INTRODUCTION

The human organism has established several layers of control mechanisms to achieve a balance between immune homeostasis and protection against pathogens. However, unfavourable accumulation of genetic risk variants, combined with environmental factors and stochastic effects during the generation of adaptive immunity can skew homeostasis towards autoimmunity. This condition is a global health problem with one of thirty individuals known to be affected with an autoimmune disorder. Autoimmunity occurs when cells of the immune system converge to facilitate the targeting of the body itself. This involves cells of the whole immune system, although central in the pathological process are self-reactive T cells of the adaptive arm. Specific mechanisms have arisen to achieve tolerance within this cell subset. Bone marrow–derived T cell progenitors will migrate to the thymus, and T cells recognizing autoantigens are removed or converted into regulatory T cells (Tregs) with suppressive capacity. However, self-reactive T cells can escape this selection by central tolerance and have access to autoantigens in the periphery. This is normally inhibited by peripheral tolerance, a process partly orchestrated by professional antigen-presenting cells (APCs), where Tregs are major players to ensure that self-reactive cells are kept in control. Autoimmunity occurs when flaws in one or more steps of the tolerance processes...
occur. Clearly, the cells involved in the aetiology of autoimmune diseases display a vast heterogeneity when it comes to function and specificity, as well as differences in location and ontogeny. This heterogeneity represents a gap in knowledge, and a hindrance for the progress of developing therapy regimes directed against the faulty immune reaction in these patients. To add to the complex picture, how distinct endogenous physiological properties and expression of specific molecules in the target organs crosstalk with the immune cells is not well characterized. This interplay between immune cells and various cell types in the target organ is probably especially important in endocrine organs, which have vital importance in hormonal homeostasis, subsequently regulating most of the body functions. Examples of autoimmune endocrine disorders are type 1 diabetes (T1D), autoimmune thyroid disease (hypo- or hyper-thyroidism) and autoimmune adrenocortical failure (autoimmune Addison’s disease (AAD)). Organ-specific disorders typically aggregate within the same individual in autoimmune polyendocrine syndromes (APS), which are yet distinct from systemic autoimmune conditions such as rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE). Symptoms of AAD can be vague, and as a result, the disease is often diagnosed after the complete destruction of the adrenal cortex. This, and restricted access to the affected organ in these patients, limits our knowledge of immunological processes occurring in the development of the disease. Thus, new ways of examining the relationship between the immune system and the target- and other non-target cells in the affected organ are important to move forward. In the last decade, we have seen an accelerating development in single-cell analysis of immune cells at the genetic, transcriptomic and protein level which can aid to better understand the heterogeneity of immune cells and such immune-organ interactions. This review aims to present single-cell technologies and their applicability in bridging this knowledge gap in autoimmune disease research and clinical practice.

2 SINGLE-CELL TECHNOLOGIES

2.1 Flow cytometry

2.1.1 The beginning of autoimmunity research at the single-cell level

Polychromatic flow cytometry (PFC) is a method that has already shed light on the immune complexity in autoimmunity by allowing simultaneous interrogation of diverse biological signatures on a single-cell level. Following the fluorescent cell photometer in 1969, the modern flow cytometer appeared in 1970s with two-colour fluorescence detection by using argon lasers instead of arc lamps as a source of excitation for fluorescence. In the following decade, in 1986, the first flow cytometry application was reported in autoimmunity research through detection and characterization of platelet alloantibodies. The identification and use of monoclonal antibodies in advanced PFC made the platform crucial for clinical research and an important diagnostic tool in immunology studies. Today, PFC is considered a user-friendly technology, especially for instruments that require minor prior operator experience (eg SH800S from SONY (www.sonybiotechnology.com), or Accuri™ and FACSMelody™ from BD (www.bdbiosciences.com)). The ability to sort cells of interest via fluorescent-activated cell sorting (FACS), together with an ever-increasing availability of antibodies and fluorophores, has contributed to the wide application in the science community today.

2.1.2 Clinical applications

The use of flow cytometry for clinical purposes is well established at present time. Importantly, the platform is suitable for multicolour immunophenotyping. The most prominent general fields where flow cytometry is in regular use are clinical immunology, haematology and oncology. One of the major common clinical use of flow cytometry in immunology is the measurement of IgG and IgM antibodies for histocompatibility cross-matching for organ transplants. PFC can also be used to assess therapy response in transplant patients by determining the amount of free CD3 receptor during anti-CD3 monoclonal antibody treatment (CLB-T3/4.A). Moreover, immunodeficiency is regularly interrogated by screening CD4 and CD8 surface markers by using the multiparametric flow technology.

The use of flow cytometry in the field of autoimmunity and alloimmunity disorder has also become wider. IgG and IgM autoantibodies are commonly measured for anti-platelet antibody detection, whereas IgG counts are measured in anti-neutrophil assays. In some cases, even immune complex screening with complement and IgG autoantibodies is done by flow cytometry. In addition, feto-maternal haemorrhage is commonly quantified by assessing haemoglobin F and maternal IgG targeting the rhesus D antigen.

