Published in final edited form as:

Cancer Immunol Immunother. 2010 June ; 59(6): 885–897. doi:10.1007/s00262-009-0815-3.

Maitake beta-glucan promotes recovery of leukocytes and myeloid cell function in peripheral blood from paclitaxel hematotoxicity

Hong Lin,

Cellular Immunology Laboratory, Division of Hematology/Oncology, Department of Pediatrics, Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10065, USA

Elisa de Stanchina,

Antitumor Assessment Core Facility, Memorial Sloan-Kettering Cancer Center, New York, NY, USA

Xi Kathy Zhou,

Division of Biostatistics and Epidemiology, Department of Public Health, Weill Medical College of Cornell University, New York, NY, USA

Feng Hong,

Integrative Medicine Service, Memorial Sloan-Kettering Cancer Center, New York, NY, USA

Andrew Seidman,

Breast Cancer Medicine Service, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY, USA

Monica Fornier,

Breast Cancer Medicine Service, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY, USA

Wei-Lie Xiao,

Department of Biological Sciences, Lehman College, City University of New York, New York, NY, USA

Edward J. Kennelly,

Department of Biological Sciences, Lehman College, City University of New York, New York, NY, USA

Kathleen Wesa,

Integrative Medicine Service, Memorial Sloan-Kettering Cancer Center, New York, NY, USA

Barrie R. Cassileth, and

Integrative Medicine Service, Memorial Sloan-Kettering Cancer Center, New York, NY, USA

Susanna Cunningham-Rundles

Cellular Immunology Laboratory, Division of Hematology/Oncology, Department of Pediatrics, Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10065, USA

Susanna Cunningham-Rundles: scrundle@med.cornell.edu

Abstract

Bone marrow myelotoxicity is a major limitation of chemotherapy. While granulocyte colony stimulating factor (G-CSF) treatment is effective, alternative approaches to support hematopoietic recovery are sought. We previously found that a beta-glucan extract from maitake mushroom *Grifola frondosa* (MBG) enhanced colony forming unit-granulocyte monocyte (CFU-GM) activity of mouse bone marrow and human hematopoietic progenitor cells (HPC), stimulated G-CSF production and spared HPC from doxorubicin toxicity in vitro. This investigation assessed the effects of MBG on leukocyte recovery and granulocyte/monocyte function in vivo after dose intensive paclitaxel (Ptx) in a normal mouse. After a cumulative dose of Ptx (90–120 mg/kg) given to B6D2F1 mice, daily oral MBG (4 or 6 mg/kg), intravenous G-CSF (80 µg/kg) or Ptx alone were compared for effects on the dynamics of leukocyte recovery in blood, CFU-GM activity in bone marrow and spleen, and granulocyte/monocyte production of reactive oxygen species (ROS). Leukocyte counts declined less in Ptx + MBG mice compared to Ptx-alone ($p = 0.024$) or Ptx + G-CSF treatment ($p = 0.031$). Lymphocyte levels were higher after Ptx + MBG but not Ptx + G-CSF treatment compared to Ptx alone ($p < 0.01$). MBG increased CFU-GM activity in bone marrow and spleen ($p < 0.001$, $p = 0.002$) 2 days after Ptx. After two additional days (Ptx post-day 4), MBG restored granulocyte/monocyte ROS response to normal levels compared to Ptx-alone and increased ROS response compared to Ptx-alone or Ptx + G-CSF ($p < 0.01$, both). The studies indicate that oral MBG promoted maturation of HPC to become functionally active myeloid cells and enhanced peripheral blood leukocyte recovery after chemotoxic bone marrow injury.

Keywords

Chemotherapy; Hematotoxicity; Paclitaxel; Beta-glucan; Bone marrow and leukocyte recovery; Hematopoietic progenitor cells

Introduction

The demonstration that accelerated dose-dense chemotherapy with sequential doxorubicin/cyclophosphamide followed by paclitaxel significantly improved clinical outcomes in breast cancer has established a new standard of care and proven the Norton–Simon hypothesis that increased frequency of cytotoxic therapy is superior to dose escalation [1, 2]. With sequential dosing, the requirement for growth factor support may be considered separately for each phase. Prophylactic granulocyte colony stimulating factor (G-CSF) is recommended with accelerated dose dense chemotherapy [3], but causes bone pain and can reduce the concentration of bone marrow progenitor cells over a significant period of time [4, 5]. Suspending the use of G-CSF growth factor support during paclitaxel treatment after the doxorubicin and cyclophosphamide components of chemotherapy has been attempted [6]. A recent study in the setting of accelerated paclitaxel treatment of early stage breast cancer showed that not giving prophylactic G-CSF was acceptable. While 40% of patients became neutropenic and 10% required secondary G-CSF, there were no treatment delays [6]. However in another study of early breast cancer treatment in which G-CSF was held during the paclitaxel phase of chemotherapy, 40% of patients did not complete therapy on time due to dose delays [7]. The patients who became neutropenic tended to be younger with a lower body surface area, to have lower absolute white blood cell (WBC) and lower absolute neutrophil counts (ANC) [7]. Paclitaxel is widely used in cancer including as first-line treatment of metastatic breast cancer [8–13]. The objective of this study was to determine if a beta-glucan extract from the *G. frondosa* mushroom that is orally active and was safely administered to breast cancer patients without inducing changes in peripheral blood counts

would stimulate hematopoiesis and enhance recovery from paclitaxel in a mouse model of dose-intensive chemotherapy [14].

