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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 regulatory T (T reg) cells is poorly understood. Here we report that human T reg (CD4+CD25+) cells inhibit the proliferative response of Th17 cells but not their capacity to secrete IL-17. However they could inhibit proliferation and cytokine production by Th1 and Th2 cells as determined by IFN- 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 RORCv2 (Th17) and FOXP3 (T regs) were enriched in the T cell fraction of lung mononuclear cells. Retrospective stratification of the patient cohort into those with chronic obstructive pulmonary disease (COPD) 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 (e.g. reviewed in ref. 1). More recently two additional sub-populations of CD4⁺ T cells have been described namely the Th17 (2, 3) and regulatory T (T reg) 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-17 producing cells are present at disease sites in many chronic inflammatory disorders where they are associated with pathology (e.g. 7, 8). Cells with this functional phenotype are also activated by certain extracellular bacteria and fungi (e.g. 11). In experimental animal models IL-17 provide 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 chronic obstructive pulmonary disease (COPD) it has been reported that IL-17 production is elevated (reviewed in 18-21).

COPD is a chronic inflammatory disease (e.g. 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 (e.g. 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 cytokines profiles (26). There are conflicting data regarding the presence of T reg cells in COPD with reports of increased, decreased or unaltered numbers of cells compared to controls

(27-30). The variability may result from differences in the site of sampling, the phenotypic markers used to define cell populations and control population of subjects used. The increased expression of IL-17A, IL-22 and IL-23 reported in the bronchial mucosa of COPD patients suggests that Th17 cells might contribute to the chronic inflammation of COPD or be a consequence of smoking (31, 32). In contrast, down-regulation of Smad7, an intracellular signalling molecule activated in the induction of Th17 cells, in bronchial biopsies suggests that Th17 are reduced in COPD (33).

Taken together these reports prompted us to investigate if CD4+CD25+ cells influence the function of Th17 cells and whether or not T regs and IL-17 producing cells are present in chronic inflammatory disease in the lung.

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. Tissue was lavaged with RPMI medium containing 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin and 5 mM EDTA to remove a macrophage-enriched cell fraction. Lavaged tissue was cut into sections and digested using 75 U/ml Collagenase type IA and 50 U/ml DNase I (Sigma) for 1.5 hours at 37 °C (shaking). Digested tissue was passed through a 70 µm cell strainer and cells washed with PBS. Mononuclear cells were separated by density centrifugation on Ficoll-paque (GE Healthcare Ltd.) and counted using a haemocytometer. Where sufficient cells were recovered, CD3⁺ cells were purified using positive magnetic isolation (Miltenyi Biotec). The purity of isolated CD3⁺ cells was >95 % in all cases.

Flow cytometry

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

Cell culture

Lung-derived mononuclear cells were stimulated with 2 μ g/ml plate-bound anti-CD3 (OKT3). CD3⁺ lung cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 (1 μ g/ml, Serotec) antibodies. 50 U/ml IL-2 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 μ g/ml) in the presence of IL-23 (10 ng/ml) and anti-IFN γ (1 μ g/ml) antibodies and autologous CD4⁻ cells. On day 3 IL-2 (50 U/ml) was added and at day 5 the culture supernatants were collected for ELISA and cells for real-time PCR. Th1 and Th2 cells were polarized in the presence of IL-12 (10 ng/ml) and anti-IL-4 antibody (1 μ g/ml) for Th1 cells or IL-4 (1000 U/ml) and anti-IL-12 (1 μ g/ml) and anti-IFN γ (1 μ g/ml) antibodies for Th2 cells. Following 5 days of polarization cells were collected and viable cells counted using trypan blue exclusion; no significant differences were noted across the cultures.

CD4⁺CD25⁺ T cells were purified from peripheral blood mononuclear cells (PBMCs) by MACS separation. First CD4⁺ cells were negatively selected using Miltenyi Biotec CD4⁺ T cell isolation kit II 130-091-155. Then purified CD4⁺ cells underwent positive selection for CD25⁺ using Miltenyi Biotec CD25 Microbeads II 130-090-445. Purity of CD4⁺CD25⁺ cells was assessed by flow cytometry and found to be routinely >90%. CD4⁺CD25⁺ were cultured (1:1) with effector Th cells (Th1, Th2 and Th17) in the

presence of anti-CD3 antibody (2 $\mu\text{g}/\text{ml}$) and irradiated CD4^+ cells as a source of APCs. Proliferation was measured by thymidine incorporation and percentage inhibition calculated as follow: % inhibition = $100 - ((\text{Treg} + \text{Th1 cpm} / \text{Th1 cpm}) \times 100)$. 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.

