host ability to control infection and inflammation in physiologic conditions.

Infection Contributes to the S100B-RAGE Up-regulated Expression by TLRs

The above findings indicate that RAGE is under the transcriptional control of HIF-1α in CF, but how S100B production is regulated during infection is not clear. Specific binding sites for NF-κB family members exist in the promoter of both human

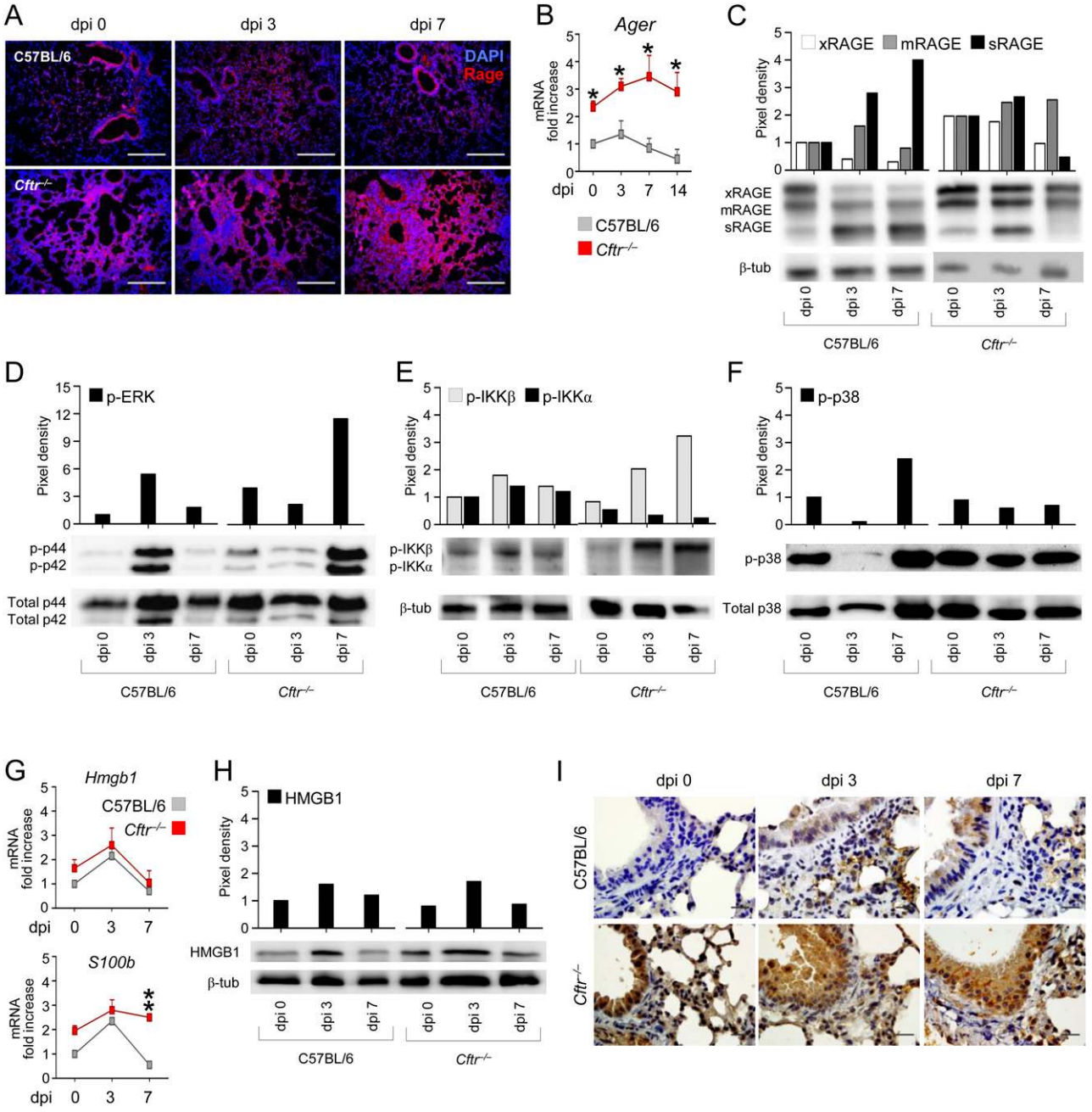

Figure 1. Receptor for advanced glycation end products (RAGE) and S100B expression are up-regulated in cystic fibrosis mice. C57BL/6 or *Cftr*$^{-/-}$ mice were infected intranasally with live *Aspergillus fumigatus* 293 conidia and assessed for (*A*) RAGE expression by immunofluorescence staining of lung, (*B*) reverse transcriptase polymerase chain reaction, and (*C*) Western blotting of total lung cells at different days postinfection (dpi). In *C*, the three major RAGE isoforms of 57.4, 52.6, and 45.1 kD recognized by N-16 antibody, namely the xRAGE, likely an additional form of membrane RAGE, the "full-length" transmembrane isoform (mRAGE), and a soluble (sRAGE) isoform are shown. (*D*) ERK p42/p44 activation, (*E*) canonical-noncanonical nuclear factor-κB activation, and (*F*) p38 phosphorylation in total lung cells from infected mice at different dpi. (*G*) *Hmgb1* and *S100b* gene expression by reverse transcriptase polymerase chain reaction. (*H*) High-mobility group box 1 (HMGB1) protein levels by Western blotting. (*I*) S100B immunohistochemistry staining in the lungs. Photographs were taken using a high-resolution Microscopy Olympus DP71 using a ×20 objective for RAGE (scale bars = 200 μm) and a ×100 objective for S100B (scale bars = 20 μm). For immunostaining, lung sections were incubated overnight with anti-S100B or anti-RAGE antibody followed by the secondary antibodies. Shown are representative images out of two independent experiments and corresponding pixel density ratio (on naive C57BL/6) normalized against β-tubulin or total proteins. Scanning densitometry was done on a Scion Image apparatus. Values represent the mean ± SD of six mice per group and are representative of three experiments. *$P < 0.05$; **$P < 0.01$, Student *t* test. CFTR = cystic fibrosis transmembrane conductance regulator.

