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Dysregulation of anti-viral function of CD8+T cells in the COPD lung: role of the PD1/PDL1 axis[1]

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At a glance commentary:

Scientific knowledge on the subject: Dysregulation of adaptive immunity is thought to be an important disease mechanism in COPD with increased numbers of cytotoxic T cells present in the lung. PD1 is a key regulator of T cell function and is associated with loss of cytotoxic function in the context of chronic infection and inflammation but the role of this axis in COPD and its association with T cell function is not known.

What this study adds to the field: This study shows that PD1 is upregulated on T cells derived from COPD patients and that PD1 expression increases following influenza infection in an experimental lung explant tissue model. In this study CD8 T cells from COPD patients also demonstrated evidence of impaired cytotoxicity. In contrast, infection-induced expression of the ligand PD-L1 on macrophages was diminished in COPD with associated increases in IFNγ expression. These observations provide evidence of dysregulation of T cell function in COPD through the PD1 axis, contributing to our understanding of mechanisms leading to the aberrant response to infection in COPD.

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Abstract

Rationale: COPD patients are susceptible to respiratory viral infections which cause exacerbations. Mechanisms underlying susceptibility are not understood. Effectors of the adaptive immune response; CD8+ T cells which clear viral infections, are present in increased numbers in lungs of COPD patients but fail to protect against infection and may contribute to the immunopathology of the disease.

Objectives: CD8+ function and signalling through the Programmed Cell Death (PD-1) exhaustion pathway was investigated as a potential key mechanism of viral exacerbation of the COPD lung.

Methods: Tissue from control or COPD patients undergoing lung resection was infected with live influenza virus *ex vivo*. Viral infection and expression of lung cell markers was analysed using flow cytometry.

Measurements and Main Results: The proportion of lung CD8+ T cells expressing PD-1 was greater in COPD(mean=16.2%) than controls(4.4%, p=0.029). Only epithelial cells and macrophages were infected with influenza and there was no difference in the proportion of infected cells between controls and COPD. Infection upregulated T cell PD-1 expression in control and COPD samples. Concurrently, influenza significantly upregulated the marker of cytotoxic degranulation (CD107a) on CD8+ T cells(p=0.03) from controls, but not from COPD patients. Virus-induced expression of the ligand PD-L1 was decreased on COPD macrophages(p=0.04) with a corresponding increase in IFNγ release from infected COPD explants compared to controls(p=0.04).

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Conclusions: This study has established a signal of cytotoxic immune dysfunction and aberrant immune regulation in the COPD lung that may explain both the susceptibility to viral infection and the excessive, inflammation associated with exacerbations.

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Introduction

Chronic Obstructive Pulmonary disease (COPD) is an irreversible progressive disease resulting in permanent loss of lung function(1, 2). It is characterised by persistent airflow limitation and innate and adaptive immune cell infiltration into the lungs. COPD patients experience recurrent viral infections accompanied with lung inflammation resulting in exacerbations which are characterised by a sudden decline in lung function, often require hospitalisation, and may result in death(3-5). CD8+ T cells, which play a key role in anti-viral immunity have been shown to be present in greater numbers in the lungs of patients with more severe COPD as measured by FEV_1(6) but these patients remain at great risk from the impacts of respiratory viral infection.

Recent studies have suggested that regulation of T cell function can occur via the T cell exhaustion pathway in response to viral infection(7). PD-L1 is the ligand for the Programmed Cell Death protein 1 (PD-1), which is a member of the CD28 family of T cell receptors. The canonical pathway of T cell activation is via antigen presentation in the context of MHC to elicit T cell receptor (TCR) activation, co-stimulation of CD28 provides a necessary signal to prevent T cell anergy(8). Contrastingly, PD-L1 binding to PD-1 causes inhibition of T cell proliferation and cytokine release(9). T cell exhaustion is a state of T cell dysfunction normally associated with chronic viral infection and cancer and is associated with prolonged stimulation of T cells due to persistent antigen presentation. However recent work has suggested that expression of PD-1 is also closely linked to T cell differentiation and can be expressed on acutely

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activated T cells but usually subsides during resolution of infection (10). The PD-1 pathway has recently been suggested as potentially relevant in COPD pathogenesis, as the presence of PD-1+ T effector cells in the blood correlated with disease severity(10). Kalathil *et al.* detected PD-1 expression in a population of blood CD4+CD127+ T cells, although there was no evidence of functional exhaustion(10). The potential for T lymphocytes to express an exhausted phenotype in the COPD lung has not yet been established. We hypothesised that T cells in the COPD lung would express an exhausted phenotype compared to cells derived from control lungs and that T cell exhaustion may account for poor responses to viral infection that may lead to COPD exacerbations.

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Materials & Methods

Ex vivo infection of lung parenchymal tissue

Resected human lung tissue was obtained from consented patients undergoing airway re-section surgery at our regional thoracic surgical unit. The collection of tissue was approved by and performed in accordance with the ethical standards of the Southampton and South West Hampshire Research Ethics Committee, LREC no: 09/H0504/109. Ex-smokers were defined as individuals who had quit smoking for >6 months. Parenchymal tissue, distant from the resection margin and any gross pathology was dissected from the lobe. Tissue was cut into 1mm^3 sections and added to a 24-well flat-bottomed culture plate before washing with Dulbecco's Phosphate Buffered Saline (DPBS – Sigma, Poole, UK). Washing of the tissue was performed by removing DPBS from the wells and replacing it with fresh DPBS, followed by unsupplemented RPMI and finally RPMI supplemented with 1% penicillin/streptomycin (both Life Technologies, Paisley, UK) and 1% gentamycin (GE Healthcare, Little Chalfont, UK). Tissue was then incubated overnight at 37ºC and 5% CO_2. *Ex vivo* infection of resected lung tissue with H3N2 X31 influenza virus (a kind gift of 3VBiosciences) was then carried out as previously described(11).

T cell and Monocyte Isolation & differentiation

CD8+ T cells and Monocytes were isolated from human peripheral blood mononuclear cells (PBMC) using MACS technology (Miltenyi Biotec, Bisley, UK)

and monocytes differentiated into macrophages by culturing for 12 days with 2 ng/ml GM-CSF.

Flow cytometry analysis

Samples were resuspended in FACS buffer (PBS, 0.5% w/v BSA, 2 mM EDTA) containing 200 µg/ml human IgG before being incubated on ice in the dark for 30 min in the presence of fluorescently-labelled antibodies as previously described(11). Flow cytometric analysis was performed on a FACSAria using FACSDiva software v5.0.3 (BD Biosciences, Oxford, UK).

RNA Isolation & RT-PCR

RNA was extracted from 25,000 flow cytometry sorted CD4+ or CD8+ lung T cells using a Stratagene Nanoprep Kit (Agilent Technologies, Stockport UK). Reverse transcription was carried out using a High Capacity cDNA Reverse Transcription Kit (Life Technologies) with random hexamers carried out according to the manufacturer's protocols. *TIM3* gene expression was analysed using TaqMan universal PCR master mix, No AmpErase® UNG in a 7900HT fast real-time PCR system instrument (all Life Technologies). Gene expression was normalized to β_2-microglobulin gene expression and quantified using the $\Delta\Delta$ C_T method.

Supernatant analyses

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IFNγ concentrations in culture supernatants were analysed by Luminex assay as per manufacturer's instructions (Bio-Rad, Hemel Hempstead, UK).

ELISpot

ELISpot for Human IFN-γ (MabTech, Stockholm, Sweden) was performed using 0.45 μm MultiScreen-IP Filter Plates (Millipore, Watford, UK) as previously described(11). Briefly, MDMs were either not infected, or were treated with 2.5 x 10^4 pfu/ml X31 Influenza A H3N2 virus at 37ºC for 2 h before washing and were then added to each well at a concentration of 5 x 10^4 cells/well and 2.5 x 10^5 monocyte-depleted PBMC or 1 x 10^5 CD8+ T cells were added to MDM-containing wells and incubated at 37ºC for a further 22 h.

Statistics

Analysis of two groups was performed using Wilcoxon's signed rank test for paired data and a Mann-Whitney U test for unpaired data. Chi-squared test and Fishers exact test were used for categorical data (GraphPad Prism v6, GraphPad Software Inc., San Diego, USA). Results were considered significant if $p<0.05$.

For full details of all methods please see supplemental data

Results

Patients

The clinical characteristics of surgical patients are presented in Table 1. COPD patients were matched with controls for age, but had a greater smoking history and lower FEV_1% predicted and greater airflow obstruction.

Lung resident T cell phenotype in COPD

Previous studies have demonstrated an increase in CD8+ T cells in the COPD lung by immunohistochemistry (6, 12). To validate our flow cytometry method we measured the proportion of CD4+ and CD8+ T cells disaggregated from the explanted lung tissue using the gating strategy outlined in Figure 1A. The proportion of CD4+ T cells was significantly lower in COPD (mean=39.3%) than controls (mean=47.3%), p=0.016 (Figure 1B). Conversely, the proportion of CD8+ T cells was greater in COPD (mean=42.7%) than controls (31.2%), p=0.004 (Figure 1C & Supplementary Data Figure E1A+B). Moreover the majority of these cells were effector memory cells (CCR7-), suggesting we are studying lung resident cells and not carry over from the blood compartment (Supplementary Data Figure E2).

Patients with COPD exhibit elevated proportions of PD1+ T cells

To investigate if the immune defect in CD8+ T cells in COPD was associated with markers of exhaustion, PD-1 expression by lung resident T cells was quantified in control and COPD patients (Figure 2). The mean proportion of CD4+ T cells

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from controls that expressed PD-1 was 1.68%, compared to a mean of 4.51% in COPD tissue (Figure 2A - p=0.07). A mean of 4.39% of CD8+ T cells from control lung tissue expressed PD-1, while a mean of 16.24% expressed PD-1 in COPD tissue (Figure 2B - p=0.0291). There was therefore evidence of a greater proportion of T cells expressing PD-1 in COPD lungs compared to control tissue.

