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Targeting the pro-inflammatory protein S100-A9 in Chronic Lymphocytic Leukemia

Angimar Uriepero[1], Melanie Mediavilla-Varela[1], Kamira Maharaj[1], Wael Gamal[1,2], María Elena Marquez[3], Eugenia Payque[3], John Powers[1], Mohammad Ammad-Ud-Din[4], Vishaal Kunta[1], Eva Sahakian[1,4], Pablo Oppezzo[3] and Javier Pinilla-Ibarz[1,4]

[1]Department of Immunology, [4]Division of Malignant Hematology, H. Lee Moffitt Cancer Center & Research Institute, Tampa, FL; [2]Department of Molecular Medicine, College of Medicine, University of South Florida, Tampa, FL.; [3]Institut Pasteur de Montevideo, Montevideo, Uruguay.

Correspondence: Angimar.Uriepero@moffitt.org

Bakcground

Since the approval of the new targeted therapies, favorable responses and longer survival can be achieved in some patients with Chronic Lymphocytic Leukemia (CLL); however, refractoriness to treatment is frequently seen and CLL is still considered an incurable disease. Therefore, to create new therapeutic options is an unmet need in this leukemia. During CLL progression, signs and symptoms of inflammation worsen and intracellular pro-inflammatory pathways are activated, providing leukemic lymphocytes with proliferative and survival advantages. Recently, our group described the presence of the S100-A9 protein in exosomes derived from CLL patients with poor therapeutic outcomes. S100-A9 is potent mediator of inflammation, tumor invasion and metastasis. This protein plays an important role in the myeloid compartment, where high expression of S100-A9 is associated with the suppressive activity of myeloid derived suppressor cells (MDSCs). In addition, S100-A9 inhibitors have shown immunomodulatory properties and are being evaluated in early phase clinical trials for multiple myeloma and prostate cancer.

We hypothesized that expression of S100-A9 in B-CLL cells render a survival advantage through activation of inflammatory pathways and modulation of the tumor microenvironment. The following specific aims are therefore proposed:

Aim 1: To assess the role of the S100-A9 protein on the crosstalk between CLL cells and their microenvironment.

Aim 2: To investigate whether S100-A9 inhibition could impair CLL progression.

Methods

- S100-A9 in primary human B-CLL cells: S100-A9 expression was evaluated by flow cytometry upon cytokine stimulation or co-culture with autologous nurse like cells (NLCs) for 24 hours. Likewise, PBMCs from CLL patients were stimulated with IL-4 +/- rhS100A9 for 72 hours, then Ki-67 expression was assessed.
- S100-A9$^{-/-}$ B-CLL cells *in vivo* model: Eμ-TCL1 and S100-A9$^{-/-}$ mice were crossed to generate a new Eμ-TCL1/S100A9$^{-/-}$ murine model. Adoptive transfer (AT) into NOD-scid IL2Rgammanul (NSG) mice were performed using 5x10^6 of isolated CD19+/CD5+ cells from aged Eμ-TCL1 and Eμ-TCL1/S100A9$^{-/-}$.
- Paquinimod (PaQ) and Tasquinimod (TasQ) treatment: 15x10^6 Eμ-TCL1 splenocytes were injected into C57BL/6 mice. PaQ and TasQ were administered at a dosage of 25 mg/Kg in drinking water ad libitum for 4 weeks. Once the treatment was completed the animals were euthanized for tissue collection. Fluorescence-activated cell sorting (BD FACS Aria II) were used to isolate CD19+ cells from the spleen of PaQ treated and control mice for mRNA NanoString study. All immunophenotyping analysis was performed via flow cytometry (FC), using a BD FACSymphony or a BD LSR II cytometer.

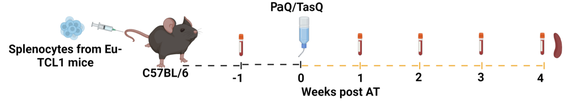

Fig. 1 Microenvironmental signals induce S100-A9 expression in primary B-CLL cells

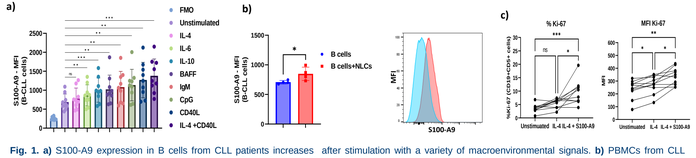

Fig. 1. a) S100-A9 expression in B cells from CLL patients increases after stimulation with a variety of macroenvironmental signals. **b)** PBMCs from CLL patients were co-culture with or without autologous NLCs for 24 hours, then S100-A9 expression was assessed by flow cytometry. **c)** PBMCs from CLL patients were stimulated with IL-4 and IL-4+hS100-A9 for 72 hours, then Ki-67 was evaluated in the malignant B cell population by FC. *** p <0.0005 ,**p <0.005,*p <0.05.

