Role of HMGB1 in regulation of STAT3 expression in CD4⁺ T cells from patients with aGVHD after allogeneic hematopoietic stem cell transplantation

Ya-jing Xu, Lin Li, Yan Chen, Bin Fu, Deng-shu Wu, Xiao-lin Li, Xie-lan Zhao, Fang-ping Chen *

Department of Hematology, Xiangya Hospital, Central South University, Changsha, Hunan, China

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A B S T R A C T

Treg/Th17 balance plays a critical role in maintaining immune homeostasis of acute graft-versus-host disease (aGVHD) patients. STAT3 is an important factor involved in the instability of Treg and the promotion of Th17. HMGB1 is a cytokine mediator of inflammation and an important chromatin protein regulating gene transcription. In this study, we found that the expressions of HMGB1 and STAT3 were higher in CD4⁺ T cells of patients with aGVHD compared with those without aGVHD, and the HMGB1 expression was positively correlated with the STAT3 expression. Simultaneously, their expressions were positively correlated with the severity of the aGVHD. We also demonstrated that HMGB1 could regulate the expression of STAT3 by modulation of its DNA methylation in CD4⁺ T cells, moreover downregulated HMGB1 in aGVHD CD4⁺ T cells could change the ratio of Treg/Th17. These data strongly suggest that HMGB1 plays a crucial role in the regulation of Treg/Th17 and progression of aGVHD.

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1. Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is one of the important measures in the treatment of hematological disorders, but acute graft-versus-host disease (aGVHD) is a frequent and unpredictable severe inflammatory complication which seriously endangers patients’ life [1–3]. aGVHD is a complex inflammatory process characterized by exaggerated inflammatory responses, during which the release of numerous proinflammatory cytokines promotes the ability of donor T lymphocytes to attack the host tissues including the skin, liver, and gastrointestinal tract [4].

Accumulated studies have demonstrated that Th17 cell differentiation accelerates the progression of aGVHD. Polarized Th17 cells can induce lethal aGVHD in mice [5]. Th17-associated cytokines including IL-17 and IL-23 were markedly increased, and closely related to the occurrence and development of aGVHD [6]. By contrast, regulatory T cell (Treg) plays a key role in the maintenance of self-tolerance in various autoimmune diseases and allosresponse [7]. Tregs contribute to the tolerance acquisition to donor antigen in solid organ transplantsations [8] and protection against the development of fatal aGVHD in a murine model [9]. Treg percentage and Foxp3 expression were significantly lower in severe aGVHD (grades 3 to 4) and mild aGVHD (grades 1 to 2) patients than in patients without aGVHD (grade 0) and healthy donors [6]. T. Fujioka et al. found that at the second week after HSCT, patients with aGVHD had sharply lower Treg frequency than those without aGVHD. And these differences were closely related with the incidence of aGVHD [10]. These provided important evidence that the balance between Th17 and Treg played a critical role during aGVHD.

Signal transducer and activator of transcription 3 (STAT3) is a member of the signal transducers and activators of transcription family which regulates gene expression involved in cell growth and division, cell movement, and apoptosis [11]. Uncontrolled STAT3 activation may lead to a variety of immune-mediated disease status. In response to the proinflammatory cytokine IL-6, activation of STAT3 in combination with TGF-β leads to increased expression of orphan nuclear receptors RORγ and RORα, signature transcription factors for Th17 cells [12–14]. Recent study has shown that STAT3 is involved in the inhibition of iTreg cell polarization and the instability of nTregs during acute murine graft-versus-host disease [15], suggesting that STAT3 may be an important regulator of Th17 and Treg cells.

High mobility group box 1 (HMGB1) protein is a DNA binding protein mainly involved in DNA replication, transcription, repair and stabilizing nucleosome [16]. Li Y. et al. found that HMGB1 can bind to Gadd45α, which may contribute to DNA demethylation of CD11a and CD70 in SLE CD4⁺ T cells [17]. Enhanced HMGB1 is reported to inhibit Treg and promote Th17 response during the immune and inflammatory process [18–20]. In the study, we investigated whether HMGB1 is involved in the regulation of abnormal expression of STAT3 in CD4⁺ T cells from aGVHD patients. We found that HMGB1 and STAT3
expressions were sharply increased in aGVHD CD4+ T cells, and their expression could be correlated with the severity of the aGVHD. We also demonstrated that HMGB1 can regulate STAT3 expression by modulating the methylation of the STAT3 promoter region. Together, our findings shed light on novel molecular mechanisms of STAT3 dysregulation in CD4+ T cells of aGVHD patients.