(GenBank: M59486) and murine (GenBank: NC_000076.5) *S100b* (37). Consistently, we have shown that *S100b* expression in airway epithelial cells is transcriptionally regulated by the sequential action of downstream MyD88- and TRIF-dependent NF-κB signaling pathways. It was induced by canonical NF-κB downstream the TLR2/MyD88/ERK pathway and inhibited by noncanonical NF-κB downstream TLR3/TRIF/p38 (37). Thus, because TLR2 signaling (52) and NF-κB–dependent gene products are increased

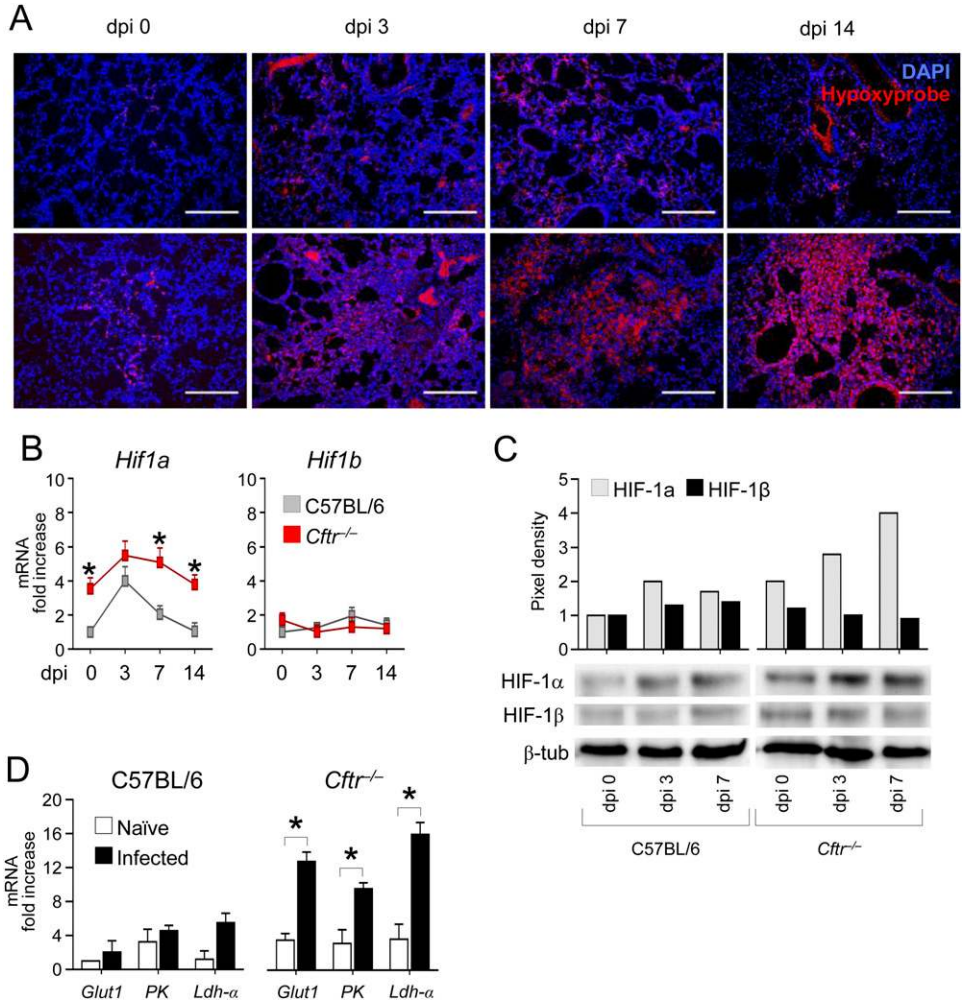

Figure 2. Hypoxia expression in cystic fibrosis mice. C57BL/6 or *Cftr*$^{-/-}$ mice were infected intranasally with live *Aspergillus fumigatus* 293 conidia and assessed for (*A*) lung levels of hypoxia by immunofluorescence with the hypoxia marker pimonidazole hydrochloride at 0, 3, 7, and 14 days postinfection (dpi); (*B*) *Hif1a* and *Hif1b* gene expression by reverse transcriptase polymerase chain reaction (RT-PCR); and (*C*) hypoxia-inducible factor (HIF)-1 protein levels by Western blotting of total lung cells at different dpi. Shown are representative images out of two independent experiments and corresponding pixel density ratio (on naive C57BL/6) normalized against β-tubulin. (*D*) Expression of HIF-1α–dependent genes, *Glut1* (the transporters glucose transport 1), *PK* (pyruvate kinase), and *LDH-α* (lactate dehydrogenase) by RT-PCR at 7 dpi. Photographs were taken using a high-resolution Microscopy Olympus DP71 using a ×20 objective. Scale bars = 200 μm. Note the increased hypoxia in uninfected cystic fibrosis mice and in lesions (*red*) during infection. Values represent the mean ± SD of six mice per group and are pooled data from three (RT-PCR) or representative of two (immunofluorescence and immunoblotting) experiments. *$P < 0.05$, Student *t* test. CFTR = cystic fibrosis transmembrane conductance regulator.

(3, 5), whereas the TLR3-TRIF pathway (53) and TRIF-dependent gene products (3, 5) are decreased in CF, increased S100B levels are likely to occur in this disease. We evaluated the effects of TLR2 or TLR3 stimulation on the expression of S100B in the lungs of CF mice treated with selective agonists alone or together, namely MALP2 and Poly(I:C). Given the feed-forward RAGE activation by RAGE ligands (54) we also evaluated RAGE expression. S100B and RAGE proteins and genes expression were increased in both WT and CF mice on TLR2 stimulation (Figures 4A and 4B). However, at variance with WT mice, S100B-RAGE expression was also increased in CF mice on concomitant TLR3 stimulation (Figure 4A) and was associated with up-regulated *S100b* gene expression (Figure 4B). These data, combined with those of Figures 1D–1F showing the increased ERK phosphorylation but decreased p38 phosphorylation in CF mice, suggest that increased TLR2-ERK signaling and defective TLR3-p38 signaling both contribute to the increased and sustained expression of S100B in CF. This may imply that respiratory fungal pathogens, through a different capacity to stimulate the different TLRs, may differently impact the S100B-RAGE expression in the lung of CF mice. This seemed to be the case, because different species of *Aspergillus*, such as *nidulans* and *terreus*, both found in patients with CF (35), showed differences in their ability to stimulate S100B and RAGE expression in the lung. At variance with *A. fumigatus*, neither *Aspergillus* species stimulated S100B and RAGE protein (Figure 4A) or gene (Figure 4C) expression in either WT or CF mice. We next assessed whether and how hypoxia adaptation may contribute to the ability of fungi to activate the S100B-RAGE axis. To this purpose, we evaluated the impact on S100B-RAGE expression of a mutant strain of *A. fumigatus* unable to grow in hypoxic conditions (41). We found that the S100B-RAGE expression induced by the mutant strain was lower as compared with the WT strain in the lung of CF and WT mice (Figures 4A–4C), a finding indicating that the failure to adapt to hypoxia may restrain the activation of the S100B-RAGE axis in infection. Therefore, pathogen recognition, and likely metabolic activity, contributes to the activation of the S100B-RAGE pathway in the CF lung by fungi.

