**Mycobacterium tuberculosis** hijacks an evolutionary recent IFN-IL-6-CEBP axis linked to monocyte development and disease severity in humans

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**Running title**  
*M. tuberculosis* hijacks IL-6R-mediated monocyte expansion in humans.

**Authors’ contribution:**
MD, EK, ELR, GL, TD, DSM, JVW and AB designed experiments and analyzed data; MD, EK, JVW, DGM, LM, GD, DP, EK, GL, TD, MAB, ELR and AB performed experiments; GB, DR, JDM contributed with critical reagents/tools/clinical samples; AB, MD and JVW wrote the manuscript.
Abstract

Following pathogen-induced injury, multicellular organisms enhance production and differentiation of leukocytes, which are important players maintaining the body homeostasis. Monocyte counts are increased during human tuberculosis but it has not been determined whether *Mycobacterium tuberculosis* (*Mtb*) can regulate myeloid commitment. We demonstrated that following exposure to laboratory or clinical *Mtb* strains, primary CD34+ cells preferentially differentiated into monocytes/macrophages, and to lesser extent granulocytes. This process required pathogen viability and while *Mtb* induced type I IFN expression and STAT1 phosphorylation in CD34+ cells, myeloid conversion did not require type I or type II IFN signaling. In contrast, *Mtb* enhanced IL-6 production by CD34+ cell cultures and IL-6R blockade abolished myeloid differentiation and decreased mycobacterial growth. Integrated systems biology analysis of transcriptomic, proteomic and genomic data of > 1,500 individuals from cohorts of healthy controls, latent and active tuberculosis (TB) patients confirmed the existence of a myeloid IL-6/IL-6R/CEBP axis associated with disease severity in vivo. Furthermore, genetic and functional analysis revealed the *IL6/IL6R/CEBP* gene module has undergone recent evolutionary selection, including Neandertal introgression, linked to systemic monocyte counts. Together, these results suggest *Mtb* hijacks an evolutionary recent IFN-IL6-CEBP feed-forward loop, increasing myeloid differentiation linked to severe TB in humans.
Hematopoiesis, the development of different blood cell lineages from hematopoietic stem cells (HSCs), is a fundamental physiological process in vertebrates. HSCs give rise to lineage-restricted progenitors that gradually differentiate into mature cells. Following cellular differentiation, single-lineage elements including erythrocytes, megakaryocytes, lymphocytes as well as myeloid cells such as monocytes and granulocytes circulate throughout the body performing diverse functions. While HSC development towards cellular lineages during homeostasis has been extensively studied\(^{1}\), the mechanisms by which how progenitors give rise to mature cells during stress responses are less comprehended. For instance, certain pathogens regulate production of blood cells by the bone marrow and it has been shown that fine-tuned regulation of cytokine-induced signals is required for differentiation of HSC into mature cell types\(^{2-4}\). For example, the protozoan parasite that causes kalazar, *Leishmania donovani*, inhabits the bone marrow of humans\(^{5}\) and induces differentiation of myeloid cells at expense of lymphoid progenitors\(^{6}\). In the same line of evidence, after experimental exposure to Gram-negative bacteria, mice display increased amounts of bone marrow-derived neutrophils, through a G-CSF–C/EBP\(\alpha\) dependent mechanism\(^{7}\). Moreover, infection by intracellular bacteria has been shown to stimulate production of circulating monocytes\(^{8-9}\). Altogether, these studies indicate vertebrate hosts respond to infection by focusing specialized cell lineages, which are highly dependent upon the interplay of cytokine-induced hematopoiesis triggered during infection. Interestingly, recent reports have demonstrated stem/progenitor cells may be infected by different classes of infectious agents such as virus\(^{10}\) and bacteria\(^{11}\), albeit at low efficiency\(^{11}\). Therefore, since many pathogens may reach the bone marrow and provide microbial-HSC interactions, it is possible that, in addition to cytokines, pathogen recognition by progenitor stem cells directly regulate cell lineage commitment providing an anti-microbial defense system. In contrast, the Red Queen hypothesis\(^{12}\) predicts such
pathogens would benefit from cell lineage commitment to establish themselves into the host.

The human pathogen *Mycobacterium tuberculosis* (*Mtb*) has been recently detected in circulating hematopoietic stem cells (Lin CD34+) from latent TB individuals\(^\text{13}\). Since *Mtb* can also gain access to the bone marrow during extra-pulmonary\(^\text{14}\) as well as active pulmonary TB\(^\text{15}\), it has been suggested that the human bone marrow is a niche/reservoir for this bacterium during active or latent infection. However, whether interactions between *Mtb* and human CD34+ cells drive cellular differentiation has not been formally demonstrated. Interestingly, during active TB, myeloid cells have been shown to display alterations such as increased numbers in the periphery\(^\text{16-17}\) and dysregulated interferon-induced transcriptional signature\(^\text{18-19}\). More specifically, several interferon-inducible genes are modulated in circulating mature neutrophils and monocytes, as opposed to lymphocytes, in active TB patients, which calls forth a possible role of these genes in TB pathogenesis\(^\text{18-20}\). In contrast, lymphocyte compartments were recently demonstrated to be contracted during progression from latent to active TB in humans\(^\text{21}\). Based on this evidence, we hypothesized that *Mtb* regulates cellular differentiation of CD34+ hematopoietic cell progenitors during TB.

**Results**

1. *Mtb H37Rv replicates in primary human HSPC cultures.* To formally investigate the dynamics of *Mtb* infection by hematopoietic stem cell progenitors (HSPCs), we have exposed peripheral blood mononuclear cells (PBMCs) from healthy donors to H37Rv *Mtb* and employed different techniques to measure bacterial infectivity by CD34+ cells. Following 4h exposure to mycobacteria (multiplicity of infection, MOI3), flow cytometry experiments confirmed fluorescent *Mtb* were associated with CD34+ cells (Figs. 1a, b and Supplementary Fig. 1a). At that time point, we observed ~69% of CD34+ and ~79%
of CD14+ associated with *Mtb* (Fig 1c). However, numbers of bacteria/CD34+ cell, by means of MFI measurements, were found to be significantly lower when compared to those seen in CD14+ cells (Fig. 1d). These data suggested that while CD34+ cells may be permissive to *Mtb* infection in vitro, only low numbers of bacteria were associated with these cells. This idea was supported by confocal microscopy analysis, which confirmed the presence of few intracellular mycobacteria in purified CD34+ cells at 4h p.i. (Supplementary Fig. 1b). These results raised the possibility that although human primary CD34+ cells can be infected by *Mtb* in vitro (Supplementary Fig. 1b) and in vivo13, this cell population displayed intrinsic resistance to *Mtb* infection as it has been reported for other bacterial species11. However, when purified CD34+ cells were exposed to *Mtb* H37Rv (MOI3) and cultivated in conditioned media22,23, bacilli numbers exhibited a significant ~1.5-log growth at 5 days post infection (dpi) (Fig. 1e). Similarly, kinyoun staining procedure confirmed the increased number of bacteria over time in the same cell cultures (Fig. 1f). Together, these data demonstrated that *Mtb* infects and replicates in primary human CD34+ cell cultures in vitro. While at 1 dpi bacilli were more associated with the surface of round cells (Fig. 1f), at 5 d.p.i. intracellular bacteria appeared to be present in cells with abundant cytoplasm (Fig. 1f), thus indicating that *Mtb*-exposed CD34+ cells displayed significant morphological alterations. These results were confirmed by Giemsa staining (Fig. 1g, arrows) which showed increased frequency of cytoplasm richer cells and indicated the possibility that *Mtb* enhances cellular differentiation in CD34+ cells.

2. Live *Mtb* induces HSPCs towards myeloid differentiation and monocyte output.

To further explore whether *Mtb* triggered cellular differentiation by human purified CD34+ cells in vitro, we first performed RNA-seq analysis of *Mtb*-exposed cells at different days post-infection and evaluated differential expression of 180 transcription factors (TFs)
previously described to be associated with differentiation of distinct hematopoietic cells\textsuperscript{24} (Supplementary Fig. 1c). Interestingly, \textit{Mtb} infection increased the expression of lineage-specific regulators of myeloid (GRAN/MONO) (\textit{SPI1}, \textit{CEBPB}, \textit{CEBPA}, \textit{EGR2} and \textit{STAT2}), but not lymphoid (B and T CELL) (\textit{GABPA}, \textit{SOX5}, \textit{TCF3}, \textit{GATA3}, \textit{LEF1}, \textit{RORA} and \textit{LMO7}) or megakaryoid/erythroid (EARLY/LATE ERY) (\textit{GATA1}, \textit{FOXO3}, \textit{NFE2}, \textit{TAL1}) differentiation (Fig. 2a). Similarly, CellRouter signature score of the GRAN/MONO gene set was found to be increased in \textit{Mtb} vs uninfected samples in all time points studied (Fig. 2b). Next, we applied a recently developed network biology-built computational platform\textsuperscript{25} which, based on classification scores, can assess the extent to which a given population resembles mature cell types. Figure 2c shows that mRNA samples from \textit{Mtb}-exposed CD34\textsuperscript{+} cells presented enrichment of monocyte/macrophage profiles, but not other mature cell populations such as lymphocytes or dendritic cells. Additionally, at day 5 post-\textit{Mtb} infection, CD34\textsuperscript{+} cell cultures from 5 different donors displayed increased frequencies of CD11b (Fig. 2d), a surface molecule expressed during myeloid differentiation\textsuperscript{26,27}. Together, these data suggested that \textit{Mtb} drives human primary CD34\textsuperscript{+} cells towards myeloid differentiation. To further approach this hypothesis, we employed multiparametric flow cytometry by measuring expression of cell surface molecules functionally associated with myeloid differentiation of human CD34\textsuperscript{+} cells\textsuperscript{28-31}. More specifically, we gated on CD34\textsuperscript{+} events and evaluated \%CD64\textsuperscript{+} cells, since it has been demonstrated that human CD64\textsuperscript{+}CD34\textsuperscript{+} progenitors generate monocytes and granulocytes\textsuperscript{30-32}. Additionally, we included CD4 in the gating strategy as a second cell surface marker of human myeloid differentiation as previously demonstrated\textsuperscript{32}. Corroborating our hypothesis, \textit{Mtb} enhanced the frequency of CD64\textsuperscript{+}CD4\textsuperscript{+}CD34\textsuperscript{+} cells (Fig. 2d-f) at 5 d.p.i., but not CD10\textsuperscript{+}CD34\textsuperscript{+} (lymphoid) or CD41a\textsuperscript{+}CD34\textsuperscript{+} (megakaryoid) progenitors (Supplementary Fig. 2a). Similarly, Lin\textsuperscript{−}CD34\textsuperscript{+} cells from human bone marrow or PBMC (Supplementary Fig. 2b,c) exposed to \textit{Mtb} displayed increased
frequency of CD4⁺CD64⁺CD34⁺ cells. Moreover, these cells were also found to display augmented levels of CD38 and HLA-DR (Supplementary Fig. 2b,c), two molecules associated with advanced stage of cellular differentiation²⁸, ³³-³⁴. In addition, frequencies of CD4⁺CD64⁺ within purified CD34⁺ cells were found to be increased in cultures exposed to live but not heat-killed (HK) H37Rv Mtb (Fig. 2e-g). These results suggested that the observed cellular phenotype was mostly due to activities of live pathogen and only partially to mycobacterial PAMPs such as TLR2 (Ara-LAM) or TLR9 (Mtb gDNA)³⁵,³⁶ agonists which induced CD38 and HLA-DR, but not CD4 and CD64 expression in CD34⁺ cells (Supplementary Fig. 3a). Importantly, increased frequency of CD4⁺CD64⁺CD34⁺ cells was also observed when cell cultures were exposed to a clinical isolate of Mtb (Fig. 2f,h), ruling out a possible genetic factor associated with the laboratory strain H37Rv.³⁷ Furthermore, CD34⁺ cell death was not enhanced by Mtb infection as demonstrated by the use of a live-and-dead probe and lactate dehydrogenase (LDH) quantification in cell culture supernatants (Supplementary Fig. 3b,c). Together, these data indicate live Mtb directs primary human CD34⁺ cells towards myeloid differentiation in vitro.

