Metformin alters human host responses to *Mycobacterium tuberculosis* in healthy subjects

Authors:

Ekta Lachmandas[1,†], Clare Eckold[2,†], Julia Böhme[3], Valerie A.C.M. Koeken[1], Mardiana Binte Marzuki[3], Bastiaan Blok[1], Rob J.W. Arts[1], Jinmiao Chen[3], Karen W. W. Teng[3], Jacqueline Ratter[1,4], Elise J. Smolders[1], Corina van den Heuvel[1], Rinke Stienstra[1,4], Hazel M. Dockrell[2], Evan Newell[3], Mihai G Netea[1,6], Amit Singhal[3,5], Jacqueline M. Cliff[2], Reinout van Crevel[1,*]

†These authors contributed equally to the manuscript

*Corresponding Author

Affiliations:

[1]Department of Internal Medicine and Radboud Center for Infectious Diseases, Radboud University Medical Center, Nijmegen, Netherlands.

[2]Department of Immunology and Infection, Faculty of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, Keppel Street, London WC1E 7HT, United Kingdom.

[3]Singapore Immunology Network, Agency for Science, Technology and Research (A*STAR), Singapore 138648, Singapore.

[4]Nutrition, Metabolism and Genomics Group, Division of Human Nutrition, Wageningen University.

[5]Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore 308232, Singapore.

[6]Human Genomics Laboratory, Craiova University of Medicine and Pharmacy, Craiova, Romania.

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Summary

Metformin has shown beneficial effects in a murine model of tuberculosis. Using in-vitro and in-vivo studies we show that metformin has beneficial effects on cellular metabolism, immune function and gene-transcription involved in innate host responses to *M. tuberculosis* in humans.

Abstract

Background

Metformin, the most widely administered diabetes drug, has been proposed as a candidate adjunctive host-directed therapy for tuberculosis, but little is known about its effects on human host responses to *Mycobacterium tuberculosis.*

Methods

We investigated in-vitro and in-vivo effects of metformin in humans.

Results

Metformin added to peripheral blood mononuclear cells from healthy volunteers enhanced in-vitro cellular metabolism whilst inhibiting the mammalian target of rapamycin (mTOR) targets p70S6K and 4EBP1, with decreased cytokine production and cellular proliferation, and increased phagocytosis. Metformin administered to healthy human volunteers led to significant down-regulation of genes involved in oxidative phosphorylation, mTOR signaling and type I interferon response pathways, particularly following stimulation with *M. tuberculosis*, and upregulation of genes involved in phagocytosis and reactive oxygen species (ROS) production was increased. These in vivo effects were accompanied by a metformin-induced shift in myeloid cells from classical to non-classical monocytes. At a functional level, metformin lowered *ex vivo* production of TNF-α, IFN-γ and IL-1β but increased phagocytosis and ROS production.

Conclusion

Metformin has a range of potentially beneficial effects on cellular metabolism, immune function and gene-transcription involved in innate host responses to *M. tuberculosis.*

Keywords: metformin; tuberculosis; host-directed therapy; anti-mycobacterial mechanisms, gene transcription

Introduction

Diabetes increases susceptibility to tuberculosis [1] and worsens tuberculosis outcome [2]. The mechanisms behind this increase in susceptibility are unclear and a role for diabetes drugs could be envisioned. In particular, the diabetes drug metformin is anti-inflammatory and inhibits pathways such as mammalian target of rapamycin (mTOR) signalling, which are important in the host defence to *M. tuberculosis* [3]. Nonetheless metformin has been demonstrated to enhance mycobacterial clearance in mice [4] and is associated with lower rates of *M. tuberculosis* infection in humans [5]. Adding to that, the use of metformin in humans has been associated with a plethora of positive effects, potentially linked to glycaemic control, such as a reduced risk of developing active TB [6, 7], lower TB mortality [8], increased TB treatment success, reduced TB-relapse [9] and enhanced culture conversion [9, 10].

Proposed mechanisms for metformin's beneficial effects include an increase in mitochondrial reactive oxygen species (mROS) and enhanced killing of *M. tuberculosis* but none of these have been investigated in humans. Importantly the mechanism of action behind metformin's effects are not clearly defined as metformin acts through several pathways including mitochondrial complex I inhibition, an increase in AMP/ATP levels leading to increased AMP activated kinase (AMPK) signaling, and decreased glucagon and mTOR signaling [11]. Lastly it is challenging to study the effects of metformin in people living with diabetes as characteristics of diabetes such as hyperglycaemia, dyslipidaemia, vitamin D deficiency and oxidative stress may all affect immune responses to *M. tuberculosis* [12].

We therefore investigated the effects of metformin in humans without diabetes. We first characterised metformin's effects on *in-vitro* responses to *M. tuberculosis* and then validated these findings *in vivo* in healthy volunteers, showing that metformin alters mTOR signaling, inhibits p38 and AKT, rewires blood cellular landscape and enhances anti-*M. tuberculosis* responses.

Methods

Healthy Volunteers and Functional laboratory assays

In the *in vivo* study 11 healthy Dutch adults were given metformin in increasing doses ending with a commonly used dose of 1000 mg twice a day. For all other in vitro experiments blood from healthy Dutch adults (estimated tuberculosis incidence 1.5/100,000) was subject to analysis in the presence or absence of metformin. Isolated PBMCs, $CD14^+$ monocytes or M1 / M2 macrophages were stimulated with *M. tuberculosis* lysate for production of tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-10, IL-17A, IL-22 and interferon gamma (IFN-γ). Proliferation of $CD4^+$ cells was measured by flow cytometry of Carboxyfluorescein succinimidyl ester (CFSE) labelled PBMCs stimulated for 6 d with *M. tuberculosis* lysate. Metabolic measurements included lactate production in stored cell culture supernatants, the NAD^+/NADH redox ratio in cell lysates, glucose consumption and mitochondrial mass and potential. Activation of downstream mTOR targets signalling was assessed by western blot of phosphorylated(p)-AMPK, p-p70 S6K, p-4EBP1, p-P38 and p-AKT . Production of Reactive Oxygen Species (ROS) was determined after incubation of whole blood or PBMCs with zymosan or *M. tuberculosis* lysate by measurement of chemiluminescence after the addition of luminol. Phagocytosis was measured in PBMCs using pHrodo® Green Zymosan Bioparticles® Conjugate and flow cytometry. *M. tuberculosis* infection was measured in PBMCs incubated with *M. tuberculosis* (H37Rv) at a multiplicity of infection (MOI) of 5 for 3 hours, lysed, and cultured on Middlebrook 7H11. Cellular viability of PBMCs was assessed by flow cytometry of Annexin V-FITC and propidium-iodide stained PBMCs.

Transcriptomics

RNA-Seq (GSE102678) analysis was performed on participants' samples pre- and post-metformin administration, directly on *ex vivo* whole blood and on isolated PBMC following incubation with *M. tuberculosis* lysate. Libraries were prepared using stranded preparation reagents from Illumina and sequenced on a NextSeq500, generating ~36-45M million 43bp paired-end reads per sample. Sequence files were aligned to the human genome and aligned reads were counted. Differentially expressed genes were determined using the R package DESeq2, and gene set analyses were performed to determine how metformin affected biological pathways *in vivo* and in the *in vitro* response to *M. tuberculosis*. qRT-PCR was performed to validate RNA-Seq and functional assay results.

