Journal of Microbiology, Immunology and Infection 55 (2022) 405—412

Available online at www.sciencedirect.com

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Original Article

Personalized profiles of antioxidant signaling pathway in patients with tuberculosis

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Received 15 April 2021; received in revised form 18 June 2021; accepted 5 July 2021
Available online 17 July 2021

KEYWORDS
NRF2;
Glutathione;
Oxidative stress;
Redox profile;
Tuberculosis (TB) patients

Abstract *Background/purpose:* The non-protein thiol glutathione is protective against infection by *Mycobacterium tuberculosis* (MTB) and, together with the transcription factor NRF2 (the nuclear factor erythroid 2-related factor 2), plays a crucial role in counteracting MTB-induced redox imbalance. Many genes implicated in the antioxidant response belong to the NRF2-signalling pathway, whose central role in the pathogenesis of tuberculosis (TB) has been recently proposed.
Methods: In this study, we measured GSH levels in blood of patients with active TB and analysed the individual NRF2-mediated redox profile, in order to provide additional tools for discriminating the pathologic TB state and addressing therapeutic interventions.

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https://doi.org/10.1016/j.jmii.2021.07.004

Results: Our findings show a systemic individual modulation of GSH and NRF2 signaling pathway in patients with TB, with a "personalized" induction of NRF2-target genes.
Conclusion: This study can provide useful tools to monitor the course of the infection and address patients' treatment.

Introduction

NRF2 (Nuclear factor Erythroid 2-related Factor 2) is a redox-sensitive transcription factor directly involved in the transcriptional activation of genes belonging to the cellular antioxidant response and responsible for the activation of different cellular processes, including metabolism, survival, differentiation and inflammation.[1–6] Under physiological conditions, the Nrf2 protein is maintained at very low levels in the cell by the negative regulator KEAP1 (Kelch-like ECH-associated protein 1), which sequesters Nrf2 in the cytoplasm leading it to CUL3 E3 ligase for ubiquitination and subsequent degradation by the proteasome.[7,8] Under stress conditions, or in presence of NRF2 inducing compounds, this negative regulation pathway is hindered, resulting in the release of Nrf2 from KEAP1 and promoting its stabilization and activation.[7–10] In response to oxidative stress, these events lead to the defensive transcriptional expression of NRF2 downstream genes, including phase II metabolism enzymes, glutathione synthesis proteins, reactive oxygen species (ROS) scavengers, and drug transporters.[11] In the innate immune system, macrophages produce an excess of ROS as defence against pathogens, such as in *Mycobacterium tuberculosis* (MTB) infection, thus causing endogenous redox imbalance.[12–14] To prevent the ROS-dependent cellular damage, infected cells induce the activation of several antioxidant pathways but, with persistence of the infection or when excessive ROS production overwhelms the antioxidant defense systems, an inefficient control of MTB toxicity may occur, thus leading to unrestrained proliferation and augmentation of mycobacterial burden.[15,16]

Indeed, the ROS overload due to the progression of the infection causes multiple reactions, including the induction of HO-1, an enzyme that catabolizes heme releasing free iron, the decrease of levels of the main ROS-scavenger glutathione (GSH), and the reduction of the enzyme GPX4, actively implicated in the lipid peroxides detoxification. All these events amplify the macrophage oxidative stress, ultimately causing the rupture of the plasma membrane, mediated by lipid peroxides, and the iron-dependent cell death by the process named ferroptosis.[17] This increases MTB replication in macrophages, further facilitating the MTB dissemination.

Therefore, it is evident that a poor antioxidant response and/or an excessive ROS production can be detrimental to the host, increasing its susceptibility and the disease severity.[18–20] Nevertheless, it has also been demonstrated a drastic suppression of macrophage death, lung tissue necrosis, and Mtb loads after treatment of infected mice with antioxidants,[17,21] thus introducing the concept of antioxidant therapy as adjuvant for TB. From all of this, the importance of monitoring oxidative stress throughout the infection and ongoing the conventional long-term treatment appears evident.

