TRANSCRIPT

Significance of the Tumor Microenvironment in Hematological Malignancies
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Slide 1
Significance of the Tumor Microenvironment in Hematological Malignancies
Dr. Louis DeGennaro:

Good afternoon. I'm Dr. Lou DeGennaro. I'm the Executive Vice President and Chief Mission Officer of The Leukemia & Lymphoma Society, and I'm here to welcome you to this year's Leukemia & Lymphoma Society's sponsored symposium.

The thing I wanted to do before we get started is to thank the corporate sponsors that have helped the Society make this program possible. They include Millennium Pharmaceuticals, Celgene Corporation and Allos Therapeutics and the grant support that they've provided goes a long way to helping the Society create high quality educational programs like this.

The other group that helps us create high quality educational programs like this is a subcommittee of the medical and scientific affairs committee of LLS, it's called the Professional Education Committee. And we're lucky to have Dr. Irv Bernstein from the Fred Hutchinson Cancer Research Center, who is the chairman of that committee, we're lucky to have him with us. And I'm going to turn the podium over to him to introduce the symposium and moderate the session.

Dr. Irwin Bernstein:

Thanks, Lou. It's a real pleasure to welcome you all here. The symposium this year is on the Significance of the Tumor Microenvironment in Hematological Malignancies. And we're particularly happy to have the opportunity to put this session on because this is a field that's been smoldering along for years, controversial about the significance. And of late, as you'll hear today, both at the fundamental level and in terms of clinical translation, significant new data is coming along. And as I believe you will see in the next few years, there'll be exponential growth in this field with highly significant data emerging. So this is a very timely occasion.

Our first speaker will tell you a lot about the microenvironment of the cell because he's been a key player in trying to decipher the interplay between the microenvironment and normal and malignant hematopoietic cells. So I'd like to introduce Sean Morrison as the first speaker. He's Director of the University of Michigan Center for Stem Cell Biology and Professor of the Department of Internal Medicine and Cell and Developmental Biology and Research Professor in the Life Sciences Institute at the University of Michigan. So he's now going to present his talk on The Hematopoietic Stem Cell Niche.

I might say that we'll have hopefully a few questions at the end of each talk, but we've reserved time at the end for more in-depth discussion with all of the speakers.

Slide 2:
The Hematopoietic Stem Cell Niche
Dr. Sean Morrison:

Thanks for the invitation to participate in the meeting. It'll be really interesting to see how these talks come together. It's a thoughtful session, having a group of speakers together that isn't often together at other kinds of meetings.

So my job this afternoon is to talk to you about what we know at this point about the nature of the hematopoietic stem cell niche.

Slide 3:

Disclosure of Conflicts of Interest

I consult for Hospira and Fate Therapeutics. I was a cofounder and stockholder of OncoMed Pharmaceuticals and I've spoken at various pharmaceutical companies in the last year. But I'm not going to talk about anything that's directly related to any of that.

Slide 4:

The nature of the hematopoietic stem cell niche remains uncertain

So you'll see a lot of suggestions in the literature about the nature of the hematopoietic stem cell niche. And I'd just like to start off by saying I think we don't know the nature of the hematopoietic stem cell niche at this point. Over the past several years the most popular model in the literature has been the idea that hematopoietic stem cells reside in an osteoblastic niche. The most popular version of which held that hematopoietic stem cells adhere by N-cadherin-mediated homophilic interactions with osteoblasts and osteoblasts secrete all the factors that regulate stem cell maintenance. And there's literally scores of papers in the literature that interpret their data through the prism of that model. But what most people I think didn't or don't recognize is that model is intuitively attractive and was repeated so many times, that I think most specialists didn't realize that many critical elements of that model had never been tested directly. And I think as we and others have tested that model directly, I think at this point the data aren't consistent with the model.

Now having said that, that doesn't mean that osteoblasts are not involved in regulating hematopoietic stem cell maintenance. They may directly or indirectly regulate hematopoietic stem cell maintenance through other mechanisms that don't necessarily involve cell-cell contact. But there's a lot of data that's emerging from our laboratory and other laboratories, suggesting the possibility of a perivascular niche in bone marrow. But again it's hypothetical because nobody has yet conditionally deleted from any cell type in the bone marrow, factors that are genetically required for stem cell maintenance. And so all of the arguments remain somewhat indirect in terms of the identity of the niche. And that's why I want to be very clear about saying that there still are many models that remain consistent with existing data.

Slide 5:

$CD150^{+}CD48^{-}CD41^{-}$ cells in bone marrow and spleen are highly purified HSCs

So we started in this area of trying to understand the environments that maintain stem cells when we found that SLAM family markers could be used to very highly purify mouse hematopoietic stem cells with simple combinations of markers. So previous to this work, the markers – you needed 12 different antibodies to purify mouse hematopoietic stem cells and 5 color flow cytometry and that made it impossible to cut sections through hematopoietic tissues and identify with precision where the stem cells were localized. And given that impossibility, the

field was left using simplified combinations of markers that generally gave very poor purity to try to localize populations that were enriched for stem cells, but in fact the vast majority of cells in those populations weren't stem cells at all. And so it left quite a lot of uncertainty about which were the stem cells and where they were localized.

But when we isolate cells that are positive for CD150 and negative for CD48, in this box, we're identifying a rare subpopulation of bone marrow cells, 45% of which, when you isolate them from bone marrow, give long-term multilineage reconstitution of irradiated mice and these markers also do a better job of purifying stem cells in other contexts where the kind of cononical historical markers did a poor job, such as from cytokine-mobilized splenocytes, where 33% of the cells in this population give stem cell activity in irradiated mice. So this made it possible for the first time to localize hematopoietic stem cells in tissue sections using a two color stain.

Slide 6:

HSCs in bone marrow are usually adjacent to sinusoids

So this is what we saw when we did that. When we identified the cells that were CD150 positive and negative for CD48, CD41 and linage markers, we found that most of the stem cells that we identified were in the trabecular zone of bones. Consistent with the idea from a number of different groups that for some reason stem cells like to be in the trabecular zone of bone and like to be at least relatively close to osteoblasts. But we only found 14% of these cells that were actually at the endosteal surface. And that really contrasted with the idea that has been prevalent in the literature, that stem cells are maintained by cell-cell adhesion with osteoblasts.

So this observation leaves two possibilities. One possibility is that at any one time there's only a small fraction of stem cells that are actually in the niche. Another possibility is that there's a small fraction of stem cells that are in an osteoblastic niche and there's other stem cells in another niche. Or the other possibility is that the niche doesn't involve direct contact with osteoblasts.

In fact, 60% of the cells that we saw were like this one, immediately adjacent to sinusoids. So the white stain is an endothelial stain, surrounding sinusoid in the bone marrow. This giant yellow cell is a megakaryocyte, which also cluster around sinusoids. And this red cell is from this highly purified stem cell population. And this, we speculated, based on this observation, that most of the cells were next to sinusoids, that there may in fact be a perivascular niche in the bone marrow. And I'll tell you more about that.

Slide 7:

HSCs in mobilized spleen were usually associated with sinusoids

When we looked in extramedullary tissues we saw the same thing. In these experiments, 62% of the cells were like these two guys, again immediately adjacent to sinusoids, when you looked at cytokine-mobilized spleen.

So whether we looked in bone marrow or in extramedullary tissues, most of the stem cells that we saw were adjacent to sinusoids.

Slide 8:

HSC localization using validated markers suggests that most HSCs are perivascular

So localization of hematopoietic stem cells with validated markers suggest that most

hematopoietic stem cells reside perivascularly, and those data contrasted then with the model that most HSCs reside on the surface of osteoblasts. So this raised the question of whether hematopoietic stem cells were regulated by osteoblasts, by direct or by indirect interactions. And so we decided to go back and directly test this osteoblastic niche model that has been so popular.

Slide 9:

Do HSCs adhere to osteoblasts via N-cadherin homotypic interactions?

So this is one popular iteration of the model, where on the top you see a hematopoietic stem cell, on the bottom is an osteoblast, and this model proposes that the hematopoietic stem cells are in physical contact with the osteoblasts via N-cadherin-mediated homophilic adhesion. And that all or many of the other factors that regulate stem cell maintenance, like angiopoietin 1, CXCL12, steel factor, are secreted by the osteoblasts and that this is the nature of the niche.

So a linchpin of this model, and in fact the inspiration for the model, was the idea that stem cells depend on this whole N-cadherin-mediated interaction with the osteoblasts. But in fact, nobody had ever shown that an N-cadherin-expressing cell could give stem cell activity.

Slide 10:

We could not detect *N-Cadherin* expression in highly purified HSCs by quantitative PCR

So when we looked at that directly, no matter what technique we used, to make a long story short, we weren't able to detect any N-cadherin expression in hematopoietic stem cells.

Here I'm showing you data by PCR, where whether we used SLAM family markers or other markers, we weren't able to amplify N-cadherin from hematopoietic stem cells, in contrast to what you see from postnatal forebrain cells, which are well established to express N-cadherin.

Slide 11:

We are not able to detect any staining of highly purified HSCs with any anti-N-cadherin antibody

We also couldn't find it by microarray analysis, by western blot, by gene-trapping, etc. We tried all of the commercially available anti-N-cadherin antibodies and while those antibodies will stain forebrain cells, we've never been able to detect staining of highly purified stem cell populations, hematopoietic stem cell populations, irrespective of what markers we used to isolate them.

Since we've published this, multiple other laboratories have since published the same thing.

Slide 12:

Conditional deletion of *N-cadherin* in vivo does not affect hematopoiesis

Of course, you can imagine that N-cadherin could be expressed at an extraordinarily low level on stem cells and still be – in a way that's hard to detect – and yet still be functionally important. So we conditionally deleted N-cadherin from hematopoietic stem cells as well as other bone marrow cells, using Mx1-cre, and whether we waited one month or five months after deletion, it never had any effect on stem cell frequency or hematopoiesis.

Slide 13:

Deletion of *N-cadherin* does not impair HSC reconstituting capacity in primary or secondary recipients

So even months after N-cadherin deletion there was no effect on blood cell counts, no effect on bone marrow cellularity, no effect on colony-forming cells, no effect on stem cell frequency, no effect on lineage composition of the bone marrow. It also didn't affect the reconstituting capacity of hematopoietic stem cells upon transplantation into irradiated mice, irrespective of whether we deleted before or after transplantation. And even if we did serial transplantation, there was no effect of N-cadherin deletion on stem cell activity, consistent with our failure to detect its expression. So then the data then don't support the idea that hematopoietic stem cells depend on N-cadherin for their maintenance.

Slide 14:

What cells express the factors that regulate HSC maintenance?

Raising a second question then, it's been presumed that all of the factors that regulate stem cell maintenance must be secreted by osteoblasts if the stem cells are maintained in physical proximity to the osteoblasts. But in fact, arguably nobody has ever looked systematically at the expression patterns of these factors, partly because the reagents available to be able to study the expression patterns haven't been good. There's not good antibodies against some of these things and in general it's very hard to localize the expression patterns of secreted factors by antibody staining.

So we've decided to try to systematically assess these expression patterns of factors that are genetically known to regulate hematopoietic stem cell maintenance, that we generated GFP or **dsRed knock-in** alleles of each of these genes. So we generated angiopoietin GFP mice, steel factor or stem cell factor GFP mice, and CXCL12 dsRed mice, so that we could look systematically through the bone marrow and see all the cells that were expressing these things, irrespective of whether we had good antibodies available, and know for the first time which cells, how many different kinds of cells express it, do osteoblasts really look like they're the major source of these factors, as has been assumed.

Slide 15:

Perivascular cells, including megakaryocytes, are the major sources of Ang-1 in the bone marrow

So I'll show you some data first on angiopoietin-1. In this case this is the only factor for which we have been able to find antibodies that stain really clearly. So I'm showing you antibody staining data here, but we see the same thing when we look at the GFP knock-in mouse. And so on the top is a negative control just showing you that this is not background staining that I'm showing you. And here's the staining for angiopoietin-1 and this is typical for what we see in the bone marrow.

The vast majority of staining for angiopoietin-1 that we see is perivascular. We see it very clearly in megakaryocytes that cluster around sinusoids in the bone marrow, but it's not just in megakaryocytes, it's also in other perivascular cells. We've not been able to detect any expression in osteoblasts in contrast to the idea that osteoblasts are the major source, but of course, that doesn't rule out the possibility that osteoblasts are a source, so we're in the process of conditionally deleting angiopoietin-1 in different bone marrow cell populations to test how it

regulates stem cells and what the critical source is.

Slide 16:
***Scf* is expressed mainly by vascular/perivascular cells throughout bone marrow**

Here's data from the SCF GFP mice. So this is a low magnification view of the entire bone marrow. And you can see that what's most striking when you look across the whole bone marrow is not staining for SCF, so MECA-32 was an endothelial marker, so you can see as David Scadden has published, that the endosteum is very highly vascularized, in contrast to this idea that there's some kind of hypoxic niche at the endosteum. And instead of seeing SCF expression concentrated at the endosteum, in fact you see SCF expression throughout the bone marrow and it's very clearly surrounding the sinusoids within the bone marrow.

Slide 17:
***Scf* is primarily expressed by vascular/perivascular cells in the bone marrow**

So here's a higher magnification view. Again, negative control at the top just to convince you that this isn't non-specific staining. Here we have an antibody that stains endothelial cells, so you can see a sinusoid here, and in the merged image you can see that the SCF GFP expression is very specifically around the sinusoids.

Slide 18:
***Scf* is primarily expressed by vascular/perivascular cells in the bone marrow**

So again this doesn't prove that perivascular cells are the physiologically important source of steel factor for stem cell maintenance, but they sure are the predominant source in the bone marrow.

Slide 19:
No detectable Scf expression by osteoblasts in bone marrow

If you look at higher magnification at bone lining cells, so this is a section through trabecular bone, here is a stain with osteopontin to identify the bone lining cells around some trabecular bone, and you don't see any SCF expression that overlaps with the osteopontin, in contrast to the idea that osteoblasts would be the major source of SCF. Instead you can see SCF very clearly around sinusoids, some of which are very close to the bone surface. So you could imagine the situation where if osteoblasts are actually secreting factors that support stem cell maintenance, that you could have cells that are around the sinusoid and close enough to an osteoblast to be influenced by factors from both. And this is part of the reason why I say that although the field has defaulted to a kind of very specific model of the niche, there remain many models that are consistent with the actual data that we have.

Slide 20:
***Cxcl12 (Sdf-1)* is expressed primarily by vascular/perivascular cells in the bone marrow**

So what about CXCL12? Well, we see the same thing with CXCL12 that I just described with Sdf-1. You don't see very clear expression at the endosteal surface among the bone lining cells, but you do see very clear expression by perivascular cells around sinusoids that are throughout the bone marrow.

Slide 21:

What can we conclude about HSC niches?

So what can we conclude based on these and other data? Well, I think the data pretty clearly indicate that hematopoietic stem cells are not maintained by N-cadherin-mediated adhesion to osteoblasts. And osteoblasts, while there's been a tendency to assume that osteoblasts must be the source of most or all factors that regulate stem cell maintenance, I think they're not the major source of all factors required for stem cell maintenance. But of course, it's important to bear in mind that they could still be a critical source of at least some factors that regulate stem cell maintenance.

Hematopoietic stem cells and the cells that produce angiopoietin-1, steel factor and CXCL 12 in the bone marrow are primarily perivascular and there doesn't appear, from our experiments, to be any single cell type that produces all the factors that regulate stem cell maintenance. We think it'll be a collaboration of multiple different cell types. The data, in our view, are pointing primarily towards perivascular sources right now. But remember, nobody has yet conditionally deleted any of these factors from specific cell types in the bone marrow. And so even though one cell type may look like the major source of a factor, that doesn't necessarily mean that another cell type is not expressing at a low level and for a variety of reasons even low level of expression could be more physiologically important for stem cell maintenance from all we know.

So what cells actually promote stem cell maintenance in vivo? And at this point I'd like to show you some data from a handful of other laboratories that have made critical contributions to this area.

Slide 22:

CXCL12-expressing perivascular cells are required for HSC maintenance (Nagasawa and colleagues)

First of all, Dr. Nagasawa and colleagues had shown independently that CXCL12 expression, just as we have seen, they have seen hematopoietic stem cells are primarily around sinusoids in the bone marrow, as are CXCL12 expressing cells. And when they express diphtheria toxin onto the diphtheria toxin receptor under the control of the CXCL12 promoter, allowing them to deplete CXCL12 expressing cells from the bone marrow, which in their hands as well appear to be primarily perivascular, they see a depletion of hematopoietic stem cells, providing some functional evidence that these perivascular cells are functionally important, but of course you have to bear in mind that, for all we know, there could be other CXCL12 expressing cells in other locations that are also depleted in these experiments.