Fig. 2 Ablation of S100-A9 in murine B-CLL cells delays disease progression

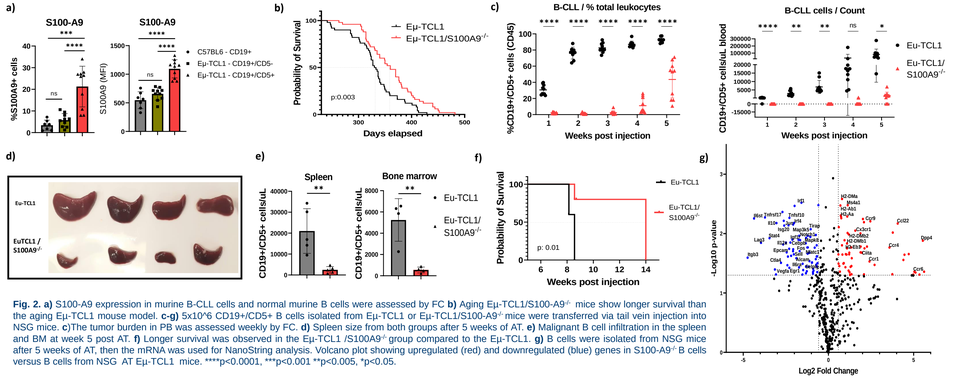

Fig. 2. a) S100-A9 expression in murine B-CLL cells and normal murine B cells were assessed by FC **b)** Aging Eμ-TCL1/S100-A9$^{-/-}$ mice show longer survival than the aging Eμ-TCL1 mouse model. **c-g)** 5x10^6 CD19+/CD5+ B cells isolated from Eμ-TCL1 or Eμ-TCL1/S100-A9$^{-/-}$ mice were transferred via tail vein injection into NSG mice. **c)**The tumor burden in PB was assessed weekly by FC. **d)** Spleen size from both groups after 5 weeks of AT. **e)** Malignant B cell infiltration in the spleen and BM at week 5 post AT. **f)** Longer survival was observed in the Eμ-TCL1 /S100A9$^{-/-}$ group compared to the Eμ-TCL1. **g)** B cells were isolated from NSG mice after 5 weeks of AT, then the mRNA was used for NanoString analysis. Volcano plot showing upregulated (red) and downregulated (blue) genes in S100-A9$^{-/-}$B cells versus B cells from NSG AT Eμ-TCL1 mice. ****p<0.0001, ***p<0.001 **p<0.005, *p<0.05.

