Journal of Molecular and Genetic Medicine
ISSN: 1747-0862

Gupta and Raghav, J Mol Genet Med 2015, 9:4
DOI: 10.4172/1747-0862.1000191

Review Article Open Access

Single Cell Transcriptomics for Autoimmune Disorders

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Received date: October 01, 2015; **Accepted date:** November 12, 2015; **Published date:** November 19, 2015

Abstract

The interplay of genetic, immunological and environmental factors is the driving force towards autoimmunity and each of these branches of biological science is necessary to identify the cause and progress of autoimmune disorders. Differential transcript abundance as an effect of environmental or epigenetic modifications may directly regulate emergence while a sustained copy number increase may drive disease progression. A precise evaluation of these transcript level differences could be the key to understand the mechanism of development and progression of autoimmune diseases however it is imperative to quantitate the subtle changes at the highest resolution. This review summarizes the studies that have explored the importance of analyzing differential transcriptome at single cell resolution, further to emphasize the importance of this approach for enhanced understanding and to identify more sensitive and specific biomarkers for autoimmune diseases.

Keywords: Transcriptomics; Autoimmune disease; Erythematosus; Major histocompatibility complex; CD8+ T cells

Description

The human body has invested enough to create a well-armed army for defense against intruders. Two well-organized lines of defense have been built up to an extent that if one fails to completely eliminate the invaders the other insinuates briskly. The interplay of these innate and adaptive immune responses proactively helps to combat diseases [1]. With the introduction of autoimmunity as an immune dysfunction that counters self, results in target tissue destruction and involves multi-organ ramification; an extensive research was initiated to understand the mechanism of immune dysfunction for identification of new therapies to treat and even prevent autoimmune diseases. More than seventy autoimmune disorders including rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus have been registered so far that are known to be a consequence of excessive immune responses. Despite their heterogeneity, autoimmune diseases share epidemiological, etiopathogenic, and clinical features [2,3]. The past two decades of research has yielded rich insights into the pathogenesis and molecular mechanisms responsible for progression of many autoimmune diseases [4-8].

The predictive onset of immunologic changes or the outcome of autoimmune disease is increasingly being employed by measuring serum auto-antibodies. An expanding spectrum of auto-antibodies has been reported for different autoimmune diseases. Besides rheumatoid factors (RF) [9,10] and anti-citrullinated peptide (anti-CCP [10]) as predictors of rheumatoid arthritis (RA), we proposed the diagnostic capacity of anti-Mannose binding lectin (anti-MBL) auto-antibodies for RA [11]. Elevated titres of anti-nuclear antibodies (ANA) are seen in patients with systemic autoimmune rheumatic diseases [12]; anti-MDA5 antibodies in myositis [13,14] and auto-antibodies to 3-hydroxy-3- methylglutaryl-coenzyme-A reductase (HMGCR) in patients with immune mediated necrotizing myopathies [15] are well recognized. The thrombotic events in anti-phospholipid syndrome are mediated by anti-phospholipid antibodies such as anti-cardiolipin antibodies (Acl [16]) and/or anti-β2 glycoprotein I (β2GPI [16,17]) and/or lupus anticoagulant (LAC [16]). Considering the fact that autoantibody signatures can often be detected prior to the onset of the disease, they have been constantly used as predictive biomarkers.

Genomics of Autoimmunity

Many studies suggest that a blend of environmental and genetic factors is responsible; both are necessary, but are insufficient alone for full articulation of an autoimmune disease. An altered combination of genetic sequences has been implicated to provide the foundation for a potential autoimmune disorder that can be triggered with an external stimulus following a stochastic event. Studies involving monozygotic twins have facilitated the understanding and have further confirmed the role of genetic factors in establishment of autoimmune disorders. Multiple gene loci have been conferred responsible for a disease outcome moreover shared pathogenesis by a single gene has been shown to be responsible for various autoimmune diseases [18].

Major histocompatibility complex (MHC) presents a predominant region in human genome with association of variants at this locus with predisposition to numerous autoimmune disorders [19]. Human MHC, known as human leukocyte antigen (HLA, class I and class II) are highly polymorphic and provide genetic restriction for T lymphocyte responses. An aberrant class II presentation of self or foreign peptides to autoreactive T lymphocytes has been suggested to play a critical role in disease specific associations [19]. Allelic heterogeneity in HLA-DRB, HLA-DR4 genes plays role in susceptibility to multiple sclerosis [20-26]; while HLA-DQ is the major disease-predisposing locus in Type 1 diabetes with DRB1*04-DQA1*0301-DQB1*0302 and DRB1* 03-DQA1*0501- DQB1*0201 haplotypes predisposing European populations to the disease [27-32]. The shared epitopes coded by HLA-DRB1 alleles is a significant genetic risk factor for rheumatoid arthritis [33]. Also shared HLA haplotypes have been implicated in different diseases highlighting their central role in mediating host inflammatory responses [19]. Others and we

have shown that a myriad of non-HLA genes like PTPN22 [34], TNF [34-36], CTLA-4 [34], MBL2 [37], PADI [34] may be prognostic for autoimmunity. Many genome-wide association studies (GWAS) have highlighted genes and pathways pertinent with autoimmune diseases [18,38] concluding that genomic studies have profound clinical applications. Hence genetic approaches to identify target or druggable genomes have been well explored since past decade [39,40].

