RYAN WILCOX: I spend most of my time thinking about the T-cell lymphomas. When I'm interacting with medical students, residents, Fellows, I actually spend the bulk of my time talking about the more common B-cell lymphomas. And so this is actually a rare opportunity for me, in which you will all be subjected to, in which I get to talk for the next 45 or 50 minutes about the T-cell lymphomas.

And with any T-cell lymphoma talk, usually the first slide looks somewhat like this one. And this just simply illustrates the natural history of these lymphomas. To put some numbers on this, last year roughly 80,000 patients were diagnosed with non-Hodgkin lymphoma. Of those, roughly 10%, or about 8,000, were T-cell-derived. The most common T-cell lymphoma is this so-called peripheral T-cell lymphoma, not otherwise specified.

And so I'll just draw your attention to the survival curves in red. And these are derived from very large studies, a very large international series, and the largest multi-center study in North America. And the point is this. For patients diagnosed with these lymphomas, the overwhelming majority will die of their disease, most within a few years of diagnosis. And so simply put, we certainly have work to do.

And so the story I'd like to share with you this afternoon really goes back to a simple question that we asked several years ago. And it's this. And it's based on the premise that a normal conventional T-cell is very much dependent upon antigen-presenting cells. That's true when T-cells are born and differentiate in the thymus. It's true for their homeostatic survival in the periphery. And that's certainly true when they encounter a antigen prior to being activated.

And so we ask the question, well, to what extent does a malignant T-cell remain dependent upon antigen-presenting cells? And there is certain circumstantial evidence for the APC's role in the T-cell lymphomas, simply because of their sheer abundance within the tumor microenvironment for most of these T-cell lymphomas. And so what you're looking at here is immunofluorescent staining. And the green are all lymphoma-associated macrophages.

And they're almost as abundant as the malignant T-cells themselves. And this isn't that extraordinary, actually. This is fairly common in many of the T-cell lymphomas.

And so we did get a fairly straightforward, simple experiment. We took peripheral blood mononuclear cells from a patient with leukemic involvement and threw them in a dish. And what you'll find is that within a matter of days, most of those malignant T-cells will undergo spontaneous cell death.

However, when you look a few days later, you'll notice these sort of-- I don't know, they sort of look like grape-like clusters to me, these clustering of malignant T-cells that will remain viable for a number of days. If you gently pipette up and down and disrupt them, what you'll find is, underneath those clusters, and being initially obscured from view, are these large, adherent cells. And this is all published. I'm not going to show you the data, suffice it to say those are simply monocyte-derived macrophages.

And if you didn't treat these monocyte-derived macrophages from this system, you accelerate the rate at which the malignant T-cells undergo cell death, suggesting that, indeed, they're providing some factor or factors that promote their growth and survival. And this is-- we don't need to go into details of this spontaneous T-cell lymphoma model. This is a mouse model. The point though is that, in a spontaneous model, you see the same dramatic expansion of lymphoma-associated macrophages, just as you do in a human PTCL.

And certainly, tumor-associated macrophages have a well-accepted and appreciated role in tumorogenesis generally, largely due to their role in the suppression of host anti-tumor immunity. But in a lymphoma context, they also play a role in directly promoting lymphoma growth and survival. And they may do that by one of a few different mechanisms, which we've simply described as a three-signal model.

Now that's-- we're not so clever. We just simply hijacked language that a basic immunologist might use to describe the rules of engagement for a normal t-cell. And any basic immunologist will tell you that signal one is of course the antigen receptor. But antigen receptor activation alone is insufficient to fully activate an I-E T-cell. You also need activation of co-stimulatory receptor or so-called signal 2. And more recently, it's been shown that, particularly for effector T-cells, you need specific cytokines to mount a robust effector response.

And so what I'm going to show you on the next slide-- and to us, this model seems aesthetically pleasing, makes it easy to communicate, clearly has therapeutic implications that we'll go through. But as the genetic landscape of these lymphomas is increasingly appreciated over the last few years, I think that's provided nice genetic evidence for this model.

And so I'm going to change the slide. But what I want you to pay attention to is many of these downstream intermediates are going to change color. All of those that change to blue are downstream signaling intermediates that are either recurrently mutated in T-cell lymphomas or otherwise genetically modified.

So here we go. So the point is a good many of them are recurrently mutated. So for example, PLC-gamma is a very important proximal signaling intermediate downstream to the T-cell receptor, recurrently mutated in possibly 20% of cutaneous T-cell lymphomas. There are some other that are a bit more interesting and novel.

