**Series name**: To Immunity and Beyond

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**Episode name:** Optimising CAR-T cell sensitivity by engineering matched extracellular sizes between CAR/antigen and CD2/CD58 adhesion complexes

**People:** Omer Dushek, Paul Klenerman

**Transcript:**

Paul

Well, welcome. We've got Omer Duchek here today. Welcome Omer.

Omer

Thank you, Paul.

Paul

Yes, pleasure to have you. And this is the first series of the podcast, which is To Immunity and Beyond. So we're just collecting some of the very best immunology going on in Oxford and trying to create a way for people to digest it. I think it's an interesting format because, as we're just saying, normally when you think about doing a review of a paper, you're thinking about the figures and it's a very visual experience, but actually if you just want to know what the impact of the paper is and the concepts, that's actually kind of, you don't need the things in front of you and it's much better described. I'm really looking forward to hearing about this great paper that you just put on bioRxiv as a preprint. Very current and I hope everybody listening will be able to access that and enjoy it, so let's get on with the podcast then. And the first question that I'm asking everybody, is just to give us a little bit about your background and how you got to do what you're doing currently.

Omer

You want to start like at birth or...

Paul

Well, at the relevant time point.

Omer

Yeah, well, OK, I guess I entered university, you know, with aspirations of going to medical school. But basically my first year I got really interested in, in physics actually and sort of decided to switch my focus into an undergraduate degree in physics. Focusing on atmospheric physics. Atomic physics and finally nuclear. You know four different projects, but actually at the end of all that, I sort of realised that to make advances in these fields, you really have to have many, many years of education, basically, and sort of work. And so I decided to try something else. And it was a course in mathematical biology that sort of changed my thinking about, and I got really inspired. And so for my PhD, I decided to go into an applied math PhD to work with someone who's applying mathematical models to study technology and I was at the University of British Columbia and it was really there that I started working on analyzing data that was coming out from T cell experiments with experimentalist in France, in Toulouse called Salvatore Valitutti. And yeah, and so I was in the summers in his lab and I really got fascinated by it. And so for my post Doc, I decided to see if I could actually go and be in an experimental lab. And I interviewed here in Oxford, actually, with Anton van der Merwe and he was happy to take the risk of having a mathematician join his lab to sort of hold pipettes. And it's really there that, you know, I learned how to do experiments with T cells. And then yeah, I guess I applied for these career development fellowships and very fortunately got lucky to get one of those and start my own lab. And it was really supported by a variety of immunologists at the time at the Dunn School. Anton was very supportive, Marion Brown was there as well. And Oreste Acuto was there as well. And they all provided, you know, support to people in my lab to help them train in experimental immunology.

Paul

Brilliant. Well, that's a fantastic story and really interesting way to interface the two bits. I mean, I think that's one of the things that Oxford potentially can offer people, is because it's so big and there's so many smart people around these interface areas that probably all the growth really happens so. And Valitutti. I seem to remember a paper from the early 90s, a very important one about serial signalling that that was quite influential at the time. Didn't really understand quite a few of the elements. If it's the same person.

Omer

Yeah, absolutely it. He was working as with Antonio Lanzavecchia at the time and he showed that a single antigen could actually serial bind multiple T cell receptors and that was one of the mechanisms that was proposed for how T cells have such high sensitivity.

Paul

Great well I'm glad he's still having an impact, a very much broader one. Well, perhaps just give us a little bit about the background to the paper that you're just pre-printing and you know what were you trying to address in the paper.

Omer

Yeah. So the paper is really focused on trying to understand why CAR T-cells have low antigen sensitivity and then to improve that. And so maybe I could take a step back and talk about why we care about that in the first place. You know CAR T-cell therapy is a form of adoptive cell therapy where patients have their T cells removed, genetically modified outside of the body to express a new antigen receptor, then re-infused back into the patient in order to make a population of T cells that can now target new antigens. And so this therapy has been wildly successful for targeting hematological cancers and the targets are often surface proteins on B cells, for example a protein called CD19 or BCMA or CD22 have all been really good targets. And these T cells then go on to kill all of the patient's B cells and have led to cures in many, many patients. The problem is that despite this initial success, many patients end up relapsing. In fact, I'm aware here in Oxford this therapy, we started giving it here about a year ago and already there's a large fraction of patients coming back now with relapse, where these B cells basically come back, and what's interesting about these patients is that they still have their CAR T-cells. And so the question was, you know, why are these CAR T-cells no longer recognizing these B cells? We now know the reason for that is that unlike recognizing antigen through the T cell receptor, the native T cell receptor, that recognition can be very sensitive. For example, it's been shown that a T cell can recognize a single peptide MHC antigen using its native T cell receptor. For CARs, we now know that you need about 100 or 1000-fold more antigens. And so what's happening on these patients is that their cancer cells simply have lower expression of the target antigen. And so in effect, these CAR T-cells no longer see these cancer cells. And so we wanted to basically fix that.

