Review Article - Narrative Review

Taiwan J Ophthalmol 2024;14:554-564

Access this article online

Quick Response Code:

Website: http://journals.lww.com/TJOP

DOI: 10.4103/tjo.TJO-D-23-00096

Single-cell transcriptomics in thyroid eye disease

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Abstract:

Thyroid eye disease (TED) is a poorly understood autoimmune condition affecting the retroorbital tissue. Tissue inflammation, expansion, and fibrosis can potentially lead to debilitating sequelae such as vision loss, painful eye movement, proptosis, and eyelid retraction. Current treatment modalities for TED include systemic glucocorticoids, thioamides, methimazole, teprotumumab, beta-blockers, and radioactive iodine; however, it has been reported that up to 10%–20% of TED patients relapse after treatment withdrawal and 20%–30% are unresponsive to mainstay therapy for reasons that have yet to be more clearly elucidated. In the past 4 years, vision researchers have harnessed high-throughput single-cell RNA sequencing to elucidate the diversity of cell types and molecular mechanisms driving the pathogenesis of TED at single-cell resolution. Such studies have provided unprecedented insight regarding novel biomarkers and therapeutic targets in TED. This timely review summarizes recent breakthroughs and emerging opportunities for using single-cell and single-nuclei transcriptomic data to characterize this highly complex disease state. We also provide an overview of current challenges and future applications of this technology to potentially improve patient quality of life and facilitate reversal of disease endpoints.

Keywords:

Gene expression, Graves' orbitopathy, single-cell RNA sequencing, single-nuclei RNA sequencing, thyroid eye disease, thyroid-associated ophthalmopathy, transcriptome

Introduction

Thyroid eye disease (TED) is a rare, debilitating autoimmune disease characterized by progressive and often irreversible orbital inflammation.[1,2] Alternatively known as Graves' orbitopathy or thyroid-associated orbitopathy, TED is estimated to affect at least 25% of patients with Graves' hyperthyroidism. It is also seen in other thyroid pathologies, such as Hashimoto thyroiditis, thyroid cancer, and subacute thyroiditis.[3,4] Potentially irreversible sequelae of TED include exophthalmos, lid retraction, diplopia, decreased visual acuity, compressive optic neuropathy secondary to increased ocular pressure, corneal ulceration due to exposure keratopathy, or pain with extraocular movement.[5] While the mainstay of treatment for active TED is systemic glucocorticoids, external-beam radiation therapy, or biologic immunomodulatory agents, treatment is often complicated by the fact that 10%–20% of patients relapse after treatment discontinuation and 20%–30% are unresponsive to glucocorticoid therapy.[6,7] Severe cases of TED are treated with orbital decompression to mitigate the functional sequelae of the disease and restore orbital anatomy and visual function.[8,9]

Although TED's immunopathogenesis is still poorly understood, it is thought that autoantigens targeting thyroid-stimulating hormone receptor and insulin-like growth factor I receptor (IGF-R1) result in the activation of orbital fibroblasts (OFs), which can upregulate the production of glycosaminoglycans such as hyaluronan, pro-inflammatory cytokines such as interferon-γ (IFN-γ), interleukin 1 β (IL-1 β), and tumor necrosis factor

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Submission: 10-07-2023
Accepted: 18-07-2023
Published: 20-10-2023

How to cite this article: Ahsanuddin S, Wu AY. Single-cell transcriptomics in thyroid eye disease. Taiwan J Ophthalmol 2024;14:554-64.

α (TNF-α), and enhance the differentiation of OFs into myofibroblasts (MYFs) and adipocytes.[10,11] The latter is thought to propagate inflammation, edema, expansion, and remodeling of retrobulbar tissue. The natural course of the disease is described by Rundle's Curve, which describes an initial phase of "active" inflammation spanning a few months to 1–2 years before reaching a peak and declining to a stable and fibrotic period.[12] In the clinical setting, the clinical activity score (CAS) determines the need for immunosuppressive treatment based on disease activity.[13] However, large gaps in knowledge remain regarding dynamic changes within the orbital microenvironment over the course of the disease process, the efficacy of various monoclonal antibodies in mitigating disease progression and potentially reversing disease endpoints, and the variables at play that contribute to the progression of thyroid pathologies such as Graves' disease to TED in certain patients as opposed to others.[14] These gaps limit more effective and targeted treatment of the disease.

In recent years, vision researchers have employed mRNA phenotyping methods to elucidate the diversity of cell types and signaling pathways driving the pathogenesis of TED. Hybridization-based microarray technologies and bulk RNA sequencing (RNA-seq) have been used to characterize complex cellular dynamics, molecular signatures, and functional pathways in TED and have also been useful for identifying circular RNA expression and long noncoding RNA and mRNA in orbital connective tissue.[15-19] These studies have helped contextualize novel clinical trials for various emerging treatments including teprotumumab,[14] tocilizumab,[20] selenium,[21] mycophenolate mofetil,[22] and azathioprine,[23] all of which have demonstrated relative efficacy in targeting certain immune cell populations implicated in the pathogenesis of TED. However, while bulk RNA-seq can be used to uncover the mechanisms by which gene expression is correlated with cellular phenotype and function in TED, it can only be used to provide an aggregate analysis of the expression profiles of large populations of cells.[24,25] Because it lacks individual cell-type resolution, bulk RNA-seq cannot be used to provide more granular assessments of gene expression dynamics in each individual cell comprising a tissue.[26] Thus, traditional RNA-seq has limited functionality for elucidating cell-to-cell variation in gene expression in highly heterogeneous periorbital tissues such as those found in TED patients.

