CHEST

Original Research

COPD

Expression of the Atypical Chemokine Receptor D6 in Human Alveolar Macrophages in COPD

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Background: **D6 is an atypical chemokine receptor involved in chemokine degradation and resolution of acute inflammatory responses in mice. Emerging evidence suggests that D6 might behave differently in human chronic inflammatory conditions. We, therefore, investigated the involvement of D6 in the immune responses in COPD, a chronic inflammatory condition of the lung.**
Methods: **D6 expression was quantified by immunohistochemistry in surgical resected lung specimens from 16 patients with COPD (FEV_1, 57% ± 6% predicted) and 18 control subjects with normal lung function (nine smokers and nine nonsmokers). BAL was also obtained and analyzed by flow cytometry, immunofluorescence, and molecular analysis for further assessment of D6 involvement.**
Results: **D6 expression in the lung was mainly detected in alveolar macrophages (AMs). The percentage of $D6^+$ AMs was markedly increased in patients with COPD as compared with both smoker and nonsmoker control subjects ($P < .0005$ for both). D6 expression was detected at both transcript and protein level in AMs but not in monocyte-derived macrophages. Finally, D6 expression was positively correlated with markers of immune activation ($CD8^+$ T lymphocytes, IL-32, tumor necrosis factor-α, B-cell activating factor of the tumor necrosis factor family, phospho-p38 mitogen-activated protein kinase) and negatively with lung function (FEV_1, FEV_1/FVC).**
Conclusions: **D6 is expressed in AMs from patients with COPD, and its expression correlates with the degree of functional impairment and markers of immune activation. Upregulation of D6 in AMs could indicate that, besides its known scavenger activity in acute inflammation, D6 may have additional roles in chronic inflammatory conditions possibly promoting immune activation.**
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Abbreviations: AM = alveolar macrophage; BAFF = B-cell activating factor of the tumor necrosis factor family; GOLD = Global Initiative for Chronic Obstructive Lung Disease; MAPK = mitogen-activated protein kinase; MDM = monocyte-derived macrophage; TNF = tumor necrosis factor

COPD is a worldwide public health problem that reduces the quality of life, increases the frequency of contact with health-care providers, causes frequent hospital admissions, and carries an increased risk of death.[1-3] Active smoking, the main risk factor for COPD development, instigates an inflammatory response in the larger bronchi and peripheral lung of all smokers. This inflammatory process is amplified and persists long after smoking cessation in those susceptible smokers who develop COPD.[4-12] The mechanisms leading to the initiation and persistence of this inflammatory immune response are not fully understood, but several studies have implicated T cells, macrophages, and neutrophils as important players in disease pathogenesis.[4-13]

Leukocyte recruitment to sites of inflammation is orchestrated by chemokines, a large family of chemotactic cytokines recognized by G protein-coupled receptors. In addition to conventional chemokine receptors able to trigger cell migration, a small family of receptors, characterized by promiscuous ligand binding and inability to promote cell migration, has been identified. These atypical chemokine receptors are believed to control chemokine bioavailability, thus

participating in the appropriate resolution of inflammatory responses and protecting from excessive tissue damage.[14,15] Examples of these receptors include duffy antigen receptor for chemokines, chemocentryx chemokine receptor, and the decoy receptor D6, a highly promiscuous receptor capable of binding, internalizing, and rapidly degrading most of the CC inflammatory chemokines.[16-21] D6 is predominately localized in intracellular vesicular pool and is rapidly mobilized to the cell surface when high levels of chemokines are present to drive them to degradative compartments.[22]

The majority of the current information about D6 biology comes from animal studies investigating resolution of acute inflammatory responses.[23-25] In this context, D6 seems to have an antiinflammatory role, as D6-deficient mice develop an exaggerated and prolonged response when challenged with various inflammatory stimuli, such as Freund adjuvant,[23] phorbol esters,[25] allergens,[26] or *Mycobacterium tuberculosis*.[27] The few studies that investigated D6 expression in human settings reported its significant upregulation in chronic inflammatory conditions, such as systemic sclerosis, inflammatory bowel disease, and cardiac allograft rejection.[28-30] Of interest in this context, there is emerging evidence that D6 activity is also required for mounting adaptive immune responses in certain experimental conditions. For example, in animal models of autoimmune encephalomyelitis and graft-vs-host disease, the absence of D6 results in impaired adaptive immune responses due to either lack of migration of dendritic cells to lymph nodes or release of myeloid suppressor cells from bone marrow.[31,32] Furthermore, in *M tuberculosis* infection, D6 and the related CC chemokines are required for an appropriate immune response, which controls pathogen growth.[27]

