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ARTICLE

Monocytic myeloid-derived suppressor cells reflect tuberculosis severity and are influenced by cyclooxygenase-2 inhibitors

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Abstract

Myeloid-derived suppressor cells (MDSCs) increase in tuberculosis (TB) and may be targets for host-directed therapy (HDT). In this study, we use flow cytometry to analyze the effects of cyclooxygenase-2 inhibitors (COX-2i) on monocytic (M)-MDSCs in blood from TB patients attending a clinical trial of COX-2i. The effects of COX-2i on M-MDSCs and mycobacterial uptake were also studied by an in vitro mycobacterial infection model. We found that M-MDSC frequencies correlated with TB disease severity. Reduced M-MDSC ($P = 0.05$) and IDO ($P = 0.03$) expression was observed in the COX-2i group. We show that peripheral blood-derived M-MDSCs successfully internalized *Mycobacterium bovis* and that in vitro mycobacterial infection increased COX-2 ($P = 0.002$), PD-L1 ($P = 0.01$), and Arginase-1 ($P = 0.002$) expression in M-MDSCs. Soluble IL-1β, IL-10, and S100A9 were reduced in COX-2i-treated M-MDSCs cultures ($P < 0.05$). We show novel data that COX-2i had limited effect in vivo but reduced M-MDSC cytokine production in vitro. The relevance of COX-2i in a HDT strategy needs to be further explored.

KEYWORDS

Mycobacteria, M-MDSC, cyclooxygenase-2 inhibitor, host directed therapy, innate immunity

1 | INTRODUCTION

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*Mtb*) is ranked as one of the top 10 causes of death worldwide.[1] The rise of multidrug-resistant TB (MDR-TB) with limited treatment options can reverse achievements in TB control and may increase the spread, morbidity, and mortality of the disease.[1] Novel therapeutic strategies to treat MDR-TB and shorten the duration of drug-sensitive TB treatment are urgently needed. Host-directed therapy (HDT) aims to modulate *Mtb*-induced inflammation and increase antimicrobial activity[2] but data on HDT in human TB are scarce.[3] Cyclooxygenase-2 inhibitors (COX-2i) have known anti-inflammatory capacity and have been proposed as candidates for HDT to limit host-meditated immune pathology in TB.[4,5]

Innate immunity is crucial in antimycobacterial defense and shaping of T cell responses.[6]

Myeloid-derived suppressor cells (MDSCs) are immunoregulatory myeloid cells that expand during pathologic conditions.[7,8] They can be divided into at least 2 subgroups; polymorphonuclear (PMN) and monocytic (M)-MDSC, both with different suppressive mechanisms.[9,10] M-MDSCs suppress both innate and adaptive immune responses in various disease settings and are therefore studied as potential HDT targets.[11,12] Accumulating evidence indicate that

Abbreviations: Arg 1, Arginase 1; BCG, Bacille Calmette Guerin; CD, cluster of differentiation; COX-2, cyclooxygenase-2; COX-2i, cyclooxygenase-2 inhibitor; ESR, erythrocyte sedimentation rate; HDT, host-directed therapy; IDO, indoleamine 2,3 deoxygenase; MACS, magnetic cell sorting beads; MDR-TB, multidrug-resistant tuberculosis; MDSC, myeloid-derived suppressor cells; MFI, mean fluorescence intensity; MOI, multiplicity of infection; Mtb, Mycobacterium tuberculosis; PD-L1, programmed death ligand 1; PGE_2, prostaglandin E_2; PPD, purified protein derivate; TB, tuberculosis; TTP, time to positivity.

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bacteria, such as mycobacteria,[13,14] staphylococci,[15] enterotoxigenic bacilli,[16] and Gram-negative pathogens,[17] trigger generation of M-MDSCs. A reduced frequency of this MDSC subset was shown in patients undergoing TB chemotherapy,[14] further strengthening the notion that disease progression in TB is associated with expansion of M-MDSCs. Thus, although data on the suppressive capacities of MDSCs have been reported mainly from studies in tumor biology, increasing evidence exist of similar effects in infectious diseases,[10] but a clear link to severity of TB disease has not yet been established. The suppressive mechanisms of M-MDSCs include amino acid depletion by Arginase-1 (Arg 1) and IDO[18] and interactions with programmed death receptor-1 and its ligand (PD-1/PD-L1).[19] As a result, MDSCs impair several T cell functions, including activation, proliferation, and cytokine production,[14,18,20] underscoring the relevance of exploring these cells as candidates for immunotherapy. M-MDSCs are also bona fide phagocytes, capable of internalizing pathogens and allow persistence, while exerting localized suppressive activity. We and others have previously shown that in humans, M-MDSC are increased in the peripheral blood and disease site in those with active TB.[14] We demonstrated that these MDSCs were immunosuppressive and promoted replication of mycobacteria, altered stability of *in vitro* TB granulomas, and provided a niche for mycobacterial survival within infected mouse lungs.[21,22]

Lessons learned from oncology may provide insight into drug targeting of MDSCs and several blocking strategies have been investigated.[23,24] In cancer, COX-2 and PGE_2 have been associated with MDSC activation, expansion, and function in cancer settings[25–27] and MDSC frequencies in tumor bearing mice were reduced after COX-2i treatment.[28] There are conflicting data on the role of COX-2 and PGE_2 in TB.[29–32] Although COX-i seems to increase bacterial load in the murine aerosol infection model, it appears that COX-i treatment improves the outcome after intravenous infection in highly susceptible mice.[31,33]

We present data from the *first-in-man* phase I clinical trial of the in vivo effects of COX-2i on M-MDSCs in peripheral blood from TB patients. We have further studied the effects of COX-2i on M-MDSC in an in vitro *Mycobacterium bovis* infection model.

