CHEST

Original Research

COPD

Cysteinyl Leukotriene 1 Receptor Expression Associated With Bronchial Inflammation in Severe Exacerbations of COPD

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Background: **Cysteinyl leukotriene 1 ($CysLT_1$) receptor expression is known to be increased in the airway mucosa of patients with asthma, especially during exacerbations; however, nothing is known of its expression in COPD.**

Methods: **We applied immunohistochemistry and in situ hybridization to endobronchial biopsies to determine inflammatory cell $CysLT_1$ receptor protein and mRNA expression in the following: (1) 15 nonsmoker control subjects (NSC), (2) 16 smokers with moderate to severe COPD in its stable phase (S-COPD), and (3) 15 smokers with COPD hospitalized for a severe exacerbation (SE-COPD).**

Results: **The total number of bronchial mucosal inflammatory cells ($CD45^+$) and those expressing $CysLT_1$ receptor protein were significantly greater in SE-COPD ($CysLT_1$ receptor protein: median [range] = 139 [31-634]) as compared with S-COPD (32 [6-114]) or NSC (16 [4-66]) ($P < .001$ for both). $CysLT_1$ receptor gene expression showed similar differences. A greater proportion of $CD45^+$ cells expressed $CysLT_1$ receptor protein in SE-COPD (median [range] = 22% [8-81]) compared with S-COPD (10% [4-32]) ($P < .03$) or NSC (7% [1-19]) ($P < .002$). In SE-COPD, the relative frequencies of $CysLT_1$ receptor-expressing cells were as follows: $tryptase^+$ mast cells > $CD68^+$ monocytes/macrophage > neutrophils > $CD20^+$ B lymphocytes = $EG2^+$ eosinophils. Moreover, there were positive correlations between the numbers of cells expressing $CysLT_1$ receptor protein and the numbers of $CD45^+$ cells ($r = 0.78$; $P < .003$) and $tryptase^+$ mast cells ($r = 0.62$; $P < .02$).**

Conclusions: **Bronchial mucosal $CysLT_1$ receptor-positive inflammatory cells are present in the bronchial mucosa in COPD in greatest number in those experiencing a severe exacerbation.**

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Abbreviations: $CysLT_1$ = cysteinyl leukotriene 1; IHC = immunohistochemistry; ISH = in situ hybridization; LTRA = leukotriene receptor antagonist; NSC = nonsmoker control subject; S-COPD = COPD in its stable phase; SE-COPD = COPD with severe exacerbation

Leukotrienes are important mediators of airway inflammation modulating recruitment of inflammatory cells, increased vascular permeability, mucus hypersecretion, and bronchoconstriction, all features of COPD and asthma. Accordingly, leukotriene receptor antagonists (LTRAs) represent a novel class of treatment of the inflammation of asthma.[1-6] While the predominant cellular profile of inflammation in COPD when stable is distinct from that of asthma,[7] the patterns of inflammation during exacerbations in asthma and COPD become more similar.[8-11]

Two pharmacologically defined G-protein-coupled leukotrienes receptors have been described: the cysteinyl leukotriene 1 ($CysLT_1$) and cysteinyl leukotriene 2 receptors.[12,13] The former is the main receptor for the actions of cysteinyl leukotrienes in the lung and a prime target for LTRAs in the treatment of airway inflammation.[12-16] Figueroa et al[12,17] first reported $CysLT_1$ receptor expression in grossly normal lung tissues resected from a nonasthmatic smoker and on peripheral blood leukocytes. It has also been demonstrated on nasal lavage cells obtained from symptomatic

patients with seasonal allergic rhinitis[14] and in human nasal mucosa from patients with perennial rhinitis.[18] We have reported increased total numbers of inflammatory cells expressing $CysLT_1$ receptor in the bronchial mucosa of mild stable patients with asthma as compared with healthy nonsmokers, with even further increases in patients with severe exacerbations of asthma. Using a simultaneous double-labeling technique, we have demonstrated in asthma that the $CysLT_1$ receptor can be expressed by a variety of inflammatory cells of distinct phenotype.[19] However, its expression has not been investigated in the so-called abnormal inflammation of either stable COPD or those experiencing an exacerbation.

We hypothesized that the increased leukocyte infiltration of COPD and, in particular, the change in character of the inflammation associated with an exacerbation would be accompanied by increases of $CysLT_1$ receptor expression. Thus, we have applied immunohistochemistry (IHC) and in situ hybridization (ISH) to endobronchial biopsies obtained from patients with COPD hospitalized and intubated as part of their emergency treatment of a severe exacerbation. Distinct inflammatory cells in the bronchial mucosa expressing $CysLT_1$ receptor protein or mRNA were identified, and their numbers counted. The results of inflammatory cell phenotypes not previously studied in exacerbations, such as macrophages, mast cells, and T and B lymphocytes, are also presented herein. The preliminary results of this study have been reported in abstract form.[20,21]

Materials and Methods

The study conformed to the Declaration of Helsinki. The study protocols were approved by the Institutional Review Board for Human Subject Research at Baylor College of Medicine and affiliated Hospital in Houston with project approval number H-4892. All subjects, and in the case of the intubated subjects their surrogates, gave informed written consent.

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Subjects

Patient demography is shown in Table 1. The study groups included the subjects examined in our previously reported study, for which we have already published the results for counts of other inflammatory end points.[8] The study groups were the same and comprised: (1) 15 nonsmoker control subjects (NSCs) with normal lung function ($FEV_1 > 80\%$ predicted; $FEV_1/FVC > 70\%$) who, under general anesthesia, were intubated as part of a surgical procedure for a nonbronchopulmonary condition (eg, hysterectomy or cholecystectomy); (2) 16 current smokers (58 ± 8 pack-years) with moderate to severe COPD (GOLD [Global Initiative for Chronic Obstructive Lung Disease] stage II and III) in its stable phase (S-COPD), (FEV_1, 42%-62% predicted and FEV_1/FVC, 44%-71%) (none of these patients had exacerbations within the preceding year); and (3) 15 current smokers (61 ± 5 pack-years) with severe COPD (FEV_1, 18%-50% predicted and FEV_1/FVC, 18%-66%) and experiencing an acute severe exacerbation (SE-COPD) (GOLD stage IV) characterized by sudden worsening of symptoms (ie, wheezing, breathlessness, chest tightness, and cough) requiring hospitalization, emergent intubation, and mechanical ventilation for respiratory failure. For details, see e-Appendix 1.