Thus, moving from several previous findings highlighting a central role for NRF2 signaling pathway in MTB infection, in this study we analyzed the expression of NRF2 and its target genes in n. 8 patients with active TB, at different stage of disease progression and antibiotics therapy. In addition, as it is well known that host cells synthesize glutathione to counteract ROS, which in its reduced form (GSH) elicits anti-mycobacterial and immune-modulating effects,[22–28] we further measured GSH levels in blood of patients with active TB, to provide an additional tool for better addressing therapeutic interventions.

Materials and methods

Subjects

48 subjects (40 healthcare workers controls and 8 patients with active TB) have been enrolled for this study. Patients with active TB were recruited at the Infectious Diseases Unit of University La Sapienza, Rome, Italy (Table 1). Symptoms concerned all admitted adult patients showing high suspicious of tuberculosis (i.e., persistent cough, fever or chills, night sweats, swollen lymph nodes, shortness of breath, fatigue and unexplained weight loss). Diagnosis of active TB was based on clinical and radiological findings and was confirmed by identification of MTB by microbiological methods (culture and nucleic acid amplification). All patients were seronegative for HIV infection and were treated with anti-TB drugs (rifampicin, isoniazid, pyrazinamide and ethambutol). Blood samples were taken after 4 (Pt#1), 7 (Pts#2 and 3), 10 (Pt#4), 11 (Pt#5), 18 (Pt#6), 32 (Pt#7), and 44 (Pt#8) days of therapy.

Table 1 Demographic data of healthy subjects and Active TB patients.

	Healthcare workers (Ctrls)	Active TB
Total subjects (n =)	40	8
Male (%)	19 (47.5%)	4 (50%)
Female (%)	21 (52.5%)	4 (50%)
Age mean (SD)	38.8 (6.9)	46.0 (20.0)

The healthy subjects were enrolled by the Unit of Occupational Medicine of Bambino Gesù Children's Hospital, Rome, Italy, a national reference hospital for children.[29] Healthcare workers (Ctrls) were selected on the basis of the absence of any risk factors for MTB exposure, a persistent negativity to the serial testing of Interferon-Gamma Release Assay (IGRA), non Calmette Guerin's Bacillus (BCG)-vaccinated, and coming from and living in a low-endemic countries for TB. Approvals by Ethical Committee of Policlinico Umberto I, University La Sapienza (Rif. CE: 5552) and of Bambino Gesù Children's Hospital (Prot. N. CO-2016-023645342018) were obtained.

GSH assay

5 ml whole blood were collected into EDTA-Vacutainer Tube (Becton Dickinson, Rutherford, NY). GSH levels have been detected by an enzymatic re-cycling assay, using the ThioStar® glutathione detection reagent (Arbor Assays, Michigan, MI, USA), and the fluorescence has been measured by an EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). After 15 min reaction, Free GSH was read, followed by the addition of a reducing mixture that converts all the oxidized glutathione, allowing the measurement of Total GSH. GSH concentrations were determined by referring to a GSH standard curve (Sigma Chemicals, St.Louis, MO, USA), and expressed as uM. Immediately after sampling, aliquots of blood have been collected and stored at −80 °C until GSH analysis.

Quantitative real-time PCR (qRT-PCR)

10 ml whole blood were collected into EDTA Vacutainer Tubes and leukocytes were isolated by adding 10% dextran and washed in phosphate buffer (PBS), until obtaining a clear pellet. Leukocytes were stored at −20 °C until RNA extraction (qRT-PCR).

500 ng RNA samples was reverse transcribed with the SuperScript™ First-Strand Synthesis system and random hexamers as primers (Life Technologies, Carlsbad, CA, USA). The expression levels of NRF2, SOD1/2, HO-1, GCL were measured by qRT-PCR in an ABI PRISM 7500 Sequence Detection System (Life Technologies, Carlsbad, CA, USA) using Power SYBR Green I dye chemistry. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method with TBP (TATA box binding protein) as housekeeping gene, and shown as fold change relative to controls. Primers used for qRT-PCR have been reported in Petrillo et al.[30]

Interferon-gamma release assay (IGRA)

