

The Toll-Like Receptor Gene Family Is Integrated into Human DNA Damage and p53 Networks

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Abstract

In recent years the functions that the p53 tumor suppressor plays in human biology have been greatly extended beyond “guardian of the genome.” Our studies of promoter response element sequences targeted by the p53 master regulatory transcription factor suggest a general role for this DNA damage and stress-responsive regulator in the control of human Toll-like receptor (*TLR*) gene expression. The *TLR* gene family mediates innate immunity to a wide variety of pathogenic threats through recognition of conserved pathogen-associated molecular motifs. Using primary human immune cells, we have examined expression of the entire *TLR* gene family following exposure to anti-cancer agents that induce the p53 network. Expression of all TLR genes, *TLR1* to *TLR10*, in blood lymphocytes and alveolar macrophages from healthy volunteers can be induced by DNA metabolic stressors. However, there is considerable inter-individual variability. Most of the *TLR* genes respond to p53 via canonical as well as noncanonical promoter binding sites. Importantly, the integration of the *TLR* gene family into the p53 network is unique to primates, a recurrent theme raised for other gene families in our previous studies. Furthermore, a polymorphism in a *TLR8* response element provides the first human example of a p53 target sequence specifically responsible for endogenous gene induction. These findings—demonstrating that the human innate immune system, including downstream induction of cytokines, can be modulated by DNA metabolic stress—have many implications for health and disease, as well as for understanding the evolution of damage and p53 responsive networks.

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Introduction

The p53 master regulator is responsive to a variety of DNA metabolic stresses resulting in induction or repression of over 200 genes as well as several LINC- and micro-RNAs [1,2]. In its role as tumor suppressor and “guardian of the genome” many of the target genes in humans influence cell cycle progression or apoptosis. Over the past decade the p53 network has been extended to transcriptional regulation of genes associated with a wide variety of biological functions including DNA repair, angiogenesis, cellular metabolism, autophagy, stem cell renewal, fertility, differentiation and cellular reprogramming. To better understand the broad role that p53 can play in human biology, we have pursued “functionality rules” for identifying target response element (REs) sequences where p53 can directly influence transactivation. Using *in vivo* transactivation systems based in yeast and human cells as well as binding in human cell extracts [3,4], we recently found that functionality of the binding consensus RRRRCWWGYYYnRRRCWWGYYY (R, pyrimidine; Y, pyrimidine; W, A or T; n, spacer of 0 to 13 bases) is greatest when

there is at most a single base spacer and if “WW” is “AT.” Furthermore, we defined functionality for half-sites (RRRCWW-GYYY) and found *in cis* synergy when another transcription factor, estrogen receptor, was bound nearby (for a description of noncanonical REs including half-sites see [3,5] and summary in [6]). Based on these functionality rules, we found that the evolution of p53 control of at least one gene family, DNA metabolism and repair, is limited to primates [7]. Furthermore, we identified single nucleotide polymorphisms in REs that are predicted to modify responsiveness of genes to p53 mediated stress [8].

Our functionality studies of canonical and noncanonical promoter p53 REs suggested that p53 may have a role in human Toll-like receptor (TLR) expression. We had reported that a single nucleotide polymorphism (ChrX:12923681, rs3761624 A/G) in the promoter of the *TLR8* gene creates an RE that can be targeted by p53 in yeast and cell line reporter systems [8] although we could not detect endogenous expression of the *TLR8* gene. In addition, the *TLR3* gene in epithelial cancer cell lines was found to be induced by p53 following exposure to 5-fluorouracil (5FU) [9].

Author Summary

Among the most prominently studied regulators of gene function is the p53 tumor suppressor, which has many roles in human biology. The transcriptional master regulator p53 directly targets expression of >200 genes. Previously, we sought to define the p53 network in terms of functionality, specifically the ability of target response element sequences (REs) to support p53 transactivation. Here we identify p53 target canonical and noncanonical REs in the family of Toll-like Receptor (TLR) innate immune response genes and establish p53 regulation of most *TLR* genes. We address p53 responsiveness in primary human lymphocytes and alveolar macrophages collected from healthy volunteers. Notably, all *TLR* genes show responses to DNA damage, and most are p53-mediated. However, there is considerable variability between individuals, suggesting that DNA and p53 metabolic stresses can markedly differ in impact on the innate immune system as well as downstream appearance of cytokines. Indeed, we report a SNP in a p53 RE within the *TLR8* promoter that alters p53 responsiveness in primary human cells. Furthermore, the p53-mediated expression of *TLRs* is unique to primates. Overall, these findings identify a new, pivotal role for the well-known human tumor suppressor p53, namely, integration of DNA damage and innate immune responses.

Innate immunity is paramount to infection and tissue injury responses [10]. The *TLR* gene family mediates innate immunity to a wide variety of pathogenic threats. The TLRs recognize conserved exogenous pathogen-associated molecular patterns (PAMPs) and endogenous danger-associated molecular patterns (DAMPs) [11], while adaptive immune receptors ensure specific antigenic responses through clonal expansion. TLR function is associated with circulating immune cells such as monocytes and dendritic cells, tissue phagocytes that include alveolar macrophages and with nonimmune cells (such as epithelial cells of gut, skin, lung, *etc.*) that are exposed to environmental injury or pathogens. The roles of TLRs in mammalian biology are continuously expanding and they are now understood to function in such diverse processes as inflammation, cell differentiation and cell survival [11]. Altered TLR function is implicated in human diseases such as systemic lupus erythematosus, inflammatory bowel disease (IBD) and cancer [11,12], and agonist/antagonist manipulations of the TLR system are being pursued to alleviate various diseases (reviewed in [13]).

Given the varied functions of TLRs, factors that regulate their expression are expected to shape immune responses [14]. However, there are few examples of *TLR* gene induction and these are limited to specific TLR-stimulus interactions [15,16]. The TLR-dependent innate immune response is thus generally considered to be “hard-wired.” Although environmental stress can influence gene expression indirectly, *e.g.*, through epigenetic mechanisms, to our knowledge there are no reports of TLR induction by environmental factors in primary human cells [15,17–20].

These collected observations led us to investigate the responsiveness of *TLR* genes as a class to common DNA stressors. We have examined expression of the entire *TLR* gene family following exposure to anti-cancer agents that induce the p53 network in primary immune cells obtained directly from human subjects as well as the impact on downstream cytokines. Agents were applied to T-lymphocytes and alveolar macrophages *ex vivo*. Prior to this there were no reports that we are aware of addressing DNA

damage-induced responses of any *TLR* genes in cells directly associated with innate immunity. As part of this study, we establish that in the evolution of the TLR responses, the p53-mediated expression of *TLRs* is unique to primates.

Results

DNA stress induces TLR gene family expression

We selected ionizing radiation (IR), 5FU and Doxorubicin (Doxo) because these agents represent a cross-section of DNA metabolic stressors, such as damage or replication inhibition, that may occur endogenously or environmentally and are well-known to activate p53 and its network of target genes. Furthermore, these agents are often employed in cancer treatments. To address TLR responses *ex vivo* in primary human immune cells, T-lymphocytes were expanded by phytohemagglutinin (PHA) stimulation of peripheral blood mononuclear cells (PBMC) freshly isolated from the blood of healthy human volunteers (see Figure S1A; for demographics of volunteers see Table 1); alternatively, T-lymphocytes were obtained from the PBMC fraction using anti-CD3 antibody-based purification as described in Figure S1B.

Table 1. Demographics of subjects in this study and genotype information for *TLR8* p53RE SNP of donor population.

Donor	Age	Gender	Race	TLR8 p53RE SNP genotype*
BS#1	31	Male	Caucasian	G
BS#2	57	Female	Caucasian	A/A
BS#4	52	Female	African American	G/G
BS#5	25	Female	Caucasian	G/A
BS#7	25	Female	Caucasian	G/G
BS#8	35	Female	Caucasian	G/A
BS#9	42	Female	Caucasian	A/A
BS#11	25	Female	Caucasian	A/A
BS#12	42	Male	Caucasian	A
BS#13	40	Male	Caucasian	A
BS#15	29	Female	Caucasian	G/A
BS#16	38	Male	Caucasian	G
BS#19	22	Male	Caucasian	G
BS#20	23	Male	Caucasian	A
BS#21	26	Female	Caucasian	G/G
BS#25	30	Female	African American	A/A
BS#26	47	Male	Caucasian	A
BS#27	23	Male	Caucasian	G
CRU1149	21	Male	Caucasian	A
CRU1161	21	Female	Caucasian	A/A
CRU1163	22	Male	Caucasian	G
CRU1164	28	Male	Caucasian	G
CRU1169	34	Male	African American	A
CRU1173	19	Male	Caucasian	A

*The *TLR8* SNP is located in the promoter region of the gene and the A allele impairs functionality of a p53 response element (rs3761624), GGCAAGATGAAACAT(*G*/A)TCA (SNP is in italics and underlined bases) [8]. Reported allele frequency for the G allele is 0.44 and for the A allele is 0.56 (the common polymorphism). The *TLR8* gene is located on the X chromosome. doi:10.1371/journal.pgen.1001360.t001

Presented in Figure 1 are the expression responses of the entire family of *TLR* genes in stimulated lymphocytes for 18 subjects (except where noted) following exposure to IR (4 Gray), 5FU (300 μ M), and Doxo (0.3 μ g/ml) (Figure 1A, 1B, and 1C, respectively). These responses are relative to no treatment controls and normalized to 18S ribosomal DNA. As additional controls, we also examined the expression of the beta-glucuronidase, *GUSB*, and actin genes (Figure S2) that are considered to be nonresponsive to chromosomal and/or p53 stress; they showed little variation after doxorubicin and nutlin exposure. The doses chosen were similar to therapeutic doses or doses commonly used in the literature. By way of comparison, we also examined the response of the cyclin-dependent kinase inhibitor gene *p21^{WAF1}* (*CDKN1A*), a prototypical p53 target gene induced by these agents in a variety of human cells. Notably, these results establish that expression of all *TLRs* can be responsive to DNA metabolic insults, even exceeding p21 induction. However, there is considerable variability in the individual responses between subjects, *TLRs*, and treatments as summarized in the “box and whiskers” presentation of Figure S2. For example, the IR induction of the ten *TLR* genes varies from 1- to over 4-fold for each *TLR* except *TLR3* (Figure 1 and Figure S2), which is not detected in the PHA-stimulated lymphocytes of most subjects. This agrees with the large differences in expression of IR-inducible genes between human lymphoblastoid cell lines [21]. The variability in gene expression among the 18 subjects can represent a continuum of responses (e.g., *TLR4* expression after IR), or exhibit more of a binary induction pattern (e.g., *TLR8* response after IR; see Figure 1). Specifically, for the *TLR8* gene, approximately half the subjects respond strongly and half exhibit a much lower response, a finding that appears to be genetically determined [8], as discussed below. Different agents also elicit differential *TLR* gene expression responses. For example, *TLR1* is responsive to IR by more than 2-fold in most subjects, but generally there is only a small level of induction in response to 5FU or Doxo.