Mechanisms promoting the persistent activation of adaptive immunity, possibly with an autoimmune component, have been described in COPD, wherein the chronic inflammation initiated by cigarette smoking may break the normal mechanisms of tolerance and promote lung damage.[10] We became interested in investigating the expression of D6 receptor in COPD, a condition in which the chronic inflammatory process may cause variable degrees of disease severity, making this condition a potential good model to study the behavior of this receptor. To this aim, D6 expression was evaluated in the lungs of smokers with COPD as well as those of smoker and nonsmoker control subjects. Furthermore, D6 was examined at both protein and mRNA levels in alveolar macrophages (AMs) obtained from BAL and in ex vivo monocyte-derived macrophages (MDMs). Finally, possible correlations with inflammatory cell infiltration and markers of immune activation in peripheral lung were examined.

Materials and Methods

Subjects undergoing lung resection for solitary peripheral carcinoma or lung volume reduction surgery for severe emphysema were recruited to the study: 16 smokers with COPD and 18 asymptomatic control subjects with normal lung function (nine smokers, nine nonsmokers). COPD was defined according to the GOLD (Global Initiative for Chronic Obstructive Lung Disease) international guidelines.[2] The study conformed to the Declaration of Helsinki, was approved by the local institutional review board (Padova Hospital Ethical Committee for Experimentation; project number: 2144P), and informed written consent was obtained for each subject undergoing surgery.

Tissue blocks were taken from the subpleural parenchyma of the lobe obtained at surgery, avoiding areas involved by tumor. Samples were fixed in 4% formaldehyde and, after dehydration, embedded in paraffin as previously described.[5-8] Sections 5-μm thick were cut and processed for immunohistochemical analysis of D6 expression (e-Appendix 1). D6 expression was quantified in AMs within alveolar spaces and in those in contact with the alveolar walls. Moreover, we investigated potential correlations between D6 expression and other immune/inflammatory parameters known to be upregulated in COPD ($CD8^+$ cells infiltrating the alveolar walls, IL-32, tumor necrosis factor [TNF]-α, B-cell activating factor of the TNF family [BAFF], and phospho-p38 mitogen-activated protein kinase [MAPK]). Details are provided in e-Appendix 1. Finally, to investigate the disease specificity, we evaluated D6 expression in other chronic inflammatory lung diseases: two autoptic samples from subjects with asthma and two explanted lungs from subjects with cystic fibrosis who underwent lung transplantation.

BAL was obtained from 10 subjects with COPD and five control smokers who underwent bronchoscopy for diagnostic assessment of suspect lung cancer. BAL cells were centrifuged, resuspended, frozen, and stored at −80°C for subsequent analyses. Immunofluorescence analysis with confocal microscopy was

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performed to detect D6, CD68, and mannose-receptor (CD206). Flow cytometry analysis was performed with CD45-PerCP, CD16-PE, or CD206-FITC and analyzed by BD FACSDiva Software (Becton, Dickinson and Company). Semiquantitative evaluation of D6 mRNA level was assessed by TaqMan Gene Expression Assay (Life Technologies). In addition, these analyses were performed in MDMs differentiated ex vivo from circulating monocytes. These cells were analyzed either in basal conditions (M0), after innate activation (TNF-α 100 ng/mL; 24 h) or after polarization to classic (M1) (100 ng/mL lipopolysaccharide plus 20 ng/mL interferon-γ) or alternative (M2) (20 ng/mL IL-4) activation.[33] Finally, D6-mediated chemokine degradation was investigated, using either the D6 ligand CCL3L1 or CXCL8/IL-8, not recognized by D6 and used here as a negative control. All details are reported in e-Appendix 1.

Group data were expressed as mean and SE or as median and range. Differences between groups were analyzed using tests for multiple comparisons: the analysis of variance or Kruskall-Wallis test. Unpaired *t* test or Mann-Whitney *U* test were subsequently carried out. Spearman rank or Pearson correlation coefficients were used to examine the association between histologic parameters as well as those with functional data. Probability values of $P < .05$ were accepted as significant.

Results

The clinical characteristics of the subjects are reported in Table 1. There were no demographic differences among the three groups of subjects, and the smoking history was similar in smokers with COPD and control smokers. Among patients with COPD, eight had severe to very severe disease (GOLD stage III-IV), and eight had mild to moderate disease (GOLD stage I-II). The values of Pa_{O_2} were significantly reduced in smokers with COPD compared with both control groups, whereas the values of Pa_{CO_2} were not significantly different. Smokers with mild to moderate COPD and control subjects with normal lung function did not receive antiinflammatory therapy (eg, oral or inhaled corticosteroids) or antibiotics within the month preceding surgery, or bronchodilators within the previous 48 h. All patients with severe to very severe COPD were treated with inhaled anticholinergic drugs and/or β_2 agonists/corticosteroids, but none with oral steroids or antibiotics.

