



Increased expression of cytokines, soluble cytokine receptors, soluble apoptosis ligand and apoptosis in dengue

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ABSTRACT

Several studies have been performed to determine biomarkers that define the risk factors to developing severe forms of dengue. In this study, the levels of TNF- α , IL-6, IL-1, IL-17, soluble interleukin-1 receptor like 1 protein (sST2), soluble TNF-related apoptosis-inducing ligand (sTRAIL), IL-12 and soluble receptors for TNF (sTNF-RI and sTNF-RII) were determined by ELISA in dengue patients and monocyte/macrophage cultures. Dengue was classified as dengue without warning symptoms (DNWS), with warning symptoms (DWWS) and severe dengue (SD). High values of IL-6, sTNFRI, sTNFRII and sST2 were observed in DWWS and/or SD and IL-12 and sTRAIL in DNWS. TNF- α and IL-17 were increased not associated to the disease severity. High production of TNF- α , IL-1 β , IL-12, IL-17, sST2 and sTRAIL and apoptosis expression were observed in dengue monocyte/macrophage cultures. This study shows that beneficial or deleterious biomarkers can be present in dengue regardless the disease severity and that monocytes may be in part the source of studied molecules.

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Introduction

Dengue virus (DENV) is a single-stranded RNA virus that causes disease in humans. DENV infection results in different clinical manifestations ranking from benign disease (Halstead et al., 1970; WHO, 2009). Due to the lack of efficient biomarkers that define the degree of severity, there is the need to find relevant biological markers of the disease. Several investigations have been focused in DENV serotypes, type of infection and risk factors to developing severe forms of the disease (Halstead et al., 1970; Monath, 1994; Guzman et al., 1990). However, there are few studies relate to serum levels of soluble biomarkers with possible beneficial or deleterious activities, during the course of dengue and their association with the severity of disease, type of infection and DENV infection.

Several cytokines such as tumor necrosis factor alpha (TNF- α), gamma interferon, interleukin (IL)-6, IL-1, IL-17, soluble interleukin-1 receptor like 1 protein (sST2) and soluble TNF-related apoptosis-inducing ligand (sTRAIL) have been associated to deleterious effect

during dengue (Halstead et al., 1970; Gagnon et al., 2002; Espina et al., 2003; Levy et al., 2010; Rachman and Rinaldi 2006; Huang et al., 2003; Wu et al., 2013; Jain et al., 2013; Amatucci et al., 2007; Tajima et al., 2007; Wajant et al., 2001), and IL-12 and soluble receptors for TNF (TNF-RI and TNF-RII) with beneficial effects (Pacsa et al., 2000; Herbein and O'Brien, 2000; Vandenabeele et al., 1995). Therefore, the aim of this study was to determine the serum levels of TNF- α , IL-6, IL-1, IL-17, sST2, sTRAIL, IL-12, TNF-RI and TNF-RII in patients infected by DENV and their association with the severity of disease, type of infection (primary, secondary) and DENV type infection. In addition, to determine the production of those biomarkers and the apoptosis expression by monocyte isolated from dengue patients and healthy controls.

Results

Hematological and biochemical parameters of dengue patients and healthy control are shown in Table 1. Decreased numbers of platelets were observed in acute DNWS, DWWS and SD patients compared to healthy controls. The lowest counts were observed in SD patients. Prothrombin time (PT) and partial thromboplastin time (PTT) were found to be increased in acute SD and PTT in acute DWWS and SD. Counts of leukocytes were decreased in acute DNWS, DWWS and SD. Liver enzymes were observed increased in

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Table 1 Age, gender and laboratory parameters of healthy controls and patients with dengue according to severity and evolution of disease.

Laboratory parameters	Control				Dengue			
	Control		DNWS		DNWS		SD	
	n	Age (years) Gender Female/male	n	Age (years) Gender Female/male	n	Age (years) Gender Female/male	n	Age (years) Gender Female/male
Leukocyte counts × 10 ³ /μl	10	18 (2–42) 6/4	12	20 (5–33) 6/6	10	12 (1–39) 6/4	8	10 (4–26) 4/4
Platelet counts × 10 ³ /μl	6.9 ± 1.3	274.5 ± 50.1	6.0 ± 1.9 ^{b,c,d}	306.0 ± 160.6 ^{b,c,d}	6.9 ± 1.3 ^{b,c,d}	6.2 ± 1.4 ^{b,c,d}	5.93 ± 1.8 ^d	164.2 ± 71.3
Hemoglobin (g/dl)	13.1 ± 1.9	13.4 ± 1.5	12.8 ± 1.5	12.6 ± 1.3	12.4 ± 1.2	11.7 ± 0.8	11.1 ± 1.7 ^b	11.8 ± 1.1
Hematocrit (%)	41.3 ± 6.0	42.8 ± 4.7	40.2 ± 4.0	39.1 ± 3.0	40.4 ± 3.1	39.1 ± 1.2	38.1 ± 5.6	37.3 ± 2.9
PT (s)	12.4 ± 0.9				12.8 ± 2.0	12.3 ± 0.4 ^d	14.7 ± 1.2 ^{a,c}	12.4 ± 0.8 ^d
PTT (sec)	30.4 ± 3.8				39.7 ± 6.2 ^a	27.9 ± 2.3 ^{c,d}	36.6 ± 5.6 ^b	29.9 ± 0.7 ^{c,d}
Glycemia (mg/dl)	78.9 ± 6.6	86.5 ± 7.9	89.2 ± 6.2	86.3 ± 7.8	92.7 ± 15.8	93.0 ± 6.7	107.4 ± 41.5 ^a	88.6 ± 15.2
Creatinine (mg/dl)	0.9 ± 0.2	0.65 ± 0.3	0.7 ± 0.2	0.7 ± 0.09	0.7 ± 0.2	0.6 ± 0.2	0.7 ± 0.1	0.7 ± 0.1
AST (IU/L)	21.2 ± 4.2	49.7 ± 22.9	23.2 ± 6.2 ^{b,c,d}	19.1 ± 3.7 ^{c,d}	156.3 ± 105 ^{a,b}	82.2 ± 41.1 ^{a,d}	233.9 ± 160.7 ^{a,b}	18.7 ± 4.32 ^{c,d}
ALT (IU/L)	23.0 ± 5.3	59.6 ± 26.1 ^a	28.8 ± 8.7 ^{c,d}	21.1 ± 4.0 ^{c,d}	146.1 ± 113 ^{a,b}	94.2 ± 59.6 ^a	152.5 ± 115.6 ^{a,b}	21.7 ± 5.9 ^{c,d}

