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CHRONIC INTERMITTENT HYPOXIA EXPOSURE PROMOTES MULTIPLE MYELOMA DEVELOPMENT
IN BLACK/6 MICE

by

Sandeep Kowkuntla

A thesis submitted in partial fulfillment of the requirements
for graduation with Honors in the Health and Human Physiology

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Thesis Mentor

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All requirements for graduation with Honors in the
Health and Human Physiology have been completed.

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Title:

Chronic Intermittent Hypoxia Exposure Promotes Multiple Myeloma Development in Black/6 Mice

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ABSTRACT

Multiple myeloma (MM) is an incurable malignancy of bone marrow plasma B cells and is associated with obesity. The mechanisms by which obesity contributes to MM risk remains unclear, however, we speculate the linking factor to be sleep apnea which we simulated by chronic intermittent hypoxia (CIH). 5TGM1 cells are a murine MM cell line derived from the KaLwRij strain that fail to routinely engraft in Black6 mice. To test the hypothesis that chronic intermittent hypoxia (CIH) promotes the development of MM, we exposed MM-resistant C57BL/6 mice to 10h/day of CIH during the light cycle (AHI=10-12/h) for 7 days, or static 21% oxygen as a control. On day 7, mice were injected with 1×10^6 GFP-labelled malignant mouse plasma cells (5TGM1) and the CIH was continued for an additional 28 days. 67% of the CIH mice developed MM, evidenced by paralysis, bone lesions in the spine, and plasmacytomas in the femur. It was also tested if the CIH effected the cells intrinsically. We hypothesized that CIH would promote the proliferation of 5TGM1 cells in culture. Two groups of 5TGM1 cells were plated in 18 gas permeable wells at a concentration of 500,000 cells/mL. One plate was placed in a plexiglass environmental control cabinet and the other plate was placed in a normoxia incubator. The incubator was maintained at 21% O₂ as a control. The environmental control cabinet, however, was maintained at an O₂ level that decreased to 10% for 90s, every five minutes, for 10 hours per day. The control plate showed normal signs of proliferation. On the other hand, the plate maintained in CIH conditions showed rapid cell death as early as Day 2 of the experiment. These results suggest that the CIH conditions do not cause growth and proliferation of the 5TGM1 cell line. Therefore, we speculate that CIH may exert its MM-promoting effects on the bone marrow stromal environment and not directly on the tumor. Future work in our laboratory will investigate the role of CIH in transforming the bone marrow microenvironment to allow for tumor cell engraftment.

Multiple Myeloma is a terminal cancer of plasma cells with a well described pre-malignant stage known as MGUS. In 2017 alone there were 30,280 new cases of MM with a death toll of 12,590 people (American Cancer Society). In the bone marrow of MM patients the plasma cells proliferate in a cancerous manner and overcrowd the healthy cells. This leads to one of the defining characteristics of MM-anemia. MM patients are known to have some telltale signs that are known by the acronym CRAB. CRAB relays that these individuals typically have hyperCalcemia, Renal insufficiency, Anemia and Bone lesions. MM is more common in African Americans and individuals with first degree relatives with MM, showing a heritability component of MM. There have been improvements in the treatment for MM over the years; however, the condition is still incurable and individuals relapse. Prior to developing MM, individuals acquire monoclonal gammopathy of undetermined significance (MGUS). MGUS is an asymptomatic precursor state to MM and has a cumulative progression rate to MM of 1-2% per year. The mechanisms that advance MGUS to MM are still unknown, but understanding these mechanisms can potentially help prevent the progression of individuals from MGUS to MM.

Multiple Myeloma associated genetic mutations are insufficient to induce MM in mice. The genetic mutations in the human genome, point mutations, which are commonly associated with multiple myeloma are activating mutations in K-Ras and N-Ras (Chng et al., 2008; Lionetti et al., 2015). It was seen in multiple mouse models that activation of K-Ras was insufficient in producing MM in these mice. These mice were then crossed with genetically tumor prone mice strain, KaLwRij, and were still not seen to progress into MM. This suggest that the genetic component of MM alone is not sufficient in being the factor for the progression from MGUS to MM. Our overarching hypothesis is that CIH increases oxidative stress, thereby accelerating B cell maturation and changing the bone marrow stromal microenvironment to support the progression of MM in the context of genetic mutations. This project will establish the role of the environmental stress CIH in cooperating with genetic markers in MM pathogenesis and the mechanisms of that cooperation, which we hypothesize are mediated by reactive oxygen species effect on the bone marrow microenvironment.

