Upregulated expression of leukocyte immunoglobulin-like receptor A3 in patients with severe aplastic anemia

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Abstract. Severe aplastic anemia (SAA) is a rare and potentially life-threatening disease characterized by pancytopenia and bone marrow (BM) hypoplasia. In a previous study by our group, increased expression of leukocyte immunoglobulin-like receptors A (LILRA), LILRA3 in myeloid dendritic cells (mDCs) and LILRA5 in CD34+ cells in SAA was detected using proteomics techniques, highlighting their potential role in disease pathogenesis. In the present study, the expression of LILRA1-6 mRNA was assessed in the BM mononuclear cells of patients with SAA using reverse transcription-quantitative (RT-q)PCR. The expression of homogenic LILRA3 and LILRA5 isoform on mDCs, as well as CD34+, CD3+CD8+, CD19+ and CD14+ cells, was detected using flow cytometry. mDCs were then induced, cultured and sorted. The expression of LILRA3 was confirmed using RT-qPCR and western blot analyses. The serum levels of soluble LILRA3 were measured using ELISA. Furthermore, the relationship between LILRA3 expression and disease severity was assessed. The results indicated increased LILRA3 mRNA expression in patients with SAA. The percentage of LILRA3+ in BM mDCs and CD34+ cells was increased. Compared with controls, the relative LILRA3 mRNA expression and the relative protein intensity were highly increased in SAA mDCs. The serum LILRA3 levels in patients with SAA were also increased. The proportion of LILRA3+CD11c+ human leukocyte antigen (HLA)-DR+/CD11c+HLA-DR+ cells was positively correlated with the ratio of LILRA3+CD34+/CD34+ cells and the expression of LILRA3 mRNA. Taken together, the expression of LILRA3 on mDCs of patients with SAA was increased, which may affect the function of mDCs. LILRA3 may have a significant role in the immune pathogenesis of SAA.

Introduction

Severe aplastic anemia (SAA) is a rare and potentially fatal disease characterized by pancytopenia and bone marrow (BM) hypoplasia (1). Patients frequently experience symptoms such as anemia, bleeding and infection, leading to a significant decrease in quality of life and eventually death (2). The abnormal activation and hyperfunction of hyper-activated effector T lymphocytes are the major immune mechanisms in the pathogenesis of primary acquired SAA (3,4). However, the etiology and pathogenesis of SAA have remained to be fully elucidated. Previous research by our group has indicated that SAA is caused by an unknown antigen substance activating dendritic cells, which increase the number and function of myeloid dendritic cells (mDCs), resulting in the hyperfunction of cytotoxic T lymphocyte (CTL) and T helper type 1 (Th1) cells. This causes the release of numerous negative hematopoietic regulatory factors. The regulatory T cells and natural killer (NK) cells that induce immune tolerance are consequently severely insufficient and damage hematopoietic target cells, leading to hematopoietic failure (5,6). In the previous study, mDCs proteomes were investigated using mass spectrometry and the CD34+ cells using isobaric tags for relative and absolute quantification (iTRAQ) labeling and a combination of multidimensional liquid chromatography and tandem mass spectrometry (7). In comparison with healthy controls, the expression of leukocyte immunoglobulin-like receptors eukocyte immunoglobulin-like receptor A3 (LILRA3) in mDCs and LILRA5 in CD34+ cells were significantly increased.

LILRs are members of the immunoglobulin superfamily that are mainly expressed on antigen-presenting cells (APCs). The genes are located on chromosome 19q13.4 (8,9). They are divided into immunosuppressive receptor LILRs (LILRB1-5) and immunoreactive receptor LILRs (LILRA1-6) according to the cytoplasmic structures and transmembrane regions (10). After binding to major histocompatibility complex I (MHC-I) ligand, it changes the autoimmune-mediated tissue damage threshold and regulates the immune responses through the inhibition or activation of cytolsis. MHC-I molecules are widely expressed on hematopoietic cells of all lineages. When
the intracellular protein fragment-short antigen peptide is presented by CTL, the LILRA-MHC-I interaction effectively stimulates T-cell proliferation and secretes inflammatory factors (11). Enhancing CTL lysis clears target cells expressing autoantigens, making the LILR a novel target for regulating CTL-mediated autoimmune diseases (12,13). LILRs are associated with autoimmune diseases, malignancies and infections (14,15). However, little is currently known about the potential role of LILRA genes in patients with SAA. The present study aimed to analyze the expression of LILRAs in patients with SAA and investigate its role in the pathogenesis of SAA by determining its impact on patient-derived mDCs and targeted BM CD34+ cells.

