Represent some of the work that my lab has been up to in the last few years. And as you will see it's quite different and perhaps different from what you expect from our prior work. But it's sort of the next wave that I, I sort of view important in building on what we have done before. So today I will talk about really different shades of cancer immunisation and some strategies that we're developing to overcome these from multiple at different angles.
So just very briefly, you know if I had to summarize sort of what our lab is doing at this point, it's really you know doing large scale genomics to understand cancer genetics using single cell genomics and things like that. But that's really the beginning. But that's really the beginning. That's really the beginning. That's really the beginning. One is immunobiology and the other one is metastatic organotropism which is really critical to consider when we use an immune base or even other therapies. The approach,
as you will see in a minute also is typically so that our questions are really inspired by clinical problems and then broken down into models. Frequently you have to develop those models or methods to study these things systematically. And ultimately, of course, the goal is to bring this back to patients. So I divided the talk into three chapters that are loosely linked to each other, as you’ll see in a moment. And I want to start with the first one because I think it really
00:01:31.726 --> 00:01:34.220 exemplifies how we can use information
from large genomic data to inform
precisely what mechanisms and sort
of things we should study in the lab.
So a few years ago we were really
interested in understanding
mechanisms of resistance to immune
checkpoint inhibitors and you know
the details don’t matter so much,
but the approach was that we use single
cell RNA sequencing in these patients.
We looked at the cancer cells specifically
and we came up with this you know
signature which we called ICR signature,
immune checkpoint resistant signature
and again the the genes don’t matter
so much but you know and I think
we learned a lot in this study but
what we were left with is a hypothesis that came out of the data
that was that this cancer cell intrinsic program was somehow conferring T cell exclusion or poor T cell infiltration
and impaired T cell activity.
But when you have these gene lists
which I know all of you have been at,
at one point in your project,
the question is how do you actually prioritize what you’re going to
look at right and and what not.
And typically what we do and this
is what we had done for this paper, we focus on something that’s plausible for which there are reagents or other practical or pragmatic reasons. But we really wanted to make this process of how we validate things a bit more unbiased and to do that we developed this method which we dubbed Perturb site seek. So basically what this method allows you to do is couple CRISPR CAS 9 perturbations with single cell RNA and protein profile using the site and a 10X genomics platform. So what you would get from this method would be that you could perturb a
gene and then ask well what does it do to the entire cells transcriptome and part of the surface proteome. And the way we use this method to validate some of those findings that we had in patients is leveraging it in in patient derived models where we had isolated in addition to doing the sequencing the cancer cells from a Melanoma patient and the tumor infiltrating lymphocytes. The advantage is that this is a fully autologous system. It doesn’t require any sort of engineering if you will to do a
Co-culture experiments into these cancer cells we generated. We generated a library that would target each of the genes that we had identified in the signature in patients. So we could test in one you know pooled experiment the relevance of any one of those 248 genes that were in the signature. And not only that but also we would get you know the transcriptome and part of the surface podium. And the premise here would be that cells that survive co-culture with their autologous T cells must harbour a perturbation that confers that mechanism. And this is exactly what we did.
And I'm just going to give you this is of course published at this point. But what this would get us is a sense of the immune fitness and the phenotype associated with this. I'm just going to give a snippet of results because I want to show you how this helped us inform kind of what we did next in the last couple of years. So here we’re looking at gene knockouts on the bottom. So genes knocked out here associated with increasing fitness against autologous T cells or tilts. And on the Y axis here we...
increased immune pressure.

So there's an effector to target ratio of 1 to the one and four to one and so on.

And as you would expect, mutations or deletions in all of the Red Arrows, all of those had been known before, right.

Mutations or deletions in those genes are strongly associated with immune evasion and they had been associated clinically with resistance to immunotherapy.

So that was really good because it validated our approach that we
00:05:24.843 --> 00:05:26.235 could recover all of those hits.

00:05:26.240 --> 00:05:27.552 Basically in one experiment,

00:05:27.552 --> 00:05:29.520 we got really interested in another

00:05:29.575 --> 00:05:30.960 hit that was less expected.

00:05:30.960 --> 00:05:33.552 That was loss of a gene called CD 58.

00:05:33.560 --> 00:05:34.160 So I’m going to get,

00:05:34.160 --> 00:05:35.756 I’m going to talk about that more.

00:05:35.760 --> 00:05:36.720 But on the flip side,

00:05:36.720 --> 00:05:38.808 we could also couple how these

00:05:38.808 --> 00:05:40.200 perturbations change the phenotype.

00:05:40.200 --> 00:05:41.664 And as you can imagine this

00:05:41.664 --> 00:05:43.330 is a huge matrix, you know,

00:05:43.330 --> 00:05:45.400 perturbation by gene expression by by,

00:05:45.400 --> 00:05:46.400 you know, protein profile.

00:05:46.400 --> 00:05:48.479 So I’m just going to show you a tiny,
tiny snippet from that which I want to use to guide you to the experiments that we did. So the way to read this here is that on the bottom you have knockouts of those genes and then on the Y axis you have a few selected features that I want to present. For example, if you knockout CD 58, then there will be no CD 58 protein left in that cell. Logical. But what was really interesting was the observation that cells that lose this gene, CD 58 had concurrently more protein by of a gene encoded.
by a protein encoded by CD 274, which of course is PDL 1. So that seemed like a double whammy. You lose something good that confers immune evasion, but then you also gain something that is a Co inhibitory ligand of course. So what is CD58? It turns out we actually don’t know that much about it in cancer. What we do know physiologically is that it is a Co stimulatory protein that ligates to CD2 on T cells, and when it does so, it can become actually the most
potent Co stimulatory protein. So loss of this gene of protein, it’s plausible that that could result in immune evasion in a number of different ways. We sought to validate this and this is work that has since been led and then published by my first MDPHD student who just graduated a few months ago. Here basically what she did is she took these Melanoma cells, she knocked out CD58 and then she Co cultured the cells with autologous T cells or engineered T cells at that point. And as you can see loss of the
gene in fact convert a better survival of these cancer cells. And when you rescue these, the gene, either it’s GPI anchored or transmembrane isoform, then you rescue the sensitivity to T cell cleaning. Furthermore, we also wanted to demonstrate that this interaction with CD2 was in fact required for this immune evasion phenotype and that, you know, CD58 loss didn’t confer a loss of fitness through some other...
mechanism that we didn’t know.

