

Tolerogenic signaling by pulmonary CD1c⁺ dendritic cells induces regulatory T cells in patients with chronic obstructive pulmonary disease by IL-27/IL-10/inducible costimulator ligand

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Background: Increased mortality rates in patients with chronic obstructive pulmonary disease (COPD) are largely due to severe infectious exacerbations. Impaired respiratory immunity is linked to the enhanced susceptibility to infections. Dendritic cells (DCs) direct host immune responses toward immunity or tolerance. Pulmonary CD1c⁺ DCs elicit robust antiviral immune responses in healthy subjects. Nevertheless, their functional specialization in patients with COPD remains unexplored.

Objective: We sought to better understand the mechanisms that suppress respiratory immunity in patients with COPD by examining the immunostimulatory and tolerogenic properties of pulmonary CD1c⁺ DCs.

Methods: We analyzed the expression of costimulatory and tolerogenic molecules by pulmonary CD1c⁺ DCs from patients with COPD (CD1c⁺DC_{COPD}) and former smokers without COPD. We isolated lung CD1c⁺ DCs and determined their ability to stimulate allogeneic T-cell responses. The suppressive effects of lung CD1c⁺ DCs and CD1c⁺ DC-primed T cells on mixed leukocyte reactions were examined. An experimental

human model of COPD exacerbation was used to investigate the levels of critical immunosuppressive molecules *in vivo*.

Results: CD1c⁺ DCs from patients with COPD hinder T-cell effector functions and favor the generation of suppressive IL-10-secreting CD4⁺ T cells that function through IL-10 and TGF-β. IL-27, IL-10, and inducible T-cell costimulator ligand signaling are essential for CD1c⁺DC_{COPD}-mediated differentiation of IL-10-producing suppressive T cells. Exposure of lung CD1c⁺ DCs from nonobstructed subjects to lungs of patients with COPD confers tolerogenic properties. IL-27 and IL-10 levels are increased in the lung microenvironment on rhinovirus-induced COPD exacerbation *in vivo*.

Conclusion: We identify a novel tolerogenic circuit encompassing suppressive CD1c⁺ DCs and regulatory T cells in patients with COPD that might be implicated in impaired respiratory immunity and further highlight IL-10 and IL-27 as potent therapeutic targets. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: Chronic obstructive pulmonary disease, dendritic cells, regulatory T cells, IL-27, IL-10, inducible costimulator ligand

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Chronic obstructive pulmonary disease (COPD) is a growing epidemic and is currently ranked fourth as a leading cause of death worldwide.¹ It is characterized by irreversible airflow limitation associated with a chronic lung inflammatory response to inhaled noxious particles or gases, mainly cigarette smoke.² Acute exacerbations represent a major cause of pulmonary deterioration and mortality in patients with COPD.³ They are often precipitated by a viral infection and complicated by secondary bacterial infections,⁴ suggesting an impaired ability of immune cells to clear pathogens.⁵ Of note, our unique human model of rhinovirus infection shows that patients with COPD have more severe lower respiratory tract symptoms and increased viral loads, which are associated with impaired IFN-β release by bronchoalveolar lavage (BAL) cells, compared with smokers.⁶ Still, the precise mechanisms underlying impaired respiratory immunity in patients with COPD remain elusive.

Dendritic cells (DCs) are potent antigen-presenting cells (APCs) directing host immune responses toward either immunity or tolerance.⁷ DCs exhibit considerable plasticity depending on the antigens they encounter, the cytokine milieu, and the context of the immune response.⁸ In human lungs various DC subsets have been identified, among which CD1c⁺ DCs represent the predominant and best characterized subset.⁹⁻¹² In healthy subjects pulmonary CD1c⁺ DCs display a higher maturation profile and

Abbreviations used

AHR: Aryl hydrocarbon receptor

APC: Antigen-presenting cell

BAL: Bronchoalveolar lavage

CD1c⁺DC_{COPD}: CD1c⁺ DCs from lung samples obtained from former smokers with COPDCD1c⁺DC_{N-SM}: DCs from lung samples obtained from never smokersCD1c⁺DC_{SM}: DCs from lung samples obtained from former smokers without COPD

CFSE: Carboxyfluorescein succinimidyl ester

CM: Conditioned medium

COPD: Chronic obstructive pulmonary disease

DC: Dendritic cell

FOXP3: Forkhead box protein 3

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

ICOS: Inducible T-cell costimulator

ICOS-L: Inducible T-cell costimulator ligand

IL-10R: IL-10 receptor

ILT4: Ig-like transcript 4

LH: Lung homogenate

LH_{COPD}: LHs obtained from patients with COPDLH_{SM}: LHs obtained from former smokers without COPD

MAF: Musculoaponeurotic fibrosarcoma oncogene homolog

NE: Neutrophil elastase

PD-1: Programmed cell death 1

PD-L1: Programmed death ligand 1

RORC: Retinoic acid-related orphan receptor C

SLPI: Secretory leukocyte protease inhibitor

TBX21: T-box 21

TCM: Tissue culture medium

Treg: Regulatory T

are superior in eliciting CD4⁺ T-cell responses compared with other DC subsets.⁹⁻¹² Furthermore, they are particularly efficient at cross-presentation of viral antigens and mount stronger antiviral CD8⁺ T cell-mediated responses.¹² In patients with COPD, CD1c⁺ DCs express high levels of costimulatory molecules that correlate with disease severity¹³; even so, their involvement in disease pathogenesis remains unexplored. Understanding the role of CD1c⁺ DCs in respiratory immunity in patients with COPD is critical for the design of effective immunotherapies aiming to ameliorate infectious exacerbations.

Here we isolated CD1c⁺ DCs from peripheral lung tissue explants, the principal affected tissue site in patients with COPD, and investigated their phenotype and functional specialization *ex vivo*. Our findings highlight unique immunosuppressive functions for lung CD1c⁺ DCs in patients with COPD. In fact, lung CD1c⁺ DCs from patients with COPD favor the differentiation of IL-10-secreting CD4⁺ T cells, which suppress other T-cell responses through IL-10- and TGF- β -dependent mechanisms. IL-27, IL-10, and inducible T-cell costimulator ligand (ICOS-L) are uncovered as critical mediators involved in CD1c⁺ DCs from patients with COPD-mediated T-cell regulation and suppressive T-cell differentiation. Moreover, *in vivo* rhinovirus infection upregulates IL-10 and IL-27 in the BAL fluid of patients with COPD but not in that of nonobstructed smokers. Collectively, we identify a novel tolerogenic circuit encompassing regulatory lung CD1c⁺ DCs and IL-10-secreting suppressive T cells in patients with COPD, and we uncover CD1c⁺ DCs as a novel therapeutic target that could be regulated to restore impaired respiratory immunity in patients with COPD.

METHODS

For more information, see the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Subjects

Lung tissue samples were obtained from patients undergoing lobectomy at the thoracic surgical clinics of Evangelismos General Hospital and Sotiria Chest Hospital in Athens, Greece, as described in [Table 1](#). For the experimental rhinovirus infection study, subjects were recruited from the Imperial College Healthcare NHS trust (St Mary's Hospital), the Royal Brompton Hospital, local general practices, and by advertisement, as previously described ([Table 1](#)).^{4,6} BAL samples were obtained before and on day 7 after rhinovirus 16 inoculation. All studies were approved by institutional review boards, and informed consent was obtained.

Human lung tissue preparation

Lung tissue was minced with scissors and incubated in digestion medium to obtain single-cell suspensions.^{10,11} Lung homogenates (LHs) were generated by using an electric rotor-stator homogenizer.

Isolation and stimulation of human lung CD1c⁺ DCs

CD1c⁺ DCs were isolated by using paramagnetic beads.^{10,11,14} Lung CD1c⁺ DCs were cultured with LPS or ovalbumin–Alexa Fluor 488 and analyzed by using flow cytometry. In other experiments CD1c⁺ DCs were pretreated with LHs in the presence or absence of secretory leukocyte protease inhibitor (SLPI) or neutralizing mAbs against IFN- γ or TNF- α and were analyzed by using flow cytometry or quantitative real-time PCR or cocultured with allogeneic naive CD4⁺ T cells. T-cell proliferation was measured based on tritiated thymidine incorporation.

Allogeneic mixed leukocyte reaction

CD1c⁺ DCs were cocultured with allogeneic naive CD4⁺ T cells (primary induction cultures) in the presence or absence of neutralizing mAbs against IL-10 receptor (IL-10R), IL-27, ICOS-L, Ig-like transcript 4 (ILT4), or programmed death ligand 1 (PD-L1). In further studies culture supernatants of lung CD1c⁺ DC–allogeneic CD4⁺ T-cell cocultures were added to fresh mixed leukocyte reactions (MLRs). Alternatively, CD1c⁺ DC–primed CD4⁺ T cells were isolated from the primary induction cultures and analyzed by using quantitative real-time PCR or flow cytometry or used in *in vitro* suppression assays. In other experiments allogeneic naive CD4⁺ T cells were cocultured with CD3-depleted PBMCs in the presence or absence of titrated numbers of lung CD1c⁺ DCs.

Autologous lung T-cell–CD1c⁺ DC assays

Lung CD3⁺ T cells were purified by using paramagnetic beads, labeled with carboxyfluorescein succinimidyl ester (CFSE), and cocultured with autologous lung CD1c⁺ DCs.

***In vitro* suppression assays**

Allogeneic naive CD4⁺ T cells were stimulated with APCs or soluble anti-CD3/anti-CD28 mAbs in the presence of CD1c⁺ DC–primed CD4⁺ T cells and/or neutralizing antibodies against IL-10R, TGF- β 1, TGF- β 2, or TGF- β 3. Alternatively, CD4⁺ T cells were isolated from a primary MLR and restimulated with allogeneic APCs in a secondary MLR in the presence or absence of CD1c⁺ DC–primed CD4⁺ T cells.

Cytokine assays

Cytokines were measured with commercially available ELISA kits or by using Luminex (Affymetrix, Santa Clara, Calif).

Quantitative real-time PCR

Total RNA was isolated and reverse transcribed with Superscript II (Invitrogen, Carlsbad, Calif). Human forkhead box protein 3 (*FOXP3*),

TABLE I. Clinical characteristics of subjects

Subject characteristics	Lung tissue studies			BAL studies		
	Never smokers	Smokers without COPD	Patients with COPD	Never smokers	Smokers without COPD	Patients with COPD
No.	7	44	41	13	12	19
Sex (M/F)	4/3	37/7	37/4	8/5	10/2	16/3
Age (y)	64 ± 13	64 ± 8	66 ± 7	58 ± 7	58 ± 6	58 ± 9
PPY	0 ± 0	76 ± 37	79 ± 27	0 ± 0	35 ± 7	41 ± 8
FEV ₁	102 ± 12	96 ± 12	68 ± 15	105 ± 8	103 ± 10	67 ± 13
GOLD stage						
I	NA	NA	14	NA	NA	2
II	NA	NA	19	NA	NA	16
III	NA	NA	8	NA	NA	1
IV	NA	NA	—	NA	NA	—

Results are presented as means ± SDs or absolute values.

