Distinct cytokine profiles of systemic-onset juvenile idiopathic arthritis-associated macrophage activation syndrome with particular emphasis on the role of Interleukin-18 in its pathogenesis

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Short title: Cytokine profile in systemic-onset juvenile idiopathic arthritis-associated macrophage activation syndrome

Key message:

1. IL-18 is an important mediator in s-JIA.
2. The cytokine release pattern is different among patients with different aetiologies of their in MAS/HLH.
3. Monitoring the cytokine profile may be useful for the evaluation of disease activity in s-JIA.

Abstract

Objectives

To compare the pro-inflammatory cytokine profiles and the cytokine kinetics in patients with secondary macrophage activation syndrome (MAS) due to systemic-onset juvenile idiopathic arthritis (s-JIA) and in both active and inactive disease states of s-JIA (but no MAS), with those demonstrated in EBV-induced hemophagocytic lymphohistiocytosis (HLH) and Kawasaki disease (KD), and to investigate the significance of interleukin (IL)-18 in the pathogenesis of s-JIA.

Methods

Five patients with MAS complicating s-JIA (MAS/s-JIA), 10 with HLH due to Epstein-Barr virus infection (EBV-HLH), 22 with Kawasaki disease (KD), and 28 healthy controls were analyzed. Cytokine concentrations (IL-18, IL-6, neopterin, tumor necrosis factor-α receptor types I and II) were quantified in serum by enzyme-linked immunosorbent assay. Results were compared with clinical features of MAS/s-JIA, including ferritin levels.

Results

Serum IL-18 concentrations in MAS/s-JIA patients were significantly higher than those in EBV-HLH or KD patients ($p<0.05$). Serum IL-6 concentrations in KD patients were significantly higher than those in EBV-HLH or MAS/s-JIA patients. Serum neopterin concentrations in EBV-HLH patients were significantly higher than those in MAS/s-JIA or KD patients. Serum IL-18 correlated positively with the following measures of disease activity: C-reactive protein, ferritin, lactate dehydrogenase, and other cytokines ($p<0.05$). Serum concentrations of IL-18 in s-JIA patients remained elevated in the

inactive phase of disease, while clinical parameters and other cytokines normalized.

Conclusions

IL-18 may be an important mediator in s-JIA. Although serum Il-18 concentrations correlated with markers of disease activity, IL-18 concentrations remained elevated even when other markers of disease activity normalized. Serum IL-18 concentration may be a promising indicator of disease activity. The cytokine release pattern in MAS/HLH is different among patients with different aetiologies. Monitoring the cytokine profile, including IL-18, may be useful for differentiation of MAS/HLH and evaluation of disease activity in s-JIA.

Introduction

Macrophage activation syndrome (MAS) is a severe, potentially life-threatening complication of childhood systemic inflammatory disorders [1,2]. It is clinically characterized by fever, hepatosplenomegaly, lymphadenopathy, profound depression of all 3 blood cell lines, deranged liver function, intravascular coagulation, and central nervous system dysfunction. Among pediatric rheumatic diseases, MAS occurs most often in children with systemic-onset juvenile idiopathic arthritis (s-JIA) and is less common in those with other rheumatic diseases, including polyarticular JIA, systemic lupus erythematosus, and Kawasaki disease (KD) [3-5]. MAS accounts for much of the significant morbidity and mortality observed with s-JIA. A variety of triggers have been implicated in the pathogenesis of MAS associated with s-JIA, including viral infections, nonsteroidal anti-inflammatory drug therapy, methotrexate, and etanercept [1,3,6]. The hallmark of this syndrome is excessive activation and proliferation of T lymphocytes and macrophages[7]. MAS bears a close resemblance to a group of hemophagocytic lymphohistiocytosis (HLH) syndromes and should be considered among the secondary causes of HLH [8,9]. Massive hypercytokinemia is strongly associated with the pathogenesis of MAS/HLH [3]; however, the kinetics of cytokine release in patients with MAS/HLH is still unclear.

Interleukin (IL)-18 was originally described as an interferon-γ-inducing factor mainly produced by activated macrophage lineage cells [10,11]. IL-18 stimulates a variety of inflammatory responses, enhances proliferation and activity of T cells and natural killer cells, and shifts helper T cell balance towards Th1 response [12,13]. IL-18 has been reported to enhance

production of Th2 cytokines and IgE and recruitment of eosinophils, suggesting that IL-18 can also regulate allergic inflammation [14]. Some reports have recently shown that serum levels of IL-18 are highly elevated in patients with s-JIA [15-17].

To assess the kinetics of cytokine release during MAS/HLH, we measured the concentrations of serum cytokines, including IL-18, IL-6, neopterin, and tumor necrosis factor (TNF)-α receptor type I (sTNFRI) and type II (sTNFRII) in patients with MAS/s-JIA. We compared them with the concentrations in patients with HLH due to Epstein-Barr virus infection (EBV-HLH) and KD, which are both characterized by prominent and systemic inflammation in children. We determined the correlation between the levels of such markers of cytokine release with measures of disease activity and severity in order to clarify the importance of IL-18 in the pathogenesis of not only MAS but also s-JIA.

