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COMPARISON OF CONDITIONING REGIMENS FOR ALVEOLAR MACROPHAGE RECONSTITUTION AND INNATE IMMUNE FUNCTION POST BONE MARROW TRANSPLANT

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Abstract

The authors compared efficiency of alveolar macrophage (AM) reconstitution from donor bone marrow post transplant following 4 chemotherapy conditioning regimens and 2 total body irradiation (TBI) regimens. TBI regimens are more effective in inducing AM reconstitution from donor marrow. However, mice conditioned with 13 Gy split-dose TBI or a dual-chemotherapy regimen (25 mg/kg busulfan $\times$ 4 days plus cyclophosphamide 100 mg/kg $\times$ 2 days) both demonstrate significant AM repopulation from donor marrow. Additionally, both protocols resulted in impaired pulmonary host defense associated with overproduction of prostaglandin E₂ and I₂ by AMs and impaired AM phagocytosis post bone marrow transplant.

Keywords

phagocytosis; prostaglandin; *P. aeruginosa*

The lung has the largest interface with the outside environment of any internal organ in the body. As such, the lung is constantly bombarded with foreign particles and pathogens. Active host defense requires the orchestration of innate immune cells and immune mediators such as cytokines, chemokines, and eicosanoids to aid in the recognition, containment, and clearance of microorganisms [1]. Unfortunately, patients who have undergone stem cell transplantation are at risk for pulmonary infections [2-5] and pneumonia remains the leading infectious cause of death following stem cell transplantation despite the implementation of numerous prophylactic strategies and advances in diagnosis and treatment [6]. These transplant patients manifest increased susceptibility to infections for periods of time (months to years) post transplantation, extending long after engraftment of leukocytes from donor sources [2, 7].

We have previously established a murine model of gram-negative bacterial infection following bone marrow transplantation (BMT) and have shown that these mice are more susceptible to pulmonary infection with the gram-negative pathogen *Pseudomonas aeruginosa* due to defects in innate immune cells. The conditioning regimen employed in

these studies involved 13 Gy of total body irradiation (TBI) followed by reconstitution with 5×10^6 whole bone marrow (BM) cells + 1×10^6 syngeneic splenic T cells. We demonstrated that AMs from these mice were >87% donor-derived by 3 weeks post BMT [4]. Despite the fact that the alveolar macrophages (AMs) were largely donor derived, the AMs were defective in their ability to both phagocytose and kill bacteria [4, 8]. In this model, AMs post BMT produced elevated amounts of prostaglandin E₂ (PGE₂), which is known to inhibit host defense [4, 8, 9]. However, one caveat to the extrapolation of these findings is that the experimental conditioning regimen utilized in our previous studies does not mimic human clinical practice. Thus, to determine the applicability of these findings to BMT performed following different conditioning regimens, we compared the level of AM reconstitution and prostaglandin production following multiple TBI and chemotherapy preparative regimens. Our results demonstrate that significant reconstitution of AMs from donor BM following either TBI or chemotherapy conditioning results in elevated PGE₂ and PGI₂ production and impaired AM function.

MATERIAL AND METHODS

Animals

Wild-type C57BL/6 (B6Ly5.1; CD45.2) mice were obtained from Jackson Laboratory (Bar Harbor, ME). B6Ly5.2 (CD45.1) mice were purchased from the Fredrick Cancer Research Facility (Fredrick, MD). Mice were housed under specific pathogen-free conditions and were monitored daily by veterinary staff. All mice were euthanized by CO₂ asphyxiation. The University of Michigan Committee on the Use and Care of Animals approved these experiments.

