Timing of microbial exposure and risk of infection-promoted acute lymphoblastic leukemia

A multifactorial mechanism has been suggested for the causation of childhood B-cell precursor (BCP) acute lymphoblastic leukemia (ALL). This proposes that common infections serve as an indirect trigger for ALL in children carrying an *in utero*-generated and clinically covert pre-malignant clone. But, critically, the model proposes that triggering reflects immune dysregulation and is dependent upon a deficit of microbial exposure and immune network priming in infancy.[1] A key premise here is that the naïve immune network of infants requires priming, principally by the gut microbiome, to ensure that later immune responses to infections are balanced and chronic inflammation avoided; a concept that has strong experimental support, especially in murine models.[2] Preliminary studies suggest that the gut microbiome of newly diagnosed children with BCP-ALL is less mature and diverse from that of age-matched controls.[3]

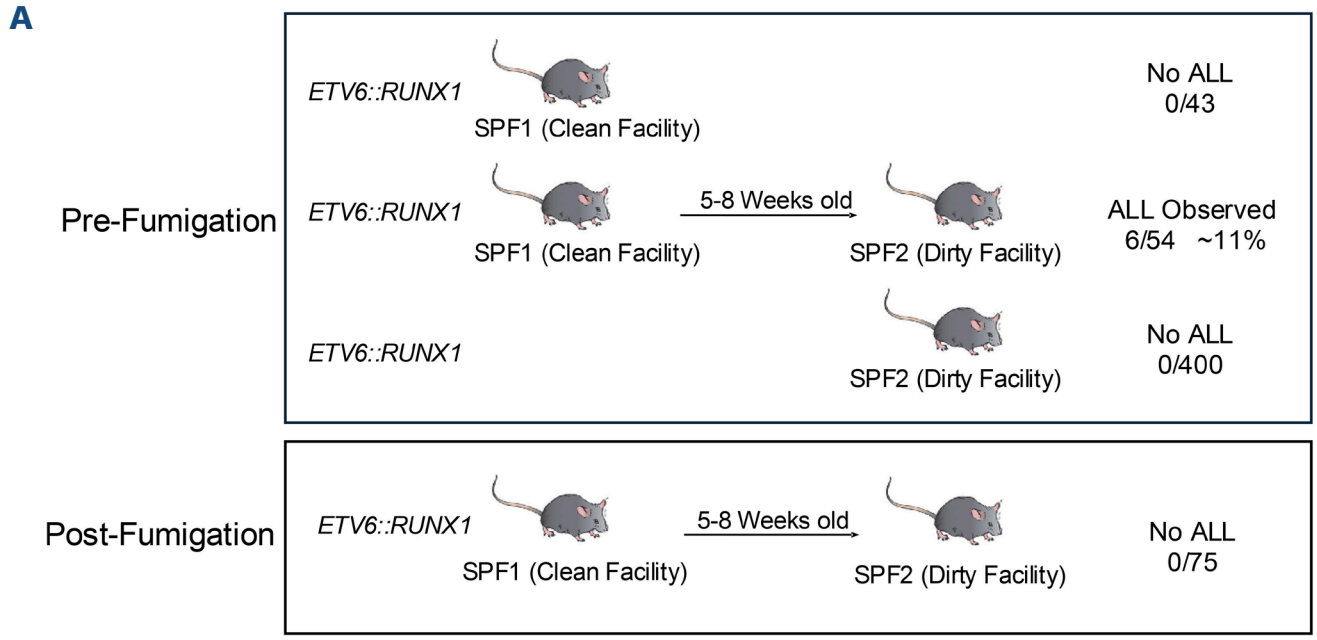
These considerations of causality in ALL have raised the possibility of prevention for the most common subtype of childhood cancer.[4,5] Pursuit of this ambitious goal would benefit from testing the causal roles of microbes in a mouse model of BCP-ALL. A mouse model of the causal hypothesis would need to test two predictions. The first is that common infections can trigger BCP-ALL in a fraction of mice engineered to express a common BCP-ALL-initiating lesion e.g., *ETV6*::*RUNX1*. The second is that this environmental trigger is only effective in the context of a deficit of early-life microbial exposures.

