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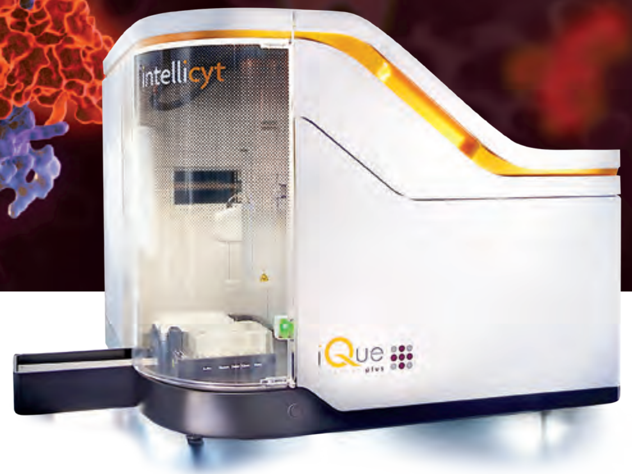

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Reprint Compendium

Precision Medicine, Volume 2:

New Targets for Cancer Immunotherapy

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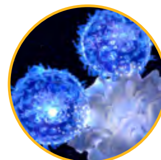
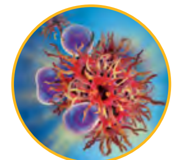
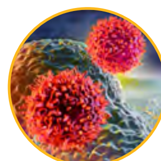


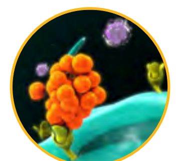
Immunophenotyping



Immune Cell Killing



Immune Cell Proliferation/Activation



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Finding the Next Bullseye

No two cancers are the same, making the disease very difficult to treat with standard therapies that have ruled the clinic for decades. Precision oncology offers great promise for improving patient outcomes because it seeks to understand the plethora of factors that drive an individual's cancer in order to develop a personalized therapy.

Approaches that harness the power of the patient's own immune system to identify and target cancer cells have shown marked promise in the clinic and have revolutionized therapeutic outlook in cancer. Most notably, tremendous research has led to widespread clinical success and adaptation of immune checkpoint blockade therapies, as well as therapies engineering T cells expressing chimeric antigen receptors that recognize tumor cells and allow tumor eradication.

In this edition of *Research Arc*, we highlight advances in potential targets that may expand applicability of cancer immunotherapy for different cancers, improve clinical responses, and surmount mechanisms of resistance. These approaches seek to harness the potential of alternative immune cells and metabolic responses in the tumor microenvironment, as well as consider the order of events in the cancer-immune cycle, among other emerging and critical factors.

Ultimately, the goal of precision medicine is to develop approaches that can target each individual's unique form of cancer. We hope that these articles that explore potential emerging targets and therapeutic avenues will inspire a richer understanding of the role of the immune system in cancer and how it can be harnessed to eventually eradicate the disease. Be sure to visit cell.com/research-arc to find other high-quality research and review content to drive your business forward.

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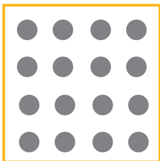

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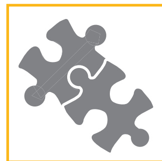

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Precision Medicine, Volume 2:

New Targets for Cancer Immunotherapy

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Cancer-Cell-Intrinsic Mechanisms Shaping the Tumor Immune Landscape — *Max D. Wellenstein and Karin E. de Visser*

RESEARCH arc

Article

Fibrinogen-like Protein 1 Is a Major Immune Inhibitory Ligand of LAG-3

Jun Wang, Miguel F. Sanmamed, Ila Datar, Tina Tianjiao Su, Lan Ji, Jingwei Sun, Ling Chen, Yusheng Chen, Gefeng Zhu, Weiwei Yin, Linghua Zheng, Ting Zhou, Ti Badri, Sheng Yao, Shu Zhu, Agedi Boto, Mario Sznol, Ignacio Melero, Dario A.A. Vignali, Kurt Schalper, and Lieping Chen

On the Cover: A 3D rendering of a dividing cancer cell. The advent of immunotherapy has allowed researchers and clinicians to develop treatments that combat cancer in new and personalized ways, with the hope that this unchecked growth can be thwarted. Image courtesy of Wild Pixel/iStock. Cover design by Kip Lyall.

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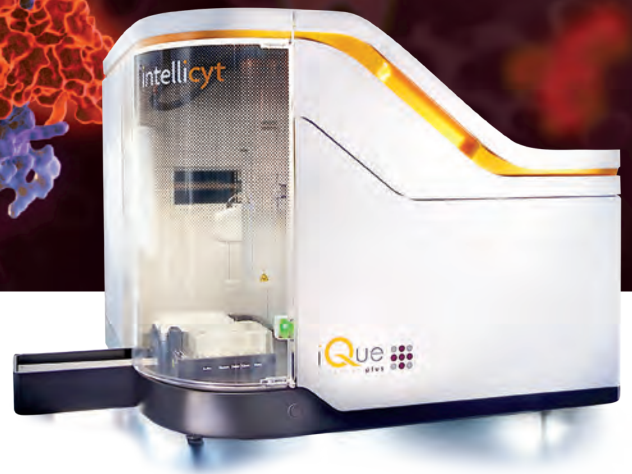

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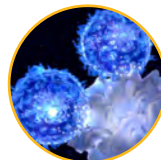

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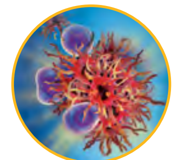

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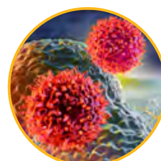

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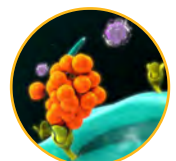

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SnapShot: Cancer Immunotherapy with Oncolytic Viruses

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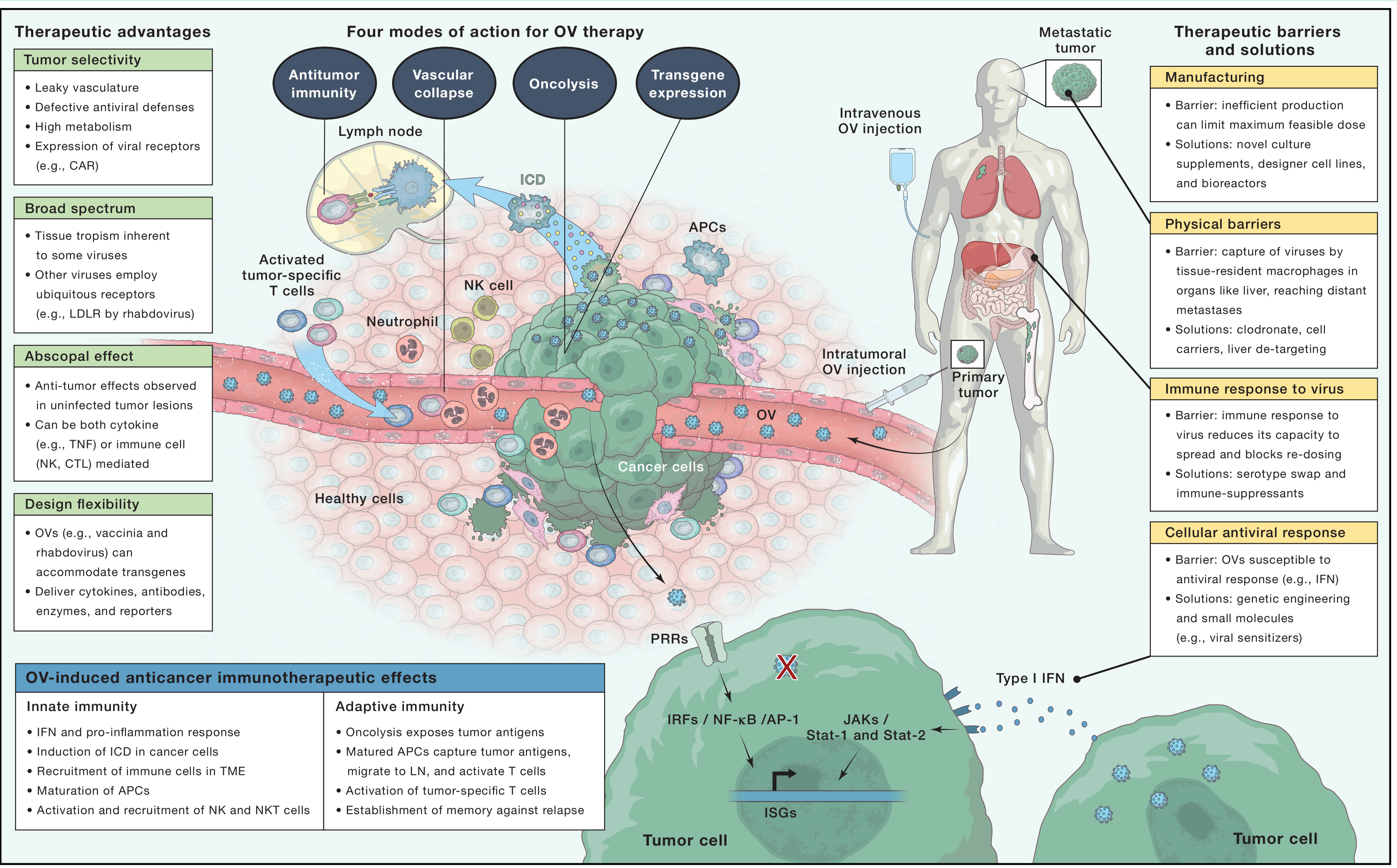
Shashi Gujar,[1] John Bell,[2,3] and Jean-Simon Diallo[2,3]
[1]Departments of Pathology, Biology, and Microbiology & Immunolgy, Faculty of Medicine, Dalhousie University, Halifax, NS, Canada; [2]Departments of Medicine & Biochemistry and Microbiology & Immunology, University of Ottawa, ON, Canada; [3]Cancer Therapeutics Program, Ottawa Hospital Research Institute, Ottawa, ON, Canada



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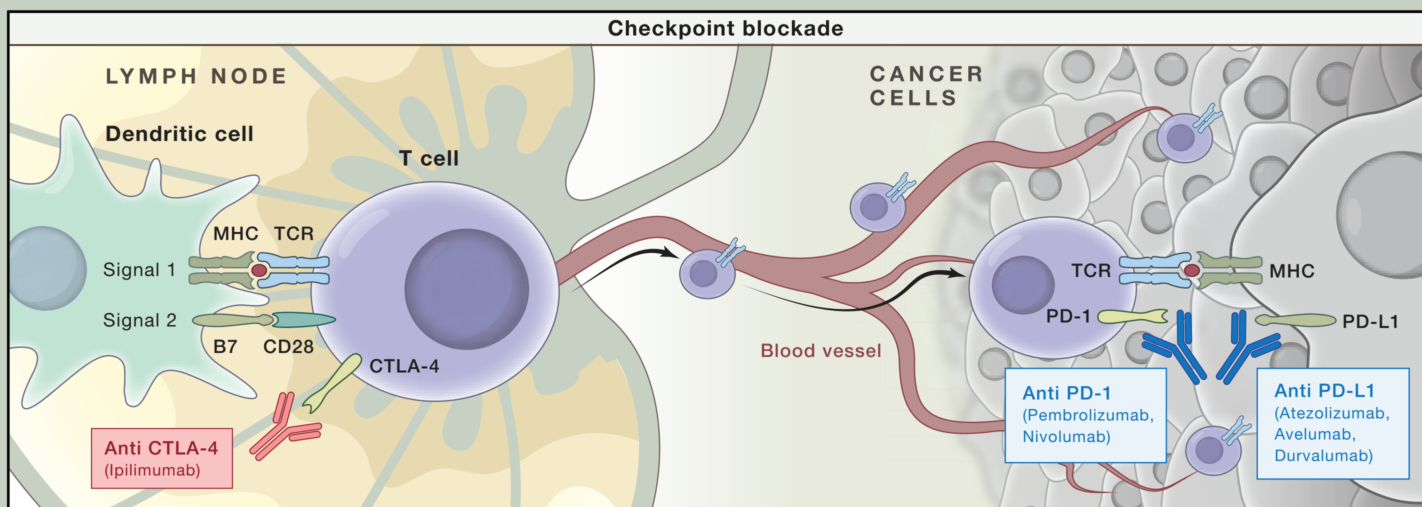
SnapShot: Immune Checkpoint Inhibitors

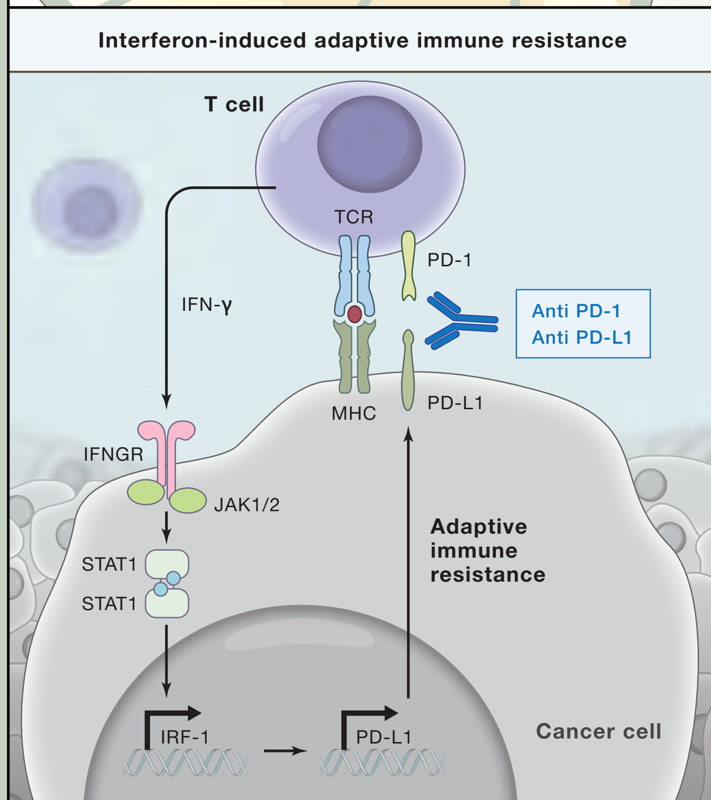
Cancer Cell

Gabriel Abril-Rodriguez[1] and Antoni Ribas[1,2]
[1]Department of Medicine, Division of Hematology-Oncology, University of California, Los Angeles, CA 90095, USA; [2]Jonsson Comprehensive Cancer Center, Los Angeles, CA 90095, USA

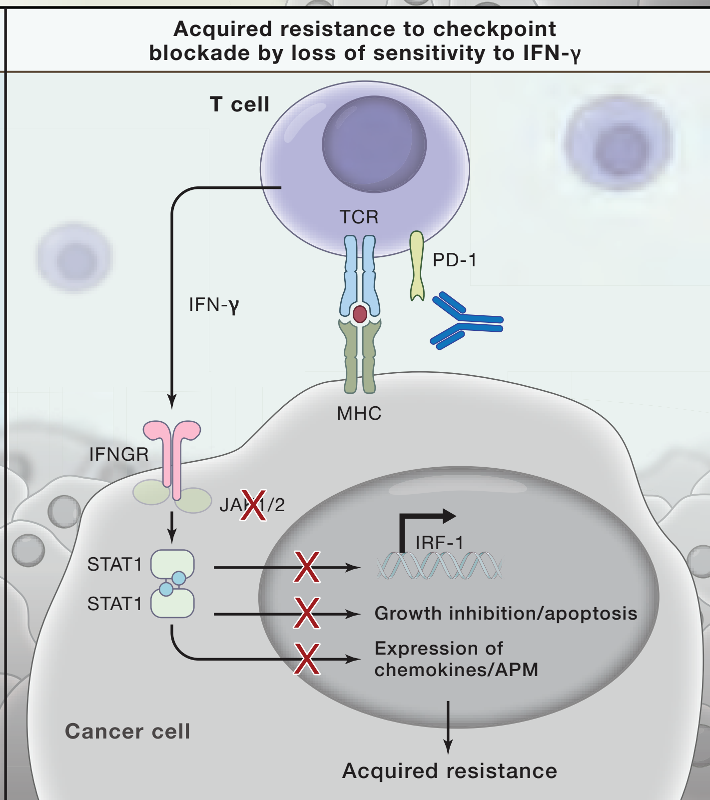
Checkpoint blockade



Interferon-induced adaptive immune resistance



Acquired resistance to checkpoint blockade by loss of sensitivity to IFN-γ



Clinically approved checkpoint inhibitors

Agent	Mechanism of action	Approved for
Ipilimumab (Yervoy)	mAb targeting CTLA-4	Metastatic melanoma
Pembrolizumab (Keytruda)	mAb targeting PD-1	Metastatic melanoma, non-small-cell lung cancer, head and neck squamous cell cancer, classical Hodgkin's lymphoma
Nivolumab (Opdivo)	mAb targeting PD-1	Metastatic melanoma, non-small-cell lung cancer, renal cell carcinoma, Hodgkin's lymphoma, head and neck cancer, urothelial carcinoma
Atezolizumab (Tecentriq)	mAb targeting PD-L1	Non-small-cell lung cancer, bladder cancer
Avelumab (Bavencio)	mAb targeting PD-L1	Urothelial carcinoma, Merkel cell carcinoma
Durvalumab (Imfinzi)	mAb targeting PD-L1	Urothelial carcinoma

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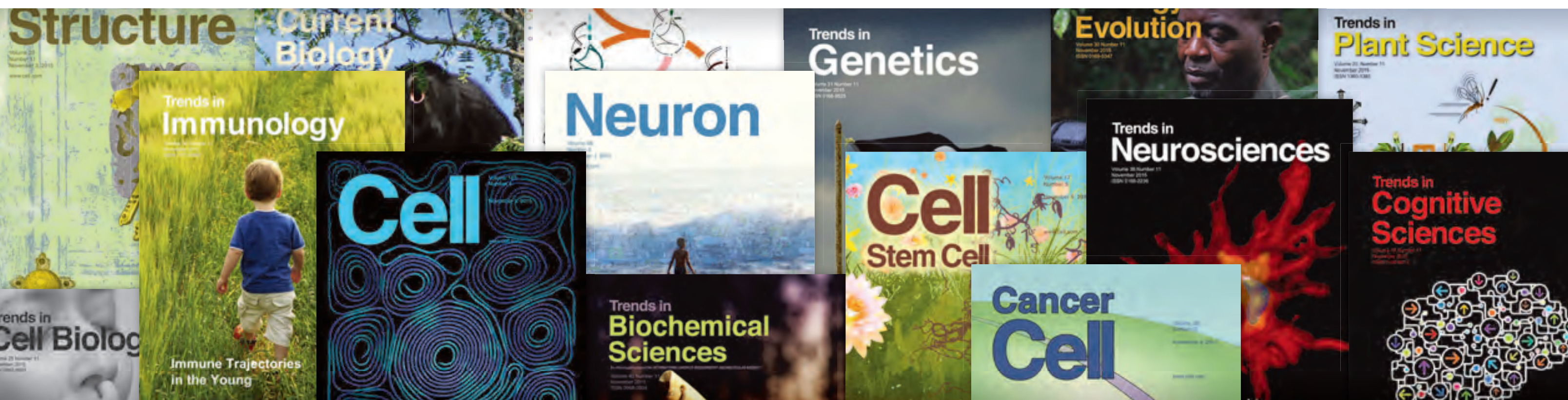

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Opinion

Challenging Standard-of-Care Paradigms in the Precision Oncology Era

Vivek Subbiah[1,*] and Razelle Kurzrock[2]

The pace of genomic and immunological breakthroughs in oncology is accelerating, making it likely that large randomized trials will increasingly become outdated before their completion. Traditional clinical research/practice paradigms must adapt to the reality unveiled by genomics, especially the need for customized drug combinations, rather than one-size-fits-all monotherapy. The *raison-d'être* of precision oncology is to offer 'the right drug for the right patient at the right time', a process enabled by transformative tissue and blood-based genomic technologies. Genomically targeted therapies are most suitable in early disease, when molecular heterogeneity is less pronounced, while immunotherapy is most effective against tumors with unstable genomes. Next-generation cancer research/practice models will need to overcome the tyranny of tradition and emphasize an innovative, precise and personalized patient-centric approach.

Highlights

The central tenet of the precision oncology paradigm requires the delivery of the right drug at the right time to the right patient.

The current model for precision oncology usually matches single agents to patients with late-stage, refractory, molecularly complex disease. This is suboptimal.

Optimizing targeted therapy requires a departure from traditional paradigms: (i) deploying gene-targeted agents early in the disease course when the tumor is less complicated at the genomic level; (ii) administration of immune-targeted therapies to patients with complex cancers harboring high tumor mutational burden; and (iii) moving from monotherapy to customized combinations.

Genomics represents the tip of the iceberg. In the future, panomic testing that includes transcriptomics, proteomics, metabolomics, and immunogenomics will paint a more complete portrait of each tumor.

Clinical Trial Paradigms in the Era of Targeted Therapies and Immunotherapies

> *"Victorious warriors win first and then go to war, while defeated warriors go to war first and then seek to win"* — Sun Tzu, The Art of War

Between 2003 and 2013, new cancer drugs approved by the European Medicines Agency (EMA) or the United States Food and Drug Administration (US FDA) produced a total mean improvement in overall survival of only 3.4 months relative to the treatments that were available in 2003 [1]. Routinely, new medicines that confer an additional survival of mere weeks with statistical *P* value victories are hailed as major breakthroughs in oncology. The randomized controlled trial (RCT), considered the gold standard for cancer clinical trials, has failed to render cures or long-term survival for the majority of patients suffering from advanced malignancies. In diseases such as metastatic pancreatic cancer, >90% of patients are dead at 2 years, despite a multitude of traditional trials [2]. The high costs of conventional trials, the large number of patients receiving futile therapy on control arms, and the lack of **biomarker** (see Glossary) selection hampers progress. In this Opinion, we critically appraise the state of standard-of-care therapies, and present an overview of current clinical trial design paradigms in the era of genomically **targeted therapies** and **immunotherapy**.

Targeted Therapies

Over 100 years ago, Paul Ehrlich introduced the concept of 'magic bullet cures' in oncology [3]. Realization of this idea remained elusive until the last decade, with the advent of drugs such as imatinib targeting the altered Bcr-Abl tyrosine kinase, which is pathognomonic of chronic myelogenous leukemia (CML). CML became a poster-child for **precision oncology**. Before the imatinib era, median survival was ~4 years; today, life expectancy for patients with CML

[1]Department of Investigational Cancer Therapeutics, Division of Cancer Medicine, Unit 0455, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA
[2]Division of Hematology & Oncology, Center for Personalized Therapy & Clinical Trials Office, UC San Diego – Moores Cancer Center, 3855 Health Sciences Drive, MC #0658, La Jolla, CA 92093-0658, USA

*Correspondence: vsubbiah@mdanderson.org (V. Subbiah).

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approaches normal, provided that treatment is started at the time of diagnosis [4]. Delaying treatment until late-stage disease (as is standard in solid tumors) renders even the breakthrough targeted therapies for CML ineffective. Other early examples of precision oncology efforts included the success of trastuzumab in Her2-positive breast cancer, and epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) inhibitors in *EGFR*- and *ALK*-aberrant lung cancers [5–7]; all of which have significantly impacted outcome, albeit not to the extent seen in CML.

In parallel, massive sequencing efforts have mapped the genome. The sequencing costs of a single human genome have dropped in a breathtaking manner, from 3 billion US dollars over a decade ago to about one thousand US dollars today. Hundreds of actionable genes have been discovered and thousands of new drugs with novel mechanisms of action, including gene-targeted agents and immunotherapy, are being identified. Yet, although we have witnessed a few remarkable triumphs by utilizing genomics, other high-throughput omics technologies such as **proteomics, transciptomics,** and **metabolomics** are in nascent stages.

Immunotherapies

Immunotherapy may be the ultimate example of a precision treatment. **Checkpoint inhibitors**, for instance, activate the immune machinery, enabling its innate ability to recognize and destroy tumors [8,9]. The immune system is both personalized and precise. Furthermore, we now realize that the immune apparatus distinguishes malignant cells from their normal counterparts because the cancer cells present neoantigens, which are produced as a result of the mutanome [10]. Additionally, specific genomic alterations, such as *PD-L1* amplification (associated with almost a 90% response rate in refractory Hodgkin's disease treated with anti-PD-1 checkpoint inhibitors) and high tumor mutational burden are greatly predictive or response [9,11–13]. Most striking is the ability of immunotherapy to induce durable complete remissions, even in patients with advanced metastatic cancer. The recent US FDA approval of **pembrolizumab**, an immune checkpoint inhibitor for **microsatellite instability** high (MSI-H) cancers across all solid tumor types (histology-agnostic approval) in pediatric and adult patients is an attestation to the power of precision medicine[i] [14–16]. This approval also demonstrates that genomics and immunotherapy are wedded to each other, and their successes epitomize the power and potential of this marriage.

Conventional Clinical Trial Paradigms

Unfortunately, conventional clinical trial strategies may not be the best way to evaluate the new generation of genomically or immune-targeted agents. Indeed, genomics has unveiled a reality that is incompatible with canonical trial design – every metastatic tumor is both unique and complex at the molecular level [17–20] (Figure 1, Key Figure, Table 1). Furthermore, drugs that are highly effective in small subpopulations of patients are not amenable to randomized trials in unselected patient populations. Under such circumstances, trials must first identify response biomarkers and then individualized combination therapy needs to be given.

The central premise of precision oncology is to offer 'the right drug for the right patient at the right time' Ironically, traditional models for clinical research are almost diametrically opposed to those needed based on the science of precision medicine: (i) in conventional models, commonalities are found between patients in order for them to receive the same drug regimen, instead of individualizing therapy; and (ii) targeted monotherapies are matched to one specific molecular alteration in a patient's tumor, rather than giving combination treatment optimally tailored to the entirety of the tumor genomic portrait. Regarding timing of therapy, genomically targeted agents are often applied to heavily pretreated patients, rather than early in the course

Glossary

Precision medicine: 'an emerging approach for disease treatment and prevention that takes into account individual variability in genes, environment, and lifestyle for each person' (definition of the National Institutes of Health, NIH); 'a form of medicine that uses information about a person's genes, proteins, and environment to prevent, diagnose, and treat disease' (definition of the National Cancer Institute, NCI).
Precision oncology: field in oncology defined by customizing treatment to an individual's molecular profile.
Biomarker: characteristic that is objectively measured or evaluated as an indicator of abnormal biological processes or pharmacological/ biological responses to a therapeutic intervention.
Randomized controlled trial: trial in which two treatment groups (an experimental group versus control group; sometimes given a placebo or a traditional therapy regimen) are compared. The only expected difference between the control and experimental groups in RCTs is the treatment effect of the experimental therapy being studied.
Genomics: study of genes.
Targeted therapy: drugs that either target molecular alterations specific to cancer cells (e.g., mutated, amplified or epigenetically up- and/or downregulated signaling proteins), or target immune cells to increase anticancer immunity.
Immunotherapy: prevention or treatment of disease with agents that stimulate the immune response of the host.
Tumor mutational burden: number of mutations in a tumor.
Vemurafenib and dabrafenib: tyrosine kinase inhibitor of aberrant BRAF.
Trametinib and cobimetinib: MEK inhibitor.
Panomics: informal name for technological fields in biology that end in omics, such as genomics, proteomics, and metabolomics.
Proteomics: study of proteins.
Transcriptomics: study of transcripts.
Metabolomics: study of metabolism.

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Key Figure

The Snowflake Theory and Changing Drug Development Paradigms

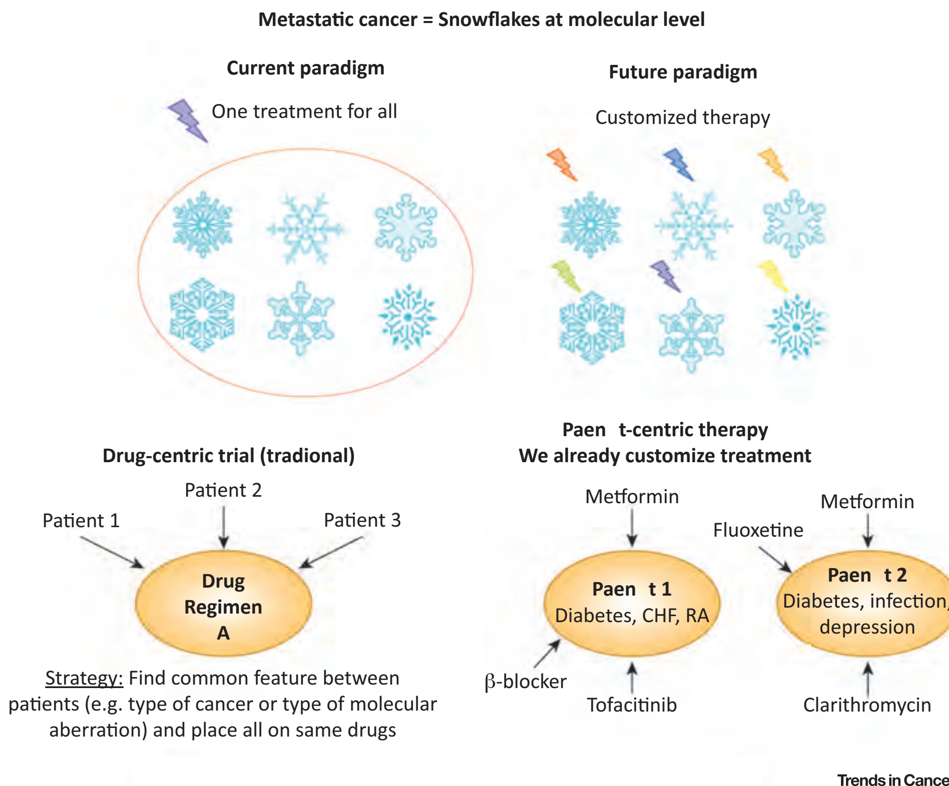

Figure 1. Top panel: cancers are akin to malignant snowflakes. No two snowflakes are identical, and it seems that it is also extremely unusual for two metastatic tumors to have the same genomic fingerprint. As it turns out, if metastatic tumors are akin to malignant snowflakes in their distinctiveness, individual tumors become the ultimate extrapolation of rare and ultra-rare tumors – n-of-one malignancies. Bottom panel: moving from drug-centric to patient-centric trials and care. If each cancer is unique and complex, precisely targeting it requires personalized combination therapy regimens. Bottom panel shows that personalized therapy is already routine in patient care outside the oncology setting. Abbreviations: CHF, congestive heart failure; RA, rheumatoid arthritis.

Drug-centric approach: approach to treatment centered on a drug or drug regimen.
Patient centric approach: approach to treatment centered on the patient.
Checkpoint inhibitor: agent that inhibits an immune checkpoint and hence can reactivate the immune system.
Pembrolizumab: antibody that works as a checkpoint inhibitor.
Microsatellite instability: microsatellites represent repeated sequences of DNA that are one to six base pairs in length. Microsatellite instability is a condition of genetic predisposition to mutation in microsatellites that results from an impaired DNA mismatch repair gene.

of the disease, when tumors are less heterogeneous, and the targeted drugs are more likely to be effective [21,22]. **Tumor mutational burden** and complexity, on the other hand, may be an advantage for immunotherapy. Importantly, standard-of-care therapies deny and/or delay evaluation of new drugs in patients with lethal cancers by making the tumors more drug resistant, impairing the immune system, and/or rendering the patients too sick to be eligible for innovative treatment.

In order to unlock the potential of precision oncology, profound changes in our traditional approaches need to occur. These changes start with universal genomic testing at the time of diagnosis of cancer [23] (Table 2) and include customizing drug combinations, with genomically targeted treatments given early in a patient's disease course, and immunotherapy using

Table 1. Redefining Clinical Trial Paradigms and Standard of Care

Subject matter	Solution	Challenge
The definition of personalized treatment is inconsistent with canonical trial/practice paradigms, where patients are grouped together based on a biologic commonality.	A patient-centered, n-of-one approach is needed to optimize therapy.	Current treatment paradigms, including precision oncology trials, are drug centered rather than patient centered.
Monotherapy is unlikely to cure patients with advanced/complex malignancies	Combination therapies needed	Matched customized combinations for n-of-one tumors require evaluation of the strategy of personalization or an algorithm for matching, rather than the drug regimens themselves
The inimitability of tumors means that each cancer is akin to a malignant snowflake – both unique and complex in its genomic portrait	Unique/complex tumors require individualized combination regimens	With 300 drugs, there are ~4.5 million three-drug regimens
Dosing of combinations of anticancer drugs has traditionally required a phase I study	Outside of oncology, patients regularly receive *de novo* combinations of drugs based on understanding impact on metabolic enzymes etc. The average oncology patient is already on eight medications, which have not been assessed together in a phase I study, but are given safely together. Dosing algorithms for anticancer drug combinations can be similarly derived from a variety of sources including the literature [57–60].	The pathway to approval and payer acceptance of drug combinations is unclear
If tumors are defined by their molecular makeup, advanced molecular tests should be considered a standard diagnostic tool for patients with cancer	Universal genomic testing of cancers	Points and counterpoints in Table 2

checkpoint inhibitors administered to patients with evolved cancers harboring high mutational burdens or microsatellite instability.

Standard of Care, Standard of Proof, and Proof of Standards

Evidenced-based, standard-of-care guidelines/pathways are promulgated by a variety of organizations and emphasize consistency[ii] [24,25]. Departure from these guidelines may leave the physician legally liable and justify insurers' refusal to pay. Yet, the standard-of-care oncology treatments are associated with >90% mortality at 2 years for some metastatic cancers.

Importantly, in their present rendition, standard-of-care pathways, by virtue of their emphasis on uniformity of management, are antithetical to precision oncology, which requires personalization of therapy. Indeed, if each patient's tumor is complex and unique, then, in order to precisely target that tumor, one must apply medicines that affect the distinct alterations of the tumor, and this requires customized treatment.

Moving Precision Oncology Forward

Precision oncology trials test feasibility of matching drugs to targeted therapy [26–29]. The evidence for this matching strategy is rapidly accumulating, both from these trials and from literature data mining [30,31]. Indeed, large meta-analyses of ~85 000 participants in phase 1, 2, and 3 studies demonstrated that biomarker selection was the single most significant independent factor predicting improvement in all outcome parameters. Of equal importance, the use of genomically targeted therapy without a biomarker produced negligible response rates, which were also worse than the results with cytotoxic agents [32–35].

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Table 2. Case for Universal Genomic Testing of Tumors: Points and Counterpoints

Points	Counterpoints	Refs
Obtaining knowledge of genetic aberrations is not worthwhile if no action can be taken in terms of treatment	Genomics is the diagnosis. Every patient with cancer deserves a diagnosis. Genetic abnormalities also predict prognosis. Genomics can also predict contraindicated drugs, e.g., EGFR therapy in *KRAS*-mutant colorectal cancer	[23]
Prohibitive cost precludes universal genomic testing	Cost of testing has decreased precipitously Financial burden of cancer therapy is massive Cost of testing for a complete diagnosis and to select appropriate therapy is tiny compared with the money squandered on ill-chosen treatments	
Genomic testing has not been validated in prospective trials	In comprehensive meta-analyses of ∼85 000 patients treated on clinical trials, genomic biomarkers were an independent factor associated with improvement of all outcome variables	[33–35]
Genomic testing may benefit only a subgroup of patients or may be germane to only rare diseases	Virtually impossible to know in advance of testing who will benefit Options that may not exist at the time of a patient's initial diagnosis may become available before the patient's disease progresses Universal genomic testing of malignancies will enable curating clinically relevant data in large databases	

The Right Drug at the Right Time for the Right Patient

The Right Drug

The discovery of *BRAF*^{V600E} mutations as a *bona fide* oncogenic driver in 50% of melanomas led to a drug development race in order to target the product of this gene. Treatment with the potent BRAF inhibitor **vemurafenib** showed high response rates leading to FDA approval in 2011 [36,37]. Since then, the BRAF inhibitor **dabrafenib** and two MEK inhibitors (**trametinib** and **cobimetinib**) have also been approved [38–40]. Yet, most patients fail to achieve complete or long-term partial remission. This is likely due to the fact that the majority of metastatic melanomas harbor several genomic alterations [41]. Hence, patients require combination therapy tailored to the biomarker portfolio of their tumor. Indeed, a recent study demonstrates that higher matching scores (number of matches divided by number of alterations) independently correlates with better outcomes [26].

The Right Time

Timing is vital in cancer therapy. Tumor complexity increases with time and under the pressure of therapy. CML epitomizes this evolution with three well-defined stages: chronic phase, accelerated phase, and blast crisis. Other cancers almost certainly undergo a similar evolution, but it is not as well delineated clinically [42]. In recent years, the clinical outcome of CML has been transformed. Three major steps enabled this transformation: (i) discovery of the underlying genetic defect (*BCR-ABL*); (ii) identification of a targeted agent (imatinib) that obviated the aberrant enzymatic activity of Bcr-Abl; and (iii) administration of imatinib to patients with newly diagnosed disease. The third step, that is treating early disease, is the one that is most frequently not addressed in solid tumors.

As an example, BRAF inhibitors in patients with *BRAF*-mutant melanoma can result in responses so remarkable that they have been designated as the oncological equivalent of the Lazarus syndrome [43]. This syndrome refers to the spontaneous return of circulation after failed attempts at resuscitation. Patients near death from melanoma can experience

dramatic tumor reduction. Unfortunately, these patients are not usually cured, and the disease almost inevitably returns after a few months and results in the patient's demise. If the experience with CML holds true, durable responses in solid tumors will require either administration of targeted agents such as BRAF inhibitors to newly diagnosed disease and/or giving customized combinations of drugs to patients with advanced disease in order to block resistance pathways.

The Right Patient (and the Right Cancer)

Most novel drugs are tested in patients who have exhausted standard-of-care therapies. At this time, not only is the cancer refractory, but the patient's performance status and biological/immune reserve may also be too poor to realistically expect the best outcomes. For these reasons, patients should be treated with novel therapies earlier in their disease course.

Advanced Cancers Are Akin to Malignant Snowflakes – Complex and Unique

No two snowflakes are identical, and it seems that it is also extremely unusual for two metastatic tumors to have the same genomic fingerprint [17–20,44] (Figure 1). For example, in 57 patients with advanced breast cancer, 216 somatic aberrations were observed (131 being distinct) in 70 different genes; no two patients had the same molecular signature [17]. A study in advanced osteosarcoma with multiple molecular profiling technologies showed similar results [20]. Furthermore, we may be viewing only the tip of the iceberg. As new technologies emerge beyond limited panel genomic sequencing, both the complexity and the individuality of tumors are likely to be amplified (Figure 2).

Customized Combination Therapy: From Drug-Centric to Patient-Centric Research and Care

One of the major stumbling blocks in precision oncology is that there are intrinsic and acquired resistance mechanisms to targeted therapy. One drug matched to a driver aberration may not realistically be expected to cure patients or achieve remissions if each tumor has distinct and complex alterations [31,41]. Other drugs must be added to overcome resistance [31,45,46]

A paradigm of individualized therapy means that the traditional way that drugs/drug regimens become standard of care no longer works. Canonical drug development paradigms are drug-centered (Figure 1). The drugs are the focus of the trial and each patient enrolled receives the same regimen, regardless of their genomic and phenotypic heterogeneity. However, if each tumor is different, we may need to test thousands of regimens in increasingly small subsets of patients. Indeed, if there are ~300 drugs in oncology, there are ~45 000 two-drug regimens and ~4.5 million three-drug regimens. The traditional clinical trial design model breaks down. However, the conundrum is solvable. Precision medicine implies patient-centered trials and care. The patient is the focus and the drugs can therefore vary from patient to patient. In this model, it is not the drug regimen that is evaluated, but rather the strategy of individualization. The question then becomes what is the standard of proof for this strategy? In the era of precision oncology, new clinical trial designs need to evaluate personalized care performance so that standard-of-care guidelines can include, emphasize, or even mandate individualized treatment.

The One-Size-Fits All Treatment Model in Oncology Is an Anomaly

In daily medical practice, physicians already use customized combinations to treat nonmalignant conditions. A patient with diabetes, congestive heart failure, and rheumatoid arthritis

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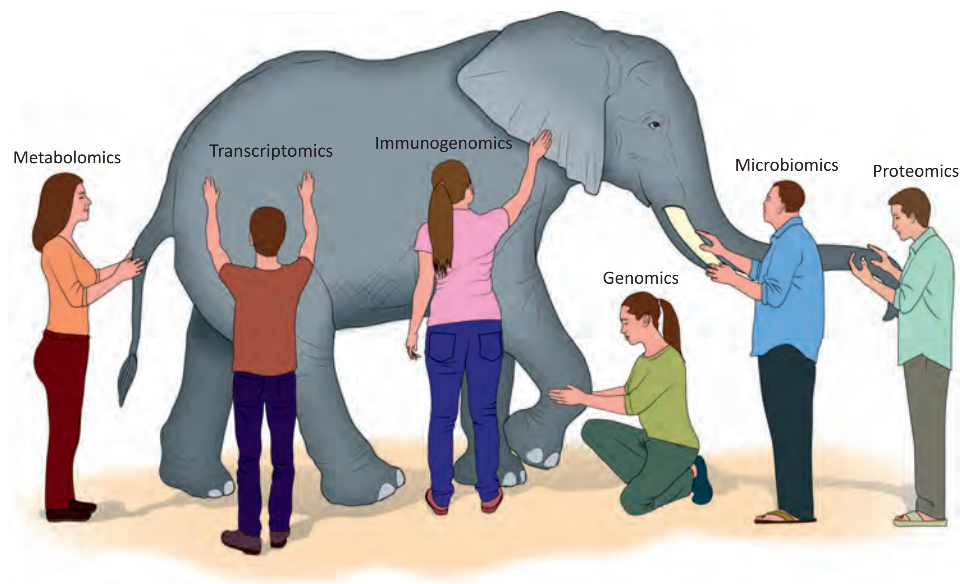

Figure 2. Six Blind Men and Elephants

Beyond genomics – transcriptomics, proteomics, and more. The comprehensive molecular profile of the not-too-distant future may include genomics, transcriptomics, proteomics, metabolomics, microbiomics, epigenomics, mutanomics, lipidomics, and immunogenotyping, and may hence predict response to multiple modalities including immunotherapy and chemotherapy [47–56]. Each of these modalities gives us a piece of the puzzle, akin to the parable of the six blind men who each touch a different part of the elephant, such as the tusk versus the trunk, and therefore have vastly different views of the elephant. Panomics testing is a requisite of comprehensive analysis and may require complex computer algorithms for data integration and computation.

receives a different set of drugs than a patient with diabetes, infection, and depression (Figure 1). The drug doses are adjusted to prevent drug–drug interactions based on known factors such as impact on metabolic enzymes. The average patient enters the oncology clinic on approximately eight drugs tailored to their specific health problems. These individualized drug combinations have never been formally tested in phase I studies; yet physicians safely and effectively administer them on a regular basis to the benefit of their patients.

In oncology, however, there is a cultural precept that, if a new drug combination has not been tested in phase I studies, it should not be used because its safety is unknown. This precept may be a legacy of the cytotoxic era, since combining cytotoxics could have serious safety concerns. However, modern anticancer agents have fewer prohibitive adverse effects and our understanding of drug combinations has grown. One size fits all is not the norm in medicine, and, since advanced cancers are heterogeneous, it should cease to be the norm in oncology care.

Immunotherapy: Yet Another Paradigm Shift

One of the most important mechanisms by which cancer cells evade the immune system is exploitation of checkpoints by the tumor to disable T cells. The PD-1/PD-L1 axis is of particular interest because of rapidly emerging data suggesting that inhibition of this checkpoint can restore anticancer immunity. Impressively, clinical responses with checkpoint inhibitors have been observed in multiple different malignancies. Remarkably, some patients with advanced tumors can achieve durable complete remission.

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Marriage of Genomics and Immunotherapy

The major predictive markers for checkpoint inhibitor response include high tumor mutational burden, either associated with microsatellite instability or not, CD8 infiltrates, and PD-L1 over-expression or amplification [9,11,12]. These markers reflect the coupling of the immune system and genomics. Once the immune system is reactivated with the use of checkpoint inhibitors, T cells must still be able to differentiate tumor cells from normal elements. T cells distinguish tumor cells from normal self in large part through presentation of neoantigens created by the mutanome. The more neoantigens, the better the chance of immune recognition. Hence, high tumor mutational burden correlates with favorable outcome after checkpoint inhibitor treatment [13]. In contrast, patients with lower number of genomic alterations appear to respond better to gene-targeted therapy [26], presumably because, in malignancies with more genomic alterations, the presence of resistance mutations abrogate the effects of treatment.

Concluding Remarks

Breathtaking advances in our understanding of genomics and the immune system have brought us to the threshold of a tipping point in cancer treatment. It appears, however, that our established models for clinical research and practice are a suboptimal fit for the reality of tumor heterogeneity (see Outstanding Questions). In order to overcome the cancer problem, it is important to break free from the tyranny of tradition, and construct novel paradigms for the management of neoplastic disease.

Outstanding Questions

Genomic sequencing is a basic diagnostic tool that delineates the underpinnings of malignancy and is therefore crucial for classifying disease, predicting prognosis, and directing therapy. If a basic precept of medicine is that each patient deserves an accurate diagnosis, should not universal genomic testing of tumors be necessary?

What adjustments to clinical trial design and regulatory and care structures are needed to move from a drug-centric approach to a patient-centric approach, wherein each tumor is prosecuted with a customized combination of drugs?

Would finding patients with identical or near-identical tumors treated in the same manner still be feasible with a new form of interrogation based on mining of large, well-annotated databases using computerized and artificial intelligence algorithms?

What is the optimal approach to identifying immunogenic, mutanome-derived neoantigens that induce a T-cell response?

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Resources

[i]https://www.fda.gov/newsevents/newsroom/pressannouncements/ucm560167.htm
FDA (2017) FDA approves first cancer treatment for any solid tumor with a specific genetic feature
[ii]https://www.nccn.org/professionals/physician_gls/f_guidelines.asp
NCCN (2016) NCCN Guidelines

References

1. Salas-Vega, S. *et al.* (2017) Assessment of overall survival, quality of life, and safety benefits associated with new cancer medicines. *JAMA Oncol.* 3, 382–390
2. Stewart, D.J. and Kurzrock, R. (2013) Fool's gold, lost treasures, and the randomized clinical trial. *BMC Cancer* 13, 193
3. Strebhardt, K. and Ullrich, A. (2008) Paul Ehrlich's magic bullet concept: 100 years of progress. *Nat. Rev. Cancer* 8, 473–480
4. Gambacorti-Passerini, C. *et al.* (2011) Multicenter independent assessment of outcomes in chronic myeloid leukemia patients treated with imatinib. *J. Natl. Cancer Inst.* 103, 553–561
5. Piccart-Gebhart, M.J. *et al.* (2005) Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. *N. Engl. J. Med.* 353, 1659–1672
6. Ciardiello, F. and Tortora, G. (2008) EGFR antagonists in cancer treatment. *N. Engl. J. Med.* 358, 1160–1174
7. Shaw, A.T. *et al.* (2013) Crizotinib versus chemotherapy in advanced ALK-positive lung cancer. *N. Engl. J. Med.* 368, 2385–2394
8. Farkona, S. *et al.* (2016) Cancer immunotherapy: the beginning of the end of cancer? *BMC Med.* 14, 73
9. Goodman, A. *et al.* (2017) PD-1-PD-L1 immune-checkpoint blockade in B-cell lymphomas. *Nat. Rev. Clin. Oncol.* 14, 203–220
10. Champiat, S. *et al.* (2014) Exomics and immunogenics: bridging mutational load and immune checkpoints efficacy. *Oncoimmunology* 3, e27817
11. Khagi, Y. *et al.* (2017) Next generation predictive biomarkers for immune checkpoint inhibition. *Cancer Metastasis Rev.* 36, 179–190
12. Patel, S.P. and Kurzrock, R. (2015) PD-L1 expression as a predictive biomarker in cancer immunotherapy. *Mol. Cancer Ther.* 14, 847–856
13. Rizvi, N.A. *et al.* (2015) Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science* 348, 124–128
14. Le, D.T. *et al.* (2017) Mismatch-repair deficiency predicts response of solid tumors to PD-1 blockade. *Science* 357, 409–413
15. Lemery, S. *et al.* (2017) First FDA approval agnostic of cancer site – when a biomarker defines the indication. *N. Engl. J. Med.* 377, 1409–1412
16. (2017) First tissue-agnostic drug approval issued. *Cancer Discov.* 7, 656
17. Wheler, J.J. *et al.* (2014) Unique molecular signatures as a hallmark of patients with metastatic breast cancer: implications for current treatment paradigms. *Oncotarget* 5, 2349–2354

18. Kurzrock, R. and Giles, F.J. (2015) Precision oncology for patients with advanced cancer: the challenges of malignant snowflakes. *Cell Cycle* 14, 2219–2221
19. Wheler, J. *et al.* (2014) Unique molecular landscapes in cancer: implications for individualized, curated drug combinations. *Cancer Res.* 74, 7181–7184
20. Egas-Bejar, D. *et al.* (2014) Theranostic profiling for actionable aberrations in advanced high risk osteosarcoma with aggressive biology reveals high molecular diversity: the human fingerprint hypothesis. *Oncoscience* 1, 167–179
21. Westin, J.R. and Kurzrock, R. (2012) It's about time: lessons for solid tumors from chronic myelogenous leukemia therapy. *Mol. Cancer Ther.* 11, 2549–2555
22. Westin, J.R. *et al.* (2012) Treatment of chronic myelogenous leukemia as a paradigm for solid tumors: how targeted agents in newly diagnosed disease transformed outcomes. *Am. Soc. Clin. Oncol. Educ. Book* 179–185
23. Subbiah, V. and Kurzrock, R. (2016) Universal genomic testing needed to win the war against cancer: genomics IS the diagnosis. *JAMA Oncol.* 2, 719–720
24. Moffett, P. and Moore, G. (2011) The standard of care: legal history and definitions: the bad and good news. *West J. Emerg. Med.* 12, 109–112
25. Markman, M. (2007) Standard of care versus standards of care in oncology: a not so subtle distinction. *J. Oncol. Pract.* 3, 291
26. Wheler, J.J. *et al.* (2016) Cancer therapy directed by comprehensive genomic profiling: a single center study. *Cancer Res.* 76, 3690–3701
27. Tsimberidou, A.M. *et al.* (2012) Personalized medicine in a phase I clinical trials program: the MD Anderson Cancer Center initiative. *Clin. Cancer Res.* 18, 6373–6383
28. Von Hoff, D.D. *et al.* (2010) Pilot study using molecular profiling of patients' tumors to find potential targets and select treatments for their refractory cancers. *J. Clin. Oncol.* 28, 4877–4883
29. Roychowdhury, S. *et al.* (2011) Personalized oncology through integrative high-throughput sequencing: a pilot study. *Sci. Transl. Med.* 3, 111ra121
30. Diamond, E.L. *et al.* (2017) Vemurafenib for BRAF V600-mutant Erdheim-Chester disease and Langerhans cell histiocytosis: analysis of data from the histology-independent, phase 2, open-label VE-BASKET study. *JAMA Oncol.* Published online November 29, 2017 https://doi.org/10.1001/jamaoncol.2017.5029
31. Hyman, D.M. *et al.* (2015) Vemurafenib in multiple nonmelanoma cancers with BRAF V600 mutations. *N. Engl. J. Med.* 373, 726–736
32. Schwaederle, M. *et al.* (2016) Precision oncology: The UC San Diego Moores Cancer Center PREDICT experience. *Mol. Cancer Ther.* 15, 743–752
33. Schwaederle, M. *et al.* (2015) Impact of precision medicine in diverse cancers: a meta-analysis of phase II clinical trials. *J. Clin. Oncol.* 33, 3817–3825
34. Schwaederle, M. *et al.* (2016) Association of biomarker-based treatment strategies with response rates and progression-free survival in refractory malignant neoplasms: a meta-analysis. *JAMA Oncol.* 2, 1452–1459
35. Jardim, D.L. *et al.* (2015) Impact of a biomarker-based strategy on oncology drug development: a meta-analysis of clinical trials leading to FDA approval. *J. Natl. Cancer Inst.* 107, djv253
36. Bollag, G. *et al.* (2010) Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. *Nature* 467, 596–599
37. Flaherty, K.T. *et al.* (2010) Inhibition of mutated, activated BRAF in metastatic melanoma. *N. Engl. J. Med.* 363, 809–819
38. Falchook, G.S. *et al.* (2012) Dabrafenib in patients with melanoma, untreated brain metastases, and other solid tumours: a phase 1 dose-escalation trial. *Lancet* 379, 1893–1901
39. Falchook, G.S. *et al.* (2012) Activity of the oral MEK inhibitor trametinib in patients with advanced melanoma: a phase 1 dose-escalation trial. *Lancet Oncol.* 13, 782–789
40. Ascierto, P.A. *et al.* (2016) Cobimetinib combined with vemurafenib in advanced BRAF(V600)-mutant melanoma (coBRIM): updated efficacy results from a randomised, double-blind, phase 3 trial. *Lancet Oncol.* 17, 1248–1260
41. Sen, S. *et al.* (2017) Co-occurring genomic alterations and association with progression-free survival in BRAFV600-mutated nonmelanoma tumors. *J. Natl. Cancer Inst.* 109, djx094
42. Nowell, P.C. (1976) The clonal evolution of tumor cell populations. *Science* 194, 23–28
43. Furmark, L. and Pavlick, A.C. (2015) BRAF inhibitors and the "Lazarus syndrome" – an update and perspective. *Am. J. Hemat. Oncol.* 11, 24–29
44. Groisberg, R. and Subbiah, V. (2017) The big, the bad, and the exon 11: adjuvant imatinib for all gastro-intestinal stromal tumors or just the ugly? *Transl. Gastroenterol. Hepatol.* 2, 81
45. Hong, D.S. *et al.* (2016) Phase IB study of vemurafenib in combination with irinotecan and cetuximab in patients with metastatic colorectal cancer with BRAFV600E mutation. *Cancer Discov.* 6, 1352–1365
46. Subbiah, V. *et al.* (2018) Dabrafenib and trametinib treatment in patients with locally advanced or metastatic BRAF V600-mutant anaplastic thyroid cancer. *J. Clin. Oncol.* 36, 7–13
47. Desai, N. *et al.* (2009) SPARC expression correlates with tumor response to albumin-bound paclitaxel in head and neck cancer patients. *Transl. Oncol.* 2, 59–64
48. Al-Ahmadie, H. *et al.* (2014) Synthetic lethality in ATM-deficient RAD50-mutant tumors underlies outlier response to cancer therapy. *Cancer Discov.* 4, 1014–1021
49. Komiya, K. *et al.* (2016) SPARC is a possible predictive marker for albumin-bound paclitaxel in non-small-cell lung cancer. *Onco. Targets Ther.* 9, 6663–6668
50. Mohni, K.N. *et al.* (2015) A synthetic lethal screen identifies DNA repair pathways that sensitize cancer cells to combined ATR inhibition and cisplatin treatments. *PLoS One* 10, e0125482
51. Subbiah, V. *et al.* (2012) Targeting the apoptotic pathway in chondrosarcoma using recombinant human Apo2L/TRAIL (dulanermin), a dual proapoptotic receptor (DR4/DR5) agonist. *Mol. Cancer Ther.* 11, 2541–2546
52. Subbiah, V. *et al.* (2011) Targeted morphoproteomic profiling of Ewing's sarcoma treated with insulin-like growth factor 1 receptor (IGF1R) inhibitors: response/resistance signatures. *PLoS One* 6, e18424
53. Subbiah, V. *et al.* (2015) STUMP un "stumped": anti-tumor response to anaplastic lymphoma kinase (ALK) inhibitor based targeted therapy in uterine inflammatory myofibroblastic tumor with myxoid features harboring DCTN1-ALK fusion. *J. Hematol. Oncol.* 8, 66
54. Jardim, D.L. *et al.* (2013) Comprehensive characterization of malignant phyllodes tumor by whole genomic and proteomic analysis: biological implications for targeted therapy opportunities. *Orphanet J. Rare Dis.* 8, 112
55. Ding, K. *et al.* (2015) Leveraging a multi-omics strategy for prioritizing personalized candidate mutation-driver genes: a proof-of-concept study. *Sci. Rep.* 5, 17564
56. Roychowdhury, S. and Chinnaiyan, A.M. (2016) Translating cancer genomes and transcriptomes for precision oncology. *CA Cancer J. Clin.* 66, 75–88
57. Nikanjam, M. *et al.* (2016) Dosing targeted and cytotoxic two-drug combinations: lessons learned from analysis of 24,326 patients reported 2010 through 2013. *Int. J. Cancer* 139, 2135–2141
58. Liu, S. *et al.* (2016) Dosing *de novo* combinations of two targeted drugs: towards a customized precision medicine approach to advanced cancers. *Oncotarget* 7, 11310–11320
59. Nikanjam, M. *et al.* (2017) Dosing immunotherapy combinations: analysis of 3,526 patients for toxicity and response patterns. *Oncoimmunology* 6, e1338997
60. Nikanjam, M. *et al.* (2017) Dosing three-drug combinations that include targeted anti-cancer agents: analysis of 37,763 patients. *Oncologist* 22, 576–584

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Feature Review

Precision Oncology: The Road Ahead

Daniela Senft,[1] Mark D.M. Leiserson,[2,6] Eytan Ruppin,[3,4] and Ze'ev A. Ronai[1,5,*]

Current efforts in precision oncology largely focus on the benefit of genomics-guided therapy. Yet, advances in sequencing techniques provide an unprecedented view of the complex genetic and nongenetic heterogeneity within individual tumors. Herein, we outline the benefits of integrating genomic and transcriptomic analyses for advanced precision oncology. We summarize relevant computational approaches to detect novel drivers and genetic vulnerabilities, suitable for therapeutic exploration. Clinically relevant platforms to functionally test predicted drugs/drug combinations for individual patients are reviewed. Finally, we highlight the technological advances in single cell analysis of tumor specimens. These may ultimately lead to the development of next-generation cancer drugs, capable of tackling the hurdles imposed by genetic and phenotypic heterogeneity on current anticancer therapies.

Trends

Genomics-driven cancer therapy benefits a subset of patients, although there are clear shortcomings to this approach.

Using genomics as a single 'biomarker' to inform therapy is insufficient to comprehensively predict efficient therapeutic approaches. By providing information about active pathways, the inclusion of transcriptomic data reveals a more comprehensive and, thus, accurate molecular profile, which likely improves the choice of therapy.

Available patient-derived functional models (e.g., organoids or patient-derived xenografts) are promising for testing multiple drugs and/or drug combinations in a clinically relevant time-frame.

Mining available data sets can allow researchers to comprehensively map the processes that drive cancer and reveal novel vulnerabilities.

Intratumor heterogeneity remains one of the biggest challenges in reaching sustained therapeutic responses to cancer treatment. Integrating additional factors (immune, metabolome, and microbiome) could pinpoint novel putative therapeutic approaches and combinational drug therapies, in an effort to overcome tumor heterogeneity.

Precision Medicine Aims to Address Inter- and Intratumor Heterogeneity

Precision medicine aims to use multiple types of data to classify patients into groups that will most likely respond to a given treatment. The identification of **biomarkers** (see Glossary) that correlate with response to therapy or function in disease initiation and/or progression (therefore representing therapeutic targets themselves) is fundamental in this process [1]. Determination of molecular biomarkers is not limited to a specific methodology, and DNA, RNA, proteins, metabolites, or microorganisms can individually, or in combination, serve as biomarkers. With cancer primarily being a genetic disease, precision oncology has largely focused on the determination of genetic biomarkers and multiple clinical trials test whether targeting these genetic alterations in cancer can prolong survival. Remarkable success in applying genomics-driven cancer therapy has been noted [2], yet, serious criticism remains regarding this genomics-focused precision oncology concept, including scientific, social, ethical, and economical aspects [2–5]. In this review, we focus on the biological rationale for precision oncology and outline current efforts and achievements of implementing precision oncology in the clinic, while highlighting promising routes to overcome the limitations of genomic-focused approaches. The current availability of screening platforms and the armamentarium of anticancer drugs now allows us to recognize and address **intertumor heterogeneity** (i.e., the different molecular characteristics observed between patients). We outline how the simultaneous assessment of genomic and transcriptomic data, combined with functional testing, can serve to overcome hurdles imposed by intertumor heterogeneity. In addition, we discuss the major limitations of prolonged response to current anticancer therapies, including **intratumor heterogeneity (ITH)**; namely, differences in the molecular make-up of tumor cells within individual patients. We have only begun to decipher and address such challenges therapeutically.

The Technical and Molecular Basis for Precision Oncology

The ability to detect mutations in a tumor sample was one of the first milestones in recognizing the genetic events that underlie the cellular transformation process, denoting an early phase of

[1]Tumor Initiation and Maintenance Program, NCI designated Cancer Center, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA 92037, USA
[2]Microsoft Research New England, Cambridge, MA 02142, USA
[3]School of Computer Sciences and

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genetic-based evidence for cancer occurrence and development. Improved technologies enabling the detection of such mutations in non-neoplastic tissues (including bodily fluids) has allowed the early detection of somatic oncogenic mutations, such as Ras mutations and hotspot p53 tumor suppressor mutations [6–8]. While these developments reflect advances made already during the 1980s, it has taken another generation to better establish the importance of mutation frequency, its variability in the transformed tissue, and its causative role. This growing understanding has been a prerequisite for the introduction of mechanism-based therapies into clinical practice. Commonly known as **targeted therapies**, these therapeutic approaches are based on small molecules or monoclonal antibodies that inhibit **oncogenic drivers** [9–14], or target genetic vulnerabilities [e.g., poly (ADP-ribose) polymerase (PARP) inhibitors in tumors with **homologous recombination deficiency** [15]]. Several years of clinical experience with targeted agents, and especially of the resistance to drugs, has led to the recognition of the central role of genetic heterogeneity and plasticity of growth-promoting signaling pathways in determining a patient's individual response. A notable example is the targeting of BRAF mutations, which are present in more than 40% of melanomas [16]. Although targeting recurrent BRAF mutation(s) by mutant-specific BRAF inhibitors demonstrated great clinical success [9,17], understanding the complex feedback and crosstalk between key players of the altered RAS/RAF/MEK/ERK signaling axis became necessary for optimizing therapy. Accordingly, in terms of clinical outcomes, combined BRAF and MEK inhibition proved superior over single-agent use [18]. Furthermore, new generations of specific BRAF inhibitors are currently in the pipeline, finely tuned to overcome mutation-driven altered signaling events in the RAS/RAF/MEK/ERK pathway [18]; these might be expected to outperform previous inhibitors of this pathway. Similar undertakings may be required to target deregulated signaling pathways arising from other mutations in different tumors, where a **driver** mutation is known, and where drugs targeting a given driver may exist.

Beyond direct targeting of genomic alterations, the impact of **differentiation hierarchies**, **epigenetic alterations** and the role of the microenvironment in driving tumor pathogenesis have become increasingly recognized. Accordingly, therapeutic approaches that aim to restore normal differentiation programs, such as all-*trans* retinoic acid in acute promyelocytic leukemia and neuroblastoma, have been developed [19]. Along these lines, drugs are and/or have been developed to reprogram epigenetic marks and restore normal gene expression programs, such as various **histone deacetylase** (**HDAC) inhibitors** [20], in addition to drugs that interfere with tumor–microenvironment crosstalk, including **angiogenesis** inhibitors [21] and immunotherapeutic agents [22].

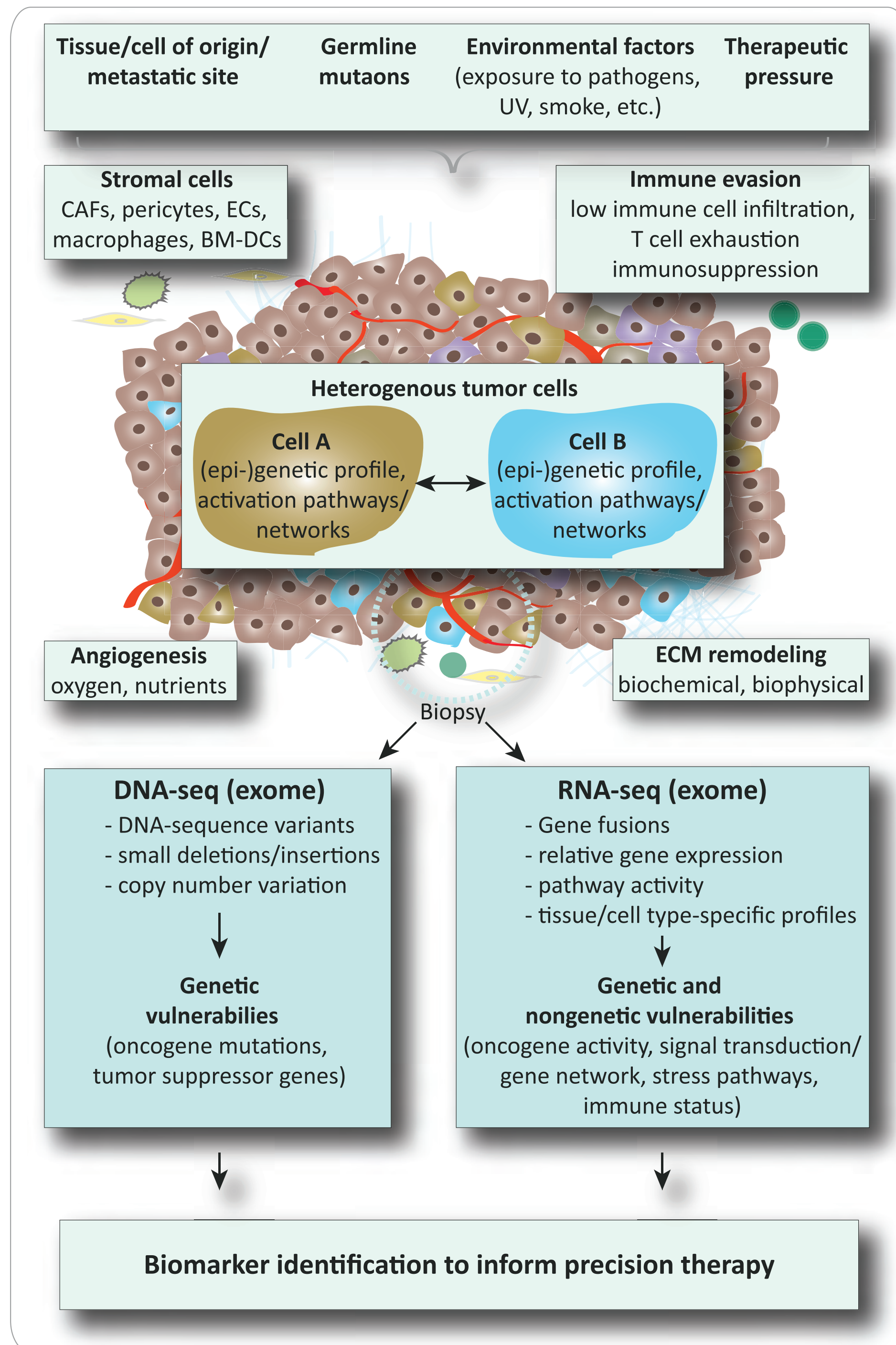
The search for cancer vulnerabilities in specific cancer types has been facilitated by numerous technological advances yielding large-scale molecular profiling of major cancer types [23,24]. This system-based analysis of tumor samples, together with massive hypothesis-based research, has significantly changed our understanding of cancer biology (Figure 1, Key Figure): carcinogenesis is generally considered to be driven by the natural selection of continuously acquired genetic and epigenetic variation in individual cells [25]. These converge on common phenotypic characteristics for cancer cells, including sustained proliferation, migration, invasion, and/or resistance to apoptosis [26]. Tissue microenvironments provide the fitness selection defining spatial and temporal changes in environmental pressures. These influence the evolutionary path of any given cancer cell, resulting in (epi-)genetically heterogeneous subpopulations. Diversity within cancer cell populations is not limited to the genome, and dynamic variations in differentiation hierarchies, transcriptional signals, and the proteomic landscape add to the **phenotypic heterogeneity** observed within tumors [27]. Indeed, cancer cells do not exist as isolated entities, but rather, engage in heterotypic interactions with stromal cells and cooperate with adjacent tumor subclones; this is important, because it can result in the increased robustness of a tumor [28].

Sackler School of Medicine, Tel Aviv University, Tel Aviv, 69978, Israel
[4]Center for Bioinformatics and Computational Biology, University of Maryland, College Park, MD 20742, USA
[5]Technion Integrated Cancer Center, Faculty of Medicine, Technion, Israel Institute of Technology, Haifa, 31096, Israel
[6]Center for Bioinformatics and Computational Biology, University of Maryland, College Park, MD 20742, USA

*Correspondence:
zeev@ronailab.net (Z.A. Ronai).

Key Figure

Determinants of Tumor Pathogenesis and Measures to Inform Precision Therapy



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Glossary

Acquired resistance: (or secondary resistance) indicates that a tumor that initially responded to therapy becomes resistant to this treatment during the course of therapy. By contrast, in intrinsic (primary) resistance, no responses against the tumor are noted upon initiation of therapy.

Actionable mutations: gene alterations that can be specifically targeted with an approved or investigational drug. The term does not provide information about drug efficacy.

Afatinib: tyrosine kinase inhibitor of EGFR (*ErbB1*), HER2 (*ErbB2*), and HER4 (*ErbB4*).

Angiogenesis: blood vessel formation.

Basket trial: histology-agnostic trial design that tests the efficacy of specific drug(s) in molecularly stratified patients. It evaluates whether a biomarker (signature) is predictive for drug response irrespective of tumor histology.

Binary alterations: binary classification of a molecular event, such as somatic mutations (present or absent), gene expression (upregulated or downregulated), or DNA methylation (hypo- or hypermethylated).

Biomarker: a molecular characteristic with a correlative or functional association with disease risk, prognosis (prognostic biomarker), or response to treatment (predictive biomarker).

Canalization evolutionary process: describes the stability of a phenotype despite variation in the genotype.

Cancer hallmarks: cellular and molecular functions required for cancer development and progression. Hallmarks are sometimes described by a set of genes that perform a specific function.

Cetuximab: anti-EGFR monoclonal antibody that binds to the extracellular domain of EGFR and prevents its dimerization.

Circulating tumor cells (CTCs): tumor cells that can be found in, and isolated from, the circulation of blood and/or lymphatic system of patients with cancer.

Circulating tumor DNA (ctDNA): circulating, cell-free tumor DNA that

Moreover, large-scale sequencing of human cancer genomes and transcriptomes have identified nearly 200 'consensus' driver genes (of which ~15% were identified primarily using DNA sequencing of cancer genomes [29]) and an additional 300 putative driver genes have been suggested [30,31]. The pathways in which these genes function are also emerging [32–36]. Coupled with the success seen using targeted therapies in certain cancer subtypes [9–14], these efforts laid the basis for precision molecular oncology: patients are treated according to the molecular make-up of their tumors rather than solely based on tumor histology, type, grade, and stage (Figure 1).

