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Could Immune Education by Microorganisms Shape Patient Response to Cancer Immunotherapy?

1. Background: Cancer Neo-antigens and Immunotherapy Outcomes

Immunotherapy presents an exciting new frontier in the treatment of cancer, offering patients the potential for sustained remission beyond survival outcomes typically produced by the treatment modalities of chemotherapy, surgery, and radiation.[1] In contrast to traditional therapies that aim to poison or burn cancer cells, immunotherapies such as PDL-1 and CTLA-4 inhibitors target receptors on patients' immune cells in order to dampen signals that might suppress immune activation, thereby invigorating the attack of immune cells on cancer cells.[2] While the effect of immunotherapy for some patients is an outcome physicians might dare describe as a "cure," only a fraction of patients obtain this tremendous benefit. Other patients experience disease progression despite immunotherapy treatment. For example, in a review by Schadendorf et al. of overall survival for 1861 melanoma patients treated with immunotherapy, only ~20% of patients survived for three years after treatment, but most of those 20% continued to survive throughout follow-up, which lasted an additional seven years.[1] Therefore, a key question in the field of immuno-oncology is: what determines how a patient's disease will respond to immunotherapy? An understanding of such determinants would not only improve clinical prognostication, but also enable physicians to tailor treatment in "precision care" to optimize patient outcomes.

Among potential predictors of immunotherapy response, tumor mutational burden (TMB), or the number of somatic mutations contained in a given cancer's genome, has shown promise. Higher TMB was found to be significantly associated with clinical benefit in response to immunotherapy among several patient cohorts of different cancer types.[3,4] A prevailing theory suggests higher TMB predicts better response to immunotherapy because high mutational burden increases the chance that a cancer cell will present mutated protein fragments, termed neo-antigens, on its surface for surveillance by immune cells. Because neo-antigens are not among the repertoire of host proteins the immune system is trained to tolerate, presentation of neo-antigens increases the chance that the immune system will recognize a cancer cell as a foreign target worthy of clearance. In support of this theory, along with high TMB, high neo-antigen load is significantly associated with clinical benefit in response to immunotherapy.[4] Neo-antigen load is typically estimated with *in silico* tools that use tumor whole-genome or whole-exome sequencing data to identify the mutation catalogue of a cancer cell and predict the binding of mutant protein fragments to host MHC molecules, resulting in a set of candidate neo-antigens. Therapies tailored to the cancer cell neo-antigens predicted by these *in silico* tools—such as dendritic cell vaccines—have shown clinical benefit.[5]

While high neo-antigen load is a favorable predictor of immunotherapy response, the underlying mechanism of this relationship remains unresolved. It may be that the sheer number of neo-antigens presented by a cancer cell is the operational determinant of clinical benefit from immunotherapy. Another possibility is that individual candidate neo-antigens vary in fitness and the operational determinant for clinical benefit from immunotherapy is whether or not a patient possesses a small number of high-fitness neo-antigens. In the latter case, high neo-antigen load might serve as a favorable indicator because increased neo-antigen load indicates a greater number of chances for the cell to produce a high-fitness neo-antigen. Although substantial research efforts have been invested in developing and refining neo-antigen prediction tools, with NetMHCpan representing the current gold standard for prediction across diverse HLA types, models of neo-antigen fitness remain limited.

Luksza *et al.* (2017) presented the existing model of neo-antigen fitness: a straightforward multiplication of two values, the first of which aims to capture binding between the neo-antigen peptide and an MHC, and the second of which aims to capture binding between the neo-antigen peptide and a TCR.[6] Peptide-MHC binding fitness was computed as the difference between the predicted binding affinity of the candidate neo-antigen (a 9- or 10-mer amino acid fragment) to MHC and the predicted binding affinity to MHC of the same 9- or 10-mer native protein fragment pre-mutation. Peptide-TCR binding fitness was computed using the alignment-based similarity of the candidate neo-antigen to positively-recognized, class-I-restricted T-cell antigens from the Immune Epitope Database (IEDB), a curated catalogue of experimental data characterizing >100,000 antibody and T cell epitopes associated with infectious diseases, allergic reactions, autoimmunity, and transplant reactions.[7] The authors state explicitly that their comparison of the candidate neo-antigen against IEDB epitopes is intended to capture the degree to which neo-antigens are "non-self" and does not presume previous exposure of the patient to the IEDB