2. Materials and methods

2.1. Subjects

Forty patients who underwent allo-HSCT from HLA-identical sibling donors at the Center for Hematopoietic Stem Cell Transplantation at Xiangya Hospital, Central South University were included in this study. The characteristics of these patients are shown in Table 1. This study was approved by the human ethics committee of the Central South University Xiangya Medical School, and written informed consent was obtained from all patients. The adoption of conditioning regimen mainly accord to the disease types and patients' general status. AML (acute myeloid leukemia) and MDS (myelodysplastic syndrome): cytarabine (2 g/m² per day, days −9 to −8), busulfan (3.2 mg/kg per day, intravenously days −7 to −5), cyclophosphamide (1.8 g/m² per day, days −4 to −3), semustine (250 mg/m², day −2). ALL (acute lymphocytic leukemia): Total body irradiation (TBI) (5 Gy per day, days −5 to −4), cyclophosphamide (1.8 g/m² per day, days −3 to −2). CML (Chronic myeloid leukemia): busulfan (3.2 mg/kg per day, intravenously days −8 to −5), cyclophosphamide (1.8 g/m² per day, days −4 to −3), semustine (250 mg/m², day −2). We use granulocyte colony stimulating factor (G-CSF) for peripheral blood hematopoietic stem cell (PBSC) mobilization. The GVHD prophylaxis regimen consisted of a combination of cyclosporine A (CsA) and short-term methotrexate (MTX). The dosage of CsA was 2.5 mg/kg/day, i.v. from day −9. The dosage of MTX was 15 mg/m² administered i.v. on day +1 and 10 mg/m² on days +3 and +6.

Assessment of aGVHD was based on clinical symptoms in accordance with commonly accepted criteria [21,22]. Organ lesions of the skin, liver, and gastrointestinal tract was staged 1 through 4 for aGVHD, patients were also assigned a grade of aGVHD (1 through 4) based on overall severity. aGVHD grade 2 was treated continuously according to the prophylaxis regimen. aGVHD grade 2 was treated with methylprednisolone (2 mg/kg/day). MTX and anti-CD25 monoclonal antibody were given to subjects intolerant of or unresponsive to methylprednisolone.

Patients were also assigned a grade of aGVHD (1 through 4) (n = 16). Peripheral blood samples were collected as soon as aGVHD was diagnosed and before therapy was begun.

2.2. Isolation, culturing and transfection of CD4+ T cells

CD4+ T cells were purified from 60 ml venous peripheral blood using human CD4 beads according to protocols provided by the manufacturer (Miltenyi, Bergisch Gladbach, Germany), and cultured in human T cell culture medium (Lonza, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. CD4+ T cells were transfected using the Human T cell Nucleofector Kit and Amaxa nucleofector (Lonza, Walkersville, MD, USA). First, CD4+ T cells were harvested and resuspended in 100 μl human T cell nucleofector solution, and then the cell suspension was mixed with either siRNA-HMGB1 or control. The mix was electrotransfected using nucleofector program V-024 in the Amaxa nucleofector. The transfected cells were cultured in human T cell culture medium and harvested for 48 h.

2.3. RNA extraction and real-time quantitative RT-PCR

Total RNA was isolated from CD4+ T cells using TRIzol Reagent (Invitrogen, Carlsbad, CA). RNA was reverse transcribed into cDNA using random primer and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Real-time PCR was performed in triplicate using the SYBR Green Master Mix (ABI Prism 7500, Foster City, CA). Human GAPDH gene was used as an endogenous control for sample normalization. The fold change was calculated using the formula $2^{-ΔΔCt}$. ΔΔCt = (Cttarget gene − Ctinternal control)sample − (Cttarget gene − Ctinternal control)control. Primers are listed in Table 2.

2.4. Western blot analysis

CD4+ T cells were lysed in protein lysis buffer containing protease inhibitor (Thermo Pierce). Lysates were centrifuged for 15 min at 14,000 g at 4 °C, and protein concentration was determined by the Bradford protein assay (Bio-Rad, CA, USA). Proteins were separated by SDS-PAGE using 8% polyacrylamide gels, and then transferred onto PVDF blotting membranes (Bio-Rad, CA, USA). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBS) buffer and immunoblotted with primary antibodies include anti-HMGB1 (Abcam, MA, USA), anti-STAT3 (Cell Signaling, BSN, USA), and anti-GAPDH (Santa Cruz, CA, USA). Band intensity was quantified using Quantity One software (Bio-Rad, CA, USA).