So to test this we repeated the same experiment that I show on the left, only this time we rescued the knockout cells with a variant of CD58 harboring and mutation K34A which is unable to actually to CD2. And as you can see when you rest, the cells continue to be resistant to D cell Co culture suggesting that this is very specific to that interaction. So one of the reasons I think this gene is not well understood is that there is no known mouse homolog.
models that we typically like to use in, you know, studying, you know, immunotherapy and so on syngenic model. So to to you know, circumvent this, what we did is we used an immunocompromised mouse that has transgenic expression of human Illinois 2, which of course is required for T cell survival in vivo. So into these animals we could implant genetically modified cancer cell lines and then adoptively transfer the mouse with the patient’s own tilts, right. So we could study this interaction.
in vivo and as you can see, the tumors that had the CD 58 loss were completely resistant to ACT. They also had an approximately hundredfold lower infiltration with T cells, validating some of the predictions that we had made in patients. And all of these effects could be rescued by RE expressing CD58 in the cancer cell. So overall this suggested that loss of CD 58 on the cancer cell conferred impaired T cell infiltration, proliferation and resistance to ACT. So coming back to that interesting interaction which I mentioned earlier, this interaction between CD58 and PDL 1.
So we did a very simple experiment in which we knocked out CD58 and simply asked how much PDL one is on the surface of these cells. And in fact, when we knocked out CD58, we found that these cells do have more CD58, excuse me, PDL one protein on the surface. And this effect again could be rescued by expressing CD58 itself. So the question then is, you know what sort of regulates this interaction and how do you go about this? Because there’s no like nothing really to help inform of where to even start, right.
What we could exclude pretty quickly is that there was no direct interaction between the proteins. So there had to be some sort of mediator that regulates that. So to do this systematically we did a genome scale loss of function screen that would show us or point us towards genes or proteins that are required for this interaction. So the design of the screen was that we took these Melanoma cell lines that express CAS nine and then we introduced the genome scale guide library to knock on every gene in the genome, let the cells, you know,
edit for a couple of weeks.

And then we sorted out the CD 58 negative or CD 58 positive cells,

sequenced the guide RNH guide RNAs in each of these pools.

And the premise here is that in the CD 58 low pool,

there must be perturbations that are somehow involved in regulating CD 58.

And this is precisely what we saw.

So here is a result of that screen.

Reassuringly,

the top hit of the screen was
knockout of CD58 itself, knockout CD58. There will be CD50 negative and a bunch of others. The one that really caught our attention, but we because we saw it also to physically interact with CD58IN in a mass spec Co IP screen, is this gene or protein called CMTM 6 super interesting? I know David had actually done some work that looked at the prognostic value of this protein, but it was unclear and I think we have the answer to why that might be So what was really interesting,
just, you know, a year or two before we had made this observation, there were two Nature papers published showing that the same gene or protein CMTM 6 was in fact required for maintaining PDL one protein on the surface. So this was a plausible, you know, logical sort of hit to go after and this is what we did. So when we knockout CMTM 6, we see a reduction in both CD58 and PDL one protein surface abundance. And when you rescue the gene CMTM 6, you rescue that and you have you bring them back to the baseline.
And to really prove that this is required for the interaction, we generated a number of additional genetic perturbations, double mutants which we rescued where we rescued only one gene at a time where we could in fact demonstrate that CMTM 6 was required for mediating this reciprocal interaction. You know the the issue with these types of down signals, right, because ultimately that’s always the goal.
So I’m going to skip a lot of data that we show in the paper. But Long story short, we identify we found that the binding sequences on CD58 and PDL one for CM takes actually CMTM 6 actually differ and so we imagined that we could leverage that knowledge. So it turns out that CMTM 6 binds to a specific amino acid domain spanning the amino acids 20 to 32. So when we scramble that area, turns out that, you know, CMTM 6 can no longer bind to PDL 1.
So our we imagined that if we could selectively disrupt the interaction between PDL one and CMTM 6 at that site, it should result in reduction in PDL 1 without affecting the levels of CD 58. And this is precisely what we saw. So we took these Melanoma cell lines, we knocked out PDL One, and then we rescued either the wild type orph or a orph where we scrambled that region that is unable to bind CMTM 6. And as you can see, the WILD type orph rescues PDL ONE expression, but the mutant does not. And then in a Co-op experiment, we could also directly show that this
00:14:19.502 --> 00:14:22.274 variant where we scramble that sequence
00:14:22.274 --> 00:14:25.030 is unable to has a significantly
00:14:25.030 --> 00:14:28.033 lower binding of Co-op to CMTM 6.
00:14:28.040 --> 00:14:31.238 So just to summarize this part,
00:14:31.240 --> 00:14:33.256 I hope I was able to show you that
00:14:33.256 --> 00:14:35.572 we were able to go from you know
00:14:35.572 --> 00:14:37.624 sequencing data to using the right
00:14:37.624 --> 00:14:39.479 functional tools to really inform
00:14:39.480 --> 00:14:42.077 precisely kind of what to go after.
00:14:42.080 --> 00:14:43.781 But you know what we were left
00:14:43.781 --> 00:14:45.239 with is actually the question,
00:14:45.240 --> 00:14:47.320 how many of these interactions do we miss,
00:14:47.320 --> 00:14:49.342 right. Every time you knockout a
00:14:49.342 --> 00:14:51.520 gene and you observe A phenotype,
00:14:51.520 --> 00:14:53.200 you know how do we know that
that’s not mediated through a number of these interactions. And the clinical or therapeutic correlate of that is, you know giving somebody a single agent immunotherapy or you know even 2 drugs and asking well what do these, what does inhibition of these two proteins do to everything else that’s going on, on the surface or within within the cell. And this is actually something we’re trying to address systematically.

