REVIEW

Single-cell approaches to dissect adaptive immune responses involved in autoimmunity: the case of celiac disease

Ida Lindeman[1,2] and Ludvig M. Sollid[1,2,3]✉

Single-cell analysis is a powerful technology that has found widespread use in recent years. For diseases with involvement of adaptive immunity, single-cell analysis of antigen-specific T cells and B cells is particularly informative. In autoimmune diseases, the adaptive immune system is obviously at play, yet the ability to identify the culprit T and B cells recognizing disease-relevant antigen can be difficult. Celiac disease, a widespread disorder with autoimmune components, is unique in that disease-relevant antigens for both T cells and B cells are well defined. Furthermore, the celiac disease gut lesion is readily accessible allowing for sampling of tissue-resident cells. Thus, disease-relevant T cells and B cells from the gut and blood can be studied at the level of single cells. Here we review single-cell studies providing information on such adaptive immune cells and outline some future perspectives in the area of single-cell analysis in autoimmune diseases.

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INTRODUCTION

Single-cell analysis has revolutionized many research fields, including studies of adaptive immune responses and autoimmunity. Here we will review such advances in the context of a particular disorder—celiac disease (CeD). We have chosen to structure this review around some common single-cell approaches used to dissect adaptive immune responses, and we start by giving a brief general overview of such approaches. We subsequently describe CeD with its clinical and immunological features. This common disorder, caused by a harmful immune response to cereal gluten proteins, presents with many autoimmune characteristics. We provide a systematic review of how each single-cell approach has advanced the understanding of the role of adaptive immune cells in this disease, before giving some future perspectives on single-cell analysis for other autoimmune diseases, highlighting lessons we have learned from studies of CeD.

SINGLE-CELL APPROACHES TO DISSECT ADAPTIVE IMMUNE RESPONSES: GENERAL PRINCIPLES

Detection of antigen-specific cells

Knowledge about the antigens being targeted in an immune response allows for the isolation of antigen-specific T cells and B cells using T- and B-cell receptor (TCR and BCR) binding ligands as baits (Fig. 1a). B cells can be detected with monovalent or multivalent BCR ligands.[1] However, due to low TCR-ligand affinities, the detection of T cells only became feasible with multimer technologies that increase the receptor avidity.[2,3]

Single-cell antigen immune receptor sequencing

The fast-developing field of sequencing technologies has revolutionized profiling of TCR- and BCR-repertoires through deep sequencing of amplified RNA or DNA from the variable (V)-region of immune receptor loci (AIRR-seq). Single-cell AIRR-seq (scAIRR-seq) adds chain-pairing information and enables enumeration of cells with identical receptors (Fig. 1b). Expression cloning of human monoclonal antibodies (hmAbs) from single disease-relevant plasma cells (PCs)[4] facilitates studies of binding strength, specificity, target epitopes and binding mode (Fig. 1c). Furthermore, full-length, paired TCR- or BCR-sequences makes it possible to study interaction of epitopes and paratopes, for instance by mutational and structural studies.

Lineage tracing

Since TCRs and BCRs are highly diverse molecules created by somatic rearrangements, they can be used to track ancestors of a single T cell or B cell, termed clones (Fig. 1d). Thus, identification of identical TCRs at different time points and tissues indicates a systemic, long-lasting immune response toward the target antigen. As B-lineage cells undergo somatic hypermutation (SHM), clonally related B-lineage cells have similar, but not necessarily identical, BCRs. Acquisition of mutations during affinity maturation can be computationally reconstructed into phylogenetic trees and analyzed at the clone or repertoire level.[5,6] Paired chain data from single cells gives the most accurate representation of lineage trees.

Flow cytometry

Flow cytometry is an invaluable tool to study cell-surface molecules and intracellular targets in a large number of single cells (Fig. 1e). Currently, more than 20 markers may be analyzed simultaneously by conventional flow cytometry.[7] Flow cytometry allows for rapid analysis of many million cells. Importantly, analyzed cells can be sorted and used for downstream analyses. However, some prior knowledge on marker expression is needed, and measurement of several markers at once requires correct compensation of

[1]KG Jebsen Coeliac Disease Research Centre, University of Oslo, Oslo, Norway. [2]Department of Immunology, Oslo University Hospital, Oslo, Norway. [3]Institute of Clinical Medicine, University of Oslo, Oslo, Norway. ✉email: l.m.sollid@medisin.uio.no

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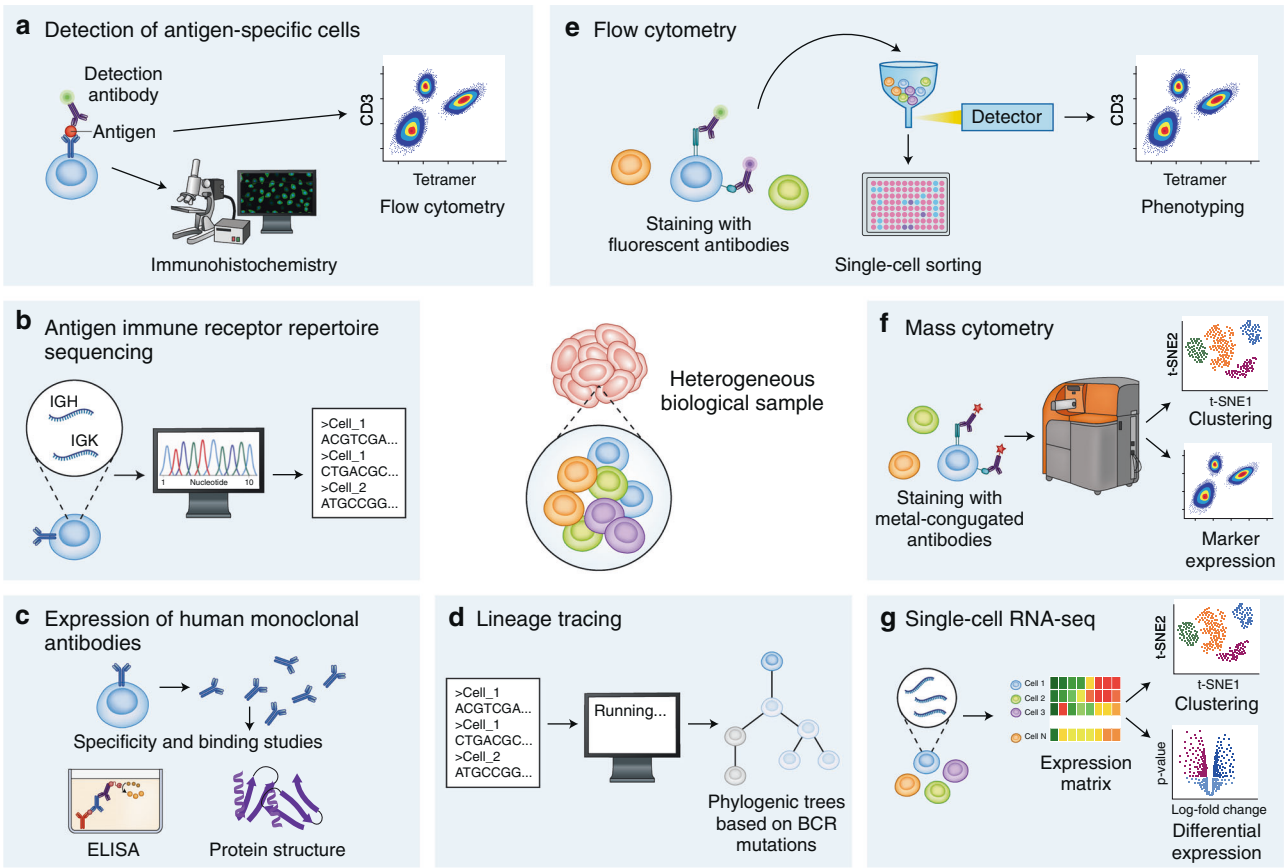

