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Neutrophils Are Required for 3-Methylcholanthrene-Initiated, Butylated Hydroxytoluene-Promoted Lung Carcinogenesis

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Abstract

Multiple studies have shown a link between chronic inflammation and lung tumorigenesis. Inbred mouse strains vary in their susceptibility to methylcholanthrene (MCA)-initiated butylated hydroxytoluene (BHT)-promoted lung carcinogenesis. In the present study we investigated whether neutrophils play a role in strain dependent differences in susceptibility to lung tumor promotion. We observed a significant elevation in homeostatic levels of neutrophils in the lungs of tumor-susceptible BALB/cByJ (BALB) mice compared to tumor-resistant C57BL/6J (B6) mice. Additionally, BHT treatment further elevated neutrophil numbers as well as neutrophil chemoattractant keratinocyte-derived cytokine (KC)/chemokine (C-X-C motif) ligand 1 (Cxcl1) levels in BALB lung airways. Lung CD11c+ cells were a major source of KC expression and depletion of neutrophils in BALB mice resulted in a 71% decrease in tumor multiplicity. However, tumor multiplicity did not depend on the presence of T cells, despite the accumulation of T cells following BHT treatment. These data demonstrate that neutrophils are essential to promote tumor growth in the MCA/BHT two-step lung carcinogenesis model.

Keywords

neutrophils; T cells; KC; lung; tumor

INTRODUCTION

The tumor microenvironment can have a profound effect on proliferation, migration, and survival of transformed cells [1–5]. A significant number of cancers are associated with chronic inflammatory conditions caused by environmental pollutants, dietary components,

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and infections. In the lung, inflammatory diseases such as asthma and chronic obstructive pulmonary disorder are associated with increased risk for lung cancer [6–8].

Cells of both the adaptive and innate immune system are frequently observed in tumors and can function in both pro- and anti-tumorigenic capacities [1]. Considerable work has focused on the role of T cell subsets in immunomodulation of cancer as these cells are frequently found to accumulate in tumors relative to the surrounding tissue. However, other immune cells such as macrophages, dendritic cells, and neutrophils have also been observed in and around neoplastic tissue. Recent evidence highlights a role for neutrophils in cancer and lung cancer specifically [9]. High levels of neutrophils and IL-8 (a neutrophil chemoattractant) have been observed in both lung biopsies and the BAL fluid of patients with bronchoalveolar carcinoma and these high levels have been correlated with poor clinical outcome [10]. Additionally, genetic association of single nucleotide polymorphisms (SNPs) in neutrophil-specific genes including myeloperoxidase (MPO), α1-antitrypsin and neutrophil elastase, have been associated with increased risk for lung cancer [11,12]. Neutrophil MPO has also been implicated in the bioactivation of polycyclic aromatic hydrocarbons such as the lung carcinogen benzo(*a*)pyrene [11]. Neutrophil-mediated inhibition of nucleotide excision base repair in an alveolar epithelial cell line has also linked this cell type to tumor progression [13,14]. Moreover, lung tumor initiation by conditional activation of oncogenic K-Ras in the mouse lung is associated with neutrophil infiltration and expression of several inflammatory cytokines, including neutrophil chemoattractants [15].

Mouse chemical carcinogenesis models have been central to elucidating the molecular events that both initiate and promote tumorigenesis. The two-stage skin carcinoma mouse model first demonstrated that carcinogen initiates tumorigenesis and further progression to carcinoma requires additional treatment with mitogenic and inflammatory promoting agents [16,17]. In the mouse lung, initiation of lung tumorigenesis with the carcinogen methylcholanthrene (MCA) results in mutation and activation of K-Ras and progression of tumors can occur by administration of various inflammatory agents [18–20]. Butylated hydroxytoluene (BHT), a once common synthetic food additive, when delivered to mice intraperitoneally, by diet or gavage, results in type I cell necrosis followed by type II cell hyperplasia and inflammatory cell infiltration into alveoli [21–23]. BHT elicits similar lung injury as other lung irritants and also exhibits strain dependent differences in the inflammatory response. BALB strain mice are particularly susceptible to inflammatory responses such as increases in lymphocyte and macrophage numbers, Cox-2 expression, and promotion of tumorigenesis by BHT, while B6 are not [22,23].

Host genetics in the mouse controls the degree of the inflammatory response to several inflammatory agents, including BHT [24]. Several mouse quantitative trait loci (QTL) that control genetic susceptibility to lung inflammation, also colocalize with QTLs that regulate lung tumor susceptibility in mice [25]. These associations suggest that genetic susceptibility to pulmonary inflammation may be a key tumor promotion step during the process of lung tumorigenesis. Collectively these data suggest that the influence of host genetics plays a critical role in lung inflammation and susceptibility to lung cancer. QTL mapping using BALB, B6, and CXB recombinant inbred strains has identified several, multi-gene containing genomic regions that are both common and distinct with other lung irritants [18,25]. However, these loci are broad and precise determination of specific susceptibility genes and gene variants is difficult. We believe that more powerful and precise gene mapping methods (e.g., Genome Wide Association Studies in multiple inbred strains) combined with a better understanding of the inflammatory mechanisms involved, will more quickly and precisely identify relevant susceptibility genes and gene variants. Our aim in this study is to identify these inflammatory mechanisms.

