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Blocking Activator Protein 1 Activity in Donor Cells Reduces Severity of Acute Graft-Versus-Host Disease through Reciprocal Regulation of IL-17–Producing T Cells/Regulatory T Cells

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A B S T R A C T

Acute graft-versus-host disease (aGVHD) is a major cause of mortality in allogeneic bone marrow transplantation. Here, the diminishing effect of activator protein 1 (AP-1) blocking with a synthetic retinoid (SR11302) on the severity of aGVHD in a murine model was investigated. MHC-mismatched strain combinations were used in vivo: C57BL/6 (H-2k^b) donors into lethally irradiated BALB/c (H-2k^d) recipients. SR11302 inhibited alloreactive T cell response in a dose-dependent manner and negatively regulated signal transducer and activator of transcription 3 (STAT3) activation. AP-1 blocking in T cells inhibited the differentiation of Th1 and Th17. Conversely, Foxp3$^+$ regulatory T cells (Treg) population dramatically expanded. Transfer of SR11302-treated donor splenocytes into lethally irradiated recipients diminished the lethality and clinical severity of aGVHD. In line with these results, AP-1 blocking in donor splenocytes exhibited reduced Th17/Th1 population and enhanced in vivo Treg population. Beneficial Treg expanding property of SR11302 was associated with the induction of Foxp3 and STAT5 transcription factor, where the inhibiting property of Th17 was achieved by suppressing the phosphorylated form of STAT3 and enhancing SOCS3. In conclusion, the preventive potential of AP-1 inhibitor in aGVHD may be accomplished by altering CD4$^+$ T cell differentiation through modulating transcription factors.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is the only curative treatment for the life-threatening hematological malignancies. However, acute graft-versus-host disease (aGVHD) is the leading complication of allogeneic HSCT and causes fatal morbidities [1]. The improvements in conditioning regimens and infectious prophylaxis have contributed to improved outcomes of HSCT. However, aGVHD remains lethal and a significant obstacle. In fact, severe aGVHD occurs in approximately 50% of recipients who receive a human leukocyte antigen–matched graft from an unrelated donor [2].

Graft-versus-host disease (GVHD) is a complex inflammatory process involving donor T cells that recognize MHC on host-derived antigen-presenting cells (APCs) and dysregulation of proinflammatory cytokines release. An overwhelming immune system attacking the recipient tissues, such as the skin and gut, by donor T cells can lead to the destruction of tissues [3]. Th1 cells and its associated cytokine, interferon-γ (IFN-γ), have been reported to play a critical role during aGVHD. However, recent research has shown IL-17–producing T (Th17) cell differentiation to affect the progression of aGVHD. In vitro, polarized Th17 cells were shown to induce lethal aGVHD [4]. On the opposite side of Th17, there is a CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells (Treg) that plays a pivotal role for the maintenance of self-tolerance in various autoimmune diseases and alloresponse [5-7]. Tregs contribute to the tolerance acquisition to donor antigen in solid organ transplantations [8] and protect against the development of fatal aGVHD in a murine model [9].

Despite clinical concerns, GVHD prophylaxis and treatment rely heavily on unspecific immunosuppressive agents. Prolonged immunosuppressive therapy increases the risk of various complications, including severe infections, which ultimately cause mortality in HSCT recipients. In addition, immunosuppressive therapy has shown to reduce the effects of T cell–mediated graft-versus-leukemia (GVL) and subsequently increase the disease relapse rate.

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The transcription factor activator protein 1 (AP-1) consists of a variety of dimers from the Fos and Jun families [10]. AP-1 is involved in cellular proliferation, death, survival, and differentiation [11]. Although AP-1 is an important transcription factor controlling the upregulation of proinflammatory cytokines, its role in the pathogenesis of aGVHD remains unknown. In the current study, the effects of SR11302 (an AP-1 blocking synthetic retinoid), by inhibiting AP-1 activity, on aGVHD severity and mortality have been investigated.