Fig. 1 Common single-cell approaches to dissect adaptive immune responses. a Antigen-specific T and B cells can be labeled using antigen-ligands as bait and visualized in situ by immunohistochemistry or ex vivo by flow cytometry or mass cytometry. **b** Various protocols are available for single-cell sequencing of antigen immune receptor repertoires from genomic DNA or RNA, yielding paired, full-length T-cell receptor (TCR) or B-cell receptor (BCR) sequences. **c** The BCR of single B cells can be expression cloned, and the resulting human monoclonal antibodies can be used to study their specificity, affinity, and binding mode. **d** BCR sequences may be used as input to computational tools for creation of phylogenetic trees to track evolving clones. **e** Flow cytometry analysis of antibody-labeled cells enables phenotyping of millions of single cells as well as sorting of single cells into plates for downstream analyses. **f** Labeling of cells with metal-conjugated antibodies allows for phenotyping of around 40 markers by mass cytometry. **g** Single-cell RNA-sequencing facilitates unbiased clustering of cells into populations based on the full transcriptional state of each cell.

fluorescent spillover between channels, which is not a trivial task. Thus, conventional flow cytometric studies have often been limited to a few markers allowing researchers to distinguish cell lineages of interest. This may change as recent progress, in particular the development of spectral flow cytometry, now allows detection of up to 40 markers in flow cytometric analysis.[8]

Mass cytometry

Mass cytometry, or cytometry by time-of-flight (CyTOF), has emerged as a potent technology enabling simultaneous analysis of ~40 markers in millions of single cells (Fig. 1f). The technology, combining the principle of flow cytometry with mass spectrometry, uses metal isotopes in place of fluorescently labeled antibodies, avoiding the need for compensation of signal spillover. Mass cytometry does however suffer from great cell loss and ideally requires prior knowledge about marker expression. Notably, the technology also allows for high-dimensional imaging.[9]

Single-cell RNA-sequencing

Single-cell RNA-sequencing (scRNA-seq) can identify and characterize subpopulations of cells in heterogeneous samples based on their full transcriptional profile (Fig. 1g).[10] scRNA-seq is especially suited for detection of rare cell types, as these cannot be identified from the average gene expression values obtained by bulk RNA-seq. In addition, scRNA-seq makes it possible to filter out contaminating cells from subsequent analyses of defined subpopulations. A plethora of library preparation protocols[11] and sequencing platforms offers large flexibility regarding throughput and sequencing depth. While no prior knowledge is needed about markers, lowly expressed genes are not always detected, and mRNA levels do not necessarily accurately represent the protein expression of a cell. scRNA-seq may however be combined with different technologies for multi-omics single-cell studies, such as index-sorting,[12] staining of proteins with barcoded antibodies,[13] or combined sequencing of transcriptomes and genomes[14] and/or epigenomes (Table 1). scRNA-seq may also be integrated with spatial transcriptomics in order to determine the spatial distribution of cell populations within a tissue and thereby identify local networks of cell communication.[15]

TCR- and BCR-sequences can be computationally reconstructed directly from scRNA-seq data,[16–23] facilitating integrated analysis of antigen specificity and phenotype. This information can also be obtained by commercial platforms, such as the Chromium Single Cell Immune Profiling solution from 10x Genomics.

CELIAC DISEASE: A FOOD HYPERSENSITIVITY DISORDER WITH MANY AUTOIMMUNE FEATURES

Celiac disease (CeD) is a common small intestinal chronic inflammatory disease driven by exposure to cereal gluten proteins

Table 1. Summary of single-cell multi-omics technologies.

Category	Measured molecules	Methods
Transcriptome and genome	RNA and gDNA	G&T-seq,[14] DR-seq,[156] SIDR,[157] TARGET-seq[158]
Transcriptome and epigenome	Poly-A mRNA and gDNA methylation	scM&T-seq,[159] scMT-seq[160]
	Poly-A RNA (nuclear) and chromatin	SNARE-seq,[161] sci-CAR,[162] Paired-seq[163]
	Poly-A RNA, gDNA methylation, and chromatin	scNMT-seq[164]
Transcriptome, genome, and epigenome	Poly-A mRNA, CNVs, and gDNA methylation	scTrio-seq[165]
Transcriptome and proteome	RNA and targeted proteins	CITE-seq,[166] PLAYR,[167] PEA/STA,[168] REAP-seq,[169] RAID,[170] INs-seq[171]
Transcriptome, epigenome, and proteome	RNA, chromatin, and targeted proteins	TEA-seq[172]
Transcriptome, genome, epigenome, and proteome	RNA, chromatin, mitochondrial DNA, and targeted proteins	DOGMA-seq[173]
Transcriptome, proteome, clonotypes, and CRISPR perturbations	Poly-A RNA, sgRNA, and targeted cell surface proteins	ECCITE-seq[174]

of wheat (gliadins and glutenins), barley (hordeins) and rye (secalins).[24] The disease varies in severity and clinical presentations with symptoms such as abdominal pain, diarrhea and malnutrition in addition to a long list of extra-intestinal symptoms.[25,26] CeD is diagnosed based on clinical, serological and histopathological data from biopsies of the proximal small intestine.[27] Patients typically present with blunted or flat intestinal villi, crypt hyperplasia and increased frequencies of lymphocytes in the lamina propria and epithelial layer.[28–30] CeD can usually be effectively treated with a life-long, strict gluten-free diet.

CeD can be classified as a food hypersensitivity disorder due to the strong immune response toward ingested gluten, but the disease also has autoimmune features such as highly disease-specific autoantibodies, targeted killing of enterocytes, and typical autoimmune genetics with a dominant role of Human Leukocyte Antigen (HLA)-genes.[31,32] The disease is virtually only seen in individuals who carry one or two of the HLA-DQ2.5, HLA-DQ2.2 or HLA-DQ8 allotypes.[33] These predisposing HLA-DQ variants are also found in many healthy subjects, so "HLA" is a necessary but not sufficient factor for CeD to develop.[34] Our current understanding of the mechanisms driving CeD is quite advanced compared to most other autoimmune diseases, and is centered around recognition of gluten-derived peptides on disease-associated HLA-DQ molecules by gluten-specific $CD4^+$ T cells.

Gluten components differ from most other ingested proteins in that they largely resist degradation by digestive enzymes due to their abundance of proline.[35] Moreover, gluten peptides are excellent targets for a posttranslational modification, termed deamidation, by the enzyme transglutaminase 2 (TG2).[36] TG2 recognizes sequence motifs frequently occurring in gluten-derived peptides to convert specific glutamine residues to glutamate. Such deamidated peptides can be good ligands for disease-associated HLA-DQ molecules, and deamidated gluten peptides (DGP) are recognized by gluten-specific $CD4^+$ T cells of CeD patients when presented on the surface of antigen-presenting cells (APCs).[37] In the lamina propria there are gluten-specific effector memory (T_{EM}) cells that drive the development of celiac lesions through secretion of proinflammatory cytokines and likely cross-talk with cytotoxic intraepithelial lymphocytes (IELs).[38,39]

CeD is also hallmarked by augmented humoral immunity, with increased numbers of lamina propria PCs and enhanced local gut immunoglobulin secretion.[40–42] Moreover, the patients have disease-specific serum antibodies to gluten[43] and TG2.[44] Antibodies to gluten is to be expected, but autoantibodies to TG2 is peculiar knowing the food-hypersensitivity nature of CeD. It is intriguing how a foreign antigen like gluten can drive autoimmune responses against TG2,[45] especially since there is little evidence for the existence of TG2-specific T cells.[38] The dual role of TG2 as the enzyme responsible for deamidation of gluten and for being the autoantigen is hardly coincidental. Production of TG2 autoantibodies conceivably can be explained by gluten-specific $CD4^+$ T cells providing help in a hapten-carrier like fashion to TG2-specific B cells via a formation of gluten:TG2 complexes.[46] The gluten-specific T cells can also provide help to gluten-specific B cells (Fig. 2). Of note, antibodies to DGP are particularly specific to the disease, and monitoring of serum antibodies to TG2 and DGP has become an essential part of the diagnostic work-up for CeD.[47] The priming of gluten-specific T cells and TG2/DGP-specific B cells likely takes place in organized lymphoid structures like the Peyer's patches, possibly with involvement of TG2 from shed enterocytes.[48] The activated T and B cells would then enter the blood circulation and home to the lamina propria, where they exert effector functions.[49]

