Cynthia Dunbar: [Music] I'm going to talk about some of our work in trying to understand hematopoiesis at a clonal level with relevance to clinical questions, particularly gene therapy and transplantation, and also the focus on models and when a non-human primate model makes sense and when it can really add to what we know and what the drawbacks can be for some of the in vitro and mirroring models. In terms of rhesus macaques' models for hematopoiesis, immunology and gene therapies, obviously, there's an incredible similarity with humans that there both an amino acid and sequence level. The hematopoietic demand, which is, what is very important for us to think about in terms of what they've evolved to react to in their environment is based on their size and lifespan. Yes, they're much smaller than humans fully grown, but they're at least in the same ballpark and their lifespan in captivity can even go up up to 30 or 40 years. That's a much different situation than potentially a small rodent who is breeding very rapidly and having a much shorter lifespan and a much different response to the environment.

Immune system characteristics are also very well understood for macaques and other non-human primates in part because of the HIV epidemic and other infectious disease stressors that have made it very important to have models that are well understood from immunologic level that are actually infectible by some of the same agents as humans. They're also non-inbred, which makes experiments a lot harder, obviously, The generation time is much longer, but at least you're looking at an immune system that is not inbred and therefore much closer to our own, and their niche characteristics and transplantation parameters are pretty similar. Practically, human cytokines, antibodies, and drugs mostly cross react. Not all, but many do, so you can translate pretty easily.

I got into working with non-human primates when I was a postdoc with Art Nienhuis early in my faculty career when we really found that as I'll talk about, results from the early gene therapy and gene marking trials, and humans really were not reflecting at all what we had seen in rodent models and in human hematopoietic regeneration study in vitro, where during my postdoc, we were very good at correcting lots of stem cells in mice and CFUs in human cultures. But when we use the same conditions in the early clinical trials, they didn't work. I was interested in using technologies for transducing hematopoietic stem regenerative cells with replication in competent integrating vectors to interrogate hematopoiesis at a clonal level, and that's what I'm going to talk about for the first part of my talk before I get into some of the more practical gene therapy applications.

Obviously, you can look at the output and prove that a cell is a stem cell that has massive self-renewal and differentiation abilities into all lineages by doing limit dilution transplants into a radiated mice. But that's what an HPSC can do under incredible replicative stress and need. When we're doing clinical transplants, we're definitely not trying to do limit dilution transplants. We're trying to get the patients as many cells as they could possibly need. For gene therapies, even more important, it's become clear over time in terms of clonal stress. That is one reason that it's very important to be able to track the cells. Based on real advances in the starting about 20 years ago in terms of using lentiviral vectors in vermin, others in San Diego and in La Jolla developing lentiviruses which are highly efficient at transducing both macaque and human HPSCs.

We developed a bar coding model where we basically used very high diversity libraries to transduce non-human primate CD34 positive cells, which is a way of basically getting rid of mature cells and having both progenitors and stem cells that are practically transducible by these libraries. These bar codes have a library ID, so we could look at multiple different libraries in the same animal if we want to look at characteristics of how we treat cells differently or different sources, and then the bar code. You can just simply use PCR to amplify the barcode and get a very quantitative read on what contributions of individual clones is over time. Most of these studies have been done with total body radiation. They're ablative transplants and autologous cells. We're not looking at the immune system in terms of its impact on this except if you have potentially a transgene that is immunogenic. But with TBI, even GFP, which most of our vectors carry is not rejected.

One barcode equals one transduced stem or progenitor cell, if you have sufficient library diversity numbers of engrafting cells, and you're also targeting a transduction efficiency that's low enough so that most of the cells that have a barcode have a single barcode, so the quantitative barcode refraction equals the contribution of the clone, and work was done with members of the lab, a series of post backs and computational students, as well as a post-op and then staff scientist, Joy Wu, who run these studies over many years. The point of these studies really has been in some cases, to follow these animals for 10-15 years to look at what happens to long-term stem cell dynamics over time. This is the data we get. This is just one way of looking at it that's easy to show, which is a heat map. Look at samples on the x-axis that are different time points and different cell lineages over time post-transplant. Basically, this is looking at the largest clones contributing to each lineage and mapping them over all the lineages. Basically, what we see is early engraftment is from lineage restricted and very transient clones in this model, just a few weeks.

By two months, generally those clones have disappeared in basically all lineages. They are replaced by very stable, multipotent, long-term repopulating clones that are variable in their level of contribution, but very stable over time. Some of these we have now followed for more than 14 or 15 years, and we don't see clonal succession. We don't see replacement, and we've seen at least starting with these animals that were basically adolescent age at the time we did the transplants for these studies, and I'm presenting here. We don't see a lot of clonal pruning, at least when they get to middle age in these models. We do see basically expansion of T cell clones that are presumably peripheral. We know that T cells are responding to things in the environment, and they are not dependent in terms of their clonal expansions on clonal expansions of hematopoietic stem progenitor cells. That was expected, and it was good to see that as an internal control.

