Dietary gluten triggers concomitant activation of CD4$^+$ and CD8$^+$ αβ T cells and γδ T cells in celiac disease

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Celiac disease is an intestinal autoimmune disease driven by dietary gluten and gluten-specific CD4$^+$ T-cell responses. In celiac patients on a gluten-free diet, exposure to gluten induces the appearance of gluten-specific CD4$^+$ T cells with gut-homing potential in the peripheral blood. Here we show that gluten exposure also induces the appearance of activated, gut-homing CD8$^+$ αβ and γδ T cells in the peripheral blood. Single-cell T-cell receptor sequence analysis indicates that both of these cell populations have highly focused T-cell receptor repertoires, indicating that their induction is antigen-driven. These results reveal a previously unappreciated role of antigen in the induction of CD8$^+$ αβ and γδ T cells in celiac disease and demonstrate a coordinated response by all three of the major types of T cells. More broadly, these responses may parallel adaptive immune responses to viral pathogens and other systemic autoimmune diseases.

autoimmunity | mucosal immunity

Celiac disease (CD) is a common autoimmune disease with an estimated prevalence of 1% among people of European ancestry. It is characterized by small intestinal mucosal injury and nutrient malabsorption in genetically susceptible individuals due to dietary gluten ingestion. CD4$^+$ T cells bearing αβ T-cell receptors (TCRs) are critical in the pathogenesis of the disease, as it occurs almost exclusively in HLA-DQ2– or HLA-DQ8–positive individuals (1, 2). CD-associated gluten peptide CD4$^+$ T-cell epitopes have been discovered, and HLA-DQ2/8–restricted gluten-reactive CD4$^+$ T cells have been identified in individuals with CD (3–5). Nonetheless, no gluten-induced enteropathy is seen in humanized mouse models expressing HLA-DQ2 and a gluten-specific TCR (6, 7), suggesting that CD4$^+$ T cells alone are unable to induce tissue damage in CD (1, 2).

An increase in intestinal intraepithelial lymphocytes (IELs), composed of both CD8$^+$ αβ T cells and γδ T cells, is a hallmark of CD. IELs are responsible for the detrimental consequences of CD, including tissue damage and lymphoma development. CD8$^+$ TCRαβ$^+$ IELs (CD8$^+$ IELs) function as effectors in protective immunity to pathogens (8), and in CD they assume a natural killer (NK)-like phenotype to kill intestinal epithelial cells in a manner independent of TCR specificity (9). In rare instances, IELs in CD may transform into enteropathy-associated T-cell lymphoma (EATL), an aggressive lymphoma with a very poor prognosis (10). EATL cells have been shown to have clonal TCRαβ or TCRγδ rearrangements, indicating that either CD8$^+$ IELs or γδ IELs may give rise to lymphoma (11, 12).

Despite intense efforts, gluten-specific IELs in CD have not been readily identified, and there is no significant genetic association of CD with any HLA class I alleles. Moreover, the cytolytic function of IELs in CD can be induced irrespective of their TCR specificity (9). Thus, although the link between dietary gluten and the CD4$^+$ response is well-established, the link between dietary gluten and the recruitment and activation of CD8$^+$ or γδ IELs in celiac disease is unknown. Furthermore, the role of the antigen specificity of IELs in CD is unclear. Here we find that CD8$^+$ and γδ T cells bearing gut-homing receptors are induced by gluten ingestion in CD patients in parallel with gluten-specific CD4$^+$ T cells, and they bear TCR sequences that indicate an antigen-focused response. This indicates that antigen-specific responses of all three of these major T-cell types play a role in this disease.

Results

Celiac disease requires the continuous presence of dietary gluten. Reintroducing dietary gluten to celiac patients who are on a gluten-free diet induces large numbers of gluten-specific CD4$^+$ T cells in the peripheral blood 6 d later (4, 5, 13). These cells express the β7 integrin receptor, indicating that they will home to the intestine (5). They also express the activation marker CD38 and lack the expression of CD62L, consistent with an effector phenotype (14). This is generally thought to represent the initiation of an immune response to gluten, and captures activated gluten-reactive CD4$^+$ effector T cells en route from mesenteric lymph nodes or gut-associated lymphoid tissue to the intestine. In an effort to better characterize the context of this immune response, we studied peripheral blood T cells in celiac patients undergoing gluten challenge by time-of-flight mass cytometry (CyTOF) (15), which allows for the independent assessment of many more cellular parameters (currently >40) than fluorescence-based flow cytometry. Indeed, we observed an increase in gluten peptide/HLA-DQ2 tetramer-positive CD4$^+$ T cells in the peripheral blood in all five HLA-DQ2$^+$ celiac patients on day 6 following gluten challenge (Fig. 1 *A* and *C*). Unexpectedly, we also observed a large increase in the number of peripheral blood CD8$^+$ αβ and γδ T cells expressing the intestinal epithelial-homing markers αE (CD103) and β7 integrins (16) and the activation marker CD38 (Fig. 1 *A* and *B* and Table S1) at this same time point. These cells were not detected in healthy HLA-DQ2$^+$ controls, who underwent oral gluten challenge after at least 1 mo on a gluten-free diet.

The kinetics with which these CD8$^+$ and γδ T cells appear is the same as that of gluten-specific CD4$^+$ T cells, peaking at day 6 after gluten challenge and declining to the baseline by day 14 (Fig. 1*C*). A similar response was also detected in two celiac patients who underwent rechallenge after returning to a gluten-free diet for at least 1 mo (Fig. 1 *A* and *B* and Table S1).

The magnitude of the peripheral blood gluten-specific CD4$^+$ T-cell response is known to be quite variable (4). Similarly, the extent of the αEβ7$^+$CD38$^+$ T-cell response varied between patients, ranging from 0.37% to 10.17% of total peripheral blood CD8$^+$ and from 0.06% to 18.61% of total peripheral blood γδ T cells (Fig. 1*B* and Table S1). One celiac patient (celiac 2) had αEβ7$^+$CD38$^+$CD8$^+$ and γδ T cells above background levels on day 0, but showed a further increase following gluten challenge.

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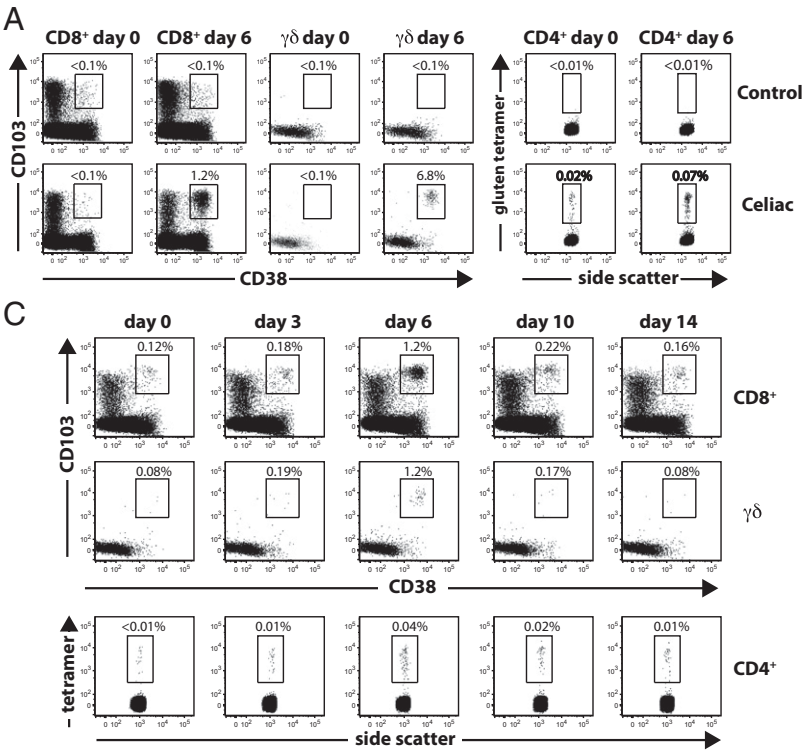


Fig. 1. Induction of activated, gut-homing CD8$^+$ αβ and γδ T cells in peripheral blood of celiac patients following oral gluten challenge. (*A*) Representative FACS analysis of CD8$^+$ αβ and γδ T-cell (*Left*) and CD4$^+$ T-cell (*Right*) response to oral gluten challenge in CD vs. nonceliac control. Expansion of CD103$^+$ (αE integrin), CD38$^+$, and gluten tetramer$^+$ CD4$^+$ T-cell populations is seen on day 6 in CD. Most CD38$^+$CD103$^+$ cells also express β7 integrin; only CD103 staining is depicted here. (*B*) Relative frequency of αEβ7$^+$CD38$^+$ CD8$^+$ T cells as a percentage of total CD8$^+$ cells (*Top*) and relative frequency of αEβ7$^+$CD38$^+$ γδ cells as a percentage of total γδ T cells (*Bottom*). (*C*) Time course showing relative percentage of CD38$^+$CD103$^+$ CD8$^+$ (*Top*), CD38$^+$CD103$^+$ γδ (*Middle*), and gluten tetramer$^+$ CD4$^+$ (*Bottom*) in the same patient at the indicated time points following oral gluten challenge. Parallel recruitment of CD38$^+$CD103$^+$ and gluten tetramer$^+$ cells peaks on day 6 before returning to baseline.

