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Refining the phenotypic and functional intraclonal complexity in chronic lymphocytic leukemia B cells uncovers a discrepancy between surface membrane IG levels and time since birth supporting the requirement for multifactorial activation and different subclonal sensitivities to treatment.

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Introduction

Chronic lymphocytic leukemia (CLL) clones can be divided into subsets based on time since last cell division. This can be determined by patients drinking deuterated water (^2H_2O) and then using flow cytometry to identify intraclonal subpopulations with reciprocal surface levels of CXCR4 and CD5. This approach distinguishes fractions enriched in recently divided "proliferative" (PF; $CXCR4^{Dim}CD5^{Bright}$); "intermediate" (IF; $CXCR4^{Int}CD5^{Int}$) and "resting" (RF; $CXCR4^{Bright}CD5^{Dim}$) cells.

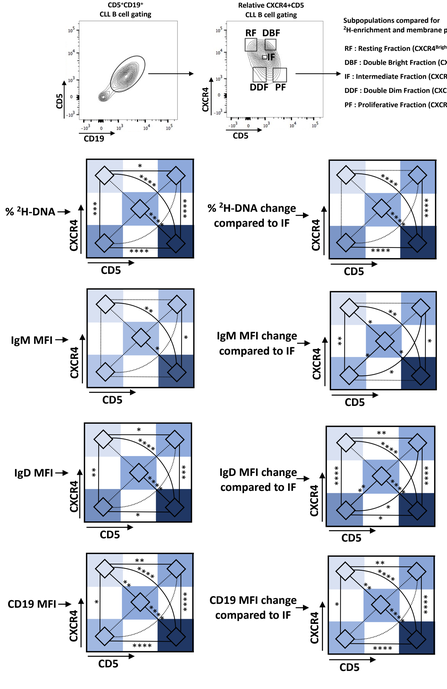
This 3-subpopulation model proposes that CLL cells, dividing in tissue proliferation centers, upregulate surface membrane (sm) CD5 and reduce smCXCR4, allowing cells to detach from the stroma and emigrate into the blood as the PF. Over time, cells of the PF transition to IF and then RF. Aging cells with higher smCXCR4 levels re-acquire the ability to migrate in the secondary lymphoid organs and some can be stimulated again and be re-born, perpetuating the cycle.

However, this model does not consider all fractions based on CXCR4/CD5 expression and how they transition from one to another. For example, the model assumes a linear and concomitant transition of smCXCR4 and smCD5 and the stimulants required to generate the "youngest" CLL cells are not clearly defined. Also, the model does not discriminate changes in smIG levels that can affect antigenic responsiveness.

We redefine the kinetics of CLL fractions and provide novel insights about their functional dynamics. Unmanipulated *ex vivo* CLL cells from 10 patients who drank ^2H_2O for 4 weeks were sorted by CXCR4/CD5 relative densities, isolating PF, IF, RF, and two previously uncharacterized fractions, "Double Dim" (DDF: $CXCR4^{Dim}CD5^{Dim}$) and "Double Bright" (DBF; $CXCR4^{Bright}CD5^{Bright}$).

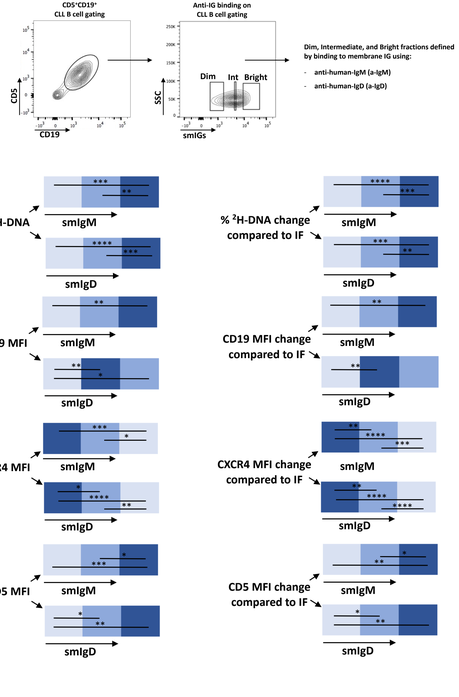
In vivo kinetics and phenotype for the intraclonal subpopulations based on CXCR4/CD5 densities

For each fraction, the amount of deuterium incorporated into cellular DNA *in vivo* was measured. The PF contained more ^2H-labeled DNA than the RF and IF. The DDF also contained more ^2H-DNA than RF. The DBF contained more ^2H-DNA than the RF, but less than the PF. Hence, using ^2H-DNA as determinant of age, a unidirectional path of phenotypic change could be defined, with the PF transitioning to DDF or IF or DBF, with the latter three moving to the RF directly or indirectly through the IF.



