

Role of Th17 Cytokines in Liver's Immune Response during Fatal Yellow Fever: Triggering Cell Damage Mechanism

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Abstract

Yellow fever (YF) is an infectious disease whose evolution and outcome are related to the host immune response pattern. We investigated the Th17 cytokine profile in the liver of humans with fatal YF. Liver tissue samples were collected from 26 patients, including 21 YF-positive and five flavivirus-negative patients with preserved hepatic parenchyma architecture who died of other causes. Samples underwent histopathological and immunohistochemical analysis to detect the Th17 profile (ROR- γ , STAT3, IL-6, TGF- β , IL-17, and IL-23). Substantial differences were found in the expression of markers between fatal YF cases and control samples with a predominance of Th17 cytokine markers in the midzonal region of the YF cases, the most affected area in the liver acinus. Histopathological changes in the hepatic parenchyma revealed cellular damage characterised mainly by the presence of inflammatory infiltrate, Councilman bodies (apoptotic cells), micro/macrovesicular steatosis, and lytic and coagulative necrosis. Th17 cytokines play a pivotal role during YF and contribute significantly to triggering the mechanisms of cell damage in the fatal outcome of severe cases.

Summary

The role of cytokines in the Th17 profile during the immune response against yellow fever virus is to enhance tissue damage and contribute significantly to triggering the mechanisms of cell damage, crucial in the fatal outcome of severe haemorrhagic yellow fever cases.

Introduction

Yellow fever (YF) is an arthropod-borne viral disease with a high fatality rate in tropical endemic areas of Africa and South America [1, 2]. The disease is caused by the YF virus (YFV), a single-stranded positive-sense RNA virus belonging to the *Flaviviridae* family, genus *Flavivirus*, whose genome encodes three structural proteins (C, M, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [3, 4]. Originally described as a viral haemorrhagic fever in clinical presentations, it presents with more severe, pansystemic viral sepsis that compromises the function of several organs, including the liver, lung, heart, kidney, spleen, and intestine [5, 6, 7].

Infection by YFV has become a major public health problem in recent years because of the occurrence of epidemics and outbreaks, the speed of dissemination, and the route of transmission. In Brazil, from 2016 to 2019, high records of epizootics and confirmed human YF cases revealed an alarming situation and a risk of re-emergence of the disease in cities with high population density [8, 9, 10].

Regarding the mechanisms of cell injury developed by the host against YFV, in the liver, it is characterized by severe tissue damage in midzonal area with hepatocyte steatosis, apoptosis, and necrosis. In the immunopathogenesis of YFV-induced liver disease, the action of Kupffer cells, dendritic cells, CD4+ T lymphocytes (Th1 and Th2), CD8+ T cells, and NK cells triggers the production of cytokines and enzymes that accentuate oxidative stress and worsen cell damage [11, 12, 13].

Because it is a disease with a pansystemic and fulminant character, growing evidence has reinforced the fact that new subpopulations of lymphocytes are determinant to potentiate the proinflammatory response in situ and induce the recruitment of leukocytes to the site of infection [14, 15]. These cells are known as Th17 cells and produce cytokines, such as IL-17 and IL-23. In addition, during the cell formation process, cytokines such as IL-6 and TGF- β participate in the process of cellular differentiation, and transcription factors such as STAT3 and ROR γ characterise their profile signature [16, 17, 18].

In general, the Th17 profile has been shown to be an important factor in the pathogenesis of several infections and is involved in the immune response to viral, bacterial, and fungal agents [19, 20, 21]. During dengue infections, the Th17 profile seems to be involved in the pathophysiology of haemorrhagic forms of the disease [22]. However, regarding the study of immunity to YF, there is still no consistent data on the role of this cytokine profile in the pathogenesis of the disease, especially in its severe forms that can evolve with marked vascular impairment, haemorrhage, and intense liver damage.

Thus, the identification of the role of new immune factors involved in the immunopathogenesis of YF is important for understanding the evolution of the infection, identification of possible therapeutic targets, and the development of more effective vaccines without adverse effects. Therefore, this study aimed to evaluate the in-situ cytokine profile of the Th17 response in human livers in fatal YF.