Materials and methods

Patients. A total of 48 patients with SAA that were admitted to the Hematology Department of Tianjin Medical University General Hospital (Tianjin, China) between June 2015 and April 2017 were enrolled. Of these, 26 were newly diagnosed, untreated patients with SAA (12 males, 14 females) with a median age of 28 years (range, 7-69 years). The other 22 patients were remission-treated patients with SAA (14 males, 8 females) with a median age of 31 years (range, 11-65 years). Patients in remission were those who improved after immunosuppressive therapy (IST, including antithymocyte globulin, cyclosporine and glucocorticoid). All the patients in remission had achieved bone marrow hematopoietic recovery and had been separated from the infusion of blood products, while some had normal peripheral blood cell counts but still required drug therapy. All subjects met the diagnostic criteria (16,17). Patients were excluded if they had complications such as iron overload, malignancy or other autoimmune diseases or if they were pregnant. The patients' clinical characteristics are provided in Table I.

All patients received immunosuppressive therapy that included rabbit anti-thymocyte globulin, cyclosporin and hematopoietic stimulating factor treatment consisting of erythropoietin, granulocyte colony stimulating factor, thrombopoietin and interleukin IL-11. Treatment efficacy was evaluated based on the Camitta standard (18).

The healthy controls (13 males and 15 females) included had a median age of 28 years (range, 24-55 years). The present study was approved by the Ethics Committee of Tianjin Medical University (Tianjin, China). Written informed consent was obtained from all study subjects.

Culture, identification and sorting of mDCs. BM mononuclear cells (BMMNCs) were extracted from patients with SAA and healthy controls using lymphocyte separation fluid (Solarbio Science & Technology) using density gradient centrifugation. BMMNCs of each subject were plated separately at a density of 2x10^5 cells/ml in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) complete medium containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% mycillin and incubated for 2 h at 37°C in an atmosphere containing 5% CO₂. Non-adherent cells were removed. The remaining cells were cultured in RPMI-1640 complete medium containing 10% fetal bovine serum, 1% mycillin, 100 ng/ml Recombinant Human Granulocyte-Macrophage Colony Stimulating Factor (cat. no. 10015-HNAH; Sino Biological) and 40 ng/ml rhIL-4. The culture conditions were 37°C and 5% CO₂. Media and cytokines were changed every two days. On day 6, rhTNF-α (1,000 µg/ml) was added to mature the mDCs for 24 h. Suspended mature mDCs in the culture supernatant were then collected on day 7. The collected cells were stained with PerCP-conjugated human leukocyte antigen (HLA)-DR (cat. no. 347364) and APC-conjugated CD11c monoclonal antibody (cat. no. 340544; all, BD Biosciences). HLA-DR+CD11c+ cells were sorted and collected using a FACS Aria flow cytometer (BD Biosciences).

Reverse transcription-quantitative (RT-q)PCR. The mRNA expression of LILRA was analyzed using RT-qPCR. BMMNCs of patients with SAA and controls were lysed using TRIzol reagent. RNA was reverse transcribed using the complementary (c)DNA Synthesis Kit (Tiangen). qPCR was performed on the BIORAD iQ5 system (Bio-Rad Laboratories, Inc.). GAPDH was used as a housekeeping gene for standardizing targeted mRNA expression. A total of 1 µl of each cDNA working solution was used with a final volume of 25 µl, which contained 12.5 µl SYBR green solution and 0.75 µl of upstream and downstream primers (concentration, 10 µM). PCR reaction conditions were as follows: LILRA1-6: 95°C for 30 sec, the indicated annealing temperature for 45 sec, 72°C for 30 sec, 45 cycles. The indicated annealing temperature and primer sequences are listed in Table II. The relative expression level of the gene of interest was calculated using the 2^-ΔΔCq method (19).