Looking at these overall lessons from these studies, like I said, we didn't see evidence for clonal exhaustion, at least starting with these younger animals. We didn't see any evidence for late appearance of "quiescent" clones that you had not started contributing basically by six months, the clones that we saw long term were all contributing. That begs the question of whether there were obviously clones that we couldn't transduce at all that were very quiescent. But given that the levels of marking stayed very stable, I don't think that is a major factor. We could also look at the frequency of long term and short term engrafting clones in this model, and there's been lots of work done on that using xenografts, putting human cells into very immunodeficient mice. But the micro environment that human cells are going into in a mouse is so different in terms of the cytokines that are present in terms of micro environmental cues like integrins, they're very inefficient in grafting in immunodeffficient mice.

We get numbers that are actually, more frequent when we look in this non-human primate model, something like one per 1,000-2,500 CD34 positive cells, or short term HSCs and one per 5,000-20,000 for long-term HSCs per mononuclear cell. It's interesting because that comes up with, based on the number of cells we gave and the number of the marking efficiency with an active stem cell pool in the neighborhood of 40-100,000 per macaque. That's interesting because about 1- years later, Peter Campbell's approach with David Kent and others were using somatic mutations as a way to map clonal dynamics in humans that were not manipulated. This nature paper and a whole series of papers since that time, and they came up with basically the same estimates for numbers of contributing cells in humans. It's interesting a paper from Peggy Goodell collaborating with Jyoti Nangalia has looked at looking at mouse hematopoiesis in the same model compared to human, and there really are a lot of differences in terms of in humans, you basically get up to your total stem cell pool numbers by about teenage dumb, and then it stays stable.

Whereas in mice, it continually expands over the entire age of the mouse. This makes it difficult to see things like clonal hematopoiesis endogenously in mice, and we'll talk more about that later. But it was very interesting to see that we basically came up with similar answers to validate the nonhuman primate model. I'm going to go off into a little bit of a tangent that may not be what people who study

hematopoiesis are usually concerned with, but I am at San Diego. I don't know if Dan Kaufman's here. I'm meeting with him later. Because we have these largish animals where we can collect lots of cells and sort them, we were looking at all lineages we could think of, and one lineage we were looking at was natural killer cells because they were there. Frankly, I didn't know anything about natural killer cells really when they started these projects. But these are lymphocytes able to kill "naturally" without priming or MHC restriction. The paradigm was that these are innate, non-adaptive, that they're responding immediately to loss of MHC on tumor cells or virally infected cells.

But the last up to 15 years, there's really been functional studies that suggest adaptive or memory functions for these cells that are really not so different in many ways from CDAT cells. They have cytotoxic functions, they have regulatory functions, and they have very important functions in placental development. They have been developed as clinical anti-cancer cell therapies unmanipulated maybe not so wildly successful yet, but very interesting work from Dan and others generating NKs from IPSCs, but more recently, engineering NK cells to be redirected by chimeric antigen receptors. They've become very interesting cell types over time. This dogma has been that NK cells are continuously produced and that they're very short lived from hematopoietic stem progenitor cells with a series of maturation steps going from CD56 bright to CD56 dim and gaining CD16 and the ability to be cytotoxic. But that makes it more difficult to imagine using these cells as adoptive cell therapies that are going to stick around for very long. Rhesus macaque and K cells, I won't spend a lot of time on this, but they have a few differences in terms of cell surface expression from human and that they lose CD56 expression almost completely when they gain CD16, unlike human cells, that stay dim.

But otherwise, they are very similar, and I won't go more into that right now. But basically, we were really surprised on these heat maps in clonal mapping, when we started looking and we saw these multipotent clones that were contributing to all lineages stably over time. Then we saw the T cell clones here that I already pointed out. But then we saw these massive clonal expansions of NK cells, specifically in "mature NK cells," the CD16 positive CD56 dim or negative NK cells. This was really a surprise. This has not been discovered previously because no one had a way of looking at clonality in NK cells because they don't rearrange any receptors. This suddenly I had to learn something about NK cells, so I went to the Karolinska for six months and did a sabbatical with Jenn and Bryson and Kelly Malburg, which was very useful. It did seem quite analogous to patterns you see with peripheral expansion of T cells. This just looks at the Shannon diversity index for different lineages in these animals.

As I said earlier, you have very stable diversity over time once you get to six months out to 20 months here for different lineages. But when you look, including immature "NK cells CD56 bright, CD16 negative." But these mature NKCs expressing CD16 were much less diverse and really were made up with very large clonal expansions. This is overall clonal contributions, and you had individual clones that were accounting for 10% or 20% of NK cell activity. Some of them, they waxed and waned over time when we looked at them and suggesting maybe they could be responding to something in the environment or just stochastic pressure from a bottleneck. They would persist for months to years, these individual clones, despite the fact that the original HPSC clone that they had been derived from, we couldn't even find most of the time, or they were very early clones of progenitors that had then disappeared. This was quite surprising and interesting. We did find that these animals reactivated CMV at the time of transplantation. They're autologous transplants. They never got sick from the CMV, but if we looked, we could find CMV reactivation. Rhesus CMV is very homologous to human CMV.

