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Harnessing the immunostimulatory properties of oncolytic reovirus for anticancer immunotherapy
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ADDENDUM **II**
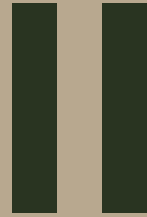
TGF-β blockade improves Reo&αPD-L1 therapy in the murine colon MC38 tumor model

BACKGROUND

In **Chapter 7**, we observed that TGF-β blockade differentially affects the efficacy of reovirus and CD3-bispecific antibody therapy (Reo&CD3-bsAbs). In the murine pancreatic KPC3 tumor model, the efficacy of Reo&CD3-bsAbs was impaired. Tumor regressions were prevented, and survival was shortened by TGF-β blockade. In contrast, in the murine MC38 colon carcinoma tumor model, TGF-β blockade significantly improved the efficacy of Reo&CD3-bsAb therapy, even leading to a 100% complete response rate. Since we also demonstrated that reovirus can enhance the efficacy of PD-L1 blockade in MC38 tumors in **Chapter 5**, we here investigated whether the efficacy of this combination therapy (Reo&αPD-L1) could also be improved by TGF-β blockade.

RESULTS & DISCUSSION

We engrafted immunocompetent C57BL/6J mice with subcutaneous MC38 tumors. Mice received TGF-β neutralizing antibodies (αTGF-β) starting a few days after tumor engraftment, and Reo&αPD-L1 therapy was administered on day 8, 11 and 14 (**Figure 1A**). Anti-TGF-β antibodies alone delayed tumor growth and induced complete tumor clearance in 10% of animals, and Reo&αPD-L1 therapy led to tumor clearance in 50% of animals (**Figure 1B-D**). However, the addition of αTGF-β to Reo&αPD-L1 therapy led to a total tumor clearance in 80% of animals and enhanced survival. Thus, the efficacy of Reo&αPD-L1 therapy can be improved by TGF-β blockade.

It is expected that PD-L1 blockade mediates its efficacy by reinvigorating dysfunctional tumor-specific T cells (1). However, the increased efficacy of Reo&αPD-L1 after αTGF-β could not be attributed to an increase in tumor-specific (Rpl18+) T cells, as similar frequencies were detected in the circulation of both groups receiving Reo&αPD-L1 therapy (**Figure 1E**). In **Chapter 7**, we also observed that Reo treatment does not improve the frequency of Rpl18$^+$ CD8$^+$ T cells, either in the circulation or in the tumor. Here, we confirmed that the frequency of Rpl18$^+$ CD8$^+$ T cells was not correlated with clinical outcome, since mice with a complete response (CR; in blue) did not always have the highest frequency of Rpl18$^+$ CD8$^+$ T cells (**Figure 1E**). Hence, we concluded that the triple combination of Reo, αPD-L1 and αTGF-β blockade demonstrates superior antitumor efficacy compared to all single arms or combinations of two arms, but that this improved efficacy was not associated with an increased frequency of tumor-specific Rpl18$^+$ CD8$^+$ T cells.

We next investigated whether antitumor immunologic memory was established by the different treatments. We therefore rechallenged the mice that completely cleared the primary tumor on the other flank and monitored tumor outgrowth (**Figure 1F**). Mice that cleared previous tumors had increased presence of Rpl18-specific T cells in the

