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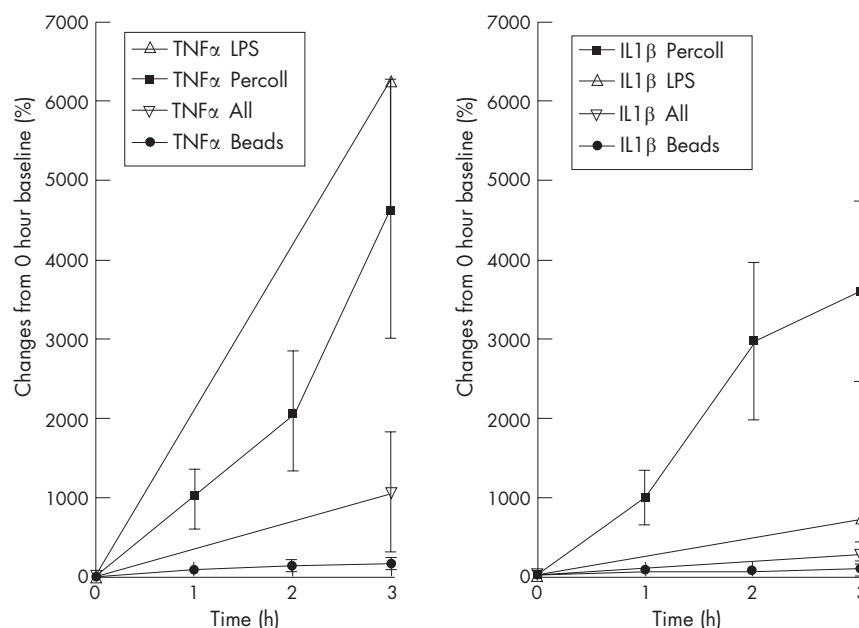
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## Macrophage enrichment from induced sputum

Since induced sputum has become a widely used non-invasive method of recovering cells from the surfaces of the bronchial airways, isolating specific cell populations will be necessary in order to learn more about their specific role in innate immunity and inflammation in the airways. Several studies have demonstrated the ability to conduct ex vivo analyses on sputum cells such as phagocytosis and surface marker measurements, but these have not been performed on isolated cell types.<sup>1-3</sup> This study demonstrates the capability to isolate sputum macrophages from human volunteers in order to advance our understanding of macrophage biology in the airways. To this end, techniques that can enrich and isolate cells without significant activation would prove extremely useful. We compared two common methods for isolating and enriching macrophages in sputum: (1) magnetic bead separation; and (2) Percoll gel density gradient centrifugation. Cell purity and markers of cell activation (mRNA tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) and interleukin-1 $\beta$  (IL1 $\beta$ )) were measured at various time points in the isolation process.

Nine healthy subjects underwent induced sputum. Sputum collection and sputum processing has been described in detail previously.<sup>4</sup> For measuring natural cell activation over time, we incubated the processed sputum cells for 3 h at 37°C and analysed mRNA TNF $\alpha$  and IL1 $\beta$  at 0 h (baseline) and 3 h. In the positive control experiment we incubated the processed sputum cells with 1 ng/ml LPS (*E coli*, Sigma). For Percoll (Amersham Biosciences) separation, 600  $\mu$ l of sputum cell suspension ( $1 \times 10^6$  cells/ml) was layered over Percoll solution (42%) and centrifuged at 560 g for 10 min. Sputum macrophages were removed and incubated at 37°C for 1, 2 and 3 h, respectively, and a pre-incubation sample was also collected. The macrophages were further pelleted and stored at -70°C. For Dynabead separation, CELlection Pan Mouse IgG Kit (Dyna, Norway) was used for immunomagnetic separation of airway macrophages coated with mouse monoclonal IgG2b HLA-DR antibody (Diatec, Norway). Bead coating and cell isolation was performed according to the protocol from the manufacturer. The isolated cells were incubated at 37°C for 1, 2 and 3 h, respectively, and a pre-incubation sample was also collected. The samples were further pelleted and stored at -70°C. Total RNA was extracted (Qiagen) from all the cell samples and reverse transcription was performed (Superscript III, Invitrogen). We used pre-developed PCR primers and probes for TNF $\alpha$  and the house-keeping gene PGK (Applied Biosystems). Specific primers and probes were designed for