CyTOF marker labelling, data acquisition and analysis

PMA and ionomycin stimulated PBMCs were stained with heavy-metal isotope-labeled antibodies (Table E1) [13], barcoded and were acquired on CyTOF 1 (Fluidigm). Samples were de-barcoded using manual gating in FlowJo and analysis of live $CD14^{+/-}CD16^{+/-}$ monocytes was carried out using the t-distributed stochastic neighbor embedding (tSNE) dimension reduction and Phenograph-based clustering algorithm [14]. See Supplementary Methods for details on Mass Cytometry and statistical analysis.

Statistics

All values are expressed as the mean ± SEM of individual samples. Unless otherwise specified data analysis was performed using GraphPad Prism Software (GraphPad Software Inc.) using paired *t*-test or Wilcoxon signed-rank test.

Study Approval

Written informed consent was received from participants prior to inclusion in the study. Experiments were conducted according to the principles expressed in the Declaration of Helsinki. Both for the *in vitro* (NL32357.091.10) and healthy volunteers (NL47793.091.14) studies ethical approval was granted by the Arnhem-Nijmegen Ethical Committee. As validation EDTA blood from 10 healthy young subjects given metformin (500 mg day 1-2) increasing to 1000 mg (day 3-8) was examined as part of a pharmacokinetic study (NL53534.091.15). The human RNA Seq study was approved by the LSHTM Research Ethics Committee (#11968).

Results

Metformin regulates cellular metabolism and cytokine production in humans

We assessed the effects of metformin on glycolytic metabolism in human cells. When added to *M. tuberculosis* lysate-stimulated PBMCs from healthy individuals metformin increased lactate production and glucose consumption (Fig. 1A and 1B) whilst decreasing the NAD^+/NADH ratio (Fig. 1C). At both therapeutic (10 – 220 μM) and experimental concentrations [15] metformin showed clear effects on cytokine production. Depending on cell type different concentrations of metformin significantly decreased *M. tuberculosis* lysate-induced (i) TNF-α, IL-10, IFN-γ and IL-17 production from PBMCs (Fig. 2A), (ii) IL-1β, IL-6 and IL-10 from M1 and M2 monocyte derived macrophages (Fig. 2B) and (iii) TNF-α, IL-1 β and IL-10 from $CD14^+$ monocytes (Supplemental Fig. S1A). At a transcriptional level metformin inhibited expression of *IL-18*, *IL-23p19* and *TGF-β1* genes (Fig. 2C). The minimal effect of metformin on cellular proliferation (Fig. 2D) is unlikely to account for the strong effects on cytokine production (Fig. 2E). Finally, although only suggestive, metformin also decreased the phosphorylation levels of the downstream mTOR targets, phospho-p70S6K and phospho-4EBP1, whilst increasing phosphorylation of its known molecular target, AMPK (Supplemental Fig. S1B). Metformin at the doses tested also had no significant effect on cellular viability (Supplemental Fig. S1C).

Transcriptional profiling reveals a metformin-related gene expression signatures in humans

Next, we investigated the *in vivo* effect of metformin. Healthy subjects took standard dose metformin and blood was drawn at several time-points before and after metformin intake

(Fig. 3A). As expected phospho-AMPK was increased in both unstimulated and *M. tuberculosis* lysate stimulated PBMCs after metformin intake (Td6 vs Td0) (Fig. 3B – 3D and Supplemental Fig. S2A). In genome-wide (unbiased) transcriptional analysis using RNA sequencing (RNAseq) on whole blood, metformin intake had no significant effects on individual genes (Supplemental Fig. S2B). Instead, a consistent metformin-mediated effect was observed on combined sets of genes (Fig. 3E), including a significant downregulation of OXPHOS and ribosome pathways and a significant upregulation of endocytosis/phagocytosis, MAPK and chemokine signaling pathways.

In PBMCs, metformin intake led to differential expression of approximately 800 genes, both in unstimulated and *M. tuberculosis* lysate stimulated cells (Supplemental Fig. S2C). In unstimulated PBMCs, metformin intake led to upregulation of genes involved in mitosis, and downregulation of genes involved in OXPHOS, adipogenesis and myc targets (Fig. 3F). In *M. tuberculosis* stimulated PBMCs, metformin intake led to suppression of genes involved in (i) signaling of cytokines such as IFN-α, IFN-γ and TNF-α, (ii) OXPHOS and (iii) mTOR (Fig. 3F) all in line with the *in vitro* effects of metformin (Fig. 1 and Fig. 2 respectively).

Cytokine responses to M. tuberculosis are suppressed by metformin in vivo

Each gene ontology (GO) group in the identified gene sets was investigated and the "response to type 1 interferon" GO set showed the most markedly reduced expression in *ex vivo M. tuberculosis* lysate-stimulated PBMCs from individuals taking metformin (Supplemental Fig. S2D). Within this GO, the expression of eight genes (Interferon-induced protein with tetratricopeptide repeats (*IFIT*) *1*, *IFIT 2* and *IFIT 3*, 2'-5'-

oligoadenylate synthase (*OAS*) *1*, *OAS2* and *OAS3*, MX dynamin like GTPase (*MX*) *1* and radical S-adenosyl methionine domain containing 2 (*RSAD2*) was more than two-fold reduced following metformin administration in cells stimulated with *M. tuberculosis* lysate for 4 hours (Fig. 4A), and to a lesser extent at 24 hours, as shown by qRT-PCR (Fig. 4A). Additionally, metformin intake led to a significant decrease in TNF-α, IL-1β, IL-6, IFN-γ and IL-17 release in response to *M. tuberculosis* lysate (Fig. 4B), with effects on cytokine production up to 21 d post metformin intake. Collectively, our results indicate that metformin inhibits *M. tuberculosis*-induced type 1 interferon response and inflammation in human PBMCs.

Metformin regulates the AKT-mTOR pathway and mitochondrial metabolism in humans

The MAPK, AKT and mTOR pathways are known to strongly influence cytokine production and so respectively the levels of phospho- and total-P38 (Fig. 4C and Supplemental Fig. S3A), phospho-AKT and phospho-4EBP1 (Fig. 4D and Supplemental Fig. S3B) were measured in PBMCs pre- and post-metformin intake. An overall decrease in the phosphorylation of all three targets were observed. Quantitative band intensity analysis showed that the ratio of p-P38 to total-P38, the levels of p-AKT/actin and p-4EBP1/actin were in most cases significantly reduced due to metformin intake (Fig. 4E). Supplemental Figs. S3C-E demonstrate the effects on phosphorylation at an individual level. For further evidence we analysed the effect of metformin on the gene expression levels of these enzymes and found a decrease in expression of AKT2 (fitting with metformin's role in homeostasis) and an increase in PRKAB2 (a regulatory subunit of AMPK) (Supplemental Fig. S3F). As AKT and mTOR are central metabolic regulators [16,

17] we investigated the effects of metformin on mitochondrial mass (Supplemental Fig. S4A). Metformin increased the mitochondrial mass of $CD14^{+}CD16^{-}$ classical monocytes as demonstrated by increased Mitotracker green median fluorescence intensity (MFI; Fig. 4F). This increase was not observed for $CD14^{-}CD16^{+}$ non-classical monocytes (Supplemental Fig. S4B). This highlights metformin mediated alterations in mitochondrial functionality in $CD14^{+}CD16^{-}$ classical monocyte which may correlate with the anti-inflammatory effect of metformin [18, 19].

Metformin modulates the peripheral monocyte landscape in humans

Metformin intake altered the number and distribution of circulating immune cells. In whole blood metformin led to a transient increase in total white blood cells (WBC) and neutrophils (Fig. 5A) without altering the relative distribution of cell types (Fig. 5B). In PBMCs metformin increased the proportion of monocytes and decreased the proportion of lymphocytes (Fig. 5C).