While rare, a few percent of cutaneous T-cell lymphomas harbor this novel translocation involving the intracytoplasmic domain of CD28, which is a co-stimulatory receptor, with the extracellular domain of CTLA-4, which is CD28's counter-part, which is actually inhibitory. But CTLA-4 has a much higher affinity for ligand than does CD28. And so T-cells were pretty clever. So you have a high-affinity receptor binding ligand, which actually activates T-cells. The first patient with that translocation described was actually treated with an anti-CTLA-4-blocking antibody, and transiently responded, despite being refractory to all previous treatments.

And so we decided to focus our attention on signal 1. And so if you accept that antigen-presenting directly promote the growth of survival of the T-cell lymphomas, we ask, well, to what extent is that dependent upon antigen-receptor engagement. And there's certainly anecdotal evidence to implicate the T-cell receptor.

Malignant t-cells very commonly lose the expression of other T-cell-associated antigens, CD2, 5, 7, and others. But for the more common T-cell lymphomas, the T-cell receptor and the associated CD3 chains are almost uniformly retained, potentially implicating the T-cell receptor. And furthermore, there are downstream signaling intermediates, like ITK, which are certainly expressed, and in many cases, in some subtypes, overexpressed.

And so we adopted the ex vivo system that I alluded to earlier. And we simply purified primary malignant T-cells, placed them in a dish. Those cells will spontaneously undergo cell death over the course of days. By Ki67, which is a marker of cell proliferation, and cells are actively cycling, under those conditions, very, very few, if any, cells are actively proliferating.

However, if you co-culture those cells with autologous monocytes, now a substantial minority of cells enter the cell cycle and are proliferating. But if you block MHC class II, which of course, is needed for engagement of the T-cell receptor, you now blunt that proliferating response.

The data here just summarizes data from multiple independent samples. And so we took this to mean that, OK, that T-cell receptor potentially be tweaked by autologous MHC. And it's doing something.

And so we next took the reverse approach, took primary samples and actively engaged the T-cell receptor with beads that are coated with anti-CD3 and anti-CD28 antibodies, so this is a very potent agonist. And when you do that in either a PTCL-NOS cell line or in primary samples, again, you see a very robust, proliferative response, which is, again, replicated in multiple samples.

And so we next looked at proximal signaling events downstream of the T-cell receptor. And this is a busy slide, lots of blots. And I won't go through all of every detail. But clearly, when you engage the T-cell receptor, what you would expect to be phosphorylated downstream of the T-cell receptor in a normal T-cell is indeed phosphorylated. And that will include, and I'll just point it out now, a proximal Tec family kinase, ITK.

And we'll come back to this later, but in primary samples, we often see some evidence for constitutive phosphorylation of that receptor in samples that are isolated and then examined immediately after isolation. And phosphorylation of that site-- it's an autophosphorylation site, and is needed for its activity, but we'll come back to that later.

We next performed gene expression profiling in primary samples after engagement of the T-cell receptor. And there were a good many genes differentially expressed, many of which, just over 1,000, are shared by these independent samples. When you do gene set enrichment analysis, you have enrichment for pathways regulating cell growth, survival, metabolism, much like you would expect in a normal T-cell.

And just as an aside, as you'll see we're going to make-- we're going to use this cell line, which unfortunately is the only PTCL-NOS cell line available. So we do that same analysis on the cell line. And the only point of this slide is, it tends to replicate many of the changes that we saw in these primary samples, so we think this is a reasonably good model.

We then validated those findings in independent samples, looking at the production of cytokines that we found in the gene expression profiling data set, and also looked at the expression of various cell-surface receptors following activation. And the point is, in independent samples, we engage a T-cell receptor. You have a dramatic increase in cytokine production and also the up-regulation of various cell-surface receptors, which in normal T-cells, are also thought to be activation-dependent. And again, T8ML-1, our cell line, looks very similar to what we observe in primary samples.

And so downstream of the T-cell receptor, a very important downstream mediator of T-cell receptor signaling is a transcription factor, NF-KB. And we won't go through all of the details. But following TCR activation, NF-KB's inhibitor, IKB, is going to be phosphorylated and ubiquitinated, degraded by the proteasome, unleashing NF-KB to translocate to the nucleus and regulate gene transcription, including genes that play a very important role in cell growth and survival.

And so we wanted to look at what was happening with NF-KB downstream to the T-cell receptor, particularly appreciating that NF-KB plays a very important role in many different lymphomas including some of the T-cell lymphomas. And indeed, perhaps not surprisingly, when we engage a T-cell receptor, we see an increase in NF-KB activation, and by that I mean its nuclear translocation. You can also visualize this by immunofluorescence. And so with engagement of the T-cell receptor, you can see a redistribution of NF-KB into the nucleus.