2 | MATERIAL AND METHODS

2.1 | Study subjects

TBCOX2 cohort: Drug-sensitive pulmonary TB patients were recruited from a randomized open label phase I clinical trial on the safety and immunogenicity of adjunctive COX-2i given as etoricoxib 120 mg once daily during the first 140 days of standard TB treatment consisting of rifampicin, isoniazid, ethambutol, and pyrazinamide (Oslo University Hospital, Norway, TBCOX2; *NCT02503839*). Peripheral blood was collected using BD CPT™ Cell Preparation Tubes (containing sodium heparin as anticoagulant) from TB patients receiving standard TB treatment (control, $n = 8$) or additional COX-2i ($n = 8$) at the start of the treatment (day 0), day 84, and 2 months after finalizing TB treatment (day 238). Clinical examination, reporting of symptoms, routine analyses of blood erythrocyte sedimentation rates (ESR), and leukocyte differential test with monocyte and lymphocyte counts as well as chest X-ray were performed at baseline. Time (days) to positive *Mtb* culture of sputum was estimated as a proxy of bacterial load. Patients were categorized by a symptom score including fever > 38°C, weight loss, cough, chest pain, or night-sweat.

ScreenTB cohort: Drug-sensitive pulmonary TB patients ($n = 15$) were recruited from a noninterventional, prospective cohort, enrolling adult participants at primary healthcare clinics in Cape Town, South Africa (*NCT03350048*) for use in the in vitro *M. bovis* infection model.

2.2 | Ethical considerations

The *TBCOX2 study* was approved by the Regional Committees for Medical and Health Research Ethics (REC) (TBCOX2 REK SØ 2015/692, EudraCT nr: 2014-004986-26). Biobank samples were collected and stored in the "Research Biobank Infectious Diseases ("Forskningsbiobank Infeksjonssykdommer" (REK 1.2006.181-S-0885, SHDNR. 09/513), Department of Infectious Diseases, OUS, Ullevål. The *ScreenTB* study was approved by the Health Research Ethics Committee of the University of Stellenbosch (N16/05/070). Written informed consent was obtained from all participants before inclusion.

2.3 | *Mycobacterium tuberculosis* detection in clinical samples

Clinical samples were incubated at 37°C for minimum 42 days in Mycobacteria Growth Indicator Tube (MGIT; BD Biosciences, NJ, USA) containing 4 ml of modified Middlebrook 7H9 broth base for qualitative detection of *Mycobacterium tuberculosis* complex antigen (*Mtb*-antigen). The instrument (BD Biosciences) scans the MGIT every 60 s for increased fluorescence that detects consumed oxygen by microorganisms with a detection level for positive sample of approximately 10^5 and 10^6 CFUs/ml.

2.4 | Flow cytometry

Flow cytometry was performed on cryopreserved PBMCs from the *TBCOX2* cohort in a blinded random-order setup. Cryopreserved PBMCs from the *TBCOX2* cohort were thawed in a water bath at 37°C and washed with prewarmed RPMI 1640 (Sigma-Aldrich, MO, USA) supplemented with 1% L-Glut, 1% penicillin streptomycin solution, and 10% FCS. Cells rested for 4 h prior to cell quantification and viability check (Muse® Cell analyzer; Merck Millipore, MA, USA). PBMCs from both *TBCOX2* and *ScreenTB* cohort ($4\text{-}7 \times 10^5$) were stained with surface markers (Supplemental Tables E1 and E2) fixed and permeabilized according to manufacturer's protocol (BD Bioscience, San Jose, CA, USA) and stained with intracellular markers. Cell acquisition was performed ($>3 \times 10^5$ events) on FACS Canto II (BD Biosciences). Instrument calibration was performed according to manufacturer's instructions and compensation adjusted using antibody – capture beads (CompBeads; BD Biosciences). Dead cells were excluded using a fixable viability dye (450; eBioscience, San Diego, CA, USA). Fluorescence minus one controls were used to determine positive and negative populations for the MDSC gating strategy (Supplemental Fig. S1). M-MDSC subsets were characterized according to the most recent standards by Bronte et al.[34] Briefly, M-MDSCs were

TABLE 1 Demographic and clinical data of the TBCOX2 cohort

	TBCOX2 cohort	
	Control ($n = 8$)	COX-2i ($n = 8$)[a]
Age (median years, range)	22 (20–27)	29.5 (19–39)
Male (%)	7 (87.5)	3 (37.5)
Origin		
Africa	6 (75)	3 (37.5)
Asia	1 (12.5)	2 (25)
Europe	1 (12.5)	2 (25)
Other	0 (0)	1[b] (12.5)
Symptoms (%)		
Cough	3 (37.5)	7 (87.5)
Night-sweat	4 (50)	4 (50)
Fever	1 (12.5)	2 (25)
Weight loss	2 (25)	3 (37.5)
Chest pain	2 (25)	3 (37.5)
Low:high symptom score[c]	3:5	3:5
Findings		
Cavity on chest X-ray (%)	2 (25)	5 (62.5)
BMI[d] (median)	20.50	20.35
ESR[e] (median mm/hour, range)	9 (1–67)	34 (3–104)
ML ratio[f] (median, range)	0.32 (0.21–1.0)	0.22 (0.13–1.36)
TTP[g] (median days, range)	12.2 (4.71–19.54)	8.17 (2.71–14.83)

[a] 1 patient in the COX-2i group had both pulmonary and extrapulmonary TB.
[b] Brazil.
[c] High = ≥2 of the following symptoms: cough, night-sweat, fever (>38°C), chest pain, and weight loss. Low = 1 symptom or asymptomatic/detected by screening.
[d] Body mass index
[e] Erythrocyte sedimentation rate.
[f] Monocyte: lymphocyte ratio.
[g] Time to positive routine Mtb culture measured as days.

characterized as human leukocyte antigen complex/major histocompatibility complex class II (HLA-DR)$^{neg/low}$, cluster of differentiation (CD)33$^+$, CD11b$^+$, and CD14$^+$. Data analysis was performed using FlowJo (Tree Star Inc, Ashland, OR, USA), expressed as frequencies or mean fluorescence intensity (MFI). Overview of sample processing and assays from the *ScreenTB* cohort are displayed in Supplemental Fig. S2. M-MDSC markers have previously been reported to change when subjected to freeze/thaw procedures and comparison between fresh and frozen MDSC markers was performed to investigate differences in protein expression upon thawing (Supplemental Fig. S3).

