Elevated serum levels of checkpoint molecules in patients with adult Still’s disease

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Abstract
Background: The interaction between galectin-9 (Gal-9) and its ligand, T cell immunoglobulin and mucin-containing-molecule-3 (TIM-3), one of the coinhibitory receptors, transduce the inhibitory signaling to regulate immune responses. The dysregulated expression of checkpoint molecules has been reported under various inflammatory or autoimmune conditions. The aim of this study is to investigate the levels of these checkpoint molecules and their associations between proinflammatory markers in patients with adult Still’s disease (ASD).

Methods: Serum samples were collected from 47 patients with active ASD, 116 patients with rheumatoid arthritis (RA), and 29 healthy controls (HCs). Serum levels of Gal-9, soluble TIM-3 (sTIM-3), and IL-18 were determined using enzyme-linked immunosorbent assay (ELISA). Results were compared with clinical features of ASD.

Results: Serum Gal-9 levels in patients with ASD (median: 21.57 ng/ml, interquartile range IQR [11.41–39.72]) were significantly higher compared to those in patients with RA (7.58 ng/ml, IQR [5.57–10.20] p < 0.001) as well as those in HCs (4.74 ng/ml, [IQR; 4.27–5.63], p < 0.001). Similarly, serum sTIM-3 levels in patients with ASD were significantly higher than those in patients with RA and HCs. Serum levels of Gal-9 or sTIM-3 showed positive correlations with IL-18 levels (Gal-9; r = 0.90, p< 0.001, sTIM-3; r = 0.78, p< 0.001) in patients with ASD. Serum levels of Gal-9 or sTIM-3 correlated with serum ferritin (Gal-9; r = 0.77, p< 0.001, sTIM-3; r = 0.71, p< 0.001) and ASD disease activity score (Pouchot’s score, Gal-9; r = 0.66, p< 0.001, sTIM-3; r = 0.67, p< 0.001). Whereas there was no significant correlation between serum Gal-9 or sTIM-3 and CRP. ASD patients with chronic arthritis phenotype had a significantly higher Gal-9/ferritin and sTIM-3/ferritin ratio than those without this phenotype. After immunosuppressive treatment, Gal-9 and sTIM-3 levels showed a significant decline in parallel to the disease activity scores.

Conclusions: Serum levels of the coinhibitory checkpoint molecules were elevated and correlated with disease activity in patients with ASD. These coinhibitory checkpoint molecule may be implicated in the autoinflammatory process seen in ASD.

Introduction
Adult Still’s disease (ASD) is a systemic inflammatory disorder characterized by spiking fever, skin rash, arthritis, and multisystem involvement [1]. ASD is considered to be an autoinflammatory disease because of the absence of autoantibodies, similar to other autoinflammatory diseases [2]. Although numerous studies have described potential biomarkers, none have been validated in clinical research, except the IL-1 family cytokine IL-18 [3, 4]. IL-18 was originally described as an IFN-γ-inducing factor primarily produced by activated macrophages [5]. IL-18 stimulates proinflammatory responses, including the activation of T cells, and shifts the Th-cell balance toward the Th1 response [6]. High levels of IL-18 have been demonstrated in patients with macrophage activation syndrome (MAS), in addition to those with ASD, and it is also believed that IL-18 is implicated in dysregulated innate immunity [7].

Galectin-9 (Gal-9) is a ligand of T cell immunoglobulin and mucin-containing-molecule-3 (TIM-3), which is expressed on Th1, Th17 and innate immune cells, providing inhibitory signals through its interaction with TIM-3 [8]. Gal-9-TIM-3 complex triggers downstream signaling contribute to the immune suppression by inducing apoptosis in T cells or NK cells [9]. These findings suggest that innate and adaptive immune responses are negatively regulated by Gal-9-TIM-3 interaction [10]. In addition, the Gal-9-TIM-3 pathway may have an important role in the pathogenesis of autoimmune or inflammatory diseases [11]. Considering that ASD is a Th1-polarized autoimmune disease [12], an impairment in the Gal-9-TIM-3 system could be associated with the pathogenesis of ASD through the dysregulation of the innate or adaptive immunity.

Therefore, to determine the involvement of checkpoint molecules in ASD, we analyzed the serum levels of these checkpoint molecules in patients with ASD in comparison with those in patients with other rheumatic disease and healthy controls (HCs). Furthermore, we studied the clinical relevance of these checkpoint molecules, including the correlations with disease activity, laboratory parameters and disease manifestation in patients with ASD.

