

Received: 2018.04.06
Accepted: 2018.05.08
Published: 2018.08.16

Increased Concentrations of Extracellular Histones in Patients with Tuberculous Pleural Effusion

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEF 1 **Xuemei Jiang***
BCDF 2 **Xiaohui Hao***
ABDF 3 **Tao Wen**
BCF 1 **Yang Jin**
BCF 1 **Meng Sun**
BCDF 4 **Hua Yang**
ABCDEFG 1 **Zongmei Wen**

1 Department of Anesthesiology, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai, P.R. China
2 Department of Tuberculosis, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai, P.R. China
3 Medical Research Center, Beijing Chao-Yang Hospital, Capital Medical University, Beijing, P.R. China
4 Shanghai Key Laboratory of Tuberculosis, Clinic and Research Center of Tuberculosis, Shanghai Pulmonary Hospital, Tongji University School of Medicine, Shanghai, P.R. China

* Xuemei Jiang, Xiaohui Hao are co-first authors

Corresponding Author: Zongmei Wen, e-mail: wzm1103@126.com; Hua Yang, e-mail: yanghua97065@163.com

Source of support: This work was supported by grants from National Natural Science Foundation of China (81400052), Beijing, P.R. China. This work was also funded by Shanghai Municipal Committee of Science and Technology (14ZR1434400, to Zongmei Wen), Shanghai, P.R. China, and by Pujiang Talent Program (14PJD030, to Zongmei Wen), Shanghai, P.R. China. This project was also supported by a grant from the Chinese National Key Project for Infectious Disease (2018ZX10722302-004 to Hua Yang)

Background: Extracellular histones have recently been suggested as critical mediators in many inflammatory diseases. However, the role of extracellular histones in tuberculous pleural effusion (TPE) is unclear. The goal of this study was to explore the potential involvement of extracellular histones in patients with TPE.





Material/Methods: Samples of pleural effusion and peripheral blood were obtained from 58 patients with tuberculosis. Extracellular histones were determined in both TPE and serum samples. Moreover, the biomarkers for cellular damage, inflammatory cell activation, and systemic inflammation including lactate dehydrogenase (LDH), myeloperoxidase (MPO), S100A8/A9, as well as multiple inflammatory cytokines were measured.

Results: Extracellular histone levels were significantly elevated in TPE (4.762 mg/mL [3.336, 7.307]) and serum samples (1.502 mg/mL [1.084, 2.478]) from tuberculosis patients as compared with the serum (0.585 mg/mL [0.285, 0.949]) from healthy controls. Notably, extracellular histones in TPE were also much higher than in serum of patients ($P=0.002$). LDH, MPO, and S100A8/A9 levels were all increased in TPE, along with a remarkable elevation of various cytokines. A correlation analysis showed that extracellular histones were positively associated with LDH, MPO, and S100A8/A9, and a panel of inflammatory cytokines in TPE.

Conclusions: These results suggest that high concentrations of extracellular histones are markedly present in TPE, which may play an inflammatory role towards the progression of tuberculosis.

MeSH Keywords: **Histones • Inflammation Mediators • Tuberculosis, Pleural**

Full-text PDF: <https://www.medscimonit.com/abstract/index/idArt/910431>

 2037  2  3  31



Background

Tuberculosis is a serious public health problem around the world, which accounts for millions of active disease cases and deaths each year [1]. Although pulmonary form is the most common manifestation of TB, the disease may also result in tuberculous pleuritis, which usually presents as an acute illness with fever, cough, pleuritic chest pain, and tuberculous pleural effusion (TPE) [1,2]. TPE is actually caused by *Mycobacterium tuberculosis* infection of the pleura and a resultant inflammatory response may result in increased pleural vascular permeability, leading to an accumulation of fluid that is enriched in proteins, and an influx of inflammatory cells into the pleural space [2,3]. TPE will accelerate an active form of tuberculosis if untreated [4]. Currently, the understanding of the inflammatory processes in TPE is limited.