Since Tang *et al.* published the first study describing single-cell RNA-sequencing (scRNA-seq) in 2009,[27] vision researchers have harnessed the technology to generate comprehensive cell atlases in a myriad of ocular disease states ranging from glaucoma,[27,28] keratoconus,[29] and Fuchs endothelial corneal dystrophy.[30] ScRNA-seq offers several key advantages over traditional bulk RNA-seq methods including its ability to uncover rare cell populations, identify regulatory networks between genes, and map the developmental trajectories of distinct cell lineages over time.[31] More recently, scRNA-seq has been used to characterize the genome-wide RNA expression of the orbital microenvironment in TED at single-cell resolution. Fang *et al.* recently investigated the significant role of Th17, RORγt, Th2, IL-21R, and IFN-γ signaling cascades in TED.[32] Li *et al.* utilized scRNA-seq to discover that lipofibroblast (LPF) expression of ras-related dexamethasone-induced 1 (*RASD1*) is integral to the pathogenesis of TED.[33] These and other studies published in the last 4 years provide unprecedented insight into complex molecular pathways and cell-cell interactions in TED, thereby yielding enormous potential for informing targeted therapeutics.

In this review, we provide a comprehensive overview of single-cell atlases detailing the transcriptional landscape of TED ranging from the active, inflammatory phase to the stable, fibrotic period in comparison to healthy controls. Using scRNA-seq data, we identify the key mediators of the disease process from the humoral and cell-mediated immune systems. We also discuss potential experimental and technical limitations in employing scRNA-seq and the advantages of using single-nuclei RNA seq (snRNA-seq) to characterize the orbital micro-environment in TED. These limitations of scRNA-seq notwithstanding, we believe that integration of transcriptomic data with clinical data can be valuable for enabling targeted and more effective therapeutic intervention. Specifically, we believe scRNA-seq can be used to develop novel immunotherapies targeting specific cytotoxic signaling pathways and immune cell interactions. In a condition that has been historically characterized by significant treatment resistance and relapse, such therapeutic strategies could potentially facilitate the reversal of disease endpoints and mitigate the need for rehabilitative surgery.

Methods

In February–March 2023, PubMed, Google Scholar, EMBOSS, and NCBI Gene Expression Omnibus databases were systematically queried for the terms "next-generation sequencing thyroid eye disease," "single cell RNA sequencing thyroid eye disease," "single cell RNA sequencing thyroid-associated orbitopathy," "scRNA-seq Graves' Orbitopathy," "Single-cell RNA seq thyroid associated orbitopathy," "thyroid eye disease transcriptomics," and "Graves' Disease transcriptome." Additional details on the literature search algorithm are included in the supplemental methods. Forty-one total publications related to transcriptomics in TED were identified and 7 articles specifically pertaining

to scRNA-seq and snRNA-seq from 2018 to 2023 were assessed. References cited within the selected articles were used to augment the literature search. Correspondences, editorial articles, and letters to the editor were excluded. Transcriptomic studies published in the foreign languages and nonoriginal review articles were also excluded from the analysis. We also manually filtered out spurious hits that pertained to Graves' disease or Hashimoto thyroiditis without any reference to the ocular complications of either disease state. A complete list of scRNA-seq studies and relevant findings is included in Table 1.

Single-cell RNA Sequencing in Thyroid Eye Disease

Characterization of the diversity of cellular phenotypes in thyroid eye disease

Vision researchers have used scRNA-seq to construct comprehensive atlases detailing the various tissue-resident and tissue-infiltrating cell types responsible for autoimmunity and retroorbital tissue remodeling in TED. These cell atlases have been used to extensively validate prior culture studies which have implicated certain cell types, such as OFs and T cells, in the pathogenesis of TED. All published scRNA-seq studies to date confirm that patients with TED harbor OFs that exhibit distinct phenotypes and functional capabilities compared to OFs found in healthy donors. In 2018, Fang *et al.* analyzed 7100 single cells obtained from both TED and control subjects and discovered that the primary immune cell types present in both groups were OFs, adipocytes, antigen-presenting cells (APCs), endothelial cells (ECs), myocytes, and lymphocytes using canonical lineage markers.[32] Li *et al.* expanded upon these findings and categorized OFs into three subtypes depending on their expression (or lack thereof) of *THY1*$^+$ and *RASD1*$^+$.[33] Wu *et al.* reiterated that OFs constitute a major cell class in TED orbital connective tissues in addition to CD8$^+$ T-cells, ECs, MYFs, monocytes, natural killer (NK) T cells, B cells, and CD4$^+$ T cells.[37]

These studies also characterized differences in cell proportions in diseased tissue compared to healthy tissue. In Fang *et al.*'s study, control retrobulbar tissue possessed fewer lymphocytes and APCs compared to TED tissues.[32] *TBX21* and *STAT1*-expressing Th1 cells, *GATA3* and *STAT6*-expressing Th2 cells, *RORA* and *STAT3*-expressing Th17 cells, *AHR*-expressing Th22 cells, and *IL2RA (CD25)*-expressing regulatory T (T_{Reg}) cells were found to be significantly upregulated in TED compared to controls, suggesting that T-cell immunoreactivity is a hallmark of TED pathogenesis. Interestingly, the orbital tissues of the TED patients included in the study had significantly elevated numbers of *CD34*$^+$-expressing OFs, macrophages expressing *CD14* and *CD68*, as well as dendritic cells expressing *THBD* (BDCA-3). NK cells were found to highly express *FCGR3A*. Orbital-infiltrating *CD34*$^+$ OFs in TED samples were identified vis-à-vis the significantly upregulated expression of *CD34* and *COL1A1*.

