Th17/Treg Imbalance in Murine Cystic Fibrosis Is Linked to Indoleamine 2,3-Dioxygenase Deficiency but Corrected by Kynurenines

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Rationale: Mutations in the cystic fibrosis (CF) transmembrane conductance regulator affect the innate epithelial immune function of the lung, resulting in exaggerated and ineffective airway inflammation that fails to eradicate pathogenic fungi. The appreciation of whether such fungi are primarily responsible for or a consequence of ineffective airway inflammation is important for future therapeutics development.

Objectives: To characterize the impact of the tryptophan/kynurenine pathway on pathogenic airway inflammation preventing effective fungal clearance in CF.

Methods: We studied the expression of indoleamine 2,3-dioxygenase (IDO), the first enzyme in the kynurenine pathway of tryptophan degradation, in human and murine CF, the impact of IDO on lung inflammation and immunity in murine CF, and the potential role of tryptophan catabolism in pathogenesis and therapy of fungus-associated lung inflammation.

Measurements and Main Results: IDO was defective in murine and human CF. Genetic and transcriptional regulatory mechanisms contributed to dysfunctional IDO activity that, in turn, correlated with imbalanced Th17/Treg-cell responses to *Aspergillus fumigatus* in murine CF. Treatments enhancing IDO function or preventing pathogenic Th17-cell activation restored protective immunity to the fungus and improved lung inflammation in murine CF.

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The respiratory tract of patients with cystic fibrosis (CF) is colonized with, in addition to bacteria, many fungi, although whether they are primarily responsible for or a consequence of ineffective airway inflammation remains controversial and largely unsolved.

What This Study Adds to the Field

Decreased tryptophan/kynurenine metabolism, as a result of defective indoleamine 2,3-dioxygenase (IDO) enzyme, was causally linked to susceptibility to *Aspergillus* infection and sensitization in murine CF through the generation of a type 17 helper T-cell/regulatory T-cell (Th17/Treg) imbalance. Treatments with IDO agonists or prevention of Th17-cell activation restored antifungal protective immunity and improved lung inflammation and function, suggesting a therapeutic potential for IDO-mimetic drugs in CF.

Conclusions: This study provides a link between tryptophan catabolism and lung immune homeostasis in murine CF, representing a proof-of-concept that targeting pathogenic inflammation via IDO-mimetic drugs may benefit patients with CF.

Keywords: cystic fibrosis; indoleamine 2,3-dioxygenase; Th17/Treg balance; aspergillosis

The respiratory system in patients with cystic fibrosis (CF) is characterized by high concentrations of neutrophils (1) and proinflammatory cytokines, including IL-17A (2–4), with reduced concentrations of antiinflammatory factors (5, 6). Type 17 helper T (Th17) lymphocytes, and other IL-17$^+$ cells—including neutrophils, γδ T cells, and natural killer T cells—are present in the airway submucosa in patients with CF, and they are presumed to account for the underlying inflammatory status in those patients (4). An exaggerated inflammatory response, relative to the actual burden of infection, is indeed responsible for much of the pathology found in CF lungs (1, 7, 8). We have shown that IL-17A/Th17 cells are at the intersection of immune protection with pathology in fungal infections (9). Indeed, Th17 cells are in balance with regulatory T (Treg) cells through the activity of indoleamine 2,3-dioxygenase (IDO), the rate-limiting enzyme in tryptophan degradation along the kynurenine pathway (10). Pathogenic Th17 cells, owing to IDO deficiency, account for the

inherent susceptibility to aspergillosis in mice with chronic granulomatous disease, because of an unopposed inflammatory response that compromises the host's ability to eradicate infection (11). These findings may be relevant in patients with CF whose respiratory tract is colonized, in addition to bacteria, by many fungi (12–14), although their involvement in respiratory infections remains controversial and largely unsolved. Indeed, although the effects of fungal colonization on lung function have remained unclear (15), colonization with *Aspergillus fumigatus* in CF may be associated with decreased FEV_1 and a more rapid decline in pulmonary function, even in the absence of allergic bronchopulmonary aspergillosis (ABPA) (16, 17), a hypersensitivity condition to *Aspergillus* colonization, affecting about 10% of people with CF (14). Because the use of inhaled corticosteroids is not apparently associated with a greater risk of *Aspergillus* infection in patients with CF, as opposed to the non-CF population (18), it has been suggested that individuals with CF may have a specific susceptibility to infection with filamentous fungi (14).

In this study, we assessed whether the inability to properly control inflammation could contribute to the susceptibility to respiratory fungal infections in CF. We tested the hypothesis that IDO deficiency may influence *Aspergillus*-dependent lung diseases in CF. Considering that tryptophan metabolites are also able to impair calcium mobilization in T cells (19), a role for IDO in the control of T-cell dysreactivity due to disturbed Ca^{2+} signaling pathways (20) is likely. We resorted to $Cftr^{tm1Unc}$ ($Cftr^{-/-}$) mice—known to mimic, to some extent, human CF (21)—to verify that defective IDO leads to unopposed Th17-cell responses to *A. fumigatus*. We found that IDO is under genetic and transcriptional control in CF, that its deficiency is present in patients with the ΔF508 mutation and experimental CF, and that it is causally linked to pathogenic Th17-cell responses to the fungus. We also obtained a proof-of-concept demonstration that targeting the Th17/Treg-cell imbalance *in vivo* through IDO restoration has curative effects. These findings pave the way to immune therapies in CF that would target pathogenic inflammation via tryptophan metabolism. Some of the results of these studies have been previously reported in the form of an abstract (22).

METHODS

Animals

Six- to 8-week-old C57BL/6 mice were purchased from Charles River (Calco, Italy). Genetically engineered homozygous $Cftr^{-/-}$ mice (23) were bred at the Cystic Fibrosis Core Animal Facility at San Raffaele Hospital (Milan, Italy). $Il10^{-/-}$ and $Indo^{-/-}$ mice were bred at the Animal Facility of the University of Perugia (Perugia, Italy). Experiments were performed according to Italian Approved Animal Welfare Assurance 245-2011-B.

Infection, ABPA, and Treatments *In Vivo*

Mice were anesthetized by intraperitoneal injection of 2.5% Avertin (Sigma Chemical Co, St. Louis, MO) before intranasal instillation of 2×10^7 *A. fumigatus* (Af293) resting conidia per 20 μl of saline. For histology, paraffin-embedded tissue were stained with periodic acid–Schiff (PAS) and Gomori, and bronchoalveolar lavage (BAL) fluid collection was done as described (24). For ABPA, mice received an intraperitoneal and a subcutaneous injection of 100 μg of *A. fumigatus* culture filtrate extract (CCFA) dissolved in Freund's incomplete adjuvant (Sigma) followed by two consecutive intranasal injections (1 wk apart) of 20 μg of CCFA (25). Additional details are reported in the online supplement.

Human Bronchial Epithelial Cells

Human bronchial epithelial (HBE) cells homozygous for the ΔF508 mutation were obtained from three lung transplants (patients with

CF) or lung resections (patients without CF) and cultured as described (26). Unpolarized cells were exposed to recombinant IFN-γ (100 U/ml; Santa Cruz Biotechnology, Santa Cruz, CA), *A. fumigatus* resting conidia at a cell-to-fungi ratio of 2:1, and 10 μg/ml poly(I:C) (Sigma-Aldrich). Cells were incubated for 18 hours at 37°C in 5% CO_2 and then stained with mouse anti-human IDO antibody clone 10.1 (Millipore, Billerica, MA) and analyzed for IDO protein expression by Western blotting and for kynurenine content by high-performance liquid chromatography.