Extracellular histones, mainly derived from dying cells or inflammatory cell infiltration, have recently been discovered as critical mediators implicated in systemic inflammation, and tissue and organ injury [5–7]. It has been described that extracellular histones may possess a variety of toxic effects including direct vascular endothelial damage [8–10], erythrocyte fragility [11], platelet aggregation and coagulation activation [12,13], as well as cytokine elevation [14,15], all of which may result in enhanced inflammation. More importantly, therapeutic modulation of extracellular histones appears to be effective in the treatment of various inflammatory injuries [6,16,17]. In this report, we sought to explore whether extracellular histones participate in the inflammatory processes associated with TPE, with a hypothesis that extracellular histones may act as inflammatory mediators involved with disease progression. Insights from this study may help diagnose, monitor TPE or improve its therapeutic strategy.

Material and Methods

Study participants

Fifty-eight patients diagnosed newly with TPE were recruited for this study. In addition, 18 healthy donors were recruited as healthy controls. The Ethics Committee of Shanghai Pulmonary Hospital, Tongji University School of Medicine (Shanghai, P.R. China) approved this study, which followed the recommendations of the Declaration of Helsinki for biomedical research regarding human subjects. Written informed consent was obtained from patients or their next of kin.

TPE was diagnosed based on: 1) typical clinical symptoms (fever and chest pain) and B-mode ultrasound showing pleural effusion; 2) high levels of adenosine deaminase (ADA) in the pleural effusion (40–80 U/L); 3) positive tuberculin test result; 4) positive histopathological examination of a pleural biopsy

specimen; and/or 5) clinical symptoms were rapidly relieved after anti-tuberculosis therapy [4]. Exclusion criteria were as follows: 1) autoimmune disease; 2) human immunodeficiency virus (HIV) infection; 3) cancer for TPE patients; 4) pregnancy; 5) ongoing infection other than pleural tuberculosis for TPE patients; or 6) any systemic diseases involving immunity [4].

Sample collection and processing

TPE from patients were collected within 24 hours of admission or on a symptomatic day before treatment. Pleural fluids were transferred to 50 mL tubes and centrifuged (3000×g for 10 min) within 1 hour of collection. Serum samples were isolated from the participants' peripheral venous blood by centrifuging at 3000×g for 10 min. All supernatants were stored at –80°C until further analyses. In addition, to avoid experimental infection with tuberculosis from TPE samples, the samples were filtered by 0.22 mm filters to remove bacteria before the analysis in a class 2 biosafety cabinet within a BSL2 laboratory.

Extracellular histones assay

We assessed extracellular histones in TPE and serum samples by using an enzyme-linked immunosorbent assay (ELISA) kit (Roche Applied Science, Germany), which uses a capturing antibody against an epitope shared by all histones and a detecting antibody against DNA [18,19]. Purified exogenous histones of calf thymus were used to produce standard curves [19].

Measurement of cell damage and inflammatory cell infiltration

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme whose activity reflects the extent of cellular damage [20]. We assessed LDH activities in these samples with a commercially available kit (Roche, Germany), according to the manufacturer's instructions. Myeloperoxidase (MPO) serves as an index for neutrophil and monocyte/macrophage infiltration, whereas the calcium-binding protein S100A8/A9 complex (S100A8/A9), which is abundantly stored in neutrophil cytoplasm, serves as another important marker for phagocyte [20,21]. Thereby, we quantified MPO and S100A8/A9 levels to analyze inflammatory cell infiltration/activation in these samples.

Assay for multiple cytokines

For detection of multiple cytokines, the ProcartaPlex™ Multiplex Immunoassay from Affymetrix eBioscience (San Diego, CA, USA) was used on a Luminex/Bioplex-200 System, which permits a simultaneous measurement of various cytokines in a single sample [9,19]. The following cytokines were measured: IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17A, IL-18, IL-21, IL-22, IL-23, IL-27, IP-10, IFN-γ, TNF-α, MCP-1, and VEGF-A.

Table 1. The characteristics of the study subjects.

