

1         **Title: Hypoxia increases neutrophil-driven matrix destruction after**  
2                         **exposure to *Mycobacterium tuberculosis***

3  
4     **Authors:** Catherine WM Ong <sup>1,2</sup>, Katharine Fox<sup>1</sup> , Anna Ettorre<sup>1</sup>, Paul T Elkington<sup>1,3</sup> & \*Jon S  
5     Friedland<sup>1</sup>

6     **Affiliations:**

7     <sup>1</sup> Infectious Diseases and Immunity, Hammersmith Campus, Imperial College London, London,  
8     UK

9     <sup>2</sup> Division of Infectious Diseases, Department of Medicine, Yong Loo Lin School of Medicine,  
10    National University of Singapore, Singapore

11    <sup>3</sup> NIHR Biomedical Research Centre, Faculty of Medicine, University of Southampton,  
12    Southampton, UK.

13

14    **Corresponding author:**

15    Jon S Friedland

16    Section of Infectious Diseases and Immunity

17    8<sup>th</sup> floor Commonwealth Building

18    Du Cane Road, London W12 0NN

19    United Kingdom

20    E-mail: [j.friedland@imperial.ac.uk](mailto:j.friedland@imperial.ac.uk)

21    Tel: 44 (0)20 8383 8521

22    Fax: 44 (0)20 8383 3394

- 1 **Key words:** Tuberculosis, hypoxia, neutrophils, matrix metalloproteinase.
- 2 Word count: 3 379
- 3 Abstract: 194 out of 200 words
- 4 Figures: 7
- 5 Supplementary figures: 3
- 6 References: 39

# 1 ABSTRACT

2 The importance of neutrophils in the pathology of tuberculosis (TB) has been recently  
3 established. We demonstrated that TB lesions in man are hypoxic, but how neutrophils in  
4 hypoxia influence lung tissue damage is unknown. We investigated the effect of hypoxia on  
5 neutrophil-derived enzymes and tissue destruction in TB. Human neutrophils were stimulated  
6 with *M. tuberculosis* (*M.tb*) or conditioned media from *M.tb*-infected monocytes (CoMTB).  
7 Neutrophil matrix metalloproteinase-8/-9 and elastase secretion were analysed by luminex array  
8 and gelatin zymography, gene expression by qPCR and cell viability by flow cytometry. Matrix  
9 destruction was investigated by confocal microscopy and functional assays and neutrophil  
10 extracellular traps (NETs) by fluorescence assay. In hypoxia, neutrophil MMP-8 secretion and  
11 gene expression were up-regulated by CoMTB. MMP-9 activity and neutrophil elastase (NE)  
12 secretion were also increased in hypoxia. Hypoxia inhibited NET formation and both neutrophil  
13 apoptosis and necrosis after direct stimulation by *M.tb*. Hypoxia increased TB-dependent  
14 neutrophil-mediated matrix destruction of Type I collagen, gelatin and elastin, the main  
15 structural proteins of the human lung. Dimethylxalylglycin (DMOG), which stabilizes hypoxia-  
16 inducible factor-1 $\alpha$ , increased neutrophil MMP-8 and -9 secretion. Hypoxia in our cellular  
17 model of TB up-regulated pathways that increase neutrophil secretion of MMPs that are  
18 implicated in matrix destruction.

# 1 INTRODUCTION

2  
3 The clinical features of tuberculosis (TB) are increasingly recognised to be mediated by  
4 the host innate immune response<sup>1-3</sup>. Proteases such as matrix metalloproteinases (MMPs) and  
5 neutrophil elastase have been implicated in TB immune-mediated pathology<sup>1,4,5</sup>. Neutrophil  
6 derived MMP-8 is increased in TB patients and is associated with clinical disease severity<sup>6</sup>,  
7 while neutrophil elastase may have anti-mycobacterial activities<sup>7</sup>. Targeting host proteases is an  
8 attractive therapeutic option to minimize the consequence of *M. tuberculosis* (*M.tb*) infection,  
9 which commonly results in cavity formation, bronchiectasis and scarring leading to patient  
10 morbidity even with appropriate antibiotic treatment<sup>8</sup>.

11  
12 Hypoxia affects diverse aspects of the host immune response in TB. Tuberculous  
13 granulomas are hypoxic both in murine models and in humans, as we demonstrated in TB  
14 patients using (18<sup>F</sup>)-MISO PET/CT scanning<sup>4,9</sup>. In TB, hypoxia initiates the angiogenesis  
15 required for the formation of granulomas<sup>10</sup>. Inhibition of vascular endothelial growth factor  
16 limits infection burden and dissemination, and improves oxygenation to granulomas by  
17 normalizing vasculature in *M. marinum*-infected zebrafish<sup>10,11</sup>. The effect of hypoxia on the  
18 pathogen is diverse. Human  $\beta$ -defensin-2 is upregulated in hypoxia in macrophages, inhibiting  
19 intracellular mycobacterial growth<sup>12</sup>. Conversely, hypoxia increases survival fitness in *M.tb* with  
20 the microbe adapting to hypoxia<sup>13</sup>, inducing a non-replicating state or dormancy to become less  
21 susceptible to anti-mycobacterial treatment<sup>14</sup>.

1 Neutrophils, which secrete MMP-8, -9 and neutrophil elastase, are abundant in the  
2 respiratory secretions of TB patients<sup>15</sup>. Neutrophil recruitment is associated with increased  
3 pathology in TB animal models, and the unique TB gene signature detected in patients reflects  
4 neutrophil activity<sup>6,16-18</sup>. In hypoxia, human neutrophils demonstrate decreased apoptosis and  
5 respiratory burst<sup>19</sup>. Hypoxia-inducible factor- $\alpha$  (HIF- $\alpha$ ), activated in neutrophils in states of  
6 hypoxia and infection, prolongs the life-span of leukocytes, reduces bacterial burden and  
7 enhances reactive nitrogen species in neutrophils prior to infection in a mycobacterial model in  
8 zebrafish<sup>20,21</sup>. Furthermore, hypoxia decreases apoptosis and increases life-span in human  
9 neutrophils<sup>22</sup>.

10

11 The relationship between hypoxia and neutrophil-driven pathology in TB has not been  
12 investigated and mechanisms by which neutrophils may increase inflammatory tissue damage are  
13 poorly defined. We hypothesize that a hypoxic environment exacerbates neutrophil-dependent  
14 immunopathology in TB. We show that hypoxia increases neutrophil MMP-8, -9 and neutrophil  
15 elastase secretion in TB, which then drive matrix destruction. Increased protease activity is  
16 dependent on the HIF-1 $\alpha$  pathway. Conversely, the production of NETs is suppressed by  
17 hypoxia. Taken together, the data show that hypoxic neutrophils increase immune-mediated  
18 pathology by up-regulating enzymes that destroy the extracellular matrix in TB by a HIF-1 $\alpha$ -  
19 dependent mechanism.

# 1 MATERIALS AND METHODS

## 2 *M.tb* culture

3 *M. tuberculosis* H37Rv was cultured in supplemented Middlebrook 7H9 medium (BD  
4 Biosciences). For infection experiments, mycobacteria were used at mid-logarithmic growth at  
5 an optical density of 0.60 (Biowave cell density meter; WPA). All *M.tb* related experiments were performed  
6 in a Containment Level 3 facility.

## 7 Cell culture and stimulation

8 Ethical approval for obtaining healthy human volunteer blood to extract human neutrophils was  
9 provided by the Outer West London Research Ethics Committee (REC reference 09/H0709/46).  
10 All research was performed in accordance with relevant guidelines/regulations, and informed  
11 consent was obtained from all participants. Whole blood were drawn in preservative-free  
12 heparin and mixed with equal volumes of 3% dextran saline to remove erythrocytes. Neutrophils  
13 were isolated from the resulting cell suspension using Ficoll-Paque density centrifugation and  
14 three rounds of hypotonic lysis. Neutrophil purity was over 95% by FACS and viability >99%  
15 by trypan blue assay. In some experiments, neutrophils were pre-incubated with 1 mM  
16 dimethyloxalylglycine, DMOG (Sigma) or HIF-1 $\alpha$  inhibitor KC7F2 (Tocris Bioscience) for 30  
17 minutes before stimulation. In all experiments involving live *M. tuberculosis* H37Rv, tissue  
18 culture medium was sterile filtered through 0.2- $\mu$ m Durapore membranes (Millipore) before  
19 removing from the CL3 laboratory, which does not remove MMPs <sup>23</sup>. Neutrophils were  
20 stimulated at 37 °C in normoxia (21% oxygen) or hypoxia (1% oxygen) in a hypoxic incubator  
21 (Galaxy 14S, New Brunswick, Eppendorf).

22 Primary human blood monocytes were prepared from donor leukocyte cones of residual  
23 cells from blood donation of healthy donors (National Blood Transfusion Service, UK). After

1 density gradient centrifugation (Ficoll Paque) followed by adhesion purification, monocyte  
2 purity was over 95% by FACS analysis. Monocytes were infected with *M.tuberculosis* at a  
3 multiplicity of infection (MOI) of 1. After incubation at 37°C for 24 hours, conditioned medium  
4 was harvested and was termed CoMTB. Media from uninfected monocytes was termed  
5 CoMCont.