epitopes themselves. When these authors categorized patients as having low fitness or high fitness based on their neo-antigen profiles, high-fitness patients had significantly higher survival rates among patients with melanoma treated with anti-CTLA-4 and among patients with lung cancer treated with anti-PD-1.* The findings of Luksza *et al.* suggest neo-antigen fitness models may enable prediction of cancer immunotherapy outcomes. The present study will explore the utility of a more nuanced neo-antigen fitness model, one that aims to capture the influence of microbial exposures on immune education.

2. Hypothesis of the Present Study

The human immune system, particularly the adaptive immune system, learns to recognize foreign adversaries and is primed to respond with increased speed and fervor upon re-exposure. On the other hand, through a carefully-orchestrated process of cellular pruning and re-programming, the immune system learns to tolerate self-epitopes and epitopes derived from the commensal microbiota, close affiliates with whom the human species has co-evolved to cement a mutually-beneficial relationship of nutrient exchange and habitat provision. The commensal microbiota outnumber cells containing human DNA in the human body by a factor of approximately ten to one.[9] Even more remarkably, there are approximately 100 bacterial genes in the collective genome of the microbiota—the metagenome—for every gene in the human genome.[10] Although not all of these gene products will be encountered by the host immune system, the scope of the host's encoded self-tolerance must be expanded substantially to accommodate the microbiota. To develop tolerance, the host immune system deliberately samples the microbiota using transepithelial dendrites of dendritic cells, transcytosis through M cells and goblet cell-associated antigen passages. Antigen-presentation of the sampled microbiota-derived epitopes then induces T-cell differentiation, resulting, among other effects, in peripheral T-regulatory cells (pTregs) specific to microbiota epitopes as well as microbiota-specific IgA+ B cells, both of which mediate immune tolerance of the microbiota.[11]

While there is believed to be some degree of geographic localization in immune education, such that pTregs specific to gut microbiota might predominantly patrol gut tissues, there is evidence to suggest that molecular mimicry between microbiota epitopes and other encountered epitopes can influence the immune response on a systemic scale. For example, Carrasco *et al.* found that individuals exposed to microbiota epitopes similar to cockroach epitopes were less likely to have a cockroach allergy, suggesting that microbiome sequences may prime host T cells to tolerate other foreign epitopes. Likewise, the long history of vaccine science demonstrates that pathogenic microbial exposures can prime the immune system to antagonize foreign epitopes.

While previous explorations of neo-antigen fitness have drawn upon known epitope structures via the IEDB, none have explicitly incorporated the concept of microbial immune education. Specifically, in addition to the physical interactions of the candidate neo-antigen peptide with MHC and TCR structures, the immune response to cancer cells may also be influenced by microbial immune education such that similarity of a candidate neo-antigen to "known foes" such as microbial pathogens could increase neo-antigen fitness and similarity of a candidate neo-antigen to "known friends" could decrease neo-antigen fitness. The present study seeks to determine whether and how similarity of candidate neo-antigens to known microbial entities might influence the immune response to cancer neo-antigens (neo-antigen fitness) and resulting patient outcomes from immunotherapy.

3. First Aim of the Present Study: Data Generation

The first aim of this study is to generate a dataset that will capture specific features of the microbial and neo-antigen landscapes for a set of patients undergoing immunotherapy for cancer treatment.