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Previous characterization of BHT-induced recruitment of inflammatory cells in the bronchoalveolar space of the mouse lung has been limited to cytological observations demonstrating increases in macrophages and lymphocytes [21,22]. In the present study, we used flow cytometry to characterize immune cell infiltrate in the airways of murine lungs after chronic exposure to BHT. We observed higher numbers of T cells, macrophages, and dendritic cells in the lungs of inflammation susceptible BALB mice, as compared to inflammation resistant B6 mice. However, unlike the other immune cell subsets, we also observed higher steady state levels of neutrophils in BALB mice as well as elevated constitutive production of the neutrophil attractant keratinocyte-derived cytokine (KC), by pulmonary CD11c+ cells. Interestingly, both neutrophil and KC levels were significantly increased in response to BHT 1 d postinjection, and returned to normal levels at day 6. Antibody-mediated neutrophil depletion in the two-step MCA/BHT carcinogenesis model led to a significant decrease in tumor multiplicity. These results illustrate a pro-tumorigenic role for neutrophils in inflammation promoted lung carcinogenesis.

MATERIALS AND METHODS

BHT Treatment and BAL Collection

Female BALB and B6 inbred mice were delivered from The Jackson Laboratory (Bar Harbor, ME) at 5 wk of age and housed in a pathogen free barrier facility. For induction of inflammation, BALB and B6 strains (at 7 wk of age) were subject to intraperitoneal administration of a 0.2 mL dose of 150 mg/kg body weight of BHT dissolved in corn oil on week 1, followed by three weekly doses at 200 mg/kg. Corn oil was used as a control in non-BHT treated animals. One or 6 d after the fourth injection, the mouse was anesthetized, and euthanized by cervical dislocation. The thoracic cavity was surgically opened, the trachea was cannulated with a 22G catheter, and the lungs were lavaged three times (1×0.7 mL, 2×1 mL) with phosphate buffered saline solution. Red blood cells were lysed with ACK lysis buffer (Lonza, Walkersville, MD), and remaining cells were collected by centrifugation (10 min at 200*g*) and counted prior to staining for flow cytometry. The supernatant was also retained for further analysis of cytokine levels. For preparation of single cell suspension from whole lung, lungs were removed, minced, and digested (RPMI 1640 + 5 U/μL DNAseI + 1 mg/mL collagenase) at 37°C for 90 min, and strained through a 70 μm filter prior to preparation for staining. The Washington University and Medical College of Wisconsin Animal Care and Use Committees approved the protocols we implemented.

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Flow Cytometry Analysis

Cells collected by BAL of the lungs of BALB and B6 mice were prepared for flow cytometry as previously described [26]. Mouse blood (10 μL) was obtained by tail bleeding and collected in ACK lysis buffer (Lonza) supplemented with 10 mM EDTA. All antibodies [CD90.2 (53-2.1), CD11b (M1/70), CD11c (N418), CD4 (RM4-5), CD8 (53-6.7), Gr-1 (RB6-8C5), Ly6G (1A8), B220 (RA3-6B2), CD19 (1D3), CD62-L (MEL-14), CD44 (IM7)] and isotype controls were purchased from BD Pharmingen (San Diego, CA) or eBioscience (San Diego, CA) conjugated with either fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), allophycocyanin (APC), or APC-eFluor®780 (APC780). Flow cytometry was performed at the High-Speed Cell Sorter Core of the Siteman Cancer Center at Washington University School of Medicine (St. Louis, MO) and at the Flow Cytometry Core at the Blood Research Institute (Milwaukee, WI).

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Cytokine Panel Analysis

The BioPlex Pro Mouse Cytokine Standard Group 1 23-plex kit (Bio-Rad, Hercules, CA) was used to perform multiplex analysis of a panel of cytokines in mouse BAL. Fluorescently

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distinct microbeads were incubated with BAL, imaged and evaluated on a Luminex®100 multiplex bio-assay analyzer (Luminex, Austin, TX). Concentrations of cytokines were calculated using the Luminex xPONENT® software package with reference to a concurrently run Bio-Plex Mouse Cytokine 23-plex standard.

Cell Isolation and KC Immunoassay

Mouse lungs were prepared as previously described [26]. Mouse CD11c microbeads (Miltenyi Biotec, Auburn, CA) were used to positively select for cells according to the manufacturer's protocol. Approximately 5×10^5 CD11c+ cells were isolated per mouse lung, and 9×10^4 cells were plated per well in a 96-well dish with complete media (RPMI 1640, 10% FBS, 1% glutamine, 0.1 mM nonessential amino acids, 50 μM β-mercaptoethanol, 100 U/mL penicillin G, 0.1 mg/mL streptomycin, 20 ng/mL granulocyte macrophage colony stimulating factor), treated with or without 1 μg/mL lipopolysaccharide (LPS), 50 μM BHT in DMSO, or DMSO control. Media was collected 18 h posttreatment and KC levels were measured using the Quantikine Mouse KC Immunoassay (R&D Systems, Minneapolis, MN).

Two Step MCA/BHT Carcinogenesis Assays

Surgically thymectomized BALB mice were obtained from The Jackson Laboratory at 5 wk of age. Mice were injected once intraperitoneally with anti-CD4 (GK1.5) and anti-CD8 (53.6.72) depleting antibodies (BioXcell, West Lebanon, NH) at 500 μg/antibody/mouse) 6 d prior to MCA injection at 7 wk of age. In the neutrophil depletion experiments mice were injected once intraperitoneally with 100 μg anti-Ly6G (1A8) or control rat IgG2a (2A3) antibodies (BioXcell), 24 h prior to each MCA and BHT injection. The two step carcinogenesis protocol involved one intraperitoneal injection of MCA (15 mg/kg body weight) in corn oil per mouse, followed by six weekly intraperitoneal injections of BHT (first injection: 150 mg/kg, next five injections: 200 mg/kg). At 20 wk post-MCA injection, mice were anesthetized and euthanized by cervical dislocation. The thoracic cavity was surgically opened to expose the lungs. The trachea was cannulated with a 22G catheter and lungs were inflated with Tellyesniczky's solution (70% ethanol, 2% formaldehyde, and 5% acetic acid) at 25 cm of pressure by gravity. Lungs were fixed overnight and the solution was exchanged to 70% ethanol the next day. Lung tumor multiplicity and size (diameter > 0.25 mm) was assessed in a blinded fashion.