Clinically Relevant 'Omics Approaches

Genomics-Driven Cancer Therapy in Clinical Testing

While at present only a small proportion of cancer patients benefit from targeted therapies, great efforts are ongoing to extend the scope of precision oncology to a broader spectrum of patients (reviewed in [2]). Massive intertumor heterogeneity has been rigorously documented through large-scale DNA and RNA sequencing, as well as **DNA copy number profiling** and **DNA methylation profiling** [e.g., **The Cancer Genome Atlas(TCGA)**, **International Cancer Genome Consortium(ICGC)**, and others] [23,24] (see Table S1 in the supplemental information online for relevant data sources). However, unexpected similarities between tumors of different tissues of origin have been uncovered, while certain tumors have been found to be more similar at the molecular level to tumors from a different tissue of origin [37]. These similarities, together with the detection of **rare variants** within well-characterized driver genes, suggest that approved targeted therapies might be effective in diverse tumor types with distinct molecular alterations [32,36]. This has resulted in the initiation of clinical programs that evaluate whether molecular profiling of patients is clinically feasible and, importantly, whether treating patients based on their genomic profiles might be beneficial relative to a given standard of care, or a physician's treatment choice (see Table 1 for examples of programs and/or studies). In addition, the identification of new putative driver genes found in a low percentage of patients with less-common cancer types or subtypes has generated several novel clinical hypotheses that await verification. Recently, Foundation Medicine reported that, in a targeted sequencing study of 63 220 tumors, more than 75% of patients presented a mutation in at least one of ten cancer driver genes, and more than 25% of patients presented a known driver mutation within these genes [38]. Accordingly, *in silico* computational studies predict that up to 90% of patients may benefit from molecularly guided therapy when biomarkers of uncertain clinical significance, as well as **off-label** and investigational drugs are considered to inform therapy [39,40]. To test this multitude of novel clinical hypotheses, new adaptive trial designs, including **basket** and **umbrella trials**, have been used [41,42] (Table 2). Basket trials are designed to test the effects of a single (or a few) drug(s) in a variety of cancer types (or possibly subtypes) using specific mutation(s) as biomarker(s). By contrast, umbrella trials are designed to test the impact of specific drugs on different mutations within the same cancer type.

Figure 1. The genetic and phenotypic characteristics of a patient's tumor are influenced by tissue- and/or cell type-specific factors, germline genetic background, lifestyle factors, as well as the number and type of previous anticancer drugs received [25,27,28]. Each individual cell is further influenced by, first, the proximity to and the integrity of the tumor vasculature; second, the biochemical and biophysical properties of the surrounding extracellular matrix (ECM); third, competing and/or cooperating interactions between individual tumor cells or tumor and stromal cells [among which are cancer-associated fibroblasts (CAFs), endothelial cells (ECs), and bone marrow-derived cells (BM-DCs)]; and fourth, antitumor immunity. These factors further shape the geno- and phenotypic properties of a tumor in a spatial and temporal manner. While the genomic analysis of a tumor biopsy at the time of diagnosis identifies genetic vulnerabilities, the inclusion of transcriptomic data holds the additional potential of identifying nongenetic vulnerabilities by considering pathway activity and the composition of the tumor microenvironment. Therefore, the integrated analysis of genomic and transcriptomic data is a valuable tool to inform precision therapy. Abbreviations: seq, sequencing; UV, ultraviolet.

can be isolated from whole blood of patients with cancer.
Clone: one or more cells derived from, and genetically identical to, a single ancestor cell. Accordingly, subclones share many of the genetic features of the initial ancestor cell, but contain additional genetic alterations.
Conditional reprogramming: technique used to establish patient-derived cell cultures from healthy or diseased (e.g., tumor) tissue.
Deep neural nets: neural network of multiple layers often used for supervised learning; at each layer, a function is applied to the input from the previous layer.
Differentiation hierarchies: relates to differences in the impact of phenotypes seen in an isogenic population of cancer cells, such as in their ability to metastasize or form tumors upon serial transplantation on immunodeficient mice (i.e., cancer stem cells).
Dimension reduction: selecting a subset of features, or combining features, from a data set (e.g., principal component analysis).
DNA copy number profiling: the genome-wide screening for gene copy number variations (gains or losses).
DNA methylation profiling: genome-wide screening for variations in DNA methylation status (hyper- or hypomethylation).
Driver: usually refers to a genetic event that is shown to elicit phenotypic changes associated with tumor initiation and progression (see 'oncogenic drivers'). In a broader definition, the term can also be used to describe nongenetic and/or non-cell autonomous alterations that can alter certain aspects of disease progression.
Epigenetic alterations: heritable trait not explained by changes in DNA sequence but by changes in gene expression. Common examples often observed in cancer cells include promoter hypermethylation or aberrant histone modifications (e.g., acetylation).
Evolutionary pressure: (or selection pressure) any change in the microenvironment (e.g., cancer therapies) that leads to a selection of clones that have a growth advantage under these new conditions.
Functional mutations: mutations that change the phenotype of a cancer cell or tumor.

The majority of these studies profile the mutation status of a few dozen or hundreds of selected genes [2]. This is based on the fact that, although whole-genome sequencing (WGS) can detect DNA sequence variants as well as focal and large chromosomal rearrangements, deletions, or amplifications, it is difficult to identify driver events within large chromosomal abnormalities. Therefore, clinically valuable sequencing approaches can be reduced to either the whole exome (WES) or targeted exomes (panel sequencing) of cancer-related genes (Figure 1). These approaches are often combined with the analysis of some well-characterized intronic regions (e.g., *ALK*, *RET1*, *ROS*, or *BCR*) that are frequently rearranged in cancer genomes [2]. Furthermore, targeted sequencing has the advantage of yielding a high **sequencing depth**, which is important to be able to infer the clonality of a detected driver event. Determining the clonal distribution of identified alterations should be a priority in precision oncology trials, given that targeting **trunk mutations** appears to be crucial to maximizing the efficacy of targeted therapies [43].

Most studies demonstrated that genomics-guided therapy improves patient outcomes when well-characterized biomarker–drug pairs, supported by strong clinical or preclinical evidence, are used (Table 1). For example, the **MOSCATO 01** trial [44], which used only last-generation drugs with high affinity to a specific target, achieved positive results, whereas the **SHIVA** trial [45], which heavily relied on everolimus, a drug weakly affecting the PI3K/AKT/mTOR pathway, indicated that genomic profiling did not result in patient benefit. Furthermore, emerging evidence suggests that targeting multiple drivers by combination therapy is superior over single-agent use [46], because patients with advanced tumors frequently exhibit multiple aberrations detected by genomic profiling. Despite these advances, several challenges remain. First, trial recruitment of patients with rare mutations is difficult [47] and only a small percentage of patients (2–5%) undergoing genomic profiling have subsequently been treated with off-label drugs or been enrolled in **genotype-matched trials** [46,48]. Noteworthy, the recent report on the positive outcomes of the MOSCATO 01 trial indicates that **match rate** can be improved (19%) when performed within a cancer center offering access to a variety of clinical trials [44]. Second, the presence of validated genetic biomarkers does not strictly predict a response to targeted therapies in different tumor types [49], given that the effect of therapies is context dependent [as seen, e.g., by the lack of response of $BRAF^{V600}$-positive colorectal cancer (CRC) to BRAF inhibitors, which show good clinical responses in melanomas carrying the same mutation [50]. On a positive note, identifying such genomic-context effects has already expanded the use of inhibitors, such as against BRAF [49] or PARP [51], and may result in the expedited approval of investigational drugs. Finally, identifying high-confidence biomarkers to guide specific drug treatments remains a challenge. One approach to expanding the biomarker landscape may be to determine the differential molecular profile of patients showing a dramatic response to targeted therapy versus nonresponsive patients. An increasing number of publications report such ‘exceptional responders’, which has led to the identification of rare genomic events likely to predict the response or resistance to targeted therapies [52–62]. Taken together, sequencing efforts of cancer genomes within clinical trials or by research initiatives, such as the TCGA and ICGC initiatives (Table S1 in the supplemental information online) are expected to improve the identification of driver mutations, as well as patient stratification strategies associated with these. This in turn may expand the scope of genomics-based precision oncology to a broader spectrum of patients.

Limitations of Using Genomics As a Single Approach for Biomarker Identification

While genomic profiling provides valuable information regarding genetic mutation, amplification, deletion, and certain epigenetic modifications (e.g., methylation), there are certain inherent limitations of using an approach that simply tests the presence or absence of genetic driver events to inform therapeutic decision-making. This includes limitations of using genomics as a single platform for biomarker identification; indeed, in some cancer types, such as prostate

Gene fusions: hybrid genes that combine parts of two or more original genes. Fusion genes originate from chromosomal rearrangements (i.e., deletions, translocations, tandem duplications, or inversions).
Genetic interaction: functional interplay between multiple genes and their corresponding gene products that impacts the cellular phenotype.
Genotype-matched trials: when a clinical trial is selected for a patient based on their genotype. This can be a study that only accepts patients with a given mutation, but also any study that either analyses a drug likely to be effective in the context of a given genotype, or inhibits a pathway that is directly linked to a mutation identified in the patient can be considered genotype matched.
Histone deacetylase (HDAC) inhibitors: HDACs remove acetyl groups from histone lysine residues, but also from nonhistone substrates. HDAC inhibitors can have not only transcription-dependent effects (e.g., relief of transcriptional repression of tumor suppressor genes), but also transcription-independent effects due to altered acetylation (and activity) of nonhistone substrates, such as those involved in cell proliferation or cell death.
Homologous recombination deficiency: a defect in double-strand break repair by homologous recombination repair associated with high levels of genomic instability. These defects were originally identified in BRCA1/2-deficient tumors, but can also be observed in the absence of BRCA mutations, a phenomenon generally referred to as ‘BRCA-ness’.
ICGC-TCGA DREAM Somatic Mutation Calling Challenge – RNA: the DREAM Challenges are crowdsourcing (open science efforts) challenges examining questions in biology and medicine. In the ‘RNA-Challenge’, leaders from ICGC and TCGA have come together to develop a Challenge to assess the accuracy of methods to work with cancer RNA-seq data.
Immune-checkpoint inhibitors: drugs that block immune-inhibitory signals (such as PD1, PD-L1, or CTLA4) expressed on and/or by tumor or immune cells. Inhibition of these factors can unleash, in some cases, a robust and durable antitumor immune response.

cancer or some pediatric malignancies, few or even no recurrent mutations have been detected, indicating that other types of **somatic variation** may be potent drivers of oncogenesis [36]. Furthermore, no genetic alterations have been found to correlate with well-characterized predictive biomarkers, such as the expression of estrogen receptor or androgen receptor in breast or prostate cancer, respectively. In addition, genomic profiling does not provide sufficient information regarding the activity of actual protein products mediating oncogenic or tumor suppressor gene functions. In other words, variations in oncogenes and/or tumor suppressor genes do not necessarily predict activation of the corresponding biological pathway, and vice versa: cancer driver pathways can be active without the presence of a mutation(s) [63]. Finally, novel biomarkers linked to nongenetic vulnerabilities, such as those involving cancer cell reliance on stress response or metabolic pathways, that may be able to predict responses to autophagy inhibitors or drugs inhibiting antioxidant enzymes need to be defined [64].

The most comprehensive approach to overcome these challenges and to elucidate cancer vulnerabilities is the simultaneous characterization of the genome, epigenome, transcriptome, proteome, and metabolome of tumors and their surrounding stroma; indeed, these are all crucial parameters to defining cellular phenotypes involved in cancer pathogenesis, as well as in characterizing responsiveness to therapy [65] (Box 1). Given that these parameters are dynamic entities (e.g., change in responses to external stimuli), they are expected to show spatial heterogeneity (geno- or phenotypic distinct **clones** may show different growth kinetics or survival rates dependent on their location). Consequently, an analysis of multiple biopsies

Box 1. Relevant 'Omics for Precision Oncology

Epigenetic profiling holds great promise in deciphering cellular states and characterizing phenotypic heterogeneity. The importance of epigenetic reprogramming in cancer is evidenced by the fact that chromatin regulators are often mutated [32,36] and widespread epigenetic changes throughout cancer genomes can be identified, intricately linked to the activity of known tumor promoters and/or suppressors, such as EGFR [200] or TP53 [201]. There are two general classes of drugs targeting the epigenome: (i) broad reprogrammers, which include inhibitors of DNA methyltransferases, histone deacetylases, or bromodomain and extraterminal motif proteins; and (ii) targeted therapies that pin specific activating mutations in DNA-modifying enzymes, such as EZH2, or in enzymes whose mutations have a profound effect on epigenetic pathways, such as IDH1/2 [20]. Currently, there are no epigenetic drug-sensitivity biomarkers that would predict the response to these approved or investigational drugs. Therefore, the addition of epigenetics in clinical practice awaits the identification of epigenetic marks that mediate distinct tumor phenotypes of clinical relevance (such as mesenchymal differentiation, stemness, dormancy, or therapy resistance) [65].

Proteomics combined with genomic data likely reveal the most accurate information on the activity state of individual genes. The proteome represents the ideal readout to define the functional state of a cell in response to internal or external perturbations, and proteogenomic analysis is being integrated in large-scale characterization efforts of the TCGA [202–204]. This integration has the power to nominate driver genes from large chromosomal deletions or amplifications and can identify new driver clusters that are not easily found in transcriptomics signatures [202,203,205]. Although TCGA analysis has long included antibody-based phosphoprotein analyses, the comprehensive proteomic characterization based on mass spectrometry increases the breadth of phosphoproteomics data and, importantly, allows for the identification of post-translational modifications beyond phosphorylation [206]. The latter may represent important biomarkers for drugs that do not target kinases, such as the identification of 'acetylation signatures' in serous ovarian cancer, and which may predict responses to HDAC inhibitors [204]. While it is expected that future technologies will provide the platform for large-scale proteomic assessment of tumor samples, current proteomic analysis requires a large amount of tissue, is costly, labor-intensive, and lacks the analytical validity and sensitivity that genomics provides.

Emerging metabolome and microbiome data are expected to provide important additions to genomics: Rewired metabolic pathways in tumors provide alternate fuel sources that can be targeted, and the mutations and/or deregulated expression of metabolic genes have been linked to tumor propensity for metastasis or therapeutic resistance [207]. Translating this knowledge in the clinic will require further preclinical analysis, especially given the differences between cancer cell metabolism *in vitro* and *in vivo* [208,209]. Microbiome-based data are a likely addition in the more distant future, which might provide novel putative biomarkers and means to monitor predicted therapeutic responses and, possibly, lead to the development of new therapeutic modalities.

'Integrated' subtype: a subtype of cancer that includes patients with tumors from multiple tissues of origin.
International Cancer Genome Consortium (ICGC): has the goal to generate comprehensive catalogs of genomic abnormalities (somatic mutations, abnormal expression of genes, and epigenetic modifications) in tumors from 50 different cancer types and/or subtypes and make the data available to the entire research community to accelerate research into the causes and control of cancer.
Intertumor heterogeneity: differences in the molecular make-up of tumor cells between patients.
Intratumor heterogeneity (ITH): differences in the molecular make-up of tumor cells within individual patients.
Match rate: frequency by which genomic alterations detected in a patient cohort can be matched to targeted therapies.
Methylome: collection of all DNA methylation markers in a single cell or a population of cells.
MOSCATO 01: prospective clinical trial evaluating whether high-throughput genomic analyses can improve patient outcomes.
'Multiview' matrix factorization: the ability to factor multiple data matrices into a lower-dimensional space, where each data matrix gives a different 'view' of the data (e.g., gene expression and DNA methylation views).
Off-label (drug use): a drug is used for a purpose not specified in the marketing authorization determined by a licensing body, such as the FDA (e.g., drug used for a different type of cancer than the one it is approved to treat).
Oncogenic drivers: genetic events associated with tumor initiation and progression, including metastasis and therapy resistance.
Orthotopic implantation: a form of xenograft experiment where (patient-derived) tumor cells are implanted into the organ of origin to maintain the tissue-specific environment.
Paclitaxel: chemotherapeutic drug that binds tubulin and inhibits the disassembly of microtubules, thereby resulting in the inhibition of cell division.
Panitumumab: monoclonal antibody that binds to and inhibits (autocrine) stimulation of the EGFR.

and longitudinal follow-up of patients would ideally be performed to predict the initial responses to therapy and to identify putative mechanisms of drug resistance. Although such comprehensive approaches are not yet feasible for routine clinical practice, current state-of-the-art technologies are already enabling the combination of at least two different 'omics platforms for cancer analysis, genomics with epigenomics, and/or transcriptomics. As discussed below, combined genomic and transcriptomic analysis, together with functional testing of 'omics-derived treatment predictions, are expected to overcome many of the challenges that current precision oncology-based trials face.

Transcriptomics As a Valuable Measure to Improve Biomarker Identification

At present, the most common way to enhance genomic information is by the inclusion of transcriptomic analyses (Figure 1). RNA sequencing (RNA-seq) technologies allow the mapping of the entire transcriptome or select gene expression networks, and are readily available and becoming economically feasible. The ability to decipher the landscape of gene expression offers important steps over acquiring genomic data alone. First, aside from the ability of RNA-seq to detect **splice variants** [66], it can also detect novel or known **gene fusions**, which have been identified as drivers of disease in a variety of rare and common tumors [66–69]. Gene fusions are promising therapeutic targets because the inhibition of fusion genes is often associated with striking efficacy, as exemplified by targeting the BCR-ABL fusion in chronic myeloid leukemia, or targeting RET, ALK, ROS, FGFR, or BRAF fusions in various malignancies (reviewed in [2]). Second, transcriptomics can provide indirect information about protein expression status; knowing that a candidate gene harboring certain mutation(s) is also expressed (and to what level) is valuable in establishing the importance and contribution of this gene to the tumor phenotype. Third, beyond providing information about the expression of tumor driver genomic variations, the inclusion of transcriptomics allows the mapping of non-oncogene vulnerabilities, and provides information about oncogenic pathway activities, even in the absence of mutated driver genes [63,70]. One such example is the *BRAF* mutation signature in colon cancer, which can be found in not only *BRAF* mutated but also *BRAF* wild-type tumors, and characterizes (in addition to the *KRAS* and *PI3K* signatures) patients resistant to EGFR inhibition [71]. The *BRAF* signature can not only serve as a resistance biomarker, but has also been recently suggested to serve as a sensitivity biomarker for mitotic poison drugs, such as vinorelbine [72]. Another notable example is that of *BRCA*-associated signatures [73], where tumors (such as breast, ovarian, or prostate tumors, among others [66,73]) sharing similar molecular signatures to *BRCA* mutant tumors may also respond to similar therapeutic approaches, even when lacking specific *BRCA* mutations [73–75]. Fourth, transcriptomes, by contrast to DNA, are tissue and cell type specific [65]; this is often considered a disadvantage for RNA analysis of bulk tumor samples because, in samples with a high proportion of stromal cells, massive computational deconvolution is necessary to extract the transcriptional profile of interest, given that all cells within the biopsy contribute to the RNA pool [65]. However, tissue specificity can provide important clinical information about tumor histology and tumor origin, which is of relevance in patients with cancer of unknown primary tumors [37]. Additionally, cell type-specific transcriptomes can reveal certain aspects of the immune status of tumor samples that may be of therapeutic relevance [76,77]. High overall mutational load within tumors (e.g., highly mutated human tumors, such as melanoma, lung cancer, or mismatch-repair deficient colon cancer) has been reported to correlate with therapeutic responses to **immune-checkpoint inhibitors** (e.g., drugs targeting CTLA-4 or the PD1/PDL1 axis) [76,77]; however, these factors have not been strictly linked, and long-term responses to checkpoint inhibition have been observed for a broad mutational spectrum of cancers [76,77]. Integrating genomic and transcriptomic data holds promise for the identification of patients who can benefit from immune-checkpoint inhibitors. For example, the transcriptomic analysis of responders to CTLA-4-blockade (ipilimumab) revealed that the expression of cytolytic effector genes (e.g., those encoding granzyme A and perforin) positively

Passenger mutations: somatic mutations with no obvious role (owing to their inability to cause phenotypic changes) in cancer.
Patient-derived xenograft (PDX): xenograft model where tumor cells (derived from a biopsy or bodily fluids) are subcutaneously or orthotopically engrafted into immune-incompetent mice, without any intermediate growth or modification in culture.
Phenotypic heterogeneity: differences in phenotypic characteristics (e.g., potential to form metastasis or resist therapy) of genetically diverse but also isogenic tumor cells.
Rare variants: genetic variations (i.e., mutations) within a particular gene that occur in $<1\%$ of patients.
Recurrent mutation: somatic mutation that occurs in a statistically significant number of times in a cohort of sequenced tumors.
Sample-level events: measurements of individual tumors, patients, or cancer cell lines (e.g., drug sensitivity).
Saturation analysis: modeling of sample complexity to determine the number of samples required to detect some event as statistically significant.
Sequencing depth: reflects the average number of times a given region has been sequenced by independent reads.
Serial passaging: serial transplantation of tumors (usually in the flank of nude mice) for tumor expansion.
SHIVA study: the first randomized, open-label, controlled Phase II study that evaluated whether targeted treatment based on molecular testing improved patient outcomes compared with conventional treatments across all tumor types.
Somatic variation: alteration (genetic, epigenetic, or signaling) occurring in a somatic (body) cell.
Splice variants: transcripts (mRNAs) resulting from the alternative splicing of different exons in genes.
Suppression interactions: phenotypic defects caused by a mutation in a particular gene are rescued by a mutation and/or deregulation in a second gene.
Synthetic rescue: type of genetic interaction where the mutation and/or deletion of one gene rescues the lethality or growth defect of a cell

correlate with patient response (complete or partial response to ipilimumab, or stable disease with overall survival >1 year by RECIST criteria) [76]. Furthermore, the expression of immune checkpoint regulators has correlated with increased patient survival [76]. In addition, transcriptomic signatures that significantly correlate with resistance to anti-PD1 therapy in melanoma have also been identified [77].

Although the practical utility of RNA-seq in the clinic has been challenging, technological advances allowing the application of RNA-seq to clinically relevant specimens (including formalin-fixed, paraffin-embedded tissues), along with efforts to benchmark data analysis pipelines (**ICGC-TCGA DREAM Somatic Mutation Calling Challenge – RNA**) [78], have set the basis to move RNA-seq into routine clinical practice. Thus, valuable transcriptomic information can be combined with genomic data to establish new blueprints that provide multidimensional insight into the characteristics of a given tumor biopsy. Such combinations can benefit from innovative computational approaches that may identify novel master regulators, not seen in either analysis alone.

Analysis Approaches to Determine Molecular Subtypes and Cancer Vulnerabilities

To overcome the challenges of intertumor heterogeneity in determining molecular-guided therapy, the identification and characterization of molecular subtypes of cancer and the mutations that drive cancer have been an urgent priority. The promise of characterization of tumors with molecular subtypes or biomarkers is twofold. The first major goal is to find molecular biomarkers of patient prognosis or effective drug treatments. The second major goal is to develop a better mechanistic model for understanding the role of the genome, transcriptome, **methylome**, epigenome, and environmental alterations of the tumor in driving its initiation and evolution. Extensive clinical efforts now provide an unprecedented view of the genomic (and transcriptomic) landscape of all advanced cancer types [79], in addition to the data sets provided by the TCGA and ICGC, which have focused on the sequencing of common cancer types early in disease progression.

The following sections describe related computational approaches; for an expanded summary of references on these and additional topics (ITH and single cell analysis approaches) the reader is referred to Tables S1–S3 in the supplemental information online.

Approaches for Tumor Subclassification

Methods for identifying molecular subtypes generally fall into two categories, based on whether data from a single platform or multiple platforms are being used. For single-platform data (e.g., gene expression), any off-the-shelf clustering algorithms can be used, although choosing the method depends on the type of data being clustered. The more challenging case is clustering patients with data from multiple platforms, especially because there is often a data type that is missing, given that not all measurements are performed in every patient. Researchers have taken multiple approaches (see references in Table S2A in the supplemental information online). Some methods search for a 'consensus' after clustering patients by each platform separately [37], or cluster with protein–protein interactions [29], or patient similarity networks [80,81]. Other methods formulate the problem as a **'multiview' matrix factorization** and **dimension reduction** (reviewed in [82]), or as a probabilistic model. In all cases, a key challenge is the selection of features from each platform as inputs to the clustering algorithms; for example, it is possible to summarize mutations, gene expression, and DNA methylation events as **binary alterations** [80], and then treat any missing data as a nonalteration event. We anticipate that recent advances in methods for learning low-dimensional representations of multiple data types, such as **deep neural nets** [83], will soon be applied in molecular classification of tumors, given the amount of molecular cancer data being produced and the successful application of

mutated and/or deleted for another gene.
Targeted therapies: drugs that either target molecular alterations specific to cancer cells (e.g., mutated, amplified or epigenetically up- and/or downregulated signaling proteins), or target immune cells to increase anticancer immunity.
Temozolomide: chemotherapeutic drug in the class of alkylating agents exerting cytotoxicity by inhibiting DNA replication.
The Cancer Genome Atlas (TCGA): a collaboration between the National Cancer Institute (NCI) and the National Human Genome Research Institute (NHGRI); it has generated comprehensive, multidimensional maps of key genomic changes in 33 types of cancer.
Trametinib: targeted therapy that specifically binds and inhibits MEK1/2.
Trunk mutations: genetic variations occurring in early tumor evolution and are thus common to most clones.
Umbrella trial: study design evaluating the efficacy of multiple drugs within a biomarker-stratified single cancer type. It allows the evaluation of multiple biomarker–drug combinations in a histology-dependent manner.

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Table 1. Studies Evaluating Feasibility and Clinical Benefit of Molecular Profiling[a]

Study	Tumor type	Screening platform	#Pts sequenced	#Pts on matched therapy	Type of therapy	Endpoint/Results
IMPACT; M.D. Anderson [193]; NCT00851032; retrospective, nonrandomized	All; advanced, refractory	Hotspot sequencing 11 genes; FISH (ALK)	1144	175	Mono and combination; Phase I therapies; N.S.	Higher ORR and longer PFS compared with unmatched therapy
IMPACT/COMPACT [48]; NCT01505400; retrospective, observational, nonrandomized	Advanced cancers and Phase I candidates	MALDI-TOF MS hotspot panel (23 genes) or targeted NGS panel (48 or 50 genes)	1640	245	N.S.; investigational agents of 277 trials, including 89 genotype-matched trials	Genotyped matched therapy improved response
PREDICT [194]; NCT02478931 retrospective, correlative, nonrandomized	All	NGS; (182 or 236 gene panel and 14 or 19 rearrangements	347	87	N.S.	More patients with SD $\geq$6 months; 45% with extended PFS of 30% compared with previous therapy
Bisgrove [195]; NCT00530192; prospective, single-arm Phase I	All; advanced, refractory	IHC, FISH, gene expression	86	68	Mono and combination; N.S.	27% with extended PFS of 30% compared with previous therapy
Genomic Profiling in Phase I [46]; NCT02437617; prospective, nonrandomized	All; advanced, refractory	Panel NGS (236 genes); standard biomarker	339	122	Mono and combination; Phase I/II therapies; N.S.	High matching score associated with higher SD $\geq$6 months/PR/CR, longer PFS and survival
MOSCATO 01 [44]; NCT01566019 prospective, nonrandomized	All; advanced, refractory	CGH array; panel NGS (WES and RNAseq in 2014 included)	843	199	Phase I drugs and off-label drugs; N.S.	33% with extended PFS of 30% compared with previous therapy
WinTher; NCT01856296; prospective, nonrandomized	All; advanced, refractory	DNA (236 genes) and RNA in tumor and normal matched tissue	To be 200	N.A.	N.S. Chosen based on DNA analysis, if no actionable mutation, then based on RNA analysis	Estimated completion in 2018
SHIVA [45]; NCT01771458 prospective, randomized	All; advanced, refractory	Panel hotspot NGS (46 genes); CNV	741	99	Erlotinib, lapatinib + trastuzumab, sorafenib, imatinib, dasatinib, vemurafenib, everolimus	Median PFS in experimental group (matched therapy) not significantly longer than in control group
NCI-MPACT; NCT01827384; prospective, Phase II randomized feasibility study	All, advanced solid tumors	NGS (4000 variants across 143 genes); activation of RAS/RAF or PI3K pathway; inactivation of DNA repair pathway	To be 700	N.A.	Carboplatin, everolimus, temozolomide, trametinib, veliparib, AZD1775	Estimated completion in 2019; outcome measures: ORR; compare genotype matched versus physicians choice

[a]Abbreviations: AZD1775, WEE1 inhibitor; CGH, comparative genomic hybridization; CR, complete response; FISH, fluorescent *in situ* hybridization; IHC, immunohistochemistry; N.A., not applicable; N.S., not specified; NGS, next-generation sequencing; ORR, overall response rate; PFS, progression-free survival; PR, partial response; Pts, patients; SD, stable disease; WES, whole-exome sequencing.

deep neural nets in areas of computer vision, natural language processing, and biology [84]. Initial molecular subtype studies have often focused on clustering samples into subtypes based on gene expression in a single cancer type, which have provided robust biomarkers and subgroups, coherent with patient survival profiles (e.g, in breast cancer [85] or CRC [86]). These studies typically reveal a more refined set of clusters than those defined by known

Table 2. Representative Basket and Umbrella Trials[a]

Study	Tumor type	Screening platform	Biomarkers tested	Drugs	Endpoint/Results
BATTLE; NCT00409968, NCT00411671, NCT00411632, NCT00410059, NCT00410189; Prospective, adaptively randomized, umbrella trial	Chemorefractory NSCLC	Non-NGS, mutation analysis, FISH, IHC	Mutation/amplification: *EGFR*, *KRAS/BRAF*, *CCND1*; protein expression: VEGF, RXR, cyclinD1	Erlotinib, sorafenib, vandetanib, erlotinib + bexarotene	Better DCR for *EGFR* + erlotinib and *KRAS/BRAF* +sorafenib
BATTLE-2; NCT01248247; 2-stage Phase II umbrella design	Advanced NSCLC	NGS; DNA, mRNA, RPPA, IHC	IHC: pAKT, PTEN, HIF1α, LKB1. Mutation: *P13KCA, BRAF, AKT1, HRAS, NRAS, MEK1, MET, CTNNB1, LKB1;*	Erlotinib (ctrl) sorafenib, MK-2206 + erlotinib; MK-2206 + selumetinib	8-week DCR
Lung-MAP; NCT02154490 randomized phase II/III umbrella design	Advanced, recurrent squamous cell lung carcinoma	FMI FoundationOne platform; IHC;	*PI3KCA, CDK4/6, CCND1/2/3, FGFR1/2/3, HGF/c-MET*	Taselisib, palbociclib, AZD4547, erlotinib, erlotinib + rilotumumab, ipilimumab, nivolumab, durvalumab	PFS, ORR, OS
I-SPY-2; NCT01042379; randomized open-label Phase II umbrella design; (adjuvant setting)	Stage III breast cancer	Conventional, MRI	HER2, hormone receptor, and Mammaprint Bayesian marker-adaptive trial designs	HER2$^+$: neratinib, MK2206 + trastuzumab, T-DM1 + pertuzumab, trebananib + trastuzumab, pertuzumab + trastuzumab; HER2$^-$: veliparib, MK2206, ganitumab + metformin, trebananib	Pathological complete response; several drugs graduated to Phase III trials [196–198]
ALCHEMIST; NCT02194738; NCT02193282; NCT02201992; NCT02595944; screening study and accrual to Phase III randomized treatment studies (adjuvant setting)	Resectable nonsquamous NSCLC	NGS, tissue and blood; germline + somatic alterations	*EGFR* mutation; *ALK* rearrangements (FISH); PD-L1 expression (IHC)	Erlotinib, crizotinib, nivolumab	OS
SAFIR02 Breast; NCT02299999; Phase II randomized umbrella design	HER2$^-$ recurrent and/or metastatic breast cancer	CGH array, hotspot sequencing	To be determined during study	AZD2014, AZD4547, AZD5363, sapitinib, selumetinib, vandetanib, bicalutamide, olaparib, durvalumab	PFS compared with standard maintenance
SAFIR02 Lung; NCT02117167; Phase II randomized umbrella design	EGFR and ALK WT recurrent and/or metastatic NSCLC	CGH array, hotspot sequencing	To be determined during study	AZD2014, AZD4547, AZD5363, sapitinib, selumetinib, vandetanib, durvalumab	PFS compared with standard maintenance
Lung MATRIX; NCT02664935 Phase II; nonrandomized umbrella design	Advanced, pretreated NSCLC	28-gene NGS platform	Mutation: *FGFR2/3, TSC1/2, LKB1, KRAS* + Rb^{WT}, *NF1, NRAS, PIK3CA, AKT1, EGFR* + $EGFR^{T790}$; LoF: *p16* + Rb^{WT}, *PTEN*; amplification: *CDK4* + Rb^{WT}, *CCDN1* + Rb^{WT}, *MET, PIK3CA*; rearranged: *ROS1*	AZD4547, AZD2014, palbociclib, crizotinib, selumetinib, AZD5363, osimertinib, durvalumab	ORR, PFS
		Hotspot-seq biomarker only; IHC	*BRAF, PIK3CA, KRAS, NRAS; PTEN, MMR*		ORR, PFS, OS

Table 2. (continued)

Study	Tumor type	Screening platform	Biomarkers tested	Drugs	Endpoint/Results
FOCUS 4 [199]; Phase II/III randomized umbrella design	Advanced/metastatic, untreated colorectal cancer			BRAFi + panitumumab +/– MEKi, aspirin, AKTi + MEKi, HER1/2/3i	
V-BASKET [49]; NCT01524978; flexible, early Phase II; basket study	Solid tumors, multiple myeloma	Mutation analysis with local method	$BRAF^{V600}$	Vemurafenib monotherapy; vemurafenib + cetuximab in CRC	Efficacy in NSCLC, ECD, and LCH
CUSTOM [47]; NCT01306045; biomarker-derived, multiarm, multihistology Phase II, basket trial	NSCLC, SCLC, thymic malignancy	NGS	Mutation: *AKT1*, *BRAF*, *EGFR*, *ERBB2*, *HRAS*, *KIT*, *KRAS*, *NRAS*, *PDGFRA*, *PIK3CA*, *PTEN;* amplification: *ERBB2*, *PIK3CA*, *PDGFRA*; fusion: *ALK*	Erlotinib, selumetinib, MK2206, lapatinib, sunitinib	Targeting EGFR and ALK offers benefit; design not feasible for most arms
NCI-MATCH; NCT02465060; nonrandomized Phase II; basket trial	Advanced, recurrent, refractory solid tumors, lymphoma, myeloma	NGS (4000 variants across 143 genes)	Mutations: *AKT1, BRAF V600; BRAF* non-*V600, BRCA1/2, cKIT, DDR2, dMMR, EGFR, EGFRT790 M, FGFR1/2/3, GNAQ/GNA11, HER2, MET ex14 sk, mTOR, NF1, NRAS, PI3KCA, PTEN, SMO/PTCH1, TSC1/2*; Translocations: *ROS1, ALK*; amplification: *CCDN1/2/3, CDK4/6, HER2, MET*; loss: *NF2, PTEN*; fusion: *NFRK*	Ado-trastuzumab emtansine, afatinib, AZD4547, AZD5363, AZD1775, osimertinib, binimetinib, crizotinib, dabrafenib + trametinib, dasatinib, defactinib, GSK2636771, larotectinib, nivolumab, palbociclib, sunitinib, TAK-228, taselisib, trametinib, trastuzumab, vismodegib	ORR

[a]Abbreviations: AZD4547, FGFR inhibitor; AZD5363, AKT1/2/3 inhibitor; AZD1775, WEE1 inhibitor; AZD2014, inhibitor of mTORC1/2; CGH, comparative genomic hybridization; DCR, disease control rate; ECD, Erdheim–Chester disease; GSK2636771, inhibitor of PI3K beta; LCH, Langerhans' cell histiocytosis; MK2206, inhibitor of AKT1/2/3; NGS, next-generation sequencing; NSCLC, non-small cell lung cancer; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; RPPA, reverse-phase protein array; SCLC, small cell lung cancer; TAK-228, dual mTORC1/2 inhibitor.

histopathological markers, with more-coherent survival profiles of the samples and/or patients composing them. While some of the molecular clusters strongly overlap with known histologically based clusters, others surprisingly comprise samples with distinct histopathological markers, but with similar transcriptomic profiles and survival rates. More recent analyses have clustered multiple platforms across multiple cancer types and, as outlined above, identified molecular similarities between tumors of different tissue of origin. For example, one study analyzed TCGA data from >3000 samples across 12 cancer types, and found that, while most cancers could be classified based on their histology, approximately 10% could be classified as belonging to an **'integrated' subtype**, that is, including cancers from multiple tissues of origin in the same subtype [37]. Furthermore, grouping samples from different tissue types yielded improved predictive power for patient prognosis, potentially reflecting the value of molecular features (such as common mutations) for predicting survival [37]. The US Food and Drug Administration (FDA) recently approved the drug pembrolizumab (an immune-checkpoint PD-1 blockade), used across many cancer types, with demonstrated effectiveness in CRC, endometrial, pancreas, thyroid, and eight other cancer types, based on the presence of a specific (mismatch-repair deficiency) signature [87]. These studies demonstrate the promise of classifying tumors using molecular features, which can give additional insights into prognosis and treatment beyond tissue of origin.

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Approaches to Identifying Genetic Drivers

While as few as three to eight somatic mutations are required to drive cancer [36], identifying the entire set of driver mutations in any given tumor is a difficult biological and computational problem. The observation that relatively few mutations occur in a significantly recurrent manner across tumors holds, despite the development of sophisticated statistical tools for evaluating the significance of mutations. Researchers have developed multiple different classes of tool that consider different information about somatic mutations, including the predicted functional impact [88] or conservation across populations [89]. Other methods attempt to classify driver mutations by identifying hotspots in the protein sequence or structure [90–92], or targets of recurrent copy number aberrations [93]. Some methods also consider side information, such as the replication timing and expression of a gene [94,95], or per patient, and/or per gene mutation rates [96] (see references summarized in Table S2B in the supplemental information online).

Despite these advances, in most cancer data sets, there is a 'long tail' of genes with infrequent mutations, where the drivers are statistically indistinguishable from **passenger mutations** [32]. One report illustrated the depth of this problem by estimating the number of samples required to detect driver mutations with a given frequency in a given cancer type through **saturation analysis** [34]. The cancer type in question was critical because of the high variance in background mutation rates in different cancers (e.g., breast cancer, prostate cancer, etc.). For example, the authors showed that up to 5300 samples would need to be sequenced to detect drivers occurring at 2% above the high background mutation rate in melanoma [34]. This presents a particular challenge for rare cancer types, especially since cancer types continue to be divided into different subtypes [34].

The observed intertumor mutational heterogeneity is widely believed to be due, in part, to mutations targeting pathways or '**cancer hallmarks**' [26], where each pathway includes multiple genes, such that many different combinations of aberrations can affect hallmark pathways and drive cancer. Thus, by uncovering the genes in these pathways, it may be possible to identify 'hidden' driver mutations in the 'long tail', that is, the set of mutations that are indistinguishable from passengers without considering prior knowledge, such as pathways. To date, researchers have developed multiple classes of method that use different side information to identify the pathways and/or hallmarks targeted in cancer (Table S2B in the supplemental information online). One group of methods searches for significantly mutated groups of genes in known pathway databases [97] and protein interaction networks [35,93,98–100]. Other methods search for **functional mutations** that co-occur with **sample-level events** [101,102], which can be viewed as a supervised learning task. These approaches have been used on cancer cell line drug sensitivity and gene dependency and/or addiction (i.e., conditional essentiality) data to generate testable hypotheses, but are less well suited for predicting coarse measurements with many factors, such as overall survival. Another promising approach has been to search for groups of genes with mutually exclusive mutations [103–107]. However, these approaches also require large sample sizes through saturation analysis [108] and, depending on the relative rate of driver and/or passenger mutations, such sample sizes can be even larger than those required by the **recurrent mutation** detection methods described above. Finally, going beyond coding region mutations, researchers are beginning to uncover recurrent and functional mutations in noncoding regions of the genome that might have a role in dysregulated gene expression, as is the case of the *TERT* promoter, shown for the first time to be mutated significantly in melanoma [109] and, more recently, in 43 tumor types [79], with significant association with poor survival in cutaneous melanoma, bladder urothelial carcinoma, and papillary thyroid cancer [79]. Larger whole-genome sequencing efforts, such as those from the ICGC, are likely to uncover more of these noncoding mutations due to increased statistical power.

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Integrating Genomic and Clinical Data

Current efforts linking genomic mutation data with clinical data to assist in therapeutic decisions build and use knowledge banks (Table S3 in the supplemental information online). These include web tools that provide data and text summaries of the frequency, mechanisms, and druggable targets of known driver mutations [110]. Multiple tools now include 'interpretations' or summaries of the driver mutations written by clinicians, including the Precision Medicine Knowledgebase (at Weill Cornell) and the Personalized Cancer Therapy knowledge base (at M. D. Anderson), or by the 'crowd' [111,112] (see references in Table S2C in the supplemental information online). A related approach recently explored leveraging existing 'omics data sets for the interpretation of variants in newly sequenced samples of acute myeloid leukemia [113]. For example, one study recently demonstrated the use of this approach by building survival models that linked genomic and clinical data, and then using these models to choose treatment (s) and predict survival for patients with newly diagnosed acute myeloid leukemia [113]. Regularized regression on both genomic and clinical features was performed on these models to predict overall survival; the authors used these to identify additional interventions that could increase overall survival, and extraneous interventions that could be removed for some patients without decreasing overall survival [113]. However, effectively integrating annotations and clinical knowledge of known variants with 'omic databases in an automated manner for the interpretation of patient molecular data, and creating features from molecular data for input into survival models, remain key challenges. This appears to be largely due to the fact that most clinical data still need to be extracted from free text, and the pertaining electronic medical record (EMR) systems are mostly not standardized.

Identifying Cancer Vulnerabilities on the Basis of Genetic Interactions

Another way to guide precision therapy is based on identifying and utilizing **genetic interactions**, in particular, by harnessing the concept of synthetic lethal interactions (SLi). SLi describe the relationship between two genes where an individual inactivation of either gene results in a viable phenotype, while their combined inactivation is lethal for the cancer cell [114,115]. SLi have long been considered a foundation for the development of selective anticancer therapies [64,114,115], which aim to inhibit the SL partner of a gene that is inactivated *de novo* in cancer cells. Given that this SLi partner gene is most likely to be inhibited only in the tumor, this treatment will primarily kill these cancer cells but not healthy ones. Thus, this offers a complementary approach for predicting patient drug responses to sequence-based cancer precision medicine strategies. This might be achieved by: (i) going beyond existing precision oncology approaches based on **actionable mutations** (i.e., a mutation that can be targeted by specific small-molecule inhibitors) in a few hundred cancer driver genes, and examining the whole genome, thereby covering all possible changes that might have occurred in a tumor. These might uncover more treatment options for patients whose tumors do not bear actionable mutations; and (ii) SLi are well poised to offer effective options for potentially treating heterogeneous tumors, impacting differentiating subclones and resulting overall in more effective tumor eradication with a reduced likelihood of drug resistance.

Given this promising potential, extensive experimental efforts have aimed to tease out the wiring of genetic interactions in cancer cells based on single (isogenic) cell lines [116–121] or on large-scale genetic knockout-based screens [122–126]. However, due to the large combinatorial space of pairwise interactions that need to be surveyed, these screens have probed only a small fraction of the coverage offered by SLi: each screen typically scans a few thousand candidate SL partners of just one 'anchor' cancer driver gene of interest (e.g., *KRAS* or *VHL*), altogether covering a mere fraction of the 500 million gene pairings in the human genome. Yet, with these screens, several SL interactions have been successfully uncovered to date; apart from examining the effect of PARP inhibitors in patients with BRCA-mutated breast and

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pancreatic tumors, a growing number of other treatments targeting SL-based cancer specific vulnerabilities are currently being clinically investigated [127].

Aiming to bypass the limitations of current experimental techniques in probing the vast space of potential SLi, various computational approaches have been developed to identify such candidate SLi (see references in Table S2D in the supplemental information online). These include applying various machine-learning methodologies to predict genetic interactions in different species [128–131], and in cancer (using yeast SLi) [119,132], utilizing metabolic modeling [133,134], evolutionary characteristics [119,129], and transcriptomic profiles [101,135], and, more recently, by mining cancer patient data [136–138] (Table S2D in the supplemental information online). One recent study evaluated the TCGA copy number and transcriptomics data to identify as candidate SLis, gene pairs that are almost never found inactivated in the same tumors [136]. The study demonstrated that gene pair interactions (a subset of which was validated in experimental screens) could be successfully used to predict the survival of patients with breast cancer in an independent data set [136]. The pair was also used to predict *in vitro* drug responses to identify novel drug repurposing indications for potentially treating renal cancer [136]. Unlike the approach of using expression and copy number data [136], an algorithm was recently developed to mine pan-cancer human tumor data and define mutation-specific SL interactions for specific cancers [139]. Its SL predictions were validated against published SL screens and one specific SL gene pair interaction between mutated *IDH1* and the gene encoding acetyl-CoA carboxylase 1 (*ACACA*) in leukemia was experimentally validated; this interaction attenuating tumor growth in **patient-derived xenografts(PDX)** [139]. Finally, certain predicted SL interactions were shown to successfully predict drug sensitivity, thus serving as biologically interpretable biomarkers of the latter [139]. Overall, while these studies have laid a solid basis for some of these genome-wide approaches, extensive research is warranted to further elucidate the potential of SLi-based approaches in precision oncology. Moreover, for clinical trials, there is an important unmet need to specifically design and test SLi-based approaches that may uncover a range of tumor-specific vulnerabilities.

The Use of *In Vitro* and *In Vivo* Models for Guiding Precision Therapy

The increased functional annotation of genetic variants of unknown significance [140], along with systematic high-throughput drug screens in 1000 cancer cell lines [141] or 1000 PDX models [142], has significantly increased our understanding of the relationship between genotype and drug sensitivity. We are beginning to understand the molecular profiles of patients responding to conventional chemotherapy, such as to **temozolomide** [143] and other DNA-damaging agents [144]. Due to their well-characterized clinical benefit in unstratified patient cohorts, these remain equally valuable therapy choices in addition to targeted therapies. With this ever-increasing number of validated biomarkers and available drugs, it is expected that molecular profiling will reveal multiple potentially actionable alterations, which may be treated with a multitude of drugs and/or drug combinations. Prioritizing predicted treatments requires functional testing, especially in cases where the drug–biomarker association has not been clinically validated. Undoubtedly, there will still be patients whose molecular analysis is either not feasible or does not reveal targetable alterations, for which alternate routes to inform therapy are necessary. For this purpose, several *in vitro* or *in vivo* patient-derived functional platforms (e.g., PDX or organoid models) have been developed that mimic the native features of tumors more closely than conventional cell culture drug-screening platforms [145].

PDX models offer one attractive approach, because tumor heterogeneity is maintained in these models at least in early passages (for a comprehensive review, see [146]). In addition, clinical studies have demonstrated remarkable correlations between drug activity in the PDX model and a patient's clinical outcome [146–149]. However, not all human tumor samples grow in mice following subcutaneous or **orthotopic implantation**, and the long time span needed for

tumor development and expansion to test multiple drugs and/or drug regimens restricts this approach to patients with a less aggressive disease course [146]. **Serial passaging** is not only accompanied by the substitution of human stroma with murine components, eventually affecting clonal evolution [146], but also results in extensive mouse colonies and, hence, logistical difficulties and rapidly expanding costs. Finally, although humanized mouse models with a (partially) competent 'human' immune system have been developed, the remaining technical and biological difficulties of generating these mice restrict the use of PDX models in studying immunotherapeutic approaches as well as the effects of immunity on the efficacy of other drugs in preclinical models [146]. Nonetheless, alternate, *in vitro* or *ex vivo* models may substitute the extensive use of PDX.

Patient-derived 3D organoids provide a practical alternative (see other models discussed in Box 2). Organoids are established by dissociating and embedding tissue in an cell-free extracellular matrix (matrigel or collagen), which can be expanded in a growth factor-enriched medium [150]. Organoids from pancreatic [151], colon [152–154], gastric [155], prostate cancer [156], and brain tumors and/or metastasis [157] have been established, and have the advantage of 3D growth of normal and cancer tissue, recapitulating copy number and mutation spectra, as well as other physiologically relevant aspects of disease progression *in vitro* [150–158]. Organoids can be established in culture from needle biopsies within a relative short time period, and have also been generated from **circulating tumor cells(CTCs)** [156]. Organoids can serve as a model system to perform high-throughput screens within a clinically relevant time frame: in a larger precision oncology study, organoids were established from fresh tissue available from 38% of 145 patients [158]. In addition, PDX models were successfully established from these organoids in 19 of 22 attempts [158]. High-throughput drug screens were performed (160 drugs, including chemotherapy and targeted therapy) in 2D cultures from four patients, and the best 'hits' (drugs that most effectively decreased cell viability *in vitro*) were verified in 3D organoid cultures. Selected treatments were then tested in combination to identify effective combination therapies. The best hits of single and combination therapies from two patients were further tested in 3D organoids and PDX models, validating tumor responses *in vivo*, and compared with the efficacy of current patient treatments [158]. In addition, potential drug

Box 2. Valuable Models for Guiding Precision Therapy

In addition to PDX and organoids, **conditional reprogramming(CR)** of patient-derived primary epithelial tumor cells or organotypic cultures are among the possibilities to test selected treatments. Patient-derived cell lines via CR can be rapidly established [210] and are suitable to screen large drug libraries [211,212], or to test drug combinations to overcome acquired resistance to targeted therapy [213]. While phenotypic features and the genetic heterogeneity of the original tumor are retained in short-term CR cultures, the enrichment of specific cell populations, including nontransformed epithelial cells in this model, requires the crossverification of pheno- and genotypic features of donor tissues and CR cells. The lack of a 3D environment may be partially overcome by culturing CR cells in sophisticated 3D artificial organotypic cultures [214], of which fully automated 1536-well high-throughput screening platforms have recently been described [215]. Although these artificial microenvironments lack the heterogeneity observed in patient tumors, they may allow testing tumor cell behaviors in the context of different organ microenvironments shown to influence drug responses [215].

In organotypic slice cultures [216] or organ explants [217], either thin slices of the tumor sample or minced tumor tissues are maintained in culture. The biggest advantage of these culture types is that they retain cancer associated stromal cells, preserve tumor–stroma interactions, signaling pathways, and gene expression profiles [218]. Improvements include the use of autologous serum and patient-specific stromal-matrix proteins to more closely resemble individual microenvironmental conditions [219], aiming to accurately predict responses to anticancer drugs. However, not all tissues are suitable to generating thin slices (e.g., soft, mucinous, or fatty tissue), where firm tissue consistency is required [220,221]. Another drawback of slice cultures is the loss of viability within 5–7 days [218]. Given that the median time frame for molecular profiling and data processing in precision oncology trials is 2–4 weeks, the method is not suitable for testing genomics-guided therapies derived from the same biopsy, unless combined with other models. Recently, organotypic slice cultures established from pancreatic ductal adenocarcinoma PDX models were used to screen against clinically relevant drug regimens in a 96-well format, demonstrating consistency between sensitivity of organotypic cultures and the clinical responses of donor patients [222].

toxicities were evaluated (e.g., **trametinib** and **afatinib** led to significant weight loss in mice) [158]. For both cases, the combination of targeted therapies was superior over standard chemotherapy [158]. This study underscores the potential use of functional screens in patients where no targeted therapies are available, and the possibility of identifying effective drugs and/or drug combinations [158]. The study further demonstrated that therapy recommendations could be retrieved within a clinically relevant time frame (between 7 and 13 weeks) [158], highlighting the importance of defining regulatory routes that might simplify off-label drug access for late-stage patients, who are often not eligible for clinical trial enrollment (see Outstanding Questions). Therefore, the combination of molecular profiling (genomics and transcriptomics) and functional testing holds promise for determining effective combination therapies for individual patients with cancer.

Clinical Management of Therapy Resistance in the Precision Oncology Era

Although genomics-guided therapy is associated with prolonged progression-free survival, patients with cancer can generally develop resistance to drugs within 6–12 months, even when trunk mutations are targeted (Figure 2). Such **acquired resistance** to targeted drugs may be explained by the selection of resistant cancer cells that are present before therapy or that are generated *de novo* as a result of genomic instability. Sequential therapy of second-, third-, and even fourth-generation inhibitors that specifically address emerging mutations within the original target (i.e., EGFR inhibitors [159–163]) or drugs targeting newly established driver mutations (e.g., MET amplification in EGFR inhibitor-resistant CRCs [164]) have been used to overcome resistance. Alternatively, combined inhibition of multiple pathways [165] or vertical pathway inhibition (targeting multiple proteins within one pathway) have been suggested to prolong progression-free survival by pre-empting resistance in a pro-active manner and inhibiting the selection of resistant clones [166]. As an example, targeted therapy has been used against BRAF and MEK in melanoma to counteract feedback regulatory loops and achieve efficient pathway inhibition [18]. Although these approaches are suitable to prolong progression-free survival, management of resistant disease remains dismal and/or short-lived, partly because multiple resistance mutations (in addition to other mechanisms) can occur simultaneously. This heterogeneity was recently demonstrated in a patient with CRC, where a MEK1 mutation was detected in a liver metastasis biopsy, conferring resistance to **cetuximab** [167]. Treatment of this patient with the combination of **panitumumab** and trametinib resulted in regression of the biopsied liver metastasis; however, other liver metastases progressed while on treatment. Analysis of **circulating tumor DNA(ctDNA)** revealed a previously unrecognized KRAS mutation, which was later found in a biopsy from a progressing liver metastasis, highlighting the challenges of combating polyclonal resistance [167]. To address these issues, more general approaches have been suggested, such as interfering with tumor evolutionary programs, for instance, by increasing genomic instability to lethal levels (e.g., PARP inhibitors in tumors with homologous recombination deficit) [25]. These have been promising strategies to exploit genome instability, as one driving force of heterogeneity, therapeutically [25]. However, even this approach is accompanied by resistance [25]. Finally, there is growing interest in applying intermittent treatment doses, or adjusting drug doses to limit the **evolutionary pressure** imposed by a given drug [25]. Such adaptive therapy may serve to maintain a drug-sensitive population, with the goal of stabilizing the tumor size rather than eliminating the tumor. Preliminary evidence for the putative benefit of such drug 'holidays' comes from patients with CRC receiving EGFR therapy [168], which was also suggested for melanoma [169,170] and, recently, breast cancer models [171]. Advances in the characterization of ctDNA now make it possible to carefully evaluate these different methods to combat genetic resistance to targeted drugs in the clinical setting [167,168,172–175]. Furthermore, the reappearance of the driver mutation or the appearance of previously undetected mutations associated with resistance to targeted therapy in the blood can enable early detection of therapy failure (before tumor

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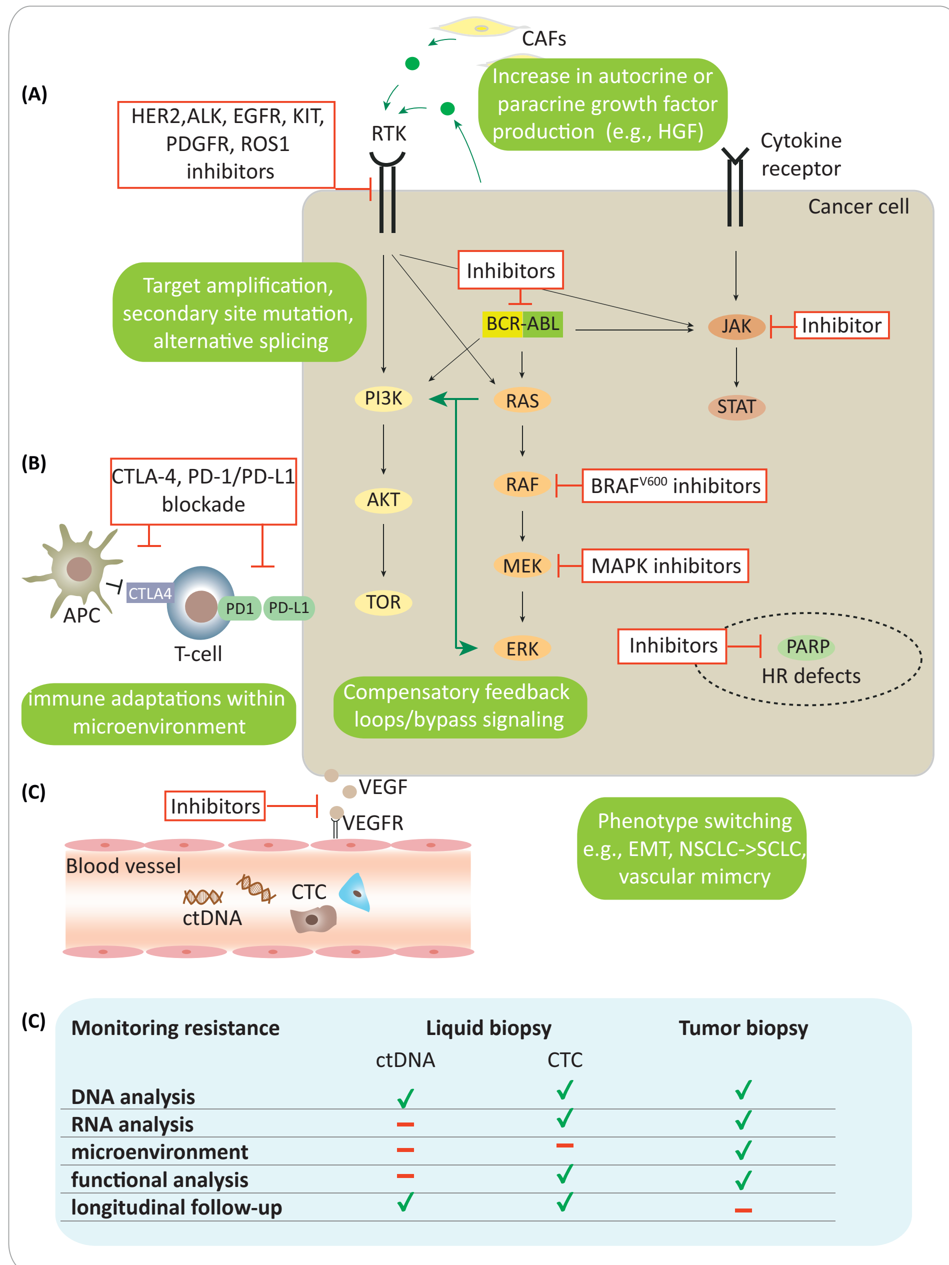

Monitoring resistance	Liquid biopsy: ctDNA	Liquid biopsy: CTC	Tumor biopsy
DNA analysis	✓	✓	✓
RNA analysis	–	✓	✓
microenvironment	–	–	✓
functional analysis	–	✓	✓
longitudinal follow-up	✓	✓	–

Trends in Molecular Medicine

Figure 2. Targeted Therapy and Mechanisms of Acquired Resistance. Major classes of current US Food and Drug Administration (FDA)-approved targeted therapies include (A) drugs targeting oncogenic drivers or drugs targeting other genetic vulnerabilities, such as poly (ADP-ribose) polymerase (PARP) inhibitors in tumors with homologous recombination (HR) deficiency; (B) drugs that aim to increase the antitumor immune response or (C) inhibit neoangiogenesis. Numerous genetic as well as nongenetic mechanisms (green boxes) of acquired resistance to targeted therapeutics are known, which likely act in concert to mediate the largely short-lived response to these drugs. (D) Routes to monitor emerging resistance as well as the suitability of liquid biopsies compared with tumor biopsies to inform second-line therapy are displayed. Abbreviations: APC, antigen-presenting cell; CAFs, cancer-associated fibroblasts; CTC, circulating tumor cell; ctDNA, circulating tumor DNA; EMT, epithelial–mesenchymal transition; NSCLC, non-small cell lung cancer; RTK, receptor tyrosine kinase; SCLC, small cell lung cancer.

imaging indicates relapse [173,174]), and might also identify new potential drivers suitable for guiding second-line therapy [167,168,172–175], a promising approach for the management of resistant disease.

In addition to the Darwinian-like evolution of genomic alterations in resistant clones under therapeutic pressure, tumors evolve resistance to therapy by adaptively rewiring transduction networks to support the signaling processes required for survival and/or tumor maintenance in a postgenomic, transient, and dynamic manner [176] (Figure 2; Tables S2F and S3 in the supplemental information online). One such example is the ability of BRAF-inhibitor sensitive $MITF^{high}/AXL^{low}$ melanoma cell populations to readily switch into a $MITF^{low}/AXL^{high}$ drug-resistant population [177,178]; these cells have been shown by single-cell RNA-seq to pre-exist in treatment-naïve samples [179]. Another example of such phenotypic plasticity was reported in $ER^{+}HER2^{-}$ breast cancers [180]. Following multiple courses of therapy, $HER2^{+}$ cells lacking gene amplification have been found to emerge in addition to $HER2^{-}$ cells in patient tumors and among CTCs; this may be indicative of nongenetic mechanisms involved in HER2 upregulation [180]. Furthermore, characterization of patient-derived CTCs revealed that, although both $HER2^{+}$ and $HER2^{-}$ CTCs maintained tumor-initiating potential in orthotopic xenograft experiments, $HER2^{+}$ cells were highly proliferative and sensitive to chemotherapy, whereas $HER2^{-}$ CTCs exhibited a slow proliferation rate, upregulated NOTCH signaling, and were chemoresistant [180]. Of note, cells could interconvert between a $HER2^{+}$-chemosensitive, and a $HER2^{-}$-chemoresistant state, and, with chemotherapy, a $HER2^{+}$ population could shift towards a $HER2^{-}$ phenotype [180]. Accordingly, targeting NOTCH in combination with chemotherapy (**paclitaxel**) suppressed tumor growth in mice, whereas either treatment alone was inefficient in limiting tumor growth [180]. Therefore, rapid interconversion of CTCs between distinct functional states may contribute to acquired resistance to therapy; consequently, it is possible that combination therapy might improve therapeutic responses and delay the onset of resistance. Other nongenetic routes to escape targeted therapy can include the epithelial–mesenchymal transition (EMT), a developmental program that is often hijacked by cancer cells [181]. Preclinical models have associated the EMT program with chemoresistance [182,183] and a subset of patients with non-small cell lung cancer (NSCLC) resistant to EGFR-targeted therapy were shown to display an increase in ‘mesenchymal’ cancer cells [184]. Additionally, tumors can also undergo ‘histological transformations’, as shown for patients with EGFR inhibitor-resistant NSCLC whose tumors converted to a small cell lung cancer (SCLC) phenotype, escaping therapy [184]. Finally, vascular mimicry, a phenomenon where tumor cells transdifferentiate into endothelial-like cells that can form matrix-rich, vascular-like, perfused channels, has also been proposed to contribute to resistance to antiangiogenic therapy, but further testing of this mechanism is required to better understand its role in resistance [185].

Multiple resistance mechanisms (genetic and nongenetic) can act in concert to confer resistance to targeted therapy (Figure 2). Indeed, in a recent melanoma study [186], analysis of patient-matched melanoma tumors biopsied before therapy and during disease progression demonstrated that, in contrast to heterogeneous genetic mechanisms that could result in acquired resistance, transcriptomic signatures were highly recurrent in serial biopsies; these indicated that a multitude of genetic and epigenetic events within the tumor compartment converged on specific genes (*c-MET*, *LEF1*, and *YAP1*) and pathways to mediate resistance, consistent with a **canalization evolutionary process** [186]. Additionally, this acquired resistance signature correlated with changes in the tumor immune microenvironment, including depletion of intratumoral $CD8^{+}$ T cells, exhaustion of tumor-reactive $CD8^{+}$ T cells, and loss of antigen presentation; these have been previously linked to resistance to anti-PD-1 salvage therapy in biopsies of patients with melanoma, in support of the presumed role of $CD8^{+}$ T cell exhaustion in the development of resistance [77,186]. Furthermore, these findings suggest that

first-line therapy with immune-checkpoint inhibitors followed by BRAF-targeted therapy upon relapse in BRAF-mutant melanomas, may be superior over BRAF-inhibitor frontline therapy, a hypothesis that is currently being tested in a clinical trial (NCT02224781).

The mere follow-up of genomic alterations in ctDNA is unlikely to result in the satisfactory management of resistant disease because adaptive mechanisms on transcriptional and signaling levels can be missed by genomic analysis alone (Figure 2). The use of CTCs may be preferred to monitor resistant disease, because they have a better overall prognostic value, provide an additional opportunity to characterizing genetic and nongenetic ITH, and are suitable for functional studies [187]. The previously described *in vitro* and/or *ex vivo* models may serve to detect relevant signaling nodes and counteract adaptive signaling by combinatorial therapy to delay onset of resistance. As suggested for the selection of first-line therapies, a comprehensive genomic, transcriptomic, and functional analysis of resistant disease is required to overcome these challenges. In that case, it may be possible to mine the increasingly available 'omics data from large cohorts of patients and identify genetic interactions that mediate network-wide signaling alterations conferring resistance. Such an approach could exploit the much less-studied type of genetic interactions, termed **synthetic rescues** (SRs), also known as **suppression interactions** [188–192]. Such SRs denote a functional interaction between two genes where the targeting of one gene is compensated by the altered activity of another gene (termed the 'rescuer gene'); this can restore and rescue cell fitness, leading to drug resistance. Similar to SLs, SR interactions could in principle be identified by mining 'omics data from large cohorts of pretreated tumor samples, taking into consideration that functional alterations might already be occurring during the natural evolution of tumorigenic populations.

Concluding Remarks: The Road Ahead

Despite the limitations of genomics-driven cancer therapy, current efforts have demonstrated that this approach has the potential to improve clinical outcomes, although at this time, only for a minority of patients. Future precision oncology treatment will need to include the broader landscape of genetic and epigenetic changes that take place in a tumor. Those include the tumor microenvironment, which comprises metabolic as well as immunological changes, in addition to the influence exerted by the microbiome. Consequently, we must understand that targeting a single pathway in a tumor is, in most cases, not sufficient to achieve a sustained response. We have outlined how the inclusion of transcriptomic data could serve to stratify patients, suggest combination therapies, or define novel vulnerabilities to improve upon current precision oncology trials (Figure 3 and Box 3). Patient-derived *ex vivo* and/or *in vivo* models can serve to identify the toxicity and efficacy of combination therapies, link genomics to drug responses, and, when such information is included in a mineable database, could serve to inform therapeutic decision-making. The limiting factor in performing multiple 'omics approaches and functional testing, aside from cost, is tissue availability, highlighting the need to improve methods to retrieve sufficient tumor material. As such, the use of CTCs is appealing, because these can be non-invasively isolated and may better reflect the prevailing ITH (see Outstanding Questions).

At present, ITH appears to be the biggest obstacle we need to overcome to achieve a sustained therapeutic response. With rapid technological advances, we are acquiring the toolbox to comprehensively characterize the complex heterogeneity of tumors at the single cell level (Box 4). However, computing the data from different platforms of 1000 cells or more remains a challenge. Among the questions that emerge is whether it will be possible to develop multiplex-based approaches, or use machine-learning techniques to compute tumor trends to predict subcluster and clonal behaviors (see Outstanding Questions).

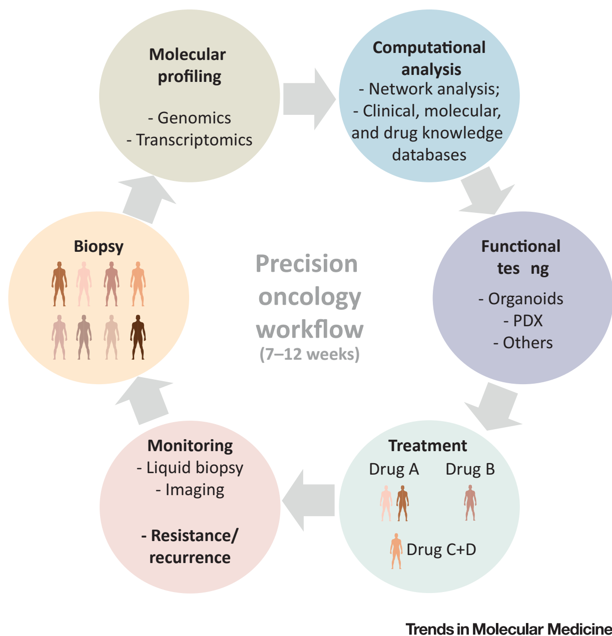

Figure 3. Precision Oncology Workflow. In this proposed precision oncology workflow, patient molecular profiles are used to suggest first-line (combination) therapies. Functional models serve to test the safety and efficacy of selected drugs or screen for drugs in cases when molecular profiling is not informative. Patients are closely monitored during the course of therapy to detect resistance. Molecular profiling, computational analysis, and functional testing of resistant tumors are repeated upon onset of resistance to determine second-line therapy options. Abbreviations: PDX, patient-derived xenografts.

Among the first series of steps needed to provide important insights into the complex nature of malignancies, the inclusion of technological advances and computational approaches in clinical trials stands out. We should aim to map and analyze the impact of complex genetic and nongenetic heterogeneity during cancer progression and therapy regimens to help pave the

Box 3. Clinicians Corner

Targeted drugs are largely based on defined drugs (small molecules or biologics) designed to inhibit specific oncogenic mutations or target key regulatory nodes that drive tumorigenesis or underlie cancer vulnerabilities. Usually, the presence of drug-specific biomarkers enables stratification of patients for therapy and monitoring drug effectiveness. Given the success of targeted therapies, together with the recognition that different tumor types share driver and/or master regulators, the use of drugs that target common regulatory nodes in a histology-agnostic manner is being evaluated in clinical trials.

Clinical experience with genomics-guided cancer therapy supports the notion that genomic profiling can improve patient outcomes. The degree of success can be associated with the ability to verify the role of a targeted mutation and/or alteration in tumor development, or vice versa; that is, whether the drug can efficiently attenuate the tumorigenic effects orchestrated by the genetic alteration.

Precision oncology cannot be limited to genetics to predict responses to therapy, neither can it be limited to a single 'omics-based approach. Multiple drivers can underlie tumor heterogeneity, which in turn can confer resistance, metastasis, and dormancy. It also requires the targeting of master regulators that are influenced by epigenetic and microenvironmental-based pathways. The inclusion of additional platforms, of which at this time the most suitable appears to be transcriptomics, (with future inclusion of metabolome and microbiome analysis), is highly desirable to identify such master regulators and design more-precise therapeutic modalities.

Advances in computational approaches to mine and integrate the multitude of data sets that become available to us are expected to allow better subclassification of patient cohorts into subpopulations able to respond to a given therapy. In addition, integration of multiple data platforms is expected to drive the identification of novel vulnerabilities, which will further add to the armamentarium of current anticancer therapies. This may likely result in newer stratification methods to identify patients who might benefit from a given precision oncology approach.

The implementation of powerful *ex vivo* or improved *in vivo* PDX models in the planning of clinical practice is encouraged. By using these models, a multitude of available drugs and predictive biomarkers might be assessed to evaluate therapeutic options, as well as their combinations and delivery sequence and/or approaches.

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Box 4. Available Tools to Decipher Intratumor Heterogeneity

ITH manifests as differences in genetic, epigenetic, and signaling networks of individual tumor cells coupled with heterogeneity within the stromal compartment [25,27]. While ITH is influenced by the inherent tumor genetic make-up, epigenetic states (influenced by the location of tumor cells) and microenvironmental factors have been recognized as being equally important in dictating the diverse cellular states that drive the primary tumor or its metastatic lesions. Those include the proximity to endothelial cells, cancer-associated fibroblasts, immune cells, as well as the nutrient and oxygen availability and biophysical properties of the extracellular matrix [28,207]. The development of single cell separation and analysis methods has provided critical insights into the complex heterogeneity of tumors, where multiple clusters of genetically [223] and (more so) phenotypically distinct populations exist. The resulting cell-to-cell variability in stemness and differentiation programs, proliferation. and quiescence markers, as well as in the expression of predictive biomarkers [179,180,224,225] define the propensity of a tumor for therapeutic resistance, metastasis, and dormancy.

Active research is being undertaken to overcome limitations in single cell methods. These include (i) improving throughput of single cell DNA- [226–228] and single cell RNA-seq [229–231] methods; (ii) limiting sampling bias, which can partially be resolved with liquid biopsies [187]; and (iii) minimizing tissue dissociation and disruption of spatial information [232].

way towards the next generation of cancer therapeutics, capable of overcoming the challenges imposed by the defying intratumor heterogeneity of cancers.

