Title

Interfering with responses: IFN fuels cancer resistance to immune checkpoint blockade

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Summary
Immune checkpoint blockade (ICB) is revolutionising cancer medicine, yet the molecular basis of resistance remains unclear. In this a recent issue of *Cell*, Benci et al demonstrate that sustained interferon signalling is central to the development of PD-L1-dependent and independent resistance to ICB.

Preview
Clinical experience with immune checkpoint blockade (ICB) has fuelled optimism but also engendered a critical question; why do these drugs fail in a fraction of patients? One explanation lies in the redundancy of T cell inhibitory receptor (TCIR) networks, evidenced by the superior efficacy of combination therapies (Larkin et al., 2015). Yet how tumours orchestrate and maintain adaptive resistance to ICB is unknown. This month, a study in Cell lead by Andy Minn demonstrates that upon prolonged interferon (IFN) exposure, melanoma cells adopt a state of Signal Transducer and Activator of Transcription 1 (STAT1)-dependent chronic resistance that is associated with epigenomic changes to the tumour, expression of interferon-stimulated genes (ISGs) and multiple TCIR ligands.

To examine the basis of resistance to ICB, Benci et al. use concurrent radiotherapy (RT) and anti-Cytotoxic T-Lymphocyte Associated Protein 4 (αCTLA-4) to treat murine B16 melanoma, a strategy that yields 20% complete responses (CR), enabling the establishment of a resistant sub line from relapsed tumours (Res499). Genetic ablation of Programmed death-ligand 1 (PD-L1) on Res499 cells fails to improve survival in the absence of ICB, but renders tumours partially sensitive to RT+αCTLA-4 (CR 40%), an effect augmented in the presence of αPD-L1 (CR 60%). The 40% late relapse in this latter experiment provides the authors with a cell line (JB2) that exhibits stable, PD-L1 independent resistance to ICB upon re-transplantation. These two melanoma lines, together with parental B16 cells provides Minn's group with a model to interrogate the mechanistic basis of PD-L1-dependent or -independent resistance *in vivo*. Subsequently, Benci et al. show that prolonged in vitro exposure to IFNγ (but not type I IFN) promotes B16 melanoma resistance to ICB in vivo, whilst IFNGR KO Res499 cells recover sensitivity, in keeping with a key role for chronic IFNγ exposure in ICB resistance. Furthermore, the authors generate IFNAR KO and dual IFNAR and IFGR (IFNA/GR) double KO Res499 cells, demonstrating a role for both prolonged IFNG *and* IFNA signalling in ICB resistance. The contribution of IFN signalling in PD-L1-independent resistance is also demonstrated by sensitising JB2 cells to ICB via ablation of IFNA/GR expression. In light of recent work demonstrating that mutations in IFNγ signalling

pathways associate with resistance to ICB in humans (Zaretsky et al., 2016), Benci's data appears paradoxical. However, the authors highlight the importance of timing, showing that ablation of IFN signalling on B16 cells potentiates an effect exclusively upon delayed scheduling of dual αCTLA-4 and αPD-L1 treatment, consistent with a role for sustained and not early IFN signalling in resistance.

To resolve the mechanisms underlying IFN signalling mediated resistance, B16 and Res499 cells were transcriptionally and epigenetically profiled pre and post IFNγ treatment. This analysis provides 2 key findings: Acquisition of resistance *in vivo* and chronic IFNγ exposure *in vitro* generate an overlapping molecular footprint, and STAT1 is both elevated and its occupancy inferred at common open chromatin regions (OCRs). Critically, functional roles for STAT1 in producing this profile and mediating resistance are then underlined by loss of the key resistance signature and ICB resistance respectively, following STAT1 ablation. To enrich functionally relevant STAT1 targets, a panel of genes that are differentially expressed between B16 and Res499 was assessed, unveiling a correlation between STAT1, multiple TCIR ligands and ISGs (TNFRSF14, LGALS9, MHCII, IFIT, MX1) in ICB resistant melanoma cells, a molecular circuit the authors dub Interferon Driven ISGs and Inhibitory Ligands (IDILs). This signature is diminished when IFNA/GR are abolished on tumours *in vivo*, and intuitively, blocking the cognate TCIRs (TIM-3, LAG-3) of two of these gene products via monoclonal antibodies (mAbs) or by knocking out TNFRSF14 via CRISPR enhances survival during αCTLA-4+PD-L1 treatment of ICB resistant tumours (Res499 and Res237 breast cancer). Perhaps most intriguing is that the effect of IFN signalling seems to be only partly explained by this constellation of TCIR ligands, since survival in mice challenged with Res499 IFNA/GR KO tumours is superior to those treated with quadruple checkpoint blockade.

To complement the examination of tumours, Benci et al subsequently measure T cell responses during ICB of mice challenged with Res499 and Res499 IFNA/GR KO or Res499 STAT-1 KO tumours. ICB in Res499 IFNA/GR KO or Res499 STAT-1 KO challenged animals leads to an increased frequency of TRP2-reactive T cells bearing high levels of PD-1 and multiple TCIRs, and enhanced $Ki67^{+}GZMB^{+}$ cells amongst $PD\text{-}1^{+}$ tumor-infiltrating lymphocytes (TILs) co-ordinately expressing TCIRs and Eomesodermin (Eomes), relative to Res499. The conclusion from these assays is that exhausted T cell populations, which accumulate during regulated anti-tumour responses, can be resurrected via targeting of IFN signalling in the tumour. The rescue of such severely exhausted T cell populations has been proposed as a rate limiting step in the success of single agent ICB(Pauken and Wherry, 2015). On this basis, the authors examine whether deletion of IFN signalling can rescue responses to αCTLA-4 or αPD-1 monotherapy. Encouragingly, delayed administration of JAK inhibitors or ablation of IFNA/GR receptor on the tumour facilitated marked and complete responses to monotherapy in resistant melanoma and breast tumours, respectively.