Results

Histopathological analysis

Histopathological changes in the liver were characterised by the presence of evident midzonal (Z2) lesions, consisting of hepatocytes with changes ranging from swelling, macro- and microvesicular steatosis, Councilman bodies (apoptotic cells), and, to a lesser or greater extent, lytic or coagulative necrosis (Table 2, Figure 1). Other changes observed in both the acinus zones (Z1, Z2, and Z3) and the PT are presented in Table 2, highlighting the presence of hyperplasia and hypertrophy of Kupffer cells, sinusoidal congestion, acinar haemorrhage, and changes in vascular structures in the central lobular vein zone (Z3) and in the PT. These changes were accompanied by acinar inflammatory infiltrate consisting of lymphocytes, plasma cells, and moderate neutrophils, especially around foci of moderate intensity lytic necrosis, congestion, haemorrhage, and tissue damage (Table 2).

Table 2

Morphological changes to the hepatic acinus (Z3, Z2, Z1) and in the portal tract (PT) in fatal cases of humans YFV-induced.

Morphological changes	Z3	Z2	Z1	PT
Cell swelling	2++	+++	+++	-
Macrovesicular steatosis	+	++	+	-
Microvesicular steatosis	++	+++	++	-
Lytic necrosis	+	++	++	-
Coagulative necrosis	+	+	+	-
Councilman bodies	+++	+++	+++	-
Kupffer cell hyperplasia	+	++	++	-
Kupffer cell hypertrophy	+	++	++	-
Edema	-	-	-	++
Congestion	-	-	-	++
Inflammatory infiltrate				
Lymphocytes	+	++	+	++
Neutrophils	+	+	+	+
Plasma cells	+	++	+	++
Eosinophils	+	+	+	+
Macrophages	-	-	-	++
Sinusoidal endothelial alteration	++	++	++	-
Alteration centrilobular vein	+++	+	+	-
Sinusoidal congestion	+	++	+	-
Cholestasis	+	+	+	+
Sinusoidal dilatation	+	+	+	-
Portal vein alteration	-	-	-	++
Portal artery alteration	-	-	-	++
Bile canaliculus alteration	-	-	-	+
Limiting plaque injury	-	-	-	+

Z3: Pericentral zone; Z2: Midzonal zone; Z1: Periportal zone; PT: Portal tract.

Analysis of the response to the Th17 profile in the hepatic parenchyma in fatal YFV cases

The pattern and immunostaining in tissues revealed by IHC showed positivity characterised by the presence of characteristic brown areas around or inside the cytoplasm, especially the cells that constitute the inflammatory infiltrate. Immunolabeling was observed in the hepatic acinus and PT and was positive for the cytokines IL-6, IL-17, IL-23, and TGF- β as well as for the transcription factors ROR- γ and STAT3 (Table 3; Figures 2 and 3). Quantitative analysis of the immunological markers for the Th17 profile in the liver of YF samples and in the negative controls was performed in the following zones: Z3, Z2, Z1, and PT. The expression of these markers was significantly more intense in Z2 among the YF samples compared to the negative control samples ($p < 0.0001$; Figure 4). The mean and standard deviation for each marker are shown between the fatal YF and normal cases (Figure 4 and Table 4).

Table 3

Quantitative analysis of markers for the Th17 profile in the liver parenchyma (Z3, Z2, Z1 and PT) in fatal cases of human YF.

