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Using single cell analysis for translational studies in immune mediated diseases: Opportunities and challenges

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ABSTRACT

The difficulty of studying small tissue samples and rare cell populations have been some of the main limitations in performing efficient translational studies of immune mediated diseases. Many of these conditions are grouped under the name of a single disease whilst there are strong suggestions that disease heterogeneity leads to variable disease progression as well as therapeutic responses. The recent development of single cell techniques, such as single cell RNA sequencing, gene expression profiling, or multiparametric cytometry, is likely to be a turning point. Single cell approaches provide researchers the opportunity to finally dissect disease pathology at a level that will allow mechanistic classifications and precision therapeutic strategies. In this review, we will give an overview of the current and developing repertoire of single cell techniques, the benefits and limitations of each, and provide an example of how single cell techniques can be utilized to understand complex immune mediated diseases and their translation from mouse to human.

1. Introduction

One of the major hurdles in studying the immune status of human diseases is the access to informative samples. Only two routes are available, biopsies for solid organs, and/or blood draw, now also called "liquid biopsy". However, both modes of sampling have inherent limitations: is the biopsy from an affected area? Is the biopsy representative of the entire organ? Will there be affected and unaffected tissue in the same sample? What control should be used? How many circulating immune cells are coming from the diseased organ? How often can the tissue and/or blood be sampled without affecting the patient?

Additionally, a consistent challenge is the low number of immune cells recovered from each sample. Up until now, most of the available and established techniques in immunology relied on bulk, population analysis that required a large number of cells defined by a limited set of markers. In very practical terms, biopsies are usually examined by immunohistochemistry, whereas peripheral blood mononuclear cells (PBMCs) are enumerated and phenotyped by flow cytometry. While immunohistochemistry investigates anatomical features, its resolution is low. Flow cytometry provides single cell resolution but is limited by the small set of phenotypic markers that can be used; this approach hinders the analysis of low frequency populations, and is ultimately only as good as the quality of the reagents used for staining (Chattopadhyay et al., 2014). In addition, these "bulk techniques" average out the signal over multiple cells, potentially obscuring rare disease-specific cells (Chattopadhyay et al., 2014). While bulk genomic techniques face the same issues, they are additionally limited in their interrogation of lymphocyte specificity as defined by T cell and B cell receptors, both of which rely on the co-expression of two chains, heavy and light for B cells, α and β for T cells, as it loses the information that pairing provides.

Antigen specificity of T and B cells is one of the most informative aspects of studying the immune system in cancer and autoimmunity as it directly links a cell to its function. Most, if not all, functionally informative gene expression observed in activated lymphocytes will be downstream of idiotypic receptor engagement. To add further complexity, heterogeneity has been observed in the gene and protein expression of cells within these populations. For resting cells, the steady state analysis demonstrates variability in single cell RNA expression that reflects stochastic gene expression, or "allele intrinsic" variability, as well as "allele-extrinsic" variability (Raj et al., 2006; Wagner et al., 2016). This variability is often significant because beyond differentiating two cells of the same type and same specificity within the

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same tissue, it may influence their functions in response to a pathogen (Haque et al., 2017). Finally, it has been shown that in humans, each patient with an autoimmune disease can exhibit progression of disease and clinical features that are unique to that individual (Coppieters et al., 2012; Roep et al., 2012; van der Helm-van Mil et al., 2005).

In this context, single cell analysis permits the interrogation of samples of small size (biopsies) and the dissection of complex mixtures of cells found in blood and tissues. The first high throughput single cell technique to be developed was flow cytometry and while it provides single cell resolution, it is limited by the small number of parameters that can be simultaneously measured. The development of flexible and cheap microfluidic systems a decade ago was a breakthrough for the single cell field. Microfluidics provided access to a single cell's transcriptome in a high throughput format and allowed the field to expand on the pioneering work of *Eberwine et al.* in 1992. In that particular study, the authors demonstrated that morphologically similar cells have distinct patterns of gene expression and that some cells had expression of several mRNAs that were not found at the population level (Eberwine et al., 1992; Grun and van Oudenaarden, 2015; Svensson et al., 2018).

Nearly two decades later, and via intermediate steps such as single cell qPCR, the first single cell RNA sequencing paper and protocol were published by Tang et al. in (2009,2010) (Tang et al., 2010, 2009). Within three years, Nature Methods declared single cell RNA sequencing (scRNAseq) the method of the year (Editorial, 2014). As proof of how far single cell RNA sequencing has come, in 2017, single cell RNA sequencing is being discussed as a tool to bridge personalized medicine with cancer diagnostics (Shalek and Benson, 2017).

However, single cell techniques still have important technical issues that require resolving including the validation of gene expression analysis at the protein level and the incorporation of spatial distribution of heterogeneous cell populations in tissues and lesions in single cell analysis.

In this review, we will briefly discuss in a non-exhaustive way the main available techniques in the single cell field, highlight the strength and weaknesses of some techniques, and discuss an approach we have taken that combines various single cell techniques to examine very small populations of cells in human biopsies and peripheral blood.

2. Important single cell technologies

While most single cell experiments isolate individual cells via flow cytometry, a single cell proteomic technique, the term "single cell analysis" most often refers to quantification of RNA and sequencing of DNA. To utilize the limited quantities of material extractable from individual cells, most single cell transcriptomic and genomic techniques rely on the ability of the polymerase chain reaction (PCR) to amplify a single, or a few molecules of DNA. Therefore, all approaches will be necessarily limited by the quality of primer pairs, the variable efficiencies of some primer pairs in multiplex reactions, the necessity to perform a reverse transcription (RT) step to examine RNA expression, and the fidelity of the RT and DNA polymerase enzymes. The addition of linkers for sequencing, bar codes for identification, and molecular identifiers for normalization can compound these technical limitations.

