IKZF1 Enhances Immune Infiltrate Recruitment in Solid Tumors and Susceptibility to Immunotherapy

Graphical Abstract

Highlights
- A network-based computational framework can identify key enhancers of immunotherapy
- IKZF1 overexpression promotes immune infiltrate recruitment in several tumor types
- IKZF1 overexpression in tumors enhances efficacy of α-PD1 and α-CTLA4 treatment
- Genomic alterations of IKZF1 predict poor prognosis and low immune infiltration

Authors
James C. Chen, Rolando Perez-Lorenzo, Yvonne M. Saenger, Charles G. Drake, Angela M. Christiano

Correspondence
amc65@columbia.edu

In Brief
Immunotherapies are promising cancer treatments but are frequently stymied by tumors that evade the immune system. Here, Chen et al. computationally identify the regulators required to hijack and reanimate immune infiltrate recruitment in tumors. They show that IKZF1 expression suppresses growth in several cancers due to enhanced immune infiltrate recruitment and significantly enhances the efficacy of α-PD1 and α-CTLA4 immunotherapies. IKZF1 genomic alterations also predict poor prognosis in patient cohorts. This work demonstrates the value of computational approaches in identifying new treatment avenues.
IKZF1 Enhances Immune Infiltrate Recruitment in Solid Tumors and Susceptibility to Immunotherapy

James C. Chen, Rolando Perez-Lorenzo, Yvonne M. Saenger, Charles G. Drake, and Angela M. Christiano

1Department of Dermatology, Columbia University Medical Center, New York, NY, USA
2Department of Systems Biology, Columbia University Medical Center, New York, NY, USA
3Department of Genetics and Development, Columbia University Medical Center, New York, NY, USA
4Department of Medicine, Herbert Irving Comprehensive Cancer Center, Columbia University Medical Center, New York, NY, USA
5Lead Contact
*Correspondence: amc65@columbia.edu
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SUMMARY

Immunotherapies are some of the most promising emergent treatments for several cancers, yet there remains a majority of patients who do not benefit from them due to immune-resistant tumors. One avenue for enhancing treatment for these patients is by converting these tumors to an immunoreactive state, thereby restoring treatment efficacy. By leveraging regulatory networks we previously characterized in autoimmunity, here we show that overexpression of the master regulator IKZF1 leads to enhanced immune infiltrate recruitment and tumor sensitivity to PD1 and CTLA4 inhibitors in several tumors that normally lack IKZF1 expression. This work provides proof of concept that tumors can be rendered susceptible by hijacking immune cell recruitment signals through molecular master regulators. On a broader scale, this work also demonstrates the feasibility of using computational approaches to drive the discovery of novel molecular mechanisms toward treatment.

INTRODUCTION

Immunotherapies are rapidly emerging as highly promising avenues of treatment across multiple cancer types, and additional research is consistently emerging that is dedicated to characterizing the complex interactions between the immune system and a growing tumor. However, studies have demonstrated that many patients do not benefit from treatments such as immune checkpoint inhibition (Topalian et al., 2015; Aggen and Drake, 2017; Emambux et al., 2018). Promoting and enhancing the efficacy of immunotherapies in additional patients and cancers, as well as the ability to predict and match patients to optimal treatments, would be invaluable scientific and medical advancements. One potential avenue for enhancement therapy is the conversion of immune-resistant tumors to an immunoreactive state by inducing immune sensitivity to cancers that have selectively acquired mechanisms to avoid immune surveillance.

The treatment paradigm of immune checkpoint inhibition specifically promotes host immune recognition of tumors, which can be countered at a molecular level by disabling the key pathways for such recognition and rendering these inhibitors ineffective (colloquially referred to as “immune evasion”). Overcoming these evasion mechanisms and restoring a host’s ability to recognize, target, and destroy tumors has opened a promising avenue of research for cancer therapy. This is particularly the case in metastatic tumors, since the ability of these tumors to spread is dependent heavily on remaining unrecognized by the immune system (Dong et al., 2002; Drake et al., 2006; Kusmartsev and Gabrilovich, 2006; Hinz et al., 2007). Tumors that express PD1 are prime candidates for PD1-mediated treatments, yet some tumors remain immune resistant. Recalcitrance to these therapies could be due to their genetics (e.g., immune evasion), mutational burden, or physical location.

In this light, immune evasion can conceptually be thought of as an inversion of autoimmunity. In autoimmunity, a host’s immune system becomes “hyperactive,” or the target tissue becomes immunoreactive, and the immune system aberrantly recognizes the host’s own tissues as foreign antigens. The immunological pathways activated in some autoimmune diseases, such as alopecia areata (e.g., cytotoxic CD8+ T cell activation), are the same pathways that are inactivated in cancers (Dunn et al., 2002; Xing et al., 2014; Chen et al., 2015). In light of this, we propose a strategy for enhancing cancer immunotherapy that entails “hijacking” the molecular mechanisms that activate the immune system in autoimmune disease and using them to essentially “tag” evasive tumors for immune-mediated destruction. We reasoned that the molecular processes that encourage active immune cell infiltration to the target organs in autoimmunity disease could be used to restore immune targeting against cancer cells.

Previously (Chen et al., 2015), we demonstrated that the master regulator IKZF1 is sufficient to induce the recruitment of immune infiltrates that result in immune-mediated cytotoxicity seen in autoimmune when expressed in target tissues. Exogenous overexpression of IKZF1 was sufficient to induce autoimmune susceptibility in unaffected cultured cells primarily through infiltrating NKG2D+, CD8+ T cells. Based on these findings, we hypothesized that tumor cohorts may achieve immune evasion via IKZF1-inactivating mutations. These tumors would then be susceptible to enhancement therapy through the restored expression of IKZF1. In this study, we sought to computationally
identify cancer cohorts whose genetic-genomic architecture (Chen et al., 2014) included IKZF1 disruptions, to validate the ability of IKZF1 to rescue or induce cytotoxic immune interactions in these tumors and to assess the enhancement of immunotherapy efficacy.