F, Female; GOLD stages, severity stages according to the European Respiratory Society/American Thoracic Society consensus statement²; M, male; NA, not applicable; PPY, pack year (smoking on average of 1 pack of cigarettes per day each year is defined as 1 pack year).

GATA3, T-box 21 (*TBX21*), aryl hydrocarbon receptor (*AHR*), musculoaponeurotic fibrosarcoma oncogene homolog (*MAF*), *IL10*, *IL27*, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA levels were analyzed (see Table E1 in this article's Online Repository at www.jacionline.org).

Statistical analysis

GraphPad Prism software (version 6; GraphPad Software, La Jolla, Calif) was used. Multiple comparisons of sampled data were made by using the Kruskal-Wallis test. The Mann-Whitney *U* test or Wilcoxon matched-pairs test was used for pairwise comparisons. A *P* value of less than .05 was accepted as significant.

RESULTS

Lung CD1c⁺ DCs in patients with COPD display a semimature phenotype *in vivo* and are hyporesponsive to stimulation with LPS *ex vivo*

CD1c⁺ DCs were analyzed in lung samples obtained from former smokers with COPD (CD1c⁺DC_{COPD}), former smokers without COPD (CD1c⁺DC_{SM}), and never smokers (CD1c⁺DC_{N-Sm}) by means of flow cytometry by gating on low autofluorescent CD1c⁺CD3[−]CD19[−] SYTOX green-negative cells (Table I).^{10,11,13,14} The numbers of CD1c⁺ DCs in lung single-cell suspensions were similar between the 3 study groups (see Fig E1 in this article's Online Repository at www.jacionline.org). Interestingly, there was a striking decrease in CD40 expression in lung CD1c⁺DC_{COPD} compared with CD1c⁺DC_{SM} and CD1c⁺DC_{N-Sm} accompanied by a decrease in CD86 levels compared with those seen in CD1c⁺DC_{N-Sm} (Fig 1, A and B). HLA-DR and CD54 expression were similar among groups (Fig 1, A and B, and data not shown). Furthermore, CD1c⁺DC_{COPD} exhibited significantly lower levels of the lymph node-homing chemokine receptor CCR7 concomitant with higher expression of CCR5, which controls DC trafficking to the lungs (Fig 1, A and B). CCR6 levels were similar in all groups studied (data not shown). Overall, our findings show that the lung CD1c⁺DC_{COPD} display a semimature phenotype *in vivo*.

To gain further insight into the functional specialization of lung CD1c⁺DC_{COPD}, we investigated their potential to endocytose antigen and to respond to maturation stimuli *ex vivo*. CD1c⁺DC_{COPD} displayed a significantly lower capacity to endocytose Alexa Fluor-conjugated ovalbumin compared with CD1c⁺DC_{SM} and CD1c⁺DC_{N-Sm} cells (Fig 1, C). Although expression of HLA-DR, CD86, and CD40 was substantially

increased in lung CD1c⁺DC_{SM} and CD1c⁺DC_{N-Sm} after exposure to LPS, lung CD1c⁺DC_{COPD} did not upregulate CD40, whereas CD86 levels were only slightly increased (Fig 1, D). Importantly, LPS-stimulated CD1c⁺DC_{COPD} produced significantly lower levels of proinflammatory TNF-α, IL-6, and IL-23 in culture supernatants, whereas they secreted higher levels of the immunomodulatory cytokines IL-10 and IL-27 (Fig 1, E).

Lung CD1c⁺DC_{COPD} possess enhanced tolerogenic properties and suppress CD4⁺ T-cell responses

The aforementioned findings led us to explore whether CD1c⁺DC_{COPD} express cell-surface inhibitory molecules, a hallmark of immunoregulatory DCs.^{15–17} ILT4, ICOS-L, and PD-L1 levels were significantly increased on CD1c⁺DC_{COPD} compared with those seen on CD1c⁺DC_{SM} and CD1c⁺DC_{N-Sm} (Fig 2, A and B). We next sought to investigate the immunostimulatory capacity of lung CD1c⁺DC_{COPD}. Priming of CD4⁺ T cells with lung CD1c⁺DC_{SM} or CD1c⁺DC_{N-Sm} resulted in substantial alloantigen-driven T-cell proliferation and IFN-γ release (Fig 2, C). In marked contrast, allogeneic CD4⁺ T cells stimulated with lung CD1c⁺DC_{COPD} exhibited poor proliferative potential and produced low IFN-γ levels (Fig 2, C).

Subsequently, we examined whether lung CD1c⁺DC_{COPD} exerted enhanced tolerogenic functions. For this, we assessed the ability of CD1c⁺ DCs to inhibit MLRs consisting of naive CD4⁺ T cells stimulated with allogeneic APCs.¹⁸ Remarkably, lung CD1c⁺DC_{COPD} suppressed robust T-cell responses induced by allogeneic APCs, whereas CD1c⁺DC_{SM} and CD1c⁺DC_{N-Sm} had no discernible inhibitory effects (Fig 2, D and E). These data highlight a dominant tolerogenic effect of lung CD1c⁺DC_{COPD} that mitigates the stimulatory capacities of fully competent APCs.

CD1c⁺DC_{COPD} favor the generation of IL-10-secreting CD4⁺ T cells

Immature DCs can hinder T-cell responses by converting naive T cells into regulatory T (Treg) cells.²⁰ Hence we sought to delineate whether CD4⁺ T cells stimulated with lung CD1c⁺DC_{COPD} acquired the characteristics of Treg cells. CD1c⁺DC_{COPD}-primed CD4⁺ T cells expressed enhanced levels of programmed cell death 1 (PD-1), PD-L1, and ICOS (Fig 3, A). Cytotoxic T lymphocyte-associated antigen 4 and HLA-G, the

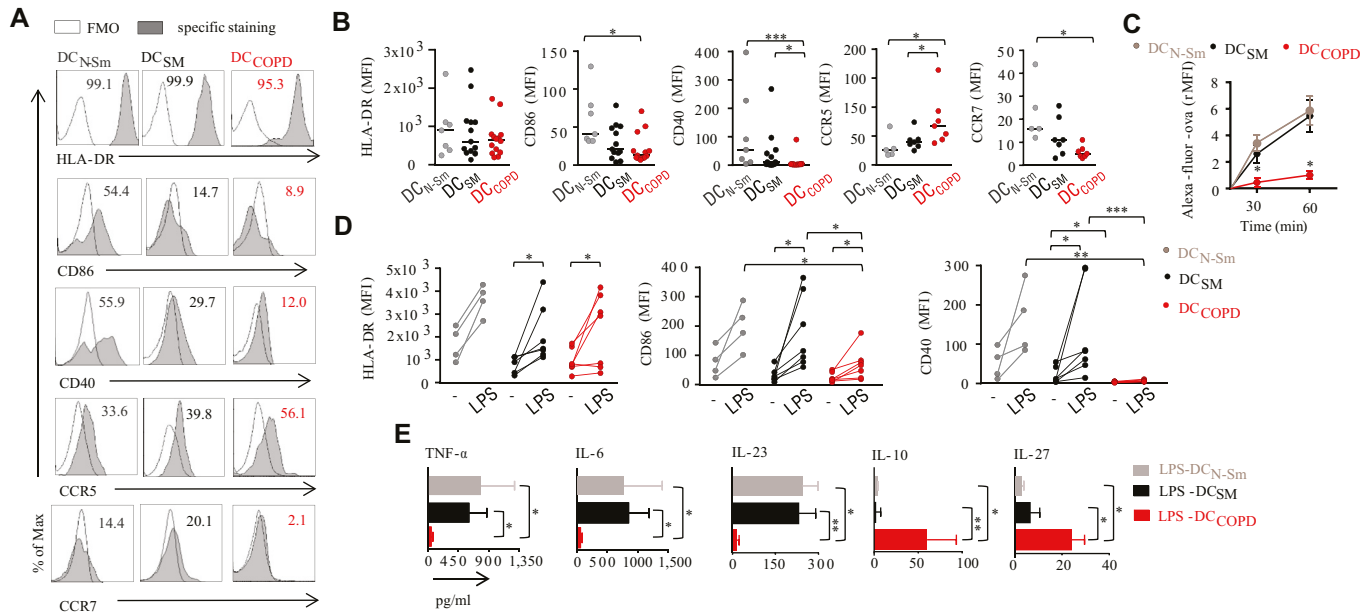


FIG 1. Characterization of CD1c⁺DC_{COPD}. **A** and **B**, Surface markers. Fig 1, **A**, Representative histogram plots relative to fluorescence-minus-one (FMO) control (percentages shown). Fig 1, **B**, Cumulative mean fluorescence intensities (MFIs; DC_{N-Sm}, n = 5-7; DC_{SM}, n = 7-13; DC_{COPD}, n = 7-14). Lines are at medians. **C**, Alexa Fluor-ovalbumin endocytosis. Relative MFI (rMFI) = (MFI^{37°C} - MFI^{4°C})/MFI^{4°C} (DC_{N-Sm}, n = 3; DC_{SM}, n = 4; DC_{COPD}, n = 4). Values are presented as means $\pm$ SEMs. **D** and **E**, Surface markers before/after LPS stimulation (DC_{N-Sm}, n = 4; DC_{SM}, n = 7; DC_{COPD}, n = 7; Fig 1, **D**) and cytokines in culture supernatants (DC_{N-Sm}, n = 3; LPS-DC_{SM}, n = 10-12; LPS-DC_{COPD}, n = 6-8; Fig 1, **E**). Values are presented as means $\pm$ SEMs. **P* < .05, ***P* < .01, and ****P* < .001.

