

TACKLING TUMORS

Turning Immune Cells into Cancer Killers

Tumors often contain a hodgepodge of cells. Some cells have genetic glitches, others don't; some obey normal growth rules, others divide out of

chasing answers, and many think the insights gained could revolutionize how we fight cancer.

The revolution has already begun.

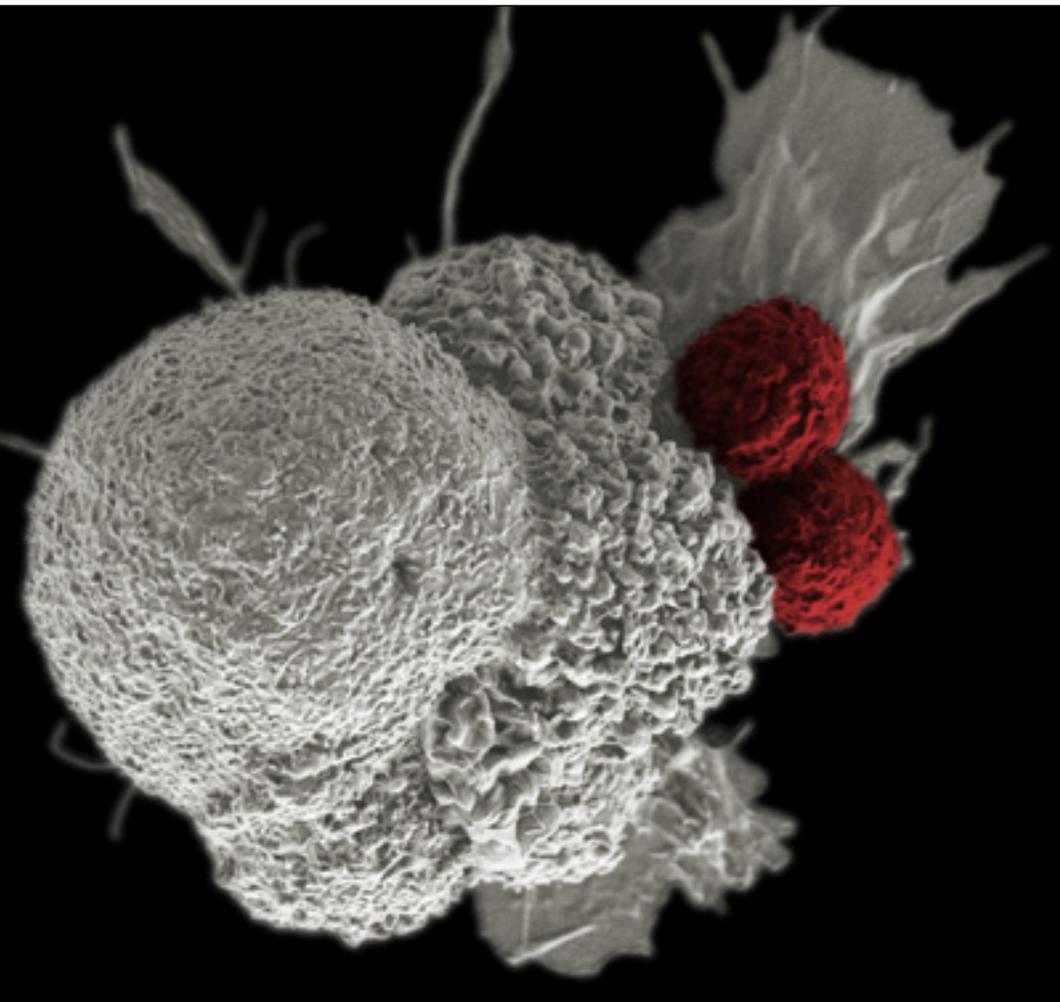
complex molecular profiling data can show how to activate key immune cells to fight cancer, why immunological cancer treatments work for some patients and not others, and which additional molecules could serve as potential targets for customized therapies to train people's immune cells to fight their own cancer.