**Figure 1** Gene expression of tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) and interleukin 1 $\beta$  (IL1 $\beta$ ) in airway macrophages in induced sputum. The 0 hour (h) Percoll suspensions are used as baseline for 1 h, 2 h and 3 h Percoll suspension (TNF $\alpha$  or IL1 $\beta$  Percoll, n = 7). The Dynabeads 0 hour suspension are used as the baseline sample for 1 h, 2 h and 3 h Dynabeads suspensions (TNF $\alpha$  or IL1 $\beta$  beads, n = 6). In addition, gene expression in the whole cell population is measured at 0 h and at 3 h with (n = 1) or without (n = 5) addition of lipopolysaccharide (LPS). The data are presented as mean (SE).

IL1 $\beta$  using ProbeLibrary (Exiqon ProbeLibrary). Quantification of mRNA was performed using the ABI Prism 7700 (Applied Biosystems), and the relative standard curve method was used to calculate the relative gene expression.

The results show that the median (range) proportion of macrophages in the pre-isolation sputum sample was 61 (34–70)%. Bead isolation produced 99 (95–99)% macrophage purity compared with 88 (85–94)% with Percoll isolation. mRNA expression of TNF $\alpha$  and IL1 $\beta$  was measured as markers of cell activation in airway macrophages (fig 1) before and after Percoll isolation, Dynabead isolation, no isolation and lipopolysaccharide (1 ng/ml) stimulation (positive control). Levels of mRNA TNF $\alpha$  and IL1 $\beta$  were significantly increased as early as 2 h using Percoll isolation compared with 0 h baseline (p = 0.02) and bead isolation (p < 0.01). For bead isolation, mRNA TNF $\alpha$  and IL1 $\beta$  expression were unchanged throughout the isolation period compared with baseline. At 3 h after bead isolation, macrophage mRNA TNF $\alpha$  expression remained near baseline levels whereas Percoll-separated macrophages showed increased activation near positive control (lipopolysaccharide) levels.

The results from this study show that sputum macrophages can be successfully isolated and enriched with a high degree of purity. Furthermore, the magnetic bead isolation technique results in higher macrophage purity and significantly less cell activation than the Percoll isolation technique. As more researchers begin to use individual sputum cell populations to describe airways cellular phenomena, data presented here will provide important technical information to achieve those research aims.

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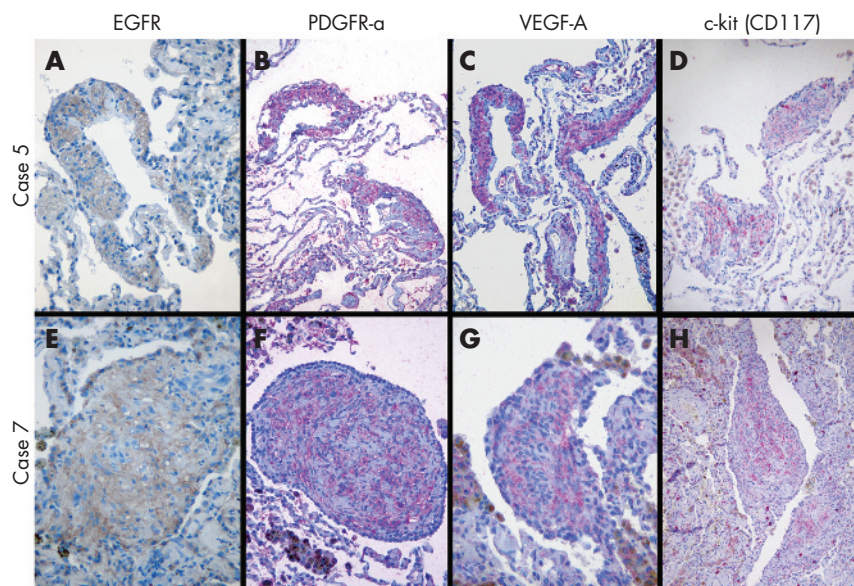
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### Lymphangioleiomyomatosis—presence of receptor tyrosine kinases and the angiogenesis factor VEGF-A as potential therapeutic targets

