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Article

Calcineurin-NFAT signaling controls neutrophils' ability of chemoattraction upon fungal infection

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Abstract

Calcineurin–nuclear factor of activated T cells (CN-NFAT) inhibitors are widely clinically used drugs for immunosuppression, but besides their required T cell response inhibition, they also undesirably affect innate immune cells. Disruption of innate immune cell function can explain the observed susceptibility of CN-NFAT inhibitor–treated patients to opportunistic fungal infections. Neutrophils play an essential role in innate immunity as a defense against pathogens; however, the effect of CN-NFAT inhibitors on neutrophil function was poorly described. Thus, we tested the response of human neutrophils to opportunistic fungal pathogens, namely *Candida albicans* and *Aspergillus fumigatus*, in the presence of CN-NFAT inhibitors. Here, we report that the NFAT pathway members were expressed in neutrophils and mediated part of the neutrophil response to pathogens. Upon pathogen exposure, neutrophils underwent profound transcriptomic changes with subsequent production of effector molecules. Importantly, genes and proteins involved in the regulation of the immune response and chemotaxis, including the chemokines *CCL2*, *CCL3*, and *CCL4* were significantly upregulated. The presence of CN-NFAT inhibitors attenuated the expression of these chemokines and impaired the ability of neutrophils to chemoattract other immune cells. Our results amend knowledge about the impact of CN-NFAT inhibition in human neutrophils.

Keywords: aspergillus, candida, chemokines, neutrophils, NFAT

1. Introduction

Neutrophils play an essential role as cells of the first response targeting pathogens at the site of infection. Furthermore, they also govern the subsequent immune response through the production of several proinflammatory cytokines and chemokines attracting other immune cells.[1,2] In particular, neutrophils recognize pathogens through pattern recognition receptors (PRRs) linked to the activation of inflammatory pathways and downstream signaling.[3] Activation via the PRRs drives neutrophil defensive responses such as degranulation, release of neutrophil extracellular traps (NETs), chemotaxis, or production of reactive oxygen species, cytokines, and chemokines.[1,4]

The pathogen recognition through PRRs induces the activation of multiple signaling pathways including calcineurin–nuclear factor of activated T cell (CN-NFAT) signaling. We and others[5–10] have shown that NFAT controls several essential functions of myeloid cells resulting in higher susceptibility to pathogens.[11,12] The initial finding of the role of NFAT in myeloid cells has been reported in macrophages and dendritic cells using mice models of fungal infections, specifically of *Candida albicans*[13] or *Aspergillus fumigatus*[5] infection. Importantly, we have reported that NFAT signaling in human monocytes coregulates the expression of important antifungal mediator pentraxin-3 (PTX-3).[14] Moreover, the secretion of cytokines and chemokines such as tumor necrosis factor α, interleukin-10, and CCL2 (MCP-1) was inhibited by CN-NFAT inhibitor cyclosporine A (CsA) upon zymosan stimulation.[14]

Vega et al.[15] reported the expression of NFAT family members in human neutrophils and showed that CN-NFAT signaling governs neutrophil expression of cyclooxygenase-2 (COX-2).[15] The 2 most commonly used CN-NFAT inhibitors, CsA and tacrolimus (FK506), inhibited the degranulation of polymorphonuclear leukocytes (PMNs).[16] Similarly, inhibition of oxidative burst assessed as production of superoxide has been reported.[17] Sasakawa et al.[18] showed the influence of CN-NFAT inhibitors on peripheral blood mononuclear cell–driven neutrophil chemotaxis.[18] Others have already connected CN-NFAT signaling in neutrophils with their infiltration to the lung during bacterial infection in mice,[19] concluding that CN-NFAT inhibition controls the expression of CXC-type chemokines, thus aggravating the lung injury upon streptococcal infection[19] and with susceptibility to fungal infections in mice.[5,8]

Our previous data showed the significant role of CN-NFAT signaling in the susceptibility of mice to fungal infection in the conditional knockout of CN in dendritic cells as well as in neutrophils.[12] These findings were further corroborated in human primary monocytes, showing the decrease in PTX-3 and cytokines and chemokines expression in CN-NFAT inhibitor–treated monocytes upon fungal ligands stimulation.[14]

Impairment of the NFAT signaling in innate immune cells has been associated with many pathologies and immune functions. Macrophages from mice lacking NFATC3 (NFAT4) expression demonstrated impaired phagocytosis and were determined as

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an important factor for higher susceptibility to polymicrobial infection assay in lungs after the cecal ligation puncture model of sepsis.[20] We recently reported profound changes in neutrophil expression and protein levels during sepsis progression.[21] Recently, a new role of CN-NFAT signaling during sepsis progression has been shown in bacterial sepsis, in which impaired NFAT in platelets promoted disease severity through increased NETs and disseminated coagulation.[22] Here, we address the specific role of CN-NFAT in human peripheral blood–isolated neutrophils, especially in the context of susceptibility to fungal infection. Furthermore, we investigate the NFAT-dependent gene expression from PMNs of patients hospitalized at the intensive care unit (ICU) with sepsis.

2. Methods

2.1 Isolation of peripheral blood human neutrophils

Neutrophils were isolated from healthy donor buffy coats (Department of Transfusion & Tissue Medicine, Brno University Hospital, Brno, Czech Republic) by a 2-step protocol. Undiluted buffy coats were layered onto PolymorphPrep (density 1.113 g/mL; Axis-shield) and centrifuged following the manufacturer's recommendations. The obtained PMN layer was purified using EasySep Direct Human Neutrophil Isolation Kit (STEMCELL Technologies) and "The Big Easy" EasySep Magnet (STEMCELL Technologies) according to the manufacturer's protocol. Isolated neutrophils were centrifuged and resuspended in X-VIVO 15 media (Lonza) without any supplementation. Neutrophils with at least 95% purity were used.

2.2 Fluorescence-activated cell sorting neutrophil purity assessment

The purity of isolated neutrophils was assessed using flow cytometry (fluorescence-activated cell sorting [FACS]). Isolated neutrophils (1×10^6 cells per sample) were stained with 2 different antibody cocktails, C1 and C2 (Table 1), in phosphate-buffered saline (PBS) + 1 mM EDTA for 30 min on ice. LIVE/DEAD Fixable Green Dead Cell Stain Kit (Life Technologies) in C1 was used to monitor cell viability. After 30 min of staining followed by the washing step, cells were fixed using IC fixation buffer (Life Technologies). Cell populations were analyzed using the FACSCanto II cytometer (BD Biosciences), and the data were analyzed using FlowJo v.10.8 (BD Life Sciences). Neutrophils were considered $CD66b^+$, $CD16^+$, and $CD11b^+$ cells, and the contamination of $CD193^+$ cells and $CD3^+$ cells was monitored. The representative gating strategy is shown in Fig. 1B.

Table 1. Antibodies used for assessment of purity of isolated neutrophils.

Antibody	Fluorochrome	Cat. no.	Manufacturer	Dilution
C1				
CD14	PE	367104	BioLegend	1:100
CD16	eF450	48-0168-42	eBioscience	1:100
CD66b	PE/Cy7	25-0666-42	eBioscience	1:25
LiveDead Green	FITC	L23101	Life Technologies	1:1000
CD107a	APC	2243100	SONY	1:100
CD63	BV510	2365160	SONY	1:100
C2				
CD66b	PE/Dzl594	2125610	SONY	1:100
CD16	eF450	48-0168-42	eBioscience	1:100
CD3	APC/Cy7	317342	BioLegend	1:50
CD11b	PE/Cy7	25-0118-42	eBioscience	1:100
CD193	APC	17-1939-42	eBioscience	1:100

2.3 Monocyte isolation

Monocytes were isolated from healthy donor buffy coats (Department of Transfusion & Tissue Medicine of the Brno University Hospital, Brno, Czech Republic) using RossetteSep Human Monocytes Enrichment Cocktail (STEMCELL Technologies) followed by layering of buffy coat diluted 1:1 with PBS onto LymphoPrep (density 1.077 g/mL; STEMCELL Technologies) density gradient medium and subsequent centrifugation according manufacturer's recommendations. An obtained layer of monocytes was washed 3 times with PBS and resuspended in X-VIVO 15 media (Lonza) without any supplementation.

2.4 *A. fumigatus* culture

A. fumigatus Af293 was cultivated on potato dextrose agar (Sigma-Aldrich) at 37 °C for 5 to 7 d. *A. fumigatus* conidia were harvested directly from agar using PBS and Tween 20 (0.05%). To obtain *A. fumigatus* swollen conidia, harvested conidia were cultivated in Sabouraud dextrose broth (Sigma-Aldrich) overnight at room temperature in flasks. To obtain *A. fumigatus* hyphae, harvested conidia were cultivated in Sabouraud dextrose liquid medium overnight at 37 °C in flasks.

