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PIAS3 suppresses acute graft-*versus*-host disease by modulating effector T and B cell subsets through inhibition of STAT3 activation

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ABSTRACT

Graft-*versus*-host disease (GVHD) caused by transplanted donor T cells remains the major obstacle of allogeneic bone marrow transplantation (BMT). Previous reports have suggested that IL-17-producing helper T (Th17) cells mediate the development of acute GVHD (aGVHD). Protein inhibitor of activated STAT3 (PIAS) inhibits the activity of the transcription factor STAT3, which is a pivotal transcription factor for Th17 differentiation. To elucidate whether PIAS3 could inhibit the development of aGVHD, pcDNA-PIAS3 or mock vector was administered in a murine model of aGVHD by intramuscular injection and subsequent electroporation. The results demonstrated that PIAS3 overexpression by pcDNA-vector administration significantly attenuated the clinical severity and histopathological severities of aGHVD involving the skin, liver, intestine, and lung. Additionally, the STAT3 activities in aGVHD target organs were suppressed by PIAS3 overexpression. Furthermore, phosphorylated (p) STAT3 activity in the spleen was profoundly attenuated in PIAS3-overexpressing GVHD mice. Interestingly, flow cytometric analysis demonstrated that the populations of $CD21^{high}CD23^{low}$ marginal zone B cells were dramatically expanded in PIAS3-overexpressing mice. PIAS3-induced inhibition of aGVHD was largely related to the downregulation of Th1 and Th17 and the upregulation of Th2 and Treg populations. Both populations of $pSTAT3^{Tyr705}$-expressing Th17 cells and B cells were significantly reduced in the spleens of PIAS3-overexpressing mice, whereas pSTAT5 activity was increased. In addition to $CD4^+CD25^+Foxp3^+$ Treg cells, the populations of $CD8^+CD25^+Foxp3^+$ Treg cells were also expanded by treatment with PIAS3. These data suggest the therapeutic potential of PIAS3 in the development of aGVHD through reciprocal regulation of Th17/Treg lineages.

1. Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is the only curative therapy with proven efficacy for the management of many hematologic malignant diseases and bone marrow failure. Allogeneic HSCT is clearly indicated for severe immunodeficiencies that are lethal in the first few years of life. However, its wide application is hampered by the development of graft-*versus*-host disease (GVHD) [1]. The development of GVHD requires escalated and prolonged immunosuppressive therapy that increases the risk of infectious complications and, ultimately, mortalities in HSCT recipients. Despite advances in the development of prophylaxis agents, acute GVHD (aGVHD; grades II–IV) occurs in 30–60% of patients after allogeneic HSCT from HLA-identical sibling donors [2]. Prevention of GVHD has been the major challenge of allogeneic HSCT. Although the pathogenesis of aGVHD remains unresolved thus far, its development is considered to be caused by mature donor T cells that recognize genetically disparate recipient antigens on APCs, resulting in the destruction of GVHD target organs, including the skin, liver, lung, and gastrointestinal tract [3,4].aGVHD is a proinflammatory process, the pathophysiology of which is

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believed to be a multistep process. Initially, host tissues are damaged by preparative chemotherapy or radiotherapy. GVHD occurs as a result of T cell activation followed by alloreactive T cell expansion and differentiation [5]. It is generally accepted that aGVHD is driven by mainly T helper 1 (Th1) cells, which produce IFN-γ and IL-2 [6]. Recent evidence has suggested that the mechanism of GVHD is more complex and might involve a new lineage of $CD4^+$ effector T cells, identified as IL-17-producing helper T (Th17) cells [7]. Th17 cells have been implicated in several inflammatory diseases [8]. Regarding GVHD pathobiology, Th17 cells play a direct role in the development of GVHD [9]. Adoptive transfer of *in vitro*-differentiated Th17 cells can induce lethal aGVHD [10]. IL-17 stimulates the production of other inflammatory cytokines, such as IL-6, IL-8, TNF-α, and vascular endothelial growth factor, supporting the proinflammatory milieu [11,12]. The signal transducer and activator of transcription (STAT)3 and RORγt are key transcription factors for Th17 differentiation from naïve $CD4^+$ T cells [13]. Prolonged activation of STAT3 is a dominant event during the development of aGVHD in target organs [14] and also plays a critical role in T-cell alloactivation [15]. Furthermore, it was reported that the STAT3 signaling pathway negatively contributes to an enhanced population of $CD4^+CD25^+Foxp3^+$ Treg cells in the development of GVHD, whereas it does not affect Th1 differentiation [16]. Previous results have suggested the identification of STAT3 protein as a potential target of treatment to prevent or inhibit aGVHD.

