Sweeney, Timothy (NIH/NCI) [E]

From: Arie Belldegrun <Arie@kitepharma.com>
Sent: Monday, January 05, 2015 7:48 PM
To: Rosenberg, Steven A. (NIH/NCI) [E]
Subject: FW: QUICK TAKE - KITE - Kite Gains Targets, Validation In Deal With Amgen - Cowen and Company

FYI – analysts interpretation of the deal

Thanks for the call today!

Arie

Arie Belldegrun, M.D.,FACS
President and CEO
Chairman, Board of Directors; Founder
Kite Pharma Inc.

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LINK TO FULL REPORT & DISCLOSURES

Biotechnology

Kite Pharma

Company Update

January 5, 2015

Price: $69.75 (01/5/2015)
Price Target: NA

OUTPERFORM (1)

Kite Gains Targets, Validation In Deal With Amgen

The Cowen Insight

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Kite and Amgen announced a partnership to develop a novel set of CAR T-cells directed at targets supplied by Amgen. Kite will receive $60MM in upfront payments, milestones, and royalties and the right to develop half of the drug candidates. We believe the deal broadens Kite's pipeline while providing validation for its platform and leading IP position. Kite remains a top pick.

Key Data

Symbol	NASDAQ: KITE
Market Cap (MM)	$2,918.1

The News: Earlier today Kite announced a collaboration with Amgen that combines Kite's CAR T-cell platform with Amgen's proprietary cancer antigen and antibody sequences. Under the collaboration, Kite will receive $60MM upfront and funding to cover all pre-clinical development. The companies will work together to develop CAR T-cells specific for multiple targets from a prespecified list. Management did not disclose the identity or number of targets on this list. However, it did disclose that all targets and antibody reagents are coming from Amgen, and that targets have already been divided 50:50 as either "Kite products" or "Amgen products". Kite is contributing its CAR T-cell manufacturing expertise and intellectual property exclusively to these targets. Following the filing of an IND, Kite will be responsible for developing its designated products. Successful development by Kite will trigger milestone payments to Amgen of up to $525MM along with tiered single-digit royalties for each product. Similarly, products designated as Amgen's will be developed at Amgen's expense. Successful development by Amgen will trigger milestone payments to Kite of up to $525MM along with tiered royalties beginning in the high single-digits and reaching double-digits for each product. A joint steering committee will facilitate the selection of CAR constructs (e.g. 2nd generation vs. 3rd generation),

election of substitute targets in the event of target failures, and coordination of clinical development programs across the partnership.

Our Take: Development of CAR T-cells is limited by the ability to (1) identify tumor specific antigens and (2) isolate antibodies specific to these antigens. Kite's ongoing collaboration with the National Cancer Institute has provided access to several targets including CD19 and others that remain outside the Amgen collaboration. Today's deal allows Kite access to a number of additional targets identified by Amgen's decades of research in oncology, as well as antibody constructs that can facilitate rapid creation of CARs. With Kite deploying its leading CAR T-cell manufacturing process and IP, the collaboration should create far more value than the sum of its parts. Importantly, we think Kite's ability to capture >50% of economics of the transaction recognizes the value Kite brings in terms of its significant IP position (and the Eshhar patent in particular) that others in the CAR space will likely also need to access.

What Is Next For KITE? Kite has submitted its corporate IND for KTE-C19's development in multicenter NHL trials, and expects to initiate a potentially pivotal Phase I/II trial in r/r DLBCL during Q1:15. Additional, potentially pivotal trials in MCL (H1:15), CLL (H2:15), and ALL (H2:15) are also planned. Data from these trials could support an initial approval of KTE-C19 as soon as 2016. Phase I/II data from Kite's first TCR product, targeting NY-ESO in solid tumors including synovial cell carcinoma, is expected to be presented at ASCO 2015.

Our Thesis on KITE Shares: Kite is a leader in the development of engineered T cells. Pivotal trials for Kite's first CD19 CAR will begin imminently. In addition, partnerships with Amgen and the National Cancer Institute should fuel a broad expansion of Kite's pipeline. Successful developments across any and all programs will further validate the engineered T cell approach and fuel significant share price appreciation.

Please see addendum of this report for important disclosures.

Sweeney, Timothy (NIH/NCI) [E]

From: Arie Belldegrun <Arie@kitepharma.com>
Sent: Monday, January 05, 2015 7:49 PM
To: Rosenberg, Steven A. (NIH/NCI) [E]
Subject: FW: STIFEL: KITE ($69.75, Buy) - Kite snags perfect partner for CAR-T target discovery in Amgen

One more analyst report FYI

Arie Belldegrun, M.D.,FACS
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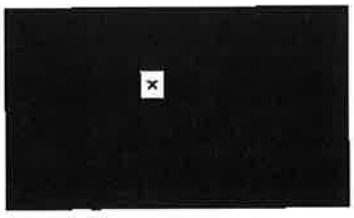
From: Lisa Burns [mailto:LBurns@burnsmc.com]
Sent: Monday, January 5, 2015 4:30 PM
To: Arie Belldegrun; Cynthia Butitta
Cc: Linda Barnes; Nancy Yu; Justin Jackson; Melissa Forst
Subject: Fwd: STIFEL: KITE ($69.75, Buy) - Kite snags perfect partner for CAR-T target discovery in Amgen

Sent from my iPhone

Begin forwarded message:

> **From:** Thomas Shrader <shradert@stifel.com>
> **Date:** 5 January 2015 7:08:07 pm GMT-5
> **To:** <lburns@burnsmc.com>
> **Subject: STIFEL: KITE ($69.75, Buy) - Kite snags perfect partner for CAR-T target discovery in Amgen**
> **Reply-To:** "Thomas Shrader" <shradert@stifel.com>



January 5, 2015

Kite Pharma, Inc.
KITE – NASDAQ
Buy

CLICK HERE FOR FULL REPORT

Kite snags perfect partner for CAR-T target discovery in Amgen

Kite today announced a development deal with Amgen to develop novel CAR-T technologies for new targets. The deal seems reasonably favorable for Kite as it involves a set number of targets that each side has "picked" from a communal pool. As a result, we believe Kite has been able to make use of Amgen's vast experience in target discovery and target validation – a key addition for a relatively new company with limited target discovery capabilities. The deal probably also validates both Kite's manufacturing and development capabilities for CAR-T products as well as Kite's accumulated intellectual property estate.

Mechanics of the deal – From the call, it sounds as if a pool of targets was assembled and the two sides took turns picking candidates they wanted. After choosing – each side will now be responsible for clinical development and commercialization of their chosen products. If chosen targets bomb for some reason, replacements can be chosen. For Kite products, Amgen is eligible for up to $525 million in milestones and tiered single digit royalties. For Amgen chosen products, Kite will be eligible for identical milestones and slightly higher royalties. Amgen will also pay Kite a one-time fee of $60 million. We have added the one-time fee to our model but not increased R&D expenses for now as we have long assumed additional targets.

Kite had unusual visibility and leverage - This is a particularly unusual deal to our eyes as we would normally not have expected Amgen to show Kite its best targets prior to the picking stage. In this case however, much of Kite's top R&D personnel recently came from Amgen, so we expect the picking may have been very equal. We view this equality of information as a huge plus for Kite in this type of deal (although we don't know that Amgen wasn't able to carve out certain targets at the start of the process). In addition, given the Kite-favoring economics of the deal, the agreement probably drives home just how impactful CAR-T and analogous technologies are viewed within the oncology community.

Target Price Methodology/Risks

We use a multiple of future earnings to derive our $71 target price for KITE. Specifically, to generate our valuation for development-stage biotech companies, we use a 30x multiple of future earnings, which represents a discount to the 20-year average earnings multiple for profitable biotech companies of 37x. Kite's valuation is driven primarily by KTE-C19, currently in Phase I/IIa testing. We apply a 27.5% discount rate, which we feel reflects the degree of uncertainty surrounding KTE-C19. With a multiple of 30x, we calculate a $71 target price based on our 2022 diluted EPS estimate of $16.61, discounted back 7.5 years.

Development risk for KTE-C19 - If Kite is not able to successfully develop the experimental CAR-T technology, we would have to lower our revenue estimates.

Competitive risk for KTE-C19 - If a competitor proves more effective or tolerable in the treatment of DLBCL, or their drugs are easier to produce, our estimates could prove optimistic.

Regulatory risk for KTE-C19 - The FDA has never approved a CAR-T-based therapy before and there exists no precedent for the approval of a genetically engineered autologous cell product. If KTE-C19 is not approved on the timeline that we envision, we would have to reduce our estimates.

Changes	Previous	Current
Rating	—	Buy
Target Price	—	$71.00
FY14E EPS	—	$(1.66)
FY15E EPS	$(1.02)	$0.20

Price (01/05/15):	$69.75
52-Week Range:	$70 – $21
Market Cap.(mm):	2,672.1
Dividend($ / %)	$0.00 / 0.0%

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Sweeney, Timothy (NIH/NCI) [E]

From: Arie Belldegrun <Arie@kitepharma.com>
Sent: Tuesday, January 06, 2015 2:39 PM
To: Rosenberg, Steven A. (NIH/NCI) [E]
Subject: FW: BWT
Attachments: BWT01062015.pdf

Hi Steve,
Please take a look at the front page Kite article.

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From: Linda Barnes
Sent: Tuesday, January 6, 2015 8:24 AM
To: Arie Belldegrun; Rizwana Sproule; Margo Roberts; David Chang; Jeff Wiezorek; Adrian Bot; Edmund Kim
Subject: BWT

BIOWORLD™ TODAY

THE DAILY BIOPHARMACEUTICAL NEWS SOURCE

JANUARY 6, 2015 BIOTECH'S MOST RESPECTED NEWS SOURCE FOR MORE THAN 20 YEARS VOLUME 26, NO. 3

IT'S NOT ABOUT THE MONEY: 'THE DEALS WE DO ARE STRATEGIC'

Isis Pharmaceuticals' shares climb as Janssen bets big in $835M gut drug deal

By Michael Fitzhugh, Staff Writer

Isis Pharmaceuticals Inc. shares (NASDAQ:ISIS) rose $6.60, or 10.7 percent, to $68.17 Monday as Johnson & Johnson-owned Janssen Biotech Inc. committed to pay it up to $835 million to discover and develop antisense drugs to treat autoimmune disorders of the gut. The deal gives Janssen options to license three therapeutic candidates in an area where it has established broad expertise with drugs such as Remicade (infliximab) while helping Isis continue to expand the breadth of antisense therapy's utility, further developing its potential for oral administration and local action, Isis

See Isis, page 3

Amgen finds CAR T ride with Kite Pharma immunotherapy alliance

By Jennifer Boggs, Managing Editor

Amgen Inc. is bolstering its immuno-oncology franchise and making its first foray into the hot chimeric antigen receptor (CAR) T-cell therapy field through an early stage deal with Kite Pharma Inc. that, if successful, could prove lucrative for both firms.

The plan is to apply Kite's CAR

See Kite, page 4

REGULATORY

Chinese regulators issue first draft guidance on biologics stability

By Cornelia Zou, Staff Writer

HONG KONG – Chinese pharmaceutical regulators will begin 2015 by taking aim at improving supervision of shelf-life studies and storage of biological products in order to enhance consumer safety.

See Stability, page 5

FINANCINGS

CANCER, HERE IS THY 'STING'

Oh boy: LADD gets $51.4M in Aduro series D financing; pancreatic phase II goes on

By Randy Osborne, Staff Writer

Tallying venture capital investments aplenty last year, Aduro Biotech Inc. chalked up more in a series D preferred

See Aduro, page 6

THE BIOWORLD BIOME

LIVE LONG AND . . . WHAT, EXACTLY?

Worm study has a skeptical view of life span extension's benefits

By Anette Breindl, Science Editor

The search for ways to increase life span is based on the idea that such an increased life span will extend the good parts of life – meaning, by and large, the

See Life span, page 7

IN THIS ISSUE

BIOSIMILARS

Extrapolation is big question for ODAC as it weighs first biosimilar

By Mari Serebrov, Regulatory Editor

Seemingly confident in the level of similarity between Sandoz Inc.'s Zarxio and Amgen Inc.'s Neupogen, the FDA has one voting question for the Oncologic Drugs Advisory Committee

See ODAC, page 8

FINANCINGS

VC flows big time with Moderna's record $450M financing

Peter Winter, BioWorld Insight Editor

There's nothing like starting the new year on a high note with news of a record venture financing. Cambridge, Mass.-based Moderna Therapeutics Inc. said it closed a whopping $450

See Moderna, page 9

FINANCINGS

Clementia Pharmaceuticals Inc., of Montreal, said it secured an additional $10 million from its current investors to support development of their lead compound palovarotene for the treatment of fibrodysplasia ossificans progressive (FOP). Led by Orbimed Advisors with participation by BDC Venture Capital, the new funds bring the total amount raised in a series A financing round to $32.5 million. The company closed on the initial $22.5 million in January last year. Palovarotene, an investigational retinoic acid receptor gamma agonist, is currently in a phase II trial in patients with FOP, a rare, severely disabling genetic disease characterized by painful, recurrent episodes of soft tissue swelling (flare-ups) and new abnormal bone formation. This process, known as heterotopic ossification, occurs in muscles, tendons and ligaments, causing significant morbidities and progressive disability. The company reports that it has established a wholly owned subsidiary, Clementia Pharmaceuticals USA Inc., in Newton, Mass., that will manage the company's operations in the U.S. (See *BioWorld Today*, Jan. 10, 2014.)

OTHER NEWS TO NOTE

Advaxis Inc., of Princeton, N.J., said it submitted an investigational new drug (IND) application to the FDA to conduct the first-in-human study of ADXS-HER2 (ADXS31-164) for the treatment of HER2 expressing solid tumors. Pending FDA's acceptance of the submission, the trial will be initiated in the first quarter and will evaluate the safety and tolerability of ADXS-HER2 in patients diagnosed with metastatic HER2-expressing solid tumors which include breast, gastric, esophageal and osteosarcoma.

Aldeyra Therapeutics Inc., of Lexington, Mass., said it submitted an investigational new drug application (IND) to the FDA to conduct phase II testing of NS2 for the treatment of Sjögren-Larsson Syndrome (SLS), a rare disease caused by mutations in fatty acid aldehyde dehydrogenase that lead to severe ichthyosis (scaly, thickened, dry skin), neurological disorders and retinal disease.

STOCK MOVERS 1/5/2014

Company	Stock in $	Change in %
Nasdaq Biotechnology	-$4.33	-0.14%
Conatus Pharmaceuticals	+$1.97	+24.35%
Galmed Pharmaceuticals	+$1.07	+18.35%
Intercept Pharmaceuticals	+$17.51	+10.98%
Isis Pharmaceuticals Inc.	+$6.60	+10.72%
Kite Pharma Inc.	+$9.14	+15.08%
Uniqure N.V.	+$2.45	+16.66%
Vitae Pharmaceuticals Inc.	+$2.65	+16.02%

Biotechs showing significant stock changes Monday

Alnylam Pharmaceuticals Inc., of Cambridge, Mass., said it filed a clinical trial application (CTA) with the Swedish Medical Products Agency to initiate a phase I trial with ALN-AS1, a subcutaneously administered investigational RNAi therapeutic targeting aminolevulinic acid synthase 1 (ALAS-1) for the treatment of hepatic porphyrias, including acute intermittent porphyria (AIP). The trial will be performed first in AIP patients who are asymptomatic "high excreters" – patients with a mutation in the porphobilinogen deaminase gene and elevated urinary aminolevulinic acid and porphobilinogen levels, but no recent symptoms of a porphyria attack – and then in AIP patients who experience recurrent porphyria attacks. The company expects to initiate the study in mid-2015, following approval of the CTA, with initial data expected to be reported in early 2016.

BIOWORLD TODAY

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Isis

Continued from page 1

CEO Stan Crooke told *BioWorld Today*.

Crooke called J&J the perfect partner, noting the company's deep understanding of inflammatory and autoimmune diseases of the gut and solid formulation experience.

The agreement covers three programs for which Isis will receive $35 million in up-front payments, including a payment to initiate human lead optimization on the first collaboration target. Isis is also eligible to receive nearly $800 million in development, regulatory and sales milestone payments and license fees for the programs.

Milestones for the programs are spread fairly evenly throughout, said Crooke, with a significant emphasis on the accomplishment of technical achievements along the way.

In addition, it will receive tiered royalties that on average are double digits on sales from any product that is successfully commercialized.

Janssen has the option to license a drug from each of the programs once a development candidate is identified. Should it exercise those options, it will assume global development, regulatory and commercialization responsibilities.

It's not the first time Isis has explored oral formulations of its drugs. In 2007, it demonstrated modest but significant bioavailability of an oral formulation of the homozygous familial hypercholesterolemia therapy Kynamro (mipomersen) – now partnered with Genzyme Corp. – that reduced apolipoprotein B100 and low-density lipoprotein-cholesterol. But while that study demonstrated the technical feasibility of oral dosing for antisense therapy, it wasn't commercially feasible at the time given the high doses required. Since then, said Crooke, the company's second-generation and generation 2.5 chemistry have boosted the potency of its antisense candidates while both lowering the required dosages and raising their bioavailability, bringing commercial viability to the fore.

The company has also explored locally targeted antisense therapies in the past with alicaforsen, an ICAM-1 inhibitor the company has since licensed to Atlantic Healthcare Ltd. Prior to that, Isis showed the drug could provide clinically significant relief to ulcerative colitis patients when delivered by way of an enema. (See *BioWorld Today*, Dec. 3, 2004.)

Though the focus of the Janssen deal is on the development of specific locally targeted oral therapies, Crooke said that "obviously everything you do along that trajectory is a step forward for oral administration in general."

While analysts had posited early last year that oral administration might be a big advantage for Kynamro competitor Aegerion Pharmaceuticals Inc.'s Juxtapid

See Isis, page 9

Kite

Continued from page 1

technology against a set of cancer targets amassed over the years by Amgen. The Thousand Oaks, Calif.-based biotech has "invested a huge amount of resources and has acquired companies and IP" to build a portfolio of cancer targets that is "second to no other companies," said Arie Belldegrun, chairman, president and CEO of Kite.

"Amgen is sitting today on a powerhouse of targets, and we're excited to have the opportunity to translate these validated targets in the CAR space," he told *BioWorld Today*.

The targets are a "tremendous addition," added David Chang, Kite's executive vice president of research and development and chief medical officer, who has more than a little familiarity with Amgen. Prior to joining Kite's executive team, Chang spent more than a decade at the big biotech, including a stint as vice president of global development and head of hematology-oncology.

News of the deal sounded good to investors, too. Kite's shares (NASDAQ:KITE) climbed steadily throughout the day to close at $69.75 Monday, up $9.14, or 15 percent. The stock has gained a whopping 165 percent since its debut in June as one of the top initial public offerings of 2014. (See *BioWorld Insight*, Jan. 5, 2015.)

CAR T-cell technology has emerged as one of the most promising approaches in the area of cancer immunotherapy – another CAR T-cell player, Juno Therapeutics Inc., also did extremely well in the initial public offering market in 2014, pricing a $264 million offering in December. (See *BioWorld Today*, Dec. 22, 2014.)

Based on the idea that engineered T cells can genetically endow a patient's own T cells with a receptor allowing them to better recognize tumor cells, the most advanced programs – Novartis AG's CTL019 and Kite's own KTE-C19 – have so far wowed investors. KTE-C19, for instance, demonstrated impressive phase I/IIa data in B-cell malignancies, and the company filed late last year to start a pivotal trial in diffuse large B-cell lymphoma (DLBCL). (See *BioWorld Today*, Aug. 27, 2014.)

But for now KTE-C19 remains the sole property of Kite. In fact, none of the firm's existing programs are included in the Amgen deal; "this is about future drug development rather than existing [development]," Belldegrun explained.

Under the terms, Kite gets $60 million up front, and Amgen also agreed to cover R&D funding costs, as Kite uses its autologous eACT platform, which genetically modifies patients' T cells to express cancer-targeting receptors that recognize and destroy cancer cells, to advance programs to the investigational new drug application (IND) stage.

And Amgen's contributions also include existing reagents, which should help speed up the preclinical process.

Each company already has pre-selected a list of targets – the specific targets and number of targets could not be disclosed – and will be responsible for filing INDs for respective programs, Chang explained.

From there, each company will be responsible for its own clinical, regulatory and commercialization activities. Santa Monica, Calif.-based Kite will be entitled to up to $525 million in milestones per Amgen program, while Amgen will be in line for the same consideration – up to $525 million in milestones – per Kite program.

For any Amgen products that make it to market, Kite will be eligible for tiered high single- to double-digit royalties. Amgen is eligible for tiered single-digit sales royalties from Kite programs.

The deal is a bit different from the typical licensing and collaboration arrangement in that "each side will get their own unencumbered assets," Chang told *BioWorld Today*.

Meanwhile, Kite is looking ahead to a series of pivotal trials this year with its anti-CD19 candidate, KTE-C19. In addition to the DLBCL study, expected to start this quarter, the firm plans trials in mantle cell lymphoma, chronic lymphocytic leukemia and acute lymphocytic leukemia. The firm, which had $195.4 million as of Sept. 30 and added another $216.6 million in a November public offering, has no plans yet to partner KTE-C19.

"In the U.S., we think we are ready to take it all the way," Chang said. Europe, however, is a different story, and Kite might pursue at least a commercialization partner there.

BUILDING AN IMMUNOTHERAPY PIPELINE

That KTE-C19 was not on the menu in Amgen negotiations was disappointing to analyst Michael Yee, of RBC Capital Markets LLC. "We would have liked to also have seen a deal on the main CD19 drug," he noted in a research report, adding that Amgen had the capability to both afford it and commercialize it outside the U.S.

But RBC is "positive" on Amgen "doing more deals and [we] would like to see more," Yee added.

In the immuno-oncology space, Amgen scored a hit last year with the accelerated approval – more than five months ahead of its May 19, 2015, PDUFA date – for Blincyto (blinatumomab), a CD19-directed CD4 T-cell engager, or BiTE, antibody, validating its 2012 $1.16 billion buyout of Rockville, Md.-based Micromet Inc. (See *BioWorld Today*, Dec. 4, 2014.)

It also has submitted applications in both the U.S. and Europe for approval of talimogene laherparepvec, an investigational oncolytic immunotherapy, for the treatment of patients with regionally or distantly metastatic melanoma. The FDA has set a PDUFA date of July 28.

Shares of Amgen (NASDAQ:AMGN) closed Monday at $157.99, down $1.90, likely due to the release of briefing documents in which FDA reviewers backed approval of a biosimilar for Amgen's blockbuster leukocyte growth factor drug Neupogen (filgrastim) developed by Novartis AG unit Sandoz International GmbH. (See the story in this issue.) //

Stability

Continued from page 1

"For biologics, this is the first guideline on stability studies. China hasn't had any related regulations before," Wei Wei, staff reviewer at the Centre for Drug Evaluation (CDE) biologics department, told *BioWorld Today*. "We want to standardize this segment; that's why we're now filling the blank with a new draft guideline."

The CDE, which operates under the CFDA, has released the "Draft Technical Guideline for Stability Studies of Biological Products," which offers the first guidance on studies that determine the shelf life and storage of biologic products in China.

According to the country's Provisions for Drug Registration, manufacturers are required to submit stability study results for the registration of new biological products. The stability study is one of the most time-consuming steps in the research and development cycle of a biological product. A comprehensive, accurate and reasonably designed stability study is crucial to the successful registration and marketing of biologics.

The CFDA released a set of 16 guidelines on stability studies of chemical drugs in 2005 but didn't issue any corresponding regulations for biologics. The CDE has been reviewing international guidelines on stability studies for biologics, in addition to researching, drafting and discussing technical key points at several meetings for years leading up to the release of the draft guideline. "This guideline gives guidance to applicants on the design and result analysis of the stability study of drug substance, finished product and intermediates of biological products that have applied for clinical or marketing approval," the draft guideline stated.

A stability study is a very important step in both new drug registration and post-market studies of biological products. The molecular structure of active pharmaceutical ingredients (API) in biological products often contains multiple active groups that are chemically unstable. Once the active groups go through hydrolysis, enzymolysis or oxidation, the structural characteristics and bioactivity of a biological product will be greatly affected.

Stability studies aim to determine the reaction to different environmental changes including temperature, humidity and light for a drug's raw ingredients, intermediates and also the finished product. The storage conditions, retest periods and expiry date of a drug are based on its stability study. The study will also help decide whether the manufacturing method, preparation formula and packaging material of a drug are appropriate.

The stability study of a biological product usually includes long-term, accelerated and stress testing components. Long-term testing is the main reference for setting the storage method and expiry date of biological products while accelerated testing and stress testing supplement the long-term study by finding the products' reaction to higher temperature and extreme conditions.

Manufacturers should have a general plan or strategy before initiating the stability study, which should include aspects such as samples, testing conditions, study items, time period, transportation and result analysis, the guideline said. Additionally, testing samples should be taken from at least three different batches.

The guideline also noted that testing conditions such as temperature, humidity, light, multigelation, vibration, oxidation and pH value can be considered for yielding primary results. Long-term, accelerated and stress testing will be planned based on the primary results.

Study items that will possibly affect the quality, safety and efficacy of biological products such as biological activity, purity and protein content need to be included in the stability testing.

The guideline suggested that the time period for stability studies should be once every three months in the first year of testing, once every six months in the second year, and once annually from the third year on. Usually, stability test results intended for clinical trial applications can support the stability studies of actual clinical studies. Stability studies conducted during application for marketing approval are also considered as the basis of the product's storage conditions and shelf life.

The transportation of biological products should also be included in the stability study. Biologics often require cold-chain storage and transportation. Manufacturers should conduct stimulation studies while taking route, vehicle, distance, time, packaging, product placement and monitoring into consideration.

Result analyses of different product batches should be consistent, the guideline stated. The overall analysis of a biological product should be used to determine its storage conditions and shelf life.

The draft guideline, released on Dec. 25, applies to biological products in general, except for gene therapy and cell therapy products that need to be studied based on their individual characteristics. Comments will be accepted through Jan. 25. //

OTHER NEWS TO NOTE

Bone Therapeutics SA, of Gosselies, Belgium, said it has started a new research project to investigate combined osteoblastic cell-matrix products for the treatment of large bone defects resulting from trauma, bone disease or surgical procedures such as bone metastasis resection. The government of the Walloon Region has granted the company €1 million of non-dilutive funding, in the form of recoverable cash advances, to finance the research. In the project, titled MXB bioprinting, the cell-matrix scaffold will be tailored to the size and form of the bone defect and will be designed to mimic the natural bone in terms of shape, structure and biomechanical properties. Once implanted, the 3D patient-tailored matrix is intended to be progressively replaced by natural bone tissue, produced by the off-the-shelf osteoblastic cells as well as by the ones from the patient that will have been recruited at the site. The cells in the matrix will also be designed to stimulate the formation of new blood vessels by releasing factors that recruit endothelial cells.

Aduro

Continued from page 1

stock financing of $51.4 million for its immuno-oncology pipeline, an amount that brings the total haul for the Berkeley, Calif.-based firm in 2014 to $106.4 million.

Last summer's series C financing brought $55 million, enough to see the company into 2016, Aduro estimated at the time. "We've expanded the scope of our activities," Stephen Isaacs, chairman and CEO of Berkeley, Calif.-based Aduro, said Monday, and the cash now on hand will "allow us to do many more things, well into 2017." (See *BioWorld Today*, June 12, 2014.)

Aduro's lead regimen of CRS-207 and Gvax Pancreas is undergoing a 240-patient phase IIb trial called ECLIPSE, a rough acronym for "Safety and Efficacy of Combination Listeria/ Gvax Pancreas Vaccine in the Pancreatic Cancer Setting."

CRS-207 is based on Aduro's platform of live-attenuated double-deleted (LADD) Listeria monocytogenes strains, genetically modified to induce potent innate and T cell-mediated immunity, specific for tumor-associated antigens. Gvax Pancreas derives from human cancer cell lines genetically modified to secrete granulocyte-macrophage colony-stimulating factor. The phase IIb trial, at 20 sites in the U.S. and Canada, follows promising results from the first phase II study, disclosed at the American Society of Clinical Oncology 2014 Gastrointestinal Cancers Symposium. (See *BioWorld Today*, Jan. 15, 2014.)

In February 2013, Aduro acquired all the Gvax assets from Biosante Pharmaceuticals Inc., of Lincolnshire, Ill., including intellectual property and cell lines. Gvax first were developed by Cell Genesys Inc., which was acquired by Biosante in 2009 following disappointing phase III results. Aduro previously had licensed rights to two Gvax vaccines – Pancreas and Prostate – for use in combination with its Listeria-based approach. The buyout covered all potential uses and included vaccines for multiple myeloma as well as breast and colon cancers, also assuming rights to the existing agreement for Gvax Melanoma. Under the terms, Aduro paid Biosante $1 million up front and pledged milestone and royalty payments. (See *BioWorld Today*, July 1, 2009.)

Listeria, an intracellular bacterium, is taken up actively by antigen-presenting cells. "If you can modify the Listeria to make it safe and effective, it takes payloads where you want them to go," Isaacs told *BioWorld Today*. "Of course, we modify the Listeria to make it safe by taking out two genes to prevent it from spreading cell to cell or getting into hepatocytes, where most of the toxicity resides."

Doing this "in combination with chemotherapy, radiotherapy, or cellular vaccines, which is what we use in the ECLIPSE trial, you get a much broader response," he said. "You get an antigen-spreading effect, and you get to use the innate side of the immune system that Listeria stimulates to not only have a profound effect against the mesothelin antigen, which we engineer in, but also against a lot of the antigens that are present on Gvax that are specific to pancreatic cancer. So you get a three-way attack." Also soon to start at Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins is a study that incorporates the PD-1 immunotherapy nivolumab (Opdivo, Bristol-Myers Squibb Co.). Isaacs said researchers are "optimistic that we will see an even more profound improvement in overall survival [OS]," with that approach.

Meanwhile, the randomized, controlled ECLIPSE experiment will evaluate the safety, immune response and efficacy of Gvax Pancreas (with low-dose cyclophosphamide) paired with CRS-207 compared to chemotherapy or to CRS-207 alone in metastatic pancreatic cancer. The primary endpoint of the trial is OS. Regulators in the U.S. have awarded the regimen breakthrough-therapy status.

Enrollment in ECLIPSE has moved "well ahead of the schedule we projected," Isaacs said, attributing the "very brisk" sign-up to an increase in median life span already shown in a study with "a very significant hazard ratio and "p" value," plus much milder side effects from Aduro's immunotherapy. Patients "feel like they have the flu for 24-36 hours," he said, predicting that enrollment will be complete in the first half of this year and top-line data will be available in the first half of 2016.

UP NEXT, DIRECT-INJECT CDNS

CRS-207's target antigen, mesothelin, is also expressed by mesothelioma tumors, and Aduro is conducting a clinical trial of the LADD compound in unresectable malignant pleural disease, testing the drug in combination with standard-of-care chemotherapy (pemetrexed and cisplatin). First data from the phase Ib experiment found that most patients showed a clinical response to the combo approach.

"Originally that was a 16-patient trial," Isaacs said, with newly diagnosed, treatment-naïve patients twice vaccinated with CRS-207, then followed by standard chemo in four to six courses, after which they were vaccinated twice more. The outcome was 94 percent disease control, 12 patients showing an objective response and three stabilizing. "That's a lot more than one sees with chemotherapy alone," Isaacs noted, adding that progression-free survival turned up at an impressive 7.5 months. "We modified the investigational new drug application and amended it so that we can enroll up to 40 patients, and we're going to start a phase II trial in the second half of this year where we randomize this therapy against chemotherapy alone," he said.

Another LADD immunotherapy, ADU-623, stimulates expression of two antigens, EGFRvIII and NY-ESO-1, associated with high-grade glioma. A phase I trial with the compound as monotherapy is under way in collaboration with the Earle A. Chiles Research Institute at Providence Cancer Center in Portland, Ore. The trial will enroll up to 38 patients previously treated with standard-of-care therapy, evaluating three dose levels.

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Life span

Continued from page 1

healthy parts. The search is on for the Fountain of Youth, not the Fountain of Endless Dotage.

But researchers at the University of Massachusetts Medical School have taken a critical look at life-extending genes in a classical animal model for longevity studies, the roundworm *Caenorhabditis elegans*, and found that well-known longevity mutations that extended both average and maximum life span often had the animals spending a prolonged period of time in decline, rather than in their prime.

The findings appeared in the Jan. 5, 2015, online issue of the *Proceedings of the National Academy of Sciences*.

The FDA has never considered aging a valid endpoint, and drug developers, too, are interested in endpoints that are quicker to reach than an increased life span would be. As a result, there is little risk that research efforts going into the clinic will backfire and turn into treatments that ultimately result in patients who live longer while being in poor health the whole time.

But the work shows that for preclinical studies to give useful clues for clinical approaches, health span will need to be directly assessed, including in mammals, whose longer life spans (the average wild-type *C. elegans* lives a mere 20 days) makes such studies more challenging and more expensive.

Senior author Heidi Tissenbaum has been skeptical for some time of the idea that extending life span implies a longer healthy life – a skepticism that was initially not widely shared, especially by granting agencies.

For a time, "we did not have a lot of success in getting this type of work funded, because people have assumed that life span and health span are correlated, and you'll never find anything" to separate the two, Tissenbaum told *BioWorld Today*.

Once they did have funding, the authors started by trying to mimic, in worms, what is known in humans as the frailty index – an overall indicator of health that measures how far below optimum an individual is functioning.

The team settled on at several factors, including how well the animals were able to recover physiologically from different types of stress, how much they moved on their own, and how quickly and how well they moved when they were forced to do so.

Tissenbaum and her team looked at those indicators in four different *C. elegans* mutants with genetic changes that extend their average and maximal life spans, to see whether the mutants lived long, healthy lives – or just long ones.

Their results were somewhat sobering. There were some parameters where some mutants stayed healthier for a greater number of days than the wild-type animals.

But frequently, the mutants became frail just as quickly as wild-type worms, meaning that the healthy part of their life was no longer than before.

And even in those instances where the mutants did stay healthy longer than wild-type animals, because they lived longer overall, they also accumulated more sick days. "No matter what, we saw the frailty extension," Tissenbaum summarized the findings.

The mutants had changes in genes that are classical life span changing genes, including daf-2, which affects insulin/ insulin-like growth factor (IGF) signaling; eat-2, which mimics caloric restriction; and others.

Other studies on the relationship between life span and health span have been mixed. Tissenbaum said that where others have come to more sanguine conclusions on the relationship between life span and health span, it has been because they have compared animals that were the same chronological age. But if one animal's expected life span is 30 days and another 60, that comparison, she pointed out, is actually comparing a young animal with a middle-age one.

In their studies, Tissenbaum and her colleagues compared the health status of the different mutants at an age that was 80 percent of their expected life spans, which led to a more critical view of how well the mutants were faring in terms of their health.

There are limits to the study, she noted – other mutations of the genes she and her team studied, or other parameters, might show a better effect on health span.

But at the very least, the data make clear that "for future research, you can't just look at life span."

The studies also showed that aging is not uniform, but a tissue-specific process. "We couldn't find a single equation that fit all the [aging] data," Tissenbaum said. Among other things, the team plans to look for tissue-specific markers of health span.

For interventions aimed at treating diseases of aging, the work implies that "we have to learn how to get the health benefits without the negative benefits," she said. "If you're not changing the rate of anything, then you're just prolonging the time . . . spent in a frail state."