The majority of D6 immunoreactivity was detected on AMs in the alveolar spaces and in those adjacent to the alveolar walls, but not in lymphatic vessels, as it has been reported before[18,29] (Fig 1). Relating the magnitude of D6 expression to the clinical status, we found that D6 expression in macrophages within the alveolar space was significantly increased in patients with COPD compared with smoker and nonsmoker control subjects. D6 expression tended to be higher in severe COPD than in mild to moderate COPD, but this difference did not reach statistical significance (e-Appendix 1). Similarly, D6 was upregulated in macrophages adjacent to the alveolar walls of smokers who develop COPD but not in smokers who maintained normal lung function (Fig 1). Conversely, D6 expression was scarce within the alveolar walls and was not significantly different among the three groups of subjects examined (0 [0-1.9] vs 0 [0-1.9] vs 0 [0-0.4] cells/mm, respectively).

The expression of D6 was not related to potential confounding factors, such as smoking status, cumulative smoking exposure, age, sex, or presence of lung cancer (e-Appendix 1). Of interest, the evaluation of D6 expression was not observed in other inflammatory disorders of the lung, such as asthma or cystic fibrosis, either in AMs or in other cells (e-Appendix 1).

To further evaluate the expression of D6 in macrophages, we analyzed BAL samples by flow cytometry, molecular analysis, and immunofluorescence. The analysis by flow cytometry showed that the great majority of BAL cells were represented by alternatively activated M2 macrophages ($CD45^+/CD68^+/CD16^+/CD206^+$) both in control smokers and patients with COPD (Fig 2). Molecular analysis showed that D6 mRNA levels were significantly increased in BAL macrophages from patients with COPD as compared with control smokers, a finding that confirms the expression of D6 observed by immunohistochemistry. Confocal microscopy confirmed the high expression of D6 in AMs of patients with COPD (Fig 2). As previously reported in other cell types,[34] D6 expression in macrophages exhibited a cytoplasmic granular pattern with a preferential colocalization with the intracellular macrophage marker CD68 rather than with the plasma membrane mannose receptor CD206 (Pearson correlation coefficient, 0.66 ± 0.03 and 0.33 ± 0.02, respectively) (Fig 2).

The expression and function of D6 were also investigated in ex vivo systems using MDMs. At variance with the in vivo setting, D6 transcript levels were barely detectable in MDMs, either in resting conditions (M0), after innate activation using TNF-α, or after polarized activation to classic (M1) or alternative (M2) macrophages (Fig 2). These results suggest that D6 expression by AM in COPD is likely dependent on microenvironmental stimuli present in the alveolar space of patients with COPD. In support of D6 activity in AMs of patients with COPD is the analysis of

Table 1—*Clinical Characteristics of the Subjects*

Characteristic	Patients With COPD	Smokers	Nonsmokers
Subjects, male (female)	13 (3)	9 (0)	5 (4)
Age, y	63 ± 2	66 ± 2	60 ± 5
Smoke, pack-y	50 ± 5	47 ± 8	…
Current (ex-smokers)	8 (8)	2 (7)	…
FEV_1, % predicted	57 ± 4[a]	102 ± 3	106 ± 6
FEV_1/FVC, %	51 ± 3[a]	79 ± 2	80 ± 1
Pa_{O_2}, mm Hg	72 ± 3[a]	86 ± 2	82 ± 2
Pa_{CO_2}, mm Hg	40 ± 1	40 ± 4	38 ± 1

Values are expressed as mean ± SE.
[a]Significantly different ($P < .05$) from smokers and nonsmokers.

