**The impact of P2X7 receptor antagonist, brilliant blue G on acute
graft-versus-host disease in mice after allogeneic hematopoietic stem cell
transplantation**

Running title: The impact of BBG on GVHD after HSCT

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Abstract

The purpose of this study was to investigate the role of P2X7R on liver inflammation in mice after hematopoietic stem cell transplantation (HSCT). Our results showed liver inflammation with neutrophils and macrophages infiltration as well as weight loss in mice after HSCT. High dose of P2X7R inhibitor administration reduced levels of IL-1 β , IL-18, caspase-1, NLRP3 as well as P2X7R, and the level of alanine transaminase (ALT) and the ratio of aspartate amino transferase (AST) /ALT compared with mice receiving low dose of BBG. Meanwhile, P2X7R blockage also reduced infiltration of macrophages and neutrophils into liver and secretion of CXCL8 and CCL2 as well as improved liver function. In conclusion, blockage of P2X7R by BBG exerts a protective effect on GVHD post HSCT and improves liver function, suggesting this receptor could be considered as an attractive target for treatment of GVHD.

Keywords: GVHD, hematopoietic stem cell transplantation, P2X7R, brilliant blue G

1 Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is considered as the most efficient strategy for the treatment of many primary immunodeficiencies and hematological tumors [1]. Despite more than 30 years of experience on allogeneic HSCT, a life-threatening complication graft-versus-host disease (GVHD) still remains a big challenge because the transplants contain mature donor T cells [2, 3]. Acute GVHD is characterized by lymphocytic infiltration and sclerosis of several target organs including liver, skin and gastrointestinal tract and is a major cause of mortality and morbidity after allogeneic HSCT [4].

If the donor T cells are activated and expanded in the process of allogeneic HSCT, proinflammatory cytokines are secreted and additional inflammatory effector cells are recruited to these sites leading to the damage of organs [5]. Several inflammatory cytokines such as interleukin-1 (IL-1), interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), IL-6 [6] and IL-18 are elevated after allogeneic HSCT either by activation of immune effector cells [7] or through direct cytotoxic effects on host tissues [8, 9]. Accumulated evidence has indicated that protein complexes termed inflammasomes activate intracellular caspase-1 which causes the maturation and cleavage of inactive pro-IL-1 β and pro-IL-18 [10]. A subgroup of the nucleotide-binding domain, leucine-rich repeat containing (NLR) proteins are considered as the key mediators of the inflammation [11]. Specially, the *in vivo* role of NLRP3 inflammasome function has been characterized as mediating caspase-1 activation via inflammasome assembly in response to pathogen infection or

danger-associated molecules, such as ATP released from dying cells [12].

Importantly, ATP levels are elevated after total body irradiation and during the development of GVHD [13]. The ATP receptor P2X7R is implicated in immune response and inflammation [14]. Binding of ATP to P2X7R leads to potassium efflux and subsequent activation of NLRP3 inflammasome as well as increased expression of CD80/CD86, followed by production of inflammatory cytokines including IL-1 β , IL-18 [13]. Given that P2X7R expression is increased at the RNA and protein levels, genetic deletion of P2X7R and blockage of the receptor plays a key role in ameliorating GVHD [15, 16]. Considering the regulation of inflammation by P2X7R, we aimed to evaluate its role in liver inflammation in mice and showed selective blockade of P2X7R ameliorated liver inflammation and improved liver function after allogeneic HSCT.

2 Materials and methods

2.1 Animals and treatment

Male C57BL6J mice were obtained from the Jackson Laboratory (USA) and housed under specific pathogen-free conditions in the Experimental Animal Center at Xuzhou Medical College (Xuzhou, China). Male BABL/c mice were purchased from Shanghai Laboratory Animal Center of Chinese Academy of Sciences (Shanghai, China). Mice were aged between 8 and 10 weeks. All animal protocols were approved by the Ethnic Committee on the Use and Care of Laboratory Animals of Xuzhou Medical College.

Hematopoietic stem cells (HSCs) were generated from C57BL/6 mice. Mice were sacrificed by cervical dislocation and then sterilized in iodophor for 5-8 min, followed by removing and cleaning all connected tissues from femurs and tibiae. Single cell-suspensions from bone marrow were collected by flushing marrow cavity of femurs and tibiae with RPMI1640 medium and then filtered through a metal screen.