2.5 | M-MDSC enrichment and in vitro *M. bovis* infection culture

Since M-MDSCs internalize both *Mtb*[21] and *M.bovis*[35] and like *Mtb*, *M.bovis* induce suppressive MDSCs,[35] we used a MDSC- mycobacterial infection model[36] that does not require biosafety level-3 containment, to assess the impact of COX-2i in mycobacterial replication control upon MDSC internalization. PBMCs from the *ScreenTB* cohort were thawed and enriched for HLADR$^-$ and CD33$^+$ using MACS magnetic bead separation (Miltenyi Biotec, Bergisch, Germany), rested in a 37°C incubator (with 5% CO_2) prior to infection. Considering that PMN-MDSCs poorly survive cryopreservation, the HLA-DR^{low} CD33$^+$ population will contain mainly M-MDSCs (see CD15 expression in Supplemental Fig. S3). Mycobacteria were washed in RPMI and passed through 27G needle to obtain a single cell suspension. Instead of etoricoxib that was used in the clinical study, but insoluble in culture conditions, the COX-2i celecoxib was chosen for in vitro studies. Celecoxib (20 μM) (Sigma–Aldrich, MO, USA) was added to the culture 1 h prior to *M. bovis* infection. As viewed in Supplemental Fig. S4, a nontoxic concentration of celecoxib was determined by evaluation of cell viability by flow cytometry. 1×10^5 M-MDSCs were infected with *M. bovis* (Bacille Calmette Guerin [BCG] Danish strain 1331) at the multiplicity of infection (MOI) of 1. Unsorted PBMCs for use in flow cytometry ($\sim 4 \times 10^5$) from the same patients were also *M. bovis* infected (MOI 0.5) for 24 h (see Section 2.4 for staining procedure). Culture supernatants were collected after 3 h infection and new media containing COX-2i was replaced to continue the culture for 24 h. Supernatants were collected for Luminex analysis and enriched M-MDSCs were subjected to CFU measurements.

2.6 | CFUs measurements from cell cultures

After 24 h infection, PBMC and M-MDSC culture plates were lysed with 100 μl ddH_2O. *M. bovis* present in the serial dilution of lysates and supernatants were plated on Middlebrook 7H11 agar (Fisher Scientific, NH, USA) plates supplemented with 10% oleic albumin dextrose catalase (Thermo-Fischer Scientific, MA, USA) and 0.5% glycerol. Total CFUs from 24 h supernatants were counted after 21 days of incubation at 37°C.

2.7 | Multiplex assay

Supernatants were harvested after 3 and 24 h infection and stored at −80°C until used. Two multiplex assays were used to measure 16 different analytes; a 14-plex measuring monocyte chemoattractant protein-1, IFN-γ, IL-1ra, IL-6, IL-10, S100 calcium-binding protein A8 (S100A8), TNF-α, GM-CSF, IL-1β, IL-4, IL-12, S100A9, IL-8, and vascular endothelial growth factor A (VEGF-A) and a 2-plex analyzing IFN-γ inducible protein (IP-10) and IL-12p70 (RnD Systems, MN, USA). Lower out of range values (OOR <) were set to zero. There were no values out of upper range (OOR >). The assay was performed on a Magpix (BioRad, CA, USA) using Bioplex Manager 6.1 software.

2.8 | Statistical analysis

Nonparametrical statistical methods were applied comparing groups in the *TBCOX2* cohort and the in vitro experiments. Correlation plots were calculated using Spearman's correlation. Cytokine profiles were analyzed by mixed-model ANOVA. Because of the exploratory nature of the trials, there was no adjustment for multiple testing. Statistical analysis was performed by SPSS statistics 25 (IBM, IL, USA) and Graphpad Prism 8 (Graphpad Software Inc, La Jolla, CA, USA).