Currently, single cell genomics techniques start by the isolation of single cells in reaction chambers, or reaction droplet, using microfluidics instruments, or in open wells in multi-well plates using fluorescence-activated cell sorting (FACS). While the latter is reasonably efficient (> 95%), the former approach is still limited in its ability to isolate single cells and not doublets; success rates vary from 60 to 90%, depending on the instrument (Holt et al., 2018).

2.1. Single-cell analysis beyond single cell RNA sequencing

The most common single cell genomics technique is single cell RNA sequencing (scRNAseq) that examines and quantifies the transcriptional landscape of a single cell (Linnarsson and Teichmann, 2016; Tang et al., 2009). Other single cell technologies examine the genome, the epigenome, the transcriptome or precisely quantify the expression of a particular set of genes. We will briefly discuss some of these techniques and direct the reader to reviews that cover each topic in more detail.

Single cell DNA sequencing studies genomic DNA and holds the potential of tracking somatic mutations, substitutions, insertions/deletions, copy number variants, and structural rearrangements (Grun and van Oudenaarden, 2015). One particularly promising use for single cell DNA genomics is to be able to sequence the entire genome of individual cancer cells to ascertain the copy number variants and/or the single nucleotide variants, gene translocation, and the rate of mutation in driver and non-driver genes within each tumor cell (Gawad et al., 2016). The determination of this tumor landscape and the intratumoral diversity, has the potential to tailor treatments that best target each group of cancerous cells (Gawad et al., 2016; Shalek and Benson, 2017). Additionally, this same approach can be used to diagnose cancer via the detection of circulating tumor cells (Gawad et al., 2016). However, for "liquid biopsy" diagnostic technique to be fully successful, the issue of whole genome amplification fidelity must be overcome. Currently, challenges of whole gene amplification include the loss of genomic coverage that can restrict de novo assembly of a cell genome, allelic dropout or imbalance, and errors during genomic amplification (Gawad et al., 2016).

Single cell epigenomics which examines epigenetic changes, has the theoretical capacity to examine DNA methylation, histone modifications, as well as changes in conformation and compaction of chromatin. As many of these techniques rely on antibodies, their sensitivity is highly linked to the quality of the antibodies used (Cheung et al., 2018). As a consequence, the number of single cell epigenetic studies are still limited but increasing. The first single cell epigenomic technique published examined DNA methylation in single mouse embryonic stem cells (mESCs), mouse sperm and mouse oocytes via reduced representation bisulfite sequencing (Guo et al., 2013). This technique queried DNA methylation at different sites, such as CpG islands, without averaging and compared the results to pooled mESCs of different cell numbers (Guo et al., 2013). A recently described technique, called epigenetic landscape profiling using cytometry by time of flight (EpiTOF), measures 8 classes of histone marks and 4 histone variants in immune cell subsets (Cheung et al., 2018) to distinguish the main cell types and lineages by assigning patterns of histone marks (Cheung et al., 2018). As a result of the technical limitations mentioned above, the major challenge of this technology is to improve the quality of the antibodies needed and to increase the low number of reads obtained by these methods that are otherwise robust techniques (Hyun et al., 2015).

Another recently published study paired assay for transposase-accessible chromatin with sequencing (ATAC-seq) and TCR sequencing at the single cell level. This study identified epigenomic signatures that were unique to clonal cancerous T cells (Satpathy et al., 2018). Applied in conjunction with single cell RNA analysis, single cell epigenomics has the potential to directly correlate epigenetic modifications to changes in gene expression (Wagner et al., 2016). Within the past few years, protocols have been developed to do exactly this including a protocol called single cell genome-wide methylome and transcription sequencing (scM&T-seq) which interrogates DNA methylation and transcriptome analysis (Angermueller et al., 2016). Additionally, another protocol has been developed by the same group that integrates chromatin accessibility with DNA methylation and transcriptome analysis in a protocol called single cell nucleosome, methylation and transcription sequencing (scNMT-seq) (Clark et al., 2018). However, it should also be noted that single cell epigenomic techniques are still primarily performed by specific labs and are very challenging. As a testimony to this assertion, no manufacturer has yet released a kit and/or protocols to perform any of these experiments.

From a technical standpoint, ***single cell transcriptomics*** are by far the most developed single cell techniques to qualitatively and quantitatively measure gene expression, and they can be carried out by most

laboratories. There are two main types of transcriptomics, the untargeted sequencing of cDNA derived from reverse transcribed RNA, and the targeted evaluation of gene expression by quantitative or digital PCR after reverse transcription. Both approaches have similar upfront drawbacks which include incomplete cell lysis, loss of RNA or inefficient reverse transcription, cell doublets, over amplification, and pre-amplification bias (Kolodziejczyk and Lonnberg, 2017; Wagner et al., 2016).

A more important issue is the loss of the spatial information for each cell following mechanical and/or enzymatic dissociation of tissues, a difficulty that can only be circumvented by laser microdissection-capture of single cells in tissue sections (Gawad et al., 2016; Nagendran et al., 2018). However, a new protocol called spatially-resolved transcript amplicon readout mapping (STARmap) has been developed to address the loss of spatial information in single cell transcriptomic analysis (Wang et al., 2018). In this method, tissue is embedded into a hydrogel matrix and cellular RNA is probed with a gene specific primer and barcoded padlock probe. Only after both bind to the RNA will there be amplification, followed by 6 cycles of sequencing to detect each padlock's 5 nucleotide code (Wang et al., 2018).