To better assess each *TLR* gene response across subjects, we portrayed the induction of *TLRs* for each subject in a format akin to a heat map, as described in Figure 1E for IR, where 2.5-fold induction is indicated in red and <2.5 fold in black (this value corresponds to the minimal *p21^{WAF1}* response for nearly all agents and subjects; see Figure S3 for other agents). Among three subjects (BS4, 5, 21), all *TLRs* were induced at least 2.5-fold by IR exposure, while only 1 (BS2), 2 (BS9) or 3 (BS8, 13) *TLRs* were induced in four others. There is no obvious pattern to the differences between *TLRs* or subjects for IR as well as for 5FU and Doxo, although it is clear that *TLR1* is generally much less responsive to the last two agents (Figure 1, Figures S2 and S3). Consistent with *TLR3* induction by DNA damage in cancer cell lines [9], its expression was induced in 5 out of 7 subjects; however, *TLR3* gene expression was not detected in 11 subjects (Figure 1 and Figure S3). Even though all *TLRs* are responsive in at least one subject, only subjects BS4, 5 and 21 exhibited high responses for most *TLRs* for all agents tested (Figure S3).

We also addressed statistically the responsiveness of the population as a whole (18 subjects, Figure 1A, 1B, 1C, except *TLR3* which was expressed only in 7 subjects) to IR, 5FU, and Doxo employing a t-test (see Material and Methods) to assess the ability of each agent to induce expression of each *TLR* gene in the population. As expected, induction of the p21 gene in the population was highly significant for all the treatments ($p < 0.0001$). While there was variation between individuals, all the *TLR* genes in the population were responsive to IR ($p < 0.0001$). A similar likelihood of responsiveness ($p < 0.0001$) was observed for 5FU and Doxo treatment for all but the *TLR1* and 7 genes ($p < 0.003$) and

TLR9 ($p = 0.011$ for 5FU and 0.053 for Doxo); however, there were substantial responses for the *TLR* 9 genes of several individuals. Collectively, these results suggest that multiple pathways may influence *TLR* expression after DNA damage, and these are specific to the mode of chromosomal stress.

p53 directly enhances TLR expression

Since p53 mediates many DNA damage responses and given our finding that all *TLRs* respond to at least one DNA metabolic disruptor, we sought to examine in more detail the ability of p53 to induce *TLRs*. PHA-stimulated lymphocytes were exposed to the p53 activator nutlin (10 μ M) to increase p53 levels. Stabilization of p53 normally occurs through stress-induced post-translational modifications affecting both p53 and MDM2 [22]. Nutlin can directly prevent p53 destruction by interfering with the MDM2-p53 interaction [23]. As expected, all treatments activated the p53 pathway in the lymphocytes of all individuals as assessed by immunodetection of p53 and p21 proteins (Figure S4; Figure 1F is a representative example). As shown in Figure 1D, Figures S2 and S3, nearly all *TLR* genes in most subjects are induced over 2.5 fold after nutlin treatment, except for *TLRs* 1 and 7. The *TLR1* gene much less responsive while the *TLR7* gene is generally repressed by p53 induction. Interestingly, *TLR7* is induced by the DNA damaging treatments, suggesting p53-independent induction mechanisms. While *TLR3* is not detected in 11 subjects, it is induced by nutlin in 5 of the remaining 7 subjects. Although there was variation between individuals, we found that most of the *TLR* genes in the population were responsive to nutlin. There was no statistically significant induction of the *TLR9* gene ($p = 0.11$); however, there was statistically significant repression of the *TLR7* gene ($p = 0.006$). The expression of all the remaining *TLR* genes was significantly induced in the population ($p < 0.003$).

A role for p53 in *TLR* induction is further indicated by the finding that co-treatment with the p53 inhibitor pifithrin-alpha [24] represses the induction of *TLRs* by IR and other agents (Figure 1G for IR; Figure 2 for Doxo, 5FU and nutlin). Notably, samples taken on two occasions from the same individuals in a 4 month-interval, blind study revealed a striking consistency in *TLR* gene expression patterns (Figure 3), thereby excluding technical or temporal variables as significant sources of variability in findings.

The p53 inducibility of most *TLRs* led us to investigate if p53 could act directly on transcription. As discussed in the Introduction, the commonly accepted consensus target RE consists of two decamers composed of (RRRC A/T A/T GYYY) separated by up to 13 bases (reviewed in [25,26]). We had established rules for predicting *in vivo* functionality of p53 REs including separation of <2 bases and dependence on a core CATG (summarized in [3,6]). Among several potential p53REs identified by *in silico* search and bioinformatic tools, we found that each *TLR* has at least one p53 target sequence within ± 5 kb of the transcription start site that is predicted to provide at least a weak-to-modest p53 responsiveness (Table 2 contains the sites predicted to have the greatest functional responses). As shown in Figure 4A, each of these target sequences (*TLR7* was not examined because we did not find any p53-like binding sequences) could support p53-driven transcription of a luciferase reporter co-transfected, along with a p53 expression plasmid, into p53 null H1299 cells. For half of the *TLRs*, the induction levels were comparable to those obtained with the moderately responsive p53 target response element of AIP.

Based on these results, we examined whether the functional REs of Figure 4A are indeed targets of induced p53 using chromatin immunoprecipitation (ChIP) analysis. We chose a representative subject BS4 whose lymphocytes exhibited strong Doxo-induced expression (Figure 1) for all *TLRs* except *TLR1* and *TLR7*. As

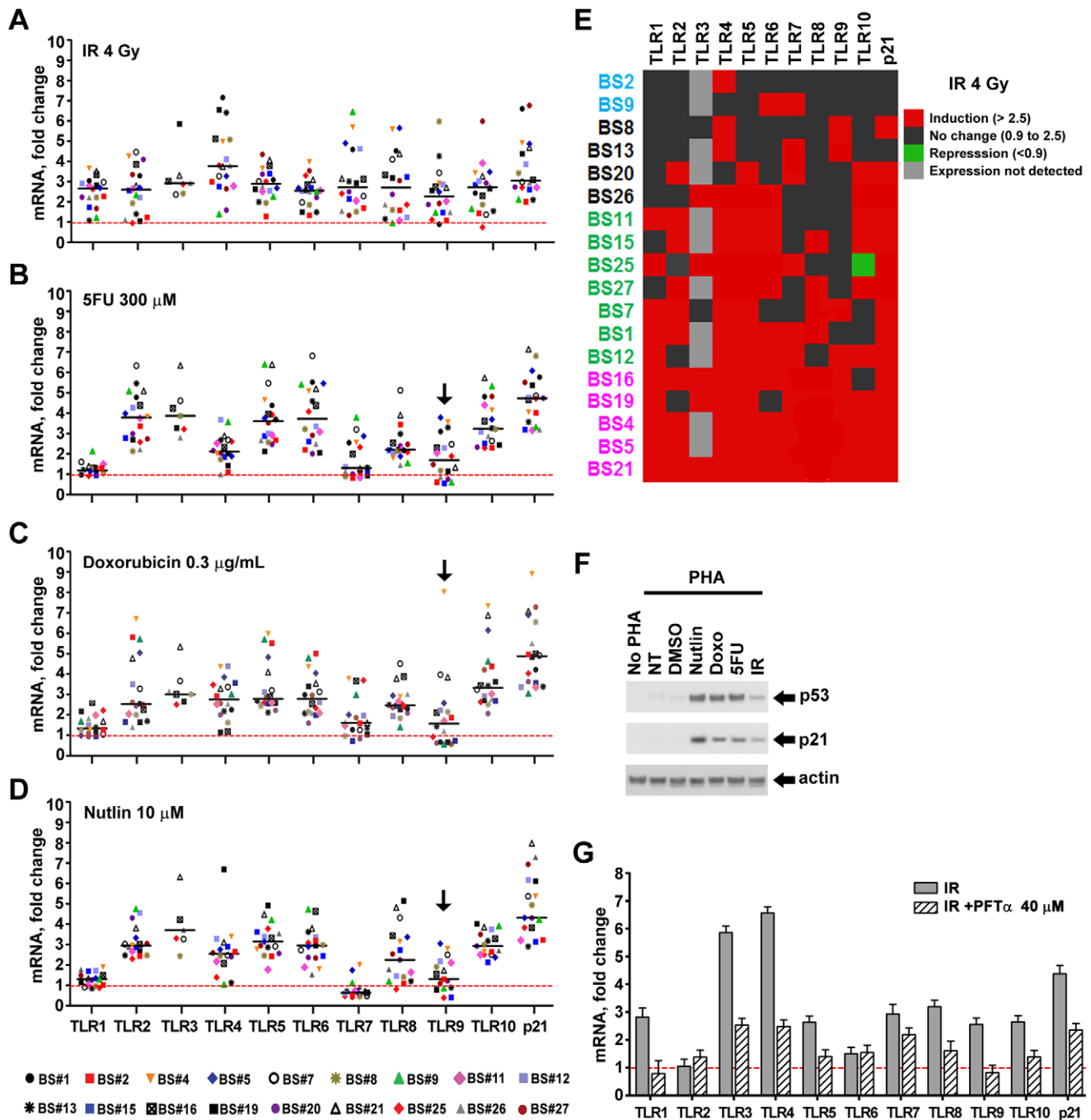


Figure 1. Induced expression of the TLR gene family in primary human T-lymphocytes by DNA stressors and activation of the p53 pathway in cells from healthy subjects. Human peripheral blood mononuclear cells (PBMC) freshly isolated from healthy subjects ($n = 17-18$) were incubated with PHA to stimulate T-lymphocyte expansion. After 48 h incubation, cells were exposed to (A) ionizing radiation (IR); (B) 5-fluorouracil (5FU); (C) doxorubicin or (D) nutlin. Cells were harvested following 24 h treatment in the presence of PHA. In the case of IR, they were harvested 24 h after exposure to 4 Gy. (Robust responses were observed under these conditions; however, further studies might reveal more optimum times and dose for primary cells.) Presented in panels A to D are mRNA expression levels of *TLRs* and *p21^{WAF1}* as compared to expression in untreated cells for each subject (the dashed red line corresponds to no change with treatment, *i.e.*, a value of 1; horizontal bars correspond to the median). Gene expression was analyzed by qPCR and normalized to 18S ribosomal RNA. *Statistical analysis in A–D.* Unless noted, the *TLR* genes of the population as a whole exhibited statistically significant change in expression induced by an agent (vs. untreated) at the $p < 0.006$ level (see text). Black arrows indicate changes that are not significantly different from the control for specific *TLR* genes in the population ($p \geq 0.05$). (E) Changes in *TLR* expression after IR of cells from all subjects (BS#) presented as a heat map. The subjects are grouped according to IR responsiveness (*i.e.*, purple, for nearly all *TLRs* being inducible and blue for people with only a few *TLRs* induced by IR). (F) p53 and p21 activation analyzed by Western blot in a representative experiment for subject BS#7 with actin as a loading control. (G) Inhibition of IR-induced *TLR* gene expression by the p53 inhibitor pifithrin- α (PFT α , 40 μ M) added to cells 2 h prior to IR and kept there after exposure. Values are relative to cells treated with DMSO represented as the dash red line, *i.e.*, a value of 1. Presented is a representative experiment with T-lymphocytes from subject BS#19.