2.1.3 Research applications

Flow cytometry was primarily applied as a diagnostic tool for haematological malignancies and for measuring DNA content but is now widely used in research in molecular biology, protein engineering, pathology, immunology and plant biology. The ability of this technique to simultaneously identify, quantify and characterize multiple subsets of immune cells helped it to emerge as an essential tool
for investigating the complexity of the immune system in health and disease. Increasing number of antibodies conjugated to fluorophores enables flow cytometry to narrow down the scale of monitoring immune dynamics. Immunophenotyping importantly allowed to define CD4 and CD8 subdivision and distinct subtypes. More molecular markers available, the more cell subtypes are defined using the flow cytometer platform to show extra layers of immune complexity in disease. An example of blood mononuclear cell immunophenotyping in APS I patients showed significant reduction of CCR5 and CXCR3 expressing helper T cells, CD16+ monocytes and regulatory T cells, suggesting altered homing of these cells to tissues in these patients. Vaccine immunomonitoring further became an important application for flow cytometer to facilitate observation of innate lymphocyte response after vaccination, and the subsequent dynamics of antigen-specific response among multiple immune cells post-vaccination. The flow cytometry assay of peptide-MHC (pMHC) tetramer staining using peptides loaded with fluorescent-tagged class I and class II HLA/MHC molecules was additionally established for the detection and isolation of antigen-specific T cells. This has been used successfully in T1D, and cytotoxic T cells (CD8+) specific for 21-hydroxylase is found present in AAD patients using HLA class I tetramer staining. However, this technology works best for strong affinity interactions, which is often not the case in autoimmunity. Dolton and colleagues thus developed an optimized peptide-MHC multimer assay for detection and isolation of autoimmune T cells bearing receptor for HLA A*02:01 and HLA B*27:05. This protocol takes advantage of protein kinase inhibitors to inhibit receptor internalization. Another innovation was the development of peptide-MHC dodecamers which reportedly detects up to fivefold more antigen-specific T-cells compared to tetramers/dextramers, even on CD4+CD8+ thymocytes. The field of autoimmune research will clearly benefit from these technological developments. The prospects of using pMHC in this research area are also addressed later in the review.

Flow cytometry is unique with capability of simultaneous identification of diverse cells and even their rare subtypes using both cell-surface markers and intracellular markers accurately. Another major flow cytometer application is intracellular cytokine staining (ICS) after in vitro stimulation. For example, such analysis has elucidated how NK cell cytotoxic activity coincides with intracellular expression of interferon gamma (IFN-γ) and surface expression of CD107a. However, also the activation state of cell pathways is critical to investigate in different disease conditions compared to control. The alteration of the signalling molecules' functions is therefore needed to be addressed and further quantified with tools of flow cytometry called 'phosphoflow' or 'phosflow'. The phosphospecific flow cytometry assay is particularly valuable for drug screening processes. This signalling modulation can potentially determine new and efficacious drug targets, exemplified by identification of antiviral ribavirin mediated STAT1 (signal transducer and activator of transcription 1) and S6 (ribosomal protein) phosphorylation state against herpes simplex virus type I (HSV-I) infection. In addition to that, single-cell network profiling or SCNF flow cytometry illuminates signalling networks in various cell types and can potentially explain autoimmune disease environments at the single-cell level.