Lymphangioleiomyomatosis (LAM) is a rare systemic disorder in women occurring either sporadically (sporadic LAM) or in association with tuberous sclerosis (TS-LAM). It is caused by proliferating smooth muscle-like LAM cells, which lead to a progressive cystic destruction of the lungs and abdominal tumours (renal angiomyolipomas and/or axillary lymph node lesions). LAM cells express receptors for oestrogen and progesterone and stain positive for HMB-45, an antibody against the melanoma-related antigen.<sup>1</sup> LAM fulfils the criteria of a neoplastic disease with enhanced proliferation,<sup>2</sup> metastasising processes,<sup>3</sup> increased migratory activity and invasiveness of LAM cells.<sup>4</sup> Currently, an effective treatment interfering with these processes does not exist. Growth factors such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) have been identified to enhance LAM and renal angiomyolipoma cell proliferation in vitro.<sup>2,5</sup> Whether LAM cells express growth factor-associated receptor tyrosine kinases and the angiogenesis factor vascular endothelial growth factor-A (VEGF-A), which represent promising targets of small-molecule and antibody therapy in neoplastic diseases, is currently unknown.

We studied immunohistochemically the expression of the following proteins by LAM cells in 10 formalin-fixed and paraffin-embedded LAM specimens: epidermal growth factor receptor (EGFR; PharmDx Kit, Dako, Hamburg, Germany), platelet-derived growth factor receptor  $\alpha$  (PDGFR- $\alpha$ ; rabbit polyclonal, Dianova, Hamburg, Germany), human epidermal growth factor receptor-2 (HER2; HercepTest, Dako), VEGF-A (clone Vg1 identifying the VEGF-A isoforms VEGF<sub>121</sub>, VEGF<sub>165</sub> and VEGF<sub>189</sub>; DCS, Hamburg, Germany) and c-KIT (CD117; rabbit polyclonal, Dako). Staining procedures were carried out according to the manufacturer's instructions, and appropriate positive and negative controls were used. A semiquantitative scoring system of the immunohistochemical reactions for all receptor tyrosine kinases, the hormone receptors and VEGF-A was applied as follows: negative, no reaction or percentage of positive cells <5%; 1, 5–25% positive cells; 2, 26–50% positive cells; 3, 51–75% positive cells; 4, >75% positive cells; +, weak staining intensity; ++, moderate staining intensity; +++, strong staining intensity. Histological severity of lung destruction was assessed using the LAM histological score.<sup>6</sup> The assessment of the LAM histological score and the immunohistochemical stainings was performed independently by two histopathologists (KE and MA). Only morphologically clear-cut, HMB-45 positive LAM lesions (nodules, cysts and diffuse LAM cell proliferations) were taken for analysis. All final decisions were made by consensus. Additionally, EGFR gene copy number per LAM cell nucleus was investigated by one histopathologist (SL) using fluorescence in situ hybridisation (FISH; LSI EGFR SpectrumOrange/CEP 7 SpectrumGreen probe, Vysis, Abbott Laboratories, Wiesbaden, Germany). The study



**Figure 1** Expression of epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor  $\alpha$  (PDGFR- $\alpha$ ), vascular endothelial growth factor-A (VEGF-A) and c-KIT (CD117) in lung lymphangioleiomyomatosis (LAM) lesions. The panel shows two pulmonary LAM specimens (case 5, A–D; case 7, E–H). Case 5 (A–D) represents predominant cystic and diffuse proliferating LAM lesions, whereas case 7 (E–H) represents predominant nodular growth pattern. The cases show a variable expression of EGFR, PDGFR- $\alpha$ , VEGF-A and c-KIT (CD117).

was approved by the local ethics committee and written informed consent was obtained from all participants or their close relatives.

In all specimens, LAM lesions were consistently positive for PDGFR- $\alpha$  and VEGF-A. EGFR-positive LAM cells were observed in seven specimens. No amplification or higher polysomy of the EGFR gene was detected. In addition to c-KIT-positive mast cells, which were sporadically present in LAM lesions and the surrounding lung tissue, LAM cells themselves were found to be positive for c-KIT in six of the specimens. HER2 was negative in all specimens (fig 1). For details, see supplementary table available online at <http://www.thorax.bmjournals.com/supplemental>.

We demonstrated that PDGFR- $\alpha$ , EGFR, c-KIT and VEGF-A as targets of currently available compounds are expressed by LAM cells. These findings imply further research in the field of small-molecule and antibody therapy in LAM.

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