DNWS: dengue without warning symptoms; DNWS; dengue with warning symptoms; SD: severe dengue; PT: Prothrombin time; PTT: Partial Thromboplastin time; AST: Aspartate transaminase; ALT: Alanine transaminase.

^a p < 0.01 vs. control.
^b p < 0.01 vs. DNWS in acute period.
^c p < 0.01 vs. DNWS in acute period.
^d p < 0.01 vs. SD in acute period.

Table 2

Dengue patients according to severity of disease, type of infection and dengue type infection.

	Dengue							
	DNWS		DWWS		SD		Total	
	n	%	n	%	n	%	n	%
Infection:								
Primary	5	42	3	30	0	0	8	27
Secondary	7	58	7	70	8	100	22	73
Viral type ^a								
DENV-1	3	37.5	2	25	0	0	5	21
DENV-2	4	50	2	25	2	25	8	33
DENV-3	1	12.5	2	25	2	25	5	21
DENV-4	0	0	2	25	4	50	6	25

DNWS: dengue without warning symptoms; DWWS: dengue with warning symptoms; SD: severe dengue.

^a Viral isolation was obtained in 24 out of 30 dengue patients.

Table 3

Total serum cytokine and soluble receptor and ligand values in dengue and healthy controls.

Cytokine ^a	Healthy control (n=10)	Dengue (n=30)
TNF-α	20.38 ± 3.24	47.36 ± 21.17 ^b
IL-1β	5.18 ± 0.45	5.80 ± 0.87
IL-6	2.21 ± 1.03	6.17 ± 2.07 ^b
IL-12	42.72 ± 9.85	86.25 ± 40.65 ^b
IL-17	3.27 ± 2.19	45.93 ± 25.11 ^b
sTNF-RI	1583 ± 458.30	3352 ± 1720 ^b
sTNF-RII	2547 ± 697.20	10,858 ± 5978 ^b
sST2	22.08 ± 5.16	10,950 ± 9248 ^b
sTRAIL	51.71 ± 8.02	95.81 ± 26.93 ^b

^a Cytokine values are expressed as pg/mL and media ± standard deviation. TNF-α: Tumor necrosis factor-alpha; IL-1β: Interleukin-1 beta; IL-6: Interleukin-6; IL-12: Interleukin-12; IL-17: Interleukin-17; sTNF-RI: soluble tumor necrosis factor receptor type I; sTNF-RII: soluble tumor necrosis factor receptor type II; sST2: Interleukin-1 receptor like 1 protein; sTRAIL: soluble tumor necrosis factor -related apoptosis inducing ligand

^b p < 0.05 vs. healthy control.

dengue patients. Incremented AST values in acute DWWS and acute and convalescence SD were observed. ALT was observed increased in DNWS, DWWS (acute and convalescence) and SD (acute and convalescence). Table 2 shows dengue patient distribution according to severity of disease, type of infection and dengue type infection. Values of total studied serum molecules were found to be increased, except IL-1β (Table 3). In general, biomarkers were found to be increased in the different grades of dengue severity (Fig. 1). High values of IL-6, sTNFRI, sTNFRII and sST2 were observed in DWWS and/or SD and IL-12 and sTRAIL in DNWS. TNF-α and IL-17 values were found to be increased compared to healthy controls, but, no differences between the different grades of the disease severity were observed. Values of studied compounds were found to be increased in the acute period of disease, reaching similar values to those observed in healthy controls in the recovery period (Fig. 2). Dengue patients having secondary infection showed increased values of sTNFRI, sTNFRII and sST2, however, TNF-α, IL-6, IL-12, IL-17 and sTRAIL values remained similar in primary and secondary infections (Fig. 3). All DENV types were capable of inducing biomarker production in infected patients. However, DENV-1 was associated to higher values of IL-12 and sTRAIL; DENV-2 to sTNFRI, sTNFRII and sST2; DENV-3 to IL-12 and DENV-4 to IL-6 and sST2 (Fig. 4).

