Decreased IL-10 accelerates B-cell leukemia/lymphoma in a mouse model of pediatric lymphoid leukemia

Briana A. Fitch,1 Mi Zhou,1 Jamilla Situ,1 Sangeetha Surianarayanan,1 Melissa Q. Reeves,2-4 Michelle L. Hemmiston,2-6 Joseph L. Wiemels,6 and Scott C. Kogan1,2

1Department of Laboratory Medicine, 2Helen Diller Family Comprehensive Cancer Center, and 3Department of Microbiology and Immunology, University of California, San Francisco, San Francisco, CA; 4Parker Institute for Cancer Immunotherapy, San Francisco, CA; 5Division of Hematology/Oncology, University of California San Francisco Benioff Children’s Hospital, San Francisco, CA; and 6Center for Genetic Epidemiology, Department of Preventive Medicine, Keck School of Medicine of the University of Southern California, Los Angeles, CA

Exposures to a wide repertoire of common childhood infections and strong inflammatory responses to those infections are associated with the risk of pediatric B-cell acute lymphoblastic leukemia (B-ALL) in opposing directions. Neonatal inflammatory markers are also related to risk by unknown mechanism(s). Here, we demonstrate that interleukin-10 (IL-10) deficiency, which is associated with childhood B-ALL, indirectly impairs B lymphopoiesis and increases B-cell DNA damage in association with a module of 6 proinflammatory/myeloid-associated cytokines (IL-1α, IL-6, IL-12p40, IL-13, macrophage inflammatory protein-1β/CCL4, and granulocyte colony-stimulating factor). Importantly, antibiotics attenuated inflammation and B-cell defects in preleukemic Cdkn2a−/−Il10−/− mice. In an ETV6-RUNX11 Cdkn2a−/− mouse model of B-ALL, decreased levels of IL-10 accelerated B-cell neoplasms in a dose-dependent manner and altered the mutational profile of these neoplasms. Our results illuminate a mechanism through which a low level of IL-10 can create a risk for leukemic transformation and support developing evidence that microbial dysbiosis contributes to pediatric B-ALL.

Introduction

The reported effects of early life infections on childhood B-cell acute lymphoblastic leukemia (B-ALL) are highly complex.1 Although prior studies have focused on the protective role of surrogates of infectious exposure, such as early day care attendance2,3 and contact with older siblings,4 more recent studies have highlighted a stimulatory role of infections within the first year of life.5,6 To develop strategies for the prevention of childhood acute lymphoblastic leukemia (ALL), there is a need to understand the underlying mechanism(s) of how infectious stimuli promote or suppress its development. Variation in the key host factors that regulate response to infection might also impact the risk of ALL.

The timing of exposure to an infectious pathogen is a hallmark of the prevailing “delayed infection” hypothesis, which proposes that a lack of common infections (vectors) in infancy can promote the emergence of childhood ALL by driving abnormal immune responses to infections contracted later in childhood.7 We have identified altered cytokine levels at birth as a risk factor for childhood ALL.8,9 These observations suggest that, in addition to the timing of early childhood infections, congenital defects in the host immune system may influence the development of childhood ALL.
Neonatal deficiency in interleukin-10 (IL-10), a key cytokine in mediating early microbial homeostasis\textsuperscript{10,11} and immune response to infection,\textsuperscript{12,13} is a strong predictor of childhood B-cell malignancies.\textsuperscript{14} Children born with low levels of IL-10 have a 25-fold increased risk for developing ALL, whereas children with inherited \textit{Il10R} deficiency have a high risk for developing B-cell non-Hodgkin lymphoma.\textsuperscript{8,15} IL-10 is a potent anti-inflammatory cytokine that suppresses myeloid cell activation, migration, and cytokine production by triggering downstream JAK/STAT signaling pathways.\textsuperscript{16} Very early onset of severe inflammatory bowel disease (IBD) is observed in children born with IL-10 and IL-10R deficiency.\textsuperscript{17} Additionally, \textit{Il10}\textsuperscript{−/−} mice have been used as a model system for IBD and colitis-associated colon cancer, in which bacterial infection and tumor-promoting inflammation drive uncontrolled immune activation.\textsuperscript{18}
double-stranded DNA breaks in intestinal cells. Of importance for understanding disease susceptibility, different mouse strain backgrounds and the particular microbiota present in different mouse colonies influence the likelihood of \( \text{II}10^{+/+} \) animals developing IBD. Although the gut is the primary site of inflammation in IL-10–deficient humans and mice, gut dysbiosis and inflammation can have distinct genotoxic effects on peripheral blood lymphocytes that might contribute to hematological
Figure 3. Antibiotic treatment response rescues preleukemic Cdkn2a<sup>−/−</sup>Il10<sup>−/−</sup> B cells from impaired development and DNA damage. Analysis of 8- to 12-week-old Cdkn2a<sup>−/−</sup>Il10<sup>−/−</sup> and Cdkn2a<sup>−/−</sup>Il10<sup>+/+</sup> mice. (A) Absolute bone marrow (BM) count of total B220<sup>+</sup>CD19<sup>+</sup> B cells and BM B-cell subsets. (B) Percent of γH2AX<sup>+</sup> cells among total BM B cells and BM B-cell subsets. (C) Schematic diagram of antibiotic (ABX) treatment and tracking of peripheral blood and BM cells in adult mice. (D) Concentration of cytokines in Il10<sup>−/−</sup>Cdkn2a<sup>−/−</sup> and controls. Lines connect values from the same mouse sampled before and after antibiotic treatment. Flow analysis of the percentage of CD11b<sup>+</sup>CD19<sup>+</sup> cells (E) and CD19<sup>+</sup>B220<sup>+</sup> cells (F) in BM. (G) Percentage of γH2AX<sup>+</sup> of CD19<sup>+</sup>B220<sup>+</sup> cells in Cdkn2a<sup>−/−</sup>Il10<sup>−/−</sup> and Cdkn2a<sup>−/−</sup>Il10<sup>+/+</sup> mice after treatment with placebo (-) or antibiotics (+) for 4 weeks. Data in (A-B) are representative of 2 experiments. Data in (D-G) were obtained from a single-cohort study. Error bars represent the mean ± standard deviation. *P ≤ .05, **P ≤ .01, ***P ≤ .001, ****P ≤ .0001. Statistical tests used are described in Methods.
Mouse models have been paramount in elucidating the infectious etiology and the development of childhood B-cell leukemia/lymphoma. However, these models have yet to elucidate the role of host immune deficiencies in preleukemic B cells and promote the development of ALL. To investigate whether host immune and microbial dysfunction can contribute to the development of childhood ALL, we crossed II10−/- mice with the robust ETV6-RUNX1 (E6R1) Cdkn2a−/- model of childhood B-ALL (previously referred to as the Cre/Ta1 Cdkn2a−/- model). We report that IL-10 deficiency leads to increased DNA damage in bone marrow B cells, this increased damage is a result of microbial dysbiosis, and IL-10-deficient animals show decreased latency of B-cell neoplastic disease. Given the association between inflammation and microbial dysbiosis in II10−/- mice, these data are compatible with such dysbiosis being a source of DNA damage that contributes to lymphoid transformation.