6

### 7 **Neutrophil elastase ELISA**

8 Neutrophil elastase concentration was measured with the Human Elastase ELISA kit (Hycult  
9 biotech, Uden, The Netherlands), with a minimum level of detection of 0.4 ng/ml. The  
10 manufacturer's protocol was followed and the plate read at 450 nm using a microplate reader  
11 ( $\mu$ Quant, Biotek Instruments, UK).

12

### 13 **Luminex array**

14 MMP-8 and -9 concentrations were analyzed by Fluorokine multianalyte profiling kit according  
15 to the manufacturer's protocol (R&D Systems) on the Luminex 200 platform (Bio-Rad). The  
16 minimum level of detection for MMP-8 and -9 was 110 pg/ml and 65 pg/ml respectively.

17

### 18 **Matrix degradation assay**

19 Type I collagen and elastin degradation was assessed using the EnzChek <sup>®</sup>  
20 Gelatinase/Collagenase Assay kit and the EnzChek <sup>®</sup> Elastase Assay kit (Molecular Probes)  
21 respectively. Samples were activated with 2 mM of 4-amino-phenyl mercuric acetate (APMA)  
22 for 1 hour at 37°C. 80 $\mu$ L of reaction buffer were added with 20  $\mu$ L of DQ collagen or elastin  
23 (Invitrogen) to a final concentration of 25  $\mu$ g/ml and 100  $\mu$ g/ml respectively. Activated samples

1 were subsequently added, and activity detected at specified times using a fluorometer (FLUOstar  
2 Galaxy).

3

#### 4 **Gelatin zymography**

5 This was performed as previously described<sup>24</sup>. Densitometric image analysis was performed  
6 using Scion Image version Beta.4.0.2.

7

#### 8 **Isolation and quantification of neutrophil extracellular traps (NETs)**

9 Human neutrophils were infected with *M.tb* at an MOI of 10, while 20 nM PMA was used as a  
10 positive control. 5 U/ml of micrococcal nuclease (Fermentas) was added in each well for 10  
11 minutes at 37°C, after which EDTA was used to halt the reaction. Supernatants were collected,  
12 sterile filtered and stored at 4°C. NETs were quantified using QuantiT PicoGreen (Invitrogen)  
13 according to manufacturer's instructions.

14

#### 15 **Real-time PCR**

16 Total RNA was extracted from  $2 \times 10^6$  neutrophils using the RNeasy Mini Kit (Qiagen).  
17 Quantitative real-time RT-PCR was performed using the OneStep RT- PCR master mix (Qiagen)  
18 according to the manufacturer's instruction on a Stratagene Mx3000P platform using 5 – 10 µg  
19 per sample. MMP-8, neutrophil elastase and β-actin primer and probe mixes were obtained from  
20 Applied Biosystems. MMP-9 forward and reverse primers were 5'-  
21 AGGCGCTCATGTACCCTATGTAC and 5'- GCCGTGGCTCAGGTTCA respectively, and the  
22 probe was 5'-FAM-CATCCGGCACCTCTATGGTCCTCG-TAMRA. To accurately determine



1 the quantitative change in RNA, standard curves were prepared from plasmids subjected to real-  
2 time PCR as above. MMP-8 and -9 data were normalized to  $\beta$ -actin detected in the same sample.

3

#### 4 **Flow cytometry**

5 Cell viability was assessed by staining neutrophils with Annexin V and propidium iodide using  
6 Annexin V-FITC apoptosis detection kit (eBioscience, Affymetrix, California, USA) and  
7 live/dead fixable stain kit (Invitrogen). Neutrophils were stimulated with 200 ng/ml  
8 staurosporine to induce apoptosis and this was used as a positive control for all experiments.  
9 Annexin V was detected on the FL-1 channel with propidium iodide and live/dead fixable dead  
10 cell stain kit on FL-3. A total of 50,000 events were gated and analysed on BD FACSCalibur  
11 flow cytometer using CellQuest. Data was analysed using FlowJo 7.6.5 (Tree Star).

12

#### 13 **Immunofluorescence microscopy**

14 Permanox chamber slides (Nunc Labtech) were coated with 0.1 mg/ml fibrinogen with or  
15 without 25  $\mu$ g/ml of DQ collagen or DQ Elastin for 60 minutes. Neutrophils were stimulated in  
16 normoxia or hypoxia, chambers removed and cover slips were placed prior to imaging. For  
17 experiments involving *M.tb*, neutrophils were infected with an MOI of 10. Samples were fixed  
18 with 4% paraformaldehyde for 30 minutes and permeabilized with 0.5% saponin for 10 minutes.  
19 Chambers were removed, slides stained with auromine-rhodamine (Merck Chemicals) and  
20 Fluoroshield Mounting medium with DAPI (Abcam) were added. Images were captured using  
21 Leica confocal microscope (Leica TCS SP5) and processed using Leica LAS AF Lite 2.6.0  
22 (Leica Microsystems, Germany) and Image J 1.43U (NIH, USA). Phagocytic index was the ratio

1 of number of neutrophils that contain at least 1 *M.tb* bacilli to the total number of neutrophils in  
2 1 field multiplied by 100%.

3

#### 4 **Statistical analyses**

5 Data were analyzed using GraphPad Prism (version 5.04, GraphPad Software). Data are  
6 expressed as mean  $\pm$  s.e.m. unless stated otherwise. All experiments are performed in technical  
7 triplicates on a minimum of 2 donors studied on separate occasions. All data points of 2 donors  
8 each in triplicate are presented. For FACS analysis, a minimum of 4 donors were studied on  
9 separate occasions with bars representing mean  $\pm$  s.e.m. Multiple intervention experiments are  
10 compared with one-way ANOVA followed by Tukey's post-test correction, kinetics experiments  
11 were analysed by 2-way ANOVA with Bonferonni post-test while continuous variables between  
12 2 sets of data are assessed using two-tailed Mann-Whitney-U test. *P* values of less than 0.05 are  
13 taken as statistically significant.

14

#### 15 **Data Availability**

16 The datasets generated during and/or analysed during the current study are available from the  
17 corresponding author on reasonable request.

# 1 RESULTS

## 2 Hypoxia increases TB-driven neutrophil collagenase (MMP-8) and neutrophil elastase 3 secretion

4 First, we investigated the effects of hypoxia on monocyte-dependent networks that drive  
5 neutrophil MMP and elastase secretion in TB. CoMTB upregulated secretion of both neutrophil  
6 collagenase MMP-8 and gelatinase MMP-9 secretion in normoxia. Hypoxia increased MMP-8  
7 secretion to 100 % at 24 hours which decreased slightly to 89% at 30 hours ( $p < 0.0001$  and  
8  $p < 0.001$  respectively) (figure 1a). MMP-9 secretion was independent of oxygen tension (figure  
9 1b). Similarly, hypoxia increased MMP-8 but not MMP-9 gene expression (figure 1c and d  
10 respectively). Neutrophils stimulated with CoMTB secreted neutrophil elastase, with hypoxia  
11 increasing the secretion by a further 76% ( $p < 0.0001$ , figure 1e). Neutrophil elastase gene  
12 expression was unchanged between normoxia and hypoxia (figure 1f).

## 14 Hypoxia does not alter viability of CoMTB-stimulated neutrophils

15 Next, we investigated whether the increase in CoMTB-dependent neutrophil MMP-8  
16 secretion in hypoxia was due to altered cellular viability. Over 30 hours, the viability of control  
17 neutrophils stimulated by CoMCont declined from  $88\% \pm 3\%$  (mean  $\pm$  s.e.m) to  $35\% \pm 7\%$  in  
18 normoxia. Viability at 30 hours was higher at  $55\% \pm 3\%$  in the presence of hypoxia in control  
19 neutrophils ( $p < 0.05$ , figure 2a). In CoMTB-stimulated neutrophils, viability similarly declined  
20 in normoxia to  $33\% \pm 5\%$ , but although viability increased to  $47\% \pm 7\%$  in hypoxia, the effect of  
21 decreased oxygen tension was less pronounced (figures 2b, S1). The proportion of apoptotic  
22 control neutrophils in normoxia at 30 hours was  $62\% \pm 7$ , with hypoxia decreasing apoptosis to

1 41%  $\pm$  3 ( $p < 0.05$ , figure 2c). CoMTB stimulation negated the effects of hypoxia on cellular  
2 apoptosis (figure 2d). Mean fluorescence intensity of Annexin V, indicating cellular death, was  
3 increased in control neutrophils at 30 hours in normoxia compared to hypoxia (CoMCont  
4 normoxia 512 A.U.  $\pm$  77 vs CoMCont hypoxia 279  $\pm$  49,  $p < 0.01$ ), but this effect was absent on  
5 stimulation with CoMTB (figure 2e). Although studying significantly more donors might  
6 generate a statistical significant difference, the effect on viability seems less pronounced in the  
7 context of CoMTB stimulation. Together, these data show that increased neutrophil MMP-8  
8 secretion in hypoxia after CoMTB stimulation was not due to increased neutrophil viability.