MATERIALS AND METHODS

Mice

Eight- to 10-week old C57BL/6 (H-2k^b, termed B6) and BALB/c (H-2k^d) mice were purchased from OrientBio (Sungnam, Korea). Foxp3-GFP knock-in mice (C57BL/6 strain) were purchased from the Jackson Laboratory (Bar Harbor, ME). The mice were maintained under specific pathogen-free conditions in an animal facility with controlled humidity (55% ± 5%), light (12/12 hours light/dark), and temperature (22°C ± 1°C). The air in the facility was passed through a high-efficiency particulate air filter system, designed to exclude bacteria and viruses. Animals were fed mouse chow and tap water ad libitum. The protocols used in this study were approved by the Animal Care and Use Committee of The Catholic University of Korea.

Bone Marrow Transplantation Model and Histopathology Scoring

Recipients (BALB/c mice) were injected i.v. with 5×10^6 bone marrow cells from donor mice after lethal irradiation with 800 cGy. To induce aGVHD, splenocytes were isolated from B6. To depleted Treg cells of donor cell, we added GFP-negative cells (non-Tregs) sorted from Foxp3-GFP mice (B6 strain). The purity of the sorted CD4$^+$Foxp3$^-$ cells was 98% to 99%, as evaluated by flow cytometry. B6 or sorted Foxp3 GFP-negative splenocytes (1×10^7 cells) were incubated with 10 μM SR11302 or control (DMSO) for 2 hours at 37°C before adoptive transfer into recipient mice. Survival after bone marrow transplantation (BMT) was monitored daily, and the clinical severity of aGVHD was assessed twice each week using a scoring system evaluating changes in 5 clinical parameters: weight loss, posture, activity, fur texture, and skin integrity [12]. Mice were sacrificed 14 days after BMT for blinded histopathological analysis of GVHD targets (skin, liver, and small and large intestine). Organs were harvested, cryo-embedded, and subsequently sectioned. Tissue sections were fixed in 10% buffered formalin and stained with H & E for histological examination.

Alloreactive T Cell Prolifereation In Vitro and Viability Studies

Splenic APC derived from B6 mice were used as allogeneic stimulators and splenic T cells from BALB/c mice were used as responder cells in a mixed lymphocyte reaction. T cell proliferation in this assay was used as a parameter of alloreactivity. Red blood cells were removed, using an ammonium-chloride-potassium lysis buffer; washed; and resuspended in complete culture medium (RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 1mM sodium pyruvate, 5×10^5 M 2-ME, 20 mM HEPES, and antibiotics [100 U/mL penicillin, 100 μg/mL streptomycin]). Aliquots of 2×10^5 CD4$^+$ T cells (responder) were cultured with 2×10^5 irradiated (2500 cGy) APC in 96-well plates containing 200 μL of complete medium at 37°C in a humidified 5% CO_2 atmosphere, pulsed with 1 μCi of [^{3}H] TdR (NEN life Science Products Inc., Boston, MA) 18 hours before harvesting, and counted using an automated harvester (PHD Cell Harvester; Cambridge Technology, Inc., Cambridge, MA). Splenic T cells were cultured with syngeneic splenic APC or allogeneic splenic APC in the absence or presence of SR11302. Results are expressed as the mean counts per minute (cpm) of triplicate samples ± standard deviation (SD). The stimulation index was calculated by comparing the antistimulators response with the anti−self-response. The effect of SR11302 on the viability of cultured murine splenocytes was determined using an MTT assay (Pierce, Rockford, IL).

Western Blot

Protein samples were separated by sodium dodecyl sulphate-polyacrylamide (SDS) gel electrophoresis and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). Membranes were stained with primary antibodies to c-Jun, c-Fos, phosphorylated signal transducer and activator of transcription (STAT3) on tyrosine 705 (pSTAT3^{Tyr705}) or on serine 727 (pSTAT3^{Ser727}), STAT3 (all from Cell Signaling, Danvers, MA), and β-actin. The HRP (Horseradish Peroxidase)-conjugated secondary antibody was then added.

