Correlation of peripheral blood monocyte and neutrophil direct counts with plasma inflammatory cytokines and TNF-α soluble receptors in the initial phase of acute pancreatitis

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Abstract

Material: The relationship between direct count of peripheral blood leucocyte populations and plasma concentrations of IL-6, IL-8, sTNFR-55 and sTNFR-75 during five initial days of acute pancreatitis was studied.

Results: Most significant relationship was found for monocytes, which correlated with sTNFR-55 ($R=0.38$, $p<0.05$) and sTNFR-75 ($R=0.41$, $p< 0.05$ and $R=0.55$, $p<0.01$ during 1st and 2nd day, respectively). Later, in days 2, 3 and 4 an interrelation between monocytes and IL-6 ($R= 0.49$ to $R=0.41$, $p <0.01$) was observed. Monocytes also correlated with IL-8 in days 2 and 3 ($R=0.41$, $p<0.05$ and $R=0.43$, $p<0.01$, respectively). Neutrophil count correlated with IL-6 in days 3 and 4 ($R=0.34$, $p<0.05$ and $R=0.56$, $p<0.01$, respectively) and with IL-8 in the 4th day only ($R=0.39$, $p<0.05$). No significant correlations of lymphocyte, eosinophil and basophil direct counts with cytokines and receptors during the initial 5 days of AP were found.

Conclusions: Observed relationships between monocyte direct counts and plasma cytokine levels reflect monocytes involvement in the development of acute pancreatitis.

Key words: acute pancreatitis, inflammatory mediators, white blood cell counts.

Introduction

Acute pancreatitis (AP) involves interstitial activation of pancreatic secretory proteases and damage of the pancreatic tissue [1,2] which process is a source of chemotactic peptides stimulating blood phagocytes [3]. Subsequently, stimulated phagocytes release a set of cytokines, including IL-1, TNFα, IL-8 and IL-6 [4], and mobilize blood neutrophils to accumulate in the inflammed pancreas. In effect, the increase of white blood cell (WBC) count is one of the most pronounced signs, reflecting functional stimulation of neutrophils and mononuclear phagocytes in developing AP [4-6]. Therefore, beside the organ-visualization methods presently used, the WBC count is still considered as a useful sign of inflammatory diseases in patients with abdominal pain [7,8]. WBC count may also indicate a severe disease course with generalized response to inflammation [9,10].

White blood cells include several populations of various cells which play different roles in the immune reaction to infection, tissue injury and tissue necrosis. Mutual interaction between inflammatory cytokines and phagocytes play a critical role in the development of AP. Several recent papers focused on the effects of AP on functional stimulation of blood lymphocytes [11-13] and monocytes [4,11,14]. These studies, employing flow cytometry, focused on specific antigens on the surface of monocytes and lymphocytes [4,11,12,14]. On the other hand, rather few studies refer to changes in direct counts of individual blood inflammatory cells and inflammatory cytokines in the course of AP. The aim of this study was to follow the assumed relationships between direct counts of the WBC populations and plasma concentration of interleukines -6 and -8 (IL-6 and IL-8) and soluble receptors of tumor necrosis factor (sTNFR55 and sTNFR75) in patients with the initial phase of AP.

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Material and methods

The studied group consisted of 56 patients (21 females and 35 males, age 22 to 92 years, mean: 52.8±16.3) admitted to the 2nd Department of General Surgery, Jagiellonian University School of Medicine in Kraków that were diagnosed as AP. All patients in the studied were admitted to the hospital no later than 48 hours after an attack of acute symptoms. Reference group for comparison was composed of 30 healthy subjects (15 females and 15 males, age 36.6±10.3 years) who were subjected to routine health check-up. These persons agreed for use of their test results for research purposes. The study was approved by the Bioethical Committee of the Jagiellonian University and all patients provided an informed consent for their participation.