Wang *et al.* and Zhang *et al.* built upon Fang *et al.*'s findings by investigating the heterogeneity within CD4$^+$ T cell subtypes.[6,32,34] By analyzing 85,265 CD4$^+$ T cells obtained from the peripheral blood of 3 treatment naïve Graves' Disease patients, 6 treatment naïve TED patients, and 4 treatment withdrawal TED patients, Wang *et al.* was able to identify six different CD4$^+$ T cell subtypes involved in TED.[6] These included central memory CD4$^+$ T cells (SELL$^+$ CCR7$^+$, 37.33%), T_{Reg} cells (FOXP3$^+$ IKZF2$^+$, 7.07%), follicular CD4$^+$ T cells (CXCR5$^+$LIMS1$^+$, 17.30%), Th17 cells (CCR6$^+$ RORC$^+$, 13.73%), intermediate differentiated cytotoxic T lymphocytes (CTLs) (KLRG1$^+$ granzyme B [GZMB]$^-$, 9.98%), and terminal effector CTLs (KLRG1$^+$ GZMB$^+$, 10.10%). The terminal effector CTLs uniquely expressed *KLRG1*, *GZMB*, *PRF1*, *GNLY*, *CX3CR1*, *FGFBP2*, *CCL4*, *CCL5*, and *IFNG*, which suggests that these cells are primarily involved in leukocyte chemotaxis, inflammation, and cytotoxicity in TED. Wang *et al.* further utilized flow cytometry and immunohistochemistry to validate that CD4$^+$ T cells in TED patients highly expressed *FGFBP2*, *GNLY*, *GZMB*, and *PRF1* compared to their Graves' disease counterparts. Of note, Wang *et al.* discovered that the CD4$^+$ CTLs in TED patients highly express *KLRG1*, a surface marker that is typically found on CD8$^+$ T cells and NK cells. In their study, CD4$^+$KLRG1$^+$ CTLs are distinctly unique to TED, suggesting that this cell type is essential for antigen-specific clonal expansion. Similar to Wang *et al.*, Zhang *et al.* also identified six different CD4$^+$ cell subtypes using scRNA-seq in 23,317 peripheral blood mononuclear cells obtained from the peripheral venous blood of TED patients with CAS $\geq$3.[34] However, their study was aimed at elucidating differential gene expression in these cell types in response to rapamycin treatment in 2 TED patients. Of note, they found that CD4$^+$CCR7$^+$SELL$^+$ T naïve-like cells, CD4$^+$SELL$^+$CD27$^+$ T central memory cells, CD4$^+$LMIS1$^+$GPR183$^+$ T effector memory cells, CD4$^+$FOXP3$^+$IKZF2$^+$ (T_{regs}), CD4$^+$CCR6$^+$CXCR3$^+$ TTh1/Th17-polarized cells, and CD4$^+$GZMA$^+$CCL5$^+$CTLs were present in the peripheral blood of TED patients both before and after rapamycin treatment.

In addition to these findings, Li *et al.* not only identified CD4$^+$ T memory (Tm) cells, CD8$^+$ Tm cells, CD8$^+$ CTL cells, CD56$^+$CD16$^+$NK2 cells, and CD56$^+$CD16$^-$ NK1 cells as the infiltrating immune cell types in the orbital connective tissues of patients with TED, but they also identified

Table 1: Summary of single-cell RNA sequencing and single-nuclei RNA sequencing studies in thyroid eye disease

Study name	Methodology	Platform	Sample source	Number of cells included in analysis	Year of publication	Molecules/pathways identified	References
Li *et al*.	ScRNA-seq	10X Genomics Chromium	Human orbital connective tissue from TED patients (*n*=10 samples) and healthy controls (*n*=10 samples)	31,353	2022	LPFs upregulate expression of *RASD1*. ACKR1^{+}ECs and adipose tissue M2 macrophages are involved in TED. IFN-γ is highly expressed by CD8^{+}CD57^{+} T cells	[33]
Fang *et al*.	ScRNA-seq	10X Genomics Chromium	Human orbital connective tissue from 3 TED patients and 3 healthy controls	7100	2019	IFN-γ and RORγt + Tbet + T cells are highly increased in TED. Th17, IFN-γ, RORγt, IL-23R, GATA3, Th2, and IL-21R pathways are all associated with TED. IL-23R and IL-1R expression is upregulated by CD34+ OFs	[32]
Zhang *et al*.	ScRNA-seq	10X Genomics Chromium	PBMCs obtained from 2 steroid-refractory GO patients prior to and after rapamycin therapy	23,317	2023	Rapamycin significantly mitigates mTORC1 pathway and the number of CD4^{+} CTLs accumulating in proximity to CD90^{+} fibroblasts in orbital connective tissues of patients with TED	[34]
Wang *et al*.	ScRNA-seq	10X Genomics Chromium	PBMCs and orbital tissue samples from 6 treatment naïve GO patients, 4 treatment withdrawal GO patients, 3 treatment naïve GH patients	85,265	2021	CD4^{+} CTLs undergo significant clonal expansion in orbital sites compared to CD8^{+} T cells and highly express cytotoxic molecules *KLRG1, GZMB, PRF1, GNLY, CX3CR1, FGFBP2, CCL4, CCL5,* and *IFNG*, which suggests that they possess chemotactic, cytotoxic, and pro-inflammatory properties	[6]
Chen *et al*.	ScRNA-seq, bulk RNA-seq	10X Genomics Chromium for scRNA-seq; Invitrogen Superscript dscDNA synthesis kit, Illumina HiSeq 3000	ScRNA-seq datasets obtained from GSE106543, GSE149652, GSE179292 and SRP226183 RNA-seq data obtained from PBMCs from 20 treatment naïve GO patients, 20 treatment-naïve GH patients	~135,936	2023	VEGF-A facilitates CD4^{+} CTL cytotoxicity by enhancing VEGF-R1/R2 signaling and the AKT/ mTOR pathway	[35]
Kim *et al*.	SnRNA-seq, bulk RNA-seq	For snRNA-seq, 10X Genomics Chromium; For RNA-seq, TruSeq Stranded Total RNA kit, Illumina NextSep 500	Intraconal fat samples from 3 healthy controls and 3 TED patients undergoing orbital surgery	7062	2021	*FABP4/5, APOE, PPARG, ADIPOQ* are all differentially expressed in orbital fat samples obtained from TED patients. PI3K/Akt, IGF-1, cAMP, AGE-RAGE signaling pathways are all highly enriched during adipocyte differentiation	[36]
Wu *et al*.	ScRNA-seq	10X Genomics Chromium	Orbital adipose/ connective tissues from 1 TED patient surgery and 1 healthy control	-	2022	IL-11Rα and IL-11 are co-expressed in OFs, indicating that IL-11 levels may serve as a potential	[37]

Contd...

