UNIVERSITY OF CALIFORNIA SAN DIEGO

The Effect of House Dust Mites and Allergic Airway Inflammation on Lung Carcinogenesis

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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2019

The Thesis of Natalie Albasha is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2019

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ACKNOWLEDGEMENTS

I would like to acknowledge Eyal Raz, M.D., and Samuel Bertin, Ph. D. for their guidance and support throughout my master's program, as well as my committee members, Douglass Forbes, Ph. D., and Li-fan Lu, Ph. D., for their support and interest in my work. I would also like to thank all the members of the Raz Lab for all the guidance they have provided over the years I spent as an undergraduate and graduate student.

ABSTRACT OF THE THESIS

The Effect of House Dust Mites and Allergic Airway Inflammation on Lung Carcinogenesis

by

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Master of Science in Biology

University of California San Diego, 2019

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House Dust Mite (HDM) is a common allergen that induces asthma and allergic airway inflammation. Unlike other lung diseases such as chronic obstructive pulmonary disorder (COPD), the relationship between asthma and lung cancer has not been well established. However, our studies conducted on HDM and its pro-inflammatory effect on the airway suggests that it has potential to act as a co-carcinogen in mouse models that are susceptible to lung cancer. We have confirmed this finding in three mouse models with lung cancer being induced differently. The lung cancer models included a urethane chemically-induced model,

another in a mouse strain genetically susceptible to lung cancer development, and the final in a genetically modified mouse model. The mechanism behind how HDM increases lung cancer development still needs further study; however, our data suggest that HDM cannot induce lung cancer on its own, but rather acts as a co-carcinogen in susceptible mouse models. We also found that IL-17A was important for the initiation of lung cancer. Furthermore, adaptive immunity was found to not play a significant role in the initiation of lung cancer development but may impact the regulation of the growth of cancer cells. Finally, phosphorylated H2AX markers indicated double stranded DNA breaks in immune cells. The consequences of this DNA damage and their impact on lung cancer development will be the subject of future studies in the lab.

INTRODUCTION

Lung Cancer

According to the American Cancer Association and the World Health Organization, lung cancer takes the lead on cancer death rates in the United States at about 160,000 deaths/year and worldwide at 1.6 million deaths/year (1,2). It is well established that smoking leads to lung cancer development with about 80% of lung cancer deaths being smokers or former smokers (3). However, approximately 20% of lung cancer deaths are made up of non-smokers and the various risk factors include exposure to radon gas, asbestos, and air pollution (4). It is also thought that chronic lung diseases and persistent lung inflammation may contribute to lung cancer development for many of those non-smokers. For instance, chronic obstructive pulmonary disorder (COPD), a chronic inflammatory lung disease, has been shown to be a risk factor for lung cancer (5). Other chronic lung diseases' impacts are less clear and still debated such as asthma's relationship to lung cancer (6).

Asthma and Lung Cancer

A meta-analysis study conducted by Qu et al. and a Cox regression analyses of data conducted by Brown et al. showed asthma to be positively associated with lung cancer development (7,8). However, a meta-regression and meta-analysis conducted by Rosenberger et al. suggested that there was no causal relationship between asthma and lung cancer (6). On the other hand, another study looking at the association between allergies and cancers found an inverse relationship between the two (9). It was hypothesized that this maybe due to the more active immune response due to the allergy that could help mitigate the development of

cancer (9). Therefore, there is no clear consensus on the relationship between asthma and lung cancer as is shown with these few examples. The reason that there is so much controversy around whether asthma affects the risk of developing lung cancer could potentially be explained by the wide range of disease heterogeneity and severity in asthma. It was not until recently that different endotypes of asthma were discovered and are now starting to be considered (10). Although asthma is historically considered to be a T helper 2 (Th2)-type of disease, there are at least two major types of allergic asthma presentation. One type falls in line with classical asthma, expressing high Th2 levels, which lead to IL-4 and IL-5 production, and ultimately results in eosinophilic airway inflammation (11). The other type of asthma has low Th2 levels but is high in T helper 17 (Th17), which leads to production of IL-17A and IL-17F and ultimately results in high neutrophilic airway inflammation (11). This distinction is important to consider because of the immunological roles that Th17 and IL-17 may have on lung cancer development.

Th17 and Lung Cancer

Th17 is a specific subset of CD4+ T-cells characterized mainly by the production of the cytokine interleukin-17 (IL-17) (12). IL-17 consists of a family of six cytokines: IL-17A through F. The most well understood cytokine is IL17-A and one of its functions is to act as a mitogen for epithelial cells (13-15). IL-17A proliferates malignant cell formation and upregulates vascular endothelial growth factor (VEGF), promoting tumor angiogenesis (16,17). Therefore, in lungs that are lined with epithelial cells, IL-17A may promote lung cancer development and metastasis (18-20). In humans, it was found that the more dangerous and fatal type of lung

cancer, non-small cell lung cancer (NSCLC) was correlated to higher IL-17 expression (21). In one mouse model study, NSCLC metastasis was shown to increase with IL-17 and to decrease metastasis in IL-17 knockout mice (22). On that account, mouse models can be useful in studying the effect that asthma with a Th17 inflammatory response may have on lung cancer.

Antigens in Asthma Mouse Models

The model antigen used to induce allergic inflammation is important to consider when studying the relationship between asthma and lung cancer. One study looked at this relationship using the widely used ovalbumin (OVA) mouse model of asthma (23). The mice treated with OVA as the antigen to induce allergic inflammation were also treated with urethane to chemically induce lung carcinogenesis. Urethane (ethyl carbamate) is a carcinogen that induces bronchioalveolar adenomas and adenocarcinomas in mice and the extent of the development depends on the genetic strain (24). Although the results showed OVA-induced asthma had no effect on lung carcinogenesis, it is important to note that OVA is an innocuous antigen that induces the classical Th2-type of allergic airway inflammation (25). Therefore, it seems as though asthma and allergic airway inflammation does not impact lung carcinogenesis. However, it would be of interest to use an antigen more relevant to human that induces both Th2 and Th17- types of allergic airway inflammation.

House Dust Mite (HDM)

House Dust Mite (HDM) is the most common allergen to cause allergic asthma worldwide (26,27). HDM is an indoor allergen that up to 80% of asthmatic human patients are

sensitized to, and that elicits a persistent inflammatory response with a mixture of predominantly Th2 but also Th17-type inflammatory response (23,28-31). HDM is a microscopic arachnoid with only 0.2-0.3 mm in length and a translucent body, making it largely invisible to the human eye (26,32). HDM can survive in all climates including high altitudes, however, it thrives in indoor environments such as ones provided by homes, as implicated by their names. It feeds on skin scales and organic debris that is partially digested by fungi, making mattresses, carpets, furniture, and bedding hospitable environments for them (27).

In order to understand the role HDM plays as an allergen, one must understand its constituents. Allergic components called Der p and Der f, which have protease activity, are highly concentrated in HDM fecal matter (26). This fecal matter is airborne and as such is easily inhalable when humans make contact with their furniture, bedding, and pillows (27). HDM itself can also be inhaled and is composed of various components that activate both innate and adaptive immunity. Its exoskeleton is comprised of chitin, fungal spores, LPS, and Der p and Der f allergens (26). The chitin comprises most of the fungal exoskeleton and acts as an adjuvant and influences both adaptive Th1, Th2, and Th17 immunity and innate immunity (26). Fungal spores on the exoskeleton consist of β-glucans, which can bind pattern recognition receptors (PRR) on dendritic cells (DCs) and other myeloid cells, ultimately impacting both innate and adaptive immunity (26). Finally, extracts from HDM contain endotoxin and gram-negative bacteria that comprise LPS which activates innate immunity and in combination with the rest of the HDM components, influences adaptive immunity (26). HDM is a complex antigen in comparison to OVA, and a more realistic allergy inducer in humans, making its effects on lungs

interesting to study (26). However, the complexity of HDM's constituents and overlapping roles in both adaptive and innate immunity make it difficult to study the mechanism of action between HDM, asthma, and lung cancer.

HDM and DNA Damage

Another reason that HDM might be of interest in the relationship between asthma and lung cancer is because of its potential to cause DNA damage. One lab recently reported that HDM can induce DNA damage through reactive oxygen and nitrogen species in lung epithelial cells (33). The authors found that HDM increased oxidative damage in mouse lungs in-vivo and then confirmed this observation in-vitro (33). Through this oxidative reactive environment, HDM was also found to induce DNA double stranded breaks which was identified through increased levels of phosphorylated Histone 2AX (H2AX), in human bronchial epithelial cells in-vitro. This observation was confirmed in human asthmatic lung tissues, which had higher levels of apoptosis and DNA repair proteins (33). Additionally, when the presence of antioxidants was examined, it was found that DNA damage by HDM could be prevented (34). Although most of their data concluded that high concentrations of HDM induce cell death and reduce cell proliferation, the possible induction of mutations as a consequence of DNA damage was not studied. These studies heavily influenced our own studies as we considered the role mutations and DNA damage play a in cancer.