Monocytes from dengue patients and healthy controls were cultured for 5 days and biomarker contents were determined. Increased contents of TNF-α, IL-1β, IL-12, IL-17, sST2 and sTRAIL

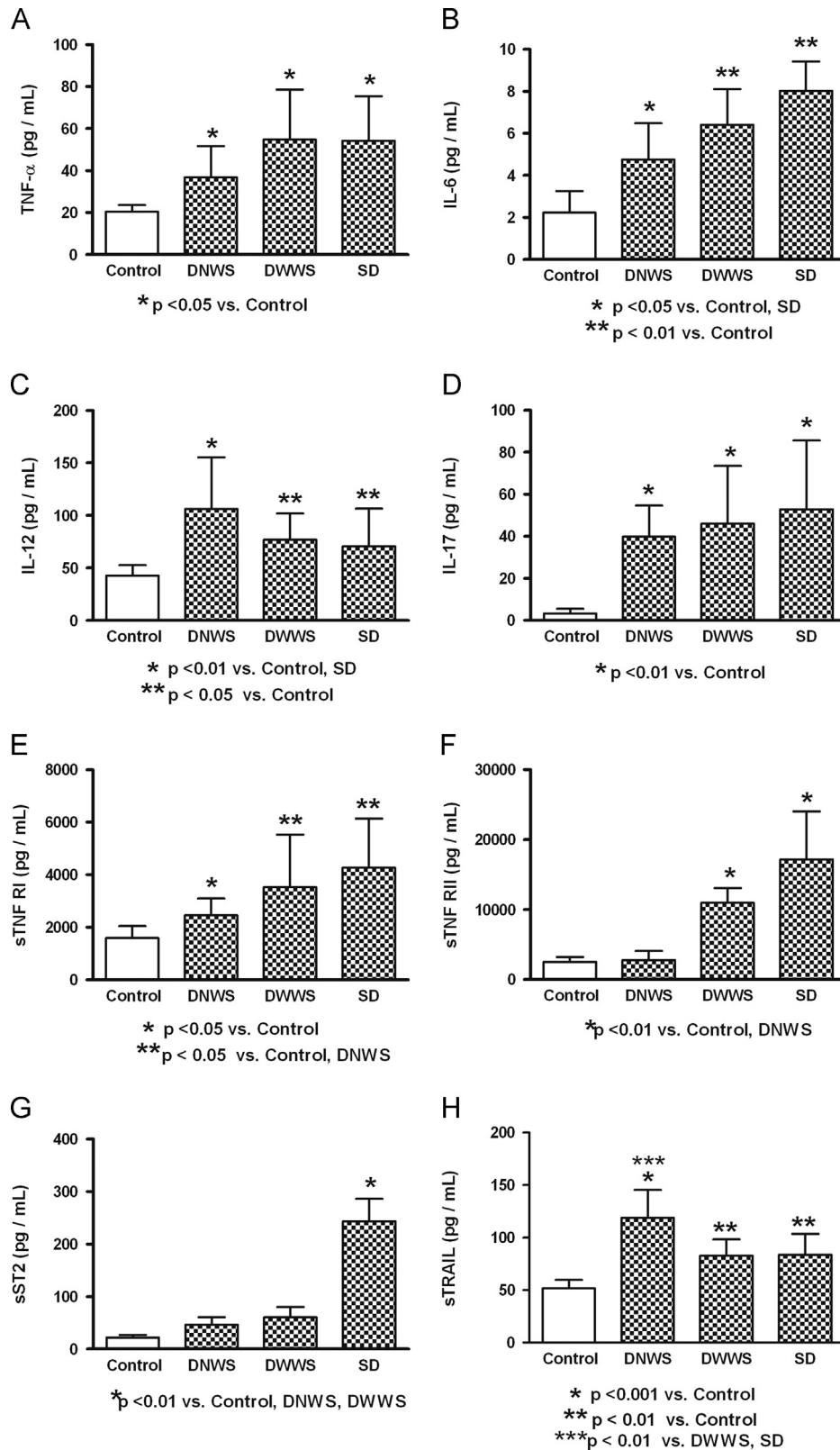


Fig. 1. Values of serum cytokines and soluble ligands and receptors for cytokines and apoptosis according to dengue severity. Dengue was classified as dengue without warning symptoms (DNWS), dengue with warning symptoms (DWWS) and severe dengue (SD). TNF- α : tumor necrosis factor- α ; IL-6, IL-12, IL-17: Interleukins-6, 12, 17; sST2: soluble interleukin-1 receptor like 1 protein; sTRAIL: soluble TNF-related apoptosis-inducing ligand; TNF-RI and TNF-RII: soluble receptors for TNF type I and II.

were observed in supernatants; however, IL-6 remained similar to controls. (Fig. 5). sTNFRI and sTNFRII were not detected by ELISA. High percentage of monocyte/macrophages from dengue patients

became apoptotic during the cultures (Fig. 6). Higher apoptotic effect was observed at day 5 of culture and similar apoptotic effect of DENV types was found (Fig. 7).

