KEYSTONE IN RIO: Breakthroughs, Predictions, and Surprises

Recent breakthroughs stole the show, but the meeting also highlighted the growing importance of systems biology and the use of sequence information in immunology and vaccinology

By Andreas von Bubnoff

The timing couldn't have been better. On the very first day of the Keystone meeting on Advancing Vaccines in the Genomics Era from Oct. 31 to Nov. 4 in Rio de Janeiro, *Science* published a series of papers matching the splashiest talks delivered at the conference: The structure of a near-native version of the HIV Envelope (Env) trimer (see cover image), and the proof that, in principle, a structure targeted by a potent neutralizing antibody can be used as a starting point to design a vaccine immunogen, at least for respiratory syncytial virus (RSV). But while these findings clearly stole the show—Novartis researcher and conference co-organizer Rino Rappuoli called them "breakthroughs," a word rarely heard from researchers—attendees also learned about an impressive array of advances in the application of systems biology to vaccine design, and how genomic sequences can be used to explore differences in immune responses to both vaccines and infections.

The fine structure of the HIV trimer

The notorious instability of the HIV Envelope trimer has long hindered efforts to obtain its molecular structure at a truly useful resolution. But an effort led by John Moore at Weill Cornell Medical College in New York has over the years identified ways to stabilize the protein without disrupting it too much, and settled on one called BG505 SOSIP.664 for further analysis. It comes from an HIV clade A founder virus (i.e. the one that initially caused infection) isolated from an infant in Kenya.

Ian Wilson and Andrew Ward from The Scripps Research Institute presented the results of the structural analysis of the BG505 trimer by X-ray crystallography and cryo-electron microscopy (EM). Wilson, who presented the X-ray crystallography work (*Science* 2013, doi: 10.1126/science.1245625), said he and his colleagues tried to grow crystals of BG505 bound to many different broadly neutralizing antibodies (bNAbs), and found that the best crystals formed when BG505 was bound to the bNAb PGT122. Ward and colleagues used cryo-EM to determine the structure of the same BG505 trimer bound to a different bNAb, PGV04 (*Science* 2013, doi: 10.1126/science.1245627; see cover image).

The structures obtained through X-ray and cryo-EM confirm and complement each other, said Ward, who presented the cryo-EM work. For the X-ray structure analysis, researchers had to shave most sugars off the trimer to crystallize it, while cryo-EM allowed them to leave on all of the sugars.

At a resolution of 4.7 Ångstroms, the X-ray structure doesn't quite reach atomic-level resolution, but Ward is confident that it's accurate because it agrees with the 5.8 Ångstrom cryo-EM structure, and with the previously determined structure of monomers of gp120, as the external part of Env is called. "This is really it," he said, adding that one challenge now is to further improve the structures to get closer to atomic-level resolution. Ward also wants to make the cryo-EM structure even more similar to the native trimer by adding back the so-called membrane proximal external region, which the researchers removed to make the trimers more

soluble and to prevent them from clumping together.

For the first time, the BG505 structures show the external part of the Env trimer in its entirety, including the relative position of the variable loops 1, 2, and 3. "The biggest thing we learned was that the epitopes are a lot more complicated than previously thought," Ward said, referring to the target sites of bNAbs. This complex environment restricts the angle an antibody can come in to bind. For example, he said, "you have to come in and navigate this very straight path in order to get to the CD4 binding site."

The studies have also taught researchers how to make other stable trimers. "[BG505] is a stable trimer, and we know that it doesn't fall apart, so it can be used as an immunogen," Wilson said, adding that the next goal is to make similar trimers from other HIV clades. "It's really the start of a new generation of immunogens and vaccines that weren't previously accessible," Ward added.

While the BG505 structures are consistent with each other and with a cryo EM structure published on Oct. 23 by Sriram Subramaniam and colleagues (*Nat. Struct. Mol. Biol.* **20**, 1352, 2013), they differ from a previous structure published by Joseph Sodroski and colleagues (*Proc. Natl. Acad. Sci.* **110**, 12438, 2013). Sodroski's structure, which has been the focus of controversy fueled by critical comments published by leading researchers, therefore remains an outlier and requires further validation of accuracy, Ward said.

