The insecticides permethrin and chlorpyrifos show limited genotoxicity and no leukemogenic potential in human and murine hematopoietic stem progenitor cells

Epidemiological and clinical studies have revealed that maternal exposure to pesticides-insecticides during pregnancy is associated with an increased risk of infant or childhood acute leukemia.[1-3] The household insecticides permethrin and chlorpyrifos, which are members of the pyrethroid and organophosphate families of pesticides, respectively, have been associated with both the induction of *MLL* rearrangements (*MLL*r) and the development of infant acute leukemia.[4-9] Despite the epidemiological association between insecticides and increased risk of leukemia, whether such insecticides act as topo isomerase II DNA-damaging poisons remains unknown and studies testing the biological plausibility of such an association are lacking. Here, we assessed the genotoxicity, induction of *MLL*r and leukemogenic potential of permethrin and chlorpyrifos by taking advantage of cutting-edge *in vitro* and *in vivo* models using prenatal, neonatal and adult hematopoietic stem and progenitor cells (HSPC). Our data suggest that the insecticides tested show no cytotoxicity, limited genotoxicity and no leukemogenic potential in human and murine HSPC *in vitro* and *in vivo*.

We initially assessed whether acute exposure to etoposide (1 μM), permethrin or chlorpyrifos (10 μM or 50 μM) induces *MLL* breaks in undifferentiated human embryonic stem cells (hESC) and CD34$^+$ HSPC derived from hESC, cord blood or adult peripheral blood (Figure 1A). Acute exposure (24 h) to either permethrin or chlorpyrifos consistently induced *MLL* breaks in 3–7% of embryonic, neonatal and adult CD34$^+$ cells in a dose-independent manner (Figure 1B, C). Embryonic and somatic CD34$^+$ cells were sensitive to the three treatments (Figure 1C). Of note, whereas embryonic and neonatal CD34$^+$ cells were slightly more sensitive to etoposide than to insecticides (Figure 1C), no differences were found for adult CD34$^+$ cells, suggesting that the potential genotoxicity of the insecticides may have a more relevant etiological impact in adult *MLL*r acute leukemia.

Chronic exposure to low doses of etoposide has been reported to induce apoptosis, *MLL* breaks and major chromosomal abnormalities in hESC.[10] To test whether this occurred after exposure to insecticides, we treated hESC for 24 h with 10 μM of either permethrin or chlorpyrifos followed by a daily "booster" dose (2 μM) for 40 days (Figure 1D). After 5 days of recovery (without treatments), *MLL*r breaks and gross genomic abnormalities were assayed by interphase fluorescence *in situ* hybridization (iFISH), G-banding and comparative genomic hybridization (Figure 1E-G). In contrast to the frequency of *MLL* breaks (3–7%) observed after acute exposure (Figure 1C), *MLL* breaks were scarcely detectable upon chronic exposure to permethrin or chlorpyrifos, suggesting a legitimate repair of the DNA damage/double strand breaks (DSB) at the *MLL* locus (Figure 1E). Likewise, karyotyping and comparative genomic hybridization analysis revealed no numerical or structural chromosomal alterations (Figure 1F) or DNA gains or losses after chronic exposure (Figure 1G). It has been previously suggested that the chromosomal topology and chromatin structure resulting from early apoptosis may represent the underlying substrate for *MLL* chromosomal translocations to occur.[11] Here, in contrast to etoposide, which did cause significant cell death at doses as low as 1 μM, neither permethrin nor chlorpyrifos induced cell death/apoptosis at doses 50-fold higher (*Online Supplementary Figure S1A*), supporting the concept that the absence of high-grade DNA fragmentation may represent a chromatin physical impediment for *MLL* DSB to fuse in-frame with a partner gene and encode an oncogenic fusion protein. Inverse polymerase chain reaction assays confirmed the absence of in-frame *MLL* fusions (*data not shown*). Overall, these data indicate that chronic exposure to insecticides neither enriches for *MLL* breaks nor generates *MLL* fusion oncogenes or gross genomic instability.

