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# Targeting Tregs in Tumors

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THIS STORY BEGAN AS an idea inspired by the observations of others and ended up with a drug in the clinic through articles in *Nature*, *Cell*, and *Nature Immunology*, and a biotech startup along the way. If you cast your mind back to the 1990s, you will recall that suppressor T cells had gone out of favor due to an inability to isolate and characterize them, essentially at the molecular level. People did not even like to use the ''S'' word! A renaissance gave birth to ''regulatory T cells'' with the seminal discovery by Sakaguchi and others (1995), published in *Journal of Immunology* in 1995, that CD25 (the interleukin [IL]-2α receptor) marked a small population of CD4+ T cells with potent suppressive activity.

There was a lot of activity in the early days with people trying to understand what regulatory T cells (Tregs) were and how they functioned. There were 2 key articles from the groups of Sakaguchi and colleagues (Takahashi and others, 1998) and Thornton and Shevach (1998) in 1998 in which they performed some critical experiments using transwell plates. These are like regular 96-well plates but include a transwell insert where you can put cells in a top or bottom compartment that are separated by a semipermeable membrane. With small pores you could get diffusion of cytokines and various agents, but you did not get transfer of cells.

The key question immunologists were asking was whether Tregs needed contact to mediate suppression? If you put effector T cells and Tregs in a well, the effector cell proliferation is prevented by the suppressor cell. But when they put Tregs in the top compartment and effector cells in the bottom compartment, there was no longer suppression. This gave birth to the term ''contact-dependent suppression.'' The whole Treg field came to this paradigm thinking that Tregs had to have physical contact with effector cells to mediate suppression.

This term stuck for some time, but over the next 10 years or so, more analysis of Treg function gave rise to the realization that these cells can and do suppress through soluble factors, specifically, inhibitory cytokines. Probably the first article of note was in 1999 by Powrie and colleagues (Asseman and others, 1999) who found that Tregs make a lot of IL-10, which constitutes a key mechanism of suppression.

She had a whole series of terrific articles particularly focusing on the importance of IL-10 in colitis and other models, highlighting, among other things, the critical role of IL-10 in Treg function. Then, of course, there was a whole series of studies showing that transforming growth factor beta (TGFβ) is also an important mechanism of Treg suppression. The emergence of cytokines as important mediators of Treg function created a bit of a quandary, because if cytokines are important, then that would infer that contact was not so critical.

Then we came onto the scene, so to speak, with our discovery of the inhibitory cytokine IL-35 (Ye and others, 2021). We were looking for other soluble mediators of Treg suppression and discovered IL-35, a heterodimeric IL-12 family member, which was published in *Nature* in 2007 (Collison and others, 2007). This got us back to the issue of how can inhibitory cytokines (IL-10, IL-35, and TGFβ) be so important for Treg function if the latter require contact to mediate suppression? We mulled over this for some time as it seemed like a contradiction. Then we had an idea. What if it is not the suppression that is contact dependent, but rather the augmentation or boosting of Treg function that was contact dependent.

A fantastic postdoc in my laboratory, Lauren Collison, who was first author on the IL-35 *Nature* article (Collison and others, 2007), took up the challenge to address this conundrum. She performed a series of experiments, which led to a really critical article published in *The Journal of Immunology* in 2009 (Collison and others, 2009). If contact between effector cells and Tregs was required for boosting, then we should see more inhibitory cytokine production from Treg stimulated in the presence of effector T cells versus their absence. Indeed, we saw that these boosted Tregs were making a lot more IL-10 and IL-35 (Collison and others, 2009).

We then asked, what if we went back to the transwell experiments of 1998 and put effector cells in the top compartment with Tregs; would that be sufficient to boost their functionality and mediate transwell suppression? We first reproduced the previous observations of Shimon and Ethan that, indeed, we had no Treg suppression across a transwell with Tregs in the top compartment and effector cells in the bottom compartment.

However, when we added effector cells with Tregs in the top compartment, now we could see great suppression of the effector cells in the bottom compartment (Collison and others, 2009). How could that happen if the Tregs are not actually contacting them? This gave rise to the notion that these effector cells in the top well were boosting Treg

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functionality so they could mediate suppression at distance through suppressive cytokines. We went on to show that we could fix the boosting effector cells in the top compartments, so they were not actually producing anything and they worked just as well.

By way of control, fixed Tregs could not boost live Tregs, so there was something special on effector cells that mediated this boosting. Then, using a variety of knockout Tregs and blocking antibodies, we could show that transwell suppression was mediated, in fairly equal measure, by IL-10 and IL-35 (Collison and others, 2009). These were key observations for us as it suggested that a key component of contact-dependent suppression was really the boosting of Treg activity rather than physical contact with effector cells being needed to mediate suppression.

This was all well and good, but it did not really tell us how Tregs were being boosted. What was the receptor–ligand interaction that was mediating this boosting. This led to terrific work from 2 postdocs in the laboratory of Greg Delgoffe and Seng-Ryong Woo. They did a bit of a tag team, because Seng-Ryong started this project and Greg finished it, which led to an article in *Nature* in 2013 describing this ligand–receptor interaction (Delgoffe and others, 2013). To begin with, we knew that it was a cell surface protein because we could fix the conventional CD4+ cells we added to boost Tregs and we could still observe transwell suppression, so that it ruled out anything secreted.