Table 1: Contd...

Study name	Methodology	Platform	Sample source	Number of cells included in analysis	Year of publication	Molecules/pathways identified	References
						biomarker of disease severity in TED	

LPFs=Lipofibroblasts, ECs=Endothelial cells, OFs=Orbital fibroblasts, GO=Graves' orbitopathy, GH=Graves' hyperthyroidism, PBMCs=Peripheral blood mononuclear cells, VEGF-A=Vascular endothelial growth factor-A, GEO=Gene expression omnibus, ScRNA-seq=Single-cell RNA sequencing, SnRNA-seq=Single-nuclei RNA sequencing, *RASD1*=Ras-related dexamethasone-induced 1, ACKR1$^+$=Atypical chemokine receptor 1, TED=Thyroid eye disease, IFN-γ =Interferon gamma, IL=Interleukin, mTOR=Mammalian target of rapamycin, mTORC1=mTOR complex 1, CD4$^+$ CTLs=CD4$^+$ cytotoxic T-lymphocytes, AKT=Protein kinase B

RASD1-expressing LPFs, atypical chemokine receptor 1 (ACKR1$^+$) ECs, and M2 adipose tissue macrophages (ATMs) as essential to the pathophysiology of TED.[33] Analysis of 31,353 single cells using canonical lineage markers demonstrated that patients with TED had six primary cell types including OFs (*CD34*$^+$*PDGFRA*$^+$*COL1A1*$^+$, 51.53%), myeloid cells (*LYZ*$^+$, 24.55%), NK cells and T cells (*CD3E*$^+$, 15.12%), ECs (*PECAM1*$^+$, 5.78%), pericytes (*MCAM*$^+$*RGS5*$^+$, 2.39%), and B cells (*CD79A*$^+$,0.64%). Specifically, NK and T cells and myeloid cells were found to be increased in TED compared to healthy controls. Interestingly, Li *et al.* was not able to identify a distinct cell cluster of adipocytes using *ADIPOQ* as a marker. In contrast to healthy controls, TED patients had increased NK and T cells (15.12% vs. 5.82%) and myeloid cells (24.55% vs. 6.66%) and significantly decreased percentages of OFs (51.53% vs. 81.83%). OFs were classified into three types based on their relative expression of *THY1* and *RASD1*: *THY1*$^+$ MYFs, *RASD1*$^+$ LPFs, and *THY1*$^+$ *RASD1*$^+$ conventional OFs (COFs) [Figure 1]. *ACKR1*$^+$ ECs were found to be significantly increased in TED compared to health. Based on marker gene expression, Li *et al.* sub-categorized ATMs into CD16$^+$ monocytes (*FCGR3A*$^+$), CD14$^+$ monocytes (*CD14*$^+$*S100A8*$^+$*S100A9*$^+$), dendritic cells (*CD1C*$^+$), and macrophages (*MARCO*$^+$).

Intraorbital cytokine and chemokine-mediated inflammation in thyroid eye disease

Several scRNA-seq studies have provided robust evidence implicating chemotactic and proinflammatory cytokines in the progression of TED. Fang *et al.* discovered that expression of cytokines IL-17A, IL-13, and IFN-γ were significantly upregulated in CD3$^+$ CD8$^-$ T cells in TED patients. Similarly, upregulation of transcription factors RORγt, RORγtTbet, and GATA3 in CD3$^+$ CD8$^-$ cells was found in TED patients compared to healthy controls.[32] Wang *et al.* identified CD4$^+$ CTL secretion of GZMB and IFN-γ as cytotoxic mechanisms and potential therapeutic targets for TED.[6] They also determined that TED-specific CD4$^+$ CTLs expressed high levels of CCL4, CCL5, CXCL8, CX3CR1, IL1B, and IFNG. Specifically, CX3CR1 and IFNG were found to be increased in TED-specific CD4$^+$ CTLs and CD4$^+$ KLRG1$^+$ T cells demonstrated significantly elevated expression of GZMB and PRF1. Wang *et al.* also discovered that in TED, the most highly enriched signatures in CD4$^+$ CTLs involve leukocyte chemotaxis, cytotoxicity, and inflammation (CCL5, CXCL8, CX3CR1, IL1B, and IFNG).

Li *et al.* noted that expression of *RASD1* by LPFs was positively correlated with expression of adipogenic transcription factors, *CEBPB, CEBPD,* and peroxisome proliferator-activated receptor γ (*PPARG)*.[33] Interestingly, they also found that CCL2, CXCL2, IL6, PTGDS, and TNFSF14 were significantly enriched in LPFs compared to COFs or MYFs in TED orbital connective tissues. *ACKR1*$^+$ ECs highly expressed CXCL2, CXCL8, CXCL14, and IL6 in TED, suggesting that this subtype of ECs play an active role in mediating leukocyte migration and inflammation in the disease process. Additionally, Li *et al.* noted that a significant source of IFN-γ were CD4$^+$ Tm cells, terminally differentiated CD8$^+$ CTLs, and CD16$^+$ and CD14$^+$ monocytes. CD4$^+$ Tm cells were found to highly express chemokines, chemokine receptors, and cytokine receptors (CXCL8, CCL8, CCR6, CCR7, IL7R, IL4R, and CCL20). Similarly, CD8$^+$ CTL cells were found to upregulate expression of various chemokine and IFN signaling-related genes. While CD57$^+$CD8$^+$ T cells expressed negligible levels of most chemokines, they upregulated expression of *CXCR1*, which plays an important role in mediating CD8$^+$ T cell migration to peripheral tissues.

