Conference Paper

Immune Regulation and Oxidative Stress Reduction by Preimplantation Factor following Syngeneic or Allogeneic Bone Marrow Transplantation

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Bone marrow transplantation (BMT), a well-established treatment for hematological diseases, is frequently hampered by graft-versus-host disease (GVHD) and/or by infections due to delay in immune restoration. Preimplantation Factor (PIF) is an embryo-derived peptide whose physiological function is to regulate local and systemic immunity and promote transplant acceptance. Synthetic PIF’s effectiveness to regulate immune response following BMT was herein examined in murine model. PIF administration reduced GVHD following allogenic BMT, decreased skin, liver, and colon inflammation and down regulated GVHD-associated gene expression in the liver. iNOS gene expression was reduced both in liver and colon. In syngeneic BMT, PIF administration reduced proinflammatory genes expression and promoted mice weight recovery up to two months after transplantation. PIF immune-regulatory effects were mediated via interaction with monocytes, resulting in decreased iNOS expression and NO secretion in-vitro. Overall, we demonstrate that by regulating immune response after BMT, PIF reduces inflammation and oxidative stress, leading to transplant success.

1. Introduction

Bone marrow transplantation (BMT) is a well-established treatment for malignant and nonmalignant hematological diseases and is performed in thousands of patients every year. Two major types of BMT are currently performed: autologous and allogeneic. Autologous refers to patients serving as their own donor. Allogeneic refers to someone else serving as the donor, and therefore, HLA matching between the donor and the recipient is required [1].

Allogeneic transplantation comes with a risk of graft-versus-host disease (GVHD) but is also associated with lower rates of malignant relapse owing to an immune-mediated graft-versus-leukemia (GVL) effect [2–4]. GVHD is a major cause of morbidity and mortality in allogeneic BMT patients, often characterized by damage to different tissues such as skin, liver, and gastrointestinal tract [5]. The early postengraftment period is associated with progressive recovery of cell-mediated immunity. Although autologous transplantation is often accompanied by more intense conditioning, immune recovery occurs more rapidly following autologous rather than allogeneic transplantation. Consequently, the infectious threat also recedes dramatically [1].

Monocytes/macrophages serve as the first line of defense against invading pathogens through generation of reactive
oxygen species, including nitric oxide (NO) and production of inflammatory cytokines such as IL-1, IL-6, TNF-α, and IFN-γ. The release of NO and inflammatory cytokines may lead to oxidative stress, systemic inflammation, and cellular dysfunction [6]. aberrant inducible nitric oxide synthase (iNOS) induction has been demonstrated to be involved in the pathophysiology of various diseases such as multiple sclerosis, colitis, and arthritis, as well as GVHD [7–10].

We view pregnancy as a perfect immune-regulatory environment and a successful transplant model. The mother's immune system accepts either a semi- or a fully-allogeneic embryo without attacking it. Immune tolerance mechanisms have been evolved during evolution to insures the maintenance of mammalian embryos [11].

Prelmplantation Factor (PIF) is an embryo-derived 15-amino acid peptide. PIF's function is to regulate local and systemic immunity. It promotes embryo implantation and placental engraftment and overall lead to acceptance, maintenance, and wellbeing of the embryo. PIF is present in viable embryo-conditioned media and in healthy pregnant maternal sera [12–14]. Synthetic PIF replicates the native peptide's biological activity [12, 15]. PIF immune-regulatory properties have been demonstrated in models of autoimmunity, including juvenile diabetes where it prevented disease while maintaining insulin production [16] and chronic neuroinflammation (EAE model), where it reversed disease/paralysis while promoting neuroregeneration [17]. PIF-imparted protection was associated with reduction in oxidative stress and protein misfolding [16].

Herein, PIF administration to regulate the immune response after both syngeneic (replicating autologous) and allogeneic BMT was examined in murine model. We report that PIF enhances recovery and protects against development of acute GVHD. PIF protective effects are mediated through interactions with monocytes, in which the reduction of iNOS expression decreases NO secretion and thereby protects against oxidative stress.

2. Material and Methods

2.1. Peptide Synthesis. Synthetic PIF (MVRKPGSANKP-SDD), scrambled PIF (GRVDPSNKMPKDIA), and conjugated PIF-FITC were obtained from Biosynthesis Lewisville, NJ USA. All peptides were purified to >95% purity documented by mass spectrometry before being tested.

2.2. Mice. C57BL/6, Balb/c, and F1 (C57BL/6xBalb/c) mice were obtained from Harlan Laboratories Ltd., Israel. The study was conducted under appropriate conditions and approved by the Institutional Animal Care and Use Committee of the Hebrew University of Jerusalem in accordance with national laws and regulations for the protection of animals.