Slide 23:

Perivascular Nestin+ mesenchymal stem cells in the bone marrow are required to maintain HSCs (Frenette and colleagues)

Paul Frenette and colleagues recently published an important paper in Nature where they found that there are nestin-positive mesenchymal stem cells that are also primarily perivascular in the bone marrow. And they did a similar experiment where they expressed diphtheria toxin receptor under the control of the nestin promoter, and when they administered diphtheria toxin to those mice, again, they depleted these nestin-positive mesenchymal stem cells and saw a

depletion of hematopoietic stem cells as well. And so again, their data support the idea of a perivascular cell that they feel is expressing multiple factors that regulate stem cell maintenance.

And it's interesting to speculate at this point about whether or not the CXCL12 expressing cells, characterized in the Nagasawa work, and the nestin-positive cells in the Frenette work, whether those are the same population of cells, whether they're different populations of perivascular cells, or whether they're partially overlapping. One of the limiting factors now in this field in trying to work out the details is that we don't have very much of a sense of how much heterogeneity there is among different perivascular cells in the bone marrow. My lab, we're just in the process of trying to identify different kinds of promoters or cre alleles that would allow us to conditionally delete genes in those populations to directly assess their contribution to stem cell maintenance. And we don't know how many different perivascular populations we'll find.

Slide 24:
Endothelial cells are essential for the recovery of HSCs after sublethal irradiation (Rafii and colleagues)

Shahin Rafii's laboratory has recently presented pretty compelling evidence that endothelial cells are critical for at least the recovery of the hematopoietic system after irradiation in the recovery of stem cell activity, because if they administer anti-VE cadherin or anti-VEGF receptor 2 antibodies, they can ablate the endothelial cells in the bone marrow and they see a failure to recover from injury and they feel that their data suggest that endothelial cells are actually secreting factors that are regulating stem cells in this perivascular niche.

Slide 25:
Osteoblasts are likely to regulate HSC localization/maintenance, though this may or may not involve cell-cell contact (Scadden and colleagues)

Of course, there's been a lot of work, which at least circumstantially suggests that osteoblasts are involved somehow in the regulation of stem cell localization and maintenance. Here I'm just showing one example from David Scadden's laboratory, where they showed that if they deleted the calcium-sensing receptor, that they saw a partial depletion of hematopoietic stem cells under steady state conditions, but even more profound, when they transplanted the calcium-sensing receptor deficient cells into irradiated mice, those cells lack the ability to reconstitute irradiated mice, perhaps because of a defect in the ability to home it into the bone marrow.

And so, of course, there could be in principle lots of different cell populations in the bone marrow that regulate calcium availability in the intracellular space, but the most obvious potential source is at the endosteum, where osteoblasts and osteoclasts are remodeling bone in a dynamic way. And so this supports the idea that osteoblasts are doing something here. And one of the fundamental questions is whether osteoblasts are directly secreting factors that promote stem cell maintenance or whether they are secreting factors that act indirectly to regulate the niche and to regulate stem cell maintenance, perhaps by influencing something like the vascularity of the bone marrow.

Slide 26:

What cells secrete the factors that promote HSC maintenance?
Conditional deletion of *Scf* from HSCs and endothelial cells using *Tie2-Cre* leads to HSC depletion

So there's a number of different laboratories that have a number of different stories, that implicate different cell populations in the bone marrow in the direct or indirect regulation of stem cell maintenance. To directly characterize the niche what we need to know is which cells are the physiologically important source of factors that actually are genetically required for stem cell maintenance. And as I mentioned earlier, none of the factors that we know are genetically required for stem cell maintenance have ever been conditionally deleted from any of these candidate niche cells in the bone marrow. And as a consequence of that, all of these arguments about the identity of the cells that create the niche are somewhat indirect.

So to try to address this directly, my lab has recently generated floxed alleles of steel factor or stem cell factor, angiopoietin-,1 and CXCL 12, and we're in the process of identifying cre alleles that are specific for all these different cell populations in the bone marrow, and we're in the process of mating together all of these mice to conditionally delete each of these genes in all of these different cell populations in the bone marrow, to assess which is this physiologically important source of the factors for stem cell maintenance.

So we're still in the process of doing the experiments and for the most part we don't know what the outcome is going to be yet. But just let me leave you with one tantalizing piece of data and that is that when we conditionally delete steel factor with Tie2-cre, which deletes in hematopoietic stem cells and endothelial cells, we see a profound depletion of hematopoietic stem cells and a profound loss of reconstituting activity.

So this leaves two possibilities for the source of steel factor or at least for a critical source of steel factor under these conditions. Either the hematopoietic stem cells are making it in an autocrine fashion or other hematopoietic cells are generating it or it's from endothelial cells.

Slide 27:
The precise nature of the HSC niche remains uncertain but it involves direct or indirect regulation by multiple vascular, perivascular, and endosteal cell types

Now from our expression pattern studies, as I've already shown you, endothelial cells could be an important source of steel factor. We're not seeing clear expression by hematopoietic cells. So we're doing more functional experiments to try to directly address that now in a functional way, but the simplest interpretation of the data that we have so far is that endothelial cells really are a critical component of the niche and one of the things that they do is secrete steel factor that regulates stem cell maintenance.

So this is going to be a complicated problem. So here's a section through the bone marrow that illustrates the complexity of the problem. There is a hematopoietic stem cell here, adjacent to a sinusoid, where there's also megakaryocytes present. You can see that that cell is close not only to endothelial cells and megakaryocytes and other perivascular cells and hematopoietic cells and it's not so far from the bone surface in this case, though we see lots of other stem cells that are, as far as we can tell, quite far from the bone surface.

So there's a lot of different cell types that could potentially be regulating the niche and influencing the maintenance of these cells. We don't think that there's going to be a single population that regulates the niche. We think there will be multiple populations, potentially

including perivascular cells as well as cells that are not perivascular, a combination of direct and indirect mechanisms, and I think the models for what constitute the niche are going to change dramatically over the next five years, as a number of different laboratories do the kinds of experiments that I've described to you and actually figure out which are the physiologically important sources of factors for stem cell maintenance.

And one take-home message for now is that I think we should be careful about interpreting ongoing experiments through the prism of the idea that the osteoblasts are really the critical source for all of these factors, because there may be many cell types in the bone marrow and even indeed more important cell types in the bone marrow, in terms of not only influencing hematopoietic stem cells, but influencing leukemic cells or other hematopoietic malignancies in that bone marrow microenvironment.

Slide 28:
Acknowledgements slide

So the discovery of the SLAM family markers that got us started was from Mark Kiel and Omer Yilmaz in my laboratory. The N-cadherin work that I mentioned briefly was done by Melih Acar and Mark Kiel in the lab. All of the ongoing work characterizing steel factor and angiopoietin-1 and Cxcl 12 is done by Lei Ding in my laboratory, a Helen Hay Whitney fellow, and I mentioned data to you from several other laboratories, including published work from David Scadden, Shahin Rafii, Paul Frenette and Takashi Nagasawa.

Slide 29:
Thank You

Thank you.

So if there are a few important questions we can take them now. Of course, there'll be a larger discussion section at the end.

Audience:

[Inaudible]

Dr. Sean Morrison:

So I think it's anybody's guess about what this all means for leukemic stem cells. One possibility is that leukemic stem cells are really far less factor-dependent than normal hematopoietic stem cells and are not as dependent on a microenvironment as the normal hematopoietic stem cells and therefore do just fine when they're localized in a lot of different places. Another possibility is that that may be true, but there may be some microenvironments that are more supportive than others, so that therapy resistance is – the cells that survive therapy are the cells that happen to find themselves in a really supportive microenvironment when the chemotherapy comes by. Another possibility is that there's a different niche for leukemic stem cells as compared to hematopoietic stem cells, that they're really depending on other factors because maybe the cell of origin is quite different, maybe they're arising from a myeloid-restrictive progenitor, and they care more about myeloid factors than the kinds of factors you'd see in a stem cell niche. So the bottom there is I think all of these questions are fundamentally important and I think they all remain open.

Audience:

Just curious, you showed a lot of normal microenvironment, but how do you think that might change after, you know, radiation, or other types of treatments and whether the niche itself might be a little more of a sort of flexible environment to respond to those genes and conditions?

Dr. Sean Morrison:

Yeah, Shelly, I think that's a fundamental question, too, and I think we don't know the answer yet. And I think again all of the possibilities that you could imagine are still on the table. Shahin Rafii has some evidence that the niche may indeed change in nature between an injured circumstances versus a steady state circumstance. But in terms of characterizing how it changes, I think that work remains to be done.

Dr. Irv Bernstein:

Well, let me introduce our next speaker, Dr. Lou Staudt, who's going to talk about Cytokines and the Microenvironment of Lymphoma. Lou is the Deputy Chief of the Metabolism Branch in the Center for Cancer Research at the NCI. He received his MD and PhD in immunology from the University of Pennsylvania. And we're fortunate that we're able to have him speak with us today.

Slide 30:
Cytokines and the Microenvironment of Lymphoma
Dr. Louis Staudt:

So I'm going to try to give you today two reasons why we should care about the tumor microenvironment. Everybody thinks they like it, but the two reasons that I'm going to try to convince you that you should is that in real clinical patients, that there is an influence of the microenvironment on their overall survival in the context of our current therapy. That will be the first part of the talk. And then I will go on to show you two unpublished stories where oncogenic changes in the malignant cell seem to, in large part, be there because of their influence on the microenvironment.

So diffuse lymphoma is our topic. It is the most common non-Hodgkin lymphoma. We're able to cure about 50% of these folks, but we're not able to cure the rest and we're unhappy about that. And we've been trying to figure out who they are and can we come up with new therapies for them.

Slide 31:
Dissecting Cancer into Molecularly and Clinically Distinct Subgroups by Gene Expression Profiling

And by gene expression profiling, as you may know, there are three now accepted subtypes of diffuse lymphoma. We call them the germinal center or GCB type, the activated B cell or ABC type, and primary mediastinal B cell lymphoma. And they all have a characteristic gene expression profiling shown here. They're clear as night and day. These are not gray areas. They're very distinct. And I won't go into this too much, but they have large differences in their

genetics.

Slide 32:
Dissecting Cancer into Molecularly and Clinically Distinct Subgroups by Gene Expression Profiling

Now clinically the ABC patients, those tumors are the bad actors. We're only curing – this is three year progression-free survival – so at best we're curing 40% of them, probably less, and GCB is coming in at around 75%, so we're not done there, but lot to do with ABCs. But you'll notice that neither of these – this subdivision does not completely capture the clinical heterogeneity of who is cured and who is not.

Slide 33:
Supervised Discovery of Genes that Influence Clinical Outcome in Cancer

And so that's what we set out to do several years ago, doing this sort of supervised analysis, where we take every gene on a microarray and look with its gene expression, whether it correlates with survival. Gene A is found more highly expressed in patients who have long survival, gene B just the opposite.

Slide 34:
Gene Expression Signatures that Predict Survival for DLBCL Treated With R-CHOP

So we did that for a set of 233 diffuse large B cell lymphomas. And then we took all of the genes that had some cutoff predicting survival and then clustered them. This is an important step. Of course, you have to worry about the multiple hypotheses you're testing, so if you confine yourself to large signatures with many genes in them, then you feel like you might be getting at areas of biology rather than statistical noise.

So the first area of biology is what I've already presented. The germinal center B cell signature was highly predictive of survival. That's shown at the top. And that's mirroring the ABC-GCB distinction. But we found two other signatures, which are called stromal-1 and stromal-2, that were both predictive of survival.

Slide 35:
Creation of a Survival Model for DLBCL Treated with R-CHOP Using Gene Expression Signatures

And you'll see that in this slide, both within ABC and within GCB patients, there is a heterogeneity for both of these stromal markers, saying that this is a cross-cutting feature that appears to be predicting survival.

Slide 36:
The Gene Expression-Based Survival Predictor Score Predicts Progression Free Survival in DLBCL Treated With R-CHOP

And so we made a predictor and I won't go into – but you average all the genes in each signature and take these signature averages and put them together into a quantitative model, where you have a so-called survival predictor score. You rank the patients according to that and divide them into quartiles for visualization. And you'll see that this predictor is pretty good now.

It has 33% survival for one quarter of the patients and 84% survival for the best quartile of patients, and the others in between. So we're capturing more of the clinical heterogeneity here.

Slide 37:
What is the Biological Basis for the Prognostic Signatures in Diffuse Large B Cell Lymphoma?

So what do these signatures show us biologically? So we simply sorted the diffuse lymphoma biopsies into the malignancy,19 positive, and the non-malignancy, 19 negative cells, and did affymetrix profiling.

Slide 38:
Survival Predictor Signatures are Derived From Malignant and Non-malignant Cells in DLBCL Biopsies

And found that the stromal-1 and stromal-2 signatures were found, in red, indicating in the non-malignant compartment, and of course, the germinal center signature was found in the malignant compartment.

Slide 39:
The Stromal-1 Signature Encodes Extracelluar Matrix Components and macrophage/myeloid-restricted Proteins

So you look at the genes in stromal-1 signature and what screams out at you is extracellular matrix, so you think collagen, collagen, collagen, you see fibronectin, you see osteonectin, the whole schmear. And so that's obviously part of it. And that's in purple. And in red are some well known markers of the myeloid lineage, especially macrophages.

Slide 40:
The Stromal-1 Signature Identifies DLBCL Tumors Enriched in Myeloid-derived Cells But Not T Cells

And you can see that if you take a previously defined monocyte signature, that it is related to this stromal score here. Whereas a Pan-T cell signature has no relationship whatsoever to it. So this about monocytes macrophages.

Slide 41:
The Stromal-1 Signature Expression in Non-malignant Tumor-infiltrating cells in Diffuse Large B Cell Lymphoma

And by in situ analysis we can see two of these, MMP9 and SPARC, lighting up histiocytic macrophage cells in these biopsies.

Slide 42:
The Stromal-1 Signature Gene SPARC is Expressed in Tumor-infiltrating Macrophages in Diffuse Large B Cell Lymphoma

And here is CD 68 as a marker of macrophages, also showing up in these biopsies. So part of this is the macrophage. But the other part is the extracellular matrix.

Slide 43:
The Stromal-1 Signature Gene Encode Components of the Extracellular Matrix in Diffuse Large B Cell Lymphoma

And here, as you may know, histologically when you look at some of these diffuse lymphomas, there is really strong fibrous threads running through them and here is lit up with fibronectin.

Slide 44:
CTGF Protein is Localized to Tumor Infiltrating Macrphages in Diffuse Large B Cell Lymphomas

Now an interesting marker that's been at the top of our list, even when we published our first profiling in 2002, was something called CTGF, connective tissue growth factor. And I didn't know anything about it, but it's a cytokine that is very important in fibrosis. And here you see that CTGF is actually lighting up in the macrophage cells in these biopsies. So what is this?

Slide 45:
Connective Tissue Growth Factor

It's a member of a so-called CCC family of secreted proteins. It binds to components of the extracellular matrix such as fibronectin, integrins, and heparin, and it is pro-fibrotic. You can just inject it into the flank of the mouse and you'll get a fibroma. And it's been implicated in the stromal reactions in various solid tumors and therefore we suspect that it might have something to do with this stromal reaction, stromal-1 reaction in diffuse lymphomas.

Slide 46:
The Stromal-2 Signature Encodes Regulators and Components of Endothelial Cells and Adipocytes

So what is stromal-2? Here we look at the genes and what also screams out is endothelial markers. We have von Willebrand's factor and a lot of the CD markers of endothelial cells. And strangely, and Sean may know about this, has some adipocyte-restricted genes and we really haven't interrogated those further.

Slide 47:
The Stromal Score Correlates with Tumor Blood Vessel Density in DLBCL Treated With R-CHOP

But when we did a quantitative measure of the micro-vessel density, shown here, and we could show a pretty good correlation with this stromal-2 signature or tumors with high stromal-2 had more – higher micro-vessel density.

Slide 48:
High Microvessel Density and Inferior Overall Survival in Diffuse Large B Cell Lymphoma

And then one of our colleagues in the LMPP, Luis Campo, led a study in which he took two large cohorts in diffuse lymphoma, on from the LMPP itself and one from a Spanish cohort, and did that quantitative measure of angiogenesis or micro-vessel density more accurately, and found that in these two cohorts there was a strong relationship of adverse survival to high micro-

vessel density.

So now this is completely fanciful and I can't do conditional knockout mice and to test any of this, alright? So give me a break. But nonetheless, I'll go with it.