The question was, there's been a lot known about NK cells and the specific phenotypes of NK cells called adaptive NK cells that seem to be very linked to CMV infection and reactivation. That was interesting. There's been this indirect evidence, as I mentioned, for these long lived adaptive memory NK cells or functional evidence, basically murine memory NK cell studies showing adoptive transfer of

long lived cells that confer specific memory to viruses or hapins. But there are many differences between murine and human or primate NK cells. There's SIV studies done by Reeves at Beth Israel showing evidence for SIV memory in experimental models of infection. Then humans post-transplantation. When you do an allogenate transplant, CMV reactivation, especially before some of the better drugs were available that are now used to prevent or control serious infections, you would see these what they were called pseudo clonal expansions of NK cells that were defined by cure, killer inhibitory receptor cell surface patterns, where you would have what looked to be these very distinct subsets of different cures being expressed on different subsets that were controlled epigenetically as if they were clonally expanding in response to CMV and the idea that you're maintaining these clones surface expression of different activating and inhibitory receptors epigenetically.

But it hadn't been linked to direct clonal studies because you couldn't do that until both sequence and studies more recently and our clonal mapping studies. We really through a bunch of different work done by collaboratively with Anon and other members of my laboratory, we really came up with this model that you were getting early engraftment with from CD34 positive short-term progenitors that were leading to CD56 bright and then dim NK cells, but that over time, in response to some small subset of these cells had the ability to gain self-renewal capacity and to clonally expand and persist very long term. It was interesting because when we looked at the beauty of being at the NIH and working with rare diseases and having freezers full of some unusual patient samples, was that I looked at a couple of clinical situations that we happened to study at the NIH that were abnormal bone marrow syndromes. One of them was, PNH, or paroxysmal nocturnal hemoglobinuria, which are somatic mutations in the GPI linking pathways, and under immune attack, individuals with mare failure or even just novo can end up with a large population of GPI negative cells and the red cells are very prone to lysis in the presence of a little acidification because they are missing some of the cell surface proteins that prevent that normally.

We looked at these P&H patients that are chimeric, basically, naturally for GPI negative and positive cells. When we looked at that, we found that even when their granulocytes were 100% GPI negative, their B cells were 100% GPI negative. Every other lineage was GPI negative except for some portion of T cells and portions of NK cells. The NK cells that were GPI negative were the ones that had these adaptive characteristics that had been reported in CMV infections that were NKG 2C positive. That was interesting that there was no correlation between the GPI negativity percentage in NK cells versus the other lineages. The other patient population is GATA2 deficiency patients, they're heterozygous, loss of function, alleles, and GATA2 transcription factor. They have a lot of issues with predisposition to AML, but they also lack immature NK cells almost completely over time. They don't have CD56 Bright. But despite that, they have a retained population of CD56 dim CD16 positive adaptive cells. That was another evidence that these cells could be peripherally self-renewing. We did a bunch of tracking studies trying to figure out where this could be going and what the progenitors were, and it seems like much of this is going on in the liver and other peripheral organs, although the majority of the adaptive and mature cells themselves are in the circulation. Are these NK clonal expansions linked to CMV? Like I said, it's very homologous to human CMV, but all wild or-

Cynthia Dunbar: Naturally, normally housed macaques become CMV positive by one year of age. Even more than humans, where something like 70, 80% of us will be positive for CMV by adulthood and macaques, it's 100%. They're running around, sharing saliva, and being cared for in troops, so they all get positive.

But there have been colonies of CMV-negative macaques that have been derived and maintained at Tulane and a number of other primate centers, mostly to be used for things like CMV vaccine studies.

I collaborated with Mitty Coer at Tulane and got some of these CMV-negative macaques and could use them for infecting them with Rhesus CMV and looking at clonality before and after. We did see marked changes in NK clonality following Rhesus CMV infection.

We did find that some of these animals, even though they were CMV-negative, still had expanded clones before we infected them, but they are positive for an EBV-like virus and other herpes viruses and are clearly otherwise have been maintained in colonies and being exposed potentially to other viruses.

We didn't get rid of these expanded clones by getting rid of CMV, but we certainly could show new clones coming up when they were naturally, experimentally infected.

More recently, Chiara Romagnani, who was an immunologist in Berlin, who I spent some time with on my sabbatical, used basically a mitochondrial mutation mapping as a way to look at clonality in human NK cells, specifically, following CMV infections or CMV reactivation post transplant.

She did show oligoclonal expansions. She did show that it was in the CD56 DM, adaptive clusters of CMV-positive individuals on the single-cell RNA-seq studies. Each clone clustered together stably over time and had a specific epigenetic signature.

That on one hand was very good because I think for the first 10 years of me reporting this stuff, people didn't believe me, although I don't know how you cannot believe these huge clones that existed. But anyway, she showed the same huge clones and published it in Nature Immunology.

These cells are now being thought of as a population of cells that could persist long-term if you wanted to use them for some therapeutic anti-cancer interventions. Chiara Romagnani has been doing some trials specifically using these adaptive NK cells as targets for adoptive cell therapies.

Enough on the NK cells, there's a lot of other peripheral studies we've been working on with those over time in terms of where these different events are occurring, and stuff on Cortes, who is a postdoc in my lab and is now an instructor-level bioinformatics, computational biology, epigenetic-focused person has been using single-cell plus conality studies to try to do better mapping of where and what events are happening to result in these expanded clones, and others are doing the same thing in human cell populations as well with these advanced sequencing approaches.