Three mature cell populations can be generated during the process of myeloid commitment: monocytes, granulocytes and myeloid-derived dendritic cells²⁹,³¹,³⁸. To investigate whether Mtb stimulated HPSC differentiation into mature myeloid populations, purified cord blood-derived CD34⁺ cells were exposed to Mtb and 10d later, surface molecules were measured by flow cytometry. Live H37Rv (Fig. 3a,e) or clinical isolate (Supplementary Fig. 3d) Mtb enhanced expression of the monocyte surface molecule CD14, confirming the observed monocyte output enrichment by CellNet analysis (Fig. 2c). Compared to control cell cultures, CD14⁺ cells induced by Mtb displayed similar MFI expression of CD11b, HLA-DR, CD64 and CD16 (Fig. 3i). However, when compared to uninfected cultures, some but not all donors presented increased frequency of CD14⁺CD16⁺ monocytes following exposure to Mtb (Fig. 3j).
Moreover, albeit not statistically significant, *Mtb* enhanced the frequency of CD16^+^CD66b^+^ neutrophils in the majority but not all samples tested (Fig. 3b,f). In contrast, heat killed *Mtb* did not stimulate monocyte or neutrophil output (Fig. 3e,f). As expected (Fig. 2a-c and Supplementary Fig. 2a), when compared to uninfected cell cultures, megakaryoid/platelet- (Fig. 3c,g), dendritic cell- (Fig. 3d,h) or erythroid- (Supplementary Fig. 3e) associated markers showed no changes after exposure to *Mtb*. Altogether, these results suggest *Mtb* selectively favors generation of monocytes, and to lesser extent neutrophils, by CD34^+^ cells in vitro.

### 3. *Mtb*-enhanced myeloid differentiation is mediated by IL-6R, but not type I or type II IFN signaling.

Cytokines are important triggers of CD34^+^Lin^-^ differentiation in vivo and in vitro^39^ and Reactome pathway analysis of genes differentially expressed between infected versus uninfected conditions displayed enrichment of “cytokine signaling in immune system” (Supplementary Fig. 1d; n=3 donors, 2 independent experiments). Among several genes, this pathway displayed a significant enrichment of *IL6* (Supplementary Table 1) and it was one of the factors present in the conditioned commercial cocktail media (SCF, TPO, IL-3, IL-6 and Flt3L) employed in our in vitro system. Moreover, cytokine receptors including *IL6R* (Fig. 4a) as well as their cytokine partners, containing *IL6* (Fig. 4b), were enriched in *Mtb*-exposed CD34^+^ cells. In addition, “interferon signaling” and “interferon alpha/beta” pathways were significantly enriched in *Mtb*-exposed CD34^+^ cells (Supplementary Fig. 1d and Fig. 5a). Together, these data suggest *Mtb* enhances IL-6 and type I IFN signaling during myeloid differentiation in vitro and therefore, we decided to further investigate both pathways during this process. *Mtb*-exposed CD34^+^ cell cultures displayed increased *IL6* expression by qPCR (Supplementary Fig. 4a) and, in some donors, *IFNA2*, *IFNB* and *IFNG* transcripts were also detected albeit at lower level relative to the former cytokine (Supplementary Fig.
4a). Interferon-stimulated genes (ISGs) such as MX1, ISG15 and IFI16 as well as IL-6R-stimulated genes such as IL1RA, GRB2 and CXCL8 were enhanced in Mtb-stimulated CD34+ cells from 5 different donors (Supplementary Fig. 4b). In addition, IL-6 protein was found to be augmented in Mtb-infected cells when compared to uninfected cell cultures (Fig. 4c) and, in experiments with exogenous IL-6 added to CD34+ cell cultures, this cytokine increased the frequency of CD4+CD64+CD34+ cells (Supplementary Fig. 4c), confirming previous findings. Altogether, these data pointed to IL-6 signaling as a possible pathway involved in Mtb-enhanced myeloid differentiation by CD34+ cells. Therefore, we next investigated whether IL-6R signaling contributed to such phenotype. We utilized neutralizing monoclonal antibodies in our in vitro system and observed that neither type I nor type II IFN signaling pathways were required to Mtb-enhanced monocyte/granulocyte conversion (Figs. 4d,e and Supplementary Fig. 4d,e). Although conditioned commercial media induced IL-6R-dependent background levels (~5%) of CD14+ monocytes and (~5%) CD66b+ granulocytes (Fig. 4f-h), neutralizing anti-IL-6Ra antibody (α-IL-6R) abolished Mtb-enhanced myeloid differentiation seen in CD34+ cell cultures (Fig. 4f-h). In contrast, megakaryoid, erythroid- or dendritic cell-associated surface molecules were unaltered in α-IL-6R-treated cell cultures (Supplementary Fig. 4f-h). Next, we asked whether IL-6R signaling was involved in pathogen growth observed during cellular differentiation. We observed that Mtb-exposed CD34+ cultures treated with α-IL-6R (Fig. 4i) presented significantly lower CFU counts when compared with infected untreated control cell cultures, while α-IFNAR2 (Fig. 4j) or α-IFN-γ (Fig. 4k) did not affect CFU counts. These results suggest Mtb hijacks IL-6R-mediated myeloid differentiation by human CD34+ cells in vitro.
4. The IL6/CEBP gene module is enriched in TB monocyte transcriptomes and correlates with systemic dissemination and disease severity. To investigate whether IL-6R signaling correlated with monocyte development and TB-associated pathology in vivo, we performed a comprehensive systems biology analysis integrating transcriptomic, proteomic and genomic data from published cohorts consisting of a total of >1500 healthy controls and patients with latent, active and disseminated TB\textsuperscript{18,21,41-43} (Supplementary Table 2). First, we investigated whether monocytes from active TB display a potential \textit{in vivo} IL-6/IL-6R signature by using Ingenuity Pathway analysis (IPA) to determine upstream regulators in CD14\textsuperscript{+} monocyte transcriptomes\textsuperscript{18}. As shown in Fig. 5a (upper panel), when compared to cells from healthy controls, IL6, IL6ST, IL6R and STAT3 were significantly enriched in transcriptomes of purified monocytes isolated from active TB patients. As reported previously\textsuperscript{18,43,44}, STAT1 and IL1B were confirmed as significant upstream regulators in active TB monocytes (Fig. 5a upper panel). Strikingly, the two top upstream regulators in TB monocytes, IRF1 and STAT1, were the only genes in common between the “Berry TB monocytes” (GSE19435, GSE19439, GSE19444), the “IL6/STAT3 pathway” and the “in vivo IFN-β” signatures (both from Molecular Signatures Database\textsuperscript{43}) (Fig. 5a lower panel), suggesting these genes might be regulated by both IL-6 and type I IFN during active TB in vivo. Since type I IFN and IL-6 share the ability to induce phosphorylation of both STAT1 and STAT3\textsuperscript{45}, we used GSEA to investigate if their downstream target genes could overlap. In addition to previously demonstrated type I IFN/STAT1\textsuperscript{18}, “IL6/STAT3 pathway” was significantly enriched in the whole blood TB disease signature (FDR-corrected p<10\textsuperscript{-4}, Supplementary Table 2). Interestingly, these pathways were also significantly enriched in a gene set recently linked to monocyte expansion in vivo, measured as monocyte:lymphocyte (ML) ratio\textsuperscript{41} (Supplementary Table 2). In that study, Naranbhai et al.\textsuperscript{41} demonstrated that genes associated with
increased ML ratio positively correlate with impaired control of mycobacterial growth in vitro, supporting a possible connection between monocyte expansion and \textit{Mtb} survival. Additionally, increased IL-6/STAT3 protein levels and changes in CD34$^+$ and CD38$^+$ homeostasis were found to be early events in TB pathogenesis (Fig. 5b upper and lower panels), as suggested by our reanalysis of the plasma proteome upon disease progression from latent to active TB$^{21}$. “IL6/STAT3 pathway”-associated proteins such as PLA2G2A, CRP, STAT3, IL-6 and CFB increased around 12 months before TB diagnosis (Fig. 5c,d), which was concomitant with significant changes in “IFN/IL6-shared” plasma markers (CXCL10, IFNAR1 and MMP9). Strikingly, these alterations preceded enrichment of the ML ratio gene set (6 months before diagnosis, $p<0.05$, Fig. 5d upper panel) and reduction of the CD34/CD38 gene markers, in agreement with our in vitro model of \textit{Mtb}-enhanced CD34$^+$ differentiation (CD34$^+$ $\rightarrow$ CD34$^+$CD38$^+$ $\rightarrow$ CD14$^+$). Moreover, fold-changes were higher for “IL6/STAT3 pathway” genes than for “IFN/IL6-shared” genes, and significantly higher than “ML ratio” genes ($p<0.05$) or “CD34/myeloid differentiation” genes ($p<0.01$) (Fig. 5d lower panel). Reciprocally, IL-6-regulated transcriptomes also significantly overlap with both type I and II IFN signaling (Supplementary Table 2, $p=10^{-3}$-$10^{-10}$) and are enriched for stem cell/HSPC/CD34$^+$ gene sets (Supplementary Table 2, $p=10^{-4}$-$10^{-10}$). Together, these results suggested that IL-6/IL-6R signaling induces expression of ISGs as well as myeloid differentiation genes during active TB in humans. In support of this concept, TFs upstream of \textit{Mtb}-enhanced ISGs such as pSTAT1, and myeloid differentiation such as CEBP family members (C/EBP$\alpha$ and C/EBP$\beta$), were found to be upregulated in CD34$^+$ cells in vitro (Supplementary Fig. 4i). Furthermore, we observed that CEBPs, \textit{IRF1} and \textit{STAT1} as well as \textit{STAT3} and \textit{ICSBP/IRF8} TFs were significantly enriched in infected CD34$^+$ transcriptomes (Fig. 5e and Supplementary Fig. 5a), which were associated with increased mycobacterial replication in vitro (Fig. 1e).
Tuberculosis pathogenesis is a convoluted process which interconnects mycobacterial dissemination, host inflammatory responses and systemic tissue pathology. Since IL-6R signaling enhanced myeloid differentiation (Fig. 4f-h) as well as \textit{Mtb} growth in vitro (Fig. 4i) and the monocyte expansion (ML ratio) gene set was found to be enriched during TB progression in vivo (Fig. 5c lower panel), we reasoned that \textit{Mtb} enhances IL-6/IL-6R signaling-mediated monocyte output, promoting immunopathology and TB disease. To further investigate this molecular link with disease severity in vivo, we examined large transcriptomic data sets of disseminated TB, which includes extrapulmonary and lymph node TB (GSE63548). The “IL6/STAT3 pathway” was found to be significantly enriched among differentially expressed genes in both extrapulmonary (FDR p=10^{-3}) and lymph node TB (FDR p=10^{-4}, Supplementary Table 2). Furthermore, downstream targets of \textit{STAT3}, \textit{CEBPB}, \textit{CEBPD} (the genes encoding C-EBPβ/NF-IL6 and C-EBPδ/NF-IL6β) as well as other transcription factors driving myeloid/monocyte commitment (\textit{SPI1}/\textit{PU1} and \textit{ICSBP}/\textit{IRF8}) (Supplementary Table 2) were significantly enriched in the \textit{Mtb}-exposed CD34^{+} cells, the Berry whole blood TB signature, the monocyte expansion (ML ratio) and the disseminated TB gene sets (Fig. 5f). On the other hand, ISRE (\textit{STAT1}/\textit{STAT2}) and \textit{IRF1}, the major upstream regulators observed in TB monocyte transcriptome (Fig. 5a) and shared between IL-6 and IFN signaling (Fig. 5b), were enriched in the Berry whole blood TB signature, in the disseminated TB and in the \textit{Mtb}-stimulated CD34^{+} cells (Fig. 5f). In addition, we found significant enrichment of \textit{CEBPB}, \textit{SPI1}/\textit{PU1} and \textit{MEIS1}, but not ISRE targets in PBMC from active TB patients with BCG vaccine failure (Fig. 5f) suggesting that activation of specific pathways downstream of these TFs may overcome BCG-mediated protection in humans. Moreover, only \textit{CEBPB} targets were significantly enriched in the monocyte expansion (ML ratio) gene set in healthy subjects (Fig. 5f), supporting its link with myeloid
differentiation during homeostasis. Interestingly, TF MEIS1, which has been demonstrated as a key player in pathogenic myeloid differentiation by leukemic cells46,47, was highly enriched in disseminated TB transcriptomes (Fig. 5f). Since the IL6/IL6R/STAT3/CEBP axis was correlated with both systemic disease dissemination and monocyte expansion, two processes associated with TB disease16,17, we next examined whether these genes might be connected to disease severity in a published cohort with detailed clinical parameters and transcriptome data18. As shown in Fig. 5g, CEBPB and IL6R transcript levels were strongly connected and significantly correlated with STAT3 and inflammatory biomarkers such as C-reactive protein and erythrocyte sedimentation rate (not shown). In addition, CEBPB transcript levels were significantly higher in Mtb-positive vs. Mtb-negative sputum smears, and strongly correlated to tissue damage (Modal X-ray grade) and total symptom count, as well as systemic clinical parameters such as lymphocyte count, albumin and hemoglobin levels (Fig. 5g). Taken together, these results suggest the IL6/IL6R/STAT3/CEBP gene module is a major hub linked to monocyte expansion during Mtb infection in vivo and is amplified in severe pulmonary and extrapulmonary disease.