### 2.2 Mass cytometry/CyTOF

The human immune system is complex and mediates its functions through specific immune cell activation and interaction, which again is dependent on the cytokine expression of the involved cells. To understand this complexity, it is crucial to achieve good single cell or cluster resolution during analysis. Flow cytometry with 20-30 parameters is considered a workhorse in this regard, but recent discoveries of new important immune markers, for example immune checkpoint molecules, have highlighted the need for multiparameter analysis at an even higher degree. This was met by the emergence of the isotope labelled mass cytometry which made it possible
to interrogate broadly cell populations before, during and after disease.\textsuperscript{45} Time-of-flight mass cytometry, or CyTOF, is a high-resolution, multiparametric single-cell technique that provides a unique opportunity to characterize complex systems at a single-cell level.\textsuperscript{46} In CyTOF, natural metal isotopes are applied to tag antibodies instead of fluorophores, reducing the spectral overlap and offering redundancy of compensation. More than 100 different parameters per cell can in theory be simultaneously measured functionally and phenotypically in contrast to the regular flow cytometry technique.\textsuperscript{44} CyTOF has clear advantages of having high resolution and possibilities for multiparametric analysis although limitations are no cell sorting functionality as the cells are destroyed in the process and the sensitivity is lower than for flow cytometry (Table 1).\textsuperscript{47} The maximum number of parameters achieved by mass cytometry surpassed polychromatic flow cytometry already within a year, with 37 parameters for assessing virus-specific T cell function and phenotype.\textsuperscript{48} Benchmarking the performance of the instrument, a study showed that CyTOF achieves consistent results comparable to the fluorescence flow cytometer when examining hematopoietic cell signalling behaviour.\textsuperscript{49} Both techniques are highly quantitative and accurate; however, PFC panel development for immune cells using more than 15 markers is laborious. Changing a single parameter may require redesign of the entire panel due to the spillover issue. Mass cytometry has far less spillover between channels, and panels are remarkably simple to develop. However, due to the limitation of the chelating polymer,\textsuperscript{50} the lanthanide ion tag antibody reporters in CyTOF are less sensitive in comparison to commonly popular fluorophore used in PFC.\textsuperscript{49,51} The maximum capacity of the polymer is limited to around 100 metal reporter ions (M\textsuperscript{3+}) that can bind to an antibody molecule.\textsuperscript{52} Autofluorescence of the cells is a known challenge by being a source of background signal in PFC, but this is very limited in mass cytometry. Furthermore, a major barrier for multicolour panel development in PFC is the sensitivity of the fluorophore, which varies about 10- to 50-fold between them. The lanthanide-tagged metal isotopes on the other hand only vary twofold in intensity and range from lower to upper mass of the cells.\textsuperscript{53} Although the acquisition speed in mass cytometry is slower compared to PFC, which especially becomes a limitation when looking at rare populations, sensitivity in PFC can also be reduced in large immune phenotyping panels due to increasing variation caused by compensation. In addition to that, the reagent of mass cytometry is inexpensive, roughly 1.5-3 US dollar per probe per test compare to 2-8 US dollar in PFC.\textsuperscript{19} Further, light-based measures of cell sizes, granularity or debris in PFC cannot be employed by mass cytometry. In addition, fluxes of small molecule, like Ca\textsuperscript{2+}, is currently unavailable.\textsuperscript{54}

CyTOF has also been utilized to determine cell cycle state in cancer while simultaneously overcoming the technological limitations of resolution in regular flow cytometry.\textsuperscript{55} Lessons can further be learned from the field of infectious disease medicine where CyTOF has been employed during human immunodeficiency virus (HIV) infection to study immune heterogeneity.\textsuperscript{56,57} Viral infections have indeed been suggested to take part in the pathogenesis of autoimmune disease,\textsuperscript{14} highlighting the importance of studying early changes during viral infections. Furthermore, the power of mass cytometry has recently been applied to dissect immune dysfunction in a pilot study including patients with different endocrine autoimmune disorders.\textsuperscript{58} Diverging immunological mechanisms in a single autoimmune disease is common and needs to be characterized precisely for better understanding of disease pathogenesis. Recent studies have used CyTOF to characterize the immune system in autoimmune T1D,\textsuperscript{59} SLE\textsuperscript{60} and RA,\textsuperscript{61,62} but very few studies have focused on autoimmune endocrine diseases. Further, pMHC dodecamers are compatible with mass cytometry and can be used to investigate antigen specificity on a broad range of lymphocyte phenotypes. In summary, mass cytometry offers broad immune phenotyping and functional analysis that is useful for autoimmune research.

### 2.3 Single-cell sequencing

#### 2.3.1 Single-cell RNA sequencing

The possibility to isolate mRNA from single cells to generate cDNA was shown as early as in 1990.\textsuperscript{63} Single-cell gene expression analysis by microarray and next-generation sequencing followed in 2003 and 2009, respectively.\textsuperscript{64,65} RNA sequencing proved to outperform microarrays when it comes to number of genes detected from single-cell libraries,\textsuperscript{65} and in the last decade, we have therefore seen the appearance of a myriad of scRNA-seq technologies and platforms, each with unique advantages (or disadvantages). These vary in isolation method for single cells (FACS, droplet-based or microfluidics), region sequenced (full length, 3' or 5'), sequencing depth (number of reads per cell), the use of unique molecule identifiers (UMIs), cDNA amplification strategy (PCR or in vitro transcription (IVT)), the use of RNA spike-in, cost per cell and finally compatibility with antibody staining\textsuperscript{66-71} (Table 2). Smart-seq2 and MARS-seq2 protocols are plate-based methods that depend on cells being sorted by FACS followed by library preparation in the individual wells.\textsuperscript{66,68} Smart-seq2, like most other scRNA-seq protocols, uses PCR to amplify cDNA after reverse transcription,\textsuperscript{66} while MARS-seq2 employs a linear in vitro transcription (IVT) strategy likely to reduce amplification bias.\textsuperscript{68} The methods also differ in coverage; while MARS-seq2 detects 3' reads at low depth, Smart-seq2 is a full-length high depth method also useful
for studying splicing events in single cells. MARS-seq2 offers UMIs while this is only available in the next-generation Smart-seq3 and not in Smart-seq2.66,72 Both techniques require more than average infrastructure in the laboratory, for example pipetting robots to achieve accurate library preparation. This has made the commercial user-friendly alternatives from 10X Genomics (droplet-based) and Fluidigm (microfluidics-based) more approachable for regular labs.

