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Research paper

Helios expression in regulatory T cells promotes immunosuppression, angiogenesis and the growth of leukemia cells in pediatric acute lymphoblastic leukemia

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ABSTRACT

Regulatory T cells (Tregs) characterized by the transcription factor forkhead box P3 (FoxP3) are crucial for maintaining immune tolerance and preventing autoimmunity. However, FoxP3 does not function alone and Helios is considered a potential candidate for defining Treg subsets. In this study, we investigated the expression and function of Helios for identifying Tregs in childhood precursor B-cell acute lymphoblastic leukemia (pre-B ALL). Our results demonstrated that patients with pre-B ALL had a higher percentage of Helios$^+$ FoxP3$^+$ CD4$^+$ Tregs. And there was a positive correlation between the expression of Helios and the suppressive function of Tregs, the risk gradation of ALL. Helios in combination with CD4 and FoxP3 may be an effective way to detect functional Tregs in pre-B ALL by promoting the secretion of transforming growth factor (TGF)-β1. Furthermore, Helios$^+$ Tregs could regulate angiogenesis in the BM niche of pre-B ALL via the VEGFA/VEGFR2 pathway. We also found Helios$^+$ Tregs decreased apoptosis rate of nalm-6 cells by up-regulating the expression of anti-apoptosis protein Bcl-2. In summary, these data strongly imply the physiological importance of Helios expression in Tregs, and suggest that the manipulation of Helios may serve as a novel strategy for cancer immunotherapy.

1. Introduction

Acute lymphocytic leukemia (ALL) is due to a disruption in the regulation of cell growth as well as the failure of the host to provoke a sufficient antitumor immune response [1]. Although many aspects of immunoregulation abnormalities in leukemia have been elucidated, the specific immunological mechanism involved in the multistep leukemogenesis remains unclear [2]. Recent studies have focused on the role of immune negative regulation in human diseases [3]. Regulatory T cells (Tregs) are known to have potent immunosuppressive abilities and play a vital role in maintaining self-tolerance [4–6]. It is postulated that FoxP3 plays an essential role in Treg function and development, but FoxP3 expression is not sufficient for stably maintaining the suppressive function and phenotype of Tregs [7,8]. FoxP3 can still be detected in activated CD4T cells, according to previous literature [9,10]. Helios, a member of the Ikaros family of genes, was reported to mediate suppression as a potential target of immunomodulation as well as a specific cell surface marker to better differentiate Tregs [11].

Helios was originally cloned from a mouse thymoma line and is mainly expressed in the T-cell lineage. Previous studies have demonstrated that Helios upregulates FoxP3 by binding to the FoxP3 promoter, and partially silenced Helios expression in Tregs resulted in decreased FoxP3 levels. In the steady state, Helios expression is largely restricted to FoxP3$^+$ Tregs [12]. These findings have led to the increased interest in Helios, which may play a critical role in controlling certain aspects of Tregs, including their suppressive function, differentiation, and survival. However, there were very few studies have addressed the regulation of Helios in ALL Tregs.

Despite the observation that immunosuppression mediated by Tregs is a key facilitator of tumor immune evasion, our current knowledge of the clear connection between tumors and abnormalities of Tregs is still incomplete. It has been suggested that peripheral immune tolerance and angiogenesis programs are closely connected and cooperate to sustain tumor growth [13]. Multiple lines of evidence have demonstrated that Tregs also can constitutively secrete VEGFA to promote a pro-angiogenic tumor milieu [14]. Given the importance of angiogenesis in bone marrow (BM) for the maintenance of leukemic clones, recognition of angiogenic and anti-angiogenic factors as well as Tregs can improve current therapeutic strategies [15].

To date, few studies have focused on Helios expressing Tregs in pediatric leukemia. In the present study, we extended the investigation to patients with pediatric precursor B-cell (pre-B) ALL, and

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demonstrated that Helios was overexpressed in a subset of $FoxP3^+CD4^+$ Tregs in ALL; also, Helios expression in Tregs are required for BM angiogenesis and the growth of leukemia cells. These observations may have important clinical implications, since $Helios^+$ Tregs may be essential for pathogenesis and angiogenesis in ALL.

2. Materials and methods

2.1. Samples

24 Peripheral blood (PB) and 5 bone marrows (BM) samples were obtained from patients who were diagnosed with pre-B ALL at Qilu Hospital, Jinan, China. All cases fulfilled the diagnostic criteria of pre-B ALL according to the Chinese Children's Leukemia Group 2008 protocol [16]. For the healthy control (HC) population, 10 PB and 5 BM consenting age-matched individuals who underwent orthopedic surgery with no history of tumors, antecedent primary hematologic abnormalities, or immunodeficiency diseases were sampled. Clinical information of the patients is listed in Supplemental Table S1. All individual participants included in the study provided written informed consent. The study was approved by the Research Ethics Committee of Qilu Hospital.

2.2. Cell culture

$CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ helper T (Th) cells were isolated using $CD4^+CD25^+$ Treg isolation kits (Miltenyi Biotec, Germany). The purity of the isolated cell population analyzed by flow cytometry was greater than 95%. Tregs were cultured in RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS) (Gibco) and 0.055 mM 2-mercaptoethanol (Gibco). Th cells were cultured in RPMI-1640 medium with 10% FBS. Nalm-6 (human pre-B ALL cell line) cells were cultured in RPMI-1640 medium containing 10% FBS. Human umbilical vein endothelial cells (HUVECs) were grown in M199 medium (Gibco) containing 15% FBS and 40 ng/ml fibroblast growth factor (Peprotech, USA). Cells were cultured at 37 °C in a 5% CO_2 atmosphere. Cell images were taken using an Olympus IX71 inverted fluorescence microscope (Olympus, Japan) and processed using the DP controller software (Olympus).