DETECTION OF ANTIGEN-SPECIFIC CELLS IN CED

Gluten-specific $CD4^+$ T cells

Historically, stimulation of gut biopsies with gluten antigen allowed for the isolation of gluten-specific $CD4^+$ T cells, and strikingly these T cells used the disease-associated HLA-DQ variants as restriction molecules.[50] The gluten-specific $CD4^+$ T cells are epitope-specific, and many different gluten epitopes exist.[51] A few epitopes, termed immunodominant, mount the strongest recall responses and also give responses in most patients.[38]

Recombinant soluble HLA-DQ molecules with bound gluten peptides represent the ligands for gluten-specific $CD4^+$ T cells, and these molecules when multimerized allow for isolation of the cells at bulk population or single-cell level (Fig. 3a).[52,53] Such HLA-DQ:gluten tetramers have been used to identify T cells specific for the immunodominant gluten epitopes DQ2.5-glia-α1a,[54,55] DQ2.5-glia-α2,[55–57], DQ2.5-glia-ω1,[54] DQ2.5-glia-ω2,[57] DQ2.5-hor3[58], or DQ8-glia-α1[53,59] from small intestinal biopsies[53,54,56,58,59] and/or peripheral blood[54–59] of untreated patients, treated patients or treated patients after gluten challenge. Bead enrichment of tetramer-binding cells furthermore enables detection of gluten-specific $CD4^+$ T cells in blood of treated patients, suggesting that tetramer analysis can be a diagnostic tool even for individuals who are already eating a gluten-free diet.[60–62]

While many studies of gluten-specific T cells have focused on a particular T-cell epitope, others have used a pool of immunodominant HLA-DQ:gluten tetramers to capture a bigger picture of the T-cell response toward gluten.[62–65] Collectively, the studies have shown that gluten-specific $CD4^+$ cells are selectively present in CeD patients, can readily be detected in the blood and gut after a short-term oral gluten challenge, and decrease in frequency in treated patients.

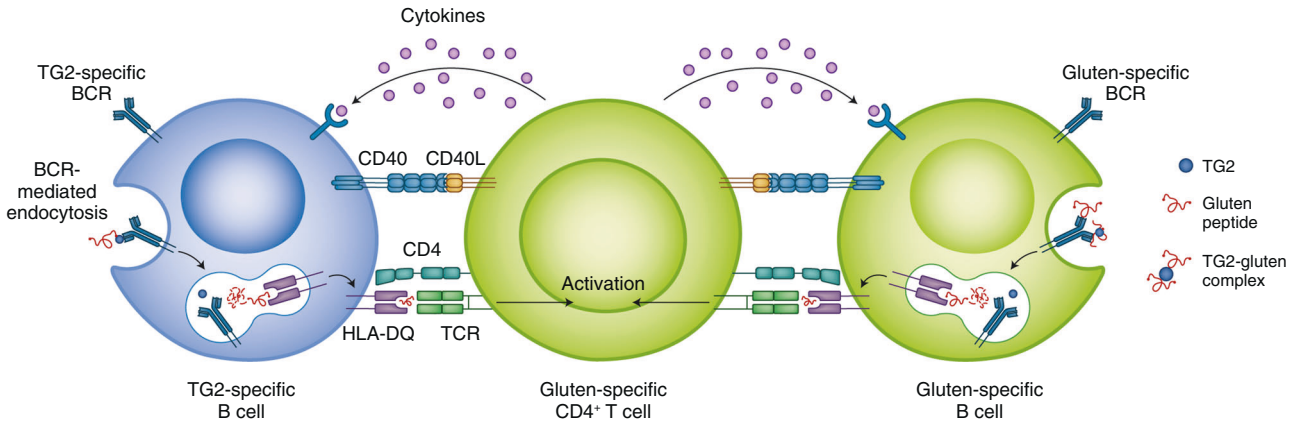

Fig. 2 T-cell and B-cell interaction in celiac disease. Conceivably, gluten-specific CD4$^+$T cells can provide help to both transglutaminase 2 (TG2)-specific and gluten-specific B cells. The help to TG2-specific B cells is possible by involvement of TG2:gluten complexes. In a hapten-carrier like fashion, TG2-specific B cells can take up such complexes by BCR-mediated endocytosis, and internalized gluten peptides containing T-cell-relevant gluten epitopes can then be presented to gluten-specific CD4$^+$T cells bound to the disease-associated HLA-DQ molecules. Gluten-specific B cells also take up gluten peptides, either alone or as part of TG2:gluten complexes, in a similar manner and present them to gluten-specific CD4$^+$T cells. Antigen presentation to the CD4$^+$T cell activates both the B cells and T cells, and leads to proliferation of both populations and induction of differentiation pathways.

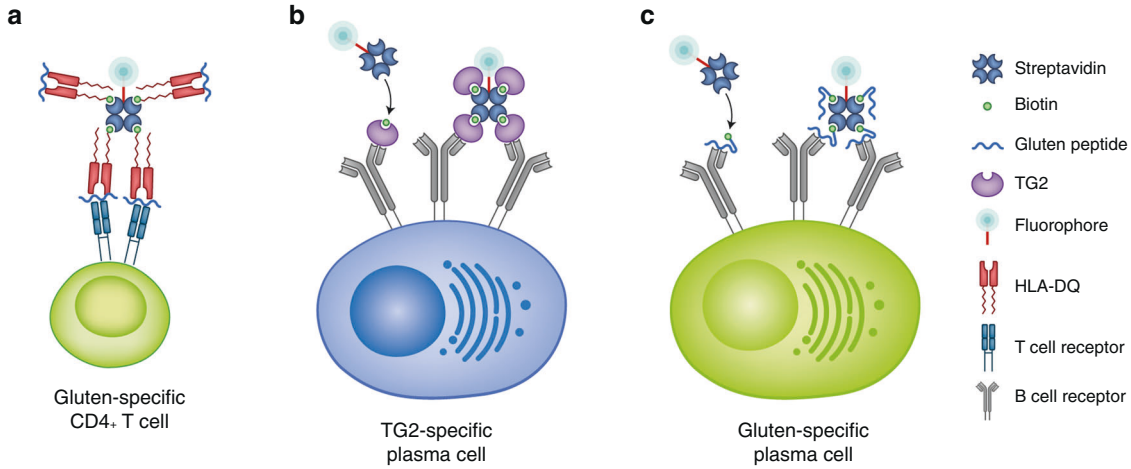

Fig. 3 Visualization of disease-relevant lymphocytes in CeD. a Staining of gluten-specific CD4$^+$T cells with pMHC class II tetramers. **b** Staining of transglutaminase 2 (TG2)-specific plasma cells with biotinylated TG2 monomers, visualized by binding of fluorescently labeled streptavidin, or TG2:streptavidin tetramers. **c** Staining of gluten-specific plasma cells immune-relevant gluten peptides as monomers or tetramers, visualized as in (**b**).