The cytokine interferon-gamma (IFN-γ), which plays an important role during the TB infection, is produced by different cells of the immune system: CD4 T-cells, CD8 T-cells and Natural Killer cells [European Center for Disease Prevention and Control. Use of interferon-gamma release assays in support of TB diagnosis. Stockholm: ECDC; 2011]. The IGRA test adopted in this study was the Quantiferon-TB Gold ® in two different releases: the QFT-GIT, which detects MTB specific antigens ESAT-6, CFP-10 and TB7.7, and the QFT Plus eliciting response for ESAT-6 and CFP-10 from CD4+ and CD8+ T lymphocytes (QIAGEN Hilden, Germany – DiaSorin Italy). After incubation for stimulation, the IFN-γ is detected by an enzyme-linked immuno-absorbent assay (ELISA) and quantified by using the QFT analysis software that performs a quality control assessment of the assay and generates a standard curve. The test is reported positive when the INF-γ level in the TB antigen tube(s) is $\geq$ 0.35 IU/ml or $\geq$ 25% of NIL value.[31–33]

Statistical analysis

Statistical analysis was performed using the GRAPHPAD/Prism 5.0 Software (San Diego, CA, USA). Statistically significant differences between groups were analyzed using Student's t-test for normally distributed variables. All data are presented as mean ± standard error. Statistical significance was defined as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, if compared to healthcare workers (Ctrls), and #p < 0.05, ##p < 0.01, ###p < 0.001, when compared to individual antioxidant profile.

All subjects enrolled for the study were tested for the risk of tuberculosis with the Interferon-gamma Release Assay (IGRA).

Results

GSH levels

Glutathione levels have been measured three times in blood of 8 patients with TB infection at different times of antibiotics therapy. Blood samples were taken after 4 (Pt#1), 7 (Pts#2 and 3), 10 (Pt#4), 11 (Pt#5), 18 (Pt#6), 32 (Pt#7), and 44 (Pt#8) days of therapy. As reported in Fig. 1A, Free GSH contents showed different contents among patients and respect to controls, with decreased levels in Pts#1, #4, #5, #7, #8, and increases in Pt#2, #3, #6. Also, the amount of Total GSH was different in patients (Fig. 1B), indicating a personalized consumption and synthesis of GSH. Three patients' samples were measured and data were reported as mean ± SEM. The statistical significance has been evaluated by Student's two-tailed t test, compared with Ctrls.

NRF2 gene expression

Glutathione, together with most of antioxidants responsible for the tissue redox defence, is strictly regulated by NRF2, whose central role in the pathogenesis of TB has been recently demonstrated.[24,25] Thus, we analyzed the NRF2 expression in leukocytes of patients, in order to evaluate if a common antioxidant response could occur. As for GSH, a specific individual response has been found for NRF2 too (Fig. 1C), with a peak of NRF2 expression in Pt#2, #3, and #4, and a decrease in patients with longer treatments. These findings, although on a small number of patients, suggest a time-dependent activation of the transcription factor, with a boost in the early days of therapy.

