NQO1 and NQO2 Regulation of Humoral Immunity and Autoimmunity

Karim Iskander, Jessica Li, Shuhua Han, Biao Zheng, and Anil K. Jaiswal

From the Departments of Pharmacology and Immunology, Baylor College of Medicine, Houston, Texas 77030

NQO1 (NAD(P)H:quinone oxidoreductase 1) and NQO2 (NAD(P)H:quinone oxidoreductase 2) are cytosolic enzymes that catalyze metabolic reduction of quinones and derivatives. NQO1-null and NQO2-null mice were generated that showed decreased lymphocytes in peripheral blood, myeloid hyperplasia, and increased sensitivity to skin carcinogenesis. In this report, we investigated the in vivo role of NQO1 and NQO2 in immune response and autoimmunity. Both NQO1-null and NQO2-null mice showed decreased B-cells in blood, lower germinal center response, altered B cell homing, and impaired primary and secondary immune responses. NQO1-null and NQO2-null mice also showed susceptibility to autoimmune disease as revealed by decreased apoptosis in thymocytes and predisposition to collagen-induced arthritis. Further experiments showed accumulation of NADH and NRH, cofactors for NQO1 and NQO2, indicating altered intracellular redox status. The studies also demonstrated decreased expression and lack of activation of immune-related factor NF-κB. Microarray analysis showed altered chemokines and chemokine receptors. These results suggest that the loss of NQO1 and NQO2 leads to altered intracellular redox status, decreased expression and activation of NF-κB, and altered chemokines. The results led to the conclusion that NQO1 and NQO2 are endogenous factors in the regulation of immune response and autoimmunity.

Quinone oxidoreductases (NQO1 and NQO2) are cytosolic flavoproteins that catalyze two-electron metabolic reduction and detoxification of quinones and derivatives. This leads to protection of cells against oxidative stress and neoplasia. However, in some cases, especially in the absence of conjugating reactions, the hydroquinones undergo oxidation to generate reactive oxygen species causing damage to cells (1, 2). NQO1-null and NQO2-null mice lacking respective gene expression showed alterations in intracellular redox status, myeloid hyperplasia, and higher susceptibility to skin carcinogenesis (3–7). Both NQO1-null and NQO2-null mice demonstrated lower expression and induction of tumor suppressor p53 (7, 8). NQO1 protects p53 from ubiquitin-independent 20S proteasomal degradation (9).

A cytokine to thymidine (C → T) polymorphism in exon 6 of human NQO1 gene produces a proline to serine (P187S) substitution that destabilizes the protein (10, 11). Individuals carrying both mutated genomic alleles are completely lacking in NQO1 activity, whereas heterozygous individuals with one mutated allele have low-to-intermediate NQO1 activity as compared with wild-type individuals (12). Approximately 2–4% human individuals are homozygous and 20–25% are heterozygous for this mutation (13–16).

The roles of genetic factors in immune deficiency and autoimmune diseases have been recognized for decades (17). B cells play a key role in regulation of immune system. B cells produce antibodies, provide support to other mononuclear cells, and contribute directly to inflammatory pathways (18). Impaired B cell production, maturation, homing, and activation are known to lead to defective immune response (19). Dysfunctional immune response and impaired apoptosis in T cells have been implicated in many immunological abnormalities including autoimmune lymphoproliferative syndrome (18, 20, 21).

In this report, we investigated the in vivo role of NQO1 and NQO2 in immune response and autoimmunity. Both NQO1-null mice as well as NQO2-null mice demonstrated lower B cells in peripheral blood, decreased germinal center response, altered B cell homing, and impaired antibody responses. NQO1-null and NQO2-null mice also showed increased susceptibility to autoimmune disease as revealed by decreased apoptosis in thymocyte and predisposition to collagen-induced arthritis. The loss of NQO1 and NQO2 led to accumulation of NADH and NRH that altered intracellular redox status. The studies also demonstrated decreased expression and lack of activation of NF-κB and altered chemokines and chemokine receptors. These results suggest that the loss of NQO1 and NQO2 leads to altered intracellular redox status that results in decreased expression and lack of activation of NF-κB. This leads to B-cell deficiency and alterations in the homing of B cells and impaired humoral immune response and autoimmunity.

