Identification of novel pathways of T cell immunosuppression in colorectal cancer
Biophysics 205 proposal, April 29, 2014

Introduction and Motivation:

There are 150,000 colorectal cancer (CRC) diagnoses per year in the United States accounting for 9% of cancer deaths (Haigis, correspondence), but there are limited effective therapeutic options available for these diagnosed patients. Almost 50% of CRCs have either a Kras or Nras mutation (Amado et al., 2008), with these patients having decreased survival compared to that of patients with WT Kras and Nras (Haigis, correspondence). Therefore, novel treatments are needed to treat this patient subpopulation.

Interestingly, an important connection between T cell infiltration into colorectal tumors and clinical outcome has been demonstrated: the presence of memory T cells in these tumors is highly predictive of survival of CRC patients (Galon et al, Pages et al). Studies have shown that immune infiltration in CRC is associated with the absence of early metastatic invasion and improved time to recurrence and overall survival (Galon et al, Pages et al). Only a subset of CRC patients exhibit an active immune response, however. Therefore, immunotherapies represent an exciting possibility for the treatment of CRC. Immunological therapies, which aim to reverse the immunosuppressive signals downstream of the negative costimulatory molecules, PD-1 and CTLA-4, have been developed. PD-1 and CTLA-4 are molecules involved in negative costimulatory pathways that prevent autoimmunity (Francisco et al, 2010), and antibody blockade of these receptors has yielded great clinical success in certain tumor types: anti-PD-1 therapy resulted in response rates of 28% of melanoma patients and 27% of renal cell carcinoma patients in a recent clinical trial (Topalian et al, 2012). But, these therapies have had little success in patients with CRC: one partial response to anti-CTLA-4 has been reported (Chung, Ki Y et al, 2010) as well as one complete clinical response to PD-1 therapy (Brahmer et al, 2012). Given the size of the study populations, though, these responders correspond to a very low percent response. Since the presence of an active immune response has such positive implications for CRC patient survival, reversing the potentially immunosuppressive tumor microenvironment in the context of CRC has great potential for improving survival.

Given the minimal clinical responses to anti-CTLA-4 and anti-PD-1 therapies in CRC, though, blockade of these immunosuppressive pathways does not appear to be sufficient to reverse the immunosuppressive environment in CRC. I hypothesize that other immunosuppressive signaling pathways also contribute to the immunosuppressive tumor microenvironment in CRC. Numerous additional immunosuppressive pathways have been implicated in preventing the immune response to tumor cells such as LAG-3 and IDO, for example (Gajewski et al, 2013, Woo et al, 2011). Recent clinical trials have demonstrated that therapies targeting CTLA-4 and PD-1 concurrently result in synergistic effects in the clinic thereby establishing that targeting multiple immunosuppressive pathways can yield greater clinical responses (Wolchok et al, 2013). Thus, identification of further mechanisms of immunosuppression in CRC in the context of anti-PD-1 or anti-CTLA-4 treatment may reveal novel targets for combinatorial therapy in hopes of achieving synergistic reversal of immunosuppression and reactivation of the anti-tumor immune infiltrate.

Specific Aims:

Specific Aim 1. To explore the effects of additional immunosuppressive pathways functioning in T cells in the CRC microenvironment, I propose an in vivo pooled shRNA screen in GFP+ T cells adoptively transferred into Fabpl-Cre; Apc^{2lox14}/+; $Kras^{LSL\text{-}G12D}$/+ mice bearing colorectal tumors that have been treated with either anti-PD-1 or anti-CTLA-4 therapy. The feasibility of such an in vivo screen was demonstrated by Zhou et al (2014).

Fabpl-Cre; Apc^{2lox14}/+; $Kras^{LSL\text{-}G12D}$/+ mice are immunocompetent, and native T cell infiltration into these colorectal tumors has been previously demonstrated (Haigis, correspondence). T cells isolated from Fabpl-Cre GFP mice will be infected with pools of shRNAs obtained from the RNAi Consortium (David Root, Broad). 4-5 shRNAs will be used per gene, and a total of approximately 15,000 genes will be screened. Each pool of T cells would be infected with virus for shRNAs against approximately 20-25 genes as well as shRNAs against luciferase and RFP as negative controls and shRNAs against PD-1 and CTLA-4 as positive controls. Each T cell should be infected with one shRNA. Prior to injection, a subset of the T cell pools would be processed for sequencing to identify the composition of the baseline population for the purpose of comparison following in vivo expansion. These pools would then be injected intravenously into mice after colorectal tumors have been

detected by colonoscopy. A pilot experiment will be performed to confirm that the adoptively transferred T cells will be recruited to the tumor and if they can be recovered by collagenase digestion of the tumor and FACS sorting for GFP. Additional pilot experiments will be performed to identify the ideal time point for examining the T cell population within the tumor. At the determined time point, tumors will be digested, GFP+ cells sorted by FACS, and the cells sequenced to determine which T cell clones are able to expand preferentially compared to the clones expressing shRNA against luciferase and RFP as negative controls. These expanded clones would reflect T cell populations that gained a survival advantage due to loss of a negative regulator. Such identified genes may be represent novel targets for reversing immunosuppression of T cells in the context of CRC.