While an ideal neo-antigen fitness model would generalize to patients of all cancer types, the present study focuses on select cancer types to optimize achievability. In order to obtain a sizeable number of patients with beneficial immunotherapy outcomes, while the minimizing the logistical challenge of total patient recruitment, a patient cohort will be recruited that includes 100 patients each from two cancer types: melanoma and colorectal

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* Snyder *et al* (2014) also examined neo-antigen fitness, using the presence of a shared amino acid tetramer as their metric of similarity between a candidate neo-antigen and an IEDB epitope, and claimed to define a signature of IEDB epitope hits that predicted improved survival and a separate signature of IEDB epitope hits that predicted reduced survival for melanoma patients treated with anti-CTLA-4.[3] Their work was subsequently criticized for its model validation methods.[8]

cancer. Melanoma was selected because of its frequent use in previous neo-antigen profiling studies, and its higher rates of immunotherapy administration and resulting survival relative to other cancer types. Colorectal cancer was selected despite lower immunotherapy administration rates and resulting survival for its direct regulation by the gut-associated immune system. Clinical data drawn from these patients, beyond typical information regarding their demographics, cancer diagnosis, treatment course, HLA type and treatment outcome according to the RECIST (Response Evaluation Criteria in Solid Tumors) classification scheme, will include thorough chart review (and oral history-taking, if possible) to assess vaccine and pathogen exposure as well as birth mode (Cesarean versus vaginal).

To capture the microbial metagenome for each patient, prior to immunotherapy treatment a stool sample will be collected and processed to extract and sequence microbial DNA, which will then be assembled into a patient-specific metagenome. Although the composition of the adult microbiota is dynamic, longitudinal studies of community composition suggest some stability is observed. In a 2013 paper, Faith *et al.* explored the persistence of bacterial strains in the gut microbiome and concluded that 60% of the approximately 200 abundant strains in each individual's gut are retained over five years.[12] Therefore, the pre-treatment profile of the microbiota will not capture all microbiota epitopes encountered over the patient's life-course, but can serve as a reasonable reference point. In addition, IgA-Seq—bacterial flow cytometry coupled with 16S rRNA gene sequencing—will be performed on these stool samples in order to identify on a genus-level those bacteria most likely to have been sampled by the host immune system. This information will be used to refine each patient-specific microbial metagenome to a subset of genes more likely to be recognized by the host immune system. While profiling the metagenome across all microbiota sites on the human body is appealing for the sake of comprehensiveness, the vast majority of that collective genome is captured by the gut microbiota alone and the gut mucosa is believed to be a primary site for immune sampling of the microbiota, so the present study focuses exclusively on profiling the gut microbial metagenome.

To characterize the neo-antigen landscape for each patient, pre-treatment biopsies will be used to obtain tumor whole exome sequencing, and sequencing of PBMCs will be used to capture the germline patient genome. Each tumor-normal pair will be processed using MuTect and Strelka to annotate SNV variant calls and in/del variant calls, respectively, in the cancer genome. The output from these algorithms will be processed through NetMHCpan, incorporating patient-specific HLA types, to generate a set of candidate neo-antigens for each patient based on binding to MHCI[†]. Additionally, RNAseq will be performed on tumor biopsy samples in order to capture gene expression levels that can be used to filter out candidate neo-antigens less likely to be expressed on a per-gene basis.

4. Second Aim of the Present Study: Predictive Modeling

The data generated under the preceding aim, coupled with selected existing data sources, will be used to create predictive models of patient outcomes (by RECIST classification). First, four features will be generated for each candidate neo-antigen.

1) Neo-antigen strength of binding to MHCI: Represented by the rank value produced by NetMHCpan for each candidate neo-antigen specific to the HLA-type of its MHCI binding partner. This value, the rank of the predicted affinity for the neo-antigen compared to a set of random natural peptides, is recommended by NetMHCpan over the raw binding affinity (nM) because it is not affected by the inherent bias of some MHC molecules towards higher or lower mean predicted affinities.

2) Neo-antigen strength of binding to TCR: Unfortunately, the field of TCR binding prediction to candidate neo-antigens is underdeveloped relative to the field of MHC binding prediction, so there is no obvious counterpart to NetMHCpan for this purpose. Although several TCR binding prediction tools exist, they typically suffer from a tradeoff between prediction accuracy and HLA-type generalizability such that tools developed using HLA-specific training and testing data have higher accuracy but cannot be cross-applied to other HLA types. One candidate algorithm that encompasses all HLA types is DeepTCR,[13] which predicts binding strength between a given peptide-MHC molecule and the host TCR based on input sequencing of patient-specific TCR alpha and beta chains, which could be taken from the whole-exome data for each patient.

