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ARTICLE

Circulating CD103$^+$ γδ and CD8$^+$ T cells are clonally shared with tissue-resident intraepithelial lymphocytes in celiac disease

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Gut intraepithelial γδ and CD8$^+$ αβ T lymphocytes have been connected to celiac disease (CeD) pathogenesis. Based on the previous observation that activated (CD38$^+$), gut-homing (CD103$^+$) γδ and CD8$^+$ αβ T cells increase in blood upon oral gluten challenge, we wanted to shed light on the pathogenic involvement of these T cells by examining the clonal relationship between cells of blood and gut during gluten exposure. Of 20 gluten-challenged CeD patients, 8 and 10 had increase in (CD38$^+$CD103$^+$) γδ and CD8$^+$ αβ T cells, respectively, while 16 had increase in gluten-specific CD4$^+$ T cells. We obtained γδ and αβ TCR sequences of >2500 single cells from blood and gut of 5 patients, before and during challenge. We observed extensive sharing between blood and gut γδ and CD8$^+$ αβ T-cell clonotypes even prior to gluten challenge. In subjects with challenge-induced surge of γδ and/or CD8$^+$ αβ T cells, as larger populations of cells analyzed, we observed more expanded clonotypes and clonal sharing, yet no discernible TCR similarities between expanded and/or shared clonotypes. Thus, CD4$^+$ T cells appear to drive expansion of clonally diverse γδ or CD8$^+$ αβ T-cell clonotypes that may not be specific for the gluten antigen.

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INTRODUCTION

Celiac disease (CeD) is a common disorder where gluten consumption causes chronic inflammation in the small intestine. The only available treatment for the disease is a gluten-free diet (GFD), which generally leads to normalization of the gut mucosal pathology[1]. The disease has strong association with certain HLA-class II allotypes, specifically HLA-DQ2.5, HLA-DQ2.2 and HLA-DQ8[2]. CD4$^+$ T cells of CeD patients selectively recognize deamidated gluten peptides in context of these disease-associated HLA-DQ molecules, strongly speaking to a central role of T cells in the pathogenesis. These gluten-specific CD4$^+$ T cells, detectable by HLA-DQ2.5:gluten tetramers, are found at high frequency in the celiac lesion of the small intestine and at much lower frequency in peripheral blood[3–6]. Several studies have reported a transient surge of gluten-specific CD4$^+$ T cells peaking at day 5–8 in blood after CeD patients in remission are exposed to gluten[7–10]. One of these studies also demonstrated that concomitant with this increase of CD4$^+$ T cells, there is an increase of gut-homing (CD103$^+$), activated (CD38$^+$) γδ and CD8$^+$ αβ T cells in the blood during gluten challenge[10]. Single-cell TCR repertoire analysis of both γδ and CD8$^+$ αβ T cells occurring in blood on day 6 after gluten challenge, revealed clonal expansion and highly focused repertoire. These results led the authors to suggest an involvement of an antigen-driven induction of certain γδ and CD8$^+$ αβ T-cell clonotypes. Relevant to this, it was reported that gut CD8$^+$ αβ T cells in CeD, mainly localised in the lamina propria though, recognize gluten peptides in the context of HLA class I molecules[11,12]. Alternative to TCR mediated activation and expansion of intraepithelial lymphocytes (IELs), it has been demonstrated that in the IL-15 rich environment of the CeD gut, T cells via their expression of NK-cell receptors can get activated and also expand by recognition of stress induced molecules on enterocytes[13–15].

To further understand the role of IELs in CeD, we wanted to examine the occurrence of CD38$^+$CD103$^+$ γδ and CD8$^+$ αβ blood T cells on day 6 of gluten challenge, as well as to compare the TCR repertoires of such blood-derived cells with that of tissue-resident T cells in the small intestine. One could expect that the activated and gut-homing T cells that increase in blood on day 6 of gluten challenge should seed the lesion and make up the majority of newly arrived IELs. Likewise, one could expect that these activated blood T cells mirror either all the IELs in the lesion, or, if responding to a distinct antigen, only a subset of these IELs. A way to investigate these matters is to determine the clonality and diversity of the TCR repertoire. A more focused and expanded TCR repertoire at peak response on day 6 or in tissue after challenge might possibly indicate a TCR-dependent induction supporting the findings by Han et al.[10]. To address these issues, we have analyzed the frequency of gut-homing, activated γδ and CD8$^+$ αβ T cells in blood on day from 20 treated CeD patients undergoing either a 14-day oral gluten challenge or a 3-day oral gluten challenge, and we have performed single-cell paired TCR sequencing of γδ and CD8$^+$ αβ T cells in blood and in gut biopies at baseline, at day 6 and day 14 in five patients that underwent the 14-day gluten challenge.

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RESULTS

Increased frequency of gut-homing, activated γδ and CD8$^+$ αβ T cells in blood corresponds with increased density of IELs after gluten re-exposure

From our previously reported gluten challenge study[16], we obtained IEL samples from eight patients whose HLA-A and HLA-B genotypes are shown in Table S1. In order to estimate the absolute number and the distribution of γδ and CD8$^+$ αβ IELs, we compared the relative number of γδ and CD8$^+$ αβ IELs of total CD3$^+$ IELs as measured by flow cytometry with the total number of IELs per 100 intestinal epithelial cells (IECs) as assessed by histological examination of parallel biopsies (Table 1). Based on this, the increase of IELs in response to gluten seemed to be predominantly due to the CD8$^+$ αβ IELs, and not by the γδ IEL subset. In contrast to a clear increase in blood on day 6 of CD103$^+$CD38$^+$ γδ and CD8$^+$ αβ T cells as was reported by Han al[10], we could only detect a distinct increase of these T-cell populations on day 6 in a subset of patients (Fig. 1a, b). The increase of CD103$^+$CD38$^+$ CD8$^+$ αβ T cells in blood (Fig. 1b) corresponded with the estimated absolute increased number of CD8$^+$ αβ IELs on day 14 (Table 1, right column,). Interestingly, both of the patients CD1340 and CD1342, who had the highest IEL count on day 14, had a clear gut-homing CD8$^+$ αβ T-cell response in blood on day 6. However, only CD1340 exhibited a γδ T-cell response in blood. Representative flow plots of the blood samples for patients CD1340 and CD1342 can be found in Fig. S1. Of note, all patients that exhibited a γδ T-cell response in blood also displayed a CD8$^+$ αβ T-cell response but not vice versa. We also investigated the expression of CD103 and CD38 on γδ and CD8$^+$ αβ IELs. As expected, virtually all IELs were CD103$^+$. Interestingly, all γδ IELs were CD38$^+$ throughout the entire challenge period in contrast to CD8$^+$ αβ IELs, where the CD38 expression increased with the ingestion of gluten (Fig. 1c, d). In the population of γδ IELs we observed bimodal expression of CD8 (Fig. 1e, f), and the fraction of γδ IELs expressing CD8 ranged from 14 to 70% (Fig. S2). When comparing this figure in treated CeD with those of untreated CeD and healthy controls[17], there was no significant differences.