doi:10.1371/journal.pgen.1001360.g001

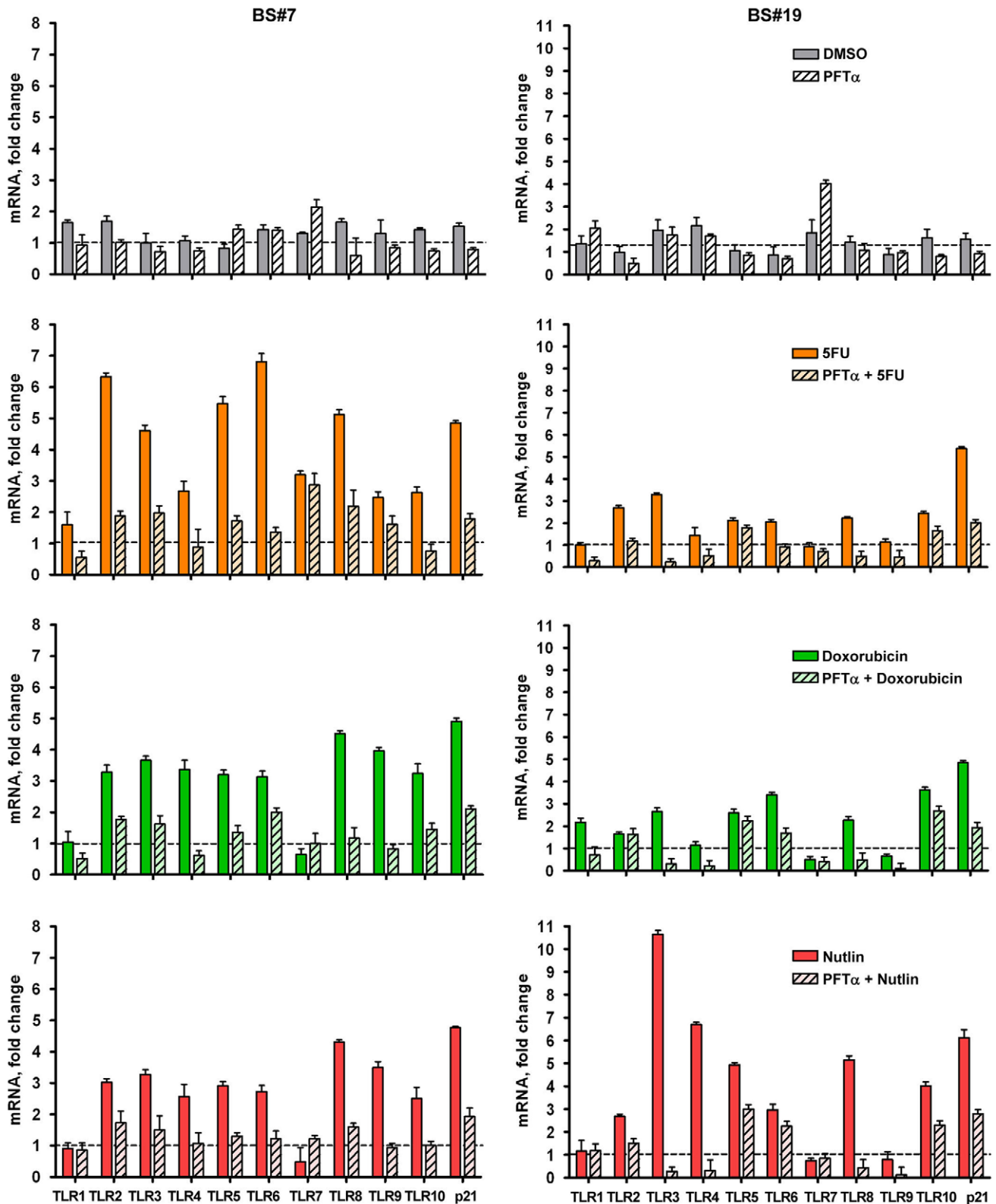


Figure 2. Inhibition of p53 activity by pifithrin-alpha dramatically reduces p53-dependent TLR induction by DNA damage and p53 activation. The p53 inhibitor pifithrin-alpha (PFT α , 40 μ M) or DMSO (control) were added to PHA-stimulated T-lymphocytes 2 h prior to Doxo (0.3 μ g/mL), 5FU (300 μ M) or nutlin (10 μ M) exposure. Following 24 h of exposure, gene expression was assessed by qPCR. Presented is the mRNA fold-change compared to untreated cells for subjects BS#7 and BS#19. Each bar represents an average of 3 PCR replicates with its standard deviation.

doi:10.1371/journal.pgen.1001360.g002

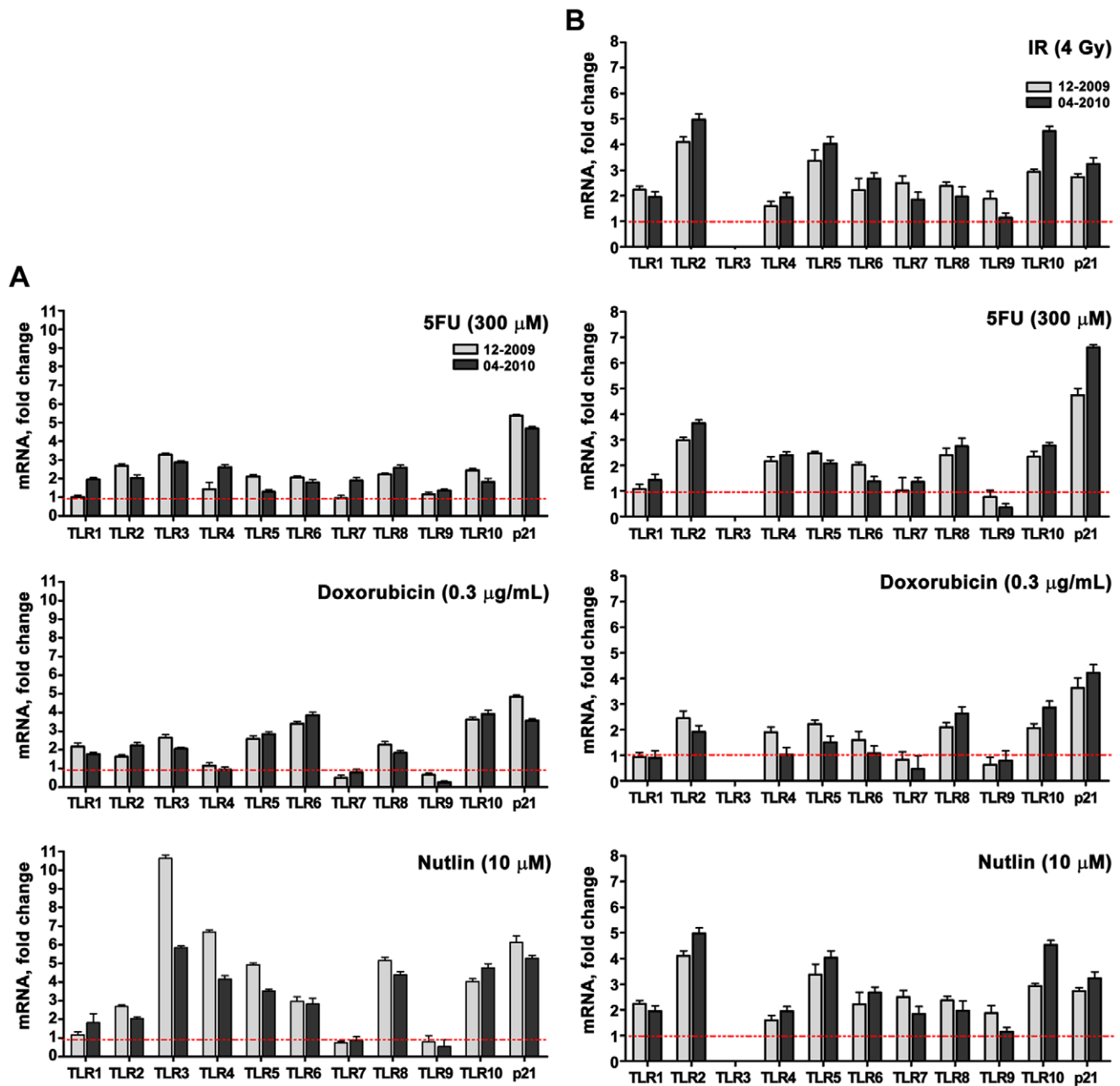


Figure 3. Induced *TLR* expression in primary human T-lymphocytes obtained from the same subjects on different days. *TLR* gene expression assessed by real time-PCR was measured in PHA-stimulated lymphocytes from 2 volunteers who were sampled twice, separated by 4 months, in a blind study as indicated in the figures (i.e., 12-2009 and 04-2010). The cells were exposed to DNA stressors or nutlin. Presented is the mRNA fold-change compared to untreated cells for patients BS#19 (A) and BS#20 (B). doi:10.1371/journal.pgen.1001360.g003

shown in Figure 4B, there is clear binding following exposure of cells to Doxo at the established p53RE on *p21^{WAF1}* as well as at the *TLR2*, 4, 5, 6, 8, 9 and 10 sequences described in Figure 4A. Thus, all *TLR* genes are subject to DNA-damage associated transcriptional regulation and most are directly targeted by p53. (We also identified other p53-like sequences in the analyzed regions; however, these were predicted to be less functional than the sequences examined). Furthermore, a direct role for p53 in *TLR* gene expression was demonstrated using p53 null SaOS2 osteosarcoma cells that have p53 under the control of a tetracycline inducible promoter [27]. Expression of wild-type

p53, but not the transcriptionally inactive G279E mutant protein, results in induction of all *TLRs* except *TLR7* (*TLR8* was not tested because it is not expressed by SaOS2 cells; Figure S5).