Western Blotting

Blots were incubated with murine anti-human IDO antibody (Millipore) or rabbit polyclonal anti-murine IDO antibodies (27). Normalization was performed with mouse anti–β-tubulin antibody (Sigma-Aldrich) and quantification was obtained by densitometric image analysis using Image Lab 3.1.1 software (Bio-Rad, Segrate, Italy).

Immunohistochemistry

Lung sections were deparaffinized and stained with phycoerythrin (PE)–anti-IL-17A (eBioscience, San Diego, CA), PE–anti-IL-10 (eBioscience), PE–anti–IFN-γ (eBioscience), rabbit polyclonal antibody to forkhead box protein P3 (FOXP3; Abcam, Cambridge, UK), fluorescein isothiocyanate (FITC)–anti-CD4 (Miltenyi Biotec, Bologna, Italy), and polyclonal anti-IDO rabbit antibodies followed by goat anti-rabbit FITC secondary antibody (Sigma-Aldrich).

Terminal Deoxynucleotidyltransferase–mediated Deoxyuridine Triphosphate Nick-End Labeling of Lung Sections

The lungs were fixed in 4% buffered paraformaldehyde, pH 7.3, for 36 hours and embedded in paraffin. Sections were deparaffinized, rehydrated, and treated with 0.1 M citrate buffer, pH 6.0, for 20 minutes in a water bath, and washed and blocked in 0.1 M Tris-HCl buffer, pH 7.5, supplemented with 3% bovine serum albumin and 20% fetal calf serum. The slides were then incubated with fluorescein-coupled dUTP and terminal deoxynucleotidyltransferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) enzyme (Roche Diagnostics, Milan, Italy) in the presence of terminal deoxynucleotidyltransferase. The samples were then washed with phosphate-buffered saline and incubated for 10 minutes at 70°C to remove unspecific binding. The sections were mounted and analyzed by fluorescence microscopy, using a ×40 objective.

Kynurenine and Tryptophan Assay

Kynurenine concentrations (micromolar) in the supernatants of 18-hour HBE cell cultures were measured by high-performance liquid chromatography (11). The kynurenine-to-tryptophan ratio was determined as described (28).

Statistical Analysis

Results are expressed as means ± SEM. Statistical analysis was performed by Student t test or analysis of variance and Bonferroni's test and analyzed with the GraphPad Prism 4.03 program (GraphPad Software, San Diego, CA). Values of P not greater than 0.05 were considered significant.

RESULTS

CF Mice Are Susceptible to *Aspergillus fumigatus* Infection and Sensitization

To assess whether IDO deficiency could affect infection and sensitization to *A. fumigatus* in mice, we evaluated the susceptibility of C57BL/6 and $Cftr^{-/-}$ mice to pulmonary aspergillosis or fungal sensitization in the ABPA model and evaluated parameters of infection, lung inflammation, and innate and adaptive Th immunity. In infection, although fungal growth was only slightly greater in $Cftr^{-/-}$ than control mice (Figure 1A), a sustained and
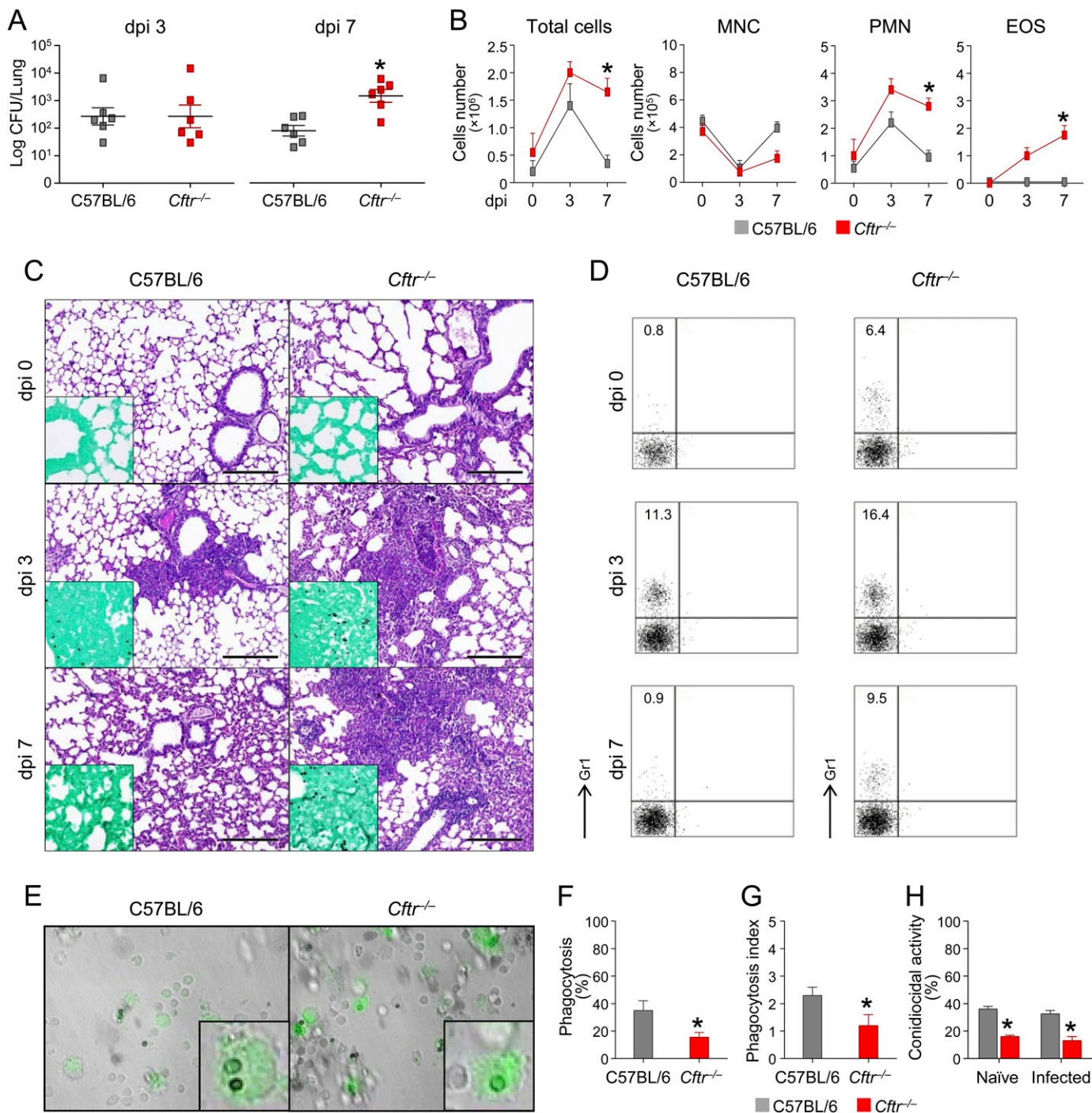
Figure 1. Cystic fibrosis (CF) mice are susceptible to *Aspergillus fumigatus* infection. C57BL/6 and *Cftr*$^{-/-}$ mice (six per group) were infected intranasally with live *A. fumigatus* conidia. (*A*) Fungal growth (log$_{10}$ cfu, mean ± SEM) in the lungs 3 and 7 days postinfection (dpi). (*B*) Bronchoalveolar lavage (BAL) morphometry at various days postinfection. Differential cell count of mononuclear cells (MNC), polymorphonuclear cells (PMN), and eosinophils (EOS) was determined on May-Grünwald Giemsa staining. Values represent the mean ± SEM of three mice per group and are representative of five independent experiments. (*C*) Lung histology (periodic acid–Schiff and, in the *inset*, Gomori staining) at various days postinfection. Photographs were taken with a high-resolution microscope (Olympus DP71) equipped with a ×20 objective and (in the *inset*) a ×40 objective. Scale bars, 200 μm. (*D*) Numbers of granulocyte-differentiation antigen-1 (Gr1)–positive cells were assessed by flow cytometry of total lung cells on various days postinfection. (*E*) Phagocytic activity, (*F*) percent ingestion of *A. fumigatus* conidia, and (*G*) phagocytosis index (mean number of ingested conidia per cell) of total lung cells exposed to live green fluorescent protein–expressing conidia for 2 hours at 37°C (representative microscopy images of two independent experiments). Data were pooled from three independent experiments. (*H*) Conidiocidal activity (percentage of colony-forming unit inhibition [mean ± SE]) of total lung cells from naive or infected mice at 3 dpi. *$P < 0.05$, *Cftr*$^{-/-}$ versus C57BL/6 mice.

