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BILIRUBIN NANOPARTICLE AS AN ANTI-INFLAMMATORY THERAPY FOR
GRAFT VERSUS HOST DISEASE

By

Sumedha Pareek, B.Tech., M.Tech.

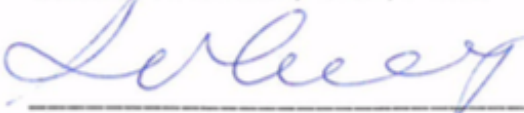
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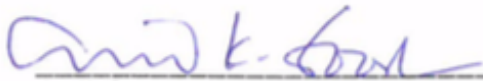
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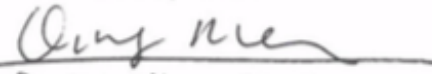
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BILIRUBIN NANOPARTICLE AS AN ANTI-INFLAMMATORY THERAPY FOR GRAFT
VERSUS HOST DISEASE

A

THESIS

Presented to the Faculty of

The University of Texas

MD Anderson Cancer Center UTHealth

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

by

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Houston, Texas

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Dedicated to Mom, Dad, Bhaiya, and Manaswi

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BILIRUBIN NANOPARTICLE AS AN ANTI-INFLAMMATORY THERAPY FOR GRAFT VERSUS HOST DISEASE

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Graft versus host disease (GvHD) caused by alloreactive donor lymphocytes is a fatal complication of hematopoietic stem cell transplant (HSCT). Myeloablative conditioning regimen, consisting of chemotherapy and/or radiation, given prior to HSCT can cause tissue damage. This non-specific tissue damage triggers cross-presentation of alloantigens to the donor immune cells, causing recruitment of leukocytes and production of inflammatory cytokines. Targeting this inflammation without affecting the anti-leukemia effects of HSCT, continues to be one of the biggest challenge in finding a therapy for GvHD. Bilirubin is a tetrapyrrole pigment, found in the blood, with natural anti-oxidative and anti-inflammatory properties. Using mouse models of various inflammatory diseases, studies by our collaborating investigators have shown that, water-soluble PEGylated bilirubin nanoparticles (BRNP) selectively accumulate at the site of inflammation and prevent further tissue damage through scavenging reactive oxygen species. Therefore, we hypothesized that BRNP treatment after myeloablative conditioning regimen can reduce clinical symptoms of GvHD by abating the initial tissue damage in HSCT. We investigated the therapeutic efficacy of BRNP using murine GvHD model. Sublethally irradiated recipient

mice (Balb/cJ) were infused with 5×10^6 bone marrow cells and 5×10^6 splenic cells from MHC-mismatched donor mice (C57/B6J) on day 1, with or without BRNP (10 mg/kg) on days 0-4. Clinical GvHD symptoms were monitored for 60 days, and mice were scored for fur, skin, posture, activity, and weight change. Untreated recipient mice (n=10) developed significantly worse GvHD (median GvHD score=3.4) compared to BRNP treated recipient mice (n=10, median GvHD score=0.3) ($p=0.0003$, Mann Whitney U Test). This translated into significantly better survival of BRNP treated mice with day 60 survival of 100% as compared to the untreated recipient with day 60 survival of 20% ($p=0.0001$, Log-rank (Mantel-Cox) Test). Histological analyses on day 8 post-transplantation, showed significantly lowered GvHD associated damage in liver, lung, skin, and gut, in BRNP treated mice as compared with untreated mice. In summary, we show that prophylactic treatment with BRNP can reduce clinical and pathological GvHD symptoms and thereby improve survival in mice. In future, we plan to investigate a treatment model of BRNP in relieving the clinical and pathological symptoms of GvHD. We also plan to explore the potential of BRNP as a drug conjugate for GvHD treatment.

Table of Contents

Approvals	Error! Bookmark not defined.
Title	ii
Dedication	iii
Acknowledgements	iv
Abstract	vi
List of Figures	x
List of Tables	xi
Chapter 1- Introduction	1
1.1 Hematopoietic Stem Cell Transplantation.....	1
1.1.1 Hematological Malignancies	1
1.1.2 Hematopoietic Stem Cell Transplantation.....	3
1.2 Graft Versus Host Disease	5
1.2.1 GvHD Signaling Cascade	6
1.2.2 Alloreactivity in Graft versus Host Disease	7
1.2.3 Existing Therapies for Graft Versus Host Disease.....	8
1.3 Bilirubin Nanoparticle	9
1.4 Hypothesis.....	12
Chapter 2- Materials and Methods	13
2.1 Mice.....	13

2.2 Hematopoietic Stem Cell Transplantation	13
2.3 Bilirubin Nanoparticle	14
2.4 Flow Cytometry Analysis.....	15
2.5 Clinical Graft Versus Host Disease Assessment.....	15
2.6 Pathological Graft Versus Host Disease	16
2.7 Serum Cytokine Quantification	16
2.8 Histopathology	17
2.9 Statistical Analysis	17
Chapter 3- Results and Analysis.....	18
3.1 Mouse Model of Graft Versus Host Disease	18
3.2 BRNP Treatment Can Increase Survival in Murine Acute Graft Versus Host Disease Model.....	24
3.3 BRNP Treatment Can Reduce Pathological Symptoms of Acute Graft Versus Host Disease.....	28
Chapter 4- Discussion and Future Direction.....	35
References.....	39
Vita	46

List of Figures

Figure 1. Mechanism of production and anti-oxidative activity of Bilirubin.....	10
Figure 2. Clinical GvHD outcome of the first strategy of murine GvHD model.....	20
Figure 3. Clinical GvHD outcome of the second strategy of murine GvHD model.....	22
Figure 4. Clinical GvHD outcome of the third strategy of murine GvHD model.....	26
Figure 5. Serum levels of pro-inflammatory cytokines IFN γ and TNF α	30
Figure 6. BRNP treatment can reduce GvHD associated tissue damage, such as lymphocyte infiltration, inflammation, and necrosis.....	31
Figure 7. BRNP treatment can reduce pathological symptoms of acute GvHD in mice.....	33

List of Tables

Table 1.....	18
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Chapter 1- Introduction

1.1 Hematopoietic Stem Cell Transplantation

1.1.1 Hematological Malignancies

Hematological malignancies, also commonly known as blood cancers, encompass all of cancer subtypes that affect the development and function of various blood cells. Blood cancers can be divided into three broad categories: Leukemia- originates in cells found in the blood and the bone marrow; Lymphoma- originates in the cells found in the lymph nodes; Myeloma- affects the production and functioning of antibody producing plasma cells. Together, the three categories of blood cancer, are responsible for approximately 10% of cancer related deaths in the United States every year (1).

Depending on patient's age, health, and stage and type of blood cancer, physician recommends specific treatment options or their combination. The first line of therapy recommendation for leukemia patients is usually chemotherapy, which targets the rapidly dividing cancer cells. Acute myeloid leukemia (AML) is the most common subtype, occurring in almost one-third of the adult patients of leukemia (1). According to recent statistics by the American Cancer Society, approximately 90% of patients with an AML subtype- acute promyelocytic leukemia (APL) and 67% of patients with other subtypes of AML, go into remission after chemotherapy induction. Despite the high remission rates, recent reports by the National Cancer Institute show that the overall 5-year survival

rates of all AML patients are the lowest among other leukemia subtypes at 28.3%.

Hematopoietic stem cells (HSCs) are unique, self-renewing stem cells that are also capable of differentiating into all lineages of cells that comprise the blood- in a process called hematopoiesis. Hematopoiesis starts during embryonic development and continues into adulthood. Over the last fifty years, there has been an exponential increase in our knowledge on the biology of HSCs (2). This breakthrough has led to development of hematopoietic stem cell transplantation (HSCT) as potentially curative therapy for various hematological disorders, especially blood cancers. In fact, five-year survival rates for leukemia in United States, have risen from 14% in the 1960 to 65% in 2014. Among the total number of allogeneic HSCTs performed around the world, about one-third are indicated for AML patients (3). According to 2006-2016 statistics by Center for International Blood and Marrow Transplant Research (CIBMTR), among approximately 13,000 AML patients, those who received stem cell transplant from their sibling as donor, had survival rates between 20-55% depending on the stage of diagnosis (early, intermediate, or late). These statistics show that HSCT offers long-term survival, especially for patients with AML. However, despite a large number of patients responding to therapy, a majority of the leukemia patients who do achieve complete remission, go on to relapse (4) or develop complications such as organ failure, infections, and graft versus host disease (5).

1.1.2 Hematopoietic Stem Cell Transplantation

Human hematopoietic system comprises of all the cell types that constitute the blood, bone marrow, and the lymphatic system. Hematopoietic stem cell transplantation is one of the most important and potentially curative therapy for leukemia and other hematological malignancies. Depending on the disease condition, recommended HSCT can be either autologous, syngeneic, or allogeneic.

In autologous HSCT, the donor is the patient themselves and it is indicated for various hematological disorders, as well as cancers. Autologous HSCT is used to reconstitute hematopoietic system for cancer patients who are given high-dose chemotherapy and/or radiation. It is recommended as treatment modality for multiple myeloma, Non-Hodgkin's lymphoma, Hodgkin's disease, acute myeloid leukemia, neuroblastoma, and ovarian cancer (6). It is also recommended for diseases that do not require the anti-tumor (also known as graft versus leukemia (GvL) effect) benefits of transplant such as aplastic anemia and bone marrow diseases, or where tumor response to chemotherapy is high, such as germ cell tumors (ovarian cancer and testicular cancer) (7). Syngeneic HSCT is similar to autologous HSCT except that the donor is patient's twin/triplet. While there is no development of graft versus host disease in syngeneic HSCT, the anti-cancer benefits are limited to high-dose chemotherapy (8).

