The Human TLR Innate Immune Gene Family Is Differentially Influenced by DNA Stress and p53 Status in Cancer Cells

Maria Shatz, Daniel Menendez, and Michael A. Resnick

Abstract

The transcription factor p53 regulates genes associated with a wide range of functions, including the Toll-like receptor (TLR) set of innate immunity genes, suggesting that p53 also modulates the human immune response. The TLR family comprises membrane glycoproteins that recognize pathogen-associated molecular patterns (PAMP) and mediate innate immune responses, and TLR agonists are being used as adjuvants in cancer treatments. Here, we show that doxorubicin, 5-fluorouracil, and UV and ionizing radiation elicit changes in TLR expression that are cell line- and damage-specific. Specifically, treatment-induced expression changes led to increased downstream cytokine expression in response to ligand stimulation. The effect of DNA stressors on TLR expression was mainly mediated by p53, and several p53 cancer-associated mutants dramatically altered the pattern of TLR gene expression. In all cell lines tested, TLR3 induction was p53-dependent, whereas induction of TLR9, the most stress-responsive family member, was less dependent on status of p53. In addition, each of the 10 members of the innate immune TLR gene family tested was differentially inducible. Our findings therefore show that the matrix of p53 status, chromosome stress, and responsiveness of individual TLRs should be considered in TLR-based cancer therapies. Cancer Res; 72(16); 3948–57. ©2012 AACR.

Introduction

The p53 tumor suppressor is a master regulatory transcriptional factor activated in response to several stress signals including DNA damage, resulting in transactivation of target genes. It functions in a variety of biologic processes including cell-cycle arrest, apoptosis, senescence, embryo implantation, and nutritional stress (1). p53 binds in vitro as a tetramer to a DNA motif composed of 2 RRRCWWGYYY decamer half-sites (R = A, G; W = A, T; and Y = C, T) separated by a spacer of up to 14 bases (2). Using a combination of yeast and human cell systems (3), we established "rules" whereby p53 was also found to function from noncanonical sequences comprising only a decamer 1/2-site or a 3/4-site (4–6). Using these rules for p53 binding and transactivation, we recently identified p53-binding sites in the promoter region of most human Toll-like receptors (TLR) and showed a direct role for p53 in regulation of this entire family of genes in primary human immune cells (7).

TLRs are a group of highly conserved integral membrane glycoproteins that recognize a variety of distinct pathogen-associated molecular patterns (PAMP; ref. 8). The recognition of PAMPs by TLR-expressing cells leads to quick and acute responses required to eliminate pathogens in the host. Upon ligand stimulation, TLRs recruit adaptor molecules MyD88, TIRAP, TRIF, and TRAM leading to activation of NF-kB, interferon responsive factors, and mitogen-activated protein kinases that, in turn, results in ligand-dependent patterns of gene expression including inflammatory cytokines, chemokines, and interferons (reviewed in ref. 8). In addition, endogenously produced ligands, referred to as damage-associated molecular patterns (DAMP) or alarmins that include self-DNA and intracellular proteins, signal tissue injury through TLRs when DAMPs are released from cells (9). In both cases TLR pathway activation mediates immune/inflammatory responses. Ten TLRs (TLR1–10) have been identified in humans. TLRs are expressed in several types of immune cells including spleen, T and B lymphocytes, dendritic cells, and macrophages (10). In addition, TLRs function in nonimmune tissues such as airway and gut epithelial cells which have direct contact with pathogens (11, 12).

Altered TLR expression has been associated with autoimmune and chronic inflammatory diseases including rheumatoid arthritis, type 1 diabetes, inflammatory bowel disease, liver diseases, rheumatoid arthritis, and systemic lupus erythematosus (13, 14). In recent years, cancer-related functions of the human TLR gene family have become apparent. Impaired expression and signaling by TLR7 and TLR9 may contribute to reduced innate immune responses during chronic viral infections and oncogenesis (15). In addition, the loss-of-function allele Asp299Gly of TLR4 is associated with breast cancer (16).
For cancer cells expressing TLRs, activation of the pathway can negatively affect growth and viability (17) including direct killing of tumor cells via induction of TNFα (18, 19) and, therefore, can be used in treatment. For instance, TLR7 agonist imiquimod is used in treating basal cell and cutaneous malignancies; also, synthetic oligodeoxynucleotides containing unmethylated CpG motifs that activate TLR9 are in clinical trials (20, 21). Stimulation of the TLR pathway in antigen-presenting cells can induce and potentiate an effective immune response against tumor cells (22–25). In addition, TLR ligands conjugated to tumor-derived proteins or immunogenic peptides are used for therapeutic vaccination to treat cancer (reviewed in ref. 26).