I'm going to come back to the gene therapies targeting hematopoietic stem cells as a more translational or clinical part of my talk.

I think it's obvious from the beginning of doing gene therapies that hematopopetic stem cells would be excellent targets to correct a whole lot of different disorders, including even CNS metabolic disorders such as adrenal leukodystrophy or metachromatic leukodystrophies, immodeficiencies, many different kinds and hemoglobinopathies, as well as Fanconi anemia and actual stem cell deficit disorders.

What has occurred to date have all been ex vivo hematopoietic stem cell gene therapies, and I'm sure most of you are aware of how this works, but for some of the biologists students who may not.

Basically, you can get your source of hematopoietic stem cells either by doing a marrow harvest or mobilizing hematopoietic cells into the circulation.

We generally use antibody selection to enrich for hematopoietic stem progenitor cells because the mature cells are not going to persist and are just going to be expensive to maintain in vitro and transduce.

Use an integrating vector, generally virus. Initially, Murine leukemia viruses were used, as I mentioned, expose them to the vector, condition the patient with something to decrease the number of endogenous cells that would compete, and transplant your transduced CD3 for positive cells and hope for clinical benefit.

This seemed like something that could be done starting back in the early to mid-1990s after the initial derivation of replication in competent retroviral vectors by Richard Mulligan and others in the 1980s. These were integrating vectors that were made replication incompetent.

They were used in pioneering clinical trials in children by Malcolm Brenner, by Mike Blais and others, and in some of our gene marking studies in adults and then Gaucher's disease in my group at the MIH.

Unfortunately, despite in vitro getting really good transduction of colony-forming units and using similar vectors in mice and having great results.

In these patients, the levels of vector-containing cells long-term was extremely low, less than 1%, not going to be clinically useful for much of anything.

But we did try to ask biologic questions at the same time, and one of the questions we asked in those early studies was whether mobilized peripheral blood cells could actually engraft long-term.

That was actually a matter of argument at the time about whether they were just providing a bridge of mature cells or if they were really engrafting. We could show long-term marking from the immobilized peripheral blood stem cell graft that was equivalent or better than from the bone marrow graft in these patients.

That was interesting biology at the same time. But it was clear that we didn't understand the underlying HSC biology well enough, the impact of in vitro culture, we didn't have the right cytokines. We didn't have the right conditions in terms of mimicking a microenvironment.

We didn't understand genetic elements that control gene expression. We had really non-predictive models, and that's when my initial postdoc mentor, and then I, when I started my independent program, really focused on setting up the non-human primate models.

We really found that how we maintain these cells during in vitro culture and transduction was super important, that prolonged ex vivo expansion, you could get loads of CD 34-positive cells, but they actually profoundly decrease their long-term engrafting ability.

We found that cytokines that were really important for supporting primitive HSCs included thrombopoietin, most importantly, and this is how I got involved in thrombopoietin in trauma past studies later on in my career, but also stem cell factor in foot three ligand.

David Williams figured out that engaging VLA 4 on HSCs with retronectin could maintain stemness and also help co-localize your HSCs and certain types of vectors such as MLV.

Basically, using these just tweaked conditions, the same vectors, we got levels of 10%-15% long-term in the peripheral blood of Macax, which was, for the first time, something that you could imagine would be clinically relevant.

This supported second-generation clinical trials that were initiated in the late 1990s, early 2000s, and the disease that was being looked at to start with was excellent SCID, which is severe combined immunodeficiency with complete lack of T cells and K cells and complete lack of functional hemeral immunity.

They have a lack of the IL2 receptor Gamma. They die in early childhood. The bubble boy, where children that were born were kept in the hospital in germ-free environments for as long as possible to try to keep them alive. But their life expectancy was very short.

French investigators led Marina Cavazzana-Calvo and Elaine Fischer used these marring retro viral

vectors with improved transduction conditions and nine of 11 of the first patients they transplanted, and they didn't use any conditioning because these patients have no T cells, and even getting a very low level of engraftment of HSCs in these very young children turned out to be basically reconstitute their immune system.

This was the first clear clinical benefit for any gene therapy, I think, worldwide, ever, which was encouraging after many years of failures.

But unfortunately, a few years later, multiple patients on those original trials, as well as a number of other trials that were using these MLV marine leukemia virus vectors with very strong internal promoter enhancers, develop leukemias.

This is the T cell counts that were normal. They were doing well, and then this is a log scale. They developed acute leukemias. They were T lymphoblastic leukemias.

The first two patients had insertions of the vector in these expanded leukemic clones that turned on the LMO2 transcription factor, which is very important in T cell development, but then it's supposed to turn off. If it's being expressed in stem cells and lymphoid progenitors, it turns out to not be a good thing.

We also, at the same time, soon thereafter, we discovered that one of our monkeys who had received transduced cells with a vector that contained a strong MLV enhancer promoter also developed a myeloid leukemia because there was argument going on initially after these cases that this was somehow related to constitutive expression of IL2 receptor Gamma, and this wouldn't actually occur in other disorders where you weren't overexpressing a signal transducing stimulatory cytokine receptor.