Targeting RAGE Has Curative Effects in Murine CF

Given the above findings, we tested the efficacy of treatments aimed at inhibiting RAGE activity in murine CF by means of administration of sRAGE to *Cftr*$^{-/-}$ mice with aspergillosis. In preliminary experiments we found that, despite the important immunomodulatory activity exhibited by sRAGE given intraperitoneally (55), local delivery of purified mouse sRAGE by means of intranasal administration had superior activity in WT mice (data not shown). We therefore subjected WT and CF mice to repeated (five times) intranasal administration of 20 μg of sRAGE, totaling a dose exceeding the concentration (~75 μg) of sRAGE found in a mouse lung (56). Mice were infected with *A. fumigatus* and then evaluated for parameters of infection and inflammation. We found that the treatment restricted the fungal growth (Figure 5A), reverted tissue inflammation (Figure 5B), decreased IL-17A and IL-4 production, and increased IL-10 in *Cftr*$^{-/-}$ mice (Figure 5C). Lung immunostaining revealed

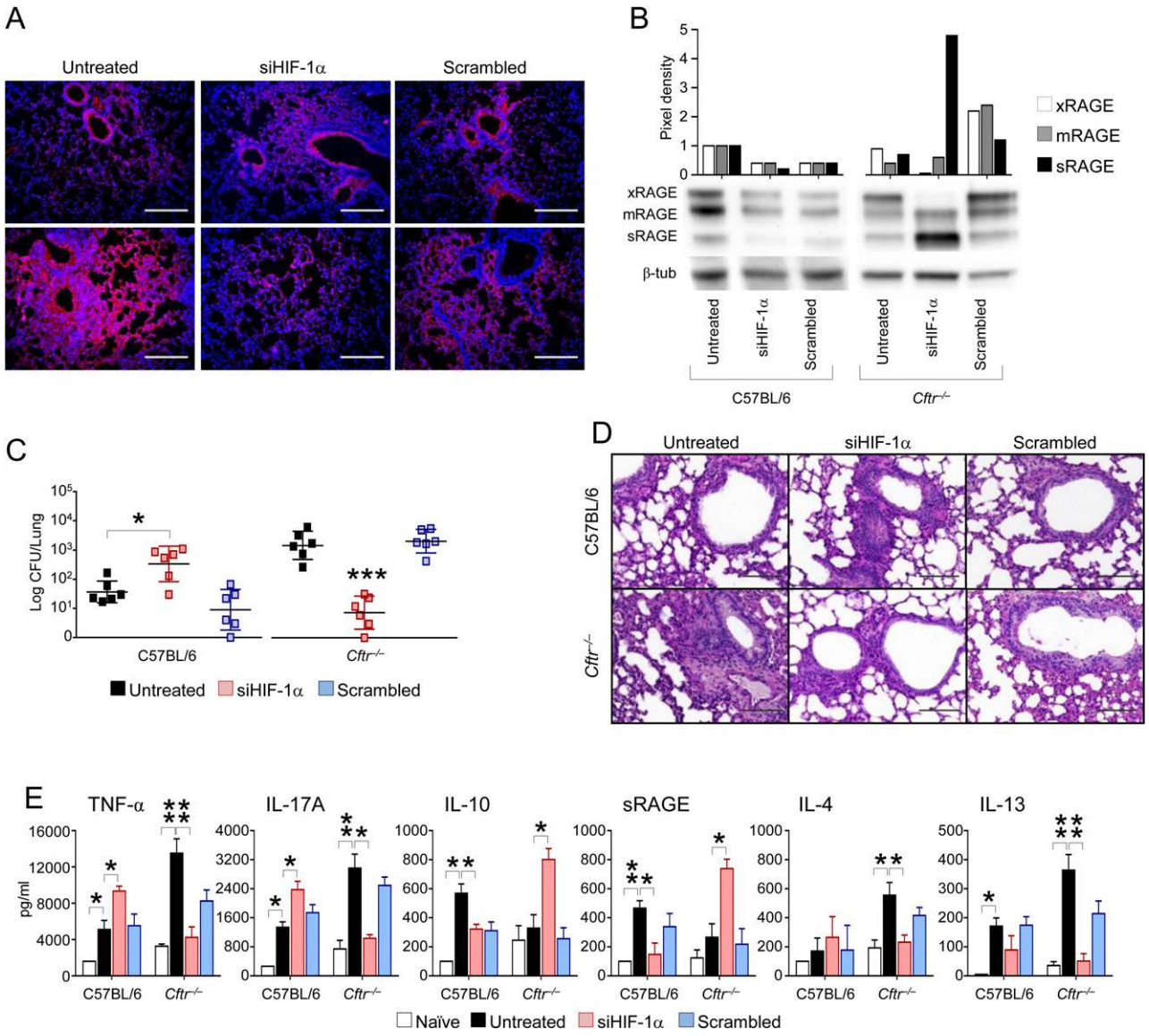

Figure 3. Hypoxia regulates receptor for advanced glycation end products (RAGE) expression in cystic fibrosis mice. C57BL/6 or *Cftr*$^{-/-}$ mice were infected intranasally with live *Aspergillus fumigatus* 293 conidia, treated with hypoxia inducible factor (HIF)-1α siRNA or scrambled siRNA, and assessed for (*A*) RAGE expression in the lungs by immunofluorescence staining, (*B*) Western blotting of the three major isoforms, (*C*) fungal growth (mean value log cfu) in the lungs, (*D*) lung histology (periodic acid–Schiff staining), and (*E*) cytokine levels (ELISA, mean value) in total lung homogenates. Assays were done at 7 days postinfection. Photographs were taken using a high-resolution Microscopy Olympus DP71 using a ×40 objective. Scale bars = 100 μm. Shown in *B* are representative blots out of two independent experiments and corresponding pixel density ratio (on untreated C57BL/6) normalized against β-tubulin. Values represent the mean ± SD of six mice per group and are pooled data from three (reverse transcriptase polymerase chain reaction) or representative of two (immunofluorescence and histology) experiments. *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$, one-way analysis of variance Bonferroni post-test. CFTR = cystic fibrosis transmembrane conductance regulator; sRAGE = soluble RAGE; TNF = tumor necrosis factor.

