Inhaled corticosteroids reduce senescence in endothelial progenitor cells from COPD patients

ONLINE DATA SUPPLEMENT

Material and Methods

Participants

Blood samples (15–48 mL) were collected from healthy non-smoking volunteers, smokers with normal lung function (forced vital capacity in 1 second (FEV₁) >80% predicted, FEV₁/forced vital capacity (FVC) >0.7) and COPD patients (FEV₁<80% predicted, FEV₁/FVC <0.7). All individuals aged 38 to 80 years, and were free from significant cardiac, renal, haematological, or other major disorders as determined by medical history, physical examination and screening investigations. All COPD patients were current or ex-smokers and were classified according to the Global initiative for chronic Obstructive Lung Disease (GOLD) criteria for severity of disease. All volunteers were stable (no chest or other infection requiring antibiotics and/or oral steroids) for at least 4 weeks. The study was approved by the Royal Marsden, Hammersmith and Queen Charlotte’s and South East Scotland Ethics Committees, and informed consent was obtained from all individuals.

Isolation and Culture of ECFC from Peripheral Blood

Peripheral blood mononuclear cells were isolated from blood samples and seeded at a density of 3–5 × 10⁷ cells per well, in complete endothelial growth medium (EGM)-2 (Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Thermo Scientific, Fisher Scientific Ltd., Loughborough, U.K.), onto six-well plates precoated with type I rat tail collagen (BD Biosciences, Bedford, MA), as previously described¹. After 24 hours, nonadherent cells and debris were aspirated, adherent cells were washed once with EGM-2 medium, and fresh EGM-2 was added to each well. Medium was changed daily for 7 days and then every 2 days. Colonies of ECFC appeared between 7 and 22 days in culture as discrete colonies of cells with cobblestone morphology and were enumerated by visual inspection using a ×4 objective lens (EVOS™ XL Core Imaging System). Endothelial cells derived from the colonies were passaged for 2–3 weeks after appearance and grown to confluence. All experiments were performed with ECFC between passage 3 and 5. There were no differences in the isolation success rate between the groups as described in supplemental material of reference.¹

Stress Induced Premature Senescence by Oxidative Stress

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We used a previously published method for inducing premature senescence by H$_2$O$_2$ (ref Stem Cells). Commercially available human umbilical vein endothelial cells (HUVEC) from pooled donors were used for our experiments (Lonza). HUVEC $1 \times 10^5$ were seeded in six-well plates and grown to 80% confluence in M199 medium (Sigma-Aldrich Company Ltd., Dorset, U.K.) plus 10% FBS. Following 1 hour pre-treatment with budesonide or control vehicle (DMSO), cells were washed twice with PBS and treated for 1.5 hours with 50 μmol/L of H$_2$O$_2$ (Sigma-Aldrich Company Ltd.). Cells were washed twice with PBS and cultured in M199 plus 10% FBS medium for three additional days, in the absence or presence of increasing doses of budesonide (Sigma-Aldrich Company Ltd) between the range $10^{-6}$M and $10^{-10}$M.

**Immunofluorescence**

Isolated ECFC or HUVEC were stained as previously described$^1$ with antibodies to vascular endothelial (VE)-cadherin (CD144) (AF938, Bio-Techne Ltd, Abingdon, Oxfordshire, U.K.), p16 and p21 (sc-65224 and sc-817 Santa Cruz Biotechnology, Insight Biotechnology Ltd., Wembley, U.K.), 53 binding protein 1 (53BP1) (4937, Cell Signaling Technology, New England Biolabs, Hertfordshire, U.K.), γ-H2AX (05-636, Millipore) and IP-10 (MA5-32674, Invitrogen Ltd). Secondary antibodies were anti-mouse AlexaFluor 647, anti-rabbit AlexaFluor 488, and anti-goat Alexa Fluor 555 (Invitrogen Ltd). Nuclei were visualized using deep red anthraquinone 5 (DRAQ5) (Biostatus Limited) or 4-6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific).

**Senescence-Associated β-Galactosidase Staining**

Senescence-associated β-galactosidase (SA-β-Gal) activity was measured with a β-Galactosidase staining kit (Senescence Detection Kit, BioVision Research Products, Mountain View, CA) following the manufacturer’s protocol. The number of blue (senescent) cells relative to the total cell number was counted in two to four different optic fields, using ×10 or ×20 objective lens. At least 200 cells were counted per sample.