**Methods**

**Mice**

B6.129P2-II10−/−/M1Cre/J mice (referred to in the text as II10−/- mice; MGI: 1857199) were purchased from The Jackson Laboratory (stock no. 002251) and crossed for >10 generations onto the FVB/N strain background. Control FVB/N mice were bred in-house, E6R1−/− Cdkn2a−/- mice (previously referred to as Cre/Ta1 Cdkn2a−/- mice28) were maintained on the FVB/N strain background. B6.129 II10−/- mice were crossed to E6R1−/− Cdkn2a−/- mice on the FVB/N background for ≥4 generations. Male and female mice were age matched for each experiment. All experiments were performed following institutional review and approval by the University of California, San Francisco Institutional Animal Care and Use Committee.

**Flow cytometry**

Cells were stained with 4',6-diamidino-2-phenylindole or Ghost Dye Violet 450 to measure viability by flow cytometry. After viability staining, cells were blocked in Fc Block or Rat IgG for 10 minutes in the dark on ice. Without washing, primary surface staining antibodies were added for an additional 20 minutes. Cells stained with biotinylated surface antibodies were then stained with fluorochrome-labeled streptavidin for 20 minutes. For intracellular staining, fixation and permeabilization were done using the BD Cytofix/Cytoperm kit per the manufacturer’s instructions. Data were acquired using a Sony SP6800 Spectral Analyzer (Sony Biotechnology) and analyzed using FlowJo software.

**Peripheral blood analyses**

For peripheral blood plasma, whole blood was obtained through submandibular bleeding and collected in EDTA-coated tubes. For cytokine analysis, tubes were centrifuged for 10 minutes at 1600g at 4°C to remove blood cells. Cytokine analysis was run on 2 technical replicate plasma samples from each mouse. A total of 32 cytokines were measured using 9-plex [IL-15, IL-18, Basic FGF, LIF, macrophage colony-stimulating factor (M-CSF), macrophage inflammatory protein-2 (MIP-2), PDGF-BB, and vascular endothelial growth factor], and 23-plex [IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17A, etoxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor], interferon-γ, KC, monocyte chemoattractant protein-1, MIP-1α, MIP-1β, RANTES, and tumor necrosis factor-α (TNF-α)] Luminex bead-based assays (Bio-Rad Laboratories).

**Antibiotic treatment**

Nutritionally complete pellets containing amoxicillin, clarithromycin, metronidazole, and omeprazole (Bio-Serv) were provided to mice for experiments shown in Figure 3C-G.

**Histology**

At the time of euthanasia, a gross necropsy was performed to identify potential sources of illness, and selected tissues were placed into 10% buffered formalin. If gross necropsy findings suggested a hematopoietic neoplasm, single-cell suspensions of involved tissues were prepared when possible and cryopreserved in medium containing 10% dimethyl sulfoxide. Formalin-fixed paraffin embedded (FFPE) tissues were stained with hematoxylin and eosin. Diagnoses were based upon gross necropsy and histopathology. Additional diagnostic information was obtained by immunophenotyping when necessary.
Cytomod data in Figure 2 used statistical tests that are previously were performed using the log-rank/Mantel-Cox test. Analysis of were calculated using the Pearson correlation. Survival analyses to investigate the impact of IL-10 deficiency on hematopoietic development, we enumerated lymphoid and myeloid lineages in the bone marrow of 8- to 12-week-old FVB/N II10−/− mice. DNA extraction

The DNeasy and QIAamp DNA FFPE Tissue kits (QIAGEN) were used to extract DNA from cryopreserved or FFPE samples from leukemic mice. DNA concentration and quality were determined by Nanodrop spectrophotometry and PicoGreen (Invitrogen).

Statistical analysis

Statistical analyses were performed using Prism 8 software (GraphPad). P values were generated using an unpaired 2-tailed Mann-Whitney U test or a paired 2-tailed Student t test for single comparisons. A χ² test was used for multiple comparisons. Correlations were calculated using the Pearson correlation. Survival analyses were performed using the log-rank/Mantel-Cox test. Analysis of Cytomed data in Figure 2 used statistical tests that are previously described.  

See supplemental Methods for additional details on cell preparation, sequencing, and analysis of sequencing.