9

## 10 **Hypoxia decreases formation of neutrophil extracellular traps (NETs) after direct *M.tb*** 11 **infection**

12 We then focused on direct *M.tb* infection of neutrophils and evaluated the secretion of  
13 neutrophil MMPs over time. Neutrophil MMP-8 and -9 secretion increased for 30 hours after  
14 *M.tb* infection, but this was not altered in the presence of hypoxia (figure 3a and b). In contrast,  
15 the release of NETs after infection with *M.tb*, was decreased by 32% ( $p < 0.001$ ) in hypoxia  
16 compared to normoxia at 4 hours, with confocal microscopy showing a decrease in NET  
17 formation (figure 3c). Neutrophil extracellular traps (NETs) are produced when neutrophils  
18 encounter *M.tb*<sup>25</sup>, and they may aid in phagocytosis of pathogens<sup>26</sup>. However, when we  
19 compared neutrophil phagocytosis of *M.tb* in hypoxia versus normoxia at 4 hours, there was no  
20 difference in the phagocytic index (figure 3d). A later time point was not used as neutrophil  
21 viability diverges.

## 1 **Hypoxia increases neutrophil viability in *M.tb*-stimulated and non-stimulated neutrophils**

2 Since the NET effect could have been due to altered cell viability, and apoptosis of  
3 unstimulated neutrophils is delayed in hypoxia<sup>19,22</sup>, we also investigated the effects of *M.tb*  
4 infection on neutrophil apoptosis and cell viability in hypoxia. Analysis of primary human  
5 neutrophils at 24 hours showed delayed apoptosis with *M.tb* infection in hypoxia (*M.tb* in  
6 normoxia 29% ± 12 s.e.m. vs hypoxia 7% ± 2,  $p < 0.05$ , figure 4a) and decreased Annexin V  
7 mean fluorescence intensity (*M.tb* in normoxia 85 A.U. ± 15 vs hypoxia 25 ± 7,  $p < 0.001$ ,  
8 figure 4b). Similarly in hypoxia, there were far fewer necrotic *M.tb*-infected neutrophils (*M.tb* in  
9 normoxia 80% ± 4 vs hypoxia 4% ± 1,  $p < 0.001$ , figure 4c) and decreased live-dead dye mean  
10 fluorescence intensity (*M.tb* in normoxia 688 A.U. ± 108 vs hypoxia 17 ± 5,  $p < 0.001$  figure 4d).  
11 The delay in neutrophil apoptosis and necrosis at 24 hours translated to a 400% increase in  
12 neutrophil viability (Annexin V and Dye negative) in hypoxia compared to normoxia (*M.tb* in  
13 normoxia 18% ± 4 vs hypoxia 92% ± 2  $p < 0.001$ , figure 4e). Apoptosis, necrosis, cell viability  
14 and mean fluorescence intensity of Annexin V and live-dead dye were all independent of *M.tb*  
15 multiplicity of infection (MOI), (figure 4 and S2). Thus, the decrease in NETs in hypoxia was  
16 not due to a decrease in neutrophil viability.

## 18 **Neutrophil-driven matrix destruction in TB is increased with hypoxia**

19 Next, we investigated the functional effect of increased neutrophil MMP-8 secretion in  
20 hypoxia on lung matrix turnover in *M.tb* infection. First, using DQ Type I collagen, which  
21 fluoresces on degradation, we demonstrated that CoMTB stimulation of neutrophils increased  
22 Type I collagen degradation and this was further increased in the presence of hypoxia (figure 5a).  
23 This reflected an increase in collagenase activity of 112% in hypoxia (normoxia 47.1 % ± 4 vs

1 hypoxia  $100 \% \pm 8$ ,  $p < 0.0001$ , figure 5b). CoMTB-stimulated neutrophil pro-MMP-9 gelatinase  
2 activity measured by zymography was also increased 35% in hypoxia compared to normoxia  
3 (normoxia  $74.1 \% \pm 6$  vs hypoxia  $100 \% \pm 4$ ,  $p < 0.001$ , figure 5c and S3).

4  
5 We further demonstrated by confocal microscopy elastin degradation in the presence of  
6 CoMTB, which was markedly increased in the presence of hypoxia (figure 6a). Using  
7 quantitative fluorescence assays to assess elastase activity, we confirmed an increase by 158% in  
8 degradation in hypoxia compared to normoxia (normoxia  $38.7 \% \pm 6$  vs hypoxia  $100 \% \pm 3$ ,  
9  $p < 0.0001$ , figure 6b). Taken together, these data show that matrix destruction in TB is increased  
10 in the presence of hypoxia.

#### 11 12 **HIF-1 $\alpha$ stabilization increases neutrophil MMP-8 and -9 secretion**

13 To investigate the mechanism by which hypoxia regulates neutrophil MMP secretion, we  
14 used dimethyloxalylglycine (DMOG) to stabilize HIF-1 $\alpha$  expression. Neutrophils pre-treated  
15 with 1 mM DMOG prior to CoMTB stimulation secreted 74 % more MMP-8 (CoMTB  $57.6 \% \pm$   
16  $4$  vs DMOG CoMTB  $100 \% \pm 5$ ,  $p < 0.0001$ , figure 7a). DMOG similarly augmented neutrophil  
17 MMP-9 secretion by 39% with CoMTB stimulation (CoMTB  $72 \% \pm 6$  vs DMOG CoMTB  
18  $100\% \pm 4$ ,  $p < 0.01$ , fig. 7b).

# DISCUSSION

In this study, we investigated the impact of hypoxia on neutrophil-dependent tissue destruction in TB and show that hypoxia increases MMP-8 and neutrophil elastase secretion in the presence of CoMTB but not with *M.tb* stimulation. These enzymes will degrade extracellular matrix rich in types 1-3 collagen and elastin, which together are the main structural components of lung tissue. We have demonstrated that pulmonary cavities in TB patients are severely hypoxic<sup>9</sup>, and the consequence of hypoxia will be increased secretion of enzymes leading to more severe immunopathology. The increase in neutrophil protease secretion was not due to altered viability of CoMTB-stimulated neutrophils in hypoxia, as viability of neutrophils in hypoxia compared to normoxia was not significantly different<sup>6,9</sup>.

Hypoxia was demonstrated to have a functional effect with increasing MMP-8 and neutrophil elastase secretion causing destruction of extracellular matrix in TB, with an increase in gene expression of MMP-8 but not MMP-9. This is possibly due to neutrophils predominantly secreting MMP-9 pre-synthesised in granules<sup>27</sup>. We observed both by confocal microscopy and quantitative fluorescence assays that in hypoxia there was increased destruction of both collagen and elastin by neutrophils stimulated via monocyte-dependent networks driven by *M.tb* infection, although hypoxia did not alter neutrophil MMP secretion in the context of direct *M.tb* stimulation. This indicates that in hypoxia, neutrophils drive tissue destruction predominantly through intercellular networks. MMPs, and specifically neutrophil-derived MMPs, are emerging as key pathological mediators in diverse destructive pulmonary pathologies ranging from emphysema to TB<sup>28-30</sup>, with our data suggesting that hypoxia is a driver of Type I collagen, gelatin and elastin destruction, the extracellular fibrils that are key components on the human

1 lung. However, HIF-1 $\alpha$  also regulates antimicrobial neutrophil granule proteases production,  
2 with HIF-1 $\alpha$  null mice demonstrating decreased neutrophil elastase, cathepsin G secretion and  
3 iNOS gene expression<sup>31</sup>. HIF acts as a master regulator of innate immune function in  
4 phagocytes and its expression is integral to the host defence mechanism<sup>32</sup>, and so the overall  
5 contribution of HIF-1 $\alpha$  to the host-pathogen interaction in human TB will be multifactorial.

6

7 Infection with pathogens such as *Staphylococcus* and *Pseudomonas* species may increase  
8 neutrophil death, in contrast to infection with intracellular organisms such as *Anaplasma*  
9 *phagocytophilum* and *Leishmania* which increase neutrophil viability<sup>33</sup>. Although we had  
10 expected neutrophil viability to be increased by *M.tb*, we found that viability was not affected in  
11 normoxia. In addition, hypoxia resulted in increased neutrophil viability, independent of *M.tb*  
12 multiplicity of infection. Our findings are in keeping with the increase in neutrophil viability and  
13 their associated mediators in cancer, COPD and bronchiectasis, states which are associated with  
14 hypoxia and inflammation<sup>29,34,35</sup>.

15

16 Since the HIF-prolyl hydroxylase (PHD) pathway is a key in regulating multiple innate  
17 immune functions in hypoxia<sup>36,37</sup>, we investigated the role of HIF in the control of neutrophil  
18 MMP secretion in TB. We found that HIF stabilization using either physiologic hypoxia or  
19 chemical-induced HIF stabilization using DMOG increased neutrophil MMP-8. This is  
20 consistent with the finding that HIF $\alpha$  stabilization in a zebrafish model delays neutrophil  
21 apoptosis and increases inflammation, and our previous finding of increased MMP-1 secretion  
22 with HIF stabilization in hypoxia and with *M.tb* stimulation<sup>9,20,22</sup>. We had previously shown



1 silencing of HIF-1 $\alpha$  led to a decrease in MMP-1 secretion<sup>9</sup>, indicating that the HIF-PHD  
2 pathway regulated MMP-1 secretion, which may also regulate neutrophil MMP secretion in TB.

