Systems Immunology Approaches to Understanding Immune Responses in Acute Infection of Yellow Fever Patients

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Highlights

- PBMC transcriptome analysis in acute YFV highlights different immune responses between survivors and deceased patients.
- Decreased expression of HLA class II genes in dendritic cells of deceased YFV patients indicates a critical impairment in antigen presentation and innate immunity.
- Elevated B cell activation markers, such as BLNK and TNFRSF13B, in fatal YFV cases suggest an overactive, potentially dysregulated B cell response.
- Increased expression of neutrophil-related genes, including DEFA1B and MMP9, in deceased YFV patients highlights neutrophils' role in aggravating inflammation and tissue damage.

No conflict of interest declared

Abstract

In the 2018 yellow fever (YF) outbreak in Brazil, we generated new transcriptomic data and combined it with clinical and immunological data to decode the pathogenesis of YF. Analyzing 79 patients, we found distinct gene expression patterns between acute YF, other viral infections, and the milder YF-17D vaccine infection. We identified a critical role for low-density, immature neutrophils in severe outcomes, marked by the downregulation of genes essential for neutrophil migration and maturation, such as PADI4, CSF3R, and ICAM1, in deceased patients. Our study also revealed complex interactions among inflammation-related genes, including increased CXCL10 and IL1R2 expression and decreased IL-1b expression in the acute phase. The diminished expression of HLA class II genes indicates impaired antigen presentation. These findings highlight the delicate balance of immune responses in YF pathogenesis and lay the groundwork for future therapeutic and diagnostic advancements.

Keywords

PBMC transcriptome; yellow fever infection; system biology analysis.

Introduction

Yellow Fever (YF) is a significant public health concern in tropical regions of Africa and South America, accounting for an estimated 29,000 to 60,000 deaths annually (Garske et al. 2014; Paules and Fauci 2017; Song and Carneiro D’Albuquerque 2019). The disease can manifest as a spectrum of clinical presentations, from self-limiting, flu-like symptoms to severe forms characterized by hemorrhage and liver disease. Johansson et al. estimated that for every reported death, there are approximately 21 other infections, comprising 12 asymptomatic cases, 8 mild cases, and 1 severe but non-fatal case (Johansson, Vasconcelos, and Staples 2014).

The host immune response significantly influences disease severity. Studying immune modulators during early stages can identify markers linked to disease processes. These markers help pinpoint individuals at higher risk of severe outcomes (Fradico et al. 2023; van de Weg et al. 2023). The pathogenesis of YF involves various immune cells—including CD4$^+$ T cells, CD8$^+$ T cells, B cells, NK cells, macrophages, and antigen-presenting cells—alongside a complex interplay of pro-inflammatory and anti-inflammatory cytokines such as TNF, IFN-γ, and TGF-β (Pulendran 2009; Quaresma et al. 2013). Prior investigations have highlighted certain immune markers in response to Yellow Fever Virus (YFV) natural infection, such as increased hepatocyte apoptosis and elevated TNF-α and IFN-γ levels in the liver of fatal cases (Quaresma et al. 2013). During the Guinea epidemic, significantly higher serum levels of cytokines, including CXCL8, TNF-α, CCL2, IL-6, CXCL10, and IL-1Ra, were observed in fatal YF cases compared to survivors, a pattern echoed in subsequent studies (T. D. Querec et al. 2008; ter Meulen et al. 2004; Meier et al. 2009; Campi-Azevedo et al. 2012).

Our understanding of the global molecular mechanisms underlying the host immune response to YFV infection remains limited. This knowledge gap is primarily due to the reliance on systems vaccinology studies focusing on YF vaccines. These live-attenuated virus vaccine investigations have provided insights into general as well as YF-specific cytokines and cell types involved in the immune response to a mild infection. Studies have shown that vaccination with the YF vaccine can induce a polyfunctional human memory CD8$^+$ T cell response, activate multiple Toll-like receptors on dendritic cells, and stimulate the production of a range of pro-inflammatory cytokines (Akondy et al. 2009; T. D. Querec et al. 2009; Pulendran 2009). However, these findings, derived from a controlled vaccine response, may not fully capture the complexity of the immune response to natural wild-type YFV infection.

This study presents the first blood transcriptomic data from patients with acute YFV infection. We employed an integrative approach, combining molecular signatures with clinical and laboratory data. This strategy enabled a nuanced examination of immune response dynamics, distinguishing between the outcomes for survivors and those who succumbed to the disease. Our analysis connected gene expression patterns with severity markers, notably the Model for End-Stage Liver Disease (MELD) score, which evaluates liver function decline. We uncovered gene signatures indicative of YFV immunopathogenesis, such as the upregulation of genes driving the antiviral interferon response and plasma B cell differentiation and the downregulation of genes essential for antigen presentation in deceased patients. We identified specific gene signatures linked to YFV pathogenesis and highlighted the potential role of low-density neutrophils in severe outcomes. Additionally, we compared immune responses to YF natural infection with those triggered by other viral infections, providing new insights into the unique aspects of YFV infection. This study advances our understanding of YF and suggests potential avenues for therapeutic interventions.