Quantitative RT-PCR

RNA was extracted using the Absolutely RNA miniprep kit (Stratagene). 125 ng RNA 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-23 receptor (Hs00332759_m1), IFN- γ (Hs00174143_m1), T-bet (Hs00203436_m1), GATA-3 (Hs00231122_m1), RORCv1 (Hs00172860_m1), RORCv1+2 (Hs01076112_m1), Fox P3 (Hs00203958_m1) and 18S (4319413E; Applied Biosystems) were used in 10 μl 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, 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

T reg cells inhibit clonal expansion of Th17 but not their ability to produce IL-17

Co-culturing polarised Th1 or Th2 cells derived from PBMCs with purified CD4+CD25+ as a source of T reg cells at a ratio of 1:1 together with immobilised anti-CD3 antibody and irradiated non-CD4+ cells as APCs, inhibited both proliferation of Th1 and Th2 cells (>75%; Fig. 1A) and cytokine production (IFN- γ and IL-5 respectively for Th1 and Th2 cells; Fig. 1B). While T reg cells significantly inhibited Th17 cell proliferation >75% (Fig.1C; $p < 0.05$) IL-17 production was not significantly altered (655.7 increasing to 875.8 pg/ml; Fig. 1D)

Phenotypic characterization of lung infiltrating mononuclear cells

Next we investigated if T regs 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 ($n = 23$) included 15 males and 8 females with an average age of 63.1 years and smoking history of 28.3 pack-years, with 8 non-smokers. Although this control group includes subjects with different disease pathologies they all had chronic inflammatory cell infiltrates in the lung. COPD patients ($n = 24$) included 18 males and 6 females with an average age of 66.5 years and smoking history of 43.7 pack-years, with only one non-smoker. There was no statistically significant difference between the number of cells recovered from lung tissue taken from non-COPD and COPD patients and there was no correlation between numbers of recovered mononuclear cells and disease severity (data not shown). Similarly, when data from smokers only were analysed there was no statistically significant difference between the number of mononuclear cells recovered from lung tissue taken from non-COPD controls and from COPD patients.

Lymphocytes from digested human lung tissue were characterised by flow cytometry. We noted that 2-80 % of lymphocytes were CD3⁺ T cells and no statistically significant differences were found between the percentages of these cells or in the percentages of CD4⁺ T cells in the lungs of smokers with and without COPD (Fig. 2A). Furthermore,

we observed that there were no statistically significant differences in the percentages of CD8⁺ T cells, NK cells, NK-T cells and B cells in the lungs of patients with COPD compared with the control group (data not shown). On average 16.9% of lymphocytes in the lung were CD4⁺ T cells and 20.4% of these CD4⁺ cells also expressed CD25 but there was no statistically significant difference in the percentage of lymphocytes that were CD4⁺CD25⁺ between patients with and without COPD (Fig. 2B).

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 (Fig. 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 (Fig. 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 (Fig. 3A & B) and transcripts for IFN- γ were detected by qRT-PCR in lung cellular infiltrates (Fig. 4A). Similarly quantitative (q)RT-PCR confirmed the presence of transcripts for IL-17A in mononuclear cells and purified CD3⁺ T cells immediately following isolation from the lung (Fig. 4A). IL-5 protein and transcripts were either absent or present at only very low levels in culture supernatants or lung infiltrate cells (data not shown). IL-22 transcripts were barely detectable in lung-derived mononuclear cells and CD3⁺ T cells in any sample (data not shown). Both IL-17A and IL-23 receptor transcripts were enriched in purified CD3⁺ T cells compared with mononuclear cells (IL17A; 6.2 and 3.9 fold increases and IL-23R; 3.5 and 1.3 fold increases for COPD and non-COPD patients respectively; Fig. 4A). Furthermore, transcripts for IFN- γ were also enriched in CD3⁺ T cells (3.6 and 2.4 fold increases for COPD and non-COPD patients, Fig. 4A). Similar fold increases were observed when only smokers were analysed (data not shown).

IL-17 producing cells are present in inflamed lung tissue

The transcription factors associated with Th1 (T-bet), Th2, (GATA-3) Th17 (RORC v2 isoform b / short isoform) and T reg (FOXP3) cells were measured in both unfractionated mononuclear cells and purified CD3⁺ T cells (Fig. 4B). Transcripts for T-bet, GATA-3, RORC and FOXP3 were all detected. Transcripts for RORC v2 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 Fig. 4B). FOXP3 and GATA3 mRNA were also enriched in the T cell fraction of lung mononuclear cells by 4.96 and 3.6 fold respectively (Fig. 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 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 (Fig. 5A). Similarly comparison of the levels of transcripts for FOXP3 and RORC showed no correlation for all subjects (Fig. 5B).

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Discussion

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

We observed that *in vitro* clonal expansion of Th17 cells is inhibited by T reg cells, which is inconsistent with the previous observation that human Th17 proliferation is resistant to T reg cell activity (34). However, in those experiments a regulatory T cell clone rather than fresh T regs was used. We also noted that IL-17 production by Th17 cells was unaffected by T regs and previous observations in mice have demonstrated that IL-17 production is unaffected or even enhanced by T reg 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/T reg cell interactions *in vivo* is in part likely to be due to functional heterogeneity of T regs (37). As regards Th1 and Th2 cells in our study T reg cells *in vitro* could inhibit proliferation and cytokine production by both subsets. This is in agreement with the experience of many other investigators (e.g. 38). In most experimental models *in vivo* there is marked polarisation of immunity and therefore the opportunity for comparative analysis of the effects of T regs on functionally distinct CD4⁺ T cell subsets has been limited. However, Th1, Th2 and Th17 cells can all induce pathology they vary in their receptiveness to T reg 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 can recruit macrophages (3). It can also increase MMP9 production (40) and transcription of MUC5A and MUC5B (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 (e.g. 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 RORCv2, 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 the IL-23 receptor 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). Here 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 GATA3 was detectable.

