Regulation of IL-17 in chronic inflammation in the human lung

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ABSTRACT

The regulation of human Th17 cell effector function by T reg cells (regulatory T-cells) is poorly understood. In the present study, we report that human T reg (CD4+CD25+) cells inhibit the proliferative response of Th17 cells but not their capacity to secrete IL (interleukin)-17. However, they could inhibit proliferation and cytokine production by Th1 and Th2 cells as determined by IFN-γ (interferon-γ) and IL-5 biosynthesis. Currently, as there is interest in the role of IL-17-producing cells and T reg cells in chronic inflammatory diseases in humans, we investigated the presence of CD4+CD25+ T-cells and IL-17 in inflammation in the human lung. Transcripts for IL-17 were expressed in mononuclear cells and purified T-cells from lung tissue of patients with chronic pulmonary inflammation and, when activated, these cells secrete soluble protein. The T-cell-specific transcription factors RORα2 (retinoic acid-related orphan receptor α2; for Th17) and FOXP3 (forkhead box P3; for T reg cells) were enriched in the T-cell fraction of lung mononuclear cells. Retrospective stratification of the patient cohort into those with COPD (chronic obstructive pulmonary disease) and non-COPD lung disease revealed no difference in the expression of IL-17 and IL-23 receptor between the groups. We observed that CD4+CD25+ T-cells were present in comparable numbers in COPD and non-COPD lung tissue and with no correlation between the presence of CD4+CD25+ T-cells and IL-17-producing cells. These results suggest that IL-17-expressing cells are present in chronically inflamed lung tissue, but there is no evidence to support this is due to the recruitment or expansion of T reg cells.

INTRODUCTION

The characteristics and functional relationship of Th1 and Th2 cells and their ability to govern selective aspects of adaptive immunity through the production of cytokines which display specific functions has been studied in detail (for example, reviewed in [1]). More recently, two additional subpopulations of CD4+ T-cells have been described, namely, the Th17 [2,3] and T reg cell (regulatory T-cell) subsets [4–6]. The former have pro-inflammatory activity [7,8], and the latter mediate suppression through the release of anti-inflammatory cytokines [9,10]. Currently, our knowledge of the interactions that occur between these subsets is incomplete.

IL (interleukin)-17-producing cells are present at disease sites in many chronic inflammatory disorders, where they are associated with pathology (for example, see [7,8]). Cells with this functional phenotype are

Key words: CD4+CD25+ T-cell, chronic obstructive pulmonary disease (COPD), cytokine, human, interleukin-17 (IL-17), lung inflammation.

Abbreviations: COPD, chronic obstructive pulmonary disease; Cy5, indodicarbocyanine; FOXP3, forkhead box P3; IFN-γ, interferon-γ; IL, interleukin; IL-23R, IL-23 receptor; NK, natural killer; PE, phycoerythrin; PBMC, peripheral blood mononuclear cell; ROR, retinoic acid-related orphan receptor; RT-PCR, real-time PCR; qRT-PCR, quantitative RT-PCR; T reg cell, regulatory T-cell.

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also activated by certain extracellular bacteria and fungi (for example, see [11]). In experimental animal models, IL-17 provides protection against respiratory infections [12–14] by inducing production of a variety of cytokines/chemokines from bronchial epithelial cells, airway fibroblasts and smooth muscle cells. This promotes the recruitment of both neutrophils and memory Th1 cells to the airways [15–17]. In human respiratory diseases, our understanding of the role of IL-17-producing cells remains limited; however, there is some indirect evidence of their involvement. For example, in asthma, cystic fibrosis, tuberculosis and COPD (chronic obstructive pulmonary disease), it has been reported that IL-17 production is elevated (reviewed in [18–21]).

COPD is a chronic inflammatory disease (see, for example, [22,23]) for which smoking is the major risk factor. However, other confounding factors may be involved, such as differences in the inflammatory processes in the lungs of COPD-susceptible and -resistant smokers [24]. Increased numbers of macrophages, neutrophils and lymphocytes are present in the lung tissue of COPD patients and could contribute to tissue destruction and lung remodelling (for example, see [22,23,25]). The function of different CD4+ subsets in COPD is largely unknown, although there appears to be a Th1 phenotype bias based on cytokine profiles [26]. There are conflicting data regarding the presence of Treg cells in COPD with or without smoking [27–30]. The variability in the phenotypic markers used to define cell populations may result from differences in the site of sampling and no patients had acute bacterial infections at the time of surgery. The study was approved by the Ethics Committee of Royal Brompton and Harefield NHS Trust and the National Heart and Lung Ethics Committee. All subjects gave informed consent.

