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14. ABSTRACT: Lung is physiologically populated by numerous immune cells, that can be skewed to support the growth of tumor cells. My main goal is to understand how cancer cells interact with the surrounding environment, creating an immunosuppressive tumor microenvironment, to promote growth. This knowledge can ultimately render new vulnerabilities that can be addressed by the development of new drugs or re-purposing of known drugs, eventually leading to an increase in survival of lung cancer patients.
Bone marrow derived macrophages (BMDM) from A/J mice show an increased expression of NRF2 and HO-1 upon stimulation with conditioned media from lung cancer cells, this increase is accompanied with increased expression of CD206, arginase, IL10 and others, showing the ability of cancer cells to skew macrophages towards a tumor promoting phenotype. Moreover, CD11 and CD3 cells were isolated from A/J mice injected with vinyl carbamate (or saline for control group). Both CD11 and CD3 cells, showed an increased expression of HO-1 2 weeks after vinyl carbamate injection. These data suggest that NRF2->HO-1 pathway is activated in macrophages early in the progression of lung cancer.

15. SUBJECT TERMS

Lung cancer, NRF2→HO-1 pathway, macrophages, clodronate liposomes

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TABLE OF CONTENTS

1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Historically, activation of the Nrf2 pathway was considered beneficial for cancer prevention. Nrf2 deficiency enhances susceptibility to carcinogens, and Nrf2 activators prevent or delay tumor development in many preclinical models, regardless of cancer type or the carcinogen used. Cells in the lung, which are continuously exposed to abundant reactive oxygen and nitrogen species as well as carcinogens, especially rely on this important defense pathway to maintain cellular homeostasis. Upon oxidative/electrophilic stress or heme accumulation, the negative regulator Keap1 is inactivated and targeted for proteasomal degradation while Nrf2 translocates to the nucleus. In the nucleus, NRF2 dimerizes with Mafs promoting the transcription of antioxidant target genes, including HO-1. Both levels of NRF2 and HO-1 (HMOX1) predict survival in lung adenocarcinoma, when KRAS is overexpressed. However, the role of NRF2 in the functionality of the immune cells within the TME in lung cancer and the role of this pathway in carcinogenesis are poorly understood. This work aims to fully understand the role of NRF2→HO-1 expressing macrophages in lung cancer progression in the presence on KRAS mutations.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Lung cancer, NRF2→HO-1 pathway, macrophages, clodronate lipossomes

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1: Immune suppressive HO-1 positive macrophages promote tumor growth in KRAS mutated lung cancer
Major Task 1B: Determine the effects of lung cancer conditioned media on the upregulation of NRF2 and HO-1 in RAW264.7 cells and BMDM from A/J and NRF2 KO mice

Major Task 1C: Assess the effects of macrophage depletion in lung cancer progression in A/J mice

Major Task 1E: Evaluate the effects of NRF2 KO macrophages chimeras on lung cancer progression

Specific Aim 2: NRF2 activated macrophages in KRAS transformed tumors decrease the activity of anti-tumor T cells, by increasing exhaustion phenotypes in cytotoxic T cells.
Major Task 2A**:** Evaluation of in vitro T cell activation by NRF2 wild type and KO macrophages

Major Task 2B: In vivo evaluation of T cell activation/exhaustion in NRF2 wild type mice and macrophage KO chimeras. N=48 (24 WT, 24 NRF2 chimeras), 6/group/time point

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

During the period here reported the major goal was to establish a robust colonies of A/J mice of both genotypes, wild type, and KO. While WT mice reproduce well with litters on average 1 month apart, and with expected numbers of pups (5-6), KO A/J mice are less productive. Typically, the first litter of pups died with the first days of birth, or if the pups survive, they are smaller in size and the number of pups/litter is on average much smaller, with only 2-4 pups per litter. Due to this unexpected trait, accruing sufficient numbers of mice for the in vivo studies is taking longer than expected.

<u>Specific Aim 1:</u> Immune suppressive HO-1 positive macrophages promote tumor growth in KRAS mutated lung cancer

Major Task 1B: Determine the effects of lung cancer conditioned media on the upregulation of NRF2 and HO-1 in RAW264.7 cells and BMDM from A/J and NRF2 KO mice – subtasks 2-5

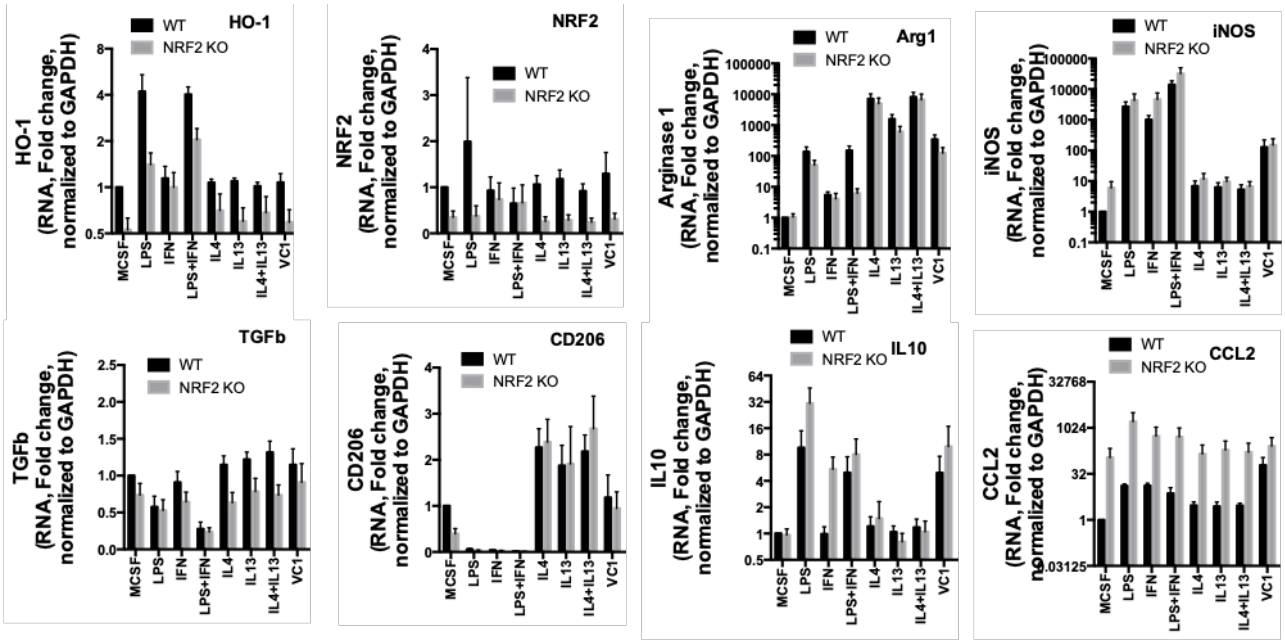

Figure 1: Conditioned media from murine lung cancer cells increases HO-1 expression in wild type (WT) bone marrow derived macrophages (BMDM). BMDM were collected from both wild type (WT) and NRF2 KO (KO) mice and cultured for 5 days with M-CSF (10 ng/ml). On day 5 media was replaced for media containing 1%FBS and LPS (1 ng/ml), interferon gamma (IFN) (10 ng/ml), IL4 (10 ng/ml), IL13 (10ng/ml) or conditioned media from murine lung cancer cells (VC1), and BMDM cultured for additional 24 hours. Conditioned media was generated by culturing VC1 cells at 70-80% confluency for 24 hours in 1%FBS media. Levels of the indicated markers were determined by RT-PCR.