RESULTS

Network Analysis Identified Cancer Cohorts Amenable to Enhancement of Checkpoint Inhibitor Therapy

The overall framework for this pipeline is detailed in Figure 1. In theory, systems biology-based analytics using regulatory networks can infer the master regulators of any modular complex trait associated to a well-defined gene-expression signature. Due to the significant relevance of immunotherapies for cancer treatment (Ascierto et al., 2010; Min and Hodi, 2014; Rizvi et al., 2015; Valecha et al., 2017; Godfrey et al., 2017) and our previous identification of master regulators activating immune infiltrate recruitment in autoimmune disease (Chen et al., 2015), we postulated that certain tumors might acquire immune evasion through the suppression of such master regulators. With access to regulatory and DIGGIT (Chen et al., 2014) networks of 15 cancers provided by The Cancer Genome Atlas (TCGA) (Verhaak et al., 2010), we sought to identify and validate patient cohorts that may be amenable to immune enhancement by identifying patients with a functional IKZF1 regulon and driver mutations in their regulatory networks.
Patients with \textit{IKZF1} disruptions can be computationally identified using integrated regulatory network algorithms (DIGGIT). This algorithm defines “activity quantitative trait loci” (aQTL) in patient tumors that are predicted to disrupt the activity of specific master regulators. This is achieved by reconstructing genetic-genomic interaction networks from patient-matched data to identify functional alterations in the genome that coincide with molecular disruption of master regulators, the particulars of which are detailed in previous publications (Chen et al., 2014; Califano, 2014) and detailed in STAR Methods. In brief, the algorithm sorts through genomic mutations and assigns them functions based on whether or not they perturb the molecular behavior of a master regulator through an accompanying gene-expression regulatory network.

Therefore, we can leverage the aQTL networks across the TCGA cancer cohorts to identify cancer subsets that have functional alterations in \textit{IKZF1}. A positive prediction by this approach indicates that (1) \textit{IKZF1} is a functional master regulator in a tested tumor, and (2) there is genomic evidence that the activity of \textit{IKZF1} is perturbed. These cancer types should therefore be tested for susceptibility to immune modulation via these master regulators. We postulated that if an aQTL for the \textit{IKZF1} regulon exists, then regulatory logic exists within the corresponding patient cohort that can potentially phenocopy the recruitment of immune infiltrates. These cancers were subsequently selected for more in-depth study and validation.

**Eight TCGA Cancers Have Targetable \textit{IKZF1} aQTLs**

We interrogated a total of 15 DIGGIT networks corresponding to the available TCGA tumor cohorts: cutaneous melanoma (SKCM), glioblastoma (GBM), head-and-neck squamous carcinoma (HNSC), lung adenocarcinoma (LUAD), thyroid carcinoma (THCA), bladder carcinoma (BLCA), lung sarcoma (LUSC), prostate adenocarcinoma (PRAD), uterine corpus endometrial carcinoma (UCEC), kidney/renal carcinoma (KIRC), colorectal adenocarcinoma (COAD), rectal adenocarcinoma (READ), liver hepatocellular carcinoma (LIHC), low-grade glioma (LGG), and stomach adenocarcinoma (STAD). For each network, we tested specifically for aQTLs enriched in the validated \textit{IKZF1} regulon that confers immune susceptibility in alopecia areata, which primarily governs the infiltration of CD8$^+$ NKG2D$^+$ cytotoxic T cells (Chen et al., 2015). The DIGGIT networks themselves were built using corresponding TCGA datasets using the standard published pipeline (Chen et al., 2014), false discovery rate (FDR) < 0.05. The overall distribution of enrichment in each tumor can be used to ascertain whether or not the regulatory and genomic network architecture of these tumors includes the candidate master regulators. Those tumors that do contain such aQTLs are potentially susceptible to immune modulation through \textit{IKZF1} as a master regulator.

The results for the DIGGIT analysis of \textit{IKZF1} are presented in Figure 2. For each tumor, the distribution of p values in the top 100 candidate aQTLs are presented (Figure 2A) in addition to a median null distribution of aQTLs defined by bootstrapping randomized gene signatures (see STAR Methods). The complete distributions represented as box-and-whisker plots are also available as Figure S1. The divergence between these two distributions was tested statistically using the non-parametric Mann-Whitney U test. The TCGA STAD cohort was omitted entirely due to an inability of the algorithm to achieve sufficient aQTL resolution.

Using this as a metric, eight cancer types were identified as having \textit{IKZF1} aQTLs: SKCM, GBM, HNSC, THCA, LUAD, LUSC, PRAD, and BLCA (FDR < 0.05).

**IKZF1 Expression Induces Immune-Mediated Cytotoxicity Molecular Programs**

Based on the DIGGIT analysis, we obtained seven human-derived tumor cell lines corresponding to the cancers bearing \textit{IKZF1} aQTLs (GBM, THCA, SKCM, PRAD, BLCA, and LUAD). Each cell line was stably transduced with an RFP (negative control) and \textit{IKZF1} constitutive-expression construct. We subsequently cultured the stable lines for use in immune-mediated cytotoxicity assays (Chen et al., 2014) using peripheral blood mononuclear cells (PBMCs) isolated from human whole-blood samples. This assay measures the relative cell death of a target cell population after exposure to immune cells, and can be used to test the hypothesis that the induction of \textit{IKZF1} will enhance tumor cell susceptibility to immune-mediated cytotoxicity. Following 8 hr of co-incubation, we observed statistically significant increases in immune-mediated cytotoxicity in \textit{IKZF1}-transfected cells compared with their corresponding RFP controls by t test (p < 0.05, Figures 2B and 2C). This was true of five of the seven lines tested: two SKCM lines, GBM, THCA, and PRAD. Conversely, the BLCA and LUAD lines tested failed to demonstrate a significant enhancement of immune-mediated cytotoxicity. These results are ranked by absolute fold change (\textit{IKZF1} versus matched RFP).

