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Exuberant Inflammation in Nicotinamide Adenine Dinucleotide Phosphate-Oxidase-Deficient Mice After Allogeneic Marrow Transplantation

Shuxia Yang,* Angela Panoskaltsis-Mortari,† Mayank Shukla,* Bruce R. Blazar, † and Imad Y. Haddad2*†

We have shown that NO and superoxide (O2−) contribute to donor T cell-dependent lung dysfunction after bone marrow transplantation (BMT) in mice. We hypothesized that inhibiting O2− production during inducible NO synthase induction would suppress oxidative/nitrative stress and result in less severe lung injury. Irradiated mice lacking the phagocytic NADPH-oxidase (phox−/−), a contributor to O2− generation, were conditioned with cyclophosphamide and given donor bone marrow in the presence or absence of inflammation-inducing allogeneic spleen T cells. On day 7 after allogeneic BMT, survival, weight loss, and indices of lung injury between phox−/− and wild-type mice were not different. However, the majority of macrophages/monocytes from phox−/− mice given donor T cells produced fewer oxidants and contained less nitrotyrosine than cells obtained from T cell-recipient wild-type mice. Importantly, suppressed oxidative stress was associated with marked infiltration of the lungs with inflammatory cells and was accompanied by increased bronchoalveolar lavage fluid levels of the chemoattractants monocyte chemoattractant protein-1 and macrophage-inflammatory protein-1β and impaired clearance of recombinant mouse macrophage-inflammatory protein-1β from the circulation. Furthermore, cultured macrophages/monocytes from NADPH-deficient mice produced 3-fold more TNF-α compared with equal number of cells from NADPH-sufficient mice. The high NO production was not modified during NADPH-oxidase deficiency. We conclude that phox−/− mice exhibit enhanced pulmonary influx of inflammatory cells after BMT. Although NO may contribute to increased production of TNF-α in phox−/− mice, the data suggest that NADPH-oxidase-derived oxidants have a role in limiting inflammation and preventing lung cellular infiltration after allogeneic transplantation. The Journal of Immunology, 2002, 168: 5840–5847.

Immunopathologic pneumonia syndrome (IPS), a noninfectious lung dysfunction after bone marrow transplantation (BMT), and graft-vs-host disease (GVHD) account for the majority of deaths after allogeneic BMT (1, 2). Reactive oxygen and nitrogen species generated during the course of irradiation, conditioning drugs, and allogeneity are implicated in the pathogenesis of tissue injury associated with IPS and GVHD (3). Total body irradiation (TBI) and commonly used conditioning drugs such as cyclophosphamide (Cy) have been shown to deplete antioxygenic enzymes and enhance the generation of superoxide (O2•−) and O2−-derived toxic species (4–7). The lung is particularly sensitive to Cy/TBI because of its oxygen-rich environment (8). In addition, allo-activated donor T cells up-regulate the expression of inducible NO synthase (iNOS) and the production of NO (9). When simultaneously generated in large amounts, NO rapidly reacts with O2− to form peroxynitrite (ONOO−), a potent oxidant and tissue-damaging nitrating species (10).

In our established murine BMT model, we observed that infusion of allogeneic T cells on the day of BMT into TBI recipients induces NO generation, and the addition of Cy to irradiation regimen facilitates T cell-dependent oxidative/nitrative stress and depletes glutathione (11, 12), possibly via the formation of ONOO− (13). Using iNOS deletion mutant mice (iNOS−/−) we reported that iNOS-derived NO amplifies T cell-dependent inflammation and mortality after allogeneic BMT (14). However, during Cy-facilitated oxidative stress mice lacking iNOS persist to generate NO-independent potent oxidants and in fact exhibited increased mortality compared with wild-type mice (14). These data indicated that cellular redox state is a main determinant of whether inhibition of NO is beneficial or detrimental after allogeneic BMT.

Phagocyte NADPH-oxidase is a multicomponent enzyme that transfers electrons from NADPH to oxygen to generate O2•− (15). The main function of NADPH-dependent oxidative stress is host defense against invading microorganisms. O2•− is a weak oxidant, but the formation of hydroxyl radicals (·OH) by the O2•−-driven fenton reaction and the formation of ONOO− by the reaction of O2•− with NO generate potent oxidants that can oxidize fluorescent probes (16). In addition, neutrophils and, to a lesser extent, monocytes can release myeloperoxidase (MPO) into the extracellular space, where it reacts with hydrogen peroxide (H2O2) and chloride to generate hypochlorous acid (HOCl), a strong oxidizing agent (17). Although oxidative stress is effective in control of infections,
not infrequently it also causes injury to host proteins, lipids, and DNA, culminating in tissue damage.

