Impact of pinworm infection on the development of murine B-cell leukemia/lymphoma in the presence and absence of *ETV6::RUNX1*

B-cell acute lymphoblastic leukemia (B-ALL) is the most common malignant disease in childhood with a peak age at 2-6 years old. A two-step model has been proposed for the development of childhood B-ALL.[1] The first step is an early somatic genetic rearrangement, such as the *ETV6::RUNX1* fusion gene, followed by a broad range of secondary mutational events driven by environmental stimuli (including infection and abnormal cytokine release from immunologically untrained cells).[1] The involvement of at least two discrete steps suggests that B-ALL may be preventable in infants with a genetically initiated step, who could be protected from harmful postnatal environmental stimuli.

In the past decades, infections have been regarded as the environmental stimuli with the most impact in the etiology of childhood B-ALL. Common pathogens may drive secondary mutations in genetically predisposed subjects.[2] Experimental models of infection can be leveraged in xenograft and animal models that closely resemble the pathophysiology of childhood B-ALL. For example, two animal studies demonstrated that transgenic mice (with the *ETV6::RUNX1* fusion or with *Pax5*$^{+/-}$ heterozygosity) only developed B-ALL when they were exposed to common infections, although with incomplete penetrance.[3,4] These past studies indicate that infections can act as important promoters of B-ALL development in the context of genetic predispositions. However, exposure to pathogens early in life via childhood contacts (daycare, microbiome) may modulate immune reactivity and decrease risk.[2] Through a serendipitous observation, we found that the impact of pinworm infection on leukemogenesis was markedly different depending on the presence or absence of a common human somatic genetic change.

Pinworms are a commonly found intestinal helminth in laboratory animals and the control of these pathogens in animal holdings is quite difficult.[3] We performed a retrospective analysis of the latency and incidence of leukemia/lymphoma of two strains of mice during and after a pinworm outbreak in a specific-pathogen-free (SPF) facility (Figure 1A). *Cdkn2a*$^{-/-}$ and *ETV6::RUNX1*$^{+}$ *Cdkn2a*$^{-/-}$ (referred to as *E6R1*$^{+}$*Cdkn2a*$^{-/-}$ in the current work and Cre^{+} TA^{+} *Cdkn2a*$^{-/-}$ in the past work[4]) mice were maintained on the FVB/N strain background and were age- and sex-matched for each survival experiment. In line with the details of our animal use protocol, body condition scoring, clinical signs, and a diagnosis of neoplasia were used, in consultation with veterinarians, to identify animals that had reached our predefined study endpoints. A gross necropsy was performed to identify potential sources of illness. Selected tissues were preserved through formalin-fixed paraffin embedding and stained with hematoxylin & eosin. Diagnoses were based upon gross necropsy and histopathology. If gross necropsy findings suggested a hematopoietic neoplasm, single-cell suspensions of involved tissues were cryopreserved in medium containing 10% dimethylsulfoxide. Additional diagnostic information was obtained by immunophenotyping when necessary. All experiments were performed following institutional review and approval by the University of California San Francisco Institutional Animal Care and Use Committee.

We previously reported that *ETV6::RUNX1* expression cooperates with *Cdkn2a* deletion to promote the development of B-ALL in mice (Figure 1B). After 2013, the leukemogenic effect of *E6R1* expression was no longer observed, as demonstrated by two independent experiments showing overlap (Figure 1C) or minimal separation (Figure 1D) between the *Cdkn2a*$^{-/-}$ and *E6R1*$^{+}$ *Cdkn2a*$^{-/-}$ survival curves. Review of the infection records in the SPF facility revealed that an outbreak of the pinworm *Aspicularis* was detected by fecal floatation testing of sentinels during the timeframe when decreased latency for leukemia/lymphoma had been observed in *E6R1*$^{+}$ *Cdkn2a*$^{-/-}$ mice in comparison with *Cdkn2a*$^{-/-}$ mice. Following the outbreak of *Aspicularis*, all mice in the room were treated with the broad spectrum antihelminthic fenbendazole. The diminished effect of *E6R1* on promoting leukemia/lymphoma development was observed after the eradication of pinworm.