However, the main limitation of all transcriptomics methods is that, as previously mentioned, they are based on PCR, and therefore inherently plagued by gene dropouts, amplification bias, and a given mutation rate that varies between polymerases. For instance, mutation rates for reverse transcriptases (RT) vary from 10^{-4} to 3×10^{-5} nucleotides polymerized, depending on the sequence and structure of the template, while thermostable DNA polymerases (DPol) range from 1 to 10×10^{-6} mutations per nucleotide added (Menendez-Arias, 2009). These same enzymes also have variable dynamic ranges. While all RTs have the potential to reverse transcribe a single copy of RNA and all DPol amplify a single copy of cDNA, buffer conditions, length of transcripts, complexity of the molecular mixtures, and biophysical parameters will all affect the outcome; the detection of single copy RNAs remains theoretical. Dynamic range issues will primarily affect quantitative PCR experiments, especially in a highly multiplexed assay. PCR-associated mutations will complicate the interpretation of sequencing data in regions of the genome bearing polymorphisms or complex recombination features such as B and T cell receptor sequences.

These experimental bottlenecks can influence the "depth" and limit the "coverage" of single cell whole transcriptome sequencing with percentages that rarely exceed 15–50% of the whole theoretical transcriptome (Haque et al., 2017; Marinov et al., 2014). If these numbers are usually sufficient for the reconstitution of the main metabolic and signaling cellular pathways, they might not be informative to dissect discrete circuits that support essential functions such as migration, cell adhesion, cytokine production, or DNA recombination in B and T cells.

The best way to circumvent these limitations is to complement scRNAseq with ***targeted single cell transcriptomics***. Directed transcriptomics utilizes single cell quantitative or digital PCR to study a panel of genes of interest. The benefits of these single cell quantitative PCR approaches are that they have the same sensitivity, specificity, reproducibility and range of detection as bulk PCR (Kolodziejczyk and Lonnberg, 2017), and thus, are very robust. Of course, they face the same technical challenges as bulk PCR, specifically PCR bias in the context of high multiplexing.

One other advantage of targeted gene expression profiling of single cells is that genes involved in one activation pathway can be measured conjointly and therefore can provide a means to validate scRNAseq experiments. A number of competing platforms are capable of achieving very similar results and have been outlined in Table 1.

2.2. The difficulties and limitations of the statistical treatment of scRNA transcriptomics experiments

For most laboratories, the main challenge of single cell techniques will be data analysis. The datasets are large, information-rich, and can

Table 1
A non-exhaustive overview of some targeted and full transcriptomic profiling techniques compiled from various sources. Each technique has strengths and weaknesses and should be selected according to the experimental questions and considerations. The Illumina-BioRad system is new and has not been exhaustively tested as other platforms have been in Ziegenhain et al. For a more rigorous examination of each listed platform, and for those not included, we refer the reader to the following papers: (Geiss et al., 2008; Kolodziejczyk et al., 2015; Valihrach et al., 2018; Ziegenhain et al., 2017). *Sensitivity is the detection of spike in controls at 1 million reads as described in Svensson et al (Svensson et al., 2017). N/A – not applicable.

Targeted or Full Transcriptome	Company	Instrument	Cell Isolation Method (from tissue source)	PCR Amplification required	Full transcript or 3'	Sensitivity* at 1 million reads	Number of genes detected per cell	Number of cells per run	Reference
Targeted	Nanostring	nCounter	RNA from sorted population or tissue RNA	No	N/A	N/A	800 genes	100 ng RNA (~10,000 cells)	Geiss et al.
Targeted	Fluidigm	Biomark	FACS sorted	Yes	N/A	N/A	96 genes	96	
Full	Fluidigm	C1 (SMART-seq 2)	Microfluidic or bulk sort followed by microfluidic single cell isolation	Yes	Full Transcript	45 molecules	9,138 genes	96	Ziegehain et al., Kolodziejczyk et al
Full	10X Genomics	Chromium	Microfluidic or bulk sort followed by microfluidic single cell isolation	Yes	3'	8 molecules	4,811 genes	4,000	Ziegehain et al., Kolodziejczyk et al
Full	Illumina/Bio-Rad	ddSeq Single Cell Isolator	Microfluidic or bulk sort followed by microfluidic single cell isolation	Yes	3'	Not assessed	Not independently verified	1,200	

be analyzed by a variety of methods that all utilize sophisticated statistical tools. However, as is the case for single cell transcriptomics, currently there is no established gold standard analysis method. Each manufacturer offers a skeleton set of tools that are rarely sufficient to produce a comprehensive analysis and/or figures. Each analysis should be tailored to a particular experiment and/or amplification protocol to take into account the amplification fold and any biases introduced during the cell isolation, library preparation and sequencing (Kolodziejczyk et al., 2015; Vallejos et al., 2017). To try to optimize reproducibility and improve the comparison of different experiments, many protocols utilize spike in controls or unique molecular identifiers (UMI). However, the detection of UMIs and spike-in controls might also vary between experiments and their utility varies depending on the sequencing technique utilized (Kolodziejczyk et al., 2015; Wagner et al., 2016).

In the absence of a gold standard protocol, there are universal steps that need to be incorporated in a data analysis pipeline (Grun and van Oudenaarden, 2015; Haque et al., 2017). The first step is to check the quality of the raw reads via programs like “fastqc” and to remove low quality data as well as trim UMIs if they have been incorporated (Stegle et al., 2015). Next, the reads should be mapped to a reference genome before expression of individual genes can be determined for each cell. In this process, PCR duplicates and cells with low yields should be removed from the analysis. Cells with low yields are defined as having a low total transcript level, a low amplification efficiency as measured by spike in RNAs, or a high spike-in to transcript ratio (Grun and van Oudenaarden, 2015). Cells with low yields may result from poor cell lysis, cells that were apoptotic before lysis, RNA loss during handling, degradation or poor sequencing efficiency. Other quality metrics may include the expression of housekeeping genes, the number of detected and aligned genes, and the ratio of aligned genes to aligned mitochondrial encoded genes (Haque et al., 2017; Kolodziejczyk et al., 2015).