persistent inflammatory response, characterized by neutrophil and eosinophil recruitment in the BAL (Figure 1B) and lung (Figures 1C and 1D), was observed in *Cftr*$^{-/-}$ mice, in which

a degree of inflammation was already visible before the infection (Figure 1C). Despite recruitment, both the phagocytic and conidiocidal activities of lung cells were reduced in *Cftr*$^{-/-}$ mice

as compared with wild-type mice (Figures 1E–1H), a finding indicative of an impaired activity of the recruited cells. Defective phagolysosome acidification in the setting of *CFTR* mutation conducive to microbial replication has indeed been described (29). In ABPA, PAS-stained sections of lung indicated a greater degree of mucous cell hyperplasia in *Cftr*^{−/−} than control mice (Figure 2A) that was associated with eosinophil infiltration (Figure 2A, *inset*) and extraordinarily high levels of total serum IgE, reaching over 20,000 pg/ml (Figure 2B), and of *Muc5* mRNA expression in the lungs (Figure 2C). These data suggest that CF mice are susceptible to *Aspergillus* infection and allergy.

Inflammatory Cytokines and Th17/Th2-Cell Responses Are Up-regulated in *Cftr*^{−/−} Mice

The heightened and unresolved inflammatory response in *Cftr*^{−/−} mice was associated with high levels of tumor necrosis factor (TNF)-α, IL-6, and IL-1β in the BAL during infection and, for TNF-α and IL-6, even before infection (Figure 3A). For the assessment of Th-cell activation, we measured levels of Th cytokines in the lungs and of the corresponding transcription factors in the draining thoracic lymph nodes. Th1 cytokines (IL-12p70/IFN-γ) and Th1-cell activation (*Tbet* expression), although higher in uninfected *Cftr*^{−/−} than wild-type mice, failed to increase in infection in *Cftr*^{−/−} mice (Figure 3B). The levels of IL-4 and IgE were higher in *Cftr*^{−/−} than in wild-type mice, both uninfected and throughout infection, whereas the levels of IL-5 and IL-13 increased in *Cftr*^{−/−} mice 1 week after infection, in concomitance with Th2-cell activation (*Gata3* expression)

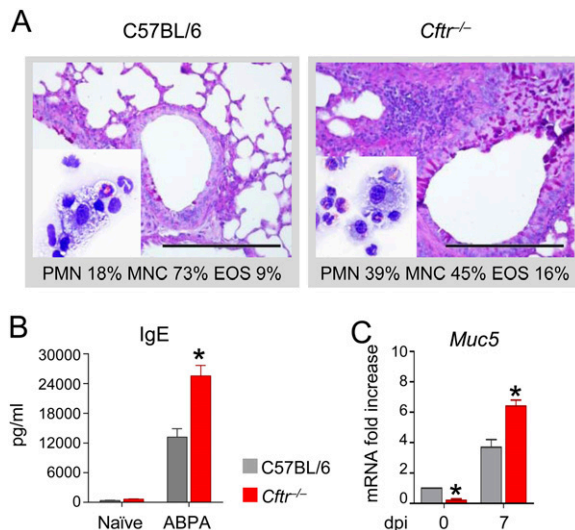PMN 18% MNC 73% EOS 9% PMN 39% MNC 45% EOS 16%

Figure 2. Cystic fibrosis (CF) mice are susceptible to *Aspergillus fumigatus* allergy. Allergic bronchopulmonary aspergillosis (ABPA) was induced by repeated injection of mice with *A. fumigatus* culture filtrate extract dissolved in Freund's incomplete adjuvant followed by intratracheal infection with resting conidia 1 week after the last injection. (*A*) Lung histology (periodic acid–Schiff staining) 1 week after the intranasal infection (numbers refer to percent mononuclear cells [MNC], polymorphonuclear cells [PMN], and eosinophils [EOS] in the bronchoalveolar lavage) (magnified in the *inset*). Photographs were taken with a high-resolution microscope (Olympus DP71) equipped with a ×40 objective (×100 in the *insets*). Scale bars, 200 μm. (*B*) Total serum IgE levels (ELISA) and (*C*) expression of *Muc5* gene (RT-PCR on lung cells) 1 week after the last injection. *P < 0.05, *Cftr*^{−/−} versus C57BL/6 mice. Data are from a representative experiment (histology) or represent pooled results from two independent experiments. dpi = days postinfection.

(Figure 3C). The Th17 cytokines IL-23, IL-17A, and IL-17F started to increase in *Cftr*^{−/−} mice 1 week after infection and remained elevated thereafter (Figure 3D), concomitant with robust Th17-cell activation (*Rorc* expression) (Figure 3D). Despite being produced by Th17 cells, IL-22 has shown autonomous protective functions against fungi (27, 30). In human CF, inducible IL-17^{+}IL-22^{+} memory cells were expanded in the draining lymph nodes (31), but low levels of *IL22* mRNA were observed in the sputum (2). We found that IL-22 production was lower in *Cftr*^{−/−} mice and was associated with progressively down-regulated expression of the aryl hydrocarbon receptor (AhR), a ligand-dependent transcription factor that mediates IL-22 production (32), in the draining lymph nodes (Figure 3E). Finally, the levels of IL-10 were consistently lower in *Cftr*^{−/−} mice than in wild-type mice, as it was with *Foxp3* expression on T cells (Figure 3F). These results indicate that, in addition to Th2-cell responses, heightened Th17-cell responses are observed in *Cftr*^{−/−} mice concomitant with defective protective antifungal responses, such as those mediated by IL-22 (30) and the Th1/Treg axis (33).

IL-17A–Producing γδ T Cells Are Expanded and Foxp3^{+} Treg Cells Are Defective in *Cftr*^{−/−} Mice

Immunostaining of lungs and phenotypic analysis of lung cells confirmed the imbalance of Th17/Treg cells in *Cftr*^{−/−} mice. We found that a population of IL-17A–producing γδ CD3^{+} T cells was expanded in infection in the lungs of *Cftr*^{−/−} mice (Figure 4A), IFN-γ^{+}CD4^{+} T cells were expanded in wild-type mice (Figure 4B), and IL-10–producing (Figure 4C) or Foxp3^{+} (Figure 4D) CD4^{+} T cells were present in wild-type more than in *Cftr*^{−/−} mice. Phenotypic analysis confirmed that γδ CD3^{+} T cells expanded (from 4.4 to 19%) in *Cftr*^{−/−} mice more than in wild-type mice (from 6.3 to 8%) (Figure 4E) and produced IL-17A and not IFN-γ, as revealed by intracellular cytokine staining (Figure 4F). CD4^{+}CD25^{+} T cells were instead present in both types of mice (Figure 4G), although they were not "*bona fide*" Treg cells as they were associated with defective IL-10 production. Thus, IL-17A–producing γδ T cells are expanded in both human (4) and murine CF whereas Foxp3^{+} Treg cells are restrained.

