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Inhibition of the Immunoproteasome Subunit LMP7 with ONX 0914 Ameliorates Graft-versus-Host Disease in an MHC-Matched Minor Histocompatibility Antigen–Disparate Murine Model

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In the current study we evaluated the effects of immunoproteasome inhibition using ONX 0914 (formerly PR-957) to ameliorate graft-versus-host disease (GVHD). ONX 0914, an LMP7-selective epoxyketone inhibitor of the immunoproteasome, has been shown to reduce cytokine production in activated monocytes and T cells and attenuate disease progression in mouse models of rheumatoid arthritis, colitis, systemic lupus erythematosus, and, more recently, encephalomyelitis. Inhibition of LMP7 with ONX 0914 in the B10.BR→CBA MHC-matched/minor histocompatibility antigen (miHA)-disparate murine blood and marrow transplant (BMT) model caused a modest but significant improvement in the survival of mice experiencing GVHD. Concomitant with these results, in vitro mixed lymphocyte cultures revealed that stimulator splenocytes, but not responder T cells, treated with ONX 0914 resulted in decreased IFN-γ production by allogeneic T cells in both MHC-disparate (B10.BR anti-B6) and miHA-mismatched (B10.BR anti-CBA) settings. In addition, a reduction in the expression of the MHC class I–restricted SIINFEKL peptide was observed in splenocytes from transgenic C57BL/6-Tg(CAG-OVA)916Jen/J mice exposed to ONX 0914. Taken together, these data support that LMP7 inhibition in the context of BMT modulates allogeneic responses by decreasing endogenous miHA presentation and that the consequential reduction in allogeneic stimulation and cytokine production reduces GVHD development.

INTRODUCTION

Immunotherapy in the form of allogeneic blood and marrow transplantation (allo-BMT) has proven to be one of the few curative treatments for patients suffering from a number of drug-resistant hematological malignancies. This therapy exploits the broad donor T cell repertoire targeting a diversity of defined and undefined allogeneic MHC antigens, non-MHC minor histocompatibility antigens (miHAs) derived from polymorphic intracellular proteins, and tumor-specific antigens presented by both MHC class I and class II molecules. Donor T cells play a central role in eliminating residual tumor cells that persist after conditioning regimens. However, they also facilitate patient immune reconstitution via homeostatic proliferation and provide first-line immunity against opportunistic infections while de novo bone marrow–derived immune cells slowly emerge after transplant. Although allo-BMT permits partial or complete remission in significant percentages of patients, it also comes with its limitations; in particular, acute graft-versus-host disease (GVHD) is a major cause of nonrelapse morbidity and mortality, affecting 40% to 60% of allo-BMT patients and accounting for 15% of deaths [1].

The proteasome is a large intracellular multicatalytic protease complex responsible for degradation of intracellular proteins and therefore critical for miHA presentation [2]. The 20S proteasome appears as a cylinder-like structure in various eukaryotes, consisting of 4 rings with 7 subunits each

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[2,3]. Three of the β subunits, designated β1, β2, and β5, bear the active centers of the 20S proteasome. In hematopoietic cells and cells stimulated with IFN-γ and tumor necrosis factor-α (TNF-α), however, the catalytic subunits LMP2 (β1i), MECL-1 (β2i), and LMP7 (β5i) replace the constitutive subunits β1, β2, and β5 and are incorporated into the immunoproteasome [2,4]. The immunoproteasome regulates antigen presentation on MHC class I molecules, which displays peptides for surveillance of pathogen-infected cells by $CD8^+$ T cells and shapes the repertoire of that T cell subset in the thymus [4,5]. In addition to this role, the immunoproteasome also regulates cytokine production by immune cells [4,6]. More recently, a number of studies have shown that the immunoproteasome also plays a crucial role in T cell–mediated autoimmune diseases [3,6,7].