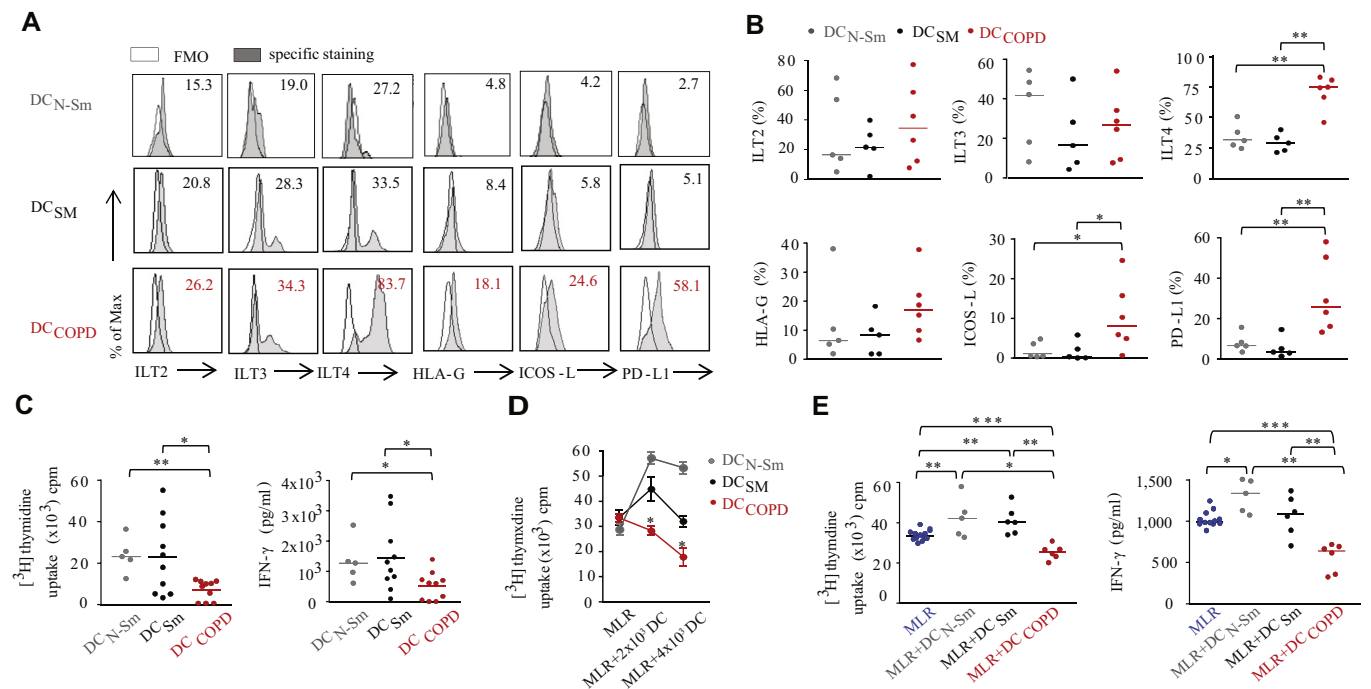


FIG 2. Lung CD1c⁺DC_{COPD} are immunosuppressive. **A** and **B**, Tolerogenic surface molecules. Representative histogram plots relative to fluorescence-minus-one (FMO) control (percentages shown; Fig 2, **A**) and cumulative data of DC_{N-Sm} (n = 5), DC_{SM} (n = 5), and DC_{COPD} (n = 6; Fig 2, **B**). **C**, Tritiated thymidine incorporation/IFN- γ by allogeneic CD4⁺ T cells stimulated by CD1c⁺ DCs (DC_{N-Sm}, n = 5; DC_{SM}, n = 10; DC_{COPD}, n = 10). **D** and **E**, Tritiated thymidine incorporation/IFN- γ by allogeneic CD4⁺ T cells stimulated by APCs (MLRs) and CD1c⁺ DCs. Fig 2, **D**, Representative (DC_{N-Sm}, n = 2; DC_{SM}, n = 2; DC_{COPD}, n = 2). Values are presented as means $\pm$ SEMs. Fig 2, **E**, Cumulative (2 $\times$ 10³ CD1c⁺ DCs; DC_{N-Sm}, n = 5; DC_{SM}, n = 6; DC_{COPD}, n = 6). Lines are at medians. **P* < .05, ***P* < .01, and ****P* < .001.

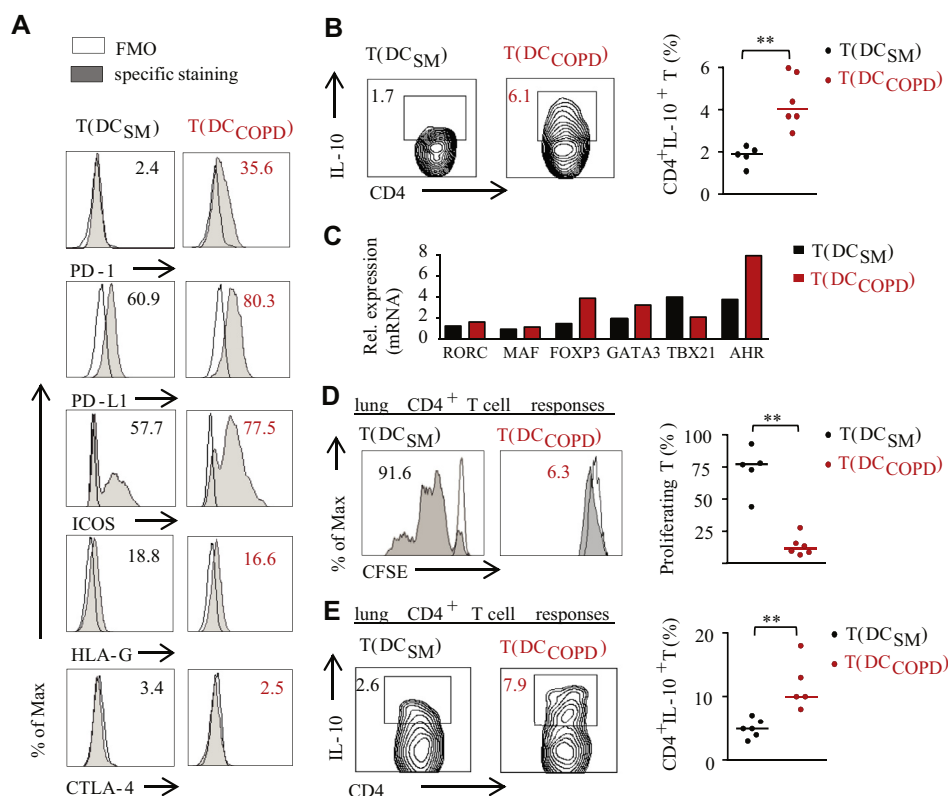


FIG 3. CD1c⁺DC_{COPD} induce IL-10-secreting T cells. **A**, Surface markers on CD1c⁺ DC-primed allogeneic CD4⁺ T cells. Representative histogram plots are shown (T[DC_{SM}], n = 3; T[DC_{COPD}], n = 3). FMO, Fluorescence-minus-one. **B**, Representative contour plots of IL-10 and summary data (right; T[DC_{SM}], n = 5; T[DC_{COPD}], n = 6). **C**, T_H cell transcriptional profile analyzed by using quantitative real-time RT-PCR. Results are presented relative to *GAPDH*. Data are representative of T[DC_{SM}] (n = 3) and T[DC_{COPD}] (n = 4). **D**, Representative CFSE plots of CD1c⁺ DC-primed autologous lung CD3⁺CD4⁺ T cells and summary data (right; T[DC_{SM}], n = 5; T[DC_{COPD}], n = 6). White-filled histograms indicate T cells alone. **E**, Representative contour plots of IL-10 by CD1c⁺ DC-primed autologous lung CD3⁺CD4⁺ T cells and summary data (right; T[DC_{SM}], n = 6; T[DC_{COPD}], n = 5). Percentages are shown on all plots. ***P* < .01.

immunoregulatory nonclassical HLA I molecule, were expressed equivalently among CD1c⁺ DC-primed T cells (Fig 3, A). We did not observe a significant difference in the frequency of CD4⁺CD25⁺Foxp3⁺ T cells among T cells exposed to CD1c⁺DC_{COPD} or CD1c⁺DC_{SM} (data not shown). Interestingly, CD1c⁺DC_{COPD}-primed CD4⁺ T cells contained a significantly increased proportion of IL-10-producing T cells (Fig 3, B). Consistently, we observed a marked increase in mRNA levels of *AHR*, a transcription factor essential for *IL10* gene expression (Fig 3, C). Moreover, the percentages of CD4⁺Tbx21⁺ T cells were slightly decreased in T cells primed by lung CD1c⁺DC_{COPD}, whereas no differences were observed in the percentages of CD4⁺retinoic acid-related orphan receptor C (RORC)⁺ T cells and CD4⁺Gata-3⁺ T cells between the 2 groups (see Fig E2 in this article's Online Repository at www.jacionline.org).

To gain insight to the *in vivo* setting of the respiratory mucosa in patients with COPD, we probed the immunostimulatory properties of CD1c⁺ DCs on autologous lung T-cell responses. Whereas CD1c⁺DC_{SM} were fully competent in driving vigorous proliferation of CFSE-labeled lung CD3⁺CD4⁺ T cells, CD1c⁺DC_{COPD} exhibited a severely impaired stimulatory capacity (Fig 3, D), which was not due to the induction of T-cell apoptosis/necrosis (measured by using 7-aminoactinomycin/Annexin V staining, data not shown). In fact, lung T cells

previously exposed to CD1c⁺DC_{COPD} proliferated in response to secondary restimulation with anti-CD3/anti-CD28, although at low levels (see Fig E3 in this article's Online Repository at www.jacionline.org). These findings might be associated with increased expression of IL-10 by CD1c⁺DC_{COPD}-primed lung T cells (Fig 3, E), whereas percentages of IFN- γ - and IL-17-producing CD4⁺ T cells were similar between CD1c⁺DC_{SM} and CD1c⁺DC_{COPD}-primed T cells (IFN- γ -producing T cells: 19.4% $\pm$ 3.4% vs 17.6% $\pm$ 5.8%; IL-17-producing T cells: 5.6% $\pm$ 0.7% vs 7.3% $\pm$ 2.3% [mean $\pm$ SEM]). We did not find IL-4-secreting CD4⁺ T cells, ruling out the possibility that CD1c⁺DC_{COPD} skewed the lung T-cell response toward T_H2 (data not shown). Similar findings were obtained for CD1c⁺ DC-primed lung CD3⁺CD8⁺ T cells (see Fig E4 in this article's Online Repository at www.jacionline.org). Overall, lung CD1c⁺DC_{COPD} have a severely impaired T-cell stimulatory capacity and drive the differentiation, expansion, or both of an IL-10-secreting CD4⁺ T-cell population.