Transcriptomics in Autoimmunity: Advantages at Single Cell Resolution

Determining the genetic makeup though can be helpful in predicting disease predisposition and progression however occurrence of geographic and occupational clustering of autoimmune patients [41-43], external factors including exposure to tobacco smoke [44,45], radiation [46,47], chemical compounds [48], epigenetic modifications [49] and infectious agents [50] play significant role in development of autoimmune disease, providing substantial evidences of the environmental involvement in these diseases. The environmental factors, dynamic changes in cellular make up of tissues and infectious challenges substantially influence gene transcription thereby affecting transcript abundance.

Cell to cell variability owing to their stochastic and deterministic nature can only be tapped by following their transcriptional states. Alterations in RNA abundance will reflect a prompt and sustained response against self-antigens as well as processes involving recurring and remitting patterns of the disease. We recently showed that CD^{8+} T cells have profound differences in their tumor activity in melanoma patients depending on the peptide vaccines with either immunodominant HLA-A*0201-restricted native peptide of the melanoma antigen Melan-AMART-126-35 (EAAGIGILTV) or single amino acid substituted analog peptide (A27L; ELAGIGILTV) [51]. This discrepancy arrives due to differential expression of transcriptome for an appropriate effector function. Thus transcriptional activity being oscillatory, adapting swiftly to the needs of environmental and physiological cues provides a powerful tool for logical exploitation to identify biomarkers describing the physiological status of a disease as well as substantiation of therapeutic interventions.

Major technological breakthroughs to study spatio-temporal differences in transcript abundance have provided an apprehensive view of disease predisposition and progression [52]. DNA microarrays have been extensively used for transcriptional profiling of many autoimmune diseases [35,53-69]. Quantitative PCR [70,71], Nanostring [72,73] and more recently next generation sequencing (NGS) technologies [74,75] have helped us to build gene expression patterns and networks for disease associations. Though the technology has advanced drastically from PCR to microarray to NGS analysis, the asymptote of genetic analyses will soon reach if we continue to analyze population of cells.

We surmise in differential transcriptome by observing subtle changes between conditions by analyzing population of cells and accepting implicitly that the constituent cells behave analogously. This averaging over population results in loss of critical information by responding cells over non-responders. Time has come that we understand and treat each cell individually. While sequencing single neuron cDNA libraries from electrophysiologically identified warm sensitive neurons [76], could characterize active adult neurons and detect rarely expressed receptors that were undetectable in population pools. Immune system players like dendritic cells (DC) are not a single cell types but a system of cells that arise from both the myeloid and lymphoid hematopoietic lineages [77]. Various DC subtypes are thought to differ in their capacity to either stimulate or inhibit the immune response [77]. Thus it is extremely important to understand how every cell of a subset responds to external or physiological stimulus, and then look for patterns in the behavior that would tell us how these cells make decisions [78,79] observed extensive bimodality in the transcriptional response of mouse bone-marrow-derived dendritic cells (BMDCs) to lipopolysaccharide by measuring RNA abundance and splicing patterns of individual BMDCs that remained previously undetected. We recently showed that single-cell gene expression profiling allows identification of qualitative differences in CD8+ T-cell responses elicited by different gene-based vaccines in melanoma patients. To this extent we as well observed that within the population of CD8+ T cells with even identical TCR clonotypes, individuals developed differential effector function depending on their gene expression pattern [51]. Moreover, analyzing CD8+ T cells at the single cell level revealed cellular heterogeneity and polyfunctionality within tumor- and virus-specific CD8+ T cell sub-populations which was previously undetected using a population of cells, demonstrating the power and promise of single-cell transcriptomics in uncovering functional diversity between cells and in deciphering cell states. Ramskold et al., [66] identified distinct gene expression patterns as well as candidate biomarkers for melanoma circulating tumor cells using single cell enabled mRNA sequencing. Even isogenic cells in culture show strong variability. Continuously changing microenvironment for individual cells in cell culture conditions has been demonstrated to propagate changes in cell-cell-matrix dialogue such that each cell assimilates subtle differences [80-83]. Recently Herderschee et al. [84] reviewed the role of single cell technologies in providing an unprecedented detail of immune responses. Moreover in their effort in analyzing in vivo transcriptional states of single cells of complex tissues to characterize cell-type compositions, Jaitin et al. [85] demonstrated the power of single-cell RNA-seq for comprehensive cellular decomposition of complex tissues.