2.5 *Candida albicans* culture

C. albicans was cultivated on Sabouraud dextrose agar (Sigma-Aldrich) at 37 °C overnight. *C. albicans* yeasts were harvested with PBS and seeded into RPMI (Life Technologies) and cultivated in culture flasks at 37 °C in the atmosphere with 5% CO_2 for 30 min to obtain germ tubes or for 90 min to obtain hyphae.

2.6 Neutrophil stimulation

Freshly isolated neutrophils were maintained in X-VIVO 15 media in concentration 2×10^6 cells/mL, unless otherwise stated. Neutrophils were incubated for 30 min and then pretreated with either 2 μg/mL CsA (Cell Signaling Technology), or0,4 μg/mL FK506 (Invivogen), for 1 h before exposure to 10^8 particles/mL heat-killed *C. albicans* (HKCA) (Invivogen) or 2×10^6 particles/mL heat-killed *A. fumigatus* (HKAF) (obtained by heat inactivation of cultured *A. fumigatus*) for 2 h. After incubation, samples were centrifuged, a conditioned medium was used for enzyme-linked immunosorbent assay (ELISA) analysis, and pellets were lysed by TRI REAGENT (Molecular Research Center) and frozen at −80 °C until RNA isolation.

2.7 Differentiation and stimulation of neutrophil-like cells

The HL-60 cell line obtained from the European Collection of Cell Culture (ECACC Cerdic) was used to differentiate neutrophil-like cells. The cell line was cultured in Iscove's Modified Dulbecco's Medium (Life Technologies) with 10% (v/v) heat-inactivated fetal bovine serum (Biowest), L-glutamine (Biowest), and penicillin/streptomycin (Biowest) at 37 °C, 5% CO_2. For differentiation into neutrophil-like cells, HL-60 cells were centrifugated and resuspended in Iscove's Modified Dulbecco's Medium with 1.25% (v/v) dimethyl sulfoxide, 10% (v/v) fetal bovine serum, L-glutamine, and penicillin/streptomycin and seeded at concentration 1×10^5. Cells were cultivated for 7 d to obtain differentiated cells and were harvested, and 10^6 cells were seeded into each well of a 12-well plate and cultivated overnight. Neutrophil-like cells were pretreated

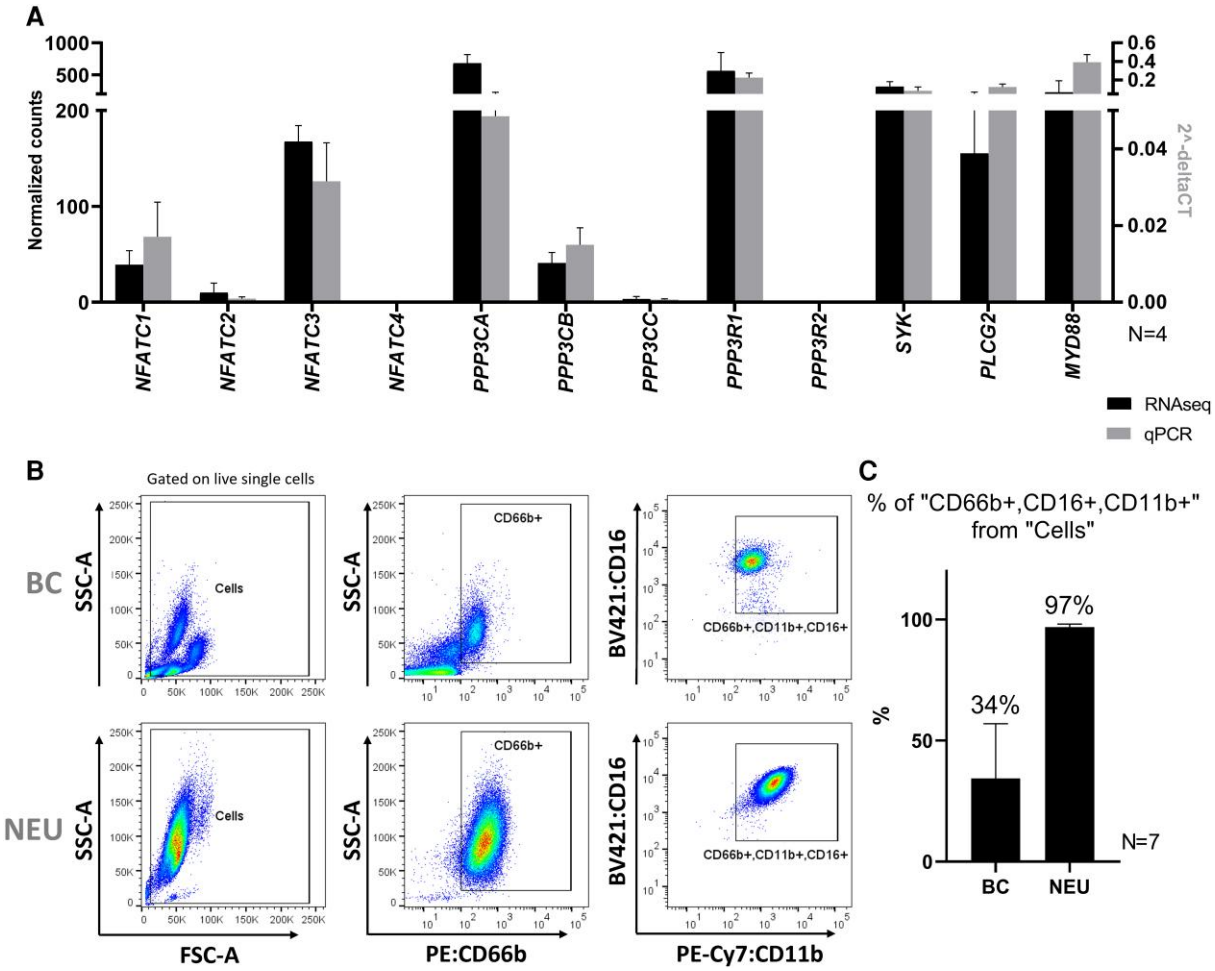

Fig. 1. Characterization of neutrophils (NEU) used in the following experiments. **(A)** Neutrophils' expression of the CN-NFAT pathway analyzed by qPCR and the expression pattern using RNAseq data obtained from nonstimulated NEU isolated from healthy donors. Columns show the mean of normalized counts with SD or mean ΔCt values, calculated as a relative expression to GAPDH. **(B)** Representative dot plots showing the gating strategy for assessing the purity of isolated NEU and comparison with blood from the buffy coat (BC) used for isolation. **(C)** Averaged purity of NEU after isolation and comparison with blood from the BC. FSC-A = forward scatter area; SSC-A = side scatter area.

with 2 μg/mL CsA (Cell Signaling Technology) or 1μM TPCA-1 (Sigma-Aldrich) for 1 h and stimulated by different life forms of *C. albicans* or *A. fumigatus* in concentration 2.5×10^6 particles/mL, or 2.5×10^6 particles/mL HKAF, or 50×10^6 particles/mL HKCA.

2.8 Enzyme-linked immunosorbent assay

Conditioned medium from neutrophil or neutrophil-like cells cultivation removed as supernatant after centrifugation was used for cytokine and protein levels determination by ELISA. Levels of PTX-3, CCL2/MCP-1, CCL3/MIP-1α, and CCL4/MIP-1β were measured in conditioned media (100 μL) using the DuoSet ELISA (R&D Systems). ELISA was performed according to the manufacturer's recommendation.

2.9 RNA isolation and quantitative polymerase chain reaction analysis of gene expression

Lysates from 2 to 6×10^6 neutrophils in 1 ml TRI REAGENT were used for the isolation of total RNA. After the addition of 20% W/W chloroform and centrifugation (12,000 *g*; 15 min; 4 °C), the upper aqueous phase was collected and mixed with an equal volume of 70% ethanol. This mixture was used for RNA isolation using an RNAeasy mini kit (QIAGEN) or Arcturus Pico pure kit (Life Technologies) according to the manufacturer's instructions. DNA contamination was prevented by 15 min of DNAse I (QIAGEN) on column digestion. RNA concentrations were determined by Nanodrop (Agilent). RNA integrity was assessed on Bioanalyzer 2100 RNA Nano 6000 chips (Agilent), and samples with acceptable RIN were used for RNA sequencing.