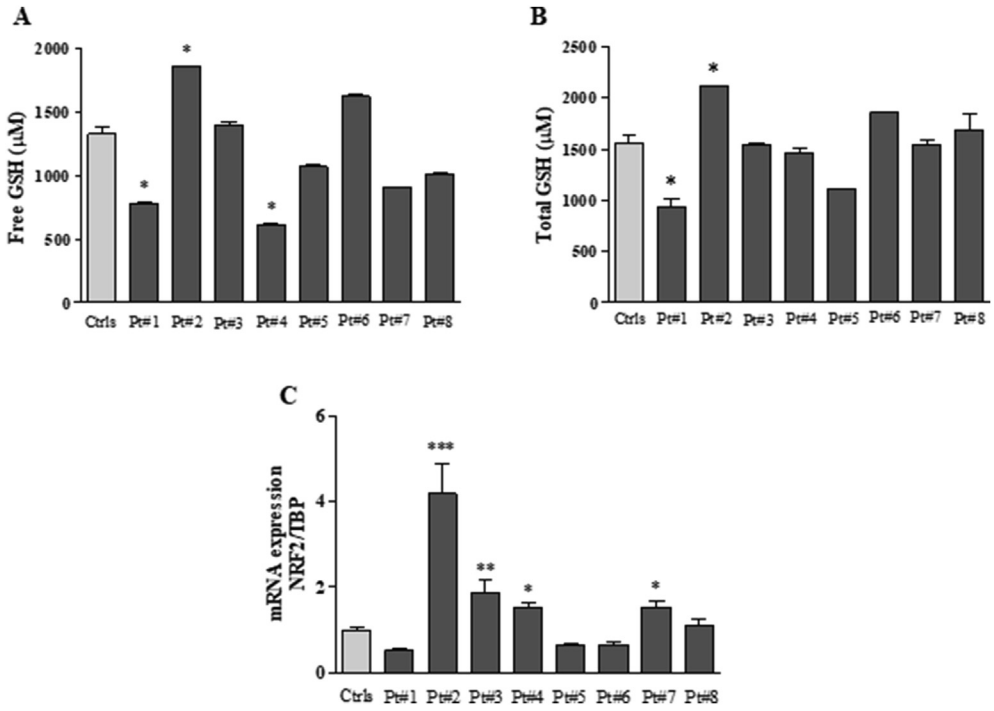

Figure 1. Systemic antioxidant status in TB patients. Free (A) and Total (B) GSH concentrations in blood of TB patients and Ctrls. qRT-PCR analysis of NRF2 gene expression in leukocytes (C). Values represent mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with Ctrls by Student's two-tailed t test.

Redox profiles

NRF2 regulates many genes of the antioxidant signaling pathway, thus we wonder if also NRF2 target genes may be differently expressed in TB patients, particularly focusing on those involved in the GSH synthesis (the glutamate-cysteine ligase, GCL), in the response to the inflammation (the heme oxygenase 1, HO-1),[34] and in those implicated in the direct neutralization of ROS (the cytosolic superoxide dismutase 1, SOD1 and the mitochondrial superoxide dismutase2, SOD2). mRNA trancripts have been measured in triplicate after 4 (Pt#1), 7 (Pts#2 and 3), 10 (Pt#4), 11 (Pt#5), 18 (Pt#6), 32 (Pt#7), and 44 (Pt#8) days of therapy, and statistical significance was evaluated by Student's two-tailed t test compared to Ctrls.

As shown in Fig. 2, mRNA transcipts exhibited different redox profiles in each patient. In particular, Pt#1 (4th day of sampling) showed low expression levels of all antioxidant markers (GSH, NRF2, SOD, GCL, HO-1), while #2 and #3, who displayed high NRF2 expression already after one week of antibiotic therapy, had all target genes down regulated respect to controls (Fig. 2A–D). Pt#2, in addition, exhibited a starting high content of GSH, likely reflecting a constitutive individual condition. Conversely, Pts #4–8, who were on treatment for a longer time (10–40 days therapy), showed up to 30-fold increase of NRF2 down-stream genes (Fig. 2A–D). These data suggest a slow activation of NRF2-target genes throughout the therapy, which also depends on the personal antioxidant response. Therefore, even if they need confirmation on a larger number of patients, we believe that our findings could open the way for a personalized medicine in this disease, with an eye on tailored combined therapies.

Discussion

The non-protein thiol glutathione is protective against infection due to MTB and, together with the transcription factor NRF2, plays a crucial role in counteracting MTB-induced redox imbalance.[17,22,24,26–28] In this study, we propose a new approach that combines the biochemical GSH determination and the expression of redox gene profiles in blood of patients with active TB.

Oxidative stress has been implicated in the pathogenesis of lung fibrosis in TB patients and several studies have demonstrated that critical antioxidants are depleted in the serum of patients.[19,20,35]

NRF2 is the main regulator of cell homeostasis, because of its role in modulating oxidative, inflammatory, and metabolic networks,[1,2,36,37] thus acquiring a key role in protecting tissues from injury caused by infections and in pneumonia pathogenesis.[38]

Using knockout mouse models, Rothchild et al.[25] demonstrated that NRF2 drives the expression of a cell-protective signature in infected macrophages, impairing the control of early bacterial growth. This host antioxidant response is ensured by the transcriptional activation of several genes belonging to the NRF2 signaling pathway. Glutathione is one of them, together with HO-1, SOD 1 and SOD2, and many others.[11]