Beta-glucans are naturally occurring polysaccharides with distinctive beta 1,3 linked and beta 1,6 linked glucose polymers that are expressed by fungi, plants including cereals, grains, mushrooms, and some bacteria. Beta-glucans are not expressed on mammalian cells and are recognized as pathogen-associated molecular patterns (PAMPS) by several types of pattern recognition receptors [15]. For leukocytes the primary receptor for beta-glucan is the C-type lectin receptor dectin-1 [15, 16]. Ligation of this receptor can trigger phagocytosis, production of cytokines and chemokines, and activation of effector cell functions according to the cell type and specific properties of the beta-glucan compound. Complement receptor 3 (CR3 or Mac-1) can also recognize beta-glucan and is involved in complement mediated hematopoietic recovery [17] and antitumor effects [18, 19]. After bone marrow injury due to chemotoxicity or radiation, CR3 and dectin-1 expression were increased on bone marrow cells and treatment with a beta-glucan enhanced both dectin-1 expression and hematopoietic recovery [17, 20].

Beta-glucans protect against myelotoxic injury from radiation and chemotherapy [21, 22]. Intravenous administration of PGG-glucan (poly 1-6 beta-D-glucopyranosyl 1,3-beta-glucopyranose) to mice after cobalt-60 radiation, enhanced recovery of bone marrow cellularity and CFU-GM activity [21]. PGG-glucan increased the levels of stromal cell-derived factor alpha (SDF-1 alpha) in plasma but not in bone marrow thereby modulating the SDF-1 gradient [23]. We reported that a beta-glucan extract from maitake mushroom, *G. frondosa* (MBG) enhanced CFU-GM activity of mouse bone marrow and human cord blood, directly stimulated neonatal monocyte production of G-CSF and spared both mouse and human hematopoietic progenitor cells (HPC) from doxorubicin toxicity in vitro [24, 25]. MBG directly enhanced HPC expansion ex vivo and promoted homing and engraftment of CD34+ cord blood cells in the nonobese diabetic/severe combined immunodeficient (NOD/SCID) mouse model of transplantation [26].

Few previous studies have examined the hematotoxic effects of paclitaxel (Ptx) in vivo in experimental models and none have assessed the dynamics of leukocyte recovery in peripheral blood by direct measurement, although this is a primary clinical correlate. To determine if MBG would hasten leukocyte recovery from Ptx in vivo, B6 D2F1 mice were given dose-fractionated Ptx to produce acute myelo-suppression without significant weight loss or morbidity comparable to clinical use. We compared orally administered MBG given during and after chemotherapy to Ptx treatment alone and to G-CSF treatment after Ptx. Leukocyte, erythrocyte, and platelet dynamics were studied in peripheral blood by daily enumeration of blood cell populations. CFU-GM colony forming activity of bone marrow and spleen and peripheral blood myeloid cell functional activity were also assessed.

Materials and methods

Chemicals and reagents

Maitake mushroom beta-glucan (MBG), also known as D fraction, is an extract from fruit body of maitake mushroom (*Grifola frondosa*), made by patented methods (Japan Pat. No. 2859843/US Pat. No.5, 854, 404) provided by Yuikiguni Maitake Corporation through the Tradeworks group, characterized by a 1,6 main chain with 1,3 branches. MBG was stored at 4°C in the dark until use. The lot of MBG used here was tested by NAMS for endotoxin by limulus amoebocyte lysate (LAL) assay and none was found (limit of detection = 0.012 EU/mg, equivalent to medium). MBG was dissolved in RPMI 1640 with 25 mM HEPES buffer and prepared at a concentration of 20 mg/mL, sterilized by filtration through 0.2 µm cellulose acetate low protein binding membrane and stored at -20°C. The stock solution was

diluted in RPMI 1640 for use. Pharmaceutical grade Paclitaxel (Ptx) was obtained from Mayne Pharma, USA. Neupogen (G-CSF) was from Amgen (Thousand Oaks, CA).

MBG characterization, composition, and purity

The fruit bodies of dried *G. frondosa* were extracted with distilled water at 121°C, and the resulting aqueous extraction was precipitated by adding ethanol for a final concentration of 45% (v/v), and after standing at 4°C for 12 h, the precipitates were removed by filtration. Additional ethanol was added to the filtrate for a final concentration of at least 80% (v/v) the solution was allowed to stand at 4°C and the resulting precipitate (MBG), was dark brown to black in color. MBG is a glucan/protein complex deduced by the positive response in anthrone reaction and ninhydrin reaction and the glucan/protein ratio was 96:4. The molecular weight is distributed around 1,000,000z as determined by gel filtration chromatography on a TSK gel GMPW.sub.XL column. The protein moiety was characterized by an automatic amino acid analyzer, as consisting of glutamic acid, aspartic acid, alanine, leucine, lysine, glycine, isoleucine, serine, valine, proline, threonine, arginine, phenylalanine, tyrosine, histidine, methionine, and cysteine.

Glycosyl composition analysis was performed by combined gas chromatography/mass spectrometry (GC/MS) of the per-*O*-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides produced from the sample by acidic methanolysis. The experiment was performed on an HP 5890 GC interfaced to a 5970 MSD, using a Supelco DB5 fused silica capillary column. Methyl glycosides were prepared by methanolysis in 1 M HCl in methanol at 80°C (18–22 h), followed by re-*N*-acetylation with pyridine and acetic anhydride in methanol. The samples were then per-*O*-trimethylsilylated by treatment with Tri-Sil (Pierce) at 80°C (0.5 h). These procedures were the same as described [27, 28]. The glycosyl composition consists of glucose, galactose, and mannose, at the ratio of 96.2, 1.5 and 2.3%, respectively, expressed as mole percent of total carbohydrate, Trace amount of ribose was also detected.