To achieve a single cell systems-level perspective of the effect of metformin on monocytes, PBMCs from pre- (Td0) and post-metformin intake (Td6) blood, were stimulated with phorbol ester and ionomycin, stained with a panel of 38 surface and intracellular cytokine markers (Table S1) and analysed using CyTOF [20]. We first verified the panel antibodies for their binding to the PBMCs (Supplemental Fig. S5) and then gated out the pure population of monocytes ($CD3^{-}CD19^{-}CD56^{-}\gamma\delta TCR^{-}Vd1^{-}VD2^{-}CD57^{-}CD161^{-}CD14^{+/-}CD16^{+/-}$) for analysis (Supplemental Fig. S6). Analysis of monocytes using tSNE in conjunction with a phenograph clustering algorithm [14, 21] identified 12 distinct

cell clusters with shared surface and intracellular marker expression characteristics (Fig. 5D and 5E). Based on the expression of CD14, CD16 and CCR2 the 12 clusters were divided into 5 monocyte subsets (Fig. 5E), illustrating significant heterogeneity among the classical and non-classical monocyte population in humans. Three out of 12 clusters were found to be significantly enriched or depleted in Td6 samples compared to Td0. These differentiated clusters included diverse activated phenotypes, i.e. $CD14^{hi}CD16^{-}MIP\text{-}1\beta^{+}IL\text{-}2^{-}TNF\alpha^{-}$ (Cluster 2, downregulated); $CD14^{hi}CD16^{-}MIP\text{-}1\beta^{+}IL\text{-}2^{+}TNF\alpha^{-}$ (Cluster 10, downregulated) and $CD14^{lo}CD16^{lo}MIP\text{-}1\beta^{+}IL\text{-}2^{-}TNF\alpha^{+}$ (Cluster 5, upregulated) (Fig. 5F). The accuracy of machine-learning automated gating when validated by manual gating indeed showed that clusters 2 and 10 were $CD14^{hi}CD16^{-}$ whereas cluster 5 was $CD14^{lo}CD16^{mid}$ (Supplemental Fig. S7A). Furthermore, when assessed for cytokine secretion by manual gating, only cluster 5 and 10 was found to express TNFα and IL-2 respectively (Fig. 5F), similar to as identified by tSNE analysis; while all three clusters (cluster 5, 10 and 2) were found to express MIP-1β (Fig. 5F) confirming the tSNE analysis. The manual gating strategy also indicated a trend towards a decreased total population frequencies of $CD14^{hi}CD16^{-}$ classical monocytes or increased $CD14^{-}CD16^{+}$ non-classical monocytes (Supplemental Fig. S7B). Collectively, our results delineate the effect of metformin on the functional capacity of heterogeneous peripheral monocytes.

Metformin enhances innate host defense pathways in exposed human leukocytes

Metformin intake showed clear effects on innate host defense mechanisms. ROS production was strongly upregulated in whole blood in samples immediately post metformin treatment (Td6), both spontaneously and upon stimulation with *M. tuberculosis*

lysate and zymosan (Fig. 6A). In line with increased ROS production in whole blood, genes involved in ROS production such as NADPH Oxidase 2 (*CYBB*), p22-PHOX (*CYBA*), *RAC1* and particularly for ROS production in neutrophils p47-PHOX *(NCF1*), p67-PHOX (*NCF2*) and p40-PHOX (*NCF4*) were strongly upregulated in blood after metformin intake (Fig. 6B). The increase in ROS did not correlate with an increase in white blood cell counts or neutrophil counts (Supplemental Fig. S8B and S8C). No increase in ROS was observed in isolated PBMCs (Supplemental Fig. S8A).

Whole blood RNAseq analysis revealed that metformin upregulated genes involved in endocytosis such as receptors (RTKs and GPCR), regulators of clathrin-mediated pit formation (*AP2*) and clathrin uncoating (Hsp70) and regulators of intracellular vesicular trafficking (Arfs, ArgGAPs and ArfGEFs) (Supplemental Fig. S9A). Increased phagocytosis following metformin intake was confirmed in a second group of healthy subjects taking metformin, using zymosan labelled beads in whole blood (Fig. 6C). The increase in phagocytosis correlated with an increase in WBC counts but not neutrophil counts (Supplemental Fig. S9B). Furthermore *in vitro* metformin pre-treated PBMCs also showed upregulated phagocytosis (Supplemental Fig. S9C). Finally, we examined the effect of metformin on the killing of *M. tuberculosis*. Out of eight subjects, metformin led to restricted *ex vivo* growth of *M. tuberculosis* in four subjects. Overall there were no significant differences (Fig. 6D). The CFU results were unaffected by normalization to monocyte numbers.

Discussion

A study in mice and retrospective human data suggest that metformin, the most widely used diabetes drug, may improve outcome of tuberculosis [4, 6, 8]. We examined how metformin modulates the peripheral immune cell distribution, its gene expression and its functional output in humans using high dimensional phenotypic and RNA analyses. Metformin administration was found to dampen pro-inflammatory cytokine production whilst promoting phagocytosis and ROS production, possibly through the generation of non-classical monocytes, which are implicated in trained innate immunity [22]. These functional changes were associated with an inhibition of the type 1 interferon pathway, and a decrease in p-AKT and p-P38 signaling and an increase in AMPK signaling. Our data are in line with increasing evidence that metformin possesses anti-inflammatory properties, considered to be mediated in part via alterations in cellular metabolism [23].

A strong effect of metformin on inflammatory cytokine signalling was observed both *in vitro* and *in vivo*. Metformin inhibited the type I interferon response by blocking the expression of interferon-stimulated genes *IFIT1*, *IFIT2* and *IFIT3*, which amongst other activities, regulate inflammatory cytokine mRNA stability, cell proliferation and apoptosis [24]. Neutrophil driven type 1 interferon signaling in blood, including upregulated *IFIT1*, *IFIT2*, *IFIT3* and genes similar to those in our data [25], but not type 1 IFNs themselves, have been identified as a signature of active tuberculosis disease [26] and inhibiting this pathway using zileuton, an arachidonic acid metabolism modulator, protects mice from tuberculosis [27]. Our data show that metformin can down-regulate the type-1 interferon pathway in humans.

ROS production and phagocytosis were increased by metformin and this was not explained by altered cell counts, suggesting that the observed effects are intrinsically mediated by metformin. This is supported by the accompanying transcriptional changes observed in both ROS and phagocytosis related genes and the increase in phagocytosis induced by metformin *in vitro*. Mechanistically, AMPK activation has been linked to phagocytosis activity as pharmacologic [28, 29] or genetic ablation [30, 31] of AMPK subunits negatively influenced phagocytosis. It will be interesting to investigate the effect of metformin on autophagy in future studies and to determine how it compares with an elegant study showing that autophagic capacity does not correlate with *M. tuberculosis* susceptibility in mice [32]. As ours is the first exploratory study of the effects of metformin on host defense *in vivo* in non-diabetic individuals future studies should examine the effect of metformin on the phagocytic capacity of specific cell types such as macrophages and dendritic cells.