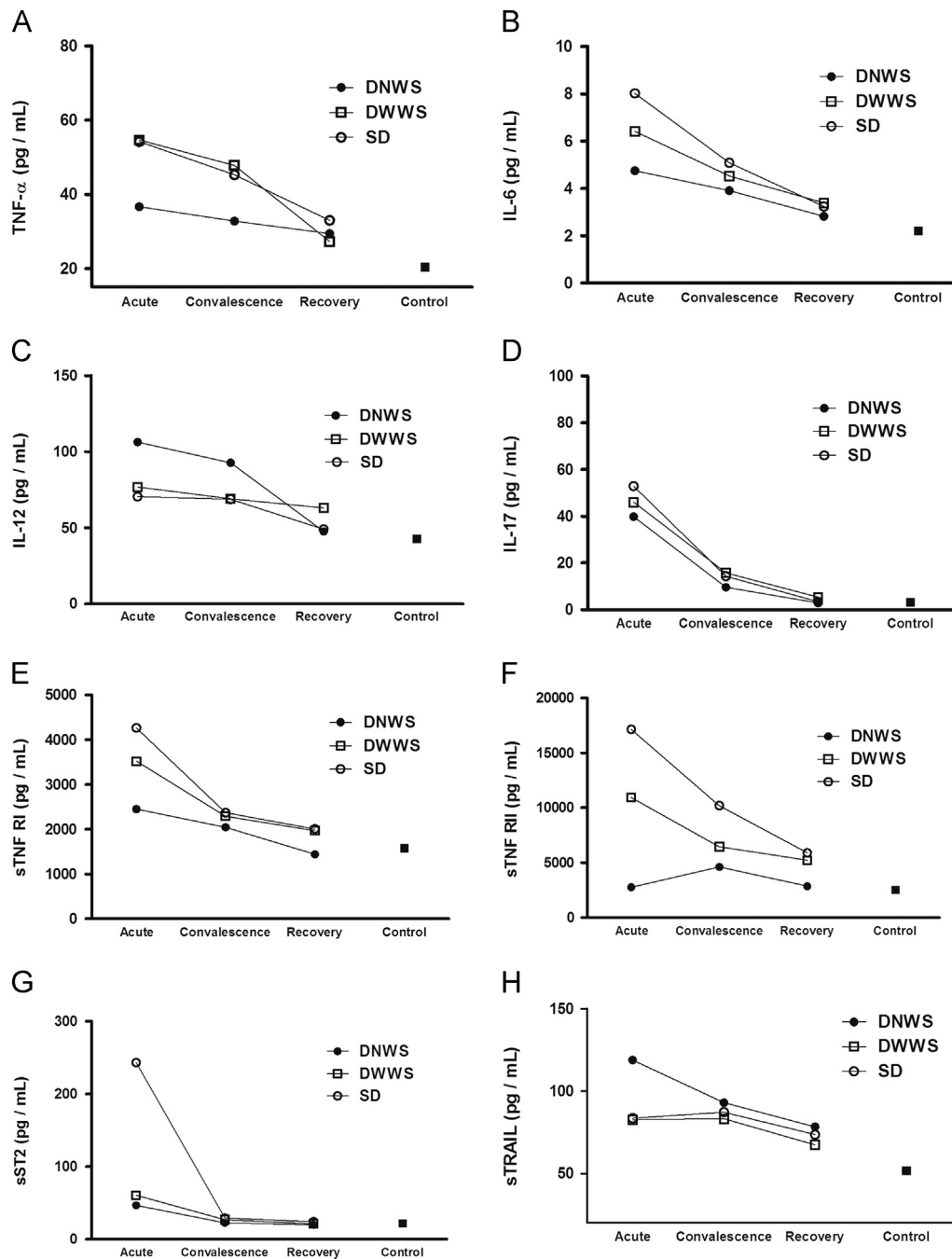


Fig. 2. Distribution of studied molecules according to phase evolution of dengue. Dengue was classified as dengue without warning symptoms (DNWS), dengue with warning symptoms (DWWS) and severe dengue (SD). TNF- α : tumor necrosis factor-alpha; IL-6, IL-12, IL-17: Interleukins- 6, 12, 17; sST2: soluble interleukin-1 receptor like 1 protein; sTRAIL: soluble TNF-related apoptosis-inducing ligand; TNF-RI and TNF-RII: soluble receptors for TNF type I and II.

Discussion

During this study several serum molecules were found to be increased in patients with dengue suggesting a complex regulation in the pathogenesis of this disease. Increased TNF- α found in dengue patients were not modulated by the severity of disease, type of infection or DENV type infection; however, TNF- α is believed to play a significant role in the pathogenesis of dengue, since elevated levels of this cytokine in the sera have been reported in infected patients (Gagnon et al., 2002; Espina et al., 2003; Levy et al., 2010). TNF- α has been associated with apoptosis and hemorrhagic manifestation in patients and in experimental dengue models (Gagnon et al., 2002; Espina et al., 2003; Levy et al., 2010; Chen

et al., 2007); however, TNF- α could have beneficial effect in dengue since can inhibit DENV replication in monocyte-derived dendritic cells (Shi et al., 2006). TNF- α has been associated to hemorrhagic manifestations of dengue (Gagnon et al., 2002; Espina et al., 2003; however, in this study, levels of TNF- α were not related to dengue severity. This finding could be related to the expression of different TNF polymorphism alleles (Chuansumrit et al., 2013; Alagarasu et al., 2013; García-Trejo et al., 2011).

High levels of IL-6 were related to DWWS and DENV-4 infection in this study, as previously observed in other groups of dengue patients (Levy et al., 2010; Butthep et al., 2012; Priyadarshini et al., 2010). High concentration of IL-6 has been implicated in the pathogenesis of DWWS and SD. This cytokine enhances the production of anti-platelet