To determine the effects of KRAS mutation on macrophage polarization and the role of NRF2→HO1pathway, bone marrow derived macrophages (BMDM) from both A/J wild type and

full body NRF2 KO were utilized. Monocytes are collected from the marrow of the femur and tibia bones and differentiated into macrophages using M-CSF (10 mg/ml) for 5 days. On day 5 media is replaced with reduced serum (1% FBS) and BMDM are further stimulated with LPS (1ng/ml), interferon (IFN) (10 ng/ml), IL4 (10ng/ml), IL13 (10 ng/ml) or conditioned media from lung cancer cells (VC1). VC1 cells were generated from A/J mice bearing lung tumors. Conditioned media is generated by culturing VC1 cells at 70-80 confluence for 24 hours in reduced serum (1% FBS). As expected, levels of HO-1 and NRF2 are higher in BMDM of WT mice compared with NRF2 KO mice, in all conditions, with exception of interferon stimulation. Likely HO-1 levels in interferon stimulation are not dependent on NRF2 activation/presence (Figure 1). To further characterize the BMDM generated, mRNA levels of macrophage polarization markers (iNOS, Arginase, CCL2, CD206, IL10 and TGFb) were analyzed. No differences were observed in the levels of iNOS, Arginase, CCL2, CD206, IL10 and TGFb between WT and KO BMDM. Levels of other markers, such as IL1b, IL33 and PDL1 are underway.

Additionally, protein levels of HO-1 and NRF2 will be tested. RAW cells (macrophage-like cells) were already treated with the same conditions as in Figure 1 and western blots will be run in the future. Flow cytometry will be used to confirm the data generated by RT-PCR for cell surface markers and cytokines.

To determine the factors that are secreted from cancer cells that lead to the increase in HO-1, BMDM were stimulated with conditioned media from two murine cell lines, VC1 and commercially available LL2 cells. Interestingly, in both cell lines the levels of HO-1 are elevated after the media is boiled for 15 minutes. Small decreases are observed when conditioned media is treated with NAC (N-acetyl cysteine), but no changes are observed with EDTA (Figure 2). NAC can act both as an antioxidant and a chelator. Both data combined suggest that cancer cells are secreting an oxidative specie that is likely conjugated with a protein waste, I do hypothesize that cancer cells are secreting heme, and that upon boiling releases iron, that can strongly activate HO-1 in macrophages. To further elucidate this, boiled conditioned media will be treated with EDTA, NAC and Iron Chelator IV, 21H7. Levels of HO-1 will be determined. Additionally, RT PCR for macrophage polarization markers (iNOS, Arginase, CCL2, CD206, IL10 and TGFb) will be analyzed.

To determine if the alteration observed in Figure 2 are reproducible at 48 hours, the same experimental strategy was used as described above. 48 hours time point is used due to the fact that some of the markers of macrophage anti-tumor or pro-tumor phenotype may take longer to be upregulated after the upregulation of HO-1, which is a "first" response mechanism. As shown in figure 3A, at 48 hours both VC-1 and LL2 conditioned media induce HO-1

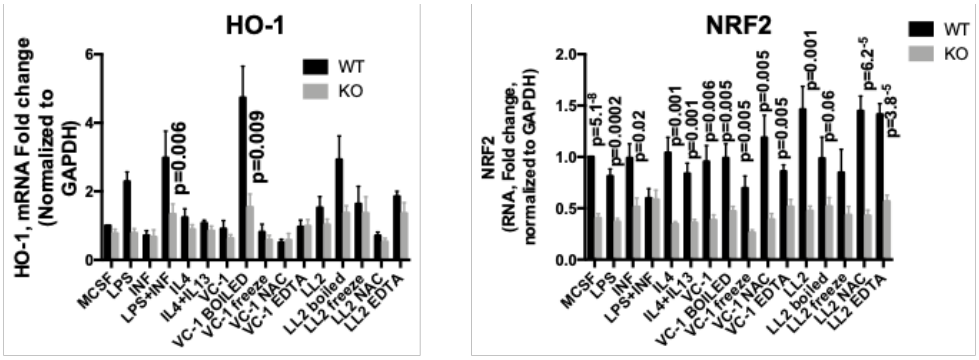

Figure 2: Boiled conditioned media from lung cancer cells increases expression of HO-1 in BMDM. Levels of HO-1 and NRF2 in wild type (WT) and NRF2 KO (KO) bone marrow derived macrophages stimulated with inflammatory cytokines or conditioned media from two murine lung cancer cells, for 24 hours. Conditioned media was boiled (15 minutes) or frozen (3 cycles of freeze/thaw) or treated with antioxidant NAC (1M) or chelator EDTA (1M).