The co-expression of PD-1 and TIM-3 has been used to identify functionally exhausted T cells in murine models(13). In contrast to PD-1, T cells isolated from tissue of either controls (n=9) or COPD individuals (n=12) did not express detectable surface TIM-3. To ensure that lack of TIM-3 detection was not due to an effect of collagenase on the lung T cells, RT-PCR experiments were performed using CD4+ and CD8+ T cells sorted from lung parenchyma. TIM-3 mRNA was not detected in either CD4+ or CD8+ T cell samples from controls or COPD patients (Supplementary Data Figure E3).

Influenza infection of lung explants

In order to assess the functional consequences of a viral infection on the activation of T cells, we utilised a previously validated *ex vivo* model of lung explant infection (11, 14, 15). In that previous study, H3N2 X31 influenza A was shown to infect epithelial cells and macrophages in both human bronchial and parenchymal tissues (11, 15). Endothelium, fibroblasts, B cells and T cells were not infected by X31(14). Therefore infection of epithelial cells and macrophages was quantified using the gating strategy outlined in Figure 3A using the expression of the influenza protein NP-1. Epithelial cells and macrophages from

inactivated (UV treated) tissue did not express NP-1. A mean of 10.38% of epithelial cells from control patients expressed NP-1 (Figure 3B) compared to 9.19% form COPD samples (p=0.77). There were also no significant difference (p=0.50) between the proportion of macrophages infected with virus from control (mean 18.12%) or COPD tissue (mean 14.45%) (Figure 3C).

T cell responses to influenza infection of lung explants

As there was no difference in the proportion of virally infected cells between healthy and COPD subjects, these data suggest that the mechanisms leading to COPD exacerbations may arise as a failure to adequately control the immune response rather than due to an increased level of infection. PD-1 expression was therefore measured to investigate differential immune responses to infection. Figure 4 shows that PD-1 is upregulated on CD4+ and CD8+ T cells in both control and COPD explants in response to X31 infection (Fig 4A & 4B). CD8+ PD-1 expression in controls increased from a mean sMFI of 165.1 to 237.7 in X31 samples (p=0.01). *Ex vivo* CD8+ T cells from COPD patients express a mean sMFI PD-1 of 231 which increased to 287.7 with X31 treatment (p=0.02). A similar pattern of expression in response to infection was seen for CD4+ T cells. Live virus induced a significantly greater fold increase (median 1.37-fold) in PD-1 expression on COPD CD8+ (p=0.0134), but not CD4+ (p=0.2847), T cells than UV-irradiated virus (Supplementary Data Figure E4C&D). The percentage of CD8+ T cells from both controls and COPD patients expressing PD-1 was also increased in response to X31 treatment. There was also a significant increase in

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the proportion of CD4+ T cell PD-1 expression from COPD patients but not controls in response to infection (Supplementary Data Figure E4A&B). PD-1 is upregulated during X31 infection, but the fold increase in expression induced by influenza in CD4+ (p=0.31) and CD8+ (p=0.27) T cells did not differ between control and COPD samples (Supplementary Data Figure E4E&F).

To analyse the functional relevance of this PD-1 upregulation, we assessed the expression of the degranulation marker CD107a in response to viral infection between controls and COPD individuals (Figure 4C&D). CD4+ T cells from both control and COPD samples significantly upregulated CD107a in response to infection (p=0.03) (Figure 4C). In contrast, whilst CD8 T cells from controls significantly upregulated (p=0.03) CD107a in response to viral infection there was not a significant upregulation by CD8 cells derived from COPD explants (Figure 4D). Taken together these results suggest that CD8 T cells in the COPD lung may have an impaired degranulation response to viral infection.

Lung macrophage expression of PD-L1 is compromised in COPD

T cells downregulate their effector functions due to ligation of PD-1 by PD-L1. To assess whether there was dysregulation of PD-L1 in our *ex vivo* model, we also measured the expression of this ligand on alveolar epithelial cells and macrophages (Figure 5) and to elucidate whether PD-L1 expression corresponded to viral infection and T cell upregulation of PD-1. The results indicated that epithelial cells express PD-L1 in human parenchyma, but its expression is lower (NI pooled sMFI = 142) than in macrophages (NI pooled

sMFI = 442.47) and is not regulated by acute X31 infection (Supplementary Data Figure E5). Macrophages, however, upregulate PD-L1 in response to infection in control samples (p=0.02) and COPD samples (p=0.02) (Figure 5A). Intriguingly, we observed lower expression of PD-L1 in COPD in response to infection compared to infected control samples (p = 0.04) Figure 5B).

As PD-L1 has been previously shown to be directly responsible for reducing CD8 T cell function in response to influenza (7), these data suggest that COPD macrophages may be unable to adequately modulate T cell activation. To assess this in the explant model we analysed the release of IFNγ into supernatants from tissue infected with X31 for 24 h (Figure 5C). Infection with influenza caused significant IFNγ release from both control (p=0.02) and COPD (p=0.004) explants (Figure 5C). However COPD explants produced significantly more IFNγ in response to influenza (mean 86 pg/ml) than infected control explants (mean 49 pg/ml, p=0.04) (Figure 5D), suggesting that the decrease in PD-L1 expression by infected macrophages does have a functional effect on T cell cytokine release.

Fluticasone does not affect CD8+ T cell PD-1 expression

In order to ensure that the effects we were seeing were not an epiphenomenon due to inhaled corticosteroid use by COPD patients, we incubated peripheral blood derived CD8+ T cells with 10^{-7} M fluticasone propionate (FP) for 24 h and analysed cell surface PD-1 expression by flow cytometry (Figure 6A). There was no significant upregulation of PD-1 on CD8+ T cells in response to fluticasone.

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Discussion

In the present study, we have demonstrated evidence of dysregulation of T cell immune function in the COPD lung. We have shown that a greater proportion of T cells express PD-1 in COPD tissue than in controls, but that this signal is not one of the canonical fully exhausted phenotype as there was no co-expression of TIM-3 at either the mRNA or protein level. However this finding was associated with evidence of diminished T cell cytotoxic degranulation responses to viral infection. Study of the virus-induced expression of the exhaustion ligand PD-L1 demonstrated that it is decreased on COPD macrophages with a corresponding increase in IFNγ release into supernatants from virally infected lung explants. These data are complex to interpret but reflect the complex regulation of T cells in the lung and interactions with the disease effects of COPD. The findings highlight that T cell regulation, of which exhaustion is an important component, is impacted upon by both aberrant T cell functionality and loss of regulatory control in the context of COPD. The consequences of these phenomena may explain the complex relationship between viral susceptibility and excessive inflammation which is the hallmark of acute exacerbations.

The increased proportion of CD8+ T cells in the lung parenchyma of COPD patients has been described and it is postulated that this may be due to their anti-viral properties(16, 17). As CD8+ T cells elicit potent anti-viral responses(18), the raised proportions of activated CD8+ T lymphocytes in COPD lungs may indicate a response to increased frequency of infection (19, 20). The localisation of T cells in a murine model of viral infection during RSV or Influenza

A virus infection resulted in recruitment of CD8+ T cells to the lungs, with virus specific T cells residing in the lungs for 30 days, with the majority of IFNγ-secreting CD8+ cells being found in lung tissues rather than in the periphery(21). Purwar et al.(22) have shown that human lung contains resident memory T cells (T_{RM}) cells independently of challenge, and these cells can secrete potent inflammatory mediators upon stimulation, underlining the importance of T_{RM} in protecting the host during infection. Recently other work using in vitro stimulation of lung derived T cells has demonstrated evidence of dysfunction in COPD. CD4+ T cells particularly in advanced disease demonstrated aberrant polarization patterns but features of exhaustion or response to direct viral infection were not explored (23).

PD-1-expressing lymphocyte populations were identified in lung parenchyma, with a greater proportion of T cells expressing this marker in patients with COPD. This expression was significantly upregulated by CD4+ and CD8+ T cells in response to infection. The inducible nature of PD-1 in response to activation was first identified by Agata *et al.*(24) and this previous work agrees with the findings presented above. PD-1 is a marker of T cell exhaustion, but it is also expressed by activated T cells which appear to be fully or partly functional (25, 26). PD-1 is upregulated by T lymphocytes in acute models of LCMV, but these are resolved before T cells display an exhausted phenotype(27). However, acute infection in the human *ex vivo* model appears to yield similar results to those of the previously reported *in vivo* murine model of influenza infection(28).

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The release of granzyme B and perforin is utilised by CD8+ T cells to induce apoptosis of virally-infected cells(29). Intracellular staining of these proteins was not performed due to variable levels of Granzyme B detection in unstimulated samples, but CD107a can be used as a surrogate marker for T cell cytotoxic degranulation(29). CD107a was upregulated by CD4 and CD8 T cells in controls but not COPD in response to influenza infection. An inability to produce cytotoxic proteins in response to viral infection infers an associated host susceptibility to infection and potentially a failure to clear the pathogen resulting in prolongation of clinical illness seen in COPD. In combination with the increased PD-1 expression in COPD, these data are consistent with the hierarchical loss of T cell function characteristic of T cell exhaustion(30). These observations suggest that there are an increased proportion of CD8+PD-1+ T cells in the COPD lung that are activated but carry important functional features of exhaustion that have an impaired ability to release cytotoxic granules.

Cytotoxic responses are predominantly associated with CD8 T cell and NK cells, but in this model CD107a was also upregulated by CD4 T cells. This finding adds to the growing literature of cytotoxic CD4 T cells in viral infection(31, 32), including influenza(33). It is unclear as to whether cytotoxic CD4+ T cells are a unique subset of T cells, or whether their killing ability is induced during an impaired CD8 response in the context of COPD and this requires further study.