Materials and Methods

Patients and samples

Serum samples were obtained from 5 patients with MAS as a complication of s-JIA, 10 with EBV-HLH, 22 with KD, and 28 age and sex matched healthy controls (HC) [age(MAS/s-JIA:5.8±6.8years control:8.8±7.3years)]. Samples from MAS/s-JIA patients were also obtained during both the active and inactive phases of the s-JIA disease, but where MAS was not present. Diagnosis of s-JIA was based on the International League of Associations for Rheumatology criteria [18]. MAS was diagnosed based on the combination of cytopenias affecting at least 2 cell lines, coagulopathy, and liver dysfunction (Table 1), according to the guidelines proposed by Ravelli et al [19]. The

criteria for the active phase of s-JIA was defined as follows: active arthritis, fever, rash, hepatosplenomegaly, generalized lymphadenopathy, active uveitis and serositis as well as increased erythrocyte sedimentation rate and C-reactive protein (CRP) levels, but where criteria noted in the guidelines for MAS proposed by Ravelli et al were not fulfilled. The criteria for the inactive phase of s-JIA on medication were as follows: no clinical symptoms which can be seen in active phase as well as normal erythrocyte sedimentation rate and C-reactive protein (CRP) levels. All patients of EBV-HLH fulfilled the diagnostic criteria for EBV-HLH [20], positivity for the EBV genome in the blood/bone marrow and other tissues (determined by polymerase chain reaction (PCR), Southern blot and/or *in situ* hybridization for EBER) and positive anti-viral capsid antigen (VCA)-specific-IgG. Diagnosis of KD was based on the classic clinical criteria [21]. Serum was separated from cells, divided into aliquots, frozen, and stored at −80 degree Celsius until use. This study was approved by the Institutional Review Board at Kanazawa University and all specimens were used after the receipt of informed consent.

Quantification of serum cytokines

Serum concentrations of IL-18, IL-6, neopterin, and TNF-α receptor (sTNFR) types I and II were evaluated by commercial enzyme-linked immunosorbent assay according to the manufacturer's instructions (IL-18:MBL, Nagoya, Japan, IL-6, TNF-α receptor types I and II :R&D Systems, Inc, Minneapolis, MN, USA, neoptrin:IBL, Hamburg, Germany).

Statistical analysis

Within-group comparisons were analyzed by the Mann-Whitney test. Correlations were expressed using the Spearman rank correlation coefficient.

For the analyzed measures, *p* values less than 0.05 were considered significant.

Results

Cytokine release in MAS patients

We determined serum concentrations of cytokines, including IL-6, IL-18, and TNF-α receptor types I and II, in patients with MAS complicated with s-JIA (MAS/s-JIA) and compared them with the concentrations in patients with EBV-HLH or KD. It is noteworthy that the magnitude of the difference in serum IL-18 concentrations between patients with MAS/s-JIA and the other patient groups was overwhelming in comparison with that of other cytokine concentrations. As shown in Figure 1, serum IL-18 concentrations in patients with MAS/s-JIA (median, 122500; range, 101000–830000 pg/ml) and active phase of s-JIA (130000, 56500-203000 pg/ml) were significantly higher than those in patients with EBV-HLH (3825, 1720–14800 pg/ml), KD (279.5, 180–560 pg/ml), and HC (140.5, 76–255 pg/ml) ($p<0.05$). Serum IL-18 concentrations in patients with s-JIA were markedly elevated even in the inactive phase of s-JIA (6025, 3730–12000 pg/ml). Other cytokines that were elevated during active disease in the MAS/s-JIA group normalized when patients were in clinical remission. Serum IL-18 concentrations were significantly higher in active s-JIA patients compared to the elevated concentrations also seen in patients with active EBV-HLH ($p<0.05$). These findings indicate that abnormal production of IL-18 appears to be highly specific for s-JIA.

Serum neopterin concentrations in patients with EBV-HLH (68, 46–135 nmol/l) were higher than those in patients with MAS/s-JIA (46, 10.5–122 nmol/l), KD

(13.5, 7–50 nmol/l), and HC (4.35, 1.8–9.5 nmol/l). Serum IL-6 concentrations in patients with KD (57, 22–310 pg/ml) were higher than those in patients with MAS/s-JIA (8.7, 5–22 pg/ml), EBV-HLH (14.3, 0.5–106 pg/ml), and HC (<3.0 pg/ml). Interestingly, serum neopterin and sTNFRII concentrations in patients with MAS/s-JIA were significantly higher than those in patients with active phase of s-JIA. Because many inflammatory cytokines are associated with the pathogenesis of MAS/HLH, we believe that monitoring the cytokine profile in combination with these cytokines might be more useful for evaluating disease activity. Consequently, we tried to represent the cytokine profile with a radar chart (Figure 2). The pattern of the cytokine profile was characteristic in each background (Figure 2)

Markedly elevated concentrations of serum IL-18 in patients with the active phase of s-JIA and MAS

To investigate the relevance of IL-18 to the pathogenesis of s-JIA, serum concentrations of IL-18 were serially monitored in all 5 cases of s-JIA (Figure 3A-E). The concentration of serum IL-18 was both rapidly and markedly rose with the development of the complication of MAS, but gradually reduced after this manifestation resolved with immunosuppressive therapy including corticosteroid and cyclosporine. However, even a few weeks after normalization of other indicators of the inflammatory reaction such as lactate dehydrogenase (LDH), IL-18 concentrations were still well above the value of HC. In case 1, MAS was frequently complicated in this phase with high concentrations of serum IL-18 (Figure 3A). Since serial monitoring of serum concentrations of IL-18 was started, the patient suffered 3 relapses but could be treated before MAS was complicated (Figure 3A). The pattern of cytokine

profile of MAS/s-JIA is similar in all cases. Serum concentrations of IL-18 in patients with s-JIA were markedly elevated even in inactive phase. The other cytokines were detected at significant concentrations in patients with MAS/s-JIA but was undetectable during remission (Figure 4).