Flow cytometry
Lung-derived cells were labelled with antibodies: CD3–PE (phycoerythrin) (#555333; BD Biosciences), CD4–PECy5 (#555348; BD Biosciences), CD8–PECy5 (indodicarbocyanine) (#555368; BD Biosciences), CD25–PE (#555432; BD Biosciences) or isotype controls. Cells were analysed using the BD Biosciences FACSCalibur flow cytometer and lymphocytes were gated for analysis. For intracellular staining of IL-17A and IFN-γ (interferon-γ), cells were stimulated overnight with PMA (100 ng/ml) and ionomycin (1 μg/ml) plus Brefeldin A (10 μg/ml) before fixation (CellFix solution; BD Biosciences) and permeabilization (FACS PERM/WASH; BD Biosciences). The cells were stained with IL-17A–PE (#12–7178; eBioscience), IFN-γ–APC (allophycocyanin) (#554702; BD Biosciences) or isotype controls before analysis on the flow cytometer.

Cell culture
Lung-derived mononuclear cells were stimulated with 2 μg/ml plate-bound anti-CD3 (OKT3) antibody. CD3+ lung cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 (1 μg/ml; Serotec) antibodies. IL-2, 50 units/ml, was added at day 3, and supernatant was collected at day 5 for ELISA analysis. Th17 cells were induced by stimulating peripheral blood CD4+ T-cells with anti-CD3 antibody (2 μg/ml) in the presence of IL-23 (10 ng/ml) and anti-IFNγ (1 μg/ml) antibodies and autologous CD4+ cells. On day 3, IL-2 (50 units/ml) was added, and at day 5, the culture supernatants were collected for ELISA and cells for RT-PCR (real-time PCR). Th1 and Th2 cells were polarized in the presence of IL-12 (10 ng/ml) and anti-IL-4 antibody (1 μg/ml) for Th1 cells or IL-4 (1000 units/ml) and anti-IL-12 (1 μg/ml) and anti-IFNγ (1 μg/ml) antibodies for Th2 cells. Following 5 days of polarization, cells were collected and viable cells counted.

**MATERIALS AND METHODS**

**Lung tissue samples**
Ethics committee approval was obtained to acquire macroscopically normal lung tissue from patients undergoing surgical resection for carcinoma. Lung tissue samples were obtained from 47 patients (Table 1), and no patients had acute bacterial infections at the time of surgery. The study was approved by the
Table 1  Clinical details of patients who donated lung tissue for the present study
N/A, data not available; FEV₁, FEV (forced expiratory volume) in 1 s; FVC, forced vital capacity; PET, position emission tomography; Control and COPD patient groups included smokers and non-smokers.

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<th>Smoking history (pack years)</th>
<th>FEV₁ (% predicted)</th>
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<th>GOLD stage</th>
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using Trypan Blue exclusion; no significant differences were observed across the cultures.

CD4+CD25+ T-cells were purified from PBMCs (peripheral blood mononuclear cells) by MACS (magnetic-activated cell sorting) separation. First CD4+ cells were negatively selected using Miltenyi Biotech CD4+ T-cell isolation kit II 130–091-155. Then, purified CD4+ cells underwent positive selection for CD25+ using Miltenyi Biotech CD25 Microbeads II 130–090-445. Purity of CD4+CD25+ cells was assessed by flow cytometry and found to be routinely >90%. CD4+CD25+ cells were cultured (1:1) with effector Th cells (Th1, Th2 and Th17) in the presence of anti-CD3 antibody (2 μg/ml) and irradiated CD4− cells as a source of antigen-presenting cells. Proliferation was measured by thymidine incorporation and percentage inhibition calculated as follows: inhibition (%) = 100–[(Treg+Th1 (c.p.m.)/Th1 (c.p.m.))×100. Cytokine production was measured by ELISA.