Bone Marrow Transplantation

Mice were given a syngeneic BMT following multiple conditioning regimens as follows. (1) *TBI + T* mice received 5×10^6 whole bone marrow (BM) cells + 1×10^6 splenic T cells post 13 Gy TBI; (2) *TBI* mice received 5×10^6 BM cells following 13 Gy TBI; (3) *dual chemo* mice received 5×10^6 BM cells following conditioning with 25 mg/kg busulfan $\times$ 4 days + 100 mg/kg cyclophosphamide $\times$ 2 days + 1 day rest; (4) *Cyclo* mice received 5×10^6 BM cells post conditioning with 100 mg/kg cyclophosphamide $\times$ 2 days + 1 day rest; (5) *Bus Lo* mice received 5×10^6 BM cells following conditioning with 50 mg/kg busulfan $\times$ 1 day + 1 day rest; (6) *Bus Hi* mice received 5×10^6 BM cells following conditioning with 25 mg/kg busulfan $\times$ 4 days + 1 day rest. In all cases, B6Ly5.2 mice (CD45.1) were used as BM donors for B6Ly5.1 (CD45.2) recipients. For TBI protocols, mice were exposed to a ¹³⁷Cs source. Busulfan and cyclophosphamide were purchased from Sigma (St. Louis, MO). Whole BM cells resuspended in Dulbecco's modified Eagle medium (DMEM) without additives were infused in a 0.2-mL volume via tail vein injection. Mice were maintained on acidified water (pH 3.5) for the first 21 days post BMT.

AM Harvest

Resident AMs from mice were obtained via ex vivo lung lavage using 20 mL of complete media containing 5 mM EDTA as previous described [8]. Cells collected in this manner were stained with modified Wright-Geimsa and were subjected to differential analysis. Greater than 85% of the cells in the lung lavage were AMs on the basis of morphology. Figure 1 shows representative examples of the differential stain of cells collected from the BAL at week 5 post TBI or the dual chemo regimen. Forward and side scatter profiles of these populations confirm these results. It should be noted that the macrophage markers F4/80 and Mac-1 are poorly expressed by AMs. Similarly, CD11b only detects 1.5% of AMs in naïve mice. In TBI + T mice, 9.3% of alveolar macrophages express CD11b. In the TBI mice, 8.2% of AMs express CD11b.

Flow Cytometry Analysis

Donor versus host reconstitution of AMs were determined by flow cytometry using CD45.1-PE (phycoerythrin) and CD45.2-FITC (fluorescein isothiocyanate) directly conjugated antibodies (Abs) purchased from BD/Pharmingen (San Diego, CA). The percent chimerism was calculated by examining 2-parameter plots (PE staining indicating donor-derived cells was displayed on the y-axis versus FITC-staining indicating host-derived cells on the x-axis). Because AMs are highly autofluorescent, gates were drawn manually for each sample to include the populations deemed to be single positive for either CD45.1 or CD45.2 (see representative examples in Figure 2). The percent chimerism was then determined by calculating %CD45.1+ divided by (%CD45.1+ plus %CD45.2+).

PGE₂ and PGI₂ Measurement

AMs (5×10^5 /ml) were cultured overnight in complete medium. After 24 hours, cell-free supernatants were collected and analyzed by specific ELISA for the production of PGE₂ and 6-keto-PGF_{1 α} (a stable metabolite of PGI₂) using kits from Cayman Chemical, Ann Arbor, MI, according to the manufacturers' instructions.

Intratracheal (IT) Infection With *P. aeruginosa*

P. aeruginosa PA01 stock was grown in tryptic soy broth (DIFCO, Detroit, MI) for 18 hours at 37°C. Bacterial concentration was determined by measuring the amount of absorbance at 600 nm compared to a predetermined standard curve. Animals were anesthetized and given the IT inoculum ($5.7 \log_{10}$) of *P. aeruginosa* as previously described [8].

Quantification of Bacterial Burden in Lung

Mice were euthanized 24 hours following *P. aeruginosa* inoculation. Lungs were collected and homogenized before 10 μ L of each lung homogenate was plated on blood agar plates using serial 10-fold dilutions and incubated at 37°C. Bacterial colonies were counted at 24 hours post IT infection as colony-forming units (CFU)/whole lung as previously described [8].

Phagocytosis Assay

AMs were enumerated by counting on a hemocytometer prior to plating. Cells were allowed to adhere to tissue culture plates in serum-free medium (SFM) for 1 hour, then were washed to remove nonadherent cells, resulting in >95% pure AM cultures as determined by modified Wright-Giemsa stain. AMs were then cultured (200,000 cells/well in half-area 96 well plates) overnight in complete medium (DMEM, 10% fetal calf serum, 1 % penicillin-streptomycin) before the ability of these cells to phagocytose unopsonized bacteria (60:1 ratio bacteria to AMs) was examined using fluorescently labeled *E. coli* bioparticles (Vybrant phagocytosis assay; Molecular Probes, Eugene, OR), following manufacturer's instructions as previously described [8]. In some assays, 5 μ M indomethacin was added during the phagocytosis assay to specifically block prostaglandin production by the AMs.