So switching gears a little bit and coming to the second chapter which is a much, much more recent chapter in the
NOTE Confidence: 0.929347958571429
00:15:25.168 --> 00:15:28.252 lab that is leveraging novel base editing tools to hopefully improve cell based immunotherapies.
NOTE Confidence: 0.929347958571429
00:15:35.040 --> 00:15:37.824 So all of you know that cell based immunotherapies are now a critical component of the treatment of many hematologic malignancies and most recently there was also an approval for the treatment of till transfer for patients with Melanoma.
NOTE Confidence: 0.929347958571429
00:15:46.123 --> 00:15:48.570 And you know the typical you know workflow is so that you take something out of the patient and don’t do something with the cells and you put them back in.
NOTE Confidence: 0.929347958571429
And in the context of CAR T cells, of course that’s taking PBMCS and putting a CAR into the cells and reinfusing them. In the context of till therapy, it is isolating tills from metastatic lesions, expand them ex vivo and then give them back to the patient so that they have in common. What they also have in common is the observation that has really emerged in the last few years is that there are very specific T cell features before you put this therapy into the patient that are strongly predictive of whether or not that
cell product is going to work. And this was published by the Rosenberg Group A few years ago. And there’s nothing shocking about some of the observation. But it really is sort of the rationale for thinking about how we can improve T cell function itself to build better cell cell therapies on top. And of course, we’re not the only ones thinking about. There’s a lot of groups take CAR T cells until therapies and engineer them in a number of different ways.
frequently in the last few years
NOTE Confidence: 0.929347958571429
what people have done is, you know,
NOTE Confidence: 0.929347958571429
for example,
NOTE Confidence: 0.929347958571429
knocking out an inhibitory receptor
NOTE Confidence: 0.929347958571429
such as CTLA 4, right?
NOTE Confidence: 0.929347958571429
And I picked one study from Carl
NOTE Confidence: 0.929347958571429
Jun’s group could have picked,
NOTE Confidence: 0.929347958571429
you know,
NOTE Confidence: 0.929347958571429
hundreds of other papers where they
NOTE Confidence: 0.929347958571429
try to improve CAR T cell therapy by
deleting some of these inhibitory receptors.
NOTE Confidence: 0.929347958571429
But the challenge with knocking
NOTE Confidence: 0.929347958571429
out a gene especially in T cells
NOTE Confidence: 0.929347958571429
is that one of the off target
NOTE Confidence: 0.929347958571429
effects of CRISPR CAS 9,
NOTE Confidence: 0.929347958571429
which is due to the double
stranded DNA breaks that take place is that you actually get a pretty high rate of aneuploidy.

So 7 to 14% of cells in a pool that you you know engineer with CRISPR CAS nine will be anemployed. And of course aneuploidy or chromosomal instability as I will talk about later is is a hallmark of cancer. And that comes with all sorts of concerns. Of course, what got me really interested in thinking about how to improve cell therapies are papers like the one that I’m showing you here from the Jonathan Powell group.
So rather than deleting a gene or over expressing a gene which comes with other issues, you know, they made a really interesting observation that is they found that some of their mice had sporadic mutations in a gene called TSE 2. The mutation itself really doesn’t matter so much. But what they were able to show is actually when you take T cells from the mouse that has this germline mutation and you adoptively transfer mice with the wild type gene that harbor Melanoma tumors, those mutant T cells are much, much more potent in eliminating not only
NOTE Confidence: 0.929933964166667
00:18:38.815 --> 00:18:41.391 melanomas but they also show this in
NOTE Confidence: 0.929933964166667
00:18:41.391 --> 00:18:44.080 context of leukemias and and other diseases.
NOTE Confidence: 0.929933964166667
00:18:44.080 --> 00:18:46.054 So what that suggested to us is
NOTE Confidence: 0.929933964166667
00:18:46.054 --> 00:18:47.839 that maybe we don’t have to,
NOTE Confidence: 0.929933964166667
00:18:47.840 --> 00:18:48.320 you know,
NOTE Confidence: 0.929933964166667
00:18:48.320 --> 00:18:49.760 take the wheels off the car.
NOTE Confidence: 0.929933964166667
00:18:49.760 --> 00:18:52.112 I mean knocking out an entire gene with
NOTE Confidence: 0.929933964166667
00:18:52.112 --> 00:18:54.559 all of its unintended consequences,
NOTE Confidence: 0.929933964166667
00:18:54.560 --> 00:18:57.320 maybe it’s sufficient to introduce very
NOTE Confidence: 0.929933964166667
00:18:57.320 --> 00:19:00.140 specific mutations in genes that will
NOTE Confidence: 0.929933964166667
00:19:00.140 --> 00:19:02.400 significantly alter T cell function.
NOTE Confidence: 0.929933964166667
00:19:02.400 --> 00:19:04.506 And this is the hypothesis that
NOTE Confidence: 0.929933964166667
00:19:04.506 --> 00:19:07.085 we sought to test in this
NOTE Confidence: 0.929933964166667
00:19:07.085 --> 00:19:09.275 project that just like in mice,
NOTE Confidence: 0.929933964166667
00:19:09.280 --> 00:19:12.059 there must be either naturally a cure
NOTE Confidence: 0.929933964166667
occurring or synthetic protein variants that may enhance T cell function and therefore may enable the production of more effective cell therapies on top. And the method that you know would be required to do that, One of the ways to do that is using base editors. So there’s different types of base editors. These are CRISPR CAS 9 dependent base editors that either citidine or adenosine deaminase linked proteins that unlike CRISPR CAS 9 don’t introduce double stranded DNA breaks but rather they induce deamination events in very specific windows guided by guide RNAs.
And ultimately in the example of citadine base editors you get C to T changes and in the context of denizine base editors you get A to G changes. So what this allows you to do is introduce at some specificity mutations at defined loci in a gene rather than knocking out the gene. And one of the challenges that that had existed with these base editors was the issue that their efficiency, especially in primary human T cells, was rather low. So we This was led by an MDPHD student in my lab.
Zach Walsh has shown all the way to your left. He took it upon himself to try to improve the efficiency of these base editors because these would be the right tools to really introduce some of these mutations in a targeted fashion. And he’s done that through a really sort of smart way of delivering the base editor. I’m not going to go through all the details, but suffice it to say, you know, we’re able to achieve extremely high efficiency, relatively speaking, between 80% and 99% with these base editors introducing very precise variants.
This is a paper that is accepted, that will be published next week. But so, you know, equipped with these methods, we then imagined, well, what do we want to actually, you know, edit in these T cells? We can’t do this on the genome scale. There’s too many bases, right? It would be an impractical experiment. So what we decided to do is rather be guided by experiments of nature. And what I mean with that is, you know,
there’s a lot of variants out there that are reported to be either definitively associated with immune, clinical, immune syndromes, either autoimmunity or immunodeficiency and everything in between or variants. And these are most of them that are variants of uncertain significance where there may be an association, but we don’t know exactly because we can’t prove each of them, you know, emotionally one at a time. So what we decided to do is put together sort of a library of 30,000 variants that are out there across 102 genes spanning all major T
cell functions and introduce all of them with these base editors in a massively parallel fashion. And then ask how each of these variants changes known hallmarks of T cell mediated anti tumor immunity including the activation of T cells, the proliferation, cytochrome production, long term expansion, persistence, etcetera. And then the premise here is that we want to identify variants from this pool that improve most and perhaps all of those favorable features that we know are important to build.
00:22:41.520 --> 00:22:44.120 good cell therapies on top.
NOTE Confidence: 0.903999559666667
00:22:44.120 --> 00:22:46.248 And so just you know a word
NOTE Confidence: 0.903999559666667
00:22:46.248 --> 00:22:47.160 about negative control.
NOTE Confidence: 0.903999559666667
00:22:47.160 --> 00:22:48.290 So in addition to the
NOTE Confidence: 0.903999559666667
00:22:48.290 --> 00:22:49.194 variants that we introduced,
NOTE Confidence: 0.903999559666667
00:22:49.200 --> 00:22:50.630 we had a number of
NOTE Confidence: 0.903999559666667
00:22:50.630 --> 00:22:51.418 different negative controls.
NOTE Confidence: 0.903999559666667
00:22:51.418 --> 00:22:52.663 I think it’s always important
NOTE Confidence: 0.903999559666667
00:22:52.663 --> 00:22:54.110 to think about these when you
NOTE Confidence: 0.903999559666667
00:22:54.110 --> 00:22:55.200 do these large scale screens.
NOTE Confidence: 0.903999559666667
00:22:55.200 --> 00:22:56.800 So here’s a distribution and
NOTE Confidence: 0.903999559666667
00:22:56.800 --> 00:22:58.894 the log fold change of negative
NOTE Confidence: 0.903999559666667
00:22:58.894 --> 00:23:01.039 controls that we had introduced.
NOTE Confidence: 0.903999559666667
00:23:01.040 --> 00:23:03.240 So these include splice acceptor
NOTE Confidence: 0.903999559666667
00:23:03.240 --> 00:23:05.000 and splice donor variants.
NOTE Confidence: 0.903999559666667
00:23:05.000 --> 00:23:07.170 Or when you when you mutate these
these splice sites then what you get is truncated proteins that get basically knocked out or lack of a better word or they get truncated proteins that that are non functional. So as you can see these are significantly depleted at a very high lock fault rate ratio, while mutations that introduce silent changes or empty window changes don’t change the distribution at all. In addition, another good control for T cells is introducing mutations that result in disruption of the
CD3 complex because the cells need the complex to be activated and proliferate and do what they do. And these are also significantly depleted and this was highly consistent between different donors that we did the screen, so we did them multiple donors. So here are a few results from that screen now and I'm going to show a couple of very selective ones. So here we are looking at the lock fold, a change of genes and the designated variants that were introduced and the you know the negative lock 10 FDR. So the higher you go the most statistically
significant things were in the screen
and again there were many sort of
expected depleted genes such as CD3
and you know row A and what have you.
But what caught our attention was this
number of these mutations that were
enriched meaning they improved T cell
function were found in the PIC three
and this time looking at the amino
acid sequence from left to right
These two genes encode for the two domains
of the immune cells specific PI3K delta.
And this is another way to look at is this
time we’re only looking at pick three CD,
this time looking at the amino
acid sequence from left to right
and where each of these mutations either enrich on the top or deplete in the screen on the bottom. And as you can see, there’s a number of enriched variants here that are associated with a favorable TC cell phenotype, you know spanning residues 524 to 529. But then this is also this other variant, the C416R that was strongly enriched. And on the flip side, we have a mutation that causes a loss of function of this gene. When you look at where these function mutations are located,
they're not anywhere in the kinase domain.