Normalization is the final step in data analysis; it will remove residual technical variations and is necessary to reveal significant biological variations. Multiple options are available for the normalization procedure. For the quantification of transcripts sequenced by single end sequencing, using the Transcripts Per One Million (TPM) metric is sufficient (Grun and van Oudenaarden, 2015). However, for transcripts that were dual end sequenced, the transcripts per one million reads per kilobase of transcript (RPKM) is the preferred method of choice (Grun and van Oudenaarden, 2015). Other quantification tools developed for bulk RNA sequencing such as Cufflinks, can be used (Tang et al., 2010, 2011). An alternative normalization tool, that is effective at removing batch effects, is down sampling, an operation that will take an identical number of transcripts from each single cell library to evaluate gene expression differences. However, this approach usually reduces the complexity of the gene expression profile comparison dramatically.

While some companies have started providing computational tools that integrate the above steps for analysis of transcriptomic data, these programs do not allow for access to and modification of the code for tailoring the analysis to your experiment. There are powerful open source programs that can be utilized but these usually require the help of an experienced bioinformatician (Haque et al., 2017).

2.3. Validation and proteomics

A rich complement to single cell RNAseq experiments is to try to position particular cells with unique gene expression profiles within the spatial context of the tissues from which they were isolated. Techniques such as single molecule in situ hybridization (smISH) and its multiplexed variant called proximity ligation in situ hybridization (PLISH), allow this translation (Nagendran et al., 2018).

In addition, beyond purely technical hurdles, the correlation between RNA levels with protein expression, while satisfactory for a large number of genes, is still highly variable within gene sets and between cell types (Vogel and Marcotte, 2012). Therefore, it is required for most gene expression profiles provided by single cell RNAseq, to follow-up with protein expression validation experiments. It should never be assumed that transcription and translation are linearly correlated; the number of outliers is large, and striking examples have been reported in immunology such as for cytokine expression; a notable example is the expression of IL-4 and IFNγ in NK T cells (Stetson et al., 2003).

Currently, there are two complementary approaches to single cell proteomics: global and targeted. The targeted approach is usually based on the detection of proteins by antibody binding; it has been extensively used and carried out by flow cytometry and mass cytometry (CyTOF) for detection. Both systems are limited by the number of proteins that can be interrogated within one experiment. While flow cytometry is usually efficient to examining 12–15 markers using fluorochrome-labeled antibodies, up to 50 markers can be examined in a mass cytometry experiment in which antibodies are labeled with metals and their natural isotopes (Bodenmiller et al., 2012; Chattopadhyay et al., 2014). In flow cytometry the need to compensate for spectral overlap limits the number of fluorophores, and therefore the number of markers that can be used for analyzing single cells (Hu et al., 2016). New spectral flow cytometers have a theoretical range of up to 25 colors (Chattopadhyay et al., 2014). Mass cytometry suffers from being a terminal analysis method as the cells are atomized and ionized before being interrogated in the time of flight chamber preventing further analysis via cell sorting as opposed to classical flow cytometry (Spitzer and Nolan, 2016). Both approaches also lose the spatial information that might be essential for the biology or pathology examined, as we have already mentioned for DNA and RNA studies. This information can only be recovered from tissues by techniques such as immunostaining or immunofluorescence which can only examine a very small set of markers at a time. Automated methods of fluorescent antibody binding-photobleaching-reprobing on tissues (Chipcytometry) and in situ mass cytometry are currently being developed to overcome this important limitation (Dijkstra et al., 2012; Schulz et al., 2018).

Another emerging targeted proteomic technique is the single cell Western blot (Hughes et al., 2014). *Hughes et al.* created a 30-μm deep polyacrylamide gel layered on top of a glass slide. The authors placed 420 microwells in a single block and patterned 16 blocks onto one slide. A single cell was placed in each well and lysed to allow separation of the proteins in an electric field like in a regular SDS-PAGE. Following that step, the proteins were coupled to the polyacrylamide gel and then probed with antibodies. In this original paper, the authors were able to probe 11 proteins successively using serial stripping and re-probing. Further developments could use multiplexing to increase the number of proteins to be tested (Hughes et al., 2014).

However, the temporal change in protein expression is still missing from the above techniques. This dimension might be accessible using a recently developed approach called fluidic force microscopy (FluidFM) which samples individual cell content by using a hollow cantilever probe pushed through the membrane by atomic force (Guillaume-Gentil et al., 2016). FluidFM can recover picoliter volumes of cytoplasm that can then be used for transcriptomic or proteomic analysis (Guillaume-Gentil et al., 2016; Zhang and Vertes, 2018). The advantage of this technique is that the spatial and temporal parameters of the single cells are known.

In any case, mass spectrometry (MS) remains the dominant method for unbiased identification and quantification of proteins. In general, there are two main approaches to single cell MS. The first approach dehydrates cells and then interrogates the cell by laser or ion beam. The most obvious limitation of this approach is that the natural state of the cell is perturbed and the temporal measurement of protein changes is not addressable (Zhang and Vertes, 2018). Different techniques, with unique strengths, have been developed including secondary ion mass spectrometry (SIMS), matrix assisted laser desorption ionization MS (MALDI-MS) and matrix free laser desorption ionization MS (LDI-MS). While SIMS can identify low mass species, both MALDI-MS and LDI-MS

are relevant for the identification of larger molecules such as proteins (Zhang and Vertes, 2018).

The second approach leaves the cell hydrated but at the cost of diminished sensitivity and lower throughput (Zhang and Vertes, 2018). However, it does allow for temporal sampling of the cell. Many of these techniques utilize sampling techniques such as capillary micro-sampling or metal coated nanospray tips for sampling. Additionally, laser ablation can also be used to generate charged molecules for the TOF chamber. In any case, sampling techniques rely on electro-spray ionization (Zhang and Vertes, 2018).

Another approach called multiple reaction monitoring MS (MRM-MS) is a more targeted proteomic approach. MRM-MS utilizes standards from representative chemical classes that have distinct fragmentation features (Cordeiro et al., 2017). Our lab has utilized this technique to specifically identify natural, low levels of endogenous α-glyosylceramides (Kain et al., 2014). The detection of known cellular proteins, for which standards are available and can be used in MRM-MS, is doable with high specificity and sensitivity; however, the technique is necessarily limited to a few proteins (Kitteringham et al., 2009).

