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1) Baker, Monya. "1,500 scientists lift the lid on reproducibility." Nature, no. 533 (May 26, 2016): 452-54. doi:10.1038/533452a.

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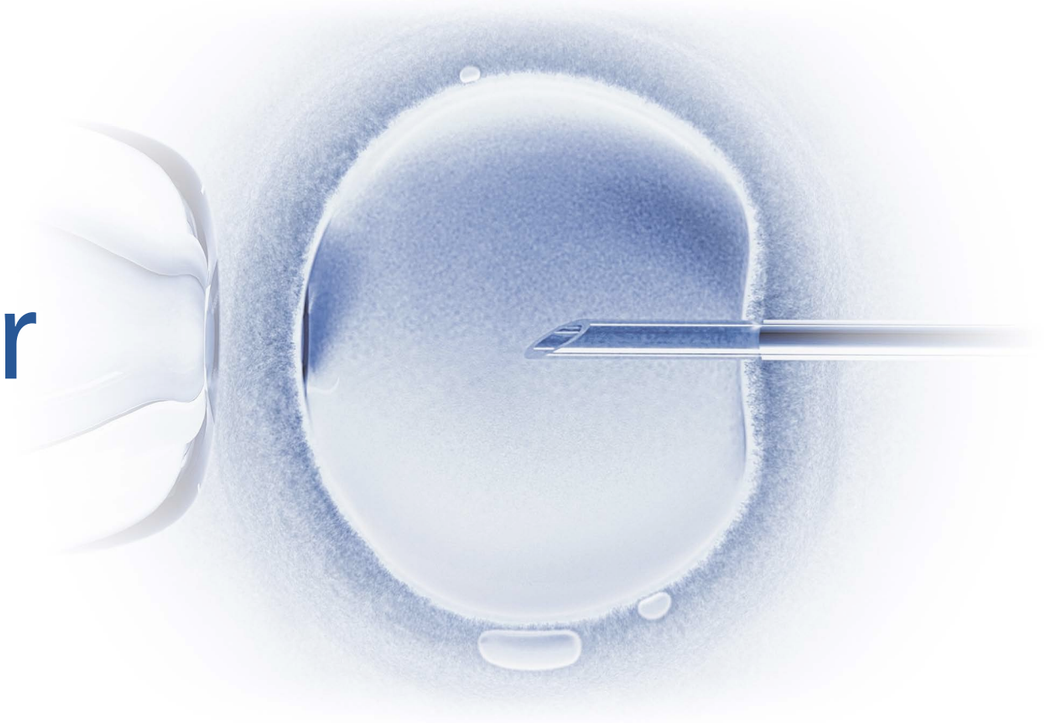

This White Paper outlines the importance of proper homeostasis maintenance in *in vitro* cell culture and oxygen levels *in vivo*.

Carl Radesovich,
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Mammalian Cell Culture and the Future of Medicine and Disease Control

Mammalian cell culture is an extremely valuable tool in the direct study of cells and tissue. Emerging regenerative medicine allows cell culture to be used as a direct therapeutic process for patients and others and serve as model systems *in vitro*. As cell culture techniques are refined, the need to simulate accurate *in vivo* conditions within an *in vitro* environment has become very important. This is especially true in applications involving stem cells, biotherapeutics and embryos.

Environmental deviations from the *in vivo* surroundings from which a particular cell is derived may lead to erroneous data, non-reproducible experiments and unwanted extensions in experimental and drug development processes.

Physiological Oxygen: Understanding Oxygen Levels *in Vivo*

Normoxia is the term most often used to describe atmospheric levels of oxygen. This

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range generally resides between 20 and 21% O_2 (160 mmHg), though most tissues do not experience oxygen levels at 20–21%.[1]

• In our lungs, oxygen levels are around 14.5% or as low as 3.4–6.8% in peripheral tissues. Physiological oxygen (2–10%) is therefore considered 'hypoxic' in respect to atmospheric oxygen.

• Most organs in the body, including the brain, liver, and pancreas, reside between 2 and 10% O_2 while certain tissues, such as the thymus and areas of the kidney, reside at oxygen concentrations lower than 1%.[2]

• Pathological tissue, like cancer, contains an oxygen gradient in which inter-cellular oxygen levels can reach below 1%.

• Hypoxic oxygen levels regulate a variety of important and normal pathological and physiological processes, such as cell differentiation, proliferation, wound healing and fetal development. Additionally, oxygen levels dictate essential events in early embryo development and stem cell niches.[3]

Table 1: Relative Oxygen from Ambient to Below Ambient

Oxygen	Description
21.0%	Oxygen in air at normal atmospheric pressure
19.9%	Oxygen in cell culture incubator operating at 5% CO_2
13.5%	Inspired oxygen pO_2 in alveoli. O_2 is affected by inflow and outflow of gases and water vapor
9.5%	Arterial blood oxygen concentration
6.5%	Approximate pO_2 at venous end or circulation

Importance of Homeostasis in Cell Culture

Maintenance of a successful *in vivo* environment within cell culture lies in the principal of properly maintaining cellular homeostasis. Cells require a delicate balance of temperature, pH, nutrients, and ions through an intricate network of cellular processes.[4]

In the early days of cell culture, scientists were able to successfully grow mammalian cells *in vitro* by exposing them to certain environmental elements that contribute to homeostasis. Today, most mammalian cells are maintained at CO_2 levels between 5–7% and supplied with a strict formulation of nutrients, pH buffers, and growth factors. These components have led to significant improvements in cell culture technology and healthier cell proliferation. Yet oxygen levels that are optimized for growth of specific cells are often overlooked.

Atmospheric air is comprised of 21% oxygen, 78% nitrogen, and 1% trace gases. Once breathed into the lungs, oxygen molecules are transported by the hemoglobin molecules on red blood cells through the bloodstream and carried to all organs in the body.

When a cell culture incubator is configured to create a typical 5% CO_2 atmosphere, O_2 levels are reduced

to 19.95%. Although this O_2 level is somewhat reduced, hypoxia is not achieved nor is it controlled to the point of reproducibility and continuity.

At the cellular level, *in vitro* oxygen plays a critical role in the regulation of a variety of cellular processes.[6] Within a given tissue, the oxygen level controls specific biological events, such as cell differentiation, cell proliferation and wound healing.

Conventionally, mammalian cells have been cultured at atmospheric oxygen levels (~20%) *in vitro*. Most mammalian cells, however, are derived from tissues that contain sub-atmospheric oxygen in the range of 2–11%.[7]

Table 2 – Known Percentages of Oxygen in Selected Human Tumors[5]

Tumor Type	Median Oxygen, Normal Tissue	Median Oxygen, Tumor
Brain	3.4%	1.7%
Head and Neck	5.3% to 6.7%	1.6% to 1.9%
Lung	5.6%	1.9% to 2.2%
Breast	6.8%	1.3%
Cervix	5.5%	1.2%
Liver	3.9%	1.8%
Pancreas		0.3%
Prostate	3.4% to 3.9%	0.3% to 1.2%
Vulva		1.3% to 1.7%
Melanoma	5.3%	1.5%
Renal	4.9%	1.3%
Rectal	6.8%	2.5% to 4.2%
Sarcoma	6.7%	1.8%

While *in vitro* cell culture remains a mainstay in the modeling of disease and physiological mechanisms, contemporary research demonstrates the detrimental effects of culturing mammalian cells at atmospheric oxygen levels.

