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CHRONIC LYMPHOCYTIC LEUKEMIA

CLL crosstalk with naïve T cells enhances the differentiation of IL-22-producing T cells and CLL -cell survival

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Patients with chronic lymphocytic leukemia (CLL) exhibit clinical findings suggesting an altered immune system, with an increased risk of infection and the development of other cancers and various autoimmune phenomena [1]. These associations are thought to be orchestrated in part by the interactions of leukemic cells with normal cells and elements in tissues, the latter referred to as the tumor microenvironment (TME). Notably, these interactions support the survival and expansion of CLL cells [2]. Most well studied is the impact of the leukemic cells on T cells, leading to alterations in T-cell subset composition, surface membrane molecule expression, immune-synapse formation, and migration, along with functional changes such as exhaustion [3, 4]. Nevertheless, the molecular mechanisms by which differentiation of naive T (Tn) cells to various memory T-cell subsets occurs in CLL and the effects of imbalances of the process on leukemic B-cell survival and disease progression are not fully understood.

Interleukin-22 (IL-22) is a member of the IL-10 cytokine family. Although originally viewed as a Th17 cytokine, IL-22 is produced by a wide variety of cells of the adaptive and innate immune systems, including $CD4^+$ T cells (Th1, Th17, Th22), $CD8^+$ T cells (Tc17, Tc22), innate T cells, natural killer subsets, and lymphoid tissue inducer cells [5]. IL-22 binds to a heterodimeric receptor comprised of IL-22Ra1 and IL-10R2, thereby activating Janus kinase 1 (Jak1), tyrosine kinase 2 (Tyk2), and Signal Transducer and Activator of Transcription 3 (STAT3). IL-22 affects innate immune responses, protective functions against pathogens, and tissue regeneration. Regarding the latter, a major target of IL-22 is non-hematopoietic epithelial cells, influencing epithelial homeostasis that contributes to host defense against extracellular pathogens [5, 6]. Additionally, due to its participation in pathways favoring cellular proliferation and survival, alterations in the balance between IL-22 and its cellular targets can result in autoimmunity and cancer [5, 6].

Both IL-22 and Th22 cells are altered in several hematologic malignancies including CLL. In newly diagnosed B-cell non-Hodgkin lymphoma (B-NHL), multiple myeloma (MM), acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL) and myelodysplastic syndrome (MDS), IL-22 and Th22 levels are higher than in healthy control subjects; levels return to normal after effective therapy [7, 8]. Interestingly in chronic myeloid leukemia (CML), the opposite is the case [9]. In CLL, augmented levels of IL-22 are associated with leukemic clones having higher numbers of CD38- and ZAP70-expressing cells, and there is a trend for correlation with unmutated *IGHV* status [10, 11]. Despite these observations, information about the Th22 populations that produce the cytokine in CLL is not available.

We first analyzed the relative and absolute numbers of $CD4^+$/IL-17^- and $CD8^+$ /IL-17^- IL-22-producing T cells (Th22 and Tc22, respectively) in Peripheral Blood Mononuclear Cells (PBMCs) of 27 patients with CLL and 7 healthy controls (HCs) (Fig. 1A). To maximize detection of cytokines, PBMCs were stimulated with Phorbol-12-Myristiate-13-Acetate (PMA) + ionomycin for 4.5 h in the presence of a Golgi blocker, and then intracellular levels were measured by flow cytometry using an IL-22-specific mAb. As expected [4], patients with CLL had significantly greater numbers of $CD3^+$ cells as well as $CD3^+CD4^+$ and $CD3^+CD8^+$ subsets (Fig. 1B). Moreover, CD4:CD8 T cell ratios in patients with CLL differed from those of HCs (1.87 vs 4.16; $P = 0.006$). In addition, CLL patients exhibited an expansion of Th22 and Tc22 cells compared to HCs (Fig. 1C).

We then asked if IL-22-producing T cells correlated with the clinical and biological characteristics of patients with CLL. Notably, higher numbers of Th22 cells associated significantly with the need for treatment (Fig. 1D) and with a shorter time to first treatment (TTFT) (Fig. 1E). As mentioned, other hematologic malignancies exhibit increased levels of circulating Th22, and higher IL-22 and Th22 levels associate with an unfavorable outcome for patients with NHL, MM, and ALL; in CLL, IL-22 has been linked with bad outcome markers like CD38, ZAP70, and IGHV-unmutated status [10, 11]. We believe this is the first report of expansion of Th22 in CLL and its association with patients' outcome.