Quite aside from the fact that no one dreams of a long life characterized by decades of ill health, that frail state is the opposite of what's needed from a societal perspective, she added. "We simply cannot afford to keep people in a frail state." //

OTHER NEWS TO NOTE

Biomedx GmbH, of Heidelberg, Germany, said it entered a collaboration agreement with **Boehringer Ingelheim GmbH**, of Ingelheim, Germany, to establish a research group focusing on the identification of therapeutic concepts for treating patients with chronic obstructive pulmonary disease (COPD). Early career scientists from leading academic institutions worldwide will be invited to submit original project proposals in the field of epigenetics and COPD. Boehringer and Biomedx will jointly select the best ideas and talent to form a new research group within Biomedx's open innovation lab. Further details of the agreement and financial terms were not disclosed.

ODAC

Continued from page 1

(ODAC) that will weigh in Wednesday on what is likely to be the first U.S. biosimilar. That question is about extrapolation.

In filing its biosimilar application last year, Sandoz requested that Zarxio (filgrastim) be approved for all five indications on Neupogen's U.S. label, including use in adult patients with nonmyeloid malignancies, marrow transplants or acute myelogenous leukemia. However, aside from healthy volunteers, the Sandoz drug was tested only in breast cancer patients. In the EU, Sandoz's filgrastim biosimilar, marketed as Zarzio, was approved in 2009 for all of Neupogen's indications. It also was approved in Australia in 2013 and in Japan last year. (See *BioWorld Today*, July 25, 2014.)

Even though the FDA is asking the extrapolation question, agency reviewers said the results of extensive pharmacokinetics (PK) and pharmacodynamics (PD) similarity studies support Sandoz's contention that there are no clinically meaningful differences in the effectiveness of Zarxio, also known as EP2006, and Neupogen for all of the indications for which Neupogen is approved in the U.S.

The discussion could direct future extrapolation decisions and possibly even guidance. To date, the FDA has issued six draft guidances on biosimilars, but none of them deals with extrapolation. In the past, agency officials have said they would look at extrapolation on a drug-by-drug basis.

Globally, extrapolation is a bit of a gray area when it comes to biosimilars. Many countries with a biosimilar path allow extrapolation when the mechanism of action is the same for the indications, but a few countries frown on granting approval for any untested indication.

Although they won't be voting on it, ODAC members also will be asked Wednesday to discuss the level of similarity between the Sandoz drug and Neupogen, which was first approved by the FDA in 1991. The FDA reviewers found that the two drugs are "highly similar, notwithstanding minor differences in clinically inactive components," according to the agency briefing document for the meeting. (Neupogen includes acetate, whereas Zarxio's formulation includes glutamate.)

LOOKING FOR GUIDANCE

While the FDA seems poised to approve Zarxio, several questions remain to be answered – primarily because the agency has yet to issue guidance on biosimilar issues such as naming and labeling. The FDA had hoped to release draft guidance on biosimilar labeling and finalize some of its earlier guidances last year, but that didn't happen.

Since biosimilars, unlike generics, are similar rather than identical to the reference drug, the labeling likely would be different, the FDA's Leah Christl said in an interview for BioWorld's report *Biosimilars: A Global Perspective of a New Market – Opportunities, Threats and Critical Strategies 2014*. As for naming biosimilars, the agency has been reviewing comments so it can develop future policies. The Biologic Price Competition and Innovation Act (BPCIA), which outlined the U.S. biosimilar path in 2010, doesn't expressly require guidance on naming, so the FDA can approve biosimilars before it issues guidance on the subject, Christl said.

In lieu of final guidance, the FDA has been meeting with sponsors and fielding questions on what's needed to develop and market specific biosimilars. The discussion at this week's ODAC meeting and information in the briefing documents can shed some light on the FDA's thinking and its advice to biosimilar sponsors.

For instance, the document details the stepwise approach Sandoz used in developing Zarxio, which is produced by recombinant technology in *Escherichia coli*. As part of its development path, Sandoz conducted five PK/PD studies and two efficacy studies, starting in 2004 when it began developing its European biosimilar. Only one of the PK/PD studies and one of the efficacy studies used the U.S.-approved Neupogen.

Thus, the briefing document also provides a look at the bridging studies Sandoz conducted to scientifically justify the relevance of data from the EU studies. The company demonstrated the analytical similarity of its biosimilar, the U.S.-licensed Neupogen and the EU-approved Neupogen by evaluating several batches of each of the three products. The biosimilar batches analyzed included those used in the EU studies.

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Moderna

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million financing that will fuel and expand its messenger RNA (mRNA) therapeutics platform across a number of therapeutic indications.

The amount raised is one of the largest in the industry's history ranking just behind the $600 million provided in 2011 by the Russian government and investors in London-based private equity firm Celtic Pharma Holdings to launch Pro Bono Bio. (See *BioWorld Today*, Sept. 13, 2011.)

New investors participating in the financing round were Viking Global Investors LP, Invus, RA Capital Management, and Wellington Management Co. LLP, as well as existing investors Astrazeneca plc and Alexion Pharmaceuticals Inc., which at the beginning of January last year also purchased 10 product options to develop and commercialize rare disease treatments. The Cheshire, Conn.-based company paid $100 million up front for the options, in addition to making a $25 million preferred equity investment in Moderna. The company is entitled to development and commercial milestone payments, as well as high single- to double-digit royalties on sales, if Alexion exercises any of the options. (See *BioWorld Today*, Jan. 14, 2014.)

Moderna emerged from stealth mode just over two years ago with a $40 million financing led by Flagship Venturelabs and a consortium of private investors and since that time the company said it has secured more than $950 million in funding through financing activities and commercial partnerships.

The company also reported active development of 45 preclinical programs in oncology, cardiovascular disease, rare diseases and infectious diseases together with its partners Astrazeneca, Alexion and the Defense Advanced Research Projects Agency (DARPA).

Astrazeneca struck its alliance with Moderna in 2013 paying $240 million up front to gain exclusive access to select any target of its choice in cardiometabolic diseases, as well as selected targets in oncology, over a period of up to five years. The pharma company has the option to pick as many as 40 drug products for clinical development, and Moderna stands to gain milestone payments related to clinical and commercial progress, along with royalties on drug sales ranging from high single digits to low double digits for each product. (See *BioWorld Today*, March. 22, 2013.)

In addition to driving its current drug discovery and development program across a series of drug modalities and therapeutic areas, Moderna is planning to use its latest investment for growth and expansion, to add more than 100 industry leaders, drug development experts and scientists to its current team of 145 employees during the coming months. //

ODAC

Continued from page 8

NEXT STEPS

While it has until May to approve Zarxio under the biosimilar user fee agreement it negotiated with industry, the FDA is under pressure to approve its first biosimilar as soon as possible. But approval may not mean an immediate launch. Amgen has filed suit against Sandoz in federal court to force the biosimilar maker to comply with the patent exchange information mandated by the BPCIA – something Sandoz has tried to "opt" out of.

While Neupogen's composition patents have expired, "Amgen has an extensive portfolio of patents relating to various aspects of the manufacture of biological products" that could be infringed, according to the lawsuit. Sandoz's biosimilar also could infringe Amgen's '427 patent claiming the use of a combination of G-CSF with chemotherapy for stem cell mobilization.

That patent was issued Dec. 19, 2000, so it has a few more years of life. Amgen wouldn't know if Zarxio infringed its other patents unless Sandoz engages in the information exchange, the Thousand Oaks, Calif.-based company said. (See *BioWorld Today*, Oct. 31, 2014.)

Since the Neupogen patents expired earlier in Europe and other countries than they did in the U.S., several filgrastim biosimilars are already on the market elsewhere. The EU alone has approved nine filgrstim biosimilars since 2008. Despite the competition, Sandoz's Zarzio ranks No. 1 in the global daily G-CSF market, claiming 30 percent of that market. It also is the first biosimilar to overtake its reference biologic. //

Isis

Continued from page 3

(lomitapide), when sales of the drugs were compared in November 2014, the route of administration turned out to be far less important than other factors, including lower U.S. prescription growth, analysts said. (See *BioWorld Today*, Nov. 3, 2014.)

But Isis is focused elsewhere: It has continued to move ahead with Genzyme, Biogen Idec Inc. and Glaxosmithkline plc, building a platform that has put the company on solid footing with a reported $73.2 million in cash and cash equivalents at Sept. 30, 2014.

Investors too are paying close attention to the company's more near-term catalysts, including phase III studies of Biogen-partnered ISIS-SMNRx for spinal muscle atrophy, GSK-partnered ISIS-TTRRx for TTR amyloidosis and the unpartnered ISIS-APOCIIIRx program, which could help patients with familial chylomicronemia syndrome.

"These days, we don't really need to do deals for dollars," said Crooke. "We're in a position where the deals we do are strategic." //

Aduro

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In non-small-cell lung cancer, Aduro has ADU-214, designed to stimulate an immune response against mesothelin and an EGFRvIII mutation common in several solid tumor malignancies. Last October, Aduro granted Janssen Biotech Inc., a unit of New Brunswick, N.J.-based Johnson & Johnson, an exclusive worldwide license to ADU-214 plus an exclusive right to develop LADD strains in lung cancer indications. Janssen provided $30 million up front, plus up to $787 million in milestone payments. The deal followed Aduro's exclusive licensing of preclinical prostate cancer candidates, including ADU-741, to Janssen in May for up to $365 million. (See *BioWorld Today*, Oct. 17, 2014.)

"There are specific strains of Listeria that we've developed for those two [glioma and lung] indications," Isaacs said. "You can take Listeria and change it around and put in different antigens that are appropriate for different types of cancer." The base, LADD strain, which is attenuated by about a thousand-fold, "acts as a DVD player, and the DVDs are the different antigens" for assorted cancer types, he said. "We're looking at a whole host of other indications that we can use with different antigens. How much we would get into partnering those now is something we're thinking about, but we do want to hold onto a lot of this for some time." In the pancreatic space, with ECLIPSE, "we're definitely looking at keeping that through data readout and maybe longer," he said.

Separately, Aduro has a program with cyclic dinucleotides (CDNs), small molecules naturally expressed by bacteria and immune cells that are known to activate the stimulator of interferon genes (STING) signaling pathway in immune cells. A central mediator of innate immune response, STING when stimulated induces the expression of various interferons, cytokines and T-cell recruitment factors that amplify and strengthen immune activity.

Aduro first made "superior CDN derivatives," Isaacs said, and then began experiments against B16 melanoma in mice. "We're in the middle of good laboratory practices toxicology tests now," and the lead compound has been manufactured, he said, forecasting that a drug candidate will enter the clinic in the second half of this year for a dose-escalation safety study, "but one always hopes you'll see hints of efficacy." First-targeted cancers are melanoma and head-and-neck, mainly because they are accessible by the direct-inject method, which could also be used in lymphoma and breast cancer. "We're also looking at ways to systemically deliver these compounds through nanoparticle formulations, or other ways to encapsulate and deliver [them]," Isaacs said. "We're also looking at potentially conjugating them to antibodies. There are lots of different ways we can see getting the CDNs to where we want them to go. The intratumoral injection is really just the start of that process."

The CDN approach is "really a standalone opportunity for us, not necessarily connected to Listeria, though there are combinations there that we are looking at, too," Isaacs said, adding that Aduro has seen "a lot of interest in the program from the players you might expect" and is considering its options.

Isaacs said Monday that he had just distributed a start-of-year memo to employees. "The nature of the message was, 'These are the good old days,'" he said. "As companies go – and I've been doing this for a long time – we're at a very positive place, with a very positive slope. Immuno-oncology is where it's at in cancer therapy today. The sky is very blue right now for us."

Eleven new investors participated in the series D financing, including Orbimed, Janus Capital Management LLC, funds managed by Franklin Advisers Inc., Jennison Associates LLC (on behalf of clients), Foresite Capital Management LLC, private investment funds advised by Clough Capital Partners L.P., and other health care investors. The Morningside group and some of the company's existing investors also participated in the financing. Leerink Partners LLC acted as placement agent in the series D round. //

OTHER NEWS TO NOTE

Celator Pharmaceuticals Inc., of Ewing, N.J., received approximately $1.94 million from the sale of its net operating losses under the New Jersey Technology Business Tax Certificate Transfer Program for the year 2014. The program enables unprofitable New Jersey-based tech and biotech firms to sell their unused net operating loss carryovers and unused research and development tax credits to unaffiliated, profitable corporate taxpayers for at least 80 percent of the value of the tax benefits. Celator remains on track to have the induction response rate data from the phase III study of CPX-351 in patients with high-risk (secondary) acute myeloid leukemia in the second quarter of 2015 and expects to have overall survival data, the primary endpoint of the study, in the first quarter of 2016.

IN THE CLINIC

Brainstorm Cell Therapeutics Inc., of New York and Petach Tikvah, Israel, finalized positive results from a 14-patient phase IIa trial of Nurown in amyotrophic lateral sclerosis (ALS) patients, saying the study achieved its primary endpoint in demonstrating that the therapy is safe and well-tolerated at doses up to 2 million cells per kilogram administered intrathecally and 48 million cells administered intramuscularly. Nearly all patients in the study experienced clinical benefit from the treatment. Of the 12 subjects with three or more months of follow-up, 92 percent experienced an improvement in the rate disease progression for the three-month period after administration of the therapy, as measured by ALS Functional Rating Score-Revised (ALSFRS) or forced vital capacity (FVC). Fifty percent had an improvement in the slope of the ALSFRS score, and 67 percent had an improvement in the slope of the percent-predicted FVC. Brainstorm shares (NASDAQ:BCLI) fell $1.75, or 23.3 percent, to $5.75 Monday after rising to a 52-week high of $8.47 Jan. 2.

OTHER NEWS TO NOTE

Celladon Corp., of San Diego, reported that it conducted initial scale-up of its viral manufacturing process for Mydicar (AAV1/SERCA2a) to commercial scale. The scale-up of primary production and downstream processing development to 2,000 liters was undertaken together with Lonza, in its facility in Houston. The achievement of completing a demonstration batch represents the first industrial-scale production of a gene therapy vector, according to Celladon.

Compugen Ltd., of Tel Aviv, Israel, reported positive initial experimental results for the first two of five in silico predicted targets for antibody drug conjugate (ADC) cancer therapy disclosed in late 2013. The two candidates demonstrate low expression levels in normal critical tissues, such as heart and liver, and higher expression in multiple cancer types, such as colorectal and prostate cancers, for which there is high unmet medical need. The results suggest that the two target candidates may serve for the development of ADC therapy in oncology. Initial validation of the remaining three candidates, and further testing of the two, is ongoing. It is expected that a therapeutic antibody discovery program against a selected ADC target will commence later this year.

Immunomedics Inc., of Morris Plains, N.J., has received fast track designation for sacituzumab govitecan, the company's lead antibody-drug conjugate (ADC), for the treatment of patients with triple-negative breast cancer (TNBC) who have failed prior therapies for metastatic disease. Sacituzumab govitecan (IMMU-132) was developed by Immunomedics by conjugating SN-38, site-specifically and at a high ratio of drug to antibody. SN-38 is the active metabolite of irinotecan, which is used to treat certain solid cancers, particularly metastatic colorectal cancers, as a part of combination therapies.

Lion Biotechnologies Inc., of Los Angeles, filed an investigational new drug application with the FDA for a phase II trial with the lead product candidate, LN-144, in the treatment of patients with refractory metastatic melanoma. LN-144 is a cell product of autologous tumor infiltrating lymphocytes derived from the patient's tumor.

Naia Ltd., of Greenbrae, Calif., disclosed its formation along with in-licensing agreements with **Amunix Operating Inc.**, of Mountain View, Calif., for its GLP-1 and GLP-2 Xten receptor agonist product candidates, plus a licensing agreement with Cedars-Sinai Medical Center in Los Angeles for a use patent for GLP-1. Assets are being pursued by two subsidiaries, Naia Rare Disease Inc. and Naia Metabolic Inc.

Oncoceutics Inc., of Hummelstown, Pa., and the University of Texas MD Anderson Cancer Center signed a strategic alliance and research collaboration agreement for the clinical development of ONC201, an anticancer drug. Preclinical studies of ONC201 have indicated there is merit in further investigation of the drug, which appears to kill cancer cells without harming healthy cells. The Oncoceutics and MD Anderson agreement will result in clinical trials in specific hematologic tumors.

Pharmamar SA, of Madrid, said **Taiho Pharmaceutical Co. Ltd.**, of Tokyo, Pharmamar's partner for Yondelis (trabectedin) development and sales in Japan, has filed a marketing authorization application that seeks approval of Yondelis for the treatment of different subtypes of advanced soft tissue sarcoma in Japan, given the clinical benefit shown in a pivotal phase II study. In November, Janssen Research & Development LLC, a unit of New Brunswick, N.J.-based **Johnson & Johnson**, submitted a new drug application seeking FDA approval of Yondelis for use in soft-tissue sarcoma, including liposarcoma and leiomyosarcoma subtypes who have received prior chemotherapy, including an anthracycline. Janssen also disclosed plans to amend the protocol for the phase III study on which the application is based.

Protalix Biotherapeutics Inc., of Carmiel, Israel, disclosed a new strategy for accelerated growth, centered on prioritizing existing and new pipeline candidates to focus on products with potentially clinically superior profiles that offer a clear competitive advantage, the company said. Candidates are targeted at indications such as Fabry disease, inflammatory bowel disease, cystic fibrosis and Gaucher disease.

Relypsa Inc., of Redwood City, Calif., said the FDA assigned a PDUFA date of Oct. 21, 2015, for patiromer for oral suspension in the treatment of hyperkalemia, or abnormally elevated levels of potassium in the blood. The company had disclosed last month that the new drug application was accepted for review, and noted that the agency was not planning an advisory committee meeting related to the compound.

Sirnaomics Inc., of Gaithersburg, Md., said its Chinese affiliate together with partner **Guangzhou Xiangxue Pharmaceutical Co. Ltd.** formally submitted an investigational new drug application (IND) to the CFDA for STP705, an anti-fibrosis RNA interference (siRNA) therapy for prevention and treatment of human skin hypertrophic scars. The anti-fibrogenic activity of STP705 has been validated in human hypertrophic scar implant models and in mouse and pig skin excisional wound and burn models. It consists of two siRNA sequences targeting two genes critically involved in fibrogenesis and packaged in a polymer nanoparticle formulation for delivery. According to the company, it's the first siRNA therapeutic candidate subject of IND filing as a type 1.1 (innovative) new drug in China. (See *BioWorld Today*, Aug. 10, 2010, and June 26, 2012.)

Soligenix Inc., of Princeton, N.J., said preliminary efficacy in an animal model of macrophage activation syndrome (MAS) has been demonstrated with its SGX94 innate defense regulator technology, significantly mitigating the pancytopenia characteristic of MAS. In animal models where MAS was induced by repeated Toll-like receptor-9 stimulation, SGX94 was shown to mitigate the disease characteristic pancytopenia by significantly increasing both white blood cell ($p = 0.01$) and platelet ($p < 0.001$) counts in the SGX94-treated animals as compared to placebo-treated animals.

OTHER NEWS TO NOTE

Symic Biomedical Inc. has formed a strategic alliance with **Nordic Bioscience A/S** to focus on the clinical development of its osteoarthritis (OA) program. The deal follows closely on the heels of the San Francisco company's $15 million series A financing earlier this month. Nordic, of Copenhagen, Denmark, has experience running clinical programs in OA for Novartis Pharma AG and Merck Serono. Under terms of the agreement, it will provide clinical development services to Symic on a shared-risk basis in exchange for a payment structure that includes service fees and equity in Symic's OA subsidiary. Symic retains responsibility for the development and commercialization of the products. Financial terms of the agreement were not disclosed. (See *BioWorld Today*, Jan. 5, 2015.)

Synageva Biopharma Corp. shares (NASDAQ:GEVA) rose $6.34, or 6.7 percent, Monday to close at $100.48, as the company announced new pipeline programs and other progress ahead of the global launch of Kanuma (sebelipase alfa) for lysosomal acid lipase deficiency and initiation of a phase I/II study of SBC-103, an investigational enzyme replacement therapy for mucopolysaccharidosis IIIB, also known as Sanfilippo B syndrome. The company said it now plans to develop SBC-105, a preclinical enzyme replacement therapy for treating rare disorders of calcification, including the first planned indication, generalized arterial calcification of infancy; will work to develop generate proof-of-concept data supporting clinical advancement of at least one enzyme targeting Hunter syndrome, Fabry disease and Pompe disease; and expects to advance other preclinical protein therapeutic pipeline programs for other rare diseases. (See *BioWorld Today*, March 7, 2014, and July 2, 2014.)

IN THE CLINIC

Cel-Sci Corp. shares (NYSE:CVM) rose 7 cents, or 11.3 percent, to 69 cents Monday, as the company reported that in 2014 it enrolled almost 200 patients with advanced primary, untreated head and neck cancer into its global pivotal phase III head and neck cancer trial for its investigational immunotherapy Multikine (leukocyte interleukin). The enrollment represented an eightfold increase over enrollment of 24 patients in 2013, the year during which the Vienna, Va.-based company dismissed its prior clinical research organization and replaced it with new CROs, Aptiv and Ergomed. The company said that it expects to enroll a total of 880 patients in about 20 countries by the end of 2015 in the world's largest phase III trial for head and neck cancer.

Cempra Inc., of Chapel Hill, N.C., reported positive topline results from a pivotal phase III trial of Solitaire (solithromycin), its oral antibiotic for the treatment of patients with community-acquired bacterial pneumonia (CABP). About three days after treatment, the drug appeared non-inferior to Bayer AG's moxifloxacin. Solithromycin also met the secondary objectives of non-inferiority in clinical success at the short-term follow-up (SFU) visit, 5-10 days after the end of therapy. The double-blind, active-controlled, global, multicenter trial enrolled 860 adult patients with moderate to moderately severe CABP. Cempra shares (NASDAQ:CEMP) rose $1.66, or 7.3 percent, to close at $24.55 Monday. (See *BioWorld Today*, Dec. 20, 2012, and Dec. 18, 2013.)

Oncomed Pharmaceuticals Inc., of Redwood City, Calif., said it dosed the first patient in its phase Ia trial testing anti-DLL4/VEGF bispecific antibody designed to have both anticancer stem cell and anti-angiogenic activity. The single-agent, open-label, dose-escalation study is enrolling patients with advanced refractory solid tumors and will assess safety, pharmacokinetics, pharmacodynamics and initial evidence of efficacy.

Pharmacyclics Inc., of Sunnyvale, Calif., said treatment with single-agent Imbruvica (ibrutinib), a Bruton's tyrosine kinase inhibitor, in treatment-naive and previously treated patients with chronic lymphocytic leukemia (CLL) resulted in a significant response rate, with 92 percent of high-risk CLL patients with deletion 17p or tumor protein 53 achieving an objective response. Results from the phase II, open-label, and 51-patient study were published in *The Lancet Oncology*. Data from the 48 evaluable patients at 24 months also showed an estimated progression-free survival rate of 82 percent. Imbruvica is partnered with Janssen Biotech Inc., a unit of New Brunswick, N.J.-based **Johnson & Johnson**.

Revance Therapeutics Inc., of Newark, Calif., said it initiated the phase II BELMONT trial, a randomized study designed to test RT002, a botulinum toxin type A for injection, in glabellar lines, commonly known as frown lines. The study will test three doses of RT002, the labeled dose of current market leader Botox (onabotulinumtoxinA, Allergan Inc.) and a placebo control. About 250 subjects will be enrolled, and the primary endpoints are the investigator's assessment of glabellar line severity at maximum frown at week 24 and median duration of effect from the date of treatment back to baseline severity.

PHARMA: OTHER NEWS TO NOTE

Bristol-Myers Squibb Co., of New York, the California Institute for Biomedical Research (Calibr) entered into a worldwide research collaboration to develop small-molecule anti-fibrotic therapies, and an exclusive license agreement that allows Bristol-Myers Squibb to develop, manufacture and commercialize Calibr's preclinical compounds resulting from the collaboration. Among the assets in Bristol-Myers Squibb's fibrosis portfolio are BMS-986020, a lysophosphatidic acid 1 (LPA1) receptor antagonist in phase II development for the treatment of idiopathic pulmonary fibrosis and a CCR2/5 dual antagonist in phase II for diabetic kidney disease. Bristol-Myers Squibb and Calibr anticipate that the transaction will close during the first quarter of 2015.

Pfizer Inc., of New York, acquired a controlling interest in Redvax GmbH, a spin-off from **Redbiotec AG**, of Zurich-Schlieren, Switzerland. The transaction provides access to a preclinical human cytomegalovirus vaccine candidate, as well as intellectual property and a technology platform related to a second, undisclosed vaccine program, Pfizer said. Terms were not disclosed.

PHARMA: IN THE CLINIC

Fennec Pharmaceuticals Inc., of Research Triangle Park, N.C., said the second sodium thiosulfate (STS) phase III study, SIOPEL 6, has fully enrolled and is now closed for recruitment. The study, which is being conducted by the International Childhood Liver Tumour Strategy Group, has enrolled 116 patients. Final protocol pre-specified independent data monitoring committee review on the first 100 patients, assessing any potential concern of an adverse effect of STS on the efficacy of the cisplatin chemotherapy, is expected this quarter. For the primary hearing endpoint analysis, protocol pre-specified interim hearing assessment of 34, 68 and 102 evaluable patients will be performed once those patients have reached 3.5 years of age.

REGULATORY FRONT

Probiomed S.A. de C.V., of Mexico City, filed a citizen's petition with the FDA protesting what it called the defamatory information Teva Neuroscience Inc. included in a citizen's petition filed last year to protect its Copaxone (glatiramer acetate) from generic competition. Claiming that Teva's July 2, 2014, petition states a series of "half-truths" on the use of Probiomed's Probioglat, a Copaxone generic approved in Mexico, the Mexican company said the petition has the "potential to damage the perceived quality of Probioglat not only in Mexico but internationally, thereby making the registration process more difficult." Probiomed requests that the FDA revise the defamatory information in Teva's petition, ask Teva to not distribute such information and take appropriate actions against those responsible for knowingly or negligently misinforming the agency. Probiomed also wants the FDA to clamp down on Teva's alleged use of its call center "to manipulate innocent people to increase its market participation."

The National Institutes of Health (NIH) is seeking comments on its draft policy promoting the use of a single institutional review board (IRB) for all domestic sites in multisite studies funded by the institutes. "When each participating institution's IRB conducts a review, the process can take many months and significantly delay the initiation" of clinical trials and recruitment, the NIH said in a notice to be published in Tuesday's *Federal Register*. The use of a single IRB in multisite studies has been shown to decrease approval times for trial protocols and may be more cost-effective than local IRB review, it added. Comments on the draft policy are due by Jan. 29.

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Sweeney, Timothy (NIH/NCI) [E]

From: Arie Belldegrun <Arie@kitepharma.com>
Sent: Sunday, January 11, 2015 2:10 AM
To: Rosenberg, Steven A. (NIH/NCI) [E]
Subject: My Thursday talk at JPM Healthcare conference
Attachments: Kite Pharma JPM Presentation 01.15.15 16X9 Version-FINAL (formatted for JPM screen).pptx

Hi Steve,

The JPM Healthcare conference is the largest podium for pharma presentations. It is geared to the business world, not to MD or scientists.

Enclosed is a copy of my upcoming presentation, to keep you in the loop and informed. Please do not distribute it out.

David, Jeff , Margo, and I will all be at the conference and my talk is at 3PM EST.

All the best,

Arie

Proprietary Information,Redacted Per Agreement

Arie Belldegrun, M.D., FACS
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Chairman of the Board; Founder
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Sweeney, Timothy (NIH/NCI) [E]

From: Arie Belldegrun <Arie@kitepharma.com>
Sent: Monday, January 19, 2015 7:43 PM
To: Rosenberg, Steven A. (NIH/NCI) [E]
Subject: Fwd: Today's news from Kite Pharma

FYI....TJ!

Making progress.

Arie Belldegrun, MD FACS
President and CEO, Chairman
Kite Pharma

www.kitepharma.com

Begin forwarded message:

Resent-From: <LisaBurnsFriendsandFamily@burnsmc.com>
From: Lisa Burns <LBurns@burnsmc.com>
Date: January 19, 2015 at 12:12:27 PST
To: The Lisa Burns Friends and Family <LisaBurnsFriendsandFamily@burnsmc.com>
Subject: Today's news from Kite Pharma

Kite Pharma Announces Presentations Highlighting Cancer Immunotherapy T Cell Manufacturing Process

SANTA MONICA, Calif., Jan. 19, 2015 (GLOBE NEWSWIRE) -- Kite Pharma, Inc., (Nasdaq:KITE), a clinical-stage biopharmaceutical company focused on developing engineered autologous T cell therapy (eACT™) products for the treatment of cancer, today announced presentations on the Company's rapid, six-day manufacturing process for the production of Kite's lead product candidate, KTE-C19. The manufacturing technology enables a patient's T cells to be genetically modified using a gammaretroviral vector to express a chimeric antigen receptor (CAR) designed to target the antigen CD19, a protein expressed on the cell surface of B cell lymphomas and leukemias. Kite recently announced filing with the FDA an investigational new drug (IND) application to conduct a Phase 1/2 clinical trial of KTE-C19 for the treatment of patients with refractory aggressive non-Hodgkin lymphoma (NHL).

Presentations taking place at this week's Scale-Up and Manufacturing of Cell-Based Therapies IV, an Engineering Conferences International conference, being held in San Diego, California, include:

Oral Presentation: "Production of autologous T cells for multi-center trials"

Presenter: Marc Better, Ph.D., Vice President, Product Sciences, Kite Pharma

Summary: To prepare for Kite-sponsored multicenter trials, Kite, in conjunction with the National Cancer Institute Surgery Branch, has developed a rapid and efficient proprietary process for the generation of anti-CD19 CAR T cells. Because refractory B cell malignancies can progress quickly, it is important to reduce the time between patient sample collection by apheresis and administration of the engineered T cells. In addition to a rapid, six-day timeframe, elements of the KTE-C19 manufacturing process include: efficient T cell stimulation and growth without anti-CD3/anti-CD28 beads; a robust closed system production, amenable to cGMP operations; rapid cell growth in serum-free cell culture medium; and cryo-preserved product that is transportable to multiple clinical sites. Further processing optimization was achieved while maintaining key parameters (including cell subset composition and T cell expansion).

Date and Time: Monday, January 19, 2015, 12:10 – 12:30PM Pacific Time

Poster Presentation: "Scale-up and Recovery of a Gammaretroviral Vector"

Authors: Timothy Langer, Marc Better, Yeuh-Wei Shen, Xiao-Chi Jia, all of Kite Pharma

Summary: Gammaretroviral vectors can be produced in large quantities and with high titers for *ex vivo* gene delivery in adoptive cell therapies. Using a process implemented at the National Cancer Institute as a basis, Kite is scaling up production of a retroviral vector to support multicenter clinical trials of an engineered T cell product. Kite's team implemented new process engineering, replacing previous filtration techniques with bioprocessing filters and allowing for streamlined manufacturing operations suitable to cGMP production.

Date and Time: Monday, January 19, 2015, 8:30 – 10:00PM Pacific Time

About Kite Pharma

Kite Pharma, Inc., is a clinical-stage biopharmaceutical company engaged in the development of novel cancer immunotherapy products, with a primary focus on eACT™ designed to restore the immune system's ability to recognize and eradicate tumors. In partnership with the NCI Surgery Branch through a Cooperative Research and Development Agreement (CRADA), Kite is advancing a pipeline of proprietary eACT™ peripheral blood product candidates, both CAR (chimeric antigen receptor) and TCR (T cell receptor) products, directed to a wide range of cancer indications. Kite is based in Santa Monica, CA. For more information on Kite Pharma, please visit www.kitepharma.com.

Forward-Looking Statements

This press release contains forward-looking statements for purposes of the safe harbor provisions of the Private Securities Litigation Reform Act of 1995. We may, in some cases, use terms such as "predicts," "believes," "potential," "proposed," "continue," "estimates," "anticipates," "expects," "plans," "intends," "may," "could," "might," "will," "should" or other words that convey uncertainty of future events or outcomes to identify these forward-looking statements. Forward-looking statements include statements regarding our intentions, beliefs, projections, outlook, analyses or current expectations concerning, among other things: our ability to manufacture and process KTE-C19; our ability to meet the demands of multicenter clinical trials; our ability to obtain and maintain U.S. Food and Drug Administration or other regulatory authority approval of, or other action with respect to, our product candidates and advancing a clinical trial of KTE-C19; and our ability to protect our proprietary technology and enforce our intellectual property rights. Various factors may cause differences between Kite's expectations and actual results as discussed in greater detail in Kite's filings with the Securities and Exchange Commission, including without limitation in its Form 10-Q for the quarter ended September 30, 2014. Any forward-looking statements that we make in this press release speak only as of the date of this press release. We

assume no obligation to update our forward-looking statements whether as a result of new information, future events or otherwise, after the date of this press release.

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Sweeney, Timothy (NIH/NCI) [E]

From: Arie Belldegrun <Arie@kitepharma.com>
Sent: Monday, February 23, 2015 9:50 AM
To: Rosenberg, Steven A. (NIH/NCI) [E]
Subject: Fwd: Celldex's Rindopepimut (Rintega(R)) Receives FDA Breakthrough Therapy Designation for the Treatment of Adult Patients with EGFRvIII-positive Glioblastoma - Yahoo Finance

FYI

Arie Belldegrun, MD FACS
President and CEO, Chairman
Kite Pharma

www.kitepharma.com

Begin forwarded message:

> **From:** Ran Nussbaum <ran@pontifax.com>
> **Date:** February 23, 2015 at 05:11:36 PST
> **To:** Rizwana Sproule <RSproule@kitepharma.com>, Cynthia Butitta <cbutitta@kitepharma.com>, Arie Belldegrun <Arie@kitepharma.com>, "Margo Roberts" <mroberts@kitepharma.com>
> **Cc:** Adrian Bot <abot@kitepharma.com>, David Chang <dchang@kitepharma.com>
> **Subject: Celldex's Rindopepimut (Rintega(R)) Receives FDA Breakthrough Therapy Designation for the Treatment of Adult Patients with EGFRvIII-positive Glioblastoma - Yahoo Finance**
>
> http://finance.yahoo.com/news/celldexs-rindopepimut-rintega-r-receives-130100842.html

_____ Information from ESET NOD32 Antivirus, version of virus signature database 11219 (20150223) _____

The message was checked by ESET NOD32 Antivirus.

http://www.eset.com

Sweeney, Timothy (NIH/NCI) [E]

From: David Chang <dchang@kitepharma.com>
Sent: Monday, March 16, 2015 8:17 PM
To: Rosenberg, Steven A. (NIH/NCI) [E]
Cc: Arie Belldegrun
Subject: Confidential
Attachments: Kite Pharma TCF press release 03.17.15.docx

Steve,

As per our discussion, I am forwarding you TCF press release that is scheduled to cross the wire tomorrow morning before the market opens.

Thanks,

David

David D. Chang, MD, PhD
Executive Vice President of R&D
and Chief Medical Officer
Kite Pharma, Inc
office: 310-622-9094
[redacted]
Personal Information,Redacted Per Agreement
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Proprietary Information,Redacted Per Agreement,Redacted Through Page 4

Sweeney, Timothy (NIH/NCI) [E]

From: Arie Belldegrun <Arie@kitepharma.com>
Sent: Thursday, March 26, 2015 5:32 PM
To: Rosenberg, Steven A. (NIH/NCI) [E]
Subject: Neoantigen Science paper
Attachments: Pages from Schumacher_galley.pdf

Hi Steve ,

Thanks for the excellent discussion today. You are not only writing the future of Immunotherapy, but now also rewiring the T-cell Immunology text books... fascinating! .