Biopsy Procedures

The biopsy specimens were taken from patients admitted to Ben Taub General Hospital (Houston, Texas). For details, see e-Appendix 1.

Nonisotopic ISH

The nucleic acid sequences of the $CysLT_1$ receptor probe have been described previously.[12] Other details as to preparation of cDNA and cRNA probes and the procedures of ISH are described in e-Appendix 1.

IHC and Double Immunohistofluorescence

These techniques have been described previously.[12] For further details of the immunostaining procedures used to detect the $CysLT_1$ receptor protein and to phenotype inflammatory cells and the indirect immunofluorescence double-staining used to identify which phenotypes of inflammatory cell expressed the $CysLT_1$ receptor, please see e-Appendix 1.

Quantification and Statistical Analyses

Cell count data were assessed first using the Kruskal-Wallis test followed by between-two-group comparisons using the Mann-Whitney *U* test. Spearman rank correlation was used as the test for correlations. *P* values of $< .05$ and $< .03$ were accepted as indicating a significant difference in Mann-Whitney *U* tests and Spearman rank correlation, respectively. For details of the methods of quantification, variability, and how we expressed the data and statistical analyses, see e-Appendix 1.

Results

Clinical Findings

The clinical characteristics of the subjects are shown in Table 1. Both FEV_1% predicted and FEV_1/FVC of the COPD groups were significantly lower than that of the NSC group. As anticipated, the lowest FEV_1% predicted and FEV_1/FVC values were found in the SE-COPD group.

Table 1—*Clinical Characteristics of Individual Patient*

Subject No.	Sex	Age, y	Smoking, pack-y	FEV_1% Predicted	FEV_1/FVC, %	$PaCO_2$, mm Hg
SE-COPD (n = 15)						
1	M	57	38	50	66	65
2	M	59	65	36	40	56
3	F	69	44	32	30	110
4	F	53	53	24	21	78
5	F	65	54	44	39	49
6	F	66	60	45	46	96
7	F	65	58	30	28	69
8	F	73	50	36	28	68
9	M	71	76	40	30	86
10	M	63	90	18	24	107
11	F	70	60	34	28	61
12	F	63	50	46	24	94
13	M	45	38	50	52	64
14	M	67	82	31	28	73
15	M	41	104	28	18	84
Mean	...	62[a]	61[a]	36[a,b]	35[a,b]	77
SEM	...	2	5	2	3	5
S-COPD (n = 16)						
16	F	52	70	43	47	N/A
17	F	47	56	42	58	N/A
18	M	58	103	42	44	N/A
19	M	71	65	53	44	N/A
20	F	52	34	62	71	N/A
21	F	40	38	55	59	N/A
22	F	63	55	60	51	N/A
23	M	61	59	46	58	N/A
24	M	63	55	58	45	N/A
25	F	73	150	48	45	N/A
26	M	59	40	57	49	N/A
27	M	62	49	53	43	N/A
28	M	61	40	50	49	N/A
29	M	59	30	54	56	N/A
30	M	57	31	53	46	N/A
31	F	68	52	59	53	N/A
Mean	...	59[a]	58[a]	52[a]	51[a]	...
SEM	...	2	8	2	2	...
NSC (n = 15)						
32	F	42	0	112	81	N/A
33	M	45	0	95	78	N/A
34	F	62	0	94	76	N/A
35	F	41	0	98	79	N/A
36	F	52	0	89	82	N/A
37	F	42	0	90	79	N/A
38	F	44	0	106	81	N/A
39	F	52	0	89	82	N/A
40	F	41	0	97	81	N/A
41	F	37	0	103	78	N/A
42	M	42	0	108	80	N/A
43	F	53	0	90	79	N/A
44	F	57	0	102	84	N/A
45	F	42	0	93	79	N/A
46	F	48	0	103	84	N/A
Mean	...	47	0	98	80	...
SEM	...	2	0	2	1	...

F = female; M = male; N/A = not available; NSC = nonsmoker control subject; S-COPD = COPD in its stable phase; SE-COPD = COPD with severe exacerbation.

[a]$P < .01$ compared with NSC.

[b]$P < .01$ compared with S-COPD (unpaired Student *t* test).

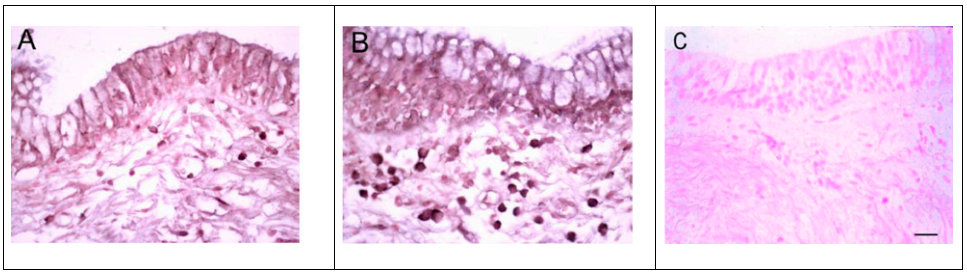

FIGURE 1. Nonisotopic in situ hybridization (ISH) of a bronchial biopsy tissue section illustrating cysteinyl leukotriene 1 ($CysLT_1$) receptor mRNA expression. The positive signal is visualized as blue/black with the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) technique. Cells are counterstained with nuclear fast red. A, A healthy nonsmoker control subject (NSC) showing relatively few positive cells in the subepithelial zone weakly expressing $CysLT_1$ receptor mRNA. B, A subject with COPD experiencing a severe exacerbation showing many strongly stained $CysLT_1$ receptor mRNA positive inflammatory cells infiltrating the subepithelial zone. C, The sense control probe shows an absence of signal in a biopsy specimen of a COPD subject experiencing an exacerbation (internal scale bar = 20 μm for A-C).