TG2-specific B-lineage cells

Detection of TG2-specific serum autoantibodies in CeD patients indicates that TG2-specific antibody-secreting cells must be present in these individuals. Using TG2 as bait, TG2-specific PCs have been detected both by immunohistochemistry of duodenal biopsy cryosections and flow cytometry of lamina propria lymphocytes (Fig. 3b). TG2-specific PCs make up on average 10% of all duodenal PCs in untreated CeD.[66] While TG2-specific antibodies were frequently identified in the intestine, serum antibody titers did not correlate with the frequency of intestinal TG2-specific PCs, indicating that part of the serum autoantibodies in CeD may be produced outside of the small intestine.[67,68] This notion was further corroborated by comparative proteomic analysis of TG2-specific (as well as DGP-specific) antibodies in serum and those produced in mucosal explants, revealing that the antigen-specific serum IgA antibodies were mainly monomers, in contrast to the typical mucosal-derived dimeric form.[69]

TG2-specific PCs are selectively found in CeD patients.[66] Interestingly, they have been detected prior to villous atrophy, and increase in frequency during overt disease.[68] This finding fits with autoantibodies commonly being present before the onset of symptoms in CeD.[26] Although TG2-specific PCs decrease in frequency on a gluten-free diet,[66–68,70,71] some cells can be identified even in well-treated patients having been gluten free for years.[67,70,71]

Little is known about TG2-specific B-cell populations in blood, presumably due to their low abundance. Rare TG2-specific memory B cells have been detected indirectly in CeD patients,[70] and we are currently characterizing such cells at a single-cell level.

Gluten-specific B-lineage cells

Gluten-specific intestinal PCs have been identified with a high staining specificity (75–80%) by using tetramerized synthetic gluten peptides as antigen (Fig. 3c).[72] Approximately 1% of intestinal IgA$^+$ PCs of untreated CeD patients bound a peptide with the sequence PLQPEQPFP, while on average 0.5% recognized a 33-mer peptide (LQLQPFPQPELPYPQPELPYPQPELPYPQPQPF).[72] The frequency of gluten-specific PCs is also dependent on gluten

exposure.[71] As with TG2-specific PCs, some gluten-specific PCs exist even after years on a gluten-free diet.[71]

ANTIGEN RECEPTOR SEQUENCING

Gluten-specific CD4$^+$ T cells

Biased TCR repertoires against specific pHLA class II complexes have been described in various diseases and can involve preferential V-gene usage, chain pairing, conserved CDR3 motifs, and/or identical TCR sequences across patients.[73,74] scAIRR-seq has revealed biased V-gene usage and chain pairing for gluten-specific CD4$^+$ T cells recognizing each immunodominant epitope. In addition, TCRs with similar V-gene usage and sequences have been detected across individuals, terming such TCRs as public receptors.[75]

The TCR repertoires of gluten-specific CD4$^+$ T cells display extensive use of public TCRs. T cells recognizing the homologous DQ2.5-glia-α1a or DQ2.5-glia-ω1 epitopes have a shared V-gene bias with preferred usage of *TRAV35*, *TRAV4*, *TRAV12-2*, and *TRBV29-1* or *TRBV20-1* and *TRBV5-1*.[54] DQ2.5-glia-α2-specific T cells preferentially use *TRAV26*, *TRAV4*, and *TRBV7*,[56,57,76,77] while T cells specific for the homologous DQ2.5-glia-ω2 epitope preferentially use *TRAV4* and *TRBV4*.[57] The DQ2.5-glia-α2-specific TCRs that use the canonical *TRAV26-1/TRBV7-2* pair moreover typically have a conserved CDR3β arginine motif.[56,57,75,76] T cells specific for the immunodominant barley-derived gluten epitope DQ2.5-hor-3 display a frequent use of *TRAV26-1* and *TRBV20-1* or *TRBV29-1*.[58] Similar V-gene biases have also been reported for DQ8-restricted gluten epitopes with DQ8-glia-α1-specific T cells preferentially using *TRBV9/TRAV26-2* with a conserved CDR3 arginine motif.[53,59]

CD8$^+$ αβ and γδ IELs

In contrast to the well-characterized gluten-specific CD4$^+$ T cells, the antigen specificities of CD8$^+$ αβ and γδ IELs that increase in frequency in CeD are still elusive.[78] A scAIRR-seq study by Han and colleagues using TCRβ or TCRγ sequencing of activated (CD38$^+$) and gut-homing (CD103$^+$) CD8$^+$ αβ T cells and γδ T cells in blood of CeD patients 6 days after a gluten challenge revealed a high degree of clonal expansion and preferential usage of *TRBV7-8*, *TRBV7-9*, and *TRBV28* among the CD8$^+$ αβ T cells and *TRDV1* (i.e. Vδ1) among the γδ T cells.[77] scAIRR-seq of γδ IELs and peripheral blood gut-homing γδ T cells showed bias toward *TRGV4* (i.e. Vγ4) and *TRDV1* usage in healthy individuals, and demonstrated that the CeD patients displayed a more diverse γδ TCR repertoire with the *TRGV4* bias being absent.[79] Interestingly, the shift in the γδ TCR repertoire was nearly identical for untreated and treated patients. Unlike γδ T cells, no irreversible change of the TCR repertoire related to CeD has been identified for CD8$^+$ αβ T cells.[80] A recent study,[80] in contrast to a previous report,[77] showed that the CD8$^+$ αβ T cell repertoire is highly polyclonal and diverse in untreated compared to treated CeD and controls.

TG2-specific B-lineage cells

A single-cell study of the BCRs of 60 intestinal TG2-specific PCs indicated a biased V-gene usage and limited number of somatic mutations.[66] These characteristic features were further investigated by high-throughput AIRR-seq of the immunoglobulin heavy-chain variable (IGHV) region in gut and blood,[70] large-scale scAIRR-seq of intestinal IgA PCs[81] and computational BCR sequence reconstruction from scRNA-seq data of intestinal PCs,[71] providing ample evidence of preferential V-gene usage and chain pairing, limited somatic mutations and affinity maturation, clonal expansion, low diversity, and skewed isotype usage.

TG2-specific PCs have a particularly prominent bias for the *IGHV5-51:IGKV1-5* pair, but there are biases also for several other heavy- and light-chain V-gene pairs such as *IGHV3-48:IGLV5-45* and *IGHV4-34:IGKV1-39* (Fig. 4a).[81] In addition, TG2-specific PCs are skewed toward the IgA isotype,[72] in particular IgA1,[69,71] compared

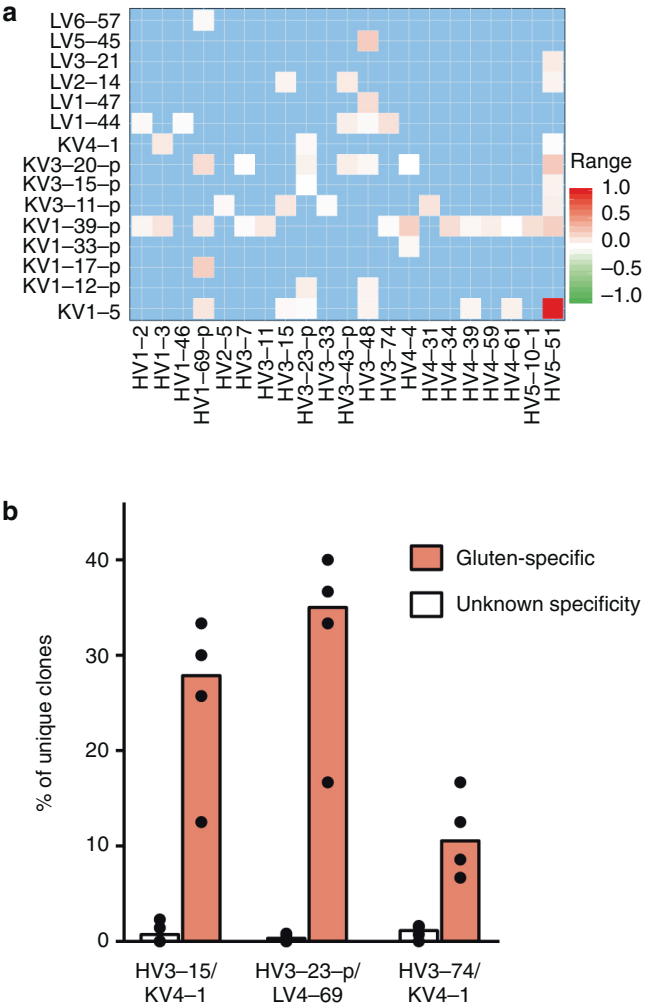

Fig. 4 Biased usage of particular V-gene pairs in TG2-specific and gluten-specific plasma cells (PCs) in celiac disease. a Preferential V-gene pairing of TG2-specific intestinal PCs compared to PCs of unknown specificity. The figure is reproduced from[81]. **b** Stereotyped V-gene pairs making up the major part of the repertoire of gluten-specific intestinal PCs. The figure is based on data published in[71]. Each dot represents a CeD patient, and bars represent the median frequency among the patients.