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References

1. Vargas, A.J. and Harris, C.C. (2016) Biomarker development in the precision medicine era: lung cancer as a case study. *Nat. Rev. Cancer* 16, 525–537
2. Hyman, D.M. *et al.* (2017) Implementing genome-driven oncology. *Cell* 168, 584–599
3. Prasad, V. (2016) Perspective: the precision-oncology illusion. *Nature* 537, S63
4. Tannock, I.F. and Hickman, J.A. (2016) Limits to personalized cancer medicine. *N. Engl. J. Med.* 375, 1289–1294
5. Voest, E.E. and Bernards, R. (2016) DNA-guided precision medicine for cancer: a case of irrational exuberance? *Cancer Discov.* 6, 130–132
6. Karnoub, A.E. and Weinberg, R.A. (2008) Ras oncogenes: split personalities. *Nat. Rev. Mol. Cell Biol.* 9, 517–531
7. Sidransky, D. (1997) Nucleic acid-based methods for the detection of cancer. *Science* 278, 1054–1059
8. Soussi, T. (2010) The history of p53: a perfect example of the drawbacks of scientific paradigms. *EMBO Rep.* 11, 822–826
9. Chapman, P.B. *et al.* (2011) Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N. Engl. J. Med.* 364, 2507–2516
10. Cobleigh, M.A. *et al.* (1999) Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J. Clin. Oncol.* 17, 2639–2648
11. Druker, B.J. *et al.* (2001) Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N. Engl. J. Med.* 344, 1031–1037
12. Kwak, E.L. *et al.* (2010) Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N. Engl. J. Med.* 363, 1693–1703
13. Lynch, T.J. *et al.* (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N. Engl. J. Med.* 350, 2129–2139
14. Saltz, L.B. *et al.* (2004) Phase II trial of cetuximab in patients with refractory colorectal cancer that expresses the epidermal growth factor receptor. *J. Clin. Oncol.* 22, 1201–1208
15. Ledermann, J. *et al.* (2014) Olaparib maintenance therapy in patients with platinum-sensitive relapsed serous ovarian cancer: a preplanned retrospective analysis of outcomes by BRCA status in a randomised phase 2 trial. *Lancet Oncol.* 15, 852–861
16. Cancer Genome Atlas, N (2015) Genomic classification of cutaneous melanoma. *Cell* 161, 1681–1696
17. Hauschild, A. *et al.* (2012) Dabrafenib in BRAF-mutated metastatic melanoma: a multicentre, open-label, phase 3 randomised controlled trial. *Lancet* 380, 358–365
18. Johnson, D.B. *et al.* (2016) Emerging targeted therapies for melanoma. *Expert Opin. Emerg. Drugs* 21, 195–207
19. Tang, X.H. and Gudas, L.J. (2011) Retinoids, retinoic acid receptors, and cancer. *Annu. Rev. Pathol.* 6, 345–364
20. Jones, P.A. *et al.* (2016) Targeting the cancer epigenome for therapy. *Nat. Rev. Genet.* 17, 630–641
21. Ferrara, N. and Kerbel, R.S. (2005) Angiogenesis as a therapeutic target. *Nature* 438, 967–974
22. Miller, J.F. and Sadelain, M. (2015) The journey from discoveries in fundamental immunology to cancer immunotherapy. *Cancer Cell* 27, 439–449
23. Cancer Genome Atlas Research, N *et al.* (2013) The Cancer Genome Atlas Pan-Cancer analysis project. *Nat. Genet.* 45, 1113–1120
24. International Cancer Genome, C *et al.* (2010) International network of cancer genome projects. *Nature* 464, 993–998
25. McGranahan, N. and Swanton, C. (2017) Clonal heterogeneity and tumor evolution: past, present, and the future. *Cell* 168, 613–628
26. Hanahan, D. and Weinberg, R.A. (2011) Hallmarks of cancer: the next generation. *Cell* 144, 646–674
27. Marusyk, A. *et al.* (2012) Intra-tumour heterogeneity: a looking glass for cancer? *Nat. Rev. Cancer* 12, 323–334

Outstanding Questions

Can the integration of transcriptomic information in clinical decision-making improve patient outcomes? There is a strong biological rationale to support this hypothesis. However, the necessary steps to enable integration of distinct data platforms and to rigorously test the clinical value of transcriptomics remain to be assessed.

How many platforms within the tumor microenvironment (immune, microbiome, and metabolome) need to be integrated to provide a multidimensional map of the complex tumor landscape? Will it allow a more accurate prediction of regulatory nodes and possible therapeutic modalities?

Preclinical patient-derived models (PDX and/or organoids) demonstrate a strong correlation with patient outcomes. Can regulatory guidelines be defined to render the use of off-label drugs in the clinic easier when based on positive outcomes taken from such models?

How many single cells (and from how many tumors) will we need to sequence to accurately depict the complex heterogeneity observed within patients? Can machine-learning approaches be used to predict the behavior of a tumor?

Are CTCs a reliable source to comprehensively map tumor heterogeneity and do they accurately define phenotypic heterogeneity of advanced disease? How can we improve the isolation of these valuable cells?

How can ITH be exploited therapeutically?

28. Tabassum, D.P. and Polyak, K. (2015) Tumorigenesis: it takes a village. *Nat. Rev. Cancer* 15, 473–483
29. Hofree, M. *et al.* (2016) Challenges in identifying cancer genes by analysis of exome sequencing data. *Nat. Commun.* 7, 12096
30. Forbes, S.A. *et al.* (2017) COSMIC: somatic cancer genetics at high-resolution. *Nucleic Acids Res.* 45, D777–D783
31. Futreal, P.A. *et al.* (2004) A census of human cancer genes. *Nat. Rev. Cancer* 4, 177–183
32. Garraway, L.A. and Lander, E.S. (2013) Lessons from the cancer genome. *Cell* 153, 17–37
33. Hofree, M. *et al.* (2013) Network-based stratification of tumor mutations. *Nat. Methods* 10, 1108–1115
34. Lawrence, M.S. *et al.* (2014) Discovery and saturation analysis of cancer genes across 21 tumour types. *Nature* 505, 495–501
35. Leiserson, M.D. *et al.* (2015) Pan-cancer network analysis identifies combinations of rare somatic mutations across pathways and protein complexes. *Nat. Genet.* 47, 106–114
36. Vogelstein, B. *et al.* (2013) Cancer genome landscapes. *Science* 339, 1546–1558
37. Hoadley, K.A. *et al.* (2014) Multiplatform analysis of 12 cancer types reveals molecular classification within and across tissues of origin. *Cell* 158, 929–944
38. Hartmaier, R.J. *et al.* (2017) Genomic analysis of 63,220 tumors reveals insights into tumor uniqueness and targeted cancer immunotherapy strategies. *Genome Med.* 9, 16
39. Dienstmann, R. *et al.* (2015) Database of genomic biomarkers for cancer drugs and clinical targetability in solid tumors. *Cancer Discov.* 5, 118–123
40. Rubio-Perez, C. *et al.* (2015) *In silico* prescription of anticancer drugs to cohorts of 28 tumor types reveals targeting opportunities. *Cancer Cell* 27, 382–396
41. Biankin, A.V. *et al.* (2015) Patient-centric trials for therapeutic development in precision oncology. *Nature* 526, 361–370
42. Rashdan, S. and Gerber, D.E. (2016) Going into BATTLE: umbrella and basket clinical trials to accelerate the study of biomarker-based therapies. *Ann. Transl. Med.* 4, 529
43. Le Tourneau, C. *et al.* (2016) Treatment algorithms based on tumor molecular profiling: the essence of precision medicine trials. *J. Natl. Cancer Inst.* 108, djv362
44. Massard, C. *et al.* (2017) High-throughput genomics and clinical outcome in hard-to-treat advanced cancers: results of the MOSCATO 01 trial. *Cancer Discov.* 7, 586–595
45. Le Tourneau, C. *et al.* (2015) Molecularly targeted therapy based on tumour molecular profiling versus conventional therapy for advanced cancer (SHIVA): a multicentre, open-label, proof-of-concept, randomised, controlled phase 2 trial. *Lancet Oncol.* 16, 1324–1334
46. Wheler, J.J. *et al.* (2016) Cancer Therapy directed by comprehensive genomic profiling: a single center study. *Cancer Res.* 76, 3690–36701
47. Lopez-Chavez, A. *et al.* (2015) Molecular profiling and targeted therapy for advanced thoracic malignancies: a biomarker-derived, multiarm, multihistology phase II basket trial. *J. Clin. Oncol.* 33, 1000–1007
48. Stockley, T.L. *et al.* (2016) Molecular profiling of advanced solid tumors and patient outcomes with genotype-matched clinical trials: the Princess Margaret IMPACT/COMPACT trial. *Genome Med.* 8, 109
49. Hyman, D.M. *et al.* (2015) Vemurafenib in multiple nonmelanoma cancers with BRAF V600 mutations. *N. Engl. J. Med.* 373, 726–736
50. Kopetz, S. *et al.* (2010) PLX4032 in metastatic colorectal cancer patients with mutant BRAF tumors. *J. Clin. Oncol.* 28, 3534
51. Kaufman, B. *et al.* (2015) Olaparib monotherapy in patients with advanced cancer and a germline BRCA1/2 mutation. *J. Clin. Oncol.* 33, 244–250
52. Al-Ahmadie, H. *et al.* (2014) Synthetic lethality in ATM-deficient RAD50-mutant tumors underlies outlier response to cancer therapy. *Cancer Discov.* 4, 1014–1021
53. Banerji, U. *et al.* (2014) Abstract LB-66: results of two phase I multicenter trials of AZD5363, an inhibitor of AKT1, 2 and 3: biomarker and early clinical evaluation in Western and Japanese patients with advanced solid tumors. *Cancer Res.* 73 (8 Suppl), LB–66
54. Cappuzzo, F. *et al.* (2006) HER2 mutation and response to trastuzumab therapy in non-small-cell lung cancer. *N. Engl. J. Med.* 354, 2619–2621
55. Hammerman, P.S. *et al.* (2011) Mutations in the DDR2 kinase gene identify a novel therapeutic target in squamous cell lung cancer. *Cancer Discov.* 1, 78–89
56. Iyer, G. *et al.* (2012) Genome sequencing identifies a basis for everolimus sensitivity. *Science* 338, 221
57. Liao, R.G. *et al.* (2013) Inhibitor-sensitive FGFR2 and FGFR3 mutations in lung squamous cell carcinoma. *Cancer Res.* 73, 5195–5205
58. Serra, V. *et al.* (2013) Clinical response to a lapatinib-based therapy for a Li-Fraumeni syndrome patient with a novel HER2V659E mutation. *Cancer Discov.* 3, 1238–1244
59. Subbiah, V. *et al.* (2014) Targeted therapy by combined inhibition of the RAF and mTOR kinases in malignant spindle cell neoplasm harboring the KIAA1549-BRAF fusion protein. *J. Hematol. Oncol.* 7, 8
60. Van Allen, E.M. *et al.* (2015) Genomic correlate of exceptional erlotinib response in head and neck squamous cell carcinoma. *JAMA Oncol.* 1, 238–244
61. Wagle, N. *et al.* (2014) Response and acquired resistance to everolimus in anaplastic thyroid cancer. *N. Engl. J. Med.* 371, 1426–1433
62. Wagle, N. *et al.* (2014) Activating mTOR mutations in a patient with an extraordinary response on a phase I trial of everolimus and pazopanib. *Cancer Discov.* 4, 546–553
63. Chen, J.C. *et al.* (2014) Identification of causal genetic drivers of human disease through systems-level analysis of regulatory networks. *Cell* 159, 402–414
64. Luo, J. *et al.* (2009) Principles of cancer therapy: oncogene and non-oncogene addiction. *Cell* 136, 823–837
65. Alizadeh, A.A. *et al.* (2015) Toward understanding and exploiting tumor heterogeneity. *Nat. Med.* 21, 846–853
66. Robinson, D. *et al.* (2015) Integrative clinical genomics of advanced prostate cancer. *Cell* 161, 1215–1228
67. Fishbein, L. *et al.* (2017) Comprehensive molecular characterization of pheochromocytoma and paraganglioma. *Cancer Cell* 31, 181–193
68. Kato, S. *et al.* (2017) RET Aberrations in diverse cancers: next-generation sequencing of 4,871 patients. *Clin. Cancer Res.* 23, 1988–1997
69. Stransky, N. *et al.* (2014) The landscape of kinase fusions in cancer. *Nat. Commun.* 5, 4846
70. Alvarez, M.J. *et al.* (2016) Functional characterization of somatic mutations in cancer using network-based inference of protein activity. *Nat. Genet.* 48, 838–947
71. Tian, S. *et al.* (2013) A combined oncogenic pathway signature of BRAF, KRAS and PI3KCA mutation improves colorectal cancer classification and cetuximab treatment prediction. *Gut* 62, 540–549
72. Vecchione, L. *et al.* (2016) A vulnerability of a subset of colon cancers with potential clinical utility. *Cell* 165, 317–330
73. Lord, C.J. and Ashworth, A. (2016) BRCAness revisited. *Nat. Rev. Cancer* 16, 110–120
74. Konstantinopoulos, P.A. *et al.* (2010) Gene expression profile of BRCAness that correlates with responsiveness to chemotherapy and with outcome in patients with epithelial ovarian cancer. *J. Clin. Oncol.* 28, 3555–3561
75. Larsen, M.J. *et al.* (2013) Classifications within molecular subtypes enables identification of BRCA1/BRCA2 mutation carriers by RNA tumor profiling. *PLoS One* 8, e64268
76. Van Allen, E.M. *et al.* (2015) Genomic correlates of response to CTLA–4 blockade in metastatic melanoma. *Science* 350, 207–211
77. Hugo, W. *et al.* (2016) Genomic and transcriptomic features of response to anti-PD-1 therapy in metastatic melanoma. *Cell* 165, 35–44

78. Ewing, A.D. *et al.* (2015) Combining tumor genome simulation with crowdsourcing to benchmark somatic single-nucleotide-variant detection. *Nat. Methods* 12, 623–630
79. Zehir, A. *et al.* (2017) Mutational landscape of metastatic cancer revealed from prospective clinical sequencing of 10,000 patients. *Nat. Med.* 23, 703–713
80. Ciriello, G. *et al.* (2013) Emerging landscape of oncogenic signatures across human cancers. *Nat. Genet.* 45, 1127–1133
81. Wang, B. *et al.* (2014) Similarity network fusion for aggregating data types on a genomic scale. *Nat. Methods* 11, 333–337
82. Meng, C. *et al.* (2016) Dimension reduction techniques for the integrative analysis of multi-omics data. *Brief. Bioinform.* 17, 628–641
83. Way, G.P. and Greene, C.S. (2017) Extracting a biologically relevant latent space from cancer transcriptomes with variational autoencoders. *bioRxiv* Published online August 11, 2017. http://dx.doi.org/10.1101/174474
84. Ching, T. *et al.* (2017) Opportunities and obstacles for deep learning in biology and medicine. *bioRxiv* Published online May 28, 2017. http://dx.doi.org/10.1101/142760
85. Curtis, C. *et al.* (2012) The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* 486, 346–352
86. Dienstmann, R. *et al.* (2017) Consensus molecular subtypes and the evolution of precision medicine in colorectal cancer. *Nat. Rev. Cancer* 17, 79–92
87. Le, D.T. *et al.* (2017) Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. *Science* 357, 409–413
88. Ng, P.C. and Henikoff, S. (2003) SIFT: predicting amino acid changes that affect protein function. *Nucleic Acids Res.* 31, 3812–3814
89. Reva, B. *et al.* (2011) Predicting the functional impact of protein mutations: application to cancer genomics. *Nucleic Acids Res.* 39, e118
90. Chang, M.T. *et al.* (2016) Identifying recurrent mutations in cancer reveals widespread lineage diversity and mutational specificity. *Nat. Biotechnol.* 34, 155–163
91. Gao, J. *et al.* (2017) 3D clusters of somatic mutations in cancer reveal numerous rare mutations as functional targets. *Genome Med.* 9, 4
92. Miller, M.L. *et al.* (2015) Pan-cancer analysis of mutation hotspots in protein domains. *Cell Syst.* 1, 197–209
93. Jia, P. and Zhao, Z. (2014) VarWalker: personalized mutation network analysis of putative cancer genes from next-generation sequencing data. *PLoS Comput. Biol.* 10, e1003460
94. Lawrence, M.S. *et al.* (2013) Mutational heterogeneity in cancer and the search for new cancer-associated genes. *Nature* 499, 214–218
95. Polak, P. *et al.* (2015) Cell-of-origin chromatin organization shapes the mutational landscape of cancer. *Nature* 518, 360–364
96. Mermel, C.H. *et al.* (2011) GISTIC2.0 facilitates sensitive and confident localization of the targets of focal somatic copy-number alteration in human cancers. *Genome Biol.* 12, R41
97. Wendl, M.C. *et al.* (2011) PathScan: a tool for discerning mutational significance in groups of putative cancer genes. *Bioinformatics* 27, 1595–1602
98. Glaab, E. *et al.* (2012) EnrichNet: network-based gene set enrichment analysis. *Bioinformatics* 28, i451–i457
99. Vandin, F. *et al.* (2011) Algorithms for detecting significantly mutated pathways in cancer. *J. Comput. Biol.* 18, 507–522
100. Vanunu, O. *et al.* (2010) Associating genes and protein complexes with disease via network propagation. *PLoS Comput. Biol.* 6, e1000641
101. Kim, J.W. *et al.* (2016) Characterizing genomic alterations in cancer by complementary functional associations. *Nat. Biotechnol.* 34, 539–546
102. Knijnenburg, T.A. *et al.* (2016) Logic models to predict continuous outputs based on binary inputs with an application to personalized cancer therapy. *Sci. Rep.* 6, 36812
103. Ciriello, G. *et al.* (2012) Mutual exclusivity analysis identifies oncogenic network modules. *Genome Res.* 22, 398–406
104. Kim, Y.A. *et al.* (2017) WeSME: uncovering mutual exclusivity of cancer drivers and beyond. *Bioinformatics* 33, 814–821
105. Leiserson, M.D. *et al.* (2016) A weighted exact test for mutually exclusive mutations in cancer. *Bioinformatics* 32, i736–i745
106. Miller, C.A. *et al.* (2011) Discovering functional modules by identifying recurrent and mutually exclusive mutational patterns in tumors. *BMC Med. Genomics* 4, 34
107. Vandin, F. *et al.* (2012) *De novo* discovery of mutated driver pathways in cancer. *Genome Res.* 22, 375–385
108. Vandin, F. *et al.* (2016) On the sample complexity of cancer pathways identification. *J. Comput. Biol.* 23, 30–41
109. Huang, F.W. *et al.* (2013) Highly recurrent TERT promoter mutations in human melanoma. *Science* 339, 957–959
110. Ainscough, B.J. *et al.* (2016) DoCM: a database of curated mutations in cancer. *Nat. Methods* 13, 806–807
111. Griffith, M. *et al.* (2017) CIViC is a community knowledgebase for expert crowdsourcing the clinical interpretation of variants in cancer. *Nat. Genet.* 49, 170–174
112. Leiserson, M.D. *et al.* (2015) MAGI: visualization and collaborative annotation of genomic aberrations. *Nat. Methods* 12, 483–484
113. Gerstung, M. *et al.* (2017) Precision oncology for acute myeloid leukemia using a knowledge bank approach. *Nat. Genet.* 49, 332–340
114. Brough, R. *et al.* (2011) Searching for synthetic lethality in cancer. *Curr. Opin. Genet. Dev.* 21, 34–41
115. Hartwell, L.H. *et al.* (1997) Integrating genetic approaches into the discovery of anticancer drugs. *Science* 278, 1064–1068
116. Lord, C.J. *et al.* (2008) A high-throughput RNA interference screen for DNA repair determinants of PARP inhibitor sensitivity. *DNA Repair (Amst)* 7, 2010–2019
117. Luo, J. *et al.* (2009) A genome-wide RNAi screen identifies multiple synthetic lethal interactions with the Ras oncogene. *Cell* 137, 835–848
118. Martin, S.A. *et al.* (2009) Methotrexate induces oxidative DNA damage and is selectively lethal to tumour cells with defects in the DNA mismatch repair gene MSH2. *EMBO Mol. Med.* 1, 323–337
119. Srivas, R. *et al.* (2016) A network of conserved synthetic lethal interactions for exploration of precision cancer therapy. *Mol. Cell* 63, 514–525
120. Wang, T. *et al.* (2017) Gene essentiality profiling reveals gene networks and synthetic lethal interactions with oncogenic ras. *Cell* 168, 890–903
121. Wang, X. *et al.* (2014) Widespread genetic epistasis among cancer genes. *Nat. Commun.* 5, 4828
122. Aguirre, A.J. *et al.* (2016) Genomic copy number dictates a gene-independent cell response to CRISPR/Cas9 targeting. *Cancer Discov.* 6, 914–929
123. Cheung, H.W. *et al.* (2011) Systematic investigation of genetic vulnerabilities across cancer cell lines reveals lineage-specific dependencies in ovarian cancer. *Proc. Natl. Acad. Sci. U. S. A.* 108, 12372–12377
124. Cowley, G.S. *et al.* (2014) Parallel genome-scale loss of function screens in 216 cancer cell lines for the identification of context-specific genetic dependencies. *Sci. Data* 1, 140035
125. Marcotte, R. *et al.* (2012) Essential gene profiles in breast, pancreatic, and ovarian cancer cells. *Cancer Discov.* 2, 172–189
126. Marcotte, R. *et al.* (2016) Functional genomic landscape of human breast cancer drivers, vulnerabilities, and resistance. *Cell* 164, 293–309
127. Beijersbergen, R.L. *et al.* (2017) Synthetic lethality in cancer therapeutics. *Ann. Rev. Cancer Biol.* 1, 141–161
128. Costanzo, M. *et al.* (2010) The genetic landscape of a cell. *Science* 327, 425–431
129. Lu, X. *et al.* (2013) Genome evolution predicts genetic interactions in protein complexes and reveals cancer drug targets. *Nat. Commun.* 4, 2124

CellPress REVIEWS

130. Madhukar, N.S. *et al.* (2015) Prediction of genetic interactions using machine learning and network properties. *Front. Bioeng. Biotechnol.* 3, 172
131. Zhong, W. and Sternberg, P.W. (2006) Genome-wide prediction of *C elegans* genetic interactions. *Science* 311, 1481–1484
132. Conde-Pueyo, N. *et al.* (2009) Human synthetic lethal inference as potential anti-cancer target gene detection. *BMC Syst. Biol.* 3, 116
133. Folger, O. *et al.* (2011) Predicting selective drug targets in cancer through metabolic networks. *Mol. Syst. Biol.* 7, 501
134. Frezza, C. *et al.* (2011) Inborn and acquired metabolic defects in cancer. *J. Mol. Med. (Berl)* 89, 213–220
135. Wang, K. *et al.* (2009) Genome-wide identification of post-translational modulators of transcription factor activity in human B cells. *Nat. Biotechnol.* 27, 829–839
136. Jerby-Arnon, L. *et al.* (2014) Predicting cancer-specific vulnerability via data-driven detection of synthetic lethality. *Cell* 158, 1199–1209
137. Park, S. and Lehner, B. (2015) Cancer type-dependent genetic interactions between cancer driver alterations indicate plasticity of epistasis across cell types. *Mol. Syst. Biol.* 11, 824
138. Szczurek, E. *et al.* (2013) Synthetic sickness or lethality points at candidate combination therapy targets in glioblastoma. *Int. J. Cancer* 133, 2123–2132
139. Sinha, S. *et al.* (2017) Systematic discovery of mutation-specific synthetic lethals by mining pan-cancer human primary tumor data. *Nat. Commun.* 8, 15580
140. Berger, A.H. *et al.* (2016) High-throughput phenotyping of lung cancer somatic mutations. *Cancer Cell* 30, 214–228
141. Iorio, F. *et al.* (2016) A landscape of pharmacogenomic interactions in cancer. *Cell* 166, 740–754
142. Gao, H. *et al.* (2015) High-throughput screening using patient-derived tumor xenografts to predict clinical trial drug response. *Nat. Med.* 21, 1318–1325
143. Nagel, Z.D. *et al.* (2017) DNA repair capacity in multiple pathways predicts chemoresistance in glioblastoma multiforme. *Cancer Res.* 77, 198–206
144. Reinhold, W.C. *et al.* (2017) DNA-targeted precision medicine; have we been caught sleeping? *Trends Cancer* 3, 2–6
145. Friedman, A.A. *et al.* (2015) Precision medicine for cancer with next-generation functional diagnostics. *Nat. Rev. Cancer* 15, 747–756
146. Byrne, A.T. *et al.* (2017) Interrogating open issues in cancer precision medicine with patient-derived xenografts. *Nat. Rev. Cancer* 17, 254–268
147. Garralda, E. *et al.* (2014) Integrated next-generation sequencing and avatar mouse models for personalized cancer treatment. *Clin. Cancer Res.* 20, 2476–2484
148. Hidalgo, M. *et al.* (2011) A pilot clinical study of treatment guided by personalized tumorgrafts in patients with advanced cancer. *Mol. Cancer Ther.* 10, 1311–1316
149. Stebbing, J. *et al.* (2014) Patient-derived xenografts for individualized care in advanced sarcoma. *Cancer* 120, 2006–2015
150. Fatehullah, A. *et al.* (2016) Organoids as an *in vitro* model of human development and disease. *Nat. Cell Biol.* 18, 246–254
151. Boj, S.F. *et al.* (2015) Organoid models of human and mouse ductal pancreatic cancer. *Cell* 160, 324–338
152. Sato, T. *et al.* (2011) Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* 141, 1762–1772
153. van de Wetering, M. *et al.* (2015) Prospective derivation of a living organoid biobank of colorectal cancer patients. *Cell* 161, 933–945
154. Weeber, F. *et al.* (2015) Preserved genetic diversity in organoids cultured from biopsies of human colorectal cancer metastases. *Proc. Natl. Acad. Sci. U. S. A.* 112, 13308–13311
155. Nadauld, L.D. *et al.* (2014) Metastatic tumor evolution and organoid modeling implicate TGFBR2 as a cancer driver in diffuse gastric cancer. *Genome Biol.* 15, 428
156. Gao, D. *et al.* (2014) Organoid cultures derived from patients with advanced prostate cancer. *Cell* 159, 176–187
157. Hubert, C.G. *et al.* (2016) A three-dimensional organoid culture system derived from human glioblastomas recapitulates the hypoxic gradients and cancer stem cell heterogeneity of tumors found *in vivo*. *Cancer Res.* 76, 2465–2477
158. Pauli, C. *et al.* (2017) Personalized *in vitro* and *in vivo* cancer models to guide precision medicine. *Cancer Discov.* 7, 462–477
159. Ercan, D. *et al.* (2015) EGFR mutations and resistance to irreversible pyrimidine–based EGFR inhibitors. *Clin. Cancer Res.* 21, 3913–3923
160. Janne, P.A. *et al.* (2015) AZD9291 in EGFR inhibitor-resistant non-small-cell lung cancer. *N. Engl. J. Med.* 372, 1689–1699
161. Jia, Y. *et al.* (2016) Overcoming EGFR(T790M) and EGFR (C797S) resistance with mutant-selective allosteric inhibitors. *Nature* 534, 129–132
162. Sequist, L.V. *et al.* (2015) Rociletinib in EGFR-mutated non-small-cell lung cancer. *N. Engl. J. Med.* 372, 1700–1709
163. Zhou, W. *et al.* (2009) Novel mutant-selective EGFR kinase inhibitors against EGFR T790M. *Nature* 462, 1070–1074
164. Bardelli, A. *et al.* (2013) Amplification of the MET receptor drives resistance to anti-EGFR therapies in colorectal cancer. *Cancer Discov.* 3, 658–673
165. Bozic, I. *et al.* (2013) Evolutionary dynamics of cancer in response to targeted combination therapy. *Elife* 2, e00747
166. Shahbazian, D. *et al.* (2012) Vertical pathway targeting in cancer therapy. *Adv. Pharmacol.* 65, 1–26
167. Russo, M. *et al.* (2016) Tumor heterogeneity and lesion-specific response to targeted therapy in colorectal cancer. *Cancer Discov.* 6, 147–153
168. Siravegna, G. *et al.* (2015) Clonal evolution and resistance to EGFR blockade in the blood of colorectal cancer patients. *Nat. Med.* 21, 795–801
169. Das Thakur, M. *et al.* (2013) Modelling vemurafenib resistance in melanoma reveals a strategy to forestall drug resistance. *Nature* 494, 251–255
170. Sun, C. *et al.* (2014) Reversible and adaptive resistance to BRAF (V600E) inhibition in melanoma. *Nature* 508, 118–122
171. Enriquez-Navas, P.M. *et al.* (2016) Exploiting evolutionary principles to prolong tumor control in preclinical models of breast cancer. *Sci. Transl. Med.* 8, 327ra24
172. Diaz, L.A., Jr *et al.* (2012) The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. *Nature* 486, 537–540
173. Girotti, M.R. *et al.* (2016) Application of sequencing, liquid biopsies, and patient-derived xenografts for personalized medicine in melanoma. *Cancer Discov.* 6, 286–299
174. Misale, S. *et al.* (2012) Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. *Nature* 486, 532–536
175. Murtaza, M. *et al.* (2013) Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. *Nature* 497, 108–112
176. Kolch, W. *et al.* (2015) The dynamic control of signal transduction networks in cancer cells. *Nat. Rev. Cancer* 15, 515–527
177. Konieczkowski, D.J. *et al.* (2014) A melanoma cell state distinction influences sensitivity to MAPK pathway inhibitors. *Cancer Discov.* 4, 816–827
178. Muller, J. *et al.* (2014) Low MITF/AXL ratio predicts early resistance to multiple targeted drugs in melanoma. *Nat. Commun.* 5, 5712
179. Tirosh, I. *et al.* (2016) Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. *Science* 352, 189–196
180. Jordan, N.V. *et al.* (2016) HER2 expression identifies dynamic functional states within circulating breast cancer cells. *Nature* 537, 102–106
181. Nieto, M.A. *et al.* (2016) EMT: 2016. *Cell* 166, 21–45
182. Fischer, K.R. *et al.* (2015) Epithelial-to-mesenchymal transition is not required for lung metastasis but contributes to chemoresistance. *Nature* 527, 472–426
183. Zheng, X. *et al.* (2015) Epithelial-to-mesenchymal transition is dispensable for metastasis but induces chemoresistance in pancreatic cancer. *Nature* 527, 525–530

184. Sequist, L.V. *et al.* (2011) Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. *Sci. Transl. Med.* 3, 75ra26
185. Dey, N. *et al.* (2015) Evading anti-angiogenic therapy: resistance to anti-angiogenic therapy in solid tumors. *Am. J. Transl. Res.* 7, 1675–1698
186. Hugo, W. *et al.* (2015) Non-genomic and immune evolution of melanoma acquiring MAPKi resistance. *Cell* 162, 1271–1285
187. Alix-Panabieres, C. and Pantel, K. (2016) Clinical applications of circulating tumor cells and circulating tumor DNA as liquid biopsy. *Cancer Discov.* 6, 479–491
188. Fong, C.Y. *et al.* (2015) BET inhibitor resistance emerges from leukaemia stem cells. *Nature* 525, 538–542
189. Miyamoto, D.T. *et al.* (2015) RNA-Seq of single prostate CTCs implicates noncanonical Wnt signaling in antiandrogen resistance. *Science* 349, 1351–1356
190. Motter, A.E. *et al.* (2008) Predicting synthetic rescues in metabolic networks. *Mol. Syst. Biol.* 4, 168
191. Rathert, P. *et al.* (2015) Transcriptional plasticity promotes primary and acquired resistance to BET inhibition. *Nature* 525, 543–547
192. van Leeuwen, J. *et al.* (2016) Exploring genetic suppression interactions on a global scale. *Science* 354, aag0839
193. Tsimberidou, A.M. *et al.* (2012) Personalized medicine in a phase I clinical trials program: the MD Anderson Cancer Center initiative. *Clin. Cancer Res.* 18, 6373–6383
194. Schwaederle, M. *et al.* (2016) Precision oncology: the UC San Diego Moores Cancer Center PREDICT experience. *Mol. Cancer Ther.* 15, 743–752
195. Von Hoff, D.D. *et al.* (2010) Pilot study using molecular profiling of patients' tumors to find potential targets and select treatments for their refractory cancers. *J. Clin. Oncol.* 28, 4877–4883
196. Park, J.W. *et al.* (2016) Adaptive randomization of neratinib in early breast cancer. *N. Engl. J. Med.* 375, 11–22
197. Rugo, H.S. *et al.* (2016) Adaptive randomization of veliparib-carboplatin treatment in breast cancer. *N. Engl. J. Med.* 375, 23–34
198. Tripathy, D. *et al.* (2015) Adaptively randomized trial of neoadjuvant chemotherapy with or without the Akt inhibitor MK-2206: graduation results from the I-SPY 2 Trial. *J. Clin. Oncol.* 33, 524–524
199. Kaplan, R. *et al.* (2013) Evaluating many treatments and biomarkers in oncology: a new design. *J. Clin. Oncol.* 31, 4562–4568
200. Liu, F. *et al.* (2015) EGFR mutation promotes glioblastoma through epigenome and transcription factor network remodeling. *Mol. Cell* 60, 307–318
201. Zhu, J. *et al.* (2015) Gain-of-function p53 mutants co-opt chromatin pathways to drive cancer growth. *Nature* 525, 206–211
202. Mertins, P. *et al.* (2016) Proteogenomics connects somatic mutations to signalling in breast cancer. *Nature* 534, 55–62
203. Zhang, B. *et al.* (2014) Proteogenomic characterization of human colon and rectal cancer. *Nature* 513, 382–387
204. Zhang, H. *et al.* (2016) Integrated proteogenomic characterization of human high-grade serous ovarian cancer. *Cell* 166, 755–765
205. Drake, J.M. *et al.* (2016) Phosphoproteome integration reveals patient-specific networks in prostate cancer. *Cell* 166, 1041–1054
206. Aebersold, R. and Mann, M. (2016) Mass-spectrometric exploration of proteome structure and function. *Nature* 537, 347–355
207. Senft, D. and Ronai, Z.A. (2016) Adaptive stress responses during tumor metastasis and dormancy. *Trends Cancer* 2, 429–442
208. Davidson, S.M. *et al.* (2016) Environment impacts the metabolic dependencies of ras-driven non-small cell lung cancer. *Cell Metab.* 23, 517–528
209. Hensley, C.T. *et al.* (2016) Metabolic heterogeneity in human lung tumors. *Cell* 164, 681–694
210. Liu, X. *et al.* (2017) Conditional reprogramming and long-term expansion of normal and tumor cells from human biospecimens. *Nat. Protoc.* 12, 439–451
211. Beglyarova, N. *et al.* (2016) Screening of conditionally reprogrammed patient-derived carcinoma cells identifies ERCC3-MYC interactions as a target in pancreatic cancer. *Clin. Cancer Res.* 22, 6153–6163
212. Saeed, K. *et al.* (2017) Comprehensive drug testing of patient-derived conditionally reprogrammed cells from castration-resistant prostate cancer. *Eur. Urol.* 71, 319–327
213. Crystal, A.S. *et al.* (2014) Patient-derived models of acquired resistance can identify effective drug combinations for cancer. *Science* 346, 1480–1486
214. Ridky, T.W. *et al.* (2010) Invasive three-dimensional organotypic neoplasia from multiple normal human epithelia. *Nat. Med.* 16, 1450–1455
215. Kenny, H.A. *et al.* (2015) Quantitative high throughput screening using a primary human three-dimensional organotypic culture predicts *in vivo* efficacy. *Nat. Commun.* 6, 6220
216. Vaira, V. *et al.* (2010) Preclinical model of organotypic culture for pharmacodynamic profiling of human tumors. *Proc. Natl. Acad. Sci. U. S. A.* 107, 8352–8356
217. Freeman, A.E. and Hoffman, R.M. (1986) *In vivo*-like growth of human tumors *in vitro*. *Proc. Natl. Acad. Sci. U. S. A.* 83, 2694–2698
218. Meijer, T.G. *et al.* (2017) *Ex vivo* tumor culture systems for functional drug testing and therapy response prediction. *Future Sci. OA* 3, FSO190
219. Majumder, B. *et al.* (2015) Predicting clinical response to anticancer drugs using an*ex vivo* platform that captures tumour heterogeneity. *Nat. Commun.* 6, 6169
220. Holliday, D.L. *et al.* (2013) The practicalities of using tissue slices as preclinical organotypic breast cancer models. *J. Clin. Pathol.* 66, 253–255
221. Naipal, K.A. *et al.* (2016) Tumor slice culture system to assess drug response of primary breast cancer. *BMC Cancer* 16, 78
222. Roife, D. *et al.* (2016) *Ex vivo* testing of patient-derived xenografts mirrors the clinical outcome of patients with pancreatic ductal adenocarcinoma. *Clin. Cancer Res.* 22, 6021–6030
223. Wang, Y. *et al.* (2014) Clonal evolution in breast cancer revealed by single nucleus genome sequencing. *Nature* 512, 155–160
224. Patel, A.P. *et al.* (2014) Single-cell RNA-seq highlights intratumoral heterogeneity in primary glioblastoma. *Science* 344, 1396–1401
225. Tirosh, I. *et al.* (2016) Single-cell RNA-seq supports a developmental hierarchy in human oligodendroglioma. *Nature* 539, 309–313
226. Gawad, C. *et al.* (2016) Single-cell genome sequencing: current state of the science. *Nat. Rev. Genet.* 17, 175–188
227. Vitak, S.A. *et al.* (2017) Sequencing thousands of single-cell genomes with combinatorial indexing. *Nat. Methods* 14, 302–308
228. Zahn, H. *et al.* (2017) Scalable whole-genome single-cell library preparation without preamplification. *Nat. Methods* 14, 167–173
229. Klein, A.M. *et al.* (2015) Droplet barcoding for single-cell transcriptomics applied to embryonic stem cells. *Cell* 161, 1187–1201
230. Macosko, E.Z. *et al.* (2015) Highly parallel genome-wide expression profiling of individual cells using nanoliter droplets. *Cell* 161, 1202–1214
231. Zheng, G.X. *et al.* (2017) Massively parallel digital transcriptional profiling of single cells. *Nat. Commun.* 8, 14049
232. Tsoucas, D. and Yuan, G.C. (2017) Recent progress in single-cell cancer genomics. *Curr. Opin. Genet. Dev.* 42, 22–32

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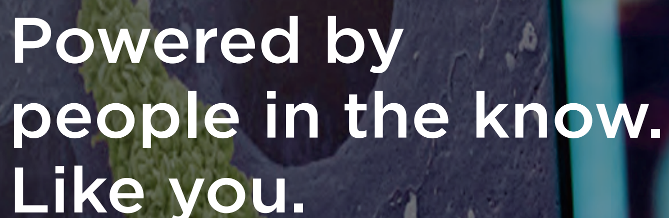

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Review

What, Why, Where, and When: Bringing Timing to Immuno-Oncology

Adrienne M. Rothschilds[1] and K. Dane Wittrup[1,2,*]

A plethora of new cancer immunotherapies are under clinical development individually and in combination for a wide variety of indications, but optimizing therapeutic outcomes will require precise consideration of timing in treatment schedule design. In this review, we summarize the current understanding of the temporal rhythms of the anticancer immune response. Lessons learned in preclinical and clinical studies begin to define a framework for incorporating duration and sequencing into immunotherapy. We also discuss key challenges and opportunities for translation of temporally programmed treatment schedules to the clinic, including alignment of immunological timescales in preclinical models and humans, and the use of current and emerging biomarkers.

Highlights

The temporal progression of response to infection and the order of events in the cancer–immune cycle indicate the existence of an endogenous temporal program for the immune system.

A growing number of examples illustrate that choice of dose schedule can either eliminate or synergistically amplify the therapeutic benefit of immunotherapeutic agents.

Treatment schedule design should take into account both duration and sequencing of combination immunotherapy agents.

New biomarkers such as circulating tumor DNA and cell counts derived from the blood can help align preclinical and clinical immunological timescales for clinical translation.

The Need for ‘When’ in Cancer Immunotherapy

Immuno-oncology is progressively becoming synonymous with clinical oncology practice. The what of cancer immunotherapy includes multiple modalities, including cytokines, antibodies, vaccines, and cell therapies – often used in combination. Recent clinical successes with combinations of **checkpoint blockade** (see Glossary) antibodies targeting **programmed cell death protein** (PD)-1 and **cytotoxic T lymphocyte associated protein** (CTLA)-4 have validated why immunotherapy is important by demonstrating the ability of the immune system to durably control tumors with cure rates of up to 58% in advanced melanoma [1]. Antibodies targeting the PD-1 pathway have been approved for 15 different indications to date and have received nine new FDA approvals so far in 2018 (https://www.cancerresearch.org/scientists/clinical-accelerator/landscape-of-immuno-oncology-drug-development). Despite these successes, many patients and tumor types still do not respond to immunotherapy or have to exit treatment due to adverse events. Numerous efforts are underway to restrict where immune activation occurs, in order to achieve improved safety and efficacy focused on tumor tissues [2–5]. However, insufficient attention has been paid so far to the critical issue of ‘when’. Successful immunotherapies of the future could orchestrate an immune response by resonating with the natural rhythms of innate and adaptive immune responses, amplifying and driving therapeutic effects by rationally timing combination immunotherapy dose schedules. Efforts invested in examination of combination dose scheduling can return qualitatively significant returns with respect to improved efficacy and decreased toxicity; all without necessitating approval of new agents. Understanding the timing of the immune system as it pertains to cancer therapy is not an insurmountable challenge; some preclinical and clinical examples provide exemplary guidance. In this review, we highlight critical aspects of therapeutic timing already understood to be significant, provide some vignettes of immunotherapies that leverage temporal aspects of immunity, and identify challenges meriting focused efforts.

Natural Rhythms of Immunity

To begin to address timing in immunotherapy treatment regimens, let us first consider examples where the duration or sequence of events is critical for innate and adaptive immune

[1]Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
[2]Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

*Correspondence: wittrup@mit.edu (K.D. Wittrup).

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activation. Changing the duration of an activating signal can lead to diametrically opposed outcomes. In the innate immune system, the duration of exposure to lipopolysaccharides (LPS) can promote dendritic cells (DCs) either to stimulate $CD4^+$ T cells to produce interferon (IFN) γ (DC LPS exposure for 8 h) or to produce interleukin (IL)-4 (DC LPS exposure for 48 h) [6]. Similarly to LPS, IFN signaling can play opposing roles in immune activation depending on duration. Brief IFN signaling is essential for immune activation (both type I and type II) [7–9], but persistent IFN signaling of either type by tumor cells can contribute to resistance to checkpoint blockade therapy [10]. Bolus intravenous administration of small cytokines can provide brief cellular stimulation, but numerous strategies are available for extended pharmacological exposure to immunomodulatory agents; namely: (i) continuous infusion (e.g., of bispecific T cell engagers) [11]; (ii) injection of cytokine fusion proteins to extend lifetime (such as **IL-2** to albumin or Fc domains) [12]; or (iii) slow cytokine release from biomaterials such as polyethylene glycol [13–15]. These precedents have demonstrated how more is not always better in immune activation, depending on the cytokine used for stimulation.

The adaptive immune system relies on precisely sequenced cues for effective $CD4^+$ and $CD8^+$ T cell activation. Dosing strategies that ignore these specific windows of opportunity may achieve lesser therapeutic effects – or worse, inadvertently suppress important effector responses. For $CD4^+$ T cells, exposure to IL-2 prior to priming and costimulation actually results in impaired activation, proliferation, and memory formation [16]. Timing of IL-2 exposure is also important for preferentially activating $CD8^+$ T cells over **regulatory T cells** since, unlike the constitutive expression in regulatory T cells, $CD8^+$ T cells only transiently express the high-affinity IL-2 receptor CD25 after T cell receptor (TCR) engagement [16]. Specifically, when $CD8^+$ T cells upregulate CD25, maximal downstream STAT5 signaling can be detected from a pulse of IL-2 exposure for 1 h *in vitro* due to IL-2 recycling; this indicates that there is an optimal time to dose IL-2 to activate $CD8^+$ T cells without extended IL-2 exposure that could activate regulatory T cells [17]. Similarly to $CD4^+$ T cells, $CD8^+$ T cells have an optimal time window for cytokine exposure. *In vitro* assays have shown that IL-12 receptor signaling is needed for a period of 24–60 h after antigen recognition and priming in order for $CD8^+$ T cells to proliferate and gain full effector function [18,19]. In addition, in a mouse model of influenza A virus infection, $CD4^+$ T cells could avoid apoptosis and transition into memory cells with exposure to antigen or IL-2, approximately 1 week after initial infection [20]. Thus, from this example among many, the memory response of adaptive immunity also requires particular temporal interactions.

Beyond the examples of the critical importance of timing in T cell cytokine responses, a temporal framework for understanding the progression of innate and adaptive events has been proposed for the anticancer immune response [21]. Since cancer often suppresses the immune system into an equilibrium phase [22,23], the first step in an immune response against cancer is the generation of antigen through initiation of a tumor cell death event [21]. Prior to treatment, the system may be asynchronous, but commencement of treatment can initiate a timed series of responses in which the duration of delays between milestones is reasonably predictable, as the following examples describe. An initiating wave of cell death can be achieved by a variety of cytotoxic agents including chemotherapy, radiotherapy (RT), and tumor-cell-targeting antibodies. Next, **antigen presenting cells** (APCs) such as DCs sample the bolus of tumor debris released in the microenvironment and traffic to lymph nodes to present antigen and prime T cells. This process is not instantaneous, and may take hours or days to align with recently characterized circadian fluxes of lymphocytes through the lymph nodes [24] to reach a maximal level of APC/T cell interactions. Immunotherapy can potentiate these processes with administration of stimulator of interferon genes (STING) agonists or cytokines such as **type I IFNs** to mature APCs, or administering immunomodulatory antibodies

Glossary

4-1BB (also known as CD137): costimulatory receptor expressed on the surface of a variety of cell types including T cells, NK cells, and DCs.

Antigen presenting cells (APCs): cells such as DCs that take up antigens and are subsequently involved in priming and costimulation of T cells.

Bispecific antibodies: antibodies that have two different target specificities because they have a different variable region on each of their two arms.

Checkpoint blockade: a class of immunotherapy involving antagonizing receptors that stop or weaken lymphocyte activity (e.g., PD-1 and CTLA-4).

Circulating tumor DNA (ctDNA): DNA released from tumor cells found systemically, often identified by known tumor mutations.

Cytokine release syndrome: a harmful condition where the body produces excess inflammation in response to immunotherapy.

Cytotoxic T lymphocyte associated protein 4 (CTLA-4): a checkpoint receptor expressed on regulatory T cells and activated $CD8^+$ and $CD4^+$ effector T cells that blocks T cell costimulation.

Duration of treatment: period of time for which a particular treatment is administered.

Immune escape: process by which cancer escapes the immune system through soluble factors that suppress immune cells or cancer cell surface receptors that prevent immune recognition.

Interleukin 2 (IL-2): an inflammatory cytokine with many functions including enhancing antibody-mediated cell killing and proliferation of lymphocytes.

Neutrophil–lymphocyte ratio (NLR): Ratio in the blood of neutrophils to lymphocytes.

Programmed cell death protein (PD)-1: checkpoint receptor expressed on the surface of T cells that inhibits T cell activation.

Regulatory T cells: type of immune cells that restrain activation of the immune system.

Sequence of treatments: order in which individual agents in combination therapies are administered.

such as **tumor necrosis factor receptor (TNFR) superfamily** agonists that can enhance signaling during T cell priming [25].

Once T cells are primed by APCs in the draining lymph nodes within the first few days after tumor cell death [26], these lymphocytes re-enter circulation until they find and can infiltrate the tumor, which can be enhanced by gradients of local chemokines such as CXCL9 or CXCL10 [27]. After the wave of infiltration of freshly primed T cells into the tumor within 4–7 days after tumor cell death, cytotoxic T lymphocytes (CTLs) engage tumor cells through their TCRs. This wave of tumor cell killing by $CD8^+$ CTLs, which can persist when checkpoint blockade antibodies block negative signaling, can then reset the clock of the anticancer immune response to begin again and even help spread the response to new tumor antigens [28]. Despite awareness of the cyclic nature of the immune response, current immunotherapy schedules are often stuck in a traditional 'persistent dose to maximum tolerated dose' perspective that squanders opportunities to exploit potential golden windows of timing.

Preclinical Examples of Duration or Sequencing in Immunotherapy

How can lessons learned about the natural rhythms of the immune system be applied to cancer immunotherapy treatment schedules? Such lessons are especially important for combination therapies (anti-PD-1 and anti-CTLA-4 [29]; anti-PD-1/CTLA-4 and IL-2 (clinical trial NCT02983045)), which are increasingly recognized as critical for preventing tumor **immune escape**. Combination therapy agents are valuable because they activate different steps of the anticancer response, yet these combination agents are most often dosed simultaneously or in treatment schedules dictated by the FDA approval process of each individual agent, instead of aligning with the underlying immune temporal dynamics. Such disruption of the naturally timed interplay of innate and adaptive immunity may harm therapeutic efficacy and lead to toxicity from futile overstimulation. A handful of preclinical and clinical studies offers vignettes of successful incorporation of timing into both the **duration of treatment** and **sequence of treatments** for combination immunotherapy (Figure 1, Key Figure).

Optimal duration of immunotherapy is essential for synergizing with the timing of the immune system. Using checkpoint blockade as an example, reports suggest that anti-PD-1 antibodies might curtail the memory response in some circumstances, as in the case of the therapeutic survival benefit of a **4-1BB** antibody, which was diminished when combined with anti-PD-1 treatment compared to anti-4-1BB treatment alone in the Eμ-myc transgenic C57BL/6 murine spontaneous B cell lymphoma model [41]. Although anti-PD-1 is generally dosed clinically every 2–4 weeks until disease progression or intolerable toxicity ensues, the extended duration of anti-PD-1 treatment merits further scrutiny if it has the potential to impair immune memory formation. Of note, a meta-analysis of the combination treatments of ipilimumab (anti-CTLA-4) and nivolumab (anti-PD-1) in the CheckMate 067 (advanced melanoma, NCT01844505) and 069 (unresectable or metastatic melanoma, NCT01927419) clinical trials showed that patients who discontinued treatment due to adverse events did not present different outcomes than patients completing the full treatment [42]. Consequently, this questions the paradigm that toxicity and efficacy are *a priori* linked, and suggests that a shorter treatment duration might not necessarily compromise efficacy.

Sequencing of immunotherapies, defined here as purposefully choosing an order of administration for individual agents, has shown success in enhancing therapeutic efficacy. Combination therapies incorporating TNFR superfamily agonists have benefitted from such approaches in several recent preclinical studies. Combination of the antitumor antibody trastuzumab (targeting HER2) with anti-CD137 agonistic antibodies, *in vitro* and in the BT474M1 cell line

Tumor necrosis factor receptor (TNFR) superfamily: group of receptors exerting related costimulatory effects on either APCs or T cells. Members of this family include 4-1BB (also known as CD137), OX40, CD40, and CD30.
Type I interferons (IFNs): inflammatory cytokines such as IFNα with many functions including activation and maturation of APCs.

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Key Figure

Preclinical and Clinical Examples of Temporally Programmed Combination Cancer Immunotherapies

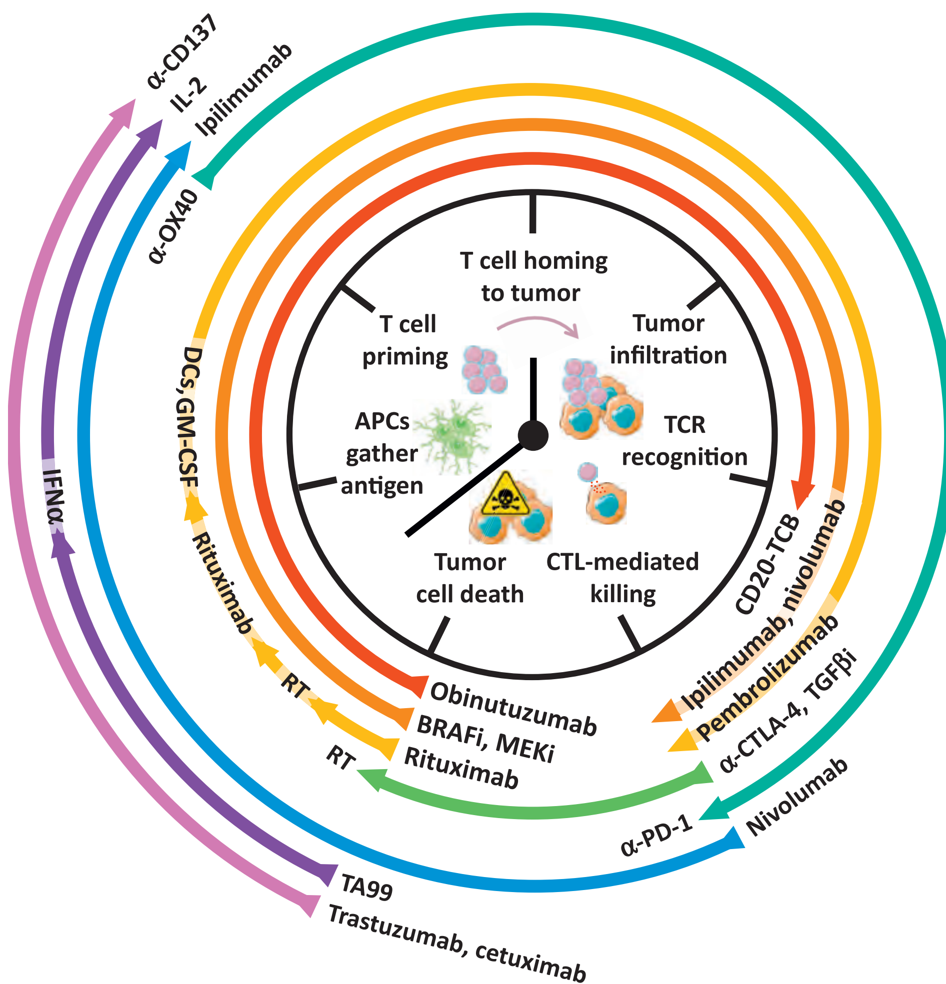

Figure 1. These therapies are superimposed along the steps of the anticancer immune response. Although many immunotherapies such as the anti-CTLA-4 antibody have the potential to affect more than one step, they are positioned coincidentally with combination partners (such as anti-PD-1 antibody treatment) to reflect the particular treatment schedule used in the reference. Placement of each arrow along the anticancer immune clock signifies the step when that particular therapy might be most useful. Although not every therapy has been iterated in combination with every other therapy in preclinical or clinical studies, the combinations that have been sequentially tested (indicated by arrows within each individual color) align a timing framework for future combination partners. The references to these examples are color-coded: red: [30]; orange: [31,32]; yellow: [33], NCT02677155; green: [34,35]; cyan: [36,37]; blue: [38]; purple: [26]; and magenta: [39,40]. Abbreviations: APCs, antigen presenting cells; CTL, cytotoxic T lymphocyte; DCs, dendritic cells; i, inhibitor; IFN, interferon; IL-2, interleukin-2; PD-1, programmed cell death protein-1; RT, radiotherapy; TCB, T cell bispecific antibody; TCR, T cell receptor.

breast cancer xenograft model injected into athymic nude Foxn1^{nu} mice, has shown that efficacy depends on dosing the anti-CD137 antibody after trastuzumab treatment, given that natural killer (NK) cells upregulate CD137 only after they initiate tumor cell death through trastuzumab [39]. The importance of delaying anti-CD137 to align with the natural rhythm of NK cell activation was also confirmed with the EGFR antibody cetuximab in peripheral blood mononuclear cells isolated from head and neck cancer patients, in which increased NK cell expression of CD137 correlated with increased percentages of EGFR-specific CD8^{+} T cells after treatment, relative to before treatment [40].

Following these data, since agonist antibodies targeting TNFR superfamily members may exert their effects mostly after tumor antigen generation, but before or during CTL-induced tumor cell killing, we posit that using these agents after a tumor targeting antibody but before checkpoint blockade makes sense. Indeed, in a PD-1 refractory, orthotopic mammary cancer mouse model, anti-PD-1 combination treatment abolished the efficacy of agonistic OX40 antibody alone [36]. However, when anti-PD-1 was given after anti-OX40 (but not concurrently or in reverse order), the two synergized, resulting in improved animal survival (Figure 2). In addition, in a syngeneic mouse TC-1 tumor model, intraperitoneal administration of anti-PD-1 antibody concurrent with anti-OX40 and an HPV16 E7 peptide vaccine decreased the number of tumor-infiltrating, E7-antigen-specific CD8^{+} T cells compared with anti-OX40 and vaccine treatment [37]. However, many ongoing clinical trials of OX40 agonist antibodies in combination with checkpoint blockade have not, to our knowledge, incorporated sequencing, which may not bode well for the predicted outcomes.

From another angle, preclinical studies in syngeneic mouse tumor models have demonstrated that staggering IFNα 2 days after the combination of IL-2 and a cytotoxic agent (such as murine anti-TRP1 antibody TA99 in the B16F10 model in C57BL/6 mice) dramatically improved survival from 0 to 60–100% relative to that with concurrent administration of all three agents [26] (Figure 2). The mechanism for the survival advantage of delayed IFNα likely involves DC maturation after (instead of before) exposure to antigens, since administration of IFNα 1 day before ovalbumin vaccination lowered the numbers of antigen-specific CD8^{+} T cells in the blood compared to no IFNα, or administering IFNα 1 day after the vaccine [26]. Toxicity in the form of 10–20% weight loss associated with this combination therapy independent of the presence of tumors, and in several mouse species, could be eliminated without affecting antitumor efficacy (in the B16F10 model in C57BL/6 mice) by IL-2 administration with or after (delayed) IFNα in this triple combination [43]. Although IFNα and IL-2 were some of the first FDA-approved immunotherapies, combinations of these two therapies with chemotherapy, compared to IFNα and chemotherapy without IL-2, exhibited increased toxicity and failed to improve efficacy in a clinical trial of metastatic melanoma (NCT00002669) [44]. Collectively, these findings suggest that properly phasing dosing regimens might potentially revive combination strategies that have failed in the past by revisiting different clinical dose scheduling.

Other preclinical studies in the CT26 colon cancer model in BALB/c mice examining combinations of immunotherapy with RT have suggested that agents such as anti-CTLA-4 used to deplete regulatory T cells as well as TGFβ inhibitors can help precondition the tumor microenvironment to respond to RT, if they are optimally delivered before RT [34,35]. For example, delivering anti-CTLA-4 7 days before RT compared with 1 or 7 days after RT in the same CT26 model system improved survival from 50% to 100% [35]. Tumor cell debris after RT-induced killing might be taken up by APCs (such as DCs, which promote a subsequent T cell response against cancer), if cells such as regulatory T cells are first depleted [45]. One way this could be accomplished is by administering anti-CTLA-4 antibodies [46]. Although we do not yet have the

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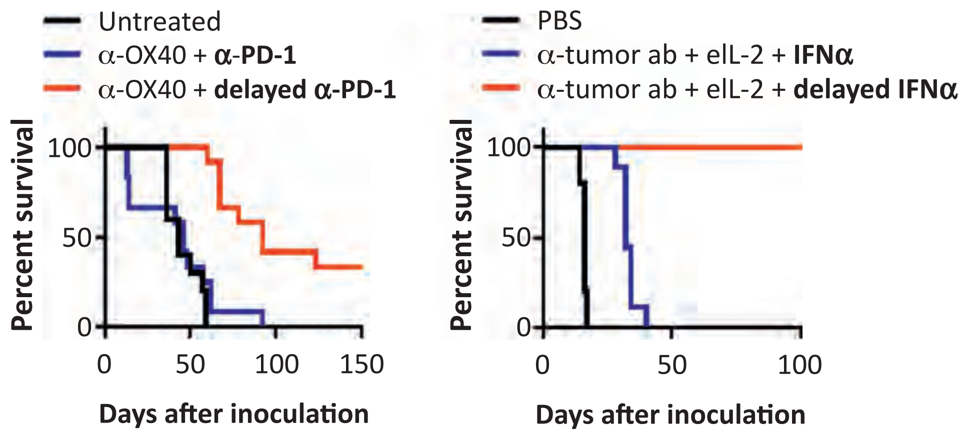

Figure 2. Sequencing Combination Immunotherapies. Two examples of sequenced combination immunotherapies demonstrate the principle that treating with all combination agents earlier does not necessarily result in an improved survival. Delayed anti-PD-1 antibody intraperitoneal treatment (days 13, 15, and 17) resulted in improved survival over concurrent treatment in combination with anti-OX40 antibody intraperitoneal treatment (days 7, 9, and 11) in the MMTV-PyMT mammary cancer model orthotopically transplanted into naïve FVB/NJ mice (day 0) [36]. In addition, delayed retro-orbital IFNα administration (days 8 and 14) resulted in improved survival relative to concurrent treatment in combination with retro-orbital antitumor antibody and IL-2 administration (days 6 and 12) in the syngeneic B16F10 melanoma model subcutaneously injected into C57BL/6 mice (day 0) [26]. Abbreviations: α-tumor ab, antitumor antibody; eIL-2, extended half-life IL-2; IFN, interferon; IL-2, interleukin-2; PBS, phosphate-buffered saline (control).

tools to precisely predict responses to temporal sequencing of every immunotherapy combination, these preclinical examples begin to indicate when along the anticancer immune response, each type of therapy might be most valuable.

Clinical Examples of Temporally Programmed Treatment Schedules

Clinical examples exploring duration or sequencing of combination immunotherapies have often been motivated by attempted avoidance of adverse events. For example, the recently published results of the CheckMate 067 trial (see above) combining ipilimumab and nivolumab for advanced melanoma documented grade 3 or 4 adverse events in 59% of combination-treated patients, which was double the rate for either individual therapy [1]. The unmet clinical need to maintain or improve combination checkpoint blockade efficacy while reducing adverse events inspired trials such as CheckMate 064. In this Phase II trial, administering nivolumab before ipilimumab resulted in a higher proportion of responding patients at week 25 compared to administering these agents in the reverse order (41% compared with 20%), although grade 3 or higher adverse events stayed high at 50% and 43% for the respective groups [38]. Interpretation of the results from CheckMate 064 is complicated because grades 3–5 adverse events were highest during ipilimumab treatment (whether before or after nivolumab) [38], but this study is still an important step towards bringing timing to the clinic. It is not just combinations of two or more immunotherapies that can benefit from sequenced approaches in the clinic. A Phase II trial showed that cycles of treatment with the BRAF inhibitor vemurafenib dosed prior to ipilimumab lowered hepatotoxicity relative to concurrent dosing of these two agents in BRAF-mutated advanced or metastatic melanoma (NCT01673854) [31]. Another ongoing Phase II clinical trial is exploring BRAF-mutated metastatic melanoma, and investigating double combinations of targeted therapies (BRAF and MEK inhibitors) sequenced with

nivolumab and ipilimumab (NCT02631447) [32]. Lastly, a Phase III clinical trial in BRAF-mutated stage III/IV melanoma is also testing combinations of BRAF inhibitors before or after nivolumab and ipilimumab (NCT02224781). Although timing studies for immunotherapy are still in their infancy, these ongoing trials will be instructive for future combinations of cytotoxic agents with checkpoint blockade, and may provide further encouragement and validation for sequenced combination therapies in general.

Challenges to Using Time for Synergizing Combination Therapies in the Clinic

Armed with the knowledge of the progression of events in the anticancer immune response, and inspired by a small number of examples of temporally designed immunotherapy treatment schedules, how can time become regularly incorporated into clinical dosing regimens? Bringing immunotherapy timing principles to the clinic necessitates confronting a few key challenges. First, we need to characterize the allometric scaling between murine and human immunological clocks – in what way, if any, do key time scales differ from mice to humans? Finding informative biomarkers to assist in monitoring the timing of responses would help ensure appropriate exploitation of these opportunities for temporal synergies.

Although caution is required in generalizing murine lessons on duration or sequencing of immunotherapies into humans, timescales of some cellular or system-level processes may be similar between the two species. In a HIV DNA vaccine system tested in humans, dosing plasmid IL-2/immunoglobulin as an adjuvant elicited optimal responses dosed 2 days after antigen DNA administration [47]. The same finding has been reflected in preclinical HIV vaccine studies in nonhuman primates [48] and mice [49]. This timing suggests a mechanism of action involving IL-2 support of freshly primed effector T cells; a process that apparently requires a similar length of time in mice and humans. One recent area where cancer immunotherapy sequencing has been successfully translated from mouse models to clinical studies is step-up-dosing, or pretreatment strategies for **bispecific antibodies**. For instance, after observing **cytokine release syndrome** in mice treated with a newly developed, extended half-life T cell bispecific antibodies against CD20, a new treatment schedule was developed. A monoclonal antibody against CD20 (obinutuzumab) administered 5–7 days prior to the bispecific antibody ameliorated toxicity in mice and cynomolgus monkeys relative to monotherapy with the CD20 bispecific antibody [30]. Indeed, this proof of concept is now in a Phase I clinical trial for patients with relapsed or refractory B cell non-Hodgkin's lymphoma (NCT03075696). Hence, the reset time for substantial clearance, and then replenishment of the CD20-positive cell pool, appears to be roughly similar across these organisms. One more example that incorporates timing into clinical immunotherapy combinations is the exciting, ongoing Phase II Lymvac-2 trial for follicular lymphoma (NCT02677155). This trial built a sequential treatment schedule on past *in vitro* [50], preclinical [51], and clinical studies (NCT01926639) [33], where intranodal injection of rituximab (anti-CD20 antibody) occurred 1 day prior to and 1 day after RT, followed by intranodal injection of autologous DCs, subcutaneous GM-CSF, and intravenous pembrolizumab, all staggered 1 day apart (NCT02677155). The immunological clock in these examples was not found to be significantly different between mice, monkeys, and humans, since the ongoing clinical trials mentioned above reflect treatment schedule timing developed in preclinical models. Thus, it may be possible to utilize emerging preclinical data to revive combinations of immunotherapies previously abandoned for lack of therapeutic index, if a rational treatment schedule design is incorporated. Examples such as these provide further validation that timescales in the order of days used to sequence combination immunotherapies in mice might be tested in monkeys for safety, and then, potentially translated to humans for efficacy and toxicity improvements in treating certain malignancies.

Another challenge in increasing confidence in moving rationally designed dosing schedules to the clinic is the development of appropriate biomarkers that could yield real-time feedback to identify optimal timing. One parameter that may hold promise as a harbinger for tumor cell death or response to therapy is **circulating tumor DNA** (ctDNA). A serum BRAF V600E ctDNA concentration spike within the first month (that subsequently disappears) after administration of tumor-infiltrating lymphocyte therapy in human metastatic melanoma has been reported to be predictive of improved survival in response to therapy, relative to a serum spike that does not disappear, or to no initial spike in BRAF V600E ctDNA [52]. Since BRAF V600E ctDNA could be released due to tumor cell death from the cellular therapy, clearance of the ctDNA after the first month may indicate that the tumor is gone or receding. With further validation under different tumor and cytotoxic settings, serum PCR for ctDNA in tumors with known mutations might be considered as a simple proxy for antigen release, and could inform appropriate timing for combination partners enhancing APC priming of T cells, such as via type I IFNs [26]. During this priming phase, activated effector T cells may upregulate members of the TNF or TNFR superfamily such as CD40L, 4-1BB, OX40, and CD30. As these proteins are cleaved by proteases and have known associations with flare-ups in certain autoimmune diseases such as rheumatoid arthritis and lupus [25,53,54], we posit that it may be feasible to monitor their concentration in the circulation; presumably, this could be evidence of immune activation in cancer as well. Biomarkers characterized from standard blood cell counts also have the potential for predictive staging of the immune response and deserve more attention. One metric termed the **neutrophil–lymphocyte ratio** (NLR), typically measured in the blood, has recently been shown to be negatively correlated with clinical response to high-dose IL-2 therapy in renal cell carcinoma [55]. A retrospective analysis of renal cell carcinoma patients treated with anti-PD-1 or anti-PD-L1 checkpoint blockade indicated that a 25% or greater reduction in the blood NLR from baseline to 6 weeks after therapy was predictive of improved clinical outcome [56]. Similarly, baseline NLR <5 has been associated with improved overall survival in response to nivolumab therapy in melanoma patients relative to baseline NLR ≥ 5 [57]. In addition, analysis of melanoma patients treated with ipilimumab has shown that a higher NLR in circulation either before treatment or increasing during treatment is associated with worsened response to therapy [58]. The rationale for this metric is that when circulating lymphocytes are higher relative to neutrophils, the anticancer immune response to immunomodulatory agents may be primed to induce T cell infiltration or prevent T cell exhaustion, and at this point, IL-2 or checkpoint blockade may be efficacious. Thus, ctDNA, inflammatory serum proteins such as TNF superfamily members, and NLR are examples of potential biomarkers that could offer a window into the patient’s immune response and inform regimens that involve timing of different combination therapy agents.

As discussed, aligning preclinical and clinical immunological timescales as well as finding biomarkers to classify the stage of the immune system are both significant challenges to the field. However, many other challenges remain to move temporally designed treatment schedules from an instantiated concept into regular clinical practice. One challenge for bioinformaticians and immunologists is to find a way to leverage the plethora of sequencing data in publicly available resources such as The Cancer Genome Atlas, especially for any studies with data at multiple time points within a given tumor type and treatment schedule. An additional challenge is that many immunotherapies are designed for optimally long pharmacological residency after administration. Taking into account the ideal time window for each drug for the immune system would mean designing (or redesigning) drugs for controlled (perhaps shorter) exposure windows without compromising biodistribution. Confronting these obstacles to incorporating rationally designed treatment schedules will require concerted efforts by clinicians, bioinformaticians, engineers, and other scientists.

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Concluding Remarks

Putting the 'when' into the cancer immunotherapy paradigm will require synthesizing existing knowledge of the progression of the anticancer immune response with timing principles established in preclinical and clinical examples, and validating new putative biomarkers to help classify stages of the immune response against tumors. Although the studies described herein begin to establish frameworks for when to administer therapeutic agents along the anticancer immune cycle, many open questions remain (see Outstanding Questions). Combinations of agents do not always act the same together as their individual components, so how can rational decisions be made about these agents instead of using a guess and test approach? Many individual immunotherapy agents have pleiotropic roles and the potential to enhance multiple steps of the immune response. Can biomarkers be developed to indicate not only the stage of the anticancer immune response, but which agent or agents would enhance that stage? How can researchers and clinicians work together to establish a set of guidelines drawing parallels between the timescales of preclinical and human immune responses for efficient clinical translation of rationally designed treatment schedules?

The time has never been more appropriate to bring these new timing concepts to the clinic. Immuno-oncology garnered international attention in 2018 with the Nobel Prize in Physiology or Medicine, but escalating drug costs and dose-limiting toxicities in regimens indicate that there is significant room for improvement. Immunotherapies such as combination nivolumab and ipilimumab are estimated to cost up to $300 000 per patient [59] and cancer care in the US is estimated to cost $174 billion in 2020 [60]. In recent immuno-oncology trials such as the CheckMate 067 trial, 39% of patients discontinued treatment due to adverse events [1]. Thus, next-generation immunotherapies incorporating timing have the potential to address these economic and toxicity roadblocks through careful duration and sequencing choices that may enable equal or better outcomes from fewer total doses. It will thus be exciting to see some of these testing strategies come into action.

Outstanding Questions

How can dose schedules optimized in mice be translated to the time scales in human patients?

What serum biomarkers might be useful for real-time assessment of ongoing therapeutic responses to fine-tune dose schedules?

Can biomarkers be developed to indicate not only the stage of the anticancer immune response, but which agent or agents would enhance that stage?

What is the best way to leverage knowledge of timing in the immune response into rational combination treatment schedule design to obviate empirical guess and test approaches?

How can researchers and clinicians work together to establish a set of guidelines drawing parallels between the timescales of preclinical and human immune responses for efficient clinical translation of rationally designed treatment schedules?