Statistical Analysis

Statistical significance was analyzed using Prism 3.0 statistical program (GraphPad Software, San Diego, CA). Comparisons among three or more experimental groups were performed by analysis of variance (ANOVA) with a post hoc Bonferroni test to determine significance between groups. A value of $P < .05$ was considered significant.

RESULTS

Optimal Repopulation of AMs Occurs at 5 Weeks Post 13 Gy Irradiation in the TBI Model

We first wanted to characterize a model for transplantation following irradiation conditioning that would result in significant reconstitution of AMs from donor BM without the inoculation of splenic T cells. In order to determine the optimal timing and dose of TBI for AM reconstitution post BMT, we first transplanted CD4S.2 mice with 5×10^6 BM cells from CD4S.1 mice following conditioning with 6.5, 8, 13, or 17 Gy TBI. The 17-Gy dose of TBI was lethal to over 60% of mice despite BM transfer. All mice survived conditioning with 6.5, 8, and 13 Gy TBI. There was a dose-dependent increase in the percent of donor-derived AMs and peripheral cells (spleen) with the highest repopulation seen at 13 Gy (Table 1). Figure 2B and C show representative flow cytometry characterization of the donor versus host reconstitution seen in the BAL at week 5 of mice following 8 and 13 Gy TBI, respectively.

To determine the optimal timing for donor-AM reconstitution, mice were subjected to TBI following 13 Gy conditioning and cells from the bronchoalveolar (BAL) and spleen ($n = 5$) were analyzed at weeks 3, 5, and 7. The percent of donor-derived AMs rose from $40\% \pm 6\%$ at week 3 to $82\% \pm 2\%$ at week 5. In that same time period, the reconstitution of the spleen with donor-derived cells rose from $71\% \pm 3\%$ at week 3 to $95\% \pm 1\%$ at week 5. At week 7, the reconstitution was no greater, and in one experiment was slightly diminished ($70\% \pm 7\%$ donor-derived AMs). Thus, week 5 following 13 Gy TBI was chosen as the optimal irradiation regimen for further study.

TBI Conditioning Is More Effective in Reconstitution of AMs from Donor BM

We next wanted to compare the effectiveness of chemotherapy conditioning regimens to irradiation conditioning regimens. B6Ly5.2 mice were used as BM donors for B6Ly5.1 recipients following the conditioning regimens noted in Table 2. Five weeks post BMT, AMs were harvested and the percent reconstitution from donor BM was calculated by determining the percent of AMs expressing the CD45.1 allele (donor) compared to the CD45.2 allele (host) by flow cytometry (Table 2). Additionally, we investigated the percent of donor versus host chimerism present in the periphery (spleen) following each BMT regimen as well as characterized the total cellularity of the BAL (of which greater than 85% of the cells were AMs by morphological assessment). Figure 2 shows representative fluorescence-activated cell sorting (FACS) 2-parameter plots to examine CD45.1- versus CD45.2-expressing cells collected from the BAL of mice at week 5 post TBI (8 Gy or 13 Gy) or dual chemo BMT. The 13 Gy TBI protocol was most efficient at reconstitution of AMs from donor BM.

Significant Reconstitution of AMs from Donor BM Is Associated with Elevated Production of PGE₂

AMs were collected from all groups of BMT mice at week 5 post BMT. AMs were cultured at 5×10^5 /mL and conditioned medium was collected after 24 hours for measurement of PGE₂ by specific ELISA (Figure 3A). AMs from untransplanted B6Ly5.2 mice were used as controls (con; *black bars*). Conditioning regimens that were associated with significant turnover of AMs from donor BM (TBI + T, TBI, or dual chemo) were also associated with elevated production of PGE₂ by AMs post BMT when compared to AMs from control mice. To determine whether this elevation was specific to PGE₂, we measured the levels of a stable metabolite of PGI₂ (6-keto PGF_{1 α}) in supernatants of AMs from control, TBI, and dual chemo mice (Figure 3B). The levels of 6-keto PGF_{1 α} were elevated in mice transplanted by both regimens; however, these levels only reached statistical significance in the cells from the TBI mice. Thus, it is likely that there is a general up-regulation of

prostanoids post BMT, but the levels of 6-keto PGF_{1α} produced are much lower than the levels of PGE₂.