A future RSV vaccine?

Peter Kwong of the Vaccine Research Center at the U.S. National Institute of Allergy and Infectious Diseases reported the design of a candidate vaccine against RSV. The strategy used could also be important for HIV vaccine design, as it shows that, in principle, structures targeted by potent neutralizing antibodies can be used as potential immunogens.

Earlier this year, Kwong and colleagues determined the crystal structure of D25, a potent neutralizing antibody to the prefusion form of RSV F, the protein the virus uses to enter its target cells.

In the new study, they used their knowledge of this structure to develop a stabilized version of the prefusion RSV F protein without the D25 antibody bound to it and used this stabilized protein to immunize mice and monkeys (*Science* **342**, 592, 2013). They found that it induces neutralizing antibodies at levels many times higher than the titers needed for protection, and at least 10 times higher than the titers induced by a post-fusion form of the RSV F protein, which is currently being developed as a vaccine candidate. Next, Kwong and colleagues plan clinical trials with the new RSV F immunogen.

Conference co-organizer Bali Pulendran from Emory University was clearly impressed with the RSV results. "This is one of the first examples of an approach where you can go from a structure through rational design to construct an immunogen that's highly immunogenic," he said.

Kwong and other researchers are trying a similar approach—using a structure bound by a highly potent antibody as an immunogen—to design an HIV vaccine. D25-like antibody responses to RSV seem very common in people, so one important message of the RSV study for the HIV field, Kwong said, is that it's important to choose an antibody as a starting point for such efforts that's not just broadly neutralizing and potent, but also commonly made by people. "It's very, very important to look at what humans make naturally at high titer. If you want a vaccine that everyone could make, see what people make," he said, adding that he is currently performing detailed analyses to find the most common bNAbs in HIV-infected people.

Good inflammation, bad inflammation

Several talks shed an interesting light on the complicated role of inflammation in vaccine responses. Glenda Canderan from Rafick Sékaly's lab at the Vaccine and Gene Therapy Institute of Florida reported that a signature of changes in inflammation-related genes in elderly people before vaccination corresponds with lower responses to flu vaccination. This suggests that age-related systemic inflammation is one reason why vaccines have less of an effect in elderly people.

But inflammation is only bad for vaccine responses if it gets out of control, Sékaly said. Normally, the body tries to keep inflammation in check by expressing certain genes (such as one called *SOCS1*) that dial it down. But in people who show lower vaccine responses to yellow fever, Sékaly didn't find such genes significantly switched on, suggesting that only inflammation that's unregulated lowers vaccine responses. This, he said, should be taken into account for vaccination strategies in developing countries, where inflammatory responses to other pathogens could lower the response to vaccination. Canderan has preliminary results that suggest that unregulated inflammation might also explain lower responses of elderly people to influenza vaccination. Next, Canderan and Sékaly want to test if reducing inflammation before vaccination can improve responses to yellow fever and hepatitis B vaccines.

Bonnie B. Blomberg of the University of Miami School of Medicine also looked at inflammation in

elderly people. She found that elderly people make more of the inflammation-mediating cytokine TNF-α, which correlates with a lower response to flu vaccination. What's more, elderly people have fewer B cells that have switched to IgG, the antibody type that's most relevant for an immune response. Blomberg's findings suggest this is probably because stimulated B cells of elderly people make less AID, an enzyme involved in antibody maturation and switching to IgG. She found that the level of AID (and TNF-α), as well as the number of switched memory B cells can predict vaccine responsiveness in elderly and young people.

Higher preexisting inflammation in elderly people might therefore lower their B-cell responses to vaccines. One possible remedy might be to restore AID function, Blomberg said. She cautioned that this might have side effects, though this could be circumvented by giving treatment only at the time of vaccination. Another strategy could be to reduce inflammation. One way of doing that without inducing side effects, she said, is surprisingly simple, at least in theory: meditation, stress reduction, a healthy diet, exercise, or social support.