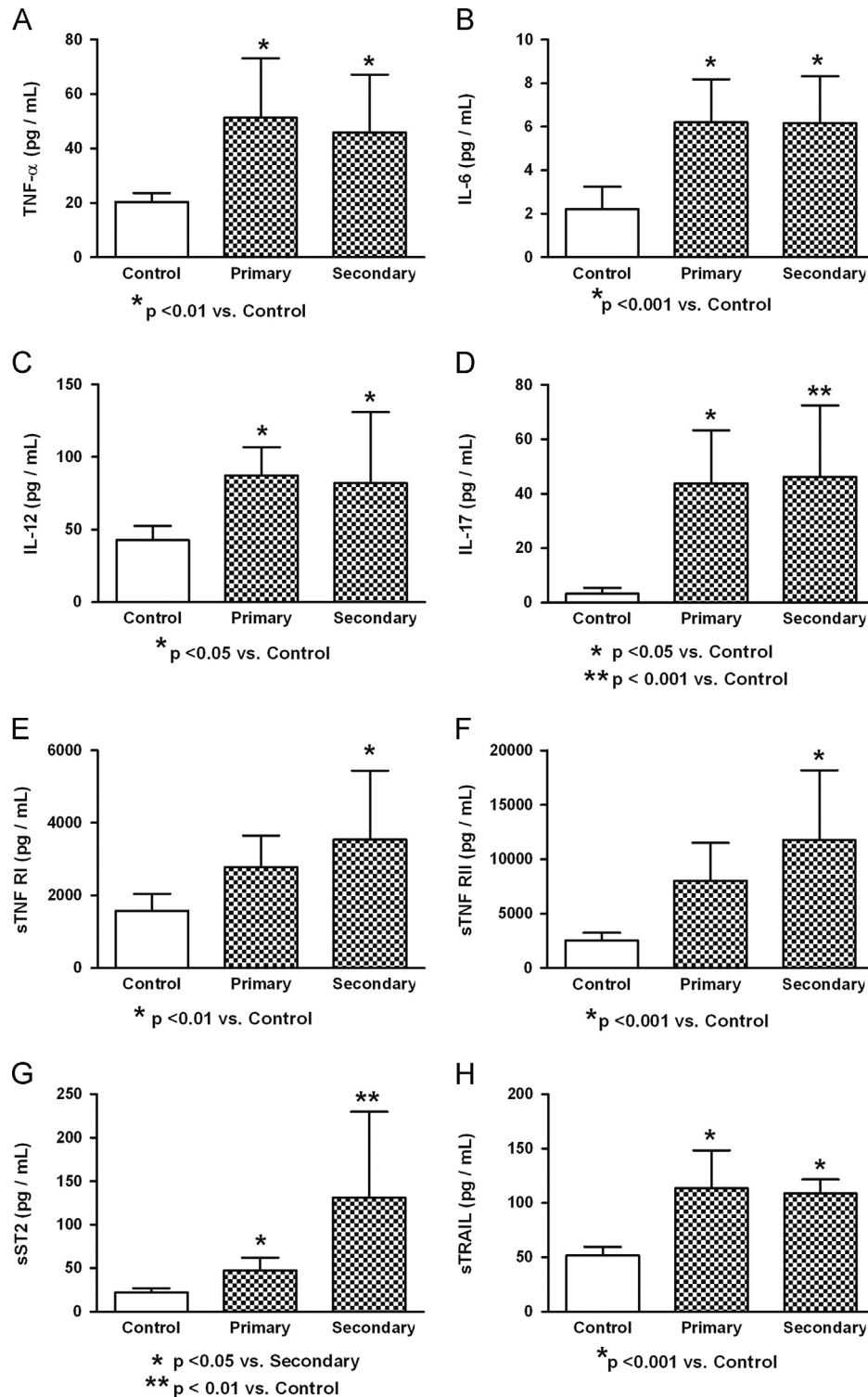


Fig. 3. Values of serum cytokines and soluble ligands and receptors for cytokines and apoptosis according to the type infection. TNF- α : tumor necrosis factor-alpha; IL-6, IL-12, IL-17: Interleukins- 6, 12, 17; sST2: soluble interleukin-1 receptor like 1 protein; sTRAIL: soluble TNF-related apoptosis-inducing ligand; TNF-RI and TNF-II: soluble receptors for TNF type I and II.

or anti-endothelial cell auto antibodies and tissue plasminogen activator, leading to plasma leakage and bleeding (Rachman and Rinaldi, 2006; Huang et al., 2003). In addition, IL-6 increases dengue virus replication in monocyte-derived dendritic cells (Shi et al., 2006).

As shown by other and in this study, high levels of IL-12 in DNWS and decreased values in hemorrhagic forms of dengue were observed, suggesting a protector role of IL-12 during severe illness, since the absence of this cytokine may be responsible for the shift

to a Th2 type response and thus for the pathogenesis of dengue hemorrhagic fever (Pacsa et al., 2000). In addition, IL-12/IL-18 induces nitric oxide synthase 2 production, which is of major importance to host resistance against DENV infection (OFagundes et al., 2011).

Increased expression of IL-17 found in this study was not associated to severity of disease, type of infection or DENV type infection. However, high IL-17 levels in children with severe