IL-10-secreting CD4⁺ T cells generated by lung CD1c⁺DC_{COPD} exhibit strong suppressive functions

Subsequently, we investigated whether T cells exposed to lung CD1c⁺DC_{COPD} acquired suppressive properties. Therefore we stimulated allogeneic naive CD4⁺ T cells with lung CD1c⁺

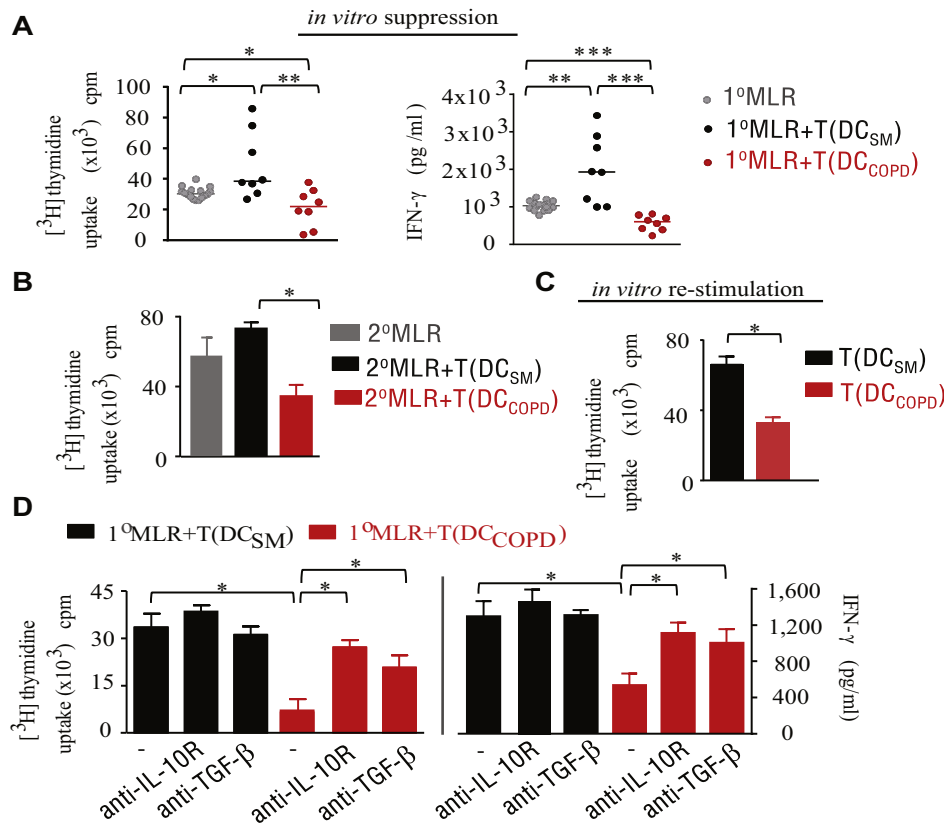


FIG 4. CD1c⁺DC_{COPD}-primed T cells suppress T-cell responses. **A**, Tritiated thymidine incorporation/IFN- γ by allogeneic CD4⁺ T cells stimulated with APCs (1°MLR) in the presence of T(DCs) (T[DC_{SM}], n = 8; T[DC_{COPD}], n = 8). Lines are at medians. **B**, Allogeneic CD4⁺ T cells were stimulated by APCs (1°MLR) and then purified and restimulated by allogeneic APCs (2°MLR) in the presence of T(DCs). **C**, T(DCs) were restimulated with anti-CD3/CD28 mAbs. **B–D**, Tritiated thymidine incorporation/IFN- γ , as in Fig 4, A. Representative (T[DC_{SM}], n = 2; T[DC_{COPD}], n = 2). Values are presented as means $\pm$ SEMs. **P* < .05, ***P* < .01, and ****P* < .001.

DCs, and then CD4⁺ T cells were added into fresh MLRs. Strikingly, CD1c⁺DC_{COPD}-primed CD4⁺ T cells, but not those stimulated with CD1c⁺DC_{SM}, induced a marked decrease in proliferation and IFN- γ production by cocultured alloreactive T responder cells (Fig 4, A). Remarkably, CD1c⁺DC_{COPD}-primed CD4⁺ T cells acquired strong inhibitory function after a single round of stimulation. Moreover, CD1c⁺DC_{COPD}-primed T cells restrained robust secondary allogeneic T-cell responses generated in a secondary MLR (2°MLR) induced by CD4⁺ T cells isolated from the primary MLR and cocultured with fresh allogeneic APCs (Fig 4, B)²⁰ and suppressed anti-CD3/CD28-driven T-cell proliferation in an APC-free system (data not shown). Notably, CD1c⁺DC_{COPD}-primed T cells did not respond to restimulation with anti-CD3/CD28 *in vitro*, a characteristic feature of Treg cells (Fig 4, C).

To delineate the molecular players involved in the *in vitro* suppressive function of CD1c⁺DC_{COPD}-primed T cells, we focused on the contribution of IL-10 and TGF- β 1. Addition of IL-10R or TGF- β 1 neutralizing antibodies in the secondary suppressive cultures reversed the inhibitory effects of CD1c⁺DC_{COPD}-primed CD4⁺ T cells on proliferation and IFN- γ release by T responders (Fig 4, D). Collectively, our findings show that T cells exposed to CD1c⁺DC_{COPD} exert strong *in vitro* suppressive function mediated by IL-10 and TGF- β 1 signaling.

IL-27, IL-10, and ICOS-L control the differentiation of suppressive T cells by lung CD1c⁺DC_{COPD}

We next sought to explore the mechanisms underlying the immunoregulatory functions of CD1c⁺DC_{COPD}. Addition of conditioned medium (CM) obtained from the supernatants of CD1c⁺DC_{COPD}-T-cell cultures to fresh MLRs induced a significant decrease in allogeneic T-cell proliferation, whereas CM from CD1c⁺DC_{SM}-T-cell cultures had no effect (Fig 5, A). Addition of IL-27 or IL-10 neutralizing antibodies in primary induction cultures partially reversed the suppressive effects of CM from CD1c⁺DC_{COPD}-T-cell cultures on MLRs, whereas addition of anti-ICOS-L had no discernible effect (Fig 5, B), suggesting the inducible T-cell costimulator (ICOS)-ICOS-L interactions are not essential for the release of immunosuppressive mediators in the CM.

Subsequently, we explored the contribution of ICOS-L, PD-L1, ILT4, IL-10, and IL-27 to the differentiation of suppressive CD4⁺ T cells by adding blocking antibodies in primary induction cultures. Priming of CD4⁺ T cells by lung CD1c⁺DC_{COPD} in the presence of anti-IL-10R, anti-IL-27, or anti-ICOS-L blocking antibodies induced a significantly impaired capacity to express IL-10 and largely abolished their suppressive effects on alloantigen-driven T-cell responses (Fig 5, C). Notably, the percentages of PD-1⁺ and ICOS⁺CD4⁺ T cells among

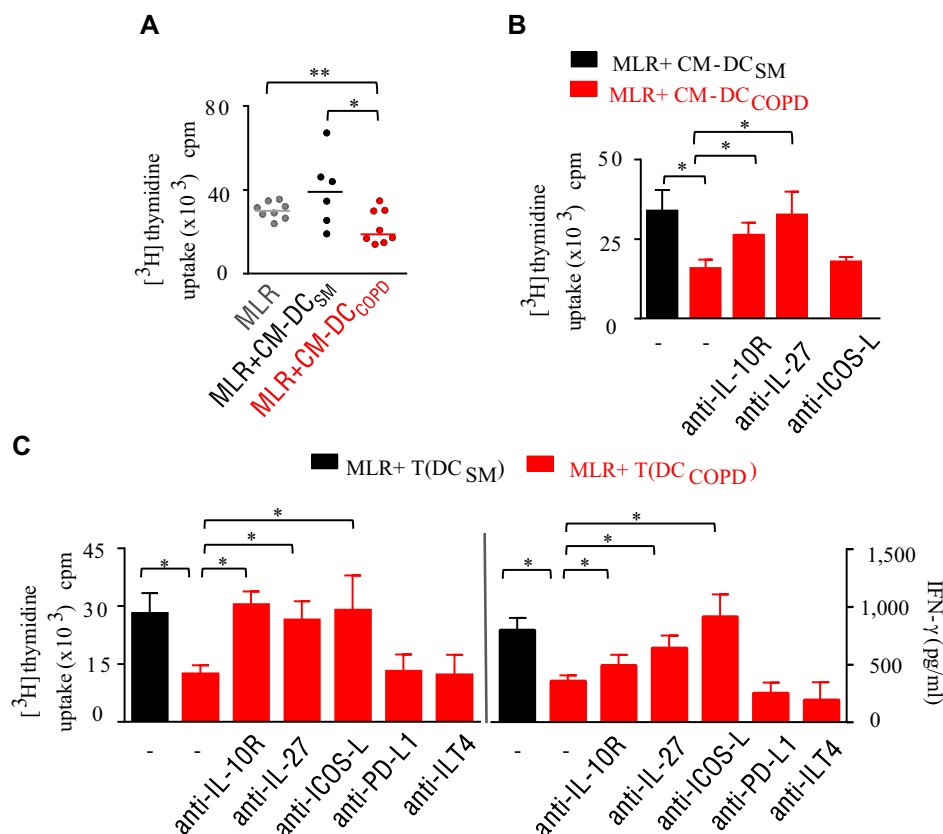


FIG 5. CD1c⁺DC_{COPD} induce Treg cells through IL-27, IL-10, and ICOS-L. **A**, CM from allogeneic CD4⁺ T cells cultured with CD1c⁺ DCs was added to fresh MLRs (CM-DC_{SM}, n = 6; CM-DC_{COPD}, n = 8). Lines are at medians. **B**, Tritiated thymidine incorporation by T cells is shown (representative: CM-DC_{SM}, n = 3; CM-DC_{COPD}, n = 3). Values are presented as means ± SEMs. **C**, CD1c⁺ DC-primed T cells generated in the presence of blocking antibodies were isolated and added to fresh MLRs, as in Fig 4, A. Tritiated thymidine incorporation/IFN-γ is shown (representative: T[DC_{SM}], n = 2; T[DC_{COPD}], n = 2). Values are presented as means ± SEMs. *P < .05 and **P < .01.

CD1c⁺DC_{COPD}-primed T cells were also decreased after inhibition of IL-10 and IL-27 signaling (see Fig E5 in this article's Online Repository at www.jacionline.org). Concomitant blocking of IL-10, IL-27, and ICOS-L showed no additive effects on responder T-cell proliferation and IFN-γ secretion (data not shown). Overall, our data suggest that IL-27, IL-10, and ICOS/ICOS-L control the generation of suppressive T cells by pulmonary CD1c⁺DC_{COPD} through intersecting pathways.