The presence of T reg cells in lung tissue was investigated by staining for CD25 expression on CD4⁺ cells and we like others observe T reg cells in chronic lung inflammation. Whether or not T reg 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, lung tissue lymphocytes and bronchial biopsies from large or small airways. Similarly the immunological parameters used as indicators of T reg 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 T regs 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 T reg cells.

Overall, this study has shown that *in vitro* IL-17 production is resistant to the inhibitory effects of T reg cells and that IL-17 producing cells and T reg 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 T reg function *in vivo* in inflammatory responses in the lung.

Author contribution:

CP and LB performed laboratory experimentation and writing paper. LD, US, SJT and PF prepared samples and data analysis, JL writing paper, PJB and MJD supervised the project, data analysis and writing paper.

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References

1. Zhu, J., Yamane, H. and Paul, W. E. (2010) Differentiation of effector CD4 T cell populations. *Annu. Rev. Immunol.* **28**, 445-489
2. Harrington, L. E., Hatton, R. D., Mangan, P. R., Turner, H., Murphy, T. L., Murphy, K. M. and Weaver, C. T. (2005) Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat. Immunol.* **6**, 1123-1132
3. Park, H., Li, Z., Yang, X. O., Chang, S. H., Nurieva, R., Wang, Y. H., Wang, Y., Hood, L., Zhu, Z., Tian, Q. and Dong, C. (2005) A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat. Immunol.* **6**, 1133-1141
4. Asano, M., Toda, M., Sakaguchi, N. and Sakaguchi, S. (1996) Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J. Exp. Med.* **184**, 387-396
5. Thornton, A. M. and Shevach, E. M. (1998) CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J. Exp. Med.* **188**, 287-296
6. Powrie, F., Leach, M. W., Mauze, S., Menon, S., Caddle, L. B. and Coffman, R. L. (1994) Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. *Immunity* **1**, 553-562
7. Langrish, C. L., Chen, Y., Blumenschein, W. M., Mattson, J., Basham, B., Sedgwick, J. D., McClanahan, T., Kastelein, R. A. and Cua, D. J. (2005) IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* **201**, 233-240
8. Pène, J., Chevalier, S., Preisser, L., Vénéreau, E., Guilleux, M. H., Ghannam, S., Molès, J. P., Danger, Y., Ravon, E., Lesaux, S., Yssel, H. and Gascan, H. (2009) Chronically inflamed human tissues are infiltrated by highly differentiated Th17 lymphocytes. *J. Immunol.* **180**, 7423–7430
9. O'Garra, A. and Vieira, P. (2004) Regulatory T cells and mechanisms of immune system control. *Nat. Med.* **10**, 801-805

10. Collison, L. W., Pillai, M. R., Chaturvedi, V. and Vignali, D. A. (2009) Regulatory T cell suppression is potentiated by target T cells in a cell contact, IL-35- and IL-10-dependent manner. *J. Immunol.* **182**, 6121-6128
11. Tesmer, L. A., Lundy, S. K., Sarkar, S. and Fox, D. A. (2008). Th17 cells in human disease. *Immunol. Rev.* **223**, 87–113
12. Happel, K. I., Zheng, M., Young, E., Quinton, L. J., Lockhart, E., Ramsay, A. J., Shellito, J. E., Schurr, J. R., Bagby, G. J., Nelson, S. and Kolls, J. K. (2003) Cutting edge: roles of Toll-like receptor 4 and IL-23 in IL-17 expression in response to *Klebsiella pneumoniae* infection. *J. Immunol.* **170**, 4432-4436
13. Higgins, S. C., Jarnicki, A. G., Lavelle, E. C. and Mills, K. H. (2006) TLR4 mediates vaccine-induced protective cellular immunity to *Bordetella pertussis*: role of IL-17-producing T cells. *J. Immunol.* **177**, 7980-7989
14. Khader, S. A. and Cooper, A. M. (2008) IL-23 and IL-17 in tuberculosis. *Cytokine* **41**, 79-83
15. Laan, M., Cui, Z. H., Hoshino, H., Lotvall, J., Sjostrand, M., Gruenert, D. C., Skoogh, B. E. and Linden, A. (1999) Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *J. Immunol.* **162**, 2347-2352
16. van den Berg, A., Kuiper, M., Snoek, M., Timens, W., Postma, D. S., Jansen, H. M. and Lutter, R. (2005) Interleukin-17 induces hyperresponsive interleukin-8 and interleukin-6 production to tumor necrosis factor-alpha in structural lung cells. *Am. J. Respir. Cell Mol. Biol.* **33**, 97-104
17. Hodge, G., Nairn, J., Holmes, M., Reynolds, P. N. and Hodge, S. (2007) Increased intracellular T helper 1 proinflammatory cytokine production in peripheral blood, bronchoalveolar lavage and intraepithelial T cells of COPD subjects. *Clin. Exp. Immunol.* **150**, 22-29
18. Scriba, T. J., Kalsdorf, B., Abrahams, D. A., Isaacs, F., Hofmeister, J., Black, G., Hassan, H. Y., Wilkinson, R. J., Walzl, G., Gelderbloem, S. J., Mahomed, H., Hussey, G. D. and Hanekom, W. A. (2008) Distinct, specific IL-17- and IL-22-producing CD4+ T cell subsets contribute to the human anti-mycobacterial immune response. *J. Immunol.* **180**, 1962-1970