But we saw this in a macaque with no transgene other than GFP. Then we began working on trying to understand what was leading to this genotoxicity. Much of this has been published. But basically, insertion site retrieval allowed us to track ametomatopoises on a clonal level and see where the insertions were.

Cole Ferguson, who I mentioned earlier, was in my lab doing a lot of this work and comparing the insertion pattern of MLV vectors versus lentiviral vectors. The lentiviral vectors were spread out along genes.

They were not all sitting near the transcription start site and seemed to less likely to turn on adjacent proto-oncogenes in terms of this pattern.

Also, HIV vectors, because you were trying to prevent recombination with endogenous HIV, had their viral promoters and enhancers completely removed, unlike the MLV vectors, where many of the vectors used the viral enhancer promoter because they were constitutive and very strong and not silenced.

Basically, we began working with lentiviral vectors in the macaque system.

They worked very well and skipped forward, many years and a lot of work by a lot of different people, but I'm very proud of one of those people is John Tisdale, who was a postdoc in my lab in the 90s and went on to lead the original successful lentiviral gene therapy trial for sickle cell anemia with Bluebird Bio and the NIH, basically adding an anti-cycling hemoglobin to counteract hemoglobin S, and very efficient transduction.

It took a while. There were a number of different cohorts in the trial, but they finally got very good engraftment following busulfan conditioning, very high-level expression of this anticycline globin.

This is basically individual patients and all the pain crises they were having before gene therapy and transplantation and then after, and you can't see any red dots. It was really no further vaso-occlusive crises.

This was approved by the FDA in 2023 for both sickle cell disease and beta thalassemia. But there remain concerns regarding genotoxicity, even the lentiviral vectors, they are integrating. Clonal expansion or transformation risk is never zero with any integrating vector, obviously.

We did see a rhesus macaque develop a myeloproliferative neoplasm and hypereosinophilia due to a lentiviral insertion activating the PLAG1 transcription factor and also leading to overexpression of stem cell factor.

This vector did have a very strong internal constitutive viral promoter that we had used, but this was a promoter being used, as I'll mention in clinical trials.

It was relevant. We had a lot of trouble getting this paper published because I think everybody it was sent to review had a conflict because they were involved with starting lentiviral viral gene therapy companies. It was very frustrating.

But we did finally publish the paper in Locker therapy, and when we presented it at ASH, the standing room only for all the venture capital people trying to get in the room and calling their brokers and saying, sell. Anyway, but https://www.nlm.nih.gov/medlineplus/ency/article/000535.htm has occurred in the sickle cell gene therapy lentiviral trial.

There was clonal vector integration in one of these patients, but it was nowhere near any growth impacting gene. They couldn't find any impact on gene expression or epigenetic marks.

There were no adjacent cancer genes, and there has been found that sickle cell disease patients, especially going through allogenic transplant with mixed chimmerism, so there's still autologous cells present or gene therapies, even if they're unsuccessful, have had evidence of coming up with AMLs due to stress with pre existing P53 mutations shown by Courtney Fitzhugh.

It may be that just the chronic marrow stress of these patients potentially added to by going through a transplant procedure is going to accelerate their risk of myeloid leukemia. But the next set of patients that it became clear that able vectors really could do this more generally is in the adrenal leuko dstrophy trials.

These patients was a clinical success, a disease that these individuals were dying very early in life from profound neurologic compromise.

Basically, with these autologous transplants done early in life with the spectra over expressing the missing enzyme, they were doing much better clinically, but seven of 67 had developed AML. This lentiviral vector had this very strong internal MLV constitutive promoter enhancer.

That was because allogenate transplant of these patients didn't do as well because it seemed like you need super physiologic expression of the in myeloid cells that are basically able to infiltrate the brain and correct the phenotype. That was why they were using this super strong promoter.

11% so far, this is out 3-5 years. It's not clear whether many more will go on to this or not, but it was definitely sobering. Having a targeted approach is obviously very attractive, and that leads us to the CRISPR and gene editing revolution.

I think you're all aware that you have a targeted guide RNA and a cast endonuclease. You get nonhomologous end joining with creation of indels or insertions or deletions that are usually very good at knocking out gene function or knocking out an epigenetic mark.

If you have DNA homologous to the cut region, either carried by a virus like AAV or double stranded DNA oligos, you can get HDR homology directed repair and potentially it seems very elegant mutation correction.

Or you could knock in a cassette that maybe into a region and replace an entire region, for instance, in a genetic disease where you have a lot of different mutations, and doing targeted correction of each one is going to be, from a regulatory standpoint, challenging. You also have the ability to think about gene therapy for dominant disorders.

Then, obviously, people like David Liu and others have gone on to design targeted guide RNA approaches to even more elegant ways to correct the genome with base editing or prime editing or epigenetic editing. This is another way you can get mutation correction.

Base editors need a bespoke editor for every mutation. You need a PAM sequence nearby. Prime editors can correct larger genomic loci are not as unlimited. But obviously, we have a lot less experience. In theory, would be safer than viral vectors due to avoidance of these semi random insertions.

But how do you predict and detect off target editing? It's clear that you can actually get chromosomal translocations when you have double stranded DNA breaks and repair. Actually, in some of our macaques that we edited, we found persistent DNA translocations after gene editing therapies.