$CysLT_1$ Receptor mRNA and Protein Expression

Subepithelial inflammatory cells stained strongly for both $CysLT_1$ receptor mRNA and protein (Figs 1, 2). These were relatively infrequent in the NSC (Figs 1A, 2A) and S-COPD groups and more frequently observed in those experiencing a severe exacerbation (Figs 1B, 2B). $CysLT_1$ receptor protein-positive inflammatory cells were also seen to have infiltrated the surface epithelium (Fig 2B). There were no signals in bronchial biopsies from the SE-COPD group stained as negative control subjects (Figs 1C, 2C).

The numbers of $CysLT_1$ receptor $mRNA^+$ and $protein^+$ cells in the S-COPD group were similar to those of the NSCs (Fig 3). However, compared with S-COPD, the values for the SE-COPD group were significantly greater for both $CysLT_1$ receptor $mRNA^+$ (4.3-fold) and $protein^+$ cells (6.3-fold) (Fig 3).

Since S-COPD and SE-COPD differed significantly as to baseline airway obstruction, we categorized subgroups based on mean values of FEV_1% predicted (e-Table 1). Among patients with S-COPD, no difference was observed in $CysLT_1$ receptor expression between those with FEV_1 below or those above the mean value. The same was true among patients with SE-COPD. Furthermore, when we evaluated patients with stable and exacerbated COPD of comparable disease severity ($40 < FEV_1 < 50\%$ predicted), we confirmed that $CysLT_1$ receptor expression was indeed increased in SE-COPD as compared with the S-COPD group patients (e-Table 1).

Quantification of Inflammatory Cells

The numbers of epithelial and subepithelial $CD45^+$ (total) leukocytes in the S-COPD and SE-COPD groups were similar, with both significantly greater than in the NSC group (Fig 4A, Table 2). Moreover, the percentage of $CD45^+$ cells expressing $CysLT_1$ receptor protein was significantly higher in the SE-COPD group than in either the S-COPD or the NSC group (Fig 4B).

Compared with the NSC group, there were greater numbers of subepithelial $CD68^+$ monocytes/macrophages in the S-COPD group and a further increase in the SE-COPD group (Fig 5A, Table 2). $CD20^+$ B lymphocytes were increased in similar fashion in both

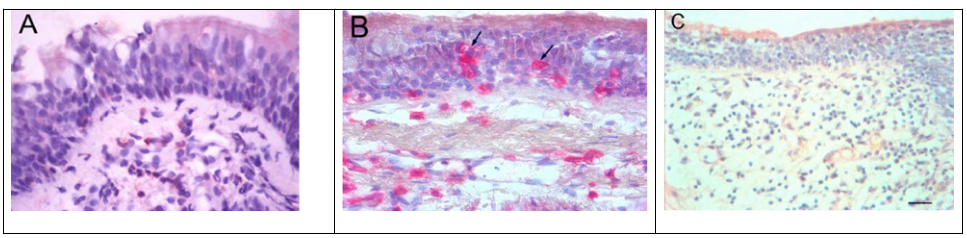

FIGURE 2. Immunohistochemistry (IHC) localizing $CysLT_1$ receptor protein in bronchial biopsy specimens. The protein is seen as red fuchsin positivity with cell nuclei counterstained blue by hematoxylin. A, A healthy NSC showing expression of $CysLT_1$ receptor protein in only a few of the subepithelial inflammatory cells. B, A subject with COPD with severe exacerbation (SE-COPD) demonstrating more $CysLT_1$ receptor protein positive inflammatory cells in both subepithelium and epithelium (arrows). C, Method control: using normal goat serum instead of anti-$CysLT_1$ receptor antibody, there is no positive stain for protein in the biopsy specimen of a COPD subject experiencing an exacerbation (internal scale bar = 20 μm for A-C). See Figure 1 legend for expansion of other abbreviations.

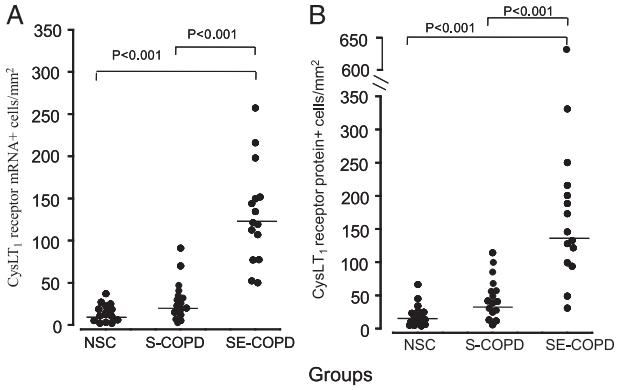

FIGURE 3. A, B, Counts for $CysLT_1$ receptor. mRNA (A) and protein-positive cells (B) in bronchial biopsy specimens of patients with NSC, S-COPD, and SE-COPD. The data are expressed as the number of positive cells per mm^2 of subepithelium. ● = Individual counts; horizontal bars = median values (Mann-Whitney *U* test). S-COPD = COPD in its stable phase. See Figure 1 and 2 legends for expansion of other abbreviations.

the S-COPD and SE-COPD groups (Fig 5B, Table 2). As reported previously,[8] subepithelial $EG2^+$ eosinophils (Fig 5C) and neutrophils were significantly more frequent only in the SE-COPD group (Table 2). In contrast, the numbers of subepithelial $tryptase^+$ mast cells were significantly fewer in both the S-COPD and SE-COPD groups (Fig 5D, Table 2). However, mast cells in the epithelium in S-COPD were significantly greater compared with both the NSC and SE-COPD groups (Table 2). In respect of T lymphocytes, both epithelial and subepithelial $CD4^+$ cells were significantly fewer in both the S-COPD and SE-COPD groups, particularly so in the former (Fig 5E, Table 2). The number of subepithelial $CD8^+$ cells was raised in the S-COPD group only (Fig 5F, Table 2). Thus, the subepithelial CD4/CD8 ratio was significantly lower in both S-COPD ($P < .0001$) and SE-COPD groups ($P < .001$) compared with the NSC group (Table 2). It was higher in SE-COPD than in S-COPD. The epithelial CD4/CD8 ratio was significantly lower in SE-COPD than that in NSC ($P < .02$) (Table 2). The rank order of frequency of subepithelial inflammatory cells in S-COPD was $CD8^+$ cells > mast cells > $CD68^+$ > $CD4^+$ > $CD20^+$ > neutrophils > $EG2^+$ eosinophils and different in SE-COPD where it was: $CD68^+$ > $CD8^+$ > $CD4^+$ > mast cells > neutrophils > $CD20^+$ > $EG2^+$ eosinophils.