Diagnosis of AP was based on typical clinical symptoms, such as abdominal pain, fever, nausea and vomiting associated with increased serum amylase activity three times above the upper reference limit (normal range: 0-220 U/L). All patients were subjected to standard physical examination followed by ultrasound examination of the abdomen. In patients diagnosed in respect of developing pancreatic necrosis, the contrast-enhanced abdominal computed tomography (CT) scans were performed at admission and after 48 hours. The severity of AP and concomitant complications were assessed according to the Atlanta Classification. 36 patients were finally diagnosed as mild AP and 20 patients as severe AP. Among 20 patients with severe AP, 7 developed organ failure and 13 local complications such as: necrosis (n=9), pseudocyst (n=3) and abscess (n=1). Seven patients underwent surgical intervention (necrosectomy and lavage) due to infected pancreatic necrosis and a deteriorating clinical condition despite ICU treatment. Five patients died. The etiology of AP included: gallstones in 35, alcohol in 16 and idiopathic origin in 5 patients.

Blood samples for standard laboratory tests and cytokine assays were collected by puncturing cubital vein in to Becton Dickinson Vacutainer tubes (Beckton Dickinson – USA) from each patients on admission and then daily for five consecutive days. Laboratory blood tests assessing patients clinical status were routinely performed at admission and then at 24, 48, 72 and 96 hours respectively. Cytokine concentrations were measured in serum. IL-8 concentration was determined with a solid phase Enzyme Amplified Sensitivity Immunoassay (IL-8 EASIA™ Biosource Europe S.A., Belgium). Concentration of IL-8 was expressed as pg/ml and minimum detectable concentration (MDC) for this test was 0.7 pg/ml. IL-6 concentration expressed as pg/ml was measured using the IL-6 EASIA™ kit (Medgenics Diagnostics S.A., Belgium) and the MDC of this assay was 2.0 pg/ml. Serum sTNFR55 (sTNFRI) and sTNFR75 (sTNFRII) were measured by an ELISA assay with monoclonal and polyclonal anti-sTNFR55 and anti-sTNFR75 antibodies (MEDGENIX COMBO sTNFRI/sTNFRII kit, Biosource Europe S.A., Belgium). Purified sTNFR55 and sTNFR75 were used to construct standard curves. The lower limits of detection for assays were 0.05 ng/ml and 0.02 ng/ml for sTNFR55 and sTNFR75, respectively.

Blood for hematological examination was collected into Vacutainer tubes with $EDTAK_2$ solution. The hematology test profile included: total erythrocyte count and erythrocyte indice values, platelet count, and direct and differential counts of neutrophils, lymphocytes, eosinophils, basophils and monocytes. Blood cell counting was performed with ABX Vega Retic hematological analyzer using 5-diff leucocyte differentiation system. Results are expressed as direct WBC count $\times 10^3/\mu l$. Individual populations of the white blood cells were counted in three separate measuring channels designed for:

1. Total white blood cells counting (WBC – channel);
2. Basophils counting (BASO – channel);
3. Lymphocyte, monocyte, neutrophil and eosinophil counting (LMNE – channel).

The ABX differential WBC counting is automatically performed by the analyzer. Differentiation of the individual cells was based on assessment of cell volume by high frequency alternative current impedance and laser light scattering mode depending on size of the nucleus and number of cytoplasmic granules (MAPS technique). At the first step of leukocyte differential counting the procedure of erythrocyte removal from the sample by addition of 500 µl of VEGALYSE™ solution was performed. The ABX Vega hematological analyzer also differentiates "large lymphocytes", registered as "Atypical Lymphocytes" (ALY) and calculates abnormal immature forms of polymorphonuclear neutrophilic granulocytes marked as "Large Immature Cells" (LIC). However, fractions of ALY and LIC cells were not followed in the present study. Results of differential percentage counts and direct counts are expressed in number of cells $\times 10^3/\mu l$ of the studied blood. The results of the automated blood counting were validated by systematic daily quality control using EightCheck-3WP; ICN blood control samples covering "normal", "high" and "low" ranges obtained from RIQAS USA. Occasional tests of random blood samples from healthy blood donors were also verified by microscopic analysis (to follow performance of the analyzer on fresh blood samples).

All variables had non-normal distribution and were presented as median and range. Mann-Whitney U-test was used to assess differences between groups and correlations between variables were calculated with Spearman coefficient. P level of <0.05 was considered statistically significant. Statistical analysis was performed with Statistica 6.0 software (StatSoft Inc., Tulsa, USA).

Results

As expected, in patients with acute pancreatitis the total WBC count values were significantly elevated in the entire observation period. The highest mean WBC count value was observed in the first day of hospitalization, and then steadily decreased in the following days (*Tab. 1*). A markedly wide scatter range of the WBC counts was observed in all days following admission. This was an apparent effect of increasing diversification of disease severity in individual patients, along with time of initial appearance of the disease symptoms.