The possibility to assess antibody staining simultaneously with RNA sequencing approaches is key in immunology research due to the already established phenotypes determined by flow cytometry. A large limitation of scRNA-seq is the lack of detection of many genes, a phenomenon referred to as dropouts, and this limits phenotyping using conventional markers. The plate-based scRNA-seq technologies, Smart-seq2 and Mars-seq, thus have an advantage in this regard, due to the possibility to index-sort cells during cell isolation by FACS. However, recent technology development combining oligonucleotide labelling of antibodies with scRNA-seq allows for phenotyping at the protein level also for droplet- and fluids-based platforms such as 10X Chromium and Fluidigm C1 (Table 2).73,74 This enables the scRNA-seq techniques with low sequencing depth to also take advantage of antibody staining prior to sequencing. For example, while FOXP3 mRNA has been reported to be detected in Tregs subsets during scRNA-seq,75 this is not always the case with low-depth sequencing technologies (unpublished observation). For mice Tregs, this can be solved by using a Foxp3-GFP mouse line and presort cells.76 For human samples on the other hand, CITE-seq can be a solution, more precisely to use extracellular protein tags, in this example CD4+CD25+CD127-, and gate the population using antibody oligonucleotide reads, and look at the RNA profile within this subset (unpublished observation). Another option is to presort Tregs prior to carrying out scRNA-seq library preparation with droplet- or fluidsics-based systems. However, this limits the sequencing to only one cell type, unless a barcoding scheme via oligonucleotide labelled antibodies is employed (also called Cell Hashing).77

The immediate strength of scRNA-seq is the inherent ability to analyse the transcriptome of individual cells in an unbiased way. Rather than using phenotypic markers to isolate or identify cell types, scRNA-seq allows for unsupervised identification of cell types and/or phenotypes present in a complex sample. Consequently, a plethora of scRNA-seq tissue atlases with relevance to autoimmune diseases have been published.78-81 Central tolerance, the process through which autoreactive thymocytes are eliminated or converted to regulatory T cells (Tregs) in the thymus, can be flawed in autoimmune diseases.82 Characterization of the healthy mouse thymus by scRNA-seq has revealed unknown heterogeneity within the medullary thymic epithelial cell (mTEC) population, and this can aid in elucidating steady-state processes in central tolerance development.78 A more recent study indeed utilized scRNA-seq to elucidate how the expression pattern of tissue-restricted genes or antigens (TRGs or TRAs) in mTECs are not stochastically expressed but rather co-expressed within gene modules.83 Further work is needed to elucidate how these modules are regulated. Single-cell analysis of the thymus would also be useful to decipher the cellular pathophysiology in transgenic models of central tolerance dysfunction, for example in mice harbouring mutations in the transcription factor autoimmune regulator AIRE. In this way, unsupervised analysis by scRNA-seq of all cells isolated from the thymus could potentially identify pathophysiological alterations not only in mTECs and T cells, but also in less ‘popular’ cell types such as thymic vascular cells, dendritic cells or macrophages.

Similarly, such characterization of the tissues affected during autoimmune disease can elucidate mechanisms of

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**Table 2.** Characteristics of common protocols and platforms for single-cell RNA sequencing

|                     | Smart-seq2 66 | Mars-seq2 68 | 10X Chromium 70 | Clontech iCell8 69 | Fluidigm C1-96 71 | Fluidigm C1-HT 71 |
|---------------------|---------------|--------------|-----------------|--------------------|--------------------|--------------------|
| Cell isolation      | FACS          | FACS         | Droplets        | Dispensing         | Microfluidics      | Microfluidics      |
| mRNA region         | Full length   | 3’           | 5’ or 3’        | Full length or 3’  | Full length        | 3’                 |
| Amplification       | PCR           | Linear IVT   | PCR             | PCR                | PCR                | PCR                |
| UMI                 | No            | Yes          | Yes             | No                 | No                 | No                 |
| RNA spike-in        | Yes           | Yes          | No              | No                 | Yes                | Yes                |
| Ab staining         | Index sorting | Index sorting | CITE/REAP-seq  | Fluorescent imaging | No                | CITE/REAP-seq      |
| Cell imaging        | No            | No           | No              | Yes                | Yes                | Yes                |
| Strengths           | High depth    | Low amplifying bias | High throughput | Live/dead cell imaging | High depth        | High throughput    |
| Limitations         | Resource intensive, no UMIs | Resource intensive, low depth | Low depth | Low Ab plex | No Ab stain | Low depth |

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Peripheral tolerance by shedding light on the complex interactions taking place between immune cells and stromal, vascular and parenchymal cells. Patients with RA develop inflamed joints and represent an autoimmune disease where cells can be collected from the affected tissue during for example synovectomy.84 Droplet-based scRNA-seq has been used to identify novel subpopulations of synovium fibroblasts in this patient group, possibly aiding to better describe the pathological processes of RA.81 Endocrine autoimmune disorders on the other hand represent diseases where access to the affected organ can be limited to post-mortem material. However, scRNA-seq of human pancreas and adrenal gland from deceased organ donors display data quality sufficient to identify cell types present.79,85 AAD is the result of autoimmune destruction of the hormone-producing cells in the cortex of the adrenal gland. Data from Han et al85 show an abundance of cells from both the innate and adaptive immune systems in the adrenal gland during steady state in mouse and human (Figure 1). Such thorough immune characterization of the steady-state condition in this tissue by scRNA-seq can be important for elucidating pathogenesis and identifying prime suspects.