Surprisingly, despite the importance of TLRs in human health, disease, and therapy, relatively little is known about their transcriptional regulation. Only a few studies suggest involvement of transcriptional factors such as PU.1, activator protein, interferon response factors, and Forkhead Box P3 in regulation of transcriptional factors such as PU.1, activator protein, interferon response factors, and Forkhead Box P3 in regulation of transcriptional factors (4,5). Therefore, differential effects on TLR gene expression might be expected from the various p53 mutations.

So far, only TLR3 was shown to be a p53 target (31). On the basis of our results with primary human cells where nearly all TLR genes are in the p53 network, we have investigated p53-dependent regulation of the entire TLR gene family in a panel of cancer cell lines and the consequences of p53 mutations on TLR expression. We focused on human cells because there are significant differences in regulation, expression, and specificity of TLRs between humans and rodents (reviewed in ref. 32), especially as relates to p53 responsiveness (7). Here, we establish that most TLR genes are responsive to p53 and genotoxic stress in human cancer cells. The considerable variability in responses of specific TLR genes that is dependent on cell line, specific p53 mutations, and stressors underscores the importance of considering these factors in TLR-based cancer treatments.

Materials and Methods

Reagents and antibodies
All reagents were from Sigma unless stated otherwise. The primary antibodies used were against p53 (DO-1), TLR5 (H1-27), actin (C-11), β-tubulin (H-300), and lamin B1 (H-90; Santa Cruz Biotecnology), p21 (SXM30, BD Biosciences Pharmigen), TLR2 (#2229, Cell Signaling).

Cell lines and treatments
The source and maintenance conditions of cell lines are described in Supplementary Materials and Methods. Where indicated, cells were treated for 24 to 48 hours with the following: Nutlin-3 (10 μmol/L), doxorubicin (0.3–0.6 μg/mL), 5-fluorouracil (5-FU, 300 μmol/L). For ionizing radiation (IR) treatment and UV, cells were irradiated at 1.56 Gy/min or UV at 1 J/m²/s, respectively. Pretreatment with the p53 inhibitor pifithrin-α (40 μmol/L) was for 4 hours. Doses of IR, UV, doxorubicin, and Nutlin-3 were chosen based on preliminary optimization as shown in Supplementary Fig. S1. None of the treatments resulted in noticeable cell death during the course of the experiments, based on visual observation.

Plasmid constructs, transfections, luciferase assays and chromatin immunoprecipitation (ChiP) assays, immunoblot, and gene expression analysis by real-time PCR were carried out as previously described (7). For details see Supplementary Materials and Methods.

Results

p53 modulates expression of TLR genes in breast adenocarcinoma and osteosarcoma cells

The impact of changes in p53 protein levels on TLR gene expression was examined in several cancer cell lines. Increases were accomplished using the p53 inducer Nutlin-3 or p53 overexpression. Nutlin-3 inhibits p53 interaction with its ubiquitin ligase MDM2 leading to p53 stabilization and accumulation (33). The p53 inhibitor pifithrin-α or RNA interference were used to reduce p53-mediated activity. Although pifithrin-α can suppress p53-mediated transactivation, the underlying mechanism of p53 inhibition remains to be established (34).

Shown in Fig. 1A are representative Western blot analysis of p53 and p21 proteins in MCF7 breast adenocarcinoma cells (wild-type p53) and an MCF7 cell line stably expressing shRNAi to p53 (designated as “vector” and “p53i”, respectively) treated with Nutlin-3 or vehicle control dimethyl sulfoxide (DMSO) for 48 hours. As expected, Nutlin-3 greatly increased expression of p53 and its target gene p21 in MCF7-vector cells and only to a small extent in MCF7-p53i cells (less than the basal level of p53 in the MCF7-vector cells). Nutlin-induced changes in expression of TLR genes in MCF7-vector and MCF7-p53i cells are presented in Fig. 1B. Expression of only 3 of the 10 TLR genes was detected in MCF7 cells under our experimental conditions: TLR2, 5, and 6. The TLR genes TLR2, 5, and 6 were induced 3.5- to 6-fold by Nutlin-3 compared with DMSO-treated control in a p53-dependent manner in MCF7 cells. There was a corresponding increase in TLR2 and TLR5 proteins in the membrane fraction of the MCF7-vector cell lysate following Nutlin-3 treatment (a reliable human TLR6 antibody was not available) as described in Fig. 1C.