MATERIALS AND METHODS

Flow Cytometry Analysis of the Blood, Bone Marrow, Spleen, and Thymus—Blood was obtained from wild-type, NQO1-null, and NQO2-null mice by cardiac stick in EDTA-coated tubes. Mice were euthanized. Femurs, spleen, and thymus were obtained. 100 μl of the blood was added to 2.5 μl of FITC-
labeled anti-CD19, 2.5 μl of PE-labeled anti-CD4, and 2.5 μl of CyChrom-labeled anti-CD8 antibodies gently vortexed and incubated on ice in the dark for 30 min. Red blood corpuscles were hemolyzed and fixed using Coulter Q-prep and analyzed using a Coulter EPICS XL-MCL flow cytometer. Femurs were cut at both ends. Bone marrow was flushed with sterile cold PBS. After two PBS washes, the cells were suspended in annexin binding buffer to a concentration of 10 × 10^6 cells/ml. Spleen cells were suspended in cold PBS using the rough surface of glass slides. Red blood cells were lysed using red blood cell lysis buffer containing 15.5 mM NH4Cl, 1 mM KHCO3, and 0.001 mM EDTA. Cells were suspended in annexin binding buffer to a concentration of 10 × 10^6 cells/ml. Thymocytes were obtained from the thymus and suspended in cold PBS using the rough surface of glass slides. Cells were then suspended in annexin binding buffer to a concentration of 10 × 10^6 cells/ml. 100 μl of cell suspensions (bone marrow, spleen, or thymus) was added to the appropriate antibodies (1 μl of annexin V-FITC, 2.5 μl of PE-labeled anti-CD19, 2.5 μl of FITC-labeled anti-CD43, 2.5 μl of FITC-labeled anti-CD25, 2.5 μl of FITC-labeled anti-IgD, 2.5 μl of FITC-labeled anti-CD19, 2.5 μl of PE-labeled anti-CD4, and 2.5 μl of CyChrom-labeled anti-CD8 antibodies). Samples were gently vortexed and incubated on ice, in the dark, for 30 min. Samples were then fixed in 4% paraformaldehyde and then analyzed using Coulter EPICS XL-MCL flow cytometer.

Thymidine Incorporation Assay of Proliferation in the Bone Marrow and Spleen Cells—The wild-type, NQO1-null, and NQO2-null mice were sacrificed, and their femurs and spleen were obtained. The bones were cut, and marrow was flushed out gently with RPMI 10% fetal bovine serum with antibiotics. Cell samples were cultured in triplicates in 96-well plates for four hours later, cells were harvested on a glass fiber filter mat using Tomtec Harvester 96. Incorporated thymidine was measured on days 21 and 42. On day 42, mice were euthanized. Sections in the paws were examined microscopically.

Microarray Analysis—We used Affymetrix GeneChip mouse expression set 430 and RNA from untreated mice bone marrow for microarray analysis. Three samples for each genotype (wild type, NQO1-null, and NQO2-null) were analyzed. Each sample included a pool of bone marrow cells from five mice. RNA samples were prepared from bone marrow cells using a Qiagen RNeasy kit. The excellent quality of the RNA samples was confirmed with an Agilent 2100 Bioanalyzer. Our core facility analyzed the samples and provided data to us. We used dChip 1.3 and GeneSpring software to analyze data. We categorized alterations in gene expression in several groups according to gene function using dChip 1.3. These categories included apoptotic...
genes such as p53, Bax, Bcl-2, caspase 2, caspase 3, caspase 8, apoptosis inhibitor 6, and C/EBP; interleukins, chemokines, and their receptors such as CXCR4, CCL9, CCR1, CXCL12, interferon γ receptor, interferon γ-induced GTPase, interleukin 7, interleukin 10, and interleukin 10 receptor; and transcription regulation genes, DNA damage response genes, and DNA replication and metabolism genes. The results for chemokines and chemokine receptors are presented as fold increase or decrease.

RESULTS

Immunological Phenotype of NQO1-null and NQO2-null Mice—Previously, we generated NQO1-null mice deficient in NQO1- and NQO2-null mice deficient in NQO2 (6, 27). An analysis of blood, bone marrow, and spleen from wild-type, NQO1-null, and NQO2-null mice was performed. Flow cytometry analysis of blood lymphocytes showed a decrease in the number of CD19+ B cells and an increase in CD4+ T cells (Fig. 1, A and B). However, NQO1-null and NQO2-null mice showed higher numbers of CD19+ cells in the bone marrow (Fig. 1C; compare with A and B). To trace different stages of B cell development in the bone marrow, we analyzed bone marrow cells for CD19 and CD43 (for pro-B cells), CD19 and CD25 (for pre-B cells), and CD19 and IgD (for mature B cells). There was no difference between wild-type, NQO1-null, and NQO2-null mice in the number of B cell progenitors, pro-B and pre-B cells (Fig. 1C). However, there was a significant increase in the
number of mature IgD⁺ B cells in the bone marrow of NQO1-null and NQO2-null mice (Fig. 1C). Apoptosis in bone marrow B cells was lower in NQO1-null and NQO2-null mice as compared with wild-type mice (Fig. 1E). In contrast to lower apoptosis in bone marrow B cells, the knock-out mice showed no difference in apoptosis of spleen B cells as compared with wild type (Fig. 1F).