Colocalization of $CysLT_1$ Receptor Protein and Inflammatory Cell Phenotypes

In SE-COPD, simultaneous double immunofluorescence labeling was applied to phenotype the inflammatory cells expressing $CysLT_1$ receptor protein in the biopsy specimens from nine SE-COPD group patients. Such double staining identified $elastase^+$ neutrophils (Figs 6A-C), $EG2^+$ eosinophils, $CD68^+$ monocytes/macrophages (Figs 6D-F), $tryptase^+$ mast cells (Figs 6G-I), and $CD20^+$ B lymphocytes (Figs 6J-L), each coexpressing $CysLT_1$ receptor protein. However, $CD3^+$, $CD4^+$ (Fig 7A), and $CD8^+$ (Fig 7B) T lymphocytes were each negative with the $CysLT_1$ receptor antisera.

Counts of cells showing colocalization for $CysLT_1$ receptor protein and mast cells, $CD68^+$ cells, neutrophils, $CD20^+$ cells, and $EG2^+$ eosinophils are shown in Table 3. The median (range) as a percentage, in rank order, of $CysLT_1$ receptor-positive cells was: mast cells > $CD68^+$ > neutrophils > $CD20^+$ = $EG2^+$

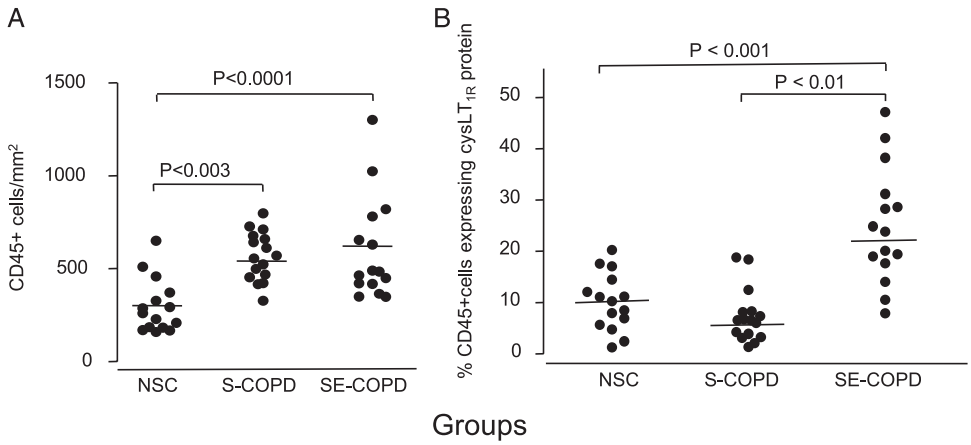

FIGURE 4. A, Counts for $CD45^+$ inflammatory cells. B, A percentage of $CD45^+$ inflammatory cells expressing $CysLT_1$ receptor protein in subepithelium of bronchial biopsy specimens of patients with NSC, S-COPD, and SE-COPD. The data are expressed as (A) the number of positive cells per mm^2 of subepithelium and (B) percentage of $CD45^+$ cells expressing $CysLT_1$ receptor protein. ● = Individual counts; horizontal bars = median values (Mann-Whitney *U* test). See Figure 1-3 legends for expansion of abbreviations.

Table 2—*Counts of Subtypes of Epithelial and Subepithelial Inflammatory Cells in Bronchial Biopsy Specimens*

	Epithelium			Subepithelium		
Cell Type	NSC (n = 15)	S-COPD (n = 16)	SE-COPD (n = 15)	NSC (n = 15)	S-COPD (n = 16)	SE-COPD (n = 15)
CD45$^+$	20 (9-64)	55 (11-95)[a]	60 (13-360)[a]	345 (143-654)	532 (356-816)[a]	607 (371-1346)[a]
CD68$^+$	10 (0-43)	18 (6-89)	13 (1-97)	35 (0-152)	120 (42-340)[a]	337 (54-674)[a,b]
Neutrophil	0 (0-0.1)	0 (0-2)	0.3 (0-8)[a]	0 (0-7)	2 (0-10)	97 (1-594)[a,b]
EG2$^+$	0 (0)	0 (0-1)	0 (0-6)	0 (0)	0 (0-10)	8.7 (0-437)[a,b]
Mast cells	1 (0-7)	6 (1-23)[a]	1 (0-10)[b]	254 (53-434)	137 (11-410)[a]	111 (31-379)[a]
CD20$^+$	0 (0-1)	0 (0-4)	0 (0-5)	6 (0-25)	10 (1-83)[a]	24 (4-465)[b]
CD4$^+$	5 (0-17)	0.9 (0.2-12)[a]	0.4 (0-11)[a]	272 (46-1198)	38 (6-440)[a]	121 (14-319)[a,b]
CD8$^+$	42 (1-117)	28 (9-55)	34 (12-51)	181 (22-499)	310 (137-852)[a]	190 (113-344)[b]
CD4/CD8	0.15 (0-5.4)	0.03 (0-1.3)	0.03 (0-0.3)[a]	1.6 (0.6-4.5)	0.13 (0-0.5)[a]	0.5 (0.1-1.5)[a,b]

Data are expressed as median (range) of numbers of cells per 0.1 mm^2 epithelial and per mm^2 subepithelial areas. See Table 1 for expansion of abbreviations.

[a]$P < .05$ or 0.01 vs NSC.