Previous investigation into PD-L1 regulation of T cell responses to respiratory viruses has focussed on expression by epithelial cells(7, 34, 35). Influenza viral infection of control and COPD tissue did not modulate PD-L1

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expression by epithelial cells, even though it was constitutively expressed at a very low level (Supplementary Figure E5). Although there was no difference in the basal expression of this ligand between controls and COPD, macrophages expressed significantly more PD-L1 in response to infection in both groups. Our previous data indicate that this PD-L1 upregulation is functionally relevant, as use of a PD-L1 blocking antibody in our influenza–infected MDM model increased production of IFNγ by autologous CD8 T cells, implicating PD-L1 in the regulation of anti-viral cytokine release(11). In the current study, infected COPD macrophages expressed significantly less PD-L1 than control macrophages, which in turn correlated with an increase in IFNγ release from infected COPD lung. Thus not only does there appear to be a defect in the antiviral cytotoxic function of T cells in COPD but also an inability to arrest the activation of these T cells by decreased PD-L1 expression. These findings are consistent with well recognised clinical phenomena of increased severity of virally infection, prolonged viral shedding and structural lung damage associated with exacerbations.

Our previous study suggested that autocrine IFNβ production by alveolar macrophages was a driver of PD-L1 expression(11). Rhinovirus-driven asthma exacerbations have been postulated to be a direct result of deficient IFNβ production by bronchial epithelial cells from asthmatics for the last decade(36). More recent work challenging COPD patients with rhinovirus to mimic an exacerbation suggests a similar defect in IFNβ production may also operate in COPD(37). Unfortunately, we are unable to directly measure IFNβ production in

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virally-challenged lung explants and so deficient production of this cytokine leading to a reduced expression of PD-L1 in response to virus in COPD remains an intriguing possibility.

This work has been performed using whole lung explants from patients undergoing thoracic surgery. This approach permits the analysis of the complex interactions between structural and immune cells and the impact of viral infection in this complex composite system. This validated model has contributed to our understanding of cellular immunity in the human lung with a clinically relevant pathogen (live influenza virus) which would be not feasible *in vivo*, but it has limitations when studying immune responses which should be considered. Firstly lung explants are isolated from the effects of immune cells trafficking from the blood and so these conclusions are valid for lung resident cells only. We feel however that, as exhaustion signalling is confined to impacting on terminally differentiated T cells, it remains a very valid model in this context. Furthermore patients donating tissue were undergoing surgery for indications including lung cancer which may impact on the findings. However only tissue distant to the tumour site was used and the COPD related effects on immunity were apparent whilst the co-morbidities leading to surgery were present in both COPD and controls. In using lung derived T cells we have been limited in the number of cells available for phenotypic analysis and hence the breadth of phenotypic markers was also constrained to a single flow cytometry panel. We have used established markers to identify T cells and their function. Cytotoxic function can be measured in a number of ways including expression of perforin and granzyme B as well as

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the marker CD107a. This latter marker has been well established as a signature of cytotoxic degranulation(7, 38). However to fully understand the dysfunctional nature of T cells further studies exploring functional killing ability and the antigen specific nature of dysfunctional T cell subsets are required. In addition the interaction with functional readouts of T cells and the response to infection with treatments such as inhaled corticosteroids and also active smoking requires further study to provide insights into how these may diminish protective immunity.

Taken together, these data indicate a dysregulation of CD8+ T cell responses to viral infection in the COPD lung. Moreover, they suggest that viral exacerbations of COPD may arise due to a combination of an already active CD8 T cell population with an impaired anti-viral action coupled with an inability to down-regulate cytokine release from these as a result of deficient PD-L1 expression. However interpretation of the observed results requires caution as it is possible that reduction of PD-L1 expression enables increased IFNγ expression which aids viral clearance in the diseased lung. Whichever the direction of effect *in vivo*, these observations have important potential implications for the therapeutic manipulation of T cell function including the use of PD-1 and PD-L1 blocking antibodies to modulate T cell activity which are in use as cancer therapies today. Further studies are required to investigate the translational potential of this approach. If this axis is modulated imprecisely, risks to the patient of pneumonitis, are already recognised(39). The key question remains as to what drives the increased expression of PD-1 in the COPD airway

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and whether cells manifesting this functional phenotype are the ones specific to pathogens or autoantigens that play a key role in the pathogenesis of COPD.

	Control	COPD	p Value
N	24	33	-
Age (years)	70.5 (61 – 76)	67 (60.5 – 74)	0.75#
Gender M/F	12 / 12	16 / 17	0.91†
BMI	27.25 (22.63 – 31.55)	23.7 (21.25 – 28.95)	0.23#
Confirmed corticosteroid use	1	9	0.0336‡
Smoker (Never/Ex/Current)	6 / 15 / 3	1 / 20 / 12	0.0671‡
Pack Years	21 (.625 – 52.5)	40 (31.25 – 62.5)	0.025#
FEV_1%	98 (91.9 – 112)	76.13 (67 – 86)	< 0.0001#
FEV_1/FVC ratio	0.776 (0.73 – 0.805)	0.601 (0.551 – 0.654)	< 0.0001#

Table 1: Clinical characteristics of surgical patients. Data are presented as median and IQR shown. Ex-smokers were defined as individuals who had stopped smoking for > 6 months prior to surgery. BMI data shown represents 16 Control and 17 COPD patients. #Mann Whitney U Test, †Chi-squared test, ‡ Fishers Exact test. Co-morbidity data can be viewed in Supplementary Table E3.

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Figure Legends

Figure 1. Flow cytometry gating strategy for T cells in lung parenchymal tissue. After resting explanted lung tissue overnight, tissue was digested with collagenase and cells analysed by flow cytometry **(A)** Unstained singlet population was obtained from digested tissue. Dead cells were excluded using LIVE/DEAD® Fixable Aqua Dead Cell Stain. Live singlet CD45+ population was then identified and from this a CD45+CD3+ T cell population. The CD45+CD3+ T cell population was divided into CD4+ and CD8+ T cells. **(A)** Proportion of CD4+ **(B)** and CD8+ T cells are gated on the live CD45+CD3+ population. Control n = 20, COPD n = 24. Median and IQR shown. Data analysed using a Mann-Whitney U-test # $p<0.05$, ## $p<0.01$.

Figure 2. Intrinsic PD-1 expression by CD4 and CD8 T cells in controls and COPD. After resting explanted lung tissue overnight, tissue was digested with collagenase and cells analysed by flow cytometry. T cells are gated on the live CD45+CD3+ population. **(A)** Proportion of CD4+ **(B)** and CD8+ T cells expressing surface PD-1. Control n = 9, COPD n = 12. Median and IQR shown. Data analysed using a Mann-Whitney U-test # $p<0.05$. Note that y axis limit is changed from 15% to 50% between **(A)** and **(B)**.

Figure 3. Infection of Epithelial cells and Macrophages from lung parenchymal tissue. After resting explanted lung tissue overnight, 1 x 10^6 pfu/ml

H3N2 X31 influenza virus or a UV-irradiated aliquot of virus (UVX31) was added for 2 h. After washing, media was replaced and the tissue was incubated for a further 22 h followed by collagenase digestion and flow cytometry analysis **(A)** Epithelial cells were identified as CD45- EpCAM+ cells. Macrophages were identified as CD45+ HLA-DR+ cells. **(B)** Proportion of Epithelial cells and **(C)** Macrophages expressing NP-1 after treatment with $1x10^6$ pfu/ml live X31 virus (X31), UV-irradiated virus (UVX31) or non-infected (NI). NP-1+ cells were classified as infected by the influenza virus. Median and IQR shown. Data analysed using Wilcoxon's signed rank test (** $p<0.01$, *** $p<0.001$) to analyse intra group variations and Mann-Whitney U-test to analyse intergroup variations. (n.s. = not significant)

Figure 4. Expression of PD-1 and CD107a by T cells in response to tissue infection by X31 influenza. (A) CD4+ **(B)** and CD8+ T cell expression of PD-1 was quantified by flow cytometry in non-infected (NI), live X31 virus infection (X31) and UV-irradiated virus (UVX31) lung samples. Control n = 9, COPD n = 12. **(C)** CD4+ **(D)** and CD8+ T cells expression of CD107a was also quantified by flow cytometry. Control n = 6, COPD n = 6. Median and IQR shown. Data analysed using a Wilcoxon-signed rank test (* $p<0.05$, ** $p<0.01$) and Mann-Whitney U-test to analyse intergroup variations.

Figure 5. Expression of PD-L1 by Macrophages and IFNγ production in response to tissue infection by X31 influenza. (A) Expression of PD-L1 by

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Macrophages was measured in non-infected (NI), live X31 virus infection (X31) and UV-irradiated virus (UVX31) samples. **(B)** Differences in PD-L1 expression between X31 control and X31 COPD samples. Control n = 9, COPD n = 11. **(C)** IFNγ was measured from supernatants of non-infected (NI), live X31 virus infection (X31) and UV-irradiated virus (UVX31) explant tissue. **(D)** Differences in IFNγ production between X31 control and X31 COPD samples. Control n = 10, COPD n = 8. Median and IQR are shown. Data in (A) & (C) were analysed using a Wilcoxon-signed rank test * $p<0.05$, ** $p<0.01$. Data in (B) & (D) were analysed using a Mann-Whitney U test # $p<0.05$, ## $p<0.01$.

Figure 6. Effect of Fluticasone Propionate on blood CD8+ T cell PD1 expression PD-1 expression was measured in non-infected blood CD8 T cells incubated in the presence or absence of 10^{-7}M FP by flow cytometry (n=4). Median and IQR are shown. Data were analysed using a Wilcoxon-signed rank test.