3

4           Pharmacologic and genetic inhibition of HIF-1 $\alpha$  had been found to decrease NET  
5 formation<sup>38</sup>. We also observed that NET formation declined in hypoxia and postulate the  
6 decrease in NET formation in hypoxia may be caused by the decrease in reactive oxygen species  
7 (ROS) during hypoxia, since ROS may regulate NET formation<sup>19,39</sup>. The decrease in NET  
8 formation in hypoxia was not due to a decrease in neutrophil viability. The consequences of  
9 decreased NET formation in TB are uncertain although somewhat unexpectedly phagocytosis of  
10 the pathogen was not decreased.

11

12           In summary, our data demonstrate that increased neutrophil MMP-8 and elastase  
13 secretion are driven by hypoxia in our cellular model of TB. Neutrophil viability is increased in  
14 the presence of hypoxia with *M.tb* stimulation. The neutrophil proteases resulted in a functional  
15 increase in destruction of the lung structural matrix components collagen and elastin. Inhibiting  
16 excessive protease secretion via modulating the HIF pathway may be a potential host-directed  
17 therapeutic approach to decrease hypoxia-driven tissue destruction in TB and improve clinical  
18 outcomes particularly in the era of rising drug resistance.

1 **Author contributions:**

2 C.W.M.O and J.S.F conceived the project. C.W.M.O., K.F and J.S.F designed the experiments  
3 and analyzed the data. C.W.M.O, K.F and A.E performed the experiments. C.W.M.O and K.F  
4 generated the data. C.W.M.O, P.T.E and J.S.F wrote the manuscript which was reviewed by all  
5 authors.

6

7 **Competing interests:** The authors declare no competing interests.

8

9 **Funding:** C.W.M.O. is funded by the Singapore National Medical Research Council. K.F is  
10 funded by the Wellcome Trust. J.S.F. and P.E. acknowledge support of the NIHR Biomedical  
11 Research Centre at Imperial College.

# 1 REFERENCES

- 2 1. Ong, C.W., Elkington, P.T. & Friedland, J.S. Tuberculosis, pulmonary cavitation, and  
3 matrix metalloproteinases. *Am J Respir Crit Care Med* **190**, 9-18 (2014).
- 4 2. Elkington, P.T., D'Armiento, J.M. & Friedland, J.S. Tuberculosis immunopathology: the  
5 neglected role of extracellular matrix destruction. *Sci Transl Med* **3**, 71ps76 (2011).
- 6 3. Dorhoi, A. & Kaufmann, S.H. Perspectives on host adaptation in response to  
7 *Mycobacterium tuberculosis*: modulation of inflammation. *Semin Immunol* **26**, 533-542  
8 (2014).
- 9 4. Reece, S.T., *et al.* Serine protease activity contributes to control of *Mycobacterium*  
10 *tuberculosis* in hypoxic lung granulomas in mice. *J Clin Invest* **120**, 3365-3376 (2010).
- 11 5. Kaufmann, S.H. & Dorhoi, A. Inflammation in tuberculosis: interactions, imbalances and  
12 interventions. *Curr Opin Immunol* **25**, 441-449 (2013).
- 13 6. Ong, C.W., *et al.* Neutrophil-Derived MMP-8 Drives AMPK-Dependent Matrix  
14 Destruction in Human Pulmonary Tuberculosis. *PLoS Pathog* **11**, e1004917 (2015).
- 15 7. Steinwede, K., *et al.* Cathepsin G and neutrophil elastase contribute to lung-protective  
16 immunity against mycobacterial infections in mice. *J Immunol* **188**, 4476-4487 (2012).
- 17 8. Dheda, K., *et al.* Lung remodeling in pulmonary tuberculosis. *J Infect Dis* **192**, 1201-  
18 1209 (2005).
- 19 9. Belton, M., *et al.* Hypoxia and tissue destruction in pulmonary TB. *Thorax* (2016).
- 20 10. Oehlers, S.H., *et al.* Interception of host angiogenic signalling limits mycobacterial  
21 growth. *Nature* **517**, 612-615 (2015).

- 1 11. Datta, M., *et al.* Anti-vascular endothelial growth factor treatment normalizes  
2 tuberculosis granuloma vasculature and improves small molecule delivery. *Proc Natl*  
3 *Acad Sci U S A* **112**, 1827-1832 (2015).
- 4 12. Nickel, D., *et al.* Hypoxia triggers the expression of human beta defensin 2 and  
5 antimicrobial activity against *Mycobacterium tuberculosis* in human macrophages. *J*  
6 *Immunol* **188**, 4001-4007 (2012).
- 7 13. Shell, S.S., *et al.* DNA methylation impacts gene expression and ensures hypoxic  
8 survival of *Mycobacterium tuberculosis*. *PLoS Pathog* **9**, e1003419 (2013).
- 9 14. Galagan, J.E., *et al.* The *Mycobacterium tuberculosis* regulatory network and hypoxia.  
10 *Nature* **499**, 178-183 (2013).
- 11 15. Eum, S.Y., *et al.* Neutrophils are the predominant infected phagocytic cells in the airways  
12 of patients with active pulmonary TB. *Chest* **137**, 122-128 (2010).
- 13 16. Nouailles, G., *et al.* CXCL5-secreting pulmonary epithelial cells drive destructive  
14 neutrophilic inflammation in tuberculosis. *J Clin Invest* (2014).
- 15 17. Mantovani, A., Cassatella, M.A., Costantini, C. & Jaillon, S. Neutrophils in the activation  
16 and regulation of innate and adaptive immunity. *Nat Rev Immunol* **11**, 519-531 (2011).
- 17 18. Berry, M.P., *et al.* An interferon-inducible neutrophil-driven blood transcriptional  
18 signature in human tuberculosis. *Nature* **466**, 973-977 (2010).
- 19 19. McGovern, N.N., *et al.* Hypoxia selectively inhibits respiratory burst activity and killing  
20 of *Staphylococcus aureus* in human neutrophils. *J Immunol* **186**, 453-463 (2011).

- 1 20. Elks, P.M., *et al.* Activation of hypoxia-inducible factor-1alpha (Hif-1alpha) delays  
2 inflammation resolution by reducing neutrophil apoptosis and reverse migration in a  
3 zebrafish inflammation model. *Blood* **118**, 712-722 (2011).
- 4 21. Elks, P.M., *et al.* Hypoxia inducible factor signaling modulates susceptibility to  
5 mycobacterial infection via a nitric oxide dependent mechanism. *PLoS Pathog* **9**,  
6 e1003789 (2013).
- 7 22. Walmsley, S.R., *et al.* Hypoxia-induced neutrophil survival is mediated by HIF-1alpha-  
8 dependent NF-kappaB activity. *J Exp Med* **201**, 105-115 (2005).
- 9 23. Elkington, P.T., Green, J.A. & Friedland, J.S. Filter sterilization of highly infectious  
10 samples to prevent false negative analysis of matrix metalloproteinase activity. *J*  
11 *Immunol Methods* **309**, 115-119 (2006).
- 12 24. Elkington, P.T., *et al.* Mycobacterium tuberculosis, but not vaccine BCG, specifically  
13 upregulates matrix metalloproteinase-1. *Am J Respir Crit Care Med* **172**, 1596-1604  
14 (2005).
- 15 25. Ramos-Kichik, V., *et al.* Neutrophil extracellular traps are induced by Mycobacterium  
16 tuberculosis. *Tuberculosis (Edinb)* **89**, 29-37 (2009).
- 17 26. Branzk, N., *et al.* Neutrophils sense microbe size and selectively release neutrophil  
18 extracellular traps in response to large pathogens. *Nat Immunol* **15**, 1017-1025 (2014).
- 19 27. Borregaard, N., Sorensen, O.E. & Theilgaard-Monch, K. Neutrophil granules: a library of  
20 innate immunity proteins. *Trends Immunol* **28**, 340-345 (2007).