Reprogramming Tumor Macrophages

Several types of immune cells, including macrophages and T-cells, turn traitorous in the face of cancer. Macrophages are scavengers. Typically they roam the body and chew up unwanted debris and dying cells, even cancerous ones. But macrophages also help with wound healing, and tumors can corrupt the scavenging cells to adopt this role inappropriately. With the switcheroo, macrophages now "see the tumor like a wound," says **Michele De Palma, PhD**, of École Polytechnique Fédérale de Lausanne (EPFL) School of Life Sciences in Lausanne, Switzerland. "They go there and help the tissue grow."

But in cancerous tissue, "healing" responses are harmful. Could macrophages be reprogrammed to act more like tumor killers?

A clue came several years ago when De Palma and coworkers discovered that macrophages at tumor sites accumulate miR-511-3p, a specific microRNA molecule (miRNA), and turn down a number of genes that are typically active in macrophages. Unlike typical RNA, miRNAs themselves don't get made into



In this pseudo-colored scanning electron micrograph, cytotoxic T cells (red) attack an oral squamous cell cancer (white) as part of a natural immune response. Source: National Cancer Institute \ Duncan Comprehensive Cancer Center at Baylor College of Medicine. Creator: Rita Elena Serda.

control. Immune cells enter the mix as well, initially swooping in to reject the tumor as they would other foreign substances. Later, however, these same cellular sentinels may inexplicably let down their guard, allowing the cancer to gain a foothold.

How do tumors outwit the body's defense system? Scientists are

Cancer treatments that harness the immune system are now a reality, and more are on the way. But with its many players and varied activities, the immune system's response to tumors, which are themselves evolving, often stymies understanding. In this setting, computational biologists are playing an important role. Plumbing

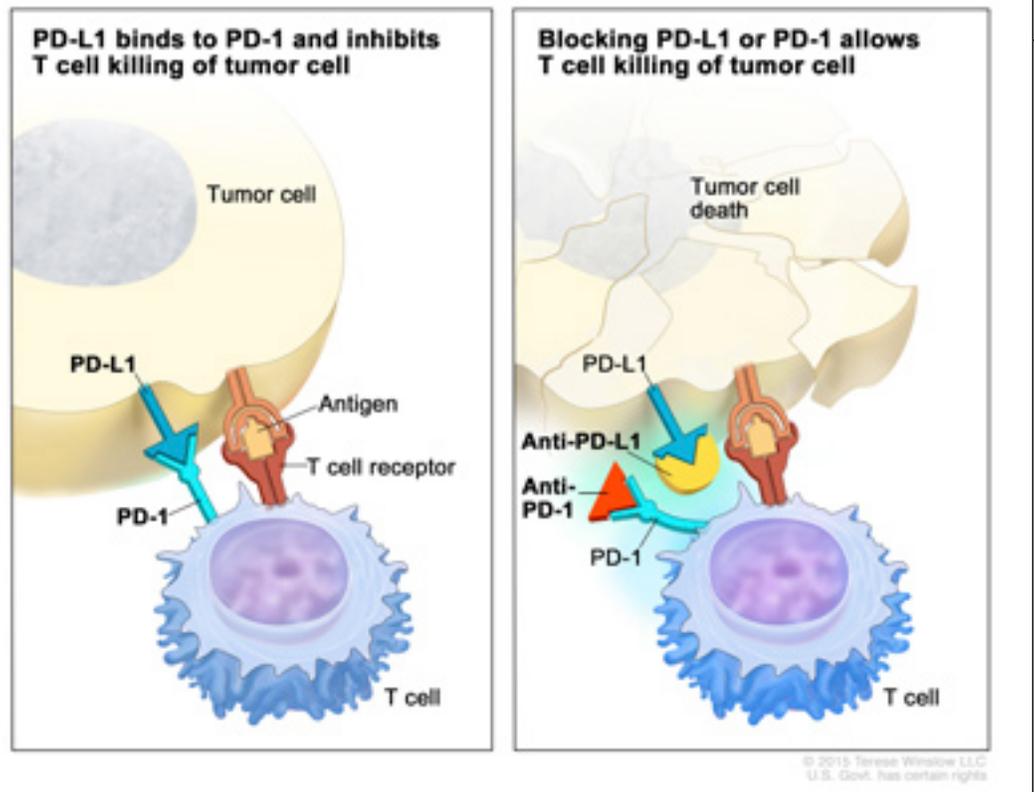
protein; instead, they dial back the activity of other genes by binding and preventing translation of their messenger RNAs (mRNAs). Discovered in the early 1990s, miRNAs play key roles in a range of developmental processes and in some human diseases—including cancer, where studies have linked changes in a cell's miRNA expression to its road toward malignancy. De Palma's finding suggests miR-511-3p regulates genes to render tumor-associated macrophages ineffective at fighting cancer.