Working Hypothesis

Due to the recent discovery that HDM can induce DNA damage in the lungs as well as a mixed Th2/Th17 immunological response, we decided to evaluate its effect in mouse models of lung cancer. We hypothesized that chronic exposure to HDM may promote lung cancer initiation and progression in susceptible mice (i.e., co-exposed to a carcinogen such as urethane, or genetically prone to develop lung cancer).

METHODS

Animals

Wild-type (WT), IL-17A Knockout (KO), and Rag2 KO mice in the C57BL/6 background were originally purchased from the Jackson laboratory and were then bred in our vivarium. $CCSP^{Cre}$ (35) and $Kras^{G12D}$ (36) in the C57BL/6 background were a gift from Dr. Chen Dong (MD Anderson cancer center). WT A/J mice were purchased from the Jackson Laboratory. All mice were kept on a 12-hour light, 12-hour dark cycle with standard chow diet and water. All experimental procedures performed on mice were approved by the University of California San Diego Institutional Animal Care and Use Committee.

In vivo protocols

- *House dust mite sensitization and challenge*

Mice were first sensitized intranasally (i.n) under general anesthesia (isoflurane) with HDM (Greer laboratories # XPB82D3A2.5; 50 mg/mouse) or its vehicle (VEH; normal saline) on day 0 and 10, and were challenged i.n twice a week for the subsequent 10 weeks with HDM (12.5 mg/mouse) or with VEH. Mice were then challenged i.n with HDM (12.5 mg/mouse) or with the VEH once a week for an additional 4 weeks (Kras model) or 16 weeks (urethane model). Mice were sacrificed 72 h after the last HDM challenge and the blood, bronchoalveolar lavage fluid (BALF) and the lungs were harvested.

- *Model 1: Urethane-induced lung cancer model*

During the 10 weeks of HDM challenge, age- and sex-matched C57BL/6 WT or IL-17 KO mice were injected intraperitoneally (i.p) once a week with urethane (Sigma # U2500; 1 mg/g of BW) as previously described. Due to their increased susceptibility to urethane, Rag2 KO mice were treated with a lower dose of urethane (0.6 mg/g of BW) but with the same number of injections. All the mice were sacrificed 26 weeks after the first urethane injection and lung inflammation and lung tumorigenesis were analyzed in the different groups.

- *Model 2: A/J mouse spontaneous lung cancer model*

A/J are derived from Balb/c mice and carry the pulmonary adenoma susceptibility allele, have high rates of Kras mutation, and have a relatively high spontaneous adenoma/adenocarcinoma incidence. We have exposed age- and sex- matched A/J mice to HDM or VEH for 10 weeks (from 9 weeks to 19 weeks of age) as described above and the occurrence of lung tumors was evaluated.

- *Model 3: Kras-induced lung cancer model*

Mice expressing an oncogenic K-ras mutation (G12D) in clara (club) cells develop spontaneous lung tumors (36). We have generated these mice by crossing K-ras^{G12D} fl/fl mice (36) with clara cell secretory protein (CCSP) cre+ mice in order to specifically induce K-ras^{G12D} expression in Clara cells, which account for 70% of the airway epithelium (35).The resulting K-ras^{G12D+} CCSP^{cre+} bitransgenic mice were exposed to HDM or VEH as described above for 9 weeks (from 5 weeks to 14 weeks of age) and the occurrence of lung tumors was evaluated.

The tumor load in each mouse was determined on H&E-stained lung tissue using Image Pro Premier 10.0 software and expressed as percentage of total lung tissue.

Bronchoalveolar lavage fluid (BALF) cellularity analysis

The lungs of mice were inflated with 1 ml of PBS and BALF was recovered and spun down. The cells were counted and loaded on slides by cytospin for Giemsa Wright staining. The slides were examined using light microscopy and BALF cellularity analysis was performed according to standard protocols (BALF protocol).

Flow Cytometry Analysis

In some experiments, the lungs of C57BL/6 and A/J mice were perfused by cardiac perfusion using 1mM EDTA containing HBSS after removal of BAL cells by lavage with PBS. Lung tissues were incubated at 37 °C for 30 minutes in the digestion solution (collagenase type 1A 0.5 mg/ml in HBSS containing 5% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin) and passed through a 100µm strainer. The cells were resuspended at the desired concentration (e.g., $1x10^6$ into 100 µL of PBS/2% FBS) in presence of anti-mouse CD16/CD32 (Fc Block; BD Biosciences) antibody and were stained for 30 min at 4 °C with antibodies against CD45 (eBioscience) and phosphorylated histone H2AX (gH2AX; Cell signaling), a pan immune cell marker and a marker of DNA repair following double-stranded DNA breaks (37), respectively. Finally, the cells were analyzed on an Accuri C6 flow cytometer and data were computed using FlowJo software (TreeStar).

Isolation of mRNA and qRT-PCR

One lung lobe out of 5 was snap-frozen in liquid nitrogen and stored at - 80°C until further processing. Isolation of total RNA was carried out with the RNeasy Mini Kit (Qiagen) following the manufacturers protocol. One µg of RNA sample was used for reverse transcription and cDNA synthesis using qScript cDNA superMix (Quanta Biosciences). Quantitative real-time PCR (q-PCR) was performed on an AB7300 (Applied Bisosytems) using PerfeCTa SYBR Green FastMix (Quanta Biosciences) according to the manufacturer' instructions. q-PCR primers for specific target genes were designed based on their reported sequences and synthesized by IDT Technologies.

Histological analysis

Four lung lobes out of 5 were fixed in 4% buffered formalin for 24h and stored in histological grade 70% ethanol. The samples were then brought to the UCSD Moores Cancer Center for paraffin embedding and sectioning. The fixed lungs were cut into 4 mm sections, placed on glass slides, and stained with hematoxylin and eosin (H&E). H&E slides were scanned on a AT2 Aperio Scan Scope (Leica Biosystems) and digitalized images were used to calculate lung tumor number (i.e., multiplicity) and load (sum of adenoma surface areas) with Aperio viewer software (Leica Biosystems). These images were then reviewed by a pathologist (Dr. Valeria Estrada M.D., Assistant Director Tissue Technology Shared Resources at UCSD Moores Cancer Center) who was blinded to the procedures.

Statistical Analysis

Data are presented as mean ± SEM. The statistical significance between two groups was determined using unpaired Student t-test with two-tailed p-values. The statistical significance between more than two groups was determined using one-way ANOVA with post hoc Bonferroni's test. All statistics were computed using PRISM software (GraphPad).

RESULTS

We first employed a widely used mouse model of lung cancer induced by the carcinogen urethane. Urethane (ethyl carbamate) is a naturally occurring substance that is formed during many fermentation processes. Urethane is also a natural constituent of tobacco and is present in tobacco smoke (38). Urethane alone is sufficient to induce lung carcinogenesis in mice, and importantly, urethane-induced lung tumors in mice closely recapitulate human lung cancers, and in particular adenocarcinomas associated with tobacco smoke (39,40). We have examined whether chronic HDM exposure makes wild-type (WT) C57BL/6 mice more sensitive to urethane-induced lung tumorigenesis following an experimental protocol detailed below in Figure 1.

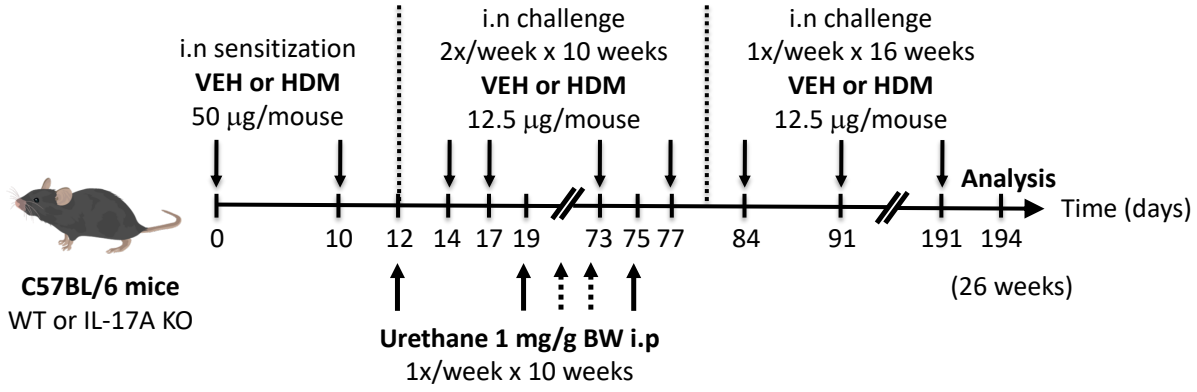

Figure 1. Schematic overview of the study design. See also Materials and Methods section for further details.

We found that WT mice treated with HDM + urethane developed severe lung inflammation as indicated by the increase in lung weight and cellularity in bronchoalveolar lavage fluid (BALF; Fig. 2A, B). These mice also developed more lung tumors than VEH + urethane-treated mice (Fig. 3B, C). To investigate the role of IL-17 in this model, we subjected

IL-17A KO mice to the same experimental protocol. Interestingly, IL-17A KO mice also developed lung inflammation (Fig. 2A, B) but only very few and lung tumors (Fig. 3B, C).