For quantitative polymerase chain reaction (qPCR) analysis, samples were diluted to the same RNA concentration and transcribed into complementary DNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Gene expression was defined by using TaqMan probes (TaqMan Gene Expression Assay; Thermo Fisher Scientific) and determined by real-time PCR analysis performed on LightCycler 480 (Roche). The Ct values of genes of interest were normalized to housekeeping gene GAPDH (ΔCt), and the relative expression of each gene of interest was calculated as $2^{-\Delta Ct}$. Used TaqMan probes are listed in Supplementary 1.

2.10 Library preparation and sequencing

Illumina sequencing libraries were prepared using the Lexogen QuantSeq FWD following the manufacturer's instructions. Sequences were barcoded using UMI Second Strand Synthesis Module for QuantSeq FWD and i5 6 nt Unique Dual Indexing Add-on Kit (Lexogen) added unique index adapter sequences

during the PCR step. QuantiFluor dsDNA System (Promega) was used for quantification followed by equimolar pooling. The sample pool was sequenced using a NextSeq 500 sequencer (Illumina) using a 75-cycle high-output cartridge, at a sequencing depth of ~13 M reads.

2.11 RNA sequencing bioinformatic analysis

Raw FASTQ files were preprocessed in an Arch Linux × 86_64 server, suitable for multithreading. All reads were subjected to quality control (FastQC) and mapped to the human genome (Ensembl GRCh38-p10). Differential expression (DE) analysis and all downstream processes were done in the R (v4.3.3; R Foundation for Statistical computing) environment by use of R/Bioconductor packages. DE analysis was done by use of the DESeq2 pipeline and was followed by Gene Ontology (GO) and gene set enrichment analysis, using the R package clusterProfiler and the desktop version of gene set enrichment analysis MSigDB, respectively. Results were visualized with the R packages ggplot2, complexHeatmap, ggrepel, fmsb, and ggVENNdiagram. Genes that demonstrated a log2 fold change $\geq 0.6|$ and a P value ≤ 0.05 were considered as significantly differentially expressed genes (DEGs). The barplots were visualized with Prism version 8 (GraphPad Software). The complete RNA sequencing (RNAseq) data are publicly available in Gene Expression Omnibus with accession number GSE259282.

2.12 Chemoattraction ability assay

The ability of neutrophils to chemoattract monocytes in different conditions was assessed using transwell inserts with 5 µm pores (cellQUART). After isolation, 1×10^6 neutrophils were seeded into a 24-well plate, pretreated with CsA for 1 h, and stimulated by HKCA for 2 h. Culture plates were centrifuged (300 *g*, 5 min, room temperature) supernatants were trashed and fresh medium was added to cells. After that, inserts with 1×10^6 monocytes stained using a Far-red cell proliferation kit (Life Technologies) were placed into wells and cocultivated for 2 h. Then inserts were trashed and plates were centrifugated again and used for imaging using a confocal microscope Zeiss LSM 780 or for flow cytometry analysis (FACS) analysis. For FACS analysis, pelleted cells were resuspended in PBS + 1 mM EDTA, harvested into 5 mL falcon tubes, washed, and stained. Cells were stained with the antibody cocktail listed in Table 2 in PBS + 1 mM EDTA for 30 min on ice. Unstained control was fixed using IC fixation buffer (Life Technologies) immediately. After 30 min of staining followed by the washing step, cells were fixed using IC fixation buffer. Cell populations were recorded using FACS Canto II cytometer (BD Biosciences) and analyzed using FlowJo v.10.8 (BD Life Sciences). $CD45^+$, $CD14^+$, and Far-red–positive cells were considered chemoattracted monocytes.

2.13 Statistical analysis

Statistical analysis was done using Prism software version 8. Data were tested for normal distribution and based on its results appropriate parametric or nonparametric statistical tests were applied. Used statistical tests are specified in the figure legends.

Table 2. Antibody cocktail for analysis of chemoattracted monocytes.

Antibody	Fluorochrome	Cat. no.	Manufacturer	Dilution
CD14	PE	367104	BioLegend	1:100
CD16	eF450	48-0168-42	eBioscience	1:100
CD66b	PE/Cy7	25-0666-42	eBioscience	1:25
CD45	BV510	304035	BioLegend	1:100

3. Results

3.1 Human neutrophils express genes necessary for CN-NFAT signaling with predominant expression of NFATC3 and NFATC1

In order to explore the importance of the CN-NFAT signaling pathway in neutrophils, we performed gene expression analysis using bulk RNAseq and qPCR from neutrophils enriched from peripheral blood–isolated PMNs of healthy donors. Our results showed that neutrophils express molecules crucial for CN-NFAT signaling (Fig. 1A). Specifically, from the calcium-dependent NFATs, *NFATC3* (NFAT4) was predominantly expressed, followed by *NFATC1* (NFAT2) (Fig. 1A). Among the calcium-sensing protein CN subunits, *PPP3CA*, *PPP3CB*, and *PPP3R1* were detected together with the signal transducers *SYK*, *PLCG2*, and *MYD88* (Fig. 1A). To ensure the maximal reliability of results, all analyses were performed with purified neutrophils. Neutrophils were gated as $CD11b^+$, $CD16^+$, and $CD66b^+$ cell population (Fig. 1B), and the average purity of the isolated cells was 97% (Fig. 1C).

3.2 NFAT signaling in human neutrophils controls the expression of chemokines

To understand the pathogen-related expression changes in human neutrophils, we stimulated the cells with HKCA and HKAF and subjected them to bulk RNAseq. Differential gene expression analysis revealed transcriptional changes of neutrophils upon 2 h of stimulation with HKCA or HKAF (Fig. 2A). Volcano plots depict an overall pattern of expressional changes shown as DEGs (Fig. 2A, B). We observed that stimulation with both pathogens caused prominent transcriptomic changes, marked by mostly an upregulation of gene expression, which was stronger in response to HKCA (Fig. 2A). CsA treatment prior to pathogen stimulation markedly attenuated the observed transcriptomic changes and caused a prominent downregulation of gene expression (Fig. 2B). GO analysis of the significant DEGs revealed that several biological processes that were significantly affected by CsA (Fig. 2C, D). Noticeably, ontologies involved in neutrophil homeostasis and chemotaxis (cell chemotaxis, cellular response to chemokine, cytokine-mediated signaling pathway, leukocyte chemotaxis, leukocyte migration, neutrophil chemotaxis, neutrophil migration, response to chemokine, and response to tumor necrosis factor) were significantly downregulated in both HKCA + CsA and HKAF + CsA conditions. Given the common features of the transcriptomic changes caused by stimulation with HKCA or HKAF, we then sought to determine the genes that were commonly upregulated by both pathogens and downregulated in the presence of CsA (Fig. 2E). We identified 61 transcripts that were uniformly upregulated by both pathogens and at the same time downregulated in the presence of CsA (Fig. 2E, F). Given the role of CsA as a CN-NFAT inhibitor, these genes were considered as NFAT dependent. Clustering of the analyzed samples based on the normalized counts of these NFAT-dependent genes demonstrated that the pathogen-stimulated samples (HKCA and HKAF) shared several similarities between themselves and clustered together. At the same time, the CsA-pretreated samples (HKCA + CsA and HKAF + CsA) clustered closer together and with the nontreated samples (Fig. 2F). This finding supported the notion that the pathogen stimulation upregulated CN-NFAT signaling, while CsA attenuated it, returning it close to the nontreated conditions. In the next step of our analysis, we sought to assign biological function to the 60 DEGs, via GO analysis (Fig. 2G). We observed that the 60 NFAT-dependent genes participated mainly in ontologies involved in cell mobility and chemotaxis. Importantly, the