Markers	Z3	Z2	Z1	PT	ANOVA ($p \leq 0.05$)
IL-17	162.2 ± 18.81	230.9 ± 22.63	145.8 ± 13.36	128.1 ± 7.200	***
Controle	42.56 ± 7.723	55.36 ± 4.323	36.80 ± 8.237	37.76 ± 3.853	
Tukey ($p \leq 0.05$)	***	***	***	***	
IL-23	140.8 ± 12.77	213.8 ± 17.54	124.6 ± 10.05	109.8 ± 7.461	***
Controle	66.24 ± 39.15	71.36 ± 52.59	62.72 ± 38.77	44.16 ± 9.371	
Tukey ($p \leq 0.05$)	***	***	***	***	
IL-6	121.1 ± 15.78	182.6 ± 17.91	104.8 ± 12.64	94.32 ± 14.69	***
Controle	45.44 ± 23.61	56.96 ± 25.21	36.80 ± 14.49	33.92 ± 4.582	
Tukey ($p \leq 0.05$)	***	***	***	***	
STAT3	133.2 ± 11.15	176.9 ± 15.22	115.7 ± 8.956	101.4 ± 8.829	***
Controle	28.16 ± 3.505	29.76 ± 2.427	25.92 ± 2.629	24.96 ± 2.907	
Tukey ($p \leq 0.05$)	***	***	***	***	
ROR γ	124.3 ± 11.20	185.1 ± 15.42	113.4 ± 7.678	96.15 ± 10.67	***
Controle	29.44 ± 2.677	32.32 ± 3.469	25.28 ± 3.469	26.88 ± 4.719	
Tukey ($p \leq 0.05$)	***	***	***	***	
TGF- β	135.9 ± 9.646	207.4 ± 17.86	119.2 ± 10.03	115.9 ± 12.10	***
Controle	63.68 ± 42.85	72.32 ± 48.31	49.60 ± 29.18	35.52 ± 9.288	
Tukey ($p \leq 0.05$)	***	***	***	***	
Z3: Pericentral zone; Z2: Midzonal zone; Z1: Periportal zone; PT: Portal tract.					

ANOVA one-way; *** $p < 0.0001$; Tukey; *** $p < 0.0001$

Table 4

Comparative analysis between the compartments studied involving the expression of Th17 profile markers in fatal cases of YF in humans.

Markers	Z2 vs Z1	Tukey <i>p</i>	Z2 vs Z3	Tukey <i>p</i>	Z2 vs PT	<i>p</i> Tukey
IL-17	230.9 ± 22.63 vs 145.8 ± 13.36	***	230.9 ± 22.63 vs 162.2 ± 18.81	***	230.9 ± 22.63 vs 128.1 ± 7.200	***
IL-23	213.8 ± 17.54 vs 124.6 ± 10.05	***	213.8 ± 17.54 vs 140.8 ± 12.77	***	213.8 ± 17.54 vs 109.8 ± 7.461	***
IL-6	182.6 ± 17.91 vs 104.8 ± 12.64	***	182.6 ± 17.91 vs 121.1 ± 15.78	***	182.6 ± 17.91 vs 94.32 ± 14.69	***
STAT3	176.9 ± 15.22 vs 115.7 ± 8.956	***	176.9 ± 15.22 vs 133.2 ± 11.15	***	176.9 ± 15.22 vs 101.4 ± 8.829	***
RORγ	185.1 ± 15.42 vs 113.4 ± 7.678	***	185.1 ± 15.42 vs 124.3 ± 11.20	***	185.1 ± 15.42 vs 96.15 ± 10.67	***
TGF-β	207.4 ± 17.86 vs 119.2 ± 10.03	***	207.4 ± 17.86 vs 135.9 ± 9.646	***	207.4 ± 17.86 vs 115.9 ± 12.10	***
Z3: Pericentral zone;Z2: Midzonal zone;Z1: Periportal zone;PT: Portal tract.						
*** <i>p</i> <0.0001; Tukey; *** <i>p</i> <0.0001.						

Discussion

Historically, YF has been considered an infectious haemorrhagic disease with a major impact on public health. In this study, we have described the relationship between histopathological changes and cellular and cytokine immune response patterns in the liver of patients who died of severe YF due to hepatic-renal failure [13, 23, 24, 25].

The results obtained in this study assessing the main liver lesions and inflammatory infiltrate agree with other authors who described inflammatory infiltrates of mild to moderate intensity and associated these with midzone injury and intense presence of Councilman bodies in hepatocytes (cells under going apoptosis) followed by macro- and microvesicular steatosis and lytic and coagulative necrosis (5,11,12,13,24,25,26). Frequently associated with these changes are swelling, hepatocyte regenerative changes, hyperplasia and hypertrophy of Kupffer cells, sinusoidal congestion, and haemorrhage of the parenchyma. The intensity of these lesions in several human studies has demonstrated a histopathological pattern of acute fulminant hepatitis with extensive acinar involvement, typically

accompanied by mild or moderate inflammatory infiltrate and disproportionate to the degree of tissue damage observed in the liver in fatal YF cases. Our findings demonstrated an increased expression of not only cytokines with a Th17 profile but also transcription factors when compared to normal controls. This increased expression was observed mainly in Z2, followed by Z3, Z1, and PT ($p > 0.0001$).