To test the *bona fide* ability of permethrin and chlorpyrifos to function as topoisomerase II poisons, and their ability to generate DNA-DSB we performed an *in vivo* complex of enzyme assays to analyze covalent genomic DNA/topoisomerase II complexes in live cells.[12] Treatment of hESC with permethrin and chlorpyrifos at 10–500 μM induced only minimal poisoning of the topoisomerase II isoforms (α and β), whereas as little as 1 μM etoposide induced significant poisoning of both isoforms (*Online Supplementary Figure S1B*). Similarly, monitoring of γ-H2AX by western blotting (*Online Supplementary Figure S1C*) or fluorescent activated cell sorting (FACS) analysis (*Online Supplementary Figure S1D-F*) revealed that the pesticides were unable to induce DSB in hESC, neonatal or adult CD34$^+$ cells at relatively high concentrations, whereas etoposide potently induced DSB that were slightly repaired to some extent over time in somatic CD34$^+$ cells but not in hESC (*Online Supplementary Figure S1F*). Overall, the data indicate that permethrin and chlorpyrifos are not topoisomerase II poisons and do not generate DNA-DSB in embryonic or somatic CD34$^+$ cells.

We next sought to determine whether prolonged *in vivo* exposure of human CD34$^+$ HSPC to pesticides induces *MLL* breaks or initiates leukemia (Figure 2A). Immunodeficient (NSG) mice xenotransplanted with cord blood-derived human CD34$^+$ HSPC were exposed for 12 weeks to permethrin or chlorpyrifos in the drinking water to mimic the environmental exposure in humans. Our target concentration for pesticide exposure was 10 mg/kg/day, and 5 mg/kg/day for etoposide. As the actual concentration to which mice are exposed will depend on their weight and their consumption of drinking water over the experimental period, we measured both parameters weekly. Results revealed an actual intake of permethrin, chlorpyrifos and etoposide only 10–20% lower than the theoretical concentrations (8.5, 9 and 4.2 mg/kg/day, respectively) (*Online Supplementary Figure S2A-C*). Importantly, the permethrin and chlorpyrifos metabolites 3-BPA and TCPy were readily detected by gas chromatography-mass spectrometry in serum and urine as soon as 48 h after pesticide exposure, confirming consistent exposure through administration of drinking water (*Online Supplementary Figure S2D*). iFISH analysis at sacrifice revealed a small but significant increase in the frequency of bone marrow-engrafted human CD45$^+$ cells harboring *MLL* breaks in etoposide-treated mice, but not in permethrin- or chlorpyrifos-treated mice (Figure 2B). FACS analysis at sacrifice revealed similar levels of human graft and normal multilineage (immature, myeloid and B-cell lymphoid) engraftment in the bone marriow and peripheral blood of etoposide-, permethrin- and chlorpyrifos-treated mice (Figure 2C, *Online Supplementary Figure S2E*), with no evidence of splenomegaly (Figure 2D). These findings indicate that chronic *in vivo* exposure to the indicated