In contrast to MCF7 cells, all TLRs except TLR8 are expressed in the osteosarcoma cell line U2OS (p53+) and most TLRs were induced by Nutlin increases in p53 (see p53 and p21 proteins in Fig. 1D). There was a 2- to 6.5-fold increase in expression of TLR2, 3, 5, 6, 9, and 10 genes by 24 hours Nutlin-3 treatment compared with the DMSO-treated control in U2OS cells (Fig. 1E). This induction was prevented by pretreatment with pifithrin-α, confirming a specific role for p53 in TLR gene expression.
Figure 1. The p53 activator Nutlin-3 induces expression of TLR genes. MCF7-vector or MCF7-p53i cells were incubated with 10 μmol/L of Nutlin-3 or 0.1% DMSO for 48 hours. Following treatment, cells were harvested and total RNA and protein were isolated. A, p53 and p21 activation were assessed by Western blot analysis. The exposure was extended long enough to allow visualization of and direct comparison between all samples. Actin was used as a loading control. B, expression of TLRs 2, 5, 6, and p21 following Nutlin-3 treatment was assessed using real-time PCR and normalized to expression of the housekeeping gene GUSB. Expression of each gene in DMSO-treated control cells was set as one. C, cell lysates were subjected to subcellular fractionation and 100 μg of protein from each membrane fraction was resolved by SDS-PAGE. Protein levels of TLR2 and TLR5 were detected by Western blotting; gel staining was used for the loading control. D, p53 and p21 induction in U2OS cells following 24 hours of incubation with Nutlin-3 was assessed by Western blot analysis. E, TLR gene expression in U2OS cells incubated with either Nutlin-3, PFTα, a combination of both for 24 hours relative to DMSO-treated control was assessed using real-time PCR. F, SaOS2 cells were transfected either with empty vector or with wild p53 or left untreated. Expression levels of p53 and p21 were examined by Western blotting 24 hours posttransfection. G, TLRs mRNA expression levels following this transfection or Nutlin-3 (10 μmol/L, 24 hours) treatment were examined by qPCR; p21 was used as a positive control. Gene expression in DMSO-treated sample was set as one. Shown are averages of 3 independent experiments. WT, wild-type.
To further investigate a direct connection between p53 and TLR gene expression, wild-type p53 was overexpressed in p53-null osteosarcoma SaOS2 cells (Fig. 1F). The pattern of induction of TLR genes differed between U2OS and MCF7 cells. At 24 hours posttransfection, the levels of TLR2–6, 9, and 10 mRNAs were increased 2- to 10-fold (Fig. 1G) relative to untransfected cells. The TLR1 and 7 gene expression was not changed and TLR8 mRNA was not detected in SaOS2 cells.

Thus, we establish that many TLR genes in cancer cells are responsive to p53. However, there are dramatic differences in the spectra of TLRs transactivated suggesting that a profile of individual cancers needs to be considered if TLR-related therapies are to be used.

**Functional evaluation of proposed p53 response elements in TLR promoters**

On the basis of rules for functional p53 binding, we identified several canonical and noncanonical p53-response elements (RE) located in the promoter vicinity of almost all human TLR genes (summarized in ref. 7; and Supplementary Table S1). Luciferase reporter constructs containing selected response elements from TLR promoter areas were transfected into SaOS2 cells in the presence (solid bars) or absence (open bars) of a vector expressing wild-type p53 and reporter induction was assessed 48 hours posttransfection. Consistent with our previous report, all proposed p53-RE sequences from the TLRs could support p53-driven transcription of a luciferase reporter. For most the levels induced were similar to the moderately responsive p53 target response element of p53-regulated apoptosis-inducing protein 1 (TP53AIP1), which was used as an internal control (Fig. 2A).