2.5. Genomic DNA extraction and bisulfite sequencing

Genomic DNA was isolated from CD4+ T cells using the TIANamp Genomic DNA Kit (TIANGEN, Beijing, China). Bisulfite conversion was performed using the EpiTect Bisulfite Kit (Qiagen, CA, USA). Three CpG islands within the STAT3 promoter region were amplified by PCR. The PCR products were next subcloned into a pGEM-T vector (Promega, WI, USA). Ten independent clones were sequenced for each of the amplified fragments. Primers used were as follows:

5’ GTGAGATTTTTTATGTATTTTATCCTTCCTAC (forward1) and

3’ CTATAGTTAATTTAATTATTATAGCTTAC (reverse1)

Table 1

<table>
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<th>Diagnosis</th>
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<tr>
<td>Number</td>
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<td>24</td>
</tr>
<tr>
<td>Median age</td>
<td>31</td>
<td>35</td>
</tr>
<tr>
<td>Sex (female/male)</td>
<td>7/9</td>
<td>8/16</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Primer sequences for real-time PCR.</th>
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</thead>
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<td>Forward primer</td>
</tr>
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</tr>
<tr>
<td>STAT3</td>
</tr>
<tr>
<td>RORγt</td>
</tr>
<tr>
<td>IL17A</td>
</tr>
<tr>
<td>IL17F</td>
</tr>
<tr>
<td>Foxp3</td>
</tr>
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<td>CTLA4</td>
</tr>
<tr>
<td>IL10</td>
</tr>
<tr>
<td>TGF-β3</td>
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<td>GAPDH</td>
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</tbody>
</table>

Primer sequences for real-time PCR.

$2^{-ΔΔCt} = (Cttarget gene − Ctinternal control)sample − (Cttarget gene − Ctinternal control)control. Primers are listed in Table 2.

5’ GTGAGATTTTTTATGTATTTTATCCTTCCTAC (forward1) and
5′ CTAAAAACACAAAAACATAAATAA3′ (reverse1); 5′ TTTAGTTGTTTTTTTTATTGGTTAGTGG3′ (forward2) and 5′ CCCACCCTACACCCCCTTCACCTATTTC3′ (reverse2); 5′ TTGAGTGGTTTTTTTTATTGGTTAGTGG3′ (forward3) and. 5′ TCCAAAAACACAAAAACATAAATAA3′ (reverse3).

2.6. Statistical analysis

Results are expressed as mean ± SD. Data were analyzed by ANOVA followed by the unpaired Student’s t-test for multiple comparisons. All analyses were performed with SPSS 16.0 software. Significance was set at p values ≤ 0.05.

3. Results

3.1. Patients

Among the 40 HSCT patients, 24 presented with aGVHD vs 16 who did not. Of the 24 patients who developed aGVHD, 6 (25%) were grade 1, 7 (29.2%) were grade 2, 7 (29.2%) were grade 3, and 4 (16.6%) were grade 4. The median day of onset of aGVHD was 43 (range: 13–82). 18 episodes of grades 2 to 4 aGVHD were treated with methylprednisolone, and 13 (72.2%) episodes were treated successfully, whereas the 5 episodes that lacked adequate response to the primary treatment were treated with intravenous MTX (10 mg per day, 1 to 2 times per week), anti-CD25 monoclonal antibody. 4 episodes showed improvement, but the remaining 1 patient had exacerbated GVHD and subsequently died.

