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A Critical Role for Macrophages in Promotion of Urethane-Induced Lung Carcinogenesis

Rinat Zaynagettdinov,* Taylor P. Sherrill,* Vasiliy V. Polouskhin,* Wei Han,* Jamie A. Ausborn,† Allyson G. McLoed,† Frank B. McMahon,* Linda A. Gleaves,* Amber L. Degreys,* Georgios T. Stathopoulos,* Fiona E. Yull,† and Timothy S. Blackwell*‡,†,‡

Macrophages have established roles in tumor growth and metastasis, but information about their role in lung tumor promotion is limited. To assess the role of macrophages in lung tumorigenesis, we developed a method of minimally invasive, long-term macrophage depletion by repetitive intratracheal instillation of liposomal clodronate. Compared with controls treated with repetitive doses of PBS-containing liposomes, long-term macrophage depletion resulted in a marked reduction in tumor number and size at 4 mo after a single i.p. injection of the carcinogen urethane. After urethane treatment, lung macrophages developed increased M1 macrophage marker expression during the first 2–3 wk, followed by increased M2 marker expression by week 6. Using a strategy to reduce alveolar macrophages during tumor initiation and early promotion stages (weeks 1–2) or during late promotion and progression stages (weeks 4–16), we found significantly fewer and smaller lung tumors in both groups compared with controls. Late-stage macrophage depletion reduced VEGF expression and impaired vascular growth in tumors. In contrast, early-stage depletion of alveolar macrophages impaired urethane-induced NF-kB activation in the lungs and reduced the development of premalignant atypical adenomatous hyperplasia lesions at 6 wk after urethane injection. Together, these studies elucidate an important role for macrophages in lung tumor promotion and indicate that these cells have distinct roles during different stages of lung carcinogenesis. The Journal of Immunology, 2011, 187: 5703–5711.

Lung cancer is the leading cause of cancer death in the United States and worldwide (1). Although genetic mutations underlie malignant transformation, the presence of mutations by themselves is not sufficient for tumor formation, and additional alterations are necessary for the development of cancer (2, 3). Among the multiple factors initiating and supporting tumor growth, inflammation plays one of the most important roles. In normal conditions, inflammation generated by the innate immune response is essential for host defense, clearance of damaged or transformed cells, and maintenance of tissue homeostasis. However, in the presence of tumors, inflammation may contribute to growth and invasion through elaboration of enzymes that induce tissue damage, release of reactive oxygen and nitrogen species that result in additional mutations, and production of angiogenic factors and cytokines that support survival of malignant cells (4). Among inflammatory cell types in the lungs, macrophages are the most abundant and perhaps the most pleiotropic. Depending on microenvironmental cues, these cells can stimulate inflammatory responses by secretion of proinflammatory cytokines or suppress immune responses by releasing high levels of anti-inflammatory cytokines such as IL-10 and TGF-β (4–7). Based on the phenotypic spectrum of these cells, Mantovani and colleagues (7) classified macrophages into two groups: “classically activated” pro-inflammatory M1 and “alternatively activated” anti-inflammatory M2. Most studies that describe the M1/M2 macrophage paradigm in tumors have been performed during progression stages of established tumors or using metastasis models. Evidence regarding the role of macrophages during early tumorigenesis is lacking. In addition, it is unclear whether carcinogen treatment affects the M1/M2 phenotype of lung macrophages during early stages of tumor formation.

In this study, we investigated the impact of macrophages during different stages of urethane-induced lung carcinogenesis. Using a method of minimally invasive, long-term alveolar macrophage depletion, we found that elimination of macrophages with repetitive doses of liposomal clodronate significantly reduced lung carcinogenesis. Depletion of macrophages during late promotion and progression stages, when there was an increase in cells polarized toward the M2 phenotype, reduced tumor angiogenesis and growth. Interestingly, depletion of macrophages during initiation and early promotion stages, when M1 polarized cells were increased, also resulted in attenuation of tumorigenesis. Early macrophage depletion reduced urethane-induced NF-κB activity and development of premalignant atypical adenomatous hyperplasia (AAH) lesions. Together, these data demonstrate separate

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Abbreviations used in this article: AAH, atypical adenomatous hyperplasia; BAL, bronchoalveolar lavage; IT, intratracheally; MDSC, myeloid-derived suppressor cell; NGL, NF-κB reporter mice.