Research has linked higher *in vitro* oxygen levels to more frequent chromosomal breaking, expression of 'stress response' genes, more oxidative damage and a weaker cell overall. Whether cultures are used for hosting or for expression of a cellular derived product, incompatible O_2 exposure impacts downstream results.

Oxygen Regulation of Hypoxic Inducible Factors in the Cell Culture Processes

Below ambient oxygen concentrations function as a signaling molecule for certain cellular events. Among the most significant cellular factor regulated by low oxygen concentrations is hypoxia-inducible factor (HIF).

HIF embraces a class of DNA-binding proteins and becomes activated at physiological oxygen levels. HIF regulates the transcription of a variety of genes critical for maintaining overall cellular

homeostasis and key pathways, such as angiogenesis, glycolysis, and erythropoiesis.

Genes regulated by HIF are important for improving cell survival and reducing cell stress which occurs physiologically during fetal development or ischemia.[8]

HIF also promotes normal physiological cell differentiation and proliferation by regulating the expression of genes that control cellular glucose uptake and metabolism. One gene, vascular endothelial growth factor (VEGF-A), is critical for the cellular process of angiogenesis (blood vessel growth) and is upregulated with the presence of HIF.

Blood vessel growth helps facilitate oxygen delivery to hypoxic tissues. VEGF also has a key role in fetal development where hypoxic microenvironments are typical.[9]

In addition, HIF has an important role in pathophysiologic or disease conditions, such as cancer, where abnormalities often create hypoxic states in certain tissues. Rapid cell division during tumor progression generates hypoxic conditions that lead to activation of HIF, which, in turn, initiates cell survival processes that maintain homeostasis and cancer growth (metastasis).[10]

Because physiological oxygen concentrations are critical for regulating a multitude of cell culture processes, atmospheric oxygen levels can create unintended side effects, such as reactive oxygen species (ROS) at higher oxygen concentrations.

ROS affect cellular processes by damaging DNA, modifying protein synthesis and function, and causing cell membrane instability.[11]

Cell cultures grown in atmospheric oxygen express a stress response gene signature which is different than cells cultured in physiological oxygen.

Consequently, using these cells for downstream cell culture experiments may have unpredictable results. Culturing cells at physiological oxygen levels brings cellular metabolism and homeostasis to a much closer *in vivo* level and activates key factors, such as HIF, which has been shown to promote cell adaptation and survival.

HIF in turn has a multitude of effects that allow cells to grow faster, live longer, stay healthier and have a more normal gene expression profile.[12]

In atmospheric oxygen, HIF is produced in low or undetectable numbers. Here, excess oxygen leads to detrimental cellular consequences, such as oxidative damage and upregulation of stress proteins.

In vitro Fertilization

With an increasing amount of research citing the benefits of culturing cells at non-atmospheric

Numerous studies have demonstrated that embryos cultured at elevated O_2 concentration levels are impacted negatively during their development, whereas culturing with low oxygen (5%) levels results in improved embryo quality.

oxygen levels, it is likely that physiological oxygen (~5%) and hypoxic (~0.1% to 2.5%) studies will increase in the future.

Researchers in the field of *in vitro* fertilization (IVF) have already realized the benefits of physiological oxygen and pioneered its use in their cultures.

Physiological oxygen is a stimulus for the expression of certain factors that play a role in embryogenesis. With the strict conditional nature of embryo development and host implantation for fertilization, IVF researchers demand an optimized *in vitro* environment for embryo cultivation.

Numerous studies have demonstrated that embryos cultured at elevated O_2 concentration levels are impacted negatively during their development, whereas culturing with low oxygen (5%) levels results in improved embryo quality.

Studies have also shown that 5% O_2 (or physiological oxygen) yields higher quality embryos, implantation and better pregnancy success than 20% O_2 (atmospheric oxygen) culturing.[13]

HIFs in Embryology

HIF is one of several factors important for the success of embryo development. During embryogenesis and organogenesis, there are many hypoxic microenvironments that form; HIF is expressed to adapt to these situations.
In one study, HIF-deficient embryos, a similar phenotype as those that might be cultured at atmospheric oxygen levels, had defects in blood vessel formation and neural-fold closure.[14] These results support the role that HIF plays in angiogenesis and cell proliferation.

Another study demonstrated the role of HIF in the proper formation of a placental architecture and its subsequent vascularization. Other studies have characterized the increased expression of HIF proteins during placental development.

Taking these studies into account suggests

that the hypoxic environment that embryos encounter normally *in vivo* is a critical factor for at least their cardiovascular-pulmonary development *in vitro*.

IVF researchers must reproduce an accurate *in vivo* environment for embryos. ROS is one variable that is diminished when culturing embryos at physiological oxygen levels.

Researchers have recognized that the deleterious effects of ROS on DNA, proteins and other biological molecules can significantly affect embryo development and eventual implantation or fertilization.[15]

Thus, growing embryos at physiological oxygen levels is beneficial for not only replicating appropriate *in vivo* cellular homeostasis, but also ensuring better integrity of the embryo's genetic profile. All of these benefits taken together can make a large difference in experimental results as well as implantation and fertilization success.

Table 3 – Contextual Oxygen and Terminology[16]

Oxygen	Pressure	Percentage	Scientific Term	Common Term
In Air	160 mmHg	21%	Atmospheric	Normoxic
pO_2 in vivo				
Lung	150 mmHg	2% to 10%	Patho-physiological	Hypoxic
Circulation	50 to 100 mmHg			
Tissue	20 to 50 mmHg			
Hypoxia	<15 mmHg	<2%	Physiologic	

Physiological Oxygen in Other Applications

The success of culturing embryos in IVF suggests that altering the oxygen levels in other applications will similarly improve cell health and growth.

The environmental principles that dictate cell differentiation and proliferation can be easily translatable to other research areas, such as stem cell research or cancer biology.

Oxygen is a factor in several metabolic and cellular pathways. Thus, to be truly comprehensive and to obtain the most relevant results, researchers studying mammalian cells should consider culturing their cells in physiologically relevant oxygen conditions.

Two major areas that have made significant headway in low oxygen studies are stem cell and cancer research.

Stem Cells

An understanding of physiological oxygen has become increasingly critical in stem cell research. Research has shown that long-term culture of stem cells at atmospheric oxygen gives rise to

chromosomal abnormalities as well as genomic and epigenomic aberrations.[17]

These mutations could lead to tumorigenesis or affected downstream results. Because many types of stem and progenitor cells often reside in a physiological microenvironment with oxygen between 1–5%, exposure to hyperoxic conditions may lead to deleterious effects.

When investigators seeking to corroborate this hypothesis cultured stem cells under physiological oxygen concentrations, the outcome was better genomic stability and maintenance of stemness in adult stem and embryonic stem (ES) cells.[18]

Physiological oxygen levels have also been shown to influence stem cell growth rates and differentiation. One study found that culturing rat bone marrow mesenchymal stem cells in 5% oxygen yielded increased proliferation and colony-forming ability than when cultured at 20% oxygen (atmospheric).[19]

Furthermore, ES cells revealed more robust growth under hypoxic oxygen conditions with a significant reduction in differentiation as compared to ES cells exposed to ambient air. Hypoxic conditions helped promote full pluripotency of embryonic stem cells.

In contrast, culturing rat peripheral and central nervous system stem cells at physiological oxygen levels promoted their differentiation into neurons. It appears that oxygen's modulation of stem cell fate is a complex mechanism dependent on the tissue. This data is an example of continuously evolving research that represents the role oxygen control plays in stem cell research.