Methods
Patients and Study design
A retrospective study of all patients diagnosed with ASD at Department of Rheumatology, Fukushima
Medical University Hospital from 1995 to 2020 was conducted. Patients included had to be 17 years old or older to be diagnosed as ASD according to the diagnostic criteria of Yamaguchi [13]. In the patient group, medical histories and clinical findings were collected by reviewing electronic medical records. The study protocol was approved by the ethics committees of the Fukushima Medical University institutional review board (No 2785). Patients were classified as having two disease pattern, the systemic or the articular manifestations as described previously [14]. Patients were finally classified as two distinct disease according to the presence or absence of chronic arthritis phenotype. As controls, 27 healthy subjects (11 males, 16 females, median age 40 years, interquartile range [IQR]; 35–49 years and 116 patients with rheumatoid arthritis (RA) were included. Among 116 patients with RA, 83 (71.6%) were female and their median age was 66 years, [IQR]; 56–74 years. The majority of the RA patients were taking disease-modifying anti-rheumatic drugs (DMARDs), mostly methotrexate (59/116, 50.9%), and biologics (38/116, 32.8%).

Clinical Investigation And Data Collection
Clinical, demographic, and laboratory features of the 47 ASD patients were analyzed. The following demographic and clinical ASD-related characteristics were collected using a standardized form; gender, date of birth, age at diagnosis, duration of disease, past or family history of rheumatic diseases, presence of Still’s disease-related rash, arthralgia, arthritis, myalgia, fever characteristics, lymphoadenopathy, and visceral involvement (serositis, liver damage). The following laboratory data were recorded: leukocyte and thrombocyte counts, hemoglobin, C-reactive protein (CRP), transaminase, lactate dehydrogenase (LDH), ferritin, and markers for hemophagocytosis (hypertriglyceridemis, hypofibrinogenemia, hemophagocytosis in the bone narrow). Each patient was assessed for the systemic score proposed by Pouchot et al. [15] for AOSD. This score assigns 1 point to each of 12 manifestations: fever, typical rash, pleuritis, pneumonia, pericarditis, hepatomegaly or abnormal liver function tests, splenomegaly, lymphadenopathy, leukocytosis > 15,000/mm$^3$, sore throat, myalgia, and abdominal pain (maximum score: 12 points). Data were collected were collected from using a stand data extraction form and were double-checked by two rheumatologists.

ELISA Methods
Serum concentrations of Galectine-9 and sTIM-3 were measured using enzyme-linked immununosorbent assay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instruction. Serum levels of IL-18 were measured using a sandwich ELISA (MBL, Nagoya, Japan) according to the manufacturer's instruction.

Statistical analysis
Results were non-normally distributed and are presented throughout the manuscript with median and 25–75th centiles [median, IQR] and were compared by the Mann-Whitney U test. Correlations between continuous variables were analyzed by the Spearman’s rank correlation test. Paired data were analyzed by paired t-test. All data entry and statistical analyses were performed using SPSS Statistics version 22.0 (IBM, Armonk, NY). In all the analyses, a 2-tailed p < 0.05 was considered statistically significant.

Results
Demographic data of patients with ASD
We evaluated the data of 47 patients with ASD (88.1% women; median age 40 years, IQR [28–56]). All patients had undergone laboratory tests, including a complete blood count, liver function tests, C-reactive protein (CRP), and ferritin. Serum samples were obtained from patients with ASD in the active state. Table 1 summarizes the baseline characteristics and the laboratory data of the patients. The principal clinical symptoms of included a high spiking fever 68.9%), skin rash (57.8%), arthralgia (57.8%), sore throat (38.9%), and splenomegaly (37.8%). Of these patients, only 3 (6.7%) were diagnosed with reactive hemophagocytic syndrome. Patients with ASD showed elevated median levels of biological markers that represent disease activity of ASD, including CRP (median 6.8 mg/dl, IQR [2.9–10.9]) and ferritin (median 1159 pg/ml, IQR [310–3887]).