ELISAs
IL-17A (R&D Systems), IL-5 and IFN-γ (BD Biosciences) paired antibody kits were used to measure cytokine concentrations in cell culture supernatants. Manufacturer’s instructions were followed.

qRT-PCR (quantitative RT-PCR)
RNA was extracted using the Absolutely RNA miniprep kit (Stratagene). RNA (125 ng) was reverse-transcribed using High Capacity cDNA Archive Kit (Applied Biosystems), TaqMan primers and probes for IL-17A (Hs00174383_m1), IL-22 (Hs01574154_m1), IL-23R (IL-23 receptor; Hs00332759_m1), IFN-γ (Hs00174143_m1), T-bet (Hs00203436_m1), GATA-3 (Hs00231122_m1), RORCv1 (Hs00172860_m1), RORCv1+2 (Hs01076112_m1), FOXP3 (forkhead box P3; Hs00203958_m1) and 18S RNA (4319413E; Applied Biosystems) were used in 10 μl-volume reactions performed on the Applied Biosystems 7500 machine. Transcripts were quantified using the comparative Ct method, normalizing to 18S RNA. For RORCv2, each sample was normalized to 18S and then calibrated to its own RORCv1+2.

Statistical analysis
Mann–Whitney U, Wilcoxon, one-way ANOVA and Spearman tests were used as appropriate. P values <0.05 were considered significant. Calculations were carried out using GraphPad Prism v4 software.

RESULTS
Treg cells inhibit clonal expansion of Th17 but not their ability to produce IL-17
Co-culturing polarized Th1 or Th2 cells derived from PBMCs with purified CD4+CD25+ as a source of Treg cells at a ratio of 1:1 together with immobilized anti-CD3 antibody and irradiated CD4− cells as a source of antigen-presenting cells. Proliferation was measured by thymidine incorporation and percentage inhibition calculated as follows: inhibition (%) = 100–[(Treg+Th1 (c.p.m.)/Th1 (c.p.m.))×100. Cytokine production was measured by ELISA.

Phenotypic characterization of lung-infiltrating mononuclear cells
Next, we investigated first whether Treg cells and IL-17-producing cells are present in chronic lung inflammation in humans, and secondly, if IL-17 levels correlated with the presence of Treg cells and whether there were differences between COPD and non-COPD patients. Mononuclear cells were isolated from lung tissue samples from patients and retrospectively classed as originating from either COPD patients or controls with unrelated lung disease (Table 1). The non-COPD patients (n = 23)
Effects of CD4+CD25+ Treg cells on proliferation and cytokine production by Th1, Th2 and Th17 cells T-cell interactions

CD4+CD25+ T-cells isolated from human peripheral blood were cultured at a ratio of 1:1 with autologous in vitro polarized populations of Th1, Th2 and Th17 cells and irradiated non-CD3 cells as a source of antigen-presenting cells and activated with anti-CD3 antibody. Proliferation was measured by thymidine incorporation and the results are expressed as percentage inhibition (A). IFN-γ, IL-5 and IL-17 production was measured by ELISA for Th1, Th2 and Th17 cells, respectively, in the CD4+ T-cell co-cultures, and results are expressed as the percentage change (B). Treg cells inhibit the proliferation of Th17 cells. Results are presented in box and whiskers and expressed as c.p.m (C). IL-17 production by Th17 cells is enhanced in the presence of Treg cells. IL-17A levels were measured by ELISA and presented as box and whiskers in pg/ml (D). Patient numbers: Th1, n = 2; Th2, n = 4; and Th17, n = 4.

Lung mononuclear cells and CD3+ T-cells produce IL-17 and IFN-γ

The expression of signature cytokines for CD4+ Th subsets, namely Th1 (IFN-γ), Th2 (IL-5) and Th17 cells (IL-17A), was investigated. Lung-derived mononuclear cells from all subjects were cultured for 5 days with plate-bound anti-CD3 antibody, and cytokine release was
measured in the cell supernatants. There was no difference in IL-17A production in patients with and without COPD (Figure 3A; median values 315.1 and 217.6 pg/ml respectively). In experiments where sufficient cell numbers allowed analysis, IL-17A was also detected in supernatants from CD3+ cells (Figure 3B); similarly, no significant difference between the groups was found. There was no marked difference in IL-17 levels between smokers and non-smokers with lung inflammation unrelated to COPD. IFN-γ was detected in cell culture supernatants of lung-derived mononuclear cells and CD3+ T-cells (Figures 3A and 3B) and transcripts for IFN-γ were detected by qRT-PCR in lung cellular infiltrates (Figure 4A). Similarly, qRT-PCR confirmed the presence of transcripts for IL-17A and 17B in mononuclear cells and purified CD3+ T-cells compared with unfractionated mononuclear cells and purified CD3+ T-cells (results not shown). IL-22 transcripts were barely detectable in lung-derived mononuclear cells and CD3+ T-cells in any sample (results not shown). Both IL-17A and IL-23R transcripts were enriched in purified CD3+ T-cells compared with mononuclear cells (6.2- and 3.9-fold increases for IL17A and 3.5- and 1.3-fold increases for IL-23R in COPD and non-COPD patients respectively; Figure 4A). Furthermore, transcripts for IFN-γ were also enriched in CD3+ T-cells (3.6- and 3.4-fold increases in COPD and non-COPD patients respectively; Figure 4A). Similar fold increases were observed when only smokers were analysed (results not shown).