Correlation between serum IL-18 concentrations and measures of disease activity in clinical course of 5 cases of s-JIA

Since the concentrations of serum ferritin, LDH, aspartate aminotransferase, and CRP are clinically used as indicators for disease activity of s-JIA, their concentrations were compared with those of IL-18. The concentrations of serum IL-18 correlated positively with each of these indicators ($p < 0.0001$; Figure 5A-D). However, even during the clinically inactive phase after remission from MAS, concentrations of serum IL-18 remained extremely elevated, although other clinical parameters were normalized.

Correlation between serum IL-18 and other cytokines in the clinical course of 5 cases of s-JIA

The concentrations of serum IL-18 correlated positively with the concentrations of serum neopterin, IL-6, sTNFRI, and sTNFRII ($p < 0.0001$; Figure 5E-H). Although the concentrations of serum IL-6 and neopterin were normalized in the inactive phase, concentrations of serum IL-18 remained significantly elevated.

Discussion

MAS is a severe, potentially life-threatening complication characterized by excessive activation of well-differentiated macrophages, resulting in fever, hepatosplenomegaly, lymphadenopathy, severe cytopenia, serious liver

disease, intravascular coagulation, and neurological involvement [1,2]. MAS bears close resemblance to a histiocytic disorder, namely secondary HLH. HLH is a better defined entity observed in a heterogeneous group of diseases, including infections, neoplasms, hematological conditions, and autoimmune disorders. It has been suggested that MAS should be replaced with the term autoimmune disease-associated reactive HLH [8,9]. The hallmark of this syndrome is excessive activation and proliferation of T lymphocytes and macrophages [7]. Massive hypercytokinemia produced by activated inflammatory cells is strongly associated with the pathogenesis of MAS/HLH; however, the kinetics of cytokine release in patients with MAS/HLH have not been analyzed completely.

In the present study, the concentrations of serum IL-18 in patients with s-JIA were markedly increased. In contrast, the concentrations of serum neopterin and IL-6 were significantly increased in patients with EBV-HLH and KD, respectively. These findings show that the pattern of cytokine release is different among patients with different conditions, although clinical characteristics bear a close resemblance.

Because many inflammatory cytokines are associated with the pathogenesis of MAS/HLH, we proposed that monitoring the cytokine profile in combination with the individual cytokines might be more useful for evaluating disease activity. Consequently, we tried to represent the cytokine profile with a radar chart (Figure 2). The pattern of the cytokine profile was characteristic for each disease (Figure 2) and similar in all cases of s-JIA/MAS (Figure 4). In the acute phase of MAS/HLH, it is often difficult to differentiate the patients' primary disease, but monitoring the cytokine profile might be very useful in

achieving early diagnosis and therapeutic decision making.

In the present study, it is noteworthy that the magnitude of the difference in serum IL-18 concentrations between patients with MAS/s-JIA and all other patients was significantly elevated in comparison with that of all the other cytokine concentrations. Serum IL-18 concentrations in patients with MAS/s-JIA and during active disease flares of s-JIA were significantly higher than those in patients with EBV-HLH and KD; these concentrations positively correlated with the measures of disease activity as well as other cytokines. Interestingly, the concentrations of serum IL-18 in patients with s-JIA dropped in the inactive phase of the disease but remained elevated compared to controls and patients with resolved EBV-HLH. Based on serial monitoring of serum IL-18 concentrations in case 1, relapses of acute flares of s-JIA and the complication of MAS occurred in this phase, indicating that careful monitoring was needed for withdrawal of immunosuppressive drugs until the concentration of serum IL-18 normalized. The concentrations of serum IL-18 increased before other clinical indicators for disease activity of s-JIA including ferritin, LDH, aspartate aminotransferase, and CRP start to increase. These findings show that serum IL-18 may be a useful biomarker to predict impending disease flares. Interestingly, the concentrations of neoptetrin and sTNFRII were significantly higher in MAS/s-JIA phase than in the acute phase s-JIA alone. These levels were also extremely high in EBV-HLH, which shows these might be a useful marker to predict the transition to MAS/s-JIA from the acute phase of s-JIA.

Our data indicate that abnormal production of IL-18 appears to be highly specific for s-JIA. However, it is still unknown what causes the induction of

extremely high IL-18 concentrations in the serum of patients with s-JIA. IL-18 is the most effective at regulating NK cell activity [22,23] and it has been reported that decreased NK cell function is found in s-JIA [24-26]. Recently, it was reported that the mechanism of the impaired NK cell function in s-JIA involves a defect in IL-18 receptor β phosphorylation [27]. Further study will be required, but non-functional IL-18/NK cell axis might be associated with the pathogenesis of s-JIA.

Some reports have recently shown that serum concentrations of IL-18 are also highly elevated in patients with adult-onset Still's disease (AOSD) [28-30]. It is still controversial whether s-JIA and AOSD are identical. It has been reported that no significant difference in clinical features such as systemic manifestations or joint lesions, or in prognosis exists between these two diseases [31]. In addition to these observations, our findings in the present study would be consistent with these two diseases being pathogenically identical.

