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Unraveling a poor-prognosis CLL subgroup: from IGHV bias and AID activation to altered T-cell profiles

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OBJECTIVE

To investigate the origins and functional impact of Activation-Induced Cytidine Deaminase (AID) expression in the IGHV-unmutated patients.

CONCLUSIONS

- Patients from Sb_1, representing 36% of U-CLL cases with earlier treatment requirements, display **a different AID mutational landscape** compared with Sb_2
- WES analysis of Sb_1 highlight **AID off-target mutations** involved in pathways related to **Interferon alpha and gamma response and KRAS signalling.**
- The percentage of **T cells with exhaustion markers** are significantly increased in **Sb_1** respect T cells of Sb_2.

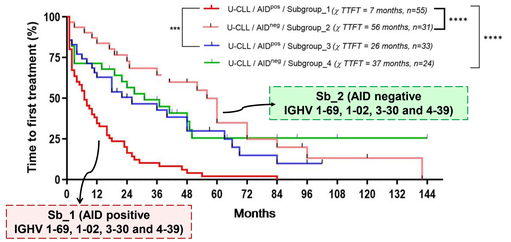
PERSPECTIVES

- Characterize at transcriptional level the different UM-CLL subgroups in order to determine the extent to which genes specifically harbour AID off-target mutations are up/down regulated in their expression, and/or how their biologic functions are affected in CLL cells.

Institut Pasteur de Montevideo

INTRODUCTION

The mutagenic **enzyme AID** (activation-induced cytidine deaminase) play a physiological role in the germinal center reaction. Nonetheless, uncontrolled expression of this enzyme could result in off-target mutations with implications in cancer development and progression (1,2). In this work we focus on AID expression levels in the peripheral blood (PB) of Unmutated-CLL **(U-CLL)** patients and analyse its association with immunoglobulin heavy chain gene **(IGHV)** rearrangements and the clinical outcome through time to first treatment **(TTFT)**. This approach led us to identify within U-CLL patients, four subgroups (Sb), with different clinical profiles: **Sb_1**, AID^{pos} with IGHV_1-69, 1-02, 3-30 and 4-39, **Sb_2**, AID^{neg} with IGHV_1-69, 1-02, 3-30 and 4-39; **Sb_3,** AID^{pos} with IGHV rearrangements different from Sb_1/2 and **Sb_4,** AID^{neg} with IGHV rearrangements different from Sb_1/2 (3). Surprised by the differences found in the TTFT between Sb_1 and 2 (similar IGHV families but different AID expression levels) we investigate the molecular mechanism that could be at the origin and during CLL progression of these two subsets. To this we performed: 1) Whole exome sequences (WES) with the goal to find a particularly AID-mutational landscape and to identify genes driver mutations associated to the early treatment need. 2) Immunophenotyping of the T cell compartment, with the hypothesis that different tumor microenvironment signalling (antigen/autoantigen, T-cells, Nurse like cells –NLC-, soluble chemokines/cytokines or others unknown molecules) could explain these different clinical profiles in U-CLL cases



Prognostic value (TTFT) of Subgroups_1, 2, 3, and 4 in U-CLL.

AID expression was assessed using a specific AID TaqMan probe and performing real-time PCR from cDNA. The assay's sensitivity and cut-off value for AID positivity was determined to be 7.000 CN, as assessed by ROC curve analysis (sensitivity and specificity >99%). The identification of IGHV family's subgroups and its association with AID expression was performed by unsupervised K-Means clustering.

TTFT curves were compared using Log-rank (Mantel-Cox) test, P values <0,0001 = **** and 0,001 = ***, n=152, U-CLL. The comparative analysis of U-CLL subgroups revealed significant differences in clinical outcomes.

METHODS

PBMCs were collected from patients with informed consent and Ethics Committee approval and stored in the GURU-LLC Biobank at the Institut Pasteur de Montevideo. Whole-exome sequencing (WES) was performed using the Agilent SureSelect capture kit and Illumina NGS technology. Raw data were quality-checked with FastQC/MultiQC, and filtered using *fastp*. Reads were aligned to the human reference genome (hg19/hg38) with BWA-MEM2. Median coverage exceeded 140x, with >88% of the exome covered at ≥50x depth. Flow cytometry analysis was performed from patients derived PBMCs.