We then measured proliferation of bone marrow cells and spleen T cells using thymidine incorporation assay. The results are shown in Fig. 1G. NQO1-null bone marrow cells showed significantly higher proliferation rate than that of wild type \( (p < 0.01) \). NQO2-null bone marrow does not proliferate significantly different from that of wild type. NQO1-null spleen cells showed significantly higher proliferation rate than that of wild type \( (p < 0.001) \). NQO2-null spleen T cells proliferate slightly faster than that of wild type \( (p < 0.05) \).

We observed that NQO1-null mice have bigger spleen than wild-type mice. We weighed the spleens of 10 wild-type, NQO1-null, and NQO2-null mice. NQO1-null mice spleen is slightly but significantly bigger than that of wild-type mice (wild type, 105 ± 8, and NQO1-null, 134 ± 10 mg; \( p < 0.05 \)). No significant difference between NQO2-null and wild type in spleen weight was observed.

We analyzed the spleen cells from wild-type, NQO1-null, and NQO2-null mice for marginal zone B cells by flow cytometry after staining for marginal zone B cell markers CD23 and CD21 (Fig. 2A). CD23⁺CD21⁺ marginal zone B cells were also lower in NQO2-null mice as compared with wild type (Fig. 2A). In same experiment, CD21⁺ cells were found...
Increased in NQO1-null mice as compared with wild-type mice. NQO2-null mice also demonstrated an increase in CD21+ cells. However, the increase was significantly lower than NQO1-null mice. The significance of the increase in CD21+ cells remains unknown.

Humoral Immune Response in NQO1-null and NQO2-null Mice—To assess the humoral immune response in NQO1-null mice and NQO2-null mice, we started by evaluating the germinal center response. Eight-week-old wild-type, NQO1-null, and NQO2-null mice were injected intraperitoneally with NP conjugated to CGG. Twelve days after immunization, spleen and bone marrow were obtained for primary immune response analysis. NP-specific AFC were quantitated by ELISPOT assay using nitrocellulose filters coated with NP-BSA 5:1 and 25:1. Labeled anti-IgM Ab and anti-IgG Ab were used to visualize NP-specific AFC. B and D, secondary humoral immune response. Seven-week-old wild-type, NQO1-null, and NQO2-null mice were injected intraperitoneally with 50 μg of NP conjugated to CGG. Eight weeks after primary immunization, mice were injected intraperitoneally with 20 μg of NP-CGG. On day 12, mice were sacrificed and analyzed for AFC. NP-specific AFC were quantitated by ELISPOT assay using nitrocellulose filters coated with NP-BSA 5:1 and 25:1. Labeled anti-IgM Ab and anti-IgG Ab were used to visualize NP-specific AFC.
week-old wild-type mice were immunized with NP-CGG as described earlier. Twelve days after immunization, spleen and bone marrow were obtained for primary immune response analysis. For another set of mice, 8 weeks after primary immunization, mice were injected intraperitoneally with 20 μg of soluble NP-CGG. Twelve days later, mice were sacrificed and analyzed for secondary immune response. Assessment of immune response was done by measuring the number of AFC. Labeled anti-IgM antibody and anti-IgG1 antibody were used to visualize NP-specific AFC. Both NQO1-null and NQO2-null mice (especially NQO1-null) showed weaker primary and secondary immune response (Fig. 3). Serum levels of NP-specific IgG were measured on days 0, 5, and 10 after the secondary immunization. NP-specific IgG were lower in NQO1-null and NQO2-null mice (Fig. 4).

Autoimmunity in NQO1-null and NQO2-null Mice—Decreased apoptosis in the thymus and bone marrow cells of NQO1-null and NQO2-null mice and increased spleen T cell proliferation in NQO1-null mice pointed to the possibility of higher susceptibility to autoimmune disease. We used the collaged-induced arthritis model to assess the susceptibility of NQO1-null and NQO2-null mice to autoimmunity. We found that NQO1-null mice developed arthritis earlier and for a longer duration than wild-type mice (Fig. 5A). Arthritis in NQO1-null mice was more severe than that in wild-type mice. This is reflected as a higher arthritis clinical score (Fig. 5B). There was no significant difference between NQO2-null and wild-type mice in the onset and severity of arthritis (Fig. 5, A and B). However, arthritis lasted longer in NQO2-null mice as compared with wild type (Fig. 5, A and B).