References

1. Wolchok, J.D. *et al.* (2017) Overall survival with combined nivolumab and ipilimumab in advanced melanoma. *N. Engl. J. Med.* 377, 1345–1356
2. Ishihara, J. *et al.* (2017) Matrix-binding checkpoint immunotherapies enhance antitumor efficacy and reduce adverse events. *Sci. Transl. Med.* 9, eaan0401
3. Park, C.G. *et al.* (2018) Extended release of perioperative immunotherapy prevents tumor recurrence and eliminates metastases. *Sci. Transl. Med.* 10, eaar1916
4. Rekers, N.H. *et al.* (2018) The immunocytokine L19-IL2: An interplay between radiotherapy and long-lasting systemic antitumour immune responses. *Oncoimmunology* 7
5. Sagiv-Barfi, I. *et al.* (2018) Eradication of spontaneous malignancy by local immunotherapy. *Sci. Transl. Med.* 10, eaan4488
6. Langenkamp, A. *et al.* (2000) Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. *Nat. Immunol.* 1, 311–316
7. Fuertes, M.B. *et al.* (2011) Host type I IFN signals are required for antitumor CD8+ T cell responses through CD8{alpha}+ dendritic cells. *J. Exp. Med.* 208, 2005–2016
8. Manguso, R.T. *et al.* (2017) *In vivo* CRISPR screening identifies Ptpn2 as a cancer immunotherapy target. *Nature* 547, 413–418
9. Yang, X. *et al.* (2014) Targeting the tumor microenvironment with interferon-β bridges innate and adaptive immune responses. *Cancer Cell* 25, 37–48
10. Benci, J.L. *et al.* (2016) Tumor interferon signaling regulates a multigenic resistance program to immune checkpoint blockade. *Cell* 167, 1540–1554.e12
11. Klinger, M. *et al.* (2012) Immunopharmacologic response of patients with B-lineage acute lymphoblastic leukemia to continuous infusion of T cell-engaging CD19/CD3-bispecific BiTE antibody blinatumomab. *Blood* 119, 6226–6233
12. Zhu, E.F. *et al.* (2015) Synergistic innate and adaptive immune response to combination immunotherapy with anti-tumor antigen antibodies and extended serum half-life IL-2. *Cancer Cell* 27, 489–501
13. Charych, D. *et al.* (2017) Modeling the receptor pharmacology, pharmacokinetics, and pharmacodynamics of NKTR-214, a kinetically-controlled interleukin-2 (IL2) receptor agonist for cancer immunotherapy. *PLoS One* 12
14. Charych, D.H. *et al.* (2016) NKTR-214, an engineered cytokine with biased IL2 receptor binding, increased tumor exposure, and marked efficacy in mouse tumor models. *Clin. Cancer Res.* 22, 680–690
15. Milling, L. *et al.* (2017) Delivering safer immunotherapies for cancer. *Adv. Drug Deliv. Rev.* 114, 79–101
16. Sckisel, G.D. *et al.* (2015) Out-of-sequence signal 3 paralyzes primary CD4(+) T-cell-dependent immunity. *Immunity* 43, 240–250
17. Su, E.W. *et al.* (2015) IL-2Rα mediates temporal regulation of IL-2 signaling and enhances immunotherapy. *Sci. Transl. Med.* 7, 311ra170
18. Curtsinger, J.M. *et al.* (2003) CD8 T cell clonal expansion and development of effector function require prolonged exposure to antigen, costimulation, and signal 3 cytokine. *J. Immunol.* 171, 5165–5171

CellPress
REVIEWS

19. Mescher, M.F. *et al.* (2006) Signals required for programming effector and memory development by CD8+ T cells. *Immunol. Rev.* 211, 81–92
20. McKinstry, K.K. *et al.* (2014) Effector CD4 T-cell transition to memory requires late cognate interactions that induce autocrine IL-2. *Nat. Commun.* 5, 5377
21. Chen, D.S. and Mellman, I. (2013) Oncology meets immunology: the cancer-immunity cycle. *Immunity* 39, 1–10
22. Dunn, G.P. *et al.* (2004) The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* 21, 137–148
23. Mittal, D. *et al.* (2014) New insights into cancer immunoediting and its three component phases – elimination, equilibrium and escape. *Curr. Opin. Immunol.* 27, 16–25
24. Druzd, D. *et al.* (2017) Lymphocyte circadian clocks control lymph node trafficking and adaptive immune responses. *Immunity* 46, 120–132
25. Ward-Kavanagh, L. *et al.* (2016) The TNF receptor superfamily in costimulating and coinhibitory responses. *Immunity* 44, 1005–1019
26. Tzeng, A. *et al.* (2016) Temporally programmed CD8α(+) DC activation enhances combination cancer immunotherapy. *Cell Rep.* 17, 2503–2511
27. Spranger, S. *et al.* (2017) Tumor-residing Batf3 dendritic cells are required for effector T Cell trafficking and adoptive T cell therapy. *Cancer Cell* 31, 711–723.e4
28. Moynihan, K.D. *et al.* (2016) Eradication of large established tumors in mice by combination immunotherapy that engages innate and adaptive immune responses. *Nat. Med.* 22, 1402–1410
29. Hodi, F.S. *et al.* (2018) Nivolumab plus ipilimumab or nivolumab alone versus ipilimumab alone in advanced melanoma (CheckMate 067): 4-year outcomes of a multicentre, randomised, phase 3 trial. *Lancet Oncol.* 19, 1480–1492
30. Bacac, M. *et al.* (2018) CD20-TCB with obinutuzumab pretreatment as next-generation treatment of hematologic malignancies. *Clin. Cancer Res.* 24, 4785–4797
31. Amin, A. *et al.* (2016) Phase II study of vemurafenib followed by ipilimumab in patients with previously untreated BRAF-mutated metastatic melanoma. *J. Immunother. Cancer* 4, 44
32. Ascierto, P.A. *et al.* (2017) SECOMBIT (sequential combo immuno and target therapy study): A three arms prospective, randomized phase II study to evaluate the best sequential approach with combo immunotherapy [ipilimumab (I)/nivolumab (N)] and combo target therapy [encorafenib (E)/binimetinib (B)] in patients with metastatic melanoma and BRAF mutation. *JCO* 35, TPS9598–TPS9598
33. Kolstad, A. *et al.* (2015) Sequential intranodal immunotherapy induces antitumor immunity and correlated regression of disseminated follicular lymphoma. *Blood* 125, 82–89
34. Young, K.H. *et al.* (2014) TGFβ inhibition prior to hypofractionated radiation enhances efficacy in preclinical models. *Cancer Immunol. Res.* 2, 1011–1022
35. Young, K.H. *et al.* (2016) Optimizing timing of immunotherapy improves control of tumors by hypofractionated radiation therapy. *PLoS One* 11, e0157164
36. Messenheimer, D.J. *et al.* (2017) Timing of PD-1 blockade is critical to effective combination immunotherapy with anti-OX40. *Clin. Cancer Res.* 23, 6165–6177
37. Shrimali, R.K. *et al.* (2017) Concurrent PD-1 blockade negates the effects of OX40 agonist antibody in combination immunotherapy through inducing T-cell apoptosis. *Cancer Immunol. Res.* 5, 755–766
38. Weber, J.S. *et al.* (2016) Sequential administration of nivolumab and ipilimumab with a planned switch in patients with advanced melanoma (CheckMate 064): an open-label, randomised, phase 2 trial. *Lancet Oncol.* 17, 943–955
39. Kohrt, H.E. *et al.* (2012) Stimulation of natural killer cells with a CD137-specific antibody enhances trastuzumab efficacy in xenotransplant models of breast cancer. *J. Clin. Invest* 122, 1066–1075
40. Kohrt, H.E. *et al.* (2014) Targeting CD137 enhances the efficacy of cetuximab. *J. Clin. Invest.* 124, 2668–2682
41. McKee, S.J. *et al.* (2017) Therapeutic efficacy of 4-1BB costimulation is abrogated by PD-1 blockade in a model of spontaneous B-cell lymphoma. *Cancer Immunol. Res.* 5, 191–197
42. Schadendorf, D. *et al.* (2017) Efficacy and safety outcomes in patients with advanced melanoma who discontinued treatment with nivolumab and ipilimumab because of adverse events: a pooled analysis of randomized phase II and III trials. *J. Clin. Oncol.* 35, 3807–3814
43. Rothschilds, A.M. *et al.* (in press) Order of administration of combination cytokine therapies can decouple toxicity from efficacy in syngeneic mouse tumor models. *OncoImmunology.*
44. Keilholz, U. *et al.* (2005) Dacarbazine, cisplatin, and interferon-alfa-2b with or without interleukin-2 in metastatic melanoma: a randomized phase III trial (18951) of the European Organisation for Research and Treatment of Cancer Melanoma Group. *J. Clin. Oncol.* 23, 6747–6755
45. Goudin, N. *et al.* (2016) Depletion of regulatory T cells induces high numbers of dendritic cells and unmasks a subset of anti-tumour CD8+CD11c+ PD-1lo effector T cells. *PLoS One* 11
46. Simpson, T.R. *et al.* (2013) Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti–CTLA-4 therapy against melanoma. *J. Exp. Med.* 210, 1695–1710
47. Baden, L.R. *et al.* (2011) Timing of plasmid cytokine (IL-2/Ig) administration affects HIV-1 vaccine immunogenicity in HIV-seronegative subjects. *J. Infect. Dis.* 204, 1541–1549
48. Barouch, D.H. *et al.* (2000) Augmentation of immune responses to HIV-1 and simian immunodeficiency virus DNA vaccines by IL-2/Ig plasmid administration in rhesus monkeys. *Proc. Natl. Acad. Sci. U. S. A.* 97, 4192–4197
49. Barouch, D.H. *et al.* (1998) Augmentation and suppression of immune responses to an HIV-1 DNA vaccine by plasmid cytokine/Ig administration. *J. Immunol.* 161, 1875–1882
50. Dhodapkar, K.M. *et al.* (2002) Antitumor monoclonal antibodies enhance cross-presentation of cellular antigens and the generation of myeloma-specific killer T cells by dendritic cells. *J. Exp. Med.* 195, 125–133
51. Kim, K.-W. *et al.* (2004) Direct injection of immature dendritic cells into irradiated tumor induces efficient antitumor immunity. *Int. J. Cancer* 109, 685–690
52. Xi, L. *et al.* (2016) Circulating tumor DNA as an early indicator of response to T-cell transfer immunotherapy in metastatic melanoma. *Clin. Cancer Res.* 22, 5480–5486
53. Caligaris-Cappio, F. *et al.* (1995) Circulating levels of soluble CD30, a marker of cells producing Th2-type cytokines, are increased in patients with systemic lupus erythematosus and correlate with disease activity. *Clin. Exp. Rheumatol.* 13, 339–343
54. Cope, A.P. *et al.* (1992) Increased levels of soluble tumor necrosis factor receptors in the sera and synovial fluid of patients with rheumatic diseases. *Arthritis Rheum.* 35, 1160–1169
55. Kuzman, J.A. *et al.* (2017) Neutrophil-lymphocyte ratio as a predictive biomarker for response to high dose interleukin-2 in patients with renal cell carcinoma. *BMC Urol.* 17, 1
56. Lalani, A.-K.A. *et al.* (2018) Change in neutrophil-to-lymphocyte ratio (NLR) in response to immune checkpoint blockade for metastatic renal cell carcinoma. *J. Immunother. Cancer* 6, 5
57. Capone, M. *et al.* (2018) Baseline neutrophil-to-lymphocyte ratio (NLR) and derived NLR could predict overall survival in patients with advanced melanoma treated with nivolumab. *J. Immunother. Cancer* 6, 74
58. Cassidy, M.R. *et al.* (2017) Neutrophil to lymphocyte ratio is associated with outcome during ipilimumab treatment. *EBioMedicine* 18, 56–61
59. Andrews, A. (2015) Treating with checkpoint inhibitors – figure $1 million per patient. *Am. Health Drug Benefits* 8, 9
60. Mariotto, A.B. *et al.* (2011) Projections of the cost of cancer care in the United States: 2010–2020. *J. Natl. Cancer Inst.* 103, 117–128

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Review

The Emergence of Natural Killer Cells as a Major Target in Cancer Immunotherapy

Fernando Souza-Fonseca-Guimaraes,[1,2,*] Joseph Cursons,[2,3] and Nicholas D. Huntington[1,2,4,*]

Immune ‘checkpoint’ inhibitors can increase the activity of tumor-resident cytotoxic lymphocytes and have revolutionized cancer treatment. Current therapies block inhibitory pathways in tumor-infiltrating CD8^{+} T cells and recent studies have shown similar programs in other effector populations such as natural killer (NK) cells. NK cells are critical for immunosurveillance, particularly the control of metastatic cells or hematological cancers. However, how NK cells specifically recognize transformed cells and dominant negative feedback pathways, as well as how tumors escape NK cell control, remains undefined. This review summarizes recent advances that have illuminated inhibitory checkpoints in NK cells, some of which are shared with conventional cytotoxic T lymphocytes. It also outlines emerging approaches aimed at unleashing the potential of NK cells in immunotherapy.

Highlights

NK cells possess the innate ability to detect transformed cells, and thus, are key to cancer immunosurveillance and antitumor immunity, particularly in hematological cancers and the control of metastatic dissemination.

Immune checkpoint inhibitors that function by enhancing cytotoxic immune responses of tumor-infiltrating lymphocytes have revolutionized the cancer therapy landscape.

Seminal discoveries in NK cell biology have culminated in recent breakthroughs with the identification of potent ‘checkpoints’ for NK cell activation, several of which may be shared with T cells.

Given the ability of NK cells to detect and destroy a range of cancerous tissues, mechanistic insight into how cancer cells regulate NK cell checkpoints and the pharmacological modulation of these checkpoints represents an unmet need for immunotherapy development.

The Success of Immune ‘Checkpoint’ Blockade (ICB): Fueling a Renaissance in Natural Killer (NK) Cell-Based Cancer Immunotherapies

Immunotherapies have arguably provided the most significant advance in cancer therapy from the last 30 years. Monoclonal antibody therapies, collectively known as ICB treatments, alleviate repression by regulatory receptors on immune effector cells, predominantly **cytotoxic CD8^{+} T lymphocytes (CTL)** (see Glossary). Metastatic melanoma is a clear example where ICB has revolutionized clinical treatment, with durable responses for over 50% of patients surviving past 4 years, compared with historical survival rates below 10% for chemotherapy/targeted therapy (CheckMate 067; NCT01844505; [1]) (Boxes 1 and 2). Despite the promise of immunotherapy, effective treatment is restricted to a subset of patients and cancer types. Robust responses are seen against tumors with both high mutational load and a high frequency of infiltrating effector lymphocytes, but tumor initiation and growth of such tumors can proceed via immunosuppression (either through changes in the microenvironment and/or the acquisition of mutations that allow immune evasion) and this must be overcome to achieve effective patient responses [2–4].

A wide array of immune cell types contribute to cancer prevention and spread through immunosurveillance. Innate immune cells, which include macrophages, dendritic cells (DC), and NK cells, contribute to spontaneous and acute antitumor responses by releasing mediators of inflammation, such as cytokines and chemokines, that activate local immune cells and recruit additional immune cells. In addition, NK cells also directly kill malignant or transformed cells by releasing cytotoxic granules to ultimately generate cell debris for capture by antigen-presenting cells, including macrophages and DCs (Figure 1, central panel). NK cells are a critical component of the innate immune system, and while they share many features with CTLs, they can detect and kill transformed cells that do not depend on specific **neo-antigen recognition**. The function of NK cells is modulated by a range of germline-encoded inhibitory and activating

[1]Molecular Immunology Division, The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3052, Australia
[2]Department of Medical Biology, University of Melbourne, Faculty of Medicine, Dentistry and Health Sciences, University of Melbourne, Melbourne, Victoria 3010, Australia
[3]Bioinformatics Division, The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3052, Australia
[4]Biomedicine Discovery Institute and the Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria 3800, Australia

*Correspondence:
guimaraes.f@wehi.edu.au
(F. Souza-Fonseca-Guimaraes) and
nicholas.huntington@monash.edu
(N.D. Huntington).

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Box 1. Current Clinical Cancer Therapies

The most effective cancer treatment at an early stage is surgical resection, while many patients with stage III disease (where the cancer has spread to draining lymph nodes) can also be treated or cured with lymphadenectomy (e.g., for melanoma, lung cancer, and breast cancer). Once cancer cells have metastasized to other parts of the body, however, therapeutic options are more limited, requiring systemic treatments such as immunotherapy or chemotherapy [110]. Current clinical therapies boost a patient's immune response against tumor cells primarily through targeting cytotoxic T lymphocytes, acting to alleviate the repressive effect of inhibitory receptors (in particular CTLA-4 and the PD-1:PD-L1 axis; see Figure 1) and reinstate optimal T cell response to neo-antigen-expressing tumor cells [4]. Other approaches showing promise in clinical trials are immunomodulatory agents that directly increase effector lymphocyte priming/activity/frequency, including treatment with stimulatory interleukins (e.g., IL-2/IL-15) or interferons, vaccination with tumor antigens (cancer vaccines), antibodies linking T cells to tumor cells (CD3-targeted bispecific antibodies), or cell therapies including autologous tumor resident T cells and chimeric antigen receptor (CAR) T cells [111–113].

Box 2. Mechanisms for Immune Checkpoint Inhibitors Used Clinically

A robust immune response must be initiated in a timely manner to effectively control pathogenic microbes. However, the immune system must subsequently be downregulated to limit damage of the surrounding healthy tissue; this is partly achieved through a series of intercellular interactions, with cellular immune checkpoints (e.g., PD-1 and CTLA-4 checkpoints). Current immunotherapies that boost a patient's immune response against tumor cells use antibodies that interfere with activation of immune checkpoints. These checkpoints form part of the physiological regulatory mechanisms that are in place to prevent the damaging effects of immune hyperactivity, and they are often hijacked by tumor cells to facilitate immune evasion. Approved therapies include: ipilimumab (anti-CTLA4); nivolumab or pembrolizumab (anti-PD-1), or atezolizumab (anti-PD-L1). While the dominant mechanism of action for CTLA4 inhibition is contentious, there is good evidence that it acts, at least in part, by modulating a natural feedback system within the lymph nodes (Figure 1B). Activation of the T cell receptor (TCR) complex by MHC-mediated antigen presentation also depends upon the TCR cofactor CD28, which is activated by CD80 on the antigen-presenting cell. This triggers the production of CTLA4, which subsequently competes for CD80, acting to downregulate T cell activation and prevent immune hyperactivation. While inhibition of these checkpoints can be advantageous for T cell-mediated tumor control, it can also contribute to autoimmunity that can arise following immunotherapy (e.g., type 1 diabetes).

receptors that allow them to recognize and respond to changes in the expression of ligands on pathogenic cells, with regulation emerging from the integrated balance of activating and inhibitory signals stemming from the NK cell–tumor interface [5]. These features are key to tumor immunosurveillance, providing NK cells with the capacity to rapidly identify newly transformed cells. Cancer cells can, however, evade NK cell detection by dysregulating this intricate balance to allow disease progression and metastasis to vital organs.

NK cells are an attractive alternative to T cell immunotherapies because they preferentially target 'altered' cells in the body, without the need for prior sensitization or knowledge of specific cancer cell antigens. In numerous preclinical studies, NK cells have shown an exceptional capacity to resist the hematogenous spread of experimental and spontaneous tumor metastases [6,7]. Clinically, NK cell activity has been inversely correlated with cancer incidence [8], and emerging evidence shows that NK cell infiltration into squamous cell lung, gastric, and colorectal carcinomas is associated with better patient outcomes [5,9]. NK cells are activated by inflammatory cytokines, immunoglobulin Fc, as well as endogenous ligands that are upregulated in response to stress (e.g., from viral infection, DNA damage, or TNF signaling). NK cells are inhibited by classical and nonclassical **human leukocyte antigen (HLA)** proteins that are highly expressed on healthy cells and engage NK cell inhibitory receptors [**killer-cell immunoglobulin-like receptor (KIR)**, CD94/NKG2A)] to prevent NK cell activation in healthy tissue [10–13] (Figure 1C). When researchers interrogated HLA-I in human melanoma biopsies, they found specific allelic losses of HLA-I in several samples [14]. Similar data have also been reported in other cancers, such as breast cancer [15], lung cancer [16], ovarian cancer [17], and others (recently reviewed in [18]). Further, in some cases when specific HLA-I molecules were present on melanoma cells, they were unable to inhibit NK cell-mediated cytotoxicity due to

Glossary

Activin-A: dimeric protein complex of two inhibin beta A subunits linked by a disulfide bond, recognized by the ALK4/ALK7 heterodimeric complex, triggering phosphorylation of SMAD2/3 signaling in response to the ligand.

Antibody-dependent cellular cytotoxicity (ADCC): innate immune system feature that can be exploited in NK cells but not in adaptive T cells; this program uses IgG antibody-mediated recognition of specific antigens in target tumor cells, followed by NK cell-dependent recognition of those antibodies and cytotoxic activation through Fc receptors (FcR).

Anti-PD-1/PD-L1 therapies: see 'immune "checkpoint" inhibitors' below.

Bispecific killer cell engager (BiKE): small molecules containing single variable portions (V_H and V_L) of an agonistic antibody to NK cell (e.g. , anti-CD16) linked to another antibody which recognizes specific tumor antigens (e.g., CD33), to maximize NK cell activation and target cell recognition.

Chimeric antigen receptor (CAR): engineered receptor proteins displaying customized responses upon recognition to specific antigens (e.g., CD19, commonly expressed in B cell lymphomas). This technology has been extensively used to maximize T cell-related adoptive therapies, and is now being investigated in NK cells.

CRISPR/Cas9: clustered regularly interspaced short palindromic repeats (CRISPR) are DNA sequences discovered in prokaryotic organisms. CRISPR-associated 9 (Cas9) is an enzyme that identifies these sequences to recognize and cleave DNA sequences complementary to the CRISPR sequences, which are used as a guide.

Cytotoxic CD8$^+$ T lymphocytes (CTL): a subset of the CD8$^+$ T cells; they are important effectors and regulators of the adaptive immune response. Cytotoxic T lymphocytes are cells with the ability to directly kill target (tumor) cells.

Epithelial–mesenchymal transition (EMT): oncogenic cellular process commonly induced by TGF-

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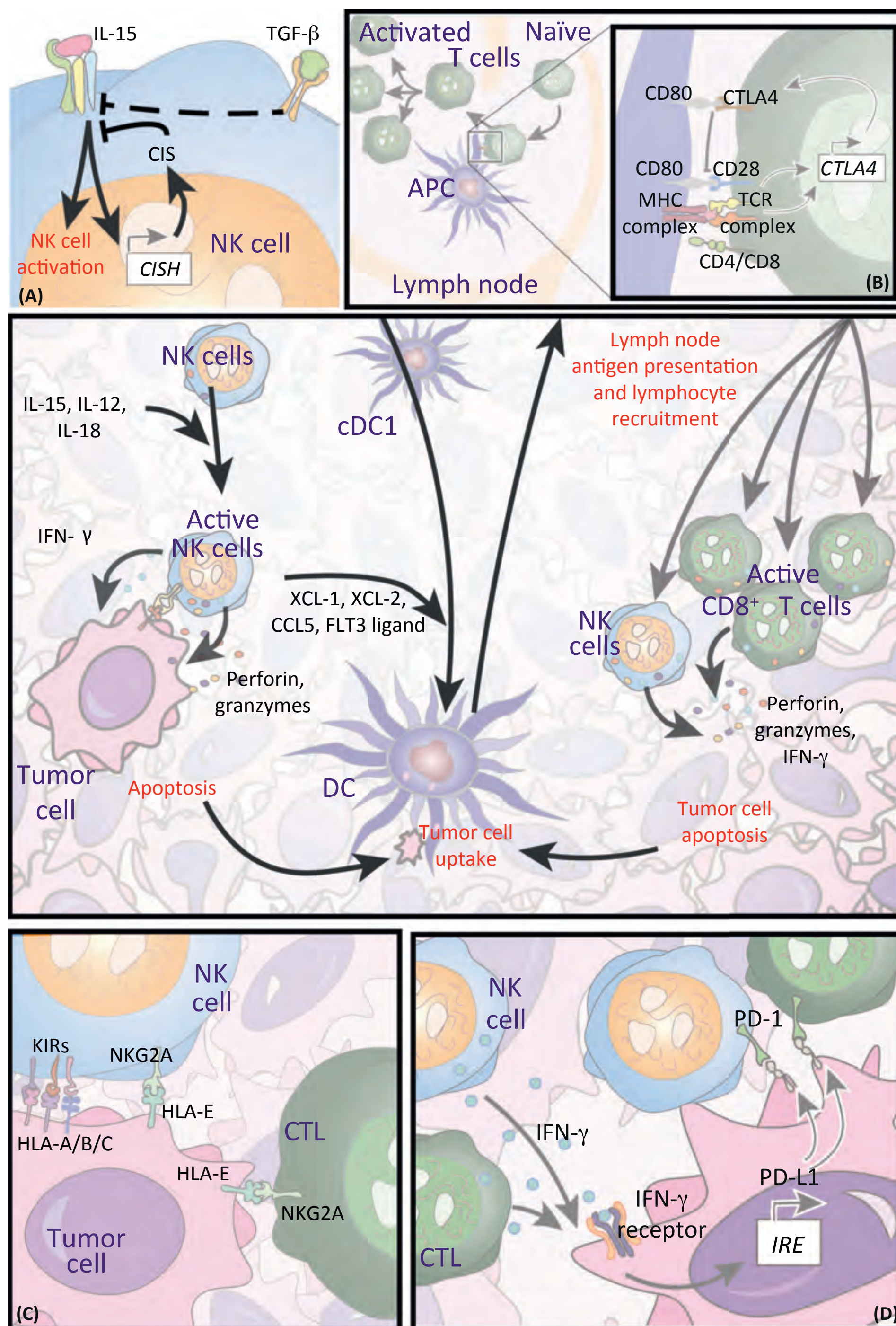

Figure 1. Natural Killer (NK) Cells Are Important Mediators of Antitumor Immunity. Middle panel: NK cells play a central role in recognizing and killing tumor cells without prior antigen recognition, and in recruiting other immune cells such as conventional type 1 dendritic cells (cDC1). These DCs can subsequently migrate to lymph nodes, mediating T cell activation and expansion, and further recruitment of effector lymphocytes to the tumor. They also uptake apoptotic tumor cells. NK and T cells secrete cytotoxic perforins and granzymes. Interferon-γ (IFN-γ) is also secreted by T cells. NK cells

(Figure legend continued on the bottom of the next page.)

β signaling, where cells from epithelial origin lose their polarity and intercellular adhesions, gaining invasive and migratory (mesenchymal) potential.

Graft-versus-host disease (GvHD): major clinical complication where donor T cells react against the recipient tissue by reacting to nonshared histocompatibility antigens.

Graft-versus-leukemia (GvL): after transplantation, allogeneic lymphocytes can trigger a strong reaction against host-derived leukemic cells, but the beneficial effects can be limited by an additional GvHD response.

Human leukocyte antigen (HLA): gene complex encoding MHC in humans.

Immune checkpoint inhibitors: drug category, often composed of antibodies (e.g., anti-CTLA-4, anti-PD-1, anti-PD-L1, etc.) that target and neutralize immune checkpoint proteins present in the tumor microenvironment, which inhibit the antitumor responses of immune cells.

Induced pluripotent stem cell (iPSC): stem cells generated from adult cells (e.g., skin or blood cells), allowing the development of a long-term source of other cell types to be engineered for therapeutic purposes.

Innate lymphoid cell (ILC)1: previously named ‘tissue resident NK cells’, these are derived from a common lymphoid progenitor (CLP), with an absence of antigen specificity and sharing characteristics with NK cells.

Invariant T cells: subsets displaying innate/effector-like characteristics, for example, mucosal-associated invariant T cells (MAIT), found in circulation and mucosal or lung tissues; they have a critical role in antimicrobial defense.

Killer-cell immunoglobulin-like receptor (KIR): family of type I transmembrane glycoproteins expressed in NK cells and some T cells regulating cytotoxicity functions by interacting with HLA I expressed in all cell types.

Major histocompatibility complex class I (MHC-I): identity protein complexes found in the cell surface of all mammalian nucleated cells. They display peptides from expected homeostatic patterns to cytotoxic T

their low expression [19]. These observations suggest that tumor evolution, or T cell immune escape, could render some malignant tumors more sensitive to NK cell killing than others, and accordingly, therapies enhancing NK cell activity or frequency within tumors deserve further attention. An increased understanding of negative regulators of NK cell activity or 'checkpoints', along with further insight into NK cell immune evasion mechanisms should lead to innovative immunotherapy strategies aimed at synergizing with current ICB, and which might further improve immunotherapy response rates. Here, we review the emerging role of NK cell-based cancer immunotherapies and highlight the potential limitations as well as advantages of this approach when compared with other therapies.

The Role of NK Cells in the Immune Activation Cascade and the Benefits of NK Cell Infiltration in Patient Survival

Over the past year, landmark studies have suggested NK cells act as a 'spark' to ignite a robust antitumor immune response through their interactions with DCs [20,21]. While NK cells have the ability to spontaneously detect and kill tumor cells, they also release chemokines and cytokines, including CCL5 (RANTES), XCL-1/XCL-2 [20], and FLT3 ligand [21], helping to recruit a subset of conventional DCs and prime their function (Figure 1, central panel). Responding DCs take up fragments of tumor cells and can subsequently migrate to secondary lymphoid organs, with antigen presentation facilitating expansion of reactive T cells. Using antibodies to deplete NK cells with genetically modified mouse strains that have NK and/or T cell deficiency (*Rag1*$^{-/-}$ and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$), one report elegantly revealed that NK cells were required for an optimal antitumor CD8^{+} T cell response by triggering the recruitment cascade for cDC1s and CTLs [20]. This NK cell-dependent increase in CD8^{+} T cell response was observed in B16F10 melanoma [21], *Ptgs1/Ptgs2*$^{-/-}$ BRAF^{V600E} melanoma and a transgene-driven breast cancer model (MMTV-PyMT) [20]. The effects of this immune signaling cascade are highlighted by patient survival effects associated with marker genes for these immune cell subsets in melanoma samples [22]. This effect can be observed with selected cell marker genes (Figure 2), although it should be noted that many NK cell and T cell marker genes are expressed across both cell types (albeit at different concentrations), and accordingly, their contributions can be difficult to distinguish in bulk tumor samples. Moreover, higher NK cell infiltration was recently associated with a significantly better response in melanoma patients treated with ICB relative to controls [21]. A mechanism by which tumor-resident NK cells may be further activated is the **STING** (stimulator of interferon genes) pathway. Using a **major histocompatibility complex class I (MHC-I)**-deficient lymphoma mouse model, the detection of cytosolic DNA in dying tumor cells by the enzyme cGAS was shown to result in cGAMP production; cGAMP was taken up by nontumor cells, activating STING, and leading to type-I

spontaneously detect and kill tumor cells and they also release chemokines and cytokines, including CCL5 (RANTES), XCL-1/XCL-2, and FLT3 ligand, helping to recruit cDC1s and prime their function. (A) The activation of NK cells depends upon the presence of local cytokines such as IL-15, IL-12, and IL-18. Resultant signaling also induces the suppressor of cytokine signaling (SOCS) family gene *CISH*, which provides negative feedback to downregulate this pathway. Other environmental cytokines such as transforming growth factor (TGF)-β can suppress the activation of NK cells through IL-15. (B) The activation of T cells by antigen presenting cells (APC) within the lymph node also triggers the expression of CTLA4, which competes with CD28 for the binding of CD80, providing a negative feedback mechanism for regulating T cell activation (inset). (C) Regulation of NK cell and T cell activity is achieved in part via binding of inhibitory cell surface receptors such as the killer-cell immunoglobulin receptors (KIRs) and NKG2A (*KLRC1*), which bind to different human leukocyte antigen (HLA) complexes on tumor cells. (D) An important component of the inflammatory response is secretion of IFN-γ, which binds to its receptor on tumor cells and induces the expression of genes with interferon-responsive elements (IREs). These genes, including those encoding programmed death-1 receptor (PD-L1) (CD274) or PD-L2 (not shown), mediate a negative feedback pathway through PD-1. While still contentious, there is evidence that PD-1 is also expressed in NK cells (see main text), and accordingly, a number of immune checkpoints are shared between T cells and NK cells and are being explored as potential therapeutic targets.

cells, which will only trigger clonal-dependent rejection and responses if these peptides are immunogenic neo-antigens or non-self. During events of oncogenesis, tumor cells routinely stop expressing the whole MHC-I complex, avoiding T cell recognition but rendering them still visible to NK cell immunosurveillance.

Myeloid-derived suppressor cell (MDSC): heterogeneous and unconventional group of immune cells derived from the myeloid lineage; they can infiltrate the tumor microenvironment and can display protective inhibitory functions against antitumor effector lymphocytes.

Neo-antigen recognition: specific antigens expressed by mutated tumor cells, discovered by tumor genomic sequencing, can trigger neo-antigen-specific T cell recognition and clonal expansion. Neo-antigen-specific T cells can be potentially generated/expanded *ex vivo* and infused back into patients as adoptive cell transfer therapy.

NKG2D: killer cell-lectin-like receptor subfamily K, member 1, is a transmembrane receptor that belongs to the CD94/NKG2 family of C-type lectin-like receptors and recognizes induced-self proteins from MIC and RAET1/ULBP families, commonly expressed on the surface of stressed or malignant cells.

Stimulatory receptors to stress ligands: the activity of effector CTLs is regulated by the balance of opposing stimulatory and inhibitory cell receptors. NK cell receptors such as NKG2D, NKp44, and NKp46 are considered activating receptors that recognize specific antigens to overcome inhibitory receptor signals to elicit cytotoxicity.

STING: intracellular DNA sensor inducing type I IFN expression when cells sense foreign (e.g., virus or intracellular parasite) or dying cell-DNA.

Trispecific killer engager (TriKE): BiKE display 'one', while a TriKE display two variable portions of an antibody of different specificity.

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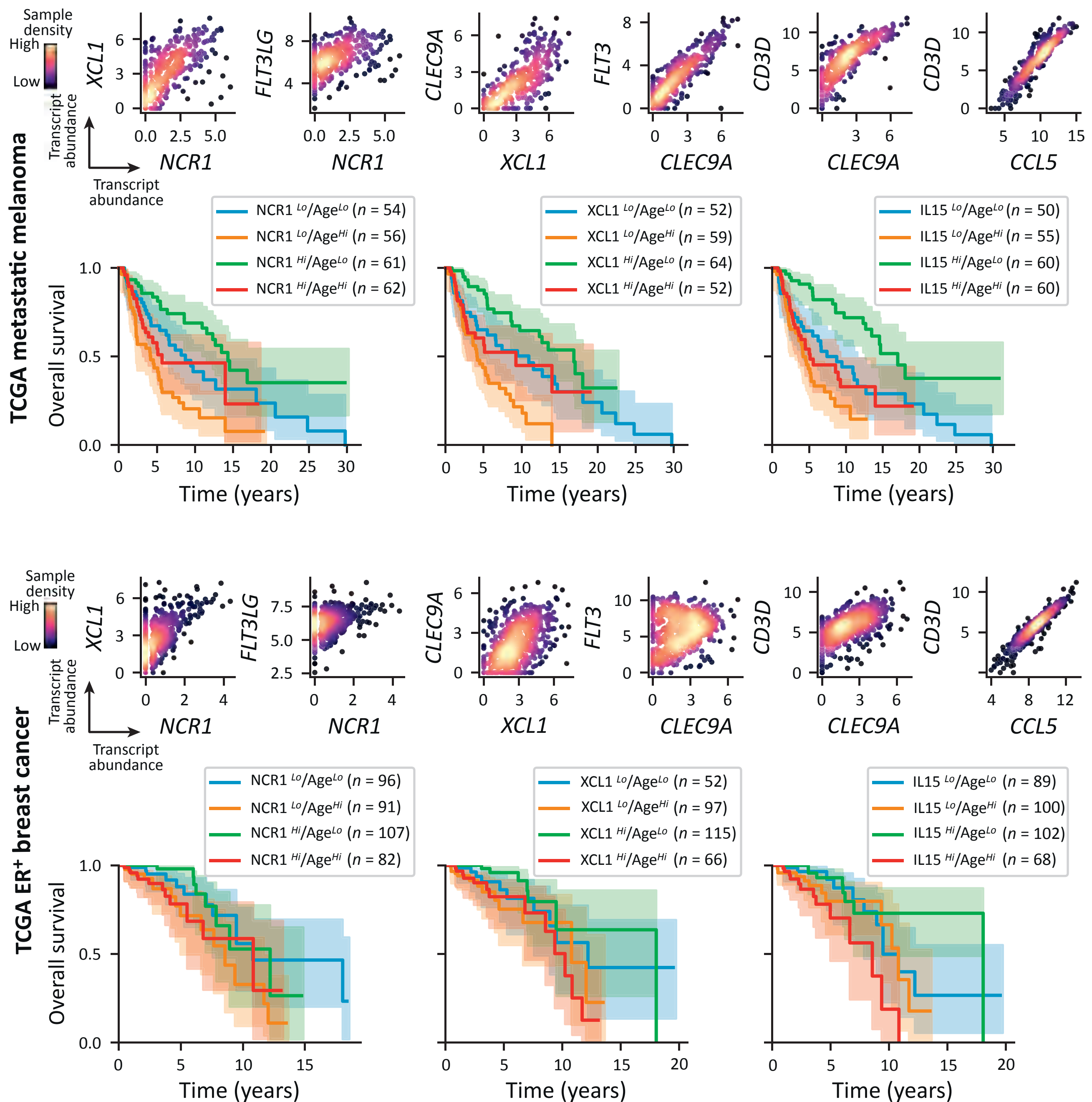

Figure 2. Natural Killer (NK) Cells and Recruited Immune Cell Types Are Associated with Improved Cancer Patient Outcomes. NK cells can play a pivotal role in the recruitment of dendritic cells (DCs) into tumors, contributing to robust antitumor immune responses. Scatter plots show the relative transcript abundance of selected marker genes within patient samples from the cancer genome atlas (TCGA) metastatic melanoma and estrogen receptor-positive (ER^+) breast cancer cohorts. The coordinated nature of immune cell recruitment is apparent in the associations between marker genes for NK cells (*NCR1*, also known as NK-p46 or *LY94*); DCs (*FLT3* and *CLEC9A*); and T cells (*CD3D*); as well as genes for secreted ligands that mediate immune cell recruitment, including putative NK secreted factors (*XCL1*, *FLT3LG*, and *CCL5*). The critical role of this intercellular signaling axis in tumor control is illustrated by Kaplan-Meier curves showing survival for patients from their respective TCGA cohorts, partitioned by age and relative abundance of indicated genes (top and bottom 40% as high/low, respectively). Data were downloaded directly from http://portal.gdc.cancer.gov/.

interferon (IFN) production. Moreover, type-I IFN has pleiotropic effects on the immune system, and in this lymphoma model, type-I IFN augmented NK cell antitumor responses [23].

In summary, therapies that increase NK cell frequency, function, and/or migration into tumors may have great potential as part of combinatorial strategies to complement T cell immunotherapies for cancer treatment. There is, however, a clear need to further improve our understanding of how lesser-studied immune subsets, such as NK cells, can function in the context of human tumor control, in order to further test these approaches.

Identification of Novel NK Cell Inhibitory Checkpoints

The Transforming Growth Factor (TGF)-β Superfamily

The TGF-β superfamily of cytokines represent a pleiotropic class of biomolecules which are essential for developmental patterning and adult wound-healing (Box 3). Signaling through this pathway appears to be dysregulated across a wide range of cancers where it can contribute to immunosuppression [24] and tumor progression through programs such as **epithelial–mesenchymal transition (EMT)** [25].

Interest in clinical modulation of the TGF-β superfamily has recently been reinvigorated [26]. Two recent studies independently showed that combination treatments of **anti-PD-1/PD-L1 therapies** with TGF-β blockade could increase CTL infiltration and antitumor responses to prevent growth and metastatic spread of both murine EMT6 breast mammary carcinoma and orthotopic colorectal cancer inoculation models [27,28]. Indeed, TGF-β small molecule inhibitors or antibodies are currently being investigated in a number of Phase I–III clinical trials [29] for: anaplastic astrocytoma (NCT00761280, trabedersen), glioblastoma (NCT00761280, trabedersen and NCT02423343, galunisertib), hepatocellular carcinoma (NCT01246986, galunisertib and NCT02423343, galunisertib), metastatic breast cancer (NCT01401062, fresolimumab), metastatic melanoma (NCT00356460, fresolimumab and NCT00844064, trabedersen), non-small cell lung cancer (NCT02423343, galunisertib; NCT00676507, lucanix; and NCT02639234, vigil), ovarian cancer (NCT02346747, vigil), pancreatic adenocarcinoma (NCT01373164, galunisertib and NCT02734160, galunisertib), and renal cell carcinoma (NCT00356460, fresolimumab).

TGF-β signaling is an important suppressor of NK cell function, where it acts to inhibit metabolism, proliferation, cytotoxicity, cytokine production, and antimetastatic functions in murine models of B16F10 melanoma, NEU15 breast cancer, and RM-1 prostate carcinoma [30]. Our group and others observed that this cytokine can induce cellular plasticity by upregulating **innate lymphoid cell (ILC)1**-like tissue residency characteristics. This includes expression of CD49a, CD69, DNAM-1, and TRAIL, and loss of eomesodermin in SM1WT1 melanoma, MCA1956 fibrosarcoma, or *de novo* 3-methylcholanthrene-induced fibrosarcoma murine tumor models, ultimately resulting in a reduction in NK cell antitumor functions [31,32].

Box 3. The TGF-β Cytokine

TGF-β is a pleiotropic cytokine with three different isoforms (TGF-β1, TGF-β2, and TGF-β3; encoded by three separate genes) and is expressed by several cell subsets present within the tumor microenvironment, including tumor cells, regulatory T cells, stromal fibroblasts, and various myeloid subsets [114]. The transmembrane receptors TGF-βRI and II are associated with intracellular serine/threonine kinases, and upon ligand binding, this initiates a signal transduction cascade that leads to phosphorylation of several SMAD-family transcription factors. This process begins with TGF-β ligand binding to TGF-βRII, which is activated, followed by subsequent recruitment and activation/phosphorylation of TGF-βRI. The final complex, consisting of TGF-β:TGF-βRII:TGF-βRI, then mediates the phosphorylation and activation of the SMAD transcription factor family, leading to its respective downstream signaling [115].

Recently, we refined an NK cell signature using the TCGA human metastatic melanoma data and showed, in agreement with recent studies [20,21], that high NK cell infiltration was associated with significantly improved overall patient survival relative to controls [22]. We found that the protective effect of NK cell infiltration was lost in melanoma samples where there was clear transcriptomic evidence of TGF-β-mediated EMT (TGF-β signature) [22]. Although pending full validation, this finding suggested that TGF-β itself, or the associated EMT process (melanoma phenotype switching) hindered the ability of NK cells and other downstream effectors to control tumor growth and metastasis. Such preclinical models show clear evidence that TGF-β suppresses NK cell effector functions, and inhibitors of this pathway can enhance endogenous tumor control and immunotherapy efficacy against certain tumors, in particular those derived from epithelial origins (e.g., breast/lung cancer, melanoma, ovarian cancer, sarcomas). Of note, the TGF-β superfamily has many additional members, such as activins and their respective receptors. A previous study reported that **activin-A** (a dimer of inhibin-β proteins) could weaken human peripheral NK cell-cytokine production *in vitro*, as well as B16F10 melanoma NK cell killing *in vivo*, at least in part through inhibitory signaling via type I and II activin receptors [33]. Extending these observations, activin-A signaling was reported to efficiently upregulate ILC1-like tissue residency features (e.g., upregulation of CD49a and CD69 while simultaneously downregulating eomesodermin) and suppress proliferation and cellular metabolism functions (lowering extracellular acidification and oxygen consumption rates) in both human or murine NK cells, although these findings await full confirmation [34]. Accordingly, activin-signaling appears to be an alternative SMAD2/3-related pathway that mediates TGF-β–like immunosuppressive effects independently of the conventional TGF-β/TGF-βRII interaction; this may provide further avenues to identify treatments that enhance the efficacy of current immunotherapies.

Regulators of Interleukin Signaling

IL-15 Signaling

NK cells are unable to survive for an extended period of time without IL-15 signaling, which promotes the sustained expression of critical antiapoptotic proteins, including MCL-1 [35]. IL-15 is known to be essential for NK cell function; it binds to IL-15Rβ⁄γ heterodimers on the surface of NK cells inducing the activation of the β⁄γ-associated JAK1 and JAK3 tyrosine kinases. This, in turn, leads to the recruitment and activation of STAT5, culminating in the transcription of STAT5-target genes required for survival and cytotoxicity, such as *Bcl2*, *GzmB*, *Idb2*, *Mcl1*, *Pim2*, and *Prf1* in murine NK cells [36–38]. IL-15 treatment will promote expansion of lymphocytes that express IL-15Rβ⁄γ, such as $CD8^+$ T cells, **invariant T cells**, and NK cells, through a JAK1/3 and STAT5-dependent signaling pathway [39,40]. The benefits of IL-15 in boosting antitumor effector lymphocytes and optimizing $CD8^+$ T cell and NK expansion is currently under clinical investigation (NCT01727076) in patients with advanced melanoma, kidney cancer, non-small cell lung cancer, as well as squamous cell head and neck cancers (HNC) [41]. Recently, the potential of IL-15 as a combinatorial treatment with CTLA-4 (ipilimumab) and PD-1 (nivolumab) blockade immunotherapies is also being clinically investigated in patients with refractory cancers that are not curable or that do not have known clinical approaches associated with potential survival benefits (NCT03388632) [42]. IL-2 is considered by some as the first effective immunotherapy for cancer, being FDA approved for kidney cancer in 1992 and melanoma in 1998 [43]. Although given the serious toxicities associated with its dosing and the modest response rate, there has been heavy investments in investigating modified/engineered IL-2 formats that address these short-comings. Engineered IL-2 formats include those with altered binding affinity to IL-2Rα versus IL-2Rβ, and altered pharmacokinetics with several formats are currently in clinical trials (recently reviewed in [44]). Furthermore, super agonists of IL-15R have been undergoing preclinical development by creating fusion

complexes between IL-15 and IL-15Rα domains that mimic the more bio-active endogenous trans-presented IL-15 format. Indeed, these highly potent IL-15 formats can overcome TGF-β-mediated inhibition of NK cell cytotoxicity against four human tumor cell lines (H460, LNCaP, MCF7, and MDA-MB-231) [45].

The kinetics of IL-15 signaling are rapid and transient and conserved between mouse and human lymphocytes, with receptor/JAK/STAT phosphorylation observed within minutes of cytokine:receptor docking and lasting for up to 1 h [46]. The SOCS (suppressor of cytokine signaling) family proteins (*Cish, Socs1-7*) are rapidly induced in response to cytokine stimulation, acting as a negative feedback to limit the duration of cytokine signaling [47,48]. *Cish* is a STAT5-target gene induced by IL-2 and IL-15, and has been previously shown to bind to the IL-2Rβ chain [49]. *Cish* is induced by IL-15 in NK cells and it regulates IL-15 responses by inhibiting JAK1 activity, subsequent NK cell activation, and antimetastatic function; thus *Cish* can be considered as an intracellular 'checkpoint' that limits IL-15-induced NK cell function [50] (Figure 1A). These murine findings are in agreement with a recent study reporting preliminarily that when *Cish* was efficiently deleted by **CRISPR/Cas9** genomic editing in human NK cells, this resulted in enhanced IL-15 signaling, proliferation, and cytotoxicity relative to controls [51]. This would seem to validate the role of *Cish* as a checkpoint in NK cell activation, providing, to our knowledge, the first evidence that human NK cells can be efficiently genome-edited using CRISPR/Cas9, although awaiting full confirmation. Given the unmet need for complementary therapies that can maximize antitumor responses and potentially synergize with current gold standard immunotherapies (e.g., anti-PD-1), IL-15-based therapies are emerging as leading immunotherapy candidates.

IL-12 and IL-18 Signaling

IL-12 is another JAK/STAT-related interleukin signaling through JAK2/STAT4. In this signaling cascade, the p35/p40 IL-12 complex binds to an IL-12Rβ2:IL-12Rβ1 heterocomplex, thus allowing STAT4 phosphorylation and subsequent translocation to nucleus, where it can exert its transcriptional activity [52]. IL-12 is a critical activating cytokine for NK cells, driving their cytokine production (e.g., IFN-γ and GM-CSF), and initial clinical studies have shown that a tolerated dose of recombinant IL-12 in advanced renal cell and colorectal carcinomas and in melanoma patients could reactivate NK cell function (e.g., increased IFN-γ production) and promote significant immune activity [53,54]. Recently, the applications of IL-12 have been revisited as a putative approach to potentiate checkpoint blockade- or adoptive cell-based immunotherapies; indeed, it can simultaneously rescue NK and/or T cell cytotoxicity and induce the development of an unconventional IL-12-dependent NK cell population *in vivo*, recognizing and controlling experimental 4T1 breast cancer-, Lewis lung carcinoma-, and B16F10 melanoma-induced metastases [55–57]. Recently, patients with recurrent ovarian carcinoma receiving intraperitoneal treatment with an IL-12 plasmid (GEN-1) administered with PEGylated liposomal doxorubicin have displayed clinical benefits without excessive toxicity [58]. Thus, IL-12-based therapy may also be a potential candidate to enhance immunotherapy with **immune checkpoint inhibitors** in combination immunotherapies.

In contrast, IL-18 is a JAK/STAT-independent interleukin related to the IL-1 family, critical for NK cell priming and IL-12-induction of IFN-γ/cytokine production [59]. Once bound to the IL-18Rα/β heterocomplex, IL-18 signals by a downstream pathway shared with toll-like receptors (TLR), leading to the recruitment of the MyD88 adaptor followed by NF-κB complex activation and metabolic priming of NK cells to enhance effector functions [60]. IL-1R8 was recently identified as an inhibitory checkpoint for IL-18 signaling in NK cells, such that deletion of IL-1R8 enhanced

NK cell metabolism and promoted hyperactivity in experimental MC38-induced liver metastases, MN/MCA1 sarcoma-induced lung metastasis, and diethylnitrosamine-induced *de novo* liver carcinogenesis [61]. Several new NK cell checkpoints have thus been revealed by studies on the JAK/STAT and NF-κB pathways, as well as the TGF-βR superfamily ligands/receptors in immune cells; it may be thus possible to target these pathways to complement current immune-therapeutic interventions.

Shared Checkpoints between T and NK cells

Ipilimumab (anti-CTLA-4) received FDA approval in 2011, followed by five additional checkpoint blockade therapies targeting the PD-1/PD-L1 members, to treat a range of different advanced cancers (including melanoma, non-small lung cancer, renal cell carcinoma, and Hodgkin lymphoma) [4]. Although blocking these inhibitory checkpoints in tumor-infiltrating lymphocytes (TILs) has revolutionized clinical practice, there are still conceptual gaps in our understanding of the mechanisms of action that underlie this practice, particularly with respect to how these checkpoints influence other immune cell subpopulations [4]. For instance, there are conflicting data on the mechanism of action of ipilimumab in depleting CTLA-4-expressing regulatory T cells versus blocking this receptor on CTLs [62–64]. NK cells have also been implicated in anti-CTLA-4 actions via **antibody-dependent cellular cytotoxicity (ADCC)** of CTLA-4 expressing melanoma cells [65]. However, while NK cells express very low levels of CTLA-4 and PD-1, a recent study using murine RMA-S or CT26-derived primary tumors, revealed that PD-1 was expressed by tumor-infiltrating NK cells, and further, anti-PD-1 therapy could improve their responsiveness and tumor control even in the absence of T cell cells [66]. In Hodgkin lymphoma, CD56^{dim} NK cells expressed higher concentrations of PD-1 than diffuse large B cell lymphoma, and PD-L1-expressing myeloid cells could efficiently suppress these PD-1^{+} NK cells *in vitro*, while anti-PD-1 reversed this effect [67]. Further evidence for the PD-1 signaling axis in NK cells comes from another study reporting PD-1 upregulation during NK cell recognition of cetuximab-opsonized HNC [68]. This was associated with an activated phenotype during ADCC, and PD-L1 binding to PD-1^{+} NK cells suppressed their activity, suggesting that blocking PD-1 could enhance NK cell-induced immunotherapy against HNC together with cetuximab. Similarly, other lymphocyte checkpoints, such as the lymphocyte-activation gene 3 (LAG3), have been previously shown to mediate an inhibitory checkpoint in T lymphocytes by overcoming TCR/CD3-induced activation [69,70], such that anti-LAG3 (or LAG3-blocking) antibodies synergistically restored T cell responsiveness in cancer when combined with anti-PD-1 antibodies in established Sa1N fibrosarcoma or MC38 tumors [71]. Our group has observed upregulation of CTLA-4, LAG-3, and PD-1 in infiltrating NK cells from established fibrosarcoma tumors in mice [31], and we believe that the effects of checkpoint-neutralizing antibodies are yet to be fully explored in the context of NK cell biology.

KIRs are a family of receptors heterogeneously expressed on NK cells, and to a lesser extent T cells, which can regulate lymphocyte cell cytotoxicity by interacting with HLA expressed on cells (Figure 1C). Inhibiting the interaction between KIRs and HLA class I molecules was previously shown to enhance NK cell-mediated control of acute myeloid leukemia (AML) and increased patient survival upon transplantation with KIR mismatched donors [72]. KIRs have been viewed as a checkpoint in cytotoxic lymphocyte activation, thus, a blocking antibody targeting KIR2DL1/2/3 was engineered to interfere with HLA-C binding. Although anti-KIR antibodies have displayed potent NK cell-mediated antitumor efficacy in preclinical models (e.g., AML [73], multiple myeloma [74], and B lymphoma experimental models [75]), and Phase I clinical trials demonstrated satisfactory safety in patients [76,77], subsequent clinical trials in AML patients have failed to show clinical effects (NCT01687387 and [78]).

A different family of paired receptors, including TIGIT [T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif (ITIM) domain] [79,80] and DNAM-1 (DNAX accessory molecule-1, or CD226) [81], bind target receptors that are weakly expressed in normal human tissues, but are often upregulated in tumor cells due to stress [e.g., poliovirus receptor (PVR or CD155), or less frequently, poliovirus receptor-related 2 (Nectin-2 or CD112 [82])]. These receptors can exhibit opposing functions (e.g., TIGIT suppresses lymphocyte activity, while DNAM-1 is activating; recently reviewed in [83]). DNAM-1 activation through PVR ligands was initially noted based on the observation that mouse and human lymphomas expressing these ligands were more susceptible to activated T and NK cell-mediated cytotoxicity, while blocking DNAM-1 would prevent killing [84]. An early study revealed that both human and murine tumor-infiltrating $CD8^+$ T cells expressed high concentrations of TIGIT, preventing DNAM-1-mediated activation by competing with the CD155 ligand, and accordingly, dual antibody therapy with simultaneous blockade of TIGIT and PD-1 could elicit $CD8^+$ T cell antitumor control [85]. A subsequent study showed that TIGIT-expressing NK cells were highly susceptible to inhibition by $CD155^+$ **myeloid-derived suppressor cells (MDSCs)**, as the presence of MDSC in co-culture systems prevented NK cell activation and cytotoxicity against target tumor cells; indeed, blocking this checkpoint restored NK cell effector function during human cytomegalovirus infection [86].

Tactile (or CD96) can also bind to CD155, although this receptor is not deemed to have a significant contribution as a checkpoint in human NK cells. It is thought that NK cell function is primarily regulated by TIGIT, as human NK cells can recover some function through TIGIT blockade, but no effect has been observed upon blockade of CD96 in $CD155^+CD96^+$ MDSC co-culture assays [86]. A role for TIGIT is supported by another study using several experimental models (murine CT26 colon cancer, 4T1 breast cancer, B16 melanoma, and MCA fibrosarcoma) to demonstrate that TIGIT blockade in NK cells, alone or in combination with other checkpoint blockade, promoted increased antitumor responses [87]. Accordingly, targeting this pathway may constitute a promising putative therapeutic strategy in certain cancers.

Transformed cells tend to upregulate a number of molecules in response to abnormal cellular stress, and many of these act as ligands for **NKG2D** (e.g., MICA, MICB, and ULBP1-6), a receptor present in both NK and T cells [88]. Although the recognition of these ligands can trigger immune responses, a recent study demonstrated that human and murine tumor cells exploit mechanisms to shed MICA/B from their surface [89]. This can prevent NK cell recognition and tumor cell binding, either by releasing the membrane-bound MICA/B expression required for NK recognition, and/or by increasing soluble MICA/B, which can bind NKG2D and limit NK cell interactions with tumor cells. To overcome this, bispecific antibodies were developed (e.g., 7C6-mIgG2a) to boost NK cell immunosurveillance by binding distinct epitopes in different regions of NKG2D ligands and preventing their shedding from the tumor cell surface; this resulted in enhanced antitumor immunity and control of metastases in B16F10 melanoma and CT26 colorectal cancer models [11,89]. However, these data are at odds with earlier preclinical work demonstrating that soluble MULT1, a high affinity mouse NKG2D ligand enhanced NK cell tumor immunity by stimulating NKG2D in distant NK cells [90]. These inconsistencies may be explained by differences in the binding affinities between NKG2D and the ligands in question. Contrasting NKG2D, NKG2A is a receptor from the same family that heterodimerizes with CD94 and acts as an inhibitory checkpoint on NK cells and CTL upon recognition of HLA-E [91] (Figure 1C). A recent study revealed that neutralizing antibodies to NKG2A promoted both NK cells and CTL effector functions in various preclinical models by enhancing killing activity and synergizing with anti-PD-1 therapy. The clinical anti-NKG2A agent (monalizumab) was also combined with cetuximab (anti-EGFR) in patients with squamous cell

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carcinoma of head and neck, in which the combined therapy response was encouraging, and reported to be superior to that of historical data on cetuximab alone (with the caveat that the comparison was done against historical data) [92]. Considering the critical role of NK cells for innate surveillance and control of cancer metastases, we believe that novel therapeutic opportunities exist for optimizing antitumor immunity and preventing cancer reoccurrence by unraveling shared mechanisms of immune checkpoint blockade across T cells and NK cells (Figure 3).

Adoptive NK Cell Therapy

Adoptive CTL therapies have been investigated over the past 30 years [93], and more recently this approach has been applied to NK cells (Figure 4). NK cells offer several advantages in the adoptive cell therapy setting, including transplantation safety. Cytokine-release syndrome (CRS) is a serious clinical complication that can be induced by allogeneic T cells responding to mismatched MHC and which can contribute to **graft-versus-host disease (GvHD)** [94]. Clinical evidence suggests that allogenic NK cells are safe, with neither CRS or GvHD being observed in various cancer patients receiving *in vitro* expanded allo-NK cells [95]. Furthermore, high-risk leukemia patients receiving allogeneic NK cells with no KIR-ligand reactivity in their donor hematopoietic graft exhibited improved donor engraftment, reduced GvHD, and reduced probability of relapse compared with grafts with KIR reactive NK cells [72]. Similarly, the introduction of grafts depleted of α/β T cells and B cells, but containing fully functional alloreactive NK cells, can contribute to an optimal **graft-versus-leukemia (GvL)** effect, where donor NK cells react strongly to HLA disparate leukemic cells [96]. Not surprisingly, multiple studies have now been performed using adoptive NK cell infusions in leukemia, often from a HLA-mismatched donor, since alloreactive NK cells will receive activating signals from stress ligands on the surface of leukemia cells without the inhibitory signals from KIRs binding matched HLA [97].

Recently, **chimeric antigen receptor** (CAR) T cell immunotherapies have also provided a powerful alternative to treat cancer in an antigen-specific manner. The CAR consists of an antigen-binding fragment fused to the T cell receptor, signaling components to generate a potent antigen-specific T cell response upon ligand binding [98]. Following highly successful clinical trials, CD19-CAR T cell adoptive therapy has been FDA approved for acute lymphoblastic leukemia and diffuse large B cell lymphoma [99]. There have been considerable drawbacks, however, with hyperactive CAR T cells leading to important side effects, such as CRS (leading to immune hyperactivation) and neurological toxicities [100]. Given these limitations, CAR NK cell products have recently been developed, showing promising antitumor efficiency and good safety profiles in terms of CRS and GvHD (reviewed in [101,102]). Preliminary observations for CD19-CAR engineered human NK cells have indicated persistence and antitumor control activity against murine or human $CD19^+$ leukemia in mouse and humanized mice settings in two independent studies [103,104]. Another recent report used **induced pluripotent stem cell (iPSC)** as an efficient off-the-shelf source of expanding NK cells to optimize the process of NK-CAR engineering, producing NK-CAR-iPSC cells *in vivo* with a remarkable efficiency that was comparable with CAR T cells in controlling $CD19^+$ leukemia progression in humanized mouse models, but with far less evident CRS [105].

Although cellular or adoptive immunotherapy and synthetic biology or cell engineering are relatively novel approaches, the infusion of genetically enhanced lymphocytes is a promising tool for the long-term treatment and prevention of cancer occurrence. Novel technologies such as CRISPR/Cas9 now provide an unprecedented and relatively simple approach to gene editing (e.g., introduction of specific CAR receptors or deletion of inhibitory checkpoints),

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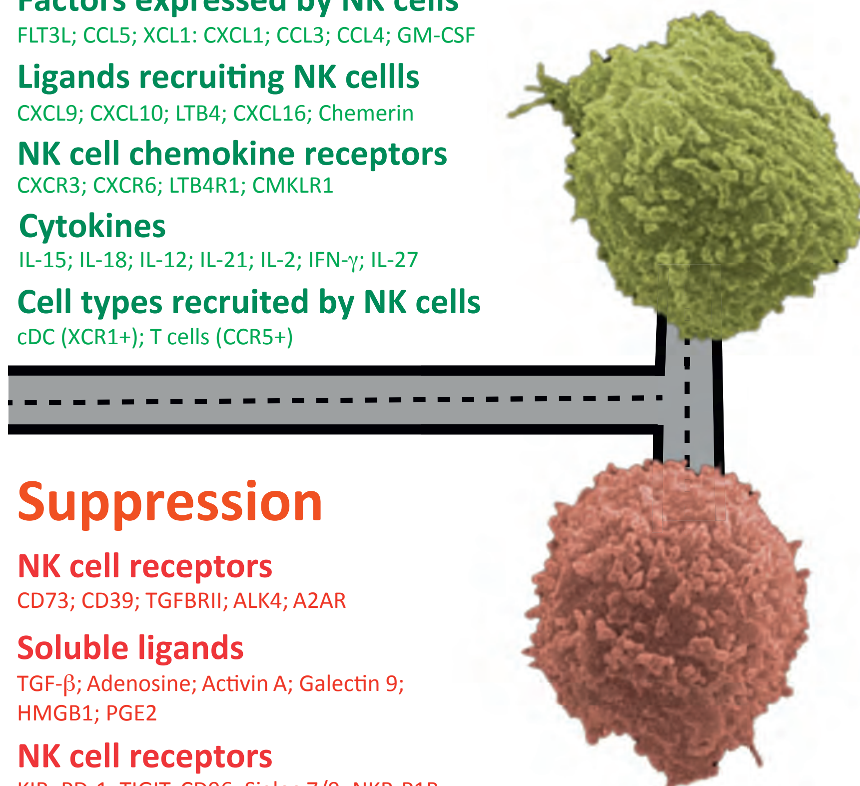

Figure 3. Natural Killer (NK) Cells at the Crossroads of Tumor Immunity. The spontaneous ability for NK cells to detect and respond to cancer cells, and recruit additional immune effectors, is controlled by numerous signals. 'GO signals' in green represent soluble and membrane factors increasing NK cell activation and tumor infiltration/inflammation. NK cell activation is controlled by soluble cytokines and membrane ligands. NK cell recruitment into tumors and the ability to recruit other effector immune cells is governed by their chemokine receptor expression and the production of chemokines. 'STOP signals' in red represent soluble and membrane factors reducing NK cell activation, fitness, viability, and ultimately, their ability to contribute to tumor immunity. NK cell suppression is controlled by soluble cytokines/growth factors and membrane ligands.

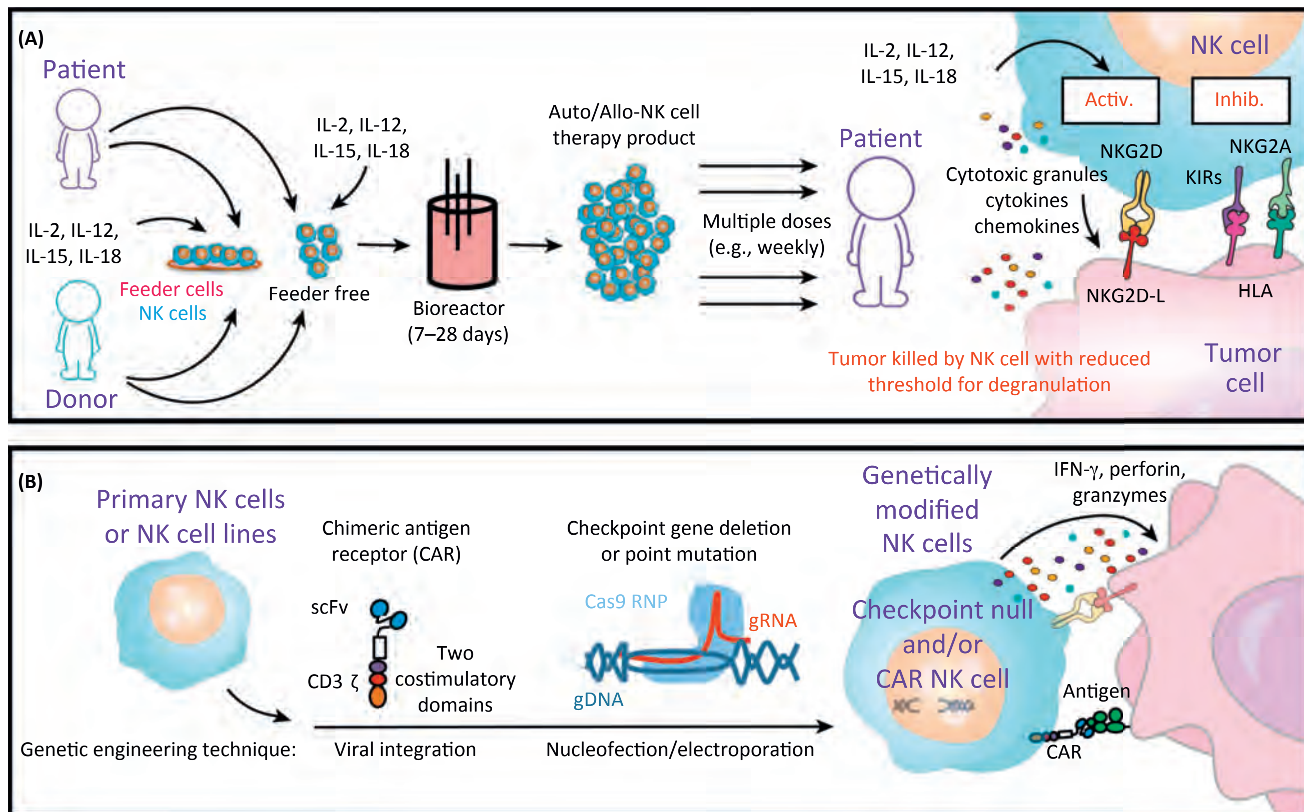

Figure 4. Adoptive Natural Killer (NK) Cell Therapies. (A) NK cells can be isolated from patients or unrelated donors and expanded *in vitro* using cytokines (IL-15, IL-2, IL-12, IL-18) alone or with a 'feeder' cell line. Clinical NK cell products typically require several weeks of *in vitro* expansion in bioreactors before adequate cell numbers are available for multiple infusions into the cancer patient. Cytokines used in this protocol not only result in NK cell proliferation but reduce their subsequent threshold for activation (Activ.) when detecting tumor cells (e.g., via NKG2D-L). This increases the likelihood of tumor killing even in the presence of inhibitory (Inhib.) signals [e.g., human leukocyte antigen (HLA)]. (B) Primary human NK cells or NK cell lines can be genetically modified to improve their activation, expansion, persistence, and tumor recognition. CAR genes can be integrated into the NK cell genome using high viral titers, whereas nucleofection technologies are emerging as an efficient approach to delete genes in NK cells via CRISPR and Cas9/gRNA ribonucleoproteins. Thus, human NK cells can be genetically modified to harbor a reduced activation threshold (e.g., deletion of a checkpoint gene) or improved tumor targeting (e.g., expression of a CAR), increasing the likelihood of tumor killing. IFN, Interferon; KIR, killer-cell immunoglobulin receptor; scFv, single-chain variable fragment.

facilitating the production of products for cell-based therapies [106]. These approaches highlight the exciting potential offered by NK cell adoptive transfer, which may be an efficient alternative to current approaches with presumably lower toxicity. Given the early stages of these technologies, however, extensive and robust testing is warranted and consideration should be given to minimizing product development costs while maximizing cellular response to ensure that basic and checkpoint research can aid in the rapid translation of such approaches.

Improved NK Cell Targeting to Cancer Cells

As exemplified above, checkpoint inhibition, cytokine-driven activation, and adoptive cellular therapies present us with possible new horizons in the application of NK cell-based immunotherapies that might enhance the efficacy and longevity of current therapies. There are a number of processes that can be considered in the field to optimize NK cell responses, such as ADCC [107]. Taking advantage of NK cell-mediated ADCC may be facilitated by novel antibody engineering approaches that can maximize NK cell responses to targets, with the development

of **bispecific killer cell engager (BiKEs) or trispecific killer engager (TriKEs) antibodies**. A BiKE is produced from the fusion of a single-chain variable fragment (scFv) to an anti-CD16 recognition site, connected with a linker to the scFv of a specific tumor-expressed antigen (e.g., CD19/CD20 for non-Hodgkin lymphomas, CD30 for Hodgkin lymphoma, or CD33/CD123 for acute myelogenous leukemia/AML), facilitating enhanced NK cell-recognition of target cells through FcR mechanisms. The novel TriKE approach consists of a modified BiKE, which also includes the cytokine IL-15 attached through a linker between the two scFv components, acting to boost NK cell function, survival, and priming by additional IL-15 signaling [108]. The overall goal of these approaches is to increase the magnitude of NK cell activation in close proximity to a tumor target. By engaging a strong activation receptor on NK cells (CD16), bridging it to a tumor target (CD19/CD33), and assuming the NK cell can also detect the target via the array of **stimulatory receptors to stress ligands** (NKG2D, NKp44, NKp46), this technology should presumably lower the threshold for NK cells to degranulate and lyse target cells. By introducing IL-15 into the TriKE, this could further reduce the activation threshold required for NK cells to kill target cells, result in more targets being killed, and ultimately reduce tumor burden; this has been shown for CD33-expressing myeloid cancers, such as AML and myelodysplastic syndrome following CD16-IL-15-CD33 TriKE-enhancement of NK cell killing kinetics [109]. Thus, while NK cells can spontaneously detect tumors, novel agents that can increase the frequency of interactions, strength, or functional quality of the NK cell:tumor interface hold promise for further exploiting NK cell-dependent cytotoxicity, especially in tumors expressing a well-defined antigen, such as CD19.

Concluding Remarks

Checkpoint inhibitors primarily block inhibitory pathways in tumor-resident T cells, however there is growing interest in other effector populations such as NK cells. In part, NK cells provide an attractive alternative to T cell immunotherapies because they preferentially target 'altered' or transformed (e.g., tumor) cells in the body without the need of prior sensitization and with a reduced risk of autoimmune disease. The innate ability of NK cells to detect cellular transformation is critical for cancer immunosurveillance, particularly in settings of metastasis or hematological cancers, and it has become clear that cancer cells produce immunosuppressive factors that can impair antitumor immune responses. Accumulating data suggests that targeting NK cells *in vivo* is achievable and may provide an alternative or complementary immunotherapy approach to the class-leading ICB. Our understanding of the dominant inhibitory/regulatory mechanisms in NK cells is still incomplete and more basic research is required to stratify which pathways are most likely to yield therapeutic benefit when successfully drugged (see Outstanding Questions). The potential for staggered or sequential immunotherapy approaches, targeting NK cells then CTLs or vice versa, to counter immune resistance in cancer is an interesting possibility. Moreover, it is possible that combination therapies targeting different cytotoxic effectors may prove to be more rational than combination therapies against the same effector population; it will thus be exciting to follow the path of future research in this arena.