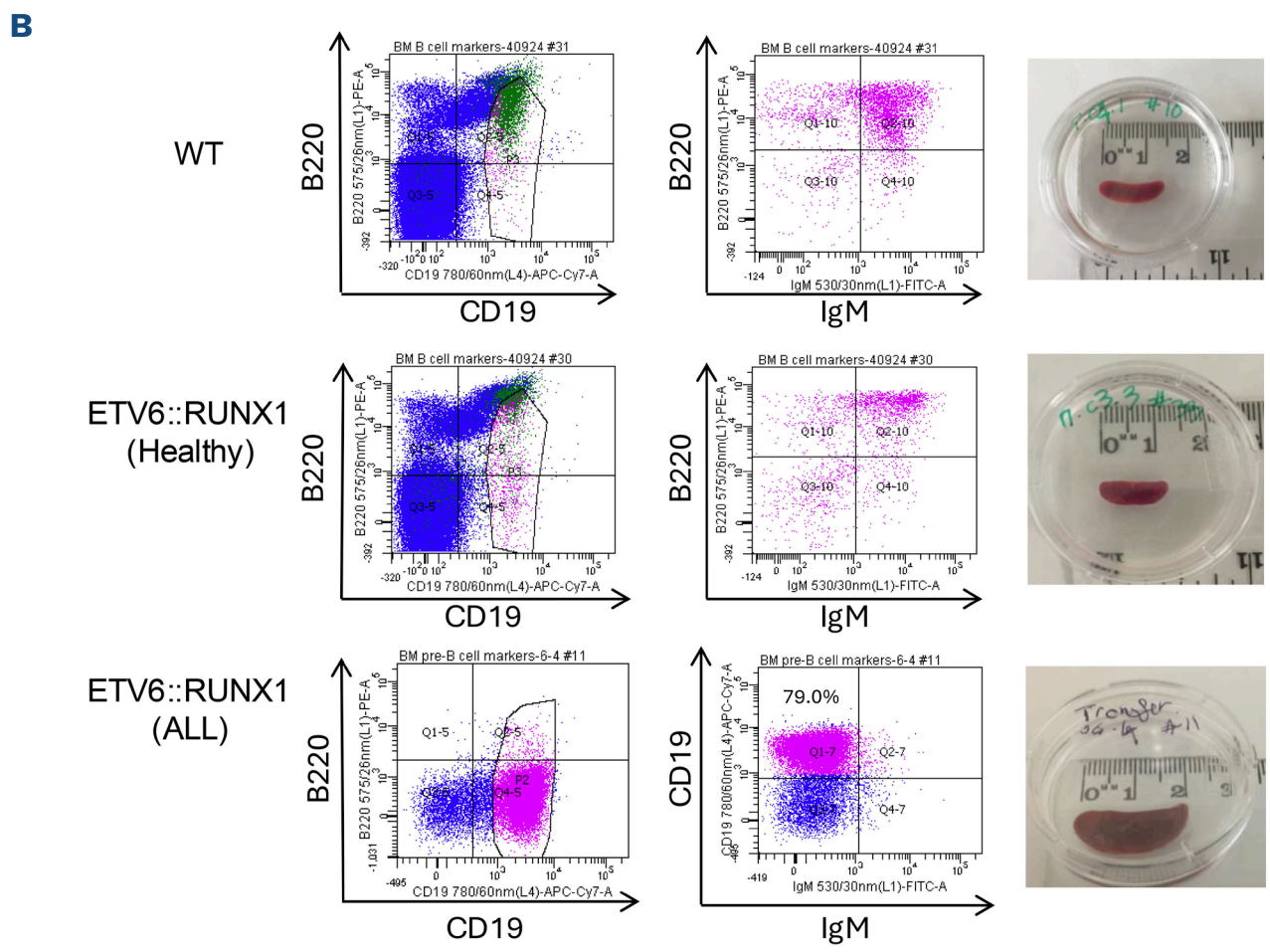
Sanchez-Garcia, Borkhardt and colleagues[6] assessed the first proposition and reported that a fraction (11%) of mice transgenic for *ETV6*::*RUNX1* or *PAX5*$^{+/-}$ develop BCP-ALL when transferred from a 'clean' housing unit to one ('dirty') with endemic microbes, endorsing the idea of infection-triggered ALL in susceptible individuals. We have confirmed this observation. In support of the second proposition, we show that *ETV6*::*RUNX1* transgenic mice do not develop ALL if exposed to the same 'leukemogenic' environment with endemic microbes from birth, or, if transferred to the 'infectious' environment after it was cleared of most endemic microbes by fumigation. All experiments were approved by and conform with the standards of the Animal Use Ethics Committee, The Institute of Cancer Research, London, UK and Home Office license requirements (PPL PP0261348, holder M Greaves). Figure 1A illustrates the overall incidence levels of ALL in these experiments. BCP-ALL was defined as an expanded population of cells with a B precursor phenotype (CD19^{+}, B220$^{+/low}$, IgM^{-}) in blood and bone marrow, clonal *IGH* rearrangement in a clinically sick mouse and, in most cases, an enlarged spleen (Figure 1B, C). A total of 54 mice were transferred from a 'clean' to a 'dirty' housing unit between 5 and 8 weeks of age. This time window is earlier than previously reported in a similar mouse model[7] and was chosen because microbiome priming of the immune system in mice is most active up until weaning from breast feeding.[8] Of those 54 mice, six (11%) became sick and were culled (age 14-23 months) and had ALL cells as defined above. A further eight (14.8%) mice were found to have an expanding clonal, BCP population following culling at 24 months and we have assumed they had subclinical ALL (*Online Supplementary Figure S1*). One mouse culled at 24 months had a large, clonal T-cell (TCRβ) population in addition to a clonal B-cell precursor population.