FIGURE 1 Seurat single-cell RNA-seq analysis of cells isolated from human and mouse adrenal gland (GEO accession number GSE134355). (A-B), Bivariate plots displaying UMAP projections of analysed human (A) and mouse (B) cells. (C-D), Heat maps displaying enriched genes (log fold change) in cell clusters from human (C) and mouse (D).
fact, a recent comprehensive study by Gawel et al\textsuperscript{86} points out how scRNA-seq combined with GWAS findings can be used to prioritize cell types for research and therapy of RA patients. Combining such human and mouse single-cell data will additionally aid in determining relevance of mice as model systems for studying the role of certain genes in autoimmune pathogenesis. This approach seems useful for endocrine autoimmune disorders also.

Unbiased characterization of immune cell compartments by scRNA-seq has also greatly improved our understanding of how immune lineages develop,\textsuperscript{80} and this will also ultimately improve our understanding of autoimmune processes. However, single-cell analysis of immune cells from actual patients with autoimmune disease represents the most promising opportunity to discover novel concepts for this disease group. Blood leucocytes are particularly relevant due to ease of sampling and isolation. For example, monocytes and dendritic cells from patients with SLE, RA and T1D have been characterised by scRNA-seq.\textsuperscript{87-88} Duterte and co-authors used a combination of flow cytometry and scRNA-seq to characterize an inflammatory circulating CD1C\textsuperscript{+}/CD14\textsuperscript{+} subset of dendritic cells in healthy individuals.\textsuperscript{88} They further demonstrated this subset to correlate with disease score in lupus patients. Similar studies have not been reported for endocrine autoimmunity such as AAD.

While single-cell analysis of mRNA expression clearly has already contributed to move the field of autoimmune research forward, the remaining non-coding RNA (ncRNA) remains as a dark spot on the single-cell autoimmune map. The ncRNA comes in different forms, with microRNA (miRNA) and long non-coding RNA (lncRNA) as the most known varieties. Their implications in immunology have already been established; macrophage expression of mir-144 during obesity has for example been shown to regulate liver oxidative stress.\textsuperscript{90} MicroRNA has also been shown to be specifically involved in autoimmunity caused by inflammatory dendritic cells.\textsuperscript{91} Further, lncRNA is known to regulate both the innate and adaptive immune systems.\textsuperscript{92} The lncRNA \textit{Flicr} is known to regulate Foxp3 expression and subsequent progression of autoimmune diabetes.\textsuperscript{93} This exemplifies the relevance for lncRNA in endocrine autoimmune disease and substantiates efforts to investigate this at the single-cell level. While some lncRNA can be detected in protocols using oligo(dT)\textsubscript{5} to select for polyadenylated transcripts,\textsuperscript{94} a dedicated protocol for single-cell analysis of small-RNA has recently been published.\textsuperscript{95} Employing these technologies to investigate non-coding RNA in immune cells from autoimmune patients may enable the detection of novel disease-specific regulation at the molecular level. Finally, immune cell gene transcription not only depends on environmental stimuli, but also the availability of chromatin due to epigenetic regulation. A widely used assay for determining chromatin accessibility is the assay for transposase-accessible chromatin by sequencing, also called ATAC-seq.\textsuperscript{96} This has been further developed for single-cell use\textsuperscript{97}; however, no studies focusing on autoimmunity have been published yet.

2.3.2 | Single-cell lymphocyte receptor sequencing

The high specificity in antigen-recognition is maintained by the B and T cells and their receptors, the B cell receptors (BCRs, also called membrane immunoglobulin (mIg)) and the T cell receptors (TCRs).\textsuperscript{98} The immunological repertoire is the collection of antigen receptor proteins, TCRs and BCRs, in an organism. To recognize any possible invading antigen, the potential to generate unique receptors through recombination mechanisms are enormous, with a theoretical number of unique TCRs estimated to \textit{10}\textsuperscript{14}-\textit{10}\textsuperscript{20}, but only a small proportion of the full repertoire is ever realized. The great diversity in the repertoire is generated through somatic recombination, also called V(D)J recombination, taking place in the primary lymphoid organs during B and T cell development. V(D)J recombination randomly combines variable, diversity and junctional gene segments in the developing immune cell, and combined with random insertion of nucleotides; this results in receptors recognizing a wide range of antigens.\textsuperscript{99} It is the complementary-determining region 3 (CDR3) of the BCRs heavy-chain and the TCRs β-chain that contain the diversity gene segment and the antigen-recognition part of the immune receptors, and that will be targeted by deep sequencing.