Statistical Analysis

All analysis of significance ($P < 0.05$) was performed by two-tailed Student's *t*-test.

RESULTS

Cell Infiltrates in Bronchoalveolar Lavage Fluid in Response to BHT

To provide further analysis of the inflammatory cell infiltrate that promotes lung tumorigenesis we utilized the BALB susceptible and B6 resistant inbred strains that have differential responses to both BHT-initiated pulmonary inflammation and BHT-promoted MCA-initiated tumorigenesis. Consistent with previous reports, immune infiltrates were present in BAL fluid collected 6 d after the final injection of four weekly injections of BHT [21,22]. Flow cytometric analysis of cell surface markers identified a BHT-dependent induction of CD11b– CD11c+ alveolar macrophages (11-fold in BALB, twofold in B6; $P < 0.05$) (Figure 1a), and additionally showed that both CD8+ CD90+ (89-fold in BALB, sixfold in B6; $P < 0.05$) and CD4+ CD90+ (42-fold in BALB, sixfold in B6; $P < 0.05$) subsets of T cells were induced for which strain dependent differences were observed for CD4 cells (Figure 1b). Additionally, we also identified increases in CD11b+ CD11c+ cells

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consistent with an inflammatory dendritic cell phenotype (eightfold in BALB, twofold in B6; $P < 0.05$) that were also BHT and strain dependent (Figure 1a). No differences in the accumulation of B220+ CD19+ B cells were observed in either strain of mice with or without BHT (data not shown). Ly6G+ GR1+ neutrophils were significantly higher in the lungs of unstimulated BALB mice than unstimulated B6 mice, but were not induced further by BHT when measured 6 d after the fourth BHT injection (Figure 1c).

T Cell Memory Phenotypes Are Enhanced in BHT Treated BALB Mice

Activated T cells exhibit tumor-promoting activities through production of cytokines, activation of B-cells and education of macrophages. Because both CD4 and CD8 T cell numbers were highly induced in the BAL, we evaluated T cell activation following the standard 4-wk course of BHT inflammation. Whole lungs were removed, digested and analyzed by flow cytometry for expression of the CD62L homing receptor and CD44 lymphocyte activation marker. Central memory phenotype (CD62L+ CD44+) activated CD4+ and CD8+ T cells were significantly elevated in BALB compared to B6 strain lungs (Figure 2a). Although resting numbers of T cells were similar in both strains (data not shown) we found that the levels of central memory phenotype T cells were twofold higher in the lungs of BHT treated BALB versus B6 mice (Figure 2a).

T Cells Are Not Required for MCA-Initiated BHT-Promoted Carcinogenesis

The observations of heightened CD4 and CD8 T cell activation and accumulation within the lungs of the susceptible BALB strain led us to ask if T cells are required for MCA-initiated BHT-promoted carcinogenesis. To answer this question we acquired BALB mice that were surgically thymectomized at 3 wk of age and thus incapable of further producing T cells. At 7 wk of age, we subjected these mice to a single intraperitoneal injection with CD4 and CD8-specific antibodies to further deplete any resident or extrathymically produced T cells, which resulted in near complete elimination of T cells from the peripheral blood (Figure 2b). We subsequently subjected T cell depleted BALB mice and wild-type BALB mice to a lung tumor promotion assay involving a single intraperitoneal injection of MCA followed by six weekly injections of BHT. At 20 wk post-MCA injection, mice were euthanized and surface tumor number and diameter was evaluated. We observed a promotion of lung tumor multiplicity from 1.3 ± 0.6 to 4.7 ± 1.5 tumors/mouse after BHT injection (Figure 2c, left panel). In the T cell depleted mice we observed a similar promotion from 0.8 ± 0.2 to 6.1 ± 1.5 tumors/mouse after BHT injection, indicating there was no significant effect of T cell depletion on MCA/BHT promoted lung tumorigenesis multiplicity ($P = 0.5$) or tumor diameter ($P = 0.8$) (Figure 2c).

Elevated KC Cytokine Levels in BAL Fluid From BALB Strain Mice

As cytokine production has been implicated in lung tumorigenesis, we assayed a panel of 23 mouse cytokines present in the bronchoalveolar lavage fluid of susceptible BALB and resistant B6 mice. While most of the cytokines were either not detected or showed no strain dependence, we did observe that KC/Cxcl1, a neutrophil chemoattractant, was higher in the lavage of untreated BALB mice as compared to untreated B6 ($P = 3 \times 10^{-5}$) (Figure 3a). However, KC levels were not significantly elevated by BHT treatment ($P = 0.7$) when measured at the standard time point 6 d after the fourth and final BHT treatment.

