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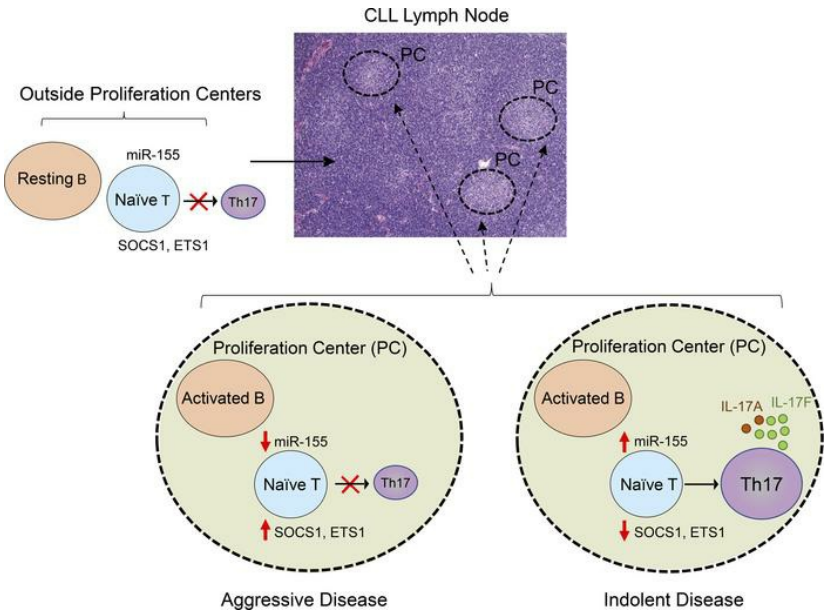
Research Article | Hematology | Immunology

Graphical abstract



JCI insight

RESEARCH ARTICLE

Activated CLL cells regulate IL-17F–producing Th17 cells in miR155-dependent and outcome-specific manners

Byeongho Jung,[1,2] Gerardo Ferrer,[1] Pui Yan Chiu,[3] Rukhsana Aslam,[1,3] Anita Ng,[1] Florencia Palacios,[1] Michael Wysota,[2] Martina Cardillo,[1] Jonathan E. Kolitz,[1,4,5] Steven L. Allen,[1,4,5] Jacqueline C. Barrientos,[1,4,5] Kanti R. Rai,[1,4,5] Nicholas Chiorazzi,[1,4,5] and Barbara Sherry[3,4,5]

[1]Karches Center for Oncology Research, Institute of Molecular Medicine, The Feinstein Institutes for Medical Research, Northwell Health, Manhasset, New York, USA. [2]Donald and Barbara Zucker School of Medicine at Hofstra/Northwell, Hempstead, New York, USA. [3]Center for Immunology & Inflammation, Institute of Molecular Medicine, The Feinstein Institutes for Medical Research, and [4]Department of Medicine, Northwell Health, Manhasset and New Hyde Park, New York, USA. [5]Department of Molecular Medicine, Donald and Barbara Zucker School of Medicine at Hofstra/Northwell, Hempstead, New York, USA.

Chronic lymphocytic leukemia (CLL) results from expansion of a $CD5^+$ B cell clone that requires interactions with other cell types, including T cells. Moreover, patients with CLL have elevated levels of circulating IL-17A$^+$ and IL-17F$^+$ $CD4^+$ T (Th17) cells, with higher numbers of IL-17A$^+$ Th17 cells correlating with better outcomes. We report that CLL Th17 cells expressed more miR155, a Th17-differentiation regulator, than control Th17 cells, despite naive $CD4^+$ T (Tn) cell basal miR155 levels being similar in both. We also found that CLL cells directly regulated miR155 levels in Tn cells, thereby affecting Th17 differentiation, by documenting that coculturing Tn cells with resting or activated (B_{act}) CLL cells altered the magnitude and direction of T cell miR155 levels; CLL B_{act} cells promoted IL-17A$^+$ and IL-17F$^+$ T cell generation by an miR155-dependent mechanism, confirmed by miR155 inhibition; coculture of Tn cells with CLL B_{act} cells led to a linear correlation between the degree and direction of T cell miR155 expression changes and production of IL-17F but not IL-17A; and B_{act} cell–mediated changes in Tn cell miR155 expression correlated with outcome, irrespective of IGHV mutation status, a strong prognostic indicator. These results identify a potentially unrecognized CLL B_{act} cell–dependent mechanism, upregulation of Tn cell miR155 expression and subsequent enhancement of IL-17F$^+$ Th17 generation, that favors better clinical courses.

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of monoclonal $CD5^+$ B cells in the bone marrow and secondary lymphoid organs that can, as the disease progresses, constitute the majority of lymphocytes in the blood (1, 2). CLL B cells communicate with numerous immune cell subsets via cell-to-cell contact and release of cytokines and other signaling molecules. These interactions, which occur in various lymphoid tissues referred to as the tumor microenvironment (TME), are essential for the survival, regulation, and expansion of the leukemic cells (3, 4).

It is well established that CLL B cells influence the function of autologous T cells (5–7), inducing defects in membrane molecule expression and adhesion, migration, and synapse formation, as well as inducing T cell exhaustion (8, 9). In addition, CLL B cells can sway the differentiation of naive $CD4^+$ T (Tn) cells to various Th cell subsets (10–12), leading to Th cell subset imbalance. The molecular mechanisms by which differentiation of Tn cells to Th cells occurs, as well as the effect of this imbalance on tumor survival and progression, are not fully understood.

Th17 cells are a subset of $CD4^+$ T cells that secrete IL-17, their signature cytokine (13). IL-17 is a family of cytokines comprising at least 5 isoforms (IL-17A through IL-17F) (14–16), and Th17 cells can produce these alone or in combination. Additionally, Th17 cells produce IL-21, IL-22, CCL20, and GM-CSF (17). Collectively, this T cell subset plays an important role in adaptive immunity through its proinflammatory properties. Moreover, its actions can be involved in autoimmune diseases and human

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cancers (13, 18). In CLL, Th17 cells appear to play a beneficial role, because patients with elevated levels of Th17A cells in the blood survive longer (11); circulating Th17 and serum IL-17A levels are higher in patients with more favorable prognostic indicators (19), and declining levels of Th17 and IL-17A are found with disease progression (20).

Differentiation of $CD4^+$ Tn cells to Th17 cells is promoted by exposure to cytokines, such as IL-6 and IL-1β (21). When patients with CLL are analyzed as a group, serum IL-6 levels are elevated and IL-1β levels are depressed (22, 23). However, when patients are divided into those with longer (≥5 years) or shorter (<5 years) time to first treatment (TTFT), patients in the former group have higher serum levels of a cluster of cytokines that includes IL-6, IL-1β, and IL-17 (24).

Several regulators, including miRNAs, control the differentiation of $CD4^+$ Tn cells to Th17 cells. One miRNA that modulates this differentiation process is miR155 (25–27). miR155 carries out a wide variety of functions, including regulating immune responses and influencing the development and function of lymphocytes (25–28), and miR155 is required for Th17 cell differentiation in mice (26). Although the precise mechanisms by which miR155 affects Tn cell differentiation to Th17 cells are not fully understood, miR155 regulates expression of several proteins that downregulate the Th17 differentiation cascade, including the transcription factors ETS proto-oncogene 1 (ETS-1) and suppressor of cytokine signaling (SOCS1). ETS-1 is a downstream mediator of IL-2, which censors genes critical for Th17 differentiation (29), and SOCS1 suppresses the JAK/STAT pathway that blocks IL-6–mediated activation of STAT3, thereby inhibiting Th17 differentiation (30–32).

Because patients with CLL can have elevated levels of Th17 cells and because CLL B cells can directly and indirectly regulate T cell activation and function, we investigated the possibility that CLL B cells influence the differentiation of Tn cells to Th17 cells, focusing on changes in miR155.

Results

Basal miR155 expression in T cells of patients with CLL and of healthy individuals. Because there are significantly higher numbers of circulating Th17 cells in patients with CLL compared with healthy, age-matched control participants (HCs), and because miR155 plays a critical role in Th17 development, we explored whether basal expression of miR155 in Th17 cells differs between patients with CLL and HCs. Analysis of miR155 expression in FACS-isolated Th17 cells from 4 patients with CLL and 4 HCs (our sorting strategy is shown in Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.158243DS1) revealed significantly higher basal expression of miR155 in CLL Th17 cells (Figure 1A).