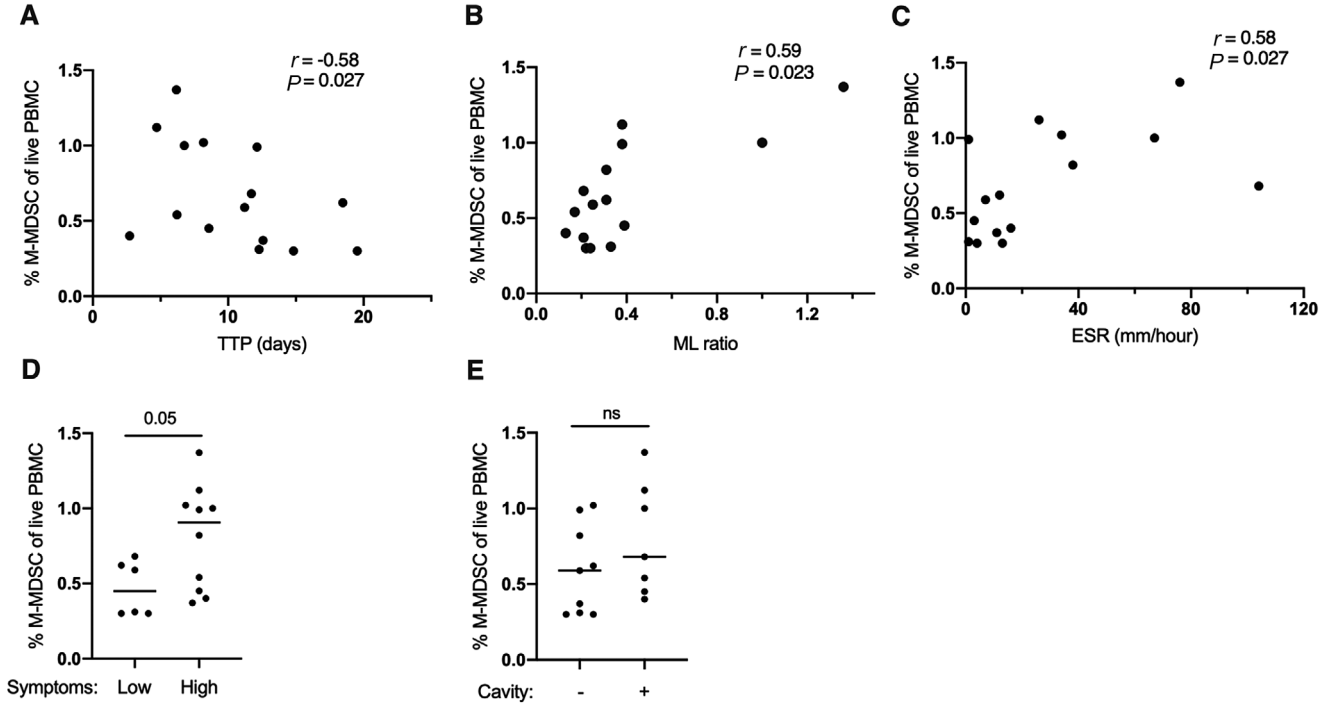

FIGURE 1 **M-MDSC frequency is associated with TB disease severity.** M-MDSC frequency (%) measured in peripheral blood from patients in the *TBCOX2* cohort ($n = 16$) at time of diagnosis is (**A**) negatively correlated with time to positive (TTP) *Mtb* cultures and positively correlated with (**B**) ML ratio and (**C**) ESR. TTP, ML ratio, and ESR data were available for 15 out of 16 patients included. (**D**) A trend of increased M-MDSC frequencies in patients with a high symptom score compared with the low symptom score. (**E**) No differences in M-MDSC frequency when comparing patients with and without lung cavities. *R* value calculated using Spearman, Graphpad Prism

3 | RESULTS

3.1 | Characterization of study participants

In the *TBCOX2* cohort, the median age was 23 (19–39) and 10 out of 16 (62.5%) were male. All were HIV negative pulmonary TB cases. See Table 1 for details concerning age, gender, ethnicity, ESR, monocyte/lymphocyte ratios (ML ratio), cavity at chest X-ray, and symptoms indicating severity of TB disease at baseline in the COX-2i intervention and control group. All TB patients responded to standard TB treatment with sputum culture conversion and clinical improvement. Patients in the intervention group have more severe TB reflected in cavitary disease, higher ESR, and shorter TTP, although with similar distribution of symptoms. However, the differences were not significant. The patients from the South-African *ScreenTB* cohort were all HIV negative pulmonary TB cases with a median age of 37 years (range 23–54) and 13 out of 15 (86.7%) were male.

3.2 | Higher M-MDSC frequencies in blood indicate more severe TB disease

M-MDSC frequencies have not yet been related to the severity of human TB disease.[37] Thus, we first investigated the association between M-MDSC frequencies in blood and TB disease severity in the *TBCOX2* cohort at the time of diagnosis. Time to positive *Mtb* culture (TTP), cavitary disease, symptom score, ESR, and ML ratio were used as markers of TB disease severity. We observed a negative association (Rho –0.58, $P = 0.03$) between M-MDSC (HLA-DR_{low}, $CD14^+$, $CD33^+$, $CD11b^+$) frequencies and TTP indicating higher bacterial load in patients with M-MDSC expansion (Fig. 1A). Supporting this finding, we observed a positive association between M-MDSC frequencies and both ML ratio (Rho 0.59, $P = 0.02$) and ESR (Rho 0.58, $P = 0.02$) (Fig. 1B/1C). There was also a trend of higher M-MDSC frequencies in patients with high symptom score ($P = 0.05$), but no association was observed with cavitary disease (Fig. 1D/1E). Interestingly, we also observed a reduction in ML ratio during TB treatment from baseline to day 238 in the control group (median 0.32 vs. 0.25, $P = 0.03$) (Supplemental Fig. S5). These findings suggest a link between TB severity and M-MDSC.

3.3 | IDO expression in M-MDSCs are reduced with adjunctive COX-2i treatment

We next assessed the effects of adjunctive COX-2i on M-MDSC frequencies and expression of COX-2, IDO, and PD-L1. In the *TBCOX2* cohort, we observed a trend of reduced M-MDSC frequencies for both groups after 238 days, most noticeable in patients with adjunctive COX-2i ($P = 0.053$) (Fig. 2A). Interestingly, IDO (MFI), responsible for tryptophan depletion and impaired T cell receptor activity, was significantly reduced in the COX-2i-treated group throughout therapy ($P = 0.03$) (Fig. 2A). In contrast, MFI of COX-2 and PD-L1 in M-MDSC did not change significantly. When comparing the COX-2i group with