In allogeneic HSCT, the donor is not the patient, but a matched or mismatched donor. Allogeneic HSCT is indicated for acute myeloid leukemia,

acute lymphoblastic leukemia, chronic myeloid leukemia, relapsed and refractory Non-Hodgkin's lymphoma, chronic lymphocytic leukemia, relapsed and refractory multiple myeloma, and some hematological disorders such as sickle-cell anemia and Wiskott–Aldrich syndrome. While allogeneic transplant has high GvL activity, it is often accompanied by inflammation in host tissue due to donor T-cell alloreactivity in a complication known as graft versus host disease (GvHD)

Allogeneic HSCT was first attempted in the 1960s, a few years after human leukocyte antigen (HLA) typing was first discovered (9). The first step in allogeneic HSCT is to find a human leukocyte antigen-matched donor. HLA is a gene system that encodes for major histocompatibility complex (MHC) proteins. The main function of the MHC protein is to present antigen in the form of peptides to the surveilling immune cells. The cytotoxic T-cells recognize MHC together with the antigen peptide, in a process known as MHC-restriction. MHC proteins are highly polymorphic, however one individual can have, at most, 12 different MHC alleles (10). High-resolution HLA-typing is employed to identify a matched donor. HLA-A, -B, and -C genes under Class I HLA encode for MHC Class I protein that is expressed on almost all the nucleated cells in the body. Whereas, HLA-DP, -DQ, and -DR encode for MHC Class II protein that are expressed only on professional antigen presenting cells such as the dendritic cells, B-cells, and monocytes (11). HLA-typing is done for HLA-A, -B, -C and -DRB1 (encodes HLA-DR β -chain) to find an 8/8 match (a complete match) or a 7/8 match (12). The allelic diversity in HLA genes across population, makes it difficult to find a complete match, especially for the minority ethnicities in USA such as the

African-Americans (19% likelihood for 8/8 match), Africans (16% likelihood for 8/8 match), as compared to White Europeans (75% likelihood for 8/8 match) according to the 2014 statistics (13).

After identifying a matched donor, the patient goes through myeloablative conditioning regimen, which consists of chemotherapy and/or radiation therapy. Conditioning regimen is given to induce immunosuppression by eliminating all the patient immune cells including any leukemia cells. Meanwhile, donor peripheral blood or bone marrow is collected and processed to obtain HSCs, which are then transplanted to the recipient. Once transplanted, donor HSCs have two major functions- firstly, to kill any remnant leukemia cells in the patient's body or the GvL effect, and secondly, to reconstitute the hematopoietic system of the patient who has undergone conditioning regimen.

1.2 Graft Versus Host Disease

Allogenic HSCT however, comes with a debilitating side-effect of acute or chronic graft versus host disease. GvHD is a state of immunological disarray caused by the donor immune system which results in inflammation and tissue damage to the host. Since 2005, NIH recommended classification into acute or chronic GvHD is based on the time of onset of GvHD and the symptoms (14). Typically, acute GvHD manifests as skin rash, increased blood bilirubin levels, gastrointestinal tract damage leading to nausea, vomiting, diarrhea, and anorexia (15). It is one of the leading cause of mortality, and some of the symptoms manifest among most patients that receive allogeneic HSCT (16). It affects approximately 30-50% and severe acute GvHD occurs in approximately

14%, of the patients that receive allogeneic transplant from an HLA-matched donor (11, 15). The current globally immunosuppressive therapies are effective in treating the symptoms, but may interfere with the GvL effect of allogeneic HSCT, thereby not reducing post-transplantation mortality (17). Therefore, there is a growing need to control the potentially lethal effects of GvHD without affecting GvL activity.

1.2.1 GvHD Signaling Cascade

The process of GvHD development starts with the administration of conditioning regimen. Conditioning regimen consists of chemotherapy and/or radiation, which are known to trigger non-specific tissue damage, especially in the gastrointestinal (GI) tract. This damage leads to release of danger signals, such as, pro-inflammatory cytokines (TNF, IL-1, and IL-6 (18)), chemokines and chemokine receptors (19), by the host tissues, and increased expression of co-stimulatory molecules on host APCs (11). Specifically, GI injury due to conditioning regimen causes systemic release of commensal gut bacteria and subsequent increase in lipopolysaccharide (LPS) and pathogen associated molecular proteins (PAMPs). This increase in danger signals, augments antigen presentation by host APC resulting in strong activation, proliferation, and differentiation of donor T-cells that play one of the most important role in GvHD-related morbidity in leukemia patients (20). The release of danger signals also causes innate immune cell activation which ultimately results in increased production of pro-inflammatory cytokines and tissue inflammation (17). GvHD manifests as skin rash and progresses towards other body organs, mainly, lung,

liver, gut, and skin. Therefore, targeted treatment, as opposed to global immunosuppression, against this host inflammation and non-specific tissue damage caused by conditioning regimen could potentially reduce GvHD while maintaining the GvL activity.

1.2.2 Alloreactivity in Graft versus Host Disease

GvHD is largely mediated by alloreactive donor T-cells. During T-cell development in thymus, immature T-cells undergo positive and negative selection. Positive selection refers to when T-cells that adequately recognize self-peptide self-MHC complex are chosen for further differentiation, while negative selection is when self-recognizing autoimmune T-cells are signaled to die. Alloreactivity refers to when the T-cells can recognize peptide and allogeneic MHC complexes that were not encountered during thymic selection (10). Alloreactive T-cells have been widely studied in the context of GvHD and whether their elimination could reduce incidence of GvHD (21). However, alloreactivity is important for development of both, GvHD and GvL effect, in allogeneic HSCT (22). In a HLA-matched HSCT, alloantigens that mediate GvHD and GvL are called minor histocompatibility antigens (miHA). MiHAs are cell-surface proteins that are associated with MHCs. Single nucleotide polymorphisms (SNPs) can translate into differences in miHAs between HLA-matched donor-host pair, especially matched unrelated donors, and are increasingly being recognized for their therapeutic potential in leukemia (23, 24). However, whether miHA differences translate into graft versus host disease is debatable (25).

1.2.3 Existing Therapies for Graft Versus Host Disease

One of the crucial aspects of therapeutic development for GvHD is preserving the GvL effect of the allogeneic HSCT. One of the widely-employed preventive therapies is reducing the intensity of myeloablative conditioning regimen given to the patient. While reduced intensity conditioning regimen considerably reduces GvHD symptoms, it also increases the burden on the donor HSCs for eliminating leukemia. Multiple studies have shown that reduced intensity conditioning fails to improve long-term survival due to high rate of leukemia relapse (17, 26). Another important step in development of GvHD is homing of T-cells to the site of tissue damage via chemokine signaling. Targeting alloreactive T-cell homing via chemokine-ligand antagonists such as CCR5 antagonists, have shown to lessen the damage to the target organs (27). However, these therapies may also interfere with recruitment of immunosuppressive T-cell subsets (17, 28). Th-1, Th-2, and Th-17 type cytokines play variable and important roles in GvHD development. Cytokine modulators such as Alpha-1 antitrypsin and cytokine IL-22 are currently under clinical trials as therapy for their protective role in GvHD (29). Many other cytokines, such as IFN γ , TNF, IL-6, and IL-23 or their modulators are being studied using murine models of GvHD (17, 18). Additionally, JAK/STAT inhibitors have also shown promise as treatment therapy for GvHD (30). JAK-STAT signaling pathway is downstream of cytokine-receptor binding, and causes activation of antigen presenting cells (APCs), which in turn results in higher APC and T-cell interaction. Therefore, preventing donor APC activation by JAK/STAT inhibition could prevent GvHD without reducing the GvL activity. Lastly, there

has been increasing interest in immunomodulatory cell therapies for GvHD. Invariant Natural Killer T-cells (iNKT cells) (31), myeloid-derived suppressor cells (MDSCs) ((32, 33), and their various subsets, are being investigated in mouse models of GvHD for their immunosuppressive role through cytotoxic T-cell interaction or production of immunosuppressive cytokines.

These are few among a large number of therapies that are currently being studied for preventing GvHD development at various points in the signaling cascade. However, we wanted to determine the effect of blocking initial inflammation by using anti-inflammatory and anti-oxidative agent, and if it could stop the signaling cascade from progressing to GvHD related end organ damage and/or death.

1.3 Bilirubin Nanoparticle

Bilirubin is a linear tetrapyrrole molecule, naturally found in human blood. It is an orange-yellow pigment, found in the liver and is produced as an end product of heme catabolism. At the end of a red blood cells' (RBC) lifetime, they undergo lysis. In this process, hemoglobin gets degraded and the heme part gets oxidized by an enzyme called heme oxygenase, forming biliverdin. Biliverdin is a greenish molecule that gets reduced by an enzyme called biliverdin reductase (BVRA), to form bilirubin. This process occurs in the reticuloendothelial cells of the liver, spleen and bone marrow. This bilirubin is ultimately carried to the liver via blood, where it performs various biological functions. Bilirubin is known for its anti-inflammatory and antioxidant properties and has been inversely correlated with cardiovascular risk (34). Bilirubin quenches reactive oxygen

species (ROS) and gets converted into biliverdin, which is converted back to bilirubin through BVRA enzyme ((35) Figure 1). This recycling property, makes bilirubin a very powerful protectant against cellular oxidative damage. Higher bilirubin levels in adults are correlated with a number of health benefits such as lower prevalence of, non-alcoholic fatty liver disease, cardiovascular disease development, colorectal cancer, and ischemia–reperfusion injury after liver transplantation among many others (36).

