Different Mitogen-Activated Protein Kinase-Dependent Cytokine Responses in Cells of the Monocyte Lineage

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Received June 25, 2007; accepted October 4, 2007

ABSTRACT

Macrophages release cytokines that may contribute to the chronic inflammation observed in pulmonary conditions such as asthma and chronic obstructive pulmonary disease. Thus, inhibition of macrophage cytokine production may have a therapeutic benefit. Human lung macrophages are a rich source of the proinflammatory cytokines, tumor necrosis factor (TNF)-α, granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-6, and IL-8, that are elevated in the bronchoalveolar lavage and sputum of subjects with respiratory diseases. Cytokine production from both monocytes and macrophages is mediated by mitogen-activated protein kinase (MAPK) pathways. This study compared the effects of a novel p38 MAPK inhibitor, N-cyano-N’-(2-[(8-(2,6-difluorophenyl)-4-(4-fluoro-2-methylphenyl)-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-yl]amino)ethyl)guanidine (PCG), and an extracellular signal-regulated kinase (ERK) pathway inhibitor, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD098059), on cytokine release from lipopolysaccharide (LPS)-stimulated human monocytes, monocyte-derived macrophages (MDM), and lung macrophages. Lung macrophages, MDM, and monocytes were stimulated with LPS, and cytokine release was measured by enzyme-linked immunosorbent assay. Immunoblots were performed to confirm p38 and ERK1/2 MAPK expression and activity. PCG inhibited TNF-α release more effectively from monocytes compared with MDM or macrophages (maximal inhibition was 99.3 ± 1.4, 62.7 ± 4.3, and 58.6 ± 6.6%, respectively; n = 7–9). PD098059 was less effective at suppressing TNF-α release from monocytes compared with MDM and lung macrophages (maximal inhibition was 37.4 ± 2.8, 70.1 ± 4.5, and 68.7 ± 5.1%, respectively; n = 7–9). The pattern of GM-CSF, IL-6, and IL-8 release was comparable with that of TNF-α. These data suggest a differential involvement for each of these MAPK pathways in macrophage cytokine production compared with monocytes.

Respiratory diseases, including asthma and chronic obstructive pulmonary disease (COPD), are associated with increased inflammatory mediators in the lung, including tumor necrosis factor (TNF)-α, granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-6, and IL-8 (Barnes, 2004a). Chronic inflammation is a key feature and is associated with macrophage accumulation in COPD. These cells can account for many of the pathophysiological features associated with COPD (Barnes, 2004b) and hence are current targets for anti-inflammatory therapy. This is particularly important because current pharmacological therapies have limited effects both in suppressing the underlying inflammation and in slowing the progression of COPD.

Several new approaches that target inflammation associated with COPD are in clinical development, including inhibition of mitogen-activated protein kinase (MAPK) pathways (Kumar et al., 2003). Of the three main MAPK pathways, p38 MAPK is implicated in chronic inflammation (Johnson and Lapadat, 2002). In animal models of lung disease, small molecule inhibitors of p38 MAPK suppress lung inflammation (Underwood et al., 2000; Haddad et al., 2001), and many are currently in Phase I and II trials for COPD (Mealy and Bayes, 2005).