The individual with the lowest detectable response (celiac 6) was an HLA-DQ8$^+$ celiac patient whose disease was diagnosed incidentally by intestinal biopsy, had equivocal antibody test results, and has always been clinically asymptomatic to gluten. Three individuals with active celiac disease, as determined by ongoing symptoms and positive autoantibody titers, were found to have αEβ7$^+$CD38$^+$CD8$^+$ and γδ T-cell proportion below background levels of 0.05% and 0.01%, respectively (Fig. S1). This aspect is similar to the absence of gluten-specific CD4$^+$ T cells in peripheral blood of patients with active celiac disease (4, 5). Also, although plasma cells secreting anti-gluten and autoantibodies are present in celiac intestinal lesions (17–19), we did not detect a similar increase in intestinal-homing B cells (not shown). This is consistent with reports indicating that tissue transglutaminase-specific B cells were undetectable in the peripheral blood of celiac patients (19, 20). In summary, dietary gluten induces the activation and concomitant peripheral blood presence of CD4$^+$ and CD8$^+$ αβ T cells and γδ T cells with gut-homing potential in celiac patients who have been on a gluten-free diet (Fig. 1 and Table S1).

Gluten-reactive CD4$^+$ T cells in the peripheral blood of celiac patients have been shown to be CD38$^+$CD62L$^-$, suggesting that they are gut-bound effector cells (7). CyTOF analysis showed that αEβ7$^+$CD38$^+$CD8$^+$ T cells are CD38$^+$, CD45RO$^+$, CD27$^-$, CD28^{low}, CD62L$^-$, and CCR7^{low} (Fig. 2). This phenotype closely resembles the phenotype of CD8$^+$ T cells isolated from duodenal tissue biopsy specimens of patients with active celiac disease (Fig. 2). CD8$^+$ T cells of this phenotype have been reported to represent differentiated effectors and, accordingly, αEβ7$^+$CD38$^+$CD8$^+$ T cells resemble peripheral blood effector memory CD8$^+$ T cells (Fig. S2) (15, 21, 22). αEβ7$^+$CD38$^+$ γδ cells are predominantly CD45RO$^+$ and CD27$^-$, mirroring intestinal γδ cells from celiac biopsies (Fig. S3). CD45RO$^+$, CD27$^-$ γδ T cells are thought to be memory cells (23).

The fact that gluten ingestion induces the activation of gluten-specific CD4$^+$ T cells in CD is well-established. However, whether or not the CD8$^+$ and γδ IELs induced in the intestine are responding to specific antigens is unknown. To address this question, we performed single-cell TCR sequencing, which provides a nonbiased means to assess the TCR repertoire without requiring expansion of T-cell clones in culture (24). Single T cells were sorted into 96-well PCR plates from peripheral blood samples of celiac patients following gluten challenge. *TCRβ* or *TCRγ* genes were amplified by a series of nested PCRs, and PCR products were directly sequenced.

We were able to perform sequencing on single T cells with high efficiency. We sorted and sequenced 90 single tetramer-positive CD4$^+$ T cells recognizing the gluten epitope DQ2-α-II from the blood of two celiac patients on day 6 after oral gluten challenge (Table S2). Sequences were successfully obtained from 77/90 (86%) of wells into which single T cells were sorted. Consistent with published sequences of DQ2-α-II–reactive T cells from blood and tissue (25), the majority (79%) of unique TCRβ sequences of individual DQ2-α-II–tetramer$^+$ T cells used TRBV7-2 and most (74%) contained the described dominant arginine in position 5 of the CDR3β loop (Table S2), thus validating our methodology.

We then sequenced αEβ7$^+$CD38$^+$CD8$^+$ and γδ T cells isolated from celiac patients on day 6 following gluten challenge. αEβ7$^+$CD38$^+$CD8$^+$ T cells, sequenced in five celiac patients, and αEβ7$^+$CD38$^+$ γδ T cells, sequenced in three celiac patients, were found to have a high degree of clonal expansion that was not observed in CD8$^+$CD45RO$^+$ control T cells (Fig. 3). αEβ7 T cells were sequenced in celiac patients who underwent repeat gluten challenge to determine whether both challenges would elicit a similar responding TCR repertoire. Indeed, identical TCRβ and TCRδ clones and similarity in frequency of common clones were found in the two gluten challenges of these patients who underwent repeat challenge after returning to a gluten-free diet for at least 1 mo (Fig. S4).

We next evaluated sequences from αEβ7$^+$CD38$^+$CD8$^+$ and γδ T cells to determine whether we could observe a convergence of TCR features among distinct TCR sequences. To evaluate convergence, we analyzed the nonredundant, unique TCR repertoire of αEβ7$^+$CD38$^+$ T cells. For a particular MHC–peptide, specific CD8$^+$ T-cell responses are often biased toward the use of a particular *TCRVβ* gene (26). We initially examined *TCRVβ* gene use. Even individuals of significantly different genetic

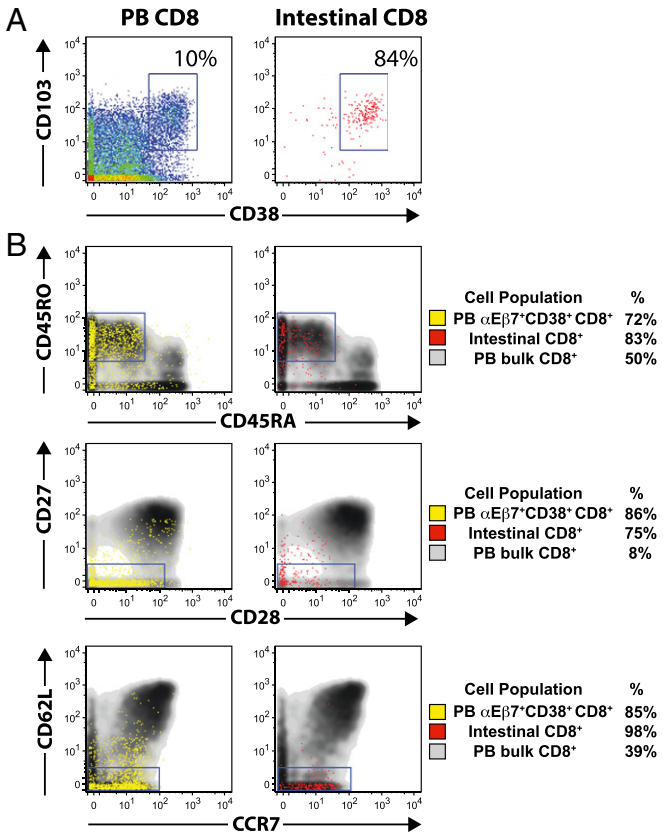


Fig. 2. Peripheral blood αEβ7$^+$CD38$^+$CD8$^+$ T cells induced by oral gluten challenge express surface markers of effector memory cells and resemble intestinal epithelial CD8$^+$ T lymphocytes from celiac mucosal biopsies. (*A*) CyTOF analysis of total peripheral blood (PB) CD8$^+$ from a gluten-challenged individual (*Left*) and total intestinal CD8$^+$ T cells from a celiac patient with active disease (*Right*) with respect to CD103 and CD38 expression. (*B*) CyTOF analyses of peripheral blood αEβ7CD38$^+$CD8$^+$ T cells (yellow) and total intestinal CD8$^+$ T cells (red) are overlaid on total peripheral blood CD8$^+$ T cells. Peripheral blood αEβ7$^+$CD38$^+$CD8$^+$ and celiac intestinal CD8$^+$ cells are predominantly CD38$^+$CD45RO$^+$CD45RA$^-$CD27$^-$CD28^{low}CD62L$^-$CCR7$^-$, consistent with an effector memory phenotype.

backgrounds share similar frequency of V gene use in their TCR repertoire, indicating that skewing within a particular population of cells is not attributable to genetic variation in baseline V gene use (27). When assessing the nonredundant TCRβ repertoire of αEβ7$^+$CD38$^+$CD8$^+$ T cells in celiac samples, we found significant overrepresentation of particular V regions in multiple celiac samples compared with unselected healthy controls (Fig. S5 *A* and *B*).