Table 1. Approximate quantification of absolute number of γδ and CD8$^+$ αβ IELs based on HE-section based IEL count and γδTCR/CD8 flow cytometry staining.

	IEL count per 100 IEC[a]		TCRγδ (% CD3 IEL)[b]		γδ IEL per 100 IEC[c]		CD8 (% CD3 IEL)[d]		CD8 IEL per 100 IEC[e]	
Patient	BL	D14	BL	D14	BL	D14	BL	D14	BL	D14
CD1342	16.2	72.4	31%	10%	5.0	7.2	65%	86%	10.5	62.5
CD1340	21.4	59.5	57%	36%	12.1	21.4	39%	60%	8.3	35.5
CD1300	15.8	52.9	14%	7%	2.2	3.6	74%	83%	11.7	43.8
CD442	26.8	56.9	20%	11%	5.5	6.2	73%	83%	19.5	47.2
CD1339	29.7	48.4	31%	32%	9.2	15.3	63%	60%	18.7	28.8
CD1299	23.9	26.5	27%	ND	6.4	ND	68%	ND	16.2	ND
CD1178	24.4	33.1	22%	29%	5.5	9.4	72%	68%	17.6	22.3
CD1298	17.9	19.1	41%	43%	7.4	8.1	56%	55%	10.0	10.5

[a]Intraepithelial lymphocyte (IEL) count based on HE-sections (taken from ref. [9]).
[b]Fraction (%) of CD3$^+$ cells stained positive to γδTCR antibody in flow cytometry (IEL sample obtained from gut biopsies).
[c]Estimation of total number of γδ IELs per 100 intestinal epithelial cells (IECs) by dividing the total number of IELs per 100 IECs by the relative number of γδTCR$^+$ CD3$^+$ cells measured by flow cytometry.
[d]Fraction (%) of CD3$^+$ cells stained positive to CD8 antibody in flow cytometry (IEL sample obtained from gut biopsies).
[e]Estimation of total number of γδ IELs per 100 intestinal epithelial cells (IECs) by dividing the total number of IELs per 100 IECs by the relative number of CD8$^+$ CD3$^+$ cells measured by flow cytometry.

Gut-homing γδ and CD8$^+$ αβ T cells in blood share TCR features with IELs

We performed single-cell paired TCR sequencing of γδ and CD8$^+$ αβ T cells in blood and IEL samples from four of these eight patients (CD442, CD1340, CD1342, CD1300). All four had a gluten-specific CD4$^+$ T-cell response[16], while only one had an increase in γδ T cells and three had an increase in CD8$^+$ αβ T cells during gluten challenge. In addition, we also obtained TCR sequences from the IEL samples of patient CD1178 who did not respond to the challenge. We primarily sorted the CD103$^+$ populations with co-expression of CD38, but in samples where there were few CD38$^+$ T cells we also index-sorted the CD103$^+$CD38$^-$ population to increase the number of potentially relevant cells from blood. This was in particular the case for the baseline blood samples (Fig. S3). For the γδ T cells, up to half of the cells sorted and sequenced were CD38$^+$ (Fig. S3a). All CD8$^+$ αβ T cells sorted from the baseline blood sample of CD1342 were CD38$^+$ while we sorted mostly CD38$^-$ cells for the other three patients at baseline (Fig. S3b). For the CD8$^+$ T cells we obtained in total paired TCR sequences from 1310 single cells giving rise to 872 CD8$^+$ αβ T-cell clonotypes. For the γδ T cells we obtained paired TCR sequences from 1200 cells giving a total of 764 γδ T-cell clonotypes.

We found that the *TRGV* and *TRDV* gene usage of CD103$^+$ γδ T cells found in blood were near identical to that of γδ IELs

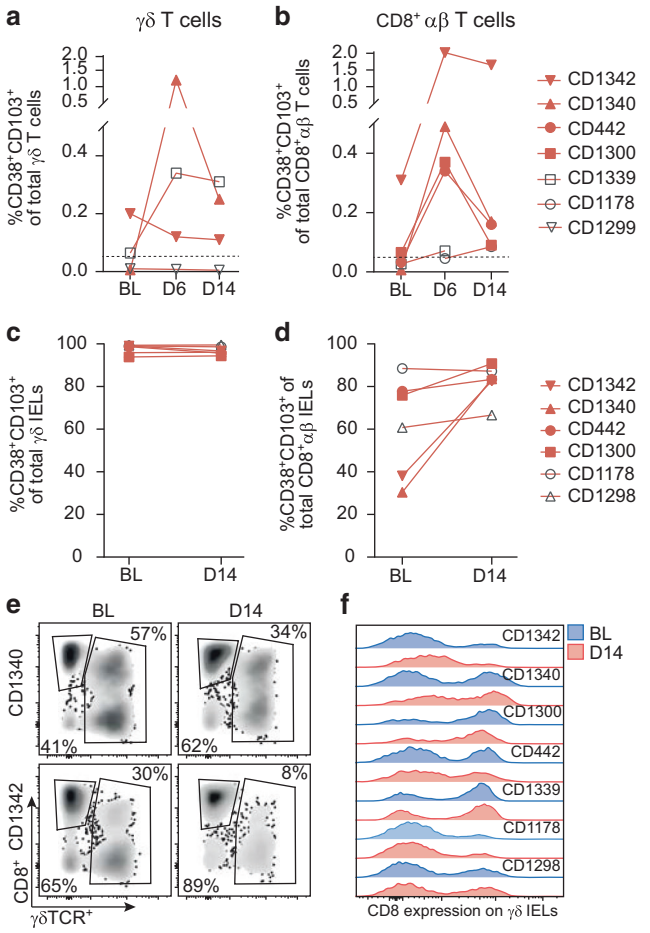


Fig. 1 γδ and CD8$^+$ αβ T cells in blood during 14-day gluten challenge in treated CeD patients. a γδ T-cell responses and **b** CD8$^+$ αβ T-cell responses. **c**, **d** Frequency of CD103$^+$CD38$^+$ intraepithelial lymphocytes (IELs), γδ T cells in c and CD8$^+$ αβ T cells in **d**. **e** Representative γδ and CD8$^+$ αβ IEL flow plots shown for CD1340 and CD1342. **f** Bimodal expression of CD8 in γδ IELs shown for different subjects at baseline (BL) and on day 14 (D14).