Histopathological evaluation of lung tumors revealed that in all the groups, most lesions have the characteristics and papillary morphology of lung adenomas (Fig. 3A). Our pathologist (Dr. Valeria Estrada, M.D) also found that 25% of WT mice treated with HDM + urethane had some lesions with the characteristic morphology of adenocarcinomas (Fig. 4A, B).

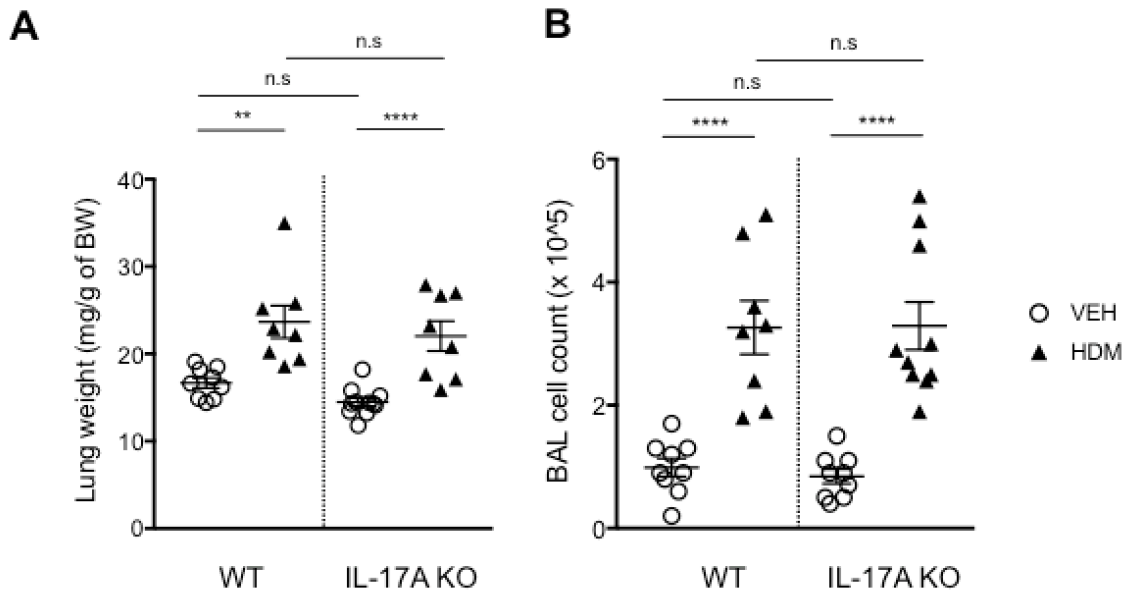

Figure 2. Inflammatory effect of chronic HDM exposure in WT and IL-17A KO mice co-treated with urethane. (A) Lung weight normalized to body weight (BW). **(B)** Bronchoalveolar lavage (BAL) total cell count. WT + vehicle (VEH; *n* = 9), WT + HDM (*n* = 8), IL-17A KO + VEH (*n* = 9) and IL-17A KO + HDM (*n* = 10). n.s: not significant, ** $P < 0.01$, **** $P < 0.0001$ (one-way ANOVA with post hoc Bonferroni's test).

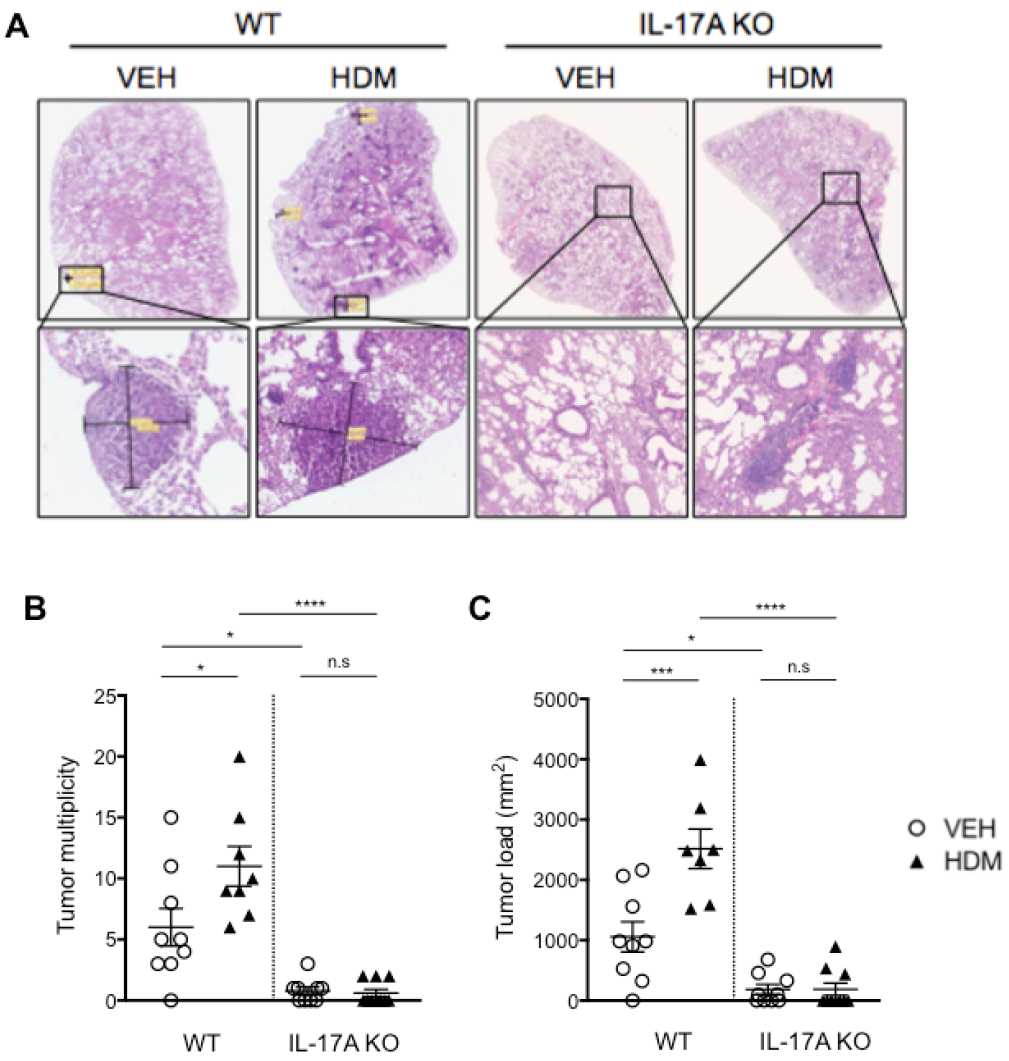

Figure 3. Effect of chronic HDM exposure on the development of lung tumors in WT and IL-17A KO mice co-treated with urethane. (A) Representative pictures of H&E staining of lung sections of WT and IL-17A KO mice treated with urethane and with VEH or HDM. One lobe per mouse is shown (overview insets) but four lobes were used for tumor quantification. The lower panels show the boxed regions at higher magnification with lesions that have the characteristic and papillary morphology of microadenomas (< 1mm in diameter). **(B)** Tumor multiplicity i.e., number of papillary adenomas per mouse (4 lung lobes). **(C)** Tumor load; i.e., sum of adenoma surface areas per mouse (4 lung lobes). The parameters in B and C were measured on 3 H&E-stained step sections of 4 lung lobes for each mouse. WT + vehicle (VEH; *n* = 9), WT + HDM (*n* = 8), IL-17A KO + VEH (*n* = 9) and IL-17A KO + HDM (*n* = 10). n.s: not significant, * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$ (one-way ANOVA with post hoc Bonferroni's test).

A

Genotype	WT	WT	IL-17A KO	IL-17A KO
Treatment	VEH	HDM	VEH	HDM
Number of mice with alveolar /bronchiolar **adenoma**	8/9 (88.9%)	8/8 (100%)	5/9 (56%)	3/10 (30%)
Number of mice with alveolar /bronchiolar **adenocarcinoma**	0/9 (0%)	2/8 (25%)	0/9 (0%)	0/10 (0%)

B

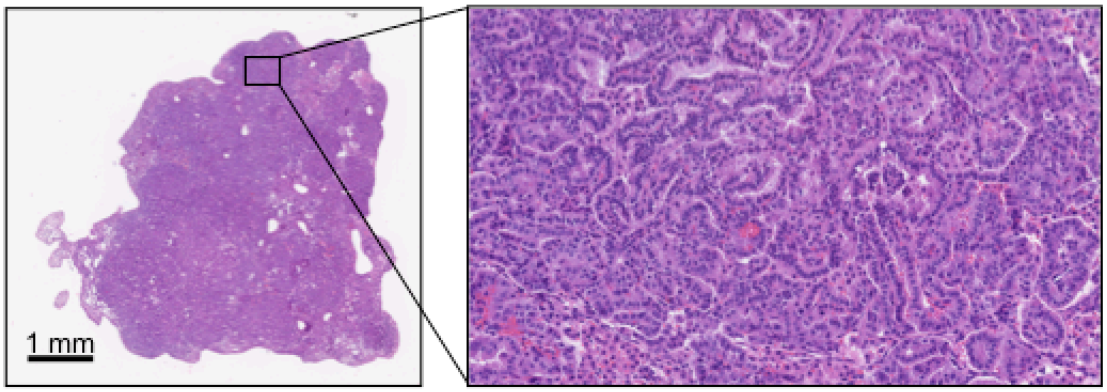

Figure 4. Lung tumor incidence in the different experimental groups. (A) Incidence of lung tumors in the different experimental groups 26 weeks after the first urethane injection. **(B)** Adenocarcinoma found in a WT mouse treated with HDM + urethane. A representative picture of H&E staining of one lobe of the lung of this mouse is shown. The right panel shows the boxed region at higher magnification.