Sleep apnea maybe culprit for the reason obese individuals have a higher risk of MM. Obesity is a morbidity that is on the rise around the world and has been correlated with the increased risk of many types of cancer including MM. If obesity rates stay consistent, by 2030, 51 percent of America's population will be obese by 2030. The physiology behind why obesity increases the chance of onset of MM is not fully understood. Similar to the genetic component, high fat diet alone again is insufficient from the development of MM in mice (Lwin et al., 2015). This led us to believe a comorbidity of obesity may need to be present in order to induce the higher risk of onset of MM. Nearly 45% of obese individuals have a complication known as sleep apnea (Young et al., 1993). Sleep apnea is when an individual has one or more pauses in breathing or shallow breaths while he or she sleeps. Breathing pauses can last from a few seconds to minutes. They may occur 30 times or more an hour (www.nhlbi.nih.gov). There is a strong correlation between severity of sleep apnea and cancer death that shows higher the severity of sleep apnea is correlated with higher frequency of death due to cancer (Miller et al., 2013; Nieto et al., 2012; Peppard and Nieto, 2013). MM and sleep apnea even have similar risk factors such as age, male sex and African American race. This leads to the hypothesis of the first experiment: if black6mice are subjected to chronic intermittent hypoxia (CIH), then the 5TGM1 cells will engraft and proliferate. CIH is the protocol used to simulate sleep apnea in the mice. If

CIH induced intrinsic effects in the MM cell line, 5TGM1, was also tested in Experiment 2. The hypothesis for Experiment 2 was as follows: if 5TGM1 cells are subjected to chronic intermittent hypoxia, then the cells will proliferate in culture.

METHODS

Experiment 1: Engraftment and Progression of Multiple Myeloma Cells in Vivo

I. Animal Model.

C57BL/6 mice and C57BL/KaLwRij were the two strains of mice we used in our experiments. All mice used in the experiment were 8 weeks olds and females. The C57BL/6 mice used in the following experiments were purchased from Jackson Laboratory in Bar Harbor, Maine. The C57BL/KaLwRij mice were bred in the animal facility at the University of Iowa in Iowa City, Iowa. All mice were housed in the same conditions: five mice per cage with food and water available at all times and with a 12 hour light cycle during day time and 12 dark cycle during nighttime. The University of Iowa Institutional Animal Care and Use Committee approved all studies involving these animals.

II. Malignant Plasma Cell Line.

5TGM1 cells are murine myeloma cells that were derived from multiple myeloma in C57BL/KaLwRij mice. Multiple myeloma occurs spontaneously in aged C57BL/KaLwRij mice with no external stimulus such as CIH being required. The 5TGM1 cells used in our experiments were genetically engineered to express green fluorescent protein (GFP). The cells were cultured in RPMI 1640 medium (ATCC modification) (GIBCO, Grand Island, New York, USA) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, Utah) and 1% penicillin-streptomycin solution (Mediatech, Manassas, Virginia) at 37°C in 5% CO₂ atmosphere.

III. Chronic Intermittent Hypoxia (CIH) Exposure

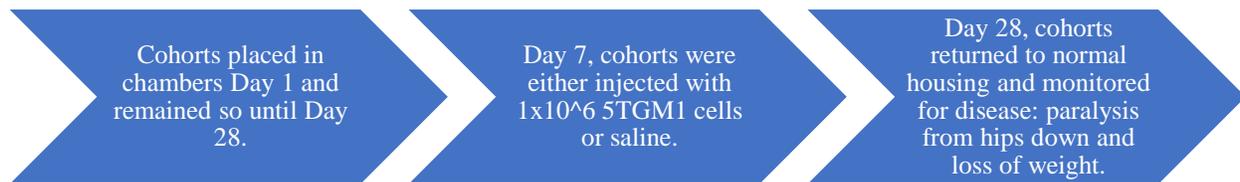
The mice experienced CIH in two plexiglass exposure chambers bought from Coy Laboratories in Grass Lake, Michigan. The CIH protocol was as follows: 10 hours of CIH during the light cycle due to the fact mice sleep during the light cycle, beginning of each cycle nitrogen was flushed into the chamber until 10% oxygen was reached within 30 seconds, 10% oxygen was maintained inside of the chamber for an additional 60 seconds, lastly oxygen is flushed into the chamber until a concentration of 21% O₂ is reached and this is maintained until the end of the 5 minute cycle. The CIH cycles are repeated for a duration of 10 hours or 120 cycles in total during the light cycle of the day. This CIH protocol produces a saturation profile that is equivalent to moderately severe obstructive sleep apnea in humans which is about 15 events per hour and an SpO₂ = 75%.



IV. Cohorts

- i. CIH with 5TGM1, n=11
- ii. CIH with saline, n= 5
- iii. Normoxia with 5TGM1, n=8
- iv. Normoxia with saline, n=11
- v. Static hypoxia with 5TGM1, n=11
- vi. Static hypoxia with saline, n= 4
- vii. KaLwRij with 5TGM1, n=10
- viii. KalwRij with saline, n=5.

