2019

Long-term treatment with the P2X7 receptor antagonist Brilliant Blue G reduces liver inflammation in a humanized mouse model of graft-versus-host disease

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Publication Details

Geraghty, N. J., Watson, D. & Sluyter, R. (2019). Long-term treatment with the P2X7 receptor antagonist Brilliant Blue G reduces liver inflammation in a humanized mouse model of graft-versus-host disease. Cellular Immunology, 336 12-19.

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Abstract

Allogeneic haematopoietic stem cell transplantation (HSCT) is a frequent curative therapy for numerous haematological malignancies. However, HSCT is limited by the occurrence of graft-versus-host disease (GVHD), with current therapies restricted to general immunosuppression. Activation of the P2X7 receptor by extracellular adenosine triphosphate (ATP) causes inflammation and tissue damage in GVHD. Short-term pharmacological blockade of P2X7 has been shown to reduce clinical disease and/or reduce inflammatory markers in allogeneic and humanized mouse models of GVHD. The current study demonstrates that long-term P2X7 blockade by intra-peritoneal injection of Brilliant Blue G (BBG) thrice weekly for up to 10 weeks did not impact human (h) peripheral blood mononuclear cell (PBMC) engraftment, predominantly T cells, in blood at 3 weeks post-hPBMC injection or in spleens at end-point in humanized mice. Histological analysis demonstrated long-term BBG treatment reduced leukocyte infiltration in the livers of humanized mice. Immunohistochemical analysis demonstrated that BBG treatment reduced liver apoptosis. Long-term BBG treatment did not alter clinical disease, mRNA expression of pro-inflammatory markers in tissues or serum human interferon (IFN)-γ concentrations. Therefore, this study demonstrates that P2X7 activation plays a role in GVHD pathogenesis in the livers of humanized mice, supporting a role for this receptor in GVHD development in HSCT recipients.

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Short title: Long-term P2X7 blockade reduces liver GVHD

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KEY WORDS: transplantation, Brilliant Blue G, hepatic graft-versus-host disease, humanized mice, lymphocyte, P2X7 receptor, purinergic signalling

ABSTRACT

Allogeneic haematopoietic stem cell transplantation (HSCT) is a frequent curative therapy for numerous haematological malignancies. However, HSCT is limited by the occurrence of graft-versus-host disease (GVHD), with current therapies restricted to general immunosuppression. Activation of the P2X7 receptor by extracellular adenosine triphosphate (ATP) causes inflammation and tissue damage in GVHD. Short-term pharmacological blockade of P2X7 has been shown to reduce clinical disease and/or reduce inflammatory markers in allogeneic and humanized mouse models of GVHD. The current study demonstrates that long-term P2X7 blockade by intra-peritoneal injection of Brilliant Blue G (BBG) thrice weekly for up to 10 weeks did not impact human (h) peripheral blood mononuclear cell (PBMC) engraftment, predominantly T cells, in blood at 3 weeks post-hPBMC injection or in spleens at end-point in humanized mice. Histological analysis demonstrated long-term BBG treatment reduced leukocyte infiltration in the livers of humanized mice. Immunohistochemical analysis demonstrated that BBG treatment reduced liver apoptosis. Long-term BBG treatment did not alter clinical disease, mRNA expression of pro-inflammatory markers in tissues or serum human interferon (IFN)-γ concentrations. Therefore, this study demonstrates that P2X7 activation plays a role in GVHD pathogenesis in the livers of humanized mice, supporting a role for this receptor in GVHD development in HSCT recipients.

1. Introduction

Allogeneic haematopoietic stem cell transplantation (HSCT) is a frequent curative method for various haematological malignancies. However, a major side effect of this treatment is graft-versus-host disease (GVHD). GVHD occurs in approximately half of HSCT recipients [1] and results in damage to the liver, gastrointestinal tract and skin, with the liver affected in approximately 50% of GVHD patients [2]. GVHD emerges due to transplanted effector T cells mounting an immune response against the host [3]. This immune response is driven by release of cytokines and danger associated molecular patterns (DAMP), which causes antigen presenting cells to release cytokines resulting in activation of T cells [4-6]. T cell activation leads to proliferation and differentiation of T cell subsets such as Th1 cells which release interferon (IFN)-γ [4, 5], and Th17 cells which release interleukin (IL)-17, resulting in inflammatory damage [7, 8]. Current therapies are largely limited to general immunosuppression through the use of steroids [9], highlighting the need for better therapies.

Targeting a pathway in one of the above stages of GVHD represents a potential therapeutic strategy. Purinergic signalling is an important pathway of immune cell signalling [10] and plays important roles in transplantation [11-13]. Of all the purinergic receptors, the P2X7 receptor is the most widely studied in immune and inflammatory responses [14]. Activation of P2X7 induces a number of downstream effects including DC activation and cytokine release [15], T cell activation [16] and inhibition of regulatory T cells [17]. Thus, given the central role of these immune processes in GVHD, blockade of P2X7 represents a potential therapeutic approach to limit GVHD development. In allogeneic mouse models the DAMP, adenosine triphosphate (ATP), is released from damaged and dying cells, to promote GVHD [18]. ATP exacerbates GVHD by activating P2X7 in allogeneic mouse models [18-20]. Short term pharmacological blockade of P2X7 with either KN-62, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid or stavudine during the first week post-transplantation can

extend survival of recipient mice in these models [18, 19]. Whilst, P2X7 blockade with Brilliant Blue G (BBG) (50 or 75 mg/kg) twice per week for 4 weeks reduces weight loss and liver GVHD in an allogeneic mouse model [20]. Finally, our group recently demonstrated that short-term blockade of P2X7 with BBG (50 mg/kg, days 0, 2, 4, 6, 8) reduced serum human (h) IFN-γ and tissue inflammation in a humanized non-obese diabetic severe combined immunodeficient IL-2 gamma receptor null (NOD-SCID-IL2Rγ^{null}; NSG) mouse model of GVHD [21].