These results, together with preclinical GVHD studies demonstrating the beneficial effects of bortezomib [8-10], a proteasome inhibitor extensively used for the treatment of multiple myeloma [11,12], led to the current evaluation of the immunoproteasome inhibitor ONX 0914 (formerly PR-957) to ameliorate GVHD. ONX 0914, an LMP7-selective epoxyketone inhibitor of the immunoproteasome, has been shown to reduce cytokine production in activated monocytes and T cells and attenuate disease progression in mouse models of rheumatoid arthritis [7], colitis [6], systemic lupus erythematosus [13], and, more recently, encephalomyelitis [3]. Inhibition of LMP7 with ONX 0914 in the B10.BR→CBA MHC-matched/miHA-disparate murine BMT model caused a modest but significant improvement in the survival of mice experiencing GVHD. Concomitant with these results, in vitro mixed lymphocyte cultures (MLC) revealed that stimulator splenocytes, but not responder T cells, treated with ONX 0914 resulted in decreased IFN-γ production by allogeneic T cells in both MHC-disparate (B10.BR anti-B6) and miHA-mismatched (B10.BR anti-CBA) settings. In addition, a reduction in the expression of the MHC class I–restricted SIINFEKL peptide was observed in splenocytes from transgenic C57BL/6-Tg(CAG-OVA)916Jen/J mice exposed to ONX 0914. These data suggested that, in the context of GVHD, immunoproteasome inhibition via LMP7 likely modulates donor T cell allogeneic responses primarily via down-regulation of host antigen presentation of miHA, an effect that preferentially impacted cytotoxic $CD8^+$ T cell activity in our BMT model.

METHODS

Mice

Breeding pairs of B10.BR-H-wk H2-T18a/SgSnJJrep (B10.BR), C57Bl/6J (B6), and C57BL/6-Tg(CAG-OVA)916Jen/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in our colony. Male CBA/J (CBA) mice were purchased from The Jackson Laboratory and used as recipients. All mice were housed in a sterile environment in microisolator cages and given autoclaved food and acidified water (pH 2.5) ad libitum. Both donor and recipient mice were used between 8 and 12 weeks of age. All protocols used in this study were approved by the Hackensack University Medical Center's Institutional Animal Care and Use Committee.

Flow Cytometry

Staining was performed using directly conjugated rat–anti-mouse mAbs (BD Pharmingen, San Jose, CA) diluted to 1:100 in FACS buffer (1% BSA with 0.02% sodium azide in 1× PBS) directed to CD4 PE (clone RM4-5), CD8 PC5 (clone 53-6.7), and B220 FITC (clone RA3-6B2). Anti-H2Kb-SIINFEKL mAb (clone eBio25-D1.16) was purchased from eBioscience (San Diego, CA). The FC500 flow cytometer (Beckman Coulter, Miami, FL) was used for all acquisitions and analyses.

Preparation of Cells for GVHD Experiments

Donor lymphocytes were prepared from pooled RBC-lysed spleens and lymph nodes enriched for T cells by a 37°C incubation with anti-CD24 mAb (J11d.2; 1:500) and guinea pig complement (C′) to deplete B cells. $CD8^+$ T cells were then depleted from the T cell–enriched population using $CD8^+$ specific mAb (3.168; 1:100) to augment the $CD4^+$ population. Anti-Thy1 mAb + C′ treated bone marrow (ATBM) cells were produced from donor mice by flushing the bone marrow cells from the femurs, followed by incubation with anti-Thy1 mAb (J1j; 1:100) and C′.

GVHD Experiments

CBA recipient mice were exposed to lethal irradiation (11 Gy, split dose, 4 hours apart) using a ^{137}Ce source (Gammacell 40 Exactor; NDS Nordion, Canada). Mice were then transplanted with 2×10^6 B10.BR ATBM cells alone or in combination with 1×10^7 enriched B10.BR T cells. ONX 0914 was administered i.v. for the day 0 to 2 treatment in our first experiment and s.c. for all subsequent treatments and experiments. Responses were comparable by either administrative route, but multiday s.c. injections were technically easier to perform. Mice were examined daily for morbidity and mortality and weighed twice weekly.