To determine if this difference reflected an intrinsic property of CLL $CD4^+$ T cells, we next quantified miR155 expression in FACS-purified $CD4^+$ Tn cells (this sorting strategy is shown in Supplemental Figure 2) from 18 patients with CLL and 8 HCs. Notably, miR155 levels in Tn cells were not significantly different between patients with CLL and the HCs (Figure 1B). Thus, in CLL, miR155 levels in $CD4^+$ Tn cells are not inherently higher, consistent with miR155 levels in mature CLL Th17 cells being influenced by the TME.

Because the clinical courses and outcomes of patients with CLL are tightly linked with the presence or absence of somatic mutations in the IGHV gene expressed by the leukemic clone (33, 34), we further compared the basal expression of miR155 in Tn cells from HCs with that from patients divided into IGHV-mutated (M-CLL) and IGHV-unmutated (U-CLL) subgroups. Basal levels of miR155 differed significantly between patients with the 2 IGHV subtypes (Figure 1B).

B lymphocytes from patients with CLL differentially modulate miR155 expression in autologous $CD4^+$ Tn cells. Because our findings indicated that miR155 levels are not inherently higher in CLL $CD4^+$ Tn cells, we postulated that the TME might regulate the expression of miR155 in Tn, ultimately leading to elevated levels of miR155 in mature CLL Th17 cells. Because the dominant cellular population in the TME is leukemic B cells, which reside in a noncycling state outside of proliferation centers or cycling within proliferation centers, we asked if resting CLL (B_{rest}) cells or preactivated CLL (B_{act}) cells could increase miR155 levels in autologous Tn cells. Therefore, FACS-purified Tn cells were cocultured with B_{rest} or B_{act} cells, and miR155 expression was quantified at day 3 (Figure 2A). To generate B_{act} cells, purified CLL B cells were stimulated in vitro with CpG plus IL-15 for 5 days (see Methods). Because unactivated CLL B cells die rapidly in vitro, the B_{rest} cell population was freshly sorted on day 0 when cocultures were initiated.

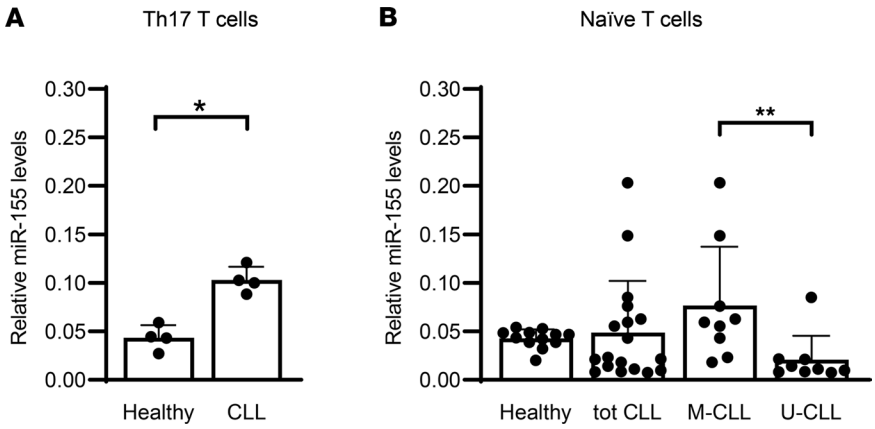

Figure 1. Comparison of miR155 expression levels in mature Th17 cells and Tn cells from untreated patients with CLL and age-matched HCs. (**A**) Relative miR155 expression (fold change relative to housekeeping gene RNU6-2) in mature $CD4^+$ Th17 cells from patients with CLL (n = 4) and HCs (n = 4). Th17 cells ($CD3^+CD4^+CD45RO^+CD161^+CCR6^+$ $CCR4^+$ $CXCR3^+$) were purified from PBMCs by FACS. *P < 0.05, ** P < 0.01 by Mann-Whitney U test. (**B**) Relative miR155 expression (fold change relative to housekeeping gene RNU6-2) in $CD4^+$ Tn cells from patients with CLL, grouped by IGHV mutation status (n = 18), and from HCs (n = 12). qPCR data were generated and analyzed using the LightCycler480 SW 1.5.1 software package and reported as relative gene expression calculated using the comparative Ct method. Friedman 1-way repeated-measure ANOVA by ranks followed by Dunn's multiple comparisons test was used for the analysis. Tot, total.

When autologous B and Tn cells from HCs were cocultured as controls, B_{rest} and B_{act} cells upregulated miR155 in Tn cells (Figure 2B), with the difference being significant for those cultures containing B_{act} cells. In contrast, cocultures of CLL B and autologous Tn cells resulted in heterogeneous changes in miR155 expression, in that leukemic B cells from some patients upregulated and others downregulated miR155 expression levels (Figure 2, C and D). Additionally, there were no significant differences in relative (Figure 2C) or normalized (Figure 2D) miR155 levels when comparing means. Of note, when inspecting the distribution (variability) between normalized miR155 levels in Tn cells after coculture with B_{rest} versus B_{act} cells, there was a significant difference (P = 0.0025 by Bartlett's test); this reflected differences in the magnitude of B_{rest} cell– versus B_{act} cell–induced miR155 changes relative to Tn cells. Collectively, these calculations suggest a fundamental difference between the effects of B_{rest} and B_{act} cells on Tn cell miR155 amount.

Because basal miR155 levels in Tn cells differed significantly between patients with M-CLL and patients with U-CLL (Figure 1B), we independently analyzed miR155 expression in Tn cells from the 2 IGHV subtypes after 3-day culture in the absence or presence of autologous B_{rest} cells or B_{act} CLL cells. Although miR155 levels in Tn cells from M-CLL cultures remained significantly higher than those from U-CLL cultures within each of the 3 experimental groups (Figure 2E), miR155 levels did not change significantly when either M-CLL and U-CLL Tn cells were cocultured with autologous B_{rest} cells or B_{act} cells (Supplemental Figure 3 and Supplemental Table 1). Finally, miR155 levels in CLL B cells, which are much higher than in T cells and affect survival (35), were hundreds of times greater than in autologous Tn cells for each M-CLL and U-CLL case analyzed (Supplemental Figure 4). This was as expected (35). Additionally, because of the clear directional heterogeneity of this result after exposure to B_{act} cells, it is unlikely that the miR155 differences observed were due to CLL B cell contamination.

B_{act} cells, but not B_{rest} cells, enhance the differentiation of CLL $CD4^+$ Tn cells to Th17 cells. We next investigated the effects that CLL B cells have on differentiation of Tn cells to Th17 cells. Cocultures were set up as described earlier but were extended for 7 additional days (Figure 3A). First, Tn cells were cultured alone or in the presence of B_{rest} cells or B_{act} cells for 3 days to allow miR155 modulation. Next, each Tn cell group that had been cultured with either B_{rest} cells or B_{act} cells was stimulated with anti-CD3– and anti-CD28–coated beads for 7 days. At various time points, supernatants and cells were collected and analyzed for secreted (by ELISA) and intracellular (by flow cytometry) IL-17 isoforms as a measure of Th17 differentiation.

When analyzing levels of secreted cytokines chronologically, minimal or negligible levels of IL-17A and IL-17F were found in day +3 supernatants. However, cytokine levels increased significantly and progressively over time (Supplemental Figure 5) when taking into account that IL-17 values on day +10 were comparable to those on day +7 despite removal of half the volume and subsequent replacement