circulation compared to naive mice, especially the group that received Reo&αPD-L1 therapy together with αTGF-β, suggesting that Rpl18-specific T-cell responses were boosted by the rechallenge with MC38 tumor cells (**Figure 1G**). While tumors grew out in 100% of naive mice, this was not the case in mice previously treated with Reo&αPD-L1 or Reo&αPD-L1+αTGF-β (**Figure 1H**).
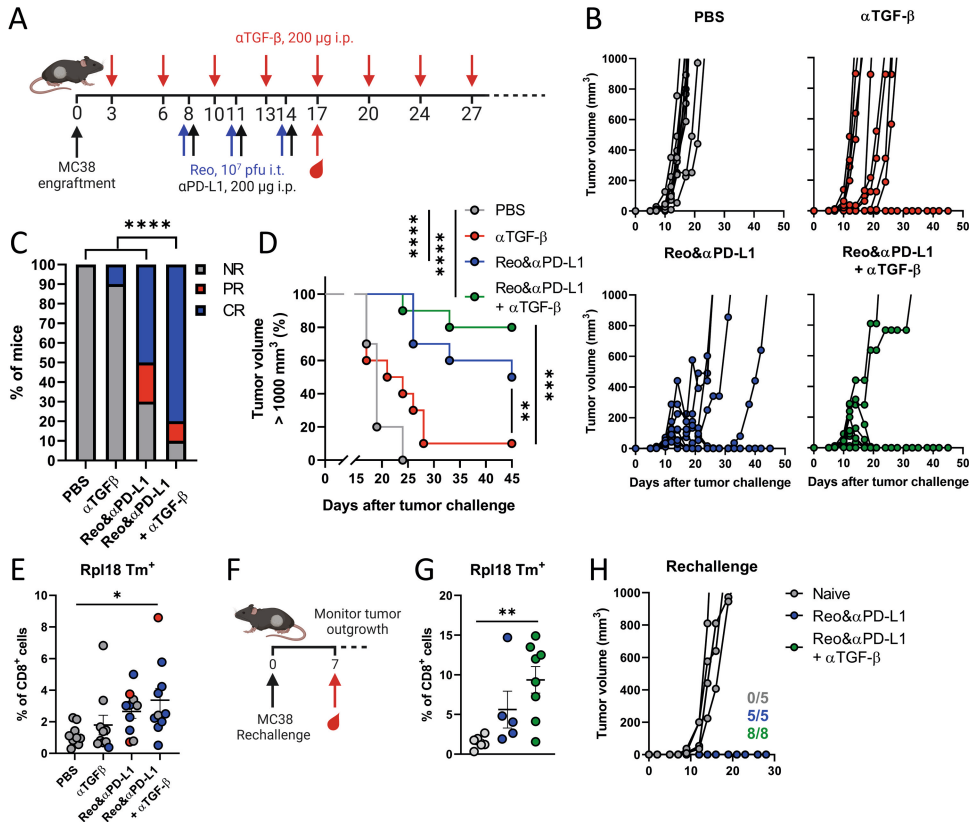
Figure 1. TGF-β blockade increases response rate after Reo&αPD-L1 in the MC38 model of colon cancer. (**A**) Overview of experiment described in (B-E). Immunocompetent C57BL/6J mice were subcutaneously engrafted with MC38 cells (5x10^5/mouse) and received TGF-β-neutralizing antibodies (αTGF-β, 200 µg/injection every 3 days) starting directly after tumor engraftment. Mice received Reo (intratumorally, 10^7 plaque-forming units/injection) and αPD-L1 (intraperitoneally, 200 µg/injection) on day 8, 11 and 14. Tumor growth was measured 3x/week. (**B**) Individual tumor growth curves of mice receiving indicated treatments. (**C**) Frequency of Non-Responders (NR), Partial Responders (PR) or Complete Responders (CR) within each treatment group. (**D**) Kaplan-Meier survival graphs of mice after indicated treatments. (**E**) Frequency of Rpl18+ CD8+ T cells in blood of mice after indicated treatments. (**F**) Design of rechallenge experiment. All CR mice from (D) were subcutaneously engrafted with MC38 tumor cells (5x10^5/mouse) in the alternate flank, and tumor outgrowth was measured 3x/week. (**G**) Frequency of Rpl18+ CD8+ T cells in blood of mice after rechallenge. (**H**) Individual tumor growth curves of mice that were rechallenged with MC38 tumor cells. Data represent mean±SEM. Significance between groups in (E) and (G) was determined using an ordinary one-way ANOVA with Tukey's multiple comparisons test. Chi-square test was used to determine statistical differences in response in (C). Log-rank tests were used to compare differences in survival in (D). Significance levels: *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

These data indicate that tumor-specific immune responses were established by our therapies, but that the efficacy of these therapies could not be explained by the increased frequency of tumor-specific Rpl18^{+} T cells. It would be interesting to investigate whether the improved protection was due to other MC38-specific T-cell responses, for instance those directed towards neo-epitope Adpgk (2). Alternatively, other cell types in the TME may be involved. For instance, tumor-associated M2 macrophages (TAMs) have been shown to hamper the efficacy of checkpoint blockade in the MC38 tumor model (3). Since anti-inflammatory M2 TAMs are induced by TGF-β (4,5), it is possible that neutralization of TGF-β hampered the function or reduced the level of these TAMs and thereby improved the efficacy of Reo&αPD-L1 therapy, without directly affecting the numbers of circulating tumor-specific T cells.

CONCLUSION

Combined, these data show that both Reo&CD3-bsAb and Reo&αPD-L1 therapy can be improved by TGF-β blockade in the MC38 tumor model. This further highlights the necessity to determine which factor(s), mechanism(s) or cell type(s) that are present in MC38 tumors permit or even contribute to this beneficial effect of TGF-β blockade, with the ultimate aim to employ this characteristic for effective viro-immunotherapy in other tumor models that are much harder to treat.

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