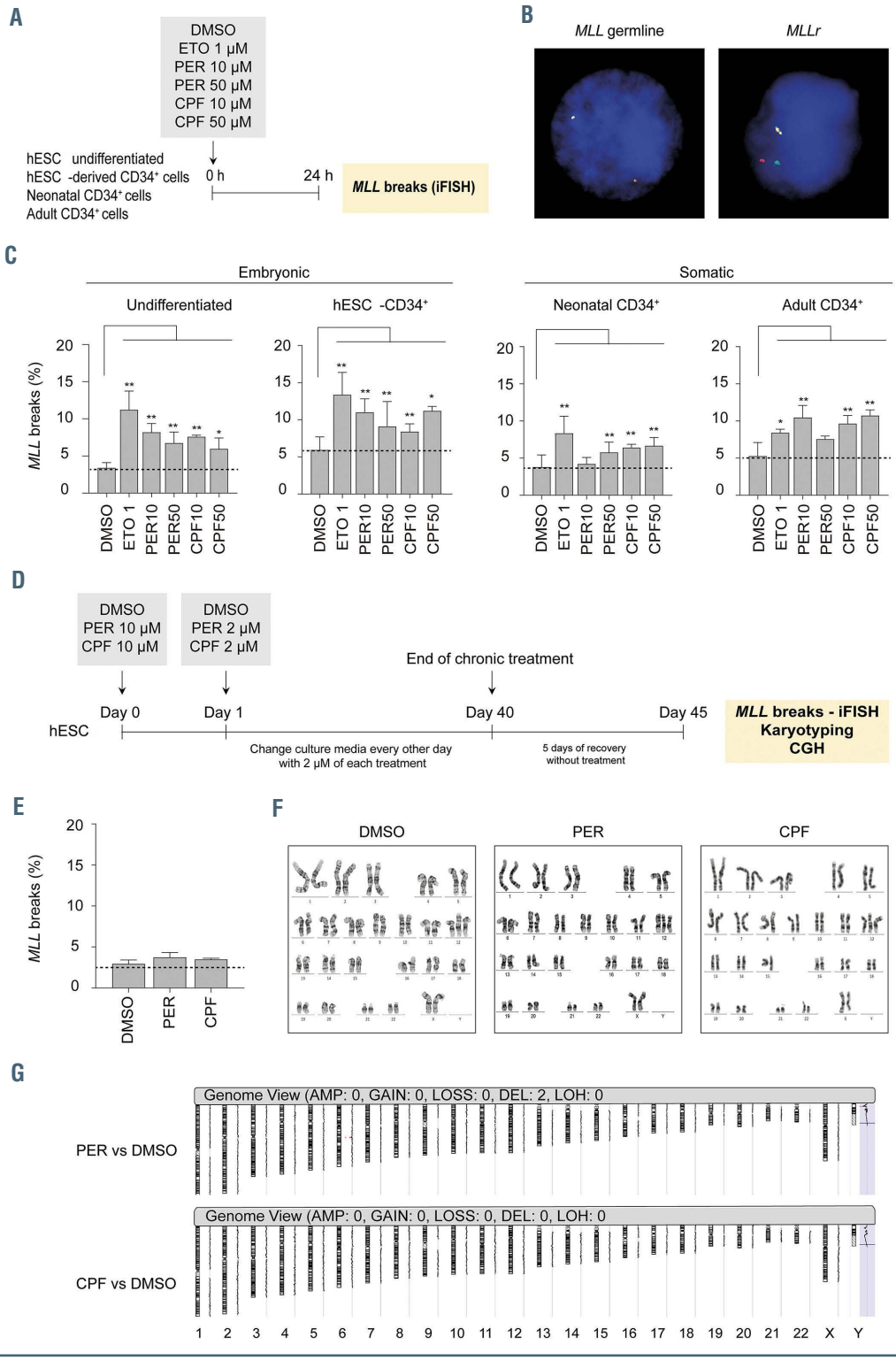

Figure 1. *In vitro* induction of *MLL* rearrangements in embryonic, neonatal and adult human CD34$^+$ hematopoietic stem and progenitor cells following acute and chronic exposure to etoposide, permethrin and chlorpyrifos. (A) Experimental design to assess the induction of *MLL* breaks in human undifferentiated embryonic stem cells (hESC) and CD34$^+$ hematopoietic stem and progenitor cells (HSPC) after 24 h single-pulse exposure to the indicated treatments (etoposide, ETO; permethrin, PER; and chlorpyrifos, CPF). (B) Representative interphase fluorescence *in situ* hybridization (iFISH) images showing *MLL* germline and rearranged (*MLLr*) human CD34$^+$ cells. (C) Frequency of *MLL* breaks in undifferentiated hESC and embryonic, neonatal and adult CD34$^+$ HSPC after 24 h single-pulse exposure to the indicated treatments (n=3 independent experiments for each cell type). Asterisks indicate statistically significant differences of a given treatment as compared with dimethylsulfoxide (DMSO, vehicle treatment) *P<0.05, **P<0.01. Dotted lines in the graphs show the percentage of *MLL* breaks in the DMSO-treated control groups. A minimum of 500 nuclei were analyzed except in some samples for which 80-400 nuclei were analyzed. (D) Experimental design to analyze the frequency of *MLL breaks* and gross chromosomal damage in hESC after continuous exposure to PER and CPF. (E) Frequency of *MLL* breaks detected by iFISH 45 days after chronic treatment with either PER or CPF (n=3 independent experiments). A minimum of 400 nuclei was analyzed per experiment. (F) Representative image of a G-banding karyotype 45 days after chronic treatment with either PER, CPF or DMSO (n=3). (G) Representative image of DNA copy number variation profiling by comparative genomic hybridization array analysis 45 days after chronic treatment with either PER or CPF. CGH: comparative genomic hybridization; AMP: amplification; DEL: deletion; LOH: loss of heterozygosity.

doses of etoposide or the pesticides fails to induce *MLL* breaks or to initiate leukemia in NSG-reconstituting human CD34$^+$ HSPC.