to the general PC population in CeD and controls.[82] The frequencies of somatic mutations correlate for heavy- and light-chain both for TG2-specific PCs and the general PC population, indicating that the low acquisition of mutations is related to the developmental history of the cell.[71,81] Typically, many of the mutations retain the physicochemical properties of the amino acid, arguing for limited affinity maturation and conservation of germline-encoded residues.[81]

Gluten-specific B-lineage cells

Gluten-specific B-lineage cells have been less studied than their TG2-specific counterparts, as they are infrequent in comparison. A study of 38 single gluten-specific PCs[72] and IGHV bulk sequencing of IgA$^+$ PCs showed repertoire features bearing striking resemblance to TG2-specific PCs, with restricted V-gene usage and limited mutations.[83] Recently, paired BCR sequences of 232 gluten-specific intestinal PCs were reconstructed from scRNA-seq data.[71] These studies point to a highly stereotypic antibody response to gluten, mostly made up of the chain pairings *IGHV3-23/IGLV4-69*, *IGHV3-15/IGKV4-1*, or *IGHV3-74/IGKV4-1* (Fig. 4b).

Mutational analysis indicated that despite correlation between mutational load in the heavy- and light-chain, their propensity to undergo SHM compared to the general PC population varies with V-gene usage.[71] This study also corroborated flow cytometry data[72] showing that gluten-specific PCs are enriched for the IgM isotype compared to TG2-specific PCs.

ANTIGEN RECEPTOR INTERACTIONS WITH ANTIGEN

TCR:pHLA-DQ binding

From single CD4$^+$ T cells of CeD patients, several disease-relevant TCR structures in ternary complexes of TCR:pMHC have been resolved, both for TCRs restricted by HLA-DQ2.5,[84] HLA-DQ2.2[85] and HLA-DQ8[53,59,86]. These studies have explained the structural basis for V-gene biases as well as the basis for the conserved CDR3β arginine motif in TCRs using *TRAV26-1/TRBV7-2*. What remains unexplained at this point is the general deamidation dependence of the gluten-specific CD4$^+$ T cells in CeD.

TG2-specific BCRs/antibodies

Limited mutation of TG2-specific antibodies indicates good affinity in the germline configuration. Indeed, reverting a few TG2-specific hmAbs to their germline sequences has demonstrated that while being reduced, the affinity to TG2 was still relatively high.[66] Thus, affinity maturation does not seem to be essential for development of CeD.

CeD patient-derived TG2-specific hmAbs were found to preferentially target the enzyme in its catalytically active conformation, and the antibodies recognize mainly four N-terminal conformational epitopes that are only accessible on soluble TG2 and correlate with *IGHV* gene usage of the antibodies.[66,87,88] Notably, the antibodies did not cross-react with other transglutaminases.[87] The structural basis for binding to TG2 has been studied with an epitope 1-specific hmAb using *IGHV5-51/IGKV1-5*, revealing several residues in both heavy- and light chain to be involved in epitope recognition.[81,89]

TG2-specific antibodies can be crosslinked by TG2 when expressed as IgD or IgM, but not IgA1 or IgG1, possibly favoring recruitment of PCs from naive or IgM memory cells.[66] Interestingly, the functional effects of antibody binding to TG2 vary depending on the epitope, potentially explaining biases toward certain epitopes.[90] For instance, antibodies recognizing C-terminal epitopes have been demonstrated to disrupt TG2-mediated crosslinking.[88] Furthermore, in vitro engineered B cells specific for N-terminal, but not C-terminal, epitopes could take up TG2-gluten complexes and act as APCs to gluten-specific T cells. Thus, the N-terminal epitope bias could result from B- and T-cell collaboration.[88]

Gluten-specific BCRs/antibodies

The epitopes recognized by gluten-specific serum antibodies have been characterized by measuring the reactivity to gluten peptide libraries.[91,92] Strikingly, the B-cell epitopes are located close to or overlapping with T-cell epitopes within the gluten protein sequences. Of note, DGPs elicit higher antibody reactivity compared to their native counterparts,[92,93] indicating a role of deamidation for epitope recognition in CeD.

Expression cloning of 38 gluten-specific hmAbs from single intestinal PCs confirmed that the antibodies typically bound gluten peptides related to T-cell epitopes, and many preferentially bound DGP.[72] While the hmAbs were not polyreactive, some exhibited cross-reactivity with different DGPs. Binding of the hmAbs to synthetic gluten peptide was blocked in the presence of patient-derived serum, suggesting recognition of common gluten epitopes.[72] A study based on the same hmAb panel demonstrated that the antibodies commonly bind to long deamidated peptide fragments harboring multivalent B-cell epitopes, TG2 recognition sequences, and various T-cell epitopes.[94] As for TG2-specific antibodies, there seems to be a correlation between V-gene usage and gluten epitope specificity.[72]

The stereotyped antibody response toward gluten has made it possible to study the structural basis for antibody recognition of DGP in CeD. Crystal structures for two antibodies using *IGHV3-23/IGLV4-69* or *IGHV3-15/IGKV4-1* reveal that germline-encoded residues in heavy- and light-chain are involved in binding to the DGP with sequence PLQPEQPFP.[83] Some of these residues may be important across a set of gluten-specific antibodies. For instance, DGP-specific antibodies using *IGHV3-15/IGKV4-1* or *IGHV3-74/IGKV4-1* share a conserved arginine residue in the heavy chain (R55), which is essential for binding of PLQPEQPFP.[71,83] R55 is nonpolymorphic in only five IGHV genes, although seven additional genes have one or more R55-containing alleles.[95] Importance of conserved germline residues could argue for a role of polymorphisms in the antibody response in CeD. As a proof of concept, it was demonstrated that a patient-derived gluten-specific antibody using the R55-containing *IGHV4-4*07* allele paired with *IGKV4-1* could not bind to DGP when mutating R55 to the allelic variant E55.[71]

LINEAGE TRACING

For a long time, it was unknown whether the circulating gluten-specific T cells were representative of the larger population of CD4$^+$ T cells residing in the lamina propria. Bulk and scAIRR-seq of tetramer-sorted CD4$^+$ T cells in CeD revealed the two compartments to be composed of nearly identical T-cell clonotypes both in untreated and treated CeD, indicating a common origin and trafficking of clones between blood and gut.[63]

Disease-relevant T-cell clonotypes in CeD are not only shared between gut and blood, but also at different time points. This has been shown for gluten-specific CD4$^+$ T cells[63] and also for CD103$^+$γδ and CD8$^+$ T cells.[96] Further, by comparing single T cells from intestinal biopsies taken years apart, gluten-specific CD4$^+$ T-cell clonotypes were demonstrated to persist for decades in CeD patients on a gluten-free diet.[63] Moreover, the recall response during gluten challenge was dominated by preexisting clonotypes. Thus, the disease-specific TCR repertoire remains stable over years in treated patients. While long-lived intestinal memory T cells exist,[97,98] occasional trace amounts of gluten likely contribute to these stable T-cell responses.