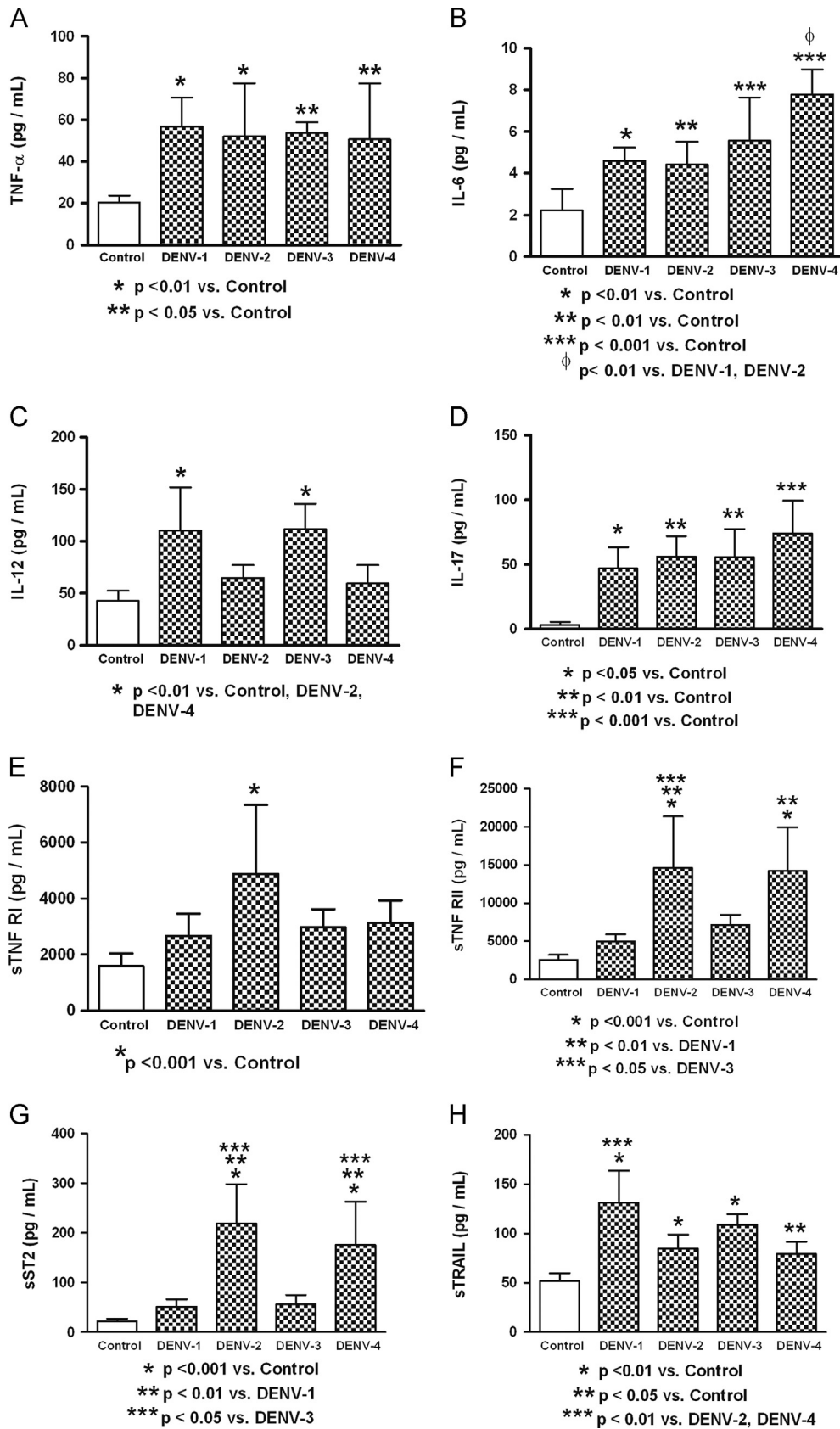


Fig. 4. Values of serum cytokines and soluble ligands and receptors for cytokines and apoptosis according to dengue virus infection. TNF- α : tumor necrosis factor-alpha; IL-6, IL-12, IL-17: Interleukins- 6, 12, 17; sST2: soluble interleukin-1 receptor like 1 protein; sTRAIL: soluble TNF-related apoptosis-inducing ligand; TNF-RI and TNF-RII: soluble receptors for TNF type I and II.

dengue, secondary infection and DENV-2 infection have been reported, suggesting a role for IL-17 in severe dengue (Jain et al., 2013). The exact role of IL-17 in the pathogenesis of severe DENV infection remains to be elucidated.

Levels of IL-1 β in dengue were similar to those observed in healthy control. This observation has been previously reported in vitro and in dengue patients by others (Levy et al., 2010; Butthep et al., 2012; Soemanto et al., 1999). These findings suggest that IL-1 β probably does not play an important role in the pathogenesis of this disease. However, this could be controversial, since, in this study, IL-1 β was observed increased in monocyte/macrophage cultures from dengue patients.

The effects of TNF- α are mediated by two specific receptors, TNF-RI and TNF-RII, which are bound to the cell surface, but by

proteolytic cleavage can be released to the environment (Herbein and O'Brien, 2000; Vandenabeele et al., 1995). In this study increased levels of sTNF-RI and RII associated to DWWS, SD and DENV-2 and DENV-4 infections were observed. Our data are consistent with previous report where increased expression of TNF-RII was associated to hemorrhagic manifestation of dengue (Bethell et al., 1998); however, the presence of these receptors in dengue could be controversial, since serum TNF-RI and RII were found elevated in dengue not associated to the severity of disease (Braga et al., 2001; Pinto et al., 1999; Hober et al., 1996). Theoretically, sTNFR I and RII can diminish the activity of TNF- α , but they may also increase its function by stabilizing the active TNF- α oligomer (Herbein and O'Brien, 2000; Vandenabeele et al., 1995). The balance between sTNFRs and TNF- α may be important to define the course of dengue infection.

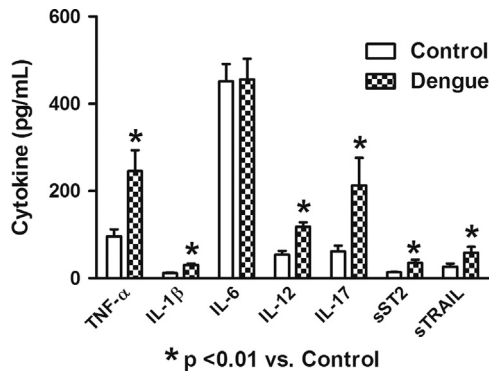


Fig. 5. Cytokines and soluble ligands and receptors for cytokines and apoptosis production by monocyte cultures from dengue patients and healthy controls. TNF- α : tumor necrosis factor- α ; IL-6, IL-1, IL-12, IL-17: Interleukins- 6, 1, 12, 17; sST2: soluble interleukin-1 receptor like 1 protein; sTRAIL: soluble TNF-related apoptosis-inducing ligand.