- 1 28. Ostridge, K., *et al.* Relationship between pulmonary matrix metalloproteinases and  
2 quantitative CT markers of small airways disease and emphysema in COPD. *Thorax* **71**,  
3 126-132 (2016).
- 4 29. Garratt, L.W., *et al.* Matrix metalloproteinase activation by free neutrophil elastase  
5 contributes to bronchiectasis progression in early cystic fibrosis. *Eur Respir J* **46**, 384-  
6 394 (2015).
- 7 30. Elkington, P.T., Ugarte-Gil, C.A. & Friedland, J.S. Matrix metalloproteinases in  
8 tuberculosis. *Eur Respir J* **38**, 456-464 (2011).
- 9 31. Peyssonnaud, C., *et al.* HIF-1alpha expression regulates the bactericidal capacity of  
10 phagocytes. *J Clin Invest* **115**, 1806-1815 (2005).
- 11 32. Nizet, V. & Johnson, R.S. Interdependence of hypoxic and innate immune responses. *Nat*  
12 *Rev Immunol* **9**, 609-617 (2009).
- 13 33. McCracken, J.M. & Allen, L.A. Regulation of human neutrophil apoptosis and lifespan in  
14 health and disease. *J Cell Death* **7**, 15-23 (2014).
- 15 34. Houghton, A.M., *et al.* Neutrophil elastase-mediated degradation of IRS-1 accelerates  
16 lung tumor growth. *Nat Med* **16**, 219-223 (2010).
- 17 35. Aliberti, S., *et al.* Quality standards for the management of bronchiectasis in Italy: a  
18 national audit. *Eur Respir J* **48**, 244-248 (2016).
- 19 36. Cheng, S.C., *et al.* mTOR- and HIF-1alpha-mediated aerobic glycolysis as metabolic  
20 basis for trained immunity. *Science* **345**, 1250684 (2014).
- 21 37. Rius, J., *et al.* NF-kappaB links innate immunity to the hypoxic response through  
22 transcriptional regulation of HIF-1alpha. *Nature* **453**, 807-811 (2008).

- 1 38. McInturff, A.M., *et al.* Mammalian target of rapamycin regulates neutrophil extracellular  
2 trap formation via induction of hypoxia-inducible factor 1 alpha. *Blood* **120**, 3118-3125  
3 (2012).
- 4 39. Fuchs, T.A., *et al.* Novel cell death program leads to neutrophil extracellular traps. *J Cell*  
5 *Biol* **176**, 231-241 (2007).

1 **FIGURE LEGENDS**

2 **FIGURE 1: Hypoxia increases MMP-8 and neutrophil elastase secretion from CoMTB-**

3 **stimulated neutrophils.** (a) Neutrophil MMP-8 and (b) MMP-9 secretion in normoxia and

4 hypoxia with CoMCont (control) and CoMTB stimulation. (c) MMP-8 gene expression is

5 increased in hypoxia at 24h. (d) MMP-9 gene expression is unchanged with hypoxia at 24h. (e)

6 Neutrophil elastase (NE) secretion is increased in CoMTB-stimulated neutrophils in hypoxia. (f)

7 Neutrophil elastase gene expression is unaffected by oxygen concentration.  $**p<0.01$ ,

8  $***p<0.001$ ,  $****p<0.0001$  by 2-way ANOVA with Bonferonni post-test. Bars represent mean

9  $\pm$  s.e.m of all data points from 2 donors each in triplicate, normalized to hypoxia CoMTB.

10

11 **FIGURE 2: CoMTB abrogates increased neutrophil viability found in hypoxia.** (a)

12 Neutrophil viability is increased in hypoxia in CoMCont (control) neutrophils. (b) Neutrophil

13 viability in the presence of CoMTB is not affected by oxygen tension. (c) Neutrophil apoptosis

14 is decreased in hypoxia in CoMCont neutrophils. (d) Neutrophil apoptosis after CoMTB

15 stimulation is independent of oxygen tension. (e) Annexin V mean fluorescence intensity is

16 increased in CoMCont-stimulated neutrophils in normoxia compared to hypoxia but not in

17 CoMTB-stimulated neutrophils, demonstrating that CoMTB negates the effect of oxygen tension

18 on neutrophil viability.  $*p<0.05$ ,  $**p<0.01$  by 2-way ANOVA with Bonferonni post-test. Bars

19 represent mean  $\pm$  s.e.m of  $n = 5$  neutrophil donors on 5 separate occasions.



1 **FIGURE 3: Hypoxia does not affect neutrophil MMP secretion in direct *M.tb* infection,**  
2 **suppresses NET formation and does not affect neutrophil phagocytosis.** (a) MMP-8 and (b)  
3 MMP-9 secretion is upregulated in *M.tb*-infected neutrophils but is independent of oxygen  
4 tension. (c) Hypoxia decreases production of NETs by *M.tb*-infected neutrophils as demonstrated  
5 by quantitative fluorescence imaging and confocal microscopy. White arrow on confocal image  
6 indicating NETs. Scale bar 20  $\mu\text{m}$ . \*\*\*  $p < 0.001$  by 2-way ANOVA with Bonferonni post-test.  
7 (d) Neutrophil phagocytosis is independent of hypoxia. Neutrophils were infected with *M.tb*  
8 MOI 10 for 4 hours in normoxia or hypoxia, fixed and stained with Auramine-Rhodamine (red)  
9 for *M.tb* and DAPI (blue) for neutrophil nuclei. Neutrophils from 10 different fields were  
10 counted and phagocytic index analysed by Mann-Whitney test. Bars represent mean  $\pm$  s.e.m of  
11 all data points from 2 donors each in triplicate normalized to hypoxia *M.tb* for MMP-8 and -9,  
12 and normalized to hypoxia PMA for extracellular DNA.

13  
14 **FIGURE 4: Hypoxia delays apoptosis, necrosis and increases neutrophil viability in *M.tb***  
15 **infection.** (a) The percentage of apoptotic neutrophils (Annexin V positive) is lower in hypoxia  
16 than normoxia for uninfected and infected cells. \* $p < 0.05$  between normoxia *M.tb* 10 and  
17 hypoxia *M.tb* 10 by 2-way ANOVA with Bonferonni post-test. (b) Mean fluorescent intensity of  
18 Annexin V positive cells was higher in cells in normoxia than in hypoxia. \*\*\*\*  $p < 0.001$   
19 between normoxia control vs. hypoxia control ; normoxia *M.tb* 1 vs. hypoxia *M.tb* 1; and  
20 normoxia *M.tb* 10 vs. hypoxia *M.tb* 10. (c) The proportion of necrotic cells stained positive for  
21 Live-Dead dye was lower in hypoxia than normoxia for uninfected and infected cells. \*\*\*\*  
22  $p < 0.001$  between normoxia control vs. hypoxia control; normoxia *M.tb* 1 vs. hypoxia *M.tb* 1; and  
23 normoxia *M.tb* 10 vs. hypoxia *M.tb* 10. (d) Mean fluorescent intensity of Live-Dead dye positive

1 cells was higher in infected and uninfected cells in normoxia than in hypoxia. \*\*\*\*  $p < 0.001$   
2 between normoxia control vs. hypoxia control; normoxia *M.tb* 1 vs. hypoxia *M.tb* 1; and  
3 normoxia *M.tb* 10 and hypoxia *M.tb* 10. (e) The proportion of viable cells is higher in hypoxia  
4 than normoxia for uninfected and infected cells, analysed by Annexin V staining. \*\*\*\*  $p < 0.001$   
5 between normoxia control vs. hypoxia control; normoxia *M.tb* 1 vs hypoxia *M.tb* 1; and  
6 normoxia *M.tb* 10 vs hypoxia *M.tb* 10. Bars represent mean  $\pm$  s.e.m of  $n = 4$  neutrophil donors  
7 on 4 separate occasions.

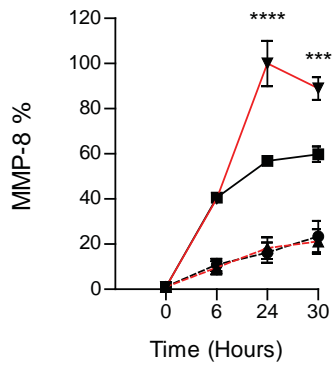
8  
9 **FIGURE 5: Hypoxia increases neutrophil-driven matrix destruction.** (a) Confocal  
10 microscopy shows increased Type I collagenase activity in CoMTB-stimulated neutrophils in  
11 hypoxia compared to normoxia. (b) Neutrophils were stimulated with CoMCont or CoMTB and  
12 breakdown of DQ collagen analysed by quantitative fluorescent assay. (c) Pro-MMP-9  
13 gelatinase activity is increased in hypoxia. MMP-9 activity by gelatin zymography with  
14 representative densitometric analysis. Zymogram representative of an experiment performed in  
15 triplicate from 2 independent experiments. Original gel is in Supplementary Figure 3. N,  
16 normoxia; H, hypoxia. \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$  by 2-way ANOVA with Bonferonni post-  
17 test. Bars represent mean  $\pm$  s.e.m of all data points from 3 donors each in triplicate normalized  
18 to hypoxia CoMTB.

1 **FIGURE 6. Elastin degradation is increased in hypoxia.** a) Neutrophils were stimulated  
2 with CoMCont or CoMTB and incubated on DQ elastin coated slides for 30 hours. b) Elastase  
3 activity is increased with CoMTB in hypoxia. Breakdown of DQ elastin analysed by  
4 quantitative fluorescent assay. \*\*\* $p < 0.01$  by 2-way ANOVA with Bonferonni post-test. Bars  
5 represent mean  $\pm$  s.e.m of all data points from 3 donors each in triplicate normalized to hypoxia  
6 CoMTB.