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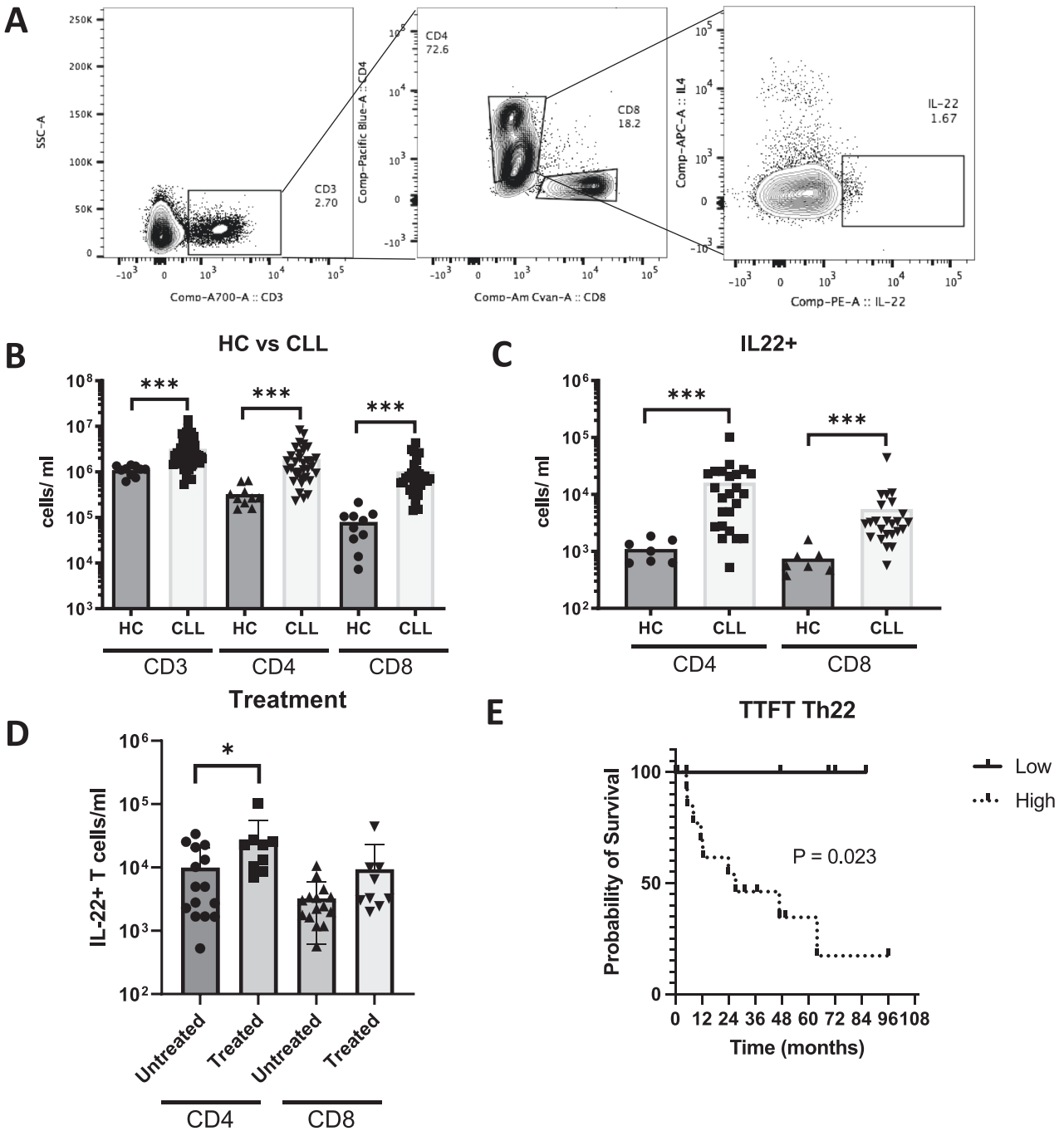

Fig. 1 Increased numbers of Th22 and Tc22 cells in peripheral blood of CLL patients correlates with shorter TTFT. A Flow cytometry marker and gating strategies to identify IL-22-producing T cells. **B**, **C** Absolute numbers of circulating $CD3^+$, $CD4^+$, $CD8^+$, $Th22^+$, and $Tc22^+$ cells from patients with CLL and gender- and age-matched healthy controls (HC). **D** Absolute counts of circulating $Th22^+$ and $Tc22^+$ cells from patients who required treatment vs. those who did not. **E** CLL patients were dichotomized into High and Low count subsets based on the numbers of IL-22-producing T cells using the Maxstat package for R-2.8.0. TTFT curves were calculated by the Kaplan-Meier method, and comparisons between groups were performed by the log-rank test. *P*-values: * < 0.05, ** < 0.01, and ***< 0.001.