Outstanding Questions

Can public tumor data be leveraged using computational methods (e.g., deconvolution or NK cell infiltration scores) to identify novel mechanisms of tumor immune evasion or activation to help tailor personalized immunotherapies for specific patient groups?

Can side effects to inhibitors of the TGF-β superfamily, JAK/STAT regulators, and/or NF-κB be limited by targeted treatment of specific immune cells rather than systemic treatment?

Given the role NK cells play by initiating a robust immune response against tumors, do they represent an 'efficient' target for immunotherapy, with indirect benefits for systemic immune activity?

Which checkpoints (either shared with T cells or restricted to NK cells) might be simultaneously targeted to maximize antitumor immune activity against advanced cancers?

Can off-the-shelf technologies allow NK cell gene editing and *ex vivo* expansion for clinical adoptive transfers at an accessible price?

Will novel gene editing technologies be able to maximize the antitumor activity of NK cells?

Will CAR-NK cells offer significant advantages along with or over CAR-T cell-based therapies for specific patient groups?

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References

1. Hodi, F.S. *et al.* (2018) Nivolumab plus ipilimumab or nivolumab alone versus ipilimumab alone in advanced melanoma (CheckMate 067): 4-year outcomes of a multicentre, randomised, phase 3 trial. *Lancet Oncol.* 19, 1480–1492
2. Binnewies, M. *et al.* (2018) Understanding the tumor immune microenvironment (TIME) for effective therapy. *Nat. Med.* 24, 541–550
3. Ribas, A. (2015) Adaptive immune resistance: how cancer protects from immune attack. *Cancer Discov.* 5, 915–919
4. Wei, S.C. *et al.* (2018) Fundamental mechanisms of immune checkpoint blockade therapy. *Cancer Discov.* 8, 1069–1086
5. Krasnova, Y. *et al.* (2017) Bench to bedside: NK cells and control of metastasis. *Clin. Immunol.* 177, 50–59
6. Sathe, P. *et al.* (2014) Innate immunodeficiency following genetic ablation of Mcl1 in natural killer cells. *Nat. Commun.* 5, 4539
7. Chockley, P.J. *et al.* (2018) Epithelial-mesenchymal transition leads to NK cell-mediated metastasis-specific immunosurveillance in lung cancer. *J. Clin. Invest.* 128, 1384–1396
8. Pasero, C. *et al.* (2015) Highly effective NK cells are associated with good prognosis in patients with metastatic prostate cancer. *Oncotarget* 6, 14360–14373
9. Souza-Fonseca-Guimaraes, F. (2016) NK cell-based immunotherapies: awakening the innate anti-cancer response. *Discov. Med.* 21, 197–203
10. Braud, V.M. *et al.* (1998) HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* 391, 795–799
11. Borrego, F. *et al.* (1998) Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. *J. Exp. Med.* 187, 813–818
12. Zitti, B. and Bryceson, Y.T. (2018) Natural killer cells in inflammation and autoimmunity. *Cytokine Growth Factor Rev.* 42, 37–46
13. Boudreau, J.E. and Hsu, K.C. (2018) Natural killer cell education in human health and disease. *Curr. Opin. Immunol.* 50, 102–111
14. Marincola, F.M. *et al.* (1994) Locus-specific analysis of human leukocyte antigen class I expression in melanoma cell lines. *J. Immunother. Emphasis Tumor Immunol.* 16, 13–23
15. Park, H.S. *et al.* (2018) Loss of human leukocyte antigen class I expression is associated with poor prognosis in patients with advanced breast cancer. *J. Pathol. Transl. Med.* Published online November 14, 2018. http://dx.doi.org/10.4132/jptm.2018.10.11
16. McGranahan, N. *et al.* (2017) Allele-specific HLA loss and immune escape in lung cancer evolution. *Cell* 171, 1259–1271
17. Norell, H. *et al.* (2006) Frequent loss of HLA-A2 expression in metastasizing ovarian carcinomas associated with genomic haplotype loss and HLA-A2-restricted HER-2/neu-specific immunity. *Cancer Res.* 66, 6387–6394
18. Aptsiauri, N. *et al.* (2018) The transition from HLA-I positive to HLA-I negative primary tumors: the road to escape from T-cell responses. *Curr. Opin. Immunol.* 51, 123–132
19. Sottile, R. *et al.* (2016) HLA class I downregulation is associated with enhanced NK-cell killing of melanoma cells with acquired drug resistance to BRAF inhibitors. *Eur. J. Immunol.* 46, 409–419
20. Bottcher, J.P. *et al.* (2018) NK cells stimulate recruitment of cDC1 into the tumor microenvironment promoting cancer immune control. *Cell* 172, 1022–1037
21. Barry, K.C. *et al.* (2018) A natural killer-dendritic cell axis defines checkpoint therapy-responsive tumor microenvironments. *Nat. Med.* 24, 1178–1191
22. Cursons, J. *et al.* (2018) A natural killer cell gene signature predicts melanoma patient survival. *bioRxiv* Published online July 23, 2018. http://dx.doi.org/10.1101/375253
23. Marcus, A. *et al.* (2018) Tumor-derived cGAMP triggers a STING-mediated interferon response in non-tumor cells to activate the NK cell response. *Immunity* 49, 754–763
24. Ganesh, K. and Massague, J. (2018) TGF-beta inhibition and immunotherapy: checkmate. *Immunity* 48, 626–628
25. Foroutan, M. *et al.* (2017) A transcriptional program for detecting TGFbeta-induced EMT in cancer. *Mol. Cancer Res.* 15, 619–631
26. Visan, I. (2018) Targeting TGF-beta in cancer. *Nat. Immunol.* 19, 316
27. Mariathasan, S. *et al.* (2018) TGFbeta attenuates tumour response to PD-L1 blockade by contributing to exclusion of T cells. *Nature* 554, 544–548
28. Tauriello, D.V.F. *et al.* (2018) TGFbeta drives immune evasion in genetically reconstituted colon cancer metastasis. *Nature* 554, 538–543
29. de Gramont, A. *et al.* (2017) Novel TGF-beta inhibitors ready for prime time in onco-immunology. *Oncoimmunology* 6, e1257453
30. Viel, S. *et al.* (2016) TGF-beta inhibits the activation and functions of NK cells by repressing the mTOR pathway. *Sci. Signal.* 9, ra19
31. Gao, Y. *et al.* (2017) Tumor immunoevasion by the conversion of effector NK cells into type 1 innate lymphoid cells. *Nat. Immunol.* 18, 1004–1015
32. Cortez, V.S. *et al.* (2017) SMAD4 impedes the conversion of NK cells into ILC1-like cells by curtailing non-canonical TGF-beta signaling. *Nat. Immunol.* 18, 995–1003
33. Robson, N.C. *et al.* (2009) Activin-A attenuates several human natural killer cell functions. *Blood* 113, 3218–3225
34. Rautela, J. *et al.* (2018) Therapeutic blockade of Activin-A improves NK cell function and anti-tumor immunity. *bioRxiv* Published online October 29, 2018. http://dx.doi.org/10.1101/454801

35. Huntington, N.D. *et al.* (2007) Interleukin 15-mediated survival of natural killer cells is determined by interactions among Bim, Noxa and Mcl-1. *Nat. Immunol.* 8, 856–863
36. Verdeil, G. *et al.* (2006) STAT5-mediated signals sustain a TCR-initiated gene expression program toward differentiation of CD8 T cell effectors. *J. Immunol.* 176, 4834–4842
37. Huntington, N.D. (2014) The unconventional expression of IL-15 and its role in NK cell homeostasis. *Immunol. Cell Biol.* 92, 210–213
38. Rautela, J. *et al.* (2018) Molecular insight into targeting the NK cell immune response to cancer. *Immunol. Cell Biol.* 96, 477–484
39. Lin, J.X. *et al.* (1995) The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. *Immunity* 2, 331–339
40. Johnston, J.A. *et al.* (1995) Interleukins 2, 4, 7, and 15 stimulate tyrosine phosphorylation of insulin receptor substrates 1 and 2 in T cells. Potential role of JAK kinases. *J. Biol. Chem.* 270, 28527–28530
41. Conlon, K.C. *et al.* (2015) Redistribution, hyperproliferation, activation of natural killer cells and CD8 T cells, and cytokine production during first-in-human clinical trial of recombinant human interleukin-15 in patients with cancer. *J. Clin. Oncol.* 33, 74–82
42. Coyne, G.H.O.S. *et al.* (2018) Phase I study of recombinant interleukin-15 in combination with checkpoint inhibitors nivolumab and ipilimumab in subjects with refractory cancers. *J. Clin. Oncol.* 36, TPS3128
43. Rosenberg, S.A. (2014) IL-2: the first effective immunotherapy for human cancer. *J. Immunol.* 192, 5451–5458
44. Spolski, R. *et al.* (2018) Biology and regulation of IL-2: from molecular mechanisms to human therapy. *Nat. Rev. Immunol.* 18, 648–659
45. Fujii, R. *et al.* (2018) An IL-15 superagonist/IL-15Ralpha fusion complex protects and rescues NK cell-cytotoxic function from TGF-beta1-mediated immunosuppression. *Cancer Immunol. Immunother.* 67, 675–689
46. Ring, A.M. *et al.* (2012) Mechanistic and structural insight into the functional dichotomy between IL-2 and IL-15. *Nat. Immunol.* 13, 1187–1195
47. Babon, J.J. and Nicola, N.A. (2012) The biology and mechanism of action of suppressor of cytokine signaling 3. *Growth Factors* 30, 207–219
48. Zhang, J.G. *et al.* (1999) The conserved SOCS box motif in suppressors of cytokine signaling binds to elongins B and C and may couple bound proteins to proteasomal degradation. *Proc. Natl. Acad. Sci. U. S. A.* 96, 2071–2076
49. Yoshimura, A. *et al.* (1995) A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors. *EMBO J.* 14, 2816–2826
50. Delconte, R.B. *et al.* (2016) CIS is a potent checkpoint in NK cell-mediated tumor immunity. *Nat. Immunol.* 17, 816–824
51. Rautela, J. *et al.* (2018) Efficient genome editing of human natural killer cells by CRISPR RNP. *bioRxiv* Published online September 6, 2018. http://dx.doi.org/10.1101/406934
52. Vignali, D.A. and Kuchroo, V.K. (2012) IL-12 family cytokines: immunological playmakers. *Nat. Immunol.* 13, 722–728
53. Leonard, J.P. *et al.* (1997) Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-gamma production. *Blood* 90, 2541–2548
54. Robertson, M.J. *et al.* (1999) Immunological effects of interleukin 12 administered by bolus intravenous injection to patients with cancer. *Clin. Cancer Res.* 5, 9–16
55. Berraondo, P. *et al.* (2018) Revisiting interleukin-12 as a cancer immunotherapy agent. *Clin. Cancer Res.* 24, 2716–2718
56. Ohs, I. *et al.* (2017) Restoration of natural killer cell antimetastatic activity by IL12 and checkpoint blockade. *Cancer Res.* 77, 7059–7071
57. Ohs, I. *et al.* (2016) Interleukin-12 bypasses common gamma-chain signalling in emergency natural killer cell lymphopoiesis. *Nat. Commun.* 7, 13708
58. Thaker, P.H. *et al.* (2017) A phase I trial of intraperitoneal GEN-1, an IL-12 plasmid formulated with PEG-PEI-cholesterol lipopolymer, administered with pegylated liposomal doxorubicin in patients with recurrent or persistent epithelial ovarian, fallopian tube or primary peritoneal cancers: an NRG Oncology/Gynecologic Oncology Group study. *Gynecol. Oncol.* 147, 283–290
59. Chaix, J. *et al.* (2008) Cutting edge: priming of NK cells by IL-18. *J. Immunol.* 181, 1627–1631
60. Kaplanski, G. (2018) Interleukin-18: biological properties and role in disease pathogenesis. *Immunol. Rev.* 281, 138–153
61. Molgora, M. *et al.* (2017) IL-1R8 is a checkpoint in NK cells regulating anti-tumour and anti-viral activity. *Nature* 551, 110–114
62. Sharma, A. *et al.* (2018) Anti-CTLA-4 immunotherapy does not deplete FOXP3+ regulatory T cells (Tregs) in human cancers. *Clin. Cancer Res.* Published online July 27, 2018. http://dx.doi.org/10.1158/1078-0432.CCR-18-0762
63. Romano, E. *et al.* (2015) Ipilimumab-dependent cell-mediated cytotoxicity of regulatory T cells *ex vivo* by nonclassical monocytes in melanoma patients. *Proc. Natl. Acad. Sci. U. S. A.* 112, 6140–6145
64. Du, X. *et al.* (2018) A reappraisal of CTLA-4 checkpoint blockade in cancer immunotherapy. *Cell Res.* 28, 416–432
65. Laurent, S. *et al.* (2013) The engagement of CTLA-4 on primary melanoma cell lines induces antibody-dependent cellular cytotoxicity and TNF-alpha production. *J. Transl. Med.* 11, 108
66. Hsu, J. *et al.* (2018) Contribution of NK cells to immunotherapy mediated by PD-1/PD-L1 blockade. *J. Clin. Invest.* 128, 4654–4668
67. Vari, F. *et al.* (2018) Immune evasion via PD-1/PD-L1 on NK cells and monocyte/macrophages is more prominent in Hodgkin lymphoma than DLBCL. *Blood* 131, 1809–1819
68. Concha-Benavente, F. *et al.* (2018) PD-L1 mediates dysfunction in activated PD-1+ NK cells in head and neck cancer patients. *Cancer Immunol. Res.* 6, 1548–1560
69. Goldberg, M.V. and Drake, C.G. (2011) LAG-3 in cancer immunotherapy. *Curr. Top. Microbiol. Immunol.* 344, 269–278
70. Hannier, S. *et al.* (1998) CD3/TCR complex-associated lymphocyte activation gene-3 molecules inhibit CD3/TCR signaling. *J. Immunol.* 161, 4058–4065
71. Woo, S.R. *et al.* (2012) Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. *Cancer Res.* 72, 917–927
72. Ruggeri, L. *et al.* (2002) Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* 295, 2097–2100
73. Romagne, F. *et al.* (2009) Preclinical characterization of 1-7F9, a novel human anti-KIR receptor therapeutic antibody that augments natural killer-mediated killing of tumor cells. *Blood* 114, 2667–2677
74. Benson, D.M., Jr *et al.* (2011) IPH2101, a novel anti-inhibitory KIR antibody, and lenalidomide combine to enhance the natural killer cell versus multiple myeloma effect. *Blood* 118, 6387–6391
75. Kohrt, H.E. *et al.* (2014) Anti-KIR antibody enhancement of anti-lymphoma activity of natural killer cells as monotherapy and in combination with anti-CD20 antibodies. *Blood* 123, 678–686
76. Benson, D.M., Jr *et al.* (2015) A phase I trial of the anti-KIR antibody IPH2101 and lenalidomide in patients with relapsed/refractory multiple myeloma. *Clin. Cancer Res.* 21, 4055–4061
77. Vey, N. *et al.* (2018) A phase 1 study of lirilumab (antibody against killer immunoglobulin-like receptor antibody KIR2D; IPH2102) in patients with solid tumors and hematologic malignancies. *Oncotarget* 9, 17675–17688
78. Vey, N.D. *et al.* (2017) Randomized phase 2 trial of lirilumab (anti-KIR monoclonal antibody, mAb) as maintenance treatment in elderly patients (pts) with acute myeloid leukemia (AML): results of the Effikir trial. *Blood* 130, 889

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79. Stanietsky, N. *et al.* (2009) The interaction of TIGIT with PVR and PVRL2 inhibits human NK cell cytotoxicity. *Proc. Natl. Acad. Sci. U. S. A.* 106, 17858–17863
80. Yu, X. *et al.* (2009) The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. *Nat. Immunol.* 10, 48–57
81. Shibuya, A. *et al.* (1996) DNAM-1, a novel adhesion molecule involved in the cytolytic function of T lymphocytes. *Immunity* 4, 573–581
82. Gao, J. *et al.* (2017) CD155, an onco-immunologic molecule in human tumors. *Cancer Sci.* 108, 1934–1938
83. Stein, N. *et al.* (2017) The paired receptors TIGIT and DNAM-1 as targets for therapeutic antibodies. *Hum. Antibodies* 25, 111–119
84. Tahara-Hanaoka, S. *et al.* (2004) Functional characterization of DNAM-1 (CD226) interaction with its ligands PVR (CD155) and nectin-2 (PRR-2/CD112). *Int. Immunol.* 16, 533–538
85. Johnston, R.J. *et al.* (2014) The immunoreceptor TIGIT regulates antitumor and antiviral CD8(+) T cell effector function. *Cancer Cell* 26, 923–937
86. Sarhan, D. *et al.* (2016) Adaptive NK cells with low TIGIT expression are inherently resistant to myeloid-derived suppressor cells. *Cancer Res.* 76, 5696–5706
87. Zhang, Q. *et al.* (2018) Blockade of the checkpoint receptor TIGIT prevents NK cell exhaustion and elicits potent anti-tumor immunity. *Nat. Immunol.* 19, 723–732
88. Schmiedel, D. and Mandelboim, O. (2018) NKG2D ligands-critical targets for cancer immune escape and therapy. *Front. Immunol.* 9, 2040
89. Ferrari de Andrade, L. *et al.* (2018) Antibody-mediated inhibition of MICA and MICB shedding promotes NK cell-driven tumor immunity. *Science* 359, 1537–1542
90. Deng, W. *et al.* (2015) Antitumor immunity. A shed NKG2D ligand that promotes natural killer cell activation and tumor rejection. *Science* 348, 136–139
91. Brooks, A.G. *et al.* (1997) NKG2A complexed with CD94 defines a novel inhibitory natural killer cell receptor. *J. Exp. Med.* 185, 795–800
92. Andre, P.D. *et al.* (2018) Anti-NKG2A mAb is a checkpoint inhibitor that promotes anti-tumor immunity by unleashing both T and NK cells. *Cell* 175, 1731–1743
93. Yang, J.C. and Rosenberg, S.A. (2016) Adoptive T-cell therapy for cancer. *Adv. Immunol.* 130, 279–294
94. Shimabukuro-Vornhagen, A. *et al.* (2018) Cytokine release syndrome. *J. Immunother. Cancer* 6, 56
95. Barkholt, L. *et al.* (2009) Safety analysis of *ex vivo*-expanded NK and NK-like T cells administered to cancer patients: a phase I clinical study. *Immunotherapy* 1, 753–764
96. Locatelli, F. *et al.* (2018) NK cells mediate a crucial graft-versus-leukemia effect in haploidentical-HSCT to cure high-risk acute leukemia. *Trends Immunol.* 39, 577–590
97. Mavers, M. and Bertaina, A. (2018) High-risk leukemia: past, present, and future role of NK cells. *J. Immunol. Res.* 2018, 1586905
98. Majzner, R.G. and Mackall, C.L. (2018) Tumor antigen escape from CAR T-cell therapy. *Cancer Discov.* 8, 1219–1226
99. Pehlivan, K.C. *et al.* (2018) CAR-T cell therapy for acute lymphoblastic leukemia: transforming the treatment of relapsed and refractory disease. *Curr. Hematol. Malig. Rep.* 13, 396–406
100. Brudno, J.N. and Kochenderfer, J.N. (2016) Toxicities of chimeric antigen receptor T cells: recognition and management. *Blood* 127, 3321–3330
101. Mehta, R.S. and Rezvani, K. (2018) Chimeric antigen receptor expressing natural killer cells for the immunotherapy of cancer. *Front. Immunol.* 9, 283
102. Daher, M. and Rezvani, K. (2018) Next generation natural killer cells for cancer immunotherapy: the promise of genetic engineering. *Curr. Opin. Immunol.* 51, 146–153
103. Liu, E. *et al.* (2018) Cord blood NK cells engineered to express IL-15 and a CD19-targeted CAR show long-term persistence and potent antitumor activity. *Leukemia* 32, 520–531
104. Oei, V.Y.S. *et al.* (2018) Intrinsic functional potential of NK-cell subsets constrains retargeting driven by chimeric antigen receptors. *Cancer Immunol. Res.* 6, 467–480
105. Li, Y. *et al.* (2018) Human iPSC-derived natural killer cells engineered with chimeric antigen receptors enhance anti-tumor activity. *Cell Stem Cell* 23, 181–192
106. Mollanoori, H. *et al.* (2018) CRISPR/Cas9 and CAR-T cell, collaboration of two revolutionary technologies in cancer immunotherapy, an instruction for successful cancer treatment. *Hum. Immunol.* 79, 876–882
107. Ochoa, M.C. *et al.* (2017) Antibody-dependent cell cytotoxicity: immunotherapy strategies enhancing effector NK cells. *Immunol. Cell Biol.* 95, 347–355
108. Davis, Z.B. *et al.* (2017) Natural killer cells unleashed: checkpoint receptor blockade and BiKE/TriKE utilization in NK-mediated anti-tumor immunotherapy. *Semin. Immunol.* 31, 64–75
109. Felices, M.S. *et al.* (2016) CD16-IL15-CD33 trispecific killer engager (TriKE) overcomes cancer-induced immune suppression and induces natural killer cell-mediated control of MDS and AML via enhanced killing kinetics. *Blood* 128, 4291
110. Sullivan, R.J. *et al.* (2018) An update on the Society for Immunotherapy of Cancer consensus statement on tumor immunotherapy for the treatment of cutaneous melanoma: version 2.0. *J. Immunother. Cancer* 6, 44
111. Lee, S. and Margolin, K. (2011) Cytokines in cancer immunotherapy. *Cancers (Basel)* 3, 3856–3893
112. Maus, M.V. *et al.* (2014) Adoptive immunotherapy for cancer or viruses. *Annu. Rev. Immunol.* 32, 189–225
113. Tang, J. *et al.* (2018) The global landscape of cancer cell therapy. *Nat. Rev. Drug Discov.* 17, 465–466
114. Kubiczkova, L. *et al.* (2012) TGF-beta - an excellent servant but a bad master. *J. Transl. Med.* 10, 183
115. Vander Ark, A. *et al.* (2018) TGF-beta receptors: in and beyond TGF-beta signaling. *Cell Signal.* 52, 112–120

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Special Issue: Cancer and the Organism

Review

Connecting the Metabolic and Immune Responses to Cancer

Thomas R. Flint,[1,2] Douglas T. Fearon,[1,3,4] and Tobias Janowitz[1,5,*]

Separate research fields have advanced our understanding of, on the one hand, cancer immunology and, on the other hand, cachexia, the fatal tumor-induced wasting syndrome. A link between the host's immune and metabolic responses to cancer remained unexplored. Emerging work in preclinical models of colorectal and pancreatic cancer has unveiled tumor-induced reprogramming of liver metabolism in cachexia that leads to suppression of antitumor immunity and failure of immunotherapy. As research efforts in metabolism and immunology in cancer are rapidly expanding, it is timely to discuss the metabolic and immunological determinants of the cancer–host interaction. We also present the hypothesis that the convergence of host metabolism and antitumor immunity may offer a platform for biomarker-driven investigations of new combination therapies.

Trends

The scope of cancer research is expanding to include the molecular circuitry of both cancer cells and non-cancer cells, as well as non-tumor tissues of the cancer host.

The current generation of immune therapies target cells of the cancer host. These therapies achieve durable remissions of advanced cancers, but the majority of patient subsets remain unresponsive.

Tumors affect their hosts' metabolism, often leading to the lethal wasting syndrome, cachexia. In recent years, the biology of cachexia has become an increasingly active field of mechanistic research, but still defies a unifying explanation.

Preclinical studies have now connected the host's metabolic and immune responses to cancer. Tumors reprogram the normal metabolic response to caloric deficiency in cachexia, leading to suppression of the antitumor immune reaction.

Cancer Is a Systemic Disease

Historically, cancer has been considered to be a consequence of systemic pathology. At his time, the physician Claudius Galen (AD 129–200) documented the consensus that cancers arose from over-abundance of a particular bodily fluid, which he designated as 'black bile' [1]. This theory remained largely unchallenged until the mid-19th century, when cancer became understood not as an aberration of its host's fluid composition, but as a consequence of acquired cellular abnormalities [2–4]. Heralded by this paradigm shift, subsequent generations of scientists proceeded to unravel the cellular and genetic basis of cancer at ever-increasing levels of molecular precision. The most relevant clinical consequence of this was the development of oncogene-targeted therapies that have in some instances proved both safe and effective [5–7].

Efforts to target the cancer cell, either through oncogene inhibition or traditional chemotherapy, are often undermined by **therapeutic resistance** (see Glossary), which may manifest either at treatment initiation or following a period of disease control. Factors that underlie this phenomenon include genomic instability and genetic heterogeneity, which fuel the emergence of therapy-resistant clones through Darwinian selection processes [8–10]. For these and other reasons, the perspective of cancer research has been widened to include the genetically stable, non-cancer cell types that contribute to the **tumor microenvironment** [11]. A variety of innovative therapies have been derived through this approach, but it is the emerging data from the current generation of **immunotherapies** [12] that have reset the focus of research and therapeutic development in cancer toward the biology of the cancer host, emphatically

[1]Cancer Research UK Cambridge Institute, University of Cambridge, Li Ka Shing Centre, Cambridge CB2 0RE, UK
[2]School of Clinical Medicine, University of Cambridge, Addenbrooke's Hospital, Cambridge CB2 0SP, UK
[3]Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA
[4]Weill Cornell Medical College, New York, NY 10021, USA

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reversing the historical trend. The most successful immunotherapies block **T-cell checkpoint molecules** that exist to curtail evolving immune reactions to foreign and self-antigens [13]. Blockade of these molecules leads to a prolonged T-cell response against cancer cells, and durable remissions in subsets of patients with melanoma, lung, renal, and urothelial cancers, often in the metastatic setting [14–17]. The mutability of the cancer genome once paved a near-inevitable pathway toward escape from treatment control. In the era of immunotherapy, this same escape mechanism has been transformed into a critical therapeutic vulnerability, because it provides target **neoantigens** for the extensive T-cell receptor repertoire of the cancer host [18–20].

[5]Department of Oncology, University of Cambridge, Addenbrooke's Hospital, Cambridge CB2 0QQ, UK

*Correspondence:
tj212@cam.ac.uk (T. Janowitz).

Host cells are critical determinants of cancer growth, but the consequences of the reciprocal influence of the cancer on its host organism are just as profound. Systemic effects of tumors on their host organism include anemia, fever, inflammation, coagulopathies, ectopic hormone secretion, neuropathies, and psychiatric disturbances. However, the weight loss condition **cachexia** is probably cancer's most prevalent and lethal systemic consequence, affecting 50% of cancer patients, and directly accounting for an estimated 22% of cancer deaths (Box 1) [21,22].

Cachexia has traditionally been dismissed by researchers and clinicians as a terminal syndrome, rather than being understood as a tractable consequence of an underlying disease process [23]. More recently, however, mechanistic and translational inquiries into cachexia are rapidly gaining momentum. The proof of principle in mouse models that reversal of wasting can be achieved and can dramatically prolong survival irrespective of tumor progression [24], and the development of an international consensus definition [25] of cachexia have increased enthusiasm for clinical trials. However, while a set of two Phase III clinical trials of anamorelin, a **ghrelin** analog designed to stimulate appetite, demonstrated feasibility of late-phase clinical trials in this challenging clinical setting, neither extended survival nor functional recovery were achieved [26]. The contemporary view of cachexia is that of a multifactorial syndrome, driven by molecular alterations within a number of organ systems including muscle, fat, gut, and brain [27,28]. The challenge is to draw such disparate threads of biological narrative into a dominant causal sequence, so as to generate therapeutic targets that are both appropriate and actionable, as well as mechanism-guided end points in clinical trials.

The host response is indispensable to the study of cancer. At one extreme, the host eradicates the tumor through the action of its immune system, and at the other, the host succumbs to the devastating effects of cachexia. These are not separate systemic phenomena though. The basis for a mechanistic connection between these two responses is provided by the clinical literature, where the deleterious effects of poor nutritional status on systemic immunity are well-documented [29]. It is thus conceivable that there exists a set of continuous variables that determine the balance between wasting and immunological clearance in cancer, and that they may be targeted for benefit of the host, and the detriment of the tumor.

Box 1. Clinical Definition of Cachexia

Cachexia is 'characterized by a negative protein and energy balance driven by a variable combination of reduced food intake and abnormal metabolism' [25].

The international consensus criteria [27] for diagnosis of cancer cachexia include any one of the following:

- Weight loss >5% over the past 6 months (in absence of simple starvation); or
- Body mass index < 20 kg/m^2 and any degree of weight loss >2%; or
- Sarcopenia (i.e., low muscle mass) as determinable by various modalities of body composition analysis [27], and any degree of weight loss >2%.

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This review examines the fields of cachexia and immunity, as they relate to cancer. The discussions of cachexia will consider to what extent its molecular features are accounted for by raised **glucocorticoid** levels and other elements of the physiological response to **caloric deficiency**. Conversely, discussions of immunity will focus on the apparent necessity of intratumoral **T-cell infiltration** for immune control of cancer. We will conclude with a discussion of recent findings [30] demonstrating how the glucocorticoid response to caloric deficiency can determine intratumoral T-cell infiltration and immune control of tumor growth in mouse models of cachexia. As a result of this connection, exciting potential lines of scientific and therapeutic inquiry may arise, and these will also be addressed.

Caloric Deficiency: The Primary Defect of Cachexia

Cachexia, by international consensus, is a clinical syndrome that is defined principally by weight loss (Box 1), and is 'characterized by a negative protein and energy balance driven by a variable combination of reduced food intake and abnormal metabolism' [25]. Reduced food intake is frequently reported in patients with cancer [31], and is considered to be a key underlying mechanism of cachexia, as well as a risk factor for progression from the **precachectic** to the cachectic state [25]. Reduced food intake is also a common finding across murine models of cachexia [30,32–35].

Reduced food intake in patients with cancer can be multifactorial, with causes including mechanical disruption of the gastrointestinal tract, paracrine dysfunction and malabsorption, nausea and vomiting due to anticancer therapy, pain, anxiety, depression, and fatigue [36]. However, it frequently manifests in the presence of none of these associated factors, and is often attributed to negative regulation of central appetite control [37], the definitive cause of which is yet to be discovered.

It has been proposed that cancer-induced reductions in food intake, combined with the energetic burden of the tumor, may lead to an energy deficit, the normal physiological response to which accounts for the cachexia syndrome [38]. Many of the biological alterations that characterize cachexia, at both systemic and tissue levels, are shared with the normal response to **caloric restriction**. In terms of whole-body metabolism, both responses in humans exhibit loss of fat and muscle mass [39], impaired glucose disposal [40], low serum levels of **leptin** [41], raised serum levels of ghrelin [42], and activation of hepatic gluconeogenesis [43]. Further parallels from studies of murine cachexia models include high levels of corticosterone (the murine equivalent of cortisol) and low levels of insulin, insulin-like growth factor 1, and thyroid hormones [44–47]. These latter changes, and in particular, those of insulin and glucocorticoids, are understood to account for the depletion of muscle and fat tissue in caloric restriction [48,49], and may therefore contribute to the depletion of these same tissues in cachexia.

The parallels of cachexia and the normal response to caloric restriction are not restricted to systemic metabolism. Recent molecular studies in mice have further highlighted the similarities of the two processes at the level of individual tissues. Muscle atrophy in response to caloric restriction, as in cachexia, can be abrogated either through myocyte-restricted knockout of the glucocorticoid receptor (GR) [50,51] or through myocyte-restricted overexpression of the super-repressor inhibitor of kappa Bα (IκBα) that potently inhibits nuclear factor-κB [52,53]. The two conditions also share multiple transcriptional features in common; in mice, these include downregulation of mRNAs for the anabolic factor insulin-like growth factor 1 [54] and upregulation of mRNAs for the catabolic factors **myostatin**, Forkhead box protein O1 (FOXO1), atrogin-1, muscle RING-finger protein-1 (MuRF1), and cathepsin L, as well as several subunits of the 20S proteasome and its 19S regulator [55–58]. *In vivo* mouse muscle transfection experiments using either dominant-negative signal transducer and activator of transcription 3 (STAT-3) or constitutively activated STAT-3 have demonstrated a dependency of myocyte

Glossary

Adipose triglyceride lipase: catalyzes the first step in triglyceride breakdown, converting one triacylglycerol to one diacylglycerol plus a fatty acid.

Autochthonous tumor: arises in the location where it subsequently grows and develops. Includes spontaneous, carcinogen-induced and genetically engineered tumors, but not transplantable or injected tumors.

Cachexia: syndrome of involuntary weight loss involving depletion of both muscle and fat tissue. It is common in patients with cancer, where it is termed 'cancer cachexia' (see clinical definition in Box 1).

Caloric deficiency: describes a state where the total of energy expenditure and energy loss from an organism exceeds its energy intake from the environment.

Caloric restriction: refers to an experimental reduction in the food intake of an organism.

Cancer-associated fibroblasts: a subpopulation of non-cancer, non-hematopoietic mesenchymal cells within the tumor microenvironment that modulate cancer progression.

CD8$^+$T-cell: a subset of T lymphocyte. In cancer, effector-differentiated CD8$^+$ T-cells produce cytotoxins in response to ligation of their T-cell receptor by cancer cells that express their specific antigenic peptides on MHC Class I molecules.

Chemotaxin: a substance that induces movement of responsive cells toward its site of production.

Circadian rhythms: cycles in physiological processes of approximately 24 h in duration. They occur spontaneously, but can also be entrained by external cues.

Clonal expansion: of lymphocytes in cancer, describes the proliferation that occurs following stimulation with antigen at the level of the tumor or lymph node. Each daughter cell shares the same antigen specificity.

Contact hypersensitivity: T helper cell-mediated immune response; in this review, a mouse model of human allergic contact dermatitis is described.

CXCR3 ligands: chemokines that potently attract CXC motif chemokine receptor 3 expressing cells including T-cells, natural killer cells, and subsets of dendritic cells. These include CXCL9, CXCL10, and CXCL11.

atrophy in cachexia on upregulated signaling of the STAT-3 transcription factor [59]. This may result from an elevation of systemic cytokines, including **interleukin-6** (IL-6), IL-11, and IL-1, which would not be observed with caloric restriction [60]. However, STAT-3 activation has also been observed in muscles from mouse models of chronic kidney disease and type I diabetes [61]. The latter observation suggests a more general role for this transcription factor in muscle catabolism that may ultimately prove to be nutrient sensitive. Reversal of muscle catabolism through administration of the soluble myostatin-family protein receptor, **sActRIIB**, drastically prolongs survival in transplantable mouse models, including the C26 model of colorectal adenocarcinoma [24]. This result challenges the hypothesis that muscle is being broken down in cachexia primarily to meet the energetic needs of the host organism. However, increases in food intake have been reported in both tumor-bearing and non-tumor-bearing mice following administration of this therapy, which may compensate for the loss of nutrients derived from muscle breakdown [62,63].

The parallels extend to adipose tissue as well, as both caloric restriction and cachexia involve activation of the rate-limiting lipase, **adipose triglyceride lipase** [33,64], as well as activation of the **thermogenic program,** as confirmed by elevated **uncoupling protein 1 (UCP-1)** expression at the mRNA and protein level in white adipocytes [65,66]. Upregulation of thermogenesis increases energy expenditure, contributing to the negative energy balance of cachexia. This may seem perverse at a time of caloric need, but this increased heat generation may be interpreted, both in the cachectic and calorically restricted setting, as an appropriate thermoregulatory response to the depletion of insulating adipose tissue [67]. Depletion of insulating tissue would also shift the location of the **thermoneutral zone**, potentially compromising single temperature-point thermoneutrality experiments that seek to exclude a primarily thermoregulatory response [68]. In the transplantable Lewis lung carcinoma mouse model, cancer cell secretion of **parathyroid hormone-related protein** has been studied using cell cultures as well as *in vivo* receptor knockout, antibody neutralization, and gain-of-function experiments. This work demonstrated that parathyroid hormone-related protein can directly act on adipocytes to upregulate the thermogenic program, leading to energy loss and muscle wasting [69,70]. Nonetheless, it remains to be clarified to what extent the browning reaction in cachexia is cause or consequence of the underlying caloric-deficient state.

One undeniable point of distinction between cachexia and caloric restriction is that both muscle wasting and survival in cachexia are resistant to conventional nutritional support [21]. There may be elements of wasting in cachexia that operate independently of caloric deficiency, but cachexia may equally exhibit an aberration in the response to caloric deficiency that prohibits conversion of raw metabolic substrates into usable energy [30]. It is reasonable to suppose that such an aberration might lead to a failure of exogenous substrate to downregulate the normal catabolic response to caloric deficiency, thus enabling a continuation of the wasting process.

Cachexia demonstrates extensive similarities to the normal response to caloric restriction at the level of whole-body metabolism, and at the level of muscle and fat tissues. Resolving the true molecular distinctions between the two processes is a priority for the field, as their correction may render weight loss reversible through conventional nutritional support.

T-Cell Infiltration: The Critical Event in Immune Control of Cancer

A similar point of mechanistic convergence can be sought for the determinants of antitumor immune control. For the adaptive immune system to generate an effective antitumor response, a number of requirements must be met. Cancer cells must express either nonmutated self-antigen or mutated neoantigens that can be recognized by the host repertoire of cytotoxic **$CD8^+$ T-cell** receptors once they are processed and presented on major histocompatibility

Ghrelin: hormone secreted by an empty stomach that acts on the hypothalamus to promote appetite.
Glucocorticoids: hormones synthesized in the zona fasciculata of the adrenal gland. They function as the effector component of the hypothalamic–pituitary–adrenal (HPA) axis. Stimuli activating the HPA axis include psychological, inflammatory, and metabolic stress. Glucocorticoids influence various aspects of metabolism, innate and adaptive immunity, as well as psychological states. Corticosterone and cortisol are the major murine and human glucocorticoids, respectively.
Hapten: a small molecule that elicits an immune response only when attached to a large carrier, such as a protein.
Immune privilege: sites within the body that tolerate the introduction of antigens without eliciting an effective immune response.
Immunogenic: defines any substance capable of producing an immune response. Not all antigens recognized by the immune system are immunogenic.
Immunotherapy: in cancer, refers to any therapy that exploits the immune system to control cancer growth.
Interleukin-6: cytokine secreted by a number of cell types including cancer cells, myeloid cells, and cancer-associated fibroblasts. A variety of metabolic and immunological roles have been described for IL-6 in cancer, including promotion of cancer cell survival, modulation of hepatic metabolism, and suppression of antitumor immunity.
Ketogenesis: essential component of the mammalian response to caloric restriction. It refers to the process of conjugation of two-carbon products to four-carbon ketone bodies such as β-hydroxybutyrate and acetoacetate. A common source of the two-carbon products is fatty acids released from adipose tissue and metabolized in the liver, where they are first subjected to beta-oxidation; however, other molecules such as certain amino acids are also ketogenic precursors.
Leptin: hormone secreted by adipocytes in the fed state; serves to inhibit appetite and thus maintain energy balance.

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complex (MHC) Class I molecules [18]. Cancer-associated antigens must then be cross-presented by a specialized population of dendritic cells, a process that is dependent on the basic leucine zipper ATF-like transcription factor 3 (BATF-3) transcription factor in mice [71,72], permitting **clonal expansion** and effector differentiation of cancer-specific cytotoxic T cells either within the tumor or within tumor-draining lymph nodes [73]. Following cytotoxic T-cell effector differentiation, these lymphocytes must be able to migrate into and/or within the tumor site, so as to ligate their T-cell receptors with cancer cell MHC Class I molecules, thus releasing their cytolytic contents [74].

An extensive preclinical literature has highlighted a number of endogenous, modulatory influences over this process in cancer. These include effects on antigen presentation from the host **microbiota** [75], effects on clonal expansion through ligation of checkpoint molecules such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) at the priming stage [76], as well as local suppressive actions of intratumoral myeloid cells [77,78], **cancer-associated fibroblasts** [79], endothelial cells [80], **T-regulatory cells** [81], cancer cells [82,83], and formation of abnormal vasculature [84]. It is clear from these studies that at each stage of the immune response to cancer, there exist physiological counter-regulatory mechanisms that in turn present opportunities for therapeutic intervention. What is not clear at this point in time is to what extent each stage of the immune response can compensate for the impairment of another step, and if there is a hierarchy of events or a dominant rate-limiting step for the immunological eradication of cancer. The resistance of the majority of solid cancers to the current generation of licensed checkpoint antagonists – anti-CTLA-4, anti-PD-L1, and anti-PD-1 – requires explanation. Responders and non-responders to these therapies are distinct in terms of their burden of predicted neoantigens [18,85], but the present literature also indicates an important role for the exclusion of T cells from areas of the tumor that contain cancer cells [74]. Stringent spatial exclusion would mean that even cancer antigen-reactive, effector-differentiated cytotoxic $CD8^+$ T-cell clones would be prevented from engaging cancer cell MHC Class I molecules with their T-cell receptors. The absence of the formation of this immunological synapse would prevent release of their cytotoxic contents and thereby, curtail anticancer activity.

Exclusion of T cells from the vicinity of their target cells is common to sites of intense **immune privilege** such as the eye, brain, and placenta. In line with these examples from normal physiology, the local infiltration of T cells, as assessed through immunohistochemistry of tumor sections of patients with cancer, has proven to be a strong, independent predictor of survival across multiple cancer types [86–89]. The stages of **immunogenic** antigen expression, antigen presentation, clonal expansion, and effector differentiation may be bypassed through mass transfer of cancer-reactive $CD8^+$ T cells, yet the efficacy of this therapy in transplantable tumor models is absolutely dependent on the ability of the transferred population to migrate into the tumor in response to local chemotactic signaling via C-X-C motif chemokine receptor 3 (CXCR3) [90]. Conversely, ectopic expression of **CXCR3 ligands** in an **autochthonous** mouse model of melanoma has been shown to lead to T-cell-dependent control of tumor growth [91], and elevated tumoral expression of the mRNA for chemokine (C-X-C motif) ligand 9 (CXCL9) is strongly predictive of responses to programmed death ligand 1 (PD-L1) blockade in clinical studies of melanoma [16]. Furthermore, epigenetically silenced expression of major **chemotaxins** has been associated with the near-absolute T-cell exclusion from the maternal–fetal interface of **ovalbumin**-immunized mice bearing ovalbumin-expressing concepti (embryos) [92]; taken together, these data suggest that evolution may have selected migration of T cells as a focus of immune regulation. It follows that cancers must exploit migratory control to evade host immunity. Studies of programmed cell death protein 1 (PD-1)–PD-L1 axis blockade provide further evidence for this, because tumor control appears inextricably linked to the magnitude of the T-cell infiltrate: initial responses in clinical studies are contingent on a

Metyrapone: Food and Drug Administration-approved drug that blocks the synthesis of corticosterone and cortisol through reversible inhibition of steroid 11β-hydroxylase.
Microbiota: refers to all microorganisms that live in or on a particular multicellular organism.
Myostatin: protein produced and secreted by myocytes that reduces muscle mass.
Neoantigen: in cancer, they are mutated peptides that are capable of being presented on host MHC molecules and may therefore elicit an immune response.
Ovalbumin: protein found in egg whites commonly used in research as a model antigen.
Parathyroid hormone-related protein: multifunctional; best known as the source of raised serum calcium in patients with cancer. Recent mouse experiments have implicated it in the development of cachexia, independent of its effect on serum calcium.
Peroxisome proliferator-activated receptor alpha: ligand-activated transcription factor essential for activation of ketogenesis in the fasting state. Mice with germ-line deletions of this protein fail to produce ketones or upregulate ketogenic enzymes when subjected to caloric restriction.
Precachectic: state of altered metabolism observed in patients prior to their fulfilling the diagnostic criteria for cachexia.
Relative hypoketonemia: reduced levels of circulating ketone bodies relative to a suitable control group. In the case of fasted tumor-bearing or IL-6-infused animals, the respective control groups would be non-tumor-bearing or saline-infused littermates.
sActRIIb: engineered, soluble form of ActRIIb; a transmembrane receptor for transforming growth factor-β superfamily members, including myostatin.
Steatohepatitis: liver inflammation with concurrent fat accumulation.
T-cell checkpoint molecules: expressed on the surface of T-cells. They interact with surface ligands on other cells to regulate T-cell function. Key examples in $CD4^+$ and $CD8^+$ T cells include CTLA-4, or PD-1, which interacts with PD-L1 on tumor cells, immune cells, and certain non-immune cells.

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pre-existing T-cell infiltrate [93], and late on-treatment progression is characterized by loss of the T-cell infiltrate [94]. Furthermore, responses in nonsensitive tumors can, in preclinical settings, be exposed through coadministration of novel therapies that act to increase the T-cell infiltrate. This appears to occur irrespective of the molecular target, and irrespective of whether its cellular source is the cancer cell, the myeloid cell, or the cancer-associated fibroblast [95–98].

Although it is unclear to what extent mechanisms of T-cell exclusion in cancer converge onto a common mechanism [99], stringent T-cell exclusion appears common to immune-privileged tumors and immune-privileged normal tissues alike, and represents a significant bottleneck in the path toward immune control of cancers [100].

Connecting Caloric Deficiency and T-Cell Infiltration in Cancer

The observations of cachexia may be accounted for by a normal response to caloric deficiency that is compounded by aberrant processing of metabolic substrates, whilst failures of immune control under checkpoint blockade are robustly associated with relative failure of T-cell infiltration. Through a mechanistic dissection of host metabolism in cachexia, our work in mice has uncovered a tumor-induced systemic immune suppression that causes loss of effector T-cell infiltration, and abolishes responses to immunotherapy [30].

In two mouse models of cancer cachexia – the ectopic, C26 colorectal tumor and an autochthonous pancreatic ductal adenocarcinoma – tumor-induced **IL-6** suppressed hepatic expression of **peroxisome proliferator-activated receptor-α (PPAR-α)**, the transcriptional master regulator of hepatic beta-oxidation and **ketogenesis** [30,101,102]. Challenging these mice with caloric deficiency led to a **relative hypoketonemia** that could not be corrected through excess provision of fatty acid substrate, and that triggered a marked rise in glucocorticoid levels [30]. This hormonal stress response was necessary and sufficient to suppress multiple markers of the intratumoral immune reaction. These included those representative of CXCR3-dependent T-cell chemotaxis (*Cxcl9*, *Cxcl10*, and *Cxcl11* mRNA), T-cell infiltration (*Cd3e* and *Cd8a* mRNA as well as the percentage of intratumoral $CD8^+$ T cells), and T-cell cytolytic activity (*Ifng*, *Gzmb*, and *Prf1* mRNA) [30]. Administration of glucocorticoids to levels lower than those which occur in cachectic mice – but comparable to the fold elevations of glucocorticoids observed in patients with pancreatic cancer and cachexia – abolished the response of an autochthonous murine pancreatic cancer to combination immunotherapy [30]. The work suggests the existence of a sequence by which cancer may alter the host metabolism to foster its own survival (Figure 1).

Systemic glucocorticoids, induced by metabolic stress, thus connect the host's metabolic and immune responses to cancer and may account for failure of immune therapies in the clinical setting. Factors from the tumor microenvironment that influence T-cell infiltration together with the response to immunotherapy have been described, examples of which include CXCL12, colony stimulating factor 1 receptor (CSF-1R), CXCR2, focal adhesion kinase (FAK), and vascular endothelial growth factor (VEGF) [95–98,103], but not one of them has been proposed to exert its effects at the systemic level. Exactly how glucocorticoids promote suppression of the antitumor immune reaction is unclear, but current data point toward a primary suppressive effect of glucocorticoids on myeloid cell production of the CXCR3 ligands, CXCL9–11, that would be indispensable for CXCR3-mediated T-cell chemotaxis into tumor sites [90]. This hypothesis is supported by the proportionate suppression of intratumoral markers of cytotoxic T-cell infiltration (*Cd3e* and *Cd8a*) and cytotoxic T-cell function (*Ifng*, *Prf1*, and *Gzmb*) in murine cachexia [30]. It is also supported by a murine study of **contact hypersensitivity**, another T-cell-dependent sterile inflammatory reaction; conditional GR knockout mice were exposed to dexamethasone-induced ear swelling in response to a **hapten** that the mice had previously

T-cell infiltration: process whereby T cells move to be within close proximity to cancer cells to establish a direct contact that is required for the effector function of cytolytic $CD8^+$ T cells.
Therapeutic resistance: exhibited by tumors that are resistant to control by a particular therapy.
Thermogenic program: in adipose tissue, refers to the pattern of mRNA and protein expression of factors associated with upregulation of mitochondrial respiration and mitochondrial uncoupling, leading to increased generation of heat energy. UCP-1 upregulation is the primary biomarker.
Thermoneutral zone: the range of temperatures for an organism where temperature regulation is achieved without regulatory changes in metabolic heat production or evaporative heat loss. It is dependent on posture, insulation, and the basal metabolic rate.
T-regulatory cells: immune suppressive subset of $CD4^+$ T lymphocytes that stains positive for the Foxp3 transcription factor.
Tumor microenvironment: describes all the cellular and non-cellular components of a tumor mass.
Uncoupling protein 1 (UCP-1): dissipates the proton gradient across the inner mitochondrial membrane, uncoupling cellular respiration and thus heat production from ATP production.

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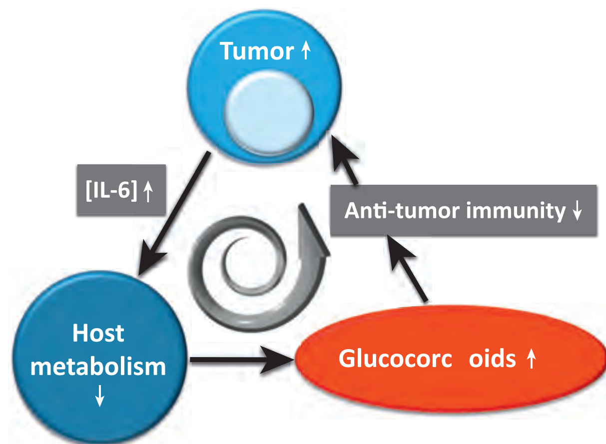

Figure 1. Proposed Sequence for the Perpetuation of Cancer in the Host Organism. The tumor reprograms host metabolism; for example, tumor-induced IL-6 can downregulate hepatic ketogenesis. In patients with cancer and anorexia, this leads to elevation of metabolic stress and circulating glucocorticoid levels. This hormonal response is sufficient to suppress antitumor immunity. IL-6, interleukin 6.

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been sensitized [104]. The data demonstrated an absolute requirement of the myeloid GR, but not the lymphocyte or epithelial GRs, in suppressing the contact hypersensitivity reaction [104].

Irrespective of the underlying mechanism by which glucocorticoids exert immune suppression, these data suggest that endogenous glucocorticoids may compromise outcomes for patients with cancer. They motivate not only further mechanistic inquiries into the regulatory mechanisms at play, but also translational inquiries into the relevance of these mechanisms for patients with cancer, each of which will be discussed below. More pressing however, they caution against pharmacological prescription of glucocorticoids as antiemetics and/or appetite stimulants, which frequently occurs in patients with cancer. Though this may still be appropriate in the palliative setting, or in managing acute or chronic autoimmune complications in the context of checkpoint immunotherapy, clinicians should be mindful that glucocorticoid administration may ultimately favor tumor progression through suppression of antitumor immunity.

Unresolved Mechanistic Questions of Host Modulation in Cancer

The discovery of endogenous glucocorticoids as a candidate continuous variable that determines the balance between wasting and immunological control of cancer, and of the factors that promote their biosynthesis, raises a number of further mechanistic questions that require evaluation. The mechanism underlying the spontaneous reduction in food intake in cachexia is a priority for future research. Reduced food intake is prevalent in patients with cancer, and reduced food intake in precachectic tumor-bearing mice has revealed the systemic consequences of tumor-induced liver reprogramming: suppressed ketogenic potential, raised glucocorticoids, and immune suppression [30]. The hypothalamus is the site of the brain where metabolic demand is transduced into behavioral change, yet it appears insensitive in cachexia to signals that would be anticipated to promote appetite; most significant of these are the low leptin levels that have been reported in human and murine studies [41,105]. Pharmacological studies of transplantable methylcholanthrene-induced sarcomas in rats suggest that this insensitivity to nutritional state may stem from the intracerebral action of serotonin and IL-1 [106], or the systemic action of tumor necrosis factor family cytokines [107]. Alternatively, it may arise from other, potentially undiscovered factors.

Of similar importance to the appetite question is the interrogation of the paradoxical response of the livers of fasted, precachectic mice to fatty acid substrate, where administration of fatty acids

has been shown to lead to a fall in ketones [30]. This result was unexpected, as suppressed ketogenic enzyme expression would instead predict a minimal increase or no change in ketones upon substrate administration. It is tempting to speculate to what extent this result models the unexplained clinical observation that administration of nutrients fails to elicit the anticipated physiological response of preserving lean mass, and fails to extend survival. It may even shed light on the observation that prolonged administration of total parenteral nutrition can cause **steatohepatitis** and liver impairment in the clinical setting. The findings on ketogenesis call for analyses of the exact mechanism by which IL-6 suppresses PPAR-α, the transcriptional master regulator of ketogenesis, but do not, at this stage, exclude other mechanisms of ketogenesis suppression. The effects of IL-6 on liver metabolism likely extend beyond the response of ketogenic enzymes to reduced food intake, and may include broad changes in the circadian regulation of insulin signaling, glucose tolerance, and lipid metabolism that have been observed with IL-6-inducing murine lung tumors [108]. Other IL-6 family proteins such as IL-11 and leukemia inhibitory factor (LIF) are elevated in cachexia, and may also play a role, perhaps converging with IL-6 onto the gp130–STAT-3 axis [60]. As the liver is an essential organ of metabolic homeostasis, its apparent metabolic dysfunction in the context of cancer-induced inflammation demands extensive, broad-based functional analyses supported by parallel clinical validation.

Downstream of substrate-refractory relative hypoketonemia, future work may investigate how the hypothalamic–pituitary–adrenal axis may be activated by reduced ketone levels, as has been demonstrated in humans subjected to insulin-induced hypoglycemia, whereby co-infusion of ketone bodies reduced the cortisol response to a given blood glucose level [109]. Another question is whether fatty acids liberated from adipose tissue that are not metabolized by the liver are instead oxidized by skeletal muscle, leading to oxidative stress, p38 activation, and an acceleration of muscle atrophy [110]. The consequences of stress-induced glucocorticoids on the intratumoral T-cell reaction might in turn be disentangled from their known effects on T cells, peripherally and in lymphoid tissue [111,112].

The immune-suppressive effects of glucocorticoids are a consistent finding in immunology, but they must be reconciled with other immunomodulatory effects of altered host metabolism in cancer. One such effect has recently been demonstrated in mouse models; specifically, brief periods of caloric restriction following transplantable tumor inoculation enhances the efficacy of $CD8^+$ T-cell-dependent chemotherapy [113,114]. This effect was dependent on starvation-induced activation of autophagy in the MCA205 sarcoma model and suppression of heme oxygenase (HO) in the 4T1 breast cancer model, being abrogated following ATG5 knockdown and HO overexpression in the respective cancer cells prior to inoculation [113,114]. HO has been independently shown to suppress rejection of murine lung allografts [115]. There are other potential ways in which reprogrammed metabolism during cancer may affect antitumor immunity. In addition to effects on glucocorticoids and cancer cells, reprogrammed metabolism during cancer may directly impact lymphocyte function by altering the availabilities of key nutrients. For example, serine restriction impairs $CD8^+$ T-cell proliferation *in vitro* and *in vivo* (transgenic mouse models of *Listeria monocytogenes* infection) [116]. Furthermore, culturing human naïve $CD4^+$ and $CD8^+$ T cells with increased l-arginine has been recently shown to promote the development of a central memory-like phenotype, as well as enhanced $CD8^+$ T-cell antitumor activity in transgenic mouse models of melanoma [117]. These results are of potential relevance to host metabolism in cancer because circulating levels of serine and arginine have been shown to be elevated in pancreatic cancer patients exhibiting cachexia as opposed to those without cachexia [118].

From another perspective, the microbiota might provide an alternative conduit by which cancer-induced reductions in food intake may affect the antitumor immune response. Indeed,

the intestinal microbiota is profoundly immunomodulatory, as demonstrated by experiments showing how the efficacy of anti-CTLA-4 immunotherapy is abolished in germ-free or antibiotic-treated mice [119], and furthermore, its phylogenetic structure is sensitive to both content and quantity of the dietary intake in humans and mice [120–122]. Consequently, the potential mechanisms by which cancer may operate outside the tumor microenvironment to affect the antitumor immune response are diverse, and need not be restricted to host metabolism.

Host-Targeted Biomarkers and Therapeutics in Cancer Immunotherapy

The dependence of preclinical immune therapies on circulating glucocorticoids provides a road map toward the development of novel mechanism-based biomarkers and therapeutics (Figure 2, Key Figure). The systemic determinants of glucocorticoid release present candidate biomarkers for patient stratification in immunotherapy. As guided by our own preclinical work, we would suggest that these candidate biomarkers include measurements of food intake, energy expenditure, IL-6 levels, ketone levels, and ketogenic potential, together with any alterations in the serum or urinary metabolomes that may signify PPAR-α dysfunction [123]. The nonmetabolic triggers of glucocorticoid release, such as disrupted **circadian rhythms** and maladaptive psychological responses to cancer, may be of equal predictive value, because the markers of the intratumoral immune reaction oscillate according to the diurnal glucocorticoid variations [30,124–127]. Once glucocorticoids are secreted by the adrenal glands, the host's diurnal exposure may be quantified through direct measurements in the serum or urine.

Key Figure

Translational Relevance of Putative Host Response Mechanisms in Cancer

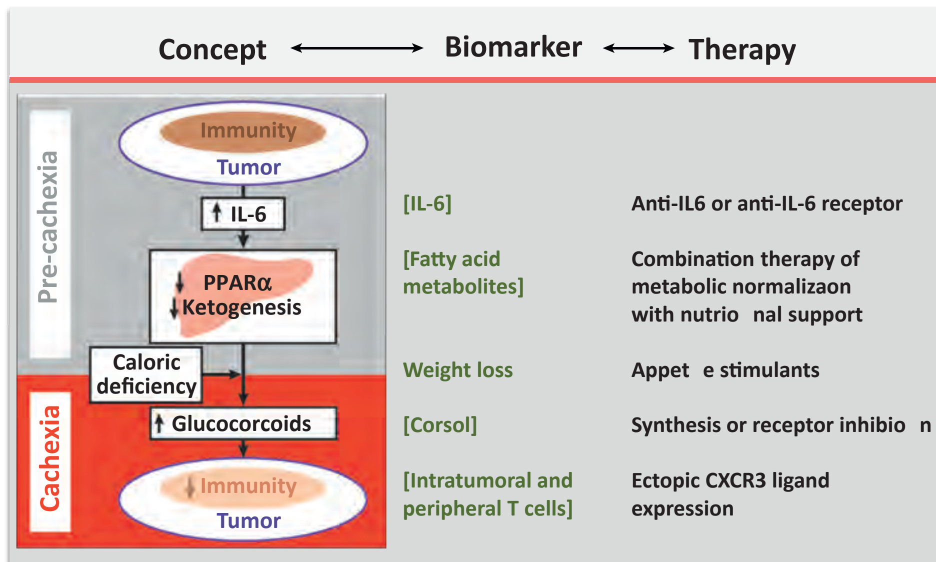

Figure 2. Based on the hypotheses posited in this article, the sequence described in the concept column might be directly translated into biomarker-driven therapeutic interventions. Examples of potential biomarkers and therapies are provided. CXCR3, CXC motif chemokine receptor 3; IL-6, interleukin 6; PPARα, peroxisome proliferator-activated receptor-α;

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The consequences of glucocorticoid signaling might be then assessed through analysis of peripheral immune cell populations [128], or through analysis of nonimmunological tissues such as adipose tissue and skeletal muscle. These indirect assessments at the tissue level may be more readily applicable to clinical practice, owing to the short (≈60 min) half-life of cortisol in plasma, and the practicalities inherent to rigorous diurnal blood sampling. In support of these putative assessments, studies of patients with B-cell lymphomas have shown that low skeletal muscle mass, a known result of glucocorticoid signaling, can be independently predictive of reduced progression-free survival in the context of combination chemoimmunotherapy [129,130]. Should such host-centric biomarkers prove to correlate with failure of immunotherapy, they might provide the essential translational support for the relevance of reprogrammed host metabolism in this clinical setting that is currently lacking. Moreover, and as with any predictive biomarker, their measurement may help minimize costly false-negatives in clinical trials through facilitating appropriate patient stratification, and ultimately serve to guide effective treatment decisions.

In terms of therapeutic approaches (Figure 2), the critical proof of principle would involve enhancing the response to immune therapy through resolution of the host's metabolic response to cancer in preclinical model systems. This might be potentially achieved by targeting IL-6 production from cancer-associated fibroblasts, which are its dominant cellular source in murine pancreatic tumors [131], or through directly targeting circulating IL-6 and/or its downstream effects on liver metabolism. Although trials of anti-IL-6 are yet to demonstrate clinical benefit [23], this therapeutic may need to be re-evaluated in the context of emerging preclinical data, and combined with either checkpoint inhibition (to potentiate its putative immunological effects) or with nutritional support (to exploit any normalization of liver metabolism that may occur). As the effects of IL-6 likely predate the onset of clinical cachexia [30], administration at early time points may also be required. As an alternative to resolving liver metabolism, the consequences of impaired ketogenic potential may be circumvented through administration of therapies that prevent cancer-induced appetite loss. Stimuli that elevate glucocorticoids may also be intercepted at a downstream level, either though prevention of glucocorticoid synthesis in the adrenal gland or through antagonism of the GR.

The feasibility of these approaches is supported by experiments in a K-RAS-driven mouse model of lung cancer where anti-IL-6 therapy increased expression of markers of a cytotoxic T-cell immune response such as intratumoral *Ifng* and *Gzmb* mRNA [132], and in multiple mouse models of pancreatic ductal adenocarcinoma where anti-IL-6 therapy synergized with anti-PD-L1 to control tumor growth and prolong survival [133]. In other studies, administration of the glucocorticoid synthesis inhibitor, **metyrapone**, was shown to suppress tumor growth in mice inoculated with a murine sarcoma virus [134]. These preclinical successes notwithstanding, the nature of systemic immune suppression in cancer, particularly following the development of cachexia, demands a multifaceted approach. The effects of caloric deficiency are compounded by a defective liver metabolism, and the release of glucocorticoids appears as an appropriate response to the nutritional state. Resolution of systemic immune suppression may therefore require correction of liver metabolism through administration of anti-IL-6 or another liver-targeted agent, in combination with nutritional support.

As we are beginning to explore response to new therapeutic approaches, we should also consider re-evaluating the classifications of cancers in a manner that is disease-site agnostic. The focus on our emerging understanding of the unifying aspects of various cancers from the perspective of immunotherapy or systemic disease may offer a guide for these attempts. A simple implementation may be to conduct classifications using binary approaches, such as high versus low neoantigen levels, high versus low T-cell tumor infiltration, high versus low IL-6, or normal versus cachectic metabolism. Through deconvolution of trial design, and attempted

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Box 2. Clinician's Corner

Oncologists and their patients are all too familiar with disease relapse or progression despite administration of anticancer therapy. This is the case for a variety of malignancies. The challenges of therapeutic failure are universal to all tumors and have been observed with chemotherapies, targeted therapies, and more recently, with immunotherapies – a renewed therapeutic strategy that can cause long-term remission. Insufficient therapies are not restricted to tumor-directed therapy, however: systemic cancer effects, such as fatigue and pain, as well as anorexia and cachexia are equally not met by sufficient therapeutic options.

Recent preclinical findings in mouse models of cancer mechanistically connect cancer cachexia, reduced anticancer immunity, and failure to respond to cancer immunotherapy. Elevated IL-6 is associated with many cancers, including nonsmall cell lung cancer and pancreatic cancer, and can induce molecular failure of hepatic ketogenesis. Such findings correlate with results from patients with pancreatic cancer and cachexia. Furthermore, in preclinical studies, such elevated glucocorticoid levels are sufficient to suppress intratumoral immunity and result in failure of cancer immunotherapy.

To transform findings such as these for the benefit of patients, clinical research to deliver proof of principle and proof of concept, as well as predictive biomarkers of therapeutic response is paramount. Efforts should include research on markers of defective host metabolism, drawn, for example, from the circulating pool of lipid and protein metabolites or hormone levels. These can include measurements such as plasma cortisol profiles, but could also rely on clinical metadata such as continued weight loss.

Ultimately, improvement of cancer therapy may be achieved by combining normalization or amelioration of metabolic stress with cancer immunotherapy. Several potential targets for combination therapy with T-cell checkpoint-targeted immunotherapy can be derived from preclinical work and should be investigated in conjunction with the experimental medicine efforts described earlier. These therapies might include administration of caloric supplementation together with either anti-IL-6 agents, glucocorticoid synthesis antagonists, or GR antagonists, so as to optimize clinical outcomes.

As a new generation of cancer therapies is being examined, we should not miss the opportunity to question and refine the methodology employed to test their efficacy. We owe it to our patients to not restrict clinical trials solely to conventional end points, but rather, we must be certain that we understand mechanistically both failure and success of single and combination immunotherapy approaches. The clinical translation of the convergence of host metabolism and host immunity may offer a new area in which to develop carefully designed clinical trials and ultimately, impactful therapeutic strategies.

isolation of critical variables, such criteria may facilitate the successful implementation of clinical treatment strategies.

Concluding Remarks

Recent trends in cancer research include expansion of focus beyond the microcosm of the cancer cell, increased recognition of the response to caloric deficiency in cachexia as a determinant of cancer outcome, and a convergence on T-cell infiltration as a requirement for response to immunotherapies. Systemic glucocorticoids represent a confluence of these trends, as they are induced by the response of precachectic mice to caloric deficiency, and act to suppress both T-cell infiltration and responses to immunotherapy. As a result, a plethora of exciting new approaches to stratifying and treating cancer patients are arising, with the focus of discovery being the host, rather than the tumor (see Outstanding Questions and Box 2). Together with the immunotherapeutic revolution, and recent insights into the manifold effects of the host microbiota, such work may resurrect the classical understanding of cancer as a disorder of its host's biology.

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Outstanding Questions

What is the mechanism that underlies the spontaneous loss of appetite in cancer-associated anorexia and cachexia?

Reduced food intake reveals the consequences of tumor-induced hepatic reprogramming in preclinical model systems, which include substrate-refractory hypoketonemia and intratumoral immune suppression.

What are the functional consequences of tumor-induced hepatic reprogramming?

Preclinical studies have defined suppressed ketogenic potential due to tumor-induced IL-6, as well as disrupted circadian control of glucose and lipid metabolism. Functional studies of substrate handling may explain why wasting in cachexia is resistant to nutritional support.

How do glucocorticoids act to suppress the antitumor immune reaction?

The antitumor immune response is sensitive even to subtle, diurnal variations in glucocorticoids. What are the cellular and molecular processes that underlie this sensitivity? Does the critical glucocorticoid-sensor reside within the tumor, within primary or secondary lymphoid tissue, or elsewhere within the body?

How do glucocorticoids interact with other immunomodulatory effects of the host metabolism in cancer?

Reduced caloric intake can promote subsequent $CD8^{+}$ T-cell-dependent tumor control under chemotherapy. It may also affect the intestinal microbiota, and circulating concentrations of critical immunometabolites. Do these changes act in concert with glucocorticoids, oppose their effects, or act at distinct phases of an immune reaction to cancer?

Can the host metabolic response to cancer provide the next generation of predictive biomarkers and therapeutic targets in immunotherapy?

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Glucocorticoids are necessary and sufficient to account for starvation-induced suppression of the immune reaction in preclinical models. Can longer-term correction of host metabolic derangements lead to improved response to immunotherapies? Can the determinants and consequences of glucocorticoid release in cancer be used to stratify patients for clinical trials?

