BRIEF COMMUNICATIONS

Toll-Like Receptor 4 in Butylated Hydroxytoluene–Induced Mouse Pulmonary Inflammation and Tumorigenesis

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Because chronic pulmonary diseases predispose to lung neoplasia, the identification of the molecular mechanisms involved could provide novel preventive, diagnostic, and therapeutic strategies. Toll-like receptors (TLRs) transduce exogenous and endogenous signals into the production of inflammatory cytokines to coordinate adaptive immune responses. To determine the role of Tlr4 in chronic lung inflammation, we compared lung permeability, leukocyte infiltration, and nuclear factor kappa B (NFκB) and activator protein 1 (AP-1) DNA binding in butylated hydroxytoluene (BHT)-treated (four weekly injections of 125–200 mg/kg each) inbred mouse strains with functional Tlr4 (OuJ and BALB) and mutated Tlr4 (HeJ and BALB^{Lps-d}). We also measured primary tumor formation in these mice after single-carcinogen injection (3-methylcholanthrene; 10 μg/kg), followed by BHT treatment (six weekly injections of 125–200 mg/kg each). Mice with functional Tlr4 had reduced lung permeability, leukocyte inflammation, and primary tumor formation (BALB^{Lps-d}, mean = 22.3 tumors/mouse, versus BALB, mean = 13.9 tumors/mouse, difference = 8.4 tumors/mouse, 95% confidence interval = 4.6 to 12.1 tumors/mouse; P = .025) compared with mice with mutated Tlr4. NFκB DNA binding activity was higher in OuJ than in HeJ mice; however, AP-1 activity was elevated in HeJ mice. To our knowledge, this is the first model to demonstrate a modulatory role for Tlr4 in chronic lung inflammation and tumorigenesis. [J Natl Cancer Inst 2005;97:1778–81]

Lung cancer has a mortality rate greater than that of breast, colorectal, prostate, and pancreatic cancers combined, largely due to the inability to diagnose early-stage disease coupled with therapeutic intransigence after metastatic spread *(1)*. Pulmonary diseases characterized by chronic inflammation, such as bronchitis, emphysema, and asthma, enhance lung cancer risk *(2–4)*. As a preclinical model system, primary mouse lung neoplasms resemble human lung adenocarcinoma in their anatomy, histogenesis, and molecular features *(5)*. The absence of functional Toll-like 125^{N} receptor 4 (Tlr4), an innate immunity gene, enhances susceptibility to lung injury caused by bacterial infections *(6,7)*. A single-nucleotide polymorphism in TLR4 has been associated with prostate cancer *(8),* and Tlr4 mediates anticancer efficacy by the streptococcal agent OK-432 *(9)*. We hypothesized that functional Tlr4 would reduce lung neoplasia in a two-stage chemical carcinogenesis mouse model in which inflammation mediates the promotion phase.

Butylated hydroxytoluene (BHT) is not carcinogenic *(10)* and is metabolized in the lungs of mice to oxidative species that cause reversible lung injury and inflammation and promote lung tumorigenesis following protooncogene activation *(11–13)*. Promotion is dependent on inflammation elicited by BHT metabolites *(14)*. C3H/HeJ (HeJ) mice have a dominant negative proline-to-histidine substitution *(15)* in Tlr4, whereas the coisogenic C3H/HeOuJ (OuJ) strain has functional Tlr4 *(15)*. C.C3H-*Tlr*$^{Lps-d}$ (BALB^{Lps-d}) mice are congenic for a 10 centimorgan region of HeJ chromosome 4 that contains Tlr4 and thus lack Tlr4 function compared with wild-type BALB/cJ (BALB) mice *(16)*.

Two protocols were used in this study based on a previous study that demonstrated a correlation, but not a causal relationship, between BHT-induced inflammation and promotion *(14)*. To study the effects of Tlr4 on inflammation (protocol 1), HeJ, OuJ, BALB^{Lps-d}, and BALB mice (6–8 weeks of age; Jackson Laboratories, Bar Harbor, ME) were injected intraperitoneally with BHT (Sigma, St. Louis, MO) once a week for 4 weeks (125–150 mg/kg for the first dose, followed by 200 mg/kg for the next three doses). Mice were killed 1 day after the last injection to assess vascular leakage of proteins into bronchoalveolar lavage fluid and 3 days after BHT injection to assess leukocyte infiltration. The right lung was lavaged, total protein concentration was determined as an indicator of hyperpermeability, and leukocyte infiltration was estimated as described previously *(17)*. To assess the role of Tlr4 in BHT-induced lung tumor promotion (protocol 2), mice were injected intraperitoneally with 10 μg 3-methylcholanthrene (MCA; Sigma)/gm of body weight or with corn oil vehicle. At this dose, MCA did not cause lung inflammation (data not shown). One week later, 150 mg of BHT/kg was administered intraperitoneally, followed by five weekly injections of 200 mg/kg each. The two additional BHT injections were given (compared with four in protocol 1) to maximize promotional efficacy; the number of tumors that arise is a linear function of the number of BHT injections *(18)*. Mice were killed 20 weeks after the MCA injection, lungs were removed en bloc and fixed in Tellyesniczky's fixative *(19)* for 48 hours, and tumors were counted using a dissecting microscope. The National Institute of Environmental Health Sciences Animal Care and Use Committee approved all protocols. Differences between groups were tested by two-way analysis of variance and Tukey a posteriori comparisons of means (SigmaStat, Jandel Scientific