RESULTS

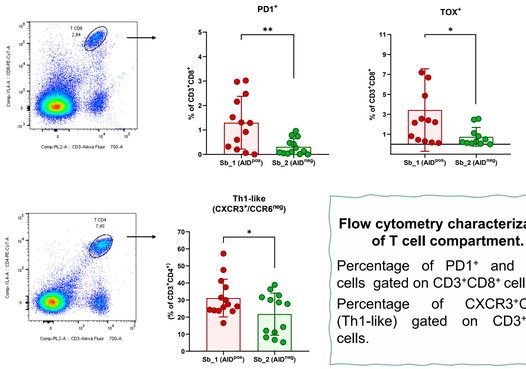
Patients from Sb_1 present more canonical and non-canonical AID off-target mutations than Sb_2 **(Fig. 1A).** These data confirm that the higher AID expression measured in the PB of Sb_1 patients is a "mirror reflex" of its activity in proliferative centers, and that this action exerts functional effects on their genome. Specific analyses of AID off-target mutations comparing Sb_1 with Sb_2 revealed a significant enrichment in genes associated with interferon alpha and gamma responses and with KRAS signalling.

After this initial approach, we analyzed genes within the three pathways of interest to identify mutations occurring exclusively in either Sb_1 or Sb_2, while discarding variants shared by both subgroups. We specifically focused on: (1) non-synonymous mutations (missense variants), (2) upstream or downstream gene variants, (3) mutations in the 3′ and 5′ UTR regions, and (4) splice donor variants. Our results revealed a higher number of unique, recurrent mutations in several patients of Sb_1 affecting key genes within the aforementioned pathways **(Fig. 1B)**. For example, within the interferon α/γ response pathway, we detected AID-associated mutations in HLA-C (9/11 patients, 81%), HLA-DRB1 (6/11, 55%), and NCOA3 (5/11, 45%), among others **(Fig. 1C).** Although the precise biological consequences of these alterations remain to be established, these findings highlight a potential role for AID-induced mutagenesis in shaping CLL progression within this patient subgroup.

Concerning the immunophenotyping, and taking into account our hypothesis about an active tumor microenvironment present in patients of Sb_1, we found in this subgroup an increase percentage of T CD8 cells expressing exhausted-related markers, such as PD1 and TOX. On the other hand, an accumulation of Th1-like cells was also found in this subgroup **(Fig.2)**.

Taking all observations into account (antigenic restriction of Sb_1, continuous AID expression, enrichment of exhausted-like CD8$^+$ T cells, Th1-like accumulation, and interferon pathway alterations driven by AID activity), we propose this scenario: Patients in Sb_1 undergo persistent antigenic or autoantigenic stimulation. This chronic activation fosters a proliferative tumor microenvironment that sustains AID expression, promotes the accumulation of off-target mutations in proto-oncogenes, and thereby accelerates disease progression. In parallel, the classical T-cell dysfunction characteristic of CLL is further exacerbated as a consequence of this persistent dynamic. Ultimately, this scenario contributes to the earlier treatment requirements observed in these patients.

Figure 2: Patients from Sb_1 show increased PD1$^+$ and TOX$^+$ T CD8 cells and Th1-like cells compared to Sb_2

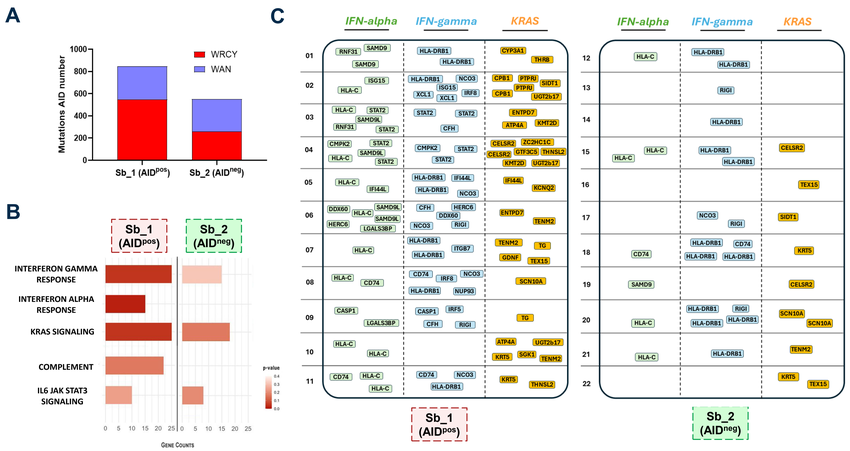


Flow cytometry characterization of T cell compartment.

Percentage of PD1$^+$ and TOX$^+$ cells gated on CD3$^+$CD8$^+$ cells.

Percentage of CXCR3$^+$CCR6$^-$ (Th1-like) gated on CD3$^+$CD4$^+$ cells.

Figure 1: Patients from Sb_1 concentrate AID off-target mutations in the IFN-α/γ and KRAS pathways



WES analysis

Panel A: Number of mutations with AID signature (canonical/WRCY and non-canonical/WAN) in Sb_1 vs Sb_2. **Panel B:** Top five pathways enrichment analysis with AID signature in Sb_1 (AID+). **Panel C:** Pathways with statical significance, discriminating by patient (01-22) genes unique mutated with AID signature in Sb_1 (left panel) vs Sb_2 (right panel).

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DISCLOSURES

The authors declare no conflicts of interest