Glutathione is an essential intracellular ROS scavenger, whose synthesis occurs by a two-step, energy dependent reaction. The first and rate-limiting step in the GSH

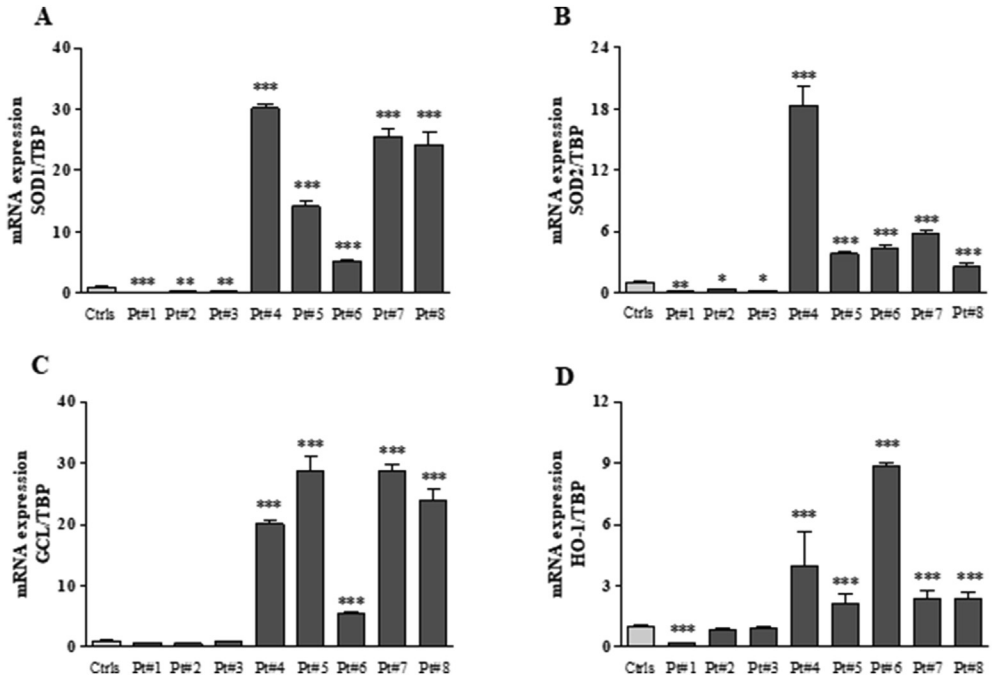

Figure 2. NRF2-related redox profiles in leukocytes of TB patients. qRT-PCR analysis of NRF2-target genes (SOD1, SOD2, GCL, HO-1) in 8 patients with active TB and 40 Ctrls (A–D). Values represent mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to Ctrls, as evaluated by Student's two-tailed t test.

synthesis is catalyzed by glutamate-cysteine-ligase (GCL), which is transcriptionally regulated by NRF2.[39,40] GSH has an important role in maintaining cellular redox homeostasis and it constitutes the most highly concentrated antioxidant within the cell.[41,42] Previously, Teskey et al.[43] reported that a virulent laboratory strain of MTB was completely cleared through supplementation with GSH or with its precursor N-acetyl cysteine (NAC), in conjunction with first-line antibiotics. Thus, despite its protective antioxidant function, GSH has also antimycobacterial and immunomodulatory activities, and monitoring its levels could be useful as indicator of the disease status.[44]

The GSH depletion was associated with increased ROS, pro-inflammatory cytokines production, and enhanced intracellular MTB survival.[26,27,44–46] Several mechanisms can be implicated in the GSH-mediated protection: i) in the form of S-nitrosoglutathione (GSNO), GSH can stabilize and release, when necessary, the nitric oxide (NO), a potent bactericidal free radical[47]; ii) GSH can also increase the cytolytic activity of natural killer (NK) cells, thereby enhancing their function against MTB[48,49]; iii) GSH is even able to modulate the release of various cytokines (such as IFN-γ), resulting in the enhancement of the Th1 cell response against MTB.[50,51]