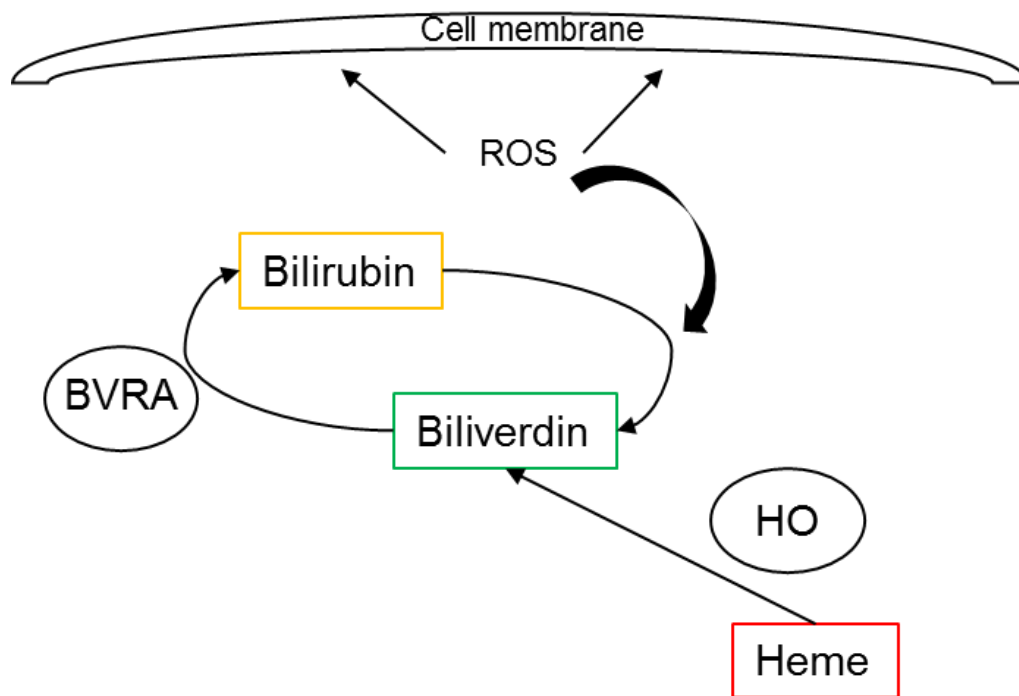


Figure 1- Mechanism of production and anti-oxidative activity of Bilirubin (inspired from Baranano et al. (35)). Heme part of the hemoglobin get oxidized to produce biliverdin, by an enzyme called heme oxygenase (HO) anchored on the endoplasmic reticulum (ER). With the help of an enzyme called biliverdin reductase (BVRA), biliverdin gets reduced to form bilirubin. BVRA regenerates bilirubin that gets oxidized to form biliverdin upon quenching of membrane bound

reactive oxygen species (ROS). This cycle enables low concentration bilirubin to quench up to 10,000 fold higher concentrations of oxidants.

Despite its well-known anti-inflammatory and anti-oxidative properties Bilirubin is rendered difficult for clinical use because of its water insolubility. In an attempt to harness the therapeutic properties of bilirubin, investigators at Korea Institute of Advanced Science and Technology (KAIST) South Korea, recently developed a water-soluble conjugate of bilirubin and polyethylene glycol (PEG), known as the bilirubin nanoparticle (BRNP). PEG forms a stable amide bond with bilirubin to form PEGylated bilirubin (PEG-BR). Since bilirubin is a lipophile and PEG is a hydrophile, the end product PEG-BR is an amphiphile, which self-assembles to form a micelle-like structure or bilirubin nanoparticle. This nanoparticle is about a 100 nm in size in freeze-dried state and retains the properties of bilirubin while being water-soluble. Upon stimulation with light, BRNP turns into photoisomer of bilirubin. Also and more importantly, upon oxidative stress such as with ROS, BRNP turns into biliverdin or oxidized fragments of bilirubin.

Recent studies by investigators at KAIST, have shown that due to its free-radical scavenging properties, BRNP selectively accumulates at the site of inflammation and tissue damage in dextran sodium sulfate induced colitis in murine model (37). In mice, pre-treated with BRNP, pathological changes associated with ischemic reperfusion were found to be significantly less as compared to mice that were treated with vehicle (38). Similarly, BRNP treatment showed significant benefits in mouse models of other inflammatory conditions,

such as asthma (39), pancreatic islet xenotransplantation (40), and even as a conjugate for anti-cancer therapy (41, 42).

1.4 Hypothesis

Therefore, we hypothesize that, BRNP treatment can reduce graft versus host disease due to its beneficial anti-inflammatory properties which act against tissue damage and inflammation in murine GvHD model.

Chapter 2- Materials and Methods

2.1 Mice

All animal experiments were conducted per The University of Texas MD Anderson Cancer Center's Institutional Animal Care and Use Committee guidelines. Six- to eight- week old female, C57/B6J and Balb/c, mice were purchased from Jackson Laboratories (Bar Harbor, ME).

2.2 Hematopoietic Stem Cell Transplantation

On day 0, 10- to 15- week old female Balb/cJ mice were given 800 cGy of Total Body Irradiation (TBI). On Day 1, age-matched donor female C57/B6J mice were dissected to obtain spleen and hind limb bones (femurs and tibiae). Spleens were pooled together in sterile 1X PBS and gently mashed. The suspension was strained through a 70 μ m cell strainer to obtain single cell suspension. Bone marrow cells were obtained by flushing the bones, with 1X PBS through a 27G needle, until they appeared white. Bone marrow cells were also similarly strained through 70 μ m cell strainer to obtain a single cell suspension. Both, spleen and bone marrow lymphocytes (without red blood cells) were counted on hemocytometer.

For our first model, we transplanted 1×10^7 RBC lysed splenocytes in mice, while the negative control group received 5×10^6 BM cells only. These mice transplanted with splenocytes, either received a single dose of 10 mg/kg BRNP or equivalent volume of vehicle (1X PBS) on day 0.

For our second model, we transplanted 1×10^6 conventional T-cells ($CD4^+$ and $CD8^+$ T-cells or Tcons) along with 4×10^6 BM cells. Starting from day 0, these mice received 3 doses of 10 mg/kg BRNP or equivalent volume of vehicle (1X PBS), every two days. The negative control mice received 4×10^6 BM cells only. Tcons were purified using MACS[®] Cell Separation technique (Miltenyi Biotec). For isolating $CD4^+$ and $CD8^+$ T-cells, 5×10^8 splenocytes from donor mice were incubated with anti-mouse $CD4$ MicroBeads (clone: L3T4, cat no. 130-117-043) and anti-mouse $CD8$ MicroBeads (clone: Ly2, cat no. 130-117-044) in magnetic associated cell sorting (MACS) buffer (PBS, pH 7.2, 0.5% human serum albumin-HSA (Sigma Life Science, cat. no. SRP6182)), and 2 mM EDTA (USB- Thermo Fisher, cat. no. 15694) for 20 minutes in dark at room temperature. Subsequently, $CD4^+$ and $CD8^+$ T-cells were isolated using LS column (Miltenyi Biotec, cat. no. 130-052-401) according to the manufacturer's specified protocol. The isolated Tcons were washed and resuspended in 1X PBS for counting and subsequent transplantation in mice.

For our third model, 5×10^6 splenocytes and 5×10^6 bone marrow cells were transplanted intravenously. The negative control group received 5×10^6 bone marrow cells only. From day 0 to day 4, mice received 5 daily doses of 10 mg/kg BRNP or equivalent volume of vehicle (1X PBS) intravenously.

2.3 Bilirubin Nanoparticle

Lyophilized BRNP was generously provided by Dr. Sangyong Jon and his laboratory at the Korea Advanced Institute of Science and Technology (KAIST), South Korea. Five milligrams of lyophilized BRNP was dissolved in 1 ml of 1X

PBS. This solution was stored at 4°C, protected from light, and used within 7 days.

2.4 Flow Cytometry Analysis

Splenocytes and bone marrow cells that were used for transplant, were stained with anti-mouse, CD3 ϵ (BioLegend, cat no. 100306), B220 (BD Pharmingen™, cat no. 553093), CD4 (BD Pharmingen™, cat no. 558107), and CD8 (BD Horizon™, cat no. 563068) antibodies for 30 minutes in dark. The cells were then washed with 1X PBS, fixed in 2% paraformaldehyde, and stored at 4°C until flow acquisition. The samples were acquired using Canto II Cell Analyzer (Beckton, Dickinson and Company, Franklin Lakes, NJ), and FlowJo version 10.3 (Tree Star, Ashland, OR) was used for analysis.

2.5 Clinical Graft Versus Host Disease Assessment

Mice were scored for clinical GvHD, by using a previously established scoring system by Cooke et al.(43). Mice were scored from 0-2 for, Weight (0: <10% loss, 1: \geq 10% <25%, 2: \geq 25%), Fur (0: normal, 1: mild to moderate, 2: severe ruffling), Posture (0: normal, 1: kyphosis at rest, 2: kyphosis impairing movement), Activity (0: normal, 1: stationary 50% of the time, 2: stationary unless stimulated), Skin (0: normal, 1: scaling paws or tails, 2: lesions). In addition, all the treatment groups were also monitored for survival, and sacrificed if they seemed inactive or non-responsive to stimuli.

