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Original article

TLR4 endogenous ligand MRP8/14 level in enthesitis-related arthritis and its association with disease activity and TLR4 expression

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Abstract

Objective. Enthesitis-related arthritis (ERA) is an inflammatory disease of childhood that lacks autoantibodies. Overexpression of surface-expressed Toll-like receptors (TLRs) has been found in ERA. Myeloid-related proteins (MRPs) 8 and 14 are calcium binding proteins that act as an endogenous ligand of TLR4. MRP8/14 levels are elevated in patients with systemic-onset arthritis. Thus we studied the role of MRP8/14 in ERA.

Methods. The study enrolled patients with ERA. Plasma and SF levels of MRP8/14 were measured by ELISA and TLR4 expression on peripheral blood and SF monocytes was measured by two-colour flow cytometry. Control plasma samples were collected from 48 blood bank donors.

Results. Of the 69 patients, 67 were male, with a mean age of 15.2 (S.D. 2.7) years and a disease duration of 5 (S.D. 3) years. Median plasma levels of MRP8/14 were higher in patients (10 862.3 ng/ml) than controls (4426.1 ng/ml, $P < 0.0001$). Patients with active disease (11 669.5 ng/ml) had higher levels as compared with inactive disease (4421.8 ng/ml, $P < 0.0001$). Plasma MRP8/14 levels decreased on follow-up after 3 months only in patients who responded to treatment ($P = 0.012$). MRP8/14 levels were negatively correlated with the frequency of $CD14^+TLR4^+$ cells ($r = -0.372$, $P = 0.02$). MRP8/14 levels were higher in SF as compared with plasma (15 858.45 ng/ml, $P = 0.024$). The frequency of $CD14^+TLR4^+$ cells was higher in SF as compared with peripheral blood.

Conclusion. MRP8/14 levels are increased in the plasma of ERA patients and are higher in those with active disease and the levels decrease in patients who respond to treatment, suggesting that it may be a good biomarker during follow-up.

Key words: juvenile idiopathic arthritis, juvenile spondyloarthritis, innate immune response, calcium binding proteins.

BASIC SCIENCE

Introduction

JIA is a group of inflammatory arthritides of unknown origin affecting children <16 years of age, of which enthesitis-related arthritis (ERA) is the most common type in India [1]. Boys are affected more often than girls, with predominant lower limb joint involvement, enthesitis and inflammatory back pain. The exact pathogenesis is unknown, however, microbes are one of the important environmental factors implicated in the pathogenesis of this disease. Autoantibodies are lacking in ERA in comparison with oligoarticular JIA and polyarticular JIA, where ANA and RF are seen, respectively [2]. All these indicate a dominant role of the innate immune system in the pathogenesis of ERA.

Toll-like receptors (TLRs) are receptors of the innate immune system that recognize pathogen-associated molecular patterns present on the microbes, producing a subsequent proinflammatory response and initiating an adaptive immune response through activation of dendritic

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cells and macrophages [3, 4]. In addition to their role in the immune response against pathogens, they also play a role in the pathogenesis of autoimmune diseases by inducing cytokine production, cell activation and subsequent tissue damage [5, 6].

Overexpression of TLRs has been reported in ERA patients [7] and stimulation of TLRs with their ligands leads to increased production of proinflammatory cytokines and MMPs by fibroblast-like synoviocytes derived from the SF of children with JIA [8]. TLRs can also be stimulated by endogenous, host-derived ligands, leading to subsequent release of proinflammatory mediators like TNF-α and sustained inflammation [5, 6]. Endogenous ligands like heat shock proteins and high-mobility group protein box-1 (HMGB-1) have been implicated in RA. Recently the levels of S100A12, a calcium binding protein (calgranulin), were reported to be elevated in patients with ERA. In contrast, the levels of soluble receptor for advanced glycation end product (sRAGE), which acts as a decoy receptor for S100A12, were found to be down-regulated in ERA. sRAGE levels negatively correlated with S100A12, ESR and swollen joint count [9]. Another calcium binding protein complex of myeloid-related proteins (MRPs) 8 (S100A8) and 14 (S100A9), expressed by granulocytes, monocytes and macrophages on activation, is an endogenous ligand for TLR4 [10, 11]. MRP8/14 levels are elevated in patients with inflammatory arthritis such as systemic-onset JIA (SoJIA), RA, SLE and PsA [12–15]. Its pathogenic role has been shown in experimental models of arthritis [16].