We also determined whether p53 induction of *TLR* genes by nutlin can lead to a corresponding increase in protein. (Nutlin was chosen because it typically led to the largest increase in p53, as shown in Figure S3.) Using western blot analysis with *TLR* specific antibodies (see Materials and Methods), the levels of *TLR2* and *TLR5* proteins were examined in the membrane fraction from stimulated lymphocytes of volunteers BS25 and B26 (sufficient cells were obtained from these subjects to enable the protein

Table 2. The p53RE-related sequence that is predicted to have the greatest function in the promoter regions (± 5 kb from transcription start site) of human *TLR* genes.

Gene	Total number p53RE related sequences *	Predicted most functional p53 RE	Chromosome coordinates**	Conserved in rodents	Tested in reporter assay
<i>TLR1</i>	11 (2)	RE#4 AGGCATGagC-tAcCATGCC	4: 38807969-38807950	NO	YES
<i>TLR2</i>	10 (3)	RE#4 AAACAAGaTCtaccctatatGGGCATGTCC	4: 154621975-154622005	NO	YES
<i>TLR3</i>	13 (2)	RE#4 AGGCATGCaC-cAACATGCC	4: 186988380-186988399	NO	YES
<i>TLR4</i>	15 (4)	RE#8 AGGCATGCTCcaGAGCAAaTCT	9: 120464256-120464277	NO	YES
<i>TLR5</i>	11 (3)	RE#4 GGGCATGgTg-GYACATGCC [^]	1: 223311442-223311461	NO	YES
<i>TLR6</i>	11 (4)	RE#6 RAGCAAGTCCactGGCAAaTCTggGGGCATGTga [^]	4: 38832459-38832492	NO	YES
<i>TLR7</i>	5 (2)	RE#2 GGGCATGTcacaatttcagattaatcaAcGCCTTGCTC	X: 12885856-12885892	NO	NO
<i>TLR8</i>	10 (7)	RE#6 AGGCAAGaTg-AAACATRTCa [^]	X: 12923665-12923684	NO	YES
<i>TLR9</i>	17 (3)	RE#14 AGGCATGgTgGtGCATGCC	3: 52273914-52273933	NO	YES
<i>TLR10</i>	6 (1)	RE#4 AGACATGTTT-GtAtATGTTT	4: 38786796-38786815	NO	YES

In lower case characters are the mismatches relative to the consensus p53 response element (RE).

The bold characters are half-site p53REs without mismatches relative to the consensus p53 RE.

The italic lower case characters correspond to the spacer sequence between p53 decamers. A lack of spacer between decamer sequences is denoted by "-".

[^]Indicates that the predicted RE contains a single nucleotide polymorphism (SNP). SNPs are indicated in bold and underline characters.

TLR5 SNP rs2192617 C/T

TLR6 SNP rs167251 G/A

TLR8 SNP rs3761624 A/G

*Number of noncanonical p53 REs is indicated inside the parenthesis.

**Chromosome coordinates based on USC Genome Browser Human Feb. 2009 (GRCh37/hg19) <http://genome.ucsc.edu/>.

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measurements; the TLR2 and TLR5 proteins were only detected in the membrane fraction as noted in the Material and Methods). Nutlin treatment resulted in a substantial increase in both proteins in the membrane fraction which corresponded well with induced expression of the *TLR 2* and *TLR5* genes, as described in Figure 5.

To address the generality of DNA damage-induced *TLR* expression and the role of p53, we also examined alveolar macrophages since they are well-known to play a pivotal role in

pulmonary innate immunity and are susceptible to DNA damage from environmental exposures including cigarette smoke and particulate matter [28–30]. The cells were collected by bronchoalveolar lavage of healthy human subjects and treated *ex vivo*. Among 6 subjects, there were considerable differences between *TLRs* with little or no induction of *TLR1* and *TLR6* by Doxo or nutlin, as described in Figure 6, and a dramatic induction of *TLR9* by Doxo, suggesting cell-specific factors that determine damage-

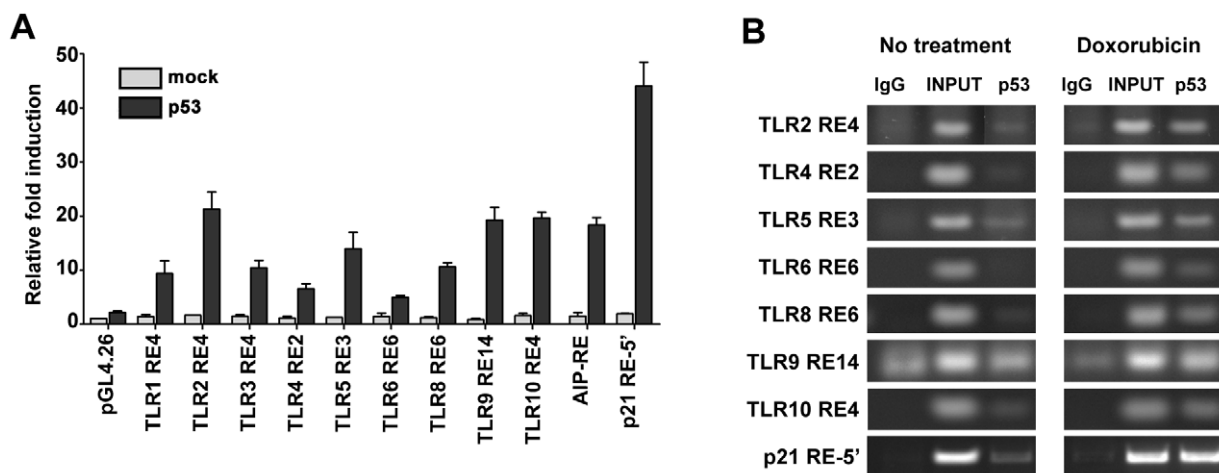


Figure 4. p53 drives expression of *TLR* family through direct binding to regulatory regions. (A) Functionality of presumptive p53 RE sequences associated with *TLRs* (sequences are described further in Table 2). H1299 cells were transfected with RE::luciferase reporter constructs in the presence (solid bars) or absence (open bars) of a vector expressing wild type p53. At 48 h post-transfection, induction of the luciferase reporter was compared with cells containing the pGL4.26 plasmid lacking p53. Presented are the average and standard deviations of 3 independent experiments. p53REs corresponding to *TLRs* 5, 6 and 8 contain SNPs as described in Table 2. (B) Occupancy of p53 at promoters of *p21* and *TLRs* 2, 4, 5, 6, 8, 9 and 10 assessed by ChIP analysis of PHA-stimulated T-lymphocytes from subject BS#4 following 24 h of doxorubicin (0.3 μ g/ml) treatment. doi:10.1371/journal.pgen.1001360.g004

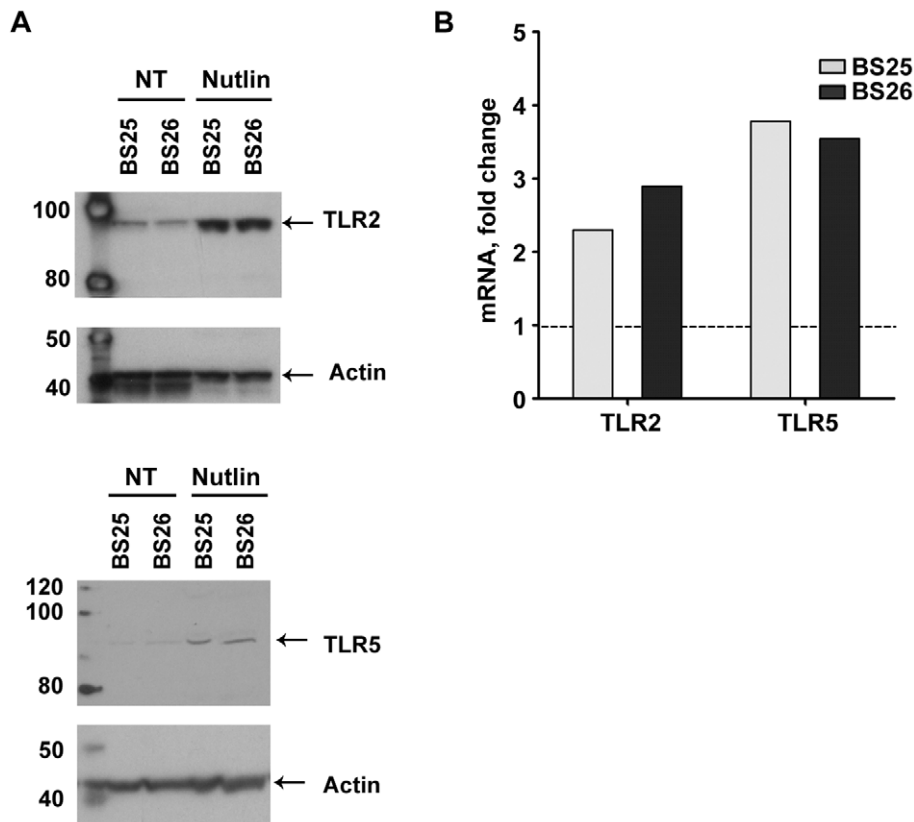


Figure 5. Nutlin induces *TLR2* and *TLR5* mRNA expression as well as proteins. (A) Induction of TLR2 and 5 protein expression in stimulated lymphocytes cells from subjects BS25 and BS26 by nutlin (10 μ M) after 24 hr post-treatment. Proteins were determined by western blot analysis using antibodies specific to TLR2 and TLR5 in the membrane fractions. Actin protein provided a loading control. (While there is little change in the amount of actin protein detected, there may be differences in the nonspecific bands.) (B) Induction of TLR2 and 5 mRNA expression in stimulated lymphocytes from subjects BS25 and BS26 (data obtained from Figure 1) by nutlin. doi:10.1371/journal.pgen.1001360.g005

TLR response profiles. In general, fewer of the *TLR* genes in the macrophages responded to Doxo and nutlin as compared to lymphocytes and the response levels were not as large.