The ability of several of these p53-REs to function as p53 target sequences was investigated further using ChIP assays of MCF7 cells treated for 24 hours with Nutlin-3 or doxorubicin, another well-established activator of p53. The p53 occupancy at the TLR promoters was assessed by ChIP analysis and quantified by SYBR Green qPCR, as described in Fig. 2B and Supplementary Fig. S1. The PCR reaction specificity was confirmed using product dissociation curve analysis and by visualization of PCR product on ethidium bromide–stained gels (Supplementary Fig. S2). Binding to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter or a region in the TLR5 promoter distant from the p53-RE provided a negative control. The binding of p53 to RE sequences in the promoter regions of the TLR2, TLR5, and TLR6 genes was enriched 3- to 6-fold at 24 hours after Nutlin-3 or doxorubicin treatments compared with the control (untreated or DMSO-treated cells). This is similar to the binding enrichment at the well-established p53-REs in p21 or PUMA promoters. Thus, as found in primary cells, TLRs can be direct transcriptional targets of p53 in cancer cells.

**Role of p53 in doxorubicin and 5-FU–induced upregulation of TLRs in MCF7 and U2OS cells**

Many clinical anticancer treatments rely on induction of DNA damage as well as p53 activation. Because several TLR ligands have been used as adjuvant treatments in combination with anticancer agents (35), profiling TLR gene expression in response to p53 in different cancer cell lines is expected to be relevant to therapeutic treatments. We, therefore, determined whether DNA damage induced by the commonly used anticancer agents doxorubicin, 5-FU, and IR can alter expression of TLR genes in cancer cell lines and what role is played by p53.

MCF7-vector and MCF7-p53i cells were treated with 0.3 μg/mL of doxorubicin (dxr) or 10 μmol/L of Nutlin-3 for 24 hours. Occupancy of p53 at promoters of TLRs 24 hours later was assessed by ChIP assay and quantified by qPCR.

Figure 2. Functional evaluation of proposed p53 response elements (RE) in TLR promoters. A, SaOS2 cells were transfected with luciferase reporter constructs containing p53-RE sequences from different TLRs in the presence (solid bars) or absence (open bars) of the vector expressing wild-type p53. At 48 hours posttransfection, induction of the luciferase reporter was assessed. Relative luciferase activity was compared with the wild-type p53 smart pool (p53i) or scrambled oligos (scr) and subsequently treated for 24 hours with doxorubicin.

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upon doxorubicin treatment which was prevented by transfection with p53 siRNA (Fig. 3D). As shown in Fig. 3E, there was a strong (9- to 11-fold) p53-dependent induction of TLRs 3 and 9. However, there also may be p53-independent induction of TLRs 2, 5, 7, and 10. The pattern of induction by 5-FU was similar to doxorubicin except that there was p53-independent induction of TLR1 (Fig. 3F). Altogether, the results show that even though an increase in p53 is sufficient to drive expression of TLRs in U2OS cells (as shown for Nutlin-3, Fig. 1), there are cell-specific as well as additional stress-specific factors that can contribute to induced expression of the TLR genes.

Doxorubicin-mediated increase in TLR5 enhances flagellin-induced cytokine responses

Next, we determined whether a p53-dependent increase in TLR expression affects functional response to TLR ligands downstream of receptor activation. MCF7 cells were treated with doxorubicin to induce TLR5 protein or left untreated for 48 hours (Fig. 4A) and then exposed to the TLR5 ligand flagellin for 3 hours. There was a significant 2- to 3-fold increase in response to flagellin in p53-positive MCF7-vector cells pretreated with doxorubicin as shown by increased production of cytokines interleukin (IL)-6 and IL-8 mRNA (Fig. 4B and C).
Importantly, this effect was p53-dependent based on reduction in the MCF7-p53i cells.

**Differential expression profile of TLR genes across cancer cell lines**

As shown earlier, several TLR genes were subject to p53 regulation in the 3 cell lines examined; however, the extent of induction and response to specific agents was variable between cell lines. Because induction of innate immune genes may be important in cancer treatments and differences between cancers could be meaningful, we expanded the panel of cell lines examined for TLR responses to DNA-damaging agents and p53 activation. Changes in TLR gene expression following 24 hours treatments with Nutlin-3, doxorubicin, 5-FU, IR, and UV (all known to activate p53) are presented in a heatmap format in Fig. 5 and in Supplementary Table S2 (conditions for exposure of MCF7 cells to Nutlin-3 and doxorubicin were modified as described in the table). The red, green, and black squares correspond, respectively, to >2.5-fold induction; less than 0.5 expression (i.e., repression) and modest or no change in expression (0.5- to 2.5-fold).