During the clinically inactive phase after remission from MAS, the concentrations of serum IL-6 and neopterin normalized. However, interestingly, the concentrations of sTNFRI and sTNFRII increased in the inactive phase. These findings suggest that TNF-α is also associated with the pathogenesis of the inflammatory process in s-JIA, not only during exacerbation but also during the clinically silent phase of this disease.

Tocilizumab (TCZ) is a humanized monoclonal antibody recently developed against the human IL-6 receptor [32]. Although the introduction of TCZ has brought about a paradigm shift in the treatment of s-JIA [32], it has been reported that some cases were complicated with MAS during treatment with

TCZ (personal communication). This finding indicates that IL-6 blocking cannot prevent the onset of MAS. It is important to analyze the kinetics of cytokine release during MAS in these cases, and the results of such analysis may give us findings that are useful in understanding the role of each cytokines in the pathogenesis of MAS and s-JIA.

In the majority of patients with active s-JIA, coagulation abnormalities and greatly elevated serum ferritin levels are observed. Some rheumatologists suggest that MAS and s-JIA are included in the same spectrum [33]. Our findings suggest that MAS and s-JIA are at different ends of the same spectrum, which is based on the significant production of IL-18 by activated macrophages[15]. The clinical course at later stages of s-JIA is highly variable. Systemic features such as fever, rash, and polyserositis tend to subside during the initial months and up to years of the disease. About half of the children with s-JIA recover almost completely, but the other half continue to show progressive involvement of additional joints. To address whether these two subpopulations have the same spectrum or not, further analysis of the kinetics of cytokine release may be useful.

In spite of the limitations, our results suggest that IL-18 plays a key role in the complex network involved in the inflammation of s-JIA and that serum IL-18 concentration is a promising indicator of disease activity. The pattern of cytokine release in MAS/HLH is different among patients with different backgrounds. Monitoring of the cytokine profile may be useful for differentiation of the primary underlying disease in patients with MAS/HLH and evaluation of disease activity in s-JIA. Some other potentially useful markers to predict MAS in s-JIA, including soluble CD163 and soluble interleulin-2

receptor have been reported [34]. Inclusion of some of these markers in cytokine profiling may improve the quality of the analysis. Further studies are needed to assess what combination of markers are the most useful for the monitoring of MAS/HLH.

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Table 1

Clinical characteristics of patients with systemic onset juvenile idiopathic arthritis during the acute phase of macrophage activation syndrome: WBC, white blood cells; CRP, C-reactive protein; ALT, alanine aminotransferase; AST, aspartate aminotransferase

Figure legends

Figure 1

Serum cytokine concentrations in different patient groups.

Serum levels of (A) IL-18, (B) neopterin, (C) IL-6, (D) sTNFRI and (E) sTNFRII from the different patient groups are shown. Bars represent median values. Statistically significant differences between each patient group are shown as, * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$.

Figure 2

Cytokine profiles with radar charts in the different patient groups.

Representative profiles of serum cytokines including, neopterin, IL-6, IL-18, sTNF-RI and sTNF-RII are shown for different patient groups. Overlayed inner green pentagons show the mean values of healthy controls.

Figure 3

Longitudinal follow up of serum IL-18 in 5 cases with MAS/s-JIA.

(A) through (E) shows the longitudinal follow up case 1 through 5, respectively, of 5 patients with MAS/s-JIA. Changes in serum IL-18 (solid lines) and LDH

(dotted lines) levels are shown in upper panels and details of therapeutic interventions are shown in the lower panels. Time points of blood draw are shown with arrows: M;MAS, A;Active phase, I;Inactive. PSL, prednisolone; mPSL, methylprednisolone; CsA, cyclosporine

Figure 4

Cytokine profiles at different s-JIA disease phases.