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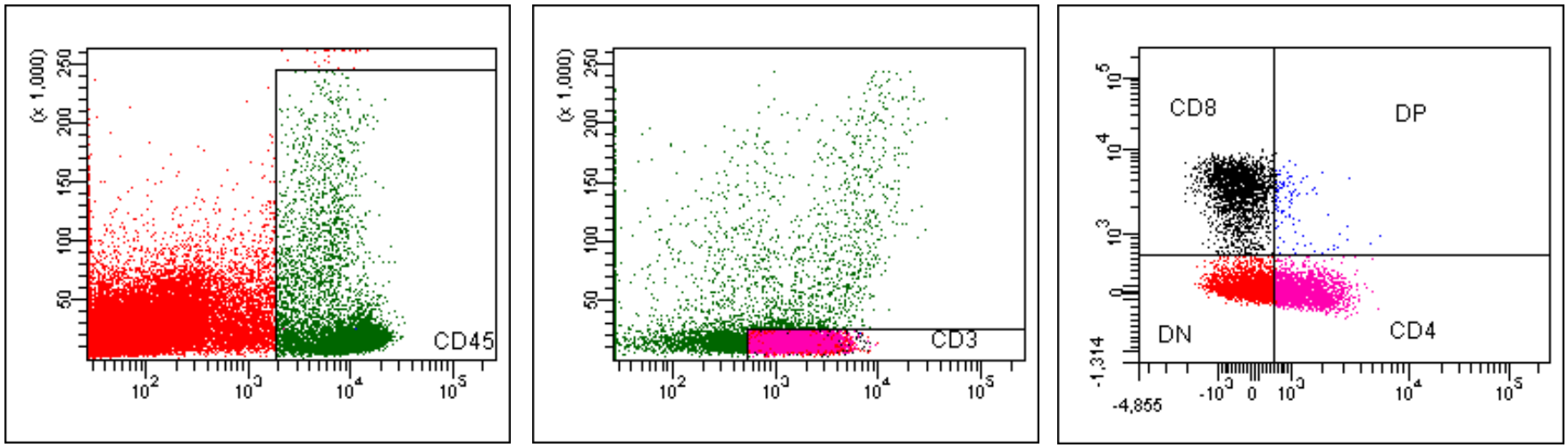

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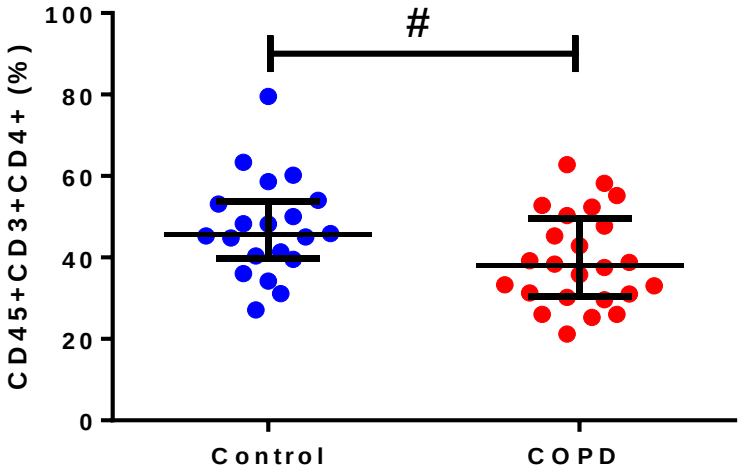

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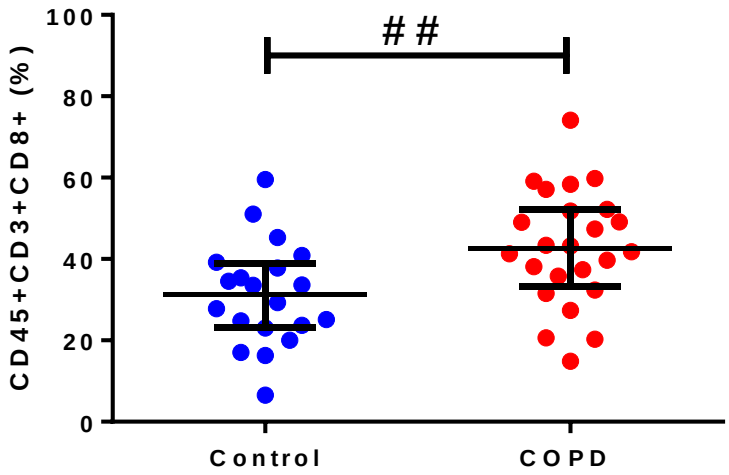

McKendry et al 2015 Figure 1

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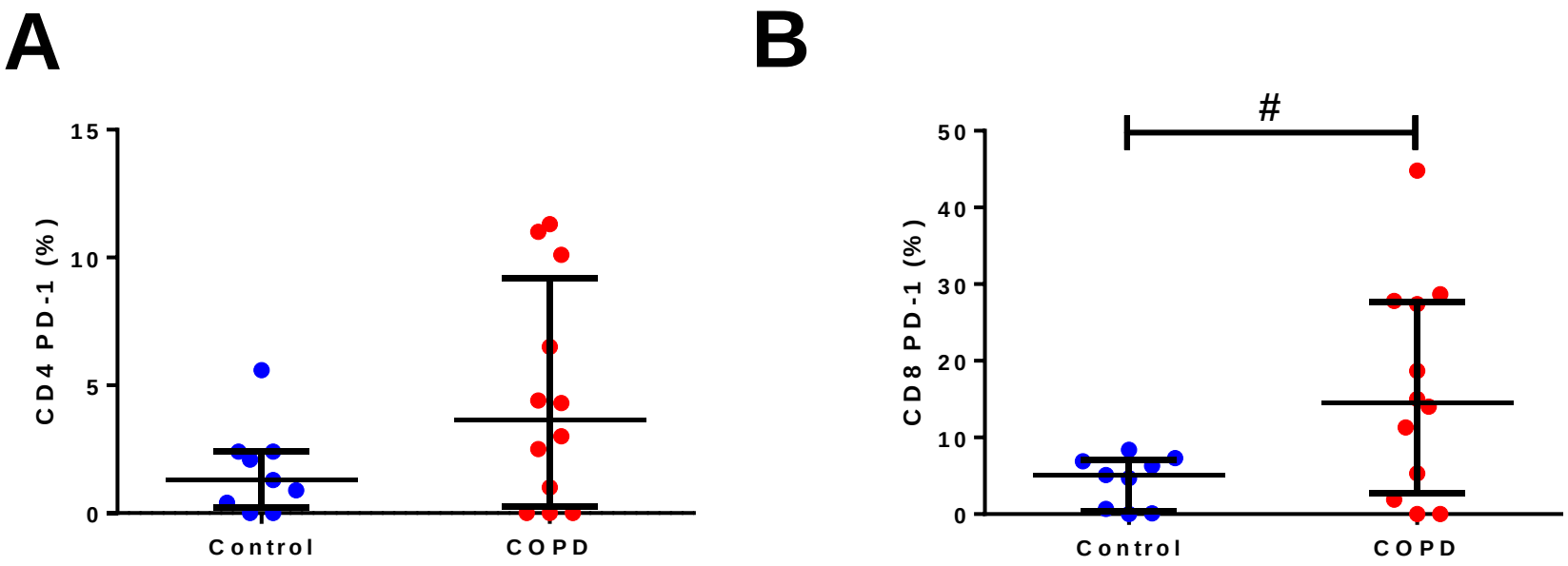
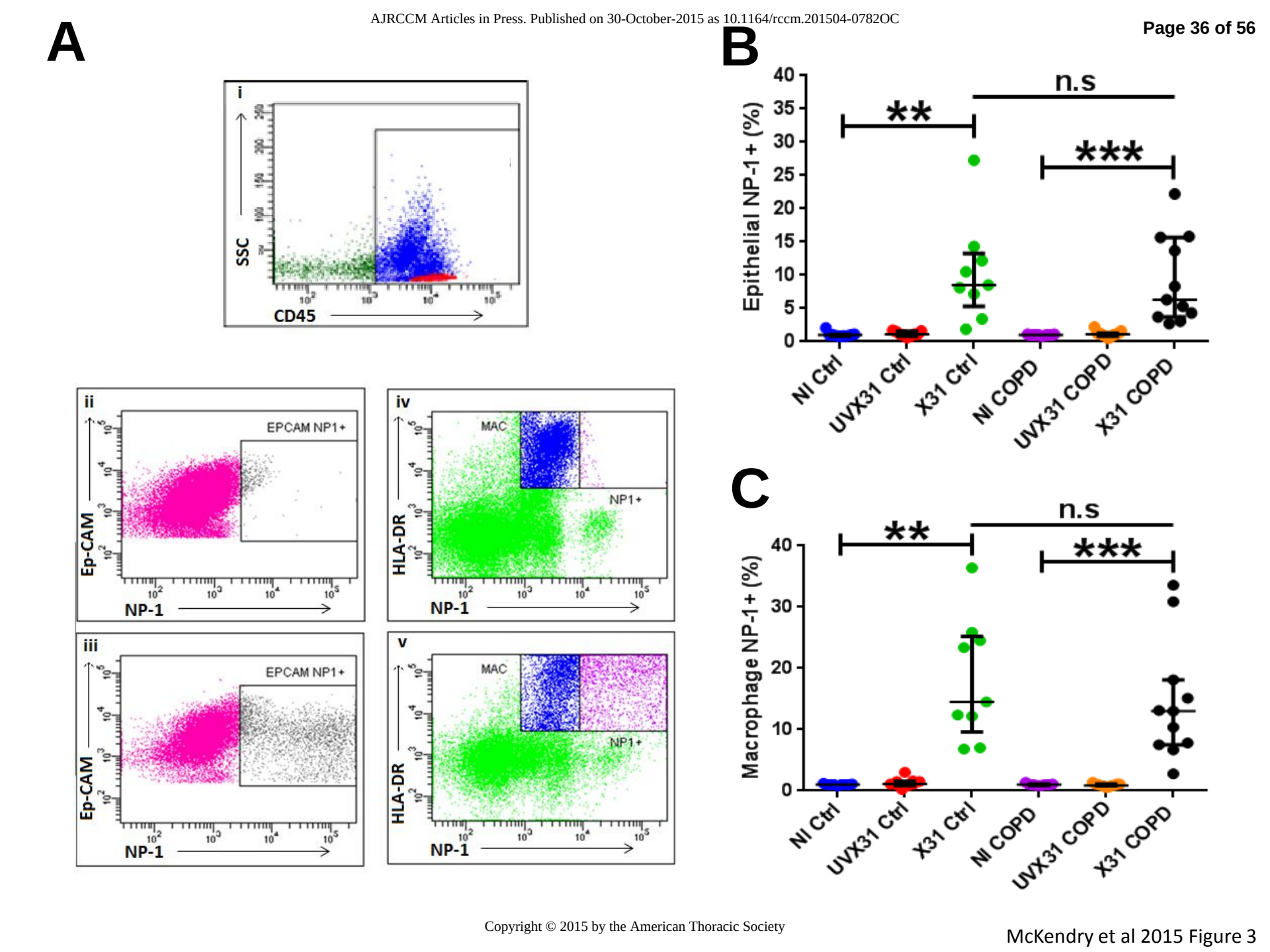