2.6 Pathological Graft Versus Host Disease

On day 0, 10-15 week old female Balb/c mice received 800 cGy TBI. On day 1, the donor C57/B6 mice were sacrificed to obtain their splenocytes and BM cells. 5×10^6 whole splenocytes and 5×10^6 bone marrow cells were transplanted intravenously to the BRNP treated and the vehicle groups. The negative control group received 5×10^6 bone marrow cells only. From day 0 (4 hours after transplantation) to day 4, the mice received 5 daily doses of 10 mg/kg BRNP or equivalent volume of vehicle (1X PBS) intravenously. These mice were sacrificed on day 8 after transplantation, and their blood, spleen, liver, lung, and skin were harvested for other downstream experiments- serum cytokine analysis and histopathology.

2.7 Serum Cytokine Quantification

Blood for each individual mouse was collected by retro-orbital vein puncture. Tubes with blood were centrifuged at 600g for 5 minutes. Serum supernatant was collected and stored at -80°C until used for analysis. BD™ Cytometric Bead Array (CBA) (cat no. 560485) was used to determine the serum cytokine concentrations of Th1, Th2, and Th17 type cytokines, namely- IL2, IL-4, IL-6, IFN- γ , TNF, IL-17A, and IL-10 proteins. Sera for all the treatment group mice was diluted 1:4 in assay diluent, incubated with mixed capture beads and subsequently washed according to the manufacturer's instructions. The samples were acquired using LSR Fortessa Cell Analyzer (Beckton, Dickinson and Company, Franklin Lakes, NJ), and FlowJo version 10.3 (Tree Star, Ashland, OR) was used for analysis.

2.8 Histopathology

Mouse organs, namely- skin (from dorsal region), liver (one lobe), lung (one lobe), small intestine (duodenum), and spleen, were fixed in 10% buffered formalin, and submitted to Research Histology Core Laboratory at The University of Texas MD Anderson Cancer Center, for haematoxylin and eosin staining. The sample slides were then evaluated by a pathologist who scored the tissues for inflammation and GvHD associated damage for each organ.

2.9 Statistical Analysis

All statistical analyses were performed using GraphPad Prism Software version 7.00 (La Jolla, California) for Windows. Data sets were analyzed using Mann-Whitney U Test with confidence level: 95%, and p values for comparisons between groups were determined. For survival analysis, Log-rank (Mantel-Cox) survival test was used. A p value less than 0.05 was considered to be statistically significant.

Chapter 3- Results and Analysis

3.1 Mouse Model of Graft Versus Host Disease

The first step to determine the anti-inflammatory and anti-oxidative effects of BRNP, was to establish a simple and reproducible murine model for acute GvHD. We chose HLA mismatch model of H-2^b C57BL/6 mice as donors and H-2^d Balb/c mice as recipients. To determine optimal transplant dose, we shortlisted different published models with their respective outcomes, as outlined in Table 1 below.

Table 1- Shortlist of published murine GvHD models- detailed for their transplant dose, radiation dose, and outcome

HLA Mismatch model- C57BL6 (H2^b) → Balb/c (H2^d)			
Cell type and dose	Conditioning Regimen	Outcome	Reference
2 x 10 ⁷ Whole Splenocytes	700 cGy	Survival between day 10-50	Margalit et al.(44)
5 x 10 ⁶ TCD-BM and 1 x 10 ⁶ Tcons	2 doses of 400 cGy	Systemic disease by Day 7-30	Schneidawind et al. (31) and Griesenauer et al. (45)
5 x 10 ⁶ BM and 5 x 10 ⁶ Splenocytes	800 cGy	Survival between day 10-40	Im et al. (46) and Wang et al.(47)
25 x 10 ⁶ GM-CSF mobilized splenocytes	900 cGy	Systemic disease by day 5-14	Kuns et al. (48)

We attempted three models with or without BRNP treatment. The first murine model (Margalit et al. (44); Table 1) received 800 cGy radiation on day 0, and a single dose of 10 mg/kg BRNP (optimized by our collaborating investigators (38)) 4 hours after radiation (Figure 2a). On day 1, the vehicle control (n=10) and the BRNP test group (n=10) of mice were transplanted with 1×10^7 RBC lysed splenocytes and 5×10^6 BM cells, whereas the negative control group (n=5) received 5×10^6 BM cells only. Mice were monitored for their weight and GvHD score every two days or until their death/ recommended euthanasia. Euthanasia was recommended at very low or no activity and/or extreme (>30%) weight loss.

The mice in this experimental group were highly inactive and lost more than 25% of their initial weight within 8 days from transplantation (Figure 2b). We observed that mice were severely kyphotic and lethargic, owing to the high dose of RBC lysed splenocytes (Figure 2c). Within 13 days, 100% of both the vehicle control and the BRNP test groups had died (Figure 2d). For our next experiment, we concluded that, donor cell dose should be lowered and BRNP dose should be increased.

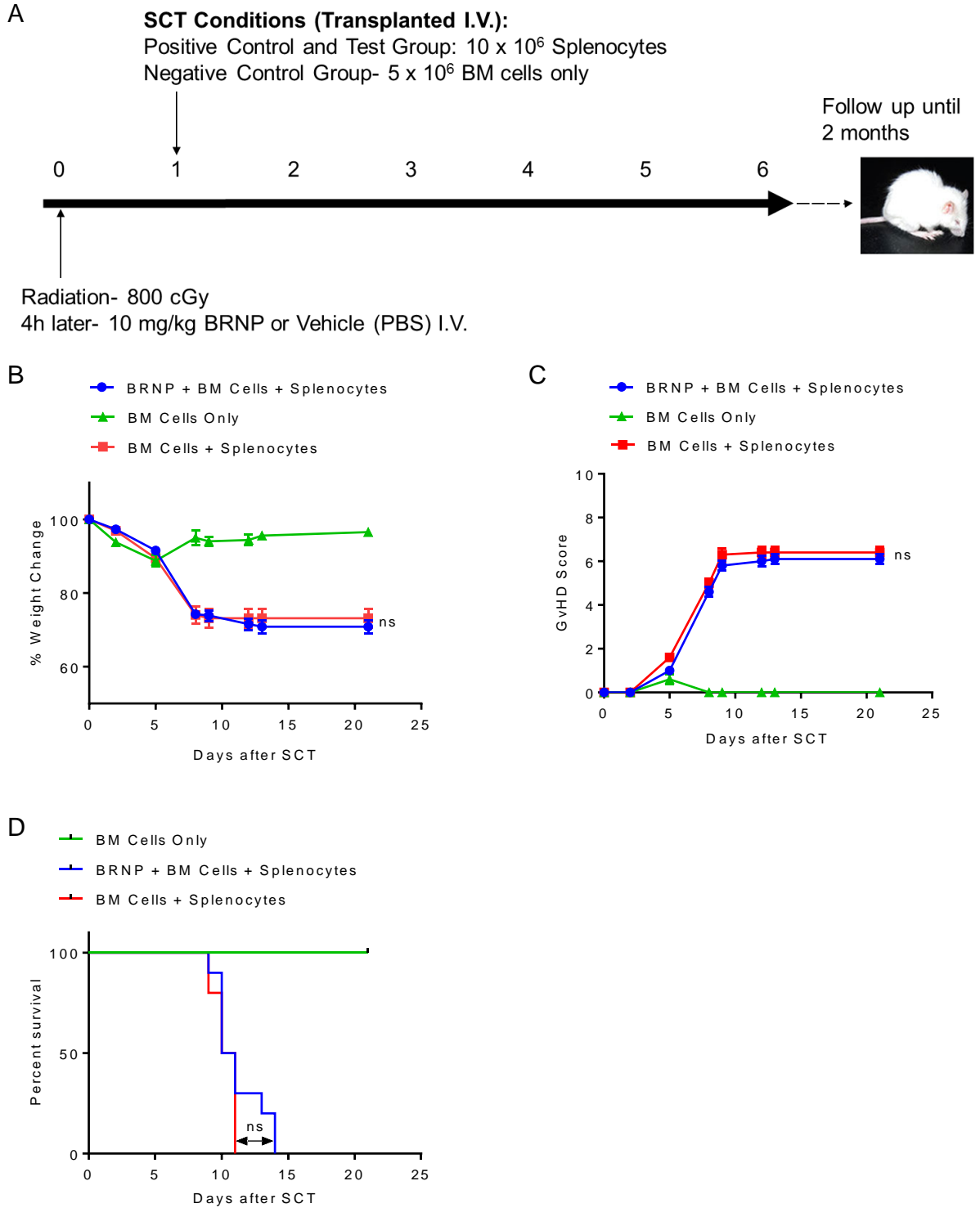


Figure 2- Clinical GvHD outcome of the first strategy of murine GvHD model. (A)- Vehicle control group (n=10), BRNP test group (n=10) and negative control group (n=5) received 800 cGy TBI on day 0. On day 1, vehicle control

group and BRNP treated group were transplanted with 1×10^7 RBC lysed splenocytes, while the negative control group was transplanted with 5×10^6 BM cells only. BRNP test group received a single dose of 10 mg/kg, 4 hours after TBI on day 0, while the vehicle control group received vehicle (1X PBS) I.V. injection. There were no significant differences between the BRNP + BM Cells + Splenocytes (test) group versus the BM Cells + Splenocytes (vehicle) group in terms of (B)- weight change, (C)- GvHD score, and (D)- Survival. “ns” stands for non-significant. p value was determined using Mann Whitney U Test for weight and GvHD score, and Mantel Cox Survival Test for survival

For our second strategy (Schneidawind et al. and Griesenauer et al. (31, 45); Table 1), we performed TBI of 800 cGy on day 0, and 4 hours later the mice were given their first dose of 10 mg/kg BRNP or equivalent volume of vehicle (1XPBS). On day 1, the recipient mice were transplanted with 1×10^6 conventional T-cells (Tcons) instead of whole splenocytes, along with 4×10^6 BM cells. On day 2 and day 4, the mice received additional dose of 10 mg/kg BRNP or equivalent volume of vehicle (1XPBS) (Figure 3a). All the treatment groups were monitored for their weight, GvHD score, and survival, for 2 months or until, their death/recommended euthanasia. Despite no significant change in weight loss (Figure 3b), this experiment was successful as we observed significantly lower clinical GvHD scores in the BRNP treated group (Figure 3c) which translated into significantly better survival outcome (Figure 3d).