The white blood cell populations which counts most significantly correlated with plasma inflammatory cytokine levels were peripheral blood monocytes. Monocyte direct counts in the initial days of AP were significantly higher than that

Table 1. **White blood cell counts and cytokines concentrations in patients with AP in five consecutive days since admission. Data are expressed as median and range. Day 1 is the day of patient admission to hospital**

Parameter studied	Patients group (N=56)					Reference group (N=30)
	Day 1	Day 2	Day 3	Day 4	Day 5	
Total WBC count (10^3/μl)	11.40* 3.20-21.20	9.70* 2.40-22.40	8.70* 3.70-17.60	7.40 3.10-19.50	7.65 3.80-18.60	5.6 3.5-11.7
Neutrophils (10^3/μl)	8.15* 2.75-16.20	6.10 1.12-16.30	5.86 2.13-15.69	4.46 1.85-13.10	5.27 2.21-14.00	5.11 4.23-6.47
Lymphocytes (10^3/μl)	1.10 0.39-3.27	1.15 0.22-2.61	1.25 0.32-3.24	1.11 0.49-2.63	1.12 0.64-2.28	1.7 1.5-3.0
Monocytes (10^3/μl)	0.75* 0.25-1.56	0.54 0.20-1.40	0.57 0.18-2.26	0.52 0.17-2.54	0.58 0.13-2.05	0.40 0.3-0.5
Eosinophils (10^3/μl)	0.11 0.00-0.70	0.13 0.00-0.55	0.18 0.05-0.56	0.18 0.03-0.56	0.18 0.04-0.49	0.20 0.05-0.25
Basophils (10^3/μl)	0.02 0.00-0.12	0.02 0.00-0.12	0.02 0.00-0.15	0.02 0.00-0.13	0.02 0.00-0.19	0.19 0.1-0.27
IL-8 (pg/ml)	47.11 0.50-1043.8	73.16*** 1.25-1204.0	21.07*** 1.14-101.5	18.06*** 1.18-118.9	18.10*** 0.28-86.07	2.25*** 0.07-4.11
IL-6 (pg/ml)	474.79*** 2.0-10000	330.19*** 2.0-4300	171.29*** 2.0-2100	83.50*** 2.0-510.0	78.71*** 2.0-332.0	8.55*** 0-10.0
sTNF55 (ng/ml)	8.93*** 0.68-73.6	7.45** 0.45-40.32	6.96** 0.98-27.28	6.26** 0.88-23.28	6.53** 0.45-21.04	2.19** 0.58-3.26
sTNFR75 (ng/ml)	14.96*** 3.56-64.51	12.26*** 2.66-46.56	11.66* 2.32-32.24	11.49*** 2.19-31.98	11.24*** 2.29-33.40	3.54** 1.67-6.40

P<0.05*; P<0.01**; P<0.001***

Figure 1. **Monocytes direct count values (mean ±SD; 10^3/μl) in patients with AP during initial five days after admission. The means did not differ significantly (p<0.05)**

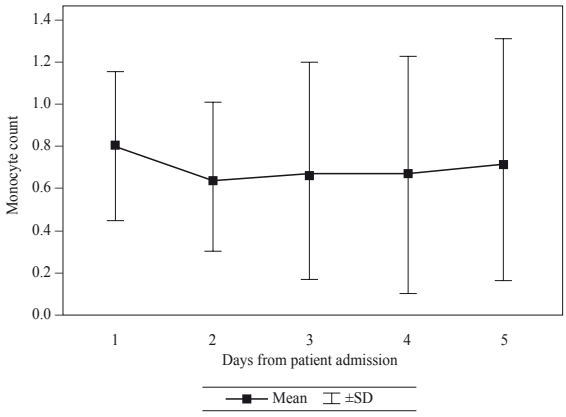
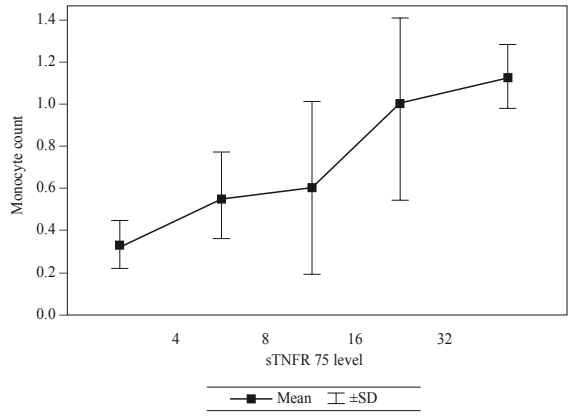