Materials and Methods

Sample collection, ethical approval, and study design

On Jan 10, 2018, a referral system was established where patients with suspected yellow fever were admitted to one of two participating institutions: the Hospital das Clínicas, University of São Paulo, and the Infectious Diseases Institute “Emilio Ribas” (both located in São Paulo, Brazil). Patients older than 18 years admitted to the hospital with fever or myalgia, headache, arthralgia, oedema, rash, or conjunctivitis were consecutively screened for inclusion in the present study (Supporting Information: Table S3). The study received ethical approval from the ethical review boards at the Institute of Infectology "Emilio Ribas" and Hospital das Clinicas, University of São Paulo (CAPPesq: 15477; CAAE: 59542216.3.1001.0068). All patients provided consent for participation, or their legal representatives did so when required, before being included in the study. Confidentiality of patient identifiable information was strictly maintained during the study. Consenting patients were included if they had travelled to geographical areas where YFV cases had been previously confirmed. Patients were confirmed to be infected by real-time PCR by detecting the YFV RNA in blood samples collected at hospital admission or tissues at the autopsy. All cases included are primary yellow fever infections, with no previous history of flavivirus infections reported. No laboratory testing was performed to confirm prior exposure to other flaviviruses (Dengue virus, Chikungunya virus, or Zika virus); the information relies solely on self-reports from each patient that consisted in not or unknown previous other flaviviruses infection. Suspected cases that tested negative for YFV RNA in blood had their diagnosis confirmed when tissue was positive for YFV RNA and presented pathological findings compatible with the disease. All patients were followed until death or for 60 days after enrollment, whichever occurred first. Peripheral blood mononuclear cells (PBMCs) were obtained by a Ficoll-Paque density gradient technique (Sigma Aldrich, St Louis), frozen, and stored at liquid nitrogen until use. The samples collected at admission (acute phase of infection) and more than 30 days post onset symptoms (convalescent phase) were submitted to load RNA virus (as described by (Kallas et al. 2019)) and RNA sequencing.

Clinical data and standard laboratory test

The clinical data and standard laboratory tests were collected and analyzed at clinical laboratories located at the Hospital das Clínicas, School of Medicine, University of São Paulo, and the Infectious Diseases Institute “Emilio Ribas”. Demographic and clinical data were age, sex, race, duration of symptoms upon admission, oliguria, bleeding, lethargy, somnolence, muscle pain, nausea, abdominal pain, fever, headache, jaundice, coluria, vomiting, pain in joints, chills, diarrhea, pain behind the eyes, cough, arthritis, shaking, dyspnea, itching, sore throat, rash, coryza, conjunctivitis, and shock. The following laboratory tests were done at admission: prothrombin time (PT), international normalised ratio (INR), activated partial thromboplastin time (aPTT), creatinine, urea, hematocrit, haemoglobin, erythrocytes, indirect bilirubin, direct bilirubin, aspartate transaminase (AST), alanine aminotransferase (ALT), and c-reactive protein (CRP). The MELD score was determined using bilirubin, INR, and creatinine values for each patient. The formula used for the score was $\mathrm{MELD} = 3.78 \times \ln(\mathrm{bilirubin}) + 11.2 \times \ln(\mathrm{INR}) + 9.57 \times \ln(\mathrm{creatinine}) + 6.43$ (Singal and Kamath 2013). We used the Spearman method to determine the correlation between two variables, considering $p\text{-value} < 0.05$ as correlated pairs. A nonparametric Wilcoxon test was applied to calculate differences between each group of samples. A proportion test was utilized to evaluate the differences in symptom prevalence among all YF patients.

Cytokine profiling

Serum/plasma samples obtained from blood of the patients were used to quantify circulating cytokines. We performed a custom 25-cytokines Milliplex Map Human TH17 Magnetic Bead Panel, immunoassay using polystyrene beads based on Luminex technology, used as recommended by the manufacturer. The 25 target cytokines were: Interleukin (IL), IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A, IL-17F, IL-17/IL-25, IL-21, IL-22, IL-23, IL-27, IL-28A, IL-31, IL-33, granulocyte macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein (MIP) 3α/CCL20, interferon (IFN)-γ, TNF-α and TNF-β. Analytes were quantified using a MAGPIX analytical test instrument, which utilizes xMAP technology (Luminex Corp., Austin, TX), and xPONENT 4.2 software (Luminex). A nonparametric Wilcoxon test was used to assess differences between the sample groups. Pearson's correlation method was applied to evaluate the correlation between two cytokines, with p-values < 0.05 indicating significant correlations.

Bulk samples preparation and RNA sequencing

RNA from PBMCs was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany), quantified using Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific), and integrity checked in a 2200 TapeStation system using RNA ScreenTape (Agilent Technologies, Santa Clara, CA). The cDNA libraries were constructed using the QuantSeq 3' mRNA-Seq Library Prep Kit for Illumina FWD (Lexogen GmbH, Austria), following the manufacturer's protocol. The concentration and the median size of the libraries were assessed by 2200 TapeStation with a DNA1000 ScreenTape (Agilent). The final pool of libraries was quantified by qPCR using the Kapa Sybr Green qPCR Kit (Roche Diagnostics, Mannheim, Germany) and subjected to a single-end sequencing (75 bp) in a NextSeq 500 Sequencing with NextSeq 500/550 High Output Kit v2.5 (Illumina, San Diego, CA).