Ultimately, applied to single cells, MS currently remains technically very challenging and difficult because of the small sample size and the rather limited sensitivity of most MS approaches (Armbrecht and Dittrich, 2017; Zhang and Vertes, 2018).

3. The frontier: determination of single cell function

The function of both B and T cells relies on the engagement of their idiotypic receptors that link specificity and activation. Therefore, the sequencing of these receptors, their re-expression, and their functional testing are required to directly link a particular cell to a particular antigen. In the context of transcriptomics studies, knowing the identity of these receptors for each cell tested permits associating a particular gene expression profile to antigen specificity (Holt et al., 2018).

The success of this approach relies on the ability to sequence both heavy and light chains, as well as α and β chains from a single B and T cell, respectively. Various approaches allow the simultaneous sequencing of both chains of each receptor; most utilize a bar-coding technique in the first round of PCR amplification (Howie et al., 2015). In addition, indexed sorted single cells can provide cell surface expression profiles that can be combined with scRNAseq, or targeted gene expression interrogation, to reveal activation pathways, and clonality via TCR sequencing. In addition to experimental methods for combining TCR and single cell RNA sequencing, a computational tool has been developed to reconstruct full length TCR sequences from single cell RNA sequencing data (Stubbington et al., 2016). Another advantage of combining TCR and transcriptomic data is that the re-expression of TCR or BCR receptors using retroviral vectors provides the opportunity to test function in vitro after re-expression in a TCR negative cell line such as BW5147.3, or in vivo after transduction of splenocytes or bone marrow derived precursors to produce retrogenic animals (Holst et al., 2006).

These techniques are powerful but remain under-utilized due to their costs and time consumption. In B cell biology, the HIV field has led the effort by re-expressing a large number of HIV-binding antibodies from patients and testing their neutralizing ability (Scheid et al., 2009; Tiller et al., 2008; Walker et al., 2009). In the T cell field, the first single cell TCR sequencing coupled with limited transcriptional profiling dates to 2014 and few publications have followed (Han et al., 2014). A recent study reported the characterization and antigen specificity of four patient derived tumor infiltrating CD8 lymphocytes using this approach (Gee et al., 2018). The very limited number of cells that could be utilized in this large study is the result of the remaining bottleneck in B and T cell characterization: the identification of the appropriate antigen, especially for T cells (Corbett et al., 2014; Keller et al., 2017; Walker et al., 2009).

4. What single cell techniques have illuminated about immune populations and sub-populations

The immune system is a panoply of diverse cell subtypes that communicate with each other and with parenchymal cells, migrate in and out of different tissue environments, help maintain tissue physiology and address or mediate tissue pathology. Understanding the interactions of diverse immune cells with each other, with tissue resident immune cells, and with parenchymal cells, is important to understand how they function in homeostasis and in disease. Given that immunologists have historically interrogated the diversity of immune cells at the single cell level via flow cytometry, it seems fitting that they pioneered other single cell techniques (Giladi and Amit, 2018).

To get a better sense of immunocyte development and function, many single cell studies have analyzed immune cell types and subtypes via scRNAseq (Gaublomme et al., 2015; Paul et al., 2015). Detailed reviews of their findings can be found elsewhere (Cheng et al., 2017; Giladi and Amit, 2018; Papalexi and Satija, 2018; Wagner et al., 2016). However, there are two major findings from single cell papers that should be highlighted: population heterogeneity and modules of gene expression.

4.1. Understanding population heterogeneity between and within cell types

One of the first studies to use scRNAseq, Jaitin et al, tested the hypothesis that sequencing bulk population of cells sorted by pre-defined markers was insufficient to take into account immune heterogeneity and plasticity while single cell RNA sequencing could more adequately define distinct cell populations (Giladi and Amit, 2018; Jaitin et al., 2014). The example they give is that dendritic cell (DC) populations have been parsed and organized by morphology, function and cell markers and yet the populations identified with this approach still retained in vivo functional heterogeneity. To test this hypothesis, the authors isolated single mouse splenocytes and profiled their transcriptome. Using hierarchical clustering, among other computational tools, they found that the transcriptionally distinct groups overlapped with previous transcriptional profiles of classically defined hematopoietic cell types (Giladi and Amit, 2018; Jaitin et al., 2014; Papalexi and Satija, 2018).

In another study, the single cell transcriptome level changes in bone marrow derived DCs (BMDCs) stimulated with LPS were examined. Sets of genes were bimodally expressed, suggesting functional heterogeneity in the BMDC population (Shalek et al., 2013, 2014). This conclusion was reinforced when the authors were able to identify early responder DCs that upregulated their gene expression sooner than other DCs, and were critical in the coordination of the response of the late responder DCs (Papalexi and Satija, 2018; Shalek et al., 2013, 2014). This heterogeneity between cell types and within individual cell types has been found in various T cell populations such as Th17, and NKT cells as well as in the developmental hierarchy of myeloid progenitors (Cohen et al., 2013; Gaublomme et al., 2015; Paul et al., 2015).

4.2. Modules of gene expression

In addition to exposing sub-population heterogeneity, scRNAseq has been able to reveal modules of gene expression, an important conceptual advance. For example, a set of 100 genes including a number of anti-viral genes most likely controlled by Irf7 and Stat2, were bimodally expressed within BMDCs and allowed to define two distinct populations (Papalexi and Satija, 2018; Shalek et al., 2013).

Similarly, scRNAseq of Tregs revealed that all Tregs expressed a core set of genes that were similar over all three definable clusters of Tregs. However, beyond that core set of genes, it appears that some upregulated genes in Tregs were specifically associated to tissue residence and function (Zemmour et al., 2018).