We also looked at its DNA binding. And this is really an ELISA-based system. So a plate is coated with NF-KB binding sequences. And when you engage the T-cell receptor using normal T-cells and control, you can appreciate a significant increase in NF-KB binding using nuclear lysate.

And likewise, a similar increase was noted using a cell line and multiple primary samples. And we also looked at a few well-established, well-described NF-KB gene targets. And as you might expect with engagement of the T-cell receptor, there's a dramatic increase in expression of NF-KB gene targets.

And so that's all fine and good, but of course what we're really interested in is its role, the T-cell receptor's role in chemotherapy resistance, because you would predict, with activation of NF-KB and turning on multiple cell growth and survival pathways, that you may confer resistance to chemotherapy. And indeed, that seems to be the case. And so again, we're using vincristine, microtubule-targeting chemotherapeutic agent often used in the up-front study in the T-cell lymphomas. And we're using a concentration that, across multiple cells, approximates the IC50.

When you activate the T-cell receptor in the presence of vincristine, you confer at least partial resistance to vincristine. And you have similar results using another agent, romidepsin. Deps Again, when you include beads, you confer at least partial resistance to romidepsin. And the same is observed when we use primary samples, demonstrating that engagement of the T-cell receptor appears to confer resistance to chemotherapy in the T-cell lymphomas.

And so we next wondered if indeed that's NF-KB-dependent, and so to answer that question, we used a pharmacologic approach with sotrastaurin, which is a PKC-theta inhibitor, which is required for NF-KB activation. And we have that data. I'm not showing it. But the point is, in the presence of this inhibitor, you significantly blunt the ability of the T-cell receptor to confer resistance to chemotherapy, implicating NF-KB downstream.

And so we next wanted to use an approach that we thought might be clinically translatable. And this is just another way of depicting T-cell receptor signaling. And I want to draw your attention to this kinase, ITK. This is a member of the Tec family of kinases, plays a very important role downstream of the T-cell receptor, has both a kinase-independent role as a scaffolding protein, bringing together many of these different players into the vicinity of the T-cell receptor, and also as a kinase-dependent role by its ability to phosphorylate and activate PLC-gamma.

And this data isn't surprising. We know malignant T-cells express ITK. When we cross-linked a T-cell receptor, it's in the neighborhood, it's in the vicinity of the T-cell receptor. And I've already shown you this data. When we engage a T-cell receptor, indeed ITK is turned on.

And so to address the extent to which TCR engagement and chemotherapy resistance in the T-cell lymphomas is ITK-dependent, we used an shRNA approach to knockdown ITK expression. And so this is showing this at the mRNA level or by Western blot. So the cell line, that T8ML-1 cell line was transduced with either a non-targeting shRNA or two independent ITK-targeting shRNA. And whether at the RNA level or the protein level, we actually have very nice knockdown.

And when we knock down ITK and then stimulate the T-cell receptor, you significantly blunt the production of various cytokines following stimulation, You're no longer able to upregulate various cell-surface receptors following TCR engagement. But of course what we're really interested is in downstream activation of NF-KB, and ultimately chemotherapy resistance.

And so we-- with loss of ITK following TCR stimulation, you see the loss of this upregulation of NF-KB. And so not surprisingly you see a similar loss of this chemotherapy-resistant phenotype following TCR activation. And so I'll walk you through this. The data is similar for this-- the data with romidepsin, on your right. And so I'll walk you through this figure.

So again, these are the same cell line transduced with non-targeting or ITK-targeting shRNA without TCR stimulation. In the presence of vincristine, you have a similar decrease in viability. As we would expect, when you engage the T-cell receptor in the controls, the wildtype and scrambled controls, they become much more resistant to chemotherapy, however, when you knockdown ITK, shown in the blue and green here, there is a dramatic reduction in that chemotherapy resistance following T-cell receptor engagement. And the data looks very similar using an alternative agent.

And so to test this in primary samples, we wanted to use a pharmacologic approach, particularly one that could be translated. And so there's a very similar story that's emerged over the past 10 years in the B-cell lymphoma world. In much the same way, we think the T-cell receptor plays a role in T-cell lymphomas.

There are some B-cell lymphomas that appear to be driven by the B-cell receptor and BTK, the ITK homolog that's needed for B-cell receptor signaling. And there's an oral tyrosine kinase inhibitor, ibrutinib, that is a potent BTK inhibitor, is now FDA-approved for multiple B-cell lymphomas. And really, in the B-cell lymphoma world this has been really a home-run drug.

And so we asked the question, given that ITK and BTK are so highly homologous, we suspected that ibrutinib may in fact, also be an ibrutinib inhibitor. And indeed, it is. And so when we engage the T-cell receptor, either our cell line or primary samples, at 1 or 5 micromolar of ibrutinib, we can see inhibition of downstream ITK-dependent signaling intermediates, including localization of NF-KB.