Using single-cell suspensions of orbital connective tissue obtained from 1 TED subject and 1 healthy control, Wu *et al.* demonstrated that expression levels of IL-11 (a cytokine implicated in cellular proliferation, migration, fibrosis) and its receptor IL-11Rα were significantly co-expressed in TED compared to a healthy control.[37] Immunofluorescence staining was used to determine that OFs were the dominant source of IL-11Rα expression, suggesting that fibroblasts are an important mediator of IL-11 expression in TED. Notably, the results from their study suggest that IL-11 expression is positively correlated with disease severity as determined by the CAS. These findings suggest that IL-11 could be a potential therapeutic target in TED, amongst several others [Table 2].

Cell-cell communication networks and signaling pathways in thyroid eye disease

To investigate the role of chemotaxis, inflammation, and cytotoxicity in TED relapse, Wang *et al.* found

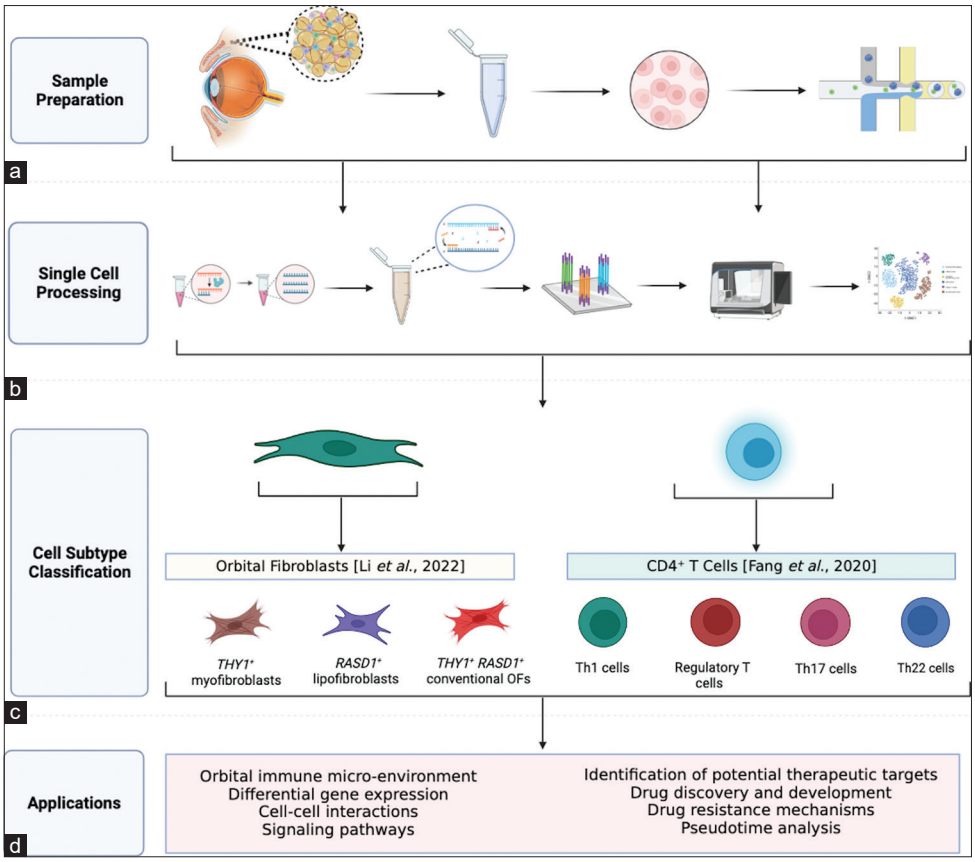

Figure 1: Schematic representation of single-cell RNA sequencing (scRNA-seq) experimental design and applications in thyroid eye disease (TED). (a) Orbital connective tissue samples from TED patients are obtained during orbital decompression surgery. Cells undergo enzymatic lysis and dissociation into individual micro-environments to release mRNA. (b) Downstream processing of mRNA includes conversion to complementary DNA (cDNA) vis-à-vis reverse transcription, cDNA amplification either by the polymerase chain reaction (PCR) or *in vitro* transcription, cDNA library preparation and sequencing, and quality control (not shown) to assess technical variability and cell viability. (c) Computational tools are then used to assess transcriptional similarity of cells, which are grouped into distinct clusters based on differential gene expression of canonical lineage markers. In two landmark TED scRNA-seq studies, orbital fibroblasts and CD4$^+$ T cell subtypes have been extensively characterized. (d) Applications of scRNA-seq in TED are wide-ranging and consist of characterizing the orbital micro-environment, uncovering cell-cell interactions, signaling pathways, and pseudotime analysis. *Created with BioRender.com*

that TED-specific CD4$^+$ CTLs were the primary source of pro-inflammatory chemokines and cytokines, as evidenced by distinctly high expression levels of *TBX21*, *EOMES*, *IFNG*, and *GZMB*.[6] To supplement this data, they also analyzed 50,082 single cells obtained from 4 TED patients before and after methylprednisolone administration and found that there was significantly decreased expression of cytotoxicity-(*GZMA*, *PRF1*, *CST7*, *GNLY*, *CTSW*, and *NKG7*), chemotaxis-(*CCL4* and *CCL5*), and inflammation-related (*DUSP1*, *FOS*, *JUN*, and *JUND*) marker genes after treatment administration. In one patient who experienced adverse effects and relapsed after treatment withdrawal, Wang *et al.* noted significantly increased expression of the aforementioned marker genes associated with cytotoxicity and chemotaxis. Interestingly, inflammation-related marker genes were found to be significantly reduced even after disease relapse. Of note, Zhang *et al.* found that treatment with rapamycin in two steroid-refractory TED patients led to significantly decreased levels of gene expression in the mammalian target of rapamycin (mTOR) signaling pathway, cell chemotaxis, and inflammation in CD4$^+$ CTL subtypes such as the Th1/Th17-polarized cells and T_{regs}.[34]

