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Article - Version of Record

Journal of Infection 89 (2024) 106359

Contents lists available at ScienceDirect

Journal of Infection

journal homepage: www.elsevier.com/locate/jinf

Bacteria and Bacterial Diseases

Monocyte transcriptome signatures of inflammation and enhanced neutrophil recruitment characterize immunopathology in the blood of tuberculosis patients

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ARTICLE INFO

Article history:
Accepted 18 November 2024
Available online 26 November 2024

Keywords:
Tuberculosis
Monocytes
Plasma immunopathology
Neutrophil granulocytes

SUMMARY

Tuberculosis (TB) is characterized by immunopathology in the blood and monocytes have been shown to be highly sensitive to plasma environment changes in TB patients. Here, we investigated TB plasma effects on 'reference monocytes' using RNA sequencing to characterize a potential immunomodulatory role of monocytes in TB. Candidate pathways induced by plasma samples from TB patients (n=99) compared to healthy controls (n=62) were analyzed for changes in signal transduction, phenotype and secreted cytokines by flow cytometry. Finally, potential implications were characterized in blood samples from corresponding patients and controls.

Reference monocytes treated with TB plasma showed an enrichment of pathways involved in inflammation and chemotaxis. Inflammatory cytokines were accompanied by enhanced phosphorylation of STAT molecules (i.e., STAT1/3/5), and strong positive correlations were detected for Interleukin (IL)-6 only in TB plasma-treated monocytes. Moreover, monocyte chemokine receptors (i.e., CCR-1, CCR-5) and pro-inflammatory chemokines (i.e., CXCL-1, CXCL-2, CXCL-8, G-CSF, CCL-2) that attract granulocytes and monocytes were significantly higher in TB plasma-treated monocytes. Notably, corresponding clinical samples also showed higher plasma levels for a subset of inflammatory cytokines/chemokines and, in particular, high IL-6 levels correlated positively with accumulation of neutrophil granulocytes in the blood of TB patients. Finally, monocytes from TB patients were characterized by increased chemokine receptor expression, higher proportions of a CCR-2^{+} subpopulation and aberrant high SOCS3 expression.

These results suggest that monocytes may play a significant role in amplifying plasma immunopathology, leading to sustained mobilization and accumulation of neutrophil granulocytes and chronic inflammation in the blood of TB patients.

Introduction

Monocytes and derived macrophage/dendritic cell subsets play a critical role in immune protection against *Mycobacterium (M.) tuberculosis* infection. As the primary host cells of *M. tuberculosis* and

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https://doi.org/10.1016/j.jinf.2024.106359

because of their involvement in granuloma formation, monocyte-derived cells fulfill crucial functions in the immune defense. Against this background, it is important to note that TB patients show changes in the monocyte population in the peripheral blood. Amongst others, monocytes differ in the phenotype and secreted cytokines in acute TB.[1–4] Due to these differences, which have been described to occur under inflammatory conditions, they were also referred to as 'inflammatory monocytes'.[5,6] Functional implications of TB pathology on blood immune cells were already documented in the last century.[7] The group of J.J. Ellner conducted ground-breaking studies demonstrating an impaired immune response in a subgroup of TB patients.[8,9] Importantly, they showed that plasma samples from TB patients can transfer immunosuppression in vitro to healthy reference immune cells.[9] Monocytes and secreted soluble factors were shown to be essential for immunosuppression in these studies,[9] highlighting the central role of this immune cell subset.

Own previous study confirmed the effects of the TB plasma environment on reference monocytes and provided evidence for functional implications.[10] Moreover, we demonstrated that plasma-induced monocyte phenotype changes largely reflect the inflammatory monocyte phenotype seen in TB patients.[4] Signal Transducer and Activator of Transcription (STAT)3 and STAT5 pathways were shown to correlate with monocyte phenotype changes and this suggested that host cytokines play a role in the plasma-dependent immunopathology of TB.[4] The present study is based on the assumption that monocytes contribute to blood pathology in TB by acting as sensors and amplifiers of *Mycobacterium tuberculosis* triggered changes in the plasma milieu. To investigate this question, we conducted a combined study based on the in vitro effects of TB/control plasma on reference monocytes and phenotyping of immune cells and peripheral blood plasma from the respective TB patient and control study groups. *In vitro* effects of blood plasma on reference monocytes were interpreted as early/initial events, while blood signatures were thought to reflect the chronic effects of TB immunopathology.

Initially, we performed global messenger RNA sequencing of TB/control plasma-treated reference monocytes to identify involved pathways and functional implications of the aberrant plasma milieu in TB patients. Selected candidates were then confirmed on the protein level on plasma-treated reference monocytes and culture supernatants. Finally, phenotyping and quantification of immune cell subsets in corresponding blood samples was performed to identify potential chronic implications on immunopathology in TB patients.

Material and methods

Study cohorts and clinical characterization

Between July 2019 and March 2022, TB patients (n=99) and asymptomatic contacts of indexed patients (controls, n=62) were recruited at four hospitals in Ghana (i.e., Agogo Presbyterian Hospital, St. Mathias Catholic Hospital, Atebubu District Hospital, Sene West District Hospital). Diagnosis of active TB was based on the patient's history, clinical examination, chest X-ray, sputum smear test and GeneXpert analyses as described.[4] All patients were included before initiation of treatment. Controls were close relatives living in the same household with indexed TB patients according to self-report and direct observation. Controls had no history of TB and showed no symptoms of TB at recruitment. Each participant donated blood for immune cell phenotyping (up to 10 mL sampled in BD Vacutainer®heparin tubes, BD) and for generation of plasma (5 mL sampled in BD Vacutainer® EDTA Tubes, BD) according to manufacturers' instructions. Given the restricted availability of samples and the necessity of matching the age and gender distribution of the control subjects in each experiment, not all experiments included all samples. The characteristics of study participants and the subsets included in different experiments are provided in Table 1.

To conduct the in vitro reference monocyte experiments, buffy coat cells were retrieved from healthy individuals at the Transfusion Medicine Department of Heinrich-Heine-University in Duesseldorf, Germany. Ethics approval for the study was obtained from the Committee on Human Research, Publication and Ethics (CHRPE/AP/023/18) at the School of Medicine and Dentistry at Kwame Nkrumah University of Science and Technology (KNUST) in Kumasi, Ghana as well as the ethics committee of the Medical Faculty of Heinrich-Heine-University Duesseldorf (ID: 5445).

The plasma milieu reference monocyte assay

The plasma milieu response of reference monocytes has been described before.[4,10] In brief, monocytes were enriched by magnetic cell sorting (EasySept Monocyte negative selection kit; Stemcell Technology) from peripheral blood mononuclear cells (PBMCs) purified from peripheral blood of healthy individuals by density gradient centrifugation (Histopaque-1077; Sigma-Aldrich) according to manufacturers' guidelines. Enriched monocytes (5×10^4 per well) were cultured in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with L-Glutamine (2 mM, Sigma Aldrich), Hepes (10 mM, Thermo Fisher Scientific), and 10% of a heterologous plasma sample from a patient with TB or a control. Monocytes were then cultured overnight at 37 °C and 5% CO_2. Thereafter, supernatants (80 µL) were harvested and stored at −80 °C (i.e., supernatant samples) until usage. Reference monocytes were incubated in 170 µL of ice-cold PBS containing 10 mM EDTA and 0.5% BSA for 30 min to detach adherent cells.

Messenger (m)RNA sequencing of plasma milieu-treated reference monocytes

Total RNA was extracted from reference monocytes after culture using the NucleoSpin RNA kit (Macherey-Nagel) following the manufacturer's instructions. Selected plasma samples, which have previously been demonstrated to exert differential effects on STAT phosphorylation (in patients with pSTAT3 levels above the median for TB patients and in controls with pSTAT3 levels below the median for TB patients), were included.[4] All samples were simultaneously processed to minimize variability. The concentration of RNA samples was determined using the Qubit RNA HS Assay (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. RNA quality was assessed by capillary electrophoresis using the 'Total RNA Standard Sensitivity Assay' (Agilent Technologies, Inc. Santa Clara, USA). All samples used for sequencing had an RNA quality number (RQN) ≥ 8. Library preparation was carried out using the VAHTS Universal stranded mRNA library prep kit V6 with polyA-capture, following the manufacturers' instructions. In brief, 100 ng of total RNA was used for mRNA capturing, fragmentation, cDNA synthesis, adapter ligation, and library amplification. The bead-purified libraries were normalized and then sequenced on the NextSeq2000 system (Illumina Inc., San Diego, CA, USA) with a read setup of SR 1×100 bp. The sequencing coverage was between 20.1 and 28.5 million reads per sample, with a median of 23.4 million reads. Conversion of BCL files to fastq files, adapter trimming, and demultiplexing were performed using the Illumina BCL Convert tool (version 4.0.3).

Data analyses of fastq files were conducted using CLC Genomics Workbench (version 23.0.4, QIAGEN, Venlo, Netherlands). The reads from all probes were subjected to adapter trimming (Illumina TruSeq) and quality trimming using the default parameters (trimming bases below Q13 from the end of the reads, allowing a maximum of 2 ambiguous nucleotides). Mapping was performed against the Homo sapiens GRCh38.107 (hg38) genome sequence. Ingenuity Pathway Analysis (IPA, QIAGEN, Venlo, Netherlands) and GraphPad Prism v10 software (GraphPad Software, La Jolla CA, USA) were used

Table 1
Characteristics of the study groups.