While oxygen regulates stem cell differentiation and genomic integrity in ways still not completely understood, researchers have been studying the effects of oxygen via HIF. Results suggest that HIFs affect stem and progenitor cell differentiation. In one study, it was shown that trophoblast stem cell differentiation in placenta

The environmental principles that dictate cell differentiation and proliferation can be easily translatable to other research areas, such as stem cell research or cancer biology.

development was altered in HIF-deficiency in mice.[20]

The lack of HIF, which represents a phenotype similar to atmospheric oxygen, resulted in stem cells differentiating into another type of cell, e.g. trophoblast giant cells instead of spongiotrophoblasts. This study suggested that physiological oxygen levels (3% in this tissue) activated HIF, which in turn pushed placental cells to differentiate into a specific fate.

Cancer and Physiological Oxygen

Oxygen levels play a key role in pathophysiological studies. Viruses, cancer, and other disease states often create hypoxic microenvironments in tissues where HIF initiates complex interactions.

In a study seeking to understand the mechanism of neurotoxicity during HIV infection, cells cultured at physiological oxygen experienced cell death from HIV's neurotoxins, while those cultured at atmospheric oxygen did not.[21] This implies that the mechanism through which HIV killed cells relied heavily on the appropriate levels of oxygen. In another study, HIF via hypoxia activation was found to downregulate ß-catenin in the Wnt signaling pathway by competitively binding to ß-catenin resulting in cell-cycle arrest and inhibition of transcriptional activity.[22]

ß-catenin and Wnt signaling have critical roles in embryogenesis, but have also been implicated in cancer development and progression when improperly regulated. These studies suggest that oxygen and HIF levels have a role in many pathophysiological processes.

As more research is conducted to understand the mechanisms of cancer and other diseases, it is critical to examine how these cells are affected by oxygen concentrations.

To achieve the most relevant results, lessons learned from IVF can be applied to other areas, such as toxicology and cancer biology. Better understanding how oxygen factors into any biological mechanism is of supreme importance for further research development.

Conclusion

With current trends moving towards incorporating oxygen into investigations, particularly in applications such as IVF, stem cell and cancer biology, more investigators are examining options associated with conventional CO_2 incubators that extend to O_2 control as well.

Due to budget restrictions, the need for a cell culture incubator with convertible performance, from conventional CO_2 control to CO_2 and O_2 control is critical.

Advances in incubator design now include the addition of multiple gas control systems based on a new generation of dual-infrared CO_2 sensors, zirconia O_2 sensors and algorithms configured to assure quick recovery of desired atmosphere levels following door openings.[23]

Coupled with both active and passive contamination mitigation, along with a range of decontamination options to meet FDA criteria, the focus of contemporary cell culture research continues to narrow on cell specificity. ■

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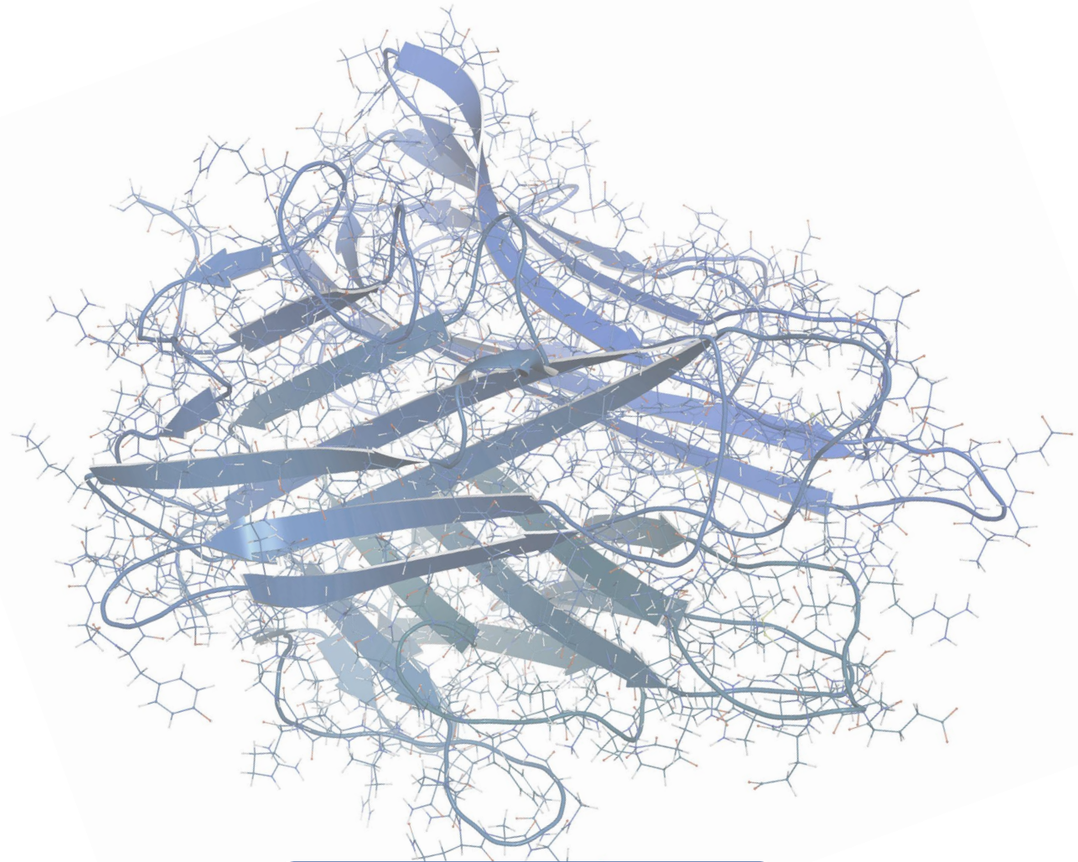
Immuno-Oncology Enhances Battlespace Awareness

Molecular Imaging Could Help Clinicians Accurately Select Patients for Treatment or Monitoring



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Ashley Jean Yeager

The immune system has two fundamental (and contrasting) jobs: spare normal cells (which carry self-antigens) and attack pathogenic cells (which carry nonself-antigens). Somewhere between normal cells and exogenous pathogens are cancer cells.

Although cancer cells are derived from normal tissues, they harbor DNA mutations that often result in tumor-specific molecules that can induce an immune response. Still, cancer cells often evade immune surveillance, as demonstrated by cancers that develop to the point of being clinically recognized, notes Lance Leopold, M.D., the group vice president for immuno-oncology development at Incyte.