Metformin intake increased *ex vivo* mycobacterial killing capacity of PBMCs in some individuals but not all. In earlier work, we found that mycobacterial survival decreased in metformin-treated human macrophages [4]. This effect of metformin was reversed by the inclusion of ROS-scavenging agents. It is possible that five days of metformin exposure *in vivo* is too short, that the effect of metformin on killing capacity of PBMCs is somewhat lost during cryopreservation, or that other cells such as neutrophils contribute to the anti-mycobacterial effects of metformin. Future studies could use bronchiolar lavage cells to investigate control mechanisms from the disease site rather than in peripheral blood. Alternatively, metformin could have subtle effects on mycobacterial killing and bigger

effects on ameliorating inflammation. Whilst pro-inflammatory cytokines are required for the control of *M. tuberculosis*, it is the balance between pro and anti-inflammatory cytokines that is important for the restriction of mycobacterial growth and prevention of overt pathology [33, 34]. Here, we found that metformin dampens the expression of pro-inflammatory cytokines whilst simultaneously enhancing anti-mycobacterial processes such as phagocytosis and ROS.

In mice, we have previously shown metformin-mediated restriction of *M. tuberculosis* outgrowth [4] although another study found no additive effect of metformin when combined to the standard tuberculosis treatment [35]. In diabetic tuberculosis patients, metformin use has been linked with more rapid culture conversion [9], particularly in patients with cavitary lung disease and high bacterial burden [10], and with better treatment outcomes [9], indicating that the net result of all the effects of metformin is enhanced mycobacterial control *in vivo*. In a cohort of 296 diabetic tuberculosis patients in Singapore [4] metformin was associated with lower mortality and a similar association was found amongst a cohort of 634 diabetic patients in Taiwan [8]. However, neither of these two cohort studies included microbiological data. The survival difference could equally be explained by the well-known beneficial effects of metformin on cardiovascular mortality or its immuno-modulating effects as found in this study. Future clinical trials in non-diabetic tuberculosis patients will help establish the effect of metformin on clinical and microbiological outcome of tuberculosis treatment.

Metformin is put forward as a candidate for host-directed therapy in tuberculosis but some caution is warranted. For example, in a model of candidemia metformin resulted in increased lethality [36]. Also, it is unknown if tuberculosis or concurrent use of anti-tuberculous drugs increase the risk of metformin-associated gastrointestinal side-effects or lactic acidosis [37]. With regard to possible drug interactions, a recent study in diabetic tuberculosis patients has shown that rifampicin increases metformin exposure, but does not alter blood glucose levels.

In summary metformin effectively modulates the balance between inflammation and effective host responses to *M. tuberculosis*. It ameliorates the pathological inflammatory responses associated with tuberculosis whilst enhancing anti-mycobacterial processes such as ROS and phagocytosis in humans.

Conflict of interest: A.S. holds the patent with respect to the use of metformin for controlling mycobacterial infection, WO2014039011A1. Other authors declared no conflict of interest.

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Corresponding Author Information:

Dr. Reinout van Crevel

E-mail: reinout.vancrevel@radboudumc.nl

Department Internal Medicine, Infectious Diseases

Radboud University Nijmegen Medical Centre

Internal code 456, P.O. Box 9101

Geert Grooteplein Zuid 8, 6500 HB Nijmegen

The Netherlands

Alternative Corresponding Author

Prof Dr Mihai Netea

E-mail: Mihai.Netea@radboudumc.nl

Department Internal Medicine, Infectious Diseases
Radboud University Nijmegen Medical Centre
Internal code 456, P.O. Box 9101
Geert Grooteplein Zuid 8, 6500 HB Nijmegen
The Netherlands

Author Contributions:

E.L. designed, performed and analysed the experiments, conducted the trial and wrote the paper. C.E., J.M.C, H.M.D. performed and analysed the RNA-seq data and wrote and reviewed the paper. V.K., B.B., R.J.W.A. and C.V.D.H helped with the trial and performed experiments. J.B. and M.B.M. performed and analysed mycobacterial killing and mitochondrial experiments. J.C., K.W.W.T., and E.N. performed and/or analysed the CyTOF data. A.S. analysed the mycobacterial experiments, CyTOF data and wrote the paper. M.G.N. and R.V.C supervised the entire study, designed experiments, conducted the trial, analysed data and wrote the paper.

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Figure Legends

Fig. 1. Metformin alters mTOR signalling axis whilst maintaining glucose regulatory effects. (A) Lactate production, (C) glucose consumption and (D) NAD+/NADH fold change in PBMCs stimulated with *M. tuberculosis* lysate in the presence or absence of 1000µM metformin for 24h, 48h, or 7d. For A, data are from two individual experiments. For A-C data are shown as means ± S.E.M. from 2-3 experiments/6- 9 donors. * $p<0.05$, ** $p<0.01$ (Wilcoxon matched-pairs signed rank test).

Fig. 2. Metformin affects the cytokine profile of human cells stimulated with *M. tuberculosis*. Cytokine production from (A) human PBMCs and (B) monocyte-derived M1 and M2 macrophages stimulated with *M. tuberculosis* lysate +/- 3–3000µM of metformin for 24h (TNF-α, IL-6, IL-1β and IL-10) or 7 d (IFN-γ, IL-17 or IL-22), and (C) cytokine gene expression in CD14+ monocytes stimulated with *M. tuberculosis* lysate +/- 3000µM metformin after 4 h (IL-18 and TGF-β1) or 24 h (IL-23p19 and IL-12p35 subunits). (D) Percentage $CD4^+$ T cell proliferation in PBMCs stimulated with *M. tuberculosis* lysate in the presence or absence of 300 µM metformin for 6 d, using CFSE labelling to track generations. (E) Radial graph representing fold-change in cytokines from PBMCs stimulated with *M. tuberculosis* lysate in the +/- 3000µM metformin, relative to stimulation in absence of metformin. Values < 1 indicate reduced cytokine production. This is indicated by projection towards the centre of the radius. For A-C and E all data (mean ± s.e.m.) are from 3 experiments/6-13 donors. For D data are (mean ± s.e.m.) from four experiments/ 7 donors. * $p<0.05$, ** $p<0.01$ (Wilcoxon matched-pairs signed rank test for A-C and Paired t-test for D.

Fig. 3. Global effects of metformin in healthy human volunteers. (A) Healthy volunteers (n =11) received an increasing dose of metformin for five consecutive days. Blood was drawn twice pre-(TdB) and several times post-metformin treatment. (B) Western blot analysis of p-AMPK in lysates of PBMCs, collected from healthy volunteers before and after metformin intake and stimulated for 2h with RPMI(-) or *M. tuberculosis* lysate (+): four representative donors are shown. (C) Quantitative relative band intensity analysis of p-AMPK between pre- (Td0) and post-metformin (Td6) periods for RPMI and *M. tuberculosis* lysate stimulation: data are mean ± S.E.M. from eight donors. (D) Fold change in p-AMPK levels between pre- (Td0) and post-metformin (Td6) periods for RPMI and *M. tuberculosis* lysate stimulation for eight donors. * $p<0.05$, ** $p<0.01$ (Paired t test). All western blot data depicted here are normalized to the loading control actin. (E) Gene set analysis from RNA-Seq data showing KEGG pathways which were differentially expressed in *ex vivo* blood samples following metformin administration. The bar length indicates the magnitude of the change of expression of the gene set. Data were analyzed using the Piano R package, and pathways with adjusted $P<0.01$ are shown. (F) Hallmark gene set enrichment and network analysis, showing gene sets up- (red) or down- (blue) regulated following metformin administration in PBMCs in either resting state or stimulated with *M. tuberculosis* lysate for 4h. The colour intensity indicates the adjusted P-value for the gene set enrichment.