	Total recruit			Ex vivo monocyte Phenotyping			Ex-vivo whole-blood phenotyping			Plasma assay (Flow cytometry)			Plasma assay (RNA sequencing)		
	TB	C	p	TB	C	p	TB	C	p	TB	C	p	TB	C	p
Number (n)	99	62		52	45		89	50		30	30		9	9	
Age (years)[a] median (range)	42 (15–80)	42 (22–75)	0.64	48 (18–76)	40 (22–75)	0.12	41 (15–80)	45 (22–75)	0.56	44 (18–69)	42 (22–75)	0.96	46 (23–76)	42 (22–75)	0.88
Gender															
male/female[b]	67/32	30/32	0.02	35/17	25/20	0.21	60/29	26/24	0.10	18/12	18/12	1	3/6	3/6	1
Diagnosis															
X-ray (suggestive/nd)	24/75			7/45			24/65			5/25			0/9		
GeneXpert (pos/neg/nd)	76/1/22	na		43/9/0	na		67/1/21	na		24/6/0	na		7/1/1	na	
Sputum culture (pos/neg/nd)	69/27/3	na		37/13/2	na		61/27/1	na		19/11/0	na		5/4/0	na	
Sputum smear AFB (neg/scanty/1+/2+)[c]	42/7/2/48			30/5/1/16			38/4/1/46			17/4/0/9			5/2/0/2		

Abbreviations: TB, tuberculosis patients; C, controls; nd, not done; neg, negative; pos, positive; AFB, Acid Fast Bacilli; na: not applicable.
[a] The Mann-Whitney U-test was used for group comparisons.
[b] The Fisher's exact test was used for gender distribution comparisons.
[c] The degree of sputum smear AFB burden was assigned to one of the four categories (neg: no AFB, scanty: 1–9 AFB, 1+: 10–99 AFB, 2+: > 100 AFB; number per 100 fields of observation).

to depict gene expression as Z scores. For functional annotation of the differentially expressed genes, we used the Database for Annotation, Visualization, and Integrated Discovery (DAVID; https://david.ncifcrf.gov). A combination of the available annotation methods was employed. These included the following: (i) functional annotation of biological processes (i.e. UP_KW_BP); (ii) gene ontology (i.e. GOTERM_BP_Direct); (iii) pathway analysis (i.e. KEGG Pathway). Significantly enriched pathways ($p < 0.05$; Bonferroni corrected) were included.

Reference monocyte phenotype and STAT phosphorylation analyses by flow cytometry

Reference monocyte phenotyping and STAT phosphorylation analyses were performed as described previously.[4] In brief, monocytes were directly processed after overnight culture in media supplemented with plasma samples. After detachment (see above) monocytes were stained on ice for 30 min in the dark in PBS/FCS 10% medium (25 µL) using fluorescently labeled antibodies against HLA-DR (PerCp-Cy5.5, Clone L243; BioLegend), CD14 (BV605, clone: M5E2; BioLegend), CCR-1 (AF488, clone 5F10B29; BioLegend), CCR-5 (PE, clone J418F1; BioLegend), CCR-2 (PE-Cy7, clone K036C2; BioLegend), and a viability dye (eFluor780; eBiosciences). For measurement of STAT phosphorylation, monocytes were fixed using true nuclear fixation buffer (100 µL; BioLegend) for 15 min at 37 °C, 5% CO_2, and permeabilized with pure methanol (130 µL) for 30 min in the dark on ice. Cells were then washed and stained with antibodies against human phosphorylated (p)STAT1 (PE-CY7, clone KIKSI0803; eBioscience), pSTAT3 (APC, clone LUVNKLA; eBioscience), pSTAT5 (PE, clone SRBCZX; eBioscience), anti-HLA-DR (PerCp-Cy5.5, Clone L243; BioLegend] for 30 min on ice. After incubation, cells were washed and measured using an LSR-Fortessa flow cytometer (BD Bioscience). FlowJo software (Version 10, Becton Dickinson) was used to analyze the data. Reference monocyte samples treated with plasma from age and sex-matched TB patients (n=30) and controls (n=30) were included in these experiments.

Quantification of immune cell subsets in whole blood samples by flow cytometry

Whole blood quantification of immune cell subsets was done by multi-color flow cytometry as described before.[11] In brief, whole blood was diluted (1:1) with RPMI1640 media. Samples were then centrifuged and stained with fluorescently labeled antibodies against human CD3 (AF700, clone SK7; BioLegend), CD45RA (APC, clone HI100; BioLegend), CD45RO (BV605, clone UCHL1; BioLegend), HLA-DR (PE-Dazzle594, clone L243; BioLegend), CD16 (APC, clone 3G8; BioLegend) and viability dye (eFluor780; eBiosciences) on ice in the dark for 30 min. After staining, red blood cells were lysed, washed, and fixed as described previously. The cells were measured using a CytoFlex S cytometer (Beckman Coulter), and the data were analyzed using FlowJo software. A representative example of the gating strategy is shown in Supplementary Figure 4a. Samples of TB patients (n=89) and controls (n=50) with comparable age and sex distributions were included in these experiments.

Monocyte phenotype characterization of TB patients and controls by multi-color flow cytometry

Cryopreserved PBMC samples were thawed, washed and stained with fluorescence-labeled antibodies as described before.[4] For characterization of monocyte phenotype and subpopulations, the following panel of antibodies against human monocyte markers was used: CCR-1 (AF488, clone 5F10B29; BioLegend), HLA-DR (PerCp-Cy5.5, Clone L243; BioLegend), CCR-5 (PE, clone J418F1; BioLegend), CCR-2 (BV421, clone K036C2; BioLegend), CD14 (BV605, clone M5E2; BioLegend), CD16 (APC, clone 3G8; BioLegend), anti-CD68/SR-D1 [Alexafluor700, clone: 298807; R and D systems], anti-CD80 [PE-

CF594, clone: 2D10; BioLegend], anti-CD11b [PE-Cy7, clone: ICRF44; BioLegend], anti-CD163 [BV510, clone: GHI/61; BioLegend] anti-CD86 [BV 650, clone: IT2.2; BioLegend] and viability dye (eFluor780; eBiosciences). After incubation, cells were washed and measured using LSR-Fortessa flow cytometer (BD Bioscience). Samples from age and sex-comparable TB patients (n=52) and controls (n=45) were included in these experiments. For SOCS3 quantification, the cells were stained (30 min, on ice and protected from light) with the following panel of antibodies: HLA-DR (PerCp-Cy5.5, Clone L243; BioLegend), CD14 (BV605, clone M5E2; BioLegend), CD16 (AF488, clone 3G8; BioLegend). The samples were then washed in PBS supplemented with 10% Fetal bovine serum (Sigma Aldrich). After fixation and permeabilization (with Fixation Buffer and Intracellular Staining permeabilization wash buffer (1X), BioLegend) according to manufacturers' instructions, the cells were stained with an antibody against human SOCS3 (C204, Immuno-Biological Laboratories, Inc.) labeled with Dy650 (DyLight™ 650 microscale antibody labeling kit; ThermoFisher). After washing in permeabilization buffer, the samples were resuspended in PBS and measured using LSR-Fortessa flow cytometer (BD Bioscience). FlowJo software (Version 10, Becton Dickinson) was used to analyze the data. A representative gating strategy is depicted in Supplementary Figure 5. Samples from age and sex-comparable TB patients (n=42) and controls (n=42) were included in these experiments.

Quantification of cytokines/chemokines in plasma and reference monocyte supernatants using flow cytometry-based bead assays

Frozen culture supernatants from plasma-treated reference monocytes or plasma samples of TB patients and controls were analyzed using a customized LEGENDplex™ Multi-Analyte Flow Assay kit (13-plex Panel). The following cytokines/chemokine candidates were measured (i.e., IL-6, IL-10, IL-1β, IL-1α, CXCL-1, CXCL-2, CXCL-8, CXCL-13, G-CSF CCL-7, CCL-20, CCL-22, CCL-24). CCL-2 measurement was performed separately using a LEGENDplex™ Human Proinflammatory Chemokine mix-and-match Panel. The samples were prepared and processed according to the manufacturer's instructions. Briefly, 12.5 µL of samples were diluted two-fold in assay buffer and incubated with premixed antibody-labeled beads for 2 h at room temperature. Streptavidin-PE was added to samples and incubated for an additional 30 min. Samples were washed and analyzed with a BD LSR Fortessa flow cytometer (BD Biosciences). Data were analyzed using LEGENDplex Data Analysis Software (Qognit. Inc). Concentrations were calculated using the respective cytokine standard. Values below the standard curve were set to the detection limit of each analyte.

Data visualization of CCR-2 expressing monocytes and candidate marker analysis

For characterization of CCR-2 positive monocytes from TB patients and controls, multiplex data analyses were performed as described in a previous study.[4] Briefly, study groups and each participant were initially assigned IDs. Viable HLA-DR positive cells were then gated and down-sampled to a maximum of 2000 cells per replicate measure using the Downsample v3.3 plugin for FlowJo, excluding CD16 and CD14 double-negative cells. All cells were included for samples with less than 2000 cells. All samples were then concatenated into one sample to compare study groups. TriMap, a method for large-scale dimensionality reduction and visualization of cell phenotype patterns, was applied. Default settings of the TriMap FlowJo plugin were used for all parameters. Analyses of CCR-2 positive subsets based on density plots were performed by back-gating clustered cells based on assigned IDs for individuals and study groups. The gating procedure is depicted in Supplementary Figure 5.

