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Exposure to sodium tungstate and Respiratory Syncytial Virus results in hematological/immunological disease in C57BL/6J mice

Cynthia D. Fastje [a,*], Kevin Harper [a], Chad Terry [a], Paul R. Sheppard [b], Mark L. Witten [a,1]

[a] Steele Children's Research Center, PO Box 245073, University of Arizona, Tucson, AZ 85724-5073, USA
[b] Laboratory of Tree-Ring Research, PO Box 210058, University of Arizona, Tucson, AZ 85721-0058, USA

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ABSTRACT

The etiology of childhood leukemia is not known. Strong evidence indicates that precursor B-cell Acute Lymphoblastic Leukemia (Pre-B ALL) is a genetic disease originating *in utero*. Environmental exposures in two concurrent, childhood leukemia clusters have been profiled and compared with geographically similar control communities. The unique exposures, shared in common by the leukemia clusters, have been modeled in C57BL/6 mice utilizing prenatal exposures. This previous investigation has suggested *in utero* exposure to sodium tungstate (Na_2WO_4) may result in hematological/immunological disease through genes associated with viral defense. The working hypothesis is (1) in addition to spontaneously and/or chemically generated genetic lesions forming pre-leukemic clones, *in utero* exposure to Na_2WO_4 increases genetic susceptibility to viral influence(s); (2) postnatal exposure to a virus possessing the ^{1}FXXKXFXXA/V^{9} peptide motif will cause an unnatural immune response encouraging proliferation in the B-cell precursor compartment. This study reports the results of exposing C57BL/6J mice to Na_2WO_4 *in utero* via water (15 ppm, *ad libetum*) and inhalation (mean concentration PM_5 3.33 mg/m^3) and to Respiratory Syncytial Virus (RSV) within 2 weeks of weaning. Inoculation of C57BL/6J mice with RSV was associated with a neutrophil shift in 56% of 5-month old mice. When the RSV inoculation was combined with Na_2WO_4-exposure, significant splenomegaly resulted ($p = 0.0406$, 0.0184, 0.0108 for control, Na_2WO_4-only and RSV-only, respectively) in addition to other hematological pathologies which were not significant. Exposure to Na_2WO_4 and RSV resulted in hematological/immunological disease, the nature of which is currently inconclusive. Further research is needed to characterize this potential leukemia mouse model.

1. Introduction

The etiology of childhood leukemia is not known. The natural history of the most common sub-classification of childhood leukemia, precursor B-cell Acute Lymphoblastic Leukemia (Pre-B ALL), provides a temporal frame which can be utilized to develop leukemogenic hypotheses. Pre-B ALL is initiated *in utero*, initially identified through a clonotypic fusion-gene [1], and may not progress to overt leukemia until the peak age at diagnosis of 2–5 years of age [2] which suggests the need for at least one additional post natal event. The most frequently occurring example of a functional fusion-gene associated with Pre-B ALL is TEL-AML1 (t(12;21)(p13;q22); ETV6-RUNX1) [3], which has been found in neonatal blood spots [4] and 1% of stored cord blood [5], which, although probably overestimates the frequency of TEL-AML1 in the general population [6], exceeds the much lower rate of diagnosed leukemia-cases [5] further indicating that leukemia is initiated *in utero*, but a second, post natal event is necessary for the development of clinically manifested ALL. Other genetic lesions associated with Pre-B ALL, rearrangements in immunoglobulin heavy-chain genes [7,8], also support a prenatal/postnatal temporal-sequence for Pre-B ALL. A prenatal/postnatal exposure sequence was utilized to test a leukemogenic hypothesis in C57BL/6J mice.

The primary mutational event occurring *in utero* has been attributed to the log phase growth of hematopoietic cells during fetal development which continues up until 2 weeks following birth [9,10]. The risk of generating a functional lesion during active fetal growth could be increased by an *in utero* exposure to a chemical and/or deficit(s) in metabolic pathways [11]. The nature of the secondary event is the subject of multiple hypotheses including

Abbreviations: Na_2WO_4, sodium tungstate ($Na_2WO_4{\cdot}2H_2O$); RSV, Respiratory Syncytial Virus.