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19. Nembrini, C., Marsland, B. J. and Kopf, M. (2009) IL-17–producing T cells in lung immunity and inflammation. *J. Allergy Clin. Immunol.* **123**, 986-994
20. Alcorn, J. F., Crowe, C. R. and Kolls, J. K. (2010) Th17 cells in asthma and COPD. *Annu. Rev. Physiol.* **72**, 495-516
21. Lane, N., Robins, R. A., Corne J. and Fairclough L. (2010) Regulation in chronic obstructive pulmonary disease: the role of regulatory T-cells and Th17 cells. *Clin. Sci.* **119**, 75-86
22. Barnes, P. J. (2007) Chronic obstructive pulmonary disease: a growing but neglected global epidemic. *PLoS Med.* **4**, e112
23. Barnes, P.J. (2008) Immunology of asthma and chronic obstructive pulmonary disease. *Nat. Rev. Immunol.* **8**, 183-192
24. Cosio, M. G. and Majo, J. (2002) Inflammation of the airways and lung parenchyma in COPD: role of T cells. *Chest* **121**, 160S-165S
25. Saetta, M., Di Stefano, A., Turato, G., Facchini, F. M., Corbino, L., Mapp, C. E., Maestrelli, P., Ciaccia, A. and Fabbri, L. M. (1998) CD8+ T-lymphocytes in peripheral airways of smokers with chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* **157**, 822-826
26. Di Stefano A., Caramori, G., Capelli, A., Gnemmi, I., Ricciardolo, F., Oates, T., Donner, C. F., Chung, K. F., Barnes, P. J. and Adcock, I. M. (2004) STAT4 activation in smokers and patients with chronic obstructive pulmonary disease. *Eur. Respir. J.* **24**, 78–85
27. Isajevs, S., Taivans I., Strazda, G., Kopeika, U., Bukovskis, M. Gordjusina, V. And Kratovska, A. (2009) Decreased FOXP3 expression in small airways of smokers with COPD. *Eur. Respir. J.* **33**, 61-67
28. Smyth, L. J., Starkey, C., Vestbo, J. and Singh, D. (2007) CD4-regulatory T cells in COPD patients. *Chest* **32**, 156-163
29. Lee, S. H., Goswami, S., Grudo, A., Song, L. Z., Bandi, V., J., Goodnight-White, S., Green, L., Hacken-Bitar, J., Huh, J., Bakaeen, F., Coxson, H. O., Cogswell, S., Storness-Bliss, C., Corry D. B. and Kheradmand F. (2007) Antielastin autoimmunity in tobacco smoking induced emphysema. *Nat. Med.* **13**, 567-569

30. Barceló, B., Pons, J., Ferrer, J. M., Sauleda, J., Fuster, A. and Agustí, A. G. (2008) Phenotypic characterisation of T-lymphocytes in COPD: abnormal CD4+CD25+ regulatory T-lymphocyte response to tobacco smoking. *Eur. Respir. J.* **31**, 555-562
31. Di Stefano, A., Caramori, G., Gnemmi, I., Contoli, M., Vicari, C., Capelli, A., Magno, F., D'Anna, S. E., Zanini, A., Brun, P., Chung, K. F., Barnes, P. J., Papi, A., Adcock, I. M. and Balbi B. (2009) T helper type 17-related cytokine expression is increased in the bronchial mucosa of stable chronic obstructive pulmonary disease patients. *Clin. Exp. Immunol.* **157**, 316–324
32. Doe, C., Bafadal, M., Siddiqui, S., Desai, D., Mistry, V., Rugman, P., McCormick, M., May, R., Sleeman, M. A., Anderson, I. A., Brightling, C. E. (2010) Expression of the Th17-associated cytokines IL-17A and F in asthma and chronic obstructive pulmonary disease. *Chest* DOI 10.1378/chest.09-3058
33. Springer, J., Scholz, F. R., Peiser, C., Groneberg, D. A. and Fischer, A. (2004) SMAD-signalling in chronic pulmonary obstructive disease: transcriptional down-regulation of inhibitory SMAD 6 and 7 by cigarette smoke. *Biol. Chem.* **385**, 649-653
34. Annunziato, F., Cosmi, L., Santarlasci, V., Maggi, L., Liotta, F., Mazzinghi, B., Parente, E., Fili, L., Ferri, S., Frosali, F., Giudici, F., Romagnani, P., Parronchi, P., Tonelli, F., Maggi, E. and Romagnani, S. (2007) Phenotypic and functional features of human Th17 cells. *J. Exp. Med.* **204**, 1849-1861
35. Lohr, J., Knoechel, B., Wang, J. J., Villarino, A. V. and Abbas, A. K. (2006) Role of IL-17 and regulatory T lymphocytes in a systemic autoimmune disease. *J. Exp. Med.* **203**, 2785–2791
36. O'Connor, R. A., Malpass, K. H. and Anderton, S. M. (2007) The inflamed central nervous system drives the activation and rapid proliferation of Foxp3+ regulatory cells. *J. Immunol.* **179**, 958-966
37. Huter, E. N., Stummvoll, G. H., DiPaolo, R. J., Glass, D. D. and Shevach E. M. (2008) Cutting edge: antigen-specific TGF beta-induced regulatory T cells suppress Th17-mediated autoimmune disease. *J. Immunol.* **181**, 8209–8213