Slide 49:
A Stromal Dependence Model of Diffuse Large B Cell Lymphoma

So here we have shown, a tumor that has maybe high stromal-1 score. It has CTGF as extracellular matrix, the fibroblasts are producing it, we think. It has high thrombospondin-2, which is an inhibitor of angiogenesis.

Slide 50:
A Stromal Dependence Model of Diffuse Large B Cell Lymphoma

It also expresses MMP9, which helps cytokines sort of have a more profound effect, and KIT ligand is actually produced. That is interesting because it can mobilize bone marrow endothelial precursor cells, which may be attracted to tumors that have this stromal-2 signature, because they produce highly characteristically SDF-1. So the whole idea is that there is an angiogenic switch in diffuse lymphoma.

Slide 51:
Survival-associated Signatures: Implications for Therapy of DLBCL

So we would conclude then that clinical trials in diffuse lymphoma need to assess these stromal markers in the context of the trial, otherwise we're taking a heterogenous disease and treating it as one monolithic entity and we won't make progress that way. We think there may be some promise, and I will be very cautious about this, with angiogenesis inhibitors in patients with this high angiogenic score or stromal-2 score. Mind you that's a small subset of the patients and the failure of some of these angiogenesis inhibitors may be because they haven't been tested in this small cohort.

Sdf-1, there are inhibitors of its interaction with CXCR4. Those could be tried as inhibiting in these bad acting tumors. Now the good acting tumors, although they're good acting, we're not curing those folks either. So perhaps we could target some trophic interactions between those macrophages and the lymphoma cell, so there might be many ways to target that with antibodies to macrophages.

And finally there are clinical inhibitors or in clinical development inhibitors of CTGF that are there for its role in fibrotic diseases such as primary pulmonary fibrosis and such. And so they may be interesting to look at as well.

Slide 52:
Genetic Aberrations in the Malignant Lymphoma Cell that May Influence the Immune Response

Alright, so now the second part of the talk is to convince you that there may be some genetic modifications to the tumor genome that influence the microenvironment.

Slide 53:
Primary Mediastinal B Cell Lymphoma

So the first story has to do with primary mediastinal lymphoma. And whether there might be an important role for some very important molecules that are inhibitory to T cell responses, namely PD-L1 and PD-L2.

Slide 54:
Molecular Similarities between PMBL and Hodgkin's Lymphoma

And what we found several years ago was that there was a striking relation by gene expression between Hodgkin's lymphoma and primary mediastinal lymphoma. And what's more, they share as their most recurrent abnormality, an amplification of a region on 9p24, chromosome 9p24, that is found in 30 to 50% of both of these types of tumors. So this is a common pathogenesis.

Slide 55:
The 9p24 Amplicon in PMBL and HL Contains Many Genes That Are Overexpressed in Association With their Amplification

And we were looking into what genes were in that interval and got together by GCH a number of tumors, defined no smaller than a 3 megabase region on 9p24 that was commonly gained or amplified, and within that region there were several genes, in red, that were over-expressed in tumors that had that amplicon and two of them were PD-L1 and PD-L2.

Slide 56:
Regulation of Lymphocyte Activation by PD-L1 and PD-L2

Now PD-L1 and 2 are B7 family members and they react with this inhibitory receptor on T cells PD-1. And if you knock out PD-1 in animals, you get a severe autoimmunity that's secondary to a defect in peripheral T cell tolerance. And what they seem to do is to inhibit activation of the T cell by T cell receptor and lead to a so-called T cell exhaustion phenotype.

Slide 57:
Thymic B Cells: the Putative Origin for Hodgkin's Lymphoma and Primary Mediastinal B Cell Lymphoma

Now primary mediastinal and Hodgkin's lymphoma are probably from a lonely B cell that resides within the thymus. So there are B cells within the thymus and they're shown here, CD20 positive cells, but they are surrounded by the CNT cells, so it may be very important for these molecules to interdict the interaction with T cells.

Slide 58:
Molecular Diagnosis of Primary Mediastinal B Cell Lymphoma By Gene Expression Profiling

And in our original profiling of primary mediastinal lymphoma, PD-L2 actually was the most characteristic gene that separated primary mediastinal from the other types of lymphomas.

Slide 59:
Molecular Diagnosis of Primary Mediastinal B Cell Lymphoma By Gene Expression Profiling

And in Hodgkin's lymphoma, we also found that PD-L2 characterized Hodgkin lymphoma cell lines as highly expressed.

Slide 60:
PD-L2 is highly expressed in both amplified and non-amplified PMBL patient samples

Now when we look at tumors, real cases now, we see high expression at the left and PD-L – in primary mediastinal tumors of PD-L2 – and that's even without the amplification, they have high expression. So this seems to be important in general for the biology.

Slide 61:
Selective Expression of PD-L1/PD-L2 in Hodgkin's Lymphoma and Primary Mediastinal B Cell Lymphoma

So here's a lymphoma cell line from primary mediastinal lymphoma. High expression of both PD-L1 and PD-L2 on the cell surface.

Slide 62:
Selective Expression of PD-L1/PD-L2 in Hodgkin's Lymphoma and Primary Mediastinal B Cell Lymphoma

If you look at a variety of primary mediastinal Hodgkin lymphoma cell lines, they all have more PD-L1 and PD-L2 than in diffuse lymphoma and mantle cell lymphoma cell lines.

Slide 63:
Does Overexpression of PD-L2 in PMBL Block T cell Activation?

So does over-expression of PD-L2 then have a functional consequence? Does it block T cell activation?

Slide 64:
Blockade of T Cell Activation by PMBL cells

So to do this we did a very simple mixing experiment between these lymphoma cell lines and a Jurkat T cell that was activated through its T cell receptor. And we see that as we titrate in these lymphoma cells, we get less activation of the CD69 marker. That's shown in a bar graph here, where we're getting this less activation.

Slide 65:
Blockade of T Cell Activation by PMBL Cells Depends on PD-L1/PD-L2 Interaction with PD-1

And when we add PD-L – the lymphoma cells. But then if we block with – block either with a PD-1 FC reagent or a PD-L2 FC reagent, we reverse this nega. So it seems that the lymphoma cells have the capacity to do this.

Slide 66:
The 9p24 Amplicon in PMBL and HL Contains Many Genes That Are Overexpressed in Association With their Amplification

Now this is not a simple story unfortunately, because this is a complex amplicon with

many other important genes. Two of them I want to highlight are JAK2 and JMJD2C.

Slide 67:
JAK2 and JMJD2C Cooperate to Block Heterochromatin and Promote PD-L1/PD-L2 Expression

And I won't be able to go into the details, this is a pretty complicated story that's coming out in Cancer Cell in the next issue, but what we feel we've demonstrated, or provided evidence for, is that both JAK2 and JMJD2C control the epigenetics of these lymphomas, in particular they counteract heterochromatin formation in these lymphomas. So you take repressive chromatin and turn it into active chromatin.

Slide 68:
JAK2 Phosphorylation of Histones Regulates Expression of PD-L2 and PD-L1

And then we've used – so what you may not know, which is in this paper, is that JAK2 actually functions in the nucleus to phosphorylate histone tails, and we then used ChIP-seq to find those targets and they're shown here. And we found that JAK2 is a target itself, JMJD2C is itself a target, MIC is a very important target, but so are PD-L1 and PD-L2. And a flavor of it is shown here. In red you see the marks for JAK2 phosphorylation at the PD-L1 and PD-L2 locus. But then if you treat with the JAK2 inhibitor you see that you lose all those marks.

Slide 69:
Opportunistic Choice of Chromosomal Loci by Cancer Amplicons

So the motto really is that the amplicon does many things. It promotes tumor survival through JAK2, tumor proliferation through JMJD2C and perhaps evasion of tumor immune surveillance through PD-L1 and PD-L2.

Slide 70:
Oncogenic Mutations that Influence Lymphoma Cytokine Secretion

Alright, so the final story then is a brand new story about recurrent oncogenic mutations in diffuse lymphoma that control cytokine production.

Slide 71:
Secretion of IL-6 and IL-10 by ABC DLBCL Cell Lines

And several years ago we noted that many of our ABC lymphomas secreted IL-6 and IL-10 at pretty substantial amounts. And that's shown at the left. Whereas the GCB cell lines did not do that.

Slide 72:
Definition of a STAT-3 High Subgroup of ABC DLBCLs

And we were able to study then the consequences of that by creating a signature of IL-6 and IL-10 signaling through JAK and STAT3 in particular. And we were able to show that there was a subset of ABC lymphomas that had a high STAT3 phenotype by this gene expression signature and one that was low. And the ones that had high STAT3 signature had high IL-6 mRNA and high IL-10 mRNA.

Slide 73:
Some ABC DLBCL Tumors Express STAT3 mRNA and phospho-STAT3 Protein

And if you look at all the tumors, the ABCs are the highest for STAT3 expression and you can see phospho-STAT3 in these malignant cells. So this is an active pathway. But we did not understand what was turning it on.

Slide 74:
Multiple Immunosuppressive Roles of IL-10

And of course, we know there's multiple immunosuppressive roles for IL-10. It can intervene in B and T cell activation. It can inhibit macrophage activation. It can inhibit dendritic cell activation. It is immunosuppressive. So this could be an obviously good strategy for a lymphoma.

Slide 75:
Control of T Helper Cell Differentiation by IL-6

IL-6 is a major cytokine that controls T helper cell differentiation with TGFβ, it promotes a Th17 phenotype. It in some degree promotes a TH2 phenotype. But it blocks a T regulatory phenotype and TH1 phenotype. So that all – so is immunomodulatory.

Slide 76:
"Achilles Heel" RNA Interference Screens to Identify New Molecular Targets in Cancer

So now how did we discover the genetic changes? Well, this relied on this genetic tool we've been using for several years, an RNA interference screen, so-called Achilles heel screen, in which we looked to identify new molecular targets in cancer.

Slide 77:
RNA interference is a normal cellular mechanism that can inactivate genes with great precision

It utilizes the RNA interference ability to inactivate genes with great precision. We've made libraries of small heparin RNAs that target thousands of genes. And we looked with those libraries at genes that are required for proliferation and survival of the cancer cells because, of course, these are new targets we think, for therapeutic development.

Slide 78:
MYAD88 and IRAK1 shRNAs Are Selectively Toxic for ABC DLBCL

So the new data is that huge hits on our screen were to MYD88 and IRAK1. Very important genes, as you'll see, in toll receptor and IL-1 receptor signaling. And in blue you see that this is the toxicity of these shRNAs for ABC lines, but in orange and other colors are a variety of GCB and Burkitt and mantle cell and multiple myeloma cell lines that are unphased by these shRNAs. But on the right side a ribosomal-targeted shRNA kills all the cells.

Slide 79:
MYD88 Signaling Downstream of Toll-like Receptors

So I've alluded to this. MYD88 is a key adapter that links most of the toll receptors, save TLR3, to many downstream pathways, through the activation of two kinases, IRAK4 and IRAK1, which then leads to TRAF6 ubiquitination activation, leading eventually to NF-κB activation, to p38 MAP kinase and JNK MAP kinase, as well as to the interferon pathway. So absolutely key mediator, immune defects in patients, without MYD88.

Slide 80:
MYD88 and IRAK1 shRNAs Are Selectively Toxic for ABC DLBCL

So here are some follow-up studies of these shRNAs where we see that when we put them into especially ABC lines in blue, we get a time-dependent killing, if we knock down __ MYD88 or IRAK1, whereas we don't see that in controlled GCB and multiple myeloma cell lines.

Slide 81:
Recurrent Mutations in the MYD88 TIR Domain in Lymphomas

Alright, so now we had this for several years actually and I was unhappy because I didn't understand why MYD88 should be necessary. So we turned another high throughput approach which is RNA sequencing, which is very effective at discovering recurrent mutations in cancer. And we found a bevy of mutations in MYD88 itself. And they're shown here, MYD88 on the top has a death domain linked to a tir domain. The death domain links it up to IRAK1 and IRAK4. The tir domain links it up to other tir domain-containing proteins such as the toll receptors themselves. And you'll see that most of the mutations are in the tir domain and many of them recurrent and most of them are in ABCs in light blue, but some did occur in GCB, Burkitt and MALT lymphomas, I'll show you.

Slide 82:
Recurrent Mutations in the MYD88 TIR Domain in Lymphomas

And the most important one is this L265P, which we had 55 mutations in ABC alone. We also had a GCB, a Burkitt lymphoma and six MALT lymphomas. So by far and away this is the most recurrent mutation, single mutation, that we've discovered in diffuse lymphoma.

Slide 83:
Location of MYD88 Mutants Within The TIR Domain

And what is it? So here's the structure of the tir domain. And you'll see in colored residues the places where there are mutations. The L265P is strangely right in the hydrophobic center of this molecule in a beta sheath. It's 100% conserved from humans to zebra fish, so it's an important amino acid, but when mutated with proline, you'll see it does really interesting things to the protein.

The other mutations are in the so-called BB loop, many of them, which has been shown previously to be involved in toll receptor interactions.

Slide 84:
Preferential Mutation of MYD88 in ABC DLBCL

So this is the important slide. We then surveyed over 350 tumors and we found 39% of the ABC tumors had MYD88 mutations. Of those, fully 29% had that one L265P mutation. The

L265P was rarely found in GCB, never in PMBL, occasionally in Burkitt, but was recurrently found in MALT lymphoma, 9%. The other mutations, the non-L2P, were found interestingly in both ABC and GCB, suggesting there be roles for this pathway also in GCB. But this tells us, this one slide, I think tells us that this MYD88 pathway is key to the pathogenesis of ABC lymphomas.

Slide 85
Addiction of ABC DLBCL Cells to MYD88 L265P

So you could ask do the cell lines that we have, depend on the mutation. In fact, every single cell line of ABC variety has a MYD88 mutation, most to L265P.

Slide 86
Addiction of ABC DLBCL Cells to MYD88 L265P

So we knocked down the endogenous gene, replaced it with either the wild type or mutant form, and we found that here when we put in the MYD88 shRNA in black, we kill the cells. If we rescue with the mutant form, they live. But if we put in the wild type they don't live. So this shows that the cells depend on this mutant form. They're addicted to this mutation.

Slide 87:
MYD88 Signaling Engages Multiple Downstream Pathways in ABC DLBCL

So what does it do? So we took one of the ABC cell lines and knocked down MYD88 and profiled the gene expression changes that ensued. And then looked in previous gene expression signatures and found a strong set of genes from an NF-κB signature, one from a JAK-STAT3 signature that we had previously developed, and also interestingly, from an interferon signature.

Slide 88:
MYD88 Signaling Activates the NF-κB Pathway in ABC DLBCL

So first the NF-κB pathway. When we take and put these mutants into a heterologous cell, a GCB cell that doesn't have NF-κB on, it turns on NF-κB by this reporter assay and by a target gene CD83. L265P in bright red is very strong, but interestingly, equally strong are two of the other recurrent mutations. So this tells us L265P may in part tell us about NF-κB ___ activation, but it doesn't explain completely why it's better than the other mutants and why it's selected more often.

Slide 89:
MYD88 L265P Induces IL-6 and IL-10 Signaling Through STAT3

So here's the cytokine aspect. We turn off both IL-6 and IL-10. We don't turn them off, we turn them down, as you'll see, when we knock down MYD88. This is not a full knock-down of MYD88. And on the right side we do rather turn off phosphorylation of STAT3. So we are affecting this pathway that I told you about.

Slide 90:
MYD88 Signaling Activates the Interferon Pathway in ABC DLBCL

Now this was interesting. One of our cell lines actually secretes type 1 interferon.

Interferon beta. And when we knock down MYD88, we turn down its expression. So obviously interferon beta itself is a potent immune modulatory agent. And if these tumors are actually spewing that out, that could have an influence both on the immune system and on the patient's well-being frankly.

Slide 91:
MYD88 L265P Associates With Phosphorylated IRAK1 and IRAK4

So here's how biochemically we think these work. When we put the L265P mutant into a heterologous cell it spontaneously forms this immune complex that can be immuno-precipitated under RIPA conditions, that involves IRAK4 and a hyper-phosphorylated slow-migrating version of IRAK1.

Slide 92:
MYD88 L265P Associates With Phosphorylated IRAK1 and IRAK4

And this shows that it is phosphorylated if we treat those immuno-precipitates with lambda phosphatase, we collapse that slow migrating band into this faster band.

Slide 93:
MYD88 L265P Associates With Phosphorylated IRAK1 and IRAK4

So we believe IRAK4 then would be phosphorylating IRAK1. And L265P, among all the mutants, is the best or maybe the only one that spontaneously forms as high level complex, which might be why it's the most recurrently selected.