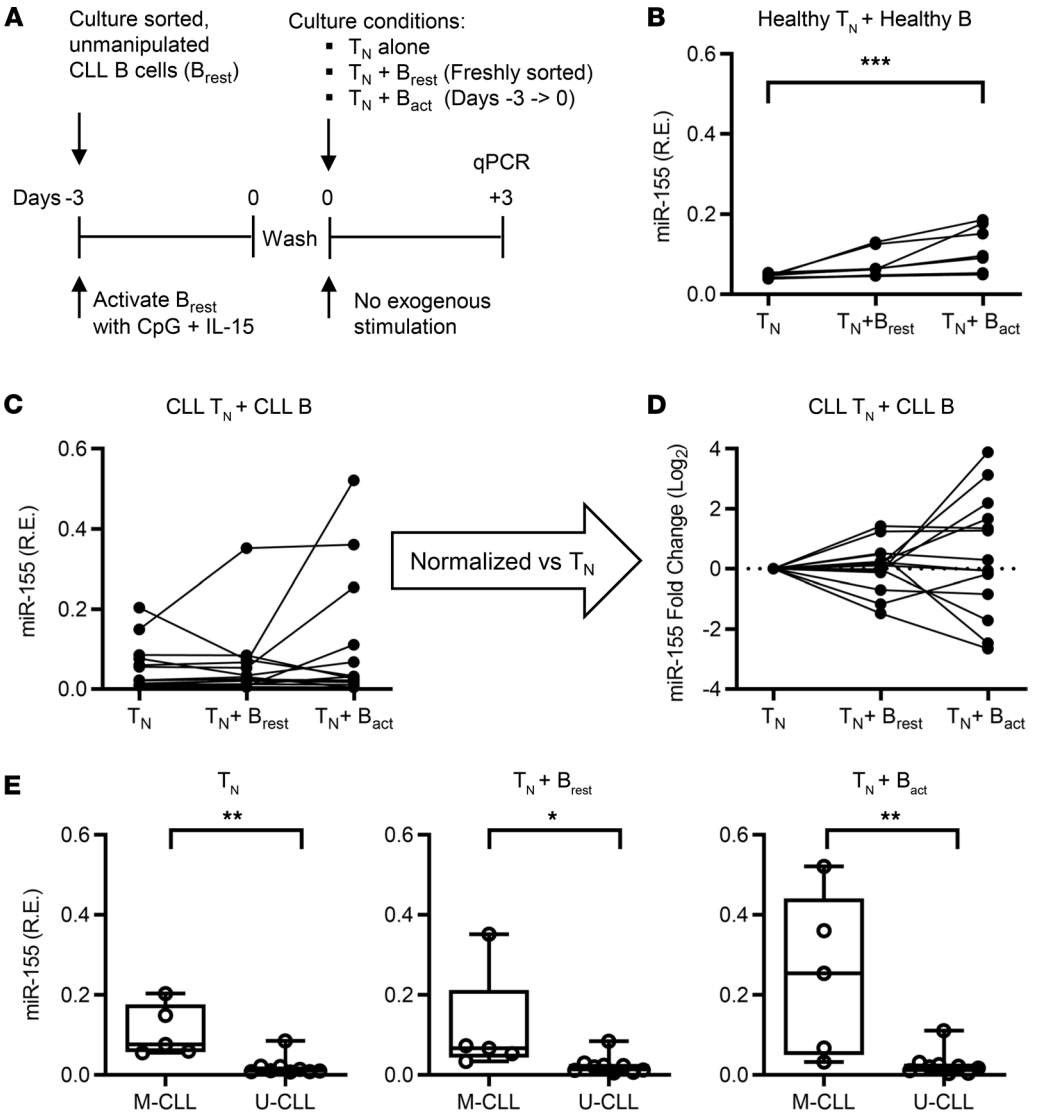

Figure 2. Modulation of Tn cell miR155 by B cells from age-matched HCs and patients with CLL. (**A**) Design and timeline of coculture experiment. B cells were initially cultured for 3 days (days –3 to 0) with CpG-ODN 2006 + IL-15. These or freshly thawed B cells were then cocultured for an additional 3 days with Tn cells (days 0 to +3) (**B**) Column scatter plot showing relative miR155 from HC Tn cells cocultured with autologous B cells. Connecting lines identify samples from the same patient. (**C**) Column scatter plot of relative miR155 from CLL Tn cells cocultured with autologous CLL B cells. (**D**) Values were normalized to Tn cells, showing miR155 expression relative to its own baseline expression. (**E**) miR155 expression was calculated and compared after 3 days of coculture. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by Friedman 1-way repeated-measure ANOVA by ranks followed by Dunn's multiple comparisons test in **B–D** and Mann-Whitney *U* test in **E**. R.E., relative gene expression.

with an equivalent amount of fresh medium on day +7. Therefore, we chose to perform subsequent analyses on day +10 supernatants.

Supernatants collected at day +10 from Tn cells plus B_{act} cell cocultures contained significantly higher levels of IL-17A (Figure 3B) and IL-17F (Figure 3C) compared with cultures of Tn cells alone or Tn cells plus B_{rest} cells. Notably, B_{act} cells led to significantly higher secretion of IL-17F than IL-17A ($P = 0.0122$). When analyzing the data based on CLL subtypes, Tn cells plus B_{act} cell cocultures derived from patients with M-CLL had significantly higher levels of both IL-17A and IL-17F ($P = 0.0073$ for both) than Tn cells cultured alone, and U-CLL showed a significant difference for IL-1F ($P = 0.0177$) and a trend toward significance for IL-17A ($P = 0.0628$) (Supplemental Table 2).

In contrast, B_{rest} cells promoted Tn cells to secrete significantly higher levels of IFN-γ than did B_{act} cells (Figure 3D), although this was the case for only M-CLL cultures ($P = 0.0117$) (Supplemental Table 2). Thus, B_{act} cells did not enhance the production of all cytokines but selectively promoted IL-17A and IL-17F

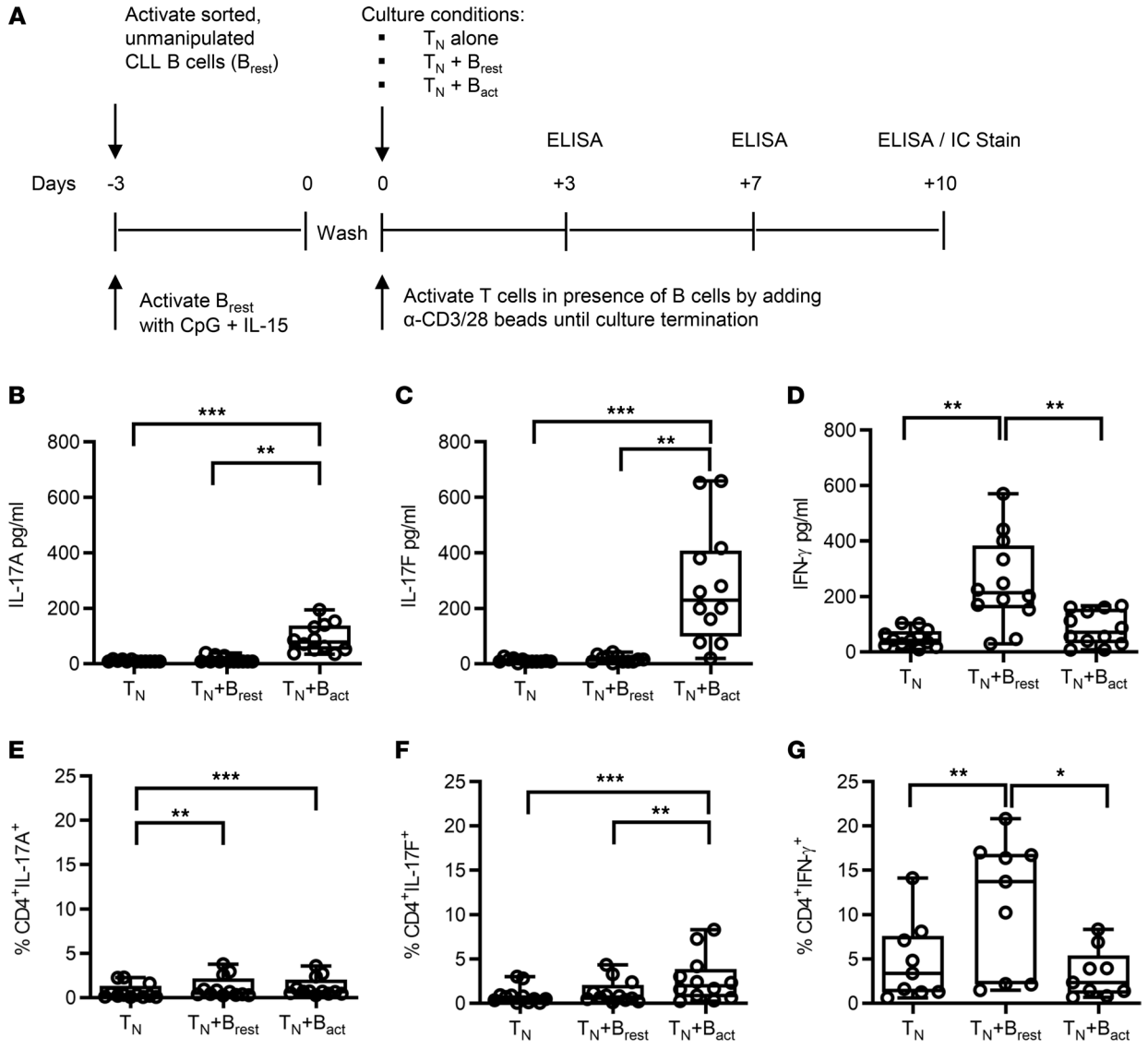

Figure 3. Effect of CLL B cells on differentiation of Tn cells to Th17A and Th17F cells. (**A**) Design and timeline of coculture. B cells were prestimulated or not, as in Figure 2 (days –3 to 0), and then cocultured with Tn cells for 3 days (days 0 to +3). At this point, the combined cell populations were stimulated with anti–CD3 and anti–CD28–coated beads for an additional 7 days (total of 10 days; days +3 to +10). ELISAs were conducted for IL-17A, IL-17F, and IFN-γ using culture supernatants collected on the days indicated. Data from analyses of supernatants and cells collected on day 10 are plotted in panels **B–G**. (**B–D**) Scatter plots with bars indicating the concentration (pg/mL) of secreted IL-17A (**B**), IL-17F (**C**), and IFN-γ (**D**) as determined by ELISA. (**E–G**) Scatter plots with bars indicating percentages of $CD4^+IL\text{-}17A^+$ cells (**E**), $CD4^+IL\text{-}17F^+$ cells (**F**), and $CD4^+IFN\text{-}\gamma^+$cells (**G**), as determined by flow cytometry. $^*P < 0.05$, $^{**}P < <0.01$, $^*P < 0.001$ by Friedman 1-way repeated-measure ANOVA by ranks followed by Dunn's multiple comparisons test. IC, intracellular.