AABBREVIATIONS: COPD, chronic obstructive pulmonary disease; TNF, tumor necrosis factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; MAPK, mitogen-activated protein kinase; MKK1, MAPK kinase; ERK, extracellular signal-regulated kinase; LPS, lipopolysaccharide; MDM, monocyte-derived macrophage; PDE, phosphodiesterase; PD098059, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; PCG, N-cyano-N’-(2-[(8-(2,6-difluorophenyl)-4-(4-fluoro-2-methylphenyl)-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-yl]amino)ethyl)guanidine; FCS, fetal calf serum; ELISA, enzyme-linked immunosorbent assay; HSP, heat shock protein; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfanylphenyl)-5-(4-pyridyl)-1H-imidazole; JNK, c-Jun amino-terminal-kinase.
Other MAPK pathways may also play a role in inflammation. The MAPK kinases (MKK1/MEK1) phosphorylate and activate the extracellular signal-regulated kinase (ERK)1 and ERK2, and this pathway is important in IL-8 release from *Haemophilus influenzae*-stimulated human epithelial cells (Wang et al., 2003). Bacterial products such as lipopolysaccharide (LPS) are also potent stimulants for cytokine release from cells of the monocyte lineage. In previous studies, we have shown that LPS stimulates the release of TNF-α, GM-CSF, and IL-8 from both peripheral blood monocytes and lung-derived macrophages (Smith et al., 2006). LPS-stimulated monocyte cytokine release was shown to be p38 MAPK-dependent; however, the effect in macrophages is less clear. This may be due to activation of alternative MAPK pathways during differentiation of monocytes toward a macrophage phenotype. Of the four isoforms of p38 MAPK, p38α is activated by inflammatory stimuli and is the target of many small molecule inhibitors. To this end, this study compared a highly selective p38α MAPK inhibitor (IC50 for p38α = 2 nM) on cytokine release from peripheral blood monocytes, monocyte-derived macrophages (MDM), and lung macrophages with an inhibitor of the ERK MAPK pathway on cytokine release from these cells. This process is important because monocytes are often used as surrogates for macrophages in many pharmacological analyses. However, regulation of cytokine release changes as monocytes differentiate into macrophages. For example, monocytes have a markedly different profile of phosphodiesterase (PDE) activities compared with both alveolar macrophages and in vitro-derived macrophages (Gantner et al., 1997), with a switch from PDE4 toward PDE3-dependent activities (Tenor et al., 1995; Gantner et al., 1997). Therefore, this present study examined the relative importance of the p38 and ERK MAPK pathways in LPS-stimulated cells of the monocyte-macrophage lineage to identify novel targets for pharmaceutical intervention and to determine whether the involvement of MAPKs is comparable between these different cell types.

### Materials and Methods

**Subjects.** Subjects providing either blood samples or lung tissue had given written informed consent. The Ethics Committee of the Royal Brompton and Harefield NHS Trust and National Heart and Lung Institute approved this study.

**Inhibitors.** PD908059 was obtained from Merck Biosciences (Nottingham, UK). The selective p38 MAPK inhibitor PCG was obtained from GlaxoSmithKline (compound patent). PCG was inactive against both MKK1 and inhibitor of IκB kinase-2 (Table 1) and against a panel of 47 tyrosine and serine/threonine kinases (see Supplemental Table 1) at concentrations of up to 10 μM (data not shown). PCG and PD908059 were both prepared in dimethyl sulfoxide at a final concentration of 0.1% (v/v).

**Isolation of Monocytes.** Blood was collected from normal healthy individuals by antecubital venipuncture into acid citrate dextrose (160 mM disodium citrate, 11 mM glucose, pH 7.4). Monocytes were isolated from peripheral blood and purified by centrifugation on discontinuous Percoll gradients followed by adherence to tissue culture plastic (Seldon et al., 1995). Monocytes (1.0 × 10⁶ cells) were plated into 24-well cell culture plates. The purity of the monocyte preparations was assessed by Kimura staining, as described previously (Smith et al., 2004). The purity of the monocyte preparation was routinely >95%.

**Monocyte-Derived Macrophages.** MDM were prepared using a modification of the method developed by Young et al. (1990). Adherent monocytes (1.0 × 10⁶/well of a 24-well plate) were cultured for 12 days in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, and 2 mg/ml GM-CSF. After 12 days, the cells were washed with RPMI 1640 medium supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

**Cell Viability.** Cell viability was determined colorimetrically by measuring the reduction of the tetrazolium salt, 3-(4-dimethylthiazol-2-yl)-2,5-diphenyloxazo-lide bromide, to formazan by mitochondrial dehydrogenases.