**Caspase-Glo 3/7 Assay**

Apoptosis was quantified by measuring caspase 3 and 7 activation, using Caspase-Glo 3/7 Assay (Promega, Southampton, United Kingdom) on a Bio-Tek Synergy HT multidetection microplate reader, following the manufacturer’s protocol.

**Western Blotting**

Western blotting was carried out as described$^1$. The following antibodies were used: p21 Waf1/Cip1 (Cell Signaling Technology, 2947), γ-H2AX (ser139) (Cell Signaling Technology, 9718), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Millipore, Watford, U.K.), α-
tubulin (Sigma-Aldrich Company Ltd.). Quantification of protein levels was performed by
densitometry and normalized against GAPDH or α-tubulin.

Real-Time Polymerase Chain Reaction

RNA was extracted from ECFC or HUVEC using the RNeasy kit (Qiagen), according to the
manufacturer’s instructions. After reverse transcription (QuantaBio qSCRIPT cDNA Supermix,
VWR Cat No. 733-1177), p21 mRNA levels were measured by quantitative real-time
polymerase chain reaction (RT-PCR) using 5ng cDNA per test well and SYBR Green technology
(Biorad iQ SYBR Green Supermix, Cat No. 1708882). Levels of p21 were normalized to
Ribosomal Protein L13a (RPL13A), which is an appropriate housekeeping gene for studies on
senescence for ECFC. All measurements were performed in triplicate. Primer sequences are
as follows: p21 forward: 5’-GCAGACCACGATGACAGATTT-3’, reverse: 5’-
GGATTAGGGCTTCCTTTGGA-3’; RPL13A forward: 5’-CTGGACCGTCTCAAGGTGT-3’, reverse: 5’-
GCCCAAGATTGCAAACCTT-3’).

Luminex assay

Human cytokines were measured in ECFC supernatant (5 non-smokers; 8 COPD patients, 3
no-ICS and 5 on ICS). 100,000 cells were seeded in 6 well-plates in 1.3 ml of normal medium
(EGM2, baseline conditions). Supernatant was collected after 48 hours.

Thirty-two human cytokines were assayed using the Luminex MAGPIX Analyzer (Austin, TX,
U.S.A.) as previously described. The mean fluorescent intensity was analysed using a five-
parameter logistic method on XLfit software v.5.3.1.3 (Guildford, Surrey, U.K.). Twenty-two
analytes were detected (please see table below: undetected analytes are displayed in grey).

| GM-CSF | IL-17A | IL-6 | RANTES |
|-------|-------|-----|--------|
| G-CSF | IL-1α | IL-7 | TNF-α |
| IFN-α-2 | IL-1α | IL-8 | TNF-β |
| IFN-γ | IL-1β | IP-10 | Eotaxin |
| IL-10 | IL-2 | MCP-1 | VEGF |
| IL-12p40 | IL-3 | MCP-3 | EGF |
| IL-12p70 | IL-4 | MIP-1α | Fractalkine |
| IL-13 | IL-5 | MIP-1β | GRO |
**Imaging and Image analysis**

At least 5 representative image stacks for quantification were captured for each sample/individual on a Zeiss LSM-780 inverted confocal laser scanning microscope using either a ×40, or ×63 oil objective. The images were analysed using FIJI image analysis software and macros were developed to quantify either the DNA damage foci per nucleus, using the DAPI or DRAQ5 fluorescent signal to create masks, or to measure the nuclear and cytoplasmic intensities for each sample.

**Statistical Analysis**

Data are expressed as mean ± SEM or ± SD as described. Statistical analysis was performed with GraphPad-Prism 9. Comparisons were performed with Mann-Whitney U test, Kruskal Wallis test followed by Dunn’s post-hoc analysis (for unpaired samples) or Friedman’s test followed by Dunn’s post-hoc analysis (for paired samples - experiments on HUVEC). The correlation of values was estimated with the Pearson $r$ correlation coefficient. Significance was defined as $p<0.05$.

**References**

We would like to include the following references some of which we were unable to include in the main manuscript due to number limitations

1. Paschalaki KE, Starke RD, Hu Y, et al. Dysfunction of endothelial progenitor cells from smokers and chronic obstructive pulmonary disease patients due to increased DNA damage and senescence. *Stem cells (Dayton, Ohio)* 2013;31(12):2813-26. doi: 10.1002/stem.1488 [published Online First: 2013/07/31]

2. McLoughlin KJ, Pedrini E, MacMahon M, et al. Selection of a Real-Time PCR Housekeeping Gene Panel in Human Endothelial Colony Forming Cells for Cellular Senescence Studies. *Frontiers in medicine* 2019;6:33. doi: 10.3389/fmed.2019.00033 [published Online First: 2019/03/28]