Paul

OK, so what you're saying is that the tumour has evolved in the face of the CAR T? But it's still theoretically a target. It hasn't lost the antigen entirely. It's just a kind of it's just crept below, it's kept its head down basically.

Omer

Yeah. I mean, there's a little bit of a debate about that because often we use flow cytometry to decide whether or not a cell is tumour positive or negative, and so many patients can look low for antigen or completely negative by flow cytometry. But again for flow we think we need about 100 antigens on the surface to detect it. So, we're not sure if they're genetically negative for the antigen or just below the detection limit.

Paul

OK. So it's driven really by a clinical problem really where we got an incredible therapy, but it's created this sort of second wave of tumours that we need to re address with better therapy.

Omer

Absolutely.

Paul

So what was your way of breaking that down into a tractable problem then?

Omer

So. You know, there was a few studies, at least two studies before our study, originally in PNAS in 2023 that showed that CARs had lower antigen sensitivity compared to the T cell receptor and they basically used an experimental system where they could directly compare a CAR and a T cell receptor and the way they did that is often to make chimeric antigen receptors that recognized peptide MHC antigen, which is not necessarily conventional, but it was just a way to compare their sensitivities. And what we could show at the time, is we basically used a system to just reproduce that effect on cells, but what we found was that if you now present the antigen on a reductionist system where you don't have any other ligands that are normally expressed on your antigen presenting cells, that sensitivity difference between the TCR and the CAR disappeared. So what it allowed us to do is to ask can some of these other cell surface proteins on T-cells contribute to the sensitivity differences that we see on cells.

Paul

So that essentially the interaction of, the recognition element is essentially maintained. But you're thinking about co-stimulation.

Omer

Exactly. And, but what we basically found by screening different co-stimulation receptors is that the T cell receptor was very good at exploiting some of these, including one known as CD2. CD2 is a co-stimulation receptor, or co-signalling receptor, also called an adhesion receptor, whose ligand CD58 is ubiquitously expressed on all the cells in our bodies. And when this receptor is engaged, it improves the T cell's ability to recognize peptide MHC antigen by 100 fold. However, when a CAR is recognising an antigen, its unable to exploit CD2 and so its sensitivity is increased by just a few fold about four fold. And so we basically trace this big difference in sensitivity in the TCR and the CAR to their ability to exploit this important adhesion molecule called CD2.

Paul

OK. So yeah, I think when I read the paper that was one of my questions really was like, well you'll explain in a minute about the CD2 finding, but there's many, many interactions going on in the cell surface, and presumably all of them could influence the sensitivity or the function of the T cell. So how do you get down to the CD2? I know it's a Dunn School favourite.

Omer

Yeah, that's right, CD2 has a long history at the Dunn School. And I mean the way that we did it initially is we looked at a number of different co-stimulation receptors that we knew were important for T cells, including CD28, 4-1BB, CD27, LFA1 for example and CD2. And out of all those, the biggest effect on antigen sensitivity in particular was CD2. They all co-stimulate. So for example, they all increase T cell activation, but CD2 is only one that enabled T cells to respond at low antigen densities.

Paul

OK. So perhaps Omer, just relating to that, there have been advances in the design of CAR Ts, some of which have taken into account the ability to co-stimulate. And maybe you could just put it in the context of those, because it sounded like listening to some talks like that problem that's already been solved by some bioengineering.

Omer

Yeah, that's right, I mean there's lots of different flavours of chimeric antigen receptors these days and a lot of work has focused on trying to change the cytoplasmic tail of the CAR to include co-stimulation directly in the CAR. So for example, second generation CARs often include CD28 co-stimulation, or 4-1BB co-stimulation, and we're aware of some studies that are including CD2 co-stimulations directly in the CAR. The issue with that is that again, although it increases the amount of cytokines the cells produce - we call

that an increase in efficacy - it tends to not actually change sensitivity very much, so it doesn't let T cells respond at low antigen densities, and we think the reason for that is related to the very specific mechanism for how CD2 functions. Its job is really to allow T cells to kind of grab the target cell, the cancer cell and hold its membrane at a precise distance from the T cell membrane, in order for its antigen receptor to directly touch the antigen.

Paul

OK so it's kind of pinning the thing down essentially, but it is actually providing an additional signal too, it’s not literally just adhesion.