In addition to their tissue-destructive effector function, NADPH-oxidase-derived reactive species can regulate cellular signal transduction pathways (reviewed in Ref. 18). For example, in a model of alcoholic liver injury, NADPH-oxidase activates the proinflammatory transcriptional factor NF-kB (19), known to up-regulate the expression of a number of genes involved in immune and inflammatory responses. In addition, O2^- and H2O2 have been reported to contribute to inflammation by increasing leukocyte adhesion to endothelium (20), by altering the intracellular redox state (21), and by induction of intracellular calcium (22). In a model of influenza-induced lung injury, transgenic mice that overexpress extracellular superoxide dismutase, an antioxidant enzyme that decreases the steady state of O2^-, exhibited resistance to injury associated with suppressed generation of oxidative stress and decreased production of TNF-a and NO (23). An in vivo anti-inflammatory role for oxidative stress has not been described.

Chronic granulomatous disease (CGD) is an inherited disorder caused by defects in NADPH-oxidase-dependent O2^- production (24). Recently, mouse models of CGD have been created by deletion of the phagocytic oxidase (phox) membrane-bound component gp91phox (25) or of the cytoplasmic component p47phox (26). phox^-/- mice are susceptible to severe bacterial and fungal infections. A second feature of CGD in humans and mice is the frequent development of inflammatory granulomas in lung, skin, liver, and the lining of gastrointestinal and genitourinary tracts (27). Although incomplete resolution of active infection has been suggested as a possible reason for granuloma formation, the rapid response to systemic steroid therapy suggests a noninfectious etiology (28). Potential mechanisms for the in vivo occurrence of inflammatory granulomas have not been clearly defined.

The purpose of this study was to investigate the role of NADPH-oxidase during noninfectious T cell-dependent inflammation after transplantation. We hypothesized that Cy/TBI phox^-/- mice given allogeneic T cells would exhibit decreased oxidative/nitritative stress but persistent NO-dependent inflammation. Our results indicate phox^-/- mice have enhanced lung cellular infiltration associated with severe activation of macrophages/monocytes. Because NO production after allogeneic transplantation in phox^-/- and wild-type mice was comparable, the data suggest a role for O2^- derived oxidative stress in modulation of the early post-BMT inflammatory events.

Materials and Methods

Mice

B10.BR (H-2^b), C57BL/6J (H-2^b), and phox^-/- mice generated by deletion of a 91-kDa subunit of the cytochrome b (gp91; backcrossed >10 generations to C57BL6 mice) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in microisolation cages in the specific pathogen-free facility of the University of Minnesota (Minneapolis, MN) and cared for according to the Research Animal Resources guidelines of our institution. For BMT, donors were 6–8 wk of age and recipients were used at 8–10 wk of age. Sentinel mice were found to be negative for 15 known murine viruses including CMV, K-virus, and pneumonia virus of mice.

Pre-BMT conditioning

C57BL/6 wild-type or phox^-/- mice received i.p. injections of Cy (Cytoxan; Bristol-Myers Squibb, Seattle, WA) 120 mg/kg per day on days -3 and -2 pre-BMT. On the day before BMT, all mice were lethally TBI (7.5 Gy) by x-ray at a dose rate of 0.41 Gv/min.

BM transplant

Our BMT and IPS generation protocols have been described previously (29). Briefly, donor B10.BR bone marrow (BM) was T cell depleted (TCD) with anti-Thy 1.2 mAb (clone 30-H-12, rat IgG2a, kindly provided by Dr. D. Sachs, Massachusetts General Hospital, Boston, MA) plus complement (Neufengen, Woodland, CA). For each experiment, a total of 5–10 recipient mice per treatment group were transplanted via caudal vein with 20 x 10^6 B10.BR TCD BM cells without spleen T cells (BM + Cy) or with 15 x 10^6 spleen T cells (BM plus spleen (BMS) + Cy) as a source of GVHD/IPS-causing T cells. Day 7 post-BMT white blood cell count was determined using a Coulter Counter (Model ZB: Coulter, Miami, FL) after lysis of RBCs by Zap-Oglibin II lytic reagent (Coulter).