We haven't had leukemias. Carl June at Penn has also reported some of these, again, without any adverse consequences yet, but it is a concern.

The other concern is that really hematopoietic stem cells don't much like double stranded DNA breaks, and they're not very controllable in terms of how they're going to come back together.

But we began using non homologous N joining editing quite early, and we were the first people to do it in macaques or non even primase models to basically look at optimization of approaches for editing of stem cells and also the disease model.

We basically can electro parade, targeted guide RNAs and cast nine into the HSPCs and do a transplant and look at a function or knockout of a locus.

We did create a macaque model for PNH, which a paroxysmal nocturnal hemoglobinuria, and I mentioned earlier, there's no mouse models that have worked for that.

This whole question about whether these PIGA deficient clones have some endogenous expansion capacity versus how they're just maybe protected from immune attack was an argument in the field for many years. We did create a PNH model in the macaques.

We didn't see any PNH clonal expansion or dominance in the absence of an immune attack. We also were interested in the RUNX1 deficiency or familial platelet disorder. This is another marrow failure syndrome with thrombocytopenia and a myeloid cancer predisposition.

The murine phenotypes, when you just knock out either both genes or one gene, both genes, they have no stem cells at all, so that's not good. But haploinsufficiency, there's no phenotype in mice.

In the monkeys, when we did do editing to knock out one allele, we got very close recapitulation of the human platelet phenotype, thrombocytopenia, platelet dysfunction, as well as an inflammatory marrow microenvironment, which is another part of the syndrome that may lead to the myeloid malignancy predisposition.

This was being funded in part by the RUNX1 Research Foundation because the families were very

interested in corrective gene therapy for RUNX1 deficiency to prevent their very high risk of leukemia.

Cynthia Dunbar: Correct every stem cell. The question was would the corrected cells dominate or would the corrected cells, be outcompeed by the mutant cells in our model where we created the mutant cells and competed them against endogenous, we found that basically the RUNX1 mutant clones dominate. A hematopoietic stem cell gene therapy approach for RUNX1 deficiency was not something you know, really made sense. That was a useful component of using our primate model to look at this.

We also worked a paper that I don't have time to talk about that we did with Saar Gill that led to this whole idea of shielding hematopoietic stem cells from CAR T cells by engineering them to lack an antigen. That you are going to treat them with like CD33 for myeloid leukemia with CAR T cells or with a bispecific antibody. Then more recently with engineered epitope shielding. We've just used the model to show that CD33 could be knocked out without any impact on human or mac neutrophil function and that anti-CD33 CAR T cells could eradicate AML in xenografts while sparing CD33 deficient HSCs. This has led to multiple clinical trials by some biotechs in AML and other malignancies.

But unfortunately, I think AML has many other things besides just CD33 as targets and are have lots of other mutations, and so far, the approach has not been very promising, and most of the companies that were engaged in that space have not been taking trials forward at this point. In the last model I'm going to talk about is age-related clonal hematopoiesis, as I age. Certainly we were interested in it. This was initially reported by Lambert Busk and others and Ben Ebert back more than 10 years ago, basically finding that there were somatic mutations that were expanded in humans with no hematologic neoplasms, with normal blood counts, but they had mutations in a set of genes, including DNMT3A, TET2, and ASXL1.

Most commonly that were also associated with human myeloid leukemias. They were all epigenetic regulators that list DNMT3A and TET2 and they had previously been linked to leukemias. It had been difficult to intensively or longitudinally study humans with clonal hematopoiesis because they really didn't want to have bone marrows all the time, although there were more and more clinics being set up, including, I think, at UCSD, for studying clonal hematopoiesis in humans.

But the preclinical models in mice, you don't ever really find these large clones with aged mice, and I think it has to do with their stem cell dynamics being very different. We first asked, do old monkeys get clonal hematopoiesis naturally? Because if they didn't, then they probably weren't a very good model. It turns out that, yes, they do. We were able to get samples from a National Aging Institute cohort of old macaques. Not easy. Most macaques are not maintained that long in captivity. They're used for other studies. They're very expensive to maintain as colonies.

But we did find the same mutations in the same types of genes in these animals at a similar incidence to in humans. That said, it might be a good model. But there are not a lot of those aged animals around, so we decided to say, okay, what if we knock out the same target genes and see what happens over time when we do an autologous transplant? We targeted DNMT3A, TET2, and ASXL1. NC34 positive cells did transplants, and especially with TET2, we saw reproducible quite rapid clonal expansion post transplantation.

If we look at TET2 altogether, but the interesting thing is it varied from animal to animal. It was really quite individually heterogeneous, which would go along with maybe differences based on gender or size or obesity or inflammation, or who knows, a non-inbred model. We did find that the bone marrow morphology was myeloid shifted. We haven't seen leukemia in any of these animals yet. We saw they were somewhat hypercellular, and we found a very hyper inflammatory cytokine profile on RNA seq and on proteomics that was different from wild type transplanted macaques.

We'd looked at could we use this as a way to look at therapies, potentially, and we looked at IL-6

inhibition because with TET2, you get over expression of the IL2 receptor on HSPCs that's released, stimulates stromal cells to make IL1 and other cytokines, and you get a vicious cycle of inflammation that would favor the mutant cells. We looked at tocilizumab in three of these animals, which blocks IL-6. We found that the clonal expansion rate did decrease when we treated for four months with tocilizumab in these animals.