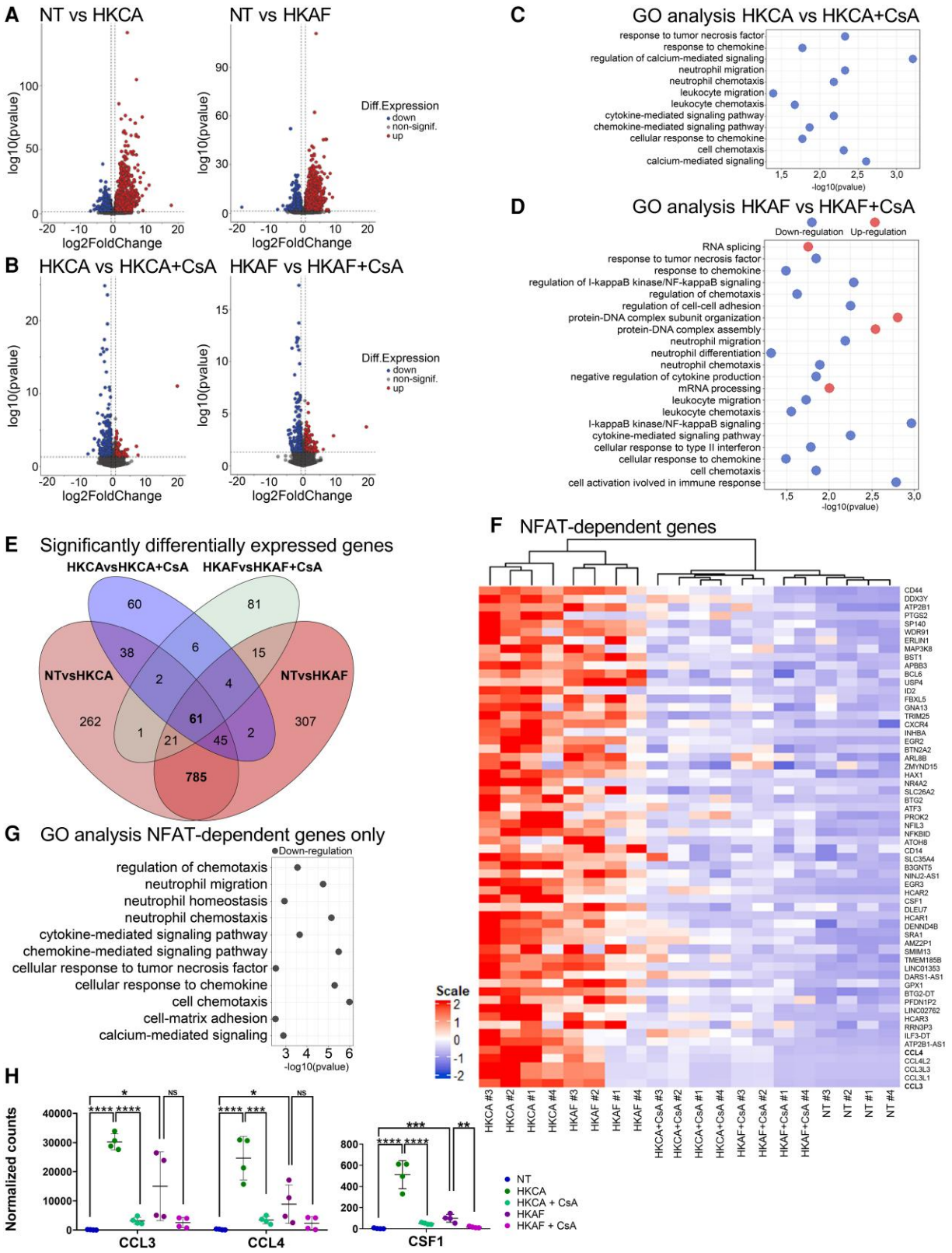

Fig. 2. CN-NFAT inhibitors affect chemokine expression in human neutrophils. **(A)** Volcano plots of the DEGs after stimulation of neutrophils with HKCA (left) and HKAF (right). **(B)** Volcano plots of the DEGs after stimulation of neutrophils with HKCA (left) and HKAF (right) in the presence of the CN-NFAT inhibitor CsA. **(C)** GO analysis of the significantly changed pathways after stimulation of neutrophils with HKCA and in the presence of CsA. **(D)** GO analysis of the significantly changed pathways after stimulation of neutrophils with HKCA and in the presence of CsA. **(E)** Venn diagram of the DEGs that are commonly and uniquely affected by the stimulation with each pathogen and by the presence of CsA. **(F)** Heatmap and sample clustering of the scaled normalized counts of the 60 genes that are uniformly upregulated by both pathogens and downregulated in the presence of CsA. **(G)** GO analysis of the genes that are uniformly upregulated upon stimulation with both pathogens and are downregulated in the presence of CsA. **(H)** Bar plots of the normalized counts for CCL3, CCL4, and CSF1 as the most abundant genes in the identified chemotactic ontologies. Bar plots are presented as mean ± SD from 4 independent biological replicates. Statistical significance was determined with 1-way analysis of variance and Tukey post hoc test: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$.

chemotactic chemokines CCL3 and CCL4 as well as the colony-stimulating factor 1 (CSF1) were present in all ontologies (Fig. 2G), suggesting a vital role for NFAT-dependent molecules in neutrophil migration, chemotaxis, and homeostasis. Finally, we sought to assess more in depth the extent of the pathogen stimulation together with the CsA effect on the expression changes of these genes (Fig. 2H). We observed a massive upregulation of all 3 of CCL3, CCL4, and CSF1 after stimulation with HKCA and a more modest one after stimulation with HKAF. In both cases, pretreatment with CsA attenuated the upregulation of all 3 genes (Fig. 2H).

3.3 Neutrophil chemokine release induced by *A. fumigatus* and *C. albicans* was inhibited by CsA

RNAseq data revealed a potential connection between CN-NFAT signaling and neutrophil chemotaxis while at the same time indicating CCL3 and CCL4 as potentially important players in these processes. Therefore, we analyzed the levels of chemokines released in response to pathogens and the potential effect of CN-NFAT inhibition by CsA. As a control, we used a TPCA-1 inhibitor in order to see the effect of nuclear factor κB inhibition on chemokine release. For this purpose, the HL-60 cell line differentiated into neutrophil-like cells was used. The cells were stimulated by different morphotypes of *A. fumigatus* and *C. albicans*. The release of chemokines CCL2, CCL3, and CCL4 induced by yeasts, germ tubes, and hyphae of *C. albicans* (Fig. 3A) and by conidia, swollen conidia, and hyphae of *A. fumigatus* (Fig. 3B) was measured using ELISA. The release of chemokines was dependent on morphotypes. Hyphae of both pathogens were the most activatory, inducing a significant increase in CCL3 and CCL4 production (Fig. 3A, B). Further, we evaluated the effect of CsA and TPCA-1 on the secretion of chemokines CCL2, CCL3, and CCL4 in response to different *C. albicans* and *A. fumigatus* morphotypes (Fig. 3C–E). Stimulation of neutrophil-like cells by yeast form and germ tube form of *C. albicans* resulted in reduced CCL2 secretion induced by TPCA-1, but the response to *C. albicans* hyphae was significantly inhibited only by CsA (Fig. 3C). On the other hand, the release of CCL3 and CCL4 was significantly inhibited only by CsA after exposure to the hyphae form of both opportunistic pathogens and the yeast form of *C. albicans* (Fig. 3D, E). Moreover, we observed a CsA inhibitory effect on CCL4 secretion after exposure to HKCA (Fig. 3E). Response to *A. fumigatus* conidia or swollen conidia was not significantly affected by CsA (data not shown). Expressional data obtained by qPCR (Fig. 3F–H) are consistent with observation at the protein level. *CCL2* expression was significantly increased in response to *A. fumigatus* hyphae and HKCA, while response to *C. albicans* hyphae was inhibited by CsA and TPCA-1 (Fig. 3F). Expression of *CCL3* was induced by *A. fumigatus* and *C. albicans* hyphae and also by HKCA, both inhibitors significantly affected response to *A. fumigatus* hyphae and HKCA, in which the CsA inhibitory effect showed strong significance (Fig. 3G). *CCL4* expression was strongly elevated in response to *A. fumigatus* and *C. albicans* hyphae, HKCA and TPCA-1 inhibited expression in response to all 3 stimuli, and CsA inhibited response to *C. albicans* hyphae and strongly also in response to HKCA (Fig. 3H).

3.4 CsA impaired the ability of neutrophils to chemoattract monocytes

The chemotactic assay was used to determine the effect of CsA on the ability of neutrophils to chemoattract other immune cells. Staining of neutrophils and monocytes using 2 different CellTrace dyes enabled us to distinguish monocytes that migrated through 5 μm pore inserts (Fig. 4A). Fluorescence microscopy images taken after 2 h of chemotactic experiment show a proportion of chemoattracted monocytes visualized in green within neutrophils visualized in red (Fig. 4A). To quantify the numbers of chemoattracted monocytes, we stained a mixture of chemoattracting neutrophils and migrated monocytes using antibodies for surface markers (Table 2) and analyzed them by flow cytometry. The representative gating strategy of monocytes can be found in Supplementary 2. The chemoattracted monocytes were defined as Far-red CellTrace$^+$ and CD14$^+$ cell population and were used to count relative chemoattraction (Fig. 4B). The ability of neutrophils stimulated by HKCA to chemoattract monocytes was significantly inhibited when neutrophils were treated with CsA prior to stimulation (Fig. 4B, C).