Most of the peptide specificity of TCRβ is determined by the CDR3 loop, which is usually positioned over the antigenic peptide (28, 29). We then determined whether convergence could be observed within CDR3β motifs, focusing on groups using *TCRVβ* genes that were overrepresented in a nonredundant sampling within a particular individual and had members that were clonally expanded. Strikingly, in αEβ7$^+$CD38$^+$CD8$^+$ T cells, we found four separate examples where identical TCRβ proteins used different DNA sequences (Fig. 4 *A* and *B*). In three of these instances, the identically convergent TCRβ occurred in the same patient, and represented a dominantly expressed TCRβ in that individual. In the other instance, the identical TCRβ occurred in different patients (Fig. 4*A*).

Additionally, within TCRVβ sequences using TRBV7-8, TRBV7-9, and TRBV28, we could identify characteristic amino acid motifs in the center of the CDR3β that were very common within celiac αEβ7$^+$CD38$^+$CD8$^+$ T cells compared with healthy reference CDR3β sequences (30) (Fig. 4). For instance, the GN motif at positions 6–7 within the CDR3 region of TCRβ clones using TRBV7-9 was highly enriched, occurring in 16 out of 40 unique (nonredundant) TCRβ clones, while occurring in only 12/9,584 of TCRβ clones using TRBV7-9 within the reference database ($P < 0.0001$) (Fig. 4 *A* and *C*). In patient 4, this motif occurred in 14 of 19 unique TCRβ clones, and 5 of these unique clones converged on two identical TCRβs. This motif also occurred in two other patients, who converged upon an identical TCRβ. TCRβ clones using TRBV7-8 similarly converged on a GT motif at position 6–7, which occurred in 17 out of 29 unique TCRβ clones, in contrast to only 43/4,546 TRBV7-8–containing TCRβ clones within the reference database ($P < 0.0001$) (Fig. 4 *B* and *C*). In all instances where the same TCR was formed using distinct VDJ rearrangements within the same patient, there were at least two nucleotide changes within the CDR3, making a PCR or sequencing error improbable.

We applied a similar analysis to αEβ7$^+$CD38$^+$ γδ T cells. Intestinal γδ T cells are appreciated to be heavily biased toward TRDV1 use (31). Consistent with this, the majority (80%, 150/188) of unique αEβ7$^+$CD38$^+$ TCRδ sequences from CD patients use TRDV1 (Table S3). We analyzed CDR3δ sequences using TRDV1 to determine whether convergent motifs could be seen in celiac patients. For comparison, we sequenced TCRδ from bulk small intestinal γδ T cells from a person without celiac disease and bulk blood γδ T cells from nine different control patients, obtaining 18,579 unique TCRδ sequences using TRDV1. The most highly expanded sequence, which was present in 76/152 total sequences, shared the CxxxxxPxLGD motif with five other unique CDR3δ sequences across two patients. This motif was rare in reference sequences, occurring in only 50/18,579 unique sequences ($P < 0.0001$; Fig. 5 *A* and *C*). We also found that the amino acid motif CxxxxxxxYWGI was highly enriched within TCRDV1$^+$ CDR3δ in αEβ7$^+$CD38$^+$ γδ cells compared with reference TCRDV1$^+$ γδ T-cell sequencing, occurring in all three celiac patients at a total frequency of 14/152 unique sequences while only present in 115/18,579 unique reference sequences ($P < 0.0001$; Fig. 5 *B* and *C*).

The high clonality of αEβ7$^+$CD38$^+$ T cells, the similarity of TCR repertoire upon a second gluten challenge, and the conservation of CDR3 motifs in different T-cell clones suggest that both CD8$^+$ αβ and γδ T cells are activated in an antigen-specific manner in response to dietary gluten.

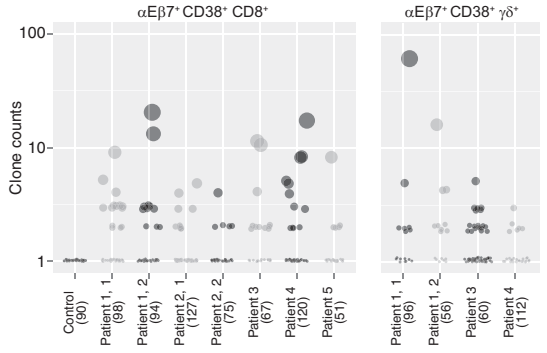


Fig. 3. Single-cell TCR sequencing of peripheral blood αEβ7$^+$CD38$^+$CD8$^+$ and αEβ7$^+$CD38$^+$ γδ T cells reveals clonal expansion upon gluten challenge in celiac disease. αEβ7$^+$CD38$^+$CD8$^+$ TCRs were sequenced in five separate patients following gluten challenge, two of whom underwent rechallenge. αEβ7$^+$CD38$^+$ γδ TCRs were sequenced in three patients, one of whom underwent rechallenge. Each individual dot represents a distinct TCR clone. The size of dots and the position along the *y* axis, plotted on a log scale, indicate the relative frequency of a particular clone. The total number of clones sequenced in each patient is indicated in parentheses.

