The Effect of MyD88 Deficiency During Graft-Versus-Host Disease

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***Abstract*—Graft-versus-host disease is the onset of an unwanted immune response in patients who have undergone bone marrow transplantation [1]. The effect of this unwanted immune response is lethal in many cases. The goal of this research is to reduce the effect of graft-versus-host disease which can possibly reduce the waiting time for an eligible bone marrow donor to appear. This paper focused on the MyD88 adaptor protein, which triggers biochemical signals that can initiate, maintain, expand, or terminate inflammatory sites [2]. Inflammatory sites are the areas where the immune cells are gathered to fight foreign pathogens [1]. Experiments were carried out wherein stem cells were extracted from C57BL/6 mice and injected into BALB.B mice. This experiment design establishes the allogeneic bone marrow transplantation because the minor histocompatibility complex gene is un-matched between the two strains of mice while the major histocompatibility complex gene is matched. We focused on the effect of MyD88 protein deficiency in transplantation recipient. The positive/negative controls for the allogeneic bone marrow transplantation and MyD88 knockout allogeneic bone marrow transplantation group were carefully observed for 28 days and assessed for survival, weight, and immune cell fraction changes. We found that MyD88 protein deficiency yields prolonged survival in graft-versus-host disease. Data also suggests that innate immunity is the dominant factor in graft-versus-host disease, not adaptive immunity. Future experiments with cytokine analysis in a similar experiment design will provide more information about the relationship between the innate immunity and the severity of graft versus host disease.**

I. Introduction

HEMATOPOIETIC stem cell transplantation (HSCT), also commonly known as bone marrow transplantation, is a treatment option for hematological and immunological defects, such as autoimmune disease and sickle-cell anaemia [3]. The general idea of HSCT is to firstly destroy the hematopoietic stem cells (HSC) as well as other immune competent cells in the patient to get rid of all defective cells. Then replace them with a donors robust HSC. The benefit of performing HSCT is that you do not have to worry about uncertainty whether treatment was effective or not. The disadvantage of HSCT is that it is an extremely risky procedure because of substantial side-effects. With current technology advances, the surgery must be complimented with lifetime post-surgery-care to prevent lethal side-effects [3]. In some cases, finding a completely MHC-matched donor is extremely difficult. In such cases, physicians can choose a non-ideal solution by performing allogeneic HSCT (usually matching at MHC locus but mis-matched at MiHC) along with supplementary immunosuppressive treatments [4]. Ultimately, in order to achieve the most effective and safest allogeneic HSCT, the injected donor graft must be free of unwanted immune cells. The major sources of unwanted immune cell are mature killer T cells. The recipients immune system will respond and reject the donors mature killer T cells due to possibly the mismatched MHC or the minor histocompatibility antigens (Minor H Ag) of the allogeneic immune competent cells [3].

The unwanted onset of an immune response after HSCT is called graft-versus-host-disease (GVHD) [3]. It was widely recognized that the activation of the adaptive immune system is the most influential factor of GVHD [1]. One possible way of GVHD occurring is when the donors mature allogeneic T cells are co-transported along with the HSC into the recipient [1]. The mature T cells gather at the inflammatory site and attack the recipients normal cells. Then, after enough time has passed for the transplanted HSC to go through T cell development, those newly matured T cells will accelerate the damaging process. In order to do damage on the recipient cells, an inflammatory site is required. Without the inflammatory site, the damage done to the recipient will significantly decrease. It is worthwhile to investigate the causes of inflammation because prevention of inflammatory site formation will lead to no T cells being activated, and thus, no GVHD. Inflammatory sites can be formed via TLR signalling pathways.

There are two major TLR signalling pathways: the MyD88-dependent pathway and the MyD88-independent pathway [1]. TLR signalling pathways are needed to convert PAMPs into communicable nucleus signals [1]. PAMPs are found on pathogens, since they are usually an indispensable component of the pathogens structure [2]. Some examples of PAMPs are lipopolysaccharides, double- and single-stranded RNA, glycoproteins, flagellin, profillin, and lipopeptides [1] When any of these PAMPs are recognized and bind to TLRs, a signal is transduced into the cell to mediate a response. In

the MyD88-dependent pathway, the TLRs utilize the MyD88 adapter protein to activate the transcription factor NF-?B. NF-?B then activates transcription, leading to the translatation of proteins necessary for the immune response. TLRs are located mainly on the cell membrane and endocytic vesicle of macrophages and dendritic cells [1].