3.5 Expression of molecules controlling immune response was impaired by CN-NFAT inhibitors

As we identified NFAT-dependent expression of molecules controlling immune response by RNAseq using CsA as an NFAT inhibitor, we used qPCR to confirm the same effect of another NFAT inhibitor FK506 on the expression of selected genes by primary human neutrophils. We focused especially on genes, that were already reported as NFAT dependent. *CCL2* expression was significantly increased by HKCA and HKAF after 2 h of stimulation, and its expression was completely prevented by both CN-NFAT inhibitors (Fig. 5A). The expression of *PTX3* was increased in response to heat-killed pathogens, but only FK506 caused significant inhibition of its expression in response to HKCA (Fig. 5B). However, in other conditions, the inhibitory effect was not consistent, although NFAT-dependent expression of *PTX3* was reported in human monocytes.[14] The same trend was observed when measuring PTX-3 levels in the medium of cultivated neutrophils using the ELISA method (Fig. 5C). HKCA stimulation caused a significant release of PTX-3; however, neither CsA nor FK506 had an inhibitory effect on the release. Another chemokine affected by CN-NFAT inhibitors was *CCL3*, whose expression was highly elevated by HKCA, and significantly inhibited by CsA and FK506 (Fig. 5D). Expression of *EGR2* and *PTGS2* (COX-2) was increased in response to HKCA and inhibited by CN-NFAT inhibitors, consistent with already published results (Fig. 5D).[8,15] The expression of *EGR1* was increased by CsA and FK506 treatment (Fig. 5D), suggesting a possible commutable role of *EGR1* and *EGR2*, when *EGR2* expressional downregulation is substituted by upregulation of *EGR1* expression. Expression of *GZMB* (granzyme B) and *TNF* was detected in all samples, with no effect of CN-NFAT inhibitors (data not shown). To confirm the NFAT-dependent production of chemokines suggested by qPCR data and results from neutrophil-like HL-60 cells (Fig. 3), we analyzed levels of chemokines CCL3 (Fig. 5E) and CCL4 (Fig. 5F), showing their elevation in response to heat-killed pathogens. Production of chemokines is abolished by CN-NFAT inhibitors CsA and FK506 with statistical significance in all combinations of heat-killed pathogen and inhibitor except CCL3 production after HKAF in the presence of FK506, but the inhibitory effect is perspicuous even in this condition (Fig. 5E, F).

These results revealed that in human neutrophils, genes involved in the regulation of the immune response were negatively affected by CN-NFAT inhibitors, rather than functional molecules of immediate response to the pathogen. NFAT-dependent expression was confirmed for CCL3 and CCL4 at protein level using ELISA.

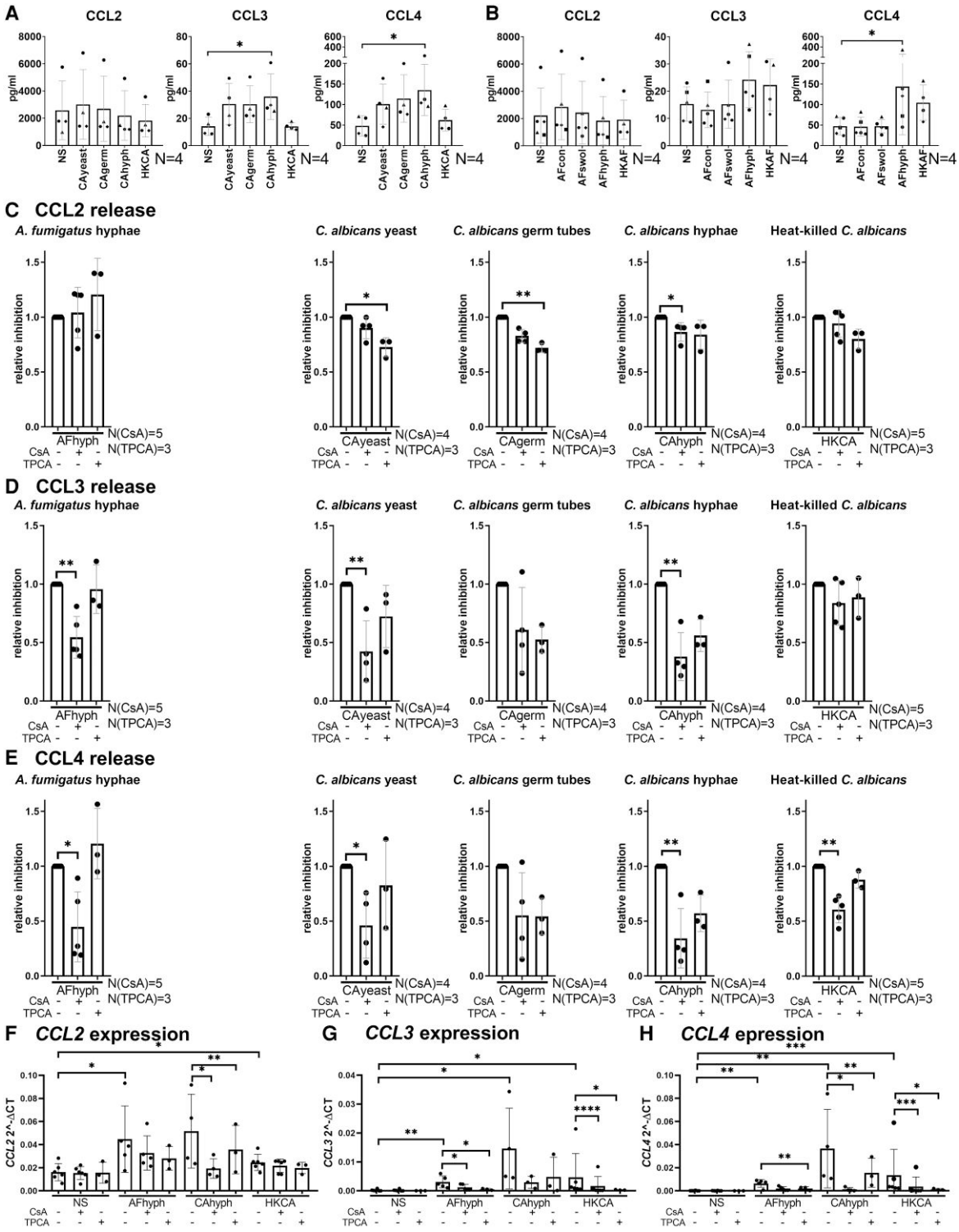

Fig. 3. Release of chemokines by neutrophil-like cells in response to the most immunogenic hyphae life form of fungal opportunistic pathogens is NFAT dependent. HL-60 cells differentiated into a neutrophil-like phenotype were stimulated for 2 h with indicated forms of *C. albicans* **(A)** and *A. fumigatus* **(B)**. The presence of chemokines CCL2, CCL3, and CCL4 was detected by ELISA. Columns represent the mean ± SD from 4 independent experiments. Stimulation with pathogens was statistically compared with nonstimulated (NS) control without treatments using a parametric ratio paired *t* test. $^{*}P \leq 0.05$. **(C–E)** HL-60 cells differentiated into neutrophil-like phenotype were stimulated with indicated forms of *C. albicans* or *A. fumigatus* with or without inhibitors. Chemokine presence in the supernatant was detected after 2h of incubation for CCL2 **(C)**, CCL3 **(D)**, and CCL4 **(E)**. Relative inhibition was calculated as a fold change of released chemokine relative to the stimulated sample. Columns represent the mean ± SD from 4 (*C. albicans* morphotypes) or 5 (*A. fumigatus* hyphae, HKCA) experiments with CsA and 3 experiments with TPCA-1 (TPCA). Nonparametric Kruskal-Wallis test was used to compare the relative inhibition of CsA or TPCA with the stimulated sample. Changes in expression of CCL2 **(F)**, CCL3 **(G)**, and CCL4 **(H)** chemokines upon stimulation with the hyphae form of *A. fumigatus* and *C. albicans* and with HKCA in the presence or absence of CsA or TPCA detected by qPCR. ΔCt is calculated as a relative expression to GAPDH. A parametric ratio paired *t* test was used to compare stimulated samples with NS control and to compare the effect of CsA and TPCA on pathogen stimulation. The number of independent experiments varies between conditions from 3 to 6 and is indicated by the number of dots in the barplots. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$.