IDO Is Defective in CF Mice

We next evaluated whether defective IDO is demonstrable in *Cftr*^{−/−} mice, either uninfected or infected with *Aspergillus*. The levels of expression of IDO protein, as revealed by Western blotting (Figure 5A) and immunofluorescence staining (Figure 5D), and of the *Ido1* gene (Figure 5B) were lower in the lungs of *Cftr*^{−/−} mice than in wild-type mice, either uninfected or infected. Similarly lower was the kynurenine-to-tryptophan ratio, a reliable indicator of IDO activity (28) (Figure 5C). The expressions of *Kmo*, *Kynu* and *Haao*, a series of genes encoding enzymes downstream of IDO (11), were also reduced in *Cftr*^{−/−} mice (Figure 5E). On assessing *Ido1* expression in purified lung dendritic cells (DCs) in response to various stimuli, we found it lower in *Cftr*^{−/−} DCs than in wild-type DCs (Figure 5F) and associated with less IL-10 and more IL-23 production (Figure 5G). Consistent with the notion that defective IDO in DCs is responsible for deregulated inflammation and immunity to the fungus (25), *Cftr*^{−/−} DCs induced IL-4 and IL-17A, more than IL-10, production on coculture with CD3^{+} T cells from wild-type mice (Figure 5H). A similar pattern of cytokine production was observed in cocultures of CD3^{+} T cells from *Cftr*^{−/−} mice with either type of DCs or on T cell receptor stimulation (Figure 5H). These findings indicated that IDO deficiency in DCs may contribute to the dysfunctional Th-cell reactivity described in CF (34–36) and
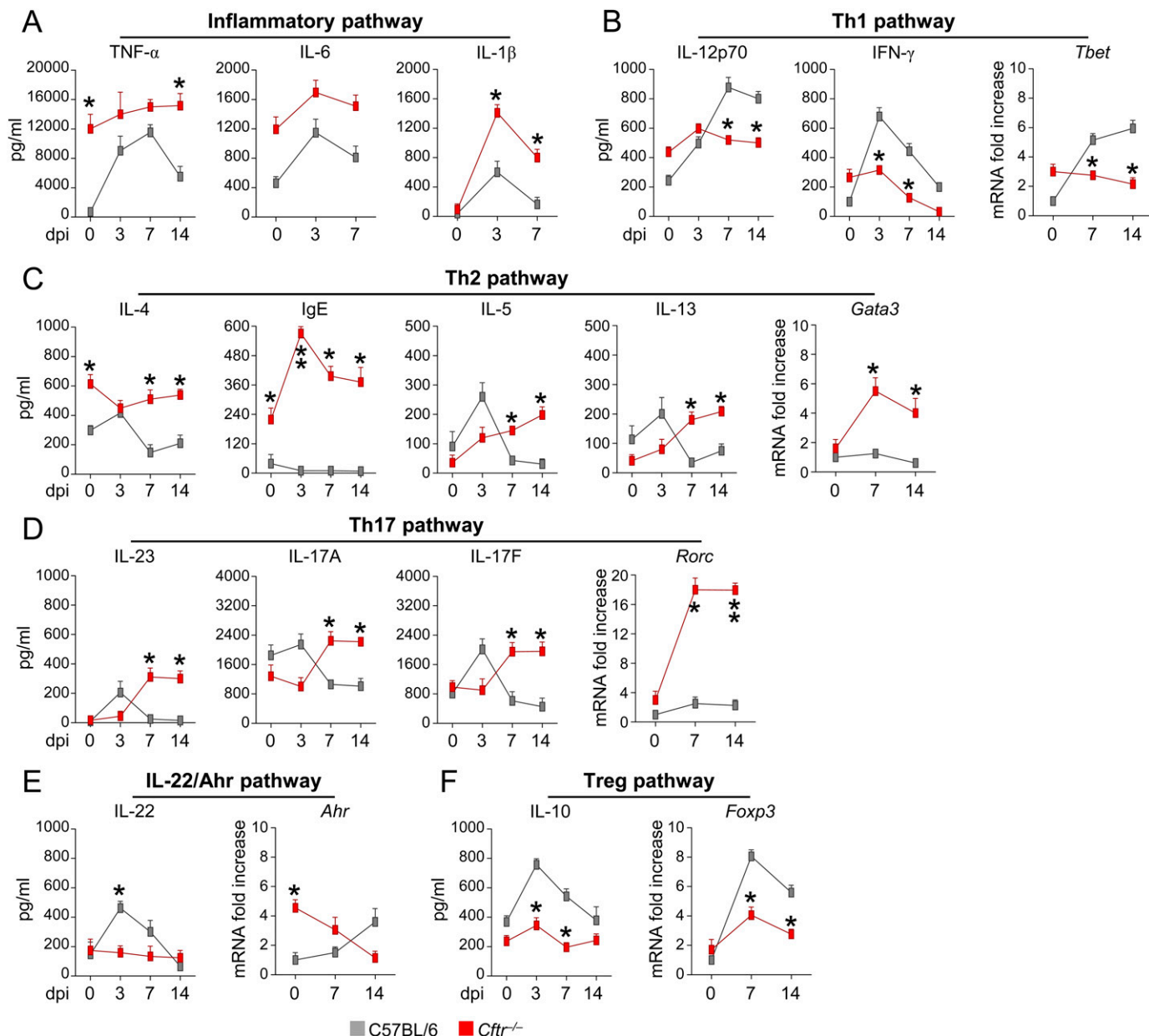
Figure 3. Inflammatory cytokines and type 17 helper T-cell/regulatory T-cell (Th17/Treg) responses are up-regulated in *Cftr*−/− mice. (*A–F*) Time course of cytokine production (mean values ± SEM, ELISA on lung homogenates) on various days postinfection (dpi) and of Th cell–specific transcripts (RT-PCR on purified CD3+ T cells from draining lymph nodes) in C57BL/6 or *Cftr*−/− mice infected intranasally with live *Aspergillus fumigatus* resting conidia. *$P < 0.05$; **$P < 0.01$, *Cftr*−/− versus C57BL/6 mice (data represent pooled results from three experiments). *Ahr* = aryl hydrocarbon receptor; *Foxp3* = forkhead box protein P3; *Rorc* = retinoic acid receptor–related orphan receptor C; *Tbet* = T box expressed in T cells; TNF-α = tumor necrosis factor-α.

prompted us to investigate the environmental mechanisms behind defective IDO activity.

IDO Expression Is Sensitive to Intracellular Ca²⁺ Flux

IDO and tryptophan metabolites are known to impair calcium mobilization in T cells (19). However, whether the enhanced intracellular Ca^{2+} flux and elevated nuclear levels of nuclear factor of activated T cells (NFAT), observed in the absence of CFTR (20, 35) and known to impact on DC functioning (37), negatively affect IDO expression is not known. We found that IDO expression in DCs was sensitive to the Ca^{2+} signaling pathway, as enhancing intracellular Ca^{2+} flux by ionomycin or blocking the calcineurin/NFAT pathway by cyclosporine A (CsA)

inhibited or promoted, respectively, IDO gene (Figure 5I) and protein (Figure 5J) expression. Thus, genetic and transcriptional regulatory mechanisms contribute to dysfunctional IDO activity that, in turn, sustains dysregulated inflammation in CF.