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but important roles for macrophages in early and late stages of urethane-induced lung tumor formation.

Materials and Methods

Animal experiments
All animal care and experimental procedures were approved and conducted according to guidelines issued by the Vanderbilt University Institutional Animal Care and Use Committee. Sex-, weight-, and age-matched 8- to 10-wk-old wild type and NF-κB reporter (NGL) mice (8) on an FVB background were used. Tumors were induced by a single i.p. injection of 1 g/kg urethane (ethyl carbamate; Sigma-Aldrich). Mice were euthanized at time points up to 4 mo after injection of carcinogen. At the time of sacrifice, lungs were lavaged, perfused, and fixed in ice-cold Bouin’s fixative solution (Sigma-Aldrich) for 24 h. After fixation, lungs were used for surface tumor number and diameter measurements, and embedded in paraffin. Tumors on the lung surface were imaged by at least two experienced readers, blinded to sample identifiers under a dissecting microscope; tumor counts were averaged and statistically analyzed. Tumor diameters were measured using Fisherbrand Traceable digital calipers (Fisher Scientific).

Depletion of macrophages with clodronate
Clodronate (dichloromethylene diphosphonic acid; Sigma-Aldrich) or sterile PBS-containing liposomes were prepared as previously described (9). Liposomes were instilled intratracheally (IT) weekly for 4 mo starting the day of urethane injection (4 mo) on days 0 and 7 after the injection of carcinogen for the depletion of macrophages during initiation and early promotion stages (early stage), and weekly starting at 4 wk after urethane for late stage promotion and progression stage macrophage depletion (late stage). For liposome instillation, mice were anesthetized and intubated using a 1-ml syringe with a 6-mm-long, 22-gauge over-the-needle catheter (Abbocath-T; Venisystems) to inject IT 100 μl liposomal clodronate or vehicle control (PBS) liposomes.

Bronchoalveolar lavage
Bronchoalveolar lavage (BAL) with total and differential cell counting was performed as previously described (10).

Biluminescent imaging
For in vivo imaging, NGL mice (8) were anesthetized, injected retro-orbitally with 1 mg d-luciferin (Biosynth AG) in 100 μl isotonic saline, and imaged using an IVIS cooled charged-coupled device (Xenogen Corporation) as described previously (8, 11). Data were collected and analyzed using Living Image v.2.50 (Xenogen Corporation) and IgorPro (WaveMetrics) software.

Tissue luciferase assay
Lungs were removed en bloc and homogenized in 1 ml lysis buffer (Promega). After pulse centrifugation, luciferase activity was measured in a Monolight 3010 Lumimeter (Analytical Luminescence Laboratory) after adding 100 μl freshly reconstituted luciferase assay buffer to 20 μl lung tissue homogenate. Results were expressed as relative light units normalized for protein content, which was measured by Bradford assay (Bio-Rad).

Histology and immunohistochemistry
Fixed lung samples were embedded in paraffin, sectioned (5 μm), stained with H&E, and analyzed by a pathologist blinded to the experimental groups for evaluation of tumor and AAh lesions in three separate sections cut at predetermined depths. GFP immunostaining was performed as previously described (12). For arginase-1 localization, lung sections were immunostained with diaminobenzidine-peroxidase detection reagents using rabbit anti-arginase-1 Ab (clone H-52; Santa Cruz Biotechnology, Santa Cruz, CA). To evaluate tumor-infiltrating vessel density, we immunostained lung sections with rat anti-mouse CD34 Abs (clone MEC14.7; BioLegend). Image analysis of digital photomicrographs was performed using Image-Pro Plus software (Media Cybernetics, Bethesda, MD).

Isolation of lung cells and flow cytometry
Perfused lungs were digested in RPMI-1640 medium containing collage- nase X1 (0.7 mg/ml; Sigma-Aldrich) and type IV bovine pancreatic D Nah (30 μg/ml; Sigma-Aldrich) to obtain single-cell suspensions. After treatment with RBC Lysis Buffer (BioLegend), single-cell suspensions were stained with Abs: CD45-Alexa Fluor 700 (30-F11) from eBioscience; CD68-FITC (FA-11), CD206-PE (MR3D3), and CD204-Alexa Fluor 647 (2F8) from AbD Serotec; and CD86 PE-Cy7 (GL1), CD11b (M1/70), and Gr1 (RB6-8C5) from BD Bioscience. Flow cytometry was performed using BD LSR II flow cytometer (BD Bioscience), and data were analyzed with FlowJo software (TreeStar). For isolation of CD11b+ cells, lungs were treated as described earlier followed by magnetic separation using anti-CD11b microbeads (Miltenyi Biotec).