Bromochloroacetaldehyde lavage

Mice were sacrificed on day 7 after BMT after an i.p. injection of sodium pentobarbital, and the thoracic cavity was partially dissected. The trachea was cannulated with a 22-gauge angiocatheter, infused with 1 ml of ice-cold sterile PBS, and withdrawn. This was repeated several times and the bromochloroacetaldehyde lavage fluid (BALF) was immediately centrifuged at 500 × g for 10 min at 4°C to pellet cells. The initial 1.5 ml of BALF was used for biochemical analysis and the remaining fluid was used to increase the yield of recovered cells.

BALF analysis

Cell-free BALF monocyte chemoattractant protein-1 (MCP-1), macrophage-inflammatory protein (MIP)-1a, MIP-1b, and TNF-a levels were determined by sandwich ELISA using murine-specific commercial kits (sensitivity, 1.5–3 pg/ml; R&D Systems, Minneapolis, MN). Nitrite in BALF was measured according to the Greiss method after the conversion of nitrate to nitrite with the reduced NADH-dependent enzyme nitrate reductase (Culbiochem, La Jolla, CA). BALF total protein was determined by the biuret amino acid (Sigma-Aldrich, St. Louis, MO) method as the standard.

Macrophage culture

The BALF cell pellets from mice in each treatment group were combined, washed twice in cold PBS, and resuspended in RPMI 1640 medium (Celox Laboratories, St. Paul, MN) containing 5% FCS, penicillin (100 U/ml), and streptomycin (100 µg/ml). Total cell number was determined with a hemacytometer. A total of 2 x 10^6 cells/well were added to flat-bottom 96-well microtiter plates (Costar, Cambridge, MA), and macrophages were allowed to adhere for 1 h at 37°C in 5% CO2 in air, followed by removal of unbound cells. More than 95% of adherent cells were macrophages. The cells were maintained in culture at 37°C for 48 h in 5% CO2 in air at termination of cell culture, supernatants were aspirated from individual culture wells for measurement of TNF-a by ELISA, nitrite by the Greiss method, and lactate dehydrogenase (LDH) by colorimetric CytoTox 96 assay (Promega, Madison, WI). Cells were washed twice with PBS and lysed with lysis solution (10×, Triton X-100; Promega), and cellular LDH release was measured. Total (supernatant plus cellular) LDH values were used to correct for possible differences in adherent cell number between groups. TNF-a and nitrite readings were adjusted accordingly using the BM group as an assigned reference value for 2 x 10^5 cells (the number of cells originally plated per well).

Macrophage-derived intracellular oxidants

Alveolar macrophages obtained from day 7 post-BMT BALF were cultured in flat-bottom 24-well plates (Costar) for 1 h followed by removal of nonadherent cells. Adherent cells (mainly macrophages/monocytes) were detached from culture plates using trypsin (0.05%; Life Technologies, Carlsbad, CA) and suspended in PBS. Cells were loaded with 2'-7'-dichloro-4',4',5',5'-tetramethylrhodamine diacetate (10 µM; Molecular Probes, Eugene, OR) for 15 min at 37°C. During loading the acetate groups are removed by intracellular esterases, trapping the probe inside the cells. Following an oxidative burst, dichlorofluorescin is oxidized to the fluorescent probe, dichlorofluorescein. Fluorescence was quantitated 30 min after loading by FACScan flow cytometer (BD Biosciences, San Jose, CA) using CellQuest applications (BD Biosciences). For each sample 5000 events were analyzed by measuring the increase in FL1 fluorescence (530 nm).

Histology and immunohistochemistry

In some animal lungs were extracted without lavage and were perfused with 1 ml PBS via the right ventricle of the heart. A mixture of 0.5–1 ml optimal cutting temperature medium (Miles Laboratories, Elkhart, IN) and PBS (3:1) was infused via the trachea into the lung. The lung was snap-frozen in liquid nitrogen and stored at -80°C. Frozen sections were cut 6 µm thick, mounted onto glass slides, and fixed for 5 min in acetone. Representative sections were stained with H&E for histopathologic assessment. After a blocking step in 10% normal horse serum (Sigma-Aldrich), sections were incubated for 30 min at 23°C with the following biotinylated mAbs
(BD Pharmingen, San Diego, CA): anti-CD4 (clone GK1.5), anti-CD8 (clone 2.43), anti-Gr-1 (clone RB6-8C5), and anti-Mac-1 (clone M1/70). Immunoperoxidase staining was performed using avidin-biotin blocking reagents, ABC-peroxidase conjugate, and diaminobenzidine chromogenic substrate (Vector Laboratories, Burlingame, CA). In control measurements the primary Ab was omitted. The sections were counterstained with hematoxylin, dehydrated, overlaid with Permount (Sigma-Aldrich), and sealed with coverslips. The number of positive CD4/CD8 (T cells), positive Mac-1 (macrophages/monocytes), and positive Gr-1 (neutrophils) cells in the blood were quantified as the percentage of nucleated cells at a magnification of ×50 (×20 objective lens). Four fields per lung were evaluated.