[b]$P < .05$ or 0.01 vs S-COPD.

eosinophils (Table 3). Seventy percent of mast cells and between 40% and 50% of CD68$^+$, CD20$^+$ cells, neutrophils, and EG2$^+$ eosinophils expressed $CysLT_1$ receptor protein (Table 3).

Associations

The numbers of subepithelial cells expressing $CysLT_1$ receptor mRNA in each group correlated positively with the number of cells expressing the receptor protein ($r = 0.62$-0.70, $P < .01$ Spearman rank correlation). There were strong positive correlations between the numbers of subepithelial CD45$^+$ leukocytes and the numbers of cells expressing $CysLT_1$ receptor mRNA and protein only within the SE-COPD group (Figs 8A, 8B). There was a significant positive correlation between the number of $CysLT_1$ receptor protein$^+$ cells and mast cells in the SE-COPD group (Fig 8C) but no significant associations between cells expressing $CysLT_1$ receptor mRNA or protein and the numbers of other inflammatory cells or FEV_1% predicted.

Infection

As described previously,[8] in the SE-COPD group, two patients had evidence of bacterial and viral infection concurrently, one patient had bacterial infection only and five had respiratory tract viral infection only, whereas six were negative for both bacteria and virus. We subdivided the SE-COPD group patients into those with and without detectable viral or bacterial infection and found that the number of epithelial CD68$^+$ cells was significantly greater in the infected group (e-Table 2). Epithelial CD8$^+$ cells and subepithelial CD20$^+$ B lymphocytes were significantly less frequent in those with infection. Finally, subepithelial $CysLT_1$ receptor mRNA$^+$ and protein$^+$ cells and other epithelial and subepithelial phenotypes of inflammatory cells were not significantly altered in those identified with infection (e-Table 2).

Discussion

This is the first demonstration that inflammatory cells in the bronchial mucosa of patients with COPD express the gene and protein for the $CysLT_1$ receptor. By comparison with NSC and subjects with stable COPD, the number of $CysLT_1$ receptor-expressing cells is significantly greater in patients with COPD experiencing a severe exacerbation. We have also determined that a number of distinct inflammatory cell phenotypes express the receptor, with their relative frequencies being tryptase$^+$ mast cells > CD68$^+$ monocytes/macrophage > neutrophils > CD20$^+$ B-lymphocytes = EG2$^+$ eosinophils. Finally, we have demonstrated that the predominant pattern of inflammatory cell phenotypes infiltrating the bronchial mucosa during a severe exacerbation is different to that in subjects with COPD when stable.

The increase in the numbers of $CysLT_1^+$ cells in exacerbations of COPD is similar to our findings in exacerbations of asthma.[19] However, whereas in stable asthma, by comparison with NSC, we have previously demonstrated increased numbers of the $CysLT_1$ receptor$^+$ inflammatory cells,[19] the trend seen herein to higher numbers of $CysLT_1$ receptor$^+$ cells in stable COPD did not achieve statistical significance. In keeping with this, the positive correlation between the increased numbers of $CysLT_1$ receptor$^+$ cells and the total number of (CD45$^+$) leukocytes was seen only in the SE-COPD group. Moreover, the proportion of all CD45$^+$ inflammatory cells that expressed the $CysLT_1$ receptor protein was significantly greater only in the SE-COPD group. The latter observation indicates that the increase of