* Corresponding author. Present address: Department of Cell Biology and Anatomy, PO Box 245044, College of Medicine, University of Arizona, Tucson, AZ 85724, USA. Tel.: +1 520 240 6983; fax: +1 520 626 2097.

E-mail addresses: cfastje@email.arizona.edu (C.D. Fastje), kharper@email.arizona.edu (K. Harper), cjt@email.arizona.edu (C. Terry), sheppard@ltrr.arizona.edu (P.R. Sheppard), mlwitten@yahoo.com (M.L. Witten).

[1] Present address: Odyssey Research Institute, 7032 E. Rosewood, Tucson, AZ 85719, USA.

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exposure to ionizing radiation, electromagnetic fields, chemicals, infection, and response to infection (reviewed in Ref. [12]). This deficit in understanding the fundamental nature of the second event has prevented case-control studies from definitively identifying factors associated with the development of childhood leukemia clusters [13]. A new approach utilizing geobiological methodologies to compare communities instead of individuals [14] has identified an exposure possibly unique to childhood leukemia clusters.

The development of two concurrent childhood leukemia clusters in Fallon, NV [15–17] and Sierra Vista, AZ [18,19] provided the opportunity to utilize new geobiological methodologies to compare these communities possessing elevated rates of childhood leukemia to each other and to geographically similar communities differing primarily in the fact that they do not possess elevated rates of childhood leukemia, to identify a potential factor which may be associated with leukemogenesis [20]. These investigations have identified atmospheric tungsten, alone or in combination with cobalt and/or arsenic, as a potential leukemogen [21–24]. *In utero* exposure to sodium tungstate (Na_2WO_4) in C57BL/6 mice significantly decreased the expression of the gene, Deleted in Malignant Brain Tumors 1 (Dmbt1), a putative tumor suppressor whose protein product aggregates bacteria and viruses in the lungs and saliva and is involved in the regulation of immune response [25]. Arsenic alone elevated expression and the combination of tungsten and arsenic significantly decreased expression of Dmbt1. Additionally, the gene microarray investigation suggested that the influence, exerted by tungsten and arsenic on immune response which could result in hematological/immunological disease, may be associated with viral defense (Table 1 [25]).

This paper presents the results of exposing C57BL/6J mice to tungsten and administering a viral challenge. The Respiratory Syncytial Virus (RSV) was selected for this challenge because the protein product of the G gene possesses the FXXKXFXXA/V peptide motif within two T-cell epitopes [28,29] which are restricted by the HLA-DP2/DP4 supertype significantly associated with Pre-B ALL diagnosed between the ages of 3 and 6 years old [30].

2. Materials and methods

2.1. Animals

Eight week-old, C57BL/6J male and female mice were purchased through an IACUC approved protocol. After acclimating to the new environment, a transponder (Bio Medic Data Systems; DAS-6006; IMI-1000) was injected at the nape of the neck of each female mouse and each mouse randomly assigned to an exposure group. The mice were housed, cared for and bred in sterile, microisolation by University of Arizona Animal Care. The study was conducted twice and the data pooled. A total of 8–10 mouse pups were obtained for each group, approximately half from each independently conducted study. The mouse pups were monitored for leukemia through a weekly examination and peripheral blood draw from a tail vein [31,32] conducted monthly during the first investigation and every other month during the second investigation. Tissues were harvested when the pups reached 5–6 months of age.

2.2. Experimental design

A hypoxic factor was originally included in these investigations to model exercise induced asthma [33,34]. However, due to inconsistent methods of administering the challenge between the two replications, the data was not included in the analysis. The experimental groups presented here consist of longitudinal controls, Na_2WO_4-only, RSV-only, and Na_2WO_4 + RSV.

