We have shown that NO and superoxide (O$_2^{-}$) contribute to donor T cell-dependent lung dysfunction after bone marrow transplantation (BMT) in mice. We hypothesized that inhibiting O$_2^{-}$ 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 O$_2^{-}$ 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.
not infrequently it also causes injury to host proteins, lipids, and DNA, culminating in tissue damage.

In addition to their tissue-destuctive 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-κB (19), known to up-regulate the expression of a number of genes involved in immune and inflammatory responses. In addition, O$_2^-$ and H$_2$O$_2$ 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 O$_2^-$, exhibited resistance to injury associated with suppressed generation of oxidative stress and decreased production of TNF-α 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 O$_2^-$ 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/microcytes. Because NO production after allogeneic transplantation in phox$^{-/-}$ and wild-type mice was comparable, the data suggest a role for O$_2^-$-derived oxidative stress in modulation of the early post-BMT inflammatory events.

Materials and Methods

Mice

B10.BR (H-2$k$), C57BL/6J (H-2$^b$), and phox$^{-/-}$ mice generated by deletion of a 91-kDa subunit of the oxidase cytochrome b (gp91; backcrossed >10 generations to C57BL/6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in microisolator cages in accordance with the 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/6J 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 Gy/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 IgG$_2$a, kindly provided by Dr. D. Sachs, Massachusetts General Hospital, Boston, MA) plus complement (Neufengger, Woodland, CA). For each experiment, a total of 5–10 recipient mice per treatment group were transplanted via caudal vein with 2 × 10$^6$ TCD BM cells without spleen T cells (BM + Cy) or with 15 × 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 ZF; Coulter, Miami, FL) after lysis of RBCs by Zap-Oglobin II lytic reagent (Coulter).

Bronchoalveolar 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 bronchoalveolar 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)-1α, MIP-1β, and TNF-α 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 (Cubibiochem, La Jolla, CA). BALF total protein was determined by the bicinchoninic acid (Sigma-Aldrich, St. Louis, MO) method with BSA 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 × 10$^5$ cells/well were added to flat-bottom 96-well microtiter plates (Costar, Cambridge, MA), and macrophages were allowed to adhere to the plate for 1 h at 37°C in 5% CO$_2$ 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% CO$_2$ in air. At termination of cell culture, supernatants were aspirated from individual culture wells for measurement of TNF-α by ELISA, nitrite by the Greiss method, and lactic dehydrogenase (LDH) by the 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-α and nitrite readings were adjusted accordingly using the BM group as an assigned reference value for 2 × 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 24 h followed by removal of nonadherent cells. Adherent cells (mainly macrophages/microcytes) were detached from culture plates using trypsin (0.05%); Life Technologies, Carlsbad, CA) and suspended in PBS. Cells were loaded with 2, 7′-dichlorofluorescein diacetate (10 μM; Molecular Probes, Eugene, OR) for 15 min at 37°C. During loading the acetate groups are removed by intracellular enzymes, 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 animals 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...
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 lung were quantitated 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. Non-specific 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; Molecular Research Center, Cincinnati, OH). 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 oxidative stress, Cy/TBI donor T cell-recipient wild-type and phox−/− mice were injected with recombinant mouse MIP-1β on day 4 after BMT. Recombinant MIP-1β (10 ng) or an equal volume of PBS was given i.p. and a cohort of phox+/+ and phox−/− mice were sacrificed 1 and 5 days 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 staining. 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 donor T cell-recipient mice 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
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 monocytic influx. Resolution power: ×50 (right panel) and ×100 (middle and left panels), equivalent to ×20 and ×40 objective lens, respectively.

phox−/− compared with wild-type mice (Fig. 4). The increased level of chemokines was dependent on infusion of T cells, because both phox−/− and wild-type mice given BM without T cells had low BALF levels of MCP-1, MIP-1α, and MIP-1β. The enhanced MCP-1 protein during NADPH-oxidase deficiency was not due to up-regulation of MCP-1 expression, because lung MCP-1 mRNA, as assessed by multiplex gene-specific relative RT-PCR, in phox−/− and wild-type recipients was similar (Fig. 5). 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 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.

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 was injected i.p. in wild-type and phox−/− mice on day 6 after allogeneic BMT, and serum MIP-1β was measured 1 and 4 h later. Consistent with BALF MIP-1β, serum MIP-1β levels in PBS-injected mice were higher in Cy/TBI donor T cell-recipient phox−/− compared with wild-type mice. Injection of rMIP-1β increased serum MIP-1β levels measured after 1 h in both wild-type and phox−/− BMS + Cy mice. However, 4 h after injection of the recombinant chemokine, MIP-1β levels in wild-type mice had returned to baseline. In contrast, MIP-1β levels in MIP-1β-treated phox−/− mice remained elevated (Fig. 6). These data establish the critical role of oxidative stress in the in vivo clearance of chemokines.
Macrophages from phox−/− 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−/− 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−/− mice

The contribution of NADPH-oxidase to the generation of oxidative and nitrative stress by monocytes/macrophages extracted from day 7 after BMT BALF was examined. The generation of strong oxidants by alveolar macrophages/monocytes was assessed using dichlorofluorescin as an intracellular fluorescent probe. Neither NO nor O2− 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 monocytes/macrophages from Cy/TBI phox−/− 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 monocytes/macrophages from BMS + Cy phox−/− 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.
and ONOO
mice given B10.BR BM (BM
phox
and

diacetate (10
obtained from day 7 after BMT BALF were loaded with dichloro
plus donor spleen T cells (BMS
type (WT) mice were preconditioned with Cy/TBI and given B10.BR BM
respectively. The early deaths and day-7 weight loss were dependent on