Serum levels of Gal-9 and sTIM-3 in patients with AOSD
Serum levels of Gal-9 were determined by ELISA in patients with ASD, patients with RA and HCs. As demonstrated in Fig. 1A, the levels of Gal-9 were significantly higher in patients with ASD (median: 21.57 ng/ml, IQR [11.41–39.72]) compared to those in patients with RA (7.58 ng/ml, IQR [5.57–10.20] p < 0.001) and HCs (4.74 ng/ml, [IQR; 4.27–5.63], p < 0.001). Similarly, serum sTIM-3 levels in patients with ASD were significantly higher than those in patients with RA or HCs (Fig. 1B).
Relationship between serum levels of Gal-9 and laboratory parameters in ASD patients

Serum levels of Gal-9 (Fig. 2A) or sTIM-3 (Fig. 2B) showed a significant correlation with serum ferritin levels (Gal-9; $r = 0.77$, $p < 0.001$, sTIM-3; $r = 0.71$, $p < 0.001$), but not with CRP levels (Fig. 3A, 3B). As shown in Fig. 4, serum Gal-9 (Fig. 4A) or sTIM-3 (Fig. 4B) levels also exhibited a positive correlation with the disease activity score (Pouchot’s score, Gal-9; $r = 0.66$, $p < 0.001$, sTIM-3; $r = 0.67$, $p < 0.001$). Positive correlations were demonstrated between serum levels of Gal-9 (Fig. 5A) or sTIM-3 (Fig. 5B) and IL-18 (Gal-9; $r = 0.90$, $p < 0.001$, sTIM-3; $r = 0.78$, $p < 0.001$) was demonstrated. To determine whether serum Gal-9 could be used to differentiate ASD phenotypes, we further analyzed the distribution of serum Gal-9 in combination with ferritin, since some ASD patients exhibited polarized to high levels of Gal-9 in the correlation with ferritin values in the two-dimensional map (Fig. 2A). All patients were subdivided into two groups based on the presence of the chronic arthritis phenotype. We then compared the serum levels of galectin-9. There was no significant difference in serum levels of galectin-9 between ASD patient with or without the chronic arthritis phenotype (Fig. 6A). Next, we calculated the ratio of Gal-9/ferritin, as some patients with ASD exhibited polarized to high levels of Gal-9 or sTIM-3 in the correlation with ferritin values in the two-dimensional map. The ratio of Gal-9/ferritin was significantly higher in patients with the chronic arthritis phenotype than in patients without this phenotype (Fig. 6B). Similarly, higher levels of sTIM-3/ferritin ratio were observed in patients with ASD with the chronic arthritis phenotype (Fig. 7A, B).

Longitudinal observation of serum levels of Gal-9 or sTIM-3

To explore the longitudinal changes in Gal-9 or sTIM-3, we included 5 patients with two longitudinal samples (at least 1 month apart). In the longitudinal study, 5 patients with active ASD were followed until they became inactive and then resampled. Serum levels of Gal-9 or sTIM-3 decreased significantly in parallel to ferritin and Pouchot’s score after immunosuppressive treatments (Fig. 8). Therefore, serum levels of Gal-9 or sTIM-3 in patients with active ASD were diminished following successful treatment and clinical improvement.

Discussion

ASD is a systemic autoinflammatory disease characterized by spiking fever, arthralgia and skin rash,
similar to systemic-onset juvenile idiopathic arthritis (sJIA) [4]. This disease is characterized by a dysregulated cytokine network [16]. Activated innate immune cells play a major role in the systemic inflammation of ASD and induces increased levels of proinflammatory cytokines, interleukin (IL)-1β and IL-18 [4]. These cytokines activate the downstream pathway and amplify the inflammatory response, including cytokine storm [17]. Activated immune cells can be regulated through the checkpoint molecules on these cells to establish immunological tolerance [18]. In particular, the negative feedback signals provided by coinhibitory receptors play an important role for the immune regulation in autoimmune disorders [19]. To our knowledge, our study represents the first attempt of investigating the involvement of these checkpoint molecules in patients with active ASD.

We demonstrated that Gal-9 levels were significantly elevated in patients with ASD and correlated with serum ferritin, one of the disease activity markers for ASD. Moreover, serum levels of Gal-9 correlated significantly with circulating IL-18 levels, an important biological signature in ASD. Our results suggest that dysregulation of the coinhibitory molecules appears to be highly specific for ASD disease activity. It is noteworthy that serum IL-18 levels correlated with the elevated levels of Gal-9. Recent studies demonstrated that Gal-9 functions as a checkpoint molecule for immune cells in maintaining the immune homeostasis [20]. However, the reason or the factor underlying the elevations of this checkpoint molecule in ASD still remains unknown.