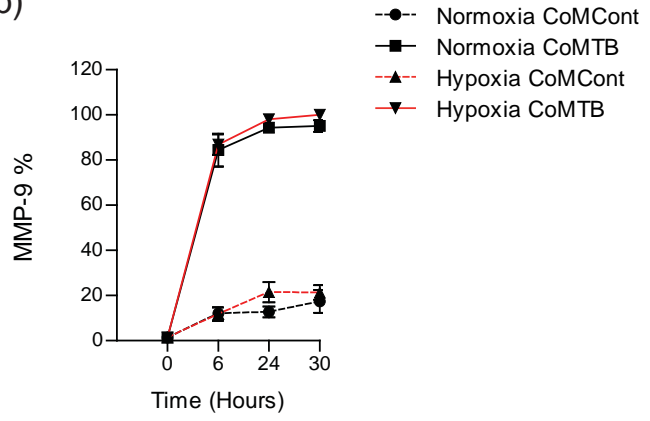
7

8 **FIGURE 7: HIF-1 $\alpha$  stabilisation modulates neutrophil MMP secretion.** HIF-1 $\alpha$  stabiliser  
9 DMOG increases (a) MMP-8 and (b) MMP-9 secretion. \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$  by 2-way  
10 ANOVA with Bonferonni post-test. Bars represent mean  $\pm$  s.e.m of all data points from 2  
11 donors each in triplicate normalized to DMOG CoMTB.

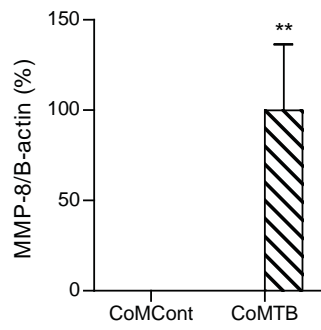
a)



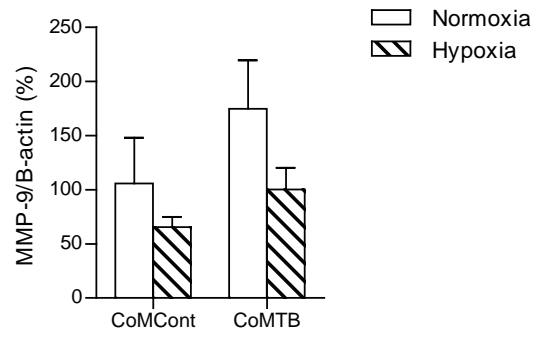
b)



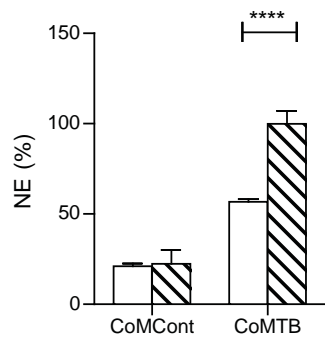
c)



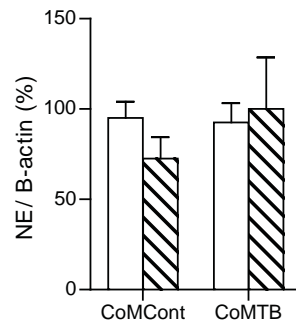
d)

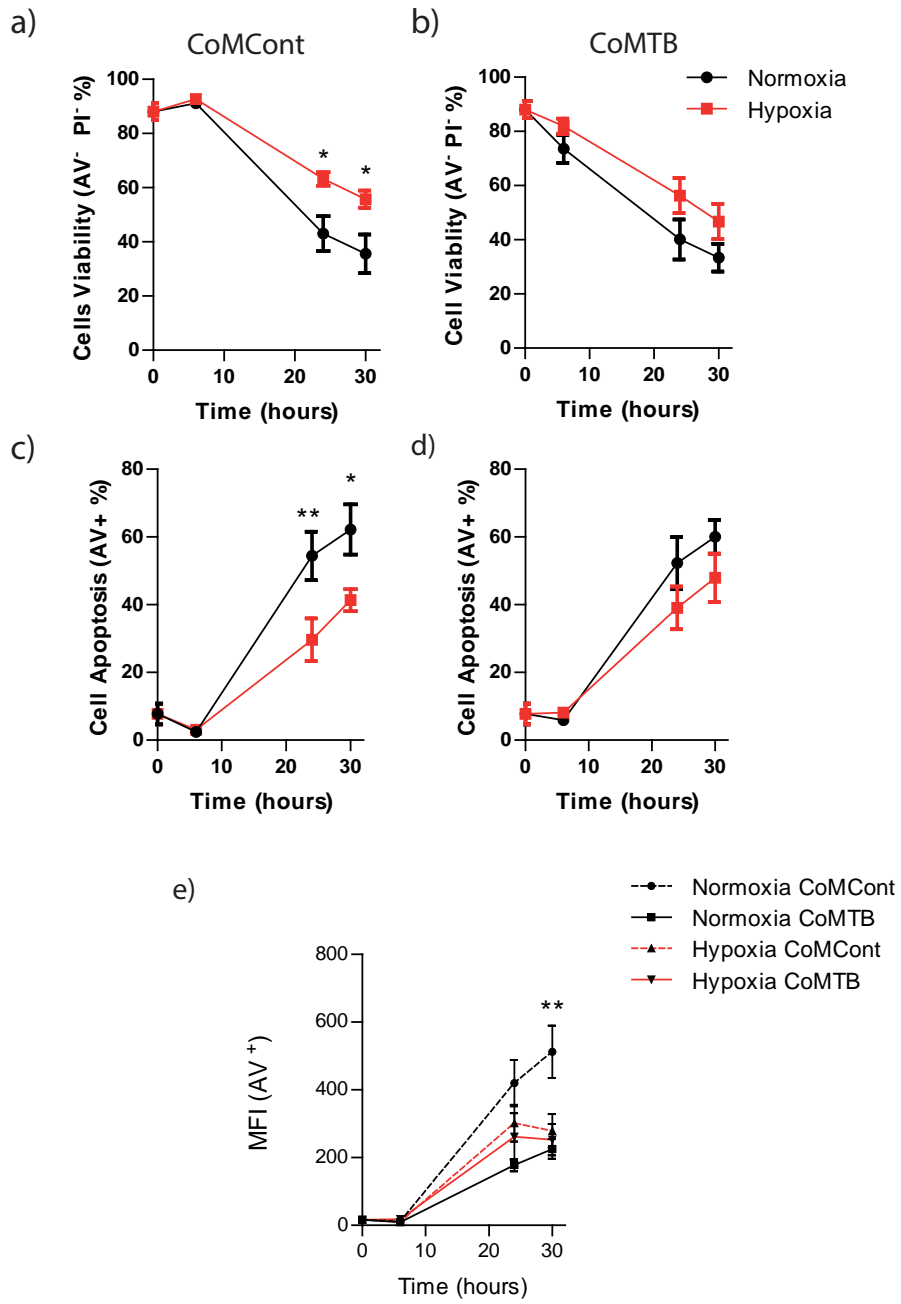


e)

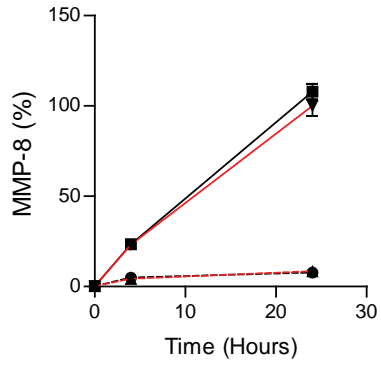


f)

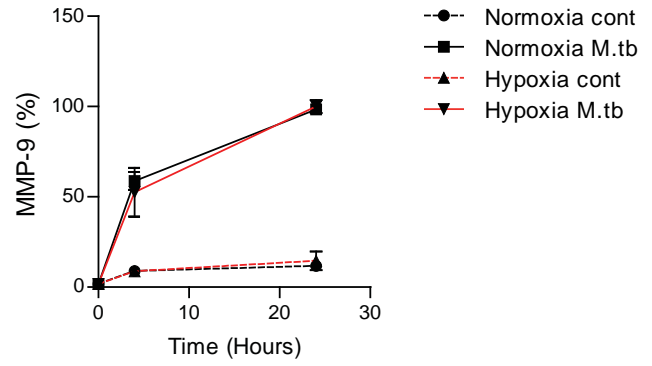




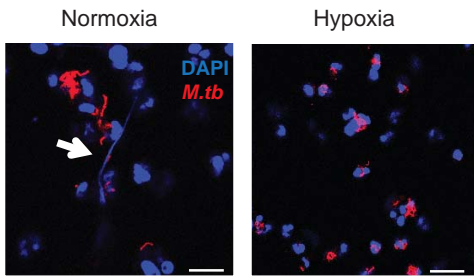
a)