**IL-17-producing cells are present in inflamed lung tissue**

The transcription factors associated with Th1 (T-bet), Th2 (GATA-3), Th17 (RORCα isoform b/short isoform) and Treg (FOXP3) cells were measured in both unfractionated mononuclear cells and purified CD3+ T-cells (Figure 4B). Transcripts for T-bet, GATA-3, RORC and FOXP3 were all detected. Transcripts for RORCα2 were highly enriched in the T-cell population (12.1- and 8.1-fold increases for COPD and non-COPD patients respectively), whereas this was not the case for T-bet (1.5- and 1.1-fold; Figure 4B). FOXP3 and GATA-3 mRNA were also enriched in the T-cell fraction of lung mononuclear cells by 4.96- and 3.6-fold respectively (Figure 4B). However, there was no statistically significant difference in the levels of transcripts between COPD and non-COPD patients.

We observed no overall differences in the characteristics of the inflammatory cell infiltrate in either group of participants. Therefore we then compared percentage of CD4+CD25+ T-cells in lung tissue and the amount of IL-17A produced by anti-CD3-stimulated lung-derived mononuclear cells for all subjects in the study but observed that there was no correlation between these two parameters (Figure 5A). Similarly, comparison of the levels of transcripts for FOXP3 and RORC showed no correlation for all subjects (Figure 5B).

**DISCUSSION**

In the present study, we have investigated human CD4+ T-cell interactions in vitro to determine whether CD4+CD25+ T-cells govern the function of Th17 cells. We then translated these findings to human disease by exploring whether IL-17-producing cells and Treg cells are present in chronic lung inflammation in humans.

We observed that in vitro clonal expansion of Th17 cells is inhibited by Treg cells, which is inconsistent with the previous observation that human Th17 proliferation is resistant to Treg cell activity [34]. However, in those
Figure 3  IL-17A and IFN-γ are produced by lung-infiltrating T-cells
Lung mononuclear cells were cultured for 5 days with plate-bound anti-CD3 antibody and 50 units/ml IL-2 added at day 3 (A). IL-17A and IFN-γ were measured by ELISA in cell culture supernatants from non-COPD controls (n = 16) and COPD patients (n = 20). Horizontal bars show means. Lung CD3+ cells were cultured for 5 days with plate-bound anti-CD3 and soluble anti-CD28 antibodies, and 50 units/ml IL-2 was added at day 3 (B). IL-17A was measured by ELISA at day 5 in the cell culture supernatant in non-COPD controls (n = 5) and COPD patients (n = 6). Horizontal bars show means.

Figure 4  Cytokine and transcription factor expression in lung mononuclear cells and T-cells from non-COPD and COPD patients
Transcripts for IL-17A, IL-23R and IFN-γ (A) and T-bet, GATA-3, FOXP3 and RORC v2 (B) in human lung mononuclear cells and purified CD3+ T-cells from non-COPD controls and COPD patients were analysed by qRT-PCR (TaqMan). Fold differences in relative transcript levels between purified T-cell and lung mononuclear cell populations are shown. Horizontal bars show means.
The presence of CD4+CD25+ T-cells in the lung does not correlate with IL-17 production

The percentage of CD4+CD25+ T-cells in the lung tissue was measured by flow cytometry, and IL-17A production by the lung mononuclear cells in culture was determined by ELISA (A). Foxp3 and Rorc2 in human lung mononuclear cells and purified CD3+ T-cells from non-COPD controls and COPD patients were analysed by qRT-PCR (TaqMan). Fold differences in relative transcript levels between purified T-cell and lung mononuclear cell populations for each transcript (B).

experiments a regulatory T-cell clone rather than fresh Treg cells was used. We also noted that IL-17 production by Th17 cells was unaffected by Treg cells, and previous observations in mice have demonstrated that IL-17 production is unaffected or even enhanced by Treg cells [35,36]. But in vivo, if administered early, these cells could prevent IL-17-induced disease by suppressing the expansion of Th17 cells [35]. The complexity of Th17–Treg cell interactions in vivo is, in part, likely to be due to functional heterogeneity of Treg cells [37]. With regards to Th1 and Th2 cells in our present study, Treg cells in vitro could inhibit proliferation and cytokine production by both subsets. This is in agreement with the experience of many other investigators (see, for example, [38]). Many immune responses in vivo are polarized, therefore the opportunity to compare the effects of Treg cells on functionally distinct CD4+ T-cell subsets has been limited. However, in one example, Th1, Th2 and Th17 cells could all induce pathology; they vary in their receptiveness to Treg-cell-mediated suppression with Th1 cells being more susceptible than Th2 cells. In contrast, Th17 cells were resistant, and their ability to induce disease was not suppressed [39].