To date, only two studies have traced the evolution of intestinal CeD-specific PC lineages at a single-cell level, revealing limited affinity maturation and frequent expansion of cells with identical BCRs within each clone.[71,81] Interestingly, PCs expressing different isotypes and longevity-associated phenotypes were found within individual clones, indicating ongoing immune responses toward TG2 and DGP involving isotype class switching and potentially reactivation of memory B cells.[71] Combined lineage tracing of PCs and memory B cells could be an important tool to answer whether reactivation of memory B cells contributes to the pool of TG2- or DGP-specific PCs. The only published study to date using this approach was at the bulk level, indicating a limited overlap between TG2-specific PCs and circulating memory B cells.[70] Clonal overlaps have also been shown by a combination of bulk IGHV sequencing of intestinal PCs and proteomic analysis of the heavy-chain CDR3 region of serum antibodies.[69] Detailed studies at single-cell resolution, preferably from multiple time points and tissues, on and off a gluten-free diet or during gluten challenge, are warranted.

SINGLE-CELL PHENOTYPING OF ADAPTIVE IMMUNE CELLS IN CELIAC DISEASE

Several studies have aimed to phenotype adaptive immune responses in CeD at a single-cell resolution to better understand their function and identify potential therapeutic targets. In recent

Table 2. Single-cell omics studies of adaptive immune cells in the small intestine and blood of adult healthy individuals and patients with celiac disease.

Reference	Sample	Donors	Target population	Staining of CeD-relevant antigen-specific cells	Technology
Healthy gut					
Nair et al.[123]	Proximal jejunum resections and/or peripheral blood	Controls	B-lineage cells	No	Mass cytometry
Celiac disease					
Atlasy et al.[129] (preprint)	Mucosal duodenal biopsies (Lamina propria and epithelium)	CeD patients Controls	Immune cells	No	scRNA-seq
Lindeman et al.[71]	Mucosal duodenal biopsies (Lamina propria)	UCeD patients TCeD patients Controls	Plasma cells	Yes	scRNA-seq (Smart-seq2)
Han et al.[77]	Peripheral blood or mucosal duodenal biopsies	UCeD patients CeD patients on gluten challenge Controls	T cells	No	Mass cytometry
van Unen et al.[121]	Mucosal duodenal biopsies and/or peripheral blood	UCeD patients TCeD patients RCDII patients EATLII patient Controls	Immune cells	No	Mass cytometry
Christophersen et al.[122]	Mucosal duodenal biopsies and/or peripheral blood	UCeD patients TCeD patients CeD patients on gluten challenge SSc patients SLE patients Influenza patients Controls	T cells	Yes	Mass cytometry

CeD Celiac disease, *UCeD* untreated celiac disease, *TCeD* treated celiac disease, *RCDII* refractory celiac disease type II, *EATLII* enteropathy-associated T cell lymphoma type II, *SSc* systemic sclerosis, *SLE* systemic lupus erythematosus.

years, powerful single-cell "omics" studies have emerged, shedding new light on T- and B-cell responses at the protein and mRNA levels (Table 2).

Assessing phenotypes by immunohistochemistry and flow cytometry

T-cell populations. Altered compositions of T-cell subtypes have been identified in the intestine and blood of CeD patients compared to controls.[99–101] An immunohistochemistry-based study demonstrated that intestinal $CD4^+$ T cells lack expression of the proliferation marker Ki-67, but express the activation marker CD25 upon gluten exposure.[102]

Gluten-specific $CD4^+$ cells. Tetramer visualization of gluten-specific $CD4^+$ T cells has allowed further dissection of the phenotypes of disease-relevant T cells in CeD. Circulating DQ2.5-glia-α1a-specific T cells of CeD patients undergoing a gluten challenge expressed integrin β7, indicating that they are gut-homing.[55] Further, phenotyping of gluten-specific $CD4^+$ T subsets in blood by tetramer staining of naive, T_{EM} and central memory (T_{CM}) cells revealed increased frequencies of gluten-specific T_{EM} cells, in particular gut-homing cells, in both untreated and treated CeD compared to controls.[61] It was reported that a large proportion of circulating gluten-specific $CD4^+$ T cells are T_{regs}, based on their expression of FOXP3 and CD39, and a paradoxical higher number of these cells in CeD patients was explained by the cells having impaired suppressive functions.[103] Others found increased frequencies of gluten-specific T_H17 cells in CeD.[104] While very few gluten-reactive T_{CM} or T_{EM} cells can be detected in controls, gluten-specific naive T cells are found in controls as well as in CeD patients.[61] Functional studies of HLA-DQ:gluten tetramer-positive cells later revealed that healthy HLA-DQ2.5$^+$ controls lack gluten-specific regulatory and memory T cells.[105]

IELs. While their antigen specificity is unknown, a fraction of IELs express Ki-67 after gluten exposure, indicating proliferation.[102] Activated ($CD38^+$), gut-homing ($CD103^+$) γδ and $CD8^+$ αβ T cells have also been detected in blood following gluten challenge.[77] $CD8^+$ αβ T cells constitutively express activating natural killer receptors,[106,107] with the highest expression of the activating receptor NKG2D found in the intestine of untreated compared to treated CeD patients.[108] While most small intestinal γδ IELs express the activating natural cytotoxicity receptors NKp46 and/or NKp44, their expression is significantly reduced in CeD patients, even after years on a gluten-free diet.[109] Furthermore, patients on a gluten-free diet have been shown to have higher frequencies of $CD8^+$ γδ IELs expressing the inhibitory receptor NKG2A and intracellular TGF-β1 compared to untreated patients.[110] While $CD8^+$ αβ IEL frequencies normalize on a gluten-free diet, this is not the case for γδ IELs.[111]

The dynamic regulation of different T-cell populations during gluten challenge is under some debate. While Han et al. and others have reported increased frequencies of both gluten-specific $CD4^+$ T cells, γδ T cells, and $CD8^+$ αβ T cells in blood after gluten challenge,[77,112] it was recently reported that not all patients respond with an increase of all three T-cell subsets in blood, although all patients with an increase of γδ and/or $CD8^+$ αβ T cells also showed elevated gluten-specific $CD4^+$ T-cell frequencies.[96]

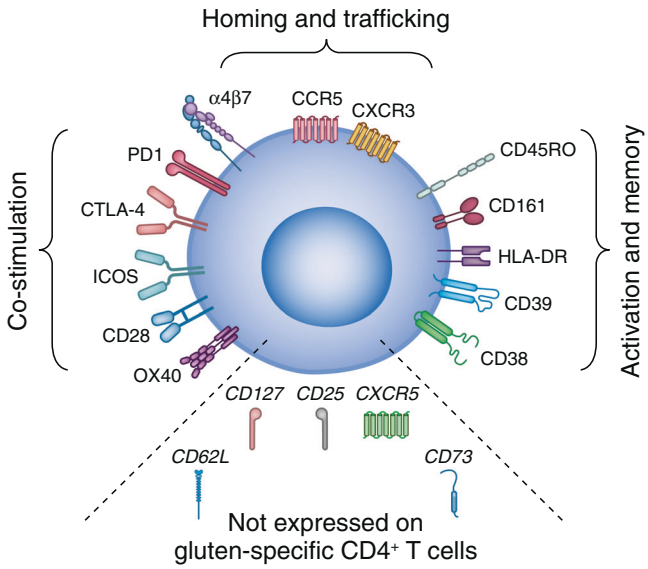

Fig. 5 Phenotypic markers expressed by gluten-specific CD4$^+$ T cells. The figure is adapted from Dahal-Koirala et al.[175] (with permission from Elsevier) and is based on data obtained by mass cytometry.[122]

The discrepancies between studies could potentially be ascribed to differences in gluten challenge and laboratory protocols, and illustrates a need for standardized challenge protocols.[113,114]

Intestinal PCs. The large population of intestinal antibody-secreting cells in CeD patients and controls are mainly end-differentiated PCs rather than proliferating plasmablasts, as they express CD138 and lack CD20 or intracellular staining for Ki-67.[4,66] PCs of the IgA and IgM isotypes express a functional BCR,[4,115] and at least a proportion of intestinal PCs retain surface expression of HLA class II[4,66,116] and costimulatory[116] molecules.