As you might imagine, since this is the catalytic sort of subunit of PR3K delta,

they are all aligned and this is a prediction from alpha fold.

They're all aligned at the interface between these two gene products of pick three CD and pick 3R1.

You know one of the expectations would be that you have higher output from the PI3K pathways.

we of course then went on to to validate these observations.

You know one of the expectations would be that you have higher output from the PI3K pathways.
So we looked at downstream signalling at phosphor akt and phosphorus 6 and we tested many different variants. But I’m just highlighting here in the red box the loss of function mutation and then the green box the gain of function mutation. And as you can see the gain of function, you see more phosphor AKT and more phosphorus 6 while it is both of these are reduced in the loss of function. In the same vein and again I highlighted that with the green boxes the gain of function and in the red boxes the loss of function.
And this is just a selection of the data. But we can see that the gain of function variant was associated with improved TNF alpha production and proliferation and so on. You know an initial validation of the of the screen. So now of course the question is can we use this information and improve cell therapies? Can they be better of cell killers? And to do this we used a simple coke culture experiment like the one that I had presented to you earlier. Only this time we engineered the T
cells to express a very specific T cell receptor against Nye cell one, which is a commonly expressed neo antigen on Melanoma.

So we can really test the specificity and then we either use the native T cells or we introduced one of a number of variants that we had identified in the screen and then Co culture them. And as you can see again the gain of function in green was strongly associated with a higher degree of Poly functionality here summarized as the fraction of cells that expressed TNF alpha, renzon B and IL 2 while the loss of function showed a reduction.
And then this also translated in improved license of Melanoma cells. So here I think you can see my cursor. Yep. So here is the gain of function variant and we're looking at the number of surviving Melanoma cells. As you can see that strongly reduced the loss of function does not enhance the activity of the T cells. What was really gratifying and this sort of closing the loop to the first part of my talk, we were also able to show that this gain of function variant was able to
overcome resistance from CD58 loss.

So we did all of these coke culture experiments, all repeated them, only this time we knocked out CD58 and then the coke culture and the C416R, the game function variant and T cells was in fact able to almost completely radical themselves and you know, without the labouring the point too much.

We also tested the same strategy in a number of different CAR T cells and a number of different CAR T cells and we find the exact same thing whether you use the CD9 CAR or CD22 CAR against different leukemia models.

Introducing these variants in the T cell that is the basis for making that
product improved their functionality and their ability to lyse these. Looking as summarise this portion of the presentation, Hope was able to show you that we are now able to base edit primary human T cells with a high efficiency that unbiased discovery of variants may be able to identify those that improve T cell function and those perhaps could be used to improve cell therapies broadly in the future. OK. So now I’m going to switch to a somewhat different area of the
lab or work in the lab that we’re doing.