V. Experimental Design



The mice were preconditioned for 7 days with either CIH, static hypoxia or normoxia in their environmental control chambers and were injected with 1×10^6 5TGM1 cells or saline intravenously through the tail on Day 7. The mice continued to experience their set condition until Day 28, when all mice were returned to normal housing. After returning the mice to their normal housing, they were monitored daily for disease symptoms such as paralysis from hips down, loss of weight and spinal deformities. The 5TGM1 cells were engineered to express green fluorescent protein in order to visualize the cells in sections of bone and spleen were used for imaging and flow cytometry.

Flow Cytometry: One Femur and one Tibia as well as half of the spleen from the euthanized mouse were used for flow cytometry. The bone marrow was flushed with PBS using a needle and syringe from the femur and the tibia. The half spleen was minced between two cover slides. Cells from bone marrow and spleen in PBS were transferred to two 50ml conical centrifuge tube (one for each) passing through disposable $40\mu\text{m}$ cell strainer. The cell suspension was centrifuged (5000rpm, 5 min), re-suspended in 10ml of ACK lysing buffer (M.K Medical, Columbia, Maryland), centrifuged and then washed with PBS. Finally, Samples were run and analyzed using Becton Dickinson LSR II (BD Biosciences, San Jose, CA).

Histology: The other femur and tibia were first decalcified by incubating it in EDTA for 2 weeks. The bones as well as the other half of the spleen were fixed and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E). Olympus BX-61 light microscope was used to examine the slides and obtain images.

3D X-ray: The tail and spine were imaged using a submicron 3D X-ray microscope; Zeiss Xradia 520 Versa. The samples were scanned at 70 kV/6 watts, a full 360° rotation over 1601 projections, one second exposure time. The projections were reconstruction into a single 3d image (CT) and reconstruction pixel size is 38 microns using ORS Visual software. Images were blindly reviewed by an independent radiologist. ImageJ software and BoneJ plugin¹⁹ was used to convert binary image into 3D mesh for quantification of bone lesions²⁰ as well as to create the local thickness map and calculate trabecular bone thickness using 3D ROI manager.

VI. *Statistics*

Odds ratio was used for the statistical analysis of Experiment 1.

Cohorts	Odds Ratio (Confidence Interval)	p-value
Normoxia with 5TGM1	1.0	
Static Hypoxia with 5TGM1	5.7 (0.5-62.7)	0.117
CIH with 5TGM1	17.5 (1.6-192.3)	0.005

Experiment 2: Effect of CIH on 5TGM1 cells in Vitro

I. *Malignant Plasma Cell Line.*

5TGM1 cells are murine myeloma cells that were derived from multiple myeloma in C57BL/KaLwRij mice. Multiple myeloma occurs spontaneously in aged C57BL/KaLwRij mice with no external stimulus such as CIH being required. The 5TGM1 cells used in our experiments were genetically engineered to express green fluorescent protein (GFP). The cells were cultured in RPMI 1640 medium (ATCC modification) (GIBCO, Grand Island, New York, USA) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, Utah) and 1% penicillin-streptomycin solution (Mediatech, Manassas, Virginia) at 37°C in 5% CO₂ atmosphere.

II. *Chronic Intermittent Hypoxia (CIH) Exposure*

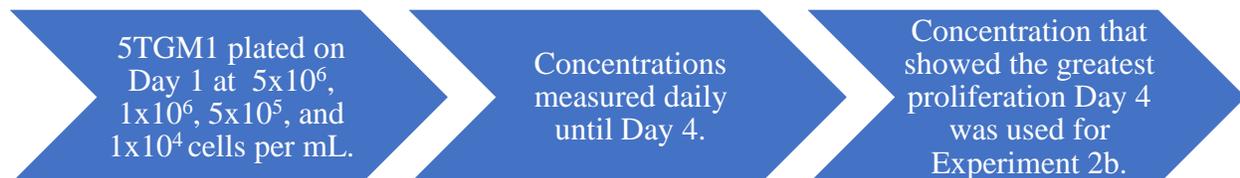
5TGM1 cells experienced CIH in a petri dish set inside of an environmental control chamber which was inside of an incubator set at normoxia with 5% CO₂. The CIH protocol was as follows: 10 hours of CIH during the light cycle due to the fact mice sleep during the light cycle, beginning of each cycle nitrogen was flushed into the chamber until 10% oxygen was reached within 30 seconds, 10% oxygen was maintained inside of the chamber for an additional 60 seconds, lastly oxygen is flushed into the chamber until a concentration of 21% O₂ is reached and this is maintained until the end of the 5 minute cycle. The CIH cycles are repeated for a duration of 10 hours or 120 cycles in total during the light cycle of the day. This CIH protocol produces a saturation profile that is equivalent to moderately severe obstructive sleep apnea in humans which is about 15 events per hour and an SpO₂ = 75%.