McKendry et al 2015 Figure 2



McKendry et al 2015 Figure 3

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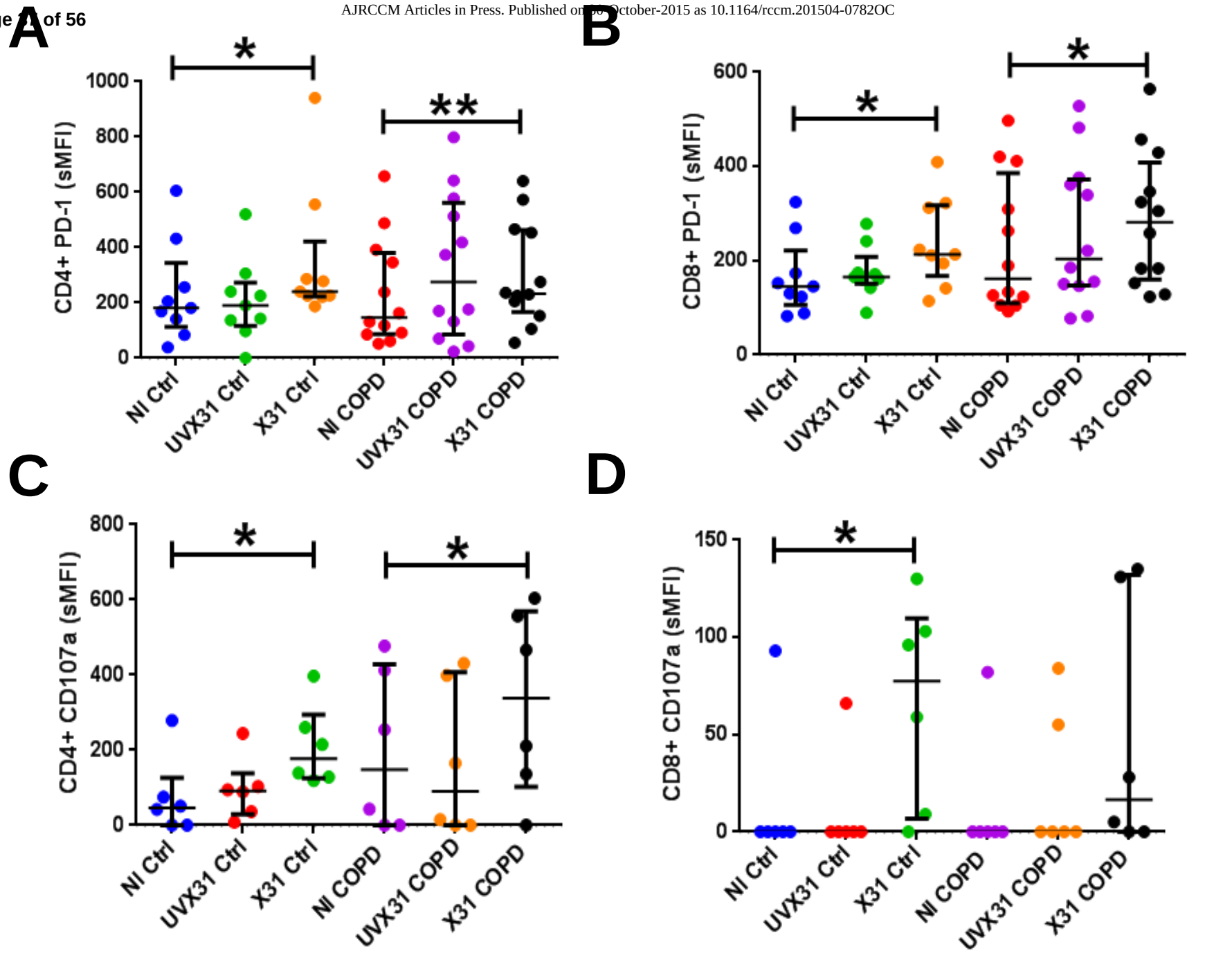
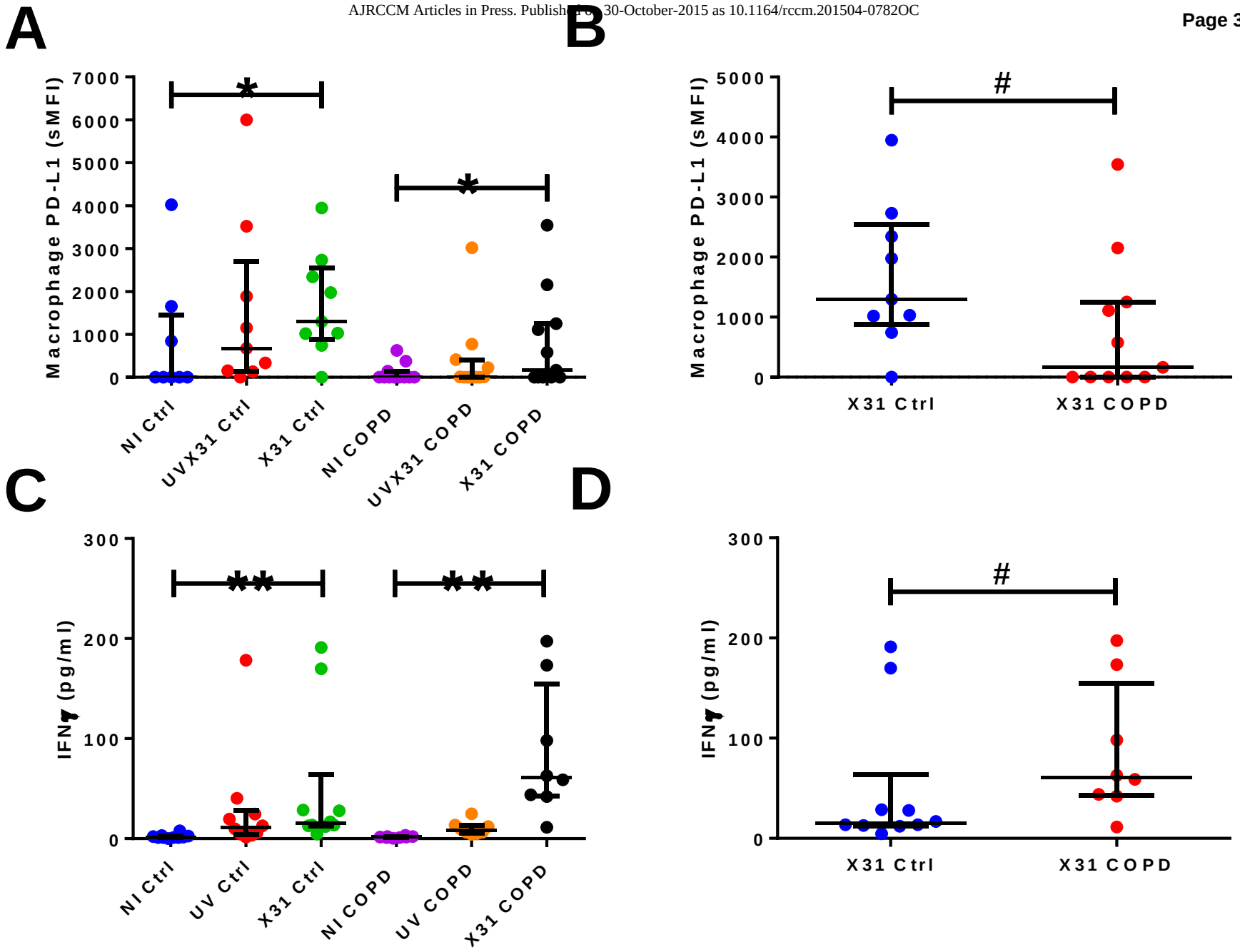


McKendry et al 2015 Figure 4



McKendry et al 2015 Figure 5

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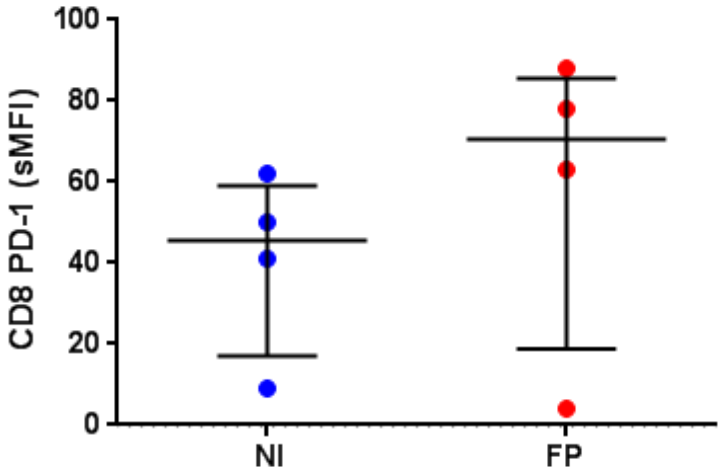

McKendry et al 2015 Figure 6

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Online supplement for:

Dysregulation of anti-viral function of CD8+T cells in the COPD lung: role of the PD1/PDL1 axis

Richard T. McKendry, C. Mirella Spalluto, Hannah Burke, Ben Nicholas, Doriana Cellura, Aymen Al-Shamkhani, Karl J. Staples, Tom M A Wilkinson.

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Methods

Ex vivo infection of resected human lung tissue

Lung tissue was obtained from patients undergoing airway re-sectioning surgery at Southampton General Hospital. The collection of tissues was approved by and performed in accordance with the ethical standards of the Southampton and South West Hampshire Research Ethics Committee, LREC no: 09/H0504/109. Parenchymal tissue, distant from the resection margin and any gross pathology was dissected from the lobe. Tissue was cut into $1mm^3$ sections and added to a 24-well flat-bottomed culture plate before washing with DPBS (Sigma, Poole, UK). Washing of the tissue was performed by removing DPBS from the wells and replacing it with fresh DPBS, followed by unsupplemented RPMI and finally RPMI supplemented with 1% penicillin/streptomycin ((both Life Technologies, Paisley, UK) and 1% gentamycin (GE Healthcare, Little Chalfont, UK). Tissue was then incubated overnight at 37ºC and 5% CO_2.