Graphical depiction and statistics

GraphPad Prism v10 software (GraphPad Software, La Jolla CA, USA) and R (version 4.3.2) were utilized for statistical analyses and graphical depictions. For RNA Sequencing, the Wald test in the CLC Differential Expression for RNA-Seq tool (version 2.8) was used for the comparison of gene expression between the study groups. The resulting p-values were corrected for multiple tests using Bonferroni correction. For all other analyses, nonparametric tests were employed due to the non-normal distribution of data, as determined by the Kolmogorov-Smirnov and Shapiro-Wilk tests. The Mann-Whitney U test was used to compare study groups. Spearman rank correlation was employed to assess the association between the parameters measured and Benjamini-Hochberg multiple correction was performed. Principal components analysis (PCA) was performed using the plotPCA function in the DESeq2 package in R. For functional annotation, Bonferroni corrected values were included. A p-value less than 0.05 was considered statistically significant.

Results

Plasma from TB patients induced higher transcription rates and distinct gene expression patterns in reference monocytes

To characterize monocyte response to plasma environment changes in human TB, we determined the RNA expression of reference monocytes after overnight culture with plasma samples from either a patient with TB (n=9) or a healthy control (n=9). For the sake of simplicity, we refer to the samples as TB or control plasma-treated monocytes throughout. RNA quantification revealed increased concentrations in monocytes treated with TB plasma (as compared to controls). This suggested generally higher transcription activity of monocytes induced by TB plasma (Fig. 1a). Comparison of gene expression patterns revealed marked differences between the study groups. 1932 genes showed significant differences between TB and control plasma-treated monocytes after adjustment for multiple testing (≥2-fold change, $p < 0.05$, Bonferroni; Fig. 1b). Most genes (n=1343) were downregulated in monocytes treated with TB plasma, possibly indicating the described suppressive effects of TB plasma.[9] In accordance, functional annotation revealed a significant enrichment of genes with lower expression involved in T-cell and NK-cell function in the presence of TB plasma (Supplementary Table 1). Signatures of regulated genes showed marked differences between the study groups and unsupervised clustering revealed high similarity among TB plasma-treated monocytes (Supplementary Figure 1a). Interestingly, two control plasma-treated samples induced monocyte gene expression patterns more like TB plasma (Supplementary Figure 1a). Principal component analyses confirmed high concurrence of TB plasma-treated samples and the similarity of two control samples to TB patients (Fig. 1c). These results suggested distinct and homogenous signatures induced in reference monocytes by plasma from TB patients.

Enrichment of gene signatures associated with inflammation and chemotaxis in TB plasma-treated monocytes

We focused on transcripts with higher expression in TB-plasma treated samples to identify pathways induced in TB disease pathology. 589 genes were significantly upregulated in TB plasma-treated monocytes. In the functional classification, 27 categories were identified in three databases, which showed significant differences between the two study groups (Supplementary Table 2). There was a notable degree of concordance between the candidates identified by functional annotation (i.e., UP_KW_BP) and gene ontology (i.e., GOTERM_BP_Direct) (Supplementary Table 2). In

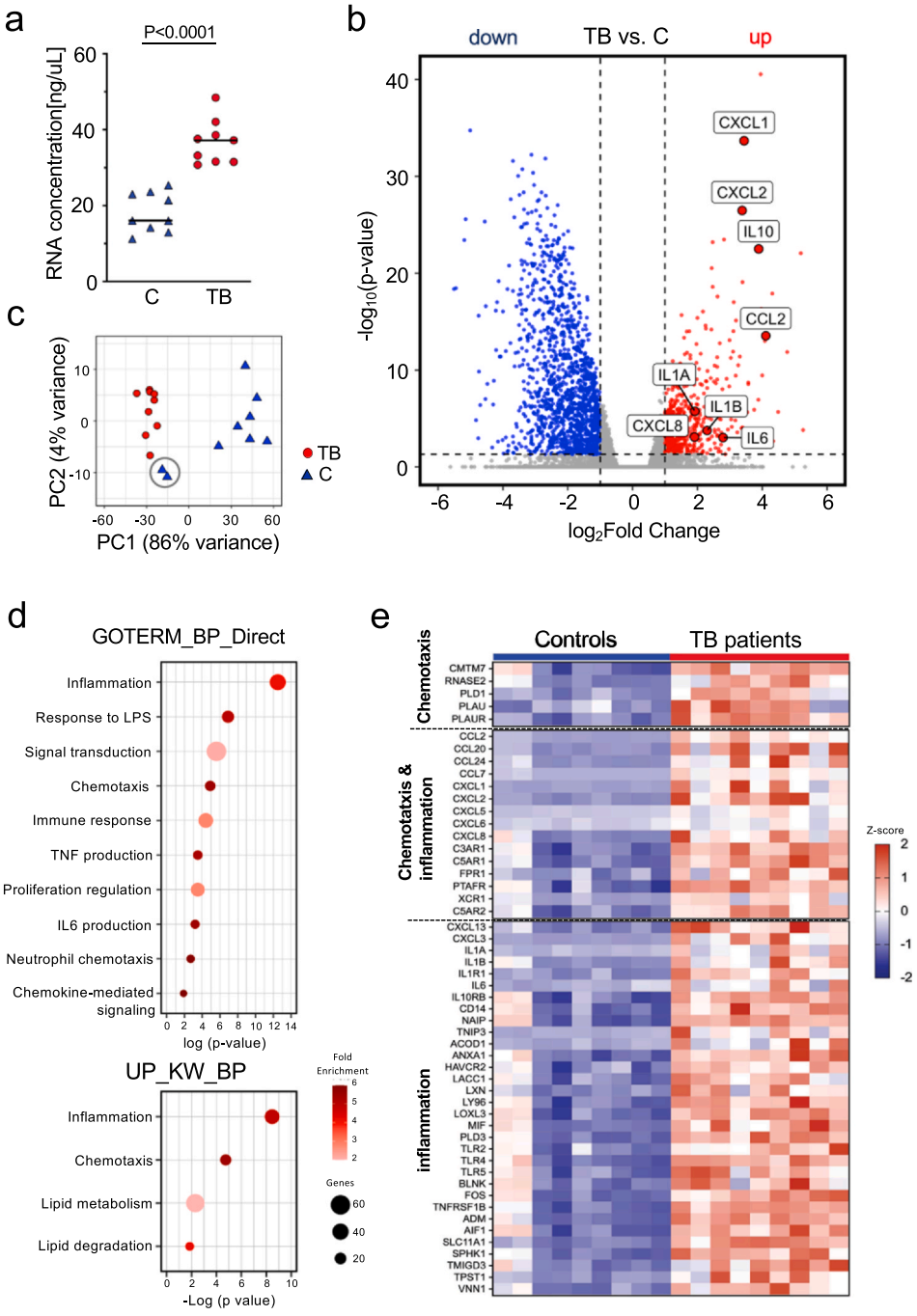

Fig. 1. *Comparison of TB and control plasma-treated reference monocytes by RNA sequencing and functional annotation.* Concomitant analysis of reference monocytes treated with samples from TB patients (n=9) and controls (n=9) has been performed for RNA sequencing. **(a)** Quantification of RNA concentrations from reference monocytes after culture containing different plasma samples. **(a, c)** Differences in symbols and colors indicate monocytes cultured with samples from TB patients (red circles) and controls (blue triangles). **(b)** A Volcano plot depicts differences in sequenced mRNAs between the study groups. Fold changes and p-values adjusted for multiple testing are shown. Thresholds of two-fold changes and p-values of < 0.05 have been applied (dashed lines) and differentially expressed mRNAs in TB sample cultured monocytes are marked (i.e., up-regulated in red, down-regulated in blue). The candidates selected by further experiments are highlighted by enlarged dots and labeled with the gene name. **(c)** Results from Principal Component Analysis (PCA) are shown as a symbol plot. Principle components 1 and 2 and the respective contribution to the variance (in brackets) are depicted. Two controls, which showed high similarity to TB patients, are highlighted by a circle. **(d)** Functional annotation of candidate genes up-regulated in TB plasma treated reference monocytes using UP_KW_BP (Uniprot keyword biological processes) and GOTERM_BP_Direct (gene onotology term biological processes direct). Only significantly enriched pathways are shown ($p < 0.05$, adjusted for multiple testing, Benjamini-Hochberg). The exact p-values (x-axis), the numbers of contributing genes (circle size) and fold enrichment (shades of red) are depicted. **(e)** Differential expression (z-scores) is shown for genes from candidate pathways of chemotaxis, and inflammation and both are shown for individual TB/control plasma samples as a heatmap. Color shading depicts individual z-scores for each gene being up-regulated (red) or down-regulated (blue).

particular, genes associated with chemotaxis and inflammation were identified by both databases, with the highest significance observed in reference monocytes treated with TB plasma (Fig. 1d). In addition, the KEGG pathway identified gene candidates associated with rheumatoid arthritis, the prototype of inflammatory diseases (Supplementary Table 1). Several chemokines were identified that contributed to the assignment of chemotaxis and a subset of chemokines involved in the chemotaxis of granulocytes and/or monocytes under inflammatory conditions was part of both biological processes/gene ontology (Fig. 1e). Moreover, key cytokines and Toll-

like receptor pathways contributed to the enrichment of inflammation-related factors in TB plasma-treated monocytes (Fig. 1e). Among the cytokines were members of the IL-1, IL-6, and IL-10 families (Fig. 1e). Interestingly, IL-10 was among the most significantly induced genes in TB plasma-treated monocytes (Fig. 1b, e) and other cytokines of the IL-10/IL-20 family as well as their receptors showed also higher levels in TB plasma-treated monocytes (Supplementary Figure 1b).