We then prospectively investigated the impact of intentional pinworm exposure on leukemogenesis. To determine whether pinworm infection could restore the leukemogenic effect of *E6R1* in the *Cdkn2a*$^{-/-}$ model, 4-week-old *Cdkn2a*$^{-/-}$ and *E6R1*$^{+}$ *Cdkn2a*$^{-/-}$ mice were transferred from an SPF facility to an *Aspicularis*-infected conventional facility (also detected by fecal floatation testing of sentinels), where they were followed for survival (Figure 1A). In the context of pinworm infection, *E6R1*$^{+}$ *Cdkn2a*$^{-/-}$ mice developed leukemia/lymphoma earlier and with a higher incidence than *Cdkn2a*$^{-/-}$ mice (Figure 1E). Together, these survival studies indicate a different impact of pinworm infection on the development of leukemia/lymphoma in *Cdkn2a*$^{-/-}$ and *E6R1 Cdkn2a*$^{-/-}$ mice.

Given the well-established role of the *E6R1* mutation in co-

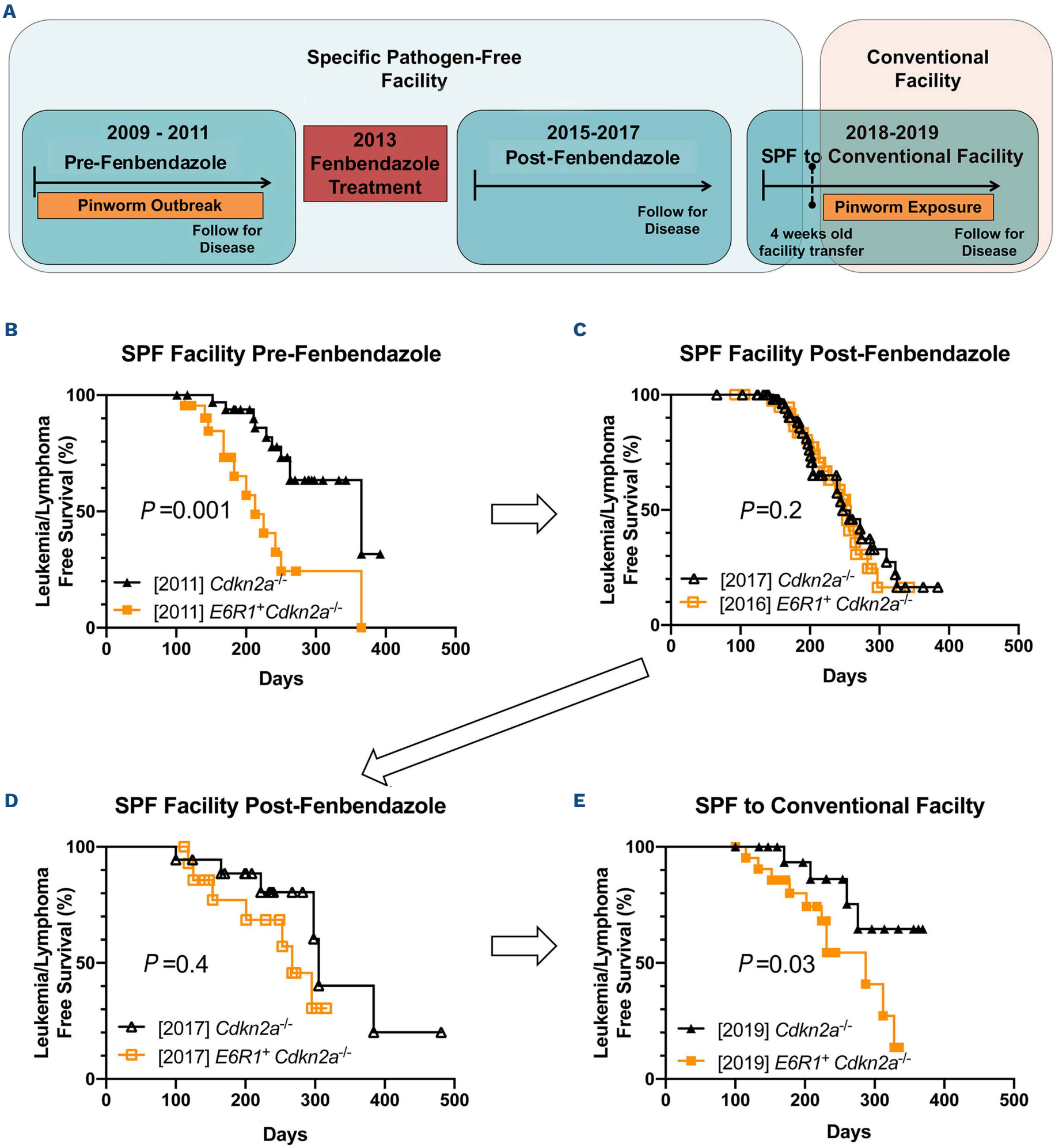

Figure 1. Pinworm exposure drives differences in leukemia/lymphoma development between *Cdkn2a*$^{-/-}$ and *E6R1*$^{+}$ *Cdkn2a*$^{-/-}$ mice. (A) Timeline of individual survival studies following *Cdkn2a*$^{-/-}$ and *E6R1*$^{+}$ Cdkn2a$^{-/-}$ mice in specific pathogen-free (SPF) and conventional facilities relative to the 2013 fenbendazole treatment. (B) Survival curves of leukemia/lymphoma development in *Cdkn2a*$^{-/-}$ (N=34) and *E6R1*$^{+}$ *Cdkn2a*$^{-/-}$ (N=22) mice housed in an SPF facility during a pinworm outbreak prior to fenbendazole treatment. Arrows indicate the chronological order of the survival studies. The year in brackets corresponds to the date of euthanasia of the last mouse to develop illness in each cohort. (C, D) Survival curves from two independent experiments of mice housed in an SPF facility after pinworm was eradicated with fenbendazole treatment. 