Therefore, given the crucial function of GSH throughout the infection, at first we compared total (Tot) and reduced (Free) GSH levels in whole blood of TB patients, in order to investigate its possible role as early systemic marker of the disease. We found that GSH, either in its active/reduced form, which reflects the GSH consumption, and as total glutathione, indicative of its synthesis, greatly depends on the individual response of the single patient. Also NRF2, which has been reported to increase in lung lesions of guinea pig during the progression of MTB infection,[12] exhibited a systemic individual responsiveness to the infection and treatment, with an apparent higher activation in the first days of infection, and antibiotic therapy, and a decrease at longer times.

The central role of NRF2 in the pathogenesis of Tuberculosis (TB) has been previously suggested by Qian et al.,[24] who proposed the transcription factor as a useful diagnostic marker to differentiate TB patients from other subjects. Recently, Rothchild et al.[25] demonstrated that MTB-infected alveolar macrophages, the first line of cells to be infected, induced the up-regulation of a protective transcriptional signature identified (by computational approaches) as the NRF2-regulated antioxidant pathway.

Nrf2 works to counteract effects of ROS, allowing for effective defence responses against MTB, but minimizing oxidative injury to the host cell.[22] Indeed, Nrf2 displays multiple activities in cell protection, and its activation stimulates the transcription of a battery of antioxidant genes (redox response), overall constituting the NRF2 signaling pathway.[23]

Given the individual response of NRF2 expression in our patients, we wonder if even the NRF2-target genes could be subjectively induced during TB infection. Thus, we analyzed GCL, HO-1, SOD1, and SOD2 mRNA transcripts in blood of patients, and we found differential redox profiles for each patient. Interestingly, a boost of genes'activation was observed in patients under about 10 days of therapy, suggesting a slowed response to early NRF2 induction.

The enzyme HO-1 is a potent antioxidant enzyme, induced]by heme accumulation,[52–54] exposure to toxic compounds,[55,56] hypoxia,[57] starvation,[58] and toll-like receptor (TLR)/cytokine-mediated cellular activation.[59–61]

The enzyme exhibits anti-inflammatory and cytoprotective effects, and increased HO-1 systemic levels were found in patients displaying severe clinical and radiographic signs of TB disease.[52,62–66]

HO-1-depleted mice were highly susceptible to MTB infection,[67] and murine and human macrophages infected *in vitro* by MTB showed a significant increase of the HO-1 production.[52] Furthermore, HO-1 levels correlated with active infection in experimental mouse and rabbit, and decreased upon antitubercular therapy.[34] In line with these previous findings, we found a consistent increase of HO-1 expression in patients collected later after diagnosis (Pt #4-#8), while HO-1 expression was lower in patients with early blood sampling (Pt#1-#3). The same trend was observed for SOD 1/2 and GCL expression, indicating a late activation of ROS neutralization mechanisms throughout the infection.

Conclusion

The identification of personalized redox signatures together with the systemic GSH determination throughout the TB infection could provide useful tools for monitoring the course of the infection and addressing patients' treatment.[68] Personalized antioxidant therapies should be hypothesized for each patient, in order to elicit the best drug effectiveness, as also suggested by a recent study trial using N-acetylcysteine as adjuvant in HIV-associated TB patients.[69] This work is a proof of concept study that needs to be confirmed with a larger group of patients and correlated to the outcome. Nonetheless, we believe that the systemic biomarkers analyzed in this study may represent an useful tool for designing targeted host-directed therapies.

Data availability

Anonymous clinical data used to support the findings of this study are available from the corresponding author upon request.

Author contributions

Conceptualization, S.P., C.M.M.., S.Z.. and F.P.; methodology and data acquisition, S.P., M.G.G.; samples collection, R.B. A.S., P.N.; data integration, S.P., P.L.R..; writing original draft, F.P., S.Z., C.M.M., E.S.B.; supervision, C.R., A.T.P. Authors have read and agreed to the published version of the manuscript.

Funding

This work has been supported by a grant from the Italian Ministry of Health (Cod. CO-2016-02364534).

Declaration of competing interest

The authors declare no conflicts of interest.

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