The hypothesis for this experiment was as follows: "*deficiency of MyD88 protein in mice under GVHD condition will not sufficiently form inflammatory environments, so that the initiation of GVHD cannot take place in. Therefore, without the inflammatory environment, activation of donor-originated T cells would not be fully initiated. As a result, the donor grafts attack will be delayed because the cells have to find different ways to trigger inflammation. Therefore, the MyD88 deficient mouse will survive longer than the allogeneic control group.*" This report has focused on the relationship between GVHD and MyD88-deficient mice to investigate on the relationship between the GVHD and the innate immune system.

II. Method & Procedure

A. Mice

'C57BL/6 strain HSC (spleen and bone marrow) into BALB.B strain model' was used because it is a well-established MHC-matched GVHD model (MiHC mismatched) [2]. This will represent the allogeneic HSCT because C57BL/6 and BALB.B genetic strains are matched at MHC-loci to each other but most other background genes are mismatched [2]. Donor and recipient mice were over 6 weeks old and caged under specific pathogen-free environment. Over the duration of experiment, no more than 8 mice were kept in one cage to prevent psychological distress.

B. Induction of GVHD

There were 3 experimental groups:

i) One positive control (allogeneic HSCT) group: C57BL/6 mouse strain into BALB.B mouse strain. The purpose of this group is to see the effect of allogeneic HSCT.
ii) One negative control (Syngeneic HSCT) group: C57BL/6 mouse strain into C57BL/6 mouse strain. The purpose of this group is to see the effect of lethal irradiation.
iii) One experimental group: C57BL/6 mouse strain into MyD88 knockout. BALB.B mouse strain. The purpose of this group is to see the effect of MyD88 knockout mouse model in allogeneic HSCT.

Recipient BALB.B mice were irradiated twice with 450 rad with a 5 hours interval. Then, the C57BL/6 donor mouses spleen and femur bone marrow cell graft were injected via I.V. into the recipient BALB.B mouse. The preparation of the spleen and bone marrow cells are as follows:

1) Spleen Cell Preparation: The extracted spleens were collected in 1× PBS (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4 at 7.4 pH) solution and stored at 4 °C up to a maximum of 4 hours. In a PBS wetted 60mm dish, an autoclaved metal mesh was used to grind the spleen. Using a pipette, the spleen-PBS solution was transferred to a 15 ml tube using cotton mesh for filtration. Then sample was centrifuged (at 1500 rpm for 3 minutes) and the supernatant was removed.

2) Femur & Tibia Bone Marrow Preparation: The extracted femur and tibia were collected in 1× PBS solution and stored at 4 °C up to a maximum of 4 hours. In a cold 100 mm dish on top of ice, a 3 ml syringe was used to extract bone marrow cells from femur and tibia. Using a pipette, the bone marrow solution was transferred to a 15 ml tube using cotton mesh for filtration. The sample was centrifuged (at 1500 rpm for 3 minutes) and the supernatant was removed.

3) Donor Graft Preparation: The total volume of graft injection was adjusted to 300 μl per mouse using 1× PBS. The graft injection was calculated and adjusted so that each mice received 2×10^7 spleen cells and 5×10^6 femur and tibia bone marrow cells.

III. Results

The Kaplan-Meier curve of mouse survival in Fig. 1 shows that the allogeneic control group declined most rapidly among three experimental designs. At day 21, less than 20% of the mice survived. The same graph also shows that the survival rate of the MyD88 deficient group declined almost as slowly as the syngeneic control group. At day 21, more than 70% of the mice survived. The Weight Change graph in Fig. 1 shows that the MyD88 deficient group and allogeneic control group had similar weight change.