IDO Deficiency Contributes to the Th17/Treg Imbalance in CF Mice

To directly prove that IDO deficiency and the ensuing imbalance between inflammatory Th17 and antiinflammatory Treg cells are causally linked to susceptibility to infection of *Cftr*−/− mice, we resorted to IDO- or IL-10–deficient mice. The inflammation (*see* Figure E1A in the online supplement) and fungal growth (Figure E1B) were both heightened in IDO or IL-10 deficiency
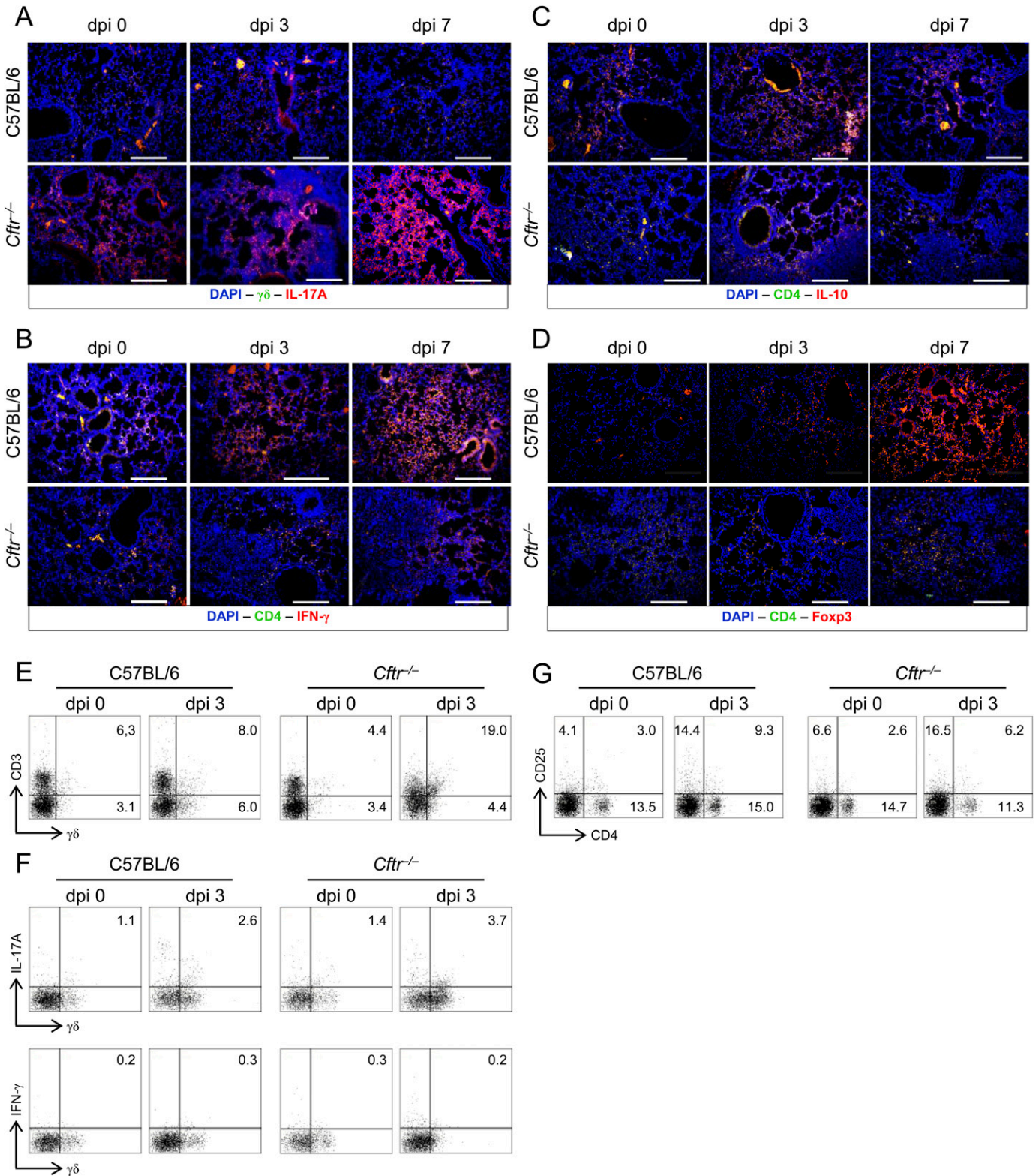
Figure 4. IL-17A–producing γδ T cells are expanded and Foxp3$^+$ regulatory T (Treg) cells are defective in *Cftr*$^{-/-}$ mice. C57BL/6 and *Cftr*$^{-/-}$ mice were infected intranasally with live *Aspergillus fumigatus* conidia. (*A*) Immunofluorescence staining of lungs 0, 3 and 7 days postinfection (dpi) with anti–IL-17A conjugated with phycoerythrin (PE) and anti–γδ conjugated with fluorescein isothiocyanate (FITC), (*B*) anti–IFN-γ PE and anti-CD4 FITC, (*C*) anti–IL-10 PE and anti-CD4 FITC, and (*D*) anti-Foxp3 PE and anti-CD4 FITC. Cell nuclei were stained blue with 4′,6-diamidino-2-phenylindole (DAPI). (*E*) Detection of CD3$^+$ γδ T cells by flow cytometry in the lungs of C57BL/6 or *Cftr*$^{-/-}$ mice either uninfected (0 dpi) or 3 days after infection (3 dpi). (*F*) Intracellular staining for IFN-γ and IL-17A in CD3$^+$ γδ T cells purified from the lungs of C57BL/6 and *Cftr*$^{-/-}$ mice. Numbers refer to percentages of positive cells. (*G*) Detection of CD4$^+$CD25$^+$ cells by flow cytometry in the lungs of C57BL/6 or *Cftr*$^{-/-}$ mice. Numbers refer to the percentages of positive cells. Representative images of two independent experiments were acquired with a ×20 objective. Scale bars, 200 μm.
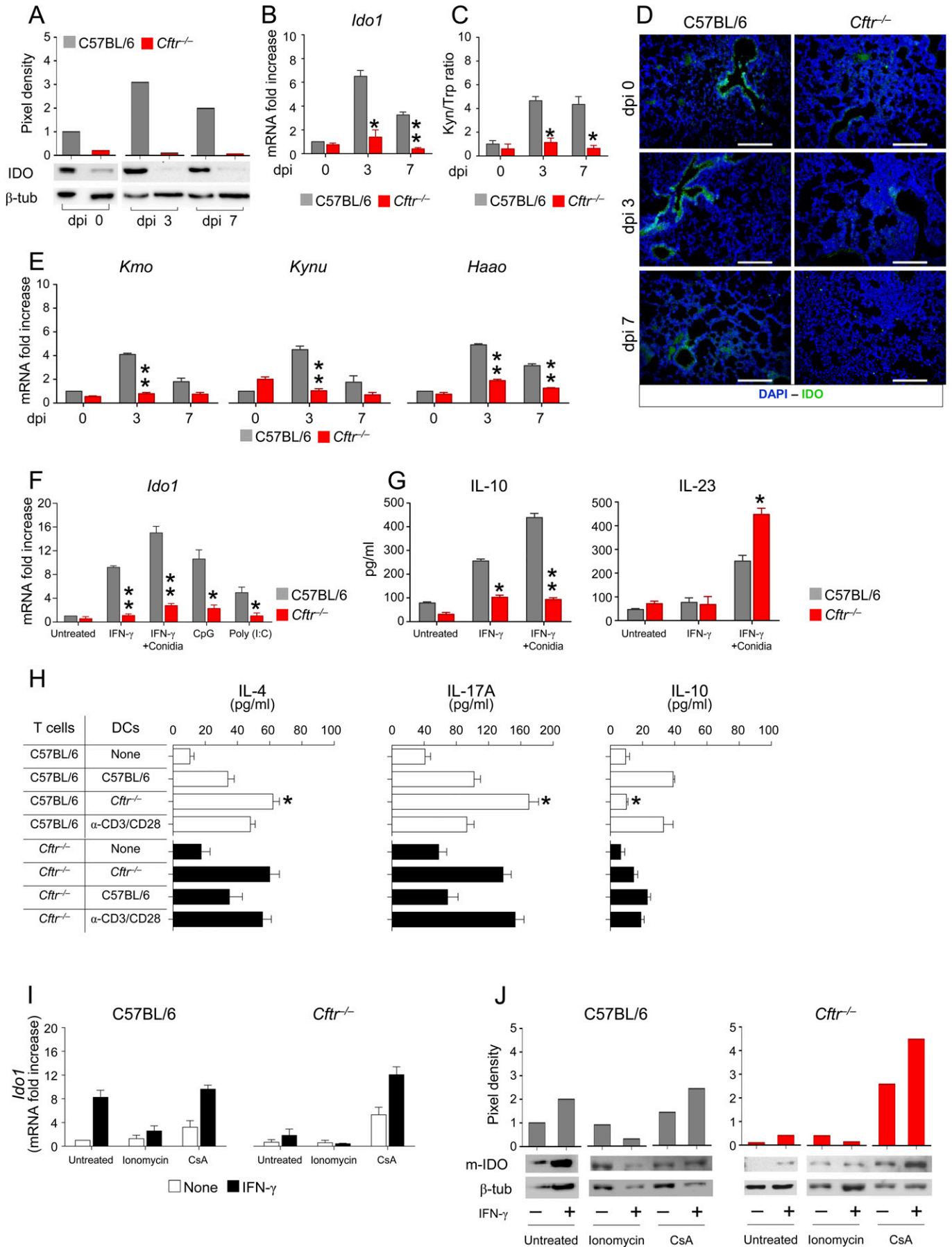
Figure 5. Indoleamine 2,3-dioxygenase (IDO) is defective in cystic fibrosis (CF) mice and contributes to T-cell dysreactivity. Mice (eight per group) were infected intranasally with live *Aspergillus fumigatus* conidia and assessed for IDO expression in the lung. (*A*) IDO protein expression was analyzed by immunoblotting with rabbit polyclonal IDO-specific antibody on whole lung lysates. Shown are representative Western blots of two independent experiments and corresponding pixel density ratio normalized against β-tubulin (β-tub). (*B*) *Indo* transcript expression (RT-PCR) in total lung cells and (*C*) the kynurenine-to-tryptophan ratio in bronchoalveolar lavage (BAL) of infected mice 3 and 7 days postinfection (dpi). (*D*) Immunofluorescence lung staining with anti-IDO antibody followed by fluorescein isothiocyanate (FITC) secondary antibody. Cell nuclei were stained blue with 4′,6-diamidino-2-phenylindole (DAPI). Representative images of two independent experiments were acquired with a ×20 objective. Scale bars, 200 μm. (*E*) Relative expression of *Kmo, Kynu,* and *Haao* (RT-PCR) in lung cells on various days postinfection. (*F*) *Indo* transcript expression (RT-PCR) and (*G*) cytokine production (ELISA) by purified lung dendritic cells (DCs) after an 18-hour exposure to the various stimuli. (*H*) Cytokine production (ELISA) in the supernatant of purified lung T cells from C57BL/6 or *Cftr*⁻/⁻ mice either unstimulated (None) or stimulated *in vitro* with plate-bound anti-CD3 and soluble anti-CD28 or with *Aspergillus*-pulsed lung DCs from either type of mice. Shown are pooled results from three independent experiments (*F* and *H*). (*I*) *Indo* transcript expression (RT-PCR) and (*J*) IDO protein expression (immunoblotting with IDO-specific rabbit polyclonal antibody and corresponding pixel density ratio normalized against β-tubulin) on purified lung DCs unstimulated (–, None) or stimulated with IFN-γ (+) and/or ionomycin or cyclosporine A (CsA) for 18 hours. Shown are representative results of two independent experiments. *$P < 0.05$; **$P < 0.01$, *Cftr*⁻/⁻ versus C57BL/6 mice or *Cftr*⁻/⁻ DCs versus C57BL/6 DCs [in (*H*)].