To determine if CLL B cells have a direct influence on the generation of IL-22-producing T cells, we sorted Tn cells and stimulated these for 6 days with anti-CD3/CD28 beads + IL-2, in the presence or absence of CLL cells (Fig. 2A). Interestingly, upon TCR signaling, Tn from patients with CLL, when cultured alone, gave rise to a significantly higher number of Th22 than Tn from HCs; suggesting a predisposition of CLL Tn to differentiate to this subset (Fig. 2B). This is consistent with Th22 differentiation being promoted by IL-23, IL-1β, TNF-α, and IL-6 [5], cytokines that are elevated in patients with CLL [12]. Notably, introducing CLL B cells into the culture promoted a significantly greater increase in Th22 cells (Fig. 2B), and an increased release of soluble IL-22 into culture medium (Fig. 2C). Worth mentioning, activated CLL cells can induce the upregulation of miR-155 in Tn, facilitating differentiation to Th17 cells [13]. Since cytokines are major inducers of Th22 differentiation, we repeated the experiment after physically separating CLL B and T cells by a membrane. This aborted the increase in Th22 (Fig. 2D), indicating a requirement for direct contact between CLL and T cells to influence Th22 levels.

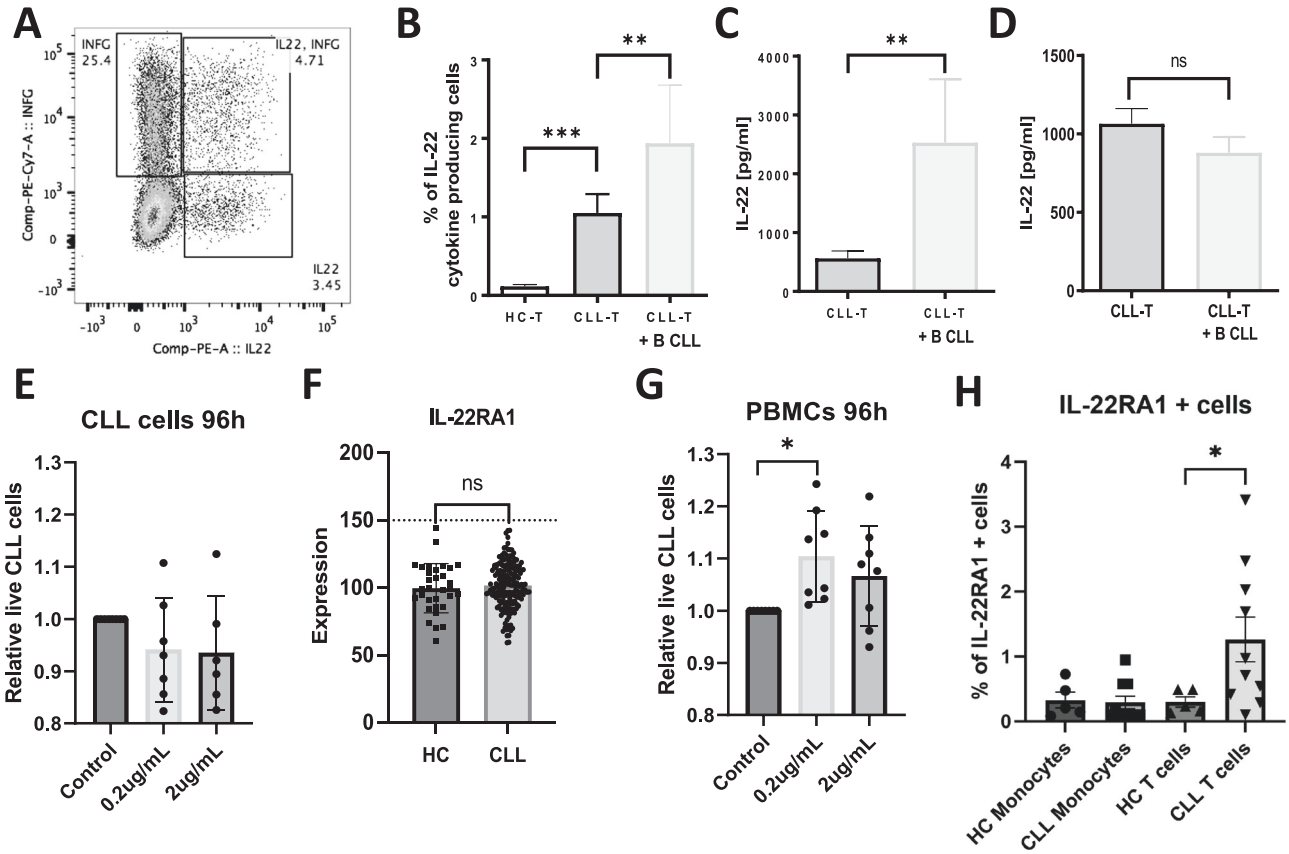

Fig. 2 CLL cells enhance naïve T-cell differentiation to Th22 cells, and IL-22 augments CLL-cell survival by microenvironmental influences. A, **B** FACS-isolated naïve $CD4^+$ T cells ($CD3^+CD4^+CD45RO^-CD62L^+$; Tn) from PBMCs of 20 CLL patients and 10 HC were stimulated in vitro with anti-CD3/CD28 beads + IL2 for 6 days, and then the percentages of IL-22 cytokine-producing cells were measured by flow cytometry. **C** Supernatants from cultures of patient T cells and patient T + autologous CLL B cells were analyzed for the levels of soluble IL-22 by ELISA. **D** Levels of IL-22 in the supernatant of $CD4^+$ Tn ($n = 5$) cultured in direct contact with CLL B cells or separated by a 0.4 µm pore membrane in a Transwell plate. **E**, **G** CLL-cell viability in 12 patient samples was evaluated in vitro by flow cytometry using FxCycle™ Violet and Annexin V staining in the presence or absence of recombinant human IL-22 (0.2–2 ug/ml) for 96 hours. **F** IL-22RA1 gene expression in samples from 32 purified normal B cells from HC and 188 CLL B cells (GSE50006). The dotted line indicates the trustable noise/signal threshold. **H** Percentage of IL-22RA1+ cells within PBMCs monocytes (CD14+ cells) and T cells (CD3+ cells) populations from five healthy controls and ten CLL patients evaluated by flow cytometry. *P*-values: * < 0.05, ** < 0.01, and ***< 0.001.