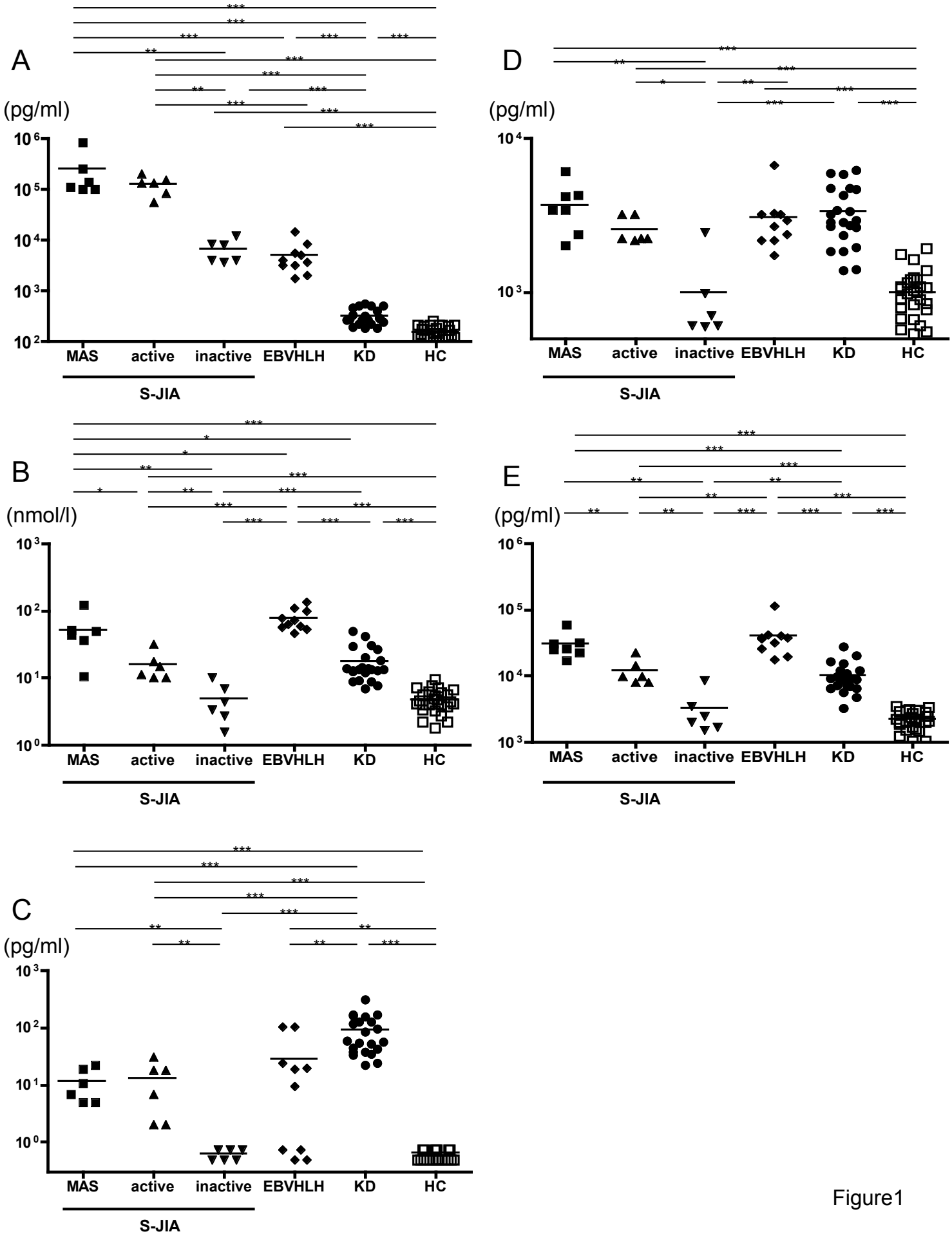
Cytokine profiles were examined by radar charts in 5 cases of s-JIA at MAS (upper panels) and inactive (lower panels) phases. Overlayed inner green pentagons show the mean values of healthy controls.

Figure 5

Positive correlations between IL-18 and other measures of disease activity.

Serum IL-18 levels were compared with other serum markers and cytokines, in 5 cases of s-JIA. A, CRP; B, AST; C, LDH; D, ferritin; E, neopterin; F, IL-6; G, sTNFRI; H, sTNFRII. Red boxes indicate the areas where IL-18 levels are increased whereas other measures remain within normal limits.

Case	1	2	3	4	5
Age (year)	11	1	15	0	2
Sex	F	M	F	M	M
Disease duration (Months)	10	1	2	0	1
Fever	+	+	+	+	+
Systemic JIA Rash	+	+	+	−	+
Arthritis	+	−	+	+	−
Hepatosplenomegaly	+	+	+	+	+
Lymphoadenopathy	+	+	+	−	+
WBC/1000µl	2.4	25.3	15.95	5.4	80
Hemoglobin g/dl	13.4	9.1	13.8	7.8	9.3
Platelets/1000µl	5.6	19.2	11.7	10.7	12.9
CRP mg/dl	17.9	10.3	2.6	0.79	4.69
ALT IU/l	26	145	2437	444	195
AST IU/l	62	241	1382	581	296
LDH IU/l	331	988	2925	1528	1318
Ferritin ng/ml	13639	1912	7000	19600	729.6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S-JIA

