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Letter to the Editors-in-Chief

Aberrant expression of NLRP3, NLRC4 and NLRP6 inflammasomes in patients with primary immune thrombocytopenia

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Immune thrombocytopenia (ITP) is a hemorrhagic autoimmune disorder that manifests as increased bleeding risk with low platelet counts [1]. The diagnosis of ITP depends on clinical characteristics and laboratory examinations, as well as on the exclusion of other causes of thrombocytopenia [2]. Typical symptoms in ITP patients include skin petechiae and bleeding in the mucosal regions, gastrointestinal or intracranial areas despite the fact that clinical features are not evident in most ITP patients [3]. Moreover, a peripheral platelet count ($< 100 \times 10^9$/L) is the most important diagnostic criterion for ITP [4]. ITP can be classified as primary form with no clear underlying cause and secondary ITP that is induced by other diseases or treatments, the incidence of which is approximately 1.9 to 6.4 per 10^5 children/year, and 3.3 to 3.9 per 10^5 adults/year, with the number progressively increasing [2].

Several studies support an essential role of proinflammatory cytokines in the pathogenesis of ITP [5,6]. Particularly, interleukin-18 (IL-18) has been reported to be increased in active ITP patients compared to healthy individuals and ITP patients with remission [7]. These observations indicate the plausible implication of inflammasome activation in the immune inflammatory response during ITP progression.

As multiprotein complexes, inflammasomes, such as the nucleotide-binding oligomerization domain (NOD)-like receptor family, pyrin domain containing 1 (NLRP1), NLRP3, NLRP6, CARD domain containing 4 (NLRC4), as well as the proteins absent in melanoma 2 (AIM2) have been identified to activate pro-IL-1β and pro-IL-18 through a caspase-1-dependent pathway. Mature IL-1β and IL-18 cytokines play an important role in the regulation of immune responses [8], providing expectant therapeutic targets for inflammasome-associated diseases using several inhibitors of individual inflammasomes. A role for inflammasome NLRP3 has been previously implicated in ITP [7], whereas little is known about the association of the level of various inflammasomes with antiplatelet autoantibody or platelet counts.

To better evaluate the role of inflammasomes in the pathophysiology of ITP, 25 blood samples from newly diagnosed primary ITP patients according to the criteria for ITP and 13 samples from healthy controls were enrolled in this pilot study [8]. The median age at onset of ITP and control cases were 54 (range, 15–77) and 34 years old (range, 23–78), respectively. No age and gender differences were observed between ITP patients and control group. Among 25 ITP patients, in 18 cases were antiplatelet autoantibody detected, while all controls were negative. These ITP patients had a median platelet count of 49×10^9/L ranging from 13 to 99×10^9/L. Meanwhile, the platelet counts in healthy individuals ranged from 174 to 373×10^9/L with a median count of 243×10^9/L. The main features of these enrolled cases were summarized in Table 1.

Blood samples were obtained into tubes containing EDTA-K_2 anticoagulant by standard clinical procedures, followed by centrifugation at 3000 rpm/min for 5 min to isolate plasma, which was stored at −80 °C until use. Then, the remaining blood components were diluted with equal volume of PBS for the following isolation of PBMC by density gradient centrifugation on Lympholyte®-H (CEDARLANE, Netherlands) at 2000 rpm for 25 min, as previously described [9]. The expression levels of inflammasomes *NLRP1*, *NLRP3*, *NLRC4*, *NLRP6*, and *AIM2* and inflammasome-associated genes *caspase-1*, *IL-1β*, and *IL-18* were comparatively measured using quantitative real-time PCR (TaKaRa, Japan) with the housekeeping gene encoding *GADPH* as an internal control. The primers used were designed and synthesized as follows: *NLRP1*, 5′-ATTGAGGGCAGGCAGCACAGAT-3′ and 5′-CTCCTTCAGGTTTCTGGTGACC-3′; *NLRP3*, 5′-GGACTGAAGCACCTGTTGTGCA-3′ and 5′-TCCTGAGTCTCCCAAGGCATT-3′; *NLRC4*, 5′-AGGTCCCACAACTCGTCAAGCT-3′ and 5′-TGCTCACACGATTTCCCGCCAA-3′; *NLRP6*, 5′-ACTGTGCCATCTGAGCAGCCTC-3′ and 5′-TCACTGAGCCTGTTGTGGAGGA-3′; *AIM2*, 5′-GCTGCACCAAAAGTCTCTCCTC-3′ and 5′-CTGCTTGCCTTCTTGGGTCTCA-3′; *Caspase-1*, 5′-GCTGAGGTTGAGATCACAGGCA-3′ and 5′-TGCTGTCAGAGGTCTTGTGCTC-3′; *IL-1β*, 5′-CCACAGACCTTCCAGGAGAATG-3′ and 5′-GTGCAGTTCAGTGATVGTACAGG-3′; *IL-18*, 5′-GATAGCCAGCCTAGAGGTATGG-3′ and 5′-CCTTGATGTCAGGAGGATTCA-3′; *GAPDH*, 5′-GTCTCCTCTGACTTCAACAGCG-3′ and 5′-ACCACCCTGTTGCTGTAGCCAA-3′. The relative expression levels of target genes were calculated by comparative Ct method and presented as $2^{-\Delta\Delta Ct}$. Detection of antiplatelet autoantibody of the plasma from control samples and ITP patients was performed by a Solid-phase Coombs Test Kit for Antiplatelet Autoantibody Assay in accordance with the instructions of the manufacturer (BoDe, China), and essentially as described [10].