We also determined that CD8+ T cells and dendritic cells (DCs) worked because we could fix them and put them in the top compartment of a transwell plate and they would boost. But obviously Tregs do not work because they are already in the well and thus could not self-boost. We also found that B cells really did not work very well. This set the landscape for us to do some mining of transcriptomic data sets, because we knew the ligand was on CD4+ and CD8+ T cells, and on DCs, but not on B cells or Tregs.

We looked for cell surface expressed molecules that fit that pattern. We thought we were going to end up with hundreds of genes to wade through but we were surprised to find it was only a handful—7 I seem to recall! Then we expressed these 7 genes to ask whether they could boost Tregs by putting transfectants in the top well of a transwell plate. Through these experiments, we discovered that Semaphorin 4a (Sema4a) was the only one that could boost Tregs (Delgoffe and others, 2013). We then did a bunch of experiments wherein we could either add in blocking antibodies to Sema4a or knockdown Sema4a in effector T cells and lost boosting activity.

We would also attach Sema4a-Ig to beads to show that this was sufficient to boost Tregs, collectively demonstrating that Sema4a was required and sufficient to boost Treg activity in a transwell, basically, to recapitulate the boosting of Treg activity we had observed previously.

This told us what was on the effector T cell/ligand side, but obviously did not tell us what the receptor was on Tregs. We thought that this was going to be quick, and that we would just knock this article out, but that was not the case. It took us quite a while to identify the receptor. Understandably, we started with the common family of receptors for semaphorins—the plexins. Semaphorins–plexin interaction is somewhat promiscuous, so we spent a lot of time going through what is a very large family using either blocking antibodies or knockout cells—trying to find a plexin that might mediate Treg boosting and were totally unsuccessful.

Then, almost serendipitously, we realized that in the early days, when people were looking for molecules that were preferentially expressed on Tregs, 1 molecule stood out, and that was neuropilin-1 (Nrp1) (Bruder and others, 2004). It seemed to be a really good marker of Tregs and at least within T cells, it seemed to be quite preferentially expressed by Tregs (Chuckran and others, 2020). Almost all regulatory T cells in mice express Nrp1, whereas expression on other T cell subsets is relatively minimal.

Nobody really knew what Nrp1 did on Tregs, so we dug into the literature. It was first identified as an important molecule for nerve development and neovasculogenesis. In fact, the Nrp1 knockout is embryonic lethal, so it is important for those early embryonic events. But its role in mediating any kind of immune function was obscure (Chuckran and others, 2020). There was 1 interesting article in *Immunity* that suggested that Nrp1 could mediate interactions between Tregs and DCs (Sarris and others, 2008).

What was known about Nrp1 was that it had 2 ligands of interest: Vegf, which you can imagine is important in neovasculogenesis, and also members of the semaphorin family. We thought, maybe this is the receptor we were looking for! So we used blocking antibodies to the semaphorin or Vegf binding parts of Nrp1 and showed that only the former could block Treg boosting in our transwell assays (Delgoffe and others, 2013). We then obtained the Nrp1 floxed mouse and crossed it to Foxp3-Cre so we could restrict Nrp1 deletion to Tregs.

As you can imagine, this all took ages. When you read the article, it seems like it all happens simultaneously, but it did not. There was about a 9-month pause in the project while we generated these mice. Once we had Nrp1-deficient Tregs, we could show that those could no longer be boosted by CD4+ T cells or DCs, really demonstrating for the first time that Nrp1 was the receptor on Tregs that boosted their suppressive capacity, and survival as we later found out (Delgoffe and others, 2013).

Now, of course, we could do all the fun things *in vivo*. We could ask the question, what is the role of Nrp1 on Tregs? Without any kind of disease induction, these mice were totally fine. They did not suffer from any Treg insufficiency (Delgoffe and others, 2013). When you have ablation of Tregs by either antibody-mediated depletion or deletion of FoxP3, which is a key transcription factor required for their development, mice get really sick with a profound systemic autoimmune condition and they do not survive much beyond a month or so.

These studies provided an early demonstration that Tregs are essential for maintaining immune homeostasis (Gavin and Rudensky, 2003; Takahashi and Sakaguchi, 2003). We were initially disappointed because we thought that if it is really important, these Tregs should be dysfunctional, and the mice would get sick. But they were not getting sick at all, even when they were 1 year old, so we thought that maybe it is just not important.

However, part of our initial incentive for looking at things that might boost Tregs is the growing appreciation that Tregs are an important inhibitory barrier for antitumor immunity. Several people, especially early work from Turk, had shown that if you deplete Tregs in tumors, you can get an almost complete collapse of tumor growth (Turk and others, 2004; Bos and others, 2013; Delgoffe and others, 2013). So we asked, do

tumors grow in our mice that have a Treg-restricted deletion of Nrp1, and that is when we saw the most amazing phenotype.