The COPD lung micromilieu endows lung CD1c⁺ DCs with tolerogenic functions

Emerging evidence suggests that a strongly inflammatory micromilieu might educate DCs to acquire immunosuppressive properties.²¹⁻²⁵ To test this, we treated lung CD1c⁺DC_{SM} with LHs obtained from patients with COPD (LH_{COPD}) or from former smokers without COPD (LH_{SM}). LH_{COPD}-exposed CD1c⁺DC_{SM} exhibited a marked increase in ICOS-L, PD-L1, and ILT4 expression accompanied by a striking increase in mRNA levels of IL-27 compared with LH_{SM}-treated CD1c⁺DC_{SM} (Fig 6, A and B). Importantly, only brief exposure of lung CD1c⁺DC_{SM} to LH_{COPD} resulted in a significantly impaired capacity of CD1c⁺DC_{SM} to stimulate allogeneic T-cell proliferation (Fig 6, C). Neutrophil elastase (NE), TNF-α, and IFN-γ are present at high levels in the lungs of patients with COPD and

can, in certain contexts, hinder blood DC *in vitro* responses.²¹⁻²⁵ Pretreatment of lung CD1c⁺DC_{SM} with LH_{COPD} in the presence of SLPI, an NE inhibitor, or TNF-α or IFN-γ neutralizing antibodies partly restored the allogeneic T-cell stimulatory potential of CD1c⁺DC_{SM}, highlighting the contribution of these molecules to the tolerogenic effects of LH_{COPD} (Fig 6, C).

Bronchoalveolar IL-10 and IL-27 levels are increased in patients with COPD at steady state and further upregulated on rhinovirus-induced COPD exacerbation *in vivo*

In light of the key roles of IL-27 and IL-10 in promoting the immunosuppressive functions of CD1c⁺DC_{COPD} *ex vivo*, we investigated their expression in lungs at baseline and during COPD exacerbations. For this, we used BAL samples generated in our well-established *in vivo* human experimental model of rhinovirus-induced COPD exacerbation.^{4,6} IL-10 and IL-27 levels were significantly increased in the BAL fluid of patients with COPD at baseline compared with those seen in never smokers, smokers without COPD, or both (Fig 7, A). Importantly, *in vivo* rhinovirus infection upregulated BAL fluid IL-10 and IL-27 levels only in patients with COPD (Fig 7, B).

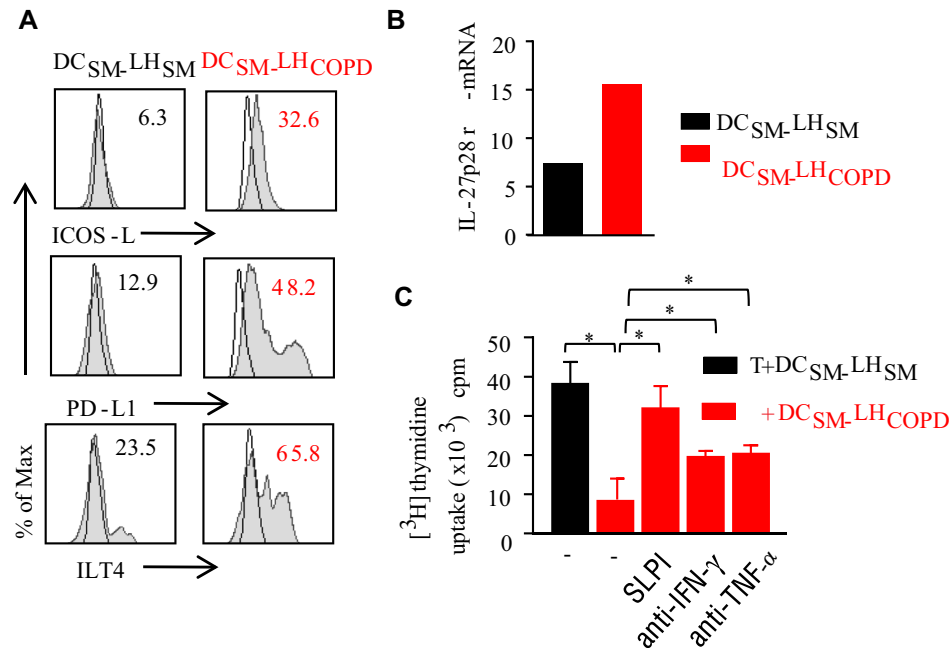


FIG 6. Exposure to a COPD lung micromilieu endows CD1c⁺DC_{SM} with tolerogenic properties. **A**, Histogram plots of tolerogenic surface markers on CD1c⁺DC_{SM} (percentages shown). **B**, Quantitative real-time PCR analysis of IL-27p28 expression relative to *GAPDH*. **C**, Allogeneic CD4⁺ T cells were stimulated with LH-treated CD1c⁺DC_{SM}, as indicated. Values are presented as means ± SEMs. Fig 6, A-C, Representative (LH_{SM}, n = 3; LH_{COPD}, n = 3). *P < .05.

DISCUSSION

Previous studies have demonstrated a key role of lung CD1c⁺DCs in shaping human antiviral CD8⁺ T-cell responses.¹⁹ Interestingly, our results uncover robust immunosuppressive functions for lung CD1c⁺DCs in the context of COPD. CD1c⁺DC_{COPD} restrain T-cell effector functions and favor the generation of suppressive IL-10-secreting CD4⁺ T cells through intersecting pathways dependent on IL-27, IL-10, and ICOS/ICOS-L signaling. Controlling these newly identified functions of lung CD1c⁺DCs in patients with COPD might emerge as a powerful tool to boost respiratory immunity and control devastating infectious exacerbations.

Lung CD1c⁺DC_{COPD} exhibit a semimature phenotype and do not secrete proinflammatory cytokines in response to maturation stimuli. Consistently, we have shown lower numbers of CD83⁺ mature DCs in the lungs of patients with COPD.^{26,27} At odds with our findings, Freeman et al¹³ reported increased expression of costimulatory molecules on CD1c⁺DC_{COPD}. This discrepancy could be due to differences in cohort characteristics, the experimental protocols used, or both. The observed decrease in costimulatory molecule expression, especially CD40, might be associated with the enhanced immunosuppressive functions of CD1c⁺DC_{COPD}.²⁸⁻³⁰ In support of this, CD40 silencing augments the tolerogenic capacity of mouse DCs during transplantation.³⁰ Pertinent to CCR7, decreased expression might be involved in the impaired capacity of CD1c⁺DC_{COPD} to endocytose and present antigens.³¹ Of relevance, low CCR7 levels on lung CD1c⁺DCs have been associated with decreased lung function in smokers.³² Apart from CD1c⁺DCs, plasmacytoid DCs might also play a role during viral infections in patients with COPD. Certain studies have shown that plasmacytoid DCs accumulate in the lymphoid follicles in the lungs of patients with COPD

compared with those of smokers without COPD.³³ Freeman et al¹³ demonstrated significant increases in CD40 and CD80 levels in patients with COPD, although there were no significant differences in lung plasmacytoid DC numbers compared with those seen in smokers without COPD.

Our findings revealed that CD1c⁺DC_{COPD} cannot stimulate allogeneic T-cell responses, a cardinal feature of immunoregulatory DCs. Importantly, CD1c⁺DC_{COPD} suppress robust MLRs, indicating that they might exert dominant tolerogenic effects *in vivo*. Notably, *in vitro* cigarette smoke exposure impairs the T-cell stimulatory capacity of human DCs, whereas *in vivo* cigarette smoke suppresses the maturation of mouse lung CD11c⁺DCs and their ability to drive T-cell proliferation in thoracic lymph nodes.³⁴⁻³⁶ Because our patients with COPD were former smokers, our findings highlight additional intrinsic defects of CD1c⁺DC_{COPD}. In support of this, our current observations demonstrate that although CD1c⁺DC functions in never smokers mostly resemble those in smokers after smoking cessation, altered CD1c⁺DC phenotypes persist in patients with COPD, as also shown by other investigators.^{37,38} In contrast to CD1c⁺DCs, pioneering studies have shown that lung CD1a⁺DCs from patients with COPD stimulate robust, potentially tissue-destructive T_H1 and T_H17 cell-mediated responses.³⁹ CD1a and CD1c expression characterize distinct human lung DC subsets in terms of phenotype and anatomic location.^{9,40} Overall, these studies propose a division of labor among lung CD1c⁺ and CD1a⁺DC subsets in patients with COPD, strengthening the rationale of targeting specific DC subtypes to control the balance between immunity and tolerance.⁸

Intriguingly, exposure of lung CD1c⁺DCs from former smokers to LH_{COPD} hinders their immunostimulatory properties and endows them with tolerogenic properties. Notably, our data

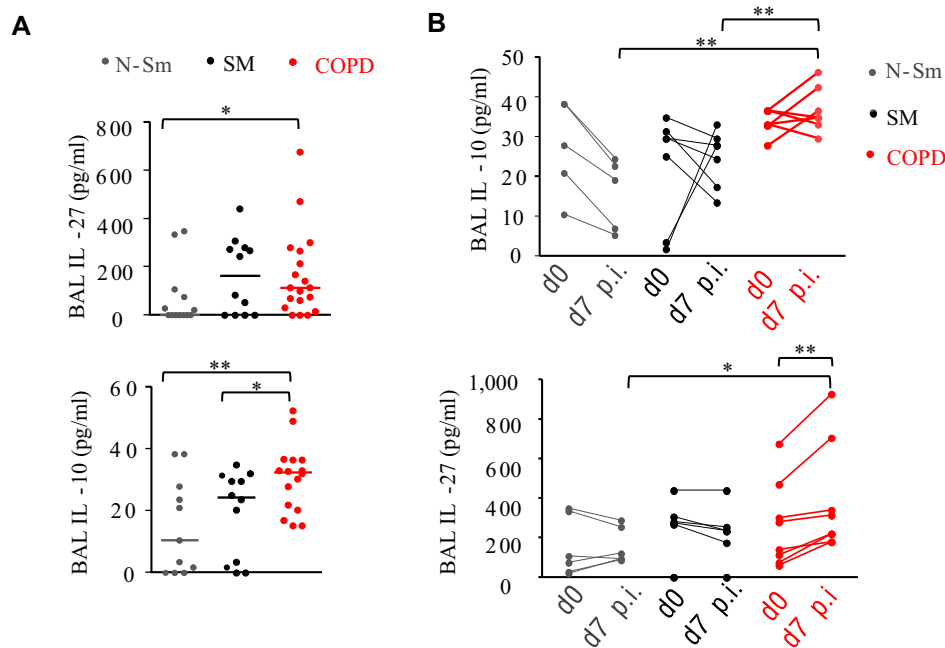


FIG 7. COPD lung IL-27 and IL-10 levels are increased at baseline and upregulated on experimentally induced viral exacerbations. **A**, IL-10 and IL-27 levels in BAL fluid of never smokers ($n = 13$; N-Sm), smokers without COPD ($n = 12$; SM), and patients with COPD ($n = 16$ -19) at baseline. **B**, BAL IL-27 and IL-10 levels at days 0 and 7 after infection (N-Sm, $n = 5$ -6; SM, $n = 7$; COPD, $n = 7$ -8). Lines are at medians. * $P < .05$ and ** $P < .01$.

showed that NE, TNF- α , and IFN- γ are critical mediators of the suppressive effects of LH_{COPD} on lung CD1c⁺ DC responses. In support of this, other investigators have shown that sputum specimens of patients with COPD dampen human DC responses *in vitro* through the release of NE.⁴¹ Collectively, it is tempting to speculate that lung-derived factors in patients with COPD foster a tolerogenic milieu that targets pulmonary CD1c⁺ DCs, impairing their immunogenic potential.