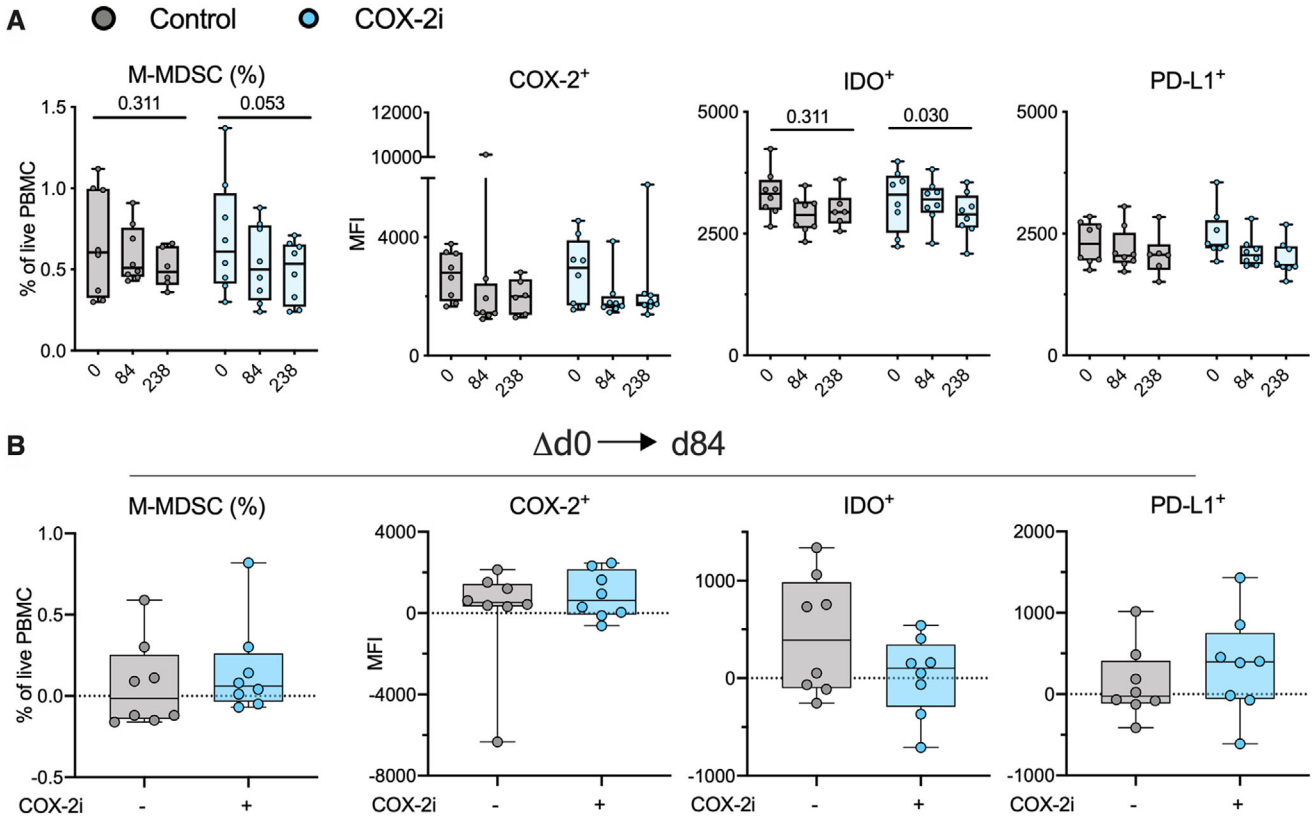

FIGURE 2 **The effect of COX-2i treatment on M-MDSC.** (**A**) Fraction of M-MDSCs in PBMC and COX-2, IDO, or PD-L1 expression in M-MDSCs (MFI; mean fluorescence intensity) before (day 0), during (day 84), and after (day 238) standard TB treatment only (gray bars, $n = 8$) or with additional adjunctive COX-2i etoricoxib (blue bars, $n = 8$) analyzed by flow cytometry. (**B**) Changes from baseline to day 84 (delta baseline–day84) of M-MDSC frequencies and COX-2, IDO, or PD-L1 expression were comparable between the control and COX-2i group. PBMC was missing from 2 patients in control group at day 238 ($n = 6$). Analysis with 3 timepoints significance was calculated using Friedman test $P < 0.05$ (median ± min–max). Comparison of intervention and control group, significance was calculated using Mann–Whitney test, $P < 0.05$

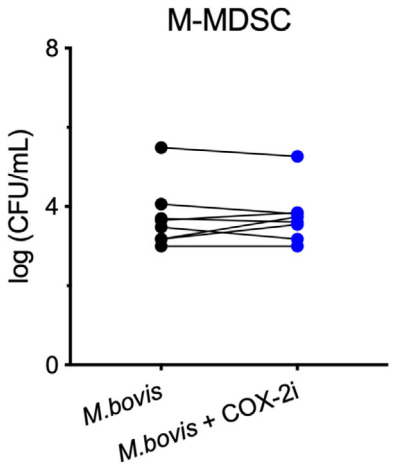

FIGURE 3 **COX-2i had no effect on bacterial load in enriched M-MDSC culture.** Enriched M-MDSCs (HLA-DR$^-$, CD33$^+$) were infected with *M. bovis* (MOI of 1). No difference in mycobacterial uptake of enriched M-MDSC (CFU) after 24 h culture was seen when comparing *M. bovis* alone ($n = 8$) or *M. bovis* + COX-2i (celecoxib) ($n = 8$) Mann–Whitney test, Graphpad Prism 8

the control group at day 84, no significant differences were observed in M-MDSC frequencies or expression (MFI) of markers neither in absolute values nor change form baseline (delta day 0–day 84) (Fig. 2B). Also, when analyzing the frequencies (%) of COX-2, IDO, and PD-L1 in M-MDSC, there were no significant differences from day 0 to day 238 (Supplemental Fig. S6).

3.4 | COX-2 inhibitor has no effect on bacterial load in the in vitro *M. bovis* infection model

We investigated M-MDSCs' capacity to regulate bacterial growth and the effects of COX-2i in an in vitro mycobacterial infection model of enriched M-MDSCs (HLA-DR$^-$ CD33$^+$) from patients included in the South African *ScreenTB* cohort before start of TB treatment. Bacterial growth measured as CFU was assessed in *M. bovis*-infected (MOI 1), COX-2i (celecoxib)-treated, and nontreated M-MDSC cultures after 24 h. We showed that human blood-derived M-MDSC successfully phagocytose *M.bovis*, but with no observed effects of celecoxib on bacterial load (Fig. 3).

3.5 | COX-2 inhibitor reduces cytokine production in in vitro *M. bovis*-infected M-MDSCs

In the mycobacterial in vitro model, we also investigated whether COX-2i (celecoxib) added to cultures influenced cytokine and chemokine