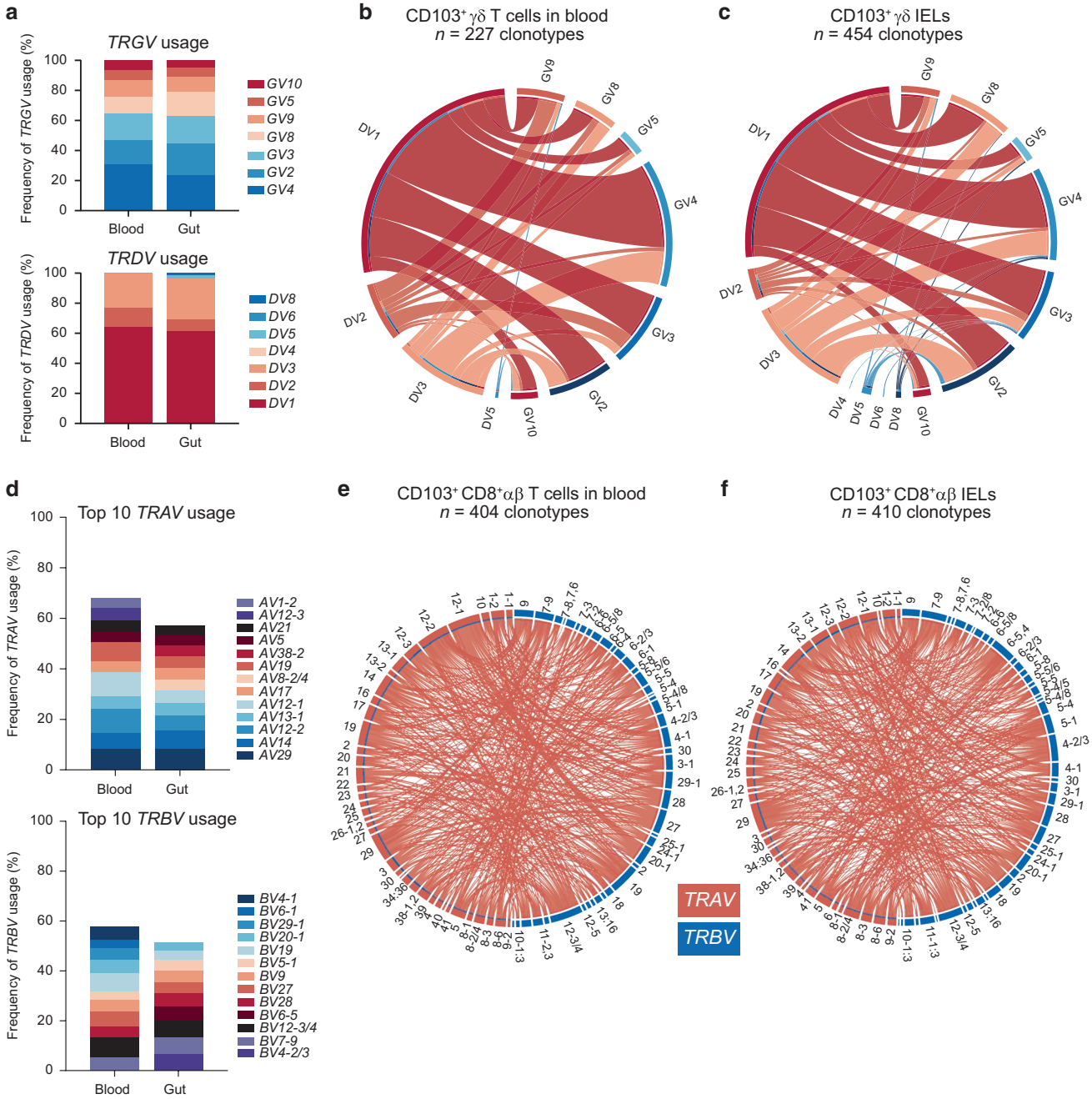


Fig. 2 V-gene usage and pairing in gut-homing γδ and CD8$^+$ αβ T cells. a The frequency of *TRGV* or *TRDV* usage in gut-homing (CD103$^+$) T-cell clonotypes from blood ($n = 227$ clonotypes) and gut ($n = 454$ clonotypes) during a 14-day gluten challenge. **b**, **c** *TRGV* and *TRDV* chain pairing in blood and gut is displayed in Circos plots where ribbons connecting chains indicate frequency of pairing (blood, $n = 227$, gut, $n = 454$ clonotypes). **d** The frequency of the top 10 used *TRAV* and *TRBV* genes in blood ($n = 404$ clonotypes) and gut ($n = 410$ clonotypes). **e**, **f** Circos plots representing chain pairing of *TRAV* and *TRBV* in blood ($n = 404$ clonotypes) and gut ($n = 410$ clonotypes).

(Fig. 2a), and the usage and pairing preference was similar to what was observed in untreated CeD[17]. Likewise, pairing preference was very similar for these γδ T cells (Fig. 2b, c). Interestingly, we found identical γδ T-cell clonotypes with and without CD8 expression in both tissue and blood samples. A complete list of all shared γδ and CD8$^+$ αβ T clonotypes across time points and tissue with CD38 and CD8 status for each patient can be found in the Supplementary File 1.

Next we looked into the V-gene usage and pairing of the CD8$^+$ αβ T cells. Focusing on the top 10 gene segments, we found that both for *TRAV* and *TRBV* these were similar between gut and blood tissues and accounted for ~50% of the overall V-gene usage in both blood and gut (Fig. 2d). This *TRBV* gene usage was similar to what has been described to be predominant *TRBV* families used in the IEL or blood compartment of healthy individuals[18]. Of note, we did not observe any pairing preference in any of the compartments (Fig. 2e, f).

γδ T-cell clonotypes: sharing across tissue and time and analysis of expanded clones

We observed extensive sharing of CD103$^+$ γδ T-cell clonotypes across tissue and time in all patients (Fig. 3a). The most expanded clonotypes contributed to clonotype sharing in several of the samples (Fig. S3a, Supplementary File 1). The clonal sharing also