Recently it was demonstrated that MRP8/14 can induce a strong proinflammatory effect on macrophages and endothelial cells through TLR4 [17, 18]. It can also have an effect on the development of autoreactive CD8 cells [19]. Even though the role of microbes is suspected in the pathogenesis of ERA, continuing disease even in the absence of infection indicates a likely role of endogenous ligands in the activation of TLRs and the persistence of disease. The present study was conducted to determine whether levels of MRP8/14 complex are elevated in patients with ERA and its relation with disease activity and TLR4 expression on monocytes in ERA patients.

Methods

Patients and controls

Consecutive patients (<18 years of age) with a diagnosis of ERA based on ILAR criteria [20], irrespective of disease activity, were enrolled in the study after obtaining informed written consent from their parents. Patients with suspected or proven infection at the time of the visit were excluded. Peripheral blood was collected from all patients, while SF was collected from patients who required IA steroid injections as a part of their treatment. Plasma from young healthy males and blood bank donors was used as a control.

Assay for TLR4 expression on peripheral blood mononuclear cells and SF mononuclear cells

Anti-CD14-FITC and biotinylated anti-TLR4/isotype antibodies were added to 100 μl of EDTA blood diluted with 100 μl PBS and incubated for 30 min in the dark at room temperature. Two millilitres of 1$\times$ RBC lysing solution was added to the tube and incubated for 15 min in the dark. The fluorescence-activated cell sorting (FACS) tube was centrifuged at 500 *g* for 5 min and the supernatant discarded, followed by washing with 2 ml of PBS. Streptavidin-PE was added to both tubes and incubated for 30 min at room temperature in the dark. PBS (100 μl) was added to the above tubes and kept at 4°C until data were acquired using the BD FACSCalibur flow cytometer (BD, Franklin Lakes, NJ, USA). Peripheral blood mononuclear cells (PBMCs) were gated from the total cell population and the median fluorescent intensity (MFI) was calculated for CD14 cells expressing TLR4. SF mononuclear cells (SFMCs) were isolated using density gradient centrifugation by layering on Histopaque for analysis and subsequently stained with anti-CD14 and anti-TLR4 and the MFI was calculated as for PBMCs.

MRP8/14

MRP8/14 was measured by sandwich ELISA (BMA Biomedicals, Rheinstrasse, Switzerland) following the manufacturer's instructions.

Statistical analysis

MRP8/14 results were expressed as median (range). All intergroup comparisons were done using non-parametric tests. Patients were grouped into active and inactive disease based on Wallace *et al.* criteria [21]. On follow-up, patients were classified as responders if they achieved inactive disease status when compared with baseline. Follow-up plasma MRP levels were compared with the baseline values among responders and non-responders. This study was approved by the institutional ethics committee of the Sanjay Gandhi Postgraduate Institute of Medical Science, Lucknow, India.

