Title

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Journal

Archives of Toxicology, 95(2)

ISSN

0340-5761

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Publication Date

2021-02-01

DOI

10.1007/s00204-020-02932-x

Peer reviewed

HHS Public Access

Author manuscript

Arch Toxicol. Author manuscript; available in PMC 2022 February 01.

Published in final edited form as:

Arch Toxicol. 2021 February ; 95(2): 693–701. doi:10.1007/s00204-020-02932-x.

Formaldehyde-induced Hematopoietic Stem and Progenitor Cell Toxicity in Mouse Lung and Nose

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Abstract

Formaldehyde (FA), an economically important and ubiquitous chemical, has been classified as a human carcinogen and myeloid leukemogen. However, the underlying mechanisms of leukemogenesis remain unclear. Unlike many classical leukemogens that damage hematopoietic stem/progenitor cells (HSC/HPC) directly in the bone marrow, FA–as the smallest, most reactive aldehyde–is thought to be incapable of reaching the bone marrow through inhalation exposure. A recent breakthrough study discovered that mouse lung contains functional HSC/HPC that can produce blood cells and travel bi-directionally between the lung and bone marrow, while another early study reported the presence of HSC/HPC in rat nose. Based on these findings, we hypothesized that FA inhalation could induce toxicity in HSC/HPC present in mouse lung and/or nose rather than in the bone marrow. To test this hypothesis, we adapted a commercially available protocol for culturing burst-forming unit-erythroid (BFU-E) and colony-forming unit-granulocyte, macrophage (CFU-GM) colonies from bone marrow and spleen to also enable culture of these colonies from mouse lung and nose, a novel application of this assay. We reported that in vivo exposure to FA at 3 mg/m^3 or ex vivo exposure up to 400 μM FA decreased the formation of both colony types from mouse lung and nose as well as from bone marrow and spleen. These findings, to the best of our knowledge, are the first empirically to show that FA exposure can damage mouse pulmonary and olfactory HSC/HPC and provide potential biological plausibility for the induction of leukemia at the sites of entry rather than the bone marrow.

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Compliance with ethical standards

Conflict of interest All of the authors declare no potential competing financial interest.

Keywords

Formaldehyde; Leukemogenesis; HSC/HPC; Colony; Toxicity

Introduction

Formaldehyde (FA) is a ubiquitous chemical extensively used in various industries (Tang et al. 2009; Duong et al. 2011). Exposure to FA mainly occurs via inhalation or skin absorption in workers in certain occupational settings including the production of FA resins and textiles, as well as embalming and medical laboratories (Zhang et al. 2009; McGwin et al. 2010). The general public is routinely exposed to FA from multiple sources, such as building materials, cigarette smoking and some manufactured wood or fabric products (Leng et al. 2019; Nikle et al. 2019). This widespread occupational and/or environmental exposure to FA raises concerns about potential adverse health effects.

The International Agency for Research on Cancer (IARC) classified FA as a Group I human carcinogen based on sufficient evidence that FA causes nasopharyngeal cancer (Cogliano et al. 2005; IARC. 2006) and is associated with myeloid leukemia (IARC 2012). A number of earlier large cohort studies on FA-exposed workers (Hauptmann 2003; Pinkerton 2004) and follow-up studies (Beane Freeman et al. 2009; Hauptmann et al. 2009) support the association between FA exposure and myeloid leukemia. Previously, we reported that FA induced hematotoxicity and chromosomal aneuploidy in the circulating myeloid progenitor cells of exposed workers (Zhang et al. 2010b; Lan et al. 2015). Additionally, we found that FA induced toxicity in bone marrow (BM) and hematopoietic stem/progenitor cells (HSC/HPC) of exposed mice via inhalation (Zhang et al. 2013b; Ye et al. 2013; Wei et al. 2017).

Most classical leukemogens (e.g. chemotherapeutic drugs and benzene) initiate leukemogenesis by directly damaging the HSC/HPC in the BM (Zhang et al. 2009, 2010a). However, FA–as the simplest and most reactive aldehyde– is unlikely to reach the bone marrow to cause toxicity and initiate leukemia, based on current studies. Several studies showed that exogenous DNA adducts were detectable in the nasal epithelium, but not in BM, of the exposed rats (Lu et al. 2010, 2011). Thus, the site and mechanism of leukemia initiation by FA remain uncertain. A number of alternative models were proposed hypothesizing potential targets (e.g. circulating HSC/HPC in blood or pluripotent nasal/oral stem cells) of inhaled FA that could initiate leukemogenesis outside of BM (Zhang et al. 2009).

Recently, a breakthrough study from Dr. Looney and his colleagues revealed that a large number of megakaryocytes along with progenitors residing in mouse lung can release platelets and circulate bidirectionally between the lung and BM (Lefrançais et al. 2017). In addition, an earlier study reported that cells dissociated from rat olfactory epithelium gave rise to multiple cells of hematopoietic lineages, including lymphoid and myeloid cells, when injected into irradiated rats (Murrell et al. 2005). Based on these discoveries that the lung and nose contain functional HSC/HPC, we primarily proposed an unconventional hypothesis (Zhang 2018). Instead of damaging HSC/HPC in BM directly, FA may induce HSC/HPC toxicity in mouse lung and nose, the portals of entry for inhaled FA exposure.