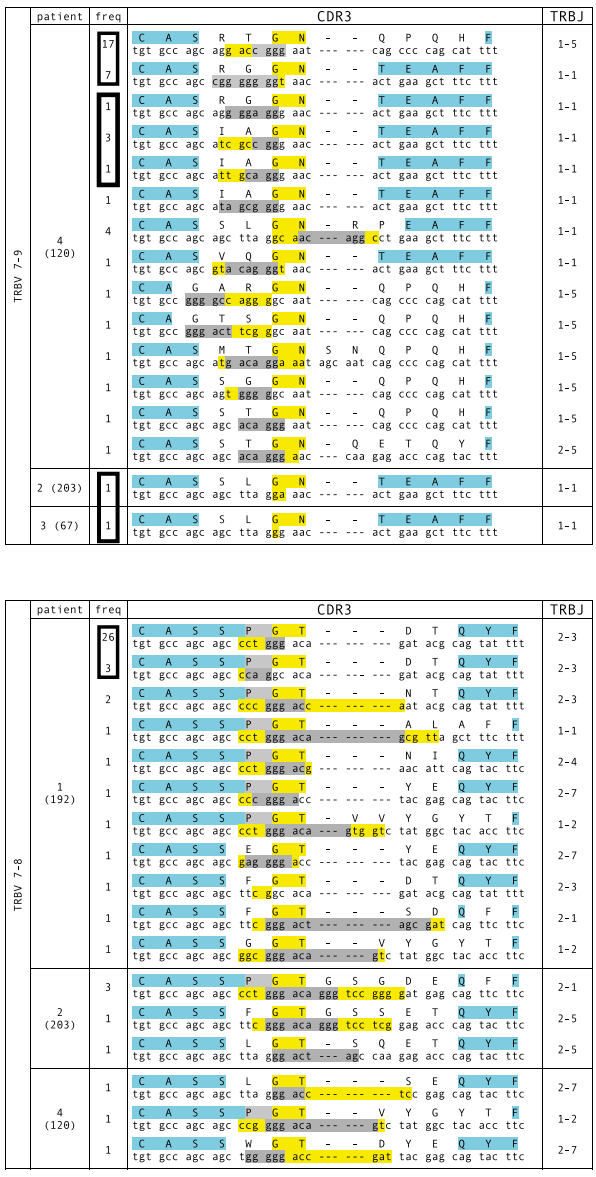
Discussion

In CD, dietary gluten induces the infiltration of T cells in the small intestine and the destruction of intestinal epithelial cells. We find that along with the induction of gluten-specific $CD4^+$ cells, the reintroduction of dietary gluten to celiac patients on a gluten-free diet induces the peripheral appearance of large numbers of activated $CD8^+$ and γδ T cells expressing gut-homing markers. These findings are consistent with the supposition that these T cells are activated and imprinted with gut-homing potential in secondary lymphoid organs by dendritic cells presenting gut-derived antigens (32). Like peripheral blood gluten-specific $CD4^+$ T cells, these cells express surface markers consistent with memory or effector cells, indicating that they are programmed as such before gut recruitment. This suggests that at least some of the pathogenic IELs in CD are purposefully activated and recruited to the gut. Importantly, these cells respond with a very focused TCR repertoire, indicating that they are selected in an antigen-specific manner before entering the intestine.

The presence of inflammation has long been postulated to promote the loss of tolerance, and prevailing models of CD pathogenesis propose that IELs are activated as a result of inflammation that is initiated by gluten-specific $CD4^+$ cells. The inflammatory cytokine IL-15 is up-regulated within celiac intestinal mucosa, and has been implicated in promoting inflammation through diverse means, including impairing regulatory T-cell generation promoting NK-like function of $CD8^+$ IELs, and enabling the expansion of IELs (9, 33). $CD8^+$ IELs have been shown to demonstrate cytotoxicity through stimulation by IL-15 and activation through NK receptors including CD94 and NKG2D (9, 34). Whereas $\alpha E\beta7^+CD38^+CD8^+$ T cells clearly show markers of effector cells and are capable of IFN-γ production, they largely do not express perforin, CD57, or higher levels of NKG2D (Fig. S6). Therefore, it is possible that tissue factors, including IL-15, are further required for cytotoxicity.

The function of γδ IELs is more poorly understood. In human CD, both cytotoxic and anti-inflammatory functions have been attributed to subsets of γδ IELs (35, 36). In mice, γδ IELs appear to be constitutively activated with high cytotoxic potential at baseline (37). However, they express both activating and inhibitory NK receptors, and it has been suggested that the combination of these NK receptors can keep the effector functions of γδ IELs in check but enable them to be readily switched on. Thus, both $CD8^+$ and γδ IEL cell populations may ultimately mediate tissue destruction through NK receptors and require tissue-derived factors. However, we find that $\alpha E\beta7^+CD38^+$ T cells express markers of differentiated effector cells before gut recruitment, and their appearance parallels the appearance of gluten-reactive $CD4^+$ T cells in blood, rather than occurring later. Also, although increased numbers of IELs and mildly increased levels of IL-15 are present in celiac patients on a gluten-free diet (38), the recruitment we describe precedes significant intestinal inflammation and tissue damage, which only reliably occur histologically after 2–4 wk of continuous gluten exposure (39). These findings suggest that IELs in CD are not simply activated as bystanders as a consequence of gut inflammation.

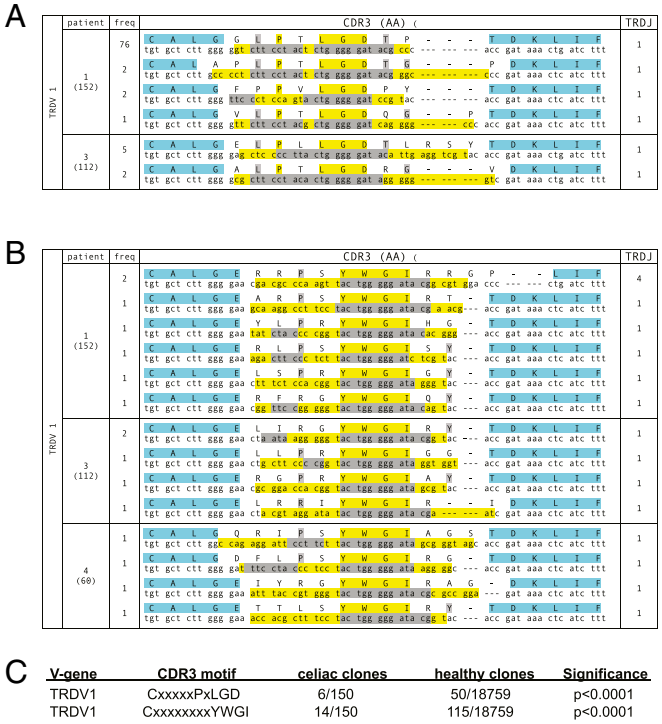
As celiac IEL populations are induced by gluten, a long-standing question has been whether their TCRs recognize gluten. Despite extensive study, gluten-derived peptide epitopes recognized by $CD8^+$ T cells in CD have not been apparent, and there is no significant genetic association of CD with particular HLA class I alleles. Therefore, it is generally thought that IELs do not mediate tissue damage through gluten recognition. Nevertheless, one group has identified a class I gluten epitope recognized by $CD8^+$ T cells isolated from CD intestinal mucosa (40). If the $\alpha E\beta7^+CD38^+CD8^+$ T cells we describe are responding to gluten, this would imply a rapid and efficient cross-presentation of gluten on MHC class I. Besides gluten, other possibilities for IEL ligands include self-antigens or infectious pathogens. The possibility of self-antigen recognition is supported by the very selective destruction of intestinal epithelial cells and the presence of autoantibodies, including antibodies to tissue transglutaminase (10, 41, 42). The role of an infectious cofactor in CD has been proposed based on epidemiologic data showing that neonatal infection seems to predispose individuals to the development of CD (43).



C

V-gene	CDR3 motif	celiac clones	healthy clones	Significance
TRBV7-9	CxxxxGN	12/36	12/9584	p<0.0001
TRBV7-8	CxxxxGT	17/28	43/4546	p<0.0001
TRBV28	CxxxF	9/37	47/1144	p<0.0001

Fig. 4. Convergent $\alpha E\beta7^+CD38^+CD8^+TCR\beta$ CDR3 motifs are found among clones within the same celiac patient and across different patients following gluten challenge. (*A* and *B*) Convergent motifs CxxxxGN (*A*) and CxxxxGT (*B*) are seen in TCRβ clones using TRBV7-9 and TRBV7-8, respectively. The frequency of each clone is indicated and the total number of T cells sequenced in the patient is indicated in parentheses. The protein sequence with the corresponding DNA sequence is shown. Within the protein sequence, yellow indicates absolutely conserved amino acids, gray indicates relatively conserved (≥50%) amino acids, and blue indicates conserved amino acids that are encoded within the V or J genes. Within the DNA sequence, nucleotides in yellow are formed through N or P addition, whereas nucleotides in gray are encoded by D genes. Boxes around frequency numbers highlight distinct clones sharing identical protein sequences. (*C*) Convergences of motifs seen in TCRβ clones using TRBV7-9, TRBV7-8, and TRBV28 are statistically significant compared with reference control TCRβ sequences.



C

V-gene	CDR3 motif	celiac clones	healthy clones	Significance
TRDV1	CxxxxxPxLGD	6/150	50/18759	p<0.0001
TRDV1	CxxxxxxxxYWGI	14/150	115/18759	p<0.0001

Fig. 5. Convergent αEβ7$^+$CD38$^+$TCRδ CDR3 motifs are found among clones within the same celiac patient and across different patients following gluten challenge. (*A* and *B*) Convergent motifs CxxxxxPxLGD (*A*) and CxxxxxxxxYWGI (*B*) are seen in TCRδ clones using TRBV1. The frequency of each clone is indicated and the total number of T cells sequenced in the patient is indicated in parentheses. The protein sequence with the corresponding DNA sequence is shown. Within the protein sequence, yellow indicates absolutely conserved amino acids, gray indicates relatively conserved (≥50%) amino acids, and blue indicates conserved amino acids that are encoded within the V or J genes. Within the DNA sequence, nucleotides in yellow are formed through N or P addition, whereas nucleotides in gray are encoded by D genes. (*C*) Convergences of motifs seen in TCRδ clones using TRBV1 are statistically significant compared with reference control TCRδ sequences.