Mice—B6D2F1 mice female, 6–8 weeks old were purchased from Jackson Laboratory, and were maintained in a pathogen-free facility at Memorial Sloan-Kettering Cancer Center (MSKCC, New York, NY) with access to fresh water and food ad libitum. Certified Rodent Diet #5053 (Lab Diet) was used. All animal experiments were approved by the MSKCC Animal Care Committee.

Treatments—Ptx was intraperitoneally injected (i.p.) at 30 mg/(kg day) over 3–7 days with cumulative dose of 90–120 mg/kg to mice. Neupogen (G-CSF) was given i.v. at 80 µg/kg at 24 h after the last dose of Ptx. MBG was administered orally at 4 mg/(kg day) or 6 mg/(kg day) (about 100–150 µg/mouse) by gavage on every experimental day in the Ptx + MBG groups. Mice were weighed each day. Blood was taken from the tail vein for complete blood cell (CBC) counts. Sacrifice was performed by the CO₂ method.

Effect of MBG on growth of MCF-7 tumor cell line and paclitaxel cytotoxicity—

Before undertaking studies to evaluate the effect of MBG on response to paclitaxel, we determined that MBG did not stimulate or inhibit the growth of tumor cell lines or affect the response to chemotherapeutic drugs [25]. Supplementary Figure 1 shows that MBG did not affect the viability of MCF-7 or inhibit the cytotoxic effect of paclitaxel.

Complete blood cell count—Approximately 20 µL of blood was taken from the tail vein after warming, for assessment of CBC by automated differential analysis using the Hemavet instrument after instrument standardization.

Bone marrow—Mouse bone marrow cells were collected as previously described [24]. Briefly, mouse bone marrow cells were collected from femoral shafts by flushing with 3 mL of cold RPMI-1640. The cell suspensions were passed up and down six times through an 18-gauge needle in RPMI-1640 to disperse clumps. Adherent bone marrow cells were removed after incubation at 37°C, 5% CO₂ for 24 h in RPMI-1640 containing 20% FBS, and non-adherent cells were collected and used as described.

Spleen—Mouse SP cells were collected with smearing between two sterile glass-slides a few times in ~3 mL RPMI-1640 medium. Cell clumps were dispersed as described above.

Colony forming unit (CFU-GM) assays—The colony-forming assay was carried out under defined conditions (StemCell Technologies Inc., Vancouver, Canada) as described previously [24]. Briefly, bone marrow cells were placed in premixed methylcellulose culture medium (Methocult M3234, StemCell Technologies Inc.; Vancouver, Canada). Final adjusted concentrations were 1% methylcellulose, 15% FBS, 1% BSA, 10 µg/mL insulin, 200 µg/mL transferrin, 10⁻⁴ M 2-mercaptoethanol and 2 mM L-glutamine. Recombinant murine IL-3 (InterGen Company, Purchase, NY), and recombinant human G-CSF (Neupogen, AMGEN, Thousand Oaks, CA) were added at 10 ng/mL and 500 ng/mL. The bone marrow cell (5 × 10⁵ cells/mL, 0.3 mL), or spleen cell (2 × 10⁶ cells/mL, 0.3 mL) suspensions were added to complete mixed culture medium (2.7 mL), vortexed, and plated in Petri dishes (Falcon, Becton–Dickinson), 1.1 mL/dish. Then all cultures were incubated in a water-saturated, 37°C, 5% CO₂ atmosphere for 7 days. CFU-GM colonies of 50 or more cells were scored by inverted microscope.

Flow cytometric oxidative burst assay—Peripheral blood was obtained by retro orbital bleeding; about 400 µL blood was collected into heparinized tubes. The respiratory burst assay was performed as described [29] with modifications. Briefly, 100 µL blood aliquots were added to each tube, wash buffer or stimuli (opsonized *E. coli*, fMLP (*N*-formylmethionyl-leucyl-phenylalanine) were added at 20 µL. Tubes were incubated in a water bath at 37°C for 10 min, then 20 µL of dihydrorhodamine (DHR-123) was added and tubes were vortexed, and then incubated for another 10 min. Red blood cells were lysed and removed after washing once. R-PE conjugated rat anti-mouse Ly-6G and Ly-6C (Gr-1, BD Biosciences) antibody was added in the presence of Mouse BD Fc Block (purified anti-mouse CD16/CD32 monoclonal antibody, 2.4G2, BD Biosciences) at 1 µg/million cells, and incubated at room temperature in the dark for 15 min. Cells were washed; supernatants removed and 200 µL of DNA staining solution was added. The samples were analyzed by flow cytometer (FACSCalibur) using CellQuest. The initial gate was set with Gr-1 to identify granulocytes/monocytes; the percentage of responding cells was then analyzed with FlowJo software.

Statistical analysis—Data are presented as mean percentage ± SD or mean counts ± SD. To identify the appropriate dose of Ptx, ANCOVA was used to examine significance of variation in average cell counts or percentage of change from baseline across all days for the cell type of interest in a treatment group and possible within-mouse correlation was adjusted by including mouse ID as a covariate. If overall significance by ANCOVA was shown, Tukey's method was used to determine between-day significance. To assess significance of variation across dose groups (including untreated) over the treatment interval, two-way ANOVA was used and then Bonferroni method was used to adjust for between-group multiple comparisons. In the studies of blood cell recovery, we calculated the decrease in cell count compared to the corresponding baseline during the treatment for each mouse. The averages of the group decreases across different treatment groups were compared using two-way ANOVA to determine significance. Two sample *t*-tests were used to compare the

difference in average percentage change at a given time point between any two treatment groups. Tukey's or Bonferroni method was used to adjust p-values for multiple comparisons where appropriate. The analyses were carried out using GraphPad Prism software version 5.01. Additional information is provided in the text.

