"Cblb-Notch1 regulation as a new target for cancer immunotherapy"

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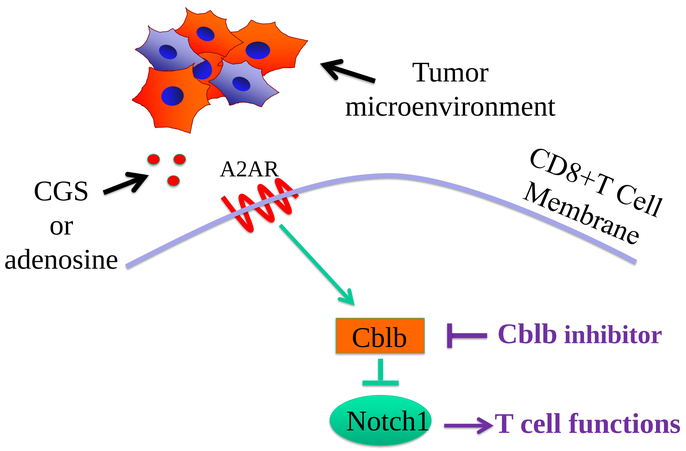
Background

- Notch1 (N1) is a transmembrane receptor that regulates proliferation, cytokine production and cytotoxic activity of CD8+ T-cells.
- One of the major functions of CD8+ T-cells is the elimination of pathogens and cancer cells within the body. However, cancer cells can evade T-cell responses, for example, by producing adenosine, an immunosuppressive metabolite.
- Adenosine blocks T-cells immune responses in part through activation of the Adenosine A2A Receptor (A2AR). Activation of this receptor by adenosine or an A2AR agonist (CGS) decreases Notch1 in CD8+ T-cells and, in turn, T-cells functions.
- It is not known how A2AR regulates N1, however, a recent study from our group identified the ubiquitin ligase Cblb as a potential negative regulator of N1.

- **Objectives:** We investigated signaling through A2AR, its effect on N1 and activation in CD8+ T-cells. The final goal of this work is to find strategies to target this new pathway for therapeutic purposes.



Methods

- Primary CD8+ T-cells were isolated from the spleens and lymph nodes of mice, activated with anti-CD3/CD28, and cultured for 72 hours with an A2AR agonist (CGS), antagonist (ZM) and a Cblb inhibitor.
- T-cells were lysed and lysates were analyzed by Immunoprecipitation and Western Blot to detect N1 protein levels
- ELISA was conducted on T-cell culture supernatants to quantify the production of Interferon Gamma (INF-gamma)
- T-cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) to measure proliferation by flow cytometry
- Tumor 3D-cell cultures (organoids) were derived from a mouse model of breast cancer and were treated with a Cblb inhibitor to test its anti-cancer activity

Results

1. A2AR stimulation increases Notch1 degradation

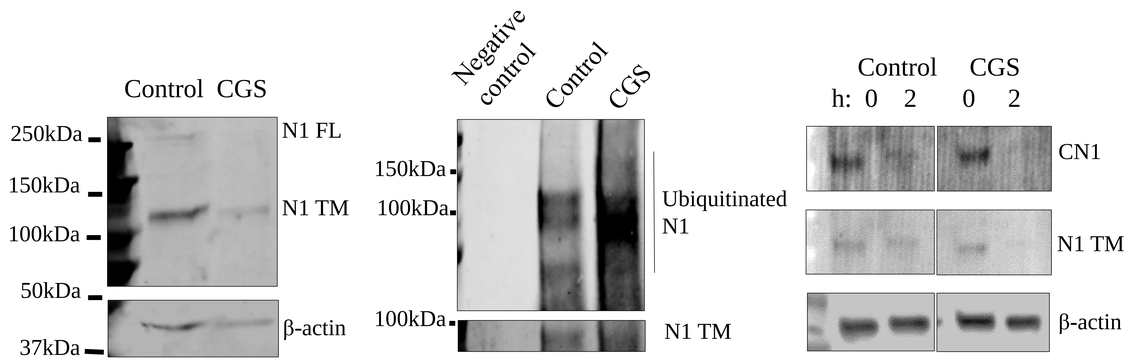


Figure 1: A2AR activation with an A2AR agonist (CGS) decreases N1 and increases its ubiquitination and degradation. Western Blot showing Full Length Notch1 (N1FL), and Transmembrane Notch1 (N1TM) protein levels in activated CD8+ T-cells untreated (control) or treated with CGS (left image). Immunoprecipitation of Notch1 and detection of Ubiquitinated Notch1 (central image). Western blot showing cleaved Notch1 (CN1) and N1TM (right image) in CD8+ T-cells treated with a protein synthase inhibitor for 0 or 2 hours.