This process through which these three T-cell subsets are synchronously mobilized and recruited to intestinal tissue clearly has implications in immunity to infections. The development of autoimmunity in CD likely represents a misdirected application of processes that are meant to be protective. Due to the well-established dependence of CD on the CD4$^+$ T-cell response, the coordinated T-cell response we describe here presumably depends upon gluten-specific CD4$^+$ T cells. In this context, multiple aspects of the effector CD8$^+$ T-cell responses to viruses have been shown to depend upon CD4$^+$ T-cell help, including the primary effector response, the generation of memory, and recruitment to sites of infection (44–47). This process has been termed "licensing," referring to the ability of CD4$^+$ T cells to license cognate effector CD8$^+$ T-cell responses. Here we speculate that CD4$^+$ T cells may be "licensing" self-antigen–specific CD8$^+$ T cells to become activated and recruited to the intestine, subsequently leading to tissue damage. This process may share mechanisms with the processes that have been described to coordinate CD4$^+$ and effector T-cell responses to viruses.

Like CD, most autoimmune diseases with HLA associations are associated with MHC class II alleles, including type 1 diabetes, multiple sclerosis, rheumatoid arthritis, and ulcerative colitis (48). Despite the association of these diseases with class II alleles rather than class I alleles, CD8$^+$ effector T cells play an important role in the pathogenesis of these diseases. For instance, although type 1 diabetes is strongly associated with class II alleles, autoreactive CD8$^+$ T cells are extensively found in inflamed diabetic islets and are appreciated to be the primary effectors driving tissue damage (49–51). Thus, the scenario we outline above for celiac disease may be generalizable to other forms of autoimmunity, in that an initial misdirected CD4$^+$ T-cell response may license effector CD8$^+$ and γδ T cells to cause tissue destruction at a particular site.

The mobilization of specific lymphocytes into the peripheral blood 6 d after antigenic challenge, as has been reported in CD (4, 5) and in the context of influenza vaccination (52), has provided an invaluable window into antigen-specific responses in human subjects. It will be interesting to see whether other such migrations are occurring at specific times in other autoimmune diseases. We also suggest that the analysis of activated T cells with gut-homing markers in the peripheral blood on day 6 after gluten challenge may be a superior method to diagnose CD in individuals currently on a gluten-free diet. An estimated 1.6 million Americans follow a gluten-free diet without an established diagnosis of CD (53). Available tests, including antibody levels and intestinal biopsy results, can be completely normal in CD patients on a gluten-free diet. Consequently, such individuals are often asked to continually eat gluten-containing foods for 2–4 wk before testing (39). This is often intolerable and precludes an accurate diagnosis. Our study shows promise in the reliable clinical diagnosis of CD with only short-term gluten exposure.

Methods

Gluten Challenge. All human sample collection was performed with informed consent under Stanford University Institutional Review Board oversight Volunteers underwent oral gluten challenge as described (4). At time of participation, all volunteers adhered to a strict gluten-free diet for at least 1 mo. After an initial blood draw, volunteers consumed four slices of white bread per day for 3 consecutive days (days 1, 2, and 3) and returned for a second blood draw on day 6. All celiac patient volunteers had a clinical diagnosis of celiac disease established by small intestinal biopsy in addition to serologic antibody testing. Five of six celiac volunteers were HLA-DQ2.5$^+$. One celiac volunteer was HLA-DQ8$^+$ according to clinical testing. Healthy HLA-DQ2.5$^+$ volunteers were either parents of children with celiac disease or individuals who endorsed gluten intolerance. Patients were tested for HLA-DQ2.5 by PCR (*SI Methods*). All healthy volunteers had a negative clinical diagnostic workup for celiac disease, and were able to comply with a gluten-free diet for at least 1 mo before participation.

Tetramer Analysis and Flow Cytometry. All FACS experiments were performed on ARIAII or LSRII instruments (Becton Dickinson). Water-soluble MHC–DQ2 molecules with covalently tethered peptides were produced in a baculovirus expression system (54). Two different MHC–DQ2.5 molecules with engineered biotinylation sites were produced with tethered deamidated T-cell epitopes of α-gliadin, DQ2-α-I (QLQPFPQPELPY) and DQ2-α-II (PQPELPYPQPE). Proteins were biotinylated, purified, and stored in PBS, 50% (vol/vol) glycerol at −20 °C. Tetramers were prepared by incubating protein with streptavidin–fluorophore conjugates (eBioscience) at a 4:1 molar ratio. Tetramer staining was performed at room temperature for 1 h using 10 mg/mL tetramer. Antibody clones used for flow cytometry are in *SI Methods*.

Intestinal Biopsy Preparation. Small intestinal biopsies were obtained with informed consent from celiac patients undergoing endoscopy at Stanford University Hospital and processed as described (55). See *SI Methods*.

CyTOF Staining and Data Acquisition. CyTOF and data acquisition were performed as described (16) on cryopreserved peripheral blood mononuclear cells or freshly isolated intestinal lymphocytes. See *SI Methods*.

CyTOF Antibody Labeling. Purified antibodies (lacking carrier proteins) were labeled 100 μg at a time according to instructions provided by DVS Sciences with heavy metal-preloaded maleimide-coupled MAXPAR chelating polymers via Pre-Load Method version 2.1 (16).

Single-Cell Sorting and TCR Sequencing. Single-cell sorting was performed using an ARIAII cell sorter (Becton Dickinson). TCR sequences from single cells were obtained by a series of three nested PCRs as described (24). The full method and TCR sequence analysis are described in *SI Methods*.

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Supporting Information

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SI Methods

HLA-DQ2.5 Testing. Subjects were tested for HLA-DQ2.5 by PCR using the following primers: DQA 5′-TCTTATGGTGTAAACTTGTACCAGTC-3′; DQA 3′-ATCAGACTGTTCAAGTTATGTTTTAGG-5′; DQB 5′-GCGTGCGTCTTGTGAGCAGAAG-3′; and DQB 3′-CCTGTCCACCGCCGCCCGTTT-5′.

Intestinal Biopsy Preparation. Three to four intestinal biopsy fragments were incubated in Roswell Park Memorial Institute medium (RPMI) with 5% FCS containing 0.5 mg/ml of Type 4 collagenase (Worthington). Cells were periodically disrupted during incubation by passing through a syringe topped with a blunt-ended 16-gauge needle. Lymphocytes were enriched through Percoll (GE Healthcare) gradient centrifugation. CyTOF staining was performed on freshly isolated lymphocytes.

Flow Cytometry. The following antibody clones were used for flow cytometry: anti-CD3 (SK7; Biolegend), anti-CD4 (RPA-T4; Biolegend), anti-CD8 (OKT8; eBioscience), anti–γδ-T-cell receptor (TCR) (MHGD04; Invitrogen), anti-CD38 (HIT2; Biolegend), anti–integrin-β7 (FIB504; eBioscience), anti-CD103 (Ber-ACT8; Biolegend), anti-CD27 (O323; eBioscience), and anti-NKG2D (1D11; Biolegend). Dead cells were excluded using a LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen).