Real-time PCR
RNA from whole-lung tissue or lung CD11b+ cells was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA). DNase-treated samples were subjected to real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems). PCR primers used were: IL-12p35—forward: 5′-TGCCTTGTTGTGTTTCTTG-3′; reverse: 5′-CAAGTAGTGGGTGTTTCTTG-3′; CCL3—forward: 5′-TGCCCTTCTGTTCTCTCT-3′; reverse: 5′-AATAATGCTTGGGTAAT-3′; IL-1β—forward: 5′-TGACGCGACCCAAAAGAGATG-3′; reverse: 5′-CAGGACAGCCAGCTTCAA-3′; mammone receptor—forward: 5′-CAAGGAAAGTGGTCAGTG-3′; reverse: 5′-CTTCATGTCCTGTTGCCAG-3′; Ym1—forward: 5′-GAGACATACCTTATTCCTGAC-3′; reverse: 5′-CCATTGCATCCATCATGTC-3′; IL-10—forward: 5′-ACCTTGCATCTGCTGGTTCG-3′; reverse: 5′-GGTTGCCAAGCCTATCGGA-3′; TNF-α—forward: 5′-AACTGTAGACCCACGTCGA-3′; reverse: 5′-GCCACCATAGTGTTGTTCTTG-3′; IL-6—forward: 5′-CACCTGGATTCCACCACTGT-3′; reverse: 5′-GACCTGGTATTGCTAATGTA-3′; IL-12p40—forward: 5′-TCCTCTGTCCTCAGTGA-3′; reverse: 5′-CTTGACCCACCTCCTTGTTGA-3′; GAPDH—forward: 5′-TGGAGGACAGGTGCTTCTCT-3′; reverse: 5′-GCCCTTGCTTACCCGTAT-3′. Relative mRNA expression in each sample was normalized to GAPDH and presented using the comparative ΔΔCt method, where ΔCt = Ct (GAPDH) – Ct (target gene).

VEGF measurement
Concentration of VEGF was assayed in cell-free BAL supernatants using ELISA (R&D Systems, Minneapolis, MN), according to the manufacturer’s instructions.

Statistical analysis
For studies comparing differences between two groups, we used unpaired Student’s t tests. For differences between more than two groups, we used one-way ANOVA with an appropriate posttest. Comparisons of chest photon counts in PBS or clodronate liposome-treated NGL mice were performed using two-way ANOVA with a Bonferroni posttest. Values are presented as mean ± SEM. A p value < 0.05 was considered statistically significant.

Results

Depletion of alveolar macrophages reduces lung tumorigenesis
To assess the role of macrophages in lung tumorigenesis, we developed a method of minimally invasive, long-term alveolar macrophage depletion using repetitive IT instillation of clodronate encapsulated liposomes, which cause selective apoptosis of macrophages (11, 13–20). Consistent with prior results (15), a single IT injection of clodronate caused a 95% reduction in macrophages obtained by BAL on day 3 with partial recovery of this cell population by 7 d (Supplemental Fig. 1A). Analysis of macrophage morphology revealed two different cell populations: intact and remnant cells. Remnant macrophages were identified as cells with a condensed, pyknotic nucleus that lack a clear cytoplasm or limiting cell membrane, indicative of dead (or dying) cells (Supplemental Fig. 1C). Although clodronate injection reduced total macrophages by 95% on day 3, only 15% of the remaining macrophages in BAL were intact, suggesting a very low number of functional macrophages. This percentage of intact macrophages increased to 53 and 79% on days 5 and 7, respectively (Supplemental Fig. 1D–E). The number and morphology of BAL macrophages in mice treated with vehicle (PBS) containing liposomes remained unchanged.

Based on these results, we used weekly IT injections of liposomal clodronate to obtain long-term reduction in macrophage numbers during the course of urethane-induced lung tumorigenesis. In these experiments, mice received a single i.p. injection of urethane and were treated weekly with liposomal clodronate for 4 mo starting the day of urethane injection. Urethane-injected mice were treated as described earlier followed by magnetic separation using anti-CD11b microbeads (Miltenyi Biotec).