III. *Experiment 2A: Determining Optimal Growth Concentration*

a. Conditions

- i. 5x10⁶ per mL
- ii. 1x10⁶ per mL
- iii. 5x10⁵ per mL
- iv. 1x10⁴ per mL

b. Experimental Design



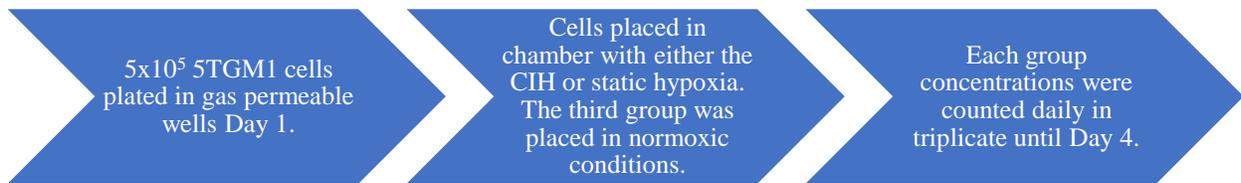
An experiment was done to determine the concentration of 5TGM1 cells that showed the greatest proliferation in a 4 day period. The different concentrations that were tested were the following: 5×10^6 , 1×10^6 , 5×10^5 , and 1×10^4 cells per mL. The cells were plated a 24 well plate with each concentration in a row of 6 wells. The concentrations were recorded daily in triplicate and the data was gathered using a hemocytometer.

IV. *Experiment 2B: Effect of CIH and Static Hypoxia in Vitro*

a. Conditions

- i. Normoxia (Static)
- ii. Chronic Intermittent Hypoxia
- iii. Static Hypoxia

b. Experimental Design



Three groups of 5TGM1 cells were plated in separate gas permeable plates on Day 1. The plate that experienced normoxia was placed in an incubator while the two other groups were placed in environmental control chambers that experienced their assigned conditions. The concentrations of each group were recorded daily in triplicate using a hemocytometer.

V. *Statistics*

Area under the curve was used for statistical analysis for Experiment 2.

Conditions	Significant	Adjusted p-value
Normoxia vs. CIH	Yes	0.0003
Normoxia vs. Static Hypoxia	Yes	<0.0001

RESULTS

Experiment 1: Engraftment and Progression of Multiple Myeloma Cells in Vivo with CIH or Static Hypoxia

The survival curve (Figure 1) shows the results of the different cohorts. None of the mice exposed to static hypoxia or CIH injected with saline died within the observation period. One mouse exposed to normoxia and injected with saline died 45 days after injection, but did not appear to have suffered weight loss or paralysis that is consistent with the 5TGM1 model. The positive control, KalwRij mice injected with 5TGM1 cells (n=10), all developed paralysis and were euthanized. The C57BL/6 mice injected with 5TGM1 multiple myeloma cells (n=8) showed resistance to development of multiple myeloma, with only one mouse developing terminal paralysis. However, 67% of the CIH exposed C57BL/6 mice and 37% of static hypoxia exposed C57BL/6 mice developed terminal paralysis. CIH exposed mice had significantly worse chance of survival compared to normoxic controls with an odds ratio of (17.5, 95% CI 1.6-192.3, p=0.005). On the other hand, static hypoxia's effect on the development of terminal paralysis was not statistically significant when compared to controls with an odds ratio of odds ratio (5.7, 95% CI 0.5-62.7, p=0.117).

Our 5TGM1 cell line expresses green fluorescent protein (GFP⁺) which allowed detection and quantification of the myeloma cell burden in both the bone marrow and spleen of mice with terminal paralysis using flow cytometry analysis (Figure 3). ***Immunoglobulin levels and distribution of GFP⁺ 5TGM1 cells in bone marrow and spleen of CIH-exposed mice were one of the indicators of multiple myeloma engraftment. Increased monoclonal immunoglobulin is also a hallmark of MM.*** Serum was drawn from the paralyzed CIH exposed and KalwRij mice and tested for monoclonal immunoglobulin production which is shown in (Figure 3). Consistent with previous findings in the model, serum IgG2b was elevated in paralyzed KalwRij mice. The multiple myeloma specific monoclonal immunoglobulin G (IgG2b) level was elevated in paralyzed CIH mice which confirms the proliferation of malignant monoclonal plasma cells in the bone marrow. Interestingly, IgG2b level in the paralyzed CIH mice was significantly lower than its level in our positive control Kalwrij mice (median IgG2b level in paralyzed CIH = 4.7 mg/ml vs 9.4 mg/ml in Kalwrij, p-value = 0.015). In the positive control, Kalwrij mice, GFP⁺ cells were highly abundant in the bone marrow and spleen. The cell content was about 50% GFP⁺ and the distribution between the two tissues was similar. Although the CIH exposed mice developed the same severity of paralysis, and appeared phenotypically like the Kalwrij mice, the tumor burden was significantly lower than in Kalwrij mice. There was also a tendency for cells to engraft preferentially in the bone marrow which is evident by the more abundant GFP⁺ cells seen in the bone marrow compared to the spleen (Figure 3). The engraftment of GFP⁺ cells is six times higher in the bone marrow than the spleen of CIH-exposed mice.