Slide 94:
Knockdown of IRAK1 is selectively toxic for ABC DLBCL Cells

So this led us to wonder whether IRAK4 was important and that had not come in our screen. But we then developed shRNAs that could effectively target IRAK4 and they killed the very same ABC lines and not the control lines.

Slide 95:
An IRAK1/4 Inhibitor is selectively toxic for ABC DLBCL Cells

And then we turned to a small molecule inhibitor of IRAK4 kinase and that actually inhibits both – all the ABC lines and not the controls.

Slide 96:
IRAK4 Kinase Activity Induces IL-6 and IL-10 Secretion

So IRAK4 kinase activity appears to be important. And when we use that inhibitor in a concentration-dependent way, we turn off both IL-6 and IL-10.

Slide 97:
MYD88 Pathway Signaling in the Pathogenesis of ABC DLBCL

So I'd like to then conclude that this is an important pathway belief for ABC, DLBCL pathogenesis, that IRAK1 and 4 and MYD88 all play a role, that mutations in the MYD88 tir domain then give you the sort of genetic smoking gun, that this is an important pathway, that this

particular mutation, L265P coordinates this high level complex, it turns on a number of downstream pathways, NF-κB, JAK, STAT, interferon, and then that leads to this cytokine release that may have immune effects and affect the symptoms of the patient, I believe. But that it opens up a wonderful therapeutic opportunity because IRAK4 kinase is high on many of the pharmaceutical companies' development list because of its important role in inflammatory and possibly autoimmune diseases. So I hope there will be some IRAK4 kinase inhibitors coming down the pike that we can test.

Slide 98:
Acknowledgements

So sum it up then, I would like to say that the initial work on the R-CHOP study was our collaborative group, The Lymphoma and Leukemia Molecular Profiling Project and was led by Georg Lenz in the lab, Lixin Rui in my lab did the work on PD-L2 and that was done in collaboration with Randy Gasgoyne, especially Christian Steidl, and then the MYD88 work was done by a number of post-docs in the lab, Vu Ngo, Ryan Young, Roland Schmitz, Sameer Jhavar, Wenming Xiao, and Kian Liu. And all of them amazingly made great contributions to that story.

Thanks a lot.

Dr. Irv Bernstein:

Thank you, Lou, that was great.

Slide 99:
Molecular Control of Leukemic Cell Infiltration into the CNS

So to move on as we progress from basic science to clinical entities, Dr. Iannis Aifantis will talk about Molecular Control of Leukemic Cell Infiltration into the CNS. Dr. Aifantis is Associate Professor of Pathology at New York University School of Medicine and Co-Director of the Cancer Stem Cell Program at the New York University Cancer Institute. He's an Early Career Investigator at the Howard Hughes Medical Institute as well. Welcome, Iannis.

Slide 100:
Disclosure of Conflicts of Interest
Dr. Iannis Aifantis:

Thank you very much for the invitation.

Slide 101:
A simplified view of hematopoiesis…..

I'm just going to give you a couple of relatively new stories that are going on in the lab. My lab is studying hematopoiesis and you've heard a lot about hematopoietic stem cell differentiation and hematopoietic progenitor differentiation from previous speakers. Basically the only thing that I think you should keep in mind at this point is that in a very naive way there's a linear way of differentiation, starting with very few progenitors that have the ability to both self-renew and differentiate.

This is a very well-studied pathway. We have a lot of details on the pathway. You've actually heard, during the first talk, that some of these details are probably not correct, but this is I

guess a challenge for the field going on. And in our case we focused a lot on the differentiation of T cells and T lymphocytes, from common lymphoid progenitors.

So basically what we know about T cells is that there is a master regulator of T cell differentiation and this is the notch signaling pathway. We don't know a lot of details about where notch is activated, but we know that it's absolutely essential, so basically if you knock out one of the four notches that we have in our body, and this is Notch 1, what happens is that you don't get any T cells at all, you don't have any thymus at all.

One of the areas of focus in my lab, is to actually see what happens when you have too much of a Notch signaling. And we and a lot of other labs during the last ten years have shown that too much Notch signaling not only gives you T cell commitment, but eventually transforms the cells, leading to something that looks a lot like T cell lymphoblastic leukemia or human T-ALL.

Slide 102:
…however, Notch *gain-of-function* leads to T cell acute lymphoblastic leukemia

I'm sure most of you know more about ALL than me, but just let me give you a very general characterization, it's mainly a pediatric disease, it's characterized by accumulation of blasts, T cell blasts in the bone marrow and blood. And today we're going to focus a lot on the ability of T cell disease to infiltrate different tissues, specifically the central nervous system.

So Notch 1 is the main culprit in this disease. Several labs have found that Notch 1 is mutated in the majority of patients that have T-ALL. And my lab has focused also on other components of the notch pathway, especially ligase, that's called Fbw7, and we have shown that it is mutated and acts as a tumor suppressor in this disease.

Slide 103:
(part 1): Why is Notch a blood oncogene?

So my talk will have two parts. The first part is based on this very simple question, why Notch is an oncogene, and focus on a specific pathway that Lou has introduced previously and that's the NF-κb pathway. And the second part will actually look at the NF-κb as an activator of adhesion events in this type of leukemia.

Slide 104:
Notch1 activation targets the NF-κB pathway

So basically our whole story started a few years ago when we found that when we activate the Notch pathway, we actually switch on an NF-κb response. Some of these genes that you see there are actually shown before in the previous talk. Today we're going to talk a lot about this first one, it's called CCR7. It's a chemokine receptor and not only is a target, but is a direct target, so sequencing results – and I'm not showing you here, shown direct interaction of NF-κb subunits with CCR7.

Other interesting mediators of adhesion are ICAM, VCAM, and a few other regulators of migration.

Slide 105:
Rapid *in vivo* induction of the NF-kB pathway in an animal model of T-ALL

So basically what this simple experiment told us was that Notch 1 activation targets the NF-κb pathway. And more recently we were able to actually see that and visualize it in vivo. We generated mice that – in which we can follow the NF-κb response using NF-κb. And when we introduced notch mutants in these mice and induced the disease, we could see very fast response and activation of the NF-κb program.

Slide 106:
The canonical NF-kB signaling pathway

So very few things because you all know about it and it was introduced before. The NF-κb pathway is controlled by a kinase complex, as the IKK kinase complex, made by NEMO, which kind of holds the whole thing together, and two active subunits, IKK-alpha and IKK-beta. There are positive regulators like a kinase that's called TAK-1, that we're going to mention it, and negative regulators, upstream and downstream. And one of these is actually CYLD which controls proteasomal degradation and activation of different subunits of the IKK complex.

Slide 107:
What is the mechanism of Notch-induced NF-kB activation?

So basically what is the mechanism of Notch induction of the NF-κb pathway? We have seen the actual IKK complex is very active in T cell leukemia and when we switch off the pathway by either gamma secretase inhibitors or specific shRNAs, we basically lose this activation of the complex. And we'll come back to that.

Slide 108:
The Notch target Hes1 is sufficient to induce NF-κB activity

So we have very suddenly found something very interesting, is that Notch can do that, but you can even go more downstream in the Notch pathway and you can have the same effect. So we focused on a gene that's called Hes1. It's a member of the Hes family, the old transcriptional repressors. And what we found is that we can actually activate the NF-κb pathway by titering in Hes1. And we can actually see that Hes1 over-expression is able to induce NF-κb activity in a lot of different types of cells. And this is controlled through the activation – sorry, through the degradation of IκBa, which is a negative regulator in this system.

Slide 109:
Hes1 facilitates IkBa degradation by regulating directly IKKb activity

So a little bit more biochemistry for us to understand where is the regulation happening. We've actually found that Hes1 and Notch have the ability, as I've shown you before, to directly activate the NF-κb kinase complex and specifically IκBβ, and by using mutated we were actually able to map the need for Hes1 upstream of this TAK-1 kinase.

Slide 110:
The expression of CYLD, a negative regulator of IKK function, is suppressed in T-ALL

So what is upstream of the IKK complex and presumably the Tac-1 kinase? We focused on two, very well studied in the NF-κb pathway, negative regulators both of them. One is called, as I've shown you, CYLD and the other A20. And we've found something really interesting, that

if you actually cluster patients that have this leukemia, over-express Notch and over-express Hes1, and you compare them with normal individuals, what you see is a suppression of expression of both of these ubiquinases.

By going further and using shRNAs to find which one of these two molecules have specific targets of the pathway, we've actually found that one of them, CYLD, is directly controlled by Notch and Hes1, and you might be able to see it here. Maybe not. But actually over-expression of nuclear active Notch leads to over-expression of Hes1 and suppression of one of the ubiquinases which is called CYLD as I mentioned before.

Slide 111:

Promoter methylation is not the mechanism of CYLD expression suppression

So how is notch able to control CYLD expression? Initially we thought that it has to do something with promoter methylation and this assay has actually brought us nowhere. So basically promoter methylation is not the mechanism that is important here.

Slide 112:

Notch signaling suppresses CYLD, though direct binding of Hes1

We went on, actually inspired by previous work on A20 mutations in several other types of leukemia and we sequenced both CYLD and L20 in T-ALL and we found no mutations at all. And to basically cut down to the chase, we found that there is actually a direct effect of the Notch pathway on CYLD expression and this is controlled through Hes1. So Hes1 is a known transcriptional repressor and what we found is that there are specific sites on enhancers that control expression of Hes1 that actually, of CYLD, that are occupied by Hes1, and this is a specific suppression. So basically Notch over-expression leads to Hes over-expression, leads to suppression of CYLD and presumably activation of the NF-κb pathway.

Slide 113:

CYLD deletion enhances NF-kB activity in a mouse model of T-ALL

To go further and prove that in a mouse model, we used the CYLD knockout and we actually activated – we've actually induced disease in these mice and in control litter mates. And what we found, and maybe you can see it up there, the first two banshifts over there are basically normal tumors that express normal levels of CYLD and the two next are actually two knockouts, showing again that activation of the NF-κb pathway can be controlled by Notch and availability of CYLD.

These tumors actually are coming faster and they were more aggressive, usually than tumors that were induced from cells that were expressing CYLD.

Slide 114:

Is IKK/NF-kB targeting a putative T-ALL therapy?

……(Is IKK signaling essential for T-ALL maintenance?)

So how important is this IKK activation and NF-κb activation for therapeutic in this type of leukemia?

Slide 115:

IKK/NEMO activity silencing is able to induce rapid T-ALL cell line death

To address that we have done two things. First of all, we tried to address it in vitro. So we assembled a bunch of T-ALL lines and T-ALL primary cells, I'll show you some cell line results here, and we used a very specific peptide inhibitor that was developed by Sankar Goss and his lab, that basically prevents the binding of NEMO to IKK alpha and beta, and inactivates the NF-κb kinase complex. So when we used this inhibitor we found a very aggressive response. We found induction of cell death in these T-ALL lines and if you use a simple microarray analysis to try to kind of pinpoint genes that are important there, you can see apoptotic genes and it was actually a very big surprise, very interesting for us, that we can see genes of the notch pathway being affected by NF-kb. So basically the opposite from what I've told you before. We still don't understand it and we try to get some insights on this regulation.

However, all of these results are in vivo, so we should take them with a grain of salt I guess.

That's why we went further and we tried to establish an in vivo system where we can test this importance of IKK activation and NF-κb activation.

Slide 116:

IKK/NEMO signaling is essential for the maintenance of T-ALL

So what we've done is that we used a conditional of IKK gamma and NEMO and we crossed it to a cre that is actually inducible. We induced the disease, and we deleted NEMO only afterwards. And as you can see, probably up there, look where it says ventral, you can see one control in four mice, two weeks after our transplantation developed the disease, and then when you switch off NEMO expression and IKK activation, you see the two mice that look much cleaner, and the other mice that actually have higher burden and eventually will go on dying.

And that was really exciting for us because it shows that NEMO function and IKK activation is indeed essential, not only for the induction, which is another thing that we can show, but the maintenance of T-ALL.

Slide 117:

NEMO/IKK silencing is inducing rapid T-ALL cell apoptosis *in vivo*

And you can see here that the response to NEMO deletion is actually very fast. This is just a few days and shows induction of apoptotic cells, death of the leukemic cells, and after a few days you can actually see peripheral lymphoid organs to look more like physiological lymphoid organs.

So all of these things suggest that notch functions as an oncogene by a lot of different ways, but one way is actually by cross-talking with the IKK and NF-κb complex through Hes1 repression of CYLD.

Slide 118:

Notch-IC expressing transplanted cells efficiently infiltrate the CNS

So the second part of my talk focuses more on the interaction of T-ALL cells with our environment. And the signals that actually tell them where to go within the body.

So basically most of you know that during diagnosis you have a percentage of T-ALL patients that present with CNS involvement, infiltration of the central nervous system, and this

percent is actually significantly higher after relapse. That was something that was first appreciated by physicians back in the 60s and the way to treat these patients was intrathecal treatments, cranial irradiation and such. Which worked very efficiently, but of course, they come with their own prices.

So my lab a few years ago had set up to actually identify mechanisms by which leukemic cells can infiltrate different tissues and specifically the central nervous system. And that's a work in progress and I'm going to show you some first results.

Slide 119:
Efficient CNS infiltration in a novel mouse model of T-ALL

So we can study T cell leukemia in mice several ways. The easiest way is just take stem cells, give them a Notch mutation and transplant them. And basically what you can see there is that you can see leukemic blasts in the blood. They all express CD4 and CD8, so they are expressing both markers of mature T cells. They and infiltration of the brain and we know that the infiltrants are actually T-ALL cells that express Notch because Notch is marked by EGFP.

So of course, somebody can tell me that this is a model that requires irradiation and that can mess up a lot of things, especially when it comes to BBB permeability. So we have generated a second model, which is more an endogenous model, so basically we take some of these notch mutations and we express them as knock-ins through – in this case the elongation factor 1 promoter. And what we can actually see is that we can see the same induction of fatal disease and infiltration of the lepto-meningeal spaces by leukemic T-ALL cells.

Slide 120:
Are there specific, Notch-responsive adhesion regulators that are important for CNS infiltration in T-ALL?

So now that we have a way to actually study disease that goes to the brain, we can actually look at what controls this response. So going back to the same experiments that I've shown you before, we have focused now on genes that are induced by Notch and they belong to either migration or adhesion families. And for reasons that we can discuss later if you want, we decided to focus on one of them, mainly because we knew that it was NF-κb target, as I've shown you in the previous part of my talk, that is called CCR7.

Slide 121:
What will happen if we switch-off CCR7 expression in leukemic cells?

So CCR7 is a chemokine receptor. It has two known ligands, CCL19 and CCL21. It's very well known in the field of immunology and specifically T cell immunology because it's very important to basically send T cells in and out the lymph nodes and other peripheral lymphoid organs.

So we basically – we decided to do what I call the stupid experiment, which is you take CCR7 deficient stem cells, you give them the Notch mutation, you transplant them and see what happens.

Slide 122:
CCR7 deletion slows down leukemia progression

And what happens what actually pretty interesting because we could see that the disease was there, it was actually sometimes even more over-presented as you can see here in a smear of peripheral blood. But these mice transplanted with CCR7 deficient stem cells, expressing Notch mutant, will actually die later than their litter mate controls.

Slide 123:
Deletion of CCR7 suppresses T-ALL CNS infiltration

And we couldn't really understand why that happened until we actually looked at the brain and you can see some brain sections here, you can see a control, next to it the infiltration from the wild type mouse, and as you can see, when you inject CCR7 deficient T-ALL cells, they just don't know how to go into the CNS. That was really interesting, really surprising for us, so we decided to follow it. And we've done a lot of work on adhesion and migration of leukemic cells.

Slide 124:
CCR7 deficiency does not affect overall T cell migration/movement

This actually shows nothing. This is just a control that shows – I think this is the spleen and you can see kind of the leukemic cells expressed in GFP Notch taking over the whole organ. And if you compare CCF7 deficient and CCF7 expressing cells, we couldn't see any differences when it comes to velocity, the way they turn, the way they arrest, the places that we go. The only thing that these guys do not know how to do is actually go to the CNS.

Slide 125:
A very specific effect: Leukemia cells just cannot get in the CNS

So if CCR7, which is a receptor, is so important to attract the cells there, what is the attractant?

Slide 126:
CCL19 (and not CCL21) is expressed in the brain

So very briefly we looked at the two known ligands of CCR7, 19 and 21, and we found that one of them, which is the CCL19, is expressed in lympto-meningeal vessels and actually on the endothelium of these vessels. And as you can see here, up there you can see some green cells, the leukemic cells, kind of creeping out these vessels into the brain. And down there you can see blue endothelial together with red CCL19, more or less expressed at the same stage.

