

Single cell immunology in cardiovascular medicine – do we know yet what we don't know?

Susanne Sattler, MSc, PhD

Affiliation:

¹ National Heart and Lung Institute, Imperial College London, London, W12 0NN, UK

Please address correspondence to:

Susanne Sattler

Imperial College London, National Heart and Lung Institute

Hammersmith Campus, ICTEM Building (room 424W2)

W12 0NN London

Tel: +44 (0)20 759 42737

Email: s.sattler@imperial.ac.uk

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As immunologist with my scientific home in cardiovascular research, I dwell in two worlds. I witness exasperation over an ever-increasing fuzziness of what defines individual immune cell types, contrasted by the excitement over the realisation that not all cardiomyocytes or endothelial cells or fibroblasts are in fact the same. Both developments have seen an unprecedented acceleration due to the recent success of single cell sequencing technology. Just a few years back, multi-channel flow cytometry got me all giddy at the sheer amount of information obtainable from just one small sample. The opportunities provided by 15 parameters per cell seemed endless. Now imagine how I feel about single cell sequencing.

Single cell sequencing technology is having a fundamental impact on cell biology and as such affects all medical research, including cardiovascular medicine. No cell type remains untouched. Fibroblasts are not just glue anymore, endothelial cells are as diverse as tissues themselves, and cardiomyocytes even differ depending on their location within the heart. Researchers in these fields are excited about the newfound heterogeneity within their favourite cell type, much as immunologists were at the advent of flow cytometry. Immunologists however are facing somewhat of an existential crisis. While other fields start to break up their cell types into distinct sub-populations, immunologists start to realise we may have already gone too far.

Humans brains learn and function by assigning categories, a lion is not a gazelle, pokeberries are not grapes. This is how we survive, learn and make sense of our environment. We do the same with scientific data, otherwise we can't grasp the meaning easily. Immunology lives off categories; a monocyte is not a macrophage, an NK cell is not a T cell. At least that's what we think, but now single cell sequencing is putting into question many of our convenient categorizations.

My team made a striking observation when analysing single cell data recently; T cells expressing the prototype B cell marker *Cd20*. After much deliberation to make sure this was not an artefact, it turned out others had previously found them too. Imagine the relief when we learned they were just wonky T cells. Order was restored because we could clearly put them back in the T cell box to analyse them together with all other T cells. However, if we keep relying this much on the expression of individual marker molecules, are we not clearly at risk of missing important (functional) information? Unfortunately, a specific function can be just as unreliable for identification of an individual cell. T helper cells for example have

long been assigned distinct identities depending on their function, i.e. the cytokines they produce. Being completely honest with ourselves, we would have noticed that - even when analysed by flow cytometry - they were not always mutually exclusive. A recent single cell study clearly shows that they are indeed a 'polarized continuum' based on their dynamic cytokine production profiles rather than distinct populations¹. At least in the gut where admittedly a lot of rather peculiar immunology happens. But I dare say, we'll see similar things in the heart² and I assure you, there are lots of people around the world digging into their datasets right now.

While T cells are still a little niche in the cardiovascular field, there is another very prominent example of how attempts to simplify our lives by rigid classification can lead us astray; M1 versus M2 macrophages. I lost count of the times I crushed people's enthusiasm with 'I'm sorry, but I will not be able to tell you if that macrophage is M1 or M2.' Unless of course they'd like to redo an *in vitro* experiment on an artificially differentiated cell type treated with a defined set of stimuli, in complete isolation, on plastic. Such experiments are essential starting points, no doubt, but immune cells do not live in isolation and the ability to respond to a plethora of ever-changing environmental cues defines their very existence. Single cell sequencing has time and again proven that there is no M1-M2 dichotomy in real-life macrophages³. Instead, they adapt their behaviour to circumstances. They do what needs to be done, there and then. Analysis at a single time point will just see one snapshot in time. Just like when we pull a strange face the moment a picture is taken. Luckily, we don't always look like that!

Like so many other mid-career immunologists today, I was taught about THE macrophage, THE B cell. T cells were cytotoxic or produced cytokines, which came in two flavours. Immunology wasn't that hard. Now, immunologists being unable to provide clear answers has turned into a running joke. One thing is clear though; we can't any longer refer to outdated concepts of distinct identities and clearly separated dichotomies, even if accompanied by a frown or wink indicating that we know very well they are wrong but have no better alternative to offer. But how then to explain to the next hopeful that the beautiful idea of M1 versus M2 macrophages is... forgive me; wrong? Function is certainly better, but we must not end up with just another kind of rigid dichotomy. Inflammatory versus regenerative sound familiar? We will need to find a way to get our ancient brains around the fact that the immune system is in constant flow with barely one cell alike the other at any one time.

The critical relevance of all this becomes evident once we move beyond basic research in the hope of harnessing the immune system for therapy. When the cardiovascular community started to appreciate that inflammation was underlying atherosclerosis, or may even decide between recovery and heart failure, excitement over a new treatment avenue took over. Immunomodulatory therapies were pushed through to cardiovascular clinical trials, but outcomes were largely disappointing. Most likely because 'Correlation is not causation.' is rarely as true as when dealing with immunological factors. Increased neutrophil numbers in the blood correlating with disease severity, do simply not tell us that neutrophils are all bad. Besides dynamic sub-populations and their equally dynamic functions, immunology is full of feedback loops, positive and negative. Right now, your friendly (and honest) next door immunologist will tell you that we most likely don't even know yet what we don't know. They

may be brave enough to mention that it is potentially a rather bad idea to suppress some immunological factor simply because it was higher in your patients compared to controls. The key to successful immunotherapy in cardiovascular disease is a much more refined understanding of the spatial and temporal dynamics of the immune response, with a focus on functional profiles rather than a broad name assigned based on selected markers.

My team is only one of many research groups trying to harness the powers of single cell sequencing to better understand immunopathology in the heart. Two exciting recent studies provided a single cell dataset on healthy⁴ and heart failure tissue in human patients⁵. While some may feel a little tired of yet another single cell publication, I eagerly await more of them, as they may be exactly what we need to get to the required temporal and spatial resolution for successful immunotherapy in cardiovascular medicine.

Conflict of Interest Disclosures: None

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