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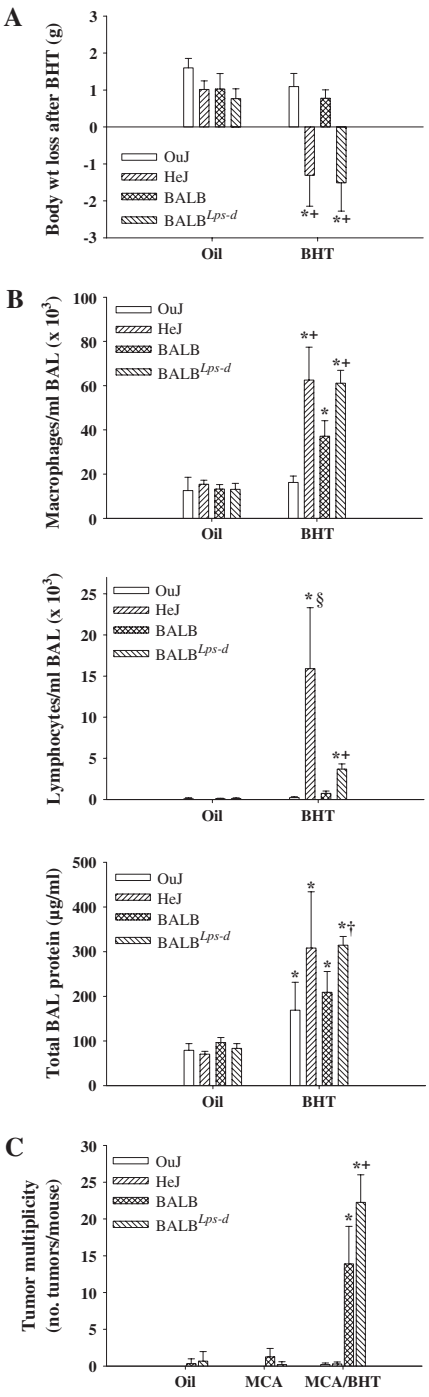

Software, San Rafael, CA). All statistical tests were two-sided; $P<.05$ was considered statistically significant.

To identify the potential mechanisms by which functional Tlr4 inibits BHT-induced injury, we examined signaling elements myeloid differentiation factor 88 (MyD88), nuclear factor kappa B (NFκB), and activator protein 1 (AP-1), all of which act downstream of TLR4 in HeJ and OuJ mice. Immunoblotting was performed using 100 μg of protein obtained from total lung homogenates, and blots were quantified (BioRad Quantity Image One Software; BioRad, Hercules, CA) as previously described *(12)*. TLR4 (goat polyclonal; L14) and MyD88 (goat polyclonal; N-19) antibodies were obtained (Santa Cruz Biotechnology, Santa Cruz, CA), and nuclear protein was prepared according to the manufacturer's instructions (Nuclear Extraction Kit; Active Motif, Carlsbad, CA). To determine transcription factor DNA binding activity, 7 μg of nuclear protein was incubated with consensus sequences for either NFκB or AP-1, and specific activity was determined with p65 NFκB (rabbit polyclonal), phosphorylated (p)-c-jun, or c-fos (both rabbit polyclonal) antibodies by colorimetric analysis (TransAm NFκB and AP-1 kits; Active Motif).