Results

B-cell lymphopoiesis is altered in II10−/− mice

To investigate the impact of IL-10 deficiency on hematopoietic development, we enumerated lymphoid and myeloid lineages in the bone marrow of 8- to 12-week-old FVB/N II10−/− knockout mice. (These mice were II10−/− Cdkn2a+/−; see sections starting at "IL-10 deficiency disrupts B-cell properties in preleukemic Cdkn2a−/− mice" regarding II10−/− Cdkn2a−/− mice.) Within the bone marrow of II10−/− mice we observed decreased CD19+ B cells, increased CD11b+ CD19− myeloid cells, and no change in CD3+ T cells (Figure 1A). The substantial loss of bone marrow B cells was consistent with II10−/− mice on the 129Sv/Ev background. A well-documented cause of bone marrow B-cell lymphopenia is the premature mobilization of pre-B cells to the periphery in response to inflammation and increased neutrophil production. Analysis of the CD19+ B220+ B-cell compartment showed a reduction in the number of pre-B cells (CD43+ IgD− IgM−) and mature recirculating (MR) B cells (IgD− IgM+), whereas the number of pro-B cells (CD43+ IgD+ IgM+) and IgM+ immature B cells (IgM+ IgD+) remained unchanged (Figure 1B). As a result of the drastic reduction in pre-B cells, pro-B cells and IgM+ B cells were increased as a percentage of CD19+ B220+ bone marrow B cells (Figure 1C). Neutrophil and monocyte numbers were increased in II10−/− bone marrow (Figure 1D); monocytes were increased as a percentage of these CD11b+ CD19− cells (Figure 1E). B-cell loss in the bone marrow was associated with changes in the spleen, including splenomegaly, increased cellularity, and expansion of mature myeloid cells, but it was not attributed to the mobilization of B cells to the spleen (supplemental Figure 1A-C). Together, these data show that a deficit of pre-B cells and MR B cells occurs concurrently with myeloid cell expansion in II10−/− mice.

Table 2. Univariate analysis comparing the cytokine profiles of 19 Cdkn2a−/− Il10−/− controls and 18 Cdkn2a−/− Il10+/+ mice

| Cytokine | Cdkn2a−/− Il10−/− (pg/mL) | Cdkn2a−/− Il10+/+ (pg/mL) | Nominal P | Corrected P | Fold change (relative to control) |
|----------|--------------------------|---------------------------|-----------|-------------|---------------------------------|
| IL-6     | 6.49                     | 98.12                     | 1.098E-06 | 3.513E-05  | 15.12                           |
| RANTES   | 134.06                   | 324.87                    | 2.226E-06 | 7.123E-05  | 2.42                            |
| G-CSF    | 219.69                   | 3783.93                   | 3.526E-06 | .0071      | 16.86                           |
| MCP-1    | 197.09                   | 570.07                    | 4.716E-06 | .0027      | 2.89                            |
| IL-17A   | 11.68                    | 106.88                    | 8.679E-06 | .0065      | 9.51                            |
| IL-12p40 | 1155.44                  | 3178.86                   | 1.701E-05 | .0066      | 2.62                            |
| MIP-1β   | 17.70                    | 54.20                     | 4.850E-05 | .0018      | 2.60                            |
| IFN-γ    | 9.20                     | 53.65                     | 6.343E-05 | .0026      | 6.83                           |
| IL-3     | 1.64                     | 6.57                      | 6.831E-05 | .0021      | 4.00                            |
| IL-1β    | 2.02                     | 5.43                      | .0001     | .0034      | 2.69                            |
| GM-CSF   | 72.45                    | 179.37                    | .0003     | .0111      | 2.48                            |
| IL-5     | 2.08                     | 24.29                     | .0004     | .0113      | 11.68                           |
| TNF-α    | 18.62                    | 70.41                     | .0006     | .0101      | 3.78                            |
| MIP-1α   | 3.96                     | 8.80                      | .0008     | .0261      | 2.22                            |
| IL-1α    | 19.88                    | 45.34                     | .0012     | .0389      | 2.28                            |
| IL-9     | 3.66                     | 23.98                     | .0022     | .0705      | 6.55                            |
| IL-12p70 | 39.63                    | 83.49                     | .0037     | .1193      | 2.11                            |
| KC       | 53.73                    | 135.20                    | .0057     | .1813      | 2.52                            |
| IL-13    | 25.79                    | 221.87                    | .0151     | .4847      | 8.60                            |
| MIG      | 115.42                   | 407.65                    | .0155     | .4966      | 3.53                            |
| MIP-2    | 6.57                     | 15.40                     | .0200     | .6401      | 2.34                            |

Eight- to 12-week-old Cdkn2a−/− Il10−/− and Cdkn2a−/− Il10+/+ mice were housed in a specific pathogen-free facility and matched for age and sex. Statistical tests used are described in Methods. P values < .05 are in bold.

Notes: Median cytokine concentrations.
Figure 4. Decreased levels of IL-10 accelerate development of B-cell disease in the E6R1 Cdkn2a−/− model. Survival curves for cancer (A) and leukemia/lymphoma development (B) in E6R1 Cdkn2a−/−IL10+/+ mice and E6R1 Cdkn2a−/−IL10−/− mice. Log-rank (Mantel-Cox) test. (C) Number of total or nonsynonymous SNVs in B-cell leukemia/lymphomas from exome sequencing of 9 E6R1 Cdkn2a−/−IL10+/+ mice and 8 E6R1 Cdkn2a−/−IL10−/− mice. Error bars represent the mean ± standard deviation. (D) Mutation spectrum representing the frequency of mutations in each context of 96 possible trinucleotide contexts in sequenced E6R1 Cdkn2a−/−IL10+/+ and E6R1 Cdkn2a−/−IL10−/− B-cell leukemia/lymphomas. (E) Model for the role of microbial dysbiosis in childhood B-cell leukemia/lymphoma. IL-10 deficiency induces microbial dysbiosis in the gut, resulting in inflammation with distal effects of B-cell deficiency and B-cell DNA damage in the bone marrow. The inflammation-associated acquisition of genetic lesions in bone marrow B cells leads to the development of B-cell leukemia/lymphoma. Antibiotics may counteract the impact of low IL-10 by restoring bacterial homeostasis in the gut.
IL-10 deficiency contributes to B-cell DNA damage

The pathogenesis of pediatric B-ALL in humans and mice is associated with RAG-mediated double-stranded DNA breaks in immunoglobulin and nonimmunoglobulin genes. Furthermore, other investigators have reported increased double-stranded DNA breaks in peripheral blood lymphocytes and small intestinal cells of II10−/− mice. To investigate whether IL-10 deficiency may contribute to bone marrow B-cell transformation, we used flow cytometry to measure γH2AX, a DNA repair protein and double-stranded DNA break marker, in B-cell progenitors from 8- to 12-week-old II10−/− mice. The percentage of γH2AX+ B cells was significantly increased in II10−/− mice relative to II10+/+ controls (Figure 1F). DNA damage in II10−/− B cells was most abundant in the pro-B cell and IgM− immature B-cell subsets (Figure 1G). Overall, our results provide evidence that IL-10 deficiency negatively selects against pre-B cells and that the few remaining bone marrow pro-B cells and IgM+ immature B cells acquire DNA damage.