2.3. Tungsten exposures

The dams were exposed to sodium tungstate ($Na_2WO_4{\cdot}2H_2O$, Acros Organics, 99 + %, ACS, Lot A0260722, IN, USA) through water (15 ppm, *ad libetum*) and aerosol. During the 45-min, 5 days/week aerosol exposures, female mice were exposed to a 187 g/L solution vacuum-drawn through a 24-port, nose-only INTOX inhalation chamber (Albuquerque, NM, USA) with an attached cascade impactor for 1 week prior to conception and 3 weeks of gestation until parturition halted exposures. Because mice possess an enhanced mucosal-elevator, a 1% deposition rate for the target particles less than 5 μm (PM_5) was utilized in calculating the concentration of the exposure solution [35,36]. (see Ref. [25] for environmental concentrations in Fallon, NV.) Pups were weaned onto Na_2WO_4-spiked water (15 ppm, *ad libetum*).

2.4. RSV exposure

At 21–35 days of age the mouse pups were lightly anesthetized and the nasal cavity inoculated with 10 μl of human RSV in medium for a total exposure of 1×10^6 pfu [37]. No pathology was observed in the mice. The hRSV A2 strain, propagated in Hep-2 cells, was a kind gift from Dr. Kevin Herrod of the Lovelace Respiratory Research Institute in Albuquerque, NM, USA.

2.5. End measures

Peripheral hematology was evaluated utilizing complete blood counts with a differential obtained from an automated HEMAVET 850 Multispecies Hematology Analyzer (Drew Scientific Inc., Oxford, CT, USA). Spleen tissue was massed and splenic ratio calculated as spleen mass per body mass. Spleens and femurs from each group were preserved in 10% formalin and submitted to Tissue Acquisition and Cellular/Molecular Analysis Shared Services (TACMASS) of the Arizona Cancer Center at the University of Arizona for embedding, slide preparation and H&E staining. Remaining spleens and femurs were stored at −70 °C for future analysis. Histopathological interpretation was conducted by University of Arizona Animal Pathology Services. Digital images were created with a Nikon LaboPhot-2 microscope Paxcam 3 camera and PAX-it Digital Image Management & Image Analysis at 20× magnification by TACMASS.

2.6. Statistical analysis

This study was conducted in the generic, tumor-resistant C57BL/6 mouse to represent a normal human population. This population was monitored for susceptible sub-group(s) possessing outlier indicators of a leukemic condition. When a population is nonparametric due to outliers and has a small *n*-value, a median is less sensitive to these outlier values and therefore, provides a better representation of the data [38]. Statistical analysis was performed with a one-tailed Mann–Whitney test utilizing Minitab version 15.1.1.0. Data are presented as medians with interquartile ranges and outliers indicated. Significance was accepted when $p \leqslant 0.05$.

3. Results

Longitudinal controls and tungsten mice did not exhibit pathological indicators. RSV inoculation within 2 weeks of weaning was associated with a neutrophil shift in 56% of 5-month old mice. When the RSV inoculation was combined with exposure to Na_2WO_4 (Na_2WO_4 + RSV), significant splenomegaly resulted (p = 0.0406, 0.0184, 0.0108 for control, Na_2WO_4-only and RSV-only,

Table 1
Description of genes associated with hematological/immunological disease and cell death in a network generated from genes differentially expressed >5-fold in spleen tissue from C57BL/6 mouse pups (study reported in Ref. [25] and gene descriptions obtained from Refs. [26] and [27]).