A prominent finding of our study is that lung CD1c⁺DC_{COPD} favor the differentiation of IL-10-secreting T cells, which suppress both naive and effector T-cell responses. In fact, CD1c⁺DC_{COPD} induce suppressor CD4⁺ T cells without exogenous cytokine supplementation, underscoring their strong immunoregulatory properties. Although suppressive CD4⁺ T cells express PD-1, PD-L1, and ICOS, their *in vitro* inhibitory function is mainly dependent on IL-10 and TGF- β production. However, the involvement of PD-1, PD-L1, and ICOS signaling in the suppressive effects of these T cells *in vivo* cannot be excluded. Notably, CD1c⁺DC_{COPD}-induced suppressive T cells inhibit allogeneic T-cell responses in a bystander fashion because in our assays suppressor and responder T cells recognize antigens on distinct APCs; nonetheless, we anticipate that T-cell suppression would be even stronger under T-cell receptor (TCR)-specific conditions.²⁰

We demonstrate that lung CD1c⁺DC_{COPD} are able to polarize autologous lung tissue-resident T cells toward IL-10-secreting CD4⁺ T cells *ex vivo*. Nevertheless, it remains unclear whether these cells are induced in the lungs of patients with COPD *in vivo*. An interesting study reported increased IL-10 expression by BAL CD4⁺ and CD8⁺ T lymphocytes in patients with COPD.⁴² It is conceivable that IL-10 released by resident lung T cells acts on lung CD1c⁺ DCs, dampening their stimulatory

function in a feed-forward tolerogenic loop. This immunosuppressive cell network might induce a regulatory phenotype in newly recruited DCs and naive T cells, amplifying the tolerogenic signals in the COPD lung microenvironment. The distinct lung microenvironment in patients with COPD might also play a key role in the antigens recognized by DCs and the ensuing immune response. For example, in patients with COPD, acute and chronic lower respiratory tract infections occur with increased frequency, and hence lung CD1c⁺DC_{COPD}, in contrast to CD1c⁺DC_{SM}, might be predominantly loaded with viral and bacterial antigens.⁴³ Although subjects reporting an acute respiratory tract infection in the 6 weeks preceding the study were excluded, low amounts of pathogenic antigens can persist in lungs of patients with COPD and loaded on CD1c⁺ DCs. Moreover, apart from foreign antigens, self-antigens can be released in lungs of patients with COPD as a result of chronic antigenic stimulation and inflammation and presented by CD1c⁺ DCs at higher frequencies compared with CD1c⁺DC_{SM}. In support of this, a strong association was observed between memory T-cell responses to self-antigens and COPD pathogenesis.⁴⁴ Furthermore, the pool of lung T cells might be different in former smokers and patients with COPD. In the case of COPD, we speculate that T cells might be predominantly specific for pathogens, self-derived antigens, or both.

In keeping with their immunosuppressive functions, lung CD1c⁺DC_{COPD} express high levels of ILT4, ICOS-L, and PD-L1 and produce IL-10 and IL-27 after stimulation *ex vivo*. Previous studies in other contexts have linked the suppressive activities of IL-27 and IL-10 to the PD-1/PD-L1 and ICOS/ICOS-L signaling pathways.^{16,17} Interestingly, we demonstrate that IL-10, IL-27, and ICOS/ICOS-L interactions are required for lung CD1c⁺ DC-mediated differentiation of IL-10-producing

suppressive CD4⁺ T cells also in the context of COPD. Moreover, CD1c⁺DC_{COPD}-primed CD4⁺ T cells express high AHR levels, which are also controlled by IL-27 signaling.⁴⁵

Of clinical relevance, BAL levels of IL-10 and IL-27 are significantly increased in patients with COPD at baseline compared with those seen in healthy control subjects. Earlier studies pertinent to pulmonary IL-10 levels in patients with COPD have generated contradictory results,^{46,47} but other groups have also found increased IL-27 levels in the BAL fluid of patients with COPD.^{48,49} Importantly, for the first time, we show that levels of these immunosuppressive cytokines are further upregulated during rhinovirus-induced COPD exacerbation *in vivo*. In support of this, animal studies have demonstrated increased IL-10 and IL-27 production by lung T cells and DCs on viral infection.⁵⁰ Overall, these data raise the intriguing possibility that IL-27 and IL-10 might be involved in the impaired antiviral responses seen in patients with COPD.

In summary, our studies uncover lung CD1c⁺ DCs as critical orchestrators of an immunosuppressive cell network in patients with COPD. We propose that strategies targeting the neutralization of surface tolerogenic molecules and/or immunosuppressive cytokines secreted by DCs, as well as blocking T-cell inhibitory molecules or T cell–secreted suppressive cytokines, might be a valuable approach for future therapeutic applications. Ultimately, we envisage a scenario in which silencing lung CD1c⁺ DCs or lung T-cell suppressive functions will restore defective immune surveillance mechanisms and lead to the control of respiratory tract infections and a decrease in mortality rates in patients with COPD.

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Key messages

- CD1c⁺DC_{COPD} express high levels of surface inhibitory molecules (ICOS-L, PD-L1, and ILT4), secrete high levels of immunosuppressive cytokines (IL-10 and IL-27), and hinder T-cell responses through the differentiation of Treg cells.
- IL-27, IL-10, and ICOS-L are required for Treg cell generation by CD1c⁺DC_{COPD}.
- IL-10 and IL-27 are upregulated on rhinovirus-induced COPD exacerbation *in vivo*.

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METHODS

Subjects

Human lung tissues were obtained from exsmokers with COPD ($n = 41$), exsmokers without COPD ($n = 44$), and never smokers without COPD ($n = 5-7$) undergoing lobectomy for isolated lung carcinoma at the thoracic surgical clinic of Evangelismos General Hospital and Sotiria Chest Hospital in Athens (Table 1). Care was taken to obtain tissues as distant as possible from the primary lesion and from the subpleural regions of the resected lung lobes to ensure sampling of the small airways and alveoli. At the time of the study and in the 6 weeks preceding the study, all subjects were free of acute symptoms suggestive of upper or lower respiratory tract infection. COPD was diagnosed by a respiratory specialist according to current guidelines.^{E1} None of the patients with COPD had received oral/systemic or inhaled corticosteroids. All studies were conducted according to the Declaration of Helsinki principles. Ethics approval was obtained by the Institutional Review Boards of Evangelismos General Hospital and Sotiria Chest Hospital. All donors provided written informed consent for the studies conducted.

In vivo human experimental rhinovirus infection

Three groups of subjects (healthy nonsmokers, smokers without COPD, and patients with COPD) were recruited from the Imperial College Healthcare NHS trust (St Mary's Hospital), the Royal Brompton Hospital, local general practices, and by advertisement for 2 studies. Details regarding the study participants, inclusion criteria, and clinical, virological, microbiological, and inflammatory outcomes have been published.^{E2,E3} All patients with COPD were GOLD stage II and not using inhaled corticosteroids, long-acting β -agonists, or long-acting antimuscarinics. Bronchoscopy was performed, and BAL samples were obtained at baseline, at least 2 weeks before rhinovirus 16 inoculation, and on day 7 after rhinovirus inoculation. Only subjects with successful infection, as confirmed by rhinovirus detection with PCR on nasal lavage, sputum, or BAL fluid, were included in the analysis.

Human lung tissue preparation

Lung tissue was extensively perfused/rinsed with tissue culture medium (TCM) to remove airway lumen cells/alveolar cells and residual blood, minced with scissors, and incubated in digestion medium in a humidified incubator at 37°C and 5% CO₂. TCM consisted of RPMI-1640 supplemented with 5% FCS, penicillin/streptomycin, L-glutamine, and 2-mercaptoethanol (Gibco, Invitrogen). Digestion medium consisted of TCM supplemented with 1 mg/mL collagenase type 2 (Roche, Genentech, South San Francisco, Calif) and 0.02 mg/mL DNase I (Roche). After 45 minutes of incubation, samples were centrifuged and resuspended in Ca²⁺- and Mg²⁺-free PBS containing 10 mmol/L EDTA for 5 minutes at room temperature on a shaker. Next, enzyme-digested fragments were passed through a 40- μ m cell strainer (BD Biosciences, San Jose, Calif) and subjected to red blood cell lysis (VersaLyse Lysing Solution, Beckman Coulter, Calif). Fifty milligrams of snap-frozen human lung tissue was homogenized with an electric rotor-stator homogenizer for 30 seconds at 4°C in 1 mL of tissue lysis buffer consisting of 137 mmol/L NaCl, 20 mmol/L Tris/HCl (pH 8.0), 5 mmol/L EDTA, and 10% glycerol 1% Triton X-100 to obtain LHs. LHs were centrifuged for 10 minutes at 14,000g at 4°C, and the supernatants were snap-frozen and stored.

Isolation and stimulation of human lung CD1c⁺ DCs

CD1c⁺ DCs were isolated from lung single-cell suspensions by using the CD1c⁺ (blood dendritic cell antigen-1⁺) human Dendritic Cell Isolation Kit (Miltenyi Biotec, Gladbach, Germany; Fig E1). The purity of the isolated CD1c⁺ DCs was consistently greater than 80%. In stimulation experiments 3×10^4 purified lung CD1c⁺ DCs were plated onto 96-well flat bottom-shaped plates and cultured with 200 ng/mL LPS (Sigma-Aldrich, St Louis, Mo) for 48 hours. Cytokine release in culture supernatants was measured by using ELISA. In other experiments purified CD1c⁺ DCs were cultured overnight with 10 ng/mL LPS and analyzed by using flow cytometry. For endocytosis assays, purified lung DCs were incubated for 1 hour at

37°C or 4°C with 200 μ g/mL ovalbumin–Alexa Fluor 488 (Invitrogen) and analyzed by using flow cytometry.