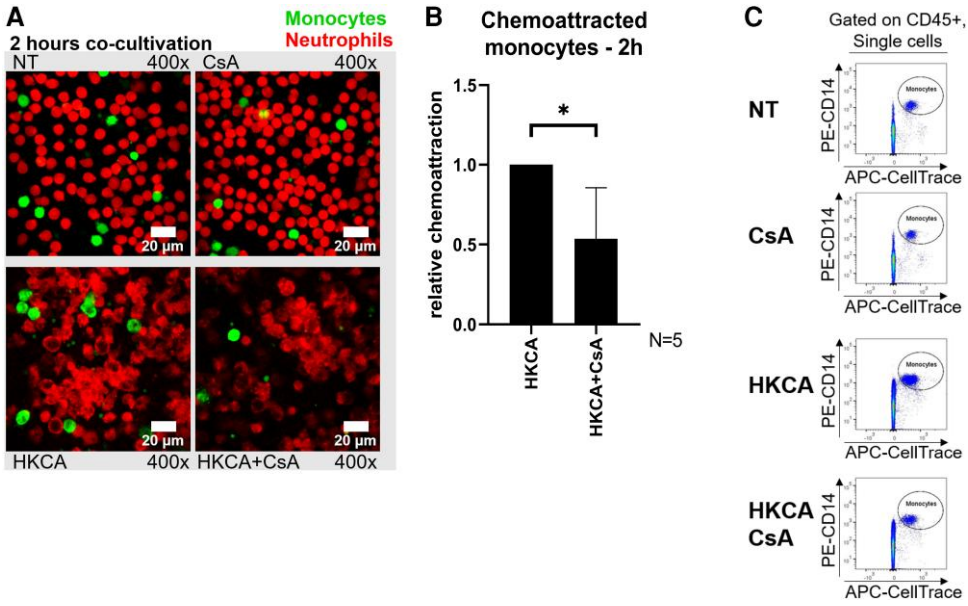

Fig. 4. HKCA-stimulated neutrophils are impaired in monocyte chemoattraction in the presence of CsA. **(A)** Confocal microscope analysis of monocytes (green) transmigrating through transwells to HKCA-stimulated neutrophils (red) in the presence or absence of CsA. **(B)** Flow cytometry quantification of $CD14^+$ and $CellTrace^+$ monocytes after 2 h transmigration to HKCA-stimulated neutrophils pretreated with CsA or controls. Relative chemoattraction was calculated as a fold change of absolute monocyte count in HKCA+CsA condition relative to the HKCA-stimulated sample, the mean ± SD is shown. A parametric ratio paired *t* test was used for statistical comparison. $^*P \leq 0.05$. **(C)** Representative gating of $CD14^+$ and $CellTrace^+$ monocytes that were chemoattracted by differently treated neutrophils.

3.6 NFAT-dependent gene expression correlated with septic patient organ function status (sequential organ failure assessment score)

We further investigated whether our previous findings could also provide a broad view of neutrophil-related changes that happen in response to the pathogens and that eventually could escalate to sepsis. For this purpose, we used an already published RNAseq dataset from PMNs of septic patients[21] and sought to identify which of our previously reported 60 NFAT-dependent genes were present in the septic patients' PMNs. For this purpose, we used the normalized counts from 11 patients, 6 of which survived until day 5 after admission to the ICU and 5 of which were deceased shortly after admission to the ICU. We showed that several CN–NFAT–regulated genes were expressed in PMNs of patients with septic shock (Fig. 6A–C). Sepsis patients' expressed genes were divided into 3 groups based on normalized counts as genes with low (Fig. 6A), medium (Fig. 6B), or high (Fig. 6C) expression. *CSF1* was identified as a gene with medium expression in the septic cohort, and interestingly, its expression significantly correlated with the sequential organ failure assessment (SOFA) score of septic patients (Fig. 6D). Although genes for chemokines were characterized as genes with low expression, we saw a trend in the correlation between the number of *CCL3*-normalized counts with the SOFA score of deceased septic patients (Fig. 6E) and normalized counts of *CCL4* gene negatively correlated with SOFA score of survived patients (Fig. 6F). These findings suggested the role of these genes in sepsis resolution.

4. Discussion

The role of NFAT in innate immunity is well established; nevertheless, the exact importance of CN-NFAT signaling varies among different cell types or pathologies. CN-NFAT signaling governs the functions of myeloid cells during their development to the control of their major immune functions,[11,23] and as such impacts the therapeutic outcome.[24] The direct impact of CN-NFAT inhibitors on neutrophil functions was poorly understood,[3] although negative impacts of CsA treatment or CN knockdown on mice neutrophils function were shown more than 10 yr ago.[8] We hypothesized that disruption of neutrophil function by CN-NFAT inhibitors can be one of the major reasons explaining the observed increased susceptibility of CN-NFAT inhibitor–treated patients to opportunistic infections. Although this adverse effect of CN-NFAT inhibitors was shown many years ago by Calne et al,[25] or by Tourneur et al.,[9] how CN-NFAT inhibitors affect human neutrophils at molecular levels and which of their functions are disrupted remains elusive.[9,25] Considering already published data about the impact of CN-NFAT inhibitors on myeloid cell function,[11,14] deep understanding of their impact on neutrophils' pathogen response can complete this knowledge. Thus, we investigated the role of CN-NFAT inhibitors on neutrophil function at the molecular level.

We and others[15] have reported the expression of NFATs family in human neutrophils. Expression of NFATC1 in human neutrophils at both messenger RNA and protein levels was already reported by Vega et al.[15] We identified *NFATC3* (NFAT4) and *NFATC1* (NFAT2) as predominantly expressed in neutrophils. This expressional pattern of the NFAT family is unique within myeloid cells[11] and differs from T cells, which express mostly *NFATC2* and *NFATC1*.[26] We also identified expression of calcineurin subunits (*PPP3CA*, *PPP3CB*, *PPP3R1*) and checked the expression of PRRs (*TLR1-TLR10*, *CLEC7A* [dectin-1]) (data not shown), which was consistent with the already known expression profile in neutrophils.[27,28] This means that neutrophils were able to activate the CN-NFAT signaling pathway upon pathogen recognition resulting in the activation of NFAT-dependent gene transcription. As demonstrated by RNAseq data, HKCA and HKAF stimulation of neutrophils resulted in massive gene upregulation, while in the presence of CsA, we detected 61 transcripts from pathogens-induced genes significantly downregulated. GO