Flow cytometric (FCM) analysis. For phenotype analysis, fresh heparinized BM samples were stained with anti-human LILRA3-FITC (cat. no. IC2574G, LILRA5-PE monoclonal antibody (mAb; cat. no. FAB6754P; both from R&D Systems), CD34-PerCP (cat. no. 340430), CD3-APC/PerCP (cat. no. 340440 or 347344), CD8-APC/PerCP (cat. no. 340584 or 347314), CD19-APC (cat. no. 340437), CD14-PE/FITC mAb (cat. no. 347947 or 347493), CD11c-APC (cat. no. 340544) and HLA-DR-PerCP mAb (cat. no. 347364; all from BD Biosciences). The staining was performed according to the manufacturer's protocol. The ratios of intracytoplasmic LILRA3*CD11c*HLA-DR*/CD11c*HLA-DR*, LILRA3*CD3*CD4*/CD4+, LILRA3*CD3*CD8*/CD3*CD8+, LILRA3*CD19*/CD19+, LILRA3*CD14*/CD14+, LILRA5*CD11c*HLA-DR*/CD11c*HLA-DR*, LILRA5*CD3*/CD3+, LILRA5*CD3*CD8*/CD3*CD8+, LILRA5*CD19*/CD19+ and LILRA5*CD14*/CD14+ cells were analyzed on a FACS Calibur flow cytometer (BD Biosciences) and CellQuest software version 3.1 software (BD Biosciences).

Western blot analysis. The mDCs of the subjects from the SAA, R-SAA and healthy control groups were collected and lysed in RIPA buffer supplemented with PMSF. The protein concentration was measured using a BCA kit. The loading volume per sample was 40 µg protein. The proteins were separated on 10% SDS-PAGE (Beijing Solarbio Science & Technology Co., Ltd.) and transferred to a nitrocellulose membrane. The membrane was blocked with 10% skimmed milk (BD Biosciences; cat. no. 232100 for 1 h at room temperature.
for 25 min in the dark. Finally, 1 ml PBS was added to each
sample tube. Subsequently, 25 µl of the fluorescence detection
reagent was added to each experimental tube and samples
were mixed thoroughly, and the samples were
incubated for 30 min at room temperature. Subsequently, 25 µl
was added, and following thorough mixing, the sample was
centrifuged at 200 x g for 5 min at room temperature. The supernatant was aspirated
to indicate a statistically significant difference.

### Results

#### Increased LILRA3 mRNA expression in patients with SAA.

The expression of transcription factor LILRA1-6 in patients with SAA and healthy controls was assessed using RT-qPCR. The results suggested that the relative expression of LILRA3 mRNA of untreated patients with SAA (3.513±3.291) and remission patients with SAA (2.451±1.767) was significantly higher than that of the controls (1.372±0.961; P<0.05). There was no significant difference between the untreated and remission groups (P>0.05). The relative expression of LILRA5 mRNA in untreated patients with SAA (1.738±0.935) and remission patients with SAA (1.671±1.308) was higher than that in the controls (1.128±0.993); however, the differences were not statistically significant. The relative expression levels of LILRA1 (1.880±1.627 vs. 1.502±1.438 vs. 2.609±2.144; P>0.05), LILRA2 (1.255±1.114 vs. 1.113±1.083 vs. 1.714±1.437; P>0.05), LILRA4 (1.808±1.751 vs. 1.625±1.588 vs. 1.213±1.124; P>0.05) and LILRA6 (2.111±2.018 vs. 1.813±1.357 vs. 1.371±1.302; P>0.05) were not significantly different between patients with SAA and the R-SAA and controls (Fig. 1).

#### Increased frequency of LILRA3 on BM mDCs in patients with SAA

The membrane was stained with anti-LILRA3 (dilution, 1:1000; cat. no. ab111562; Abcam) and anti-β-actin antibodies (dilution, 1:1000; cat. no. 4970; Cell Signaling Technology, Inc.) for 2 h at 4°C. After washes with tris-buffered saline containing Tween-20, the membrane was incubated with a secondary antibody (cat. no. ZB-2305; dilution, 1:10,000; goat anti-mouse, Zhongshanjingqiao) for 1 h at room temperature. The bands were visualized with the Super ECL Plus Detection Reagent (Applygen Technologies Inc.). Protein levels were normalized to β-actin.