To further extend the above observations [18-21] to humans, the current study investigated the effect of long-term P2X7 blockade with BBG (50 mg/kg; thrice weekly for up to 10 weeks) in a humanized NSG mouse model of GVHD. Similar to our previous study [21], injection of BBG did not impact human leukocyte engraftment, weight loss, clinical score or survival. Notably, this regime reduced leukocyte infiltration and apoptosis in the livers of mice, supporting a role for P2X7 activation in GVHD pathogenesis in the liver.

2. Materials and Methods

2.1. Humanized mouse model of GVHD

Experiments with human blood and mice were approved by the Human and Animal Ethics Committees, respectively (University of Wollongong, Wollongong, Australia). Humanized NSG mice were established as described [21]. Briefly, hPBMCs, isolated by density centrifugation using Ficoll-Paque PLUS (GE Healthcare; Uppsala, Sweden), were injected intra-peritoneally (i.p) (10 x 10^6 hPBMCs/mouse) into female NSG mice aged 5-7 weeks (Westmead Animal Research Facility, Westmead, Australia) housed at the University of Wollongong. Mice were injected i.p. 2 hours later with 200 µL of saline (saline group) or saline containing BBG (Sigma-Aldrich; St Louis, MO, USA) (50 mg/kg; BBG group), then

thrice weekly (every second or third day apart) for up to 10 weeks. At 3 weeks post-hPBMC injection, engraftment was examined by immunophenotyping of tail vein blood. Mice were monitored up to 10 weeks for signs of clinical GVHD using a scoring system, giving a total clinical score out of 10 as described [21] and euthanized at 10 weeks post-hPBMC injection, or earlier if the clinical score was ≥ 8 or weight loss was ≥ 10%, as per the approved ethics protocol.

2.2. Immunophenotyping by flow cytometry

Tail vein blood and spleen cells were obtained from mice and lyzed with ammonium chloride potassium buffer and immunophenotyped as described [21] with the addition of an allophycocyanin conjugated mouse anti-hCD56 monoclonal antibody (clone: B159) (BD, San Jose, CA, USA). Data was collected using a BD Fortessa-X20 Flow Cytometer using band pass filters of 525/50 for fluorescein isothiocyanate, 586/15 for R-phycoerythrin, 695/40 for peridinin chlorophyll protein and 670/30 for allophycocyanin. The relative percentages of cells were analyzed using FlowJo software v8.7.1 (TreeStar Inc.; Ashland, OR, USA) (Fig. S1 and Fig. S2).

2.3. Histological, immunohistochemical and image analysis

Some formalin-fixed tissue sections (5 μm) were stained with haematoxylin and eosin (POCD; Artarmon, Australia) for histological analysis. Other formalin-fixed tissue sections were stained with either rabbit anti-hCD3 mAb (clone: EP449E) (Abcam, Cambridge, UK) and haematoxylin as described [21] or an *In situ* Apoptosis Detection Kit (Abcam) as per the manufacturer's instructions. Total numbers of leukocyte and $hCD3^+$ T cell infiltrates and apoptotic cells in livers were quantified from captured images (Leica DMRB microscope, Wetzlar, Germany) using FIJI is just ImageJ (FIJI) software [22]. Data is represented as the total number of cells measured per field of view. Epidermal thickness of skin sections was measured as described [23].

2.4. Quantitative real-time PCR

RNA was isolated using TRIzol reagent (Thermo Fisher Scientific) and converted to cDNA using the qScript cDNA Synthesis Kit (Quanta Biosciences, Beverly, MA, USA) as per the respective manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed as described [21] with the addition of a human tumor necrosis factor (TNF)-α (Hs01113624_g1) primers (Thermo Fisher Scientific). Human gene expression was normalized to cDNA from hPBMCs, directly isolated from a human donor. Murine gene expression was normalized to cDNA from a spleen of a BALB/c mouse [24].

2.5. ELISAs

Serum was obtained from mice as described [21], and hIFN-γ and hIL-17 concentrations determined using respective Ready-Set-Go! ELISA Kits (eBioscience, San Diego, CA, USA) as per the manufacturer's instructions.

2.6. Statistical Analysis

Data is given as mean ± standard deviation (SD). Statistical differences were determined using Student's t-test for single comparisons or one-way analysis of variance (ANOVA) with Tukey's post-hoc test for multiple comparisons. Weight and clinical score over time were compared using a repeated measures two-way ANOVA with Tukey's post-hoc test for multiple comparisons. Survival (median survival time; MST) was compared using the log-rank (Mantel-Cox) test. Mortality was compared using Fisher's exact test. All statistical analyses and graphs were generated using GraphPad Prism 5 for PC (GraphPad Software, La Jolla, CA, USA). $P < 0.05$ was considered significant for all tests.