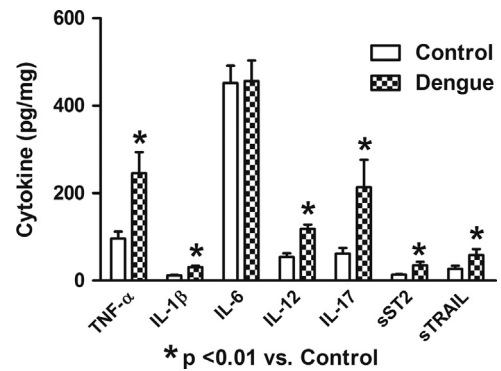


Fig. 7. Apoptosis induction by the different dengue virus types in monocyte/macrophage cultures. Increased expression of apoptosis in days 3 and 5 of culture, regardless virus type was observed.

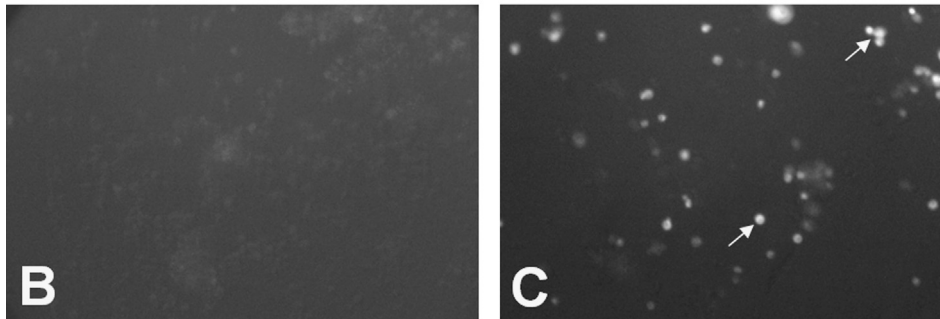
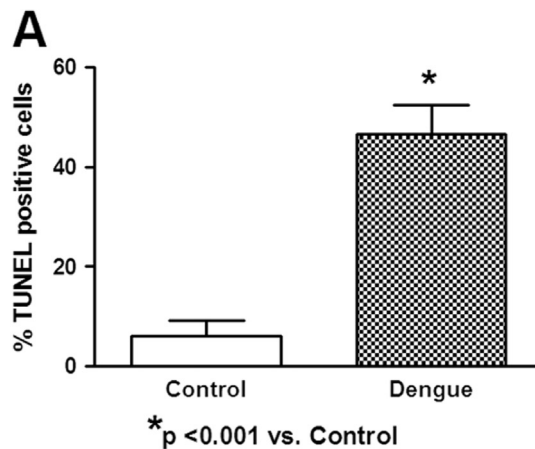


Fig. 6. Apoptosis expression in day 5 of monocyte/macrophage cultures from dengue patients and healthy controls. (A) Percentage of TUNEL positive cells in cultures. (B) TUNEL immunofluorescence staining from healthy control cultures. (C) TUNEL immunofluorescence staining from dengue monocyte/macrophage cultures. Arrows show apoptotic positive cells.

Soluble sST2 has been recently reported to be elevated in the serum of patients with DENV infection and it was correlated with secondary infections and with severe forms of the disease (Guerrero et al., 2013; Houghton-Trivino et al., 2010; Becerra et al., 2008). In this study sST2 levels were observed increased in SD, secondary infection and in DENV-2 and DENV4 infections. sST2 function during DENV infection remains unknown. Previous reports have suggested that sST2 could be involved in the inflammatory and Th2 immune responses (Amatucci et al., 2007; Tajima et al., 2007). However, sST2 could act as an anti-inflammatory mediator, by inhibition of Toll-like receptor signaling (Sweet et al., 2001; Brint et al., 2004) or inhibition of I- κ B degradation (Takezako et al., 2006). Therefore, elevated sST2 levels found in dengue patients may be an indication of the immune hyperactivation and/or down-regulation of inflammation. High levels of cytokines such as TNF- α and IL-6 found in this study could induce sST2 (Kumar et al., 1997; Tajima et al., 2003).

TRAIL is expressed on the cell membrane and signals apoptosis via the death domain-containing receptors TRAIL-R1 and TRAIL-R2. In this study, high values of sTRAIL were observed in DNWS patients and DENV-1 infection. DNWS is a benign form of dengue and the apoptotic effect of sTRAIL could diminish the viral replication (Everett and McFadden, 1999) during the disease. In addition, high levels of sTRAIL were associated to high production of interferon- α , a cytokine with antiviral effect (Gandini et al., 2013). These data support the role of sTRAIL inducing mild form of dengue mediate by apoptosis and antiviral cytokines. Our results showed that monocytes/macrophage isolated from dengue patients were capable of producing elevated concentration of sTRAIL in culture supernatants, suggesting a role of these cells in the increased serum concentration of sTRAIL found in dengue patients and a role of sTRAIL in the increased apoptosis found in those cultures. It was not determined viral presence in monocytes cultures, but, since *in vitro* infection of human primary monocytes, B cells and dendritic cells with DENV induces high levels of sTRAIL (Gandini et al., 2013; Becerra et al., 2009), we cannot rule out a role of cell-virus infection in sTRAIL production.

The source of cytokines, soluble receptors and ligands found increased in dengue patients could come from several cell types (Gagnon et al., 2002; Chen et al., 2007; Shi et al., 2006). Monocytes from dengue patients produced several cytokines found in patient's serum (TNF- α , IL-12, IL-17, sST2 and sTRAIL); however, failed to produced others (sTNFRI and sTNFRII), suggesting a partial role of monocytes in the serum content of those molecules.