BALB/c mice were randomly divided into 4 groups: normal control (n = 10), GVHD1 (n = 16), GVHD2 (n = 16) and GVHD3 (n = 16). Allogeneic HSCs transplant were performed as previously described [17]. BALB/c host mice in three GVHD groups were irradiated with 7.5 Gy (0.67 Gy/min) and injected with 5×10^6 HSCs and 5×10^6 spleen cells via tail vein within 24 h. Day of HSCs injection was assigned as day 0. After cell transplantation, 0.45 ml of BBG (Brilliant Blue G-250, AnaSpec) was injected intraperitoneally into mice in GVHD2 (50 mg/kg) and GVHD3 (75 mg/kg) respectively. Equal volume of sterile saline was given to mice in GVHD1. Treated mice were received intraperitoneal injection twice every week to day 28. Animals were kept on antibiotic water (gentamicin-ceftriaxone sodium) for the first 28 days. After 28 days, lineage-specific chimerism of transplanted mice was measured by staining bone marrow cells with FITC-anti-H-2K^b and cell lineage markers.

2.2 Flow cytometric analysis

For circulating HSCs assay, peripheral blood was incubated with fluorescein isothiocyanate (FITC)-anti-CD45, phycoerythrin (PE)-anti-H-2K^b, FITC-anti-H-2K^d

(BD Biosciences). Streptavidin-Texas red conjugate and propidium iodide were used in analysis. All analyses were performed on a modified dual laser FACSCantoII flow cytometer with FACSDiva software for data analysis.

2.3 Measurement of weight loss

After confirmation of the successful HSCT, the survival and phenotype of mice were monitored daily and body weight was measured weekly. Weight loss of every mice after HSCT was measured.

2.4 Measurement of liver function

Liver function was determined by measuring serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), as well as AST/ALT using IDEXX VetTest (IDEXX Laboratory, Maine, US).

2.5 Measurement of chemokines

Levels of CXCL8 and CCL2 in the peripheral blood were measured by commercial ELISA kits according to the manufacturer's protocol (Abcam, Cambridge, England).

2.6 H&E staining and immunohistochemical staining

Histopathological specimens from liver and colon were obtained on days 7, 14, 21 and 28 after HSCT, fixed in 4% formaldehyde solution for 24 h and embedded in paraffin. For all samples, 4- μ m slices were stained with H&E for histological examination. For immunohistochemistry of IL-1 β and P2X7R, sections were rehydrated, washed with TBS (150 mmol/L NaCl supplemented with 50 mmol/L Tris) and blocked for one hour with TBS containing 10% FCS and 1% BSA, followed by

incubation with TBS containing 1% BSA and primary antibodies for IL-1 β and P2X7R for 16h. Tissue sections were washed twice with TBS and then HRP-conjugated anti-IgG secondary antibodies were added to TBS. After washing twice, nuclear counterstaining was done with Mayer hematoxylin and images were analyzed with Nikon eclipse 90i digital microscope.

2.7 Western blot

Whole proteins extracted from liver of mice were separated on 10% SDS-PAGE and then transferred to NC membrane, followed by blotting with rabbit anti-mouse myeloperoxidase (MPO), CD68 or P2X7R antibody. The membranes were visualized using HRP-conjugated anti-rabbit secondary antibody and enhanced chemiluminescence. Image J software was applied to quantify the intensity of positive band.

2.8 Real-time quantitative PCR

Total RNA was isolated from liver and colon using Trizol (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized using M-MLV (Invitrogen, Carlsbad, CA, USA). Real-time PCR was conducted using LC480 SYBR Green Master Mix (Roche Diagnostics, Germany) on a LightCycler 480 system following cycling conditions: 94 °C for 2min, then 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 35 s, end elongation for 2 min. The following primer sequences were used:

5'-AAGCTGTACCAGCGGAAAGA -3'/5'-CCTGCAAAGGGAAGGTGTAGT for P2X7R, 5'-TCTGCACCCGGACTGTAAAC-3'/5'-CATTGTTGCCCAGGTTTCAGC-3' for NLRP3, 5'-ACTGCTATGGACAAGGCACG-3'/5'-CCTGCCAGGTAGCAG

TCTTC-3' for Caspase-1, 5'-GAAATGCCACCTTTTGACAGTGAT-3'/5'-CAGCCACAATGAGTGATACTGC for IL-1 β , 5'-TCAGAAGACTCTTGCGTCAAC-3'/5'-GGGTTCACCTGGCACTTTGAT for IL-18, 5'-GTTAAGCAGTACAGCCCCA-3'/5'-GTCTGGCCTGTATCCAACAC for HPRT. Each sample was repeated for three times. PCR product specificity was confirmed by a melting-curve analysis and the relative mRNA expression was examined using $2^{-\Delta\Delta C_t}$ method.