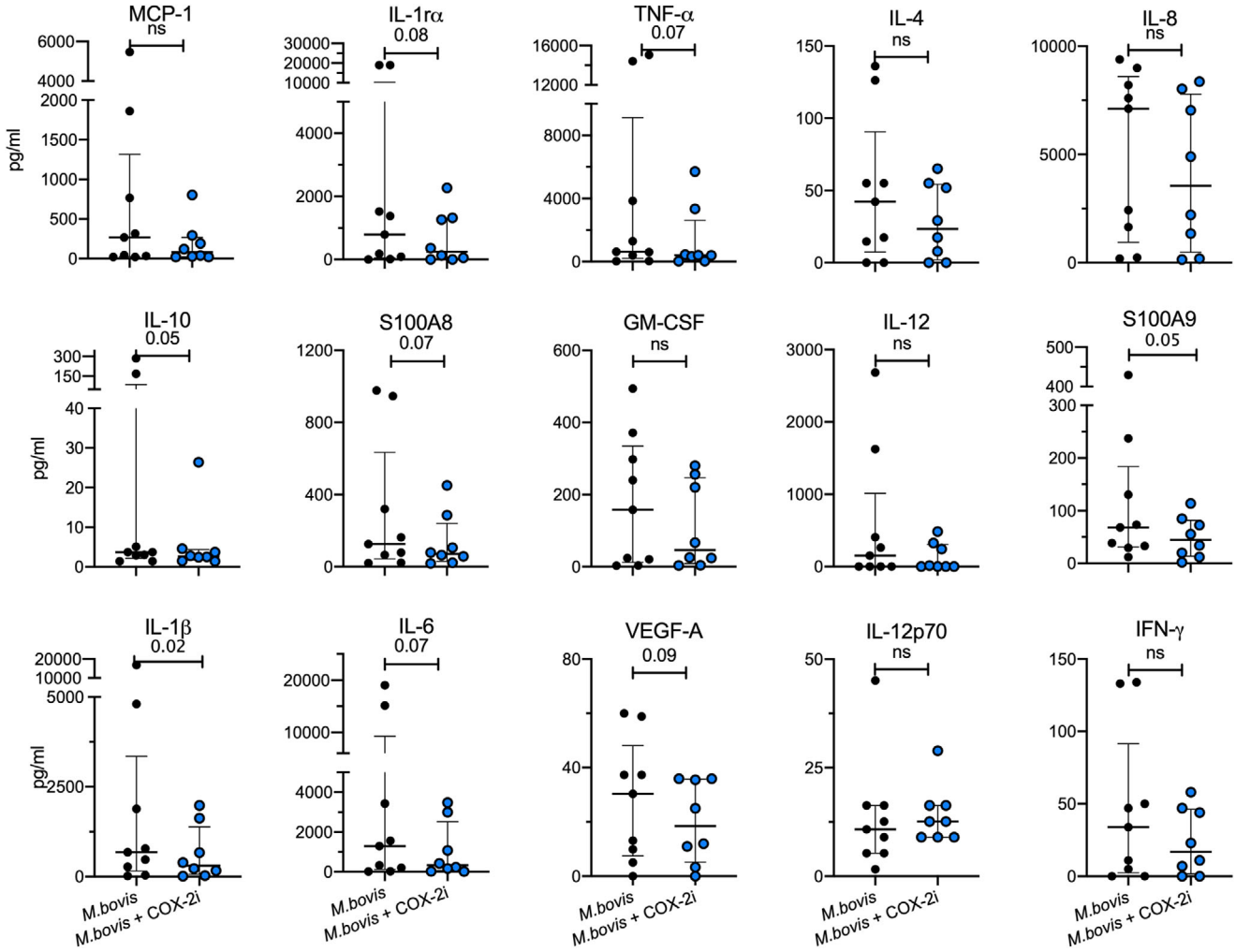

FIGURE 4 **Cytokine production in infected M-MDSCs is influenced by the COX-2i celecoxib**. Soluble markers were measured in the supernatants from *M. bovis*-infected M-MDSCs cultures without (*M. bovis*) and with celecoxib (*M. bovis* + COX-2i) for 24 h ($n = 10$). Plots are shown as median with interquartile range (IQR). Significance calculated using mixed model ANOVA, * significance $P < 0.05$

production from enriched infected M-MDSCs using multiplex analysis on culture supernatants. From the 16 soluble markers measured, 15 were registered within a detectable range (Fig. 4). IL-1β ($P = 0.02$), S100A9 ($P = 0.05$), and IL-10 ($P = 0.05$) demonstrated reduced concentrations in the presence of celecoxib in the cell culture compared with controls. We also observed a trend in reduction of IL-1ra ($P = 0.08$), IL-6 ($P = 0.07$), S100A8 ($P = 0.08$), TNF-α ($P = 0.07$), and VEGF-A ($P = 0.09$) indicating a functional role of COX-2 in M-MDSCs during infection. Cytokine levels did not correlate with CFU from the corresponding cultures (r value range = −0.50 to 0.60, $P = > 0.1$), but several relevant cytokines for M-MDSC function was reduced when COX-2i was added to the cultures.

3.6 | In vitro mycobacterial infection increases expression of M-MDSC suppressive markers

To investigate the effect of mycobacterial infection and COX-2i on M-MDSCs and their suppressive phenotype, M-MDSCs from the in vitro mycobacterial infection model were analyzed by flow cytometry. COX-2 expression in M-MDSCs was significantly increased in infected compared with the uninfected cell cultures (97.0% ± 4.4 vs. 3.9% ± 4.5, $P = 0.002$). Further, COX-2 expression was slightly reduced in response to celecoxib compared with the *M.bovis*-infected cultures with no celecoxib ($P = 0.039$) (Fig. 5). In contrast, Arg-1 expression in M-MDSCs increased with *M. bovis* infection but more strikingly after celecoxib treatment ($P = 0.002$). PD-L1 expression was already at a high level in uninfected M-MDSCs with a further increase after mycobacterial infection, but no significant effects of celecoxib were observed. Finally, IDO expression in M-MDSCs did not change in response to either *M. bovis* infection or celecoxib. These findings suggests that M-MDSC activity is induced by *M. bovis* and that COX-2 and Arg expression is influenced by COX-2i.