Functional Tlr4 inhibited BHT-induced weight loss, inflammation, and MCA/BHT-induced tumor formation. Statistically significantly greater weight loss was observed in Tlr4-mutated HeJ and BALB$^{Lps\text{–}d}$ mice compared with their respective Tlr4 normal controls (HeJ versus OuJ, difference = −6.7%, 95% confidence interval [CI] = −10.2% to −3.1%, $P = .006$; BALB$^{Lps\text{–}d}$ versus BALB, difference = −7.9%, 95% CI = 11.2% to −4.6%, $P<.002$) (Fig. 1, A.) Survival rates were 100% for OuJ, BALB, and BALB$^{Lps\text{–}d}$ mice and 62% for HeJ mice (95% CI = 39.0% to 85.0%; $P = .032$). In addition, bronchoalveolar lavage fluid from HeJ and BALB$^{Lps\text{–}d}$ mice contained more protein, alveolar macrophages, and lymphocytes than that from OuJ and BALB mice, respectively (Fig. 1, B), suggesting a protective role for TLR4 in lung inflammation and permeability. Lung tumors did not develop in MCA/BHT-treated OuJ or HeJ mice because these strains contain a tumor resistance allele at the Kras locus *(20)*. Tumor-sensitive BALB mice contain one copy of a 37-bp intronic sequence in Kras *(21),* whereas two copies are found in resistant strains, such as OuJ and HeJ *(22)*. The results described herein confirm earlier findings in C3H mice *(23)*. Tumor multiplicity increased 60% in BALB$^{Lps\text{–}d}$ mice compared with that in BALB mice (means = 22.3 and 13.9 tumors/mouse, respectively; difference = 8.4 tumors/mouse, 95% CI = 4.6 to 12.1 tumors/mouse; $P = .025$) (Fig. 1, C), but no differences in overall tumor size or morphology were noted. Interestingly, increases in numbers of pulmonary lymphocytic aggregates were found in Tlr4-mutated mice compared with wild-type mice following MCA or MCA/BHT treatment, although the importance of these cellular aggregates remains unclear.

Chronic BHT treatment increased the protein levels of TLR4 and the MyD88 adaptor protein responsible for TLR4-mediated signal transduction *(24)* 3 days after the final BHT injection in OuJ mice (Fig. 2, A). Although TLR4 protein levels increased in the HeJ mice, the levels remained statistically significantly lower than those in OuJ mice, and no changes in MyD88 concentration were found in HeJ mice (Fig. 2, A). BHT treatment led to elevated p65 NFκB transcriptional activity in both strains at 3 days (Fig. 2, B), but binding was statistically significantly greater in OuJ mice compared with HeJ mice (Fig. 2, B). Compared with HeJ mice, a larger increase in p50 and p65 NFκB DNA binding was found in OuJ mice after 3 days (electromobility shift assay) (data not shown). NFκB activation can be proapoptotic *(25);* hence, it can inhibit tumor formation by altering the balance between apoptosis and proliferation. In contrast with the increase in NFκB activation observed in the TLR4 wild-type mice, AP-1 transcriptional activity

Fig. 1. Comparisons of butylated hydroxytoluene (BHT)– or 3-methylcholanthrene (MCA)/BHT–induced pathologies in strains of mice with normal (OuJ and BALB) or mutated (HeJ and BALB$^{Lps\text{–}d}$) Toll-like receptor 4 (Tlr4). **A**) Body weight changes after a single BHT injection. Means and 95% confidence intervals (CIs) are presented; n = 10–19 mice/experimental group. *, $P<.001$ compared with oil vehicle; +, $P<.001$ compared with OuJ and BALB controls. **B**) Total bronchioalveloar lavage fluid (BAL) macrophages, lymphocytes, and protein recovered from OuJ, BALB, HeJ, and BALB$^{Lps\text{–}d}$ mice after chronic BHT treatment. Means and upper 95% CIs are presented; n = 6–18 mice/experimental group. *, $P<.001$, and §, $P = .016$ compared with oil vehicle; +, $P<.001$ compared with OuJ and BALB controls; †, $P = .002$ compared with BALB control. **C**) Tumor multiplicity (the total number of tumors per total number of mice) in OuJ, BALB, HeJ, and BALB$^{Lps\text{–}d}$ mice 20 weeks after oil, MCA, or MCA/BHT treatment. Tumor incidence (the total number of mice with tumors) was 100% in BALB and BALB$^{Lps\text{–}d}$ mice in response to MCA/BHT treatment and <70% in the control treatment groups, as shown previously in this promotion model *(13)*. Means and upper 95% CIs are presented; for all strains, n = 3–5 mice per oil and MCA experimental groups, n = 14–16 OuJ and HeJ mice per MCA/BHT experimental group, and n = 8–9 BALB and BALB$^{Lps\text{–}d}$ mice per MCA/BHT experimental group. The MCA/BHT protocol was repeated once in BALB and BALB$^{Lps\text{–}d}$ mice. *, $P<.001$ compared with MCA and oil vehicle groups; +, $P<.025$ compared with BALB mice. No tumors were found in OuJ or HeJ mice in oil and MCA experimental groups. Differences between groups were tested using two-way analysis of variance and Tukey a posteriori comparisons of means; all statistical tests were two-sided.

