SNUH Harvard collaboration proposal

Dennis L. Kasper, MD -Harvard Medical School

Specific Aims

Since only a subset of cancer patients respond to ICI therapy, research into possible approaches to enhance immunological therapy of cancer has received a lot of attention. In a series of seminal studies, gut microbiome composition was highlighted as a strong predictor of response to ICI therapy. Treatment with single strains of commensal bacteria have not been reported to be successful. Moreover, commensal bacteria are known to induce multiple diverse immunomodulatory effects in their host, which could result in unwanted side-effects that are independent of ICI therapy. Since organism growth cannot be controlled, the use of a live organism risks over-growth or niche competition in the recipient's microbiome, which can disrupt the delicate equilibrium between bacteria in the gut. However, it has been demonstrated that fecal microbiome transplants (FMTs) from ICI-responder patients to non-responder patients could increase response of non-responders to ICI treatment. FMT is a treatment that lacks a mechanistic understanding and very likely, even in successfully treated patients, works by different mechanisms in many or most patients. There had been little mechanistic insight into how gut microbes modulate efficacy of check-point blocking therapy until our recent publication **(Park, et al Nature 2023**). By carefully sorting through a complex human microbiota that in mice facilitated check point blocking therapy, we found that colonization of GF mice with a single strain, *Coprobacillus cateniformis*, was sufficient to promote anti-tumor immunity in non-responders to anti-PD-L1 or PD-1 therapy and that mechanistically *C. cateniformis* colonization suppresses PD-L2 on dendritic cells in both the MLNs (mesenteric lymph nodes) and tumor dLNs (draining lymph nodes). Our data suggested that *C. cateniformis* promotes response to anti-PD-L1 by suppressing PD-L2 and that this signal may originate in the MLNs and travel to the tumor dLNs.

Our preliminary immunochemical studies indicate the molecule from *C. cateniformis* that is responsible for the immune modulation of the PD-L2/RGMb pathway is a lipid. We have now found that this lipid from a gram- positive bacteria modulates the expression of PD-L2 through innate immune signaling mediated by TLR4. Colonization of previously germ-free mice with *C. cateniformis* significantly reduced PD-L2 expression in CD11b+ cells and CD11c+ cells in the tumor dLNs and reduced PD-L2 expression in CD11c+ cells in the MLNs. Additionally, in vitro treatment of bone-marrow-derived dendritic cells (BMDCs) with soluble surface extracts of *C. cateniformis* downregulated PD-L2 expression, and this suppression of PD-L2 was reduced in MYD88 and TLR4 knockout DCs. Using the insight that lower PD-L2 levels facilitated PD-L1 or PD-1 therapy in mice and that this was impacting signaling thru RGMb on T cells, we determined that monoclonal blocking antibodies to RGMb or PD-L2 in conjunction with PD-L1 or PD-1 therapy were sufficient to induce tumor regression in mice previously resistant to PD-1 or PD-L1. In this proposal we will define in detail the bacterial molecule responsible for initiating this event, the innate receptor through which this molecule is signaling, and the initiating signaling mechanisms responsible for decreasing PD-L2 expression on DCs.

Specific Aim !,Determine the lipid that promotes the efficacy of ICI therapy

1a. Scale up the HPLC separation of the *C. cateniformis* lipid extract
1b. Determine the structure of the lipid down-regulating PD-L2 in BMDCs
1c. Chemically synthesize the lipid
1d. Validation of lipid efficacy in tumor models

Specific aim 2: Characterize the TLR4 recognition and signaling of the lipid down-regulating PD-L2.

2a. Pull-down of FLAG-tagged TLR4 complexed with the lipid and other lipid binding proteins
2b. Immunoprecipitation of native TLR complexed with the lipid and other lipid-binding proteins
2c. Pull-down of the biotinylated lipid complexed with TLR4 and other lipid-binding proteins
2d. CRISPR-Cas9 KO of the target proteins in BMDCs and confirm the KO cells no longer respond to the lipid

Small Molecule Therapeutics for HPV Associated Cancers

Peter M. Howley, M.D.

Professor, Department of Immunology

Harvard Medical School

Approximately 5% of cancers are caused by high-risk human papillomaviruses. Although effective preventive vaccines will reduce this cancer burden significantly over the next several decades, they have no therapeutic effect for those infected who remain at risk for HPV cancers. HPV-associated cancers are dependent upon the viral E6 and E7 oncogenes. The oncogenic function of E6 relies on its ability to induce p53 degradation. This is an activity and pathway that my laboratory discovered in the 1990s. We found that E6 hijacks a cellular E3 ligase (E6AP) and directs it to ubiquitinate p53. Thus, p53 is generally wildtype in HPV-associated cancers, and p53 stabilization induces apoptosis and/or results in senescence.