ALL cells would be expected to have genetic alterations that functionally complement *ETV6*::*RUNX1*.[9] We attempted to expand, by transplantation into NSG mice, three bone marrow samples of putative ALL diagnosed in mice transferred into the 'dirty' unit. Only one sample regenerated *in vivo* in sufficient numbers for whole-genome sequencing analysis. This post-transplant sample had the same *IGH* rearrangement as the primary sample and, in addition, a deletion in *Ebf1*, and a large deletion on chromosome 10 that included both *Btg1* and *Arid5b* (*Online Supplementary Figure S2A*). Also detected in the primary mouse sample by genomic polymerase chain reaction, these deletions are found in diagnostic samples from patients with BCP-ALL and are frequently associated with *ETV6-RUNX1* gene fusion.[10]

In contrast, no *ETV6*::*RUNX1* mice (0/400) from a sequential series of cohorts bred and maintained in the 'dirty' facility over a number of years (with sustained endemic infections throughout) developed ALL. Although we did not routinely test for subclinical ALL in these otherwise healthy mice following culling at 24 months, we note that those mice analyzed did exhibit polyclonal rearrangements within the *IGH* locus (*Online Supplementary Figure S2B*). When mice bred in the 'clean' facility were transferred into the 'dirty' facility following fumigation and microbial depletion of the latter (following the coronavirus [COVID] pandemic) no cases of ALL were recorded (0/75) (*Online Supplementary Figure S3A)*.

We compared the spectrum of microbes present in the 'dirty' facility before (i.e., when associated with ALL) with that after fumigation (i.e., in the absence of ALL) and also with the microbes reported to be present in the Spanish facility in which BCP-ALL also developed following mouse transfer.[7] Only one microbe was present in association with ALL in both 'dirty' facilities in London and Spain – this