To further characterize the transcriptional heterogeneity of OFs, LPFs, and ECs in TED, Li *et al.* found that expression of genes involved in extracellular matrix remodeling (*COL1A1*, *DCN*, *MMP2*, *COL12A1*, *CTHRC1*, and *POSTN*), inflammation (*CXCL14*), and muscle contraction (*TAGLN* and *ACTA2*) were highly enriched in *THY1*$^+$ MYFs.[33] *RASD1*$^+$ was found to be primarily expressed by LPFs, which also highly express genes associated with inflammation (*IL6*, *CXCL2*, *CCL2*, *PTGDS*, and *ICAM1)* and adipogenesis (*CEBPB*, *CEBPD*, *PPARG*, *PLIN2*, *PTGDS*, and *APOD*). Expression of *PIM1*, a gene associated with a variety of cancers, was found to be implicated in the promotion of the LPF phenotype. In contrast to both *RASD1*$^+$ LPFs and *THY1*$^+$ MYFs, *THY1*$^+$ *RASD1*$^+$-COFs displayed negligible expression levels of these marker genes. LPFs in TED were not only found in slightly increased numbers compared to healthy controls, but they were also noted to highly express genes implicated in cellular

Table 2: Potential therapeutic targets and therapeutic agents in thyroid eye disease identified by single-cell RNA sequencing

Therapeutic target	Proposed agent(s)	Mechanism of action	Potential benefit in TED	References
IGF-1R	Teprotumumab (FDA-approved), VRDN-001 and VTERDN-002[38] (Phase I/II clinical trials); linsitinib (Phase II clinical trial), lonigutamab (Phase I clinical trial)	Teprotumumab, VRDN-001, VTERDN-002 are monoclonal antibodies targeting IGF-1R; linsitinib and lonigutamab are anti-IGF-1R inhibitor	Reduction of orbital fibroblast adipogenesis and differentiation	[36]
TNF	Infliximab, adalimumab	Monoclonal antibodies targeting TNF	Mitigation of leukocyte migration, inflammation, and glycosaminoglycan hyaluronan production	[33]
IL-1	Anakinra	IL-1R antagonist	Reduction in inflammation	[6]
IL-6	Tocilizumab	Monoclonal antibody targeting IL6-R	Reduction in inflammation	[33]
IL-11	BI 765423 (Phase I clinical trial)	Monoclonal antibody targeting IL-11	Reduction of orbital connective tissue fibrosis	[37]
PPAR-γ	Selective PPAR-γ modulators	PPAR-γ antagonists	Mitigation of orbital connective tissue fibrosis and expansion	[33,36]
IFN-γ	Rapamycin, tacrolimus, cyclosporine	mTOR and calcineurin inhibitors	Reduction in inflammation	[6,32,33]
TGF-β	Lerdelimumab, GC1008	Monoclonal antibodies targeting TGF-β	Reduction of orbital fibrosis	[33]
VEGF	Aflibercept (Phase II clinical trial)	VEGF inhibitor	Reduction in angiogenesis	[33]
mTORC1	Rapamycin	mTOR complex 1 inhibitor	Reduction in orbital inflammation, adipogenesis, and fibrosis by targeting $CD4^+$ CTL expression of mTORC1	[34]

IGF-1R=Insulin-like growth factor 1 receptor, TNF=Tumor necrosis factor, IL=Interleukin, PPAR-γ=Peroxisome proliferator-activated receptor gamma, IFN-γ=Interferon gamma, TGF-β=Transforming growth factor beta, VEGF=Vascular endothelial growth factor, mTOR=Mammalian target of rapamycin, mTORC1=mTOR complex 1, $CD4^+$ CTLs=$CD4^+$ cytotoxic T-lymphocytes

proliferation and activation (*JUNB, JUND, MYC,* and *PIM1*) and cellular growth (*IGF1)*. ECs that were found to highly express *ACKR1*$^+$ were found to upregulate expression of genes essential to inflammation, antigen presentation, cell adhesion, and angiogenesis such as *VCAM1*, *IL1R1*, *SELE*, *CSF3*, *ITGAV*, and *SELP*. In addition to expression of genes involved in inflammation and cytokine signaling discussed earlier, ATMs were found to highly upregulate expression of genes related to lipid metabolism (*CEBPB*, *APOE*, and *FABP5*), fatty acid transport (*CD36*), and the PPAR signaling pathway. Interestingly, despite exhibiting an anti-inflammatory M2 phenotype, these ATMs still highly expressed inflammatory genes during TED.

In accordance with prior findings, Li *et al.* was able to demonstrate that there are increased interactions between MYFs and LPFs with $ACKR1^+$ ECs in TED as well as $ACKR1^+$ ECs and OFs compared to healthy subjects.[33] Interactions between immune cells and OFs were significantly increased vis-à-vis IL6/IL6 receptor, transforming growth factor β (TGFβ)/TGFβ receptor, and chemokines/chemokine receptors. A synergistic relationship between OFs and ECs was observed with OFs producing vascular endothelial growth factor (VEGF), which acted on ECs, and ECs producing platelet-derived growth factor, which promoted OF proliferation. Chen *et al.* expanded upon these findings by noting that $CD4^+$ CTLs with enhanced cytotoxicity significantly upregulated expression of VEGF-VEGFR1 signaling pathway and the AKT pathway.[35]