All of the experiments were conducted at least in triplicate with consistent results. The data were processed with GraphPad Prism 6 and SPSS 16.0 statistics software, and descriptive data were presented as median (range) for continuous variables. The data of differential expression between the groups of ITP and control cases from qRT-PCR

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Table 1
The relevant characteristics of primary ITP patients and HC.

Clinical features	ITP	HC
Number	25	13
Gender (M/F)	15/10	6/7
Age (range)	54 (15–77)	34 (23–78)
Antiplatelet autoantibody (−/+)	7/18	13/0
Platelet count (range) ($\times 10^9$/L)	49 (13–99)	243 (174–373)

Note: M/F, male/female; ITP, immune thrombocytopenia; HC, healthy controls.

assay were analyzed with unparied Student's *t*-tests with the results presented as the mean ± SEM. The Spearman correlation coefficients were used for correlation analyses of various target genes with other variables. The p values < 0.05 were considered significant.

We assessed the transcriptional status of various inflammasomes in primary ITP patients by qRT-PCR. As shown in Fig. 1, the transcriptional levels of *NLRP3* and *NLRC4* in patients with newly diagnosed ITP were markably higher than that in healthy controls, whereas the expression level of *NLRP6* was notably lower in ITP patients than that in controls with statistical significance (Fig. 1A–C). Additionally, ITP patients displayed slight higher mRNA levels of *NLRP1* and *AIM2* as no difference was observed compared with controls (data not shown). These findings suggested differential expression pattern of various inflammasomes in ITP patients; nevertheless, further study is needed to explore the likely cell types influencing this aberrant expression of these inflammasomes in the development of this disorder.

Caspase-1 could be activated by intracellular inflammasomes after receiving external stimuli. To find out the potential alteration of caspase-1 during the development of ITP, the caspase-1 levels in the PBMC of ITP patients and healthy controls were comparatively measured using qRT-PCR. It revealed a remarkably increased expression of *caspase-1* in ITP patients when compared to healthy controls, showing statistical difference ($p = 0.003$) (Fig. 1D). Consistent with the expression profile of caspase-1, significantly higher levels of *IL-1β* and *IL-18*, the processing and secretion of which could be regulated by