and correlated with low levels of IL-10 production and high levels of IL-17A (Figure E1C). Moreover, directly targeting the IL-17A/Th17 axis by intranasal delivery of siIL-17A (small interfering RNA against IL-17A) ameliorated inflammatory pathology (Figure E1D) and the Th17/Treg-cell imbalance (Figure E1E) in *Cftr*⁻/⁻ mice. Thus, the Th17/Treg cell imbalance due to IDO deficiency is causally linked to *Aspergillus*-induced inflammation in *Cftr*⁻/⁻ mice.

Restoration of IDO Has Curative Effects in Murine CF

Given the previously described findings, we tested the efficacy of IDO restoration therapies in *Cftr*⁻/⁻ mice with aspergillosis by subjecting them to treatment with IFN-γ, CsA, and replacement therapy with a mixture of L-kynurenines, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid downstream in the IDO pathway (11). Each treatment reversed tissue inflammation (Figure 6A) and damage (Figure 6B) and readdressed the Th17/Th2/Treg-cell balance in *Cftr*⁻/⁻ mice (Figures 6C and 6D). Treatment with IFN-γ and CsA also induced IDO protein (Figure 6E) and gene expression (Figure 6F) and, together with kynurenines, the expression of genes encoding enzymes downstream of IDO (Figure 6G). These findings expand on our previous data showing the effective treatment of experimental lung inflammation with IDO inducers (11) and reveal a novel mechanism of action for CsA in targeting pathogenic lung inflammation in CF.

IDO Is Defective in Patients with CF

Defective tryptophan uptake, eventually leading to low kynurenine production, has been demonstrated in human bronchial epithelial (HBE) cells from patients with CF (26). To assess whether defective IDO could also be demonstrated in HBE cells from patients with CF, we evaluated IDO protein expression in HBE cells from three patients with CF (26) and non-CF control subjects on exposure to *Aspergillus* conidia and IFN-γ (38) or poly(I:C), known IDO inducers (27). IDO protein expression and function were lower in mutant than in control cells, with and without IFN-γ, as revealed by immunofluorescence staining (Figure 7A), Western blotting (Figure 7B), and decreased levels of secreted kynurenines (Figure 7C). Poly(I:C) restored, albeit partially, IDO expression and function in CF HBE cells (Figures 7A–7C), suggesting that, like in murine CF, nongenetic control over IDO expression may occur in human CF.

DISCUSSION

The results of this study show that decreased tryptophan/kynurenine metabolism may contribute to the inflammatory process in CF. The finding that IDO deficiency was causally linked to

a Th17/Treg-cell imbalance responsible for *Aspergillus*-mediated lung inflammation in experimental CF strongly suggests that IDO may contribute to lung dysfunction and inflammation in CF patients with aspergillosis. Preliminary single-nucleotide polymorphism (SNP) analysis revealed that genetic variants affecting the function of IDO were present in patients with CF and significantly modulated predicted FEV₁ values, a finding consistent with the role of IDO in lung function normalization during lung transplantation (19). However, further studies are needed to establish the possible link between the *IDO* SNPs and *Aspergillus* diseases in human CF.