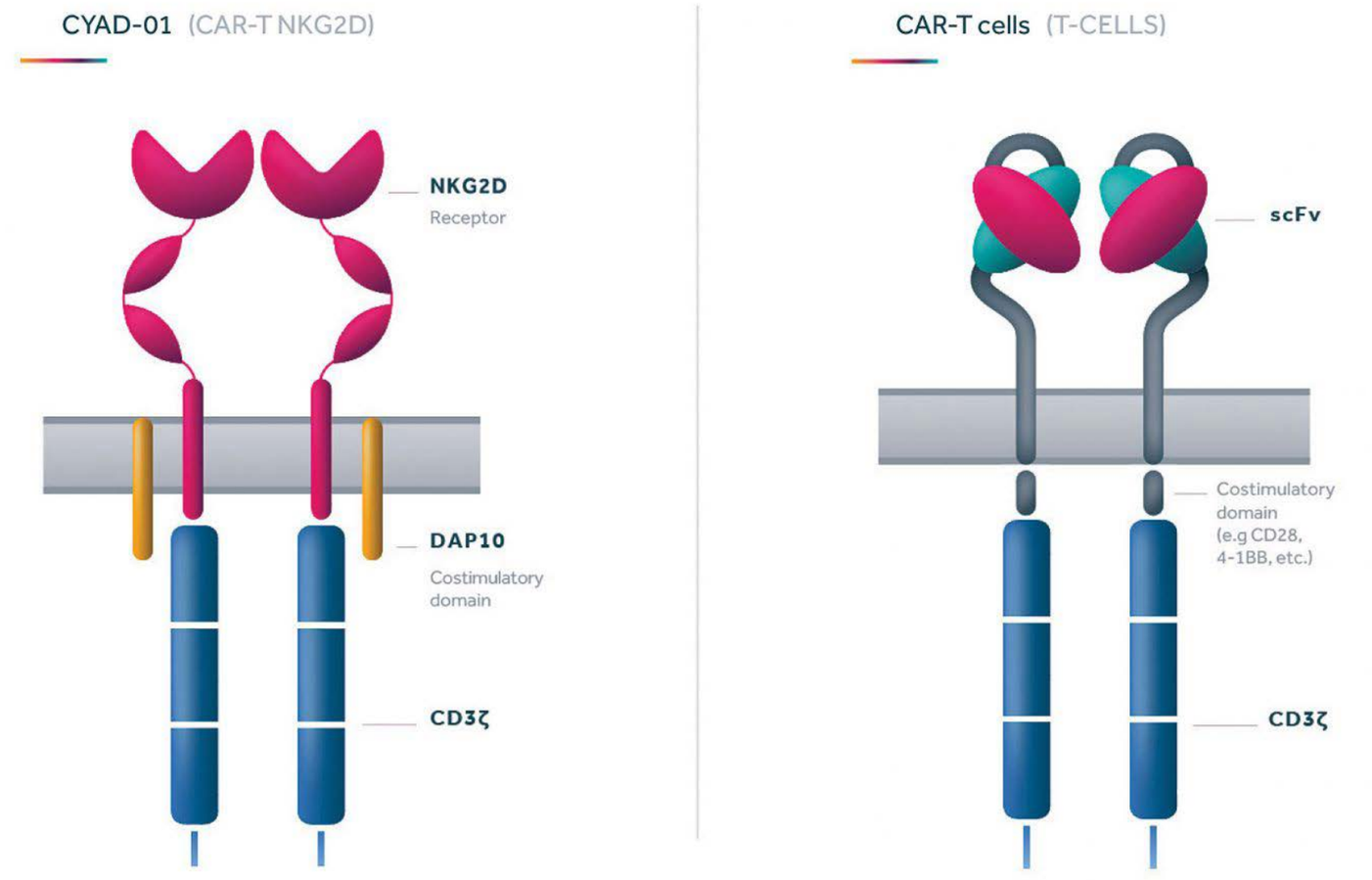
Scientists are now developing therapeutics to target the pathways that help cancer cells evade an immune response. From inhibiting key checkpoints that regulate immune activity, to developing new chimeric antigen receptor

T cells (CAR-T), to using molecular markers to predict responses to immunotherapies, researchers are working to make cancer treatments as effective as possible. Some of the work was presented at Immuno-Oncology 360°, a conference recently held in New York.

Turning T Cells against Cancer

A key immune checkpoint pathway, PD-L1/PD-1, now validated as a target for several approved therapies, regulates a T-cell surface receptor that inhibits T-cell activation. Targeting this interaction can coax T cells to attack cancer cells. That's the basis for Merck & Co.'s PD-1 inhibitor Keytruda® (pembrolizumab), which was approved in 2014 by the FDA and is now used to treat forms of lung cancer and bladder cancer along with melanoma and classical Hodgkin lymphoma. While this drug has proven useful for treating canacer patients, it is effective in only a subset of patients, leaving researchers looking for other pathways to target in combination with the PD-1 inhibitors.

Indoleamine-pyrrole 2,3-dioxygenase 1 (IDO1) is another regulator of immune-cell activity. It



CYAD-01 (CAR-T NKG2D) is the lead CAR-T cell approach that is being developed by Celyad. The technology is based on preclinical work carried out by Professor Charles Sentman at Dartmouth College (USA), who demonstrated that T cells engineered to express the Natural Killer Receptor Group 2D (NKG2D) receptor fused with the CD3ζ chain of the T cell receptor complex can drive impressive antitumor activity against established tumors in mouse models.

was first discovered to have a potent role in immune tolerance between a mother and fetus. This enzyme is commonly expressed and results in the depletion of a key nutrient required for T-cell activation and function, tryptophan, Dr. Leopold says. Inhibiting IDO1 reverses the inhibition of T cells, facilitating immune surveillance and tumor destruction.

The inhibition of IDO1 is the goal of epacadostat, an investigational drug developed by Incyte. The company has been running a Phase III trial to evaluate the efficacy, safety, and tolerability of a combination of epacadostat and pembrolizumab in patients with metastatic melanoma.[1]

Based on other trial response rates in patients, Dr. Leopold says the company has "hope and optimism" that the combination treatment will produce results superior to those obtained with pembrolizumab alone. Data from the Phase III clinical trial should be available later this year.

A Novel, Nonspecific CAR-T

NKG2D ligands are proteins that are absent from, or present only at low levels on, the surface of normal cells, but they are overexpressed by infected, transformed, senescent, or stressed cells. Researchers at Celyad, building off work by Dartmouth researcher Charles L. Sentman, Ph.D., are testing CAR-T cells with engineered NKG2D receptors to see if the modified cells can recognize and latch on to any of the eight NKG2D ligands expressed by stressed cells.

By fusing an NK cell-surface receptor—the Natural Killer Group 2D protein—to the CD3ζ cytoplasmic domain of the T-cell receptor, the researchers have developed a novel CAR T cells outfitted with this CAR could attack multiple types of cancer, says David Gilham, Ph.D., the vice president for research and development at Celyad, based in Belgium.

What could be quite advantageous about this approach is that other cell types in the tumor environment will also signal they are stressed, so the therapy could target these cells as well, making it harder for the tumor to survive. The downside, Dr. Gilham notes, is that many healthy cells may have these stress surface ligands expressed as well, so the scientists have to be very careful to ensure those cells aren't being targeted and killed by the CAR-T cells, too.

In Phase I clinical trials,[2,3] the treatment has proven to be safe, suggesting the CAR-T cells are attacking the cancerous cells and not healthy ones. Patients received no pretreatment with chemotherapy drugs before the infusion of the CAR-T cells, which is usually standard protocol.

Dr. Gilham states that research in animal models had suggested that this kind of pretreatment was not necessary, so researchers have been treating cohorts without it. The goal, he continues, is to test the CAR-T cells' safety first and foremost. And, he adds, the researchers want to ensure any effects of the treatment are due to the CAR-T cells themselves, not something administered at the same time as the CAR-T construct.

Preconditioning treatments may be added in future trials. "We're very cautious about how to proceed," Dr. Gilham admits. "But we're encouraged by what we've seen to date."

Determining Whether Patients Will Benefit

Immunotherapy has revolutionized how doctors treat the disease. However, not all patients respond to the new therapies. Each individual's immune system is wired slightly differently. Consequently, some people are more sensitive to cancer while others are less so. Recognizing these differences in sensitivity, researchers are now looking for biomarkers that can predict which patients will respond to treatment.