Figure 2. **Interrelation between monocyte mean values (10^3/μl) and the respective means of sTNFR75 (ng/ml) on the 2nd day after admission. The bars represent SD for the calculated mean values. The same relationship is expressed by regression equation: monocytes (10^3/μl)=0.066+0.184 x $\log_2$ sTNFR75 (ng/ml). The correlation coefficient between monocytes and sTNFR75 (R=0.55; p<0.01)**



in healthy patients (*Tab. 1*). The scatter range of monocyte counts increased in days following admission (*Fig. 1*). There was an evident interrelation between the sTNFR75 and direct monocyte count (*Fig. 2*). On the 2nd day after admission, this relationship in terms of regression manifested as the following equation: monocytes (10^3/μl) =0.066+0.184 x $\log_2$ sTNFR75 (ng/ml). Monocyte direct count significantly correlated with both sTNFR55 and sTNFR75 in the initial two days of hospitalization (*Tab. 2*). Then, in days 2, 3 and 4 there was a significant interrelation between monocytes and IL-6 (*Tab. 2*). This interrelation manifested as a highly significant correlation in days 2 and 3 (R=0.49, p<0.01) and at day 4 (R=0.41, p<0.05) (*Fig. 3*). There was also a moderate correlation with IL-8 at days 2 and 3, respectively (*Tab. 2*, *Fig. 4*).

The total WBC count significantly correlated with IL-6 plasma level in days 2, 3, 4 and 5 after admission; whereas IL-8 correlated with WBC count in days 2 and 3 only (*Tab. 3*). Also sTNFR55 correlated with WBC count in days 3 and 4 only. On the other hand no significant correlation in the entire 5-days observation period was found for WBC count and sTNFR75. Also, none of the cytokines and receptors studied correlated with the WBC count in the first day of observation (*Tab. 3*). Relationship between IL-6 levels and WBC counts in day 3 was expressed as regression equation: WBC (10^3/μl) =6.131+2.111 x $\log_{10}$ IL-6 (pg/ml).

***Table 2*. Correlations of direct monocyte counts with proinflammatory cytokine plasma levels in patients with AP during five consecutive days since admission**

Observation day	Spearman rank correlation coefficient R			
	sTNFR-55	sTNFR-75	IL-6	IL-8
1	0.38 (p=0.007)	0.41 (p=0.005)	0.26 (p=0.055)	0.22 (p=0.121)
2	0.38 (p=0.007)	0.55 (p<0.001)	0.49 (p<0.001)	0.41 (p=0.005)
3	0.25 (p=0.077)	0.20 (p=0.199)	0.49 (p<0.001)	0.43 (p=0.002)
4	0.24 (p=0.095)	0.25 (p=0.078)	0.41 (p=0.003)	0.26 (p=0.057)
5	0.23 (p=0.103)	0.26 (p=0.057)	0.25 (p=0.077)	0.26 (p=0.058)

Observation day 1 is the day of admission

***Table 3*. Correlations of total WBC counts with cytokine plasma and soluble TNF-α receptors levels in patients with AP during five consecutive days since admission**

Observation day	Spearman rank correlation coefficient R			
	sTNFR-55	sTNFR-75	IL-6	IL-8
1	0.18 (p=0.201)	0.16 (p=0.264)	0.13 (p=0.376)	0.12 (p=0.406)
2	0.12 (p=0.410)	0.23 (p=0.100)	0.30 (p=0.033)	0.27 (p=0.049)
3	0.31 (p=0.026)	0.21 (p=0.144)	0.44 (p=0.001)	0.29 (p=0.041)
4	0.34 (p=0.016)	0.20 (p=0.174)	0.39 (p=0.005)	0.16 (p=0.262)
5	0.26 (p=0.058)	0.19 (p=0.189)	0.39 (p=0.005)	0.22 (p=0.120)