Figure1

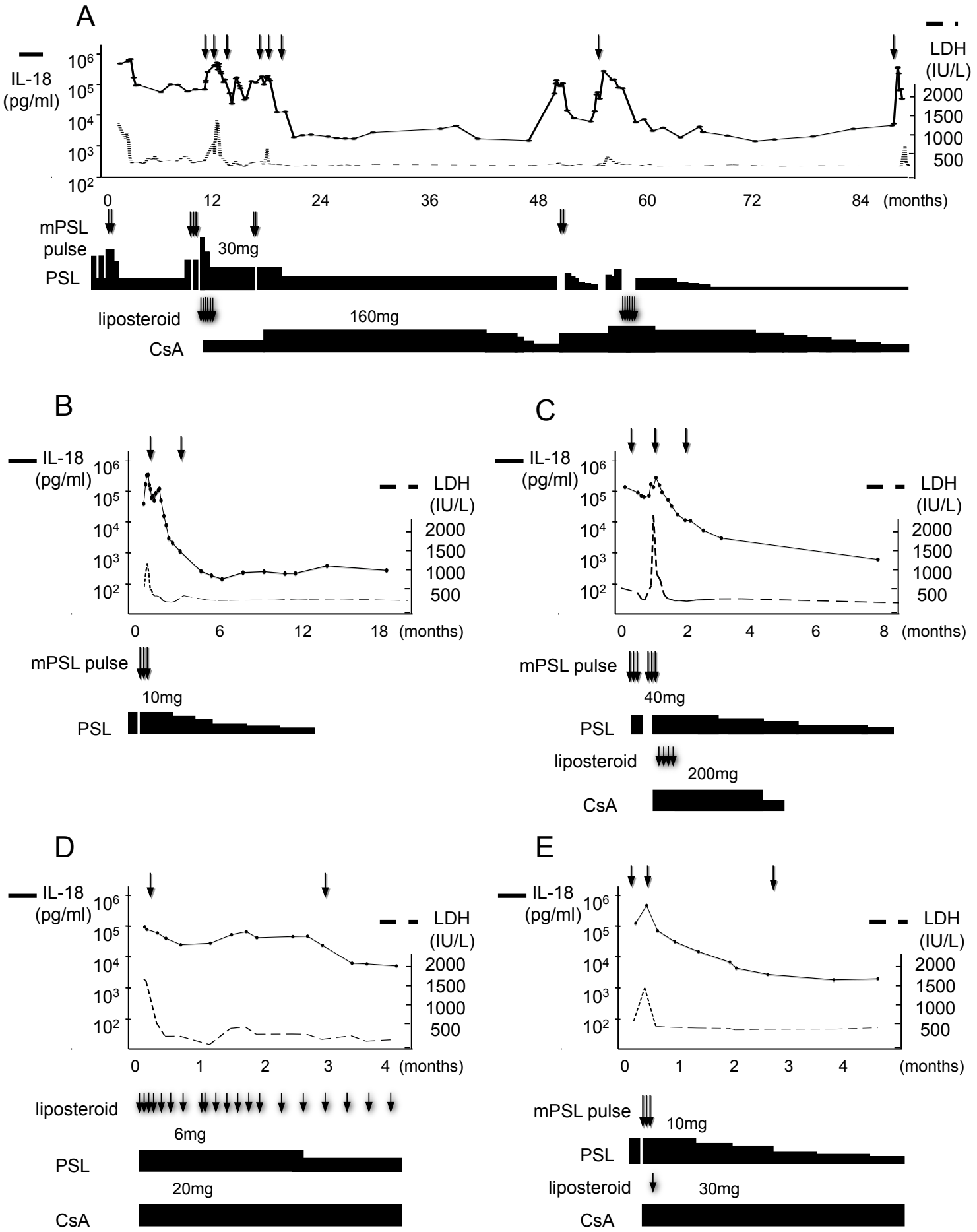

A
IL-18 (pg/ml)
LDH (IU/L)
10^6
10^5
10^4
10^3
10^2
2000
1500
1000
500
0
12
24
36
48
60
72
84
(months)
mPSL pulse
30mg
PSL
liposteroid
160mg
CsA
B
IL-18 (pg/ml)
LDH (IU/L)
0
6
12
18
(months)
mPSL pulse
10mg
PSL
C
IL-18 (pg/ml)
LDH (IU/L)
0
2
4
6
8
(months)
mPSL pulse
40mg
PSL
liposteroid
200mg
CsA
D
IL-18 (pg/ml)
LDH (IU/L)
0
1
2
3
4
(months)
liposteroid
6mg
PSL
20mg
CsA
E
IL-18 (pg/ml)
LDH (IU/L)
0
1
2
3
4
(months)
mPSL pulse
10mg
PSL
liposteroid
30mg
CsA