The first biomarker assays approved by the FDA targeted PD-L1 expression, but because tumor biology and immunity are complex, a single type of biomarker is not sufficient. That has led investigators to conduct biomarker investigations spanning protein expression, genomics, and transcriptomics. For example, researchers are looking at the correlation between genomic markers and PD-L1 expression.

Maher Albitar, M.D., senior vice president, chief medical officer, and director of research and development at NeoGenomics Laboratories, is leading a study of genes implicated in non-small cell lung cancer (NSCLC) and colorectal cancer. Dr. Albitar's group is evaluating whether these genes, which include *FGFR1, FGFR2, FGFR3, SRC, JAK3, ERBB4, ERBB2, TP53, and SMAD4,* correlate with PD-L1 expression.

The investigators found that PD-L1 expression is significantly lower in colorectal cancer compared with *NSCLC*. They also determined that in *NSCLC* patients, *PD-L1* expression is significantly higher in tumors with mutated *TP53*. So, there's a correlation between *TP53* mutation in *NSCLC* and the expression of PD-L1, but not in colorectal cancer, even though the tumor types had similar rates of *TP53* mutation.

"This suggests a possible difference in the mechanism of regulating PD-L1 expression between the two tumor types," the team wrote in a poster[4] available on the company's website. Negative correlations between PD-L1 expression and *EGFR* in lung cancer and *BRAF* in melanoma have also been identified, along with positive correlations between PD-L1 expression and Met amplification in lung cancer and between PD-L1 expression and the lack of TP53/Ras mutations in colorectal cancer, Dr. Albitar notes in a related webinar.[5]

Biomarkers Through Imaging

Molecular imaging is a noninvasive way to evaluate tumors and, as a result, could help clinicians accurately select patients for immuno-oncology treatments or monitor their responses. Current imaging technology falls short of this promise, though. It does not allow doctors to evaluate trafficking of activated T cells into tumors or how treatment affects the tumor.

At Imaging Endpoints, Ronald L. Korn, M.D., Ph.D., the company's founder, chairman, and chief medical officer, has been working with colleagues to integrate positron emission

tomography (PET), magnetic resonance imaging (MRI), and other techniques into a system that can quantify how well cancer treatments work. In one study, the team examined whether features of ferumoxytol (FMX) iron nanoparticles in tumors, identified by quantitative MRI, may predict tumor lesion response to the FDA-approved nanoliposomal irinotecan treatment for pancreatic cancer.

In tumors, the uptake of FMX nanoparticles is similar to the uptake of the drug, suggesting that the FMX nanoparticles could offer a way to monitor how well the drug shrinks tumors. In *Clinical Cancer Research*,[6] Dr. Korn and colleagues report, "higher FMX levels were associated with greater reduction in lesion size after nal-IRI treatment," suggesting that quantitative FMX-MRI "may serve as a predictive biomarker for nanoparticle-based drug delivery."

PET imaging with 2-deoxy-2-[fluorine-18]fluoro-D-glucose integrated with CT scanning, called 18F-FDG PET/CT, may also be an option. Study results show that such imaging can be useful for tracking tumors in advanced pancreatic cancer patients treated with the chemotherapy drugs nab-paclitaxel plus gemcitabine.

PET/CT imaging successfully tracked changes in tumor metabolic activity at 6 and 12 weeks, the researchers report, noting that PET analysis could be used to determine how effective other investigational agents are in treating advanced pancreatic cancer.[7] The team is working on others too, such as the anticancer agent RRx-001 in conjunction with the MRI contrast agent, Gd-LC7-SH.[8]

"It's exciting times for the whole field," Dr. Gilham says. ■

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Cell Therapy Production Moves to Factory Floor

Assembly Lines Are Preparing to Churn out Safe, Reliable, and Affordable Constructs

Meghaan M. Ferreira, Ph.D.

Brown hair frames the face of a vibrant, rosy-cheeked girl who has two reasons to consider the day, May 10, 2017, a good day to celebrate. One reason is no more remarkable than another birthday, her 12th. The other reason, however, is worthy of all the news coverage it received. A chalkboard held by the girl, one Emily Whitehead, said it all: "I am 5 years cancer free!"

Emily survived a terminal diagnosis of relapsed or refractory acute lymphoblastic leukemia (r/r ALL) after receiving CAR-T (chimeric antigen receptor T-cell) therapy during Phase I trials at the Children's Hospital of Philadelphia. Her miraculous story has inspired hope and fortified the campaign for cell therapies.

The year 2017 marked a milestone not just for Emily but for the cell therapy industry, as the FDA approved its first CAR-T therapy and Novartis launched Kymriah™ commercially. According to Pascal Touchon, senior vice president and global head, cell and gene, Novartis Oncology, "Delivering this therapy to patients

4X-image/Getty Images

Novartis declares that its Kymriah suspension for intravenous transfusion is the first FDA-approved chimeric antigen receptor T-cell (CAR-T) therapy. Kymriah, which was granted priority review for treatment of adult patients with relapsed or refractory diffuse large B-cell lymphoma, is manufactured at Novartis' Morris Plains, NJ, facility. Each dose uses the patient's own cells.

required an entirely new approach to both the supply chain and manufacturing."

Regenerative medicine has shifted the health-care paradigm away from alleviating symptoms and toward potentially curative approaches that address the underlying disease, but the continued success of these revolutionary therapies relies on establishing new manufacturing paradigms. Manufacturing cell therapies safely, reliably, and affordably at a commercial scale will require ingenuity in the design of custom equipment, facilities, and workflows.

Scalable Solutions

"When stem cells were discovered, there was a surge of hope," said Nina Bauer, Ph.D., associate director, commercial development, Lonza, as she chronicled the evolution of cell therapies, which began as an endeavor to cure diseases like Alzheimer's and Parkinson's. While the industry has made little headway against these giants, it has demonstrated the potential for allogeneic stem cell therapies to treat, and perhaps cure, a variety of other diseases like macular degeneration and type 1 diabetes.

Although allogeneic stem cell therapies, which use the same product batch to treat multiple patients, allow a traditional scaleup manufacturing model, expanding these sensitive cells to create a sufficient quantity for therapeutic applications while maintaining viability, pluripotency, and functionality presents a major challenge.

"The cells are very sensitive," explained Antoine Heron, Ph.D., head of cell and gene therapy commercial development, MilliporeSigma. "Very limited changes in your process could lead to differentiation of the cells, and then you will lose your product."

Over the past two years, Dr. Heron and Frank Edenhofer, Ph.D., a professor of anatomy and cell biology at the University of Würzburg, have collaborated on a scalable solution for manufacturing induced pluripotent stem cells (iPSCs). After culturing iPSCs for two weeks in a 3-L bioreactor, they achieved a 125-fold increase in cell number—essentially doubling the process yields reported in

GE Healthcare and CCRM (Centre for Commercialization of Regenerative Medicine) scientists explain GE's cell processing equipment while giving a tour of CCRM's laboratories in Toronto, Canada. Funded by grants totaling $40 million from GE Healthcare and Canada's FedDev program, the facilities substantiate Toronto's bid to become "Stem Cell City."

the literature with a final yield of 2 billion cells/L. Further characterization by immunocytochemistry, flow cytometry, karyotype analysis, and differentiation into all three germ layers confirmed that the human iPSCs retained their pluripotency and normal karyotype after expansion.