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treated with PBS-containing “empty” liposomes and mice treated with urethane alone were used as control groups. At the time of harvest (4 mo after urethane and 6 d after the final clodronate dose), mice treated with clodronate exhibited a significant reduction in total BAL cells (Fig. 1A). Total and intact macrophages were reduced by clodronate treatment, whereas the relatively small number of neutrophils and lymphocytes in BAL were similar between clodronate and PBS liposome-treated animals (Supplemental Fig. 2A, 2B). Depletion of macrophages during the 4 mo of lung carcino genesis resulted in a marked reduction in the number of lung surface tumors in comparison with the control groups (Fig. 1B). In addition, the mean diameter of surface tumors was significantly reduced after macrophage elimination (Fig. 1C).

To confirm the finding of fewer lung tumors in macrophage-depleted mice, we performed histological examination of lungs sectioned at three predetermined depths. Microscopic evaluation of tumor appearance revealed both adenomas and AAH lesions (Supplemental Fig. 2C). Similar to surface tumors, the number of adenomas per lung section from urethane-injected mice was 3.6-fold lower after macrophage depletion (Fig. 1D). At the same time, we found no differences in AAH lesions between clodronate- or PBS liposome-treated animals (4.39 ± 0.50 versus 4.33 ± 0.33 AAH lesions per H&E-stained lung section, respectively). Our results indicate that urethane-induced lung tumorigenesis is highly dependent on the presence of alveolar macrophages.

**Induction of lung tumorigenesis by urethane changes the phenotype of lung macrophages**

Because macrophage polarization has been implicated in modulation of tumor progression, we characterized the phenotype of lung macrophages after urethane treatment. We measured expression of CD204 (scavenger receptor) and CD206 (mannose receptor) as markers of the M2 phenotype in the population of CD45⁺CD68⁺ lung macrophages by flow cytometry (Fig. 2A). As shown in Fig. 2B, the percentage of macrophages positive for CD204 and CD206 was significantly decreased during the first 2 wk after urethane injection, followed by an increase at 6 wk. To confirm the shift in polarization of lung macrophages, we isolated CD11b⁺ cells from lungs during weeks 1–6 after urethane injection and assessed mRNA expression for IL-12, CCL3, and IL-1B as markers of M1 macrophages and mannose receptor, Ym1, and IL-10 as indicators of cells with M2 characteristics (7). We found up-regulation of M1 markers on CD11b⁺ cells at weeks 2–3 after urethane, including increased IL-12 at weeks 2–3 and increased CCL3 and IL-1B at week 3 (Fig. 2C–E). Expression of M2 markers mannose receptor and Ym1 were increased by week 6 after urethane, but IL-10 was not changed at any time point (Fig. 2F–H). To examine the distribution of M2 polarized macrophages in the lungs, we immunostained lung sections from mice harvested at different time intervals after urethane exposure using anti-arginase-1 Abs. We chose arginase-1 as the M2 marker for these studies because of the availability of an Ab with good sensitivity for immunohistochemistry. We detected few positively stained macrophages in lungs from naive mice (Fig. 2J); however, we observed a large increase in arginase-1⁺ cells at 6 wk after carcinogen exposure (Fig. 2K). Because arginase-1 can be expressed by myeloid-derived suppressor cells (MDSCs), as well as macrophages (21), we investigated whether an increase in MDSCs (defined as CD11b⁺Gr1⁺ cells) could account for the increase in arginase-1⁺ cells identified at 6 wk after urethane injection. We did not detect a significant increase in MDSCs in the lungs at this time point (5.97 ± 1.50% of total CD45⁺ leukocytes in urethane-treated mice versus 4.85 ± 0.79% in naive mice), supporting the conclusion that increased arginase-1 staining was related to increased numbers of M2 polarized macrophages. Together, these studies indicate a shift in macrophage phenotype after carcinogen treatment. Depending on the measurement used, there appears to be a shift toward M1 macrophage marker expression during the first 2–3 wk after urethane treatment, followed by a shift toward increased M2 polarization by 6 wk.