And so now, we then looked at NF-KB activation again in either-- this is by DNA binding using either a cell line or primary samples. And when you include ibrutinib, particularly at the higher concentrations, which are admittedly not clinically achievable, you see a significant inhibition of NF-KB DNA binding. There's also a loss of NF-KB target gene expression, which isn't surprising, loss of upregulation of various cell-surface receptors, cytokine production. Again, none of this is surprising.

But here's the important data. And so this is in primary lymphoma cells. Again, using a concentration of romidepsin that approximates the IC50, we engage the t-cell receptor. We promote their survival. However, if we include ibrutinib, that's blunted, that's largely blocked, suggesting that we can block TCR-mediated chemotherapy resistance with an ITK inhibitor, ibrutinib.

And so we wanted to translate this into an in vivo model. And to do that, there are unfortunately very few mouse models in the T-cell lymphoma world, and so we used this model, which is basically a spontaneous peripheral t-cell lymphoma that developed in an aged mouse. But it's very useful, in that it can be successfully adoptively transferred in recipient mice. It's very aggressive. After adoptive transfer, mice have to be sacrificed within two or three weeks.

This is sort of the histology of a normal spleen at low and in high power. But in these lymphoma-bearing mice, I think you can appreciate complete loss of the normal architecture of the spleen. These cells look very atypical under higher power. As you would expect, they are CD3-positive, will also not only involve the spleen, lymph nodes, liver, they will eventually replace the bone marrow.

And these are clonal. They're CD8-positive. They express a clonal TCR V-Beta chain, V-Beta 2. And they really have a immunophenotype that you might expect for a PTCL. And for our purposes, that would include the expression of GATA-3.

In vitro, these cells behave much like primary samples do. When you isolate those cells, put them in a dish, in this case for 48 hours, these really do not like being removed from their microenvironment. They undergo spontaneous cell death.

So by Annexin V PI staining, very few viable cells remaining after 48 hours. You accelerate that by including some ibrutinib in vitro. However, if you engage the T-cell receptor, you promote their survival. And as you might predict, that is blunted with ibrutinib.

And so we wanted to look at this in vivo. And so to do that, we treated mice with a combination chemotherapy alone, ibrutinib alone, or the combination of the two. And then assessed disease bulk by spleen and liver weights.

And this is a very aggressive model. Some of these mice will become spontaneously ill and have to be sacrificed due to spontaneous splenic rupture. And so that's what the arrow is meant to indicate. And I know it's hard to appreciate probably as it's projected. And you'll note the different time points that these mice have been sacrificed.

The point is, chemotherapy alone tends not to do much. This lymphoma model, they're quite resistant to conventional chemotherapy. Likewise, ibrutinib alone doesn't have much activity. However, the combination of the two, you see a significant reduction in disease bulk, again supporting the notion that by blocking T-cell receptor signaling with an ITK inhibitor, you increase their sensitivity to standard chemotherapy.

So thus far, to summarize, I tried to convince you that APCs within the tumor microenvironment engage the antigen receptor initiating a signaling cascade which includes activation of NF-KB, presumably upregulation of multiple growth and survival factors, ultimately culminating in chemotherapy resistance. Now, it's going to appear that I'm about to take a left-hand turn, and take you somewhere out in left field. So bear with me for a few slides. And in just a few minutes, I'll bring us back to where we need to be, OK?

So peripheral T-cell lymphomas come in many flavors, some more aggressive than others. Approximately 60% of T-cell lymphomas in North America are specified, meaning it's very easy for a pathologist to look at that particular lymphoma and say, OK, it falls into that bucket. Unfortunately, about 40%, the remaining PTCLs, fall into this waste basket. They're a hodgepodge of unspecified T-cell lymphomas. And as you can imagine, when you have an entity, the most common T-cell lymphoma in North America, and it's a hodgepodge of different lymphomas, that has really impeded progress in the field.

And so initially, I attributed this to the sheer heterogeneity of these lymphomas. And so it's very challenging for pathologists to appropriately classify these. And I say until recently, because just recently, I discovered this paper. And they showed that they could train a pigeon to do as well a job in making a call as the pathologist.

And what I want to point out is that the first author on this, these are pathologists. So I don't know if these are just, I mean, if this is a low self-esteem issue. I don't know if these are just lazy pathologists.

In any event, if nothing else, when you walk away, you can take this with you. And you have something to harass all your pathology friends with. And I can assure you I've made good use of this.