We next assessed whether chronic exposure to pesticides during embryonic development induces *Mll* breaks in developing murine HSPC. To do this, CD1 male and female mice were mated and pregnant CD1 females were exposed to etoposide (10 mg/kg/day) or to pesticides (20 mg/kg/day) in drinking water from gestational day 0.5 to day 21 (Figure 3A). No significant differences were found between treatment groups for the number of pups per litter at birth (range, 11–18) or sex distribution (*Online Supplementary Figure S3A, B*). The mothers and one-half of the litter were euthanized at weaning to analyze the impact of etoposide and pesticides on the *Mll* locus and on hematopoietic homeostasis, and the

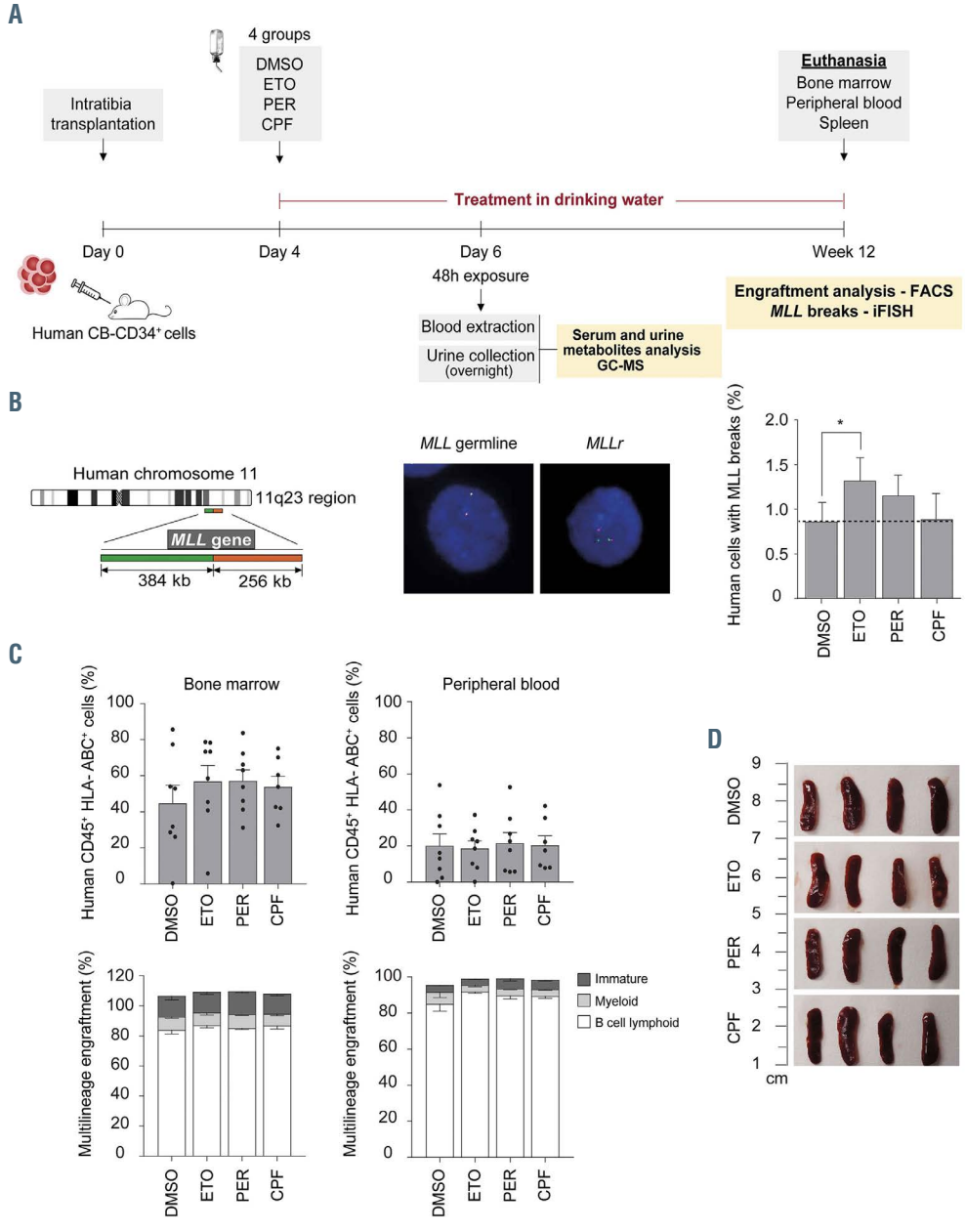

Figure 2. Continuous exposure to permethrin or chlorpyrifos fails to induce *MLL* breaks or leukemia in NSG-reconstituting human CD34$^+$ hematopoietic stem and progenitor cells. (A) Experimental design to determine whether prolonged *in vivo* exposure of human CD34$^+$ hematopoietic stem and progenitor cells (HSPC) to insecticides induces *MLL* breaks or can initiate leukemia. In brief, 32 NSG mice were irradiated and cord blood-derived human CD34$^+$ cells were transplanted into the bone marrow 6–8 h later. Four days later, mice were divided into four groups (8 mice/group) to initiate exposure in drinking water to etoposide (ETO, 5 mg/kg/day), permethrin (PER, 10 mg/kg/day), and chlorpyrifos (CPF, 10 mg/kg/day) or 0.1% dimethylsulfoxide (DMSO). The presence of the PER and CPF metabolites 3-BPA and TCPy in urine and serum was analyzed 48 h later by gas chromatography-mass spectometry. Mice were sacrificed for interphase fluorescence *in situ* hybridization (iFISH) and