Antiinflammatory drugs have been show to be beneficial in CF patients with ABPA (39). However, adverse effects may limit therapy with oral corticosteroids and ibuprofen (40), and long-term azithromycin therapy has been associated with *Aspergillus* colonization (41). Thus, to optimize antiinflammatory therapy, it is necessary to further unravel the pathomechanisms of *Aspergillus* disease in CF (42). We found that, in addition to heightened Th2-inflammatory responses (43, 44), Th17 cells are activated in response to the fungus and contribute to the state of chronic lung inflammation in experimental CF. Reduction of the inflammatory response and promotion of more efficient pathogen clearance were achieved by antagonizing Th17-cell responses, a finding consistent with previous observations on *Pseudomonas aeruginosa* infection in *Cftr*⁻/⁻ mice (45). As pathogenic Th17-cell responses are also associated with candidiasis (9), it is not surprising that children with CF had high prevalence of colonization by *Candida albicans* (46), whose sensitization has been shown to contribute to ABPA (41). In addition to inhibiting IDO (47), IL-17A was reported to increase fungal adaptation and virulence (48) as well as expression of nucleotide-binding oligomerization domain (NOD)–like receptors (49) that may contribute to unrestrained inflammation in CF through sustained inflammasome activation (50). Thus, restraining Th17-dependent inflammation may represent a targeted general therapy in CF (4, 51). However, one novel finding of this study is that replacement therapy with kynurenines also limits the effects of a deregulated inflammatory process in experimental CF. Perhaps more importantly, complete cure and reversal of the hyperinflammatory CF phenotype were achieved by administration of recombinant IFN-γ, a known IDO inducer, or supplemental kynurenines. Targeting IDO resulted in enhanced production of IL-10 and expression of Foxp3, both markers of Treg activity in *Aspergillus* infection and allergy (52). IL-10 production is defective in patients with CF (53) and heterogeneity in the *IL10* gene may influence the response to *A. fumigatus* infection and the occurrence of *A. fumigatus*–related lung disease in patients with CF (54). *IL10* gene therapy was indeed successful in dampening fungal allergy in experimental CF, but side effects were observed on prolonged secretion of
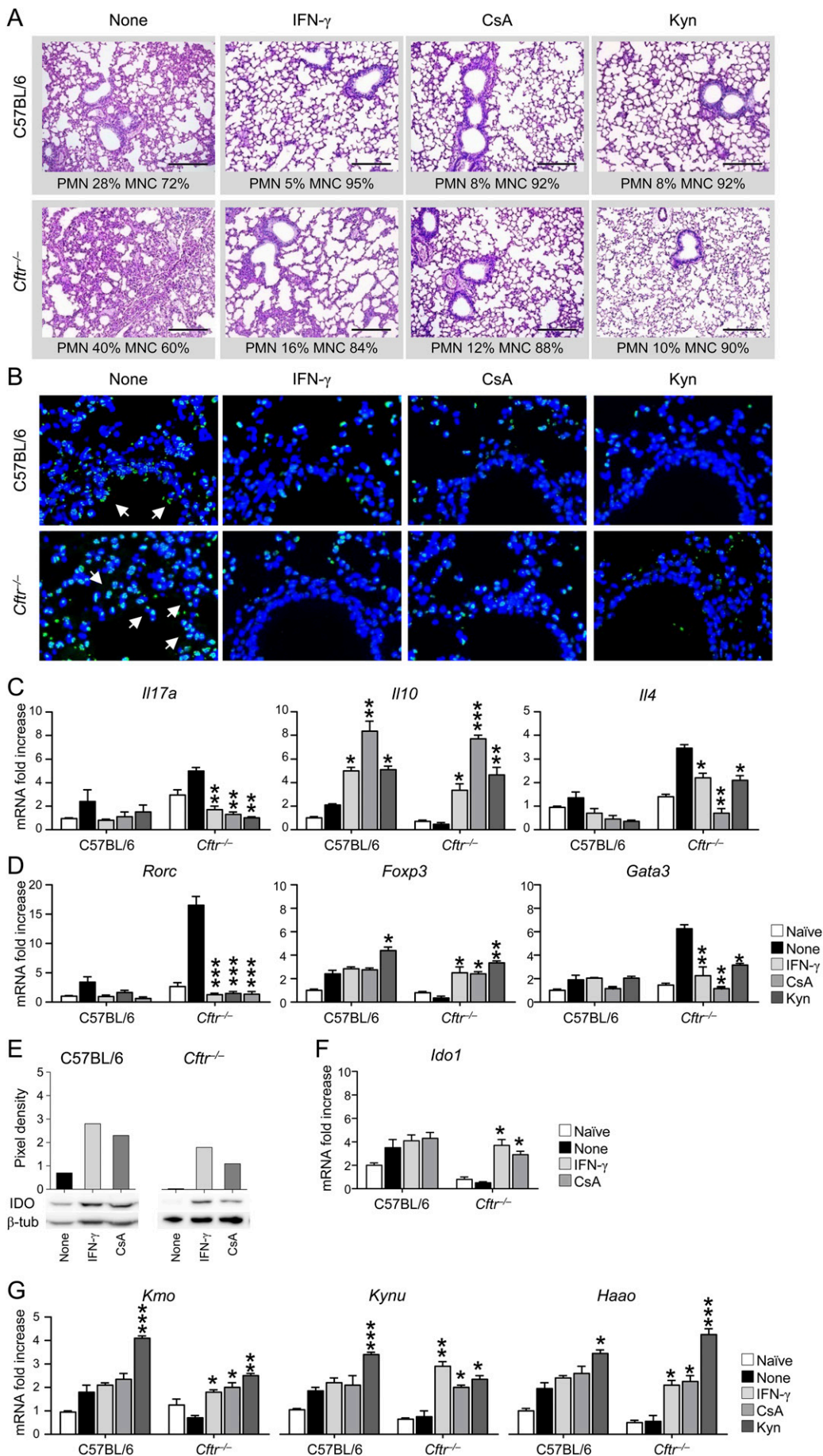
Figure 6. Restoration of indoleamine 2,3-dioxygenase (IDO) has curative effects in murine cystic fibrosis (CF). Mice (six per group) were intranasally infected with *Aspergillus* conidia and treated daily with IFN-γ, cyclosporine A (CsA), or a mixture of L-kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid, starting 3 days before and up to 7 days after infection. (*A*) Lung histology (periodic acid–Schiff staining) and bronchoalveolar lavage morphometry (%, mean ± SEM, of mononuclear cells [MNC] or polymorphonuclear cells [PMN]). Photographs were taken with a high-resolution microscope (Olympus DP71) equipped with a ×20 objective. Scale bars, 200 μm. (*B*) Increased deposition of DNA on lung parenchyma cells on staining by terminal deoxynucleotidyltransferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL), resulting in bright DNA staining. *Arrows*, DNA deposits. Representative staining from two independent experiments is shown. The images were acquired with a fluorescence microscope equipped with a ×40 objective. (*C*) Cytokine and (*D*) transcription factor gene expression were assessed by RT-PCR on total lung cells or purified CD3$^+$ T cells from the thoracic lymph nodes, respectively. (*E*) IDO protein expression (immunoblotting with rabbit polyclonal IDO-specific antibody on lung whole cell lysates) and (*F*) Indo and (*G*) kynurenine pathway gene expression (RT-PCR on total lung cells) in mice infected and treated as described previously. Scanning densitometry was done with Image Lab 3.1.1 software. Shown are representative Western blots of two independent experiments and corresponding pixel density ratio normalized against β-tubulin ((β-tub). *$P <$ 0.05,**$P <$ 0.01, ***$P <$ 0.001, treated versus untreated (None) mice.
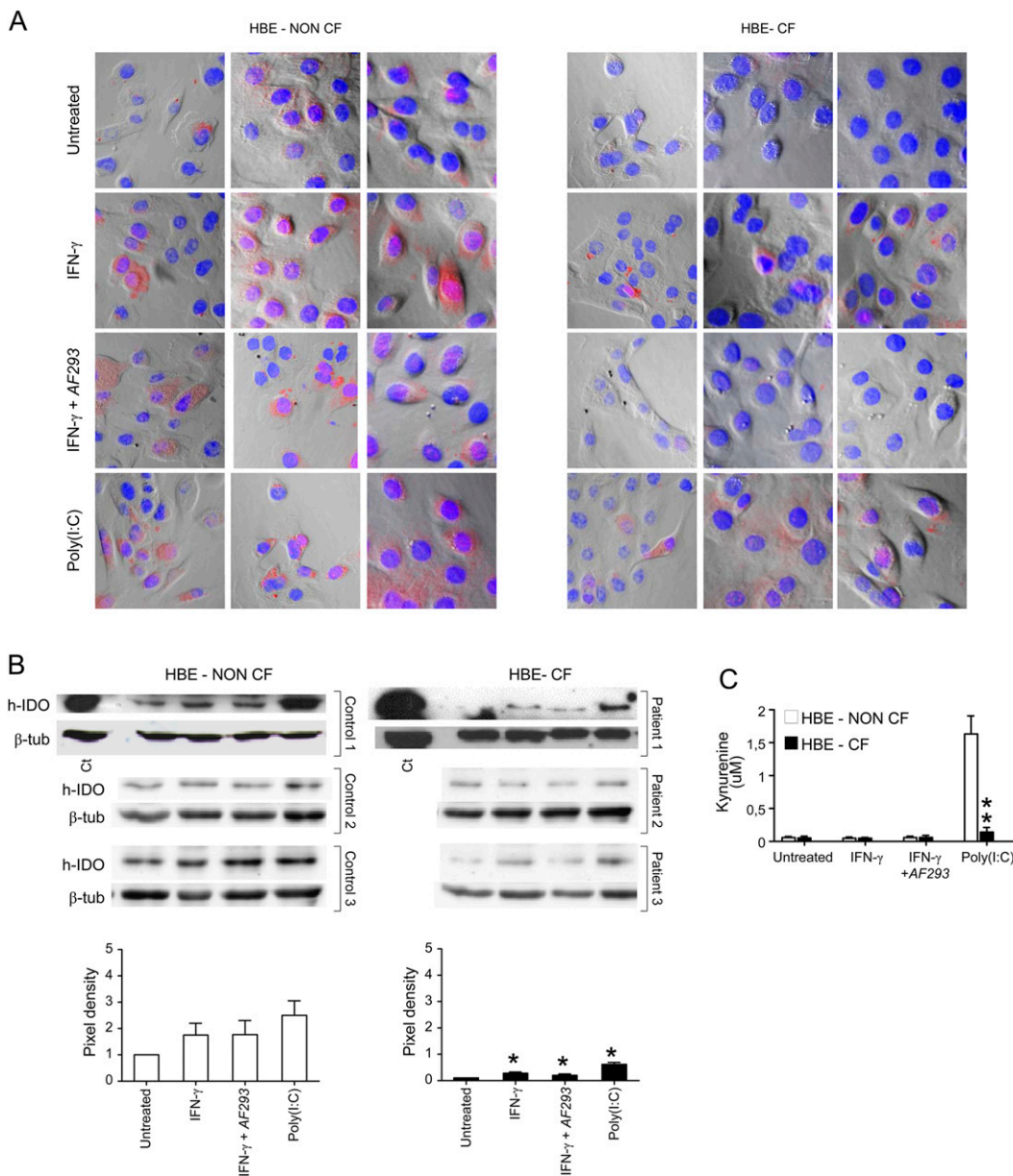
Figure 7. Indoleamine 2,3-dioxygenase (IDO) expression in patients with cystic fibrosis (CF). Human bronchial epithelial (HBE) cells homozygous for the ΔF508 mutation and control cells (n = 3) were exposed to human recombinant IFN-γ and/or *Aspergillus fumigatus* (Af293) conidia or poly(I:C) for 18 hours at 37°C in 5% CO_2. (*A*) Cultures growing on culture slides were fixed and incubated with anti-IDO antibodies. Images were acquired with an Olympus BX51 fluorescence microscope with a ×40 objective and analySIS image-processing software (Olympus). 4',6-Diamidino-2-phenylindole (DAPI) was used to detect nuclei. Shown are representative images of two independent experiments. (*B*) IDO protein expression was analyzed by immunoblotting with IDO-specific antibody on HBE cells homozygous for the ΔF508 mutation and control cells (n = 3). Scanning densitometry was done with Image Lab 3.1.1 software. Shown are representative Western blots of two experiments and the corresponding pixel density ratio (mean ± SEM of three values) normalized against β-tubulin (β-tub). (*C*) Kynurenine levels (by high-performance liquid chromatography) in supernatants of HBE cells stimulated as described previously (mean ± SEM of three values). *$P < 0.05$; **$P < 0.01$, HBE-CF versus HBE–non-CF.