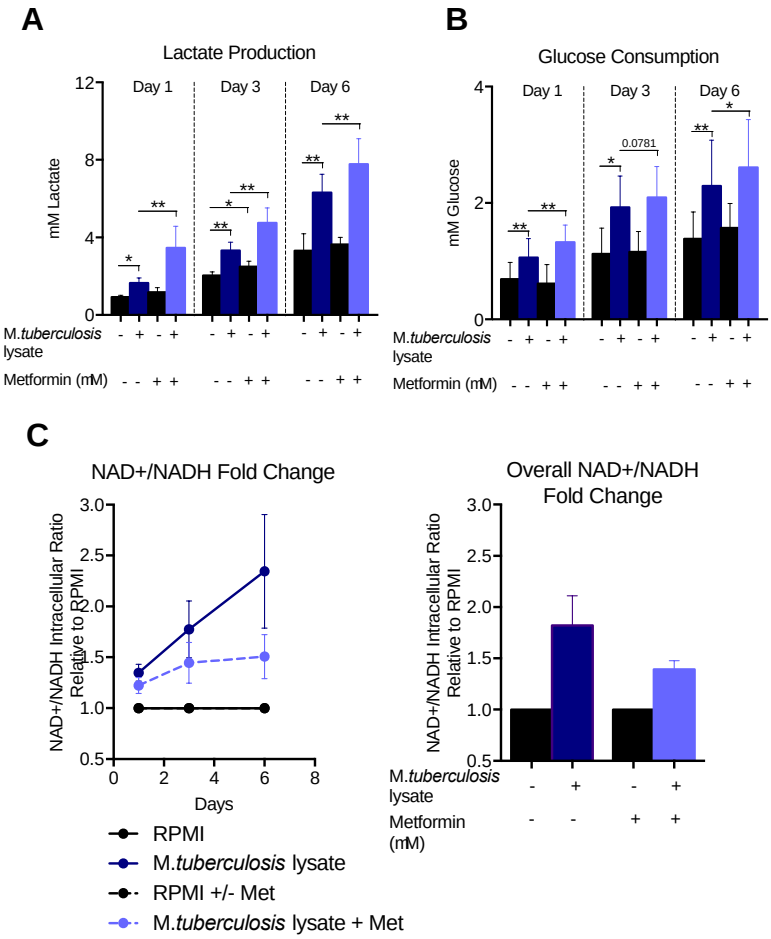
Fig. 4. Metformin intake in healthy volunteers affects cytokine production via P38 and AKT inhibition. (A) Expression of eight genes in the “response to type 1 interferon” Gene Ontology group in PBMCs stimulated with *M. tuberculosis* lysate *in vitro* for 4 or 24 h,

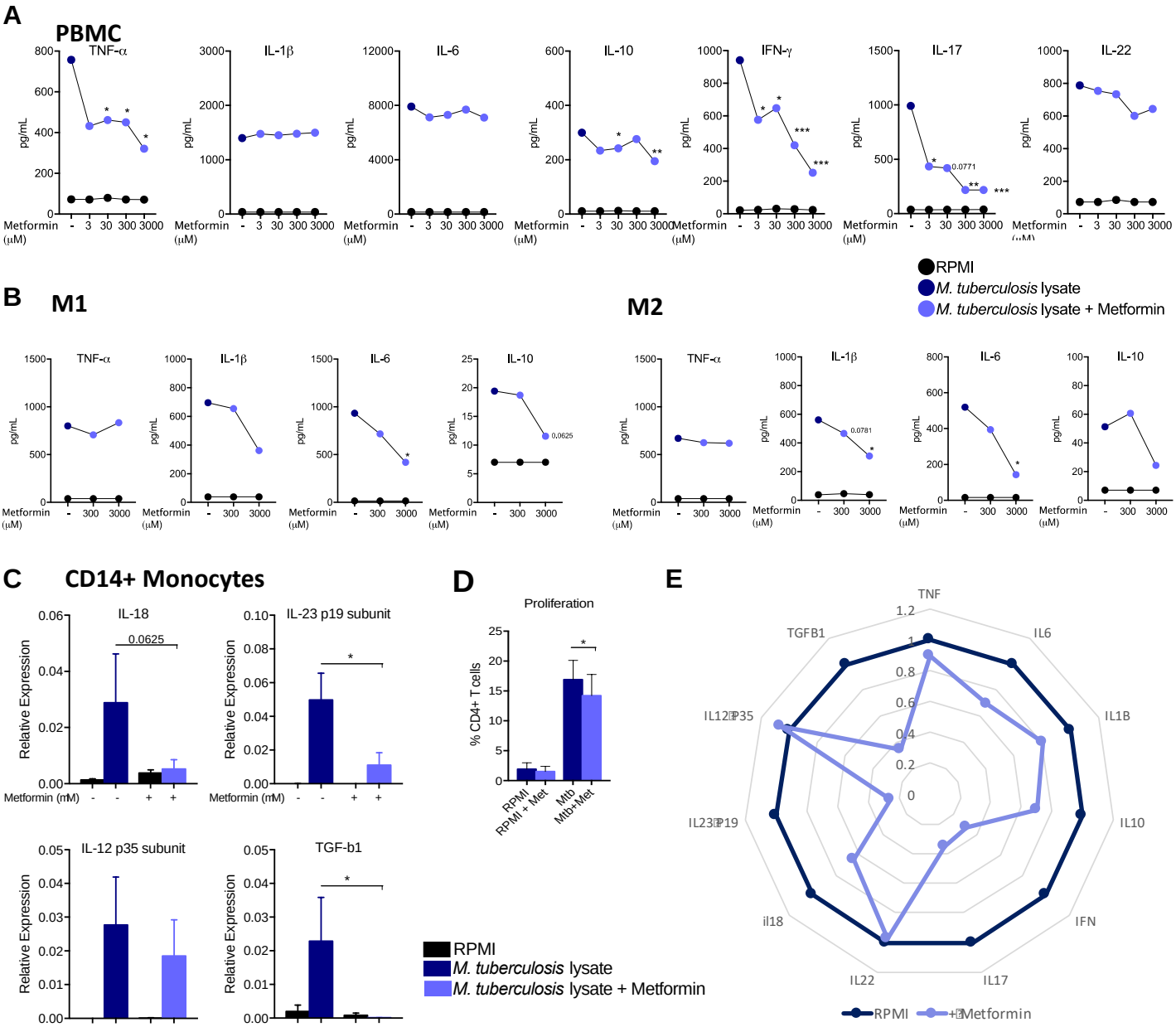
before and after *in vivo* metformin administration in healthy volunteers. Expression measured by RNA-Seq (4hr) and qRT-PCR (4 and 24hr). (B) Cytokine production from isolated PBMCs stimulated with *M. tuberculosis* lysate 24h (TNF-α, IL-6, IL-1β and IL-10) or after 7d (IFN-γ, IL-17 or IL-22) in the presence of 10 % pooled human serum before and after metformin intake. (C) Western blot analysis of p-38 and Total p38 and (D) p-AKT and p-4EBP1 levels in lysates of PBMCs stimulated for 2h RPMI (-) or *M. tuberculosis* lysate (+) from healthy volunteers before and after metformin intake. Data are representative of four of eight measured donors from the trial. All western blot data depicted here are normalized to the loading control actin. (E) Fold change in p-38/Total p38 levels, p-AKT/actin or p-4EBP1/actin between pre- (Td0) and post-metformin (Td6) periods for RPMI and *M. tuberculosis* lysate stimulation. (F) Mitochondrial mass assessment in $CD14^{+}CD16^{-}$ monocytes: left panel – overlay of before and after metformin from same individual, right panel - MFI of MitoTracker Green from n=3 samples. Grey – FMO control. * $p<0.05$, ** $p<0.01$ (Paired t test). All western blot data (mean ± S.E.M.) are representative of a total of eight donors presented in (C) or (D) or Supplementary Fig. 3A or 3B.