Neutrophils respond rapidly to sites of inflammation and are prime effectors of the early phase response. Hence, we additionally tested an earlier time point to test if levels were stimulated 1 d postinjection. Interestingly, we observed a significant increase in the percentage (0.2% vs. 2.6%, $P = 0.03$) and number of neutrophils (~30-fold, $P = 0.03$) in the BAL fluid of BALB mice treated 1 d after the fourth and final BHT injection (Figure 3b). We did not observe BHT stimulated neutrophil levels in B6 mice (Supplementary Figure 1).

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We also observed a threefold increase in KC levels in the BAL of BALB mice ($P = 0.03$) (Figure 3c).

BALB CD11c+ Cells Express High Levels of KC

Lung macrophages and dendritic cells are prolific producers of inflammatory cytokines. As both populations of cells express the adhesion marker CD11c+ we assessed ex vivo KC production in microbead purified CD11c+ cells from the lung tissue of BALB and B6 mice. CD11c cells were cultured with or without BHT or LPS (as a positive control) for 18 h, and KC levels in the media were measured by ELISA. KC production by CD11c+ cells was eightfold higher in untreated BALB versus B6 CD11c+ cells ($P = 0.001$) (Figure 3d). Direct addition of BHT to CD11c+ cell cultures did not enhance KC production [BALB ($P = 0.6$) or B6 ($P = 0.4$)] (Figure 3d).

Neutrophils Are Required for MCA-Initiated BHT-Promoted Carcinogenesis

Higher homeostatic numbers of neutrophils in the airways of BALB mice, stimulation of neutrophil numbers 1 d post-BHT, in addition to the observation that BALB lung CD11c+ cells produce higher amounts of KC/Cxcl1, led us to directly test the role of neutrophils in the tumor promotion assay in vivo. To do so, we depleted neutrophils in BALB mice 1 d prior to MCA- and prior to each of six weekly BHT-injections of the carcinogenesis protocol. We demonstrated that anti-Ly6G antibody effectively depletes neutrophils in the peripheral blood, BAL fluid and whole lung when tested 1 wk postinjection (Figure 4a). Furthermore, whole lung digests indicated that anti-Ly6G antibody specifically depleted neutrophils and not alveolar macrophage, dendritic or T cells (Supplementary Figure 2a and b). Twenty weeks postcarcinogen, lung tumor multiplicity and size was evaluated and a representative set of lungs were photographed (Figure 4b and c). As compared to tumor promotion in control IgG-treated BALB mice (0.6 ± 0.2 to 5.0 ± 0.9 tumors/mouse), tumor promotion in Ly6G-treated BALB mice was greatly reduced (0.3 ± 0.2 to 1.4 ± 0.5 tumors/mouse) indicating that neutrophil depletion had a strong effect on tumor multiplicity in the MCA/BHT lung tumor promotion model ($P = 0.005$). Average tumor diameter was not significantly reduced in the neutrophil depleted mice (0.55 ± 0.05 mm/tumor to 0.46 ± 0.05 mm, $P = 0.37$) (Figure 4b, right panel).

DISCUSSION

Our data show that neutrophils play a key role in MCA-initiated BHT-promoted lung carcinogenesis, as depletion of neutrophils caused a significant reduction of tumor multiplicity. We did not observe a reduction in tumor diameter suggesting that neutrophils are likely important in the early initiation and promotion stages rather than later growth and progression stages. Additionally, it is important to note that neutrophil depletion was only performed during these early stages. Somewhat surprisingly, depletion of T cells did not affect tumorigenesis as there is a strong response to BHT. It is important to note that the literature is varied with examples of T cell dependent and independent tumorigenic processes [27–30], including ones evaluating carcinogenic processes in the lung [31,32]. We also observed the accumulation of memory-phenotype T cells populations in both strains following BHT treatment raising the possibility that local pulmonary inflammation induces the nonspecific bystander activation of T cells. Interestingly, bystander activation for both memory-phenotype CD4+ and CD8+ T cells can be potentiated by IFN-γ [33,34], an inflammatory mediator recently shown to accumulate in the BAL fluid of a BHT-induced chronic lung inflammation model [35].

Previous studies have demonstrated that BHT administration results in high levels of lymphocytes in the alveolar space when measured 6 d posttreatment [21–23]. Here we

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demonstrated that both CD4+ and CD8+ T cell subsets are increased, and not B cells. We also confirmed observations of increased pulmonary macrophage levels as previously described by Bauer et al. [21], and additionally detected increased numbers of lung cells with dendritic marker phenotype. The precise role of each immune cell subset in pulmonary carcinogenesis remains undefined. A previous report implicated macrophages as mediators of the tumor promotion process through depletion studies involving chlorinated water [22]. However, the specificity of chlorinated water for macrophages is unclear as it may deplete bone marrow myeloid progenitor populations that give rise to macrophages, dendritic cells, and neutrophils. Importantly, Ly6G is not expressed on common myeloid progenitors and the Ly6G 1A8 antibody clone used in this study to deplete neutrophils has been shown by us and others not to target monocyte derived cells that give rise to macrophages or dendritic cells [36]. However, it is still conceivable that depletion of macrophages, a source of KC, could potentially reduce neutrophil numbers in the airway.

The requirement for neutrophils to drive tumorigenesis in our model is consistent with enhanced neutrophil and KC levels in bronchoalveolar carcinoma patients with poor outcome [10]. Data from our model suggests that high KC levels produced by lung tissue-resident BALB CD11c+ cells may enhance susceptibility to pulmonary carcinogenesis, by maintaining high levels of neutrophil trafficking into the lung. Existence of significant numbers of tissue-resident neutrophils in the lung have been hypothesized to be a tradeoff between pathogen elimination at the expense of increased susceptibility to chronic inflammation [37]. We also have shown that BHT temporally induces KC and neutrophil levels 1 d post-BHT treatment. These levels return to normal after 6 d. This is consistent with recent studies showing similar kinetics of KC and neutrophil levels, caused by V_2O_5, another tumor promoter of MCA tumorigenesis [38].