3. Results

3.1. BBG does not impact engraftment of human cells in NSG mice

To determine if long-term P2X7 blockade can ameliorate disease development in a humanized mouse model of GVHD, NSG mice injected with hPBMCs were subsequently injected with the P2X7 antagonist BBG [25] at 50 mg/kg or with saline thrice weekly for up to 10 weeks. To first investigate if human cell engraftment was affected by BBG treatment, flow cytometric analysis of blood was conducted at 3 weeks post-hPBMC injection (Supplementary Fig. 1). Mice injected with BBG demonstrated a similar frequency of human leukocytes (13.2 ± 7.6% $hCD45^+$ $mCD45^-$ cells, n = 10) compared to mice injected with saline (control) (9.7 ± 7.3% $hCD45^+$ $mCD45^-$ cells, n = 7) (P = 0.3561) (Fig. 1a). T cells comprized the majority of $hCD45^+$ cells, and the frequencies of T cells did not vary between mice injected with BBG (93.2 ± 4.4% $hCD3^+$ $hCD56^-$ cells, n = 10) or saline (91.6 ± 5.5% $hCD3^+$ $hCD56^-$ cells, n = 7) (P = 0.5188) (Fig. 1b). In all mice, there was a small population of $hCD3^+$ $hCD56^+$ cells, potentially natural killer T (NKT) cells [26], but the frequency of these cells did not vary between mice injected with BBG (3.1 ± 3.5% $hCD3^+$ $hCD56^+$ cells, n = 10) or saline (3.3 ± 2.7% $hCD3^+$ $hCD56^+$ cells, n = 7) (P = 0.8768) (Fig. 1c). Both groups of mice also demonstrated engraftment of a small proportion of natural killer (NK) cells ($hCD3^-$ $hCD56^+$), which did not significantly differ between mice injected with BBG (1.0 ± 1.0% $hCD3^-$ $hCD56^+$ cells, n = 10) or saline (2.3 ± 2.0% $hCD3^-$ $hCD56^+$ cells, n = 7) (P = 0.1067) (Fig. 1d). Finally, there was a small population of cells that were neither T, NKT nor NK cells; the frequency of which did not vary between mice injected with BBG (2.7 ± 2.5% $hCD3^-$ $hCD56^-$ cells, n = 10) or saline (2.8 ± 2.8% $hCD3^-$ $hCD56^-$ cells, n = 7) (P = 0.9854) (Fig. 1e).

Splenocyte analysis at end-point (Supplementary Fig. 2) showed human leukocytes comprized the majority of total murine and human leukocytes. Mice injected with BBG

exhibited a similar frequency of human leukocytes (76.7 ± 9.7% $hCD45^+$ $mCD45^-$ cells, n = 10) compared to mice injected with saline (71.4 ± 16.2% $hCD45^+$ $mCD45^-$ cells, n = 7) (P = 0.4308) (Fig. 1f). Similar to engraftment at 3 weeks post-hPBMC injection, T cells comprized the majority of human leukocytes in the spleen, with mice injected with BBG (86.4 ± 15.7% $hCD3^+$ $hCD56^-$ cells, n = 10) or saline (96.1 ± 4.9% $hCD3^+$ $hCD56^-$ cells, n = 7) demonstrating similar frequencies of T cells (P = 0.1390) (Fig. 1g). Again, there was a small population of $hCD3^+$ $hCD56^+$ cells, and the percentage of these cells did not vary between mice injected with BBG (1.3 ± 1.7% $hCD3^+$ $hCD56^+$ cells, n = 10) or saline (0.9 ± 0.3% $hCD3^+$ $hCD56^+$ cells, n = 7) (P = 0.5154) (Fig. 1h). Few mice demonstrated engraftment of NK cells in spleens at time of euthanasia, which did not significantly differ between mice injected with BBG (0.7 ± 1.5% $hCD3^-$ $hCD56^+$ cells, n = 10) or saline (0.1 ± 0.1% $hCD3^-$ $hCD56^+$ cells, n = 7) (P = 0.3064) (Fig. 1i). Finally, there was a remaining population of cells that were neither T, NKT nor NK cells; the frequency of which did not vary between mice injected with BBG (10 ± 12.6% $hCD3^-$ $hCD56^-$ cells, n = 10) or saline (3.0 ± 5.1% $hCD3^-$ $hCD56^-$ cells, n = 7) (P = 0.1868) (Fig. 1j).

Human T cell analysis ($hCD3^+$) in spleens at end-point (Supplementary Fig. 2) revealed that mice injected with BBG or saline contained similar frequencies of $hCD4^+$ T cells (71.3 ± 13.4%, n = 10 vs. 75.6 ± 8.4%, n = 7, respectively, P = 0.5345) and $hCD8^+$ T cells (17.9 ± 8.3%, n = 10 vs 14.6 ± 5.2%, n = 7, respectively, P = 0.4500) (Fig. 1k). In mice injected with BBG or saline, the percentages of $hCD4^+$ T cells were significantly greater than that of $hCD8^+$ T cells (both $P < 0.0001$ and $P < 0.0001$, respectively) (Fig. 1k).

3.2. BBG does not prevent clinical GVHD in NSG mice

For up to 10 weeks, mice were monitored for weight loss and signs of GVHD. All mice injected with BBG or saline exhibited weight loss from 4 weeks, with similar weight loss between groups over the course of the study (P = 0.2853) (Fig. 2a). Both groups of mice

began to show signs of mild GVHD from day 35, and mice injected with BBG or saline had similar clinical scores at end-point (5.8 ± 1.7, n = 10 vs. 5.9 ± 2.0, n = 7, respectively; P = 0.9494), and mean clinical scores over 10 weeks (P = 0.1286) (Fig. 2b). Similar survival was also observed between mice injected with BBG (mortality of 90%, n = 10) or saline (mortality of 86.7%, n = 7) (P = 1.000) (Fig. 2c). Mice injected with BBG or saline also demonstrated similar MSTs (45.5 days vs. 52.0 days, respectively) (P = 0.3014) (Fig. 2c).