TGF- β is a cytokine that has been investigated in the immunopathogenesis of YF, which has a dichotomous and pleiotropic character, exercising multiple functions (5,12,14,26). Thus, in addition to being considered a potent inducer of apoptosis, it also regulates tissue repair [26, 27, 28]. It is important to emphasize that, relevant to its activity, the activation of signalling pathways, in addition to the response triggered by SMADs, adds up to a scenario that streamlines the tissue repair process. In the studied fulminant cases, where cell damage is accentuated due to inflammation and tissue hypoxia and in an attempt to repair this process, resident cells such as Kupffer cells induce the activation of an alternative pathway with their main representative M2 phenotype [29, 30, 31, 32]. Therefore, it is worth noting that, in this scenario, TGF- β can induce the production of components that regulate the structuring of the extracellular matrix, including collagen I, II, and III, undulin, fibronectin, laminin, elastin, proteoglycans, and hyaluronan, suggesting that cytokines contribute to tissue remodelling [33, 34].

This may be directly related to the participation of IL-6, a cytokine included in our study that, in addition to participating together with TGF- β in the differentiation process of the Th17 profile, can also stimulate myofibroblasts and hepatocytes to synthesise collagen, which is important for the formation of the reticular fibres that make up the sinusoid cords [17, 35, 36]. Against flaviviruses, a central and striking characteristic of the protein corresponds to the potentiation of the acute inflammatory response and how it has a practical effect on the dynamics of the endothelium. In cases suggestive of fulminant hepatitis caused by DENV, it has been shown that cytokines can orchestrate the increased expression of IL-8, which serves as a chemotactic factor attracting leukocytes to the infection foci. In addition, in cases of haemorrhage, vasculopathy, and thrombocytopenia, cytokine expression is positively correlated with endothelial injury triggered by the action of IL-6 as well as adhesion molecules and other pro- and anti-inflammatory cytokines [37, 38, 39]. A marked histopathological characteristic in the hepatic parenchyma of the studied cases is the presence of haemorrhagic necrosis, indicating that, in situ, the performance of IL-6 in addition to other immune factors is fundamental to generating a harmful environment in the hepatic parenchyma of fatal YF cases.

Another interesting issue is the presence of transcription factors that appear in our study, such as STAT3, considering that, in previous studies conducted with flaviviruses, it was observed that non-structural proteins, such as NS1 of DENV-2, interact with transcription and increase the expression of IL-6 and TNF- α , consequently aggravating the inflammatory process in haemorrhagic conditions and further reinforcing the role of both STAT3 and ROR γ as central markers for inducing the construction of the Th17 profile [40, 41].

IL-17 is highly expressed in fatal cases of YF. Our data indicate that IL-17 is involved in the secretion of cytokines and chemokines with an effect on neutrophils, inducing recruitment, activation, and migration

to the site of infection. The presence of neutrophilic infiltration, especially close to the foci of lytic necrosis, is probably in response to cell necrosis and by the action of certain cytokines such as IL-17, which contribute to the presence of these cells in the liver, thus reinforcing the role of Th17 response in the evolution of YFV infection [22, 42]. Other approaches with DENV reported that, in the absence of IL-22, IL-17 could be instrumental in increasing the expression of CXCL1/KC, CCL5, IFN- γ , IL-6, and caspase 3; NK cell activity; and recruitment of TCD8 cells, indicating that IL-17 may be a key cytokine in the cascade of inflammatory responses and cell death in the liver, consequently in the pathogenesis [43].

IL-23 is considered to stabilise the differentiation and maturation of Th17 cells [44]. In a study investigating the mechanisms of cell damage in the central nervous system of fatal cases and microcephaly by ZIKV, both IL-17 and IL-23 were found not only with increased expression, but also with the Th1 profile to accentuate inflammation [20].