38. O'Connor, R. A., Taams, L. S. and Anderton, S. M. (2009). Translational minireview series on Th17 cells: CD4⁺ T helper cells: functional plasticity and differential sensitivity to regulatory T cell-mediated regulation. *Clin. Exp. Immunol.* **159**, 137-147
39. Stummvoll, G. H., DiPaolo, R. J., Huter, E. N., Davidson, T. S., Glass, D., Ward, J. M. and Shevach, E. M. (2008) Th1, Th2, and Th17 effector T cell-induced autoimmune gastritis differs in pathological pattern and in susceptibility to suppression by regulatory T cells. *J. Immunol.* **181**, 1908–1916
40. Prause, O., Bozinovski, S., Anderson, G. P. and Linden, A. (2004) Increased matrix metalloproteinase-9 concentration and activity after stimulation with interleukin-17 in mouse airways. *Thorax* **59**, 313–317.
41. Chen, Y., Thai, P., Zhao, Y. H., Ho, Y. S., DeSouza, M. M. and Wu, R. (2003) Stimulation of airway mucin gene expression by interleukin (IL)-17 through IL-6 paracrine/autocrine loop. *J. Biol. Chem.* **278**, 17036–17043
42. Sonnenberg, G. F., Nair, M. G., Kim, T. J., Zaph, C., Fouser, L. A. and Artis, D. (2010) Pathological versus protective functions of IL-22 in airway inflammation are regulated by IL-17A. *J. Exp. Med.* doi 10.1084/jem.20092054
43. Ivanov, II., McKenzie, B. S., Zhou, L., Tadokoro, C. E., Lepelley, A., Lafaille, J. J., Cua, D. J. and Littman, D. R. (2006) The orphan nuclear receptor ROR γ directs the differentiation program of proinflammatory IL-17⁺ T helper cells. *Cell* **126**, 1121-1133
44. Hodge, G., Nairn, J., Holmes, M., Reynolds, P. N. and Hodge, S. (2007) Increased intracellular T helper 1 proinflammatory cytokine production in peripheral blood, bronchoalveolar lavage and intraepithelial T cells of COPD subjects. *Clin. Exp. Immunol.* **150**, 22-29
45. Grumelli, S., Corry, D. B., Song, L. Z., Song, L., Green, L., Huh, J., Hacken, J., Espada, R., Bag, R., Lewis, D. E. and Kheradmand, F. (2004) An immune basis for lung parenchymal destruction in chronic obstructive pulmonary disease and emphysema. *PLoS Med.* **1**, e8

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46. Tzanakis, N., Chrysofakis, G., Tsoumakidou, M., Kyriakou, D., Tsiligianni, J., Bouros, D. and Siafakas, N. M. (2004) Induced sputum CD8+ T-lymphocyte subpopulations in chronic obstructive pulmonary disease. *Respir. Med.* 98, 57-65

Figure legends

Table 1. Patient details

Clinical details of patients who donated lung tissue for this study (N/A = data not available; FEV1 = forced expiratory volume in 1 second; FVC = forced vital capacity). Control and COPD patient groups included smokers and non-smokers.

Figure 1. The effects of CD4+CD25+ T reg 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* polarised populations of Th1, Th2 and Th17 cells and irradiated non-CD3 cells as a source of APCs and activated with anti-CD3 antibody. Proliferation was measured by thymidine incorporation and the results are expressed as % 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 % change (B). T reg cells inhibit the proliferation of Th17 cells. Results are presented in Box and whiskers and expressed as cpm (C). IL-17 production by Th17 cells is enhanced in the presence of T reg 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.

Figure 2. Percentage of CD3+CD4+ and CD4+CD25+ T cells in lung tissue

Flow cytometric analysis of the lung CD4+ cell populations. CD3+CD4+ T cells are presented for non-COPD controls (squares, n=23) and COPD (triangles, n=23) patients (A) and CD4+CD25+ T cells for non-COPD controls (circles, n=8) and COPD (triangles; n=6) patients (B). Horizontal bars show means.

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 U/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; circles) and COPD patients (n = 20; squares). Horizontal bars show means. Lung CD3⁺ cells were cultured for 5 days with plate-bound anti-CD3 and soluble anti-CD28 antibodies and 50 U/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; circle symbols) and COPD patients (n = 6; triangle symbols). 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 quantitative RT-PCR (TaqMan). Fold differences in relative transcript levels between purified T cell and lung mononuclear cell populations are presented. Horizontal bars show means.