Results

Paclitaxel-induced leukopenic mouse model

To determine the dose of Ptx required for induction of acute hematotoxicity that also permitted spontaneous recovery, fractionated dosing at 10–30 mg/kg was given three times every other day to groups of mice. After doses greater than 15 mg/kg were found effective, we compared cumulative doses of 60 and 90 mg/kg to untreated mice ($n = 4$ each group). Serial CBCs were obtained from each mouse. Mean absolute numbers of leukocytes, neutrophils, and lymphocytes are shown in Fig. 1. Variation was evaluated by one-way ANCOVA and within mouse correlation was included as the covariate. Pair-wise differences were evaluated by Tukey's post-test. Treatment group variation was assessed by two-way ANOVA over the interval followed by between-group comparisons using Bonferroni post-tests. As shown in Fig. 1, leukocyte, neutrophil, and lymphocyte numbers did not decline in the untreated group.

Leukocytes—A cumulative Ptx dose of 90 mg/kg, but not 60 mg/kg, produced significant changes in leukocyte numbers (one-way ANCOVA; $p < 0.0001$). Compared to baseline, leukocyte numbers after 90 mg/kg Ptx were lower on day 4 ($p < 0.05$), day 5 ($p < 0.01$) and 3 days after Ptx was stopped on day 8 ($p < 0.05$) by Tukey's post-tests. Differences across treatment groups were significant (two-way ANOVA, $p < 0.0001$). The 60 mg/kg Ptx group had fewer leukocytes on days 3, 4 ($p < 0.0001$) or day 5 ($p < 0.05$) and the 90 mg/kg Ptx group had lower leukocyte numbers on days 3, 4, and 5 ($p < 0.0001$), compared to untreated mice (Bonferroni). Rebound in leukocytes occurred later after the higher Ptx dose compared to the lower dose.

Neutrophils—After Ptx at 60 mg/kg the overall drop in neutrophil counts across all days was significant ($p < 0.01$, ANCOVA) but no pair-wise comparisons were significant. In contrast at 90 mg/kg, Ptx caused a greater overall change in neutrophil numbers ($p < 0.0001$, ANCOVA) and counts were significantly lower on days 4 and 5 compared to baseline ($p < 0.001$, $p < 0.0001$, Tukey's). Significant variation among treatment groups was observed ($p < 0.0001$, two-way ANOVA). Compared to the untreated group, neutrophils were more reduced after the higher dose of Ptx ($p < 0.001$) and the counts were still down on day 8 ($p < 0.01$).

Lymphocytes—Lymphocyte numbers varied significantly across time only after 90 mg/kg Ptx ($p < 0.001$, ANCOVA). Lymphocytes were lower on day 8 compared to baseline ($p < 0.05$, Tukey's). Overall between-group variation was also significant ($p < 0.0001$, two-way ANOVA). Lymphocyte numbers were lower over days 3, 4, and 5 ($p < 0.001$) after 90 mg/kg compared to the untreated group and on day 8 compared to 60 mg/kg Ptx ($p < 0.001$, Bonferroni).

Effect of MBG on recovery of hematopoietic progenitor CFU-GM activity after Ptx

Recovery of peripheral blood counts after chemotherapy depends upon emergency bone marrow hematopoiesis after chemotoxic injury. To determine if MBG would enhance CFU-GM activity in bone marrow and spleen after Ptx treatment, mice ($n = 4$ each group) received four doses of Ptx at 30 mg/(kg day) every other day for a cumulative dose of 120 mg/kg. Another group received the same Ptx dose and were also given daily oral MBG at 4

mg/(kg day), starting on the first day of Ptx and throughout the experimental period. The choice of initial dose level was based on our previous study showing that 4 mg/kg was sufficient to enhance homing and engraftment of human CD34+ cells in a xenograft model [26]. Bone marrow and spleen cells were collected for CFU-GM colony forming assays performed ex vivo 2 days after the last Ptx injection. For each sample, colony counts were obtained from cultures plated in quadruplicate. Measurements from the same mouse were considered to be in the same cluster, and repeated measures ANOVA was used to compare the CFU-GM numbers in each tissue after log transformation was applied.

Figure 2a shows that Ptx + MBG treatment led to higher CFU-GM activity in both bone marrow and spleen compared to Ptx alone ($p < 0.001$, $p = 0.002$, respectively). We then increased the dose of MBG to 6 mg/(kg day) with the same Ptx regimen. As shown in Fig. 2b, bone marrow from Ptx + MBG treated mice had greater CFU-GM activity compared to Ptx alone ($p = 0.003$). Compared to Ptx-alone, Ptx + MBG at 6 mg/kg also increased CFU-GM activity in the spleen, but differences were not statistically significant ($p = 0.07$) due to greater variation within the group and smaller sample size.

Effect of MBG and G-CSF on dynamics of leukocyte recovery after Ptx treatment

Since MBG enhanced bone marrow and spleen CFU-GM activity after Ptx, we addressed the relationship to the dynamics of peripheral blood leukocyte recovery. Treatment with Ptx + MBG or Ptx plus G-CSF was compared to Ptx-alone in three groups ($n = 6$, each group). 30 mg/(kg day) Ptx was given over three consecutive days (90 mg/kg cumulative dose) to each group. Mice given Ptx + MBG received 6 mg/(kg day) orally each day from day 1 of chemotherapy and daily thereafter. The Ptx + G-CSF group received one dose of intravenous G-CSF (80 μ g/kg), 24 h after the last Ptx dose. Changes in absolute numbers of leukocytes (white blood cell count) from baseline were evaluated from post-day 1 to post-day 8 after chemotherapy. For each mouse, percent change in counts from baseline was calculated. Change from baseline across days within a treatment group was assessed by ANCOVA and within-mouse correlation was included as appropriate. Paired t -tests were used to compare any two time-points. Differences among treatment groups across the experimental period were assessed by two-way ANOVA followed by two sample t -tests for pair-wise comparisons. Tukey's or Bonferroni methods were used as appropriate.