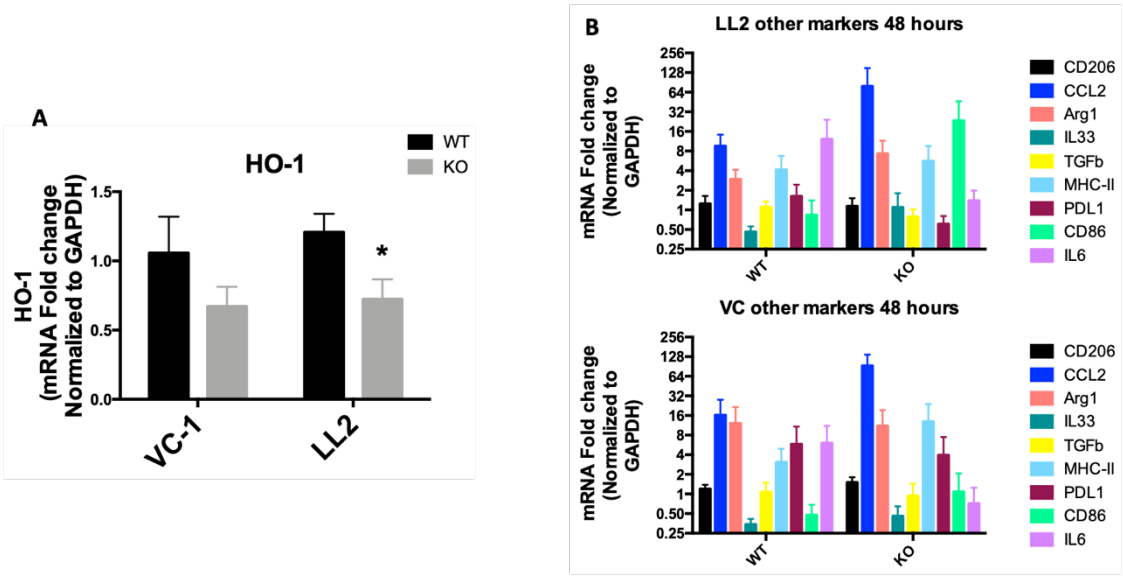

Figure 3: Conditioned media from murine lung cancer cells increases HO-1 expression in wild type (WT) bone marrow derived macrophages (BMDM). (A) Levels of HO-1 in wild type (WT) and NRF2 KO (KO) bone marrow derived macrophages stimulated with inflammatory cytokines or conditioned media from two murine lung cancer cells, for 48 hours. (B) Conditioned media also modifies other BMDM markers (n=5-2). Conditioned media was generated by culturing VC1 or LL2 cells at 70-80% confluency for 24 hours in 1%FBS media. Levels of the indicated markers were determined by RT-PCR. *p<0.05 LL2 WT vs LL2 KO

expression in WT BMDM when compared with NRF2KO BMDM. Additionally, other markers, such as CCL2 and IL6 are differently expressed between WT and NFF2 KO BMDM. (Figure 3B). CCL2 appears to be more predominantly expressed in NRF KO BMDM, this effect is in accordance with past studies that show that NRF2KO mice develop lung cancer more rapidly and with higher tumor burden when compared with WT mice, and this increase is accompanied by an increased infiltration of myeloid cells. Il6 follows the opposite changes as CCL2. IL6 expression decreases in NRF2 KO macrophages. As observed, at 24 hours, at 48 hours, boiled conditioned media further increases the expression of HO-1 and a reduction is observed when BMDM are treated with NAC (Figure 4A). Moreover, IL6 expression is also increased when conditioned media is boiled and reduced when BMDM are treated with NAC (Figure 4B). This suggests that the factor produced by cancer cells that is

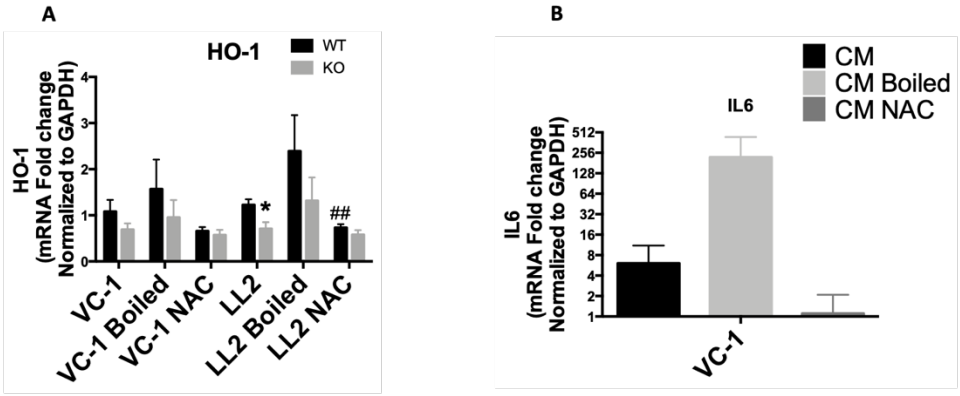

Figure 4: Boiled conditioned media from lung cancer cells increases expression of HO-1 and IL6 in BMDM. (A) Levels of HO-1 in wild type (WT) and NRF2 KO (KO) bone marrow derived macrophages stimulated with inflammatory cytokines or conditioned media from two murine lung cancer cells, for 48 hours. Conditioned media was boiled (15 minutes) or treated with antioxidant NAC (1M), n=6. (B) Levels of IL6 in wild type (WT) bone marrow derived macrophages stimulated with inflammatory cytokines or conditioned media from VC-1 murine lung cancer cells, for 48 hours (n=2). Conditioned media was boiled (15 minutes) or treated with antioxidant NAC (1M), n=2. * p=0.022, LL2 WT vs LL2 KO; ## P=0.0076 LL2 WT vs LL2 NAC.

responsible for HO-1 elevation is likely to upregulate IL6 as well. This observation is in accordance with the literature that has shown that IL6 can be in part regulated by NRF2.

Subtask 6: Isolate CD11b positive cells from lungs of A/J WT and NRF2 mice after stimulation with vinyl carbamate. Lungs will be collected every week for 8 weeks. CD11b cells will be characterized by RT-PCR and/or western blot.

To determine the evolution of HO-1 expression in macrophages in the lung after tumorigenesis initiation with vinyl carbamate, lung of A/J mice were collected at 1, 2, 4, and 8 weeks after the last vinyl carbamate injection. Vinyl carbamate is a carcinogenic compound that will lead to the formation of lung adenocarcinomas in A/J mice approximately 12 weeks after administration. Typically, 2 injections are given to 7–8-week-old A/J mice with 1 week of interval. Saline injections were done as controls. Mice were sacrificed and lungs were collected 1, 2, 4, and 8 weeks after the last vinyl carbamate injection. The right lobe was store in buffed formalin for further analysis by IHC. The remain lobes were lysed using a mixture of collagenase, dispase and DNAse, to obtain a single cell suspension. Cells were counted to normalize to the recommended concentrations before incubating with the respective reagents for magnetic isolation of CD11 and CD3 cells. CD11 and CD3 cells isolated from each mouse (4 females and 4 males per group) were tested for HO-1 levels by RT-PCR. Levels of HO-1 were similar in vinyl carbamate and saline stimulated mice in CD11 cells. In CD3 cells HO-1 levels were elevated compared to saline on weeks 2 and 4 after stimulation. To compare the evolution of the HO-1 levels over time, all CD11 and CD3 samples from mice stimulated with vinyl carbamate, levels were compared with cells isolated at week 1. Both CD11 and CD3 cells show an increase in the levels at week 2 and decreases at weeks 4 and 8 after vinyl carbamate stimulation (Figure 5). To determine if HO-1 elevation is preceded by NRF2 increase, NRF2 levels were determined (Figure 6A). Additionally, based on the BMDM experiments, levels