Characteristics	TB patients	Healthy controls
Sample sizes	58	18
Sex (Male/Female)	39/19	10/8
Age (years)	43.67±2.8	40.50±3.4
Blood		
ADA (U/L)	17.63±6.9	14.23±5.7
Total protein (g/L)	26.7±9.2	21.4±6.5
TPE		
ADA(U/L)	68.9±8.4	
Total protein (g/L)	42.3±7.6	

Statistical analysis

GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA) was used for statistical analysis. Unless otherwise stated, all values were expressed as medians and interquartile ranges. Data were analyzed by 2-tailed Student's *t*-test or one-way analysis of variance (ANOVA). Correlations between variables were evaluated using Spearman's rank correlation analysis. Results were considered statistically significant when $P < 0.05$.

Results

Patients' characteristics

Tuberculosis patients were 43.67±2.8 years old, including 39 males and 19 females. Healthy controls were 40.50±3.4 years old. The participants' baseline characteristics are shown in Table 1. No patient had severe tuberculosis such as hematogenous disseminated pulmonary tuberculosis, or tuberculous meningitis. All patients were HIV-negative, and no patient had been diagnosed with immunodeficiencies. Furthermore, clinical symptoms of all patients were rapidly relieved after 1 month of anti-tuberculosis chemotherapy.

Extracellular histone levels are elevated in TPE

We first detected extracellular histone levels in TPE and serum samples. We found that extracellular histones in TPE samples (median value, 4.762 mg/mL [3.336, 7.307]) were significantly higher than in serum samples from tuberculosis patients (median value, 1.502 mg/mL [1.084, 2.478], $P < 0.001$) or the serum from normal controls (median value, 0.585 mg/mL [0.285, 0.949], $P < 0.001$) (Figure 1). In addition, the levels of extracellular histones were also remarkably higher in serum of tuberculosis patients than in the serum of normal controls ($P = 0.002$).

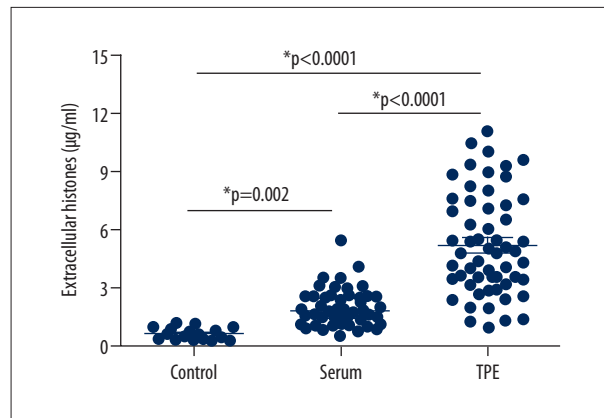


Figure 1. Extracellular histone levels are elevated in tuberculous pleural effusion (TPE). ELISA analysis showed that the concentrations of extracellular histones were significantly higher in TPE as compared with the serum from the corresponding tuberculosis patients or the healthy controls. Data are expressed as median (interquartile range).

High levels of LDH, MPO, and S100A8/A9 in TPE

Previous research has suggested that extracellular histones may originate from necrotic and apoptotic cells or from inflammatory cell infiltration/degradation [6,22]. Thus, we checked cellular origins of extracellular histones by measuring LDH, MPO, and S100A8/A9 activities in these samples. We found that LDH, MPO, and S100A8/A9 were all remarkably elevated in TPE as compared with the serum of tuberculosis patients or the serum of healthy controls (Figure 2). In contrast, MPO was also significantly elevated in the serum of tuberculosis patients as compared to healthy controls ($P = 0.008$), whereas no statistical difference was detected in LDH and S100A8/A9 levels between the serum samples of tuberculosis patients and normal controls (both $P > 0.05$). Notably, there was a positive correlation of extracellular histones with LDH ($r = 0.3267$, $P = 0.028$), MPO ($r = 0.6525$, $P < 0.0001$), and S100A8/A9 ($r = 0.5108$, $P = 0.0017$), thereby suggesting that the increased extracellular histones were possibly released from damaged pleural mesothelium or the degradation of inflammatory cells during tuberculosis infection.