[†] MHCII analyses could also be performed but are likely to reflect a minority of cancer neo-antigens.

3) Neo-antigen similarity to "known foes": To define a set of known foes, this study will obtain the IEDB dataset of known immunogenic epitopes, and filter in only epitopes with documentation of positive assays in human samples, with MHC Class I, associated with infectious diseases. To refine this set of foes to plausibly-known foes, geographically-restricted, low-prevalence infectious disease epitopes (e.g., Crimean-Congo hemorrhagic fever) would be filtered out, and the remaining epitopes would be categorized as A) vaccine-associated (e.g., influenza, rubella), B) high prevalence (e.g., *Neisseria gonorrhoeae*, rhinovirus), or C) historically-relevant (e.g., plasmodium falciparum, *Mycobacterium tuberculosis*). While patients recruited in the United States are unlikely to have lifetime exposure to the historically-relevant pathogens, it would be interesting to examine whether including the latter category might improve prediction, through some inter-generational selective pressure that would advantage individuals whose immune molecules were capable of strong recognition of those epitopes. Having defined a set of known foes, similarity will be quantified in the following way. For a given neo-antigen, alignment scores will be generated between that candidate neo-antigen and all known foes using BLOSUM62 similarity scoring and very strong gap penalties, with retention of only the maximal score.

4) Neo-antigen similarity to "known friends": The microbiota metagenome generated for each patient will be refined to only include immune-sampled microbes' genes using IgA-Seq information. IgA-Seq will reveal the sampled genera, then existing database information (see description of the MDICD below) regarding the genes contained in the genomes of each genus, will be used to select for sampled microbes' genes. To further restrict this set of genes from the metagenome of immune-sampled microorganisms to plausibly-immunogenic gene products, KEGG annotations will be generated for each gene in the metagenome, and only sequences from KO categories that represent microbial surface molecules will be retained (e.g., ABC transporters, the Phosphotransferase system). Using this set of known friend sequences, maximum similarity scores would be generated for each candidate neo-antigen following the approach described for known foes.

Because the ultimate aim of these models is to predict outcomes on a per-patient basis, the features on a per-neo-antigen basis described above must be collapsed to single set of features per patient. This will be done using two overall schemes, to capture, respectively, the hypothesis that immunotherapy outcome is determined by a small set of strong candidates, and the hypothesis that characteristics of the entire landscape of neo-antigens determine immunotherapy outcomes. In the first scheme, neo-antigens would be ranked within each patient, a small number of the highest-ranking neo-antigens would be selected, and a central tendency metric such as a simple mean could be used among those selected neo-antigens to create patient-level values for each of the four features described above. In the second scheme, a metric such as the sum or mean could be taken for all neo-antigens from a given patient to create patient-level values for each of the four features described above.

With the resulting patient-level feature set, several modeling approaches (SVM, Random Forest, Naïve Bayes, Logistic Regression) would be trained and tested using six-fold cross-validation on the entire dataset to generate AUC metrics of model performance.

In this way, the data generated in this study will be used to evaluate the hypothesis that neo-antigen comparison to microbial epitopes offers predictive utility by improving performance relative to a simpler model, akin the Luksza model above, constructed using only information regarding predicted MHC and TCR binding. Furthermore, these data will be used to evaluate the relative performance of models trained on neo-antigen-landscape characteristics versus models trained using a small set of higher-fitness neo-antigens for each patient. This study will also compare the success of models built using as known foes: vaccine-associated and high-prevalence pathogens only, or these two categories of known foes, plus historically-relevant pathogens. Furthermore, the study will compare the success of models built for melanoma-only versus colorectal cancer-only patients, to determine whether direct regulation by the gut-associated immune system (in CRC) versus primarily-systemic immune regulation (in melanoma) might increase the degree of influence of immune education on neo-antigen fitness.