Blomberg noted that there are two types of inflammation. The bad sort is chronic inflammation, which leaves the immune system less room to respond to a vaccine with a proper, acute inflammatory innate immune response. In chronic inflammation, Blomberg said, B cells can become refractory to an antigenic stimulus. "You want to make an inflammatory response when you get the bug [or vaccine], but not before," she said.

One question researchers are still grappling with is why in the Step trial, people with preexisting immune responses to Ad5, the vector used in that trial, showed increased HIV infection risk. Alan Aderem of the non-profit Seattle Biomedical Research Institute reported that one day after vaccination with the Step trial vaccine MRKAd5, people with preexisting immunity to Ad5 activated fewer inflammation-related genes, suggesting that insufficient activation of appropriate "danger signals" by the vaccine may have something to do with the increased HIV infection risk in this population.

Certain genetic defects can also modulate inflammation in people, which can lead to serious problems, according to findings by Dan Kastner of the National Human Genome Research Institute of the NIH. Kastner and his colleagues have established a cohort of about 1,900 patients with so-called autoinflammatory diseases whose causes are unknown in most of the patients.

The cohort includes two young children with an extremely rare combination of symptoms: recurrent high fever and strokes. To find the reason for this, Kastner and colleagues sequenced the part of the genomes of the two children and their parents that codes for proteins. Assuming that both copies of a gene need to be mutated to cause the disease, they found that both children shared a mutation in two copies of only one gene, which could explain the disease.

The defect was in a gene called *CECR1*, which encodes a protein called ADA2 that is thought to be important for the development of certain white blood cells. To test whether mutations in *CECR1* can cause strokes, Kastner and his colleagues inhibited the expression of a *CECR1*-related gene in zebrafish embryos. And indeed, they found that the fish developed strokes within 48 hours and had impaired white blood cell development, effects that could be reversed by introducing the normal version of the human gene into the embryos.

They then used these clues to take a closer look at the defects in the children and found that they, too, had impaired development of a certain type of white blood cell that keeps inflammation in check, in addition to inflammation around blood vessels and problems with the integrity of their blood vessel walls. The combination of these problems could explain the development of fever and strokes in these children.

These insights have already led to the identification of additional cases, including one child whom Kastner and his colleagues first learned about on the MSNBC web site. The North Carolina boy suffered from unexplained strokes and his doctor, a pediatric neurologist, told the host of the "Today" show that he had never seen a case like this before. "He was sure that there was no other case like it in the United States," Kastner said. "Well, we had four [cases], so we called him up and sure enough, that child also had two mutations in this gene."

The findings may eventually lead to treatments of the condition, Kastner said, for example by replacing the missing protein by gene therapy. They could also shed light on the cause of adult strokes, because Kastner and colleagues found one mutated copy of the *CECR1* gene in two adult brothers, both of whom had strokes in their 70s.

HLA alleles and immune responses

Small variations in a tiny part of the genome that encodes HLA molecules can have dramatic consequences for the quality and vigor of responses to both vaccines and infections. That's because

immune cells use HLA molecules on their surface to present tiny parts of immunogens called peptides to activate CD4+ and CD8+ cells, which play key roles in both the antibody and cellular immune response.

Differences in the exact sequence of these HLA molecules matter, because they determine which parts of an immunogen HLA molecules present to CD4+ and CD8+ T cells. Researchers have found that such differences can result in different responses to vaccination. Tomer Hertz of the Fred Hutchinson Cancer Research Center in Seattle reported evidence suggesting that certain HLA alleles made some vaccine recipients in the RV144 trial—the only one to have detected any measure of vaccine-induced protection from HIV—more likely to be protected from HIV. He told attendees that HIV virions that could infect RV144 volunteers despite vaccination were more likely to have mutations in peptides that are preferentially presented by an HLA class I variant called A*02, which presents HIV peptides to activate CD8+ T cells. This suggests that vaccinees with HLA A*02 were more likely to be protected in RV144.