Omer

Yeah, that's right. I mean in in the early studies, people have argued that CD2 had an adhesion function like integrands and a co-stimulation function like CD28. It seems like it can do both. And that’s partly why we think it's actually quite an important receptor. It's quite important that we get CARs to exploit this receptor.

Paul

So that seems like the you've got yourself through a series of conceptual ideas and experiments to the nubbins of it, which is that you want to improve CD2 function in the context of a CAR T. Just talk us through the experiments and what you found, because it's very cool, I thought, the way you did it.

Omer

Yeah. So, I mean, we started this project really by knowing, based on previous work, that CD2 binding to CD58 aligns the two membranes at about 14 nanometres, which is exactly the same distance as a T cell receptor binding peptide MHC. But now we had to re-engineer that size to accommodate chimeric antigen receptors who had an extracellular dimension that we didn't really know. And they target diverse antigens. And so the CAR antigen distance we are, we reasoned, would actually vary quite a lot depending on the CAR that's used and the target antigen. And so what we decided to do was to try to synthetically modify CD2 in order to change the interface height. And we did that initially by doing what other people did, which is to try to introduce IG domains, and other sort of structured domains into the protein. And all we could do there is really reduce the activity of CARs and that kind of, you know, took a few years to realise and then we reason that it might make sense to try to change the extracellular dimension. Not by an IG domain, which is about 3.7 nanometres in height, but actually by sub 3.7 nanometre, sort of, increments and that's where we use this other protein called CD43, which is a mucin. So we use

different amounts of its extracellular domain in order to elongate CD2. And remarkably, what we found was that if you elongate CD2 it reduces the ability of T cells to recognize antigens through their T cell receptor, because of course you're elongating the membrane so you reduce the activity of CD2, but it actually improved the ability of T cells to recognize antigen through CARs. And what was also quite interesting was that the degree to which we had to elongate CD2 varied depending on the extracellular hinge of the CAR. Or the antigen that we were targeting, or the epitope on the antigen that we were targeting. And so as a result, we found that as a different elongation of CD2 was really required to optimize the sensitivity of these different receptors.

Paul

OK, because each individual CAR receptor is slightly different and the obviously the thing they're targeting, they can be any size really.

Omer

Exactly, yeah.

Paul

OK. And actually just while we're on the subject then you mentioned that you would reduce a sensitivity for the regular TCR. So would that help with any of the kind of potential off-target effects of having... because these CAR T still have an existing TCR unless people have gone to the trouble of knocking them out.

Omer

Yeah, yeah, that's a good point. I mean, it's an interesting point that I haven't really thought about. When you're using T cells in an autologous therapy, there hasn't been really any cross reactivities associated with the TCR, but it's true that if you were going to use it as an allergenic therapy, you would then introduce a novel TCR into patients that could cause reactivity and so you're right. If you then reduce the function of CD2 on the TCR, you potentially would reduce any of those potential cross reactivities.

Paul

OK, but your main objective is just to kind of solve the problem of the lack of sensitivity and you describe that in a lovely way, that that you kind of have to micro-design the thing to, kind of, optimise, and there's a presumably some little Goldilocks zone where...

Omer

Yeah, exactly.

Paul

....where too much or not enough are bad and you just get it right and you get a quite a marked peak in function. So how much of an effect were you able to achieve with that?

Omer

Yeah. So we basically managed to produce elongated CD2s that improve the sensitivity of CARs targeting peptide MHC to the same level as the TCR. And so we think that that's been, you know giving us a lot of encouragement that you can have the same high sensitivity as a TCR with a completely synthetic receptor, provided you're able to match, you know the size of CD2 to give that synthetic receptor good adhesion function. We also managed in the paper to produce a CAR, a new CAR which we call a compact CAR. And what this compact CAR has is basically a shorter extracellular domain, and it's actually an extracellular domain that we engineered to perfectly match that of the TCR. And that compact CAR now has also the same sensitivity as a TCR when using wild type CD2. Again to a peptide.

Paul

OK, so you've got 2 methods for achieving the same ends, OK. And have people tried to shorten the CAR receptor before in order to achieve a similar thing?

Omer

Yeah. So it's been quite interesting. I mean, there's been some studies that have changed the extracellular hinge of the CARs. And the issue with those studies is that as a whole, they can be a little bit paradoxical because sometimes reducing the hinge improved sensitivity, sometimes it made it worse and conversely increasing the extracellular domain of the CAR sometimes improved it and sometimes reduced it and we think the reason for that is that it all depends on the relative matching to wild type CD2 CD58, so you could, like you said, there's this kind of Goldilocks and you could basically, you know, overshoot or undershoot potentially, in that way, and it's worth also adding that the second issue and this has been really a big issue with studying sensitivity is that it's very difficult to change the surface density of your target antigen when it's a folded, you know, when it's a structured protein on the cell surface and as a result, a lot of these claims on sensitivity have actually come from inference, where you sort of, you know, you actually have one antigen density and you're just looking for CARs with different hinges and whether or not they perform better or worse, but it's been very, very difficult to change the surface density of, for example, CD19 on the surface of B cells.