Figure 5. The presence of CD4⁺CD25⁺ T cells in the lung does not correlate with IL-17 production

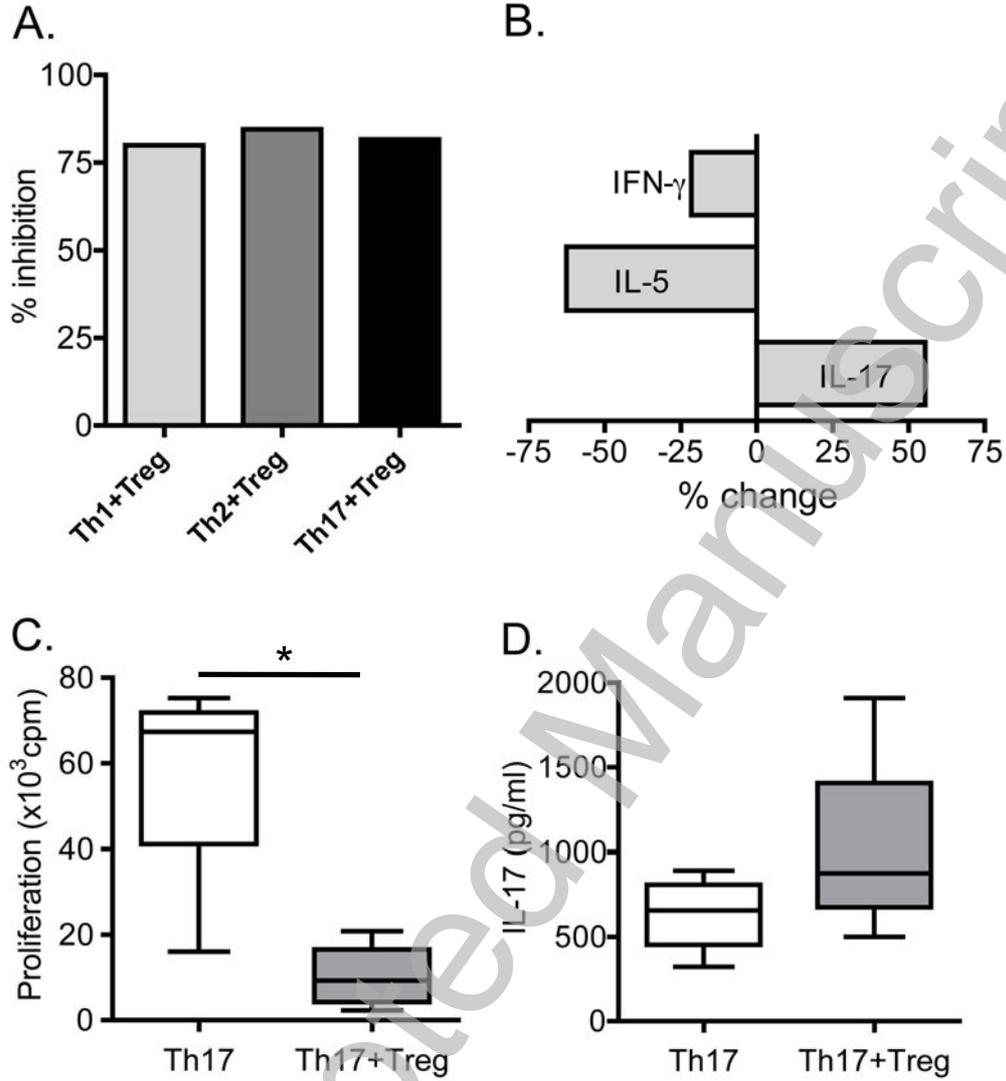
The percentage of CD4⁺CD25⁺ T cells in the lung tissue was measured by flow cytometry and IL-17A production by the lung mononuclear cells in culture by ELISA (A). FOXP3 and RORC v2 in human lung mononuclear cells and purified CD3⁺ T cells from Non-COPD controls and COPD patients were analysed by quantitative RT-PCR (TaqMan). Fold differences in relative transcript levels between purified T cell and lung mononuclear cell populations for each transcript (B).