3.3. BBG reduces leukocyte infiltration and apoptosis in livers of NSG mice

Short-term BBG has been shown to partly reduce leukocyte infiltrates, predominantly T cells, and apoptosis in GVHD target organs including liver, small intestine and skin in humanized NSG mice [21]. Therefore, to assess if long-term BBG treatment could reduce leukocyte infiltration and damage, target tissues from humanized NSG mice were analyzed via histology. There were no histological differences in the small intestines or skin, including epidermal thickening, of BBG- and saline-injected mice (Supplementary Fig. 3). However, livers from mice injected with BBG demonstrated reduced histological damage with less apoptotic cells (Fig 3a). Image analysis demonstrated a significant 52% reduction in leukocytes in livers from BBG-injected mice (517 ± 244 cells/field of view; n = 9) compared to saline-injected mice (1082 ± 324 cells/field of view; n = 6) (P = 0.0020) (Fig. 3a).

To determine the identity of the cells above (Fig. 3a), immunohistochemistry using an anti-hCD3 mAb was conducted. Image analysis demonstrated a 55% reduction in $CD3^+$ cell infiltration in livers from BBG-injected mice (117 ± 157 cells/field of view; n = 4) compared to saline-injected mice (263 ± 404 cells/field of view; n = 4), however this difference did not reach statistical significance (P = 0.5263) (Fig. 3b). Moreover, the number of $hCD3^+$ T cells detected (Fig. 3b) represented only 24% of the total leukocytes detected by histology (Fig. 3a). Finally, an *In Situ* Apoptosis detection kit was used to confirm that there was reduced apoptosis in the liver. Image analysis demonstrated there was a significant 57% decrease in

apoptotic cells in livers from BBG-injected mice (65 ± 32 cells/field of view; n = 4) compared to saline-injected mice (150 ± 47 cells/field of view; n = 4) (P = 0.0244) (Fig. 3d).

3.4. BBG does not impact cytokine expression in the liver of NSG mice

IFN-γ, IL-17 and TNF-α are important pro-inflammatory cytokines implicated in GVHD pathogenesis [7, 8]. To investigate if long-term BBG treatment alters these cytokines in the liver, hIFN-γ, hIL-17 and hTNF-α expression were analyzed by qPCR. hIFN-γ expression was similar in mice injected with BBG (2.8 ± 0.8, n = 7) or saline (3.0 ± 1.6, n = 7) (P = 0.7347) (Fig. 4a). There was a two-fold increase hIL-17 in mice injected with BBG (4.0 ± 5.2, n = 7) compared to mice injected with saline (1.8 ± 1.4, n = 7), but this did not reach statistical significance (P = 0.2940) (Fig. 4b). hTNF-α expression was low, and similar in mice injected with BBG (0.1 ± 0.1, n = 7) or saline (0.1 ± 0.1, n = 7) (P = 0.7754) (Fig. 4c).

Short-term BBG treatment reduces serum IFN-γ in allogeneic [18] and humanized [21] mouse models of GVHD. To determine if prolonged BBG treatment could alter the amount of these cytokines, serum hIFN-γ and hIL-17 concentrations in humanized NSG mice were assessed by ELISA. hIFN-γ was present in the serum of all mice (Fig. 4d). In contrast to previous data involving short-term BBG treatment [21], serum hIFN-γ concentrations were similar in mice injected with BBG (15.8 ± 10.7 ng/mL, n = 10) or saline (14.9 ± 8.4 ng/mL, n = 7) (P = 0.8462) (Fig. 4d). hIL-17 was not detected in the serum of any mice injected with BBG (n = 10) or saline (n = 7) using an ELISA with a reported sensitivity of 4 pg/mL (eBioscience).

P2X7 expression is significantly increased in livers of mice with allogeneic GVHD [18]. Therefore, to determine the impact of P2X7 blockade on expression of hP2X7 and mP2X7, livers of humanized NSG mice were analyzed by qPCR. There was a four-fold increase in

relative hP2X7 expression in mice injected with BBG (8.7 ± 14.8, n = 7) compared to mice injected with saline (2.0 ± 1.5, n = 7), however this did not reach statistical significance (P = 0.2547) (Fig. 4e). Relative mP2X7 expression was similar in mice injected with BBG (0.07 ± 0.02, n = 7) or saline (0.07 ± 0.01, n = 7) (P = 0.9986) (Fig. 4f).

4. Discussion

The current study demonstrated that a long-term regime of P2X7 blockade using BBG (50 mg/kg i.p. thrice weekly for up to 10 weeks) can reduce liver inflammation and apoptosis in a humanized mouse model of GVHD. Saline-injected control mice exhibited characteristic leukocyte infiltration and apoptosis in the liver, consistent with previous observations in humanized NSG mice [27, 28]. The long-term regime of BBG reduced leukocyte infiltrates, including human T cells, into the livers compared to control mice. This reduction was greater than our previous study using a short-term regime of BBG (50 mg/kg i.p. on days 0, 2, 4, 6, 8) in humanized NSG mice [21]. Leukocyte infiltrates are also reduced in livers from allogeneic mice following a long-term regime of BBG (50 or 75 mg/kg i.p. twice weekly for four weeks) [20]. The long-term BBG regime in the current study also reduced apoptosis in livers, an effect that was greater than the previous short-term BBG regime in humanized NSG mice [21]. Similarly, the long-term BBG regime in allogeneic mice also reduced inflammatory damage [20]. The reduction in leukocyte infiltrates and apoptosis in livers is also similar to other GVHD therapies in humanized mice [28-30]. Notably, P2X7 blockade is an efficacious therapy in drug-induced inflammatory liver damage [31, 32]. Thus, the current and past [20] studies indicate that P2X7 is important for liver GVHD development and that P2X7 represents a potential therapeutic target in HSCT to prevent this form of GVHD.