These cell lines were then sent for gene-expression profiling via RNA sequencing (RNA-seq). We tested each cell line for enrichment of the validated \textit{IKZF1} immune-recruitment regulon (Chen et al., 2015) in gene set enrichment analysis (GSEA) (Subramanian et al., 2005). Figure 2D details the results of each GSEA. Across all available cell lines, the transduction of \textit{IKZF1} resulted in statistical enrichment of the \textit{IKZF1} immune-recruitment regulon compared with RFP counterparts at FDR < 0.05 (Subramanian et al., 2005), indicating that the DIGGIT-predicted tumor cohorts in fact contain the regulatory logic associated with our characterized \textit{IKZF1} aQTL, and thus may be amenable to the rescued expression of \textit{IKZF1} to enhance immune infiltrate recruitment.

**IKZF1 Expression in Mouse-Derived Melanomas Suppresses Melanoma Growth**

Since the human melanoma (SKCM) TCGA cohort had the most significant candidate driver predictions across the entirety of this network analysis (the strongest DIGGIT aQTLs, correlations with prognosis, and with immune infiltration marker panels), we turned to a syngenic melanoma model using C57/B6 mice to perform \textit{in vivo} characterization of the role of \textit{IKZF1} as a master regulator of immune-recruitment enhancement. B16F10 spontaneous melanomas are isolated from immunocompetent mice and can be reintroduced as oncogenic subcutaneous grafts in the C57/B6 background via subcutaneous injection. This tumor line does not normally express \textit{IKZF1} (see Figure S2A), making it ideal for testing overexpression of \textit{IKZF1}. We cultured B16F10 tumor cells and stably transduced a constitutively active
IKZF1 construct (or RFP control). We grafted $5 \times 10^4$ cells into B6 mice and tracked the development of tumors over 21 days (Figure 3A). We observed a statistically significant reduction in tumor size in mice grafted with IKZF1-producing B16F10 tumors as compared with the RFP controls. The difference in tumor growth was detectable as early as 17 days post graft and was stable until the end of the time course. Conversely, there was no significant reduction in growth when this experiment was conducted in immunocompromised nude mice (Figure S2B), indicating that an active immune system is necessary for the tumor-suppression phenotype. Viability of the mice was not affected by the injections except one, which was excluded from analysis (Figure S2C).

We performed *in vitro* cytotoxicity assays using PBMCs isolated from whole blood of individual mice against both IKZF1- and RFP-B16F10 to quantify IKZF1-dependent immune-mediated toxicity. Upon completion of the time course, whole blood was isolated from three RFP- and four IKZF1-grafted mice. PBMCs were isolated for cytotoxicity assays using the B16F10 stably transfected cells (Figure 3B). PBMCs from each mouse were tested against both IKZF1- and RFP-transduced cells, and in six of seven samples there was a statistically significant increase in immune-mediated cytotoxicity in IKZF1 tumors compared with RFP controls. The last sample exhibited a notable and concordant increase, but fell marginally short of statistical significance by t test (FDR < 0.1 but $p < 0.05$). Taken as a whole, there was a statistically significant increase in immune-mediated cytotoxicity in IKZF1-B16F10 cells compared with RFP controls ($p = 2.79 \times 10^{-5}$). This effect was not dependent on the PBMC source (whether the PBMC donor initially received an IKZF1 or RFP xenograft). Instead, the only predictor of increased immune activity was whether or not the tumor itself expressed IKZF1.

**Increased CD8+ T Cell Infiltrates Found in B16F10 Grafts with IKZF1 Expression**

After the completion of the time course, we collected the remaining tumors and performed RNA-seq profiling to quantify the
Figure 3. Results of a Syngenic Melanoma Mouse Model Testing Immune-Recruitment Enhancement Function of IKZF1

(A) Composite (n = 5 per arm) tumor growth curves of B16F10 melanomas in a syngenic, immunocompetent B6 mouse background. Tumors were stably transfected with either RFP- or IKZF1-expressing constructs and subcutaneously injected into mouse flanks. Tumor growth was recorded over the course of 21 days. There was a statistically significant separation in tumor growth solely based on the expression of IKZF1 (****p < 0.01).

(legend continued on next page)
molecular effects of IKZF1 transduction. We quantified the relative abundance of infiltrating immune cells within the tumor mass based on the RNA-seq consensus of each immune cell type, each defined by a unique molecular biomarker panel derived from tumor infiltrate studies (Bindea et al., 2013; Jabbari et al., 2016). These panels were cross-checked with the gene-expression profiles of ungrafted, in vitro cultured tumors to ensure exclusivity (provided in Supplemental Information). Figure 3C displays the consensus fold change of each immune cell type and the p value associated with it following Fisher’s integration. In particular, four categories of immune markers—T cell markers, T helper type 1 (Th1) markers, natural killer (NK) cell markers, and CD8+ markers—were at least one order of magnitude more statistically significant than the others, as well as displaying an average 10- to 20-fold change increase in IKZF1-treated tumors. These markers were all highly increased in IKZF1-expressing tumors, suggesting selective increases in infiltration of these specific immune cell populations in the tumors. We included in vitro cultured IKZF1 and RFP B16F10 cells as negative controls, and these samples did not register any reads to these markers, indicating that these cells reflect immune infiltrates from the mouse host (Tables S2 and S4).