fluorescence activated cell sorting (FACS) analysis after 12 weeks of continuous treatment. (B) *Left*, scheme depicting the human chromosome 11 and the 11q23 region where the *MLL* probe hybridizes. *Middle*, representative iFISH image showing human cells with germline *MLL* or *MLL* rearrangement (*MLLr*). *Right*, percentage of human CD45$^+$ cells harboring *MLL* breaks detected by iFISH at sacrifice. A minimum of 500 nuclei per sample was analyzed, except in one mouse from the DMSO group, for which only 216 nuclei could be analyzed. (C) Upper panels show the percentage of human engraftment (CD45$^+$HLA$^-$ABC$^+$ cells) in bone marrow and peripheral blood. Lower panels show the relative proportion of immature (CD34$^+$), myeloid (CD33$^+$) and B-cell (CD19$^+$) populations within human engraftment in bone marrow and peripheral blood. (D) Representative macroscopic images of spleens at sacrifice for each experimental group. GC-MS: gas chromatography-mass spectrometry.

remaining half of the litter was maintained for 32 weeks for analysis at adulthood (Figure 3A). iFISH analysis revealed that exposure to permethrin or chlorpyrifos during pregnancy failed to induce *Mll* breaks in bone marrow Lin$^-$Kit$^+$ progenitors from mothers, pups or adult offspring, whereas etoposide exposure induced *Mll* breaks in a small but significant proportion of Lin$^-$Kit$^+$ progenitors in the mothers and adult offspring, but not in the pups (Figure 3B, C, *Online Supplementary Figure S3C*).

Finally, we analyzed whether prenatal exposure to etoposide or pesticides affects the hematopoietic homeostasis of mothers and pups (at weaning) and adult offspring. Analysis at sacrifice revealed no significant differ-