Increased concentrations of multiple cytokines in TPE

It has been demonstrated that high concentrations of extracellular histones could aggravate systemic inflammation in patients [9,23]. To assess the severity of systemic inflammation in TPE, we measured a panel of multiple cytokines. It showed that there were totally 12 cytokines markedly increased in TPE as compared with the corresponding serum samples or the controls (Figure 3). Moreover, there were 4 cytokines (IL-6, IL-10, IL-27, and IFN- γ) observed to be different in serum samples

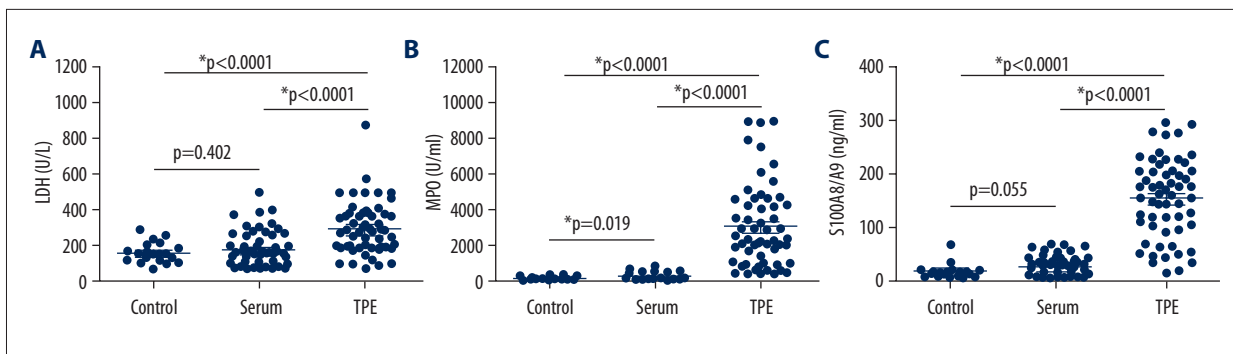


Figure 2. The possible sources of extracellular histones in tuberculous pleural effusion (TPE) and serum samples. (A) Lactic dehydrogenase (LDH), a marker reflecting degree of cellular damage, (B) myeloperoxidase (MPO), an index representing neutrophil/monocyte infiltration, and (C) the calcium-binding protein S100A8/A9 complex (S100A8/A9), an important marker for phagocyte activation were quantified. The results showed that LDH, MPO, and S100A8/A9 level were all remarkably increased in TPE as compared to the serum of the tuberculosis patients or the healthy controls. Variables were expressed as median (interquartile range).

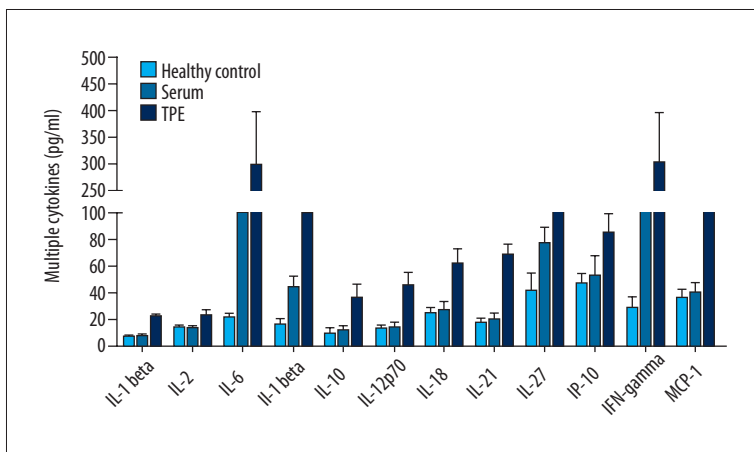


Figure 3. The concentrations of multiple cytokines were increased in tuberculous pleural effusion (TPE). Multiplex immunoassay for a panel of multiple cytokines was performed. Compared to the corresponding tuberculosis patients' serum or serum from healthy controls, a total of 12 cytokines was higher in TPE with significant differences (all $P < 0.05$). Variables were expressed as median (interquartile range).