These results were confirmed at the protein level by flow-cytometry analyses. Post-treatment residual tumors were excised and processed for flow (see STAR Methods) to quantify the relative CD8+ immune cell infiltrate. Cells were stained for CD4 and CD8; in these studies we observed an approximately 3-fold enrichment of CD8+ cells (p < 0.05) in the infiltrate in IKZF1-expressing tumors compared with the corresponding RFP controls (Figure 3D). Paired t-test analysis of the CD8 and CD4 populations independently also indicated statistically significant increases in CD8+ cells (p < 0.03) and CD4 cells (p < 0.02). We additionally analyzed markers for T regulatory cell populations (FOXP3), B cells (CD19), NK cells and CD8+, NK cells (NK1.1), and dendritic cells (CD11c). There were statistically significantly elevated levels of NK1.1+ cells, as a significant suppression of CD19+ cells in the extracted infiltrates.

**IKZF1 Expression Suppresses Tumor Growth in Combination with α-PD1 and α-CTLA4 in Melanoma**

While the previous experiments established the mechanistic relevance of IKZF1 as a master regulator, we sought to further investigate its potential translational implications. We repeated the mouse experiments, this time incorporating individual and combinatorial treatments of α-PD1 and α-CTLA4 antibodies (Figure 3E). Photos of mice from each arm are provided in Supplemental Information. The quantification of tumor mass is also provided in linear and logarithmic scale (for improved resolution of the smaller tumors). Administration time points of α-PD1 and/or α-CTLA4 is indicated by arrows at the provided dosages.

Over the 21-day time course, we observed that IKZF1 expression was sufficient to phenocopy the kinetics of PD1 inhibition, such that they are statistically indiscernible (both are statistically significant from controls at p = 0.05). Individual CTLA4-inhibitor treatment had no statistically differentiable effect on tumor growth, but was enhanced by IKZF1 expression. Combinatorial therapy of PD1 and CTLA4 inhibitors resulted in the eventual suppression of tumor growth, but this was not observable until post-graft day 14. Notably, any combination of IKZF1 overexpression with a drug treatment regimen significantly enhanced the regression rates of B16F10 growth, and tumors began following a regressive curve as early as 8 days post graft compared with RFP controls. In particular, α-CTLA4 treatment alone was largely ineffective compared with controls, but produced a significantly enhanced effect when combined with IKZF1. Inclusion of IKZF1 treatment with both α-PD1 and α-CTLA4 resulted in complete tumor regression by day 18, and significantly enhanced the rate of regression throughout the entire time course compared with α-CTLA4 and α-PD1 alone. Composite data plots in linear and log scale for each treatment are available in Figure S2D, with representative photos shown in Figure S2E.

**IKZF1 Immune Rescue Is Validated in Three Additional Mouse Cancer Lines**

As a final in vivo test of both the positive and negative predictive value of the computational approach, we repeated the mouse model experiments in two tumor models that we predicted would be recalcitrant to IKZF1, renal (READ/RENGA), and colorectal (COAD, CT26) tumors, as well as a second model predicted to be sensitive to IKZF1, prostate (PRAD, MyC-CaP). READ and COAD were shown by the DIGGIT analysis to either have unmethylated or inactive (unexpressed) IKZF1 in their genetic-genomic networks, suggesting that introduction of IKZF1 would have minimal effect. Conversely, PRAD showed statistically enriched aQTLs disrupting IKZF1 activity, and our in vitro assays in human lines suggested that IKZF1 expression would enhance infiltration in a manner similar to SKCM. Our experiments validated our predictions in both scenarios: the prostate tumors recapitulated the results observed with the B16F10 line (Figure S3, left panels), and were only observed in immunocompetent mice (Figure S3, right panels). We were able to recapitulate the effectiveness both of IKZF1 independently and in combination with PD1 and CTLA4.
CTLA4 inhibitors in PRAD. In contrast, both the READ and COAD tumor lines failed to suppress tumor growth individually (IKZF1 only) or enhance PD1 and CTLA4 inhibition. Taken together, these in vivo studies bring the predictive accuracy of the algorithm to 2/2 positive and 2/2 negative.

IKZF1 Overexpression Induces Significant Changes in Expression of Immune Markers in Human Tumors

Since our data indicated the overall activation of immune infiltrate recruitment signals, we returned to the RNA-seq data of the human tumors lines (first described in Figure 2, available in Table S2) and tested for an association of IKZF1 overexpression and subsequent changes in cytokine or membrane-bound signaling molecules. This was defined by comparing each IKZF1-transfected line to its respective RFP control, taking all genes that were differentially expressed by at least 20% and performing naïve functional annotation enrichment analysis. This was done independently for each tumor type and the results were integrated for comparative viewing in Figure 4. Blue nodes represent individual genes that were differentially expressed, and linked yellow nodes denote the cancers in which association was detected. The differential expression profiles of all tumors tested were significantly enriched in membrane-bound signaling molecules (p < 1 × 10^{-20} corrected), as well as in immunoglobulin, immune response, immune activation, and cytokine annotation categories (p < 1 × 10^{-15} corrected). This list includes human leukocyte antigen, interferon, and chemokine signature genes, a full list of which is available in Table S3. The overall
description, commonalities, and divergence in the effects of IKZF1 on the expression of these genes across the cancer types can be seen in Figure 4. This analysis details the molecular enrichment of immune markers associated with IKZF1 expression in human tumors, providing insight into how the master regulator may mediate an increase in immune-mediated cytotoxic sensitivity.