Their innovative strategy eliminates microcarriers or special surface coatings by culturing the iPSCs in small cell aggregates formed from single cells. They maintained cell viability and functionality by optimizing critical parameters, like aggregate size and agitation rate, to ensure delivery of oxygen and essential nutrients while also minimizing shear stress. According to Dr. Heron, their work "suggests that iPSCs can be cultured in stirred suspension for at least 49 days, possibly even longer, while remaining remarkably viable and pluripotent, which is definitely a strong foundation for a scalable manufacturing process."

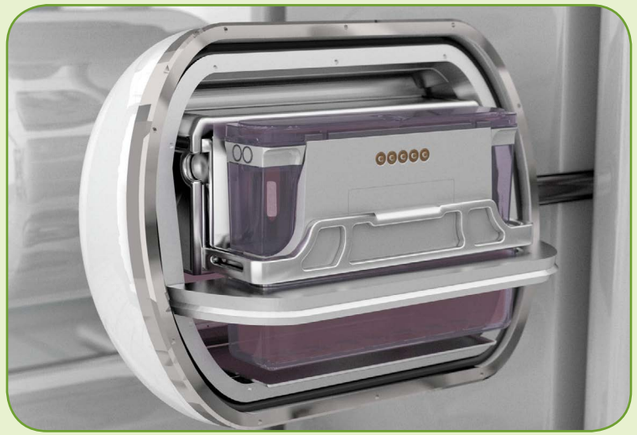
Taking the World by Storm

While allogeneic therapies follow a traditional scaleup model, albeit at a smaller scale than conventional biologics, autologous therapies, which are customized for each patient, require an unconventional "scale out" model. "From a manufacturing standpoint, producing autologous therapies at scale seemed ridiculously challenging," remarked Phil Vanek, general manager, cell therapy growth strategy, GE Healthcare Life Sciences.

The apprehension around manufacturing custom therapies dissipated when CAR-T therapies, based on pioneering research conducted by Carl H. June, M.D., at the University of Pennsylvania, entered the clinic as a cancer treatment.

"These therapies were taking the clinical world by storm," recalled Dr. Vanek. "Patients that typically had a less than 10% chance of survival suddenly had remission rates above 80%. It was that clinical success that made the entire industry take note and say, 'okay, if these therapies really work, how can we manufacture them for everyone who needs them?'"

Bridging the gap between scientific discovery



Cocoon technology, currently under development by Lonza in collaboration with Octane Biotech, is based on an automated GMP-in-a-box concept for autologous cell therapy manufacturing. This image zooms in on the Cocoon instrument, which houses a patient-specific, presterilized, disposable cassette that provides a system of interlinked modules for processes such as seeding, expansion, feeding, and harvesting.

and industrial manufacturing meant tackling the labor-intensive, multistep workflow used to genetically modify patients' T cells with a new *modus operandi*. GE Healthcare's strategy simplifies this workflow by connecting multiple unit operations using semiautomated subroutines to increase overall efficiency and reduce the need for manual intervention.

In addition to offering semiautomated, closed systems for cell isolation, expansion, and harvesting, GE Healthcare's portfolio includes an integrated end-to-end solution for cryogenically preserving cell therapies with Asymptote cryopreservation technology that includes VIA Freeze™ controlled rate freezers, automated thawers, and my.Cryochain software.

This past January, Cellular Biomedicine Group, a clinical-stage biopharmaceutical firm, announced its plans to configure part of its facility in Shanghai using GE Healthcare's FlexFactory platform to commercialize its CAR-T therapies. According to Ger Brophy, Ph.D., general manager, cell therapy, GE Healthcare, the scalable, start-to-finish cell therapy manufacturing platform could cut in half the 18 months it typically takes a company to prepare a facility.

In addition to equipment, GE Healthcare's FlexFactory provides logistical solutions like Fast Trak training programs. Developed in collaboration with the Centre for Commercialization of Regenerative Medicine (CCRM), these programs aim to get staff up and running as quickly as the facility itself.

Anticipating a Metamorphosis

According to Dr. Bauer, autologous therapies now constitute approximately 60% of the cell therapy industry. Lonza responded to these turning tides by partnering with Octane Biotech, a regenerative medicine company, in 2015. Octane's automated bioreactor, Cocoon™, performs core functions necessary for autologous cell therapy production, including cell seeding, expansion, feeding, and harvesting. "We call it a GMP-in-a-box system," said Dr. Bauer.

Since adopting the technology, Lonza has worked with Octane to integrate additional

unit operations, such as magnetic bead separation and electroporation, into the all-in-one production platform. Cocoon controls gas and temperature levels for the cells, which are introduced into a plastic cassette that slides into the bioreactor. The automated system moves cells between multiple chambers that perform separate steps of the production workflow inside the sterile cassette.

Sterile, closed environments are paramount for cell therapy manufacturing, but it's not just adventitious bacterial or viral contamination that can have serious, even life-threatening, consequences. Cross-contamination from other cell therapies produced in the same facility could also adversely affect patient safety. One strategy to reduce the risk for cross-contamination segregates autologous therapies during manufacturing using small, individual manufacturing suites, but this space-intensive approach requires larger, more expensive facilities.

The closed, single-use, disposable cassettes at the nucleus of Cocoon provide a segregated, self-contained system that minimizes the risk of cross-contamination without requiring individual manufacturing suites. In addition, Octane has designed rotating stands, nicknamed "trees," that hold 8–10 Cocoon bioreactors at once. The overall design resembles a futuristic orchard for regenerative medicine with rows of stands suspending the pod-shaped bioreactors. The small footprint, approximately 9 square feet per tree, enables production of up to 10 therapies simultaneously, potentially making its space-efficient design one of the technology's biggest virtues.

Is Hope Enough?

Whether it's enabling a patient's T cells to recognize tumor antigens or replacing dysfunctional beta islet cells, regenerative medicine promises more than disease management—it promises cures. In addition, according to Dr. Bauer, "It's the curative aspect that brings a lot of hope to the cell therapy space." However, the complexities involved in designing, manufacturing, and administering these therapies to patients at a commercial scale begs the question: Is hope enough?

"These are probably some of the most complex products that we've dealt with over the years," commented Carl Burke, Ph.D., new platform integration, pharmaceutical development and manufacturing sciences, Janssen Research & Development. In December, 2017, Janssen announced its intent to develop, manufacture, and commercialize a CAR-T therapy for multiple myeloma in collaboration with Chinese company Legend Biotech.

Janssen has developed custom equipment and disposables to close and automate their cell-manufacturing processes, but equipment constitutes only a portion of the overall costs and logistical challenges involved in cell therapy manufacturing. Dr. Burke also emphasized the importance of developing close partnerships with suppliers to ensure both the quantity and quality of materials, including custom disposables, custom media, and viral transduction vectors, as well as collaborating with healthcare facilities to make sure they have the appropriate infrastructure to receive, store, and track these sensitive, expensive therapies once they reach the hospital dock.

While the approval and commercial launch of the first CAR-T therapies, Kymriah™ (Novartis) and Yescarta™ (Gilead Sciences), marked an important milestone for the cell therapy industry, the $475,000 price tag for a one-time dose of Kymriah caused some sticker shock.