Activation of p53 influences PAMP-induced TLR cytokine responses

Induction of cytokines is a prototypical functional response that is downstream of TLR activation [11,31,32], and increases in *TLR* expression can enhance PAMP or DAMP-induced signaling and innate-immune mediated effects. As shown in Figure 7, pretreatment of freshly isolated CD3+ T-lymphocytes (see Figure S1) with nutlin to induce *TLR2* by p53 resulted in 2- to 5-fold increased expression of interleukin *IL-1* and *IL-8* by the TLR2 ligand PAM3CSK4, suggesting that *TLR* induction by p53 can directly affect innate immune function. (The TLR2 induced cytokine response was examined because of our observation that T-lymphocytes from all subjects experience nutlin-induced *TLR2* expression, as shown in Figure 1 and Figure S3). The magnitude of cytokine induction varied between subjects (see Figure S6), suggesting that other factors, besides p53-induced gene expression, affect innate immune responsiveness.

TLR8 promoter SNP determines nutlin and IR responsiveness

Our previous report [8] of a SNP in a potential p53 response element of the *TLR8* promoter (AAACAT(G/A)TCa; see Table 1)

provided a unique opportunity to directly assess the relationship between p53 and *TLR* expression. While we had established large differences in the potential p53-responsiveness of the two SNP sequences [8]; see Figure 4A for the “positive”, p53-responsive G-allele), their cellular impact could not be assessed because the *TLR8* gene was not expressed in the human cell systems previously examined. Unlike the results with cell lines, we observed a dichotomous response in *TLR8* expression in lymphocytes following IR and nutlin treatment (Figure 1), and in alveolar macrophages following nutlin exposure (Figure 6), with some subjects showing low *TLR8* induction and others robust induction. We, therefore, determined which p53 response element alleles were present and their relationship to *TLR8* induction. Since *TLR8* is located on the X-chromosome, males carry only a single copy and females 2 copies (the specific alleles for each volunteer are described in Table 1). As shown in Figure 8 and Figure S3, the ability of nutlin and IR to induce *TLR8* correlates well with the presence of the G-allele. The frequency of this allele in our study is 0.43 (13/28, which corresponds to the frequency in the general population (Table 1). Importantly, *TLR8* induction was always high when only G-alleles were present (both alleles in females or the single allele in males) and absent if there were only A-alleles. However, among the 3 female subjects that were heterozygous for these alleles, only one responded poorly to both nutlin and IR. Possibly, the variability between female subjects heterozygous for the SNP is related to X chromosome inactivation.

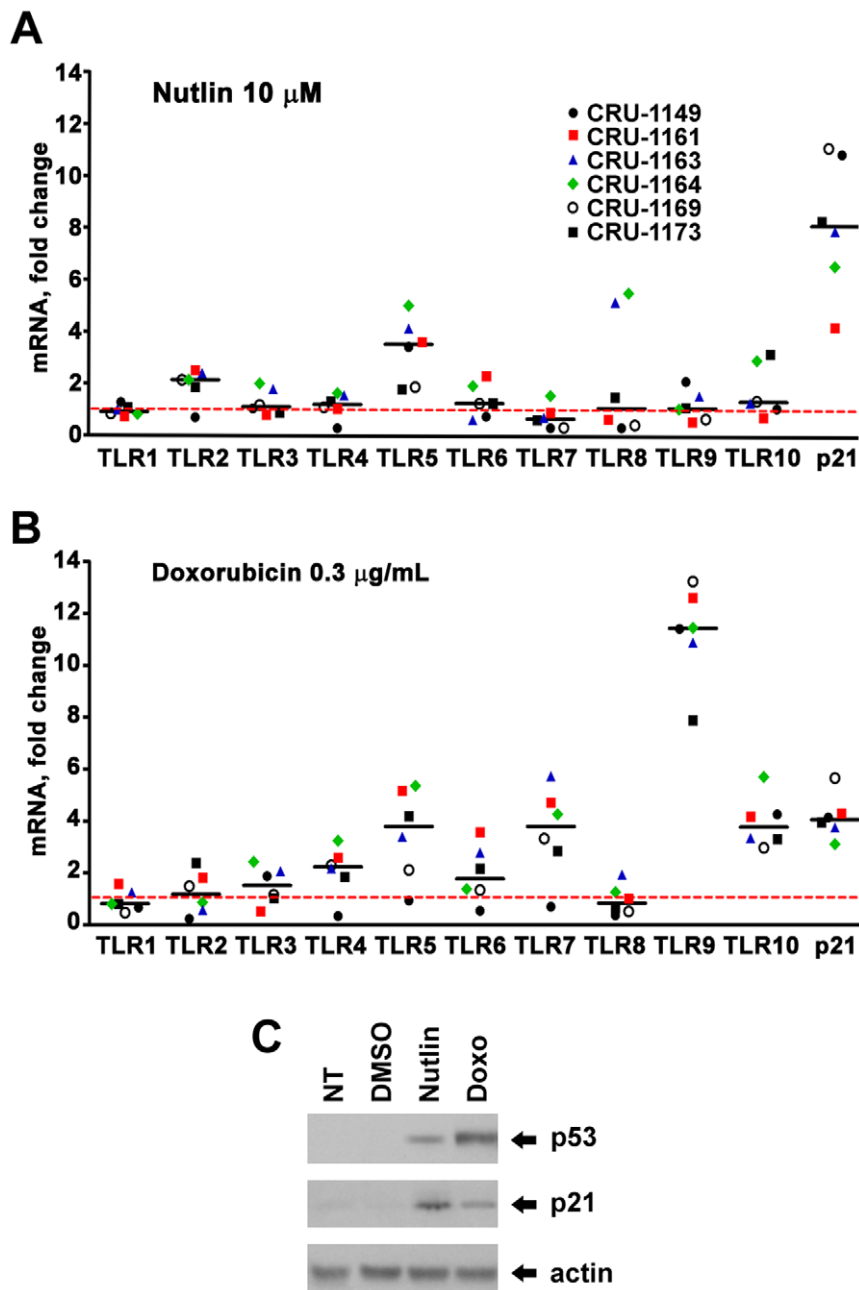


Figure 6. Induced expression of *TLR* gene family in human alveolar macrophages by DNA stressors and activation of the p53 pathway. Alveolar macrophages obtained by bronchoalveolar lavage of normal, healthy human subjects were incubated for 24 h in the presence of (A) nutlin or (B) Doxo. *TLR* gene expression was analyzed by qPCR and is presented as fold-change compared to untreated cells. Horizontal bars correspond to the median. (C) Activation of p53 pathway (p53 and p21) in alveolar macrophages following 24 h of incubation with nutlin or Doxo as analyzed by Western blots.

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While these results demonstrate differences in the impact of the polymorphic alleles, the differences do not extend to 5FU or Doxo, suggesting that other p53-related target sequences are responsible for the induction or involvement of additional p53-independent mechanisms (Figure 8 and Figure S3). Notably, these findings with *TLR8* provide the first direct demonstration, to our knowledge, in humans of the ability of a specific RE in a p53 target gene to drive transcription. The SNP-associated differences in expression may provide opportunities to identify other factors in human primary tissue that determine the ability of specific REs to support p53-

driven transcription [33,34]. Although there are SNPs in the REs of *TLR5* and *TLR6*, as described in Table 2, they are predicted to have no functional impact (see [6–8]).

Evolution of TLR gene family responsiveness to p53

Our results with primary human cells have been confirmed for DNA damage induction of *TLRs* (to be presented elsewhere) in several human cell lines including the previously reported *TLR3* [9]. However, as shown in Figure S7, they do not extend to murine cells. The *TLR* responses to Doxo, 5FU and IR were found to be

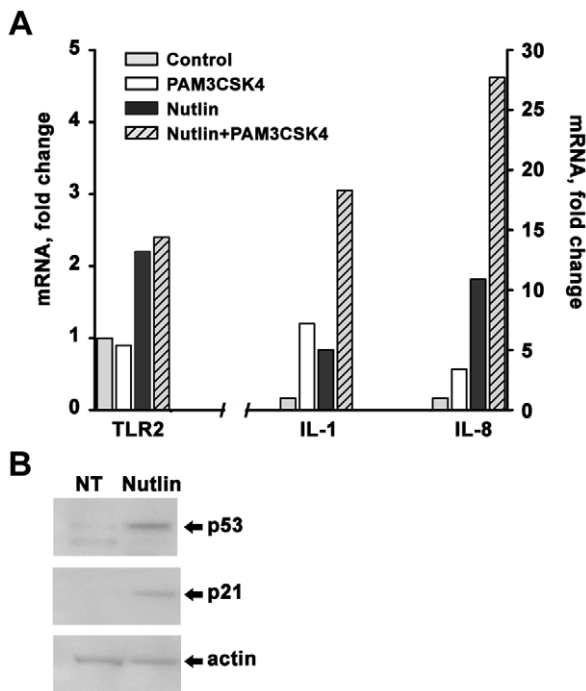


Figure 7. Induction of p53 sensitizes freshly isolated CD3⁺ cells to PAMP stimulation. (A) *TLR2* and cytokine (*IL-1* and *IL-8*) expression in CD3⁺ lymphocytes incubated for 20 h with nutlin (10 μ M) or DMSO and then exposed for 4 h to the TLR2 ligand PAM3CSK4 (1 μ g/ml). Presented are representative results for subject BS#37. Gene expression was analyzed by qPCR and presented as fold-change compared to untreated cells. (B) Activation of p53 pathway by 24 h of nutlin treatment in CD3⁺ cells isolated from peripheral blood as determined by Western blot analysis. doi:10.1371/journal.pgen.1001360.g007

small in mouse peritoneal elicited macrophages, bone marrow-derived macrophages, and embryonic fibroblasts (MEFs; except for *TLR9* in MEFs). The low induction level appears to be p53-independent based on results with nutlin and on the similar responses in p53-positive and -null MEFs. These results are consistent with the lack of sequence conservation between humans and rodents of functional p53 response elements in *TLR* genes (Figure S8) even though the coding sequences are well-conserved. Although we were able to identify p53 RE-related sequences in the vicinity of the transcription start site of several mouse *TLRs*, none were predicted to be functional p53 targets.