secretion, with the latter being greater than the former. Notably, relative to Tn cells, B_{rest} cells did not induce significant differences in the day +10 levels of IL-17A, IL-17F, or IFN-γ in cultures of patients with M-CLL and patients with U-CLL, although the levels of IL-17A and IFN-γ secreted by Tn cells plus B_{rest} cells trended higher in patients with M-CLL than those with U-CLL (Supplemental Table 2).

Next, to determine if differences observed in supernatant fluids reflected an increase in the numbers of cells secreting each cytokine or the same number of cells secreting different cytokine amounts, cells from each culture were analyzed for intracellular IL-17A, IL-17F, and IFN-γ by flow cytometry (for sorting strategy, see Supplemental Figure 6). Significantly higher percentages of both $CD4^+$ Th17A and Th17F cells were seen when Tn cells were cocultured with B_{act} cells. This was the case for both IL-17A and IL-17F from M-CLL cell cultures ($P = 0.0117$ and $P = 0.0045$, respectively) as well as U-CLL cell cultures ($P = 0.0183$ and $P = 0.0117$, respectively (Supplemental Table 2). In comparison, for cocultures of Tn cells with B_{rest} cells, only the percentages of $Th17A^+$ cells were higher (Figure 3, E and F). Finally, it was notable that B_{rest}

cells significantly promoted higher percentages of $CD4^+$ IFN-γ^+ cells (Figure 3G); this held true for U-CLL ($P = 0.0281$), but not M-CLL, cell cultures (Supplemental Table 2). Thus, in general, intracellular cytokine levels defined by flow cytometry mirrored secreted levels measured by ELISA, suggesting that both the number of cells making IL-17 and the amount made by these cells were increased by B_{act} cell interactions.

miR155 expression influences the magnitude and direction of the change in IL-17F but not IL-17A. To solidify the link between miR155 levels and Th17 generation, we correlated the degree of change in Tn cell miR155 expression levels with IL-17A, IL-17F, and IFN-γ protein amounts in supernatants from +10-day Tn cells plus B_{act} cell cocultures. Notably, the change in miR155 levels in Tn cells after coculture with activated CLL B cells correlated with the absolute levels of IL-17F in supernatants (Figure 4A). This was the case for patients with U CLL and trended for patients with M-CLL (Supplemental Figure 7, E and F). Moreover, correlations with IL-17F were linear, with greater increases in miR155 after B_{act} cell coculture correlating with higher IL-17F levels, and greater decreases in miR155 correlating with lower IL-17F levels. miR155 changes also correlated with normalized (fold change Tn cells plus B_{act} cells versus Tn cells) levels of IL-17F (Figure 4B). The correlation with normalized IL-17F trended toward significance for U-CLL but not M-CLL (Supplemental Figure 7, G and H). Notably, a significant correlation with the change in miR155 was not observed for either absolute or normalized levels of IL-17A (Figure 4A and Supplemental Figure 7, A–D). Additionally, miR155 changes did not correlate with IFN-γ absolute (Supplemental Figure 8, A–C) or normalized levels (Supplemental Figure 8D).

Inhibition of miR155 blocks ETS1 and SOCS1 production and inhibits Th17 generation, defining a mechanistic link between miR155 and the generation of Th17 cells. In normal immune responses, the transcription factors ETS1 and SOCS1 downregulate the Th17 pathway (29, 30). Additionally, and especially relevant here, each is a target of miR155 (36, 37). Therefore, to assign a direct link between CLL B cell–induced miR155 levels and Th17 differentiation from Tn cells, we used a locked nucleic acid complementary to miR155 to specifically inhibit this miRNA. The efficacy of the inhibitor was first documented by treating naive CLL T cells with the active, inhibitory (miR155 locked nucleic acid [LNA]) or inactive (control LNA) reagent and then quantifying ETS1 and SOCS1 levels by qPCR. Treatment with miR155 LNA significantly increased ETS1 expression until 3 days after treatment initiation (Figure 5A) and SOCS1 expression for 1 day (Figure 5B). In contrast, no change in expression of either transcription factor was observed in cells exposed to the control LNA or no LNA. Similar results were obtained with naive T cells from HCs (data not shown).

We next examined the effects of miR155 inhibition on IL-17 production by pretreating Tn cells with either the inhibitory or the control LNA and then coculturing the 2 pretreated Tn cells sets with B_{act} cells for 10 days (Figure 5C). Untreated Tn cells cultured alone were included for comparison. Preincubation with miR155 LNA significantly decreased secretion of IL-17A (Figure 5D) and IL-17F (Figure 5E) compared with exposure to control LNA. Notably, IL-17F levels were reduced to baseline (Tn cells alone). However, conspicuously, IL-17A levels in the group treated with the inhibitory LNA remained significantly higher than baseline, although well below levels observed in Tn cells plus B_{act} cells cultured in the presence of the inactive LNA (Figure 5D). These findings support the data in the previous section (Figure 4B) and strongly suggest that miR155 has a greater influence on the production of IL-17F than of IL-17A.

Levels of miR155 and IL-17F, but not IL-17A, correlate with disease progression. Finally, we determined if the difference (normalized fold change) in miR155, IL-17A, and/or IL-17F levels between Tn cells plus B_{rest} cells and Tn cells plus B_{act} cell cultures correlated with disease progression and patient course and outcome. We chose to use fold change as opposed to the change in absolute values of the miR155, IL-17A, and/or IL-17F at the end of the coculture period because, in patients, Tn cells do not exist in isolation but are among leukemic B cells throughout the course of the disease, and the degree of CLL B cell activation correlates with patient outcome (38).

When the fold change in miR155 elicited by B_{act} cell versus B_{rest} cell incubation was correlated with disease progression and patient survival, we found a correlation with TTFT (Figure 6A) and overall survival (OS) (Figure 6B). However, this was the case only for patients with U-CLL (for TTFT data, see Supplemental Figure 9A; for OS data, see Supplemental Figure 9B). Additionally, absolute levels of miR155 at the end of the coculture period correlated with TTFT (Supplemental Figure 10A) and OS (Supplemental Figure 10B).

Moreover, when we divided patients into groups based on the direction of miR155 change (i.e., increasing or decreasing fold changes in miR155 relative to Tn cells plus B_{rest} cells) the direction of