We developed a live cell, image-based high-throughput screening platform to identify molecules that stabilize p53 and/or affect viability in the HPV-positive HeLa cervical cancer cell line (Martinez-Noel et al., 2021). We utilized a HeLa cell reporter cell line stably expressing a bicistronic mRNA that encodes a transcriptionally inactive mutant p53 (R273C) fused to the monomeric red fluorescent protein mRuby (mRuby-p53) as well as histone 2B (H2BC11) fused to the green fluorescent protein SGFP2 (H2B-SGFP2). We chose to utilize the R273C transcriptionally inactive p53 mutant because it acts in a dominant negative manner by being incorporated into a p53 tetramer also containing endogenous wildtype (wt) p53, precluding wt p53 binding to DNA and interfering with cell cycle arrest and cell death as a consequence of p53 transcriptional activity. We validated the robustness and potential of this screening assay by assessing the activities of approximately 6,500 known bioactive compounds at the ICCB-Longwood in a study that was published in 2021 (Martinez-Noel et al., 2021). We have also used this screening platform to identify miRNAs that stabilize p53 when introduced into HPV positive cells (Martinez-Noel et al., 2022).

With the support of a QFASTR grant from Harvard, we completed a screen of the 50,000 compound ChemBridge 2020 library at the ICCB-Longwood. From the initial 52 ‘hits’, we performed validation experiments in non-reporter HPV positive cancer cell lines and determined HPV specificity by comparing activities in HPV-positive cancer cells and HPV-negative cancer cells harboring wt p53. We now have 3 compounds that are structurally related that stabilize p53 in Hela cells with HPV-specificity.

Potential collaborative projects:

(1) Since the screen was a phenotypic screen, we need to identify the cellular target(s) of these small molecules. Use proteomic approaches to identify the cellular targets and the pathways involved.
(2) Expand the screen to a natural products screen to identify natural product hits. This would be done using the Natural Products Library from the National Cancer Institute, and involve a collaboration with scientists at the NCI.
(3) Participate and perform whole genome CRISPR screens (knock out, activator, and inhibitor) to identify genes that are involved in the E6/E6AP/p53 pathway my laboratory discovered. Such genes could serve as targets for HPV-specific therapeutics.

Small Molecule Therapeutics for HPV Associated Cancers

Peter M. Howley, M.D.

Professor, Department of Immunology

Harvard Medical School

Approximately 5% of cancers are caused by high-risk human papillomaviruses. Although effective preventive vaccines will reduce this cancer burden significantly over the next several decades, they have no therapeutic effect for those infected who remain at risk for HPV cancers. HPV-associated cancers are dependent upon the viral E6 and E7 oncogenes. The oncogenic function of E6 relies on its ability to induce p53 degradation. This is an activity and pathway that my laboratory discovered in the 1990s. We found that E6 hijacks a cellular E3 ligase (E6AP) and directs it to ubiquitinate p53. Thus, p53 is generally wildtype in HPV-associated cancers, and p53 stabilization induces apoptosis and/or results in senescence.

We developed a live cell, image-based high-throughput screening platform to identify molecules that stabilize p53 and/or affect viability in the HPV-positive HeLa cervical cancer cell line (Martinez-Noel et al., 2021). We utilized a HeLa cell reporter cell line stably expressing a bicistronic mRNA that encodes a transcriptionally inactive mutant p53 (R273C) fused to the monomeric red fluorescent protein mRuby (mRuby-p53) as well as histone 2B (H2BC11) fused to the green fluorescent protein SGFP2 (H2B-SGFP2). We chose to utilize the R273C transcriptionally inactive p53 mutant because it acts in a dominant negative manner by being incorporated into a p53 tetramer also containing endogenous wildtype (wt) p53, precluding wt p53 binding to DNA and interfering with cell cycle arrest and cell death as a consequence of p53 transcriptional activity. We validated the robustness and potential of this screening assay by assessing the activities of approximately 6,500 known bioactive compounds at the ICCB-Longwood in a study that was published in 2021 (Martinez-Noel et al., 2021). We have also used this screening platform to identify miRNAs that stabilize p53 when introduced into HPV positive cells (Martinez-Noel et al., 2022).

With the support of a QFASTR grant from Harvard, we completed a screen of the 50,000 compound ChemBridge 2020 library at the ICCB-Longwood. From the initial 52 ‘hits’, we performed validation experiments in non-reporter HPV positive cancer cell lines and determined HPV specificity by comparing activities in HPV-positive cancer cells and HPV-negative cancer cells harboring wt p53. We now have 3 compounds that are structurally related that stabilize p53 in Hela cells with HPV-specificity.

Potential collaborative projects:

(1) Since the screen was a phenotypic screen, we need to identify the cellular target(s) of these small molecules. Use proteomic approaches to identify the cellular targets and the pathways involved.
(2) Expand the screen to a natural products screen to identify natural product hits. This would be done using the Natural Products Library from the National Cancer Institute, and involve a collaboration with scientists at the NCI.
(3) Participate and perform whole genome CRISPR screens (knock out, activator, and inhibitor) to identify genes that are involved in the E6/E6AP/p53 pathway my laboratory discovered. Such genes could serve as targets for HPV-specific therapeutics.

Relevant Publications (PDFs attached):

Martinez-Noel, G., Szajner, P., Kramer, R.E., Boyland, K.A., Sheikh, A., Smith, J.A., and Howley, P.M. (2022). Identification of MicroRNAs That Stabilize p53 in Human Papillomavirus-Positive Cancer Cells. J Virol *96*, e0186521.

Martinez-Noel, G., Vieira, V.C., Szajner, P., Lilienthal, E.M., Kramer, R.E., Boyland, K.A., Smith, J.A., and Howley, P.M. (2021). Live cell, image-based high-throughput screen to quantitate p53 stabilization and viability in human papillomavirus positive cancer cells. Virology *560*, 96-109.