Since recent studies indicated that pulmonary inflammation plays a critical role in the initiation and progression of lung cancer (41,42), we further examined the pulmonary inflammatory response to HDM. A more thorough analysis of lung sections (H&E staining as shown in Fig. 3), revealed that WT mice treated with HDM + urethane developed severe airway inflammation with increased immune cell infiltration in the lung parenchyma.

In line with the increased total cell count in the BALF of mice treated with HDM + urethane (Fig. 2B), Giemsa Wright staining and differential cell count of the BALF revealed increased numbers of lymphocytes, monocytes/macrophages, eosinophils and neutrophils in the BALF of mice treated with HDM + urethane compared with VEH + urethane-treated mice (Fig. 5).

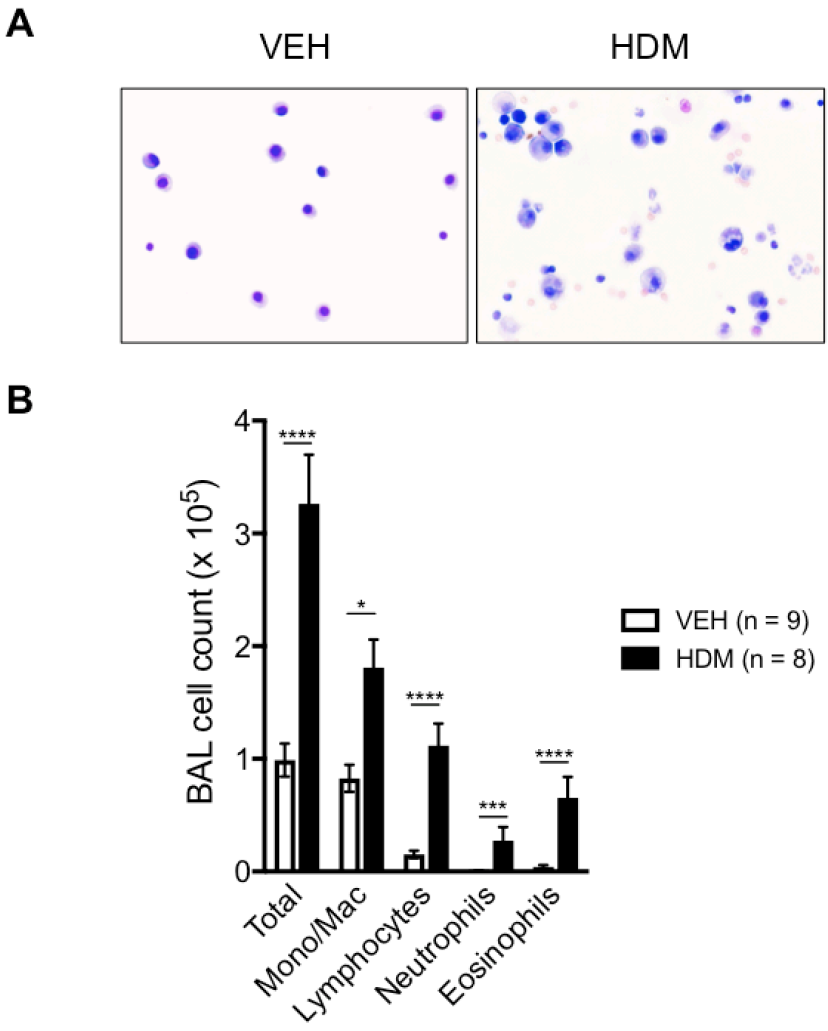

Figure 5. Analysis of BALF cellularity in WT mice 26 weeks after the first urethane injection and 72h after the last HDM or VEH challenge. (A) Representative images of Cytospin slides and BALF cell count in mice treated with VEH or HDM. **(B)** Bronchoalveolar lavage (BAL) total cell count and differential cell count. WT + Vehicle (VEH; *n* = 9), WT + HDM (*n* = 8). * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$ (one-way ANOVA with post hoc Bonferroni's test).

To further evaluate lung inflammation in these mice, we used one liquid nitrogen snap-frozen lobe of the right lung and analyzed the expression of various pro- and anti-inflammatory cytokines by qRT-PCR. As shown below in Figure 6A, we found a significant increase in expression of IL-10, IL-13, IL-33, TGFb1 and IFNg mRNA levels in the lungs of WT mice treated with HDM + urethane compared with VEH + urethane-treated mice. However, we did not observe an increase in IL-17A mRNA or its inducers such as IL-1b, IL-6 or IL-23 at this late time point (26 weeks). Additionally, IL17A, IL-1b, and IL-6 protein levels were not significantly different between VEH/HDM groups as measured through an ELISA performed on BALF and blood serum (data not shown). One possible explanation for this could be that long-term exposure to HDM drives the induction of immunological tolerance to HDM antigens as previously reported by others (43). Interestingly, in addition to cytokines, we found increased mRNA expression of several cell proliferation markers (e.g., cyclin D1, c-Myc) and genes involved in the DNA damage response (e.g., Parp1, Dnapkcs) in the lungs of HDM + urethane-treated mice (Fig. 5B and 5C).