2016-2017: *Cdkn2a*$^{-/-}$ (N=58) and *E6R1*$^{+}$ *Cdkn2a*$^{-/-}$ (N=40); 2017: *Cdkn2a*$^{-/-}$ (N=18) and *E6R1*$^{+}$ C*dkn2a*$^{-/-}$ (N=15). (E) Survival curve from one experiment in which *Cdkn2a*$^{-/-}$ (N=20) and *E6R1*$^{+}$ *Cdkn2a*$^{-/-}$ (N=22) mice were housed in an SPF facility for 4 weeks, then transferred to a conventional facility for exposure to pinworm bedding. The log-rank (Mantel-Cox) test was applied to the survival curves.

operating with radiation, chemicals, and infectious exposures to promote the development of lymphoid malignancies,[3,4,6,7] we had hypothesized that pinworm infection would promote leukemogenesis and have a stronger effect in *E6R1⁺ Cdkn2a⁻/⁻* mice than in *Cdkn2a⁻/⁻* mice. We examined this hypothesis by aggregating the survival cohorts from the studies shown in Figure 1. Mice In a pinworm-free facility mice (cohorts shown in Figure 1C, D) were aggregated to serve as pinworm-free controls (Figure 2A). For comparison, survival cohorts of pinworm-infected mice (Figure 1B, E) were also aggregated. Consistent with individual experiments, only pinworm-infected mice demonstrated a statistically significant difference in the development of leukemia/lymphoma between *E6R1⁺ Cdkn2a⁻/⁻* mice and *Cdkn2a⁻/⁻* animals (Figure 2B).

In contrast to our hypothesis, leukemia/lymphoma‐free survival curves revealed that pinworm infection elicited a protective effect in *Cdkn2a⁻/⁻* mice (Figure 2C) that was not observed in *E6R1⁺ Cdkn2a⁻/⁻* mice (Figure 2D). In pinworm-infected *Cdkn2a⁻/⁻* mice, the median latency of leukemia/lymphoma development increased from 305 days to 365 days (Table 1). In contrast, pinworm infection was associated with a decrease in the median latency of leukemia/lymphoma in *E6R1⁺ Cdkn2a⁻/⁻* mice from 253 days to 231 days (Table 1). Although there was an effect on latency, the incidence of leukemia/lymphoma was not affected by pinworm infection (*Online Supplementary*