Inflammation in II10−/− mice is associated with B-cell DNA damage

Inflammation is a well-established driver of DNA damage in human cancer and II10−/− mouse models of cancer. We hypothesized that the expression of proinflammatory cytokines in the plasma of II10−/− mice would be associated with the level of DNA damage in developing B cells. To test this hypothesis, we measured the concentration of 32 cytokines that are involved in T helper 1 (Th1) cell, Th2 developing B cells. To test this hypothesis, we measured the concentration of 32 cytokines that are involved in T helper 1 (Th1) cell, Th2, cell, Th17 cell, and immune responses. The following 6 cytokines were significantly increased in II10−/− mice: IL-1α, IL-6, IL-12p40, IL-17, G-CSF, and MIP-1β (CCL4) (corrected P value < .05; Table 1). Although previous work reported a bias toward the expression of the Th1 cell–related cytokines interferon-γ and TNF-α in II10−/− mice, we demonstrated that myeloid growth factors and proinflammatory cytokines can predominate the peripheral blood inflammatory response in II10−/− mice (Figure 1H).

We then tested whether patterns of inflammation were associated with abnormal B-cell numbers and DNA damage in II10−/− mice. During complex immune responses, such as inflammation, cytokines may change in characteristic patterns. Therefore, we applied clustering analysis to our cytokine data set to determine whether cytokine clusters were more significantly associated with B-cell DNA damage and proliferation compared with individual cytokines. To this end, we used CytoMod, an unsupervised approach to clustering cytokines across individuals. We then used linear regression to determine whether there was an association between cytokine modules and altered B-cell number and DNA damage. P values were adjusted using the Benferroni-Holm method to obtain the family-wise error rate (FWER). We chose FWER < 0.05 as a cutoff for statistical significance. Of the 5 cytokine modules that were derived from normalized cytokine concentrations (Figure 2A), plasma module 5 (PM5) was associated with an increased percentage of DNA damage in bone marrow B cells and decreased numbers of pre-B and MR B cells (Figure 2B). PM5 contained the following cytokines: IL-1α, IL-6, IL-12p40, IL-13, G-CSF, and MIP-1β (Figure 2A). Our results demonstrate that, in the setting of IL-10 deficiency, proinflammatory cytokines are associated with B-cell loss and DNA damage. These results also indicate that IL-10 deficiency can contribute to the aberrant expression of the proinflammatory cytokine IL-6, which is a causal factor for childhood B-ALL, as previously reported in neonatal blood and demonstrated more recently in mice.

IL-10 deficiency disrupts B-cell properties in preleukemic Cdkn2a−/− mice

We then investigated the role of IL-10 deficiency in the Cdkn2a−/− mouse model of B-cell leukemia/lymphoma. For preleukemic studies, Cdkn2a−/− mice were II10+/+ or II10−/− (referred to herein as Cdkn2a−/−II10+/+ or Cdkn2a−/−II10−/−). Cdkn2a codes for the p18INK4a and p19ARF (p14ARF in humans) tumor suppressor proteins, which are key regulators of the cell cycle. Cdkn2a loss is found in one third of cases of human ALL and deletion of this gene in mice can induce B-ALL. Deletion of II10 in Cdkn2a−/− mice had a similar effect on inducing pre-B-cell loss and B-cell DNA damage as did II10 deletion in wild-type Cdkn2a mice (Figure 3A). However, we did not detect a particular B-cell subset with increased DNA damage (Figure 3B). Although age-matched II10+/+ and II10−/− mice were not assessed side by side with Cdkn2a−/− II10−/− and Cdkn2a−/−II10+/− mice, it is worth noting that the fold change between II10−/− and control mouse B-cell numbers (Figure 1A vs Figure 3A) and total B-cell DNA damage (Figure 1F vs Figure 3B) was similar across these different experiments. In addition, Cdkn2a−/−II10−/− mice shared patterns of inflammation with II10−/− mice: 15 cytokines were statistically significantly increased in Cdkn2a−/−II10−/− mice (Table 2), including the 6 cytokines that were increased in II10−/− mice. Therefore, the effects of IL-10 deficiency on B-cell lymphopoiesis and inflammation were present in the Cdkn2a−/− mouse model of B-cell leukemia/lymphoma.

We observed variable penetrance of inflammation, as defined by excessive proinflammatory cytokines and corresponding increased monocytes, in II10−/− and Cdkn2a−/−II10−/− mice. To assess the potential for inflammation to drive B-cell abnormalities, 8- to 12-week-old preleukemic Cdkn2a−/−II10−/− mice were stratified into 2 groups based on the presence or absence of increased inflammatory cells relative to control Cdkn2a−/−II10+/+ mice. A comparison of Cdkn2a−/−II10−/− mice with normal myeloid counts with those with elevated monocytes or elevated neutrophils (supplemental Figure 2A) revealed that abnormalities in B-cell count and DNA damage were only present in Cdkn2a−/−II10−/− mice with elevated monocytes (supplemental Figure 2B-C). These results indicate that IL-10 deficiency depletes and damages bone marrow B cells in a manner that might rely on inflammatory monocytes. These results suggest that inflammatory monocytes in Cdkn2a−/−II10−/− mice could be responsible for abnormalities in developing B cells, as previously reported for splenic marginal zone B cells.