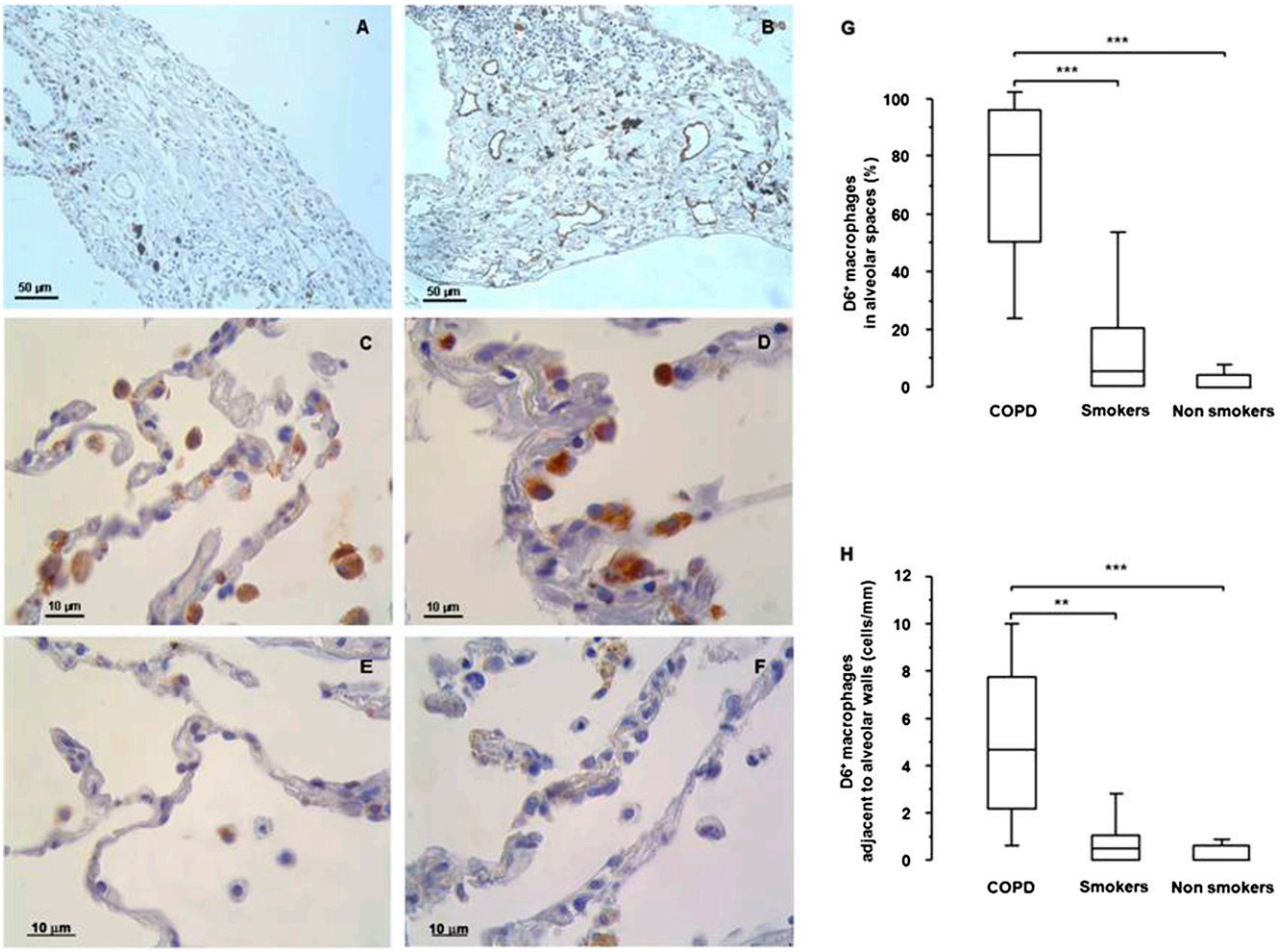

FIGURE 1. A, B, Expression of D6 in smokers with COPD was not detected in lymphatic vessels (A), even in the subpleural regions where they are abundant as identified by podoplanin immunostaining (B). C-F, By contrast, D6 expression in smokers with COPD was prominent in alveolar macrophages (AMs) both in those within the alveolar spaces (C) and in those adjacent to alveolar walls (D) and almost absent in AMs of smoker (E) and nonsmoker control subjects (F). D6 and podoplanin staining are in brown. G, H, Quantification of D6 expression in AMs within the alveolar spaces and in those adjacent to the alveolar walls, respectively. In each box plot, the box bottom and top denote the 25th and 75th percentiles, respectively, the solid line is the median, and the brackets are the 10th and 90th percentiles. *P* values are calculated using the Kruskall-Wallis test ($P < .05$ for both) followed by Mann-Whitney *U* test (*$P < .05$, **$P < .01$, and ***$P < .005$).