**Cell Treatments.** Lung macrophages, MDM, and monocytes were incubated in RPMI 1640 medium supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin with test compounds (PCG, PD908059) for 1 h before adding a submaximal concentration of LPS. The inhibitors were diluted in RPMI 1640 medium supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin containing 0.1% dimethyl sulfoxide (v/v). None of the test compounds had an effect on the viability of any of the cell types examined.

**Cytokine Measurements.** Cell-free supernatant was removed 20 h poststimulation of cells with LPS, and the cytokines were measured by enzyme-linked immunosorbent assay (ELISA). TNF-α and GM-CSF were measured using matched pair antibodies from R&D Systems Europe Ltd. (Abingdon, Oxfordshire, UK), and IL-6 and IL-8 were detected using matched pair antibodies from BioSource (Nivelles, Belgium) according to the manufacturer's instructions. The detection limit of the IL-8 detection assay was 31 pg/ml, and the detection limits for the GM-CSF, TNF-α, and IL-6 assays were 15.5 pg/ml.

**Western Immunoblot Analysis.** Cells were prepared for Western Blotting as described previously (Smith et al., 2004). The anti-heat shock protein (HSP)-27, anti-phospho-HSP-27, anti-p42/p44, and anti-phospho-p42/p44 were purchased from New England Biolabs (Hitchin, UK).

**Statistical Analyses.** Data points and values in the text and figure legends represent the mean ± S.E.M. of n independent determinations using cells from different donors. Concentration-response curves were analyzed by least-squares, nonlinear iterative regression with the GraphPad Prism curve-fitting program (GraphPad Software Inc., San Diego, CA), and EC₅₀ values were subsequently interpolated from best-fit curves. Data were analyzed using the
Kruskal-Wallis test followed by the Dunn’s multiple comparison test. Values of $p < 0.05$ were considered significant.

**Results**

**Effect of LPS on the Release of Cytokines from Human Lung Macrophages.** To evaluate the inhibitory activity of the p38 and ERK MAPK inhibitors on inflammatory mediator release by monocytes, MDM, and lung macrophages, submaximal concentrations of LPS were selected, and TNF-α, GM-CSF, IL-6, and IL-8 were detected in the cell culture supernatants after 20-h stimulation (Table 2). There was no basal release of cytokine from any cell type, with the exception of IL-8 release from lung macrophages (83.58 ± 39.78 ng/ml; $n = 10$).

**Effect of the p38 MAPK Inhibitor PCG on LPS-Stimulated Cytokine Release from Human Monocytes, MDM, and Lung Macrophages.** The novel p38 MAPK inhibitor PCG suppressed LPS-induced cytokine release in a concentration-dependent manner from all of the cell types studied (Fig. 1). The EC$_{50}$ values of the p38 MAPK inhibitor PCG for the suppression of TNF-α and GM-CSF release from monocytes were significantly different from those of both MDM and lung macrophages (Fig. 1; Table 3), with monocytes being more sensitive to inhibition by PCG. The EC$_{50}$ values of the p38 MAPK inhibitor PCG for the suppression of IL-6 release from monocytes were significantly different from those of MDM (Table 3). For all of the cytokines measured, the maximal inhibition at 10 μM and the EC$_{50}$ values of PCG were similar for MDM and lung macrophages (Table 3). In all

**TABLE 2**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Monocytes</th>
<th>MDM</th>
<th>Lung Macrophages</th>
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<tbody>
<tr>
<td>TNF-α</td>
<td>2.70 ± 0.39</td>
<td>2.23 ± 5.64</td>
<td>4.74 ± 9.21</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>2.23 ± 5.64</td>
<td>0.47 ± 0.07</td>
<td>1.89 ± 0.37</td>
</tr>
<tr>
<td>IL-6</td>
<td>35.44 ± 5.12</td>
<td>30.20 ± 2.34</td>
<td>37.54 ± 12.88</td>
</tr>
<tr>
<td>IL-8</td>
<td>111.32 ± 26.28</td>
<td>47.38 ± 8.74</td>
<td>206.08 ± 47.42</td>
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</tbody>
</table>