Genomic Alterations in IKZF1 aQTLs Are Predictive of Decreased Immune Infiltrates in TCGA Human Cancer Cohorts

In this study, the application of DIGGIT to 15 cancer cohorts from TCGA identified the presence of targetable master regulators using a combination of transcriptional networks and genomic mutation architecture. Copy-number variations (CNVs) were used as the primary genomic feature. The algorithm predicts genomic mutations that drive aberrant master regulator activity by linking specific CNVs to coordinated regulatory changes through a network. These fCNVs (functional CNVs) can be clustered into co-mutated loci that reflect typical minimal-common-regions of mutations that are observed in human patients (Chen et al., 2014). This effectively creates small numbers of mutation sets that can be used as biomarkers for association studies in human cohorts (Figure 5A). In this instance, we identified two sets of mutations in the affected cohorts (Figure 5B), accounting for 30%–50% of patients in each tumor cohort (Figure 5C). The frequency of the IKZF1 aQTL in these tumors is conducive to an immune infiltrate analysis (>10% of patients for sufficient statistical power). By segregating patients into subgroups based on whether or not an individual had evidence of genomic alterations that affect master regulators of immune infiltrate recruitment, genomic disruptions of IKZF1 can then be tested for association of molecular and physiological outcomes.

We therefore aimed to establish an association between tumor immune infiltration and mutations in IKZF1 aQTLs under the hypothesis that tumor samples bearing such mutations will...
have lower levels of infiltrating signal. Since our primary interest in IKZF1 is as a recruiter of CD8+ NKG2D+ T cells (Xing et al., 2014; Chen et al., 2015), we extracted the appropriate CD8, T cell, and cytotoxic marker signatures from Bindea et al. (2013) and performed a naive random forest clustering across these patients to ascertain whether or not a difference in these signatures was detectable. The clustering identified ten partitions (11 total clusters) based on the Bindea signatures for these three marker panels (Figure 5D) that could be organized into patients with high and low signals of immune infiltration. Using this classification, we detected a statistically significant enrichment of patients bearing IKZF1 aQTLs in the three lowest pan-signal clusters (1, 3, and 4), odds ratio = 1.52 and p = 1.49 × 10^-4 by Fisher’s exact test. Cluster 2 was included in the high-signal group due to the abundance of T cell markers, despite the relative lack of CD8 and cytotoxic markers. This result indicates that patients with CNVs affecting IKZF1 are significantly more likely to have the lowest levels of immune infiltrate signal (a complete description of all immune types is available in Figure S4 and Table S5).

The difference in immune infiltrate levels was quantified using gene expression across each of the cancers by relative fold change (Figure 5E). Normalized fold changes show statistical changes in individual immune marker panels. The SKCM, BLCA, and GBM cohorts demonstrated the most significant shifts in all three immune marker panels, while LUAD/LUSC and HNSC showed statistically significant changes in CD8 and T cell markers. These results suggest that the candidate aQTL loci have the strongest evidence for influencing immune infiltration in the SKCM, GBM, and BLCA cohorts.

**Decreased IKZF1 Activity Predicts Recurrence and Poor Prognosis in an Independent Cohort of Human Melanoma and across TCGA Cancer Cohorts**

We analyzed an independent clinical melanoma cohort with expression data (Sivendran et al., 2014) for immune infiltrates to analyze the predictive value of IKZF1 expression. These samples were assayed for immune markers and correlated to clinical response and recurrence rates. In this case, comparative analysis was done by binning the patients in order of IKZF1 expression and comparing the upper and lower 30% against each other. In this comparison, we found that decreased IKZF1 expression was significantly associated with tumor recurrence (FDR < 0.05, Figure 6A), and was also predictive of overall suppression in detectable infiltrates measured by immune biomarker panels (Figure 6B).

Returning to the TCGA datasets, we segregated all patients into two cohorts defined by whether or not each patient had CNVs affecting IKZF1 activity as previously defined (set 1, Figure 6C and set 2, Figure 6D). Cohorts of CNV-bearing patients were tested against their corresponding negative control cohort (no CNVs) for various clinical survival outcomes. Across all tests, we found that patients with CNVs in at least one gene within these loci presented with significantly shorter median survival and higher mortality rates than patients with no mutations at these loci. The set of genes (listed in Supplemental Information) that are directly linked to IKZF1 activity and decreased immune infiltration are also sufficiently predictive of poor prognosis regardless of any other mitigating factor. They represent the most clinically relevant candidate loci that perturb the IKZF1 regulon, and therefore serve as both genomic indicators of susceptibility to IKZF1 modulation and ideal candidates for further functional studies and drug discovery.

**DISCUSSION**

An analysis of DIGGIT networks across the TCGA data was able to identify patient subsets across six cancers that display significant evidence of IKZF1 aQTLs. While straightforward, this result has two key corollaries. First, it indicates that a genomic alteration can be functionally linked specifically to the molecular behavior associated with regulation of infiltrate recruitment in cancer cohorts (identifying immunoreactive versus immune-resistant tumors). Second, it provides an actionable target for enhancement of immune therapies (converting immune-resistant tumors to an immunoreactive state).

In terms of translational applications, these findings add to a growing body of literature proposing master regulators not only as biomarkers of important pathophysiology, but also as targets for drug therapy development. IKZF1 is a key regulator of a complex molecular signature governing immune infiltrate recruitment, making it both a predictor of response to therapy and an excellent candidate for future drug targeting. Computational modeling of drug mechanism of action using similar analytic pipelines allows for the matching of compounds to patients based on the key master regulators governing their particular molecular pathologies (Woo et al., 2015), allowing for the possibility of identifying patients with cancers bearing IKZF1-inactivating mutations and planning treatments accordingly, e.g., combinatorial therapy with a compound that activates the IKZF1 regulon.