At the time of lung tumor assessment (4 mo after urethane injection), the percentage of lung macrophages expressing both CD204 and CD206 was not significantly increased in comparison with naive mice (Fig. 2F). Interestingly, macrophages positive for arginase-1 were found only within the tumor and peritumor areas, but not in normal-appearing alveoli (Fig. 2L, 2M). These data suggest that in the presence of fully formed tumors, M2 polarized macrophages are limited to areas of tumor formation.

**Elimination of alveolar macrophages during “early” and “late” stages of urethane-induced tumorigenesis reduces lung tumor formation**

Based on information regarding timing of macrophage polarization described in the previous section, we sought to determine the role of macrophages in tumor formation during early (M1 predominant) and late (M2 predominant) time periods. Therefore, we treated mice with clodronate liposomes during the first 2 wk posturethane for “early-stage” macrophage depletion or starting at 4 wk posturethane for “late-stage” macrophage depletion (Fig. 3A). Animals were sacrificed at 4 mo posturethane. As expected, elimination of macrophages during the late stage significantly reduced the numbers of macrophages in BAL at 4 mo (Fig. 3B, 3C). These changes were accompanied by a decrease in number and diameter of lung surface tumors (Fig. 3D, 3E). Histopathological examination of lung sections confirmed the reduced numbers of tumors after depletion of macrophages during the late stage (Fig. 3F–H).

**FIGURE 1.** Depletion of alveolar macrophages attenuates urethane-induced lung tumorigenesis. A, Number of total cells in BAL from mice injected with urethane only (Ureth) or treated with weekly IT injections of empty (PBS) liposomes or clodronate (Clod) liposomes for 4 mo after urethane. B, Number of lung surface tumors and (C) tumor diameter in mouse lungs. D, Histological assessment of the number of tumors per H&E-stained lung section from tumor-bearing mice treated with weekly IT injections of PBS liposomes or clodronate liposomes for 4 mo. Data are presented as mean ± SEM of 6 mice for the urethane only group, 6 mice for the PBS group, and 10 mice for the clodronate group. *p < 0.05 compared with the other groups.
3F), with no difference in the number of AAH lesions compared with the PBS liposome group (Fig. 3G). These findings were similar to those obtained from experiments in which macrophages were depleted during the entire 4 mo. However, to our surprise, we found that elimination of macrophages during the early stage had similar effects on BAL cellularity, the number and size of surface tumors, and histological assessment of lung tumors as compared with late-stage macrophage depletion (Fig. 3B–G).

Although the numbers and sizes of tumors after early-stage macrophage depletion were slightly greater than in animals having depletion of cells during late-stage tumorigenesis or during the entire 4-mo experiment, the differences were not statistically significant. Together, our findings demonstrate that macrophages are required for urethane-induced lung carcinogenesis during both “early” and “late” stages, and elimination of macrophages during either period markedly attenuates tumor formation.

**Depletion of lung macrophages during later stages of lung carcinogenesis reduces vascular growth in tumors**

Because macrophages represent a primary source of proangiogenic proteins in the tumor microenvironment (22–24), we measured the concentration of VEGF in BAL from control and macrophage-depleted mice. The level of VEGF in BAL of urethane-injected mice was reduced by depletion of macrophages during “late” carcinogenesis and by depletion of macrophages throughout the experiment (Fig. 4A). We also assessed tumor angiogenesis by immunostaining for an endothelial cell marker of new blood vessels, CD34 (25). Although the number of blood vessels infiltrating tumors was not significantly different in macrophage depletion groups compared with controls, the total vessel area in tumors was significantly reduced in both macrophage depletion groups compared with the PBS liposome-treated control group (Fig. 4B–D). These data suggest that, in our model, macrophages are not required for the appearance of new tumor-infiltrating blood vessels, but rather are critical for supporting the growth of new and existing vessels.