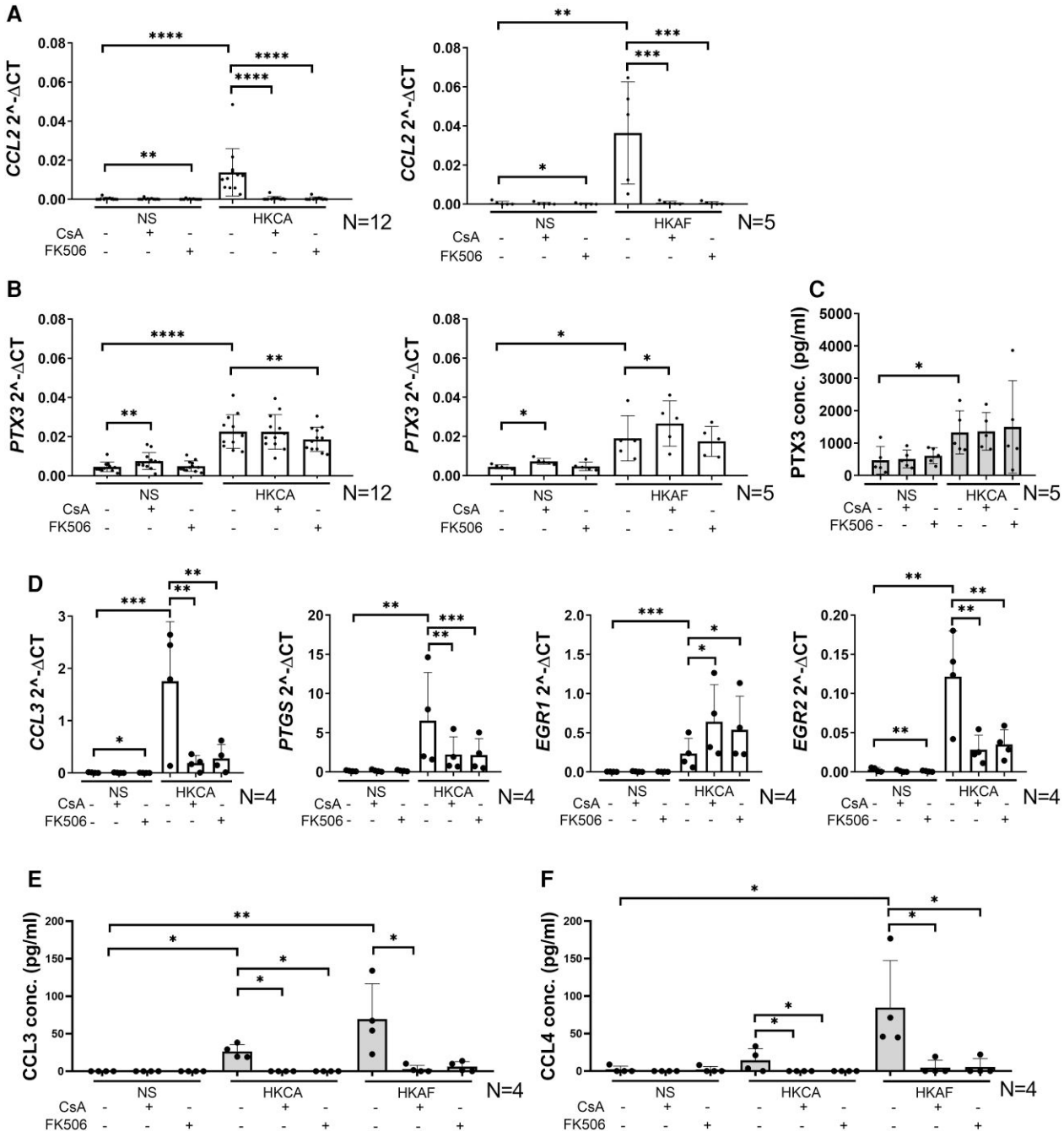

Fig. 5. In response to fungal pathogens, neutrophils initiate the expression of chemokines and other immunoregulatory molecules in an NFAT-dependent manner. Expression of *CCL2* **(A)** and *PTX3* **(B)** by human neutrophils was analyzed using qPCR after 2 h stimulation by HKCA or HKAF in the presence or absence of CN-NFAT inhibitors CsA and FK506. **(C)** PTX-3 concentration in a medium of neutrophils stimulated for 2 h by HKCA with or without CsA and FK506 was detected by ELISA. **(D)** Expression of *CCL3*, *PTGS2*, *EGR1*, and *EGR2* in response to HKCA and the impact of CsA and FK506 on their expression was analyzed by qPCR. Stimulation with pathogens was compared with nonstimulated (NS) control without any treatments using a parametric ratio paired *t* test; the statistical significance of expressional change with inhibitors treatment was compared with the condition with stimulation by the relevant pathogen using a parametric ratio paired *t* test. ΔCt is calculated as a relative expression to GAPDH in all qPCR experiments. Levels of CCL3 **(E)** and CCL4 **(F)** in a conditioned medium of human neutrophils were measured using ELISA after 2 h of cultivation with heat-killed pathogens in the presence or absence of CsA or FK506. Nonparametric Kruskal-Wallis test with multiple comparisons was used to evaluate stimulation with the pathogen and to compare the stimulated sample with respective samples with inhibitors. Columns represent the mean ± SD from 4 independent experiments. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$.

analysis of NFAT-dependent genes revealed biological processes connected with the chemotaxis and genes for CCL3, CCL4, and CSF1, which were shared by all affected processes. The high impact of 2 CN-NFAT inhibitors, CsA and FK506, on CCL3 and CCL2 expression was observed by qPCR analysis after stimulation of neutrophils by HKCA or HKAF. While NFAT-dependent expression of CCL2 was already reported in human monocytes[14] and macrophages,[29] we reported CCL3 and CCL4 NFAT-dependent expression in human neutrophils for the first time. Although neutrophil extravasation elicited by CCL3 was reported to be dependent on PI3Kγ signaling, it was not connected with the NFAT pathway.[30] Neutrophil-secreted CCL3 was shown to be essential

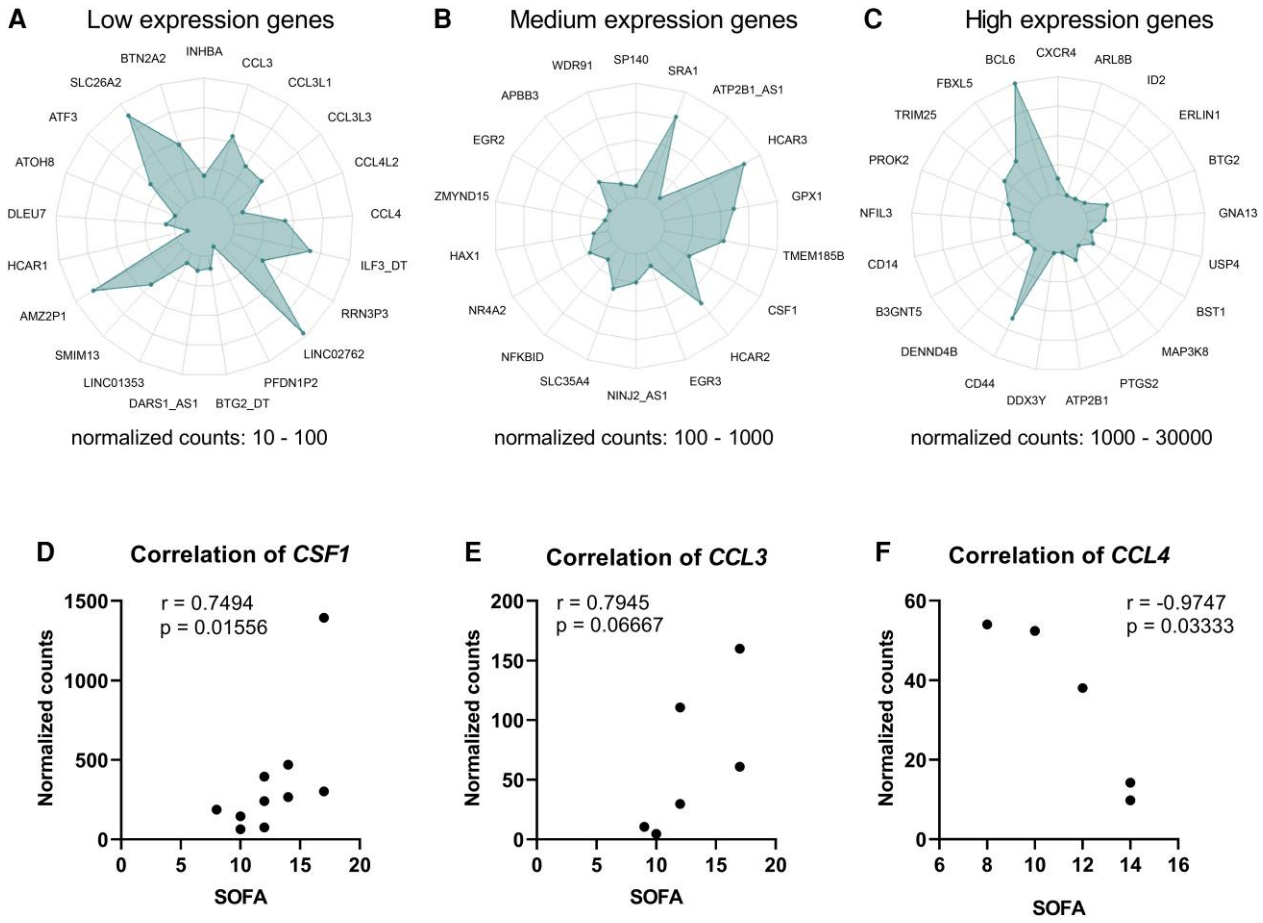

Fig. 6. NFAT-dependent genes are expressed in septic patients and correlate with organ function SOFA scores. **(A–C)** Spider plots of the expression of the 60 NFAT-dependent genes in septic patients. Genes were divided based on the means of normalized counts as genes with low (10 to 100 mean normalized counts) **(A)**, medium (100 to 1,000 normalized counts) **(B)**, and high expression (more than 1,000 normalized counts on average) **(C)**. **(D–F)** Spearman's correlation plots of the normalized counts of *CSF1* **(D)**, *CCL3* **(E)**, and *CCL4* **(F)** with the SOFA scores of septic patients. Numbers of normalized counts of *CSF1* correlated with SOFA score of septic patients **(D)**. Normalized counts of *CCL3* correlated significantly with the SOFA scores of only the deceased septic patients **(E)**, while normalized counts of *CCL4* correlated significantly with the SOFA score of only the day 5 survivors **(F)**.