Tissue was infected and analysed according to according to the method described by Staples et al(1). After resting overnight the media was replaced with serum-free RPMI supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 250 ng/ml fungizone. Influenza A virus strain X31 was supplied at a concentration of 4×10^7 pfu/ml (a kind gift of 3VBiosciences). Inactivated virus (UVX31) was prepared by exposure of the X31 to an ultra-violet (UV) light source for 2 h. X31 or UVX31 at a concentration of 1×10^6 pfu/ml was added to designated wells, with control non-infected wells. Tissue was incubated at 37ºC and 5% CO_2 for 2 h to allow for infection of cells residing in the tissue. Supernatant was removed and tissue was washed three times with unsupplemented RPMI in order to remove excess virus from the wells. Serum-free RPMI supplemented with 100 U/ml penicillin,

100 µg/ml streptomycin, 2 mM L-glutamine and 250 ng/ml fungizone was added to the wells and tissue was incubated at 37ºC and 5% CO_2 for a further 22 h.

Protocol used to digest tissue was adapted from Holt *et al* (2). Briefly, Tissue was added to a solution of pre-warmed unsupplemented RPMI and 0.5 mg/ml collagenase type I (Sigma) for digestion. A magnetic stirrer was added to the solution in order to mechanically disaggregate tissue. Collagenase digestion occurred at 37ºC for 15 min. This tissue digestion protocol was optimised using blood-derived cells to ensure there was no effect on the major T cell markers expressed on naive blood cells. After digestion the solution was filtered through a 35 µm pore straining cap into 5 ml round-bottomed polypropylene FACS tubes (BD Biosciences) in preparation for FACS analysis.

Monocyte Isolation & differentiation

Human peripheral blood mononuclear cells (PBMC) were isolated from heparinised blood by centrifugation on Ficoll-Paque® (GE Healthcare, Little Chalfont, UK). Monocytes were then isolated from the PBMC using CD14+ microbeads (Miltenyi-Biotec, Bisley, UK) according to the manufacturer's instructions. Isolated monocytes were resuspended in complete RPMI supplemented with 2 ng/ml GM-CSF (R&D Systems, Abingdon, UK). MDM were then washed extensively with basal RPMI before addition of virus RS-RPMI. Collection of samples for this part of the study was approved by the Southampton and South West Hampshire Research Ethics Committee (reference: 08/H0504/138). CD8+ T cells were isolated from the monocyte-depleted PBMC that resulted from this separation step using CD8+ microbeads (Miltenyi-Biotec) before freezing at -80°C in 10% (v/v) DMSO/HI-FBS for use in later ELISpot analysis.

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59

60 **Infection of lung macrophages and MDM**

61 Influenza A virus strain X31 was supplied at a concentration of 4 x 10^7 pfu/ml (a kind
62 gift of 3VBiosciences). Inactivated virus (UVX31) was prepared by exposure of the
63 X31 to an ultra-violet (UV) light source for 2 h. Macrophages were incubated for 2 h
64 with no virus, or 4000 pfu (lung) or 500 pfu (MDM) of X31 or UVX31. Supernatants
65 were harvested (T-2), the cells washed three times, the final wash was harvested
66 (T0) and fresh RS media was added to the MDMs. Cells were then washed and
67 incubated for a further 22 h at 37°C, 5% CO_2. After a further 22 h, supernatants were
68 harvested (T22) for cytokine analysis and cells collected and immediately analysed
69 by flow cytometry. For phenotypic characterisation of influenza infected
70 macrophages, cells were removed from culture plates using a non-enzymatic cell
71 dissociation solution (Sigma).

72

73 **Flow cytometry analysis**

74 Samples were resuspended in FACS buffer (PBS, 0.5% w/v BSA, 2 mM EDTA)
75 containing 200 µg/ml human IgG (Sigma).

76 Lung explants:

77 Single cell suspensions derived from collagenase digestion were incubated on ice in
78 the dark for 30 min with the following antibodies: Phycoerythrin-CF594 (PE-CF-594)-
79 conjugated anti-CD45, Peridinin-Chlorophyll Protein-Cyanine 5.5 (PerCPCy-5.5)-
80 conjugated anti-EpCAM-1 (CD326), and Allophycocyanin-Cyanine 7 (APC-Cy7)-
81 conjugated anti-HLA-DR, Phycoerythrin (PE)-conjugated anti-PD-L1, PE-Cy7
82 conjugated anti-CD3, PerCPCy5.5-conjugated anti-CD4 and APC-Cy7-conjugated
83 anti-CD8 (All BD Biosciences, Oxford, UK). Appropriate isotype and fluorescence-

matched control antibodies were added in a sample of the cells to aid gating of cell populations. After washing, intracellular staining for viral nucleoprotein (NP)-1, was performed using BD Cytofix/Cytoperm kit according to manufacturer's instructions, and AlexaFluor 488 (AF488)-conjugated anti-NP1 antibody (HB-65, a kind gift of 3VBiosciences). The gating strategy is shown in Figure 1A.

MDMs & blood T cells:

Isolated macrophages were incubated on ice in the dark for 30 min with the following antibodies: PE-conjugated anti-PD-L1, Allophycocyanin (BD Biosciences or appropriate isotype controls. After washing, intracellular staining for viral nucleoprotein (NP)-1, was performed using BD Cytofix/Cytoperm kit according to manufacturer's instructions, and AlexaFluor 488 (AF488)-conjugated anti-NP1 antibody (HB-65, a kind gift of 3VBiosciences).

Flow cytometric analysis was performed on a FACSAria using FACSDiva software v5.0.3 (all BD).

ELISpot

Monocyte-depleted PBMC were defrosted at room temperature and suspended in RS RPMI. MDMs were removed from 24-well culture plate using non-enzymatic cell dissociation solution (Sigma) and transferred to sterile 1.5 ml Eppendorf tubes. Cells were centrifuged at 400 g, 4ºC, 5 min before resuspension in serum-free (SF) RPMI containing L-glutamine, penicillin/streptomycin, and fungizone. MDMs were either not infected, or were treated with 2.5 x 10^4 pfu/ml X31 Influenza A H3N2 virus at 37ºC for 2 h before washing and resuspending in RS RPMI.

ELISpot for Human IFN-γ was then performed using 0.45 µm MultiScreen-IP Filter Plates (Millipore, Watford, UK) following manufacturer's instructions (MabTech,

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Stockholm, Sweden). Briefly, coating antibody (1-DIK) was diluted to 15 µg/ml in sterile DPBS and was added to the plate before overnight incubation at 4ºC. Plates were then washed five times with sterile DPBS before replacement with SF RPMI for 30 min at RT. The SF-media was then removed and MDM were added to each well at a concentration of 5 x 10^4 cells/well and 2.5 x 10^5 monocyte-depleted PBMC or 1 x 10^5 CD8+ T cells were added to MDM-containing wells and incubated at 37ºC. After 22 h, the plate was washed five times with sterile DPBS + 0.05% Tween20 (Sigma). Detection antibody (7-B6-biotin) was diluted to 1 µg/ml in sterile DPBS + 0.5% FBS and was added to the plate which was then incubated at RT. After 2 h, the plate was washed five times with sterile DPBS before addition of Streptavidin-ALP (diluted 1:1000 in sterile DPBS+0.5% FCS) and incubation for 1 h at RT. Plates were then washed with sterile DPBS before replacement with substrate solution (BCIP/NBT diluted 1:1:8 in sterile H_2O). Plate was incubated at RT for 2-5m until clear spots were visible. At this point wells were washed five times with dH_2O and allowed to dry at RT. Spot development was analysed using an AID EliSpot Reader (Germany) and AID EliSpot Software (Germany).

In initial experiments, no IFNγ staining was seen in wells containing infected MDM or lymphocytes alone (1). Peripheral blood T cells do not appear to be infected when exposed to X31 (1).

RNA Isolation & RT-PCR

RNA was extracted from T cells using a Stratagene Microprep Kit (Agilent Technologies, Stockport, UK). Reverse transcription was carried out using a High Capacity cDNA Reverse Transcription Kit (Life Technologies) with random hexamers carried out according to the manufacturer's protocols. *TIM3* gene expression was

analysed using TaqMan universal PCR master mix, No AmpErase® UNG in a 7900HT fast real-time PCR system machine (all Life Technologies). Gene expression was normalized to β_2-microglobulin gene expression and quantified using the $\Delta\Delta$ C_T method.

Supernatant analyses

IFNβ concentrations in culture supernatants were measured by ELISA according to the manufacturer's instructions (MSD, Gaithersberg, USA). Culture supernatants were analysed by Luminex assay for IFNγ as per manufacturer's instructions (Bio-Rad, Hemel Hempstead, UK).

Statistics

Analysis of two groups was performed using Wilcoxon's signed rank test for paired data and a Mann-Whitney U test for unpaired data. Chi-squared test and Fishers exact test were used for categorical data (GraphPad Prism v6, GraphPad Software Inc., San Diego, USA). Results were considered significant if $p<0.05$.