5. Recent genetic changes link IL6/IL6R/CEBP axis, monocyte expansion and TB pathogenesis in humans. The “type I IFN” signature found in active TB18, shared with the IL-6-regulated gene set (this study), comprises a number of well-characterized ISGs with cross-species antiviral activity (IRF1, PKR/EIF2AK2, OAS and APOBEC family members), which have been undergoing strong purifying selection during primate evolution48,49. Moreover, recent studies have also suggested a primordial role for RNA viruses in driving recent human evolution, considering selective Neandertal introgression in a large number of antiviral effector molecules50,51. We thus investigated the IL6/IL6R/CEBP signaling pathway, its partial overlap with type I IFN signaling and its link
to monocyte expansion from an evolutionary perspective. We hypothesized that these gene modules maintained during successful antiviral hominid evolution are linked to monocyte expansion and human susceptibility to severe TB. As shown in Fig. 6a, both the “specific” IL6/C/EBPβ pathway (upper left panel) and the partially overlapping IL-6/IFN STAT1/STAT3 pathways (upper right panel, STRING protein-protein interaction p<10^{-16} for both) displayed highly variable levels of conservation when compared to Homo sapiens (Fig. 6a lower panel). IL-6, IL-6R and C/EBP family members C/EBPα, C/EBPβ and C/EBPδ differ substantially throughout primate evolution and even among closely related hominids (Pan troglodytes and Gorilla gorilla), while both STAT1 and STAT3 remain largely conserved in most mammals, birds and reptiles (Fig. 6a lower panel). Upon analysis of cross-species type I IFN regulation from the ‘mammalian interferome’ database^{18}, we found that highly conserved mammalian ISGs significantly overlapped with the TB disease signature^{18,20}, as well as the IL6/STAT3 signaling pathway (Supplementary Table 2). As expected, the “IFN/IL6-shared” genes CXCL10, CXCL9, STAT1, STAT2 displayed higher fold-changes upon type I IFN treatment across all 10 species (Fig. 6b). Interestingly, the “IL6/STAT3 pathway” genes IL6, STAT3 and SOCS3 were also significantly upregulated while IL6R was significantly down-regulated (Fig. 6b) when cells from 10 species were exposed to type I IFN. Among CD34/myeloid pathway genes, ICSBP/IRF8 and CD38 were strongly upregulated, but only in 4/10 and 2/10 species, respectively, while ELF1 was homogenously and significantly upregulated in 9/10 species (Fig. 6b). These results suggest that type I IFN consistently regulates expression of IL-6 signaling and myeloid-associated genes in different species. However, among the entire IL6/IL6R/CEBP/CD34 myeloid gene set, CEBPB was the top most variable ISG across mammalian evolution (CV >1000%, Fig. 6c). Remarkably, type I IFN-mediated transcriptional upregulation of both CEBPB and CEBPD (previously
identified as IL6-specific TFs, NF-IL6 and NF-IL6β, respectively) was absent in all mammals investigated and significant only in humans (Fig. 6c, inset). These results were validated in an independent, published data set52, showing primate-specific (humans and macaques) upregulation of CEBPB and CEBPD (in contrast to rodents), upon stimulation with double-stranded RNA, one of the strongest type I IFN inducers (Supplementary Fig. 5b). Thus, transcriptional upregulation of C/EBP-β/C/EBP-δ by synergistic IL6- and type I IFN-signaling seems to be a relatively recent event in mammalian and primate evolution. In contrast, IFN-inducibility of both IL6 and STAT3 transcripts is conserved in most mammals, including humans (Fig. 6c, inset). Moreover, conserved mammalian ISGs49 also significantly overlapped with the monocyte expansion (ML ratio) gene set (Supplementary Fig. 5c). While “IFN/IL6-shared” genes displayed larger effect sizes for ML ratio, as compared to “IL6-specific pathway” genes (Fig. 6d left panel), the opposite was observed for absolute monocyte counts (Fig. 6d lower right panel). These data recapitulate the IL6/IL6R-mediated effect on monocyte expansion we observed in CD34+ cells in vitro (Fig. 4f-h). Hence, analysis of evolutionary pressure on the IL6/CEBP/CD34 myeloid gene module reveals a “feed-forward” loop in the crosstalk between type I IFN and IL-6 signaling through CEBPB and CEBPD, which appeared to be recently acquired in mammalian evolution. The latter was confirmed by a significant enrichment of primate-selected genes in this pathway (Supplementary Table 2). Since genetic susceptibility and transcriptional response to intracellular pathogens has also shown significant links to Neandertal introgression, mainly in populations of European and Asian descent populations50,53, we next explored enrichment for Neandertal introgression in the IL6/IL6R/CEBPB/CD34 myelopoietic gene module. As shown in Figure 6e (Venn diagram), three genes with Neandertal introgression in the ML ratio gene set were significantly upregulated in Mtb-stimulated CD34+ cells (OAS1, OAS2,
MT2A), while 7 genes shared between Neandertal and ML ratio gene sets were not differentially expressed in Mtb-exposed CD34+ cells (DUSP16, FCGR2A, IFITM1, PPARC1B, TNFSF13B, GRK5 and CD8A). OAS1, OAS2 and MT2A transcripts had significantly higher effect sizes upon ML ratios, corresponding to monocyte expansion, as compared to other introgressed genes (p<0.05) and to all other genes shown to regulate ML ratio \textit{in vivo} (p<0.001, Fig 6e right panel). Moreover, a recently identified introgressed Neandertal \textit{trans} e-QTL (rs5743618)\textsuperscript{50} has been associated to pediatric TB susceptibility in the Chinese Han population\textsuperscript{54}. Biological pathway analysis of genes significantly regulated in \textit{trans} of rs5743618 revealed IL-6/STAT3 signaling as significantly enriched (FDR p=4.6x10\textsuperscript{-8}) and \textit{IL6} as the most connected gene (Supplementary Table 2 and Fig. 6f). Finally, when considering genome-wide human variation, three lines of evidence support our hypothesis of a pathogen-selected \textit{IL6/IL6R/CEBP} gene module regulating monocyte expansion \textit{in vivo}. First, several large GWAS studies (a total of >200,000 individuals from different ethnic backgrounds) have identified single-nucleotide polymorphisms (SNPs) in or adjacent to \textit{IL6R}, \textit{CEBPA-CEBPD-CEBPE} and \textit{ICSBP/IRF8} genes (ranked in Fig. 6f and detailed in Supplementary Table 3) as significantly associated to blood monocyte counts. Second, gene-specific z-scores for human adaptation measured by Daub et al.\textsuperscript{55} in 51 different populations worldwide have revealed polygenic adaptation to pathogens. All genes in our proposed \textit{IL6/IL6R/CEBP} myeloid differentiation module (except \textit{CXCL10}) had positive z-scores (Fig. 6f and Supplementary Table 3), indicating relatively higher levels of population differentiation, with the highest z-scores for \textit{CEBPB, IL6} and \textit{IL6ST}, respectively. In addition, IL6/STAT3 signaling (but not type I IFN signaling) was significantly enriched among the top 500 genes with extreme positive adaptation z-scores (Supplementary Table 3). Third, a strong genetic component significantly predicts
IL-6 production stimulated by *Mtb* in both human purified macrophages and PBMCs from a large cohort of healthy controls$^{56}$ with both GWAS and immunophenotyping (Supplementary Fig. 5d). More importantly, the genetic factor defined by Bakker et al.$^{56}$ as variance in cytokine production explained by genetics (corrected for age and gender) was significantly stronger for IL-6, as compared to IL-1β, IL-22 and IFN-γ, considering 10 different bacterial and viral stimuli (Supplementary Fig. 5d). As compiled in Fig. 6f, our multi-level analysis revealed significant genetic links shared between IL-6/IL-6R/CEBP signaling, CD34+ myeloid differentiation, monocyte homeostasis and *Mtb* pathogenesis, which have undergone mammalian, primate and recent human selection, including Neandertal introgression and worldwide population-specific adaptation.