The immunological repertoire was previously analysed by flow cytometry and by the immunoscope method, also known as CDR3 spectratyping, comparing the lengths and distribution of the CDR3 region.\textsuperscript{100} However, flow cytometry could not accurately recognize all the different V genes, and neither of the methods would quantify the number of immune cells bearing the same receptor or reveal the specific sequence of the CDR3 regions. By using new sequencing technology, it is possible to target the CDR3 region of the immune receptors, revealing the different V and J genes with the same affinity, and give a quantitative picture of the immunological repertoire in one person.\textsuperscript{101} At the single-cell resolution, this reveals the combined receptor chains, for example the α and β chain composing the T cell receptor in one single T cell.\textsuperscript{102} Sequencing can be performed both at the DNA and RNA levels, either from peripheral blood, sorted immune cells or cells from organs. Along with the development of single-cell sequencing, these methods have also been applied for immune cell receptor analysis. Single-cell sequencing of the immunological repertoire has the same selection of different sequencing method with their different depth of sequencing, and variation in the length of the reads. Further, integrating analysis of the immunological repertoire by peptide-MHC multimers with mRNA single-cell sequencing offers the possibility to associate antigen specificity to
transcriptional dynamics, hence broaden our understanding of the molecular mechanisms of immune cell plasticity, and a powerful method for tracking the clonal evolution of lymphocytes applicable to the study of immunity, autoimmunity and cancer. In the cancer-field, these methods are used both in monitoring disease and in analysing the activation status of the T cells. In autoimmune diseases like Sjögren's syndrome, activated Th1 and Th17 cells from patients showed restricted clonal diversities to unique motifs. Also relevant to autoimmune diseases is the analysis of human T cell repertoire formation, indicating a preferential positive selection to autoimmune diseases is the analysis of human T cell repertoire formation, indicating a preferential positive selection of shared human cross-reactive CDR3s, and that the TCR repertoire formation, indicating a preferential positive selection of shared human cross-reactive CDR3s, and that the TCR repertoire formation, indicating a preferential positive selection of shared human cross-reactive CDR3s, and that the TCR repertoire formation, indicating a preferential positive selection of shared human cross-reactive CDR3s, and that the TCR repertoire formation, indicating a preferential positive selection of shared human cross-reactive CDR3s, and that the TCR repertoire formation, indicating a preferential positive selection of shared human cross-reactive CDR3s, and that the TCR repertoire formation, indicating a preferential positive selection of shared human cross-reactive CDR3s, and that the TCR.

2.4 | Single-cell Imaging

2.4.1 | Classical imaging

Fluorescence microscopy or immunohistochemistry-based techniques have been largely used through the last three decades, but the single-cell resolution of these methods remains greatly compromised. With the unfolding of how complex the immune apparatus is, tissue imaging at the cellular and subcellular level became crucial to reveal the pathogenesis and molecular level detection in organ-specific autoimmune disorders. In situ hybridization (ISH) was first described in 1969 to provide microscopic localization of nucleic acid (DNA, mRNA and microRNA), whereas immunohistochemistry (IHC) is used to detect, localize and quantitate protein (antigen) in preserved tissue. Immunohistochemistry is considered convenient and reliable but ISH offers more precise localization of nucleic acids. IHC is light microscopy based and semi-quantitative due to chromogenic detection. Fluorescence in situ hybridization (FISH) is considered a pivotal and powerful tool in molecular biology and cytogenetic research but suffers for false-positive and false-negative results. The limitations of these methodologies have paved the way for innovative development suited to improve in situ analysis.

2.4.2 | Advanced imaging techniques

Imaging mass cytometry (IMC), or Hyperion, is an advanced development of CyTOF. Hyperion image mass cytometry facilitates imaging of fresh frozen and fixed tissue sections or cell smears using more than 30 protein markers at subcellular resolution. Simultaneous detection of 37+ markers with 135 additional channels for more in-depth parameter at 500 nm resolutions is now available. Digital spatial profiling (DSP) is another single-cell technique based on fluorescence in situ hybridization (FISH) that works on regions of interest of a tissue sample in a slide up to 600 microns. The resolution allows to capture images of 96 proteins and over 1000 RNA targets to obtain high plex analysis. Multiplexed ion beam imaging (MIBI) is the first multiplexed solution with ion beam imaging, instead of a laser, commercialized as MIBIscope. It allows for higher resolution compared to IMC, with a theoretical limit of 200 nm, which gives opportunities for imaging at the subcellular level and rescanning for image construction. Co-detection by indexing (CODEX) has recently been developed by scientists from Stanford University. They showed great resolution of 40 markers used simultaneously, converting fluorescence microscope to capture tissue images using oligonucleotide barcode sequences as reporters, conjugated with antibodies. Other imaging methods are currently being developed for commercial use. CyCIF/t-CyCIF, or cyclic immunofluorescence, is an imaging technique published in 2018 that can collect up to 60-plex images. Multiparameter imaging cell screen or MICS or MACSima works at single-cell resolution on tissues using fluorescence microscopy. More than hundred proteins can be imaged in a single run by cycling of each marker. These methods may have importance particularly in autoimmunity research to portray the interactions between the immune system and the affected tissues (Table 3).

