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Immune Checkpoint Modulation by Urease-Mediated Alkalization

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INTRODUCTION

Immune checkpoint proteins, such as cytotoxic T lymphocyte antigen (CTLA)-4 and programmed death (PD)-1, downregulate T cell responses in order to prevent autoimmunity and inflammation. However, tumor cells that overexpress the ligand for PD-1, PD-L1, inhibit the activity of local T cells, providing a survival benefit to the tumor cells. Antibodies that target PD-1 (nivolumab, pembrolizumab) increase T cell activity and have provided remarkable outcomes in melanoma and lung cancer patients, and are now approved for clinical use. We describe a novel method to reactivate T cells by reducing PD-L1 expression on tumor cells and PD-1 expression on CD8+ T cells using the previously described antibody-urease conjugate, L-DOS47. L-DOS47 is currently in Phase I/II testing for treatment of non-small cell lung cancer. It is prepared by conjugating urease to the camelid single domain antibody specific for human CEACAM6. The immunoconjugate specifically targets and delivers urease to CEACAM6-expressing cancer cells, where the urease enzyme converts urea into ammonia. The ammonia increases the pH of the tumor microenvironment *in situ*. In this study, L-DOS47 and urea were used to increase the pH of culture media for *in vitro* studies. The effects on tumor cell PD-L1 expression and T cell PD-1 expression were monitored by flow cytometry. T cell cytokine production was evaluated by ELISA.

RESULTS

L-DOS47 + urea treatment reduces PD-L1 expression on IFNγ-stimulated MDA-MB-231 breast cancer cells

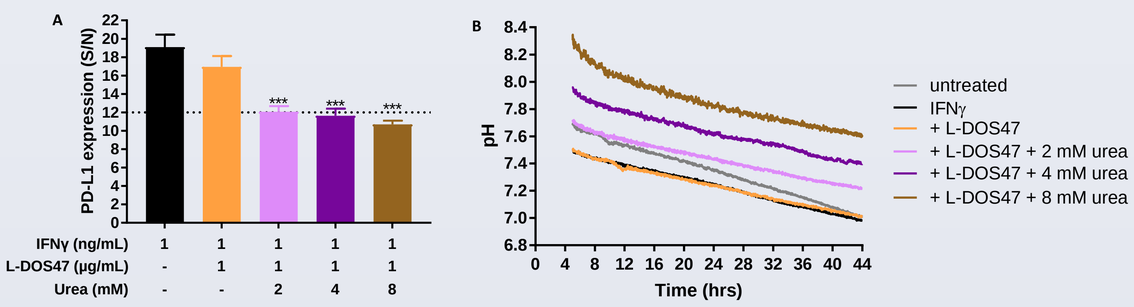

Figure 1: (A) Untreated MDA-MB-231 cells express a moderate level of PD-L1, as determined by flow cytometry (dashed line). Treatment with IFNγ for 2 days increases PD-L1 expression. Additional treatment with L-DOS47 and urea, but not L-DOS47 alone, significantly reduces PD-L1 expression to the level of untreated cells.*** p = 0.0005-0.001 compared to IFNγ treated cells. (B) The pH of each sample was monitored continuously throughout the experiment using a PreSens SensorDish® and SensorDish® Reader. It takes approximately 4-5 hours for the sensors and culture media to equilibrate, thus only pH measurements taken after T = 5 hrs are reported. Treatment with L-DOS47 + urea increases the pH of the media in a dose dependent manner. L-DOS47 alone has no effect.