For nitrotyrosine (NT) staining, BALF cells were centrifuged onto glass slides, permeabilized, and fixed with methanol at -20°C for 7 min. Endogenous peroxidase activity was quenched by treatment with 0.3% H2O2 in cold methanol for 30 min followed by three washes with PBS. Nonspecific binding was blocked with 10% goat serum for 30 min. The primary Ab, polyclonal anti-NT Ab (Upstate Biotechnology, Lake Placid, NY), at 0.01 mg/ml in 10% goat serum and 2% BSA in PBS was applied to the cells for 30 min. Control measurements included rabbit polyclonal IgG (Upstate Biotechnology) and NT Ab in the presence of excess NT (10 mM; NT block). To visualize specific NT Ab binding, sections were incubated with secondary Ab, goat anti-rabbit IgG conjugated with HRP (1/500 dilution) followed by the addition of 3,3′-diaminobenzidine (Vector Laboratories) chromogenic substrate. The sections were counterstained with hematoxylin, dehydrated, overlaid with Permount (Sigma-Aldrich), and sealed with coverslips. Cells were considered NT positive based on the presence or absence of the brown reaction product in the cell cytoplasm.

**Multiplex quantitative RT-PCR**

Total RNA was extracted from whole lungs obtained on day 7 after BMT using the guanidium thiocyanate-phenol-chloroform method (Tri-Reagent; Sigma-Aldrich). Reverse transcriptase was performed using a cDNA synthesis kit (First-Strand cDNA Synthesis kit; Amersham Pharmacia Biotech, Uppsala, Sweden). MCP-1 cDNA were amplified using mouse MCP-1 gene-specific primers with 18S rRNA as an internal control (Gene Specific Relative RT-PCR; Ambion, Austin, TX). The PCR products were electrophoresed through 1% agarose gel and amplified cDNA bands were visualized by ethidium bromide staining. MCP-1 PCR product was included as positive control. Densitometry was used in relative semiquantitative assessment of RT-PCR product (NIH Image; Scion, Frederick, MD).

**Chemokine clearance**

To assess the clearance of chemokines from the circulation after allogeneic transplantation in the presence or absence of NADPH-oxidase-derived oxidant stress, Cy/TBI donor T cell-recipient wild-type and *phox*-/- mice were injected with recombinant mouse MIP-1β on day 6 after BMT. Re- combinant MIP-1β (10 ng) or an equal volume of PBS was given i.p. and a cohort of *phox*+/+ and *phox*–/– mice were sacrificed at 1 and 4 h after MIP-1β/PBS administration (n = 3 per time point). Serum MIP-1β was determined by sandwich ELISA (R&D Systems).

**Lung weights**

Mice were sacrificed on day 7 after BMT and the thoracic cavity was partially dissected. To maximize use of mice, the right lung (bi-lobed) was used for weight determinations while the left lobe was processed for tissue analysis. For each mouse, the wet weight was determined immediately after removal from the thorax. Lungs were dried overnight to a constant weight at 80°C followed by determination of dry weights and wet/dry weight ratio was calculated. No correction for extravascular blood content was attempted in the calculations.

**Statistical analysis**

Results are expressed as means ± SEM. Data were analyzed by ANOVA or Student’s t test. Statistical differences among group means were determined by Tukey’s Studentized test. Values of *p* ≤ 0.05 were considered statistically significant.