4 | DISCUSSION

The MDR-TB epidemic calls for efforts to look beyond antibiotics to discover new and alternative treatment options such as HDT.[2,3]

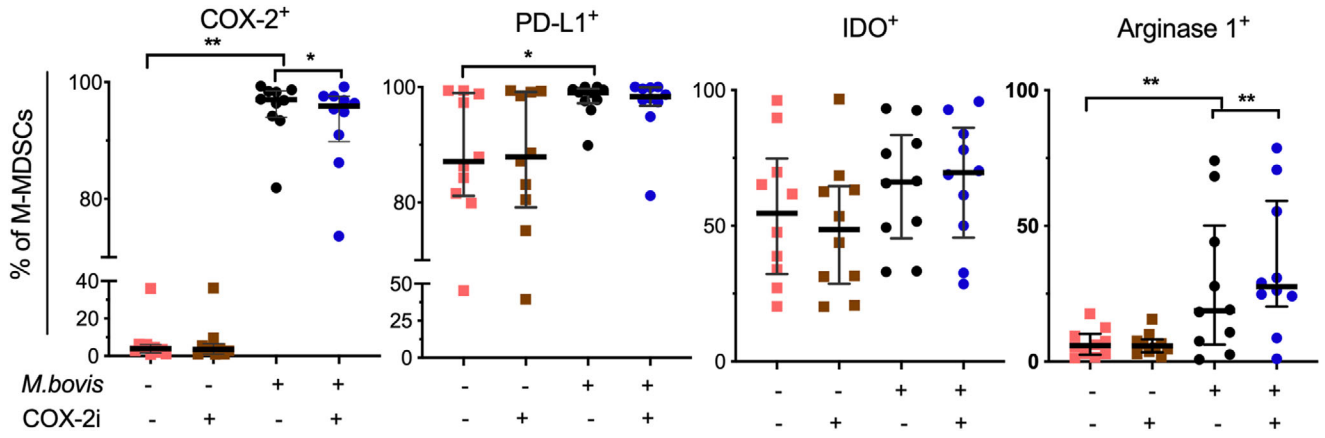

FIGURE 5 **Increased activity of M-MDSCs during *M. bovis* infection.** Expression of COX-2, IDO, PD-L1, and Arginase-1 in uninfected (pink), uninfected with celecoxib (brown), *M. bovis*-infected (gray), and *M. bovis*-infected with celecoxib (blue) cultured cells. M-MDSCs gated as HLA-$DR_{low/neg}$, $CD33^+$, $CD11b^+$, $CD14^+$, $CD15^-$ from PBMC. Plots are shown as median ± min–max, $n = 10$. Statistical significance calculated with Wilcoxon test: * significance $P < 0.05$, ** significance $P < 0.01$

COX-2i are used in several inflammatory diseases,[38] and have proven to reduce MDSC-mediated immune suppression in cancer.[28,39] It is known that M-MDSCs expand at the periphery in those with active TB disease.[14] Our novel data show that the severity of TB disease is clearly linked to increasing levels of M-MDSCs. We present, for the first time, data on peripheral blood M-MDSCs from a cohort of patients with active TB disease participating in a phase I clinical trial of adjunctive COX-2i supported by a mycobacteria in vitro infection model. We found no convincing differences in the frequency of M-MDSCs between the TB groups with and without COX-2i treatment. One may hypothesize that adjunctive COX-2i etoricoxib has a functional impact as shown by a more apparent decline in a single M-MDSC suppressive marker. Our findings of reduced cytokine levels in in vitro-infected M-MDSC cultures treated with the COX-2i celecoxib are also suggestive of qualitative changes in the M-MDSC population. Further research is however required to provide evidence on the effect of COX-2i on MDSC suppressive function.

We have previously shown that MDSCs, enriched through HLA-DR^{low}/$CD33^+$ isolation from blood of TB patients, demonstrate immunosuppressive potential by reducing T cell function.[14] In this study, high M-MDSC frequencies at TB diagnosis were associated with shorter TTP and clinical parameters as increased ML ratio, elevated ESR, and a high symptom score. It has previously been demonstrated that recently TB-exposed subjects display higher circulating levels of M-MDSCs compared with the controls with remote TB exposure and that M-MDSC frequencies decrease during TB treatment.[14] Increased MSDC activity during acute TB might also be necessary to prevent excessive inflammation and tissue damage. However, in several cancers, the extent of inflammation/disease, such as tumor burden and disease stage, correlates with M-MDSC frequencies and inhibition of MDSC is of great potential in cancer immunotherapies.[40,41] Our data support a linkage between M-MDSCs and TB disease severity, underscoring the relevance of M-MDSCs as HDT target in TB.

We report a trend of reduced M-MDSC frequencies and M-MDSC IDO expression from baseline to day 238 during TB therapy, most noticeable in patients receiving adjunctive COX-2i treatment. IDO inhibits adaptive host immune responses by metabolizing essential amino acids required for T cell activation.[18] High IDO expression is also associated with poor immune responses to *Mtb* in mouse models.[42] Here, we did not observe any difference in M-MDSC frequencies or markers when comparing the COX-2i group and controls during the first 84 days of treatment, although etoricoxib could be measured in plasma from all participants (data not shown). A trend in reduction of several M-MDSC markers was observed in both study groups. A reason for the significant reduction of IDO in the COX-2i group could be ascribed to the finding that the group randomized to adjunctive COX-2i, appeared to have more severe TB disease, as illustrated by cavitary disease, higher median ESR, and lower median TTP than controls. Thus, cation must be taken when interpreting the results. A likely hypothesis to explain the findings is that standard TB treatment may be the major cause of reduced M-MDSC frequencies and their suppressive capacity, as reported by others.[43,44] Still, COX-2i could accelerate this process as COX-2i reduced IDO expression in M-MDSCs in cancer cell lines and a mouse model.[27,45] This suggests that COX-2 may influence the expression of IDO in M-MDSCs, which is one of the immune suppressive mechanisms possessed by M-MDSCs.

Although there are conflicting reports about the effect of adjunctive COX-2i in murine TB,[31] acute TB infection in animal models suggests a beneficial role under high inflammatory conditions.[31,33,46,47] In human studies, aspirin treatment in TB meningitis reduced new brain infarcts and related deaths, possibly through thromboxane A2.[48] COX-2-specific inhibitors are currently under investigation as treatment to prevent immune reconstitution inflammatory syndrome (TB-IRIS) (*NCT02060006*). As in the present study, results from a whole blood bactericidal model in healthy volunteers showed no antimycobacterial effects of COX-2i without or in combination with anti-TB drugs.[49] However, larger clinical trials will be better suited to explore and evaluate the potential beneficial effect of adjunctive COX-2i treatment in TB.