that treatment with sRAGE also decreased the local expression of S100B (Figure 5D) and, most importantly, of RAGE (Figure 5E). Thus, these findings indicate that sRAGE, by preventing S100B from binding to RAGE, may prevent excessive inflammation promoted by feed-forward RAGE activation (37). In contrast, consistent with the ability of the S100B-RAGE axis to restrain pathogen-induced inflammation in physiologic conditions (37) and, more generally, with the multiple roles played by RAGE in executing the signal transduction mechanisms initiated by ligand binding (57), treatment with sRAGE, while decreasing the local fungal growth (Figure 5A), failed to ameliorate lung inflammation (Figure 5B) and to affect the IL-17A/IL-4/IL-10 production in WT mice (Figure 5C).

RAGE and S100B Expressions Are Up-regulated in CF Mice with *Pseudomonas aeruginosa* Infection

To provide evidence that the sustained activation of the HIF-1α–RAGE pathway in CF also occurs in infection by the most common CF pathogen, *P. aeruginosa*, we analyzed the expression of HIF-1 subunits, S100B, and RAGE in the lung of WT and CF mice with chronic *P. aeruginosa* infection. We found that, associated with an higher bacterial burden (Figure 6A), the expression of HIF-1α gene (Figure 6B) and protein (Figure 6C), S100B, and RAGE protein by immunofluorescence (Figure 6D) and Western blotting (Figure 6E) and gene by reverse transcriptase polymerase chain reaction (Figure 6F) were all increased in infected CF mice.

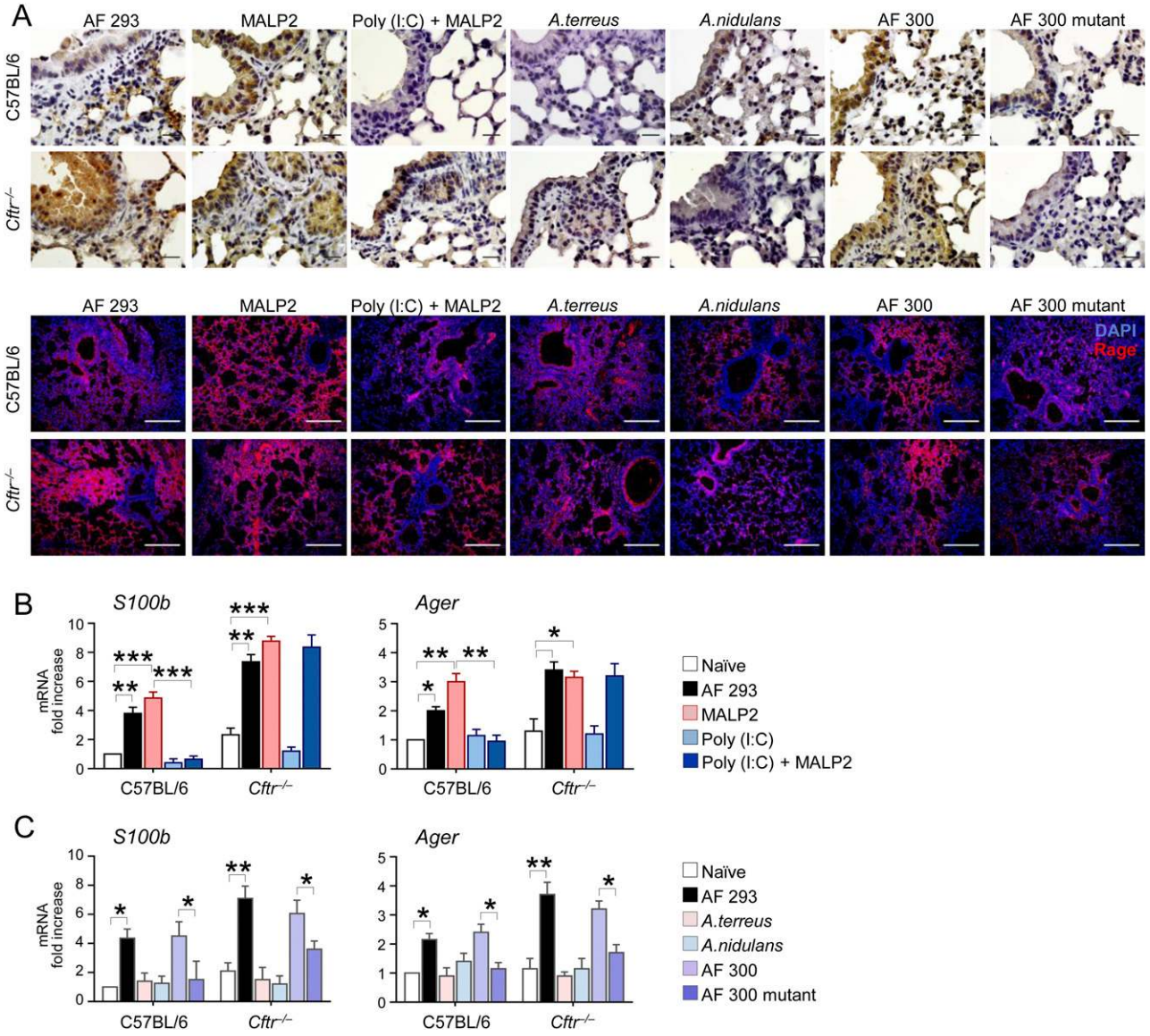

Figure 4. Toll-like receptors contribute to the S100B–receptor for advanced glycation end products (RAGE) up-regulated expression in cystic fibrosis mice. C57BL/6 or *Cftr*$^{-/-}$ mice were infected intranasally with live condia of *Aspergillus fumigatus* strains 293, 300, the 300 mutant, *A. nidulans*, and *A. terreus* or treated with MALP2 or Poly (I:C) intranasally. (*A*) Expression of S100B and RAGE by immunohistochemistry and immunofluorescence staining, respectively, at 3 days postinfection. Photographs were taken using a high-resolution Microscopy Olympus DP71 using a ×20 objective for RAGE (scale bars = 200 μm) and a ×100 objective for S100B (scale bars = 20 μm). (*B* and *C*) Expression of *S100b* and *Ager* gene by reverse transcriptase polymerase chain reaction on total lung cells at 3 days postinfection. Values represent the mean ± SD of six mice per group and are pooled data (reverse transcriptase polymerase chain reaction) or representative (immunofluorescence and histology) of two experiments. *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$, one-way analysis of variance Bonferroni post-test. CFTR = cystic fibrosis transmembrane conductance regulator.