for the recruitment of dendritic cells in a mouse model of *Leishmania major* infection.[31] Charmoy et al.[31] also showed a transient impact on the development of a protective immune response by influencing T helper 1 cell immune response and suggested the role of CCL3 in the regulation of immune response to pathogens.[31] RNAseq and qPCR data also showed NFAT-dependent expression *PTGS2* (COX-2) and *EGR* genes, which is consistent with already published observations in mice neutrophils[8] and for COX-2 also in human neutrophils during CN-NFAT inhibitor–dependent IgE-driven allergic response.[15]

Invasive fungal infections are serious life-threatening complications after organ or hematopoietic cells transplantation.[32–36] *Candida spp.* and *Aspergillus spp.* are the most common opportunistic pathogens affecting immunocompromised patients.[34,37] Antifungal defense is mainly governed by phagocytes and neutrophils that have an undoubted role as the most common ones,[38–40] as disseminated fungal infections are most common in neutropenic patients.[41]

Considering that CCL2, CCL3, and CCL4 are among the most important as well as abundant chemokines produced by neutrophils, and these chemokines are critical for their ability to chemoattract other immune cells as well as more neutrophils to the site of infection.[2,42] We used different life forms of *C. albicans* and *A. fumigatus* to stimulate neutrophil-like (HL-60) cells. Differentiated HL-60 cells have neutrophil-like phenotype and also neutrophils' functional properties as the ability to form NETs, produce reactive oxygen species, phagocyte, and are capable of chemotaxis, as reviewed by Blanter et al,[43] and they also have granules consisting of PTX-3.[43,44] We monitored HL-60 differentiation by CD11b marker and we also provided pilot experiments to assess their ability to interact with fungal pathogens and respond by increased expression of selected genes, as shown in Supplementary 3. It is known that the 3 morphotypes of *C. albicans* and *A. fumigatus* differ in polysaccharide (i.e. β-glucan) content, and therefore are differently recognized by the immune system[12,45,46] due to different interactions with host PRRs including dectin-1.[47] The morphogenic transformation from yeast to hyphae not only allows fungi to penetrate tissues, but also has consequences for their immunogenicity, as fungi change the content of surface polysaccharides during this morphological transformation.[45,48,49] The heat-killed pathogens, although dead, can still be used as dectin-1 agonists, as shown by HKCA recognition by dectin-1[50] by HKAF causing dectin-1–mediated response of macrophages.[49] We confirmed that the hyphae of both pathogens were the most activatory in terms of chemokine release response. Only CsA significantly inhibited chemokine response to the hyphae form of pathogens, while we did not observe any statistically significant inhibition by TPCA-1 at the protein level. Expression of

chemokines was affected by CsA and TPCA-1, confirming the shared contribution on its regulation by NFAT and nuclear factor κB pathway. These observations highlight that chemokine release in response to fungal opportunistic pathogens was crucially dependent on the NFAT pathway. We further showed on primary human neutrophils that CsA and FK506 have similar effects on the expression of selected genes, including genes for chemokines, in which the effect of both inhibitors was confirmed at protein level for CCL3 and CCL4 chemokines. Although these 2 drugs (CsA, FK506) interact with different immunophilins in cells and are used at different concentrations, they eventually inhibit calcineurin and by that prevent NFAT dephosphorylation and activation. Interestingly, Borges et al[51] reported the NFATC2 (NFAT1)-independent impact of FK506 on neutrophil migration using in vivo mice model of sepsis.[51] Our data do not corroborate with their finding, possibly due to the more prominent expression of NFATC1 and NFATC3 in human monocytes and neutrophils, while our data showed only very weak expression of NFATC2.[11,15] Borges et al[51] focused on the CXCL2 chemokine and its receptor CXCR2, and here we provided a broader molecular context showing a more complex regulation network of CN-NFATs.[51] We and others[51] have suggested that FK506 treatment results in impairment of neutrophil migration, which can support previously published results on human monocytes[14] and results of this study.

To confirm the defects in the ability of neutrophils to chemoattract other immune cells, we performed a chemotaxis assay using transwells with monocytes. C-C motif chemokines primarily mediate the migration of monocytes,[52–54] but it was also shown that they are involved in the chemoattraction of neutrophils creating a positive feedback loop.[55–58] Our results clearly showed that HKCA-activated neutrophils chemoattract monocytes within 2 h, but chemoattraction was inhibited when neutrophils were treated by CsA prior to HKCA stimulation. This assay modeled the behavior of neutrophils at the site of the infection, in which they need to properly react to the pathogens in order to attract other immune cells and more neutrophils to eliminate the invading pathogens.[59,60]

Neutrophil status and activation play a key role in sepsis and COVID-19 resolution and markedly determine clinical outcomes.[21,61] A variety of markers are connected with their disease-associated dysfunctional status, diverging from recently proposed hepcidin as a marker of septic shock,[62] to cytokines, as interleukin-18 was connected with cardiovascular inflammation after COVID-19,[63] or chemokines.[64,65] As we showed the role of CN-NFAT signaling in proper neutrophil response to pathogens, its impact in septic conditions should be considered. We took advantage of the access to samples from the septic patients' cohort, in which we have recently focused on the dysfunctional activation of PMNs, and reanalyzed published RNAseq data.[21] This allowed us to show that genes identified using CN-NFAT inhibitors as NFAT dependent in our experimental setup were also expressed by PMNs during sepsis. By correlating the expression of selected genes with the SOFA score of septic patients, we showed the relevance of NFAT-dependent genes in septic conditions and its possible connection with sepsis severity. We showed that the numbers of normalized counts of *CSF1* correlated with the SOFA score of septic patients and that *CCL4*-normalized counts correlated negatively with the SOFA score of septic survivors, while *CCL3* showed the opposite trend in deceased patients. These findings are even more clinically relevant considering that CCL3 was shown to be essential to the host resistance even against bacterial sepsis.[64] Furthermore, CCL3 and CCL4 are important biomarkers in sepsis.[65] CCL4 serum levels were shown to be a predictive marker of good prognosis of pediatric septic shock patients, as its serum levels of 140 pg/mL or less, when obtained within 24 h of admission, predicted a very high likelihood of survival in pediatric septic shock.[66] CCL4 was also one of the supposed biomarkers to discriminate children with sepsis from nonseptic disease conditions (e.g. clinical malaria and other febrile conditions).[67] Moreover, CCL4 and CCL2 plasma levels significantly differ in 2 groups of septic patients with and without bacteremia.[68]

In conclusion, we have provided evidence that the CN-NFAT signaling pathway is necessary for the proper response of human neutrophils to opportunistic fungal pathogens. When neutrophils were treated with CN-NFAT inhibitors, the profound changes in gene expression, including genes for CCLs, caused impairment of neutrophils' ability to chemoattract other immune cells. Alongside with previously published data by Greenblatt et al.[8] using mice neutrophils and others describing the role of NFAT in myeloid cells, our article amends knowledge about the NFAT role in human neutrophils. These findings have high clinical relevance, helping to understand the impact of CN-NFAT inhibitors on the immune system and suggesting that the high susceptibility of CN-NFAT inhibitor–treated patients to opportunistic infections is caused by impairment of myeloid cell functions due to decreased NFAT activity. Another clinical consequence of the presented data can be found under septic conditions, as NFAT-dependent genes were expressed by PMNs from septic patients, and some of them correlated with sepsis severity. Our data revealed the vital role of CN-NFAT signaling in the functionality of neutrophils. The negative effects of CN-NFAT inhibitors should be more seriously considered within their clinical applications. In sum, we showed that the therapeutic CN-NFAT inhibition impacts important functions of the neutrophils with a potential impact on increased susceptibility to infections in these vulnerable patients.

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Author contribution

O.V. designed and performed the experiments, analyzed primary and aggregated data, and wrote the manuscript. I.P. and V.B. performed an analysis of RNAseq data and their interpretation. K.B. and I.A. provided methodic support. M.H.-K. designed experiments, analyzed aggregated data, provided methodical support and secured funds, and wrote the manuscript. G.V. and T.Z. performed some experiments, analyzed primary data, and provided methodic support. J.F. conceived and supervised the project, designed experiments, analyzed aggregated data, secured funds, and wrote the manuscript. All authors revised the manuscript.

Supplementary material

Supplementary materials are available at *Journal of Leukocyte Biology* online.

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Conflict of interest

The authors declare no conflict of interest.

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