Lactic acid treatment increases PD-L1 expression on IFNγ-stimulated MDA-MB-231 breast cancer cells

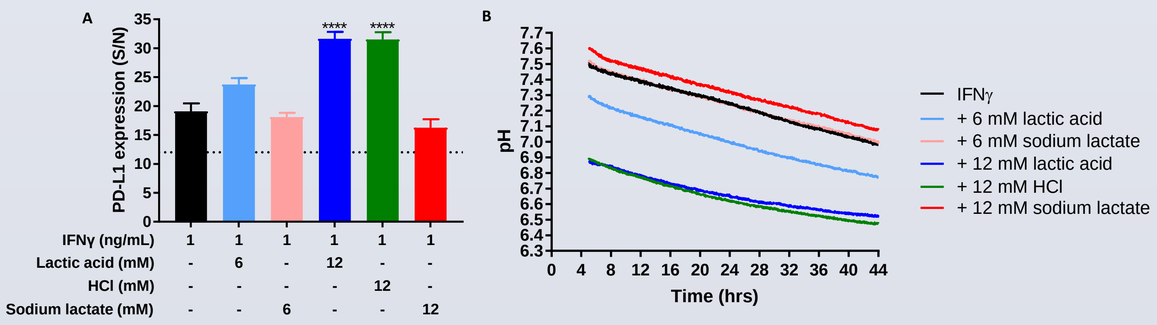

Figure 2: (A) Treatment with 12 mM lactic acid or 12 mM HCl significantly increases PD-L1 expression on IFNγ-stimulated MDA-MB-231 cells. 12 mM sodium lactate has no effect. **** p = 0.0001 compared to IFNγ treated cells. (B) Treatment with lactic acid or HCl decreases the pH of the media in a dose dependent manner. Sodium lactate has a minimal effect.

L-DOS47 + urea treatment restores low PD-L1 expression on lactic acid and IFNγ-treated MDA-MB-231 breast cancer cells

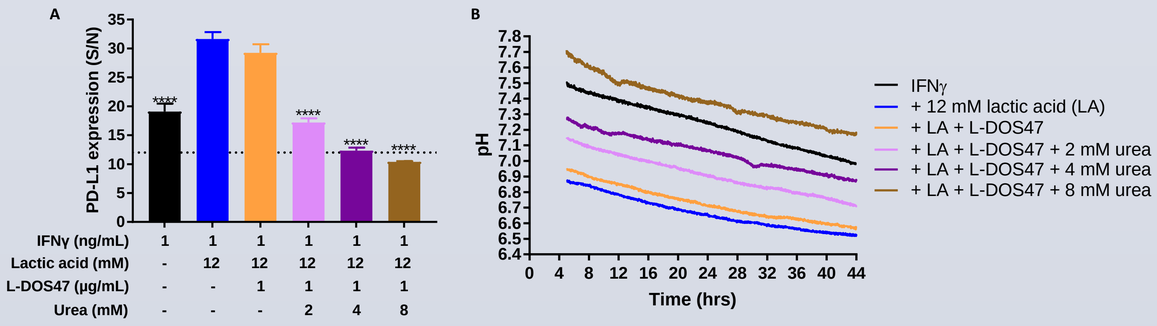

Figure 3: (A) Treatment with L-DOS47 + urea significantly decreases PD-L1 expression on MDA-MB-231 cells treated with IFNγ and 12 mM lactic acid. L-DOS47 alone has no effect. **** p = 0.0001 compared to cells treated with 12 mM lactic acid. (B) L-DOS47 + urea treatment increases the pH of the media of cells treated with 12 mM lactic acid in a dose-dependent manner. L-DOS47 alone has minimal effect.

Lactic acid treatment has no effect on PD-L1 expression on IFNγ-stimulated SKOV-3 ovarian cancer cells