Quality control, mapping, and annotation processes

Raw single-end reads were preprocessed for quality control. Sequencing quality was assessed before and after adapter trimming using the program FastQC (Andrews 2010). The Trimmomatic software version 0.39 (Bolger, Lohse, and Usadel 2014) was used to remove adapters sequence trimming the 5′, and 3′ ends with a mean quality score below 25 (Phred+33) and discard reads shorter than 36 bp after trimming. We used the parameters “LEADING:3 TRAILING:3 SLIDINGWINDOW:4:20 MINLEN:36”. After preprocessing, the high-quality reads were mapped into the reference genome Homo sapiens (version GRCh38) with the bowtie2 program (version 2.2.5) (Langmead and Salzberg 2012). To quantify the gene abundance of the mapped reads in each sample, we used the featureCounts tool from the R/Bioconductor package Rsubread (Liao, Smyth, and Shi 2019) with the following parameters: GTF.featureType = “gene”, GTF.attrType = “gene_id”, isPairedEnd = FALSE, minOverlap = value 1, allowMultiOverlap = FALSE, countMultiMappingReads = FALSE. Normalization of the gene counts was performed with counts per million normalization (CPM) and quantile normalization, which accounts for differences in library size.

Molecular degree of perturbation and gene co-expression module

Sample quality and the molecular degree of perturbation (MDP) between groups were assessed with the R/Bioconductor package MDP (Gonçalves et al. 2019), using the normalized gene expression for survivor and non-survivor at the acute phase, which was compared to the convalescent group. A nonparametric Wilcoxon test was used to assess sample MDP scores between patients in the convalescent phase and between deceased and surviving patients.

RNA-seq Differentially Expressed Genes Analysis

The identification of differentially expressed genes (DEGs) between groups was carried out with the R/Bioconductor package EdgeR (Robinson, McCarthy, and Smyth 2010; McCarthy, Chen, and Smyth 2012), using independent 2-class t-tests. P values were submitted to a false-discovery rate (Benjamini-Hochberg procedure) correction, and the statistical significance was defined by the criteria, adjusted p-value ≤ 0.05 and fold-change > 2.

Pathway Enrichment Analysis

Regarding the pathways that may be related to the progression of the disease, a Gene Set Enrichment Analysis (GSEA) was performed using as ranks of the acute vs. convalescent and deceased vs. survivor comparison. A set of Blood Transcriptional Modules (BTM), previously identified by our group (Li et al. 2014) through large-scale network integration of publicly available human blood transcriptome, were used as the gene sets.

Co-expression analysis

We identified and analyzed co-expression modules using the R/Bioconductor package CEMiTool (Russo et al. 2018). We used the log2CPM normalized gene expression matrix to identify the gene co-expression modules. The CEMiTool default parameters were followed. The biological meaning of the co-expression modules was explored by performing additional analysis with CEMiTool, the GSEA, the Over-Representation Analysis (ORA), and the network analysis. The GSEA was conducted to identify the induced and repressed modules in the three phenotypes: 1) patients in the acute phase deceased; 2) patients in the acute phase survived; and 3) convalescent patients. To identify the gene sets enriched in each module, we performed an ORA providing the Blood Transcriptional Modules (BTM) (Li et al. 2014) as gene sets. We provided Protein-Protein Interaction (PPI) data as a gene interactions file to build the network.

Cell proportion estimation

The cell proportion was estimated using the ABsolute Immune Signal (ABIS) deconvolution tool (Monaco et al. 2019) and 29 human immune cell types were characterized by RNA-seq and flow cytometry. A Spearman's rank correlation coefficient > 0.5 was used to associate cell types with clinical parameters.

Virus-related published infection studies

For the Dengue virus infection, we obtained differentially expressed gene tables from GEO id number GSE94892 processed by (Banerjee et al. 2017). Peripheral blood mononuclear cells (PBMCs) in clinically and virologically well-characterized patients with mild and severe dengue infection were used for Illumina HiSeq 2500 sequencing. The authors defined the group of samples A, B, and C, corresponding to Dengue Fever, Dengue Haemorrhagic Fever, and Dengue Shock Syndrome, respectively. The DEGs were identified with an adjusted p-value < 0.05 and fold-change > 2. We analyzed and published a bulk RNA sequencing for CHIKV patients' infection, and we used them (Bioproject number PRJNA507472) for viruses' comparison. Blood samples were collected from subjects reporting arbovirus-like symptoms, and total RNA was extracted and sequenced using the HiSeq Illumina platform. The gold standard bulk sequencing pipeline was used as described in the publication (Soares-Schanoski et al. 2019). The DEGs were identified with an adjusted p-value < 0.05 and fold-change > 2. The COVID-19 study was obtained from (Xiong et al. 2020) under the accession number CRA002390. The DEGs were provided in the supplementary data from the author's paper. Peripheral blood mononuclear cells in COVID-19 patients were sequenced in the MGISEQ-2000 platform (MGI, Shenzhen, P. R. China). The DEGs followed the criteria adjusted p-value < 0.05 and fold-change > 2.