Fig. 1. Effect of p38 MAPK inhibitor PCG on cytokine release from monocytes, MDM, and lung macrophages. Human monocytes (■, $n = 4–8$), MDM (○, $n = 4–8$), and lung macrophages (○, $n = 4–8$) were treated with increasing concentrations of the p38 MAPK inhibitor PCG for 30 min before stimulation with LPS. Supernatants were harvested after 20 h, and TNF-α (A), GM-CSF (B), IL-6 (C), and IL-8 (D) were measured by ELISA. Data are presented as percentage of inhibition ± S.E. * represents $p < 0.05$ for differences between monocytes and MDM; † represents $p < 0.05$ for differences between monocytes and lung macrophages.
of the cell types examined, the efficacy of the p38 MAPK inhibitor on LPS-stimulated cytokine release was greater for TNF-α and GM-CSF compared with IL-6 and IL-8 (Table 3).

Effect of the MKK1 Inhibitor PD098059 on LPS-Stimulated Cytokine Release from Human Monocytes, MDM, and Lung Macrophages. PD098059 exerted similar inhibitory effects on LPS-induced cytokine release from all of the cells studied (Fig. 2). There were no differences in the potency of PD098059 among monocytes, MDM, or lung macrophages for TNF-α or GM-CSF (Table 4). The EC_{50} values of PD098059 for the suppression of IL-6 release from monocytes were significantly different from those of MDM (p < 0.05), and EC_{50} values of PD098059 for the suppression of IL-8 from monocytes were significantly different from those of lung macrophages (p < 0.05). The MDM and lung macrophages responded similarly to PD098059 for inhibition of cytokine release.

Effect of PCG and PD098059 on LPS-Induced p38 and ERK MAPK Activation. The weak effect of the p38 MAPK inhibitor PCG in MDM and lung macrophages prompted an assessment of the ability of LPS to activate, and PCG to inhibit, the p38 MAPK signaling pathway in these cells. Phosphorylation of HSP-27 was used to measure p38 MAPK activation. LPS-induced phosphorylation of HSP-27 was inhibited in a concentration-dependent manner by PD098059 in both MDM and lung macrophages (Fig. 3, C and D). In both cell types, the highest concentration of PD098059 tested (30 μM) inhibited phosphorylation of p42/44 and was similar to that in unstimulated cells. Expression of total p42/44 was unaffected by incubation of MDM or macrophages with LPS or PD098059 (Fig. 3, C and D). Therefore, the MKK1/2 MAPK inhibitor is effective at blocking the downstream signaling events leading from ERK MAPK activation in MDM and lung macrophages.

To demonstrate the selectivity of the MAPK inhibitors used in this study, phosphorylation of HSP-27 and p42/44 was measured in the presence of the highest concentration of PCG and PD098059. The p38 MAPK inhibitor suppressed LPS-induced phosphorylation of HSP-27 but did not affect phosphorylation of p42/44 in MDM and lung macrophages (Fig. 4). Specificity was further confirmed as PD098059 suppressed phosphorylation of p42/44 but did not affect phosphorylation of HSP-27 (Fig. 4). Neither MAPK inhibitor suppressed the expression of total HSP-27 or p42/44. To determine whether the differential effects of ERK pathway inhibition observed in these cell types could be due to alterations in protein expression, Western blots were performed using monocytes, MDM, and macrophages. However, there were no differences in the level of expression of either p42 or p44 between these cell types (Fig. 5).

Discussion

In COPD, macrophages have been implicated in disease pathogenesis. Therefore, it is important to investigate the regulation of inflammatory mediators released from these cells. Inflammation in COPD and asthma is associated with an increase in cytokines, including TNF-α, GM-CSF, IL-6, and IL-8. Current therapies for COPD have limited effects in suppressing the inflammatory response. In a similar manner, in steroid-resistant asthma, hence, there is a requirement for novel therapy (Barnes, 2006; Fujimoto et al., 2006).