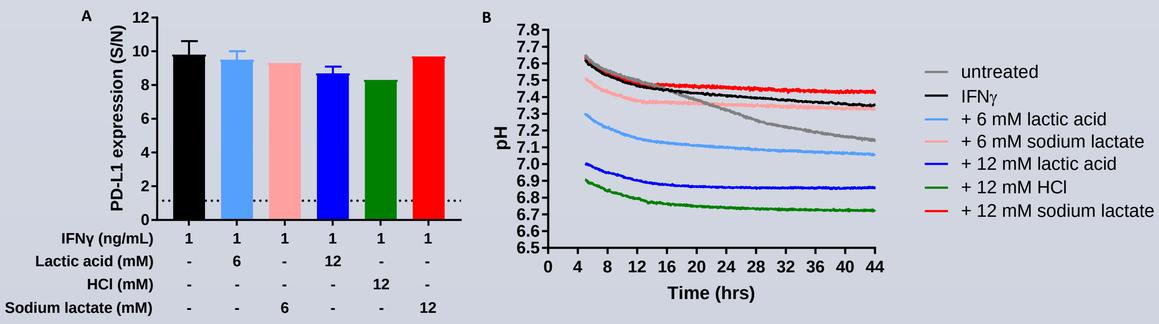

Figure 4: (A) Treatment with 12 mM lactic acid, 12 mM HCl or 12 mM sodium lactate has no effect on PD-L1 expression on IFNγ-stimulated SKOV-3 cells. (B) Treatment with lactic acid or HCl decreases the pH of the media in a dose dependent manner. Sodium lactate has minimal effect.

L-DOS47 + urea treatment increases IL-2 and IFNγ production by activated CD8+ T cells

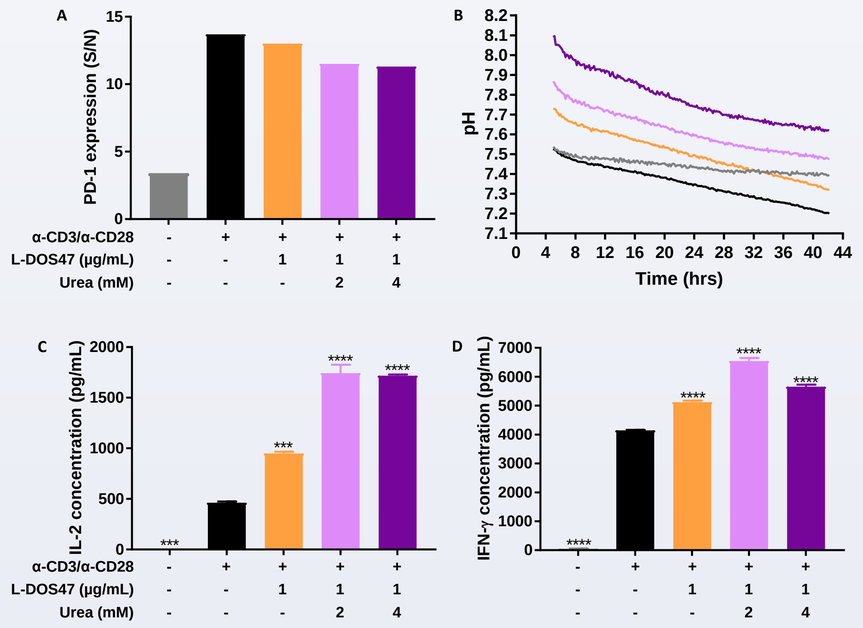

Figure 5: CD8+ T cells were purified from donor PBMC using negative selection methods. Cells were activated with α-CD3/α-CD28 Dynabeads® and IL-2 for 3 days before the initiation of the experiment. (A) PD-1 levels were evaluated by flow cytometry. Activated CD8+ T cells express PD-1. Levels are relatively unchanged upon treatment with L-DOS47 ± urea. (B) Treatment with L-DOS47 ± urea increases the pH of the media in a dose dependent manner. (C) Treatment with L-DOS47 ± urea significantly increases IL-2 and (D) IFN-γ production from activated CD8+ T cells as measured by ELISA.