**Results**

**phox**–/– mice have exaggerated cellular lung infiltration after allogeneic BMT**

To examine the role of host phagocyte NADPH-oxidase during the early inflammatory response and oxidative stress after allogeneic BMT, conditioned B6 wild-type and *phox*–/– mice were given B10.BR donor spleen T cells at time of BMT. BALF return volumes collected on day 7 after transplantation were similar in all groups (>90% of instilled volume). BALF from Cy/TBI donor T cell-recipient *phox*–/– mice contained a significantly higher total number of inflammatory cells compared with wild-type mice (Fig. 1A). This increase of cellularity in BALF from *phox*–/– mice was not due to an increased number of inflammatory cells in the blood (Fig. 1B). Furthermore, H&E-stained lung sections from *phox*–/– Cy/TBI-conditioned mice given donor T cells revealed severe interstitial infiltration with inflammatory cells (Fig. 2). As determined by immunohistochemistry, the increased number of lung-infiltrating cells in *phox*–/– mice were positive for Mac-1, CD4, and CD8 surface markers, consistent with enhanced monocyctic and lymphocytic influx during NADPH-oxidase deficiency (Fig. 3). Cy/TBI NADPH-oxidase-deficient and -sufficient mice given BM without T cells did not exhibit significant cellular infiltration in the lung.

**phox**–/– mice have increased expression of the CC chemokines MCP-1, MIP-1α, and MIP-1β**

Previous data showed that up-regulation of MCP-1 on day 7 after BMT BALF and lung parenchyma of T cell-recipient mice preceded lung infiltration with host monocytes, whereas increased expression of MIP-1α and MIP-1β was accompanied by infiltration with donor T cells (30). We reasoned that measurement of MCP-1, MIP-1α, and MIP-1β in BALF of T cell-recipient *phox*–/– mice may clarify, at least in part, the exuberant influx of monocytes and T cells into the lungs. Day 7 after allogeneic BMT MCP-1, MIP-1α, and MIP-1β levels were significantly higher in the BALF of...
and sufficient

phox−/− mice exhibit impaired clearance of rMIP-1β from the circulation after allogeneic BMT

To confirm that oxidative stress facilitates chemokine clearance, recombinant mouse MIP-1β or PBS were injected i.p. in wild-type and phox−/− mice on day 6 after allogeneic BMT, and serum MIP-1β levels were measured 1 and 4 h later. Consistent with BALF MIP-1β levels in PBS-injected mice, MIP-1β levels in wild-type mice were higher in Cy/TBI donor T cell-receptor phox−/− compared with wild-type mice (Fig. 4). Taken together, these data are consistent with decreased clearance instead of increased production as the cause of the elevated chemokine BALF levels from Cy/TBI T cell-recipient phox−/− mice.

FIGURE 2. Mice deficient in phagocytic NADPH-oxidase (phox−/−) exhibit increased cellular lung infiltration after allogeneic transplantation. H&E and Mac-1 immunostaining of frozen lung sections taken on day 7 after BMT from Cy/TBI-conditioned phox−/− C57BL/6 mice given TCD BM from B10.BR mice (BM + Cy), or phox+/+ and phox−/− C57BL/6 mice given B10.BR spleen T cells in addition to BM (BMS + Cy). A large number of lung-infiltrating cells were Mac-1-positive, consistent with mononuclear influx. Resolution power: ×50 (right panel) and ×100 (middle and left panels), equivalent to ×20 and ×40 objective lens, respectively.

FIGURE 3. NADPH-oxidase deficiency enhances the influx of monocytes and T cells in the lung after allogeneic transplantation. Expression of Mac-1, CD4, CD8, and Gr-1 was determined by immunoperoxidase staining with biotinylated mAbs. Cy/TBI C57BL/6 NADPH-oxidase-deficient and -sufficient mice were given B10.BR TCD BM with spleen donor cells (BMS + Cy). Lung tissues were harvested on day 7 after BMT. Data are expressed as the percentage of nucleated cells expressing the surface marker in the lung as determined by counting four fields per lung section under light microscope. Shown are mean values ± SE from two to three mice per group per experiment from two representative experiments. *, p < 0.05 vs controls (nonirradiated and nontransplanted). †, p < 0.05 comparing the effects of NADPH-oxidase deficiency in each group.