Hematopoiesis is a process whereby HSC/HPC undergo differentiation into mature blood cells (Orkin and Zon 2008; Jagannathan-Bogdan and Zon 2013). HSC continuously replenish the blood system through a series of lineage differentiation steps and the early steps involve the development of the myeloid and lymphoid cell lineages (Kondo 2010). The myeloid lineage includes erythrocytes (E), granulocytes and macrophages (GM) and megakaryocytes (M), while the lymphoid lineage includes T lymphocytes, B lymphocytes and natural killer cells (Kondo et al. 2003).

In the present study, we aimed to examine FA-induced toxicity in HSC/HPC from mouse lung and nose, in both in vivo (inhalation) and ex vivo experiments. We applied a colony assay from StemCell Technologies™ to evaluate the growth and number of HSC/HPC in multiple mouse tissue types, including lung, nose, bone marrow and spleen. Two colony types, burst-forming unit-erythroid (BFU-E) and colony-forming unit, granulocytes and macrophages (CFU-GM), predominantly are cultured from committed myeloid stem/progenitor cells using this assay.

Methods

Animals and mouse tissues

Specific pathogen-free male BALB/c mice (5 weeks old) were purchased from Hubei Experimental Animal Center (Wuhan, China) (in vivo) and Jackson Laboratory (Bar Harbor, ME) (ex vivo), respectively. They were housed in the Experimental Animal Center of Central China Normal University (in vivo) and Northwest Animal Facility of University of California, Berkeley (ex vivo) and maintained under a 12 h daily light cycle. All mice had free access to the clean food and water. They were adapted to the environment for one week before any treatments. All procedures involving animals were conducted in accordance with Institutional Animal Care and Use Committee of Central China Normal University and Animal Care and Use Committee of University of California, Berkeley.

Four types of tissue including BM, spleen, lung and nose were selected for the downstream colony assay. As the two main sites containing HSC/HPC, BM and spleen were chosen as control tissue types. According to previous findings that functional HSC/HPC are also present in lung (Lefrançais et al. 2017) and nose (Murrell et al. 2005), these two tissues were chosen as experimental tissues. Theoretically, the amount of HSC/HPC in lung and nose would be much lower than in BM and spleen. In order to acquire adequate cell numbers for downstream (colony) analyses, the lung and nose tissues from 3 mice in each treatment group were combined.

FA inhalation exposure (in vivo)

FA inhalation exposure was conducted in Central China Normal University. BALB/c mice were randomly divided into two groups, vehicle group (control) and FA exposed group. Briefly, the mice in each group were exposed to ambient air prepared from double-distilled (DD) water or 3 mg/m^3 FA prepared from 10% formalin (Sigma-Aldrich, StLouis, MO), using an environmentally controlled 8.4 L glass chamber. During exposure, the air FA concentration in the chamber was monitored every 2 h using a Gaseous FA Analyzer 4160–2

(Interscan, SimiValley, CA). Air temperature, humidity and ventilation rate were maintained at 23 ± 0.5°C, 45 ± 5.0% and 1.5 L/min, respectively. Mice were exposed from 9 a.m. to 5 p.m., 8 h/day, 5 days/week, for 2 weeks (Fig. 1a). Two researchers independently conducted two experiments and the results are shown as experiment I (Expt. I) and experiment II (Expt. II).

Biological sample preparation (in vivo)

On the 13th day of treatment, mice were anesthetized by intraperitoneal injection of 100 mg/kg (body weight) pentobarbital sodium and then sacrificed by cervical dislocation. BM, spleen, lung, and nose tissues were collected. BM cells were flushed from femurs with ice-cold Iscove's Modified Dulbecco's Medium (IMDM) containing 1% penicillin-streptomycin and 2% fetal bovine serum (FBS). Spleens and lungs were rinsed 3–4 times with IMDM containing 1% penicillin–streptomycin and 2% FBS and cells were flushed out with the same media using 1mL syringes with needles (23 gauge). Nasal tissue, mainly olfactory mucosa from the nasal septum, were cut into pieces after opening the nasal cavity. Nasal cells were isolated and harvested by digestion using 0.25% trypsin at 37°C for 45 min. The collected cells were then filtered through 40 μM strainers and the erythrocytes were lysed by red blood cell (RBC) lysing reagent (150 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA). Lung, nose, BM and spleen cell counts were determined using a blood cell analyzer (Motenu MTN-21, Changchun, China).