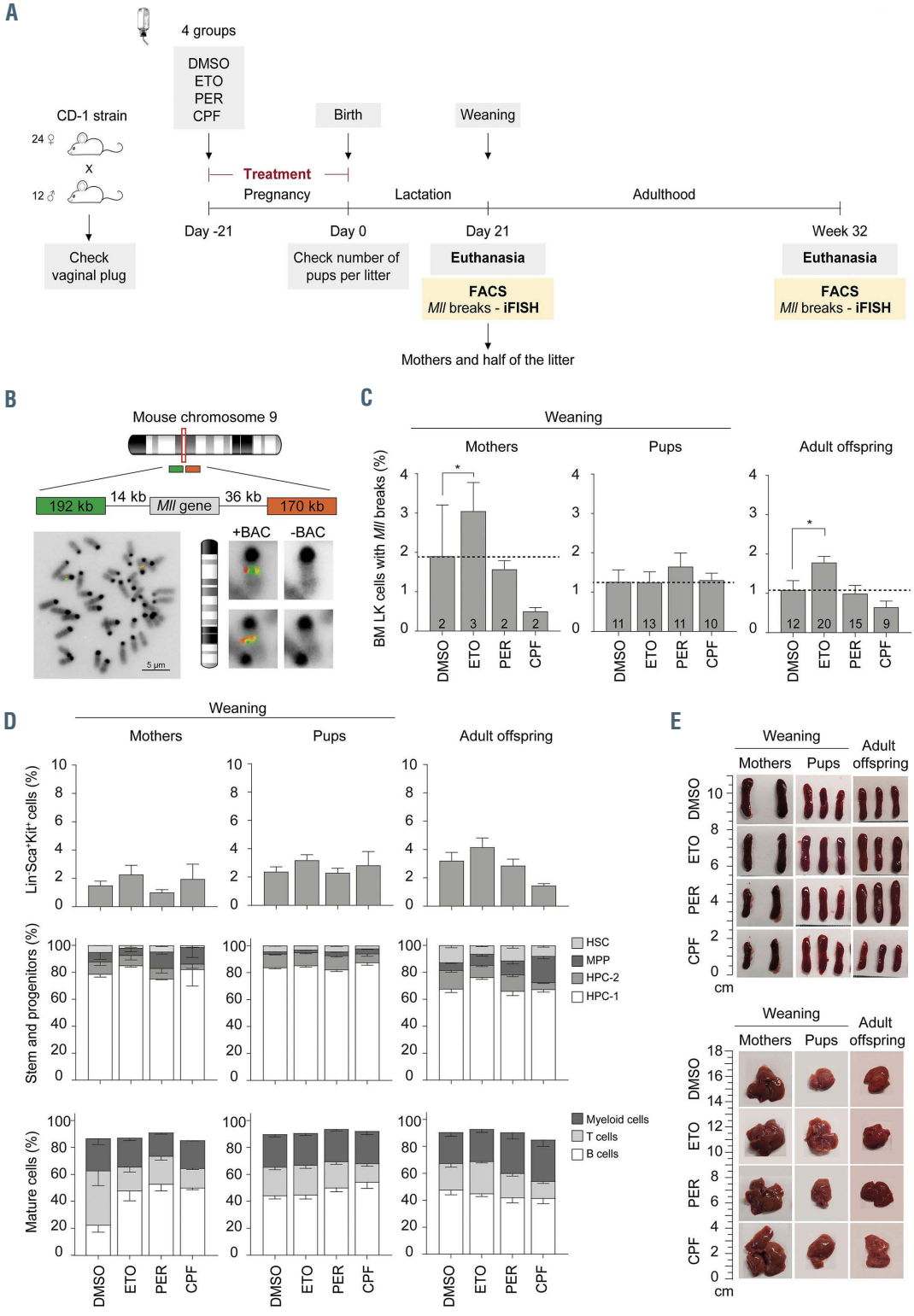

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Figure 3. Continuous exposure to permethrin or chlorpyrifos during pregnancy fails to induce *Mll* breaks in bone marrow progenitors or alterations in the hematopoietic homeostasis of mothers, pups or adult offspring in mice. (A) Experimental design to determine whether prenatal exposure to etoposide (ETO), permethrin (PER) or chlorpyrifos (CPF) induces *Mll* breaks or hematopoietic alterations in mothers, pups or adult offspring in mice. In brief, pregnant CD1 female mice were exposed to PER (20 mg/kg/day), CPF (20 mg/kg/day), ETO (10mg/kg/day) or 0.1 % dimethylsulfoxide (DMSO) from day 0 to day 21 of gestation. The number of pups per litter and their sex distribution were analyzed at birth. The mothers and one-half of the litter were analyzed at weaning while the remaining one-half of the offspring were maintained for 32 weeks for analysis in adulthood. (B) *Upper panel*, scheme depicting mouse chromosome 9 and the region where mouse *Mll* probes hybridize (UCSC, GRCm38/mm10). *Lower left panel*, a representative fluorescence *in situ* hybridization (FISH) image of a mouse metaphase and presence of fluorescence signals in both *Mll* alleles. *Lower right panel*, a zoom image of mouse chromosome 9 by DAPI banding, with and without the BAC fluorescence signal revealing the *Mll* gene localization. (C) Percentage of mouse bone marrow (BM) LK cells with *Mll* breaks detected by interphase FISH at sacrifice. DMSO and ETO were used as negative and positive controls, respectively. The numbers in bars indicate the number of mice analyzed. (D) Fluorescence activated cells sorting (FACS) BM analysis of the Lin^- $Sca\text{-}1^+$ Kit^+ (LSK) subpopulation (upper panels), hematopoietic stem and progenitor cell subsets (middle panels), and mature cells (lower panels) in mothers, pups and adult offspring. (E) Representative macroscopic images of spleens (upper panel) and livers (lower panel) at sacrifice of mothers, pups and adult offspring exposed to the indicated treatments. HSC: hematopoietic stem cells; MPP: multipotent progenitors; HPC: hematopoietic progenitor cells.