But you know the common theme is that we are interested in what causes immune evasion and lack of response to immunotherapies. And the reason we got into this, as you all of you know, brain metastasis are a common problem across cancers, but very common in Melanoma. In fact, the incidence is probably as high as 75% in patients who have advanced disease. And while the combination of immunotherapies are you know, showing efficacy against brain metastasis, there’s still a lot of work to do.
You know one of the reasons for that is that those regimens are very toxic and despite the activity in some patients we still see forms of Immunivision that seem to be pretty distinct in the brain. So the motivation was really to study a brain metastasis but I’ll show you how that sort of got us into this field of chromosomal instability. So a couple years ago we published a paper in this was led by Jana Johannes and Yiping postdocs in my lab where we asked a simple question in patients, what is the difference between an...
untreated brain metastasis and an untreated extracranial metastasis.

What we didn’t want is any sort of therapeutic intervention in between.

We’re really interested just in the salient biology which has been quite poorly described actually in patients compared to other you know areas in Melanoma at least.

And I just want to point out a couple of sort of results from this paper.

The first one is when we compare Melanoma brain Mets MBM versus extracranial Mets ECM, we found that the brain Mets were had a higher fraction of the genome altered FGA and that is a surrogate or a
NOTE Confidence: 0.86158250875
00:31:12.697 --> 00:31:14.917 process called chromosomal instability.
NOTE Confidence: 0.86158250875
00:31:14.920 --> 00:31:16.336 What is chromosomal instability?
NOTE Confidence: 0.86158250875
00:31:16.336 --> 00:31:18.760 It is a hallmark of cancer.
NOTE Confidence: 0.86158250875
00:31:18.760 --> 00:31:21.935 It’s rather broadly seen across almost every solid tumor.
NOTE Confidence: 0.86158250875
00:31:21.935 --> 00:31:24.237 And one of the ways by which chromosomal instability can arise is through errors that cancer cells make during anaphase, where they don’t segregate chromosomes properly so that one of the data cell you know is left with more and the other one with less material.
NOTE Confidence: 0.86158250875
00:31:27.413 --> 00:31:29.804 And one of the ways by which chromosomal instability can arise is through errors that cancer cells make during anaphase, where they don’t segregate chromosomes properly so that one of the data cell you know is left with more and the other one with less material.
NOTE Confidence: 0.86158250875
00:31:32.400 --> 00:31:34.280 where they don’t segregate chromosomes properly so that one of the data cell you know is left with more and the other one with less material.
NOTE Confidence: 0.86158250875
00:31:34.280 --> 00:31:36.759 cell you know is left with more and the other one with less material.
NOTE Confidence: 0.86158250875
00:31:36.759 --> 00:31:38.959 right, and chromosomal civility, sort of the perpetual dynamic.
NOTE Confidence: 0.86158250875
00:31:42.960 --> 00:31:44.396 The end product of this is aneuploidy, right, and chromosomal civility, sort of the perpetual dynamic.
NOTE Confidence: 0.86158250875
00:31:44.396 --> 00:31:46.191 sort of the perpetual dynamic.
NOTE Confidence: 0.86158250875
The process that gives rise to that.

The extra material in the one of the data cells is frequently packaged, if it survives, is frequently packaged in so-called micronuclei.

So one of the ways to quantify chromosomal instability more functionally beyond just genomics is actually look at the frequency of those micronuclei. And this is what we did.

This is in the same study where we had cell lines that were derived from either a brain or an extracranial metastasis from the same individual.

We enumerated the rate of micronuclei.
and as you can see the one from the brain in fact had more micronuclei compared to the one that came from a lymph node in this case. And when we put these cells back into animals in immunocompromised mice, those cells in fact are more likely to cause brain metastasis in the mouse than those that come from outside the brain. The second sort of a key result from the study when we looked at the microenvironment was the observation that brain that appeared to have a much more rhotomogenic myelod compartment as you can see both from the single
cell data to the left here. And then also we validated this in two independent patient cohorts by Multiplex immunofluorescence. So this is all in Melanoma.

The question then of course is you know what about other common cancers that frequently you know metastasize to the brain, the most common one in terms of prevalence is non small cell lung cancer. So naturally we're interested in asking do some of these concepts also apply to non small cell lung cancer. And the answer is and this is sort of in an analogous study that we're trying to publish right now.
We also asked the same question in that disease and this time though we had a lot more data. This time we could leverage data from the MSK impact cohort where we had genomic data that was linked with the location where the disease. So there’s lots of primary tumors and then a bunch of different metastatic sites and then all the way on the right here you see again brain metastasis have the highest fraction of genome altered, again a surrogate for chromosomal instability. We went on to validate this and
a couple of additional published cohorts that are out there as well as in a very large cohort of nearly 9500 patients where we had whole exome and RNA sequencing. Through an industry collaboration, we find the exact same observation that brain Mets are more unstable than extracranial Mets which are more unstable than the primary tumor. So clearly this process seems to be important in conferring a sort of aggressive phenotype and perhaps also in modulating the immune environment in an unfavorable way.
But it’s kind of an obscure concept to study, right, because it’s such a perpetual dynamic process. How do you go about actually studying that? I think the first question that we as ourselves is well what is a good model to use to study this and what is a good model that we could sort of easily identify, right. So the way we approach this problem is we looked at public data again TCGAACR genie, CP tag and we asked which subsets of lung non small cell lung cancer.
are particularly chromosomally unstable and are defined by very distinct genomic subsets. And it turns out that one particular mutation or loss in a gene called LKB one also known as STK 11 was across the board associated with a higher rate of chromosomal instability. What you also need to know about this particular subset of non small cell lung cancer which is common is that these patients virtually never respond to immunotherapy. So this is from a paper published from MD Anderson where they looked at patients with or without
00:35:44.277 --> 00:35:46.197 mutations or deletions in SDK 11.
00:35:46.200 --> 00:35:47.800 And as you can see here in red,
00:35:47.800 --> 00:35:50.340 these patients do extremely poorly
00:35:50.340 --> 00:35:52.880 in response to PD1 inhibition.
00:35:52.880 --> 00:35:56.048 And lastly,
00:35:56.048 --> 00:36:00.920 this particular subset happens
00:36:00.920 --> 00:36:03.356 to also more frequently be
00:36:03.360 --> 00:36:06.656 associated with brain metastasis.
00:36:06.656 --> 00:36:10.038 So you know, with this information,
00:36:10.040 --> 00:36:14.190 we believe that this particular subset is a
00:36:14.253 --> 00:36:16.598 really good archetypical sinhi chromosomally,
00:36:16.600 --> 00:36:18.040 that that we want to understand better.
another MDPHD student in my Lac lab, taking upon herself a couple of years ago and she started with a couple of very simple experiments. We brought in a few human cell lines which are shown here and two of them are LKB 1 deficient, the other one LKB 1 proficient. And we did imaging and enumerated the rates of these micronuclear. I’m showing you here 2 exemplary ones. And as we would predict from the genomic data, the LKB 1 deficient subset in fact had more of these micronuclear, suggesting that there are in fact
more chromosomally unstable.