Histopathology and 3D X-ray of bone in CIH exposed mice. We used 3D X-ray to scan the lumbar and tail vertebral bones from the paralyzed mice of the CIH exposed cohort and normoxia control cohort. The images were then blindly reviewed by an independent investigator with radiology expertise. More lytic bone lesions were seen in the paralyzed CIH mice cohort compared to the normoxia control cohort (median number of lesions in paralyzed (CIH = 25 vs 9 in normal controls, p-value = 0.015). A similar trend was seen regarding bone thickness as well (median bone thickness in paralyzed CIH = 175 μ m vs 236 μ m in normal controls, p-value = 0.015). The bone marrow of mice in the normoxia control cohort and CIH cohort were made into

slides that were stained with hematoxylin and eosin and then sent for histopathological examination, which showed sheets of monotonous malignant plasma cells with eccentric nuclei and dark chromatin infiltrating the bone marrow cavity to different degrees in Kalwrij mice versus paralyzed CIH-exposed mice. In the case of Kalwrij mice, the bone marrow cavity was completely obliterated with sheets of malignant cells, while in the paralyzed CIH-exposed mice areas of scattered patches of malignant plasma cells were found that ranged from 0% to 90% of the total area of the bone marrow sections (Figure 4).

Experiment 2: Effect of CIH and Static Hypoxia on growth of 5TGM1 cells in culture

5TGM1 cells grown in 21% oxygen proliferated to double their original concentration over the 3 day period (5×10^5 vs $11.6 \pm 1.1 \times 10^5$ cells/mL, $p=0.002$). There was no change in the concentration of cells grown in CIH with an oxygen nadir of 10% at Day 3, while cell concentration decreased in 10% and 1.5% static hypoxia and CIH with an oxygen nadir of 1.5% ($0.5 \pm 0.4 \times 10^5$ cells/mL, $p=0.006$; $1.2 \pm 0.3 \times 10^5$ cells/mL, $p=0.006$; and $2.2 \pm 0.6 \times 10^5$ cells/mL, $p=0.04$, respectively) (Figure 5, Top). An evaluation of the area under the curve showed cell concentration for both CIH and both static hypoxia conditions were reduced compared to the normoxic control (Figure 5, Bottom).

DISCUSSION

Our study demonstrates that although CIH does not promote myeloma cell growth and proliferation in culture, in live animals CIH creates a hospitable environment permitting the engraftment and growth of malignant plasma cells in the bone marrow of otherwise myeloma resistant C57BL6 mice.

Clinical studies have shown an association between elevated body mass index (BMI) and risk of development of multiple myeloma. However, the underlying mechanism for this well documented link has not been fully understood. A study by Lwin et al. examined the effects of high fat diet on the development of MM in C57BL6 mice. Mice in their study were fed with high fat diet for 5 weeks and then injected with 5TGM1 MM cells. These mice only developed a MGUS-like condition as evidenced by elevated serum para-protein level which reverted to control levels on cessation of the high fat diet. Obstructive sleep apnea (OSA) is a condition that is closely associated with obesity. The risk factors for OSA are nearly identical to the risk factors for MM such as the elderly above 65 years old, men, and with high body mass index^{1, 12, 21}. Given these similarities between both conditions, we set to study the effect of CIH which is a characteristic of obstructive sleep apnea on MM development. The CIH protocol used in the experiments mimicked the hypoxia pattern seen in moderate OSA patients. When the MM resistant C57BL6 mice were exposed to CIH and then injected with 5TGM1 MM cells, these mice developed a phenotype of symptomatic MM like that observed in the Kalwrij mice. This phenotype is characterized by paralysis, bone lesions, decreased bone thickness, elevated monoclonal immunoglobulin IgG2b production and sheets of monotonous malignant plasma cells detected on the histological examination of H&E stained bone marrow slides and quantified using flow cytometry. Kalwrij is a mice strain that is susceptible to MM and that is why they served as the positive control and from which the 5TGM1 MM cells were originally derived⁶⁻⁸.