2.3. Cell Isolation. BM mononuclear cells were collected by flushing the femur and tibia bones with Dulbecco's phosphate-buffered saline (PBS) (Biological Industries Ltd., Israel). Splenocytes were collected by crushing spleens through 70 μm screens into PBS. The mononuclear cells were isolated after centrifugation on a Ficoll-Paque gradient.

Dendritic cells and monocytes were isolated as previously described [18]. T cells were negatively selected from splenocytes using MACS separation magnetic beads (Miltenyi Biotec).

2.4. Bone Marrow Transplantation (BMT). Recipient mice underwent lethal whole-body irradiation by single dose of 1000 rads (semi-allogeneic and autologous) or 700 rads (allogeneic). A day after irradiation, the mice were reconstituted with donor bone marrow cells and spleen cells administered to the tail vein.

2.5. Clinical Examination. Mice were monitored daily for loss of weight, diarrhea, ruffled skin, and survival as previously described [19]. GVHD disease score, based on all of these factors, was calculated as well [5].

2.6. PIF Administration. PIF or PIF scrambled (PIFscr) used as control was dissolved in PBS. 1 mg/kg/day solvent peptide or PBS was administered from the same day of the BMT for two weeks using Alzet osmotic pumps (Model 1002, Durect Corp., CA, USA), implanted subcutaneously.

For cells-culture experiments, PIF was added to the medium at different concentrations.

2.7. Cell Activation and Proliferation. For mixed lymphocyte reaction (MLR), irradiated splenocytes from donor C57BL/6 mice were cultured together with splenocytes from Balb/c mouse and anti-CD3 antibody (BioLegend) for 4 days. Different concentrations of PIF were added to the culture media 24 h after the initial incubation. For co-culture experiments, BM-derived monocytes were cultured together with sorted T cells. Proliferation was measured by H3 thymidine incorporation.

2.8. Flow Cytometry Analysis. Cells were incubated with FITC-PIF conjugates 15 min prior to h incubation with antibodies against markers for immune-cell populations: anti-CD3 PE (eBioscience), anti-CD19 APC (BD Pharmingen), and anti-CD11b APC (SouthernBiotech). In blood samples, lysis buffer was added in order to eliminate red blood cells in the sample. Flow cytometry was performed using the MACSQuant analyzer.

2.9. RNA Extraction and PCR Analysis. Tissue samples and cells were collected and preserved in RNA Save (Biological Industries, Israel). RNA was extracted using the TRIzol reagent protocol (Invitrogen, CA, USA), and cDNA was synthesized. Liver cDNA from autologous and semi-allogeneic experiments was tested using “mouse inflammatory response and autoimmunity” microarray plate, and Real-Time PCR was performed according to the manuactory protocol. Data was analyzed using the SABiosciences Web-based PCR array data analysis software. cDNA from allogeneic experiments and from RAW264 cells was detected for iNOS gene transcript levels using TaqMan Gene Expression
assay kits (Applied Biosystems). Mouse HPRT-1 was used as a housekeeping gene transcript. Primers and probes were purchased from Invitrogen. Data was analyzed by StepOne software version 2.2 (Applied Biosystems).

2.10. Nitrite Measurement. RAW 264.7 macrophage cells line were cultured in DMEM medium for 4-5 days. PIF (200 nM) was added to the culture media at day 0 and/or 3 of the experiment. For cell activation, LPS was added for the last 24 h of the experiment. Nitrite accumulation, an indicator of NO production, was measured in cell culture supernatants using the Griess reagent.

2.11. Histological Analysis. Skin, liver, and colon samples were obtained from mice and fixed in 4% neutral-buffered formalin. Samples were then embedded in paraffin, cut into 10-micron thick sections, and stained with hematoxylin and eosin.

2.12. Statistical Analysis. Data of the mouse GVHD experiments and the comparison of gene expression were analyzed by using Students t-test or chi-square analysis. Data from syngeneic BMT experiments was analyzed by using ANOVA analysis. Histological score and in-vitro data were analyzed using nonparametric Mann-Whitney test. Significance was set at $P < 0.05$.

3. Results and Discussion

3.1. PIF Protects against GVHD after Semi-/Full Allogeneic BMT. GVHD is characterized by damage to different tissues like the skin, liver, and gastrointestinal tract [5] and may lead to progressive weight loss and mortality.