We also got 2 cell lines from Quoc Wong at NYU where he had, you know, established the the KP model, you know which have the K Ras mutation and P53 mutant. But on top of that they had developed a model with deletion of LKB one and derived A syngenic cell lines. So we got those into the lab as well and find that the LKB 1 deficient line in fact was more chromosomally unstable. So this seems to be shared between both the human and the available best available mouse models.
So now coming back for a second

NOTE Confidence: 0.86652967233333

to these micronuclei,

NOTE Confidence: 0.86652967233333

as I mentioned earlier they are

NOTE Confidence: 0.86652967233333

you know extra material that are

NOTE Confidence: 0.86652967233333

engulfed in these mini nuclei.

NOTE Confidence: 0.86652967233333

But the the,

NOTE Confidence: 0.86652967233333

the envelope of these micronuclei

NOTE Confidence: 0.86652967233333

is very rupture prone.

NOTE Confidence: 0.86652967233333

So the DNA within those is released

NOTE Confidence: 0.86652967233333

at some rate into the cytosol which

NOTE Confidence: 0.86652967233333

is an absolute no go

NOTE Confidence: 0.86652967233333

when it comes to you know normal

NOTE Confidence: 0.86652967233333

immunity where you know our bodies

NOTE Confidence: 0.86652967233333

this is highly conserved are

NOTE Confidence: 0.86652967233333

trained to sense DNA in the cytosol

NOTE Confidence: 0.86652967233333

from all sorts of infections for
example and respond to that.

And and you know cells do that very efficiently through several pathways, perhaps the most important one being the C gas sting pathway.

So here C gas senses cytosolic DNA and well lights off convert, convert this DNA to C gam which binds to sting and ultimately triggers a cascade that typically will result in the production of type 1 interferons which of course a very potent antiviral and anti tumor activity. Now this is only true when you activate the pathway very briefly.
but this is not really what we see in cancer, right? Because chromosomal instability is perpetual, this pathway is tonically activated. And it turns out that when you do that, you actually flip the entire pathway on its head. And this is what I want to demonstrate in the next few slides and how we might be able to use this information to target this process. So when you tonically activate the pathway, you in fact see less type 1 inference production and you see a more aggressive phenotype sort of flipping the pathway on its head.
So we wanted to test this hypothesis and we did a couple of simple experiments. First, we asked are LKB 1 deficient cells in fact less capable of activating type 1 interferon related pathways. And this is what we have these cell lines that we stimulated with double stranded DNA as sort of surrogate for chromosomal instability. And then we looked at a couple of key downstream nodes from in the C gasting pathway, phosphor TBK one and phosphor IR 3.
As you can see the LKB 1 proficient Syn low cell lines are able to do that rather efficiently while the LKB 1 deficient lines do not. This results in a significant impairment in the LKB 1 deficient lines with respect to a couple of important Type 1 interference. I'm just showing you a couple selected ones. So suggesting that these, the Syn high state in fact confers impaired production of type 1 interference. So how can we now prove that it is impaired this impairment? So one way to do this is by modulating either up or down the
rate of chromosomal instability. And one way to do this is by over expressing a variety of different genetic constructs which had been previously established. So in this example we can take a cell line that is highly chromosomally unstable at baseline LKB 1 deficient and over express a gene called MCAC, also known as KF2C, which improves the segregation fidelity that cells have when they undergo A chromosome. Segregation in so many words reduce the number of errors that these cells make.
and therefore on a population level, the rate of chromosomal instability, which is again measured here as the number of frequency of micronuclei. So when you suppress chromosomal instability, that alone is sufficient to rescue the ability of these cells to again produce type 1 interference. You can do the converse experiment where you take a cell line that is relatively chromosomally stable and express them with a different construct that makes them more unstable, as shown here again as a measure of micronuclei. And that alone is sufficient to reduce
their ability to produce type 1 interference, suggesting that it is really sin that’s driving the ability or inability of these cells to properly signal through this pathway and produce type 1 interference. The other way to approach this of course is to imagine either deleting genetically or pharmacologically inhibiting C gas. If you don’t have C gas, then you won’t be able to tonically activate the pathway downstream.
00:41:59.409 --> 00:42:01.557 deletion of pharmacological inhibition,
NOTE Confidence: 0.872830699375
00:42:01.560 --> 00:42:03.810 you might be able to allow this thing to
NOTE Confidence: 0.872830699375
00:42:03.810 --> 00:42:06.568 come back to an equilibrium where you can
NOTE Confidence: 0.872830699375
00:42:06.568 --> 00:42:08.535 leverage its physiological function which
NOTE Confidence: 0.872830699375
00:42:08.535 --> 00:42:10.680 is producing these important cytokines.
NOTE Confidence: 0.872830699375
00:42:10.680 --> 00:42:12.696 So we tested this in a number
NOTE Confidence: 0.872830699375
00:42:12.696 --> 00:42:13.560 of different ways.
NOTE Confidence: 0.872830699375
00:42:13.560 --> 00:42:16.668 One is by deleting Cgas and then
NOTE Confidence: 0.872830699375
00:42:16.668 --> 00:42:19.296 stimulating the cells with the with
NOTE Confidence: 0.872830699375
00:42:19.296 --> 00:42:22.117 its natural product that is CGAM O,
NOTE Confidence: 0.872830699375
00:42:22.120 --> 00:42:22.945 we delete Cgas.
NOTE Confidence: 0.872830699375
00:42:22.945 --> 00:42:25.154 We let the cells sort of relax for
NOTE Confidence: 0.872830699375
00:42:25.154 --> 00:42:27.143 a week or two and then we ask was
NOTE Confidence: 0.872830699375
00:42:27.208 --> 00:42:29.469 that enough to bring the pathway to
NOTE Confidence: 0.872830699375
00:42:29.469 --> 00:42:31.403 an equilibrium and stimulate it and
NOTE Confidence: 0.872830699375
00:42:31.403 --> 00:42:33.329 show that they’re now again able
00:42:33.329 --> 00:42:35.440 to produce type 1 interference.