ELISA. Serum levels of soluble LILRA3 were measured using an ELISA reagent kit (cat. no. SEB387Hu; Cloud Clone). According to the manufacturer's protocols, the samples were measured and read with a BioTek ELX800 microplate reader (BioTek Corp.) at a wavelength of 450 nm.

Cytokine detection. A cytokine detection kit (Human Th1/Th2 subsets detection kit) was used for this analysis (cat. no. P010001; Sajiishengwu). The venous blood samples were collected in EDTA anticoagulation tubes and centrifuged at 1,000 x g for 20 min for later use. Standards were configured with the following concentrations: 10, 20, 40, 80, 150, 312, 625, 1,250, 2,500 and 5,000 pg/ml. The captured microsphere mixture was centrifuged at 200 x g for 5 min at room temperature. The supernatant was aspirated, the same volume of microsphere buffer as the aspirated supernatant was added, and following thorough mixing, the sample was incubated for 30 min at room temperature. Subsequently, 25 µl of the solution was added to each experimental tube and the sample was vortexed. A total of 25 µl of standard product and 25 µl of the sample to be tested was added in the same sample tube. Subsequently, 25 µl of the fluorescence detection reagent was added to each experimental tube and samples were thoroughly mixed and incubated at room temperature for 25 min in the dark. Finally, 1 ml PBS was added to each experimental tube, which was then centrifuged at 200 x g for 5 min at room temperature. The supernatant was aspirated and 100 µl PBS was added for fluorescence detection on the flow cytometer (BD Biosciences; FACSCanto II). This was performed according to the manufacturer's instructions.

### Table I. Clinical characteristics of the patients.

| Item          | Untreated SAA (n=26) | R-SAA (n=22) |
|--------------|----------------------|--------------|
| Age (years)  | 28 (7-69)            | 31 (11-65)   |
| ANC (x10^9/l)| 0.58±0.38            | 3.01±1.76    |
| Hb (g/l)     | 58.14±23.32          | 127.19±38.64 |
| PLT (x10^9/l)| 18.31±11.62          | 118.84±70.31 |
| Ret%         | 0.41±0.29            | 2.89±1.97    |
| Therapy      | Not previously treated except for transfusions | IST |
| Duration (months) | 2 (1-3)            | 30 (7-109)   |

Values are expressed as the mean ± standard deviation or median (range). Age and duration values are mid (min, max). R-SAA, remission-treated severe aplastic anemia; ANC, absolute neutrophil count; PLT, platelets; Hb, hemoglobin; Ret, reticulocyte; IST, immunosuppression therapy.

### Statistical analysis

Statistical analyses were performed using SPSS 22.0 software (IBM Corp.). Data analyses were performed with GraphPad Prism 5.0 software (GraphPad Software, Inc.). The normality of the distribution was proven using a Kolmogorov-Smirnov test. The mean ± standard deviation was used to represent normally distributed data. Comparisons between two independent samples were performed using the t-test. The rank-sum test was used to analyze data with a non-normal distribution and the median and interquartile range were used to represent the data. Age and duration values are mid (min, max). For correlation tests, Spearman's rank correlation was used. P<0.05 was considered to indicate a statistically significant difference.
the relative intensity of LILRA3 protein in mDCs of the untreated SAA group was significantly increased (P<0.05; Fig. 3B).

**Increased percentage of LILRA3 on BM CD34+ cells in patients with SAA.** Untreated (47.05±32.07%) and remission (39.86±19.46%) patients with SAA had increased percentages of intracytoplasmic LILRA3+CD34+ cells compared with the controls (14.26±14.00%; P<0.05). The percentages of BM LILRA5+CD34+ cells were not different between patients with SAA and healthy controls (P>0.05). Furthermore, the
percentages of LILRA3+ and LILRA5+ cells among the CD14+, CD19+ and CD8+ lymphocytes in patients with SAA were not different compared with those of the healthy controls (P>0.05; data not shown).