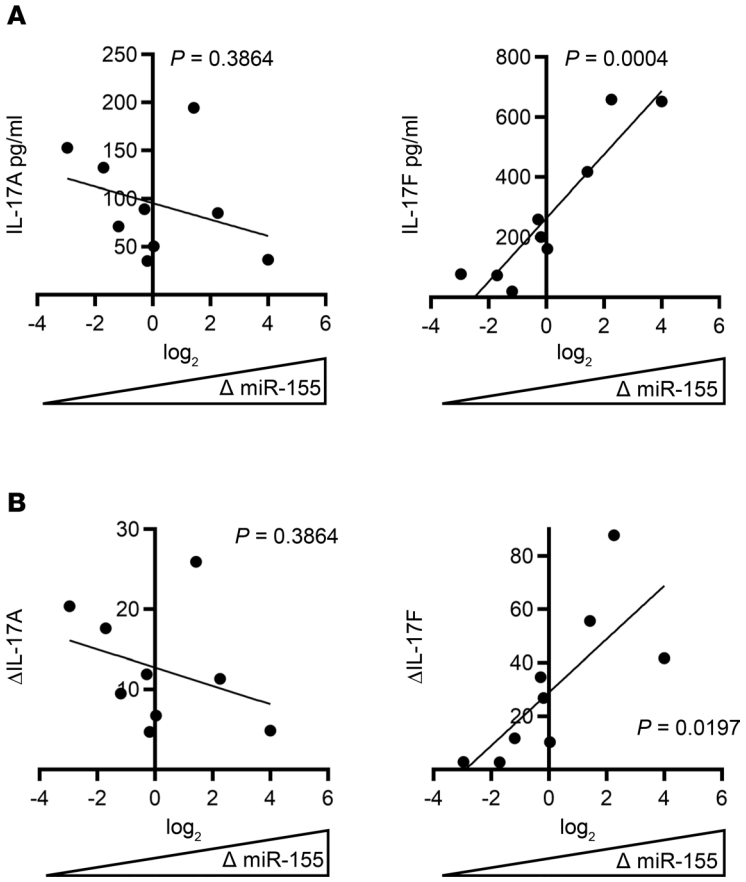

Figure 4. Correlations between miR155 and IL-17 isoforms. (**A**) Scatter plot of the change in miR155 expression between B_{act} and B_{rest} cells versus the absolute levels of secreted IL-17A and IL-17F at day 10 of Tn cells plus B_{act} cell coculture as determined by ELISA. (**B**) Scatter plot of the change in miR155 versus the changes in IL-17A and IL-17F between B_{act} and B_{rest} cells after normalization to Tn cells. The listed *P* values were determined from linear regression. Equation for calculating ΔmiR155, ΔIL-17A and ΔIL-17F is: fold change (log2) of Tn + B_{act} – fold change (Log2) of Tn + B_{act}. Δ, change.

change correlated significantly with both TTFT and OS, with increasing levels associated with longer TTFT (Figure 6C) and OS (Figure 6D) and decreasing levels with shorter TTFT (Figure 6C) and OS (Figure 6D). Notably, median TTFT of patients with increasing miR155 levels was longer than for patients with M-CLL (138 months versus 77 months, respectively) and median TTFT of patients with decreasing miR155 levels was comparable with that of patients with U-CLL (58 months versus 60 months, respectively) (Supplemental Figure 11, A and B). Median survival of patients with increasing miR155 levels was comparable to that of patients with M-CLL (138 months for both) and median survival of patients with decreasing miR155 levels was comparable to that of patients with U-CLL (67 months versus 71 months, respectively) (compared Supplemental Figure 11C with Supplemental Figure 11D). As expected, there was a significant difference in OS between M-CLL and U-CLL cases (Supplemental Figure 11, B and D).

When examining the relationship of secreted IL-17A and TTFT, neither the relative change in IL-17A levels (Figure 6E) nor the absolute levels in supernatants of Tn cells plus B_{act} cell cultures (Supplemental Figure 12A) correlated with TTFT. Conversely, for secreted IL-17F, both the normalized relative-change values (Figure 6F) and the absolute levels (Supplemental Figure 12B) correlated with TTFT ($P = 0.0001$ and $P = 0.0058$, respectively). When IL-17A– and IL-17F–producing cells were analyzed by flow cytometry (Supplemental Figure 13, A–D), only the normalized relative change in IL-17F correlated with TTFT.

Thus, increased miR155 levels in Tn cells induced by coculturing with activated CLL B cells correlate with longer TTFT and OS. However, this is not the case for both IL-17 isoforms, because only IL-17F, but not IL-17A, production and secretion are linked to improvements in both clinical outcome parameters.

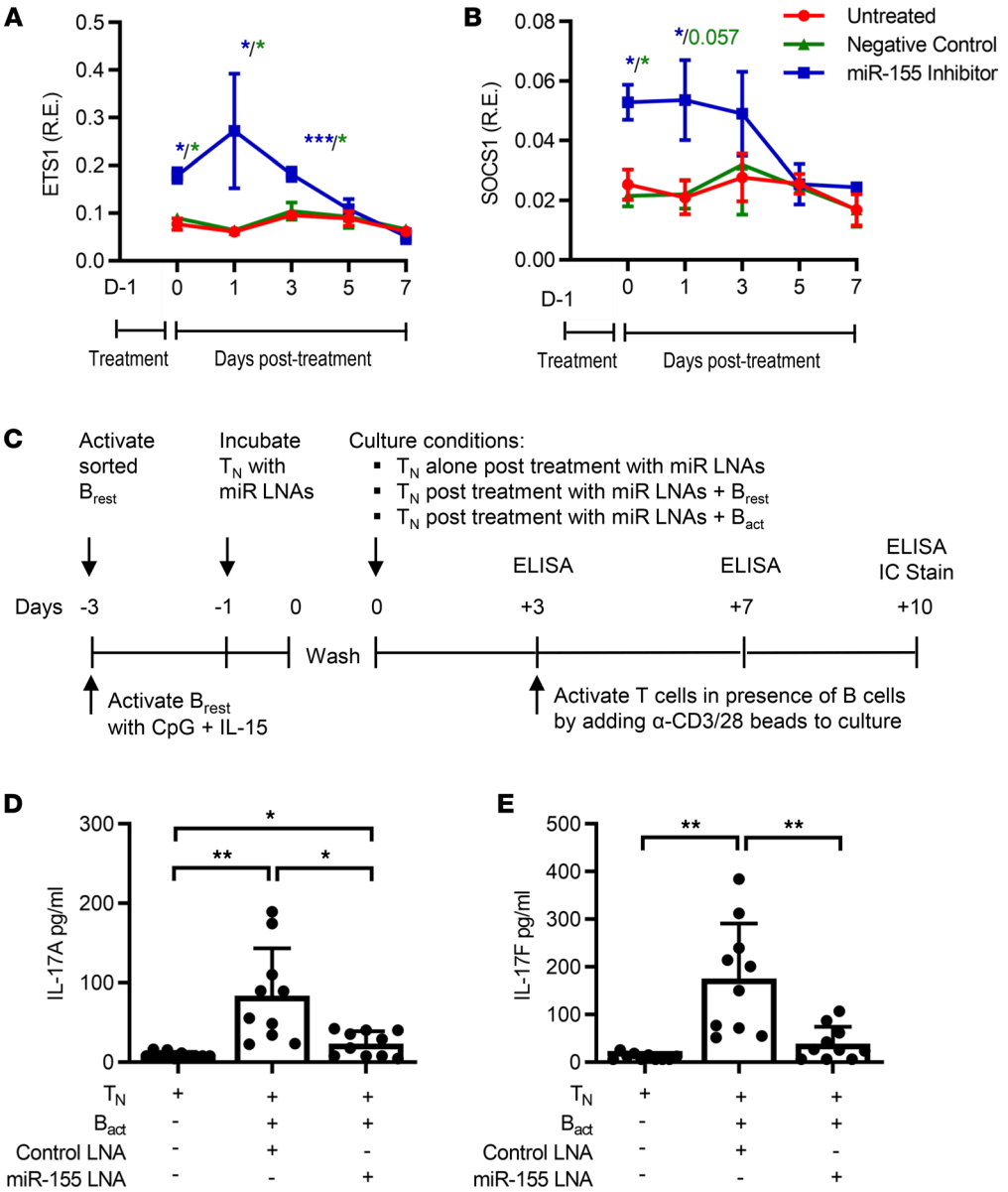

Figure 5. Effect of miR155 inhibition on IL-17 secretion. (**A** and **B**) Expression of ETS1 (**A**) and SOCS1 (**B**) by CLL T cells at various time points after 1 day of treatment with miR155 inhibitor or inactive control. Cells were washed on 1 day and expression of ETS1 and SOCS1 was measured by qPCR. (**C**) Design and timeline of coculture. B cell prestimulation for 3 days (days –3 to 0), as shown in Figure 2. At day –1, Tn cells were pretreated with miR155 inhibitor or control for 1 day and washed prior to start of coculture. Coculture conditions of Tn cells plus B cells were as shown in Figure 3. (**D** and **E**) Concentration of IL-17A (**D**) and IL-17F (**E**) in coculture supernatants at day +10 of coculture measured by ELISA. Coculture conditions are defined with presence (+) or absence (–) of the listed factors. $*P < 0.05$, $**P < 0.01$, $*P < 0.001$ by Friedman 1-way repeated-measure ANOVA by ranks followed by Dunn's multiple comparisons test. R.E., relative gene expression.

Discussion

Crosstalk between CLL B cells and other cells within the TME is essential, bidirectional, and affects patient outcome (3, 39–41). Understanding the complexity of these interactions might reveal new pathways for therapeutic targeting in this still incurable disease. Here, we investigated changes of miR155 expression in Tn cells mediated by CLL B cells; the influence of B cell activation status on the process; the relationship of miR155 changes on the differentiation of Tn cells to Th17A- and Th17F-producing cells; and the effects of the magnitude and direction of miR155 changes on the production and secretion of IL-17A and IL-17F and the correlation of these induced cytokines with disease progression and patient outcome.