First, we tested the prophylactic effect of PIF on GVHD after semiallogeneic transplantation (C57BL/6 to C57BL/6 x Balb/c F1). Low dose PIF (1 mg/kg/day) administered for only 2 weeks reduced GVHD-related symptoms 4 and 6 weeks after BMT (Figures 1(a) and 1(b), resp.) and increased survival three months after therapy (Figure 1(c)). We have selected a semi-allogeneic model, since the clinic haploidentical donor (parent to child) is the final transplantation option. However, we also confirmed that PIF induced significant protective effects against more severe forms of GVHD using a fully allogeneic model (C57BL/6 to Balb/c). Similar to the results in the semi-allogeneic model, PIF significantly improves GVHD score in this severe model as well (Figure I(d)). In the semi-allogeneic model, PIF-treated mice exhibited significantly less skin ulceration (Figure I(e)) and lymphocytic infiltration into the liver (Figure I(f)) [20]. In the full-allogeneic model, histological analysis of the colon showed that PIF treatment significantly reduced ulceration (Figure I(g)) [20]. Overall our data suggests that PIF offers protection against damage in major clinical disease target organs, such the skin, liver, and gut.

3.2. PIF Down-Regulates GVHD-Associated Gene Expression after Allogeneic BMT. The decrease in lymphocytic infiltration to the liver of PIF-treated mice encouraged us to evaluate levels of inflammatory genes expression within the liver. Liver cDNA samples from semi-allogeneic transplanted mice were analyzed. In PBS control mice, the RNA level of expression was found to be increased (more than 3 fold) in 58% out of 84 inflammatory genes tested, while in PIF treated mice, only 20% of the inflammatory genes were elevated, compared with naive mice (data not shown). PIF treatment significantly prevented or reduced the upregulation of GVHD-associated cytokines and chemokines expression, as shown in Figures 2(a) and 2(b), respectively. GVHD is associated mainly with Th1 and Th17 immune response and increased levels of inflammatory cytokines [21–23]. In our model, PIF downregulated IFN-γ, IL-1β, IL-8r, and TNF-α gene expressions (Figure 2(a)) which are associated with Th1 response. Although IL-17 expression was not directly examined in the liver, PIF was found to down-regulate the expressions of IL-6, IL-23a, IL-23R, CCR4, TNF-α, IL-22, and CCL20 (Figures 2(a) and 2(b)) which are involved in the Th17 pathway. iNOS-induced NO secretion is a major element in oxidative stress-induced damage. Langrehr et al. demonstrated in 1992 that the level of NO in rat serum can serve as a marker of rejection after histoincompatible tissues transplants [24]. Subsequently, iNOS activity was associated with GVHD pathogenesis in mouse models [10, 25], and high levels of NO in the serum were described in GVHD patients [26]. In our model, among the genes that were found to be associated with liver GVHD, we found very high expression of iNOS gene in the control mice, which was almost abolished in PIF-treated mice (Figure 2(c)). Remarkably, this phenomenon was further confirmed in both liver and colon of full-allogeneic BMT model (Figures 2(d) and 2(e), resp.), where GVHD is more severe. Prevention of the marked elevation in iNOS in GVHD target organs by PIF treatment supports the premise that PIF can control this critical inflammatory pathway.

3.3. PIF Down-Regulates Inflammatory Genes Expression and Enhances Body Weight Recovery after Syngeneic BMT. Autologous BMT is most commonly used in the treatment of malignant diseases to facilitate intensive antineoplastic cytotoxic therapy. The intensive therapy may lead to systemic inflammation and acute toxicity to different organs. In addition, during the neutropenic period, the patient is exposed to a variety of infections [1]. Rapid recovery of the hematological transplant function would improve the outcome of such treatment. We examined PIF effect on lethally irradiated C57BL/6 mice following reconstitution with syngeneic bone marrow and spleen cells, as a model for autologous BMT. Our results demonstrate that PIF-treated mice recover their body weight to normal levels starting from 10 days after transplantation and during at least two months after transplantation, unlike control mice (Figure 3(a)).

Number of chemokines such as CCL2, CCL3, CCL4, CCL5, CXCL2, CXCL9, and CXCL10 are secreted from hepatic resident cells and participate in the direction of inflammatory response in the liver [27]. PIF treatment prevented the elevated liver expression of some of these chemokines and the inflammatory cytokine IL-1β (Figure 3(b)). For example,
PIF reduced the expression of CCL4 and CCL5 which are inducers of acute liver injury. Moreover, PIF reduced the expression of CXCL2, which together with IL-1β causes neutrophil chemotaxis, leading to ROS release and hepatocyte necrosis [27]. Interestingly, the level of CCL2 was not decreased upon PIF treatment. Although this chemokine is associated with liver inflammation, a regulatory function and hepatoprotective effect via resolution of inflammation were previously suggested [27].

Improved body weight recovery and local regulation of organ inflammation may shorten the vulnerable period after intensive conditioning and therefore reduce morbidity and
mortality following autologous BMT. These promising results encourage us to further examine the possibility of using PIF treatment for the management of radiation-induced sickness which we currently pursue as well.