The researchers wondered if shutting down miRNAs could shift macrophage behavior so they would fight tumors instead of ignore them. In a more recent study, they designed experiments to find out.

These studies used mice engineered for two traits: the tendency to grow cancerous tumors and an absence of DICER, an enzyme that is necessary for miRNAs to mature. Essentially, most miRNA activity is blocked in the macrophages of these mice. The result: a radical change in the macrophages' gene expression profiles and behavior. DICER-deficient tumor macrophages became "very nasty to tumors," DePalma says. They behaved like macrophages fighting a bacterial infection. And they didn't just battle the tumor alone—they recruited cytotoxic T cells to attack and eliminate the tumor, De Palma's team reported in June 2016 in *Nature Cell Biology*.

The team didn't stop there. Knowing macrophages have several hundred miRNAs, and having established that shutting them off turns bystander macrophages into tumor killers, De Palma wanted to find out which specific miRNAs were responsible for this turnaround—and that's where bioinformatics came in.

They used two approaches, one with mouse data and another with human data. In the first, the team isolated tumor-associated mouse macrophages with and without DICER and compared their



In cancerous cells, checkpoint proteins, such as PD-L1 on tumor cells and PD-1 on T cells, help keep immune responses in check. The binding of PD-L1 to PD-1 prevents T cells from "seeing" the tumor cell, allowing the cancer to grow unchecked (left panel). Blocking the PD-1/PD-L1 interaction with an immune checkpoint inhibitor (anti-PD-L1 or anti-PD-1) unshields the tumor cell so T cells can kill it (right panel). Source: National Cancer Institute. Printed with permission © 2015 Terese Winslow LLC, U.S. Govt. has certain rights.

transcriptomes to identify differentially expressed genes. This allowed them to determine which mouse mRNAs are targeted by specific miRNAs. The second strategy, which was developed in collaboration with co-author **Chia-Huey Ooi, PhD**, a bioinformatician at Roche in Basel, Switzerland, involved analyzing 171 publicly available blood samples from patients with acute myeloid leukemia (AML). For each sample, the researchers determined mRNA/miRNA signatures—whenever gene A gets expressed, miRNA B goes up—and used those signatures to predict which miRNAs potentially regulate the gene signature of macrophages in the DICER-deficient mouse tumor models.

Both approaches identified Let-7 as one of the miRNAs responsible for reprogramming macrophages into tumor tolerators. Wet-lab experiments confirmed the finding: When the researchers restored Let-7 miRNA activity in DICER-deficient tumor macrophages—in which the absence of DICER causes a broad miRNA shutdown—the macrophages reverted to ignoring the

tumor. Spurred by the new findings, the researchers are now working on using nanoparticles to block DICER or Let-7 activity in tumor-associated macrophages.