Biological sample preparation and FA treatment (ex vivo)

The ex vivo experiment was conducted at University of California, Berkeley. After one-week adaption, mice were euthanized by CO_2 inhalation. BM cells were flushed from femurs with phosphate buffered saline (PBS). Lung, nose and spleen cell samples were prepared following the same protocol as for the in vivo experiments. Briefly, all three tissues were removed from mice and rinsed with PBS to remove debris. The tissues were minced finely and digested enzymatically using digestion buffer (2 mL PBS + 10 μL Dnase + 0.25 mg liberase per tissue) at 37°C for 45 min. The collected cells were then filtered through 40 μM strainers, subjected to RBC lysis, resuspended and counted using Vi-CELL™ Cell Counter for Cell Viability Analyzer (Beckman-Coulter, Brea, CA). Lung, nose, BM and spleen cells were plated (see below for cell density) and treated with 0, 50, 100, 200, 400 μM FA (Thermo Fisher Scientific, Waltham, MA) for 1 h at 37°C (Fig. 1b).

Myeloid progenitor colony formation assay both in vivo and ex vivo

Cells harvested from all tissues after treatment and preparation were seeded in MethoCult™ GF M3434 (StemCell Technologies™,Vancouver,Canada), a methylcellulose-based medium enriched in the recombinant cytokines including erythropoietin (EPO), in 30 mm petri dishes. GF M3434 supports the generation of colonies from stem or progenitor cells that are present. According to the protocol of StemCell Technologies™ and the results of our pilot study, the optimal seeding concentrations by tissue type were as follows: lung 1×10^6 cells/dish, nose 3×10^5 cells/dish, BM 5×10^4 cells/dish, and spleen 4×10^5 cells/dish, with 1.2 mL medium per dish. Cells were plated in duplicate and cultured for 10–12 days at 37°C. Two colony types, BFU-E and CFU-GM, were predominantly cultured using medium in this colony forming unit (CFU) assay.

Colonies, defined as containing more than 50 cells, were verified and counted under the microscope by two researchers after 10–12 days culture based on colony phenotypes indicated by StemCell Technologies™ instructions.

Statistical analysis

Data are presented as mean ± SEM. GraphPad Prism 6.0 was used for the statistical analysis. Student's t-test was used to test the significance of differences between groups. P_{trend} was analyzed with linear regression. Statistical significance was considered to be $p < 0.05$.

Results

Culture of BFU-E and CFU-GM colonies from mouse lung and nose

BFU-E and CFU-GM, two major colony types formed from more committed myeloid progenitors, were cultured from BM and spleen, major reservoirs of blood cell precursors. In addition, both colony types were cultured from the lung and nose of mice and were shown to be morphologically similar to those cultured from BM and spleen (Fig. 2).

FA exposure in vivo decreased the formation of both BFU-E and CFU-GM colonies in lung and nose as well as in BM and spleen

A greater number of BFU-E (Fig. 3a) than CFU-GM colonies (Fig. 3b) were cultured from all four tissue types in two independent experiments (Expt. I and Expt. II). FA exposure (3 mg/m^3) significantly reduced BFU-E colony formation from lung in Expt. II only (Fig. 3a A1, $p < 0.05$) and from nose in both experiments (Fig. 3a A2, $p < 0.001$ and $p < 0.05$, respectively), compared to unexposed controls. FA exposure significantly reduced CFU-GM colony formation from lung in experiment Expt. II only (Fig. 3b B1, $p < 0.05$) and from nose in Expt. I only (Fig. 3b B2, $p < 0.05$), compared to control.

FA exposure significantly reduced BFU-E colony formation from BM in both Expt. I and II (Fig. 3a A3, $p < 0.01$ and $p < 0.001$, respectively) and from spleen in Expt. II (Fig. 3a A4, $p < 0.001$). FA exposure significantly reduced CFU-GM colony formation from BM in both Expt. I and Expt. II (Fig. 3b B3, $p < 0.001$ and $p < 0.01$, respectively) and from spleen in Expt. I and Expt. II (Fig. 3b B4, $p < 0.05$ and $p < 0.01$, respectively).

Ex vivo FA exposure significantly decreased the formation of both BFU-E and CFU-GM colonies in nose, BM and spleen but not in lung

We conducted ex vivo experiments to confirm the HSC/HPC toxicities induced by FA inhalation. FA treatment (0 – 400 μM) statistically reduced the formation of both BFU-E (Figu. 4a) and CFU-GM (Fig. 4b) colonies from mouse nose, BM and spleen ($p_{trend} < 0.05$). However, the decrease of colonies in the lung was not statistically significant ($p_{trend} > 0.05$). Further, 400 μM FA induced a significant ($p < 0.05$) reduction in BFU-E from mouse nose, BM and spleen (Fig. 4a A2–A4) and in CFU-GM from spleen (Fig. 4b B4).