3.2. Expression levels of HMGB1 and STAT3 in CD4+ T cells from aGVHD patients

In the early days, HMGB1 was identified as a DNA-binding protein that functions as a structural co-factor critical for proper transcriptional regulation [23]. STAT3 is an important factor by regulation of the balance between Treg and Th17 involved in the pathogenesis of aGVHD [24]. Inhibition of the STAT3 activity could repress the development of aGVHD [25]. In this part, we investigated whether HMGB1 was involved in the regulation mechanism of abnormal expression of STAT3 in CD4+ T cells from aGVHD patients. We measured the mRNA and protein expression levels of HMGB1 and STAT3 in CD4+ T cells from 24 HSCT patients with aGVHD and 16 HSCT patients without aGVHD (the purity of CD4+ T cells from 60 ml venous peripheral blood: 96.9% ± 2.1%, and the absolute numbers: 3.12 ± 1.25 × 10⁶). The results from real-time quantitative RT-PCR and western blot showed that HMGB1 (Fig. 1A, C and D) and STAT3 (Fig. 1B, C and E) expressions were significantly up-regulated in patients with aGVHD compared with patients without aGVHD. Furthermore, the expressions of HMGB1 and STAT3 were even higher in severe aGVHD (grades 3 to 4) patients compared with that of patients with mild aGVHD (grades 1 to 2). We also did the correlation analysis between HMGB1 and STAT3 expression in CD4+ T cells from patients with aGVHD. Result showed that the expressions of HMGB1 and STAT3 were significantly positive correlated (Fig. 1F and G). Together, our results suggest that patients with aGVHD manifest a high expression of HMGB1 and STAT3 compared to patients without aGVHD, and their expression could be correlated with the severity of the aGVHD.
3.3. DNA methylation of STAT3 promoter in CD4+ T cells from patients with aGVHD

To investigate whether the promoter region of STAT3 was aberrantly DNA methylated in CD4+ T cells from patients with aGVHD, we did bisulfate sequencing analysis for a region (−730 to +770) flanking the STAT3 promoter, which contains three CpG islands (Fig. 2A). The STAT3 promoter was found to be significantly hypomethylated in CD4+ T cells from patients with aGVHD as compared with patients without aGVHD (Fig. 2B), and the methylation of STAT3 was even lower in severe aGVHD (grades 3 to 4) patients compared with that of patients with mild aGVHD (grades 1 to 2). Further, we found that the STAT3 promoter DNA methylation was negatively correlated with its mRNA level through the correlation analysis (Fig. 2C). In addition, we found that the HMGB1 protein levels were inversely correlated with the methylation status of the STAT3 promoter (Fig. 2D). These data strongly suggest that the abnormally elevated HMGB1 may contribute to the DNA hypomethylation of the STAT3 promoter in CD4+ T cells of aGVHD patients.

3.4. Downregulation of HMGB1 can reduce STAT3 expression by promoting its DNA methylation in aGVHD CD4+ T cells

To further demonstrate the impact of HMGB1 on STAT3 expression, we repress HMGB1 expression by transfection with siRNA-HMGB1 in CD4+ T cells from aGVHD patients. Real-time quantitative RT-PCR analysis showed that siRNA-HMGB1 transfection rendered much lower expression of HMGB1 and STAT3 mRNA in aGVHD CD4+ T cells (Fig. 3A), and similar results were obtained by western blot analysis (Fig. 3B and C). Further, bisulfate sequencing analysis revealed DNA hypermethylation in the STAT3 promoter after transfected with siRNA-HMGB1 in aGVHD CD4+ T cells (Fig. 3D). Overall, these data suggest that HMGB1 plays a crucial role in the regulation of STAT3 expression by modulation of its DNA methylation in CD4+ T cells of aGVHD patients.

3.5. Expression of Th17 and Treg associated genes in aGVHD CD4+ T cells after downregulation of HMGB1

In order to detect the influence on Th17 and Treg after downregulation of HMGB1 in aGVHD CD4+ T cells, we assessed the expression of RORγt, IL17A, IL17F related to Th17 and Foxp3, CTLA4, IL10, and TGF-β related to Treg by real-time quantitative RT-PCR. We observed decreased mRNA level of RORγt, IL17A, IL17F (Fig. 4A), and increased mRNA level of Foxp3 and CTLA4 (Fig. 4B). There were no significant differences in IL10 and TGF-β mRNA expression (Fig. 4B).

4. Discussion

Acute graft-versus-host disease (aGVHD) is a primary T cell mediated severe complication of allogeneic hematopoietic stem cell
transplantation (allo-HSCT) [26, 27], which is associated with high morbidity and mortality due to the damage to target organs including the skin, liver, and gastrointestinal tract [4].