Antibiotic-mediated suppression of the inflammatory milieu promotes recovery of B-cell development and diminishes B-cell DNA damage in II10−/− mice

Mice housed in specific pathogen–free facilities commonly harbor Helicobacter spp. In II10−/− mice, microbial dysbiosis of
Helicobacter can spontaneously induce colitis and lead to an expansion of splenic inflammatory monocytes/macrophages and marginal zone B cells. We reasoned that such previously described inflammatory responses to microbial dysbiosis may also be an underlying cause of B-cell deficiency and elevated B-cell DNA damage in the bone marrow of II10−/− mice.

We investigated whether blocking microbial-driven inflammation could limit abnormalities in B-cell number and DNA damage in preleukemic mice. To this end, we administered a 4-week regimen of antibiotics that target Helicobacter spp. or a placebo treatment to Cdkn2a−/−/II10−/− and Cdkn2a−/−/II10+/+ mice (Figure 3C). A multiplex Luminex assay was used to monitor cytokine concentrations in the peripheral blood before and after antibiotic treatment. Strikingly, antibiotic treatment reduced several proinflammatory cytokines that were associated with B-cell deficiency and B-cell DNA damage, including IL-1α, IL-6, and G-CSF (Figure 3D). We next assessed the impact of antibiotics on the frequency of immune cells and the extent of DNA damage in B cells residing in the bone marrow. As expected, the expansion of myeloid cells within the bone marrow compartment of Cdkn2a−/−/II10−/− mice was attenuated by antibiotics (Figure 3E). Antibiotic treatment also partially abrogated the decrease in B-cell number (Figure 3F) and remarkably reversed the increased B-cell DNA damage (Figure 3G) that was observed in placebo-treated Cdkn2a−/−/II10−/− mice. These results support a role for microbial dysbiosis and inflammation in promoting B-cell loss and B-cell DNA damage in the bone marrow of preleukemic II10−/− mice.

Decreased levels of IL-10 accelerate B-cell neoplasms in E6R1 Cdkn2a−/− mice

Children born with low levels of IL-10 were found to have a 25-fold increased risk for developing ALL. Whether this association reflects causality had not been previously tested. Although Cdkn2a−/− mice develop B-cell leukemia/lymphoma, a greater proportion of Cdkn2a−/− mice develop this malignancy in the presence of E6R1. Therefore, E6R1 Cdkn2a−/− mice were used to determine whether low levels of IL-10 promote the development of B-cell leukemia/lymphoma. We followed a cohort of E6R1 Cdkn2a−/− mice with wild-type IL-10 (E6R1 Cdkn2a−/−/II10+/+) for the development of disease alongside cohorts of mice with decreased IL-10 expression (E6R1 Cdkn2a−/−/II10−/−) or no IL-10 expression (E6R1 Cdkn2a−/−/II10+/−). We did not observe a significant difference in the incidence of leukemia/lymphomas among our survival cohorts (supplemental Table 1). Nevertheless, time-to-illness analysis revealed that a complete loss of IL-10 in E6R1 Cdkn2a−/−/II10−/− mice significantly decreased cancer-free survival (Figure 4A) and leukemia/lymphoma-free survival (Figure 4B) relative to E6R1 Cdkn2a−/−/II10+/+ controls. The median time to leukemic lymphoma was 140 days in E6R1 Cdkn2a−/−/II10−/− mice vs 220 days in E6R1 Cdkn2a−/−/II10+/+ mice (P = 6.54 × 10−6, Mann-Whitney U test; supplemental Table 2). For E6R1 Cdkn2a−/−/II10−/− mice, the median time to leukemia/lymphoma was intermediate at 174 days (E6R1 Cdkn2a−/−/II10−/− vs E6R1 Cdkn2a−/−/II10+/+ mice, P = .01, Mann-Whitney U test; supplemental Table 2), supporting a dose-dependent relationship between decreased levels of IL-10 and early onset of leukemia/lymphoma (supplemental Figure 3). Of note, we were not able to subtype disease in all mice; however, consistent with previous work, B-cell leukemia/lymphoma was diagnosed in the preponderance of animals identified as having leukemia/lymphoma. These data demonstrate that low levels of IL-10 have a causal role in decreasing the time to leukemia/lymphoma development in the E6R1 Cdkn2a−/− model.

IL-10 loss increases the frequency of C>G and C>T mutations in B-cell neoplasms

We next sought to determine whether the increased levels of B-cell lesions observed in preleukemic II10−/− mice corresponded to an increased mutational burden or altered mutational pattern in II10−/− B-cell leukemia/lymphomas. We used whole-exome sequencing to characterize the number and type of mutations in B-cell leukemia/lymphomas from 9 E6R1 Cdkn2a−/−/II10−/− mice and 8 E6R1 Cdkn2a−/−/II10+/− mice. Total and nonsynonymous single-nucleotide variants (SNVs) were similar in number between E6R1 Cdkn2a−/−/II10−/− and E6R1 Cdkn2a−/−/II10+/− B-cell leukemia/lymphomas (Figure 4C). The total number of C>G and C>T mutations was increased in E6R1 Cdkn2a−/−/II10−/− samples (Figure 4D), although we did not find evidence for enrichment of inflammation-related mutational signatures delineated in the COSMIC mutational signatures database (version 2). Notably, C>T mutations are also elevated in solid tumors from mice with IL-10 deletions. SNVs related to human cancer were found in a number of genes (supplemental Table 3), with synonymous Hoxa9 mutations in 63% (5/8) of E6R1 Cdkn2a−/−/II10−/− B-cell leukemia/lymphomas. Similar synonymous mutations in HOXA9 have been found in several lymphoid neoplasm cell lines with a high pathogenicity score. Although synonymous changes have been considered non-pathogenic in the past, recent data suggest that such mutations have the potential to cause pathogenic effects. We also observed mutations in Jak3, Cdkn1a, and Dnm2 (supplemental Table 3), which have human orthologs that are mutated in human leukemias. The Jak3V670A mutation was present in 3 of 17 B-cell leukemia/lymphomas and is a homolog of human JAK3V674A. Mirroring the heterogeneity of human leukemias, other canonical cancer SNVs were identified in our cohort; however, there was little overlap in SNVs among samples. Overall, our results demonstrate that decreased levels of IL-10 cause acceleration of lymphoid malignancies with increased C>G and C>T mutations in the E6R1 Cdkn2a−/− model of B-cell leukemia/lymphoma.