Here, we showed that five of seven (GBM, two different SKCM, PRAD, and THCA) tested tumor types that were predicted to be responsive to IKZF1 therapy (immuno-reactive tumors with IKZF1 in their regulatory logic) did indeed respond in in vitro assays with a physiologically observable output (increased cytotoxicity). The results of this study draw parity between GBM and SKCM cancers, which are usually considered disparate using more classical oncological definitions. This finding suggests that cancers traditionally considered completely disparate may have common therapeutic targets from a regulatory perspective. The RNA-seq analysis and in vitro assays demonstrate that IKZF1 expression in the tumor masses enhances immune infiltration, supporting the predictions drawn by our network analysis.

The overexpression of a single master regulator was sufficient to induce the activation of a complex immune infiltrate recruitment signature (Chen et al., 2015) across several predicted tumor types, resulting in enhanced molecular interactions with cytotoxic immune cells. This in turn identifies IKZF1 as the minimum regulator required to coordinate a complex molecular phenotype (Margolin et al., 2006; Lefebvre et al., 2010), and makes for an ideal candidate for further mechanistic studies. It should be noted that one predicted cell line, TCCSUP, demonstrated no clear statistical shift in the in vitro immune-mediated cytotoxicity assays. This could be due to unidentified mutations downstream of IKZF1 in the signaling pathway, or an
insufficiency of the in vitro assay to reflect the necessary biological conditions to elicit a measurable response. NCI-H441 responded with a suppression of tumor immune sensitivity. While this is clearly not ideal from a clinical treatment perspective, the network analysis sufficiently identified that **IKZF1** was a functional node in the regulatory network—the analysis was not conducted to include directionality in the molecular predictions.

These results translated into the syngenic mouse melanoma model. Stably transfected RFP and **IKZF1** B16F10 tumor cells were maintained on low-level selection, sorted by fluorescence-activated cell sorting, and grafted into the appropriate strain of immunocompetent B6 mice. Across all experiments there was a statistically significant separation in tumor growth detectable as early as 15–16 days post injection. Importantly, this was not observed when the experiment was repeated in immunocompromised nude mice. The statistically inseparable survival outcomes of **IKZF1**- and RFP-expressing melanomas in nude mice suggested that an active immune system is necessary for the suppression phenotype.

These experiments also demonstrated that the function of these master regulators is mediated through the tumor itself, rather than the accompanying host immune system. This is evidenced by the identification of somatic driver mutations throughout the TCGA cohorts as well as the exogenous **IKZF1** expression restricted entirely to the tumor, and is further supported by the in vitro cytotoxicity assays. Across all trials, the trend was the same: the defining predictor of enhanced cytotoxicity was whether or not the melanoma itself expressed **IKZF1**; whether or not the PBMCs had been previously exposed to an **IKZF1** tumor was irrelevant.

From a translational perspective, **IKZF1** expression in melanomas provided synergistic enhancement of both α-PD1 and α-CTLA4 inhibition. This observation adds to the growing body of research suggesting the promise of leveraging master regulators to drive novel clinical and therapeutic development. **IKZF1** exogenous expression alone is sufficient to phenocopy the tumor suppression following PD1 inhibition, and facilitates efficacy of CTLA4 inhibition that is not observed in individual treatment. Although the effect size of PD1 treatment itself

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**Figure 6. Prognostic Studies in Human Cancer Cohorts Using **IKZF1** as a Biomarker**

(A) An independent human melanoma cohort aimed at studying recurrence rates shows that recurring tumors are statistically associated with lower levels of **IKZF1** expression (*p < 0.05*).

(B) Patients in the cohort with low **IKZF1** expression associate with overall significant suppression of infiltrate biomarker panels (red dots).

(C and D) In TCGA patients, and across all available cancers, CNVs in the candidate aQTLs (C, set 2; D, set 4) predict suppression of **IKZF1** activity, and are solely sufficient to predict poor clinical outcomes by Kaplan-Meier prognostics (red line, mutation bearing aQTLs; blue line, no mutations) and by overall total survival rates. Data are plotted as the mean ± SEM.
that exogenous expression of or relevant regulator. In functional mouse models, we observed pathology via these same master regulators (Xing et al., 2014; Chen et al., 2015) and reflect the same molecular pathways that have been reported as suppressed in cancer. This analysis provides compelling evidence of the specificity of these master regulators, since only these select populations were significantly enriched as infiltrates in the IKZF1 tumors.

Finally, demonstrating both the positive and negative predictive values of these approaches, we showed that two cancer types that we predicted to be completely non-responsive to IKZF1 expression showed no response to overexpression in in vivo mouse models. CT26 (COAD) and RENCA (READ) were predicted by network analysis not to have IKZF1 as a functional or relevant regulator. In functional mouse models, we observed that exogenous expression of IKZF1 in these tumors indeed had no effect on tumor growth or aggressiveness independently, nor did they enhance the efficacy of PD1 or CTLA4 inhibitors. Across TCGA cancer cohorts and an independent melanoma cohort, disruptions of the IKZF1 regulon were sufficient to predict poor prognosis, high mortality rates, and recurrence of tumors.

On a broader scale, this work also demonstrates the feasibility of using systems biology approaches to drive the discovery of novel molecular mechanisms that drive complex molecular pathologies. Further development would allow for refinement of this approach to individual patient targeting both by studying the exact biological mechanism of the IKZF1 immune-recruitment phenotype, and by extending the analysis to search for recruitment master regulators of other immune cell types. For the analysis specifically of the IKZF1 aQTL, the potential amenable cohort size across the TCGA ranged from 25% to 60%, indicating significant potential for clinical development and broad application.

We have established the precedent that computational methods such as DIGGIT provide a framework that can contribute to multiple key goals of precision medicine. Adding to the emerging body of work focused specifically on using master regulators to identify actionable targets, we show that these approaches can be leveraged not only to identify master regulators that affect tumor cell-autonomous physiologies, e.g., mesenchymal transformation (Carro et al., 2010), but also to discover novel interaction networks that mediate immune infiltration in cancer. As a corollary, we can identify patient cohorts that may be amenable to specific treatment regimens. Network-based approaches have repeatedly demonstrated their promise in driving the development of precision medicine, and our work highlights the potential of identifying and leveraging master regulators with the goal of enhancing cancer immunotherapy.