I learned that the Science online will be published ahead of the AACR meeting and ahead of the printed addition. Will have the exact day earlier next week.

All the best,

Arie Belldegrun, M.D.,FACS
President and CEO
Chairman, Board of Directors; Founder
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REVIEW

Neoantigens in cancer immunotherapy

Ton N. Schumacher[1]* and Robert D. Schreiber[2]*

The clinical relevance of T cells in the control of a diverse set of human cancers is now beyond doubt. However, the nature of the antigens that allow the immune system to distinguish cancer cells from noncancer cells has long remained obscure. Recent technological innovations have made it possible to dissect the immune response to patient-specific neoantigens that arise as a consequence of tumor-specific mutations, and emerging data suggest that recognition of such neoantigens is a major factor in the activity of clinical immunotherapies. These observations indicate that neoantigen load may form a biomarker in cancer immunotherapy and provide an incentive for the development of novel therapeutic approaches that selectively enhance T cell reactivity against this class of antigens.

Immunotherapies that boost the ability of endogenous T cells to destroy cancer cells have demonstrated therapeutic efficacy in a variety of human malignancies. Until recently, evidence that the endogenous T cell compartment could help control tumor growth was in large part restricted to preclinical mouse tumor models and to human melanoma. Specifically, mice lacking an intact immune system were shown to be more susceptible to carcinogen-induced and spontaneous cancers compared with their immunocompetent counterparts (*1*). With respect to human studies, the effects of the T cell cytokine interleukin-2 in a small subset of melanoma patients provided early clinical evidence of the potential of immunotherapy in this disease. In 2010, the field was revitalized by a landmark randomized clinical trial that demonstrated that treatment with ipilimumab, an antibody that targets the T cell checkpoint protein CTLA-4, improved overall survival of patients with metastatic melanoma (*2*). As a direct test of the tumoricidal potential of the endogenous T cell compartment, work by Rosenberg and colleagues demonstrated that infusion of autologous ex vivo expanded tumor-infiltrating lymphocytes can induce objective clinical responses in metastatic melanoma (*3*), and at least part of this clinical activity is due to cytotoxic T cells (*4*). Importantly, recent studies demonstrate that T cell–based immunotherapies are also effective in a range of other human malignancies. In particular, early phase trials of antibodies that interfere with the T cell checkpoint molecule PD-1 have shown clinical activity in tumor types as diverse as melanoma, lung cancer, bladder cancer, stomach cancer, renal cell cancer, head and neck cancer, and Hodgkin's lymphoma (*5*). Based on the relationship between pretherapy CD8+ T cell infiltrates and response to PD-1 blockade in melanoma, cytotoxic T cell activity also appears to play a central role in this form of cancer immunotherapy (*6*).

An implicit conclusion from these clinical data is that in a substantial fraction of patients, the endogenous T cell compartment is able to recognize peptide epitopes that are displayed on major histocompatibility complexes (MHCs) on the surface of the malignant cells. On theoretical grounds, such cancer rejection epitopes may be derived from two classes of antigens. A first class of potential cancer rejection antigens is formed by nonmutated proteins to which T cell tolerance is incomplete—for instance, because of their restricted tissue expression pattern. A second class of potential cancer rejection antigens is formed by peptides that are entirely absent from the normal human genome, so-called neoantigens. For the large group of human tumors without a viral etiology, such neo-epitopes are solely created by tumor-specific DNA alterations that result in the formation of novel protein sequences. For virus-associated tumors, such as cervical cancer and a subset of head and neck cancers, epitopes derived from viral open reading frames also contribute to the pool of neoantigens.

As compared with nonmutated self-antigens, neoantigens have been postulated to be of particular relevance to tumor control, as the quality of the T cell pool that is available for these antigens is not affected by central T cell tolerance (*7*). Although a number of heroic studies provided early evidence for the immunogenicity of mutation-derived neoantigens [reviewed in (*8*)], technology to systemically analyze T cell reactivity against these antigens only became available recently. Here, we review our emerging understanding of the role of patient-specific neoantigens in current cancer immunotherapies and the implications of these data for the development of next generation immunotherapies.

Exome-guided neoantigen identification—process considerations

A large fraction of the mutations in human tumors is not shared between patients at

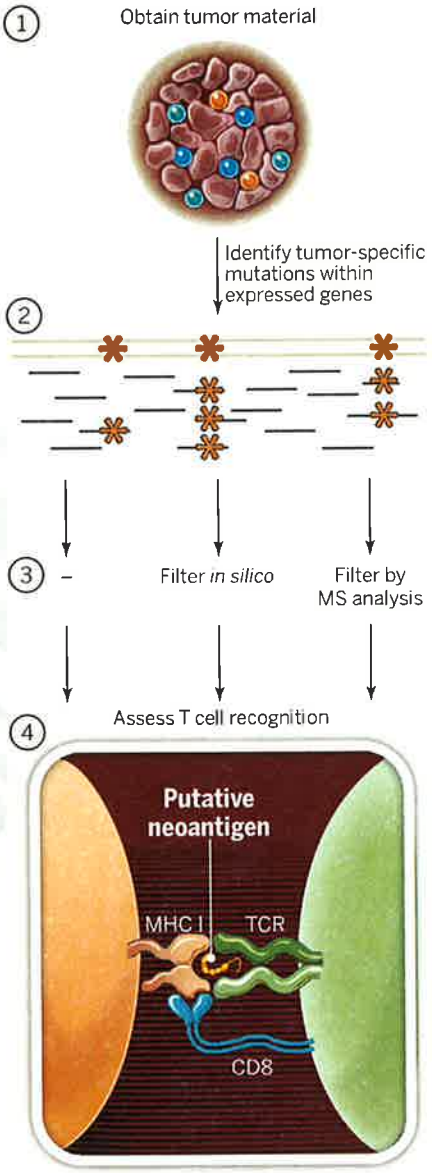

Fig. 1. Cancer exome-based identification of neoantigens. (**1**) Tumor material is analyzed for nonsynonymous somatic mutations. When available, RNA sequencing data are used to focus on mutations in expressed genes. (**2**) Peptide stretches containing any of the identified nonsynonymous mutations are generated in silico and are either left unfiltered (*16, 17*), filtered through the use of prediction algorithms [e.g., (*10–13*)], or used to identify MHC-associated neoantigens in mass spectrometry data (*15, 20*). Modeling of the effect of mutations on the resulting peptide-MHC complex may be used as an additional filter (*20*). Resulting epitope sets are used to identify physiologically occurring neoantigen-specific T cell responses by MHC multimer-based screens (*13, 22*) or functional assays [e.g., (*11, 12*)], within both CD8+ [e.g., (*11–13, 19, 39*)] and CD4+ (*16, 18*) T cell populations. Alternatively, T cell induction strategies are used to validate predicted neoantigens [e.g., (*10, 20*)].

[1]Division of Immunology, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX, Amsterdam, Netherlands. [2]Department of Pathology and Immunology, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110, USA.
*Corresponding author. E-mail: t.schumacher@nki.nl (T.N.S.); schreiber@immunology.wustl.edu (R.D.S.)

MS no: RVaaa4971/CJH/IMMUNOLOGY

meaningful frequencies and may therefore be considered patient-specific. Because of this, technologies to interrogate T cell reactivity against putative mutation-derived neoantigens need to be based on the genome of an individual tumor. With the development of deep-sequencing technologies, it has become feasible to identify the mutations present within the protein-encoding part of the genome (the exome) of an individual tumor with relative ease and thereby predict potential neoantigens (*9*). Two studies in mouse models provided the first direct evidence that such a cancer exome-based approach can be used to identify neoantigens that can be recognized by T cells (*10*, *11*). In brief, for all mutations that resulted in the formation of novel protein sequence, potential MHC binding peptides were predicted, and the resulting set of potential neoantigens was used to query T cell reactivity. Subsequent studies have demonstrated that cancer exome-based analyses can also be exploited in a clinical setting, to dissect T cell reactivity in patients that are treated by either tumor-infiltrating lymphocyte (TIL) cell therapy or checkpoint blockade (*12*, *13*). Furthermore, following this early work, the identification of neoantigens on the basis of cancer exome data has been documented in a variety of experimental model systems and human malignancies (*10*–*22*).

The technological pipeline used to identify neoantigens in these different studies has varied substantially, and further optimization is likely possible (Fig. 1). Accepting the limitations of probing the mutational profile of a tumor in single biopsy (*23*), the genetic analysis of the tumor itself can be considered a robust process. Specifically, based on the analysis of neoantigens previously identified by other means, the false-negative rate of cancer exome sequencing is low—i.e., the vast majority of neoantigens occur within exonic sequence for which coverage is sufficient (*24*). At the same time, it is apparent from unbiased screening efforts—in which the entire collection of identified mutations was used to query T cell reactivity—that the vast majority of mutations within expressed genes do not lead to the formation of neoantigens that are recognized by autologous T cells (*16*, *17*). Because of this, a robust pipeline that can be used for the filtering of cancer exome data is essential, in particular for tumors with high mutational loads.

How can such filtering be performed? With the set of mutations within expressed genes as a starting point, two additional requirements can be formulated. First, a mutated protein needs to be processed and then presented as a mutant peptide by MHC molecules. Second, T cells need to be present that can recognize this peptide-MHC complex. In two recent preclinical studies, presentation of a handful of predicted neoantigens by MHC molecules was experimentally demonstrated by mass spectrometry (*15*, *20*), and this approach may form a valuable strategy to further optimize MHC presentation algorithms. At the same time, the sensitivity of mass spectrometry is presently still limited, thereby likely resulting in a substantial fraction of false negatives. For this reason, but also because of logistical issues, implementation of this approach in a clinical setting is unlikely to happen soon. Lacking direct evidence for MHC presentation, as can be provided by mass spectrometry, presentation of neoantigens by MHC class I molecules may be predicted using previously established algorithms that analyze aspects such as the likelihood of proteasomal processing, transport into the endoplasmic reticulum, and affinity for the relevant MHC class I alleles. In addition, gene expression levels (or perhaps preferably protein translation levels) may potentially also be used to help predict epitope abundance (*25*).

Although most neoantigen identification studies have successfully used criteria for epitope prediction that are similar to those previously established for the identification of pathogen-derived epitopes [e.g., (*12*, *13*)], Srivastava and colleagues have argued that neoantigens in a transplantable mouse tumor model display very different properties from viral antigens and generally have a very low affinity for MHC class I (*14*). Although lacking a satisfactory explanation to reconcile these findings, we do note that the vast majority of human neoantigens that have been identified in unbiased screens do display a high predicted MHC binding affinity (*24*, *26*). Likewise, minor histocompatibility antigens, an antigen class that is conceptually similar to neoantigens, are correctly identified by classical MHC binding algorithms (*27*). Moreover, the mutations that were identified in a recent preclinical study as forming tumor-specific mutant antigens that could induce therapeutic tumor rejection when used in tumor vaccines (*15*) were not predicted to be significant using the Srivastava approach. Another potential filter step that has been suggested examines whether the mutation is expected to improve MHC binding, rather than solely alter the T cell receptor (TCR)–exposed surface of the mutant peptide. However, with examples of both categories in both mouse models and human data, the added value of such a filter may be relatively modest (*11*, *15*, *20*, *26*). For MHC class I restricted neoantigens, conceivably the biggest gain in prediction algorithms can be made with respect to identification of the subset of MHC binding peptides that can successfully be recognized

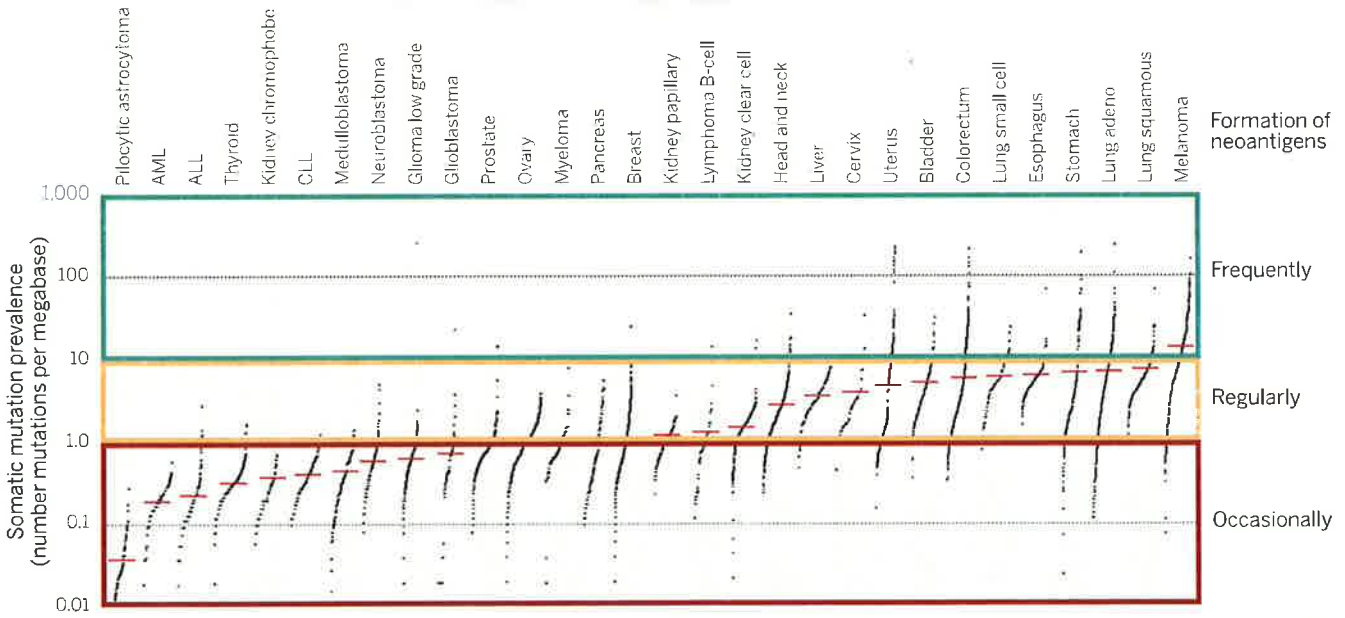

Fig. 2. Estimate of the neoantigen repertoire in human cancer. Data depict the number of somatic mutations in individual tumors. Categories on the right indicate current estimates of the likelihood of neoantigen formation in different tumor types. Adapted from (*50*). It is possible that the immune system in melanoma patients picks up on only a fraction of the available neoantigen repertoire, in which case the current analysis will be an underestimate. A value of 10 somatic mutations per Mb of coding DNA corresponds to ~150 nonsynonymous mutations within expressed genes.

by the TCR repertoire. With respect to this, the nature of the central TCR-exposed residues of MHC-bound peptides has been shown to be associated with peptide immunogenicity (*28*). By the same token, alterations at these sites may potentially be picked up by the immune system more readily (*20*). However, a substantial further experimental effort is required to evaluate to what extent algorithms that predict immunogenicity can facilitate the identification of MHC class I–restricted neoantigens. For MHC class II–restricted neoantigens, it will be important to obtain a better understanding not only of peptide immunogenicity but also of the basic factors that determine the efficiency of epitope presentation.

Size and nature of the neoantigen repertoire

Large-scale analyses of neoantigen-specific T cell reactivity have now been carried out for a substantial number of patients, mostly in melanoma (*12*, *13*, *16*, *17*). With the caveat of a potential selection bias toward patients with a clinical benefit upon immunotherapeutic intervention, these analyses provide a first estimate of the frequency with which the immune system recognizes the neoantigens that are formed as a consequence of mutations. The first and arguably most important conclusion that can be drawn from these analyses is that the T cell–based immune system reacts to both MHC class I–restricted (*12*, *13*, *17*) and MHC class II–restricted neoantigens (*16*) in a large fraction of melanoma patients. The second conclusion that can be drawn from these analyses is that only a very small fraction of the nonsynonymous mutations in expressed genes in these tumors leads to the formation of a neoantigen for which CD4+ or CD8+ T cell reactivity can be detected within tumor-infiltrating lymphocytes.

What do these observations mean for the potential formation of neoantigen repertoires in other human malignancies? Most human melanomas have a mutational load above 10 somatic mutations per megabase (Mb) of coding DNA, and this is apparently sufficient to lead to the frequent formation of neoantigens that can be seen by T cells. Based on these data, formation of neoantigens that can potentially be recognized by autologous T cells is expected to also be common for other tumors with a mutational load above 10 somatic mutations per Mb (corresponding to approximately 150 nonsynonymous mutations within expressed genes) (Fig. 2). This group contains a sizable fraction of high-prevalence tumor types such as lung cancer and colorectal cancer. If formation of neoantigens is a frequent event in tumors with mutational loads above 10 somatic mutations per Mb, many tumors with a mutational load of 1 to 10 per Mb may still be expected to carry neoantigens that can be recognized by T cells. However, as based on the fact that even for melanomas with a mutational load around 10 mutations per Mb, T cell reactivity is not always observed ((*16*), tumor types with a mutational load below 1 mutation per Mb appear less likely to commonly express neoantigens that can be recognized by autologous T cells.

Although this analysis provides a useful first sketch of the expected relevance of neoantigens in different tumor types, three important factors should be taken into account. First, by relying on the presence of preexisting T cell reactivity as a readout, the human studies carried out to date will only detect neoantigens that were immunogenic during in vivo tumor outgrowth (either spontaneously or boosted by therapy). It is conceivable that not all tumor-expressed neoantigens induce an autologous T cell response—for instance, because they are not efficiently cross presented. In addition, at least in preclinical models, there is evidence for immunodominance of tumor antigens, where the immune system becomes so fixated on particular antigens that it ignores other antigens that are both present and detectable in the tumor (*29*). If only a fraction of the available neoantigens would normally elicit T cell reactivity, the analyses carried out to date may underestimate the actual neoantigen repertoire. As a second consideration, it is important to realize that the formation of neoantigens is a probabilistic process in which each additional mutation increases the odds that a relevant neoantigen is created. Thus, in this "neoantigen lottery," there will be cases in which despite a high mutational load, neoantigen-specific T cell reactivity is lacking or, vice versa, in which a tumor with only a handful of mutations will express an MHC class I- or class II–restricted neoantigen. Third, although we here make a prediction with regard to the frequency with which neoantigens that can potentially be recognized by the TCR repertoire are formed, it should be kept in mind that the presence of a neoantigen does not equal the induction of T cell reactivity. Human tumors vary substantially in the composition of their microenvironment, and this is likely to influence the ability of the T cell pool to respond to mutated antigens. Related to this, from a conceptual point of view, therapeutic manipulation of T cell reactivity would seem particularly attractive for tumor types that do express large numbers of antigens but in which the tumor micro-environment hinders the activation of the T cells that recognize them.

What are the characteristics of mutation-derived neoantigens in human cancer, both with respect to the genes from which they are derived and the frequency with which they occur within the patient population? In an ideal world, neoantigens would be derived from essential oncogenes and occur in large patient groups, to both reduce the likelihood of escape and facilitate clinical interventions that enhance T cell reactivity against them. Clearly, T cell responses do sometimes occur against MHC class I–restricted (*30*) and MHC class II–restricted neoantigens in validated oncogenes that are shared between subgroups of patients (*31*). At the same time, it is apparent that, at least in melanoma, the bulk of the neoantigen-specific T cell response is directed toward mutated proteins that are essentially unique to that tumor and that are unlikely to play a key role in cellular transformation (Fig. 3, top and bottom) (*16*). A direct implication of this bias in neoantigen-specific T cell reactivity toward patient-specific passenger mutations is that the targeting of defined neoantigens will likely require the development of personalized immunotherapies.

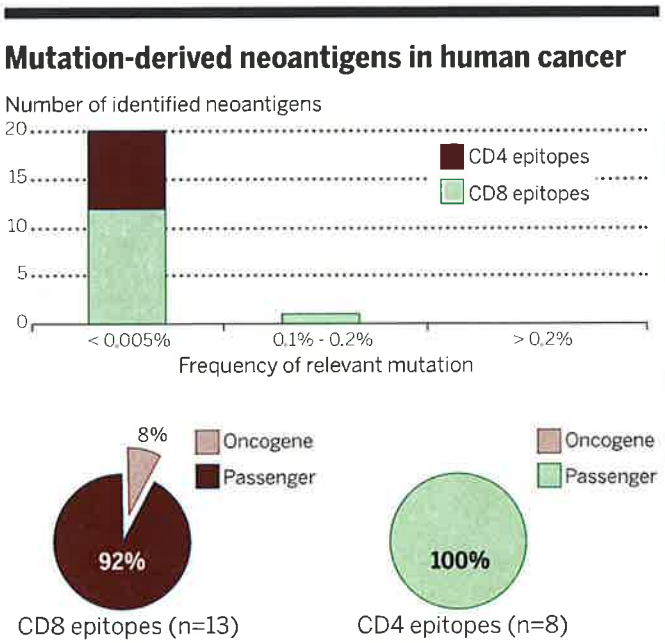

Fig. 3. Characteristics of melanoma neoantigens. (**Top**) For a group of CD4+ T cell neoantigens (8 epitopes) and CD8+ T cell neoantigens (13 epitopes) identified by cancer exome based screens, the frequency of mutation of that residue in a cohort of ~20,000 human tumor samples (*51*) is depicted. (**Bottom**) For the same group of CD4+ T cell and CD8+ T cell neoantigens, the fraction of encoding mutations that occurs within known oncogenes (*52*) is depicted.

Extrinsic influences on the tumor antigenic landscape

The neoantigen repertoire expressed in a clinically apparent cancer may have been substantially influenced by the developing tumor's interaction with the immune system that occurs even before it becomes clinically apparent. This is the process of "cancer immunoediting" that has been well documented in preclinical cancer models (*1*, *32*, *33*). In its most complex form, cancer immunoediting may occur in three phases: elimination, in which the innate and adaptive immune systems work together to recognize a developing tumor and

destroy it before it becomes clinically apparent; equilibrium, in which residual occult tumor cells not destroyed in the elimination phase are held in a state of tumor dormancy as a consequence of adaptive immune system activity and undergo "editing"; and escape, in which edited tumor cells are no longer recognized or controlled by immune processes, begin to grow progressively, induce an immunosuppressive tumor microenvironment, and then emerge as clinically apparent cancers. Recent work has demonstrated that T cells play a major role in shaping the immunogenicity of developing cancers—i.e., "edit" tumor immunogenicity—and exert this effect by at least two mechanisms. First, T cells can shape tumor antigenicity/immunogenicity through an immunoselection process by destroying tumor cells that express strong tumor-specific mutant antigens, leaving behind tumor cells that either express weaker antigens (some of which may still be mutant tumor antigens) or are incapable of expressing antigens (e.g., those that have developed mutations in antigen processing or presentation) (*11*). Second, chronic T cell attack on a tumor has been shown to silence expression of certain tumor-specific antigens through epigenetic mechanisms in a preclinical model (*34*). Strikingly, a recent study, based on analysis of thousands of Cancer Genome Atlas solid tumor samples, showed that, in particular in colorectal cancer, mutated peptides predicted to bind to autologous MHC class I molecules are less frequent than expected by chance, an observation that is consistent with immune-based selection (*35*). By extension, the combination of cell extrinsic forces such as cancer immunoediting and the stochastic nature of epitopes arising from tumor-specific mutations, may help drive the heterogeneous mutational—and by inference, antigenic—landscapes that have been noted in certain tumors (*23*). As such, the antigenic heterogeneity of tumors might explain some of the differences in response that individual patients display to checkpoint blockade therapy. Individuals who develop durable responses to checkpoint blockade may be those whose tumors retain sufficient antigenicity to render them sensitive to the heightened immune function that accompanies cancer immunotherapy, despite not being controlled by naturally occurring antitumor immune responses.

Role of neoantigens in cancer immunotherapy

On theoretical grounds, two factors should determine the relative importance of neoantigens and nonmutated self-antigens in the effects of cancer immunotherapies such as checkpoint blockade and TIL therapy: first, the frequency with which T cell responses against the two antigen classes occur; second, the relative potency of T cell responses specific for the two antigen classes. Recent work in mouse models using transplantable carcinogen-induced cancers has demonstrated that checkpoint blockade alters both the quality of the neoantigen-specific intratumoral T cell response [as reflected by common- and treatment-specific changes in gene expression in CD8+ TILs isolated from tumor-bearing mice treated with antibodies to CTLA-4 (anti–CTLA-4) and/or antibodies to PD-1 (anti–PD-1)] and the magnitude of this T cell response (seen with CTLA-4 or combined CTLA-4/PD-1 blockade but not with PD-1 blockade only) (*15*). Because the neoantigens identified in this model serve as cancer rejection antigens, these data provide compelling evidence that checkpoint blockade acts at least in part through neoantigen-specific T cell reactivity in this setting. However, in the case of human melanoma, where autochthonous tumors may be in contact with the immune system for years, the situation is more complicated. As discussed above, T cell reactivity against neoantigens is common in melanoma. Furthermore, a case report has shown that such reactivity can be enhanced by anti–CTLA-4 treatment (*13*). However, T cell reactivity against nonmutated shared antigens is also observed in the majority of melanoma patients, and broadening of this T cell response has been documented following both TIL therapy and anti–CTLA-4 treatment (*36*, *37*). Thus, although the murine data show that neoantigen-specific T cell reactivity can be critical to the effects of checkpoint blockade, the human data are presently only consistent with this possibility.

What other data are available with respect to this issue? If recognition of neoantigens is an important component of cancer immunotherapy, one would expect tumor types with high numbers of mutations to be characterized by strong T cell responses and to be particularly sensitive to immunotherapy. Furthermore, also within a given tumor type, response rate should correlate with mutational load. Evidence for a role of neoantigens in driving the strength of the intratumoral T cell response is provided by the observation that the presence of CD8+ T cells in cancer lesions, as read out using RNA sequencing data, is higher in tumors with a high mutational burden (*38*). Furthermore, an extensive analysis by Hacohen and colleagues has demonstrated that the level of transcripts associated with cytolytic activity of natural killer cells and T cells correlates with mutational load in a large series of human tumors (*35*). With respect to the effects of immunotherapy in tumors with different mutational loads, in non–small cell lung cancer patients treated with anti–PD-1, mutational load shows a strong correlation with clinical response (*22*). Likewise, in melanoma patients treated with ipilimumab, an antibody to CTLA-4, long-term benefit is also associated with a higher mutational load, although the effect appears less profound in this setting (*39*). A striking observation in the latter study has been that the predicted MHC-binding neoantigens in patients with a long-term clinical benefit were enriched for a large series of tetrapeptide motifs that were not found in tumors of patients with no or minimal clinical benefit. An appealing interpretation of these data is that the neoantigen-specific T cell response is preferentially directed toward a subset of mutant sequences, something that could facilitate bioinformatic identification of neoantigens for therapeutic targeting. However, analysis of the sequence properties of human neoantigens identified in other studies does not show the profound bias toward these tetrapeptide signatures that would be predicted if their role were central in the tumor-specific T cell response (*40*), and conceivably the identified tetrapeptide motifs play a different role.

The genetic damage that on the one hand leads to oncogenic outgrowth can also be targeted by the immune system to control malignancies.

It will be valuable to extend the analysis of genomic determinants of tumor cell sensitivity to cancer immunotherapeutics to other malignancies. However, because of the probabilistic nature of neoantigen generation, mutational load will by itself always remain an imperfect biomarker, even in a situation in which neoantigen reactivity is the sole tumor-specific T cell reactivity that is relevant to tumor control. Furthermore, the formation of tumor-specific antigens is only one of a number of essential conditions for a successful immune attack on cancer cells, a concept that is well described by the cancer-immunity cycle introduced by Chen and Mellman (*41*). As an example, genetic inactivation of the β_2-microglobulin subunit of MHC class I molecules is a relatively frequent event in some tumor types (*42*). In addition, a recent analysis of genetic alterations that are present in tumors with high immune activity provides evidence for a series of other escape mechanisms (*35*). In such cases, in which the cancer-immunity cycle is disrupted at another site, the number of neoantigens produced is unlikely to still be of much relevance. Because of this interdependence of different phases of the cancer-immunity cycle, the combined use of assay systems that report on these different phases appears warranted.

Arguably the most direct data on the relevance of neoantigen-specific T cells in human tumor control comes from a small number of clinical studies that involve infusion of defined T cell populations or infusion of TCR-transduced T cells. Encouragingly, a recent case report demonstrated regression of a metastatic cholangiocarcinoma by infusion of a CD4+ T cell product that was highly enriched for reactivity against an MHC class II–restricted neoantigen (*18*). Combined with the observation that, at least in melanoma, CD4+ T cell recognition of neoantigens is a frequent event (*16*), these data underscore the potential clinical relevance of MHC class II–restricted neoantigens. Comparison of the clinical effects of TIL therapy with that of T cells modified with TCRs recognizing different shared antigens can also be considered informative. Infusion of T cells modified with TCRs directed against the gp100 and MART-I

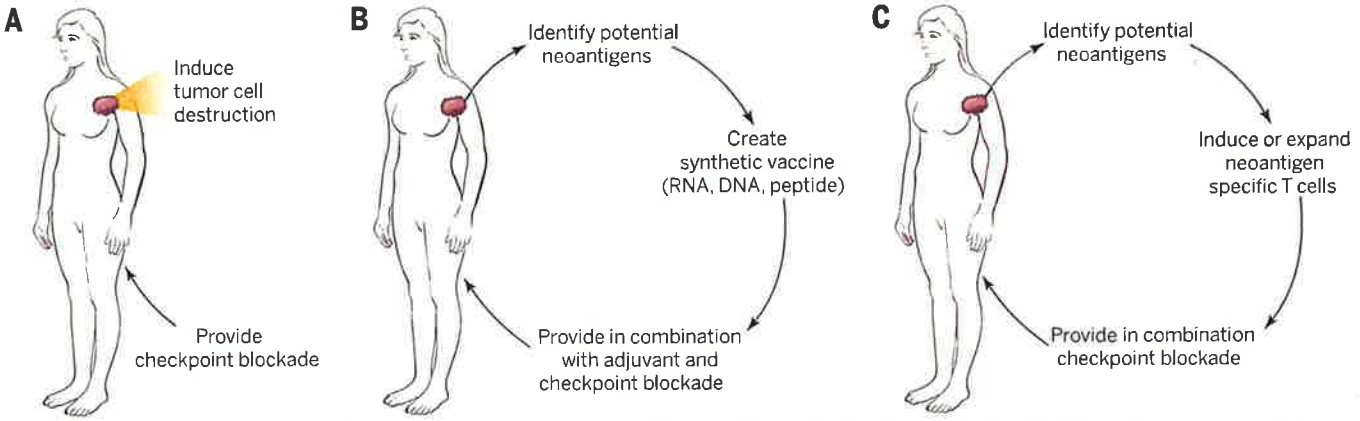

Fig. 4. Strategies to target the patient-specific neoantigen repertoire. (**A**) Immunotherapy is given in combination with interventions such as radiotherapy that enhance exposure to autologous neoantigens. (**B**) Potential neoantigens are identified as in Fig. 1 steps 1 to 3, a patient-specific vaccine is produced, and this vaccine is given together with adjuvant and T cell checkpoint-blocking antibodies. (**C**) Potential neoantigens are identified as in Fig. 1 steps 1 to 3, T cells that are specific for these neoantigens are induced or expanded in vitro, and the resulting T cell product is given together with T cell checkpoint-blocking antibodies.

melanocyte differentiation antigens, a prominent class of self-antigens in melanoma, shows a relatively modest clinical effect that is accompanied by substantial on-target toxicity against healthy melanocytes (*43*). Because this toxicity is relatively infrequent in TIL therapy, these data strongly suggest that T cell reactivity against the melanocyte differentiation antigens is not a major driver of the antitumor activity of this therapy. At the same time, there is data showing that T cell products directed against NY-eso-1, one of the nonmutant self-antigens from the family of cancer/germline antigens that show very limited expression in healthy tissue, can display substantial antitumor activity (*44*, *45*). Thus, although the available data support the notion that T cell recognition of neoantigens contributes substantially to the effects of the currently used immunotherapies, it would not be justified to dismiss a potential contribution of T cell responses against a subset of nonmutant antigens. A direct comparison of the antitumor activity of neoantigen-specific and self-antigen–specific T cells obtained from individual patients would be useful to further address this issue.

Therapeutic use of the patient-specific neoantigen repertoire

Based on the fact that, at least in tumors with high mutational loads, the amount of DNA damage is sufficient for the immune system to see one or multiple epitopes as foreign, it becomes of interest to stimulate neoantigen-specific T cell responses in cancer patients. Such stimulation can obviously only be of value if the strength of the neoantigen-specific T cell response is otherwise a limiting factor in tumor control. Human data on this important issue are lacking. However, in mouse models, vaccination with defined neoantigens has been show to result in increased tumor control (*10*, *14*, *15*, *20*), providing sufficient rationale for the clinical development of neoantigen-directed therapeutics. Because the majority of possible neoantigens are specific to the individual being treated (Fig. 3), such therapeutic approaches will in most cases entail personalized immunotherapies that either exploit the antigen repertoire in the tumor cells themselves, or information on that repertoire, as obtained by tumor sequencing (Fig. 4). As a first approach, a combination of checkpoint-blocking antibodies with therapeutic interventions—such as tumor radiotherapy, oncolytic viruses, or autologous tumor cell vaccines—that can increase neoantigen exposure to the T cell-based immune system may be synergistic (Fig. 4A). As a downside, as compared to molecularly defined vaccines, the neoantigens released by such strategies will be diluted by the large amount of nonmutant peptides that are also present. In addition, control over the maturation signals received by antigen-presenting cells is relatively limited. Nevertheless, because of the relative ease of clinical development of some of these combination therapies, extensive testing of such therapies is warranted.

To allow a more defined targeting of the neoantigen repertoire in human tumors, two alternative approaches should be considered, in both cases relying on sets of potential neoantigens as identified by sequencing of tumor material (Fig. 4, B and C). First, synthetic vaccines may be produced that contain or encode a set of predicted neoantigens. Although still a substantial departure from the classical pharmaceutical model, clinical development of such personalized vaccines is within reach (*46*–*48*). Mouse model data support the clinical translation of this approach, and the two most pressing questions appear to be (i) whether our ability to predict the most relevant neoantigens is already sufficiently advanced and (ii) how such vaccines may best be administered. Second, the information obtained from tumor sequencing may be used to create neoantigen-specific T cell products in vitro. This may either involve the expansion of neoantigen-specific T cell populations that can already be detected within tumor tissue or in blood or the de novo induction of such cells.

Regardless of the strategy used to enhance neoantigen-specific T cell reactivity, it will likely prove important to target multiple neoantigens simultaneously in order to prevent tumor escape by editing of the mutated epitope concerned (*1*). In addition, it may be prudent to avoid the targeting of mutations in gene products that are seen by the immune system in autoimmune disease to avoid induction of or exacerbation of cancer-associated autoimmune disease (*49*).