Discussion

Cellular stress and the inflammatory response are intricately linked pathways subject to endogenous and exogenous challenges. Here, we provide the first evidence that DNA stressors may also modulate inflammatory responses at a fundamental level in primary human cells, namely, by altering *TLR* expression. All members of the human *TLR* gene family tested (*TLR1-10*) are responsive to at least one disruptor of chromosome metabolism. While there are considerable variations between individuals, for each *TLR* gene there is a group of several subjects that is responsive to at least one of the stressors (5FU, doxorubicin, IR and/or nutlin). Note, for example, that there is at most a low level of *TLR1* induction by 5FU and nutlin in the primary cells from 18 subjects and there is even a general repression of *TLR7* by nutlin. We thus propose that, contrary to the paradigm that innate

immunity is hard-wired, the TLR system in humans actually has a complex, robust responsiveness to environmental conditions that challenge the integrity of the genome. We establish that induction of *TLRs* by DNA damage is a class effect that in many cases can be mediated by p53 (including repression of *TLR7*) and prevented by the p53 inhibitor pifithrin. In support of these findings, several potential p53 binding sites were identified in the proximity of the transcription start sites for all *TLRs*, except *TLR7*. The new p53REs were identified using functionality rules to predict p53RE responsiveness [6,7]. These were confirmed with reporter assays and by the binding of p53 in human lymphocytes following stress activation. In addition the functional *TLR8* SNP in the p53 target response element directly confirms a role for p53. Interestingly, for *TLR2* and *TLR10*, the most functional sites were predicted to be noncanonical, containing only a half-site p53RE (*i.e.*, one decamer). Previously, the genes *FLT1* [35] and *RAP80* [36] were shown to be directly controlled by p53 through half-site REs and several other target genes have been identified that are predicted to be regulated by noncanonical p53 RE (summarized in [6]).

The inclusion of *TLR* genes expands the universe of genes and biological functions that fall within control of the p53 master regulator. The addition of the set of *TLR* genes identifies a biological gene niche regulated by the p53 master regulator that is distinct from rodents whose *TLR* genes appear to lack functional p53 REs. It is interesting that a similar niche was identified for all the DNA metabolic genes that are p53-responsive in humans in that none of them respond to p53 in rodents [7,36]. p53 provides a rapid, integrated signaling mechanism for increasing or maintaining gene responses to acute and chronic DNA damage stress. In a more general sense, we suggest that the evolutionary inclusion of sets of genes with related functions may provide an efficient means of dealing with sudden, temporary challenges that can result from DNA damage and/or DNA damage itself may be a modulator of broader biological threats, as for the case of infection.

We speculate that there may be feedback loops that integrate this newly identified role for p53 in DNA damage and innate immune responses, as described in Figure 9. In this scheme, DNA damage from environmental agents or potentially from TLR-elicited reactive oxygen species (ROS) may amplify the responsiveness of the innate immune system by promoting *TLR* upregulation. p53 itself displays a variety of roles in mediating ROS signals [37,38]. On the other hand, *TLR* upregulation may also sensitize tissues to maladaptive aseptic inflammation in the setting of environmental injury. For example, tissue injury is reported to induce inflammation through release of DAMPs that act upon *TLR2*, *TLR4*, and *TLR9* [39,40]. We speculate that, during tissue injury, upregulation/activation of TLRs may serve as a cell-fate counterbalance to p53-mediated pro-apoptotic responses by leading to activation of the pro-survival factor NF- κ B [39]. This may be particularly relevant to cancer therapy, as stimulation of TLR5, 7, 8, and 9 have been shown to modify cellular radio- and chemoresistance [41,42]. These findings demonstrating that p53 can increase an inflammatory response differ from the generally held view relating to the antagonistic affect of p53 on inflammation directed by NF- κ B [41]. However, the mechanism here is quite different in that it involves the p53-mediated increase in a receptor that translates ligand interactions into cytokine responses. Our results may be particularly relevant to diseases in which variations in T-cell function can impact pathogenesis, such as autoimmune disease, asthma, and IBD. For example, recent reports suggest that intestinal inflammation can induce genotoxicity in circulating leukocytes [43], while increased DNA damage is also detected in lymphocytes obtained from rheumatoid arthritis patients [44]. The heightened pro-inflammatory status in such

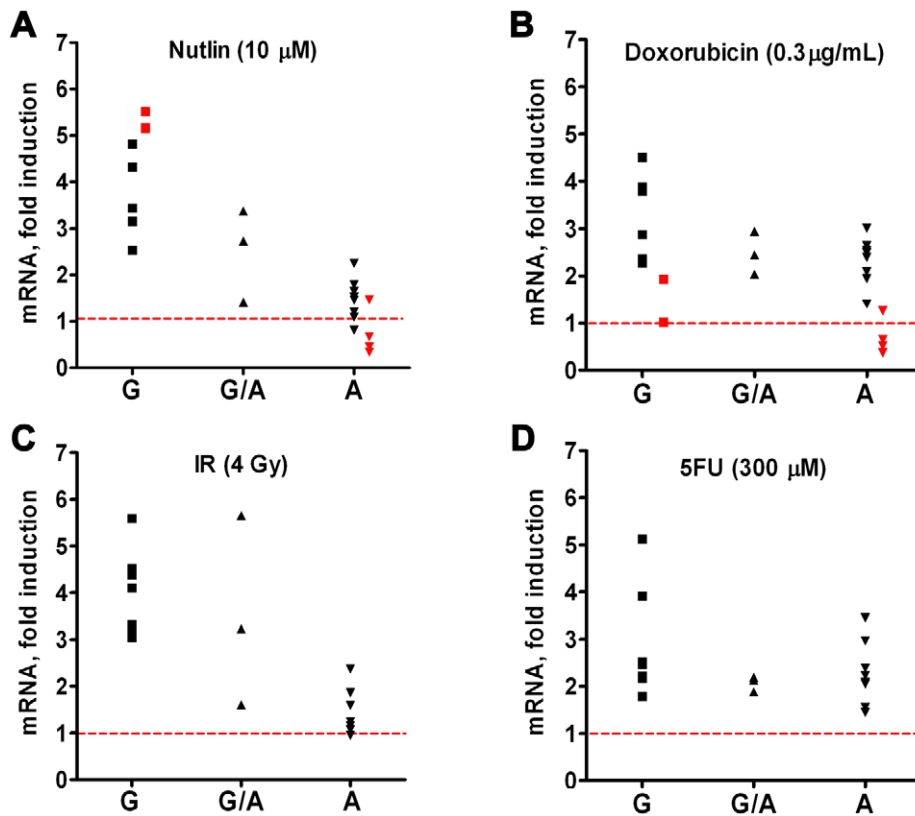


Figure 8. Ability of p53 to drive *TLR8* expression in primary human cells depends on SNP in p53 response element of *TLR8* promoter. The regulatory region of *TLR8* was amplified by PCR and the product was analyzed for the presence of the A and/or the G version of the p53 RE. Presented are SNP genotypes and the expression levels of *TLR8* following 24 h of treatment with (A) nutlin, (B) IR, (C) Doxo and (D) 5FU. Since the gene is X-linked, males have one copy and females have two copies of the allele. Presented are results with stimulated lymphocytes (black symbols) or alveolar macrophages (red symbols). doi:10.1371/journal.pgen.1001360.g008

patients, as well as the common finding of systemic or multi-organ inflammation during exacerbations of autoimmune disease, might be mediated by circulating immune cells which have suffered DNA damage during passage through inflamed tissues.

Beyond the *TLR* gene and cell subset variability in response to DNA damage and p53 activation, we also demonstrate considerable inter-individual variability. This variability may be relevant to inter-individual differences in susceptibility to a wide spectrum of diseases and therapies. Our results suggest that various anti-cancer agents may yield different patterns of responses across *TLRs* and between subjects. Since TLR ligands are increasingly used as adjuvants for vaccines (TLR4 and TLR9) and cancer treatment (TLR3/7/9) (reviewed in [45]), the ability to detect and predict genetically determined inter-individual variability in *TLR* induction may prove a useful therapeutic tool. Future studies are warranted to determine whether single agents such as nutlin, or even factors that induce chromosome stress, may serve as useful immune adjuvants through manipulation of *TLR* expression in human subjects.

Materials and Methods

Lymphocyte isolation

Healthy adult volunteers were recruited to the NIEHS Clinical Research Unit and underwent phlebotomy. Subjects were excluded if they had a history of recent infection, were on anti-inflammatory medications, or tested positive for hepatitis B, C or

HIV. Up to 300 ml of whole blood were withdrawn from an antecubital vein into citrated tubes. Lymphocytes were isolated using percoll (Sigma) and anti-CD3-coupled Magnetic Beads (Miltenyi Biotec) as per manufacturer's protocol. Cell purity was >98% after percoll/magnetic bead isolation based on flow cytometry. We maintained lymphocytes in RPMI supplemented with 10% FBS. For T cell stimulation, cells were activated with phytohemagglutinin-M (PHA, Invitrogen, 3% vol/vol) for 72 h. The total number of lymphocytes available per treatment conditions after PHA was typically around 10 million cells or less. Cells were treated starting at 48 h post PHA addition and cell cultures were harvested 24 h later. Freshly isolated CD3+ cells were treated with nutlin for 20 h or DMSO as a vehicle control, then washed and exposed to TLR1/2 ligand PAM3CSK4 (1 μg/ml) at 1×10^6 cells/ml. Total yield of CD3+ cells from a single subject was typically around 10–15 million cells or less. Protocol and procedures were approved by the NIEHS Institutional Review Board.