What these studies confirmed at the single cell level and in granular

details is the inherent plasticity of T cells (Giladi and Amit, 2018; Papalexi and Satija, 2018; Shalek et al., 2013, 2014). Further, single cell studies will likely aid our understanding of the signaling pathways that support immune plasticity and its role in physiology and pathology.

5. An approach we have taken

While many groups have used single cell methods to decipher the complexity of immune cells and their lineages, fewer have used them to understand pathways leading to disease; the exception is in cancer biology where the approach has been taken to define lineages and subtypes in tumors and tumor immune infiltration (Li et al., 2017; Papalexi and Satija, 2018; Zheng et al., 2017).

Our laboratory has focused its interest in understanding the role of the immune response in autoimmunity and in chronic inflammatory diseases. Given that our focus is to identify and define molecules and signaling pathways that can be targetable therapeutically, we adopted single cell approaches to address the low number of cells that are often isolated from human blood and biopsies. Most of our studies have been focused on T cells and have been built on the same stepwise strategy: 1) use an MHC tetramer to access antigen-specific T cells, 2) profile the main T cell activation pathways by targeted transcriptomics, 3) confirm this result by whole scRNAseq and eventually discover additional pathways in the process, 4) sequence αβ TCRs and re-express pairs in hybridoma cells to confirm specificity, 5) assign a cell surface phenotype, a transcriptional profile, and a TCR usage to a single cell (Fig. 1). This information can then be used and expanded to evaluate the precise role and pathogenicity of T cells in disease. Because the same experiment can be carried out in animal models and humans, this approach is a remarkable tool for translational studies. Below we will briefly discuss one of these examples that we presented at the CD1-MR1 International workshop.

5.1. The role of MAIT cells in ulcerative colitis

Ulcerative colitis, one of two major forms of inflammatory bowel diseases along with Crohn disease, is a chronic inflammatory condition that localizes to the colonic mucosa (Ungaro et al., 2017). While its etiology is unknown, it is currently thought that an environmental trigger in individuals with genetic predisposition leads to a self-reinforcing inflammatory process that damages the mucosa leading to pain, bleeding, and colon resection in the most severe cases (de Souza and Fiocchi, 2016; Ungaro et al., 2017).

Based on histologic evidence, it is believed that T cells play an important role in UC pathogeny, but no particular T cell has been directly implicated (Ungaro et al., 2017). We have focused our attention on one T cell subtype that seems uniquely suited to participate in UC; the mucosal associated invariant T (MAIT) cell. MAIT cells are innate like CD8 T cells that recognize vitamin B2 derivatives in the context of MR1, a major histocompatibility complex (MHC) class 1b molecule (Ussher et al., 2014). The supply of vitamin B2 in humans derives from the microbiota with a majority of bacteria competent for the production of riboflavin (Ussher et al., 2014). MAIT cells are enriched in mucosal tissues, especially the lung and the colon (Kurioka et al., 2016). Upon activation, they can release pro-inflammatory cytokines such as IFN-γ, TNF-α, and IL-17, one of the most important tissue homeostasis cytokines in the gut (Ussher et al., 2014).

To add to the circumstantial scenario that could link bacteria, MAIT cell activation, and mucosal damage, the efficacy of anti-TNF-α therapy increases the suspicion that MAIT cells are implicated in disease progression (Neurath, 2017). In addition, it has been shown in two studies that MAIT cell frequency while reduced in the blood of patients, compared to the normal population, were increased in the UC lesions (Haga et al., 2016). However, the direct evidence that could link MAIT cells and disease is lacking.

We hypothesized that gene expression profiles of MAIT cells in inflamed lesions would be different from MAIT cells residing in adjacent normal tissue and surmised that gene expression pathway mapping could lead to a precise placement of MAIT cells in the pathology of UC. The overall design of the study consisted in examining single MAIT cells of UC patients in blood, inflamed and uninflamed regions of the colon, after MR1 tetramer cell sorting and transcriptomics.

With classic techniques, we confirmed that MAIT cells were reduced in the peripheral blood of UC patients, as a percentage of peripheral blood CD3+ CD8 + T cells, compared to normal blood donors, while MAIT cells had an increased frequency in inflamed colonic mucosa compared to patient matched normal colonic mucosa (Fig. 2).

The targeted gene expression profiling of 96 genes covering homing and adhesion receptors, cell surface activation markers, cytokines, and activating and inhibitory signaling molecules, was performed on isolated MAIT cells from each tissue compartment using the Fluidigm Dynamic Array. The data was first analyzed using the manufacturer software package, and then using dimensionality reduction tools such