Impaired AM Phagocytosis Characterizes Both the TBI and Dual Chemo Regimens

We next wanted to determine whether AM phagocytosis would be similarly impaired following either a TBI or chemo BMT protocol. For these studies, we chose the TBI regimen to compare to the dual chemo regimen because both protocols utilized the same donor inoculum. AMs were harvested from control, TBI, or dual chemo mice at week 5 post BMT. AMs were plated and left untreated or treated with a 5-*fLM* dose of indomethacin, an inhibitor of cyclooxygenases and prostaglandin production. FITC-labeled bacteria were added and phagocytosis was measured after 2 hours (Figure 4A). AMs from both TBI and dual chemo BMT mice were similarly defective in their ability to engulf bacteria. Inhibition of prostaglandin synthesis by the AMs improved phagocytosis to control levels in both groups of BMT mice. As previously demonstrated [8], indomethacin treatment was also able to augment phagocytosis by control AMs, but indomethacin treatment of BMT samples only restored phagocytosis to levels of control AMs in the absence of indomethacin. Thus, overproduction of PGE₂ (and possibly PGI₂) likely contributes to the impaired AM phagocytosis noted in these mice regardless of conditioning regimen employed.

Both TBI and Dual Chemo BMT Mice Display Impaired Pulmonary Host Defense

To determine whether host defense was impaired following either conditioning regimen, control (untransplanted), TBI BMT, or dual chemo BMT mice were infused with 5.7log₁₀ *P. aeruginosa* and colony counts in the lungs were determined 24 hours later (Figure 4B). Whereas control mice showed evidence of bacterial clearance, both the TBI and dual chemo BMT mice were similarly deficient in their ability to clear the bacterial challenge. These results mirror our previous findings using the TBI + T conditioning regimen at week 3 post BMT [4, 8].

DISCUSSION

Pulmonary complications, particularly infections remain the leading cause of morbidity and mortality in patients undergoing hematopoietic stem cell transplant despite advances in prophylaxis [2, 3, 7]. This is likely due, in part, to innate immune defects noted in AMs of patients post transplant [10]. Our previous work established a murine model of BMT (TBI + T), which was characterized by overproduction of PGE₂, impaired AM phagocytosis and diminished host defense [4, 8]. However, the TBI + T conditioning regimen used in our earlier studies does not mimic clinical practice [2, 11–13]. Additionally, the TBI + T conditioning regimen results in substantial reconstitution of AMs at an earlier time point (week 3) [4] than occurs when T cells are not added to the donor inoculum ([14] and the present results). Thus, we wanted to assess the effect of other conditioning regimens on AM reconstitution and function.

TBI conditioning was superior to all tested methods of chemotherapy conditioning for the reconstitution of AMs from donor BM. Only the dual chemo regimen of 25 mg/kg busulfan × 4 days followed by 100 mg/kg cyclophosphamide × 2 days resulted in significant AM reconstitution from donor BM. AM reconstitution was only seen in situations where there was also extensive peripheral reconstitution as measured by spleen cell chimerism. Previous studies have suggested that approximately 20% of AMs are capable of extensive replication and colony formation in vitro, a population termed alveolar macrophage colony-forming cells (AL-CFCs) [15]. This population of cells is moderately resistant to ionizing irradiation [16] and likely represent a local lung progenitor cell for AMs. Using fractionated low-dose irradiation schemes, Tarling and colleagues demonstrated that when the AL-CFC population

was not ablated, that AMs were repopulated by host cells; presumably the lung-resident AL-CFC [17]. However, high-dose irradiation schemes (>9 Gy) ablated the AL-CFC population and resulted in AM repopulation by donor BM cells [17]. Our present results agree with these observations and confirm that 8 Gy TBI results in approximately 36% AM reconstitution from donor BM, whereas high-dose irradiation (13 Gy) results in ~80% AM repopulation from donor marrow. Furthermore, these data suggest that of the chemotherapy regimens tested, only the dual chemo regimen resulted in significant AM repopulation from donor BM. We speculate that the 13 Gy TBI and dual chemo regimens likely ablate the AL-CFC local lung progenitors. Alternatively, these results may suggest that AM reconstitution from donor BM requires significant lung injury/inflammation induced by either TBI or chemotherapy. A previous report by Maus and colleagues suggests that lung inflammation is required for efficient exchange of AM populations from circulating BM-derived progenitors [18]. In support of this idea, the regimens that result in significant AM repopulation are also associated with greater cellularity in the BAL.