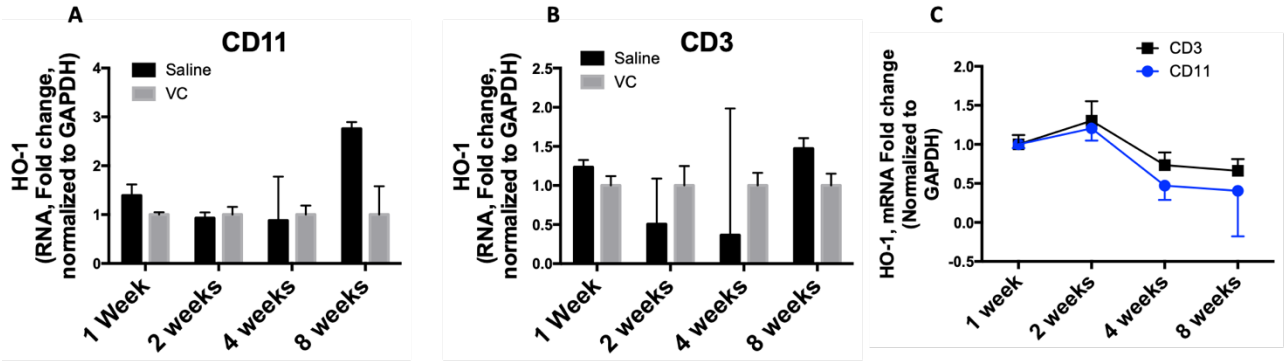

Figure 5: Vinyl carbamate stimulation increases the expression of HO-1 in CD11 and CD3 cells. HO-1 levels on CD11 and CD3 cells isolated from lungs of A/J mice after vinyl carbamate stimulation. 7-8 weeks old A/J mice were injected with vinyl carbamate or saline on days 1 and 7. Lungs were collected and digested to obtain a single cell suspension. CD3 and CD11 cells were isolated by magnetic cell sorting. HO-1 levels in CD11 positive (A) or CD3 positive (B) cells from lungs stimulated with saline or vinyl carbamate (VC) after 1, 2, 4 and 8 weeks after the last vinyl carbamate injection. All values are normalized to the respective saline. (C) HO-1 mRNA levels over time in CD11 and CD3 positive cells, week 1 was assumed as 1. (n=4, females)

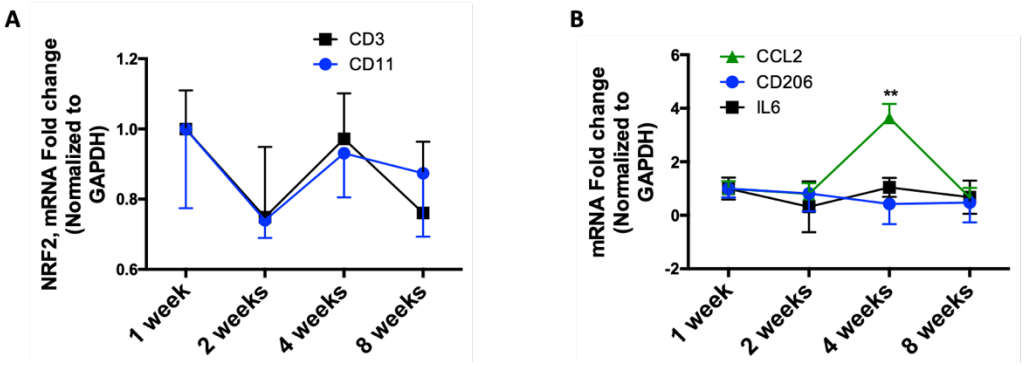

Figure 6: Vinyl carbamate stimulation increases the expression of NRF2 in CD11 and CD3 cells, and CCL2 in CD11b cells. NRF2 levels on CD11 and CD3 cells isolated from lungs of A/J mice after vinyl carbamate stimulation. 7-8 weeks old A/J mice were injected with vinyl carbamate or saline on days 1 and 7. Lungs were collected and digested to obtain a single cell suspension. CD3 and CD11 cells were isolated by magnetic cell sorting. (A) NRF2 levels in CD11 positive or CD3 positive cells from lungs stimulated with saline or vinyl carbamate (VC) after 1, 2, 4 and 8 weeks after the last vinyl carbamate injection. All values are normalized to the respective saline. (B) CCL2, CD206 and IL6 mRNA levels over time in CD11 positive cells, week 1 was assumed as 1. **p<0.01 CCL2 at 4 weeks compared with the other time points. (n=4, females)

of IL6, CCL2 and CD206 were analyzed (Figure 6B). NRF2 higher point is at one week after the last vinyl carbamate injection, followed by HO-1 at 2 weeks and CCL2 at 4 weeks.

Male mice were enrolled and collected RT-PCR is currently being performed. Additionally, 2 extra groups were added to test if the elevation of HO-1 occurs rapidly after the first vinyl carbamate injection, since no significant increase was observed one weeks after the second vinyl carbamate injection. Mice were sacrificed and CD11b and CD3 cells were collected from the lungs as described above, 72hours and 1 week after the first vinyl carbamate injection. RT-PCR is currently being performed to analyze levels of HO-1 and other activation markers for CD11b and CD3 cells.

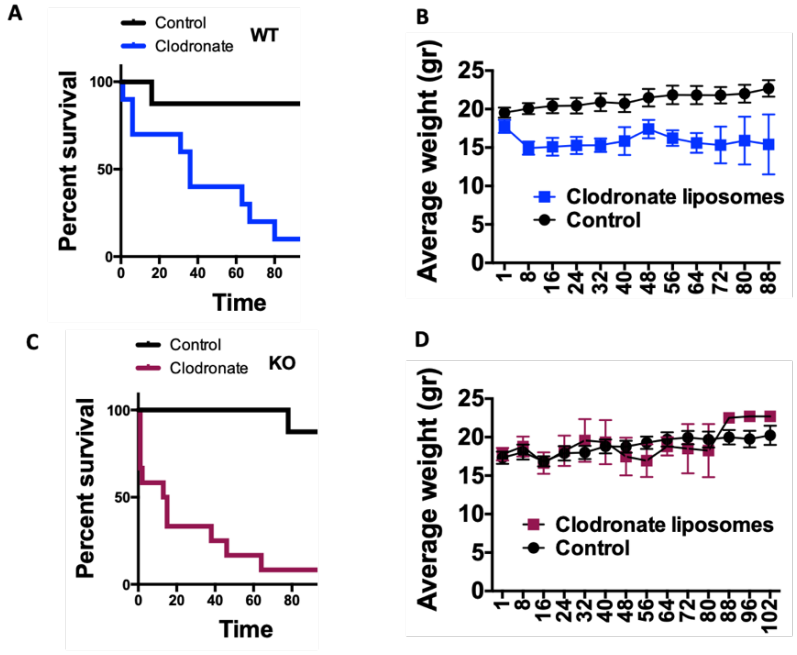

Figure 7: Survival of A/J wild type (WT) (A) and NRF2 KO (KO) (C) mice when injected with clodronate liposomes. 7-8 weeks old A/J mice were injected with clodronate liposomes or control liposomes on day 1 followed by vinyl carbamate on day 2. Vinyl carbamate was again injected on day 7 and clodronate liposomes or control liposomes were injected twice weekly until the end of the study (12 weeks). Average weight over time for WT (B) and KO mice (D). N=8-12.