OK, so several years ago, we asked that question, and you'll recall from your basic immunology, that after naive T-cells encounters antigen, it can, under the influence and the context in which the antigen is provided, can differentiate down a number of different pathways under the influence of dominant transcription factors that control the fate of that particular T-cell subset, each of which is characterized by the expression of relatively subset-specific cytokines.

And so to make a long story short, in a cohort of PTCL-NOS, we observed that those T-cell lymphomas can be subtyped into two dominant subtypes, the first of which is enriched for GATA-3 and some of its gene targets. The second is enriched for the Th-1-associated transcription factor T-bet and some of its gene targets. And this is important, because when you stratify PTCL-NOS patients GATA-3 expression, it appears to make a big difference in patient outcome.

And so, at least in our cohort, if you had a PTCL-NOS that's GATA-3-positive, there were no long-term disease-free survivors in that group. This turns out to be a multivariate analysis independent of conventional risk factors in PTCL. When we were doing this work, we had the opportunity to interact with members of the International T-cell Lymphoma Project, which is a large, international, multi-center study. They were in the process of accumulating additional cases for, really, a more robust gene expression analysis.

And they found the same thing. In hundreds of samples, they found one subset that was enriched for GATA-3's gene targets, an alternative subset enriched for-- and this is T-bet-- for T-bet's gene targets. And those survival curves look fairly similar to the ones I've just shown you. When these two papers came out, in the year since, there have been two additional single-center reports finding the same or similar results. Again, if your PTCL-NOS is GATA-3-positive, those patients tend to do very poorly. And so we're moving away, I think, in the PTCL world, away from PTCL not otherwise specified to, hopefully, PTCL specified.

Now, a few comments about GATA-3-- so we know, at least in normal T-cells, GATA-3 is regulated by the T-cell receptor. It is itself an NF-KB target gene. It plays an important role in regulating the homeostatic survival of particular T-cell subsets, particularly CD8-positive T-cells. And its gene targets, at least in normal T-cells, have been described. And the number of gene targets regulated by GATA-3 rivals what you would see for c-myc in a B-cell lymphoma.

And so we asked, which was really a crazy question at the time, well, is it possible that GATA-3, downstream of NF-KB, is playing a dominant role in regulating chemotherapy resistance? And of course, we were bolstered in asking that question because we already knew the clinical data. But really, that's kind of a crazy question, because NF-KB regulates so different growth and survival pathways, so to think that if you knocked out just one that would make a significant impact seemed a bit far-fetched at the time. But we just felt compelled to answer that question.

And so as a way of initially addressing that question, we asked, well, if we stimulate the T-cell receptor, is GATA-3 upregulated? And indeed, it appears to be, both in cell lines and in patient samples compared to unstimulated cells. We engage in T-cell receptor, there's a significant upregulation of GATA-3 expression. And that's just a summary of multiple patient samples. As you might predict, it's ITK-dependent.

And so when we look at our cells in which ITK has been knocked down, the first thing that you'll notice is in unstimulated cells, shown here in the open histogram, there's a slight reduction in GATA-3 expression when you knock down ITK. But more striking, when you engage the T-cell receptor, when you would expect to see upregulation in the absence of ITK, absolutely no upregulation of GATA-3 expression. It's absolutely dependent upon ITK.

And you can make a similar point using a ibrutinib. And so we did that in primary samples. And again-- and this is what's interesting. And I pointed out that Western blot many slides ago. In many of the primary samples isolated ex vivo, when you analyze them immediately, there is some evidence for some low-level activation of ITK, because you remember ITK was phosphorylated, even in the absence of stimulation. And so maybe that explains why, when you add ibrutinib, not only do you block the upregulation of GATA-3, but GATA-3 expression remains at a level that is below what you would expect basally in the unstimulated cell. And again, this just summarizes the data in multiple samples.

And so we suspected that GATA-3-- and this just slide is an attempt to summarize a large amount of data. But the point is, this is, yes, largely transcriptional and NF-KB-driven. That doesn't appear to be the only answer. It's not due to changes in mRNA or protein stability. There also seems to be a role, probably mTOR-dependent, downstream of TCR activation, in increasing the translation rate of the GATA-3 transcript.

So we next asked the question, and this is the meat, and that is, to what extent, if we blunt GATA-3's ability to be upregulated following engagement of the T-cell receptor, do we inhibit chemotherapy resistance? And so to do that, we generated these cell lines, in this case, transduced with GATA-3-targeting shRNA. And we were initially a bit disappointed, because with those shRNA, we certainly do not get complete loss of GATA-3 expression.

Compared to the controls here, in unstimulated cells on the histogram, there's just a slight reduction in GATA-3 expression. But what was useful for our purposes, is when you stimulate the T-cell receptor in these cell lines, you do get some upregulation of GATA-3, however, it is to an extent that is comparable to the unstimulated controls.