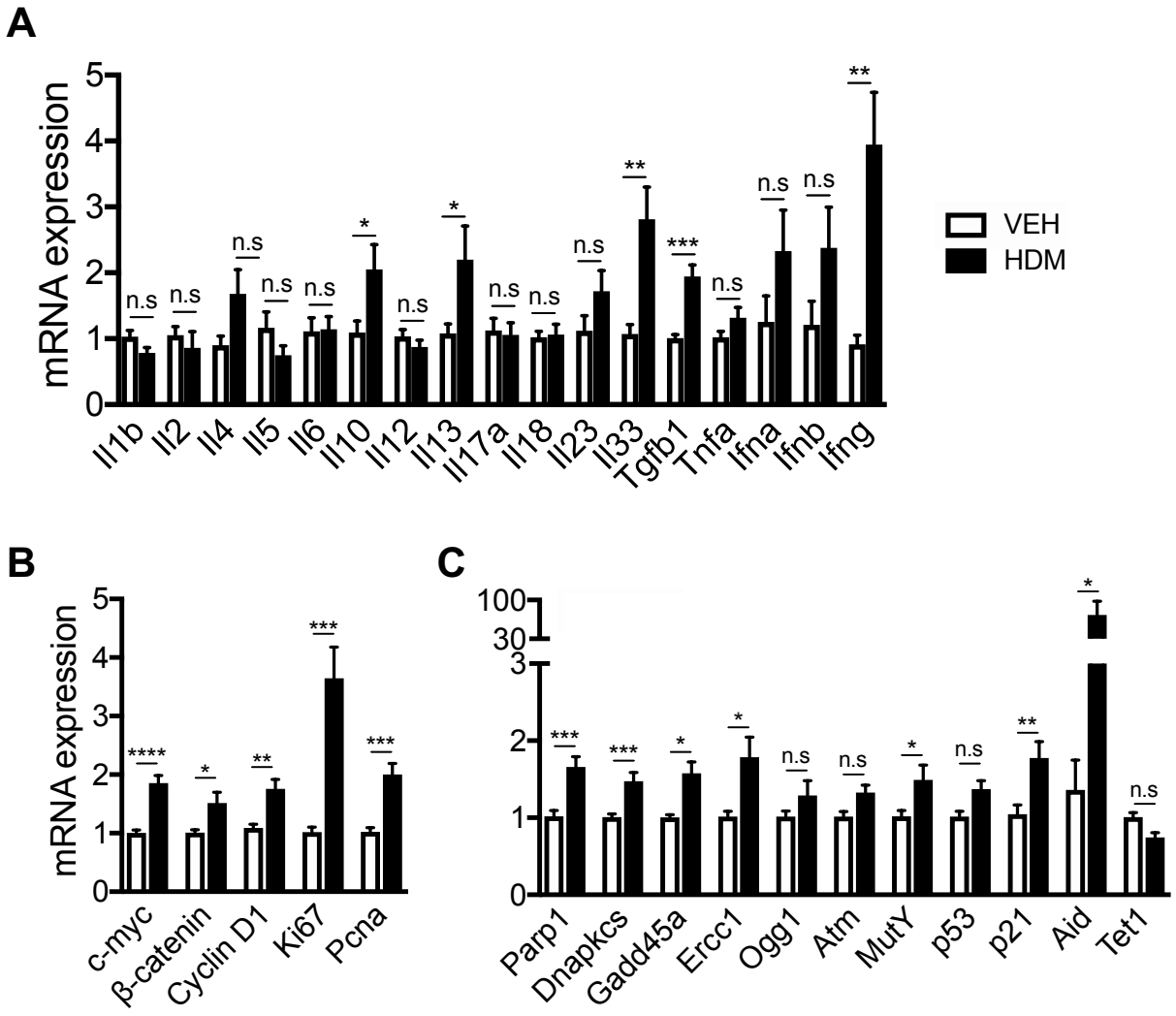

Figure 6. Increased expression of pro- and anti-inflammatory cytokines, cell proliferation markers and DNA damage response genes in the lungs of WT mice treated with HDM + urethane. The relative mRNA expression level of several **(A)** pro- and anti-inflammatory cytokines. **(B)** Cell proliferation markers and **(C)** DNA damage response genes were analyzed by qRT-PCR in lung homogenates of WT mice treated with urethane and with VEH or HDM. The expression level of GAPDH, a housekeeping gene, in the VEH group was used as reference and assigned to the value of 1. Because the severe lung inflammation in HDM-treated mice as compared to VEH-treated mice may affect the normalization of some of our qPCR data, we used Cytokeratin 19, an epithelial cell-specific gene, instead of an ubiquitous housekeeping gene such as GAPDH to assess the expression of epithelial cell markers in B and C. Mean ± SEM (*n* = 8-9 mice/group); n.s: not significant, * *P* <0.05, ** *P* <0.01, *** *P* <0.001, **** *P* <0.0001 (two-tailed Student's t-test).

To evaluate the potential contribution of adaptive immunity to the tumor-promoting effect of HDM, we treated Rag2 KO mice, which lack mature T and B cells, with urethane and with or without HDM, and compared the number of lung tumors in the two different groups 26 weeks after the first urethane injection as described for WT mice in Figure 1. However, because of their increased susceptibility to urethane, Rag2 KO mice were treated with a lower dose of urethane than the one used in WT mice (see Materials and Methods). We found that HDM-induced inflammation (increased lung weight and increased total cell count in BALF) was completely inhibited in Rag2 KO mice (Fig. 7A and 7B). Nevertheless, we still observed more tumors in Rag2 KO mice treated with HDM + urethane as compared with control mice treated with VEH + urethane (Fig. 7D-E). However, the effect of HDM on the tumor load (the sum of tumor surface areas per mouse) in these animals was abolished. Taken together these observations suggest that adaptive immunity is dispensable for the effect of HDM on tumor initiation but rather important for its effect on tumor growth.

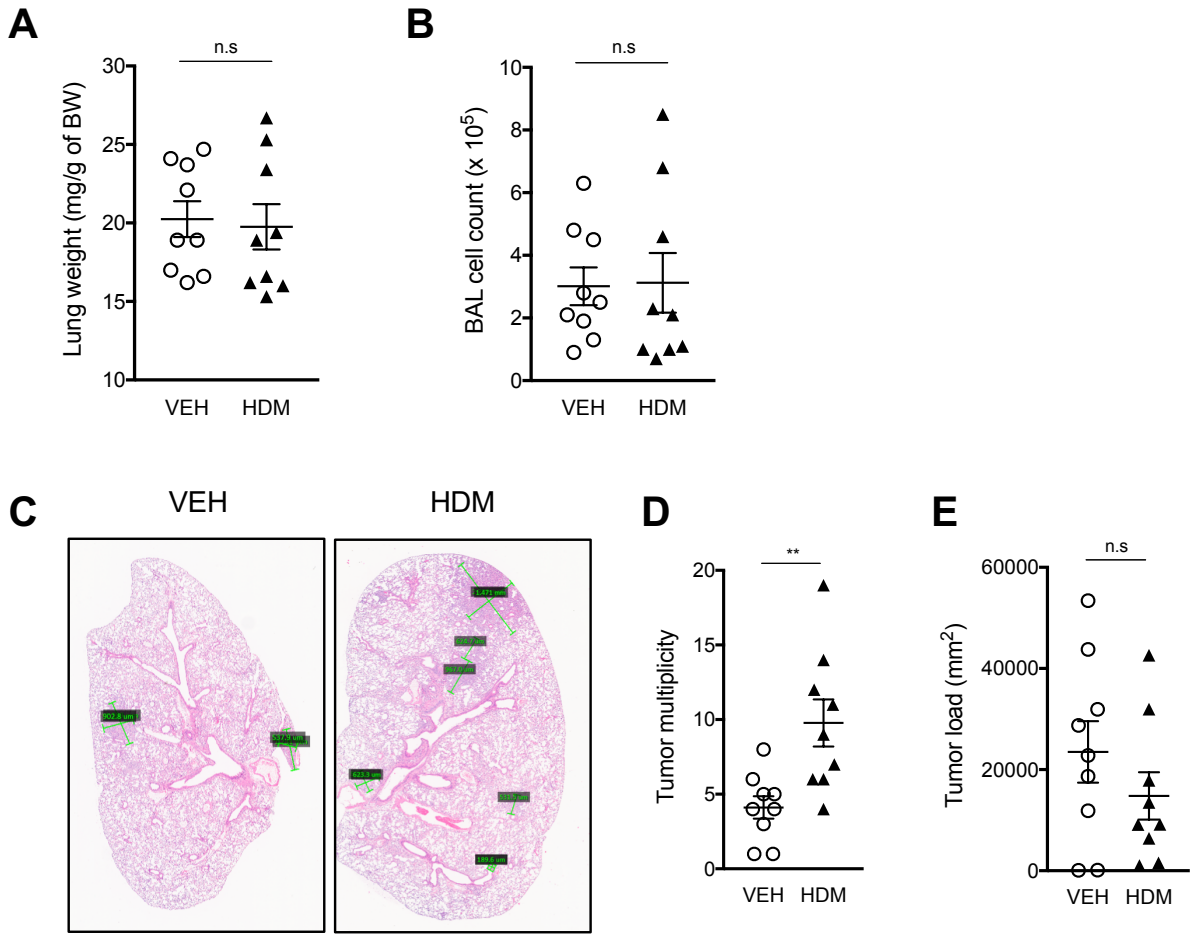

Figure 7. Effect of chronic HDM exposure in Rag2 KO mice co-treated with urethane. Age- and sex-matched Rag2 KO mice were randomly assigned to two groups and were treated with HDM or with the VEH for 26 weeks in presence of low dose urethane co-treatment. **(A)** Lung weight. **(B)** Bronchoalveolar lavage (BAL) total cell count. **(C)** Representative pictures of H&E staining of lung sections. **(D)** Average number of lung tumors areas per mouse, as measured on 3 H&E-stained step sections of 4 lung lobes for each mouse. Mean ± SEM (n = 9 mice/group); n.s: not significant, ** *P* <0.01 (two-tailed Student's t-test).

Next we asked the question of whether HDM accelerates the growth of urethane-induced tumors or if it leads to the formation of new tumors. To test whether HDM alone can induce lung tumor formation in the absence of urethane, we treated WT C57BL/6 mice with HDM or VEH for 26 weeks as described in Figure 1 but without urethane co-treatment, and we assessed lung inflammation and tumor incidence as described above. We found that chronic

exposure to HDM alone induces lung inflammation as judged by the increase in lung weight and cellularity in the BALF (Fig. 6A, B), but it is not sufficient to induce lung tumor development in WT C57BL/6 mice, at least within the 26-week follow-up period (Fig. 6C, D).

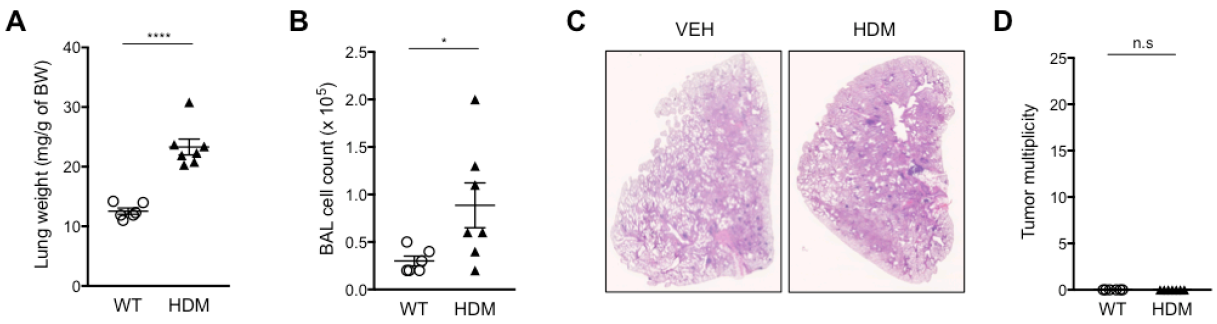

Figure 8. Effect of chronic HDM exposure in WT C57BL/6 mice in absence of urethane co-treatment. Age- and sex-matched wild-type C57BL/6 mice were randomly assigned to two groups and were treated with HDM or VEH for 26 weeks in absence of urethane co-treatment. **(A)** Lung weight normalized to body weight (BW). **(B)** BAL total cell count. **(C)** Representative pictures of H&E staining of lung sections. **(D)** Average number of lung adenomas per mouse as measured on 3 H&E-stained step sections of 4 lung lobes for each mouse. Mean ± SEM (*n* = 6-7 mice/group); n.s: not significant, * *P* <0.05, **** *P* <0.0001 (two-tailed Student's t-test).