The current study demonstrated that a long-term regime of BBG did not impact clinical disease in humanized mice. This result is similar to that of the short-term BBG regime in this model [21] but differs to that of a long-term BBG regime in the allogeneic mouse model of GVHD above [20]. In the latter study, BBG prevented weight loss, with a stronger effect observed with 75 mg/kg BBG compared to 50 mg/kg BBG, but clinical score and survival were not reported. Nevertheless, since the larger dose of 75 mg/kg significantly reduced histological GVHD and weight loss compared to the dose of 50 mg/kg in this allogeneic mouse model, investigation of this or larger doses in a humanized mouse model are warranted. Finally, it should be noted that administration of BBG into humanized NSG mice caused rapid weight gain, the reasons for which remain unknown. However, P2X7 blockade with BBG or *P2RX7* gene deficiency also induces weight gain in C57BL/6 mice within one week [33]. This previous study hypothesized that a consensus motif similar to Janus kinase 2 within the C-terminus of P2X7 may be a substrate of protein-tyrosine phosphatase 1B, which has known roles in obesity [34]. Thus, blockade of P2X7 may induce an initial weight gain in mice via modulation of this phosphatase.

The long-term regime of BBG in the current study did not detect any differences in the expression of hIFN-γ, hIL-17, hTNF-α or mP2X7 in the livers of humanized mice. There was a two-fold, non-significant increase in hP2X7 in the liver, similar to the 1.7-fold increase in the short-term regime [21]. These results are somewhat unexpected given the reduced leukocyte infiltration and apoptosis in the livers of these mice. This suggests that other molecules may be controlling T cell migration and T cell-mediated damage in the livers of these mice. Alternatively, the similar expression of these molecules may simply reflect the comparison of livers at end-stage disease, but this does not explain observed differences in leukocyte infiltration and apoptosis. Analysis of liver samples earlier in disease progression may reveal further differences between BBG- and saline-treated mice.

Short-term [21] but not long-term BBG treatment (the current study) reduces serum hIFN-γ in humanized mice. The reason for this difference remains unknown, but suggests long-term P2X7 blockade negates any benefits observed with short-term P2X7 blockade in relation to serum hIFN-γ. This suggests that P2X7 blockade shortly after hPBMC engraftment reduces the development of hIFN-γ-producing T cells in humanized mice, similar to P2X7 deficiency reducing IFN-γ production early in disease development in allogeneic mice [35]. However, P2X7 blockade at later stages of engraftment and GVHD development may promote development of hIFN-γ-producing T cells in humanized NSG mice. Alternatively, comparison of serum hIFN-γ concentrations between the current and previous [21] studies indicates that the long-term regime with saline reduced serum hIFN-γ compared to the short-term saline regime. This suggests long-term injections of saline may reduce some disease parameters in this humanized mouse model of GVHD. In support of this, injection of saline is used in other inflammatory disease models to rescue mice from weight loss [23, 36].

Finally, the current study demonstrates that a long-term BBG regime does not affect engraftment of donor human cells in NSG mice. Although the clinical benefits of this regime were limited, this indicates long-term P2X7 blockade may permit reconstitution of the immune system, an essential aim of HSCT [37]. In the current study, the majority of engrafted leukocytes were T cells, consistent with previous studies [21, 27, 38]. Our previous study demonstrated that B cells are not present in the blood or spleens of humanized NSG mice, but the identity of the remaining human leukocytes remained unknown [21]. In the current study, NK cells constituted a portion of the remaining leukocyte population, consistent with similar frequencies of NK cells in humanized NSG mice observed by others [39, 40]. NK cells contribute to the graft-versus-leukemia effect but not GVHD in both mice [41] and humans [42], suggesting that NSG mice injected with hPBMCs could be potentially used to study the graft-versus-leukemia effect. Importantly, BBG did not affect NK cell

engraftment suggesting P2X7 blockade may not impact graft-versus-leukemia immunity, another key aim of HSCT. The current study also demonstrated, for the first time, that humanized NSG mice engraft a small proportion of NKT cells [43], although in the absence of additional markers these cells may represent activated T cells instead or in part [44]. NKT cells suppress GVHD in allogeneic HSCT in mice [45-47] and higher numbers of NKT cells in humans correlates to reduced GVHD [48, 49]. BBG did not alter the percentage of these cells indicating that P2X7 blockade does not affect engraftment of NKT cells. Thus, humanized NSG mice may help elucidate the role of human NK and NKT cells in GVHD. It should be noted that a limitation of the current study was that the flow cytometric analyzes did not include gating of singlets or dead cell exclusion using a viability dye. Thereby increasing the likelihood of small errors in determining the proportions of human leukocyte subsets especially rarer subsets such as NK and NKT cells.

The current study demonstrated that long-term P2X7 blockade does not impact human leukocyte engraftment, weight loss, clinical score or mortality associated with GVHD. However, this regime can reduce leukocyte infiltration and apoptosis in the liver. Therefore, this study demonstrates that P2X7 activation plays a role in GVHD pathogenesis in the livers of humanized mice, supporting a role for this receptor in GVHD development in HSCT recipients. Moreover, given that long-term P2X7 blockade does not affect donor human leukocyte engraftment or appear to have any other adverse effects, P2X7 may represent a potential therapeutic target to reduce liver GVHD in HSCT recipients. Nevertheless, given that long-term P2X7 blockade appears to have similar benefits compared to short-term P2X7 blockade in humanized NSG mice, the design of future mouse studies or clinical trials exploring the therapeutic benefits of P2X7 blockade in preventing GVHD need to consider the value of extended treatments with P2X7 antagonists.