These mice were incredibly resistant to a whole series of different tumor types (Delgoffe and others, 2013). Yet they did not get any autoimmune or inflammatory conditions, not only before being given tumors, but even once they had cleared the tumors. Thus there was a uniquely selective requirement for Nrp1 on Tregs in this inflammatory tumor microenvironment, suggesting that Treg control of normal immune homeostasis in the periphery did not require boosting. In other words, this kind of tonic functionality of Tregs was more than sufficient to maintain that immune homeostasis, and it was only when you put Tregs in the challenging tumor microenvironment that they needed this extra help through Nrp1.

A whole series of biochemical analyses showed that Nrp1 binds to phosphatase and tensin homolog, which is a phosphatase that regulates AKT activity, and that AKT activity is critical for regulating the nuclear translocation of a transcription factor called FoxO, which had been shown to be very important for regulating and promoting FoxP3 activity (Delgoffe and others, 2013). Not so much whether FoxP3 is active, but the type of activity that it has. We also found that when you take Tregs that lack Nrp1 out of tumors, they do not lose FoxP3 expression or even much of their transcriptional identity.

In other words, they do not seem to become unstable. When people talk about unstable Tregs, they are thinking about FoxP3 loss and, therefore, loss of Treg function and identity. However, Nrp1-deficient Tregs seemed to maintain normal FoxP3 expression, but they could no longer suppress (Delgoffe and others, 2013). They had a substantial reduction in their regulatory program, downregulating IL-10, IL-35, and CD73. They lose quiescence and survival factors, such as Klf2 and Bcl2, which are important for maintaining their regulatory identity and survival, and instead, start upregulating molecules that promote T effector programs such as RORγt, IRF4, T-bet, and interestingly IFNγ.

Although these Nrp1-deficient Tregs start to look more like conventional effector T cells, they do not lose their whole T reg identity, because they still maintain FoxP3, so in some ways, this was sort of an ideal physiological situation as there has always been the concern that if Tregs lose FoxP3, they might become autoreactive because of the fact that they recognize self better. Tregs that lose Nrp1 lose their suppressive capacity but do not become dangerous to the host, which is probably why they do not cause the severe autoimmune and inflammatory phenotype typically seen if you deplete Tregs.

This led us to coin the term 'Treg fragility' to refer to this state where Tregs maintain FoxP3 but lose their suppressive activity. The next obvious question was what drives this fragile phenotype. This took a while to work out, but we were kind of intrigued by the fact that when we looked at our transcriptomic data from the *Nature* article, the one thing that seemed to be really prominent was IFNγ.

So a graduate student in my laboratory, Abby Overacre-Delgoffe, did a whole series of experiments and found that IFNγ was driving this fragile phenotype, detailed in an article published in *Cell* (Overacre-Delgoffe and others, 2017). We could go 1 step further to show that if we made a mouse model where Tregs lacked the IFNγ receptor, they could no longer be made fragile. Not only could they no longer be made fragile, but also mice that would normally respond well to anti-PD1 immunotherapy and clear tumors now could no longer respond to anti-PD1.

This gave rise to the idea that in mice, or maybe even patients given immunotherapy, one of the things that may have to happen is that Tregs lose some of their activity to allow effector cells to get hold of a productive antitumor response and clear the tumor (Overacre-Delgoffe and Vignali, 2018). This gave rise to the notion that IFNγ-driven fragility was a potentially important component of effective antitumor immunity.

These stories were very much focused on Nrp1 in Tregs, but I would be remiss if I gave the sense that that is the only place that Nrp1 is expressed. It is expressed quite extensively on myeloid cells, and macrophages in particular, and I think that very little has been done in terms of understanding its role there (Chuckran and others, 2020). The other tangential thing we did was to look at the role of Nrp1 in CD8+ T cells, which we published last year in *Nature Immunology* (Liu and others, 2020). That article showed that Nrp1 on CD8+ T cells functioned like a checkpoint in a way similar to PD1 and TIM3 but seemed to be much more important for memory T cell development than effector function.

Finally, from the *Nature* article, we got really interested in the possibility that Nrp1 could be an important therapeutic target, especially if we could recapitulate the tumor phenotype we observed in mice that had a Treg-restricted Nrp1 deficiency. In other words, could we substantially reduce suppressor activity of Tregs selectively in tumors without impacting their control of immune homeostasis. We had some hint that this might be possible because as part of the *Nature* study, we also did some Nrp1 blocking antibody experiments, either alone or in combination with anti-PD1, and could show that Nrp1 antibodies gave rise to reduced tumor growth, and they could combine with anti-PD1 to reduce tumor growth to an extent that is greater than either of the monotherapies.

Also, interestingly, we showed in Abby's *Cell* article that although most healthy human subjects do not express Nrp1 on their Tregs, a high percentage of intratumoral Nrp1+ Tregs can be found in patients with melanoma and head and neck cancer, and that this seems to correlate with poor prognosis (Overacre-Delgoffe and others, 2017). With all of this in mind, I was one of several who cofounded a company called Potenza Therapeutics, which developed a therapeutic antibody to Nrp1. That company was bought by Astellas Pharma, which has taken this antibody into the clinic. It is in Phase I studies now so we will see what happens!

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