Apoptosis is a regulatory feature of the immune system. In response to viral infection, apoptosis may occur as a pathogen-directed mechanism of viral dissemination and immune escape or may represent an appropriate host response for limiting virus replication (Everett and McFadden, 1999; Myint et al., 2006). Apoptosis seems to be a relevant mechanism in cell damage during dengue (Espina et al., 2003; Levy et al., 2010; Myint et al., 2006; Mosquera et al., 2005; Morchang et al., 2011). In this study, cultured monocytes from patients with dengue showed high expression of apoptosis. In this regard, it has been reported that DENV is capable of inducing apoptosis in experimental infection of monocyte line (Klomporn et al., 2011), suggesting that by intrinsic way, dengue virus can induce apoptosis (Myint et al., 2006; Morchang et al., 2013; Netsawang et al., 2010). In addition, cytokines induced by dengue infection in monocytes and other cell types can lead to apoptosis (Espina et al., 2003; Levy et al., 2010; Morchang et al., 2011; Leong et al., 2007). In this study, monocytes from dengue patients produced high levels of TNF- α , and sTRAIL and were not capable of producing sTNFRs; these conditions can induce apoptosis. In this regard, high levels of TNF- α in dengue patients has found to be associated to apoptosis (Espina et al., 2003; Levy et al., 2010; Klomporn et al., 2011; Jaiyen

et al., 2009). The presence of this cytokine in monocyte/macrophage cultures could lead to apoptosis and the action of TNF could be enhanced, since sTNFRs were absent. In addition, the action of sTRAIL on cell membrane TNF receptors can contribute to higher expression of apoptosis (Wajant et al., 2001).

In conclusion, the response to dengue virus infection in this study was characterized by the production of several cytokines, soluble ligands and receptors, and the overall picture appears to be complex. The presence of beneficial or deleterious biomarkers is not related to disease severity, type of infection or DENV infection; therefore, specify profiles for dengue were not observed. The balance between protective and pathologic molecules instead of only their presence, could lead to define the course of dengue. Apoptosis seems to be an important mechanism of cell damage in dengue as reflected by the high percentage of monocytes triggered for apoptosis. This study defines the presence of beneficial and deleterious molecules during dengue infection, however, the precise role and interactions of those molecules remain to be studied in association with clinical outcomes.

Materials and methods

Patients

Thirty male and female patients (1–39 years old) presenting clinical diagnosis of dengue according to WHO criteria (WHO, 2009) were studied. Dengue patients were classified as dengue without warning symptoms (DNWS), dengue with warning symptoms (DWWS) and severe dengue (SD). In addition to suggestive clinical diagnosis, dengue virus infection was confirmed either by the presence of serum anti-dengue antibodies or by virus isolation. Blood samples were taken during the acute phase (1–6 days after the onset of the symptoms), during the convalescent phase (7–26 days after the onset of the symptoms) and during the recovery phase. Dengue immune response was considered as primary or secondary by serum immunoglobulin pattern as determined by ELISA (Diagnostic Automation, Inc., Calabasas, USA). In addition, the acute-phase samples were subjected to virus isolation through cell culturing with the C6/36 mosquito cell line obtained from *Aedes albopictus* (Tesh, 1979). Virus serotypes were determined by indirect immunofluorescence using monoclonal antibodies. Patients with cardiovascular, hematological, hepatic, lung, kidney and autoimmune diseases, hypertension, diabetes, cancer, pregnancy, treated with steroids or immunosuppressors and infections with bacteria or other virus were excluded from this study. The blood samples obtained from similar age and sex healthy individuals ($n=10$; age: 2–42 years old) were used as controls. Samples from patients and controls were stored at -70°C until used and tested for studied molecules and biochemical parameters. The Ethics Committee of Instituto de Investigaciones Clínicas Dr. Américo Negrette and FONACIT (Caracas, Venezuela) approved this study protocol and written informed consent was obtained from all patients or parents prior blood collection in accordance with the ethical standards of Declaration of Helsinki (1964).

Laboratory studies

IL-1 β , IL-6, TNF α and IL-12 contents were measured using a commercially available ELISA kits (Thermo Scientific, MA, USA) as well as IL-17, sTRAIL, sST2, sTNF-RI and sTNF-RII (R&D Systems, Inc., MN, USA). The results are expressed as pg/ml or pg/mg of cellular protein.

Monocyte/macrophage cultures

Mononuclear leukocytes were obtained from heparinised venous blood from 8 seronegative healthy adult donors and 8 dengue patients during the acute phase by density gradient centrifugation in Hystopaque 1.077 (Sigma Chemical Co. St. Louis MO, USA). Cells were cultured in supplemented RPMI 1640 and incubated at 37 °C and 5% CO₂. After 3 hour-incubation, adherent cells were enriched by washing away unattached cells. After 5 days supernatants were collected and biomarker contents were determined as described. Cells were sonicated for protein determination. Culture purity was assessed by an FITC-conjugated anti-human CD14 monoclonal antibody (Sigma Chemical Co., St. Louis, MO, USA) and by a microscopy with epifluorescence system (Zeiss, Germany). Apoptosis was determined in attached cells after 3 and 5 days of culture by the TUNEL assay.

TUNEL assay

Cultured monocyte/macrophages from studied groups were fixed with 1% paraformaldehyde in PBS and permeabilized with acetic acid: ethanol. The percentages of apoptotic cells were assessed by TUNEL reaction using the In situ Apop Tag kit (Chemicon International; USA and Canada) according to the manufacturer's instructions.

Statistical analysis

Values were expressed as mean ± SD. The significance of differences was tested by ANOVA and the Bonferroni post hoc test. Two tailed $p < 0.05$ values were considered statistically significant.

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