Acknowledgements

Funding was provided by the Illawarra Health and Medical Research Institute and Molecular Horizons (University of Wollongong). We thank the technical staff of the Illawarra Health and Medical Research Institute, the Westmead Research and University of Wollongong Animal Facilities for assistance. We thank Stephen Fuller (Nepean Hospital) and Winny Varikatt (Westmead Hospital) for histological advice, and Stephen Alexander (Children's Hospital at Westmead) for providing NSG mice.

Disclosures

None.

Author Contributions

N.J.G., R.S. and D.W. designed the experiments. N.J.G. performed the experiments, analyzed the data, prepared the figures and wrote the manuscript. R.S. and D.W. supervized the project, reviewed the data and edited the manuscript.

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Figures

Figure 1. Long-term BBG treatment does not affect engraftment of human cells in a humanized mouse model of GVHD

(a-k) NOD-SCID-IL2Rγ^{null} (NSG) mice were injected intra-peritoneally (i.p.) with 10 x 10^6 human (h) peripheral blood mononuclear cells (hPBMCs), and subsequently with saline (n = 7) or saline containing Brilliant Blue G (BBG) (50 mg/kg) (n = 10) thrice weekly. The percentages of human leukocytes in (a-e) blood at 3 weeks post-hPBMC injection and (f-k) spleens at end-point were determined by flow cytometry. (a, f) hCD45$^+$ leukocytes are expressed as a percentage of total mCD45$^+$ and hCD45$^+$ leukocytes. (b, g) hCD3$^+$ hCD56$^-$, (c, h) hCD3$^+$ hCD56$^+$, (d, i) hCD3$^-$ hCD56$^+$ and (e, j) hCD3$^-$ hCD56$^-$ cells are expressed as a percentage of total hCD45$^+$ leukocytes. (k) hCD4$^+$ and hCD8$^+$ T cell subsets are expressed as a percentage of total hCD3$^+$ leukocytes. Data represents group means ± SD; symbols represent individual mice; *** $P < 0.0001$ compared to corresponding hCD8$^+$ T cells.

Figure 2. Long-term BBG treatment does not affect disease development in a humanized mouse model of GVHD

(a-c) Saline- and BBG-injected humanized NOD-SCID-IL2Rγ^{null} (NSG) mice (Fig. 1) were monitored for (a) weight loss, (b) clinical score (using a scoring system, giving a total clinical

score out of 10), and (c) survival for up to 10 weeks. Data represents (a, b) group means ± SD or (c) percent survival (saline $n = 7$; BBG $n = 10$).

Figure 3. Long-term BBG treatment reduces leukocyte infiltration and apoptosis in livers of a humanized mouse model of GVHD

(a-c) Livers from saline- and BBG-injected humanized NOD-SCID-IL2Rγ^{null} (NSG) mice (Fig. 1) at end-point were stained with (a) haematoxylin and eosin, (b) anti-human (h) CD3 monoclonal antibody and haematoxylin or (c) terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick-end and methyl green. (a-c) Images were captured by microscopy; representative images from one mouse per group shown; bar represents 100 μm. Image analysis was used to quantitate the mean number of cells per field of view from three different captured images per mouse. Data represents group means ± SD (saline n = 4-6; BBG n = 4-9); symbols represent individual mice; * $P < 0.05$ or ** $P < 0.01$ to saline.

Figure 4. Long-term BBG treatment does not affect serum or liver inflammatory markers in a humanized mouse model of GVHD

(a-f) cDNA from livers of saline- and BBG-injected humanized NOD-SCID-IL2Rγ^{null} (NSG) mice at end-point (Fig. 1) were used to assess the relative expression of human (h) (a) interferon (IFN)-γ, (b) interleukin (IL)-17, (c) tumor necrosis factor (TNF)-α, (e) hP2X7, and (f) murine (m) P2X7 by qPCR. (d) Concentrations of serum hIFN-γ from mice at end-point were analyzed by ELISA. (a-f) Data represents group means ± SD (saline $n = 7$; BBG n = 7-9); symbols represent individual mice.