References

1. Grant, M. (2000) *Galen on Food and Diet,* Routledge
2. Hajdu, S.I. *et al.* (2015) A note from history: landmarks in history of cancer, part 1. *Cancer* 121, 2480–2513
3. Mukherjee, S. (2010) *The Emperor of All Maladies: A Biography of Cancer,* Scribner
4. Virchow, R. and Chance, F. (1860) *Cellular Pathology As Based upon Physiological and Pathological Histology,* John Churchill
5. Chabner, B.A. and Roberts, T.G., Jr (2005) Timeline: chemotherapy and the war on cancer. *Nat. Rev. Cancer* 5, 65–72
6. Lynch, T. *et al.* (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non–small-cell lung cancer to gefitinib. *N. Engl. J. Med.* 350, 2129–2139
7. Chapman, P.B. *et al.* (2011) Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N. Engl. J. Med.* 364, 2507–2516
8. Gerlinger, M. *et al.* (2012) Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N. Engl. J. Med.* 366, 883–892
9. Kemper, K. *et al.* (2015) Intra- and inter-tumor heterogeneity in a vemurafenib-resistant melanoma patient and derived xenografts. *EMBO Mol. Med.* 7, 1104–1118
10. Kobayashi, S. *et al.* (2005) EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N. Engl. J. Med.* 352, 786–792
11. Hanahan, D. and Weinberg, R.A. (2011) Hallmarks of cancer: the next generation. *Cell* 144, 646–674
12. Wolchok, J. and Chan, T. (2014) Cancer: antitumour immunity gets a boost. *Nature* 515, 496–498
13. Pardoll, D.M. (2012) The blockade of immune checkpoints in cancer immunotherapy. *Nat. Rev. Cancer* 12, 252–264
14. Powles, T. *et al.* (2014) MPDL3280A (anti-PD-L1) treatment leads to clinical activity in metastatic bladder cancer. *Nature* 515, 558–562
15. Brahmer, J.R. *et al.* (2012) Safety and activity of anti–PD-L1 antibody in patients with advanced cancer. *N. Engl. J. Med.* 366, 2455–2465
16. Herbst, R.S. *et al.* (2014) Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature* 515, 563–567
17. Topalian, S. and Hodi, F. (2012) Safety, activity, and immune correlates of anti–PD-1 antibody in cancer. *N. Engl. J. Med.* 366, 2443–2454
18. Rizvi, N.A. *et al.* (2015) Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science* 348, 124–128
19. Schumacher, T.N. and Schreiber, R.D. (2015) Neoantigens in cancer immunotherapy. *Science* 348, 69–74
20. Le, D.T. *et al.* (2015) PD-1 blockade in tumors with mismatch-repair deficiency. *N. Engl. J. Med.* 372, 2509–2520
21. Tisdale, M. (2009) Mechanisms of cancer cachexia. *Physiol. Rev.* 89, 381–410
22. Dewys, W.D. *et al.* (1980) Prognostic effect of weight loss prior to chemotherapy in cancer patients. *Am. J. Med.* 69, 491–497
23. Fearon, K.C.H. *et al.* (2012) Cancer cachexia: mediators, signaling, and metabolic pathways. *Cell Metab.* 16, 153–166
24. Zhou, X. *et al.* (2010) Reversal of cancer cachexia and muscle wasting by ActRIIB antagonism leads to prolonged survival. *Cell* 142, 531–543
25. Fearon, K. *et al.* (2011) Definition and classification of cancer cachexia: an international consensus. *Lancet Oncol.* 12, 489–495
26. Temel, J.S. *et al.* (2016) Anamorelin in patients with non-small-cell lung cancer and cachexia (ROMANA 1 and ROMANA 2): results from two randomised, double-blind, phase 3 trials. *Lancet Oncol.* 17, 519–531
27. Petruzzelli, M. and Wagner, E.F. (2016) Mechanisms of metabolic dysfunction in cancer-associated cachexia. *Genes Dev.* 30, 489–501
28. Argilés, J.M. *et al.* (2014) Cancer cachexia: understanding the molecular basis. *Nat. Rev. Cancer* 14, 754–762
29. Keusch, G.T. and Farthing, M.J.G. (1986) Nutrition and infection. *Annu. Rev. Nutr.* 6, 131–154
30. Flint, T.R. *et al.* (2016) Tumor-induced IL-6 reprograms host metabolism to suppress anti-tumor immunity. *Cell Metab.* 24, 672–684
31. von Haehling, S. and Anker, S.D. (2014) Treatment of cachexia: an overview of recent developments. *J. Am. Med. Dir. Assoc.* 15, 866–872
32. Tsoli, M. *et al.* (2012) Activation of thermogenesis in brown adipose tissue and dysregulated lipid metabolism associated with cancer cachexia in mice. *Cancer Res.* 72, 4372–4382
33. Das, S.K. *et al.* (2011) Adipose triglyceride lipase contributes to cancer-associated cachexia. *Science* 333, 233–238
34. Sanchís, D. *et al.* (1998) Skeletal muscle UCP2 and UCP3 gene expression in a rat cancer cachexia model. *FEBS Lett.* 436, 415–418
35. Bing, C. *et al.* (2000) Increased gene expression of brown fat uncoupling protein (UCP) 1 and skeletal muscle UCP2 and UCP3 in MAC16-induced cancer cachexia. *Cancer Res.* 60, 2405–2410
36. Esper, D.H. and Harb, W.A. (2005) The cancer cachexia syndrome: a review of metabolic and clinical manifestations. *Nutr. Clin. Pract.* 20, 369–376
37. Laviano, A. *et al.* (2008) Neural control of the anorexia-cachexia syndrome. *Am. J. Physiol. Endocrinol. Metab.* 295, E1000–E1008
38. Friesen, D.E. *et al.* (2015) Modeling the energetic cost of cancer as a result of altered energy metabolism: implications for cachexia. *Theor. Biol. Med. Model* 12, 17
39. Keys, A. *et al.* (1950) *The Biology of Human Starvation,* University of Minnesota Press
40. Cersosimo, E. *et al.* (1991) The effect of graded doses of insulin on peripheral glucose uptake and lactate release in cancer cachexia. *Surgery* 109, 459–467
41. Simons, J. *et al.* (1997) Plasma concentration of total leptin and human lung-cancer-associated cachexia. *Clin. Sci. (Lond)* 93, 273–277
42. Wolf, I. *et al.* (2006) Adiponectin, ghrelin, and leptin in cancer cachexia in breast and colon cancer patients. *Cancer* 106, 966–973
43. Leij-halfwerk, S. (2000) Weight loss and elevated gluconeogenesis from alanine in lung cancer patients. *Am. J. Clin. Nutr.* 71, 583–589
44. Svaninger, G. *et al.* (1986) Thyroid hormones and experimental cancer cachexia. *J. Natl. Cancer Inst.* 77, 555–561
45. Tanaka, Y. *et al.* (1990) Experimental cancer cachexia induced by transplantable colon 26 adenocarcinoma in mice. *Cancer Res.* 50, 2290–2295
46. Lowe, W.L. *et al.* (1989) Regulation by fasting of rat insulin-like growth factor I and its receptor. Effects on gene expression and binding. *J. Clin. Invest.* 84, 619–626
47. Chan, J.L. *et al.* (2003) The role of falling leptin levels in the neuroendocrine and metabolic adaptation to short-term starvation in healthy men. *J. Clin. Invest.* 111, 1409–1421
48. Duncan, R.E. *et al.* (2007) Regulation of lipolysis in adipocytes. *Annu. Rev. Nutr.* 27, 79–101
49. Rooyackers, O.E. and Nair, K.S. (1997) Hormonal regulation of human muscle protein metabolism. *Annu. Rev. Nutr.* 17, 457–485
50. Braun, T.P. *et al.* (2013) Cancer- and endotoxin-induced cachexia require intact glucocorticoid signaling in skeletal muscle. *FASEB J.* 27, 3572–3582
51. Hu, Z. *et al.* (2009) Endogenous glucocorticoids and impaired insulin signaling are both required to stimulate muscle wasting under pathophysiological conditions in mice. *J. Clin. Invest.* 119, 3059–3069

52. Cai, D. *et al.* (2004) IKKβ/NF-κB activation causes severe muscle wasting in mice. *Cell* 119, 285–298
53. Lee, D. and Goldberg, A.L. (2015) Muscle wasting in fasting requires activation of NF-κB and inhibition of AKT/mTOR by the protein acetylase, GCN5. *J. Biol. Chem.* 290, 30269–30279
54. Costelli, P. *et al.* (2006) IGF-1 is downregulated in experimental cancer cachexia. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 291, R674–R683
55. Roberts, E.W. *et al.* (2013) Depletion of stromal cells expressing fibroblast activation protein from skeletal muscle and bone marrow results in cachexia and anemia. *J. Exp. Med.* 210, 1137–1151
56. Allen, D.L. *et al.* (2010) Myostatin expression is increased by food deprivation in a muscle-specific manner and contributes to muscle atrophy during prolonged food deprivation in mice. *J. Appl. Physiol.* 109, 692–701
57. Nie, Y. *et al.* (2009) STAT3 inhibition of gluconeogenesis is downregulated by SirT1. *Nat. Cell Biol.* 11, 492–500
58. Lecker, S.H. *et al.* (2004) Multiple types of skeletal muscle atrophy involve a common program of changes in gene expression. *FASEB J.* 18, 39–51
59. Bonetto, A. *et al.* (2012) JAK/STAT3 pathway inhibition blocks skeletal muscle wasting downstream of IL-6 and in experimental cancer cachexia. *Am. J. Physiol. Endocrinol. Metab.* 303, E410–E421
60. Bonetto, A. *et al.* (2011) STAT3 activation in skeletal muscle links muscle wasting and the acute phase response in cancer cachexia. *PLoS One* 6, e22538
61. Zhang, L. *et al.* (2013) Stat3 activation links a C/EBPδ to myostatin pathway to stimulate loss of muscle mass. *Cell Metab.* 18, 368–379
62. Akpan, I. *et al.* (2009) The effects of a soluble activin type IIB receptor on obesity and insulin sensitivity. *Int. J. Obes.* 33, 1265–1273
63. Busquets, S. *et al.* (2012) Myostatin blockage using actRIIB antagonism in mice bearing the Lewis lung carcinoma results in the improvement of muscle wasting and physical performance. *J. Cachexia Sarcopenia Muscle* 3, 37–43
64. Villena, J.A. *et al.* (2004) Desnutrin, an adipocyte gene encoding a novel patatin domain-containing protein, is induced by fasting and glucocorticoids: ectopic expression of desnutrin increases triglyceride hydrolysis. *J. Biol. Chem.* 279, 47066–47075
65. Petruzzelli, M. *et al.* (2014) A switch from white to brown fat increases energy expenditure in cancer-associated cachexia. *Cell Metab.* 20, 433–447
66. Fabbiano, S. *et al.* (2016) Caloric restriction leads to browning of white adipose tissue through Type 2 immune signaling. *Cell Metab.* 24, 434–446
67. Nedergaard, J. and Cannon, B. (2014) The browning of white adipose tissue: some burning issues. *Cell Metab.* 20, 396–407
68. Kingma, B. *et al.* (2012) The thermoneutral zone: implications for metabolic studies. *Front. Biosci* E4, 1975–1985
69. Kir, S. *et al.* (2016) PTH/PTHrP receptor mediates cachexia in models of kidney failure and cancer. *Cell Metab.* 23, 315–323
70. Kir, S. *et al.* (2014) Tumour-derived PTH-related protein triggers adipose tissue browning and cancer cachexia. *Nature* 513, 100–104
71. Broz, M.L. *et al.* (2014) Dissecting the tumor myeloid compartment reveals rare activating antigen-presenting cells critical for T cell immunity. *Cancer Cell* 26, 638–652
72. Hildner, K. *et al.* (2008) Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science* 322, 1097–1100
73. Thompson, E.D. *et al.* (2010) Tumor masses support naive T cell infiltration, activation, and differentiation into effectors. *J. Exp. Med.* 207, 1791–1804
74. Joyce, J. and Fearon, D. (2015) T cell exclusion, immune privilege, and the tumor microenvironment. *Science* 348, 74–79
75. Vétizou, M. *et al.* (2015) Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. *Science* 350, 1079–1084
76. Leach, D.R. *et al.* (1996) Enhancement of antitumor immunity by CTLA-4 blockade. *Science* 271, 1734–1736
77. Pylayeva-Gupta, Y. (2012) Oncogenic kras-induced GM-CSF production promotes the development of pancreatic neoplasia. *Cancer Cell* 21, 836–847
78. Ruffell, B. *et al.* (2014) Macrophage IL-10 blocks CD8+ T cell-dependent responses to chemotherapy by suppressing IL-12 expression in intratumoral dendritic cells. *Cancer Cell* 26, 623–637
79. Kraman, M. *et al.* (2010) Suppression of antitumor immunity by stromal cells expressing fibroblast activation protein-{alpha}. *Science* 330, 827–830
80. Motz, G.T. *et al.* (2014) Tumor endothelium FasL establishes a selective immune barrier promoting tolerance in tumors. *Nat. Med.* 20, 607–615
81. Serrels, A. *et al.* (2015) Nuclear FAK controls chemokine transcription, Tregs, and evasion of anti-tumor immunity. *Cell* 163, 160–173
82. Chang, C.H. *et al.* (2015) Metabolic competition in the tumor microenvironment is a driver of cancer progression. *Cell* 162, 1–13
83. Iwai, Y. *et al.* (2002) Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc. Natl. Acad. Sci. U. S. A.* 99, 12293–12297
84. Hamzah, J. *et al.* (2008) Vascular normalization in Rgs5-deficient tumours promotes immune destruction. *Nature* 453, 410–414
85. Snyder, A. *et al.* (2014) Genetic basis for clinical response to CTLA-4 blockade in melanoma. *N. Engl. J. Med.* 371, 2189–2199
86. Zhang, L. *et al.* (2003) Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N. Engl. J. Med.* 348, 203–213
87. Galon, J. *et al.* (2006) Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 313, 1960–1964
88. Ali, H.R. *et al.* (2014) Association between CD8+ T-cell infiltration and breast cancer survival in 12 439 patients. *Ann. Oncol.* 25, 1536–1543
89. Naito, Y. *et al.* (1998) CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. *Cancer Res.* 58, 3491–3494
90. Mikucki, M.E. *et al.* (2015) Non-redundant requirement for CXCR3 signalling during tumoricidal T-cell trafficking across tumour vascular checkpoints. *Nat. Commun.* 6, 7458
91. Hong, M. *et al.* (2011) Chemotherapy induces intratumoral expression of chemokines in cutaneous melanoma, favoring T-cell infiltration and tumor control. *Cancer Res.* 71, 6997–7009
92. Nancy, P. *et al.* (2012) Chemokine gene silencing in decidual stromal cells limits T cell access to the maternal-fetal interface. *Science* 336, 1317–1321
93. Tumeh, P.C. *et al.* (2014) PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature* 515, 568–571
94. Chen, P.-L. *et al.* (2016) Analysis of immune signatures in longitudinal tumor samples yields insight into biomarkers of response and mechanisms of resistance to immune checkpoint blockade. *Cancer Discov.* 6, 827–837
95. Zhu, Y. *et al.* (2014) CSF1/CSF1R blockade reprograms tumor-infiltrating macrophages and improves response to T cell checkpoint immunotherapy in pancreatic cancer models. *Cancer Res.* 74, 5057–5069
96. Feig, C. *et al.* (2013) Targeting CXCL12 from FAP-expressing carcinoma-associated fibroblasts synergizes with anti-PD-L1 immunotherapy in pancreatic cancer. *Proc. Natl. Acad. Sci. U. S. A.* 110, 20212–20217
97. Steele, C.W. *et al.* (2016) CXCR2 inhibition profoundly suppresses metastases and augments immunotherapy in pancreatic ductal adenocarcinoma. *Cancer Cell* 29, 832–845
98. Jiang, H. *et al.* (2016) Targeting focal adhesion kinase renders pancreatic cancers responsive to checkpoint immunotherapy. *Nat. Med.* 22, 851–860
99. Fearon, D.T. (2017) Immune-suppressing cellular elements of the tumor microenvironment. *Annu. Rev. Cancer Biol.* 1, 241–255

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100. Tang, H. *et al.* (2016) Facilitating T cell infiltration in tumor microenvironment overcomes resistance to PD-L1 blockade. *Cancer Cell* 30, 500
101. Mandard, S. *et al.* (2004) Peroxisome proliferator-activated receptor alpha target genes. *Cell. Mol. Life Sci.* 61, 393–416
102. Kersten, S. *et al.* (1999) Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. *J. Clin. Invest.* 103, 1489–1498
103. Shrimali, R.K. *et al.* (2010) Antiangiogenic agents can increase lymphocyte infiltration into tumor and enhance the effectiveness of adoptive immunotherapy of cancer. *Cancer Res.* 70, 6171–6180
104. Tuckermann, J.P. (2007) Macrophages and neutrophils are the targets for immune suppression by glucocorticoids in contact allergy. *J. Clin. Invest.* 117, 1381–1390
105. Bing, C. *et al.* (2001) Cachexia in MAC16 adenocarcinoma: suppression of hunger despite normal regulation of leptin, insulin and hypothalamic neuropeptide Y. *J. Neurochem.* 79, 1004–1012
106. Laviano, A. *et al.* (2000) Effects of intra-VMN mianserin and IL-1ra on meal number in anorectic tumor-bearing rats. *J. Investig. Med.* 48, 40–48
107. Torelli, G.F. *et al.* (1999) Use of recombinant human soluble TNF receptor in anorectic tumor-bearing rats. *Am. J. Physiol.* 277, R850–R855
108. Masri, S. *et al.* (2016) Lung adenocarcinoma distally rewires hepatic circadian homeostasis. *Cell* 165, 896–909
109. Amiel, S.A. *et al.* (1991) Ketone infusion lowers hormonal responses to hypoglycaemia: evidence for acute cerebral utilization of a non-glucose fuel. *Clin. Sci. (Lond)* 81, 189–194
110. Fukawa, T. *et al.* (2016) Excessive fatty acid oxidation induces muscle atrophy in cancer cachexia. *Nat. Med.* 22, 666–671
111. Tarcic, N. *et al.* (1998) Restraint stress-induced thymic involution and cell apoptosis are dependent on endogenous glucocorticoids. *J. Neuroimmunol.* 82, 40–46
112. Dhabhar, F.S. *et al.* (1995) Effects of stress on immune cell distribution. Dynamics and hormonal mechanisms. *J. Immunol.* 154, 5511–5527
113. Pietrocola, F. *et al.* (2016) Caloric restriction mimetics enhance anticancer immunosurveillance. *Cancer Cell* 30, 147–160
114. Biase Di, S. *et al.* (2016) Fasting-mimicking diet reduces HO-1 to promote T cell-mediated tumor cytotoxicity. *Cancer Cell* 30, 136–146
115. Minamoto, K. *et al.* (2005) Reciprocal regulation of airway rejection by the inducible gas-forming enzymes heme oxygenase and nitric oxide synthase. *J. Exp. Med.* 202, 283–294
116. Ma, E.H. *et al.* (2017) Serine is an essential metabolite for effector T cell expansion. *Cell Metab.* 25, 345–357
117. Geiger, R. *et al.* (2016) L-Arginine modulates T cell metabolism and enhances survival and anti-tumor activity. *Cell* 167, 829–842.e13
118. Ubhi, B.K. *et al.* (2012) Targeted metabolomics identifies perturbations in amino acid metabolism that sub-classify patients with COPD. *Mol. Biosyst.* 8, 3125–3133
119. Viaud, S. *et al.* (2013) The intestinal microbiota modulates the anticancer immune effects of cyclophosphamide. *Science* 342, 971–976
120. Zhang, C. *et al.* (2013) Structural modulation of gut microbiota in life-long calorie-restricted mice. *Nat. Commun.* 4, 2163
121. Furet, J. *et al.* (2010) Differential adaptation of human gut microbiota to bariatric surgery-induced weight loss. *Diabetes* 59, 3049–3057
122. David, L.A. *et al.* (2014) Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 505, 559–563
123. Zhen, Y. *et al.* (2007) Metabolomic and genetic analysis of biomarkers for PPARα expression and activation. *Mol. Endocrinol.* 21, 2136–2151
124. Sephton, S.E. *et al.* (2000) Diurnal cortisol rhythm as a predictor of breast cancer survival. *J. Natl. Cancer Inst.* 92, 994–1000
125. Greer, S. *et al.* (1979) Psychological response to breast cancer: effect on outcome. *Lancet* 314, 785–787
126. Kettner, N.M. *et al.* (2016) Circadian homeostasis of liver metabolism suppresses hepatocarcinogenesis. *Cancer Cell* 30, 909–924
127. Papagiannakopoulos, T. (2016) Circadian rhythm disruption promotes lung tumorigenesis. *Cell Metab.* 24, 324–331
128. Dimitrov, S. *et al.* (2009) Cortisol and epinephrine control opposing circadian rhythms in T cell subsets. *Blood* 113, 5134–5144
129. Chu, M.P. *et al.* (2015) Skeletal muscle radio-density is an independent predictor of response and outcomes in follicular lymphoma treated with chemoimmunotherapy. *PLoS One* 10, e0127589
130. Go, S.-I. *et al.* (2016) Prognostic impact of sarcopenia in patients with diffuse large B-cell lymphoma treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. *J. Cachexia Sarcopenia Muscle* 7, 567–576
131. Öhlund, D. *et al.* (2017) Distinct populations of inflammatory fibroblasts and myofibroblasts in pancreatic cancer. *J. Exp. Med.* 214, 579–596
132. Caetano, M.S. *et al.* (2016) IL6 blockade reprograms the lung tumor microenvironment to limit the development and progression of K-ras-mutant lung cancer. *Cancer Res.* 76, 3189–3199
133. Mace, T. *et al.* (2016) IL-6 and PD-L1 antibody blockade combination therapy reduces tumour progression in murine models of pancreatic cancer. *Gut* http://dx.doi.org/10.1136/gutjnl-2016-311585
134. Rettura, G. *et al.* (1973) Metyrapone-inhibited oncogenesis in mice inoculated with a murine sarcoma virus 2. *J. Natl. Cancer Inst.* 51, 1983–1985

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Cancer-Cell-Intrinsic Mechanisms Shaping the Tumor Immune Landscape

Max D. Wellenstein[1] and Karin E. de Visser[1,*]
[1]Division of Tumor Biology & Immunology, Oncode Institute, Netherlands Cancer Institute, 1066 CX Amsterdam, the Netherlands
*Correspondence: k.d.visser@nki.nl
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Owing to their tremendous diversity and plasticity, immune cells exert multifaceted functions in tumor-bearing hosts, ranging from anti-tumor to pro-tumor activities. Tumor immune landscapes differ greatly between and within cancer types. Emerging evidence suggests that genetic aberrations in cancer cells dictate the immune contexture of tumors. Here, we review the current understanding of the mechanisms whereby common drivers of tumorigenesis modulate the tumor immune milieu. We discuss these findings in the context of clinical observations and examine how cancer-cell-intrinsic properties can be exploited to maximize the benefit of immunomodulatory therapies. Understanding the relationship between cancer cell-intrinsic genetic events and the immune response may enable personalized immune intervention strategies for cancer patients.

Introduction

The recognition of cancer as a genetic disease is more than a century old and stems from observations by David von Hansemann and Theodor Boveri that cancer cells display chromosomal abnormalities (Boveri, 1914; von Hansemann, 1890). In the early 20th century, Francis Rous revealed that retroviruses could drive sarcoma formation in chickens (Rous, 1911). Many decades later, in 1970, the Rous sarcoma virus was found to carry a gene called *v-Src*, the first oncogene to be identified (Duesberg and Vogt, 1970; Stehelin et al., 1976). Concurrently, it was discovered that not only activation, but also inactivation of so-called tumor suppressor genes (TSGs) can lead to tumorigenesis (Knudson, 1971). (Proto-)oncogenes and TSGs regulate essential cellular processes like cell cycle, apoptosis, migration, and survival, and genetic aberrations that lead to dysregulation or loss of function of these genes can result in malignant transformation. The generation of transgenic mice carrying an activated oncogene, also called *oncomice*, in the 1980s and TSG knockout mice in the 1990s further substantiated the notion that oncogene expression or loss of TSGs in normal mammalian cells leads to cancer development (Adams et al., 1985; Donehower et al., 1992; Hanahan et al., 2007; Stewart et al., 1984). The dependency of cancers on these dysregulated genes was demonstrated in genetically engineered mouse models (GEMMs) in which de-activation of oncogenes or re-expression of TSGs in fully established tumors led to rapid tumor regression (Fisher et al., 2001; Jain et al., 2002; Moody et al., 2002; Ventura et al., 2007). These insights into the causal role of genetic aberrations in cancer initiation and progression spurred the long-held belief that tumorigenesis is entirely driven by cancer-cell-intrinsic genetic traits. However, over the past couple of decades, this dogma has been challenged by new experimental evidence demonstrating that genetic aberrations alone are required, but not sufficient, for a cancer to develop. Like a seed needing fertile soil for successful germination, cancer cells only survive and develop into invasive tumors in an environment that provides sufficient nutrients and oxygen, and that lacks strong cytotoxic signals. In this review, we will focus on one of the most influential cancer cell-extrinsic regulators of cancer biology, the immune system.

Similar to its physiological function, the immune system exerts multifaceted tasks in tumor-bearing hosts, with different immune cells playing different and sometimes opposing roles. The composition and function of immune cells in tumors differs greatly between, but also within, cancer types. For example, of the breast cancer subtypes, triple-negative breast cancer (TNBC) presents with highest levels of tumor-infiltrating lymphocytes (TIL) and macrophages (Medrek et al., 2012; Stanton et al., 2016). Striking differences in relative leukocyte composition between different tumor types were observed in a study that integrated gene expression and clinical outcome data of over 18,000 human tumors (Gentles et al., 2015). Moreover, this study revealed considerable variation in intratumoral presence of certain immune cell subsets and how these were associated with cancer-specific outcomes. For example, whereas memory CD4^{+} T cells were associated with adverse outcome in bladder cancer patients, they correlated with favorable outcome in lung adenocarcinoma patients (Gentles et al., 2015), suggesting that differences in immune profile are not only phenotypically distinct but are also of functional consequence. But what determines this substantial variation in immune contexture between different tumors? Given the surge of interest in utilizing immunomodulatory drugs for the treatment of cancer patients, it is critical to understand the underlying tumor characteristics that dictate the inter-tumor heterogeneity in immune landscapes and to use this knowledge for rational decision-making in the clinical use of immunomodulatory strategies.

In this review, we will discuss recent insights into how cancer cell-intrinsic properties can dictate the immune landscape of tumor-bearing hosts. Specifically, we will examine which genetic aberrations correlate with immune cell composition in human tumors. Next, we will discuss the current knowledge on oncogene- and TSG-dependent signaling pathways that underlie the differential crosstalk of cancer cells with the immune system as identified in genetically engineered mouse tumor models (GEMMs). Finally, we will discuss how the growing insights into

these mechanisms may open new avenues for personalized immune intervention strategies for cancer patients.

Genetic Makeup Influencing the Immune Contexture of Tumors—Observations from the Clinic

In 1863, the German pathologist Rudolf Virchow was the first to hypothesize a link between the development of tumors and the inflammatory state of their anatomical location (Balkwill and Mantovani, 2001). Around the same time, William Coley, pioneer of cancer immunotherapy, demonstrated that some patients displayed tumor regression after being injected with immune stimulatory *Streptococcus pyogenes* cultures (Coley, 1893). Nowadays, it is fully established that inflammation can be causally linked with human cancers and that the immune infiltrate of human tumors contains prognostic and predictive information (Diakos et al., 2014; Gentles et al., 2015). Moreover, cancer immunotherapy has revolutionized cancer treatment (Yang, 2015), illustrating that immune cells can be harnessed successfully to destroy tumors in a proportion of cancer patients. Recently, studies have started to explore the cancer cell characteristics—including the genetic makeup—that play a critical role in dictating the heterogeneity in immune landscape between different tumors. Studies aimed at assessing the link between the genetics of human tumors and the immune infiltrate can be roughly divided into three categories: (1) studies that have assessed the extent of the mutational load of tumors with T cell abundance, specificity and activity, (2) studies that have linked distinct molecular tumor subtypes with a certain immune landscape, and (3) studies that have focused on the association between defined oncogenic driver mutations or loss of TSGs and parameters of the inflammatory tumor microenvironment. In this section, we will discuss the findings of these three different strategies to assess the impact of genetic events on the crosstalk with the immune system.

The core function of the adaptive immune system is to recognize and destroy cells expressing non-self-antigens, while not responding to self-antigens. Because cancers arise from host cells, these cancer cells, with the exception of viral-associated cancers, do not express the typical immunogenic foreign antigens as seen in infections. The recent clinical breakthrough of immune checkpoint inhibitors has fueled studies aimed at identifying the tumor antigens that are recognized by effective antitumor immune responses. This resulted in the hypothesis that a higher mutational load of a tumor will inevitably result in more "foreign" peptide presentation and consequently higher immunogenicity of the tumor. Mutations and other genomic rearrangements in cancer cells can encode for neo-antigens, antigens uniquely expressed by the tumor, that when presented by MHC molecules can potentially be recognized by the endogenous T cell repertoire (Schumacher and Schreiber, 2015). Indeed, neo-antigen-specific T cells have been observed in melanoma patients (Lennerz et al., 2005; Linnemann et al., 2015; Robbins et al., 2013; van Rooij et al., 2013; Wölfel et al., 1995) and tumor types with a relatively high mutational burden, such as melanoma, non-small cell lung cancer (NSCLC) and microsatellite-instable (MSI) tumors display increased T cell influx and have an overall better response rate to immunotherapeutics compared to tumors with a lower mutational load (Le et al., 2015; Rizvi et al., 2015; Van Allen et al., 2015). Nevertheless, there is a substantial number of patients with good response and low mutational load and vice versa (Balli et al., 2017; Charoentong et al., 2017; Hugo et al., 2016; Rizvi et al., 2015; Robinson et al., 2017; Spranger et al., 2016). These observations suggest that for some tumors the mutational burden of tumors can serve as a quantitative measure for T cell abundance and likelihood to respond to immune checkpoint inhibitors. However, there are clearly additional determinants of the immune contexture in tumors besides mutational load.

Distinct molecular subtypes of human cancers can be associated with a defined immune composition and activation state in the tumor microenvironment. Several cancer types can be subtyped based on their molecular and genetic profile, thus forming separate classes within a given tumor type, often with distinct progression characteristics and treatment regimens. For example, breast tumors can be classified as Luminal A (ER/PR^+, $HER2^-$), Luminal B (ER/PR^+, $HER2^{+/-}$), HER2-enriched ($HER2^+$), and triple-negative/basal-like ($ER/PR/HER2^-$) (Parker et al., 2009). It has been reported that $CD8^+$ T cells preferentially infiltrate in triple negative tumors and those patients with high intratumoral T cell abundance show better disease-free survival (Chen et al., 2014; Medrek et al., 2012; Savas et al., 2016; Stanton et al., 2016). Breast tumors that express hormone receptors or HER2 are more frequently infiltrated by $FoxP3^+$ regulatory T cells (Tregs) compared to other subtypes, suggesting dependency on these receptors in the establishment of an immunosuppressive milieu (Decker et al., 2012; Jiang et al., 2015). Accordingly, the presence of Tregs in breast tumors predicted metastatic progression and poor survival (Jiang et al., 2015; Liu et al., 2011). For other cancer types, such as colorectal cancer, glioblastoma, and head and neck cancer, similar subtype-specific tumor immune infiltrates have been observed (Becht et al., 2016; Doucette et al., 2013; Keck et al., 2015; Wang et al., 2017) (Table 1). These clinical observations indicate that different molecular subtypes of tumors can be characterized by distinct immune landscapes. However, due to the complex nature that underlies molecular subtypes, the exact genes and mechanisms that determine this immune heterogeneity cannot be distilled from these studies.

A growing body of clinical observations indicates that defined oncogenic driver mutations and loss of TSGs in human cancers are also correlated with changes in immune composition and immunotherapy response. For example, loss of *NF1* in glioblastomas associated with an increase in macrophages in the tumor (Wang et al., 2017). Another study showed that loss of heterozygosity (LOH) or mutation of *TP53* in ER-negative and basal-like breast tumors is associated with decreased intratumoral expression of a cytotoxic T cell signature and poor survival (Quigley et al., 2015). These studies indicate that a single TSG can be associated with the immune composition of the tumor, across different tumor subtypes, and therefore might be a dominant driving force of immune influx. Furthermore, in pancreatic ductal adenocarcinoma (PDAC), expression of genes associated with cytotoxic T cell function and immune checkpoint molecules was inversely linked with amplification of *MYC*, *NOTCH2*, and *FGFR1*, but not with mutational load (Balli et al., 2017). The reduced expression of cytolytic immune response markers in these *MYC*-, *NOTCH2*-, and *FGFR1*-amplified tumors was observed across the different PDAC subtypes (Bailey et al.,

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Table 1. Clinical Observations on Tumor Subtype and Genotype-Immunophenotype Relations

Determinant of tumor immune landscape	Cancer type	Immune cell subset	Effect on therapy/disease outcome	Reference
Tumor subtype				
CMS1	CRC	↑ Cytotoxic T cells[a]	Overall favorable response to immune checkpoint blockade	(Becht et al., 2016)
Mesenchymal	Glioblastoma	↑ Immunosuppressive cells[a] ↑ T effector cells[a]	NA	(Doucette et al., 2013)
		↑ Macrophages, neutrophils[a]		(Wang et al., 2017)
Triple-negative/basal-like	Breast cancer	↑ $CD8^+$ T cells, macrophages	High $CD8^+$ T cell abundance gives high overall survival	(Chen et al., 2014; Medrek et al., 2012; Savas et al., 2016; Stanton et al., 2016)
ER/PR/HER2$^+$		↑ Tregs	High Treg abundance gives poor overall survival	(Decker et al., 2012; Jiang et al., 2015; Liu et al., 2011)
Inflamed/mesenchymal HPV$^{+/-}$	HNSCC	↑ $CD8^+$ T cells[a]	NA	(Keck et al., 2015)
Mutated oncogenes or tumor suppressor gene				
TP53 loss or mutation	ER$^-$ & basal-like breast cancer	↓ Cytotoxic T cells[a]	Poor survival	(Quigley et al., 2015)
	Pan-cancer	↓ Cytotoxic T, NK cells[a]	NA	(Rooney et al., 2015)
MYC, NOTCH2, FGFR1 amplification	PDAC	↓ Cytotoxic T cells[a]	NA	(Balli et al., 2017)
MYC amplification	Neuroblastoma	↓ T cells[a]	NA	(Layer et al., 2017)
PIK3CA, MET mutations	Pan-cancer	↑ Cytotoxic T, NK cells[a]	NA	(Rooney et al., 2015)
BRAF mutations	Thyroid cancer	↑ Immunosuppressive cells[a]	NA	(Charoentong et al., 2017)
RAS mutations		↑ T cells[a]		
VHL, STK11 mutations	Pan-cancer	↓ Macrophages[a]	NA	(Rooney et al., 2015)
NF1 loss	Glioblastoma	↑ Macrophages	NA	(Wang et al., 2017)

Abbreviations: CRC, colorectal cancer. HNSCC, head and neck squamous cell carcinoma. PDAC, pancreatic ductal adenocarcinoma. NA, Not assessed.
[a]Immune cell composition based on gene expression signatures.

2016; Balli et al., 2017) and suggests that aberrant expression of oncogenic pathways also dominantly impacts the composition of the pancreatic tumor microenvironment (Table 1).

Genetic aberrations in tumors can also influence the T cell response by altering expression levels of immune checkpoint molecules by cancer cells. In a cohort of lung adenocarcinoma patients, accumulation of p53 in tumor cells, which is indicative of mutations in *TP53*, correlated with increased *PD-L1* expression, while mutant EGFR tumors were characterized by low expression of *PD-L1* (Cha et al., 2016). In contrast, another study showed that EGFR mutated lung tumors have high levels of *PD-L1* (Akbay et al., 2013), demonstrating that the role of mutant EGFR in regulating PD-L1 expression is still under debate. In metastatic neuroblastoma, amplification of *MYCN* correlated with low expression of *PD-L1* and a reduced T cell gene-expression signature in the tumor compared to *MYCN*-normal tumors (Layer et al., 2017). Moreover, *MYCN* overexpression inversely correlated with natural killer (NK) cell-activating factors such as NKG2D in primary human neuroblastoma cell lines (Brandetti et al., 2017). In addition, resistance to anti-PD-1 treatment in melanoma and MSI CRC patients correlated with mutations in JAK1/2 (Shin et al., 2017). Using human melanoma cell lines, it was shown that JAK1/2 mutations led to an impaired IFN signaling pathway-mediated PD-L1 expression, suggesting that also JAK-STAT signaling is involved in regulating immune checkpoint expression. These findings indicate that screening for expression of certain oncogenes or loss of function of specific TSGs might be exploited to improve the stratification of cancer patients for therapeutic targeting the PD-1/PD-L1 axis.

The link between the genetic makeup of tumors and their immune contexture was further strengthened by recent high-throughput next generation sequencing (NGS) studies, which allow an unbiased assessment of the genetics of tumors in parallel with high-resolution mapping of the tumor immune landscape. By correlating an RNA-based metric of immune cytolytic activity (mainly associated with T and NK cell function) with genetic data from the Cancer Genome Atlas (TCGA) dataset, it was shown that immune activity varies substantially across tumor types (Rooney et al., 2015). Consistent with the concept that a higher mutational load increases tumor immunogenicity, there was a positive correlation between adaptive immune activation gene signatures and mutational load across tumor types

(Rooney et al., 2015). Interestingly, this study also revealed that expression of genes associated with cytotoxic immune activation was elevated in tumors with mutations in *PIK3CA* or *MET*, while *TP53* mutant tumors displayed low levels of these genes (Rooney et al., 2015). Additionally, mutations in *VHL* and *STK11* associated with reduced macrophage signatures (Rooney et al., 2015). In another study into genotype-immunophenotype relationships, it was found that BRAF-mutated thyroid tumors were characterized by infiltration of immunosuppressive cells, while the RAS-mutated subtype contained higher T cell influx and displayed downregulation of MHC molecules, despite comparable mutational load (Charoentong et al., 2017). Accordingly, oncogenic mutations also link with response to immunotherapy. Using human datasets to predict response to anti-CTLA-4 therapy in melanoma patients, it was demonstrated that mutations in oncogenes such as *KRAS*, *ATM*, and *mTOR* correlated with good immunotherapy response for some tumor types (Ock et al., 2017). These studies demonstrate that NGS studies can reveal relationships between cancer-associated genes, activation of immune cells and response to immunotherapies in a high-throughput and high-resolution manner.

Together, these observations suggest that mutational load, tumor subtype, and aberrant expression of oncogenes and TSGs highly impact the tumor microenvironment. Interestingly, for certain tumors, the tumor driver genes, mutational load, and subtype are intrinsically linked, as for example aberrant expression of *BRCA1* impairs the DNA damage repair machinery and therefore has consequences for the mutational load of a tumor. However powerful, these genotype-immunophenotype studies in human cancers leave several questions open. Due to the descriptive nature of these analyses, these studies do not yield mechanistic insights into causal relationships between tumor genetics and the immune composition. From a therapeutic perspective, it is important to assess whether a causal link between tumor genetics and immune contexture exists and to elucidate the underlying molecular mechanisms, since this would open new avenues for personalized immune intervention strategies. Of note, the above described clinical studies often rely on the analysis of a small tumor biopsy at a given time point, and therefore may overlook intratumoral heterogeneity and tumor evolution. For these reasons, mechanistic studies in relevant GEMMs that mimic the development, heterogeneity and progression of human tumors in an immune-proficient setting are key to understand how cancer cell-intrinsic properties can dictate the tumor immune landscape (Kersten et al., 2017). In the next sections, we will discuss recent insights into these mechanisms and how these insights can be translated into personalized immune intervention strategies. Given the growing interest in the role of the immune system in tumorigenesis, we anticipate that more pathways will be uncovered in the years to come.

NF-κB and p53: Central Nodes in Cancer Cell-Mediated Changes in the Inflammatory Microenvironment

The mechanisms by which oncogenes and TSGs orchestrate the inflammatory tumor microenvironment are now being uncovered. Specific cancer-associated genes, besides driving cancer cell-intrinsic programs, also change the secretome of cancer cells and thereby change the immune microenvironment (Figure 1, Table 2). One notable example is NF-κB, a transcription factor that controls cell survival and proliferation, but also production of inflammatory cytokines. For example, NF-κB signaling promoted tumor development in the *Kras*$^{LSL\text{-}G12D/+}$*;Trp53*$^{F/F}$ lung adenocarcinoma model (Meylan et al., 2009). Interestingly, NF-κB activity was increased upon loss of p53, and restoration of p53 expression reduced its activity. Cancer cell-intrinsic NF-κB inactivation resulted in increased intratumoral immune cell influx and impaired lung cancer formation in *Kras*$^{LSL\text{-}G12D}$*;Trp53*$^{F/F}$ mice (Meylan et al., 2009), showing a link between loss of p53, NF-κB pathway activation, and an inflammatory tumor microenvironment. As one of the most frequently mutated genes in cancer (Kastenhuber and Lowe, 2017), the tumor suppressor p53 can potentially regulate the immune infiltrate in a wide variety of tumor types, through its interactions with NF-κB or otherwise. Indeed, the control of the pro-inflammatory NF-κB pathway by p53 appears to be occurring across cancer types (Cooks et al., 2014). For example, in the *Pgr-cre;Cdh1*$^{F/F}$*;Trp53*$^{F/F}$ mouse model for endometrial cancer, the combined loss of E-cadherin and p53 resulted in increased NF-κB activity, which correlated with elevated cytokine expression and increased influx of macrophages, as compared to deletion of either gene alone (Stodden et al., 2015). However, in another mouse model in which endometrial tumorigenesis is driven by loss of PTEN, loss of p53 did not alter neutrophil influx into early lesions (Blaisdell et al., 2015), suggesting that this effect might be context dependent. Together, these and other studies show that NF-κB, key regulator of immune signaling in the tumor microenvironment, is controlled by p53. In several tumor models, loss of p53 activates the NF-κB pathway, stimulates the production of cytokines and other pro-inflammatory mediators from cancer cells, which through paracrine interactions modify the immune contexture.

Studies in mouse models in which chemical-induced inflammation drives malignant conversion and progression show that the NF-κB-mediated inflammatory response can also be a driving force of tumorigenesis in p53-knockout models. For example, azoxymethane (AOM)-induced colonic tumorigenesis was enhanced in *Villin-cre;Trp53*$^{F/F}$ mice that harbor p53 deletion in intestinal epithelial cells, as compared to mice with p53 proficient intestinal epithelial cells (Schwitalla et al., 2013). Mechanistic studies in these mice revealed that loss of p53 impaired the removal of pre-neoplastic transformed cells and induced NFκB-dependent cytokine production, thus driving an inflammatory tumor microenvironment (Schwitalla et al., 2013). Importantly, genetic ablation of IKKβ, a protein involved in NF-κB activation, in cancer cells or myeloid cells, reduced tumor proliferation and invasion, demonstrating that NF-κB signaling in p53 null cancer cells or in surrounding myeloid cells plays a fundamental role in tumor progression (Schwitalla et al., 2013).

A critical feature of p53 biology in cancer not addressed in these studies is its wide variety of both activating and inactivating mutations, leading to very diverse and sometimes even opposing functions (Muller and Vousden, 2014). How one of these p53 mutations affects NF-κB activation, was addressed in a gain-of-function (GOF) mutant *p53*^{G515A} mouse model that was repeatedly exposed to dextran sodium sulfate (DSS) to stimulate colitis-induced colorectal cancer (CRC) (Cooks et al., 2013). Repair of DSS-induced damaged tissue was impaired in

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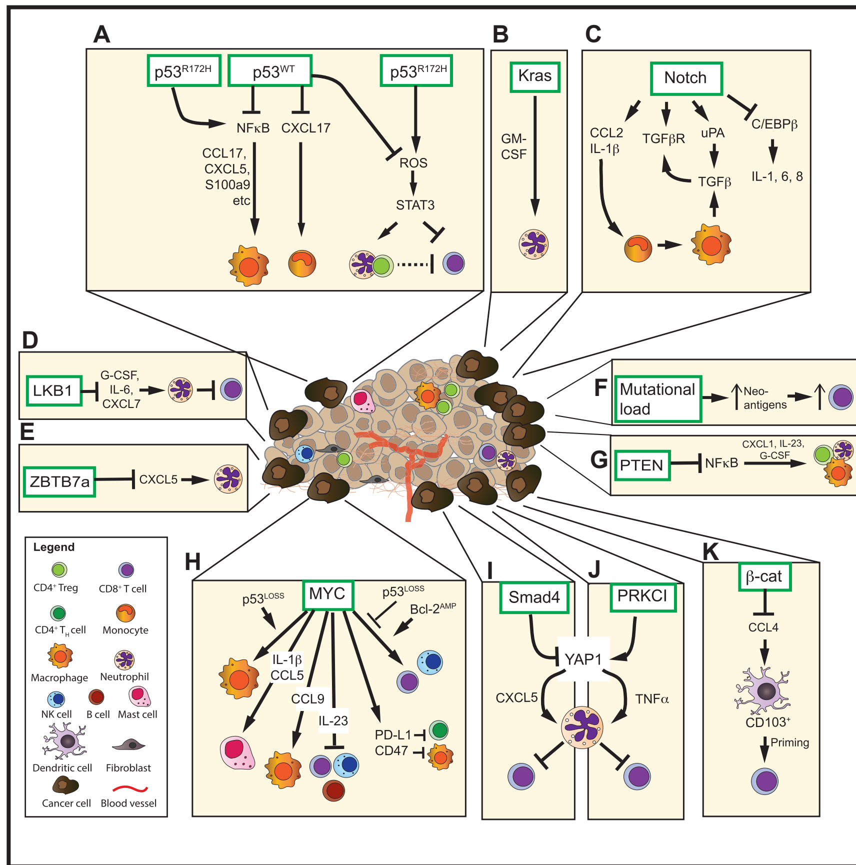

Figure 1. Cancer Cell-Intrinsic Signaling Pathways that Shape the Tumor Immune Landscape

(A) The p53 pathway can modulate the immune microenvironment of the tumor by regulating NF-κB signaling, that is generally activated by loss or loss-of-function (LOF) mutation of p53. This results in increased cytokine production by tumor cells and recruitment and activation of immune cells, such as macrophages (Cooks et al., 2013; Schwitalla et al., 2013). In addition, by activating ROS, mutant p53 can induce JAK-STAT signaling and thereby increase macrophage, neutrophil, and CD4$^+$ T cell frequencies in the tumor, while concurrently reducing CD8$^+$ T cell levels (Wörmann et al., 2016).
(B) Mutant KRAS can increase GM-CSF by cancer cells and thereby promote neutrophil recruitment to the tumor (Pylayeva-Gupta et al., 2012).
(C) Activated Notch signaling can signal to monocytes and macrophages by driving CCL2 and IL-1β expression. Notch also drives TGF-β receptor and uPA expression, of which the latter is involved in activating macrophage-derived TGF-β, thus inducing a growth promoting signaling loop (Shen et al., 2017). Notch can also limit the anti-tumor immune response by inhibiting C/EBPβ and thereby limiting expression of IL-1, IL-6, and IL-8 (Hoare et al., 2016).
(D) Loss of tumor suppressor gene LKB1 can drive production of G-CSF, CXCL7, and IL-6 by the tumor, which promotes neutrophil recruitment, which can block anti-tumoral cytotoxic T cells (Koyama et al., 2016).
(E) ZBTB7a blocks CXCL5 production by binding its promoter, and loss of ZBTB7a therefore can lead to CXCL5-mediated neutrophils recruitment (Bezzi et al., 2018).
(F) High mutational load in tumors can increase the number of neo-antigens and thus potentially increase neo-antigen-specific T cell responses.
(G) PTEN can negatively regulate NF-κB signaling. Therefore, loss of PTEN increases NF-κB-mediated expression of cytokines and growth factors that drive macrophage, neutrophil, and Treg accumulation in the tumor (Ying et al., 2011).
(H) MYC can regulate macrophage recruitment, which is promoted by p53 loss (Yetil et al., 2015). Additionally, by inducing CCL5 and IL-1β, MYC can promote mast cell recruitment and activation (Shchors et al., 2006; Soucek et al., 2007). MYC can also induce CCL9 and IL-23 expression, the former of which induces macrophage recruitment, while the latter limits NK, T, and B cell accumulation in the tumor (Kortlever et al., 2017). MYC can also inhibit CD4$^+$ T cells and macrophages by regulating PD-L1 and CD47 expression on tumor cells (Casey et al., 2016). Lastly, the anti-tumor NK- and CD8$^+$ T cell-response to MYC amplified tumors can be counteracted by additional loss of p53 in the tumor, while amplification of Bcl-2 promotes anti-tumor immunity (Schuster et al., 2011).
(I) SMAD4 can suppress YAP1 signaling, and loss of SMAD4 in tumors therefore drives YAP1-mediated CXCL5 production, which recruits immunosuppressive neutrophils (Wang et al., 2016).
(J) PRKCI amplification can also induce YAP signaling. Activation of YAP1 here induces TNFα-mediated recruitment and activation of immunosuppressive neutrophils (Sarkar et al., 2017).
(K) Activated Wnt signaling via β-catenin can limit the priming of CD8$^+$ T cells by suppression of CCL4 production, which would otherwise activate CD103$^+$ DCs (Spranger et al., 2015).

p53^{G515A} mice. Combined with enhanced NF-κB activity and extended inflammation, this led to an increase in colorectal tumor incidence in mice (Cooks et al., 2013). In addition, *p53^{G515A}* mutant intestinal organoids derived from these mice showed increased tumor necrosis factor alpha (TNF-α) and CXCL1 production when compared to *p53$^{-/-}$* cells, which could be reverted by NF-κB knockdown (Cooks et al., 2013). In line with these experimental findings, expression of mutant *TP53* correlated with NF-κB expression in human CRC patients (Cooks et al., 2013). These findings show that this GOF mutant p53 induces aberrant NF-κB interactions, leading to different inflammatory phenotypes than observed after loss of p53. In a mouse model for pancreatic cancer, p53^{R172H} has been reported to elicit similar immune phenotypes as loss of p53. *Kras^{G12D};p53^{R172H}* mutant mouse pancreatic tumors drive inflammatory responses via ROS and JAK2-STAT3 activation (Wörmann et al., 2016). Here, both p53^{R172H} mutant and p53-deficient tumors displayed similar STAT3-dependent immune evasion and accelerated tumor growth, which both could be reversed by pharmacological targeting of JAK-STAT signaling (Wörmann et al., 2016). These findings indicate that different mutations of p53 can shape the tumor microenvironment in a distinct manner. In future studies, it would be interesting to systematically dissect the differences between gain- and loss-of-function p53 mutations on NF-κB interactions and the immune landscape of the tumor. Altogether, these studies demonstrate the profound role of p53-mediated regulation of key immune signaling pathways such as NF-κB and STAT signaling, and its downstream effects on the tumor immune landscape.

MYC: A Key Controller of the Immune Microenvironment

The MYC oncogene is one of the most frequently amplified oncogenes in several tumor types, such as lymphoma, breast cancer, and NSCLC (Beroukhim et al., 2010). As a transcription factor,

Table 2. Genetic Aberrations Influencing the Immune Landscape of Tumors

Gene	Genetic aberration	Consequence for intratumoral immune cells	Signaling involved	Tumor type	Tumor model	Reference
AKT	Loss	Macrophages ↓	AKT deletion decreases tumorigenesis by reducing pro-tumorigenic Wnt-producing macrophages in the tumor	Liver cancer	*Alb-cre;Pten*$^{F/F}$ and *Alb-cre;Pten*$^{F/F}$*;Akt2*$^{F/F}$	(Debebe et al., 2017)
ATR	Deletion	Macrophages ↑	NA	Melanoma	*Tyr::ER*^{T2}*;Braf*$^{V600E/+}$*;Pten*$^{F/F}$ and *Tyr::ER*^{T2}*;Braf*$^{V600E/+}$*; Pten*$^{F/F}$*;ATR*$^{F/F}$	(Chen et al., 2017)
		B cells ↑, CD8$^+$ T cells ↓				
β−catenin	Amplification	CD8$^+$ T cells ↓	Active β-catenin inhibits CCL4, thus inhibiting CD8$^+$ T cell priming by CD103$^+$ DCs.	Melanoma	*Tyr:cre-ER;Braf*$^{LSL-V600E/+}$*; Pten*$^{F/F}$ and *Tyr:cre-ER; Braf*$^{LSL-V600E/+}$*;Pten*$^{F/F}$*; LSL-CAT-STA*	(Spranger et al., 2015)
CKIα	Loss	Macrophages ↓	Loss of CKIα triggers an inflammatory SASP. Subsequent loss of p53 or p21 leads to inflammation-accelerated tumorigenesis.	CRC	*Villin-cre;CKIa*$^{F/F}$, *Villin-cre; CKIa*$^{F/F}$*;p21*$^{-/-}$ and *Villin-cre;CKIa*$^{F/F}$*;Trp53*$^{F/F}$	(Pribluda et al., 2013)
EGFR	Mutation	Macrophages, neutrophils ↑	NA	NSCLC	*Ccsp-rtTA;TetO-Egfr*^{L858R}	(Busch et al., 2016)
		CD8$^+$ T cells ↓				
		CD8$^+$ T cells ↓	EGFR pathway activates PDL1 expression in bronchial epithelial cells		*Ccsp-rtTA;TetO-EGFR*^{T790M}, *EGFR*$^{T790M/L858R}$ and *EGFR*$^{exon\ 19\ del/T790M}$	(Akbay et al., 2013)
FAK	Amplification	Macrophages, neutrophils, monocytes ↑	Potentially via STAT3 signaling.	PDAC	*p48-Cre;Kras*$^{LSL-G12D/+}$	(Jiang et al., 2016)
		CD3$^+$ T cells ↓, Tregs ↑	Potentially due to immunosuppressive myeloid cells			
FGFR	Activation	Neutrophils ↑	FGFR drives mTOR signaling, which causes increase in G-CSF production, driving neutrophil expansion, thus promoting tumor progression	Breast cancer	*MMTV-Wnt1*, *MMTV-Wnt1-iFGFR* and *MMTV-cre; Trp53*$^{F/F}$*;Pten*$^{F/F}$	(Welte et al., 2016)
IFNAR1	Mutation	NK cells ↓, neutrophils ↑	Inactivating mutant of IFNAR1 promotes the establishment of an immunosuppressive microenvironment and tumor progression	CRC	AOM-DSS induced	(Katlinski et al., 2017)
		CD8$^+$ cells ↓				
KRAS	Mutation	Myeloid cells ↑	NA	NSCLC	*Kras*$^{LSL-G12D/+}$ and *Kras*$^{LSL-G12D/+}$*;Trp53*$^{F/F}$	(Busch et al., 2016)
		T cells (CD8$^+$, Treg, γδ T cells) ↑				
LKB1	Loss	Neutrophils ↑, Macrophages, CD4$^+$, CD8$^+$ T cells ↓	Loss of *Lkb1* leads to an increase in CXCL7, G-CSF and IL-6, which drive neutrophil increase. Neutrophils decrease IFNγ$^+$ T cells in the tumor.	NSCLC	*Kras*$^{LSL-G12D/+}$ and *Kras*$^{LSL-G12D/+}$*;Lkb1*$^{F/F}$	(Koyama et al., 2016)
mTOR	Amplification	NK cells, macrophages ↑	mTOR regulates IL-1α levels, and IL-1α activates NFκB, thus driving SASP and immune cell recruitment	Liver cancer	Hydrodynamic tail-vein injection of *NRAS*^{G12V}	(Herranz et al., 2015; Laberge et al., 2015)
		T, B cells ↑	mTOR activates tumor suppressive SASP			

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Table 2. *Continued*

Gene	Genetic aberration	Consequence for intratumoral immune cells	Signaling involved	Tumor type	Tumor model	Reference
MYC	Loss	Macrophages, neutrophils ↓	NA	Pancreatic cancer	*RIP1-Tag2 and TRE-Omomyc;CMVrtTA;RIP1-Tag2*	(Sodir et al., 2011)
	Amplification	Mast cells ↑	MYC activation drives IL-1β and CCL5 expression, leading to an influx of mast cells in the pancreatic tumor	Pancreatic cancer	*pIns-mycER^{TAM};RIP7-bcl-xL*	(Shchors et al., 2006; Soucek et al., 2007)
		$CD4^+$ T cells ↓	Regulates expression of CD47 and PDL1	T-ALL	MYC T-ALL s.c. transplanted cell line, *Eμ-tTA/tet-O-MYC, LAP-tTA/tet-O-MYC*	(Casey et al., 2016)
		Macrophages ↑ NK cells ↓	MYC drives expression of CCL9, which recruits macrophages, and IL-23, which limits NK recruitment	NSCLC	*Kras$^{LSL-G12D}$;Rosa26-LSL-MycER^{T2}*	(Kortlever et al., 2017)
		T, B cells ↓	MYC drives expression of IL-23, which excludes T and B cells from the tumor			
NOTCH	Amplification	Macrophages ↑	NOTCH activates CCL2 and IL-1β production by tumor cells thus increasing pro-tumoral monocytes and macrophages	Breast cancer	4T1, MDA-MB-231 cell lines and *RBPJκ^{IND}-MMTV;MMTV-PyMT*	(Shen et al., 2017)
		T cells ↓	NOTCH represses CEBP/β leading to impaired clearance of senescent cells and subsequent liver tumor development	Liver cancer	Hydrodynamic tail-vein injection of NRAS^{G12V}	(Hoare et al., 2016)
NRAS	Mutation	Neutrophils, monocytes, NK cells, macrophages, DCs ↑	NRAS mutation induces SASP, thus recruiting immune cells and $CD4^+$ T cell-mediated clearance of tumor cells.	Liver cancer	Hydrodynamic tail-vein injection of *Nras^{G12V}* and *Nras$^{G12V/D38A}$*	(Kang et al., 2011)
		$CD4^+$ T cells ↑	NRAS-induced senescent cells are cleared by $CD4^+$ T cells			
p53	Mutation	Myeloid cells ↑	Mutant p53 activates NFκB and thus drives cytokine production and inflammation-associated tumor progression	CRC	DSS-induced	(Cooks et al., 2013)
	Loss	Neutrophils, macrophages ↑	Potentially via dysregulation of NFκB	Lung cancer	*Kras$^{LSL-G12D/+}$* and *Kras$^{LSL-G12D/+}$;Trp53$^{F/F}$*	(Meylan et al., 2009)
		Macrophages ↑	Loss of p53 leads to an impaired intestinal epithelial barrier, thus triggering intestinal microflora-mediated immune activation via NFκB.	CRC	*Villin-creER^{T2};Trp53$^{F/F}$* and AOM-induced	(Schwitalla et al., 2013)
		Macrophages, monocytes, neutrophils ↑	STAT3-mediated establishment of an immunosuppressive microenvironment	PDAC	*Ptfa1-cre;Kras$^{LSL-G12D/+}$*, *Ptfa1-cre;Kras$^{LSL-G12D/+}$;p53$^{F/F}$* and *Ptfa1-cre;Kras$^{LSL-G12D/+}$;p53$^{R172H/+}$*	(Wörmann et al., 2016)
		Monocytes ↑	p53 transcriptionally regulates CXCL17, and loss of p53 leads to an increase of CXCL17, thus recruiting monocytes to the tumor	Prostate cancer	*Pb-cre;Pten $^{F/F}$;Trp53$^{F/F}$*	(Bezzi et al., 2018)

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Table 2. Continued

Gene	Genetic aberration	Consequence for intratumoral immune cells	Signaling involved	Tumor type	Tumor model	Reference
PRKCI	Amplification	NK cells ↓, $CD11b^+Gr1^+$ cells ↑	PRKCI activates YAP1, inducing TNFα to promote an immunosuppressive microenvironment	High-grade serous ovarian carcinoma	*Pax8-cre;tetO*$^{LSL\text{-}PRKCI}$; *Pten*$^{F/F}$;*Trp53*$^{F/F}$ with inducible loss of PRKCI and cell lines derived from these tumors	(Sarkar et al., 2017)
		$CD8^+$ T cells↓	PRKCI amplification induces immunosuppressive neutrophils, thus reducing $CD8^+$ T cells			
PTEN	Loss	$CD11b^+Gr1^+$ cells ↑	PTEN loss activates NFκB and thereby expression of CXCL1, G-CSF, IL-23	PDAC	*p48-Cre; Kras*$^{LSL\text{-}G12D}$; *Pten*$^{F/+}$	(Ying et al., 2011)
		$CD8^+$ T cells ↓	Loss of PTEN promotes resistance to T cell killing by inhibiting autophagy	Melanoma	Cell line inoculation models and *Tyr:CreER; Pten*$^{F/F}$;*Braf* $^{V600E/+}$	(Peng et al., 2016)
RAS	Mutation	$CD11b^+Gr1^+$ cells ↑	Via GM-CSF production by tumor cells	PDAC	*Kras*^{G12D} inoculation model	(Ancrile et al., 2007; Pylayeva-Gupta et al., 2012)
RB	Loss	Macrophages ↓	NA	SCLC	*Rb1*$^{F/F}$;*Trp53*$^{F/F}$	(Busch et al., 2016)
SMAD4	Loss	Neutrophils ↑	SMAD4 loss increases YAP1-mediated CXCL5 expression, thus driving immunosuppressive neutrophils.	Prostate cancer	*Pb-cre;Pten*$^{F/F}$;*Trp53*$^{F/F}$	(Wang et al., 2016)
		$CD8^+$ T cells, Tregs ↓	PRKCI amplification induces immunosuppressive neutrophils, thus reducing $CD8^+$ T cells and Tregs			
ZBTB7a	Loss	Neutrophils ↑	p53 transcriptionally regulates SOX-9, and loss of p53 leads to an increase of SOX-9, which in turn activates CXCL5, thus recruiting neutrophils to the tumor	Prostate cancer	*Pb-cre;Pten* $^{F/F}$;*Trp53*$^{F/F}$	(Bezzi et al., 2018)

Abbreviations: NA, Not assessed. SASP, Senescence-associated secretory phenotype. CRC, Colorectal cancer. NSCLC, Non-small cell lung cancer. PDAC, Pancreatic ductal adenocarcinoma. T-ALL, T cell acute lymphoblastic leukemia. SCLC, small cell lung carcinoma.
Listed here are the cancer cell-intrinsic genetic aberrations that result in a change in innate and adaptive immune contexture as demonstrated in genetically engineered mouse models.

MYC regulates many essential processes in the cell. In addition, recent studies revealed that it also has a strong hold on the tumor immune landscape (Figure 1, Table 2). Using the *RIP1-Tag2;TRE-Omomyc;CMV-rtTA* pancreatic β-cell cancer mouse model, in which treatment with doxycyclin induces expression of a dominant-negative MYC mutant, it was shown that inhibition of endogenous MYC in established islet tumors resulted in tumor regression, which was accompanied by a marked decrease in infiltrating macrophages and neutrophils (Sodir et al., 2011). This study illustrates that although MYC is not an oncogenic driver in this tumor model, its endogenous expression is crucial for tumor progression and has a profound effect on the inflammatory microenvironment. In another transgenic β-cell cancer mouse model carrying a switchable form of the MYC oncoprotein in the pancreas, forced expression of MYC in β-cells resulted in pancreatic cancer formation (Shchors et al., 2006). Importantly, Myc activation stimulated production of the potent pro-inflammatory cytokines CCL5 and interleukin-1β (IL-1β) by β cells, which facilitated tumor angiogenesis and recruitment of pro-tumoral mast cells to the tumor (Shchors et al., 2006; Soucek et al., 2007). These studies demonstrate that MYC can drive tumor progression at least in part through orchestrating pro-tumoral inflammatory conditions.

The effects of MYC signaling on the tumor microenvironment may not be limited to pancreatic cancer alone. In the *Eμ-tTA-TRE-Myc* mouse lymphoma model, inactivation of MYC in established tumors resulted in a marked decrease in intratumoral macrophages (Yetil et al., 2015). It would be of interest to assess whether the same MYC-controlled inflammatory mediators are involved in lymphoma and pancreatic cancer. Interestingly, upon additional loss of p19ARF, but not p53, MYC-dependent regulation of macrophage recruitment is not observed (Yetil et al., 2015), suggesting that the ability of MYC to control recruitment of immune cells to tumors can be counteracted by other aberrantly expressed genes. This is also illustrated by the observation that the spontaneous anti-tumor T and NK cell response in the *Eμ-MYC* lymphoma model could only be elicited when Bcl-2 was overexpressed, but not when p53 was deleted (Schuster et al., 2011). How p53 loss counteracts MYC activity in modulating the tumor microenvironment however remains a subject of future research.

MYC can also control the immune landscape of tumors by regulating expression of immune checkpoint molecules. In the *Eμ-tTA/tet-O-MYC* lymphoma model and cell lines with switchable *MYC* expression, MYC increased the expression of both PD-L1 and the "don't eat me" receptor CD47 on cancer cells by binding directly to their respective promoters (Casey et al., 2016). Exogenous overexpression of PD-L1 and CD47 on cancer cells limited the $CD4^+$ T cell and macrophage recruitment to the tumor. Moreover, MYC inactivation downregulated CD47 and PD-L1 expression and induced tumor regression, while exogenous overexpression of PD-L1 and CD47 in cancer cells enhanced disease progression (Casey et al., 2016). Although not experimentally proven, this study suggests that MYC may facilitate tumor immune escape by induction of immune checkpoints. Similarly, a MYC amplification-dependent T cell-poor environment has also been reported in human neuroblastomas, but in these tumors genomic amplification of N-MYC inversely correlated with *PD-L1* expression, possibly due to MYC-induced suppression of interferons and pro-inflammatory signaling pathways (Layer et al., 2017). These studies show that MYC activation in tumors can control immune checkpoint molecules and T cell influx, but the underlying mechanisms may differ between tumor types.

Another mechanism by which MYC regulates the immune phenotype of tumors was recently demonstrated in the $Kras^{G12D}$-driven lung adenocarcinoma model. Here, conditional MYC amplification resulted in a rapid decrease of intratumoral B, T, and NK cells, and an increase in macrophages (Kortlever et al., 2017). Mechanistically, MYC amplification led to increased expression of IL-23 by cancer cells, which inhibited B, T, and NK cell recruitment, and increased expression of CCL9, which recruited and activated macrophages in the tumor. These macrophages inhibited T cells, while also promoting angiogenesis. Interestingly, these tumors rapidly acquired dependency on MYC amplification, and MYC de-activation resulted in tumor regression in an NK cell-dependent fashion (Kortlever et al., 2017). These findings suggest that targeting MYC in tumors would be an attractive therapeutic strategy to unleash anti-tumor immunity. While MYC is as of yet not directly targetable, indirect therapeutic strategies emerge. One such strategy targets the epigenetic modulators DNA methyl transferases (DNMTs) and histone deacetylases (HDACs). Combined treatment of NSCLC mouse models with DNMT and HDAC inhibitors reduced MYC expression, increased CCL5 levels, decreased macrophage influx, and increased cytotoxic T cell influx and inhibited tumor growth (Topper et al., 2017). This study demonstrates that indirect targeting of MYC might prove therapeutically beneficial by limiting tumor growth and reversing immune evasion. However, this study did not formally exclude a direct effect of the epigenetic modulators on the immune system.

These studies show that in addition to the key role MYC has in tumor cell-intrinsic processes, this transcription factor can exert a wide variety of functions to modulate both the innate and the adaptive immune landscape of several tumor types. While MYC is not directly targetable, insights into these mechanisms open up new ways to target MYC-regulated signaling.

Other Genetic Determinants of the Tumor Immune Landscape

The effect of oncogenes and TSGs on the tumor immune landscape is not just limited to the abovementioned genes and pathways; several other genetic events and downstream immune effects have been described (Figure 1, Table 2). One example is the impact of the Ras oncogene on tumor-associated myeloid cells. Mutated Ras strongly induces expression of IL-6 and IL-8 in *in vitro* models (Ancrile et al., 2007; Sparmann and Bar-Sagi, 2004). These Ras-controlled cytokines have been reported to facilitate myeloid cell infiltration and tumor progression (Ancrile et al., 2007; Sparmann and Bar-Sagi, 2004). Furthermore, $Kras^{G12D}$-induced changes in cytokine expression resulted in accumulation of $CD11b^+Gr1^+$ immunosuppressive cells in a variety of tumor models, including pancreatic and lung cancer (Ji et al., 2006; Pylayeva-Gupta et al., 2012; Wislez et al., 2006). Ablation of one of the $Kras^{G12D}$-induced cytokines, GM-CSF, in tumor cells impaired immunosuppressive cells from entering pancreatic tumors and consequently resulted in an increase in $CD8^+$ T cells (Pylayeva-Gupta et al., 2012). These

studies demonstrate the causal relationship between Ras oncogenic signaling pathways, immune-stimulatory transcription programs and immune landscape. Another study revealed a role for adherence junction protein α-catenin in inflammatory signaling. In the *K14-Cre;α-catenin$^{F/F}$* mouse model for skin squamous cell carcinoma (SCC), loss of α-catenin activates NFκB and its downstream inflammatory target genes, such as IL-1β and IL-6, and stimulates SCC, thus again linking tumor-initiating oncogenic events with NF-κB-mediated immune signaling (Kobielak and Fuchs, 2006). Likewise, by comparing the *Pdx1-cre;Kras$^{LSL-G12D}$* and the *Pdx1-cre;Kras$^{LSL-G12D}$;Pten$^{+/F}$* mouse models for pancreatic cancer, it was demonstrated that loss of *Pten* resulted in increased activation of the NF-κB pathway, driving expression of several immune regulators by cancer cells, such as G-CSF, IL-23 and CXCL1 (Ying et al., 2011). *Pten* loss and the downstream NF-κB activation not only accelerated tumor progression, but also influenced the frequency of intratumoral neutrophils, monocytes, and Tregs (Ying et al., 2011). Another study showed a profound role for the *STK11/LKB1* tumor suppressor in NSCLC. Comparing *Kras$^{G12D/+}$* with *Kras$^{G12D/+}$;Lbk1$^{-/-}$* mice, it was found that loss of *Lkb1* resulted in increased IL-6 production, which resulted in higher intratumoral and systemic immunosuppressive neutrophil levels (Koyama et al., 2016). Indeed, blockade of IL-6 resulted in increased levels cytotoxic CD8^{+} T cells and tumor control (Koyama et al., 2016). Although not all of these studies elucidated the functional consequence of the altered immune landscape on tumor growth, they demonstrate that a wide variety of cancer-driving mutations can dictate the composition of the tumor microenvironment.

Collectively, studies pertaining to cancer cell-intrinsic pathways and immune contexture are gaining ground and have identified various cancer-driving genes that orchestrate diverse immune landscapes in the tumor. Thus far, many of these studies have been relatively biased and focused on a single genetic pathway in a single mouse tumor model. A more systematic assessment of immune cell populations in relation to tumor genotypes was recently performed in two studies. One compared four independent lung cancer GEMMs: *Ccsp-rtTA;TetO-Egfr^{L858R}*, *Rb1$^{F/F}$;Trp53$^{F/F}$*, *Kras$^{LSL-G12D/+}$* and *Kras$^{LSL-G12D/+}$;Trp53$^{F/F}$* models, representing molecularly distinct human SCLC and NSCLC subtypes (Busch et al., 2016). This approach revealed key differences in immune cell content between the different tumor genotypes, such as that *Egfr^{L858R}*-driven tumors showed lower frequencies and activation of CD8^{+} T cells compared to *Kras*-driven tumors, whereas NK cells in *Kras*-driven tumors, but not EGFR mutants, show downregulation of activation markers (Busch et al., 2016). A second study compared the *Pb-cre;Pten$^{F/F}$;Zbtb7a$^{F/F}$*, *Pb-cre;Pten$^{F/F}$;Trp53$^{F/F}$* and *Pb-cre;Pten$^{F/F}$;Pml$^{F/F}$* prostate cancer models and observed profound differences in composition of the tumor microenvironment (Bezzi et al., 2018). Mechanistic studies revealed distinct chemokine production by tumors controlled by loss of Zbtb7a, p53, or Pml and blockade of the respective signaling pathways impaired innate immune cell recruitment and tumor progression. These studies demonstrate the powerful potential of GEMMs in identifying the complex mechanisms that control the tumor microenvironment and potential for immunomodulatory therapeutic intervention based on genetic aberrations in the tumor. With the rapid developments in mouse model-generating techniques (Huijbers, 2017), future systematic approaches in GEMMs may increasingly reveal causal genotype-immunophenotype relationships, and its impact on tumor progression.

The Role of Oncogene-Induced Senescence in Promoting an Inflammatory Tumor Microenvironment

A cancer-cell-intrinsic pathway in which many of the above-mentioned cancer-driving genes are involved and that strongly influences the intratumoral immune landscape is cellular senescence. In a process called oncogene-induced senescence (OIS), precancerous cells undergo cell-cycle arrest upon activation of oncogenic signaling. Cellular senescence is a physiological program that can be activated in response to cellular stress and aging, leading to an essentially irreversible cell proliferation arrest (Muñoz-Espín and Serrano, 2014). Senescent cells can persist and actively secrete cytokines and other inflammatory and growth-promoting factors, a process called the senescence-associated secretory phenotype (SASP) (Pérez-Mancera et al., 2014). Through their SASP, senescent cells can exert a significant, and sometimes opposing, impact on the immune landscape of the tumor. SASP can lead to immune-mediated clearance of pre-malignant cells, or via stimulation of chronic inflammation promote tumor progression. Below we discuss how oncogenes and TSGs, via SASP activation, shape the inflammatory microenvironment.

Several oncogenes and TSGs have been linked with SASP activation (Figure 2). The p53 pathway plays an important role in the induction of OIS. This was demonstrated by the induction of senescence and tumor clearance upon doxycyclin-mediated activation of p53 in a *Hras^{G12V};TRE.shp53* inoculation model for liver cancer (Xue et al., 2007). Activation of p53 did not lead to tumor cell death in a cell-autonomous manner, but rather neutrophils, macrophages and NK cells were recruited to these tumors by activated SASP and removed the senescent cells (Xue et al., 2007). Indeed, maintenance of WT p53 was a prerequisite of senescence induction, as also observed in other tumor models (Cooks et al., 2013; Pribluda et al., 2013). Because NF-κB is a key transcription factor in SASP activation (Chien et al., 2011), the regulation of NF-κB by the p53 pathway might play an important role in SASP regulation. In colorectal tumor models, Wnt signaling can also regulate SASP induction. *Villin-creER^{T2};CKI$\alpha^{F/F}$* mice, which display hyper-activated Wnt signaling due to loss of *CKIα*, exhibit growth arrest of colorectal tumors and induction of senescence, paired with an inflammatory response (Pribluda et al., 2013). SASP is maintained upon additional p53 deletion in this model, however, it dissociates from growth arrest while the inflammatory response continues, resulting in inflammation-accelerated tumorigenesis (Pribluda et al., 2013). These findings illustrate that depending on the genetic makeup of cancer cells, the senescence-associated inflammatory response can result in two opposing outcomes: tumor inhibition or tumor promotion. In addition to p53 and Wnt, mTOR signaling was shown to induce SASP in CRC and prostate cancer cells *in vitro* (Laberge et al., 2015). mTOR inhibition by rapamycin decreased mTOR-induced SASP and decreased influx of macrophages, T, B, and NK cells into inoculated Nras^{G12V} mutant liver tumors (Herranz et al., 2015). These studies suggest

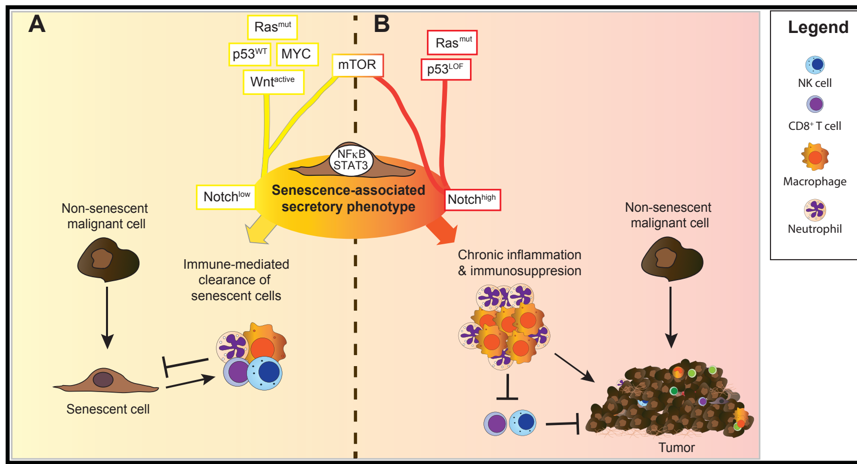

Figure 2. Relationship between Genetic Events in Cancer Cells, the Dynamic Aspects of SASP and the Immune System

(A) Oncogene-induced senescence (OIS), in combination with WT p53, activated MYC, low Notch signaling, active Wnt signaling, activated RAS, or active mTOR signaling induces a senescence-associated secretory phenotype (SASP) that leads to the recruitment and activation of macrophages, neutrophils, NK cells, and CD8$^+$ T cells that clear senescent cells and thus limit tumorigenesis.