Paul

OK. So actually you need an assay for what would reflect the breakthrough cells. Something with kind of low levels of expression, where we're actually putting the thing to the test.

Omer

Yeah, absolutely. And that's one of the technologies that we developed as part of our study. We developed a system where we could basically have a cell – in this case, we use CHO cells that don't have most of the normal proteins that our target cells express – and on a surface we put a protein called Spy Catcher which was developed here in Oxford by Mark Howarth many years ago and by having this protein on the cell surface, what it allowed to do is take purified antigens like CD19 that are fused to a spy tag. And now we could add it to different concentrations and do a titration basically on the cell surface. And so within just a few minutes we get a panel of cells that have extremely low levels or different levels into the extremely low regime of the surface proteins and then we could add our T cells and assess their ability to recognize, you know, target cells that have, you know, ostensibly as low as one CD19 molecule on their cell surface.

Paul

That's fantastic and super impressed by the kind of range of technologies to break it down. And I'm sure the readers of the paper will get the full picture when they when they look at it all. I guess the few kind of other things struck me reading it. So one was about kinetic segregation, which of course is another Dunn School favourite and perhaps you could just describe the model. You know, you know much more about it than I do. And then it struck me that there might be some kind of things that relate to the model that have come out of your study.

Omer

Yeah, absolutely. So you know kinetic segregation is a model that was proposed by Anton van de Merwe and Simon Davis here in Oxford, and it's a model that tries to explain how extracellular antigen binding to the T cell receptor initially causes intracellular signalling. And this model now we believe applies to many other receptors including these chimeric antigen receptors. And the way this model works is that it proposes that when T cells recognize antigen, a close contact is initially formed, and it's largely, we think, mediated by CD2/CD58 interactions and this close contact now has about an intermembrane distance of about 14 nanometres. And at this close contact these very large phosphatases that we know are on the cell membrane are excluded. These molecules have very large extracellular domains that exceed 14 nanometres, and so by definition, they can't really be in this close contact. What that does is it generates a kind of kinase rich region within this

close contact such that if a T cell receptor for example goes into this region and binds ligand, it can stay in there sufficiently long to become phosphorylated and...

Paul

because you're excluding all the kinases from the region, all the phosphatases, sorry...

Omer

Excluding all the phosphatases. Yeah, exactly.

Paul

Of course. Sorry. Concentrating the kinases, yeah.

Omer

However, if a TCR doesn't like it, it doesn't bind any ligand, it just diffuses out and gets dephosphorylated and no triggering happens. And so you know, the way that we think about this model as applying to CARs is that, you know, if you now elongate this close contact, which we've done now by you know, increasing the size of CD2, eventually we believe that we would no longer be able to segregate CD45. And so in this paper, we actually collaborated with Simon Davis' lab and what they showed is that if you take our T cells that we've engineered to have elongated CD2, they actually do indeed have reduced phosphatase exclusion, if the elongation is sufficiently long. However, for more modest elongations, what we do find is that CD45 continues to be excluded. And you get improved CAR antigen interactions within these contacts.

Paul

So they're not so long that the... CD45 is very long, so I guess you'd only really get into trouble when it got to roughly the same length as CD45.

Omer

Yeah, we believe so. That's right.

Paul

So the segregation model lives on.

Omer

It does.

Paul

Yeah. In fact, you reinforced it. Everybody's happy.

Omer

Yeah, exactly.

Paul

Great. So I suppose from the clinical point of view, there's an enormous amount of evolution that can go on in these tumours. You already described some of it. So have you got a feel for how you might address that? Presumably a tumour could also modulate some of its expression of some of these co-stimulatory receptors.

Omer

Yeah, absolutely. There's already documented evidence that in a variety of tumours, CD58, the ligand for CD2, is actually down-regulated as well. And so I think the way that this should be approached of course is to try to deploy as much of your arsenal as you can, you know, from the get-go. And so for example, we think improving antigen sensitivity from the start will hopefully reduce relapse in these patients initially, but of course if they become antigen negative completely, or if they down-regulate CD58, we then have to kind of re-engineer our CARs now to target other antigens.

