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Erratum to "Magnetic Field Emulations of Small Inhibitor RNA: Effects on Implanted GL261 Tumors in C57BL/6 Immune Competent Mice", [Open Journal of Biophysics, 2024, 14, 339-354]

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The original online version of this article (Xavier A. Figueroa*, Gabriel Vogeli, B. Michael Butters (2024) "Magnetic Field Emulations of Small Inhibitor RNA: Effects on Implanted GL261 Tumors in C57BL/6 Immune Competent Mice", Open Journal of Biophysics, 14, 339-354, https://doi.org/10.4236/ojbiphy.2024.144013) unfortunately contains a mistake. The authors wish to correct the errors.

Keywords: Erratum

In the process of reviewing the results of our publication (Magnetic Field Emulations of Small Inhibitor RNA: Effects on Implanted GL261 Tumors in C57BL/6 Immune Competent Mice) post publication, it was drawn to our attention that our analysis of our Western blots was missing an internal adjustment for our referenced house-keeping gene (β-actin) step for proper quantification.

In our band quantification section (2.6 Band Quantification), we used the values of the band density normalized to the known positive control value. What we did not carry out was the normalization of the protein value of the tumor to the β-actin levels for the sample. This key step adjusts the value of the expressed target protein to an accepted internal reference standard.

This missing step has resulted in changes to our analysis and protein expression level assessments for **Figure 4**, **Figure 5** and **Figure 6**, including changes to sections 3.2. Additionally, this correction has altered parts of our Discussion (4. Discussion) and Conclusion (5. Conclusion) sections of our publication.

Here we submit this erratum to correct the record to the readers of the Open Journal of Biophysics. Corrected and updated text is marked in red for the correc-

tions to the article.

2.6. Band Quantification

The films were scanned with a laser densitometer (Model PDSI, Molecular Dynamics Inc., Sunnyvale, CA). The scanner was checked for linearity prior to scanning with a calibrated Neutral Density Filter Set (Melles Griot, Irvine, CA). Phoretix 1D v11.2 was used for lane selection, background subtraction, band selection and quantification of band volume. All band intensity values are normalized to the positive protein control for each Western blot. The normalized values of each protein are adjusted to the β-actin expression levels for each lane.

3.2. Effects of the A2 Signal on CTLA4, Pcdc-1, Ki67, CD4, CD8 and Caspase 3 Expression in Tumor Tissue

Tumor samples were snap frozen in liquid nitrogen at time of collection and prepared for delivery to a third party, independent protein analysis laboratory (Kendrick Labs). Tumors were randomly selected from the A2 WAV and control group (N = 10 per group) and the samples blinded. The staff at Kendrick Labs were unaware of the exposure that each tumor experienced.

Western blots of protein expression for CTLA4 and Pcdc-1 (PD-1) are displayed in https://www.emulatetx.com/s/Supplement-2-PAGE-Western-Blots.pdf (pages 2 - 5). A total of 40 ug of protein lysate were loaded per lane for each tumor. A laser densitometry measure was applied to the entire Western blot and the band values were calibrated to the positive control band for each specific protein labeled. Each protein band was adjusted to the β-actin expression level of the sample. The band density values were derived for the bands identified between the upper and lower red lines. The individual results from the measured bands for each lane are displayed in **Figure 4(a)** (CTLA4) and **Figure 4(b)** (Pcdc-1). The average results for the protein expression values are displayed in **Figure 4(c)**.