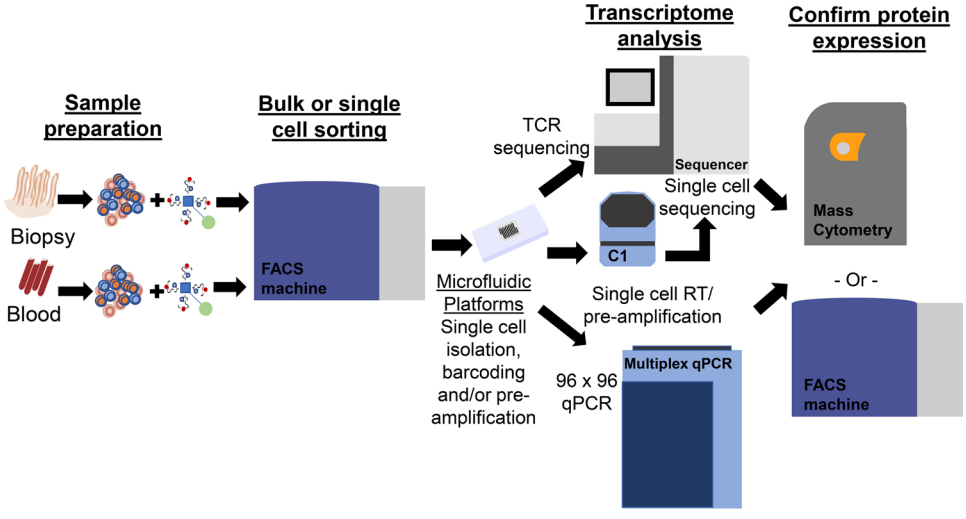

Fig. 1. An integrated workflow to identify differentially regulated genes and their protein expression. Our laboratory utilizes a single cell workflow, integrated with conventional immunology techniques, to sort disease specific cells, identify dysregulated genes and confirm protein expression from patient samples. Cells are isolated from blood via ficoll, and tissue via enzymatic dissociation. Single cells are indexed sorted via FACS. Pre-amplified cDNA libraries were created with pooled primers for 96 genes, for analysis by single cell qPCR, as well as primers for all αβ TCR pairs. Gene expression profiling is assessed utilizing the Dynamic Array, a microfluidic platform, and the Biomark (Fluidigm). TCR sequencing libraries are pre-amplified utilizing Fluidigm's Access Array, while sequencing is performed on an Illumina MiSeq. A C1 library preparation system is used, post tetramer coupled FACS sorting, for pre-amplification of single cell transcriptomes that are intended for Illumina sequencing. Validation experiments are performed by mass cytometry, classic flow cytometry or multispectral flow cytometry. Our approach differs from other single cell studies in that we have chosen to study particular cell types, isolated from various tissues, via single cell transcriptomics. This approach provides the antigen specificity, gene expression profiling and protein expression for each cell. In contrast, many other single cell studies immediately start with scRNAseq of bulk isolated cells from complex tissues such as the spleen or bone marrow.

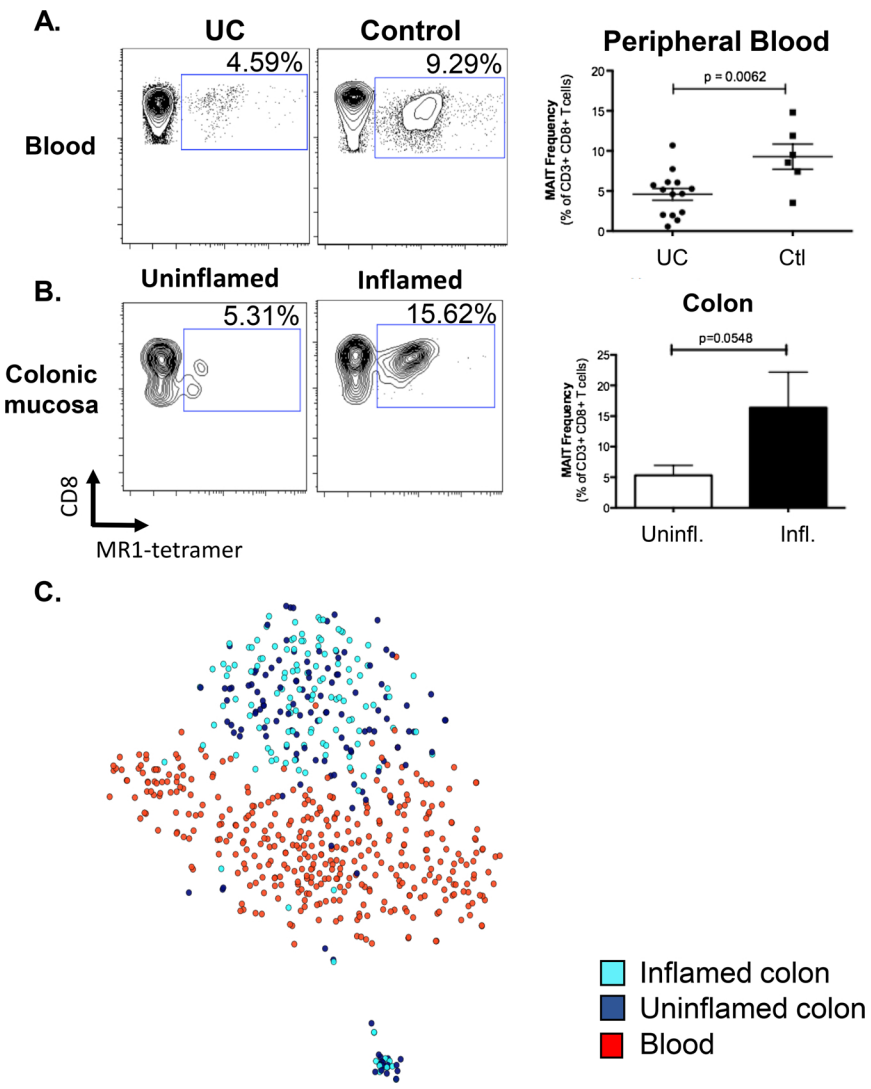

Fig. 2. Utilizing single cell approaches to decipher the role of MAIT cells in ulcerative colitis. A) MR1-tetramers, paired with conventional flow cytometry, was used to quantify MAIT cells as a percentage of CD3+ CD8 + T cells. Numbers were significantly decreased in UC patients (4.59%) as compared to normal blood donors (9.29%). B) Utilizing the same approach as detailed for blood, MAIT frequency was found to be increased in inflamed colonic biopsies (15.62%) when compared to uninflamed adjacent mucosa (5.31%). C) tSNE analysis of the targeted gene expression profiling of MAITs isolated from inflamed colonic mucosa, uninflamed colonic mucosa, and blood resident MAITs. 96 genes were quantified for expression using a multiplexed, single cell qPCR system. tSNE analysis demonstrated that MAIT gene expression is tissue specific. Additionally, it demonstrated the ability of targeted transcriptomic approaches in elucidating heterogeneity within cell populations. Following the confirmation of heterogeneity, single cell RNA sequencing will be used to identify genes, and gene modules that are unique for each tissue specific MAIT population. Abbreviations: UC, Ulcerative colitis; Ctl: Controls/Healthy donors; Uninfl: Uninflamed; Infl: Inflamed.

as tSNE and k-means clustering. This first layer analysis demonstrated that blood and tissue resident MAIT cells could be transcriptionally differentiated from each other (Fig. 2). CCR2, CCR1 and CCR5 were upregulated on blood MAIT cells whereas NF-kB, Nur77 and ICOS, as well as IRF4 and ZEB2 were upregulated on colonic MAIT cells, suggesting local TCR stimulation. A small number of genes was also differentially expressed between inflamed and normal colonic mucosal MAIT cells. These transcriptional signatures will be confirmed by antibody staining and cytometry analysis.

