

Exclusion criteria

Patients in the following categories were excluded: i) Smoking within 3 months prior to screening or total smoking history 10 pack years, ii) Pregnant or lactating females or those at risk of pregnancy, iii) Patients taking >20mg of prednisolone daily, iv) Hospitalisation for asthma or exacerbation requiring systemic corticosteroid therapy within 3 months of the screening visit, v) History of life-threatening asthma, defined as an asthma episode that required intubation and/or was associated with hypercapnia, respiratory arrest and/or hypoxic seizures within the 6 months prior to screening, vi) Pre--bronchodilator FEV1 < 40% predicted, vii) Patients in whom omalizumab therapy was conventionally contraindicated or should be used with caution according to the omalizumab SmPC.

Immunofluorescence

Bronchial biopsies were fixed in 4% PFA for 2 hours at room temperature, washed overnight in PBS containing 15% sucrose, embedded in O.C.T. compound (Tissue-Tek®, VWR International LLC) and stored at -80°C. Eight micron thick sections were cut using a cryostat (Bright OTF 5000, Bright Instruments Co Ltd), air dried overnight onto Polysine slides (Thermo Scientific) and stored at -80°C. PFA fixed sections were thawed at room temperature for 30 minutes and rehydrated in PBS for 5 minutes.

Total tryptase⁺ mast cells, CD20⁺ B cells and CD138⁺ plasma cells, IgE⁺ cells and cells of these phenotypes co-expressing IgE were sought and enumerated from digital images of the sections by two observers ignorant of the origin of the sections, using single and double immunofluorescence and confocal microscopy. Sections (18 sections per subject: 9 at baseline and 9 at time A) were stained with primary antibodies diluted in PBS containing 1% normal human serum and 1% normal goat serum (antibody buffer) or PBS/0.1% saponin for anti-CD20cy [mouse anti-CD138 (1:50; Dako), mouse anti-human tryptase (1:100, Dako), mouse anti-human CD20cy (1:120, Dako), and rabbit anti-IgE (1:500, Dako) and incubated overnight at room temperature. Excess antibodies were washed off in PBS (5 min X 3 times), then the sections incubated with anti-mouse-IgG-FITC (1:200; Life Technologies) and anti-rabbit-IgG-Alexa Fluor 594 (1:200; Life Technologies) diluted in antibody buffer for 1 hour at room temperature in the dark. After washing with PBS, the sections were mounted with Prolong Gold Antifade Reagent with Dapi blue nucleic acid background stain (Life Technologies). The images were captured using a Nikon Eclipse Ti-E inverted confocal microscope (Nikon Imaging Centre, King's College London). The images were processed under identical conditions of illumination using ImageJ (<http://rsb.info.nih.gov/ij/>) and the total numbers of positively stained cells (red fluorescing CD20⁺, CD138⁺ and tryptase⁺ cells and green fluorescing IgE⁺ cells and those expressing both red and green fluorescence measured confocally) counted and expressed as per mm²

of the entire area of the submucosa. The typical mean co-efficient of variability between the sections was 67.46% for tryptase⁺ mast cells and 93.01% for CD138⁺ plasma cells.

Immunohistochemistry

Bronchial biopsies were fixed, frozen and stored as described in the immunofluorescence section. Total BMK-13⁺ cells were sought and enumerated using single immunohistochemistry ~~stain~~ and confocal microscopy. Sections were stained with BMK-13 (1:30, Abcam) ~~diluted~~ in PBS containing 5% normal human serum and incubated overnight at room temperature. Excess antibodies were washed off in PBS (5 min x 3 times), then the sections ~~were~~ incubated with Rabbit-anti Mouse secondary antibody (1:50, ~~;~~ Dako) in PBS containing 5% normal human serum for 30 mins at room temperature in ~~athe~~ humidifier chamber. After washing with PBS, the sections were incubated with Mouse anti-AP-APAAP (1:30, AbD Serotec) in PBS containing 5% normal human serum for 30 minutes at room temperature and then washed again with PBS (5 min x 3 times). Fast Red solution, prepared by dissolving Fast Red tablets (Sigma) in dH₂O, was applied to the sections for 20 mins or until ~~a suitable~~ colour change was observed under the microscope. ~~The~~ Fast Red reaction was stopped with H₂O for 5 minutes. Hematoxylin was applied for 30 ~~seconds~~, then ~~rinsed off~~ ~~the staining~~ ~~stopped~~ under running water for 5 minutes. The slides were then dried for an hour, mounted with Glycerol Gel (Dako) and dried

overnight. The images were captured using Nikon Eclipse Ti-E inverted confocal microscope (Nikon Imaging Centre, King's College London). The images were processed under identical conditions of illumination using ImageJ (<http://rsb.info.nih.gov/ij/>) and the total numbers of BMK-13 positively stained cells (red colour) counted and expressed as per mm² of the entire area of the submucosa.