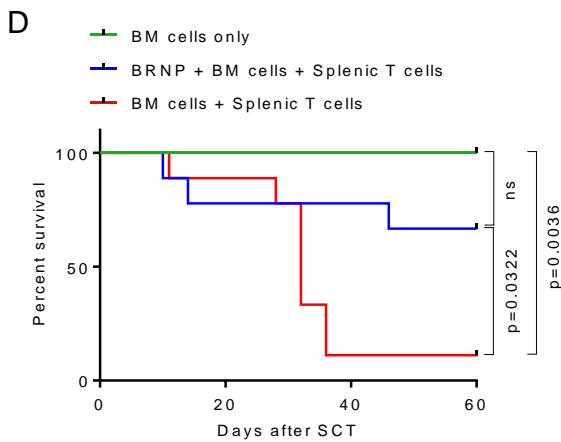
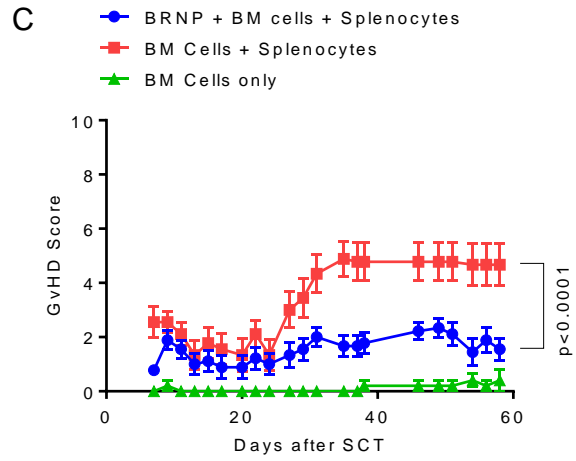
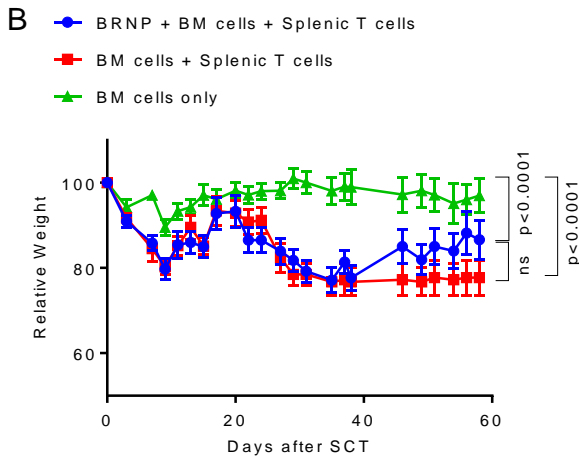
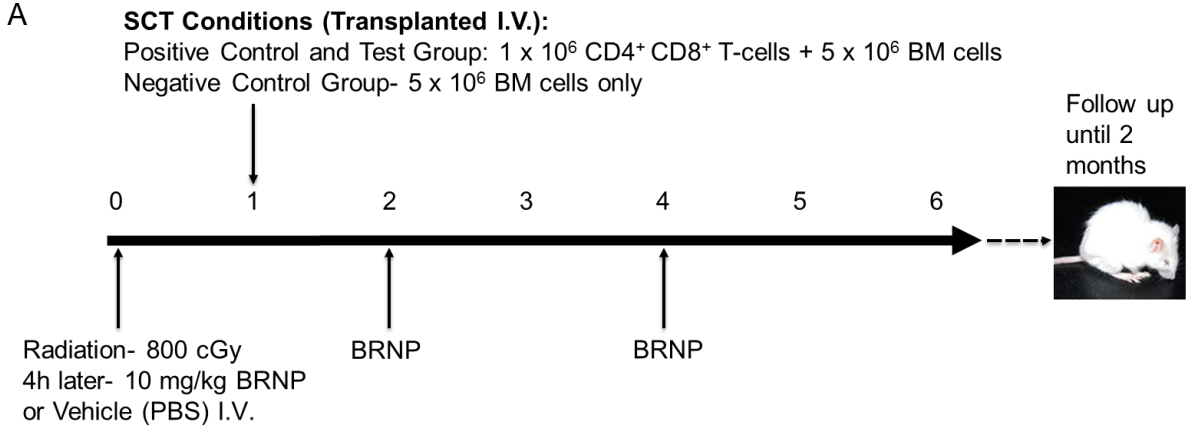


Figure 3- Clinical GvHD outcome of the second strategy of murine GvHD model. (A)- Vehicle group (n=9) and the BRNP treated group (n=9) mice were given 800 cGy TBI on day 0, and transplanted with 1×10^6 Tcons and 4×10^6 BM cells from C57/B6 mice on day 1. On day 0, 2, and 4, the BRNP treated group mice were i.v. treated with 10 mg/kg dose of BRNP, while the vehicle control group received equivalent volume of 1X PBS. The negative control group (n=5) mice received 4×10^6 BM cells only. No significant changes between the BRNP + BM Cells + Splenocytes and BM Cells + Splenocytes groups were seen in (B)- Weight, however, BRNP + BM Cells + Splenocytes group of mice has significantly lower (C) GvHD score ($p < 0.0001$, Mann Whitney U Test), which translated into better (D) survival ($p = 0.0322$; Mantel-Cox Survival Test). The median survival for BRNP test group at day 60 was 65% as compared with vehicle control in which only 11% of the mice survived. Error bars represent standard error of mean.

In other studies by our collaborating investigators they have reported that doses as high as 150 mg/kg BRNP (15-fold higher than our current dose) are non-toxic to mice (10 mg/kg). Therefore for our next strategy, we decided to increase the number of BRNP injections in order to maximize the anti-inflammatory and anti-oxidative activity against GvHD. While several factors such as radiation dose, donor recipient strains, and pathogens associated with colony influence the GvHD outcome, the most important factor is the T-cell dosage (49). To test the efficacy of BRNP as an anti-inflammatory molecule, we wanted to establish a rather simplified murine model with reliable GvHD outcome.

3.2 BRNP Treatment Can Increase Survival in Murine Acute Graft Versus Host Disease Model

We tested the anti-inflammatory and anti-oxidative benefits of BRNP treatment in murine acute GvHD by using our third model (Im et al. and Wang et al. (46, 47); Table 1), which was consistently reproducible. For our third strategy, we gave 800 cGy TBI to the recipient mice on day 0, followed by intravenous administered of 10 mg/kg BRNP, 4 hours later. On day 1, we transplanted 5×10^6 whole splenocytes and 5×10^6 BM cells in the vehicle control and the BRNP test groups, whereas the negative control group was transplanted with 5×10^6 BM cells only. BRNP test group mice were given 5 daily doses of 10 mg/kg intravenous BRNP from day 0-4 while the vehicle control group similarly received vehicle (1X PBS) injections (Figure 4a).

Similar to our second strategy for HSCT, we observed that after an initial weight loss within the first week (Figure 4b) for all the three treatment groups, only the BRNP treated group and the negative control mice swiftly recovered. The vehicle control group continued to show faster weight loss till around day 13 and a delayed recovery as compared with BRNP treated or the negative control groups. We observed that while the BRNP treated mice continued to maintain their weight between 90-100% of their initial weight, the vehicle control mice kept getting significantly worse ($p=0.0001$; Mann Whitney U Test) after their initial recovery. Similar to the weight loss trend, we observed significantly lower GvHD score in BRNP treated mice as compared to the vehicle control group ($p=0.0003$, Mann Whitney U Test; Figure 4c). Despite being a subjective method

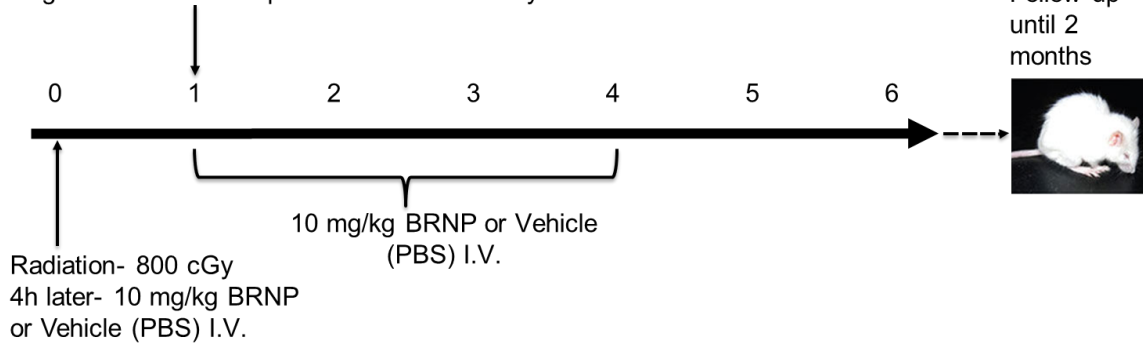
of scoring, GvHD score provides a crucial measurement of clinical GvHD development in mouse model. The initial peak in GvHD score at around day 15 for vehicle control mice, can be partly attributed to weight loss due to gut toxicity caused by radiation treatment. The BRNP treated group, however, did not seem to develop a similar extent of radiation toxicity, as their GvHD score did not increase. Although the vehicle control mice did recover from the initial weight loss, they quickly started to show clinical symptoms again at around day 20. At the same time, the BRNP treated group also showed a slight increment in their GvHD score, but it was still less than their vehicle control counterparts. While the untreated mice, continued to show symptoms through their lifetime, the BRNP treated group, recovered around day 30, and did not show GvHD symptoms anymore. Lower GvHD score and weight loss, translated into significantly higher survival ($p=0.0001$; Log-rank (Mantel-Cox) survival test) in BRNP treated mice, as compared with the vehicle control mice (Figure 4d). At day 60, 100% of the BRNP treated mice had survived, while for the vehicle control group, only 20% of the mice survived.

