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Impaired function of regulatory T-cells in patients with chronic obstructive pulmonary disease (COPD)

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ABSTRACT

Anti-inflammatory pathways affecting chronic obstructive pulmonary disease (COPD) are poorly understood. Regulatory T-cells (Tregs) are important negative regulators of T-cell activity and hence were investigated in COPD patients in this study. We hypothesised that functional defects in Tregs may promote increased inflammation contributing to the pathogenesis of COPD.

Peripheral blood mononuclear cells (PBMC) were isolated from patients with stable COPD and age-matched non-smoking controls. Treg-mediated suppression of memory non-Treg ($Foxp3^{-}CD45RO^{+}$) $CD4^{+}$ T-cell activation was analysed by comparing PBMC responses to staphylococcal enterotoxin-B (SEB) pre- and post-depletion of Tregs ($CD25^{+}CD127^{low}CD4^{+}$ T-cells) by fluorescence-activated cell sorting (FACS). Activation of T-cells was assessed by HLA-DR expression. Levels of secreted cytokines were measured by ELISA.

Depletion of Tregs increased SEB-induced activation of $Foxp3^{-}CD45RO^{+}$ $CD4^{+}$ T-cells in samples from 15/15 healthy controls (demonstrating Treg-mediated suppression) and 9/14 COPD patients (Fisher's test, $p = 0.017$). A screen of clinical data associated a failure of Treg-mediated suppression in the remaining five COPD patients with a higher body mass index (BMI) (33–38 kg/m^2) compared to patients with unimpaired Treg function (20–32 kg/m^2).

In conclusion, we demonstrate impaired Treg-mediated suppression of $CD4^{+}$ T-cell activation in a subset of COPD patients, all of whom had high BMI. Obesity and/or perturbed homeostasis of Treg subsets may explain this defect and therefore contribute to increased inflammation observed in COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is amongst the top 5 causes of global morbidity and mortality (Mannino et al., 2002). It is characterised by persistent inflammation, airflow obstruction and progressive destruction of lung tissue (Hogg et al., 2004; Rivera et al., 2008). COPD is regarded as a multi-systemic disorder with several co-morbidities (*e.g.* vascular and ischaemic heart disease) (Barnes and Celli, 2009; Sinden and Stockley, 2010). Biomarkers of systemic inflammation such as soluble tumour necrosis factor receptor (sTNFR) are elevated in COPD patients (Takabatake et al., 2000).

It is increasingly recognised that T-cells play a key role in regulating inflammation in COPD. The presence of activated T-cells in the lungs of COPD patients who have ceased smoking is

Abbreviations: BMI, body mass index; BALF, bronchoalveolar lavage fluid; BSA, bovine serum albumin; COPD, chronic obstructive pulmonary disease; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; IFN, interferon; IL, interleukin; MFI, mean fluorescence intensity; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; sTNFR, soluble tumour necrosis factor receptor; SEB, Staphylococcus enterotoxin-B; TGF, transforming growth factor; Tregs, regulatory T-cells.

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consistent with a role for T-cells during the acute phase of lung injury (Chrysofakis et al., 2004; Cosio et al., 2009). Infiltration of cytotoxic $CD8^+$ T-cells into the airways of COPD patients may promote destruction of lung tissue (Majo et al., 2001; Roos-Engstrand et al., 2009). Proportions of circulating $CD8^+$ T-cells producing interferon (IFN)γ or TNFα in response to stimulation by phorbol myristate acetate and ionomycin are increased in COPD patients (Paats et al., 2011). Increased numbers of IL-17-producing cells in the bronchial submucosa and alveolar wall of COPD patients may promote neutrophilic airway inflammation characteristic of COPD (Alcorn et al., 2010; Chu et al., 2011a; Di Stefano et al., 2009).

Despite evidence of the importance of T-cells in COPD pathogenesis, mechanisms underlying T-cell dysregulation in COPD are unclear. Regulatory T-cells (Tregs) negatively regulate T-cell responses and are important in preventing autoimmune diseases or excessive inflammatory responses (Sakaguchi et al., 2009; Walker and Sansom, 2011). Accumulations of Tregs have been described in peripheral blood, bronchoalveolar lavage fluid (BALF), lung-derived lymphoid follicles and the large airways of COPD patients (Isajevs et al., 2009; Plumb et al., 2009; Smyth et al., 2007; Vargas-Rojas et al., 2011). However, other studies have reported normal frequencies of Tregs in peripheral blood (Barcelo et al., 2008) or decreased levels of Tregs in other tissue sites including the alveolar walls and small airways of COPD patients (Chu et al., 2011b; Isajevs et al., 2009).