The TLR2, 3, and 9 genes were subject to damage/p53-mediated induction in almost all p53-expressing cell lines. Reintroduction of functional wild-type p53 in p53-null cancer cells (colon carcinoma HCT116 p53-, osteosarcoma SaOS2, and lung cancer H1299) also led to induction of most TLRs in at least 2 of the 3 cell lines evaluated. Clearly, there is considerable variation in responsiveness of the TLR genes between agents across the many cell lines tested. Importantly, induction is largely dependent on p53.

**Effect of p53 mutations on TLR expression**

Having established that wild-type p53 can regulate expression of TLR genes, we assessed the potential for mutant p53 to activate endogenous TLR genes as mutants that retain function are frequent in cancers. The osteosarcoma SaOS2 p53-null cells were transiently transfected with vectors that express p53 mutant proteins at comparable levels (Fig. 6A).

Seven cancer-associated p53 mutants were analyzed, and the TLR expression results are presented as a heatmap in the Fig. 6B. Included is a brief description of the functional transactivation status for each p53 mutant based on yeast reporter assays (36, 37). Also presented is the frequency of these mutations in human somatic and germline tumors. The levels of mRNA from all the TLR genes were evaluated 24 hours posttransfection. Consistent with our previous findings, TLR8 expression was not detected. B2M and p21 gene expression were used as respective negative and positive controls for p53 dependency.

As shown in Fig. 6, 3 patterns of gene expression were observed. Mutants A138V, P151H, and G279R retain the ability to drive expression of most TLRs at levels similar to wild-type p53. However, both A138V and P151H have decreased ability to drive expression of TLR3 gene, a strong target of the wild-type protein. The P151H was unable to induce TLR4. A fourth mutant in this group, G279R, had a profile similar to wild-type for TLRs 1, 2, 3, and 4 but reduced capacity to transactivate TLRs 5, 9, and 10 along with p21.

A much greater change-of-spectrum for TLR expression was observed for M133T and R337H. The former exhibited limited transactivation for TLR4 and TLR5 only. The R337H mutant showed a clear change-in-spectrum of TLR expression when compared with wild-type p53. In the third group, the cancer-
mutants were also evident for the p53 target gene as well as effects on endogenous expression of the cancer-associated p53 mutants can have dramatically different increased levels of p53. In support of this, we showed that opportunities in TLR-based cancer treatments, especially as associated loss-of-function hotspot p53 mutants R175H and G245S were transcriptionally inactive toward all TLR genes as well as p21. Differences in transactivation profiles for the p53 mutants were also evident for the p53 target gene p21. Thus, cancer-associated p53 mutants can have dramatically different effects on endogenous expression of the TLR family of genes and potentially affect their transcriptional response to stress and downstream signaling.

Discussion

Here, we show that expression of most members of the TLR gene family in human cancer cells can be responsive to p53 activation and genotoxic stress. This is expected to increase opportunities in TLR-based cancer treatments, especially as therapies may include both DNA-damaging agents and increased levels of p53. In support of this, we showed that treatment of p53-positive MCF7 cells with doxorubicin before activation of TLR5 by its ligand flagellin leads to increased downstream expression of cytokines IL-6 and IL-8. Interestingly, treatment of breast cancer cells with flagellin was recently shown to reduce cell proliferation in culture and xenograft growth in mice. This effect was in part mediated by flagellin-induced soluble factors (38). Similarly, a combined treatment with flagellin along with a class I phosphoinositide 3-kinase inhibitor resulted in delayed tumor growth and increased animal survival in several mouse models (39). Therefore, an increased expression of cytokines in response to Nutlin-3/flagellin combination might predict better treatment outcome.

We have shown that there is considerable variation in transcriptional responses of specific TLR genes between cell lines and stressors. For example, TLR3 expression was induced by all genotoxic agents tested in almost all the p53-positive cell