We observed that the survival of BRNP treated mice in our third model of acute GvHD (Figure 4d; 100% mice survived until day 60), is better than their survival in second strategy (Figure 3d; 65% mice survived until day 60). This could be attributed to either change in the transplantation strategy, or to the increase in number of BRNP injections. However, in both the strategies, 11% and 20% of the vehicle control mice survived by day 60, indicating that both strategies were capable of forming similar extent of GvHD. Therefore, increasing

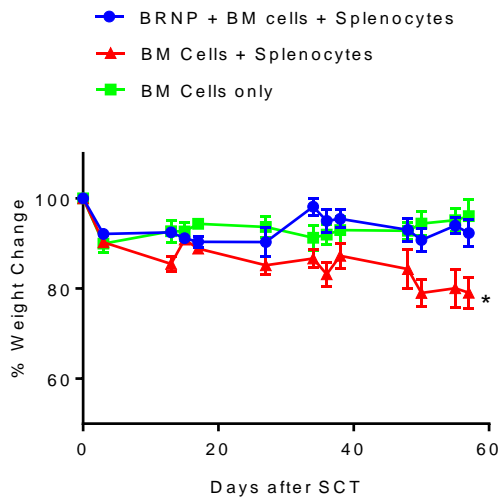
the dose of BRNP, may have led to the higher survival in BRNP test group, indicating the potential beneficial effects of BRNP against GvHD development.

A SCT Conditions (Transplanted I.V.):

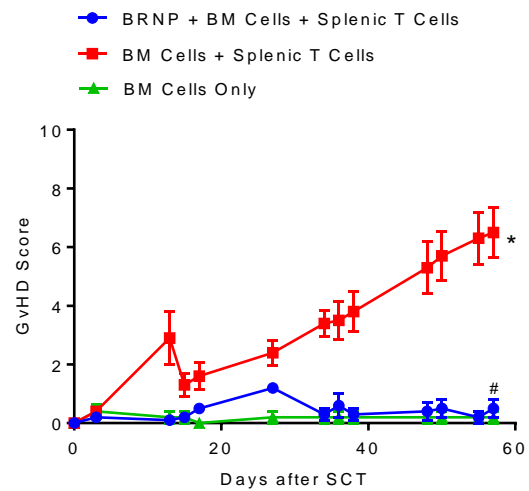
Positive Control and Test Group: 5×10^6 Splenocytes + 5×10^6 BM cells
 Negative Control Group- 5×10^6 BM cells only



B



C



D

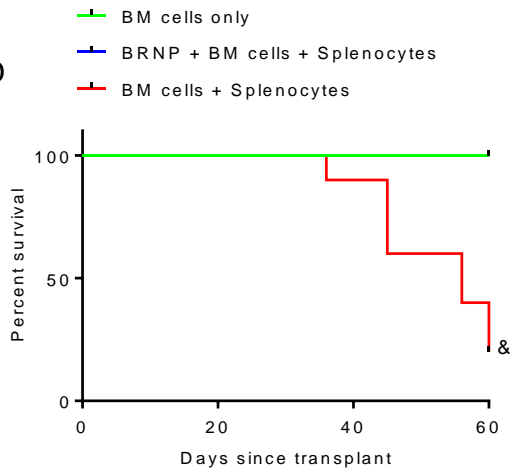


Figure 4- Clinical GvHD outcome of the third strategy of murine GvHD model. Shown here is cumulative data from N=2 independent experiments. (A)- Vehicle control group (n=10) and the BRNP test group (n=10) mice were given 800 cGy TBI on day 0, and transplanted with 5×10^6 BM cells and 5×10^6 splenocytes from C57/B6 mice on day 1. On day 0 to 4, the BRNP test group mice were given 10 mg/kg intravenous dose of BRNP, while the vehicle control group received similar vehicle (1X PBS) injections. The negative control group (n=5) mice received 5×10^6 BM cells only. As compared with BM Cells + Splenocytes group, the BRNP + BM Cells + Splenocytes group showed significantly lower (B)- Weight loss (* $p < 0.0001$, Mann Whitney U Test) (C) GvHD score (* $p < 0.0001$, Mann Whitney U Test), which translated into better (D) survival ($p = 0.0003$; Mantel-Cox Survival Test). Error bars represent standard error of mean. * $p < 0.0001$ (Mann Whitney U Test) between BRNP + BM Cells + Splenocytes and BM Cells + Splenocytes groups. # $p = 0.0208$ (Mann Whitney U Test) between BRNP + BM Cells + Splenocytes and BM Cells only groups. & $p = 0.0003$ (Mantel-Cox Survival Test) between BRNP + BM Cells + Splenocytes and BM Cells + Splenocytes groups.

3.3 BRNP Treatment Can Reduce Pathological Symptoms of Acute Graft Versus Host Disease

To determine the protective role of BRNP treatment on pathological symptoms of acute GvHD, we performed histopathological analyses on GvHD target organs such as the liver, lung, gastrointestinal tract (GI), and skin. We performed SCT on three treatment groups, with our previously optimized third strategy, and on day 8 we sacrificed the mice to collect their GvHD target organs along with blood and spleen.

We performed cytokine bead array on blood sera collected from all the treatment group mice. Serum levels for pro-inflammatory cytokine IFN γ (Figure 5a) and TNF (Figure 5b) were trending lower in the BRNP test mice (n=5) as compared to vehicle control mice (n=4), however they were not found to be significantly different. Higher serum levels of these pro-inflammatory cytokines are associated with higher organ damage which result in more severe onset of acute GvHD (18). We also analyzed other Th1, Th2, Th17 cytokines such as IL2, IL-4, IL-6, IL-17A, and IL-10. However, due to limitations in the cytokine bead array, values lower than 20 pg/ml are not accurately detectable. And therefore, we were unable to measure the serum cytokine levels of these proteins in our treatment groups.

A previous GvHD study showed that, transplant engraftment in mice, with 4×10^6 MHC-mismatch splenocytes after 800 cGy radiation, happens within 6 days after transplant (50). Another study with different acute GvHD mouse model shows that histopathological changes are most apparent at around 7 days after

transplantation (compared between day 5, day 7, and day 21) (51). Indeed, within 24 hours after HSCT in mice, the donor T-cells migrate to secondary lymphoid organs for alloantigen priming before migrating to the GvHD target organs (49). Based on these studies, we justified sacrificing the three treatment groups, along with naïve control, on 8th day after transplantation.

Manifestation of skin inflammation and rash is one of the first symptoms of GvHD development after allogeneic transplantation. In agreement with our hypothesis, we observed significantly lower inflammatory cell infiltration in skin of the BRNP treated mice as compared with vehicle control (Figure 6, Figure 7a). Lung and liver damage is one of the hallmarks of GvHD development. We observed significantly less inflammation in lung (Figure 6, Figure 7b) and liver (Figure 6, Figure 7c) in BRNP treated mice when compared with vehicle control mice. Lastly, the duodenum part of the gastrointestinal tract shows significantly lesser necrosis in BRNP treated mice as compared to the vehicle control (Figure 6, Figure 7d), thus proving our hypothesis that BRNP treatment can reduce pathological symptoms of GvHD.

Quantitative evaluation of GvHD associated organ damage shows significantly lower lymphocyte infiltration in hair follicles (Figure 7a; $p=0.0079$; Mann-Whitney Test) indicating lower skin damage in mice treated with BRNP. We also observed significantly lower perivascular inflammation in lung (Figure 7b), and central vein inflammation in liver (Figure 7c). GI tract damage is one of the hallmarks of GvHD, and we found significantly lower single-cell necrosis in

the BRNP treated group as compared with vehicle control. BRNP treated mice showed close similarities with the negative control group in all histology scores.