2.3. Flow cytometry

To measure the percentage of Tregs, the following fluorochrome-conjugated antibodies to surface markers were used: FITC anti-CD4 (OKT4, 11-0048, eBioscience) and PE/Cy5 anti-CD25 (BC96, 302608, BioLegend). APC anti-FoxP3 (PCH101, 17-4776, eBioscience) and PE anti-Helios (22F6, 12-9883, eBioscience) were detected with the FoxP3/Transcription Factor Staining Buffer Set (00-5523, eBioscience). Multi-color flow cytometry use fluorescence minus one (FMO) control for determining backgroud signal.

To measure intracellular cytokine production, Tregs were stimulated for 4 h at 37 °C with 12-Otetradecanoylphorbol-13-acetate (PMA, 50 ng/ml, Multiscience, China) and ionomycin (1 ug/ml, Multiscience) in the presence of monensin (0.75 μl/ml, eBioscience). The cells were fixed, permeabilized, and stained for APC anti-FoxP3, PE anti-Helios, as well as PE anti-human latency associated peptide (LAP/TGF beta 1) (FNLAP, 12-9829-41, eBioscience) antibody, separately. Flow cytometry was performed using the Guava easyCyte 8HT flow cytometer (Millipore, MA), and the data were analyzed using FlowJo version 7.6.2.

2.4. Cell transfection

The lentiviral vectors GV358-enhanced green fluorescent protein (EGFP)-Helios (NM_016260) (Lenti-Helios) and GV358-EGFP (Lenti-NC) were developed by Shanghai GeneChem Company. The viral concentrate was diluted to infect PB-derived Tregs at a multiplicity of infection of 50. Infected Tregs were cultured in X-VIVO 15 medium (Lonza, Swiss) supplemented with 100 U/mL of rIL-2 and CD3/CD28 beads (bead-to-cell ratio of 1:1) (Invitrogen, USA). A successful transduction was confirmed by flow cytometry after 72 h. The cells were maintained and allowed to grow for another 3–5 days.

Tregs were transfected with siRNA-FAM against Helios (siRNA-Helios) or with a nontargeting control (siRNA-NC) (GenePharma, China) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The sequences of the siRNA-Helios were listed as follows: sense, 5′-GGAAGAUUGUAAGGAACAATT, anti-sense, 5′-UUG UUCCUUACAAUCUUCCTT. A successful transfection was confirmed by flow cytometry after 24 h.

After 72 h, gene expressions (Helios, FoxP3, TGF-β1, IL-10, CTLA-4, IFN-γ, SATB1, and TNFRSF1B) were confirmed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) or flow cytometry. Part of the transfected Tregs were next tested for their immunosuppressive function by carboxyfluorescein diacetate succinimidyl ester (CFSE). The culture supernatant of the transfected Tregs were obtained by centrifugation, which was then used in an endothelial-tube formation assay.

2.5. Cell apoptosis detection and CFSE-based suppression and proliferation assay

Apoptosis was quantified using the BD Apoptosis kit (BD Bioscience, USA) with Annexin V/PI double staining. The flow cytometry analyses were performed using a guava easyCyte 8HT flow cytometer.

The suppressive capacity of the Treg populations were detected as follows: Th cells were labeled with 5 μM CFSE and incubated for 48 h. Tregs transfected with Lenti-Helios or siRNA-Helios (1×10^5 cells/well) were then added to the wells and co-cultured with Th cells (at 1:2 ratio) for 72 h, separately. Proliferation of Th was rated by flow cytometry. The proliferation index of Th cells was inversely proportional to suppression capability of Tregs. Modfit FLT software was used for data analysis.

2.6. Endothelial-tube formation assay

Growth factor-reduced Matrigel (BD Biosciences, USA) was allowed to polymerize in a 96-well plate at 37 °C for at least 30 min. HUVECs (5×10^3 cells/well) were suspended in 100 μl medium conditioned by the culture supernatant of above $Helios^{high}$ Tregs, $Helios^{low}$ Tregs or normal Tregs (NC). After incubation for 6 h at 37 °C, capillary-like structures in the Matrigel were photographed under Olympus IX71 inverted fluorescence microscope. The length of the tubes was measured using the Image J software. After incubation for 24 h, total RNA was extracted from HUVECs to test pro-angiogenic genes (IL-6, IL-17a, and lipopolysaccharide-induced CXC chemokine [LIX]), anti-angiogenic genes (IFN-γ, TGF-β, and IL-10), Neuropilin 1, CCL28, and CCL22.

2.7. qRT-PCR analysis

Total RNA was extracted from samples using Trizol reagent (Invitrogen). First-strand cDNA was synthesized using 1 μg of total RNA in a 20 μl of reverse transcriptase reaction mixture using the ReverTra Ace qPCR RT Master Mix kit (TOYOBO, Japan) with primers. qRT-PCR was performed on an ABI Prism 7500 sequence detection system using Thunderbird SYBR qPCR Mix (TOYOBO) in a 20 μl of reaction mixture. Data were analyzed by Sequence Detection Software 1.4 (Applied Biosystems, CA). The control group set to one. The used primer sequences are presented in Supplemental Table S2.

2.8. Western blot

Western blotting was performed as previously described [10]. Antibodies against VEGFA (ab46154, Abcam Inc. Cambridge, MA, USA,