It has been considered that the initiation and facilitation of ASD are primarily driven by innate immune cells, among which macrophage activation plays a major role in the pathogenesis of ASD through the amplification of inflammatory responses [4]. Coinhibitory receptors are important for the regulation of inflammation and autoimmunity [10]. TIM-3 is an immune checkpoint molecule expressed on Th1 and Th17 cells and induces tolerance of T effector cells [21]. TIM-3 is one of these coinhibitory receptors expressed on immune cells and regulates inflammatory or autoimmune responses [22]. We speculate that inflammatory stimuli not only exacerbates the proinflammatory processes but also promotes the expression of anti-inflammatory molecules in patients with ASD. Recent studies have shown that Gal-9 regulates autoimmunity in lupus model mice [23]. Arikawa et al. reported that treatment with Gal-9 in an arthritis model repressed macrophage activity, resulting
in the reduction of proinflammatory cytokine expression and the upregulation of anti-inflammatory cytokine expression [24]. These findings suggest that Gla-9 may affect the inflammatory process as an anti-inflammatory mediators.

Based on our observations, it can be concluded that the Gal-9-TIM-3 pathway is activated in ASD patients. Gal-9-TIM-3 pathway also might be relevance in immune regulation, because this coinhibitory pathway triggers the apoptosis of TIM-3 expressing immune cells [9]. However, this pathway can be modulated by the soluble form of TIM-3 which is shedded form TIM-3 expressing immune cells [25]. Membrane bound TIM-3 can be cleaved from the cell surface by a disintegrin-like and metalloproteinase with thrombospondin type 1 motifs (ADAMTS) 10 or ADAMTS17, yielding a soluble TIM-3 ectodomain [26]. Although the major function of the membrane bound TIM-3 is to limit the immune responses, sTIM-3 is considered to interfere with the function of membrane bound TIM-3 [27]. Gal-9-TIM-3 interaction may result in T cell exhaustion, on the contrary, sTIM-3 seems to have alternative effects against this feedback mechanism. However, a soluble form of a receptor may not always result in blocked receptor. Further functional study is needed to explore the role of the Gal-9-TIM-3 pathway on the autoinflammation that is responsible for the pathogenesis of ASD.

Recent studies suggest the presence of a dichotomy between the two major phenotypes of ASD, i.e., a systemic pattern and chronic articular pattern with chronic arthritis and joint damage [28]. In the present study, Gal-9 levels did not differ among patients exhibiting the chronic arthritis phenotype. Our data demonstrated that serum Gal-9 levels highly correlated with serum ferritin levels in patients with ASD; however, some patients with ASD had high levels of Gal-9, which were not in parallel with serum ferritin levels, in the combined distribution pattern of Gal-9 and ferritin. The ratio of Gal-9/ferritin was significantly higher in patients with chronic arthritis phenotype than in patients without this phenotype. It has been shown that galectin-9 (Gal-9) inhibits the development of collagen-induced arthritis (CIA) [29]. These findings suggest that Gla-9 may affect the inflammatory arthritis process as an anti-inflammatory mediators. The relative polarized upregulation of Gal-9 levels observed in patients with ASD with the chronic arthritis phenotype may reflect the augmented status of the induction of anti-inflammatory coinhibitory systems to limit the arthritis in ASD. The sample
size of patients with ASD with different phenotypes is relatively small in this study. Further research with a larger sample size is required to evaluate the effects of these checkpoint molecules on disease activity of ASD.

There were several limitations in this study. The sample size was relatively small, particular in subgroup analyses for comparison in terms of the systemic or articular form. We did not compare these molecules with other febrile disorders. All patients with ASD and HCs in this study were Japanese, and hence additional studies in other ethnic groups are required to verify our findings. The mechanism through which the Gal-9-TIM-3 pathway contributes to the pathogenesis of ASD was not clarified. Further research involving a large sample size is required to evaluate the usefulness of these markers in patients with ASD. Nevertheless, our findings suggest that the Gal-9-TIM-3 pathway is involved in the pathophysiology of ASD reflecting immune activation or disease phenotype of ASD.

