Investigation of combinatorial innate immunotherapy with chemotherapy to enhance responses in colon cancer

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The purpose of this UGP proposal was to obtain preliminary data to elucidate the ability of different TLR agonists, alone and in combination with one another and chemotherapy, to induce regression of solid tumors. The specific objectives of this proposal were to: 1) evaluate the ability of a TLR7/8 and/or TLR4 agonist, individually and in combination with one another and the chemotherapy cyclophosphamide, to induce growth arrest or remission of tumors and 2) to dissect the underlying mechanism of action of these compounds on the immune system and in the tumor microenvironment (TME). In support of these aims two in vivo studies of similar structure were done (Figure 1).

In the initial study two escalating doses of the TLR4 agonist CRX-527 and the TLR7/8 agonist CRX-727 were assessed alone and in combination with one another and/or the chemotherapy cyclophosphamide (cytoxan). Mice bearing CT-26 colon tumors on their flanks were treated 3x 1/week. Toxicity in the combination groups after the first administration necessitated 2-fold decrease in compound concentration upon reinjection (i.e. from 50/10 µg at week 1 to 25/5 µg at weeks 2 and 3). All groups without the inclusion of the chemotherapy reached high tumor burden quickly, though addition of CRX-527 did appear to slow the growth of tumors (Figure 2, upper). Cyclophosphamide on its own was able to diminish tumors but upon cessation of treatment, tumors began to rapidly regrow (Figure 2, lower left, red line). Addition of higher dose CRX-527 or the combination of CRX-527/CRX-727 to the chemo, induced immunological memory responses capable of maintaining low tumor levels (green, light red or yellow lines). While CRX-527 synergized with chemotherapy to decrease tumor burden, the greatest effect on tumor decrease was the addition of both TLR4 and TLR7/8 agonists (Figure 2, lower right, yellow and light red).

A follow up study was done with a lower dose of cytoxan and compounds to assess if greater synergy would be seen at lower dose. Interestingly, decreasing the chemotherapy does by two-fold did not greatly diminish the tumor ablation ability as was expected. With this lower chemotherapy dose though it was again seen that tumor clearance by chemo was a transient phenotype that rebounded upon cessation of treatment (Figure 3, lower left, red line). Interestingly, in this study the lower doses of the TLR4 agonist appear to be less efficacious, while the TLR7/8 agonist (CRX-727) was actually more efficacious (Figure 3, lower right). With either 5 or 1 µg CRX-527 there was no synergistic benefit to chemotherapy-induced tumor burden reduction or induction of immunological memory (i.e. tumors again rebounded in size after treatment). However, with the lowest dose of CRX-727 (1 µg) there was the greatest synergy and immunological memory when combined with chemotherapy (Figure 3, lower figures). This interesting phenotype of increased activity of the TLR7/8 agonist alone at lower doses suggests a clear dose-dependent activity of all of these compounds. Whereby titrating one or both is necessary to maximize response. In addition to assessing the TLR4 and TLR7/8
compounds in this study a novel compound was also included – UM-1024, a purported Mincle agonist. Mincle is a disparate innate receptor thought to be responsible for driving a Th17 response. While the role of Th17 effort cells in cancer therapy is not clearly elucidated and often conflicting, we chose to include this molecule to being to elucidate this further. Interestingly while the compound on its own had no effect at tumor reduction, combination with the chemotherapy was synergistic. 50% of the mice were cured of the tumor when given this combination regimen (Figure 3, lower right). Though not as efficacious as low dose TLR7/8 this compound presents an intriguing path of study and should be investigated more thoroughly.

In addition, in this study spleens were harvested from mice upon sacrifice (either because to tumor burden or study end) and evaluated for immunophenotyping. Analysis of this data is complicated as different groups were harvested/stained/analyzed at different times based on tumor burden and it appears there were differences in staining across these groups making cross group comparison inaccurate. However, when the median tumor volume of the end groups (those that made it to day 49 of the study, i.e. those with CTX) was plotted against the percentage of several different immune cell subtypes, some correlations were garnered. Unexpectedly no correlation between the percentage of M1 macrophages or Treg cells and tumor burden were found (Figure 4A and E). Negative correlation (i.e. more tumor was seen with less cells) was seen for M MDSC, CD8 and CD4 T cells (Figure 4D, F and G), while the increased presence of M2 or PMN MDSC was positively correlated (i.e. more tumor with more cells) (Figure 4B and C). This data would suggest that to drive successful tumor clearance suppression the PMN myeloid derived suppressor and the M2 macrophage subsets must be diminished with augmentation of CD4 and CD8 T cell subsets.

**Extramural Grant Submissions**

In continuation of this work, and fulfillment of UGP requirements, a U01 entitled ‘Investigation of combinatorial innate immunotherapy with chemotherapy to enhance responses in colon cancer’ was submitted to the National Cancer Institute in Jan of 2018 (application identifier 238684). Review is expected in late May 2018.