TB plasma-induced cytokines amplify an inflammatory response via STAT pathways in monocytes

Since RNA sequencing indicated strong effects of TB plasma on cytokines secreted by monocytes, we next analyzed the supernatants of treated monocytes to measure the cytokine candidates (i.e., IL-1α, IL-1β, IL-6, IL-10). Supernatants of TB plasma-treated (n=30) or control plasma-treated (n=30) monocytes were analyzed using a custom-designed cytometric beads assay. All four cytokines, IL-1α, IL-1β, IL-6, and IL-10, had significantly higher concentrations in the supernatants of TB plasma-treated monocytes (Fig. 2a). Higher cytokine concentrations in supernatant may affect reference monocytes in an autologous manner. This was even more likely as the receptors specific for IL-1, IL-6, and IL-10 also showed increased RNA expression in monocytes treated with TB plasma (Fig. 1e; Supplementary Figure 1b). IL-6 and IL-10 induce Jak/STAT signaling pathways that have been shown to contribute to the effects of plasma milieu before.[4] Therefore, we next measured STAT1, STAT3, and STAT5 phosphorylation in treated reference monocytes. TB plasma induced significantly higher levels of phosphorylated (p) STAT1, pSTAT3, and pSTAT5, and the strongest induction was seen for pSTAT3 and pSTAT5 (Fig. 2b). STAT3 and STAT5 phosphorylation was concomitantly detected in a subset of monocytes (Supplementary Figure 2) and proportions of pSTAT3/pSTAT5 double-positive monocytes were also significantly higher in TB plasma-treated monocytes (Fig. 2c). To address the question of potential autologous cytokine effects on signaling pathways, we next determined associations between cytokine concentrations and STAT phosphorylation in both study groups. TB plasma-treated samples showed a strong positive correlation for almost all comparisons (Fig. 2d). However, only moderate correlations were detected between cytokines and pSTATs in control plasma-treated monocytes (Fig. 2d). Especially, IL-6 concentrations were strongly associated with pSTAT1 and pSTAT3 expression in the presence of TB plasma but not control plasma (Fig. 2d). These results indicated autologous effects of secreted cytokines on STAT phosphorylation in TB plasma-treated reference monocytes.

Monocyte chemokine receptors and chemokines for the recruitment of granulocytes are induced by TB plasma

Chemotaxis pathways were strongly involved in monocyte transcriptome changes and aberrant chemokine receptor expression has been described to characterize monocyte pathology in TB patients.[2] Therefore, we next compared the influence of TB plasma on the expression of monocyte chemokine receptors and secreted chemokines in supernatants of reference monocytes by flow cytometry. A special focus was on three chemokine receptors (i.e., CCR-1, CCR-2, and CCR-5), which play a role during inflammatory conditions.[12] TB plasma induced higher protein expression of CCR-1 and CCR-5 on reference monocytes as compared to control plasma (both $p < 0.0001$) whereas CCR-2 was not different (Fig. 3a). Several chemokines associated with the recruitment of immune cells under inflammatory conditions were found to be higher in the monocyte transcriptome of TB plasma-treated monocytes (Fig. 1b, e). CBA-based analyses of selected chemokine candidates by flow cytometry confirmed differences in the supernatants between the study groups (Fig. 3b; Fig. 3c; Supplementary Figure 3). In particular, three chemokines involved in the recruitment of granulocytes, namely CXCL-1, CXCL-2, and CXCL-8, showed markedly higher concentrations (in some cases above the maximum detection limit) in the supernatants of monocytes treated with TB plasma (Fig. 3b). Moreover, two key factors of bone marrow granulocyte and monocyte mobilization under inflammatory conditions, i.e., G-CSF and CCL-2, showed higher concentrations in supernatants from monocytes treated with TB plasma as compared to controls (Fig. 3c).

Correlation between inflammatory signatures and chemokine receptor expression/chemokine secretion in TB plasma-treated monocytes

Next, we analyzed possible interrelations between differentially expressed chemokines and chemokine receptors for monocytes treated with plasma samples from both study groups. CXCL-8 showed a positive correlation with all other regulated chemokines and receptors (Fig. 3e). CXCL-1 and CXCL-2 correlated strongly with each other independent of TB or control plasma sample treatment (Fig. 3d). In contrast, correlations of CXCL-1 and CXCL-2 with CCR-1 expression were only detected in monocytes treated with TB plasma (Fig. 3d). Moreover, CXCL-1 correlated with the mobilizing factors, G-CSF and CCL-2, only in the presence of TB plasma (Fig. 3d).

Interestingly, the inclusion of IL-6 – which was associated with STAT signaling only in TB plasma-treated monocytes (Fig. 2d) – also showed strong correlations with CXCL-1 and CXCL-2 only in the presence of TB plasma (Fig. 3d). These results rendered an amplifying role of IL-6 secreted by monocytes in the presence of TB plasma and induced expression of inflammatory chemokines likely.

Blood plasma and immune cell profiles in TB patients

The plasma milieu assay described in the previous paragraphs determines early responses of reference monocytes to TB plasma. To determine whether potential implications of plasma pathology are also detectable in the blood of TB patients, we next analyzed the candidate cytokines/chemokines in plasma samples of TB patients (n=30) and controls (n=30). Inflammatory signatures were detected in plasma and the key cytokines, i.e., IL-6, IL-10, IL-1α, and IL-1β, showed higher concentrations in plasma samples from TB patients (Fig. 4a). Chemokines, expressed under inflammatory conditions, which were all markedly different in the plasma milieu assay, showed a more heterogeneous picture in plasma samples. Of the chemokines that recruit granulocytes, only CXCL-8 showed higher concentrations in the plasma of TB patients compared to healthy controls, while CXCL-1 and CXCL-2 were similar between the study groups (Fig. 4b). In particular, the results differed from those of the plasma milieu assay with regard to the chemokines involved in the mobilization of granulocyte and monocyte populations from the bone marrow. CCL-2, the crucial chemokine for the mobilization of monocytes under inflammatory conditions, had comparable concentrations between the study groups (Fig. 4c). Conversely, the concentrations of the granulocyte-mobilizing factor (G-CSF) were also elevated in the plasma of patients with TB (Fig. 4c). These results suggested chronic inflammation as well as ongoing mobilization of granulocytes from the bone marrow in TB patients.

To address the question if mobilization of granulocytes affected the blood picture of TB patients, we next performed whole blood phenotyping using flow cytometry to quantify the different immune cell populations in corresponding study groups of TB patients (n=89) and controls (n=50). Gating procedures are provided in Supplementary Figure 4a. Immune cell subsets differed markedly between the study groups (Fig. 5a). Granulocyte proportions were significantly higher in the blood of TB patients and this was accompanied by lower proportions of lymphocytes (CD3-positive and CD3-negative; Fig. 5b). Monocyte proportions were comparable