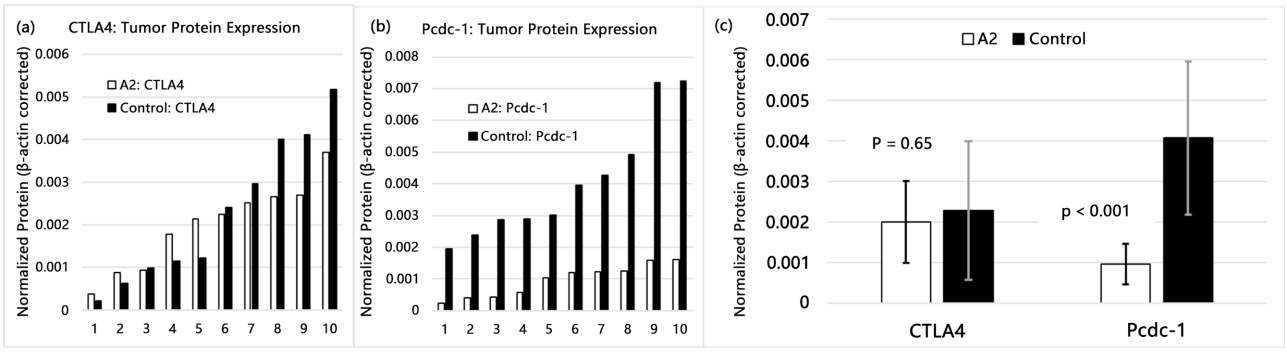

Figure 4. Plotted values of normalized band densities (adjusted for β-actin expression levels) for CTLA4 and PD-1 Western blots. (a) CTLA4 densitometry values are normalized to the positive loading control (40 ug) for each lane and adjusted for β-actin expression levels; (b) Pcdc-1 densitometry values are normalized to the positive loading control (40 ug) for each lane adjusted for β-actin expression levels; (c) average densitometry values for each protein blotted (a = 0.05). Error bars are standard deviation.

As displayed on **Figure 4(a)**, CTLA4 expression was reduced ~~consistently~~ in the

A2 WAV exposure group, when compared to the Control group but did not reach statistical significance. In **Figure 4(b)**, Pcdc-1 expression was ~~only~~ **reduced in ~~half of the~~** all the tumors exposed to the A2 signal ~~tumors~~ when compared to the Control group. Pcdc-1 expression was significantly reduced. Comparing the average reduction in CTLA4 and Pcdc-1 protein expression demonstrated a significant reduction in the Pcdc-1 ~~CTLA4~~ group ($P < 0.001$ ~~$P = 0.017$~~), but not in the CTLA4 ~~Pcdc-1~~ group (**Figure 4(c)**).

Figure 5(a) and **Figure 5(b)** plot the individual tumor sample values for Caspase 3 and Ki 67, respectively. **Figure 5(c)** plots the average band densities of Caspase 3 and Ki67. The Caspase 3 values in the A2 group were significantly greater than the control group ($P < 0.001$). ~~trending towards significance, but neither A2 WAV or the Control group demonstrated a statistically significant difference~~. Western blots of protein expression for Caspase 3 and Ki67 are displayed in https://www.emulatetx.com/s/Supplement-2-PAGE-Western-Blots.pdf (pages 6 - 7, 14 - 15).

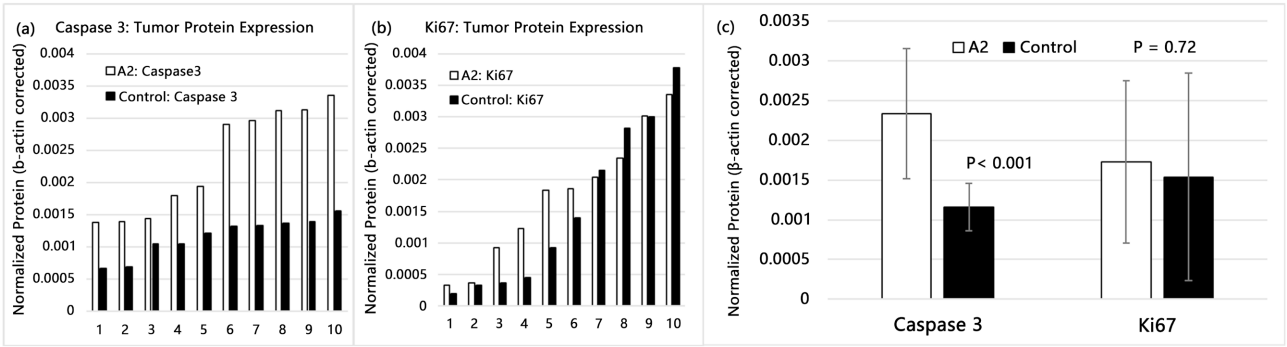

Figure 5. Plotted values of normalized band densities (adjusted for β-actin expression levels) for Caspase 3 and Ki67 Western blots. (a) Caspase 3 densitometry values are normalized to the positive loading control (40 ug) for each lane and adjusted for β-actin expression levels; (b) Ki67 densitometry values are normalized to the positive loading control (40 ug) for each lane and adjusted for β-actin expression levels; (c) average densitometry values for each protein blotted. Error bars are standard deviation.

