CANCER IMMUNOTHERAPY

Tumour heterogeneity impairs immunogenicity in mis-match repair deficient tumours.

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In brief
DNA mismatch repair deficiency (MMRd) is associated with elevated tumour mutational burden (TMB) and exceptional immunotherapy responses, yet a fraction of MMRd patients fail to experience clinical benefit. In this issue of Nature Genetics Wescott et al. propose that high intra-tumoural heterogeneity (ITH) can offset immunogenicity associated with sporadic MMRd, highlighting a new potential mechanism of immunotherapy failure in MMRd non-responders.

The association of TMB with response to immune checkpoint blockade (ICB) has been consistently demonstrated within immunogenic tumour types and across pan-cancer studies[1], underscoring recent FDA approval as a predictive biomarker. Whilst clinically useful this association is imperfect, in part due to high TMB non-responding tumours (e.g. glioblastoma) or other patient groups, including a subset of patients with MMRd.

MMRd is a feature of more than 10 cancer types but most prevalent in endometrial (30%), colon (15%) and gastric (9-22%) cancers. Both hereditary (arising from germline MMR gene mutations e.g. in Lynch syndrome) and sporadic (caused by somatic mutations or promoter methylation in MMR genes) MMRd causes instability in microsatellites, repetitive regions of the genome preferentially sensitive to DNA repair defects. This microsatellite instability (MSI) leads to an average 10-100-fold increase in TMB (e.g. 30-fold in colorectal cancer)[2] and drastically improves immunotherapy response rates relative to MMR proficient (MMRp) tumours[2]. The mechanisms underlying ICB failure amongst the non-responding MMRd demographic are incompletely understood. Exploring this important knowledge gap led Westcott et al to study the interplay between clonal evolution, immune surveillance and T cell activation in experimental models of MMRd cancer.

Although autochthonous genetically engineered mouse models (GEMMs) of cancer have been critical for studying tumourigenesis[3], the most widely used models do not fully recapitulate tumour immune responses[4]. This lack of immunogenicity is likely due to significantly lower tumour mutation burden (TMB) in mouse models compared to patients [5,6]. Cancer transplant models have also been a valuable tool for studying tumour immune responses, although these models have led to some debate over whether neoantigen intratumour heterogeneity[7] or overall tumour mutation burden[8] determine immunogenicity.

Herein, the group began by establishing *Kras^{G12D}; Trp53^{KO}* (*KP*) mutant lung and *Apc* mutant colorectal mouse models of cancer, which allowed for tissue-specific tumour induction and targeted knockout of components of the MMR machinery via CRISPR-Cas9 or an *Msh2* (an MMR component frequently mutated in cancer) conditional knockout mouse allele. The tumour mutational profiles of these mice recapitulated the mutational signatures of human MMRd colon cancer, to a greater extent than previous models.

The 4-5-fold increase in TMB conferred by *Msh2* ablation did not alter disease severity, increase T cell infiltration, or enhance response to combination ICB (anti-CTLA-4 + anti-PD-1) in the lung model. To reverse spontaneous immunity, the group then depleted T cells continuously and observed no change in tumour grade or burden in the wild type or *Msh2* KO (*Msh2ko*) animals, suggesting that T cells were not controlling progression of MMRp or MMRd tumours. These data supported the notion that MMRd did not induce an underlying T cell response in this model.

The clonal makeup of tumors and cell lines revealed that most mutations were subclonal. Next, the authors performed single-cell cloning experiments with re-expression of the MMR gene *Msh2* prior to subcloning. Significantly more somatic mutations were observed in all single-cell clones compared with parental lines, suggesting a significant number of subclonal mutations present in the single-cell clones were not captured in bulk sequencing of tumors. The authors concluded that the novel MMRd tumour models harbour high subclonal tumor mutational burden. Importantly, these experiments also highlighted that bulk sequencing of mouse tumours was likely insufficient for accurate assessment of intra-tumor heterogeneity.