Supplementary Figures

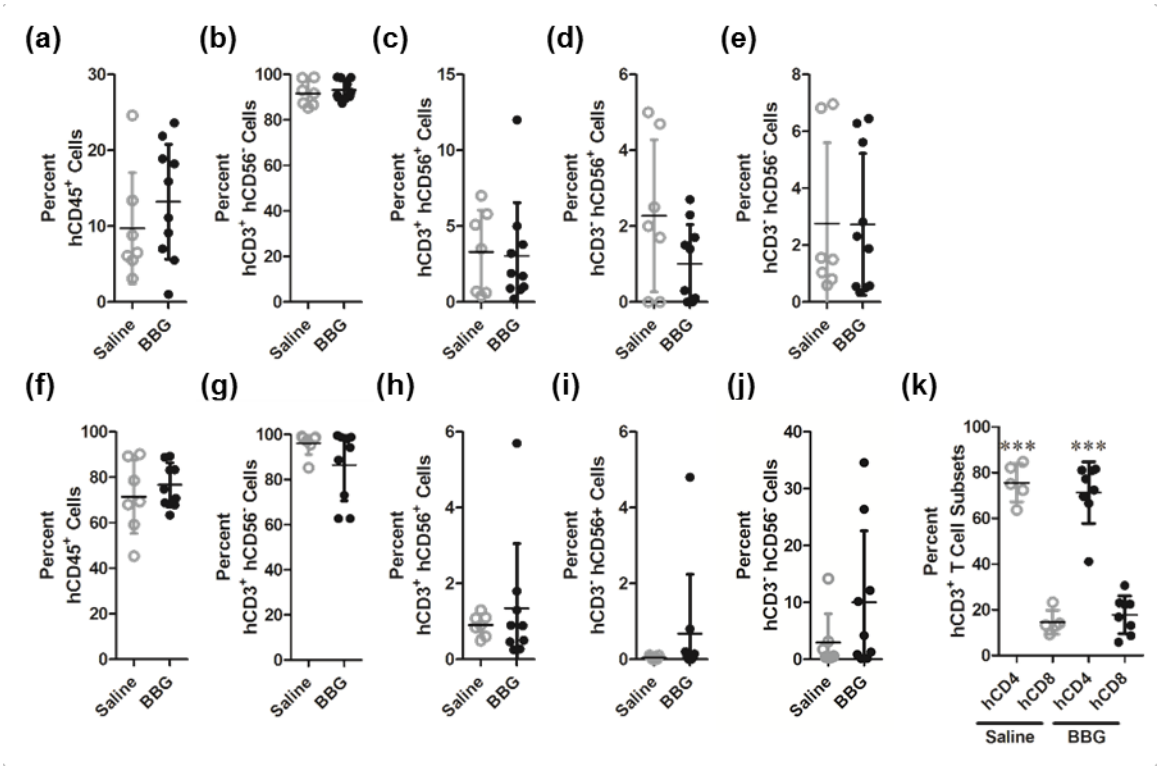
Figure S1. Flow cytometric gating of leukocytes in blood and spleens from humanized mice

(a-b) NOD-SCID-IL2Rγ^{null} (NSG) mice were injected intra-peritoneally with 10 x 10^6 human (h) peripheral blood mononuclear cells, and subsequently with saline or saline containing

Brilliant Blue G (BBG) (50 mg/kg) thrice weekly. (a) Blood cells and (b) splenocytes from humanized NSG mice were labeled with isotype control or CD-specific monoclonal antibodies. (a, b) Leukocytes, initially gated by forward scatter (FSC-A) and side scatter (SSC-A), were analyzed to determine the percentages of human leukocytes ($hCD45^+$ $mCD45^-$), human T cells ($hCD45^+$ $mCD45^-$ $hCD3^+$ $hCD56^-$), human NK cells ($hCD45^+$ $mCD45^-$ $hCD3^-$ $hCD56^+$), human NKT cells ($hCD45^+$ $mCD45^-$ $hCD3^+$ $hCD56^+$), human non-T/NK/NKT cells ($hCD45^+$ $mCD45^-$ $hCD3^-$ $hCD56^-$), and (spleen only) human $CD4^+$ T cell ($hCD3^+$ $hCD4^+$ $hCD8^-$), and human $hCD8^+$ T cell ($hCD3^+$ $hCD4^-$ $hCD8^+$) subsets.

Figure S2. Long-term BBG treatment does not affect intestinal or skin damage in a humanized mouse model of GVHD

(a-b) NOD-SCID-IL2Rγ^{null} (NSG) mice were injected intra-peritoneally (i.p.) with 10 x 10^6 human (h) peripheral blood mononuclear cells (hPBMCs), and subsequently with saline or saline containing Brilliant Blue G (BBG) (50 mg/kg) thrice weekly. (a) Small intestines and (b) skin from hPBMC-injected mice injected with saline or BBG at end-point were stained with haematoxylin and eosin. Images were captured by microscopy; representative images from one mouse of seven saline- or 10 BBG-injected mice. (b) Image analysis was used to quantitate the mean epidermal thickness from three different skin images per mouse. Data represents group means ± SD (saline $n = 6$; BBG $n = 7$; $P = 0.2174$); symbols represent individual mice.