Finding Neoantigens

Whereas De Palma's experiments showed macrophages could be reinvigorated to lure killer T cells to tumor sites, existing cancer immunotherapy drugs spur T cells into action by targeting immune checkpoint molecules found on their surface. These therapies have brought lasting relief to former U.S. president Jimmy Carter and other patients with previously incurable cancers.

Key to developing these drugs was the discovery that a T-cell checkpoint molecule called PD-1 recognizes PD-L1 proteins on the surface of some cancer cells and tumor-infiltrating immune cells, particularly macrophages. Interaction between these molecules creates a "stealth shield" for the tumor, preventing the immune system from seeing it, explains **Richard Chen, MS, MD**, chief scientific officer at Personalis, a Silicon Valley genomics company. Checkpoint

inhibitors prevent those molecular interactions and break the stealth shield, making the tumor visible to immune cells.

But there's a vexing problem: Checkpoint blockade doesn't work for many eligible cancer patients. Even when a drug succeeds in "unshielding" a tumor, there's no guarantee a cell's particular collection of tumor peptides will actually trigger an immune response. "Each tumor is unique and complex," Chen says, and determining a tumor's immunogenicity poses a difficult problem.

To gauge which patients will respond to immunotherapy, Chen's team is using bioinformatics and machine-learning approaches to probe their tumor's genetic mutations and determine how the tumor is evading the immune system.

Tumor-specific peptides are a key determinant of immunogenicity. As tumor cells accumulate mutations, some give rise to tumor-specific mutant peptides, or neoantigens, that the immune system considers foreign. Studies have shown that tumors with more neoantigens stimulate stronger immune responses.

Personalis has developed a platform called ACE ImmunoID™, which combines DNA and RNA profiling to gauge a tumor's mutations and neoantigen load. While existing assays can screen for certain proteins known to be important for immunogenicity, such as PD-1 and PD-L1, Personalis' method achieves higher specificity and sensitivity. The patented technology optimizes chemistry and probes to fill in common sequencing gaps in DNA and RNA. In addition, it uses computational algorithms to predict, from the sequencing information, which mutations could result in neoantigens that are likely to bind major histocompatibility (MHC) proteins—the set of cell surface proteins that help the immune system recognize foreign molecules. Interaction with MHC is a key prerequisite for a neoantigen to be immunogenic, Chen says, so people with MHC-binding neoantigens are more likely to benefit from checkpoint blockade therapies.

The prediction algorithm uses a

machine-learning approach that includes neural networks trained using experimental data on thousands of peptides that do or don't bind well to MHC molecules. With enough input data, the machine-learning algorithm can learn to predict whether a new peptide is likely to bind to MHC. Strong neoantigens identified by this method could also help researchers design tumor-specific vaccines. "You'd have a completely personalized therapeutic," Chen says.

Similar tools are under development elsewhere. Researchers at the La Jolla Institute for Allergy and Immunology in California have created the Immune Epitope Database Analysis Resource (www.iedg.org), a collection of tools for predicting and analyzing immune epitopes in the context of T- and B-cell responses. Another group led by scientists at the Technical University of Denmark has developed NetMHC (<http://www.cbs.dtu.dk/services/NetMHC/>), which uses artificial neural networks to estimate the affinity of user-submitted peptide sequences to specific MHC alleles.

Probing Transcriptomes

Other researchers are taking a different approach to understanding why checkpoint blockades work in some patients and not others. Perhaps, they say, it has to do with the specific nature of a tumor's heterogeneity, including differences in the T cells that are present. Gene expression profiles of tumors as a whole can't address that. "If you take tumor tissue and grind it up for sequencing, all you detect is a mixture of signals," says **Benjamin Izar, MD**, an oncology fellow working with **Levi Garraway, MD, PhD**, at the Dana-Farber Cancer Institute, Boston, and at the Broad Institute of MIT and Harvard. "It's hard to say where the signal came from and what it means in the context of the tumor."