**Depletion of alveolar macrophages during early carcinogenesis blocks NF-κB activation and reduces AAH lesions**

In a previous study, we demonstrated an important role for the NF-κB signaling pathway in urethane-induced lung carcinogenesis (10). In that study, we found that NF-κB was transiently activated in epithelial cells and macrophages of tumor-prone mouse strains. In this study, we used NGL mice (FVB strain background) in which a synthetic NF-κB–dependent promoter drives the expression of a GFP-luciferase fusion protein (8). NGL mice were injected with urethane and IT clodronate or PBS liposomes on day
0, followed by weekly treatments for 4 mo. Repetitive noninvasive bioluminescent imaging of luciferase activity was performed immediately before each treatment with liposomes. Measurement of photon emission from the chest of mice treated with PBS liposomes revealed increased NF-κB–dependent luciferase activation during early stages of carcinogenesis with maximal values on day 14 (Fig. 5A, 5B). Depletion of alveolar macrophages with clodronate substantially attenuated the increased NF-κB activity observed between days 7 and 21. Analysis of luciferase activity in lung homogenates confirmed a significant attenuation of NF-κB activity after clodronate treatment (Fig. 5C). To confirm that these differences reflected lung-specific NF-κB activation, we treated NGL mice with clodronate or PBS liposomes on days 0 and 7 after injection of urethane and harvested lungs on day 14. In this experiment, treatment with clodronate significantly reduced total BAL cells and alveolar macrophages (Supplemental Fig. 3A, 3B). On lung sections from these mice, immunohistochemistry for GFP showed a marked reduction in NF-κB–dependent GFP expression in the entire tissue section, particularly in airway epithelium (Fig. 5D). The finding of attenuated NF-κB activity in airway epithelial cells after clodronate injection supports the conclusion that elimination of macrophages impairs proinflammatory signaling between macrophages and epithelial cells, thus reducing urethane-induced NF-κB activity in the entire lung compartment.

To investigate the impact of macrophage depletion on inflammatory mediator production in the lungs, we measured mRNA expression of several inflammatory mediators in the lungs after treatment with clodronate or PBS liposomes on days 0 and 7. At day 11 posturethane, IL-12 and iNOS expression were reduced after clodronate treatment and IL-6 showed a trend toward reduced expression (Fig. 5E–G). In contrast, expression of TNF-α and IL-10 was unchanged compared with the PBS liposome-treated control group (Fig. 5H, 5I). Although the precise mechanisms by which macrophages promote early-stage tumorigenesis are uncertain, inflammatory mediator production may contribute to a protumorigenic microenvironment.
We then asked whether “early” macrophage depletion and impaired NF-κB activation would result in a reduction of AAH lesions at 6 wk after urethane treatment, a time point at which AAH lesions become detectable in this model (26). In these studies, we depleted macrophages with clodronate on days 0 and 7 after urethane injection (weeks 1 and 2, “early stage”) and on weeks 4 and 5 (“late stage”), and harvested mice at week 6 (Fig. 6A). As shown in Fig. 6B, the number of AAH lesions was markedly reduced in mice after early-stage macrophage depletion compared with mice with late-stage macrophage depletion and mice treated with PBS liposomes. Thus, our data indicate that early macrophage depletion impairs NF-κB activation and early AAH formation, likely resulting in fewer tumors at 4 mo.

In a separate experiment, BAL was performed on untreated control mice, mice treated with clodronate alone on days 0 and 7, and urethane-treated mice injected with clodronate or PBS liposomes on days 0 and 7. At 6 wk after initiation of treatment, depletion of macrophages during the early stage after urethane injection resulted in similar numbers of total BAL cells compared with untreated (naive) mice and mice treated with clodronate alone (Fig. 6C). However, mice treated with PBS liposomes in the early stage after urethane injections had increased BAL macrophages compared with the other groups (Fig. 6D). These results suggest that the long-term reduction in lung macrophage numbers observed in mice subjected to the early-stage macrophage depletion protocol posturethane (compared with urethane-treated controls)
was not due to prolonged effects of liposomal clodronate. Rather, we suspect that the prolonged reduction in BAL macrophages after early-stage macrophage depletion resulted from attenuation of the initial inflammatory response and subsequent differences in tumor multiplicity.

Discussion

These studies demonstrate that macrophages are essential for promotion of carcinogen-induced lung tumors during both early (tumor initiation and early promotion) and late (late promotion and progression) stages of tumorigenesis. Although depletion of alveolar macrophages during either early or later stages reduces tumor size and multiplicity, the mechanisms involved appear to be different. Later stage macrophage depletion decreases VEGF concentration in BAL and reduces vascular growth in lung tumors. In contrast, early-stage macrophage depletion, before the onset of M2 polarization, reduces urethane-induced NF-κB activation in the lungs, alters inflammatory mediator expression, and impairs formation of pre-malignant AAH lesions. Together, these studies indicate that macrophage support development and maintenance of a protumorigenic microenvironment in the lungs in a variety of ways. As a result, a sustained presence of macrophages throughout the course of tumorigenesis is required for maximal tumor formation.