The precise mechanisms of how neutrophils promote tumorigenesis and what role KC expressing CD11c+ cells play in lung carcinogenesis, has yet to be determined. The hypothesis is that BHT administration enhances KC levels and results in recruitment of neutrophils into the lung. Neutrophils are capable of synthesizing a variety of proteases such as cathepsin G, proteinase-3, gelatinase B, collagenase, and elastase, and can also produce reactive oxygen and nitrogen species through MPO activity that can cause genotoxic events such as double-stranded DNA breaks. We hypothesize that BHT induction of neutrophils likely increases these activities in the lung and directly influences genotoxicity and signaling pathways in epithelial cells. Interestingly, a recent study showed a neutrophil’s ability to directly deliver elastase into tumor cells, cause IRS-1 cleavage and enhanced tumor cell proliferation [39]. Additionally, inflammatory conditions can promote the transformed state through effects on angiogenesis [40]. Neutralizing antibody to Cxcr2 (receptor for both KC and Mip2) inhibited heterotopically established lung tumors through a reduction in angiogenesis [41]. In a xenograft model of lung cancer, oncogenic K-Ras-dependent IL-8 production was required for tumor vascularization and growth, in addition to neutrophil infiltration [42]. These data are similar to observations that neutralization of Cxcr2 in Kras-LA1 transgenic mice affected tumor progression through induction of apoptosis of vascular endothelial cells [43]. We have also observed that anti-Cxcr2 antibody delivery to BALB mice effectively depletes neutrophils in the BAL fluid (data not shown). However, whether this is sufficient to inhibit tumorigenesis is unknown and thus remains to be tested.

Our study and a growing body of literature suggest that targeting neutrophils may have therapeutic value in treatment of cancer [44]. However, systemic neutrophil depletion is not a practical approach, as this would seriously compromise the response towards infection. Targeting the IL-8 and CXCR2 receptor signaling pathway is one alternative avenue. Reparixin, a CXCR2 inhibitor, inhibits neutrophil recruitment into the lung in animal models of acute lung injury [45], and is currently in human trials of lung and kidney

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transplantation. Future use of this molecule is likely in preclinical cancer models of prevention and treatment. Alternatively, targeting neutrophil-specific activities such as MPO and elastase is another approach. Sivelestat, a specific inhibitor of neutrophil elastase has shown efficacy in amelioration of acute lung injury [46].

In conclusion, our study identifies neutrophils, as important mediators of inflammation promoted carcinogenesis in the lung and supports further investigation into the molecular mechanisms by which neutrophils enhance susceptibility and promotion of pulmonary carcinogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MPO	myeloperoxidase
MCA	methylcholanthrene
BHT	butylated hydroxytoluene
QTL	quantitative trait loci
KC	keratinocyte-derived cytokine
APC	allophycocyanin

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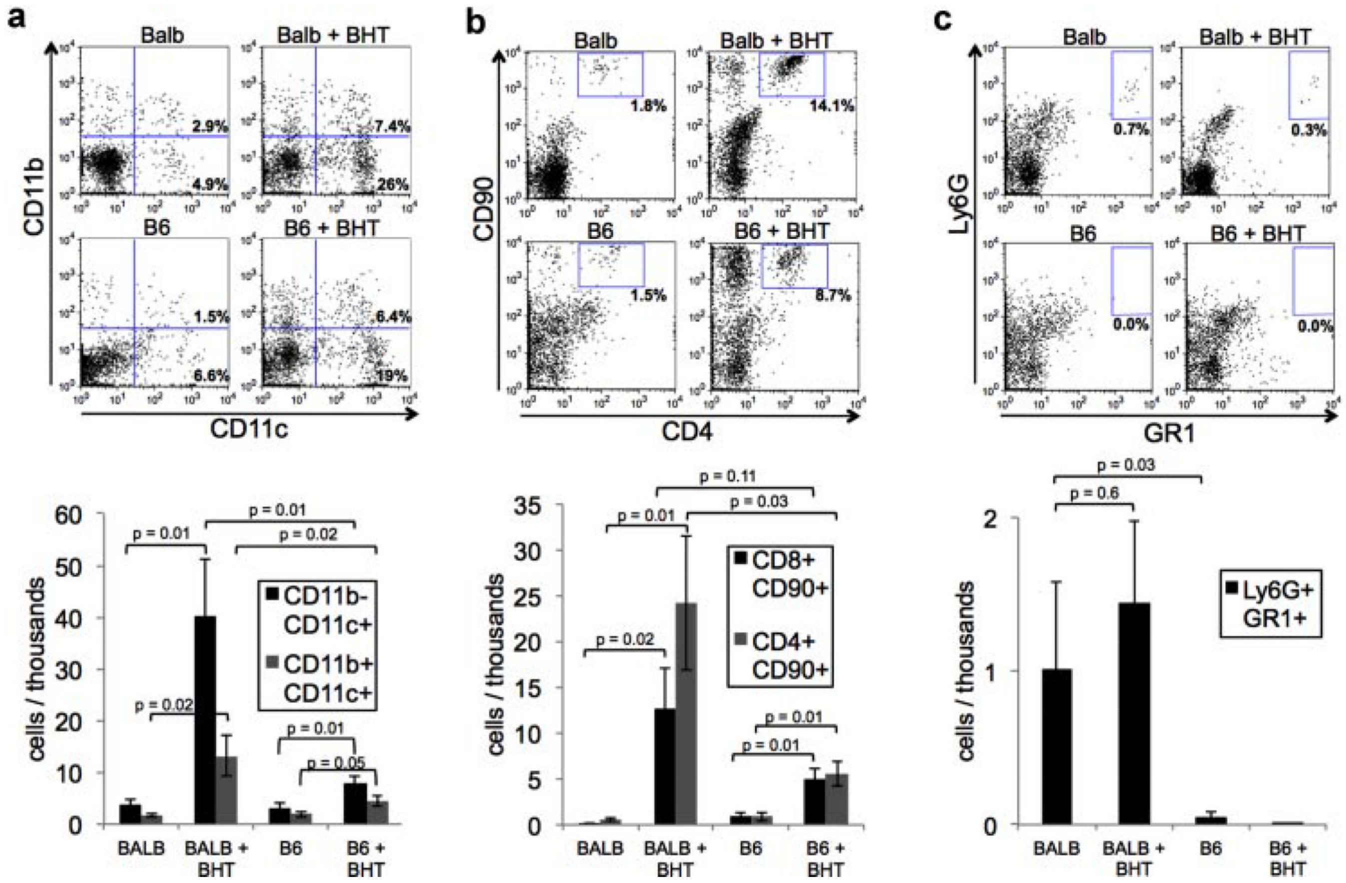