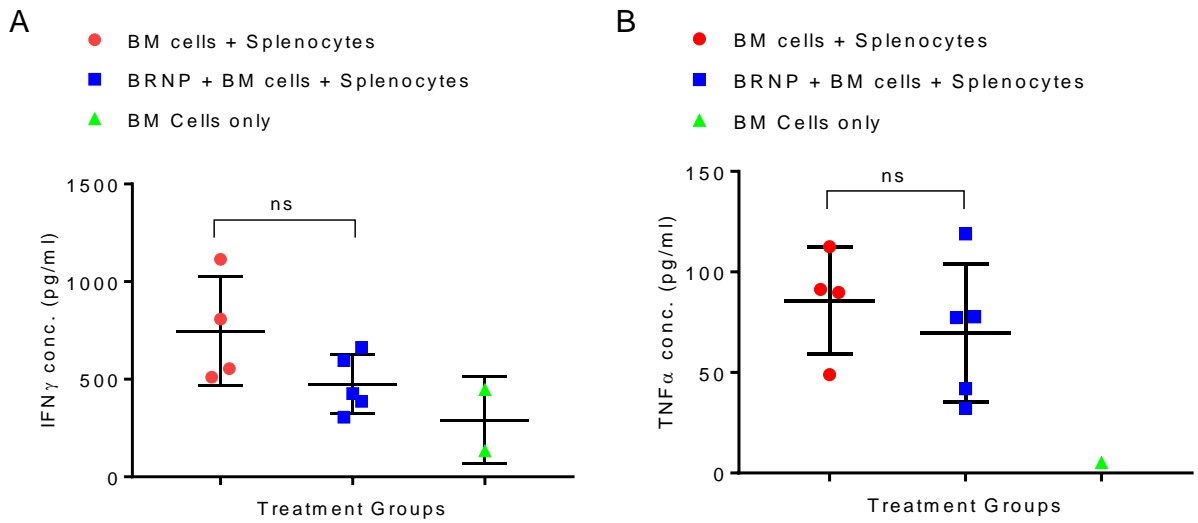


Figure 5- Serum levels of pro-inflammatory cytokines IFN γ and TNF α . Cytokine analysis shows a trending decrease in pro-inflammatory cytokines (A)- IFN γ and (B)- TNF in the blood serum collected on day 8 post transplantation. Each dot represents individual mouse for all treatment groups. The error bars represent standard deviation. ns= Non-significant (Mann-Whitney U Test).

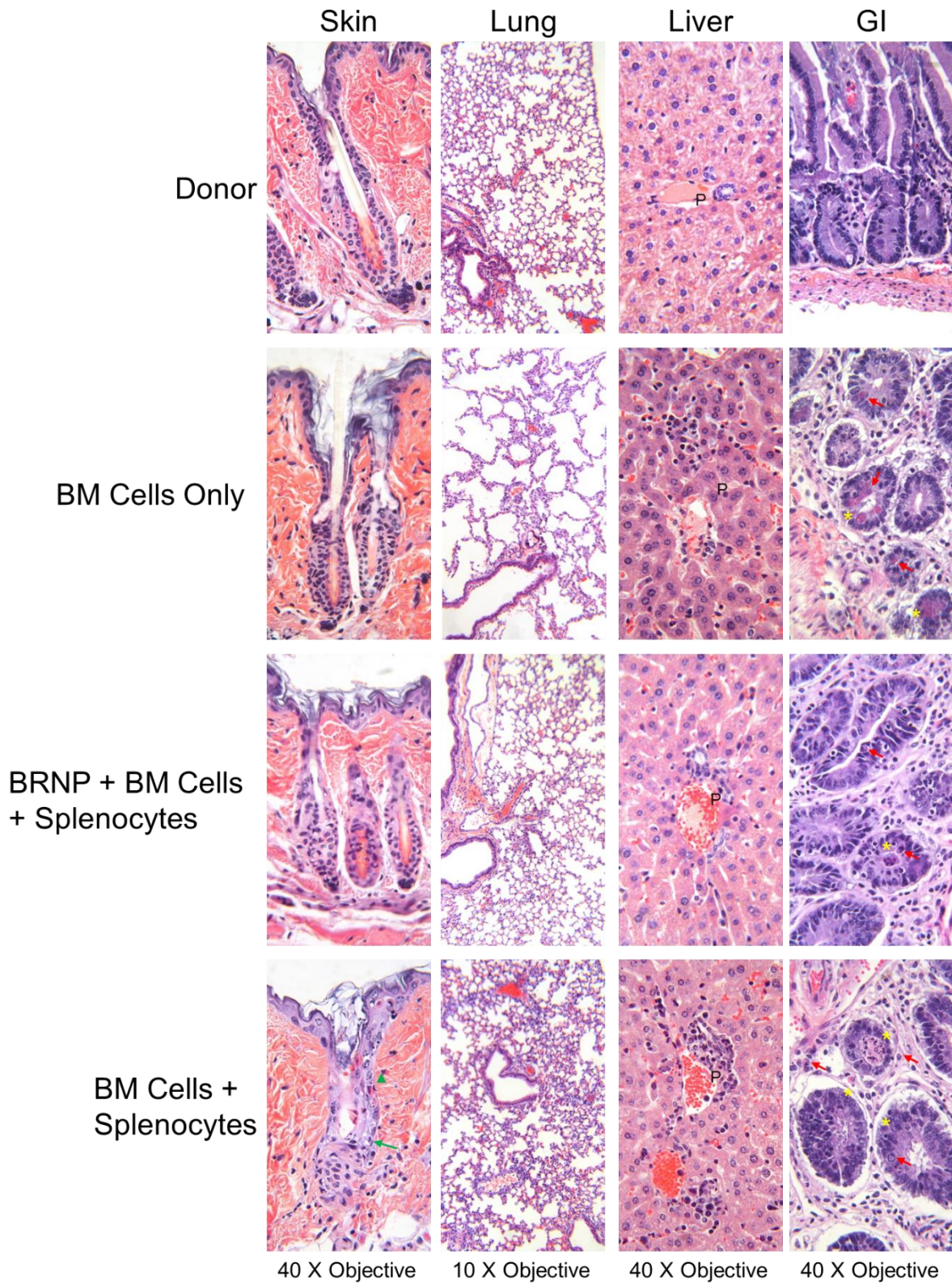


Figure 6- BRNP treatment can reduce GvHD associated tissue damage, such as lymphocyte infiltration, inflammation, and necrosis. Shown here are representative images of hematoxylin and eosin (H&E) staining for different GvHD target organs, namely, liver, lung, skin, and GI. Skin- In BM Cells + Splenocytes panel, green arrow shows an example of inflammatory cell infiltration, and the green arrow head shows an example of single-cell necrosis, which is significantly less in the BRNP treated mice. Lung- Donor (naïve control) and the BRNP treated mice show the least inflammation, as compared to the BM Cells + Splenocytes and BM Cells only group. Liver- Symbol P in the liver panel for all the treatment groups represents portal tract. Compared with BM Cells + Splenocytes group, the BRNP treated mice show fewer inflammatory cells, while donor mouse shows normal portal vein with no inflammation. GI- Red arrows show single-cell necrosis in the glandular epithelium and the yellow asterisk (*) show necrotic cells in crypt lumen. BRNP treated mice showed lesser GI single-cell necrosis as compared to BM Cells + Splenocytes group.