In addition, in a study on the hepatitis B virus, IL-23 could modulate the M1 phenotype and induce the production of IL-1 β , IL-6, TNF- α , and IFN- γ and, consequently, ROS [45]. An interesting issue in this scenario is consistent with the mechanisms that culminate in tissue damage, whereas, in these cases, the presence of IL-23 would be necessary to induce the production of VEGF by activating the JAK/STAT3 pathway.

Finally, our results indicate that the Th17 profile in situ contributes significantly to the exacerbation of the inflammatory response observed in the livers of fatal YF cases, and that this process is accentuated in the hepatic parenchyma, resulting in cell damage and cell death, as well as enhancing the typical haemorrhagic diathesis phenomena in YF caused by endothelial cells injury.

Methods

Study design

An analytical cross-sectional study was carried out with liver samples from the biobank of the Section of Pathology of the Evandro Chagas Institute of the Ministry of Health of Brazil from 2000 to 2016.

Tissue samples

Liver tissue samples from 21 deceased patients with a confirmed diagnosis of YFV infection by clinical, epidemiological, histopathological, immunohistochemical, and molecular criteria were included in this study. For the control group, tissue samples from five patients who had died from non-infectious or inflammatory causes were selected and showed no histopathological changes or positivity for other hepatotropic viruses, according to the death verification service in the city of Belém in the state of Pará, Brazil.

Liver samples were obtained by viscerectomy, fixed in 10% buffered formalin, embedded in paraffin, and sectioned into 5- μ m sections using a microtome. The sections were stained using the hematoxylin–eosin technique, reticulin, Masson trichrome, and Perls to assess morphological changes. Additional 5- μ m

sections were obtained and mounted on salinized slides for subsequent immunohistochemical staining with specific monoclonal antibodies.

Histology and semi-quantitative analysis

Histological analysis was performed using an Axio Imager Z1 microscope (Zeiss, Oberkochen, Germany) model 456006 (400x magnification). Each zone of Rappaport's hepatic acinus (Z1: periportal zone, Z2: Midzonal zone, and Z3: Pericentral zone) and portal tract (PT) was evaluated using a semi-quantitative scale from 0 to 4 (0: absent, 1: mild, 2: moderate ++, 3: intense +++, 4: very intense ++++) to classify the degree of tissue damage observed.

Immunohistochemistry

Immunostaining of the hepatic tissues with antibodies specific for ROR- γ , STAT3, IL-6, TGF- β , IL-17, and IL-23 (Table 1) was performed using the biotin–streptavidin–peroxidase method. Briefly, the tissue samples were dewaxed in xylol and hydrated in ethyl alcohol at concentrations of 90%, 80%, and 70%. Endogenous peroxidase was blocked with 3% hydrogen peroxide (H₂O₂) for 45 min. Antigens were retrieved by incubation in citrate buffer (pH 6.0) for 20 min at 90°C. Non-specific proteins were blocked by incubating the sections in 10% skim milk for 30 min.

The primary antibodies (Table 1) were diluted in 1% bovine serum albumin (Sigma Aldrich, Saint Louis, USA) for 14 h, and then the secondary biotinylated antibody LSAB (DakoCytomation, Glostrup, Denmark) was added in an oven for 30 min at 37°C. For visualisation, the specimens were treated with a chromogenic solution composed of 0.03% diaminobenzidine and 3% hydrogen peroxide. Histological sections were washed in distilled water, counterstained with Harris hematoxylin for 1 min, dehydrated in ethanol (70%, 80%, and 90%), and deparaffinized in xylene.

Table1. Antibodies used in the study of Th17 profile in liver of fatal cases YFV-induced.

Markers	Reference	Dilution
ROR- γ	Abcam/ab219496	1:100
STAT3	Abcam/ab76315	1:100
IL-6	Abcam/ab6672	1:100
TGF- β	Abcam/ab190503	1:100
IL-17	Abcam/ab79056	1:100
IL-23	Abcam/ab45420	1:100

Quantitative analysis and photo-documentation

The markers used to characterise the in-situ Th17 profile were visualised using an Axio Imager Z1 microscope (Zeiss). Immunostaining results were evaluated quantitatively by randomly selecting ten

fields in the hepatic parenchyma of the fatal YF or negative control cases for viewing at high magnification. Each field was subdivided into 10 × 10 areas delimited by a 0.0625 mm² grid.