Leukocytes—Treatment with 90 mg/kg Ptx-alone caused a marked reduction in leukocyte numbers. Overall changes from baseline were significant ($p < 0.0001$ by ANCOVA). As shown in Fig. 3a, numbers declined by 70% on post-day 1. Differences compared to baseline were significant for post-days 1–4 ($p < 0.0001$). Counts improved by post-day 5 and 8 compared to post-day 1 ($p < 0.001$) but were not restored to baseline levels.

Leukocyte numbers were also reduced in both Ptx + MBG and Ptx + G-CSF groups. Change from baseline was significant across time for both ($p < 0.0001$). Leukocytes were lower over the first four post-Ptx days for both groups but recovery began earlier compared to Ptx-alone. For the Ptx + MBG group, leukocytes were reduced on post-days 1–3 ($p < 0.01$), or 4 ($p = 0.05$) compared to baseline. By post-day 5, levels increased compared to post-days 1–3 ($p < 0.005$), or 4 ($p < 0.03$). For the Ptx + G-CSF group, leukocytes were reduced through post-day 4 with average counts below baseline. Compared to baseline, counts were lower on post-day 1 and 2 ($p < 0.0001$), 3 ($p < 0.001$), and 4 ($p < 0.05$). After Ptx + G-CSF, leukocyte numbers increased by post-day 5 compared to post-day 1 and 2. By post-day 8, the average count was significantly higher compared to day 1, 2, 3 ($p < 0.0001$), 4 ($p < 0.001$) or 5 ($p = 0.04$).

Comparison among the treatment groups showed that the maximum decrease (nadir) in leukocytes was least in the Ptx + MBG group. Differences were significant compared to Ptx-

alone ($p = 0.024$) and to Ptx + G-CSF ($p = 0.031$). As shown in Fig. 3b, on post-day 2 the effect of G-CSF was not observed, while the ameliorating effect of MBG on Ptx treatment was evident. The decline in leukocytes after Ptx + MBG was less than after Ptx-alone ($p < 0.05$) or Ptx + MBG ($p < 0.01$). As shown in Fig. 3c, by the eighth day after Ptx treatment, mice in both the Ptx + MBG group and the Ptx + G-CSF group had higher leukocyte counts compared to Ptx-alone ($p < 0.001$).

Neutrophils—Neutrophil numbers declined sharply from baseline following Ptx treatment alone. The overall change from baseline was significant ($p < 0.0001$, ANCOVA) as shown in Fig. 4a. The mean decline from baseline was 87% at post-day 1 ($p < 0.0001$, Tukey's). By post-day 5, recovery had begun and neutrophil levels were higher compared to post-days 1, 2, and 3 ($p < 0.05$). Baseline levels were achieved by post-day 8.

Neutrophil counts declined in the Ptx + MBG group. The change from baseline was significant ($p < 0.0001$ by ANCOVA) and neutrophil levels were reduced compared to baseline on post-treatment days 1–4 ($p < 0.05$, Tukey's). By post-day 5 average neutrophil counts had recovered to baseline level and were higher compared to post-day 1–3 ($p < 0.05$) or 4 ($p = 0.09$). In the Ptx + G-CSF group, neutrophil numbers showed significant overall variation ($p < 0.0001$, ANCOVA). By post-day 5, the neutrophil level was greater than on post-day 1 or 2 ($p < 0.05$).

Comparison of average neutrophil counts across treatment groups showed significant variation across the 8-day period ($p < 0.0001$, two-way ANOVA). We also compared the treatment groups over the period of early recovery from chemotherapy (baseline to day 5). Average neutrophil counts varied significantly ($p < 0.0001$, two-way ANOVA). The decrease in neutrophil level in the early recovery period was greater in the Ptx-alone group compared to either Ptx + G-CSF ($p < 0.01$) or Ptx + MBG ($p < 0.05$) treated groups on post-day 5. See Fig. 4b. On the eighth post-Ptx day, as shown in Fig. 4c, the neutrophil counts in the Ptx + MBG group were higher compared to Ptx-alone ($p < 0.04$), and comparable to Ptx + G-CSF treatment.

Lymphocytes—Lymphocytes were reduced by more than 50% in all treatment groups after chemotherapy. Overall change from baseline was significant in the Ptx-alone group ($p = 0.001$, ANCOVA). Decline from baseline was significant on post-days 1, 2 ($p < 0.001$, Tukey's) and 3, 4 ($p < 0.01$). While neutrophils were increased on post-day 5 compared to post-day 1 or 2 ($p < 0.01$), baseline levels were not achieved, even by post-day 8 in the Ptx-alone group. See Fig. 5.

Lymphocytes declined on post-day 1, 2, or 3 when compared to baseline in the Ptx + MBG group ($p < 0.001$, $p < 0.02$, respectively, Tukey's). By post-day 4 lymphocytes were not statistically different from baseline. By post-day 5, levels were higher than on post-days 1–4 ($p < 0.0001$, Tukey's). After Ptx + G-CSF, lymphocyte recovery began on post-day 4, and by post-day 5 these levels were higher compared to post-days 1–3 ($p < 0.05$, Tukey's) or 4 ($p = 0.06$). Overall differences in lymphocyte numbers across treatment groups were also significant ($p < 0.01$, two-way ANOVA). Lymphocyte levels were higher in the Ptx + MBG group but not the Ptx + G-CSF treated group compared to Ptx alone on post-day 5 ($p < 0.01$, Bonferroni).