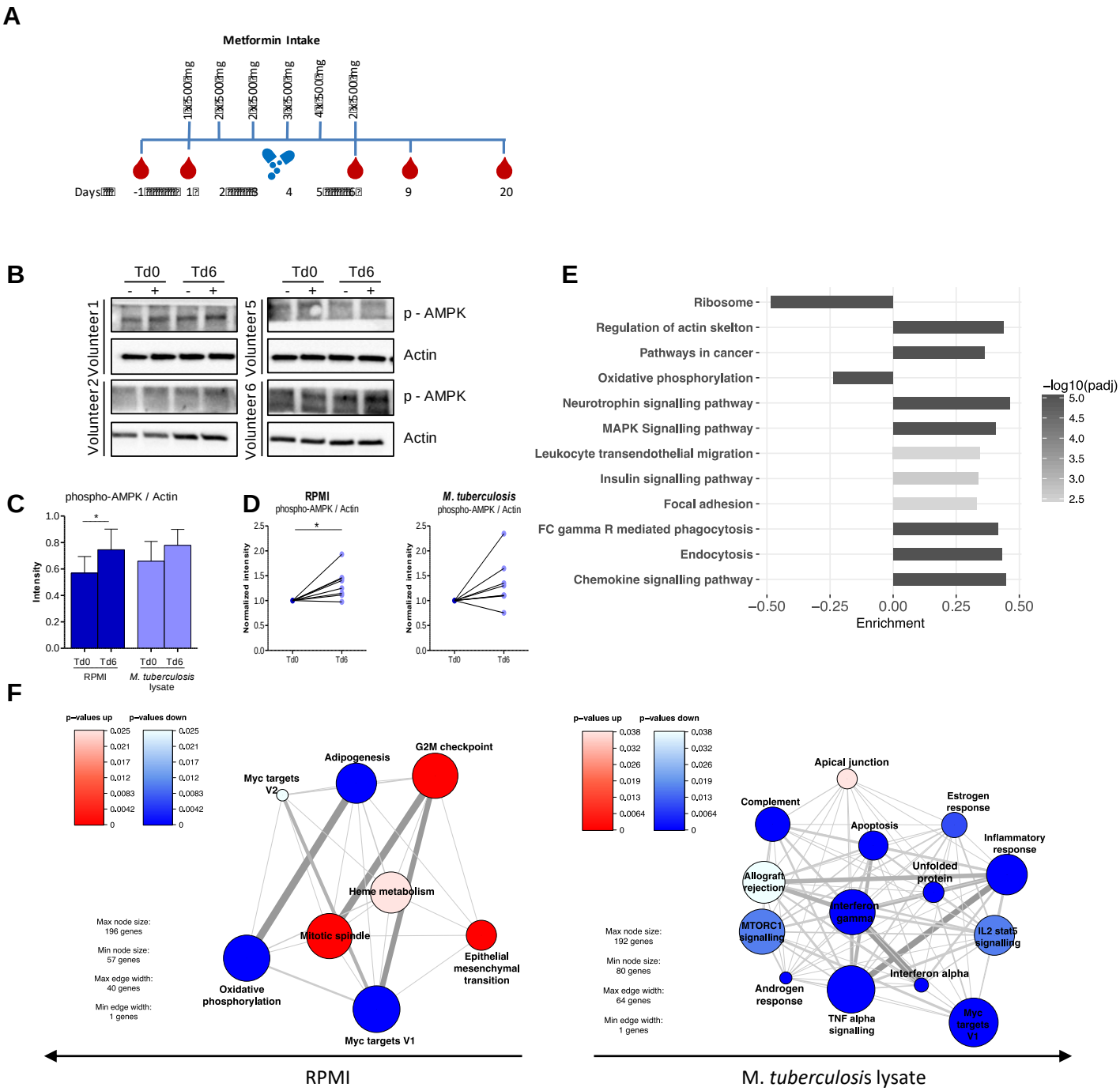
Fig. 5. Metformin intake in healthy volunteers alters the blood cellular composition landscape. Analysis of leukocyte counts plotted (A) as raw cell counts for whole blood, (B) as percentage of total counts for whole blood and (C) as percentage of total counts for isolated PBMCs. (D) Cryo-preserved PBMCs before (Td0) and after (Td6) metformin intake were stimulated with PMA-ionomycin and analysed by mass cytometry. tSNE analysis of single-cell data from blood monocytes of analyzed samples. Cells were

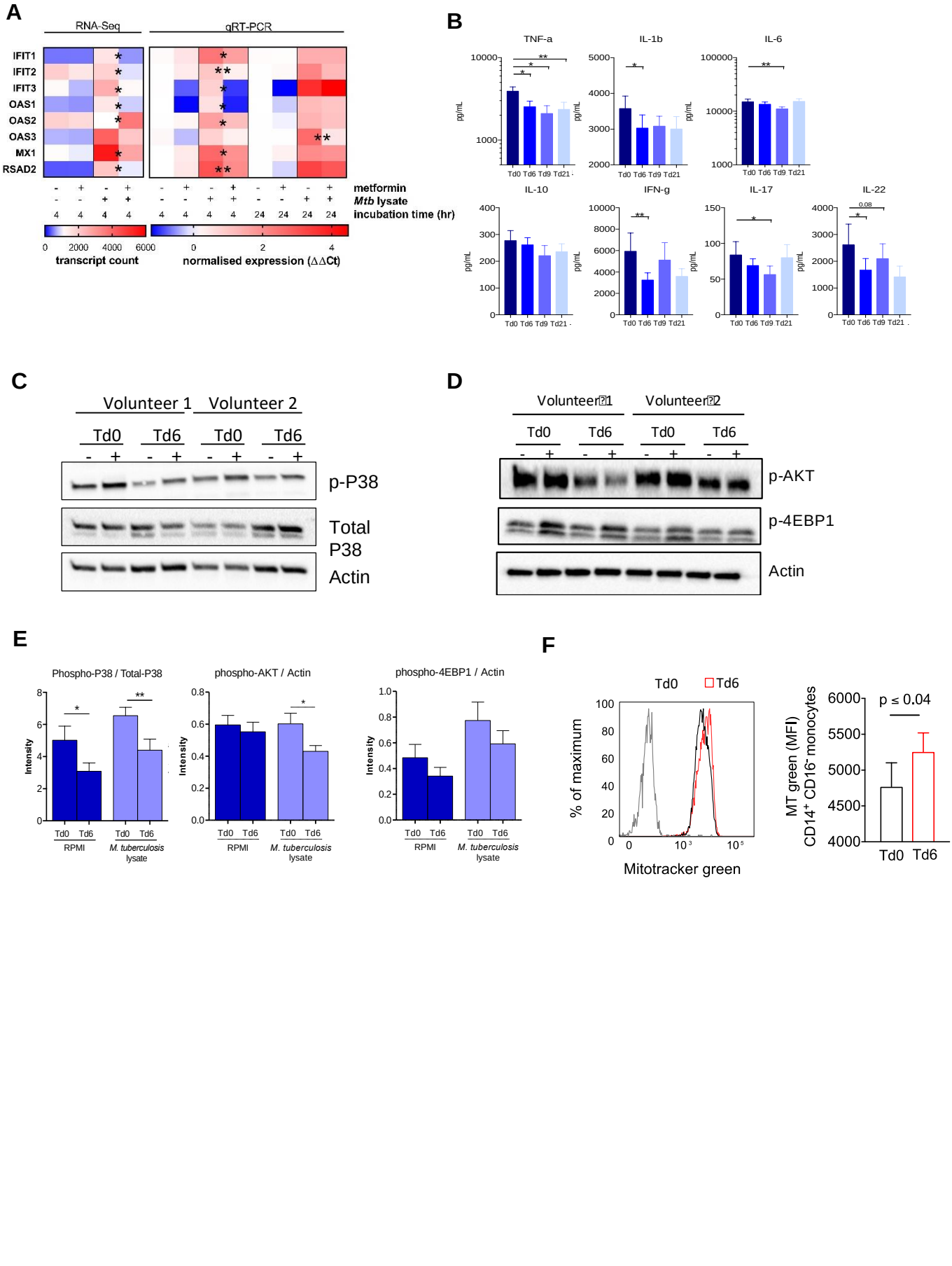
plotted and color-coded by the 12 ‘unsupervised’ phenograph clusters. (E) Heat-plot summary of average median expression of each marker analysed for the 12 clusters identified. 12 clusters are divided into five subsets based on the expression of CD14, CD16 and CCR2. (F) Mass cytometry data was analyzed by manual gating strategy. The 3 differentially regulated monocyte clusters were overlayed to assess the expression of cytokines. Table on right indicates the depiction of (in terms of + and -) which cluster express which cytokine based on the manual gating strategy.