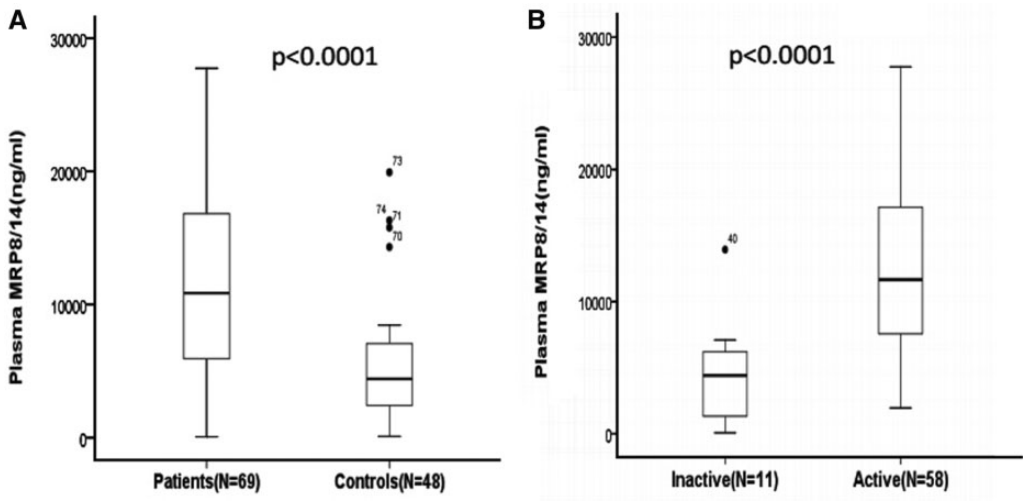
Results

Among 69 patients with ERA there were 67 boys and 2 girls. Follow-up samples were obtained from 26 patients 3–4 months after the first sample collection. Their mean age at presentation was 15.2 (S.D. 2.7) years, mean age at onset was 10.3 (S.D. 2.7) years and mean duration of disease was 5 (S.D. 3) years. Fifty-eight patients were receiving NSAIDs, 12 patients MTX, 12 patients low-dose prednisolone, 4 SSZ and 2 HCQ. Patients with follow-ups were not different from other patients with respect to the duration of disease and treatment received.

Plasma MRP8/14 levels

Median plasma MRP8/14 levels were higher in patients (10 862.3 ng/ml) compared with controls (4426.1 ng/ml, $P < 0.0001$; Fig. 1). SF MRP8/14 levels were analysed in

Fig. 1 Box plot showing plasma MRP8/14 levels.



(**A**) Patients and controls. (**B**) Active and inactive patients.

10 patients. MRP8/14 levels were higher in SF [15 858.4 (4731–28 176) ng/ml] than plasma ($P = 0.024$). There was no correlation of plasma MRP8/14 levels with disease duration.

TLR4 expression on PBMCs and SFMCs

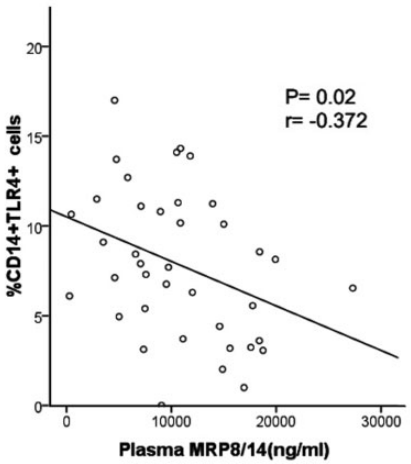
TLR4 expression on monocytes among PBMCs was analysed in 52 patients and on paired SF samples from 10 patients. Analysis of paired samples showed a significantly higher MFI for SFMCs [1409.5 (S.D. 890.6)] than PBMCs [946.1 (S.D. 798.9), $P = 0.005$]. The percentage of $CD14^+TLR4^+$ cells was also higher in SFMCs [18.7 (S.D. 4.5)] compared with PBMCs [6.0 (S.D. 4.0), $P < 0.0001$].

Association of plasma MRP8/14 with disease activity and TLR4 expression

Patients were grouped into active and inactive disease based on Wallace *et al.* criteria [21]. Eleven patients fulfilled the criteria for inactive disease. Plasma MRP8/14 levels were higher in patients with active disease [11 669.5 (1946–27 748) ng/ml] compared with patients with inactive disease [4421.8 (80–13 938.2) ng/ml, $P < 0.0001$; Fig. 1]. Plasma MRP8/14 levels correlated inversely with the frequency of $TLR4^+CD14^+$ cells in the PBMC gate ($P = 0.02$, $r = -0.372$; Fig. 2).

Of the 26 patients, 8 fulfilled the criteria for inactive disease at the follow-up visit while 18 did not achieve inactive disease or had a flare-up of disease. The median plasma levels of MRP8/14 decreased from 11 197.5 ng/ml to 3784 ng/ml ($P = 0.012$) in patients who responded to treatment. However, there was no significant difference noted from baseline values (11 145.5 ng/ml) among non-responders (10 843.38 ng/ml, $P = 0.347$; Fig. 3).

Fig. 2 Scatter plot showing the correlation of plasma MRP8/14 levels with the percentage of $CD14^+TLR4^+$ cells in the PBMC gate.