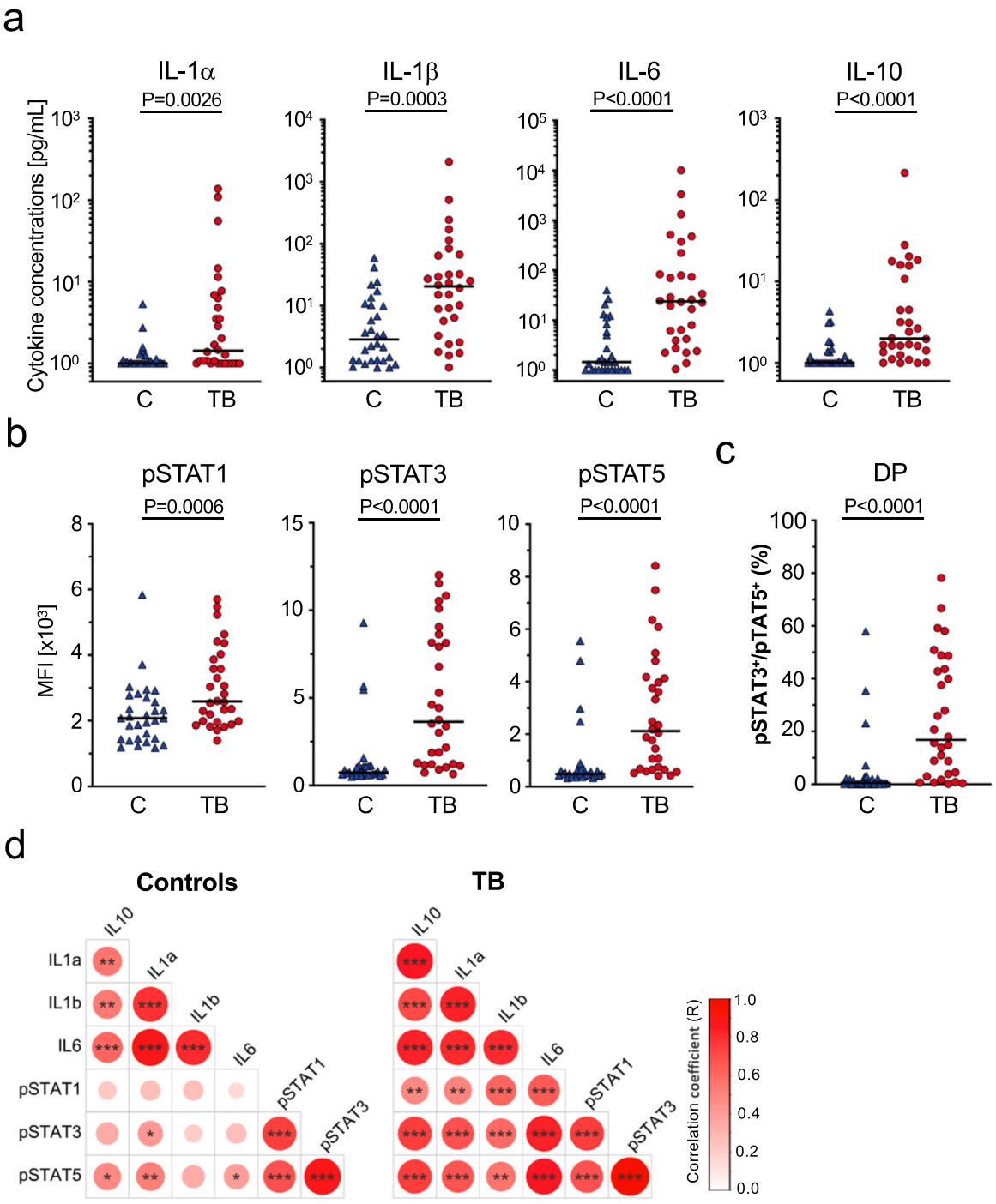

Fig. 2. *Inflammatory cytokine signatures and induced STAT phosphorylation in TB/control plasma-treated reference monocytes.* Concomitant characterization of reference monocytes (n=2) each cultured with different samples from TB patients (n=30) and controls (n=30) has been performed. **(a)** Selected candidate cytokines released under inflammatory conditions were measured in the supernatants using a custom-designed cytometric bead assay and flow cytometry. **(b, c)** Phosphorylation of STAT family members is depicted. Median fluorescence intensities (MFI) of phosphorylated (p)STAT1, STAT3, and STAT5 **(b)** and proportions of pSTAT3/pSTAT5 double-positive monocytes (DP) **(c)** are provided. **(a, b, c)** Symbol plots are depicted and differences in symbols and colors indicate monocytes treated with samples from TB patients (red circles) and controls (blue triangles). Nominal p-values calculated with the Mann-Whitney U-test are given. P-values below 0.05 were considered significant. **(d)** Correlation matrices of candidates are shown for both study groups. Symbol sizes and color shading indicate correlation indices (R-values) calculated using the Spearman rank test. P-values were adjusted for multiple testing according to Benjamini-Hochberg and are indicated as asterisks for *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

between the study groups (Fig. 5a; Supplementary Figure 4b). Granulocyte subset analyses revealed that mainly CD16-positive (i.e., neutrophil granulocytes) were enriched in TB patients whereas CD16-negative granulocyte subpopulations showed similar proportions between the study groups (Fig. 5c). Correlation analyses were then performed between the altered plasma cytokine/chemokine concentrations and the proportion of granulocytes. CXCL-8 and G-CSF concentrations were positively correlated with the proportion of granulocytes in all participants (Supplementary Figure 4c). Nevertheless, the correlation coefficients were relatively low (CXCL-8: r=0.46, p=0.0004; G-CSF: r=0.53, $p < 0.0001$) and no correlation was observed in the subgroups (Supplementary Figure 4c). In addition, there was a strong positive correlation between IL-6 concentrations and the proportion of granulocytes/neutrophil granulocytes in the

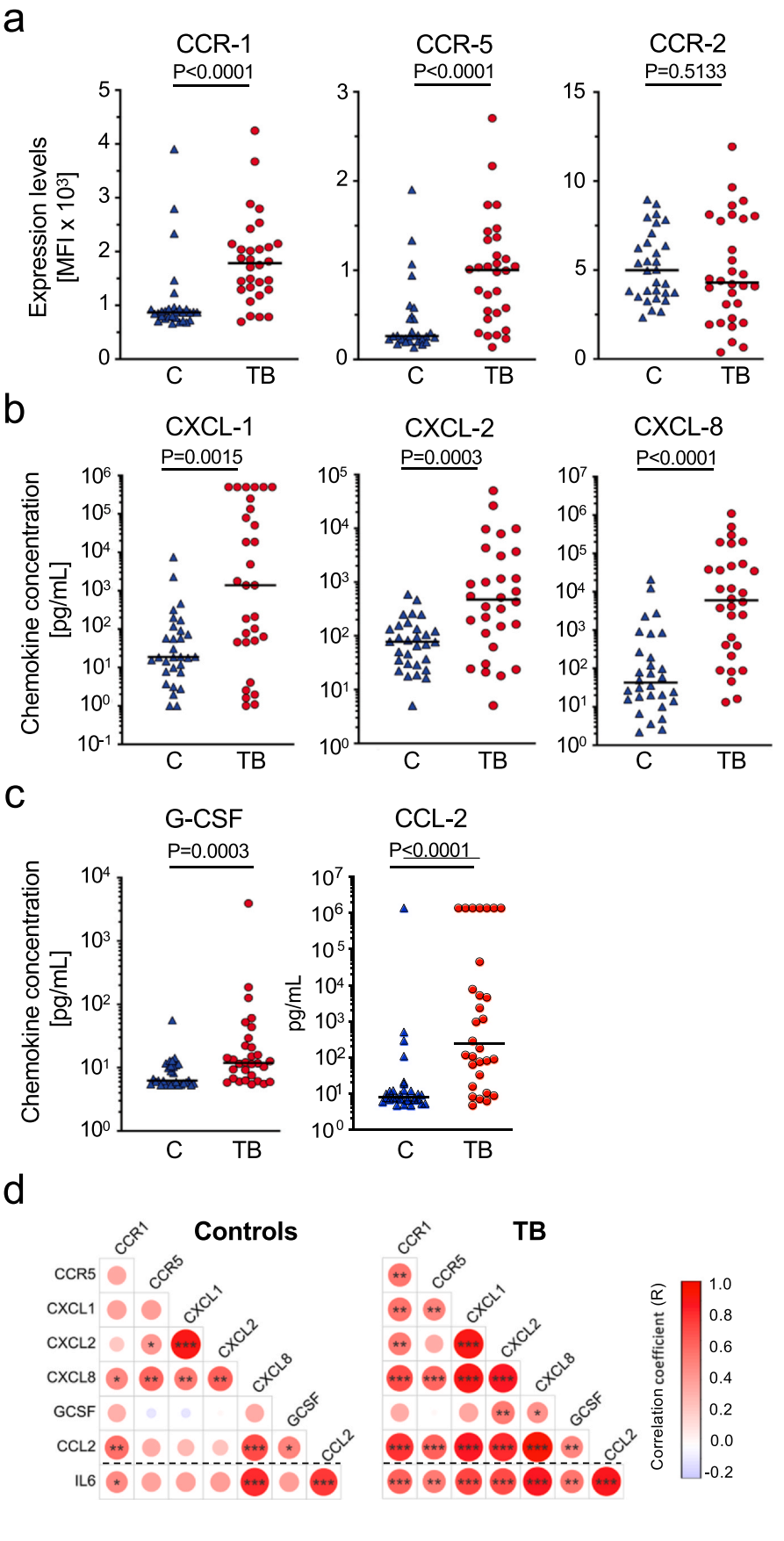

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Fig. 3. *Chemokines and chemokine receptors in TB/control plasma-treated reference monocytes and supernatants.* Concomitant characterization of reference monocytes (n=2) each cultured with different samples from TB patients (n=30; red circles) and controls (n=30; blue triangles) has been performed. **(a, b)** Chemokine receptors were measured in reference monocytes by flow cytometry. MFI values for individual samples are given for both study groups. Chemokines involved in the recruitment **(b)** or mobilization **(c)** of granulocytes/monocytes under inflammatory conditions were measured in the supernatants by flow cytometry using a custom-designed cytometric bead assay. Nominal p-values calculated with the Mann-Whitney U-test are given. P-values below 0.05 were considered significant. **(d)** Correlation matrices of chemokines/chemokine receptors and the key inflammatory cytokine IL-6 are shown for both study groups. Symbol sizes and color shading indicate correlation indices (R-values) calculated using the Spearman rank test. P-values adjusted for multiple testing according to Benjamini-Hochberg and are indicated as asterisks with *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