**Discussion**

Emerging evidence have suggested that *Mtb* establishes an infectious niche in the human bone marrow during active pulmonary TB$^{13}$. This idea is corroborated by independent observations showing that human mesenchymal and hematopoietic stem cells were found to contain viable bacilli during *Mtb* infection$^{13,15}$. Notably, elevated circulating monocyte and decreased lymphocyte numbers have been previously associated with active TB$^{16,17,41,57}$. In the present study, we observed that *Mtb* hijacks and enhances IL-6R-mediated myeloid differentiation by human primary CD34+ cells in vitro. The IL-6/IL-6R signaling-associated molecules such as C/EBPβ, C/EBPδ, STAT3 and their downstream targets were significantly enriched in cell transcriptomes from active TB patients. This pathway positively correlated with TB disease severity and systemic disease. Therefore, our data expands previous studies and raise a novel scenario in which *Mtb* skews myeloid development, mediated by IL-6 signaling, as a key step in TB pathogenesis.
While *Mtb* consistently enhanced IL-6 expression by purified CD34⁺ cell cultures in all donors, type I IFN was detected in some but not all donors. However, ISGs were highly enriched in the *Mtb*-exposed samples suggesting that either low amounts of type I IFN were produced in infected cell cultures and/or IL-6 itself induces STAT1 activation by CD34⁺ cells⁵⁸. Furthermore, these results indicate *Mtb* is a potent stimulus to induce ISGs in primary human CD34⁺ cells, similar to what has been reported for human macrophages⁴³. Additionally, the “interferon inducible gene signature” induced by *Mtb* in circulating mature myeloid cells, which has been largely demonstrated to be associated with TB disease severity¹⁸-²⁰, was also seen in *Mtb*-exposed CD34⁺ cell cultures in vitro (Supplementary Fig. 5e). However, while IL-6R signaling was involved in *Mtb* growth by CD34⁺ cells under cellular differentiation, type I or type II IFN signaling was not. Although the ISG set was enriched in *Mtb*-exposed CD34⁺ cells, our data suggest type I or type II IFN signaling do not mediate *Mtb*-induced monocyte development in vitro. Interestingly, in our previously characterized cohort of multiple sclerosis patients⁵⁹,⁶⁰, IFN-β therapy in vivo did not significantly change monocyte or lymphocyte counts, nor did it increase the ML ratio after three months of treatment in patients with documented clinical response (Supplementary Fig. 5f), supporting the concept that type I IFN by itself is not sufficient to cause monocyte expansion in vivo.

It has not been determined how mature myeloid cell populations from active TB patients acquire the “ISG” signature. While monocytes and other cells may encounter *Mtb*-associated inflammatory stimuli in infected tissues (e.g.: lungs and liver), our data raises the possibility *Mtb* may activate these cells during their development in the bone marrow. We have not directly addressed whether circulating monocytes/granulocytes acquire their phenotype in the bone marrow, during development of myeloid progenitors in vivo. Nevertheless, a recent study by Norris and Ernst⁶¹ demonstrated increased monocyte egress from the bone marrow in a murine model of *Mtb* infection. Considering
virulent $M_{tb}$\textsuperscript{15} and attenuated mycobacteria (BCG) can easily access the bone marrow and stimulate IL-6\textsuperscript{62-64} it is possible that individuals draining higher amounts of $M_{tb}$ into the bone marrow display increased inflammatory alterations including IL-6R-mediated myelopoiesis, upregulation of IFN-stimulated responses and increased disease severity. Indeed, we found that enrichment of the IL6/IL6R/STAT3/CEBP axis positively correlated with systemic disease such as lymph node and extrapulmonary TB (Fig. 5f,g). In line with our observations, IL-6, C/EBP\textbeta{} and SPI1/PU1 have been previously reported to be associated with TB susceptibility\textsuperscript{65,66}. Moreover, several independent studies have also indicated a detrimental role of “IFN and IFN-induced genes” during $M_{tb}$ infection in human TB\textsuperscript{18,20,21,67,68} and murine models\textsuperscript{69}. Our results expand these previous studies, revealing a link between the IL6/IL6R/CEBP gene module to monocyte development, mycobacterial dissemination and TB disease severity. Moreover, as evidenced from Scriba et al\textsuperscript{41} and re-analyzed in the present study (Fig. 5d), both IFN and IL6 pathways are early events in TB pathogenesis, detectable in the plasma proteome >6 months before diagnosis. Nevertheless, a pivotal role for IL6/IL6R signaling has not been evident from previous “omics” approaches. While IL-6 signaling partly overlaps with type I IFN responses (Fig. 5a-c), mostly due to their shared ability to activate STAT1 and STAT3\textsuperscript{46}, whole blood transcriptomic analyses are predominated by an “IFN-inducible neutrophil signature”\textsuperscript{18}. Therefore, the high numbers of neutrophils in the blood possibly mask differential expression of other gene sets in less frequent populations, such as monocytes, monocyte subsets and, in particular, Lin$^-$CD34$^+$ cells.

Interestingly, most ISGs present in the whole blood Berry TB signature\textsuperscript{18}, the Scriba et al. TB plasma proteome\textsuperscript{41} and the Naranbhai et al. ML ratio gene set\textsuperscript{41} displayed cross-species type I IFN-induction throughout mammalian evolution (Fig. 6b-d). In spite of a shared STAT1/STAT3 activation by type I IFN and IL-6, homeostatic
activation of CEBP-β is mostly IL-6-specific, as evidenced by data mining and STRING analysis (Fig. 6a), in keeping with its original description as NF-IL6\textsuperscript{70}. Across species, CEBPB is the top most variable ISG of the entire IFN/IL6/CD34/myeloid gene set (Fig. 6c). However, in addition to conserved type I IFN-induced IL6 and STAT3 transcription in the species studied, IFN-inducibility of CEBPB and CEBPD mRNA appeared to be developed during hominid evolution. Our data support the concept that Mtb hijacks a feed-forward loop between IL-6/IL-6R/STAT3 and IFN/IFNAR/STAT1/IRF1, activating the C/EBP gene module during myeloid differentiation in vivo (Fig. 6g). Furthermore, this process is linked to monocyte expansion and disease severity. While this study points to an important role for human CEBPs in TB disease, further experiments will define the precise molecular mechanisms of each CEBP family member, specifically CEBP-β and CEBP-δ, during Mtb infection.

We postulate that IL-6R-mediated myeloid differentiation in the bone marrow is used by Mtb to establish an infection niche in the human host, contributing to disease severity and pathogen dissemination. Alternatively, since the lung may be a reservoir for hematopoietic progenitors\textsuperscript{71}, Mtb could also active extra-marrow myelopoiesis during natural infection. While these questions merit directly investigation, nonetheless, it has been shown that anti-IL-6R treatment does not increase immunopathology nor tissue Mtb H37Rv loads in Balb/c mice\textsuperscript{72} corroborating a hypothetical use of IL-6R blockade in severe human TB\textsuperscript{73}. In summary, our results reveal a recent evolutionary link between myeloid differentiation, TB severity and IL6-IL6-R/CEBP signaling, which represents a novel host-directed target for therapeutic intervention in a major human disease.
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Materials and Methods

Reagents. *Mtb* Ara-LAM was obtained from BEI Resources and used at 5 µg/mL. *Mtb* H37Rv genomic DNA was obtained from 28 days colonies growing in Löwenstein–Jensen medium by CTAB method as previously described\(^7\). Recombinant human (rh) IL-6 was purchased from Immunotools. Anti-IFNAR2A (clone MMHAR-2, PBL) and anti-IFN-γ (clone B27, Immunotools) neutralizing antibodies were used at 1 and 10 µg/mL, respectively and anti-IL-6R (Tocilizumab, Roche) was used at 1 µg/mL. Fluorescent dye Syto24 was obtained from Thermo Fisher Scientific.