(a)
Percent hCD45+ Cells
(b)
Percent hCD3+ hCD56- Cells
(c)
Percent hCD3+ hCD56+ Cells
(d)
Percent hCD3- hCD56+ Cells
(e)
Percent hCD3- hCD56- Cells
(f)
Percent hCD45+ Cells
(g)
Percent hCD3+ hCD56- Cells
(h)
Percent hCD3+ hCD56+ Cells
(i)
Percent hCD3- hCD56+ Cells
(j)
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(k)
Percent hCD3+ T Cell Subsets
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hCD4
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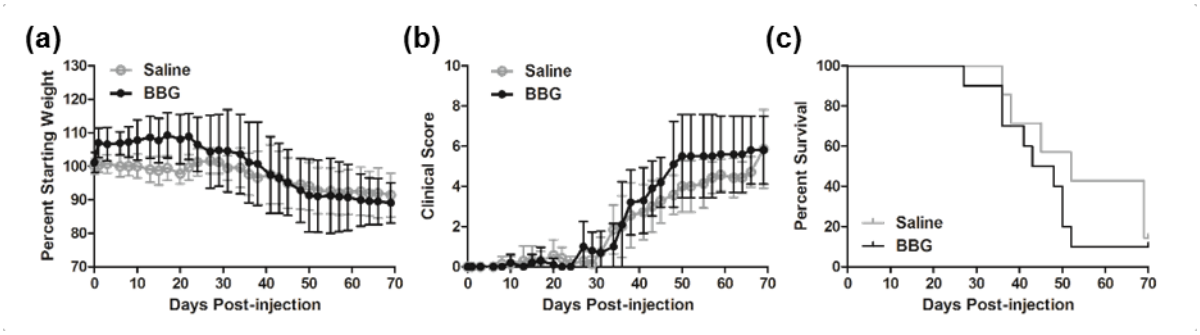
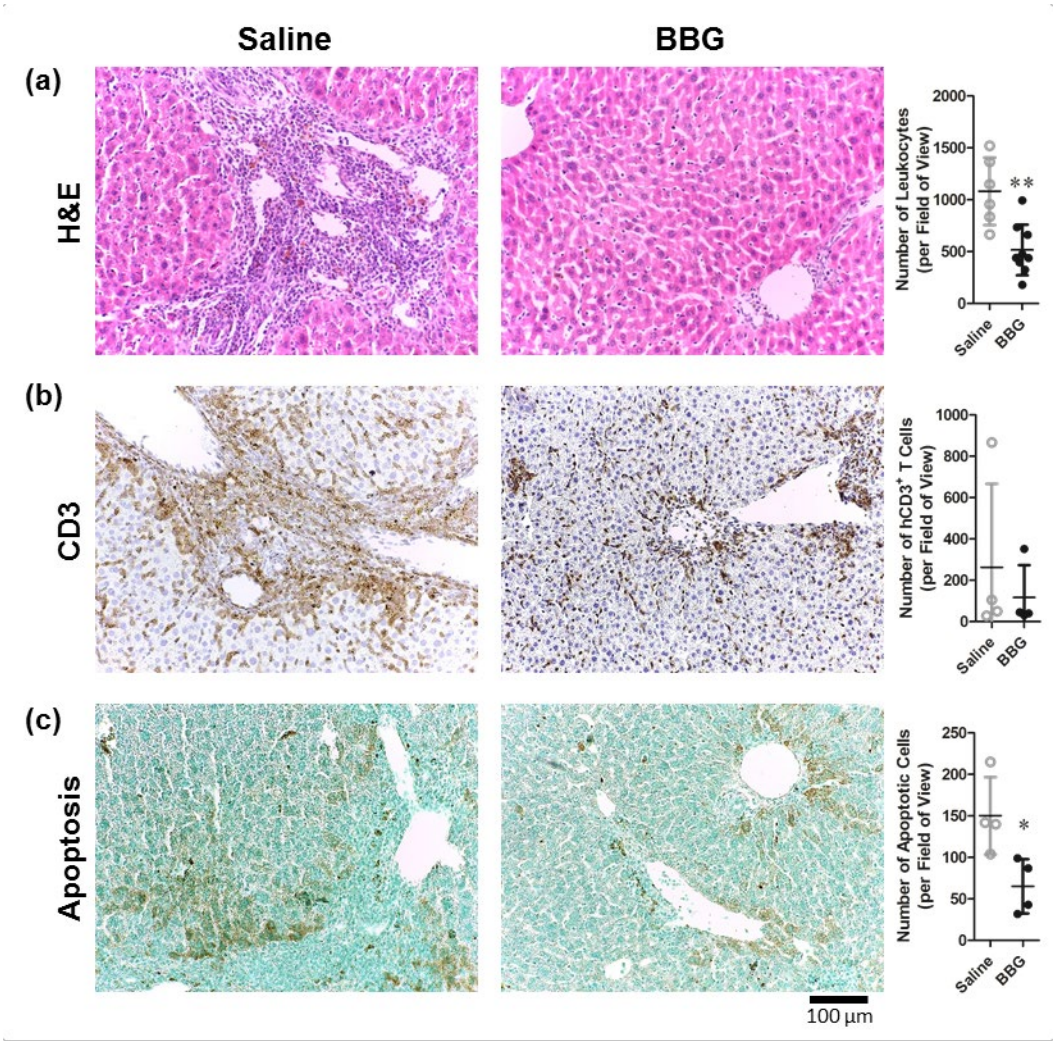
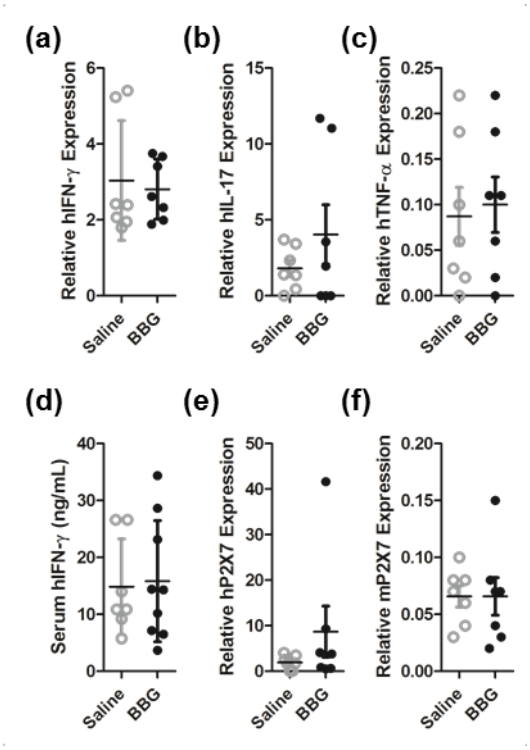
Saline
BBG

Long-term P2X7 receptor blockade reduces liver GVHD



Long-term P2X7 receptor blockade reduces liver GVHD




(a)
Relative hIFN-γ Expression
0
2
4
6
Saline
BBG
(b)
Relative hIL-17 Expression
0
5
10
15
Saline
BBG
(c)
Relative hTNF-α Expression
0.00
0.05
0.10
0.15
0.20
0.25
Saline
BBG
(d)
Serum hIFN-γ (ng/mL)
0
10
20
30
40
Saline
BBG
(e)
Relative hP2X7 Expression
0
10
20
30
40
50
Saline
BBG
(f)
Relative mP2X7 Expression
0.00
0.05
0.10
0.15
0.20
Saline
BBG