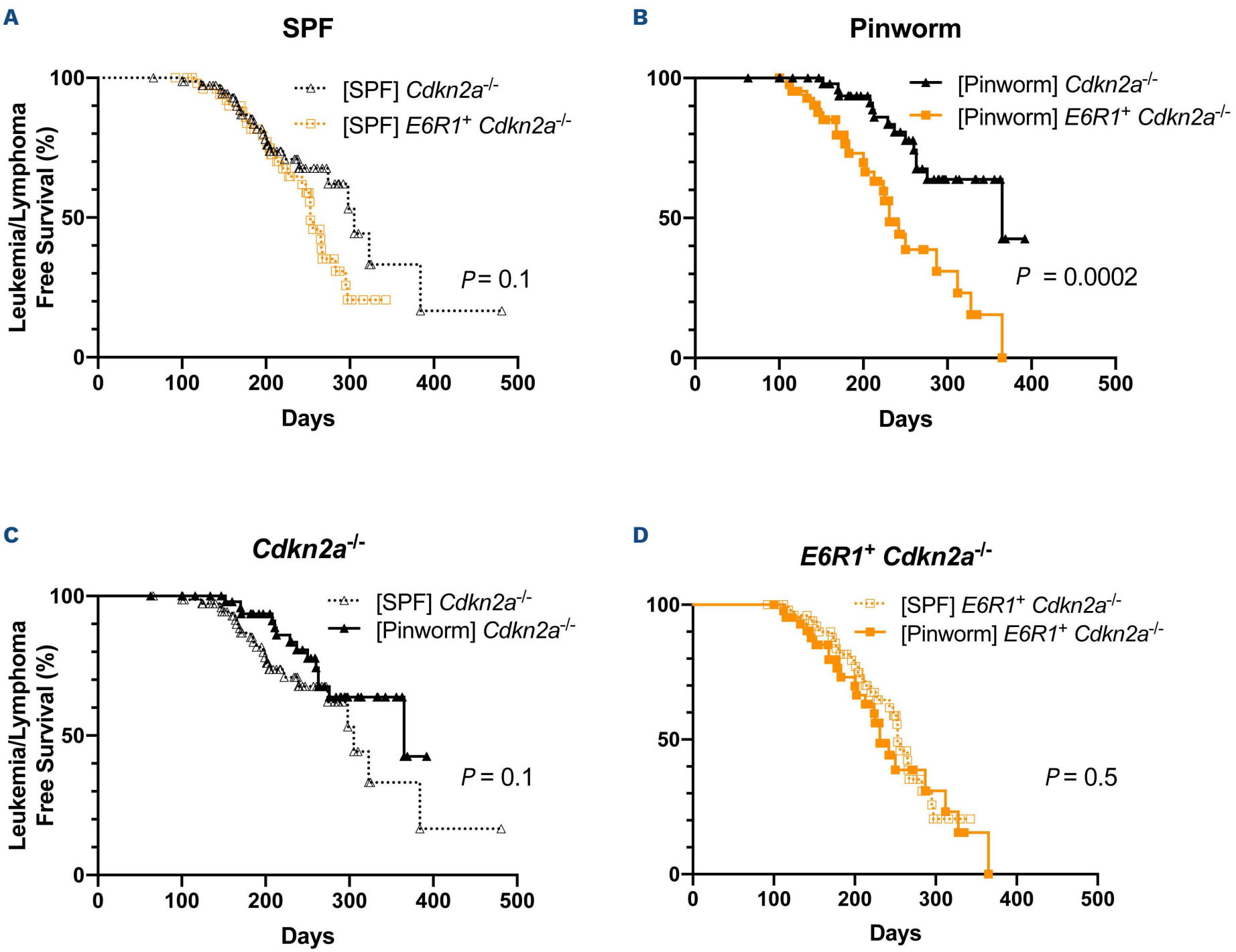

Figure 2. Impact of pinworm exposure on leukemia/lymphoma development in the absence and presence of *E6R1*. Cumulative survival curves showing combined data from Figure 1 of four independent experiments of mice housed in a pinworm-free (SPF) facility or pinworm-infected facility. Leukemia/lymphoma-free survival for (A) SPF-housed (open symbols) and (B) pinworm-exposed (filled symbols) *Cdkn2a⁻/⁻* mice (black triangles) and *E6R1⁺ Cdkn2a⁻/⁻* mice (orange squares). Leukemia/lymphoma-free survival for genotype-matched (C) *Cdkn2a⁻/⁻* mice and (D) *E6R1⁺ Cdkn2a⁻/⁻* mice. SPF *Cdkn2a⁻/⁻* mice (N=76), SPF-housed *E6R1⁺ Cdkn2a⁻/⁻* mice (N=55), pinworm-exposed *Cdkn2a⁻/⁻* mice (N=54), and pinworm-exposed *E6R1⁺ Cdkn2a⁻/⁻* mice (N=44). The log-rank (Mantel-Cox) test was applied to the survival curves.

Table 1. Median latency of development of malignancy in *Cdkn2a*$^{-/-}$ and *E6R1*$^{+}$ *Cdkn2a*$^{-/-}$ mice housed in pinworm-free or pinworm-infected facilities.

Malignancy	Median latency, days			
	SPF *E6R1*$^{+}$*Cdkn2a*$^{-/-}$ N=55	Pinworm *E6R1*$^{+}$*Cdkn2a*$^{-/-}$ N=44	SPF *Cdkn2a*$^{-/-}$ N=76	Pinworm *Cdkn2a*$^{-/-}$ N=54
Cancer	248	202	218	259
Leukemia/lymphoma	253	231	305	365

SPF: specific pathogen-free facility (i.e., free of pinworm).