ences in the proportions of mature cells (myeloid, T and B) in peripheral blood or total $Lin^-Sca^+Kit^+$ progenitors, hematopoietic stem cells, multipotent progenitors or hematopoietic progenitor cells in the bone marrow between treatments in any group (Figure 3D, *Online Supplementary Figure S3D*). Similarly, prenatal exposure to etoposide or pesticides did not affect the hematopoietic homeostasis in peripheral blood, as determined by absolute numbers of white blood cells, red blood cells and platelets (*Online Supplementary Figure S3E*). Lastly, no evidence of splenomegaly or hepatomegaly was observed in mothers, pups or adult offspring (Figure 3E). Our results thus suggest that chronic exposure to permethrin or chlorpyrifos during pregnancy does not induce *Mll* breaks in bone marrow progenitors or alterations in the hematopoietic homeostasis of mothers, pups or adult offspring.

A unique strength of the present study is the cutting-edge *in vivo* models employed to assess the genotoxicity and leukemogenesis potential of etoposide, permethrin or chlorpyrifos. The NSG mice model was established to mimic the adult exposure associated with occupational risk, whereas the CD1 mice model attempted to mimic prenatal exposure to topoisomerase II poisons and insecticides suggested to be involved in the etiology of infant leukemia. Continuous exposure to permethrin or chlorpyrifos in both models failed to induce *MLL* breaks or alterations in hematopoietic homeostasis, confirming the *in vitro* results of limited genotoxicity and no leukemogenic potential of permethrin or chlorpyrifos in human and murine HSPC after chronic exposure. The fact that *MLL* breaks are acutely induced by permethrin or chlorpyrifos but are not sustained upon long-term chronic exposure *in vitro* or *in vivo* indicates a legitimate repair of the DNA damage/DSB in the *MLL* locus. Of note, although long-term *in vivo* exposure to etoposide did induce *MLLr* in some hematopoietic progenitors, it failed to initiate leukemia in either *in vivo* models, in line with a previous study confirming that *in utero* exposure to etoposide did not trigger the development of leukemia in either $Atm^{+/+}$ or $Atm^{-/-}$ mice.[13] The eventual development of overt leukemia might depend on the survival and proliferative advantage of minor *MLLr* pre-leukemic clones, targeting the right cell-of-origin, on stromal bone marrow interactions and also on the acquisition of secondary cooperating oncogenic alterations. The clearance and lack of selection of *MLLr* clones is consistent with the development of *MLLr* treatment-related acute leukemia in adults or infant leukemia in only a rare subset of patients exposed to topoisomerase II poisons.

Our results clearly suggest that permethrin and chlorpyrifos induce *MLL* breaks in human HSPC across ontogeny. However, such insecticide-induced DNA-DSB are successfully repaired, and do not involve chromosomal translocations encoding *MLL* fusions. This explains their limited genotoxicity and no leukemogenic potential and reinforces why these compounds are still considered as non-classifiable carcinogens. Linking environmental or genotoxic exposure to causal and/or functional *MLL* chromosomal translocations has long been controversial,[11] and the cutting-edge *in vitro* and *in vivo* cellular models employed in the present study also have obvious limitations. For instance, site-specific cleavage within the *MLL* break cluster region (bcr) has been shown to be induced by either topoisomerase II poisons but also genotoxic chemotherapeutic agents which do not target topoisomerase II and even by non-genotoxic stimuli of apoptotic cell death. In addition, *MLL* chromosomal translocations have been linked to higher-order chromatin fragmentation that occurs during the initial stages of apoptosis, suggesting that the generation of *MLL* chromosomal translocations (and likely others) are part of a generalized acute apoptotic response-mediated higher-order chromatin fragmentation which ultimately renders a chromosome topology and chromatin structure prone to chromosomal DNA exchanges.[14] This is further supported by the ambiguity of *MLL* translocations partnering with a large number of different chromosomal loci.

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