Alveolar macrophage isolation

Healthy, nonsmoking male volunteers, 18 to 40 yr of age, underwent fiberoptic bronchoscopy with lavage to procure alveolar macrophages. The screening procedures for each subject included a medical history, physical examination, and routine hematologic and biochemical tests. None of the subjects had a history of asthma, allergic rhinitis, chronic respiratory disease, or cardiac disease. Subjects were excluded from the study if they had

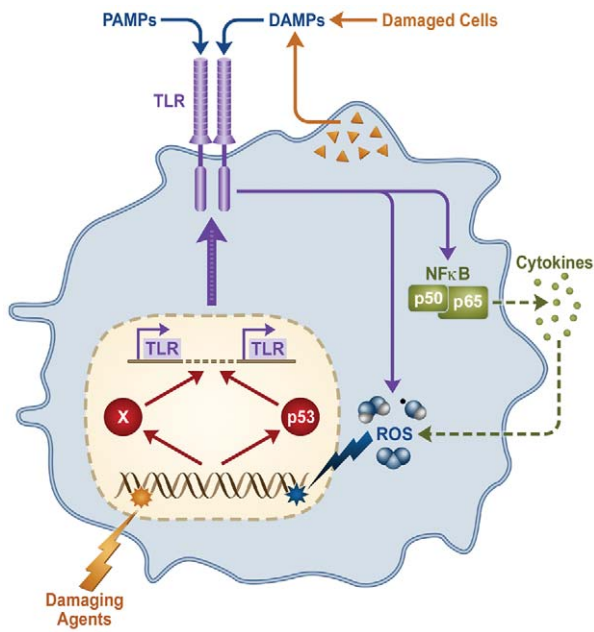


Figure 9. Model describing expression loop between p53 and the TLR gene family in response to chromosome and inflammation stresses. DNA metabolic stress is induced by both environmental factors and endogenous sources, such as host-derived reactive oxygen species (ROS) generated downstream of Toll-like Receptors and pro-inflammatory cytokines. DNA damage/stress activates p53-dependent and independent (“X”) pathways that in turn induce expression of TLRs. Increased TLR expression sensitizes the cell to both exogenous, pathogen-associated molecular patterns (PAMPs) and endogenous, damage-associated molecular patterns (DAMPs) released during tissue injury which, in this loop, lead to further ROS.
doi:10.1371/journal.pgen.1001360.g009

suffered a recent acute respiratory illness and were asked to avoid exposure to air pollutants such as tobacco smoke and paint fumes. A fiberoptic bronchoscope was wedged into a segmental bronchus of the lingula. Six 50-ml aliquots of sterile saline were instilled and immediately aspirated. The procedure was repeated on the right middle lobe, again using 300 ml of saline. Samples were put on ice immediately after aspiration and centrifuged at 300 x g for 10 min at 4–8°C. Cells from all aliquots were pooled, washed twice with RPMI 1640, and re-suspended in RPMI 1640 at 1.0×10^6 /mL. Total yield was typically around 10–15 million cells or less. Around 2.0×10^6 cells per well were seeded in a 12 well plate. After 2 hr, cells were washed twice with warm PBS and 2 ml of growing media was added to each well. Cells were then treated with nutlin or doxorubicin. Cells were harvested 24 hr post-treatment. The protocol and consent form were approved by the University of North Carolina School of Medicine Committee on the Protection of the Rights of Human Subjects. Prior to participation in the study, subjects were informed of the procedures and potential risks and each signed a statement of informed consent.

Cancer cell cultures

H1299 lung cancer cells (American Type Culture Collection) were routinely maintained following standard conditions and procedures for culturing mammalian cells. All cultures were incubated at 37°C with 5% CO₂. p53 tetracycline inducible SaOS2 TET-off cell lines expressing the wild-type or the G279E mutant protein were cultured as described previously [27]. p53 expression was kept “off” by 2 mg/ml doxycycline (Clontech). To induce the p53 expression, cells were washed 3X with phosphate-

buffered saline (PBS) and placed in medium lacking doxycycline during 24 h.

Reagents and treatment conditions

For drug treatment and p53 activation, cells were incubated with doxorubicin (Sigma 0.3 μg/mL), nutlin3 (Sigma, 10 μM) and 5-fluorouracil (Sigma, 300 μM). For ionizing radiation treatment, cells were irradiated at 1.56 Gy/min from a Shepherd cesium irradiator in PBS at room temperature at final dose of 4 Gy. Where indicated cells were also pretreated 2 h with p53 inhibitor pifithrin-alpha (Sigma, 40 μM).

RNA isolation and gene expression analysis

Total RNA was isolated by RNEasy kit (Qiagen). Real-time PCR was performed in triplicate with Taqman PCR Mix (Applied Biosystems) in the 7000 ABI sequence Detection System (Applied Biosystems). All human and mouse primers were purchased from Applied Biosystems (information available upon request). For PHA stimulated lymphocytes and alveolar macrophages expression of TLR genes was normalized to 18S ribosomal RNA gene while for freshly isolated CD3+ T cells, the glucuronidase-beta gene was used for normalization.

Luciferase reporter assays

Pairs of complimentary oligonucleotides for the desired p53RE from selected TLRs and containing restriction sites were cloned into the open reading frame of firefly luciferase pGL4.26 plasmid (Promega) previously double digested by Xho I/Kpn I restriction enzymes. The identity of the inserts was confirmed by DNA sequencing. Luciferase activity was measured 48 h after Fugene6-mediated co-transfection of the TLR p53RE constructs in the presence of p53 (pC53-SN3) or empty vector pCMV NEO-BAM3 along with pRL-TK Renilla as a transfection efficiency control into p53 null H1299 cells, as previously described [5]. Forty-eight hours post-transfection extracts were prepared using the Dual Luciferase Assay System (Promega) following the manufacturer’s protocol and luciferase activity was measured on a Victor Wallac multilabel plate reader (PerkinElmer). Relative luciferases activities for each construct was defined as the mean value of the firefly luciferase/Renilla luciferase ratios obtained from 4 independent experiments performed in triplicate.

Immunoblot analysis

Whole cell extracts were quantified using the Bradford protein assay kit and gamma globulin as a reference standard (BioRad). For TLR protein detection, cellular pellets were subjected to subcellular protein fractionation (Thermo Scientific) following the manufacturer’s instructions and protein was quantified using BCA protein assay kit (Thermo Scientific). For TLR western blot analysis ~30 μg of total membrane fraction was used, while for the analysis of other proteins ~25 μg of total cell extract were used. As expected, we did not detect TLR2 and TLR5 in the cytosolic fractions; therefore, those data are not included. Proteins were resolved on 4–12% BisTris NuPAGE and transferred to polyvinylidene difluoride membranes (Invitrogen) and were visualized with primary antibodies followed by horseradish peroxidase-conjugated goat anti-mouse or donkey anti-goat immunoglobulin (Santa Cruz Biotechnology) through the use of enhanced chemiluminescence reagents (Amersham Biotechnology). The primary antibodies used in these studies were against p53 (DO1, Santa Cruz Biotechnology), p21 (SXM30, BD Biosciences Pharmingen) and Actin (C-11 Santa Cruz Biotechnology). The following is the list of TLR antibodies tested in this study in order

to detect TLR protein expression in whole cell extracts as well as membrane and cytosol protein fractions: TLR3 ab24185 and TLR10 ab45088 from Abcam, Inc.; TLR1#2209, TLR2#2229, TLR7#2633 and TLR9#2254 from Cell Signaling. We also used a Toll-like receptor detection kit that includes antibodies for all human TLRs (TLR1 to TLR10 antibodies from ProSci, Inc., as well as TLR3-4H270 and TLR5-H1-27 antibodies from Santa Cruz Biotechnology. The TLR3-IMG-315A, TLR4-IMG6370A antibodies were from IMGENEX. Only the TLR2 (Cell Signalling) and TLR5 (Santa Cruz) gave clear results. Attempts to detect other TLRs with these antibodies were unsuccessful and appear to be a general problem with TLR antibodies from our collective experience. One of the antibodies enabled us to detect induction of full length TLR4 by nutlin; however, those results are not presented due to the appearance in both untreated and treated samples of nonspecific bands.

Chromatin Immunoprecipitation (ChIP) assays

ChIP assays were done as previously described [35] using ChIP kits (Millipore). Approximately 40×10^6 PHA stimulated lymphocytes were used for each experimental sample. Cell lysates were sonicated using conditions that yield chromatin fragments 200–500 bp long. One microgram of DO-7 p53-specific monoclonal antibody (BD Biosciences Pharmingen) was used per ChIP assay. As a negative control, mouse Ig (Santa Cruz Biotechnology) was used. PCR amplifications were performed on immunoprecipitated chromatin using primers to amplify specific regions on the *TLRs* promoters (sequence information available upon request). The PCR cycles were as follows: initial 10 min Taq polymerase (Invitrogen) at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The PCR products were then run on a 1.8% agarose gel.

Flow cytometry analysis

Cells were resuspended in 100 μ l of PBS and incubated with 5 μ l of fluorescent antibody per sample for 30 min, then washed and fixed with 0.5% paraformaldehyde. The fluorescence intensity was evaluated using a Becton Dickinson LSR II Flow Cytometer. All antibodies used for FACS were from BD Pharmingen.

Genotyping

SNPs were assessed by three different approaches. In RFLP assays, genomic DNA was extracted from Percoll-isolated lymphocytes by DNeasy kit (Qiagen). For the SNP in the *TLR8* p53RE#6 (AGGCAAGATGAAACAT(G/C)TCA), the G-SNP creates a unique restriction cutting site for NspI (R CATG Y). PCR was performed with 100 ng of DNA, 50 pmol of each primer, 1.5 mM MgCl₂, 1 μ l 10 \times PCR buffer, and 0.0125 U of Taq (Invitrogen). After 10 min at 94°C, 35 cycles were repeated as follows: 94°C 30 s, 60°C 30 s, and 72°C 35 s; this cycling was followed by a final extension at 72°C for 10 min. The PCR product was digested with 5 U Nsp I (New England Biolabs, Ipswich, MA), at 37°C for 4 h. Since Nsp I recognizes the polymorphic sequence, a G allele is demonstrated by the presence of two fragments 109 and 69 bp in a gel. The A allele is revealed by the presence of a single 177 bp band. The following primers were used for amplifying the region containing the p53RE on *TLR8* promoter region:

Forward: 5'-TCATAACAAGGTGTTCCACAGTC-3'

Reverse: 5'-ATCTGGCCCTTTACAGAAAAAGTT-3'.

The status of this SNP was determined also by using a Taqman SNP genotyping assay. All primers were purchased from Applied Biosystems (Assay ID:C_27497635_10). For direct sequencing the

region containing the p53RE was first PCR amplified using the following pairs of primers:

Forward: 5'-TTGAATTCCCTTAGGGTGTGA-3',

Reverse: 5'-AAACTGCCCTTCGATTATTATTATTACA-3'

This was followed by running the samples on a TEA-agarose gel. The expected product (397 bp) was cut out and cleaned using QIAquick gel extraction kit (QIAGEN). The sequencing reactions used Big dye (Applied Biosystems) per manufacturer recommendations and the following primers:

Forward: 5'-TCATAACAAGGTGTTCCACAGTC-3

Reverse: 5'-ATCTGGCCCTTTACAGAAAAAGTT-3'

Mouse primary cell culture

p53^{+/+} and *p53*^{-/-} mouse embryonic fibroblasts (MEFs) were cultured in DMEM media and 10% of FBS. Female C57BL/6 mice were purchased from Jackson Laboratories. All experiments were performed in accordance with the Animal Welfare Act and the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals after review of the protocol by the Animal Care and Use Committee of the National Institute of Environmental Health Sciences. For murine peritoneal macrophage harvests, mice were injected i.p. with 2 ml of 4% Brewer's thioglycollate and euthanized 96 h later. The peritoneum was washed with 10 ml ice cold PBS three times. Cells were centrifuged (1,000x RPM, 6 minutes, 4°C) and washed twice with sterile PBS. Peritoneal exudate macrophages were resuspended in DMEM/0.1% FBS, counted, and plated at 2×10^6 cells/well in a 12-well plate. Cells were allowed to settle for 2 h (37°C/5% CO₂) before replacing media with DMEM complimented with 10% FBS.