Concluding remarks

Based on data obtained over the past few years, it is plausible that neoantigen-specific T cell reactivity forms a major "active ingredient" of successful cancer immunotherapies. In other words, the genetic damage that on the one hand leads to oncogenic outgrowth can also be targeted by the immune system to control malignancies. Based on this finding, it will be important to engineer therapeutic interventions by which neoantigen-specific T cell reactivity is selectively enhanced. Because of the tumor-restricted expression of the antigens that are being targeted, these personalized cancer immunotherapies offer the promise of high specificity and safety. Conceivably, the boosting of neoantigen-specific T cell reactivity that can be achieved with such personalized immunotherapies will further increase the spectrum of human malignancies that respond to cancer immunotherapy.

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From: Dr. Arie Belldegrun <arie@belldegrun.com>
Sent: Monday, March 30, 2015 4:27 PM
To: 'Justin Jackson'; Rosenberg, Steven A. (NIH/NCI) [E]; David Chang; Jeff Wiezorek
Subject: FW: ACT review article
Attachments: June et al. Adoptive cellular therapy.pdf

FYI, "joint" Kite, Novartis Juno paper.....

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IMMUNOTHERAPY

Adoptive cellular therapy: A race to the finish line

Carl H. June,[1]* Stanley R. Riddell,[2]* Ton N. Schumacher[3]*

Adoptive T cell transfer for cancer, chronic infection, and autoimmunity is an emerging field that shows promise in recent trials. Using the principles of synthetic biology, advances in cell culture and genetic engineering have made it possible to generate human T cells that display desired specificities and enhanced functionalities compared with the natural immune system. The prospects for widespread availability of engineered T cells have changed dramatically, given the recent entry of the pharmaceutical industry to this arena. Here, we discuss some of the challenges—such as regulatory, cost, and manufacturing—and opportunities, including personalized gene-modified T cells, that face the field of adoptive cellular therapy.

INTRODUCTION

Adoptive cell transfer (ACT) is a term coined by Billingham and colleagues to describe the transfer of lymphocytes to mediate an effector function (*1*). Presently, there are three types of therapies that are advancing on a path toward regulatory approval (Fig. 1): tumor-infiltrating lymphocytes (TILs) as well as chimeric antigen receptor (CAR) and T cell receptor (TCR) engineered T cells. TILs have been developed with slow but continuing progress over several decades, primarily at the National Cancer Institute. Recently, an international phase 3 randomized trial began for treating patients with metastatic melanoma with TILs (NCT02278887). A number of pharmaceutical and newly formed biotechnology companies are now commercializing various forms of ACT, including TIL therapies (Table 1).

In contrast to TILs, gene-transfer–based strategies have been developed to overcome the consequences of immune tolerance on the tumor-specific T cell repertoire. These approaches redirect T cells to tissues by the transfer of CARs composed of antibody-binding domains fused to T cell signaling domains, or transfer of TCR α/β heterodimers. The infusion of gene-modified T cells directed to specific targets offers the possibility to endow the immune system with reactivities that are not naturally present and has the added benefit of the rapid onset of action that is usually seen with cytotoxic chemotherapy or with targeted therapies, contrasting to delayed effects observed with vaccines and some of the T cell checkpoint therapies.

Currently, most trials are using α/β T cells for ACT. However, investigators are exploring the use of numerous lymphocyte subsets—including γ/δ T cells, invariant natural killer (NK) T cells, NK cells, and T helper 17—for their specialized functions in various clinical settings of cancer and chronic infection. For indications involving autoimmunity, tolerance induction, prevention of organ graft rejection, and treatment of graft-versus-host disease (GVHD), regulatory T cells (T_{reg} cells), including natural and induced T_{reg} cells, are being tested. Myeloid-derived suppressor cells and regulatory B cells, which have anti-inflammatory properties involving mechanisms distinct from T_{reg} cells, have also been proposed as novel forms of ACT (*2*, *3*). In this Perspective, we review the status of ACT and the rapidly emerging role of the biotechnology industry in the race to accelerate the development and promote the widespread availability of this new form of cellular therapy that has demonstrated efficacy treating patients with refractory life-threatening cancers.

ACT is generally considered in the context of cancer, typically leukemias and melanoma (Table 1). It is interesting to note from a historical perspective that some of the first forms of ACT involving gene-modified T cells were conducted two decades previously in patients with advanced HIV-1/AIDS (*4*). Many of the results from trials conducted in patients with AIDS have informed current concepts in the field of cancer, as exemplified by the demonstration that CAR T cells could survive for more than a decade in HIV/AIDS patients (*5*). These initial trials were done in order to control drug-resistant forms of HIV-1 infection. However, the current challenge in the field is to develop cellular therapies with the potential to eliminate the reservoir of HIV-1 that is resistant to current antiviral therapies (*6*). The field has been energized by an extraordinary experiment conducted by Gero Hütter and colleagues in Berlin in a patient who has apparently been cured of HIV infection after an allogeneic hematopoietic stem cell transplant and ACT from a homozygous C-C chemokine receptor type 5 (CCR5) Δ32 donor (*7*). There are several approaches to induce a cell-intrinsic resistance to HIV-1 infection and to target the reservoir of HIV-1 by gene-modified ACT and cytotoxic T lymphocytes (CTL) (*8*, *9*).

Cancer immunotherapies that target T cell checkpoints, such as cytotoxic T lymphocyte–associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) (*10*), rely on the ability of the endogenous T cell compartment to recognize the tumor as foreign because of the epitopes it carries. TIL therapy likewise relies on an intrinsic tumor recognition capacity of the T cell compartment, and checkpoint therapies and TIL therapy may therefore be assumed to have potential for a similar set of human cancers. Notably, recent work suggests that T cell recognition of neoantigens that are created as a consequence of tumor-specific mutations forms a major component of the clinical activity of checkpoint therapies (*11*, *12*), and clinical activity of these therapies may therefore be highest in tumors with a high mutational load. Adoptive therapy with gene-modified T cells has the potential to address an entirely different need by creating a tumor-specific T cell compartment that is otherwise lacking in patients (Fig. 1). As such, gene-modified ACT has potential for tumor types that may not be responsive to T cell checkpoint or TIL therapies, such as most cancers occurring in children and many of the hematological malignancies. In addition, gene-modified ACT addresses a different critical node in the "cancer–immunity cycle," the series of stepwise events required for an anticancer immune response to lead to cancer cell eradication (*13*). Furthermore, T cell checkpoint therapies and gene-modified ACT have the potential to work synergistically.

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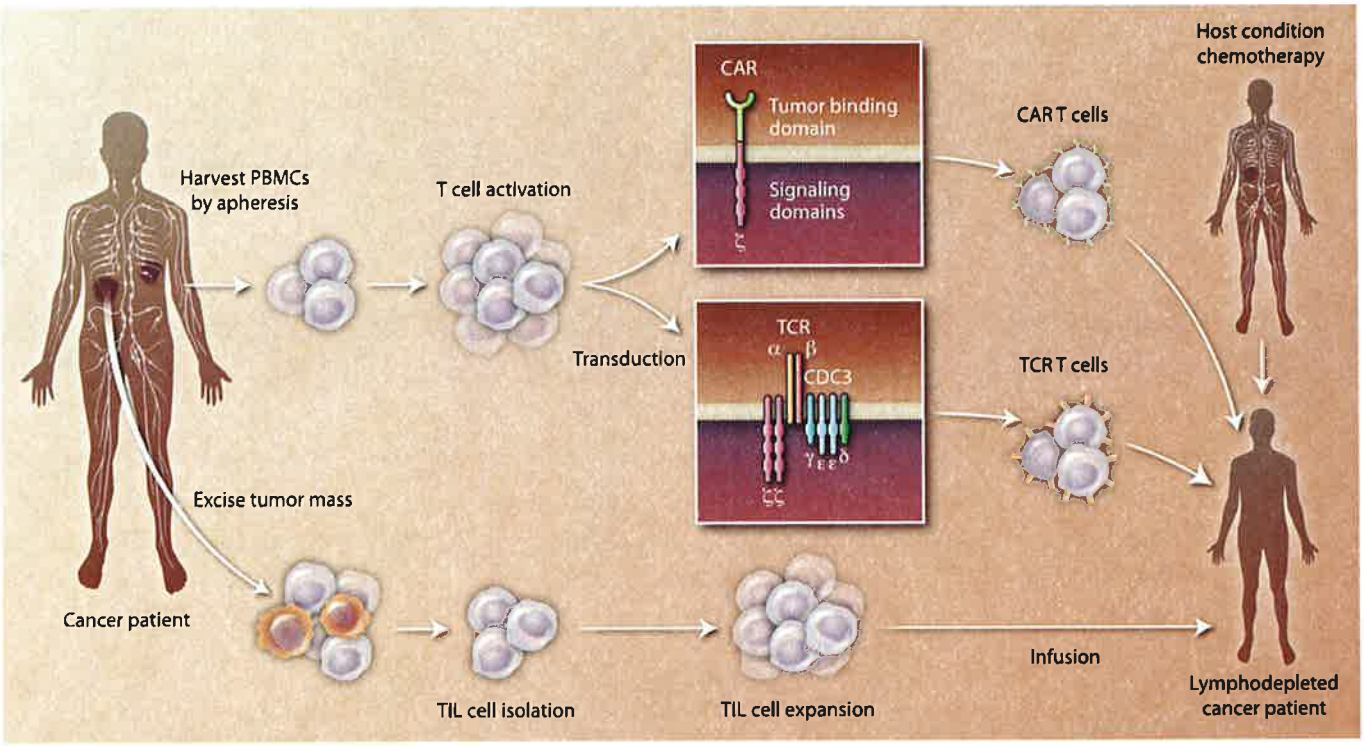

Fig. 1. Adoptive cell therapy is currently represented by three general approaches. TILs are produced after surgical excision of tumor and enrichment and expansion of TILs from a disaggregated tumor biopsy sample. TCR- and CAR-modified T cells are produced from peripheral blood lymphocytes in a manufacturing step that includes introduction of the desired receptor through viral or nonviral methods in order to engineer cells. Patients often receive a lymphodepleting chemotherapy regimen before infusion. PBMC, peripheral blood mononuclear cell.

SOURCE OF CARS AND TCRS

Most of the chimeric antigen receptors currently used to create gene-modified T cells are derived from mouse antibodies, and both antibody and T cell responses against CARs have been observed in clinical trials (*14*, *15*). Furthermore, the extent of this problem may presently be underestimated because the most visible trials in the area have involved the targeting of the B cell compartment—a clinical setting in which transgene-specific humoral immunity will be less of an issue than in settings in which the humoral immune system is left intact. To minimize the impact of transgene-specific immune responses on the activity of introduced cells, the use of humanized or fully human antibodies obtained from mice transgenic for the human immunoglobulin (Ig) loci forms an obvious solution. Clinical trials with fully human CARs have only recently opened (NCT02209376 and NCT01837602). In addition, it may be beneficial to engineer the CAR format so that the formation of nonhuman sequences at the domain fusion sites is also avoided.

By the same token, immunogenicity of nonhuman TCR sequences has been described in a subset of patients treated with TCR-modified T cells—in this case, involving antibody recognition of mouse TCR variable domains (*16*). Here again, the isolation of receptors from the human T cell repertoire or from mice that carry a humanized TCR repertoire is likely to be an effective solution (*17*). In the case of TCRs, the source from which the receptor is obtained will also influence the likelihood of off-target toxicity: the recognition and destruction of normal tissues that express a different epitope from that of the targeting agent. From a conceptual point of view, the T cell pool from a human lymphocyte antigen (HLA)–matched individual should be considered the safest source of TCRs, but the quality of the available TCR pool is likely capped by T cell tolerance for many antigens. The breadth of the available repertoire will be—roughly in order—greater in HLA-transgenic mice, in T cell pools from HLA-mismatched individuals, and in the in vitro TCR display systems that avoid T cell tolerance altogether. However, the safe use of the latter type of technologies is only feasible when rigorous assay systems are in place that can screen against unwanted cross-reactivity.

TOXICITY FROM ACT

In accord with expectations, toxicities from ACT have increased as the therapies have become more potent. Although TILs have generally been safe (as with other forms of autologous cellular therapy), both on-target and off-target recognition of normal tissue can occur with engineered T cells. For instance, on-target toxicity has been reported in patients treated with T cells engineered with a TCR that is specific for the carcinoembryonic antigen, resulting in severe inflammatory colitis developed from expression of target antigen in normal colon (*18*). With B cell–directed forms of ACT with CARs, commonly observed on-target toxicities have been B cell aplasia and cytokine release syndrome (*19*). Severe cardiac toxicity was reported owing to off-tumor and off-target recognition of titin after ACT with T cells expressing an affinity-engineered TCR that was originally specific for melanoma-associated antigen 3 (MAGE A3) (*20*). Methods involving computational and bio-

Table 1. Pharmaceutical and biotechnology companies in the ACT space. ACT applications are shown for cancers, infections, and GVHD.

Company	Technology/cell type	Indication
	Cancer	
Lion Biotechnologies	TIL (autologous)	Metastatic melanoma
Autolus	CAR (autologous)	Unspecified
Novartis	CAR (autologous) targeting CD19	Pediatric and adult ALL, diffuse large B cell lymphoma, non-Hodgkin's lymphoma (NHL)
Juno Therapeutics	CAR (autologous) targeting CD19, TCR (autologous) targeting Wilms tumor protein (WT-1)	Adult and pediatric ALL, NHL, adult acute myeloid leukemia (AML), non–small cell lung cancer (NSCLC)
Cardio3 Biosciences	CARs targeting NK cell p30-related protein (NKp30); NK group 2, member D (NKG2D); B7 homolog 6 (B7H6)	Range of hematological malignancies and solid tumors
Cellular Biomedicine Group	CARs targeting CD19, CD20, CD30, and EGFR	Range of hematological malignancies and solid tumors
CARsgen	CARs targeting glypican-3 (GPC-3)	Hepatocellular carcinoma
Celgene/Bluebird	CAR (autologous)	Range of hematological malignancies and solid tumors
Kite Pharma/Amgen	CAR (autologous) targeting CD19, TCR	Relapsed or refractory ALL
Cellectis/Servier/Pfizer	CAR (allogeneic, UCART 19)	CLL, ALL, and AML in preclinical stage, phase 1 for B cell leukemia to be initiated in 2015
GSK/Adaptimmune	TCR (autologous) targeting the cancer testis antigen NY-ESO-1 and other targets	Trials in multiple myeloma (MM), melanoma, sarcoma, and ovarian cancer
Janssen/Transposagen	CAR (allogeneic)	Unspecified
Unum Therapeutics/Sanofi-Genzyme	Antibody-coupled TCR (autologous)	Unspecified
Ziopharm Oncology/Intrexon	CAR	Unspecified
Opus Bio	CAR (autologous) targeting CD22	Pediatric and adult ALL and NHL, CD22 licensed to Juno
Takara Bio (Japan)	CAR (autologous) targeting CD19, TCR, MAGE-A4	NHL, esophageal cancer
Bellicum Pharmaceuticals	CAR (autologous) targeting CD19 with a proprietary safety switch to mute unwanted adverse events, such as cytokine release syndrome	Potential hematological malignancies and solid tumors
Cellular Therapeutics Ltd (UK)	CAR (autologous)	Metastatic melanoma, esophago-gastric cancer
Cell Medica (UK)	Virus-specific T cells (allogeneic) targeting Epstein-Barr virus antigen	Advanced NK/T cell lymphoma
Celdara Medical	CAR (autologous) targeting NKG2D	AML, advanced myelodysplastic syndrome (MDS), MM
Catapult Cell Therapy (UK)	TCR (autologous) targeting WT-1–overexpressing cells	AML, MDS
Medigene (Germany)	TCR (autologous)	Hematological malignancies
TheraVectys (France)	CARs (autologous) targeting CD19, CD33, and CD123	ALL, CLL, AML
BioNTech AG (Germany)	TCR, CAR (autologous)	Solid tumors (ovarian, endometrial, lung)
CARsgen (China)	CAR (autologous) targeting GPC-3 expressed in hepatocellular carcinoma; other CARs	Liver, lung, and brain cancers
FF CanVac	Virus-specific T cells (autologous)	Head and neck cancer
Apceth	Genetically engineered mesenchymal stem cells (MSC) (autologous)	Advanced, recurrent, or metastatic gastrointestinal cancer
Pocastem	Genetically engineered MSCs	Solid tumors (head and neck, brain)
TVAX Biomedical	Antigen-specific T cells (autologous)	Solid tumors (brain, kidney)
TC Biopharm (Scotland)	γ/δ T cells (autologous)	Melanoma
Immunovative Therapies (Israel)	Activated T cells (allogeneic)	Hematological malignancy, prostate cancer, breast cancer, glioblastoma, colorectal cancer with liver metastases, kidney cancer, NSCLC
CytoVac (Denmark)	Activated T cells/NK cells (autologous)	Glioblastoma, prostate cancer, pancreatic cancer
Conkwest	CAR NK cell line	AML
Coronado Biosciences	Activated NK cells (autologous)	AML
	HIV/Infection	
Calimmune	CCR5 knockdown $CD4^+$ T cells and stem cells	HIV
Cell Medica (UK)	Cytomegalovirus (CMV) infection after allogeneic hematopoietic stem cell transplant (HSCT)	CMV infection
Sangamo Biosciences	CCR5-mutated $CD4^+$ T cells and stem cells	HIV
Stage Therapeutics (Germany)	CMV-specific donor lymphocytes	CMV infection
Takara Bio (Japan)	mRNA interferase MazF (autologous) endoribonuclease–modified $CD4^+$ T cells	HIV
	GVHD	
Kiadis Pharma (Netherlands)	Allo-depleted T cells (allogeneic)	Facilitate early immune reconstitution without life-threatening (acute) GVHD in leukemia patients (ALL, AML, MDS) undergoing HSCT
Miltenyi Biotec GmbH/Prometheus Laboratories (Germany)	T_{reg}-enriched infusion (allogeneic) + low-dose IL2	Steroid-refractory chronic GVHD

logical approaches are being developed to predict off-target recognition by engineered TCRs (*21*).

Apart from toxicity consequent to the reactivity pattern of the introduced CAR or TCR itself, it is expected that autoimmunity and inflammation will sometimes result from the infusion of ex vivo–activated autologous lymphocytes. Current experimental trials exclude patients with active autoimmune disorders, so the incidence of immunopathology may rise when ACT achieves broad usage in the community. Severe side effects from CTLA-4 and PD-1 antagonism occur with relatively high frequency, especially upon combined checkpoint blockade (*22*, *23*), and we expect that this will occur with ACT unless, for example, steps are taken to edit out endogenous TCRs. In mice, the inflammatory consequences of immunotherapy are more severe in aged mice than in young mice and in obese rather than in thin mice (*24*). This may also happen in humans, and relevant to this is the observation that GVHD occurs more frequently and is more severe in aged rather than young patients (*25*).

A potential safety concern related to ACT with engineered T cells is integration-related insertional mutagenesis and cellular transformation—events previously demonstrated with engineered hematopoietic stem cells. To date, transformation of human lymphocytes has not been reported after ACT (*5*, *19*), and the incidence can be calculated to be less than one event per 1000 patient years of exposure to engineered T cells, an event rate that is lower than that reported for cytotoxic chemotherapy (*26*). The low genotoxicity with ACT may be due to cell-extrinsic mechanisms that control T cell homeostasis (*27*).

THE EXPANDING TOOLBOX FOR GENETIC ENGINEERING

Novel technologies that enable targeted alterations of the genome to modify or regulate cellular functions provide an opportunity for improving both the efficacy and safety of ACT. Zinc-finger nucleases (ZFNs) and transcription activator–like effector nucleases (TALENS) that rely on customized DNA binding proteins, and the natural bacterial CRISPR-Cas9 system of RNA-guided nucleases, can introduce DNA double-strand breaks at specific sites and lead to disruption of a gene sequence or provide a site for targeted gene insertion (*28*, *29*). ZFNs and TALENS have been used to disrupt endogenous TCR genes, and the first clinical application of ZFNs to disrupt expression of the HIV co-receptor CCR5 in $CD4^+$ T cells was reported recently (*30*–*33*).

Efficient genome editing paves the way for additional applications in ACT. The importance of T cell–intrinsic regulatory molecules such as CTLA-4 and PD-1 in suppressing beneficial tumor-reactive T cell responses has been established by using antibodies targeting these pathways (*34*–*36*). Selective editing of PD-1 or CTLA-4 genes in adoptively transferred T cells might similarly enhance efficacy without the side effects of systemic antibody blockade. Other regulatory pathways that inhibit T cell function locally in the tumor microenvironment have been revealed by introducing pooled short hairpin RNA (shRNA) libraries into tumor-specific T cells used in ACT, and this provides previously unidentified targets for gene editing, including intracellular targets that are not amenable to antibody-mediated blockade (*37*). A potential caveat of editing regulatory genes in T cells is that these molecules serve context-dependent roles in normal physiology, and permanent disruption, even in a subset of T cells, may have unforeseen consequences.

Genes can also be introduced into T cells in order to enhance their ability to localize at tumor sites and to function in the immunosuppressive tumor microenvironment. The introduction of chemokine receptor genes in T cells that bind chemokines produced by tumors can enhance T cell migration into tumors (*38*), and expression of dominant-negative transforming growth factor–β (TGF-β) receptors renders T cells resistant to the local inhibitory effects of TGF-β (*39*). Engineering T cells to secrete interleukin-12 (IL-12) induces a programmatic change in myeloid cells in the tumor microenvironment to promote tumor destruction, while avoiding the systemic toxicity of IL-12 (*40*).

Modifying T cells by means of gene editing or insertion to enhance therapeutic potency should coincide with attention to the safety of transferred T cells. Transgenes that provide for conditional cell suicide have been developed and can rapidly reverse acute or long-term toxicities of ACT. These include cell-surface molecules, such as CD20 or truncated epidermal growth factor receptor (EGFR), that are recognized by clinically approved monoclonal antibodies that mediate antibody-dependent cellular cytotoxicity (*41*, *42*). Herpes simplex virus thymidine kinase (HSV-TK) confers sensitivity of dividing T cells to ganciclovir and has been used effectively to eliminate transferred T cells that cause GVHD after allogeneic hematopoietic stem cell transplantation, although this approach is limited in immunocompetent hosts by immune responses to the viral TK (*43*). A nonimmunogenic suicide construct that consists of human caspase-9 fused to a modified domain of the human FK506-binding protein can induce cell death through exposure to a synthetic dimerizing drug, AP1903. The administration of AP1903 rapidly and completely reversed clinical manifestations of GVHD that occurred after T cell administration (*44*), suggesting that this "safety switch" approach may be sufficiently rapid to abrogate unexpected immediate toxicities of ACT.

FROM UNIVERSAL T CELLS TO PERSONALIZED ACT

Current approaches to gene-modified T cell therapy are personalized in the sense that a patient-specific cell product is created but generic in the sense that the same receptor is used for larger patient groups. As extensions to this, strategies to develop universal T cell products and to develop patient-specific receptors have recently been proposed.

Approaches toward universal T cell therapy aim to allow the widespread application of gene-modified T cell therapy at a lower cost (Fig. 2A). With respect to the creation of such universal T cells, several substantial barriers need to be overcome. First, alloreactivity within the endogenous TCR pool leads to GVHD when HLA-mismatched donor-derived T cells are used for therapy. By the same token, recognition of donor-cell allo-determinants by the patient's T cell pool leads to rapid rejection of infused cells unless additional measures are taken. Genome engineering technologies make it feasible to create T cell products in which one or both of the endogenous TCR chains have been inactivated, allowing a more comprehensive editing of T cell specificity and consequent avoidance of allo-reactivity (*30*, *31*, *33*). In addition, such inactivation of both the endogenous TCR α and β chains avoids the formation of the mixed TCR dimers that have been shown to cause GVHD in mouse models (*45*). With respect to technologies to suppress rejection of the infused cells, inactivation of donor major histocompatibility complex genes could potentially be used to prevent T cell–mediated rejection (*46*) but may at the same time trigger NK

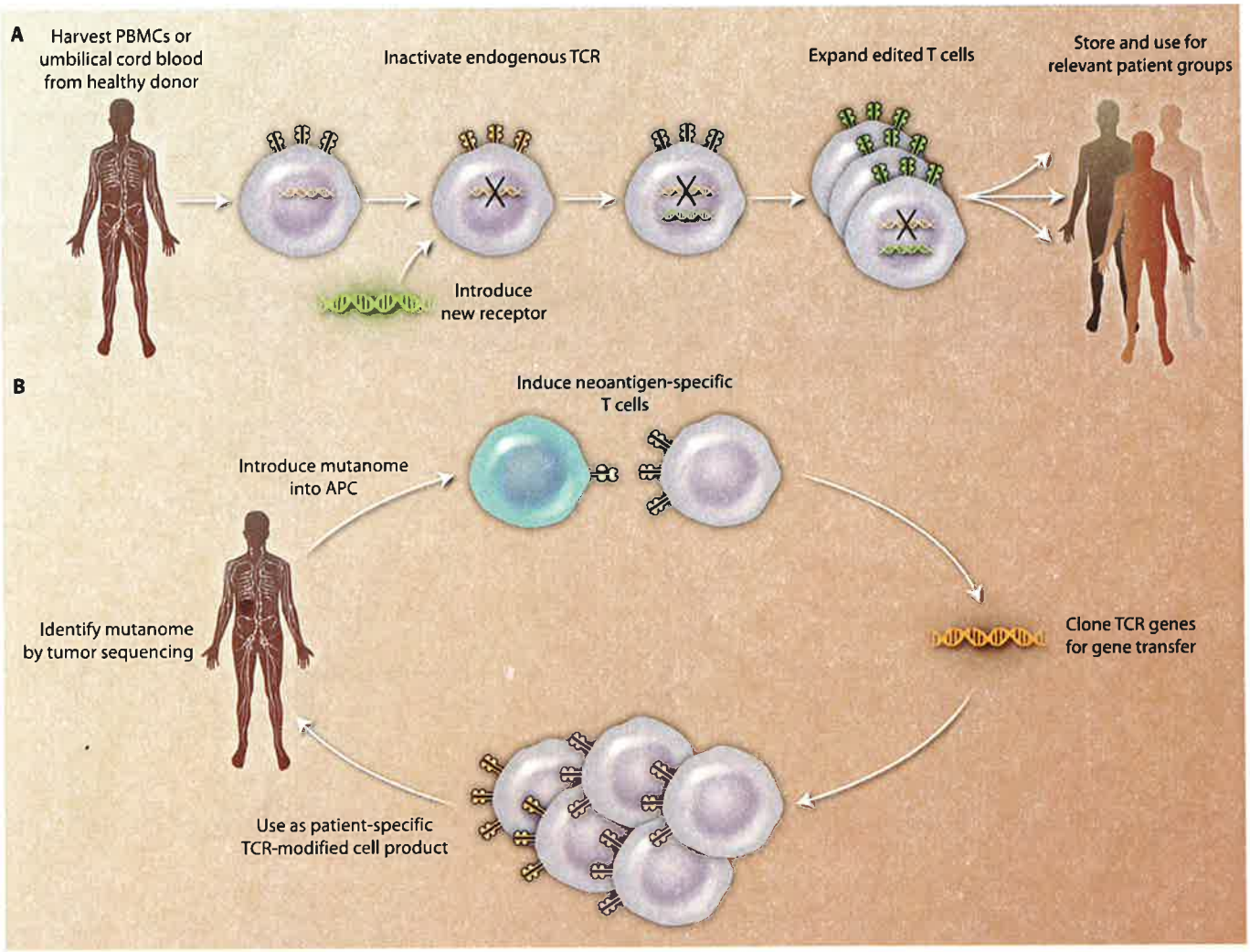

Fig. 2. From universal to highly personalized gene-modified ACT. (**A**) Universal T cells in which the endogenous TCR has been replaced by a CAR or TCR as "off-the-shelf" ACT products. Expression of the endogenous TCR can be eliminated through genetic editing. (**B**) Targeting the patient-specific mutanome by gene-modified ACT. Tumor-specific mutations are expressed in antigen-presenting cells (APCs), and the TCR repertoire is isolated from the responding T cells. The desired tumor-specific TCRs can be isolated and introduced into T cells for later ACT.

cell recognition. Conceivably, development of approaches that render infused cells selectively insensitive to immunosuppressive drugs may form a superior alternative.

At present, the number of antigens that can safely be targeted by TCRs or CARs is still limited to a handful. To increase the antigenic targets that are available to gene-modified T cell therapy, approaches to obtain receptors that are reactive against patient-specific neoantigens may be of interest (Fig. 2B). Recent work has shown that in human melanoma, both $CD8^+$ and $CD4^+$ T cell recognition of neoantigens occurs frequently (*11*, *47*, *48*). And based on overlap in mutational loads, formation of neoantigens that can be recognized by T cells can be expected in several other high-prevalence human tumors (*47*). In case the endogenous T cell pool generally "picks up" on the majority of neoantigens presented by an individual tumor, isolation of the relevant TCRs from the autologous T cell pool may be a way to boost immune reactivity against this class of antigens. Alternatively, it seems possible that in some human tumor types, priming of an endogenous T cell response may be inefficient. In such cases, it may be attractive to exploit antigen-presenting cells that express the patient-specific mutanome so as to induce such reactivities.

From a safety perspective, the targeting of the patient-specific neoantigen repertoire is highly appealing. However, it remains to be established for which tumor types neoantigen-specific TCRs can readily be obtained, and the logistic hurdles—with respect to regulation, timelines, and projected costs—are substantial.

TRANSLATIONAL BOTTLENECKS AND CHALLENGES

Therapeutically effective T cells can be derived from tumor infiltrates in melanoma patients; however, the peripheral blood is the preferable site for obtaining T cells for genetic modification for ACT because of the ease of procurement. To date, the focus has been on genetically modifying α/β T cells without regard to subset or differentiation status. However, α/β T cells are present in

functionally heterogeneous $CD4^+$ and $CD8^+$ subsets that differ in frequency, phenotype, transcriptional profile, and effector function. Current models suggests progressive differentiation from antigen-inexperienced naïve cells (T_N) to $CD62L^+$ central memory (T_{CM}), $CD62L^-$ effector memory (T_{EM}), and effector (T_E) T cell subsets, with loss of proliferative capacity and acquisition of effector function (*49*–*51*). Treatment efficacy after adoptive transfer of endogenous or genetically redirected tumor-reactive T cells correlates best with the ability of transferred T cells to proliferate and persist in vivo, suggesting that selection of T_N and/or T_{CM} may provide greater therapeutic potency. The optimal composition of $CD4^+$ and $CD8^+$ subsets for ACT may also differ depending on the malignancy being treated. Unfortunately, the lack of rapid, cost-effective, and efficient clinical-grade cell-selection devices and procedures currently impedes the evaluation of therapeutic T cell products derived from distinct T cell subsets.

A challenge for all cell therapies, including T cell therapy, is the need to develop cost-effective and efficient manufacturing and delivery capabilities. The sipuleucel-T (Provenge®) dendritic cell vaccine for prostate cancer developed by Dendreon demonstrated that cell therapies could be manufactured and delivered to physicians but illustrated that efficacy needed to be high to justify the cost and complexity and to compete with more easily administered pharmaceuticals. ACT has been pioneered in academic laboratories for which the resources to develop closed robotic automated systems for cell selections, genetic modification, and expansion are not readily available. The recent influx of biotechnology and pharmaceutical companies into cell-based therapeutics should accelerate automation to reduce cost and improve feasibility and delivery (Table 1). Off-the-shelf genetically modified tumor-specific T cells from allogeneic donors could further diminish the manufacturing burden for ACT, in case the immunologic barriers to this approach can be overcome.

The ability to redirect T cells with previously unidentified TCRs and CARs is increasing the types of malignancies that can be targeted with ACT. In the case of CARs, few targets that are exclusively expressed by tumor cells have been identified. The potential for—and consequences of—on-target recognition of normal cells can be evaluated in animal models, providing that the expression patterns are identical to humans (*52*). Logic gates, such as dual targeting with split receptor systems, may be used to improve the selectivity of tumor cell recognition by CAR-T cells for targets expressed on tumor and a subset of normal cells (*53*).

As the clinical applications of ACT expand, it will be important to identify biomarkers that predict success. Analysis of tumor biopsies before therapy might identify signatures that predict susceptibility to ACT or define interventions that may be necessary to improve therapeutic efficacy. The ability of T cells to proliferate and/or persist in vivo has correlated with therapeutic efficacy after ACT for viral diseases and cancer. Thus, analysis of the functional properties of engineered T cells before transfer and their fate and function after transfer could provide insights into optimal compositions of ACT for therapeutic efficacy. Combining ACT with checkpoint-blocking antibodies, vaccines, and targeted drug therapies is supported by studies in animal models (*54*, *55*) and is beginning to be investigated in clinical trials.

The development of ACT, particularly with genetically modified T cells, has occurred predominantly in the United States. ACT with TILs for melanoma, CARs targeting CD19, TCRs for cancer, and gene-edited T cells for HIV have advanced to phase 2 clinical trials (NCT02228096, NCT01567891, NCT02348216, and NCT02225665), and it is likely that one or more of these T cell therapies will obtain eventual U.S. Food and Drug Administration (FDA) approval (Table 1). Regulatory agencies in Europe have not had the same experience in this field, and given the early success of this approach, these agencies are likely to be inundated with new applications and challenged by patient demand. The complexity of ACT makes it vital to educate patients and physicians regarding the appropriate indications and the particular toxicities and their management so as to avoid preventable adverse outcomes. New therapeutic technologies including ACT are expensive, and this will present additional challenges regarding reimbursement that are best overcome by clearly demonstrating therapeutic value and cost-effective outcome as compared with those of alternative therapies.

SUMMARY

Advances in genetic engineering have reinvigorated efforts to engineer T cells to be tumor-reactive to treat advanced human malignancies through adoptive transfer. Remarkable success in patients treated on trials at academic centers has enticed unprecedented interest from the biotechnology and pharmaceutical industry (Table 1), which is now rapidly advancing these approaches for FDA approval and accelerating research and development to safely apply ACT to a broad range of human diseases, from acute lymphoblastic leukemia (ALL) to glioblastoma to HIV. The field faces numerous scientific, regulatory, and economic obstacles and challenges in educating clinicians in the use of ACT. Surmounting these obstacles will require collaboration between academia and biotechnology in order to ensure that therapy with engineered T cells is established as a viable approach for common human malignancies. Results in cancer are likely to pave the way to ACT as a new approach for infections and autoimmunity.

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Sweeney, Timothy (NIH/NCI) [E]

From: David Chang <DChang@KitePharma.com>
Sent: Thursday, April 02, 2015 4:19 PM
To: Rosenberg, Steven A. (NIH/NCI) [E]
Subject: FW: Intrexon Signs Cooperative Research and Development Agreement (CRADA) with the National Cancer Institute (NCI) for RheoSwitch Controlled IL-12 Cancer Therapies Using T cell Receptors (TCR) Derived from Peripheral Blood
Attachments: Intrexon Signs Cooperative Research and Development Agreement.pdf

From: Mulé, James J. [mailto:James.Mule@moffitt.org]
Sent: Wednesday, April 01, 2015 1:39 PM
To: Arie Belldegrun; Arie (arie@belldegrun.com); 'abelldegrun@mednet.ucla.edu'; David Chang
Subject: FW: Intrexon Signs Cooperative Research and Development Agreement (CRADA) with the National Cancer Institute (NCI) for RheoSwitch Controlled IL-12 Cancer Therapies Using T cell Receptors (TCR) Derived from Peripheral Blood

In case you have not seen this... Jim

From: Marie Rossi [mailto:publicrelations@intrexon.com]
Sent: Wednesday, April 01, 2015 4:19 PM
To: Mulé, James J.
Subject: Intrexon Signs Cooperative Research and Development Agreement (CRADA) with the National Cancer Institute (NCI) for RheoSwitch Controlled IL-12 Cancer Therapies Using T cell Receptors (TCR) Derived from Peripheral Blood

Good afternoon,

Today Intrexon Corporation (NYSE: XON), a leader in synthetic biology, announced that it has signed a Coop Development Agreement (CRADA) with the National Cancer Institute (NCI), part of the National Institute development of adoptive T cell therapies utilizing the RheoSwitch Therapeutic System® (RTS®) platform for the trea malignancies. The principal goal of the CRADA is to develop and evaluate improved adoptive cell transfer-bas (ACT) using NCI proprietary methods for the identification of autologous peripheral blood lymphocytes (PBL) occurring anti-tumor activity combined with Intrexon's RTS® gene switch for introducing spatially and temporally c 12 (IL-12) expression.