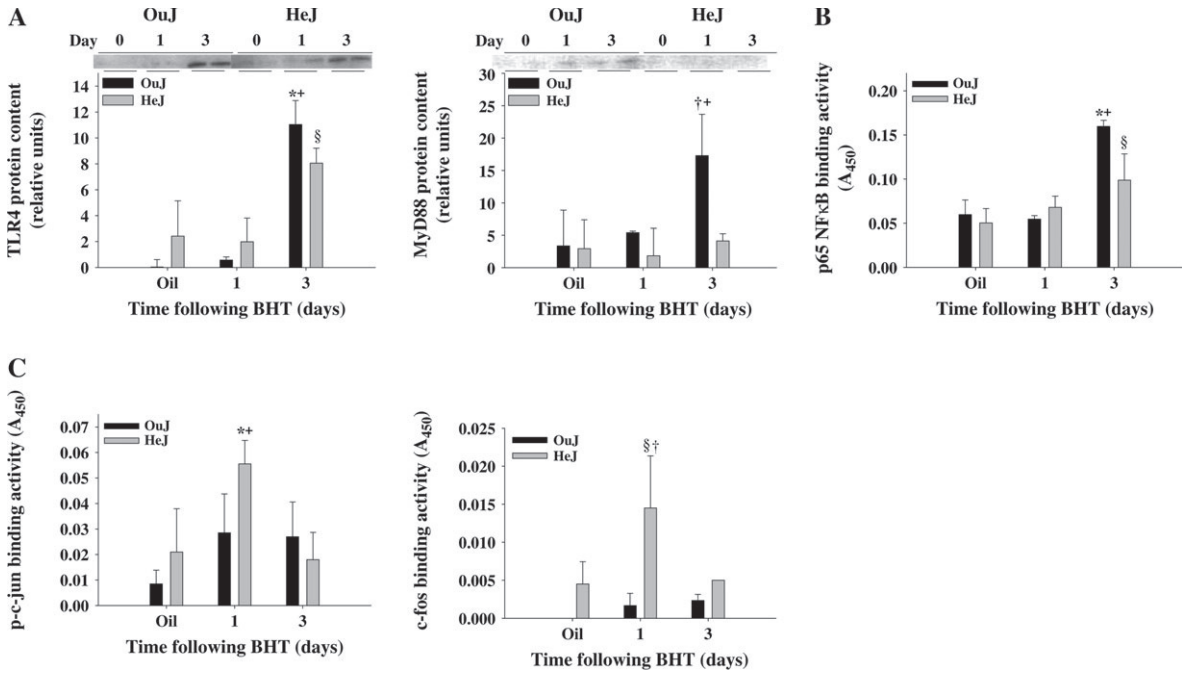

Fig. 2. Toll-like receptor 4 (TLR4) signaling after chronic butylated hydroxytoluene (BHT) treatment in C3H/HeOuJ (OuJ) compared with C3H/HeJ (HeJ) mice. **A**) Quantification of immunoblots for TLR4 and myeloid differentiation factor 88 (MyD88, adaptor protein) to estimate protein expression in whole-cell lung homogenates. Means and upper 95% confidence intervals (CIs) are presented; n = 3 mice per treatment. *, $P<.001$, §, $P = .025$, †, $P = .004$ compared with oil vehicle. +, $P = .009$ compared with HeJ mice. **B**) p65 nuclear factor kappa B (NFκB) transcription factor activity in OuJ and HeJ mice indicated by measurement of A_{450} (Trans-Am NFκB kit). Means and upper 95% CIs are presented. *, $P<.001$, and §, $P = .004$ compared with oil vehicle. +, $P<.001$ compared with HeJ mice. **C**) Nuclear phosphorylated (p)-c-jun and c-fos activator protein 1 (AP-1) transcription factor activity in OuJ and HeJ mice indicated by measurement of A_{450} (Trans-Am AP-1 kit). Means and upper 95% CIs are presented. *, $P<.001$, and §, $P = .007$ compared with oil vehicle. +, $P<.001$, and †, $P = .027$ compared with OuJ. Differences between groups were tested using two-way analysis of variance and Tukey a posteriori comparisons of means; all statistical tests were two-sided.

was elevated in HeJ but not OuJ mice 1 day following BHT treatment, as evidenced by increased p-c-jun and c-fos binding to the AP-1 response element (Fig. 2, C). Therefore, differential transcriptional activities following BHT treatment of mice depend on the presence or absence of functional Tlr4. The functional importance of the different transcription factor kinetics observed is not clear.

These novel findings, in a chronic pulmonary inflammation model, demonstrate the importance of Tlr4 in chronic disease states. Our results suggest that Tlr4 inhibits lung carcinogenesis by inhibiting tumor promotion and that it may be an effective target for alternate preventive, diagnostic, and therapeutic strategies.

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NOTES

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