Mycobacteria cultures. The virulent laboratory H37Rv *Mtb* strain and the clinical *Mtb* isolate (CS267) were maintained in safety containment facilities at LACEN and UFSC as described elsewhere\(^7\). Briefly, *Mtb* was cultured in Löwenstein-Jensen medium (Laborclin) and incubated for 4 weeks at 37ºC. Prior to use, bacterial suspensions were prepared by disruption in saline solution using sterile glass beads. Bacterial concentration was determined by a number 1 McFarland scale, corresponding to 3 x 10\(^8\) bacteria/mL.
Subjects samples, cells and *Mtb* infections. This study was approved by the institutional review boards of Universidade Federal de Santa Catarina and The University Hospital Prof. Polydoro Ernani de São Thiago (IRB# 89894417.8.0000.0121). Informed consent was obtained from all subjects. Peripheral blood and bone marrow mononuclear cells were obtained using Ficoll-Paque (GE) in accordance with the manufacturer’s instructions. Briefly, blood collected in lithium-heparin containing tubes was further diluted in saline solution 1:1 and added over one volume of Ficoll-Paque reagent. The gradient was centrifuged for 40 min at 400 x *g*, 20ºC. The top serum fraction was carefully removed, the mononuclear fraction was harvested and washed once in a final volume of 50 mL of saline solution for 10 min at 400 x *g*, 20ºC. Subsequently, cell pellet was suspended and washed twice with 20 mL of saline solution for 10 min at 200 x *g*, 20ºC, to remove platelets. Cells were then suspended to the desired concentration in RPMI 1640 (Life Technologies) supplemented with 1% fresh complement inactivated (30 min at 56ºC) autologous serum, 2 mM *L*-glutamine (Life Technologies), 1 mM sodium pyruvate (Life Technologies) and 25mM HEPES (Life Technologies). Human Cord Blood (CB) purified CD34+ cells from 5 different donors were obtained from STEMCELL Technologies and resuspended in StemSpan Expantion Media – SFEM II (STEMCELL Technologies) according to manufacturer’s instruction. Optimal cell density for replication was 5 x 10⁴ CD34+ cell/mL. Following 4 days of expansion, cells were washed and diluted in SFEM II media without cytokine cocktail to the desired concentration. Culture purity was assessed by FACS and showed more than 90% of CD34+ events after expansion. For *in vitro* infection experiments, 1 McFarland scale was diluted in media to fit the desired multiplicity of infection (MOI). For each experiment, bacteria solution was plated in Middlebrook 7H10 agar (BD Biosciences) supplemented with 10% Oleic Acid Albumin Dextrose Complex (OADC) and incubated at 37ºC to confirm initial bacteria input. In a set of experiments, 1 McFarland scale was
incubated with 500 nM of Syto24 dye as described previously74. In some experiments, H37Rv Mtb was heat killed (HK) at 100°C for 30 min. In cytokine/cytokine neutralizing experiments, cells were pretreated with anti-IFNAR2 (1 µg/mL), anti-IFN-γ (10 µg/mL) or anti-IL-6R (1 µg/mL) for 1h and exposed to Mtb (MOI3). Following different time points post-infection, cells were harvest and centrifuged at 400 x g for 10 min, 20°C. Supernatants were then stored at -20°C, cells washed once in sterile saline solution and lysed by using 200 µL of 0.05% Tween 80 solution (Vetec) in sterile saline. Cell lysates were diluted in several concentrations (10^{-1} to 10^{-5}), plated onto Middlebrook 7H10 agar (BD Biosciences) supplemented with OADC 10% and incubated at 37°C. After 28 days, colony-forming units (CFU) were counted and results were expressed graphically as CFU/mL.

**Microscopy experiments.** After different time points post-infection, cells were washed and fixed with PFA 2% overnight at 4°C. Subsequently, cells were washed with sterile water solution and adhered into coverslips by cytopsin centrifugation. Samples were then fixed with methanol for 5 min, washed with sterile water and stained with carbol-fuchsin (Sigma) for 2 min. Samples were washed once with sterile water and counterstaining was done with methylene blue dye (Sigma) for 30 sec. Coverslips were fixed in slides with Permount mounting medium (Sigma) and examined using Olympus BX40 microscope and digital camera Olympus DP72. Syto24-stained Mtb was visualized in CD34⁺ cells by using confocal fluorescence-equipped inverted phase contrast microscope and photographed with a digital imaging system camera. Briefly, 1 x 10^5 CD34⁺ cells were seeded in 24-well plate and infected with Mtb syto24, MOI3, for 4 h. Further, cells were washed, fixed with PFA 2% and adhered into coverslip by cytopsin centrifugation. For nucleus visualization, cells were stained with Hoechst 33342 (Immunocchemistry technologies) for 2h. Cells were after washed and mounted for analysis in Leica DMI6000 B confocal microscope.
**Immunoblotting.** CD34+ cells were seeded at 3 x 10^5 cells in 24-well plate and infected with *Mtb* (MOI3). After 5 days of infection, cells were centrifuged at 4°C, pellet was lysed using M-PER lysis buffer (Thermo Fisher Scientific) containing protease inhibitors (Complete, Mini Protease Inhibitor Tablets, Roche) and protein extracts were prepared according to manufacturer’s instructions. For Western blot, 15 µg of total protein were separated and transferred to nitrocellulose difluoride 0.22 µm blotting membranes. Membranes were blocked for 1 h with TBST containing 5% w/v BSA and subsequently washed three times with TBST for 5 min each wash. Further, membranes were then probed with anti-pSTAT1 Y701 1:1000 (M135 – Abcam), anti-STAT1 1:1000 (SM1 – Abcam), anti-C/EBPβ 1:250 (sc-150 – Santa Cruz) or anti-β-Actina 1:5000 (8226 – Abcam) primary antibodies diluted in 5% w/v BSA, 0.1% tween 20 in TBS, at 4°C with gentle shaking overnight. Membranes were washed with TBST, incubated in secondary HRP-linked Ab for 2 h at room temperature, washed and chemiluminescence developed using ECL substrate (Pierce). Relative expression was normalized with β-actin control and pixel area was calculated using ImageJ software.

**Flow cytometry.** PBMC and bone marrow mononuclear cells were seeded at 5 x 10^5 cells per well in a final volume of 200 µL. After 4 h of resting at 37°C with 5% CO₂, cells were infected with *Mtb* (MOI3) for 72h, unless indicated otherwise. Cells were detached from the plate by vigorous pipetting, centrifuged at 450 x g for 10 min and washed twice in saline solution and stained with fixable viability stain FVS V450 (BD Biosciences) at the concentration 1:1000 for 15 min at room temperature. Cells were then washed with FACS buffer (PBS supplemented with 1% BSA and 0.1% sodium azide) and incubated with 10% pooled AB human serum at 4°C for 15 min. The following antibodies were used in different combinations for staining:

Staining of human CD34+ in PBMC: anti-Lin1(CD3, CD14, CD16, CD19, CD20,CD56) (FITC, clones MrP9, NCAM 16, 3G8, SK7, L27, SJ25-C1), anti-CD34 (PE,
PE, clone 581), anti-CD34 (FITC, 8G12), anti-CD34 (PerCP, clone 581), anti-HLA-DR (PE-Cy7, clone L243), anti-HLA-DR (Bv510, clone G46-6), anti-CD38 (APC, clone HIT2), anti-CD4 (APC-Cy7,GK1.5), anti-CD64 (Bv421, clone 10.1), anti-CD10 (FITC, clone HI10A), anti-CD14 (V450, clone MoP9), anti-CD14 (Alexa488, clone M5E2) were added at titrated determined concentration and incubated for 40 min at 4ºC.

Staining of CB CD34+ cells: anti-CD34 (PE, clone 581), anti-CD11b (clone), anti-CD4 (APC-Cy7, clone GK1.5), anti-CD64 (Bv421, MoP9), anti-CD14 (V450, clone MoP9) anti-CD14 (Alexa488, clone M5E2), anti-CD66b (PE, clone G10F5), anti-BDCA1 (APC-Cy7, clone L161), anti-CD41a (FITC, clone 6C9), anti-BDCA2 (APC, clone 201A), anti-BDCA3 (Bv510, clone 1A4), anti-Clec9A (A700, clone FAB6049P), anti-CD123 (PE, clone 7G3), anti-CD16 (APC, clone 3G8) were added at titrated determined concentrations and incubated for 40 min at 4ºC. All cells were subsequently washed with FACS buffer and resuspended in 2% PFA. Cells were acquired on BD FACS Verse with FACSuite software. Analysis were performed using FlowJo software v. 10.1 (TreeStar).

**Real-time quantitative PCR.** Total RNA was extracted from CD34+ cells exposed or not with *Mtb*. RNA was extracted after 1, 3 and 5 days of infection using TRIzol reagent (Thermo) according to manufacturer's instruction. Using 1 µg of RNA, cDNA was produced with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and 2 µL of 1:8 diluted product was used to the quantitative PCR reaction in a final volume of 10 µL. qPCR reactions were performed using the primers for: IFNA2A F: 5’-TTGACCTTTGCTTTACTGGT-3’, R: 5’-CACAAGGGCTGTATTTCTC-3’. IL6 F: 5’-CCACACAGACAGCACCAC-3’, R: 5’-AGGTTGTTTTCTGCCAGTG-3’. IFNG F: 5’-TCAGCTCTGATGTTTGGG-3’, R: 5’-GTTTCCATTATCCCGCTACATCTGAA-3’. IL1B F: 5’-ACAGATGAACTGCTCCTCTC-3’, R: 5’-GTCGGAGATTTCGATGCTGGG-3’. IFI16 F: 5’-ACTGAGTACAACAAAGCCATTTGA-3’, R: 5’-TTGTGACATTGTCTGTCAC-3’. MX1 F: 5’-ATCCTGGGATTTTGGGGGCTT-3’, R:
5'-CCGCTTGTCGCTGGTGTCG-3'. ISG15 F: 5'-TCCTGGTGAGGAATAACAAGGG-3', R: 5'-CTCAGCCAGAACAGGTCTGTC-3'. CSF1 F: 5'-GGAGACCTCGTGCCAAATTA-3', R: 5'-TATCTCTGAGCGCATGGTG-3'. CSF2 F: 5'-CTCAGAAATGTTTGACCTCCAG-3', R: 5'-TGACAAGCAGAAAGTTCCCTCAG -3'. CXCL8 F: 5'-
GAGGTGATTGAGGGACCAC-3', R: 5'-CACACCTCTGCACCCAGTTT-3'.