Discussion

The prevailing delayed infection hypothesis regarding the infectious etiology of childhood leukemia posits that a lack of exposure to common pathogens during the first years of life raises the risk of leukemia. There is extensive support for a role of aberrant immune responses to common childhood infections in promoting leukemia development. However, the hypothesis that congenital defects in the host immune response can modulate the immune response to childhood infections, promoting the risk of childhood leukemia, has gained support by more recent studies. We observed that decreased levels of IL-10 caused a dose-dependent acceleration in the development of B-cell neoplasms in the E6R1 Cdkn2a−/− model of ALL. This finding provides in vivo evidence for a causal role of IL-10 deficiency in the development of B-cell neoplasms, building upon previous epidemiological studies that showed that low neonatal IL-10 is an ALL risk factor and that congenital genetic deficiencies in IL10 and IL10R are correlated with childhood B-cell leukemia.
Our data in preleukemic mice point to an indirect relationship between IL-10 deficiency and the impairment of B-cell development. In cohorts of Il10−/− mice with spontaneous onset of inflammation, none of the Il10−/− mice with normal monocyte counts (associated with a lack of inflammation) developed a deficit or extensive DNA damage within the bone marrow B-cell compartment. A role for inflammation in B-ALL development has been supported by several studies demonstrating that proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, can drive in vitro mutagenesis.37 Selection of preexisting oncogenic mutations in B-cell progenitors,52 and in vivo B-ALL development in Pax5−/− mice.53 Given that IL-10 deficiency leads to the upregulation of a 6-cytokine module that is associated with B-cell DNA damage, chronic inflammation triggered by IL-10 deficiency may foster a favorable environment for B-cell transformation and leukemogenesis.

An additional mechanism for B-cell transformation and leukemogenesis might involve microbial-dependent inflammation caused by enterocolitis in Il10−/− mice. Gut bacteria in mice can drive VDJ recombination in lamina propria B cells54 and regulate hematopoiesis in the bone marrow.55 The attenuation of inflammation and B-cell DNA damage in antibiotic-treated Il10−/− mice indicates that microbial dysbiosis might underlie the genotoxic effects observed in bone marrow B cells. A different role of microbes, in protecting against childhood ALL, has been supported by human studies showing an association between newborns delivered through scheduled elective cesarean section and increased childhood ALL risk.56,57 In view of these human data, a study of all mouse models with intact IL-10 found that antibiotics increased leukemogenesis.58 The commonality between our findings and past studies is that childhood ALL development might be related to various states of microbial dysbiosis caused by excessive bacterial growth or deprivation of early microbial exposures. This is consistent with a concept, recently presented by Mel Greaves,49 that a well-balanced microbiome in early life that promotes immunologic homeostasis may prevent childhood ALL.

Our work has limitations. We observed that low IL-10 levels accelerated development of B-cell neoplasms using a mouse model in which a combination of E6R1 expression with Cdkn2a deletion results in a relatively high incidence of leukemia/lymphoma.27 This penetrant development of disease may have limited our ability to detect the impact of IL-10 levels on disease incidence. In our model, large numbers of cells are susceptible to transformation (ie, initiated); hence, acceleration of disease may reflect low IL-10 levels increasing the probability of transformation (ie, progression). Nonetheless, studies of the impact of IL-10 levels in a low-penetrance model of lymphoblastic leukemia will be an important complement to our work. In addition, we do not yet have sufficient data to estimate the frequency at which low IL-10 levels are likely to be a contributory factor to cases of human pediatric leukemia/lymphoma. Further, although our mouse model system enabled us to identify that low IL-10 levels can lead to increased B-cell DNA damage and to an altered mutational spectrum, DNA damage studies of preleukemic cells, as well as sequencing of human ALLs from children with well-characterized cytokine profiles, are needed to assess whether similar phenomena occur in human leukemogenesis. With regard to the possibility of leukemia prevention, we showed that antibiotics can suppress B-cell DNA damage but did not demonstrate that such treatment could reverse leukemia acceleration.

Future prospective studies that are designed to characterize the development of the gut microbiome and immune system in children predisposed to ALL could further clarify the complex relationship among genetics, immune disorders, and microbial imbalances to ultimately guide clinical interventions aimed at decreasing ALL risk in children.

Acknowledgments

The authors thank Sony Biotechnology for providing guidance on the design of antibody staining panels for spectral flow cytometry and use of the Sony SP6800 Spectral Analyzer. They also thank Todd Whitehead and Kamir Hiam for useful discussions.

This work was supported by National Institutes of Health National Cancer Institute grants R01-CA185058 (S.C.K. and J.L.W.) and F31-CA221157 (B.A.F.).

Authorship

Contribution: B.A.F., M.L.H., J.L.W., and S.C.K. conceived the study; B.A.F., M.Z., J.S., S.S., M.O.R., M.L.H., and S.C.K. designed the study; B.A.F., M.Z., J.S., and S.S. performed experiments and analyzed data; and B.A.F. wrote the manuscript with contributions by S.C.K. and M.Z. All authors interpreted data, reviewed the work critically, and revised the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

ORCID id: S.C.K., 0000-0002-2395-8479.

Correspondence: Scott C. Kogan, Helen Diller Family Comprehensive Cancer Center and Department of Laboratory Medicine, University of California San Francisco, 513 Parnassus Ave, Room S-561, Box 0451, San Francisco, CA 94143-0451; e-mail: scott.kogan@ucsf.edu.

References

1. Hwee J, Tait C, Sung L, Kwong JC, Sutradhar R, Pole JD. A systematic review and meta-analysis of the association between childhood infections and the risk of childhood acute lymphoblastic leukaemia. Br J Cancer. 2018;118(1):127-137.