Pseudotime analysis

Pseudotime analysis, otherwise referred to as trajectory inference, has been widely employed in scRNA-seq research to investigate dynamic changes in cellular gene expression in continuous biological processes using advanced statistical methods such as principal component analysis or uniform manifold approximation and projection.[39] To date, three scRNA-seq studies have utilized pseudotime analysis to infer underlying functional changes of various cell types based on changes in gene expression. Li *et al.* conducted pseudotime analysis to investigate potential transitions between OF populations in TED.[33] They found that in TED, there is a significant shift towards LPF differentiation from COFs and MYFs (MOFs). Expression of *FABP4*, *CEBPB*, *PLIN2*, *RASD1*, *FABP5*, *CEBPD*, *PPARG*, and *PIM1* was positively correlated with LPF differentiation. In orbital connective tissues of patients with TED, $CD16^+$ monocytes were noted to evolve into dendritic cells and macrophages. Zhang *et al.* determined that rapamycin treatment resulted in increased concentrations of intermediate differentiated $CD4^+$ CTLs and decreased concentrations of terminal effector $CD4^+$ CTLs.[34] This suggests that rapamycin can inhibit the terminal differentiation of $CD4^+$ CTLs, thereby mitigating cytotoxicity and inflammation. Pseudotime analysis in Chen *et al.*'s study

demonstrated that CD4+ CTLs with lower cytotoxicity expressed higher exhaustion signatures and higher levels of *GZMA*, *PDCD1*, *LAG3*, *TIGIT*, and *CTLA4* at different differentiation stages.[35]

Experimental Considerations and Limitations of Single-cell RNA Sequencing in Thyroid Eye Disease

While scRNA-seq can provide comprehensive transcriptome-wide insights into tissue heterogeneity and cell-specific gene expression, there are several experimental and data analysis challenges to consider. A critical bottleneck to scRNA-seq research is single cell isolation, since the methodology employed to isolate individual cells can alter transcriptional expression due to inadvertent RNA degradation, stress-induced transcriptional alterations during cellular dissociation, or failure to isolate cells embedded within collagenous matrix.[24,40] Li *et al*. noted that their scRNA data could not detect the presence of adipocytes (*ADIPOQ*+) potentially because of their larger cell size and their propensity for lysis compared to other cell types.[33] Additionally, studies are hindered by their inability to discern the functional significance of marker genes that are expressed at very low levels.[36] Data quality is markedly reduced in fixed or frozen samples, which poses a logistical challenge for researchers when there is uncertainty regarding the timing of sample procurement. Depending on the exact method used to prepare and isolate single cells from a given sample, significant variations in sensitivity and accuracy may occur, which poses notable challenges for interpretation of data obtained from complex, heterogenous tissues.[41] As a result of this technical limitation, the extent to which *in vitro* scRNA-seq datasets correspond with *in vivo* conditions in humans remains to be more clearly elucidated. Additional limitations to consider include scRNA-seq's prohibitively high cost[31] and the need for high coverage sequencing to ensure adequate quantification of transcript abundance per cell.[26] Due to its highly complex nature, analysis of single-cell data requires more in-depth knowledge of mainstream analysis platforms such as Seurat in R,[42] Scanpy in Python,[43] and Scater in R.[44]

Single-nuclei RNA Sequencing inThyroid Eye Disease

While scRNA-seq has furthered our understanding of cell-specific dynamics involved in the orbital micro-environment of TED, further insights have been garnered using an alternative methodology, snRNA-seq.[41] Instead of isolating whole cells, snRNA-seq uses isolated nuclei to quantify relative transcript abundance. SnRNA-seq can be utilized to enhance detection of rare cell populations and cells embedded in collagenous matrix from frozen tissues or tissues that are challenging to dissociate.[45] This can be particularly useful to study orbital connective tissue in TED because OFs are known to significantly upregulate production of collagen and glycosaminoglycans in the extracellular matrix. Additionally, published snRNA-seq methodologies address the challenge of preserving mRNA integrity using scRNA-seq by flash-freezing tissue immediately after dissection, thereby preventing new gene transcription from occurring and minimizing any confounding effects incurred by single cell isolation on mRNA expression levels.[46] In contrast to the enzymatic dissociation employed in scRNA-seq, snRNA-seq employs tissue homogenization, which has been demonstrated to reduce RNA degradation during the nuclei isolation step. Wu *et al.* has demonstrated that snRNA-seq methodologies harbor comparable sensitivity levels to scRNA-seq methodologies if all mapped intronic reads are included in the analysis.[47]

To date, there is only one study utilizing snRNA-seq to elucidate the pathophysiological mechanisms underpinning TED at single-nuclei resolution. Kim *et al*. employed snRNA-seq on intraconal fat samples to identify highly expressed genes in OFs undergoing *in vitro* orbital adipogenesis and differentiation.[36] They found that undifferentiated OFs in TED displayed similar gene expression profiles of control OFs. However, during adipogenic differentiation of OFs, expression of *FABP4/5*, *APOE*, *PPARG*, and *ADIPOQ* was highly enriched, mirroring the gene expression profiles of OFs obtained from TED orbital connective tissue. Pseudotime analysis demonstrated that OFs transitioned into adipocyte stem cells and then into mature adipocytes. Early in the differentiation process, OFs were found to highly express *IGF1*, *IGF1R*, *ZEB1*, *FGF7*, and *SFRP2*. Because this study identified enrichment of PI3-AkT, AGE-RAGE, insulin resistance, PPAR signaling, and fatty acid synthesis pathways in OFs undergoing adipogenesis, this study has important implications for developing therapeutic strategies targeting adipogenesis early in TED. While prior studies have shown that teprotumumab can improve proptosis and diplopia by modulating the immune response, Kim *et al.*'s study suggests that a potential reason for its utility in TED is its targeting of the IGF-1R pathway, which is highly enriched early on in OF adipogenesis.

Similar to scRNA-seq, there are several limitations to consider when using snRNA-seq. More so than isolated single cells, isolated nuclei are prone to aggregating together, which can subsequently contribute to inflated doublet counts.[48] Careful sample preparation involving sample agitation must be undertaken to minimize the occurrence of doublet rates.[41] Similarly, because only

the genetic material within the nucleus is isolated for analysis, the overall starting genetic material isolated per nucleus tends to be lower on average compared to scRNA-seq.[48] Various research groups have proposed including mapped intronic reads in the analysis in order to enhance the recovery of genes expressed by immune cell populations.[49,50] Furthermore, insights regarding the functional significance of genes that are expressed at low levels cannot be made using existing analytical approaches.