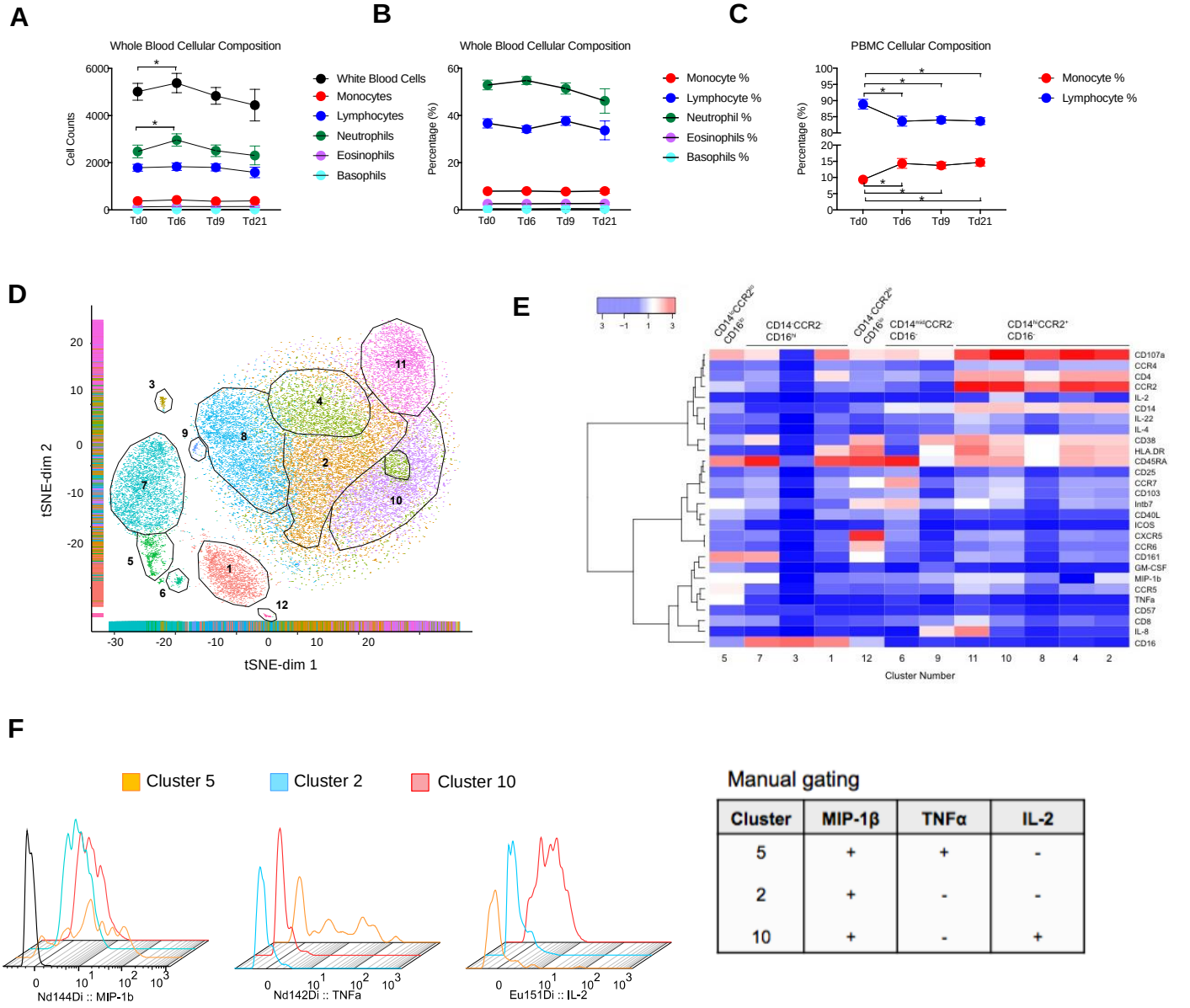
Fig. 6. Metformin intake in healthy volunteers affects ex-vivo anti-mycobacterial defence mechanisms but not *M. tuberculosis* outgrowth. (A) ROS production as measured by luminol-reaction from whole blood from pre- and post-metformin treated volunteers unstimulated (RPMI) or stimulated with *M. tuberculosis* lysate (Mtb) or zymosan. Data are representative of 11 individual donors. Bars representing the fold-change of Td6, Td9 or Td21 over Td0 for each individual donor are superimposed with grey dots representing the mean ± s.e.m. (B) Expression of six genes encoding key NADPH oxidase proteins for ROS production were assessed in ex vivo blood by RNA-Seq before and after administration of metformin in the healthy volunteers. * $p<0.05$, ** $p<0.01$ (Wilcoxon matched-pairs signed rank test). (C) Net phagocytosis of pHrodo conjugates in healthy volunteers given metformin for seven days. Lysed blood was incubated with the pH rodo suspension for 2 h in a non-CO2 elevated incubator at 37°C before measuring fluorescence. (D) Colony forming units (CFU)/mL between 24 h or 48 h and 3 h of infection of PBMCs from pre- and post-metformin treated volunteers infected with mycobacteria. Data was normalised to monocyte count.