Decreased NT immunostaining in BALF cells from
Forty-seven of 48
phox
−/− Cy/TBI T cell-recipient mice and 48 of
51 wild-type mice survived at least until day 7 after BMT (p >
0.05). Post-BMT weight loss in
phox
−/− and wild-type mice was also similar. On day 7 after BMT, weight loss was 25 ± 1% and
24 ± 0.5% of baseline in wild-type and
phox
−/− mice, respectively. The early deaths and day-7 weight loss were dependent on

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 dichlorofluorescin-diacetate (10 μM) for 15 min at 37°C. Generation of intracellular oxidants was determined 30 min after loading by measuring intracellular fluorescence of dichlorofluorescein, the oxidized product of dichlorofluorescin, 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.

Discussion
The main findings of these studies are that mice lacking NADPH-oxidase exhibit increased inflammation despite suppression of oxidative/nitrative stress after cytoxan, irradiation, and allogeneic BMT. Inflammation was characterized by markedly enhanced cellular infiltration with T cells and monocytes and increased activation of alveolar macrophages/monocytes. Enhanced donor T cell-dependent inflammation was observed in the absence of positive surveillance cultures, suggesting that NADPH-oxidase has a role in limiting the early inflammatory responses after BMT.

CGD mice are known to sequester abnormally large numbers of inflammatory cells following Ag exposure. For example, tracheal instillation of sterile Aspergillus fumigatus resulted in extensive neutrophil infiltration, but the reasons for the increase influx of cells in the lungs are incompletely understood (32). Our previous studies show donor T cell-dependent induction of the CC chemokines MCP-1, MIP-1α, and MIP-1β after transplantation (30). In the current study, chemokine levels were higher in BALF from Cy/TBI donor T cell-recipient
phox
−/− compared with wild-type mice. The increased MCP-1 protein during NADPH-oxidase deficiency was observed in the absence of increased expression of MCP-1 mRNA, consistent with inefficient MCP-1 clearance and subsequent accumulation in the lungs. Oxidant-induced inactivation of chemokines was confirmed using recombinant mouse MIP-1β injected in
phox
−/− and
phox
+/+ mice on day 6 after allogeneic BMT. Persistent elevation of MIP-1β levels in the serum of MIP-1β-treated
phox
−/− mice compared with similarly treated wild-type mice strongly supports a critical role for oxidative stress in clearance of chemokines, and indicate oxidants may have an important anti-inflammatory function in vivo.

Clark (33) reported that the ability of formyl-methionyl peptide to attract inflammatory cells is abolished in the presence of PMA-stimulated neutrophils obtained from healthy volunteers, but not patients with CGD. Notably, incubation of PMA-stimulated normal neutrophils with the MPO inhibitor, azide, and catalase, but not superoxide dismutase, prevented chemotactic factor inactivation, suggesting a more important role for MPO-catalyzed H2O2/HOCl than O2− and ONOO− in the inactivation process. These results may clarify the reason for the Cy-facilitated elevation of lung and BALF MCP-1, MIP-1α, and MIP-1β levels despite ONOO− generation in Cy/TBI mice infused with allogeneic T cells (30). Alternatively, ONOO− has been shown to up-regulate chemokine gene expression (34), offsetting the potential of ONOO− to inactivate MCP-1 as reported in an in vitro system using chemically synthesized ONOO− (35). Taken together, we hypothesize that the lack of NADPH-oxidase-derived oxidative stress during donor T cell-dependent inflammation limits the clearance of oxidant-sensitive chemotactrant proteins, leading to exaggerated migration of inflammatory cells into the lungs.

Not only were lung-infiltrating cells increased in number, but monocytes/macrophages obtained from day 7 after BMT BALF of T cell-recipient
phox
−/− mice also secreted more TNF-α on a per cell basis than similarly treated controls. Because we previously reported that NO amplifies T cell-dependent TNF-α production after allogeneic BMT (14), we initially expected to find increased

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. $O_2^*$ is known to limit the steady state of NO (36), and scavengers of $O_2^*$ 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 NADPH-oxidase mutant mice. Cy/TBI lethally irradiated on day $-1$, and infused with B10.BR TCD BM plus donor spleen T cells (BMS + Cy). BALF collection and lung weights were performed on day 7 after BMT. Values are means $\pm$ SE for $n = 6-10$ mice per group.

FIGURE 10. A model of the causes of enhanced inflammation in NADPH-oxidase mutant mice. Cy/TBI phox$^{-/-}$ mice given allogeneic T cells exhibit suppressed production of NADPH-oxidase-derived oxidative/nitritative stress but persistent production of NO, which may amplify donor T cell-dependent inflammation. Inhibition of ONOO$^-$, $\cdot$OH, and HCl impairs the clearance of chemokines that attract monocytes and T cells and activate inflammatory cells. Exaggerated production of inflammatory mediators may lead to the formation of noninfectious inflammatory lesions.

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 $O_2^*$ by xanthine oxidase and NO by iNOS. Although our results show decreased oxidative burst and nitritative 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/microcyte-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$^{p47/-}$ 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 $O_2^*$ 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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