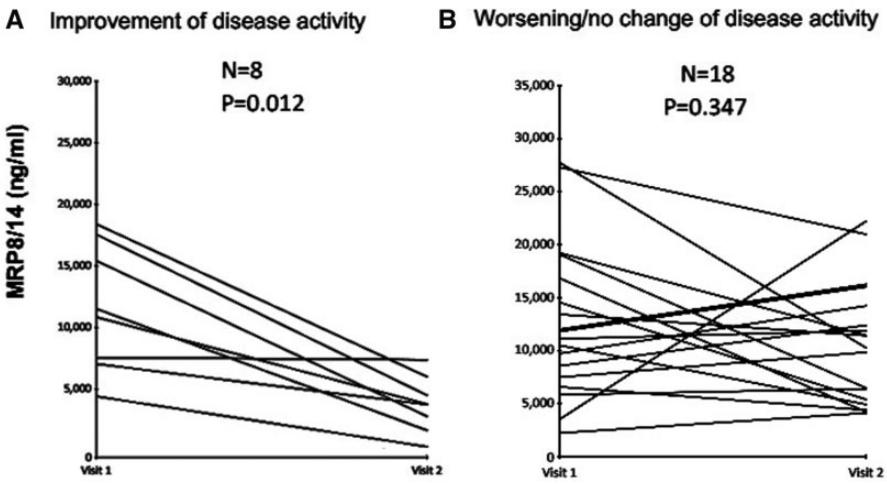


Discussion

The data suggest that MRP8/14 levels are elevated in the plasma and SF of ERA patients, plasma MRP levels correlate with disease activity and levels are reduced in patients who show clinical improvement. Further, they have a negative correlation with TLR4 expression.

Similar to the present study, increased plasma levels of MRP8/14 have been reported in patients with SoJIA, RA, SLE and PsA, suggesting a role in mediating inflammation or an increase subsequent to inflammation [12–15]. The levels of MRP8/14 in SoJIA are much higher compared with patients with infections, acute leukaemia and

FIG. 3 Paired values of MRP8/14 in individual patients.



(**A**) Responder group. (**B**) Non-responder group.

neonatal-onset multisystem inflammatory disease (NOMID) [22]. In SLE patients, significant elevations of MRP8/14 levels were seen compared with patients with primary SS and healthy controls [14].

Plasma MRP8/14 levels were nearly three times higher in those with active disease. SoJIA patients with active disease have nearly 20 times higher levels than those with inactive disease and more than 10 times higher during flares [23]. This difference between SoJIA and ERA may be due to marked activation of neutrophils in SoJIA, whereas in ERA, monocytes may be the major producers of MRP8/14 rather than neutrophils. This is further supported by the higher median value of MRP8/14 in SoJIA.

SF levels of MRP8/14 were higher than in plasma in the present study. An earlier study among patients with oligoarticular JIA found 10 times higher levels in the SF as compared with serum [10]. Another study comparing serum, SF and synovial tissue expression of MRP8/14 in patients with PsA, RA and SpA showed significantly higher expression in synovial tissue [15].

A decrease in the levels of MRP8/14 in patients who responded to treatment and a lack of such a pattern among non-responders suggests that it may be a good biomarker for follow-up. In SoJIA serum levels increased during flares when compared with inactive disease, suggesting a potential role in assessing disease activity and prediction of flares [23]. Higher amounts of MRP8/14 can activate cells by binding to TLR4, leading to the release of proinflammatory cytokines. These mediators can lead to increased disease activity. In a trial of MTX withdrawal, high MRP8/14 levels at the time of drug withdrawal were associated with an increased risk of flare in JIA [24]. However, there were only 10 patients with ERA in this study. Further prospective follow-up studies are required to establish its role as a predictor of flares in ERA. A recent study found higher MRP8/14 levels to be a predictor of response to MTX [25].

An inverse relation was noticed between TLR4 expression on PBMCs and plasma MRP8/14 levels. This could be due to down-regulation of TLR4 expression due to continuous stimulation by its ligand MRP8/14. The strengths of our study are inclusion of a large number of patients with ERA, estimation of both MRP8/14 and TLR4 expression and longitudinal follow-up. The major limitations of our study are a heterogeneous patient population as regards the duration of disease, use of drugs and fewer SF samples.

Thus to conclude, our data suggest that MRP8/14 levels are increased in the plasma and SF of ERA patients, plasma MRP8/14 correlates with disease activity and levels decrease on follow-up in patients who respond to treatment. A prospective study with a longer follow-up will help establish the role of MRP8/14 in monitoring disease activity and predicting flares.

Rheumatology key messages

- Plasma levels of myeloid-related proteins (MRPs) 8 and 14 are increased in enthesitis-related arthritis (ERA).
- ERA patients with active disease have higher levels of MRP8/14.
- ERA patients who attain inactive disease show a reduction in MRP8/14 levels.

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