Intestinal PCs can, despite previous beliefs, survive for years. Notably, retrospective carbon-14 dating of bulk-sorted populations based on the cell surface proteins CD19 and CD45 has revealed three populations of short-, intermediate-, and long-lived PCs with a median age of 0, 11, and 22 years, respectively.[117] Along the same theme, it was recently demonstrated that most PCs in untreated CeD, in particular TG2- or DGP-specific PCs, have a short- or intermediate-lived phenotype, possibly reflecting the large expansion of PCs in the celiac lesion.[71] This was true also for patients staying gluten free for a few years, while patients treated for more than a decade mainly had long- and intermediate-lived TG2- or DGP-specific PCs. The high frequency of short-lived TG2- or DGP-specific PCs in patients treated for several years was surprising, and possibly indicates that these patients unknowingly may have been exposed to minute amounts of gluten.[118]

Memory B cells. No study has characterized the phenotype of memory B cells in CeD.

Regulatory B cells. Regulatory B cells (B_{regs}) have been implicated in various autoimmune conditions.[119] Although TG2- or gluten-specific B_{regs} have yet to be identified in CeD, the general population of peripheral blood CD24^{hi} CD38^{hi} B_{regs} has been shown to be larger in CeD patients compared to controls or patients with the autoimmune disease Hashimoto's thyroiditis.[120] The same authors have proposed that these B_{regs} may be functionally defective in CeD based on their limited ability to produce interleukin (IL)-10 upon in vitro stimulation with CpG.

Phenotyping by mass cytometry

A study by van Unen et al.[121] used mass cytometry to analyze the overall composition of innate and adaptive immune cells in intestinal biopsies and paired PBMCs from healthy individuals and patients with inflammatory intestinal diseases, including CeD. The study confirmed previous reports of an increased γδ IEL population in CeD, and further identified differences in innate lymphocyte populations.

T cells. A limited number of mass cytometry studies have focused on the T-cell response in CeD. This technology was used in a study on activated and gut-homing γδ and CD8$^+$ αβ T cells after gluten challenge.[77] A later study combined HLA tetramer staining with mass cytometry to phenotype gluten-specific CD4$^+$ T cells.[122] Interestingly, this study revealed that gluten-specific CD4$^+$ T cells have a distinct phenotype with upregulation of activation markers CD38, CD161, CD28, HLA-DR and OX40 as well as CXCR3, CD39, and PD-1 both in the gut and blood (Fig. 5). Remarkably, the same CD4$^+$ T-cell phenotype was seen in patients with systemic sclerosis or systemic lupus erythematosus, suggesting that disease-driving CD4$^+$ T cells in autoimmune conditions with unknown target antigens could be identified based on their phenotype.

B-lineage cells. To date, no studies using mass cytometry to dissect B-cell responses in CeD have been published. Although the study by van Unen et al. contained some B-lineage cells, it lacked markers to dissect the B-lineage response. While studies of antigen-specific B-lineage cells in CeD are warranted, characterization of B-cell subsets and their marker expression in the healthy intestine and blood may serve as a reference.[123] Mass cytometry has already been used in combination with staining of antigen-specific cells to profile rotavirus-specific intestinal and circulating B-lineage cells.[123]

Single-cell RNA-sequencing

scRNA-seq has the potential to build on previous bulk RNA-seq studies of CeD[122,124–128] by dissecting the cellular composition of celiac lesions, identifying rare cell types potentially involved in disease pathology, and excluding contaminating cells from detailed analyses of specific cell populations. With the goal of characterizing the composition of all immune cells in the celiac lesion, Atlasy and colleagues sequenced immune cells from duodenal biopsies from celiac patients and controls.[129] Corroborating previous findings, the authors reported increased numbers of total lamina propria immune cells and CD3$^+$ IELs in untreated CeD patients compared to controls.

T cells. scRNA-seq studies of T cells with known antigen specificity in CeD are currently lacking; the only RNA-seq data available for gluten-specific T cells is from bulk populations, largely agreeing with mass cytometry data.[122] While no information on antigen specificity is included, the preprint by Atlasy et al. found the majority of CD4$^+$ T cells to differ transcriptionally in CeD patients and controls. Furthermore, most of the epithelial and lamina propria CD8$^+$ T cells had an activated phenotype in CeD compared to controls, with expression of cytotoxic activity-associated genes such as *KLRC2* and *KLRD1*.

B-lineage cells. A recent scRNA-seq study of intestinal PCs in CeD and controls largely corroborated bulk RNA-seq data,[124] and revealed that TG2- and DGP-specific PCs differ to some extent from PCs of unknown specificity.[71,124] At least a subset of the PCs, regardless of antigen specificity, expressed low levels of mRNA transcripts for HLA class II and costimulatory molecules. Interestingly, the general intestinal PC population in CeD patients may differ transcriptionally from controls.[71,124] Differences between

PCs in untreated and treated CeD and controls may indicate potentially pathogenic roles of PCs, including non-disease-specific ones, in CeD.

PCs typically have been defined as $CD27^{high}$, in addition to high expression of CD38 or CD138. Likewise, CD27 has been widely used as a memory B cell marker. These definitions are however changing, as $CD27^{-/dim}$ B-cell subsets are gaining more interest,[130,131] especially in the context of autoimmune diseases. Indeed, our RNA-seq studies revealed that *CD27* expression is lower in TG2- or DGP-specific PCs compared to other PCs, as well as in PCs of CeD patients compared to controls. Enrichment of $CD27^{-/dim}$ TG2-specific PCs and circulating memory B cells has also been observed at the protein level (our unpublished results).

T-CELL FUNCTIONS IN CED

Helper cell functions

The pile of evidence resulting from extensive CeD research points to gluten-specific $CD4^+$ T cells as key players in CeD pathogenesis by providing help to B cells for antibody production[132] and by supporting cytotoxic IELs that kill enterocytes.[38] Bulk RNA-seq data of HLA-DQ:gluten tetramer-staining cells of the gut lesion revealed expression of *IL21* and *CXCL13*, cytokines hallmarking follicular helper cells providing B-cell help,[133] yet a lack of CXCR5 expression speaks against a germinal center function of these cells.[122] Of note, the interaction of T cells with B cells does not only affect the B-cell side resulting in antibody production, it also leads to T-cell activation and clonal expansion. Studies of transfectants where CeD patient-derived TCRs and BCRs were introduced demonstrated that gluten-specific T cells can be activated by TG2-specific B cells in the presence of TG2:gluten complexes.[66]

Regulatory T cell function

T_{regs} are a subset of $CD4^+$ T cells, important for maintaining peripheral tolerance. It has therefore been speculated that in the healthy state a response toward gluten is kept in check by T_{reg} activity.[134–136] While expression of the transcription factor FOXP3 is a hallmark of regulatory T cells in mice, robust markers for human T_{regs} are lacking. T_{regs} in humans are often characterized as $FOXP3^+ CD25^+ CD127^{low}$, albeit FOXP3 can be expressed by activated human $CD4^+$ T cells without regulatory functions.[137] A report of high frequencies of gluten-specific $CD4^+$ memory T cells in CeD expressing typical T_{reg} markers following stimulation of blood samples with gluten is puzzling. These cells displayed an impaired suppressive function after in vitro expansion, suggesting that T_{reg} dysfunction may play a role in CeD.[103] The results of the study contrast extensive phenotyping of gluten-specific T cells by mass cytometry, which revealed that while some gluten-specific T cells express FOXP3, these cells do not express CD25.[122] This discrepancy between studies could be explained by the finding that in vitro stimulation of gluten-specific $FOXP3^+ CD25^-$ T cells induce CD25 in these cells.[122]

Healthy HLA-DQ2.5 expressing individuals have rare $CD4^+$ T cells in blood that stain with HLA-DQ:gluten tetramers. These cells do not express FOXP3 or CD25 suggesting that they are not T_{regs}.[105]