2.9 Statistical analysis

All data were shown as Mean $\pm$ SD. Differences between groups at different time point was analyzed by two-way ANOVA with Bonferroni post-tests using GraphPad Prism software (version 6.0). A *P* value less than 0.05 was considered statistically significant.

3 Results

3.1 Gross characterization of GVHD in transplanted mice

To investigate the role of inflammasome, GVHD model was established via infusion of HSCs with spleen cells into BALB/c mice. Lineage-specific chimerism of transplanted mice after HSCT was measured by flow cytometry and we found that after 15 days it was $(98.23 \pm 5.46) \%$ (Figure 1A). The results suggested the success of HSCT. Mice in control group showed normal spirit and activity, but dispirited and decreased sign developed starting from 4 to 8 days in mice post HSCT and regained 10 days post HSCT. In light of possibility that P2X7R may be involved in liver inflammation, P2X7R inhibitor, BBG was administrated into mice during HSCT to

evaluate whether P2X7R blockage could ameliorate liver inflammatory injury. After administration of BBG at different dosage, protein level of P2X7R was decreased when compared with normal liver, suggesting the successful blockage of P2X7R (Figure 1B).

3.2 The effect of BBG on body weight of mice

In attempt to investigate the function of P2X7R and inflammasome in GVHD, BBG was used to block the function of P2X7R. After mice were treated with different dose of BBG, weight loss as a surrogate parameter for GVHD severity was calculated (Figure 2). Decreased weight changes were observed two weeks after BBG administration compared with mice receiving saline. Meanwhile, the results showed reduced GVHD severity in mice receiving 75 mg/kg BBG compared with that receiving 50 mg/kg BBG on day 14 ($P < 0.05$), 21 and 28 ($P < 0.01$).

3.3 The impact of BBG on liver function

To further explore the impact of BBG on liver function, two parameters ALT and AST/ALT were detected in mice. As shown in Figure 3, the mean level of ALT was significantly decreased after administration of 50 mg/kg and 75 mg/kg BBG compared with GVHD group receiving saline on day 21 and 28 after HSCT ($P < 0.01$). However, the difference of ALT level between two groups receiving different dosage of BBG did not reach statistical significance (Figure 3). The ratio of AST/ALT was obviously decreased after administration of 50 mg/kg BBG on day 14 and 21 ($P < 0.05$), but the ratio of AST/ALT in mice receiving 75 mg/kg BBG did not change. Notably, the ratio of AST/ALT was significantly decreased in mice that received 75

mg/kg BBG compared with 50 mg/kg BBG ($P < 0.05$). Taken together, these results showed that improved liver function after blockage of P2X7R after HSCT.

3.4 Histological characterization of effect of BBG on liver

Because GVHD is a systemic process in humans, several organs including liver are affected in mice subjected to HSCT. Histologically, hyperplasia, necrosis and lymphocytic infiltrate were observed in liver (Figure 4A). Notably, the histological signs of liver were reduced in mice that received BBG compared with saline, as well as in mice receiving 75 mg/kg BBG compared with 50 mg/kg BBG (Figure 5A). Meanwhile, abnormal structure of liver lobule, coagulative necrosis of hepatocytes, liver sinusoid narrowing and inflammatory cells such as neutrophil granulocyte and macrophage infiltration were observed after HSCT, which suggested liver inflammatory injury occurred after allogenic HSCT (Figure 4B). Histologic evaluation demonstrated reduced GVHD severity after administration of high dose of BBG compared with low dose.

To evaluate the type of inflammatory cell infiltration into liver after inhibition of P2X7R, liver was isolated from mice for western blot analysis of the expression level of CD68 and MPO. As shown in Figure 4C, protein levels of CD68 and MPO were significantly decreased in mice administrated with 75 mg/kg BBG compared with that received 50 mg/kg BBG. Specifically, the change trend of CD68 and MPO was similar as the results of H&E staining analysis.