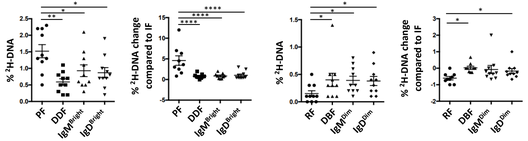
In vivo kinetics and phenotype for the intraclonal subpopulations based on IgM and IgD densities

Since BCR signaling is fundamental for CLL proliferation, we analyzed the densities of smIgM, smIgD (smIGs) and smCD19 for each subpopulation, finding higher smIGs and smCD19 densities on fractions with higher ^2H-DNA incorporation. Accordingly, we measured ^2H-DNA in fractions with low, intermediate, and high levels of smIGs, confirming that intraclonal subpopulations with high smIGs/CD19 divided more recently.



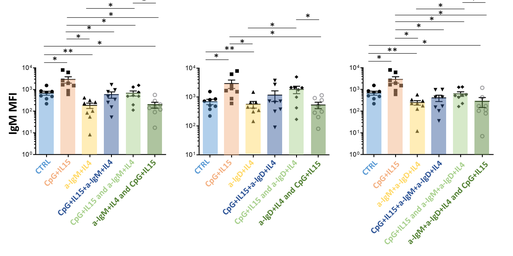
Comparison of the CXCR4/CD5 and the IgM and IgD methodologies for selection/enrichment of the most recently divided cells.

In the context of the ^2H-DNA-enriched fraction defined by the CXCR4/CD5 and the smIgM and smIgD density techniques, the PF subset display the most substantial enrichment. Likewise, when examining the fraction with the least ^2H, the RF subset shows the least amount of enrichment.



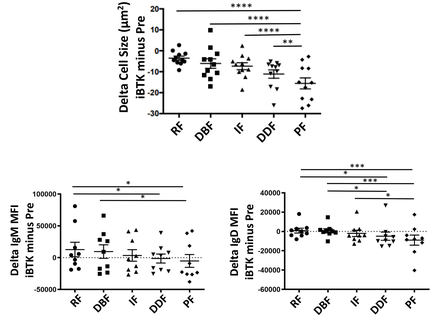
Specific chronological combinations of stimuli via TLR9 and smIGs lead to increased smIGs densities on recently divided cell

Notably, these findings are not consistent with cell division being uniquely initiated by BCR engagement since that lowers smIGs levels. Therefore, we tested if engagement of TLR9 or CD40L would affect mIG densities on CLL cells. After stimulation of 32 CLL clones via TLR9 or CD40L or smIGs pathways, an increase in smIGs and CD19 was observed for the former two, while the latter led to their downregulation. Interestingly, chronological combinations of stimuli via TLR9 and smIGs showed that increased IG density required TLR9 stimulation before or concurrently with the latter. Thus, recently-divided cells might have experienced multifactorial stimulation in defined chronologies



Ibrutinib differentially affects the various intraclonal CXCR4/CD5 and smIG fractions.

Finally, the 5 intraclonal fractions sorted from CLL patients, before and during ibrutinib treatment *in vivo*, displayed diverse intraclonal changes in smIG densities and metabolic activation, with ^2H-enriched and higher smIG density cells being more affected.



Conclusions

These data define additional CXCR4/CD5 subpopulations of divergent ages, phenotypes, and sensitivities to treatment, suggesting that CLL B-cell kinetics are more complex than the current model describes. This complexity originates in secondary lymphoid organs, where serial stimulation delivered by the BCR and other pathways generates the young PF and possibly the DDF that, once in the blood, continues to age to the quiescent RF fraction. Combining CXCR4/CD5 with smIG/CD19 densities might better resolve the complexity of the time from last cell division in leukemic cells, thereby allowing better understanding of the underlyng biological mechanisms that give rise to subpopulations differing in biologic function and susceptibility to therapies. Since each cell within a clone appears to traverse these stages, the unique biologic features at each phase might represent novel processes for potential therapeutic targeting, alone or in combination.