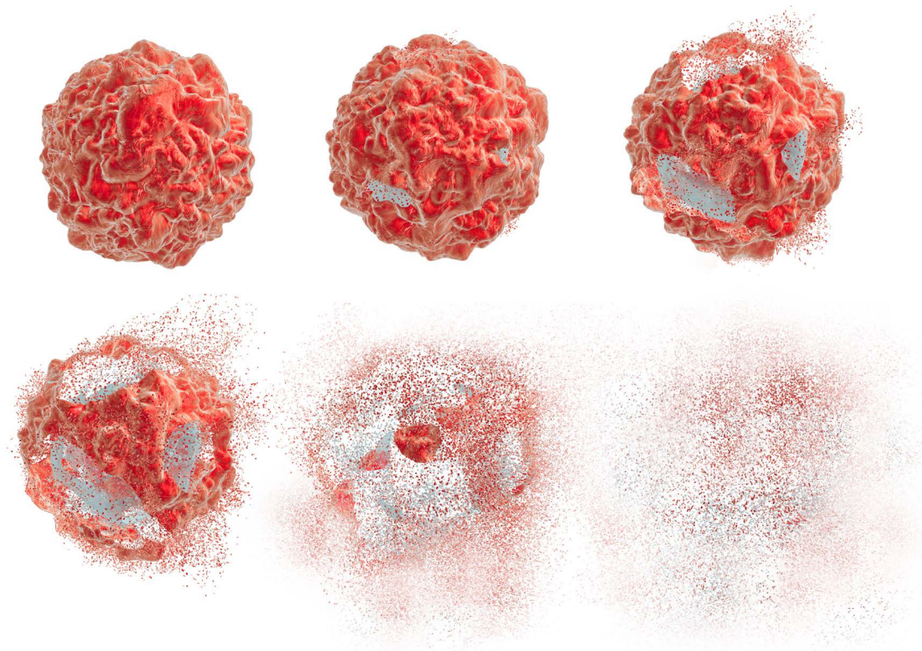
"Ensuring that patients have access to these therapies once they are approved by regulatory authorities is paramount," said Sanjaya Singh, Ph.D., global head, Janssen BioTherapeutics, Janssen Research & Development. "The healthcare community—industry, payers, providers—all need to work together to figure out how best to create a sustainable environment that supports patients and further innovation."

In an effort to improve patient access, Novartis has contracted an outcomes-based pricing approach for Kymriah with the Centers of Medicare and Medicaid. In the unprecedented arrangement, Novartis will receive payment only if the patient responds to treatment within one month, and since Novartis' price only applies to its current indication for children and young adults with r/r ALL, the company could initiate indication-specific pricing in the future. These pricing models could set the precedent for future CAR-T therapies.

Increasing the market size for cell therapies would allow manufacturers to implement more efficient and cost-effective strategies. Novartis estimates that only 600 patients per year will be eligible to receive treatment for the current indication, but the company has studies underway to assess Kymriah's effectiveness in diffuse large B-cell lymphoma and r/r follicular lymphoma. They also have several CAR-T therapies in development for glioblastoma, ovarian cancer, and mesothelioma, and demonstrating clinical success in solid tumors is considered the next big milestone for the industry.

"I am 5 years cancer free." Emily's chalkboard is emblematic of the hope inspired by CAR-T therapies. While the clinical success of cell therapies has taken the world by storm, delivering these therapies to the world will require a miraculous story of manufacturing ingenuity. However, as philosopher Bernard Williams once said, "There was never a night or a problem that could defeat sunrise or hope." ■

Local Immunotherapy Shrinks Tumors Near and Far



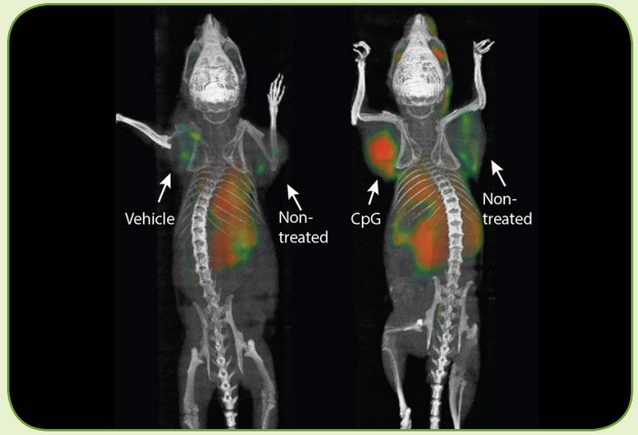
Inject locally, treat globally. That's the gist of a new cancer immunotherapy strategy called *in situ* vaccination. It has three steps: (1) Deliver immunoenhancing agents to a tumor site. (2) Trigger an immune response among "prescreened" T cells, that is, T cells that have already shown that they can infiltrate the tumor. (3) Watch the T cells attack cancer throughout the body.

In situ vaccination was developed by Stanford University scientists who believe that their approach, which involves local application of tiny amounts of immunoenhancing agents, could serve as a rapid and relatively inexpensive cancer therapy. These agents? An innate immune system activator called CpG, and a stimulatory antibody called anti-OX40.

The scientists, led by oncologist Ronald Levy, M.D., also emphasize that their approach is unlikely to cause the adverse side effects often seen with body-wide immune stimulation.

"When we use these two agents together, we see the elimination of tumors all over the body," said Dr. Levy. "This approach bypasses the need to identify tumor-specific immune targets and doesn't require wholesale activation of the immune system or customization of a patient's immune cells."

KATERYNA KON/Science Photo Library/Getty Images



Scientists devised a pinpointed immunotherapy regimen—intratumoral delivery of a TLR9 ligand with OX40 activation to ramp up T-cell responses—that eradicated tumors throughout the body in mice. [Sagiv-Barfi et al., *Science Translational Medicine*, 2018]

Details about the new approach appeared January 31 in the journal *Science Translational Medicine*, in an article entitled "Eradication of Spontaneous Malignancy by Local Immunotherapy." The article describes how *in situ* vaccination was tested in mice with transplanted lymphoma tumors in two sites on their bodies. Just one tumor was injected with the two test agents, but both the treated and the untreated tumors regressed.

"...The combination of unmethylated CG–enriched oligodeoxynucleotide (CpG)—a Toll-like receptor 9 (TLR9) ligand—and anti-OX40 antibody provided the most impressive results," wrote the article's authors. "Low doses of CpG injected into a tumor induce the expression of OX40 on CD4+ T cells in the microenvironment in mouse or human tumors. An agonistic anti-OX40 antibody can then trigger a T cell immune response, which is specific to the antigens of the injected tumor."

In 87 of 90 mice, the cancers were eradicated. Although the cancer recurred in three of the mice, the tumors again regressed after a second treatment. The researchers saw similar results in mice bearing breast, colon, and melanoma tumors.

"I don't think there's a limit to the type of tumor we could potentially treat, as long as it has been infiltrated by the immune system," Dr. Levy stated.

Some immunotherapy approaches rely on stimulating the immune system throughout the body. Others target naturally occurring checkpoints that limit the anticancer activity of immune cells. Still others, like the chimeric antigen receptor (CAR) T-cell therapy recently approved to treat some types of leukemia and lymphomas, require a patient's immune cells to be removed from the body and genetically engineered to attack the tumor cells. Many of these approaches have been successful, but they each have downsides—from difficult-to-handle side effects to high cost and lengthy preparation or treatment times.

"All of these immunotherapy advances are changing medical practice," Levy noted. "Our

approach uses a one-time application of very small amounts of two agents to stimulate the immune cells only within the tumor itself. In the mice, we saw amazing, body-wide effects, including the elimination of tumors all over the animal."