We then examined directly lung tissue from patients with chronic lung inflammation for CD4+CD25+ T-cells and IL-17 and found that both were present. IL-17 plays an important role in neutrophil responses in the lung and by increasing CCL2 (CC chemokine ligand 2) can recruit macrophages [3]. It can also increase MMP9 (matrix metalloproteinase 9) production [40] and transcription of MUC5A (mucin 5A) and MUC5B (mucin 5B) [41]. Collectively, this has led to speculation that IL-17 may contribute to COPD pathogenesis, although direct evidence of this is limited. Immunohistological analysis of bronchial biopsies has revealed elevated numbers of IL-17-expressing cells in the submucosa of stable COPD patients and smokers [31,32]. Rather than immunostaining, we isolated mononuclear cells from human lung tissue and determined the ability of activated cells to produce IL-17 protein. We detected IL-17A transcripts in mononuclear and CD3+ cells and protein in their supernatants immediately following isolation from the lung, but the levels were not significantly different between COPD and non-COPD patients. As levels of IL-17 are elevated in asthma, cystic fibrosis and in response to cigarette smoke alone (see, for example, [19–21]), it is not surprising that we observed increased IL-17 in both COPD and non-COPD-related inflammation. In experimental models in vivo, IL-17 expression in the lung can be either protective or pathogenic by inhibiting IL-22 [42]. However, for human chronic lung inflammation, in the absence of infection, the role of IL-17A has not been resolved. Enriched expression of Rorc2, the transcription factor associated with Th17 cells [43], was observed in the CD3+ T-cell fraction of both COPD and non-COPD patients, but since IL-17 can be produced by a variety of different T-cell subsets, it cannot be assumed that only Th17 cells have been recruited or expanded in the lung. A similar argument applies to the increased level of transcripts for IL-23R that were detected in the CD3+ cell population. Thus, the presence of IL-17-producing cells in lung tissue may be a generic response of chronic inflammation.

Increased expression of IFN-γ has been reported in lung-tissue-derived lymphocytes and in bronchoalveolar fluid and sputum of COPD patients [33,44–46]. In the present study, we detected IFN-γ transcripts in both unfractionated mononuclear and CD3+ T-cells in the COPD and non-COPD patients. In contrast, little or no IL-5 was present, although expression of GATA-3 was detectable.

The presence of Treg cells in lung tissue was investigated by staining for CD25 expression on CD4+
cells, and we, like others, observe Treg cells in chronic lung inflammation. Whether or not Treg cell numbers are increased or decreased in COPD is controversial [27–30]. The lack of consensus may arise from experimental differences, for example, the analysis of different tissues, namely, BALF (bronchoalveolar lavage fluid), lung tissue lymphocytes and bronchial biopsies from large or small airways. Similarly, the immunological parameters used as indicators of Treg cells may differ between the studies. Their presence may be an attempt to resolve inflammation and through the production of IL-10 suppress Th1 cytokines and inhibit neutrophil recruitment. We also detected IL-17-producing cells; thus, it seems that Treg cells do not prevent their expansion or recruitment to the lungs. Furthermore, since we failed to observe a correlation between CD4+CD25+ T-cells and IL-17 in the lung, we cannot attribute production of IL-17 to any activity of Treg cells.

Overall, the present study has shown that in vitro IL-17 production is resistant to the inhibitory effects of Treg cells and that IL-17-producing cells and Treg cells are both present in human lung tissue in chronic inflammation. However, there is no direct evidence indicating that IL-17 production is linked to Treg cell function in vivo in inflammatory responses in the lung.

**AUTHOR CONTRIBUTION**

Carol Pridgeon and Laurence Bugeon performed the laboratory experiments and wrote the paper. Louise Donnelly, Ursula Straschil, Susan Tudhope and Peter Fenwick prepared the samples and analysed the data. Jonathan Lamb wrote the paper. Peter Barnes and Margaret Dallman supervised the project, analysed the data and wrote the paper.

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