(B) Loss or loss-of-function mutations in p53, or activated RAS, Notch, or mTOR signaling can lead to an alternative SASP that also attributes to a chronic inflammatory state that establishes an immunosuppressive tumor microenvironment. Immunosuppressive macrophages and neutrophils limit NK and CD8$^+$ T cell-mediated anti-tumor response and thus promote tumorigenesis. NF-κB and STAT3 signaling in senescent cells is key in SASP induction.

that targeted therapies, such as rapamycin, may reduce tumor-induced inflammation, but potentially also reduce senescent tumor cell clearance by infiltrating immune cells, thus demonstrating the complexity of targeting SASP. Nonetheless, these studies reveal the essential role of oncogenes and TSGs in SASP induction and the potential of targeting these genes to revert tumor-promoting SASP.

The composition of SASP mediators secreted by senescence cells is dynamic and experimental evidence points toward NOTCH1 as one of the master regulators controlling this SASP diversity. In Nras^{G12V} mutant tumor models, Nras^{G12V}-induced senescence was accompanied by fluctuations in endogenous Notch expression levels (Hoare et al., 2016). Ectopic expression of active Notch in an Nras^{G12V}-dependent oncogene-induced senescence liver model increased cancer progression in a non-cell-autonomous fashion (Hoare et al., 2016). In this model, Notch levels determined the composition of the SASP and subsequent immune function. Notch inhibited lymphocyte-mediated clearance of senescent cells through repression of C/EBPβ. Reversely, inhibition of Notch during senescence led to an increase of lymphocyte-mediated senescent cell clearance (Hoare et al., 2016). This Notch-dependent cytokine production and shaping of the immune phenotype of tumors was also demonstrated in breast cancer, where tumor-intrinsic Notch signaling increased monocyte and macrophage accumulation by increasing expression of IL-1β and CCL2 (Shen et al., 2017). These studies demonstrate that immune cell influx can be strongly influenced by SASP, but also that the activity of cancer cell-intrinsic genes play important roles in determining the spectrum of inflammatory mediators produced within the tumor. Indeed, in the *Ptf1a-cre;Kras$^{LSL-G12D/+}$* mouse model for pancreatic cancer, genetic deletion of *RelA*, the gene that encodes the NF-κB subunit p65, abrogated senescence and SASP, thus enhancing progression of pancreatic tumors (Lesina et al., 2016). While reducing SASP, *RelA* deletion led to a marked increase in immunosuppressive cells and decreased T cell activation in the pancreata of these mice (Lesina et al., 2016). Therefore, in these tumors, the cancer-immune cell crosstalk is not limited to SASP.

The infiltrating immune cells can also impact senescence itself. In *Pten*-induced senescent prostate tumors, CD11b$^+$Gr-1$^+$ cells can actively counteract SASP by producing IL-1 receptor antagonist (Di Mitri et al., 2014). Additionally, senescence programs in tumor-associated stromal cells also impact tumorigenesis through modulation of immune responses. In a carbon tetrachloride (CCl_4)-induced liver fibrosis model, p53 activity in hepatic stellate cells (HSCs) limits fibrosis and cirrhosis, and reduced liver tumorigenesis in mice treated with CCl_4 and diethylnitrosamine (DEN) (Lujambio et al., 2013). Here, wild-type p53 cooperated with NF-κB to induce senescence and SASP in HSCs, which induced a tumor-inhibiting phenotype in macrophages. Loss of p53 in stromal HSCs changed their secretome, induced the polarization of macrophages toward a tumor-promoting phenotype and accelerated inflammation-induced hepatocellular carcinoma (Lujambio et al., 2013), indicating that also stromal cell-intrinsic p53 controls tumorigenesis via modulation of the immune system.

Collectively, depending on the tumor type and oncogenic wiring, the activated SASP-related genes and downstream inflammatory profile may differ, resulting in a wide spectrum of immune responses that range from tumor-promoting chronic inflammatory responses to immune-mediated clearance of cancer cells (Figure 2). Deeper mechanistic insights into the causal relationship between genetic events in cancer cells and the dynamic aspects of SASP may open new avenues for therapeutic intervention. Indeed, this is exemplified by a study showing that the efficacy of docetaxel could be enhanced by pharmacologically targeting *Pten*-loss-induced SASP in a transgenic prostate tumor model (Toso et al., 2014). Important to note however, is that senescent cells are not the only cells actively secreting inflammatory mediators in the tumor, and the cytokine milieu and its net effect on the immune landscape is not only determined by SASP. Therefore, it is of key importance to delineate how the tumor-promoting aspects of SASP can be reverted, while enhancing the tumor-limiting aspects.

Mechanisms of Cancer-Cell-Intrinsic Regulation of Parameters of the Cancer Immunity Cycle and Immune Checkpoint Blockade Response

As discussed above, the mutational load of tumors is one of the determinants linked with responsiveness to immune checkpoint inhibition. The expectation is that many other parameters,

including the activation of certain oncogenes or inactivation of TSGs, are associated with therapeutic benefit as well, and that they may differ per tumor (sub)type. As of yet, preclinical studies focused on unlocking the relationship between tumor genetics and response to immunotherapy are still relatively limited, however, the concept is emerging that genetic events in cancer cells dictate various aspects of the tumor-immunity cycle (Chen and Mellman, 2013), such as activation of immunosuppressive myeloid cells, induction of immune checkpoint molecule expression, regulation of DC activation and T cell priming, and induction of tumor resistance to T cell attack.

One such genetic event is mutation in the serine/threonine-protein kinase ATR. ATR is a DNA damage sensor and is frequently mutated in melanoma. It has been reported to influence important parameters of immunotherapy response, such as intratumoral T cell influx and expression of immune checkpoints. Transgenic expression of an ATR LOF mutant in the *Tyr::CreERT2; Braf^{V600E};Pten$^{F/F}$* model for melanoma diminished T cell influx in the tumor, while increasing B cells and macrophages (Chen et al., 2017). This was associated with an increase in expression of *Arginase 1*, *CD206*, and *PD-L1* in the tumor, suggesting a more T cell suppressed environment. Cyclin-dependent kinases (CDKs)—essential regulators of the cell cycle—have also been shown to be involved in immune checkpoint regulation. In medulloblastoma (MB) cell line inoculation models, the anti-tumor function of CD4$^+$ T cells depends on disruption of CDK5 in MB cells (Dorand et al., 2016). In this model, CDK5 is required for PD-L1 expression by MB cells, as CDK5 is a repressor of IRF2 and IRF2BP2, that both regulate IFN-γ-mediated PD-L1 expression (Dorand et al., 2016). Additionally, it was recently shown that the activating Ras^{G12V} mutation can cause stabilization of *PD-L1* mRNA via activation of MEK (Coelho et al., 2017). However, the functional relevance of these changes for immunotherapy and disease progression in relation to ATR, CDK5, and RAS remains unaddressed in these studies.

Another mechanism by which tumor cells may regulate immunotherapy response is via establishment of an immunosuppressive microenvironment. Overexpression of PRCKI, a protein kinase, is frequently observed in a variety of cancer types, including high-grade serous ovarian carcinoma (Sarkar et al., 2017). Upon conditional overexpression of PRKCI in the *Pax8-rtta;TetO-Cre;Trp53$^{F/F}$;Pten$^{F/F}$* mouse model for ovarian cancer, tumors upregulated TNF-α, as a result of which tumors were strongly infiltrated by immunosuppressive neutrophils, thus decreasing CD8$^+$ T cell influx (Sarkar et al., 2017). This TNF-α-mediated neutrophil recruitment was dependent on PRKCI-induced YAP1—a key transcriptional regulator and oncogene—signaling in cancer cells (Sarkar et al., 2017). Likewise, by comparing *Pb-cre4;Pten$^{F/F}$* with *Pb-cre4;Pten$^{F/F}$;Smad4$^{F/F}$* prostate cancer mouse models, a strong YAP1-dependent influx of neutrophils was observed upon cancer cell-intrinsic Smad4 loss (Wang et al., 2016). Here, Smad4 loss caused YAP1-mediated upregulation of CXCL5 in tumor cells. This in turn recruited CXCR2$^+$ neutrophils, which suppressed the CD8$^+$ T cell response to the tumor (Wang et al., 2016). These studies show that Smad4 and PRCKI both function as inducers of immunosuppression via cancer cell-intrinsic YAP signaling and that YAP inhibitors—which are currently in preclinical development—may prove beneficial to alleviate T cell suppression. Collectively, these studies show that oncogenic pathway activation can significantly impact on parameters of the cancer-immunity cycle. However, the functional consequences of these genetic changes on immunotherapy response have not been addressed in these studies. Focal Adhesion Kinase (FAK) activity in cancer cells has also been identified as an important regulator of immunosuppression in the tumor microenvironment, and its impact on immunotherapy efficacy has been addressed experimentally. FAK amplification was observed in the *p48-Cre;Kras$^{LSL-G12D}$; Trp53$^{F/+}$* model for PDAC, and therapeutic targeting of FAK improved survival by alleviating the immunosuppressive microenvironment, mainly by reducing macrophages, monocytes, and neutrophils in the tumor (Jiang et al., 2016). This held true for cancer-cell-specific ablation of FAK, indicating that immune cell changes occur via FAK targeting in cancer cells. Importantly, inhibition of FAK synergized with anti-CTLA-4/anti-PD-1 combination immunotherapy (Jiang et al., 2016), indicating that interference with this cancer cell-intrinsic signaling pathway renders tumors sensitive to immunotherapy.

DC activation and T cell priming can also be influenced by cancer cell-intrinsic signaling pathways. Using the *Braf^{V600E}; Pten$^{-/-}$;CAT-STA* mouse model for melanoma, which expresses constitutively active β-catenin, it was revealed that β-catenin signaling prevented expression of CCL4 by cancer cells, resulting in suppression of recruitment of CD103$^+$ DCs and impaired priming and intratumoral accumulation of T cells (Spranger et al., 2015). As a consequence, β-catenin-active tumors failed to respond to anti-CTLA-4/anti-PD-1 treatment. In line with these data, active WNT/β-catenin signaling in human metastatic melanomas correlated with absence of a T cell gene expression signature (Spranger et al., 2015). This study highlights the importance of cancer cell-intrinsic WNT/β-catenin signaling in immune evasion of tumors, and suggests that targeting the WNT pathway may improve the therapeutic benefit of immune checkpoint inhibition in tumors with active β-catenin signaling.

Some oncogenes and TSGs have been demonstrated to regulate immune checkpoint molecule expression in a cell-autonomous fashion, and thus influence response to immunotherapy. In EGFR-driven lung cancer mouse models, EGFR mutation caused rapid induction of an immunosuppressive tumor microenvironment (Akbay et al., 2013). The EGFR mutant lung tumors displayed increased expression of immune checkpoint molecules such as PD-1 and PD-L1, which led to an increased sensitivity to anti-PD-1 monotherapy in these tumor-bearing mice. In line with these pre-clinical findings, EGFR pathway activating mutations in human lung tumors, and not the other prevalent driver mutation KRAS^{G12V}, correlated with PD-L1 expression (Akbay et al., 2013). Intriguingly, another study reported KRAS mutant lung tumors in patients treated with anti-PD-1 to have higher PD-L1 levels relative to EGFR mutated tumors (Garon et al., 2015), potentially mediated by KRAS-induced stabilization of PD-L1 (Coelho et al., 2017). The different levels of PD-L1 regulation by mutated oncogenes and the underlying mechanisms will therefore be an important topic of future research.

Similarly, PTEN status is implicated in immunotherapy response due to its ability to render cancer cells resistant to T cell attack. In a cohort of melanoma patients, PTEN loss correlated with low TIL influx and poor response to anti-PD-1 therapy

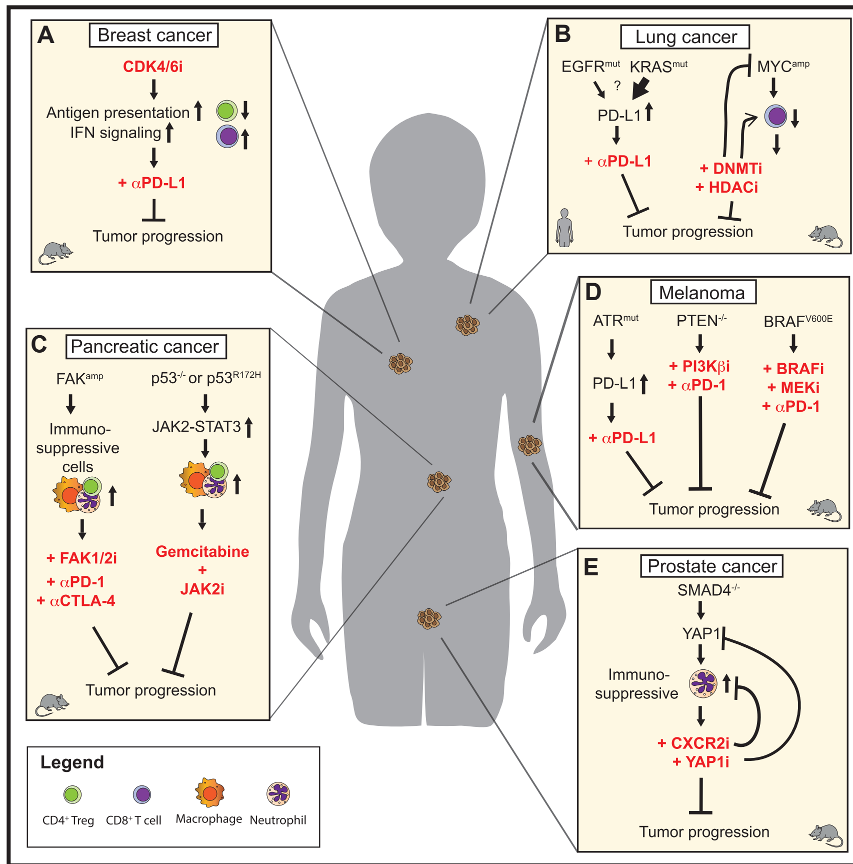

Figure 3. How to Exploit the Genetic Makeup of Individual Tumors to Allow for Patient-Specific Immune-Based Therapeutic Interventions

Maximizing therapeutic efficacy by rational selection of targeted drugs and immunomodulatory compounds based on the genetics of the tumor. Examples depicted here are mainly based on pre-clinical intervention studies, with therapeutic modalities highlighted in red. For every example a mouse or human symbol is used to depict what is based on clinical or pre-clinical evidence.

(A) In breast cancer, CDK4/6 inhibition increases antigen presentation, interferon signaling, and $CD8^+$ T cell levels, while decreasing Tregs in the tumor. Combined with anti-PD-L1 treatment, this leads to a marked tumor regression (Goel et al., 2017).

(B) In EGFR mutant lung cancer, PD-L1 has been described to be upregulated, increasing the sensitivity to anti-PD-L1 therapy (Akbay et al., 2013). KRAS mutation in lung cancer can also drive PD-L1 expression, to a higher extent than EGFR mutation (Garon et al., 2015). In MYC-driven lung tumors, combined inhibitors against HDAC and DNMT both target MYC and $CD8^+$ T cells, thus limiting tumor growth (Topper et al., 2017).

(C) Pancreatic tumors with FAK amplification show an accumulation of immunosuppressive cells in the tumor. FAK1/2 inhibitors alleviate this, and combined with anti-PD-1 and anti-CTLA-4 treatment limit tumor progression (Jiang et al., 2016). Pancreatic tumors with p53 loss or mutation establish an immunosuppressive microenvironment by JAK-STAT signaling. Targeting JAK2 in combination with gemcitabine reduces tumor burden (Wörmann et al., 2016).

(D) In melanoma, ATR loss-of-function mutation increases PD-L1 and thereby potentially sensitizes these tumors to anti-PD-L1 treatment. In PTEN null melanomas, the resulting activated AKT signaling can be reduced by PI3Kβ inhibitors, which in combination with anti-PD-1 limits tumor growth (Peng et al., 2016). Combining MEK and BRAF inhibitors in $BRAF^{V600E}$ mutant melanoma also synergize with anti-PD-1 treatment (Hu-Lieskovan et al., 2015).

(E) In prostate tumors with loss of SMAD4, YAP1-mediated immunosuppressive neutrophil recruitment can be counteracted by YAP1 inhibitors or anti-CXCR2 treatment (Wang et al., 2016).

(Peng et al., 2016). Using xenograft mouse models for melanoma, it was shown that PTEN loss in cancer cells reduced T cell influx, and resulted in reduced autophagy, leading to resistance to T cell-mediated killing (Peng et al., 2016). Treating PTEN null tumors with a PI3Kβ inhibitor, thus reducing the dysregulated AKT activity in these tumors, improved response to anti-PD-1 therapy, highlighting a potential therapeutic approach for PTEN null melanoma in controlling resistance to anti-PD-1 therapy.

Altogether, these studies show that aberrant signaling pathways in cancer cells can impact the anti-cancer immune response and the response to immune checkpoint inhibition (Figure 3). One aspect that needs to be taken into account when using GEMMs to model human cancers with high mutational load, is that the mutational load in transgenic mice may not correspond to that of the human tumors, due to the strong driver mutations engineered in these mice. This could be overcome by for example exposing early melanoma lesions to UV irradiation, or early lung lesions to carcinogens. The drawback however, is that this may not result in clonal antigens and the mutational spectrum may be highly variable from one mouse to the next. Alternatively, transgenic models that are prone to generate high mutational load tumors can be used, such as those with mutations in DNA repair machinery, or mutations can be engineered in a tissue-specific manner. This would allow for physiological modeling and therefore correct assessment of pre-clinical immunotherapeutic strategies in an immunocompetent setting.

Targeting Genetic Pathways to Unleash Anti-Tumor Immunity

One major theme that emerges from the aforementioned studies is that many targeted therapies, specific for hyperactive signaling pathways, are likely to also exert a major impact on the immune contexture of tumors. Most targeted drugs initially induce very strong anti-cancer effects in patients, however, the rate of durable clinical responses is disappointingly low (Groenendijk and Bernards, 2014). Given the previously unrecognized impact of these targeted drugs on the immune landscape of tumors, the question arises whether we can rationally induce a favorable immune environment in tumors or even sensitize tumors to immunomodulatory drugs by selective usage of targeted therapy. In this regard, we can learn from the growing number of pre-clinical studies that have addressed the impact of targeted drugs on the immune microenvironment of tumors and their response to immunotherapy. For example, as described above,

BRAF mutant thyroid tumors are characterized by infiltration of immunosuppressive cells (Charoentong et al., 2017), raising the question of whether inhibition of mutant BRAF in thyroid cancer would induce a more favorable immune contexture. Indeed, combined targeting of $BRAF^{V600E}$ and SRC increased influx of $CD8^+$ T cells, B cells, and macrophages and reduced tumor growth in an orthotopic inoculation model for anaplastic thyroid cancer (Vanden Borre et al., 2014). Also in patients with $BRAF^{V600E}$ mutated metastatic melanoma, BRAF inhibition with vemurafenib enhanced melanoma antigen presentation by cancer cells, increased cytotoxic T cell influx, and decreased immunosuppression (Frederick et al., 2013). This is in line with findings in $BRAF^{V600E}$ melanoma mouse models in which BRAF inhibition improved adoptive T cell therapy (Koya et al., 2012) and BRAF inhibition combined with MEK inhibition synergized with anti-PD-1 treatment (Hu-Lieskovan et al., 2015). These studies indicate that therapeutic targeting of cancer cell-intrinsic oncogenic driver mutations can be exploited to induce a favorable immune environment and thus sensitize tumors to cancer immunotherapy.

Other targeted therapies have also been reported to exert strong effects on the cancer-immune cell crosstalk. For example, CDK4/6 inhibitors were originally designed to selectively inhibit cell-cycle progression, but emerging experimental evidence reveals that part of the therapeutic benefit of these inhibitors lies in their anti-tumor immunity promoting capacity. In the *MMTV-rtTA/tetO-HER2* mouse model for breast cancer, treatment with the CDK4/6 inhibitor abemaciclib leads to tumor regression by inducing anti-tumor immunity (Goel et al., 2017). *In vitro* studies revealed that CDK4/6 inhibition increased antigen presentation and production of type III interferons by cancer cells, which induced $CD8^+$ T cell proliferation and activation (Goel et al., 2017). Simultaneously, CDK4/6 inhibition reduced systemic and intra-tumoral regulatory T cell numbers, which occurred independent of the presence of a tumor. Both the effect of the CDK4/6 inhibitor on antigen presentation by cancer cells and the impact on regulatory T cells was dependent on inhibition of the RB-E2F-DNMT1 axis (Goel et al., 2017). Importantly, by modulating the immune microenvironment, anti-CDK4/6 treatment improved response to anti-PDL1 in *MMTV-rtTA/tetO-HER2* mice (Goel et al., 2017). Also, in an *in vitro* small molecule screen, CDK4/6 inhibitors were identified to directly enhance T cell activity. Mechanistically, CDK4/6 inhibition resulted in de-repression of NFAT activity in T cells, resulting in increased T cell accumulation in lung tumors of $Kras^{LSL\text{-}G12D}$;$Trp53^{F/F}$ mice, which synergized with immune checkpoint inhibition (Deng et al., 2018). These two studies illustrate that CDK4/6 inhibitors, which were originally developed to induce cell-cycle arrest in cancer cells, work in part by counteracting tumor immune evasion. This is a result of combined targeting of cancer cell-intrinsic pathways, changing parameters of the cancer-immunity cycle, and direct targeting of T cells.

Targeted therapies have also been reported to affect the abundance and function of myeloid cells in tumor-bearing hosts, since the signaling pathways targeted by these drugs also play functional roles in the immune system (Muñoz-Fontela et al., 2016). For example, neutrophils in the *Hgf-Cdk4*^{R24C} model for melanoma and cell line inoculation models impair the anti-tumor $CD8^+$ T cell response (Glodde et al., 2017). In this study, cMET inhibition enhanced the efficacy of adoptive cell transfer and immune checkpoint therapies by direct targeting of immunosuppressive neutrophils that express the cMET receptor (Glodde et al., 2017). However, targeting cMET-expressing neutrophils in another study promotes tumor progression (Finisguerra et al., 2015), highlighting the complex model-dependent and dual role of neutrophils in cancer biology (Coffelt et al., 2016). Likewise, it has been reported that the depletion of immunosuppressive $CD11b^+Gr1^+$ cells as a bystander effect of other targeted therapies, for example by ITK/BTK-inhibitor ibrutinib, benefits the response to immunotherapies in cell line inoculation models for breast cancer and melanoma (Sagiv-Barfi et al., 2015; Stiff et al., 2016). Ibrutinib can also reprogram macrophages, relieve immunosuppression, and facilitate $CD8^+$ cytotoxicity in PDAC-bearing mice (Gunderson et al., 2016). These studies highlight that targeted drugs can impact the immune contexture of tumors via their working mechanism on cancer cells, which indirectly changes the immune landscape, and via their direct effect on immune cells. Insights into the complexity of the combined effect of these targeted drugs on the cancer cells and tumor microenvironment will help us to maximize the therapeutic benefit of targeted drugs in combination with immunomodulatory strategies (Figure 3).

Conclusions and Future Directions

From the studies discussed in this review it has become clear that activation of oncogenes or loss of TSGs not only exert an intrinsic influence on the fate of cancer cells, but can have profound effects on tumor-host interactions. Commonly mutated genes that lie at the basis of tumorigenesis can actively participate in recruitment, activation, or dampening of the immune system. This could in part explain the heterogeneity between and within tumor types in immune infiltration and activation. From a clinical perspective, these insights will help identify patients that would or would not benefit from immunomodulation. Moreover, identifying the mechanisms underlying the causal relationship between the genetic makeup of tumors and their immune landscape may identify novel targets for anti-cancer immunomodulatory therapies. The studies presented here likely only reveal the tip of the iceberg. Most studies focus on one particular oncogene or TSG, and the majority of research is concentrated on the primary tumor. This leaves the effect on the systemic immune milieu and metastasis largely unaddressed. With increasingly sophisticated methodologies to generate mouse models that closely mimic the genetics and biology of human cancer and approaches to analyze tumors in depth, it will be possible to screen for a multitude of genetic and epigenetic alterations and their effect on the immune system. *In vivo* genetic manipulation will be key to delineate the spatiotemporal regulation of the tumor immune landscape, both in the primary as well as the metastatic lesion. This knowledge will help maximize the potential of immunomodulatory therapeutics for cancer patients and provide rationale for personalized combination therapies based on the genetic profile of tumors.

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REFERENCES

Adams, J.M., Harris, A.W., Pinkert, C.A., Corcoran, L.M., Alexander, W.S., Cory, S., Palmiter, R.D., and Brinster, R.L. (1985). The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. Nature *318*, 533–538.

Akbay, E.A., Koyama, S., Carretero, J., Altabef, A., Tchaicha, J.H., Christensen, C.L., Mikse, O.R., Cherniack, A.D., Beauchamp, E.M., Pugh, T.J., et al. (2013). Activation of the PD-1 pathway contributes to immune escape in EGFR-driven lung tumors. Cancer Discov. *3*, 1355–1363.

Ancrile, B., Lim, K.H., and Counter, C.M. (2007). Oncogenic Ras-induced secretion of IL6 is required for tumorigenesis. Genes Dev. *21*, 1714–1719.

Bailey, P., Chang, D.K., Nones, K., Johns, A.L., Patch, A.M., Gingras, M.C., Miller, D.K., Christ, A.N., Bruxner, T.J., Quinn, M.C., et al.; Australian Pancreatic Cancer Genome Initiative (2016). Genomic analyses identify molecular subtypes of pancreatic cancer. Nature *531*, 47–52.

Balkwill, F., and Mantovani, A. (2001). Inflammation and cancer: back to Virchow? Lancet *357*, 539–545.

Balli, D., Rech, A.J., Stanger, B.Z., and Vonderheide, R.H. (2017). Immune Cytolytic Activity Stratifies Molecular Subsets of Human Pancreatic Cancer. Clin. Cancer Res. *23*, 3129–3138.

Becht, E., de Reyniès, A., Giraldo, N.A., Pilati, C., Buttard, B., Lacroix, L., Selves, J., Sautès-Fridman, C., Laurent-Puig, P., and Fridman, W.H. (2016). Immune and Stromal Classification of Colorectal Cancer Is Associated with Molecular Subtypes and Relevant for Precision Immunotherapy. Clin. Cancer Res. *22*, 4057–4066.

Beroukhim, R., Mermel, C.H., Porter, D., Wei, G., Raychaudhuri, S., Donovan, J., Barretina, J., Boehm, J.S., Dobson, J., Urashima, M., et al. (2010). The landscape of somatic copy-number alteration across human cancers. Nature *463*, 899–905.

Bezzi, M., Seitzer, N., Ishikawa, T., Reschke, M., Chen, M., Wang, G., Mitchell, C., Ng, C., Katon, J., Lunardi, A., et al. (2018). Diverse genetic-driven immune landscapes dictate tumor progression through distinct mechanisms. Nat. Med. *24*, 165–175.

Blaisdell, A., Crequer, A., Columbus, D., Daikoku, T., Mittal, K., Dey, S.K., and Erlebacher, A. (2015). Neutrophils Oppose Uterine Epithelial Carcinogenesis via Debridement of Hypoxic Tumor Cells. Cancer Cell *28*, 785–799.

Boveri, T. (1914). Zur Frage der Entstehung Maligner Tumoren. Gustav Fischer *1914*, 1–64.

Brandetti, E., Veneziani, I., Melaiu, O., Pezzolo, A., Castellano, A., Boldrini, R., Ferretti, E., Fruci, D., Moretta, L., Pistoia, V., et al. (2017). MYCN is an immunosuppressive oncogene dampening the expression of ligands for NK-cell-activating receptors in human high-risk neuroblastoma. OncoImmunology *6*, e1316439.

Busch, S.E., Hanke, M.L., Kargl, J., Metz, H.E., MacPherson, D., and Houghton, A.M. (2016). Lung Cancer Subtypes Generate Unique Immune Responses. J. Immunol. *197*, 4493–4503.

Casey, S.C., Tong, L., Li, Y., Do, R., Walz, S., Fitzgerald, K.N., Gouw, A.M., Baylot, V., Gütgemann, I., Eilers, M., and Felsher, D.W. (2016). MYC regulates the antitumor immune response through CD47 and PD-L1. Science *352*, 227–231.

Cha, Y.J., Kim, H.R., Lee, C.Y., Cho, B.C., and Shim, H.S. (2016). Clinicopathological and prognostic significance of programmed cell death ligand-1 expression in lung adenocarcinoma and its relationship with p53 status. Lung Cancer *97*, 73–80.

Charoentong, P., Finotello, F., Angelova, M., Mayer, C., Efremova, M., Rieder, D., Hackl, H., and Trajanoski, Z. (2017). Pan-cancer Immunogenomic Analyses Reveal Genotype-Immunophenotype Relationships and Predictors of Response to Checkpoint Blockade. Cell Rep. *18*, 248–262.

Chen, D.S., and Mellman, I. (2013). Oncology meets immunology: the cancer-immunity cycle. Immunity *39*, 1–10.

Chen, Z., Chen, X., Zhou, E., Chen, G., Qian, K., Wu, X., Miao, X., and Tang, Z. (2014). Intratumoral $CD8^+$ cytotoxic lymphocyte is a favorable prognostic marker in node-negative breast cancer. PLoS ONE *9*, e95475.

Chen, C.F., Ruiz-Vega, R., Vasudeva, P., Espitia, F., Krasieva, T.B., de Feraudy, S., Tromberg, B.J., Huang, S., Garner, C.P., Wu, J., et al. (2017). ATR Mutations Promote the Growth of Melanoma Tumors by Modulating the Immune Microenvironment. Cell Rep. *18*, 2331–2342.

Chien, Y., Scuoppo, C., Wang, X., Fang, X., Balgley, B., Bolden, J.E., Premsrirut, P., Luo, W., Chicas, A., Lee, C.S., et al. (2011). Control of the senescence-associated secretory phenotype by NF-κB promotes senescence and enhances chemosensitivity. Genes Dev. *25*, 2125–2136.

Coelho, M.A., de Carné Trécesson, S., Rana, S., Zecchin, D., Moore, C., Molina-Arcas, M., East, P., Spencer-Dene, B., Nye, E., Barnouin, K., et al. (2017). Oncogenic RAS Signaling Promotes Tumor Immunoresistance by Stabilizing PD-L1 mRNA. Immunity *47*, 1083–1099.e6.

Coffelt, S.B., Wellenstein, M.D., and de Visser, K.E. (2016). Neutrophils in cancer: neutral no more. Nat. Rev. Cancer *16*, 431–446.

Coley, W.B. (1893). The treatment of malignant tumors by repeated inoculations of erysipelas: with a report of ten original cases. Am. J. Med. Sci. *105*, 487–511.

Cooks, T., Pateras, I.S., Tarcic, O., Solomon, H., Schetter, A.J., Wilder, S., Lozano, G., Pikarsky, E., Forshew, T., Rosenfeld, N., et al. (2013). Mutant p53 prolongs NF-κB activation and promotes chronic inflammation and inflammation-associated colorectal cancer. Cancer Cell *23*, 634–646.

Cooks, T., Harris, C.C., and Oren, M. (2014). Caught in the cross fire: p53 in inflammation. Carcinogenesis *35*, 1680–1690.

Debebe, A., Medina, V., Chen, C.Y., Mahajan, I.M., Jia, C., Fu, D., He, L., Zeng, N., Stiles, B.W., Chen, C.L., et al. (2017). Wnt/β-catenin activation and macrophage induction during liver cancer development following steatosis. Oncogene *36*, 6020–6029.

Decker, T., Fischer, G., Bücke, W., Bücke, P., Stotz, F., Grüneberger, A., Gropp-Meier, M., Wiedemann, G., Pfeiffer, C., Peschel, C., and Götze, K. (2012). Increased number of regulatory T cells (T-regs) in the peripheral blood of patients with Her-2/neu-positive early breast cancer. J. Cancer Res. Clin. Oncol. *138*, 1945–1950.

Deng, J., Wang, E.S., Jenkins, R.W., Li, S., Dries, R., Yates, K., Chhabra, S., Huang, W., Liu, H., Aref, A.R., et al. (2018). CDK4/6 Inhibition Augments Antitumor Immunity by Enhancing T-cell Activation. Cancer Discov. *8*, 216–233.

Di Mitri, D., Toso, A., Chen, J.J., Sarti, M., Pinton, S., Jost, T.R., D'Antuono, R., Montani, E., Garcia-Escudero, R., Guccini, I., et al. (2014). Tumour-infiltrating Gr-1+ myeloid cells antagonize senescence in cancer. Nature *515*, 134–137.

Diakos, C.I., Charles, K.A., McMillan, D.C., and Clarke, S.J. (2014). Cancer-related inflammation and treatment effectiveness. Lancet Oncol. *15*, e493–e503.

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Jr., Butel, J.S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature *356*, 215–221.

Dorand, R.D., Nthale, J., Myers, J.T., Barkauskas, D.S., Avril, S., Chirieleison, S.M., Pareek, T.K., Abbott, D.W., Stearns, D.S., Letterio, J.J., et al. (2016). Cdk5 disruption attenuates tumor PD-L1 expression and promotes antitumor immunity. Science *353*, 399–403.

Doucette, T., Rao, G., Rao, A., Shen, L., Aldape, K., Wei, J., Dziurzynski, K., Gilbert, M., and Heimberger, A.B. (2013). Immune heterogeneity of glioblastoma subtypes: extrapolation from the cancer genome atlas. Cancer Immunol. Res. *1*, 112–122.

Duesberg, P.H., and Vogt, P.K. (1970). Differences between the ribonucleic acids of transforming and nontransforming avian tumor viruses. Proc. Natl. Acad. Sci. USA *67*, 1673–1680.

Finisguerra, V., Di Conza, G., Di Matteo, M., Serneels, J., Costa, S., Thompson, A.A., Wauters, E., Walmsley, S., Prenen, H., Granot, Z., et al. (2015). MET is required for the recruitment of anti-tumoural neutrophils. Nature *522*, 349–353.

Fisher, G.H., Wellen, S.L., Klimstra, D., Lenczowski, J.M., Tichelaar, J.W., Lizak, M.J., Whitsett, J.A., Koretsky, A., and Varmus, H.E. (2001). Induction and apoptotic regression of lung adenocarcinomas by regulation of a K-Ras transgene in the presence and absence of tumor suppressor genes. Genes Dev. *15*, 3249–3262.

Frederick, D.T., Piris, A., Cogdill, A.P., Cooper, Z.A., Lezcano, C., Ferrone, C.R., Mitra, D., Boni, A., Newton, L.P., Liu, C., et al. (2013). BRAF inhibition is associated with enhanced melanoma antigen expression and a more favorable tumor microenvironment in patients with metastatic melanoma. Clin. Cancer Res. *19*, 1225–1231.

Garon, E.B., Rizvi, N.A., Hui, R., Leighl, N., Balmanoukian, A.S., Eder, J.P., Patnaik, A., Aggarwal, C., Gubens, M., Horn, L., et al.; KEYNOTE-001 Investigators (2015). Pembrolizumab for the treatment of non-small-cell lung cancer. N. Engl. J. Med. *372*, 2018–2028.

Gentles, A.J., Newman, A.M., Liu, C.L., Bratman, S.V., Feng, W., Kim, D., Nair, V.S., Xu, Y., Khuong, A., Hoang, C.D., et al. (2015). The prognostic landscape of genes and infiltrating immune cells across human cancers. Nat. Med. *21*, 938–945.

Glodde, N., Bald, T., van den Boorn-Konijnenberg, D., Nakamura, K., O'Donnell, J.S., Szczepanski, S., Brandes, M., Eickhoff, S., Das, I., Shridhar, N., et al. (2017). Reactive Neutrophil Responses Dependent on the Receptor Tyrosine Kinase c-MET Limit Cancer Immunotherapy. Immunity *47*, 789–802 e789.

Goel, S., DeCristo, M.J., Watt, A.C., BrinJones, H., Sceneay, J., Li, B.B., Khan, N., Ubellacker, J.M., Xie, S., Metzger-Filho, O., et al. (2017). CDK4/6 inhibition triggers anti-tumour immunity. Nature *548*, 471–475.

Groenendijk, F.H., and Bernards, R. (2014). Drug resistance to targeted therapies: déjà vu all over again. Mol. Oncol. *8*, 1067–1083.

Gunderson, A.J., Kaneda, M.M., Tsujikawa, T., Nguyen, A.V., Affara, N.I., Ruffell, B., Gorjestani, S., Liudahl, S.M., Truitt, M., Olson, P., et al. (2016). Bruton Tyrosine Kinase-Dependent Immune Cell Cross-talk Drives Pancreas Cancer. Cancer Discov. *6*, 270–285.

Hanahan, D., Wagner, E.F., and Palmiter, R.D. (2007). The origins of oncomice: a history of the first transgenic mice genetically engineered to develop cancer. Genes Dev. *21*, 2258–2270.

Herranz, N., Gallage, S., Mellone, M., Wuestefeld, T., Klotz, S., Hanley, C.J., Raguz, S., Acosta, J.C., Innes, A.J., Banito, A., et al. (2015). mTOR regulates MAPKAPK2 translation to control the senescence-associated secretory phenotype. Nat. Cell Biol. *17*, 1205–1217.

Hoare, M., Ito, Y., Kang, T.W., Weekes, M.P., Matheson, N.J., Patten, D.A., Shetty, S., Parry, A.J., Menon, S., Salama, R., et al. (2016). NOTCH1 mediates a switch between two distinct secretomes during senescence. Nat. Cell Biol. *18*, 979–992.

Hu-Lieskovan, S., Mok, S., Homet Moreno, B., Tsoi, J., Robert, L., Goedert, L., Pinheiro, E.M., Koya, R.C., Graeber, T.G., Comin-Anduix, B., and Ribas, A. (2015). Improved antitumor activity of immunotherapy with BRAF and MEK inhibitors in BRAF(V600E) melanoma. Sci. Transl. Med. *7*, 279ra41.

Hugo, W., Zaretsky, J.M., Sun, L., Song, C., Moreno, B.H., Hu-Lieskovan, S., Berent-Maoz, B., Pang, J., Chmielowski, B., Cherry, G., et al. (2016). Genomic and Transcriptomic Features of Response to Anti-PD-1 Therapy in Metastatic Melanoma. Cell *165*, 35–44.

Huijbers, I.J. (2017). Generating Genetically Modified Mice: A Decision Guide. Methods Mol. Biol. *1642*, 1–19.

Jain, M., Arvanitis, C., Chu, K., Dewey, W., Leonhardt, E., Trinh, M., Sundberg, C.D., Bishop, J.M., and Felsher, D.W. (2002). Sustained loss of a neoplastic phenotype by brief inactivation of MYC. Science *297*, 102–104.

Ji, H., Houghton, A.M., Mariani, T.J., Perera, S., Kim, C.B., Padera, R., Tonon, G., McNamara, K., Marconcini, L.A., Hezel, A., et al. (2006). K-ras activation generates an inflammatory response in lung tumors. Oncogene *25*, 2105–2112.

Jiang, D., Gao, Z., Cai, Z., Wang, M., and He, J. (2015). Clinicopathological and prognostic significance of FOXP3+ tumor infiltrating lymphocytes in patients with breast cancer: a meta-analysis. BMC Cancer *15*, 727.

Jiang, H., Hegde, S., Knolhoff, B.L., Zhu, Y., Herndon, J.M., Meyer, M.A., Nywening, T.M., Hawkins, W.G., Shapiro, I.M., Weaver, D.T., et al. (2016). Targeting focal adhesion kinase renders pancreatic cancers responsive to checkpoint immunotherapy. Nat. Med. *22*, 851–860.

Kang, T.W., Yevsa, T., Woller, N., Hoenicke, L., Wuestefeld, T., Dauch, D., Hohmeyer, A., Gereke, M., Rudalska, R., Potapova, A., et al. (2011). Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. Nature *479*, 547–551.

Kastenhuber, E.R., and Lowe, S.W. (2017). Putting p53 in Context. Cell *170*, 1062–1078.

Katlinski, K.V., Gui, J., Katlinskaya, Y.V., Ortiz, A., Chakraborty, R., Bhattacharya, S., Carbone, C.J., Beiting, D.P., Girondo, M.A., Peck, A.R., et al. (2017). Inactivation of Interferon Receptor Promotes the Establishment of Immune Privileged Tumor Microenvironment. Cancer Cell *31*, 194–207.

Keck, M.K., Zuo, Z., Khattri, A., Stricker, T.P., Brown, C.D., Imanguli, M., Rieke, D., Endhardt, K., Fang, P., Brägelmann, J., et al. (2015). Integrative analysis of head and neck cancer identifies two biologically distinct HPV and three non-HPV subtypes. Clin. Cancer Res. *21*, 870–881.

Kersten, K., de Visser, K.E., van Miltenburg, M.H., and Jonkers, J. (2017). Genetically engineered mouse models in oncology research and cancer medicine. EMBO Mol. Med. *9*, 137–153.

Knudson, A.G., Jr. (1971). Mutation and cancer: statistical study of retinoblastoma. Proc. Natl. Acad. Sci. USA *68*, 820–823.

Kobielak, A., and Fuchs, E. (2006). Links between alpha-catenin, NF-kappaB, and squamous cell carcinoma in skin. Proc. Natl. Acad. Sci. USA *103*, 2322–2327.

Kortlever, R.M., Sodir, N.M., Wilson, C.H., Burkhart, D.L., Pellegrinet, L., Brown Swigart, L., Littlewood, T.D., and Evan, G.I. (2017). Myc Cooperates with Ras by Programming Inflammation and Immune Suppression. Cell *171*, 1301–1315.e14.

Koya, R.C., Mok, S., Otte, N., Blacketor, K.J., Comin-Anduix, B., Tumeh, P.C., Minasyan, A., Graham, N.A., Graeber, T.G., Chodon, T., and Ribas, A. (2012). BRAF inhibitor vemurafenib improves the antitumor activity of adoptive cell immunotherapy. Cancer Res. *72*, 3928–3937.

Koyama, S., Akbay, E.A., Li, Y.Y., Aref, A.R., Skoulidis, F., Herter-Sprie, G.S., Buczkowski, K.A., Liu, Y., Awad, M.M., Denning, W.L., et al. (2016). STK11/LKB1 Deficiency Promotes Neutrophil Recruitment and Proinflammatory Cytokine Production to Suppress T-cell Activity in the Lung Tumor Microenvironment. Cancer Res. *76*, 999–1008.

Laberge, R.M., Sun, Y., Orjalo, A.V., Patil, C.K., Freund, A., Zhou, L., Curran, S.C., Davalos, A.R., Wilson-Edell, K.A., Liu, S., et al. (2015). MTOR regulates the pro-tumorigenic senescence-associated secretory phenotype by promoting IL1A translation. Nat. Cell Biol. *17*, 1049–1061.

Layer, J.P., Kronmüller, M.T., Quast, T., van den Boorn-Konijnenberg, D., Effern, M., Hinze, D., Althoff, K., Schramm, A., Westermann, F., Peifer, M., et al. (2017). Amplification of N-Myc is associated with a T-cell-poor microenvironment in metastatic neuroblastoma restraining interferon pathway activity and chemokine expression. OncoImmunology *6*, e1320626.

Le, D.T., Uram, J.N., Wang, H., Bartlett, B.R., Kemberling, H., Eyring, A.D., Skora, A.D., Luber, B.S., Azad, N.S., Laheru, D., et al. (2015). PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. N. Engl. J. Med. *372*, 2509–2520.

Lennerz, V., Fatho, M., Gentilini, C., Frye, R.A., Lifke, A., Ferel, D., Wölfel, C., Huber, C., and Wölfel, T. (2005). The response of autologous T cells to a human melanoma is dominated by mutated neoantigens. Proc. Natl. Acad. Sci. USA *102*, 16013–16018.

Lesina, M., Wörmann, S.M., Morton, J., Diakopoulos, K.N., Korneeva, O., Wimmer, M., Einwächter, H., Sperveslage, J., Demir, I.E., Kehl, T., et al. (2016). RelA regulates CXCL1/CXCR2-dependent oncogene-induced senescence in murine Kras-driven pancreatic carcinogenesis. J. Clin. Invest. *126*, 2919–2932.

Linnemann, C., van Buuren, M.M., Bies, L., Verdegaal, E.M., Schotte, R., Calis, J.J., Behjati, S., Velds, A., Hilkmann, H., Atmioui, D.E., et al. (2015). High-throughput epitope discovery reveals frequent recognition of neo-antigens by CD4+ T cells in human melanoma. Nat. Med. *21*, 81–85.

Liu, F., Lang, R., Zhao, J., Zhang, X., Pringle, G.A., Fan, Y., Yin, D., Gu, F., Yao, Z., and Fu, L. (2011). $CD8^+$ cytotoxic T cell and $FOXP3^+$ regulatory T cell infiltration in relation to breast cancer survival and molecular subtypes. Breast Cancer Res. Treat. *130*, 645–655.

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Lujambio, A., Akkari, L., Simon, J., Grace, D., Tschaharganeh, D.F., Bolden, J.E., Zhao, Z., Thapar, V., Joyce, J.A., Krizhanovsky, V., and Lowe, S.W. (2013). Non-cell-autonomous tumor suppression by p53. Cell *153*, 449–460.

Medrek, C., Pontén, F., Jirström, K., and Leandersson, K. (2012). The presence of tumor associated macrophages in tumor stroma as a prognostic marker for breast cancer patients. BMC Cancer *12*, 306.

Meylan, E., Dooley, A.L., Feldser, D.M., Shen, L., Turk, E., Ouyang, C., and Jacks, T. (2009). Requirement for NF-kappaB signalling in a mouse model of lung adenocarcinoma. Nature *462*, 104–107.

Moody, S.E., Sarkisian, C.J., Hahn, K.T., Gunther, E.J., Pickup, S., Dugan, K.D., Innocent, N., Cardiff, R.D., Schnall, M.D., and Chodosh, L.A. (2002). Conditional activation of Neu in the mammary epithelium of transgenic mice results in reversible pulmonary metastasis. Cancer Cell *2*, 451–461.

Muller, P.A., and Vousden, K.H. (2014). Mutant p53 in cancer: new functions and therapeutic opportunities. Cancer Cell *25*, 304–317.

Muñoz-Espín, D., and Serrano, M. (2014). Cellular senescence: from physiology to pathology. Nat. Rev. Mol. Cell Biol. *15*, 482–496.

Muñoz-Fontela, C., Mandinova, A., Aaronson, S.A., and Lee, S.W. (2016). Emerging roles of p53 and other tumour-suppressor genes in immune regulation. Nat. Rev. Immunol. *16*, 741–750.

Ock, C.Y., Hwang, J.E., Keam, B., Kim, S.B., Shim, J.J., Jang, H.J., Park, S., Sohn, B.H., Cha, M., Ajani, J.A., et al. (2017). Genomic landscape associated with potential response to anti-CTLA-4 treatment in cancers. Nat. Commun. *8*, 1050.

Parker, J.S., Mullins, M., Cheang, M.C., Leung, S., Voduc, D., Vickery, T., Davies, S., Fauron, C., He, X., Hu, Z., et al. (2009). Supervised risk predictor of breast cancer based on intrinsic subtypes. J. Clin. Oncol. *27*, 1160–1167.

Peng, W., Chen, J.Q., Liu, C., Malu, S., Creasy, C., Tetzlaff, M.T., Xu, C., McKenzie, J.A., Zhang, C., Liang, X., et al. (2016). Loss of PTEN Promotes Resistance to T Cell-Mediated Immunotherapy. Cancer Discov. *6*, 202–216.

Pérez-Mancera, P.A., Young, A.R., and Narita, M. (2014). Inside and out: the activities of senescence in cancer. Nat. Rev. Cancer *14*, 547–558.

Pribluda, A., Elyada, E., Wiener, Z., Hamza, H., Goldstein, R.E., Biton, M., Burstain, I., Morgenstern, Y., Brachya, G., Billauer, H., et al. (2013). A senescence-inflammatory switch from cancer-inhibitory to cancer-promoting mechanism. Cancer Cell *24*, 242–256.

Pylayeva-Gupta, Y., Lee, K.E., Hajdu, C.H., Miller, G., and Bar-Sagi, D. (2012). Oncogenic Kras-induced GM-CSF production promotes the development of pancreatic neoplasia. Cancer Cell *21*, 836–847.

Quigley, D., Silwal-Pandit, L., Dannenfelser, R., Langerød, A., Vollan, H.K., Vaske, C., Siegel, J.U., Troyanskaya, O., Chin, S.F., Caldas, C., et al. (2015). Lymphocyte Invasion in IC10/Basal-Like Breast Tumors Is Associated with Wild-Type TP53. Mol. Cancer Res. *13*, 493–501.

Rizvi, N.A., Hellmann, M.D., Snyder, A., Kvistborg, P., Makarov, V., Havel, J.J., Lee, W., Yuan, J., Wong, P., Ho, T.S., et al. (2015). Cancer immunology. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. Science *348*, 124–128.

Robbins, P.F., Lu, Y.C., El-Gamil, M., Li, Y.F., Gross, C., Gartner, J., Lin, J.C., Teer, J.K., Cliften, P., Tycksen, E., et al. (2013). Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. Nat. Med. *19*, 747–752.

Robinson, D.R., Wu, Y.M., Lonigro, R.J., Vats, P., Cobain, E., Everett, J., Cao, X., Rabban, E., Kumar-Sinha, C., Raymond, V., et al. (2017). Integrative clinical genomics of metastatic cancer. Nature *548*, 297–303.

Rooney, M.S., Shukla, S.A., Wu, C.J., Getz, G., and Hacohen, N. (2015). Molecular and genetic properties of tumors associated with local immune cytolytic activity. Cell *160*, 48–61.

Rous, P. (1911). A Sarcoma of the Fowl Transmissible by an Agent Separable from the Tumor Cells. J. Exp. Med. *13*, 397–411.

Sagiv-Barfi, I., Kohrt, H.E., Czerwinski, D.K., Ng, P.P., Chang, B.Y., and Levy, R. (2015). Therapeutic antitumor immunity by checkpoint blockade is enhanced by ibrutinib, an inhibitor of both BTK and ITK. Proc. Natl. Acad. Sci. USA *112*, E966–E972.

Sarkar, S., Bristow, C.A., Dey, P., Rai, K., Perets, R., Ramirez-Cardenas, A., Malasi, S., Huang-Hobbs, E., Haemmerle, M., Wu, S.Y., et al. (2017). PRKCI promotes immune suppression in ovarian cancer. Genes Dev. *31*, 1109–1121.

Savas, P., Salgado, R., Denkert, C., Sotiriou, C., Darcy, P.K., Smyth, M.J., and Loi, S. (2016). Clinical relevance of host immunity in breast cancer: from TILs to the clinic. Nat. Rev. Clin. Oncol. *13*, 228–241.

Schumacher, T.N., and Schreiber, R.D. (2015). Neoantigens in cancer immunotherapy. Science *348*, 69–74.

Schuster, C., Berger, A., Hoelzl, M.A., Putz, E.M., Frenzel, A., Simma, O., Moritz, N., Hoelbl, A., Kovacic, B., Freissmuth, M., et al. (2011). The cooperating mutation or "second hit" determines the immunologic visibility toward MYC-induced murine lymphomas. Blood *118*, 4635–4645.

Schwitalla, S., Ziegler, P.K., Horst, D., Becker, V., Kerle, I., Begus-Nahrmann, Y., Lechel, A., Rudolph, K.L., Langer, R., Slotta-Huspenina, J., et al. (2013). Loss of p53 in enterocytes generates an inflammatory microenvironment enabling invasion and lymph node metastasis of carcinogen-induced colorectal tumors. Cancer Cell *23*, 93–106.

Shchors, K., Shchors, E., Rostker, F., Lawlor, E.R., Brown-Swigart, L., and Evan, G.I. (2006). The Myc-dependent angiogenic switch in tumors is mediated by interleukin 1beta. Genes Dev. *20*, 2527–2538.

Shen, Q., Cohen, B., Zheng, W., Rahbar, R., Martin, B., Murakami, K., Lamorte, S., Thompson, P., Berman, H., Zúñiga-Pflücker, J.C., et al. (2017). Notch Shapes the Innate Immunophenotype in Breast Cancer. Cancer Discov. *7*, 1320–1335.

Shin, D.S., Zaretsky, J.M., Escuin-Ordinas, H., Garcia-Diaz, A., Hu-Lieskovan, S., Kalbasi, A., Grasso, C.S., Hugo, W., Sandoval, S., Torrejon, D.Y., et al. (2017). Primary Resistance to PD-1 Blockade Mediated by JAK1/2 Mutations. Cancer Discov. *7*, 188–201.

Sodir, N.M., Swigart, L.B., Karnezis, A.N., Hanahan, D., Evan, G.I., and Soucek, L. (2011). Endogenous Myc maintains the tumor microenvironment. Genes Dev. *25*, 907–916.

Soucek, L., Lawlor, E.R., Soto, D., Shchors, K., Swigart, L.B., and Evan, G.I. (2007). Mast cells are required for angiogenesis and macroscopic expansion of Myc-induced pancreatic islet tumors. Nat. Med. *13*, 1211–1218.

Sparmann, A., and Bar-Sagi, D. (2004). Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. Cancer Cell *6*, 447–458.

Spranger, S., Bao, R., and Gajewski, T.F. (2015). Melanoma-intrinsic β-catenin signalling prevents anti-tumour immunity. Nature *523*, 231–235.

Spranger, S., Luke, J.J., Bao, R., Zha, Y., Hernandez, K.M., Li, Y., Gajewski, A.P., Andrade, J., and Gajewski, T.F. (2016). Density of immunogenic antigens does not explain the presence or absence of the T-cell-inflamed tumor microenvironment in melanoma. Proc. Natl. Acad. Sci. USA *113*, E7759–E7768.

Stanton, S.E., Adams, S., and Disis, M.L. (2016). Variation in the Incidence and Magnitude of Tumor-Infiltrating Lymphocytes in Breast Cancer Subtypes: A Systematic Review. JAMA Oncol. *2*, 1354–1360.

Stehelin, D., Varmus, H.E., Bishop, J.M., and Vogt, P.K. (1976). DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. Nature *260*, 170–173.

Stewart, T.A., Pattengale, P.K., and Leder, P. (1984). Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/myc fusion genes. Cell *38*, 627–637.

Stiff, A., Trikha, P., Wesolowski, R., Kendra, K., Hsu, V., Uppati, S., McMichael, E., Duggan, M., Campbell, A., Keller, K., et al. (2016). Myeloid-Derived Suppressor Cells Express Bruton's Tyrosine Kinase and Can Be Depleted in Tumor-Bearing Hosts by Ibrutinib Treatment. Cancer Res. *76*, 2125–2136.

Stodden, G.R., Lindberg, M.E., King, M.L., Paquet, M., MacLean, J.A., Mann, J.L., DeMayo, F.J., Lydon, J.P., and Hayashi, K. (2015). Loss of Cdh1 and Trp53 in the uterus induces chronic inflammation with modification of tumor microenvironment. Oncogene *34*, 2471–2482.

Topper, M.J., Vaz, M., Chiappinelli, K.B., DeStefano Shields, C.E., Niknafs, N., Yen, R.C., Wenzel, A., Hicks, J., Ballew, M., Stone, M., et al. (2017). Epigenetic Therapy Ties MYC Depletion to Reversing Immune Evasion and Treating Lung Cancer. Cell *171*, 1284–1300 e1221.

Toso, A., Revandkar, A., Di Mitri, D., Guccini, I., Proietti, M., Sarti, M., Pinton, S., Zhang, J., Kalathur, M., Civenni, G., et al. (2014). Enhancing chemotherapy efficacy in Pten-deficient prostate tumors by activating the senescence-associated antitumor immunity. Cell Rep. *9*, 75–89.

Van Allen, E.M., Miao, D., Schilling, B., Shukla, S.A., Blank, C., Zimmer, L., Sucker, A., Hillen, U., Foppen, M.H.G., Goldinger, S.M., et al. (2015). Genomic correlates of response to CTLA-4 blockade in metastatic melanoma. Science *350*, 207–211.

van Rooij, N., van Buuren, M.M., Philips, D., Velds, A., Toebes, M., Heemskerk, B., van Dijk, L.J., Behjati, S., Hilkmann, H., El Atmioui, D., et al. (2013). Tumor exome analysis reveals neoantigen-specific T-cell reactivity in an ipilimumab-responsive melanoma. J. Clin. Oncol. *31*, e439–e442.

Vanden Borre, P., Gunda, V., McFadden, D.G., Sadow, P.M., Varmeh, S., Bernasconi, M., and Parangi, S. (2014). Combined BRAF(V600E)- and SRC-inhibition induces apoptosis, evokes an immune response and reduces tumor growth in an immunocompetent orthotopic mouse model of anaplastic thyroid cancer. Oncotarget *5*, 3996–4010.

Ventura, A., Kirsch, D.G., McLaughlin, M.E., Tuveson, D.A., Grimm, J., Lintault, L., Newman, J., Reczek, E.E., Weissleder, R., and Jacks, T. (2007). Restoration of p53 function leads to tumour regression in vivo. Nature *445*, 661–665.

von Hansemann, D. (1890). Ueber asymmetrische Zelltheilung in Epithelkrebsen und deren biologische Bedeutung. Virchows Arch. Path. Anat *119*, 299–326.

Wang, G., Lu, X., Dey, P., Deng, P., Wu, C.C., Jiang, S., Fang, Z., Zhao, K., Konaparthi, R., Hua, S., et al. (2016). Targeting YAP-Dependent MDSC Infiltration Impairs Tumor Progression. Cancer Discov. *6*, 80–95.

Wang, Q., Hu, B., Hu, X., Kim, H., Squatrito, M., Scarpace, L., deCarvalho, A.C., Lyu, S., Li, P., Li, Y., et al. (2017). Tumor Evolution of Glioma-Intrinsic Gene Expression Subtypes Associates with Immunological Changes in the Microenvironment. Cancer Cell *32*, 42–56 e46.

Welte, T., Kim, I.S., Tian, L., Gao, X., Wang, H., Li, J., Holdman, X.B., Herschkowitz, J.I., Pond, A., Xie, G., et al. (2016). Oncogenic mTOR signalling recruits myeloid-derived suppressor cells to promote tumour initiation. Nat. Cell Biol. *18*, 632–644.

Wislez, M., Fujimoto, N., Izzo, J.G., Hanna, A.E., Cody, D.D., Langley, R.R., Tang, H., Burdick, M.D., Sato, M., Minna, J.D., et al. (2006). High expression of ligands for chemokine receptor CXCR2 in alveolar epithelial neoplasia induced by oncogenic kras. Cancer Res. *66*, 4198–4207.

Wölfel, T., Hauer, M., Schneider, J., Serrano, M., Wölfel, C., Klehmann-Hieb, E., De Plaen, E., Hankeln, T., Meyer zum Büschenfelde, K.H., and Beach, D. (1995). A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. Science *269*, 1281–1284.

Wörmann, S.M., Song, L., Ai, J., Diakopoulos, K.N., Kurkowski, M.U., Görgülü, K., Ruess, D., Campbell, A., Doglioni, C., Jodrell, D., et al. (2016). Loss of P53 Function Activates JAK2-STAT3 Signaling to Promote Pancreatic Tumor Growth, Stroma Modification, and Gemcitabine Resistance in Mice and Is Associated With Patient Survival. Gastroenterology *151*, 180–193.e12.

Xue, W., Zender, L., Miething, C., Dickins, R.A., Hernando, E., Krizhanovsky, V., Cordon-Cardo, C., and Lowe, S.W. (2007). Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. Nature *445*, 656–660.

Yang, Y. (2015). Cancer immunotherapy: harnessing the immune system to battle cancer. J. Clin. Invest. *125*, 3335–3337.

Yetil, A., Anchang, B., Gouw, A.M., Adam, S.J., Zabuawala, T., Parameswaran, R., van Riggelen, J., Plevritis, S., and Felsher, D.W. (2015). p19ARF is a critical mediator of both cellular senescence and an innate immune response associated with MYC inactivation in mouse model of acute leukemia. Oncotarget *6*, 3563–3577.

Ying, H., Elpek, K.G., Vinjamoori, A., Zimmerman, S.M., Chu, G.C., Yan, H., Fletcher-Sananikone, E., Zhang, H., Liu, Y., Wang, W., et al. (2011). PTEN is a major tumor suppressor in pancreatic ductal adenocarcinoma and regulates an NF-κB-cytokine network. Cancer Discov. *1*, 158–169.

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Fibrinogen-like Protein 1 Is a Major Immune Inhibitory Ligand of LAG-3

Jun Wang,[1] Miguel F. Sanmamed,[1] Ila Datar,[2] Tina Tianjiao Su,[1] Lan Ji,[1] Jingwei Sun,[1] Ling Chen,[3] Yusheng Chen,[4] Gefeng Zhu,[1] Weiwei Yin,[5] Linghua Zheng,[1] Ting Zhou,[1] Ti Badri,[1] Sheng Yao,[1] Shu Zhu,[1] Agedi Boto,[1,2] Mario Sznol,[6] Ignacio Melero,[7] Dario A.A. Vignali,[8,9] Kurt Schalper,[2] and Lieping Chen[1,3,6,10,*]

[1]Department of Immunobiology, Yale University, New Haven, CT 06511, USA
[2]Department of Pathology, Yale University, New Haven, CT 06510, USA
[3]Immunotherapy Institute, Fujian Medical University, Fuzhou, Fujian 350108, China
[4]Provincial Clinical Medical College, Fujian Medical University, Fuzhou, Fujian 350108, China
[5]Key Laboratory for Biomedical Engineering of Ministry of Education, College of Biomedical Engineering and Instrument Science, Zhejiang University, Hangzhou 310027, China
[6]Department of Medicine (Medical Oncology), Yale University, New Haven, CT 06510, USA
[7]Department of Immunology and Immunotherapy, University of Navarra, Pamplona 31008, Spain
[8]Department of Immunology, University of Pittsburgh, Pittsburgh, PA 15213, USA
[9]Tumor Microenvironment Center, UPMC Hillman Cancer Center, Pittsburgh, PA 15232, USA
[10]Lead Contact
*Correspondence: lieping.chen@yale.edu

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SUMMARY

Lymphocyte-activation gene 3 (LAG-3) is an immune inhibitory receptor, with major histocompatibility complex class II (MHC-II) as a canonical ligand. However, it remains controversial whether MHC-II is solely responsible for the inhibitory function of LAG-3. Here, we demonstrate that fibrinogen-like protein 1 (FGL1), a liver-secreted protein, is a major LAG-3 functional ligand independent from MHC-II. FGL1 inhibits antigen-specific T cell activation, and ablation of FGL1 in mice promotes T cell immunity. Blockade of the FGL1-LAG-3 interaction by monoclonal antibodies stimulates tumor immunity and is therapeutic against established mouse tumors in a receptor-ligand inter-dependent manner. FGL1 is highly produced by human cancer cells, and elevated FGL1 in the plasma of cancer patients is associated with a poor prognosis and resistance to anti-PD-1/B7-H1 therapy. Our findings reveal an immune evasion mechanism and have implications for the design of cancer immunotherapy.

INTRODUCTION

Lymphocyte-activation gene 3 (LAG-3, CD223) is a transmembrane protein primarily found on activated T cells (Anderson et al., 2016; Andrews et al., 2017; Triebel et al., 1990). LAG-3 protein consists of four extracellular immunoglobulin (Ig)-like domains (D1–D4) with high homology to CD4 (Triebel et al., 1990). LAG-3 expression can be upregulated by interleukin (IL)-2 and IL-12 on activated T cells (Annunziato et al., 1996, 1997; Bruniquel et al., 1998), where it mainly functions as a receptor that delivers inhibitory signals (Huard et al., 1994, 1996; Workman et al., 2002a). LAG-3 negatively regulates the proliferation, activation, effector function, and homeostasis of both $CD8^+$ and $CD4^+$ T cells, as shown in LAG-3 knockout mice and antibody studies (Huard et al., 1994; Workman et al., 2002a, 2002b, 2004; Workman and Vignali, 2003, 2005). LAG-3 may represent an "exhaustion" marker for $CD8^+$ T cells similar to PD-1 in response to repetitive antigen stimulation in chronic viral infections or cancers (Blackburn et al., 2009; Chihara et al., 2018; Grosso et al., 2007, 2009; Matsuzaki et al., 2010; Williams et al., 2017). Additionally, LAG-3 is also constitutively expressed on a subset of regulatory T cells and contributes to their suppressive function (Camisaschi et al., 2010; Gagliani et al., 2013; Huang et al., 2004). Currently, monoclonal antibodies (mAbs) that block the interaction of LAG-3 with its canonical ligand, MHC-II, are being evaluated for their antitumor activity in clinical trials (Anderson et al., 2016; Ascierto et al., 2017; Rotte et al., 2018).

The major ligand that mediates the immune suppressive functions of LAG-3, however, remains controversial. Initial studies by Baixeras et al. (1992) showed an interaction between MHC-II and LAG-3 via a cell-cell adhesion assay, which was further extended by studies indicating LAG-3 fusion protein binding to $MHC\text{-}II^+$ B cell lines (Huard et al., 1995, 1996). However, there is a lack of direct evidence for the protein-protein interaction between LAG-3 and MHC-II. MHC-II was proposed to interact with LAG-3 through the residues on the membrane-distal, top face of the LAG-3 D1 domain (Huard et al., 1997). Functionally, the MHC-II-CD4 interaction supported helper T cell activation, while overexpression of LAG-3 downregulated antigen-dependent $CD4^+$ T cell responses *in vitro* (Workman and Vignali, 2003). However, several mAbs that do not block the binding of LAG-3 to MHC-II nonetheless promoted T cell functions. For example, C9B7W, a specific mAb against the murine LAG-3 D2 domain, enhanced the proliferation and effector functions of T cells *in vitro* and *in vivo* (Workman et al., 2002b, 2004; Workman and Vignali, 2005). This antibody also increased the accumulation and effector function of tumor-specific $CD8^+$ T cells in

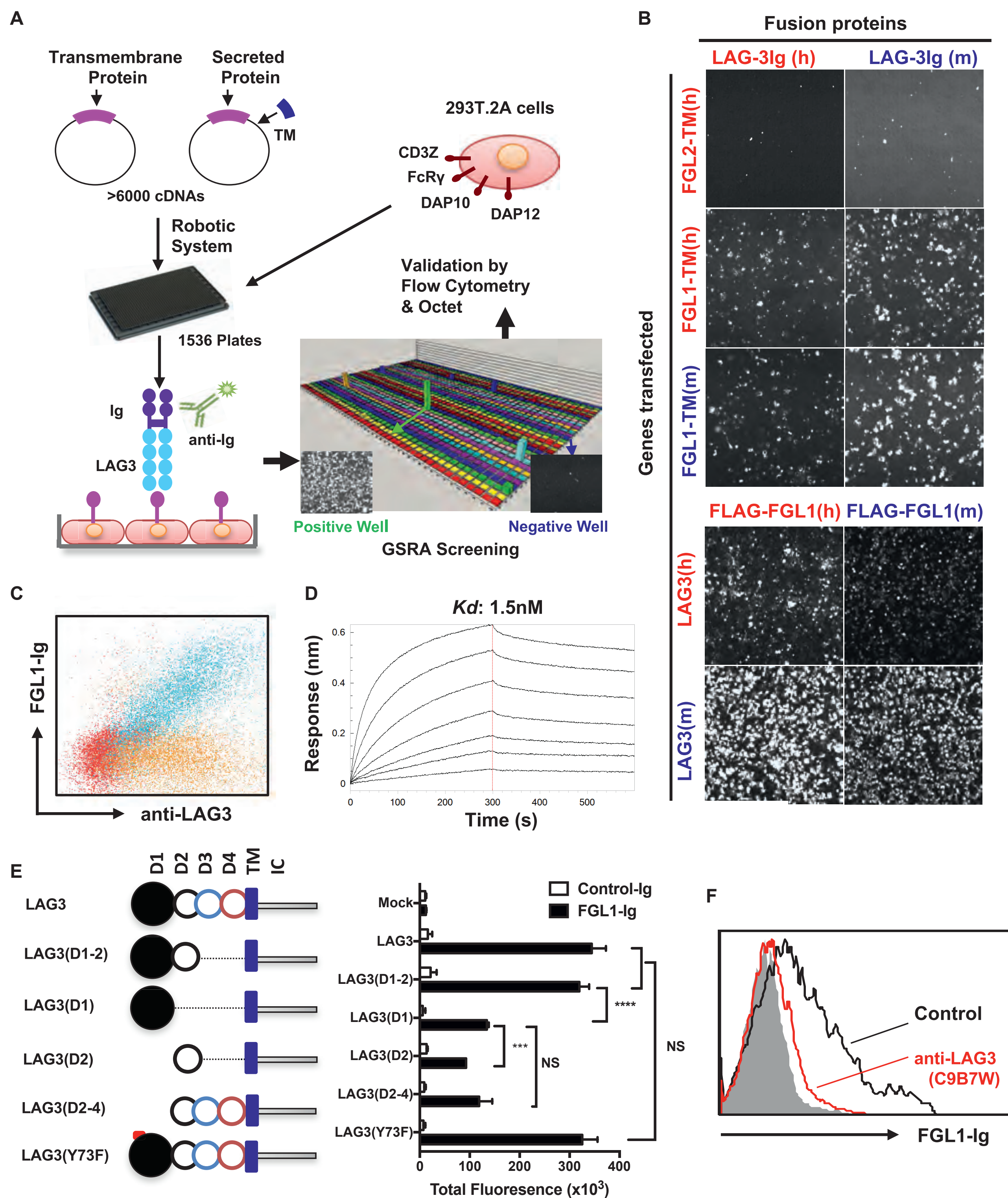

Figure 1. Identification of FGL1 as a Binding Partner of LAG-3 in the GSRA System

(A) Schematic representation of the GSRA system. Individual plasmids of genes encoding both transmembrane and secreted proteins were transfected into 293T.2A cells (see STAR Methods and Table S1) in 1,536-well plates. LAG-3-Ig as well as fluorescence labeled anti-Fc mAb were added into each well for rapid detection of LAG-3-Ig binding. Human Fc receptors served as internal positive controls within each plate. Positive hits were confirmed by flow cytometry or Octet bio-layer interferometry. TM, transmembrane domain.