between tuberculosis patients and healthy controls. A correlation analysis indicated that the concentrations of extracellular histones were positively correlated with most of the detected cytokines, which are actually classical markers for systemic inflammation (Table 2). Taken together, these results indicated that high concentrations of extracellular histones were closely associated with the inflammatory processes in TPE, which may aggravate disease progression.

Discussion

The primary purpose of this study was to explore the possible involvement of extracellular histones in TPE. Until recently, extracellular histones, high mobility group box-1 protein (HMGB1), mitochondrial DNA, and formyl peptides are known to act as damage-associated molecular pattern (DAMP) molecules that can enhance inflammatory response in addition to pathogen [22]. Of these, extracellular histones were specifically discovered as a novel type of tissue-damaging products implicated

in many inflammatory insults [11,22–24]. Histones are basic unit structure components of chromatin [6,22]. Besides having nuclear function, histones can be liberated into extracellular milieu after massive cell apoptosis or necrosis and turn out to be endogenous danger signals [6]. Mounting evidence unravels many biological effects of extracellular histones such as direct cytotoxicity by disrupting cell membranes and causing calcium influx, platelet aggregation, and coagulation activation, and cytokine surge, which is, more importantly, responsible for enhanced inflammatory state [5]. In addition, increased concentrations of extracellular histones have also been clinically associated with disease progression and outcome in patients with sepsis, cancer, stroke, organ injury, anemia, virus/bacterial infections, and autoimmune diseases [6,7,11,18,23–25]. It is known that tuberculosis-related pleural effusions may result from the infiltration of the pleural space by *M. tuberculosis* antigens or bacilli [2]. However, it is uncertain whether extracellular histones are involved in disease progression related to TPE.

Table 2. Correlation of extracellular histones with various variables.

	TPE patients (n=58)	
	r	P
LDH	0.3267	0.028
MPO	0.6525	<0.0001
S100A8/A9	0.5108	0.002
IL-1 β	0.3923	0.008
IL-6	0.6104	<0.0001
IL-10	0.5835	<0.0001
IL-17A	0.3284	0.021
IL-18	0.4218	0.005
IL-21	0.4083	0.005
IL-27	0.5723	<0.0001
IP-10	0.4635	0.003
IFN- γ	0.6012	<0.0001
MCP-1	0.3756	0.015

* p<0.05 was considered to be statistically significant.

Our results showed that extracellular histones were significantly increased in TPE in contrast to those in corresponding blood or blood from healthy controls. TPE samples have higher concentrations of extracellular histones than the corresponding serum samples in these patients, indicative of a more severe pathological status in TPE. We were thus intrigued to check cellular origins of extracellular histones in TPE, and we observed that the elevated concentrations of extracellular histones may be originated from damaged pleural mesothelium as well as massive inflammatory cell infiltration during tuberculosis infection. However, other origins of extracellular histones still need to be explored.

High concentrations of extracellular histones in TPE may play a toxic role associated with disease progression, because they are known to possess many biological effects, particularly inflammation-promoting effects [5]. We checked systemic inflammation in TPE by measuring multiple cytokines and observed that a couple of canonical pro-inflammatory cytokines were remarkably increased, demonstrating the occurrence of enhanced inflammation in TPE. A further correlation analysis indicated a positive correlation between high levels of extracellular histones and high levels of inflammatory cytokines in TPE. Collectively these studies provide evidence supporting that

elevated extracellular histones in TPE during the course of tuberculosis may accelerate systemic inflammation.