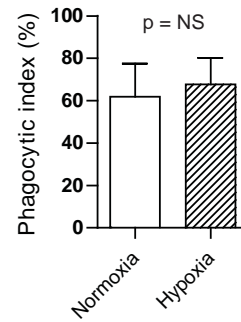
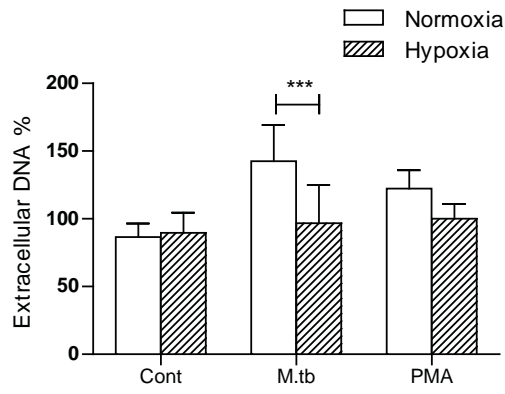
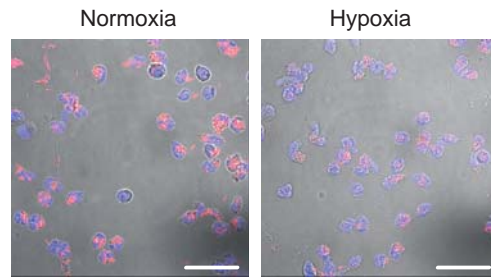
b)

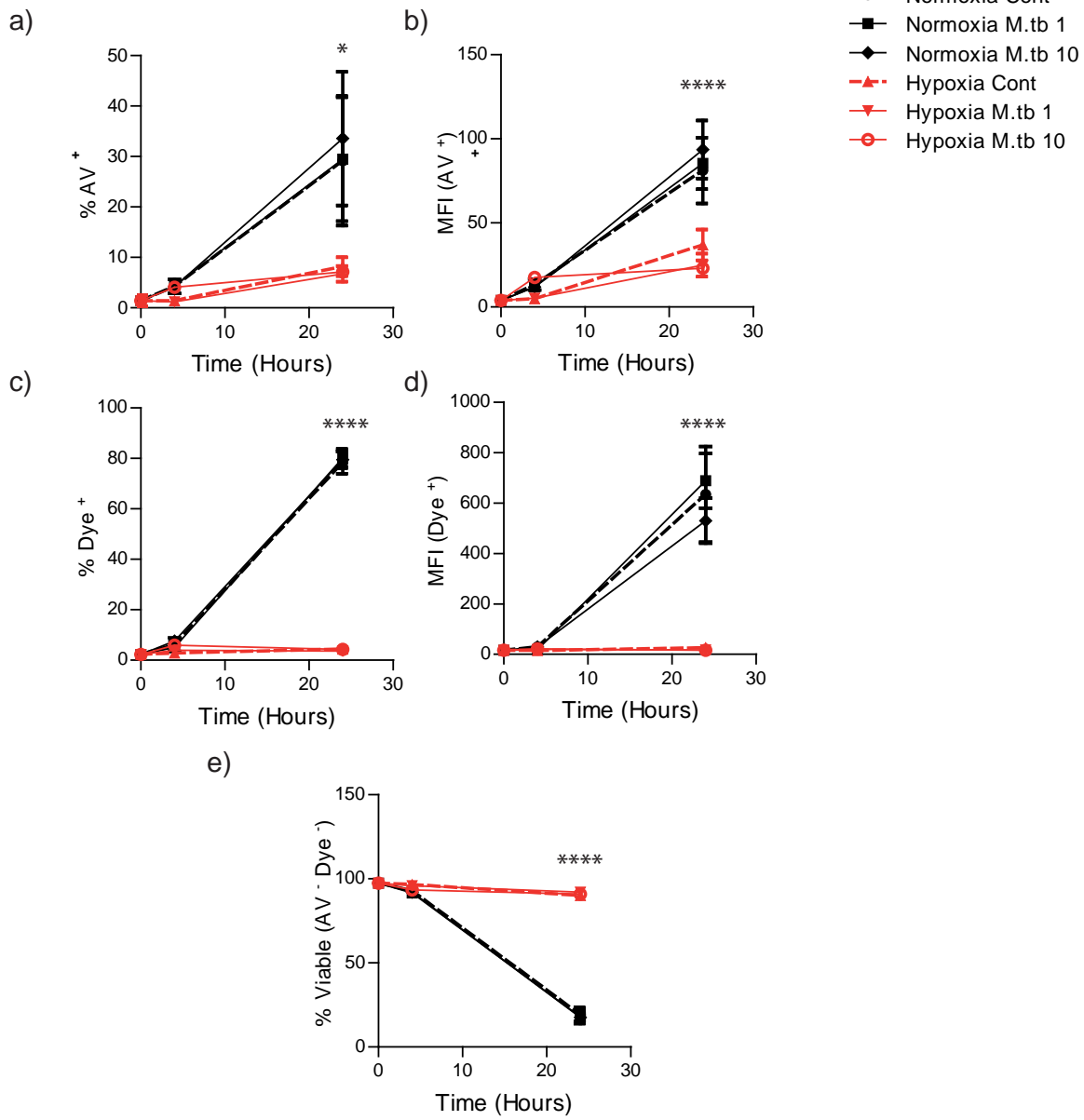


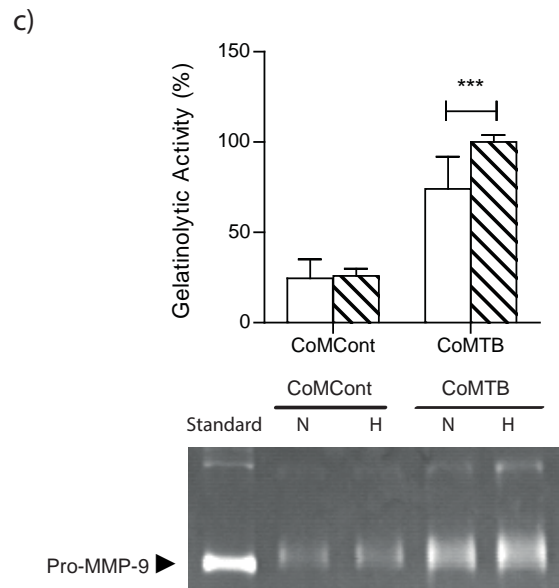
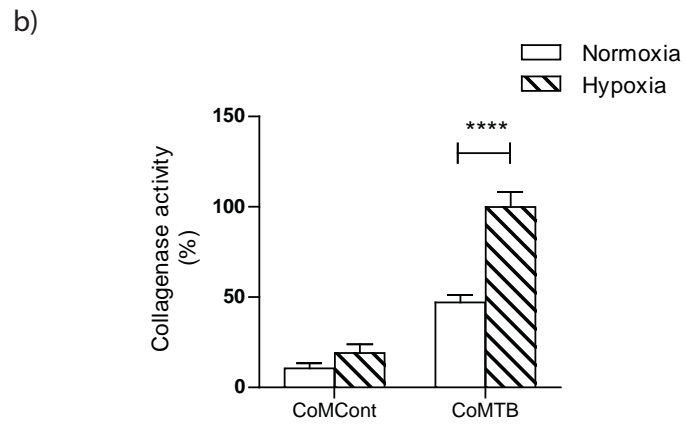
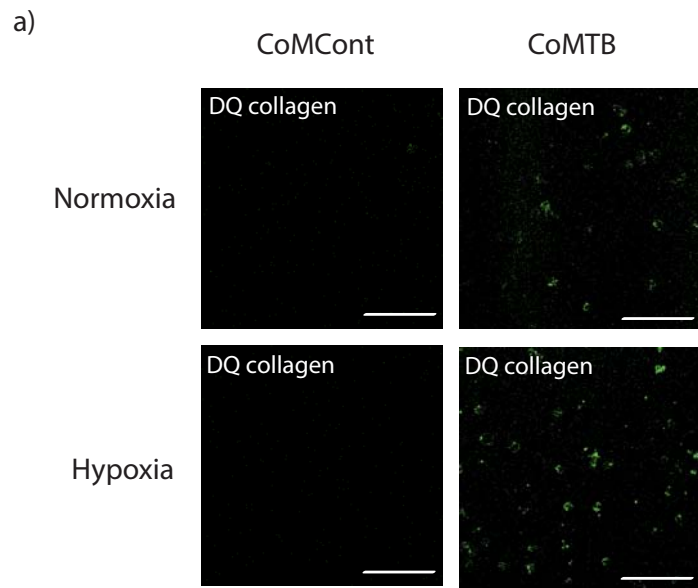
c)