Tumour immunoediting is a powerful evolutionary force that shapes ITH, which led the team to postulate that immunoediting may play a role in their system. To examine this, they depleted T cells in their models and observed significant increases in the clonal (but not overall) mutational burden, and the cancer cell fraction of neoantigens in comparison to non-T cell depleted MMRd mice. No decrease in clonal mutation burden was observed in tumours treated with ICB, although these tumours showed a decrease in subclonal mutation burden, possibly because of a lowered threshold for subclonal elimination, or compensatory subclonal expansion. These data suggested that immunoediting contributes to ITH through a decrease in the relative fraction of clonal mutations in these mice.

Given the importance of mutational architecture within their model, the authors aimed to identify specific immunogenic neoantigens fuelling T cell responses. They conducted mass-spec immunopeptidomics on single-cell clones derived from the parental *Msh2*ko tumour lines, resulting in the identification of five epitopes with demonstrable immunogenicity across *in vivo* and *in vitro* assays.

Next, the authors undertook an elegant series of clonal mixing and transplant experiments to test whether reducing clonal heterogeneity enhanced T cell immunogenicity (**Fig.1**). The clonal fractions of a specific neoantigen-expressing clone (M5) was titrated in a background of the remaining clones from the parental *Msh2*ko line, and transplanted into the lungs of syngeneic mice. The effect of decreasing clone composition (from 100% to 12.5%) on neoantigen-specific T cell response was measured using a major histocompatibility complex

(MHC) Multimer specific to an immunogenic neoepitope from the M5 clone. Transplantation of a monoclonal tumour (100% M5), in combination with ICB, elicited potent neoantigen-specific T cell expansion in the tumour and draining lymph nodes with reactive cells exhibiting an effector phenotype, positive for the cytotoxic molecule GZMB and negative for TCF1, a marker of resting and/or progenitor cells. Neoantigen-specific T cell expansion and effector differentiation both decreased with diminishing M5 clonal fraction, which likely reflected sub-optimal priming via antigen insufficiency in transplants with lower M5 clonal fraction. Notably, when the same, high number of M5 cells was transplanted there was no difference if their clonal fraction represented 1.0 or 0.5, implying that a minimal threshold of neoantigen clonality was driving an optimal response and that this was insensitive to the presence of irrelevant clones.

To analyse whether there was an analogous role for ITH in patients, Westcott et al used a dataset of ICB-treated MMRd advanced gastric and colon cancers. Here, they found that clonal (but not subclonal) neoantigen burden and a low ITH index was associated with improved response and progression-free survival.

These observations represent a significant and important advance in our understanding of the determinants of tumour immunogenicity within and beyond MMRd and support a growing appreciation of ITH as a central variable[7,9,10]. A key conclusion is that total neoantigen-expressing cellularity may be rate limiting for eliciting an effective T cell response. This implies that in contexts of high TMB (or possibly larger tumour burden) a cellularity threshold is more easily reached, and ITH less influential on immunogenicity. Given that the MMRd models represent the lower end of TMB gains over MMRp counterparts seen in the clinic (4-5-fold relative to 25- fold or higher[11]) the role of ITH could arguably be accentuated. However, although the clinical dataset was relatively small, the clinical analysis suggests a role for ITH in patients with high TMB. It is possible that ITH may impact priming less in the context of a high TMB, but an effector phase response focused on subclonal neoantigens still facilitates tumour escape by subclonal evolution, ultimately manifesting in poorer outcome. Furthermore, in high TMB tumours where antigen presentation is impaired by HLA loss or defective co-stimulation, ITH may regulate already limited availability of key epitopes.

As the authors state, the fate of a given response is reliant upon a complex network involving other interdependent immunological variables such as epitope dominance, CD4 help, Treg infiltration or the presence of tertiary lymphoid structures. An important inference is that other immunogenomic variables may become decisive in a TME poised at the tipping point of a neoantigen-cellularity threshold. Taken together, an immunological context that is conducive towards generating clonal neoantigen-specific T cell pools which are sensitive to immunotherapy (e.g. dysfunctional, PD-1+), but can elicit effector function (GZMB) and retain progenitor potential (e.g. TCF1) continues to be viewed as optimal[12].