Figure 1.

Total hematopoietic cell infiltrates in bronchoalveolar lavage fluid in response to BHT. BALB and B6 mice were injected four times weekly with BHT or with corn oil vehicle alone ($n = 8$ per group). Six days after the last injection, BAL fluid was collected and cells were stained for cell surface marker analysis. Live cells were selected by forward- and side-scatter profile. Populations of (a) dendritic cells (CD11b+ CD11c+) and alveolar macrophages (CD11b− CD11c+), (b) CD4 T cells (CD4+ CD90+) and CD8 T cells (CD8+ CD90+), and (c) neutrophils (Ly6G+ GR1+) were quantified. Dot plots for a representative set of animals is shown. Two-tailed statistical *t*-tests were performed. Error bar, standard error of the mean.

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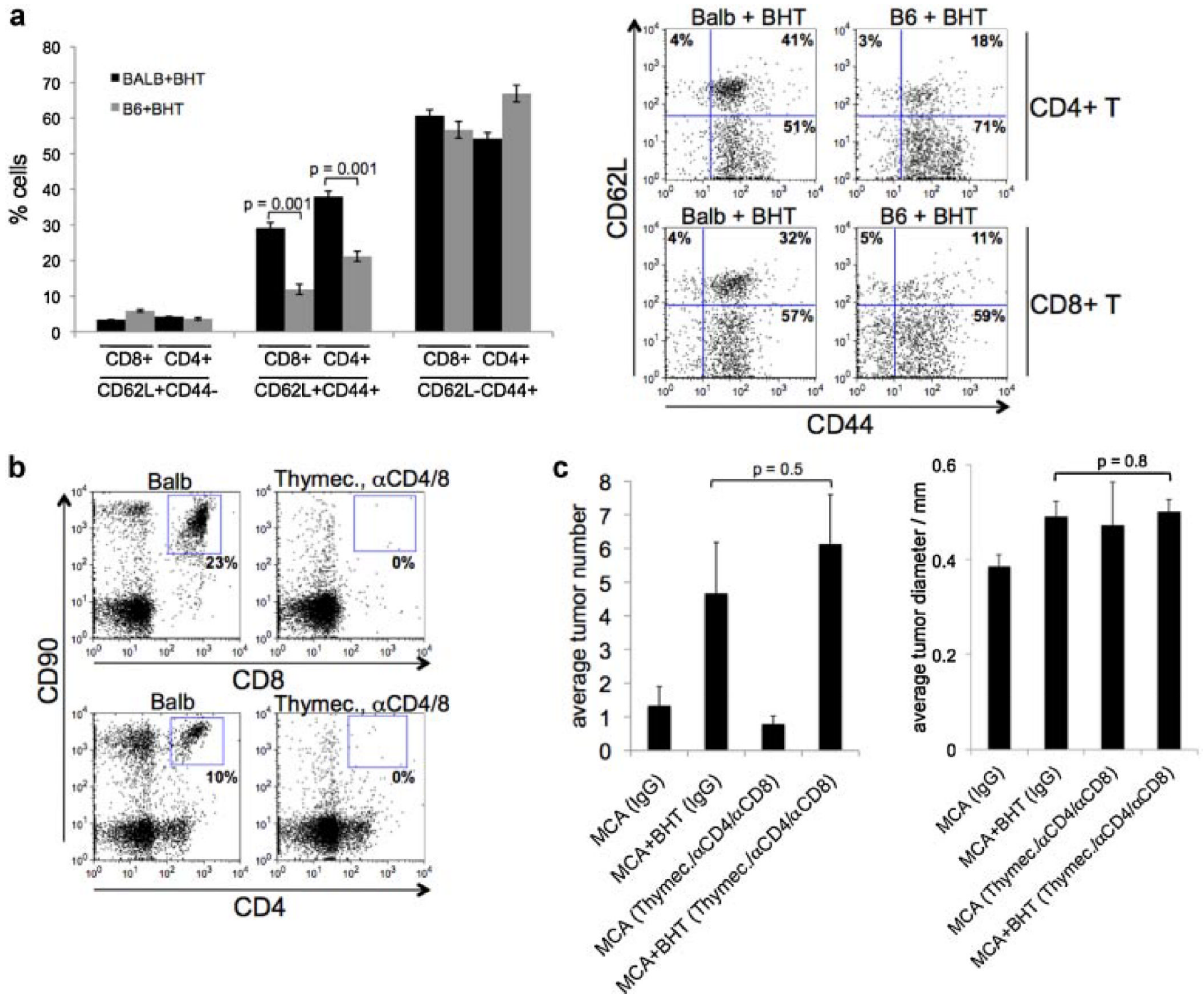

Figure 2.