Paul

OK. Actually, that makes a lot of sense from a virological point of view, where if you target two or three regions of the virus, either by T cells or drugs, you've got a much better chance of cure or suppression than...

Omer

Yeah, absolutely. And people are now developing CARs that target from the get-go two different antigens on the on the leukemic B cells.

Paul

On the same CAR or as a pool of CARs?

Omer

They're doing both. There's some studies that are looking at 2 independent CARs, that target two different antigens, and some are taking one CAR and just adding multiple antibody fragments, you know, to its extracellular domain.

Paul

So there's plenty of work to do, and perhaps I could just ask you. Because you've spun out a company and it's on the preprint, which sounds very exciting. That's presumably complementing, expanding on, or how does it relate to this piece of work?

Omer

Yeah, exactly. So as a basic science lab, we've kind of shown the principle of how we think you can modulate antigen sensitivity for these CAR T-cells. And we've largely tested it with these toy antigens in a very controlled setting, but of course we now want to exploit that to improve these CAR T-cells basically to urgently treat these relapsed patients and hopefully treat and hopefully prevent relapse from happening in the first place. And we thought the best way to do that is to actually set up a spin out company and we were very fortunate that Phil Jakeman, who is the CEO of the company now at the time, was actually at Oxford Science Enterprises. And he had this fantastic job of talking to Oxford academics to kind of see what, you know, new research might be able to be translated. And I think he basically picked up that there could be some, you know, useful technology here and working with him we managed to make a business case, and secure some venture capital from Oxford Science Enterprises, to set up MatchBio, which is now focused on deploying this technology to a variety of different cancer indications.

Paul

Great. So are you going to work with other companies then that that generate the CAR-Ts, or are you going to be generating your own CAR-Ts, for therapy?

Omer

I think I at the moment, the kind of plan is a bit open and we're very fortunate that investors have allowed us to kind of keep options open. What we want to do now is again show proof of principles to a number of different antigen targets, and then we'll see which what are the most promising leads.

Paul

Then that sounds incredibly exciting to go from, essentially a kind of structural biology approach to something so translatable in one move through more or less one piece of work. Brilliant.

Omer

Yeah absolutely. I mean part of it, as you know, is that I think T cell immunologists these days are kind of at the right time at the right place. There's so much infrastructure already that makes translation of T cell work much easier.

Paul

Do you think, I mean, it seems a little bit from the outside that the CAR-Ts, you know, have got this really important role now in in haematological tumours, but they're not necessarily making such an impact on solid tumours. Are any of the features of your model relevant to that issue or are there other things entirely about the biology that we need to think about?

Omer

Yeah. I mean, it's a very good question. I think the jury is still a little bit out on exactly why they're failing in solid tumours. Lots of fantastic hypotheses which people are now exploring and I think, my guess is that there won't be kind of one solution to this. It might be a combination of different technologies that will have to come together to enable them to hit solid tumours, but certainly I think that low antigen density you know can be an issue and improving that I imagine would help in in rapidly killing...

Paul

Yeah, I mean, I guess if it's kind of an 'every T cell can do a bit of a better job' than you should be kind of pushing in the right direction.

Omer

Yeah, exactly.

Paul

Great. Well, that that's been very exciting. And perhaps the last question would be, apart from the company which you've described, where does it all go next? I mean, obviously you're waiting for reviewers' comments and so forth. But what would the plans be for the future?

Omer

Well, I think I mean we sort of stumbled on the activity of CD2 on these CAR T-cells. But as you mentioned, T cells express a whole host of these co-stimulation and co-inhibition receptors and we sort of think of them in this kind of binary classification and what our lab is really interested in trying to understand is, in some sense, go beyond this binary classification and sort of sub-classify these molecules according to whether they selectively improve sensitivity or efficacy. We also heavily study antigen discrimination. You know, this ability of T cells to differentiate between self and non-self antigen, and we want to see whether some of these molecules can modulate the activity of discrimination. And we also study what we call antigen adaptation. You know, this observation that when T cells see persistent antigen they stop responding, and we previously identified some co-

stimulation molecules that can allow T cells to overcome that. And again, we're developing assays to very quickly screen these molecules for these activities using this platform that I mentioned where we could couple proteins directly on the cell surface, that's allowing us effectively to generate antigen presenting cells with any combination and concentration of surface molecules within just a few minutes. And using that platform, we can now finally identify the quantitative phenotype of these of these co-stimulation and co-inhibition molecules.

Paul

Well, thank you very. I think if people hear that, they'll be knocking on your door. So hopefully you'll get some visitors soon from around the campus. Thank you very much, Omer, and good luck with the paper.

Omer

Thanks so much, Paul.