Effects of Ptx, MBG and G-CSF on monocytes, erythrocytes, and platelets

The relative effects of MBG and G-CSF compared to Ptx-alone on peripheral blood monocytes, erythrocytes, hemoglobin and platelets counts were also examined, and differences were found.

Monocytes—Monocyte numbers declined in all treatment groups but were restored by post-day 8. Overall percent change from baseline across the experimental period was significant for all ($p < 0.0001$, ANCOVA). For both Ptx-alone and Ptx + MBG groups, the lowest point occurred on post-day 3 when monocyte counts were $77.6 \pm 13\%$ below baseline for Ptx-alone and $84.9 \pm 7.3\%$ below baseline for the Ptx + MBG group. For the Ptx + G-CSF group, counts were lowest on post-day 2 at $90.5 \pm 4.0\%$ below baseline. Variation among treatment groups across was significant ($p < 0.0001$, two-way ANOVA). Monocyte levels were more suppressed in the Ptx + G-CSF group compared to the Ptx-alone group on post-day 4 ($p < 0.001$ and post-day 5 ($p < 0.01$, Bonferroni).

Erythrocytes and hemoglobin—For all groups, the maximum drop in mean absolute RBC counts occurred on post-day 5 and levels did recover to baseline. Variation in average absolute erythrocyte number was significant for each treatment group. ($p < 0.0001$). The baseline mean absolute RBC count in the Ptx-alone group was 10.4 ± 0.3 K/ μ L, dropped to 5.1 ± 1.7 K/ μ L at post-day 5 ($p < 0.0001$), and rose to 7.9 ± 0.9 K/ μ L by post-day 8. The baseline RBC count in the Ptx + MBG group was 11.1 ± 0.4 K/ μ L, and fell to 5.6 ± 1.0 K/ μ L at post-day 5 ($p < 0.0001$) but improved to 7.9 ± 0.5 K/ μ L on post-day 8. The Ptx-G-CSF group's pretreatment RBC count was 11.3 ± 0.3 K/ μ L, declined to 4.8 ± 1.1 K/ μ L ($p < 0.0001$) on post-day 5 and was 7.4 ± 1.0 K/ μ L on post-day 8. The baseline hemoglobin was similar in all groups (14.6 ± 0.4 g/dL in Ptx-alone; 15.53 ± 0.5 g/dL in Ptx + MBG; 15.2 ± 0.9 g/dL in Ptx + G-CSF) and dropped to a nadir of about 8 g/dl in all groups on post-day 5.

Platelets—The changes in platelet numbers were normalized based on each individual baseline count. Platelets increased within in all groups after Ptx; overall variation in percent change from baseline across time after Ptx treatment was significant within each group ($p < 0.0001$). In the Ptx-alone group, platelet levels increased to $76.5\% \pm 25.6$ above baseline at post-day 3 ($p < 0.001$, Tukey's). For the Ptx + G-CSF group mean platelet levels were always significantly above baseline after post-day 3 ($p < 0.001$, Tukey's) and in the Ptx + MBG group after day 1 ($p < 0.001$, Tukey's).

Recovery of neutrophil and monocyte function after Ptx treatment

To determine the functionality of myeloid cells after Ptx treatment and the effects MBG or G-CSF treatment, we measured production of reactive oxygen species (ROS) in Gr-1+ myeloid cells. After brief exposure of whole blood to opsonized *E. coli* and the chemotactic peptide *N*-formyl-Met-Leu-Phe (fMLP) ex vivo, fluorescence of ROS positive cells was detected by the oxidation of the dihydro-rhodamine substrate. Blood samples were collected from each group 4 days after the last dose of Ptx and again on post-day 11. Figure 6a, shows the percentage of ROS+ cells in the three treatment groups on post-day 4. The responses varied significantly within each treatment group by test stimulus ($p < 0.0001$, ANOVA, each group). The responses of the three treatment groups showed across-group variation (two-way ANOVA, $p < 0.001$). The response to *E. coli* was stronger in the Ptx + MBG group compared to the Ptx-alone group or to the Ptx + G-CSF group ($p < 0.01$ for each, Bonferroni post-test). Response to fMLP was stronger in the Ptx + MBG group compared to the Ptx + G-CSF group ($p < 0.05$). Post-day 4 was 3 days after G-CSF injection for the Ptx + G-CSF group. The ROS responses of normal, untreated mice were also studied. As shown in Fig. 6a, untreated mice produced a significantly greater ROS response to *E. coli* than did mice treated with Ptx alone or with Ptx + G-CSF ($p < 0.01$ for both). In contrast ROS response in the Ptx + MBG group was equal to that of untreated mice. Interestingly the fMLP response at post-day 4 was higher in the Ptx + MBG group compared to normal untreated mice ($p < 0.01$).

On post-day 11 the ROS response to *E. coli* was equivalent in all treatment groups. For each of the treatment group significant variation across the two time points was observed ($p < 0.001$, one-way ANOVA for each). See Fig. 6b. The ROS response to *E. coli* increased ($p < 0.0001$) on post-day 11 compared to 4, while response to fMLP decreased ($p < 0.0001$) in the Ptx-alone group. Unstimulated ROS production was unchanged. For both Ptx + MBG treated and Ptx + G-CSF groups, the percentage of ROS producing cells also increased on post-day 11 compared to post-day 4 ($p < 0.0001$) in response to *E. coli*. However, responses to fMLP varied overall across the groups ($p < 0.01$ one-way ANOVA). The Ptx + MBG treated group showed a higher response to fMLP compared to both the Ptx-alone group and the Ptx + G-CSF group ($p < 0.05$ Tukey's). See Fig. 6b. The ROS response of untreated mice to *E. coli* was now much lower compared to each of the three treatment groups ($p < 0.0001$).