2.5 | Single-cell data analysis

2.5.1 | Cytometry data analysis

The rapid development of single-cell techniques with its increased number of parameters has resulted in a need for analysis tools for increasingly complex data. The traditional flow cytometry data analysis is based on the ‘manual’ bi-axial gating for identifying cellular subsets of interest. FlowJo (www.flowjo.com) is a popular analytical tool used for flow cytometry data that can group the samples by density-based gating upon user choice to minimize the self-fluorescence. The software is user-friendly and has different visual outputs integrated, like bivariate plots with the limitation of displaying a few parameters, or histograms for univariate presentation. More importantly, phenotyping of cells with biaxial gating in for example FlowJo requires prior knowledge, although more advanced multiparameter analysis tools have become available. These advanced tools often require more bioinformatic knowledge; however, plugins are becoming available for more user-friendly platforms such as FlowJo or Cytobank (www.cytobank.org). The latter is a cloud-based platform that functions in a highly automated way with advanced bioinformatics tools while having repository possibility. In addition, intracellular signalling studies done by phosphoflow are also covered.
in the Cytobank analysis platform. Integration of advanced bioinformatics algorithms, like SPADE, CITRUS, viSNE and FlowSOM, allows Cytobank to offer a wide range of analysis possibilities. It is important to remember that both flow cytometry and mass cytometry multiparametric data are well compatible with such multivariate analysis tools, regardless the complexity or size of the dataset. Multivariate analytical tools can be unsupervised and unbiased but also be supervised based on the hypothesis. Most of the tools rely upon clustering algorithms, dimensionality reduction methods and trajectory inference. Examples of such algorithms are explained below.

SPADE, or spanning-tree progression of density-normalized event (www.cytospade.org), is a clustering algorithm that clusters cells into subsets based on the specified markers by using a minimum spanning-tree algorithm. Expression of the specified marker can then be visualized for each cluster by colour. CITRUS is another hierarchical clustering algorithm developed for mass cytometry data. The tool is very thorough and reliably compares diseased to control based on the marker intensities. CITRUS is particularly helpful to generate dendrograms for visual presentation of new findings or predicting models. FlowSOM is one of the fastest clustering methods, which measures F-scores and a self-organizing map (SOM) to identify the cell population with reference to manual gating results. Another clustering algorithm PhenoGraph is also used to detect communities from complex cell mixtures. Interconnected cells are phenotypically considered as similar based on the weighted edges and clustered by a community detection algorithm for network analysis.

Furthermore, the inter-cluster relationship is also analysed and represented by a Scaffold or Force-directed map. Scaffold is a landmark-based reference map used for multivariate data sets to identify alike cells. The algorithm has the unsupervised discovery possibility but requires a reference map. Force-directed maps are also a type of Scaffold map for progression analysis for de-differentiation.

High-dimensional data are often difficult to interpret and visualize, and the heterogeneity can become unclear when many parameters are combined. Thus, dimensionality reduction techniques can help to map data into a lower dimensional space consisting of two or three dimensions. The advantage of dimensional reduction is the straightforward visualization of the structure of multidimensional data with information at single-cell level. The t-distributed stochastic neighbour embedding (t-SNE) algorithm used in dimensionality reduction analysis is, in contrast to principal component analysis (PCA), a non-linear transformation method. The two most common tools used in cytometry based on t-SNE are viSNE and ACCENSE. Importantly, the unbiased analysis with the viSNE algorithm can discover unexpected changes in marker expression and identify new phenotypes.

How cells develop or mature is important for the appreciation of the complexity of our immune system. The interest in trajectory inference analysis is therefore growing, also due to the measurement of cellular processes at the single-cell level (eg cell cycle, cell differentiation and cell activation). Through such measurements and marker expression profiles, advanced tools can model trajectories of cell development and the transition between different cell states. The application Wanderlust is an example used for trajectory tracking of cell development and has a wide use. Wanderlust maps multiparameter events along a one-dimensional trajectory based on the most probable position in such a continuum. The algorithm is limited by assumptions that the trajectory is one-dimensional and that the sample contains cells of all stages in this continuum (which may not be true for the sample). Some other advanced analytical tool should also be considered to use for multidimensional data analysis, such as flowClust clustering available in R and Bioconductor (www.bioconductor.org), trajectory inference PHATE used in Matlab or Python. Gemstone (www.vsh.com) is for creating a two-dimensional summary of the multidimensional single-cell data utilizing the relative cell population from one to another to organize and visualize by probability state modelling.