M-MDSCs function as a reservoir and facilitate *Mtb* replication contributing to disease pathogenesis.[21] We report for the first time that human blood-derived M-MDSCs successfully phagocytose mycobacteria in short-term in vitro cultures. This has previously been shown in murine models where M-MDSCs harbor *Mtb* and promotes tissue damage[22] and in in vitro differentiated M-MDSCs from human PBMC.[21] Although *M. bovis* and not *Mtb* was used in our in vitro assay, they are closely related pathogens with 99% homology at genome level and *M. bovis* has been used as a model pathogen for human TB.[50,51] Furthermore, *M. bovis* administered as vaccination in mice has shown to induce M-MDSC suppression of early T cell responses and facilitate *M. bovis* persistence.[35] BCG vaccinated rhesus macaques also showed an increase in M-MDSC at injection site highlighting important aspects in TB vaccine research.[52]

We measured reduced levels in the cultures with celecoxib of all soluble cytokines and chemokines analyzed, significant for IL-1β, IL-10, and S100A9. M-MDSCs are a paracrine source of both IL-10 and S100A9, important for expansion, development, and function of M-MDSCs.[53,54] IL-1β is secreted in the tumor microenvironment causing accumulation of MDSCs.[55] Interestingly, a S100A9 inhibitor was recently proposed as a HDT target as it reduced MDSC frequency in *Mtb*-infected mice.[56] IL-1 confers host resistance by preventing formation of type I interferons in an aerosol-infected mouse model.[32] Although IL-1 could come from other sources than MDSCs in the mouse study, this demonstrates the complexity of host immune responses in TB. Taken together, the reduction of important cytokines for M-MDSC function by COX-2i may indicate a beneficial effect of adjunctive COX-2i for the host.

Our data suggests that celecoxib has no effect on bacterial growth in vitro, as measured by CFU in the mycobacterial infection model. This is in line with a recent study testing celecoxib in a whole-blood bactericidal activity model.[49] Thus, either COX-2i does not influence bacterial killing in our M-MDSC model or the COX-2i concentrations, infection dose or incubation time of 24 h was not appropriate to detect any possible effects. Still, in our in vitro mycobacterial assay, infection induced important proteins related to M-MDSC function such as COX-2, Arg-1, and PD-L1, which may facilitate mycobacterial persistence through its immune suppressive machinery. Unexpectedly, we observed a significant increase of Arg-1 in the presence of COX-2i in M-MDSCs indicating a link to COX-2 expression in both cell types. This is in conflict with some cancer studies that state that COX-2i blocks Arg-1 expression[57] and possibly reflects different regulation of the arachidonic acid pathway in various disease models.

Certain limitations should be considered in this study. In the clinical trial, sample size was limited due to the phase I design not aiming to study M-MDCSs as primary endpoint and small effects could therefore be difficult to discover. Also, although the patients were randomized, the COX-2i group seems to have more severe TB disease at baseline reflected in higher ESR and cavity compared with the control group that may have influenced the results. Peripheral blood was the source for M-MDSCs providing limited information on cells at the disease site. Furthermore, M-MDSC phenotyping has been extensively debated,[34,58] and a more recent recommendation has been published[59] after we planned and conducted our experiments, which will be part of our future investigations on MDSCs. Due to the heterogenic nature of MDSCs comprising different immature myeloid cells, our M-MDSC population is likely to contain other MDSC subtypes and possibility other non-MDSCs. Other recently emerged subtypes are early-stage MDSC (eMDSC) characterized as lineage negative (including CD3, CD14, CD15, CD19, CD56) HLA-DR- CD33+[34] and it is possible that our enriched MDSC (HLA-DR- CD33+) population used in in vitro infection assay contains eMDSC. As reported by others,[60,61] we observed alterations in MDSC protein expression when comparing fresh and frozen samples, especially CD15 expression defining PMN-MDSCs and this subset was therefore not investigated in this study. Thus, as PMN-MDSC poorly survives freezing,[61] but M-MDSC investigations have proven to be reliable in cryopreserved samples,[62] we assessed the in vivo impact of COX-2i on M-MDSC frequency and function on longitudinal collected frozen cells. Some markers of M-MDSC such as CD11b and CD14 show stability in cryopreserved samples.[62] Thus, since all blood samples were treated equivalently, we believe our data comparing patient groups are valid although data must be interpreted with caution as for every study based on frozen material. In the in vitro *M. bovis* infection model, due to limited cell numbers, we were not able to sort monocytes for comparison of CFU that could be of importance as COX-2i could affect the uptake and proliferation of *Mtb* in monocytes. Also, the 24 h in vitro infection time point was selected as the accepted time for one round of mycobacterial replication. The COX-2 effects are probably dependent on doses as well as time kinetics in such assays and longer infection time should be further studied. Finally, in our infection assay, M-MDSCs were infected with a MOI of 1, which may not be physiologically relevant in peripheral blood due to low frequency of M-MDSC; however, this ratio could be relevant in the context of granuloma, although the role of M-MDSC is not yet clear.[37]

In conclusion, in TB disease, the fine-tuned balance between efficient immune responses and suppressive mechanisms leading to *Mtb* clearance or containment and protecting the host from severe tissue damage is crucial. This study denotes a link between M-MDSCs and TB disease severity. COX-2 affects both pro- and anti-inflammatory pathways and adjunctive COX-2i treatment had limited effects on M-MDSC characteristics. However, the in vitro *M. bovis* infection model suggests a potential effect of COX-2i on M-MDSC function through alteration in important cytokines. Further studies are needed to conclude if COX-2 or M-MDSCs could serve as targets for HDT in TB disease.

AUTHORSHIP

A.M.D.R., N.D.P., and M.J.J. contributed with the concept and design of the study. Funding was arranged by A.M.D.R., G.W., and N.D.P. K.T., S.J., N.D.P., and A.M.D.R. recruited the participants for the study. M.J.J. and N.D.P. acquired the data. M.J.J. and N.D.P. performed the statistical analyses. M.J.J., K.T., S.J., N.D.P., R.M., and A.M.D.R. carried out the analysis and interpretation of the data. M.J.J. and A.M.D.R. drafted the

manuscript. K.T., S.J., N.D.P., A.M.D.R., R.M., and G.W. performed the critical revision of the manuscript and intellectual content. All authors have reviewed and approved the final manuscript.

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DISCLOSURES

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

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