Table S1). Of note, pinworm exposure had a modest impact on leukemia/lymphoma-free survival curves of Cdkn2a$^{-/-}$ mice (Figure 2C) and *E6R1*$^{+}$ *Cdkn2a*$^{-/-}$ mice (Figure 2D), but the cumulative bidirectional effect of pinworm resulted in a significant difference between these two mouse strains (Figure 2B). Because *Cdkn2a*$^{-/-}$ mice are susceptible to other cancers in addition to leukemia/lymphoma, we examined cancer-free survival curves: there was no difference between C*dkn2a*$^{-/-}$ and *E6R1*$^{+}$ *Cdkn2a*$^{-/-}$ mice in SPF conditions (*Online Supplementary Figure S1A*), but there was a statistically significant difference in the presence of pinworm (*Online Supplementary Figure S1B*). Interestingly, pinworm was associated with divergent responses: protection in *Cdkn2a*$^{-/-}$ mice (*Online Supplementary Figure S1C*) and a promotion in *E6R1*$^{+}$ *Cdkn2a*$^{-/-}$ nice (*Online Supplementary Figure S1D*). Neither *E6R1* (*Online Supplementary Figure S2A, B*) nor pinworm exposure (*Online Supplementary Figure S2C, D*) had a statistically significant impact on the development of solid tumors. Taken together, this work demonstrates a protective effect of an infection on leukemia/lymphoma development of *Cdkn2a* null mice in the absence of the *E6R1* mutation, but that this protection is reversed in the presence of this common prenatally acquired genetic change.

The increased latency of leukemia/lymphoma in pinworm-exposed *Cdkn2a*$^{-/-}$ mice is a novel *in vivo* demonstration of a protective role of pathogenic infection in a mouse model of B-ALL. These results build upon past findings, in which immune stimulation with CpG, a TLR9 agonist, protected against B-ALL in Eu-ret mice.[5] Exposure to microbes that are capable of priming the early immune system to respond appropriately to infections has long been associated with reduced risk of childhood B-ALL.[1,2] Interestingly, pinworms have immunomodulatory properties, and their decades-long decline in western societies is inversely correlated with the rising incidence of B-ALL and other childhood immune disorders.[6] While the current study does not characterize the immune response to pinworm exposure in *Cdkn2a*$^{-/-}$ mice, pinworm infections are well-described to stimulate anti-inflammatory immune responses that are characterized by the production of the cytokine interleukin-10 (IL-10) and T-regulatory cells.[7] As IL-10 is a protective factor for B-cell leukemia/lymphoma in humans[8] and mice,[9] it is possible that pinworm-induced IL-10 and/or pinworm-induced microbial diversity provide protection against B-ALL in specific genetic settings. In addition to parasites, viral and bacterial pathogens are also capable of supporting the gut microbiome and inducing production of IL-10 and T-regulatory cells.[10] It will therefore be interesting to determine whether the protection garnered from parasitic infection can be acquired through other infectious pathogens that stimulate similar immune pathways.

The gut microbiome is well known for shaping immune responses through structural components or metabolites of its constituent bacteria[11] and has recently been identified as a player in the development of B-ALL.[12] Considering the impact of helminth infections in shaping the composition of the gut microbiome,[13,14] it is likely that pinworm exerts a protective effect in *Cdkn2a*$^{-/-}$ mice by enhancing microbial diversity. Although the timing of pinworm introduction varied (from birth [Figure 1B] or from weaning age [Figure 1E]), we did not find evidence for a role of timing differences in leukemia/lymphoma development. We instead observed a strong genetic-environmental interaction, in which *E6R1* expression completely inhibited the protective effect of pinworm exposure in *Cdkn2a*$^{-/-}$ mice. This result may be explained by the recently described ability of *E6R1* to induce a state of microbial dysbiosis[12] or the well-established role of *E6R1* in converting B-cell precursors into B-ALL.[2] Pinworm and probiotic interventions that are currently being investigated for the prevention of childhood autoimmune disorders may also have the capacity to prevent childhood B-ALL.[2] Future studies should be aimed at understanding how genetics and infectious exposures interact to affect the gut-immune axis. This will be an important step toward leveraging the full potential of preventative strategies in children who are genetically predisposed to B-ALL.

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Disclosures

No conflicts of interest to disclose.

Contributions

BAF and SCK conceived the study. BAF, MZ, and SCK designed the study. BAF, MZ, and JS performed experiments and analyzed data. BAF wrote the manuscript with contributions from SCK and MZ. All authors interpreted data, reviewed the work critically, and revised the manuscript.

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Data-sharing statement

The original data and protocols are available to other investigators without restriction.

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