Results

Clinical Outcomes and Biochemical Markers in Yellow Fever Infection

We recruited 81 patients acutely infected with the YFV (Figure 1A). Among these patients, 26 died from the disease within the first 30 days of symptoms (32.9%), and 53 survived (67.1%). We excluded two patients (2.5%) who died after 30 days of post-onset symptoms from the analyses. Seventy-nine participants were included in the final analysis. Blood samples were collected during the acute phase (between 3 and 16 days after the onset of symptoms). Additionally, we collected blood samples from surviving patients during the convalescent phase (more than 30 days post-symptom onset), referred as "non-infected controls" (Figure 1A).

Patients presented diverse symptoms; however, none demonstrated a statistical association with mortality (Figure S1). Analysis of blood parameters during the acute phase revealed pronounced liver injury (elevated levels of AST, ALT, indirect and direct bilirubin), renal impairment (increased creatinine and urea), and coagulopathy (increased PT, INR, and aPTT) compared to non-infected control patients (Figure 1B) (similar findings reported by (Kallas et al. 2019; Bailey et al. 2020)). Levels of AST were higher in deceased patients in the initial days post-onset of symptoms compared to survivors, while indirect bilirubin levels were higher in later days post-onset symptoms (Figure 1C). C-reactive protein (CRP) levels and YFV viral load did not significantly differ between survivors and deceased patients (Figure 1D). To assess the severity of these patients, we calculated a Model for End-Stage Liver Disease (MELD) score based on levels of bilirubin, INR, and creatinine (Malinchoc et al. 2000). As expected, deceased patients exhibited a higher MELD score compared to convalescent patients and survivors (Figure 1D).

Dissecting the Transcriptional Landscape of YFV infection

To better understand the molecular mechanism of the disease, we first investigated the blood transcriptome of deceased and surviving patients during acute and convalescent phases. Initially, we calculated the molecular degree of perturbation (MDP score) of each sample (Gonçalves et al. 2019) to assess the molecular heterogeneity of the disease. At the transcriptome level, we found that deceased patients had higher MDP scores when compared to survivors (Figure S2A). Interestingly, the MDP score correlated with the MELD score, AST levels, and INR (Figure S2B-D), but not with viral load or CRP (data not shown).

Next, we identified gene signatures correlated with each one of the three factors associated with the immunopathogenesis of YFV infection analyzing the MDP scores of surviving and deceased patients (Supporting Information: Table S1). This information was taken from the Human Gene Atlas (Su et al. 2002) and we filtered each signature for highly expressed genes in six immune blood cell populations. As expected, transcription factors that mediate cellular responses to interferons, such as STAT1, STAT2, and IRF7, were associated with viral load across different cell types (Figure 2 and Figure S3). Interestingly, however, the MELD score was negatively correlated with HLA class II genes (HLA-DRA, HLA-DPA1, HLA-DMB, HLA-DPB1) in dendritic cells (Figure 2), suggesting an impairment in the innate immune system of patients with greater disease severity. Similarly, in CD8$^+$ T cells, patients with a higher MELD score exhibited lower levels of the CCL5 gene (RANTES), which plays a crucial role in homing and migration of effector and memory T cells during acute infections, and ITGAL (CD11A), which potentially regulates effector CD8$^+$ T cell activation and differentiation as well as antigen-specific central memory development in response to infection (Figure 2).

We further detailed the impact of global expression changes caused by YFV infection, we performed a differential expression analysis between patients in the acute phase of the disease and those in the convalescent phase (Supporting Information: Table S2). We also compared the expression profile of patients who died from the disease to those who survived (Supporting Information: Table S2). We noticed that many genes were differentially expressed (DEGs) in both analyses (Figure 3A). Several genes encoding proteins related to the antiviral interferon response and the differentiation of B cells into antibody-secreting cells had increased expression in the acute phase of the disease and in patients who died from YF (Figure 3B and 3C). On the other hand, genes associated with antigen presentation, such as CD74 and several HLA class II genes, had decreased expression in patients who died from the disease (Figure 3C). Although the gene encoding the pro-inflammatory protein CXCL10 (IP10) was increased in the acute phase of the disease and associated with death, the gene encoding IL-1b had decreased expression (Figure

3A). The gene encoding the IL1R2 receptor, which binds to IL-1 and acts as a decoy receptor that inhibits the activity of IL-1 ligands, was also increased in the acute phase and in patients who died from YF. We validated these findings using Luminex plasma assays to measure IL1B and IL6 levels across different disease stages. IL1B plasma levels were significantly lower in deceased and survivor patients compared to individuals in the convalescent phase (Figure 3D). Although IL6 levels were not significantly altered between groups (Figure 3E), a significant positive correlation between IL6 and IL1B was found in deceased patients (R = 0.45, p = 0.025), but not in survivors or convalescent individuals (Figure 3F). These data suggest that the role of inflammation and inflammasome-related pathways (or its suppression) during the first days of symptoms may be crucial to the outcome of the disease.