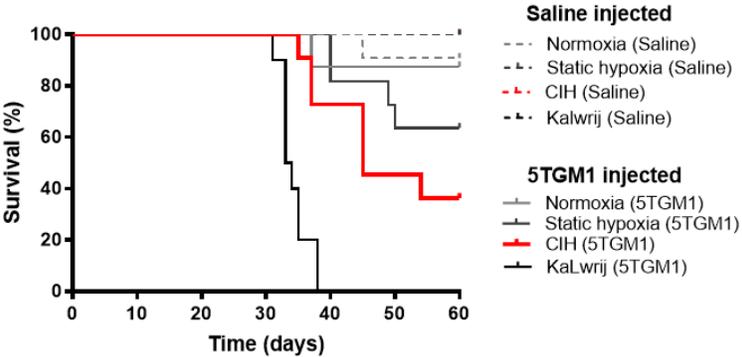
How might CIH change the bone marrow microenvironment to promote the engraftment of MM cells? In vitro CIH did not promote 5TGM1 cell proliferation; suggesting that the results we observed in experiment 1 were produced by CIH influencing the bone marrow microenvironment and/or altering the interaction of 5TGM1 cells with the host immune system. Tumor angiogenesis is hypothesized to be one of the mechanisms by which CIH promotes MM tumor development. A key transcriptional factor for several genes involved in angiogenesis in both normal physiological settings and in neoplastic settings²² is hypoxia-inducible factor (HIF). HIF upregulates the transcription of angiogenic growth factors like vascular endothelial growth factor (VEGF) leading to the proliferation, migration, assembly and lumen acquisition of endothelial cells and increased angiogenesis. Another mechanism that was hypothesized as a mechanism by which CIH promotes MM tumor development is that CIH alters the host immune response to cancer. It has been shown that intermittent hypoxia functionally changes tumor associated macrophages which in turn enhances tumor proliferation, migration, invasion, and extravasation properties²³. CIH was, also, shown to act as a pro-inflammatory stimulus because it induces interleukin (IL)-6 expression which is a pro-inflammatory cytokine involved in T-cell proliferation, B-cell differentiation, and monocyte maturation²⁴. A study by Dechow et al. shows IL-6's involvement in the pathogenesis of MM. In the study IL-6/IL-6 receptor activation starts downstream signaling process that induce a disease strongly resembling human MM²⁵. While our study establishes the link between CIH exposure and development of MM, further studies are needed to fully investigate and understand the underlying mechanisms of the linkage.

Why do CIH-exposed mice experience enhanced bone damage despite a lower tumor burden?

Quantification of both flow cytometry and ELISA monoclonal IgG2b showed that CIH exposed mice had significantly lower tumor burden compared to Kalwrij mice. However, the phenotype developed by both cohorts of mice strains were identical. This suggests that multiple myeloma cells in CIH exposed mice were more aggressive. Studies of solid tumors have shown that intermittent hypoxia increases the tumor’s aggressiveness, and metastatic properties^{13, 26}. A possible explanation can be that CIH selects for the most aggressive, apoptosis-resistant population of tumor cells. Interestingly, we also observed that myeloma cells preferentially populated the bone marrow with minimal number of myeloma cells detected in the spleen in the CIH exposed mice cohort. In the Kalwrij mice cohort, the myeloma cells equally populated the spleen and the bone marrow. Myeloma cells specific engraftment in the bone marrow may be caused by a special interaction between bone marrow stromal cells treated with CIH and the myeloma cells. The importance of this study is significant because if sleep apnea does directly or indirectly promote engraftment of MM cells, then it may be prevented by being more cautious of sleep apnea symptoms and treating sleep apnea.

Limitations: In our study, we established a link between CIH exposure and MM development, but we were not able to answer all the questions that arose about the underlying mechanisms. In the future studies, we are going to address the effects of CIH on angiogenesis in the bone marrow and its effect on the host immune system.

Figures



	Odds Ratio (Confidence Interval)	p-value
Normoxia (5TGM1) vs Normoxia (Saline)	1.0	
Static Hypoxia (5TGM1) vs Normoxia (Saline)	5.7 (0.5-62.7)	0.117
CIH (5TGM1) vs Normoxia (Saline)	17.5 (1.6-192.3)	0.005

Figure 1: Kaplan Meier Survival Curve with Statistics. Top: Kaplan-Meier curve showing survival of all mice cohorts. Among the black6 mice, CIH exposed cohort with 5TGM1 injection experienced worst survival rate. Bottom: Odds ratio for developing paralysis as a result of myeloma is significantly higher in CIH exposed mice compared to controls under normal O₂.