Because C57BL/6 mice are resistant to spontaneous and carcinogen-induced lung tumors (44), we next evaluated the effect of chronic exposure to HDM alone in a lung tumor susceptible mouse strain. A/J mice carry the pulmonary adenoma susceptibility allele, have high rates of Kras mutation, and have a relatively high spontaneous adenoma/adenocarcinoma incidence (45,46). We exposed age- and sex- matched A/J mice to HDM or VEH for 10 weeks as shown below in Figure 8A and we evaluated the occurrence of lung tumors in the two experimental groups. We identified that chronic exposure to HDM alone is sufficient to induce the formation of lung adenomas in A/J mice (Fig. 8B-F).

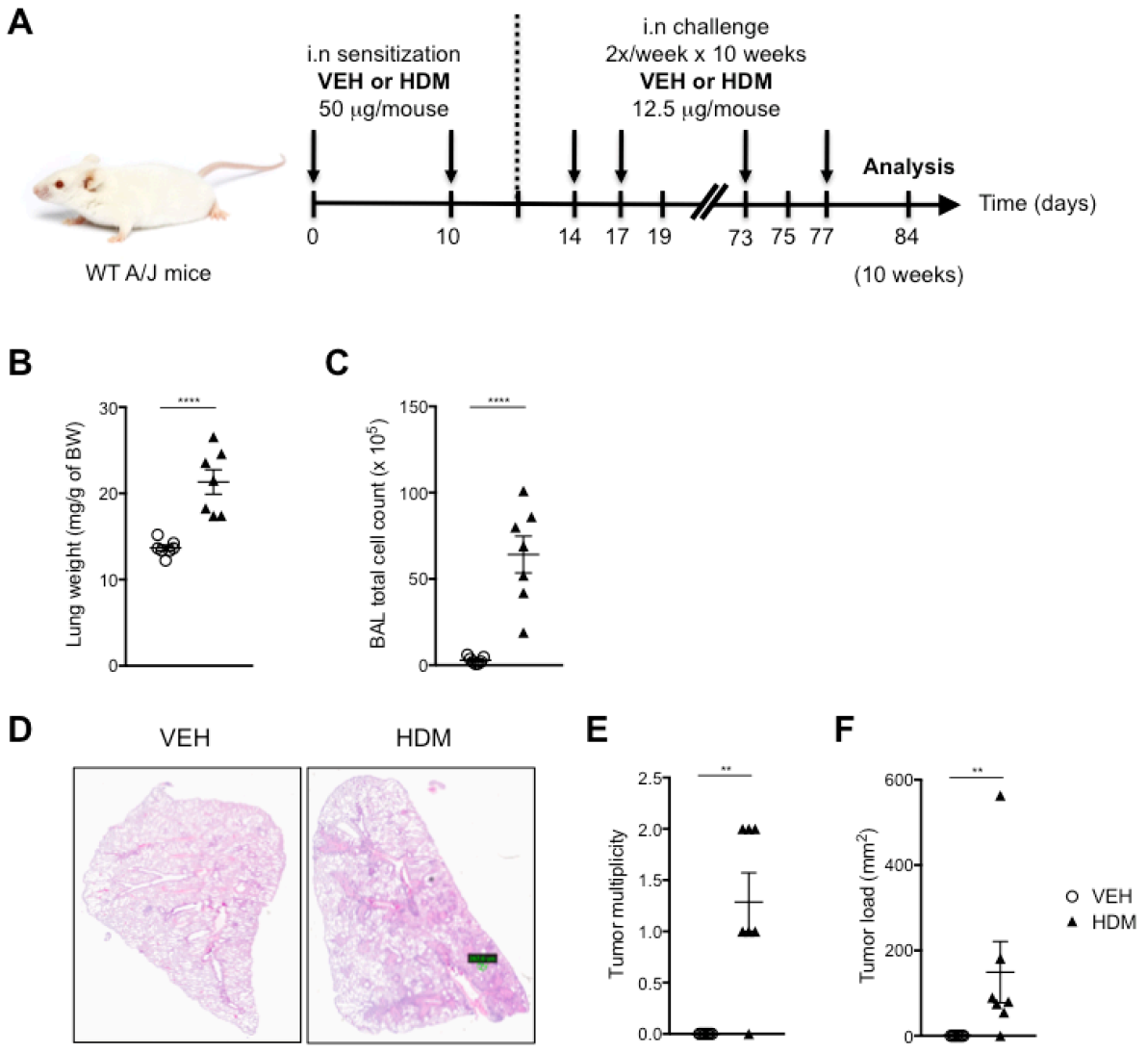

Figure 9. Effect of chronic HDM exposure in absence of urethane treatment in the lung tumor susceptible A/J mouse strain. (A) Schematic overview of the study design. Age- and sex-matched wild-type A/J mice were randomly assigned to two groups and were treated with HDM or VEH for 10 weeks in absence of urethane co-treatment. **(B)** Lung weight normalized to body weight (BW). **(C)** BAL total cell count. **(D)** Representative pictures of H&E staining of lung sections. **(E)** Average number of lung adenomas per mouse. **(F)** Average tumor load (sum of adenoma surface areas) per mouse. The parameters in E and F were measured on 3 H&E-stained step sections of 4 lung lobes for each mouse. Mean ± SEM (*n* = 7 mice/group); n.s: not significant, * *P* <0.05, ** *P* <0.01, *** *P* <0.001, **** *P* <0.0001 (two-tailed Student's t-test).

To further evaluate the effect of HDM on tumor initiation in A/J mice, we performed another experiment at an earlier time point in order to evaluate the effect of short-term exposure to HDM that precedes the formation of lung tumors. To evaluate the potential induction of DNA damage by HDM, we treated a small cohort of A/J mice with HDM or VEH (n = 3-4 mice/group) for 15 days as shown in Figure 9A. We then determined by flow cytometry the number of cells positive for CD45 (a pan immune cell marker) and phosphorylated histone H2AX (gH2AX; a marker of DNA repair following double-stranded DNA breaks (DSBs) (37)) as described in the Materials and Methods section. In line with the results from Chan *et al* (33,34), we observed that HDM induces double-strand breaks in the lungs *in vivo*. However, in contrast to their findings, we observed that the vast majority of the DSBs are in CD45+ (immune) cells and not in CD45- (non-immune) cells, which include lung epithelial cells (Fig. 10B, C).