IL1RA F: 5'-ATGGAGGGAAGATGTGCCTGTC-3', R: 5'-
GTCCTGTCTTTCTGTCTCGTC-3'. GRB2 F: 5'-
GAAATGCTTAGCAACAGCGGCA-3', R: 5'-TCCACTTGGAGACCCGTCG-3'.

**RNA isolation and sequencing.** Total RNA from CB CD34+ cells exposed to *Mtb* in vitro and PBMCs from either healthy controls or patients with latent or active TB were isolated using TRIzol LS (Invitrogen; 10296010). RNAseq libraries were prepared using the Nugen Ovation Trio low input RNA Library Systems V2 (Nugen; 0507-08) according to the manufacturer’s instructions by the Nucleomics Platform (VIB, Leuven, Belgium). Pooled libraries were sequenced as 150 bp, paired-end reads on an Illumina HiSeq 2500 using v4 chemistry.

**RNAseq data quality assessment and differential expression analyses.** Illumina sequencing adapters and reads with Phred quality scores lower than 20 were removed with Trimmmomatic (0.36). Trimmed reads were aligned to *H. sapiens* reference genome (hg38) by STAR (2.6.0c). Aligned reads were mapped to genes using featureCounts from the Subread package (1.6.1). Genes with reads of less than 3 were removed. Library based normalization was used to transform raw counts to RPKM and further normalized using the edgeR TMM normalization (3.10.0). Data were then transformed using the limma voom function (3.36.2), prior to batch correction using ComBat (sva 3.28.0). Negative binomial and linear model-based methods were used for differential
expression analysis, using packages edgeR and limma packages. Differentially expressed genes (DEGs) were calculated with t-statistics, moderated F-statistic, and log-odds of differential expression by empirical Bayes moderation of the standard errors.

**CellNet and CellRouter analysis.** We applied CellNet to classify RNA-seq samples as previously described. Raw RNA sequencing data files were used for CellNet analysis. The analysis pipeline is based on the R statistical programming language. We used R version 3.4.1, CellNet version 0.0.0.9000, Salmon version 0.8.2 and the corresponding index downloaded from the CellNet website. We used CellRouter to calculate signature scores for each sample based on cell-type specific transcriptional factors collected from literature. Specifically, for this analysis, we normalized raw counts by library size as implemented in the R package DESeq2. We then plotted the distributions of signature scores across experimental conditions. Moreover, we used CellRouter to identify genes preferentially expressed in each experimental condition and used those genes for Reactome pathways enrichment analysis using the package Enrichr version 1.0.

**Systems biology analysis.** Ingenuity Pathway Analysis (IPA) software was used to perform the initial pathway/function level analysis on genes determined to be differentially expressed in transcriptomic analysis (Ingenuity Systems, Red Wood City, CA). Uncorrected p-values and absolute fold-changes were used with cut-offs of p<0.05 (monocyte transcriptomes from active TB patients) or p<0.01 (differentially expressed genes in *Mtb*-exposed CD34+ cells and all publicly available datasets from GEO). Differentially expressed genes were sorted into gene networks and canonical pathways, and significantly overrepresented pathways and upstream regulators were identified. Additional pathway, GO (Gene Ontology) and transcription factor target enrichment analysis was performed using GSEA (Gene Set Enrichment Analysis, Broad Institute Molecular Signatures Database (MSigDB)) and WebGestalt (WEB-based GEne SeT AnaLysis Toolkit). Gene sets from GO, Hallmark, KEGG pathways, WikiPathways and
Pathway Commons databases, as well as transcription factor motifs, were considered overrepresented if their FDR-corrected p-value was smaller than 0.05. To validate our compiled IL6/IL6R/CEBP and CD34+ myeloid differentiation gene modules, we used STRING (version 10.5) protein-protein interaction enrichment analysis (www.string-db.org), using the whole human genome as background. Principal component analysis, correlation matrices, unsupervised hierarchical (Euclidean distance) and k-means clustering were performed using XLSTAT and visualized using MORPHEUS (https://software.broadinstitute.org/morpheus/).

**Data processing and statistical analyses.** Data derived from in vitro experiments was processed using GraphPad Prism 6 software and analyzed using unpaired t test, one-way ANOVA or two-way ANOVA according to the experimental settings. Data from experiments performed in triplicate are expressed as mean ± SEM. Non-parametric tests (Mann-Whitney, Spearman correlation) were used for clinical data (sputum bacillar load, modal X-ray grade, symptom count) and molecular data that were not normally distributed. Pearson correlation was used for molecular data with a normal distribution. Statistical significance was expressed as follows: *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001.

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Figure legends

Fig. 1 | *Mtb* H37Rv infects human CD34+ cells and proliferates in cell cultures in vitro. PBMC from healthy donors were exposed to Syto24-labeled *Mtb* H37Rv (MOI3, Supplementary Fig. 1a) for 4h. **a**, Representative flow cytometry contour plots of gating strategy to analyze *Mtb* association within PBMC CD34+ cells. **b**, Live Lin−CD34+ events gated in (a) were analyzed for *Mtb*-Syto24 MFI. Black line: Uninfected control. Blue, orange and purple lines represent samples from three different donors. **c**, Frequencies and **d**, MFI of *Mtb*-Syto24+ events within CD34+ or CD14+ cell populations. Results are means ± SEM of data pooled from 3 independent experiments, n = 10 healthy donors. **e**, Purified CB CD34+ cells were exposed to *Mtb* H37Rv (MOI3) for different time points and CFUs from cell culture lysates were enumerated in 7H10 media. Results are means ± SEM of data pooled from 5 independent experiments, ***p ≤ 0.001 between experimental and control groups. **f**, Kinyoun staining of CD34+ cells after 1d and 5d of infection. Arrows indicate cells associated with bacilli. Experiments shown are representative of three performed. **g**, Representative Giemsa staining of CD34+ cells of a 5d culture. Arrow indicates cytoplasm-rich monocytoid cells in *Mtb*-infected cultures. Experiments shown are representative of three performed.

Fig. 2 | Live *Mtb* induces human CD34+ cells towards myeloid differentiation in vitro. Purified CD34+ cells from healthy donors (Stemcell technologies, n = 3) were exposed to *Mtb* H37Rv (MOI3) for different time points and mRNA-seq was performed as described in methodology section. **a**, Heatmap of the mRNA expression (z-score) of transcription factors involved in cell lineage commitment24. **b**, Signature score of data
from a by employing CellRouter analysis. c, Heatmap from mRNA data of uninfected vs 
*Mtb* infected cultures analyzed by CellNet as described in methodology section. d, 
Purified CB CD34<sup>+</sup> cells were exposed to *Mtb* H37Rv (MOI3) for 5 days and flow 
cytometry was performed. Graph represents frequencies of CD11b<sup>+</sup> events in uninfected 
(open circles) vs *Mtb*-infected cultures (blue circles) from four independent experiments. 
** p≤0.01 between experimental and control groups. e, Purified CB CD34<sup>+</sup> cells were 
exposed to *Mtb* H37Rv, Heat-killed (HK) *Mtb* H37Rv or *Mtb* clinical isolate 267 (MOI3) 
for 5 days and flow cytometry with the gating strategy was performed. f, Representative 
contour plots show frequencies of CD4<sup>+</sup>CD64<sup>+</sup> events within CD34<sup>+</sup> events. 
CD34<sup>+</sup>CD4<sup>+</sup>CD64<sup>+</sup> events of polled data from f were plotted to generate bar graphs g 
and h. Results are means ± SEM of data pooled from 4 independent experiments g and 
2 independent experiments h. ** p≤0.01 between experimental and control groups.

Fig. 3 | *Mtb* infection increases monocyte output from CD34<sup>+</sup> cells in vitro. Purified 
CB CD34<sup>+</sup> cells were exposed to *Mtb* H37Rv or HK *Mtb* H37Rv (MOI3) for 10 days and 
flow cytometry was employed to determine the frequencies of a, monocytes (CD14<sup>+</sup>), b, 
neutrophils (CD16<sup>+</sup>CD66b<sup>+</sup>), c, megakaryocytes/platelets (CD41a<sup>+</sup>) and d, classical 
myeloid dendritic cells (BDCA1<sup>+</sup>CD14<sup>low</sup>) in uninfected and *Mtb*-infected CD34<sup>+</sup> cell 
cultures. Graphs show frequencies of e, CD14<sup>+</sup>, f, CD16<sup>+</sup>CD66b<sup>+</sup>, g, CD41a<sup>+</sup> and h, 
BDCA1<sup>+</sup>CD14<sup>low</sup> events in uninfected (open circles), *Mtb*-infected (blue diamonds) or HK 
*Mtb*-exposed (red diamonds) cell cultures at day 10. Each symbol represents one 
individual experiment. Results are means ± SEM of data pooled from 3 - 9 independent 
experiments. ** p≤0.01 between experimental and control groups. i, Histograms show 
the expression of CD11b, HLA-DR, CD64 and CD16 in CD14<sup>+</sup> events from a. Black 
dashed lines: Uninfected control. Blue solid lines: *Mtb*-infected group. Data 
representative of 5 independent experiments. j, Frequency of CD14<sup>+</sup>CD16<sup>+</sup> events in
Mtb-exposed cell cultures after 10d. Contour dot plot of CD14^+CD16^+ frequencies from one representative donor. Open circles: Uninfected control. Blue circles: Mtb-infected group. Each symbol represents an individual experiment. Pooled data of eight independent experiments, n = 5 different donors. p=0.076 between experimental and control groups.