Fig. 3 S100-A9 inhibitors prolong the survival of CLL bearing mice

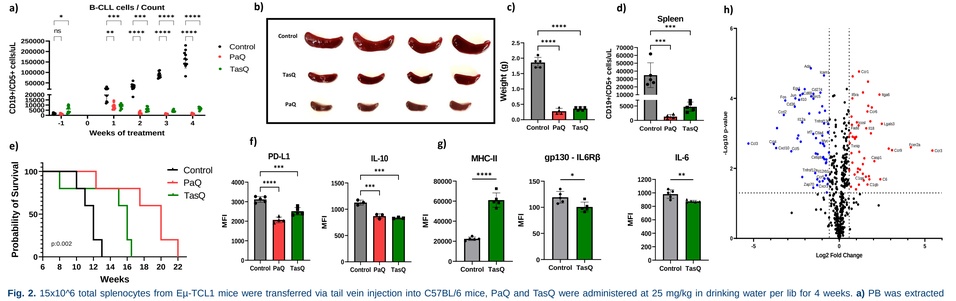

Fig. 2. 15x10^6 total splenocytes from Eμ-TCL1 mice were transferred via tail vein injection into C57BL/6 mice, PaQ and TasQ were administered at 25 mg/kg in drinking water per lib for 4 weeks. **a)** PB was extracted weekly, and the tumor burden was assessed by flow cytometry. Our results show a significant reduction in the total number of malignant cells since week 1 in the treatment group compared to the vehicle. **b-c)** Representative picture and spleen size after 4 weeks of treatment. **d)** Spleens show less infiltration of B-CLL cells in mice treated with both S100-A9 inhibitors. **e)** Mice treated with PaQ or TasQ show a longer survival compared with the control group. **f)** CD19+CD5+ B cells from treated mice show less IL-10 and PD-L1 expression measured by flow cytometry (MFI) **g)** CD19+CD5+ B cells from PaQ treated mice show higher expression of MHC-II and lower expression of both IL-6 and the active subunit of the IL-6 receptor **h)** Volcano plot showing differential gene expression in B cells from PaQ versus vehicle using NanoString PanCancer Immune Profiling. ****p<0.0001, ***p<0.001 **p<0.005, *p<0.05

Fig. 4 S100-A9 inhibitors reverse the immunosuppressive tumor microenvironment in Eμ-TCL1 adoptive transfer mice

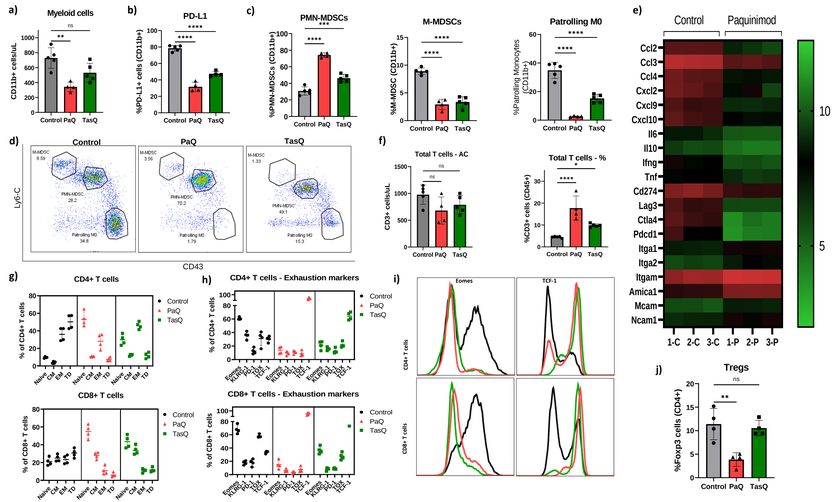

Fig. 4 a) Eμ-TCL1 AT mice were treated with PaQ, TasQ or vehicle for 4 weeks. Spleens from both groups were collected for flow cytometry and NanoString analysis. **a)** Absolute count of total myeloid cells **b)** PD-L1 expression was downregulated in myeloid cells from mice treated with PaQ and TasQ **c-d)** The fractions of M-MDSCs and patrolling monocytes were significantly reduced after treatment with S100-A9 inhibitors, while the PMN-MDSCs were increased. **e)** CD11b+ cells were isolated from control and PaQ treated mice, then the mRNA was used for NanoString analysis. Heatmap showing the most differential expressed genes among the two groups **f)** Fraction and absolute count of total T cells in the spleen **g)** Phenotypic characterization of CD4+ and CD8+ T cells in the spleen after the treatment **h)** Expression of exhaustion markers in the CD4+ and CD8+ T cells subsets in the spleen from the three mice group i) Representative histograms from flow cytometry data showing Eomes and TCF-1 expression in T cells from the three treatment groups **j)** Percentage of T regulatory (Treg) cells were significantly decreased after *in vivo* treatment with PaQ. .

Conclusions

- Microenvironmental signals promote S100-A9 expression in primary B-CLL cells.
- S100-A9 in combination with IL-4 significantly increases Ki-67 expression in B cells from CLL patients.
- Leukemic cells from Eμ-TCL1 show higher S100-A9 than normal B cells.
- Eμ-TCL1/S100A9$^{-/-}$ mice have longer survival in comparison with Eμ-TCL1 mice.
- Murine S100A9$^{-/-}$ B-CLL cells grow slower than leukemic CD19+CD5+ lymphocytes with a WT S100-A9 protein.
- Pharmacological inhibition of S100-A9 significantly reduces tumor burden and prolongs survival in the Eu-TCL1 adoptive transfer model.
- B cells and myeloid cells from mice treated with PaQ show significantly lower expression of inflammatory cytokines.
- Eμ-TCL1 AT mice show a less immunosuppressive TME after treatment with S100-A9 inhibitors.