IL-10 (55). In contrast, the low-frequency Treg-cell population observed in CF patients with ABPA could be increased by treatment with vitamin D (36), a finding emphasizing the usefulness of targeting Tregs to prevent or treat lung pathology.

One interesting observation of the present study is the reciprocal cross-regulation between IDO and Ca^{2+} mobilization in DCs. On the one hand, IDO and kynurenines have been shown to impair calcium mobilization of T cells (19); on the other, we found that IDO expression in DCs is sensitive to intracellular Ca^{2+} levels, a finding consistent with the effects of calcineurin/NFAT signaling on DCs (37). Thus, altered Ca^{2+} signaling pathways in CF cells not only contribute to T-cell dysreactivity (20) but also limit IDO functioning in tolerogenic DCs. Considering that IDO expression on epithelial cells (ECs) was similarly sensitive to intracellular Ca^{2+} levels (data not shown), these data further point to the critical role ECs may have in response to *A. fumigatus* in CF (56). The mechanisms underlying the regulation of IDO by intracellular Ca^{2+} signaling is presently under investigation. Preliminary observation failed to show a consensus binding sequence for NFAT on the *IDO* promoter, a finding indicating

that other components of the Ca^{2+} signaling pathway are involved. Irrespective of the molecular mechanism, the finding that inhibition of the Ca^{2+}–calcineurin–NFAT signaling pathway with CsA restored IDO activity is novel and is consistent with the ability of CsA to inhibit Th17-cell activation (57) and to improve clinical signs and symptoms in patients with CF (58). As calcineurin inhibition is also known to impair virulence in *A. fumigatus* (59), these findings point to the successful manipulation of this pathway to improve outcome in CF patients with aspergillosis.

Within the priority areas identified for CF (60), the present study identifies a novel pathogenic mechanism that may contribute to CF lung disease, provides indications for immunogenetic screening of *IDO1* and kynurenine levels as biomarkers that reflect CF pathophysiology, and suggests new therapeutics in CF lung diseases. Current CF treatments that target respiratory infections, mucociliary clearance, and nutritional status are associated with improved pulmonary function and reduced exacerbations (61, 62). Within the aberrant pulmonary inflammation seen in response to the fungus in the setting of *CFTR* mutation, a role for antifungals to halt progressive CF lung disease has been

suggested (56). With the exception of the first randomized controlled clinical trial showing the nonclinical benefit of itraconazole in patients with CF (63), there are at present no other randomized controlled trials to evaluate the use of antifungal therapies (64) or prevent resistance to antifungal therapy in people with CF (13, 65). Our study clearly indicates that genetic and epigenetic mechanisms collude to impair IDO activity and promote Th17-dependent inflammatory pathology in CF. Thus, the inability to properly handle respiratory fungal pathogens could be primarily responsible for the state of chronic inflammation seen in patients with CF, and this predicts that combination strategies in CF should target primary inflammation along with pathogen eradication.

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