Potential Involvement of Low-Density Neutrophils in Yellow Fever Pathogenesis

Co-expressed genes are often functionally related or involved in the same biological process (Simone et al. 2021). We used CEMiTool to identify modules of genes with similar expression patterns (Russo et al. 2018). Among the 8 modules identified, we found that the activity of modules M5 and M6, which respectively contained genes related to plasma B cells and type I interferon response, was increased in deceased patients when compared to those who had already recovered from the disease (Figure S4A and S4B). In contrast, the activity of modules M2 and M3, which contained genes related to TLR signaling and inflammation, was decreased in patients who died from YF (Figure S4A and S4B). Within module M3 (Figure S4C), the TLR2 gene (which was not differentially expressed in any comparison) was identified as a hub in the co-expression network. It has been shown that the live-attenuated YFV strain of the YF-17D vaccine can activate Dendritic Cells through TLR2 to elicit the production of pro-inflammatory cytokines and IFN-a (T. Querec et al. 2006).

An interesting hub in module M2 was PADI4, which was also significantly down-regulated in deceased patients compared to survivors and convalescent patients (Figure S4C). PADI4 encodes one of the enzymes that convert arginine to citrulline residues. This protein promotes the decondensation of chromatin during the innate immune response to infection in neutrophils, being essential for the formation of neutrophil extracellular traps (NETs). Additionally, CSF3R and ICAM1 (are down-regulated in deceased patients compared to survivors) are also present in module M2. Both genes encode proteins essential for the adhesion, migration, and development of mature neutrophils (Yang et al. 2005; Dwivedi and Greis 2017). Although the role of neutrophils in severe YFV infection remains unclear (Fontoura, Rocha, and Marques 2021), our findings suggest that these cells play an essential role in the pathogenesis of the disease.

Our bulk RNA-seq data represents the transcriptome of a mixture of peripheral blood mononuclear cells (PBMCs). In the density gradient centrifugation method, high-density granulocytes (neutrophils, basophils, and eosinophils) are removed from the low-density fraction of PBMCs. However, low-density neutrophils, which are considered immature forms of neutrophils, can often be found in the PBMC fraction (Blanco-Camarillo, Alemán, and Rosales 2021) and are increased in certain pathological conditions such as sepsis, chronic infections, and autoimmune diseases (Schenz et al. 2021). In order to investigate the role of this type of neutrophil in the pathogenesis of the disease, we initially ran a deconvolution analysis using immature neutrophil signatures (Monaco et al. 2019) to estimate the relative frequency of this cell type in the PBMCs of YF patients. While 76% (16 out of 21) of YF patients who died from the disease had estimated immature neutrophil levels above 1%, this level was only seen in 50% (10 out of 20) of patients who survived (Figure 4A). Comparing the estimated levels of low-density neutrophils directly, we noticed a significant increase in patients who died compared to those who survived (Figure 4B). We then analyzed complete blood count data from patients and confirmed that the levels of immature neutrophils are associated with death from the disease, as well as the levels of mature neutrophils (Figure 4C, (Kallas et al. 2019)). Taken together, these results show for the first time that low-density neutrophils may have an essential role in driving NET production and inflammation in severe disease.

Comparative Analysis of Transcriptome Changes in Acute YFV Infection and Other Viral Infections

In addition, we investigated whether the signatures associated with death from YF could also be related to other viral infections. To do this, we compared the list of 276 genes with increased or decreased expression in the blood of YF patients who died from the disease compared to the convalescent phase with the differentially expressed genes in the blood of: (1) patients infected with the Dengue virus who had or did not have shock syndrome (1,341 genes, (Banerjee et al. 2017)); (2) patients infected with the Chikungunya virus compared with healthy controls (4,957 genes, (Soares-Schanoski et al. 2019)); and (3) patients with severe COVID-19 compared with healthy controls (704 genes, (Xiong et al. 2020)). Most of the genes (161 genes, 58.3%) in the YF signatures were not shared by infection with other viruses, suggesting that most genes are specifically associated with the YF pathophysiology. As expected, several genes related to the antiviral response had increased expression in YF and other viruses (Figure 5). Similarly, key genes related to inflammation such as PTGS2 (COX2), A2M, and KLRB1 or associated with immunity like MATK and PZP had decreased expression in patients who died from YF and in patients with COVID-19 or infected with CHIKV (Figure 5).

Some genes had the opposite direction between the signatures of YF and other viruses. AKAP12 is a scaffold protein that is related to liver injury (Wu et al. 2022) and liver fibrosis (Lee et al. 2018) and showed increased expression in patients who died from YF but decreased in patients with COVID-19 or infected with CHIKV (Figure 5). Genes related to complement activation (C4B), lymphocyte homing (CCR7), or associated with immunoglobulins (FAM30A, IGHM, and IGLV10-54) (Lima et al. 2019) also had increased expression in patients who died from YF but decreased in patients infected with CHIKV (Figure 5). Among the genes with altered expression in patients who died from YF, several genes related to viral immunity had increased expression in other viruses, including RASGEF1B, ADA2, HLA-DQB2, DPYD, CD83, IL1RAP, and IVNS1ABP (Figure 5).