High-mobility group box 1 (HMGB1), originally described as a non-histone and highly mobile DNA-binding protein, can regulate proper transcription as a structural co-factor [28]. HMGB1 polymorphisms are associated with aGVHD after allogeneic hematopoietic cell transplantation [29]. HMGB1, as a key mediator of inflammation leading to both acute and chronic organ injury, can directly bind to TLR4 or format complexes with endogenous or exogenous molecules in extracellular or cytoplasm space to trigger inflammation [30–32]. The HMGB1–IL–23–IL–17–IL–6–Stat3 axis plays a pivotal role in tumor development in murine models of melanoma, and blocking any portion of this axis will attenuate melanoma tumor growth [33]. In this study, we found that the expressions of HMGB1 and STAT3 in CD4+ T cells from patients with aGVHD are higher compared to patients without aGVHD. And the HMGB1 expression was positively correlated with the STAT3 expression. Simultaneously, their expressions were positively correlated with the severity of the aGVHD. These indicated that HMGB1 may be one of the main causes of elevated STAT3 in CD4+ T cells from patients with aGVHD.

Li Y. et al. have elucidated that HMGB1 was involved in the pathogenesis of lupus by binding to Gadd45a, and leading to DNA demethylation in CD4+ T cells [17]. In our study, we elucidated that the abnormally elevated HMGB1 contributes to the DNA hypomethylation of the STAT3 promoter in CD4+ T cells of aGVHD patients. We demonstrated that HMGB1 plays a crucial role in the regulation of STAT3 expression by modulating its DNA methylation in CD4+ T cells of aGVHD patients.

Th17 cells were characterized by the production of IL-17A, IL-17F, IL-21, and IL-22 [34], and its associated cytokines IL-21, IL-22, IL-23, and IL-6 have been reported to play a pivotal role during aGVHD [35–38]. Studies in murine models showed that Th17 plays a pathogenic role in aGVHD [39, 40]. In humans, some retrospective reports have shown that circulating and/or tissue localized Th17 cells can be increased in aGVHD [41, 42]. The steroid receptor-type nuclear receptor RORγt is an essential factor for the differentiation of Th17 cells [43], and the induction of RORγt is dependent on STAT3 [44]. Furthermore, STAT3 and RORγt could bind to the IL17 promoter directly, and collaboratively promote the production of IL-17 [45]. In our study, the expression of STAT3 was sharply reduced, after downregulation of HMGB1 in aGVHD CD4+ T cells. And RORγt, IL17A, and IL17F mRNA levels were significantly decreased, which indicated that HMGB1 is pivotal in the regulation of Th17 in aGVHD.

CD4+ CD25+ Foxp3+ regulatory T cells (Tregs) contribute to maintaining and regulating immune tolerance to self-antigens. Treg cells possess high potency to suppress immune responses mainly by contact-mediated direct inhibition of effector T cells and secretion of suppressive cytokines such as IL-10, TGFB and CTLA4. Accumulating studies have demonstrated that the decreasing frequencies of Tregs are associated with a higher incidence of aGVHD in patients who have undergone allo-HSCT [46–48]. Foxp3 is a member of the forkhead/winged-helix family of transcription factors and acts as a “master” regulator in the development and suppressive function of Tregs. Foxp3 controls Treg functions by inhibiting IL-2 transcription and upregulating CTLA4 levels [49]. A recent study showed that Foxp3 could form a cooperative complex with NF-AT to up-regulate the expression of CTLA4 [50]. Arian Laurence et al. have demonstrated that transcription factor STAT3 can promote the instability of nTreg cells and limit the generation of iTreg cells, and Foxp3 expression was increased in STAT3-deficient naive CD4+ T cells [15]. Visibly, STAT3 is an important negative regulator in Foxp3 expression and Treg function. From our results, we found that the elevated HMGB1 contributes to the overexpression of STAT3 in CD4+ T cells of aGVHD patients. In order to assess the influence on Tregs after downregulation of HMGB1 in aGVHD CD4+ T cells, we detected the expression of genes related to Treg, including Foxp3, CTLA4, IL10 and TGFB. We observed decreased expression of STAT3 and increased mRNA level of Foxp3 and CTLA4. But there were no significant differences in IL10 and TGFB expression. Therefore, our data suggested that HMGB1 could restrain Treg function by regulation of STAT3 in CD4+ T cells of aGVHD patients.

5. Conclusions

In summary, our studies have demonstrated that HMGB1 could regulate the expression of STAT3 in CD4+ T cells via modulation of its DNA methylation, and subsequently promote induction of Th17 lymphocytes and inhibit differentiation of Treg cells, resulting in disease progression of aGVHD. Thus, HMGB1 may serve as a potential molecular marker assisting our diagnosis and assessment of aGVHD after allo-HSCT.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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References

