

Insights regarding the structure of a protein called Nef (blue) have helped Thomas Smithgall's team understand how it attaches to cellular proteins and turns them on. Smithgall is hopeful that blocking Nef could help stop HIV from reemerging in patients.



A TEAM HUNTS DOWN  
LATENT HIV  
BY GAVIN JENKINS

# NEF BUSTERS

**J**ohn Alvarado is pretty good at scooping up salt grain-size Nef protein crystals. This task is like fishing out leaves from a swimming pool—except, well, a lot harder. Alvarado’s “skimmer” is a metal rod, about as big as a pen, with a 20-micrometer diameter hoop attached; he needs to magnify the tip to even see his hoop. Alvarado is a PhD research assistant professor of microbiology and molecular genetics at the University of Pittsburgh and an x-ray crystallographer. When he was a grad student at Purdue University, he made his own tool by supergluing nylon loops to rods.

This meticulous and usually grueling approach to studying proteins is not a job for over-caffeinated scientists with jittery hands. The crystalline nuggets can stick to the bottom of the slide or slip around in the solution.

Even with more than 15 years of experience, Alvarado might take eight hours to capture 20 crystals. He estimates that 10 percent of the crystals he harvests are physically damaged and not suitable for the next step—shooting x-rays through the crystals to see the diffraction patterns.

X-ray crystallography gives scientists a picture that offers clues to an atomic protein structure. Imagine, you aren’t shown a prism, but you are shown the pattern of light that emerges after a lightbeam is shone through the prism. From that pattern, you are expected to figure out the shape of the prism. That’s akin to what x-ray crystallographers do as they solve the structure of proteins.

The outermost surface of a protein creates an electron cloud that diffracts or scatters x-rays. (The more ordered the protein molecules packed inside the crystal, the higher the resolution of the diffracted rays—and that means more structural clues are revealed.) By applying the diffraction data, scientists can connect the dots about protein structure by determining where each amino acid must be.

Alvarado’s expertise in this field has made him a key member of Thomas Smithgall’s laboratory team, a group that’s applying newly found knowledge about Nef to figure out how to stop HIV from hiding from the immune system.

In 2016, Smithgall’s lab landed a spot on the BELIEVE (Bench to Bed Enhanced Lymphocyte Infusions to Engineer Viral Eradication) project. Funded by the National Institutes of Health (NIH) with a five-year, \$28 million grant, this 18-center consortium aims to find ways to eliminate hidden HIV reservoirs through a cell therapy approach that will focus on augmenting natural immunity specific to each patient.

This tactic has been borrowed from breakthroughs in cancer treatment showing how the immune system can fight back when strengthened. Smithgall, the William S. McEllroy Professor and chair of microbiology and molecular genetics, notes that, like cancer, HIV can come back. Because of hidden viral reservoirs, HIV will reemerge if a patient stops taking antiretrovirals—even after years of treatment.

“It’s fair to say this is a big part of what AIDS research is right now: defining the so-called latent viral reservoir and then coming up with approaches to wipe it out,” Smithgall says.

To figure out how to eradicate these reservoirs, Smithgall says that his team has taken on an “agonizingly slow process” of experimentation. One morning this summer, Smithgall, Alvarado, and Haibin Shi, a PhD research assistant professor who moved to Pittsburgh from Tianjin, China, gathered in a corner of the lab and laughed about how “extremely interesting and frustrating” it is to search for a cure that has eluded scientists for more than 30 years.

But Alvarado later adds that driving to the lab, located off Technology Drive along the Monongahela River bank, can feel like “going to playtime every day. . . . It’s always exciting, and it’s never the same.”

And despite the vexation and intrigue inherent in his work, Smithgall, 57, always seems to be smiling. (He’s a native of Reading, Pa., who studied and trained at the University of Pennsylvania and the NIH.) In his large corner office, the size of which he likes to joke about, there’s a bookcase filled with gifts from PhD students who have worked with him through the years. One student drew a portrait of him; others have added to his growing Penguins and Pirates bobblehead collection; some Chinese students have presented him with bottles of baijiu, a grain alcohol.