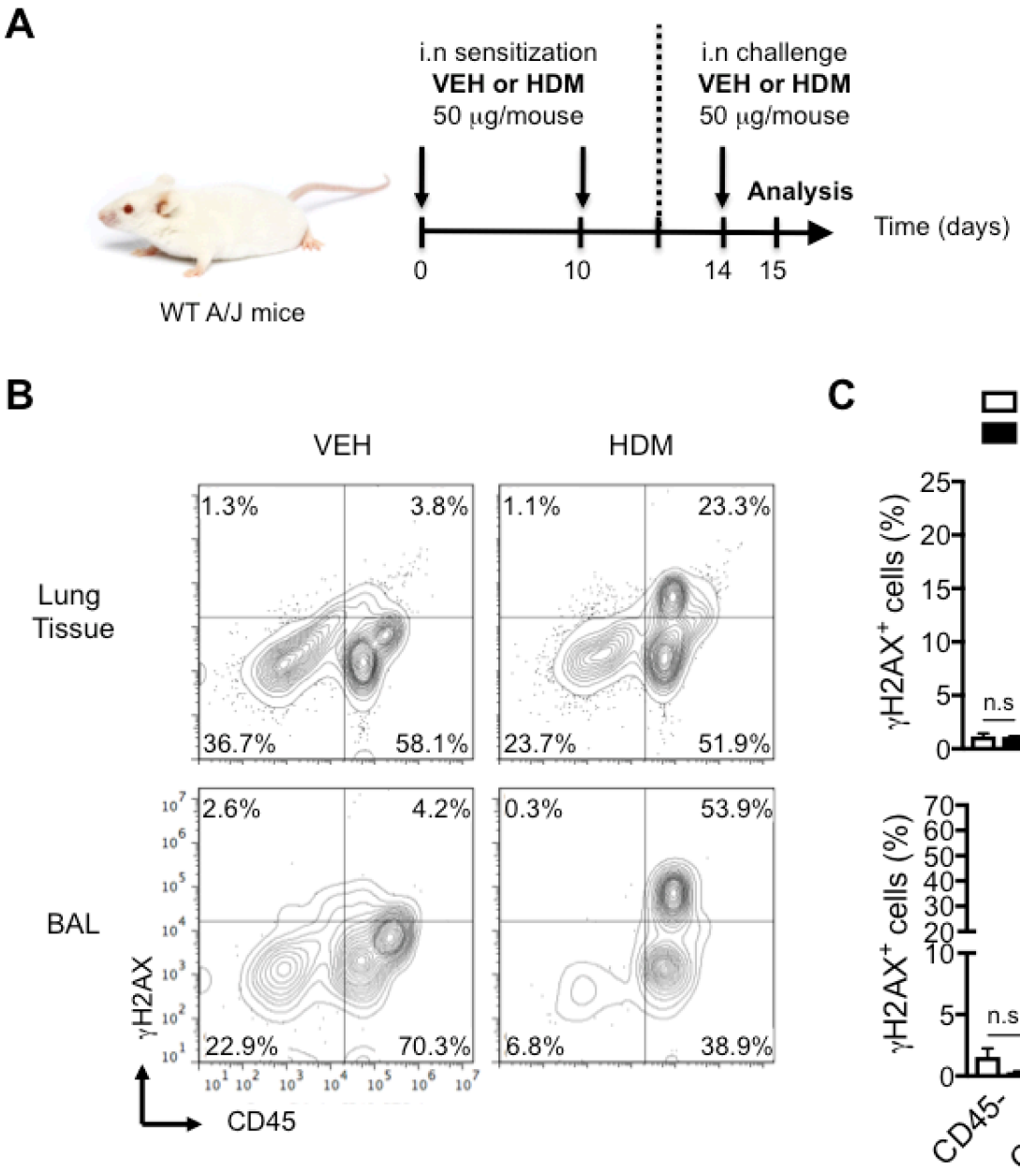

Figure 10. Effect of Short-Term HDM Exposure in A/J mice. (A) Schematic overview of the study design. Age- and sex-matched wild-type A/J mice were randomly assigned to two groups and were treated with HDM or VEH for 15 days. **(B)** Representative plots of flow cytometry staining for CD45 and gH2AX in lung tissue and in the BALF. **(C)** Average % of CD45+gH2AX+ and CD45-gH2AX+ per mouse. Mean ± SEM (*n* = 3-4 mice/group); n.s: not significant, *** *P* <0.001, **** *P* <0.0001 (two-tailed Student's t-test).

Finally, to more fully evaluate the potential clinical significance of our mouse data generated in the urethane-induced lung cancer model and in the spontaneous lung cancer model in A/J mice, we have evaluated the effect of chronic HDM exposure in K-ras^{G12D+} CCSP^{cre+} mice that are genetically engineered to develop lung tumors (36). K-ras mutation is the most common driver mutation in patients with non-small-cell lung cancer (NSCLC) and confers a poor prognosis (47,48). We treated age- and sex-matched treated K-ras^{G12D+} CCSP^{cre+} mice with HDM or VEH for 9 weeks following a protocol similar to the one described in Figure 9A for A/J mice, and evaluated the occurrence of lung tumors in the two experimental groups. As observed in previous mouse models, chronic HDM exposure induces severe lung inflammation (Fig. 11A, B). Interestingly, HDM also increases the tumor load in this model. Because of the diffuse infiltrative nature of the lung tumors in this model, the tumor load in each mouse was determined on H&E-stained lung tissue using an image analysis software (see Materials and Methods). Tumor load in this model was expressed as percentage of total lung tissue (Fig. 11C, D).

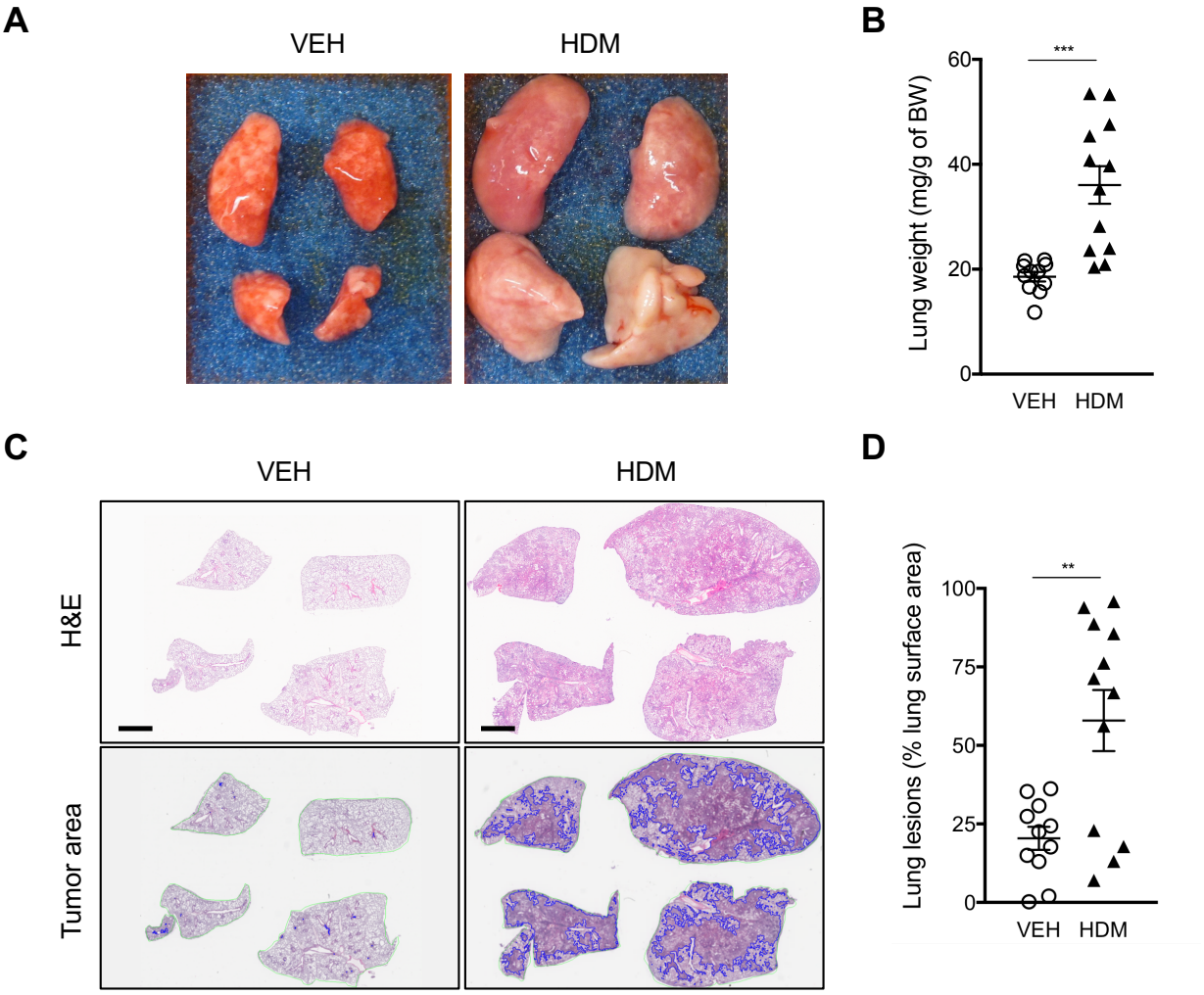

Figure 11. Effect of chronic HDM exposure in the K-ras^{G12D+} CCSP^{cre+} model. (A) Age- and sex-matched K-ras^{G12D+} CCSP^{cre+} mice were randomly assigned to two groups and were treated with HDM or VEH for 9 weeks in absence of urethane co-treatment. **(B)** Lung weight normalized to body weight (BW). **(C)** Representative pictures of H&E staining of lung sections. The lower panels are the same images as above panels after software analysis of tumor area. The area within the blue borders was considered positive for lung tumor, which include papillary hyperplasia, adenomas, and adenocarcinomas. **(D)** Average tumor area per mouse calculated on H&E slides as shown in C. The parameters in C and D were measured on 3 H&E-stained step sections of 4 lung lobes for each mouse. Mean ± SEM (*n* = 11-12 mice/group); n.s: not significant, * *P* <0.05, ** *P* <0.01, *** *P* <0.001, **** *P* <0.0001 (two-tailed Student's t-test).

Collectively, our data suggest that the effects of HDM are not limited to the induction of allergic airway inflammation and asthma. Indeed, we found in three different mouse models of lung cancer that HDM promotes lung tumor development. In addition, our data suggests that innate and adaptive immunity may play complementary roles in promoting lung tumor initiation and growth, respectively. Finally, we identify that HDM induces DNA damage in lung immune cells. Its physiological consequence and the targeted cell population(s) in our models will be the subject of future investigations in the lab. Therefore, our study may have identified a novel role for HDM as a potential respiratory carcinogen or co-carcinogen.

DISCUSSION

Since it is one of the most fatal cancers, identifying new risk factors to prevent lung cancer is important. The long establishment of lung cancer's association with smoking is well understood, however, the other risk factor for the lung cancer, including those responsible for lung cancer death in non-smokers are less understood. In the current literature, asthma's relationship to lung cancer is controversial, however recent discovery about asthma endotypes may explain this controversy (10). Similarly the type of antigen used in mouse models to study the potential association between asthma and the risk of lung cancer is important. For instance, OVA produces a strong Th2 inflammatory response while HDM elicits a mixture of Th17/Th2 inflammatory response (25). Therefore the fact that no association was found by using the OVA-model of asthma and the urethane model of lung cancer may be imputed to the innocuity of OVA as an antigen (23). We employed the same model of lung of cancer and a similar protocol to induce asthma and airway inflammation but with HDM as the antigen instead of OVA and actually found an increase in tumor development compared with control mice treated with urethane alone. This finding could potentially be explained by the fact that HDM is a more potent antigen than OVA due to its complex structure and by its capacity to induced a mixed Th2/Th17 inflammatory response.