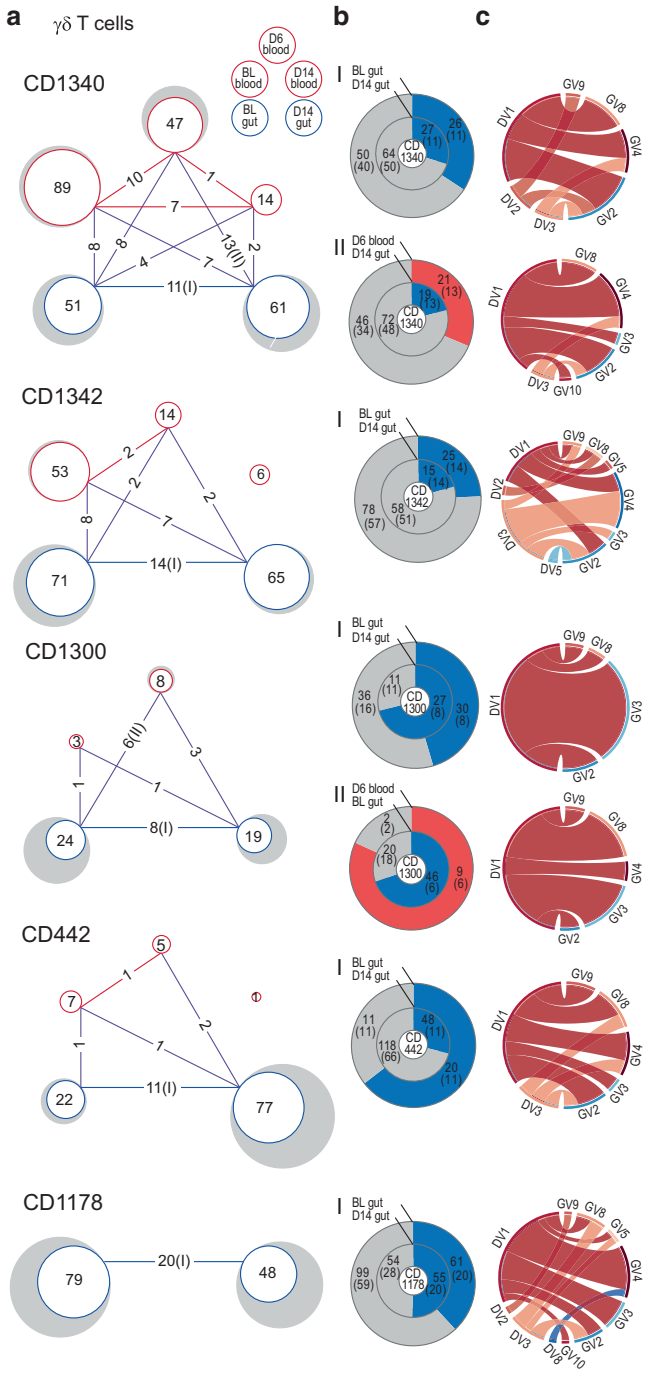


Fig. 3 Sharing of CD103$^+$ γδ T-cell clonotypes across tissue and time. a All samples per patient are displayed as area-proportional circles relative to sample size and shared clonotypes between different samples are connected with a line. Gut samples are displayed in blue and blood samples in red circles with the number of total clonotypes. Grey circles proportionally represent the sample size based on the number of cells. The number of shared clonotypes is denoted on the connecting lines and samples compared in **b** and **c** are indicated in parentheses. Samples with >20% clonotype sharing, >5 clonotypes and shared with >1 gut samples are displayed as (**b**) donut charts to include the total number of cells of shared (in red for blood and blue for gut) and non-shared (in grey) clonotypes and (**c**) Circos plots to visualize the paired V-gene usage of TCRγ and TCRδ of the shared clonotypes.

included a large fraction of T cells that were negative for CD38. It was only patient CD1340 who had a clear γδ T-cell response in blood at day 6. For this subject we observed clonal sharing between all time points and tissues (Fig. 3a), and the highest degree of sharing was observed between day 6 in blood and day 14 in gut. Here, 13 out of 47 clonotypes (28%) at day 6 was shared with γδ IELs at day 14. Further we compared more carefully the samples with most sharing (>20%, >5 shared clonotypes and shared with gut tissue) to look for the contribution of expanded clonotypes and the data are depicted as donut charts in Fig. 3b. We observed a high degree of sharing between the gut samples at baseline and day 14 for all five patients except CD1342, and the clonal sharing was dominated by expanded clonotypes compared to the non-shared clonotypes (Fig. 3b). The shared cells for CD1340 between day 6 and day 14 also were from expanded clones (Fig. 3b). In contrast to CD1340 and despite the small number of sequenced cells due to no clear γδ response for patient CD1300 at day 6 in blood, six out of eight γδ clonotypes were shared with γδ IELs at baseline.

Next we wanted to analyze the paired V-gene usage of the TCRγ and TCRδ chains of the shared clonotypes displayed as Circos plots (Fig. 3c). The shared clonotypes have the same dominating *TRDV1* usage but variable *TRGV* usage for all patients, similar to the overall observations shown in Fig. 2b and c. Focusing on the expanded clones (>2) and the CDR3s at day 6, no similarities in the CDR3 across the clonotypes could be discerned (Table 2). Neither did we find an overrepresentation of previously identified CeD-associated sequence motifs, specifically the H-J1 motif[19] or motifs reported by Han et al.[10], among these expanded clones nor clonotypes present in blood on day 6. Altogether, these findings demonstrate that diverse CD103$^+$ γδ T-cell clonotypes found in the IEL compartment are commonly present in the circulation regardless of gluten challenge.

CD8$^+$ αβ T-cell clonotypes: sharing across tissue and time and analysis of expanded clones

To investigate clonal relationship between CD103$^+$CD8$^+$ αβ T cells in blood with those of CD8 IELs, we conducted a similar analysis as for the γδ T cells. Three out of the four CeD patients with paired blood and gut samples showed clonal sharing across tissues, and this included sharing prior to gluten challenge (Fig. 4a). Similar to the γδ T cells, the most expanded CD8$^+$ αβ clonotypes could be found in multiple samples of the same patient (Fig. S3b). The four CeD patients examined had a CD8$^+$ T-cell response at day 6 in blood, but we observed some interesting differences in their T-cell responses at the clonal level in these patients. Altogether, 36% of the clonotypes made up 70% of the cells at baseline and these were also present in the IEL compartment at day 14 as expanded clones (Fig. 4b). CD1340 and CD1300 had the highest degree of clonal sharing between day 6 in blood and in gut at day 14, while CD442 did not have many shared clonotypes despite a comparable number of sequenced cells. Unexpectedly, compared to what was observed for γδ IEL clonotypes, CD8$^+$ αβ IEL clonotypes showed a much lower degree of sharing in the tissue. This observation was also similar for CD1178 who did not respond to the gluten challenge (Figs. 3a and 4a).

Similar to the γδ T cells, we wanted to explore if there were any particular TCR features between the expanded T-cell clonotypes that were present in blood and in gut tissues. The paired V-gene usage for the shared clonotypes visualized as Circos plots in Fig. 4c did not demonstrate any TCR pairing preference across the shared CD8$^+$ αβ clonotypes. Similarly, when assessing the most expanded T-cell clones at day 6, we could not demonstrate any similar TCR features between different clonotypes within a patient or across patients (Table 3). Looking at the CDR3β motifs CxxxxGN (with *TRBV7-9*) and CxxxxGT (with *TRBV7-8*) as reported by Han et al.[10],

Table 2. Top shared and most expanded CD103$^+$ γδ T-cell clonotypes on day 6.