Table 1. Patient details

Sample	Gender	Age	Smoking history (pack years)	FEV1 (% predicted)	FEV/FVC	GOLD stage	Clinical information
16	M	61	70	93.0	0.72	control	N/A
17	M	52	34	87.9	0.78	control	Chronic pneumonia, Bronchiectasis
22	M	75	15	93.3	0.75	control	Non-small cell lung cancer
25	M	71	smoker (py N/A)	75.0	N/A	control	Carcinoid tumour?
26	M	24	0	90.2	0.84	control	Bronchiectasis, parenchymal fibrosis middle lobectomy
27	F	87	31	112.0	1.50	control	Lung cancer - unspecified (?non-small cell lung carcinoma)
29	F	82	15	79.8	0.74	control	Metastatic malignant fibrous histiocytoma
31	M	9	0	73.0	0.90	control	Bronchiectasis
34	F	60	25	85.0	0.80	control	Mass and primary lung tumour on PET scan
37	M	44	0	117.0	0.87	control	Squamous cell cancer
39	F	63	0	94.0	0.89	control	Adenocarcinoma
40	M	60	50	90.6	0.71	control	Squamous cell carcinoma
47	M	70	50	59.0	0.77	control	Non-small cell lung cancer
48	M	75	40	98.6	0.71	control	Adenocarcinoma
49	M	79	50	N/A	>0.7	control	Adenocarcinoma
51	F	76	0	104.0	0.94	control	Cancer?
52	M	N/A	80	97.6	0.70	control	Adenocarcinoma
53	F	59	0	66.9	0.89	control	Mucinous adenocarcinoma
55	M	56	0	119.8	0.92	control	Squamous cell carcinoma, lung nodules, previous neck carcinoma
56	F	71	0	88.0	0.82	control	Pulmonary metastases from primary bowel carcinoma
58	M	70	30	99.0	0.85	control	Cancer
62	F	75	40	74.0	0.86	control	Cancer
65	M	68	93	56.7	0.75	control	Non-small cell lung cancer
10	F	82	0	86.2	0.59	1	N/A
18	M	76	44	86.9	0.61	1	Cancer
21	M	83	60	82.2	0.53	1	Lower left lobe lesion
23	M	63	30	82.0	0.63	1	Small cell carcinoma
30	F	47	N/A	91.0	0.68	1	Squamous cell carcinoma
32	M	60	N/A	94.5	0.59	1	Right interbronchial squamous cell carcinoma
57	F	79	40	99.3	0.69	1	Non-small cell lung cancer, basal-like cancer
63	M	69	83	94.7	0.69	1	Non-small cell lung cancer, poorly differentiated adenocarcinoma
64	M	56	80	92.0	0.66	1	Non-small cell lung cancer, Squamous cell carcinoma, pulmonary fibrosis
9	M	N/A	50	57.4	0.43	2	Adenocarcinoma
11	M	64	49	64.0	0.49	2	Large cell undifferentiated carcinoma
12	M	69	26	63.0	0.47	2	Squamous lung cancer
15	F	62	24	76.6	0.57	2	Adenocarcinoma
20	M	67	30	60.5	0.52	2	Squamous cell carcinoma
38	M	61	50	66.0	0.57	2	N/A
41	M	67	5	76.6	0.66	2	Squamous cell carcinoma
42	F	69	40	59.0	0.52	2	Lung cancer
44	M	72	50	66.6	0.56	2	Adenocarcinoma, non-small cell carcinoma
45	M	54	30	51.8	0.67	2	Non-small cell lung cancer
54	M	79	>20	72.0	0.62	2	Adenocarcinoma
59	M	70	56	72.0	0.53	2	T2N1 carcinoma
28	M	56	30	49.3	0.47	3	Mass in left upper lobe - unknown histology
36	F	58	40	47.0	0.57	3	Adenocarcinoma
46	M	67	100	45.6	0.41	3	Non-small cell lung cancer

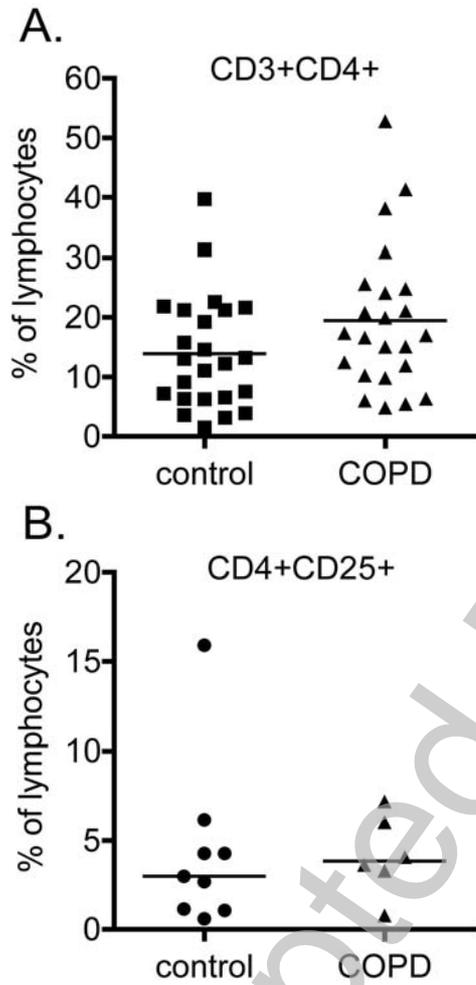
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Figure 1



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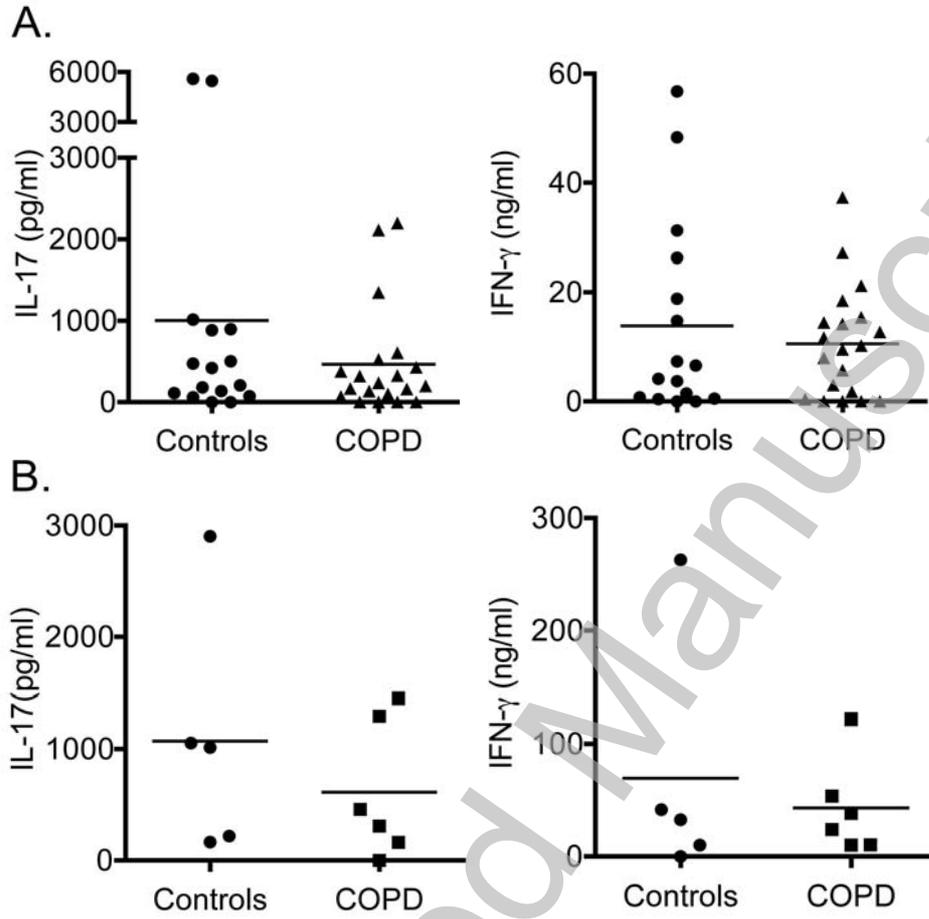
Figure 2