Discussion

Paclitaxel was discovered in the National Cancer Institute (NCI) screening program as a natural product extract from the Pacific yew tree, *Taxus brevifolia*, with activity against a broad range of tumor types [30], especially breast, ovarian, and lung cancer. Ptx lacks cumulative toxicity [31] and is widely used both for anti-tumor activity and mobilization of peripheral blood stem cells in cancer patients [32]. Although the mechanism of anti-cancer effect involves induction of tubulin polymerization preventing formation of the mitotic spindle, Ptx also causes apoptosis at doses that do not affect tubulin [33, 34]. The primary toxicity of Ptx is leukopenia, mainly neutropenia [35]. Pharmacodynamic studies in the rat have shown that time course of paclitaxel exposure affected critical parameters of hematopoiesis specifically the production, maturation, and lifespan of precursor cells and mature neutrophils [36]. Related modeling studies in patients suggest that neutrophil progenitor cells remain sensitive to paclitaxel in the early maturing phase [37]. Ptx reduces mesenchymal stem cell proliferation and causes a partial arrest of these cells at the G(2) phase of the cell cycle [38]. The overall effect of Ptx treatment in the non tumor bearing host is acute hematotoxic injury that leads to stimulation of G-CSF, the major regulator of neutrophilic granulocytes [39] and to rebound leukocytosis. G-CSF and GM-CSF stimulate colony formation by primitive hematopoietic stem cells and can synergize with other growth factors such as IL-1 alpha to enhance recovery from chemotoxicity [40].

This is the first study to examine the dynamics of leukocyte recovery in peripheral blood after Ptx treatment in vivo in a normal mouse. Ptx alone led to suppression of peripheral blood white blood cell counts below baseline levels for more than 8 days. Maitake mushroom beta-glucan (MBG) or G-CSF treatment stimulated earlier recovery of leukocytes and increased the numbers of neutrophils and lymphocytes above baseline by post-Ptx day 5. Giving oral MBG throughout chemotherapy was as effective as a single dose of G-CSF given i.v. after the last dose of Ptx in reducing leukopenia in this model. Doses of G-CSF in the range of 10–250 $\mu\text{g/kg}$ have been shown to enhance peripheral blood cell counts when given to normal mice without prior chemotherapy [41, 42]. MBG enhanced hematopoietic progenitor cell CFU-GM activity 2 days after Ptx treatment and increased leukocyte recovery in peripheral blood 2 days later. Other investigators have shown that bone marrow CFU-GM content is directly linked to recovery of peripheral blood cells [43]. We showed that myeloid cell function is compromised by Ptx treatment and that MBG, but not G-CSF, treatment restored the oxidative burst response to normal levels after treatment. Clinical studies have shown that chemotherapy reduces peripheral blood leukocyte oxidative burst activity [44], and increases leukocyte apoptosis [45]. Reduced neutrophil function is associated with greater risk of infection after chemotherapy [46]. We used *E. coli* as the physiologically relevant test stimulus. Paclitaxel is known to suppress leukocyte locomotion and bacterial killing through reduction of membrane fluidity but was previously reported to

enhance ROS response after fMLP stimulation in vitro [47–50]. Our results show that Ptx significantly reduces this function in vivo and are in agreement with clinical observations [44]. Mice are known to have a large pool of functionally mature neutrophils compared to humans that are released in response to stress, [51] suggesting that Ptx treatment impairs functional maturation. MBG stimulates production of G-CSF in human cord blood cells, as we reported previously [25]. Ito et al. have shown that maitake D fraction enhanced G-CSF in the mouse and granulocyte mobilization into the spleen [52]. Clinical studies have shown that G-CSF treatment after chemotherapy does not enhance ROS activity [53]. Therefore activation of ROS activity appears to be distinct and novel property of MBG in addition to promotion of hematopoietic progenitor cell maturation and mobilization of leukocytes into the periphery.

Comparatively few investigations have used in vivo models to study hematopoiesis in both bone marrow and the peripheral blood compartments after paclitaxel chemotherapy [36, 54–56]. Compared to other studies, the doses of Ptx given here were higher (90–120 mg/kg compared to 60 mg/kg) and the period over which the doses were given was longer (three daily doses or every other day for 7 days compared to two doses over 1.5 h [54, 55, 57]. Romero-Benitez observed a greater suppressing effect on the erythroid lineage cell numbers in bone marrow compared to myeloid lineage cells [54]. We found that the maximum loss of erythrocytes in peripheral blood occurred later compared to lymphoid or myeloid cells after dose-intensive Ptx. While the recovery of myeloid cells was enhanced in the Ptx treated mice that also received G-CSF or MBG, the course and magnitude of Ptx mediated suppression of RBC numbers and hemoglobin levels were not affected. Platelet numbers were strikingly increased in all mice receiving Ptx and this was also not affected by either concurrent MBG or therapeutic G-CSF. Megakaryocytes are relatively resistant to Ptx. Pertusini et al. have reported that Ptx treatment was associated with increased serum thrombopoietin (Tpo) levels and also demonstrated that Ptx could stimulate Tpo production in bone marrow stromal cells in vitro [58].

The enhancing effect of MBG on fMLP response shown here may be relevant to the anti infective activities reported for several beta-glucans. This formylated tripeptide is released by bacteria at the site of infection, and signals via the FPR receptor to induce migration of phagocytic cells such as neutrophils, macrophages, and monocytes [59]. Soluble beta-glucan has been shown to have a pro chemotactic effect on human neutrophil migration in response to fMLP leading to directional rather than random migration and to involve Mac-1 (CD11b/CD18) or CR3 [60]. CR3 contains two distinct binding sites, one that mediates adhesion and a lectin-like domain that binds polysaccharides such as beta-glucan. Both CR3 and dectin-1 receptors are expressed on hematopoietic cells and have been implicated in the recovery of bone marrow cells after radiation or chemotherapy.