00:42:35.440 --> 00:42:37.316 And this is precisely what we find.

00:42:37.320 --> 00:42:39.904 So after a few days of deleting C gas,

00:42:39.904 --> 00:42:42.080 genetically stimulating the cells with C
gaps or stimulating sting in this case,

00:42:42.080 --> 00:42:44.372 we see that these cells regained their
ability to produce Type 1 inference.

00:42:44.372 --> 00:42:46.719 And this is both true in the human as
well as in the mouse models that we use.

00:42:46.720 --> 00:42:48.974 And lastly we,

00:42:48.974 --> 00:42:50.959 we tested these concepts also in vivo.

00:42:50.960 --> 00:42:52.282 And this is both true in the human as

00:42:52.282 --> 00:42:54.758 well as in the mouse models that we use.

00:42:54.760 --> 00:42:56.948 And lastly we,

00:42:56.948 --> 00:42:59.720 So here we use the KL,

00:42:59.720 --> 00:43:01.760 the LKB 1 deficient line and

00:43:01.760 --> 00:43:04.610 implanted them into B6 mice.
We treated these animals either with isotype control or with an anti PD1 antibody and just like in patients there is absolutely no response to PD1 inhibition and these LKB1 deficient Synthi tumors. Deleting Cgas alone was sufficient to partly reduce the growth rate of these tumors but also significantly sensitized them to PD1 inhibition. The converse experiment we also did is taking the KP cell line which are relatively sensitive to PD1 inhibition as you can see here and make them more chromosomally unstable. And this time we show that sin...
00:43:46.295 --> 00:43:48.859 elevating sin alone is sufficient to render them resistant to immunotherapy.

00:43:54.094 This is work that we have done in collaboration with Sam Bakum, the other Bakum and Chrissy Hong a postdoc in his lap.

00:44:05.258 Of course we’re now interested in moving these concepts closer to patients. The problem is that there is no known, you know, soluble human selective CS inhibitor.

00:44:10.742 So we work with our medicinal chemists to actually develop such an inhibitor.

00:44:18.880 And what I’m showing you here is the REDUCT redacted structure of that compound.
And on the bottom, the functional assay that we use to determine the activity of the compound. We're looking at the levels of CGAMP where we treated the cell lines that are we've you know I've shown you throughout this talk with this new compound and we see that we can pretty potently suppress the production of C gas meaning the activity of C gas. And just treating the cells with the C gas inhibitor alone results in reconstitution of these set cell lines to phosphorylate TBK one and IR three more efficiently.
and ultimately result in an improved ability to produce these important cytokines.

So with that, I want to summarize this last part of my talk.

Hope I was able to show you that, you know not all metastases are created equal.

Brain meds are quite distinct genomically.

That tonic activation of the C gas thing pathway through sin is bad and results in suppression of Type 1 interference that can be rescued through genetic modulation of sin or inhibition or deletion of C gas.

And that we are, you know,
very interested in moving this into the clinic using AC gas inhibitor of course. And I, you know, mentioned the people who have done the work throughout, but this is their entire lab. I want to think and of course all of the collaborators, both nationally and international collaborators who we are lucky to work with and the funding sources that support this work happy to take questions. Thank you Ben for a wonderful talk. I think you’re very deserving of the plaque. While people think what they want to ask.
I’m going to ask you a question about the CD2 CD 58 access. If you up regulate CD2, do you get reciprocal up regulation of CD 58 because that might be another way to approach. If you up regulate CD2 because you were focused on the tumor cell with CD 58, but can you manipulate it via the T cell? Yes. So we we did the other way around also. So we when we I guess I should talk here. So so we did the other way around where we knocked out CD2 and rescued it in the T cells.
The interesting part there is of course deletion of CD2 is basically leads to non responsiveness irrespective of CD 58 status. But the interesting observation and and and that might be an artifact of the system is when you over express CD2 through an ORPH, then you see actually suppression of CD 58. So we can’t just change the T cell in that case. Yeah.

All right. Questions. And I’m sure there’s some online there 50 something people I saw some have left bones. I didn’t think the microphones working. OK Chen. Yeah.
I can click the very interesting work. I have a question about the third part.

You have to show those results in primary tumor setting.

Have you looked at in metastasis setting in brain metastases setting?

No, because we don’t have the right model for it.

And I think that everyone who works on trying to understand brain metastasis, this was sort of how we got interested in this.

One of the challenges is that to my knowledge at least, there isn’t a good immunocompetent
model that has a sufficiently high rate of developing brain metastasis in a number of different ways, at least through sort of natural metastatic routes. So we haven’t been able to actually directly study this in brain metastasis for that reason. I assume some of the young models are LKB. One loss, maybe some of those could be positive, even if you do an LV injection or an intracarotic injection,
they don’t see the brain for some reason which we don’t really understand.

But what was the second part? You had another part to your question? That’s yes.

So, I think it’s just a limitation right now in terms of the available models.

Since I’m sitting next to Chen, I’ll take advantage to ask a quick question. So the CMTM 6 in the first part of the talk is interesting, but the binding region that you identified is in the extracellular domain. I thought it was a stabilizer.
binding to the cytoplasmic domain

and the transmembrane protein, but

it’s actually the extracellular domain and it has two

extracellular domains which we we we also mutated and and shown in the paper that both extracellular domains of CMTM 6 are required for stabilizing both PDL one as well as CD 58.

So it’s clearly the extracellular domains that are necessary.

It’s a stabilization phenomenon that is a function of CMTM 6.