In another set of experiments purified CD1c⁺ DCs were pretreated for 4 hours with TCM supplemented with a 30% volume of LHs in the presence or absence of SLPI (75 μ g/mL; R&D Systems, Minneapolis, Minn) or of neutralizing antibodies against IFN- γ (25 μ g/mL) or TNF- α (25 μ g/mL; BioLegend, San Diego, Calif) or immunoglobulin control. CD1c⁺ DCs were extensively washed and cocultured with allogeneic naive CD4⁺ T cells. T cells were isolated from PBMCs by using the human naive CD4⁺ T Cell Isolation Kit II (Miltenyi Biotec). The purity of the naive CD4⁺ T-cell preparation exceeded 95%. LH-exposed CD1c⁺ DCs (2×10^3) were cocultured in 96-well round-bottom plates with 6×10^4 allogeneic naive CD4⁺ T cells for 7 days. The same healthy donor was used in all experiments. T-cell proliferation was measured by using tritiated thymidine incorporation. LH-exposed CD1c⁺ DCs were analyzed by means of flow cytometry and quantitative real-time PCR.

Allogeneic MLR

Allogeneic naive CD4⁺ T cells (6×10^4) were isolated and stimulated with 2×10^3 lung CD1c⁺ DCs as above (primary induction cultures). Cell-culture supernatants were collected for cytokine analysis. T-cell proliferation was measured as above. In other experiments CD1c⁺ DC–primed CD4⁺ T cells were isolated from the MLRs by using the human Pan T Cell Isolation Kit II (Miltenyi Biotec) and analyzed by using quantitative real-time PCR or flow cytometry. In blocking experiments neutralizing mAbs against IL-10R (20 μ g/mL), IL-27 (15 μ g/mL; both from R&D Systems), ICOS-L (20 μ g/mL), ILT4 (20 μ g/mL), or PD-L1 (20 μ g/mL; all from eBioscience, San Diego, Calif) or immunoglobulin control were added in the primary induction cultures. In other experiments CM obtained from the supernatants of lung CD1c⁺ DC–allogeneic CD4⁺ T-cell cocultures was added to fresh MLRs consisting of naive allogeneic CD4⁺ T cells and APCs.

In another set of experiments 2×10^4 allogeneic naive CD4⁺ T cells were cocultured with mitomycin C–treated CD3–depleted PBMCs (APCs) isolated by using anti-CD3 paramagnetic beads (Miltenyi Biotec) in the presence or absence of titrated numbers of lung CD1c⁺ DCs. After 5 days, T-cell proliferation was measured as above, and supernatants were collected for cytokine analysis.

Autologous lung T cell–CD1c⁺ DC assays

Lung CD3⁺ T cells were isolated from single-cell suspensions by using the human Pan T Cell Isolation Kit II (Miltenyi Biotec). Lung CD3⁺ T cells had a purity of greater than 80%. CD3⁺ T cells were labeled with 1 μ mol/L CFSE (Invitrogen) and cocultured in 96-well round-bottom plates with autologous lung CD1c⁺ DCs at an 8:1 ratio for 5 days. T-cell phenotype was analyzed by using flow cytometry, and proliferation was evaluated by means of CFSE analysis. In other experiments CD1c⁺ DC–primed CD3⁺ T cells were isolated, washed, and restimulated with soluble anti-CD3 and anti-CD28 mAbs (both at 1 μ g/mL; BD Biosciences, San Jose, Calif) for 48 hours. T-cell proliferation was measured as above.

In vitro suppression assays

For *in vitro* suppression assays, allogeneic naive CD4⁺ T cells (10^4) were cocultured with APCs (10^4) in the presence or absence of CD1c⁺ DC–primed CD4⁺ T cells (10^4). After 5 days, T-cell proliferation was measured with tritiated thymidine incorporation, and cell-culture supernatants were collected. In some experiments neutralizing antibodies against IL-10R (20 μ g/mL) or TGF- β 1, TGF- β 2, or TGF- β 3 (15 μ g/mL; from R&D Systems) or immunoglobulin control were added in secondary suppressive cultures.

In experiments investigating T-cell hyporesponsiveness, CD1c⁺ DC–primed CD4⁺ T cells were isolated from primary induction cultures, as described above, and restimulated with 1 μ g/mL soluble anti-CD3 and 1 μ g/mL soluble anti-CD28 mAbs (BD Biosciences). T-cell proliferation was measured as above.

In other experiments CD4⁺ T cells were isolated from a primary MLR (1°MLR) and restimulated with allogeneic APCs in a secondary MLR

(2°MLR) in the presence or absence of CD1c⁺ DC–primed CD4⁺ T cells. After 5 days, T-cell proliferation and cytokine release were analyzed as above. Naive CD4⁺ T cells and APCs were isolated from the same 2 healthy donors in all experiments.

In another set of experiments, 10⁴ CD1c⁺ DC–primed CD4⁺ T cells were cocultured with 10⁴ autologous naive CD4⁺ T cells stimulated with 1 µg/mL soluble anti-CD3 and 1 µg/mL soluble anti-CD28 mAbs (BD Biosciences). T-cell proliferation and cytokine release were analyzed as above.

Cytokine assays

Cytokines were measured in cell-culture supernatants by using commercially available ELISA kits for human IFN-γ, IL-6, TNF-α, and IL-12 (eBioscience). Human IL-10 and IL-27 levels were measured by using Luminex in cell-culture supernatants and by using commercially available ELISA kits (R&D Systems) in the BAL fluid.

Flow cytometry

Whole lung cells and isolated lung CD1c⁺ DCs were stained with antibodies against human CD1c, CD19, CD80, CD86, CD40, HLA-DR, CD54, CCR5, CCR6, CCR7 (all from BD Biosciences), ICOS-L, PD-L1, HLA-G, ILT2, ILT3 (eBioscience), ILT4 (Beckman Coulter, Brea, Calif), and PD-L2 (BioLegend). T-cell phenotype was analyzed by using antibodies against human CD3, CD4, CD8 (BD Biosciences), PD-1, PD-L1, ICOS, HLA-G, cytotoxic T lymphocyte–associated antigen 4, CD69, Foxp3, RORC, TBX21 and GATA-3 (eBioscience). Expression of intracellular IL-10, IFN-γ, and IL-17 (BD Biosciences) was evaluated after cell stimulation for 5 hours with 10 ng/mL phorbol 12-myristate 13-acetate and 250 ng/mL ionomycin (Sigma-Aldrich). In the last 3 hours of activation, 1 µL/mL of Brefeldin A (BD Biosciences) was also added to the culture. For intracellular IL-10 expression, cells were also stimulated with 4 µg/mL immobilized anti-CD3 and 2 µg/mL soluble anti-CD28 (Becton Dickinson) for 24 hours before treatment with phorbol 12-myristate 13-acetate and ionomycin, as above. Fluorescence-activated cell sorting acquisition was performed with the cytometer Cytomics FC500 (Beckmann Coulter), and data were analyzed by using WinMDI (Bio Soft Net, <http://en.biosoft.net>) and FlowJo (Tree Star, Ashland, Ore) software.

Quantitative real-time PCR

Total RNA was isolated in TRI Reagent (Sigma-Aldrich) and, where necessary (cell number <10⁵), with the NucleoSpin RNA XS kit (MACHEREY-NAGEL, Bethlehem, Pa), according to the manufacturers' protocols. When TRI Reagent was used, RNA samples were treated with RQ1 RNase-Free DNase (Promega, Madison, Wis) to eliminate contaminating genomic DNA. RNA samples were then reverse transcribed with Superscript II (Invitrogen), according to the manufacturer's recommendations. The expression of human *FOXP3*, *GATA3*, *TBX21*, *AHR*, *MAF*, *IL10*, *IL27*, and *GAPDH* mRNA was analyzed with SYBR Green Master mix (Bio-Rad Laboratories, Hercules, Calif; Table E1). The relative expression of human genes over the expression of *GAPDH* was calculated by using the 2^{−ΔΔC_t} method.

Statistical analysis

GraphPad Prism software version 6 (GraphPad Software) was used for statistical analysis. Nonparametric tests were applied. Multiple comparisons of sampled data were made by using the Kruskal-Wallis test. The Mann-Whitney *U* test or Wilcoxon matched-pairs test was used for pairwise comparisons. A *P* value of less than .05 was accepted as statistically significant. When cumulative data are shown, the data are derived from independent donors, and each value represents a single donor, whereas when representative data are depicted, values are shown from replicate experiments by using samples from the same donor.

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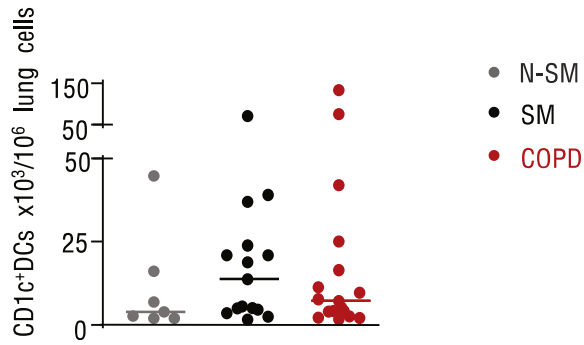


FIG E1. Numbers of CD1c⁺ DCs among 10⁶ total lung cells in never smokers (N-SM; n = 7), former smokers without COPD (SM; n = 15), and patients with COPD (n = 16) are shown. Each *dot* represents a single donor. *Lines* are at medians.