Patient + Clone ID	Total clone size	Tissue	Time-point	Clone size	TRGV // TRGJ // CDR3γ	TRDV // TRDD // TRDJ // CDR3δ	Dual TRG/TRD
CD1340							
2	9	Blood	BL	2	TRGV4 // TRGJP1 // ATWDGPGATGWFKI	TRDV1 // TRDD2 // TRDJ1 // ALGELASSYGRWGRATDKLI	
			D6	4			
			D14	1			
		IEL	BL	2			
3	8	Blood	BL	1	TRGV4 // TRGJP1 // ATWSTTGWFKI	TRDV1 // TRDJ1 // ALGERCHGDTLVKPDKLI	
			D6	4			
		IEL	BL	1			
			D14	2			
14	5	Blood	D6	3	TRGV4 // TRGJP1 // ATWDGVPQTGWFKI	TRDV1 // TRDD2 // TRDJ1 // ALGERYGGPYKTGGYLRTDKLI	
		IEL	D14	2			
7	4	Blood	D6	2	TRGV2 // TRGJ2 // ATWDGYYKKL	TRDV1 // TRDD2 // TRDJ1 // ALGELSVLQKLGDTTLTYTDKLI	
		IEL	BL	1			
			D14	1			
CD1342							
13	3	Blood	D6	2	TRGV8 // TRGJP2 // ATWDNWIKT	TRDV3 // TRDD3 // TRDJ1 // AFTGGPYTDKLI	
		IEL	D14	1			
17	2	Blood	BL	1	TRGV9 // TRGJP // ALWDITGRKKIKV	TRDV1 // TRDD2 // TRDJ1 // ALGDPHFNTGVNFRWGPRKLI	TRGV8 // TRGJ2 // ATKSYKKL
			D6	2			
CD1300							
1	27	Blood	D6	3	TRGV9 // TRGJP // ALWEAELGKKIKV	TRDV1 // TRDD3 // TRDJ1 // ALGGSSKHGGYASYTDKLI	TRGV3 // TRGJ1 // ATWDRPDYKKL
		IEL	BL	8			
			D14	16			
2	17	Blood	D6	2	TRGV2 // TRGJP2 // ATWDGRGSDWIKT	TRDV1 // TRDD3 // TRDJ1 // ALGGTWGGTQKFFPYTDKLI	TRGV8 // TRGJ1 // ATWDSRVYYKKL
		IEL	BL	11			
			D14	4			

we did not observe these in our dataset. A third motif, CxxxF (with *TRBV28*), that was reported by the same authors, were present in 6 clonotypes from gut samples of patient CD1178 (Table S1). Of note, the 6 *TRBV28*-expressing clonotypes had TCRα sequences with unique *TRAV* and *TRAJ* genes. Overall, these findings demonstrate that CD103$^+$ CD8$^+$ αβ T cells with an activated phenotype in blood are clonally expanded and shared with those in the IEL compartment without any TCR or TCR commonality standing out as shared across tissue and time or representing expanded clones.

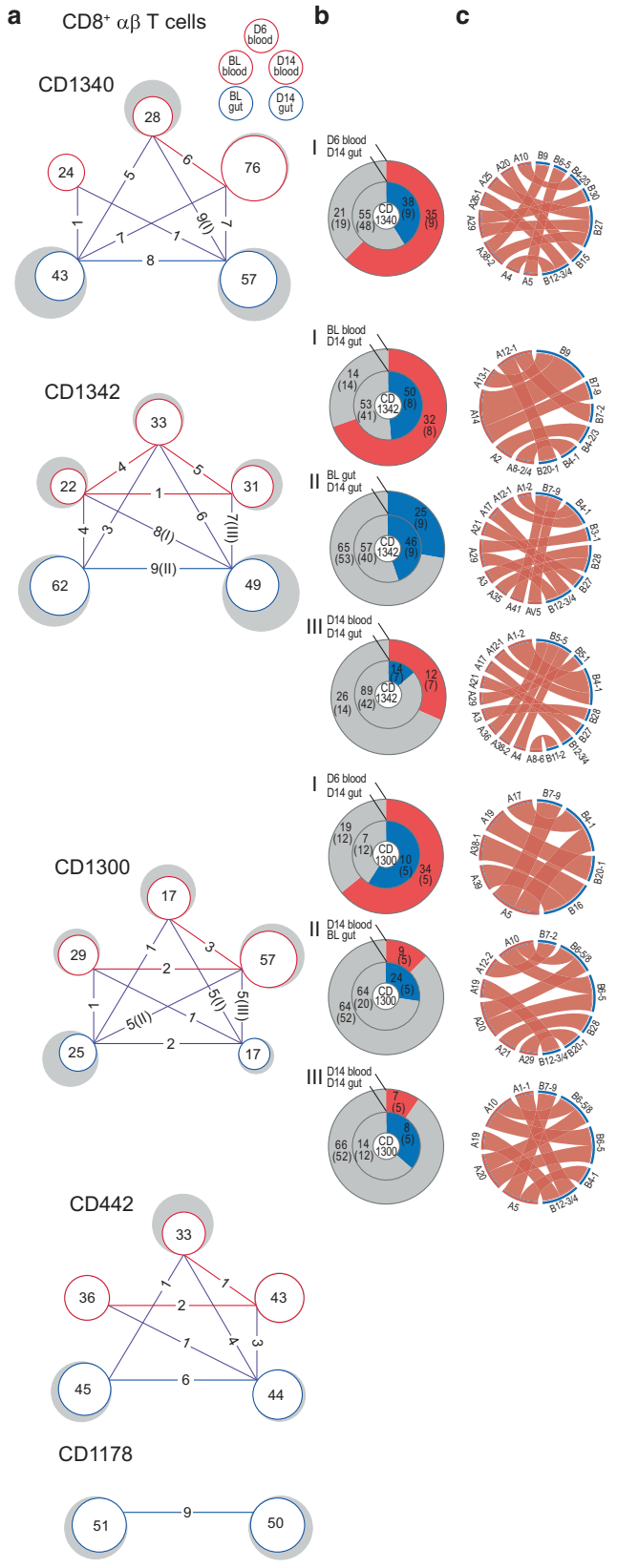
The frequency of gut-homing, activated γδ and CD8$^+$ αβ T cells correlates with the frequency of CD4$^+$ gluten-specific T cells in blood on day 6

In contrast to previous reports[10,20], we could detect an increase of CD38$^+$ CD103$^+$ γδ and CD8$^+$ αβ T cells on day 6 only in a subset of patients. On the other hand, we have previously reported an increase of gluten-specific CD4$^+$ T cells following gluten challenge in the majority of treated CeD patients[7,9]. To further explore the connection between these different CD8, CD4 and γδ T-cell responses in blood on day 6, we analyzed blood from another 12 treated patients that underwent a 3-day gluten challenge. Here, 10 out of the 12 patients responded to challenge according to the elevation of HLA-DQ2.5:gluten tetramer$^+$ CD4$^+$ T cells on day 6 compared to baseline. Similar to the 14-day gluten challenge study, we did not observe an increased frequency of CD103$^+$CD38$^+$ CD8$^+$ and γδ T cells on day 6 in all patients. Strikingly, we observed a range in responses of all three T-cell populations, and correlation analyses demonstrated that the degree of the different T-cell responses on day 6 were highly correlated (Fig. 5).