We also analyzed the thymus of wild-type, NQO1-null, and NQO2-null mice for CD4+CD8−, CD4+CD8+, and CD4−CD8+ populations and for apoptosis. No significant difference between wild-type, NQO1-null, and NQO2-null mice in thymic cell population was observed (Fig. 5C). However, there is a significant decrease (about one-half; p < 0.01) in apoptosis in NQO1-null thymocytes as compared with wild type (Fig. 5D). NQO2-null thymocytes also showed significantly lower apoptosis (p < 0.05) (Fig. 5D).

Alteration in Intracellular Redox Status, Decreased Expression, and Activation of NF-κB and Altered Chemokines and Chemokine Receptors—The analysis of bone marrow NADH, NAD, NRH, and NR showed a significant increase in NADH:NAD ratio in NQO1-null and NRH:NR ratio in NQO2-null mice (Fig. 6A). Similar analysis also showed a significant increase in NADPH:NADP ratio in NQO1-null mice as compared with wild-type mice (data not shown). We used electrophoretic mobility shift assay to investigate the expression and LPS activation of NF-κB in wild-type, NQO1-null, and NQO2-null mice. The results are presented in Fig. 6B. Decreased NF-κB binding to DNA was observed with bone marrow nuclear extract from NQO1-null mice as compared with wild-type mice (compare lanes 3 and 5). LPS treatment demonstrated a significant increase in NF-κB binding to DNA in wild-type mice (compare lanes 3 and 4). The LPS-mediated activation of NF-κB binding was more or less not observed in NQO1-null mice (compare lanes 5 and 6). The decreased binding of NF-κB and lack of LPS activation of NF-κB binding to DNA was also observed in NQO2-null mice (data not shown). However, the magnitude of difference was lower in NQO2-null than in NQO1-null mice. The results on the increase in NADH:NAD and NRH:NR ratios and lower NF-κB binding and lack of LPS activation of NF-κB binding to DNA were also observed in spleen and thymus of NQO1-null and NQO2-null mice (data not shown). Microarray analysis of bone marrow from untreated wild-type, NQO1-null, and NQO2-null mice were performed. The microarray analysis revealed alterations in chemokines and chemokine receptors associated with the loss of NQO1 and NQO2 in respective null mice (Fig. 6C). Specially noted were chemokine (CXCl motif) ligand 12, receptor 4, and receptor 1. Interestingly, the alterations noted were of lower
showed weaker primary and secondary antibody response. This involved in the homing of B cells to the bone marrow (28).

CXCr4 and CXCL12 are the chemokine receptor and ligand (CXCL12), 20% in NQO1-null and 5% in NQO2-null mice.

The experiments in this study, for the first time, establish a NQO1 relationship to immune response and autoimmunity. A, NAD(P)H:NAD(P) ratio in bone marrow of wild-type, NQO1-null, and NQO2-null mice. Femurs were surgically removed, and pyridines were extracted with chloroform and analyzed by HPLC procedures as described under "Materials and Methods." The data are shown only for NADH/NAD. B, electrophoretic mobility shift assay. One million bone marrow cells were untreated or treated with 10 μg/ml LPS for 30 min. Nuclear extracts were prepared, and NF-κB binding was analyzed by electrophoretic mobility shift assay. Only the shifted bands are shown. C, microarray analysis. RNA from wild-type, NQO1-null, and NQO2-null mice bone marrow were used to perform microarray analysis. The differences in selected chemokines and receptors are listed as compared with wild-type mice.

magnitude in NQO2-null mice as compared with NQO1-null mice.

**DISCUSSION**

The experiments in this study, for the first time, establish a physiological role of NQO1 and NQO2 in control of immune response and autoimmunity. The NQO1-null and NQO2-null mice showed impaired humoral immune response. The magnitude of impairment in humoral immune response was of higher magnitude in NQO1-null mice than NQO2-null mice. Phenotypic analysis of NQO1-null and NQO2-null mice showed a decrease in the number of B cells in the blood and an increase in the bone marrow, whereas no change in B cell number was observed in the spleen. Further investigations revealed that mature B cells and not pre- and pre-B cells increased in bone marrow of NQO1-null and NQO2-null mice. Since the maturation of B cells to IgD positive mature B cells takes place in the peripheral lymphoid organs, spleen, and lymph nodes (not in the bone marrow), the increase in IgD positive mature B cells in the bone marrow most likely resulted due to the increased homing of mature B cells into the bone marrow. Microarray analysis of the bone marrow showed increased chemokine (CXC motif) receptor 4 (CXCR4) and chemokine (CXC motif) ligand 12 (CXCL12), 20% in NQO1-null and 5% in NQO2-null mice. CXCR4 and CXCL12 are the chemokine receptor and ligand involved in the homing of B cells to the bone marrow (28).