Statistical analysis

The data were stored in a Microsoft Excel 2007 spreadsheet and analysed using GraphPadPrism 5.0. The numerical variables were expressed as the mean, median, standard deviation, and variance. One-way ANOVA, Tukey's test, and Pearson correlation were also applied; results were considered statistically significant at $p < 0.05$.

Declarations

Ethical aspects

All methods were carried out in this study are in accordance with relevant guidelines and regulations of the Ministry of Health Ethics Committee (CONEP). In addition, all experimental protocols carried out in this study were approved by a the institutional ethics committee, and was approved by the Research Ethics Committee of the Evandro Chagas Institute (IEC) (number 2,462,701), Rodovia BR-316, km-07, Ananindeua, Pará, Brazil, according to the recommendations in the resolution of the National Health Council No. 466/2012, and this study was performed in accordance with the 1964 Helsinki 254 Declaration and later versions. As all liver samples were obtained during surveillance of fatal cases the written informed consent was waived by the IEC Ethics Committee (decision number 2,462.701) since all biological samples of patients were obtained during public health actions years before this study be performed.

Availability of data and materials

The database used and/or analysed during the current study is not publicly available (to maintain privacy) but can be available from the corresponding authors on reasonable request.

Authors contribution

Designed the study: MISD, JASQ, JRS, and PFCV

Performed lab tests: MLGC, JRS, JCL, CCHM, FAO, CAMS, VSCM, LCS, FSSV, RSSA, ACRC, VCAG, LFMF, and LCM

Furnished reagents: MISD, JASQ, and PFCV

Drafted the manuscript: MLGC, JRS, MISD, JASQ, and PFCV

Final version review and agreed submission: all authors

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Figures

Figure 1

Histopathological analysis in Z3, Z2, Z1, and PT zones in the hepatic parenchyma of fatal YF and control cases. (A) Macrovesicular steatosis (yellow arrow), Councilman bodies (black arrow), coagulative necrosis (circle). (B) Macrovesicular steatosis (yellow arrow), microvesicular steatosis (green arrow), intense hemorrhagic necrosis (circle). (C) Mild coagulative necrosis and steatosis (circle). (D) Presence of inflammatory infiltrate in the PT (circle); (E–H) preservation of the hepatic parenchyma (Z3, Z2, Z1 and PT) in control cases (circles).