Slide 127:
CCL19 deletion inhibits T-ALL brain infiltration

So if CCL19 is expressed there, is it important? So we use the CCL19 knockout now as a host, not as a donor, and what you see is that if you transplant now wild type leukemic cells, once more these cells do not know how to go into the brain, that's why we could not see any filtration either in the brain or the spine of these mice.

Slide 128:
Is CCR7 important for the CNS infiltration of human T-ALL cells?

So all of these data I've shown you are mouse data, are similar controls appearing in

human T-ALL, by assessing primary human T-ALL samples and also cell lines, we've seen that a lot of them, close to 80% of them expressing CCR7. Some of them very high. Very few of them very low. And just for simplicity reasons, I'm just going to show you a few experiments with one kind of classic line, expressing very high levels of CCR7, and another one, which is the only one which was negative for CCR7.

So when you take these two lines and you inject them in a mouse, what you see is that the CCR7 expressing line is very able to actually infiltrate the central nervous system. The CCR7 non-expressing line is actually unable to do that.

Slide 129:
CCR7 expression influences human T-ALL cell CNS infiltration ability

What is interesting is that the CCR7 positive line can go into the CNS, in this case is, I think, the lower part of the spine, and actually engulf the CNS at some time, and destroy it. That's why a lot of these mice in this cohort are actually paralyzed, the time that they we analyzed them.

So the question here is: Is CCR7 expression sufficient now to target human T-ALL cells into the CNS? So kind of the opposite from the deletion experiment that I've shown you before.

Slide 130:
Spinal Cord Infiltration is Also Dependent on CCR7 Expression

So in this experiment what you see is that on the left hand side is the CCR7 negative line, that doesn't know how to go to the brain. If you now come with the lenti-virus and express CCR7, you actually target them very efficiently, both through the brain and the spine.

Slide 131:
CCR7 ectopic expression is targeting T-ALL cells to the CNS

So what we're doing currently is we are focusing more on human disease and either human lines or primary cells and we're using shRNAs to control CCR7 expression or CC19 expression, and I'll just show you one preliminary example, but very interesting here.

Slide 132:
Silencing of CCR7 expression in human T-ALL leads to decrease of CNS infiltration potential

On the upper hand you basically have a human T-ALL line that knows to go to the CNS very efficiently. And in the lower part you basically have the same line with much lower levels of CCR7, that now doesn't appear in the CNS, kind of supporting the mouse results that I've shown you before.

Slide 133:
Down to a gene: Specific Notch targets are important for disease progression:

So basically what I've told you today is that we are trying to go down to the gene or down to the pathway and find specific notch targets that are important for different stages of disease progression. I've shown you that CCR7 is a notch target that is essential for an infiltration of the central nervous system. I've shown you that NF-κB is essential definitely for the survival of the cells, but also we believe for migration. And we think that – and this is the way that we are trying

to follow now – that if you mess up with the cross-talk between CCR7 and its ligands, you could actually affect the ability of these leukemic cells to infiltrate the central nervous system.

Slide 134:
http://www.aifantislab.com

So the work that I've shown you was done by a few people in the lab, Severine has done all the notch work. Sylvia and Jelena have done the CCR7 work that I've shown you at the end. And we couldn't do all of these things without some of our collaborators that are down there.

Slide 135:
Thank You

Thank you very much.

Dr. Irv Bernstein:

Why don't we go on, as we progress towards more disease-focused and preclinical and even experiments, in which stromal tumor relationships might be better understood or perturbed, leading to therapeutic effects.

The next talk, Dr. Michael Rettig will present Clinical and Translational Studies of Stroma-Leukemia Interactions. Dr. Rettig is a Research Assistant Professor of Medicine in the Section of Bone Marrow Transplant in Division of Oncology at Washington University School of Medicine.

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Slide 136:
Clinical and Translational Studies of Stroma-Leukemia Interactions
Dr. Michael Rettig:

I'll try to take you through John's slides. His wife was admitted to the hospital last week, so he needs to stay in St. Louis and help with her.

Slide 137:
Disclosure of Conflicts of Interest

So both Dr. DiPersio and I have received honoraria from Genzyme, just to disclose that.

Slide 138:
Stem Cell Mobilization: AMD3100 + G-CSF

So as we've heard today, Dr. DiPersio has been – we've been interested in normal stem

cell mobilization for transplantation. Normal stem cells are tethered into the bone marrow and interact with the extracellular matrix, osteoblasts, osteoclasts, as we heard. Leukemia stem cells probably also use a number of these pathways. CXCR4 interaction with SDF-1, VLA4 on stem cells interacting with VCAM1 and fibronectin, KIT ligand with its receptor, and then selectins with the selectin ligands in the marrow.

In normal stem cell mobilization, GCSF is the most commonly used agent and studies by Matt Christopher and Dan Link at our institution have shown in CXCR4 deficient mice you get no mobilization by GCSF, so it's definitely dependent on the CXCR4/SDF-1 axis. And G-CSF probably acts multiple ways by activating proteases that cleave some of these ligands. And then also by working through, it looks like a monocyte-derived cell intermediate, to down-regulate SDF-1 mRNA and osteoblasts in the marrow.

So we've been studying a new drug, plerixafor. I think on some of John's slides it's also listed as AMD3100 or Mozobil®. It's a small molecule, CXCR4 antagonist. In the first ten slides or so I want to go through some of our data in normal stem cell mobilization and show that the stem cells mobilized by these two agents are different. And then after that, talk about mobilization of leukemia cells.

Slide 139:

NHL Patients (%)[a] Achieving ≥ 5 × 10^6 CD34+ Cells/kg by Apheresis Day – ITT Population

So these are results from non-Hodgkin's lymphoma patients, the Phase III studies that led to the approval of plerixafor for use in combination with GCSF. In this Phase III trial patients were randomized to receive either plerixafor in yellow or placebo in blue. And you're looking at the number of patients that achieved greater than 5,000,000 CD34+ cells per kilogram, by apheresis day 1, 2, 3 and 4. So these patients are treated with GCSF four days. The night of the fourth day they're either treated with plerixafor or placebo. And what you can see is that on day 5, the first apheresis, there's about a six-fold increase in the number of patients who reached 5,000,000 CD34+ cells collected versus only 4% for placebo. And then that increases over apheresis days. But the amazing thing is that even after four apheresis days you still don't attain the number of CD34+ cells as you do in a single day of collection with the combination of GCSF and plerixafor.

This data shows the total number of CD34+ cells collected. Absolute CD34+ cell numbers also increase six-fold upon the addition of plerixafor. So clearly there's a population of CD34+ cells that are not mobilized by G that are upon treatment with plerixafor.

Slide 140:

Overall Survival of Patients Receiving Plerixafor-Mobilized Allografts (n = 38)

And then in separate studies in allogeneic transplantation, we've been looking at what happens when you transplant just the plerixafor mobilized graft. And so this shows the overall survival of 38 patients in an HLA matched sibling transplant. So these grafts were only mobilized with plerixafor and no GCSF. And the point of this slide is that we can mobilize long term repopulating stem cells with plerixafor only.

Slide 141:

AMD-ALLO: GVHD (n = 38)

One concern with plerixafor only mobilized grafts is there's even two-fold more T cells than in a GCSF mobilized graft. And so we've been interested in following the rates of acute and chronic graft-versus-host disease. And shown on the left is the incidence of acute GVHD, similar to historical controls, about 40% grade 2 to 4. And then about 20% grade 3 to 4, severe acute GVHD. And then the incidence of chronic GVHD has also been about 40%. So not significantly worse than what you would see with GCSF.

Slide 142:
Heat Map of 18 gene signature Red=A/G>2; Green A/G<0.5

For the allogeneic trial, in the first eight donors, which are indicated by pair 1 through 8, we collected a backup graft. Patients were initially mobilized with plerixafor. Those grafts were collected and transplanted. But we also collected a backup of the exact same patients with GCSF and collected CD34+ cells. So this is just RNA profiling, comparing the CD34 cells isolated after plerixafor or GCSF.

And in red are genes that were consistently expressed, greater than – over background, over 500, at two-fold higher levels in the plerixafor mobilized grafts. And in green would be two-fold higher in the GCSF mobilized 34 cells.

What we can find is that they separate. The CD34+ cells are different. There were 11 genes that were two-fold higher upon mobilization with plerixafor. And there were seven genes that were consistently expressed higher in the GCSF mobilized 34 cells. So it's just additional data that the subsets mobilized by G-CSF and plerixafor are different.

Slide 143:
Co-expression of CD45RA on $CD34^{+}$ cells identifies the $CD34^{dim}$ subset

And then we've done some phenotyping studies, shown by CD45RA and CD34 on the Y-axis here. These are three donors that are mobilized with plerixafor and then three donors mobilized with GCSF.

So the CD34 high and RA negative cells are more primitive than your common myeloid progenitors. The RA positive, CD34 high cells, would be more of your GMP, granulocyte-monocyte progenitors. And then there's a population of CD34 dim, CD45RA high cells, that are significantly enriched upon mobilization with plerixafor, compared to GCSF. These are unrelated donors. And this is significantly demonstrated down here where yellow is GCSF and blue are the eight donors mobilized with plerixafor only. So these are the matched pairs. So definitely different subsets of 34 cells are being mobilized by plerixafor.

Slide 144:
Normal Bone Marrow Microenvironment

So in addition to looking at just the CXCR4-SDF-1 axis, we've also started looking at other axes. BIO5192 is a small molecule inhibitor of VLA4. So we've started studies in both normal mobilization and leukemia mobilization when we target this axis. And we've also started studies looking at selectin inhibitors. But we won't present any data on those today.

Slide 145:

Kinetics of Murine Progenitor Mobilization in Response to BIO5192 and Plerixafor

So this is normal mouse CFU progenitor mobilization versus time after injection. And in red is plerixafor, where we see maximum mobilization of CFUs approximately three hours after treatment. And in yellow is a single injection of a BIO5192, which is the VLA4 antagonist. This was published by Pablo Ramirez in our group in Blood last year. And so you see significant mobilization, actually no different than plerixafor, with the VLA4 antagonist alone. It's again further indication that potentially different subsets are mobilized when you attack these different tethers in the marrow.

Slide 146:
Additive Mobilization of Murine Progenitors After Combination of Plerixaflor SC and BIO5192 IV

And the interesting thing is that this mobilization was additive, so in red again is plerixafor alone and in yellow is BIO5192 alone and green is the combination. And the combinations mobilized significantly more mouse CFUs compared to either agent alone.

And so I think these data are important as we start to go towards deciding what's the best way to potentially chemo-sensitize leukemia cells. We're mobilizing different subsets of cells.

Slide 147:
Hypothesis of chemosensitization

So based on this work in normal HSCs, we had the following hypothesis; that the interaction of leukemia cells with the bone marrow stroma may provide a survival benefit to the leukemia cells, as we've heard about today.

So our thought was that if we could interrupt this interaction, it may enhance the sensitivity to chemotherapy or potentially even radiation therapy. So there have been a number of studies, at least 13 different clinical trials, using GCSF or GMCSF as a priming agent. And those have only shown modest results. A little bit, Lowenberg's group showed a little bit of a difference in a subset of patients in disease-free survival.

But now that we know that AMD and GCSF are additive or synergistic, we are wanting to explore, if we could maybe increase this priming.

Slide 148:
High-penetrance mouse model of acute promyelocytic leukemia with very low levels of PML-RARα expression

So we first used a model developed by Tim Ley at our institution, in which the human PML-RAR-**α** trans gene was targeted to a single allele of the murine cathepsin locus, so it's an APL model with PML-RAR**α**. Ninety to 100% of these mice developed leukemia and died between 150 and 400 days after transplant – or after modification.

So we can harvest and bank these APL cells and adoptively transfer them into secondary recipients or into different mice. And upon transfer, those mice will develop a rapidly fatal leukemia and usually die within three weeks.

Slide 149:
APL ENGRAFTMENT

So we've used this model to try to begin to test our chemo-sensitization effect with these different mobilizing agents. So we genetically modified the APL cells with firefly luciferase, so that we could track them by BLI. And this is just a representative mouse, showing a ventral view or a dorsal view at the indicated days. So similar to human AMLs, these APL cells traffic initially to the marrow, indicated by the fibula and tibia here and the sternum, the back. And then they expand in the marrow and the spleen. And then generally by about two to three weeks there's a massive proliferation and these mice die from blast crisis.

There it is. Spleens get huge.

Slide 150:
Effect of direct contact between APL and stromal cells on APL viability

So we've started to look at the effect of stroma just in vitro on these APL cells. And this slide is just looking at direct contact, the effect of direct contact of stroma on APL cells in vitro. And so this should be Annexin V+. About 30% of the APL cells in the absence of stroma are Annexin V+. And when you culture these APL cells for 2 days in the presence of stroma, the percentage of Annexin V+ goes down to less than 2%. And then when you treat these APL cells either in the absence or presence of stroma, with chemotherapy, either Ara-C or daunorubicin, you see a dramatic increase over – nearly 90% of the APL cells in the absence of stroma are Annexin V+ in both cases with Ara-C and daunorubicin, and the stroma almost completely protects those cells from apoptosis in vitro.

Slide 151:
Effect of stroma on APL proliferation and spontaneous apoptosis

And then Pablo used CFSE to try to look at the mechanism of this protection in vitro. So on the Y, this is the percentage of CFSE high cells. Basically the number of APL cells that have not divided. And on the right hand graph here is in the absence of stroma. So over two days, over 75 to 80% of the APL cells proliferate. And there's an increase in the number of Annexin V+. What you can see in the presence of stroma is that the number of APL cells that proliferate is significantly reduced, suggesting that the stroma is driving quiescence. And that may mediate their chemo-protective effect.

Slide 152:
Reduced Proliferation of APL Cells in the Presence of M2-10B4 Stromal Cells

The lab used BrdU and 7-AAD to look at the cell cycle of APL cells in the absence or presence of stroma. So in the absence of stroma, indicated in yellow, about 30% are G0/G1, 50 to 60% are in S phase, and 10 to 15% are in G2+M. Then when you put the APL cells on stroma, we see a significant reduction in the number of cells in S phase. The number of cells in S phase become less than 20%, and a concomitant increase in the number of cells in G0/G1 and G2+M. So this is consistent with his CFSE data, showing inhibition of proliferation and sort of driving quiescence of these APL cells in the presence of stroma. And again that would favor chemo protection.

Slide 153:
Effect of stroma on mTOR pathway phosphorylation

Pablo also started looking at some of the signaling mechanisms that are being driven by the stroma. This is a western blot looking at phospho-S6 and total S6 and then 4E-BP1, phospho 4E-BP1, and total E-BP1. These are all part of the mTOR pathway signaling through RAS and ERK and also through PI3 kinase and AKT.

So pretty dramatically in the presence of stroma these APL cells have very high levels of pS6. This controls translation, which provides pro-survival and anti-apoptotic signals. And this phosphorylation could be inhibited by rapamycin. We didn't see really any differences in 4E-BP1 phosphorylation levels in the presence or absence of stroma.

Slide 154:
Increased sirolimus-induced APL apoptosis in presence of stromal cells

So then he next looked at just what effect would rapamycin alone have on APL survival in vitro. Again looking at Annexin V+, 5% in the absence of rapamycin and then increasing doses of rapamycin. So in the absence of stroma, indicated in blue, apoptosis increased to approximately 15% over two days. Pretty remarkably there was a significant increase in the amount of APL cells that were Annexin V+ in the presence of rapamycin, peaking at 10 nanomolar. So when these APL cells are in contact with stroma, it seems like this mTOR pathway is critical for their survival and disruption of that could potentially increase efficacy of chemo treatment.

Slide 155:
AMD3100 Mobilization of mAPL

So we next started to use this APL model in vivo and we published this in Blood last year and these studies were led by Bruno Nervi in John's lab. The graph on the left is normal mouse CFU mobilization vs time after AMD. And you can see, as I showed earlier, peak mobilization of normal CFU is by three hours. And down below is the time after AMD in leukemic mice. These were non-leukemic mice. We can track these leukemia cells by their aberrant co-expression of GR-1 and murine-CD34. And you can see before treatment, 1% of the cells in circulation are APL. This percentage increases, so these APL cells are mobilized by AMD, increases to 6% at three hours. This is a total of five mice. And it's significantly increased over background. So just like normal CFUs, we're able to mobilize these murine APL cells with the CXCR4 antagonist.