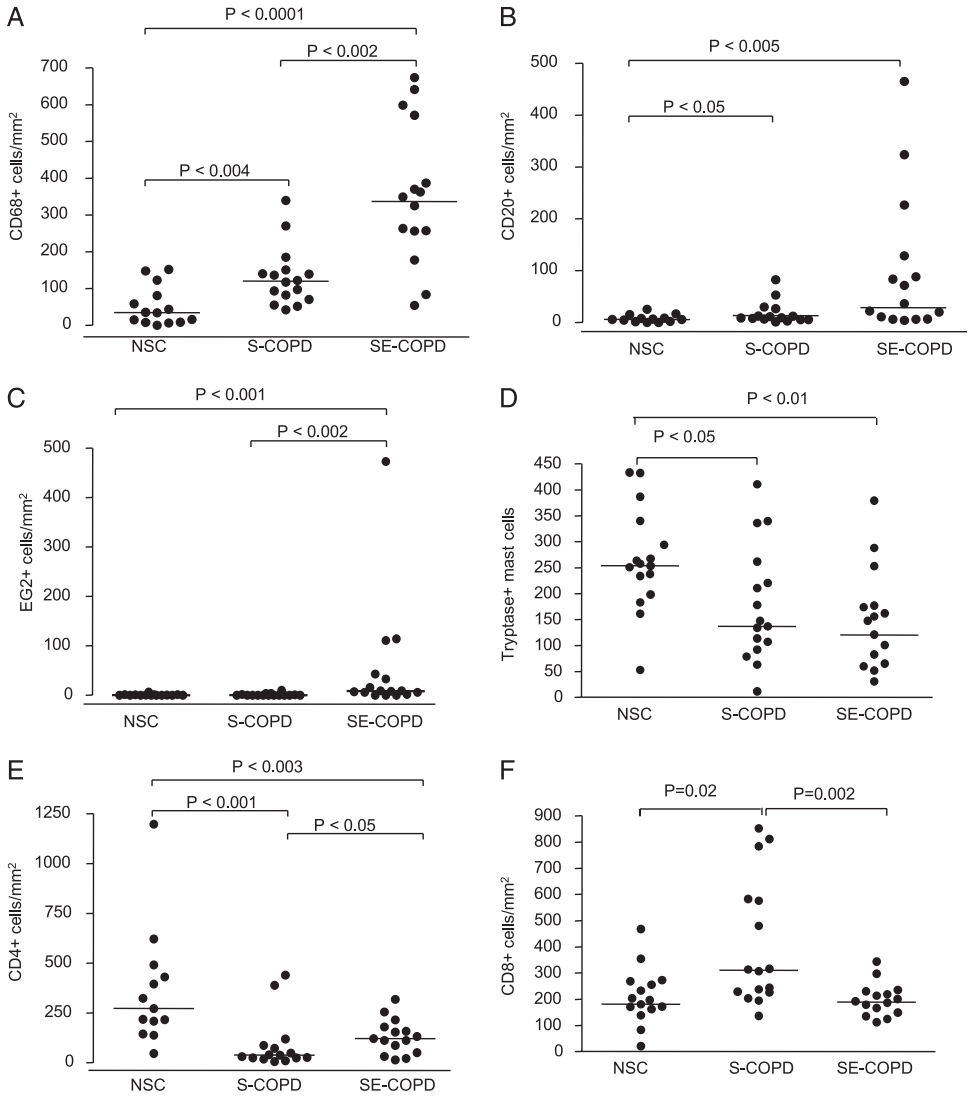

FIGURE 5. Graphs of counts for inflammatory cells in subepithelium of bronchial biopsy specimens of patients with NSC, S-COPD, and SE-COPD. A, $CD68^+$. B, $CD20^+$. C, $EG2^+$ eosinophils. D, Tryptase$^+$ mast cells. E, $CD4^+$. F, $CD8^+$. The data are expressed as the number of positive cells per mm^2 of subepithelium. ● = Individual counts; horizontal bars = median values (Mann-Whitney *U* test). See Figure 1-3 legends for expansion of abbreviations.

$CysLT_1$ receptor$^+$ cells was due not only to an overall increased recruitment of inflammatory cells but also the result of an upregulation of its expression by the distinct inflammatory cell phenotypes making up the $CD45^+$ population.

In order to determine which cell types expressed the receptor, we applied a simultaneous double-labeling technique in patients experiencing an exacerbation and demonstrated that, similar to asthma,[19] bronchial mucosal mast cells, monocytes/macrophages, eosinophils, neutrophils, and $CD20^+$ B lymphocytes, but not $CD4^+$ and $CD8^+$ T lymphocytes, each express the $CysLT_1$ receptor. Moreover, we found that mast cells, $CD68^+$ macrophages, and neutrophils formed the predominant populations of $CysLT_1$ receptor-expressing cells (Table 3).

We hypothesize that the increase of $CysLT_1$ receptor$^+$ cells in SE-COPD, but not S-COPD, is likely due to alteration of the predominant pattern of leukocyte infiltration. In support of this, we have demonstrated that unlike S-COPD, which is predominately due to more $CD8^+$ cells, the numbers of $CD68^+$ monocytes/macrophages became the predominant cell type in SE-COPD. Moreover, the numbers of $CD68^+$ cells, neutrophils, and $EG2^+$ eosinophils, in SE-COPD were significantly greater compared with S-COPD, whereas the number of subepithelial $CD8^+$ cells in the SE-COPD group was significantly less than that seen in S-COPD. Thus, alterations in the relative frequency of inflammatory cell types favoring $CD68^+$ cells, neutrophils, and $EG2^+$ eosinophils would be expected to lead to an increase in

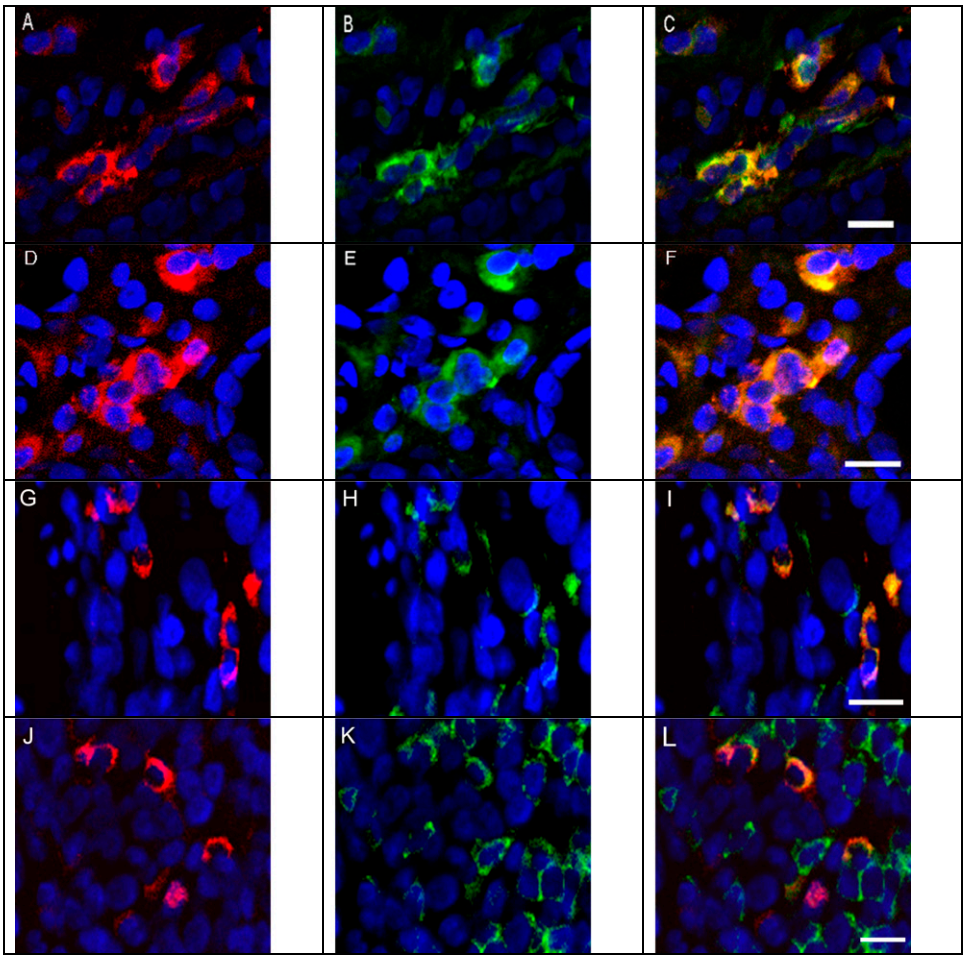

FIGURE 6. Double immunofluorescence staining to demonstrate colocalization of $CysLT_1$ receptor to inflammatory cells of distinct phenotype in a bronchial biopsy specimen from a patient with SE-COPD. A, D, G, and J, $CysLT_1$ receptor protein immunopositivity is illustrated with Texas Red fluorescence. B, E, H, K, Neutrophil elastase$^+$ neutrophils (B), CD68$^+$ monocytes/macrophages (E), tryptase$^+$ mast cells (H), and CD20$^+$ B lymphocytes (K) shown by the green fluorescence of fluorescein isothiocyanate (FITC). C, F, I, L, Coexpression of $CysLT_1$ receptor seen as yellow fluorescence in each case in neutrophils (C), monocytes/macrophages (F), mast cells (I) and CD20$^+$ cells (L) (internal scale bars = 10 μm for A-L). Nuclei are counterstained blue with 4′,6-diamidino-2-phenylindole (DAPI). See Figure 1 and 2 legends for expansion of other abbreviations.