Molecules[a]	Name; known function(s)
ABCB1B, **ACAA1**	Acetyl-Coenzyme A acyltransferase 1; beta oxidation system of the peroxisomes
ANGPTL4	Angiopoietin-like 4; inhibition of vascular activity preventing metastasis
ANKRD25	KN motif and ankyrin repeat domains 2; matrix remodeling
ATG7, Cbp/p300	Autophagy related 7 homolog; fusion of peroxisomal and vacuolar membranes
CDKN2A, cyclic AMP, **DCTN4**	Dynactin 4; linking dynein and dynactin to the cortical cytoskeleton
Dehydroepiandrosterone sulfate, ↓DUSP2	Dual specificity phosphatase 2; Regulates mitogenic signal transduction by dephosphorylating both Thr and Tyr residues on MAP kinases ERK1 and ERK2
↓DUT (includes EG:1854)	Deoxyuridine triphosphatase; nucleotide metabolism - produces dUMP, the immediate precursor of thymidine nucleotides; decreases intracellular concentration of dUTP preventing incorporation of uracil into DNA
↓GABARAP	GABA(A) receptor-associated protein; interaction with the cytoskeleton
GH1, *HMGA2*	High mobility group AT-hook 2; transcriptional regulator, cell cycle regulation
HMGC52	Not found
Hydrogen peroxide, ↓IRF3	Interferon regulatory factor 3; Functions as a molecular switch for antiviral activity
LGP2	DEXH (Asp-Glu-X-His) box polypeptide 58; Participates in innate immune defense against viruses
↓LIPE, MCHR1	Lipase, hormone-sensitive; primarily hydrolyzes stored triglycerides to free fatty acids; steroid hormone production
PHF20	PHD finger protein 20; possible transcription factor
↓PPAP2A	Phosphatidic acid phosphatase type 2A; dephosphorylating lysophosphatidic acid (LPA) in platelets which terminates signaling actions of LPA.
PPARG, ↓RAD23A	RAD23 homolog A; post-replication repair of UV-damaged DNA
↓RPL21	Ribosomal protein L21; ribosomal protein that is a component of the 60S subunit
SLC31A2	Solute carrier family 31 (copper transporters), member 2; low-affinity copper uptake
SLC7A11	Solute carrier family 7, (cationic amino acid transporter, y + system) member 11; anionic form of cystine is transported in exchange for glutamate
SLCO1, *SLCO1B3*	Solute carrier organic anion transporter family, member 1B3; Mediates the Na(+)-independent transport of organic anions such as methotrexate
SOD2	Superoxide dismutase 2, mitochondrial; destroys toxic radicals normally produced within the cells
↑TP53INP1	Tumor protein p53 inducible nuclear protein1; promotes p53/TP53 phosphorylation on 'Ser-46' and subsequent apoptosis in response to double-strand DNA breaks
↓UQCRH	Ubiquinol-cytochrome c reductase hinge protein; component of the ubiquinol-cytochrome c reductase complex that is part of the mitochondrial respiratory chain
↓WDR5	WD repeat domain 5; contributes to histone modification; as part of the MLL1/MLL complex, it is involved in methylation and dimethylation at 'Lys-4' of histone H3
ZMYND19	Zinc finger, MYND-type containing 19; binds to the C terminus of melanin-concentrating hormone receptor-1

[a] Genes in italic were up-regulated and genes in bold were down-regulated in the gene microarray analysis. Genes in capitals were differentially expressed less than 5-fold with the direction of the arrow indicating whether they were up or down regulated.

Table 2
Five-month old mice demonstrating pathology following post-natal exposure to RSV-only (RSV) and to Na_2WO_4 + RSV (W + RSV). Control and Na_2WO_4-only groups demonstrated no pathology.

Group	Gender	WBC (k/μl)	Neutrophils (k/μl)	Lymphocytes (%)	Other hematopathology	Spleen/body (g)
RSV	Female	9.08	4.05	42.83	None	0.1022/21.1
RSV	Male	8.06	4.15	43.03	None	0.0835/22.7
RSV	Male	6.5	2.46	57.95	None	0.0914/25.0
RSV	Female	8.6	2.92	57.64	None	0.0818/19.5
RSV	Female	4.68	2.6	38.88	None	0.0826/19.2
W + RSV	Female	12.4	2.54	73.84	Leukocytosis	0.1282/21.5
W + RSV	Female	20.68	15.79	15.55	Anemia, leukocytosis	0.2384/17.1
W + RSV	Female	[a]	[a]	[a]	[a]	0.252/17.2
W + RSV	Female	4.22	1.42	55.80	Monocyte shift-only	0.1771/23.6

[a] Mouse expired prior to blood draw.

respectively) in addition to other hematological pathologies which were not significant. The splenomegaly present in a subset of mice in the Na_2WO_4 + RSV group was accompanied by true neutrophilia, progressive anemia, and morbidity/death (Table 2).