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Results

	Control	COPD	p Value
N	20	24	-
Age (years)	68 (60.25 – 74.75)	67 (59.75 - 74)	0.89#
Gender M/F	9 / 11	12 / 12	0.741†
BMI	28 (24.55 – 32.1)	23.85 (21.45 – 27.7)	0.08#
Confirmed corticosteroid use	1	7	0.0544‡
Smoker (Never/Ex/Current)	5 / 11 / 4	1 / 16 / 7	0.13‡
Pack Years	20 (0.9 – 55.25)	40 (29 – 56.25)	0.11#
FEV_1%	95 (85.45 – 112.2)	76.1 (64.56 – 88.88)	< 0.001#
FEV_1/FVC ratio	0.752 (0.727 – 0.8074)	0.612 (0.524 – 0.668)	< 0.0001#

Supplementary Table 1: Clinical characteristics of surgical patients shown in Figure 1 B+C. Data are presented as median and IQR shown. Ex-smokers were defined as individuals who had stopped smoking for > 6 months prior to surgery. BMI data shown represents 13 Control and 16 COPD patients. #Mann Whitney U Test, †Chi-squared test, ‡ Fishers Exact test

	Control	COPD	p Value
N	10	14	-
Age (years)	70.5 (58.75 – 76.5)	67 (61.25 – 72.25)	0.627#
Gender M/F	4 / 6	6 / 8	1.00†
BMI	22.4 (21.7 – 28.9)	28.3 (22.6 – 36.4)	0.9#
Confirmed corticosteroid use	1	4	0.3577‡
Smoker (Never/Ex/Current)	2 / 7 / 1	0 / 8 / 6	0.065#
Pack Years	40 (1.5 – 56)	40 (34.38 – 66.25)	0.35#
FEV_1%	98 (93 – 114)	73 (62 – 78.5)	< 0.001#
FEV_1/FVC ratio	0.747 (0.747 – 0.797)	0.605 (0.523 – 0.642)	< 0.0001#

Supplementary Table 2: Clinical characteristics of surgical patients shown in Figures 2-5. Data are presented as Median and IQR shown. Ex-smokers were defined as individuals who had stopped smoking for > 6 months prior to surgery. BMI data shown represents 3 Control and 5 COPD patients. #Mann Whitney U Test, †Chi-squared test, ‡ Fishers Exact test

Control (24)	COPD (33)
24 Lung cancer	33 Lung cancer
2 Hypertension	5 Hypertension
2 Hypercholesterolaemia	4 Hypercholesterolaemia
2 Arthritis	4 Arthritis
1 Hypothyroidism	3 Hypothyroidism
2 Hysterectomy	3 Hysterectomy
1 Hip replacement	2 Hip replacement
2 Appendectomy	1 Appendectomy
1 Abdominal aortic aneurysm	1 Abdominal aortic aneurysm
1 Varicose veins	1 Varicose veins
1 Irritable Bowel Syndrome	1 Irritable Bowel Syndrome
1 Throat polyps	1 Throat polyps
1 Skin cancer	1 Skin cancer
2 Type 2 Diabetes	1 Type 1 Diabetes

Supplementary Table 3: Common comorbidities of surgical patients. Number indicates incidence in each group. Numbers do not sum as each patient can have more than one co-morbidity.

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Supplementary Figure Legends

Supplementary Figure E1. CD4+ and CD8+ T cell population in human lung parenchymal tissue, Unstained singlet population was obtained from digested tissue. Dead cells were excluded using LIVE/DEAD® Fixable Aqua Dead Cell Stain. Live singlet CD45+ population was then identified and from this a CD45+CD3+ T cell population. The CD45+CD3+ T cell population was divided into CD4+ and CD8+ T cells. **(A)** Control samples **(B)** and COPD samples displaying their paired T cell populations. Control n = 20, COPD n = 24.

Supplementary Figure E2. Flow cytometry gating strategy for memory CD4+ and CD8+ T cells. Naïve and Tmem cell populations in blood and tissue. PBMCs were isolated from blood using Ficoll-Paque density centrifugation. Tissue was prepared as previously described and digested in 0.5 mg/ml collagenase solution for 15 min. **(A+C)** T cell populations were gated on a singlet CD3+CD4+CD8- population or **(B+D)** a singlet CD3+CD4-CD8+ population. **(A)** CD4+ memory populations and **(B)** CD8+ populations in blood. **(C)** CD4+ memory populations and **(D)** CD8+ populations in lung parenchymal tissue. Flow cytometry dot plots representative of at least 5 independent experiments.

Supplementary Figure E3. RT-PCR gene expression of TIM-3 by T cells isolated from lung tissue. T cells were gated on the live CD45+CD3+ population. **(A)** RT-PCR was performed with 2.5 x 10^4 CD4+ **(B)** or CD8+ T cells sorted from control or COPD lung parenchyma. ΔΔCt value calculated using B2M housekeeping gene expression. n = 6

Supplementary Figure E4. Proportion of CD4+ and CD8+ T cells which express PD-1 in response to tissue infection by X31 influenza. (A) Proportion of CD4+ **(B)** and CD8+ T cells which expressed PD-1 was quantified by flow cytometry in non-infected (NI), live X31 virus infection (X31) and UV-irradiated virus (UVX31) lung samples. **(C)** The fold increase in PD-1 expression (sMFI) of CD4+ **(D)** and CD8+ T cells between NI and X31-infected samples and NI and UVX31 (UV)- treated samples from COPD patients (n=12). **(E)** The fold increase in PD-1 expression (sMFI) of CD4+ **(F)** and CD8+ T cells between NI and X31 samples was also calculated for Control samples (n = 9) and COPD samples (n = 12). Median and IQR shown, Data analysed using a Wilcoxon-signed rank test * $p<0.05$, ** $p<0.01$.

Supplementary Figure E5. PD-L1 expression by epithelial cells during X31 infection of lung parenchyma. $1cm^3$ sections of tissue were treated with UVX31, X31 or NI for 2 h before a 22 h incubation. Proportions of epithelial cells expressing PD-1 was quantified by flow cytometry. Control n = 9, COPD n = 11. Median and IQR shown.

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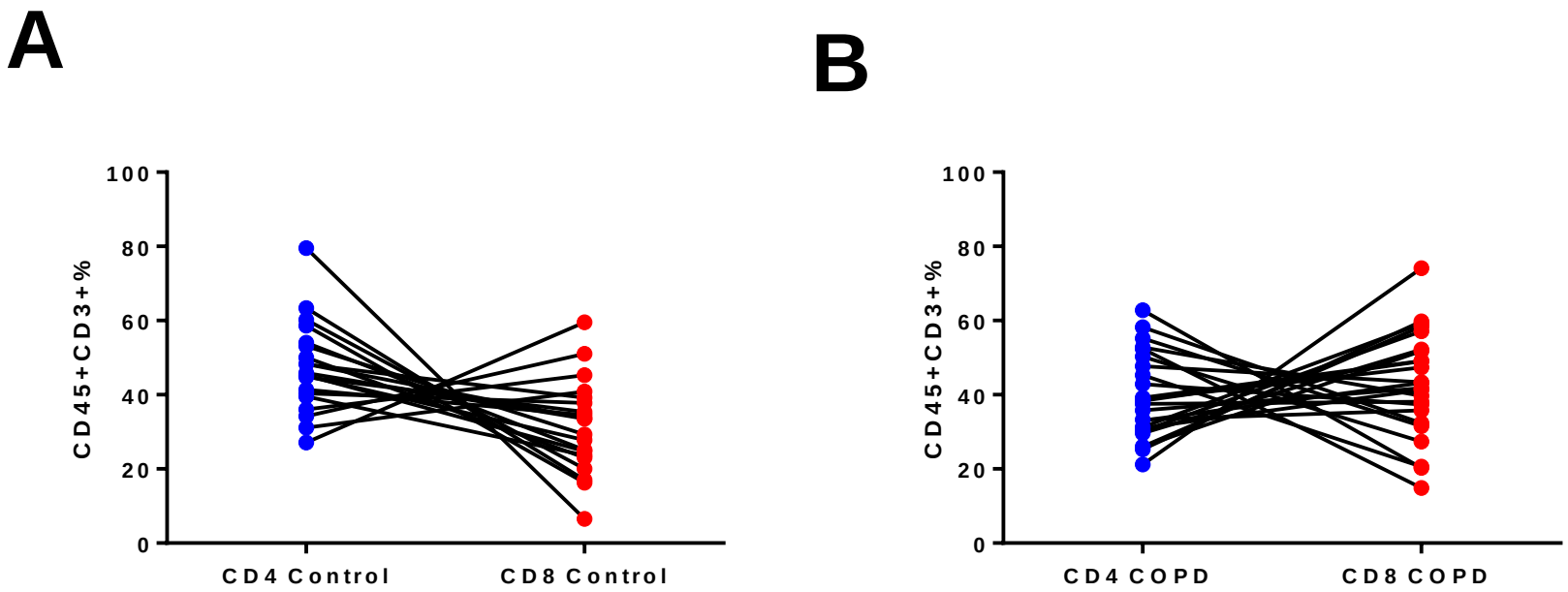