This work supports the concept that tumour sequencing strategies which can accurately measure ITH that factor in both TMB and neoantigen clonality might optimise future predictors of ICB response. Therapeutically, if a low cellularity threshold restrains ICB responses this could be overcome by vaccination or adoptive cell therapies, in particular, those directing responses to clonal neoantigens. In addition, strategies to therapeutically limit ITH by inhibiting endogenous drivers of diversification may be advantageous.

Approval of neoadjuvant ICB for the first-line treatment of unresectable MMRd cancers will provide an opportunity to further explore how de-repressed T cell responses shape ITH in this setting. Sophisticated mouse models of cancer, like those developed by Wescott et al. will be important in testing hypotheses that emerge from such ongoing clinical evaluation, including patient groups with superior[13,14]and inferior responses. In future work it may be interesting to parse the contributions of total TMB, insertion-deletion mutational burden[15] and ITH on immunogenicity in these tumour models.

Critically, these data raise concerns regarding therapeutic strategies that aim to indiscriminately raise TMB or drive subclonal neoantigens later in cancer evolution. Based on these data and past work[7,9,10], such approaches might result in impaired immune responses.

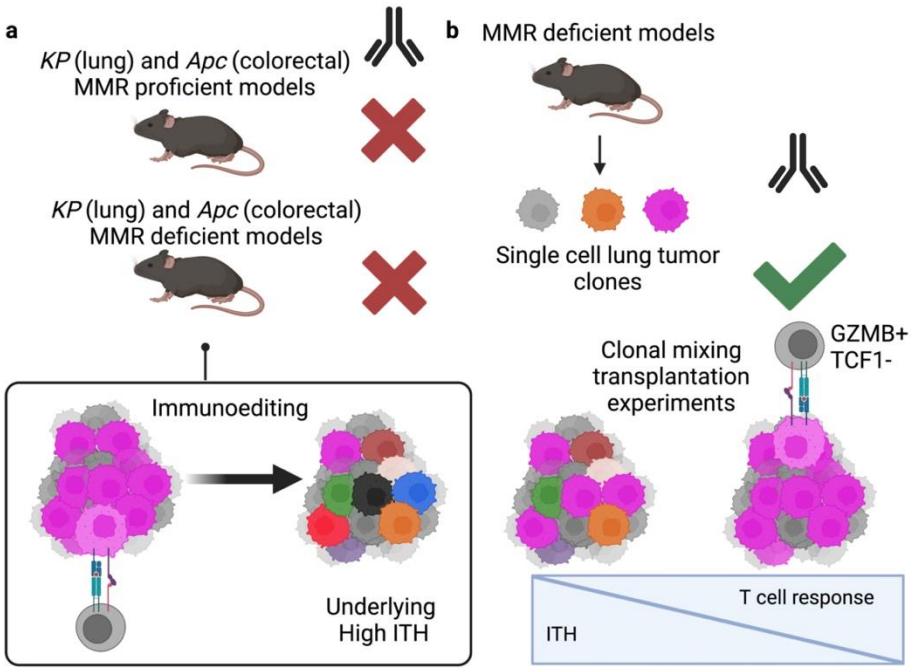

Figure 1. Overview of the study a, Westcott et al generated novel MMRd mouse models through Msh2 ablation in lung (*KP*) and colorectal (*Apc*) models. Both MMR proficient and deficient mice were non-immunogenic and failed to respond to anti-PD-1 anti-CTLA-4 immune checkpoint blockade. Underlying ITH in these models was driven by immunoediting. **b,** Clonal mixing experiments of clones derived from parental lung tumor lines reduce ITH and elicit neoantigen specific T cell responses characterised by GZMB+TCF1- effectors.

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