So when we then do the experiment using those cells exposed to chemotherapy, in the controls, following TCR engagement, there is a dramatic increase in cell viability. But that is significantly blunted if you prevent that cell's ability to up regulate GATA-3 after engagement of the T-cell receptor. And so we actually thought this was a bit surprising, because it suggests that you have one downstream target of NF-KB. It's not the only answer, certainly, but it's playing a dominant role in conferring resistance to chemotherapy.

And so we then did the reverse experiment. In the interest of time, I won't go through it. But if you take some of these cell lines and overexpress GATA-3, you make them more resistant to chemotherapy. We then wanted to look at GATA-3 in isolation, particularly in vivo. And so to do that, we transducer T-cell lymphoma lines, with various genetic backgrounds including loss of P53. And these are cells transduced with different GATA-3 shRNA.

And in vitro, when you knockdown GATA-3, you make them more sensitive to chemotherapy. When we do this in vivo-- so these are T-cell lymphoma line, again, transduced with either non-targeting, or in this case, two different targeting shRNA. The one shown in red is the shRNA that gives very robust knockdown of GATA-3. And you'll get a sense for that in a upcoming slide.

But in the presence, in-- when these xenografts are treated with chemotherapy, there is some delay in tumor growth in the controls. But when you knockdown GATA-3, they're much more sensitive to chemotherapy. And particularly with the cells in which GATA-3 knockdown is much more striking, you get complete disappearance of those xenografts. And we'll come back to that, I think, in another slide.

We've looked at this in multiple cell lines using multiple agents. And the only point of this busy slide is, certainly for some cell lines exposed to some chemotherapeutic agents, GATA-3 knockdown doesn't appear to do much. But what we never see is where GATA-3 knockdown makes them less sensitive to chemotherapy. And so we thought this was fairly persuasive.

And so I'm going to go back to this experiment, because when we were treating these mice with chemotherapy, we knockdown GATA-3, tumors go away. At around 30, we thought we were a pretty good mouse oncologists, right? Maybe these mice are cured of their T-cell lymphoma. But unfortunately, when you stop treatment, those tumors fairly quickly recur, which is, unfortunately, what we often see in patients.

So we went back, and we explanted those tumors. Now, prior to treatment, this is what GATA-3 expression looks like. So again, in this tumor, their GATA-3, very high. And we can knockdown GATA-3 expression to varying degrees with that particular shRNA of very robust knockdown. However, after treatment, in this group, the tumors that recur have now regained GATA-3 expression. They're now GATA-3-high, suggesting that there's a selection pressure imposed by chemotherapy for GATA-3 expression.

And so we looked at this in paired clinical samples. Unfortunately, we didn't have many samples. Here's a representative example. So this is an initial diagnostic biopsy, pre-chemotherapy. We would classify this particular case as being GATA-3-negative, because there's very little GATA-3 expression. However, at the time of disease relapse, this tumor is now GATA-3-positive. And so when we looked at the five serial biopsies that we had available, there was a clear enrichment for GATA-3 expression.

We then looked at things a bit differently and asked a different question. And that is, if we took our PTCL-NOS cohort that we previously published, stratified them by GATA-3 expression-- and all of these patients were treated similarly up-front with multi-agent anthracycline-based chemotherapy. And then we looked at the rate of primary refractory disease, which simply means the patient's lymphoma progressed during treatment with chemotherapy or quickly relapsed within six months following the completion of chemotherapy.

And if you're PTCL-NOS with GATA-3 positive, the rate of primary refractory disease was approximately 50%, compared to approximately 10% for those tumors that were GATA-3-negative. And as you can imagine, that was highly significant, so again, we think provides direct evidence that GATA-3 confers chemotherapy resistance in the T-cell lymphomas.

And so I only include this slide to make the point that, while we've been focused on the cell-autonomous role of GATA-3, it also plays a non-cell-autonomous role by regulating the production of cytokines, which in turn regulate constituents of the tumor microenvironment. And so we thought about how we might go about targeting this pathway. We've already talked a bit about ITK inhibition. I won't go to more details.

You can think about targeting NF-KB. I've already mentioned that the inhibitor of NF-KB is regulated by the proteasome, so proteasomal inhibition is an established-- one way to potentially inhibit NF-KB. And we showed in both cell lines and primary samples, perhaps not surprisingly, that indeed, an oral proteasome inhibitor does inhibit the proteasome of T-cell lymphomas. T-cell lymphomas really don't like being exposed to proteasome inhibition. And yes, it inhibits NF-KB.