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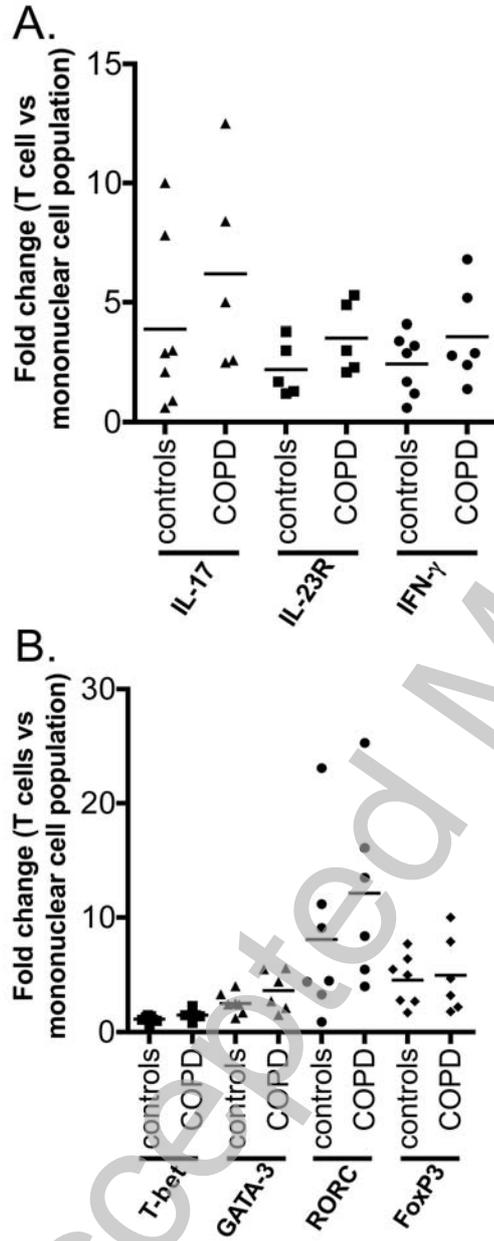
Figure 3



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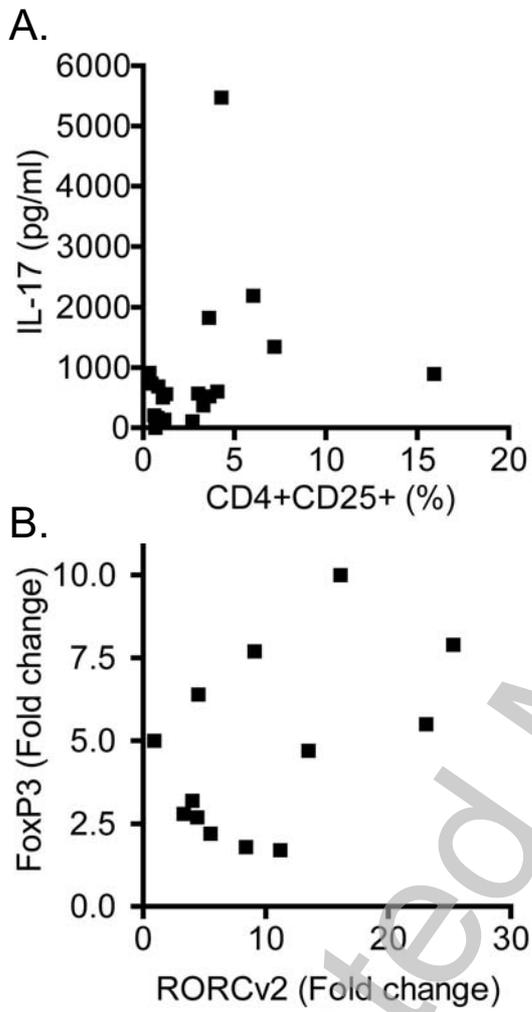
Figure 4



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Figure 5



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