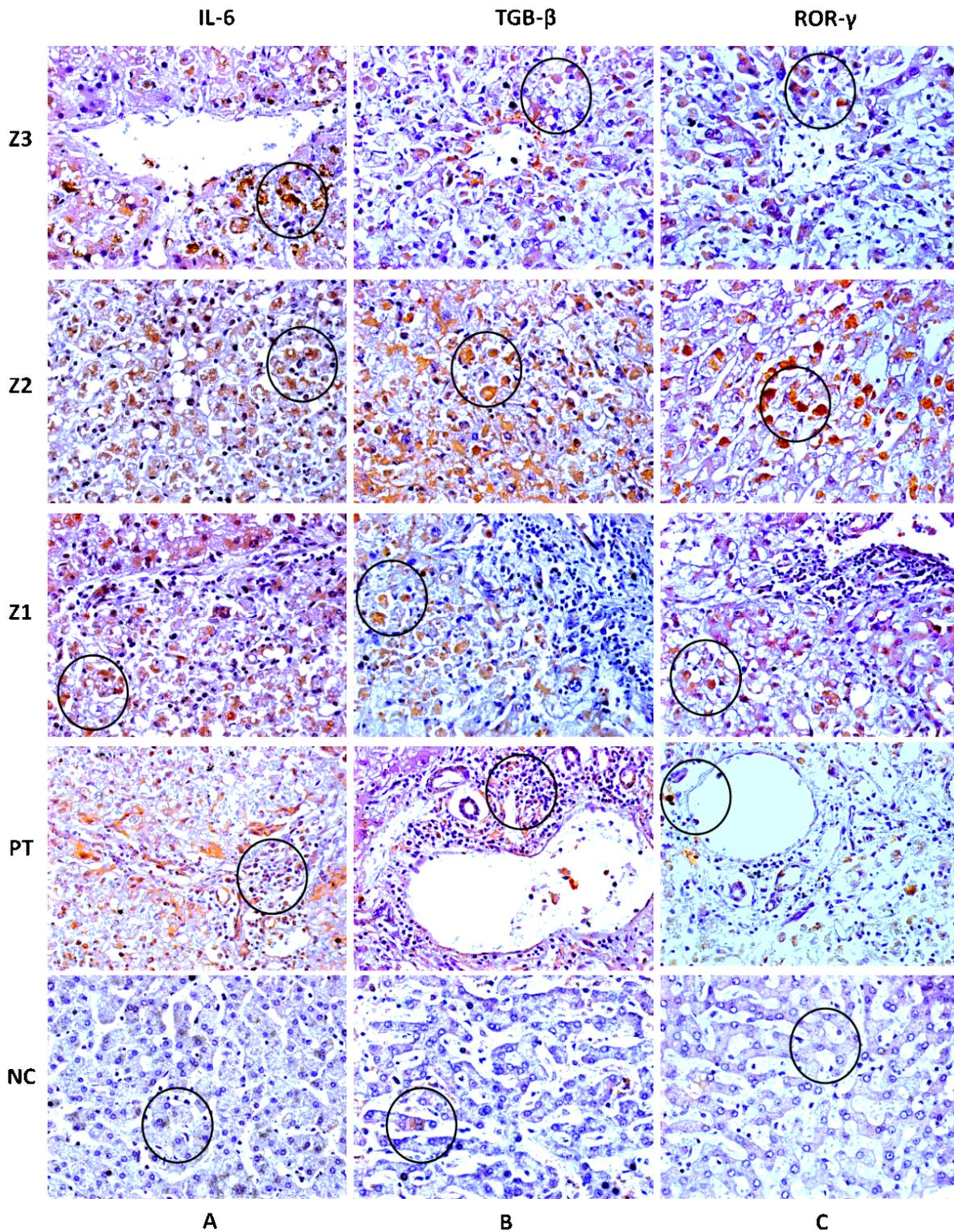


Figure 2

Positive immunohistochemistry for IL-6, TGF- β , and ROR- γ in zones Z3, Z2, Z1, and PT in the liver parenchyma of fatal YF and control cases. Immunolabeling for IL-6 (circle) in Kupffer cells (A-Z3), hepatocytes (A-Z2 and A-Z1), and the inflammatory infiltrate (A-PT). (B) Immunolabeling for TGF- β (circle) in hepatocytes (B-Z3, B-Z2, B-Z1), and in the inflammatory infiltrate (B-PT). (C) Immunomarking for ROR- γ (circle) in hepatocytes (C-Z3, C-Z2, C-Z1), and T cells (C-PT). Absence of labeling for IL-6 and

preservation of the liver parenchyma (A-NC), slight labeling for TGF- β and ROR- γ in Z2, and preservation of the liver parenchyma (B-NC and C-NC).

Figure 3

STAT3, IL-17, and IL-23 positive immunohistochemistry in zones Z3, Z2, Z1, and PT in the liver parenchyma of fatal cases of human YF and control. (A) Immunolabeling for STAT3 in hepatocytes (circle) (A-Z3, A-Z2, and A-Z1) and in the inflammatory infiltrate (A-PT). (B) Immunolabeling for IL-17 in hepatocytes (circle) (B-Z3, B-Z2, and B-Z1), and in the inflammatory infiltrate (B-PT). Immunolabeling for IL-23 in hepatocytes (circle) (C-Z3, C-Z2, and C-Z1), and inflammatory infiltrate in (C-PT). Light marking for STAT3 in Z2 and preservation of the hepatic parenchyma (Circle) (A-NC), absence of marking for IL-17 and IL-23, and preservation of the hepatic parenchyma (circle) (B-NC and C-NC).