Additional information can be found in the attached press release. Please contact me if you have any questions.

Best Regards,
Marie

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INTREXON®

Intrexon Signs Cooperative Research and Development Agreement (CRADA) with the National Cancer Institute (NCI) for RheoSwitch® Controlled IL-12 Cancer Therapies Using T cell Receptors (TCR) Derived from Peripheral Blood

GERMANTOWN, MD, April 1, 2015 – Intrexon Corporation (NYSE: XON), a leader in synthetic biology, today announced that Intrexon has signed a Cooperative Research and Development Agreement (CRADA) with the National Cancer Institute (NCI), part of the National Institutes of Health, for the development of adoptive T cell therapies utilizing the RheoSwitch Therapeutic System® (RTS®) platform for the treatment of solid tumor malignancies. The principal goal of the CRADA is to develop and evaluate improved adoptive cell transfer-based immunotherapies (ACT) using NCI proprietary methods for the identification of autologous peripheral blood lymphocytes (PBL) possessing naturally occurring anti-tumor activity combined with Intrexon's RTS® gene switch for introducing spatially and temporally controlled interleukin-12 (IL-12) expression.

RTS® technology enables transcriptional regulation of a wide variety of therapeutic genes upon dosing of an oral activator ligand veledimex, including *in vivo* modulation of IL-12 gene expression with a broad dynamic range. As the first gene switch employed in the clinic to enable dose-dependent cytokine expression and offer the ability to administer or withdraw veledimex for continued treatment cycles, the RheoSwitch® platform provides the opportunity to tailor solutions for patient-specific therapeutic effects. Intrexon will genetically modify PBL using vectors that encode IL-12 under RTS® inducible control. Lead anti-tumor ACT/PBL/IL-12 cell therapy candidates will then be clinically evaluated by NCI in patients with metastatic cancer.

Under the CRADA, Steven A. Rosenberg, M.D., Ph.D., Chief of the Surgery Branch in the Center for Cancer Research at the NCI, will be the Principal Investigator for the study, and Gregory Frost, Ph.D., Senior Vice President and Head of Intrexon's Health Sector, will serve as co-investigator.

"Dr. Rosenberg and his colleagues at the NCI Surgery Branch have extensive experience in the clinical translation of tumor-targeting peripheral blood products for cancer treatment," said Dr. Frost. "Together with our molecular and cell engineering capabilities, we believe the research programs under this CRADA have the potential to accelerate development of targeted and controllable adoptive therapies for patients suffering with advanced stage malignancies."

About Intrexon Corporation

Intrexon Corporation (NYSE: XON) is a leader in synthetic biology focused on collaborating with companies in Health, Food, Energy, Environment, and Consumer sectors to create biologically-based products that improve the quality of life and the health of the planet. Through the Company's proprietary UltraVector® platform and integrated technology suite, Intrexon provides its partners with industrial-scale design and development of complex biological systems delivering unprecedented control, quality, function, and performance of living cells. We call our synthetic biology approach Better DNA®, and we invite you to discover more at www.dna.com.

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Safe Harbor Statement
Some of the statements made in this press release are forward-looking statements. These forward-looking statements are based upon our current expectations and projections about future events and generally relate to our plans, objectives and expectations for the development of our business. Although management believes that the plans and objectives reflected in or suggested by these forward-looking statements are reasonable, all forward-looking statements involve risks and uncertainties and actual future results may be materially different from the plans, objectives and expectations expressed in this press release.

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Sweeney, Timothy (NIH/NCI) [E]

From: Arie Belldegrun <Arie@kitepharma.com>
Sent: Monday, April 06, 2015 10:28 AM
To: Rosenberg, Steven A. (NIH/NCI) [E]
Subject: Fwd: Kite Pharma Announces Publication in Science of Cancer Immunotherapy Articles Authored by Lead Collaborators at the National Cancer Institute and the Netherlands Cancer Institute

FYI

Thank you for everything,

Arie Belldegrun, MD FACS
President and CEO, Chairman
Kite Pharma

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Begin forwarded message:

From: "Kite Pharma, Inc." <jjackson@burnsmc.com>
Date: April 6, 2015 at 05:04:01 PDT
To: <Arie@kitepharma.com>
Subject: Kite Pharma Announces Publication in Science of Cancer Immunotherapy Articles Authored by Lead Collaborators at the National Cancer Institute and the Netherlands Cancer Institute

Kite Pharma Announces Publication in Science of Cancer Immunotherapy Articles Authored by Lead Collaborators at the National Cancer Institute and the Netherlands Cancer Institute

SANTA MONICA, Calif., April 6, 2015 (GLOBE NEWSWIRE) -- Kite Pharma, Inc. (Kite) (Nasdaq:KITE), a clinical-stage biopharmaceutical company focused

treatment of cancer, today announced articles being published in the current issue of *Science*, one article by the Company's Cooperative Research and Development Agreement (CRADA) collaborators at the National Cancer Institute (NCI) and the second article by the Netherlands Cancer Institute (NKI). The first article, "Adoptive Cell Transfer as personalized immunotherapy for human cancer," was authored by Steven A. Rosenberg, M.D., Ph.D., Chief of Surgery at the NCI, and his colleague Nicholas P. Restifo, M.D., Senior Investigator at NCI's Surgery Branch. The second article, "Neoantigens in cancer immunotherapy," was written by Professor Dr. Ton N. M. Schumacher, Ph.D., Deputy Director of NKI, and Robert D. Schreiber, Ph.D., Director, Center for Human Immunology and Immunotherapy Programs, at Washington University School of Medicine. Professor Schumacher also serves as Chief Scientific Officer of Kite Pharma EU.

In their article, Drs. Rosenberg and Restifo reviewed the development of adoptive cell therapy, including the advent of genetically modified T cells and their promising results in patients with multiple tumor types. Last month, Kite announced an expansion of its CRADA with the Surgery Branch at the NCI, led by Dr. Rosenberg. The amendment encompasses emerging areas of research in the immune response to tumor neoantigens, in addition to new T cell receptor (TCR) and chimeric antigen receptor (CAR) product candidates targeting solid tumors.

Also, in March, Kite acquired T-Cell Factory B.V. (TCF™), a privately held Dutch company founded by preeminent scientists, including Professor Schumacher and Professor Dr. Dirk H. Busch, M.D., of the Technische Universität München (TUM). The acquisition of TCF greatly expands Kite's TCR product platform to discover and develop TCR-based product candidates for the treatment of solid tumors, complementing Kite's CAR pipeline. TCF was renamed Kite Pharma EU and provides a base for Kite to build its global presence.

In their review article, Professor Schumacher and Dr. Schreiber described breakthrough patient-specific neoantigen research, including the targeting of multiple neoantigens to augment patient responses. According to the authors, "Because of the tumor-restricted expression of the antigens that are being targeted, these personalized cancer immunotherapies offer the promise of high specificity and safety. Conceivably, the boosting of neoantigen-specific T cell reactivity that can be achieved with such personalized immunotherapies will further increase the spectrum of human malignancies that respond to cancer immunotherapy."

"Our alliances, including with CRADA collaborator NCI and now with NKI, are essential to building and growing our pipeline of promising clinical and preclinical programs," said Arie Belldegrun, M.D., FACS, Chairman, President and Chief Executive Officer. "Based on our progress, there are six ongoing trials under our CRADA with the NCI and we anticipate initiating four pivotal trials of our anti-CD19 CAR product candidate, KTE-C19, in 2015. This momentum is built on strong foundations and relationships."

About Kite Pharma, Inc.

Kite Pharma, Inc., is a clinical-stage biopharmaceutical company engaged in the development of novel cancer immunotherapy products, with a primary focus on eACT™ designed to restore the immune system's ability to recognize and eradicate tumors. Kite is based in Santa Monica, CA.

Cautionary Note on Forward-Looking Statements

This press release contains forward-looking statements for purposes of the safe harbor provisions of the Private Securities Litigation Reform Act of 1995. Kite may, in some cases, use terms such as "predicts," "believes," "potential," "proposed," "continue," "estimates," "anticipates," "expects," "plans," "intends," "may," "could," "might," "will," "should" or other words that convey uncertainty of future events or outcomes to identify these forward-looking statements. Forward-looking statements include statements regarding Kite's intentions, beliefs, projections, outlook, analyses or current expectations concerning, among other things: the success and timing of the ongoing and anticipated clinical trials for Kite's current product candidates, including statements regarding the timing of initiation of the KTE-C19 clinical trials, the ability and willingness of the NCI to continue research and development activities relating to eACT™ pursuant to the CRADA; and the ability to expand Kite's pipeline of TCR-based product candidates through the acquisition of TCF. Various factors may cause differences between Kite's expectations and actual results as discussed in greater detail under the heading "Risk Factors" in the Form 10-K for the year ended December 31, 2014. Any forward-looking statements that Kite makes in this press release speak only as of the date of this press release. Kite assumes no obligation to update its forward-looking statements whether as a result of new information, future events or otherwise, after the date of this press release.

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To: Rosenberg, Steven A. (NIH/NCI) [E]
Subject: FW: Science
Attachments: Sharma and Allison Science 2015.full.pdf; Garrett Science 2015.pdf; Joyce and Fearon Science 2015.pdf; Rizvi Mutational Burden in PD1 response Science 2015.pdf; Rosenberg & Restifo Science 2015.full.pdf; Schumacher and Schreiber Science 2015.pdf

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REVIEWS

The future of immune checkpoint therapy

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Immune checkpoint therapy, which targets regulatory pathways in T cells to enhance antitumor immune responses, has led to important clinical advances and provided a new weapon against cancer. This therapy has elicited durable clinical responses and, in a fraction of patients, long-term remissions where patients exhibit no clinical signs of cancer for many years. The way forward for this class of novel agents lies in our ability to understand human immune responses in the tumor microenvironment. This will provide valuable information regarding the dynamic nature of the immune response and regulation of additional pathways that will need to be targeted through combination therapies to provide survival benefit for greater numbers of patients.

The field of immune checkpoint therapy has joined the ranks of surgery, radiation, chemotherapy, and targeted therapy as a pillar of cancer therapy. Three new immune checkpoint agents have now been approved by the U.S. Food and Drug Administration (FDA) for the treatment of melanoma, and there is a high expectation that these agents, and others in this class, will also be approved over the next several years for treatment of patients with lung cancer, kidney cancer, bladder cancer, prostate cancer, lymphoma, and many other tumor types. The antibody against CTLA-4 ipilimumab was approved in 2011, and two antibodies against PD-1 (pembrolizumab and nivolumab) were approved in 2014. These drugs represent a radical and disruptive change in cancer therapy in two ways. First, they do not target the tumor cell, but target molecules involved in regulation of T cells, the soldiers of the immune system. And, perhaps in a more radical shift, the goal of the therapy is not to activate the immune system to attack particular targets on tumor cells, but rather to remove inhibitory pathways that block effective antitumor T cell responses. Immune checkpoint therapy, with anti-CTLA-4 having longer follow-up than other agents, leads to durable clinical responses that can last a decade and more, but only in a fraction of patients. There are ongoing studies to identify predictive biomarkers with which to select patients for treatment with a particular agent, but the complexity of the immune response has made this difficult.

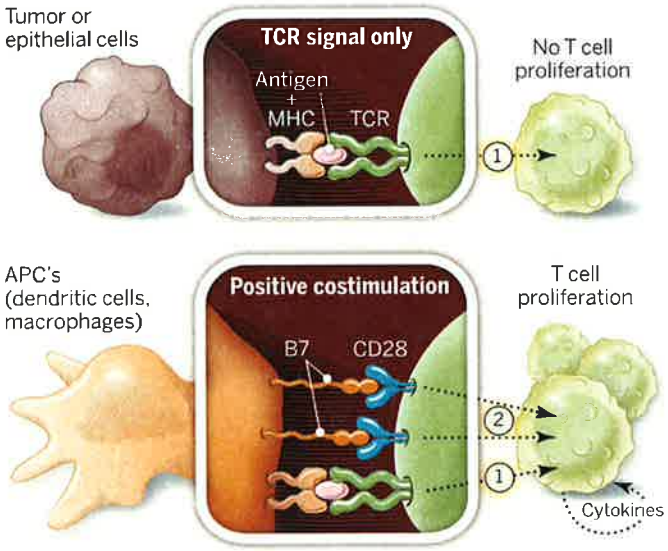

Fig. 1. Activation of T cells requires two signals. T cell activation occurs only after interaction between T cell receptor (TCR) and antigen in the context of MHC (signal 1) plus CD28 costimulation (signal 2).

In the past two decades, remarkable advances in basic science have led to new strategies for the treatment of cancer, which are justifiably generating optimism that it may soon be possible to cure a subset of patients with some types of cancer. We now have detailed knowledge of the molecular basis of cancer to allow a more "personalized" treatment based on genomic sequencing of an individual's cancer cells to identify specific mutations in genes. These mutations can then be targeted with compounds to block the downstream pathways that drive cancer development and progression. Therefore, each specific mutation serves as the predictive biomarker for selecting patients for treatment with a given agent. For example, patients with melanoma whose tumors harbor the BRAFV600E mutation, which enables constitutive activation of the BRAF signaling pathway, would be selected to receive treatment with an agent that inhibits BRAF (*1*, *2*). These targeted therapies have led to promising clinical responses, albeit generally of short duration, in patients whose tumors express the appropriate target biomarker.

The clinical success of genomically targeted agents laid the foundation for other cancer therapies, including the prerequisite to identify predictive biomarkers for selection of patients for treatment. Eventually, as the field of cancer immunotherapy found clinical success with agents based on a greater understanding of how to unleash T cell responses by targeting immune checkpoints, it became clear that the framework used for identification of predictive biomarkers for genomically targeted agents would present a challenge. As opposed to mutated genes in tumors that permanently mark a tumor, the immune response is dynamic and changes rapidly. Therefore, the issue facing the field of cancer immunotherapy may not be the identification of a single biomarker to select a subset of patients for treatment. Instead, we must assess the effectiveness of an evolving immune response, define the immune response that contributes to clinical benefit, and then, hopefully, drive every patient's immune response in that direction through combination therapies.

Tumor microenvironment: Cancer cells and host immune responses

Tumors are composed of many cell types, including the cell of origin with genetic alterations and a myriad of other cells, such as fibroblasts, endothelial cells, and eventually, perhaps, a variety of immune cells. Initially the immune infiltrates may be scarce, but eventually may contain natural killer (NK) cells and macrophages with lytic capacity and, perhaps most importantly, T cells. T cells attack tumor cells that express tumor-specific antigens in the form of complexes of tumor-derived peptides bound to major histocompatibility complex (MHC) molecules on the cell. The tumor antigens can be derived from oncogenic viruses, differentiation antigens, epigenetically regulated molecules such as cancer testes antigens, or neoantigens derived from mutations associated with the process of carcinogenesis (*3*). T cells survey the microenvironment and become activated when tumor antigens are recognized. They then proliferate and differentiate, ultimately leading to the T cell's ability to attack and destroy cells that express relevant antigens. However, regulation of T cell responses is an extremely complex process consisting of both stimulatory and inhibitory cell intrinsic signaling pathways, which limit T cell responses against cancer and prevent eradication of tumors.

Recognition of antigen-MHC complexes by the T cell antigen receptor is not sufficient for

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activation of naïve T cells—additional costimulatory signals (*4*, *5*) are required that are provided by the engagement of CD28 on the T cell surface with B7 molecules (CD80 and CD86) on the antigen-presenting cell (APC) (Fig. 1). Expression of B7 molecules is limited to subsets of hematopoietic cells, especially dendritic cells, which have specialized processes for efficient antigen presentation. With the exception of certain lymphomas, cancer cells do not express B7 molecules, and hence are largely invisible to the immune system. This can be overcome by an inflammatory response, such as the killing of tumor cells, which permits APCs, such as dendritic cells, to take up antigen and present antigen bound to MHC along with B7 molecules for effective activation of T cells.

After encountering tumor antigen in the context of B7 costimulation, initially in tumor-draining lymph nodes, tumor-specific T cells may acquire effector function and traffic to the tumor site to mount an attack on the tumor. Infiltration of T cells into the tumor microenvironment is a critical hurdle that must be overcome for an effective antitumor immune response to occur. However, once T cells are in the tumor microenvironment, the success of the assault is determined by their ability to overcome additional barriers and counter-defenses they encounter from the tumor cells, stroma, regulatory T cells, myeloid-derived suppressor cells, inhibitory cytokines, and other cells in the complex tumor microenvironment that act to mitigate antitumor immune responses.

In the 1980s, tumor antigens from human melanomas were found to elicit T cell responses (*6*), which drove efforts to use vaccination strategies to mobilize the immune system to attack cancer. The vaccines generally consisted of some form of the antigen (for example, peptide or DNA vaccines), as well as additional components to enhance responses (for example, cytokines). While there were anecdotal successes, in hundreds of trials there was scant evidence of reproducible clinical responses (*7*). This failure to induce effective immune responses by attempting to turn T cell response "on" with antigenic vaccines led many to become skeptical of the potential of immunotherapy as a strategy for cancer treatment.

Regulation of T cell responses

Further insights into the fundamental mechanisms that regulate early aspects of T cell activation may provide one of many possible explanations for the limited effectiveness of these early vaccine trials. By the mid-1990s, it was becoming clear that T cell activation was even more complex, and in addition to initiating proliferation and functional differentiation, T cell activation also induced an inhibitory pathway that could eventually attenuate and terminate T cell responses. Expression of *ctla-4*, a gene with very high homology to CD28, is initiated by T cell activation, and, like CD28, CTLA-4 binds B7 molecules, albeit with much higher affinity. Although CTLA-4 was first thought to be another costimulatory molecule (*8*), two laboratories independently showed that it opposed CD28 costimulation and down-regulated T cell responses (*9*, *10*). Thus, activation of T cells results in induction of expression of CTLA-4, which accumulates in the T cell at the T cell–APC interface, reaching a level where it eventually blocks costimulation and abrogates an activated T cell response (Fig. 2).

Based on knowledge of the function of CTLA-4, we proposed that blocking its interaction with the B7 molecules might allow T cell responses to persist sufficiently to achieve tumor eradication. We hypothesized that this could be achieved by releasing the endogenous immune responses, perhaps even without specific knowledge of the antigenic targets of those responses or even the type of cancer. We also proposed that combination treatment with an antibody against CTLA-4 and agents that directly killed tumor cells to release antigens for presentation by APCs to T cells would improve antitumor responses. Our hypotheses were tested in many different experiments in mice (*11*–*15*), with data generated to support the concept, leading to the development of ipilimumab, an antibody against human CTLA-4 for clinical testing. Ipilimumab led to considerable improvement in overall survival for patients with metastatic melanoma (*16*, *17*), which led to FDA approval in 2011.

The preclinical successes of anti-CTLA-4 in achieving tumor rejection in animal models and the ultimate clinical success opened a new field of immune checkpoint therapy (*18*, *19*). It is now known that there are many additional immune checkpoints. Programmed cell death–1 (PD-1) was shown in 2000 to be another immune checkpoint that limits the responses of activated T cells (*20*). PD-1, like CTLA-4, has two ligands, PD-L1 and PD-L2, which are expressed on many cell types. The function of PD-1 is completely distinct from CTLA-4 in that PD-1 does not interfere with costimulation, but interferes with signaling mediated by the T cell antigen receptor (*4*). Also, one of its ligands, PD-L1 (B7-H1), can be expressed on many cell types (Fig. 2), including T cells, epithelial cells, endothelial cells, and tumor cells after exposure to the cytokine interferon-γ (IFN-γ), produced by activated T cells (*21*). This has led to the notion that rather than functioning early in T cell activation, the PD-1/PD-L1 pathway acts to protect cells from T cell attack.

Immune checkpoint therapy in the clinic

Ipilimumab, a fully human antibody to human CTLA-4, entered clinical trials in the late 1990s and early 2000s. As predicted, tumor regression was observed in patients with a variety of tumor types. Phase I/II trials showed clinical responses in

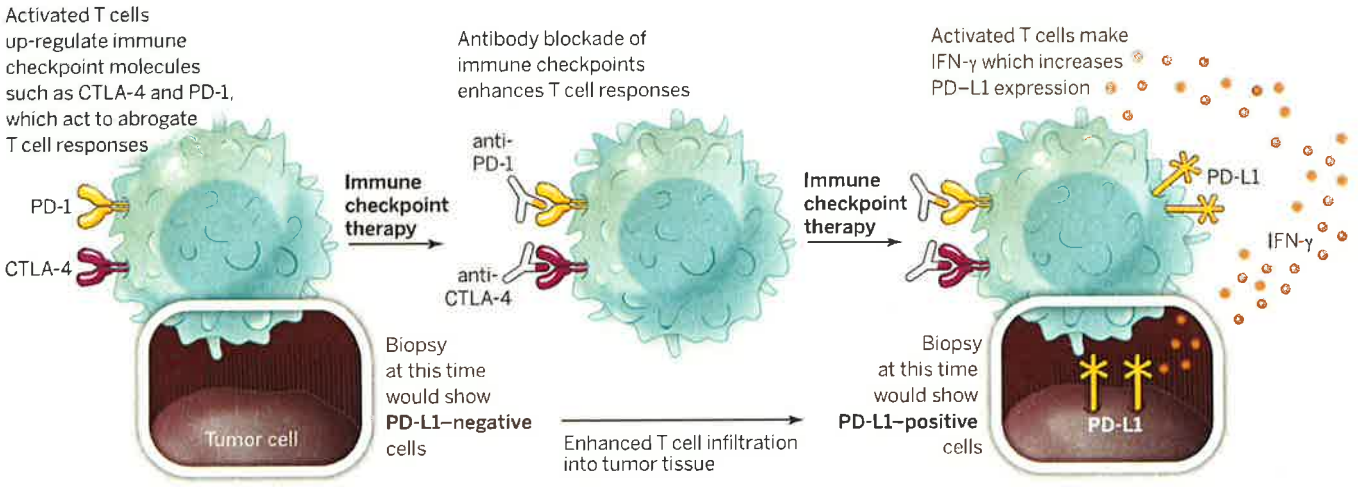

Fig. 2. Blockade of immune checkpoints to enhance T cell responses. After T cell activation, T cells express immune checkpoints such as CTLA-4 and PD-1. A biopsy of tumors taken from patients before treatment with immune checkpoint therapy (so prior to infiltration of activated T cells into tumor tissues) may indicate lack of PD-L1 expression. However, upon T cell activation, T cells can traffic to tumors, up-regulate expression of immune checkpoints such as CTLA-4 and PD-1, and produce cytokines such as IFN-γ, which leads to expression of PD-L1 on tumor cells and other cells, including T cells, within the tumor tissues.

patients with melanoma (*22*), renal cell carcinoma (*23*), prostate cancer (*24*), urothelial carcinoma (*25*), and ovarian cancer (*26*). Two phase III clinical trials with anti-CTLA-4 (ipilimumab) were conducted in patients with advanced melanoma and demonstrated improved overall survival for patients treated with ipilimumab (*16, 17*). Importantly, durable responses were observed in about 20% of patients living for more than 4 years, including a recent analysis indicating survival of 10 years or more for a subset of patients (*27*).

Antibodies targeting the PD-1/PD-L1 axis have also shown clinical responses in multiple tumor types. Anti-PD-L1 antibodies led to tumor regression in patients with melanoma, renal cell carcinoma, non–small cell lung cancer (*28*), and bladder cancer (*29*). Phase I clinical trials with anti-PD-1 (nivolumab) demonstrated similar clinical responses (*30*). Recently, a large phase I clinical trial with the anti-PD-1 antibody MK-3475 was shown to lead to response rates of ~37 to 38% in patients with advanced melanoma (*31*), with a subsequent study reporting an overall response rate of 26% in patients who had progressive disease after prior ipilimumab treatment (*32*), which led to FDA approval of MK-3475 (pembroluzimab) in September 2014. A phase III trial of a different anti-PD-1 antibody (nivolumab) also showed clinical benefit in patients with metastatic melanoma. In this trial, the objective response rate was 40% and overall survival rate was 72.9% for patients treated with nivolumab as compared to an objective response rate of 13.9% and overall survival rate of 42.1% for patients treated with dacrabazine chemotherapy (*33*). Nivolumab received FDA approval in December 2014 as a treatment for patients with metastatic melanoma. In addition, nivolumab was FDA-approved in March 2015 for patients with previously treated advanced or metastatic non–small cell lung cancer based on a phase III clinical trial, which reported an improvement in overall survival for patients treated with nivolumab as compared to patients treated with docetaxel chemotherapy.

That CTLA-4 and PD-1 regulate distinct inhibitory pathways and have nonoverlapping mechanisms of action suggested that concurrent combination therapy with both might be more efficacious than either alone. This was indeed shown to be the case in preclinical studies in murine models (*34*). In 2013, a phase I clinical trial with anti-CTLA-4 (ipilimumab) in combination with anti-PD-1 (nivolumab) demonstrated tumor regression in ~50% of treated patients with advanced melanoma, most with tumor regression of 80% or more (*35*). There are ongoing clinical trials with anti-CTLA-4 plus anti-PD-1, or anti-PD-L1, in other tumor types, with preliminary data indicating promising results, which highlight this novel combination as an effective immunotherapy strategy for cancer patients.

Tissue-based immune monitoring: Anti-CTLA-4 therapy

Properly designed presurgical or tissue-based trials, where treatment is administered before surgical resection of tumors, can provide valuable insight into the cellular and molecular mechanisms of immune checkpoint therapy by providing sufficient tissues to conduct a battery of analyses. Data gathered from analysis of tumor tissue can then guide rational searches for relevant markers in the blood. We designed the first presurgical clinical trial with anti-CTLA-4 (ipilimumab), which was administered to 12 patients with localized bladder cancer prior to radical cystectomy (*36*). The endpoints of this study were safety and access to samples for immune monitoring. We did not view this trial as a neoadjuvant study, which administers therapy prior to surgery for clinical benefit, but as a presurgical study to provide mechanistic insights regarding the impact of anti-CTLA-4 therapy on the tumor microenvironment. Unexpectedly, the trial enabled us to detect a clinical signal for anti-CTLA-4 as a therapeutic agent for patients with bladder cancer since three patients had no residual tumors identified within the cystectomy samples. This trial was also successful in establishing the safety of anti-CTLA-4 in the presurgical setting, which would be important for future trials, and obtaining patients' matched tumor and blood samples for immune monitoring. This work laid the foundation for using presurgical trials as an important tool to evaluate human immune responses in the tumor microenvironment, which should be included in the current paradigm of phase I, II, and III clinical trials.

> ***"Because of the very nature of immune checkpoint therapy, the development of pharmacodynamic, predictive, or prognostic biomarkers faces unique challenges."***

The collection of fresh tumor samples at the time of surgery can provide sufficient tissue for genetic, phenotypic, and functional studies, as well as material for immunohistochemical (IHC) analyses, which can provide extensive insight into the biologic impact of the immunotherapy agent on the tumor microenvironment. For example, high-quality mRNA can be obtained for gene expression studies comparing posttreatment tumor tissues to pretreatment tumor tissues or untreated samples obtained from a stage-matched control group of patients. These types of studies allow unbiased analyses of the samples to identify novel genes and pathways that are affected by therapy. In our ipilimumab trial, gene array data revealed that most of the differences between treated and untreated samples could be attributed to pathways involved in T cell signaling, which is not surprising given the large increases in T cell infiltrates in tumor tissues after CTLA-4 blockade (*25, 26*). The most pronounced difference was an increase in T cells that express inducible costimulator (ICOS), a T cell surface molecule that is a closely related member of the extended CD28/CTLA-4 family. We confirmed our gene expression studies by flow cytometry. $ICOS^+$ T cells were increased in tumor tissues from patients treated with ipilimumab (*36*). The increase in the frequency of $ICOS^+$ T cells in tumor infiltrates was accompanied by similar increases in the blood. These data, coupled with other studies, showed that an increase in the frequency of $ICOS^+$ CD4 T cells served as a pharmacodynamic biomarker of anti-CTLA-4 treatment (*37*).

To test our hypothesis that $ICOS^+$ CD4 T cells might play a role in the therapeutic effect of CTLA-4 blockade, we conducted studies in mice. In wild-type C57BL/6 mice, anti-CTLA-4 treatment resulted in tumor rejection in 80 to 90% of mice, but in gene-targeted mice that were deficient for either ICOS or its ligand, the efficacy was less than 50% (*38*). The loss of efficacy of CTLA-4 blockade in the absence of an intact ICOS pathway indicates the critical importance of ICOS to the therapeutic effects of treatment with anti-CTLA-4 antibodies. The important role played by ICOS in the effectiveness of CLTA-4 blockade suggested that providing an agonistic stimulus for the ICOS pathway during anti-CTLA-4 therapy might increase its effectiveness. To test this notion, we conducted studies in mice to provide an agonistic signal through ICOS in combination with CTLA-4 blockade. We found that combination therapy resulted in an increase in efficacy that was about four to five times as large as that of control treatments (*39*). Thus, ICOS is a stimulatory checkpoint that provides a novel target for combination immunotherapy strategies. Antibodies for ICOS are being developed for clinical testing, which are expected to start within the next year.

Whereas some presurgical and tissue-based trials are focused on evaluating human immune responses in the tumor microenvironment, other studies have focused on evaluating components of the cancer cells that may contribute to clinical benefit with anti-CTLA-4. Genetic analyses of melanoma tumors revealed that higher numbers of mutations, termed "mutational load," and creation of new antigens that can be recognized by T cells as a result of these mutations, termed "neoantigens," correlated with clinical responses to anti-CTLA-4 therapy (*3, 40*). These studies provide a strong rationale to integrate genetic analyses of the tumor with immune profiling of the tumor microenvironment for a more comprehensive evaluation of mechanisms that contribute to clinical responses with anti-CTLA-4 therapy.

Tissue-based immune monitoring: Anti-PD-1/PD-L1 therapy

Given that immune checkpoint therapy only benefits a fraction of patients, there are ongoing efforts to identify predictive biomarkers that could be used to select patients for treatment.

Because the PD-1 ligand PD-L1 (and sometimes PD-L2) can be expressed on tumor cells and immune cells in the tumor microenvironment, there have been efforts to use expression of PD-L1 as a criterion for selecting patients for treatments with antibodies targeting the PD-1/PD-L1 pathway.

The initial phase I trial with anti-PD-1 therapy (nivolumab) reported that PD-L1 expression on tumor cells, measured on pretreatment archival samples by immunohistochemical (IHC) methods, may potentially serve as a predictive marker to indicate which patients would benefit from treatment (*30*). Patients with PD-L1–positive tumors (≥5% staining for PD-L1 on tumor cells) had an objective response rate of 36% (9 of 25 patients) whereas patients with PD-L1–negative tumors did not show any objective clinical responses (0 of 17 patients). However, in subsequent trials, some patients whose tumors were deemed to be PD-L1–negative had clinical responses to anti-PD-1 and anti-PD-L1 treatments with either tumor regression or stabilization of disease. For example, on a phase I trial with anti-PD-1 (nivolumab), patients with PD-L1–positive tumors had an objective response rate of 44% (7 of 16) and patients with PD-L1–negative tumors had an objective response rate of 17% (3 of 18) (*41*). Although PD-L1 expression in tumor tissues does correlate with higher response rates, it is not predictive for clinical benefit. Furthermore, current data indicate that the differences in response rates do not translate to differences in survival benefit. For patients with metastatic melanoma who received treatment with nivolumab on a phase III trial, the median overall survival had not been reached for either PD-L1 subgroup, and both subgroups had improved overall survival as compared to patients who received dacarbazine chemotherapy (*33*).

In a phase I study of anti-PD-L1 (MPDL3280A), patients with bladder cancer were considered to have PD-L1–positive tumors if their pretreatment archival tumor samples contained ≥5% PD-L1–positive tumor-infiltrating immune cells (*29*). Twenty-one patients with PD-L1–positive tumors were enrolled onto the trial prior to enrollment of patients with PD-L1–negative tumor samples. Data were reported after a minimum of 6 weeks of follow-up. An objective response rate of 43.3% (13 out of 30 patients) and stable disease rate of 26.7% (8 of 30) was reported for patients with PD-L1-positive tumors, which was compared to an objective response rate of 11.4% (4 of 35 patients) and stable disease rate of 37.1% (13 of 35) for patients with PD-L1–negative tumors. Because the patients with PD-L1–positive tumors received treatment for a longer period of time as compared to patients with PD-L1–negative tumors, it is unclear if the difference in response rates in this study was due to PD-L1 expression or time on treatment. However, for patients with metastatic bladder cancer whose disease had progressed after first-line chemotherapy and in a setting where there are no approved second-line treatments, an objective response rate of 11% and stable disease rate of 37.1% are clinically relevant.

Similarly, in another phase I study of anti-PD-L1 (MPDL3280A) in multiple tumor types, objective response rates were reported as 46% in the cohort of patients whose tumors had the highest PD-L1-expression, 17% in the cohort of patients whose tumors had moderate expression of PD-L1, 21% in the cohort of patients whose tumors had minimal PD-L1 expression, and 13% in the cohort of patients whose tumors had no detectable level of PD-L1 expression (*42*). Thus, this trial also showed that patients whose tumors were deemed as PD-L1–negative can have objective responses. Interestingly, the cohort of patients whose tumors were categorized as moderate expression of PD-L1, which correlates with PD-L1-positive status, had objective responses (17%) and median progression-free survival (18 weeks) that were similar to the objective responses (21%) and median progression-free survival (17 weeks) of the cohort of patients whose tumors had minimal expression of PD-L1, which correlates with PD-L1–negative status. Additional studies will be needed to determine whether PD-L1 expression in the tumor microenvironment affects survival outcomes for patients treated with anti-PD-L1.