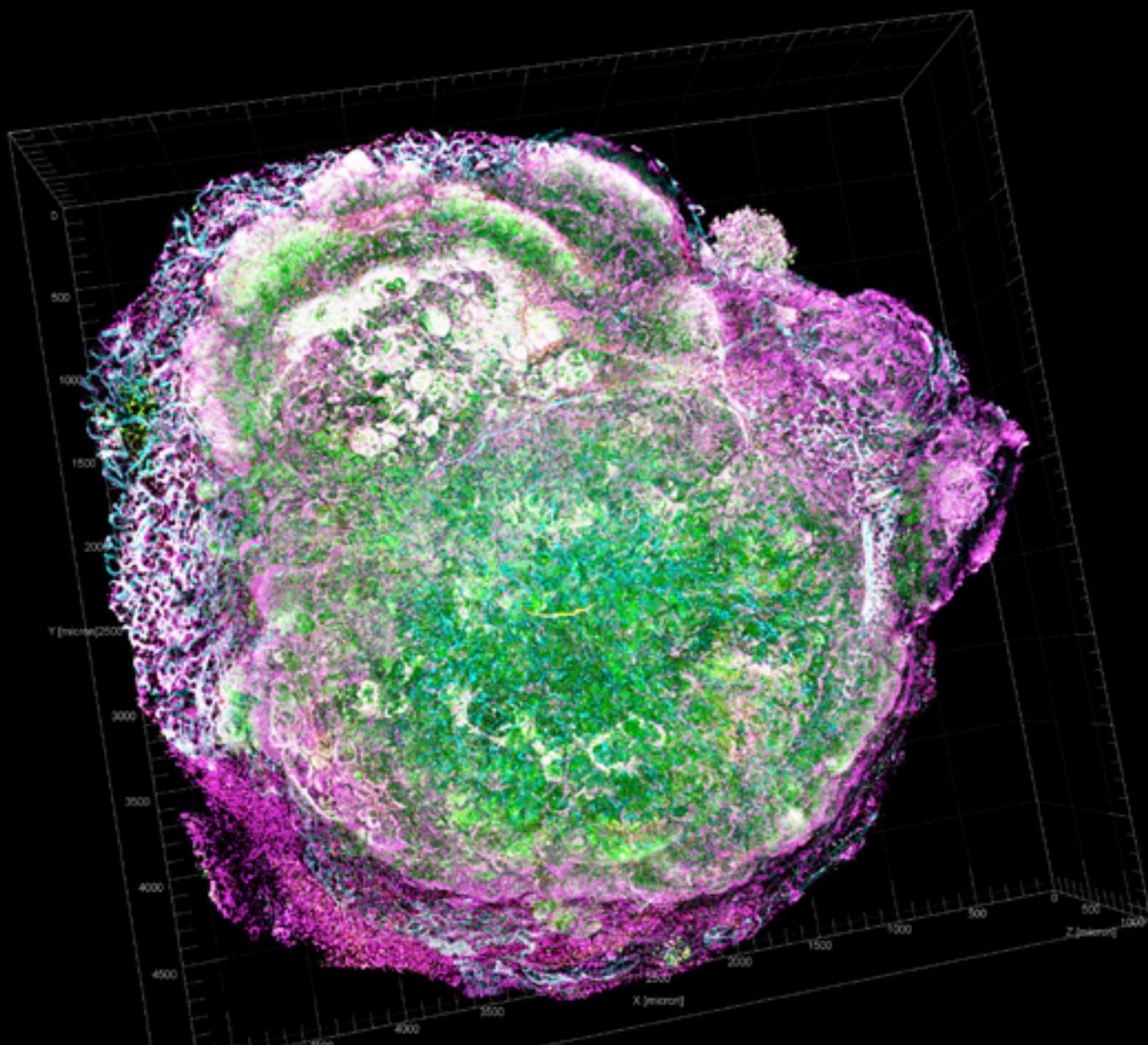
That's why Izar and Garraway teamed up with colleagues **Aviv Regev** and **Itay Tirosh** to perform single-cell RNA sequencing not just on cancerous cells but non-malignant types including immune