Even in this infection, sRAGE could be detected in WT mice 2 weeks after the infection but not in CF mice (Figure 6E) and administration of sRAGE greatly reduced the bacterial burden (Figure 6A) and RAGE expression (Figure 6B) in CF mice. These data suggest that the S100B-RAGE axis is up-regulated in *P. aeruginosa* infection in CF mice and are fully consistent with the up-regulated expression of TLR2 (58) and impaired expression of TLR3 (53) observed in this infection.

RAGE and S100B Are Up-regulated in Human CF

To assess whether RAGE was up-regulated in human CF by hypoxia, we evaluated protein expression in bronchial epithelial cells (HBE) from a patient with CF (7) on 4-hour exposure to *A. fumigatus* conidia, S100B, or HMGB1 in hypoxic or normoxic conditions. Immunofluorescence staining revealed that RAGE expression was detected in CF-HBE kept at normoxic conditions (Figure 7A), either untreated or in response to the different stimuli. However, RAGE expression markedly increased in these cells in hypoxic conditions in response to S100B and, to a lower degree, to conidia or HMGB1 (Figure 7B). The number of cells expressing RAGE also increased in CF-HBE on stimulation in normoxic (Figure 7C) or hypoxic (Figure 7D) conditions. Therefore, RAGE expression in human CF is sensitive to hypoxia and induced by S100B. In patients with CF, *AGER* expression was up-regulated in lung cells (Figure 7E), sRAGE levels reduced (Figure 7F), and S100B levels increased (Figure 7G) in expectorates, these findings suggesting hyperactivation of the S100B-RAGE axis and the relative failure to produce sRAGE in these patients.

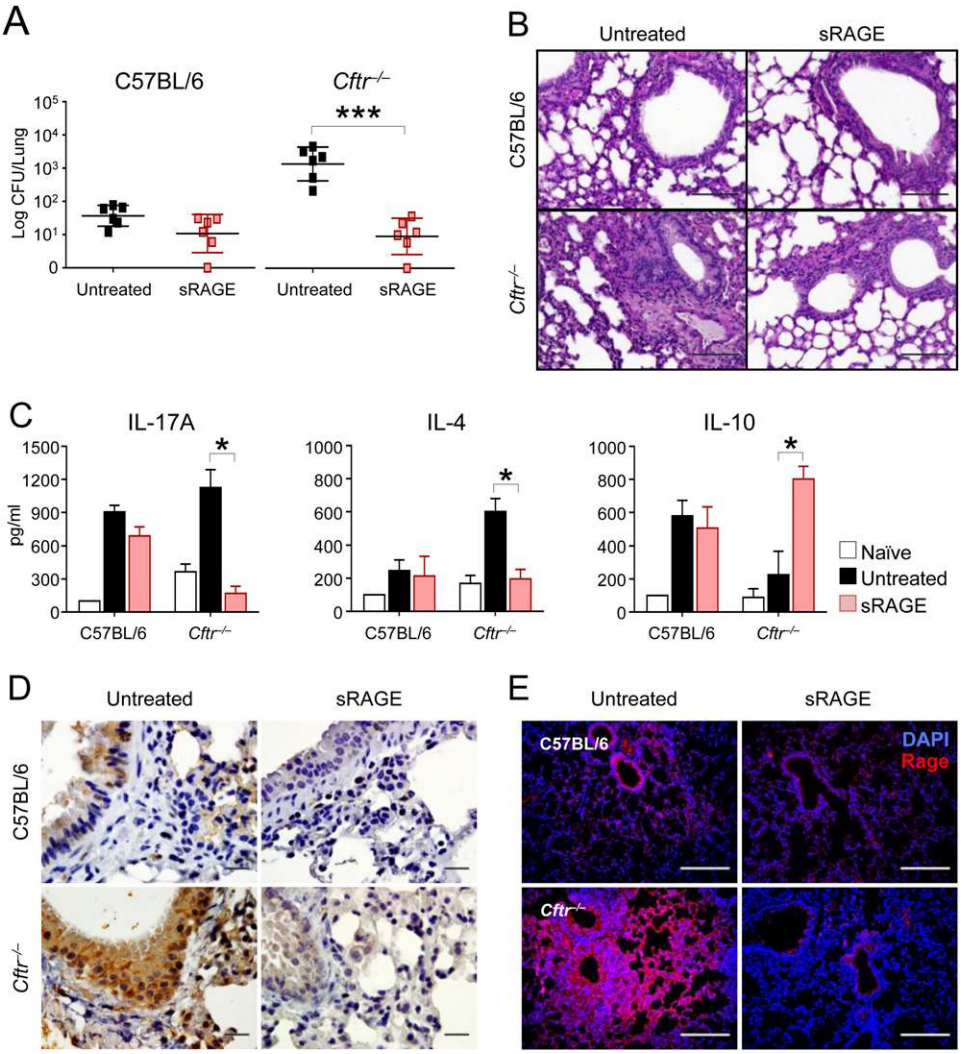

Figure 5. Treatment with soluble receptor for advanced glycation end products (sRAGE) restrains inflammation in cystic fibrosis mice. C57BL/6 or *Cftr*$^{-/-}$ mice were infected intranasally with live *Aspergillus fumigatus* 293 conidia and treated with sRAGE intranasally before the assessment at 7 days postinfection of (*A*) fungal growth (mean value log cfu), (*B*) lung histology (periodic acid–Schiff staining), (*C*) cytokine levels (ELISA, mean value) in total lung homogenates, and (*D*) S100B and (*E*) RAGE protein expression by immunohistochemistry or immunofluorescence staining, respectively. Photographs were taken using a high-resolution Microscopy Olympus DP71 using a ×40 objective. Scale bars = 100 μm. Immunohistochemistry or immunofluorescence images were taken using a high-resolution Microscopy Olympus DP71 using a ×20 objective for RAGE (scale bars = 200 μm) and a ×100 objective for S100B (scale bars = 20 μm). Values represent the mean ± SD of three mice per group and are pooled (cfu and ELISA) or representative of three experiments. *$P < 0.05$, ***$P < 0.001$, Student *t* test and one-way analysis of variance Bonferroni post-test. CFTR = cystic fibrosis transmembrane conductance regulator.