Figure 5. Genotoxic stress and p53 activation differentially affect expression of TLR1 to 10 genes across a variety of cancer cell lines. Presented is a heatmap of changes in TLR gene expression after the following treatments: 24 hours incubation with Nutlin-3, 10 μmol/L; doxorubicin, 0.6 μg/mL; and 5-FU, 300 μmol/L. Cells were also exposed to IR, 10 Gy; UV, 20 J/m² and harvested 24 hours after the treatment. MCF7 cells were exposed to Nutlin-3, 10 μmol/L, and to 0.3 μg/mL doxorubicin in MCF7 cells for 48 hours. Gene expression is relative to the untreated control cells, set as one. The red, green, and black squares correspond, respectively, to >2.5-fold induction; less than 0.5 expression (i.e., repression) and modest or no change in expression (0.5- to 2.5-fold). The values are averages of 3 to 6 independent experiments.
lines examined as well as by overexpression of p53 protein in p53-null cells. TLR3 was previously shown to induce apoptosis in cancer cells (18, 40), suggesting that a combination of TLR3 ligands with genotoxic drugs could be beneficial for treatment of tumors containing wild-type p53. Alternatively, because DNA damage can induce TLR9 expression in p53-dependent and -independent manners, it may be possible to amplify TLR9-based treatment by genotoxic agents regardless of tumor p53 status. Similarly, enhanced expression of TLR1 and TLR7 may be associated with cell type as well as with DNA damage response mechanism. We did not see a dependence on p53 status for treatments leading to enhanced TLR7. The TLRs 2, 4, 5, and 6 appeared to be less subject to induction across the cell lines examined; however, the induction generally required the presence of functional p53. Previously, we reported that a wide diversity in responses was also seen for p53-induced expression of TLR genes in stimulated lymphocytes from healthy individuals (7). Similar differences between individuals in terms of transcriptional response to IR have been observed in derived lymphoblastoid cell lines (41). Therefore, variability between cell lines may originate from either interindividual or tissue type-dependent difference.

The altered function p53 mutations can result in cellular responses that impact genome stability, repair, replication, and programmed cell death (36, 42). Single amino acid changes in p53 that differentially impact transactivation may alter the efficacy of chemotherapeutic agents and diversify cell responses to stress (43). Here, we evaluated the effect of several p53 mutants associated with both somatic and germline tumors, particularly breast and colon cancers [Fig. 5 and the IARC database R15 (ref. 30, http://www-p53.iarc.fr/)], on expression of the TLR genes. The changes in TLR transactivation patterns, including change-of-spectrum, are consistent with our previous report in which each of mutant was tested for transactivation at 11 validated human p53-REs using a yeast-based system (4).

While the classic loss-of-function mutants R175H and G245S were unable to induce any TLR gene, the profile of TLR-expressed genes for the remaining 5 p53 mutants was altered. The R337H mutation resulted in the most dramatic changes including loss or gain of transactivation for different TLRs (Fig. 5). Located on the surface of the tetramerization domain of the p53 protein, the R337H mutation is considered to have a subtle functional transactivation change causing a pH-dependent effect on folding (44). The differential response of TLR
genes to this p53 mutant, which is associated with pediatric adrenal cortical carcinoma, could inform treatments that are TLR ligand–based. In our study, we observed that the B37TH p53 mutant might have the ability to induce expression of TLR2. Other factors also may play a role in the regulation of the adrenal response as shown for TLR9 and its relation with sepsis response in the presence of bacterial DNA (45). These observations support the view that the specific status of p53 should be considered in designing adjuvant cancer therapies that use TLR pathways.

Overall, we suggest that a profile of TLR gene expression patterns in specific tumors in response to p53 and DNA-damaging agents combined with knowledge of p53 expression and mutation status in these tumors can be an important tool in cancer diagnosis and in strategies that target TLR pathway for cancer therapy. For example, the presence of a p53 mutation that can change a damage-induced TLR expression pattern could determine which TLR agonists or antagonists to use for specific tumors as well as the choices of p53 adjuvant inducers of TLRs. Also, we suggest that there are unique opportunities to capitalize on chromosome stress responses in human cancer therapies that might not be apparent from studies in other organisms as the responsiveness of the human set of TLR genes to p53 is unique to primates (7).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: M. Shatz, D. Menendez, M.A. Resnick
Development of methodology: M. Shatz, D. Menendez, M.A. Resnick
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Shatz, D. Menendez
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Shatz, D. Menendez, M.A. Resnick
Writing, review, and/or revision of the manuscript: M. Shatz, D. Menendez, M.A. Resnick
Administrative, technical, or material support (i.e., reporting and organizing data, constructing databases): D. Menendez, M.A. Resnick
Study supervision: D. Menendez, M.A. Resnick

Acknowledgments
The authors appreciate discussions with David Schwartz at early stages of work and critical comments of Carl Anderson, Michael Fessler, and Stavros Garantziotis during manuscript development.

Grant Support
The study was supported by NIEHS intramural research funds, project Z01-ES065079.

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Received December 28, 2011; revised May 17, 2012; accepted May 19, 2012; published OnlineFirst June 6, 2012.

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