Like the TBI regimen, the dual chemo protocol was also the only chemotherapy-based protocol to result in significant secretion of PGE₂ by AMs post BMT. Similarly, metabolites of PGI₂ were also elevated in AMs from the TBI regimen (and to a lesser extent in the dual chemo regimen), although the levels of this metabolite were expressed at lower levels than PGE₂. This finding suggests that conditioning for BMT may induce an up-regulation in synthesis of all prostanoids. As PGI₂ and PGE₂ both signal through G-protein-coupled receptors and both induce cyclic adeno-sine monophosphate (cAMP) elevation, the downstream effects of either PGE₂ or PGI₂ signaling on host defense would be expected to be similarly inhibitory [9, 19].

The elevations in PGE₂ synthesis are likely not contained to just the donor-derived AMs post BMT. We previously published that PGE₂ levels were elevated in alveolar epithelial cells as well as in the peritoneal cavity following the TBI + T regimen [8]. We have confirmed these findings in the TBI regimen as well (data not shown). Furthermore, in a preliminary experiment where donor versus host AMs were sorted following 8 Gy of TBI, via expression of CD4S.1 versus CD4S.2 and analysis of PGE₂ levels in each population, the production of PGE₂ was significantly elevated in both the donor and host-derived AMs when compared to AMs from control mice; however, the production of PGE₂ by donor-derived AMs was about 3-fold greater. Thus, BMT conditioning likely results in global increases in prostanoid production in cells of both donor and host origin. Although it is not clear exactly what the trigger for elevated prostanoid production is post BMT, we speculate that oxidative stress or reactive oxygen species, a known inducer of PGE₂ [20], may play a role following the TBI and dual chemo regimens.

We next wanted to determine whether the elevated levels of PGE₂ following BMT with either the TBI or the dual chemo regimen would result in similar alterations in AM phagocytosis and host defense. We chose the TBI and the dual chemo model for analysis because they utilized the same donor inoculum and these conditioning regimens mimic human transplantation protocols [11-13]. Five weeks post BMT, AMs from BMT mice conditioned with either regimen were similarly defective in phagocytosis when compared to control AMs and this inhibition could be reversed by the addition of the prostaglandin synthesis inhibitor, indomethacin, to the cultures. The impaired AM function in both the TBI and dual chemo regimens led to similar defects in pulmonary clearance of a gram-negative infection as well. Of note, we previously demonstrated that indomethacin treatment in vivo could restore host defense to mice following the TBI + T regimen [8]. Thus, we conclude that lung-myeloablative conditioning regimens (whether TBI or chemotherapy based), which result in significant turnover of AMs from donor BM, are associated with elevated production of prostanoids by these AMs, impaired AM function, and diminished host

defense. As the conditioning regimens utilized in these studies mimic clinical practice, these results suggest that these findings may be extrapolated more broadly. Interestingly, elevated levels of PGE₂ have been noted in the plasma of patients undergoing autologous BMT or autologous stem cell transplantation [21]. The conditioning regimens differed among the patients (TBI versus chemo versus combination), but PGE₂ levels were elevated in all cases [21]. Thus, these results may be widely applicable to stem cell transplantation in humans as well.

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FIGURE 1.

Differential analysis of BAL cells post BMT. BAL cells were collected from TBI or dual chemo BMT mice at week 5 post transplant. BAL cells were cytospun and stained with modified Wright-Geimsa stain. Greater than 85% of the cells collected from the lung lavage had the characteristic morphology of AMs at this time point.

FIGURE 2.