Protein inhibitor of activated STAT3 (PIAS3) was initially identified as an endogenous molecule that inhibits DNA binding of STAT3 [17]. PIAS3 is known to inhibit gene induction through activated STAT3 by blocking the DNA binding activity of the transcription factor. In addition, PIAS3 has also been linked to the inhibition of phosphorylation of STAT3 [18]. The *in vivo* function of PIAS3 as a key molecule in suppressing the microphthalamia transcription factor (MITF)-induced transcriptional activity has been demonstrated [19,20]. PIAS3 activates TGF-β/Smad transcriptional responses by forming a complex with Smad and increases SUMO-E3 ligase activity [21,22].

Therefore, we hypothesized that a PIAS3-enhancing strategy would suppress the development of aGVHD in an experimental murine model. To elucidate the mechanism by which PIAS3 exerts a therapeutic effect, we investigated whether PIAS3 affects T-cell subset populations, particularly the Th17/Treg imbalance, and B cell subsets. In the present study, we identified the preventive potential of PIAS3 in the development of aGVHD in a murine model through modulation of T- and B-cell subpopulations.

2. Materials and methods

2.1. Animals

Female BALB/c (BALB/c, H2d) and C57BL/6 (B6, H2b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were between 8 and 10 weeks of age at the start of the experiments. The mice were maintained under specific pathogen-free conditions in an animal facility with controlled humidity (55 ± 5%), light (12/12 h light/dark), and temperature (22 ± 1 °C). The air in the facility was passed through a HEPA filtration system designed to exclude bacteria and viruses. Animals were fed mouse chow and tap water *ad libitum*. All experimentation was carried out in strict accordance with the guidelines of the Animal Care and Use Committee of The Catholic University of Korea. The protocol was approved by the Animal Research Ethics Committee of the Catholic University of Korea (permit number: 2012-0155-01), which conforms to all National Institutes of Health of the USA guidelines.

2.2. Hematopoietic stem cell transplantation procedures

The bone marrow transplantation (BMT) procedure was performed as described previously. BALB/c (H2d) mice were used as recipients. Recipient mice received total body irradiation (400 cGy 32 split dose, total 800 cGy). Animals were followed by the infusion of 5×10^6 total bone marrow cells intravenously with 1×10^7 splenocytes (as a source of allogeneic T cells) from allogeneic donor C57BL/6 (B6, H2b). All experiments were conducted with 12 mice per group. The recipient mice were randomly divided into the control group and therapy group. One days after GVHD induction, the mice were injected intramuscular injection of 50 μg of the pCMV-PIAS3 vector in the both (left and right) leg with electrical stimulation (electroporation) using a 31-gauge needle insulin syringe. Recipients were monitored twice a day for survival, weight loss, and the degree of clinical GVHD was assessed during the 30 day observation period using a scoring system that summed changes in five clinical parameters: weight loss, posture, activity, fir texture, and skin integrity. Survival of mice was monitored by daily observation, and the day of death was recorded as the day the mouse spontaneously died. Animals were humanely sacrificed when they exhibited the euthanasia GVHD criteria (greater than 20% weight loss or animals that received a score of. 6.5 or higher).

2.3. Immunohistochemistry and histopathologic analysis of GVHD target organs

The small intestine and skin from GVHD control and PIAS3 vector-treated mice of the BMT model were fixed in 10% formalin and embedded in paraffin. Small intestine and skin tissues were then sectioned at 7-μm thickness, deparaffinated using xylene, dehydrated through a gradient of alcohols, and then stained with H&E. GvHD was scored by two trained pathologists blinded to the treatment groups as previously published histopathology scoring system [23,24]. Endogenous peroxidase activity was quenched with 3% H_2O_2 in methanol. Immunohistochemistry was performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). Tissues were incubated with the first primary anti-mouse antibody STAT3 overnight at 4 °C. The primary antibodies were detected using a biotinylated secondary linking antibody, followed by incubation with streptavidin–peroxidase complex for 1 h. The final color product was developed using the DAB chromogen (Dako, Carpinteria, CA, USA). Positive cells were counted, and the results were expressed as means ± SD.

2.4. $CD4^+$ T cell isolation and differentiation

$CD4^+$ T cells were isolated from spleen using $CD4^+$ T cell isolation kits (Miltenyi Biotec) according to the manufacturer's instructions. The purity of the isolated $CD4^+$ T cells was >95%. Isolated $CD4^+$ T cells were stimulated with plate-bound anti-CD3 (0.5 μg/ml); and soluble anti-CD28 (0.5 μg/ml) for 72 h in 24-well plates. Th17 cell differentiation was induced by treatment with, anti-IFN-γ (4 μg/ml), anti-IL-4 (4 μg/ml), TGF-β (2 ng/ml), and IL-6 (20 ng/ml), for 72 h.

2.5. Transfection and expression of PIAS3 and mock overexpression vectors

To generate PIAS3 overexpression vector, PIAS3 cDNA was purchased from Korea Human Gene Bank, Medical Genomics Research Center, KRIBB, Korea and subcloned into the Kpn1 and Xho1 sites of pcDNA3.1+ (Invitrogen). The mock, PIAS3 vector constructs were transfected using an Amaxa 4D-Nucleofector X unit according to