Observation day 1 is the day of admission

***Figure 3*. A linear regression for interdependence between monocyte direct count (10^3/μl) and IL-6 levels (pg/ml) in plasma on the 3rd day after admission. The calculated regression is expressed by equation: monocytes (10^3/μl) =0.153+0.401 x $\log_{10}$ IL-6 (pg/ml)**

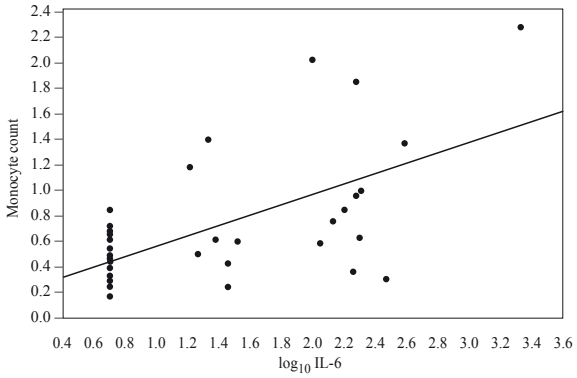
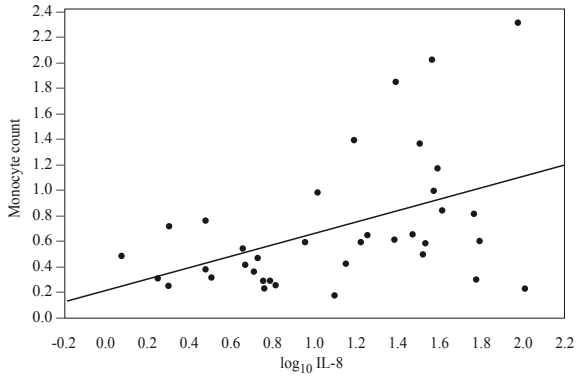


***Figure 4*. Regression for interdependence between monocytes direct count (10^3/μl) and IL-8 (pg/ml) on the 3rd day after admission. The calculated regression is expressed by the equation: monocytes (10^3/μl)=0.219+0.439 x $\log_{10}$ IL-8 (pg/ml)**



Neutrophils which compose a major fraction of the WBC count tended to follow the total WBC count changes. A markedly wide scatter range of the neutrophil direct counts in days following admission were observed. The direct neutrophil counts showed an increasing correlation with IL-6 in days 3 and 4 (R=0.34, p<0.05 and R=0.56, p<0.01, respectively); while IL-8 correlated with direct neutrophil count in the 4th day only (R=0.39, p<0.05).

The lymphocyte counts in AP are present in *Tab. 1*. Though patients with AP had generally lower direct lymphocyte count values than healthy persons, no significant trend of changes in the studied days was observed. Also, no significant changes in eosinophil and basophil direct counts in patients with AP during the initial 5 days after admission were found. Moreover, no significant correlations between lymphocyte, eosinophil and basophil direct counts and cytokines or soluble receptors studied were found.

Discussion

The initial phase of AP development is difficult to study due to inevitable delay between the onset of disease symptoms and presentation of patients in the admission room. Mean delay time of the studied patients was about 24 hours. To study interrelation between inflammatory cytokine levels and individual WBC subsets collection of a group of patients both with mild and severe course of AP was necessary. Therefore, our AP group represents the whole range of symptoms related to developing inflammation, broad enough to disclose existing correlations between the parameters studied. Such correlations may remain unnoticeable in studies concerning mechanisms of inflammation in acute pancreatitis, especially in experimental animals representing uniform severity and course of disease. As expected, results of carried out studies have shown some regularity in relationship between subsets of white blood cell populations, plasma inflammatory cytokines and soluble TNFα receptors.

The most pronounced correlation was found for monocytes which significantly correlated with sTNFR55 and sTNFR75 in the first and second observation days; and then with IL-6 in days 2, 3 and 4. Monocyte count also correlated with IL-8 in days 2 and 3, respectively (*Tab. 2*). The initial correlations with sTNFR55 and sTNFR75 are replaced by correlations with IL-8 and IL-6 that dominated in days 3 and 4. These results are in accordance with results of other studies indicating that blood monocytes play a main role in the enhancement of the inflammatory process and in the development of life threatening systemic inflammatory reaction syndrome (SIRS) [15]. The assay of peripheral blood monocyte direct count provides immediate information on the cytokine output in the developing inflammation.