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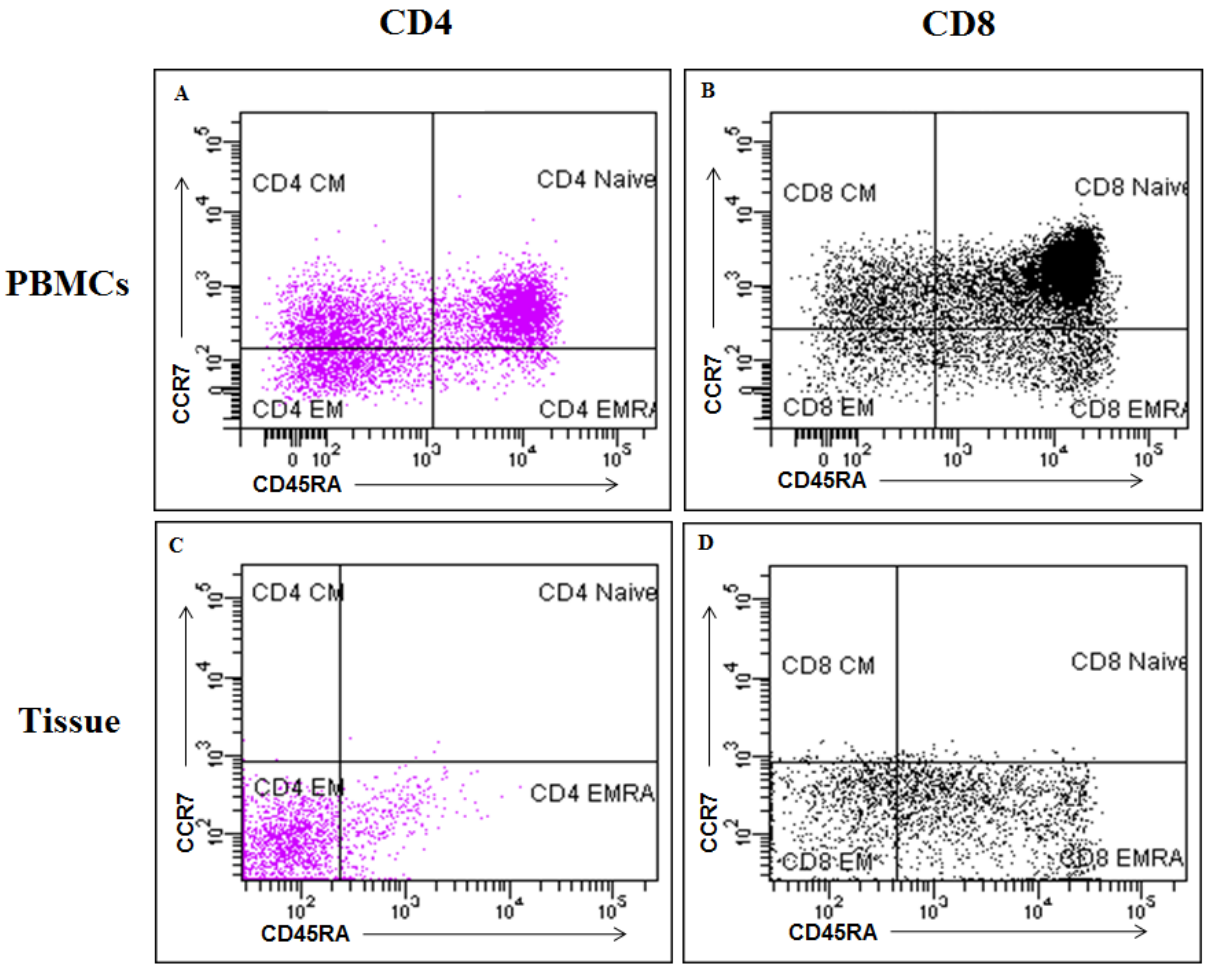

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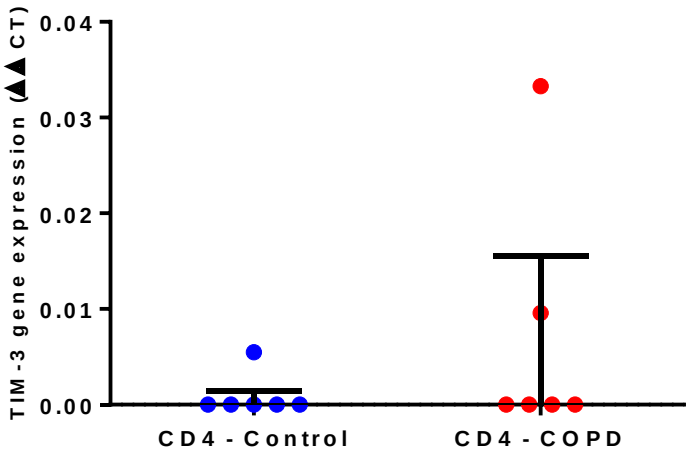

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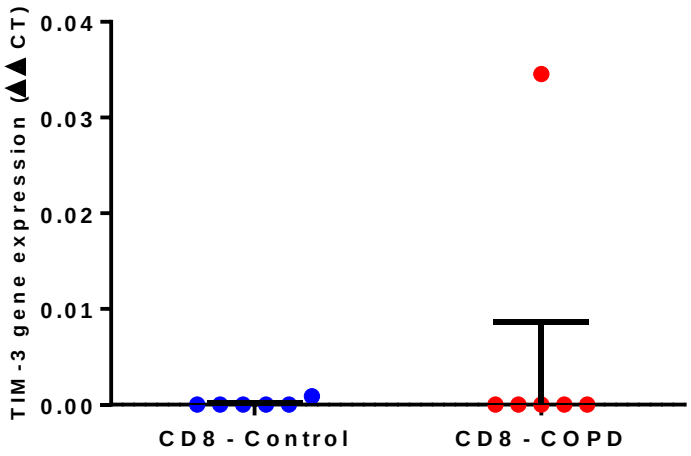

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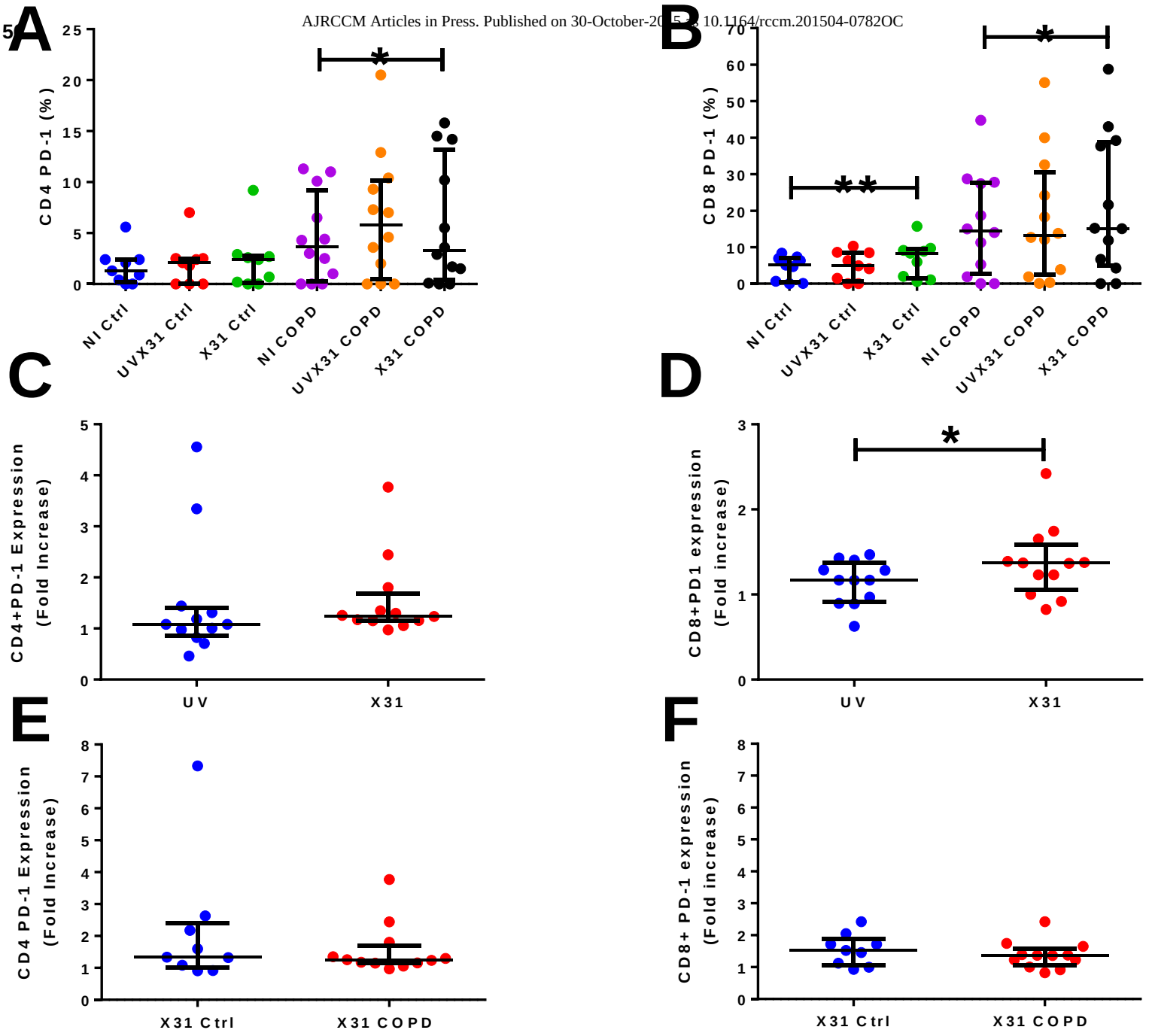

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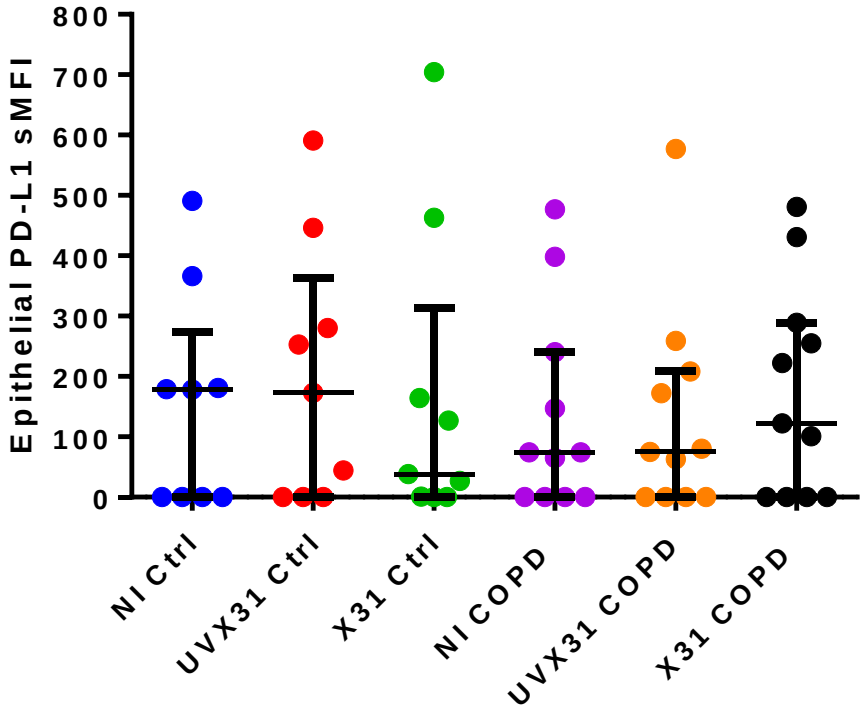

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