Discussion

Detection of HSC/HPC in BALB/c mouse lung and nose using a modified CFU assay

The CFU assay has been widely applied in research to evaluate the growth of HSC/HPC (North et al. 2007; Tomar et al. 2010; Kobayashi et al. 2010; Zhang et al. 2013a). Basically, when cultured in a semi-solid matrix supplemented with appropriate growth factors and/or cytokines, each individual stem or progenitor cell, the so-called CFU, proliferates and differentiates to form one colony. Using the CFU assay, extensive studies have investigated the growth of HSC/HPC residing in peripheral blood, BM and spleen (Ratajczak et al. 2010; Loh et al. 2010; Lafuse et al. 2013). Moreover, our previous studies revealed HSC/HPC toxicity induced by benzene and FA in the peripheral blood of exposed workers (Lan et al. 2004; Zhang et al. 2010b) and in BM of FA-inhaled mice (Ge et al. 2020; Wei et al. 2017). However, the CFU assay was not previously applied to study the growth of HSC/HPC present in the lung and nose. To our knowledge, this is the first study to do so.

We adapted the CFU assay from StemCell Technologies™ to culture BFU-E and CFU-GM colonies from HSC/HPC present in mouse lung and nose for the first time. We utilized BALB/c mice for comparability with our previous studies of FA-induced toxicity (Zhang et al. 2013b; Wei et al. 2017). Thus, our findings have confirmed the presence of viable HSC/HPC in lung and nose in a second mouse strain. The presence of hematopoietic precursors in lung (Lefrançais et al. 2017) and nose (Murrell et al. 2005) was originally reported in mice (C57/BL6) and rats (RT7.2-positive and RT7.2-negative), respectively. Notably, this novel detection of viable HSC/HPC was successful at laboratories both in China and in the U.S. as well as both in vivo and ex vivo.

Colony numbers differed across tissues and type of experiment

In the present study, many more BFU-E than CFU-GM colonies were generated from all tissues in the in vivo experiments, which is similar to what we previously reported in our studies of human cells treated with FA (Zhang et al. 2010b) and workers exposed to benzene (Lan et al. 2004). However, our previous in vivo study in BALB/c mice, conducted using a different type of media, produced a higher level of CFU-GM than BFU-E from BM (Wei et al. 2017). On the other hand, similar numbers of both colony types were generated from the ex vivo experiments. This could have arisen, in part, from the different tissue preparation protocols used in the in vivo and ex vivo experiments, especially the utilization of different digestive enzymes. As expected, fewer colonies were cultured from lung and nose than from BM and spleen, the main sites of HSC/HPC and hematopoiesis, though more colonies were generated from nose than from lung in our experiments both in vivo and ex vivo. It is unclear if this reflects actual numbers of HSC/HPC present in those tissues, thus, more investigations are warranted.

FA exposure in vivo and ex vivo damaged HSC/HPC in lung, nose, BM and spleen

Leukemogenesis is an evolutionary process which may be initiated by the transformation of primitive HSC into leukemic stem cells (LSC) (Passegue et al. 2003; Renneville et al. 2008). The LSC arises from the accumulation of multiple genetic events, such as genomic alterations and/or chromosomal aberrations (Dash and Gilliland 2001; Renneville et al.

2008). Previously, our epidemiologic study in China showed a significant elevation of leukemia-specific chromosomal aneuploidy in the circulating myeloid progenitor cells of FA-exposed workers (Zhang et al. 2010b; Lan et al. 2015). Correspondingly, our previous animal studies indicated FA-induced toxicity in mouse BM (Zhang et al. 2013b; Ye et al. 2013; Wei et al. 2017). Consistent with previous findings, it was confirmed in the current study that FA exposure damaged the HSC/HPC in BM. Furthermore, for the first time, we have shown that FA exposure impaired the formation of colonies from HSC/HPC in spleen, again supporting the role of FA in causing hematotoxicity in leukemia-relevant tissues.

In the present study, inhalation of 3 mg/m^3 FA decreased the formation of both BFU-E and CFU-GM colonies in mouse lung and nose, demonstrating that FA can exert toxicity to pulmonary/olfactory HSC/HPC. In addition, FA exposure ex vivo suppressed colony formation in nose tissue in a dose-dependent manner, while the decreased colony formation was not statistically significant in lung. A strength of this study is the general consistency between the in vivo and ex vivo experiments both of which showed the nose to be more sensitive than lung to FA toxicity. Possibly, this could reflect a higher dose of FA via a shorter inhalation route and/or the greater inhalation intake through nose than lung in mice (Fig. 5). However, due to the relatively low yield of colonies from lung, further studies are needed to confirm this difference in sensitivity. In summary, our findings provide direct evidence to support our hypothesis that FA may induce leukemogenesis without reaching BM, by damaging HSC/HPC at the sites of entry, nose and lung.