Conclusions
Our results indicated that serum levels of Gal-9 and sTIM-3 were elevated in patients with ASD and could be implicated with the dysregulated immune network in ASD. Serum levels of Gal-9 and sTIM-3 correlated with serum IL-18, in patients with ASD. Furthermore, serum levels of Gal-9 and sTIM-3 correlated with disease activity in ASD patients. Investigation of these check point molecules may facilitate the development of tools to diagnose and assess disease activity of ASD.

Abbreviations
ASD
adult Still’s disease
ADAMTS
a disintegrin and metalloproteinase with a thrombospondin type 1 motif
Gal-9
galectin-9
IL-18
interleukin-18
Th1
T helper 1
RA
rheumatoid arthritis,
sTIM-3
soluble T cell immunoglobulin domain and mucin-3, Th1 = T helper 1

Declarations

Ethical Approval

Ethical approval for this study (No. 2785) was provided by the Ethics Committee of Fukushima Medical University.

Consent for publication

Not applicable

Availability of supporting data

Not applicable

Competing interests

KM has received research grants from Chugai, Pfizer, and AbbVie. Rest of the authors declares that they have no competing interests

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Authors’ contributions

YF, TA, NM, JT, SS, HM, MF, ES, HK, HW were involved in acquisition of clinical data. YF and KM drafted manuscript.

YF, KM carried out the biochemical studies, participated in the sequence alignment and drafted the manuscript. AK, KM participated in the sequence alignment and drafted the manuscript.

TK, AK, KM participated in the design of the study, FY performed the statistical analysis. All authors read and approved the final manuscript.

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Table
Due to technical limitations, Table 1 is provided in the Supplementary Files section.

Figures
Figure 1

Serum levels of Gal-9 (A) and sTIM-3 (B) in patients with ASD. Serum levels of Gal-9 (A) and sTIM-3 (B) in ASD patients (n=46) were significantly higher compared to those in RA patients (n=116) or healthy subjects (n=27). Results were presented with median plus 25-75th centiles [median, IQR] and were compared by the Mann-Whitney U test.
Figure 2

Relationship between serum levels of checkpoint molecules and ferritin in patients with ASD. A: Correlation analysis of serum levels of Gal-9 and ferritin showed a significant positive correlation in ASD patients. B: Correlation analysis of serum levels of sTIM-3 and ferritin showed a significant positive correlation in ASD patients.
Figure 3

Relationship between serum levels of checkpoint molecules and CRP in patients with ASD. A: Correlation analysis of serum levels of Gal-9 and CRP did not show a significant correlation in ASD patients. B: Correlation analysis of serum levels of sTIM-3 and CRP did not show a significant correlation in ASD patients.
Correlation between serum levels of checkpoint molecules and disease activity score (Pouchot’s score) in patients with ASD. A: Correlation analysis of serum levels of Gal-9 and disease activity scores (Pouchot’s score) showed a significant positive correlation in ASD patients. B: Correlation analysis of serum levels of sTIM-3 and disease activity scores (Pouchot’s score) showed a significant positive correlation in ASD patients.
Figure 5

Relationship between serum levels of checkpoint molecules and IL-18 in patients with ASD.

A: Correlation analysis of serum levels of Gal-9 and IL-18 showed a significant positive correlation in ASD patients. B: Correlation analysis of serum levels of sTIM-3 and IL-18 showed a significant positive correlation in ASD patients.
Figure 6

Serum levels of Gal-9 in ASD patients with or without chronic arthritis phenotype. A: We compared serum levels of Gal-9 between ASD patients with or without chronic arthritis phenotype. There was no significant difference in serum levels of Galectin-9 between ASD patients with and without chronic arthritis phenotype. B: The ratio of Gal-9/ferritin was significantly higher in ASD patients with chronic arthritis phenotype compared to those without chronic arthritis phenotype.
Serum levels of sTIM-3 in ASD patients with or without chronic arthritis phenotype. A: We compared serum levels of sTIM-3 between ASD patients with or without chronic arthritis phenotype. There was no significant difference in serum levels of sTIM-3 between ASD patients with and without chronic arthritis phenotype. B: The ratio of sTIM-3/ferritin was significantly higher in ASD patients with chronic arthritis phenotype compared to those without chronic arthritis phenotype.
Figure 8

Longitudinal changes of serum Gal-9 (A) or sTIM-3 (B) concentrations in 5 patients with ASD before and after immunosuppressive treatments. Paired samples from the same subjects were compared by paired T test.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

Table1.pdf