Our study identified unique alterations in the gene expression profiles of patients who succumbed to Yellow Fever (YF). Notably, two genes crucial for B cell development and activation - BLNK and TNFRSF13B (TACI) - showed exclusive changes in YF cases. We also observed a unique up-regulation of genes encoding hemoglobin components (HBA2, HBD, HBM) in those who died from YF. These genes may reflect precocious anemia that triggers innate immune responses by acting as damage-associated molecular patterns (Bozza and Jeney 2020). Additionally, we identified several genes exclusively up-regulated in YF cases that are key to neutrophil function. These include DEFA1B, a defensin released from specific granules upon activation to combat pathogens; MMP9, a protein involved in neutrophil migration by degrading extracellular matrix proteins; PRTN3, a serine protease that assists neutrophil migration by breaking down extracellular matrix components; and S100P, a protein that, upon release, acts as a signal to recruit and activate immune cells. Our analysis revealed unique and specific immune response pathways, both innate and adaptive, that may be associated with YF severity.

Discussion

This study investigated the clinical, biochemical, and transcriptional responses of 79 patients with acute YFV infection. We observed the expression of genes such as STAT1, IRF7, PADI4, HLA-DRA, HLA-DPA1, and CCL5, which were linked to the severity of YFV infection (T. D. Querec et al. 2008; Hou et al. 2017; Azamor et al. 2021). These genes, important for immune response and inflammation regulation (Qing and Liu 2023; Saheb Sharif-Askari et al. 2021; Tolomeo, Cavalli, and Cascio 2022), align with previously documented biochemical markers of severe liver damage, renal impairment, and coagulopathy (Bailey et al. 2020; Kallas et al. 2019; Chen et al. 2016). The early elevation of AST levels (Chen et al. 2016) and subsequent rise in indirect bilirubin levels (Wouthuyzen-Bakker et al. 2017) in deceased patients suggest a pattern of organ damage closely related to gene dysregulation. We also identified a potential impairment in the immune system's antigen presentation capabilities, indicated by the negative correlation between the MELD score and HLA class II gene expression in dendritic cells. This may reflect viral modulation of host immune functions (Lin et al. 2022) or inefficient presentation of viral antigens by certain HLA molecules (Augusto et al. 2023). The observed decrease in genes crucial for T cell migration and memory, such as CCL5 and ITGAL, in cases with higher MELD scores (Lim et al. 2010), underscores disrupted immune processes critical for infection response.

Our findings on systemic inflammation during YFV infection revealed no elevation of key markers like IL-1b, IL-6, and TNF during the acute phase. Both transcriptomic and cytokine profiling confirmed a decrease in IL-1b, highlighting its role in modulating the immune response. Lower levels of IL-1a, IL-1b, and IFN-γ in deceased patients, along with a significant elevation in IL1R2, a decoy receptor that inhibits IL-1, suggest a dysregulated immune response contributing to disease severity. In contrast, a study by Pelletier et al. (Pelletier et al. 2021) identified a substantial upregulation of pro-inflammatory cytokines, including IFNα2a, IFN-β, IL-6, and IFN-γ, in patients with severe outcomes, possibly contributing to the cytokine storm observed in such cases. These findings point to a complex immune response, where IL1R2 might play a dual role by suppressing essential inflammatory responses, allowing viral persistence while modulating inflammation. Such regulatory mechanisms, although protective against excessive inflammation, might undermine effective viral control (Casanova and Abel 2021). This balance between pro-inflammatory and regulatory responses emphasizes the need for therapeutic strategies targeting both immune suppression and enhancement.

Interleukin-27 (IL27) was another key cytokine upregulated in YF-infected patients. Known for its anti-inflammatory effects on Th17 cells (Yoshida and Hunter 2015), IL27 may modulate immune responses against YFV by inhibiting Th17-driven inflammation (Jouhault et al. 2023). It has also been shown to activate innate antiviral proteins, offering protection against flavivirus infection (Kwock et al. 2020).

We also connected YFV pathogenesis to the increase of low-density neutrophils, commonly seen in conditions like sepsis and chronic infections (Tay, Celhar, and Fairhurst 2020; Sun et al. 2022; Dumont et al. 2024). These immature neutrophils, released during emergency granulopoiesis, exhibit impaired functions, leading to vascular inflammation and potentially contributing to the severe complications observed in fatal cases of YFV (Galis and Khatri 2002; Trivioli et al. 2022). YFV-infected patients exhibited distinct transcriptional signatures compared to those infected with dengue, Chikungunya, and COVID-19, indicating that YFV triggers unique immune pathways.

In this study, we acknowledge that our cohort was exclusively male, with an average age of 42 years, limiting the generalizability of our findings across genders and age groups. Although we have observed a primary YFV infection and unique transcriptome profile, the study lack of possible co-infection with other flaviviruses (such as Dengue, Chikungunya, and Zika viruses) measured by molecular techniques. The relatively small sample size may have reduced the statistical power of the analysis. Additionally, we were unable to collect samples from individuals without YFV exposure as endemic controls, so we used convalescent-phase patients as controls to minimize inter-group variability. It is important to emphasize that our transcriptomic analysis primarily serves as a screening tool, identifying gene expression patterns but not fully elucidating the underlying mechanisms. Bulk sequencing, while useful for identifying general immune responses, may not capture the full heterogeneity of immune cell populations. Single-cell sequencing, which provides a more detailed view of immune cell dynamics, represents an important direction for future research.