chemokine degradation, which shows a decrease in the level of the D6-ligand CCL3L1, whereas the concentration of the non-D6 ligand CXCL8/IL-8 was not modified (Fig 2). These results are suggestive of an active degradation of the CCL3L1 by D6 in AMs. To gain some insight into the mechanisms sustaining D6 selective induction on AMs in vivo, we analyzed the relationship between the expression of D6 and markers of immune activation previously reported to be increased in patients with COPD. Data for this analysis were available from previous studies and are reported in the e-Appendix 1. When all subjects were considered as a group, D6 expression was positively correlated with the number of $CD8^+$ cells infiltrating the alveolar walls ($r = 0.42$, $P = .02$) and with markers of immune activation (IL-32: $r = 0.79$, $P < .0001$; TNF-α: $r = 0.74$, $P = .0002$; BAFF: $r = 0.55$, $P = .003$; phospho-p38 MAPK: $r = 0.60$, $P = .003$) (Fig 3). Conversely, no correlation was found with the number of neutrophils infiltrating the alveolar walls. Finally, D6 expression was negatively correlated with lung function parameters (FEV_1: $r = -0.59$, $P = .0008$; FEV_1/FVC: $r = -0.67$, $P = .0001$). The majority of these correlations remained significant when nonsmoking subjects were excluded from the analysis (IL-32: $r = 0.76$, $P = .0002$; TNF-α: $r = 0.60$, $P = .006$; BAFF: $r = 0.40$, $P < .05$; phospho-p38 MAPK: $r = 0.58$, $P = .01$; FEV_1: $r = -0.46$, $P = .02$; FEV_1/FVC: $r = -0.62$, $P = .002$). The correlations with expression of IL-32 and TNF-α remained significant even when considering patients with COPD only ($r = 0.67$, $P = .01$; and $r = 0.73$, $P = .01$, respectively). Of interest, when patients with COPD were subcategorized according to the relative expression of D6 (above or below the median value), all patients with COPD with high levels of D6 were clustered within high levels of IL-32 and TNF-α,