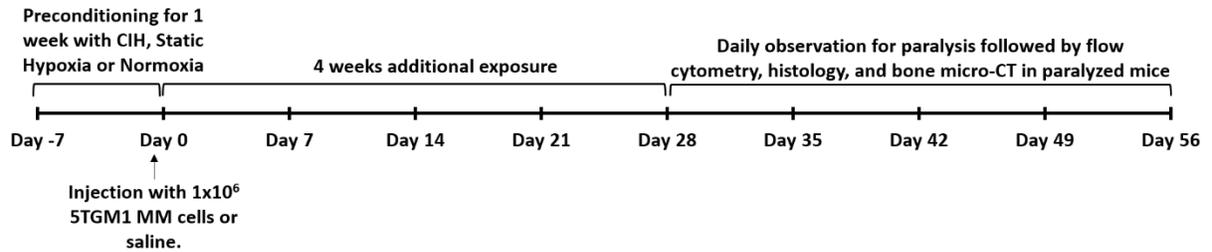


Figure 2: Experiment 1 Timeline. Timeline for experiment 1 included one week of preconditioning the mice with their assigned protocols (CIH, static hypoxia, normoxia). On day 0, seven days since start of preconditioning, mice were injected with either 5TGM1 cells or PBS. The mice then continued their protocol in exposure chambers for 4 more weeks. On day 28, the mice were returned to normal housing where they were under daily observation for signs of paralysis. Paralyzed mice were euthanized, and tissues were collected for examination using flow cytometry, histology, and radiology.

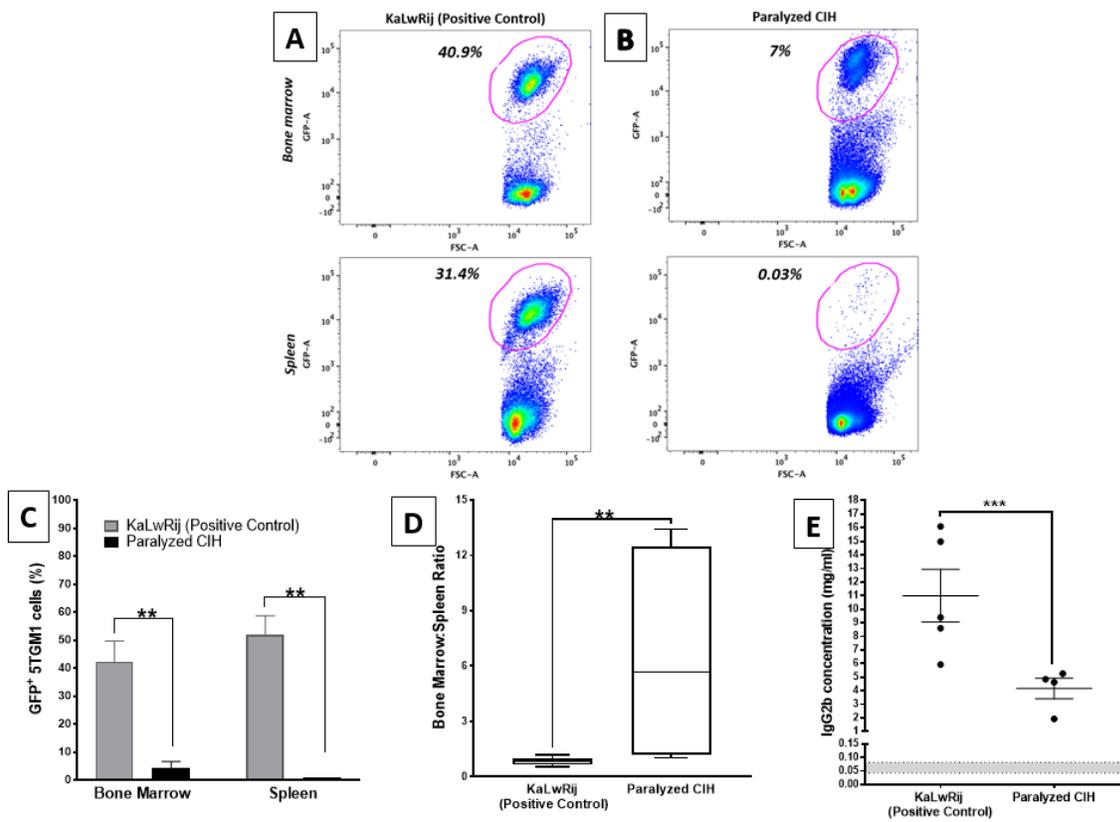


Figure 3: Flow Cytometry. Right: Flow cytometry shows GFP⁺ 5TGM1 MM cells in the bone marrow (top) and spleen (below) of a paralyzed C57BL/6 mouse exposed to CIH. Left: Flow cytometry showing GFP⁺ 5TGM1 cells in the bone marrow (top) and spleen (below) of a paralyzed Kalwrij mouse. Bottom: Tumor cell burden was higher in the bone marrow and spleen of Kalwrij mice compared to paralyzed CIH exposed mice. The myeloma cells in bone marrow to spleen ratio showed equal distribution in Kalwrij mice, but in CIH exposed C57BL/6 mice it was seen that tumor engraftment favored the bone marrow.