On the basis of data reported thus far, it seems fair to conclude that expression of PD-L1 in tumor tissues should not be used as a predictive biomarker for selection or exclusion of patients for treatment with either anti-PD-1 or anti-PD-L1 antibodies. In a study of primary and

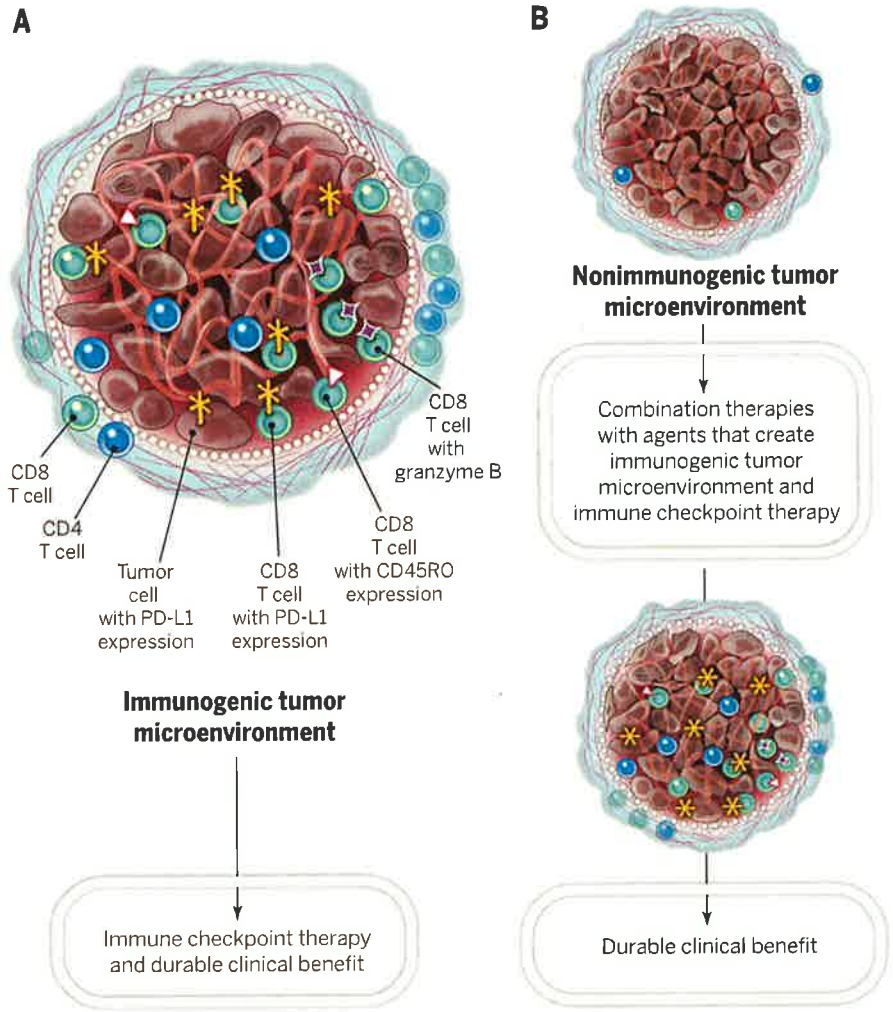

Fig. 3. Potential characteristics of immunogenic and nonimmunogenic tumors. (**A**) Tumor tissue depiction indicating tumor cells and an invasive margin (dotted line), which may delineate separation of tumor cells from stromal components. Evaluation of tumor tissues may reveal an immunogenic tumor microenvironment consisting of many immunologic markers, including CD8 T cells, CD4 T cells, PD-L1, granzyme B, and CD45RO, which may be effectively treated with immune checkpoint therapy to elicit clinical benefit. (**B**) Tumor tissues that lack expression of many immunologic markers may indicate a nonimmunogenic tumor microenvironment, which may require combination therapies consisting of an agent to create an immunogenic tumor microenvironment plus an immune checkpoint agent to further enhance the immune response for clinical benefit.

metastatic melanoma samples, many taken from the same patient, it was shown that PD-L1 expression was discordant between primary tumors and metastases and between intrapatient metastases. In addition, patients whose tumor tissues were positive for both PD-L1 expression and infiltration of T cells were found to have improved overall disease-specific survival as compared to patients who had only one of the two features or lacked both features (*43*). Similarly, in a study with anti-PD-1 (pembrolizumab), it was reported that while expression of PD-L1 in pretreatment tumor tissues correlated with clinical outcomes, the preexisting density of CD8 T cells in the invasive margin of the tumor was more predictive of clinical response to anti-PD-1 (*44*). These data suggest that PD-L1 expression in the tumor is most compelling when it is observed in the context of an active T cell response, and that the ongoing T cell response itself, not PD-L1 expression, is the key factor.

Taken together, these data indicate the complexity of determining the PD-L1 status of a patient's tumor by examination of a single pretreatment tumor sample (Fig. 2). It also raises questions as to whether clinical decisions regarding treatment of patients who have failed conventional therapies and for whom no other treatments are available should be based on static assessment of PD-L1 expression in pretreatment tumor samples.

However, in some settings, expression of PD-L1 in tumors is constitutive and is neither associated with T cell infiltration nor induced by IFN-γ. In these settings, assessment of PD-L1 expression in tumor tissues may be very useful in guiding treatment. In Hodgkin's lymphoma, Reed-Sternberg cells are known to harbor amplification of chromosome 9p24.1, which encodes PD-L1 and PD-L2 and leads to their constitutive expression. Anti-PD-1 (nivolumab) was shown to elicit an objective response rate of 87% in a cohort of 20 patients with Hodgkin's lymphoma (*45*). Therefore, in the setting of Hodgkin's lymphoma, and possibly other malignancies that harbor amplification of chromosome 9p24 or up-regulate PD-L1 or PD-L2 in response to an oncogenic signal, the expression of these ligands may indeed serve as a predictive biomarker.

In addition to evaluation of PD-L1 expression, tumor tissues can also be studied to identify patterns of expression of multiple immunologic components, including other checkpoints and their ligands. T cells that coexpress PD-1 together with other inhibitory molecules such as LAG-3 or Tim-3 may be even more profoundly hyporesponsive than those expressing PD-1 alone and indicate the need for the blockade of multiple checkpoints (*46*, *47*). Given the complexity of regulation of T cell responses by multiple signaling pathways, both negative and positive, it will be necessary to determine the patterns of expression of the receptors, as well as the ligands on T cells, tumor cells, myeloid cells, and other components of the tumor microenvironment, for development of combination strategies with greater clinical benefit.

Additional biomarkers that play a role in antitumor responses elicited by anti-PD-1 therapy and anti-PD-L1 therapies may also be identified through genetic analyses of tumor cells. Similar to previous reports with anti-CTLA-4 therapy, higher numbers of mutations, including mutations in DNA repair pathways, with subsequent increase in numbers of neoantigens, was found to correlate with clinical responses in patients with non-small cell lung cancer who received treatment with anti-PD-1 (pembrolizumab) (*48*). These data highlight the complex interplay between cancer cells and the immune system, which will need further elucidation, to guide rational development of combination therapies.

Combination therapy to increase clinical benefit

Given the dynamic nature of immune responses to tumors and the complexity of regulation of expression of multiple immune checkpoints and their ligands, it may be difficult to rely on any single immunologic biomarker to select patients for treatment. It may be necessary to evaluate multiple components within the tumor microenvironment, which may enable us to distinguish between an immunogenic (hot) tumor microenvironment (Fig. 3A) that is comprised of infiltrating T cells, cytokines such as granzyme B, memory T cell markers such as CD45RO and PD-L1 expression versus a non-immunogenic (cold) tumor microenvironment that lacks these components (Fig. 3B). Patients whose tumors are immunogenic would be treated with immune checkpoint therapy to elicit durable clinical benefit but, patients whose tumors are non-immunogenic would receive combination therapies designed to create an immunogenic tumor microenvironment that would respond to treatment with subsequent durable clinical benefit (Fig. 3).

Substantial data already exist to indicate that certain combination therapies may overcome the limitations of anti-CTLA-4 and anti-PD-1/PD-L1 monotherapies. For example, anti-CTLA-4 seems to drive T cells into tumors, resulting in an increase in the number of T cells and a concomitant increase in IFN-γ. This, in turn, can induce expression of PD-L1 in the tumor microenvironment, with subsequent inhibition of antitumor T cell responses, but also increase the chance of benefit from anti-PD-1 and anti-PD-L1 therapies. Therefore, combination treatment with anti-CTLA-4 plus anti-PD-1 or anti-PD-L1 should enable the creation of an immunogenic tumor microenvironment with subsequent clinical benefit for patients regardless of whether their pretreatment tumor tissues have infiltrating T cells or express PD-L1. Data from a recent phase I clinical trial with anti-CTLA-4 (ipilimumab) plus anti-PD-1 (nivolumab) demonstrated that patients with metastatic melanoma had similar response rates in the setting of concurrent therapy regardless of PD-L1 expression in pretreatment tumor tissues (*35*). For patients with PD-L1–positive tumors, the objective response rate was 46% (6 of 13 patients), which was similar to the objective response rate of 41% (9 of 22 patients) for those patients with PD-L1–negative tumors. Similar data were reported for a combination study with anti-PD-1 (nivolumab) plus anti-CTLA-4 (ipilimumab) in patients with metastatic renal cell carcinoma (mRCC) (*49*).

Conventional cancer therapies (Table 1) may also lead to tumor cell death and release of antigens to initiate activation of T cells, which may then migrate into tumor tissues. Therefore, combination studies with these conventional agents and immune checkpoint therapies should create an "immunogenic" tumor microenvironment with subsequent clinical benefit for patients. There are multiple ongoing trials with radiation therapy in combination with anti-CTLA-4 or anti-PD-1/PD-L1 antibodies, which will provide valuable information regarding schedule, safety, and efficacy of these combinations for future studies (*50*, *51*). In addition, combination treatment with anti-PD-1 (nivolumab) plus pazopanib or sunitib in patients with mRCC resulted in promising clinical responses, with response rates that were similar across all patients regardless of PD-L1 expression in pretreatment tumor tissues (*52*).

Other combination strategies, such as vaccines plus anti-CTLA-4 (ipilimumab), are also being developed and have shown promising results in patients with pancreatic cancer, which has

Table 1. Potential agents for combination therapy. List of some conventional cancer therapies, inhibitory immune signals and stimulatory immune signals that can be considered for combination strategies to improve antitumor responses and durable clinical benefit.

CONVENTIONAL THERAPIES	INHIBITORY IMMUNE SIGNALS	STIMULATORY IMMUNE SIGNALS
Chemotherapy	CTLA-4	ICOS
Radiation	PD-1/PD-L1	OX40
Surgery	LAG-3	41BB
Genomically targeted	TIM-3	Vaccines
Anti-angiogenic	VISTA	Cytokines
Hormonal	BTLA	Oncolytic virus

been consistently viewed as a nonimmunogenic tumor type (*53*). Combination treatments are also being developed to enable blockade of multiple inhibitory pathways, such as LAG-3 (*54*, *55*), TIM-3 (*56*, *57*), VISTA (*58*, *59*), and BTLA (*60*, *61*), or blockade of an inhibitory pathway while providing an agonistic signal through a stimulatory pathway, such as ICOS (*39*), OX40 (*62*), 41BB (*63*), vaccines (*24*, *53*), cytokines (*64*), and oncolytic virus (*65*). The development of these combinations and others are critical for driving antitumor immune responses in many cancer patients, even those who are deemed to have nonimmunogenic or PD-L1–negative tumors.

Discussion

Because of the very nature of immune checkpoint therapy, the development of pharmacodynamic, predictive, or prognostic biomarkers faces unique challenges. Agents that block immune checkpoints unleash dynamic and complex immune responses. Anti-CTLA-4 antibody overcomes a block in essential costimulatory signals that are required for activation of both naïve T cells and resting clones, whereas PD-1/PD-L1 blockade seems to remove a barrier to the function of T cells later in the response and in the tumor tissue. Therefore, there is a fundamental difference in the predictive value of preexisting tumor inflammation for PD-1/PD-L1 and CTLA-4 blockade. The existence of a T cell infiltrate and select biomarkers, such as expression of PD-L1, which indicate a "hot" tumor microenvironment, does correlate with clinical benefit for patients treated with anti-PD-1 or anti-PD-L1. However, in the setting of a "cold" tumor microenvironment, it seems that anti-CTLA-4 therapy can drive T cells into the tumor and induce expression of PD-L1, thus creating a tumor microenvironment that may be responsive to anti-PD-1 or anti-PD-L1 therapy, which provides a strong rationale for combination therapy.

There are many ongoing efforts to identify predictive biomarkers of immune checkpoint therapy. It may be that germline differences in immune genes and pathways or host microbiome may affect host immune responses and clinical outcomes in the setting of immune checkpoint therapy. Also, the nature of the tumor itself can also affect the outcome of immune checkpoint therapy. Tumor types differ considerably in their mutational load, which may affect the number of neoantigens that can serve as targets of antitumor T cell responses (*66*). Patients with tumors at the high end of the mutational spectrum may be more likely to respond to immune checkpoint therapy. For example, anti-PD-1 therapy was thought to be ineffective against colon cancer, but it appears that colon cancer with microsatellite instability, and consequently a higher overall mutational load, may be responsive to treatment with anti-PD-1 (*67*). However, this concept may not hold true for all tumor types, because patients with kidney cancer, which has relatively low numbers of mutations, have had notable clinical responses to immune checkpoint therapy (*28*, *30*).

There are multiple immunologic pathways, both positive and negative, with new checkpoints and ligands that emerge as an immune response develops. Because of the constant evolution of an immune response, it is unlikely that a single immunologic biomarker can be identified at baseline that can predict responses to any agent. It will probably be necessary to develop panels of markers based on patterns of expression of relevant markers, and use these to guide development of combination therapies that will increase the response rate. These combinations will not be limited to agents that target immune checkpoints, because it is apparent that small molecules that target signaling pathways involved in cancer can affect antitumor immune responses (*68*). This can occur at the level of the T cells by enhancing activation signals, but also at the level of the tumor by inducing tumor antigen expression and presentation, thus making the tumors more susceptible to T cell killing. The goal then should be to use panels of markers to guide development of combination therapies, and then examine tumor tissues for changes in markers elicited by the combinations to guide decisions about additional treatment to further increase efficacy, and, hopefully, durable clinical responses.

Immune checkpoint therapies and combination strategies with immunotherapy have provided cancer patients with novel treatments that have the potential to elicit durable control of disease and even cures. The specificity, adaptability, and memory response that are inherent to the immune system give us the opportunity to measure multiple components, not just a single biomarker, that can be targeted over time to provide curative treatments for many patients. The ability of an activated immune response to generate a diverse T cell repertoire that adapts to heterogeneous and genetically unstable tumors and the persistence of memory T cells with specificity for tumor antigens, which provide efficient recall responses against recurrent disease, make it absolutely essential to expand our efforts to find rational combinations to unleash antitumor immune responses for the benefit of cancer patients. Properly done, it seems likely that cures for many types of cancer will soon become reality.

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REVIEWS

T cell exclusion, immune privilege, and the tumor microenvironment

Johanna A. Joyce[1]* and Douglas T. Fearon[2,3]*

Effective immunotherapy promotes the killing of cancer cells by cytotoxic T cells. This requires not only that cancer-specific T cells be generated, but also that these T cells physically contact cancer cells. The coexistence in some patients of cancer cells and T cells that recognize them indicates that tumors may exhibit the phenomenon of immune privilege, in which immunogenic tissue is protected from immune attack. Here, we review the evidence that stromal cells of the tumor microenvironment mediate this restriction by excluding T cells from the vicinity of cancer cells. Overcoming this T cell checkpoint may thus enable optimal immunotherapy.

The microenvironment of tumors contains numerous cell types in addition to cancer cells, which include bone marrow–derived inflammatory cells, lymphocytes, blood vessels, fibroblastic cells, and the extracellular matrix composed of collagen and proteoglycans (*1*, *2*). The importance of a stromal microenvironment, especially one that has characteristics of a "wound" or regenerating tissue, has been recognized for at least a century (*3*), but its possible role in blunting an immune attack of cancer cells awaited the discovery of adaptive cellular immunity. In 1960, Klein and colleagues found that when mice developed primary methylcholanthrene-induced sarcomas, they also developed an antitumor immune response mediated by lymph node cells to a secondary challenge comprising cancer cells derived from the primary tumor (*4*). The paradoxical and critical finding of the study was that this anticancer immune response did not control the growth of the primary tumor, despite its ability to prevent the establishment of a secondary tumor comprising cancer cells derived from the primary tumor. In traditional immunological terminology, the primary tumor evaded immune control by establishing an immune-privileged microenvironment that is functionally analogous to that of certain normal tissues, such as the eye (*5*).

Unambiguous evidence for the inability in humans of a systemic immune response to eliminate immunogenic cancer cells was provided by Boon's studies 30 years later of the antigens that elicit specific $CD8^+$ T cell responses in melanoma patients (*6*). Cloned $CD8^+$ T cells from a melanoma patient were used to identify the antigen expressed by that patient's cancer: MAGE-A1. The explicit demonstration of the coexistence of a progressing melanoma with melanoma-specific T cells in this patient implicitly raised the question of why the T cells did not control the growth of the cancer. Immunoediting, or the elimination of immunogenic cancer cells (*7*), could be excluded, which left the possibility of immune suppression by the tumor microenvironment (TME). Despite this evidence that the presence of antigen-specific $CD8^+$ T cells alone may not be sufficient for the control of cancer, a major pharmaceutical company recently conducted phase III trials in patients with non-small cell lung cancer (NSCLC) of the clinical efficacy of vaccination with the MAGE-A3 antigen (MAGRIT, NCT00480025). The study did not meet its primary end point of extending disease-free survival and was discontinued in 2014. Moreover, Rosenberg and colleagues reported evidence of disease recurrence in melanoma patients despite very high levels of vaccine-induced circulating T cells and no evidence of antigen loss by the cancer cells (*8*).

The discovery of melanoma-specific T cells in patients led to another strategy to increase the frequency of cancer-specific T cells in patients, that of adoptively transferring large numbers of in vitro expanded tumor-infiltrating lymphocytes (TILs). As discussed elsewhere in this issue of *Science* (*9*), this approach has shown some efficacy, which has been of major importance to the field by serving as proof that the immune system has the potential to control cancer (*10*). However, adoptive T cell therapy (ACT) with TILs has not had the dramatic success of ACT with virus-specific $CD8^+$ T cells to immunodeficient bone marrow transplant recipients with cytomegalovirus infection (*11*) or Epstein-Barr virus–associated lymphoproliferative disorders (*12*). Differences in the microenvironments of virally infected tissues and cancers may account for these distinct outcomes, with the latter being immune-suppressive. Another important point of comparison is that the TME of solid cancers is likely to be fundamentally different to that of the leukemias, in which clinical trials of ACT with T cells expressing chimeric antigen receptors, so-called CAR T cells, have demonstrable efficacy (*9*). These findings raise the possibility that increasing the frequency of cancer-specific T cells, by whatever means, may be more effective if combined with an approach that alters the immune-suppressive TME.

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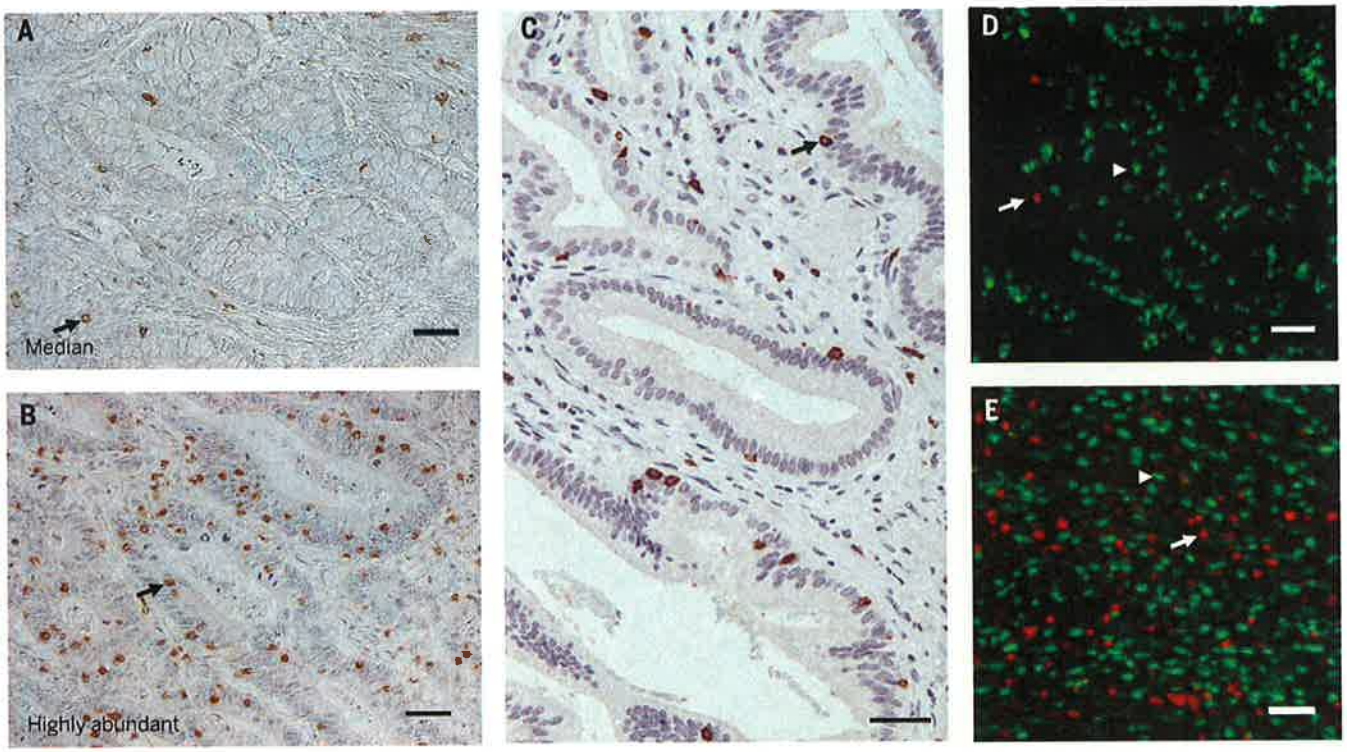

Fig. 1. Exclusion of T cells from human and mouse adenocarcinomas. (**A** to **C**) $CD3^+$ T cells are identified by immunoperoxidase stains of [(A) and (B)] human colorectal (*82*) and (C) human pancreatic ductal adenocarcinomas , demonstrating the presence of few [(A) and (C)] and many (B) intraductal T cells. (**D** and **E**) $CD3^+$ T cells and $p53^+$ cancer cells are identified by use of immunofluorescent stains of pancreatic ductal adenocarcinomas taken from (D) untreated mice and (E) mice that have been treated for 24 hours with the CXCR4 antagonist, AMD3100, demonstrating that T cell exclusion can be regulated by CXCR4 signaling (*29*). Scale bars, 50 μm. Arrows indicate examples of $CD3^+$ T cells, and arrowheads indicate examples of $p53^+$ cancer cells.

The more recent strategy of enhancing the function of effector T cells by targeting immunoregulatory membrane receptors has been successful in subsets of patients with melanoma, NSCLC, urothelial bladder cancer, and renal cell cancer (*13*–*18*). The therapeutic effect of blocking antibodies to the immune checkpoint regulators cytotoxic T-lymphocyte–associated protein 4 (CTLA-4) and the programmed cell death protein 1 (PD-1)/PD-L1 receptor-ligand pair is covered in detail elsewhere in this issue of *Science* (*19*), and we briefly discuss them here because these therapies relate to the TME. For example, in the mice, anti-CTLA-4 therapy leads to clearance from the tumor of $Foxp3^+$ regulatory T cells (T_{reg} cells) (*20*), which may impair the functions of effector T cells at that site (*21*). Cancer cells—as well as infiltrating monocytic cells, including dendritic cells (DCs) and macrophages—express PD-L1 (*16*, *17*, *22*, *23*), which suppresses the proliferative and effector responses of T cells by engaging the inhibitory PD-1 receptor on these cells. Nevertheless, it has become apparent that even if these T cell checkpoint antagonists overcome some of the immune-suppressive effects of the TME, there may be other, more fundamental inhibitory reactions in the TME to explain why most patients—especially those with microsatellite stable colorectal cancer (CRC), ovarian cancer, prostate cancer, and pancreatic ductal adenocarcinoma (PDA)—rarely exhibit objective responses to these therapies (*14*, *15*, *24*).

A clue to the nature of this dominant immune suppression mediated by the TME comes from studies that have examined the spatial relationship of $CD8^+$ effector T cells to cancer cells in three of the tumors that did not respond to anti-PD-1/anti-PD-L1: CRC, ovarian cancer, and PDA (Fig. 1). In 1998, the exclusion of $CD8^+$ T cells from the vicinity of cancer cells in CRC was shown to correlate with a poor long-term clinical outcome (*25*), an observation that was confirmed and extended by Galon and colleagues in 2006 (*26*). Exclusion of T cells from the vicinity of cancer cells was also found in ovarian cancer (*27*, *28*) and PDA (*29*). Thus, the tumor immunology field provided evidence more than 10 years ago that the TME can limit the capacity of T cells to accumulate among cancer cells. It is reasonable to conclude that until this problem is circumvented, the full potential of other approaches to T cell–mediated tumor immunotherapy, such as augmenting the numbers and function of cancer-specific T cells, may not be realized.

Fortunately, studies over the past several years have begun to explain how this form of immune suppression is mediated. Preclinical studies in mouse models of cancer now implicate the major stromal cell types of the TME, cancer-associated fibroblasts (CAFs) and myelomonocytic cells, including several subsets of cells within the general designation of myeloid-derived suppressor

Table 1. Myelomonocytic cells and CAFs control the accumulation of T cells.

TUMOR	TARGET	CELL TYPE AFFECTED BY THERAPEUTIC INTERVENTION	REFERENCE
B16 melanoma-GM-CSF	CCR2	Monocytes	(*30*)
PDA	GM-CSF	MDSCs	(*31*)
PDA	GM-CSF	MDSCs	(*32*)
Cervical, Breast	CSF-1R	Monocytes, TAMs	(*33*, *36*)
PDA	CXCR4	Likely T cells (CXCL12 is produced by CAFs)	(*29*)
PDA	CSF-1R	Monocytes, TAMs	(*34*)
Prostate	CSF-1R	Monocytes, TAMs	(*35*)

cells (MDSCs) and tumor-associated macrophages (TAMs), as being responsible for restricting the accumulation of T cells in the vicinity of cancer cells (*29*–*36*) (Table 1). As would be predicted, overcoming this restriction revealed the antitumor effects of a T cell checkpoint antagonist that had been ineffective when administered as monotherapy. Moreover, as will be discussed, the tumor vasculature also plays an active role in restricting T cell entry into the TME. Fortunately, for each immune suppressive element of the TME there are therapeutic entities that are potentially suitable for administration to patients.

Control by the TME of the extravasation of T cells from the circulatory system into tumors

After the priming of cancer-specific T cells in the lymph nodes that drain the tumor, these T cells traffic via the circulatory system to the tumor. Studies have shown that the TME may regulate the accumulation of T cells in tumors at the initial step of their interaction with local blood vessels. Given that many other immune cells that compose the TME are nonetheless able to extravasate from the circulation (*1*), there must be means by which these distinct cell types are differentially recruited into the tumor. One mechanism for cellular discrimination comes from the release of chemokines that preferentially recruit certain immune cell types over others. Another is the capacity of the TME to posttranslationally alter chemokines. For example, the production of reactive nitrogen species by MDSCs within the TME induces nitration of CCL2 (N-CCL2), which

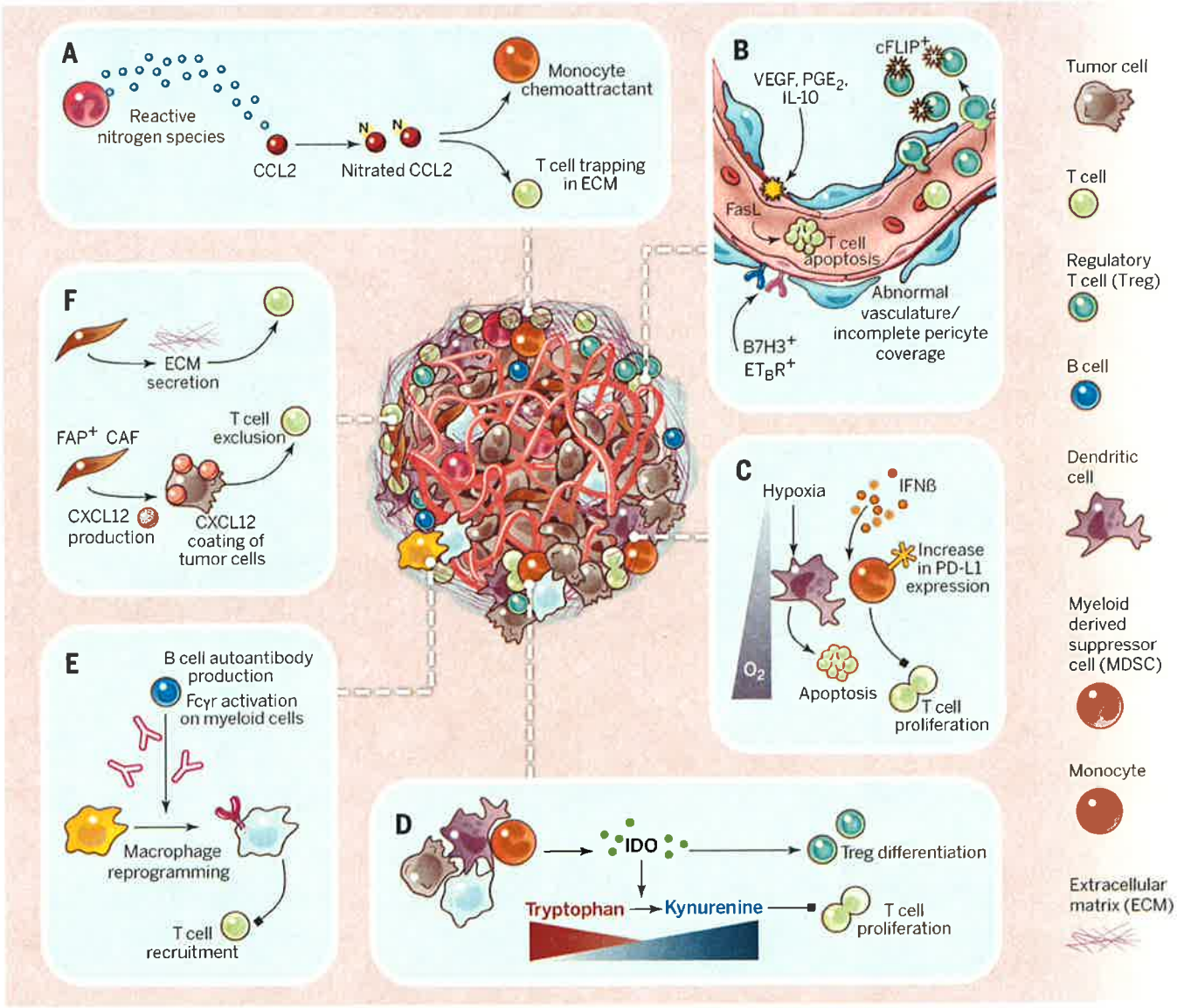

Fig. 2. Mechanisms of TME-driven immune suppression. A plethora of noncancerous cells in the TME regulate the infiltration, accumulation, and proliferation of T cells in tumors, with representative examples shown here. (**A**) T cell recruitment can be blocked by nitration of the chemokine CCL2, resulting in T cell trapping in the stroma. (**B**) The tumor vasculature plays a complex role in preferential recruitment of other immune cells over T cells, in part through endothelial cell (EC)–specific expression of FasL, ET_BR, and B7H3. (**C**) PD-L1 expression can be up-regulated in myelomonocytic cells, in addition to tumor cells, and is driven in part by hypoxic conditions in the TME and the production of cytokines, such as IFNβ. (**D**) The aberrant production of metabolites in the TME, such as the pathway regulated by IDO, can result in a multitude of effects directly on T cell functions and indirectly via other cells such as T_{reg} cells. (**E**) B cells can regulate the phenotype of TAMs resulting in suppression of CD8 cells. (**F**) Cancer-associated fibroblasts (CAFs) have multiple functions in the TME, in part through extracellular matrix (ECM)–mediated T cell trapping and CXCL12-regulated T cell exclusion.

results in the trapping of T cells in the stroma that surrounds tumor cells of human colon and prostate cancers (Fig. 2A) (*37*). In contrast, N-CCL2 still attracts monocytes, potentially contributing to the differential recruitment of these distinct immune cell types in vivo. Inhibitors of CCL2 nitration enhanced the accumulation of TILs in the corresponding animal models and resulted in improved efficacy of ACT.