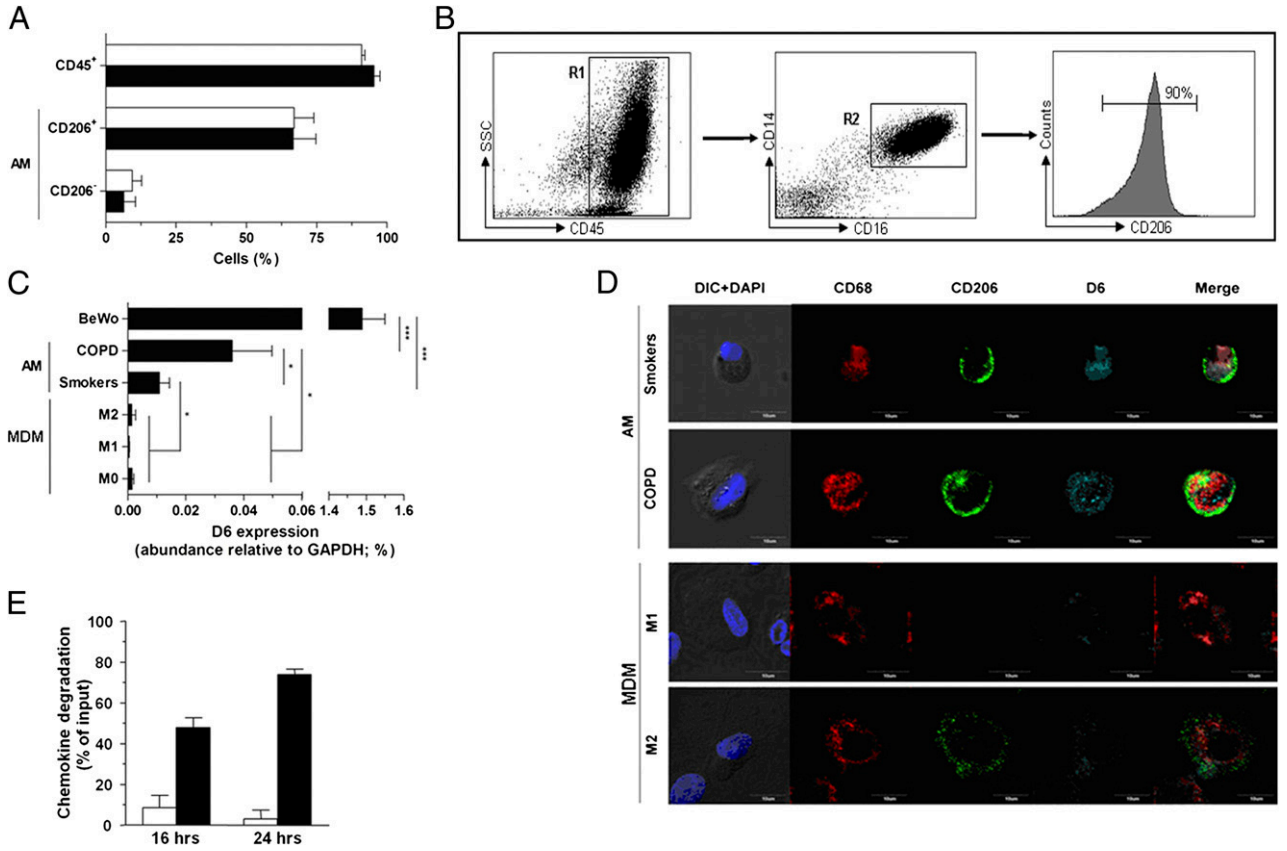

FIGURE 2. A, flow cytometry analysis of BAL cells in COPD (black columns) and control smokers (white columns). B, Gating strategy to identify human leukocyte population from BAL. The analysis was based on CD45 staining to define total leukocytes (R1); human macrophages were identified as $CD16^{+}/CD14^{+}$ subpopulation (R2). Finally, CD206 relative expression was calculated in R2-gated macrophages. Doublets and dead cells were excluded prior to analysis. C, D6 mRNA expression by real time reverse transcriptase polymerase chain reaction in human choriocarcinoma BeWo cells (positive control), in human BAL samples of patients with COPD and control smokers, and in ex vivo differentiated MDMs in resting conditions (M0) or after polarized activation (M1 and M2). The amount of D6 was calculated as relative abundance of D6 expression over GAPDH. *P* values are calculated using the Student *t* test ($*P < .05$ and $***P < .005$). D, confocal microscopy of AMs isolated from COPD and control smokers and ex vivo polarized MDMs. Cells were stained for CD68 (red), CD206 (green), and D6 (cyan). Cellular and nuclear morphology are shown by Nomarski technique (DIC) and DAPI staining (blue), respectively. E, chemokine degradation assay in BAL cells of patients with COPD. Total BAL cells were incubated with 1 nM CCL3L1 (black columns) or 1 nM CXCL8/IL-8 (white columns) at 37°C for the indicated time points. Results are indicated as 100 minus the percentage of intact chemokine, measured by enzyme-linked immunosorbent assay test. DAPI = fluorochrome 4′,6-diamidino-2-phenylindole; DIC = differential interference contrast; GAPDH = glyceraldehyde phosphate dehydrogenase; MDM = monocyte-derived macrophage; SSC = side scatter detector. See Figure 1 legend for expansion of other abbreviation.

whereas levels of these cytokines were much more scattered in patients with COPD with low D6 expression (Fig 3).

DISCUSSION

The present study demonstrates an increased expression of the atypical chemokine receptor D6 in peripheral lung from smokers with COPD but not in smoking subjects who did not develop the disease and nonsmoker control subjects. The expression of D6 was almost exclusively present in AMs, as confirmed by immunohistochemical, molecular, and confocal microscopy analysis, and was correlated with markers of immune activation. Conversely, D6 was barely expressed in ex vivo differentiated macrophages and could not be induced by either innate activation by TNF-α or classic (M1) or alternative (M2) activation pathways.

The observation of increased D6 expression in the lungs of patients with COPD is in keeping with the few previous reports that investigated D6 in human chronic inflammatory conditions. Indeed, these studies reported D6 upregulation in intestinal lymphatic vessels in Crohn's disease-ulcerative colitis[29] as well as in peripheral blood mononuclear cells in systemic sclerosis.[28] In contrast with previously reported localization of D6 in lymphatic vessels in mice and in some human studies,[18,29] we found that in COPD D6 expression was predominantly, if not exclusively, detected in AMs, an observation that we confirmed in both surgical specimens and BAL. These results are in keeping with the findings of a recent study showing that D6, which was found to be upregulated in cardiac allograft