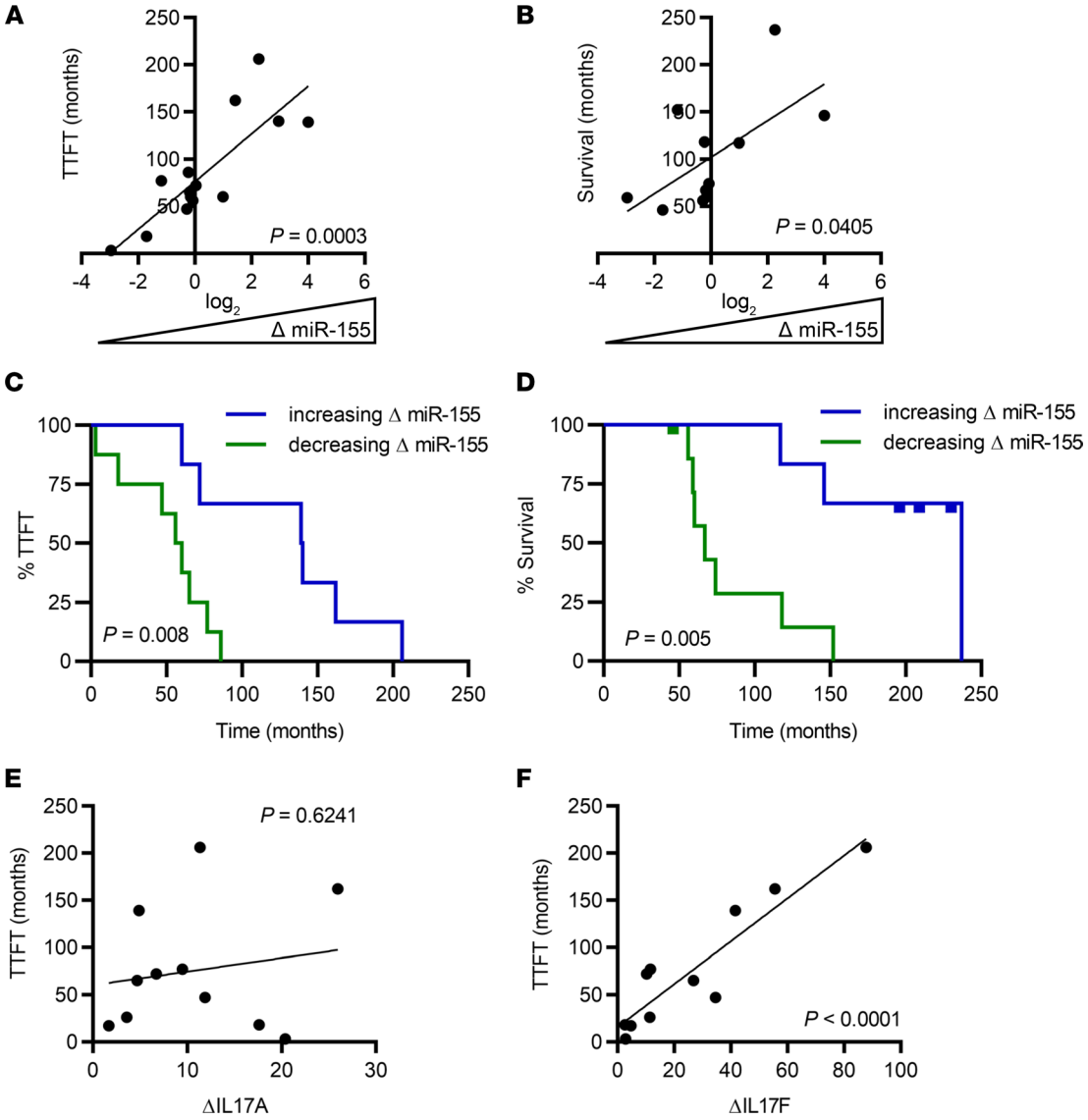

Figure 6. Correlations between miR155, IL-17, and patients' courses and outcomes. (**A**) Scatter plot of change in (Δ) miR155 and patient TTFT. (**B**) Scatter plot of change in miR155 and patient OS. (**C**) Kaplan-Meier plot of miR155 groups based on median values and corresponding patient TTFT. (**D**) Kaplan-Meier plot of miR155 groups based on median values and corresponding patient OS. (**E** and **F**) Scatter plot of TTFT and the change in IL-17A (**E**) and IL-17F (**F**) secreted in Tn cells versus Tn cells plus B_{act} cell cultures (normalized levels). *P* values are noted and were determined by linear regression and log-rank tests.

T cell subset skewing is controlled by a complex set of regulatory pathways, 1 of which involves miR155. This miRNA promotes Tn cell differentiation to the Th1, Th17, and Treg subsets and is required to skew maturation of Tn cells toward Th17 and away from Th2 (25, 42–44). Thus, pathways that modulate miR155 expression in Tn cells have the potential to affect the phenotype and function of the Th cell compartment. In this study, we showed that, although miR155 levels in Tn cells from HCs and patients with CLL as a group are similar, miR155 levels in mature, circulating Th17 cells from patients with CLL are significantly higher, suggesting that the regulation of miR155 may differ in the disease. Coculture of Tn cells with autologous B cells led to elevated levels of miR155 in both patients with CLL and HCs, but miR155 regulation appeared different between the 2 groups. HC B cells uniformly and significantly increased miR155 expression in autologous Tn cells, with the state of B cell activation only affecting the magnitude of the induced change. In contrast, in CLL, the effect of B_{rest} cells on miR155 expression in Tn cells was negligible, and the effect of B_{act} cells was heterogeneous among donors, both in the extent and direction of the response. This heterogeneity, in turn, was associated with marked variability in the capacity of CLL B cells, in the resting and activated states, to promote or inhibit miR155 levels in Tn cells and

affect their differentiation to Th17 and Th1 and their secretion of associated cytokines. In this regard, for B_{rest} cells, there were minimal changes in miR155 and IL-17 levels, whereas B_{act} cells changed both more consistently and considerably in some patients. This diversity in T cell responsiveness to activated CLL B cells related to the IGHV mutation status of the leukemic B cell clone, a feature that predicts a patient's clinical course and outcome. Notably, B_{act} cells from all but 1 patient with M-CLL led to increases in miR155 by autologous Tn cells and in IL-17 levels by the generated Th17 cells.

It should be noted that when looking at the population of patients with CLL as a whole, the correlation between miR155 and IL-17 secretion holds true only when considering the change in miR155 expression between Tn cells cultured alone and Tn cells cocultured with activated CLL B cells (by comparative Ct). Consistent with this, the absolute levels of miR155 expressed in Tn cells after coculture with B_{act} cells did not correlate with the amount of IL-17 secreted. Thus, the actual quantity of miR155 among individual patients does not appear to play the key role; rather, it is the fold change between the level of miR155 from Tn cells cultured alone and the resultant level of Tn cell miR155 upon T and B cell coculture (normalized levels) that is the main influencing factor. The precise reason for this is unclear, although the relative change in miR155 expression might alter the existing homeostatic balance within a cell more significantly than the change in miR155 absolute levels. Additionally, miR155 might be necessary but not sufficient for Th17 differentiation with other factors derived from Tn and CLL B cell (B_{rest} cells and B_{act} cells) or interactions affecting Th17 differentiation.

A notable finding in our studies was the more efficient maturation to Th17F than to Th17A cells and the more abundant secretion of IL-17F than IL-17A upon exposure to activated CLL B cells. Although there is no evidence addressing whether this occurs in response to signals delivered or molecules elaborated by activated CLL B cells or is an intrinsic property of Tn cells, or a combination of both, we have shown that it is not due to an inherently higher level of miR155 in CLL Tn cells. Rather, because there are greater numbers of circulating Th17F cells in CLLs than in healthy, age-matched HCs (45), there might be an inherent predisposition for Tn cells to more readily differentiate into Th17F, as suggested (45). In this regard, we did not find significant differences between ex vivo levels of IL-17A– and IL-17F–producing $CD4^+$ cells in this cohort of patients with CLL.