So the function of CMTM 6, I didn’t get to the into this in detail is it’s it shuttles,
it shuttles its cargo through the recycling endosome. So typically when proteins are bound to CMTM 6, they are shuttled back and recycled back to the cell membrane. When you delete CMTM 6, you can show that they’re more preferentially lysosomally degraded. Oh, thank you. Very fantastic talk. I’m extremely interested in the LKP one seems like it’s leading to chromosome’s instability. But however in my impression now
00:50:35.542 --> 00:50:37.230 we think chromosome instability
NOTE Confidence: 0.808377375
00:50:37.230 --> 00:50:39.880 leads to more like mutation burden,
NOTE Confidence: 0.808377375
00:50:39.880 --> 00:50:42.931 which is a good thing for you know
NOTE Confidence: 0.808377375
00:50:42.931 --> 00:50:44.919 response to to immunotherapy.
NOTE Confidence: 0.808377375
00:50:44.920 --> 00:50:47.468 But in your story seems like it’s
NOTE Confidence: 0.808377375
00:50:47.468 --> 00:50:50.576 on the other way and also you know
NOTE Confidence: 0.808377375
00:50:50.576 --> 00:50:53.486 some point that we know the PD1
NOTE Confidence: 0.808377375
00:50:53.486 --> 00:50:55.316 blocking antibody is approved for
NOTE Confidence: 0.808377375
00:50:55.316 --> 00:50:57.772 all cancer which has the MSI high,
NOTE Confidence: 0.808377375
00:50:57.772 --> 00:50:59.236 you know your tumors.
NOTE Confidence: 0.808377375
00:50:59.240 --> 00:51:01.074 So I wonder if you have any
NOTE Confidence: 0.808377375
00:51:01.074 --> 00:51:02.200 comments between on this.
NOTE Confidence: 0.808377375
00:51:02.200 --> 00:51:06.625 And also my second question is when you I,
NOTE Confidence: 0.808377375
00:51:06.625 --> 00:51:09.235 I know you’re planning to put
NOTE Confidence: 0.808377375
00:51:09.235 --> 00:51:12.277 the C gas inhibitor on clinic,
NOTE Confidence: 0.808377375
00:51:12.280 --> 00:51:13.056 what’s your,
what’s the approach in your mind
because now we know there’s a lot
of steam possibly agonist those
So seems like you know,
I don’t know if you ever discussed with
Thomas Kiosky because he’s a you know,
yeah thing guy.
I am very happy that you asked both of
these questions because I think it’s
really important to clarify a couple
of things and maybe I went too fast.
So to the first point, yes,
TMB to some extent is associated weekly,
but it is associated with
response to checkpoint inhibition.
What I was talking about here is not TMB,
it's not, you know the number
of non synonymous, you know mutations throughout the genome.
When we talked about chromosomal instability,
a different form of genome instability that
is rather characterized by large changes,
large structural changes,
loss of chromosome arms or gains
loss of entire chromosomes, etc.
What we were actually able to show,
and that was on the slide but I
got over it probably too quickly,
is that in this particular case of LKB 1,
the mutation is not associated with a difference in TMB. It's only associated with a difference in the rate of chromosomal instability, at least the genetic surrogate. So I think these are two different forms of genome instability that are very different. OK.

And then the second part is you know with respect to sting, those structures don’t work so well in patients, right? That’s the reality,
The response rate to a sting agonist is relatively low. There are and I had some of them myself, there are some patients who are exquisitely responsive to them, but the vast majority of patients do not. And I think that the work that I presented here actually supports why that might be right because when you have this tonic activation of the pathway, as I said, you sort of flip the pathway on its head. And so if you throw a sting agonist on there, you know it probably doesn’t do very much right to the at least
00:53:34.207 --> 00:53:35.641 to the cancer cell and perhaps
00:53:35.641 --> 00:53:37.438 it might make the problem worse,
00:53:37.440 --> 00:53:40.240 right if if you stimulating the wrong clade,
00:53:40.240 --> 00:53:42.879 if you will of the downstream signaling.
00:53:42.880 --> 00:53:44.800 What I'm suggesting is that we
00:53:44.800 --> 00:53:46.932 might be able to, you know,
00:53:46.932 --> 00:53:49.446 rescue some of these in agonists,
00:53:49.446 --> 00:53:51.864 but they need to be combined
00:53:51.864 --> 00:53:53.680 in the proper way.
00:53:53.680 --> 00:53:56.200 So you could imagine that if you,
00:53:56.200 --> 00:53:58.272 you know, inhibit sea gas right in
00:53:58.272 --> 00:54:01.117 a in a cell line or in a patient,
00:54:01.120 --> 00:54:01.936 if you will,
00:54:01.936 --> 00:54:03.840 then you allow this pathway to come
00:54:03.897 --> 00:54:05.738 back to an equilibrium, you know,
00:54:05.738 --> 00:54:07.621 and then coming in with the sting
NOTE Confidence: 0.874233872352941
00:54:07.621 --> 00:54:09.158 agonist might be more fruitful.
NOTE Confidence: 0.874233872352941
00:54:09.160 --> 00:54:11.182 So I don’t think these are
NOTE Confidence: 0.874233872352941
00:54:11.182 --> 00:54:12.193 mutually exclusive strategies,
NOTE Confidence: 0.874233872352941
00:54:12.200 --> 00:54:13.104 but I think that,
NOTE Confidence: 0.874233872352941
00:54:13.104 --> 00:54:13.556 you know,
NOTE Confidence: 0.874233872352941
00:54:13.560 --> 00:54:15.330 the work suggests that just
NOTE Confidence: 0.874233872352941
00:54:15.330 --> 00:54:17.100 throwing sting agonists on things
NOTE Confidence: 0.874233872352941
00:54:17.167 --> 00:54:19.400 will is unlikely to be beneficial.
NOTE Confidence: 0.490145155
00:54:28.240 --> 00:54:31.320 David, differential output.
NOTE Confidence: 0.330621972
00:54:34.120 --> 00:54:35.760 How’s my treatment quoted? How’s
NOTE Confidence: 0.47446849
00:54:35.760 --> 00:54:39.040 the self burgering out? Atomic CS activity.
NOTE Confidence: 0.6040632
00:54:45.120 --> 00:54:46.320 Fortunately, somebody figured
NOTE Confidence: 0.6040632
00:54:46.320 --> 00:54:48.120 that out. It wasn’t me,
NOTE Confidence: 0.742325126
00:54:48.120 --> 00:54:49.920 but it was Sam’s lab.
NOTE Confidence: 0.742325126
00:54:49.920 --> 00:54:51.960 They published a a paper last
year in Nature that where they show that tonic activity of sting preferentially induces ER stress. And through ER stress you basically you know a tonic ER, ER stress in this case resulted in the chromatostatic clade. And they've shown beautifully in vitro models, in fibroblast models that it doesn’t take very much of tonic activation to get to that point where you know if you stimulate once you get a really nice Type 1 interferon.
But even after two or three stimulations over a couple of days you very quickly sort of you know, tacky for LAX on that pathway and you start activating the bad counterpart which is in this case the chronic ER stress activity. OK.

I'm going to give the last question to Ben and Lou. Perfect.

We did not, we actually looked at that. We did not actually look at that. That was one of my students just said I'm going to throw it on the flow cytometer.
We don’t see, we don’t see changes and see the 58 level depending on Syn.

Ben, thank you for a wonderful talk and be around for a few minutes if anyone has extra questions. Thank you.