IL-22 interacts with IL-22RA1 to activate the JAK/STAT pathway. Although the expression of IL-22RA1 is mostly restricted to non-hematopoietic cells, CLL cells have been reported to aberrantly express IL-22RA1 [14]. Thus, we tested if the supplementation of human recombinant IL-22 to cultures of CLL cells impacted their viability. After 96 h, we did not detect augmented viability of FACS-purified CLL B cells cultured with different concentrations of IL-22 (0.2 and 2 ug/ml; Fig. 2E). To determine if this lack of a direct response to IL-22 was due to the absence of IL-22RA1, we probed expression of IL-22RA1 in CLL cells from bulk and single-cell RNA-seq data from independent cohorts studied in our laboratory. This search did not document expression of IL-22RA1 (not shown). In addition, we interrogated an available large gene expression dataset derived by microarray from a cohort of almost 200 CLL patients (GSE50006). The IL-22RA1 signal in that dataset was lower than threshold for trustable noise/signal, indicating that the amount of IL-22RA1 in CLL and sorted normal B cells is very low or not present (Fig. 2F, Supplementary Fig. 1). Moreover, none of the patients with CLL exhibited higher levels than the threshold or the normal counterparts. These findings support our observation that IL-22 does not directly affect survival of CLL cells and suggests that enhanced survival is mediated by other cells in the TME. Consistent with the latter, an increase in CLL viability was observed when unseparated PBMCs from patients with CLL were cultured with IL-22 (0.2 ug/ml) (Fig. 2G). Thus, it appears that either IL-22 acts on normal hematopoietic cells in the blood or that a non-leukemic population is needed for CLL cells to influence the response to the cytokine. This is analogous to the finding that myeloid cells and T cells can express IL-22RA1 in the inflammatory milieu found in patients with COVID-19 [15]. In this regard, we have identified a small fraction of T cells in CLL patients that express surface membrane IL-22RA1 (Fig. 2H); IL-22 will promote JACK-STAT activation and induce gene expression changes, consistent with these cells providing survival signals to CLL B cells (Fig. 2G). Additionally, although an increase in IL-22RA1 expression was not observed on monocytes (Fig. 2H), we cannot rule out that other myeloid cells and other monocyte-derived populations, such as nurse-like cells, could also express an IL-22 receptor and thereby provide additional survival signals to CLL cells.

Together, these results suggest a previously unrecognized positive loop involving IL-22-producing T cells, CLL B cells, and T cells in the TME that contributes to the maintenance of the leukemic clone and influences patient outcomes. Deciphering this complex interplay within the CLL TME might provide insights that could inform future therapeutic strategies.

DATA AVAILABILITY

The datasets analyzed for this study can be found in the Gene Expression Omnibus (GEO), expression arrays GSE50006, and scRNAseq GSE165087.

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AUTHOR CONTRIBUTIONS

GF, FP, PYC, and KW performed experiments. GF and NC designed the study, analyzed the data, interpreted the data, and wrote the manuscript. ABC, SC analyzed data. FP, BS, and SC helped interpret data. JCB, JEK, SLA, and KRR provided samples. All authors read and approved the final manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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