Lactic acid increases PD-1 expression and decreases IFNγ production by activated CD8+ T cells

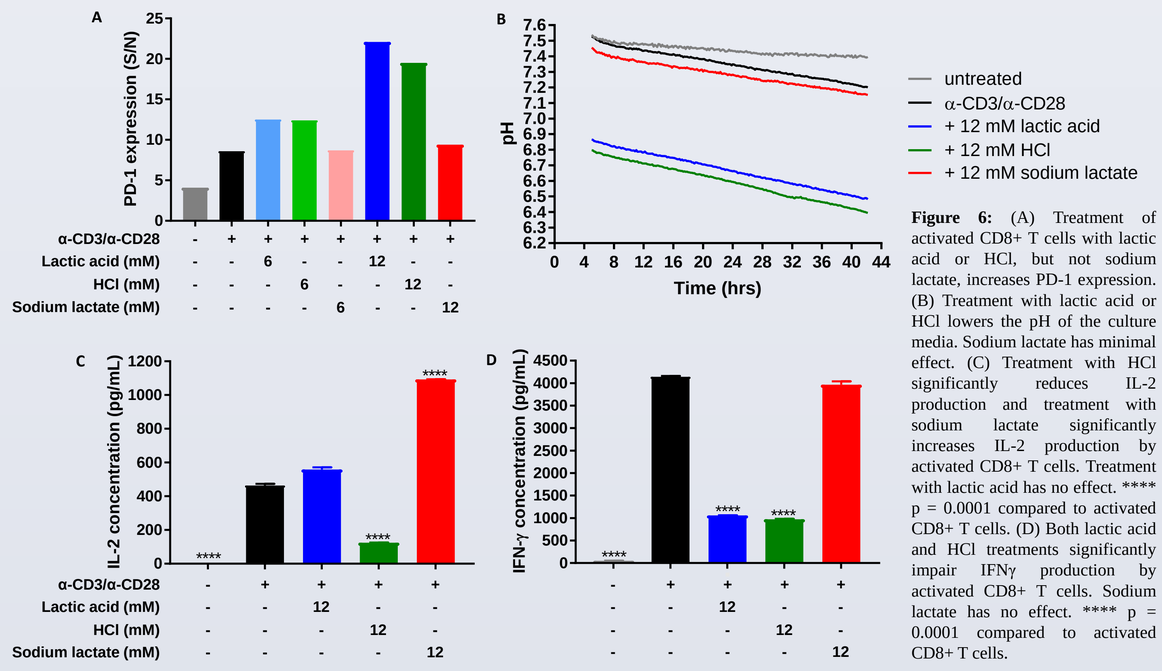

Figure 6: (A) Treatment of activated CD8+ T cells with lactic acid or HCl, but not sodium lactate, increases PD-1 expression. (B) Treatment with lactic acid or HCl lowers the pH of the culture media. Sodium lactate has minimal effect. (C) Treatment with HCl significantly reduces IL-2 production and treatment with sodium lactate significantly increases IL-2 production by activated CD8+ T cells. Treatment with lactic acid has no effect. **** p = 0.0001 compared to activated CD8+ T cells. (D) Both lactic acid and HCl treatments significantly impair IFNγ production by activated CD8+ T cells. Sodium lactate has no effect. **** p = 0.0001 compared to activated CD8+ T cells.

L-DOS47 + urea treatment of lactic acid-cultured, activated CD8+ T cells reduces PD-1 expression, increases IL-2 production and restores high IFNγ production