2. Urayama KY, Buffer PA, Gallagher ER, Ayoub JM, Ma X. A meta-analysis of the association between day-care attendance and childhood acute lymphoblastic leukaemia. Int J Epidemiol. 2010;39(3):718-732.

3. Rudant J, Lightfoot T, Urayama KY, et al. Childhood acute lymphoblastic leukaemia and indicators of early immune stimulation: a Childhood Leukemia International Consortium study. Am J Epidemiol. 2015;181(8):549-562.
4. Hjalgrim LL, Rostgaard K, Hjalgrim H, et al. Birth weight and risk for childhood leukemia in Denmark, Sweden, Norway, and Iceland. *J Natl Cancer Inst.* 2004;96(20):1549-1556.

5. Chang JS, Tsai C-R, Tsai Y-W, Wiemels JL. Medically diagnosed infections and risk of childhood leukaemia: a population-based case-control study. *Int J Epidemiol.* 2012;41(4):1050-1059.

6. Crouch S, Lightfoot T, Simpson J, Smith A, Ansell P, Roman E. Infectious illness in children subsequently diagnosed with acute lymphoblastic leukemia: modeling the trends from birth to diagnosis. *Am J Epidemiol.* 2012;176(5):402-408.

7. Greaves M. Infection, immune responses and the aetiology of childhood leukaemia. *Nat Rev Cancer.* 2008;8(3):193-203.

8. Chang JS, Zhou M, Buffer PA, Chokalingam AP, Metcalf C, Wiemels JL. Profound deficit of IL10 at birth in children who develop childhood acute lymphoblastic leukemia. *Cancer Epidemiol Biomarkers Prev.* 2011;20(8):1736-1740.

9. Seegaard SH, Røstgaard K, Skogstrand K, Wiemels JL, Schmiegelow K, Hjalgrim H. Neonatal inflammatory markers are associated with childhood B-cell precursor acute lymphoblastic leukemia. *Cancer Res.* 2018;78(18):5458-5463.

10. Glocker E-O, Kotlarz D, Boztug K, et al. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *N Engl J Med.* 2009;361(21):2033-2045.

11. Johanson P, Fasth A, Ek T, Hammarsten O. Validation of a flow cytometry-based detection of γ-H2AX, to measure DNA damage for clinical applications. *Cytometry B Clin Cytom.* 2017;92(6):534-540.

12. Raedler D, Illi S, Pinto LA, et al. IL10 polymorphisms influence neonatal immune responses, atopic dermatitis, and wheeze at age 3 years. *J Allergy Clin Immunol.* 2013;131(3):789-796.

13. Genovese F, Mancuso G, Cuzzola M, et al. Role of IL-10 in a neonatal mouse listeriosis model. *J Immunol.* 1999;163(5):2777-2782.

14. Couper KN, Blount DG, Riley EM. IL-10: the master regulator of immunity to infection. *J Immunol.* 2008;180(9):5771-5777.

15. Neven B, Mamessier E, Bruneau J, et al. A Mendelian predisposition to B-cell lymphoma caused by IL10R deficiency. *Blood.* 2013;122(23):3713-3722.

16. Moore KW, de Waal Malefyt R, Coffman RL, O’Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol.* 2001;19(1):683-765.

17. Zhu L, Shi T, Zhong G, Wang Y, Chang M, Liu X. IL-10 and IL-10 receptor mutations in very early onset inflammatory bowel disease. *Gastroenterol Res.* 2017;10(2):65-69.

18. Kanneganti M, Mino-Kenudson M, Mizoguchi E. Animal models of colitis-associated carcinogenesis. *J Biomed Biotechnol.* 2011;2011:342637.

19. Almana Y, Mohammed R. Current concepts in pediatric inflammatory bowel disease; IL10/IL10R colitis as a model disease. *Int J Pediatr Adolesc Med.* 2019;6(1):1-5.

20. Frick A, Khare V, Paul G, et al. Overt increase of oxidative stress and DNA damage in murine and human colitis and colitis-associated neoplasia. *Mol Cancer Res.* 2018;16(4):634-642.

21. Ray A, Basu S, Gharai RZ, et al. Gut microbial dysbiosis due to *Helicobacter* drives an increase in marginal zone B cells in the absence of IL10 signaling in macrophages. *J Immunol.* 2015;195(7):3071-3085.

22. Westbrook AM, Wei B, Braun J, Schiestl RH. Intestinal inflammation induces genotoxicity to extraintestinal tissues and cell types in mice. *Int J Cancer.* 2011;128(8):1815-1825.

23. Westbrook AM, Wei B, Hacke K, Xia M, Braun J, Schiestl RH. The role of tumour necrosis factor-α and tumour necrosis factor receptor signalling in inflammation-associated systemic genotoxicity. *Mutagenesis.* 2012;27(1):77-86.

24. Yamamoto ML, Maier I, Dang AT, et al. Intestinal bacteria modify lymphoma incidence and latency by affecting systemic inflammatory state, oxidative stress, and leukocyte genotoxicity. *Cancer Res.* 2013;73(14):4222-4232.

25. Swaminathan S, Klemm L, Park E, et al. Mechanisms of clonal evolution in childhood acute lymphoblastic leukemia. *Nat Immunol.* 2015;16(7):766-774.

26. Rodriguez-Hernández G, Hauer J, Martín-Lorenzo A, et al. Infection exposure promotes *ETV6-RUNX1* precursor B-cell leukemia via impaired H3K4 demethylases. *Cancer Res.* 2017;77(18):4365-4377.

27. Papaeunmanui E, Rapado I, Li Y, et al. RAG-mediated recombination is the predominant driver of oncogenic rearrangement in *ETV6-RUNX1* acute lymphoblastic leukemia. *Nat Genet.* 2014;46(2):116-125.

28. Li M, Jones L, Gaillard C, et al. Initially disadvantaged, TEL-AML1 cells expand and initiate leukemia in response to irradiation and cooperating mutations. *Leukemia.* 2013;27(7):1570-1573.