The ability of B_{act} cells to preferentially promote the generation of Th17 cells making IL-17F might be important from a functional perspective. Specifically, although IL-17A and IL-17F exert overlapping activities that cooperate with other proinflammatory cytokines to amplify inflammatory responses (16), the 2 isoforms also can carry out discrete functions (46, 47). Furthermore, the cytokine secretion profiles of Th17Fs are different from those of Th17As. For example, Th17F cells release lower levels of IL-10 and GM-CSF and higher levels of IFN-γ than do Th17As. (46). These cytokine differences could influence CLL B cell survival and growth.

In this study, we activated CLL B cells with CpG oligodeoxynucleotide (CpG-ODN) plus IL-15, a stimulation that is primarily T cell independent and mediated by the TLR9 signaling pathway. We chose this combination because it is an especially effective way to activate CLL B cells and induce cell division and survival (48). However, CLL B cells can be activated in other ways. Because of the documented differences in B cell receptor structure that characterize CLL cells, the best studied stimulant is B cell receptor engagement, with or without IL4. Paradoxically, this is a less efficient activator of CLL cell proliferation than is CpG-ODN plus IL-15 (48). In addition, CLL B cells can be turned on by interactions with activated T cells, a pathway that can be mimicked in vitro by exposure of B cells to a cocktail of CD40L plus IL4. Because our main goal was to maximally activate the majority of CLL clones to investigate the effects of B_{act} cells versus B_{rest} cells on Tn cells, we did not address either of the latter pathways. However, future studies should assess whether activation triggered by B cell receptor or CD40L engagement, alone or in combination, mediates a similar enhancement in miR155 expression and subsequent Th17 generation, and if so, whether this correlates with patient prognostic indicators.

Collectively, our findings are somewhat at odds with our previous demonstration that longer TTFT, reflective of a more indolent disease course, correlates with higher absolute numbers of circulating IL-17A–expressing Th17 cells (11, 19). However, it is important to recognize that our present study addresses the effects of CLL B cells on the generation of Th17 cells from Tn cells and not their effects on existing, committed Th17 cells, the consequences of which might differ. In this regard, the ratio of Tn cells to T memory cells in CLL and in aging humans, in general, changes considerably over time, with the $CD4^+$ T cell population becoming predominantly composed of memory cells (9). Another possible explanation for this apparent conundrum is

the complex interactions that occur in the TME, where Tn and B cells can interact with other cell types such as macrophages, DCs, and NK cells that might influence the maturation of Tn cells toward IL-17A or IL-17F (49–51). These interactions are very likely not replicated in our in vitro culture systems. Additionally, the different profile of Th17 subsets found in the blood (high Th17A) versus in our culture system (high Th17F) might reflect a difference in selective migration and localization of Th17F versus Th17A cells in vivo. It is important to note that published studies quantifying Th17 levels in vivo have focused primarily on Th17A and not Th17F cells. This is especially relevant because, as mentioned, Th17 cells can produce both IL-17A and IL-17F (52). Apropos, others have demonstrated selective production or inhibition of IL-17A and IL-17F in B and T cell cocultures, suggesting a differential regulation of the 2 isoforms (53). This might be especially relevant and consistent with 2 of our current findings (i.e., that the degree of change in miR155 in Tn cells after coculturing with B_{act} cells strongly correlates with normalized levels of IL-17F, but not IL-17A, and that specific inhibition of miR155 by an miR155 LNA more significantly reduces levels of IL-17F than IL-17A in B_{act} cell cocultures). Additionally, it is also important to note that previous studies have been limited to the blood, a compartment where CLL B cells do not receive the same signals as they do in secondary lymphoid tissues or bone marrow (54). Thus, our present in vitro study more approximates the situation that a Tn cell could encounter in a "proliferation center," a site where CLL B cell activation occurs (55) and where B cell expansion occurs in patients (56).

Our findings are also surprising in light of our previous observation that enhanced levels of CLL cell activation and replication in vivo correlate with inferior clinical courses (38, 57) and the observation of others that the numbers of proliferation centers link with worse clinical outcomes (58, 59). In this regard, the type of patient with CLL and their stage of the disease should be considered. Here, we found that patients with the "less aggressive" M-CLL type were more likely to have a greater increment in miR155 over baseline, the specific parameter that correlates with TTFT. Additionally, considering that activated CLL cells can secrete higher levels of IL-6 and IL-1β, which are both Th17-skewing cytokines (22, 60, 61), they might more efficiently promote Th17 generation (62), thereby mirroring our finding that coculturing Tn cells with B_{rest} cells does not lead to the generation of Th17 cells, whereas coculturing with B_{act} cells does. Furthermore, B cells from patients with progressive disease secrete lower levels of these 2 cytokines as compared with patients with nonprogressive CLL (23, 24, 63), suggesting that CLL B cells in patients with better outcomes create a TME more favorable for Th17 generation and action, reflected by differences in magnitude of cytokine secretion. Finally, it is again important to reiterate that in the present study, we correlated the effects of CLL B cells on the commitment of Tn cells with the Th17 lineage and did not address the effects on established Th17 cells.

Although we report that activated CLL B cells from a subset of patients with CLL with better clinical outcomes promote Th17 generation in vitro, there are several potential confounding factors and technical limitations to consider. One is that we used purified populations of CLL B cells and Tn cells to decipher the direct interaction(s) between these 2 cell populations. Although this approach allows for more precise conclusions, it does not account for the potential influence of other types or subsets of cells that are present in the TME in vivo. Also, in our coculture system, B_{act} cells likely release cytokines that could alter miR155 expression in Tn cells. One is IL-6, a mediator that is elevated in the sera of patients with CLL (45, 64) and is released by CLL B cells, especially upon activation (45). IL-6 also can drive the differentiation of Tn cells to Th17 cells (52, 65) and can induce STAT3 phosphorylation in T cells and subsequent upregulation of miR155 expression (65, 66).

Despite these considerations, our work offers the first direct evidence, to our knowledge, that CLL B cells influence the skewing of Tn cells toward a Th17 phenotype in patients with distinct disease courses and outcomes and that the mechanism whereby this occurs involves miR155. The former is consistent with findings in mice that B1 ($CD5^+$) B lymphocytes induce the differentiation of $CD4^+$ T cells to Th17 cells (67) and that the normal, human, cellular equivalent of CLL B cells could be a $CD5^+$ B cell (68).

Of special relevance, CLL B cells from the subset of patients with better clinical outcomes (i.e., those with M-CLL) are more likely to elicit this effect than leukemic B cells from patients with poor clinical outcomes (i.e., those with U-CLL). Furthermore, we show that CLL B cell enhancement of miR155 expression in Tn cells is most pronounced when using CLL B cells from patients with better clinical outcomes, irrespective of IGHV mutational status. Heterogeneity in the molecular and functional features of the leukemic B cells among patients with CLL is a well-documented phenomenon, both biologically

and clinically. In our study, we successfully link the 2, correlating heterogeneous responses at the molecular level to the protein and functional levels, and linking such biological responses with disease course and outcome. Together, these results identify what appears to be a previously unrecognized pathway that may explain why certain patients with CLL exhibit indolent disease and suggest that manipulation of this pathway in patients with CLL with poor prognostic features might improve outcomes.

Methods

Study samples. Samples from 30 patients with CLL, identified according to the International Workshop on CLL diagnostic criteria (69), and 8 age-matched HCs were used. Clinical and laboratory characteristics of the patients with CLL are described in Supplemental Table 3. All samples used in this study were obtained from patients who were not treated at the time of blood sample collection, although they were treated at some point thereafter. Anonymized, age-matched blood samples from HCs collected by leukapheresis were obtained from the New York Blood Center.

Study design. Experiments were designed to investigate the mechanisms by which CLL B cells influence the differentiation of autologous Tn cells to Th17 cells and to determine whether the findings correlate with clinical course of the disease. The design represents a series of controlled laboratory experiments. CLL and HC B and T cell populations were purified from cryopreserved PBMCs by flow cytometry sorting and cultured alone or in combination. miR155 levels were analyzed by qPCR. Th17 cells were quantified by intracellular flow cytometry and ELISA, as described below. Because of the requirement for many PBMCs from patients with CLL (>200 million) to allow for the isolation of the requisite number of purified Tn cells, the choice of samples and their use for each assay were based on material availability (Supplemental Table 3).

Blood processing and isolation of Th17 cells, Tn cells, and naive B cells. PBMCs from whole blood (from patients with CLL) or leukapheresed blood (blood from HCs) were isolated using Ficoll-Paque (GE Healthcare) density gradient centrifugation. Purified PBMCs were suspended in FBS and DMSO (Sigma-Aldrich) and stored in liquid nitrogen until use.