That's all very nice, not very surprising. But for our purposes, we were most struck that, indeed, with proteasome inhibition, we had inhibit GATA-3 expression in primary samples. And so onto that data, we initiated a clinical trial with an oral proteasome inhibitor. Unfortunately, there was only a single PTCL-NOS patient enrolled on that clinical trial, as many different PTCL and CTCL, cutaneous T-cell lymphoma, subtypes were included in that trial, but the point is, that single PTCL-NOS patient was an exceptional responder.

Here's a pretreatment scan. After two cycles of therapy there's been a reduction. And after four cycles of therapy, he's achieved a complete remission. For reference, for this patient who is not transplant-eligible, median anticipated overall survival is just under six months.

He did well for a year on this therapy before relapsing. He had an isolated relapse at a previous site of disease with this inguinal lymph node, underwent radiation. He remains without evidence of disease to this day and is walking around without therapy.

And so we were rather encouraged by the result, but again, it was only a single patient. When we went back and looked at the phase I data, there were seven patients, PTCL-NOS patients, treated in the two phase I studies with this agent. Of those seven, two responded. So that's a overall response rate then of 3/8, which, in the T-cell lymphoma world, is a signal.

And so an important question in that trial was, OK, we've performed. If your tumor was GATA-3-positive, you had an on-treatment biopsy after one month of therapy. And when we look at NF-KB expression, I think, in contrast to the pretreatment biopsy, where you see very nice nuclear staining of NF-KB, with treatment can see a loss of nuclear NF-KB and redistribution to the cytoplasm. When we looked at GATA-3 expression, while there is certainly not loss of GATA-3 expression, in comparison to the pretreatment biopsy which is GATA-3, very bright, there does indeed seem to be some inhibition of GATA-3 expression.

And so moving downstream, well, how might you think about targeting GATA-3? Because transcription factors are historically difficult to target. And so as a way of beginning to wrap our minds around that, we wanted to have a way of accessing GATA-3's ability to bind as gene targets. And we chose three gene targets which we don't believe anyone would refute. They're very well-established GATA-3 gene targets. They emerge in our gene expression data sets in PTCL-NOS. We've shown that they're absolutely GATA-3-dependent in the T-cell lymphomas.

And then we examined the ability to GATA-3 to bind those gene targets using chromosome immunoprecipitation. And so when we do ChIP with GATA-3 which is shown in the gray here, clearly GATA-3 is binding its anticipated gene targets. We can replicate that in primary-- that was data from a cell line. We can replicate that in primary samples, including fresh-frozen and formalin-fixed paraffin embedded tissue.

In the interest of time, I'm not going to go through the details about HDAC inhibition. The point though is this. HDAC inhibitors-- so HDACs are histone deacetylases, but they also play a role in deacetylating many non-histone proteins, including transcription factors. And in the early preclinical experience with HDAC inhibitors demonstrated particular sensitivity of T-cell lymphoma lines. So that led to this fairly rapid translation through phase I and phase II of HDAC inhibitors in the T-cell lymphomas. And so currently, there are three FDA-approved HDAC inhibitors available for use in various T-cell lymphomas.

And interestingly, GATA-3 is self-acetylated. If you look at gene expression profiling studies of T-cell lymphoma lines treated with an HDAC inhibitor, it appears that there's preferential inhibition of Th2, meaning GATA-3-dependent cytokines. And so we wondered if HDAC inhibitors, potentially by tweaking the acetylation state of GATA-3, is in some way impairing its ability to bind its gene targets. And so we did this initial experiment.

This is data from a T-cell lymphoma line. And after a very brief few-hour exposure with belinostat, an HDAC inhibitor, there was complete loss of GATA-3's ability to bind its gene targets. And we see this with all of the currently available HDAC inhibitors. I'm just showing you the belinostat data.

We then did this in primary samples. So primary samples, in vitro, again, treated with belinostat, there was a loss of GATA-3 binding by ChIP to its targets. And we took the same patient, a month after treatment, with vorinostat, another HDAC inhibitor, and similar result compared to pretreatment, after one month of treatment with an HDAC inhibitor, there was an inhibition of GATA-3 binding its gene targets.

And so we thought, well, this hypothesis seems very obvious, right? You're inhibiting a deacetylase. That then means that there should be an increase in GATA-3 acetylation following treatment with an HDAC inhibitor. And structurally-- the crystal structure of GATA-3 bound to DNA is known. And that seemed to make sense to us.

But long story short, we were completely wrong. It's just the opposite. And we demonstrate that through this IP study. When we IP acetylated lysine, blot for GATA-3, or do the reverse, IP GATA-3, blot for acetyllysine, in the presence of belinostat, there is a loss of GATA-3 acetylation.