29. Cohen L, Fiore-Gartland A, Randolph AG, et al. A modular cytokine analysis method reveals novel associations with clinical phenotypes and identifies sets of co-signaling cytokines across influenza natural infection cohorts and healthy controls. *Front Immunol.* 2019;10:1338.

30. Gomes-Santos AC, Moreira TG, Castro-Junior AB, et al. New insights into the immunological changes in IL-10-deficient mice during the course of spontaneous inflammation in the gut mucosa. *Clin Dev Immunol.* 2012;2012:560817.

31. Cain D, Kondo M, Chen H, Kelsoe G. Effects of acute and chronic inflammation on B-cell development and differentiation. *J Invest Dermatol.* 2009;129(2):266-277.

32. Kang Y-J, Yang S-J, Park G, et al. A novel function of interleukin-10 promoting self-renewal of hematopoietic stem cells. *Stem Cells.* 2007;25(7):1814-1822.

33. Martín-Lorenzo A, Auer F, Chan LN, et al. Loss of Pax5 exploits Sca1-BCR-ABL" susceptibility to confer the metabolic shift essential for pB-ALL. *Cancer Res.* 2018;78(10):2669-2679.
34. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144(5):646-674.

35. Oft M. IL-10: master switch from tumor-promoting inflammation to antitumor immunity. *Cancer Immunol Res.* 2014;2(3):194-199.

36. Berg DJ, Davidson N, Kühn R, et al. Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. *J Clin Invest*. 1996;98(4):1010-1020.

37. Beneforti L, Dander E, Bresolin S, et al. Pro-inflammatory cytokines favor the emergence of ETV6-RUNX1-positive pre-leukemic cells in a model of mesenchymal niche. *Br J Haematol*. 2020;190(2):262-273.

38. Mullighan CG, Goorha S, Radtke I, et al. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature*. 2007;446(7137):758-764.

39. Serrano M, Lee H, Chin L, Cordon-Cardo C, Beach D, DePinho RA. Role of the INK4a locus in tumor suppression and cell mortality. *Cell*. 1996;85(1):27-37.

40. Fox JG, Ge Z, Whary MT, Erdman SE, Horwitz BH. *Helicobacter hepaticus* infection in mice: models for understanding lower bowel inflammation and cancer. *Mucosal Immunol*. 2011;4(1):22-30.

41. Sato Y, Takahashi S, Kinouchi Y, et al. IL-10 deficiency leads to somatic mutations in a model of IBD. *Carcinogenesis*. 2006;27(5):1068-1073.

42. Catalogue of Somatic Mutations in Cancer (COSMIC). HOXA9 Gene. https://cancer.sanger.ac.uk/cell_lines/gene/analysis?ln=HOXA9. Accessed 17 March 2020.

43. Sun H, Yu G. New insights into the pathogenicity of non-synonymous variants through multi-level analysis. *Sci Rep.* 2019;9(1):1667.

44. Mullighan CG, Zhang J, Harvey RC, et al. JAK mutations in high-risk childhood acute lymphoblastic leukemia. *Proc Natl Acad Sci USA*. 2009;106(23):9414-9418.

45. Ding L-W, Sun Q-Y, Tan K-T, et al. Mutational landscape of pediatric acute lymphoblastic leukemia [published correction appears in *Cancer Res*. 2017;77(8):2174]. *Cancer Res*. 2017;77(2):390-400.

46. Oshima K, Khiabanian H, da Silva-Almeida AC, et al. Mutational landscape, clonal evolution patterns, and role of RAS mutations in relapsed acute lymphoblastic leukemia. *Proc Natl Acad Sci USA*. 2016;113(40):11306-11311.

47. Abbas T, Dutta A. p21 in cancer: intricate networks and multiple activities. *Nat Rev Cancer*. 2009;9(6):400-414.

48. Greaves M. The ‘delayed infection’ (aka ‘hygiene’) hypothesis for childhood leukaemia. *The Hygiene Hypothesis and Darwinian Medicine*. 2009:239-255.

49. Greaves M. A causal mechanism for childhood acute lymphoblastic leukaemia [published correction appears in *Nat Rev Cancer*. 2018;18:526]. *Nat Rev Cancer*. 2018;18(8):471-484.

50. Wiemels J. Perspectives on the causes of childhood leukemia. *Chem Biol Interact*. 2012;196(3):59-67.

51. Nielsen AB, Zhou M, de Smith AJ, et al. Increased neonatal level of arginase 2 in cases of childhood acute lymphoblastic leukemia implicates immunosuppression in the etiology. *Haematologica*. 2019;104(11):e514-e516.

52. Henry CJ, Casás-Selves M, Kim J, et al. Aging-associated inflammation promotes selection for adaptive oncogenic events in B cell progenitors. *J Clin Invest*. 2015;125(12):4666-4680.

53. Isidro-Hernández M, Mayado A, Casado-García A, et al. Inhibition of inflammatory signaling in Pax5 mutant cells mitigates B-cell leukemogenesis. *Sci Rep.* 2020;10(1):19189.

54. Wesemann DR, Portuguese AJ, Meyers RM, et al. Microbial colonization influences early B-lineage development in the gut lamina propria. *Nature*. 2013;501(7465):112-115.

55. Yan H, Baldrige MT, King KY. Hematopoiesis and the bacterial microbiome. *Blood*. 2018;132(6):559-564.

56. Marcotte EL, Thomopoulos TP, Infante-Rivard C, et al. Caesarean delivery and risk of childhood leukaemia: a pooled analysis from the Childhood Leukemia International Consortium (CLIC). *Lancet Haematol*. 2016;3(4):e176-e185.

57. Wang R, Wiemels JL, Metayer C, et al. Cesarean section and risk of childhood acute lymphoblastic leukemia in a population-based, record-linkage study in California. *Am J Epidemiol*. 2017;185(2):96-105.

58. Vicente-Dueñas C, Janssen S, Oldenburg M, et al. An intact gut microbiome protects genetically predisposed mice against leukemia. *Blood*. 2020;136(18):2003-2017.