Very small study, obviously, but it was a proof of concept of the ability to use these animals for that. I think I'm getting towards the end of my time, but I did want to just talk about one more project, which is this whole issue of the plethora of gene therapy approaches we have now to HSC modification. We have lentiviral gene addition where we have many years of follow up, clinically effective, but the concern of genotoxicity, we have CRISPR non-homologous end joining to reactivate BCL11A.

To inactivate BCL11A and turn fetal hemoglobin production on that vertex had approved at the same time as lentiviral gene addition is also very clinically effective with shorter follow up, but so far looks safe. Then we have HDR gene correction, for instance, sickle cell disease, which Matt Porteous and a biotech took forward into patients pretty rapidly because in xenographs it worked pretty well. Initiated a clinical trial. But we have experience and we have expectations, and they don't tend to always match up.

We were interested in looking at HDR gene editing and its impact on hematopoietic stem cell dynamics and on clonality, etc. We were knocking in a barcoded cassette, and we did a competitive repopulation design where we took one arm, and we just barcoded a little lentiviral vector, where we have lots of data, and we took another half the cells and did HDR editing in the presence of a cassette that had a different barcode library. The one thing to remember when you're doing HDR, I mean, we got reasonable efficiency 10-20% of HDR in these animals.

But look at how many endos you end up with to get that. You have to think about what knocking out the gene is going to do at the same time as you're knocking into some cells and hemoglobinopathies. That could be an issue. The number that actually had HDR and HDR in both alleles was really pretty low. But we found that the animals that we gave graphs that had HDR editing recovered their platelet counts much more slowly. This was concerning, requiring multiple platelet transfusions never reached normal range, even though the cell numbers and viability looked okay if we looked at the bulk CD34 population.

This is around the time that graphite that was accompanied doing the HDR sickle cell disease trial treated N of one patients who had persistent thrombocytopenia, required a trauma pad, and the trial was put on hold, and the company dissolved, and they went back to the lab to work on this some more. But in xenographs that had not been a big issue, but I'm not convinced xenographs were actually looking at true long term stem cells. I think HDR is really quite tough on cells. This is looking at clonality.

Look at the diversity index for the lentiviral bar coated arms, very high, very low for the HDR arms. You can just see a few clones that we could detect long term as opposed to lentiviruses when we had thousands. Really quite different. This is even in the presence of a P-53 pathway inhibitor used during the editing that various people developed to say if we can inhibit this transiently, maybe we won't have as much damage. Just showing the difference, this is a log scale. GFP positive cells from the lentivirus very stable over time, and INDELs very stable over time.

Nonhomologous end joining was okay, but the HDR cells were really very low. If you're interested in this work, there's more in the paper, but I do think it shows that especially for new technologies, I think NHP models are pretty important for testing totally new approaches before you go into patients because xenographs are really not telling you how their counts are going to recover or how many clones there are necessarily. Ex vivo gene therapies, there's lots of hurdles to implementation.

Less than 60 patients have been treated since the Bluebird and Vertex approvals for sickle cell disease and thalassemia despite the really astounding clinical efficacy. There's patient related barriers. There's issues with HSPC collection. They often need multiple rounds of mobilization and apheresis because in presence of inflammation, mobilization is inefficient. This is unacceptable to patients being away from their families, being away from their jobs, lack of hospital capacity and expense. There's concern over infertility and secondary malignancies when you're using busulfan for conditioning.

They also mucositis, all the side effects of high dose chemotherapy. Then there's the socioeconomic barriers. In the US reimbursement system it's 1.5 to over $3 million per patient. That's obviously pretty difficult to imagine supporting widely and where sickle cell disease and thalassemia are more prevalent in the rest of the world there's just no way in terms of both the infrastructure and the funding. What are some of the potential solutions? One approach that at least maybe would take away some of the non-hematologic toxicities of transplant is antibody based conditioning, which has been pursued for more than 20 years, either targeting C KIT or CD45 with people at Stanford and Dave Skadden in Boston.

Work that was done collaboratively mainly by John Tisdale showed that CD117 ADCs were efficacious in macaques for getting very high level engraftment of gene edited cells, and we could show the animals were still fertile. C KIT, there was concern over that because WW mice, which are deficient in that pathway for both alleles are infertile. But we took edited female, edited male, bred them together, and there was no problem. We had babies. But there's been unexpected toxicities in the Magenta original clinical trial that was done in actually acute myeloid leukemia. It wasn't for transplant.

It was using an ADC against CD117 expressing AMLs to patients and the dose escalation died. Not clear really from what. They were heavily pre-treated patients, but Magenta shut down. The data has never been published, and it is really unfortunate. There's also a Jasper company was started out of Stanford that is a non-ADC. It's a CD117 non-conjugated antibody that uses ADC seed to wipe out stem cells. It works for situations where you don't need much ablation like an X skid, but Jasper has also cut back their pipeline significantly.