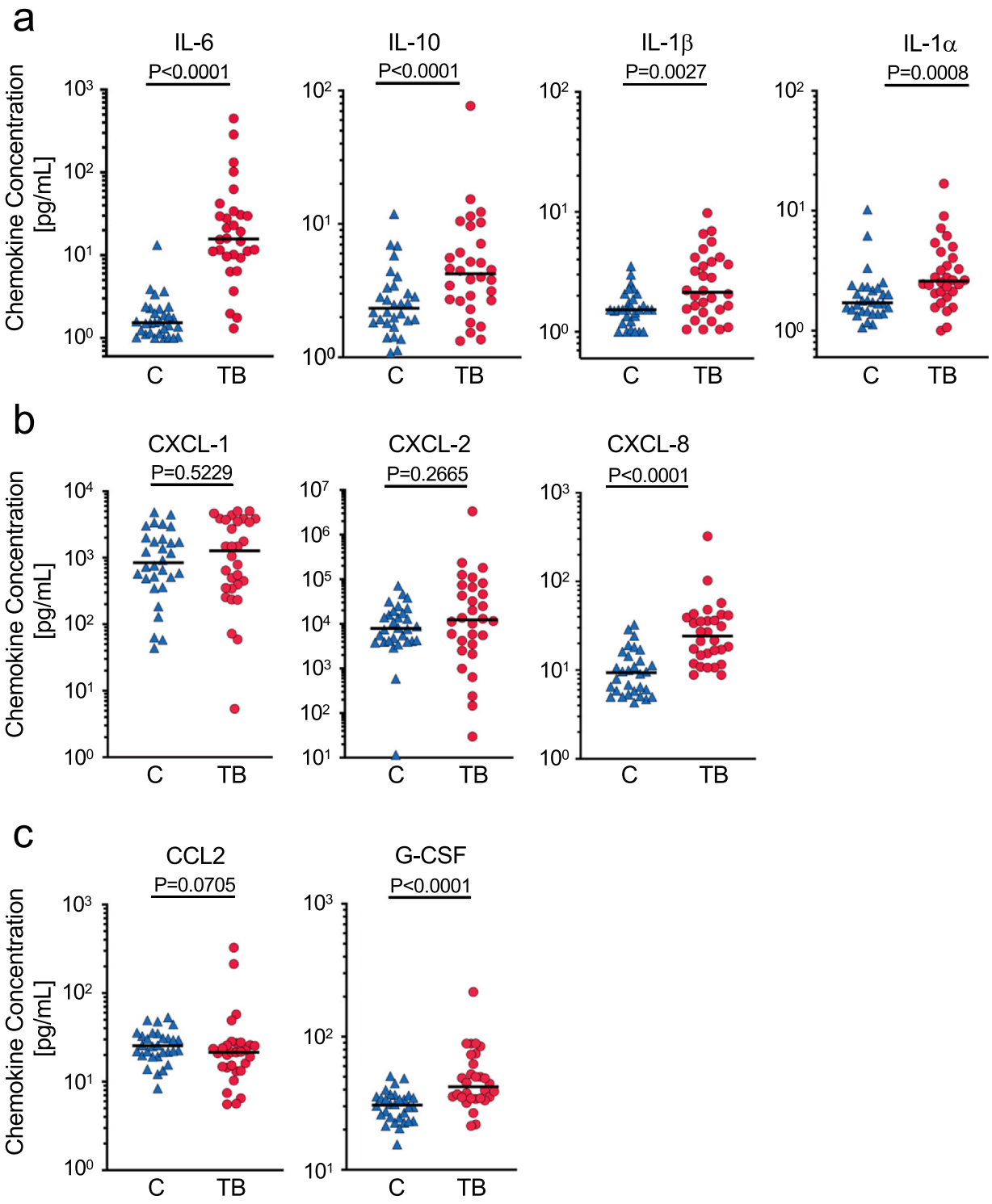

Fig. 4. *Cytokines and chemokines in plasma samples from TB patients and controls.* Selected candidate cytokines **(a)** and chemokines **(b, c)** released under inflammatory conditions were measured in plasma samples from TB patients and controls using a custom-designed cytometric bead assay. Symbol plots are depicted, and different symbols/colors indicate plasma samples from TB patients (red circles; n=30) and controls (blue triangles; n=30). Nominal p-values calculated with the Mann-Whitney U-test are given. P-values below 0.05 were considered significant.

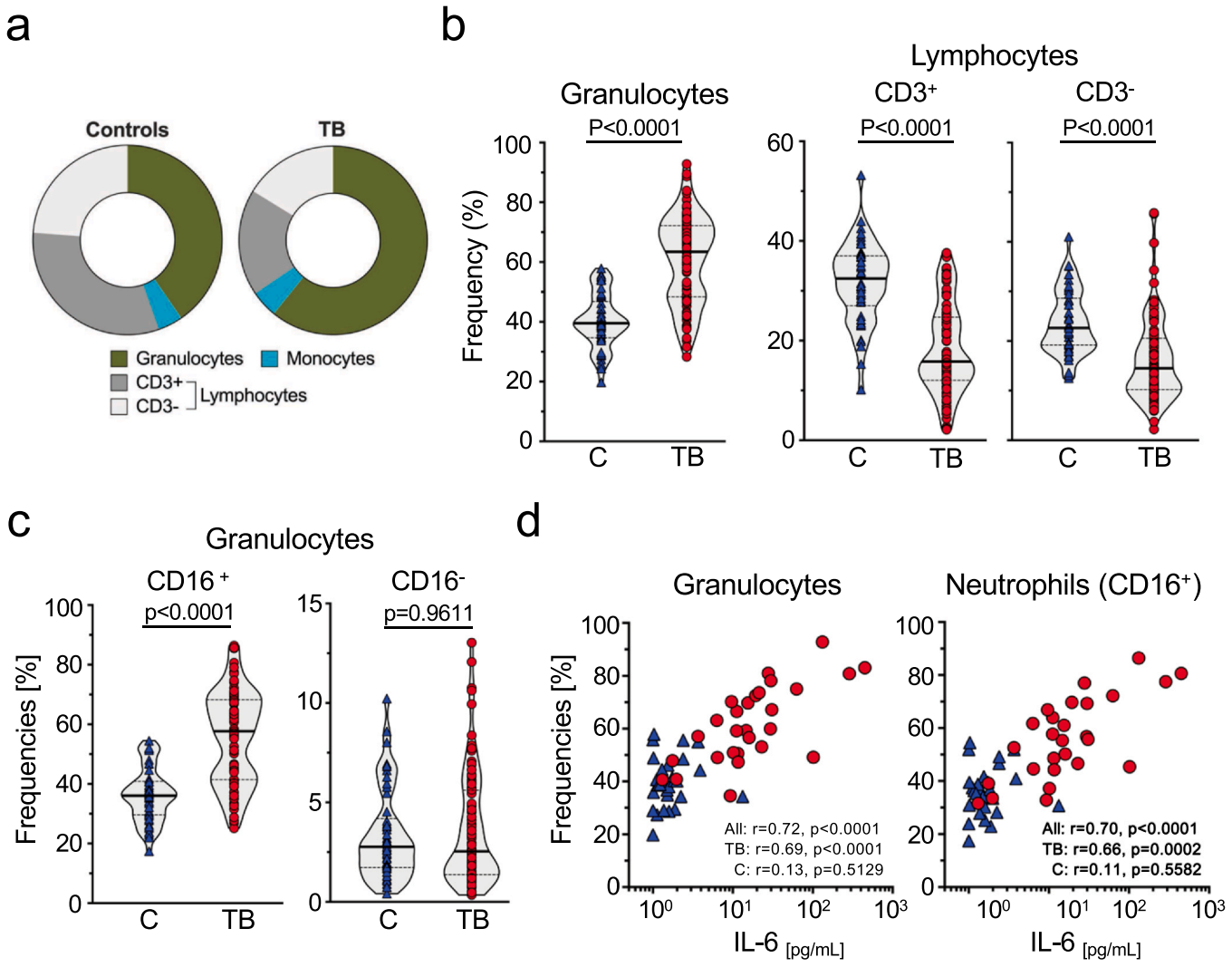

Fig. 5. *Differences in immune cell subsets in the peripheral blood from TB patients and controls.* Proportions of different immune cell populations (i.e., granulocytes, monocytes, lymphocytes) as well as subpopulations were measured by flow cytometry and are depicted as pie charts **(a)** or violin symbol graphs **(b, c)**. Different symbols/colors indicate plasma samples from TB patients (red circles; n=89) and controls (blue triangles; n=50). Nominal p-values calculated with the Mann-Whitney U-test are given. **(d)** Correlations between granulocyte (left graph) and CD16-positive (neutrophil) granulocyte frequencies (right graph) with IL-6 plasma concentrations for both study groups (TB patients, red circles, n=28; controls, blue triangles, n=28) are depicted. The Spearman Rank Test was used for correlation analyses and nominal p-values are given. P-values below 0.05 were considered significant.

blood (r=0.72, $p < 0.0001$; r=0.70, $p < 0.0001$, respectively). In contrast to CXCL-8/G-CSF, IL-6 also correlated with granulocyte/neutrophil granulocyte proportions in the subgroup of TB patients (r=0.69, $p < 0.0001$; r=0.66, p=0.0002, respectively) but not in controls (Fig. 5d). This suggested that chronic inflammation - characterized by persistently high IL-6 plasma levels - contributes to the ongoing mobilization and accumulation of neutrophil granulocytes in TB patients.

An inflammatory phenotype and enriched proportions of CCR-2/CCR-5 positive cells characterize monocytes from TB patients

Monocyte proportions in the blood were comparable between the study groups (Supplementary Figure 4b) although higher CCL-2 concentrations were found in TB plasma-treated reference monocyte supernatants. To analyze if TB-plasma induced CCL-2 was of biological relevance, we next characterized the phenotype of monocytes with a special focus on chemokine receptors in TB patients (n=52) and healthy controls (n=45). As in the plasma assay, CCR-1 and CCR-5 expression levels were significantly higher on monocytes from TB patients as compared to controls (Fig. 6a). Notably, and in contrast to plasma-treated reference monocytes, we also detected increased expression of CCR-2 on monocytes from TB patients (Fig. 6a). Since CCR-2 is the exclusive receptor of CCL-2, we considered recruitment of CCR-2 positive monocytes at early stages of *M. tuberculosis* infection (before clinical onset) a possible explanation. Hence, we next classified monocytes based on CCR-2 expression and found higher proportions of CCR-2 positive cells within monocyte populations from TB patients as compared to controls (Fig. 6b; Supplementary Figure 5). These results suggested an accumulation of CCR-2-positive monocytes recruited before the onset of symptomatic TB.