3.4. PIF Regulates Immune Response through Monocytes. To gain a better understanding of PIF effect on the immune response, we examined whether PIF could also modulate allogeneic activation in-vitro. Therefore, a mixed lymphocyte reaction (MLR) assay of Balb/c and C57BL/6 splenocytes was carried out. PIF reduced proliferation in a dose-dependent manner (Figure 4(a)). In order to identify which cells mediate PIF's effect, we first examined PIF’s association with various immune cell populations using FITC-labeled PIF. We found that PIF binds mostly monocytes (CD11b\(^+\) cells) as opposed to T or B cells (CD3 and CD19, resp.), as shown in Figure 4(b). Monocytes are part of the first line of defense of the innate immunity and also serve as antigen presenting cells (APCs), which direct the adaptive immune response. Addition of PIF to the culture media of bone marrow-derived monocytes significantly reduced proliferation of activated T cells when added in co-culture (Figure 4(c)).

The expression of iNOS in activated macrophage following inflammation or infection has been well characterized and accepted as a vital component of the host's adaptive response [28, 29]. Based on our in-vivo results, we have also evaluated PIF's influence on iNOS expression in-vitro. Following PIF treatment, LPS-activated RAW 264.7

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**Figure 2:** PIF down-regulates GVHD-associated gene expression after semi/full allogeneic BMT. (a) mRNA expression of cytokines and cytokine receptors involved in liver GVHD after semi-allogeneic BMT. (b) mRNA expression of chemokine and chemokine receptors involved in liver GVHD after semi-allogeneic BMT. (c) iNOS mRNA expression in the liver after semi-allogeneic BMT. (d) iNOS mRNA expression in the liver (Mann-whitney: \(P < 0.05\)) and (e) the colon after full-allogeneic BMT.
Figure 3: PIF down-regulates inflammatory gene expression and enhances body weight recovery after syngeneic BMT. (a) Mice weight from day 0 to day 72 after syngeneic BMT. The difference between PIF and PBS treated groups is significant starting at day 11 (ANOVA: $P = 0.03$). (b) mRNA expression of cytokines and chemokines involved in liver GVHD after syngeneic BMT.

Figure 4: PIF regulates immune response through monocytes. (a) FACS analysis of bone marrow cells (upper panel) and blood cells (lower panel) stained with FITC-labeled PIF or PIFscrb and antibodies against CD3 (T-cell marker), CD19 (B-cell marker), and CD11b (monocytes and granulocytes marker). The % of specific binding is the % of PIF binding cells minus % of PIFscrb (nonspecific) binding cells; summary of 2 experiments. (b) Thymidine proliferation assay of mouse T cells in co-culture with bone marrow-derived monocytes treated with PIF; summary of 4 experiments. The results are shown as % of control. (Mann-Whitney: $P \leq 0.001$). (c) Nitric Oxide secretion from RAW cells treated/nontreated with PIF for 3 days and activated with LPS for 24 h. One representative experiment is shown out of three performed. (d) iNOS mRNA expression in RAW 264.7 cells treated/nontreated with PIF for the indicated and activated with LPS for 24 h.
macrophage cell line reduces iNOS gene expression (Figure 4(d)) and nitric oxide secretion (Figure 4(e)).

These results indicate that PIF intervention in the immune response relies principally on the influence of APC and nitric oxide secretion (Figure 4(e)). Overall, this work sets the basis for PIF treatment in allograft and autotransplantation as well as in radiation-induced disease. PIF use in a clinical setting is warranted and is in late-phase planning.

4. Concluding Remarks

In this work, we show that short-term, low-dose synthetic PIF administration, in both allogeneic and syngeneic transplantation, regulates immune response thereby preventing deleterious acute GVHD and radiation-induced symptoms. These results indicate that PIF intervention in the immune response relies principally on the influence of APC and nitric oxide secretion (Figure 4(e)).

Authors’ Contribution

Eytan R. Barnea and Reuven Or participated equally to this paper.

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PIF is a proprietary compound owned by BioIncept, LLC. Dr. Eytan R. Barnea is its (uncompensated) Chief Scientist. Dr. J. H. Barnea is its (uncompensated) President and CEO and is the majority shareholder. Hadassah Hospital, Hebrew University received unrestricted grants from BioIncept, LLC (to Reuven Or). Yale University received unrestricted grants from BioIncept, LLC (to Michael J. Paidas). Yehudith Azar, Dr. J. H. Barnea is its (uncompensated) President and CEO and is the majority shareholder. Hadassah Hospital, Hebrew University received unrestricted grants from BioIncept, LLC.

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