Cytotoxic functions and other functions by IELs

$CD8^+$ αβ IELs. IELs normally contribute to immune protection of the gut by preventing pathogen entry into the epithelium by maintaining barrier integrity. In CeD, however, the expanded population of $CD8^+$ αβ IELs is thought to be responsible for killing of epithelial cells, contributing to the formation of the disease lesion. The cytokine interleukin IL-15 is considered particularly important for the expansion of the $CD8^+$ αβ IELs[138,139], yet other cytokines, like IL-2 and IL-21, are likely also implicated.[140,141]

It has been suggested that $CD8^+$ IELs in CeD are activated in a TCR-independent fashion by binding of ligands to activating natural killer receptors.[38] This hypothesis is supported by the observed polyclonal TCR repertoire and lack of V-gene bias of $CD8^+$ IELs in CeD,[80] shift in expression toward activating natural killer receptors in untreated CeD,[110] and ability to respond to stress signals via activating natural killer receptors independent of the TCR specificity.[136,138] Thus, it is probable that $CD8^+$ IELs sense intestinal stress and inflammation, become licensed to kill enterocytes by help of pro-inflammatory $CD4^+$ T cells, and upregulate their cytolytic activity.

γδ IELs. γδ IELs play an important role in immune surveillance of the intestinal epithelium.[142] Their role in CeD pathogenesis is poorly understood. IELs are likely recruited to the intestinal epithelium in CeD as a response to stress or damaged epithelial cells. It has been suggested that a subset of γδ IELs, characterized by expression of CD8 and NKG2A, may play a regulatory role in CeD, based on their ability to limit the cytotoxic potential of IL-15-induced $CD8^+$ αβ IELs.[110] Further it was demonstrated that gluten-induced inflammation leads to depletion of naturally occurring $Vγ4^+/Vδ1^+$ IELs with innate cytolytic properties and specificity for enterocyte-expressed butyrophilin-like (BTNL) molecules BTNL3/BTNL8.[109] Reduced enterocyte expression of BTNL8 and loss of $Vγ4^+/Vδ1^+$ IELs was found to be accompanied by the expansion of gluten-sensitive, interferon-γ-producing $Vδ1^+$ IELs that do not recognize BTNL3/BTNL8 and which potentially have a pathogenic role.

B-CELL AND PLASMA-CELL FUNCTIONS IN CED

Antibody secretion

While autoantibodies may have a clear pathogenic role in some autoimmune diseases, it is uncertain whether antibodies specific to TG2 or gluten are pathogenic as circulating immunoglobulins.[143,144] The finding that gluten-specific PCs are enriched for IgM-expressing cells compared to TG2-specific PCs[71,72] could have implications for the possible pathogenicity of these antibodies in CeD, as IgM antibodies can activate complement through the classical pathway.[145]

Antigen presentation

While circulating immunoglobulin may not be pathogenic in CeD, immunoglobulin as BCR on antigen-presenting B cells likely plays an important pathogenic role. In CeD the interaction between antigen-presenting B cells and T cells probably happens in organized lymphoid structures, like Peyer's patches, isolated lymphoid follicles or mesenteric lymph nodes. The rapid decline of serum antibody levels when patients commence a gluten-free diet[146,147] and the limited degree of SHM in TG2-specific[66,70,71,81] and DGP-specific[71,72,83] PCs at the same time argues for short-lived germinal center or extrafollicular responses.[148] Whether there is also interaction of B-lineage cells with T cells in the lamina propria is still an open question. At this site, PCs dominate and there are very few B cells present.[149] PCs have been thought to downregulate surface BCR and HLA class II expression, and the cells have hence not been considered as APCs. Importantly, however, IgA- and IgM-expressing PCs, by contrast to IgG-expressing PCs, retain functional cell-surface BCR.[4,115] A low/medium expression of HLA class II molecules detected both on the protein and mRNA level in a proportion of intestinal PCs,[71,116,124] and an expression of costimulatory molecules[116] thus raise the possibility that the expanded lamina propria PC population may act as APCs in CeD. By use of pHLA-specific hmAbs it was demonstrated that PCs are the dominating cell type presenting gluten peptides on their surface.[116] Thus, intestinal PCs have the machinery needed to present relevant T-cell epitopes on HLA-DQ molecules on their surface. Importantly, actual T-cell recognition of and activation by PC-displayed gluten peptides is yet to be demonstrated.

Cytokine and chemokine secretion

It is unknown whether B-lineage cells secrete molecules contributing to the development of celiac lesions, as the most abundant B-lineage cells in CeD are PCs, whose functions beyond antibody secretion are not well studied. Recent literature contains some reports of cytokine and chemokine secretion by PCs, particularly by regulatory PCs.[119] While there is no evidence of intestinal regulatory PCs in CeD by scRNA-seq, at least a subset of intestinal PCs, regardless of specificity, produce mRNA for *IL15* and *IL16*,[71,124] and small populations of PCs express high levels of *CCL3* and *CCL4*.[71] This is interesting, given the assumed vital role of IL-15 in CeD pathogenesis,[138,139] and as IL-16 attracts and activates $CD4^+$T cells.[150] It is however not known if mRNA expression of these cytokines translates to expression of the secreted cytokines; translation of *IL15* is for instance under extensive regulation,[151–153] and isoforms with different functions and subcellular localization exist.[153,154]

CONCLUDING REMARKS AND FUTURE DIRECTIONS

In this review we have discussed how single-cell approaches have provided invaluable knowledge about the specificity, binding mode, phenotype, and function of antigen-specific adaptive immune cells in the well-characterized disease CeD. As community efforts such as the Human Cell Atlas[155] have gained substantial interest in recent years, we expect that new mass cytometry and scRNA-seq studies will continue to build the basis for detailed maps of the phenotypes of T- and B-cell populations found in various tissues of autoimmune patients and healthy individuals. Single-cell multi-omics studies will likely contribute to new knowledge about CeD pathogenesis and may uncover novel potential therapeutic targets. Of note, integration of spatial transcriptomics data with scRNA-seq and protein expression could reveal networks of communicating immune cells and their interactions with epithelial and stromal cells in the celiac lesion, improving our understanding of the mechanisms underlying CeD pathology.

The lack of knowledge of the disease-driving antigens remains a major hurdle for further progress in understanding the pathogenesis of many autoimmune diseases. Single-cell analysis may well provide help to overcome this hurdle. The finding of a rare phenotypic profile in $CD4^+$T cells that is shared across diseases may offer an avenue to define the disease-driving T cells and the disease-driving antigen in diseases where this is unknown.[122] Further, single-cell analysis allows to define clonally expanded populations of T and B cells. In the lesion of the disease, culprit T and B cells are likely to belong to the clonally expanded cells. If there among clonally expanded cells are stereotypic patterns shared across patients, the likelihood that these cells are disease-relevant would increase. For instance, if the autoantigen of celiac disease was unknown, single-cell sequencing of PCs in the celiac lesions probably would have identified clonally expanded *IGHV5-51/IGKV1-5* PCs. Expression cloning of such *IGHV5-51/IGKV1-5* monoclonal antibodies, with subsequent identification of their antigen ligands, would then identify TG2 as the target antigen. If also clonally expanded T cells exist, showing a productive interaction for transfectants armed with TCRs and BCRs of the clonally expanded cells would represent a further step toward identifying the disease-driving antigen. Further certainty would come if the $CD4^+$T cells use disease-associated HLA allotypes as their restriction element. So far, we lack examples that demonstrate the success of such approaches. Notwithstanding, we are sure we have just seen the beginning of single-cell analysis of autoimmune conditions. Exciting years lie ahead of us.

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AUTHOR CONTRIBUTIONS

I.L. and L.M.S. drafted and revised the manuscript. I.L. created the figures.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Correspondence and requests for materials should be addressed to Ludvig M. Sollid.