Time-of-Flight Mass Cytometry Staining. Time-of-flight mass cytometry (CyTOF) and data acquisition were performed as described (1). Antibodies used for mass cytometry included: anti–integrin-β7 (FIB504; Biolegend), anti-CD103 (B-Ly7; Biolegend), anti-γδTCR [using anti-Phycoerythrin (PE) (PE001; Biolegend) as secondary, with PE-labeled γδTCR (SA6.E9; Invitrogen) as primary], anti–IL-17 (BL168; Biolegend), anti–IL-4 (8D4-8; Becton Dickinson), anti–IL-10 [using a biotinylated primary antibody (JES3-12G8; Biolegend), and heavy metal-conjugated streptavidin as secondary as described (1)], anti-CD5 (UCHT2; Biolegend), anti-CD25 (M-A251; Becton Dickinson), anti-CD95 (DX2; Biolegend), and anti–CTLA-4 (BNI3; Becton Dickinson). All other antibody clones used for CyTOF can be found in Newell et al. (1). Cryopreserved peripheral blood mononuclear cells (or freshly isolated intestinal lymphocytes) were thawed and washed with complete RPMI before overnight recovery at 37 °C. Cells were transferred to 96-well plates (or tubes), washed, and resuspended in cytometry buffer [PBS, 0.05% sodium azide, 2 mM EDTA, 2% (vol/vol) FCS] for staining as previously described (1). For stimulation, all cells were cultured for 3 h at ∼15×10^6 per mL in complete RPMI [10% (vol/vol) FCS] plus 1× brefeldin A (eBioscience), 1× monensin (eBioscience), 2.5 μg/mL anti-CD107a, 1.25 μg/mL anti-CD107b, and 10 μM TAPI-2 (VWR International). For phorbol-12-myristate-13-acetate+ionomycin stimulation, 150 ng/mL PMA + 1 μM ionomycin was added to the cells.

At the end of the 3-h stimulation, cells were pipetted vigorously to remove adherent cells from the plate and transferred to 96-well plates (or tubes), washed, and resuspended in cytometry buffer. The cells were incubated for 30 min on ice with a prepared mixture of metal-conjugated surface-marker antibodies at concentrations found to be effective in prior antibody tests. After surface staining, cells were washed once and resuspended in 20 μM ^{115}In-loaded maleimido-monoamine-DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) in PBS [a sulfhydryl-reactive trivalent cation-chelating bifunctional ligand; Macrocyclics B-272; mixed with a 0.5 molar ratio of ^{115}InCl and stock solution dissolved in L-buffer (DVS Sciences) at 1 mM, stable at 4 °C, and working much like commercially available amine-reactive LIVE/DEAD staining reagents (Invitrogen)]. Cell fixation using paraformaldehyde (Electron Microscopy Sciences), DNA labeling with an iridium-containing DNA intercalator (DVS Sciences), intracellular staining, washing, and data acquisition was performed as described (1).

TCR Sequencing. A series of three nested PCRs was performed on single sorted T cells. For the first reaction, reverse transcription and preamplification were performed with a One-Step RT-PCR Kit (Qiagen) using multiplex PCR with multiple Vβ or Vδ region primers and a Cβ or Cδ region primer. When necessary, base degeneracy was incorporated into the primers to account for TCR polymorphism and ensure amplification of all known functional TCRVβ or TCRVδ and TCRCβ and Cδ regions identified in the international ImMunoGeneTics information system (IMGT) database (www.imgt.org). Next, an aliquot of the first reaction was used as a template for the second PCR using a set of multiple internally nested TCRVβ or TCRVδ primers and an internally nested Cβ or Cδ primer with a HotStarTaq DNA Polymerase Kit (Qiagen). The second set of TCRV region primers also incorporated base degeneracy when needed and contained a common 23-base sequence at the 5′ end to enable further amplification with a common 23-base primer. The third and final PCR was performed on an aliquot of the second reaction using a primer containing the common 23-base sequence (incorporated into the second set of Vβ primers) and a third internally nested Cβ or Cδ primer using HiFi Hotstar DNA polymerase (Qiagen). Amplified PCR products were treated with ExoSAP-IT (Affymetrix) and sequenced using primers from the final PCR from both ends. Primer sequences for TCRβ sequencing can be found in Su et al. (2). Primer sequences for TCRδ can be found in Table S4.

TCR Sequence Analysis. TCR sequence analysis was performed with VDJFasta (3). Segment classification was performed to reference segment databases from the IMGT. CDR3 from all domains was extracted and translated using TCR-specific profile hidden Markov models, constructed from 95% nonredundant concatenations of IMGT V, D, and J segments. TCR junctional analysis was performed using IMGT/V-Quest (www.imgt.org/). A dataset of 165,291 naive $CD8^+$ TCRβ sequences (4) was used as a control for CDR3β convergence. To generate TCRδ reference sequences, between 10^5 and 10^6 TCR$\gamma\delta^+$ T cells from the peripheral blood (PB) of eight different individuals and intraepithelial lymphocytes (IELs) from one individual were sorted by flow cytometry. RNA was extracted using an RNeasy RNA extraction kit (Qiagen). RNA from each of these samples was amplified and sequenced using the primers described above. Sequencing was performed by using an Illumina MiSeq platform after incorporation of Illumina paired-end adapters through PCR. As a control for TCRδ convergence, 18,579 total unique TCRδ sequences using TRDV1 were used. Motif enrichment was evaluated by comparing the observed vs. expected frequency of 2-mer and 3-mer motifs within CDR3β or CDR3δ clones using the same V region. Enrichment is represented as the odds of encountering enrichment of the motif in the reference dataset to the degree observed in the selected set. The significance of motif enrichment was evaluated by using the Fisher's exact test with the Bonferroni

correction such that P values <0.05/<howmany> = 1e−4 were considered to be statistically significant. Analysis was performed in R version 2.11.1. The most statistically significant examples are illustrated.

1. Newell EW, Sigal N, Bendall SC, Nolan GP, Davis MM (2012) Cytometry by time-of-flight shows combinatorial cytokine expression and virus-specific cell niches within a continuum of CD8(+) T cell phenotypes. *Immunity* 36(1):142–152.
2. Su LF, Kidd BA, Han A, Kotzin JJ, Davis MM (2013) Virus-specific CD4(+) memory-phenotype T cells are abundant in unexposed adults. *Immunity* 38(2):373–383.
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4. Warren RL, et al. (2011) Exhaustive T-cell repertoire sequencing of human peripheral blood samples reveals signatures of antigen selection and a directly measured repertoire size of at least 1 million clonotypes. *Genome Res* 21(5):790–797.

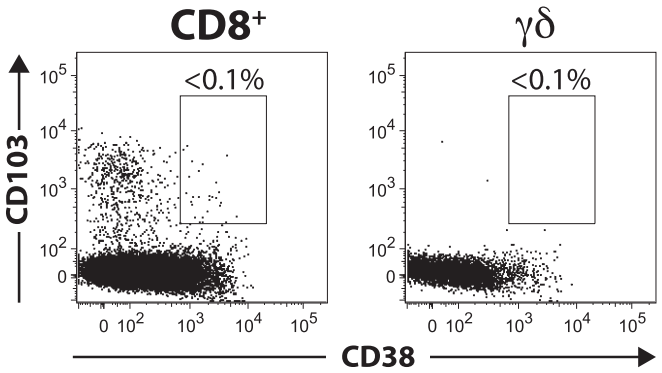


Fig. S1. Patients with active celiac disease (CD), as determined by ongoing symptoms and positive autoantibody titers, have αEβ7$^+$CD38$^+$CD8$^+$ and γδ T-cell proportions below background levels of 0.05% and 0.01%, respectively. Representative FACS analysis of CD8$^+$ αβ and γδ T cells with respect to CD103 (αE integrin) and CD38.