Should this research endeavor suggest that microbial immune education is an informative consideration for immunotherapy outcomes, it would be valuable to know whether patient-specific microbiota sequencing offers critical added prediction benefit over microbial metagenome databases as a more accessible, inexpensive and generalized approach to capturing the microbiota metagenome. To this end, this study will compare the success of models built across all patients using as the microbial metagenomic landscape: 1) patient-specific time-of-treatment metagenomic sequencing versus 2) a reference gut microbial metagenome from the Microbial Database Integrated Gene Catalogue (MDIDC)—a database assembled from >1,200 sequenced samples across healthy

individuals from three continents that was refined to a consensus set of 9.9M genes through de-replication of orthologs. The MDIDC is also annotated with gene-occurrence frequencies across individuals for easy refinement to a gene set that is highly conserved across individuals.[14]

It is infeasible to obtain microbiota sequencing from the patients in the present study that would capture the community composition that was present in the patients' younger years. However, some authors suggest the strongest influence of the microbiota on host immune education may take place in the first years of life, when microbiota communities are strongly influenced by birth mode and slowly evolve to adult community composition over the course of 2-3 years. Instead, alternative known friend sequence sets will be generated using existing microbiota metagenomic sequencing from infant microbiota communities in the literature.[15] These reference sets will not be specific to the patients in the present study, but will be assigned to patients based on their actual birth mode as an approximation of the patients' early-life communities. Then, the accuracy of the resulting models for immunotherapy outcomes will be compared to performance when the models were trained on reference database metagenomic sequences for adult gut microbial communities.

Finally, the present study will also examine whether or not a personalization of the "known-foes" sequence comparison set to entities of confirmed exposure on the basis of patient history would offer an improvement in prediction accuracy.

5. Third Aim of the Present Study: Validation of Predicted Neo-antigen Immunogenicity

If these prediction tools have clinically-relevant utility in their ability to differentiate among patients who will respond well versus poorly to immunotherapy, it is not of paramount importance whether or not the hypotheses embedded within them are scientifically correct. Nevertheless, it would be valuable to directly evaluate the hypothesis that the structural similarity of a neo-antigen to known friends and foes will influence its potential immunogenicity.

Using the techniques described in Tran *et al.,* the immunogenicity of a small subset of predicted cancer neo-antigens for a subset of patients will be quantified using neo-antigen specific stimulation of patient-derived T-cells and subsequent quantification of T-cell response using IFN-gamma secretion, enzyme-linked immunospot (ELISPOT) assay, and up-regulation of the T cell activation marker 4-1BB using flow cytometry.[16] According to the authors, these approaches offer orthogonal information about antigen-specific T cell responses.

Specifically, from each of 20 randomly-selected patients, three of their cancer neo-antigens will be randomly selected from each of the following three groups: 1) the lowest 10% of candidate neo-antigens ranked by fitness[‡] (incorporating the four components outlined above), 2) the middle 10% of ranked neo-antigens, and 3) the top 10% of ranked neo-antigens. In total, nine neo-antigens per each of the 20 patients will be tested, resulting in 180 total assays. A regression analysis will be performed on the resulting data to examine the relationship between predicted and actual immunogenicity for the neo-antigens.

6. Summary

Immunotherapy can offer life-saving results to a subset of patients with cancer, but our ability to predict patient responses to immunotherapy is limited. Previous studies suggest cancer neo-antigen profiling could hold predictive value but the relationship between high neo-antigen load and benefit from immunotherapy has not yet been resolved. The present study extends beyond existing models of neo-antigen fitness—which emphasize only peptide binding to MHC and TCR—to incorporate a consideration of immune education through microbial exposures, including both encounters with pathogenic organism epitopes and the resident microbiota. The models developed in this study could be used to shed light on several outstanding questions about the immune response to cancer cells, and contribute to improving precision oncology patient care.

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‡ I admittedly do not describe here an exact formula for neo-antigen fitness. The construction of this formula will be informed by the results of the described modeling work, and will likely include differential weighting of the four components outlined here, and possibly exclusion of certain neo-antigens entirely if their values for any one feature fall below certain thresholds.