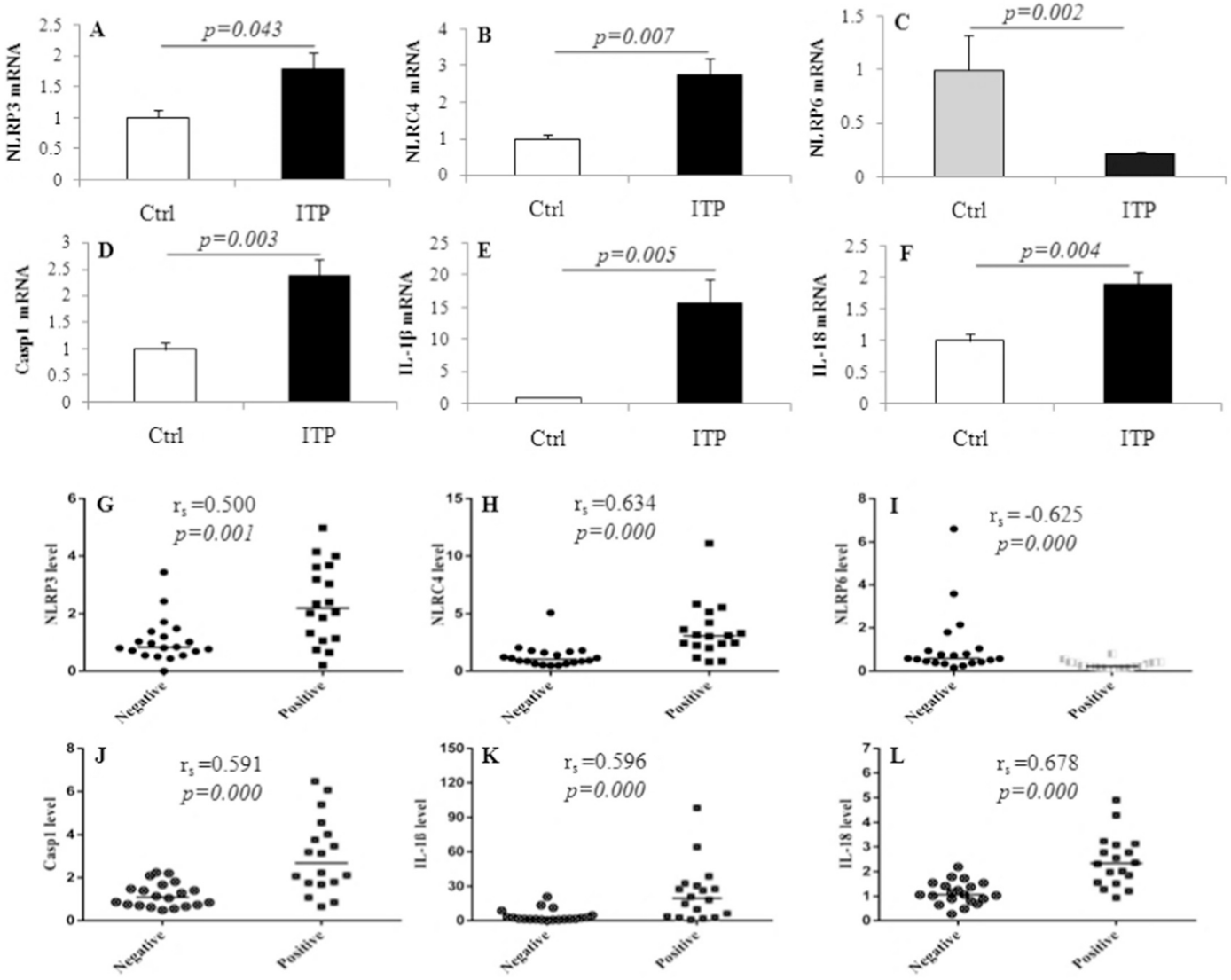

Fig. 1. Differential expression assay of inflammasomes and inflammasome-associated genes, as well as their association with antiplatelet autoantibody in ITP patients. A, The expression of *NLRP3* mRNA; B, the expression of *NLRC4* mRNA; C, the expression of *NLRP6* mRNA; D, the expression of *Casp1* mRNA; E, the expression of *IL-1β* mRNA; F, the expression of *IL-18* mRNA; G, the association of *NLRP3* mRNA with antiplatelet autoantibody; H, the association of *NLRC4* mRNA with antiplatelet autoantibody; I, the association of *NLRP6* mRNA with antiplatelet autoantibody; J, the association of *Casp1* mRNA with antiplatelet autoantibody; K, the association of *IL-1β* mRNA with antiplatelet autoantibody; L, the association of *IL-18* mRNA with antiplatelet autoantibody; the solid line within each group represents the median of the relative mRNA level. The Spearman's correlation coefficient and p value were labeled above. The $p < 0.05$ was considered to be statistically significant. Negative, negative antiplatelet autoantibody; Positive, positive antiplatelet autoantibody in ITP patients; *Casp1*, *caspase-1*.

activated caspase-1, were noticeable in patients with newly diagnosed ITP than controls (Fig. 1E, F). These data indicated that elevated expression of *caspase-1*, as well as subsequent *IL-1β* and *IL-18* might be attributed to the activation of inflammasomes *NLRP3* and *NLRC4*.

To address the potential correlations of differentially expressed inflammasomes with positive antiplatelet autoantibody, a total of 38 subjects enrolled in this study were divided into positive (18 cases) and negative (20 cases) antiplatelet autoantibody and employed into Spearman correlation analysis. The results showed that significant correlation of positive antiplatelet autoantibody with the relative mRNA level of *NLRP3* ($r_s = 0.500$, $p = 0.001$), *NLRC4* ($r_s = 0.634$, $p = 0.000$), *NLRP6* ($r_s = -0.625$, $p = 0.000$) (Fig. 1G–I), *NLRP1* (data not shown), but not *AIM2* (data not shown) were noticed in total subjects, despite the fact that no correlation with statistical significance was observed between differentially expressed inflammasomes and positive antiplatelet autoantibody in ITP patients (data not shown). These results demonstrated positive correlations of *NLRP3*, *NLRC4*, *NLRP1* level and negative correlation of *NLRP6* level with positive antiplatelet autoantibody in a cohort comprising both ITP patients and healthy controls.