Even if the appropriate chemotactic signals for the extravasation and recruitment to the tumor of T cells are present, the vasculature can override their effects and actively exclude T cells (Fig. 2B), a function that may distinguish between the effector T cells and other leukocyte populations, such as T_{reg} cells and myeloid cells. Insights into the mechanism of how this might occur have come from studies comparing T cell–rich and T cell–poor tumors. These studies revealed that the apoptosis inducer Fas ligand (FasL) is expressed in the tumor vasculature of multiple tumor types, including ovarian, colon, prostate, breast, bladder, and renal cancer (*38*). In tumors with high levels of endothelial FasL, there are few $CD8^+$ T cells but abundant T_{reg} cells, which may be protected against FasL-mediated killing by their relatively high expression of the apoptosis inhibitor, c-FLIP. Accordingly, in preclinical models FasL inhibition resulted in a substantial increase in the influx of tumor-rejecting T cells relative to T_{reg} cells, which led to T cell–dependent tumor suppression. FasL expression itself is induced by the TME-derived immunosuppressive factors vascular endothelial growth factor (VEGF), prostaglandin E_2 (PGE_2), and interleukin-10 (IL-10), suggesting that multiple

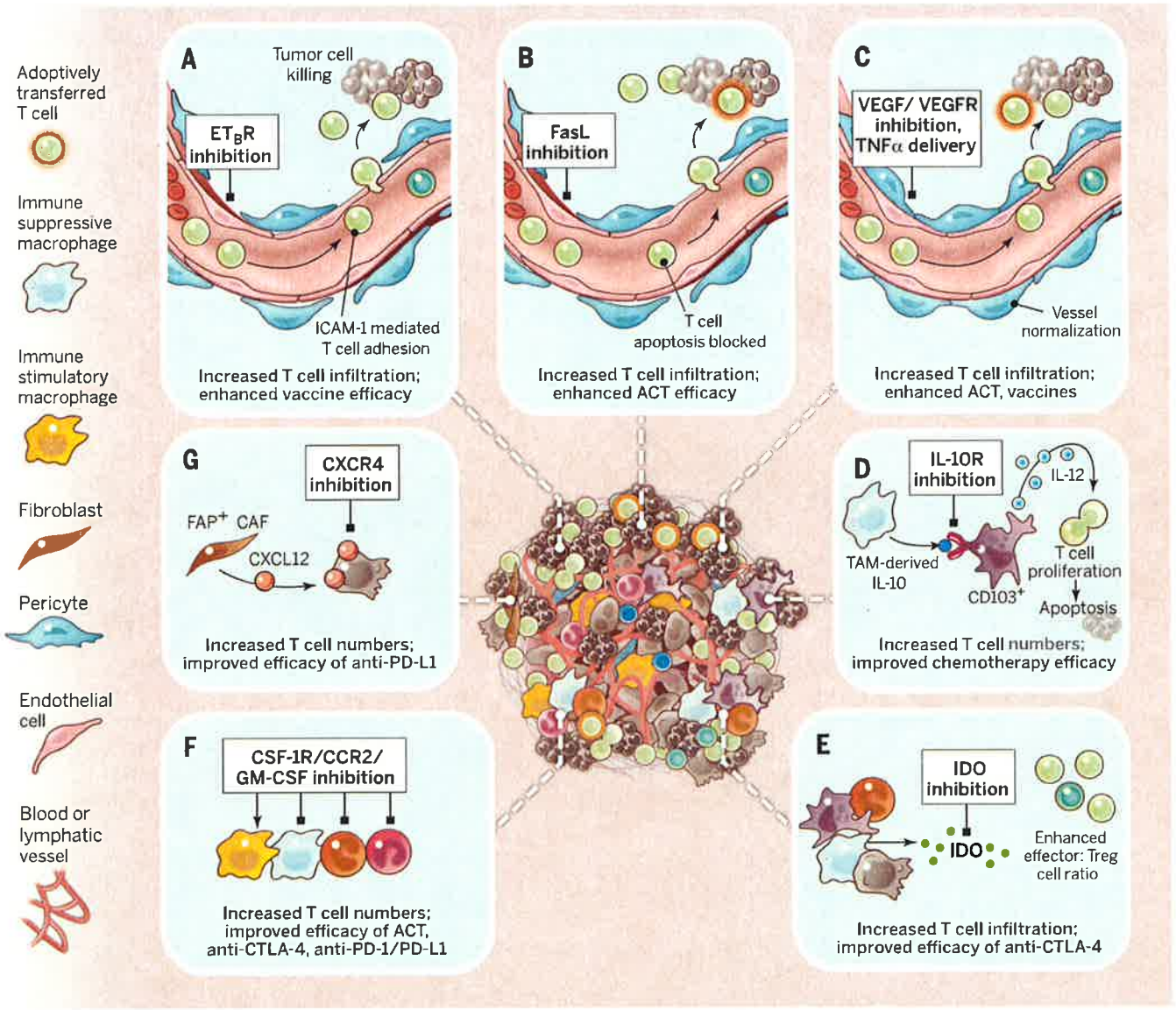

Fig. 3. Therapeutic strategies to overcome immune suppression in the TME. A number of vascular-targeted therapies result in increased T cell infiltration and improved efficacy of different immunotherapies such as adoptive cell therapy and anticancer vaccines. These include (**A**) ET_BR inhibition, (**B**) FasL inhibition, and (**C**) VEGF/VEGFR/TNFα inhibition. (**D**) Dendritic cells (DCs) can have opposing functions in the TME, either supporting or suppressing tumor development. $CD103^+$ DCs have an immune stimulatory function, resulting in IL-12 secretion and T cell replication when the immune-suppressive cytokine receptor IL-10R is inhibited. (**E**) IDO inhibition has multiple effects on TILs, including augmenting T cell expansion and preventing their differentiation into T_{reg} cells. (**F**) Various myelomonocytic cells suppress T cell numbers and/or functions; this suppression can be relieved by inhibition of a number of cytokine signaling pathways indicated here, resulting in depletion or reeducation of these cells in the TME. Further information is provided in Table 1. (**G**) Inhibition of CXCL12/ CXCR4 downstream of FAP^+ CAFs in the TME leads to T cell accumulation and increased efficacy of anti-PD-L1 therapy.

networks of cellular interactions may converge to establish immune tolerance. In ovarian cancer elevated VEGF levels, and expression of the immune regulatory ligand B7H3 (CD276), or the endothelin B receptor (ET_BR) on tumor vessels correlates with decreased T cell infiltration and worse clinical outcome (*27, 39, 40*). Pharmacological inhibition of ET_BR increased T cell adhesion to endothelial cells in an intercellular adhesion molecule-1 (ICAM-1)–dependent manner, resulting in significantly enhanced TIL numbers in mice and a corresponding tumor response to an otherwise ineffective anticancer vaccine (Fig. 3A) (*40*). Similarly, FasL inhibition also improves the efficacy of ACT (Fig. 3B) (*38*). The improved efficacy of these distinct TME-directed immunotherapies was not as a consequence of a more effective systemic antitumor immune response but could be attributed to increased effector T cell infiltration into tumors.

Attention has also been focused on anti-angiogenic therapies as a potential means to enhance the efficacy of immunotherapy (*41*). Anti-angiogenic inhibitors targeting VEGF and its receptor VEGFR2, which are approved for clinical use in multiple cancers (*42*), induce vascular normalization. This, in turn, increases TILs and improves the efficacy of ACT and cancer vaccines in preclinical models (Fig. 3C) (*43, 44*). In relation to the next section of this Review, VEGF impairs the maturation of DCs (*45*), so that anti-VEGF therapy has an additional means by which it could enhance intratumoral immune responses. Further support for the importance of vascular normalization has come from the finding that deleting the regulator of G-protein signaling, Rgs5 (*46*), reduced vessel leakiness and hypoxia, enhanced T cell infiltration into mouse pancreatic neuroendocrine tumors, and prolonged animal survival. Therefore, from an immunotherapeutic perspective, vascular normalization is likely to be more efficacious than anti-angiogenic therapies that result in vessel destruction, as exemplified by the differential effects of delivering the pro-inflammatory cytokines interferon-γ (IFN-γ) versus tumor necrosis factor-α (TNF-α). Only targeted delivery of the latter, which was reported to normalize tumor blood vessels and increase $CD8^+$ T cell infiltration, enhanced vaccine and ACT therapies (Fig. 3C) (*47, 48*).

TME-mediated regulation of the local replication of T cells within tumors

The extravasation of cancer-specific T cells into the tumor is a necessary, but not sufficient, step in the immune control of cancer. For effective immune killing of cancer cells, these T cells must also locally replicate to further increase their frequency, avoid being killed themselves by hostile elements of the TME, and overcome barriers that restrict their distribution to the stroma and away from cancer cells. The TME affects all three of these intratumoral T cell responses.

Although the site of the self-renewing T cells that are clonally expanding in response to cancer cell–associated antigens is likely to be the draining lymph nodes, the enrichment of cancer-specific effector T cells within the tumor relative to their frequency in the periphery indicates that replication of effector T cells within the tumor also occurs. Findings in preclinical models suggest that the TME may be the major site of clonal expansion of cancer-specific T cells (*49, 50*), and that the $CD8^+$ T cell replicative response at this site is orchestrated by the $CD103^+$, Baft3-dependent DC, which can efficiently cross-present cancer cell antigens (*51, 52*). The dependence of T cell–mediated tumor regression on the intratumoral presence of $CD103^+$ DCs suggests that therapeutic interventions that enhance their numbers or capacity for driving T cell replication in the TME may contribute to tumor control. Among such strategies are antibodies to the IL-10R, which in a mouse model of mammary carcinoma neutralized the effects of IL-10 produced by TAMs, relieved the suppression of IL-12 production by intratumoral DCs, and improved the $CD8^+$ T cell–dependent antitumor effects of chemotherapy (Fig. 3D) (*53*). A similar outcome was achieved by neutralizing CSF-1, which impaired the intratumoral accumulation of TAMs (*32, 33*). Yet another strategy is the administration of antibody-IFN-β complexes, targeted against oncogenic receptors, such as EGFR, that activate intratumoral DCs for cross-presentation of antigen to $CD8^+$ T cells (*54*). Tumor eradication resulted when PD-L1, which also was induced by IFN-β acting on DCs, was neutralized, demonstrating the recurring theme in the immune system that activating stimuli prompt compensatory inhibitory responses. DC function also may be adversely affected by the hypoxic conditions characteristic of the TME, which induces PD-L1 expression on DCs and other myelomonocytic cells (Fig. 2C) as a result of HIF-1α binding directly to a hypoxia-responsive element in the PD-L1 promoter (*55*). Even the aerobic glycolysis of cancer cells may antagonize local immune reactions via its increased production of lactate, which induces the M2 polarization of TAMs (*56*). An M1 to M2 phenotypic transition of intratumoral macrophages has also been reported after the induction of cancer cell apoptosis in human and mouse gastrointestinal stromal tumors by the administration of the KIT oncoprotein inhibitor, imatinib (*57*). It should be noted that the designation of M1 and M2 polarization states undoubtedly represent an oversimplification of the complexity of macrophage biology (*58*) and that at least six different TAM subpopulations have been reported (*59*). Therefore, descriptors of TAM phenotypes in the TME are likely to be most informative in investigating and therapeutically targeting these cells.

In addition to altering T cell replication indirectly via effects on myeloid cells, the TME may directly impair intratumoral T cell proliferation. Indole 2,3-dioxygenase (IDO)—which can be expressed by DCs, MDSCs, and cancer cells—catabolizes tryptophan and generates kynurenine (Fig. 2D). Both the deprivation of tryptophan and the generation of its metabolic product inhibit clonal expansion (*60, 61*). IDO also promotes the conversion of naïve T cells to T_{reg} cells and increases IL-6 expression, which augments MDSC functions (*62*). Accordingly, IDO1 genetic deficiency is associated with reduced tumor burden and metastasis and enhanced survival in mouse models of lung and breast cancer (*62*). The therapeutic potential of inhibiting IDO, in combination with the T cell checkpoint antagonist anti-CTLA-4, has been demonstrated in the B16 melanoma model and was associated with increased accumulation of intratumoral T cells (Fig. 3E) (*63*). Last, the capacity of IDO to block the reprogramming of T_{reg} cells to helperlike cells by suppressing the loss of the transcription factor Eos, and the corresponding transcriptional program it regulates, exemplifies another means by which this enzyme promotes immune suppression within the TME (*64*).

Control by the TME of the viability of T cells within tumors

The TME can also limit the viability of T cells. Both IDO and PD-L1 not only may impair the intratumoral proliferation of effector T cells but may also induce apoptosis of these cells. Products of myelomonocytic cells that cause the apoptosis of T cells include FasL, TNF-α, and TNF-related apoptosis inducing ligand (TRAIL). In addition to these known effectors of death, previously unidentified pathways that control the viability of intratumoral T cells may be discovered by innovative, unbiased approaches. For example, an in vivo, pooled short hairpin RNA screen identified Ppp2r2d as a key regulator promoting T cell apoptosis and suppressing T cell proliferation within the TME (*65*).

Interventions that target intratumoral TAMs and MDSCs can also lead to reduced tumor burdens in preclinical models, in both T cell–dependent and T cell–independent ways. For instance, inhibiting chemokine receptor type 2 (CCR2) (*30*), colony-stimulating factor-1 receptor (CSF-1R) (*33, 34, 36*), and granulocyte macrophage colony-stimulating factor (GM-CSF) (*31, 32*) in preclinical models of melanoma, pancreatic, breast, and prostatic carcinoma increased intratumoral T cells and controlled tumor growth, especially when combined with anti-CTLA-4 or anti-PD-1/PD-L1 (Table 1 and Fig. 3F). Although these studies did not determine whether the increases in T cells were a consequence of enhanced viability or replication, they emphasize again how elements of the TME regulate the accumulation of effector T cells. Inhibition of CSF-1R in a preclinical model of proneural glioblastoma multiforme and in patient-derived glioma xenografts increased survival and caused regression of established tumors in an apparent T cell–independent manner that correlated with the reprogramming of macrophages away from an M2 phenotype (*66*). Similarly, an activator of TAMs, an agonistic antibody to CD40, when administered in combination with the chemotherapeutic drug gemcitabine, suppressed the growth of mouse PDA in a T cell–independent manner (*67*), suggesting that macrophages alone, when appropriately stimulated, may have potent anticancer functions. B cells have also been shown to regulate the phenotype of TAMs in the squamous cell carcinoma TME (Fig. 2E) (*68*). Correspondingly, B cell depletion reprogrammed TAMs, thus relieving their suppression of CD8 cells and enhancing chemotherapy efficacy. Another example

of how the antitumor effects of macrophages can be used therapeutically is an autochthonous mouse model of melanoma in which the melanoma-killing capability of these cells was revealed by depleting T_{reg} cells and neutralizing IL-10 (*69*). TAMs would also be the mediators of the antitumor effects of antibodies (*70*) and genetically engineered ligands (*71*) that interact with CD47 on cancer cells to prevent the CD47/signal regulatory protein-α (SIRPα) signaling system from suppressing the phagocytosis of antibody-coated cancer cells.

The TME regulates spatial distribution of T cells within tumors

Increased numbers of intratumoral, cancer-specific T cells will be of little import if T cells are restricted to the stroma and prevented from accumulating in the vicinity of cancer cells. CAFs, which may be identified by their expression of the membrane protein fibroblast activation protein-α (FAP), have been shown to have two means by which they can mediate this restriction, the first of which is a physical exclusion mediated by the extracellular matrix that they produce (Fig. 2F). Live cell imaging of lung tumor tissue slices from patients revealed active T cell motility in regions of loose fibronectin and collagen, whereas T cells migrated poorly in dense matrix areas surrounding tumor nests (*72*). When either collagenase was added to reduce matrix rigidity, or the chemokine CCL5 was experimentally produced by tumor cells, there was increased T cell movement out of the stromal regions and into contact with cancer cells.

The second means by which FAP^+ CAFs exclude T cells involves their biosynthesis of CXCL12 (Fig. 2F). Conditionally depleting these cells from the stroma of an ectopic, transplanted tumor (*73*) and of an autochthonous PDA (*29*) allowed preexisting cancer-specific T cells to rapidly control tumor growth and revealed the antitumor effects of anti-PD-L1. However, depleting FAP^+ stromal cells is not a reasonable therapeutic option unless the depletion can be limited to the TME because these cells carry out essential functions in several normal tissues (*74*). The recent report of "reprogramming" these cells in the TME by administration of a vitamin D analog (*75*) may be one means of circumventing this problem. Another may be to block their immune suppressive mechanism. In a preclinical mouse model of PDA, FAP^+ CAFs produce the chemokine CXCL12, which is bound by the PDA cancer cells, which had been previously reported for cancer cells in human PDA, CRC, and ovarian cancer (*76*–*78*). Because FAP^+ stromal cells also accumulate in nontransformed, inflammatory lesions, this "coating" of cancer cells may reflect a means by which "injured" epithelial cells protect themselves from adaptive immune attack. Administering an inhibitor of CXCR4, the receptor for CXCL12, to the PDA-bearing mice caused the rapid accumulation of T cells among cancer cells, arrest of tumor growth, and tumor sensitivity to anti-PD-L1 (Fig. 3G) (*29*). How the cancer cell–bound CXCL12 excludes T cells has not yet been shown, although the mechanism must involve either T cells or myelomonocytic cells because they, and not cancer cells or FAP^+ CAFs, express CXCR4 in this model.

Conceptual challenges and therapeutic opportunities

Among the challenges that remain for understanding the immune suppressive roles of the TME, three are foremost: comprehending the mechanisms by which the TME excludes T cells, determining whether the TME of primary and metastatic tumor sites differ, and assessing the potential clinical efficacy of interventions that affect the TME. The preclinical studies in mice that showed that inhibiting CCR2, CSF-1/CSF-1R, GM-CSF, or CXCR4 improved immune control of tumor growth also showed that these interventions shared a capacity for increasing the frequency of T cells among cancer cells (Fig. 3). Because targeting CCR2 and CSF-1/CSF-1R diminishes the accumulation of CCR2-expressing cell types, including bone marrow–derived TAMs and DCs, one must conclude that at least one function of these cells is to suppress the accumulation of intratumoral T cells. However, given that these cells are distributed in both the stromal and cancer cell regions of tumors, it is not readily apparent how they can selectively exclude T cells only from the vicinity of the cancer cells. On the other hand, the distribution of intratumoral CXCL12, which is associated with cancer cells, does correlate, albeit inversely, with that of T cells, so that the hypothesis that CXCL12 is involved with T cell exclusion would be reasonable and is supported by the antitumor outcome of inhibiting CXCR4. Even here a mechanism that may account for this effect of CXCR4, other than T cell "repulsion" (*79*), is not apparent. For the moment, then, one may only suggest that because CSF1R- and CCR2-dependent cells and CXCR4 signaling are both required for the exclusion of T cells, they are elements of a single pathway that mediates this dominant immune suppressive process.

Regarding the TME of metastatic sites, most preclinical and clinical analyses to date have been restricted to primary tumors. It has been noted earlier that mice in which an immune response has been induced by growth of a primary methycholanthrene-induced sarcoma prevent the establishment of a secondary tumor by these sarcoma cells (*4*). In a preclinical model of spontaneous melanoma, cancer cells were found to disseminate early but to remain in a dormant state that was mediated, at least in part, by $CD8^+$ T cells (*81*). Consistent with this report of immune-induced metastatic dormancy is a study that found metastases in another mouse model that grew rapidly in association with the exclusion of $CD8^+$ T cells (*81*). A challenge will be to determine whether the immune-suppressive intensity of the TMEs of metastatic lesions may vary, with dormant metastases being dominated by immune control and growing lesions exhibiting immune suppression.

With respect to clinically assessing the effects of altering the TME for the purpose of increasing the frequency of intratumoral effector T cells, the academic oncologist already has several agents available that are specific for the same targets in humans that have regulated this process in mouse cancers: IDO inhibitors, CSF-1R inhibitors, CCR2-specific antibodies, and an inhibitor of CXCR4. Examples of each are already in clinical trials in human cancer patients, usually as monotherapies. There is an obvious rationale to combine those agents that are found to augment the intratumoral accumulation of effector T cells with therapies that improve the response of T cells to TCR ligation, such as antibodies to PD-1 and PD-L1, or increase the overall frequency of cancer-specific T cells, such as vaccines and ACT.

Last, recognition of the function of the TME in excluding T cells prompts an interest in the identity of the normal biological circumstance that is responsible for the development of this phenomenon. Tumor immunologists currently consider mutated genes to be the major source of antigens in cancer cells that T cells respond to, but some cancers that have a low mutational burden may elicit cancer-specific $CD8^+$ T cells, as exemplified by the mouse model of PDA (*29*). Is it possible that nontransformed epithelial cells in regenerating tissues also express immunogenic neoantigens, a circumstance that would select for an immune suppressive microenvironment? The frequent occurrence of the immune suppressive elements of the TME, myelomonocytic cells, and FAP^+ stromal fibroblasts in regenerating tissues is consistent with this conjecture and merits further investigation.

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REVIEWS

Cancer and the microbiota

Wendy S. Garrett[1,2,3,4]

A host's microbiota may increase, diminish, or have no effect at all on cancer susceptibility. Assigning causal roles in cancer to specific microbes and microbiotas, unraveling host-microbiota interactions with environmental factors in carcinogenesis, and exploiting such knowledge for cancer diagnosis and treatment are areas of intensive interest. This Review considers how microbes and the microbiota may amplify or mitigate carcinogenesis, responsiveness to cancer therapeutics, and cancer-associated complications.

The relationship between cancer and microbes is complex. Although cancer is generally considered to be a disease of host genetics and environmental factors, microorganisms are implicated in ~20% of human malignancies (*1*). Microbes present at mucosal sites can become part of the tumor microenvironment of aerodigestive tract malignancies, and intratumoral microbes can affect cancer growth and spread in many ways (*2*–*6*). In counterpoise, the gut microbiota also functions in detoxification of dietary components, reducing inflammation, and maintaining a balance in host cell growth and proliferation. The possibility of microbe-based cancer therapeutics has attracted interest for more than 100 years, from Coley's toxins (one of the earliest forms of cancer bacteriotherapy) to the current era of synthetic biology's designer microbes and microbiota transplants. Thus, interrogation of the roles of microbes and the microbiota in cancer requires a holistic perspective.

The ways in which microbes and the microbiota contribute to carcinogenesis, whether by enhancing or diminishing a host's risk, fall into three broad categories: (i) altering the balance of host cell proliferation and death, (ii) guiding immune system function, and (iii) influencing metabolism of host-produced factors, ingested foodstuffs, and pharmaceuticals (Fig. 1). Assigning microbial communities, their members, and aggregate biomolecular activities into these categories will require a substantial research commitment. This Review discusses how microbes and the microbiota may contribute to cancer development and progression, responsiveness to cancer therapeutics, and cancer-associated complications.

Microbial contributions to carcinogenesis

Of the estimated 3.7×10^{30} microbes living on Earth (*7*), only 10 are designated by the International Agency for Cancer Research (IACR) as carcinogenic to humans (*1*). Although most of these carcinogenic microbes colonize large percentages of the human population, only a subset of affected individuals develop cancer, because host and microbial genotypes influence cancer susceptibility.

Tumors arising at boundary surfaces, such as the skin, oropharynx, and respiratory, digestive, and urogenital tracts, harbor a microbiota, which complicates cancer-microbe causality. Enrichment of a microbe at a tumor site does not connote that a microbe is directly associated, let alone causal, in disease. Rather, microbes may find a tumor's oxygen tension or carbon sources permissive and take advantage of an underused nutritional niche. Decreased abundances of specific microbes may also place a host at enhanced risk for cancer development at sites local or distant from this microbial shift. Thus, rigorous frameworks for interpreting tumor-associated microbiota data are essential (*2*).

Oncomicrobes, shifting the balance of when to die and when to grow

Bona fide oncomicrobes—microbes that trigger transformation events in host cells—are rare. Beyond the 10 IACR-designated microbes, there are a handful of other microorganisms with robust but fewer aggregate data supporting their role in human carcinogenesis. As many of these and their carcinogenic mechanisms have been recently reviewed (*2*–*6*, *8*), select activities representing common pathways by which microbes influence cancer will be highlighted.

Human oncoviruses can drive carcinogenesis by integrating oncogenes into host genomes. Human papillomaviruses (HPV) express oncoproteins such as E6 and E7. Data from recent genomic analyses of HPV^+ cervical cancers suggest that viral integration also selectively triggers amplification of host genes in pathways with established roles in cancer (*9*).

Microbes also drive transformation by affecting genomic stability, resistance to cell death, and proliferative signaling. Many bacteria have evolved mechanisms to damage DNA, so as to kill competitors and survive in the microbial world. Unfortunately, these bacterial defensive factors can lead to mutational events that contribute to carcinogenesis (Fig. 2). Examples include colibactin encoded by the *pks* locus [expressed by B2

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REVIEWS

Adoptive cell transfer as personalized immunotherapy for human cancer

Steven A. Rosenberg* and Nicholas P. Restifo*

Adoptive cell therapy (ACT) is a highly personalized cancer therapy that involves administration to the cancer-bearing host of immune cells with direct anticancer activity. ACT using naturally occurring tumor-reactive lymphocytes has mediated durable, complete regressions in patients with melanoma, probably by targeting somatic mutations exclusive to each cancer. These results have expanded the reach of ACT to the treatment of common epithelial cancers. In addition, the ability to genetically engineer lymphocytes to express conventional T cell receptors or chimeric antigen receptors has further extended the successful application of ACT for cancer treatment.

Adoptive cell therapy (ACT) has multiple advantages compared with other forms of cancer immunotherapy that rely on the active in vivo development of sufficient numbers of antitumor T cells with the functions necessary to mediate cancer regression. For use in ACT, large numbers of antitumor lymphocytes (up to 10^{11}) can be readily grown in vitro and selected for high-avidity recognition of the tumor, as well as for the effector functions required to mediate cancer regression. In vitro activation allows such cells to be released from the inhibitory factors that exist in vivo. Perhaps most importantly, ACT enables the manipulation of the host before cell transfer to provide a favorable microenvironment that better supports antitumor immunity. ACT is a "living" treatment because the administered cells can proliferate in vivo and maintain their antitumor effector functions.

A major factor limiting the successful use of ACT in humans is the identification of cells that can target antigens selectively expressed on the cancer and not on essential normal tissues. ACT has used either natural host cells that exhibit antitumor reactivity or host cells that have been genetically engineered with antitumor T cell receptors (TCRs) or chimeric antigen receptors (CARs). With the use of these approaches, ACT has mediated dramatic regressions in a variety of cancer histologies, including melanoma, cervical cancer, lymphoma, leukemia, bile duct cancer, and neuroblastoma. This Review will discuss the current state of ACT for the treatment of human cancer, as well as the principles of effective treatment that point toward improvements in this approach.

A brief history of ACT

Very little was known about the function of T lymphocytes until the 1960s, when it was shown that lymphocytes were the mediators of allograft rejection in experimental animals. Attempts to use T cells to treat transplanted murine tumors were limited by the inability to expand and manipulate T cells in culture. Thus, ACT used transfer of syngeneic lymphocytes from rodents heavily immunized against the tumor, and modest growth inhibition of small established tumors was observed (*1*, *2*). In early preclinical studies, the importance of host inhibitory factors was suggested by findings that lymphodepletion using either chemotherapy or radiation before cell transfer enhanced the ability of transferred lymphocytes to treat established tumors (*3*, *4*).

The ability to use ACT was facilitated by the description of T cell growth factor [interleukin-2 (IL-2)] in 1976, which provided a means to grow T lymphocytes ex vivo, often without loss of effector functions (*5*). The direct administration of high doses of IL-2 could inhibit tumor growth in mice (*6*), and studies in 1982 demonstrated that the intravenous injection of immune lymphocytes expanded in IL-2 could effectively treat bulky subcutaneous FBL3 lymphomas (*7*). In addition, administration of IL-2 after cell transfer could enhance the therapeutic potential of these adoptively transferred lymphocytes (*8*). The demonstration in 1985 that IL-2 administration could result in complete durable tumor regressions in some patients with metastatic melanoma (*9*) provided a stimulus to identify the specific T cells and their cognate antigens involved in this cancer immunotherapy. Lymphocytes infiltrating into the stroma of growing, transplantable tumors were shown to represent a concentrated source of lymphocytes capable of recognizing tumor in vitro, and studies in murine tumor models demonstrated that the adoptive transfer of these syngeneic tumor-infiltrating lymphocytes (TILs) expanded in IL-2 could mediate regression of established lung and liver tumors (*10*). In vitro studies in 1986 showed that human TILs obtained from resected melanomas contained cells capable of specific recognition of autologous tumors (*11*), and these studies led in 1988 to the first demonstration that ACT using autologous TILs could mediate objective regression of cancer in patients with metastatic melanoma (*12*).

Populations of TILs that grow from tumors are generally mixtures of $CD8^+$ and $CD4^+$ T cells with few if any major contaminating cells in mature cultures. The ability of pure populations of T lymphocytes to mediate cancer regression in patients provided the first direct evidence that T cells played a vital role in human cancer immunotherapy. However, responses were often of short

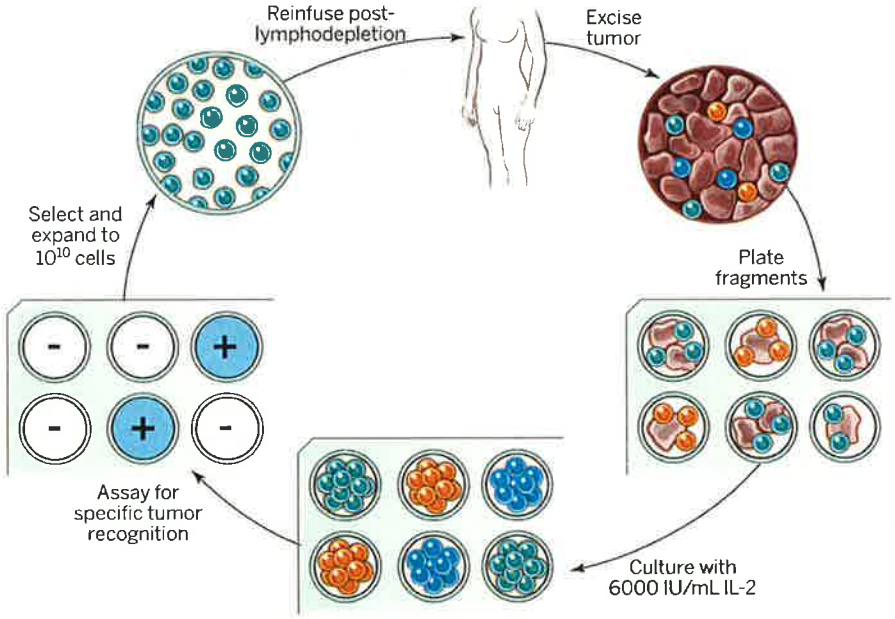

Fig. 1. General schema for using the adoptive cell transfer of naturally occurring autologous TILs. The resected melanoma specimen is digested into a single-cell suspension or divided into multiple tumor fragments that are individually grown in IL-2. Lymphocytes overgrow, destroy tumors within 2 to 3 weeks, and generate pure cultures of lymphocytes that can be tested for reactivity in coculture assays. Individual cultures are then rapidly expanded in the presence of excess irradiated feeder lymphocytes, OKT3, and IL-2. By approximately 5 to 6 weeks after resecting the tumor, up to 10^{11} lymphocytes can be obtained for infusion into patients.

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duration, and the transferred cells could rarely be found in the circulation just days after administration. A critical improvement in the application of ACT to the treatment of human cancer was reported in 2002, when it was shown that lymphodepletion using a nonmyeloablative chemotherapy regimen administered immediately before TIL transfer could lead to increased cancer regression, as well as the persistent oligoclonal repopulation of the host with the transferred antitumor lymphocytes (*13*). In some patients, the administered antitumor cells represented up to 80% of the $CD8^+$ T cells in the circulation months after the infusion.

Lymphocyte cultures can be grown from many tumor histologies; however, melanoma appeared to be the only cancer that reproducibly gave rise to TIL cultures capable of specific antitumor recognition. The stimulus to more widely apply ACT to treat multiple human cancers led to studies of the genetic engineering of lymphocytes to express antitumor receptors. Following mouse models (*14*), it was shown for the first time in humans in 2006 that administration of normal circulating lymphocytes transduced with a retrovirus encoding a TCR that recognized the MART-1 melanoma-melanocyte antigen could mediate tumor regression (*15*). Administration of lymphocytes genetically engineered to express a chimeric antigen receptor (CAR) against the B cell antigen CD19 was shown in 2010 to mediate regression of an advanced B cell lymphoma (*16*). These findings of the use of either naturally occurring or genetically engineered antitumor T cells set the stage for the extended development of ACT for the treatment of human cancer.

ACT using TILs is an effective immunotherapy for patients with metastatic melanoma

Adoptive cell therapy using autologous TILs is the most effective approach to induce complete durable regressions in patients with metastatic melanoma (Table 1). The general approach for growing and administering human TILs is shown in Fig. 1. The resected melanoma specimen is digested into a single-cell suspension or divided into multiple tumor fragments that are individually grown in IL-2. Lymphocytes overgrow, destroy tumors within 2 to 3 weeks, and give rise to pure cultures of lymphocytes that can be tested for reactivity against tumors, if available, in coculture assays. Individual cultures are then rapidly expanded in the presence of excess irradiated feeder lymphocytes, an antibody targeting the epsilon subunit within the human CD3 complex of the TCR, and IL-2. By ~5 to 6 weeks after resecting the tumor, up to 10^{11} lymphocytes can be obtained for infusion into patients. A substantial increase in cell persistence and the incidence and duration of clinical responses was seen when patients received a lymphodepleting preparative regimen before the cell infusion (*13*). It might be possible to optimize the intensity or duration of the lymphodepletion that is employed, but the most frequently used lymphodepleting preparative regimen consists of 60 mg/kg cyclophosphamide for 2 days and 25 mg/m^2 fludarabine administered for 5 days followed by cells and IL-2 given at 720,000 IU/kg to tolerance (Fig. 2). In a pilot study in the Surgery Branch, National Cancer Institute (NCI), objective cancer regressions by RECIST criteria (Response Evaluation Criteria in Solid Tumors) were seen in 21 of 43 patients (49%), including 5 patients (12%) who underwent complete cancer regression (*13*). When 200 or 1200 centigray (cGy; 1 Gy = 100 rads) total-body irradiation (TBI) was added to the preparative regimen in pilot trials of 25 patients each, objective response (OR) rates of 52 and 72% were seen, including 20 and 40% complete regressions. However, there were no statistically significant differences in the OR rates between preparative regimens (*13*, *17*). Twenty of the 93 patients (22%) in these trials had complete regressions, and 19 (20%) have not experienced recurrences at follow-up times of 5 to 10 years and are probably cured. A prospective randomized study comparing the chemotherapy preparative regimen alone versus chemotherapy plus the addition of 1200 cGy TBI in 101 patients was recently concluded at the NCI, National Institutes of Health (NIH), and results are pending.

In the combined experience of the treatment of 194 patients using TILs grown from individual melanoma fragments at the NCI (Bethesda, Maryland), 107 patients (55%) have shown ORs. Similar OR rates to TIL therapy have been reported by multiple groups, including those from the Moffitt Cancer Center (Tampa, Florida) (38% OR rate) (*18*), the MD Anderson Cancer Center (Houston, Texas) (48% OR rate) (*19*), and the Ella Cancer Institute (Ramat Gan, Israel) (40% OR rate) (*20*) (Table 1).

There is no relation between the bulk of disease or the site of metastases and the likelihood of achieving a complete cancer regression (*17*). Of the 34 complete responders thus far seen in the two trials at the NCI, only one has recurred, and only one patient with complete regression received more than one treatment. The brain is not a sanctuary site, and regression of brain metastases has been observed (*21*). Prior treatment with targeted therapy using the Braf inhibitor vemurafenib (Zelboraf) does not appear to affect the likelihood of having an OR to ACT treatment in patients with melanoma. ACT can also be effective after other immunotherapies have failed. Of the 194 patients treated in the NCI trials, OR rates in patients who had no prior therapy or who progressed through IL-2, antibody to cytotoxic T lymphocyte–associated protein 4 (anti–CTLA-4), anti-PD1, or Braf inhibitors were 48, 63, 42, 50, and 43%, respectively.

Lymphodepletion appears to be an important component of ACT, and mouse models have shown that lymphodepletion given before cell transfer can increase the effectiveness of treatment more than 10-fold. In the clinic, the persistence of T cells was once a rarity (*22*), but in trials conducted after the initiation of lymphodepleting therapy, adoptively transferred T cells could comprise the majority of the peripheral blood $CD8^+$ cells 1 month after transfer (*13*). The cellular basis of the effect of lymphodepletion is complex and still not completely understood. In mouse models, myeloid-derived suppressor cells and $CD4^+$ FoxP3 regulatory T cells can be found at high levels in tumors in vivo and can depress immune responses in the mouse tumor microenvironment (*23*). In accord with these preclinical findings, preparative chemotherapy in humans severely depletes lymphocytes and myeloid cells from the circulation at the time of cell infusion, although the rate of reappearance of FoxP3 inhibitory T cells after lymphodepletion was inversely correlated with clinical response (*24*).

Lymphodepletion prior to T cell transfer is followed by immune reconstitution

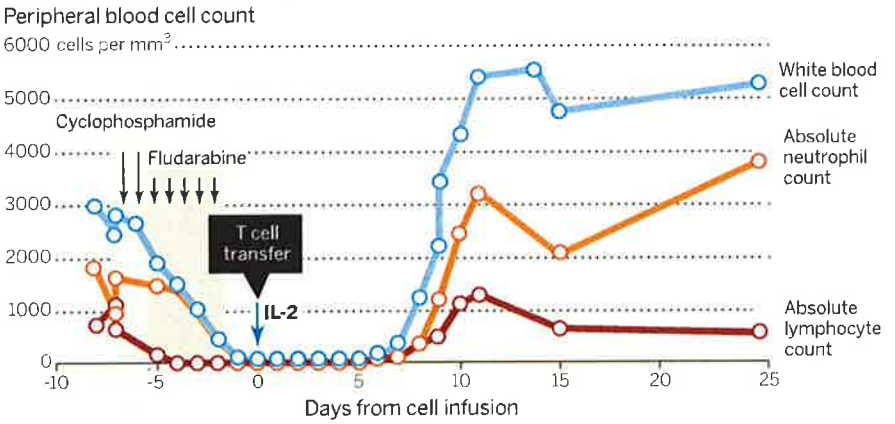

Fig. 2. A substantial increase in cell persistence and the incidence and duration of clinical responses is observed when patients received a lymphodepleting preparative regimen before the cell infusion. The most frequently used lymphodepleting preparative regimen consists of 60 mg/kg cyclophosphamide given for 2 days and 25 mg/m^2 fludarabine administered over 5 days, followed by T cells and IL-2 administration.