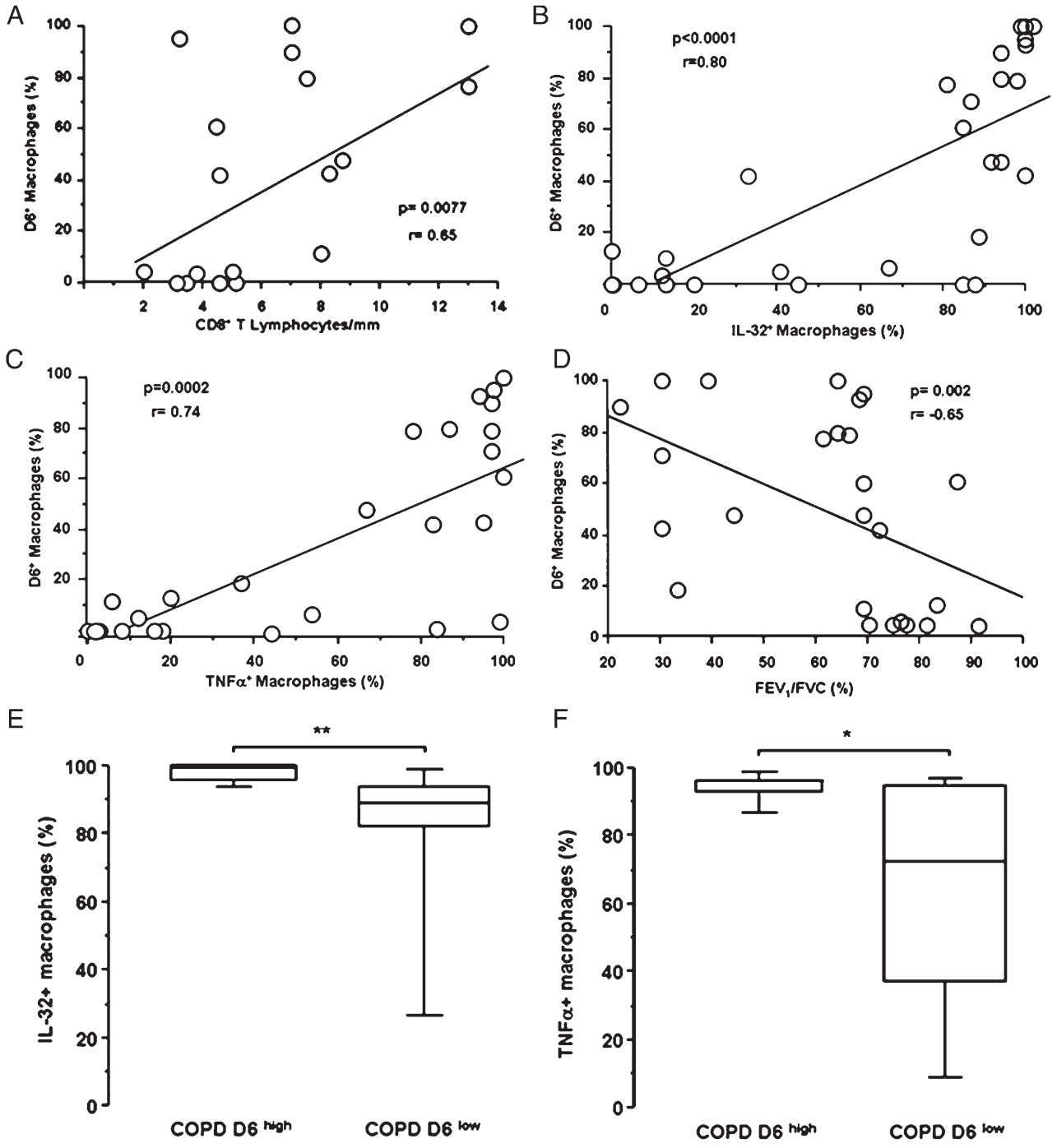

FIGURE 3. A-D, When all subjects were considered as a group, D6 expression was positively correlated with the markers of immune activation: $CD8^+$ cells infiltrating the alveolar walls (A), IL-32 (B), and TNF-α (C) and negatively correlated with lung function parameters (FEV_1/FVC) (D). E, F, When stratified according to the expression of D6 (above or below the median value), patients with COPD with high D6 levels showed levels of IL-32 and TNF-α significantly higher than patients with COPD with low D6 levels in which the levels of these cytokines were scattered. *P* values (Mann-Whitney *U* test): $*P < .05$, $**P = .01$. TNF = tumor necrosis factor.

rejection,[30] was predominantly localized to graft-infiltrating macrophages. Furthermore, in that study, the degree of D6 upregulation was related to the severity of the rejection. These findings suggest that macrophages are important determinants of the fate of adaptive immune responses and that D6 may have an important role in this setting. Of interest, in our study, increased expression of D6 was observed in clusters of macrophages within the alveolar spaces as well as in macrophages that were in close contact with the alveolar walls. In this site, macrophages can modify the function of type 2 pneumocytes, which normally

suppress immune inflammatory responses within the lung and maintain immunologic tolerance.[35,36]