Slide 156:
Mobilization of leukemia cells increases the efficacy of anti-leukemic chemotherapy

So then we went to the chemo sensitization experiment and this is the design. So Bruno put 10 million APL cells into syngeneic mice and then this is the design of the experiment. So control mice were left untreated and then mice were treated with Ara-C alone on day 12 or day 13 at 500 milligrams per kilogram. And then to test the effect of mobilization, another cohort of mice were treated with AMD immediately before Ara-C and three hours afterwards, so we bracketed the chemotherapy injection on both day 12 and 13. Then we also looked at doxorubicin with the same schema, but I won't show any of that data. It was similar to the Ara-C. And then as a control we also just looked at the effect of AMD alone on these APL cells.

Slide 157:
AMD3100 enhances effect of chemotherapy

So this is the BLI, so the total photons, so we're tracking total leukemia burden in the different cohorts and these are representative BLI images. So in yellow are the control mice. And so before any chemotherapy or AMD treatment, the mice all had similar levels of leukemia burden on day 12 before injection. And the leukemia was primarily in the spleen and marrow at that time point, as I showed earlier. And then as these mice went on, the control mice and the mice treated with AMD, shown in yellow and magenta here, had a dramatic increase in total BLI signal, primarily driven within the spleen, and they'd die of blast crisis by day 18 or day 19.

The Ara-C group, you see a significant reduction in BLI signal, four days after you started chemotherapy, indicated in the green here. This was significantly different than the untreated mice. But then those mice relapsed and all died by day 25 or so, so increased BLI signal.

And then remarkably when we combined the Ara-C with the AMD3100, we see a prolonged inhibition of APL cell growth, so a better kill of the APL cells upon the combination. And I don't think John included the survival slide in here, but these mice survived approximately ten to 12 days longer than the Ara-C only group and that was significant in this very aggressive leukemia model.

Slide 158:

Phase I/II Study of AMD3100 + MEC in Relapsed or Refractory AML

So based on these encouraging preclinical data, we started a clinical trial and we've completed this clinical trial. So it was patients with relapsed AML, either primary relapsed or refractory AML. And their ECOG score had to be > 2 – or < 2. And then the age range was listed here. And the important thing for this clinical trial is we limited the patients enrolled to a blast count of less than 30,000/mm^3. In some mice with really high leukemia burdens, we would see the death of mice upon mobilization due to leukostasis. So we were concerned at the start of this trial, so we limited patients to a blast count of less than 30,000/mm^3.

And then the design was on day zero we treated patients with AMD only and did our correlative studies to see what effect AMD alone had on mobilization of the blast cells. And beginning on day 1 through day 5, we mobilized with AMD3100 and then treated with a combination of mitoxantrone, etoposide, and cytarabine, standard chemotherapy, four hours after AMD.

In the Phase I portioned we started at a dose of 80 micrograms per kilogram of AMD and then increased that to 240.

Slide 159:

Patient Characteristics (n=49)

This just summarizes the patient characteristics. The average age of the 49 patients enrolled in the trial was 51. Equal numbers of male and female. Eight of the patients had favorable cytogenetics, 28 had intermediate and 13 had poor cytogenetics. Eight patients had secondary AML, three of those were therapy-related and then five were previous MDS or MPD. Eleven of the patients had the FLT-3 tyrosine kinase mutation, ten of those were IDT, 19 were unmutated and 17 were not known. And then eight of the patients had undergone previous transplantation.

Slide 160:

Treatment Indication n (%)

So AML outcome is based on the length of the patient's initial complete remission and the number of prior salvage therapies. So we enrolled 13 patients that had a complete remission lasting less than six months. These patients normally do very poorly. Thirteen patients had a first complete remission that was between six and 12 months. And then ten patients that had a complete remission longer than 12 months. There were two patients that were in first relapse and the second salvage, second round of chemo, and one patient that was second relapse. And then the ten primary refractory, eight patients with one induction and two patients with two inductions.

Slide 161:
Mobilization of AML Blasts

So this slide just shows the mobilization of white cells, total white cells, and CD45 dim side-scatter low blasts. And what we see is about a two- to three-fold on average increase in white cell count and CD45 dim and side-scatter low blasts. And then in nine – I think it was actually 11 – nine are shown here, where we have informative cytogenetics. We did FISH and calculated the number of positive cells before, six and 24 hours after treatment. This flow data was at six hours.

And what we see is a pan-mobilization. We do not see any preferential mobilization of the AML cells. It's a pan-mobilization of everything.

Slide 162:
Surface CXCR4 Expression & Response

Others have previously reported that high CXCR4 expression on AML blasts confers a poor prognosis. And this is just looking at baseline bone marrow biopsies and the peripheral blood of patients that either had progressive disease in our trial or had a complete remission. And similar to other studies, our patients that did not respond to the AMD-MEC therapy generally had significantly higher expression of CXCR4 compared to those that did respond to our treatment. So we were really hoping that with these high CXCR4 expressing patients, by targeting with a specific CXCR4 antagonist, that we could potentially cure some of these high CXCR4 expressors that have a poor prognosis.

Slide 163:
CXCR4 Expression

We do see an increase in CXCR4 expression on blast cells after mobilization. This is a CD45 dim side-scatter low plot of one representative patient, and CXCR4 expression with the 1D9 clone antibody, which is not inhibited by plerixafor. In black is the isotype control, pre-treatment in red, six hours after treatment in green, and blue is twenty-four hours after treatment. You can see in this patient, really high expression of CXCR4, 24 hours after treatment with AMD3100.

And this is a summary of all 35 patients that we were able to do this flow on, looking at the 1D9 clone in yellow, so on average we see about a two-fold increase in CXCR4 expression, after mobilization that peaks at six hours, which is the peak of mobilization. And remains elevated at 24 hours. And then a different clone, shown in white, is the 12G5 antibody of CXCR4, which is inhibited by plerixafor, and this indicates that plerixafor was bound to these AML cells and so that's why you see decreased 12G5 binding. And then as a control we also looked at

interleukin-3 receptor expression. It did not change. And then VLA4, measured by CD49-D and that also did not change after mobilization.

So we see a dramatic increase in CXCR4 expression after treatment with AMD3100 in these patients.

Slide 164:
Transwell Migration Assays

So we wanted to see if that CXCR4 was functional, so we performed transwell migration experiments towards Sdf-1. In the absence of Sdf, either pre, so AML blasts before treatment, and then six hours after mobilization, harvested from the peripheral blood, there's no migration. But we see significantly increased migration of the AML blasts towards Sdf-1 at six hours, compared to baseline. So this increased CXCR4 expression on AML blasts is functional CXCR4, as indicated by migration. And there was a correlation between the increase in CXCR4 mean fluorescence intensity or CXCR4 expression and the amount of migration.

Slide 165:
Safety & Toxicity

There was no evidence of hyperleukocytosis in these patients. It was a very safe therapy. Median time to recovery of absolute neutrophil counts was 26 days. Platelets was 26 days, that's similar to traditional MEC chemotherapy. As far as adverse events, there were no dose-limiting toxicities in the Phase I. And the adverse events were primarily hematologic with febrile neutropenia. There were two early deaths due to complications of sepsis. So it was well tolerated.

Slide 166:
Response Evaluation (n=49)

And just the response and evaluation. Three patients were treated at the lowest dose, three patients were treated at 160 micrograms per kilogram, and then 43 patients were treated at the target dose of 240 micrograms per kilogram. So overall 49 patients were treated and 46 were evaluable.

And so we looked at complete remissions and complete remissions with incomplete blood count recoveries, indicated by CRI. So one of the three patients at the lowest dose of AMD3100 had a CR, for a response rate of 33%. We also observed one CR in the intermediate dose, and then 17 CR and three CRIs at the highest dose for an overall CR and CRI rate of approximately 50%.

Slide 167:
Response Evaluation (240mcg/kg cohort)

And then since in AML, the outcome is based on the length of your first complete remission and the number of prior salvage therapies, we broke those – our patients into four different groups. So these generally have the best outcomes with the long initial complete remission and these patients have the worst prognosis. So we enrolled no patients in this group. And we compare historically – we have to compare it to historical controls. All patients from M.D. Anderson trial data or patients, if you just focus historical and traditional salvage, which would be similar to our MEC therapy.

So patients in the CR, one to two years, we enrolled nine patients and had six CRs for a rate of 86% in these patients, and that compares favorably to the historical control. Our highest number of patients were in complete remission less than one year. And their first salvage, we had a total of 12 CRs for a response rate of CR and CRI at 41, which is about double that of the historical control. And then only four patients in the worst prognosis group where we had one response.

Slide 168:
Conclusions

So the conclusions from this clinical trial are that plerixafor can be safely administered in combination with cytotoxic chemotherapy in patients with AML. The effects of CXCR4 blockade are observed in the AML blasts in vitro and in vivo after treatment with AMD3100. We see increased CXCR4 due to inhibition, and probably prevention of the receptor cycling, following treatment. And the CR and CRI rate of 50% compares favorable to the historical controls.

Slide 169:
Normal Bone Marrow Microenvironment

So just the last couple of slides. Some of our unpublished work, looking at targeting these other pathways, these other tethers that potentially keep the leukemia stem cell in the protective environment.

Slide 170:
Mobilization of mouse APL in vivo by the VLA-4 inhibitor, BIO5192

So this just shows our APL cells mobilization again, following treatment with the VLA4 inhibitor. We're able to see about a six-fold increase in APL mobilization with treatment with the VLA4 inhibitor alone.

Slide 171:
G-CSF suppresses mature osteoblasts

And this is work from Adam Greenbaum in Dan Link's lab, where they use the Col2.3 transgenic mice driving GFP. GFP is expressed in osteoblasts, indicated by green on the endosteal region. In untreated mice you see GFP expressing cells lining the bone. Following treatment with GCSF, this is unpublished data, you see a loss of osteoblasts. Dan has previously reported this observation, just not with this system.

Slide 172:
APL Chemosensitization by G-CSF & BIO5192

So we started preclinical studies where we combine looking at the effect of GCSF priming in our APL model and BIO5192 priming with Ara-C. So GCSF was given over four days with Ara-C each day and BIO5192 was similar to the AMD experiments, two days with two injections.

Slide 173:
BIO5192 and G-CSF Enhance the Effect of Chemotherapy

So untreated mice or mice treated with just BIO5192 or just GCSF, died by day 21. And

then mice treated with either the two days of Ara-C or the four days of Ara-C die later compared to untreated mice. And then treatment with BIO5192 alone, this wasn't statistically significant, we repeated this and John doesn't have this slide, but it is prolonged, not quite as much as AMD alone, survival is. And the remarkable thing with GSCF, we've actually cured some of these mice with APL and that's never happened with AMD3100 alone or BIO5192 and that's indicated in yellow.

Slide 174:

BM day +120 NOG Mice Phenotyping of Primary AML

So Ibraheem Motabiin the lab is starting to work with primary AML cells in NOD scid gamma mice. And this just shows 120 days after injection, that the human AML cells in the marrow are 33 positive expressed high levels of VLA4, high levels of CXCR4 and the interleukin-3 receptor.

Slide 175:

Effect of AMD31000 and G-CSF on primary human AML mobilization in NOG Mice

And he's able to mobilize these cells. So this is before AMD and after AMD. So we're able to see mobilization of human AML cells in the NOD mice. But not much mobilization with GCSF alone, which is interesting. We don't completely understand that.

Slide 176:

Phase I/II Study of G-CSF + AMD3100 + MEC in Relapsed or Refractory AML

So we've proposed the next trial, where it appears that both G and AMD target CXCR4, but they're clearly different and they're synergistic when you put them together. So our next trial we would like to look at the combination of G and A. So we'll do two days of GCSF alone and then G and A, where we can do our correlative studies again and then make sure that this is tolerated okay and then we will treat with the MEC chemotherapy, similar to our original trial.

Slide 177:

NOG Model of Human G2 ALL

And then we've also started work in ALL, using Lapidot's G2 ALL model, where the ALL cells traffic also to the marrow and were able to mobilize these ALL cells with AMD. So we want to see if they chemo-sensitize.

Slide 178:

Hypothesis: **Interruption of Stroma-leukemia cell contact and/or inhibition of stroma-induced signaling in leukemia cells will result in proliferation, apoptosis, differentiation and *sensitization to genotoxic stresses such as chemotherapy***

So just in general, signals derive to a leukemia cell and a normal stem cell provide anti-apoptosis, anti-proliferative, probably anti-differentiation signals from the extracellular matrix, from vascular niches, endosteal niches. So our approach has been to target, try to disrupt these tethers and see if we can chemo-sensitize. And I think we're getting there slowly. But there's clearly other approaches where you could potentially target the signaling molecules as we've shown with some of the rapamycin data.

Slide 179:
Acknowledgements

So all this was done in John's lab. I tried to indicate who did which work. Bruno and Pablo did all the APL stuff. Mark Schroeder did some of the newer work with GCSF and AMD. Kyle McFarland did all the phenotyping in the trial. Thanks to Tim Ley for the APL model, Dan Link and Adam Greenbaum for the osteoblast data. Geoff Uy wrote the AMD MEC trials. And then Dave Piwnica-Worms in our imaging center and then Genzyme for supplying the drug.

Slide 180:
Thank You

I'll stop there. Thanks.

Dr. Irv Bernstein:

Thank you. Continuing in the vein of translational research talks in this area of tumor microenvironment, the next presenter, and final, is Dr. Irene Ghobrial. She's at the Dana-Farber Cancer Institute, where she's Assistant Professor in the Department of Medicine at Harvard Medical School, and she'll speak on Cell Trafficking in Multiple Myeloma.

And after her talk, for the afficionados of this subject, we'll have the speakers up here, we might ask them some more difficult questions.

Slide 181:
Cell Trafficking in Multiple Myeloma
Dr. Irene Ghobrial:

Good evening, everyone, and thank you so much for this opportunity to present our data.

What I'll do is I have the opportunity now that everyone else has presented all those model systems and I'll take you now through multiple myeloma and how we've applied some of those concepts into understanding the biology, as well as also taking it into translation and taking it into clinical trials for patients with myeloma.

Slide 182:
Disclosure of Conflicts of Interest

This is my conflict of interest.

Slide 183:
Multiple Myeloma is a dynamic interaction of MM cells with TME

So multiple myeloma is a plasma cell disorder of the bone marrow and in general we used to think that it's only in the bone marrow and there is not really a dynamic trafficking of those cells. But if you think carefully about it, 70%, even more, of the patients have small circulating cells that are continuously going from the bone marrow into the peripheral blood and finding a new place into another bone marrow. So by definition myeloma is really a metastatic model and by the time we see our patients and diagnose them, they have multiple lytic lesions and in fact this is multiple metastatic sites. And it happens probably by one original site of plasma cells that grows, induces egress of those myeloma cells out of the bone marrow into the peripheral blood,

and by doing that they go on and find another new home and go into the bone marrow and make a new metastatic niche. And for us to understand better myeloma dissemination or myeloma growths and multiple lytic lesions, we have to understand this dynamic process of exits from the bone marrow and entry into the bone marrow or cell trafficking.

Slide 184:
Model systems of studying MM

Now these are some of the methods that we've used and I have to give a lot of credit for Ken Anderson who has done a lot of work on the tumor microenvironment in multiple myeloma and really trying to show that it's not just the myeloma tumor clone itself, but really an interaction between the tumor clone and the microenvironment, whether it's the stromal cells, endothelial cells, osteoclasts and osteoblasts that you heard about in the previous speakers' presentations, but what we're trying to do is not only take that genetic spark or those static interactions between those cells, we're trying in my lab to take them into a dynamic interaction. What happens when stroma and myeloma cells interact together, what happens when those cells home into the new bone marrow microenvironment and how do those cells go on home and adhere and then proliferate into that microenvironment.

Slide 185:
Blood Vessel

So this is just a very simplistic cartoon of what happens for the homing process when a myeloma cell enters into the circulation and tries to find a new bone marrow niche. And it's very similar to what you've already heard about for stem cells or for leukemia cells, where they really need adhesion molecules, selectin molecules and then also chemokines like Sdf-1 or Cxcl 12 that you heard about. And this process occurs through the rolling – the initial steps that require selectins, then adhesion that requires integrins, and then you go on to chemokine attraction into the bone marrow niches and you've already heard that there may be no vascular niche or perivascular niche and endosteal niche, but these actually are interchanging in a way right now. And then these cells will adhere there in the stromal cells and then start to proliferate.

Slide 186:
IN VIVO FLOW CYTOMETER

So for us to understand, this was actually used, a model system that was developed at Mass General with Charles Lin, where you image the cells as they home in the circulation, using an in vivo flow cytometry. And at the same time you can do in vivo confocal imaging and you've seen a little bit of the in vivo confocal imaging from the other presenters.