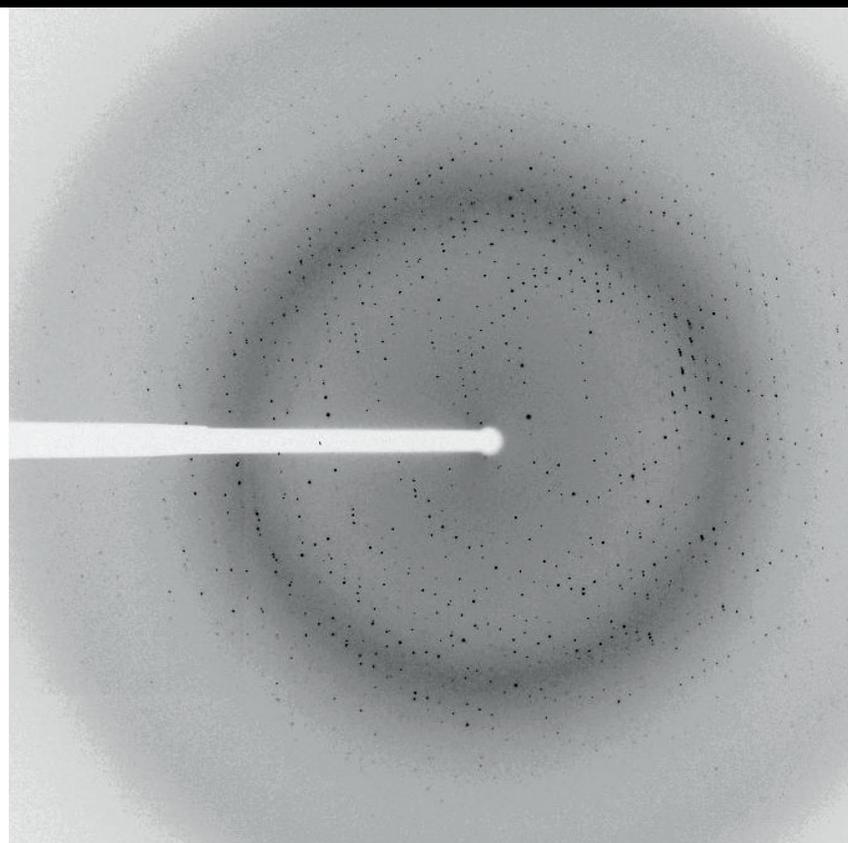
One of his favorite gifts to show off is on a shelf near the floor. On an August morning, he’s laughing before he can slide out the book: a former PhD student’s bound thesis with a yellow and black cover that reads, *Nef for Dummies*.

*For Dummies* is a series of textbooks that takes a complex subject and explains it in a digestible way for a common reader to understand. Like *Auto Repair for Dummies*.

Nef (negative regulatory factor) is an HIV accessory protein that helps the virus block the host cell’s communication with the body’s immune system. Smithgall has been examining Nef since Pitt recruited him in 1998; and earlier this year, his team reported on a synthetic molecular compound that, during lab tests, inhibits Nef and reestablishes communication between the infected cell and the immune system. With Nef inhibited, the immune system was able to kill the HIV-infected cell. Smithgall’s group tested 250,000 compounds over an 18-month period before finding one that seems to stop HIV from hiding from the immune system. The lab’s role in BELIEVE centers on Nef.

Unfortunately, *Nef for Dummies* doesn’t actually exist, but Smithgall would probably enjoy writing such a text.

He explains that after HIV infects an immune cell known as a CD4 T cell, Nef removes a molecule called MHC-1 from



**LEFT:** These crystals took more than three months to grow. They are crystallized versions of the Nef protein as it binds to a kinase in the immune system of its host. Nef is an HIV-1 accessory protein that shuts down the immune response to HIV-1. Inhibiting Nef may help patients use their own immune systems to fight latent HIV reservoirs. **RIGHT:** The high quality of the crystal and the diffraction patterns it created on this x-ray crystallograph allowed John Alvarado in Smithgall's lab to figure out Nef's structure as it binds to the kinase.

the cell's surface in a process called immune escape. Typically, the immune system has a mechanism to recognize virally infected cells and ignore uninfected ones; but Nef eliminates this line of communication, preventing the immune system from being activated despite the presence of HIV.

"The promise of developing drugs or inhibitors for Nef is that they could restore, or perhaps prevent Nef from downregulating, MHC-1," says Smithgall. And once viral antigens have been restored to the cell surface, the body's immune system could wipe out the virally infected cells.

After reporting the first drug leads for Nef in 2013, Smithgall's lab garnered attention from researchers who wanted to team up. Smithgall credits Douglas Nixon, chair of microbiology, immunology, and tropical

medicine at George Washington University, with putting together the BELIEVE project. The partnerships are already paying off.

Earlier this fall, Smithgall and Lori Emert-Sedlak, a PhD in pharmacology and a research assistant professor in his lab, coauthored a paper in the *Journal of Clinical Investigation* with Mario Ostrowski at the University of Toronto. The paper shows that Nef inhibitors discovered by the Smithgall team can indeed reverse Nef-mediated immune escape by HIV-infected cells from patients.

Emert-Sedlak coordinated the screening campaign with Paul Johnston in the School of Pharmacy, and she estimates that they tested 50 to 70 plates of 300 compounds each day. The process took 18 months because they had to continually make and test new protein batches and then perform follow-up tests for

promising compounds to be sure the results could be replicated.

As exciting as their discoveries are, it's a long road from a proof of concept inhibitor to something that can go into a pill, notes Smithgall. The team found compounds with useful inhibiting properties. But that doesn't mean the compounds can be absorbed orally, get along with the liver, or meet other stringent requirements of a new drug.