Major Task 1C: Assess the effects of macrophage depletion in lung cancer progression in A/J mice

To determine if macrophages with HO-1 upregulation are important players in lung carcinogenesis and lung cancer progression, clodronate liposomes were used to deplete macrophage population. A single clodronate liposome ip injection was administered before stimulation with vinyl carbamate. Clodronate liposome administration is continued until the end point (12 weeks), with 2 doses a week. Vinyl carbamate is administered twice with 1 week interval. Control/empty

liposomes are used as control.
Unexpectedly, the combination of vinyl carbamate and clodronate liposomes was lethal in both wild type and NRF2 KO mice. On average, wild type mice receiving both clodronate and vinyl carbamate survive for 36 days and KO NRF2 mice survive 14 days after the first injection of clodronate liposomes (Figure 4). Only one mouse on each cohort survives until the end point of 12 weeks after the first clodronate liposome injection. Macrophages were successfully depleted in the two surviving mice. Wild type mice receiving clodronate liposomes show a minor weight loss in the first week that is not recovered over time. NRF2 KO mice do not show a difference between the mice receiving clodronate liposomes and mice receiving control liposomes.
To overcome the lethality observed mice will be given one initial dose of clodronate liposomes on day one and a dose of vinyl carbamate on day 2. Mice will be allowed to recover for 1 week and the clodronate liposome administration will be reestablished. A single dose of vinyl carbamate will be used, since is sufficient for tumor induction. Mice will be given additional nutritional support in the form of gel food, to increase food and water intake.
After action was taken to reduce the mortality of mice receiving clodronate liposomes, with reduce dosages and administration, mortality was still observed. This led to the conclusion that these mice are increased susceptible to complete reduction of the myeloid population. Four mice receiving clodronate liposomes survived until the end of the study and the lungs were collected for IHC, flow cytometry and flash frozen. Flow cytometry analysis (Figure 8) showed significant increases in CD4 and CD8 T cells as well as their activation (CD69). Myeloid cell populations showed an increase in some activation markers in some populations. Inflammatory monocytes (iMono) showed an increase in the expression of IA-IE (MHC-II) and PDL1 expression. Eosinophils (Eosiph) also showed and increased expression of IA-IE and PDL1. Histologically the lungs of mice receiving clodronate liposomes are extremely different from the mice receiving control liposomes, blinded evaluation is being performed by a pathologist.

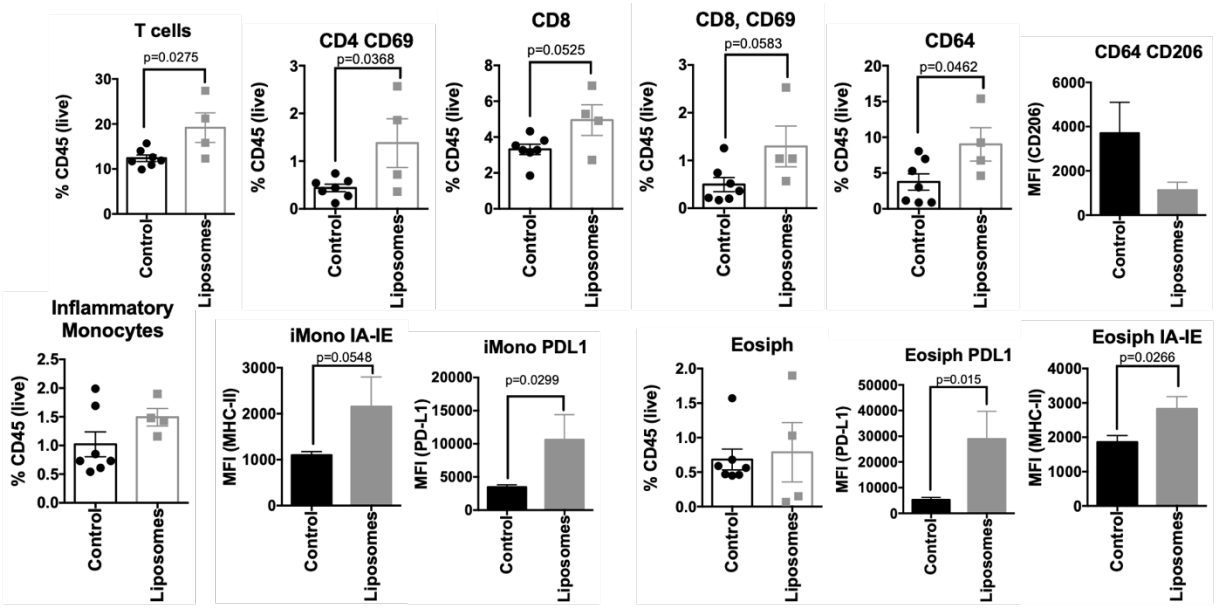

Figure 8: Immune cell changes in A/J wild type (WT) mice injected with clodronate liposomes analyzed by flow cytometry. 7-8 weeks old A/J mice were injected with clodronate liposomes or control liposomes on day 1 followed by vinyl carbamate on day 2. Vinyl carbamate was again injected on day 7 and clodronate liposomes or control liposomes were injected twice weekly until the end of the study (12 weeks). Flow cytometry was performed in the 2 right lobes of the lung.