It is known that *AGER* is a modifier gene of lung disease severity in CF. Indeed, the *AGER*-429T/C polymorphism was associated with an increased lung disease severity in CF and was able to modulate RAGE expression *in vitro* (26). We assessed whether the *AGER* -374T/A polymorphism, known to lead to an overexpression phenotype (59) and associated with susceptibility to aspergillosis in stem cell transplant recipients (38), might be correlated with lung inflammation in CF (Table 1). *AGER* expression was not only up-regulated in patients with CF bearing the *AGER* -374T/A polymorphism (Figure 7E) but also contributed to higher levels of IgE (Figure 7H) in both patients with atopy and ABPA-sensitized patients (*see* Figure E2) independently from the type of ΔF508 mutation (data not shown), a finding indicating that RAGE hyperactivation may contribute to lung allergic inflammation in human CF that may eventually facilitate sensitization by *Aspergillus* spp.

DISCUSSION

In the present study, we used *in vivo* and *in vitro* models to show that sustained expression of RAGE and its ligand S100B exerted a proximal role in the chronic inflammatory state in CF, such that targeting the RAGE pathway *in vivo* restored lung immune homeostasis and measurement of sRAGE levels could be a useful biomarker for RAGE-related pathogenic inflammation in patients with CF. Thus, our study expands on previous findings showing that RAGE expression is up-regulated on CF airways neutrophils (4) and contributes to disease severity (25, 26). This is consistent with the opposing role of RAGE signaling in acute inflammation, where it stimulates host's proinflammatory events and in conditions of persistent elevations of endogenous ligands where it promotes chronic pathogenic inflammation (29, 37). Additionally, we provide evidence for the contribution of hypoxia and TLRs to the sustained activation of this inflammatory pathway, being RAGE up-regulated by hypoxia and S100B by infection by TLRs. This may represent a unifying conceptual framework within which to accommodate the vicious cycle of airways infection and inflammation in CF. Because RAGE down-regulates neutrophil survival and functions in murine aspergillosis (37), this may explain the seemingly apparent paradox of failure to eliminate pathogen in the face of an heightened inflammatory response in CF. Additionally, the activation of the Th17 pathway downstream RAGE (37) may further impair through a positive feed-back loop the host's antimicrobial resistance by increasing RAGE expression (60) and promoting fungal growth and virulence in a host-autonomous fashion (61). As a matter of fact, we have already shown that treatments with IL-17A antagonists restored lung immune homeostasis and antimicrobial resistance in experimental CF (7).

Our study shows that, in addition to HMGB1, whose levels are significantly elevated in bronchoalveolar lavage fluids of patients with CF (62), S100B also contributes to excessive lung