Implication and Conclusion

To address the controversy over the association of myeloid leukemia and FA exposure in humans, we hypothesized that FA inhalation could damage HSC/HPC in the lung and/or nose (Zhang 2018), and that these damaged cells might travel to bone marrow to initiate leukemogenesis (Fig. 5). Our hypothesis has been tested in a mouse model in the current study. A colony assay was adapted to examine FA-induced toxicity in HSC/HPC presented in mouse lung and nose in comparison with BM and spleen. We first successfully cultured two main type of colonies, BFU-E and CFU-GM, and then detected the FA-reduced growth of colonies from lung and nose in vivo and ex vivo. Our findings suggest that nasal stem and progenitor cells may be another target of FA exposure. This is also supported by previous studies, for example, FA induces DPCs and other toxicities in nasal passages of animals (Lu et al. 2010, 2011; Moeller et al. 2011) as well as nasal and oral mucosa of exposed humans (Ballarin et al. 1992). Potentially, FA-induced DNA damage and lesions in the pluripotent stem cells in nose could result in damaged olfactory HSC/HPC travelling to the BM, acquiring a subsequent mutation, and initiating leukemogenesis.

In conclusion, for the first time, we have demonstrated the induction of HSC/HPC toxicity in mouse lung and nose by FA exposure both in vivo and ex vivo, providing biological plausibility to an alternative model of leukemia induction outside of the BM. It could also deepen and improve our understanding of chemically-induced leukemogenesis.

Acknowledgements

This project was partially supported by NIEHS Superfund research grant P42ES004705 and Chau Hoi Shuen Foundation Women in Science Program Award at University of California at Berkeley (L Zhang), as well as the National Key Research and Development Program of China (2017YFC0702700) (R Li). The content is solely the responsibility of the authors and does not necessarily represent the official views of NIEHS and other funding agencies. We thank Dr. Zhe Liu from University of California, San Francisco (UCSF) at Dr. Looney's lab for her valuable suggestions on the protocol of lung cell extraction. YZ is a recipient of the China Scholarship Council for a graduate fellowship to UC Berkeley for two years (201706770048).

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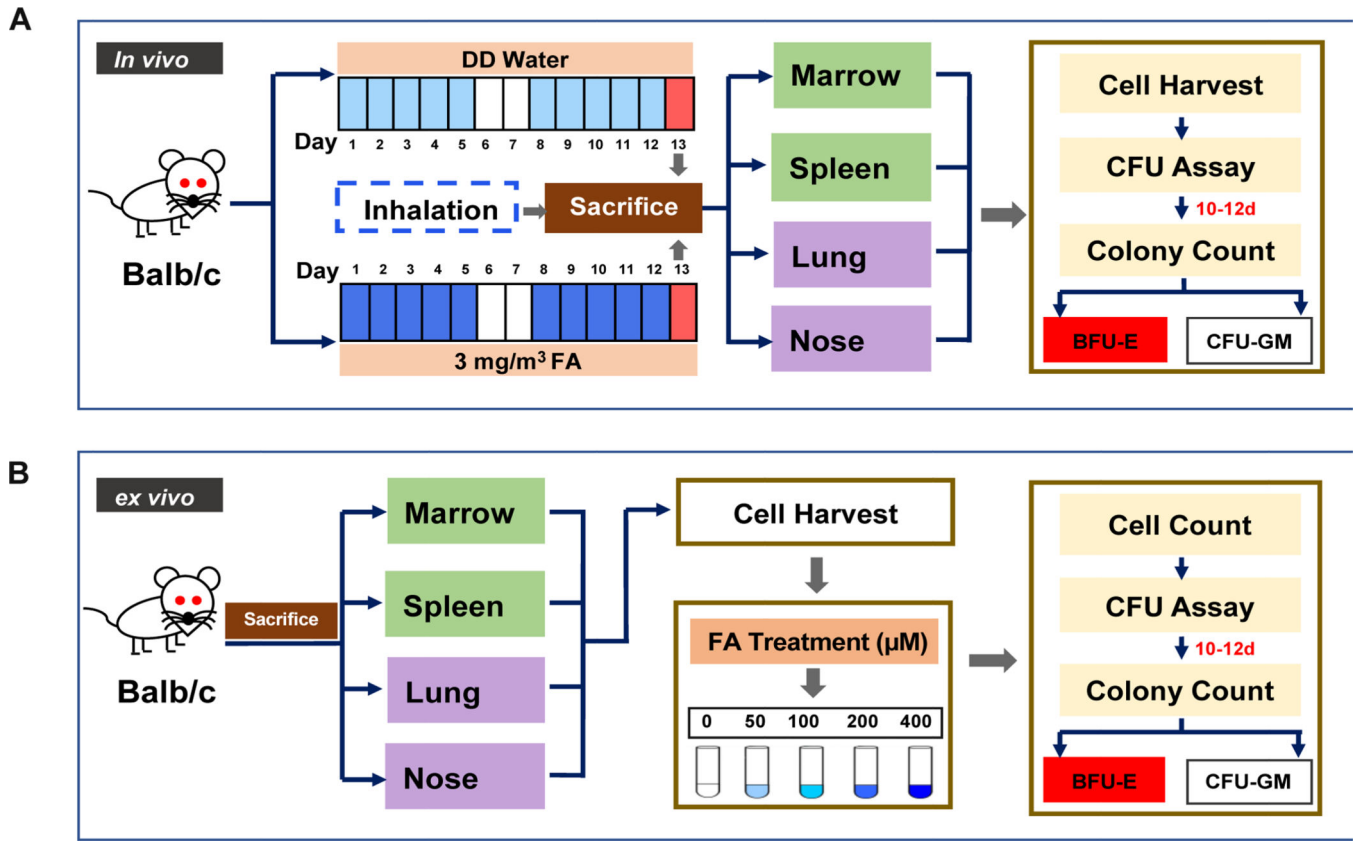