For bone marrow-derived macrophages (BMM), marrow was flushed from femoral and tibial bones using bone marrow media (DMEM/2 mM L-glutamine/10% L929-conditioned medium/10% FBS). Cells were spun down (2200x RPM, 5 min, 4°C), brought up in 1 ml sterile ACK buffer, incubated 4°C for 1 min after which 10 ml PBS was added. Cells were spun as above, resuspended in bone marrow medium, counted, and plated at 1×10^6 cells/well in a 12-well plate. Cells were cultured at 37°C and 10% CO₂ in 2 ml bone marrow medium/well and fed on Day 5 with addition of 1 ml medium/well. Experiments were performed on Day 6. At 24 h post-treatment, cells were harvested for RNA extraction.

Statistical methods

To examine statistically whether the average mRNA fold-change at each locus in the population sampled differed from 1 for the various exposures, we applied one-sample Student's t tests to log-transformed values of mRNA fold change. The logarithmic transformation helps the data meet the distributional assumptions for the t test. This procedure, in effect, tests the null hypothesis that the geometric mean mRNA fold change at the locus is equal to 1 against the two-sided alternative that the geometric mean differs from 1.

Supporting Information

Figure S1 Characterization of cell populations obtained from peripheral blood of healthy subjects. Presented are FACS profiles of peripheral blood mononuclear cells (PBMC) obtained by percoll gradient separation from subject BS#8. Cells were either stimulated by PHA, yielding predominantly T-lymphocytes (A) or (B) the CD3+ cells were isolated from PBMC by magnetic beads conjugated to anti-CD3 antibody. The amount of monocytes in these preparations based on CD14 staining did not exceed 1.4%. Cells were stained with fluorescent anti-CD3 antibody to visualize

surface marker expression. The X-axis identifies the CD3 fluorescence intensity.

Found at: doi:10.1371/journal.pgen.1001360.s001 (0.54 MB TIF)

Figure S2 Induced expression of *TLR* gene family in primary human T-lymphocytes via DNA stressors and activation of the p53 pathway. Freshly isolated human peripheral blood mononuclear cells (PBMC) from healthy subjects ($n = 17-18$) were incubated with PHA to stimulate T-lymphocyte expansion. After 48 h, cells were exposed to (A) ionizing radiation (IR); (B) 5-fluorouracil (5FU); (C) doxorubicin or (D) nutlin. Cells were harvested following 24 h treatment (24 h after IR exposure). Presented in panels A–D are mRNA expression levels as compared to expression in untreated cells (the dashed line is a value of 1; *i.e.*, no change) measured by qPCR and normalized to 18S ribosomal RNA. In this “boxes and whiskers” diagram, the limits of the 2nd and 3rd quartiles of observed values (*i.e.*, the middle 50% of observations) are at the ends of the box; the median is the horizontal line within the box; “+” is the average. Maximum and minimum values are at the ends of the “whiskers” and outliers (see Figure 1) are presented as dots. For *TLR3* only subjects that expressed *TLR3* (see Figure 1) are presented. The outliers were determined using the method of Grubbs (also called the Extreme Studentized Deviate, or ESD, method which determines a value that is unlikely to have come from the same Gaussian population as the other values in the group [Barnett et al.]. There is little difference in the expression of housekeeping genes *GUSB* and actin following treatment with doxorubicin (S2E) or nutlin (S2F). (Only 14 of the 18 subject samples in Figure 1 had sufficient material to be able to include *GUSB* and actin analysis.) Furthermore, for *TLR* genes that are not responsive to DNA damage or p53 induction (*i.e.*, *TLR1* with nutlin treatment), there is a nearly constant ratio across the individuals tested, as seen in these graphs. The basal levels of expression of the *TLR* genes, while low, are clearly measurable. [Barnett V, Lewis T, Rothamsted V. “Outliers in statistical data” in the Wiley Series in Probability and mathematical statistics. Applied Probability and Statistics, John Wiley & Sons, 1994.]

Found at: doi:10.1371/journal.pgen.1001360.s002 (1.19 MB TIF)

Figure S3 Heat map presentation of *TLR* expression after DNA damage stress and p53 activation in PHA-stimulated human T lymphocytes. A modified microarray heat map graphic style was used to represent the fold-change in mRNA expression for *TLR* and *P21* genes in PHA-stimulated human lymphocytes from 18 subjects (BS#) after 24 h of treatment with nutlin, ionizing radiation, 5-fluorouracil (5FU) or doxorubicin at the doses indicated. The subjects were grouped by colors according to their responses to nutlin as indicated in the top part of each heatmap. An arbitrary cut-off value for induction, no change and repression was based on *P21* gene expression responses to nutlin treatment. BS#1 sample was not treated with nutlin (yellow squares). The genotype for the SNP associated with the putative p53RE in the promoter of *TLR8* is also presented for each subject.

Found at: doi:10.1371/journal.pgen.1001360.s003 (2.52 MB TIF)

Figure S4 Activation of p53 pathway in PHA-stimulated T-lymphocytes from 18 healthy subjects. T-lymphocytes generated from PBMC, as described above, were treated with nutlin (10 μ M); doxorubicin (Doxo, 0.3 μ g/mL); 5-fluorouracil (5FU, 300 μ M); ionizing radiation (IR, 4Gy); DMSO (vehicle control) or left untreated (NT). Following 24 h treatment, cells were harvested and subjected to Western blot analysis. Activation of the p53 pathway was assessed by induction of p53 and p21 protein levels. Actin levels provided loading control. The “BS#” corresponds to individual subjects as described in Figures S2 and S3.

Found at: doi:10.1371/journal.pgen.1001360.s004 (1.82 MB TIF)

Figure S5 *TLR* expression in p53 inducible SaOS2 cell line. *TLR* gene expression was assessed by real-time PCR as previously described [27] using SaOS2 TET-off inducible cell lines expressing WT or the transcriptionally inactive mutant G279E p53 protein after removing doxycycline from the media to induce p53. The gray bar corresponds to cells grown in the presence of doxycycline (*i.e.*, no p53 expression). Presented is the mRNA fold-change compared to the parental SaOS2 TET off cells lacking p53 expression vectors, represented by the dashed line, *i.e.*, a value of 1. Each bar represents an average of 3 replicates with its standard deviation.

Found at: doi:10.1371/journal.pgen.1001360.s005 (0.46 MB TIF)

Figure S6 Induction of p53 pathway sensitizes freshly isolated CD3+ cells to stimulation by TLR2 ligand PAM3CSK4. Presented is cytokine gene expression in isolated CD3+ lymphocytes incubated for 20 h with nutlin or DMSO and then exposed for 4 h to the TLR2 ligand PAM3CSK4 (1 μ g/ml). Gene expression changes for lymphocytes from subjects BS#19, #22, #26, and #29 were analyzed by qPCR and are presented as fold-change compared to untreated cells. Each bar represents an average of 2 replicates.

Found at: doi:10.1371/journal.pgen.1001360.s006 (0.84 MB TIF)

Figure S7 Expression of the *TLR* gene family in mouse cells in response to DNA stressors and activation of the p53 pathway. A panel of mouse cells was exposed to doxorubicin (0.3 μ g/ml); 5-fluorouracil (5FU, 300 μ M); ionizing radiation (IR, 4Gy); nutlin (10 μ M); DMSO (vehicle control); transfected with p53 expression plasmid or left untreated (NT). TLRs and *P21* gene expression were analyzed by qPCR and presented as fold-change compared to untreated cells in (A) peritoneal macrophages, (B) bone marrow-derived macrophages, (C) MEF *p53* +/+, and (D) MEF *p53* –/–. Presented is the average of 2 or 3 experiments. Each experiment was done in triplicate; presented is the standard deviation.

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Figure S8 Sequence conservation between primates and rodents for putative p53 response elements that are predicted to be functional in the human toll-like receptor genes. Presented are multispecies alignments for 9 primates (blue box) and 8 rodents (red box). Sequence alignments were derived from the BLAT alignment tool of the USC genome browser (Human Feb. 2009 (GRCh37/hg19); <http://genome.ucsc.edu/>). The human p53 response element flanked by 10 bp was used as reference sequence. For the human reference p53 RE sequence (second line), mismatches with respect to the commonly accepted p53 consensus binding sequence (p53CBS) site are in red characters. For each species, the characters highlighted in red are mismatches relative to the reference human p53RE sequence. Mismatches relative to the reference human p53RE sequence but matches relative to the p53CBS are shown in highlighted characters. Highlighted in green are base transition changes (*i.e.*, purine to purine or pyrimidine to pyrimidine) relative to the human p53RE, while highlighted in blue are changes relative to a mismatch in the original human p53RE that restore the base relative to the p53CBS. Characters in light blue color are the bases corresponding to the spacer between p53 decamers. Mismatches versus the human reference spacer sequence are highlighted in pink. Based on display chains between alignment configurations from the USC genome browser, which enables the display of gaps between alignment blocks in the pairwise alignments, the following conventions are used: 1) Single dashed line: no bases in the aligned species. This may be due to a lineage-specific insertion between the aligned blocks in the human genome or a lineage-specific deletion between the aligned blocks in the aligning species. 2) Double dashed line: aligning species has one or more unalienable bases in the gap region. This may be due to excessive evolutionary

distance between species or independent indels in the region between the aligned blocks in both species. 3) Blank space: sequence is not available in the database for the corresponding species. “N” denotes a gap in the genomic sequence. Additional details: 4) SNPs in the p53RE are highlighted in light green. For p53 REs containing SNPs, additional information about the SNP frequencies are presented. 5) The corresponding chromosome coordinates for each p53 RE are provided. For the *TLR5*, *TLR6* and *TLR9* genes, different transcription start sites (TSS) of the associated transcripts are shown (Ensembl, current release 57 - Mar 2010, <http://www.ensembl.org>).
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Conceived and designed the experiments: DM MS SG MBF MAR. Performed the experiments: DM MS KA. Analyzed the data: DM MS SG MBF MAR. Contributed reagents/materials/analysis tools: DM SG MBF MAR. Wrote the paper: DM MS SG MBF MAR.