This study examined the respective roles of the p38 and ERK MAPK pathways in the production of inflammatory cytokines from monocytes, in vitro differentiated macrophages, and lung-derived macrophages. We have previously investigated the effect of two different p38 MAPK inhibitors on cytokine release from lung macrophages from patients with COPD compared to control subjects (Smith et al., 2004), in which inhibition of p38 MAPK was less effective at inhibiting LPS-induced lung macrophage cytokine release compared with monocytes. In the present study, we have extended these observations by examining the effect of a novel p38 MAPK inhibitor (EC_{50} 2.5 nM for p38-α) in parallel with an ERK MAPK pathway inhibitor. In all of our studies, MDM and lung macrophages responded similarly. The present study confirms that of Carter et al. (1999), where LPS activated both ERK and p38 MAPK in human alveolar macrophages.

It has been previously shown that levels of mRNA for TNF-α and IL-6 are up-regulated in human monocytes by LPS and that this expression could be inhibited by the p38 MAPK inhibitor SB203580 (Carter et al., 1999). Likewise, we have shown that PCG is highly effective at suppressing LPS-stimulated TNF-α, GM-CSF, and IL-6 release from mono-
cytes. PCG also significantly reduced TNF-α, GM-CSF, and IL-6, but not IL-8, production from LPS-stimulated human lung macrophages and MDM. However, the ability of PCG to suppress cytokine release in these cell types differed for each of the cytokine outputs. Similar findings have been reported by Westra et al. (2004) who showed that the IC₅₀ values for inhibition of IL-8 release from monocytes with a p38 MAPK inhibitor were greater than those required to suppress TNF-α and IL-1β.

Compared to monocytes, the efficacy of PCG on cytokine release from lung macrophages and MDM was reduced. These differences could not be explained by a lack of p38 MAPK activation or lack of efficacy of the inhibitor in these cells, because phosphorylation of HSP-27 was completely suppressed at 10 μM PCG. We have previously found a differential effect of p38 MAPK inhibitors on lung macrophage and monocyte cytokine release and suggested that this could be related to an increase in expression of p38-α MAPK in lung macrophages (Smith et al., 2006) or an associated or

![Fig. 2. Effect of MKK1 MAPK inhibitor PD098059 on cytokine release from monocytes, MDM, and lung macrophages. Human monocytes (□, n = 4–5), MDM (●, n = 4–5), and lung macrophages (○, n = 4–6) were treated with increasing concentrations of the MKK1 MAPK inhibitor PD098059 for 30 min before stimulation with LPS. Supernatants were harvested after 20 h, and TNF-α (A), GM-CSF (B), IL-6 (C), and IL-8 (D) release was measured by ELISA. Data are presented as the percentage of inhibition ± S.E. * represents p < 0.05 for differences between monocytes and MDM; † represents p < 0.05 for differences between monocytes and lung macrophages.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>EC₅₀ μM</th>
<th>Maximal Inhibition %</th>
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</thead>
<tbody>
<tr>
<td>Monocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>5.1 ± 1.7</td>
<td>37.43 ± 2.76 (n = 5)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>6.7 ± 3.6</td>
<td>96.42 ± 3.66 (n = 4)</td>
</tr>
<tr>
<td>IL-6</td>
<td>34.6 ± 1.3*</td>
<td>32.78 ± 6.27 (n = 4)</td>
</tr>
<tr>
<td>IL-8</td>
<td>17.3 ± 14.0</td>
<td>43.82 ± 9.57 (n = 4)</td>
</tr>
<tr>
<td>MDM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>2.0 ± 1.4</td>
<td>70.58 ± 4.54 (n = 5)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>3.7 ± 1.1</td>
<td>94.40 ± 3.76 (n = 4)</td>
</tr>
<tr>
<td>IL-6</td>
<td>5.2 ± 3.0</td>
<td>60.60 ± 4.43 (n = 4)</td>
</tr>
<tr>
<td>IL-8</td>
<td>2.1 ± 0.5</td>
<td>57.35 ± 2.41 (n = 4)</td>
</tr>
<tr>
<td>Lung Macrophages</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>11.4 ± 3.7</td>
<td>68.67 ± 13.11 (n = 6)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>3.2 ± 1.2</td>
<td>95.54 ± 2.80 (n = 6)</td>
</tr>
<tr>
<td>IL-6</td>
<td>9.2 ± 5.7</td>
<td>63.20 ± 2.45 (n = 5)</td>
</tr>
<tr>
<td>IL-8</td>
<td>55.3 ± 12.7*</td>
<td>64.58 ± 5.78 (n = 3)</td>
</tr>
</tbody>
</table>