Fig. 1.
Experimental design and workflow. a Experiment in vivo. b Experiment ex vivo

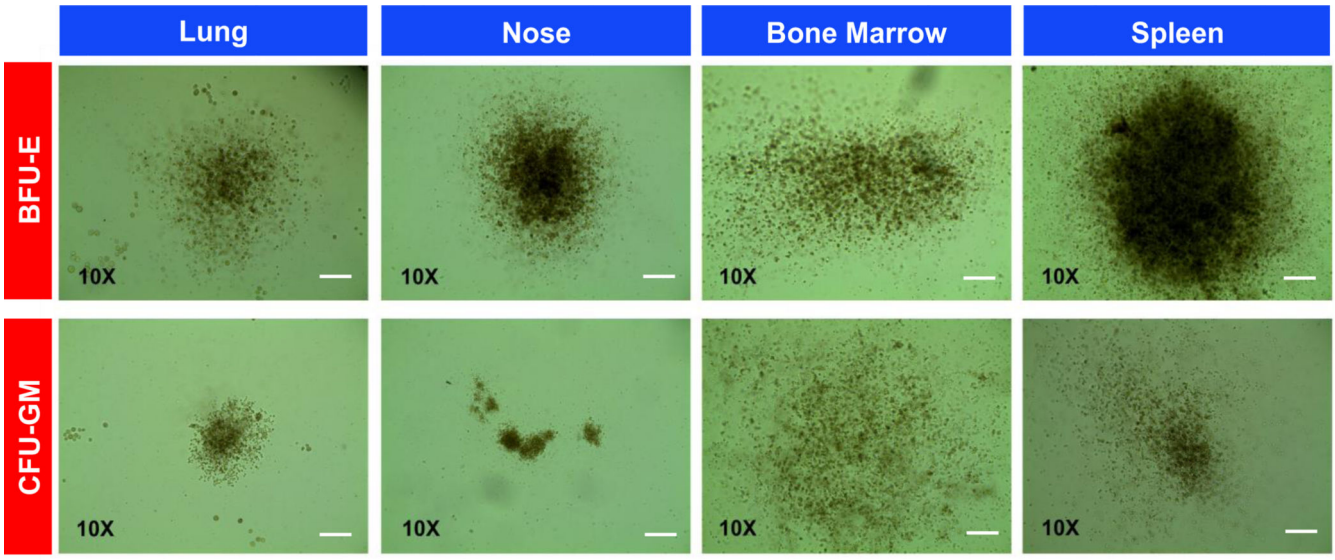

Fig. 2.

BFU-E and CFU-GM colonies cultured from the lung, nose, BM and spleen of BALB/c mice. Based on a guideline from StemCell Technologies™, erythroid cells tend to grow together forming a “wrinkled” cluster (BFU-E) in which individual cells cannot be distinguished. In contrast, in the CFU-GM colonies, individual cells are “phase-bright” or “clear” in appearance, enabling the identification of this colony type