To date, no study has examined the function of Tregs in the context of COPD and it is unknown whether Tregs from COPD patients are able to suppress inflammation. Therefore, we analysed Treg function by comparing peripheral blood mononuclear cell (PBMC) responses to a T-cell stimulus, staphylococcal enterotoxin-B (SEB) pre- and post-Treg depletion.

Methods

Study groups

Fifteen previous smokers (>15 pack-years and ceased smoking >5 years earlier) with stable COPD were recruited from a dedicated COPD clinic (Table 1). The diagnosis and severity of COPD was established by a respiratory physician according to the global initiative for chronic obstructive lung disease (GOLD) criteria (stages II–IV) (Rabe et al., 2007). All COPD patients had been treated with anticholinergics, long-acting beta agonists and inhaled corticosteroids for >3 months prior to participating in the study. No patients were receiving systemic corticosteroids or had diabetes, neuromuscular, allergic or rheumatologic disease. Age-matched healthy non-smokers with no evidence of COPD and normal spirometry were included as controls. The study was approved by the Ethics Committee of Royal Perth Hospital and all participants gave informed consent.

PBMC isolation and immunophenotyping

Blood samples were collected into lithium heparin tubes. PBMC were isolated by Ficoll gradient centrifugation. Plasmas were stored at −80 °C. PBMC were resuspended in sterile 1% bovine serum albumin (BSA)/phosphate buffered saline (PBS). One million PBMC were stained with CD3-APC-H7, CD4-PerCP-Cy5.5, CD25-FITC, CD127-PE, CD45RO-PE-Cy7 and HLA-DR-APC antibodies (BD Biosciences, San Jose, CA, USA) for immunophenotyping. 500,000 events were acquired using a BD FACS Canto II cytometer and analysis was performed with FlowJo v5.7.2 software (Tree Star, Ashland, OR, USA).

Treg depletion

Ten million PBMC were stained with CD3-APC-H7, CD4-PerCP-Cy5.5, CD25-FITC and CD127-PE antibodies, and $CD3^+CD4^+CD25^+CD127^{low}$ cells (Tregs) were depleted using a BD FACS ARIA II cell sorter (Fig. 1B). The average sort purity was 80%. Unstained PBMC were passed through the cell sorter as a control for the effects of this procedure. Treg-depleted and non-depleted PBMC (1×10^6 cells/mL) were cultured for 40 h in culture media alone (10% foetal calf serum/RPMI) or with 1 μg/mL SEB (Sigma–Aldrich, Sydney, Australia). Supernatants were stored at −80 °C. Cells were then resuspended in 1% BSA/PBS and surface-stained with CD3-APC-H7, CD4-PerCP-Cy5.5, CD45RO-PE-Cy7 and HLA-DR-APC. Intranuclear staining with Foxp3-PE (BD Biosciences) was performed using the BD Pharmingen™ Human Foxp3 buffer set. 250,000 events were acquired and analysed as described above.

Cell doublets were excluded based on the forward scatter height and area (FSC-H *vs.* FSC-A) followed by gating on lymphocytes based on forward scatter and side scatter areas (FSC-A *vs.* SSC-A) and then $CD4^+$ T cells based on co-expression of CD3 and CD4 (Fig. 1A). For sorting, $CD25^+CD127^{low}$ cells were gated from $CD4^+$ T-cells (Fig. 1B). Following 40 h culture of PBMC and Treg-depleted PBMC with SEB, memory non-Treg $CD4^+$ T-cells were identified as $CD45RO^+Foxp3^-$ cells gated from $CD4^+$ T-cells (Fig. 1E).

Quantification of soluble biomarkers by ELISA

Plasma sTNFR1 level was measured by ELISA (R&D Systems, Minneapolis, MN, USA). Concentrations of IFNγ, interleukin (IL)-6, IL-10, transforming growth factor (TGF)β (BD Biosciences) and IL-17 (eBioscience, San Diego, CA, USA) were measured in culture supernatants.

Statistical analyses

Non-parametric Mann–Whitney tests were used for comparisons between groups. Correlations were assessed using

Table 1
Study demographics and immune phenotypes.

	COPD patients	Healthy controls	*p*-value
N	14	15	–
Gender, males/females	8/6	8/7	–
Age (years)	72 (61–83)	62 (53–84)	0.14
FEV_1 (% predicted)	45 (20–60)	92 (80–106)	<0.001
FEV_1 (litres)	0.97 (0.41–2.07)	2.75 (2.18–3.51)	<0.001
FEV_1/FVC (%)	48 (38–55)	92 (88–96)	<0.001
BMI	26 (20–38)	25 (21–29)	0.19
Plasma sTNFR1 (pg/mL)	1526 (751–2365)	996 (608–1424)	0.002
Expression of HLA-DR on $CD4^+$ T-cells[a]	444 (149–959)	241 (150–494)	0.02

Data presented as median (range). FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; BMI, body mass index.
[a] Presented as mean fluorescence intensity (MFI).