3.1. Peripheral hematology

3.1.1. Neutrophilia

Na_2WO_4-only, RSV-only and Na_2WO_4 + RSV demonstrated significantly elevated neutrophil counts as compared to the longitudinal controls ($p = 0.0162, 0.0081, 0.0059$ for RSV-only, Na_2WO_4-only and Na2WO4 + RSV, respectively). However, the first and third quartiles for all experimental groups were within the normal range (0.1–2.4 k/μl) except for RSV-only whose third quartile was 2.76 k/μl. Additionally, Na_2WO_4 + RSV possessed an extreme outlier (Table 2). No significant differences were demonstrated between RSV-only, Na_2WO_4-only and Na_2WO_4 + RSV.

The percentage of neutrophils contributing to the total WBC count was significantly greater for all three exposure groups as compared to the controls. However, the first and third quartiles for all experimental groups were within the normal range (6.6–38.9%) except for RSV-only whose third quartile was 44.67%. Additionally, Na_2WO_4 + RSV possessed an extreme outlier for whom 76.3% of the WBC was neutrophils. No significant differences were demonstrated between Na_2WO_4-only, RSV-only and Na_2WO4 + RSV (Fig. 1A).

3.1.2. Total white blood cell counts

While the percentage of neutrophils increased significantly, there was no associated increase in WBC counts for mice ex-

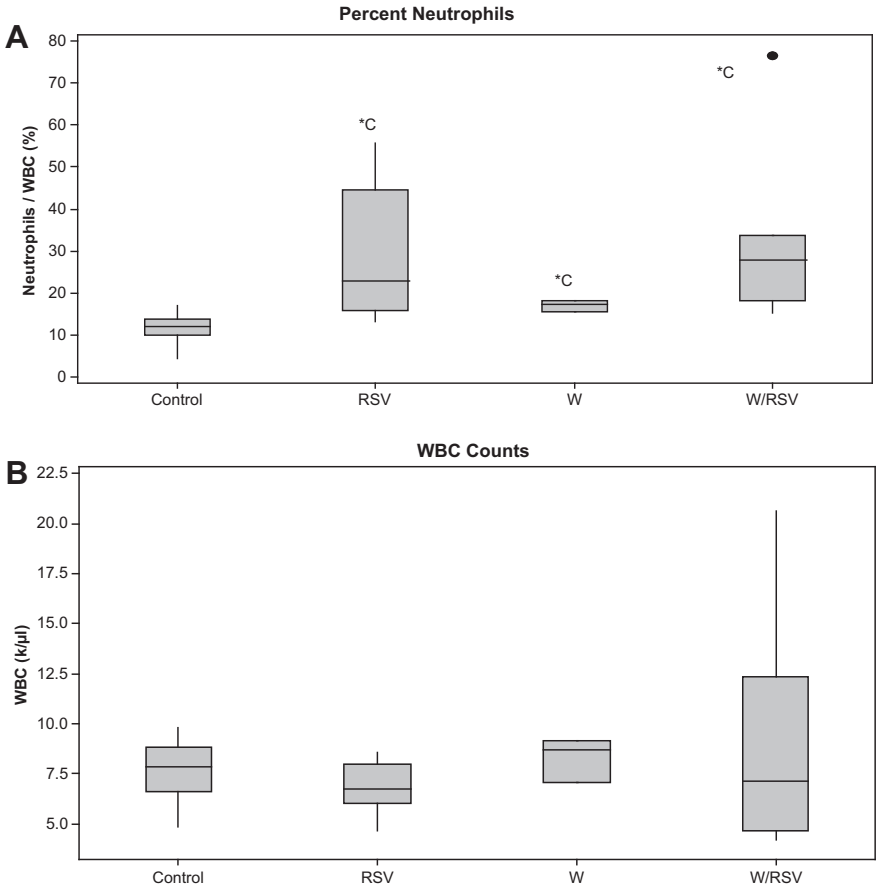

Fig. 1. Percent neutrophils (A) and WBC counts (B) for 5-month old C57BL/6 mice exposed to W while *in utero* and/or to the Respiratory Syncytial Virus within 2 weeks of weaning. First and third quartiles with a median bar, whiskers and extreme outliers (●) are indicated. *C indicates a significant difference from the longitudinal controls ($p = 0.0015$, 0.0265, 0.0015 for RSV, Na_2WO_4, and Na_2WO_4 + RSV, respectively).