The other approaches in vivo gene therapies, potentially using viral vectors or lipid nanoparticles to deliver gene editing machinery to HSPCs. There's a lot of work going on that academically and in biotech right now, but it is very challenging in terms of HSPCs or needles in a haystack. T cells are pretty easy. You have a lot of T cells in your blood that you can put CRTs in with in vivo gene therapies, and there's already been clinical data presented in myeloma for in vivo CAR T generation. But HSPCs are much more difficult in finding the right target.

Andre Larochelle at NIH, who originally trained in my lab has been working with Mpl, the thrombopoietin receptor is a very good potential target. Just because I've run out of time, I'm going to skip this part. I just retired from the NIH, full disclosure, partly planned because I knew I was going to be president of ASH, and I thought I needed to be able to advocate to the NIH and to the FDA and the CDC when I wasn't a government employee. It turns out that was really prescient given what's happened with a different administration where I really wouldn't have been able to speak out.

I am still at the NIH in terms of Emeritus and finishing up some research projects and doing career development. But my "full-time job" now for the next year and a half is to be president of Ashton to advocate for what we need to try to keep hematology science going in the next couple of years. But I just want to thank all the people I trained in my lab over the years. I just gave a thing of the night when I retired and I looked up everybody, and there's a lot of people. But thanks to all of them, I had the individuals for each study, I think, on each slide. My outside collaborators also quite extensive, and this is pictures of everybody over time.

But I'm going to take the last couple of minutes to give a plug for what ASH is doing and to keep you informed about how you can get involved. Starting in spring of 2025, things obviously got pretty crazy, and especially in sickle cell disease where many grants were being canceled by DOGE, given like if you did a word search, you're going to find mRNA or you're going to find African American words in all those grants. A lot of them were canceled. We set up this fight for hematology that had a number of different components, advocacy greatly increasing our advocacy footprint, not just federally, but also in the statehouse.

Getting patient stories and investigator stories about the impact of what was going on and was having on them, especially Medicaid cuts for sickle cell disease and work requirements for Medicaid, which is just not feasible for many patients with severe sickle cell disease, amplifying the voice on social media and fundraising. I'm happy to send this to anybody. You can find this on the fight to hematology on the ASH website in terms of following some of the things we're doing.

But I think most importantly for investigators is that the last two years we've basically taken an extra $12 million from our reserves for two years to try to fill in some of the gaps in terms of funding research grants, both career development type grants, as well as filling in for RO ones, especially in areas that were cut in health outcomes research and sickle cell disease. We basically have grant programs at ASH for basically every level of training, whether MD clinical research or PhD in translational research or basic research. An extra 1 million for student and trainee grants, an extra 4 million in 92 recipients for ASH scholar awards.

People have been funded through that mechanism, but we need more. That's an extra 22. Then mid career and beyond what we used to call Bridge grants, which we stood up originally during the last financial crisis in science in 2008. But that required a pink sheet from NIH that you showed how you would use the money to show how you're going to respond. Can't even get pink sheets anymore. Now we changed the name, and they're called ARCH grants and Treating Fairly grants for M Career and Beyond, and we have an extra 37 recipients with 6 million.

We're going to keep this going as long as we can and as long as we need to. But therefore, we need people to support ASH through the ASH Foundation in terms of fundraising, which is going to be a major focus of mine, both during my presidency and I think after I finish because we were very lucky at the time all this happened that ASH had pretty good reserves because there have been so many new drugs in hematology over the last 10 years, and new drugs mean lots of pharma wants to come to the meeting and exhibit and pay us and sponsor symposia on the corporate day before the meeting.

We had accumulated money at the right time, even coming out of COVID, which was quite unusual. Most societies were not in good shape coming out of COVID, and we actually were. We felt very strongly we had to quickly try to do something. If anybody has questions about these grant programs, wants to apply, wants to know the timeline. It's all on the website, but I'm happy to also connect any of you to the right people on the ASH staff and donate to the ASH Foundation. Probably trainees in the room, that's probably not quite the right. But if you happen to start a startup next year and get an IPO, we can tell you what to help do with your money. Thanks very much.

Catriona Jamieson: You can see what I champion Dr. Dunbar is for all of us. I had a question about VAMP4. In the first three patients on the sickle cell anemia let globin gene study, there was integration next to VAMP4, and that's important for NK function. I think I've been totally brainwashed by Dan to think that NK cells may be important for immune surveillance. Do you think that may have had something to do with it because it would deregulate VAMP4 through integration?

Cynthia Dunbar: I don't believe they could find any dysregulation.

Catriona Jamieson: No. They just said it integrated next to it. I think they left it.

Cynthia Dunbar: But they did the book, and they couldn't find any impact on expression Looking at individual colonies and I don't think they did single cell. Again, there's so many things that people want to do with all those cells. Also the research side of Bluebird disappeared and all the people like Melissa Bonner that were there. It's been a little challenging, but John has looked and we just have not seen a problem when you have the enhancer globin enhancer that's only turned on during erythro development.

Now, there is splicing abnormalities that have been reported right in the first thalassemia patient, where there was a benign clonal expansion then disappeared in Michel Sadelain and Sloan Kettering's first patient. There was a myeloproliferative type gene that had abnormal splicing, but it went away and that has not been seen again. But we signed a lot of abnormal splicing with the plague that integration it was splicing events that were the problem, not just simply up regulation by general enhancer activity.