ELISpot Assay

Millipore (Billerica, MA) 96-well multiscreen plates with Immobilon-P membrane were coated with affinity-purified anti-mouse IFN-γ mAb (AN-18; 10 μg/mL; eBioscience) at 4°C overnight. Responder cell populations were prepared by harvesting peripheral lymph nodes and enriching for T cells by B cell depletion and, when necessary, $CD8^+$ enrichment by CD4 depletion. RBC-lysed splenocytes were irradiated (15 Gy) and used as stimulators. Compound treated groups were incubated for 1 hour at 37°C, 5% CO_2, with either .1% DMSO vehicle, 300 nM ONX 0914 (a LMP7-selective concentration [7]) solubilized in .1% DMSO, or 125 nM PR-825 (a β5 subunit selective concentration [6]) solubilized in .1% DMSO, followed by 3 washes with cRPMI before plating.

Coated ELISpot plates (Millipore, Billerica, MA) were washed and blocked for 1 hour at 37°C, 5% CO_2 with cRPMI before plating the cells in an R:S ratio of 1:2 in cRPMI (RPMI 1640 media [Mediatech, Herndon, VA] containing 10% FBS [Hyclone, Logan, UT], 2 mM L-glutamine, penicillin [50 IU/mL[/streptomycin [50 μg; Mediatech] and 2-ME) for 48 hours at 37°C, 5% CO_2. After incubation, the plates were washed with 1× PBS containing .01% Tween-20 (Sigma-Aldrich, St. Louis, MO).

Biotinylated anti-mouse IFN-γ mAb (clone R4-6A2; 1 μg/mlL; eBioscience) was incubated for 2 hours in the dark at room temperature, followed by Tween-20 washes as described above. The plate was then incubated with a 1:1000 dilution of Avidin-HRP (eBioscience) in 1× PBS for 1 hour at room temperature. 3,3′-Diaminobenzidine tetrahydrochloride (Sigma-Aldrich) substrate solution was used to develop the plate for 10 minutes at room temperature, followed by washing and spot enumeration using an Immunospot plate reader, software version 3.2 (Cellular Technology Limited, CTL, Shaker Heights, OH). Spot counts from a minimum of 4 wells per experiment were averaged and statistical analysis performed. Mouse strains and T cell populations are indicated for each experiment in the appropriate figure legend, as cited below.

Antigen Presentation Assay

Spleens from C57BL/6-Tg(CAG-OVA)916Jen/J mice were harvested and RBCs lysed. Splenocytes were either left untreated or treated with .1% DMSO or 300 nM ONX in .1% DMSO for 1 hour at 37°C, 5% CO_2. After treatment, cells were washed 3 times and plated with cRPMI at 37°C, 5% CO_2. SIINFEKL expression was analyzed by flow cytometry at 24 and 48 hours post-treatment.

Histopathology

At days 5 and 12 post-transplantation, GVHD target organs (liver, spleen, small and large intestine) were harvested into 4% paraformaldehyde, which was washed and replaced with 70% ethanol after 24 hours. Samples were sent to the Digital Imaging and Histology Core at Rutgers-NJMS Cancer Center (Newark, NJ) for paraffin embedding, sectioning, slide preparation, and H & E staining. GVHD scoring was performed by a veterinary pathologist. A semiquantitative scaling from 0 to 5 was implemented based on histopathological findings, as follows: normal = 0, minimal = 1, mild = 2, moderate = 3, moderately severe/marked = 4, and severe/high = 5. A half score was considered for the findings that fell between severities described earlier. Cumulative histopathology scores were calculated based on the sum of individual changes of various parameters in each organ (villous blunting, crypt cell hyperplasia, crypt cell apoptosis, and mucosal and submucosal inflammation in the small intestine; Goblet cell depletion, crypt cell hyperplasia, crypt cell apoptosis, and mucosal and submucosal inflammation in large intestine; and bile duct degeneration and inflammation in liver). Each experiment consisted of 3 to 4 mice per group. Images were visualized using an Olympus BX61 microscope (Waltham, MA) and images captured with CellSense Standard software (Olympus, Waltham, MA).