Our studies suggest that MBG could synergize with G-CSF in supporting functional myeloid cell recovery. MBG clearly enhanced mobilization of myeloid cells into blood since we observed a shortened time to leukocyte recovery in peripheral blood after chemotherapy. G-CSF is widely given intravenously for the collection of hematopoietic progenitor and stem cells for transplantation in neoplastic diseases. Mobilization occurs via attenuation of the SDF-1 chemokine receptor, CXCR4 axis and a single dose of G-CSF substantially decreases SDF-1 and increases expression of CXCR4 in the bone marrow [61]. The process involves upregulation of matrix-degrading metalloproteinases (MMPs) MMP2 and MMP9, neutrophil elastase, cathepsin G, and complement components but varies according to the type of activator [61–63]. MBG could enhance the effect of G-CSF. Another glucan, PGG beta glucan, when given intravenously mobilized stem cells in the wild type mouse, increased MMP-9 and plasma levels of SDF-1, and synergistically enhanced G-CSF mobilization [23]. Unlike PGG beta glucan, MBG also stimulates production of G-CSF [25]. G-CSF

mobilization requires activation of the classical immunoglobulin (Ig)-dependent complement cascade pathway. Mice that lack Ig, like immunodeficient patients, are poor stem and progenitor cell mobilizers in response to G-CSF but do mobilize in response to zymosan or glucan via Ig independent activation of complement [62, 64]. Therefore MBG might enhance the response of poor as well as normal mobilizers to G-CSF. Taken as a whole, the present studies demonstrate that beta-glucans have potential use in the support of bone marrow recovery after cancer chemotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The study was supported by NIH NCI R25 105012 Collaborative Program in Nutrition and Cancer Prevention; NIH NCCAM and ODS: 1-P50-AT02779 Botanical Research Center for Botanical Immunomodulators, and the Children's Cancer and Blood Foundation.

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Fig. 1.

Paclitaxel induction of leukopenic mouse model: Ptx was given at cumulative doses of 60 and 90 mg/kg. Complete blood counts (CBCs) were compared to untreated mice ($n = 4$ each group) over 10 days. Comparison of mean absolute counts of leukocytes, neutrophils, and lymphocytes are shown. The arrows on the top in each chart indicate days of Ptx injection. Data are shown as group mean absolute counts $\pm$ SD

Fig. 2.

MBG enhancement of CFU-GM activity after Ptx: 2 days after the last Ptx injection, bone marrow (BM) and spleen (SP) cells were collected for ex vivo colony forming unit assays (CFU). (a) Ptx + MBG 4 mg/(kg day) treated mice had significantly higher CFU-GM counts in BM and SP ($p < 0.001$, $p = 0.002$, respectively) compared to Ptx alone by ANOVA. (b) Ptx + MBG 6 mg/(kg day) led to increased CFU-GM in BM ($p = 0.003$), and showed a trend towards increase in SP

Fig. 3.

Effect of MBG on leukocyte recovery after Ptx: (a) the decrease in white blood cell (WBC) count was less in the Ptx + MBG group compared to Ptx-alone ($p = 0.024$), or after Ptx + G-CSF ($p = 0.031$). (b) At 2 days post-Ptx, the decline WBC in the Ptx + MBG group was less compared to Ptx-alone ($p < 0.05$), or G-CSF ($p < 0.01$) since the effect of G-CSF was not evident 24 h after injection. (c) On day 8 post-Ptx, both Ptx + MBG and Ptx + G-CSF groups had similar mean WBC counts that were higher compared to Ptx-alone ($p < 0.001$)

Fig. 4.

Effect of MBG on neutrophil recovery after Ptx: **(a)** Ptx decreased neutrophil counts in all groups that lasted until post-Ptx day 5 for the Ptx + MBG and Ptx + G-CSF groups. **(b)** On post-Ptx day 5, neutrophils were less decreased in the Ptx + MBG ($p < 0.05$) and Ptx + G-CSF ($p < 0.01$) groups compared to Ptx alone. **(c)** On day 8 post-Ptx, neutrophil counts had rebounded far above baseline after both Ptx + MBG and Ptx + G-CSF but not after Ptx-alone

Fig. 5.

Effect of MBG on lymphocyte recovery after Ptx: Ptx reduced lymphocyte numbers in all groups compared to the baseline ($p < 0.0001$). By post-day 5, lymphocyte counts were higher than baseline for the Ptx + MBG ($p < 0.01$) but not the Ptx + G-CSF group. On post-day 8 counts were higher than baseline for both Ptx + MBG and Ptx + G-CSF groups ($p < 0.01$) but not for Ptx-alone

Fig. 6.

MBG promoted early recovery of myeloid function: **(a)** Peripheral blood Gr-1+ granulocyte/monocyte production of reactive oxygen species (ROS) was tested ex vivo 4 days after Ptx by flow cytometry. Response to *E. coli* in the Ptx-alone and Ptx + G-CSF groups was lower compared to untreated mice ($p < 0.01$ for both). ROS response was higher in the Ptx + MBG group compared to Ptx alone ($p < 0.01$) or Ptx + G-CSF ($p < 0.01$), and equal to untreated mice. Response to fMLP stimulation was higher in the Ptx + MBG group than either untreated mice ($p < 0.01$) or the Ptx + G-CSF group ($p < 0.05$). **(b)** At post-Ptx day 11 ROS response to *E. coli* was now equal in all treated groups and higher than in untreated mice ($p < 0.0001$). However, fMLP response in the Ptx + MBG group was higher compared to Ptx-alone or Ptx + G-CSF groups ($p = 0.013$ and $p = 0.014$, respectively)