* p < 0.05 differences between TNF-α and IL-6.
† p < 0.05 differences between TNF-α and IL-8.
‡ p < 0.05 differences between GM-CSF and IL-6.
# p < 0.05 differences between GM-CSF and IL-8.
differential regulation of cytokine transcription and translation in these cells (Carter et al., 1999). However, the present study suggests that regulation of cytokine release changes from being a more predominant p38 MAPK-mediated effect in monocytes to a more ERK-dependent effect after differentiation to a macrophage phenotype. It is noteworthy that ERK protein expression did not alter during differentiation of monocytes toward macrophage phenotype.

Inhibition of LPS-induced GM-CSF production via the ERK pathway was similar for both monocytes and macrophages. However, PD098059 was more effective at inhibiting TNF-α and IL-6 from lung macrophages and MDM compared to monocytes. Thus, the importance of the different MAPK pathways in LPS-induced cytokine release seems to switch as circulating monocytes differentiate into macrophages. The ability of PD098059 to suppress LPS-stimulated GM-CSF production concurs with previous studies in both monocytes (Meja et al., 2000) and alveolar macrophages (Koch et al., 2000).
2004). This finding is important because many studies use monocytes as a surrogate for macrophages to investigate novel therapeutics, and yet these cells are not representative of lung macrophages in terms of regulation of cytokine release. In contrast, this study demonstrates that MDM after 12 days in culture more closely reflects the signaling pathways observed in lung macrophages.

In summary, the p38 MAPK inhibitor, PCG, suppressed LPS-stimulated TNF-α release from monocytes more effectively than it did from both MDM and lung macrophages. In contrast, PD098059 was more effective at inhibiting LPS-stimulated TNF-α and IL-6 release from both MDM and lung macrophages compared to monocytes. It remains possible that the c-Jun amino-terminal-kinase (JNK) and the nuclear factor-κB pathways may be involved in LPS-induced cytokine release from lung macrophages and monocytes. However, the differential effects of ERK and p38 inhibition observed between these cell types could not be accounted for by off-target effects for PCG or PD098059 on the JNK or nuclear factor-κB-dependent pathways (data not shown). However, the absence of selective inhibitors for the JNK pathway prevents investigations of this pathway at the present time. Although there are numerous reports citing SP600125 as a selective JNK inhibitor, this compound also inhibits 13 other protein kinases with a similar or greater potency than JNK1/2 (Bain et al., 2003). In an attempt to study the role of JNK, we used cell-permeable inhibitor peptides of JNK (Zhang et al., 2003), although these peptides had no effect in our system (data not shown). Therefore, the role of JNK remains unclear. In this study, we have shown that complete inhibition of macrophage cytokine release is not achievable with either a p38 MAPK inhibitor or an MKK1 inhibitor. However, simultaneous inhibition of both pathways can completely suppress TNF-α and IL-6 release from alveolar macrophages (Carter et al., 1999). These findings suggest that the development of molecules that target both the p38 and ERK MAPK pathways may have added value to target macrophage-driven inflammation, as seen in COPD.

Acknowledgments

We thank Peter Goldstraw (Department of Cardiothoracic Surgery, Royal Brompton Hospital) for providing human lung specimens for macrophage isolation.

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