the overall number of inflammatory cells expressing the $CysLT_1$ receptor in SE-COPD.

Interestingly, by comparison with NSCs and those with S-COPD, the median number of subepithelial mast cells was lowest in the SE-COPD group, yet, with a wide range in their number (ie, 31-379/mm^2). Therefore, in these cases, the positive association found shows that those with more mast cells infiltrating in their bronchial mucosa had higher numbers of $CysLT_1$ receptor$^+$ cells. The finding in SE-COPD that approximately 35% of $CysLT_1$ receptor$^+$ cells were mast cells and that 70% of mast cells expressed the receptor indicates that mast cells would have contributed, at least in part, to the overall increase in $CysLT_1$ receptor$^+$ cells. However, due to relatively low mast cell numbers, we suggest that it is instead the increased mix of CD68$^+$ cells, neutrophils, EG2$^+$ eosinophils, and CD20$^+$ cells that has resulted in the overall increase of inflammatory cells expressing the receptor in severe exacerbations of COPD. Unexpectedly, we did not find such associations between numbers of $CysLT_1$ receptor$^+$ cells and numbers of CD68$^+$ cells, neutrophils, EG2$^+$ eosinophils, and CD20$^+$ cells individually in the SE-COPD group. This may be due to the smaller contribution of these individual cell types to the total number of $CysLT_1$ receptor$^+$ cells. Furthermore, we found in earlier studies of exacerbations that the relative proportions of the inflammatory cell phenotypes do alter, probably determined in large part by the specific initiating agent (eg, viral, bacterial, pollutant) but also due to subject to subject variability.[8,22] In support of this, the ranges for subepithelial neutrophil and eosinophil numbers found in the present study

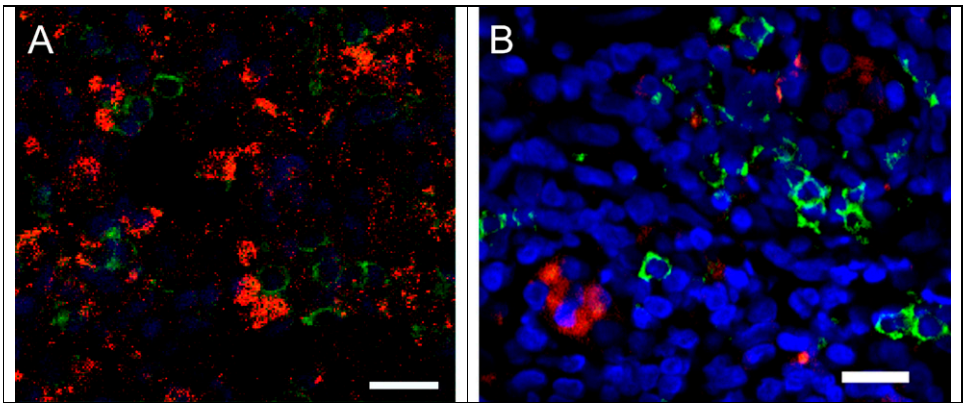

FIGURE 7. A, B, Double immunofluorescence staining for $CysLT_1$ receptor is illustrated with Texas Red fluorescence and $CD4^+$ (A) and $CD8^+$ T lymphocytes (B) are shown by green FITC fluorescence. In this case, there are no yellow fluorescent double-labeled cells. Nuclei are counterstained blue with DAPI (internal scale bar = 10 μm for A and B). See Figure 1 and 6 legends for expansion of abbreviations.

were wide (ie, 1-594 and 0-437/mm^2, respectively). Thus, while we found that the total number of $CysLT_1$ receptor-expressing cells was positively associated with the number of $CD45^+$ inflammatory cells as a whole, the association with each individual cell subtype was less evident.

The Potential for Cysteinyl LTRAs in COPD

The $CysLT_1$ receptor is the target receptor for the antiinflammatory actions of LTRAs, which have a clinically useful role in the treatment of the allergic pattern of inflammation associated with asthma and allergic rhinitis. Their potential for benefit in other respiratory conditions such as COPD has been less well considered.[23] The likely clinical effectiveness of LTRAs in inflammatory conditions other than asthma will probably depend on the extent of synthesis and release of leukotrienes as well as the relative numbers and predominant phenotype of inflammatory cells expressing the $CysLT_1$ receptor. While not the most frequently found cell type herein, the mast cell was a predominant inflammatory cell phenotype expressing the $CysLT_1$ receptor. It is known that mast cells are a major source of proinflammatory leukotrienes,[24] that cigarette smoke stimulates chemokine release from these cells,[25] and that mast cell products have the capacity to alter both secretion of mucus and lung function.[26,27] However, as the overall increase in the number of cells expressing the $CysLT_1$ receptor in exacerbations is due mainly to increased numbers of macrophages and neutrophils (which we demonstrate also express this receptor), we suggest that LTRAs would not only effectively act to modulate mast cell function but also the effector functions of all other inflammatory cell types expressing this receptor in COPD. Consideration should be given for the potential to modulate $CysLT_1$ receptor-mediated responses via receptor blockade[28] in the altered inflammatory response we have described in severe exacerbations of COPD.