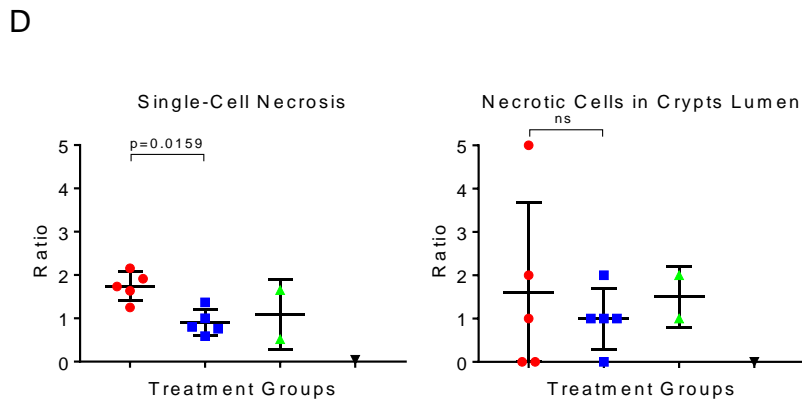
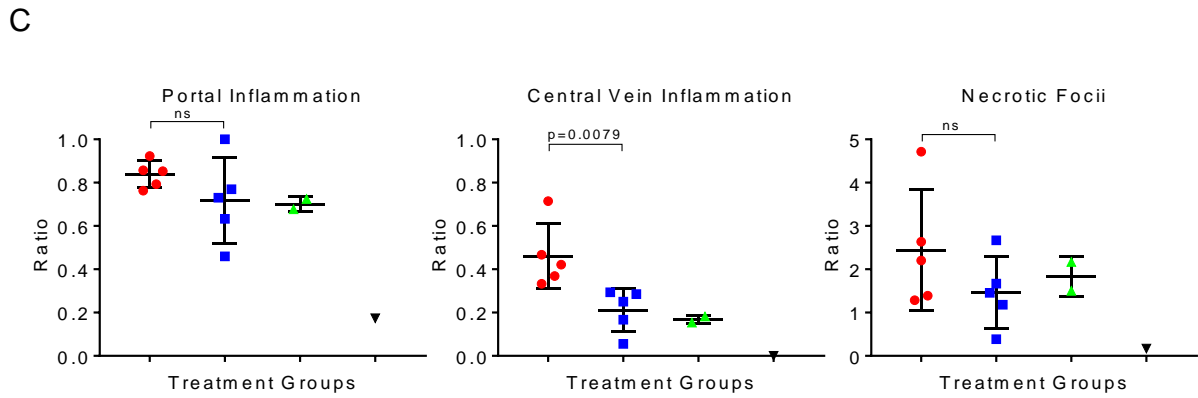
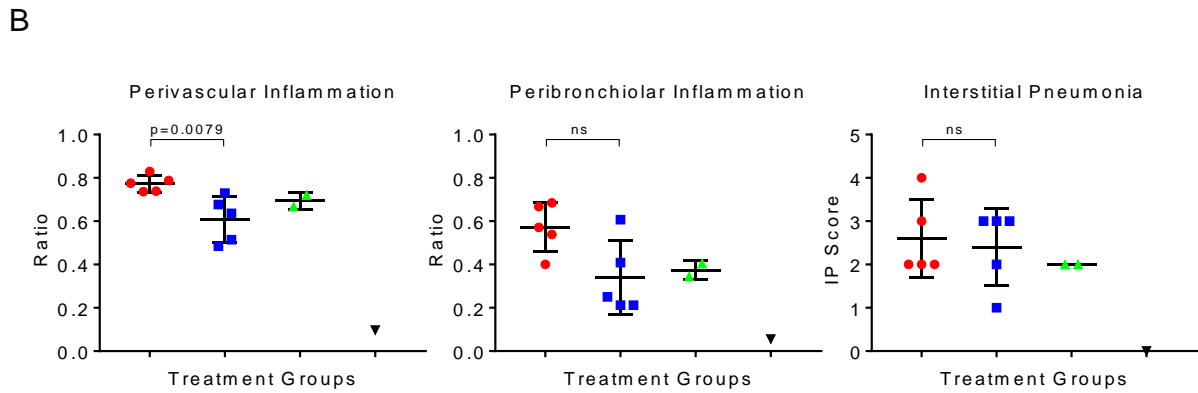
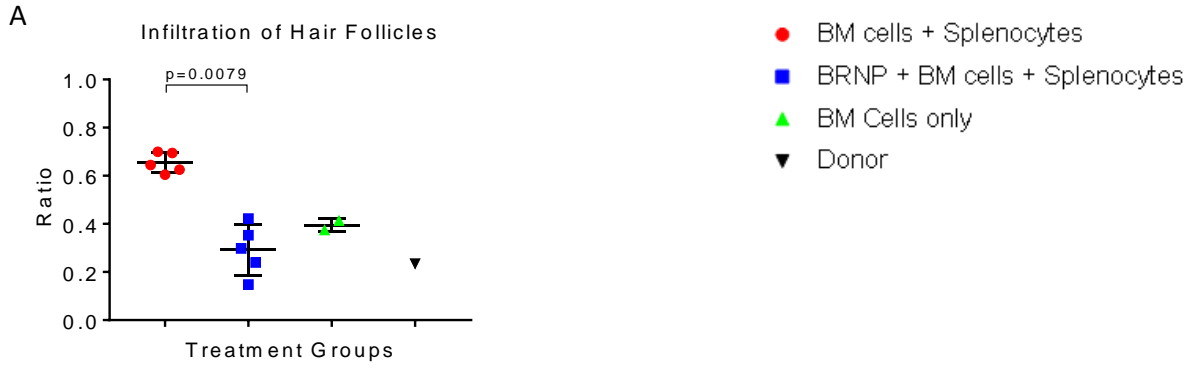


Figure 7- BRNP treatment can reduce pathological symptoms of acute GvHD in mice. Shown here is a quantitative evaluation of histopathology of acute GvHD performed by a board-certified pathologist. Pathological symptoms in different GvHD target organs such as (A) infiltration of hair follicles in skin, (B) perivascular inflammation, peribronchiolar inflammation, and interstitial pneumonia in lungs, (C) portal vein inflammation, central vein inflammation, and necrotic foci in liver, and (D) single cell necrosis and necrotic cells in crypt lumen of the GI. Histology ratio on y-axis for inflammatory conditions was measured by dividing the number of affected areas with total number of the areas observed under a 40X objective. Interstitial pneumonia scoring for lungs was done by evaluating the percentage of affected area under 10X objective and assigning a score from 1-4 with 4 being the most affected (Scoring: 1= $<25\%$, 2= $\geq 25\%$ to $<50\%$, 3= $\geq 50\%$ to $<75\%$, 4= $\geq 75\%$). Similarly for liver, the number of necrotic foci counted were normalized with the number of evaluated areas observed under 10X objective. Lastly, single cell necrosis or necrotic cells in crypt lumen for GI, were quantified by normalizing the counted number of necrotic cells with the number of areas evaluated. Dot-plots for all the treatment groups were plotted along with a single donor used as naïve histopathological control and statistics were performed using Mann-Whitney U Test.

Chapter 4- Discussion and Future Direction

Most therapies such as steroids that are currently in clinic as first line treatment for GvHD are globally immunosuppressive, which leads to compromise in the GvL effect of the allogeneic HSCT. Other prophylactic therapies such as reduced conditioning regimen are good for reducing GvHD symptoms, however because of being non-myeloablative, it comes with a higher risk of infections and/or leukemia relapse (26). Several studies continue to explore and understand the immune cell interactions that happen after the allogeneic HSCT to identify potential therapeutic targets for GvHD. There have been a few studies that look at the role of anti-oxidative agents, such as hydrogen therapy (52), green tea extracts (53), and ROS scavenging compounds such as cyclopentylamino carboxymethylthiazolyindole (NecroX-7) (46) as treatment for GvHD related organ damage using murine models of GvHD. However, there is a critical unmet need for clinically relevant, scalable, and non-toxic therapies for GvHD that do not affect the GvL outcome.

Bilirubin is a very effective and powerful antioxidant pigment found naturally in mammalian blood serum. Several human and mouse studies show that higher bilirubin level in blood serum is associated with protection against, cardiovascular diseases, non-alcoholic fatty liver disease, colorectal cancer, and ischemia–reperfusion injury after liver transplantation (36). Bilirubin has shown to be an effective antioxidant immunomodulator as treatment for experimental autoimmune encephalomyelitis (54, 55). However, elevated blood serum levels of bilirubin can be toxic due to its water insolubility. Hyperbilirubinemia may

indicate liver damage and can be neurotoxic in new born infants. More importantly, elevated bilirubin levels are found in patients that develop acute GvHD after allogeneic HSCT.

To maximize the therapeutic potential and reduce the toxicity of bilirubin, investigators at KAIST conjugated polyethylene glycol with bilirubin, which resulted into an amphiphilic molecule that self-assembles to form stable, non-toxic (at concentration as high as 150 mg/kg), and water soluble bilirubin nanoparticles. Polyethylene glycol is an FDA approved active ingredient. Conjugation with polyethylene glycol or PEGylation is used to improve the pharmacokinetic activity of various FDA approved drugs such as peginterferon alfa-2a for hepatitis C treatment (56). However, conjugation with PEG, especially near active site of the drug, may cause decrease in the drug activity in some cases (57). PEGylation of bilirubin has been shown to not affect the characteristic antioxidative and light sensitive properties of bilirubin, in both *in vitro* and *in vivo* experiments. Moreover, BRNP treatment has shown benefits in various mouse models of inflammatory diseases (37-41).

In this study, we looked at clinical and pathological benefits of BRNP as a prophylactic therapy for GvHD. Through our pre-clinical mouse model of GvHD we show that five daily intravenous doses of 10 mg/kg BRNP treatment significantly reduces, GvHD symptoms, associated weight-loss, and improves overall survival. Pathological damage associated with the onset of GvHD was also found to be significantly less in skin, liver, lung, and GI. We found significantly lesser lymphocyte infiltration and inflammation in these organs,

which may have contributed to the higher overall survival. We believe that BRNP treatment was able to reduce the initial non-specific tissue damage associated with conditioning regimen and continued to reduce tissue damage after HSCT. Lower tissue damage could translate into reduced antigen presentation to donor cytotoxic T-cells, which ultimately lessens the cytokine production and end-organ damage. We performed serum cytokine analysis, and saw a trend towards lower inflammatory cytokine production in the BRNP treated GvHD mice. Overall, our results show that prophylactic BRNP treatment in mouse GvHD model, is beneficial in reducing the symptoms and improving overall survival.

In future studies, we want to assess a treatment model of BRNP in reducing GvHD symptoms and improving overall survival. Through our mouse GvHD model, we plan to evaluate the role of post-transplantation BRNP treatment in alleviating GvHD symptoms without affecting the GVL activity of HSCT. In the treatment model, it would also be interesting to analyze immunomodulatory effects of BRNP by performing flow cytometry analysis for various immune cells and subsets, on the GvHD target organs.

Bilirubin nanoparticles dissociate into water-soluble photoisomers of bilirubin upon light ($\lambda = 450 \text{ nm}$ or 650 nm) stimulation (41). BRNP has also been shown to accumulate specifically at the site of tissue damage in a mouse colitis model, (37). Utilizing these properties of the nanoparticles, our collaborating investigators at KAIST, have successfully tested BRNP as a nano drug-carrier for anti-cancer therapy (42). Therefore, after establishing BRNP in a treatment model for GvHD, a future extension of this study would be to evaluate BRNP as

a drug conjugate for existing therapies for GvHD. Our first step would be to check for the accumulation site of the BRNP-drug conjugate in the mouse model, and test whether BRNP can successfully transport the drug to the site of organ damage. Eventually, we would compare clinical and pathological symptoms of GvHD along with overall survival using our mouse GvHD model treated with BRNP-drug conjugate and BRNP alone. Our hypothesis is that BRNP-drug conjugate can selectively reduce tissue damage, thereby reducing the global toxicity of the drug and improving the GvHD outcome.

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