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This is further corroborated by the subsequent RNA-seq analysis. We observed statistically significant, elevated consensus levels of infiltrating CD8+ T cell, Th1 cell, and NK cell populations uniquely in the mouse tumors expressing IKZF1. These are the primary cell types that were originally implicated in alopecia areata pathology via these same master regulators (Xing et al., 2014; Chen et al., 2015) and reflect the same molecular pathways that have been reported as suppressed in cancer. This analysis provides compelling evidence of the specificity of these master regulators, since only these select populations were significantly enriched as infiltrates in the IKZF1 tumors.

Finally, demonstrating both the positive and negative predictive values of these approaches, we showed that two cancer types that we predicted to be completely non-responsive to IKZF1 expression showed no response to overexpression in in vivo mouse models. CT26 (COAD) and RENCA (READ) were predicted by network analysis not to have IKZF1 as a functional or relevant regulator. In functional mouse models, we observed that exogenous expression of IKZF1 in these tumors indeed had no effect on tumor growth or aggressiveness independently, nor did they enhance the efficacy of PD1 or CTLA4 inhibitors. Across TCGA cancer cohorts and an independent melanoma cohort, disruptions of the IKZF1 regulon were sufficient to predict poor prognosis, high mortality rates, and recurrence of tumors.

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Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **CONTACT FOR REAGENT AND RESOURCE SHARING**
- **EXPERIMENTAL MODELS AND SUBJECT DETAILS**
- **METHOD DETAILS**
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- **QUANTIFICATION AND STATISTICAL ANALYSIS**
- **DATA SOFTWARE AND AVAILABILITY**

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes five figures and six tables and can be found with this article online at https://doi.org/10.1016/j.cels.2018.05.020.

**ACKNOWLEDGMENTS**

We thank Dr. Charles Karan and the High-Throughput Screening Facility at Columbia University Medical Center for the human cancer cell lines used in this project. We thank Drs. Andrea Califano, Wendy Chung, Ali Gharavi, Ronald Wapner, and Krzysztof Knylik for their insightful discussions related to the work. J.C.C. was supported by the Columbia University Herbert and Florence Irving Medical Center Dean’s Precision Medicine Research Fellowship (UL1TR001873). These studies utilized the resources of the EpiCURE Skin Disease Research Core Center (P30AR069632) as well as the Flow Cytometry Shared Resource and the Human Immune Monitoring Core of the Herbert Irving Comprehensive Cancer Center (P30CA013696).

**AUTHOR CONTRIBUTIONS**

The conceptual framework for this project was developed by J.C.C. and A.M.C. J.C.C. implemented and performed the computational analysis and associated analysis. Y.M.S. provided an independent human melanoma cohort data and assisted with its analysis. C.G.D. provided expertise for immune cell analysis and interpretation of immunotherapy data. The manuscript was written by J.C.C. and A.M.C., and all authors participated in editing of the final manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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by cytotoxic T lymphocytes and is reversed by JAK inhibition. Nat.
Med. 20, 1043–1049.
## STAR★METHODS

### KEY RESOURCES TABLE

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<td><strong>Experimental Models: Cell Lines</strong></td>
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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Angela Christiano (amc65@cumc.columbia.edu).

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Seven-week old female C57BL6/J and athymic mice (Ncrnu/nu) were purchased from The Jackson Laboratory or Taconic Farms respectively. All animals were kept under a controlled environment of temperature and humidity and a 12h light/dark cycle. Experimental procedures were in compliance with a Columbia University Institutional Animal Care and Use Committee approved protocol.

The following human-derived cancer cell lines used in this study were obtained courtesy of the Drug Screening Facility at Columbia University along with relevant documentation (through the ATCC): 8505c (thyroid), LNCaP (prostate), D04 (melanoma), SKCM-MEC2 (melanoma), TCCSUP (bladder), NCIH441 (lung); and SF210 (glioblastoma) from UCSF. Cell lines were cultured, passaged, and maintained in accordance with the guidelines provided in these documents (see Key Resources Table for details). All relevant information on each individual line is detailed in the associated, publicly available documents. Mouse B16F10 lines were made available through Dr. David Owens and the EPICURE Core at Columbia University Medical Center, MyCCap from Dr. Charles Drake, and RENCA and CT26 from the ATCC. B16F10 was cultured with DMEM 10%FBS, MYCCAP and CT26 with RMI1640 10%FBS, all stored at 37°C, 5% CO2.
**Cell Culture and Transductions**

The IKZF1 and RFP cassette genes were subcloned for transduction into a modified pLOC expression vector bearing a multiple cloning site. This standard vector is the same backbone that has been used in previous studies. Subcloning was performed and colonies were validated by Sanger sequencing via GeneWiz (https://www.genewiz.com/en).

Transfections were performed using the JetPRIME (http://www.polyplus-transfection.com/products/jetprime/) reagent according to manufacturer protocols, 1:2 DNA:reagent ratio. Transfections were tested in target cell lines and 293T cells (hosts for lentivirus production) and validated by PCR and fluorescence microscopy.

Lentiviral transfections into 293T were done with the pMD2.G and pCMV8.74 (Addgene plasmid #12259, Addgene plasmid #22036) packaging plasmids, scaling to 3ug of target plasmid for a 10cm plate in serum-free media (3mL overnight). The following day and 24 hours after transfection, virus-bearing media was syringe filtered and viral particles were precipitated overnight at 4C using Peg-IT viral precipitating media (https://www.systembio.com/products/lentivirus-production/virus-concentration-and-titering/peg-it-virus-precipitation-solution/). Viral titer was aliquotted and pelleted for dry-pellet storage at -80C or use in experiments.