2. A2AR stimulation decreases T-cells cytotoxic activity

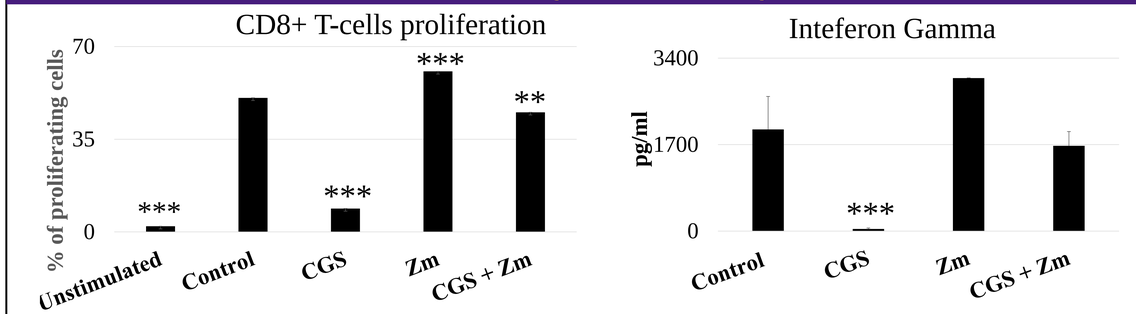
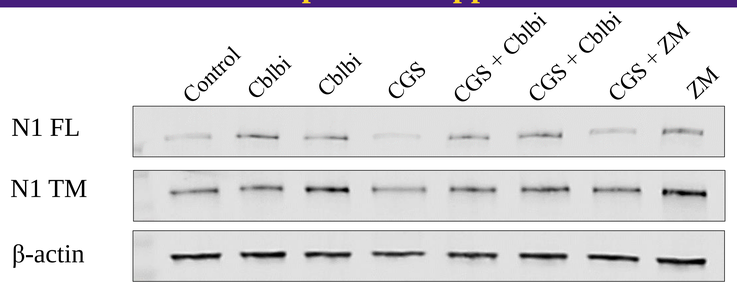


Figure 2: A2AR activation by CGS significantly decreases proliferation and the cyrtokine Interferon-Gamma production. The suppressive effect of CGS is rescued by an A2AR antagonist (Zm), which blocks A2AR. The first graph shows the percentage of proliferating cells measured by flow cytometry in unstimulated or activated CD8+ T-cells treated with CGS and Zm (left image). The second graph shows the amount of Interferon-Gamma measured by ELISA in the same conditions (right image). Bars show averages +/- standard deviation (n = 3). Groups were compared using two tailed T-test with equal variance. **p<0.01; ***p<0.001.

3. CBLB inhibition rescues Notch1 from A2AR-dependent suppression

Figure 3: Inhibition of CBLB with an experimental pharmacologic inhibitor rescues N1 protein level from CGS-induced suppression. Western blot showing N1FL, N1TM, and β-actin from top to bottom, respectively. CD8+ T-cells were treated with Cblb inhibitor (Cblbi), CGS, ZM and their combination.



4. CBLB inhibition restores cytotoxic activity

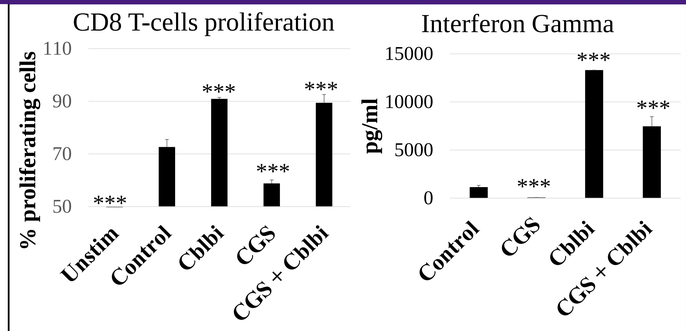


Figure 4: CBLB inhibitor restores proliferation and Interferon-Gamma production from CGS-induced suppression. The graphs show the percentage of proliferating cells measured by flow cytometry and the amount of Interferon-Gamma measured by ELISA in unstimulated or activated CD8+ T-cells treated with Cblb inhibitor, CGS and their combination. Bars show averages +/- standard deviation (n = 3). Groups were compared using two tailed T test with equal variance. ***p<0.001

5. CBLB inhibition shows anti-tumor activity

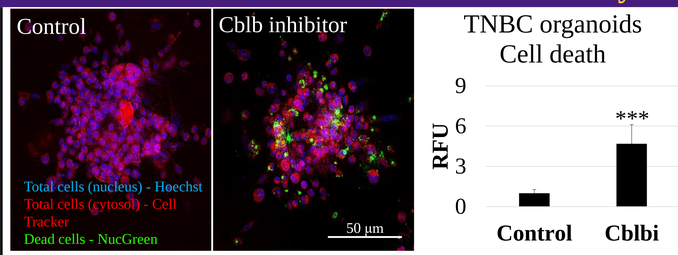


Figure 5: CBLB inhibitor induces cell death in organoids derived from a triple-negative breast cancer (TNBC) mouse model. The panel shows tumor-derived organoids untreated (control) or treated with Cblb inhibitor. Organoids were stained with ell-membrane permeable dyes (blue-red) to mark all cells and a cell-membrane impermeable dye to mark dead cells (green). The graph shows the relative fluorescence intensity (RFU) of the dead cell staining as a measure of cell death. Bars show averages +/- standard deviation (n = 3). Groups were compared using two tailed T-test with equal variance. ***p<0.001.

Conclusions

Our data indicate that modulation of signaling through the A2AR is capable of manipulating N1 levels through CBLB and, in turn, T-cell functions in CD8+ T-cells. Importantly, CD8+ T-cells treated with drugs that increase N1 activation showed enhanced T-cell functions and anti-tumor responses, suggesting that this pathway is a promising new target for cancer immunotherapy.

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