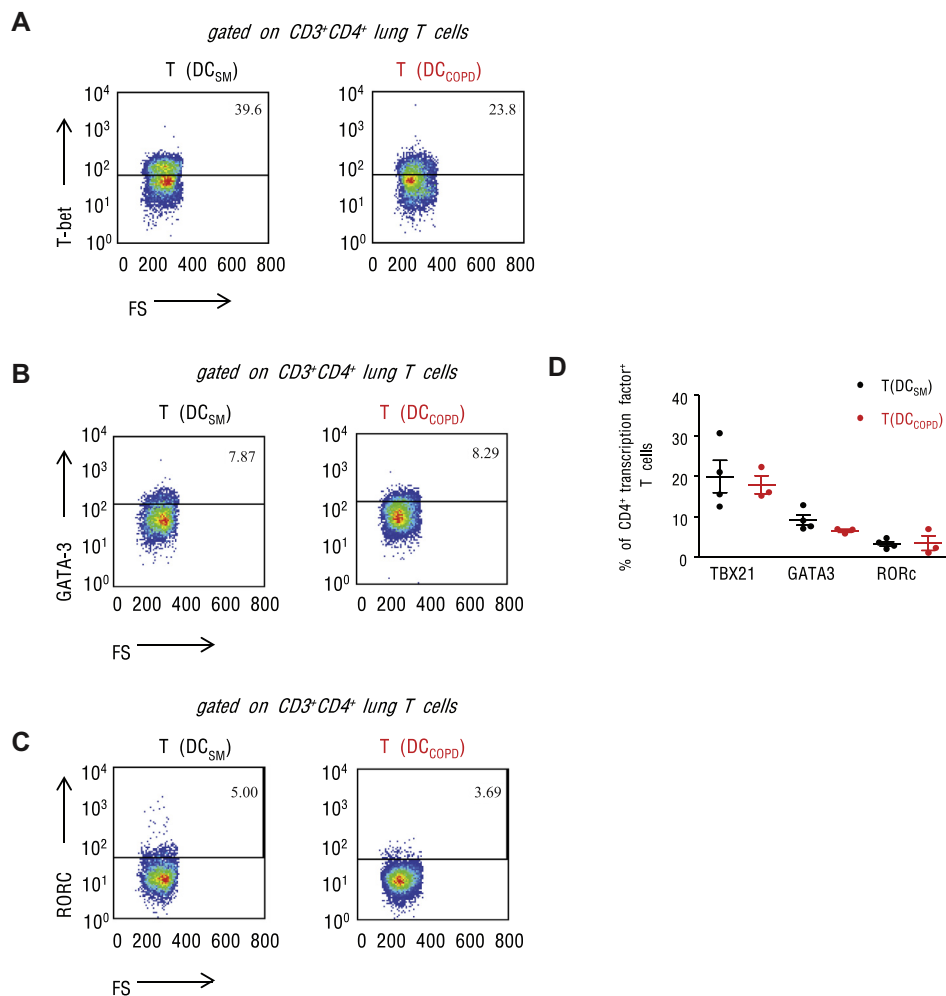


FIG E2. Fluorescence-activated cell sorting analysis of CD1c⁺ DC-primed T cells. **A-C**, Representative density plots showing transcription factor expression gated on CD3⁺CD4⁺ T cells are shown, as indicated (T[DC_{SM}], n = 4; T[DC_{COPD}], n = 4). **D**, The percentages of TBX21⁺, GATA-3⁺, and RORC⁺ cells on gated CD4⁺ T cells are shown as well (right).

lung CD3⁺ T cell responses

restimulation

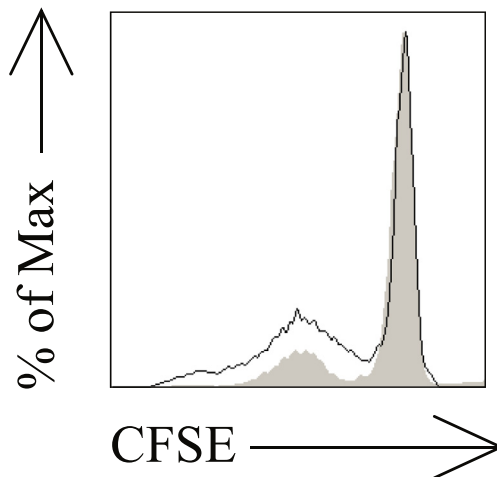
T(DC_{COPD})

FIG E3. CD1c⁺DC_{COPD}-exposed lung T cells respond poorly to secondary polyclonal stimulation *in vitro*. Lung CD1c⁺DC_{COPD} were cocultured with CFSE-labeled CD3⁺ T cells isolated from the same lung tissue explants. CD3⁺ T cells were isolated, washed, and restimulated with anti-CD3 and anti-CD28. T-cell proliferation was assessed by measuring CFSE dilution. *Gray-filled histograms* represent lung T cells cultured with autologous CD1c⁺DC_{COPD}. *White-filled histograms* represent CD1c⁺DC_{COPD}-primed lung T cells restimulated with anti-CD3 and anti-CD28. Data are representative of 3 independent experiments.

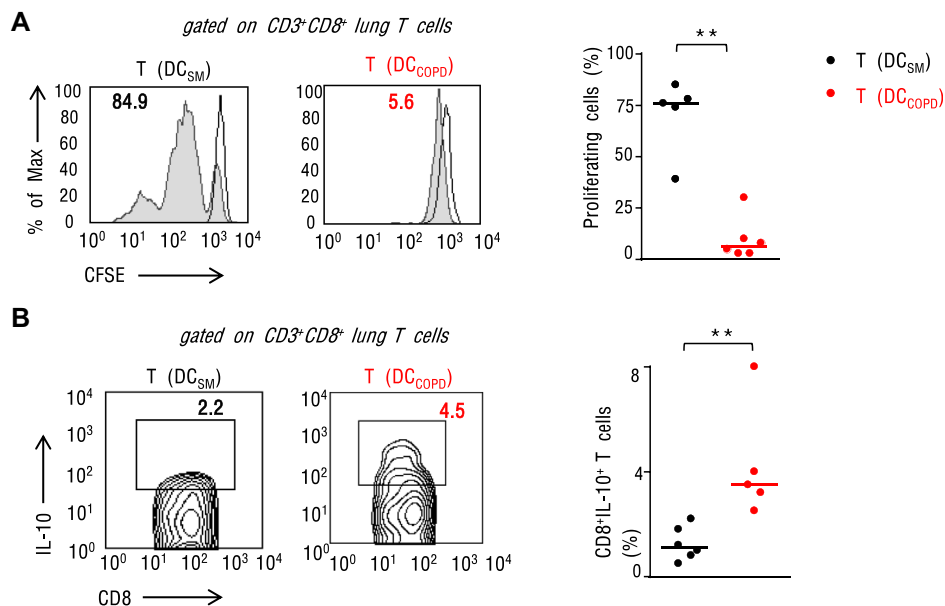


FIG E4. Lung $CD1c^+DC_{COPD}$ do not stimulate autologous lung-resident $CD8^+$ T-cell responses. **A**, Lung $CD1c^+DC_{SM}$ or $CD1c^+DC_{COPD}$ were cocultured with CFSE-labeled $CD3^+$ T cells isolated from the same lung tissue explants. $CD3^+CD8^+$ T-cell proliferation was assessed by measuring CFSE dilution. Representative CFSE plots are shown (left), and numbers in plots indicate percentages of $CFSE^{-/low}$ cells. White-filled histograms represent T cells cultured in the absence of DCs. Summary data (right) show percentages of proliferating T cells from 11 independent experiments (T [DC_{SM}], $n = 5$; T [DC_{COPD}], $n = 6$). **B**, Intracellular IL-10 expression on gated lung $CD3^+CD8^+$ T cells stimulated as above is shown. Representative contour plots are shown (left), and numbers in plots indicate percentage of positive cells. Summary data (right) show percentages of $CD8^+IL-10^+$ T cells from 11 independent experiments (T [DC_{SM}], $n = 6$; T [DC_{COPD}], $n = 5$). Lines are at medians. Statistical significance was obtained by using the Mann-Whitney U test: ** $P < .01$.

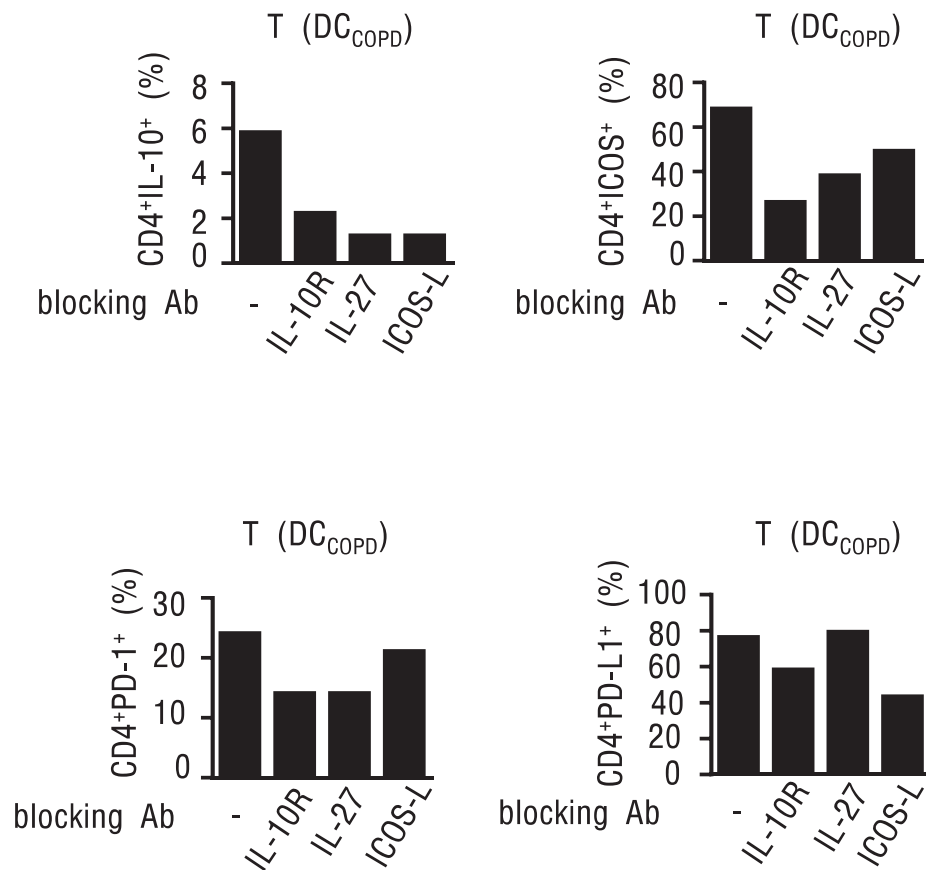


FIG E5. IL-10, ICOS, and PD-1 expression on CD4⁺ T cells primed by lung CD1c⁺DC_{COPD} depends on IL-27, IL-10, and ICOS-L signaling. Purified lung CD1c⁺ DCs of patients with COPD were cocultured with allogeneic naive CD4⁺ T cells, as indicated. T(DC_{COPD}) were isolated and analyzed by using flow cytometry. Percentages of IL-10⁺, ICOS⁺, PD-1⁺, and PD-L1⁺ cells on gated CD4⁺ T cells are shown. Data are representative of 2 independent experiments.

TABLE E1. Primer sequences used for quantitative real-time PCR analysis

Genes	Forward primer	Reverse primer
<i>GAPDH</i>	GCAAATTCCATGGCACCGT	TCGCCCCACTTGATTTTGG
<i>FOXP3</i>	GAAACAGCACATTCCCAGAGTTC	ATGGCCCAGCGGATGAG
<i>GATA3</i>	GCGGGCTCTATCACAAAATGA	CTCTCCTGGCTGCAGACAGC
<i>TBX21</i>	GATGCGCCAGGAAGTTTCAT	GCACAATCATCTGGGTACATT
<i>RORC</i>	GCAGCGCTCCAACATCTTCTC	GCACACCGTTCCCACATCTC
<i>AHR</i>	CCGTGTGCGATGTATCAGTGC	GCCTGGCAGTACTGGATTGT
<i>MAF</i>	ACGAGAAGTTGGTGAGCAGC	TTCCAAAATGTGGCGTATCC
<i>IL27</i>	AAGCTGCTCTCCGAGGTTC	GGAAGGTCAGGGAAACATCA