T cell memory phenotypes are enhanced in BHT treated BALB mice, however T cells are not responsible for MCA-initiated BHT-promoted carcinogenesis. (a) BALB mice injected with BHT exhibit higher levels of central memory phenotype T cells compared to B6 mice. BALB and B6 mice ($n = 3$ per group) were injected four times weekly with BHT. Six days later, whole lungs were digested and stained for flow cytometry analysis. Cells were selected by forward- and side-scatter, and gated on CD45+ CD4+ CD90+ or CD45+ CD8+ CD90+. The percentage of naïve (CD44– CD62L+), central memory (CD44+ CD62L+), and effector memory (CD44+ CD62L–) T cells was determined. Dot plots for a representative set of animals is shown. (b) Thymectomized and anti-CD4/8 treated mice are depleted for T cells as assayed in blood. (c) T cell depletion does not affect tumor multiplicity or tumor diameter in BALB mice. Mouse lung tumor nodules were enumerated (left panel) and diameter measured (right panel) across the indicated groups ($n = 15$ per group). Two-tailed *t*-tests were used to evaluate *P* values. Two-tailed statistical *t*-tests were performed. Error bar, standard error of the mean.

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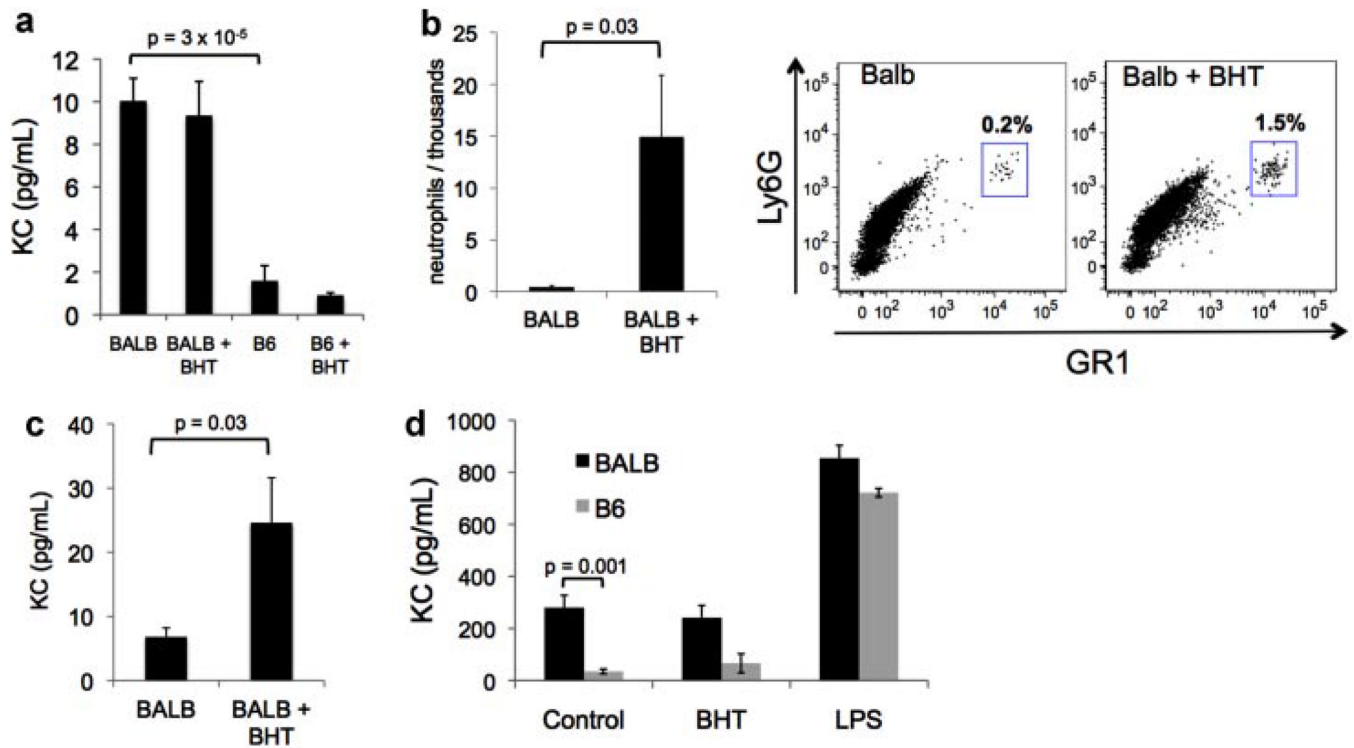

Figure 3.