Emert-Sedlak and Shi, together with research technologist Li Chen, make up the frontline team that conducts the first phases of testing potential drugs, or analogs, for Nef inhibition. (Analog synthesis is done in partnership with the Fox Chase Chemical Diversity Center, in Doylestown, Pa., and is funded through an NIH Small Business Technology Transfer Research Grant directed

by Smithgall.) Emert-Sedlak also developed many of the assays that the lab is using now to assess drug candidates. The team had been conducting experiments from cells easily grown in culture and infected with HIV. It's now testing cells derived from blood from both healthy donors and HIV patients.

"These cells are as relevant as we can get before going into animal models," Emert-Sedlak says. (She notes the CD4+ T cells from patients can be difficult to work with since they have a "very defined and short life span.")

Shi manages a new surface plasmon resonance (SPR) detector, which gives real-time data on the interaction of each analog with Nef. The team is looking for an inhibitor that

binds quickly and remains bound to Nef.

Compounds that show promise move to a second phase where they are crystalized and x-rayed by Alvarado. At the same time, they are tested on mouse models by Sherry Shu, a PhD research assistant professor.

Smithgall wants the members of his team to understand and appreciate how much HIV/AIDS research has changed. He encourages everyone who works in his lab to read David France's book, *How to Survive a Plague*, about the epidemic's early years, so they don't lose sight of the groundwork that came before them or the devastating number of deaths attributed to HIV—about 35 million people. Now his lab may help lead the way to a cure.

"This is what we're working for," he says.

Advancements in technology have accelerated progress in HIV research, and there are reminders of this throughout Smithgall's lab. Some seem subtle, like the SPR detector that Shi excitedly points out during a tour.

Other signs are more obvious, like the "nice robot," as Alvarado calls it. To make protein crystals, Alvarado mixes 200 nanoliters of crystallization solution with 200 nanoliters of purified protein. In just five minutes, the robot drops the mixture into 96 wells. Alvarado used to do this part by hand—that task alone would take him a full eight-hour day.

Later, he'll sit down at a microscope and look at the drops for crystal nuggets to scoop for x-ray experiments. Crystals might form overnight or take weeks or months to grow.

While at Purdue in the 1990s, Alvarado didn't think his x-ray crystallography skill set translated well to where HIV/AIDS research was then. He says no one studied HIV or any other retroviruses at Purdue at the time, and, unlike other viruses that interested him, not much was known about the structure of HIV. Alvarado notes that the HIV envelope protein structure was only just determined in 2012.

"I did not want to study HIV because it was too complicated," Alvarado says.

After graduating from Purdue, Alvarado landed a postdoc position at Albert Einstein College of Medicine, where he analyzed proteins in a herpes virus. In 2009, when he accepted a job in Smithgall's lab, Alvarado still considered HIV to be a daunting field. But he was confident in his level of expertise, and with good reason. His insights regarding the structure of Nef have helped Smithgall's team understand how it attaches to cellular proteins and turns them on.

Now Alvarado is working on determining the three-dimensional structure of Nef when it is bound to an inhibitor. Smithgall says that figuring this out will offer unprecedented insight regarding how their compounds work. He adds that solving this structure would also give his team a clearer path to developing a generation of compounds that can be tested in animal models and ultimately in people.

Alvarado is optimistic about where the team is headed, saying, "We're close." ■

## TICK SPIT FOR THE TICKER

**Even when they are on antiretroviral therapy, people living with HIV are almost twice as likely to suffer from cardiovascular disease than those without the virus. However, researchers from Pitt and the National Institute of Allergy and Infectious Diseases (NIAID) report that a chemical in tick saliva may help these patients.**

**The researchers found that HIV patients had an elevated number of immune cells called monocytes; these cells were producing high levels of a protein called tissue factor, which is linked to inflammation and blood clotting. The monocytes were present regardless of how well a patient's HIV was being controlled with antiretroviral therapy.**

**A team led by Irini Sereti, an MD at the Laboratory of Immunoregulation at the NIAID, exposed blood samples to Ixolaris, an experimental drug based on an anticoagulant found in tick saliva. Sereti's group found that the compound shut off tissue factor activity in monocytes.**

**Pitt's team, led by Ivona Pandrea, MD/PhD professor of pathology, focused on the causes of inflammation in HIV patients and primate models, a topic Pandrea has been examining for a decade. The cause isn't the same with each patient, but Pandrea says one of the most common explanations for inflammation stems from what happens in the gut shortly after infection. The virus destroys immune and epithelial cells there, allowing bacteria to spread through the bloodstream.**

**"Of course this bacteria stimulates a lot of immune cells and creates a vicious cycle, because stimulating more cells [creates] more targets for the virus, more virus replication, then more gut problems," says Pandrea.**

**Hypercoagulation (too much clotting) also causes inflammation, and Pandrea notes that this is often misinterpreted to mean problems in pulmonary arteries. But it's a tissue problem as well as a peripheral one, she says. Microscopic clotting can show up in small blood vessels in the kidney, lung, gut, or brain.**

**"When we see these problems, we don't know if they have a common cause or if they occur independently," Pandrea says. "I think hypercoagulation may be a good explanation for connecting these comorbidities." —GJ**