In conclusion, this study presents a comprehensive integrative analysis, combining molecular data with clinical and laboratory parameters to identify novel molecules that may play crucial roles in acute YFV infection. These findings could serve as markers for therapeutic targets or for evaluating disease severity in future YFV outbreaks.

Author contributions

Conceptualization, A.N.A.G., P.R.C., E.G.K., and H.I.N.; Data curation, A.N.A.G.; Formal Analysis, A.N.A.G., F.M.M., V.E.M., F.M.F., J.D.A.A., A.P.V., P.C.G.D.C., and H.I.N.; Funding acquisition, E.G.K., and H.I.N.; Investigation, A.N.A.G., R.P.S., C.O.M., J.G.A.C.R., D.M.F., E.G.K., and H.I.N.; Methodology, A.N.A.G., P.R.C., M.V.T., C.A.C., M.P.M., J.Z.C.D., C.G.T.S., L.G.F.A.B.E.Z., A.M., N.B.C., C.H.V.M., R.B., A.C.F., V.E.M., F.M.F., J.D.A.A., A.P.V., P.C.G.D.C., E.G.K., and H.I.N.; Project administration, E.G.K.; Resources, A.N.A.G., P.R.C., M.V.T., C.A.C., M.P.M., J.Z.C.D., .C.G.T.S., L.G.F.A.B.E.Z., A.M., N.B.C., C.H.V.M., R.B., A.C.F., E.G.K., and H.I.N.; Software, A.N.A.G., and H.I.N.; Supervision, E.G.K., and H.I.N.; Validation, A.N.A.G., P.R.C., M.V.T., C.A.C., M.P.M., J.Z.C.D., C.G.T.S., L.G.F.A.B.E.Z., A.M., N.B.C., C.H.V.M., R.B., A.C.F., V.E.M., and H.I.N.; Visualization, A.N.A.G., V.E.M., F.M.F., J.D.A.A., A.P.V., P.C.G.D.C., and H.I.N.; Writing – original draft, A.N.A.G., F.M.M., and H.I.N.; Writing – review & editing, A.N.A.G., .P.R.C., .M.V.T., C.A.C., M.P.M., J.Z.C.D., C.G.T.S., L.G.F.A.B.E.Z., A.M., N.B.C., C.H.V.M., R.B., A.C.F., F.M.M., V.E.M., F.M.F., J.D.A.A., A.P.V., P.C.G.D.C., R.P.S., C.O.M., J.G.A.C.R., D.M.F., E.G.K., and H.I.N.

Data availability statement

The complete set of raw sequences for each assay generated in the HiSeq Illumina 1500 were deposited at the NCBI through the BioProject: PRJNA1017409 and the BioSample Range SAMN37395281 to SAMN37395335. The raw and normalized count matrix were deposited at the NCBI GEO through accession number: GSE243442.

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Declaration of interests

The authors declare no competing interests.

No conflict of interest declared

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Figures legend

Graphical abstract

No conflict of interest declared

Figure 1. Patient Sample Distribution and Laboratory Indicators. (a) Distribution of deceased (orange dots) and survivors (purple dots) during the acute phase of YFV infection, indicating which assays were performed or data collected at each time point: transcriptome, symptoms, laboratory tests, and viral load measurement. The date of death is indicated by a red cross. Additional samples from surviving patients, collected post-recovery, are denoted as convalescent samples (blue dots), with a pink triangle indicating the hospital discharge date. Viral loads are represented by squares on the left, in copies per mL. (b) Fold-change in deceased and surviving patients' laboratory parameter values relative to those in convalescent samples. Parameters are related to bleeding (PT [prothrombin time], INR [international normalized ratio], aPTT [activated partial thromboplastin time]), renal insufficiency (urea, creatinine), anemia (hematocrit, hemoglobin, erythrocytes), liver dysfunction (total bilirubin, indirect bilirubin, direct bilirubin), and hepatic injury (ALT [alanine transaminase], AST [aspartate aminotransferase]). (c) AST and indirect bilirubin levels for deceased and surviving patients compared with convalescent patients during the first 14 days post-symptom onset. (d) CRP levels, viral load, and MELD (Model for End-Stage Liver Disease) score for each patient

outcome, for both non-infected and infected (deceased and survivor) patients. A nonparametric Wilcoxon test was applied to calculate differences between each group of samples.

Figure 2. Clinical Parameter-associated Signatures in Plasmacytoid Dendritic Cells and CD8$^+$ T Cells. The network was constructed using the correlation between gene expression (normalized to patients in the convalescent phase) and MELD score, C-reactive protein (CRP), or viral load (VL) values. Correlated genes (Spearman's rank correlation coefficient > 0.5) that are also expressed in dendritic cells or CD8$^+$ T cells according to the Human Gene Atlas were selected as nodes. The red line indicates a positive correlation, and the blue line indicates a negative correlation between genes (gray circles) and parameters (black rectangle).