And indeed, Hertz found that the vaccine efficacy was 54% in RV144 vaccinees with the A*02 allele, but only 3% in the vaccinees without A*02. Because cells prefer HLA A*02 to present HIV peptides from the V2 part of HIV's Envelope protein to CD8+ T cells, V2-specific CD8+ T-cell responses could be responsible for some of the protection observed in RV144. (So far, much of the focus on the modest reduction in risk induced by the vaccine regimen has been on effects associated with non-neutralizing antibodies to the same part of the HIV envelope.)

This is puzzling because CD8+ T cells kill infected cells and are therefore not usually thought to prevent infection, but to control the virus in people who are already infected. "The explanation for what happened in RV144 might be more complicated than what we imagine," said Hertz, but added that Jerome Kim and colleagues recently reported mucosal CD8+ T-cell responses in RV144 vaccinees. Such responses might have had a role in preventing infection, since mucosal tissues are the first sites exposed to the incoming virus.

Another explanation, Hertz said, is what immunologists call "cross-presentation:" One arm of the immune system (the cellular CD8+ T-cell response) hands over immunogens to stimulate the other arm (the CD4+ T-cell activated antibody response). In this case, MHC class I A*02 molecules could bind V2 HIV Env peptides, internalize them, and hand them over to class II MHC molecules, which activate CD4+ T helper cells to stimulate a V2-specific antibody response. Others have recently reported evidence that this kind of cross-presentation is indeed possible, Hertz said.

Systems biology: The genome as crystal ball

Attendees also heard a lot of talks about systems biology, an emerging branch of biology where researchers measure certain parameters of biological "systems" in their entirety to glean insights about the immune system. For example, researchers use so-called microarrays to measure changes in the activity of all genes shortly after vaccination to predict whether a vaccine candidate is likely to work.

A few years ago, Pulendran and his colleagues were the first to report that a signature of gene expression changes a few days after yellow fever vaccination can be used to predict the level of the later adaptive T- and B-cell immune responses to that vaccine.

Those and similar studies on other vaccines have raised the question of whether each signature is specific to the vaccine in question, or if there is perhaps a more general signature that can be used to predict responses to different vaccines. At the meeting, Pulendran reported that signatures that predict responses to the same vaccine class tend to be similar, but differ from signatures predicting responses to a different vaccine class. For example, the signatures that correlate with immune responses to the carbohydrate components of the two meningococcal vaccines are similar to each other, but differ from those that correlate with responses to yellow fever and other live viral vaccines (*Nat. Immunol.* 2013, doi: 10.1038/ni.2789).

Aderem also reported that he has used a "systems" approach to predict vaccine responses in rhesus macaques. He measured gene expression changes two weeks after the animals received a vaccine that contained Gag, Pol, and Nef proteins from the simian immunodeficiency virus (SIV), and used this information to predict lower viral load after SIV challenge one year later with about 85% accuracy. He said he and his team analyzed pairwise combinations of vaccine response genes and used machine learning, where computers learn from repeated analyses of data how to focus on the most relevant information, to limit the data-crunching this entailed.

Systems biologists are also interested in predicting adverse effects of vaccines. For example, almost half of the 374 children who were vaccinated in a trial that tested an inactivated whole H5N1 flu virus vaccine in the 2007/2008 flu sea-

son had fever after the first of two vaccinations and, as a result, the vaccine was not approved by the Japanese health authorities.

Junichi Ito of the National Institute of Biomedical Innovation in Japan described a way to predict whether these flu vaccine recipients would develop fever. He measured the expression of most of the 2,000 known microRNAs (small RNA molecules that regulate gene expression) in serum samples that had been taken before the vaccination from 85 of the children, and found 73 miRNAs that showed a different expression level in the children who developed fever compared with the ones who didn't. These miRNAs can therefore be used as a biomarker to predict whether vaccinees will develop fever in other trials, he said.

Thomas Scriba of the South African Tuberculosis (TB) Vaccine Initiative, meanwhile, has been trying to predict whether people with latent TB infection are likely to develop active TB disease. One third of the world's population, or about two billion people, are estimated to have latent TB infection, he said; of those, about 10% will develop active TB at some point in their lives. Every year, nine million people develop the active disease, and 1.5 million die.