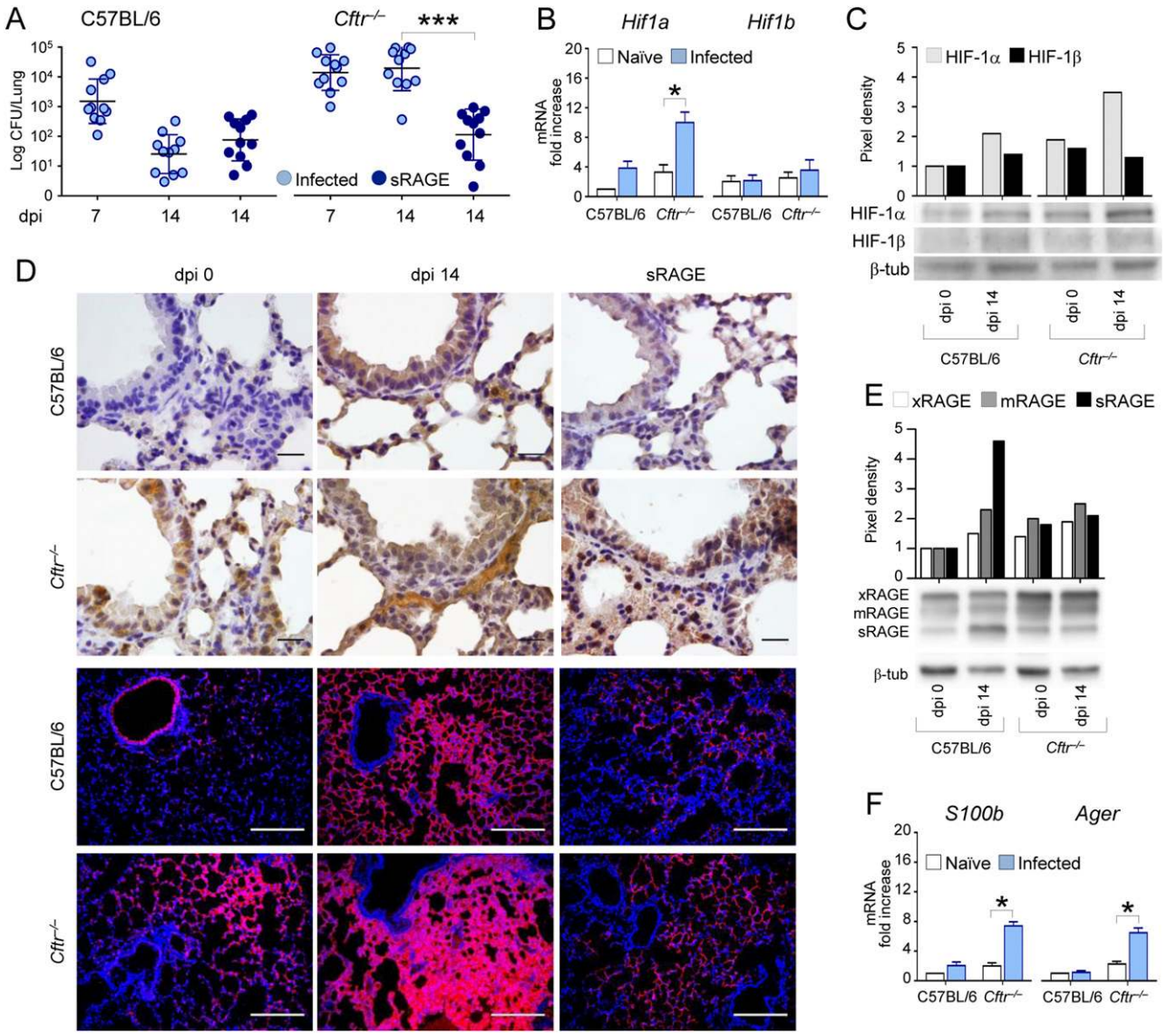

Figure 6. Receptor for advanced glycation end products (RAGE) and S100B expression are up-regulated in *Pseudomonas aeruginosa* infection. C57BL/6 or *Cftr*$^{-/-}$mice were intratracheally infected with 5×10^6 cfu/agar beads of *P. aeruginosa* before the assessment at 7 and 14 days postinfection (dpi) of bacterial burden (*dots* represent individual measurements of cfu per lung, and *horizontal lines* represent median values). (*A*) Hypoxia-inducible factor (HIF)-1α and HIF-1β gene (*B*) and protein (*C*) expression; S100B and RAGE expression by (*D*) immunofluorescence, (*E*) Western blotting, and (*F*) reverse transcriptase polymerase chain reaction on total lung cells. Mice were treated intranasally with 20 μg of soluble (s) RAGE every other day, starting the day before and up to13 days after the infection. Photographs were taken using a high-resolution Microscopy Olympus DP71 using a ×20 objective for RAGE (scale bars = 200 μm) and a ×100 objective for S100B (scale bars = 20 μm). Values represent the mean ± SD of six mice per group and are pooled (cfu and reverse transcriptase polymerase chain reaction) or representative (immunofluorescence) of two experiments. *$P < 0.05$, ***$P < 0.001$, Student *t* test and one-way analysis of variance Bonferroni post-test. CFTR = cystic fibrosis transmembrane conductance regulator.

inflammation in CF during fungal and bacterial pneumonia, likely through feed-forward RAGE activation (37). Elevated levels of S100B have been observed in certain immunomediated diseases (40), a finding also consistent with the ability of S100B to form complexes with TLR ligands, thus affecting the activity of the partner receptor (37). In this regard, through binding to nucleic acids, intracellular S100B was able to activate the TLR3-TRIF–dependent pathway that, by transcriptionally down-regulating *S100b* gene expression (37), inhibited the S100B-RAGE axis and contributed to resolution of inflammation in fungal pneumonia. This predicts that TLR3 hypofunctioning, which has been reported in patients with CF (53), may contribute to the degree of inflammation in response to respiratory infections in these patients and suggests that immune screening for TLR3 functional activity would help identify those patients with CF at risk of developing inflammatory pathology in infections. Although several studies suggested that CF and non-CF epithelia have similar expression of TLRs (52, 63), alterations in receptor localization resulting in different TLR-dependent signaling between epithelial and myeloid cells have been described (5).

One interesting observation of the present study is that, in addition to the up-regulated expression of the S100B-RAGE axis, sRAGE expression and production were down-regulated in murine and human CF. As in various chronic pulmonary diseases (30), sRAGE deficiency was linked to sustained inflammation in murine CF that could be reversed on sRAGE administration.