DISCUSSION

In this study with oral gluten challenge of CeD subjects, we observed a response of HLA-DQ2.5:gluten tetramer$^+$ integrin β7$^+$ effector-memory CD4$^+$ T cells in the majority of the subjects and less frequent responses of (CD38$^+$CD103$^+$) γδ and CD8$^+$ αβ T cells in blood. All of the subject who had increase CD38$^+$CD103$^+$ γδ T cells, also had an increase in CD38$^+$CD103$^+$ CD8$^+$ αβ T cells. Thus, there was a correlated and hierarchical pattern of the responses. We further observed that circulating CD103$^+$ γδ and CD8$^+$ T cells were clonally shared with the IELs in CeD, and this was so even prior to the gluten challenge. Diverse clonotypes were present among the CD103$^+$ γδ and CD8$^+$ T cells in patients who had surge of cells in blood on day 6. Thus, gluten-specific CD4$^+$ T cells appear to drive expansion of diverse γδ or CD8$^+$ αβ T-cell clonotypes in CeD. In blood on day 6 almost all patients had increase in HLA-DQ2.5:gluten tetramer$^+$ integrin β7$^+$ effector-memory CD4$^+$ T cells and fewer had increased in CD38$^+$CD103$^+$ γδ T cell and CD38$^+$CD103$^+$ CD8$^+$ αβ T cells.

In line with our previous findings[17], we found that the *TRGV/TRDV* usage in gut IELs in CeD patients undergoing gluten challenge is similar to that observed in untreated CeD. Moreover, we observed that the CD103$^+$ γδ T cells from the blood have a near identical V-gene usage and chain pairing to that of resident γδ IELs. Focusing on the top 10 used *TRAV* and *TRBV* genes due to the high TCRαβ diversity, we found that the usage of both *TRAV*



and *TRBV* genes were similar in blood and gut. It may be that the similar V-gene usage only reflect that certain V genes are more frequently used than others. In fact, the top 10 *TRBV* genes used here correspond well to the reportedly used *TRBV* families in gut

Fig. 4 Sharing of CD103+ CD8+ αβ T-cell clonotypes across tissue and time. a All samples per patient are displayed as area-proportional circles relative to sample size and shared clonotypes between different samples are connected with a line. Gut samples are displayed in blue and blood samples in red circles with the number of total clonotypes. Grey circles proportionally represent the sample size based on the number of cells. The number of shared clonotypes is denoted on the connecting lines and samples compared in **b** and **c** are indicated in parentheses. Samples with >20% clonotype sharing, >5 clonotypes and shared with >1 gut samples are displayed as (**b**) donut charts to include the total number of cells of shared (in red) and non-shared (in grey) clonotypes and (**c**) Circos plots to visualize the paired V-gene usage of TCRα and TCRβ of the shared clonotypes.

and blood of healthy individuals[18]. Moreover, at the level of chain pairing we did not observe any pairing preference of TCRαβ neither in blood nor in gut. Apart from the CxxxF motif carried with *TRBV28* in CD8+ αβ T cells, we did not observe the CeD conserved motifs reported by Han et al.[10] or Mayassi et al.[19] in our dataset. A limited number of clonotypes examined with our single-cell sequencing approach and the use of cryopreserved samples could be a reason for these disparate results, but we find this unlikely as Han et al.[10] also sequenced single cells from cryopreserved samples. A limited number of sequences were also examined by Mayassi et al.[19]. For the motifs carried by the CD8+ αβ T cells, the representation of study subjects with different HLA class I allotypes could be a confounder. Information on the HLA class I allotypes of the study subjects of Han et al.[10] is not available, but our study subjects carried typical CeD HLA-A and HLA-B alleles[21] with seven out of seven being HLA-B8 and five out of seven being HLA-A1. Given this HLA profile of the patients, the lack of sharing of T-cell clonotypes between the patients is likely not explained by HLA heterogeneity of the study subjects.

Our observation of bimodal expression of CD8 in the γδ IEL population is in keeping with previous observations[22]. Among human IELs the CD8αβ heterodimer is dominating with 10% of the cells expressing the CD8αα homodimer[23]. Our staining with anti-CD8α will include both cell populations. The role of the CD8 molecule in γδ T cells is to best of our knowledge unknown. Our observation that identical γδ T-cell clonotypes are present with and without the expression of CD8 suggests that the acquisition or loss of CD8 could be a regulated process in these cells.

Our group has previously shown that gluten-specific CD4+ T-cell clonotypes are shared between blood and gut tissue[4]. We wanted to determine if the same phenomenon occurs for γδ and CD8+ αβ T cells. In our previous study[17], we highlighted the importance of accurate clonotype assignments as we observed many identical TCRγ sequences that paired with different TCRδ sequences. In addition, the presence of dual TCR sequences even further strengthen the correct annotation of clonotypes. With this in mind, it was remarkable to detect sharing of γδ and CD8+ αβ T-cell clonotypes across blood and gut tissues, also prior to gluten challenge. The degree of clonal sharing between the different samples appeared to be higher for γδ T cells compared to CD8+ αβ T cells, especially for the sharing of IEL clonotypes. As the CD8+ IELs in CeD are believed to have an oligoclonal TCR repertoire[24,25], one could expect to observe a higher degree of sharing of CD8 IEL clonotypes. On the contrary, CD8+ IELs in CeD patients appear to have a much more diverse TCR repertoire than previously believed. These findings suggest that clonal sharing is a common feature that is independent of continuous gluten exposure. The sharing of γδ and CD8+ αβ T-cell clonotypes across blood and gut tissue could possibly relate to the extraintestinal manifestations of CeD, but this possibility remains speculative.

Relevant to our observation of clonal sharing between gut tissue and blood, it was reported in a recent paper that small

Table 3. Top shared and expanded CD103$^+$ CD8$^+$ αβ T-cell clonotypes on day 6.