Flow Cytometry

Mononuclear cells were immunostained with various combinations of the following fluorescence-conjugated antibodies: CD4, CD25, Foxp3, IFN-γ, IL-4, and IL-17. These cells were also intracellularly stained with the following antibodies: IL-4 (BD Biosciences, San Jose, CA), IL-10 (Biolegend, San Diego, CA), IL-17, and Foxp3 (eBioscience, San Diego, CA). Before intracellular staining, the cells were restimulated for 4 hours with 25 ng/mL PMA and 250 ng/mL ionomycin (Sigma, Saint Louis, MO) in the presence of GolgiSTOP (BD Biosciences, San Jose, CA). Intracellular staining was conducted using an intracellular staining kit (eBioscience) according to the manufacturer's protocol. Harvested cells were stained with surface mAb, fixed, permeabilized, and stained for anticytokine mAb. Flow cytometric analysis was performed on a FACS Calibur cytometer (BD Biosciences). Splenocytes isolated from B6 mice were stimulated with (1 μg/mL) anti-CD3 mAb and (1 μg/mL) of anti-CD28 mAb (BD PharMingen, CA) for 72 hours. Cells were stained first with mAbs to CD4, CD25, inducible costimulator (ICOS), glucocorticoid-induced TNF-related (GITR), and programmed death-1 (PD-1), follow by mAbs to cytotoxic T lymphocyte antigen 4 (CTLA-4) and Foxp3 using the regulatory T Cell Staining Kit (eBioscience). All analyses were based on control cells incubated with appropriate isotype control to adjust background fluorescence.

Measurement of Cytokines

The concentrations of cytokines in culture supernatants were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (Duoset; R&D Systems, Lille, France) as described by the manufacturer.

Reverse Transcription–polymerase Chain Reaction Analysis

Total RNA (2 μg) was reverse transcribed into cDNA using a transcription kit (TaqMan Reverse Transcription Reagents; Applied Biosystems, Darmstadt, Germany). The resulting cDNA was amplified by PCR using Foxp3 sense (5′- GGC CCT TCT CCA GGA CAG A-3′) and antisense (5′- GCT GAT CAT GGC TGG GTT GT-3′) primers and SOCS3 sense (5′- CGC CTC AAG ACC TTC AGC TC-3′) and antisense (5′- CTG ATC CAG GAA CTC CCG AA-3′) primers. PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide.

Confocal Staining

Spleen tissue was obtained 14 days after BMT and was snap-frozen in liquid nitrogen and stored at −80°C. Tissue cryosections (7 μm thick) were fixed with acetone and stained using PE-labeled anti-IFN-γ, IL-4, IL-17, or Foxp3 Ab (eBioscience), FITC-labeled anti-pSTAT3^{Tyr705}, pSTAT3^{Ser727}, pSTAT5, PD-1, or GITR (eBioscience), PerCP−labeled anti-CD4 Ab (Biolegend), or APC-labeled anti-CD25 Ab. In all experiments, background staining was established using isotype-matched PE -, or FITC-conjugated isotype control. After incubation overnight at 4°C, stained sections were analyzed using a confocal microscopy system (LSM 510 Meta. Zeiss, Gottingen, Germany).

Statistical Analysis

Data are presented as mean ± SD. Data comparisons between more than 2 groups and between 2 groups were performed with Kruskal-Wallis test and Mann-Whitney *U*-test or Student *t* test, respectively. To assess the Gaussian distribution and the equality of variance, the Shapiro-Wilk test and Leven test were used, respectively. The overall survival rate in aGVHD was evaluated using the Kaplan-Meier estimate and compared using the log-rank test. Weight data were analyzed at each time point. Statistical analysis was performed using the SPSS statistical software package (standard version 16.0; SPSS, Chicago, IL). *P* values less than .05 (2-tailed) were considered significant.

RESULTS

AP-1Blocking Modulates Alloreactive T Cell Responses In Vitro

Before the in vitro experiment, cell viability with increasing treatment doses of SR11302 was assessed using the MTT colorimetric assay. The results showed that SR11302 treatment up to 10 μM did not show any significant toxicity to murine splenocytes (Figure 1A). To determine the impact of AP-1 activity on the proliferative capacity of donor CD4$^+$ T cells in response to alloantigens, T cell alloreactivity with treatment of SR11302 was measured by incorporating [^{3}H]-thymidine. Proliferative responses to B6 splenic APC (allogeneic stimulator) were measured in BALB/c splenic T cells (responder cells) and were compared with those of BALB/c APC (syngeneic stimulator). BALB/c T cells that were incubated with B6 splenic APC in the presence of SR11302 displayed decreased alloreactivity when compared with those in the absence of SR11302 in a dose-dependent manner