Table 1. Selected clinical trials of ACT for the treatment of human cancer. CLL, chronic lymphocytic leukemia; ALL, acute lymphocytic leukemia; CR, complete response; HPC, human papillomavirus; allo-HSCT, allogeneic hematopoietic stem cell transplantation; DLBCL, diffuse large B cell lymphoma; EBV, Epstein-Barr virus. Dashes indicate not applicable.

CELLS USED FOR ACT	YEAR	CANCER HISTOLOGY	MOLECULAR TARGET	PATIENTS	NUMBER OF ORS	COMMENTS
Tumor-infiltrating lymphocytes*	1998	Melanoma (*12*)		20	55%	Original use TIL ACT
	1994	Melanoma (*88*)		86	34%	
	2002	Melanoma (*13*)		13	46%	Lymphodepletion before cell transfer
	2011	Melanoma (*17*)		93	56%	20% CR beyond 5 years
	2012	Melanoma (*19*)		31	48%	
	2012	Melanoma (*18*)		13	38%	Intention to treat: 26% OR rate
	2013	Melanoma (*20*)		57	40%	Intention to treat: 29% OR rate
	2014	Cervical cancer (*89*)		9	33%	Probably targeting HPV antigens
	2014	Bile duct (*44*)	Mutated ERB2	1	–	Selected to target a somatic mutation
In vitro sensitization	2008	Melanoma (*90*)	NY-ESO-1	9	33%	Clones reactive against cancer-testes antigens
	2014	Leukemia (*91*)	WT-1	11	–	Many treated at high risk for relapse
Genetically engineered with CARs	2010	Lymphoma (*16*)	CD19	1	100%	First use of anti-CD19 CAR
	2011	CLL (*68*)	CD19	3	100%	Lentivirus used for transduction
	2013	ALL (*70*)	CD19	5	100%	Four of five then underwent allo-HSCT
	2014	ALL (*92*)	CD19	30	90%	CR in 90%
	2014	Lymphoma (*71*)	CD19	15	80%	Four of seven CR in DLBCL
	2014	ALL (*93*)	CD19	16	88%	Many moved to allo-HSCT
	2014	ALL (*94*)	CD19	21	67%	Dose-escalation study
	2011	Neuroblastoma (*78*)	GD2	11	27%	CR2 CARs into EBV-reactive cells
Genetically engineered with TCRs	2011	Synovial sarcoma (*81*)	NY-ESO-1	6	67%	First report targeting nonmelanoma solid tumor
	2006	Melanoma (*15*, *32*)	MART-1	11	45%	

*Molecular targets of TIL in melanoma appear to be exomic mutations expressed by the cancer (*39*, *40*, *44*)

Levels of homeostatic cytokines, which promote T cell proliferation and survival, are dramatically induced upon lymphodepletion (*25*) in mouse models. In humans, lymphodepletion leads to the appearance in the circulation of the T cell growth factor IL-15, which serves to promote the expansion of the transferred cells in the absence of competing endogenous lymphocytes (*26*). Further, lymphodepletion can enhance the translocation of commensal microflora across mucosal barriers in the mouse, and this can enhance the effect of ACT by stimulating Toll-like receptors (*27*) to activate antigen-presenting cells (APCs). These preclinical results have highly affected clinical translation, and it seems likely that immune ablation will be a part of future cell-based treatments in patients with cancer.

Adoptive cell therapy is a "living" treatment, and administered lymphocytes can expand more than 1000-fold after administration. Studies in mouse models, including those involving the injection of human cells into immunodeficient animals, have emphasized the importance of the differentiation state of the infused cells (*28*, *29*). The phenotypic and functional status of less differentiated murine cells is highly positively correlated with their ability to eliminate vascularized tumor in vivo. These findings are in accordance with the high positive correlation between the persistence of the transferred TILs in the circulation of patients at 1 month and with the induction of partial and complete clinical responses (*17*). Further, one clinical study showed a strong correlation between expression of the phenotypic marker CD27, which is associated with cells early in their differentiation pathway, and clinical response (*17*). The presence of longer telomeres as a correlate of clinical response was seen in one study (*17*) but not in another (*18*).

The observation that melanoma TILs can mediate durable, complete, and probably curative cancer regression in patients with metastatic melanoma has raised considerable interest in the possible use of TILs for the treatment of multiple cancer types. Although TILs can be grown in vitro from virtually all tumors, only melanomas consistently give rise to TILs with antitumor reactivity. In an attempt to gain insight into the possible extension of ACT to the treatment of other common cancers, extensive studies of the antigens recognized by TILs have been pursued.

Melanoma TILs recognize the products of cancer mutations

Early studies identified two nonmutated melanoma-melanocyte differentiation proteins, MART-1 and gp100, that were often recognized by melanoma TILs (*30*, *31*). Melanocytes in the skin, eye, and ear express the MART-1 and gp100 proteins, and yet toxicity targeting these proteins was not seen in the majority of patients treated with TILs who underwent complete cancer regression. In contrast, when a high-affinity TCR against MART-1 or gp100 was inserted into lymphocytes used for ACT, profound eye and ear toxicity was often seen in the absence of antitumor activity, which suggests that the reactivity against melanoma-melanocyte antigens was not the decisive target resulting in the in vivo antitumor activity of melanoma TILs (*32*).

A study of exomic mutation rates in more than 3000 tumor-normal pairs revealed that the frequency of nonsynonymous mutations varied more than 1000-fold across different cancer types (*33*). Pediatric cancers exhibited mutation frequencies as low as 0.1/Mb, whereas melanomas and lung cancers often exceeded 100 mutations/Mb.

The suggestion that mutations might be targets of immune recognition of tumor cells has been around for some time (*34*). The responsiveness of melanoma to a variety of immunotherapy approaches such as ACT, IL-2, anti–CTLA-4, and anti–PD-1 suggested that peptide epitopes encoded by the large number of mutations in melanoma might be the targets of TIL therapy (*35*). Support for this hypothesis comes from recent observations that anti–PD-1 can mediate ORs not only in patients with melanoma but also in patients with lung and bladder cancer, the two tumor types closest to melanoma with a high frequency of mutations (*36*). A patient successfully treated with anti–CTLA-4 generated circulating T cells that recognized a distinct mutation in the melanoma (*37*). Another study suggested that increased numbers of exomic mutations in a cancer correlated with better outcomes (*38*).

New approaches using whole-exomic sequencing of tumor-normal pairs in patients with melanoma have consistently identified nonsynonymous cancer mutations recognized by autologous TILs that mediated complete cancer regressions (*39*, *40*). However, not all expressed mutations can be recognized by T cells. Proteins incorporating the mutations must be processed to short peptides of ~9 amino acids for major histocompatibility complex (MHC) class 1 and a bit longer for MHC class 2; these peptides are then presented on the cell surface. One approach to identify the immunogenic mutations that we have taken is to identify 21- to 25–amino acid polypeptides, each one containing a mutated amino acid flanked by 10 to 12 normal residues. Using peptide-MHC binding algorithms, these polypeptides can then be scanned to identify peptides with high binding to individual MHC molecules of the patient. The top-predicted binding peptides are then synthesized and tested for recognition by coculture with TILs that mediated cancer regression. This method depends on the accuracy of peptide-MHC binding algorithms, which are often inadequate for many of the less frequent MHC molecules (*39*).

An alternate method eliminates the need for predicted peptide binding to MHC and enables the screening of all candidate peptides on all MHC loci in a single test (*40*) (Fig. 3). As above, minigenes, rather than polypeptides, are constructed that encode each mutated amino acid flanked by 10 to 12 amino acids. Strings of 6 to 20 minigenes are then linked into tandem minigenes, and these DNA constructs are subsequently cloned into an expression plasmid and in vitro transcribed to RNA, which is electroporated into the patient's autologous APCs. These APCs present all mutated peptides capable of being processed and binding to any of the patient's class 1 or class 2 MHC molecules. Culture of the patient's TILs with these APCs can identify the tandem minigene as well as the individual minigene responsible for tumor recognition. Using these approaches, TILs from 21 patients with melanoma that responded to ACT identified 45 mutations presented on a variety of class 1 and class 2 MHC molecules. Thus far, every mutation recognized by TILs was distinct (i.e., each from a different expressed protein), with none shared by another melanoma in the set studied. These findings provide suggestive evidence that melanoma TILs capable of mediating antitumor responses were recognizing random somatic mutations in the cancer. In many cases, multiple mutations were recognized by an individual TIL population. The concept that cancer regressions after immunotherapy are the result of targeting mutations explains why patients can experience tumor regression without autoimmune sequelae. Conversely, the ineffectiveness of the vast number of therapeutic cancer vaccines that targeted nonmutated self-proteins can also be explained (*41*, *42*). Whereas strong reactivity to self-antigens causes autoimmune toxicity, vaccines against self-antigens trigger the expansion of low-affinity TCRs against self-proteins that escaped negative selection in the thymus. This raises the possibility that vaccines targeting mutated immunogenic epitopes may be much more effective. The specific targeting of individual mutated antigens in a patient's cancer presents a daunting problem for widespread therapeutic application of ACT but also presents an opportunity to develop treatments for multiple cancer types. Schumacher and Schreiber discuss additional aspects for targeting mutated antigens in this issue (*43*).

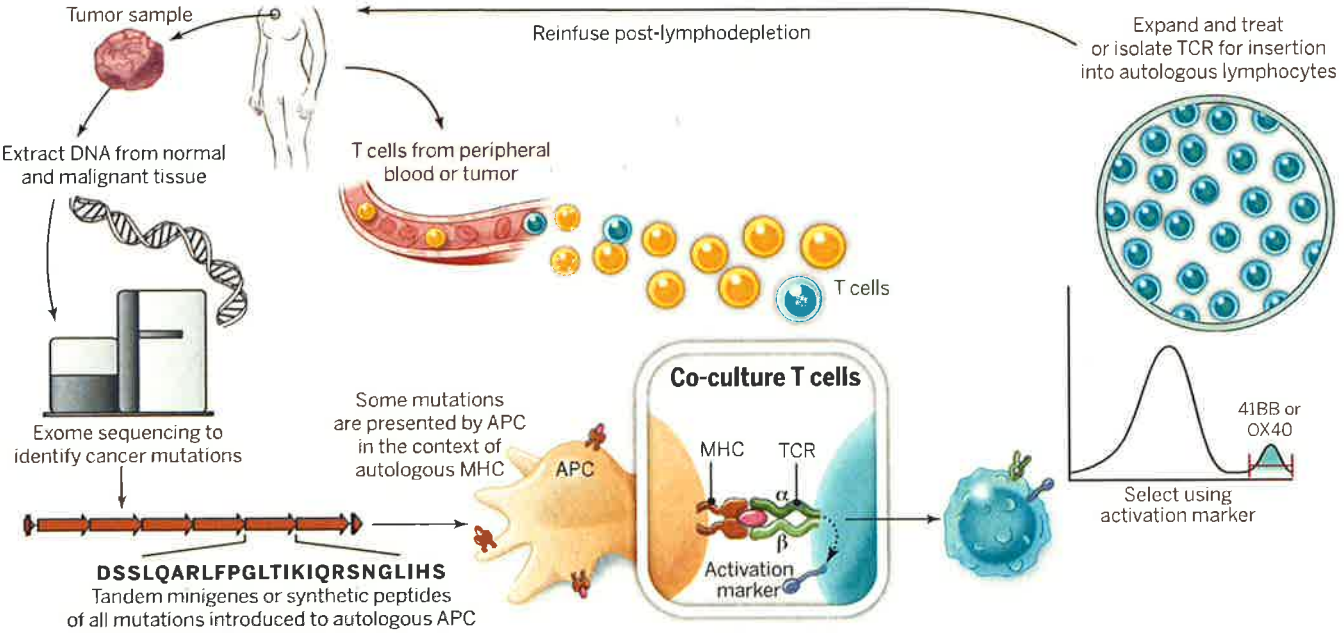

Fig. 3. A "blueprint" for the treatment of patients with T cells recognizing tumor-specific mutations. The sequences of exomic DNA from tumor cells and normal cells from the same patient are compared to identify tumor-specific mutations. Knowledge of these mutations can then be used to synthesize either minigenes or polypeptides encoding each mutated amino acid flanked by 10 to 12 amino acids. These peptides or minigenes can be expressed by a patient's autologous APCs, where they are processed and presented in the context of a patient's MHC. Coculture of the patient's T cells with these APCs can be used to identify all mutations processed and presented in the context of all of a patient's MHC class I and class II molecules. The identification of individual mutations responsible for tumor recognition is possible because T cells express activation markers, such as 41BB ($CD8^+$ T cells) and OX40 ($CD4^+$ T cells), when they recognize their cognate target antigen. T cells expressing the activation marker can then by purified using flow cytometry before their expansion and reinfusion into the tumor-bearing patient.

TILs from common epithelial cancers can also recognize cancer mutations

A recent report has shown that the mutated antigens in a nonmelanoma epithelial cancer can give rise to immune responses, despite the low number of mutations in these cancers (*44*). Exomic sequencing of a metastatic cholangiocarcinoma in a patient who had progressed through multiple chemotherapies revealed 26 nonsynonymous mutations. Tandem minigenes that encoded each mutated amino acid and its flanking sequences were constructed and electroporated into the patient's APCs. CD4 cells from TIL cultures from this patient's tumor recognized the ERBB2IP mutation restricted by the MHC class 2 antigen HLA-DQ O6. ERBB2IP is a tumor suppressor that binds to ERBB2 and attenuates downstream RAS/ERK signaling. Despite the lack of an objective clinical response to the administration of bulk autologous TILs in this patient, administration of TILs that were selected to contain more than 95% ERBB2IP mutation-reactive TILs mediated a dramatic regression of liver and lung metastases ongoing beyond 1 year. This result provides compelling evidence that mutation-reactive T cells are capable of mediating in vivo tumor regression in patients with this epithelial cancer. Further, the findings suggest that this treatment approach may be suitable for patients with other common epithelial cancers that are not normally considered to be immunogenic.

Mutations that are targeted may be driver mutations essential for the malignant phenotype of the cell, or alternatively, the TILs may contain reactivity against multiple immunogenic passenger mutations, which would decrease the likelihood that the loss of any individual antigen would subvert the clinical antitumor response. TIL populations can be highly polyclonal and thus are likely to be capable of potentially recognizing multiple antigens simultaneously. Given their curative potential, it seems likely that TILs are able to recognize antigens expressed by cancer stem cells. Although some of the mutations are probably driver mutations because they are found in expressed genes associated with known oncogenic pathways (e.g., mutated β-catenin), many of the targets of TILs may well be passenger mutations.

Genetic engineering of lymphocytes for use in ACT

In an attempt to broaden the reach of ACT to other cancers, techniques were developed to introduce antitumor receptors into normal T cells that could be used for therapy (Fig. 4). The specificity of T cells can be redirected by the integration of genes encoding either conventional alpha-beta TCRs or CARs. CARs were pioneered by Gross and colleagues in the late 1980s (*45*) and can be constructed by linking the variable regions of the antibody heavy and light chains to intracellular signaling chains such as CD3-zeta, often including costimulatory domains encoding CD28 (*46*) or CD137 to fully activate T cells (*47*, *48*). CARs can provide non-MHC–restricted recognition of cell surface components and can be introduced into T cells with high efficiency using viral vectors.

An important question confronting the use of genetically engineered cells in the ACT of cancer involves selection of the ideal human T cell subpopulation into which the gene should be introduced, as well as the selection of appropriate antigenic targets of the introduced TCRs or CARs. Preclinical studies in mouse models strongly suggest that improved antitumor responses are seen when T cells in early stages of differentiation (such as naïve or central memory cells) are transduced (*49*), a result supported by studies in monkeys showing improved in vivo persistence of infused central memory compared with effector memory cells (*50*). $CD8^+$ T cells can be categorized into distinct memory subsets based on their differentiation states. We and others have found that $CD8^+$ T cells follow a progressive pathway of differentiation from naïve T cells into central memory and effector memory T cell populations [summarized in (*51*)]. $CD8^+$ T cells paradoxically lose antitumor T cell functionality as they acquire the ability to lyse target cells and to produce the cytokine interferon-γ, qualities thought to be important in their antitumor efficacy (*52*). The differentiation state of $CD8^+$ T cells is inversely related to their capacity to proliferate and persist (*52*–*54*). These

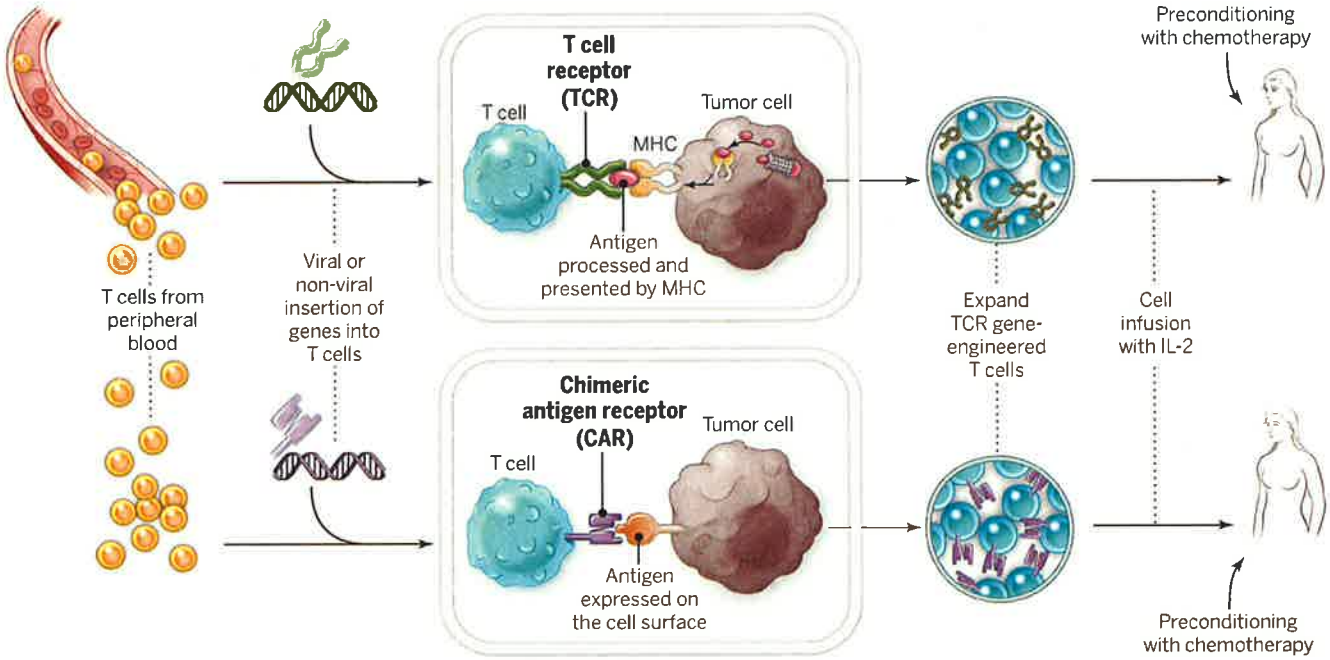

Fig. 4. Gene-modification of peripheral blood lymphocytes. In an attempt to broaden the reach of ACT to other cancers, techniques are being developed to introduce antitumor receptors into normal T cells that could be used for therapy. The top panel shows the insertion of a conventional TCR into a patient's T lymphocytes, followed by the expansion and infusion back into the patient. The bottom panel shows the insertion of a CAR into a patient's T cell, followed by the expansion of these cells and their re-infusion. TCRs and CARs are fundamentally different in their structures and in the structures that they recognize. TCRs are composed of one α chain and one β chain, and they recognize antigens that have been processed and presented by one of the patient's own MHC molecules. CARs are artificial receptors that can be constructed by linking the variable regions of the antibody heavy and light chains to intracellular signaling chains (such as CD3-zeta, CD28, 41BB) alone or in combination with other signaling moieties. CARs recognize antigens that do not need to be MHC-restricted, but they must be presented on the tumor cell surface.

findings may be clinically relevant, and younger T cells are statistically positively correlated with clinical effectiveness in ACT trials (*17*). It seems clear that, like many organ systems in the body, $CD8^+$ T cells can exist in a stem cell–like state, capable of clonal repopulation. Human T memory stem cells express a gene program that enables them to proliferate extensively and can further differentiate into other T cell populations (*29*).

Much of the existing work in cancer immunotherapy has focused on $CD8^+$ T cells. However, $CD4^+$ T cells can also efficiently promote tumor rejection. $CD4^+$ T cells do not merely enhance $CD8^+$ T cell function, but they also play a more direct role in tumor elimination. This notion has been validated recently in humans (*44*). The roles that $CD4^+$ T cells play in the antitumor immune response crucially depend on their polarization, which is determined by their expression of key transcription factors. $CD4^+$ cells can destroy tumor cells, and recent evidence suggests that adoptively transferred T helper 17 cells can promote long-lived antitumor immunity (*55*).

Toxicity of ACT when targeting antigens shared by tumors and normal tissue

The marked potency of T cells enables the recognition of minute levels of antigen expressed on normal cells. Thus, targeting normal, nonmutated antigenic targets that are expressed on normal tissues but overexpressed on tumors has led to severe on-target, off-tumor toxicity in patients. Suitable antigens to target are those presented exclusively on the cancer or, alternatively, on normal cells that are not essential for survival.

The first successful application of ACT using genetically engineered lymphocytes treated 17 patients with metastatic melanoma using autologous T cells transduced with a weakly avid human TCR recognizing the MART-1 melanoma-melanocyte differentiation antigen (*15*). Two patients experienced objective partial regressions of metastatic melanoma, and in both patients the transferred cells could be found in the peripheral blood 1 year after cell infusion. This approach was expanded to 36 patients with metastatic melanoma who received high-avidity TCRs that recognized either the MART-1 or gp100 melanoma-melanocyte antigens (*32*). Although objective cancer regressions were observed in 30 and 19% of patients who received the MART-1 or gp100 TCR, respectively, severe off-tumor, on-target toxicity was seen in the skin, eyes, and ears of patients due to the expression of melanocytes in these organs. These findings coincided with severe eye toxicity seen in mice when targeting melanocyte antigens and provided an early demonstration of the power of T cell therapy (*56*). The treatment of patients with renal cancer using T cells encoding a CAR against carbonic anhydrase 9, which is overexpressed in renal cancer, led to severe liver toxicity due to expression of this antigen in biliary duct epithelium (*57*). A high-affinity TCR against the carcinoembryonic antigen was used to treat patients with metastatic colorectal cancer that expressed high levels of this antigen (*58*). All three patients experienced life-threatening colitis and colonic hemorrhage that precluded further use of this TCR, even though one patient exhibited a partial response of liver metastases. Unexpected toxicities can also result when previously unknown cross-reactivities are seen that target normal self-proteins expressed in vital organs. MAGE-A3, a cancer-testes antigen to be discussed in more detail below, is not known to be expressed in any normal tissues. However, targeting an HLA-A*0201–restricted peptide in MAGE-A3 caused severe damage to gray matter in the brain, resulting in two deaths because this TCR recognized a different but related epitope expressed by MAGE-A12, expressed at very low levels in the brain (*59*). It should also be noted that CARs are capable of toxicity against self-antigens as well. Acute pulmonary toxicity resulting in death was observed after infusion of CAR T cells specific for ERBB2, which seemed likely due to the recognition of low levels of this antigen on pulmonary epithelium (*60*).

Several groups have attempted to affinity-enhance TCRs by altering amino acids in the antigen-combining sites of the TCR (*61*, *62*). By removing the protective effects of negative thymic selection that eliminate high-affinity TCRs against normal proteins, these modified TCRs could potentially recognize new and unrelated determinants. Two patients (one with multiple myeloma and one with melanoma) were treated with an HLA-A1–restricted MAGE-A3–specific TCR whose affinity was enhanced by this site-specific mutagenesis, and both experienced fatal cardiogenic shock due to the recognition of an HLA-A1–restricted peptide derived from an unrelated protein, titin, present in cardiac muscle (*63*). Thus, methods aimed at enhancing the affinities of TCRs can be fraught with problems of unexpected toxicities, which remain difficult to predict. Of course, the same pitfalls of unexpected toxicities may apply to the use of novel CARs.

Targeting antigens expressed on cancers and nonessential human tissues

Cancers that express target molecules shared with nonessential normal organs represent potential targets for human cancer immunotherapy using ACT. A prominent example of such an antigen is the CD19 molecule expressed on more than 90% of B cell malignancies and on B cells at all stages of differentiation, excluding plasma cells. Following preclinical work by many groups [summarized in (*64*–*67*)], the first successful clinical application of anti-CD19 CAR gene therapy in humans was reported in 2010 (*16*). Administration of autologous cells expressing the anti-CD19 CAR to a patient with refractory lymphoma resulted in cancer regression in a patient who remains progression-free after two cycles of treatment ongoing 4 years after treatment. Multiple groups have now shown the effectiveness of ACT targeting CD19 in patients with follicular lymphoma, large-cell lymphomas, chronic lymphocytic leukemia, and acute lymphocytic leukemia (*68*–*72*). On-target toxicity against CD19 results in B cell loss in the circulation and in the bone marrow and can be overcome by the periodic administration of immunoglobulin infusions. Substantial toxicity can be seen by the excessive release of cytokines by CAR-expressing cells, and thus, careful selection of the lymphodepleting preparative regimen and the cell dose is required to safely apply ACT targeting CD19, as well as many other antigens now under experimental study (*72*).

Dramatic regressions of lymphomas and leukemias with ACT have elicited considerable enthusiasm, although most reports contain fewer than 20 patients, and fewer than 200 patients have been treated worldwide. The introduction of CARs into lymphocytes has mainly used gammaretroviruses and lentiviruses, although nonviral approaches such as transposon-transposase systems (*73*) and CRISPR-cas (CRISPR, clustered regularly interspaced palindromic repeat) technology to introduce genes are also being explored (*74*). The single-chain antibody governs recognition of the antigen to be targeted, although the T cell is activated via the CD3-zeta chain signaling domain. In addition to the zeta chain, a variety of costimulatory molecules have been employed in retroviral constructs such as CD27, CD28, CD134, CD137, or ICOS that can profoundly influence the function of the CAR [reviewed in (*64*–*66*)]. Optimization of these costimulatory domains is a subject of active study. The results of CAR therapy for B cell malignancies might be confounded by the sensitivity of lymphomas and leukemias to the preparative chemotherapy regimen. Thus, delineation between the effects of the preparative therapy and those of the CAR T cells needs to be considered.

Multiple other B cell antigens are being studied as targets, including CD22, CD23, ROR-1, and the immunoglobulin light-chain idiotype expressed by the individual cancer (*65*). CARs targeting either CD33 or CD123 have been studied as a therapy for patients with acute myeloid leukemia, though the expression of these molecules on normal precursors can lead to prolonged myeloablation (*75*). BCMA is a tumor necrosis factor receptor family protein expressed on mature B cells and plasma cells and can be targeted on multiple myeloma (*65*). The Reed-Sternberg cell expresses CD30, and this target is being explored as a treatment for patients with refractory Hodgkin lymphoma (*75*–*77*).

Although CARs are being successfully applied to the treatment of hematologic malignancies, the lack of shared antigens on the surface of solid tumors that are not also expressed on essential normal tissues has severely limited the application of CARs to the treatment of solid tumors. Thyroglobulin is a potential target for some patients with thyroid cancers because thyroglobulin is present only in the thyroid gland and not on solid tissues. Neuroblastomas express GD2, which has been targeted by CARs (*78*). Mesothelin has also been forwarded as a potential target, although it is also expressed on normal tissues, including cells in the pericardium and pleural and pertitoneal linings (*79*). A search is ongoing for other tissue-specific surface antigens expressed on tissues that are not essential for survival.

Cancer-testis antigens are a family of intracellular proteins that are expressed during fetal development but have highly restricted expression in adult normal tissues (*80*). There are more than

100 different members of this family of molecules whose expression is epigenetically up-regulated from 10 to 80% of cancer types using highly sensitive techniques. However, initial enthusiasm for targeting cancer-testes antigens has been tempered by the lack of high levels of protein expression of these antigens. Approximately 10% of common cancers appear to express enough protein to be suitable targets for antitumor T cells. There are low levels of some cancer-testes antigens expressed on normal tissues, and this can lead to untoward toxicities. The NYESO-1 cancer-testes antigen has been targeted via a human TCR transduced into autologous cells (*81*). ORs were seen in 5 of 11 patients with metastatic melanoma and 4 of 6 patients with highly refractory synovial cell sarcoma.

Looking to the future of ACT for the treatment of cancer

The continued development of ACT, as well as other immunologic approaches to the treatment of cancer, depends on the identification of suitable targets for immunologic attack. Although CARs have been successful in the treatment of hematologic malignancies and are likely to soon join the mainstream of oncologic treatment, the ability to treat common epithelial solid cancers, which account for ~90% of all cancer fatalities, is severely limited by the lack of suitable targets exclusive to cancer. Extensive searches for monoclonal antibodies that can recognize distinct determinants on the surface of solid cancers but not normal tissues have been in progress for more than 30 years, but few suitable determinants have been found. The EGFRvIII mutation on ~40% of high-grade glioblastomas is a rare example of a shared-surface mutation, and attempts to target this molecule using CARs are in progress (*82*). Shared mutations in intracellular proteins involved in oncogenesis—such as Braf in melanomas and Kras in pancreatic and other solid cancers—would be ideal ACT targets using conventional alpha-beta TCRs, though immunogenic epitopes have not yet been identified in these molecules. Driver and random somatic mutations occurring in many solid cancers may represent excellent targets for the treatment of solid tumors.

Opportunities to improve ACT involve the identification and development of specific antitumor T cells with the functional properties optimal for tumor destruction (*83*). One approach under active evaluation is the growth of cells under conditions that enable in vitro proliferation while limiting differentiation, such as the use of IL-21 or inhibitors that target the kinase AKT (*84*, *85*). Improved specific lymphodepleting preparative regimens and better design of the transducing vectors, including the incorporation of optimal costimulatory molecules, are likely to improve clinical results. Introduction of genes encoding other molecules such as the cytokine IL-12, which can profoundly alter the tumor microenvironment to favor antitumor immunity, has shown substantial promise in animal models (*86*). Enhanced methods for regulating the expression of these highly potent cytokine genes would be an important part of incorporating them into clinical treatment. The incorporation of "suicide" genes that can enable destruction of the transferred cells could add an extra level of safety when exploring genetic changes in lymphocytes (*87*).

Adoptive cell therapy is a more complex approach to the delivery of cancer treatment than many other types of immunotherapy and has often been criticized as impractical and too costly for widespread application. The need to develop highly personalized treatments for each patient does not fit into the paradigm of major pharmaceutical companies that depend on "off-the-shelf" reagents that can be widely distributed. However, curative immunotherapies for patients with common epithelial cancers will probably dictate the need for more personalized approaches. Several new biotechnology companies have arisen to meet the need to expand a patient's lymphocytes, and detailed genetic analysis of individual tumors is already commonplace at large academically affiliated medical centers. Although multiple commercial models have been proposed, widespread application of ACT will probably depend on the development of centralized facilities for producing tumor-reactive TILs or genetically modified lymphocytes that can then be delivered to the treating institution. The effectiveness of treatment will need to trump convenience of administration in the application of new effective approaches to cancer immunotherapy.

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Adoptive cell transfer as personalized immunotherapy for human cancer

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REVIEWS

Cancer and the microbiota

Wendy S. Garrett[1,2,3,4]

A host's microbiota may increase, diminish, or have no effect at all on cancer susceptibility. Assigning causal roles in cancer to specific microbes and microbiotas, unraveling host-microbiota interactions with environmental factors in carcinogenesis, and exploiting such knowledge for cancer diagnosis and treatment are areas of intensive interest. This Review considers how microbes and the microbiota may amplify or mitigate carcinogenesis, responsiveness to cancer therapeutics, and cancer-associated complications.

The relationship between cancer and microbes is complex. Although cancer is generally considered to be a disease of host genetics and environmental factors, microorganisms are implicated in ~20% of human malignancies (*1*). Microbes present at mucosal sites can become part of the tumor microenvironment of aerodigestive tract malignancies, and intratumoral microbes can affect cancer growth and spread in many ways (*2*–*6*). In counterpoise, the gut microbiota also functions in detoxification of dietary components, reducing inflammation, and maintaining a balance in host cell growth and proliferation. The possibility of microbe-based cancer therapeutics has attracted interest for more than 100 years, from Coley's toxins (one of the earliest forms of cancer bacteriotherapy) to the current era of synthetic biology's designer microbes and microbiota transplants. Thus, interrogation of the roles of microbes and the microbiota in cancer requires a holistic perspective.

The ways in which microbes and the microbiota contribute to carcinogenesis, whether by enhancing or diminishing a host's risk, fall into three broad categories: (i) altering the balance of host cell proliferation and death, (ii) guiding immune system function, and (iii) influencing metabolism of host-produced factors, ingested foodstuffs, and pharmaceuticals (Fig. 1). Assigning microbial communities, their members, and aggregate biomolecular activities into these categories will require a substantial research commitment. This Review discusses how microbes and the microbiota may contribute to cancer development and progression, responsiveness to cancer therapeutics, and cancer-associated complications.

Microbial contributions to carcinogenesis

Of the estimated 3.7×10^{30} microbes living on Earth (*7*), only 10 are designated by the International Agency for Cancer Research (IACR) as carcinogenic to humans (*1*). Although most of these carcinogenic microbes colonize large percentages of the human population, only a subset of affected individuals develop cancer, because host and microbial genotypes influence cancer susceptibility.

Tumors arising at boundary surfaces, such as the skin, oropharynx, and respiratory, digestive, and urogenital tracts, harbor a microbiota, which complicates cancer-microbe causality. Enrichment of a microbe at a tumor site does not connote that a microbe is directly associated, let alone causal, in disease. Rather, microbes may find a tumor's oxygen tension or carbon sources permissive and take advantage of an underused nutritional niche. Decreased abundances of specific microbes may also place a host at enhanced risk for cancer development at sites local or distant from this microbial shift. Thus, rigorous frameworks for interpreting tumor-associated microbiota data are essential (*2*).

Oncomicrobes, shifting the balance of when to die and when to grow

Bona fide oncomicrobes—microbes that trigger transformation events in host cells—are rare. Beyond the 10 IACR-designated microbes, there are a handful of other microorganisms with robust but fewer aggregate data supporting their role in human carcinogenesis. As many of these and their carcinogenic mechanisms have been recently reviewed (*2*–*6*, *8*), select activities representing common pathways by which microbes influence cancer will be highlighted.

Human oncoviruses can drive carcinogenesis by integrating oncogenes into host genomes. Human papillomaviruses (HPV) express oncoproteins such as E6 and E7. Data from recent genomic analyses of HPV^+ cervical cancers suggest that viral integration also selectively triggers amplification of host genes in pathways with established roles in cancer (*9*).

Microbes also drive transformation by affecting genomic stability, resistance to cell death, and proliferative signaling. Many bacteria have evolved mechanisms to damage DNA, so as to kill competitors and survive in the microbial world. Unfortunately, these bacterial defensive factors can lead to mutational events that contribute to carcinogenesis (Fig. 2). Examples include colibactin encoded by the *pks* locus [expressed by B2

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