Patient + Clone ID	Total clone size	Tissue	Time-point	Clone size	TRAV // TRAJ // CDR3α	TRBV // TRBD // TRBJ // CDR3β	Dual TRA/TRB
CD1340							
2	21	Blood	D6	11	TRAV20 // TRAJ26 // AVQVNRGQNFV	TRBV15 // TRBJ2-7 // TRBD1 // ATSPMTGGEQY	
			D14	2			
		IEL	BL	2			
			D14	6			
3	22	Blood	D6	9	TRAV25 // TRAJ43 // AVNNDMR	TRBV27 // TRBJ1-2 // TRBD1 // ASSPGTGLDYGYT	
		IEL	BL	1			
			D14	12			
1	36	Blood	D6	6	TRAV26-1 // TRAJ20 // ISWASNDYKLS	TRBV12-3 // TRBJ1-2 // TRBD2 // ASSPSPGAWYGYT	
			D14	1			
		IEL	BL	18			
			D14	11			
6	7	Blood	D6	2	TRAV10 // TRAJ34 // VVSALSNTDKLI	TRBV4-2 // TRBJ2-1 // TRBD2 // ASSPLESYNEQF	
			D14	2			
		IEL		3			
7	4	Blood	D6	2	TRAV38-2/DV8 // TRAJ48 // AYSDFGNEKLT	TRBV27 // TRBJ2-7 // TRBD2 // ASSLSLAGDHEQY	
			D14	1			
		IEL		1			
CD1342							
4	11	Blood	BL	1	TRAV38-2/DV8 // TRAJ57 // AIPQDLAQGGSEKLV	TRBV5-5 // TRBD2 // TRBJ1-2 // ASSLGSVFNYGYT	
			D6	6			
		IEL	D14	4			
1	33	Blood	BL	9	TRAV3 // TRAJ8 // AVRDSQSGFQKLV	TRBV28 // TRBD2 // TRBJ2-2 // ASSLRDPGELF	
			D6	3			
		IEL	BL	2			
			D14	19			
6	5	Blood	D6	2	TRAV12-1 // TRAJ17 // VLYGAAGNKLT	TRBV20-1 // TRBD1 // TRBJ2-1 // SGGTGSHEQF	
			D14	2			
		IEL		1			
12	4	Blood	BL	1	TRAV19 // TRAJ8 // ALSEANTGFQKLV	TRBV5-1 // TRBD1 // TRBJ2-3 // ASSLEGQHTDTQY	
		IEL	D6	3			
17	3	Blood	D6	2	TRAV2 // TRAJ15 // AVEDENQAGTALI	TRBV4-1 // TRBD2 // TRBJ2-7 // ASSQAPGNEQY	
		IEL	D14	1			
CD1300							
2	17	Blood	D6	12	TRAV5 // TRAJ45 // AESGGYSGGGADGLT	TRBV7-9 // TRBD2 // TRBJ2-1 // ASSLLSPQRDNEQF	
			D14	1			
		IEL		4			
6	10	Blood	D6	8	TRAV19 // TRAJ36 // ALSEARRTGANNLF	TRBV20-1 // TRBD2 // TRBJ1-6 // SATETGFYNSPLH	
		IEL	D14	2			
7	10	Blood	D6	8	TRAV17 // TRAJ49 // ASLDASFTGNQFY	TRBV4-1 // TRBD2 // TRBJ2-7 // ASRYTGTSVYEQY	
		IEL	D14	2			
4	5	Blood	D6	3	TRAV5 // TRAJ15 // AEPGQAGTALI	TRBV4-1 // TRBD1 // TRBJ2-3 // ASSRGPLSTDTQY	
			D14	1			
		IEL		1			
8	4	Blood	D6	3	TRAV39 // TRAJ57 // GGSEKLV	TRBV16 // TRBD1 // TRBJ1-1 // ASSPTAASEAF	TRAV38-1 // TRAJ54 // APGLLIQGAQKLV
		IEL	D14	1			
CD442							
2	17	Blood	D6	16	TRAV12-1 // TRAJ6 // VVNMEGGSYIPT	TRBV5-8 // TRBD1 // TRBJ1-3 // ASSFTGSGNTIY	
		IEL	D14	1			
–	5	Blood	D6	5	TRAV21 // TRAJ16 // AVEDGQKLL	TRBV12-3 // TRBD1 // TRBJ2-1 // ASSLDSSYNEQF	
–	4	Blood	D6	4	TRAV5 // TRAJ11 // AVDSGYSTLT	TRBV6-2 // TRBD1 // TRBJ2-3 // ASSPTGGNTQY	

Table 3. continued

Patient + Clone ID	Total clone size	Tissue	Time-point	Clone size	TRAV // TRAJ // CDR3α	TRBV // TRBD // TRBJ // CDR3β	Dual TRA/TRB
1	4	Blood	D6	2	TRAV13-2 // TRAJ41 // AETRSGYALN	TRBV5-5 // TRBD1 // TRBJ2-7 // ASSLRPSLEQY	
		IEL	BL	1			
			D14	1			
6	3	Blood	D6	2	TRAV14/DV4 // TRAJ57 // GGSAQGGSEKLV	TRBV24-1 // TRBD1 // TRBJ2-5 // ATSDSTGVETQY	TRAV24 // TRAJ20 // AFEVSNDYKLS
		IEL	D14	1			

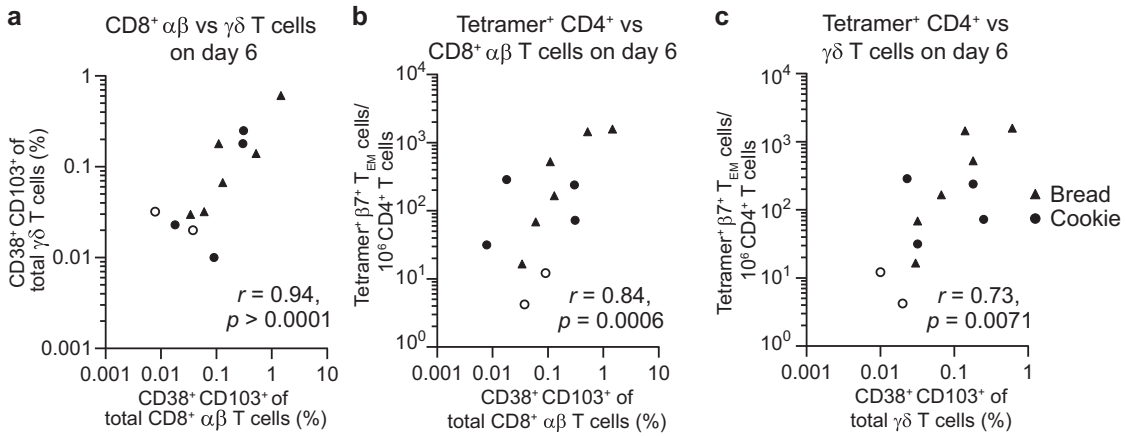


Fig. 5 Correlation analyses between the different T-cell responses in blood on day 6 after 3-day challenge with cookie or bread. **a** CD103$^+$CD38$^+$ CD8$^+$ αβ and CD103$^+$CD38$^+$ γδ T cells. **b** HLA-DQ2.5:gluten tetramer$^+$ integrin β7$^+$ effector-memory CD4$^+$ T cells and CD103$^+$CD38$^+$ CD8$^+$ αβ T cells. **c** HLA-DQ2.5:gluten tetramer$^+$integrin β7$^+$ effector-memory CD4$^+$ T cells and CD103$^+$CD38$^+$ γδ T cells. Patients challenged with cookie or bread are depicted with circles and triangles, respectively. Two patients who did not respond to the gluten challenge (determined by tetramer response) are indicated with open circles. *P* value (p) was calculated by Pearson correlation coefficient.

intestinal resident memory T cells recirculate in the blood of mice[26]. Furthermore, these resident T cells that rejoined the blood displayed a preference for homing back to their original tissue upon reactivation. While this study was performed in mice, our data indicate that a similar circulation of resident T cells may occur in humans as well, however further studies are needed are prove this concept. Taken together, these observations based on single-cell sequencing support the notion that the activation of clonally diverse CD8$^+$ αβ T cells in blood and gut of CeD subjects might be inflammation-induced, and where IL-15 and NK-cell receptors on the T cells could be involved.