posed to RSV-only. No group demonstrated a significant increase in WBC counts as compared to the controls or to each other. First and third quartiles for all experimental groups were within the normal range (1.8–10.7 k/µl) except for Na_2WO_4 + RSV whose third quartile was 12.4 k/µl (Fig. 1B). A greater *n*-value would be needed to obtain significance. Additionally, a confounding variable of age at RSV inoculation is thought to be influencing the data. Pups inoculated at 21 days as opposed to 35 days of age demonstrated greater pathology. For the subgroup of Na_2WO4 + RSV mice exhibiting pathology, leukocytosis began at 2–3 months of age and continued until death/morbidity at 5–6 months of age.

3.2. Splenomegaly

Splenic ratios for mice exposed to Na_2WO_4-only or RSV-only did not vary significantly from the controls or from each other. Splenic ratios for Na_2WO4 + RSV mice were significantly larger as compared to all other groups ($p = 0.0406$, 0.0184, 0.0108 for control, Na_2WO_4-only and RSV-only, respectively) (Fig. 2). The colors/hues of the spleens were not consistent in mice demonstrating splenomegaly and were not the same color as the control spleens.

3.3. Histology

Histologic slide preparation and interpretation was conducted with both spleen and bone marrow tissues from two Na_2WO_4 + RSV mice exhibiting the greatest degree of splenomegaly and compared with a longitudinal control mouse. The control

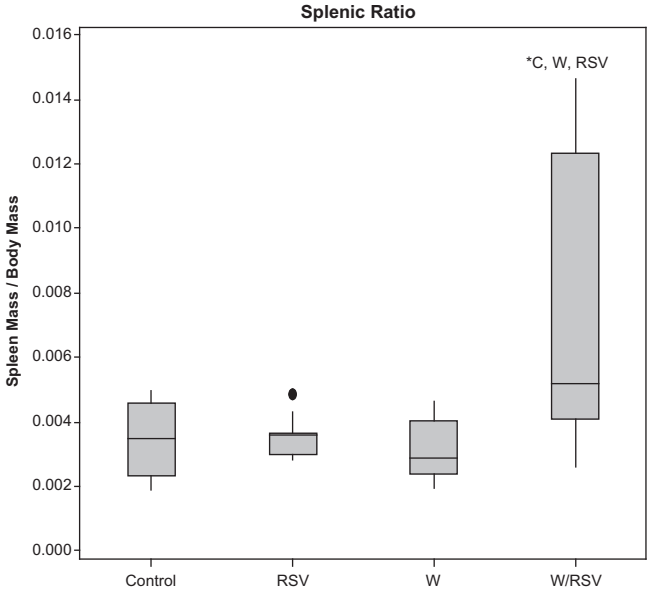

Fig. 2. Splenic ratios for 5-month old C57BL/6 mice exposed to W while *in utero* and/or to the Respiratory Syncytial Virus within 2 weeks of weaning. First and third quartiles with a median bar, whiskers and extreme outliers (●) are indicated. *C indicates a significant difference from the longitudinal controls, *W from Na_2WO_4, *RSV from Respiratory Syncytial Virus.

exhibited a 1:1 ratio of erythropoietic and granulopoietic precursor cells with all stages represented. Both Na_2WO_4 + RSV mice demon-

strated consistent histology, a 1:20 ratio of erythropoietic to granulocytic precursors with all stages represented (Fig. 3). Additionally, marked thrombopoiesis was reported in both spleen and bone marrow tissues.

4. Discussion

The primary, statistically significant result in this study is the occurrence of splenomegally ($p = 0.0406$, 0.0184, 0.0108 for control, Na_2WO_4-only and RSV-only, respectively) as a result of combined exposure to both Na_2WO_4 and RSV. Pathological presentation associated with the splenomegaly varied, primarily presenting as true neutrophilia, but also as lymphocytosis and monocytosis. The mice may have differing pathological conditions all of which produced splenomegaly. This is supported by the fact that the enlarged spleens differed from each other and from the controls in appearance.