A
Lactate Production
Day 1
Day 3
Day 6
mM Lactate
12
8
4
0
M.tuberculosis lysate
Metformin (mM)
B
Glucose Consumption
mM Glucose
4
2
0
0.0781
C
NAD+/NADH Fold Change
NAD+/NADH Intracellular Ratio Relative to RPMI
3.0
2.5
2.0
1.5
1.0
0.5
0
2
4
6
8
Days
RPMI
M.tuberculosis lysate
RPMI +/- Met
M.tuberculosis lysate + Met
Overall NAD+/NADH Fold Change


A
PBMC
TNF-α
IL-1β
IL-6
IL-10
IFN-γ
IL-17
IL-22
pg/mL
Metformin (μM)
- 3 30 300 3000
B
M1
M2
TNF-α
IL-1β
IL-6
IL-10
pg/mL
Metformin (μM)
- 300 3000
RPMI
M. tuberculosis lysate
M. tuberculosis lysate + Metformin
C
CD14+ Monocytes
IL-18
IL-23 p19 subunit
IL-12 p35 subunit
TGF-b1
Relative Expression
Metformin (mM)
- - + +
D
Proliferation
% CD4+ T cells
RPMI
RPMI + Met
Mtb
Mtb+Met
E
TNF
IL6
IL1B
IL10
IFN
IL17
IL22
il18
IL23P19
IL12P35
TGFB1
RPMI
+Metformin

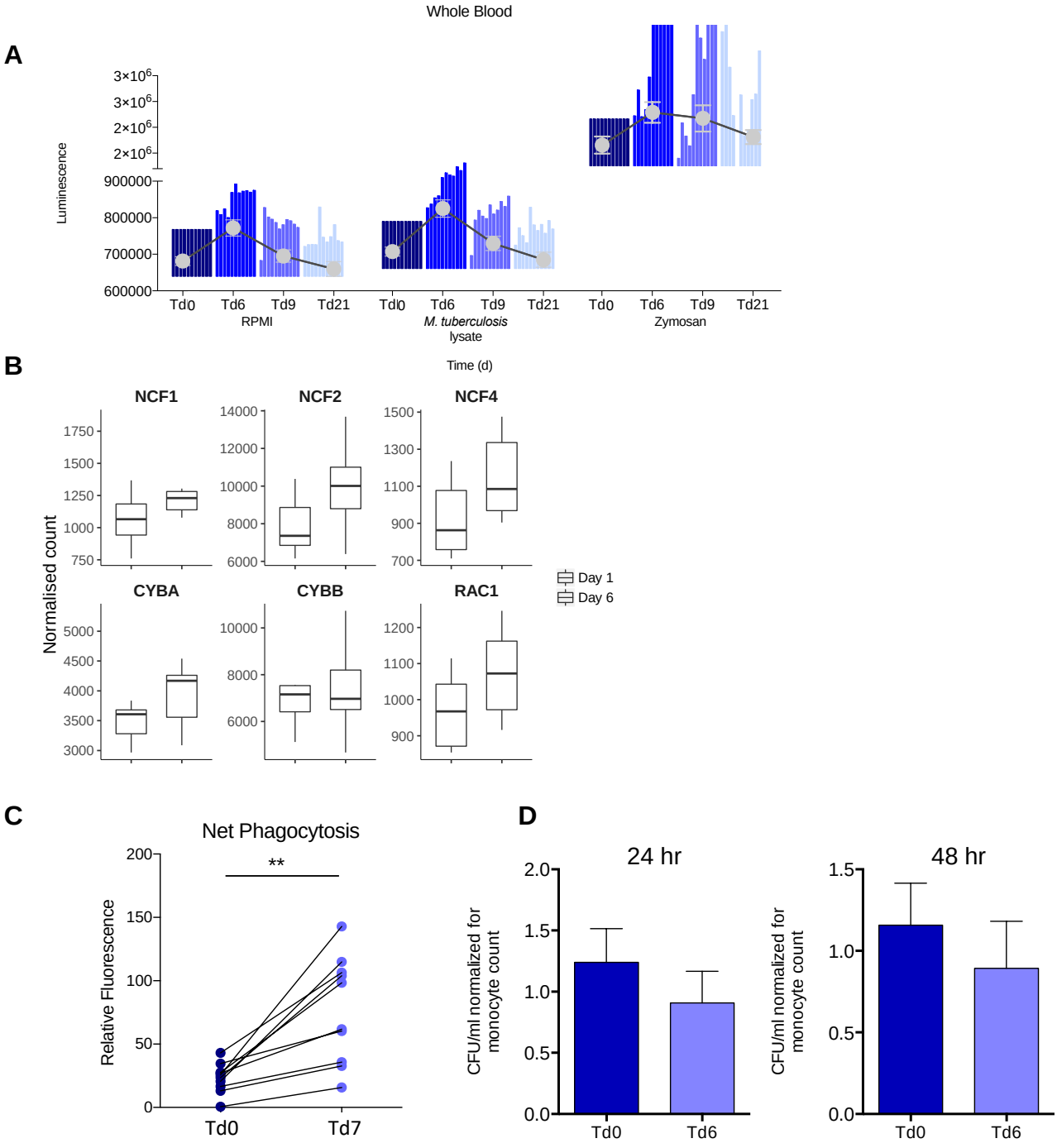

A
Metformin Intake
1x500mg
2x500mg
2x500mg
3x500mg
4x500mg
2x500mg
Days
-1
1
2
3
4
5
9
20
B
Td0
Td6
Volunteer 1
Volunteer 2
Volunteer 5
Volunteer 6
p - AMPK
Actin
p - AMPK
Actin
C
phospho-AMPK / Actin
Intensity
RPMI
M. tuberculosis lysate
D
RPMI
M. tuberculosis
phospho-AMPK / Actin
Normalized intensity
E
Ribosome
Regulation of actin skelton
Pathways in cancer
Oxidative phosphorylation
Neurotrophin signalling pathway
MAPK Signalling pathway
Leukocyte transendothelial migration
Insulin signalling pathway
Focal adhesion
FC gamma R mediated phagocytosis
Endocytosis
Chemokine signalling pathway
−log10(padj)
Enrichment
F
p−values up
p−values down
Max node size: 196 genes
Min node size: 57 genes
Max edge width: 40 genes
Min edge width: 1 genes
Adipogenesis
G2M checkpoint
Myc targets V2
Heme metabolism
Mitotic spindle
Epithelial mesenchymal transition
Oxidative phosphorylation
Myc targets V1
RPMI
Max node size: 192 genes
Min node size: 80 genes
Max edge width: 64 genes
Min edge width: 1 genes
Apical junction
Complement
Estrogen response
Apoptosis
Inflammatory response
Allograft rejection
Unfolded protein
Interferon gamma
MTORC1 signalling
IL2 stat5 signalling
Androgen response
Interferon alpha
TNF alpha signalling
Myc targets V1
M. tuberculosis lysate


A
RNA-Seq
qRT-PCR
IFIT1
IFIT2
IFIT3
OAS1
OAS2
OAS3
MX1
RSAD2
metformin
Mtb lysate
incubation time (hr)
transcript count
normalised expression (ΔΔCt)
B
TNF-a
IL-1b
IL-6
IL-10
IFN-g
IL-17
IL-22
pg/mL
Td0 Td6 Td9 Td21
C
Volunteer 1
Volunteer 2
Td0
Td6
p-P38
Total P38
Actin
D
p-AKT
p-4EBP1
Actin
E
Phospho-P38 / Total-P38
phospho-AKT / Actin
phospho-4EBP1 / Actin
Intensity
RPMI
M. tuberculosis lysate
F
Td0
Td6
% of maximum
Mitotracker green
MT green (MFI) CD14+ CD16- monocytes
p ≤ 0.04



Manual gating

Cluster	MIP-1β	TNFα	IL-2
5	+	+	-
2	+	-	-
10	+	-	+


A
Whole Blood
Luminescence
3×10^6
3×10^6
2×10^6
2×10^6
900000
800000
700000
600000
Td0
Td6
Td9
Td21
RPMI
M. tuberculosis lysate
Zymosan
B
Time (d)
NCF1
NCF2
NCF4
CYBA
CYBB
RAC1
Normalised count
Day 1
Day 6
C
Net Phagocytosis
**
Relative Fluorescence
200
150
100
50
0
Td0
Td7
D
24 hr
48 hr
CFU/ml normalized for monocyte count
2.0
1.5
1.0
0.5
0.0
Td0
Td6