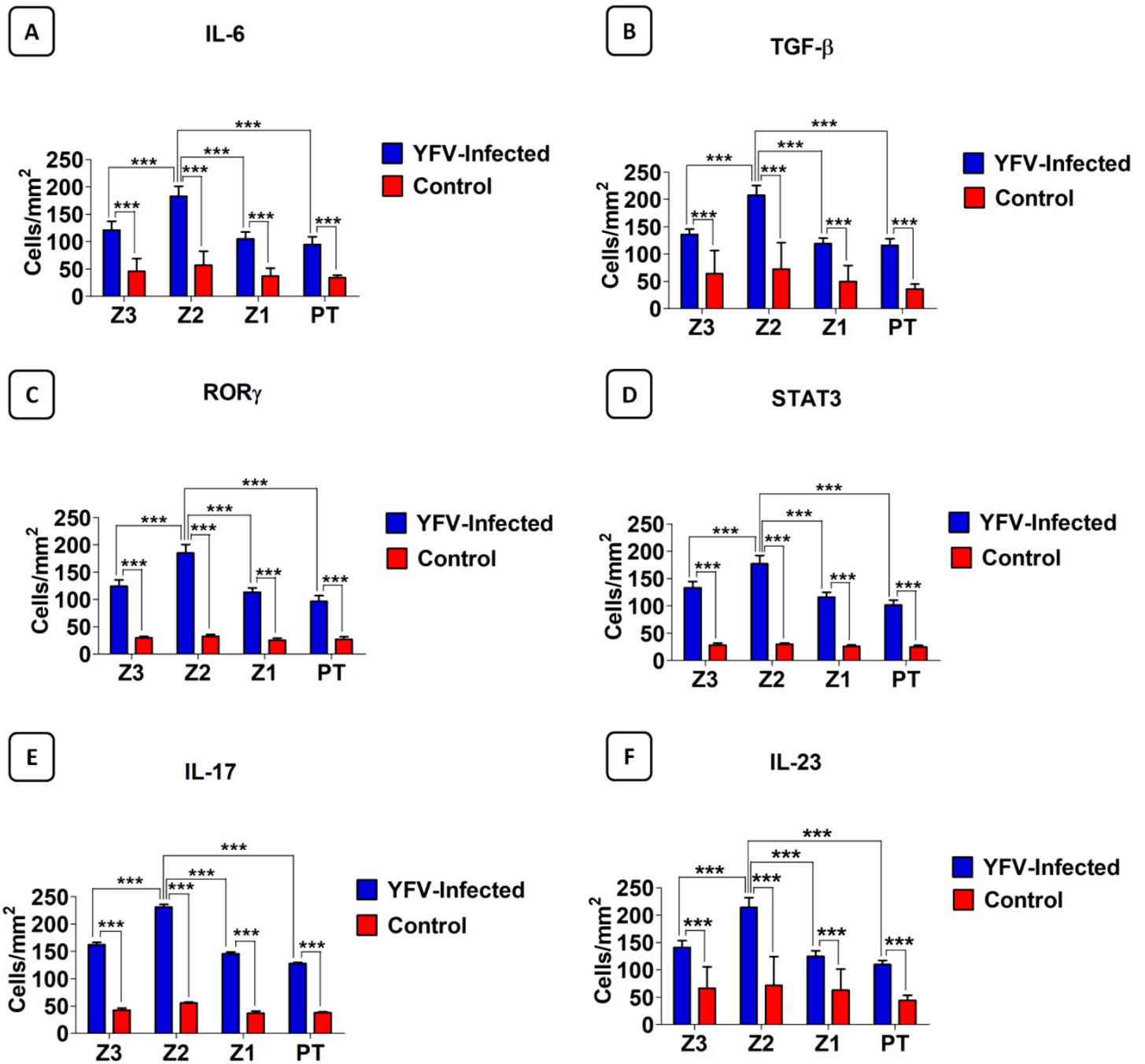


Figure 4

Quantitative analysis for IL-6 (A), TGF- β (B), ROR- γ (C), STAT3 (D), IL-17 (E), and IL-23 (F) in zones Z3, Z2, Z1 and PT in the liver parenchyma of fatal cases of human YF compared to negative control.

Tukey***p<0.0001; ANOVA***p<0.0001