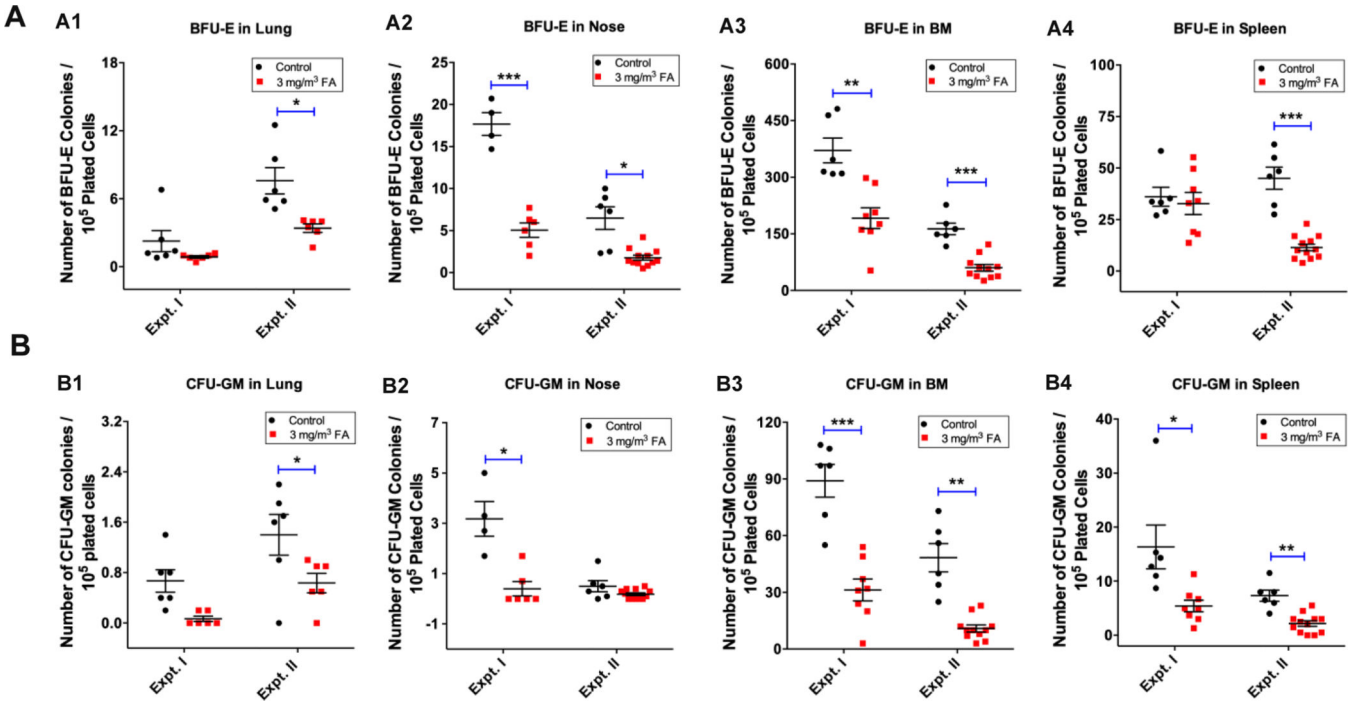

Fig. 3.

The formation of both BFU-E and CFU-GM colonies from all four tissues (lung, nose, BM and spleen) of BALB/c mice exposed to vehicle or 3 mg/m^3 FA in vivo. The experiments were conducted independently by two researchers and the results are shown as Expt. I and Expt. II. Each tissue type was cultured in duplicate. Each data point represents one replicate of each tissue type. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, as compared to control

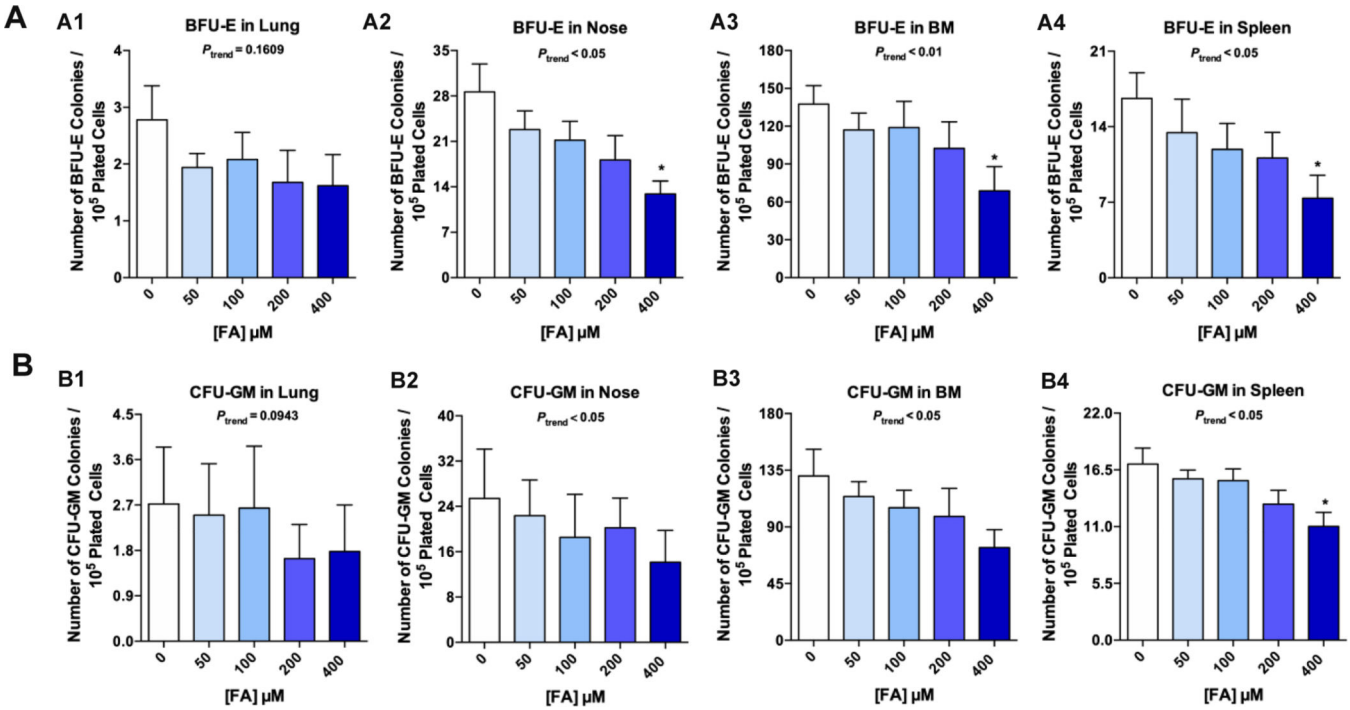

Fig. 4.