Compared with peripheral blood, TPE is enriched with proteins and cells, so it is not straightforward to identify one or several mediators or cells associated mostly with TPE [26]. Wang et al. reported that T helper type 17 cells were markedly increased in TPE, resulting in an elevation of their effective cytokines including IL-6, IL-17, and IFN- γ [27]. Conversely, Chen et al., demonstrated that the frequency of Th17 cells in patients with active tuberculosis was lower than patients with latent tuberculosis infection or healthy controls [28]. More studies are required to solve these discrepancies. Indeed, many available research reports focused solely on the role of single cytokine in TPE. For example, Wang et al. reported that IL-27 was elevated in TPE and may serve as a sensitive biomarker for disease diagnosis [29]. High levels of IFN- γ have also been documented in TPE, which may play an immuno-regulatory role in the development of TPE [30]. Measurement of IFN- γ is likely to be a useful diagnostic tool for TPE [31]. Dong et al. disclosed that IL-35, as a novel anti-inflammatory and immunosuppressive cytokine, was increased in TPE and may play a pathological or targetable role for TPE [4]. So far, we described for the first time an inflammatory role of extracellular histones in TPE, which may help unravel the inflammatory processes associated with TPE, because extracellular histones appear to be an upstream factor implicated in systemic inflammation. In addition, targeting extracellular histones have been demonstrated to be protective in many inflammatory injuries [6], thereby leading us to hypothesize that histone-targeted interventions may have potentials in the treatment of TPE.

Conclusions

High concentrations of extracellular histones were predominantly present in TPE as compared with the serum from the same patients or healthy controls, and massive cellular death or inflammatory cell degradation may be the origins of extracellular histones. Extracellular histones may aggravate the inflammatory processes involved with TPE. Extracellular histones may be considered as a potential biomarker for TPE, and could be used to diagnose, monitor or improve the prognosis of TPE. More studies are needed to explore their functions.

Conflict of interest

None.

References:

- Vorster MJ, Allwood BW, Diacon AH et al: Tuberculous pleural effusions: Advances and controversies. *J Thorac Dis*, 2015; 7(6): 981–91
- Zhai K, Lu Y, Shi HZ: Tuberculous pleural effusion. *J Thorac Dis*, 2016; 8(7): E486–94
- Srinivasan L, Ahlbrand S, Briken V: Interaction of *Mycobacterium tuberculosis* with host cell death pathways. *Cold Spring Harb Perspect Med*, 2014; 4(8): pii: a022459
- Dong X, Yang J: High IL-35 pleural expression in patients with tuberculous pleural effusion. *Med Sci Monit*, 2015; 21: 1261–68
- Allam R, Kumar SV, Darisipudi MN et al: Extracellular histones in tissue injury and inflammation. *J Mol Med (Berl)*, 2014; 92(5): 465–72
- Chen R, Kang R, Fan XG et al: Release and activity of histone in diseases. *Cell Death Dis*, 2014; 5: e1370
- Silk E, Zhao H, Weng H et al: The role of extracellular histone in organ injury. *Cell Death Dis*, 2017; 8(5): e2812
- Perez-Cremades D, Bueno-Beti C, Garcia-Gimenez JL et al: Extracellular histones disarrange vasoactive mediators release through a COX-NOS interaction in human endothelial cells. *J Cell Mol Med*, 2017; 21(8): 1584–92
- Lv X, Wen T, Song J et al: Extracellular histones are clinically relevant mediators in the pathogenesis of acute respiratory distress syndrome. *Respir Res*, 2017; 18(1): 165
- Kumar SV, Kulkarni OP, Mulay SR et al: Neutrophil extracellular trap-related extracellular histones cause vascular necrosis in severe GN. *J Am Soc Nephrol*, 2015; 26(10): 2399–413
- Kordbacheh F, O'Meara CH, Coupland LA et al: Extracellular histones induce erythrocyte fragility and anemia. *Blood*, 2017; 130(26): 2884–88
- Alhamdi Y, Toh CH: The role of extracellular histones in haematological disorders. *Br J Haematol*, 2016; 173(5): 805–11
- Liaw PC, Ito T, Iba T et al: DAMP and DIC: The role of extracellular DNA and DNA-binding proteins in the pathogenesis of DIC. *Blood Rev*, 2016; 30(4): 257–61
- Bosmann M, Grailer JJ, Ruemmler R et al: Extracellular histones are essential effectors of C5aR- and C5L2-mediated tissue damage and inflammation in acute lung injury. *FASEB J*, 2013; 27(12): 5010–21
- Allam R, Darisipudi MN, Tschopp J et al: Histones trigger sterile inflammation by activating the NLRP3 inflammasome. *Eur J Immunol*, 2013; 43(12): 3336–42
- Wygrecka M, Kosanovic D, Wujak L et al: Antihistone properties of C1 esterase inhibitor protect against lung injury. *Am J Respir Crit Care Med*, 2017; 196(2): 186–99
- Chaaban H, Keshari RS, Silasi-Mansat R et al: Inter-alpha inhibitor protein and its associated glycosaminoglycans protect against histone-induced injury. *Blood*, 2015; 125(14): 2286–96
- Ashar HK, Mueller NC, Rudd JM et al: The role of extracellular histones in influenza virus pathogenesis. *Am J Pathol*, 2018; 188(1): 135–48
- Wen Z, Lei Z, Yao L et al: Circulating histones are major mediators of systemic inflammation and cellular injury in patients with acute liver failure. *Cell Death Dis*, 2016; 7(9): e2391
- Zhang Y, Wen Z, Guan L et al: Extracellular histones play an inflammatory role in acid aspiration-induced acute respiratory distress syndrome. *Anesthesiology*, 2015; 122(1): 127–39
- Walscheid K, Heiligenhaus A, Holzinger D et al: Elevated S100A8/A9 and S100A12 serum levels reflect intraocular inflammation in juvenile idiopathic arthritis-associated uveitis: Results from a pilot study. *Invest Ophthalmol Vis Sci*, 2015; 56(13): 7653–60
- Marsman G, Zeerleder S, Luken BM: Extracellular histones, cell-free DNA, or nucleosomes: differences in immunostimulation. *Cell Death Dis*, 2016; 7(12): e2518
- Xu J, Zhang X, Pelayo R et al: Extracellular histones are major mediators of death in sepsis. *Nat Med*, 2009; 15(11): 1318–21
- Yang R, Zou X, Tenhunen J et al: HMGB1 and extracellular histones significantly contribute to systemic inflammation and multiple organ failure in acute liver failure. *Mediators Inflamm*, 2017; 2017: 5928078
- Szatmary P, Liu T, Abrams ST et al: Systemic histone release disrupts plasmalemma and contributes to necrosis in acute pancreatitis. *Pancreatology*, 2017; 17(6): 884–92
- Tong ZH, Shi HZ: Subpopulations of helper T lymphocytes in tuberculous pleurisy. *Tuberculosis (Edinb)*, 2013; 93(3): 279–84
- Wang T, Lv M, Qian Q et al: Increased frequencies of T helper type 17 cells in tuberculous pleural effusion. *Tuberculosis (Edinb)*, 2011; 91(3): 231–37
- Chen X, Zhang M, Liao M et al: Reduced Th17 response in patients with tuberculosis correlates with IL-6R expression on CD4+ T Cells. *Am J Respir Crit Care Med*, 2010; 181(7): 734–42
- Wang W, Zhou Q, Zhai K et al: Diagnostic accuracy of interleukin 27 for tuberculous pleural effusion: Two prospective studies and one meta-analysis. *Thorax*, 2018; 73(3): 240–47
- Zhao J, Shiratori B, Chagan-Yasutan H et al: Secretion of IFN-gamma associated with Galectin-9 production by pleural fluid cells from a patient with extrapulmonary tuberculosis. *Int J Mol Sci*, 2017; 18(7): pii: E1382
- Keng LT, Shu CC, Chen JY et al: Evaluating pleural ADA, ADA2, IFN-gamma and IGRA for diagnosing tuberculous pleurisy. *J Infect*, 2013; 67(4): 294–302