Just why some people come out of latency while others don't is not understood, Scriba said. But microarray analysis, he said, could be used to identify markers that can help predict whether latently infected people will develop active infection, which would make prevention and treatment of TB much easier.

To see if this is possible, Scriba, in collaboration with Aderem and others, used microarray analysis to measure gene expression changes in blood cells taken from 6,363 adolescents with latent TB in South Africa five times over a period of two years. They found that more than 1,200 genes were differentially expressed in 35 people with TB infection who developed TB disease during that time, compared with 70 people with TB infection who didn't develop disease. Scriba said he could use this information to predict the development of active TB six months in advance with up to 80% accuracy.

The accuracy was lower for earlier time points, but still had excellent predictive value up to 18 months before active TB developed, Scriba said. He hopes the analysis could allow prophylactic treatment of high risk groups and enable researchers to preferentially enroll high risk people into efficacy trials of TB vaccines or treatments.

Because the "systems" approach involves measuring everything, researchers are not constrained by their preconceptions of what to expect, which is why systems biology can also lead to unexpected insights, something that was perhaps best illustrated in a talk by Pulendran.

When Pulendran and his colleagues measured global gene expression changes in response to flu vaccination, they found that the upregulation of a gene called *TLR5* one week after vaccination correlated with the level of the subsequent antibody response to the vaccine. That surprised Pulendran, because TLR5 is a receptor that senses bacterial flagellin, which is not present in viruses. So, at first, Pulendran and colleagues thought the flu vaccine they were studying might be contaminated with bacteria.

Turned out it wasn't. Further investigation revealed that mice without *TLR5*, or without bacteria in their gut, showed less differentiation of plasma cells, the cells that produce antibodies. This suggests that the sensing of our own gut bacteria by TLR5 might help induce the antibody response to vaccines, and that things that disturb them, like antibiotics, might be harmful to some vaccine responses.

A few years ago, Pulendran made another surprising observation: When he used microarray analysis to study the immune response to the yellow fever vaccine, he found that early activation of a gene called *GCN2* in the blood was correlated with the magnitude of the later adaptive $CD8^+$ T-cell response to the vaccine (*Nat. Immunol.* **10**, 116, 2009). Pulendran and colleagues also showed that *GCN2* was required for the adaptive immune response in mice.

While it was known that the protein encoded by *GCN2* is activated in response to amino acid starvation of cells in response to stress, the link of *GCN2* to the adaptive immune response was not known before. At the meeting, Pulendran reported that he has now elucidated the mechanism of how GCN2 activates the adaptive immune response: It induces autophagy, in which stressed cells start digesting themselves to generate energy. If this happens in dendritic cells, it enables the cells to better present antigens to $CD4^+$ and $CD8^+$ T cells, which results in a better immune response (*Science* 2013, doi: 10.1126/science.1246829). This mechanism could explain in part why yellow fever vaccine elicits such a powerful immune response.

These studies are an example that systems biology can actually lead to new insights, Pulendran said. "I never knew what GCN2 was, and no one knew that it could play such an important role in immunity in this way," he said. In his final remarks to the attendees, Rappuoli, a vaccinologist who is head of research at Novartis Vaccines and Diagnostics, agreed. While systems biology has until recently been in a validation phase, in which researchers sim-

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ply confirmed things that were already known, he said, Pulendran's results show that the field is ready to discover new things.

He ended the meeting with a glimpse of the future. Systems biology, he said, might eventually help accelerate the development of candidate vaccines—which, he noted, can take about 15 years to move from bench-top to clinic. "That's a long time," he said, adding that while clinical trials today test, say, 10 parameters in 10,000 people, systems biologists might be able to find ways to do the same thing with much less effort. "I want to leave you with [a] dream for vaccine development," he said. "If we could use [this] technology [to test] ten people [with] 40,000 data points per person to predict what's going to happen, probably vaccine development would be much faster." ■