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Figure 3. Transcriptomic Insights into Acute Infection and Disease Outcome. (a) Relationship between differentially expressed genes in deceased patients versus surviving patients and those during the acute phase versus the convalescent phase. Circles represent the log2 fold-change of genes that were up-regulated (red) or down-regulated (blue) in both comparisons. (b) Gene Set Enrichment Analysis (GSEA) using the log2 fold-change and adjusted p-value as rank and Blood Transcription Module (BTM) as gene sets. Circles represent the NES (Normalized Enrichment Score) of BTMs that were enriched with up-regulated (red) or down-regulated (blue) genes. (c) Network of antigen presentation (I) (M71), antiviral IFN signature (M75), and plasma cells and B cells, immunoglobulins (M156.0) gene sets. The edges of the BTM network were defined in our previous publication (Li et al. 2014). Boxplots displaying (d) IL1B and (e) IL6 plasma protein levels (pg/mL) in deceased (orange dots), survivors (purple dots), and convalescent individuals (blue dots). Differences between groups were assessed using the non-parametric Wilcoxon test. P-values are shown for significant comparisons. (f) Correlation between IL1B and IL6 plasma levels in deceased, survivor, and convalescent patients. A scatter plot shows IL1B vs IL6 levels, with different groups highlighted: deceased (orange), convalescent (blue), and survivors (purple). Pearson's correlation coefficient (R) and p-values are indicated for each group. The shaded regions represent the 95% confidence interval for the linear model fit to the data.

Figure 4. Levels of low-density neutrophils are increased in deceased YF patients. We used the ABsolute Immune Signal (ABIS) deconvolution tool (Monaco et al. 2019) to estimate the frequency of low-density neutrophils in the PBMC RNA-seq dataset. (a) Number of patients who survived or died from YF with ABIS fraction for low-density neutrophils over 1% (black). (b) ABIS fraction for low-density neutrophils estimated in patients who survived (purple) or died (orange) from the disease. (c) Number of immature neutrophils and neutrophils from Complete

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Blood Count (CBC) analysis of patients who survived (purple) or died (orange) from the disease. The Wilcoxon test was used to assess the statistical significance shown in each plot.

Figure 5. Comparing signatures associated with infection by Yellow Fever, Dengue, Chikungunya or SARS-CoV-2. The nodes represent the genes that were up-regulated (red nodes, top part) or down-regulated (blue nodes, bottom part) in patients who died of Yellow Fever compared with those who survived during the acute phase of the disease and who were also differentially expressed in infection by another virus. Red and blue edge colors indicate the genes up- or down-regulated by some virus, respectively.

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Figure S1. Summary of Symptoms in Yellow Fever Patients. The prevalence of each symptom among Yellow Fever (YF) patients was calculated as the frequency of occurrence relative to the total patient cohort. Fisher's exact test was employed to determine the prevalence disparities of symptoms between deceased patients and survivors. Additionally, a proportion test was utilized to evaluate the differences in symptom prevalence among all YF patients. No statistically significant differences were observed for any of the symptoms when comparing outcomes.

Figure S2. Assessing the Molecular Degree of Perturbation in Deceased and Surviving Patients. (a) The MDP score was calculated for each sample (black dots) using normalized expression values and outcome information, with the reference group consisting of patients in the convalescent phase. A nonparametric Wilcoxon test was used to assess sample MDP scores between patients in the convalescent phase and between deceased and surviving patients. Spearman correlation analysis comparing sample MDP score and (b) MELD score, (c) AST laboratory parameter, and (d) INR laboratory parameter. MDP (Molecular Degree of

Perturbation); MELD (Model for End-Stage Liver Disease); AST (Aspartate Aminotransferase); INR (International Normalized Ratio).

Figure S3. Signatures associated with Clinical Parameters in Immune Cell Populations. The network was constructed using the correlation between gene expression (normalized to patients in the convalescent phase) and MELD score, C-reactive protein (CRP), or viral load (VL) values. Correlated genes (Spearman's rank correlation coefficient > 0.5) that are also expressed in Natural Killer cells, B cells, monocytes, or CD4$^+$ T cells according to the Human Gene Atlas were selected as nodes. The red line indicates a positive correlation, and the blue line indicates a negative correlation between genes (gray circles) and parameters (black rectangle).

Figure S4. Modular Co-expression Analysis of YFV Infection. (a) Gene Set Enrichment Analysis (GSEA) of the 8 co-expression modules identified by CEMiTool in patients in the convalescent phase and in acute infection who either survived or died from the disease. The size and color are proportional to the Normalized Enrichment Score (NES). The rankings used in the GSEA analysis were obtained through z-score normalization per gene, followed by averaging the values across the 3 different groups. (b) Over-representation analysis between genes from each module and genes in the BTMs. An adjusted P-value cut-off of 0.05 was used. A representative BTM was selected for the bar chart. (c) Protein-protein interaction networks between genes in modules M2 and M3. Highly connected genes (hubs) are marked and were identified by the CEMiTool.