3.5 The impact of BBG on secretion of CXCL8 and CCL2

Two important chemokines responsible for the recruitment of monocytes and

neutrophils, CXCL8 and CCL2 in peripheral blood were also detected in liver (Figure 5). The results showed that protein levels of CXCL8 and CCL2 started to dramatically increase from d7 and reached maximum on d14 in GVHD group, but reduce significantly after receiving BBG. Notably, levels of CXCL8 and CCL2 were also decreased after administration of 75 mg/kg BBG compared with 50 mg/kg BBG, indicating increased level of CXCL8 and CCL2 might recruit monocytes and neutrophils to liver, leading to inflammation.

3.6 Molecular characterization of the effect of BBG on GVHD

To explore the mechanisms of GVHD at the molecular and cellular levels, the cytokines produced by liver from mice administrated with BBG and controls were measured. IL-18 and IL-1 β , two powerful pro-inflammatory cytokines with pleiotropic activities, have been proved to play key role in the regulation of inflammation. Level of IL-18 in liver was increased after administration of 50 mg/kg BBG on day 7 ($P < 0.05$) compared with 75 mg/kg but decreased significantly on day 14 ($P < 0.01$) (Figure 6A). In terms of IL-1 β levels in liver (Figure 6B), it was significantly lower after receiving 75 mg/kg BBG compared with mice that were administrated with 50 mg/kg BBG ($P < 0.01$) post HSCT. Given blockage of P2X7R by BBG, expression of P2X7R was significantly increased on day 14 after administration of 75 mg/kg BBG, but decreased on the following days (day 21-28) (Figure 6C, $P < 0.05$), which was consistent with the results of western blot analysis for P2X7R. When compared with mice receiving 50 mg/kg of BBG, mice in the group received 75 mg/kg of BBG showed decreased level of NLRP3 on day 7, 14 and 21 (P

< 0.05), but no significant differences were observed on day 28 (Figure 6D). Similarly, when compared with mice that received control saline, mice receiving 50 mg/kg BBG showed increased caspase-1 expression on day 7 and day 14, but decreased on the following days. The significantly lower level of caspase-1 was present in liver of mice that were administrated with 75 mg/kg BBG compared with that receiving 50 mg/kg BBG (Figure 6E, $P < 0.05$). However, the time point showing lower level of cytokines was different after BBG was given to mice with GVHD, but the trend of change was similar. Meanwhile, immunohistochemical examination indicated significantly decreased expression of IL-1 β and P2X7R after administration of 75 mg/kg BBG compared with the group receiving 50 mg/kg BBG (Figure 6F). These data indicated that blockage of P2X7R has a protective effect on GVHD through regulation of the expression of inflammatory cytokines.

4 Discussion

Allogeneic hematopoietic stem cell transplantation (HSCT) is widely considered as an effective strategy for treatment of many malignancies including myeloma, leukemia or autoimmune diseases [18, 19]. However, HSCT-related complications including GVHD and hepatic veno-occlusive disease are observed. Given the critical role of P2X7R in the regulation of inflammation, whether it is involved in liver inflammation after HSCT remains poorly understood. In this study, we characterized the impact of BBG on severity of GVHD after HSCT as well as on a molecular level, and found that P2X7R ablation in GVHD mouse model produced significant

improvements in the key functional and molecular disease parameters in liver. We chose BBG because it blocked the receptor of P2X7R that is increasingly considered as an important molecule in the pathogenesis of GVHD [13, 20]. Furthermore, we also found high dosage administration of BBG (75 mg/kg) had protective effect on liver inflammation compared with low dosage. This wide therapeutic impact reflects convergence of P2X7R blockage on pathological mechanisms, thus offering a promising new approach in treating GVHD.