HDM is a complex organism that is widely prevalent in the environment, and largely known for its ability to induce asthma. However, our studies have shown that the effect of HDM is not limited to inducing asthma and that it can also promote lung cancer development in mice that are susceptible to lung cancer. Through studying several mouse strains, we found HDM to

act as a co-carcinogen to inducing lung cancer. We studied the impact HDM had on three lung cancer models, one was a chemically-induced model through urethane in WT C57BL/6 mice, the second was the A/J mouse strain which is genetically susceptible to develop spontaneous lung cancer, and the last was C57BL/6 mice which were genetically modified to express an oncogenic form of K-ras (K-ras^{G12D}) in lung epithelial cells. In all three of these models, we compared the effect of intranasal treatment with HDM or its vehicle (control saline) and found that HDM consistently increased tumor multiplicity. In order to see whether HDM acts as a carcinogen on its own, we treated WT C57BL/6 mice with VEH and HDM in the absence of urethane, and found that although it was inducing severe lung inflammation, it was not able to induce lung tumors within the follow-up period (26 weeks). Interestingly, we got different data when using a different mouse strain. Indeed, we found that HDM alone was able to stimulate the gross of lung adenomas in A/J mice within 10 weeks of treatment. The difference in results between C57BL/6 and A/J mice can most likely be explained by the fact that C57BL/6 and A/J are respectively, resistant and susceptible to the development of lung tumors (44). Our data and the previous studies led us to conclude that HDM cannot induce lung cancer in mice on its own, but acts as a co-carcinogen in susceptible mice.

HDM is made up of fungal and bacterial constituents with protease activity (26). Thus, exploring why we observed increased lung cancer is not a simple task. The recent distinction between endotypes of asthma along with the difference in lung cancer development between treatment with either OVA or HDM as an antigen led us to explore the effects that the Th17/IL-17 inflammatory response may have had. Based on previous studies and the function of IL-17A

as a mitogen for epithelial cells, it seemed to be a potential culprit for the increase in lung cancer incidence (13-15). For this reason, we decided to examine if knocking out IL-17A in mice would impact lung cancer development. When WT mice were treated with HDM + urethane, there was an increase in tumor multiplicity and tumor load as compared to mice treated with VEH + urethane. However, when IL-17 KO mice were used, both the HDM/VEH + urethane groups had little to no tumors. Therefore, our data suggest that IL-17A promote lung cancer development in this model. This finding is in agreement with other studies that have explored the role of IL-17A in other mouse models of lung cancer (21,22,49). However, it is not known whether it plays a role in the initiation and/or the growth phase of tumor development. Our data also do not allow us to conclude whether the tumor-promoting effect of HDM is dependent on IL-17A because the deletion of IL-17A reduces the formation of all the tumors, including those in VEH + urethane-treated mice. Therefore, it is unclear whether HDM or urethane requires this factor.

We measured the levels of IL-17A in both experimental groups at 26 weeks of age and found no significant difference in lung tissue by qRT-PCR and in the BAL and serum by ELISA. Considering the data from our IL-17A KO mice, it is plausible that IL-17A has to be present at an earlier time point than the one we looked at. Our data leads us to believe that IL-17A is important for the initiation of lung cancer as opposed to the growth of the cancer cells. This theory aligns with an observation that we made with the IL-17A KO mice. Although there were very few lung tumors in these mice, when there were, their average size was similar to the average size of the tumors found in WT mice (data not shown). This leads us to speculate that

IL-17A's presence is necessary in order to initiate/facilitate the formation of these tumor cells. Future experiments will evaluate the effect of HDM on the induction of IL-17A at an earlier time point (e.g., 2-3 weeks), which precede lung tumor formation. Although it has been reported that HDM can induce a Th17 inflammatory response, the cells that produce IL-17 are not clearly defined. To identify the cellular source(s) of IL-17A in response to HDM exposure, we will treat IL-17A eGFP reporter mice that we are currently breeding in our vivarium with HDM or VEH, and we will determine the number of GFP+ DCs, macrophages, neutrophils, NK cells, abT cells (CD4 and CD8), gdT cells and ILC3 by flow cytometry as previously described (50). Further studies will also be conducted in A/J mice that allow us to focus on HDM's effects in the absence of urethane for example using IL-17A neutralizing antibodies. The shorter latency of tumor development in this model (10 weeks) will also allow us to determine whether IL-17A neutralization using antibodies affects preferentially the initiation and/or the growth phase of lung cancer development in response to HDM.

Due to the complexity of HDM's components, both innate and adaptive immune mechanisms are being activated during the allergic inflammation. To understand the role of adaptive immunity in lung cancer development, we used Rag2 KO mice and treated them with HDM/VEH and urethane. Rag2 KO mice are known to develop significantly more epithelial tumors (35% gastrointestinal, 15% lung), even when raised on broad-spectrum antibiotics in a pathogen-free facility (51). In accordance with this phenomenon, we found that a lower dose of urethane was necessary in order to induce tumor formation in these mice. The increase in tumor multiplicity but lack of effect on tumor load suggested that adaptive immunity did not

play a role in the initiation of the tumor development, but that it is important for the regulation of tumor growth. It has been shown that long-term exposure to HDM can cause an increase in Foxp3+ regulatory T cells in the lungs (43). Immunosuppression by Foxp3+ regulatory T cells can lead to the inhibition of cancer immunosurveillance by cytotoxic T-cells, which can suppress the immune system from detecting cancerous cells, and allow cancer cells to proliferate (52,53). Some of our own preliminary data also found increased *Foxp3* mRNA by qRT-PCR (preliminary data not shown) and may in part explain how the absence of regulatory T-cells absence in Rag2 KO mice abolished the effect of HDM on tumor load as observed in WT mice. Ultimately, our studies showed that adaptive immunity is not important for lung tumor initiation as Rag2 KO mice treated with HDM + urethane still develop more lung tumors control mice treated with VEH + urethane. Therefore, we assume that T cells are not the main the cellular source of IL-17A in the initiation phase of lung cancer development in our model. However, it is likely that adaptive immunity plays a role in later stages such as the growth of lung cancer cells.

Other studies have shown that long-term exposure to HDM to increase IL-10+ alveolar macrophages (43). This falls in line with our own data where increased macrophages were found in BALF and was the predominant type of immune cell present in the BALF. Recent studies have shown that macrophages play important roles in lung cancer development (54). Two studies have shown that the depletion of alveolar macrophages led to decreased adenocarcinomas in chemically-induced lung cancer mouse models, including the urethane model that we used (54,55). Macrophages have various functions that include pro-inflammatory cytokine secretion or anti-inflammatory activity by secretion of cytokines such as

IL-10 and TGFb. These two opposite functions of macrophages often classify them into two categories, M1 are “classically activated” and pro-inflammatory, while M2 are “alternatively activated” and anti-inflammatory (56). It was reported that depletion of M1 macrophages in the early stages of cancer development lead to less tumor initiation while depletion of M2 macrophages in late stages led to reduced tumor angiogenesis and tumor progression (54). Our own data found an increase in IL-10 and TGFb mRNA by qRT-PCR, which are markers for M2 macrophages. We will therefore further investigate whether the inflammatory response induced by HDM promotes M2 macrophages differentiation.

Inflammation has been shown to act synergistically with DNA damage in order to induce mutations driving cancer development (57). Due to recent studies showing that HDM induces double stranded DNA breaks in lung epithelial cells in-vitro and in-vivo (33), we also evaluated the possible induction of DNA damage in our models. Surprisingly enough, after performing flow cytometry, we found that most of the double stranded DNA breaks, as indicated by the phosphorylation of H2AX, were found in immune cells. Future studies in the lab will investigate what type of immune cells were exhibiting the DNA damages, and our preliminary data that is not shown points in the direction of macrophages. The consequences of the DNA damages as well as the component(s) within HDM that is (are) responsible for its tumor promoting effect will be the subject of future investigations in the lab.

CONCLUSION

The relationship between lung cancer and asthma as a chronic lung disease and risk factor is not well understood. HDM is one of the most common allergens and is a major cause of allergic rhinitis and asthma, and as our data suggest, a potential respiratory carcinogen or co-carcinogen. If our data generated in mice are confirmed in humans, chronic HDM exposure may present a new risk factor for developing lung cancer in susceptible individuals (e.g., smokers or individuals exposed to other environmental carcinogens). This new finding may have important implications for lung cancer prevention efforts especially in individuals sensitized to HDM and exposed to other air pollutants. These studies may also lay down the foundation for preventive HDM immunotherapy strategies for at-risk populations. Finally, our investigations may further demonstrate the therapeutic potential of blocking IL-17A for the treatment of lung cancer.

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