So let me just show you this model system. You can inject myeloma cells, either fluorescently labeled or primary patient samples also fluorescently labeled into the vein or you can put them into the femoral head, for example, and then try to see metastases. And then using the same mouse you can put the ear – and because you can actually see the vessels of the ear of the mice easily, you can focus a flow cytometry machine and see those circulating fluorescent cells as they circulate through the circulation. And if you have more cells circulating in the peripheral blood, then that could be an egress, out from the bone marrow into the blood, and if you see less and less of those cells in the circulation, then they may be homing into the bone

marrow.

At the same time you can also open the scalp area and now you can see the calvarial bone and this was also most of the imaging systems that you've seen already, where you can now see in vivo confocal imaging or live confocal imaging of those cells as they enter into the bone marrow niches.

Slide 187:
Normal bone marrow architecture

Now here is some of that normal bone marrow architecture and this is again some stacking of those 3-D images, showing you the first – in blue, these are just the bone areas, and then as you go in, and this is again the call 2.3 mice that you've heard already about, where the osteoblasts are GFP labeled and here we've labeled the blood vessels with a red dye , and you can here very closely the association between the blood vessels and the osteoblasts. Again this was presented very nicely by David Scadden in a Nature paper, showing you that the osteoblasts or the endosteal niche has a lot of blood vessel contact near it and it's not really a hypoxic area as you heard before.

Slide 188:
Homing in MM

Now let me show you a little bit what happens to myeloma because we had not worked before on the trafficking of myeloma cells. If you inject myeloma cell lines or patient samples into the tail vein as I've showed you before, and track those cells every minute through the in vivo flow cytometer, you can find that within one hour most of those cells will exit from the circulation and we went on to further show that they are not trapped in the lungs or liver or spleen, but they actually do home into the bone marrow niches.

Slide 189:
Homing of MM cells and engraftment in first 72 hrs

And these are some of those early pictures of injecting only 10,000 cells of myeloma cells and looking at two hours or six hours or 24 hours and then 72 hours, as you can see here in those images, and trying to look at those myeloma cells and do they home towards the vascular areas or the endosteal niches. And this was very similar to what has been done before by David Scadden for normal stem cells.

Slide 190:
Homing of MM cells (cont'd)

And it shows here, those white dots are actually those myeloma cells, at early time-points, at two hours even you can see some of those in close association with the vascular areas that are in red. And they're not very close to the green fluorescent areas which are the osteoblasts here that you can see. Now if you do this by quantification, then you can actually see how close they are to the bone or to the blood vessels and then to the osteoblasts. And it shows you that most of them were clustered very closely to the perivascular area and less so to the endosteal area, which again goes very well to the previous speakers' discussions about how the tumor cells can be very close to the perivascular areas.

Slide 191:
Homing of MM cells (cont'd)

Now if you follow those mice further and try to see how does it grow into the bone marrow niches and you can see these are some of those plasma cytomas growing into the bone marrow of those mice, and it starts replacing the whole architecture of the bone marrow and inducing more and more tumor growth in those areas.

Slide 192:
In vivo confocal imaging detects surviving cells after bortezomib therapy.

And here, this is some of that comparison between confocal imaging, bioluminescence imaging, that most of us will use, and then immunohistochemistry. And it shows you that confocal imaging may be a lot earlier in detecting tumor growth compared to bioluminescence, so you can see here by the time we start seeing bioluminescence positive, we already have huge tumor growth and already metastasis of those areas of the bone marrow when we see by confocal microscopy.

So this model system can be used actually for early stages of the disease like MGUS stages, going on to develop active myeloma or progressive myeloma.

Slide 193:
In vivo confocal imaging detects surviving cells after bortezomib therapy.

It can also be used for minimal residual disease. Our biggest problem right now post-treatment of myeloma is when we transplant our patients we still have minimal disease that causes relapse three years or four years after. And can we better understand why is there minimal residual disease in myeloma, and by doing that we've actually injected bortezomib in some of our mice and made sure that by bioluminescence you have a negative signal, so you think that you're in a complete remission, but if you look carefully at the positive GFP signal, you still have some GFP signals and this time it's not close to the vascular niches, it's actually away from it.

So again, giving us an idea that minimal residual disease may not be something present in the tumor clone itself, but may actually be a component of the microenvironment effect and where those cells hide in the bone marrow and how to get them out of there.

Slide 194:
Close interaction of MM cells with the bone marrow

Again through this system you can see that we have a close interaction with the microenvironment. This is just showing you neo-angiogenesis and new blood vessel formation in that system. And as you decrease the tumor size you can still see that the blood vessel formations are still present there and have not changed significantly.

Slide 195:
Stroma-myeloma interaction and cell trafficking and metastasis

So with that I'll take you through now some of the model systems and how do we define what happens with cell trafficking in myeloma. And you take it from biology to the clinic, like you've heard from the other presenters. And I'll take you through separate areas of my talk here.

Some are the surface expression receptors that you've heard a lot about, the integrins, the chemokine receptors, especially CXCR4 and Sdf-1 axis. And then I'll take you through downstream signaling Rho/Rac signaling pathways and a little bit about TORC1 and TORC2 and then finally I'll take you through hypoxia and then in the last few slides, a little bit about what we're doing with epigenetics and how does that regulate cell trafficking and cell progression in multiple myeloma.

Slide 196:
CXCR4 in regulating homing of MM

So let's go through CXCR4. You heard from all the presenters before that the chemokine receptor CXCR4 and, of course, now CXCR7, which is the other new receptor for Sdf-1 are very important for cell trafficking of normal stem cells, as well as leukemia cells, that you've heard already from the previous presenters. So can we say the same thing for multiple myeloma?

Here we've done some previous work showing that CXCR4 is highly expressed on the surface of multiple myeloma cells and it responds very well to Sdf-1 signaling. By the confocal microscopy here, you can see that the CXCR4 internalizes in response to Sdf-1. And if you inhibit CXCR4 by plerixafor or AMD3100, you have less homing into the bone marrow niches.

Slide 197:
Mobilization of MM cells by AMD3100

These are controlled – these are cells that were pretreated with the CXCR4 inhibitor and you can see the same image here by in vivo flow cytometry. The control cells home very fast and within one hour you only have 20% circulating cells, while those that were pretreated for two hours with plerixafor circulated for a longer time in the peripheral blood, and by doing so they have less homing potential into the bone marrow.

Slide 198:
Mobilization of MM cells by AMD3100

However, we know that most of our myeloma cells, by the time we diagnose our patients, are already in the bone marrow, so the part of homing may be either a very early event in MGUS or maybe a very late event when you're trying to prevent further disease progression. But what do you do with those patients who have active disease where the cells are already in the bone marrow?

And we came to the point well, if you can also inhibit CXCR4, you can do the same thing that you see in stem cells. You can de-adhere those cells from the stroma and mobilize them or at least have that concept of de-adhesion and then by doing so you can disrupt that interaction with the stromal cells and having more sensitization to therapy.

Slide 199:
Mobilization of MM cells by AMD3100

So here we have cells that were taken from patient samples, plasma cells, labeled fluorescent dye, CD34 labeled with a different fluorescent dye from the same patients, we injected them into the same mouse and waited for three days for them to engraft well and then started giving the mice plerixafor. And looked at the peak mobilization time of those stem cells and

myeloma cells in those mice.

Slide 200:
Mobilization of MM cells by AMD3100

And you can see here that we had the higher mobilization of the myeloma cells at two hours and less so at the six hour time point, which was a little bit different than the stem cells that we injected, the primary stem cells that we injected from those patient samples.

If you follow up at the two hour time point, at different days, you can see that you have a high mobilization of those myeloma cells that goes on even by day four, while your stem cell mobilization decreases significantly by day three and day four.

Again, this was in our model system with stem cells taken from patients with myeloma and it was a little bit different than what you have seen already in the previous presentations.

Now we say well, potentially we can actually target this area where you have minimal stem cell mobilization, highest myeloma mobilization, and potentially give those patients, when there is maximum de-adhesion, another drug like bortezomib, that can have a higher sensitivity when those cells are not adherent to the stroma. And in fact, we looked at the combination of bortezomib and plerixafor together, if they would have any effect on colony formation assays, and they did not. So we could easily give that and we would not have a detrimental effect on the stem cells when they're being mobilized.

Slide 201:
Phase I/II trial of AMD3100 (plerixafor) in combination with bortezomib in MM

But the question to prove that concept or that hypothesis was can you induce better response when you have a combination of plerixafor and bortezomib in multiple myeloma. And here you can see, again by bioluminescence imaging, that if you have control mice treated with a vehicle or treated with plerixafor alone, you have no effect on the tumor growth of those xenograft models with myeloma. And you can see it by bioluminescence here. If you have bortezomib alone, then you have a nice effect here, but it does not cure those mice and you still have residual disease. If you use the combination of plerixafor and bortezomib, you have a better tumor – or less tumor growth in those mice, and a better survival in those mice. Indicating again the same concept of chemo-sensitization. If you just de-adhere those cells, make them mobilize into the peripheral blood or just even de-adhere them, then they will become more sensitive to therapy.

And this shows you bone marrow biopsies from those mice at different time points and again it comes to that concept you don't have to mobilize them all into the peripheral blood, but just de-adhere them. This combination here of plerixafor and bortezomib was the best with the tunnel assay for apoptosis and had the least number of CD138 bone marrow cells in the bone marrow as well as in the liver and spleen, again indicating that that combination was better than bortezomib alone in those mice.

If you look at the component of the circulating cells that are de-adherent and have become mobilized into the peripheral blood, and we inject into mice AnnexinV as a marker of apoptosis and see if it marks those cells, then we can do by in vivo flow cytometry again the number of GFP positive cells, which are alive, or the number of AnnexinV positive cells, which are apoptotic, and

quantify how many cells that are circulating in the blood are apoptotic or not.

So here you can see again with plerixafor alone you don't have too many apoptotic cells, with bortezomib alone you have some apoptotic cells, but you increase it significantly when you have the combination of plerixafor and bortezomib in the circulating cells. And you can see it also by confocal imaging here of this blood vessel with some apoptotic tumor cells, that you see here, while the other GFP positive viable cells are still in the microenvironment or in the tumor niche.

Slide 202:
Schedule and dose escalation

With that in mind, we have initiated the Phase I clinical trial of the combination of plerixafor and bortezomib in patients with multiple myeloma. These are relapsed or relapsed refractory patients with myeloma. And here, this is the dose escalation that you can see with those patients. And we went on beyond the FDA approved dose for patients with stem cells at the 240 micrograms per kg, we're right now at level 5-B here, where you can see that we are at the 320 micrograms per kg injected in those patients with myeloma and given at the different schedule where we're trying to give them for three days, the plerixafor, and then add the bortezomib on day 3 based on our preclinical setting.

Slide 203:
Mobilization of MM and CD34 cells in the peripheral blood

Now I'll show you some preliminary data and the clinical data will be presented in a poster, so please come and see it on Saturday. But this is some of the initial data from the blood taken from those patients at different time points. And you can see on the first day where they're getting plerixafor alone, zero hours, two hours, four hours, and you can see already myeloma tumor mobilization early on, at 1.5-fold increase or percent increase, sorry, of the cells in the peripheral circulation. And then it goes away when you start giving bortezomib into those mice. Especially when you give the higher doses of bortezomib, then you lose completely that tumor mobilization, again indicating that you are inducing apoptosis of those tumor cells circulating in the peripheral blood.

This is the CD34 and we have no effect on the mobilization of CD34 with the combination of plerixafor and bortezomib.

Slide 204:
CXCR7 in Multiple Myeloma

Now I'll take you through other things. This was a proof-of-concept of CXCR4 and how it can be used as a chemo-sensitization method. But we know now that CXCR4 is not the only receptor for Sdf-1 and that there is another receptor. It used to be called RDC1, now called CXCR7, and this is also very important in adhesion and angiogenesis, especially for tumors.

So here we show you that CXCR7 is also expressed in myeloma as well as endothelial cells obtained from myeloma patients. And this is some work where we're showing it has a very significant effect on adhesion to HUVEC cells, endothelial cells, as well as to stromal cells. And if you do the same concept of homing, which is a mechanism of adhesion, you can see that by inhibiting CXCR7 you have an effect on the homing of myeloma cells towards the bone marrow microenvironment.

Slide 205:
Rho and Rac in MM

If I take you further downstream from the surface expression into signaling pathways, what happens? And you've already heard a lot about the PI3 kinase pathway and it's downstream into AKT and mTOR, but there are other kinases that are very important and here we're talking about the GTPases, including the Rho and Rac pathway. And these could be very important for migration and adhesion. And here we dissected the signaling pathways of Rho and Rac in myeloma and found that Rho, with an inhibitor in Rac which is downstream of Rho, is very important for the migration and chemotaxis or chemo-attraction of myeloma cells. While if you regulate ROCK, in GTPase, you can actually have an effect on both migration and adhesion with inside-out signaling through VLA-4. And it's very important for that integrin interaction.

Slide 206:
Rho and Rac in MM

And here, this is some of that work through inhibition in myeloma cell lines, in patient samples, in adhesion assays or migration assays in vitro, as well as filloidin expression, you can see that we have a very nice inhibition of adhesion to fibronectin or to stromal cells in myeloma patients and cell lines, as well as effects in migration, especially with the ROCK inhibitor and the Rac inhibitor, but no effects of adhesion with the Rac inhibitor.

And then you further downstream dissect where exactly those pathways are regulated in myeloma and you can see that Roc inhibition can affect, while Rac inhibition can affect LMPK.

Slide 207:
Rho and Rac in MM

Now why would that be important? It would be important because there are now clinically developed agents that are targets in Rho and Rac and this could be useful for signaling in multiple myeloma. With the same concept we have now a TORC inhibitor and we know that rapamycin as a TORC1 inhibitor can have effects, as you've heard already before, from the other presenters. But TORC1 may not be alone sufficient to inhibit regulation of migration or chemotaxis or even adhesion in myeloma and you may need inhibition of both TORC1 and TORC2 that regulate AKT to have that effect.

Slide 208:
TORC1/TORC2 regulation in MM

And you can see here, this is work that will be presented in an oral presentation on Monday, so please go see it, by Patricia Maiso from our group, that shows you that when you have adherence of the myeloma cells to stromal cells, rapamycin can have an effect, but IMK128, which is a TORC1, TORC2 inhibitor that we're testing right now, has a significant effect in inhibiting adhesion and by doing so, it can actually inhibit more the proliferation. So myeloma cells when they're adherent to stroma, they have a higher proliferation index, but when you have that new inhibitor that affects both TORC1 and TORC2, you can have a significant effect on the proliferation of cells that are in co-culture with stromal cells, much more than rapamycin alone.

And here you can show the same thing of in vivo homing where the IMK128, the inhibitor

of TORC1 and TORC2, had the best effect of inhibiting homing of those cells. And when you co-culture stromal cells or IL-6 and IGF-1, you have a high activation of AKT and phospho-4 ABP1, which again are very important to be inhibited by this drug.

Again, why is this important? We already have a Phase I trial going on right now with IMK128 in multiple myeloma, trying to specifically target this, where you not only inhibit homing and adhesion, but also you would inhibit proliferation and survival and we know that these two concepts go hand in hand, that you cannot prevent only invasion or adhesion, but you can also inhibit proliferation of those cells.

Slide 209:
Selectins in MM

Now let's go on to selectins. And selectins are very important for the early initiation part of the rolling process before they adhere. And here I show you some work that's still unpublished, where we've looked at the selectins and the selectin ligands in multiple myeloma and we've looked at selectin ligand, the P selectin ligand being highly expressed on myeloma cells, but not on normal plasma cells. And this is a tissue microarray of immunohistochemistry here for multiple plasma cells from different bone marrows. And you can see that the PSGL-1, P-selectin ligand, is highly expressed on those myeloma cells.

Now if you inhibit the PSGL-1 either by specific antibodies, by siRNA, or by a new drug from Glycomimetics called GMI-1070, you can have a significant inhibition of adhesion as well as adhesion to endothelial cells and trans-endothelial migration.

Slide 210:
The role of P-selectin ligand in cytoskeletal signaling and homing in MM cells

And here, this is some of the in vivo work where we can see again by in vivo flow cytometry, that control cells home very rapidly to the bone marrow, while the mice that are injected with the GMI-1070 the myeloma cells stay in the peripheral circulation, and by doing so you can prevent homing and you can also now put the drug like bortezomib and prevent those cells from going into the bone marrow, but also inducing cytotoxicity in them.

This is confocal imaging of those mice at the 15 minute time point, control and GMI and it doesn't show very well, but you have more cells in the control bone marrow and less cells in the GMI.

And we went on to look at signaling downstream, where you add P selectin ligand or you add HUVEC cells with that co-culture effect, and you can have a very significant effect, not only on selectin effects, but also on the integrin effects and downstream to cofilin and phospho-SRC.

