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Anti-inflammatory effects of interleukin-35 in acquired aplastic anemia

Wei Yu [1], Meili Ge [1], Shihong Lu, Jun Shi, Xingxin Li, Jizhou Zhang, Min Wang, Jinbo Huang, Yingqi Shao, Zhendong Huang, Jing Zhang, Neng Nie, Yizhou Zheng *

State Key Laboratory of Experimental Hematology, Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Science & Peking Union Medical College, 288 Nanjing Road, Tianjin 300020, PR China

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ABSTRACT

Interleukin (IL)-35 is a novel regulatory cytokine primarily produced by regulatory T cells. Accumulating evidence has established that IL-35 plays an important role in the regulation of immune homeostasis, but little is known regarding the function of IL-35 in acquired aplastic anemia (AA). The aim of the present study was to investigate the expression of IL-35 and its effects on T cell response in AA. Our study demonstrated that significantly decreased plasma levels of IL-35 in AA were closely correlated with disease severity. *In vitro* stimulation experiment further confirmed the anti-inflammatory effects of IL-35, including suppressing the proliferation of $CD4^+$ and $CD8^+$ effector T cells, inhibiting the secretion of interferon-γ, tumor necrosis factor-α and IL-17 and promoting the production of transforming growth factor-β by peripheral blood mononuclear cells from patients with AA. Furthermore, we established that IL-35 inhibited the differentiation of type 1 T cells and T helper 17 cells but promoted the differentiation of type 2 T cells. Accordingly, the expression of T-bet and RORγt was inhibited while the expression of GATA3 was induced after IL-35 treatment. In summary, our findings suggested that decreased IL-35 might contribute to the loss of immune-tolerance and be critically involved in the pathogenesis of AA.

1. Introduction

Acquired aplastic anemia (AA) is an immune-mediated bone marrow (BM) failure syndrome characterized by pancytopenia in the peripheral blood (PB) and hypoplasia in the BM [1]. Although the exact pathogenesis of AA is not yet understood, destruction of hematopoiesis mediated by an aberrant immune response is considered to be the main cause [2–5]. In recent years, abnormal immunity, particularly T-cell immune and aberrant cytokine profiles, have been widely studied in the pathophysiology of AA. In addition to the abnormally polarized T helper (Th) 1 and cytotoxic T (Tc) 1 cells secreting immoderate inhibitory hematopoietic cytokines, such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ, decreased numbers and impaired immunosuppressive function of circulating regulatory T cells (Tregs) and elevated numbers of Th17 cells have been demonstrated to play vital roles in the pathogenesis of AA [6–13]. The dysfunction of antigen-presenting cells and other potent pro-inflammatory cytokines also contributed to the activation of T cells and subsequent inhibition of hematopoiesis [14–16].

Interleukin (IL)-35, the newest member of the IL-12 family, is a heterodimeric cytokine composed of the IL-12 p35 subunit and the IL-27 Epstein–Barr virus-induced protein 3 (EBI3) subunit. Distinctive from its siblings, IL-35 has been identified as a potentially immunosuppressive cytokine primarily secreted by Tregs, and to a lesser extent by activated dendritic cells and macrophages [17–19]. Recently, IL-35, but not IL-10 or transforming growth factor-β (TGF-β), was considered to be the essential effector molecule for Treg-mediated suppression [18].

More importantly, accumulating evidence has suggested that IL-35 exerted various critical roles in the immune response. IL-35 could inhibit the proliferation of conventional T cells and the differentiation of $CD4^+$ T cells into Th1 or Th17 effector cells but promote the proliferation of Tregs [20–22]. In addition, IL-35 could significantly reduce the production of Th1-type cytokines, such as IFN-γ, TNF-α and IL-2, and Th17-type cytokines [20,21]. Besides, Wang *et al.* [23] reported that IL-35 induced the expansion

Abbreviations: AA, aplastic anemia; BM, bone marrow; CR, complete response; EBI3, Epstein–Barr virus-induced protein 3; IFN-γ, interferon-γ; IL, interleukin; IST, immunosuppressive therapy; Non-SAA, non-severe AA; PB, peripheral blood; PBMCs, peripheral blood mononuclear cells; Tc, cytotoxic T; TGF-β, transforming growth factor-β; Th, T helper; TNF-α, tumor necrosis factor-α; Tregs, regulatory T cells; VSAA, very severe AA.

* Corresponding author.
E-mail address: zheng_yizhou@hotmail.com (Y. Zheng).

[1] Wei Yu and Meili Ge contributed equally to this work.

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of human regulatory B cells and inhibited B cell proliferation. More surprisingly, IL-35 has been shown to induce a potent regulatory population, named inducible IL-35-producing Tregs (iT_R35 cells), which were strongly suppressive and stable without the expression of the Treg transcription factor forkhead box P3 (Foxp3) [24]. Based on these pleiotropic effects of IL-35 in immune regulation, some researchers have indicated that IL-35 was involved in the development and exacerbation of several inflammatory diseases, such as encephalomyelitis and inflammatory bowel disease [18,25].

As yet, the involvement of IL-35 in the pathogenesis of AA is not determined. To investigate the possible role of IL-35 in the pathogenesis of AA, we detected the expression level of IL-35 and evaluated its *in vitro* effects on the proliferation, differentiation and secretion of cytokines in peripheral blood mononuclear cells (PBMCs) of patients with AA. Our findings suggested that decreased levels of IL-35 might contribute to the loss of immunological self-tolerance in patients with AA, which further broadened our understanding of AA.