Donor versus host reconstitution of BAL post BMT. Mice were conditioned with either 8 Gy or 13 Gy of TBI or with the dual chemo regimen and were transplanted with 5×10^6 BM cells. BAL was performed 5 weeks post transplant and cells were analyzed for expression of CD45.1 (PE-donor AMs) versus CD45.2 (FITC-host AMs) to differentiate donor versus host reconstitution. (A) Representative dot plot showing unstained cells post 13Gy TBI. Two-parameter plots for unstained samples were similar for other BMT regimens as well. (B) Representative dot plot for mice post 8 Gy TBI. In this example, 39% of the cells are donor derived. (C) Representative dot plot of BAL from mice post 13 Gy TBI. In this example, 95% of the cells are donor derived. (D) Representative dot plot of BAL from mice post dual chemo BMT. In this example, 53% of the cells were donor derived.

FIGURE 3.

Prostanoid secretion by AMs post BMT. AMs were collected via ex vivo lung lavage from control (untransplanted), TBI + T, TBI, dual chemo, cyclo, Bus Lo, or Bus Hi conditioned BMT mice. Cells were cultured at $5 \times 10^5/\text{mL}$ for 24 hours and PGE₂(A) or 6-keto PGF_{1α} (B) was measured in cell-free supernatants by specific ELISA ($n = 5$; *** $P < .001$, * $P < .05$ compared to control AMs).

FIGURE 4.

AM function and host defense post BMT. (A) AMs were harvested from control (untransplanted), TBI, or dual chemo BMT mice and plated at 200,000 cells per well in half-area 96-well plates. Fluorescently labeled *E. coli* were added to each well and cells and bacteria were incubated for 2 hours. In some wells, a final concentration of 5 μ M indomethacin was added as a prostaglandin synthesis inhibitor. Fluorescence of engulfed bacteria was determined by fluorimetry in the presence of trypan blue to quench extracellular fluorescence, $n = 6$. The # symbol signifies $P < 0.05$ vs. non-indomethacin-treated cultures. Other P values are compared to the Concolumn. (B) Control (untransplanted), TBI, or dual chemo BMT mice were inoculated with $5.7 \log_{10}$ *P. aeruginosa* at week 5 post BMT. Lungs were harvested 24 hours later and CFU were determined via colony counts. Dotted line represents inoculated dose. Control mice demonstrate clearance of the bacteria within 24 hours whereas BMT mice do not ($n = 5$).

TABLE 1

Percent Chimerism of AMs and Spleen Following Various Doses of Irradiation at Week 5

Irradiation dose	Percent donor-derived AMs	Percent donor-derived spleen cells
6.5 Gy	0.7 ± 0.1	Not tested
8 Gy	36.2 ± 4.2	87.6 ± 0.2
13 Gy	82.3 ± 2.4 *	94.9 ± 1.1 *

* $P < .005$ between 8 and 13 Gy treatments.

TABLE 2

Reconstitution of AMs Following Various Conditioning Regimens

Abbreviation	Conditioning regimen	Donor inoculum	% donor-derived AMs (week 5)	% donor-derived spleen cells	Total number of AMs in BAL ($\times 10^4$)
TBI + T	6.5 Gy $\times$ 2 (3 h separation)	5×10^6 whole BM cells + 1×10^6 splenic T cells	88 ± 4.5	98.3 ± 0.4	164 ± 14
TBI	6.5 Gy $\times$ 2 (3 h separation)	5×10^6 whole BM cells	80.5 ± 5.8	94.9 ± 1.1	144 ± 45
Dual chemo	25 mg/kg busulfan $\times$ 4 days + 100 mg/kg cyclophosphamide $\times$ 2 days + 1 day rest	5×10^6 whole BM cells	56 ± 6.2	72.3 ± 2.1	136 ± 43
Cyclo	100 mg/kg cyclophosphamide $\times$ 2 days + 1 day rest	5×10^6 whole BM cells	<1%	4.64 ± 0.5	76 ± 12
Bus Lo	50 mg/kg busulfan $\times$ 1 day + 1 day rest	5×10^6 whole BM cells	<1%	6.17 ± 0.70	64 ± 15
Bus Hi	25 mg/kg busulfan $\times$ 4 days + 1 day rest	5×10^6 whole BM cells	2.2 ± 0.8	48.5 ± 2.3	32 ± 7

Note. By ANOVA for AM percent reconstitution: TBI + T versus TBI alone $P =$ nonsignificant; TBI versus Dual chemo $P < .05$; TBI versus cyclo $P < .001$; TBI versus Bus Lo $P < .001$; TBI versus Bus Hi $P < .001$.