**Increased serum LILRA3 levels in patients with SAA.** Patients with SAA had increased serum levels of LILRA3 in comparison with those of the controls. The serum LILRA3 levels of untreated patients with SAA (9.466±2.629 ng/ml) were significantly higher than those of the remission patients with SAA (4.591±2.958 ng/ml; P<0.05) and controls (3.682±2.695 ng/ml; P<0.05; Fig. 3B).

**Frequency of LILRA3 is closely associated with clinical characteristics and cytokine levels in patients with SAA.** To determine whether the frequency of LILRA3 may identify a pathogenically distinct subset of patients with SAA or whether it simply reflects disease activity, clinical indices and evidence of disease activity in BM were compared with the expression of LILRA3.

In the SAA group, there were positive correlations between the proportion of LILRA3+CD11C+HLA-DR+ and the expression of LILRA3 mRNA (r=0.330 and 0.344, respectively; P<0.05). There were negative correlations between the ratio of LILRA3+CD34+ and the platelet count and hemoglobin levels (r=-0.497 and -0.427, respectively; P<0.05; Fig. 4A).

The levels of LILRA3 mRNA and serum cytokine TNF-α (r=0.636, P<0.05) and IFN-γ (r=0.618, P<0.05) in untreated
patients with SAA were positively correlated. There was no significant correlation between LILRA3 mRNA levels and serum cytokine IL-4 (r=0.356, P>0.05) or IL-10 (r=0.411, P>0.05) in untreated patients with SAA (Fig. 4B).

**Discussion**

The present study was the first to indicate an alteration of BM LILRAs in patients with SAA. SAA is a type of BM failure mediated by abnormal cellular immunity. To date, the etiology and pathogenesis of SAA have remained to be fully elucidated. Previous studies by our group have confirmed that various immune cells and cytokines constitute an abnormal immune status in patients with SAA (20,21), including the involvement of Th1/Th2 subset imbalances, hyperfunctional CTLs, insufficiencies in the regulatory T and NK cell populations and negative hematopoietic cytokines (22,23). These subsequently induce excessive apoptosis of CD34+ HSCs and inhibit hematopoietic colony formation through the perforin, granzyme B, Fas/FasL and the TNF-related apoptosis-inducing ligand pathways (13). Furthermore, an in-depth study of its pathogenic mechanism suggested that both the numbers and function of mDCs were significantly increased in the peripheral blood from individuals with SAA (5,6,24). mDCs secrete IL-12, a major stimulator, which induces the differentiation of naive CD4+ T cells into the Th1 phenotype (25). Th1 cells were reported to be overly activated subsequent to the excessive secretion of cytokines such as IFN-γ and IL-2, which promote CD8+ CTLs in patients with SAA (26). Thus, it may be inferred that the pathogenesis of SAA is associated with improved function and increased number of mDCs.

Although the understanding of the immune pathogenesis of SAA gradually improved over numerous years of research, the specific antigens, mDCs and T cells involved remained unclear. Previous studies by our group have elucidated that the immune cascade activation of SAA is closely relevant to the upregulation and hyperfunction of mDCs, which may be the initiating factor resulting in the activation of subsequent immune responses. Liu et al (6) detected changes in protein components of BM mDCs in the SAA group by two-dimensional electrophoresis analysis. Compared with normal controls, the expression of LILRA3 in mDCs of patients with SAA increased. Coincidentally, Qi et al (7) also reported on the alterations in protein expression levels of BM CD34+ cells in the SAA group determined by iTRAQ analysis. Compared with that in healthy participants, the expression of LILRA5 in CD34+ cells of patients with SAA also increased.