BHT induces KC and neutrophil levels 1 d posttreatment. (a) Levels of KC are higher in BAL fluid from susceptible BALB mice compared to resistant B6 mice as measured 6 d post-fourth and final BHT injection. (b) Neutrophil numbers increase 1 d post-BHT injection in the BAL fluid of BALB mice collected 1 d postinjection ($n = 4$ per group, $P = 0.03$). Dot plots for a representative set of animals is shown. (c) Levels of KC increased threefold in BAL fluid ($n = 4$ per group, $P = 0.03$) in response to BHT 1 d post-BHT. (d) CD11c+ dendritic cells and alveolar macrophages isolated from BALB mouse lungs and cultured in vitro produce more KC compared to those from B6 mouse lungs. Treatment of isolated cells with BHT (50 μM BHT) for 18 h did not affect KC expression. LPS (1 μg/mL) was used as a positive control. Two-tailed statistical *t*-tests were performed. Error bar, standard error of the mean.

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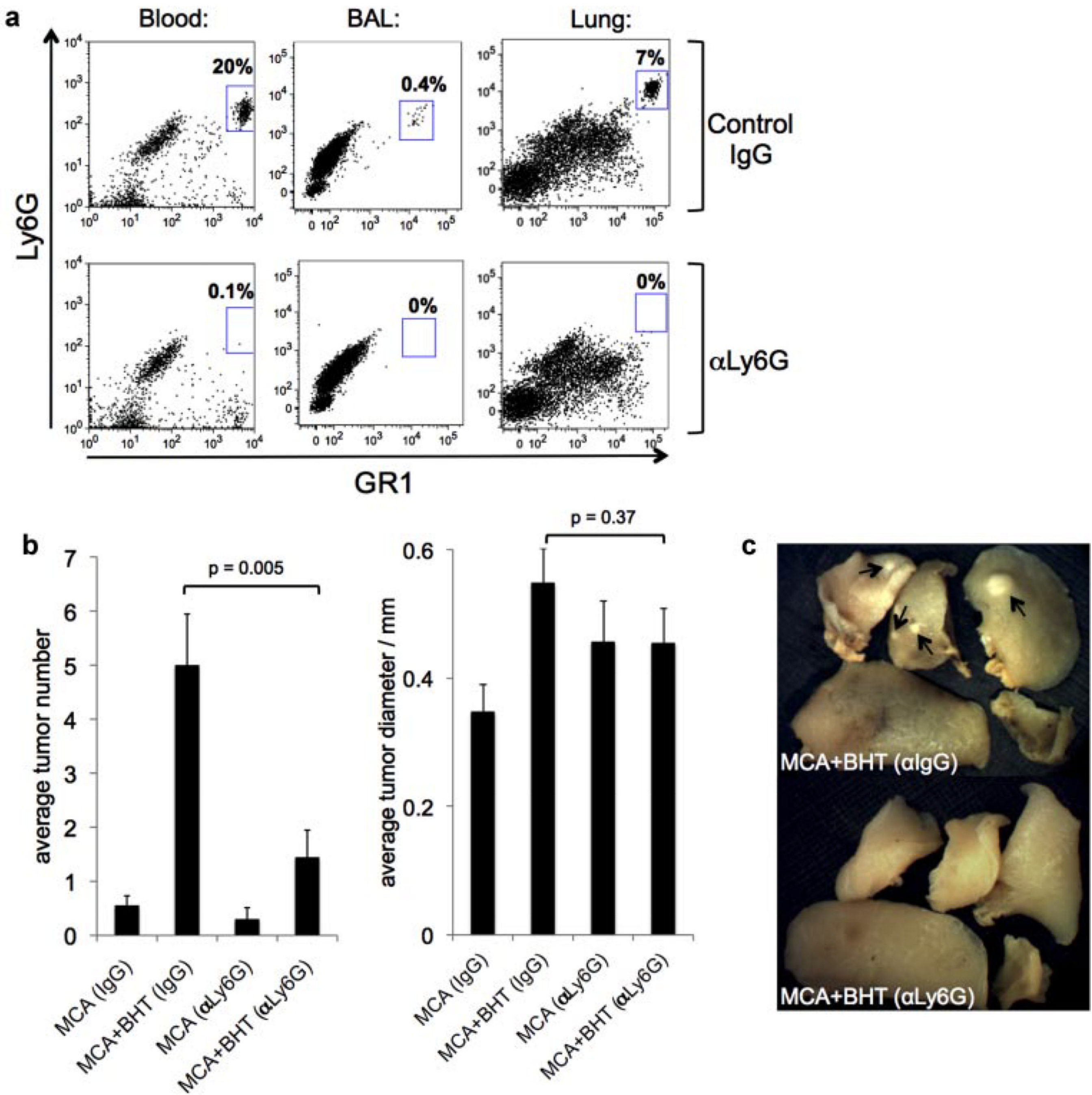

Figure 4.

Neutrophils are required for MCA-initiated BHT promoted carcinogenesis. (a) Single intraperitoneal injection of anti-Ly6G (100 μg) antibody effectively depletes neutrophils 1 wk later, as measured in the blood, BAL fluid, and whole lung. (b) Neutrophil depletion inhibits tumor multiplicity but not tumor diameter in BALB mice. Mouse lung tumor nodules were enumerated (left panel) and diameter measured (right panel) across the indicated groups ($n = 10$ per group). (c) Lungs from anti-Ly6G and control IgG (right panel) treated mice (20 wk post-MCA + BHT) were fixed in Tellyesniczky's solution, stored in 70% ethanol, and had their lobes separated for tumor enumeration. Arrows indicate the

location of tumors. Two-tailed statistical *t*-tests were performed. Error bar, standard error of the mean.

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