FIGURE 4. Increased expression of the CC chemokines MCP-1, MIP-1α, and MIP-1β in BALF from phox−/− mice after allogeneic transplantation. C57BL/6 phox−/− and wild-type mice were preconditioned with Cy/TBI and given B10.BR TCD BM without spleen cells (BM + Cy) or with 15 × 10^6 spleen cells (BMS + Cy) on the day of BMT. BALF was collected on day 7 after BMT. Chemokine expression was determined by sandwich ELISA. Mean values ± SE are indicated for 10–15 mice per group collected from two separate experiments. *, p < 0.05 vs BM + Cy. †, p < 0.05 comparing the effect of NADPH-oxidase deficiency on chemokine levels.
Macrophages from phox<sup>−/−</sup> mice exhibit enhanced production of TNF-α but not NO

To determine whether NADPH-oxidase deficiency also altered the production of inflammatory mediators by macrophages/monocytes, equal number of cells obtained from day 7 after allogeneic BMT BALF were cultured for 48 h and supernatant was assessed for TNF-α and nitrite, the stable byproduct of NO metabolism. Macrophages/monocytes from Cy/TBI phox<sup>−/−</sup> mice given donor T cells (BMS + Cy) produced ~3-fold more TNF-α than cells from BMS + Cy wild-type mice (Fig. 7A). In contrast, nitrite levels in the supernatant of the same macrophages were not different (Fig. 7B). Similarly, day 7 after BMT BALF from T cell-recipient Cy/TBI mice lacking phagocytic NADPH-oxidase contained significantly higher levels of TNF-α, but not nitrite plus nitrate, than BALF from T cell-recipient wild-type controls (data not shown).

Macrophage/monocyte-derived oxidative/nitrative stress in phox<sup>−/−</sup> mice

The contribution of NADPH-oxidase to the generation of oxidative and nitrative stress by macrophages/monocytes extracted from day 7 after BMT BALF was examined. The generation of strong oxidants by alveolar macrophages/monocytes was assessed using dichloroﬂuorescein as an intracellular fluorescent probe. Neither NO nor O<sub>2</sub>· is able to oxidize dichloroﬂuorescin. In contrast, ONOO· and other strong oxidants such as ·OH and HOCl oxidize dichloroﬂuorescin to form the highly fluorescent product dichloroﬂuorescin (31). Generation of oxidants was dependent on infusion of donor T cells because macrophages from irradiated mice given BM without T cells exhibited baseline fluorescence (data not shown). Compared with cells from Cy/TBI wild-type mice given donor T cells, the majority of macrophages/macrophages from Cy/TBI phox<sup>−/−</sup> T cell-recipient mice exhibited lower levels of fluorescence, quantified by flow cytometry (Fig. 8).

Intracellular nitrative stress was assessed by detection of antigenic sites related to NT. Nitration of macrophages/macrophages from BMS + Cy phox<sup>−/−</sup> mice was less than cells from BMS + Cy NADPH-oxidase-sufficient mice. Nitration was specific because staining was completely blocked in the presence of excess Ag, 10 mM NT (Fig. 9). These data indicate that the majority of alveolar monocytes/macrophages generate fewer oxidants and nitrating species during NADPH-oxidase deficiency after allogeneic transplantation.

Survival, weight loss, and lung injury

The effects of NADPH-oxidase deficiency on day 7 after BMT survival, weight loss, and indices of lung injury were determined.
FIGURE 8. Decreased production of oxidants by the majority of macrophages/monocytes from mice lacking phagocytic NADPH-oxidase (phox−/−) after allogeneic transplantation. C57BL/6 phox−/− and wild-type (WT) mice were preconditioned with Cy/TBI and given B10.BR BM plus donor spleen T cells (BMS + Cy). Alveolar macrophages/monocytes obtained from day 7 after BMT BALF were loaded with dichloro-fluorescein, the oxidized product of dichloro-fluorescence of dichloro-fluorescein, using flow cytometry. Forward- and side-scattered light (FSC and SSC) of cells from phox−/− (left inset) and wild-type (right inset) after allogeneic transplantation was similar. Shown is a representative experiment of duplicate samples from wild-type and phox−/− BMS + Cy mice; results were reproduced two times.