Finally, a conditional probability or stochastic model can be generated using DREMI (conditional-Density Resampled Estimate of Mutual Information) analysis for pathway construction. The combination of statistical tests like correlation analysis in addition to machine learning techniques can also be used on complex multiparametric data to discover disease mechanisms, drug targets or specific immunity responses. However, a major drawback of multiparametric antibody-based cytometry analysis is generation of noise that can hamper the process of answering the scientific questions. Therefore, care must be taken to initially hypothesize the number of parameters necessary to measure for answering the scientific question and translate the findings into new knowledge.

2.5.2 scRNA-seq analysis

A major difference between cytometry data and data generated by sequencing RNA from single cells is the 10- to 100-fold increase in the number of parameters measured by the latter (ie genes, also called features). The number of genes detected per cell varies depending on cell type and protocol used, with 2-3000 genes detected for the low-depth technologies and 4-8000 genes detected for the high depth technologies. This has triggered the development of designated analysis tools, most of which are carried out in R or Python. However, user-friendly software with intuitive graphical user interfaces (GUI) is also becoming available (for example
SeqGeq by FlowJo). After bioinformatic demultiplexing and mapping of reads, uniquely mapped UMI counts or read counts are arranged in a gene expression matrix that serve as the common starting point for most downstream analysis tools. General considerations to be made prior to downstream analysis includes which combination of quality control criteria to use (total read count, mitochondrial reads, RNA spike-ins or number of genes detected), and whether to include all genes detected or only variable genes.

For dimensionality reduction, it has been common to use t-SNE analysis. Another recent development suitable for scRNA-seq data is the uniform manifold approximation and projection (UMAP) algorithm. Compared to t-SNE, this algorithm performs faster, produces less crowded projections and displays more meaningful immune cell clusters both for scRNA-seq and mass cytometry data. For clustering scRNA-seq data, there are different types of algorithms available and in use. Common examples are the hierarchical algorithm BackSPIN and the graph-based clustering that is used in Seurat. Hierarchical algorithms may struggle to identify rare cell types in a data set, and iterative analysis may be necessary to accurately identify cell types, while graph-based clustering on the other hand is better capable of detecting clusters consisting of few cells or to identify proliferating cells (unpublished observation).

Once cell types have been annotated through cluster analysis, it can be meaningful to make trajectory inference. A widely used algorithm for determining cellular trajectories is pseudotemporal ordering by Monocle. A major pitfall when assessing trajectories is to include cell types that are not related biologically. Careful consideration by the user is therefore necessary on beforehand. Molecular mechanisms active during T cell apoptosis or T cell differentiation into Tregs in the thymus could for example be elucidated by such analyses. It is also possible to investigate how cell types may interact through ligand-receptor pairs. How such ligand-interactions occur in thymus or affected tissues during autoimmune attack could be investigated by ligand-receptor interaction assessment of scRNA-seq data. This analysis alone will not prove biological relevance in autoimmunity, but rather open new avenues of hypothesis-driven research.

### 3 CONCLUSIONS

The complexity of the immune system has been known for decades, but new knowledge that shines a light on immune cell heterogeneity is still being discovered. Expression of the same markers by macrophages and dendritic cells did for example blur the distinction between them a decade ago, but this has now been resolved thanks to single-cell approaches such as mass cytometry and scRNA-seq. By a twist of fate, a recent study employed scRNA-seq to unravel how cDC2s put on a macrophage cloak during inflammation by expressing CD64. This shows how the field of immunology is in constant movement and in symbiosis with single-cell technology advancement, and points towards a more digital future with larger emphasis on single-cell and bioinformatic analysis. Especially, autonomous approaches to identify immune patterns.
associated with disease are likely to become important. This is likely to revolutionize the knowledge of immunological disorders, including endocrine autoimmunity. For example, new methods such as scRNA-seq allows for unbiased investigation of many cell types in an organ undergoing immune attack. This facilitates the study of the crosstalk or interplay between the immune cells and the target tissue. In this way, a sound hypothesis-driven study can produce hypothesis-generating results that may move the field forward. However, unlike cancer where biopsies are often accessible, tissue availability can be more challenging in endocrine autoimmune disease. Since patients with endocrine autoimmune disorders often are identified after the organ is destroyed, relevant biopsies are difficult to obtain. Research therefore also relies on good animal models, with ample tissue availability, to proceed to identification of novel disease mechanisms and assessment of immune therapy efficacy. But mechanisms found in animal models need to be verified in humans before proceeding to clinical trials and the final goal to improve patient health.

ACKNOWLEDGMENTS
The authors acknowledge the KG Jebsen Foundation, University of Bergen, the Western Norway Health Authorities and the Norwegian Research Council for funding.

CONFLICT OF INTERESTS
The authors declare no conflict of interest.

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**How to cite this article:** Sulen A, Islam S, Wolff A, Oftedal BE. The prospects of single-cell analysis in autoimmunity. *Scand J Immunol*. 2020;92:e12964. https://doi.org/10.1111/sji.12964.