Slide 211:
GMI-1070 induces sensitization to bortezomib therapy in vivo

And we went on to do the same thing, that if you inject GMI-1070 in the mice and add bortezomib to it, you have that chemo-sensitization effect that you not have with GMI alone or with bortezomib alone. And you have a survival benefit when you have those drugs together. So better tumor regression and better survival event and hopefully we will have clinical trials using GMI-1070 in combination with bortezomib in multiple myeloma.

Slide 212:
Role of hypoxia in MM

Now I'll take you now through another idea, which is the role of hypoxia. And we know that hypoxia is very important for solid tumors, so as tumors grow beyond the vasculature that they have, they become more and more hypoxic. And by doing that they actually up-regulate HIP1α, which is a transcription factor, and then they actually can prevent apoptosis. They're capable of preventing apoptosis even in that hypoxic environment.

Not only that, they're capable of metastasizing and there has been very nice work done before where hypoxic cells when metastasized, whether they're breast cancer or other cancers in solid tumors, to other areas, so hypoxia may be very important as a trigger of cell metastasis.

And we started to say well, would that be also important in myeloma? We know that the bone marrow microenvironment is hypoxic in multiple myeloma and in normal bone marrows actually they are hypoxic. And our initial hypothesis was as the tumors grow, as you get those plasma cytomas in the bone marrow, they become more and more hypoxic. And it starts triggering that egress, that event for those cell dissemination, that event for the cells to start going into the peripheral blood, finding a new bone marrow niche that is less hypoxic and by doing so they inhibit their integrins and cadherins, they go into the peripheral blood, they up-regulate their CXCR4, so that they can home now to new areas. And although those areas are termed here normoxic, they're not really normoxic, but they're gradients of different hypoxia. And here you have much more hypoxia in the bone marrow that has tumor growth or myeloma growth.

And that work was also presented previously by another paper by Karen Vanderkirk in Myeloma, where it showed that if you use a hypoxia drug CH302, which is an alkylating agent that actually gets activated in hypoxia, it will go and target those myeloma cells. So you can use hypoxia for your benefit because you can push into it a drug that will go into the hypoxic areas and get activated there.

Slide 213:
Role of hypoxia in regulating egress of MM cells from the bone marrow to new bone marrow sites.

Now how do we prove that hypoxia is important for cell trafficking or for cell metastasis in multiple myeloma? What we did is we took different mice by bioluminescence that have different areas of tumor growth. So some of them have minimal tumor growth and some of them are very heavily disseminated already. And we looked at the level of hypoxia in the myeloma cells in the bone marrow and in the peripheral blood and tried to correlate whether there is a dissemination into the peripheral blood.

And what we did is we injected pimonidazole, which gets uptaken into the hypoxic cells, so you can actually look at the relative tumor burden by bioluminescence, relative to the myeloma cells that are hypoxic. And you can see that there is a correlation between tumor burden and hypoxia and myeloma cells in the bone marrow. We then looked at the circulating tumor cells and see if there is a correlation between them or not.

And here you can see that as the tumor burden increases, at first you don't have much circulation of the cells. But then you come to a certain point, a trigger point, and then you have an increase, a significant increase of the number of circulating cells.

But this was not a direct correlation with the tumor burden. So we started to say well, does

it correlate then with hypoxia, can hypoxia be one of those triggers that induces mobilization or egress of the myeloma cells. And here you can see that when you look at the hypoxia level in the bone marrow of myeloma cells in the bone marrow versus circulating myeloma cells, you have a direct correlation between those, indicating potentially that as hypoxic cells go on more and more in the bone marrow, they trigger a point where they can start egressing or going out into the peripheral circulation, and by doing that they will induce cell metastasis and go on to another bone marrow niche.

Slide 214:
Graphic

And here we show you how to prove that. We've looked at hypoxic myeloma cells or hypoxic stromal cells and start to see if they have an inhibition of adhesion. So you can see that normoxic myeloma cells would have a very nice adhesion to stroma, but if you use hypoxic myeloma cells that adhere less to normoxic stroma, and if you use a hypoxic stroma, everything, whether it's normoxic or hypoxic, will adhere less, indicating that this hypoxic stroma does not really want more and more tumor cells to go into it, but it actually prevents further tumor growth into it, because it's already packed with tumor cells. And the same for the hypoxic cells that are myeloma cells, if they are into a bone marrow microenvironment, they want to egress and have less adhesion and then egress to another area.

And we further show that this is mainly dependent on N-cadherin. You can see here some of the western blot of hypoxic myeloma cells or normoxic myeloma cells. Same thing with luminescence. And then looking also at the stromal cell compartments, so not only at the myeloma cells, but the stromal cells, whether they're normoxic or hypoxic, and showing you that N-cadherin would be very important.

We also looked at it in vivo in those mice, looking at level of hypoxia versus cadherin, and you can see that there was an inverse correlation. The more hypoxia you have, the less cadherins you have. And the less of adhesion those cells have, indicating that they want to egress.

Slide 215:
Graphic

Now once they egress and they have a higher CXCR4 expression, based on prior work and based on our work, they want to home now to new bone marrow areas. And we tried to prove that by injecting either normoxic cells or hypoxic myeloma cells into the mice and trying to see if they will home faster or slower into the bone marrow.

And you can see here that the hypoxic myeloma cells homed much faster into the bone marrow, indicating that they had higher CXCR4 expression and that would lead them into the bone marrow much faster.

In fact, if you actually inhibit the CXCR4 by AMD3100 or plerixafor, you can reverse that effect of hypoxia completely and prevent those cells from homing into the bone marrow. These are some of those bone marrow confocal imaging areas, again showing you that there is more hypoxic cells that homed within 15 minutes into the bone marrow niches.

And again showing you that areas that are already full of myeloma cells, down-regulate their Sdf-1, so that you have less of the myeloma cells going into those same areas that are already packed, and they will look for other areas of normal bone marrow.

And again, this is just a quantification of if you either inhibit Sdf-1 or you inhibit AMD3100, you reverse that effect of migration and homing towards the microenvironment.

Slide 216:
Hypoxia induces resistance to therapy

We're also working on understanding how hypoxia can induce resistance in multiple myeloma. Those areas that are packed with myeloma cells and are hypoxic are probably resistant to chemotherapy and this is further work that is ongoing right now, especially for minimal residual disease, but I don't have time to talk about it.

Slide 217:
MicroRNA Expression identifies miR 15-a and 16-1 in MM

In the last few slides I'll show you what we're doing for epigenetics. So I took you from the surface expression into signaling pathways, into now epigenetics and potentially genetic regulation of cell trafficking in multiple myeloma. And I'll just take you through some of that data where we're looked at the micro RNA regulation in multiple myeloma and how that regulates cell trafficking.

Our original work was done by Aldo Roccaro, looking at micro RNA expression for filing of myeloma samples or healthy donors, showing that there was a huge difference in the level of micro RNA 15a and 16-1 in myeloma samples compared to healthy control, where they were down-regulated.

Now this is important because miR-15a and 16-1 are present on the 13q chromosome. And again, this is in 50% of patients with myeloma, they have 13q deletion, so potentially this could be an important tumor suppressor area, that's deleted in multiple myeloma.

And this is just confirming it by QPCR.

Slide 218:
miRNA-15a and -16-1 modulates proliferation and cell cycle of MM cells

But why would that be important for cell trafficking because proliferation and trafficking also work together. Here we show you that if you induce miR-15a and 16-1 in myeloma cells, you can actually have less cell cycle arrests, you have survival of those myeloma cells less with here the proliferation assay, showing significant inhibition when you induce miR-15a and 16-1 and less cell cycle regulation and survival pathway regulation.

But not only that, you can also regulate the microenvironment. So from the tumor cells you have less VEGF secretion and less angiogenesis in those tumor cells and less TNF expression.

Slide 219:
miRNA 15-a and 16-1 regulate NFkB in tumor cells, angiogenesis and tumor growth in vivo

Now we're looking at it in the other way around where miR-15a and 16-1 are in the stroma and how they regulate the tumor cells and regulate that microenvironment.

Slide 220:
Conclusion

So I hope in the last few minutes here I showed you that cell trafficking is very important for multiple myeloma and that process of having multiple lytic lesions in myeloma is really a continuous dynamic process of entry and exit from the bone marrow into the blood, again into the bone marrow. And by trying to regulate that through integrins, cadherins, or selectins, or even downstream or chemokine receptors or downstream signaling or even epigenetics, you can really target myeloma, either by preventing the early progression stages or preventing minimal residual disease, but also even in the active cases, where you can induce chemo-sensitization and you can have so many different areas that you can target now, that we used to not target before. And hopefully with that you can prevent that interaction of the stroma and myeloma microenvironment.

Slide 221:
Acknowledgement

So with that I'll stop. I'd like to acknowledge so many people who work with us. Of course, our leader Ken Anderson, our group, Nikhil Munshi, Barrett Rollins, Paul Richardson, Ruben Carrasco. The group who does all the animal work, Dr. Andrew Kung, Dr. Charles Lin, who has the confocal imaging. A wonderful lab team. And please try to see their presentations in ASH this year. A wonderful clinical team who does the clinical trials. And then our support, including The Leukemia & Lymphoma Society.

Thank you.

Slide 222:
Question-and-Answer Session
Dr. Irv Bernstein:

Let's have the speakers come up and let's see if we have some discussion or we've learned everything we're going to learn. So if you have questions go to the microphone and we'll see how it plays out.

Audience:

My question relates to the non-selectin mobilization…[inaudible]

Dr. Michael Rettig:

That was a big worry. In the mouse models we haven't looked specifically at DNA damage, but in mouse models treated with mobilization and chemo, we see similar platelet and neutrophil recovery, really no death due to bone marrow failure per se up front. I don't know that it's justified yet up front, I'm not a clinician. But I think that's our initial thought. And definitely we were worried about the effect on normal stem cells, are you going to cause graft failure or just a loss of hematopoiesis. And we haven't seen that in any of the 48 patients in this trial.

Audience:

But you looked at markers like…[Inaudible]

Dr. Michael Rettig:

Yes, so we've proposed that in the next trial. We did a lot of flow phenotyping in the

original trial, but we didn't look at markers for DNA damage.

[Audience Q?]:
Do you see any anosmia in the patients where you treat with the combination of plerixafor and chemotherapy?

Dr. Michael Rettig:
What do you mean? I'm not sure.

[same audience member]:
So the neural stem cells in the forebrain give rise to new neurons that migrate into the olafactory bulb and that process is also regulated by Cxcl-12. And so one prediction is that you could have an increased ability beyond what chemotherapy normally has on disrupting their ability to learn or remember new odors.

Dr. Michael Rettig:
Yeah, that's a great point.

[Same audience member]:
It would be interesting to look and see whether you notice any effect like that in the patients.

Dr. Michael Rettig:
To my knowledge there hasn't been – but I'm sure that they haven't looked.

Audience:
[Inaudible]

[?]:
There could be heterogeneity within the stem cell pool. There could be a subset of stem cells that localizes to an endosteal niche and a different subset that localizes to a vascular niche. All of those possibilities remain open. Another possibility is that we have not studied the localization of stem cells after irradiation. We've only looked at it under steady state conditions and maybe it's different after irradiation. One of the points that Shahin Rafii makes is that one of the things that irradiation does is it destroys the vasculature. And so it would disrupt any vascular niche that exists, raising the possibility that the cells may depend more on osteoblasts potentially, after injury. These are all open questions that remain to be determined.

Audience:
[Inaudible]

Dr. Michael Rettig:
I think what it is that we've washed the cells and we've probably washed away the plerixafor in preparation for the migration assay. That's what I think. We're looking at that. But

my gut feeling is that in the expression levels, with the 12G5 and 1D9, we see that difference because that's a lice and a wash whole blood assay. But then to do the transwell migration studies, you know, ACK lysine induced extensive washing before setting those up, and so potentially you could remove the inhibitor. That's our initial thought, but the suggestions you indicated also could be possible.

Audience:
[Inaudible]

Dr. Michael Rettig:
We haven't done the VLA4 and AMD. We've done the VLANG and that does seem to improve, similar to G. We haven't done the VLA4NA chemo-sensitization yet.

Audience:
[Inaudible]

[?]:
So that's what held us up a little bit, is that we couldn't definitively knock down any particular TLR and have an effect on the samples. Having said that, we know that many cases you have combined action of several TLRs and we didn't do the combined knockouts, which we're now doing. So I think whenever we find these mutations it's a fair bet that other tumors have done the same thing in a different way and I just don't have any evidence, but it's a very valid point. And at the moment I don't know whether this MYD88 mutant even needs a TLR, maybe it spontaneously assembles itself without a platform, but I kind of imagine that it does need a TLR. So we'll have to work on that.

Audience:
[Inaudible]

[?]:
Some of the others might, absolutely. Good point.

Audience:
[Inaudible]

Dr. Iannis Aifantis:
No, I didn't, but I'm not sure if they have looked for CNS invasion. We haven't done that. There's a study out there on notch activation and CNS invasion that suggests that there is correlation of these two, but for CCR7 I don't know.

Audience:
[Inaudible]

Dr. Iannis Aifantis:

So we've tried some B-AAL models, BCR-ABL induced in others, and we have done the same experiments. They're not CCR7-mediated. So we think it could depend on the oncogene that you use or even the type of leukemia itself. So that's the only model that we've tried so far.

Audience:
[Inaudible]

Dr. Iannis Aifantis:
I agree with you. I think that somebody should look and compare relapsed versus diagnosis samples, a few of them. The only interesting thing that I've seen out there on CNS and CCR7 is that you know that the normal T cells that are playing a immuno-protective role in the CNS, they're all CCR7, there are very few, but they're all CCR7 high. So I think that could be a general mechanism for T cell homing into the CNS. Relapsed versus diagnosis, I don't know yet.

Audience:
[Inaudible]

Dr. Irene Ghobrial:
We're looking actually at this specifically right now. We're actually doing a knockdown of every isozyme and trying to see which one is very important for migration, which one is important for adhesion, as well as proliferation. So we are doing that work right now.

Audience:
[Inaudible]

Dr. Irene Ghobrial:
Yeah, so again this is very early hypothesis. When we did those confocal images, we found that the leftover cells that were after treatment were not close enough to the blood vessels, but again, it's very early data, hypothesis-generating. I think we need a lot of work to try and understand what exactly is going on with those.

Audience:
[Inaudible]

Dr. Irene Ghobrial:
So it might be related, again, if you think that those cells are hypoxic, away from the blood vessel area, and that's why they are resistant, that could be an area of interest. But we're still looking at it to see if that's true or not.

I had one question for the group about diurnal variations in cell trafficking and I think we did not address that, especially when we're now doing some treatment with plerixafor, should we do it morning or evening and how does that ___ and so on? Any comment?

Dr. Iannis Aifantis:
I guess you should do it the same time of the day. [Laughter]

Dr. Irene Ghobrial:

Yeah, but what's the best timing, based on that diurnal variation? I mean, there was a beautiful paper on the stem cell trafficking with diurnal variations.

[?]:

Paul Frenette has shown that there's a diurnal variation in the rate of trafficking from the bone marrow. And so it would make sense to test it, daytime and at nighttime, and see empirically what the effect is.

Dr. Irv Bernstein:

So I'll take the last question ___ in the long run, this notion of therapy by disrupting the stromal interactions with hematological malignancies, ___.

[?]:

Well, it's entirely possible that the environmental factors that the leukemia-initiating cells depend on could be different than hematopoietic stem cells in critical ways. So if the leukemic stem cells depended on factors that were more similar to a myeloid restrictor progenitor, for example, then you could target those things and probably have normal hematopoiesis do quite well because perhaps you wouldn't disrupt the stem cells at all. These are all open questions, but I don't think there's reason to be pessimistic about the potential.

Dr. Irv Bernstein:

I wouldn't be pessimistic, but it's certainly an area that requires investigation to understand the potential for this field. This is certainly the group to do it.

Dr. Irene Ghobrial:

We're lucky in myeloma that that's not a problem because even with some of our therapeutic agents like bortezomib and so on, we don't have a pure effect on stem cells, so we could potentially have that kinetic difference. And we saw a kinetic difference of mobilization between myeloma cells and normal stem cells.

Dr. Michael Rettig:

[Inaudible] .. that these AML cells, trying to get something that was indicative of a leukemia stem cell. And it didn't turn out specifically. So I don't think we know that one specific marker. And I think that's why John ___ potentially target multiple pathways up front and signaling. But yeah, we don't have the one marker, at least in AML.

Dr. Irv Bernstein:

With that, let me thank all the speakers for a great conference. And let me thank the bravest of our audience for sticking it out to the very end. I thank all of you and I thank The Leukemia Society.

END