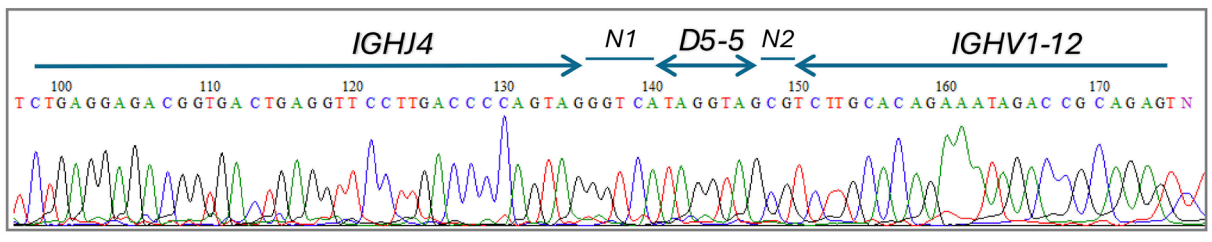


Figure 1. Leukemia in *ETV6::RUNX1* mice transferred from a SPF-1 to SPF-2 facility. (A) Cartoon to show the setup and incidence of acute lymphoblastic leukemia (ALL) for the 'housing switch' model for *ETV6::RUNX1*$^{+}$ mice (B6CBA) kept in SPF-1 (clean) or SPF-2 (dirty) conditions or switched from SPF-1 to SPF-2 (top panel). A similar protocol was followed after fumigation of the SPF-2 facility (bottom panel). The *ETV6::RUNX1* transgene was driven by the human β-globin promoter and lymphoid lineage specificity achieved via the immunoglobulin (*IGH*) heavy chain enhancer.[14] (B) Immunophenotype and respective spleen size of a representative *ETV6::RUNX1*$^{+}$ mouse with ALL. A wild-type (WT) and a healthy *ETV6::RUNX1*$^{+}$ mouse are shown for comparison. The B220 *versus* CD19 FACS plots identify the B-lineage populations and the expanded CD19^{+}/IgM^{-} population of the leukemic mouse confirms the presence of a B-lineage precursor clone (CD19^{+}, B220$^{+/low}$, IgM^{-}). The average spleen size of WT and polyclonal *ETV6::RUNX1*$^{+}$ mice was 1.6±0.2 cm. The average spleen size of *ETV6::RUNX1*$^{+}$ mice with *IGH* clonal ALL was 2.0±0.6 cm. (C) DNA sequence of a clonal *IGH* gene rearrangement in BCP from a typical *ETV6::RUNX1*$^{+}$ mouse with overt leukemia. Case-specific random nucleotide insertion (*N1, N2*) occurs between the respective V, D and JH joins.[15] (All mice with phenotypic BCP-ALL displayed an identical clonal *VNDNJ* rearrangement in both the bone marrow and spleen).

was murine norovirus (MNV) (Table 1). We confirmed by polymerase chain reaction analysis that mice fecal samples were MNV-positive during the relevant time period before COVID (Figure 2A). This suggests that MNV should be explored as a candidate agent for triggering ALL in these mouse models. Pinworm was also lost from our 'dirty' facility following fumigation. Although not recorded as present in the Spanish facility, there is evidence that pinworm exposure can accelerate development of ALL in a different mouse model, in which both the initiating and secondary mutations are already present, resulting in a high penetrance of ALL.[11] There is no suggestion that MNV or pinworm are likely triggers for BCP-ALL in children in whom respiratory viruses have been implicated by epidemiological studies.[1] Nevertheless, a mouse model in which ALL could be reproducibly induced by a non-transforming infectious agent would aid the assessment of potential intervention measures.

The causal model of BCP-ALL implicates deficiencies of the gut microbiome in infants.[1] In support of this, incidence rates of ALL in a mouse model substantially increased following antibiotic exposure within a 'clean' facility.[12] The authors concluded that a dysbiotic microbiome rather than exposure to infections was sufficient to drive the development of BCP-ALL in *Pax5*$^{-/-}$ or *ETV6*::*RUNX1*$^{+}$ mice. If correct this would contradict the two-step causal model proposed for BCP-ALL where epidemiological observations support the idea of an exogenous, microbial trigger.[1] We note the antibiotic experiment[12] was not carried out in a germ-free facility and therefore involvement of endemic microbes cannot be ruled out.

We explored the diversity and composition of the gut microbiome by bacterial 16s sequencing in a small number of mice maintained in our 'clean', 'dirty' and post-fumigation 'dirty' facilities (Figure 2B). Firstly, and in contrast to the findings of Vicente-Dueñas *et al.*[12] the microbiome of our *ETV6*::*RUNX1* mice was no different in diversity from that in wild-type mice with the same genetic background.