We and others previously demonstrated that macrophages can support tumor growth in a variety of animal models, including lung cancer, melanoma, breast cancer, and ovarian cancer models (11, 13, 14, 16, 17, 19, 20). In addition to animal model data, there is a strong correlation between macrophage density in tumors, microvessel counts, and relapse-free survival in humans with lung cancer (27). Importantly, prior studies have been performed using established cancer cell lines and metastasis models, or were carried out during progression stages when tumors were already established. Little is known regarding the role of macrophages in lung tumor promotion, but the prevailing opinion has been that during tumor initiation and early promotion, macrophages primarily function to kill and remove malignant clones through their cytotoxic and phagocytic activities. In contrast, our studies indicate that macrophages have important protumorigenic functions within the first few weeks after urethane injection. Early-stage macrophage depletion by clodronate injection on days 0 and 7 posturethane almost completely eliminated AAH lesions in the lung at 6 wk after urethane treatment, indicating a critical role for macrophages in early tumor promotion. Our findings suggest that macrophages promote tumorigenesis by enhancing survival and/or proliferation of mutated cells in the first few weeks after urethane treatment.

In these studies, we found that elimination of macrophages blocks NF-κB activation in the lungs of urethane-treated mice, which occurs transiently during the first 2–3 wk after urethane treatment (10). We previously reported that urethane induces tumors only in mouse strains where NF-κB is activated, and that inhibition of NF-κB in airway epithelial cells reduces lung tumorigenesis (10). These findings were recently corroborated and expanded by other reports demonstrating a requirement for NF-κB signaling in tobacco smoke and oncogenic Kras-induced lung carcinogenesis (28–30). In addition, mice with impaired NF-κB activation in myeloid cells showed reduced tumor size and multiplicity in a lung tumor model in which mutant Kras-expressing mice were exposed to tobacco smoke (28). It has been suggested that chronic inhibition of NF-κB signaling in tumor-associated macrophages switches them to an activated phenotype that is cytotoxic to tumor cells (31). Together, available data indicate that the NF-κB pathway is an important regulator of lung tumorigenesis in mouse models and may play cell-specific roles in epithelial and myeloid cells. Our studies contribute to this emerging picture by showing that cross talk between macrophages and epithelial cells is required for NF-κB activation in lung epithelium after urethane exposure. As we have previously shown, NF-κB signaling impacts survival of epithelial cells after urethane treatment through expression of antiapoptotic mediators like Bcl-2 (10). Therefore, we speculate that early macrophage depletion impacts tumorigenesis by depriving epithelial cells of crucial survival and proliferation signals via NF-κB pathway signaling.

Classically activated M1 macrophages have been reported to play important roles in host defense and tumor resistance (7, 22, 32). In lungs, M1 polarization of macrophages has been associated with prolonged survival time in patients with nonsmall cell lung cancer (33–35). Despite reported antitumor roles for M1 polarized macrophages, persistent activation of these cells may lead to tissue damage, and thereby support malignant transformation (5, 16, 36). Proinflammatory cytokines produced by M1 macrophages such as IL-6 and TNF-α have been reported to protect initiated epithelial cells from apoptosis and promote the proliferation of these cells (37–40). Other inflammatory cells recruited by macrophages, particularly neutrophils, can impact malignant transformation of epithelial cells through release of reactive oxygen and nitrogen species (5, 16, 41, 42). Our studies support the concept that the overall impact of macrophages is protumorigenic throughout the entire course of carcinogen-induced tumor formation.