FIGURE 9. Decreased NT immunostaining in BALF cells from phox−/− mice after allogeneic transplantation. Cells were obtained from day 7 after BMT BALF of C57BL/6 Cy/TBI-conditioned wild-type (WT) mice given B10.BR BM (BM + Cy), and Cy/TBI-conditioned wild-type and phox−/− mice given BM plus 15 × 10⁶ donor spleen T cells (BMS + Cy). BALF cells were centrifuged onto glass slides and incubated with nonspecific rabbit IgG, NT Ab, or NT Ab in the presence of 10 mM NT (NT block). Shown is a representative figure; data were reproduced two times.
NO production by macrophages from phox\(^{-/-}\) mice. \(O_2^\cdot\) is known to limit the steady state of NO (36), and scavengers of \(O_2^\cdot\) can enhance NO production (37). However, NO generation in BALF and by macrophages from Cy/TBI T cell-recipient phox\(^{-/-}\) mice and genetically matched controls were not significantly different. We concluded that although NO may have contributed to the early donor T cell-dependent inflammatory responses, another NO-independent factor (or factors) is responsible for exaggerated inflammation and enhanced production of macrophage-derived proinflammatory cytokines in phox\(^{-/-}\) mice. Of note is that chemokines have been shown to contribute to inflammatory cell activation. For example, MIP-1\(\alpha\) can stimulate TNF-\(\alpha\) production during acute lung injury in rats (38).

In contrast to our study, van der Veen et al. (39), using a model of experimental allergic encephalomyelitis, reported that mice lacking NADPH-oxidase exhibit NO-dependent suppression of T cell proliferation associated with improvement in clinical score and brain histopathology. NO is known to inhibit T cell immune responses in vivo (40). A potential explanation for the lack of antiproliferative T cell effects of NO in our model is the complete MHC mismatch, which supercedes the inhibitory effects of NO.

Weight loss, BALF indices of lung injury, wet lung weights, and wet: dry lung weights ratio between phox\(^{-/-}\) and wild-type mice after allogeneic BM were not different. A potential reason for persistence of lung dysfunction during NADPH-oxidase deficiency is lung infiltration with high numbers of activated inflammatory cells capable of secreting oxidant-independent tissue-damaging mediators, such as perforin, TNF-\(\alpha\), and a variety of metalloproteinasases and proteases. Alternatively, NADPH-oxidase-independent generation of oxidative stress in phox\(^{-/-}\) mice may result in persistent injury after allogeneic transplantation. For example, Kubo et al. (41) postulated that persistence of cobra venom factor-induced permeability edema in the lungs of CGD mice is caused by ONOO\(^-\), formed during the simultaneous production of \(O_2^\cdot\) by xanthine oxidase and NO by iNOS. Although our results show decreased oxidative burst and nitrative stress by the majority of monocytes/macrophages of mice lacking NADPH-oxidase, we cannot rule out in vivo formation of oxidative stress via endothelial cell-derived xanthine oxidase and nonphagocytic NADPH-oxidase (42). However, CGD mice are unable to clear infections, presumably because of inadequate generation of in vivo oxidative stress (43). Taken together, we favor the hypothesis that the exuberant inflammatory response in NADPH-oxidase-deficient mice is the main culprit responsible for abolishing the potentially tissue-protective effects of decreased macrophage/monocyte-dependent oxidants.

We used mice lacking membrane-bound component of NADPH-oxidase (gp91). Although differential susceptibility of gp91 and p47 NADPH-oxidase-deficient mice to the lethal effects of hyperoxia has been suggested (44), it is important to note that p47 knockout mice also manifest exuberant inflammation following i.p. injection with the sterile irritant thioglycolate (26). Notably, Koay et al. (45) recently reported increased neutrophil influx and elevated MIP-2 levels in lung tissue from LPS-treated p47\(^{-/-}\) mice compared with wild-type mice. Enhanced inflammation occurred despite inhibition of LPS-induced NF-\(\kappa\)B activation in NADPH-oxidase-deficient mice.

In summary, we have shown that mice lacking NADPH-oxidase exhibit exuberant migration and activation of inflammatory cells into the lungs after allogeneic transplantation. Based on our previous data (14), we initially hypothesized that inflammation in phox\(^{-/-}\) during inhibition of phagocyte NADPH-oxidase-derived oxidants is NO dependent. However, NO production in phox\(^{-/-}\) and phox\(^{+/+}\) was similar, consistent with NO-independent exacerbation of early inflammatory responses in mice lacking NADPH-oxidase after allogeneic BMT. Data indicate that oxidative stress facilitates the clearance of chemokines that contribute to the initiation and sustenance of donor T cell-dependent inflammation after BMT (Fig. 10). These results may also explain why CGD patients develop inflammatory lesions in the absence of infection. Further studies will be necessary to determine whether scavenging extracellular \(O_2^\cdot\) without eliminating NADPH-oxidase will result in modulation of the severe early inflammatory response and attenuation of IPS injury following allogeneic transplantation.

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References