Th17 cell isolation. Th17 cells were isolated from thawed PBMCs by FACS after an initial selection of $CD3^+$ cells using the EasySepHuman CD3 Positive Selection Kit (StemCell Technologies). Then, $CD3^+$ cells were washed, resuspended in Dulbecco's PBS without magnesium or calcium, supplemented with 2% FBS at a concentration of 2×10^7 cells/mL, and stained for 30 minutes at 4°C in the dark with anti–CD4-APC H7 (BD Biosciences, clone RPA-T4), anti–CD45RO-FITC (BD Biosciences; clone:UCHL1), anti–CD161-PE (Miltenyi Biotec; clone 191B8), anti–CCR6-PE-Cy7 (BioLegend; clone G034E3), anti–CCR4-APC (R&D Systems; clone 205410), and anti–CXCR3-PerCPCy5.5 (BD Biosciences; clone 1CG). Immediately prior to sorting, PI (Millipore Sigma) was added to cells to achieve a final concentration of 200 ng/mL to allow identification of dead cells. Th17 cells ($CD3^+CD4^+CD45RO^+CD161^+CCR6^+CCR4^+CXCR3^+$) were collected according to the sorting strategy outlined in Supplemental Figure 1.

Tn and B cell isolation. Tn cells were isolated from thawed PBMCs by FACS after an initial depletion of B cells using the EasySep Human CD19 Positive Selection Kit II (StemCell Technologies); the latter process was carried out per the manufacturer's protocol except that only 80% of the suggested volume of Ab and magnetic-bead solution was used to allow some B cells to remain for subsequent isolation by FACS. Next, cells were washed, resuspended in Dulbecco's PBS without magnesium or calcium (GE Healthcare Life Sciences) at a concentration of 1×10^8 cells/mL, and stained for 30 minutes at 4°C in the dark with anti–CD4-FITC (BD Biosciences, clone SK3), anti–CD19-PE-Cy7 (BioLegend; clone HIB19), anti–CD62L-PE (eBioscience Inc., clone DREG-56), and anti–CD45RO-eFluor450 (eBioscience Inc., clone UCHL1). The sorting strategy is outlined in Supplemental Figure 2. FACS-purified Tn cells ($CD4^+CD19^-CD62L^+CD45RO^-$) and CLL B cells ($CD4^-CD19^+$) were used in subsequent experiments.

Coculture of naive $CD4^+$ T cells with and without CLL B cells. $CD4^+$ Tn cells were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 25 mM HEPES buffer in round-bottom, 96-well plates for 3 days, alone or with CLL B cells enriched from PBMCs, using the EasySep Human CD19 Positive Selection Kit II at a ratio of 5:1 (B cells to $CD4^+$ Tn cells). The leukemic B cells were either freshly isolated B_{rest} cells or preactivated for 3 days in vitro by culturing with CpG ODN (CpG ODN 2006, 1 μg/mL; Invivogen) and IL-15 (10 ng/mL; R&D Systems) at 2×10^6 cells/mL. Three days after the initiation of coculture (day 0), T cells were again isolated by FACS and lysed in TRIzol LS (Thermo Fisher Scientific) to isolate mRNA and determine miR155

mRNA levels by qPCR. For cytokine analyses, long-term cocultures were established as described above, but on day 3, half the medium was removed and supplemented with fresh medium containing Dynabeads Human T Activator CD3/CD28 (Thermo Fisher Scientific) at a 1:10 bead to cell ratio, and cultures were returned to the incubator. Supernatants were collected at days 7 and 10 for ELISA, and cells were collected at day 10 for intracellular cytokine analysis by flow cytometry. Fresh medium was supplemented to replenish removed medium at day 7.

For miR155 inhibition studies, Tn cells were treated with 300 nM hsa-miR155-5p miRCURY LNA miRNA Power Inhibitor or miRCURY LNA miRNA Power Inhibitor Control A (Qiagen) for 24 hours. Cells were then washed and cocultured for 10 days, following the coculture method described in the coculture protocol above. Supernatants were collected for ELISA and cells for flow cytometry at day 10. The control LNA was reported by Qiagen to have no hits with >70% homology to human, mouse, or rat miR155 sequences. The efficacy of miR155 LNA was confirmed by qPCR of ETS1 and SOCS1, downstream targets of miR155.

RNA isolation and RT-qPCR for miR155 expression. RNA was isolated from the lysates as described in the previous paragraphs, using Direct-zol RNA MicroPrep Kit (Zymo Research) and reverse transcribed to cDNA using miScript II RT Kit (Qiagen). RT-qPCR assays were performed in triplicate with miScript SYBR Green PCR Kit (Qiagen) and Hs_miR155_2 miScript Primer Assay (Qiagen). RNA, U6 small nuclear 2 (RNU6-2), was used as an endogenous control using Hs_RNU6-2_11 miScript Primer Assay (Qiagen). Threshold values reported in Figure 1 were obtained using the Roche LightCycler 480 (Roche Life Science) and threshold values reported in all relevant subsequent figures were obtained with the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Other data reported in Figure 1 were analyzed using LightCycler 480 SW 1.5.1, and Sequence Detection System 2.4, RQ Manager 1.2.1 was used in analyses shown in all relevant subsequent figures. qPCR data are reported as relative gene expression calculated using the comparative Ct method or as fold change calculated using the comparative Ct method, as indicated in the figure legends.

Intracellular staining and flow cytometry analysis of IL-17A, IL-17F, and IFN-γ. After 10 days of coculture, as described above, cells were collected, washed once with PBS, and surface antigens identified using anti–CD4-FITC and anti–CD19-PE-Cy7 for 30 minutes at 4°C in PBS. Cells were then washed with PBS and stimulated for 4.5 hours with 10 ng/mL PMA, 250 ng/mL ionomycin, and 3 μM BD GolgiStop Protein Transport Inhibitor (BD Biosciences). Stimulated cells were washed, fixed, and permeabilized with the Fixation/Permeabilization Solution Kit (BD Biosciences). Intracellular cytokines were identified with anti–IL-17A–PerCP-Cy5.5 (BD Biosciences; clone N49-653), anti–IL-17F–PE (Thermo Fisher Scientific; clone SHLR17), and anti–IFN-γ-APC (eBiosciences Inc.; clone 4S.B3). Flow cytometry was conducted with an LSR II (BD Biosciences), and data were analyzed by FlowJo software, version 10.5.3.

ELISA assays. Supernatants collected from cocultures described above were frozen and stored at –20°C. Immediately prior to analysis, supernatants were thawed and analyzed for IL-17A, IL-17F, and IFN-γ using the DuoSet ELISA kits (R&D Systems).

Statistics. Because experiments like these have not been published to our knowledge, we could not use existing data to carry out a formal power analysis. Therefore, sample size for both patients with CLL and HCs was selected on the basis of results from initial studies. All experimental conditions were tested in replicate. In all experiments, samples from patients with CLL and from HCs were treated and analyzed in parallel.

Groups of 2 were compared using the Mann-Whitney *U* test or Wilcoxon matched-pairs test, as indicated in the figure legends. Groups of 3 (i.e., Tn cells, Tn cells plus B_{rest} cells, Tn cells plus B_{act} cells) were compared using Friedman's 1-way repeated-measure ANOVA by ranks followed by Dunn's multiple comparisons test, repeated measures 1-way ANOVA with Geisser-Greenhouse correction followed by Tukey's multiple comparisons test, or unpaired ANOVA, as indicated in the figure legends. TTFT was defined as the time, in months, between diagnosis and initial therapy. Correlations were tested for significance using linear regression, and groups of the Kaplan-Meier plot were compared using the log-rank test. Statistical analyses were performed using GraphPad Prism 8.4.1. *P* values of less than 0.05 were considered significant.

Study approval. All procedures in this study were approved by The Feinstein Institutes for Medical Research/Northwell Health Office of the Institutional Review Board (approval no. 08-202A). All study participants provided informed, written consent.

Author contributions

BS and NC conceived of the study. BJ, NC, and BS determined the methodology and study design. BJ, GF, PYC, MW, MC, and BS contributed to data acquisition. BJ, GF, FP, AN, RA, NC, and BS performed data analysis and interpretation. KRR, JEK, SLA, JCB, NC, and BS contributed resources. NC, BS, KRR, and RA handled funding acquisition. BJ, NC, and BS wrote the manuscript, and all authors approved its final version.

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Address correspondence to: Barbara Sherry or Nicholas Chiorazzi, The Feinstein Institutes for Medical Research, 350 Community Drive, Manhasset, New York 11030, USA. Phone: 516.562.3402; Email: BSherry@Northwell.edu (BS). Phone: 516.562.1090; Email: NChizzi@Northwell.edu (NC).

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