Several lines of evidence are used to directly highlight the translational applicability of their pre-clinical findings. Amongst these, human homologues of the ISG and TCIR ligand genes expressed in Res499 were shown to significantly correlate with STAT1 expression in primary melanoma lysates, an association that is accentuated when PD-L1 levels are high. Also, low expression levels of 2 ISGs (IFIT1 and MX1) that form part of the chronic IFN resistance signature in mouse melanoma are shown to associate with improved clinical response to anti-PD-1.

Although the pleotropic anti-tumour effects of IFN signalling are well described both in vitro and in vivo, the clinical efficacy of interferon as an anti-cancer therapy is limited. Evidence of prolonged recurrence-free survival in patients with melanoma treated in the adjuvant setting does not convincingly translate to an overall survival benefit in meta-analysis (Wheatley et al., 2003). Similarly, in advanced disease, IFN treatment in combination with chemotherapy has resulted in improved response rates in some studies, without impacting on overall survival (Kaufmann et al., 2005). Emerging evidence of the immunoregulatory effects of IFN signalling presented here and elsewhere (Teijaro et al., 2013; Wilson et al., 2013) sheds light on these findings.

The basis of resistance to ICB likely involves several interdependent, patient and tumour-specific variables; i) The density of the targeted axis (receptor and ligand) and its relative contribution to suppression of T cell priming and effector function ii) the (neo)antigenic landscape of the tumour iii) the cellular and metabolic constitution of the tumour microenvironment (TME) iv) the differentiation and/or exhaustion status of tumour reactive T cells v) the intrinsic sensitivity of tumour cells to cytolytic effector mechanisms and vi) disease stage and/or duration and treatment history (Schietinger and Greenberg, 2014). The report by Benci et al connects several of these parameters to highlight a potentially clinically relevant component of tumour immune regulation. This phenomenon is perhaps best termed chronic resistance, defined as a STAT1-dependent, stable, epigenetically distinct tumour state, reached through prolonged IFN exposure and typified by ISG signatures and multiple TCIR ligand expression.

The core feature of this report is that chronic IFN signalling portends expression of multiple TCIR ligands. However, lower efficacy of quadruple ICB relative to IFNA/G KO signifies the existence of novel inhibitory pathways, with corresponding candidate genes likely part of the wider chronic resistance program. In this regard, the contribution of type I IFNs in resistance (alluded to by the activity of single IFNAR KO) is tantalising and could be further resolved via deletion of ISGs in mouse models (e.g. MX1 or IFIT1), and/or corroborative *in silico* analysis to determine whether single nucleotide polymorphisms in this pathway associate with responses to ICB. The manuscript frames an emerging complex paradigm of IFNs in tumour immunology. Whilst intact tumour signalling via STAT1 permits enhanced antigen presentation (type I IFN) and susceptibility to cytotoxic potential or HLA-class II restricted recognition (IFNGR), antagonising chronic resistance ostensibly neutralizes regulation via recognised and novel immunomodulatory axes (Figure 1).

As the authors suggest, scheduling of pharmacological intervention will require careful consideration. To this end, stratification of ICB non-responding or relapsed patients by neoantigen (NA) burden may best predict outcome using Jak inhibitors, with chronic resistance likely confined to patients with a larger number of NAs. This patient group is highlighted in the current report where progression is observed consistent with both high mutational burden and higher levels of MX1 and IFIT1. In keeping, several groups have observed STAT1 signatures in well infiltrated tumours, a marker which we found to be preferentially expressed in tumours of lung adenocarcinoma patients with high clonal NA burden (McGranahan et al., 2016). Determining whether the chronic resistance program is active within non-responding patients of this strata will add weight to the current report

and support a rationale for Jak inhibition in similar demographics. In view of the potential unwanted effects, alternative strategies to bypass chronic resistance may be explored. In this respect, positive co-stimulation (e.g. via 41BB, OX40, ICOS) and/or gamma chain cytokines may be obvious candidates. In spite of these predictions there may be some utility in antagonizing presumably lower levels IFN signalling in the context of poorly immunogenic tumours, given the effectiveness in B16 melanoma.

Advances in ICB research continue to elucidate key molecular pathways that govern resistance. The report by Benci et al reveals sustained IFN signalling as one such circuit, suppressing potentially curative anti-tumour T cell responses via established and novel immune regulatory networks. This mechanism of chronic resistance is amenable to existing pharmacologics, reasserting that collaborative efforts in T cell immunology, cancer cell biology, genetics and bioinformatics are providing drug targets of potential clinical relevance. However, whilst the approach is attractive, the complex pro- and anti-tumour effects of interferon signalling will require a cautious approach to future translational efforts.

Figure Legend

Figure1: Antagonising chronic resistance via IFN signalling blockade. Whilst chronic signalling via IFNs promotes the upregulation of PD-L1, ISGs and TCIRs on tumour cells fuelling resistance to immunotherapy, targeting this pathway requires a deeper understanding of the multiple activities of IFN in cancerous and normal cells. Targeting chronic IFN signalling can revert resistance via downregulation of PD-L1, ISGs and TCIR ligands on the tumour but it could also negatively impact antigen processing and presentation by tumour and antigen presenting cells, in a time dependent manner.

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