Upon further reflection-- of course, you're always smarter when you prove yourself wrong, right-- GATA-3 is one member of a larger family. There are six GATA family members. Three of them are very well-described, and it's very well appreciated that their acetylation is needed in order to see binding of their gene targets. And so it seemed consistent with what's known about other members of the family.

And so this is a model. And so we believe that p300, the-- it's a histone acetyltransferase. It also acetylates hundreds of other non-histone proteins. It's a known binding partner of GATA-3. We believe p300 is acetylating GATA-3 basally, but in the presence of HDAC inhibitors, in some way, its function or ability to bind GATA-3 is impaired, and so GATA-3 is no longer acetylated, consequently no longer able to bind its gene targets.

So as an initial way of getting at this, we used a specific inhibitor of p300's histone acetyltransferase activity. And when you inhibit p300's acetyltransferase activity, you can appreciate, again, by that same IP sort of approach, inhibition of GATA-3's acetylene. And as you might predict then, you also see a loss of binding to its DNA targets. And you see the anticipated loss in target gene expression.

Now, p300 is a very large protein, multiple domains. And that includes a so-called bromodomain. Bromodomains are protein modules designed to bind protein sequences that contain an acetylated lysine residue. And p300's bromodomain has previously been implicated in binding other transcription factors.

And p300 itself is also acetylated. And there's a dramatic increase in its acetylation following HDAC inhibition, which is thought to actually increase its acetyltransferase activity. So we speculate that acetylation of its bromodomain may impair its ability to bind GATA-3.

And so, in an effort to test that, we used a specific bromodomain inhibitor, which is specific for the p300 bromodomain. And when we do so-- and that's this compound right here. And when we do so, we see a loss of GATA-3's binding to its gene targets and a corresponding loss of target gene expression.

And so that data, combined with our previous experience with ixazomib, has led to a clinical trial combining ixazomib with the HDAC inhibitor romidepsin. That will be conducted by the Big Ten Cancer Research Consortium. And Suma Devata will be the local PI here.

Certainly, this wouldn't have been possible without many collaborators. We're not afraid to pursue questions that take us well outside of our comfort zone. And that's only possible in an environment like this one, where there are collaborators who know much more than we do and are generous with their time.

And last but not least, that's the lab. And with that, I'll thank you for your attention, and certainly be happy to try to answer any questions.

[APPLAUSE]

MODERATOR: I wonder how long it took you to prepare for this talk. That was quite a tour de force to take us through an enormous amount of immunology. That was fabulous. So are there any questions? Yes, we've got one.

AUDIENCE: Early on in the talk, you talked about how you found [INAUDIBLE]

RYAN WILCOX: Yeah, and that wasn't as clear--

MODERATOR: Want to repeat the question for the [INAUDIBLE]--

RYAN WILCOX: Oh yeah, thank you. So the question was, in the in vivo mouse model, combining ibrutinib with chemotherapy, do you actually see a survival benefit? And we-- you do. And that wasn't as clear on the slide. When That were sacrificing those mice, you might have-- it was-- probably didn't project very well, but those mice were being sacrificed at a later time point. But this is such an aggressive model that that survival advantage is measured in days. Yes?

AUDIENCE: In cell cultures, you're activating the lymphoma with anti-CD3 plus anti-CD28.

RYAN WILCOX: Right.

AUDIENCE: I wonder, is that second signal important in vivo? And if it is, could you think about doing the somewhat counter-intuitive thing of masking CD28 [INAUDIBLE] since we have a biologic that does that in, say, rheumatoid arthritis?

RYAN WILCOX: Yes, so that's a very good question. And the reason we-- so the question was, is there a role for CD28, because you note that we used anti-CD3 and CD28 for many of these experiments. And we wanted to do that, because within the tumor microenvironment, certainly, CD28 ligands, B7-1, B7-2, are abundant. so we we're trying to replicate, perhaps, what a malignant T-cell sees in the native microenvironment.

And so we're actually-- and I was getting to that at the end of some of the slides that I skipped over. But we're certainly very interested in understanding what other co-stimulatory receptors, including CD28, what role they might play in the T-cell lymphomas, because certainly, you're quite right. Both in the rheumatology world and also in the tumor immunotherapy checkpoint blockade world, there are plenty of agents available to block some of those receptors.

AUDIENCE: So why [INAUDIBLE]

RYAN WILCOX: Historically, they've just-- it's been difficult to design agents to directly inhibit or otherwise target transcription factors, so conventional wisdom is that they're difficult to target. And if you look at MYC for example, MYC has been known to be a very important driver in the B-cell lymphoma world now for decades. And really, there are no clinically available direct agents that directly target MYC.