To further characterize this monocyte subset, we analyzed the expression of selected monocyte markers described to play a role in TB and performed data complexity reduction and visualization of study group differences by TriMap (for details see methods section). The gating procedure of CCR-2-positive monocytes is shown as Supplementary Figure 5. Interestingly, two populations (termed CCR-2_{Pop1} and CCR-2_{Pop2}) were identified and these contributed to approx. 90% of all CCR-2-positive monocytes (Fig. 6c). Concatenated data sets suggested higher proportions of CCR-2_{Pop2} within monocytes from TB patients whereas the CCR-2_{Pop1} was seemingly more frequent in controls (Fig. 6c). Comparison of study groups showed

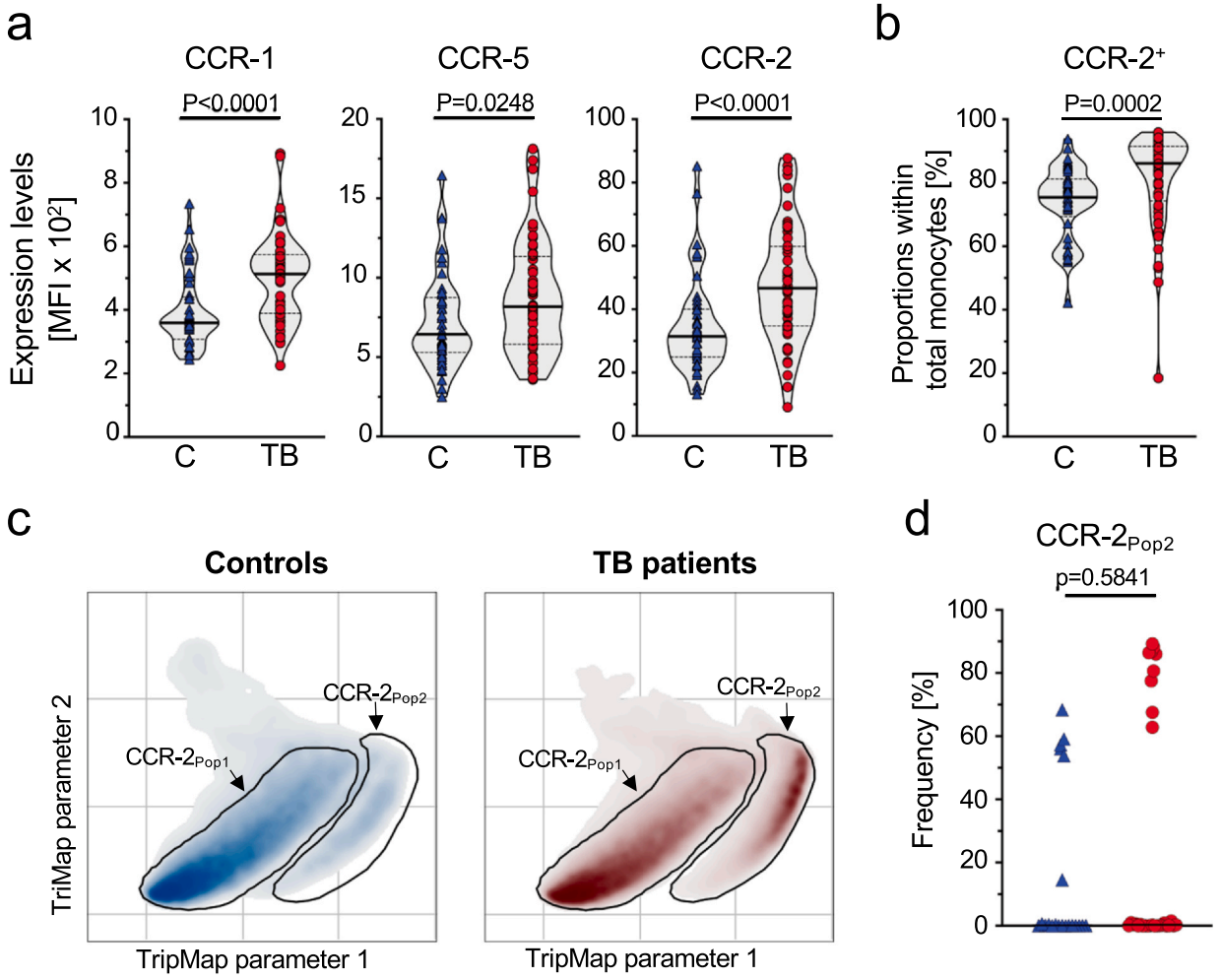

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Fig. 6. *Characterization of monocyte phenotype and CCR2⁺ subsets in the peripheral blood from TB patients.* Monocyte phenotype analyses were done using flow cytometry in PBMCs from TB patients and controls. **(a, b)** Chemokine receptor expression patterns were depicted as mean fluorescence intensity (MFI) **(a)** or proportions of CCR-2 positive cells within monocytes **(b)**. **(c)** CCR-2 positive monocytes were characterized for monocyte marker expression (i.e., HLA-DR, CD11b, CD14, CD16, CD68, CD80, CD86) and visualized using TriMap based complexity reduction for each study group separately. Density plots are depicted for both study groups and the main two subpopulations within CCR-2 positive cells were gated (i.e., CCR-2_{Pop1}, CCR-2_{Pop2}). **(d)** Relative frequencies of CCR-2_{Pop2} within CCR-2-positive monocytes are shown for each participant and both study groups as a symbol plot. **(e)** Stacked histograms show MFI expression differences for all included markers on both, CCR-2_{Pop1} and CCR-2_{Pop2}, subsets. **(f)** Suppressor Of Cytokine (SOCS)-3 expression in monocytes from both study groups is shown. Samples from TB patients (n=42) and controls (n=42) have been included. Nominal p-values calculated with the Mann-Whitney U-test are given. P-values below 0.05 were considered significant.

that only subgroups of TB patients and controls had high proportions of CCR-2_{Pop2} and these were not significantly different (Fig. 6d). However, phenotype comparison of CCR-2_{Pop2} monocytes between the study groups showed markedly higher expression of several markers with relevance in TB immunopathology exclusively on CCR-2_{Pop2} monocytes from TB patients (Fig. 6e). These results suggested that mobilization of CCR-2-positive inflammatory monocytes by CCL-2 is a transient feature that happens at before clinical onset of TB.

Higher SOCS3 expression in monocytes from TB patients

Our results indicate a continuous mobilization and accumulation of neutrophil granulocytes in acute TB patients, whereas the mobilization of monocytes in TB patients is not continuous. Since the enrichment of neutrophils is associated with high IL-6 plasma levels, we assumed that the sensitivity of monocytes from TB patients to IL-6 might be impaired. A similar mechanism has been described for T cells in TB patients and aberrant high SOCS3 expression has been identified as the potential inhibitor.[13] Hence, we analyzed SOCS3 protein levels in monocytes from TB patients (n=42) and healthy controls (n=42) and detected higher SOCS3 expression in monocytes from patients as compared to healthy controls (Fig. 6f). These results suggested that high SOCS3 expression and potential inhibitory effects on IL-6-dependent STAT3 signaling may impair the recruitment of CCR-2 positive monocytes in TB patients.

Discussion

In the present study, global changes in gene transcription, phenotype and cytokine secretion were analyzed in monocytes treated with TB (or control) plasma samples. The in vitro response of monocytes to the TB plasma milieu was then compared with the phenotype and composition of immune cell subsets in corresponding samples from TB patients and healthy controls. Inflammatory cytokines and chemokines were strongly induced by TB plasma and especially markers of chronic inflammation, like IL-6, showed aberrant high expression in the plasma of TB patients. Ongoing granulocyte mobilization and enrichment in the blood of TB patients was seen and this was associated with high IL-6 plasma levels.

Plasma pathology in a subset of TB patients has been described already in the last century.[9] The group of J.J. Ellner demonstrated that in vitro culture of immune cells from healthy individuals with plasma from TB patients conferred impaired functional responses including decreased proliferation and T-cell cytokine expression.[9,14] Monocytes were identified as central effector cells and suppressive features were found in monocytes from TB patients.[9,15–18] Notably, RNA sequencing of reference monocytes in the present study also revealed a significant enrichment of genes involved in T-cell function, with lower expression in the presence of TB plasma. Despite the use of non-contact magnetic cell sorting to purify the reference monocytes prior to use, the resulting samples exhibited a purity of approximately 80–90%. The presence of non-monocytes (mainly T cells) in the culture can therefore be explained. It is reasonable to assume that the lower expression of T-cell genes reflects the suppressive effect of TB plasma on T cells. Given the well-described immunosuppressive role of IL-10 in TB,[19] higher levels of IL-10 secretion by reference monocytes in the presence of TB plasma may be causative of T-cell suppression. However, further investigation is required to ascertain whether a relationship exists.

Mycobacterial components with immunomodulatory functions were identified[8] but the detection and functional assignment of such a component in plasma remains to be done. If mycobacterial components are responsible for the effect, these must also be effective at very low concentrations, as only a small amount of plasma is required to modulate the reference monocytes. In the present study, a 1 in 10 dilution of TB plasma in a medium was sufficient to confer monocyte modulation. This argued for a highly active molecule that can induce an amplifying reaction in reference monocytes. The fact that diluted plasma was effective also excluded a direct effect of increased TB plasma cytokines (e.g., IL-1α, IL-1β, IL-6, IL-10) since the dilution will result in a final concentration at or below physiological cytokine plasma levels. Hence, these host cytokines are unlikely triggers of plasma pathology but our results suggested that induced secretion of inflammatory cytokines in reference monocytes potentially boosted the effect in an autologous way. In accordance, STAT signaling pathways were shown to be strongly associated with monocyte phenotype changes and secreted cytokines. IL-6 and IL-10 are well-known inducers of STAT3 signaling and both were associated with STAT3/5 signaling in reference monocytes. These results were in accordance with previous studies that showed high constitutive STAT3 phosphorylation in T cells and monocytes from TB patients.[13,20] However, the cytokines responsible for the high expression of pSTAT3/5 in reference monocytes are not sufficiently confirmed and additional functional tests (e.g., blocking of cytokine receptors) are required to confirm the assumed mechanisms. Furthermore, blocking signaling pathways, such as Jak/STAT, would be important to determine the role of alternative pathways.