Thus to our belief, synchronization of cells by isolating with identical characteristics followed by an experimental protocol and interpreting data is nothing but a concocted extrapolation. Hence given the inherent stochasticity and heterogeneity of multicellular tissues, single cell transcriptomics is essential to understand the biological functions.

For autoimmune disorders, being inherited but driven by environmental cues, it makes it imperative to view their real time spatio-temporal dynamics at a single cell resolution. The major challenge that needs to be addressed in the studies involving autoimmune disorders is finding predictive biomarkers of the disease. For the purpose, the transcript signatures of individual cells will not only hasten an unbiased discovery of overlooked key molecules mediating robust effects but will also enable identification of circulating rare cell population that may be stimulating neighbor cells for the visible disease outcome. Blood has always been a preferred tissue for gene expression analysis studies to identify potential biomarkers and clues for disease pathogenesis. However blood only provides a snap shot of the intricate immune networks operating in the body. In autoimmune disorders like juvenile rheumatoid arthritis where the cells migrate from the blood and accumulate at the site of inflammation [86,87], sampling the site of inflammation like synovial fluid, tissue cells (biopsy) will provide better and detailed view of specific immune reaction. Again these sites are reservoirs of diverse cell types with lineage abundances shadowing the few deterministic

cells. Understanding the functional relevance of these cells can only be possible following an analysis at single cell level enhancing the sensitivity and specificity of the identified biomarker. Covey et al. [88] clearly highlighted the significance of single cell analysis by network profiling in biological characterization of autoimmune diseases like systemic lupus erythematosus and rheumatoid arthritis benefiting clinical medicine and drug development. Using multiparameter single-cell measurements Candia et al. [89] identified phenotypic differences between healthy and diseased patients, as well as between Behçet's disease and sarcoidosis, the diseases that share many common features and are difficult to diagnose.

Understanding the progression of an autoimmune disorder by identifying classifiers that distinguish different clinical forms or activity phases of the disease is as intriguing as finding diagnostic markers and poses another challenge in the field of research. Finding stringent predictors at distinct stages can be challenging if the analysis masks important information pertaining to population level analysis. It is also important to decipher the processes influencing initial tolerance breakdown and to distinguish them from those responsible for perpetuating autoimmune pathology. Analyzing and averaging over a population of cells that includes many distinct intermediate differentiation states will compromise over absolute results and will alter differentiation kinetics pertinent with the disease progression. This highlights the importance and necessitates the use of transcriptomics in understanding autoimmunity at the single cell resolution.

Further the prognostic competence of the proposed biomarker needs to be validated before compliance and RNAi mediated in vitro protocols are often exploited for the purpose. Snijder et al., [90] while analyzing population context of RNAi screens have confirmed that the cell population averaging can be misleading in interpreting even a perturbation phenotype and the methods that have the depth to measure activities at single-cell resolution can only overcome this issue, further acknowledging the potential of single cell analysis.

Patients with autoimmune disorders like rheumatoid arthritis, psoriasis, and inflammatory bowel disease are prescribed with disease modifying drugs or anti-TNF therapies. Very often the patients undergo remission, however occasionally they show a progressive relapse [91,92]. It will be interesting to follow patterns of gene expression and regulated gene networks during the course of the disease as well as in patients proceeding towards clinical remission.

Moreover understanding the discrete genetic modifications and the consequent signaling mechanisms leading to an occasional relapse as well as determining the efficiency with which the patient responds towards the drug will be of paramount importance. It is thus fundamental to understand the gene expression signatures that will allow us to discriminate between clinical phases of relapse and remission between patients eventually substantiating the efficacy of therapeutic interventions. A well-targeted intervention requires a more complete map of the cellular mechanisms and genes underpinning self-tolerance, thus single cell enabled NGS will be beneficial in this regard to not only discern overlooked transcripts but to also identify distinguished cells.

Moreover clustering together of analyzed single cells according to qualitative and quantitative differences in their transcript expression patterns manipulating the cellular signaling mechanisms will help us answer the key question of why only a subset of cells are responders and not the entire cell population.