Previously, anemia and/or thrombocytopenia were observed in approximately 10% of mice exposed to ammonium paratungstate [25]. Although we did not observe evidence of suppressed hematopoiesis in the Na_2WO_4-only mice during this particular study, this may be the result of a low n-value ($n = 8$). The significantly elevated neutrophil counts as compared to the controls were within the normal range. No pathology was observed in the Na_2WO_4-only mice in this study.

This study provides additional evidence that post-natal exposure to RSV can promote shift neutrophilia several months after exposure ($p = 0.0015$ as compared to controls, but not significantly different from Na_2WO_4-only or Na_2WO_4 + RSV) with most hematological measures remaining within normal parameters. When this RSV-induced T-cell activation was combined with exposure to Na_2WO_4, progressive anemia, leukocytosis and splenomegaly resulted culminating in morbidity/death in a subset of the mice.

4.1. Neutrophilia

Two different types of neutrophilia were observed. RSV-only and Na_2WO_4-only mice demonstrated significantly elevated neutrophil counts ($p = 0.0015$, 0.0265 for RSV-only and Na_2WO_4-only, respectively) compared to control mice without an associated increase in total leukocytes (shift neutrophilia). Na_2WO_4 + RSV mice demonstrated significant neutrophilia compared to controls ($p = 0.0015$), which included a corresponding increase in total leukocyte counts (true neutrophilia). Because all exposures were completed by 5 weeks of age and the blood draws were performed on all mice, the Na_2WO_4-only and RSV-only groups of mice at 5 months experienced the same level of stress as controls and therefore, the significant difference in neutrophil counts is probably not associated with chronic stress. RSV has been reported to indirectly activate neutrophils through cytokines and inflammatory agents released by infected cells [39] and to prolong the survival of airway epithelial cells by delaying the cells' death through posttranslational degradation of p53 [40]. Commonly deleted in cancers, p53 is a tumor suppressor functioning as a regulator of apoptosis in damaged cells.

4.2. Diagnosis

The diagnostic guidelines, Bethseda Proposals for Classification of Non-lymphoid Hematopoietic Neoplasms in Mice, indicates that the first level of screening for a leukemic condition in the peripheral blood of a previously healthy mouse would be the appearance of any combination of blasts, anemia, thrombocytopenia, or neutropenia,