NQO1-null and NQO2-null mice (especially NQO1-null) showed weaker primary and secondary antibody response. This was demonstrated by the lower number of NP-specific AFC in the spleen and bone marrow and lower serum NP-specific IgG after primary and secondary immunization with NP-CGG. Germline center response was also weaker, especially in NQO1-null mice. All these observations led to the conclusion that the loss of NQO1 or NQO2 (NQO1 more than NQO2) resulted in impaired humoral immune response, suggesting that NQO1 and NQO2 are significant endogenous factors in regulation and proper functioning of immune response.

T cell tolerance. T cell tolerance includes elimination of autoreactive T cells (negative selection), mostly by apoptosis (29). Lower apoptosis in the thymus of NQO1-null and NQO2-null mice might compromise T cell tolerance. This might have allowed autoreactive T cells to escape negative selection and thus increased susceptibility to develop autoimmune disease. These findings together pointed toward the possibility of increased susceptibility in NQO1-null or NQO2-null mice to develop autoimmunity. Indeed, NQO1-null mice developed arthritis earlier and for a longer duration than wild-type mice in collagen-induced arthritis model. NQO2-null mice did not show significant difference in autoimmunity from wild-type mice in the same experiment. The higher sensitivity of NQO1-null mice to induce arthritis could be due to impaired T cell tolerance in the lymphoid organs as a result of decreased apoptosis and increased proliferation of T cells. This is supported by the lower apoptosis seen in the thymus and bone marrow cells of NQO1-null mice and the increased proliferation in bone marrow cells and splenic T cells. The decreased apoptosis and increased proliferation in the lymphoid organs of NQO2-null mice were milder than that in NQO1-null mice.

The above observations raised an interesting question regarding the mechanism of NQO1 and NQO2 regulation of immune response and autoimmunity. The loss of NQO1 and NQO2 led to alterations in intracellular redox status. This was due to accumulation of reduced NADH in NQO1-null mice and NRH in NQO2-null mice. Alterations in the redox status of the cells presumably changed transcription and/or modifica-
tion of factors including the loss of expression and lack of LPS activation of NF-κB and alterations in chemokines (including CXCr4 and CCL12). The redox modulation of NF-κB and chemokine/receptor is reported earlier (35). LPS has been shown to cause apoptosis in B cells by activating NF-κB (36), CD4 + CD8 + thymocytes, and lymphoid organs (32). Failure of activation of NF-κB might have contributed to reduced apoptosis in thymocytes in NQO1-null and NQO2-null mice. The alterations in intracellular redox status combined with lower expression and lack of activation of NF-κB might have altered the homing of B cells and reduced antibody responses. The changes in B cells were translated to decreased primary and secondary immune response. Decreased apoptosis and increased proliferation of thymocytes contributed to autoimmune in NQO1-null mice.

The results on impaired immune response and autoimmunity in NQO1-null and NQO2-null mice are extremely significant and have major impact on human health. This is since 2–4% of human individuals are homozygous for C → T mutation in the NQO1 gene, leading to proline to serine substitution, and totally lack the NQO1 protein (10, 11). In addition, greater than 20% individuals are heterozygous and carry one mutated NQO1 allele. These individuals lack 50% NQO1 protein. The NQO1 homozygous, and heterozygous mutant individuals are expected to have impaired immune response and are at risk for autoimmune diseases. This is the first study toward genotyping human individuals for lack of NQO1 and problems associated with impaired immune response and autoimmunity.

In conclusion, NQO1 and NQO2 are important endogenous factors in regulation of immune response and autoimmunity. The loss of NQO1 or NQO2 (especially NQO1) results in impaired humoral immune response and higher susceptibility to autoimmune diseases. The alterations in intracellular redox status due to the loss of NQO1 and NQO2 presumably led to changes in expression and induction of factors including NF-κB, chemokines, and chemokine receptors. These changes resulted in altered B cell homing, reduced B cell function, and decreased apoptosis of thymocytes. These changes led to compromised immune response and autoimmunity. The detailed mechanisms of the role of NQO1 and NQO2 role in regulation of immune response and autoimmunity await future investigations.

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