To account for potential sources of false positives and false negatives, spleens would also be removed from the mice, dissociated, and the GFP+ cells isolated for FACS sorting for sequencing and comparison to intratumoral T cell populations, as a secondary screen. This would allow for the elucidation of shRNAs that cause a general proliferative advantage that is not dependent on the reversal of an immunosuppressive pathway in the tumor microenvironment (possible false positives). Additionally, some shRNAs may disrupt general T cell migration or homing to the tumor site, so these shRNAs may be false negatives if they target immunosuppressive pathways relevant to the tumor microenvironment that also impact broader T cell function. The absence of T cells infected with these shRNAs in the splenic T cell population would be an indicator of this possibility. If the pilot experiments and positive controls confirm that the system is functional, but no shRNAs yield expansion of T cell populations, then this would suggest that T-cell intrinsic immunosuppressive pathways other than the PD-1 and CTLA-4 pathways may not be biologically relevant in the CRC tumor microenvironment. In this case, I would explore the possibility that other immune cell types could be involved in suppressing T cell function in these tumors.

Specific Aim 2. To evaluate the potential therapeutic value of hits identified in the initial screen, individual genes would be validated, and their effects on survival determined. To validate hits identified by the pooled shRNA screen, smaller pools of shRNAs with additional hairpins per target gene (ideally up to 15 per gene) would be infected into T cells and the in vivo experiment would be repeated with the smaller pool of targets. Hits would be considered validated if the T cell clones with target shRNA are able to expand to a greater extent than shRNA luciferase and shRNA RFP control cells. The increased number of hairpins per target gene is necessary to control for the possibility of off-target effects of the shRNAs. Validation of a hit will require that multiple shRNA hairpins yield expansion of their respective T cell subpopulation in the tumor.

Upon validation of hits, each will be assessed for potential therapeutic benefit by determining if there is a survival benefit caused by the knockdown of the particular target in T cells adoptively transferred into animals +/- CTLA4/PD-1 Ab treatment: the ability of T cell clones infected with the target(s) of interest to slow tumor progression would be compared with that of T cell clones infected with shRNA luciferase or shRNA RFP. Tumor progression will be evaluated by measurement of tumor volume and overall survival. Histopathological effects of adoptive transfer of the shRNA-targeted T cell populations with loss of the target gene will be assessed by fixing of CRC tumors and staining by immunohistochemistry for applicable markers for characterization of both tumor and immune cells.

Specific Aim 3. After validation of hits identified in the initial pooled shRNA screen, the possible mechanisms underlying the reversal of T cell immunosuppression will be investigated further in order to evaluate their therapeutic potential. I propose two approaches to address these mechanistic questions: first, an in vitro single cell assay to determine if the loss of the negative regulation increases T cell cytotoxicity against colorectal cancer cells and, second, gene expression profiling by mRNA sequencing of the infected T cells recovered from the tumors to examine changes in gene expression in these T cells compared to shRNA luciferase and shRNA RFP control T cells.

The activity of the enriched populations of T cells will be evaluated using an in vitro single cell assay in which a single isolated T cell will be co-cultured with an isogenic CRC line isolated from tumor-bearing mice to determine the cytotoxic activity of the T cells, as has been previously demonstrated to be feasible by Liadi et al (2013) and Varadarajan et al (2013). This assay will allow for the determination of if the survival advantage of the particular T cell clone coincides with functionality as assayed by T cell cytotoxicity against the colorectal cancer cells or if these cells are nonfunctional, possibly due to T cell anergy. This is an important therapeutic consideration as anergy is a known mechanism of tumor escape from the immune system (Gajewski et al,

2013). Furthermore, these assays will also be used to profile the cytokines secreted by these T cell subpopulations, another means of evaluating the potential functional activity of these cells.

mRNA sequencing of clonal populations of the T cells infected with the shRNA against validated targets versus that of the control shRNA luciferase and shRNA RFP cells will enable the determination of differential gene expression between these cell populations using the method described by Oshlack et al (2010). GOseq analysis will then be used to identify enriched or depleted pathways, which might yield insight into the mechanisms by which loss of the target leads to reversal of immunosuppression.

Conclusions:

The need for new therapies for the treatment of CRC is pressing, and novel approaches to immunotherapies designed to reverse immunosuppressive pathways within the tumor microenvironment are a potential therapeutic target for this tumor type. Using an in vivo shRNA screen in T cells, I hope to identify negative regulators of T cell activation, which could be new therapeutic targets to reactivate T cells present in the CRC microenvironment. Single cell in vitro cytotoxicity assays and RNA-seq will be utilized to understand the mechanism by which these knockdowns result in increased T cell survival in vivo.

A major limitation of this study design is that it is restricted to the discovery of targets involved in T cell-dependent mechanisms of immunosuppression. Other cells types, both immune and stromal, could certainly be involved in immunosuppression in the colorectal cancer tumor microenvironment as well. In order to identify such targets, the alternative screening approaches could possibly be applied to other cell types.

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