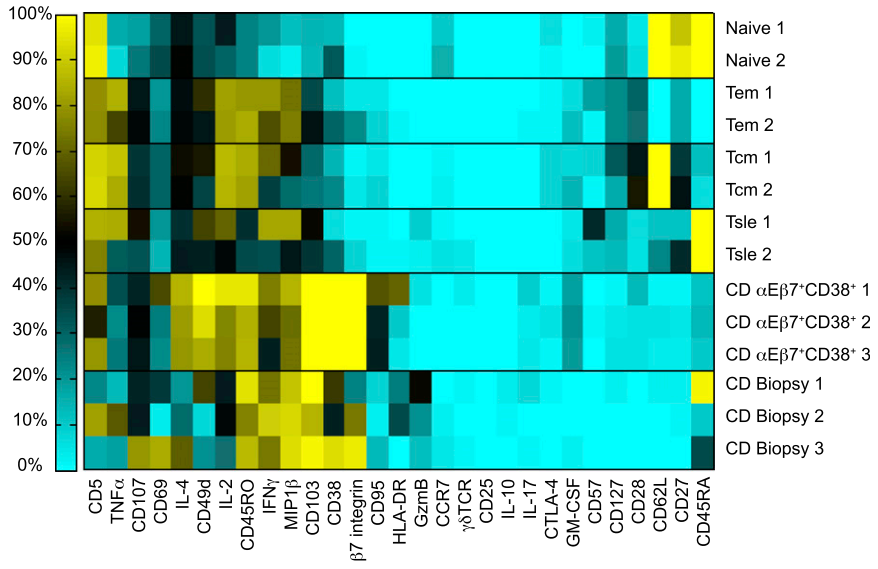


Fig. S2. Phenotype and functional capacity of αEβ7$^+$CD38$^+$CD8$^+$ T cells resemble effector memory cells and CD8$^+$ IELs. Functional capacities of the indicated cell types with respect to the indicated markers are plotted as a heat plot. The color scale is shown (*Left*) and indicates the percentage of cells expressing the indicated marker. Cells were stimulated with PMA and ionomycin and analyzed for the indicated cell-surface or intracellular markers. Cells were segregated based on stringent criteria based on the gates indicated: naive (CD45RA$^+$CD27$^+$CD62L$^+$CCR7$^+$), effector memory (Tem; CD45RA$^-$CD27$^-$CD62L$^-$CCR7$^-$), central memory (Tcm; CD45RA$^-$CD27$^+$CD62L$^+$CCR7$^+$), short-lived effector (Tsle; CD45RA$^+$CD27$^-$CD62L$^-$CD28$^-$), celiac PB (CD3$^+$αEβ7$^+$CD38$^+$CD8$^+$), and celiac biopsy (CD3$^+$CD8$^+$). All blood samples analyzed are from celiac patients on day 6 following gluten challenge. Biopsy samples are from different celiac patients with active celiac disease including villous blunting and IEL expansion by histologic examination.

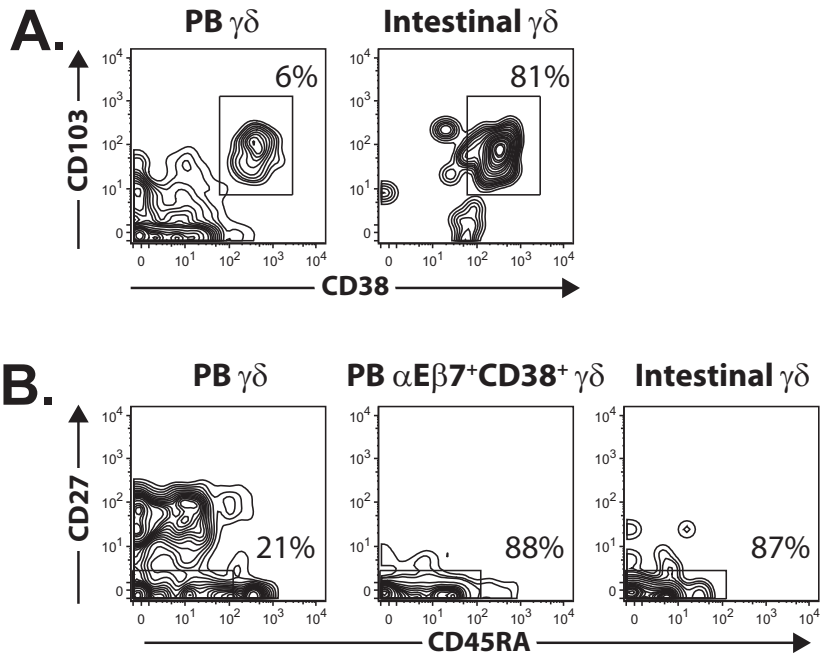


Fig. S3. Peripheral blood αEβ7$^+$CD38$^+$ γδ T cells induced by oral gluten challenge express surface markers of memory cells and resemble intestinal epithelial γδ T lymphocytes from celiac mucosal biopsies. (*A*) CyTOF analysis of total peripheral blood γδ and total intestinal γδ T cells with respect to CD103 and CD38 expression. (*B*) CyTOF analysis of total peripheral blood γδ, αEβ7$^+$CD38$^+$ γδ, and total celiac intestinal γδ with respect to CD27 and CD45RA expression. αEβ7$^+$ CD38$^+$ γδ and celiac intestinal γδ T cells are predominantly CD27$^-$ and CD45RA$^-$, consistent with a memory phenotype.

A.

Patient 1 αEβ7$^+$CD38$^+$ CD8	TCRβ CDR3	Freq 1 (98)	Freq 2 (94)
	CASSPGTDTQYF	9	20
	CSVEMNTEAFF	2	13
	CASSFGGELFF	5	1
	CASSAGHPEQFF	3	3
	CASSNLRQGAAGNTIYF	3	3
	CASSQEEQGAFYEQFF	3	3
	CASTEGQAEAFF	3	2
	CASSLINTEAFF	3	1
	CSVDGNYLTDTQYF	3	1
	CAWSVKTLRRADTQYF	1	3
	CASRIQGEGSPLHF	2	1
	CASSLASVGSTEAFF	1	2
	CASEMDANTGELFF	1	1
	CASSPFSGDYYEQYF	1	1

B.

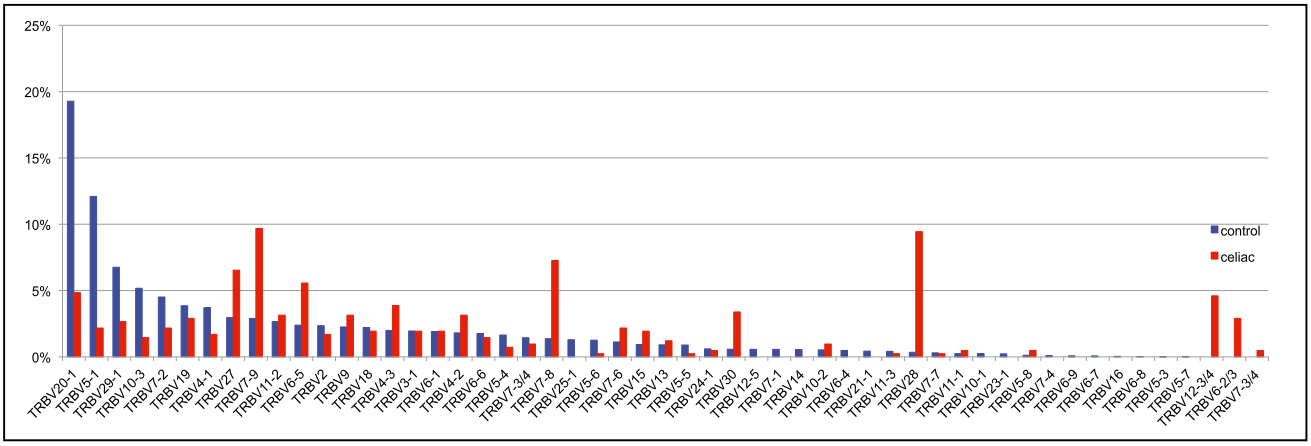
Patient 2 αEβ7$^+$CD38$^+$ CD8	TCRβ CDR3	Freq 1 (127)	Freq 2 (75)
	CASSYDVRSGNYEQYF	5	1
	CASSVGGVQPQHF	4	2
	CASNLAGGSNEQFF	2	1
	CASSKLDSGYTF	1	1
	CASSLGRVEAFF	1	1
	CASSLSQGGHNEQFF	1	1
	CASSPTSGRTTSYEQYF	1	1
	CASSQDGGTYNEQFF	1	1
	CASSSTPGGLWYGYTF	1	1
	CASTAGFNQPQHF	1	1

C.