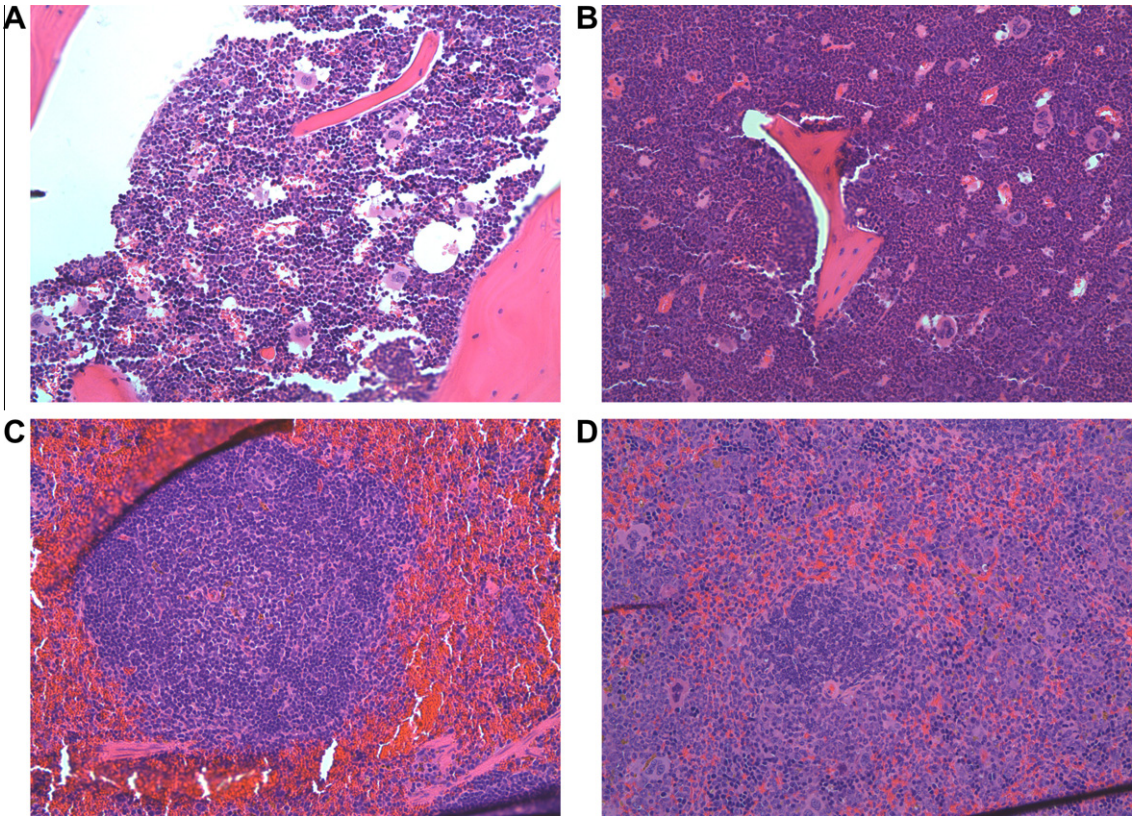

Fig. 3. Spleen and bone marrow images obtained from H&E slides with 20× magnification (A) control femur with bone marrow (B) Na_2WO_4 + RSV femur with bone marrow (C) control spleen and (D) Na_2WO_4 + RSV spleen.

unless there is a neutrophilic component defined as any combination of ⩾20% of leukocytes are neutrophils in bone marrow, ⩾20% of leukocytes are neutrophils in the spleen, or ⩾5 times the normal quantity of neutrophils in peripheral blood [32]. A Na_2WO_4 + RSV mouse presented with anemia only, but also met the criteria for a neutrophilic component. The masses of other organs were not recorded and no notation was made indicating abnormal liver appearance. The sternum and femur appeared white. A diagnostic determination cannot be ascertained with the current information. Although not reported in this study, mice in the Na_2WO_4 + RSV + Hypoxia group demonstrated anemia, thrombocytopenia, neutrophilia and/or lymphocytosis, but no blasts in the peripheral blood were observed (unpublished data).

Either a viral infection or a chronic, severe bacterial infection can produce lesions similar to those observed in the bone marrow and spleen tissue samples. The two mice from which the tissue images were obtained demonstrated progressive anemia suggesting they may have been immunocompromised, and they both possessed bacterial abscesses. Two additional female mice were cage mates and had normal hematology reports with spleen and body masses similar to the controls, further suggesting that the two mice presenting with anemia/splenomegaly may have been immunocompromised providing an opportunistic environment. Further research is needed to identify and characterize the Na_2WO_4 + RSV-associated hematological/immunological disease(s) presenting with significant splenomegaly.

This investigation made no attempt to control the presence/absence or character of any initial genetic lesion in hematopoietic cells in the mice. This potential leukemia mouse-model should be characterized as to the molecular and genomic characteristics of the proliferating cells, and whether these or a similar cell compartment exists which will propagate a leukemia-like condition to confirm or negate the sole presence of a reactive condition. Additionally, these investigations should be conducted in a susceptible-mouse model harboring TEL-AML1$^+$ hematopoietic stem cells [41]. This concept suggests that by controlling the initial mutation and cell type, the final hematopoietic neoplasia may also be controlled.

5. Conclusion

This study provides evidence indicating exposure to Na_2WO_4 and RSV produces a possible leukemia mouse model. However, further research is needed to characterize the model.

6. Conflict of interest statement

Sheppard and Witten have provided documents, data, and testimony in case CV03-03482, Richard Jernee et al. vs Kinder Morgan Energy et al., Second Judicial District Court of Nevada, Washoe County, which is related to the childhood leukemia cluster of Fallon, NV.

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