Figure3

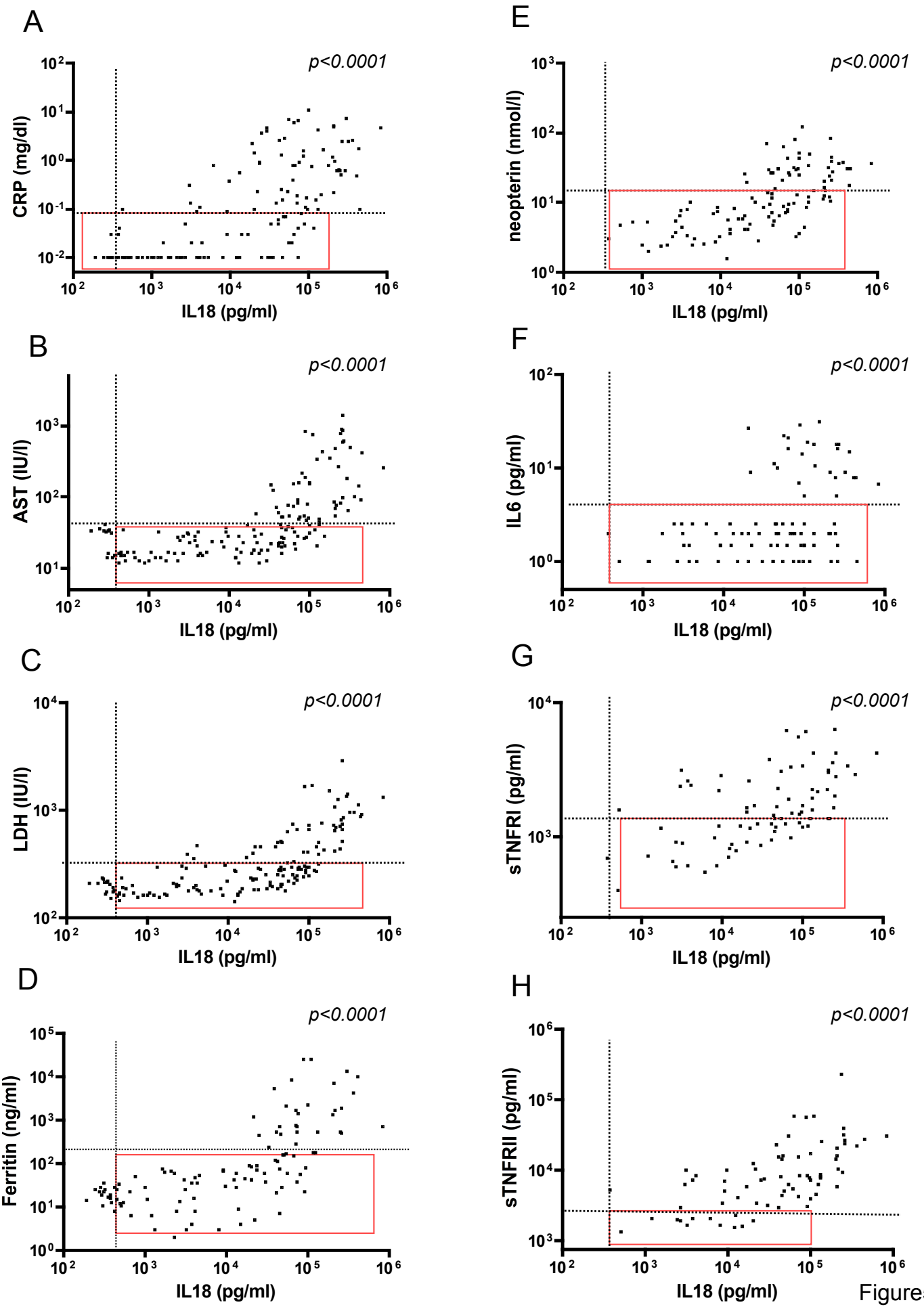

A
p<0.0001
CRP (mg/dl)
IL18 (pg/ml)
B
p<0.0001
AST (IU/l)
IL18 (pg/ml)
C
p<0.0001
LDH (IU/l)
IL18 (pg/ml)
D
p<0.0001
Ferritin (ng/ml)
IL18 (pg/ml)
E
p<0.0001
neopterin (nmol/l)
IL18 (pg/ml)
F
p<0.0001
IL6 (pg/ml)
IL18 (pg/ml)
G
p<0.0001
sTNFRI (pg/ml)
IL18 (pg/ml)
H
p<0.0001
sTNFRII (pg/ml)
IL18 (pg/ml)