The formation of both BFU-E and CFU-GM colonies from all four tissues (lung, nose, BM and spleen) of BALB/c mice exposed to vehicle or FA ex vivo. The experiments were independently repeated 5 times for lung, BM and spleen, and 4 times for nose. Data at each dose were merged from 4–5 repeated experiments. Each tissue type was cultured in duplicate. The average of the duplicate for each tissue type represents each individual experiment. Data are presented as mean ± SEM. *$p < 0.05$ as compared to control

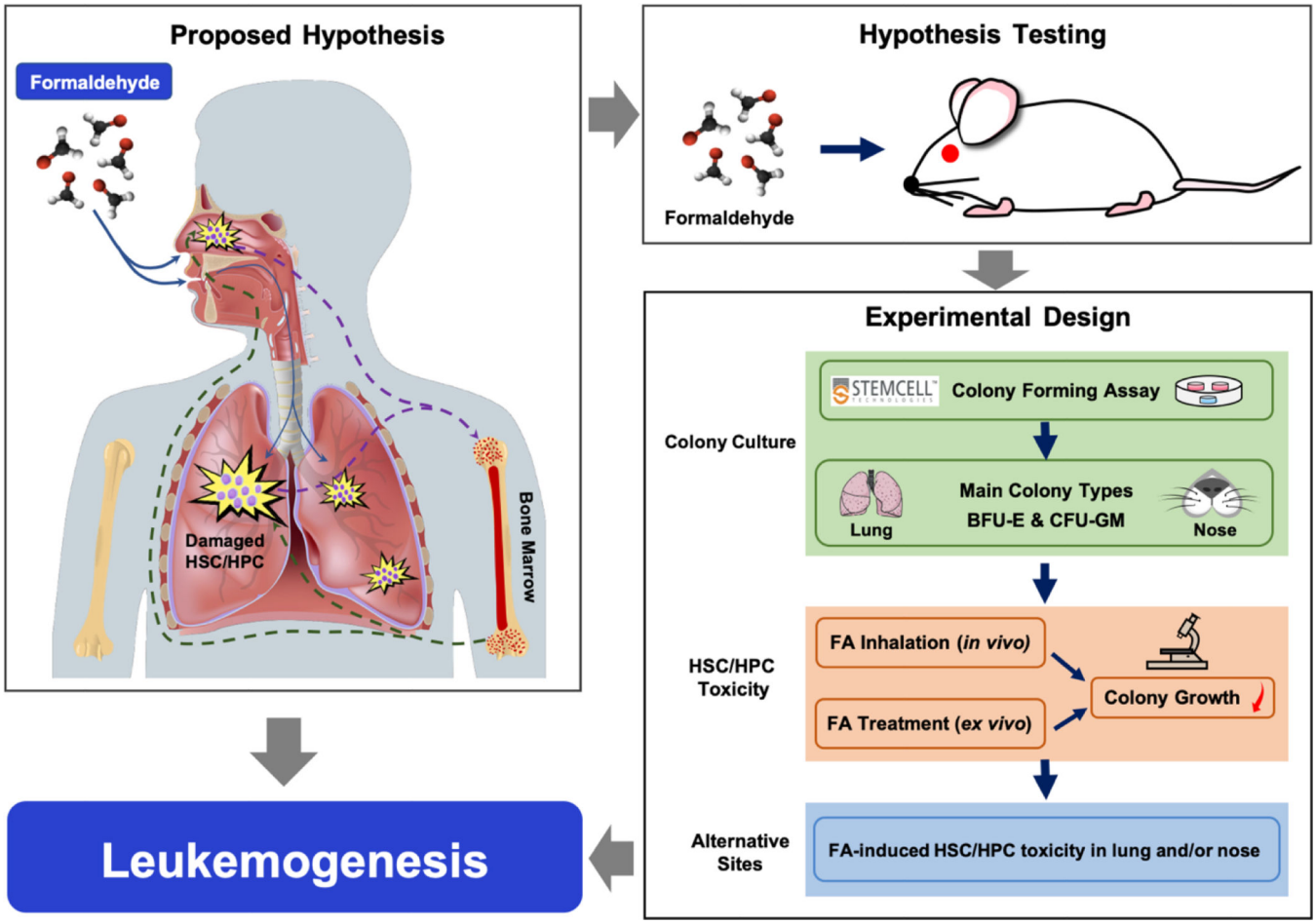

Fig. 5.

Overview of our study hypothesis, testing model and experimental design for detection of HSC/HPC toxicity induced by FA in mouse lung and nose. (Note: The image of human background figure is adapted from https://www.pngwing.com.)