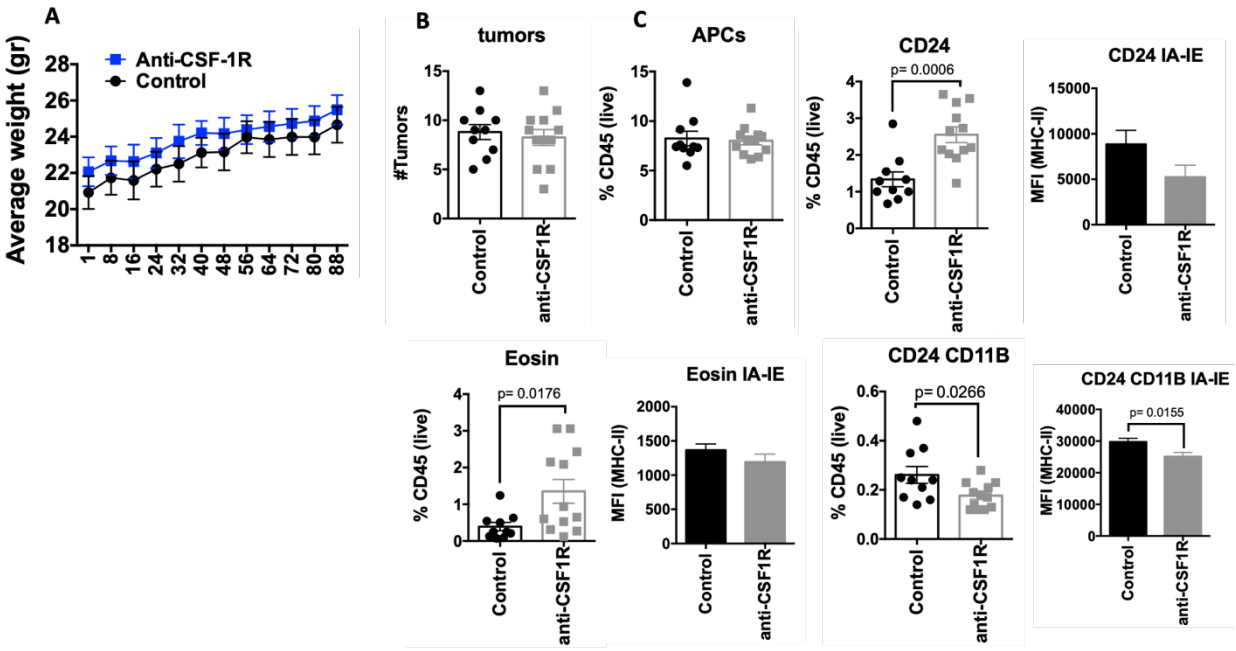

Figure 9: Weight, tumor count and flow cytometric changes in dendritic cells (CD24 and CD24, CD11b) and eosinophils (eosin) . 7-8 weeks old A/J mice were injected with vinyl carbamate on day 1 and day 7. Anti-CSF-1R was started 6 weeks after the first vinyl carbamate injection. Anti-CSF-1R and isotype control were injected ip 3 times a week for 6 weeks (300ug/mouse). (A) Average weight over time for mice receiving either control isotype or anti-CSF-1R. (B) Number of tumors on sections of H&E sections. (C) Percentages of antigen presenting cells, dendritic cells (CD24 and CD24, CD11b) and eosinophils and activation marker IA-IE (MHC-II). N=10-12.

Due to the mortality and toxicity of clodronate liposomes, a different approach as used to reduce activation of myeloid cells instead of depletion. For this anti-CSF1R was used. A/J mice were stimulated with vinyl carbamate, and anti-CSF1R was started 6 weeks after the first vinyl carbamate injection. Anti-CSF1R or the isotype were administered i.p. at 300ug/mouse 3 times a week, for 6 weeks. No change on weight was observed during administration of anti-CSF-1R (Figure 9A). Lungs were collected at 12 weeks after the first vinyl carbamate injection. Lungs were collected for flow cytometry, histology and flash frozen. No changes in the number of total tumors (Figure 9B) on histology was observed, however, grading and size are still to be evaluated. Flow cytometry (Figure 9C) showed an increased in CD24 expressing antigen presenting cells, and in eosinophils (Eosin), but no increase in the expression of activation

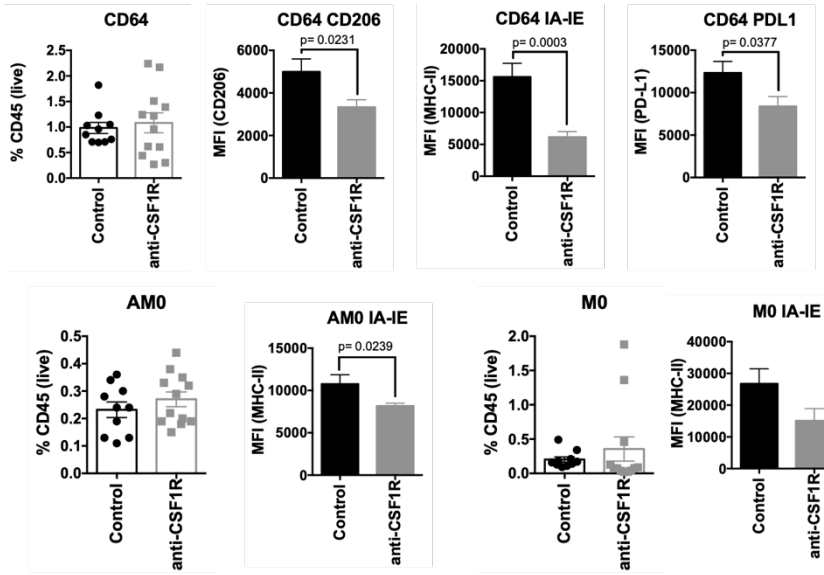

Figure 10: Reduction of activation markers in lung macrophages from mice treated with anti-CSF-1R. Mice were treated was in figure 9 and flow cytometry was done as described. CD64 represents the general antigen presenting macrophage population, AM0 the alveolar macrophages (CD64, CD11c) and M0 the interstitial macrophages (CD64, CD11b). N=8-12.