Future Directions

Its technical limitations notwithstanding, scRNA-seq has demonstrated previously underestimated heterogeneity of cell subtypes and molecular signaling pathways implicated in the immunopathogenesis of TED. Considerable benefit can be derived from future scRNA-seq studies that investigate potential differences in gene expression signatures based on patient demographic characteristics such as gender, race, and ethnicity. With the Federal Drug Administration's approval of teprotumumab anti-insulin-like growth factor 1 receptor (anti-IGFR) to treat TED in 2020, it would be valuable to utilize scRNA-seq to elucidate the mechanisms by which certain patients are recalcitrant to treatment (nonresponders) while others are more responsive to therapy (responders). Such studies could also investigate whether different T-cell subtypes infiltrate retroorbital connective tissues at different stages of the disease process and whether relative proportions of different cell subtypes are influenced by thyroid status.[32]

Future studies investigating the efficacy of acute versus long-term use of antiproliferative agents (mycophenolate sodium) and biologics (rituximab [anti-CD20]) that are presently thought to mitigate the functional sequelae of disease progression would also be of considerable value. For instance, Wang *et al.*'s identification of $CD4^+$ CTLs as a unique cell type involved in the progression of TED suggests that there may be value in developing therapeutic strategies targeting the mTOR pathway, which is essential for the differentiation of both $CD4^+$ and $CD8^+$ T cells [Table 2].[6] Targeting mTOR could also facilitate selective mitigation of the expression of key cytotoxic molecules such as GZMB, PRF, and IFN-γ. While the use of immunotherapy is limited in TED, such studies would be of enormous value to better understand how immune modulating agents can be used to slow the natural course of the disease, reduce the probability of vision-threatening complications, and mitigate the need for rehabilitative surgery. Future studies ought to include patients with long-standing, chronic TED to better elucidate how transcriptional heterogeneity of the immune microenvironment varies with time.[32]

A third future application of scRNA-seq in studying TED lies in the use of spatially resolved transcriptomics (SRTs), an emerging technology that can enhance our understanding of spatiotemporal tissue organization and its impact on functionality of individual cell types in diseased tissue *in situ*.[51,52] SRT technologies can be used to visualize the relative abundance and distribution of RNA transcripts by approximate location in a given sample of orbital connective tissue. What would be particularly advantageous when applying single-cell spatial transcriptomics methodologies to TED would be to reconstruct spatial expression patterns of resident and tissue-infiltrating immune cell types, OFs, and ECs. While SRT methods such as 10X Genomics Visium and SLIDE-seq provide less resolution than fluorescence *in situ* hybridization-based methods, the benefits of using such technologies lies in the fact that they can measure transcript abundance and heterogeneity across a larger number of genes by spatiotemporal location.[40] Use of such technology could provide meaningful insight into the physical interactions between different cell types, which may be just as important as molecular signals for determining cell fate and behavior. Thus, using *in vitro* model systems like organoids that permit mechanical control of the three-dimensional environment will be just as important for understanding single-cell dynamics in diseased tissue. Both murine and human organoid-based model systems have already been used to model Graves' hyperthyroidism[53] and can be used to further study cellular dynamics in orbital connective tissues with TED.

Additionally, we believe that single-cell proteomics (scProteomics) will become increasingly critical for determining the correlation (or lack thereof) between alterations in mRNA expression and protein concentration.[54] mRNA concentrations in a cell are highly dynamic and can be transient, and not all mRNA is translated into protein. While current scProteomics methodologies are limited by throughput and depth of coverage, integrating data generated using deep visual proteomics with clinical data could potentially provide novel insights into aberrant protein expression of single cells in their spatial and subcellular context in TED.[55] Notably, single-cell profiling of living cells that are still viable at the time of profiling using Live-Seq can also provide downstream molecular and functional insights that are missed by traditional scRNA-seq profiling methods.[56]

Conclusions

This review summarizes recent findings employing scRNA-seq methodologies to elucidate the orbital micro-environment in TED. Despite the technology's limitations, scRNA-seq has provided meaningful and clinically significant insights into the diversity of

cellular phenotypes and cytotoxic pathways implicated in TED pathogenesis. ScRNA-seq has been used to characterize the complex interplay between humoral and cell-mediated mechanisms of disease. It has also been used to describe with unprecedented resolution the dynamics of T-cell immunoreactivity as a hallmark of TED pathogenesis. The promise of scRNA-seq in further studying TED lies in its ability to potentiate novel, targeted therapeutic development for this clinically heterogenous condition.

Data availability statement

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Financial support and sponsorship

This manuscript was in part funded by a Grant in Aid of Research from Sigma Xi, The Scientific Research Honor Society (G20230315-5685) and an unrestricted grant from Research to Prevent Blindness and by an NEI core grant (P30-EY026877) given to the Stanford Department of Ophthalmology.

Conflicts of interest

The authors declare that there are no conflicts of interests of this paper.

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Supplementary Information

We have included below additional details describing the literature search algorithm used to identify the relevant publications for this review article.

Methods

The keywords listed in the Methods section were divided into two groups. Group 1 terms were disease terminology-related and included "ScRNA seq thyroid eye disease" OR "ScRNA seq Graves' Orbitopathy" OR "ScRNA seq thyroid-associated orbitopathy." The MeSH terms "single nuclei RNA seq" and "SnRNA-seq" were also used with the corresponding disease terminology to ensure that all relevant studies were included for review. Group 2 terms were human vs. animal model-related and included "ScRNA seq human thyroid eye disease" OR "single cell RNA seq murine thyroid eye disease" OR "single cell RNA sequencing animal models thyroid Graves' Orbitopathy."

The literature search was performed using the following algorithm: search for any terms in group 1 (disease terminology-related) in combination with each of the terms in Group 2 (model-related).