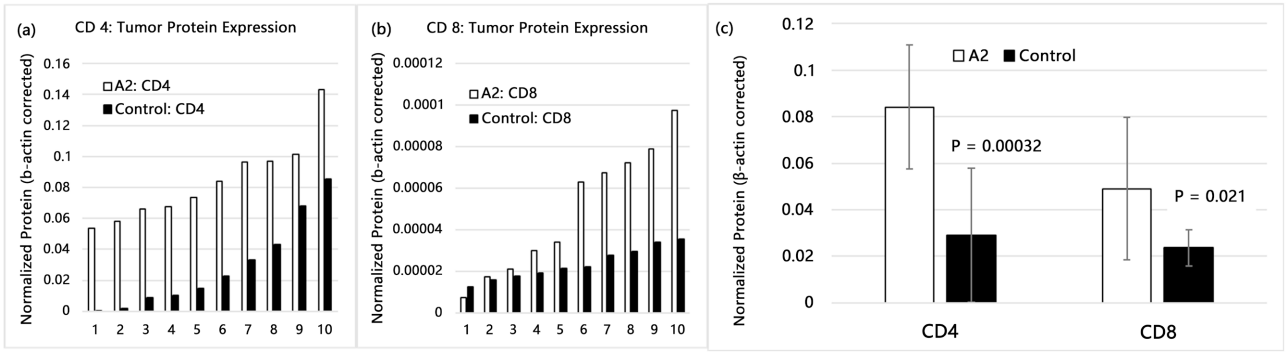

Figure 6. Plotted values of normalized band densities (adjusted for β-actin expression levels) for CD4 and CD8 Western blots. (a) CD4 densitometry values are normalized to the positive loading control (40 ug) for each lane and adjusted for β-actin expression levels; (b) CD8 densitometry values are normalized to the positive loading control (40 ug) for each lane and adjusted for β-actin expression levels; (c) average densitometry values for each protein blotted. Error bars are standard deviation.

Figure 6(a) and **Figure 6(b)** plot the individual values for CD4 and CD8 ex-

pression. **Figure 6(c)** plots the average band densities of CD4 and CD8. Both CD4 and CD8 values in the A2 group were significantly greater than the control group ($P < 0.00032$) & $P = 0.021$, respectively). ~~trending towards significance, but neither A2 WAV or the Control group demonstrated a statistically significant difference~~. Western blots of protein expression for CD4 and CD8 are displayed in https://www.emulatetx.com/s/Supplement-2-PAGE-Western-Blots.pdf (pages 8 - 11).

4. Discussion

The rationale for reducing the expression of key immune checkpoint receptors, such as CTLA-4 and PD-1 with siRNA, is based on the success of the CTLA-4 and PD-1 targeting immunoglobulin therapies [20]-[22].

We hypothesized that reducing the expression of CTLA-4 and PD-1 should result in a more effective immune response that produces a slow-down in tumor growth.

The rationale for running Western blots on tumor proteins with 10 mice per group was done to reduce cost and to produce an even sampling of the protein measures in tumors. Randomly selecting tumors from both groups allowed for unbiased detection of total protein change with balanced arms.

The exposure of the A2 signal, which is a magnetic field recording of the siRNAs targeting the murine CTLA-4 and PD-1 mRNA, resulted in the statistically significant reduction of tumor volume and reduction of ~~CTLA-4~~ Pcdc-1 receptor protein expression in the tumors (**Figure 4(c)**). The differential contribution of the sources of the ~~CTLA-4~~ Pcdc-1 receptor protein is unknown, as both tumor and WBCs express ~~CTLA-4~~ Pcdc-1 receptor. In either case, an observed decrease in ~~CTLA-4~~ Pcdc-1 receptor expression was measured in the A2 exposed group, similar to the reported reduction of EGFR mRNA expression using the same technology [10]. The CTLA-4 protein levels were trending towards lower expression in the A2 exposure group.

~~The PD-1 expression profile was mixed, with half of the tumors that were exposed to the A2 signal expressing a lower amount of PD-1 versus control (Figure 4(b), right). The other tumors showed the opposite effect, with greater PD-1 expression than controls. The difference in the subsets of Figure 4(b), left (between the controls and A2 groups) are statistically significant (data not shown), but when looked in aggregate, there is no significant difference. We are unsure as to why this occurred, as CTLA-4 expression was reduced significantly, in all tumors analyzed.~~

The lack of consistent ~~PD-1~~ CTLA-4 receptor protein reduction may explain the differences observed between the absolute tumor volume changes reported by our mouse study and the result from Mukthavaram *et al.*'s article [7]. The Kesari study demonstrated a stronger inhibition of tumor growth using the A2 signal than ours. Although both were statistically significant in effect, the magnitude was less in our hands. Genetic background differences may play a role, as the mouse

strains used in the UCSD study [7] and the IDRI study (ours) came from two different vivariums. Unfortunately, Mukthavaram *et al.* do not report on the expression of CTLA-4, PD-1 or CBCs in their publication.