The assessment of isolated cells from BAL in our study showed that D6 mRNA levels were significantly increased in smokers with COPD as compared with smokers with normal lung function, a finding that confirms the expression of D6 observed by immunohistochemistry. Interestingly, D6 localized in vivo in human AMs expressing high levels of the mannose receptor, a marker of the alternatively activated anti-inflammatory macrophages (M2). This finding invites the speculation that the expression of D6 might be related to the described antiinflammatory function of the M2 AMs. Contrary to our findings on macrophages from the alveolar compartment of the lung, we found that D6 was barely expressed in monocyte-derived macrophages and that it could not be induced by either innate, classic (M1), or alternative (M2) activation pathways. These observations suggest that there ought to be some tissue-specific factors, at least in chronic inflammatory states, that induce D6 expression in macrophages in vivo, as suggested by our findings in COPD and by the results in cardiac allograft rejections.[30]

An interesting observation in our study was that the expression of D6 in AMs was related to markers of immune activation, such as the infiltration of $CD8^+$ T lymphocytes, the phosphorylation of p38-MAPK, and the expression of the proinflammatory cytokines IL-32, TNF-α, and BAFF.[37-39] Furthermore, all patients with COPD with high levels of D6 also showed high levels of IL-32 and TNF-α. The described upregulation of D6 in chronic inflammatory conditions, such as COPD, Crohn's disease, and chronic allograft rejection, together with its correlation with markers of disease severity and immune activation, does not go along with its described antiinflammatory role in acute inflammation in animal models. It is possible that D6 upregulation may represent a failed mechanism to restrain inflammation in COPD. However, it seems more likely that, beyond its essential role in acute innate responses, D6 may act differently in chronic inflammatory states with an adaptive immune component.[32] In favor of this possibility are the findings in a mouse model of autoimmune encephalomyelitis in which $D6^{-/-}$ mice were resistant to the induction of the disease.[31] Furthermore, in the same study, the adoptive transfer of $D6^{-/-}$ mice T cells transferred disease poorly as compared with wild-type $D6^{+/+}$ animals, suggesting impaired T-cell priming in the $D6^{-/-}$ mice.[31] Intriguingly, since D6 is expressed by dendritic cells and B cells in vitro and modulates the induction of IL-17 and antibody production,[40] it has been suggested that D6 may be critically involved in the initiation of adaptive immune responses, possibly affecting antigen presentation.[41] In this context it can be hypothesized that, since D6 traffics continuously between the cell membrane and the endolysosomal compartment to internalize inflammatory cytokines, it may return loaded with antigenic peptides. All these results seem to point to the possibility that in chronic inflammatory states, upregulation of D6, which expression might initially increase in the attempt to restrain the inflammatory response, may eventually promote immune activation and disease progression. These possibilities would go along with the finding that in humans D6 has been found to be increased in diseases with a likely autoimmune component, like cardiac allograft rejection, Crohn's disease-ulcerative colitis, and COPD, but not in asthma and cystic fibrosis, in which the autoimmune component does not seem to be important.

A possible criticism of our findings could be that the presence of lung cancer in most of our patients may have influenced our results. We were careful to examine only areas free of disease and distant from tumor, and moreover, since lung cancer was present even in smoker and nonsmoker control subjects, we believe that our findings are valid. Furthermore, patients with COPD undergoing lung volume reduction surgery, who did not have lung cancer but had severe COPD, had higher levels of D6 expression than control subjects with lung cancer. Finally, although our study did not fully address the functional consequences of D6 expression in AMs, we believe these results have the potential to provide the clinical framework for proper functional investigations.

In conclusion, the atypical chemokine receptor D6 is expressed in human AMs, is upregulated in COPD, and is correlated with markers of disease severity and immune activation. These data suggest that, besides its known scavenger activity in acute inflammation, D6 probably has additional functions in chronic inflammatory conditions, possibly promoting immune activation.

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Author contributions: Drs Bazzan, Saetta, Turato, Mantovani, Bonecchi, and Locati are guarantors of the manuscript.

Dr Bazzan: contributed to study conception and design, experimental analysis, result interpretation, and manuscript drafting for important intellectual content.

Dr Saetta: contributed to study conception and design, result interpretation, and manuscript drafting for important intellectual content.

Dr Turato: contributed to study conception and design, result interpretation, and manuscript drafting for important intellectual content.

Dr Borroni: contributed to experimental analysis, results interpretation, and manuscript drafting.

Ms Cancellieri: contributed to experimental analysis and manuscript drafting.

Dr Baraldo: contributed to experimental analysis, result interpretation, and manuscript drafting for important intellectual content.

Dr Savino: contributed to experimental analysis, result interpretation, and manuscript drafting.

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Additional information: The e-Appendix can be found in the "Supplemental Materials" area of the online article.

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