LILRs are a family of immune-modulatory proteins that are localized on human chromosome 19 in the region 19q13.4 (9), principally expressed on NK, myeloid, T and B cells. Receptors on LILRs modulate the maturation of DCs and affect the antigen that presents DC functions, thus regulating T-cell proliferation. LILRs are progressively known as the critical regulators of innate immune responses that act
through the threshold modulation and amplitude of lymphoid and myelomonocytic cell activation (27). LILRA3 is a soluble molecule belonging to a household of highly homologous cell surface receptors, principally expressed through mono-myeloid cells (9). LILRA3 is unique as such that it lacks cytoplasmic and transmembrane domains, and thus, is an exclusively secreted protein. MHC class I molecules and Nogo 66 are candidates for high-affinity binding with LILRA3 ligands. The functions of LILRA3 remain to be fully elucidated. However, studies suggested that it may act as a significant function in the pathogenesis of autoimmune disorders. Serum LILRA3 protein levels are significantly increased and are one of the strongest independent markers of disease severity in multiple sclerosis. Serum IL-10 and IFN-γ are positively correlated with LILRA3 levels and negatively associated with serum TNF-α and LILRA3 in patients with multiple sclerosis (12). Serum LILRA3 concentrations are also significantly upregulated in patients with rheumatoid arthritis (28), systemic lupus erythematosus (29) and Sjögren’s syndrome (30), and positively correlated with disease activity and severity. In the present study, the expression of LILRAs in patients with SAA was investigated. LILRA3 mRNA expression was increased in BMMNCs of patients with SAA, whereas others (LILRA1, -2, -4 and -6) were not significantly different. Compared with healthy controls, the results verified that the percentage of intracytoplasmic LILRA3+ on CD11C+HLA-DR+ mDCs, the relative intensity of LILRA3 protein in mDCs and the expression of LILRA3 mRNA in mDCs were markedly enhanced in patients with SAA. Simultaneously, the percentages of intracytoplasmic LILRA3+ in CD34+ cells were also augmented, which positively correlated with its expression in mDCs. These results suggested that LILRA3 was abnormally present upstream of SAA in mDCs and that there was an abnormal expression of downstream targets in CD34+ cells. The present results

Figure 4. (A) LILRA3 expression was closely associated with the clinical characteristics of patients with SAA. (B) Correlations of LILRA3 mRNA with cytokines TNF-α, IFN-γ, IL-4 and IL-10 in patients with untreated severe aplastic anemia. LILRA, leukocyte immunoglobulin-like receptors A.
suggested that the expression of LILRA3 was increased in patients with SAA and that this was positively correlated with disease progression, activity and response to therapy.

The expression of LILRA3 mRNA was higher in patients with new SAA and remission SAA in comparison with the control group, but there were no differences between new SAA and remission SAA. Although the expression of LILRA3 in remission SAA was not significantly different from that of untreated SAA, the expression of LILRA3 on mDCs of patients with remission SAA was lower than that in the untreated SAA group. The reason may be that compared with the normal control group, the cytokine storm period in the remission SAA group was not over, but it was smaller than that in the patients with untreated SAA. No statistically significant difference in the expression of LILRA3 was obtained between SAA patients with short-term remission (<12 months) and long-term remission (>12 months). A significant risk of relapse was reported with rapid tapering of cyclosporine in patients with remission SAA and the treatment should be continued for a long time until the immunoreaction is completely back to normal (20). LILRA3 upregulated the transcription of IL-1A, IL-1B and IL-6 and modulated the expression of co-stimulatory molecules and MHC in B-cells and monocytes. Signaling via LILRs affects the maturation and activation of DCs, maintains the antigen-presenting properties of DCs and regulates immune responses (27,30,31). Thus, it may be possible that LILRA3 is involved in the CTLs-mediated autoimmune pathogenesis of SAA and antagonizing LILRA3 may be a novel strategy for addressing disease prevention and progression. However, the present results provide insight into several hypotheses about the pathogenesis of SAA. Future studies delineating the exact mechanisms of LILRA3 in the pathogenesis of SAA are warranted.

In conclusion, the present results suggested that LILRA3 was highly expressed and positively correlated with disease progression in the mDCs and CD34+ cells of patients with SAA. Taken together, the present results indicate that LILRA3 is an important participant in the pathogenesis of SAA and a potential therapeutic target.

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Availability of data and materials

Not applicable.

Authors’ contributions

RF and ZS designed the study and revised the manuscript. HY, HL and YZ performed experiments, analyzed data and wrote the initial draft of the manuscript. HW, WQ, CL, ZL, SG, YS and JT contributed to the experiments and the collection of patients' features. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was in compliance with the Declaration of Helsinki and was approved by the Ethics Committee of Tianjin Medical University General Hospital (Tianjin, China). Written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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