cells and connective tissue from patient tumors. "We wanted a broad, unbiased reflection of what is actually in the tumor," Izar says of their study published April 2016 in *Science*. In total the team analyzed 4,645 cells in tumors collected from 19 people with melanoma skin cancer. Some patients had never been treated for their cancer. Others had taken a drug designed to target melanoma cells with a specific mutation. Still others had received immune checkpoint inhibitors. The sequencing process yielded thousands of transcriptomes. The data were complex and noisy, says Izar. So they used various statistical and machine-learning methods to visualize and interpret the data.

Several interesting features jumped out when the researchers analyzed the transcriptomes of T cells, whose presence and function at tumor sites has been shown to predict responses to immune checkpoint therapies. Many of the tumor T cells expressed markers of "exhaustion," says Izar. Unlike normal cytotoxic T cells that help control cancer by recognizing key molecules on the surface of tumor cells, some T cells lose their fighting power, and this exhaustion is marked by transcriptional changes in specific genes.

Still, it can be hard to distinguish activated T cells from exhausted ones. Analyzing gene expression at the single-cell level could help identify better markers for truly exhausted T cells because those might be the patients who will respond to immunotherapies, says Izar.

In a paper published in August 2016 in *Genome Biology*, another Boston team, led by **X. Shirley Liu, PhD**, at the Dana-Farber Cancer Institute, also used RNA-sequencing data to evaluate the clinical impact of immune cells in various types of cancer. However, instead of directly measuring transcriptomes, the team analyzed published data from over 10,000 RNA-sequencing samples across 23 cancer types from The Cancer Genome Atlas. They developed a computational algorithm called TIMER (Tumor IMMune Estimation Resource) that estimates the



The heterogeneity of the tumor microenvironment plays a crucial role in allowing cancer to grow and evade destruction. This image of a mouse model for HER2-positive breast cancer uses a novel imaging technique called transparent tumor tomography that three-dimensionally illuminates the tumor microenvironment at a single-cell resolution. HER2 (green), Ki-67 (red), PD-L1 (purple), immune cells (yellow), and endothelial cells (cyan). Source: National Cancer Institute \ Univ. of Chicago Comprehensive Cancer Center. Creator: Steve Seung-Young Lee.

tumors' immune-cell composition and correlates the immune cells' presence and gene expression with clinical outcomes. The analysis found, unexpectedly, that the abundance of CD8 cytotoxic T cells does not always correlate with expression of CTLA4, an immune checkpoint protein sometimes targeted in checkpoint blockade immunotherapies. The researchers think this might explain why some patients don't respond to CTLA4-blocking treatments despite expressing high levels of CTLA4.

Though T cells and macrophages have been a big focus, other immune cells can also determine how well a patient responds to cancer immunotherapy

drugs. Using a computational approach called CIBERSORT, researchers led by Stanford oncologist **Ash Alizadeh** characterized the cell composition of around 18,000 human tumors by surveying their gene expression profiles. Their analysis, reported in a 2015 *Nature Medicine* paper, found complex relationships between 22 immune subset signatures and overall survival across 25 cancer histologies. For example, they found that people whose tumors contained high numbers of plasma cells (a type of immune cell) had a better prognosis, while those with a high concentration of neutrophils (another type of immune cell) tended to have a worse

outlook. The findings could be used to find new targets for cancer therapies—or to help predict patients' chances of responding to some existing treatments.

Using these and other diverse approaches, scientists hope to refine and identify additional molecular signatures in patient tumors to help predict responses to immunotherapies. But it won't be easy. "It's different from a cholesterol test where you measure one entity and you're done," Chen says. "When you're talking about genomics-based diagnostics there's significant complexity in the informatics and sequencing. There are multiple dimensions to the problem." □