There is a common notion that an increase in the number of neutrophils, which are directly involved in the pancreas injury process [16-18], reflects severity of developing acute pancreatitis [5,9,19]. In our studies, neutrophils correlated with plasma IL-6 and IL-8 levels in days 3 and 4. This is in accord with data indicating that IL-8 is the cytokine responsible for neutrophil stimulation in the inflammatory site [20,21]. However, in our studies the neutrophils – IL-8 interrelation was less pronounced than that of monocytes and sTNFR55 and IL-6, as well as the neutrophils – IL-6 relationship .The total WBC counts correlate similarly to that of neutrophils with IL-8, IL-6 in days 2 and 3 and with IL-6 in day 4. This is in accord with the phenomenon that in acute inflammation the WBC count increase is mostly due to the increase in neutrophils direct count. Neutrophil count (as well as total WBC count) increase in the later disease phase when IL-6 and IL-8 stimulation dominates. This may reflect the evolution of pancreas injury, which (in humans) usually leads to necrotic lesions at 48 hours after onset of pancreatitis acute symptoms [25]. Neutrophils react secondarily to monocytes and macrophages – releasing the TNFα, IL-1β, IL-6 and IL-8 [3,18] as primary mediators of developing inflammation [4]. On the other hand, neutrophils are the main source of IL-8 in the later phase of the inflammatory process [3].

De la Mano et al. [22] studied the role of circulating inflammatory blood cells in pathogenesis of AP in rats subjected to pancreatic duct obstruction. The authors noticed a significant increase in direct neutrophil and monocyte counts, which peaked at the 6th hour after pancreatic duct obstruction and returned to normal thereafter. The activation of circulating monocytes was reflected by CD11b antigen expression and the TNF output. Lymphocytes, as well as CD4+ and CD8+ cell subsets increased at the earlier stages after pancreatic duct obstruction and progressively decreased thereafter. The TNFα level increased in the 12th h after inducing AP and was paralleled by a spontaneous production of TNFα by monocytes; while no TNFα neither IL-10 were produced by circulating T cells. This study indicates a central role of peripheral blood monocytes in the systemic inflammatory response induced by severe AP caused by pancreatic duct obstruction. Blood monocytes in AP increase interleukin 1 receptor expression and decrease the HLA-DR surface antigen expression both in the initial phase of disease and in the subsequent days of the disease. Onset of AP is accompanied by low levels of anti-inflammatory cytokines IL-4, IL-10 and IL-13. Moreover, the severity of the disease is related to the concentration of IL-6 and IL-10 at the admission time [13,14]. Development of acute pancreatitis depends on contradictory action of stimuli enhancing the inflammatory process, derived from activated neutrophils infiltrating the injured pancreas [23] and on action of anti-inflammatory cytokines attenuating release of IL-8 and TNFα [24]. Usually the activity of the inflammatory process is regulated by a feed-back type relationship between the pro-inflammatory cytokine IL-1β and anti-inflammatory IL-10 [25]. However, a high concentration of neutrophil deriving mediators originating from the inflammatory core in the pancreas may eventually abolish this regulatory mechanism, leading to an uncontrolled general inflammatory reaction. Therefore, monocytes are both the source of pro-inflammatory cytokines and the effectors cells reacting to various inflammatory stimuli [4,11,26].

In acute pancreatitis, a decrease in direct lymphocyte count indicates the development of severe course of disease [11], while increase in neutrophile/lymphocyte ratio above 5.3 predicts pancreatic necrosis. In patients with severe acute pancreatitis with pancreatic necrosis, activated lymphocyte subset CD19+ was significantly higher (46±16.6% versus 26.4±14.6%) then in mild acute pancreatitis [11]. This effect, however, was observed when severe complications of disease were already fully developed.

Results obtained in this study indicating increase in concentration of inflammatory IL-6 and soluble plasma TNF-α receptors in early phase of AP are in accord with recent findings that AP development involves counteraction between primary proinflammatory stimuli with systemic release of anti-inflammatory cytokines [14-16]. This process influences mobilization of blood inflammatory cells and the described relationships may add to symptoms disclosing development of “severe acute pancreatitis”.

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