** P-value <0.001

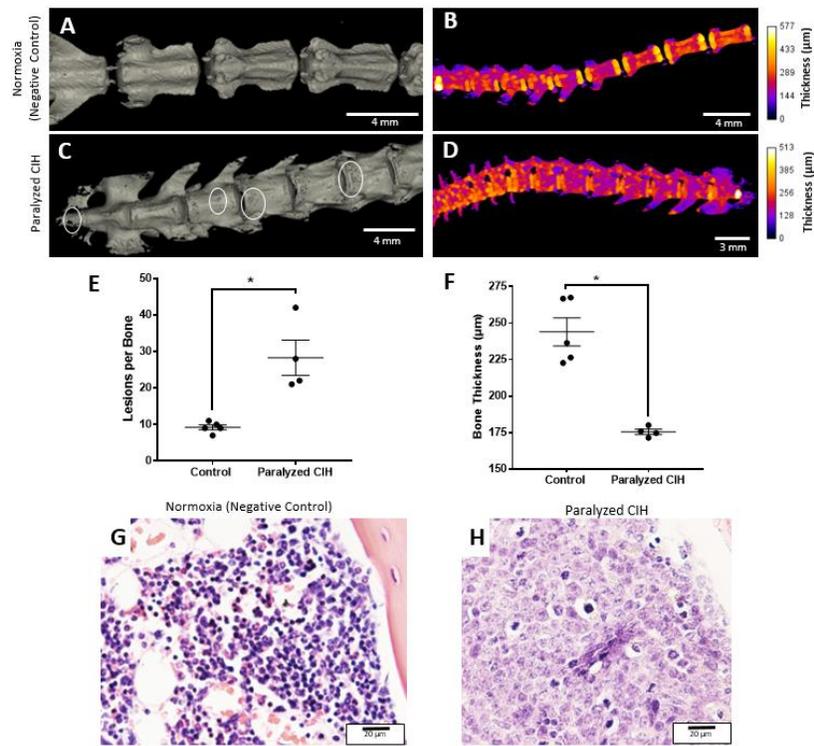


Figure 4: Bone Damage on Micro-CT. A: 3D bone surface mesh image used for bone lesions counting (left), and bone thickness heat map image (right) of the tail and spine of a control mouse B: 3D mesh (left), and bone thickness heat map images (right) of the tail and spine of a CIH exposed mouse C: Bone thickness comparison showing decreased bone thickness in the CIH exposed cohort $p=0.004$ D: CIH exposed cohort had higher number of bone lesions compared to control $p=0.047$ E: hematoxylin & eosin stained slide of the bone marrow of a CIH exposed mouse showing malignant plasma cells infiltrating the bone cavity F: hematoxylin & eosin stained slide showing normal bone marrow of a control mouse.

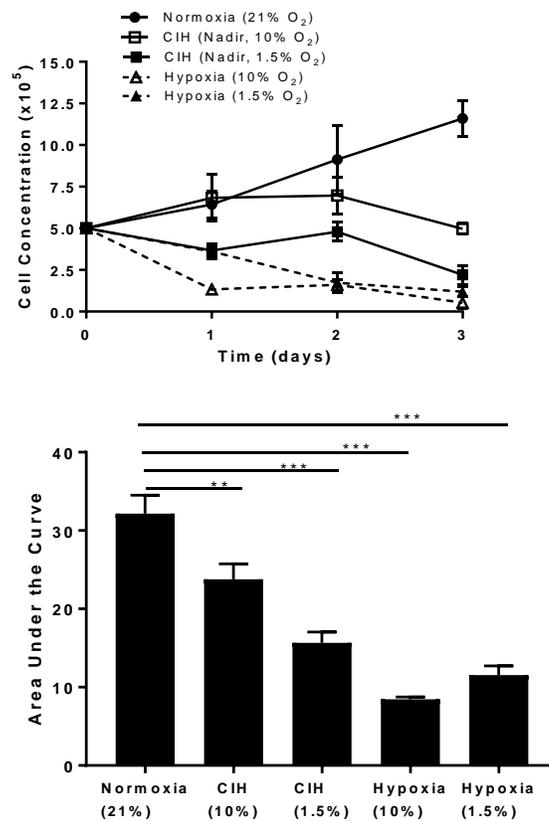


Figure 5: Cell Growth. Above: Changes in 5TGM1 cell concentration over the time of 3 days while exposed to different O₂ conditions was significantly different. Normoxia is the normal oxygen condition at 20.9%; cells double within 72-hours. CIH (10%) is chronic intermittent hypoxia fluctuating between 20.9% and 10% O₂ concentration, CIH (1.5%) is chronic intermittent hypoxia fluctuating between 20.9% and 1.5% O₂ concentration, static hypoxia is exposed to 10% O₂; the three groups showed lower cell growth compared to control. Below: area under the curve showing cells exposed CIH (10%), CIH (1.5%), and static hypoxia had less growth compared to normoxia.

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