**Fig. 4** | *Mtb* enhances IL-6R-mediated myeloid differentiation in vitro. Purified CB CD34^+ cells were exposed to *Mtb* H37Rv (MOI3) for different time points and mRNA-seq was performed as described in methodology section. a, Heatmap (z-score) of differentially expressed interleukin receptor genes. b, Heatmap (z-score) of differentially expressed interleukin genes. Shown is average mRNA expression of three different donors from two independent experiments. c, Supernatant IL-6 measurements from unexposed or *Mtb*-exposed CB CD34^+ cell cultures at day 1 and 5 p.i. Open circle: uninfected control. Blue circle: *Mtb*-infected group. Results are means ± SEM of data pooled from 8 independent experiments. **p≤0.05 between 1d experimental and control groups. Purified CB CD34^+ cells were treated with d, anti-IFNAR2 (1 µg/ml) or e, anti-IFN-γ (10 µg/ml) and then exposed to *Mtb* H37Rv (MOI3) during 10d for determination of CD14^+ monocyte frequencies. Results are means ± SEM of data pooled from 2 independent experiments. f, Representative contour plots of CD14^+ monocytes in CD34^+ cell cultures exposed to *Mtb*, in the presence or absence of anti-IL6R (Tocilizumab, 1 µg/ml) for 10d. g, Results shown are means ± SEM of data pooled from 3 independent experiments from f. **p≤0.01 between uninfected and *Mtb* group and #p≤0.05 between *Mtb* and *Mtb*+anti-IL6R-treated group. h, Results shown are means ± SEM of data pooled from 3 independent experiments showing frequency of CD66^+CD16^+ neutrophils in *Mtb*-infected cell cultures in the presence or absence of anti-IL6R. CD34^+ cell cultures were treated as in d-f with i, anti-IL6R, j, anti-IFNAR2 and k, anti-IFN-γ and then
exposed to *Mtb* CFUs for different time points and CFU enumerated as described in Material and Methods. Results are means ± SEM of data pooled from 4 independent experiments. **p≤0.01 between *Mtb* and *Mtb*+anti-IL6R at 7d.

**Fig. 5** An *IL6/IL6R/STAT3/CEBPB* gene module is enriched in active TB transcriptome and proteome and correlates with myeloid differentiation and disease severity. 

**a,** Upper panel: upstream regulators significantly enriched by causal Ingenuity Pathway Analysis (IPA) in monocyte transcriptomes from patients with active TB (GSE19443), ranked by activation z-score, p-values are corrected for genome-wide testing (FDR). Lower panel: IRF1 and STAT1 are the top upstream regulators shared between the Berry et al. TB disease signature (GSE19435, GSE19439, GSE19444), the IL6/STAT3 pathway (Hallmark GSEA) and the “human in vivo IFN-β signature” (GSEA HECKER_IFNB1_TARGETS). 

**b,** Upper panel: overlap between the Berry et al. TB disease signature, IL6/STAT3 pathway (GSEA) and the active TB plasma proteomic signature⁴¹. Lower panel: significant STRING protein-protein interaction network (p<10⁻¹⁶) for IFN/IL6 shared genes (green marbles) and IL6/STAT3 genes (red marbles), clustering separately by k-means. 

**c,** Upper panel: significant linear increase over time before active TB diagnosis in plasma proteome⁴¹ for “CD34/myeloid” (yellow), “IL6/STAT3” (orange) and “IFN/IL6-shared” (blue) clusters found in **b.** Lower panel: monocyte/lymphocyte (ML) ratio gene set members defined by Narabhai et al.⁴² over time before active TB diagnosis in plasma proteome⁴¹. 

**d,** Upper panel: increased “IL6/STAT3” cluster protein expression precedes monocyte expansion markers (ML ratio gene set) in the TB plasma proteome. Lower panel: data as in **d** shows significant higher fold-changes for “IL6/STAT3” vs. “IFN/IL6-shared” cluster members. 

**e,** Transcription factor enrichment analysis (GSEA) of differentially expressed genes (determined by RNA-seq) in *Mtb*-exposed CD34⁺ cells in vitro (n = 3 donors). 

**f,** Multi-cohort transcription
factor enrichment analysis (GSEA) of large transcriptomic data sets shows overlap between TB active disease signature\textsuperscript{18}, in vitro CD34\textsuperscript{+} \textit{Mtb} signature at 24h, in vivo monocyte expansion (ML ratio)\textsuperscript{42}, disseminated TB (lymph node, GSE63548), as well as TB patients with BCG vaccine failure (GSE20716). \textbf{g,} Transcriptional analysis of whole blood shows significant correlation of \textit{CEBPB} transcripts with \textit{IL6R} and \textit{STAT3} transcript levels, mycobacterial positivity in sputum smears, C-reactive protein (CRP), tissue damage (Modal X-ray grade), symptom count as well as systemic clinical parameters (lymphocyte count, albumin and haemoglobin levels), in patients with tuberculosis. Results shown are from UK cohort described in ref. 18 (n = 30 patients with latent and active TB) P-value<0.05, ** p-value<0.01, *** p-value<0.001, **** p-value<0.0001.

\textbf{Fig. 6 | Evolutionary recent and human-specific genetic adaptation link \textit{IL6/IL6R/CEBP axis with monocyte expansion and TB pathogenesis. a,} Upper panel: STRING protein-protein interaction networks centred around \textit{CEBPB} (left) and \textit{IL6R} (right, both p<10\textsuperscript{-16}), k-means clustering shows \textit{CEBPA}, \textit{CEBPB} and \textit{CEBPD} myeloid TF (green marbles, left panel) and “IFN/IL6 shared” genes (green marbles, right panel) form separate clusters. Lower panel: Heat map showing \textit{CEBPB} vs. \textit{IL6R} networks generated by STRING co-occurrence protein conservation scores across primates, mammals, birds and reptiles. Note only \textit{CEBPB} and \textit{CEBPA} differ strongly among hominids, while \textit{CEBPA-CEBPB-CEBPD} vary significantly throughout primate and mammalian evolution, as compared to highly conserved \textit{STAT3/STAT1} amino acids. \textbf{b,} Highly conserved type I IFN upregulation of “IFN/IL6-shared” genes from humans to birds (derived from http://isg.data.cvr.ac.uk/), as compared to “IL6/STAT3” and “CD34/myeloid differentiation” genes. \textbf{c,} \textit{CEBPB} displays high variation in type I IFN transcriptional regulation across human-mammalian-bird
evolution. *Inset, CEBPB and CEBPD* selectively acquired type I IFN upregulation in humans (filled circles); ** p-value<0.01 and * p-value<0.05 represent CEBPB and CEBPD values, respectively for humans versus the other species. ** Left panel: both IFN-regulated and IL6-regulated transcripts display significantly higher effect size on monocyte/lymphocyte (ML) ratio as compared to all other ML ratio gene set members. Right panel: IL6-regulated transcripts have significantly higher effect sizes on absolute monocyte counts, as compared to IFN-regulated transcripts (p<0.05). ** Left panel: overlap between human genes with significant Neandertal introgression, genes differentially expressed in *Mtb*-exposed CD34+ cells (CD34+ DE) and the “ML ratio” gene set. Right panel: OAS1, OAS2 and MT2A transcripts presented significantly higher effect sizes upon ML ratios, corresponding to monocyte expansion, as compared to other introgressed genes (p<0.05) and to all other genes shown to regulate ML ratio in vivo (p<0.001). ** Compiled functional, proteomic, transcriptomic and genomic evidence for an IL6/IL6R/CEBP gene module linking CD34+ myeloid differentiation to TB pathogenesis and disease severity. For enrichment analysis (GSEA) and differential gene expression, ranks were determined as 0-1-2-3 according to quartiles; z-scores were obtained from Daub et al.55 p-value<0.05, ** p-value<0.01, *** p-value<0.001, **** p-value<0.0001. ** Proposed model for *Mtb* hijacking of IL-6-mediated CD34+ myeloid differentiation pathways using an IFN/IL6 feed-forward loop (details in the text).

**Supplementary Figure Legends**

S1 – *Mtb*-CD34+ interactions and signaling pathways associated with CD34+ cell differentiation. ** Gating strategy demonstrating H37Rv stained for Syto24 as measured through the FL1 channel. b, Confocal microscopy showing a CD34+ cell infected with Syto24-stained H37Rv. Nuclei = DAPI/blue. ** Mtb = Syto24/green. c, Heat map showing z-score values of 180 transcription factors expressed by CD34+ cells.
exposed to *Mtb* (MOI3) at days 1, 3 and 5 post-infection. **d,** Enrichment of Reactome pathways based on gene signatures derived from each experimental condition. Enrichment of Reactome pathways based on gene signatures derived from each experimental condition. Gene signatures were composed by genes with log2 fold change > 0.75 when comparing one experimental condition versus all others. The size and color of the circles are proportional to the -log10 of the adjusted p-value.

**S2 – *Mtb* induces myeloid progenitors in bone marrow and peripheral blood mononuclear cells.** **a,** Frequencies of CD10⁺CD34⁺ and CD41a⁺CD34⁺ cells in uninfected vs *Mtb*-infected cell cultures. Dot plots of **b,** bone marrow and **c,** peripheral blood samples from 2-4 healthy subjects showing frequencies of CD4⁺CD64⁺CD34⁺ and CD38⁺HLADR⁺CD34⁺ cells in uninfected vs *Mtb*-infected cell cultures.

**S3 – Purified mycobacterial agonists partially activate CD34⁺ cells.** **a,** CD34⁺ cells were exposed to AraLam (10 µg/ml) or gDNA (10 µg/ml) and 5 days later, CD34⁺ cells were gated and CD38, HLADR, CD4 and CD64 MFI calculated. Cell death analysis by means of **b,** frequencies of cell permeability dye (FVS+) and **c,** LDH quantities detected in the supernatants from uninfected of *Mtb*-exposed CD34⁺ cell cultures 5 dpi. **d,** Frequencies of CD14⁺ cells during 10-day exposure to a clinical isolate of *Mtb*. **e,** The erythroid cell marker CD235a was measured by flow cytometry in *Mtb*-exposed and control CD34⁺ cells 10 dpi.

**S4 – IL6/IFN gene expression and transcription factor activation during myeloid differentiation by *Mtb*-exposed CD34⁺ cells.** CD34⁺ cells were exposed to *Mtb* (MOI3) and at days 1, 3 and 5 p.i., qPCR was performed for quantification of **a,** IL6/IFN
cytokines and b, ISGs and IL-6-induced genes. c, CD34+ cells were stimulated with or without recombinant IL-6 for 5 days and frequencies of CD4+CD64+CD34+ cells were measured by flow cytometry. CD66b+CD16+ neutrophil frequencies in 10-day culture of Mtb-exposed CD34+ treated with d, anti-IFNAR2 (1 µg/ml) or e, anti-IFN-γ (10 µg/ml) blocking antibodies. f, CD41+ megakaryocytes, g, CD235+ erythrocytes and h, BDCA1+CD14low myeloid DC frequencies in 10-day culture of Mtb-exposed CD34+ treated with anti-IL-6R blocking or control antibodies. i, Western blotting for pSTAT1 (Y701), total STAT1, C/EBPα, C/EBPβ and actin was performed as described in the methodology.