Cancers often exist in a kind of immunological limbo. Immune cells like T cells recognize the abnormal proteins often present on cancer cells and infiltrate to attack the tumor. However, as the tumor grows, it often devises ways to suppress the activity of the T cells. Dr. Levy's method works to reactivate the cancer-specific T cells by injecting microgram amounts of two agents directly into the tumor site.

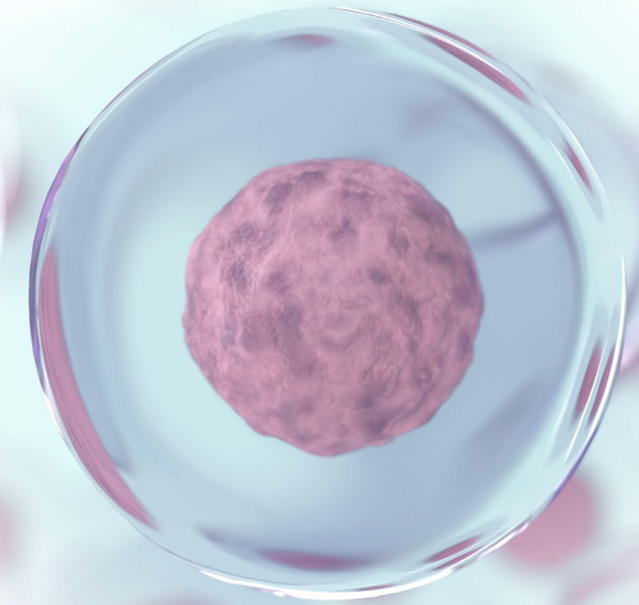
"This is a very targeted approach," Dr. Levy explained. "Only the tumor that shares the protein targets displayed by the treated site is affected. We're attacking specific targets without having to identify exactly what proteins the T cells are recognizing."

To explore the specificity of the T cells stimulated by *in situ* transplantation, the study's lead author, Idit Sagiv-Barfi, Ph.D., transplanted two types of tumors into the mice. She transplanted the same lymphoma cancer cells in two locations, and then she transplanted a colon cancer cell line in a third location. Treatment of one of the lymphoma sites caused the regression of both lymphoma tumors but did not affect the growth of the colon cancer cells.

One of the agents is currently already approved for use in humans; the other has been tested for human use in several unrelated clinical trials. A clinical trial was launched in January 2018 to test the effect of the treatment in patients with lymphoma.

The current clinical trial is expected to recruit about 15 patients with low-grade lymphoma. If successful, Levy believes the treatment could be useful for many tumor types. He envisions a future in which clinicians inject the two agents into solid tumors in humans prior to surgical removal of the cancer as a way to prevent recurrence due to unidentified metastases or lingering cancer cells, or even to head off the development of future tumors that arise due to genetic mutations like *BRCA1* and *2*. ■

CAR-Engineered Blood Stem Cells Offer New Hope for Long-Term HIV Gene Therapy



Researchers in the U.S. have created chimeric antigen receptor (CAR)-engineered hematopoietic stem/progenitor cells (HSPCs), which successfully engrafted in pigtail macaques infected with simian human immunodeficiency virus (SHIV) and generated virus-targeting cytotoxic T cells that destroyed infected cells. Reporting in *PLOS Pathogens*, the researchers, from the University of California, Los Angeles (UCLA), the Fred Hutchinson Cancer Research Center, and the University of Washington, say that that the CAR-engineered HSPCs persisted in the animals for more than two years, suggesting that their approach could successfully be used to treat infections such as HIV that can re-emerge after many years of suppression or dormancy. "This demonstrates for the first time the safety and feasibility of HSPC-based therapy utilizing an HIV-specific CAR for suppressed HIV infection," they write. Their published paper is entitled "Long-Term Persistence and Function of Hematopoietic Stem Cell-Derived Chimeric Antigen Receptor T Cells in a Nonhuman Primate Model of HIV/AIDS."

CAR T cells represent a "powerful immunotherapy" for treating different forms of cancer and have shown promise as an approach for treating

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Previous studies by Dr. Kitchen's team using humanized mice had demonstrated that HSPCs modified with a protective CD4 CAR could successfully differentiate into CD4CAR-expressing T cells and suppress HIV.

HIV infection, notes senior UCLA author Scott G. Kitchen, Ph.D., and colleagues. However, current methods have focused on engineering peripheral T cells, which may not persist over the long term and so fail to tackle cancers or infections that can reappear months or years after initial treatment. In contrast, HSPCs can successfully engraft in recipients over long periods of time. Thus, HSPCs engineered to carry HIV-targeting CARs could feasibly provide a lifetime supply of CAR T cells that continue to tackle any re-emerging infection. "Stem cell-based expression of CARs contributes a long-lived source of these cells, capable of providing lifelong immune surveillance against recrudescent virus," the authors comment. "In contrast, adoptive transfer of CAR-modified T-cell products must overcome barriers including immune exhaustion, limited trafficking to tissues, and lack of functionality at these sites." An additional benefit of CAR-based therapy using modifying HSPCs is that the engineered blood-forming cells will undergo normal T-cell differentiation and selection, "eliminating potentially self-reactive T cells and increasing the potential for the development of immunological memory."

HIV infects immune cells by interacting with the host cell's CD4 molecule. Previous studies by Dr. Kitchen's team using humanized mice had demonstrated that HSPCs modified with a protective CD4 CAR could successfully differentiate into CD4CAR-expressing T cells and suppress HIV, "suggesting a high degree of feasibility in redirecting immunity with an HSPC-based approach." For their primate studies, the researchers engineered HSPCs taken from pigtail macaques with a protective CD4 chimeric antigen receptor (C46CD4CAR), which effectively "hijacks the essential interaction between the virus and the cell surface molecule CD4," to direct HSPC-derived T cells against the infected cells. The engineered HSPCs were then reimplanted back into the same host animals, which were subseqently infected with SHIV. Twenty-four weeks following infection, the animals were given combination antiretroviral therapy (cART) for another 28 weeks to suppress the virus, after which the cART was withdrawn.

The studies confirmed that the CAR gene-modified HSPCs successfully engrafted in the primates and

persisted for nearly two years. The CAR-expressing HSPCs also expanded in response to SHIV infection and differentiated into SHIV-targeting effector T cells. Interestingly, the level of CAR T-cell response mirrored the SHIV antigen level. "Intriguingly, we found that CAR cells contracted during cART treatment during lower levels of antigen expression, followed by a rapid expansion after cART withdrawal, mimicking a memory response," the team writes. "As a result, CAR animals had decreased viremia during post-cART viral rebound as compared to control animals."

Encouragingly, the CAR cells were found in multiple lymphoid tissues, including those of the gut, which is a major site for HIV replication and persistence, and there was no evidence of toxicity associated with the treatment. Compared with control animals, macaques transplanted with the autologous CAR-engineered HSPCs exhibited much lower SHIV mRNA levels in the lymph nodes, gut, and brain. "These results set the stage for future attempts to eradicate viral infection and provide more effective immune surveillance for HIV, using optimized CAR vectors and combinatorial approaches," the team notes. " … we demonstrate that HIV/SHIV-specific CAR cells possess strong antiviral activity even at low levels....These cells should act as sentinels, generating a robust immune response to reactivated infected cells months or years after they are introduced, without requiring expression in a high percentage of immune cells."

The researchers say that while their studies could also lay the foundation for the development of CAR-engineered HSPCs against other diseases, "Importantly, these findings have broad implications beyond HIV. Additional preclinical studies should be performed to explore HSPC-expressed CARs against other infectious diseases and cancer in greater detail." ■

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