The microbiomes from *ETV6*::*RUNX1* mice in the 'dirty' facility were significantly more diverse than those from mice in the 'clean' facility or in the 'dirty' facility after fumigation. Mice transferred from the 'clean' to the 'dirty' facility acquired a more diverse gut microbiome (Figure 2B). Whole-genome sequencing or shotgun sequencing would be required for detailed analysis of the composition of the bacterial species, but we note that anti-inflammatory, short-chain fatty acid (SCFA)-producing genera[13] were more numerous in the 'dirty' facility (*Online Supplementary Figure S3B*).

All mouse models of human disease have limitations. Mice are genetically uniform with very different developmental time frames, metabolic rates, diets, gut microbiomes and environments compared to humans. Most transgenic models of BCP-ALL have the leukemia-initiating lesion present and expressed in effectively all B lineage cells of the mouse in marked contrast to the rare or clonal *ETV6*::*RUNX1* 'pre-malignant ' cells in children prior to a diagnosis.[1] One additional limitation of our mouse model

Table 1. Presence of infectious organisms and leukemia.

Micro-organism	LONDON		SALAMANCA[a]
	Period 1 ALL^{+} (before fumigation)	Period 2 ALL^{-} (after fumigation)	ALL^{+}
BACTERIAL species			
Helicobacter sp.	+	+	+
P. pneumotropica-HeyL	+	+	-
P. pneumotropica-Jawetz	+	+	-
*P. mirabilis**	+	-	-
*Campylobacter sp.**	+	-	-
*S. aureus**	+	+	-
*K. oxytoca**	+	-	-
*Aspiriculus tetraptera**	+	-	-
VIRUSES			
MHV*	-	-	+
MNV (Norovirus)[#]	+	-	+
PARASITIC species			
Pinworm	+	-	-
Trichomonas	+	+	+
*S. muris**	+	-	-
Demodex sp. (mites)*	+	-	-
*E. histolytica**	+	-	-

[a]Refer to Rodriguez-Hernandez G, *et al.*[7] *Sporadic presence other organisms are endemic. [#]Murine norovirus was the only microbe present in association with acute lymphoblastic leukemia in both 'dirty' facilities in London and Spain, but not following fumigation. ALL: acute lymphoblastic leukemia; MHV: murine hepatitis virus; MNV: murine norovirus.