Table 3—*Proportion of $CysLT_1$ Receptor Protein$^+$ Cells Coexpressing Inflammatory Cell Markers*

Cell Type	% $CysLT_1$ Receptor Protein$^+$ Cells Coexpressing Cell Markers	% Cell Marker$^+$ Cells Coexpessing $CysLT_1$ Receptor Protein
Mast cells	35 (20-65)	70 (45-90)
$CD68^+$	27 (3-41)	50 (32-85)
Neutrophils	20 (10-48)	38 (25-46)
$EG2^+$	4 (1-12)	42 (32-58)
$CD20^+$	4 (2-15)	48 (40-75)

Data are expressed as median (range). Proportion of $CysLT_1$ receptor protein$^+$ cells coexpressing inflammatory cell markers and each inflammatory cell type coexpressing $CysLT_1$ receptor protein in nine patients with COPD with severe exacerbations. $CysLT_1$ = cysteinyl leukotriene 1.

Limitations

All patients with SE-COPD had received IV steroids at admission. However, none of the subjects, in the NSC and S-COPD groups, had received oral or inhaled glucocorticosteroids in the month preceding the study. Yet by comparison, our data in the SE-COPD group show greater numbers of $CysLT_1$ receptor$^+$ cells, $EG2^+$ eosinophils, neutrophils, and $CD68^+$ cells despite IV steroid. These findings indicate that at least at the time the biopsies were taken (5-26 h after IV steroid was given), steroid did not significantly reduce the inflammatory response and infiltration of $CysLT_1$ receptor$^+$ cells into the airway mucosa of patients with COPD experiencing a severe exacerbation. We acknowledge that in this difficult area of research, there are unavoidable practical limitations imposed upon the design and interpretation

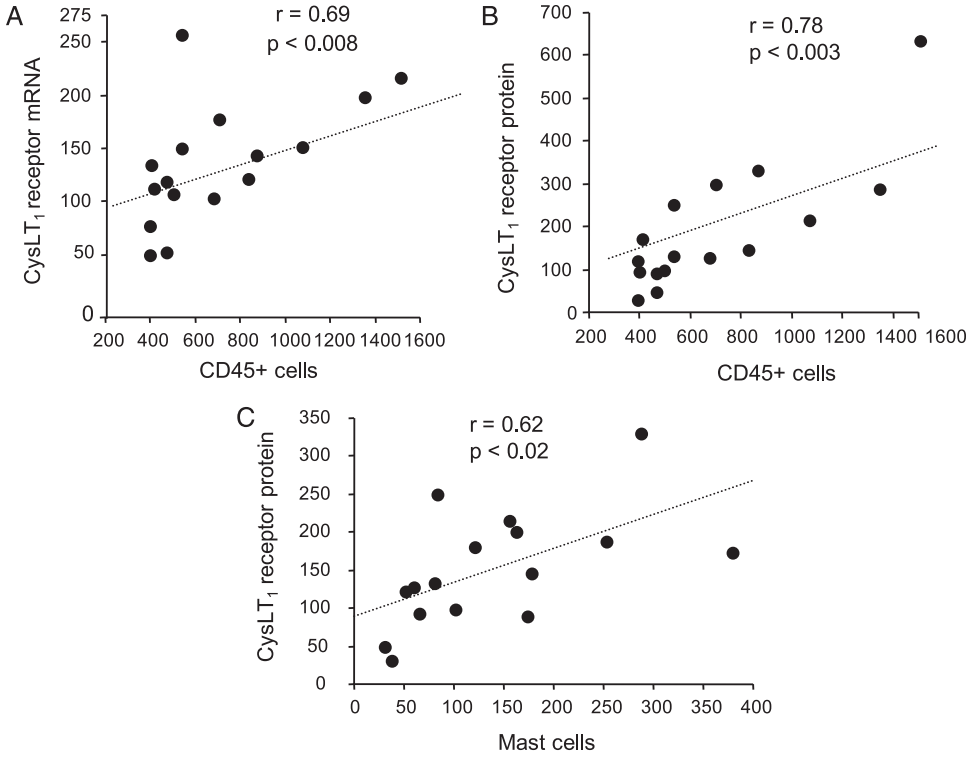

FIGURE 8. A-C, Correlations between the numbers of subepithelial. $CD45^+$ inflammatory cells (A, B) or (C) $tryptase^+$ mast cells and $CysLT_1$ receptor $mRNA^+$ or $CysLT_1$ receptor $protein^+$ cells in the SE-COPD group, expressed as the number of cells/mm^2 of subepithelium (Spearman rank correlation; n = 15). See Figure 1 and 2 legends for expansion of abbreviations.

of a study such as ours involving human subjects. These include age, effects of glucocorticosteroids, intubation, and determination of the presence or absence of infection.[8]

CONCLUSION

We demonstrate for the first time that the $CysLT_1$ receptor is expressed by a variety of inflammatory cell phenotypes infiltrating the bronchial mucosa of COPD subjects, in greatest numbers in those experiencing a severe exacerbation. We find novel inflammatory changes in exacerbations of COPD: Macrophages and B lymphocytes increase, whereas mast cells and $CD4^+$ T lymphocytes decrease. Our data support consideration of the potential for LTRAs as add-on treatment aimed at reducing the bronchial inflammation associated with severe exacerbations of COPD.

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Dr Bandi: contributed to subject recruitment, performing the bronchial biopsy, collecting relevant clinical data, and approving the manuscript.
Dr Qiu: contributed to performing the immunohistochemical staining, preparing figures, and approving the manuscript.
Dr Figueroa: contributed to supplying the $CysLT_1$ receptor cDNA probe and antisera, study design, data interpretation, and approving the manuscript.
Dr Evans: contributed to study design, data interpretation, and revising and approving the manuscript.
Dr Barnes: contributed as co-principal investigator and approving the manuscript.
Dr Guntupalli: contribute to recruiting subjects, performing the bronchial biopsy, collecting relevant clinical data and approving the manuscript.
Dr Jeffery: initiated and served as the principal investigator for the study, oversaw data analysis, and revised the manuscript.
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Additional information: The e-Appendix and e-Tables can be found in the "Supplemental Materials" area of the online article.

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