During the course of lung tumor development, lung macrophages shift their polarization to the M2 phenotype, particularly in and around tumors. Redente and colleagues (43) demonstrated a shift in polarization of lung macrophages from M1 toward M2 at 3 wk after urethane exposure in A/J mice. In FVB mice, we found an increase in M1 polarized macrophages during the first 2–3 wk after urethane, followed by an increase in M2 cells by 6 wk after urethane. Therefore, reduction of macrophages during later stages of tumor development has a disproportional effect on cells with M2 characteristics. In contrast with M1 polarized macrophages, alternatively activated (M2) polarized macrophages have been shown to support tumor growth (4, 7, 22, 44). Mice that lack IFN-γ exhibit larger lung tumors because of M2 macrophage polarization, whereas IL-4R knockout mice have smaller tumors (44). Because M2 polarized macrophages are known to secrete proangiogenic cytokines, these cells can support tumor progression through increased vascularization of neoplastic lesions. Based on this concept, M2 polarization of macrophages has been suggested as an important prognostic factor for many tumors, including lung cancer (4, 7, 22, 32, 34, 35). Although the level of VEGF was lower in BAL from macrophage-depleted mice in our studies compared with controls, the density of microvessels within the tumors was not decreased. However, elimination of alveolar macrophages significantly reduced the size of vessels in lung tumors. These data suggest that, in the urethane model, alveolar macrophages are dispensable for formation of new blood vessels in tumors but are required for the growth of new and existing vessels. Thus, an important protumorigenic aspect of lung macrophages appears to be modulation of vascular growth.

Macrophages are very plastic cells and, depending on the microenvironmental cues, may exhibit diverse functions (45). We identified macrophages’ phenotype as M1 or M2 based on FACS analysis, mRNA expression for a panel of markers, and immunohistochemistry for arginase-1. Although we found phenotypic differences in macrophages over time in response to urethane injection, a diverse population of macrophages was present in the lungs at each time point studied. In addition, partial macrophage phenotypes that do not fit neatly within the M1/M2 paradigm and cells that express low levels of both M1 and M2 markers appear to...
be common. Therefore, although it is tempting to try to explain the role and function of cell types based on simple constructs, global interventions involving macrophages or other complex cell types impact cells with a spectrum of functions and characteristics. Therefore, these interventions are likely to result in complex changes to the local microenvironment.

Accumulating evidence supports a strong association between airway inflammation and lung cancer, particularly in patients with chronic obstructive pulmonary disease (46). Retrospective studies have suggested that treatment with anti-inflammatory drugs such as corticosteroids reduce the incidence of lung cancer (47). Macrophages are prominently increased in the airways of chronic obstructive pulmonary disease patients (27); therefore, these cells may be involved in mediating increased lung cancer risk in these individuals. Because our studies suggest that macrophages may be involved in mediating increased lung cancer risk in these individuals. Because our studies suggest that macrophages may be important for inflammation in lung tumorigenesis (47).

The authors have no financial conflicts of interest.

References
ONLINE DATA SUPPLEMENT

A CRITICAL ROLE FOR MACROPHAGES IN PROMOTION OF URETHANE-INDUCED LUNG CARCINOGENESIS.


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Figure S1. Depletion of alveolar macrophages by clodronate. A) The number of BAL macrophages in mice on days 1-7 after single intratracheal injection of PBS containing liposomes (black bar) or clodronate (open bar) liposomes. B) Identification of intact (open bars) and remnant (black bars) macrophages after single treatment with clodronate liposomes. Data are presented as mean ± SEM of 3 mice per group. C) Representative microphotograph of normal (red arrow) and remnant (black arrow) macrophages in BAL.
**Figure S2.** Weekly treatment with liposomal clodronate reduces the number of BAL macrophages at 4 months after injection of urethane. A) The number of intact macrophages and B) remnant macrophages (Rem MΦ), neutrophils (Neutr) and lymphocytes (Lymph) in BAL from mice injected with urethane and treated with weekly injection of PBS (black bar) or clodronate (open bar) liposomes for 4 months. Mice were euthanized 6 days after the last liposome injection. Data are presented as mean ± SEM of 7 mice for the PBS group and 10 mice for the clodronate group, *=p<0.05 compared to the PBS group. C) Photomicrographs of H&E stained lung sections from tumor bearing mice treated with weekly IT injections of PBS liposomes (left panels) or clodronate liposomes (right panels) for 4 months. Bars: 100μM.
Figure S3. Treatment with clodronate after urethane injection significantly reduces the number of total inflammatory cells in the lungs and lung M1 macrophages. A) Total BAL cells, B) differential cell counts in BAL assessed at day 14 after urethane injection in mice treated with PBS or clodronate liposomes. Animals were given PBS or clodronate liposomes on days 0 and 7 after injection of urethane, n=5 per group, *p<0.05 compared to PBS group.