For transduction, pellets were resuspended in the appropriate media for each cell line and placed over 50-60% confluent cells in six-well format. A total of 1mL of virus-bearing media was used per transduction. Transduction was repeated 24 hours later with fresh viral titer. Following infection, cells were placed on blasticidin selection at 5ug/ml for seven days. After selection, cells were maintained on 1ug/ml blasticiticin. Prior to amplification for grafting, cells were sorted for GFP production (IKZF1) or RFP and GFP production (RFP) to maximize purity.

**RNAseq Processing and Analysis**

Total RNA samples were isolated from cell populations frozen in TRIzol after experimentation. All samples were isolated and purified simultaneously using RNA prep kits available through Qiagen. RNA concentration and quality was assessed by Bioanalyzer via the core facilities at Columbia University and submitted to the Genome Center at Columbia University for processing. RNAseq results were retrieved from the Center in accordance to their standard QC and analytic pipeline.

Subsequent analyses for differential expression and Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005) were conducted using standard analytic packages available in R via Bioconductor. In all circumstances an alpha of 0.05 was used for the threshold defining differential expression to better facilitate network-based analysis (Lefebvre et al., 2010). GSEA (Subramanian et al., 2005; Woo et al., 2015) was conducted using this data and the validated IKZF1 regulon provided in (Chen et al., 2015).

**Mouse Model and Methods**

Subcutaneous melanomas were generated by s.c. Injection of 5x10^4 B16F10 tumor cells [obtained from Dr. David Owens at the Columbia University Skin Disease Resource-Based Center (epiCURE)], with or without the forced expression of human IKZF-1, into the right the flank of C57BL/6 or NCr nude mice. For the immunotherapy experiments, animals were randomly assigned to different groups and starting on day 5 received three i.p. injections (three days apart) of either anti PD1 (Rat IgG2a anti-CD279, clone RMP1-14), anti CTLA4 (Mouse IgG2b anti CD152, clone 9D9) monocolonal antibodies or the corresponding isotype controls (Clones 2A3 and MPC-11 respectively), all from Bioxcell. Tumor size was measured daily with a caliper, and their volumes calculated by using the ellipsoid formula (d<sup>2</sup>xD/3)x0.52, where D represents the greatest diameter and d represents the smallest diameter. Mice were sacrificed when tumor exceeded 20 mm in diameter.

**Tumor Cell Isolation**

Subcutaneous B16 melanoma tumors were excised from the animals at the end of the experiments. Tumor infiltrating lymphocytes were prepared in a discontinuous Percoll (GE Healthcare) gradient by collecting the interface between the 40 and 80% layers.

**Flow Cytometry and Antibodies**

The anti-mouse antibodies CD19-FITC (clone eBio1D3), NK1.1-PE (clone PK136), Foxp3-APC (clone FJK-16s) were purchased from eBioscience. Anti-CD11c-PE/DazzleTM 594 (N418), CD45-Brilliant violet 605 (30-F11) CD4-Brilliant Violet 510 (GK1.5), and CD8a-Brilliant Violet 711 (53-6.7) were from BioLegend. Intracellular staining for Foxp3 was performed using the Foxp3-Transcription factor staining buffer set (eBioscience) following the manufacturer’s protocol. Cells were acquired on a Fortessa flow cytometer (BD Biosciences) and data were analyzed using FCS Express 6 flow software (DeNovo software).

**Cytotoxicity Assays**

Whole blood PBMCs were isolated using Histopaque (https://www.sigmaaldrich.com/catalog/product/sigma/10771?lang=en&region=US) precipitation using blood provided by healthy donors via the clinic at Columbia University. Isolation was performed the day before cytotoxicity experiments to ensure viability. Transduced target cell lines were cultured as described above in 10cm plates.

Cytotoxicity was assayed using the Cytotox96 kit available from Promega (https://www.google.com/search?q=cytotox&oq=cytotox&aq=chrome.6957/0i2/6965l2/6961.1103|0j4&sourcied=chrome&ie=UTF-8), as has been done in previous work. The target:PBMC ratio was set to 1:100 and performed in technical quadruplicates in a 96-well plate. Standard manufacturer protocol was followed otherwise.
QUANTIFICATION AND STATISTICAL ANALYSIS

Unless otherwise specified for specific methods (which will be detailed in their respective publications), a statistical threshold of \( p < 0.05 \) corrected for multiple hypothesis testing (False Discovery Rate or Bonferroni correction as indicated) was used. All figures report mean and SEM as the error bars except the box-and-whisker plots which report median, IQR, and 5\(^{th}\)/95\(^{th}\) percentiles. All relevant numbers are disclosed in the corresponding figure legends and sections. Mann-Whitney U-test was used to identify whole shifts in aQTL distribution. All categorical comparisons were done by Fisher’s Exact Test. Statistical significance in survival was determined using KM survival curves and associated statistics. Differences in cytotoxic sensitivity were assayed by T-test. Gene Set Enrichment Analysis was conducted using bootstrapped, shuffled data to generate random distributions (10k iterations), which were used to compute the normalized enrichment score as detailed in previously published literature. For flow quantification and CD4+ CD8+ ratios, the paired T-test was used. For tumor growth, two-way ANOVA with Bonferroni’s post-test was used to determine significant differences between treatment groups.

DATA SOFTWARE AND AVAILABILITY

All RNA-seq data generated specifically in this study are publicly available at the Gene Expression Omnibus under accession number GEO: GSE111201. All TCGA data used are available through their respective data portal. Independent melanoma cohorts used in this study are available as supplemental materials via Sivendran et al. (2014). All algorithms used in this study are publicly available through Bioconductor R packages.