3. Rossios C, Pavlidis S, Gibeon D, et al. Impaired innate immune gene profiling in airway smooth muscle cells from chronic cough patients. *Bioscience reports* 2017;37(6) doi: 10.1042/bsr20171090 [published Online First: 2017/08/27]

**References for the effect of glucocorticoids on vascular function**

4. Goodwin JE, Feng Y, Velazquez H, et al. Endothelial glucocorticoid receptor is required for protection against sepsis. *Proceedings of the National Academy of Sciences of the United States of America* 2013;110(1):306-11. doi: 10.1073/pnas.1210200110 [published Online First: 2012/12/19]

5. Goodwin JE, Zhang X, Rotllan N, et al. Endothelial glucocorticoid receptor suppresses atherogenesis- brief report. *Arteriosclerosis, thrombosis, and vascular biology* 2015;35(4):779-82. doi: 10.1161/atvbaha.114.304525 [published Online First: 2015/03/27]
6. Zhou H, Mehta S, Srivastava SP, et al. Endothelial cell-glucocorticoid receptor interactions and regulation of Wnt signaling. JCI insight 2020;5(3) doi: 10.1172/jci.insight.131384 [published Online First: 2020/02/14]

7. Zielińska KA, Van Moortel L, Opdenakker G, et al. Endothelial Response to Glucocorticoids in Inflammatory Diseases. Frontiers in immunology 2016;7:592. doi: 10.3389/fimmu.2016.00592 [published Online First: 2016/12/27]

8. Cruz-Topete D, Oakley RH, Cidlowski JA. Glucocorticoid Signaling and the Aging Heart. Frontiers in endocrinology 2020;11:347. doi: 10.3389/fendo.2020.00347 [published Online First: 2020/06/13]

Reference on the therapeutic doses of budesonide

9. Miller-Larsson A, Jansson P, Runström A, et al. Prolonged airway activity and improved selectivity of budesonide possibly due to esterification. American journal of respiratory and critical care medicine 2000;162(4 Pt 1):1455-61. doi: 10.1164/ajrccm.162.4.9806112 [published Online First: 2000/10/13]

References on IP-10 and cardiovascular disease

10. George PM, Oliver E, Dorfmuller P, et al. Evidence for the involvement of type I interferon in pulmonary arterial hypertension. Circulation research 2014;114(4):677-88. doi: 10.1161/circresaha.114.302221 [published Online First: 2013/12/18]

11. Heller EA, Liu E, Tager AM, et al. Chemokine CXCL10 promotes atherogenesis by modulating the local balance of effector and regulatory T cells. Circulation 2006;113(19):2301-12. doi: 10.1161/circulationaha.105.605121 [published Online First: 2006/05/10]

12. Ide N, Hirase T, Nishimoto-Hazuku A, et al. Angiotensin II increases expression of IP-10 and the renin-angiotensin system in endothelial cells. Hypertension research : official journal of the Japanese Society of Hypertension 2008;31(6):1257-67. doi: 10.1291/hypres.31.1257 [published Online First: 2008/08/22]

References on the use of corticosteroids and inhaled corticosteroids in COVID-19

13. Ramakrishnan S, Nicolau DV, Jr., Langford B, et al. Inhaled budesonide in the treatment of early COVID-19 (STOIC): a phase 2, open-label, randomised controlled trial. The Lancet Respiratory medicine 2021;9(7):763-72. doi: 10.1016/s2213-2600(21)00160-0 [published Online First: 2021/04/13]

14. Yu LM, Bafadhel M, Dorward J, et al. Inhaled budesonide for COVID-19 in people at high risk of complications in the community in the UK (PRINCIPLE): a randomised, controlled, open-label, adaptive platform trial. Lancet (London, England) 2021;398(10303):843-55. doi: 10.1016/s0140-6736(21)01744-x [published Online First: 2021/08/14]

15. Edalatifard M, Akhtari M, Salehi M, et al. Intravenous methylprednisolone pulse as a treatment for hospitalised severe COVID-19 patients: results from a randomised controlled clinical trial. The European respiratory journal 2020;56(6) doi: 10.1183/13993003.02808-2020 [published Online First: 2020/09/19]

16. Horby P, Lim WS, Emberson JR, et al. Dexamethasone in Hospitalized Patients with Covid-19. The New England journal of medicine 2021;384(8):693-704. doi: 10.1056/NEJMoa2021436 [published Online First: 2020/07/18]