Both CTLA-4 and PD-1 are expressed in a wide variety of tumors [23] [24], but the exact mechanism by which the immune system induces tumor cell death or tumor cell growth arrest (apoptosis, necrosis or cell cycle inhibition) appears to be dependent on multiple factors.

Total tumor protein expression, specifically Caspase 3 and Ki67 (**Figure 5**), suggests that the observed reduction in tumor growth was ~~not~~ due primarily to apoptosis,~~but~~ and not by a reduction in the rate of tumor cell division~~and potentially increased necrosis. Although the~~ The proteins levels did ~~not~~ reach statistical significance between the A2 and Control groups for Caspase 3, with the A2 groups showing an increase in expression, ~~Ki67, the protein expression differences of Caspase 3 was trending to significance. This trend suggesting a lower level of apoptosis in the A2 exposure group than the control group.~~ indicating an increase in apoptosis. The levels of Ki67 expression were equivalent across both A2 and control, indicating no significant differences in the rate of cell division between tumors.

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In our study, we demonstrated that exposure to the A2 signal significantly (**Figure 3**, Lymph #) lowers lymphocyte levels when compared to the Control group. When an analysis of the ratio between polymorphonucleocytes to lymphocytes (PLR), eosinophils to lymphocytes (ELR) and platelets to lymphocytes (PlaLR) was done, there was a trend towards significance (**Table 1**). Increases in the ratios between these sub-populations suggest that decreasing the expression of CTLA-4 and Pcdc-1 produce changes in the immune cell numbers.

These alterations may be specific to our mechanism of action, a reduction in CTLA-4 and Pcdc-1 expression, as compared to the infusion of immunoglobulins that target CTLA-4 or Pcdc-1. The change in lymphocyte counts in our study could be indicative of an effective tumor response when CTLA-4 and Pcdc-1 receptor expression is reduced. Studies in CTLA-4 conditional KO mice (tamoxifen suppressor) demonstrate a reduction of lymphocytes [27] after tamoxifen induced KO after several weeks. The report by Paterson *et al.* parallels the response we measure when CTLA-4 knock-down occurs with our A2 signal.

The reduced lymphocyte cell counts (**Figure 3**) correspond with ~~a trend towards a lower~~ an increased expression of the CD4+ and CD8+ antigen cell-surface markers, as measured in Western blot analysis (**Figure 6(c)**) of the tumors analyzed. CD4+ and CD8+ T-cells are primary effector cells involved in tumor infiltration and the primary targets of the co-inhibitory and co-stimulatory axis of current immune therapies [28]. Reduction in blood lymphocyte counts coincides with the trend observed in the CD4+ and CD8+ protein expression in the tumor and is supported by reports of GL261 mouse models treated with an anti-PD-1 antibody [1]. Although we did not directly measure the relative number of CD4+

and CD8+ T-lymphocytes between the A2 and control group, the reduction in lymphocytes could indicate an effect produced by the CTLA-4 and Pcdc-1 reduction via the signal, indicating increased infiltration of T-cells into the tumors.

5. Conclusion

Here we demonstrate the ability to target a significant reduction in the expression of at least one immune-check point inhibitor receptor (Pcdc-1 ~~CTLA-4~~) in a mouse model. The exposure to the magnetic field emulations of siRNA targeting murine CTLA-4 and PD-1 resulted in significant reduction in tumor volume in the A2 exposure group as compared to the control group, replicating the results from the Kesari laboratory [7]. A significant reduction in lymphocytes was observed in the A2 group which correlated with a trend in the ~~lowered~~ increased expression of CD4 and CD8 protein in tumor samples. No safety signals, grossly or in CBCs, were observed in mice. Our results mirror the observed safety profile of the NAT-105 clinical trial. Further clinical research is supported by our preclinical and clinical results.

Added References

[1] Dai, B., Qi, N., Li, J. and Zhang, G. (2018) Temozolomide Combined with PD-1 Antibody Therapy for Mouse Orthotopic Glioma Model. *Biochemical and Biophysical Research Communications*, **501**, 871-876. https://doi.org/10.1016/j.bbrc.2018.05.064