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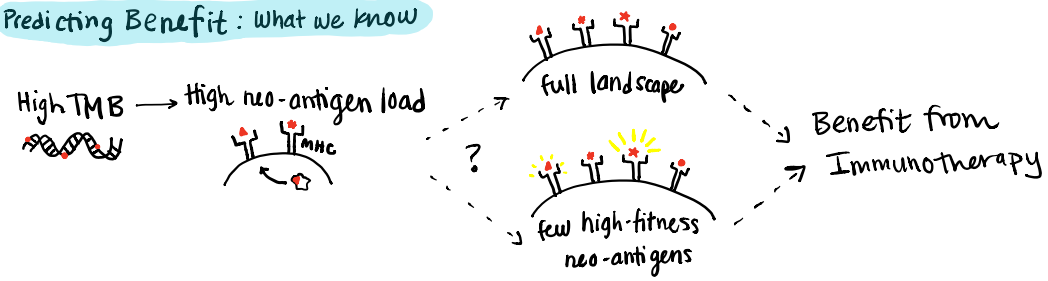
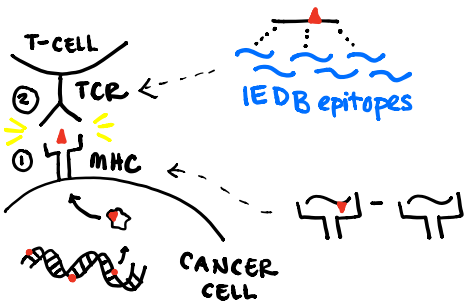
Predicting Benefit: What we know



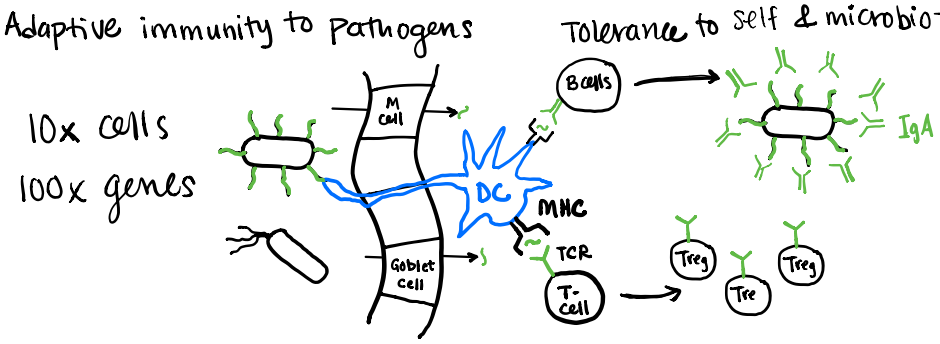
Existing Neo-Antigen Fitness Model

$$\text{Fitness of cancer clone} = \max_{i \in \text{clone}} (\text{fitness of neo-antigen } i)$$

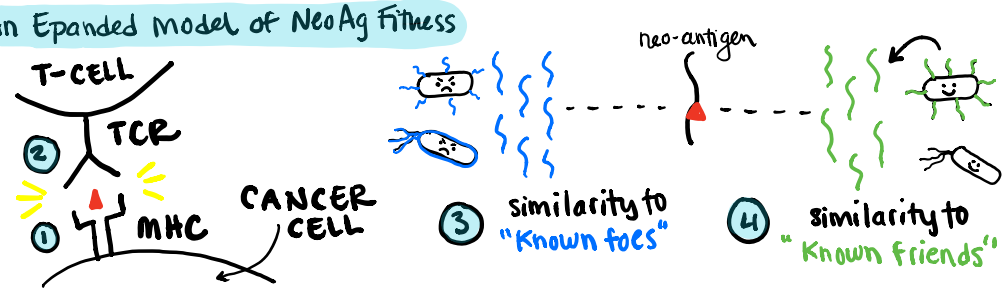
$$\text{fitness of neo-antigen } i = \text{TCR binding} \times \text{MHC binding}$$



Immune Education by Microorganisms



An Epanded Model of NeoAg Fitness



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