Figure5

Figure 2

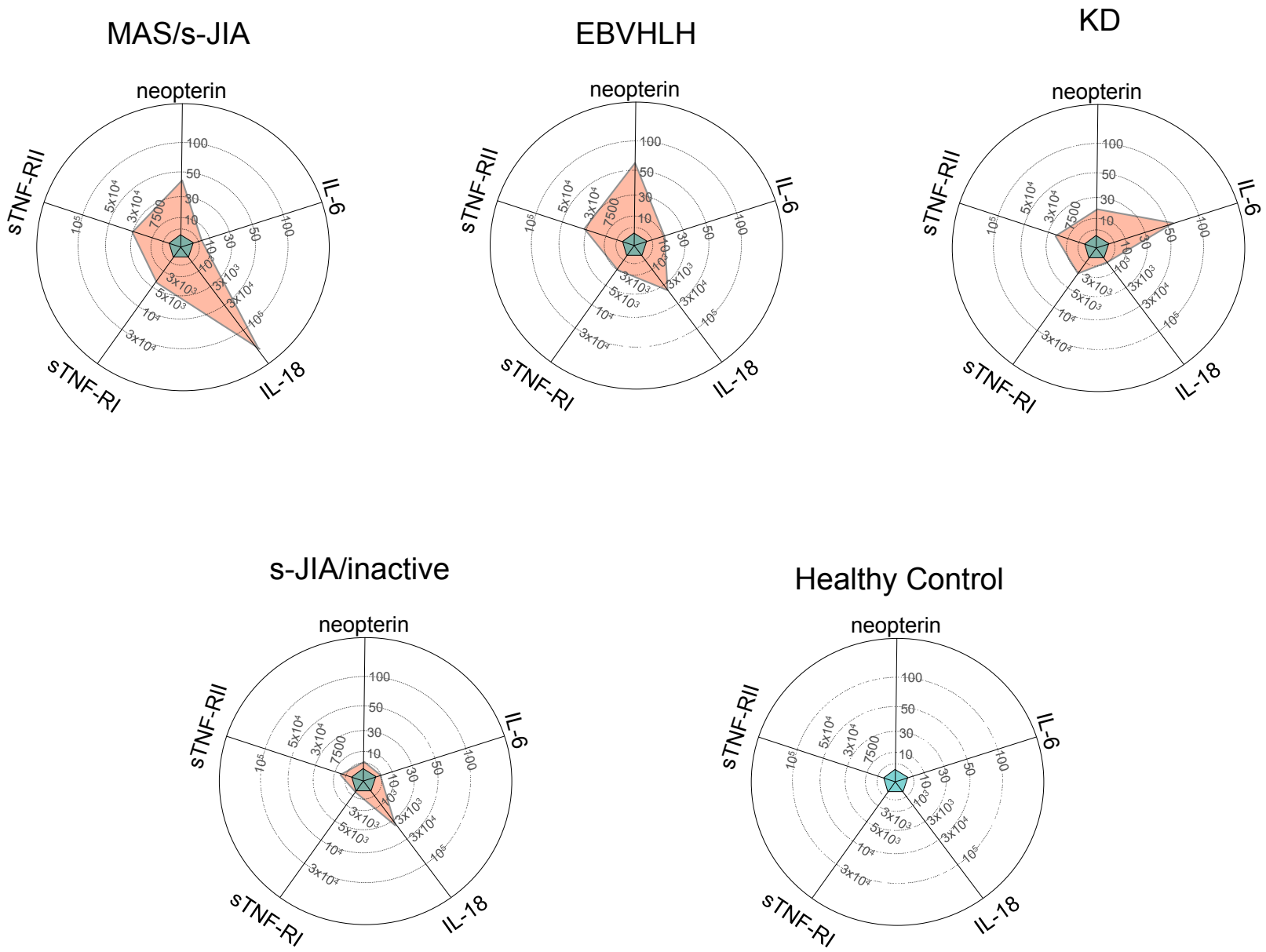
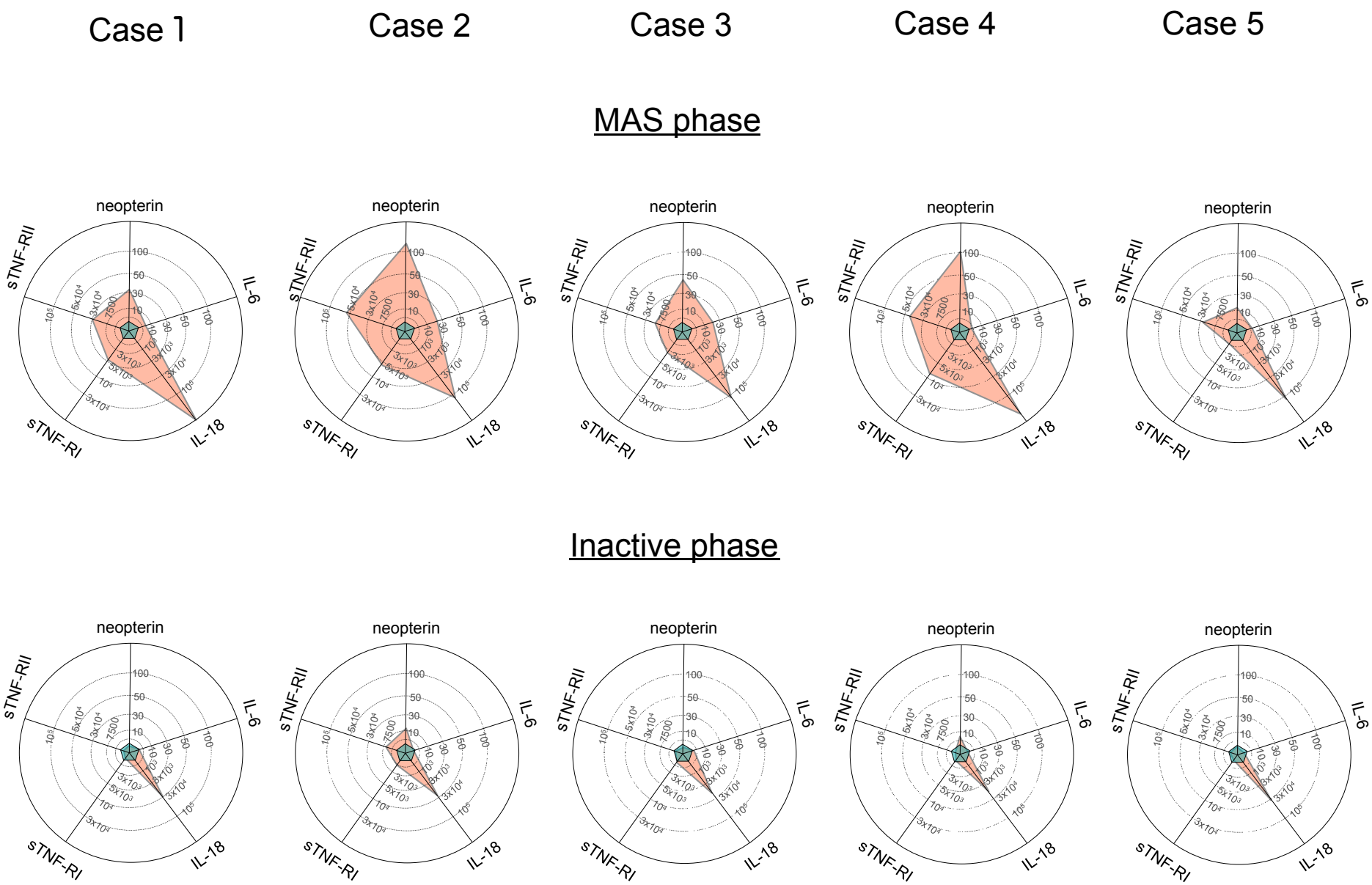

MAS/s-JIA
neopterin
IL-6
IL-18
sTNF-RI
sTNF-RII
EBVHLH
neopterin
IL-6
IL-18
sTNF-RI
sTNF-RII
KD
neopterin
IL-6
IL-18
sTNF-RI
sTNF-RII
s-JIA/inactive
neopterin
IL-6
IL-18
sTNF-RI
sTNF-RII
Healthy Control
neopterin
IL-6
IL-18
sTNF-RI
sTNF-RII

Figure 4


Case 1
Case 2
Case 3
Case 4
Case 5
MAS phase
Inactive phase
neopterin
IL-6
IL-18
sTNF-RI
sTNF-RII