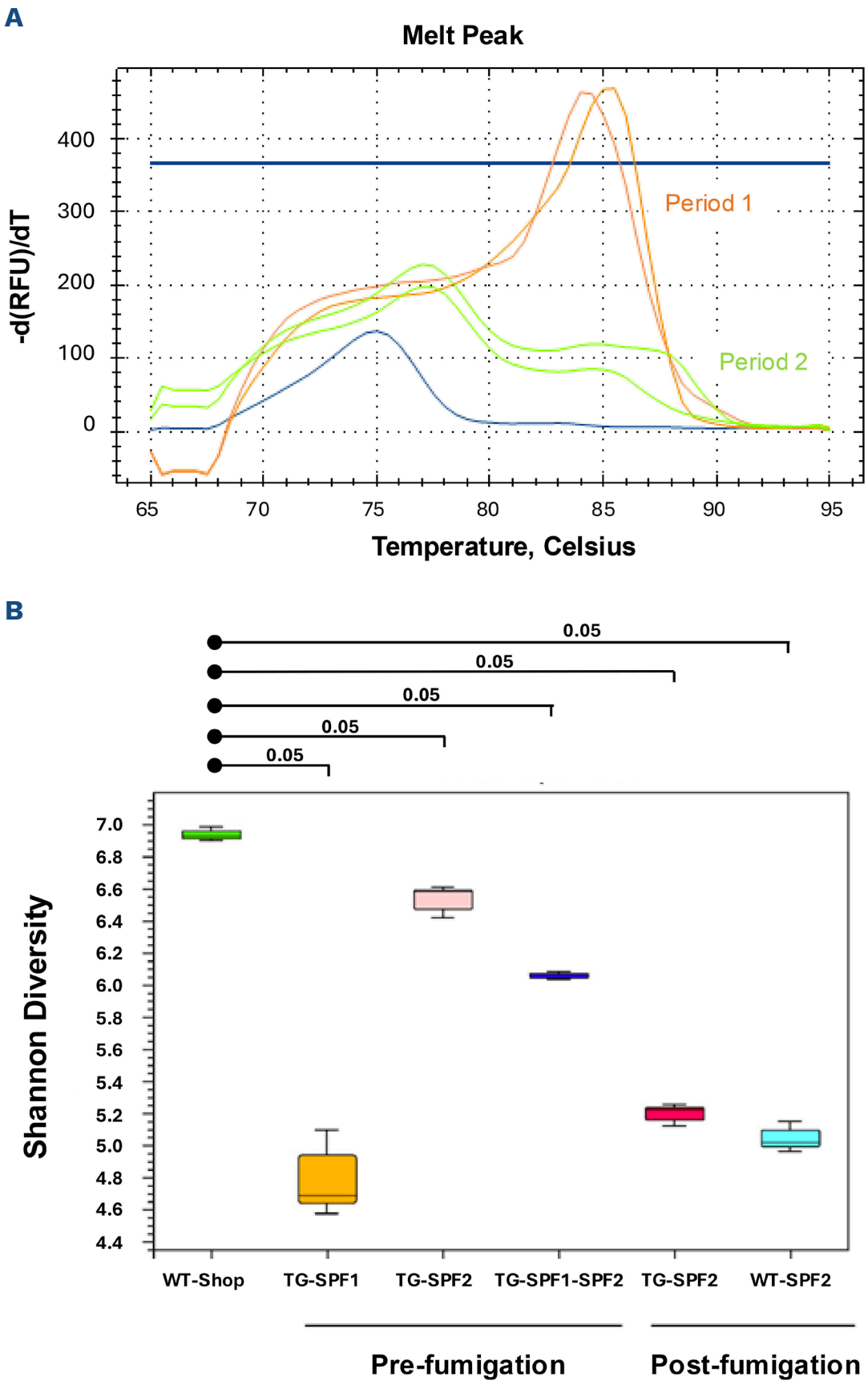


Figure 2. Gut microbiota in SPF-1 and SPF-2 facilities before and after fumigation. (A) Presence of murine norovirus (MNV) in mouse stools before fumigation of the SPF-2 facility. SYBR green PCR Melt Curves were used to detect the presence of MNV in cDNA prepared from individual mouse stools collected from the SPF-2 facility at two different timepoints. "-d(RFU)" is the change in fluorescence and "dT" is the change in temperature. The orange trace (Period 1) shows two stools collected in 2018, before fumigation; the green trace (Period 2) shows two stools collected in the post-COVID period (2024) and after fumigation. The thin blue trace is a water control. (B) Shannon diversity index of gut microbiota across genotypes and facility environments. All mice were 8-9 weeks of age. Boxplots represent Shannon entropy values for commercially sourced wild-type mice (WT-Shop) compared to facility-raised transgenic (TG) and wild-type (WT) cohorts. Facility groups are categorized by fumigation status: Pre-fumigation (TG-SPF1, TG-SPF2, TG-SPF1-SPF2) and Post-fumigation (TG-SPF2, WT-SPF2). The WT-Shop group exhibited significantly greater microbial diversity compared to all facility-born groups (Kruskal–Wallis P=0.01), with all pairwise comparisons against WT-Shop showing significance (P<0.05). Data are presented as box-and-whisker plots, N=3 per group.

and other similar ones[6,7] is that the resultant ALL had long latencies and were diagnosed in aging adults.

Accepting the above caveats, the data provide some support for the proposed causal model of BCP-ALL.[1] They confirm that endemic, non-pathological infections may trigger BCP-ALL in genetically susceptible mice. They further suggest the possibility that mice may be protected from infection-triggered ALL if exposed to those infections from birth and/or if benefiting from a richer, immune priming, anti-inflammatory gut microbiome. Studies such as these will, hopefully, encourage further exploration of the possible prevention of BCP-ALL, perhaps by microbiome boosting.

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Contributions
MG and AMF designed the study and co-wrote the paper. VC, ES, JP and PJ-B carried out the *in vivo* mouse investigations. ES analyzed bacterial 16s and diversity measures. All authors read and approved the final manuscript.

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Data-sharing statement
The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

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