Challenges of Single Cell Transcriptomics

Though in relatively naïve stage, single cell transcriptomics will revolutionize our understanding of the functional identity of each cell of a subset (Figure 1). Methods employed to pick up single cells are debatable and are a matter of personal and or practical preference however we believe that the methods should be rapid and at near-physiological conditions. Handpicking of neuronal cells has been demonstrated by Morris et al., [93], we used flowcytometry based sorting of each melanoma antigen specific CD8+ T cell directly in 96 well plates [51,94,95], laser microdissection or micromanipulation [96] and microfluidics for isolation of single cells has been extensively exploited [97-102]. Lengthier protocols to isolate single cells may lead to alterations of gene expression patterns as well as cell death.

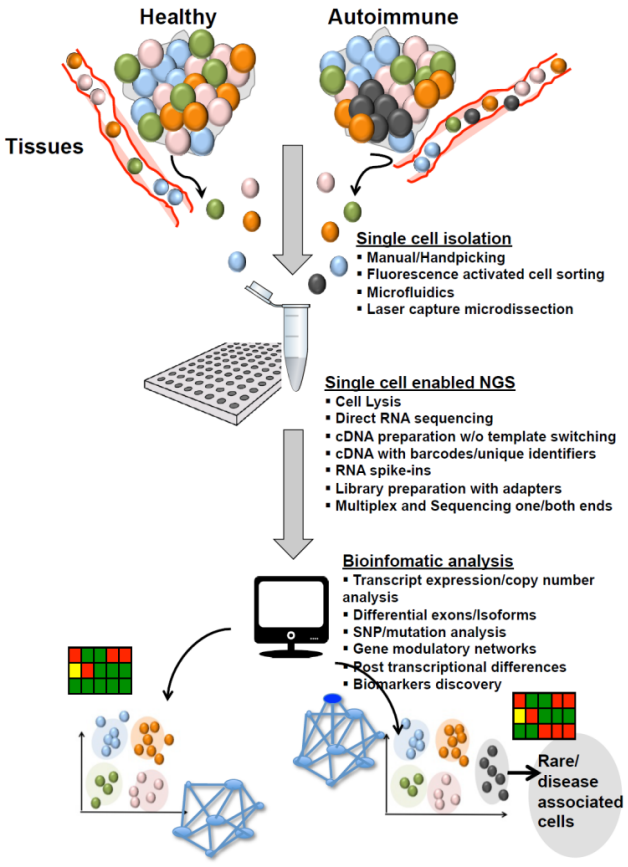

Figure 1: Protocol for single cell transcriptomics.

Nonetheless the first question that puzzles is 'how many cells'? Even using as few as ten cells, single cell sequencing studies have demonstrated the potential to distinguish complex heterogeneity [103]. Since most methods employ linear amplification of transcripts for a statistical readout of relative abundance, a better number will definitely provide conclusive results. However, for analyzing rare or low-abundance cells, tag-based sequencing of 5' or 3' e nds provides a scaled up assay for better estimates of transcript counts [80,103-105]. Still the numbers can be enormous. To address the issue, efforts have

been put together by researchers to build cost effective as well as high-throughput methodologies [103-112]. Multiplexing individual cells marked with specific barcodes for NGS work wonders to overcome the cost barriers (Soumillon et al.). NGS though not cheap will no longer remain the most expensive part of the protocol.

Following a well-planned experiment the next step is to generate meaningful data to build disease associated gene networks for effective perturbations necessitate another brainstorming engagement. To begin with, the use of RNA spike-ins [113], incorporating a unique identifier into every molecule prior to amplification [105,114]; direct sequencing of single molecules of RNA from single cells [26,83] can overcome the concept of technical noise in these single cell experiments thereby enabling hand picking of relevant transcripts. Moreover, factors such as heterogeneity of starting populations owing to the cell source, sample collection and processing methods as well as analysis platforms may well contribute to the differences observed between transcriptomic studies. However if each cell is analyzed individually in its particular time and space, there are better chances of reproducible representation of cell population.

Conclusion

Knowing the unique transcript signature of each cell of a subset in its particular time and space will allow us to disclose predisposition potential, predict the vivacity with which the cell can respond to a stimulus and reconstruct cell lineage trees with very high precision. Considering the ease of data collection and interpretation, it is mandatory and increasingly important to collect meaningful information and single cell enabled analysis can only be effective in this regard. It is the method of present and future. However, extending single-cell analyses beyond the transcriptome is indeed an area of interest. Since epigenetics modifications have been implicated in various autoimmune diseases [49] methods pertaining to single cell epigenetics would be of increasing priority to understand transcriptional regulation and build regulatory networks for better perturbation strategies. Fessenden [115] in her article has very evidently summarized that examining epigenetic modifications in single cells will allow researchers to establish the differences among the mosaic of cells as well as the functional consequences of those differences for development and disease states. Besides these protein-gene interactions, it is necessary to identify the cell specific protein-protein interactions to define the discrete protein interactome responsible for pathogenesis and progression of autoimmune disorders [116-119].

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