To further assess the potential factor associated with differentially expressed inflammasomes, the clinical parameter of platelet counts from all enrolled subjects were introduced using Spearman correlation analysis. The results revealed noticeable correlation of lower platelet counts with higher *NLRC4* ($r_s = -0.435$, $p = 0.006$) level, lower *NLRP6* ($r_s = 0.470$, $p = 0.003$) level, as well as higher *AIM2* level, but non-significant higher *NLRP3* and *NLRP1* levels (data not shown). These findings implied that aberrant expression of inflammasomes might play an important role in the pathogenic process of increased platelet clearance.

To ascertain the associations of inflammasome-associated genes with positive antiplatelet autoantibody and lower platelet counts, the laboratory and clinical parameters from the enrolled cases were statistically analyzed. Spearman correlation analysis illustrated no statistical correlation between inflammasome-associated genes and positive antiplatelet autoantibody in ITP patients, which is consistent with that of differentially expressed inflammasomes mentioned above (data not shown). However, apparent correlations of positive antiplatelet autoantibody with the relative higher mRNA level of *caspase-1* ($r_s = 0.591$, $p = 0.000$), *IL-1β* ($r_s = 0.596$, $p = 0.000$) and *IL-18* ($r_s = 0.678$, $p = 0.000$) were observed in all enrolled subjects (Fig. 1J–L). Additionally, lower platelet counts correlated well with the higher level of *caspase-1* ($r_s = -0.473$, $p = 0.003$), *IL-1β* ($r_s = -0.632$, $p = 0.000$) and *IL-18* ($r_s = -0.451$, $p = 0.004$) (data not shown), respectively. Taken together, these data demonstrated positive and negative correlations of the inflammasome-associated genes with positive antiplatelet autoantibody and lower platelet counts, respectively.

In conclusion, this study shows a differential expression pattern of various inflammasomes in ITP patients compared to controls. While acknowledging the limitations of the sample size that precludes significant associations when considering only ITP patients, analyses of controls and patients suggest positive and negative correlations of specific inflammasomes and their associated genes with positive antiplatelet autoantibody and lower platelet counts, respectively, indicating the involvement of aberrantly expressed inflammasomes in the development of ITP and providing a conceivable target for ITP therapy.

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Competing interests

All authors declare no competing interests.

Ethics statement

Written informed consent was obtained from all individual participants included in the study according to the Declaration of Helsinki (1964), and the study was approved by the local Medical Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine, Zhejiang, China.

References

[1] D.B. Cines, V.S. Blanchette, Immune thrombocytopenic purpura, N. Engl. J. Med. 346 (2002) 995–1008.
[2] M. Swinkels, et al., Emerging concepts in immune thrombocytopenia, Front. Immunol. 9 (2018) 880.
[3] G. Moulis, et al., Epidemiology of incident immune thrombocytopenia: a nationwide population-based study in France, Blood 124 (2014) 3308–3315.
[4] E. Lo, S. Deane, Diagnosis and classification of immune-mediated thrombocytopenia, Autoimmun. Rev. 13 (2014) 577–583.
[5] A.M. Rocha, et al., The levels of IL-17A and of the cytokines involved in Th17 cell commitment are increased in patients with chronic immune thrombocytopenia, Haematologica 96 (2011) 1560–1564.
[6] H. Zhou, et al., Interleukin 27 inhibits cytotoxic T-lymphocyte-mediated platelet destruction in primary immune thrombocytopenia, Blood 124 (2014) 3316–3319.
[7] J. Qiao, et al., Elevated expression of NLRP3 in patients with immune thrombocytopenia, Immunol. Res. 64 (2016) 431–437.
[8] B.K. Davis, et al., The inflammasome NLRs in immunity, inflammation, and associated diseases, Annu. Rev. Immunol. 29 (2011) 707–735.
[9] J. Xie, et al., Changes in follicular helper T cells in idiopathic thrombocytopenic purpura patients, Int. J. Biol. Sci. 11 (2015) 220–229.
[10] J.A. Lown, J.G. Ivey, Evaluation of a solid phase red cell adherence technique for platelet antibody screening, Transfus. Med. 1 (1991) 163–167.

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