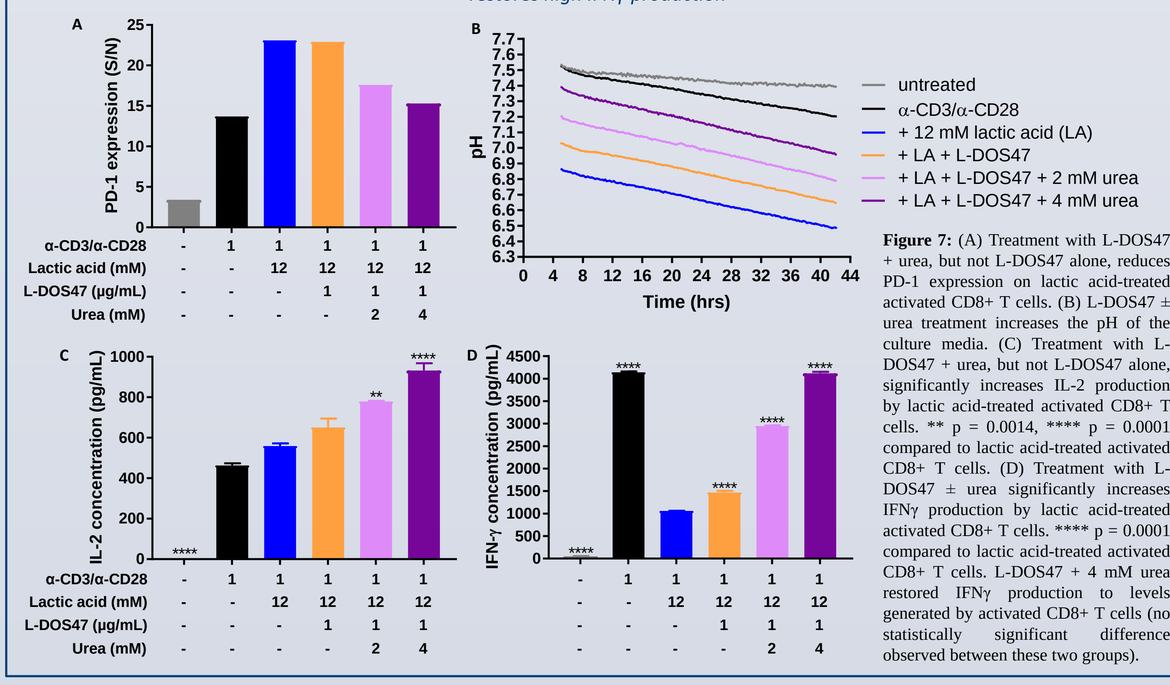

Figure 7: (A) Treatment with L-DOS47 + urea, but not L-DOS47 alone, reduces PD-1 expression on lactic acid-treated activated CD8+ T cells. (B) L-DOS47 ± urea treatment increases the pH of the culture media. (C) Treatment with L-DOS47 + urea, but not L-DOS47 alone, significantly increases IL-2 production by lactic acid-treated activated CD8+ T cells. ** p = 0.0014, **** p = 0.0001 compared to lactic acid-treated activated CD8+ T cells. (D) Treatment with L-DOS47 ± urea significantly increases IFNγ production by lactic acid-treated activated CD8+ T cells. **** p = 0.0001 compared to lactic acid-treated activated CD8+ T cells. L-DOS47 + 4 mM urea restored IFNγ production to levels generated by activated CD8+ T cells (no statistically significant difference observed between these two groups).

CONCLUSIONS

- Treatment of IFNγ-stimulated MDA-MB-231 breast cancer cells with lactic acid reduces the pH of the media and increases PD-L1 expression. Treatment with L-DOS47 + urea reduces PD-L1 expression to levels observed on untreated cells.
- It is interesting to note that PD-L1 levels are unchanged on similarly treated SKOV-3 ovarian cancer cells. Future comparative analysis of pH sensitive and pH insensitive cells will help to delineate the mechanism involved.
- Activated CD8+ T cells express PD-1 and secrete IL-2 and IFNγ. Incubation with lactic acid increases PD-1 expression, has little effect on IL-2 production, and significantly impairs IFNγ production.
- Treatment of lactic acid-cultured CD8+ T cells with L-DOS47 + urea increases IL-2 production, lowers PD-1 expression and restores IFNγ production to levels observed on untreated activated cells.
- L-DOS47 treatment represents a novel method to reduce acid-induced immunosupressive PD-1/PD-L1 interactions, by lowering expression of PD-1 and PD-L1 on T cells and tumor cells, respectively.

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