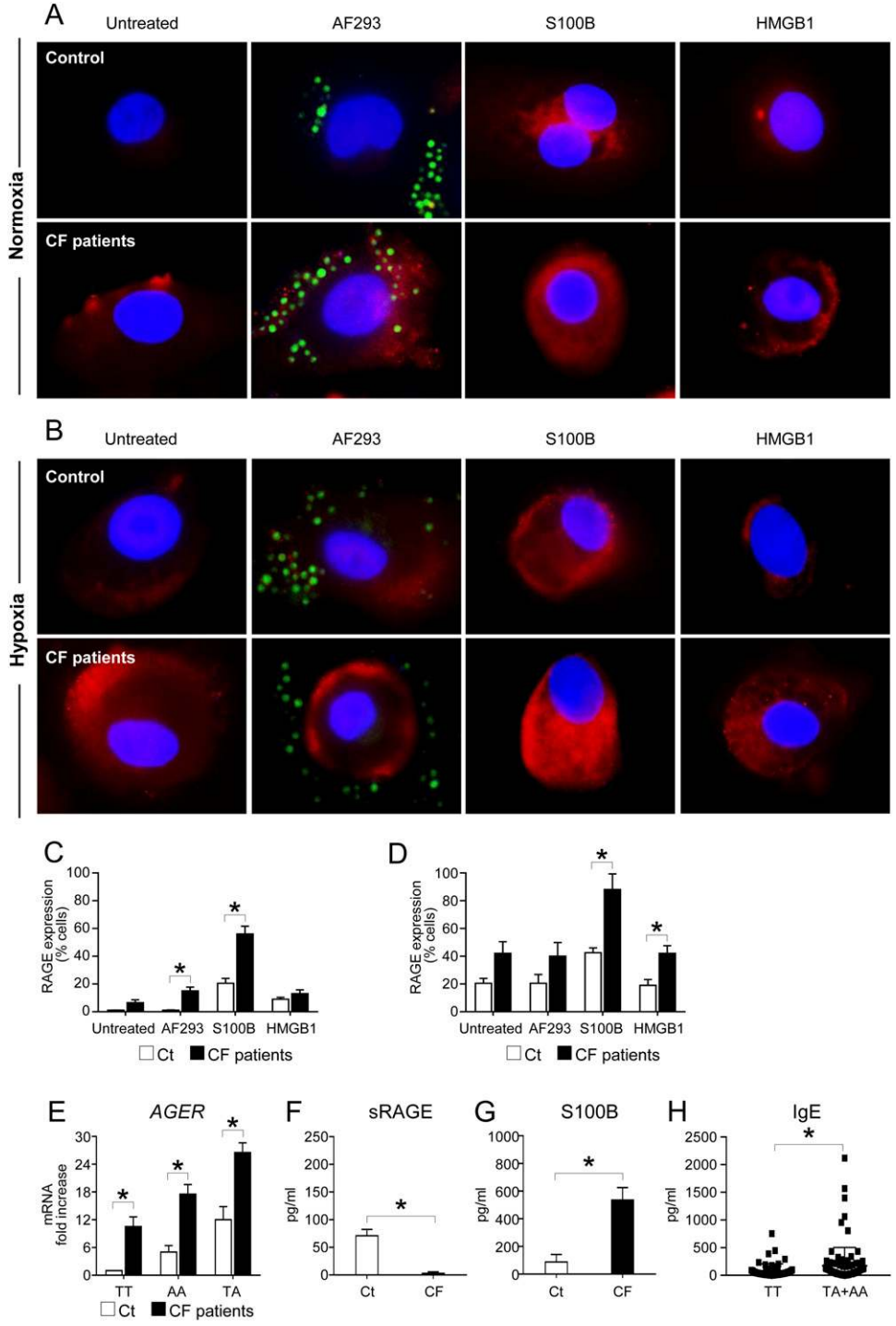

Figure 7. Receptor for advanced glycation end products (RAGE) and S100B are up-regulated in human cystic fibrosis (CF) by hypoxia. (*A* and *B*) Human bronchial epithelial cells homozygous for δF508 mutation and control (Ct) cells were cultured in normoxic or hypoxic conditions and exposed to *Aspergillus fumigatus* conidia at cells:fungi ratio of 2:1, 4 nM S100B or 400 μM high-mobility group box 1 (HMGB1). Cells were incubated for 18 hours at 37°C in 5% (normoxic) or 0.05% CO_2. Cultures growing on culture slides were fixed and incubated with anti-RAGE antibody. Images were acquired using the Olympus BX51 fluorescence microscope with a ×100 objective and the analySIS image processing software (Olympus). DAPI was used to detect nuclei. Representative images of two independent experiments from three patients homozygous for δF508 mutation and Ct cells. (*C*) Number of human bronchial epithelial cells with positive RAGE expression in normoxic conditions or (*D*) hypoxic conditions (mean ± SD, determined by fluorescence microscopy) (n = 2). (*E*) Cellular *AGER* expression by reverse transcriptase polymerase chain reaction, levels of (*F*) soluble (*s*) RAGE, (*G*) S100B, and (*H*) total IgE in expectorates from Ct or patients with CF carrying the TT, TA, or AA genotypes (n = 8 for each genotype) of the *AGER* -374T/A gene. **P* < 0.05, Student *t* test.

Thus, similar to what was observed in several experimental models (64), the administration of sRAGE as a ligand decoy may have beneficial effects in reducing chronic inflammatory stresses thereby thwarting tissue injury in patients with CF. Additionally, decreased levels of sRAGE may be useful as a biomarker of ligand-RAGE pathway hyperactivity and inadequate endogenous protective response, thus providing a powerful complement to risk stratification and identifying potential therapeutic targets and/or biomarkers of RAGE activity in CF, as already suggested for other diseases (33).

Understanding the full plethora of RAGE alternative splicing and its regulation will be central to exploiting the therapeutic manipulation of RAGE in human diseases (54). In this regard, we have found that sRAGE production was increased by HIF-1α inhibition, a finding suggesting that HIF-1α may not only bind to the RAGE promoter to promote RAGE transcriptional activity (22), but also affects the expression of the different RAGE isoforms. However, how the different RAGE isoforms expression is regulated in humans is poorly understood (65). For sRAGE, putative mechanisms include the actions of ADAMs (a disintegrin and metalloprotease) (66, 67) and a naturally occurring splice variant resulting in an "endogenous secretory" form of the soluble receptor (68). Irrespective of the putative mechanisms through which HIF-1α may regulate sRAGE expression, hypoxia may affect RAGE expression through nontranscriptional mechanisms, including the release of advanced glycation end products activating

TABLE 1. CLINICAL CHARACTERISTICS OF PATIENTS WITH CYSTIC FIBROSIS

Characteristics	N = 277
Sex, % male	46.6
Age, yr	13.5 ± 11.0
FEV_1, % predicted	85.1 ± 26.4
FVC, % predicted	90.6 ± 22.1
Height, cm	149.9 ± 23.2
Weight, kg	47.8 ± 18.5
BMI	20.3 ± 3.9
CFTR mutation, %	
ΔF508 homozygous	19.5
ΔF508 heterozygous	38.6
Other	31.1

Definition of abbreviations: BMI = body mass index; CFTR = cystic fibrosis transmembrane conductance regulator.

Continuous variables are expressed as mean ± SD.

RAGE (69) and the activation of Th17 cells (16, 17) promoting RAGE expression (60). In addition, inhibition of IDO protein level and activity under hypoxia (18) may further contribute to up-regulation of Th17 cell response, and hence RAGE activity in CF. Therefore, treatments with IL-17A antagonists or IDO-promoting agents to restore lung immune homeostasis and antimicrobial resistance (7) may also include an activity on RAGE that may further add to their therapeutic activity. Similarly, the protective effects of cyclosporin A in murine CF (7) may not only result from decreased calcineurin activity in lung but also encompass the inhibition of HIF-1α transcriptional activity (70) or a combination of these factors.

The finding that blocking HIF-1α by siRNA ameliorates inflammation in CF mice suggests that targeting hypoxia could down-regulate RAGE and Th17 activity and restore IDO function. Of interest, HIF-1α dimerizes with HIF-1β protein, also known as aryl hydrocarbon nuclear translocator, which is an important binding partner for the aryl hydrocarbon receptor (71), an environment-sensing transcription factor with important immunomodulatory properties in the lung (72). Thus, hypoxia, by decreasing aryl hydrocarbon receptor activity in the lung, may further impact lung inflammation, as suggested by our own preliminary experiments. However, the potential for impairment of essential immune functions by HIF-1α inhibition has to be carefully evaluated and weighed (20, 73). In fact, despite that hypoxia and immune signaling pathways are connected in the lung at a number of levels (74), further investigations are needed to establish a causal relationship between hypoxia and development of pathogenic inflammation in CF, considering that hypoxia is a rather late event in CF lung and that regional, rather than global, hypoxia within the mucous deposits may likely contribute to *P. aeruginosa* metabolic activity (75) and antibiotic resistance in anaerobic conditions (76).

It has been suggested that adaptation to hypoxia may contribute to fungal virulence (36, 77) by cell wall assembly (78) and secondary metabolite production (49, 77). We have found that, consistent with the finding that a plethora of genes and proteins are transcriptionally and post-transcriptionally regulated by hypoxia on *Aspergillus* (77), adaptation to hypoxia may contribute to the activation of S100B-RAGE axis in fungal pneumonia. Of interest, we found differences, among different *Aspergillus* species, in the ability to up-regulate the S100B-RAGE axis, a finding that could help discriminate saprophitic versus nonsaprophitic fungal growth in the lung of patients with CF.

Collectively, we have identified a novel molecular pathway that contributes to the heightened inflammation in CF and provided evidence that this pathway could be a useful therapeutic target and biomarker of lung inflammation in this disease.

Author disclosures are available with the text of this article at www.atsjournals.org.

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