Targeted gene expression analysis was coupled with paired TCR αβ sequencing which showed differences in β chain usage between normal and inflamed tissue on the first set of patients examined. This targeted transcriptomic approach identified cellular heterogeneity and as these studies are expanded to larger groups of patients to reach statistical power, MAIT cells from lesions and normal tissues will also be investigated and compared using scRNAseq, and other complementary single cell techniques, to dissect the unique features and spatial relationships of MAIT cells present in damaged tissue (Fig. 3).

6. Conclusion

In this review, we have discussed the different techniques available in single cell analysis, highlighted the strengths of some techniques to uncover intra- and intercellular heterogeneity and the difficulties in single cell data analysis. Most importantly, we have made the case for integrating a graded, focused single cell methodology into translational studies to better parse the complexity of the immune response as well as to optimally utilize small amounts of tissue obtained from patients. Utilizing tetramer sorted cells and targeted transcriptomic analysis, we have demonstrated that MAIT cells in different tissue compartments express specific chemokine receptors and different activation genes. Paired with single cell TCR sequencing, we have also been able to demonstrate differences in clonality of MAIT cells in inflamed, uninflamed and blood MAIT cells.

Additionally, we have demonstrated how this integrated single cell approach can be used to isolate low frequency, disease specific T cells from the blood of T1D patients and demonstrate that only a fraction of these cells has an activated gene expression profile. We anticipate profiling more cells, as well as patients, via targeted and unbiased transcriptomic techniques will reveal specific pathways that not only will be targeted for treatment but also utilized to improve the specificity and sensitivity of T1D diagnosis.

With increased development and refinement, single cell approaches should be able to bridge translational and basic sciences with the ultimate benefit of providing new targets for diagnosis and therapy.

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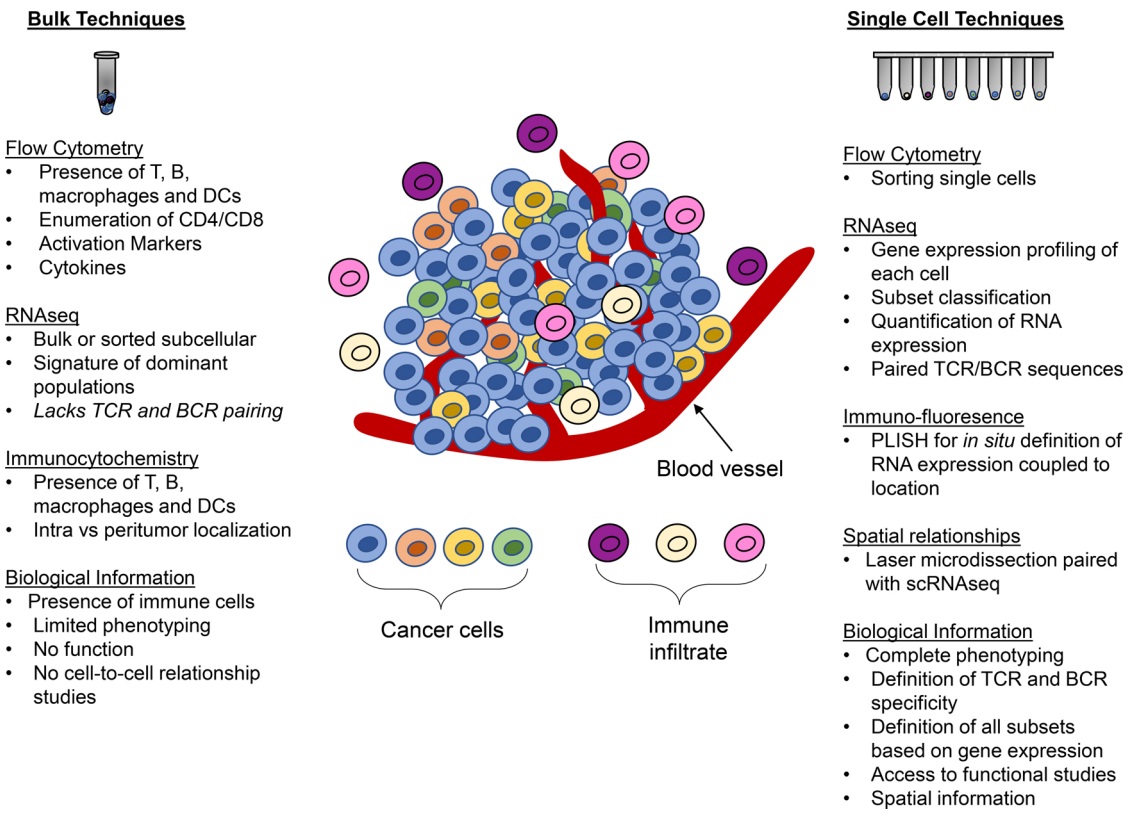

Fig. 3. Schematic representation and comparison of the analysis of tumor infiltrating immune cells by bulk and single cell techniques.

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Update

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Corrigendum

Corrigendum to "Using single cell analysis for translational studies in immune mediated diseases: Opportunities and challenges" [Mol. Immunol. 103 (2018) 191–199]

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