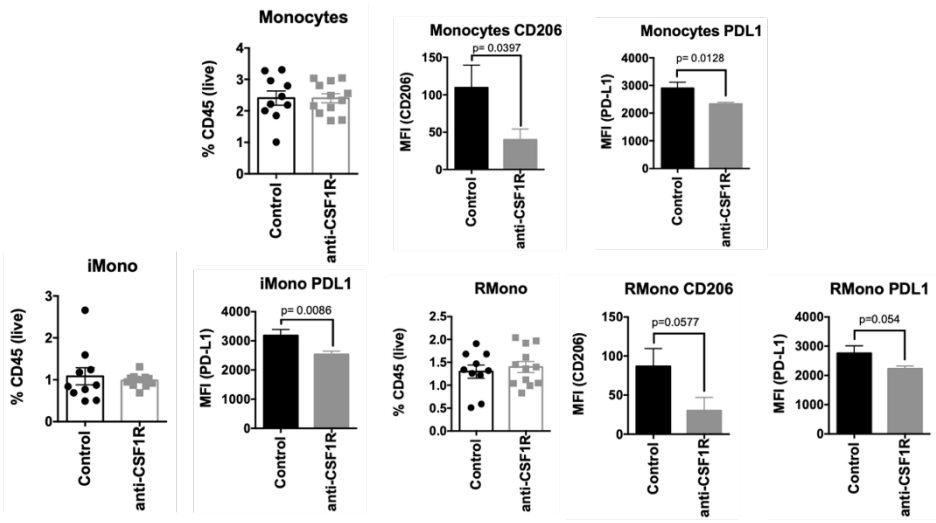

Figure 11: Reduction of activation markers in lung monocytes from mice treated with anti-CSF-1R. Mice were treated was in figure 9 and flow cytometry was done as described. Monocytes represents the general IA-IE negative, CD11b positive population. iMono represent infiltrating/inflammatory monocytes (Gr1 positive) and RMono represent resident monocytes (CD11c positive). N=8-12.

markers, such as IA-IE (MHC-II), moreover, CD24, CD11B cells were significantly reduced, as well the expression of IA-IE in this subpopulation in lungs receiving anti-CSF-1R.

The general macrophage population (CD64) did not show a change in the infiltrating numbers (Figure 10), as expected, once anti-CSF-1R targets a cytokine receptor. However, as expected, CD64 positive population shows a decrease in all the analyzed markers of activity (CD206, IA-IE, PDL1) (Figure 10). The two subpopulations of CD64, alveolar macrophages (AM0) and interstitial macrophages (M0) don't show change in the number of infiltrated cells between control and anti-CSF1R but show a decrease of expression of IA-IE (MHC-II).

The monocyte population also reduced expression of CD206 and PDL1 in the lung of mice treated with anti-CSF-1R (Figure 11). With the monocyte population, both subpopulations of inflammatory monocytes (iMono) and resident monocytes (rMono), a reduction in the expression of PDL1 was observed (Figure 11).

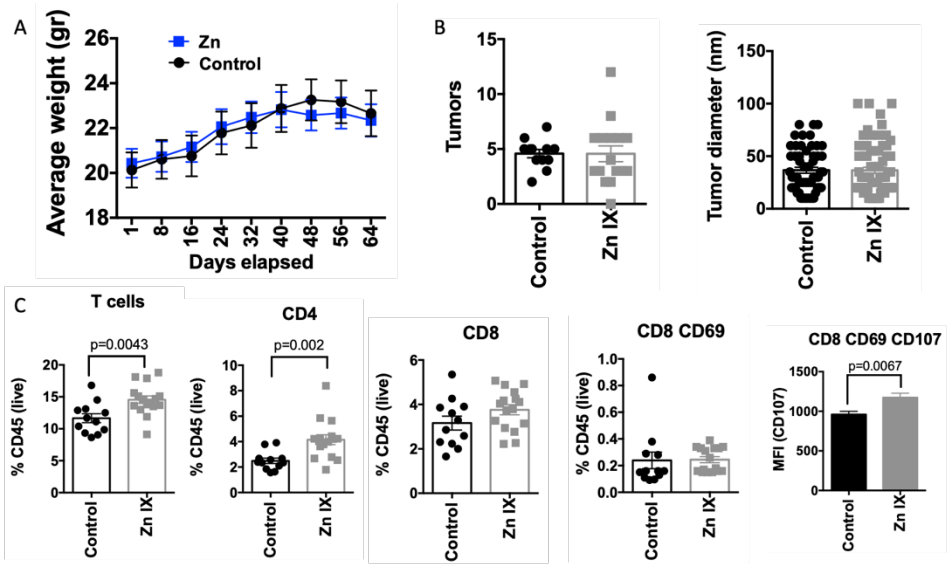

Figure 12: Weight, tumor count and diameter and flow cytometric changes in T cells of mice treated with zinc (II) propoporphyrin IX (Zn IX). 7-8 weeks old A/J mice were injected with vinyl carbamate on day 1 and day 7. Zinc (II) propoporphyrin IX was started 6 weeks after the first vinyl carbamate injection. Zinc (II) propoporphyrin IX was injected ip every 2 days for 2 weeks at 50 mg/Kg, control mice received a DMSO control solution. (A) Average weight over time for mice receiving either control DMSO or zinc (II) propoporphyrin IX. (B) Number of tumors and approximate size on sections of H&E sections. (C) T cells percentages and activation (CD69 and CD107) measured by flow cytometry. N=12-16.

Within subtask 1C it was also proposed to treat A/J mice with zinc (II) propoporphyrin IX after vinyl carbamate stimulation. Zinc (II) propoporphyrin IX is an HO-1 inhibitor, that

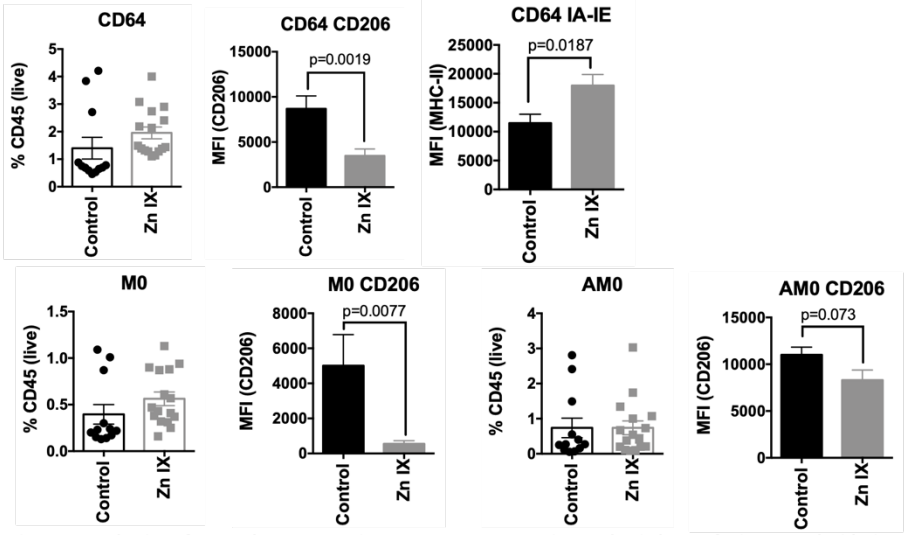

Figure 13: Reduction of macrophages expressing CD206 tumor promoting marker in lungs of mice treated with zinc (II) propoporphyrin IX (ZN IX) . A/J mice were stimulated with vinyl carbamate and treated with zinc (II) propoporphyrin IX has described in figure 12. CD64 represents the general antigen presenting macrophage population, AM0 the alveolar macrophages (CD64, CD11c) and M0 the interstitial macrophages (CD64, CD11b). N=12-15.

has shown anti-tumor and immune modulatory effects previously. A/J mice after stimulation with vinyl carbamate, were treated with zinc (II) propoporphyrin IX for 2 weeks, every 2 days at 50mg/Kg. Zinc (II) propoporphyrin IX treatment had no influence on mice weight (Figure 12A). At the end of the study lungs were collected and used for flow cytometry evaluation of immune cells, flash frozen for later assessment of cytokines and immune markers by RT-PCR and preserved in 10% buffered formalin for histology. Histological assessment of number of tumors and size did not show a difference between mice treated with control-DMSO or zinc (II) propoporphyrin IX. This is likely due to the short time of treatment and the fat that the mice were sacrificed only 8 weeks after the first vinyl carbamate injection (Figure 12B).