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several tumor models (Grosso et al., 2007; Woo et al., 2012). The effects of C9B7W mAb on T cells are largely similar, if not identical, to those produced by LAG-3 genetic deficiency (Woo et al., 2012; Workman and Vignali, 2005). A recent study also showed that anti-LAG-3 mAb that do not block MHC-II binding could still stimulate T cell activation and anti-tumor activity (Cemerski et al., 2015). Given that LAG-3 also suppresses the function of $CD8^+$ T cells and natural killer (NK) cells, which do not interact with MHC-II (Anderson et al., 2016), these studies raise the possibility that the immunological functions of LAG-3 might be mediated via an unknown ligand.

Here, we report that fibrinogen-like protein 1 (FGL1) is a major functional ligand of LAG-3. FGL1 belongs to the fibrinogen family with high amino acid homology to the carboxyl terminus of the fibrinogen beta- and gamma-subunits, but it does not have the characteristic platelet-binding site, cross-linking region, and thrombin-sensitive site necessary for fibrin clot formation (Yamamoto et al., 1993). Under normal physiological conditions, FGL1 protein is primarily secreted from hepatocytes and contributes to its mitogenic and metabolic functions (Demchev et al., 2013; Hara et al., 2001; Li et al., 2010; Liu and Ukomadu, 2008; Yamamoto et al., 1993; Yan et al., 2002). The immunological function of FGL1, however, remains unknown. Our results demonstrate that FGL1 is a major inhibitory ligand for LAG-3, revealing a new mechanism of immune evasion.

RESULTS

FGL1 Is an MHC Class II-Independent High-Affinity Ligand of LAG-3

We employed our genome-scale receptor array (GSRA) technology to search for LAG-3 binding protein(s) using an immunoglobulin (Ig) Fc-tagged LAG-3extracellular domain fusion protein (LAG-3-Ig) (Figure 1A). The GSRA is a semi-automatic gene expression and detection system for rapidly identifying protein-protein interactions, which has been modified from our previous report (Yao et al., 2011). In this updated system, individual human cDNA encoding transmembrane and secreted proteins (upon addition of a transmembrane domain) were overexpressed on the surface of 293T cells. Several adaptor genes were also expressed in 293T (293T.2A cells) to facilitate protein expression on the cell surface (Figure 1A). An Ig-tagged protein of interest can then be screened for interaction(s) using the GSRA system in a high throughput fashion by the mix-and-read laser scanning macro-confocal fluorescent plate reader. The current version of the GSRA contains over 90% of annotated genes encoding human transmembrane ($\sim$5,600) and secreted ($\sim$1,000) proteins (Table S1). FGL1 was identified as a major binding protein for LAG-3-Ig in the GSRA system (Figures 1B and S1A). The FGL1-LAG-3 interaction is conserved across species in both human and mouse (Figure 1B). This interaction was further validated by flow cytometry, as indicated by a linear association between FGL1-Ig and anti-LAG-3 staining on LAG-3^+ cells (Figure 1C). The FGL1-LAG-3 interaction was shown to have a K_d value of $\sim$1.5 nM by Octet bio-layer interferometry analysis (Figure 1D). Using an SEC650 size exclusion column, the purified recombinant FLAG-tagged FGL1 (FLAG-FGL1) showed an oligomeric state (peak 1-2) and a dimeric peak (peak 3) (Figure S1B), which was validated via size exclusion chromatography with multi-angle light scattering analysis (SEC-MALS, data not shown). We observed stronger binding of the oligomeric forms of FLAG-FGL1 (peak 1-2) than the dimeric form (peak 3) to immobilized LAG-3-Ig in the Octet analysis (Figure S1C). In addition, the slow disassociation rate hints at a stable interaction between FGL1 and LAG-3 in both human and mouse (Figures 1D and S1D). FGL2, a homolog of FGL1 previously implicated in Treg functions (Shevach, 2009), as well as other fibrinogen domain-containing family members such as angiopoietin-related proteins, did not bind LAG-3 (Figure 1B and data not shown), indicating that the FGL1-LAG-3interaction is highly specific.

FGL1 is composed of a coil-coil domain (CCD) and a fibrinogen-like domain (FD) (Yamamoto et al., 1993). Through domain deletion studies, we demonstrated that the FD, but not CCD, is responsible for LAG-3 binding (Figure S1E). The LAG-3 protein consists of four Ig-like extracellular domains, D1–D4 (Huard et al., 1997; Triebel et al., 1990) (Figure 1E, left). The deletion of the D3–D4 domain in LAG-3 did not affect FGL1 binding, while either D1 or D2 alone partially decreased the binding (Figure 1E, right), suggesting that both D1 and D2 contribute to the FGL1-LAG-3 interaction. A single point mutation (Y73F) in the C′ strand of LAG-3 D1 domain was previously shown to disrupt MHC-II binding (Huard et al., 1997; Workman et al., 2002a). However, this mutation did not affect FGL1-Ig binding (Figure 1E, right), indicating that the FGL1-LAG-3 interaction is non-redundant with MHC-II-LAG-3 binding. Furthermore, pre-incubation of

(B) Image of the FGL1-LAG-3 interaction in GSRA system. 293T.2A cells were transfected with human (h) or mouse (m) FGL1-TM or full-length LAG-3 as indicated on the y axis. Human FGL2-TM was included as a negative control. The indicated fusion proteins shown on the x axis were added to the culture to evaluate binding to the transfectants by the cellular detection system (CDS).

(C) Representative flow cytometry dot plot of FGL1-Ig binding to mouse LAG-3^+ 293T.2A (blue) or mock cells (red). Control Ig binding to mouse LAG-3^+ 293T.2A is also shown (brown).

(D) Representative Octet sensorgrams showing various amounts of FLAG-tagged mouse FGL1 (starting from 10 μg/mL, 2-fold serial dilutions) binding to immobilized mouse LAG-3-Ig.

(E) Schematic representation of constructs coding full-length mouse LAG-3, LAG-3 Y73F mutant, or LAG-3 with different extracellular domain deletions (left). LAG-3 full-length protein consists of four extracellular Ig domains (D1–D4), the transmembrane domain (TM), and intracellular domain (IC) (left). Quantification of FGL1-Ig binding to 293T.2A cells transfected to express LAG-3 with domain deletion/mutation (right). Data were analyzed by CDS software and presented as the mean ± SEM. ***$p < 0.001$, ****$p < 0.0001$, NS, not significant by Student's t test.

(F) FGL1-Ig binding to mouse LAG-3^+ 293T.2A cells in the presence of control mAb (black line) or anti-LAG-3 (red line) by flow cytometry. Cells stained with control Ig (shadow) served as a negative control.

All data are representative of at least two independent experiments.

See also Figure S1.

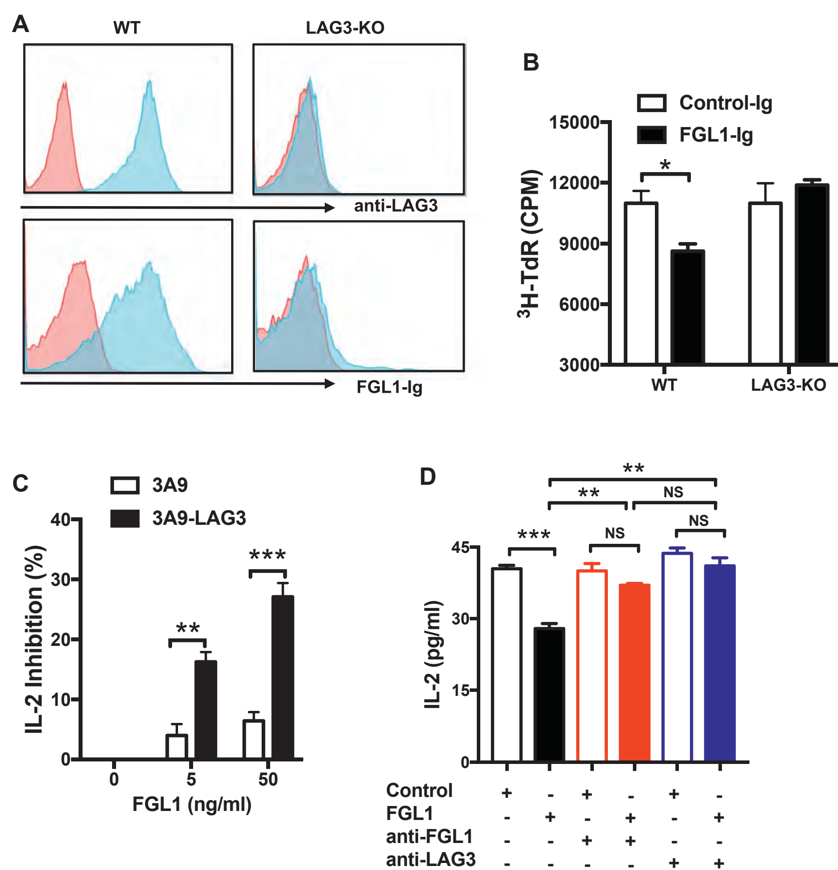


Figure 2. FGL1 Mediates LAG-3-Dependent T Cell Suppression

(A) Splenic T cells from WT or LAG-3-KO mice were activated by immobilized anti-CD3 mAb for 24 hr, stained with anti-LAG-3 mAb or FGL1-Ig fusion protein (blue) or control antibody/Ig (red), and analyzed by flow cytometry.

(B) Splenic T cells from WT or LAG-3-KO mice were activated by immobilized anti-CD3 mAb at suboptimal concentration in the presence of soluble FGL1-Ig or control-Ig (5 μg/mL) for 3 days before the addition of ^{3}H-dTR. Thymidine incorporation of proliferated T cells was analyzed 16 hr later.

(C) The 3A9-LAG-3 or parental 3A9 mouse T cell hybridoma cells were co-cultured with LK35.2 B cell line in CellGenix serum free medium in the presence of HEL peptide and the indicated concentrations of FLAG-tagged FGL1. Shown is the normalized % of inhibition on the IL-2 levels in the supernatant at 24 hr normalized to levels with 0 ng/mL FGL1.

(D) The 3A9-LAG-3 mouse T cell hybridoma cells were co-cultured with LK35.2 B cell line in the presence of HEL peptide, FLAG tagged FGL1 (50 ng/mL), anti-FGL1, or anti-LAG-3 mAb (1 μg/mL). Shown are the IL-2 levels in the supernatant at 24 hr.

Data are representative of at least two independent experiments and are presented as the mean ± SEM. *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$, NS, not significant by Student's t test.

See also Figure S2.

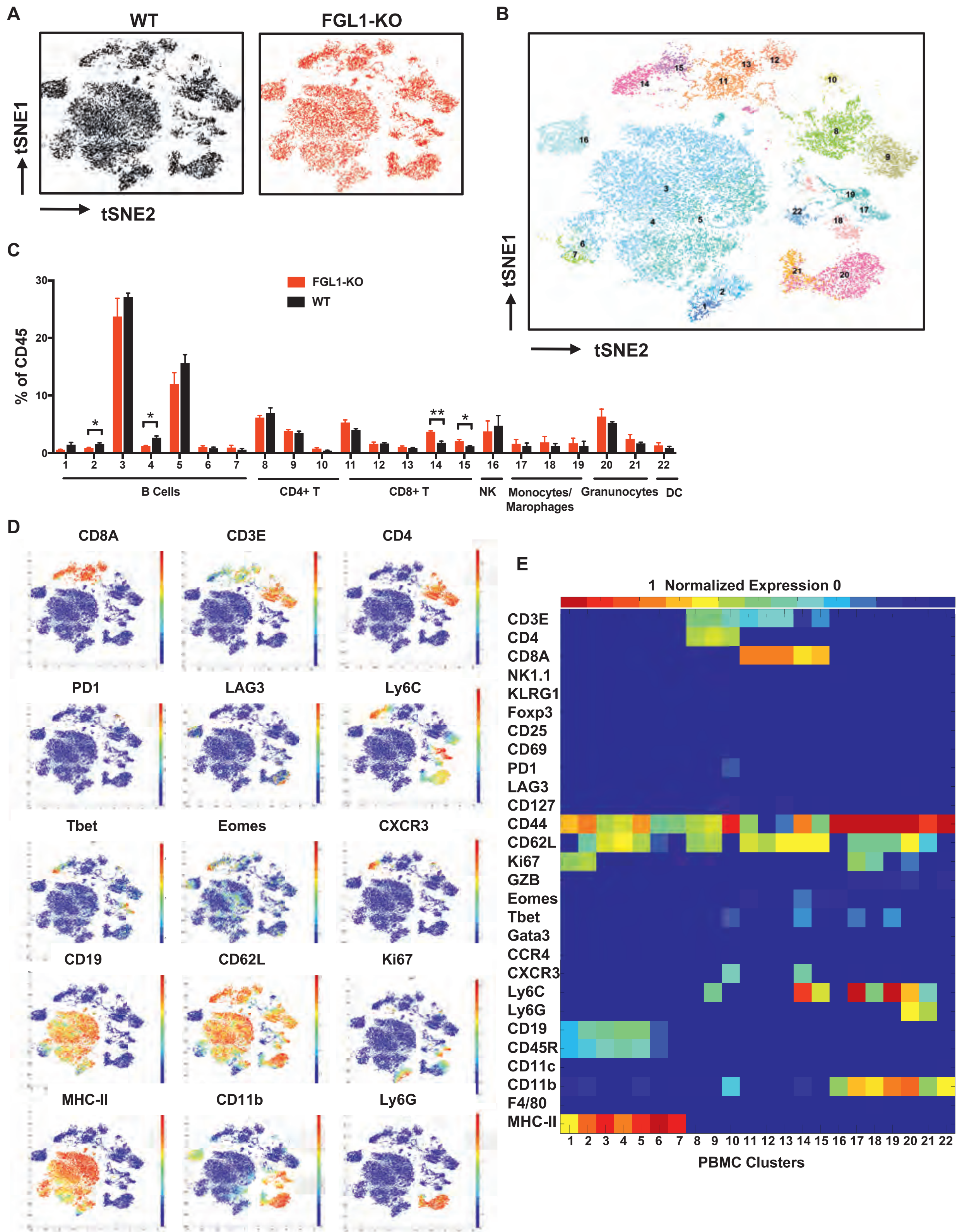
LAG-3^{+} 293T cells with C9B7W, an anti- LAG-3 mAb that binds the LAG-3 D2 domain without blocking the FGL1-MHC-II interaction (Andrews et al., 2017; Cemerski et al., 2015; Workman et al., 2002b), led to complete abrogation of FGL1-LAG-3 binding (Figure 1F). Finally, LAG-3^{+} cells stained with MHC-II (I-A^{b}) fusion protein did not show a significant decrease in binding even in the presence of a 100-fold excess of FGL1-Ig (Figure S1F). Taken together, our results indicate that FGL1 interacts with LAG-3 in an MHC-II-independent manner, and this interaction involves the FGL1 fibrinogen-like domain and the LAG-3 D1–D2 domain.

FGL1 Inhibits Antigen-Mediated T Cell Responses via LAG-3 *In Vitro* and *In Vivo*

LAG-3 is not found on resting T cells other than a subset of Tregs but can be upregulated under various antigen stimulation conditions (Baixeras et al., 1992; Triebel et al., 1990; Workman et al., 2002b). FGL1-Ig fusion protein did not bind resting T cells that express minimal LAG-3 levels (data not shown), although it did bind activated T cells from wild-type (WT) but not from LAG-3-KO mice, as determined via flow cytometry analysis (Figure 2A). Inclusion of FGL1-Ig partially suppressed WT splenic T cell proliferation under suboptimal anti-CD3 stimulation, but this suppression was diminished using LAG-3-KO splenocytes (Figure 2B), indicating that the suppressive effect of FGL1-Ig is dependent on LAG-3. Similarly, FGL1 better suppressed the antigen-specific induction of IL-2 from a murine LAG-3 overexpressing 3A9 T cell line (3A9-LAG-3) in a dose-dependent fashion compared to the parental 3A9 cell line with low endogenous LAG-3 expression (Figure 2C). We generated a mAb specific for mouse FGL1 (clone 177R4) that blocks FGL1-Ig binding to LAG-3^{+} 293T cells in a similar manner to anti-LAG-3 mAb C9B7W (Figures S2A and S2B). Both mAbs abrogated the suppression of FGL1 on IL-2 production from 3A9-LAG-3 cells (Figure 2D). Upon CD8^{+} OT-1 transgenic T cell transfer into syngeneic mice and subsequent immunization with chicken ovalbumin peptide antigen, administration of anti-FGL1 mAb 177R4 significantly promoted antigen-specific OT-1 T cell activation in a manner similar to anti-LAG-3 mAb, as determined by increased plasma levels of tumor necrosis factor alpha (TNF-α) and interferon (IFN)-γ (Figures S2C and S2D). Thus, our results support that FGL1 is an inhibitory ligand of LAG-3.

Fgl1-Deficient Mice Slowly Develop Spontaneous Autoimmune Symptoms

We generated a *Fgl1* gene knockout mouse strain (FGL1-KO) on the C57BL/6 background using an agouti color gene modified mouse ESC line (JM8) (Pettitt et al., 2009). In WT mice, *Fgl1* mRNA was detected in the liver but not in other organs or hematopoietic cells (Figure S3A). Soluble FGL1 was also detected in mouse blood (Figure S3B) as previously reported (Liu and Ukomadu, 2008). In contrast, FGL1 was not detected in the plasma or liver of FGL1-KO mice via specific sandwich ELISA and western blot analysis, respectively (Figures S3B and S3C).



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FGL1-KO mice have an overall normal appearance, organ size, and litters, indicating that FGL1 does not globally affect the development and growth of mice. However, up to 40% (8/20) of FGL1-KO mice developed spontaneous dermatitis at the age of 8 months or older, showing lymphocyte infiltration in the dermis (Figures S3D and S3E). At 14–16 months of age, 5/8 female, but not male mice, had elevated levels of anti-double-stranded DNA autoantibodies in their plasma compared to WT mice (Figure S3F). These findings are consistent with the role of FGL1 as an immune suppressive molecule. To evaluate overall changes in the immune system of this KO strain, we profiled mouse peripheral blood cells by mass cytometry (CyTOF), a single cell high dimensional analysis tool using 32 metal-conjugated mAbs to determine immune cell lineages as well as functional molecules. A recently described unsupervised clustering method named x-shift was also employed (Samusik et al., 2016). Analysis of the total $CD45^+$ hematopoietic cells revealed 22 distinct cell type or subsets (clusters), with small but significant increases in central memory-like $CD8^+$ T cells subsets (cluster 14–15, $CD44^+$ $CD62L^+$ $Ly6C^+$ $CD127^{med}$ $Tbet^+$ $Eomes^+$) and decreases in two B cell subsets (cluster 2 and 4) (Figures 3A–3E). All other clusters were similar in FGL1-KO compared to WT mice (Figures 3A–3E). There were no major differences in T cells or myeloid cell subsets in peripheral lymphoid tissues including the spleen or liver (data not shown).

These findings indicate that endogenous FGL1 does not affect mouse development and growth, although it may participate in regulating autoimmunity and immune homeostasis in aged mice.

Fgl1 Silencing Promotes T Cell Immunity against Tumor Growth in Mouse Models

FGL1-KO and LAG-3-KO mice were inoculated subcutaneously (s.c.) with syngeneic murine MC38 colon cancer cells. Similar to LAG-3-KO mice, FGL1-KO mice showed significantly slower tumor growth in comparison to WT mice (Figure 4A). Whereas all of the WT mice reached an endpoint (average mean tumor diameter of 15 mm) within 60 days, ~50% of FGL1-KO or LAG-3-KO mice were tumor-free beyond 200 days upon MC38 inoculation (Figure 4B). Similarly, both anti-FGL1 and anti-LAG-3 mAbs significantly controlled tumor growth of established MC38 murine colon (Figure 4C) and Hepa1-6 murine liver cell lines inoculated s.c. in syngeneic C57BL/6 mice (Figure S4A). In contrast, the anti-FGL1 and anti-LAG-3 mAb antitumor effect was abrogated in Rag1-KO C57BL/6 mice, which are devoid of T and B cells (Figure 4D). Consistent with these findings, depletion of either $CD8^+$ or $CD4^+$ T cells by specific mAbs completely eliminated the anti-tumor effect of both anti-FGL1 and anti-LAG-3 mAb in the MC38 tumor model, indicating that the anti-tumor effect of these mAbs is dependent on both $CD8^+$ and $CD4^+$ T cells (Figure S4B).

To exclude the possibility that additional ligands for LAG-3 are functionally redundant to FGL1 and contribute to the anti-tumor effect of the anti-LAG-3 mAb, we tested the effect of anti-LAG-3 mAb in FGL1-KO mice. While the anti-LAG-3 mAb suppressed MC38 tumor growth in WT mice, this anti-tumor effect was completely eliminated in FGL1-KO mice (Figure 4E). The effect of anti-FGL1 was also dependent on LAG-3, as this mAb did not have additive effects on tumor growth in LAG-3-KO mice (Figure 4F). Therefore, the anti-tumor effect of anti-FGL1 mAb is dependent on LAG-3, whereas the effect of anti-LAG-3 relies on FGL1 but not MHC-II or other LAG-3 ligands. Altogether, our findings support FGL1 as a major ligand for LAG-3 to induce T cell suppressive function and immune evasion.

The deficiency of FGL1 significantly reduced MC38 tumor growth while spleen size and the number of lymphocytes in either tumor-draining or non-tumor-draining lymph nodes remained similar (Figure S4C). Analysis of tumor-infiltrating leukocytes (TIL) in tumors excised on day 17 from FGL1-KO and WT mice by mass cytometry revealed a significant increase of $CD45^+$ leukocytes in FGL1-KO tumors (Figure S4D). In 22 clusters across $CD45^+$ cells (Figures 5A and 5B), we found a significant expansion of $CD44^+$ $CD62L^-$ $PD\text{-}1^+$ $Gata3^+$ effector memory-like CD4+ TIL (cluster 2 and 3), as well as $CD44^+$ $Ly6C^+$ memory-like $CD8^+$ (DeLong et al., 2018; Pihlgren et al., 1996; Walunas et al., 1995) TIL populations (clusters 8–10) in FGL1-KO tumors (Figures 5B–5E). In contrast, Treg (cluster 1), NK (cluster 11), or B cells (cluster 14) did not change significantly (Figures 5B–5E). Interestingly, a natural killer T (NKT) population (cluster 12) was highly expanded in the FGL1-KO tumors, in comparison with a significant decrease of $F4/80^+$ $CD11b^+$ $MHC\text{-}II^+$ $CD11c^{med}$ tumor-associated macrophages (cluster 15) (Figures 5C–5E). Confirming this data, we also observed a significant increase in the absolute number of leukocytes ($CD45^+$ cells), $CD8^+$, and $CD4^+$ TIL per mg of tumor tissues in mice treated with anti-FGL1 or anti-LAG-3 mAbs compared to control treated mice (Figure S4E). Furthermore, there was a significant increase in activation or functional markers, such as CD69, Ly6C, granzyme B (GZB), CD4, and FAS, in $CD4^+$ or $CD8^+$ TIL from anti-FGL1 or anti-LAG-3 treated mice (Figure S4F). Our results indicate that silencing the FGL1-LAG-3 interaction by either genetic knockout or antibody blockade promotes tumor immunity by stimulating T cell expansion and activation preferentially in the tumor microenvironment.

FGL1 Is Upregulated in Human Cancers

FGL1 mRNA and protein expression is largely limited to the liver and pancreas of human normal tissues according to the BioGPS tissue microarray database and proteome analysis (Kim et al., 2014) (Figure S5A). Meta-analysis of the Oncomine databases revealed the upregulation of *FGL1* mRNA in human solid tumors including lung cancer, prostate cancer, melanoma, and

Figure 3. Immune Cell Phenotyping of FGL1-KO Mice

(A) Density t-SNE plots of an equal number of $CD45^+$ compartment in the peripheral blood from WT and FGL1-KO mice (n = 3).

(B) t-SNE plot of $CD45^+$ compartment overlaid with color-coded clusters.

(C) Frequency of clusters grouped by indicated immune cell subsets. Data were shown as the mean ± SEM. $^*p < 0.05$; $^{**}p < 0.01$ by unpaired t test.

(D) t-SNE plot of $CD45^+$ compartment overlaid with the expression of selected markers.

(E) Heatmap displaying normalized marker expression of each immune cluster.

See also Figure S3.

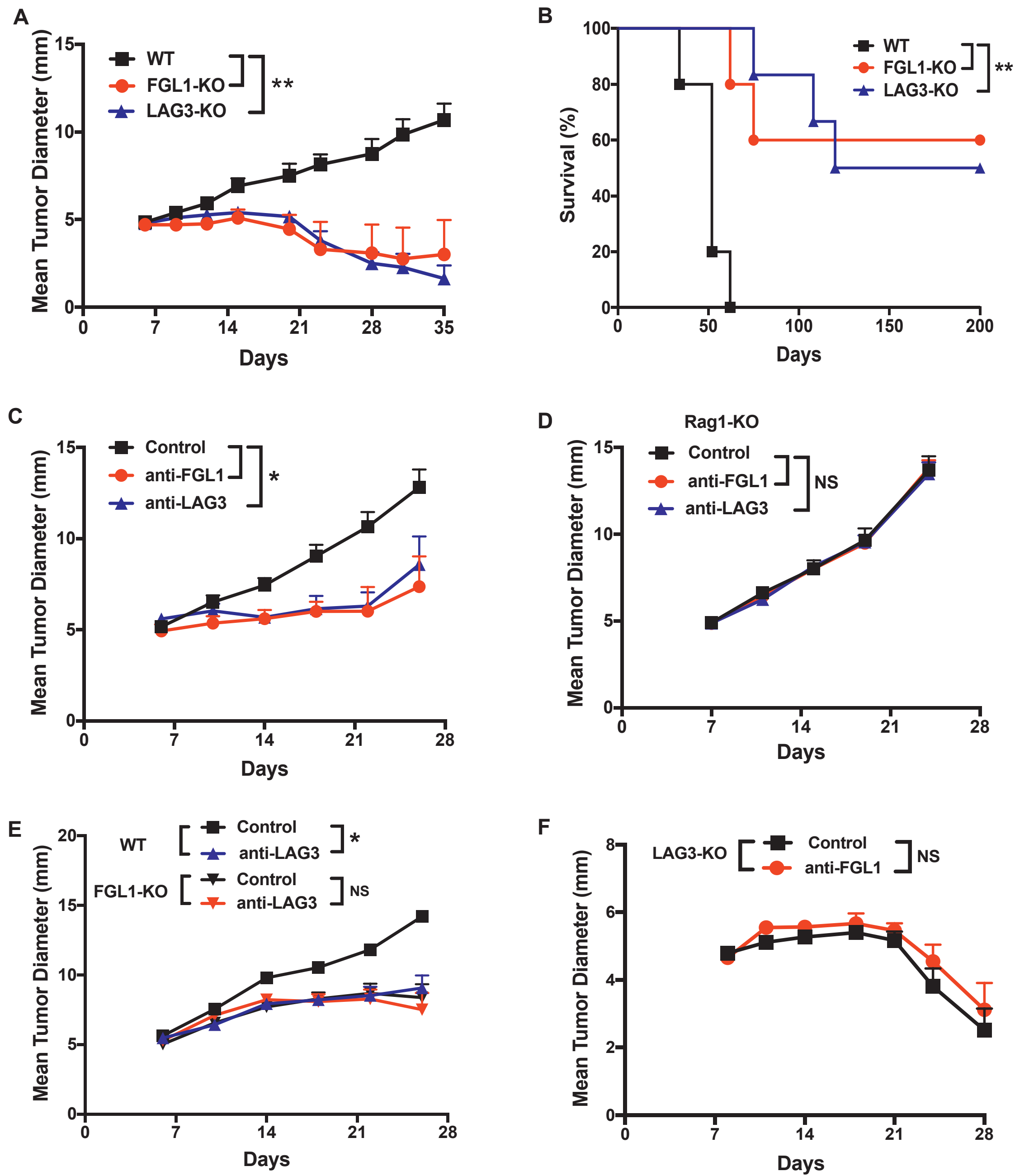

Figure 4. Ablation of the FGL1-LAG-3 Interaction Inhibits Tumor Growth in Mouse Models

(A and B) FGL1-KO, LAG-3-KO, or WT littermates were inoculated with MC38 cells (0.5 × 10^6/mouse). The mean tumor diameters (A) and survival (B) of mice in each group (n = 6) are shown.

(C and D) B6 (C) or Rag1-KO (D) mice were inoculated with MC38 cells (0.5 × 10^6/mouse) at day 0 and treated with anti-FGL1, anti-LAG-3, or control mAbs every 4 days from day 6 to day 18. The mean tumor diameters in each group (n = 6) are shown.

(E) WT or FGL1-KO mice were inoculated with MC38 cells and were treated with anti-LAG-3 or control mAb as in (C). The mean tumor diameters in each group (n = 6–8) are shown.

(F) LAG-3-KO mice were inoculated with MC38 cells and were treated with anti-FGL1 (n = 8) or control mAb (n = 7) as in (C). The mean tumor diameters in each group are shown.

Data were representative of at least two independent experiments and are shown as the mean ± SEM. *$p < 0.05$; **$p < 0.01$; NS, not significant. (A) and (C–F) by two-way ANOVA; (B) by log-rank test.

See also Figure S4.

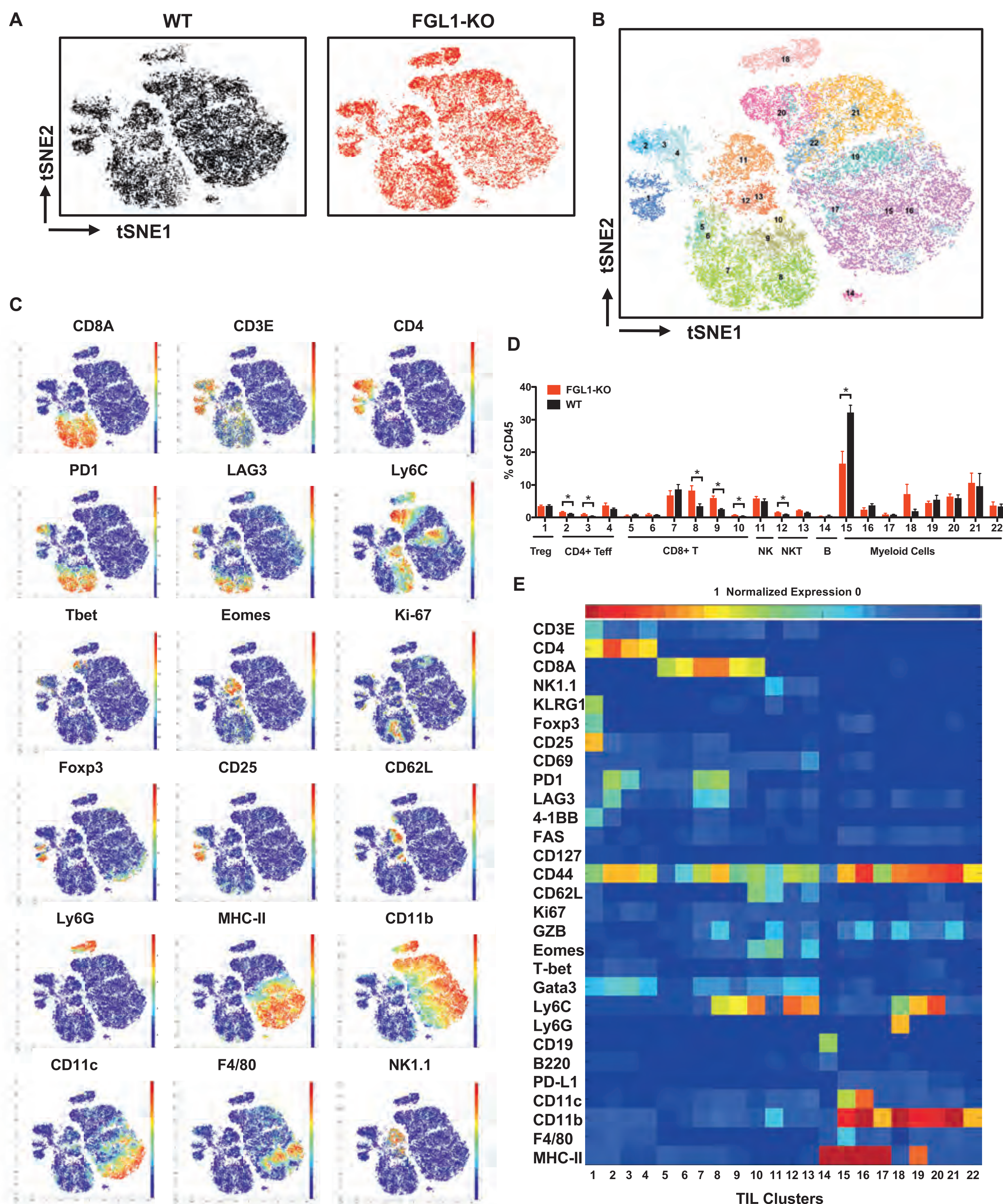

Figure 5. Expansion of Tumor-Infiltrating T Cell Populations in FGL1-KO Mice

(A) Density t-SNE plots of an equal number of $CD45^+$ MC38 tumor-infiltrating leukocytes in WT (n = 4) and FGL1-KO (n = 5) mice. Size of unsupervised clusters denotes the relative number of cells in that grouping.

(B) t-SNE plot of tumor infiltrating leukocytes overlaid with color-coded clusters.

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colorectal cancer compared to normal tissues, with the highest percentage of upregulation (8/23, or 35%) in lung cancer datasets, while its expression appeared to be downregulated in pancreas, liver, and head and neck cancers (Figure S5B). Furthermore, *FGL1* is one of the most upregulated genes in The Cancer Genome Atlas (TCGA) database for lung adenocarcinoma (Figure S5C; Table S2). *FGL1* is also significantly upregulated in prostate or breast cancer but is downregulated in liver cancer within this database (Figure S5C).

We established a multiplex quantitative immunofluorescence (QIF) assay to detect FGL1 protein expression on cells and tissues. In addition, a quantitative sandwich ELISA was also established to detect secreted FGL1 in human plasma (see STAR Methods). A 293T line constitutively expressing FGL1 and human tissue samples were utilized to standardize the assay in FFPE preparations and establish the signal detection threshold (Figure S6A). As expected, levels of FGL1 protein were highest in a cell line transfected to express the *FGL1* gene (Figure S6A) and human liver (data not shown), but low or undetectable in mock-transfected cells (Figure S6A) or samples from human testis and skeletal muscle (data not shown). We then evaluated the localized expression of FGL1 in 275 non-small cell lung carcinomas (NSCLC) presented in tissue microarray format (cohort #1, from Yale University, also see Table S3) by simultaneous staining of FGL1 and pan-cytokeratin using multiplex QIF staining. In NSCLC, FGL1 protein was found localized in tumor cells (the pan-cytokeratin-positive) with minimal expression in the stromal compartment (the pan-cytokeratin-negative) (Figure 6A) and no expression in paired normal lung tissues (Figure S6B). Tissue FGL1 levels showed a continuous distribution in this cohort and ~15% of specimens from NSCLC patients showed elevated expression (Figure 6B) which was associated with a significantly decreased 5-year overall survival (Figure 6C). Interestingly, there was no association between FGL1 and B7-H1 (PD-L1) expression levels, but high FGL1 in tumor tissue was significantly associated with high LAG-3 levels (Figure S6C). In addition, we also found significantly higher plasma FGL1 levels in NSCLC patients compared to healthy donors in two independent cohorts (see also Table S3): cohort #2 (n = 18) from University of Navarra, Pamplona, Spain (Figure 6D) and cohort #3 (n = 56) from Fujian Medical University, Fuzhou, China (Figure S6D). Of note, there was no difference in plasma FGL1 levels among NSCLC patients with or without metastasis as well as liver injury (Figure S6E). Furthermore, in cohort #2, we found a positive association of tumor FGL1 QIF scores and plasma FGL1 levels (data not shown). Our findings indicate that FGL1 is upregulated in human cancers, especially in NSCLC.

High Plasma FGL1 Is Associated with Poor Outcomes in Patients with Anti-PD Therapy

To test if FGL1 acts independently from the B7-H1-PD-1 pathway to suppress tumor immunity, we evaluated the association between the baseline plasma FGL1 levels and the efficacy of the B7-H1-PD-1 blockade therapy (anti-PD therapy) in metastatic NSCLC patients. In cohort #2 (see also Tables S3 and S4), we found that higher plasma FGL1 levels were associated with worse overall survival in NSCLC patients treated with anti-PD therapy (hazard ratio [HR] = 6.8, 95% confidence interval [CI] = 1.1–42 and p value = 0.04) (Figure 6E). Similar results were observed in an independent cohort (cohort #4, from Yale University, see also Tables S3 and S4) of metastatic melanoma patients (n = 21) treated with anti-PD-1 mAbs (HR = 7.9; 95% CI = 2.2–27.4 and p value <0.001) (Figure 6F). Our results suggest that the FGL1-LAG-3 interaction is independent from the B7-H1-PD-1 pathway and could potentially contribute to the resistance of anti-PD therapy in human cancers.

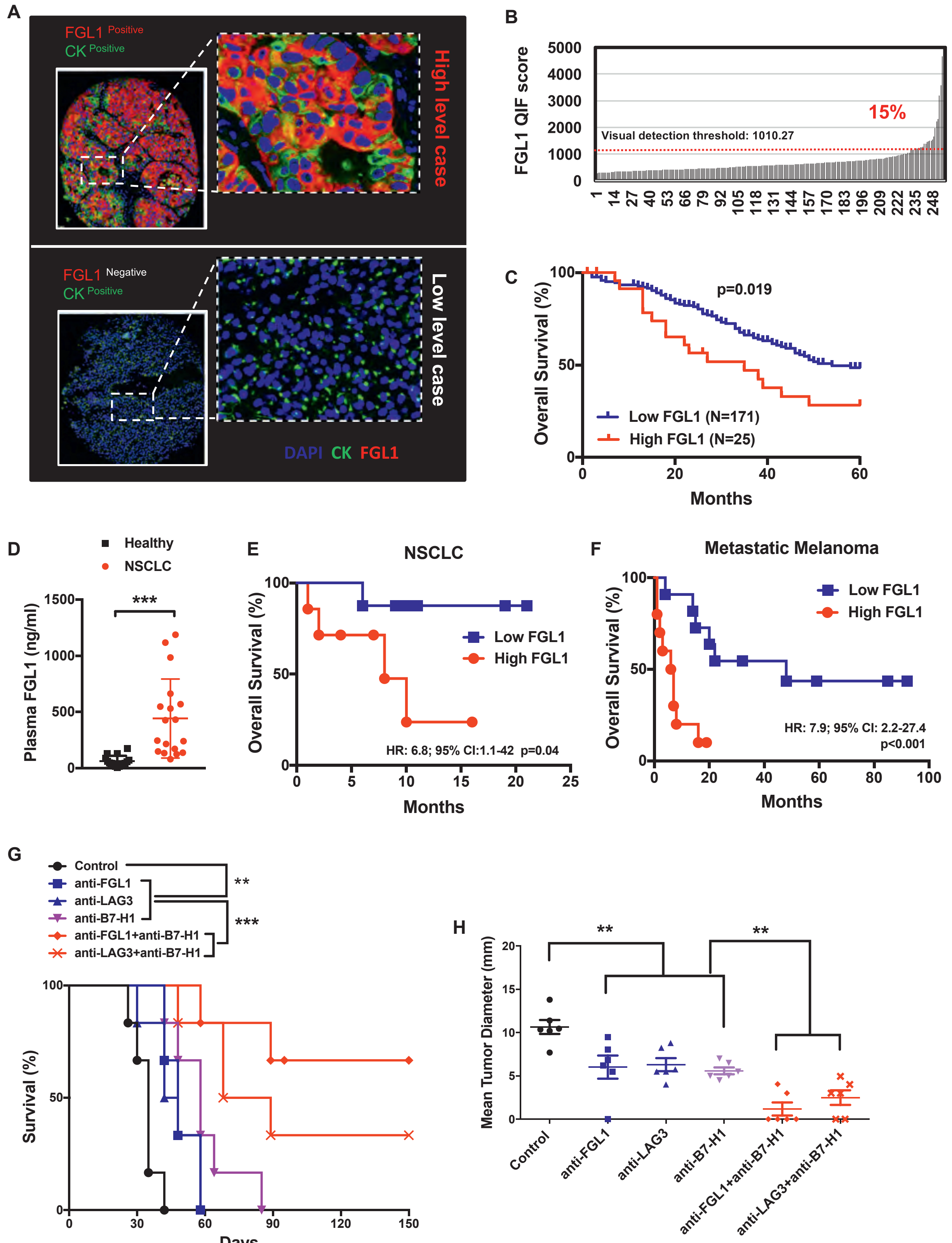
We further tested the role of the anti-FGL1 or anti-LAG-3 in the presence of the B7-H1-PD-1 pathway blockade using the MC38 tumor model. Mice were inoculated s.c. with MC38, and established tumors at day 6 were treated with the mAbs. When applied individually, anti-FGL1, anti-LAG-3, or anti-B7-H1 mAb slowed tumor growth and minimally prolonged survival (Figure 6G). However, anti-FGL1 or anti-LAG-3 mAb in combination with anti-B7-H1 mAb significantly improved survival (Figure 6G) and decreased tumor burden (Figure 6H) compared to single mAb treatment. A significant proportion of mice (>30% of mice) treated with the combination therapy were free of tumor for over 150 days (Figure 6G). Our results suggest that the FGL1-LAG-3 pathway is an independent tumor immune evasion mechanism, and blockade of this interaction may synergize with anti-PD therapy.

DISCUSSION

In this study, we have identified and characterized FGL1 as a major ligand of LAG-3 that is responsible for its T cell inhibitory function in a receptor-ligand interdependent manner both *in vitro* and *in vivo*. Genetic ablation or mAbs blocking the FGL1-LAG-3 interaction enhanced T cell responses and promoted anti-tumor immunity. With limited expression in the majority of normal tissues, FGL1 is upregulated in several human cancers and is associated with a poor prognosis and therapeutic outcome. Together, our findings support the FGL1-LAG-3 pathway as an immune escape mechanism and a potential target for cancer immunotherapy.

Physiological functions of FGL1 are not well understood. Soluble FGL1 protein can be detected in the blood plasma of healthy donors at the ng/mL level, while *FGL1* mRNA can only be detected in liver and pancreas across a large panel of normal tissues (Figure S5A), suggesting that FGL1 may be produced by the liver and/or pancreas and subsequently released into the bloodstream. In addition to the reported function in hepatocyte regeneration and metabolism, our findings reveal for the first time a prominent role of FGL1 in the negative regulation of

(C) t-SNE plot of tumor infiltrating leukocytes overlaid with the expression of selected markers.
(D) Frequency of clusters grouped by indicated immune cell subsets. Data were shown as the mean ± SEM. *p < 0.05 by unpaired t test.
(E) Heatmap displaying normalized marker expression of each immune cluster.
See also Figure S4.



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inflammatory immune responses. FGL1-KO mice spontaneously developed several autoimmune symptoms including dermatitis and anti-dsDNA autoantibodies (Figures S3D–S3F). However, these symptoms developed only in the aged but not in newborn or young adult mice, indicating that endogenous FGL1 is not a major regulator for self-tolerance but may suppress environmentally induced inflammation. Interestingly, liver is considered an immune-privileged organ, as allogeneic liver transplants can survive longer without immune suppressive agents (Horst et al., 2016). The underlying mechanism for this immune privilege, however, is largely unknown. We did not observe significant liver inflammation in aged FGL1-KO mice (data not shown), perhaps due to a lack of LAG-3 expression on resting T cells in the liver. While FGL1 may play a local role in maintaining the tolerogenic environment of the liver, its secretion as a soluble factor allows for potential cross-talk between the liver and other peripheral tissues that may help fine-tune systemic inflammation. This normal physiological function of FGL1 may be hijacked by several solid tumors that increase FGL1 expression to suppress local anti-tumor immunity. In this context, immune evasion may be mediated by high levels of FGL1 in the tumor microenvironment through the interaction with LAG-3 specifically expressed on tumor-infiltrating T cells. Our results in mouse tumor models indicate a preferential activation of T cell immunity in the tumor microenvironment upon FGL1-LAG-3 blockade while the effect of this blockade in systemic immune suppression is minimal (unpublished data), suggesting a major role for FGL1 in immune suppression of the tumor microenvironment.

Our results support FGL1 as a major ligand for the T cell inhibitory function of LAG-3. First, FGL1-LAG-3 represents a high-affinity interaction that is specific and physiological, as indicated by fusion protein binding experiments involving primary T cells from WT or *Lag3*-deficient mice. Second, FGL1 mAb has similar effects to anti-LAG-3 on the stimulation of T cell responses and antitumor effect in our *in vitro* and *in vivo* experiments. Furthermore, anti-FGL1 mAb has no antitumor effect in LAG-3-deficient mice while anti-LAG-3 mAb likewise loses efficacy in *Fgl1*-deficient mice. Finally, our preliminary studies indicate that adult *Fgl1*-deficient mice are also prone to the induction of autoimmune diseases (unpublished data), a phenotype similar to LAG-3-deficient mice (Bettini et al., 2011; Woo et al., 2012).

To date, at least four different proteins have been reported to interact with LAG-3 including MHC-II, galectin-3, LSECtin, and α-synuclein. Galectin-3 and LSECtin have potential roles in T cell regulation, while α-synuclein is possibly involved in the neurological function of LAG-3 (Kouo et al., 2015; Mao et al., 2016; Xu et al., 2014). The interaction modality of galectin-3 and LSECtin to LAG-3 are less known, but both molecules have previously been shown to have several other binding partners (Kizuka et al., 2015; Li et al., 2009; Liu et al., 2004; Stillman et al., 2006; Tang et al., 2010). It remains to be shown whether their roles in the suppression of T cell-responses and antitumor immunity are dependent on LAG-3. Although MHC-II is the first identified ligand for LAG-3, the detailed biochemistry and affinity of this interaction is still unclear. Given that FGL1 does not compete with MHC-II for LAG-3 binding (Figure S1F), this opens the possibility for the existence of a FGL1-MHC-II-LAG-3 trimolecular complex—open questions include the signaling outcome of FGL1 versus MHC-II upon interaction with LAG-3 and how this complex could contribute to T cell suppression. A detailed stoichiometry analysis may be required to understand how soluble FGL1 triggers cell surface LAG-3 to transmit signals for T cell suppression, which is currently unknown. We found that FGL1 could form oligomers, and these oligomeric forms of FGL1 bound to LAG-3 much better than the dimeric form (Figures S1B and S1C), implicating that oligomeric FGL1 may be required for T cell suppression. The presence of native oligomeric FGL1 may also explain our results in the Octet assay showing a high-affinity interaction of purified soluble FGL1 with LAG-3. Thus, increased avidity of FGL1, most likely through oligomerization, but potentially through other mechanisms such as attachments to the extracellular matrix, may facilitate its interaction with LAG-3 *in vivo*. Currently, several MHC-II blocking anti- LAG-3 mAbs are being evaluated in clinical trials for the treatment of advanced human cancer. Preliminary data of these trials showed minimal or modest effect as a single agent (Ascierto and McArthur, 2017; Ascierto et al., 2017). Based on our findings, a possible interpretation for the clinical results could be that these mAbs block the MHC-II-LAG-3 interaction but do not block FGL1-LAG-3 binding. Thus, these mAbs may still allow FGL1 to transmit inhibitory signals to LAG-3, leading to an incomplete blockade of LAG-3-mediated immune suppression. Our findings warrant careful re-evaluation of

Figure 6. Upregulated FGL1 in Human Cancers Is Associated with a Poor Prognosis

(A) Representative immunofluorescence staining of FGL1, DAPI (for nuclear counterstain), and pan-cytokeratin (CK) in FGL1-positive or -negative NSCLC cancer sections.

(B and C) FGL1 expression as indicated by quantitative immunofluorescence (QIF) staining in NSCLC cancer tissues from cohort #1 (see also Table S3). (B) Distribution of FGL1 expression and (C) association of high or low FGL1 expression with overall survival of the patients. The QIF visual detection threshold (1010.27) was used as a cutoff as indicated by dotted line in (B).

(D) The baseline plasma FGL1 levels were determined by ELISA in cohort #2 (see also Table S3) of NSCLC cancer patients (n = 18) and healthy donors (n = 16). Data were presented as the mean ± SEM. ***p < 0.001 by Student's t test.

(E and F) Kaplan-Meier plots of overall survival stratified by median baseline plasma FGL1 levels in NSCLC (cut-point: 336.5 ng/mL) and melanoma (cut-point: 114 ng/mL) patients treated with single-agent anti-PD-1 therapy in NSCLC (D, cohort #2, n = 18) and melanoma (E, cohort #4, n = 21). See also Tables S3 and S4.

(G and H) B6 mice were inoculated s.c. with MC38 cells (0.5×10^6/mouse) at day 0, followed by the treatment with anti-FGL1, anti-LAG-3, or control mAb (n = 6 per group) every 4 days from day 6 to day 18. In some groups, mice were also treated with a single dose of anti-B7-H1 (10B5) at day 6.

(G) Survival of the mice is shown. Survival analysis was conducted by log-rank test, **p < 0.01; ***p < 0.001. The presented data is representative of at least two independent experiments.

(H) Tumor sizes are shown as the mean tumor diameter ± SEM at day 22. **p < 0.01 by Student's t test.

See also Figures S5 and S6 and Table S2.

therapeutic strategies that aim to block the immune inhibitory function of LAG-3.

Our findings support that the FGL1-LAG-3 pathway maybe an important immune evasion mechanism and could contribute to current cancer immunotherapy efforts for several reasons. Our studies indicate that FGL1 is a major ligand for LAG-3 to suppress T cell responses and constitute a new target for immune modulation. Furthermore, upregulation of FGL1 on tumor cells but not in normal tissues (Figure S5) may allow for a highly tumor-selective targeting of antibody therapy. In addition, tumor model studies using FGL1-KO mice demonstrate that FGL1 has a potent immune suppressive effect on anti-tumor immunity that is dependent on LAG-3. FGL1-LAG-3 interaction may also affect the generation of memory T cells as shown by our CyTOF data of increased memory-like CD4+ and CD8+ T cells in TILs from FGL1 KO mice during tumor growth (Figure 5) and an association of low FGL1 level with long-term survival of cancer patients upon anti-PD therapy (Figure 6E and 6F). Moreover, FGL1 may be a potential biomarker to predict the outcome of anti-PD therapy, since high plasma FGL1 levels are associated with a worse response to anti-PD therapy in NSCLC and melanoma patients (Figures 6E and 6F). Lastly, FGL1 blockade also synergizes with anti-B7-H1 blockade in animal models (Figures 6G and 6H), suggesting that FGL1 and anti-PD dual-blockade may be an alternative treatment for patients who are resistant to anti-PD therapy. In summary, our findings identify a functional interaction of the LAG-3 pathway and reveal a possible mechanism that tumors may employ for immune evasion, with important implications for developing next generation cancer immunotherapies.

STAR★METHODS

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

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SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and four tables and can be found with this article online at https://doi.org/10.1016/j.cell.2018.11.010.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.W., M.F.S., and Lieping Chen; Methodology, J.W., M.F.S., I.D., T.T.S., L.J., Ling Chen, J.S., S.Y., and G.Z.; Software, J.W., M.F.S., T.T.S., I.D., W.Y., T.Z., and S.Z.; Formal Analysis, J.W., M.F.S., T.T.S., I.D., W.Y., A.B., and Ling Chen; Investigation, J.W., M.F.S., T.T.S., L.J., I.D., Ling Chen, J.S., and T.B.; Resources, L.Z., T.Z., S.Y., M.S., I.M., D.A.A.V., Y.C., K.S., and Lieping Chen; Writing – Original Draft, J.W. and Lieping Chen; Writing – Review & Editing, J.W., Lieping Chen, T.T.S., M.F.S., D.A.A.V., M.S., I.M., and K.S.; Visualization, J.W., M.F.S., and Lieping Chen; Supervision, Lieping Chen; Project Administration, J.W. and Lieping Chen.; Funding Acquisition, Lieping Chen.

DECLARATION OF INTERESTS

Lieping Chen is a consultant or advisory board member and receives consulting fees from Pfizer, Vcanbio, and GenomiCare; is a scientific founder of NextCure and TAYU Biotech; and has sponsored research grants from Boehringer Ingelheim and NextCure. There is a patent application pending related to this work.

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REFERENCES

Anderson, A.C., Joller, N., and Kuchroo, V.K. (2016). Lag-3, Tim-3, and TIGIT: co-inhibitory receptors with specialized functions in immune regulation. Immunity *44*, 989–1004.

Andrews, L.P., Marciscano, A.E., Drake, C.G., and Vignali, D.A. (2017). LAG3 (CD223) as a cancer immunotherapy target. Immunol. Rev. *276*, 80–96.

Annunziato, F., Manetti, R., Tomasévic, I., Guidizi, M.G., Biagiotti, R., Giannò, V., Germano, P., Mavilia, C., Maggi, E., and Romagnani, S. (1996). Expression and release of LAG-3-encoded protein by human CD4+ T cells are associated with IFN-gamma production. FASEB J. *10*, 769–776.

Annunziato, F., Manetti, R., Cosmi, L., Galli, G., Heusser, C.H., Romagnani, S., and Maggi, E. (1997). Opposite role for interleukin-4 and interferon-gamma on CD30 and lymphocyte activation gene-3 (LAG-3) expression by activated naive T cells. Eur. J. Immunol. *27*, 2239–2244.

Ascierto, P.A., and McArthur, G.A. (2017). Checkpoint inhibitors in melanoma and early phase development in solid tumors: what's the future? J. Transl. Med. *15*, 173.

Ascierto, P.A., Melero, I., Bhatia, S., Bono, P., Sanborn, R.E., Lipson, E.J., Callahan, M.K., Gajewski, T., Gomez-Roca, C.A., Hodi, F.S., et al. (2017). Initial efficacy of anti-lymphocyte activation gene-3 (anti–LAG-3; BMS-986016) in

combination with nivolumab (nivo) in pts with melanoma (MEL) previously treated with anti–PD-1/PD-L1 therapy. J. Clin. Oncol. *35*, 9520.

Baixeras, E., Huard, B., Miossec, C., Jitsukawa, S., Martin, M., Hercend, T., Auffray, C., Triebel, F., and Piatier-Tonneau, D. (1992). Characterization of the lymphocyte activation gene 3-encoded protein. A new ligand for human leukocyte antigen class II antigens. J. Exp. Med. *176*, 327–337.

Bettini, M., Szymczak-Workman, A.L., Forbes, K., Castellaw, A.H., Selby, M., Pan, X., Drake, C.G., Korman, A.J., and Vignali, D.A. (2011). Cutting edge: accelerated autoimmune diabetes in the absence of LAG-3. J. Immunol. *187*, 3493–3498.

Blackburn, S.D., Shin, H., Haining, W.N., Zou, T., Workman, C.J., Polley, A., Betts, M.R., Freeman, G.J., Vignali, D.A., and Wherry, E.J. (2009). Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. Nat. Immunol. *10*, 29–37.

Bruniquel, D., Borie, N., Hannier, S., and Triebel, F. (1998). Regulation of expression of the human lymphocyte activation gene-3 (LAG-3) molecule, a ligand for MHC class II. Immunogenetics *48*, 116–124.

Camisaschi, C., Casati, C., Rini, F., Perego, M., De Filippo, A., Triebel, F., Parmiani, G., Belli, F., Rivoltini, L., and Castelli, C. (2010). LAG-3 expression defines a subset of CD4(+)CD25(high)Foxp3(+) regulatory T cells that are expanded at tumor sites. J. Immunol. *184*, 6545–6551.

Cemerski, S., Zhao, S., Chenard, M., Laskey, J., Cui, L., Shukla, R., et al. (2015). T cell activation and anti-tumor efficacy of anti-LAG-3 antibodies is independent of LAG-3 – MHCII blocking capacity. J. Immunother. Cancer *3* (*Suppl 2*), 183.

Chapoval, A.I., Ni, J., Lau, J.S., Wilcox, R.A., Flies, D.B., Liu, D., Dong, H., Sica, G.L., Zhu, G., Tamada, K., and Chen, L. (2001). B7-H3: a costimulatory molecule for T cell activation and IFN-gamma production. Nat. Immunol. *2*, 269–274.

Chen, L., Ashe, S., Brady, W.A., Hellström, I., Hellström, K.E., Ledbetter, J.A., McGowan, P., and Linsley, P.S. (1992). Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. Cell *71*, 1093–1102.

Chen, L., McGowan, P., Ashe, S., Johnston, J., Li, Y., Hellström, I., and Hellström, K.E. (1994). Tumor immunogenicity determines the effect of B7 costimulation on T cell-mediated tumor immunity. J. Exp. Med. *179*, 523–532.

Chihara, N., Madi, A., Kondo, T., Zhang, H., Acharya, N., Singer, M., Nyman, J., Marjanovic, N.D., Kowalczyk, M.S., Wang, C., et al. (2018). Induction and transcriptional regulation of the co-inhibitory gene module in T cells. Nature *558*, 454–459.

DeLong, J.H., Hall, A.O., Konradt, C., Coppock, G.M., Park, J., Harms Pritchard, G., and Hunter, C.A. (2018). Cytokine- and TCR-mediated regulation of T cell expression of Ly6C and Sca-1. J. Immunol. *200*, 1761–1770.

Demchev, V., Malana, G., Vangala, D., Stoll, J., Desai, A., Kang, H.W., Li, Y., Nayeb-Hashemi, H., Niepel, M., Cohen, D.E., and Ukomadu, C. (2013). Targeted deletion of fibrinogen like protein 1 reveals a novel role in energy substrate utilization. PLoS ONE *8*, e58084.

Dong, H., Strome, S.E., Salomao, D.R., Tamura, H., Hirano, F., Flies, D.B., Roche, P.C., Lu, J., Zhu, G., Tamada, K., et al. (2002). Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. Nat. Med. *8*, 793–800.

Gagliani, N., Magnani, C.F., Huber, S., Gianolini, M.E., Pala, M., Licona-Limon, P., Guo, B., Herbert, D.R., Bulfone, A., Trentini, F., et al. (2013). Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. Nat. Med. *19*, 739–746.

Grosso, J.F., Kelleher, C.C., Harris, T.J., Maris, C.H., Hipkiss, E.L., De Marzo, A., Anders, R., Netto, G., Getnet, D., Bruno, T.C., et al. (2007). LAG-3 regulates CD8+ T cell accumulation and effector function in murine self- and tumor-tolerance systems. J. Clin. Invest. *117*, 3383–3392.

Grosso, J.F., Goldberg, M.V., Getnet, D., Bruno, T.C., Yen, H.R., Pyle, K.J., Hipkiss, E., Vignali, D.A., Pardoll, D.M., and Drake, C.G. (2009). Functionally distinct LAG-3 and PD-1 subsets on activated and chronically stimulated CD8 T cells. J. Immunol. *182*, 6659–6669.

Hara, H., Yoshimura, H., Uchida, S., Toyoda, Y., Aoki, M., Sakai, Y., Morimoto, S., and Shiokawa, K. (2001). Molecular cloning and functional expression analysis of a cDNA for human hepassocin, a liver-specific protein with hepatocyte mitogenic activity. Biochim. Biophys. Acta *1520*, 45–53.

Hirano, F., Kaneko, K., Tamura, H., Dong, H., Wang, S., Ichikawa, M., Rietz, C., Flies, D.B., Lau, J.S., Zhu, G., et al. (2005). Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. Cancer Res. *65*, 1089–1096.

Horst, A.K., Neumann, K., Diehl, L., and Tiegs, G. (2016). Modulation of liver tolerance by conventional and nonconventional antigen-presenting cells and regulatory immune cells. Cell. Mol. Immunol. *13*, 277–292.

Huang, C.T., Workman, C.J., Flies, D., Pan, X., Marson, A.L., Zhou, G., Hipkiss, E.L., Ravi, S., Kowalski, J., Levitsky, H.I., et al. (2004). Role of LAG-3 in regulatory T cells. Immunity *21*, 503–513.

Huard, B., Tournier, M., Hercend, T., Triebel, F., and Faure, F. (1994). Lymphocyte-activation gene 3/major histocompatibility complex class II interaction modulates the antigenic response of CD4+ T lymphocytes. Eur. J. Immunol. *24*, 3216–3221.

Huard, B., Prigent, P., Tournier, M., Bruniquel, D., and Triebel, F. (1995). CD4/major histocompatibility complex class II interaction analyzed with CD4- and lymphocyte activation gene-3 (LAG-3)-Ig fusion proteins. Eur. J. Immunol. *25*, 2718–2721.

Huard, B., Prigent, P., Pagès, F., Bruniquel, D., and Triebel, F. (1996). T cell major histocompatibility complex class II molecules down-regulate CD4+ T cell clone responses following LAG-3 binding. Eur. J. Immunol. *26*, 1180–1186.

Huard, B., Mastrangeli, R., Prigent, P., Bruniquel, D., Donini, S., El-Tayar, N., Maigret, B., Dréano, M., and Triebel, F. (1997). Characterization of the major histocompatibility complex class II binding site on LAG-3 protein. Proc. Natl. Acad. Sci. USA *94*, 5744–5749.

Kim, M.S., Pinto, S.M., Getnet, D., Nirujogi, R.S., Manda, S.S., Chaerkady, R., Madugundu, A.K., Kelkar, D.S., Isserlin, R., Jain, S., et al. (2014). A draft map of the human proteome. Nature *509*, 575–581.

Kizuka, Y., Kitazume, S., Sato, K., and Taniguchi, N. (2015). Clec4g (LSECtin) interacts with BACE1 and suppresses Aβ generation. FEBS Lett. *589*, 1418–1422.

Kouo, T., Huang, L., Pucsek, A.B., Cao, M., Solt, S., Armstrong, T., and Jaffee, E. (2015). Galectin-3 shapes antitumor immune responses by suppressing CD8+ T cells via LAG-3 and inhibiting expansion of plasmacytoid dendritic cells. Cancer Immunol. Res. *3*, 412–423.

Li, Y., Hao, B., Kuai, X., Xing, G., Yang, J., Chen, J., Tang, L., Zhang, L., and He, F. (2009). C-type lectin LSECtin interacts with DC-SIGNR and is involved in hepatitis C virus binding. Mol. Cell. Biochem. *327*, 183–190.

Li, C.Y., Cao, C.Z., Xu, W.X., Cao, M.M., Yang, F., Dong, L., Yu, M., Zhan, Y.Q., Gao, Y.B., Li, W., et al. (2010). Recombinant human hepassocin stimulates proliferation of hepatocytes in vivo and improves survival in rats with fulminant hepatic failure. Gut *59*, 817–826.

Liu, Z., and Ukomadu, C. (2008). Fibrinogen-like protein 1, a hepatocyte derived protein is an acute phase reactant. Biochem. Biophys. Res. Commun. *365*, 729–734.

Liu, W., Tang, L., Zhang, G., Wei, H., Cui, Y., Guo, L., Gou, Z., Chen, X., Jiang, D., Zhu, Y., et al. (2004). Characterization of a novel C-type lectin-like gene, LSECtin: demonstration of carbohydrate binding and expression in sinusoidal endothelial cells of liver and lymph node. J. Biol. Chem. *279*, 18748–18758.

Mao, X., Ou, M.T., Karuppagounder, S.S., Kam, T.I., Yin, X., Xiong, Y., Ge, P., Umanah, G.E., Brahmachari, S., Shin, J.H., et al. (2016). Pathological α-synuclein transmission initiated by binding lymphocyte-activation gene 3. Science *353*, aah3374.

Matsuzaki, J., Gnjatic, S., Mhawech-Fauceglia, P., Beck, A., Miller, A., Tsuji, T., Eppolito, C., Qian, F., Lele, S., Shrikant, P., et al. (2010). Tumor-infiltrating NY-ESO-1-specific CD8+ T cells are negatively regulated by LAG-3 and PD-1 in human ovarian cancer. Proc. Natl. Acad. Sci. USA *107*, 7875–7880.

Pettitt, S.J., Liang, Q., Rairdan, X.Y., Moran, J.L., Prosser, H.M., Beier, D.R., Lloyd, K.C., Bradley, A., and Skarnes, W.C. (2009). Agouti C57BL/6N embryonic stem cells for mouse genetic resources. Nat. Methods *6*, 493–495.

Pihlgren, M., Dubois, P.M., Tomkowiak, M., Sjögren, T., and Marvel, J. (1996). Resting memory CD8+ T cells are hyperreactive to antigenic challenge in vitro. J. Exp. Med. *184*, 2141–2151.

Rotte, A., Jin, J.Y., and Lemaire, V. (2018). Mechanistic overview of immune checkpoints to support the rational design of their combinations in cancer immunotherapy. Ann. Oncol. *29*, 71–83.

Samusik, N., Good, Z., Spitzer, M.H., Davis, K.L., and Nolan, G.P. (2016). Automated mapping of phenotype space with single-cell data. Nat. Methods *13*, 493–496.

Schalper, K.A., Brown, J., Carvajal-Hausdorf, D., McLaughlin, J., Velcheti, V., Syrigos, K.N., Herbst, R.S., and Rimm, D.L. (2015). Objective measurement and clinical significance of TILs in non-small cell lung cancer. J. Natl. Cancer Inst. *107*, dju435.

Shevach, E.M. (2009). Mechanisms of foxp3+ T regulatory cell-mediated suppression. Immunity *30*, 636–645.

Sica, G.L., Choi, I.H., Zhu, G., Tamada, K., Wang, S.D., Tamura, H., Chapoval, A.I., Flies, D.B., Bajorath, J., and Chen, L. (2003). B7-H4, a molecule of the B7 family, negatively regulates T cell immunity. Immunity *18*, 849–861.

Stillman, B.N., Hsu, D.K., Pang, M., Brewer, C.F., Johnson, P., Liu, F.T., and Baum, L.G. (2006). Galectin-3 and galectin-1 bind distinct cell surface glycoprotein receptors to induce T cell death. J. Immunol. *176*, 778–789.

Tang, L., Yang, J., Tang, X., Ying, W., Qian, X., and He, F. (2010). The DC-SIGN family member LSECtin is a novel ligand of CD44 on activated T cells. Eur. J. Immunol. *40*, 1185–1191.

Triebel, F., Jitsukawa, S., Baixeras, E., Roman-Roman, S., Genevee, C., Viegas-Pequignot, E., and Hercend, T. (1990). LAG-3, a novel lymphocyte activation gene closely related to CD4. J. Exp. Med. *171*, 1393–1405.

van der Maaten, L., and Hinton, G. (2008). Visualizing data using t-SNE. J. Mach. Learn. Res. *9*, 2579–2605.

Walunas, T.L., Bruce, D.S., Dustin, L., Loh, D.Y., and Bluestone, J.A. (1995). Ly-6C is a marker of memory CD8+ T cells. J. Immunol. *155*, 1873–1883.

Williams, J.B., Horton, B.L., Zheng, Y., Duan, Y., Powell, J.D., and Gajewski, T.F. (2017). The EGR2 targets LAG-3 and 4-1BB describe and regulate dysfunctional antigen-specific CD8+ T cells in the tumor microenvironment. J. Exp. Med. *214*, 381–400.

Woo, S.R., Turnis, M.E., Goldberg, M.V., Bankoti, J., Selby, M., Nirschl, C.J., Bettini, M.L., Gravano, D.M., Vogel, P., Liu, C.L., et al. (2012). Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. Cancer Res. *72*, 917–927.

Workman, C.J., and Vignali, D.A. (2003). The CD4-related molecule, LAG-3 (CD223), regulates the expansion of activated T cells. Eur. J. Immunol. *33*, 970–979.

Workman, C.J., and Vignali, D.A. (2005). Negative regulation of T cell homeostasis by lymphocyte activation gene-3 (CD223). J. Immunol. *174*, 688–695.

Workman, C.J., Dugger, K.J., and Vignali, D.A. (2002a). Cutting edge: molecular analysis of the negative regulatory function of lymphocyte activation gene-3. J. Immunol. *169*, 5392–5395.

Workman, C.J., Rice, D.S., Dugger, K.J., Kurschner, C., and Vignali, D.A. (2002b). Phenotypic analysis of the murine CD4-related glycoprotein, CD223 (LAG-3). Eur. J. Immunol. *32*, 2255–2263.

Workman, C.J., Cauley, L.S., Kim, I.J., Blackman, M.A., Woodland, D.L., and Vignali, D.A. (2004). Lymphocyte activation gene-3 (CD223) regulates the size of the expanding T cell population following antigen activation in vivo. J. Immunol. *172*, 5450–5455.

Xu, F., Liu, J., Liu, D., Liu, B., Wang, M., Hu, Z., Du, X., Tang, L., and He, F. (2014). LSECtin expressed on melanoma cells promotes tumor progression by inhibiting antitumor T-cell responses. Cancer Res. *74*, 3418–3428.

Yamamoto, T., Gotoh, M., Sasaki, H., Terada, M., Kitajima, M., and Hirohashi, S. (1993). Molecular cloning and initial characterization of a novel fibrinogen-related gene, HFREP-1. Biochem. Biophys. Res. Commun. *193*, 681–687.

Yan, J., Ying, H., Gu, F., He, J., Li, Y.L., Liu, H.M., and Xu, Y.H. (2002). Cloning and characterization of a mouse liver-specific gene mfrep-1, up-regulated in liver regeneration. Cell Res. *12*, 353–361.

Yao, S., Zhu, Y., Zhu, G., Augustine, M., Zheng, L., Goode, D.J., Broadwater, M., Ruff, W., Flies, S., Xu, H., et al. (2011). B7-h2 is a costimulatory ligand for CD28 in human. Immunity *34*, 729–740.

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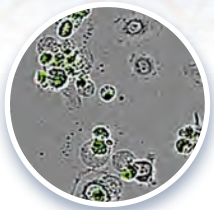

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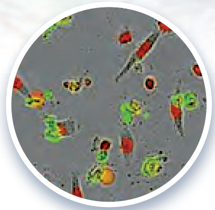

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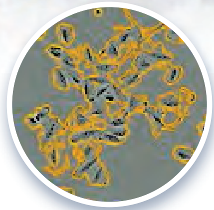

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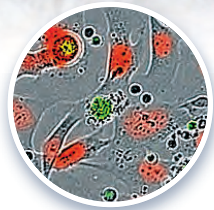

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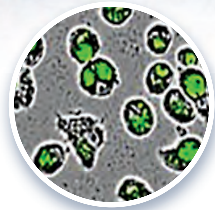

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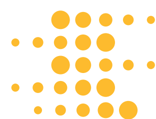

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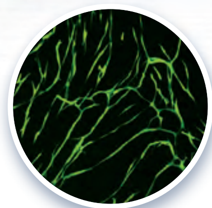
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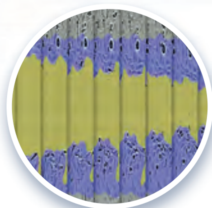
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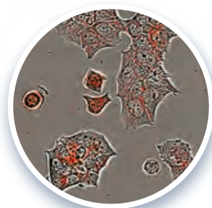
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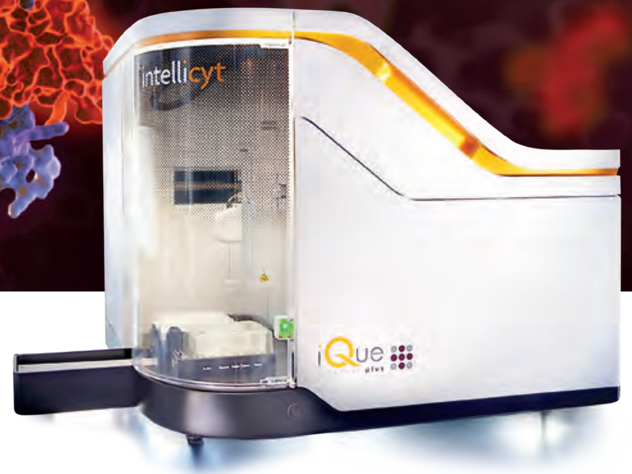

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