S5 – ISG/CEBP gene expression and regulation during myeloid differentiation by Mtb-exposed CD34+ cells. a, Purified CB CD34+ cells were exposed to Mtb H37Rv (MOI3) for different time points. Heat maps showing z-score values of CEBP family members expressed by CD34+ cells exposed to Mtb (MOI3) at different time points. b, Primary dermal fibroblasts from humans, macaques and mice were stimulated for 8h in vitro with dsRNA analog (polyI:C) and CEBP family member transcript levels were quantified by RNAseq (expressed as fold-change over unstimulated cells). Raw data obtained from Hagai et al.52 (https://scb.sanger.ac.uk/#/base/main). c, Overlap between conserved mammalian ISGs (as in Fig. 6 c,d), the “IL6/STAT3 pathway” and the “ML ratio” gene set defined by Narabhai et al.42 (upper right panel). d, Data plotted as genetic factor, which is the variance of cytokine secretion explained by genetics corrected for age and gender56. from monocyte-derived IL-6, TNF, IL-1β and lymphocyte-derived IL-17, IL-22 and IFN-γ. Cells were exposed to bacterial and viral stimuli (LPS, PolyI:C, Influenza, E. coli, B. burgdorferi, M. tb, C. burnetii, S. aureus) and monocyte-derived IL-6, TNF, IL-1β as well as lymphocyte-derived IL-17, IL-22 and IFN-γ measured66. e,
Purified CB CD34+ cells were exposed to \textit{Mtb} H37Rv (MOI3) for different time points. Heat maps showing z-score values of ISGs expressed by CD34+ cells exposed to \textit{Mtb} (MOI3) at different time points. f, Lymphocyte and monocyte counts, as well as monocyte/lymphocyte (ML) ratio before and after 3 months of IFN-\(\beta\) treatment in 7 MS patients previously described as IFN-\(\beta\) responders\textsuperscript{59,60}.
Figure 1

(a) Flow cytometry plots showing SSC-A, FSC-H, and Gated on Singlets and Live.

(b) histograms of H37Rv Syto24 gated on Lin-CD34+.

(c) Graphs showing Mtb syto24 (% and MFI) for CD34+ and CD14+.

(d) Graphs showing Mtb syto24 MFI for CD34+ and CD14+.

(e) Bar graph showing CFU/mL [log10] for 4h, 1d, 3d, and 5d.

(f) Images showing Mtb growth over 1d and 5d.

(g) Images showing Mtb growth under Uninf. and Mtb conditions.
Delgobo et al. Figure 2

Panel a: Heatmap showing the expression of various genes in different time points (1d, 3d, 5d) of uninfected and Mtb-infected conditions.

Panel b: Scatter plot depicting the signature scores for B cell, Gran/Mono, HSC/Early Ery, Late Ery, and T cell across different time points.

Panel c: Schematic diagram illustrating the process from HSPCs to CellNet analysis.

Panel d: Graph showing the percentage of CD11b+ cells in uninfected and Mtb-infected conditions.

Panel e: Flow cytometry plots for gating on singlets and live cells.

Panel f: Gating strategy for CD34+ cells.

Panel g: Bar graph showing the percentage of CD4+CD64+ cells in uninfected, H37Rv, HK H37Rv, and Mtb-CS267 conditions.

Panel h: Bar graph showing the percentage of CD4+CD34+ cells in uninfected and Mtb CS267 conditions.
Figure 3

(a) Uninf. vs. Mtb
(b) Gated on CD14+
(c) Gated on CD66b-
(d) BDCA1

Uninf. Mtb HK Mtb

CD14+ cells (%)

CD66b+CD16+ (%)

CD41a+ (%)

BDCA1+CD14+ (%)

**

Gated on CD14+

-- Uninf. --- Mtb

CD11b HLA-DR

CD64 CD16

CD14+CD16+ (%)

p=0.076
### Table

| Upstream Regulator | z-score | p-value  |
|-------------------|---------|----------|
| IRF1              | 3.251   | 2.23E-09 |
| STAT1             | 2.526   | 0.0048   |
| IL6               | 2.342   | 0.000062 |
| IL1B              | 2.048   | 0.000000236 |
| JAK1              | 1.998   | 0.0000728 |
| IL6ST             | 1.982   | 0.0138   |
| JAK2              | 1.66    | 0.000198 |
| IL6R              | 1.432   | 0.0161   |
| LAM               | 1.342   | 0.0000137 |
| IL10              | 1.033   | 0.0000553 |
| STAT3             | 0.467   | 0.000025 |
| CSF2              | 0.099   | 0.000117 |
| IL6ST             | -1.249  | 0.0144   |
| SOCS3             | -3.092  | 0.000716 |

### Diagrams

**Figure 5**

- **a**: Upstream Regulator Activation
- **b**: Berry TB, IL6/STAT3, IFNβ in vivo
- **c**: Berry TB, IL6/STAT3, IFNβ in vivo
- **d**: Fold-change TB plasma proteome
- **e**: Log P-value
- **f**: Enrichment of TF targets
- **g**: Sputum smear

**Abbreviations**

- CD34/myeloid
- IL6/STAT3
- IFN/IL6-shared
- Berry TB
- Plasma TB
- IFN-β
- CEPRP
- C-reactive protein
- Hemoglobin
Figure 6

**a**

De novo analysis of CD34+ DE and ML Ratio between Neandertal and modern human samples. Heatmap visualization of conservation across species. Top rows show rank z-score, with genes on the left and species on the top. The color represents % a.a. conservation, with max = red and min = blue.

**b**

Box plot showing the mean IFN-induced log FC (across 10 species) for IFN/IL-6 shared, IL-6/STAT3, and CD34/myeloid.

**c**

Mean IFN-induced log FC across species, showing the CV (%)/IFN-regulation.

**d**

Bar chart showing ML ratio effect size for different conditions: IFN/IL-6, IL-6, other, and ML ratio effect size.

**e**

Venn diagram showing the number of CD34+ DE and ML Ratio between Neandertal and modern human samples.

**f**

Heatmap showing the enrichment of genes with high z-score in CD34+ DE and ML Ratio.

**g**

Steady State and Active TB pathways in Monocytes, indicating the role of IL-6 and IL-6R in TB dissemination and severity.
Delgobo et al. Supplementary Figure S1

a

SSC-A

FSC-A

FL-1

--- H37Rv

--- H37Rv-syto24

b

MERGE/DIC

Mtb-syto24

ROESCHT

5µm

5µm

c

d

Reactome pathways

- Downstream signal transduction
- Signaling by FGFR1
- Signaling by PDGF
- NGF signaling via TRKA from the plasma membrane
- Signaling by FGF1
- Downstream signaling of activated FGFR1
- Downstream signaling of activated PDGF
- Downstream signaling of activated PDGF
- Phospholipase C-mediated cascade, FGFR1
- Signaling by Type 1 Insulin-like Growth Factor 1 Receptor (IGF1R)
- IRS-related events triggered by IGF1R
- IGF1R signaling cascade
- Interaction between L1 and Ankyrin
- EPHA-mediated growth cone collapse
- Negative regulation of FGFR4 signaling
- SHC-mediated cascade, FGFR4
- Axon guidance
- FGFR4 ligand binding and activation
- Cell surface interactions at the vascular wall
- Adherens junctions interactions
- C3(1) mediated glycosylation
- G alpha (i) signaling events
- Neuronal system
- Transmission across Chemical Synapses
- O-glycosylation of TSR domain-containing proteins
- P2X7 receptor activation, signaling and aggregation
- Defective B3GALT1 causes Peter’s plus syndrome (Pts)
- ADF/cofilin-mediated microtubule disassembly
- RIG-I/MDM2 mediated induction of IFN-alpha/beta pathways
- Negative regulators of RIG-I/MDM2 signaling
- ISG15 antiviral mechanism
- Antiviral mechanism by IFN-beta stimulated genes
- Interferon-gamma signaling
- Interferon-alpha/cytokine signaling
- Cytokine signaling
- Synthesis of pyrophosphates in the cytosol
- Class I/II (MHC) antigen processing and presentation
- Interferon transport
- Bcl2 family proteins
- Telement maintenance
- Inositol phosphate metabolism
- Extension of Telomeres
- O2/C02 exchange in erythrocytes
- Erythrocytes uptake carbon dioxide and release oxygen
- Erythrocytes uptake oxygen and release carbon dioxide
- Innate immunity
- Cytokine signaling
- Immunoregulatory interactions between a Lymphoid and a Histocytic cell
- Chemokine receptors bind chemokines
- GPCR ligand binding
- Peptide-signal-binding receptors
- Extracellular matrix organization
- Cytokine signaling in immune system
- Immune System

Uninf. 1d 3d 5d
Uninf. Mtb Uninf. Mtb Mtb

-log10 (p-value)

5

10

15
Supplementary Figure S3

(a) Graphs showing MFI/CD34^+ for CD38, HLA-DR, and CD64 for Uninf., AraLAM, and gDNA.

(b) Scatter plot showing gating on singlets with SSC-A vs. FVS and percentage of FVS^+ cells for Uninf., Mtb, and HK Mtb.

(c) Bar graph showing LDH release (O.D. 490 nm) for Uninf. and Mtb.

(d) Flow cytometry plots and bar graph showing percentage of CD14^+ cells for Uninf. and Mtb-CS267.

(e) Flow cytometry plots and bar graph showing percentage of CD235a^+ cells for Uninf. and Mtb.
Supplementary Figure S4

**a**

Fold increase over uninfected (2^(-ΔΔCt))

**b**

Fold increase over uninfected (2^(-ΔΔCt))

**c**

Gated on CD34+

Media +
Cytokine cocktail

CD64

CD4

**d**

Neutrophils (%)

**e**

Neutrophils (%)

**f**

CD41a^+ (%)

CD235a^+ cells (%)

**g**

BDCA1^+CD14^low cells (%)

**h**

**i**

Uninf. Mtb

pSTAT1

STAT1

β–Actin

Uninf. Mtb

C/EBPα

C/EBPβ

β–Actin
Delgobo et al. Supplementary Figure S5

**a**

Heatmap showing gene expression changes over time (1d, 3d, 5d) in response to infection.

**b**

Bar graph showing fold change in gene expression for various genes.

**c**

Venn diagram illustrating overlap in gene expression between samples.

**d**

Box plot comparing variance explained by genetics in Mtb-stimulated PBMC and Mtb-stimulated macrophages.

**e**

Heatmap showing expression changes of different immune-related genes over time.

**f**

Graph showing cell counts and ML ratio changes over time with IFN-β therapy.