Flow cytometry of lungs of mice treated with zinc (II) propoporphyrin IX showed and increase in T cell infiltration to the lungs, more specifically and increase in CD4 T cells and increase in the activation of CD8 T cells (CD107) (Figure 12C). The number of macrophages infiltrating the lungs did not change between mice treated with control-DMSO or zinc (II) propoporphyrin IX, however, significant reductions in the tumor promoting marker CD206 was observed in all macrophages (CDC64) and in interstitial macrophages (M0) and alveolar macrophages (AM0) (Figure 13). Moreover, the same macrophage populations showed and increased expression of IA-IE (MHC-II), an anti-tumor related marker in macrophages. The monocyte population showed and increased infiltration in inflammatory monocytes (iMono), and a decrease expression of CD206 is all populations, as observed for macrophages (Figure 14). Moreover, in general monocytes showed an increased expression of PDL1 and IA-IE, markers associated with an anti-tumor phenotype (Figure 14).

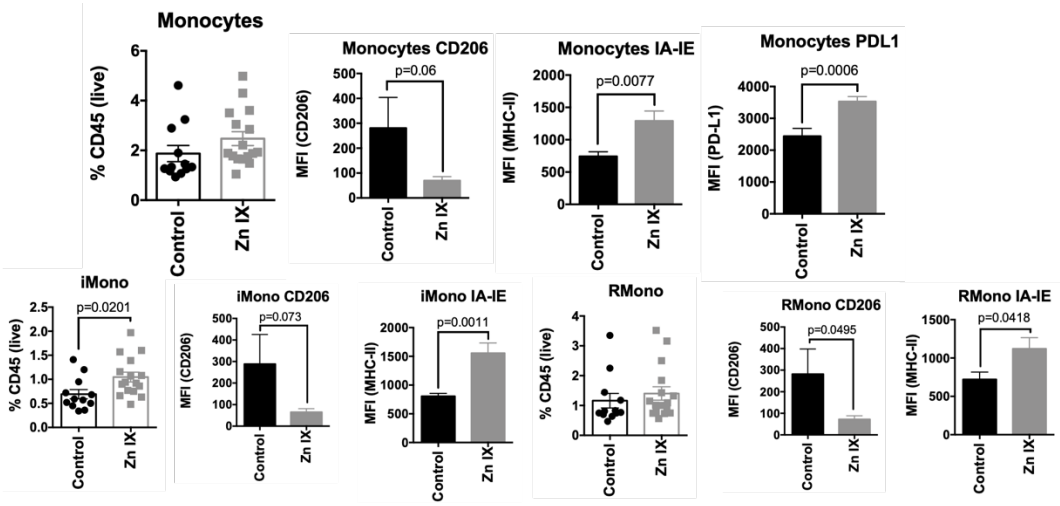

Figure 14: Reduction of tumor promoting marker CD206 in lung monocytes from mice treated with zinc (II) propoporphyrin IX. Mice were treated was in figure 12 and flow cytometry was done as described. Monocytes represents the general IA-IE negative, CD11b positive population. iMono represent infiltrating/inflammatory monocytes (Gr1 positive) and RMono represent resident monocytes (CD11c positive). N=12-16.

Major Task 2A: Evaluation of in vitro T cell activation by NRF2 wild type and KO macrophages

Subtask 1: Establish the co-culture protocol for NRF2 wild type and NRF2 KO BMDM with wild type T cells

These co-culture systems were established, and cells collected. RT-PCR and flow cytometry will be run.

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to Report

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

Only tasks remain are the RT-PCR analysis of all mice collected, tumor burden analysis, and IHC to confirm the findings of flow cytometry.
Major Task 1E: Evaluate the effects of NRF2 KO macrophages chimeras on lung cancer progression and Major Task 2B: In vivo evaluation of T cell activation/exhaustion in NRF2 wild type mice and macrophage KO chimeras. N=48 (24 WT, 24 NRF2 chimeras), 6/group/time point will be performed at Indiana University cancer center within their specialized working group (https://cancer.iu.edu/research/shared-facilities/pmtc/ivt.html). These tasks will be accomplished during the no -coast exemption.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Nothing to Report

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report

5. CHANGES/PROBLEMS: *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Toxicity in the combination of vinyl carbamate and clodronate liposomes: reduce the 2 doses of vinyl carbamate to 1 dose; and allow mice time to recover before administering clodronate liposomes after vinyl carbamate. Gel food is now provided to increase mice nutritional uptake. Depletion was repeated using anti-CSF-1R.

Reduced breading ability in A/J NRF2 KO mice: increase breading

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Difficulties and maintaining student worker in the summer: Most of mouse colony work is done by students, however, students are rarely available in the summer months (May to September). This reduces the ability to process samples and enroll mice necessary for the objectives.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Due to mortality of animals receiving both clodronate liposomes and vinyl carbamate, nutritional support is now provided in the for of gel food. Gel food allow the animal to have access to enriched nutrients and water.

Significant changes in use of biohazards and/or select agents

Nothing to report

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to report

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the

Nothing to report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*

Researcher Identifier (e.g. ORCID ID): *1234567*
Nearest person month worked: *5*

Contribution to Project: *Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support: *The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name: *Ashley Rowell*
Project Role: *Student Worker*
Researcher Identifier (e.g. ORCID ID): *n/a*
Nearest person month worked: *3*

Name: *Lizbeth Lockwood*
Project Role: *Technician*
Researcher Identifier (e.g. ORCID ID): *n/a*
Nearest person month worked: *1*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
<u>Organization Name:</u>
<u>Location of Organization: (if foreign location list country)</u>
<u>Partner's contribution to the project</u> (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ebrap.org/eBRAP/public/index.htm*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil/Pages/Resources.aspx) should be updated and submitted with attachments.*

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.