2. Materials and methods

2.1. Patients

We analyzed PB samples from 80 patients with AA (median age 35 years, 42 male and 38 female) and 50 age-matched healthy controls (median age 33 years, 24 male and 26 female). Written informed consents were obtained from all patients and controls. This study was approved by the Ethics Committees of the Institute of Hematology, Chinese Academy of Medical Sciences and Peking Union Medical College according to the guidelines of the Declaration of Helsinki (Ethics number: KT2014005-EC-1). The diagnosis and severity classification of patients with AA were established according to the criteria of Camitta *et al.* [26], including 25 patients with non-severe AA (Non-SAA), 29 patients with SAA, and 26 patients with very severe AA (VSAA). Among all of the AA patients, 65 were untreated before enrollment and 15 were in complete response (CR) after immunosuppressive therapy (IST) consisting of antithymocyte globulin and cyclosporine A. Patients with comorbidities, such as active infection or secondary to other autoimmune diseases, were excluded from our study.

2.2. Cell culture and proliferation assays

PBMCs were isolated from whole blood samples by Ficoll-Hypaque density-gradient centrifugation. Parts of the isolated PBMCs from AA patients and controls were used to measure the expression of EBI3 and p35 mRNA. In the proliferation assays, aliquots of 2×10^5 freshly isolated cells were cultured in complete RPMI 1640 medium (containing 10% fetal calf serum, 1% glutamine and 1% penicillin/streptomycin) for 72 h at 37 °C in a 5% CO_2 with or without 100 ng/mL IL-35 (Sino Biological Inc, Beijing, P.R. China), and meanwhile stimulated with 5 μg/mL PHA (Sigma, St Louis, MO, USA) and 5 ng/mL rIL-2 (Peprotech, Rocky Hill, NJ, USA). BrdU (Roche Molecular Biochemicals, Mannheim, Germany) was added during the last 18 h of culture. Then, the cells were harvested and their proliferation was measured according to the manufacturer's instructions.

In some experiments, aliquots of 1×10^6 isolated PBMCs in 1 mL complete RPMI 1640 medium were cultured as described above. After incubation, the absolute number of total cells was evaluated by direct cell counting, the percentage of $CD4^+$ and $CD8^+$ T cells was measured by flow cytometry and the remaining cells were used for extraction of total RNA. For the detection of intracellular cytokines, 50 ng/ml of phorbol 12-myristate 13-acetate (Sigma), 1 μg/mL of ionomycin (Sigma) and 3 μg/mL brefeldin A (eBioscience, San Diego, CA, USA) were added during the last 5 h of culture after incubation with or without IL-35 for 48 h.

2.3. Flow cytometry

Cultured cells were harvested and washed twice with phosphate-buffered saline by centrifugation at 300 g for 7 min. To measure the percentage of $CD4^+$ and $CD8^+$ T cells, cells were labeled with FITC-conjugated anti-CD4 antibody, APC-conjugated anti-CD8 antibody and the appropriate isotype controls (Biolegend, San Diego, CA, USA) according to the manufacturer's instructions. The cells were resuspended with 1% paraformaldehyde for flow cytometry analysis. For the detection of intracellular cytokines, the cells were first incubated with FITC-conjugated anti-CD3 and APC-conjugated anti-CD8 antibodies (Biolegend) at room temperature for 20 min. Subsequently, the cells were fixed with Fixation Buffer (Biolegend), followed by permeabilization with Permeabilization wash buffer (Biolegend). The cells were then stained with PE-conjugated anti-IL-4, PE-conjugated anti-IL-17, PE-Cy7-conjugated anti-IFN-γ and isotype control antibodies (Biolegend) at room temperature for 30 min, washed twice, and resuspended with 1% paraformaldehyde for analysis. Data acquisition was performed on a Canto II flow cytometer (BD Biosciences) and analyzed using FlowJo software (Version 7.6).

2.4. Real-time polymerase chain reaction (PCR)

Total RNA of freshly isolated or cultured PBMCs was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The reverse transcription reactions were performed using the *TransScript* First-Strand cDNA Synthesis Supermix (TransGen Biotech, Beijing, P.R. China) according to the manufacturer's procedure. Real-time PCR was performed with the 2 × SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) using the Applied Biosystems Gene Amp 7500 sequence detection system. The sequences of PCR primers were listed in Table 1. The relative quantity of target mRNA expression was calculated relative to the expression of β-actin using the $2^{-\Delta\Delta Ct}$ method.

2.5. ELISA assays for cytokines

The plasma and culture supernatants were stored at −80 °C until the ELISA assays were performed. Plasma IL-35 was measured using ELISA kits (Uscn Life Science Inc., Missouri City, TX, USA) according to the manufacturer's instructions. The concentrations of IFN-γ, TNF-α, IL-17, IL-10 and TGF-β in the culture supernatants were also measured using ELISA kits (NeoBioscience Technology, Shenzhen, P.R. China) according to the manufacturer's instructions.

2.6. Statistical analysis

All analysis was performed using SPSS version 16.0 software (SPSS Science). The results were expressed as mean ± SEM unless

Table 1
Primer sequences for real-time polymerase chain reaction.

Gene	Forward sequence (5′ → 3′)	Reverse sequence (5′ → 3′)
p35	TCCTCCCTTGAAGAACCGGA	TGACAACGGTTTGGAGGGAC
EBI3	GCAGCAGACGCCAACGT	CCATGGAGAACAGCTGGACAT
T-bet	TGACCCAGATGATTGTGCTC	TATGCGTGTTGGAAGCGTTG
GATA3	CAGCACAGAAGGCAGGGAGT	AGGCGTTGCACAGGTAGTGTC
RORγt	GTGCGTGTTAGGATGTGCCG	GTGGGAGAAGTCAAAGATGGA
FoxP3	GTGGCATCATCCGACAAGG	TGTGGAGGAACTCTGGGAAT
β-actin	GGCACCCAGCACAATGAAG	CGTCATACTCCTGCTTGCTG