Crucially, P2X7R up-regulation has increasingly been found in many diverse pathologies such as transplant rejection, pain [21], duchenne muscular dystrophy [22] and limb-girdle muscular dystrophy type 2B [23]. Regarding the mechanisms by how P2X7R blockade protected liver, these might involve both direct effects in mice cells showing over-activation of this receptor and reduced inflammation. Considering reduced inflammation, cells in liver and colon chronically released high levels of ATP that resulted in immune cell infiltrations contributing to GVHD progression [13]. Growing evidence has indicated P2X7R has unique capability to respond to high ATP levels and to activate inflammation by releasing pro-inflammatory cytokines [24]. Previous report has suggested the potential therapeutic role for P2X7R antagonists in the treatment of inflammatory diseases [25]. Indeed, in mice receiving high dosage of BBG, we found a significant decrease in inflammatory signature and reduced numbers of infiltrating leukocytes in liver and colon. Moreover, we also discovered reduced NLRP3 and Caspase-1 mRNA levels and protein levels.

A previous study demonstrated that stimulation of P2X7R triggers the rapid

assembly of inflammasome signaling complexes including apoptosis-associated speck-like protein containing a caspase-recruitment domain [26] and NLRP3 [27], which acts as signaling platforms to drive the proteolytic activation of caspase-1 and maturation of IL-1 β . Consistent with these results, administration of P2X7R antagonist induced down-regulation of some cytokines and reduced GVHD-related injury in the liver. Several lines of evidence have indicated a role of negative regulators of ATP responses in controlling the severity of GVHD, because ATP is highly concentrated in the cell but low concentrations in the extracellular space, thus it maybe considered as a potent danger signal when the cell experience stress or damage [28]. Blockade of P2X7R could modulate ATP function in mice subjected to GVHD through several pathways such as Toll-like receptor and nucleotide-binding oligomerization domain-like receptor pathways that contribute to the activation of different types of innate immune cells.

Furthermore, reduced inflammatory cells such as neutrophils and macrophages infiltration into liver were shown in mice receiving high dose of P2X7R inhibitor compared with low dose group. To further confirm the role of P2X7R in liver inflammation, the ratio of the serum AST/ALT levels which is often used as a parameter to evaluate liver function were calculated. Abnormal levels of ALT and AST/ALT in patients with GVHD indicate dysfunction of liver. In the current report, we found that animals receiving high dose of BBG showed a significant decrease of ratio of AST/ALT, suggesting the important role of BBG in liver recovery.

In summary, we have demonstrated that administration of high dosage of

antagonist to P2X7R can ameliorate GVHD after HSCT. Moreover, high dose of P2X7R could significantly reduce the levels of cytokines, NLRP3, caspase-1 together with reduce inflammatory cells infiltration and improve liver function. Given that specific P2X7R antagonists have been used in human trials for several conditions, these might be promising for the treatment of GVHD.

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Figure legends

Figure 1 GVHD model assessed by flow cytometry analysis and western blot analysis after HSCT. (A) The proportion of H-2Kd cells in the hematopoietic stem cells of mice that survived for extended period in the spleen cell group. (B) Meanwhile, liver was isolated from sacrificed mice and proteins were extracted for western blot analysis of the expression of P2X7R, β -Tubulin was served as an internal control.

Figure 2 Weight changes of animals in the different groups. Mice receiving 75 mg/kg BBG compared with 50 mg/kg BBG experience less-pronounced weight loss. $*P < 0.05$; $**P < 0.01$.

Figure 3 Concentration of alanine transaminase (ALT) and the ratio of aspartate aminotransferase (AST)/ALT in blood serum of mice treated with brilliant blue G (BBG) at different dosage. $*P < 0.05$; $**P < 0.01$.

Figure 4 Liver pathology and western blot analysis of inflammatory cells infiltration in mice after HSCT. Mice in normal, GVHD, 50 and 75 mg/kg group were sacrificed at indicated time points and then liver were isolated for pathology analysis (A and B). Meanwhile, liver were isolated and protein were extracted for western analysis of expression of CD68 and MPO in liver (C). $*P < 0.05$; $**P < 0.01$; NS, not significant.

Figure 5 Secretion levels of chemokines. At indicated time points after HSCT, peripheral blood was obtained for the measurement of secretion of CXCL8 and CCL2 by ELISA kits. $*P < 0.05$; $**P < 0.01$.

Figure 6 the levels of cytokines IL-18 (A), IL-1 β (B), P2X7R (C), NLRP3 (D) and caspase-1 (E) in liver of mice treated with brilliant blue G (BBG). Analysis was

conducted by quantitative PCR. Immunohistochemical staining for P2X7R and IL-1 β were performed on liver biopsies and correlated with the severity of GVHD (F). * $P < 0.05$; ** $P < 0.01$.

Figures

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6