Patient 1 αEβ7$^+$CD38$^+$ γδ	TCRδ CDR3	Freq 1 (96)	Freq 2 (56)
	CALGGLPTLGDTPTDKLIF	59	17
	CALCLLADWGYTDKLIF	5	1
	CALGELRSLLHLHWGIRTDKLIF	1	4
	CALGDGGGFYTSRVLGGYAFVTTDKLIF	2	2
	CALGELPYWALRGADKLIF	2	1
	CALGGSGISYVGILGKLIF	1	2
	CALGEFFPRYWGTTYTDKLIF	1	1
	CALGELQPRYWGRRFDKTKLFF	1	1
	CALGFPPVLGDPYTDKLIF	1	1

Fig. S4. Identical αEβ7$^+$CD38$^+$CD8$^+$ and αEβ7$^+$CD38$^+$ γδ TCR clones reappear in celiac patients upon repeat gluten challenge. (*A* and *B*) Identical αEβ7$^+$CD38$^+$ CD8$^+$ TCRβ clones are reencountered upon repeat gluten challenge within the same patient. All TCRβ sequences reoccurring in the same individual upon repeat challenge are shown. CDR3β motif and frequency are indicated. (*C*) Identical αEβ7$^+$CD38$^+$ TCRδ clones are reencountered upon repeat gluten challenge within the same patient. All TCRδ sequences reoccurring in the same individual upon repeat challenge are shown. CDR3δ motif and frequency are indicated.

A.



B.

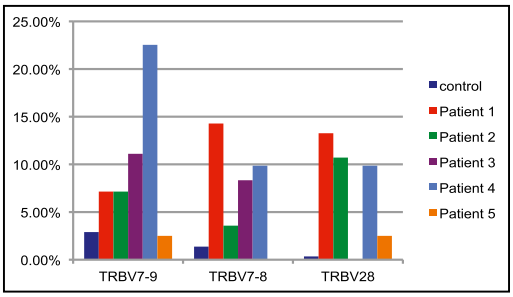


Fig. S5. Certain TRBV genes are overrepresented in a nonredundant sampling of $\alpha E\beta 7^{+}CD38^{+}CD8^{+}$ T cells in some celiac patients compared with controls. (*A*) Relative frequency of TRBV gene use in unique (nonredundant) TCR clones in celiac patients compared with a reference database of sequences shows overrepresentation of TRBV7-9, TRBV7-8, and TRBV28 in celiac patients vs. controls. (*B*) Relative frequency of TRBV7-9, TRBV7-8, and TRBV28 use in unique TCR clones in individual celiac patients compared with controls.

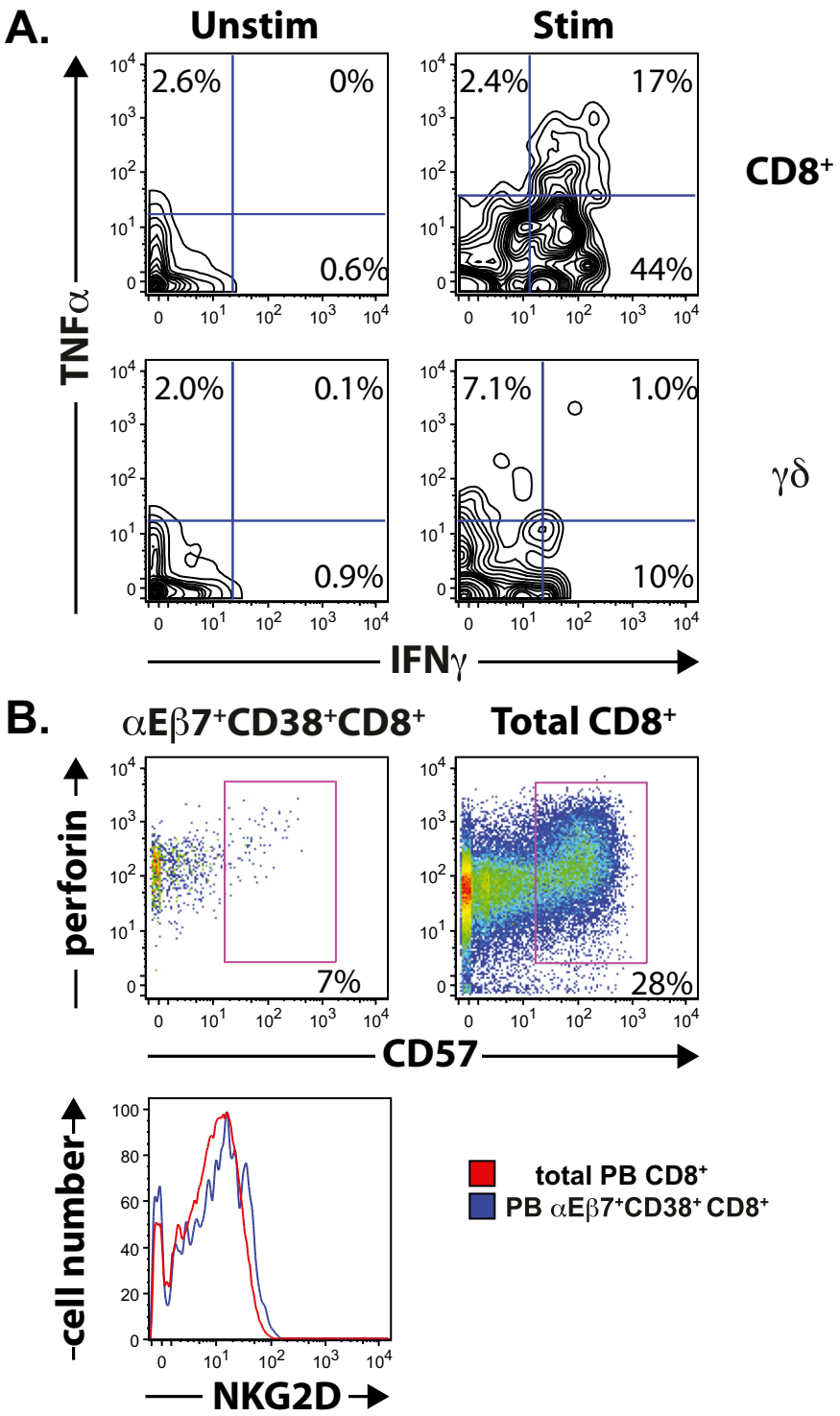


Fig. S6. αEβ7$^+$CD38$^+$CD8$^+$ T cells can produce IFNγ but do not express higher levels of perforin or NKG2D relative to total blood CD8$^+$ T cells. (*A*) Stimulated αEβ7$^+$CD38$^+$CD8$^+$ T cells but not αEβ7$^+$CD38$^+$CD8$^+$ γδ T cells are able to produce TNFα and IFNγ in response to stimulation with PMA and ionomycin. (*B*) αEβ7$^+$ CD38$^+$CD8$^+$ T cells do not express higher levels of perforin or NKG2D than total CD8$^+$ T cells.

Table S1. Quantification of peripheral blood $\alpha E\beta7^+CD38^+CD8^+$ and $\alpha E\beta7^+CD38^+$ $\gamma\delta$ T cells in celiac patients and control individuals following gluten challenge

Table S1

All six celiac patients but none of the controls exhibit induction of peripheral blood $\alpha E\beta7^+CD38^+CD8^+$ and $\alpha E\beta7^+CD38^+$ $\gamma\delta$ T cells on day 6 following oral gluten challenge. Numbers indicate $\alpha E\beta7^+CD38^+CD8^+$ or $\alpha E\beta7^+CD38^+$ $\gamma\delta$ T cells as a percentage of total blood $CD8^+$ or $\gamma\delta$ T cells.

Table S2. Single-cell TCR sequencing of α-II-gliadin tetramer-positive T cells shows most clones use TRBV7-2 and share a consensus arginine at position 5

Table S2

CDR3β sequences from two patients with indicated V and J use and frequency. Use of TRBV7-2 and consensus arginine are highlighted in bold.

Table S3. Summary of single-cell TCRβ and TCRδ sequencing

Table S3

CDR3β and CDR3δ sequences from all patients tested with indicated V and J use and frequency.

Table S4. Primers used for TCRδ sequencing

Table S4

TCRδ was amplified using a series of nested PCRs as described (*SI Methods*) (1).

1. Su LF, Kidd BA, Han A, Kotzin JJ, Davis MM (2013) Virus-specific CD4(+) memory-phenotype T cells are abundant in unexposed adults. *Immunity* 38(2):373–383.