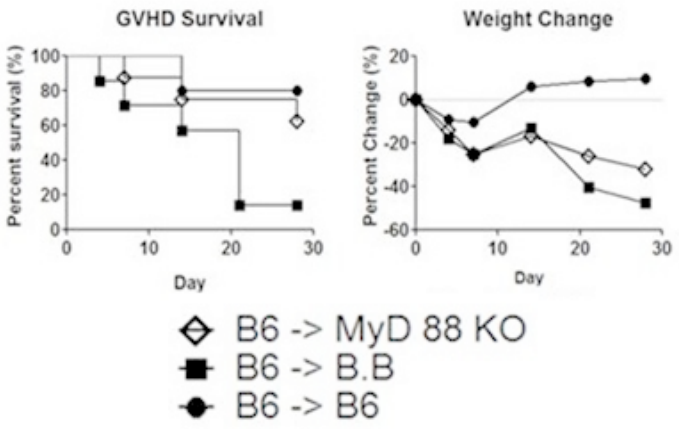

Fig. 1. Kaplan-Meier curve of mouse survival (titled: GVHD Survival) and weight change of the 3 experimental groups over 28 days. Due to the fast injection of the donor graft, a mouse in the allogeneic control group died on the first day.

Generally, graphs show that fractions rapidly increase until day 4, then, either rapidly decreased or maintained its fraction except for the macrophage and the granulocytes. The Macrophage Change graph and Granulocyte Change graph in Fig. 2 show that they continued to increase rapidly and reached the highest fraction (90%) by day 7. The macrophages, granulocytes, dendritic cells, B cells, and CD4+ T cells showed distinct difference in positive and negative control groups.

The Dendritic Cell Change graph in Fig. 3 shows an unusual fraction change. The dendritic cells in the MyD88 deficient group mice expectedly showed fraction change patterns as similar to allogeneic control group until day 14. But after day 14, the dendritic cell fraction notably increased and eventually reached at similar fraction as the syngeneic control group.

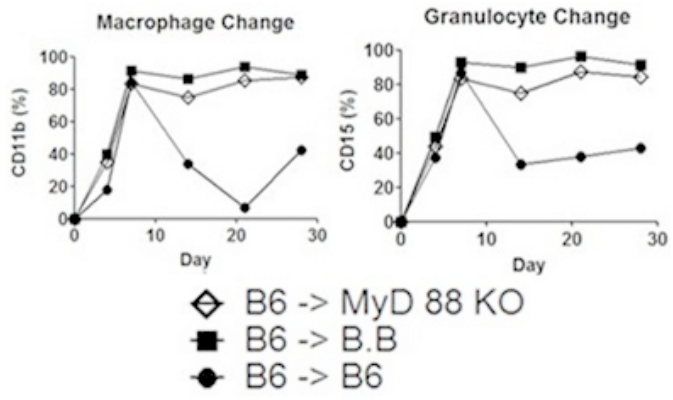

Fig. 2. Change in the fraction of innate immune cells. Each graph shows the change that occurred in three different experimental groups over 28 days using FACS analysis. The result of 8 mice in each experimental group was collected in to a solution together to acquire the mean value.

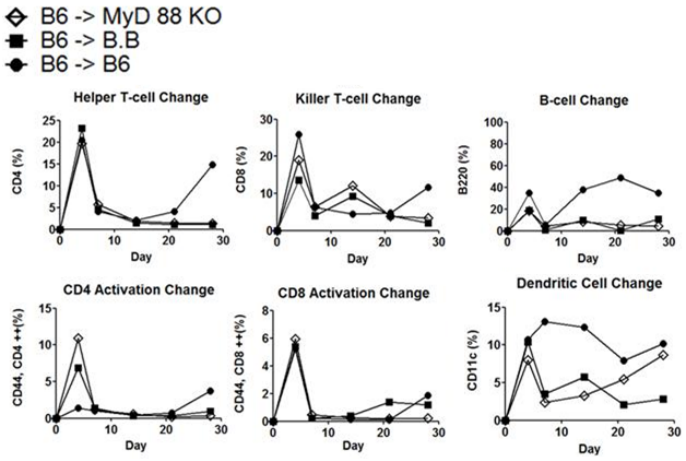

Fig. 3. Change in the fraction of adaptive immune cells. Each graph shows any changes that occurred in three different experimental groups over 28 days using FACS analysis. The result of 8 mice in each experimental group was collected in to a solution together to acquire the mean value.