High expression levels of SOCS3 in monocytes from TB patients were detected in this study. A substantiate number of cytokines induce SOCS3 via the Jak/STAT pathway, with SOCS3 functioning as a feedback inhibitor for select cytokine receptors.[21] We have previously described that constitutive STAT3 phosphorylation positively correlates with increased levels of SOCS3 in T cells from TB patients,[13] and high levels of pSTAT3 in monocytes from TB patients was found by others.[20] It is therefore reasonable to assume that aberrant high Jak/STAT signal transduction is the cause of the high SOCS3 levels in monocytes in the present study. This finding strengthened the assumption for a role of aberrant STAT pathway signaling in TB pathology. The central role of STAT3 and SOCS3-mediated regulation for immune protection against *M. tuberculosis* infection is well established.[22] In myeloid cells, an anti-inflammatory function of STAT3/SOCS3 is assumed, which is mainly based on the finding that IL-6 signaling is inhibited by SOCS3, while the signaling of IL-10 - another inducing cytokine of phosphorylated STAT3 - is not inhibited.[22] In accordance, recent studies on the genetics of IL-6 and IL-6 receptors found a protective role of IL-6 promoting polymorphisms against TB.[23,24] These findings seem to contradict the results of the present study, and a possible explanation for this could be differences in the role of cis- and trans-signaling of IL-6 (reviewed in[25]). Differences in the involvement of soluble IL-6 receptor complexes that can trigger an IL-6 response in IL-6 receptor-negative cells (trans-signaling) could therefore contribute to the inflammatory or anti-inflammatory effects of IL-6.[25] This may explain the apparent contradiction. Moreover, it is

reasonable to assume that the role of IL-6 can be interpreted as a double-edged sword, whereby both an excessively high and a low expression of IL-6 can have a negative effect on the immune response against *M. tuberculosis*. This assumption is corroborated by the finding that IL-6 has been found to be a marker of inflammation, disease severity and treatment response in the peripheral blood of patients with TB.[13,26–30]

Chemotaxis pathways – and especially chemokines secreted under inflammatory conditions – were strongly induced in reference monocytes by TB plasma. Among these were candidates involved in the mobilization and recruitment of granulocytes. Granulocyte chemoattractant proteins (i.e., CXCL-1, CXCL-2, CXCL-8) and G-CSF, a key factor of granulocyte mobilization in the bone marrow, were strongly induced and secreted by monocytes treated with TB plasma. Higher CXCL-8 and G-CSF concentrations were also detected in the plasma from TB patients as expected.[31,32] The functional relevance of these chemokine differences was confirmed by the finding of higher granulocyte proportions in the blood of TB patients. Granulocyte enrichment in the blood of TB patients was shown before.[33] The role of granulocytes in TB has long been controversial and these innate immune cells have been described as contributing to both immune protection and disease progression.[34] Controversial results may at least partly reflect the differential roles of granulocyte subsets since the contribution of eosinophils to host resistance and neutrophils to disease pathology were described.[35] The role of neutrophilia in TB disease pathology, however, has been clearly shown.[36] Neutrophilia is associated with hyperinflammation and severe lung damage in TB patients.[37] Moreover, mortality rates are increased in TB patients with neutrophilia.[38] Here we provide evidence that especially neutrophil ($CD16^+$) granulocyte proportions were higher in TB patients and detected a correlation between IL-6 plasma concentrations and granulocyte proportions in TB patients. This finding indicated a potential involvement of IL-6 in the mobilization and recruitment of granulocytes. This hypothesis was corroborated by prior research, most notably by Florentin et al.[39]. They observed a correlation between elevated plasma IL-6 levels and the proportion of granulocytes in the lungs and peripheral blood of patients with pulmonary hypertension.[39] Furthermore, they demonstrated in an animal model that IL-6 contributes to granulocyte accumulation by promoting the mobilization of granulocytes from the bone marrow through the induction of CX_3CR-1 expression on granulocytes.[39] In contrast to IL-6, the plasma concentrations of CXCL-8 and G-CSF showed only moderate correlations with the proportion of granulocytes in the blood. One possible explanation could be time course effects. Considering the known effects - especially of G-CSF - in inducing granulocyte mobilization from the bone marrow, it is reasonable to assume that high CXCL-8/G-CSF plasma concentrations may precede granulocyte accumulation. This would explain the moderate correlation when the parameters are measured at the same time.

The cytokines/chemokines with higher plasma concentrations in TB patients (i.e., IL-1α, IL-1β, IL-6, IL-10, CXCL-8, G-CSF) have been identified as potential markers for characterizing disease severity and for monitoring treatment response in TB patients. This has been previously demonstrated for high plasma IL-6 concentration in TB patients.[29] Given the high heterogeneity of individual cytokine/chemokine plasma levels, combined results of different markers may also prove to be optimal biomarkers. Such biomarkers may prove beneficial in shortening chemotherapy and in measuring treatment efficacy in individual TB patients.

Phenotype changes of blood monocytes in human TB are well described and our previous studies demonstrated the important role of the aberrant plasma environment for monocyte pathology.[4,10] Higher expression of CCR-1 and CCR-5 on monocytes has been demonstrated[2] and this has been confirmed for TB plasma-treated reference monocytes and monocytes from TB patients in the present study. Notably, higher CCR-2 expression was not induced by TB plasma but a subset with high CCR-2 expression was enriched within the monocyte population in the blood of TB patients. A deeper understanding of the functions of CCR-2-positive monocytes is crucial for advancing our knowledge of the implications of these cells in the context of TB pathology. Nevertheless, it is an established fact that the phenotype of monocytes in vitro is highly flexible. The expression of monocyte subset markers (e.g. CD14/CD16) is subject to significant regulation in vitro (with or without stimulation), which presents a challenge in the identification of monocyte subsets detected *ex vivo*. In light of the aforementioned considerations, the isolation of CCR-2 positive monocytes by fluorescence-activated cell sorting prior to in vitro analysis of functional differences would be a prerequisite for this approach.

Moreover, the results of longitudinal studies investigating the monocyte phenotype will elucidate whether aberrant chemokine receptor expression normalizes during the treatment of TB. CCL-2 is the key chemokine for mobilization of monocytes from the bone marrow under inflammatory conditions and CCR-2 is the exclusive receptor for CCL-2 (reviewed in[12]). Since CCL-2 was strongly induced in reference monocytes treated with TB plasma but showed no differences between plasma samples from the study groups, we assumed that mobilization of monocytes from the bone marrow by CCL-2 was induced previously but was not ongoing in TB patients. This could explain the fact that although a general enrichment of granulocytes was observed in TB patients, the general monocyte percentage did not differ between the study groups. A potential mechanism underlying changes in the mobilization could involve IL-6 and its feedback inhibitor SOCS3. Since granulocyte recruitment was associated with IL-6 plasma levels, higher SOCS3 levels detected in monocytes may cause impaired IL-6 response as it has been shown for T cells from TB patients.[13] Future studies will have to address the question whether the mobilization of CCR-2 positive monocytes is occurring early during *M.* tuberculosis infection causing changes in monocyte subpopulations detectable in TB patients at clinical onset.

The present study is limited by the moderate size of the experimental groups and the necessity to utilize reference monocytes for the characterization of alterations in the plasma environment in TB. The utilization of disparate reference monocytes for each plasma environment experiment resulted in a reduction in the number of plasma samples per experiment. Consequently, RNA sequencing was only feasible with a restricted number of samples (n=18, nine per study group), due to the necessity of simultaneous sample processing. Due to the moderate size of the study groups for RNA sequencing and the limited statistical power, samples were pre-selected based on differential STAT phosphorylation in previous experiments.[4] This approach introduced a potential bias in the identification of factors induced by STAT signaling pathways. However, subsequent experiments were performed on all available samples to ensure the integrity of the sequencing results and the relevance of the identified signaling pathways.

Taken together, the present study strengthened the hypothesis that changes in the plasma milieu are drivers of a hyperinflammatory immune response in TB. Monocytes contributed to the harmful recruitment of neutrophils and secreted key factors of inflammation, which, like IL-6, are associated with impaired T-cell response and disease severity. This study strongly argues for host-directed treatment to modulate the harmful effects of chronic inflammation with potential long-term effects of TB. We propose SOCS3 and IL-6 receptor inhibition as potential host-specific therapeutic strategies for TB treatment. However, given the multiple functions of SOCS3 and the described differences in IL-6 signaling (discussed above), we conclude that further upfront characterization of the underlying mechanisms is required.

Funding

This study was supported by the German Research Foundation (DFG, JA 1479/9-1) and the graduate school molecules of infection

(MOI)-4 funded by the Jürgen Manchot Foundation. The funders had no role in study design, data collection, data analysis, decision to publish, or preparation of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank all study participants, study nurses, and physicians for supporting this investigation. Computational infrastructure and support were provided by the Centre for Information and Media Technology at Heinrich Heine University Düsseldorf.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jinf.2024.106359.

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