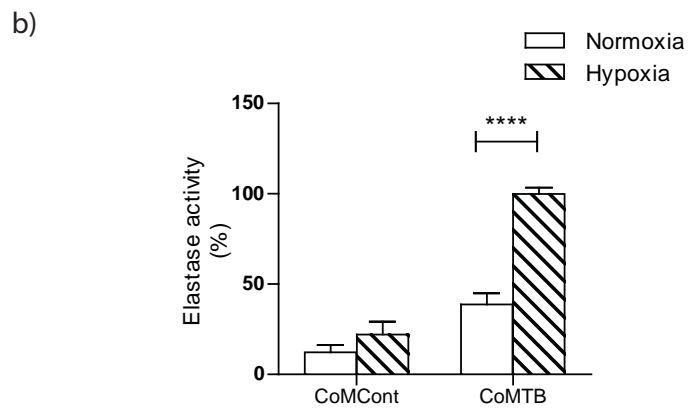
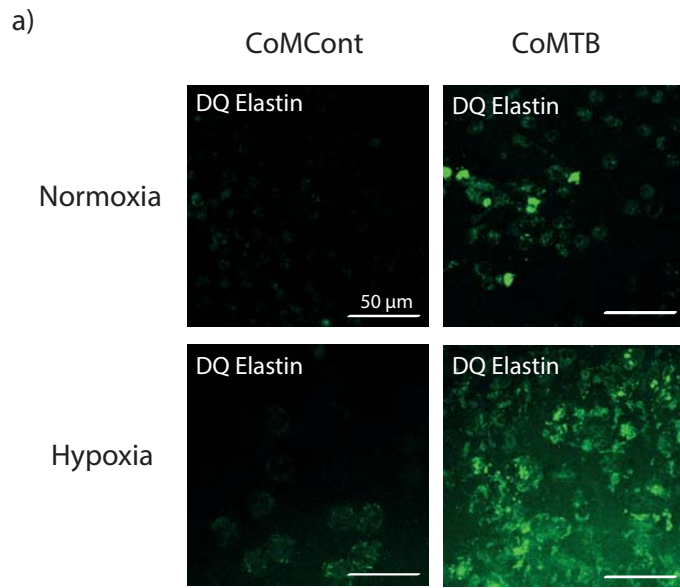
d)



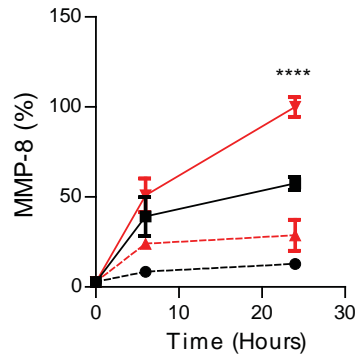




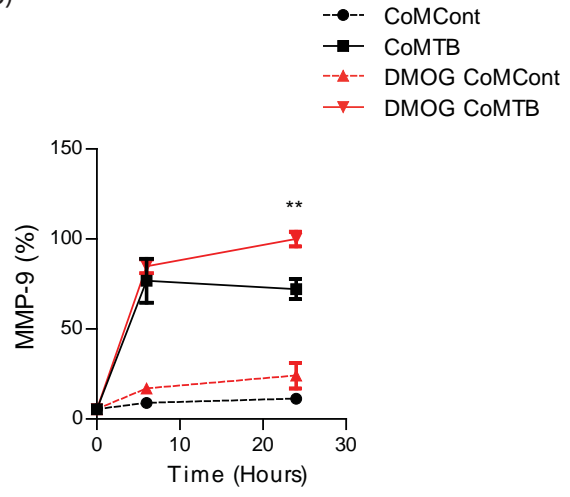




a)

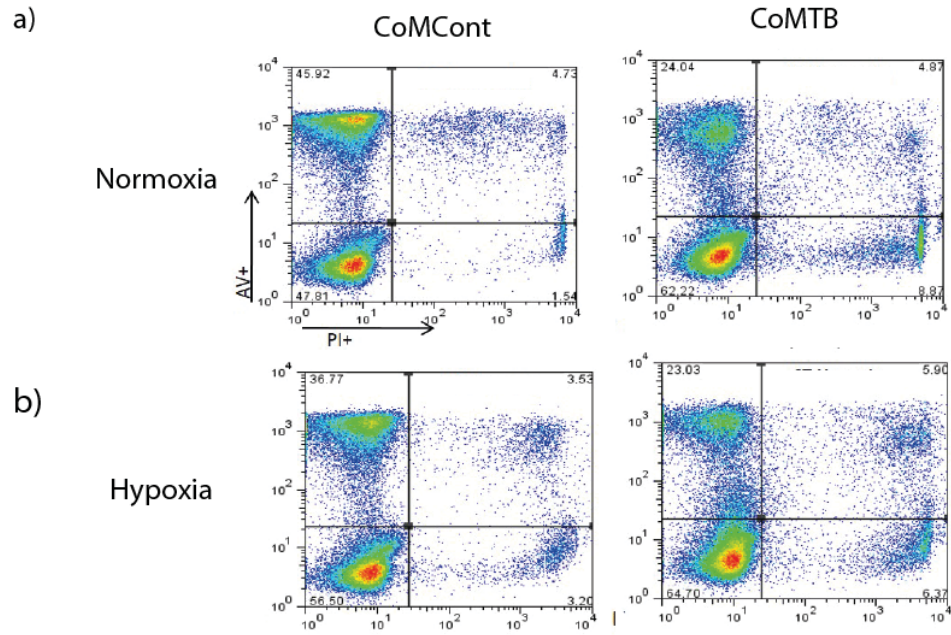


b)

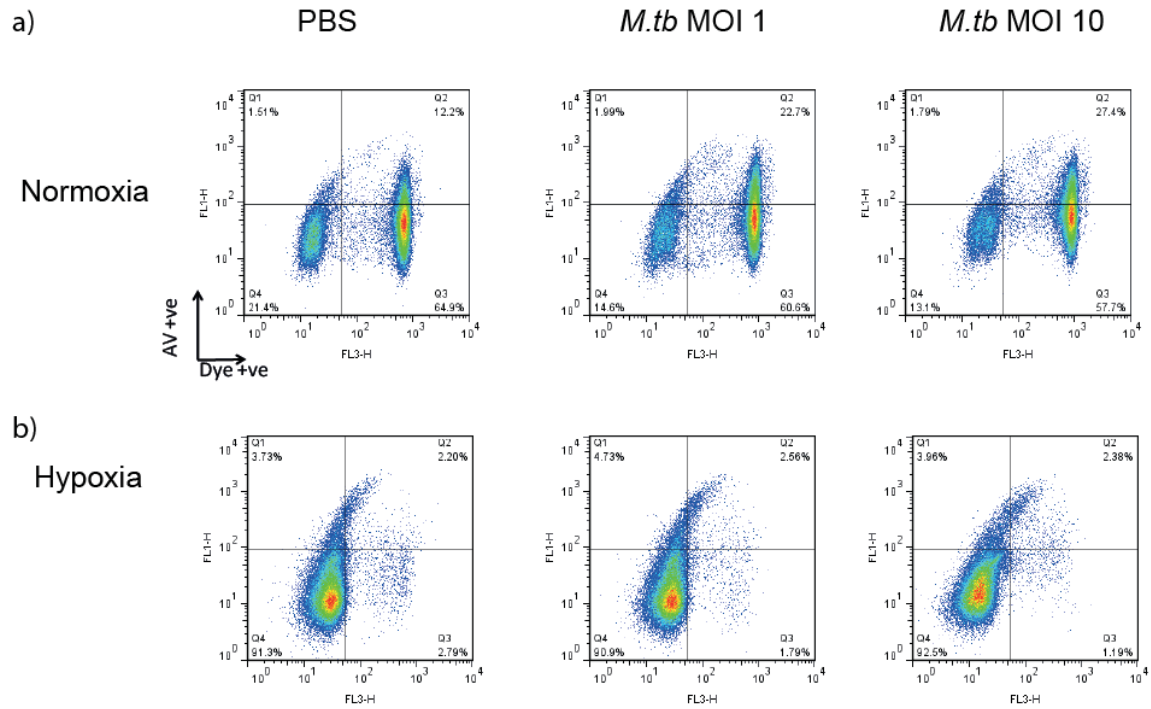


**Hypoxia increases neutrophil-driven matrix destruction after  
exposure to *Mycobacterium tuberculosis***

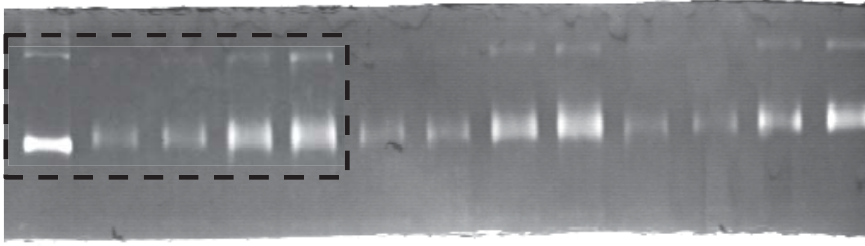
**Authors:** Catherine WM Ong, Katharine Fox , Anna Ettore, Paul T Elkington & Jon S Friedland



**FIGURE S1:** Hypoxia does not alter neutrophil viability with CoMTB stimulation. (a, b) Representative FACS plot of neutrophils stimulated with CoMCont or CoMTB for 30 hours and stained with Annexin V and propidium iodide. 50,000 events were collected in the gate of live cells for all FACS plots.



**FIGURE S2.** Hypoxia prolongs neutrophil viability in the absence and presence of *M.tb* infection. (a, b) Representative FACS plot of neutrophils stimulated with PBS, *M.tb* MOI 1 or *M.tb* MOI 10 in normoxia or hypoxia at 24 hours. In normoxia, *M.tb* infection reduced cell viability, but in hypoxia viability was increased. 50,000 events were gated. Plots representative of 4 donors.



**FIGURE S3:** Uncropped version of representative gelatin zymogram shown in main figure 5c.

Dashed lines correspond to areas where gel was cut.