IV. Discussion

The hypothesis was supported, however the data show there are more factors that need to be considered. For instance, the MyD88 knockout group lived longer than the allogeneic control group. This pattern suggests that MyD88 deficient mice must hold mechanisms that keep them alive longer but still be severely affected by GVHD.

MyD88 is largely expressed in macrophages and dendritic cells [1]. It functions to send signals to transcription factors in the nucleus to stimulate the production and secretion of inflammatory signals [1]. Although most granulocytes do not normally express MyD88 proteins, granulocytes do play a wide range of roles during inflammation. Therefore it is logical to see the macrophages and the granulocytes in allogeneic HSCT groups (Fig. 2) maintain very high fraction as shown in our results **??**. We see that the high fraction of both B cells and T cells demonstrate that the inflammatory site activity is much higher in the allogeneic HSCT models than in the syngeneic models due to the GVHD effect.

Macrophages have TLR receptor and MyD88 protein that can activate MyD88-dependent pathway. The main job of macrophages is to clean up after metabolic or immune activity via phagocytosis. Macrophages phagocytose pathogens and waste products after apoptosis or necrosis. Granulocytes (including all: neutrophil, basophil, eosinophils, mast cell, and natural killer cells) act in many different roles in creating inflammatory sites [1]. Noting the above two characteristics of macrophages and granulocytes, we propose that the high fraction of macrophages and granulocytes show high incidence of inflammation throughout the body. The fraction of the innate immune cells which generate inflammatory sites in allogeneic HSCT group is about 45% higher than in syngeneic HSCT group, which strongly suggests that innate immunity is dominant in allogeneic HSCT during GVHD.

The MyD88 deficient group shows a unique pattern in the dendritic cell fraction that is different than the allogeneic HSCT control group. The fraction of dendritic cell in the MyD88-deficient group is much higher in quantity than in the allogeneic HSCT control group. But, we do not anticipate that this pattern possesses any importance because the fraction differences between all three groups are very small anyways. However some counter-opinion may argue that as dendritic cells are known to act as messenger cells, it is realistically possible that the small quantity of dendritic cells can result in significant consequences to the whole immune system. That is why the importance of this fraction pattern needs to be confirmed in future experiments. Once biochemistry of this pattern is explained in future experiments, more in-depth understanding of the relationship between the innate immune system and the adaptive immune system will be gained.

B cells play an important role in adaptive immunity by producing antibodies [5]. These antibodies are widely used for immune attack via neutralization of epitopes, direct necrosis or signalling indicators to other attacking cells [5]. The fraction of B cells in the syngeneic HSCT group is almost 5 fold greater than the allogeneic HSCT group. This is the opposite outcome in comparison to expected outcome and warrants investigation in future studies.

The results described in this paper present important and strong evidence to show that innate immune response is highly active and a dominant factor in GVHD. However, the design of this experiment could not provide reasoning for the dendritic cell and B cell fraction patterns observed in GVHD. Future experiments should investigate the reason for the B cell fraction being very high in the syngeneic HSCT group but not in the allogeneic HSCT group. As well, the reason for MyD88 deficient groups dendritic cells fraction pattern to change from being similar to allogeneic control group into being more-like syngeneic control group.

V. Conclusions

Our data provided evidence to suggest that during GVHD, the number of the innate immune cells dominantly outnumbers the adaptive immune cells. Therefore, it is more accurate to say that the innate immune system is more important than the

adaptive immune system during GVHD. Our results propose that the innate immune strategies (inflammation without T-cell involvement) are most influential in GVHD. When the MyD88 protein is absent in GVHD, the adaptive immune system tries to compensate for the loss of its messenger function by producing more dendritic cells. But, we presumed that increased dendritic cell is a very minor effect and has a minor contribution to the outcome of GVHD. On the contrary, the cells that show significant contribution to the outcome of GVHD are: macrophages, granulocytes (neutrophils, eosinophils, basophils, mast cell) and B cells. This report has identified and is suggesting several possible research directions to study GVHD within MyD88 deficiency. No specific immune mechanism regarding MyD88 deficiency was found in this report. The future experiments should be focused on investigating cytokine secretion in blood or different organs because the study of cytokines can tell us the intensity and location of inflammation. The results found in this experiment have offered a new perspective in thinking about GVHD; in particular, that it is possible to utilize the innate immune system to suppress GVHD.

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