In conclusion, our gluten challenge study has revealed that there is γδ and CD8$^+$ αβ clonotype sharing between blood and gut tissues and that this sharing is seen prior to gluten consumption. We found no evidence that gluten-induced immune activation in CeD involves particular patient-shared γδ or CD8$^+$ αβ T-cell clonotypes. Our observations suggest that CD4$^+$ T cells employing the disease-associated HLA-molecules as restriction elements can drive expansions of clonally diverse γδ and CD8$^+$ αβ T cells.

METHODS

Human subjects and study design

14-day gluten challenge. Twenty treated CeD patients were recruited to a clinical study with the aim to determine whether an oral gluten challenge of 14 days is sufficient to demonstrate mucosal changes in well-treated CeD patients[16]. Duodenal biopsies were collected prior to and at day 14 of the gluten challenge. In addition to histological assessments, eight biopsies were collected to investigate immune cells in the small intestine by flow cytometry. Blood was drawn and collected throughout the gluten challenge; at baseline, day 6 and day 14. The detailed information of the clinical study can be found elsewhere[16]. For this study, we obtained complete or partly complete sets of biological samples from eight of the patients from the 14-day gluten challenge study.

In order to obtain single-cell suspension of the lamina propria cells of the duodenal biopsies, we first treated the biopsies with EDTA for 10–20 min at 37 °C in order to remove the epithelial layer. Subsequently, the biopsies were treated with collagenase for 30–60 min, at 37 °C with rotation. The initial isolation step allowed us to separate the intraepithelial lymphocyte (IEL) from the lamina propria cells, and the samples were cryopreserved prior to further analysis.

Peripheral mononuclear blood cells (PBMCs) were isolated using a density gradient method with Lymphoprep and directly subjected to tetramer staining and magnetic bead enrichment, as described in Sarna et al.[16]. The tetramer-enriched sample were further analysed while the depleted PBMC fraction were cryopreserved until further use. For patient CD1300, we suspected baseline and day 6 samples were mixed up prior to sorting due to the presence of a distinct population of CD38$^+$CD103$^+$ T cells only in the baseline sample. This patient was a clear responder on all other parameters. This suspicion of sample mixup was later confirmed when this patient participated in a later gluten challenge study. Hence, the data presented here for blood samples on baseline and day 6 for CD1300 have been swapped.

3-day gluten challenge. For further analysis of the different T-cell responses on day 6 in blood, we had access to blood samples from another 12 treated CeD patients that underwent a 3-day

gluten challenge. Six of the patients ate a gluten-containing, FODMAP-free, cookie for 3 days and has been described previously[8]. The other half ate 4 slices of white bread for 3 days. Blood was collected in BD Vacutainer Cell Preparation Tubes (CPT) and processed and cryopreserved as described[8]. Upon analysis, the frozen samples were thawed and enriched for gluten-specific T cells using a cocktail of five HLA-DQ2.5 tetramers. In parallel, the depleted PBMC fraction was used for analysis of CD8$^+$ and γδ T-cell populations.

Surface marker staining, flow cytometry analysis and single-cell sorting

The frozen tetramer-depleted PBMCs from 14-day gluten challenge study were first thawed and filtered. We then stained the cells with PE-conjugated anti-γδTCR for 20 min followed by an anti-PE bead enrichment step due to low frequency of CD103$^+$ γδ T cells (<1% of total γδ T cells). The γδTCR-enriched sample was used for sorting of γδ T cells while the depleted fraction was used to sort CD8$^+$ αβ T cells. The samples were then stained with a cocktail of the following antibodies: CD8-PerCP-Cy5.5 (clone SK1, BioLegend), CD27-PE-Cy7 (clone LG.7F9, eBioscience), CD38-FITC (clone HB7, BioLegend), CD103-APC (clone B-ly7, eBioscience), CD4-APH-H7 (clone SK3, BD Biosciences), CD3-eVolve605 (clone OKT3, eBioscience). The depleted fraction was additionally stained with γδTCR-PE (clone 5A6.E9, Invitrogen), and a dump channel consisting of CD11c-Pacific Blue (clone B-ly6, BD Biosciences), CD14-Pacific Blue (clone M5E2, BioLegend), CD19-Pacific Blue (clone HIB19, BioLegend) and CD56-Pacific Blue (clone MEM-188, BioLegend). Cryopreserved IELs were thawed and filtered. They were directly stained without bead enrichment with a similar panel to the blood samples. The exceptions were the use of epithelial antigen-FITC (clone BerEP4, DAKO) and CD38-PE-Cy7 (clone HIT2, eBioscience).

For the additional 3-day gluten challenge studies, the cryopreserved PBMCs were thawed as described above and the samples were first subjected to PE-conjugated tetramer bead enrichment. These cells were then analysed as described[8] whereas the depleted PBMC fraction was labelled with the same panel of antibodies as described above but without γδTCR-enrichment prior to flow cytometry analysis on FACS AriaIII. Data was analysed using FlowJo software v10 (Flowjo LLC). Gating strategy is provided in Supplementary Fig. 4.

Library preparation for single-cell γδ and αβ TCR sequencing

Single-cell TCR sequencing of γδ and αβ T cells were performed as described[4,17]. After preparing separate γδ and αβ libraries, we combined the two libraries and sequenced them in the same Illumina Miseq sequencing run. Sequencing was performed at the Norwegian Sequencing Center and sequence data deposited at the European Genome-Phenome Archive (EGA) under the accession number EGAS00001004484.

TCR repertoire analysis

Data processing and analysis was performed as described in[4,17]. Briefly, obtained reads from Illumina sequencing were pre-processed using selected tools from the pRESTO toolkit[27]. Using an in-house Java program the γδ and αβ TCR sequences were subjected to further filtration and processing as previously described[4,17]. Only sequences with >50 reads were included in further analysis. We allowed for dual chains with a maximum of three chains in total. For both *TRG/TRD* and *TRA/TRB* sequences from each patient, cells were classified to the same clonotype when having identical V and J gene as well as identical nucleotide CDR3 region.

Circos plots were generated using the online circos tool (http://circos.ca/circos_online). Other graphs were made in Graphpad Prism 8 and Adobe Illustrator.

Statistics

Integrated statistical tools in Graphpad Prism 8 was used for statistical analyses.

Study approval

The studies were approved by the Regional Committee for Medical and Health Research Ethics South-East Norway (2010/2720, 2012/341 and 2013/1237), and registered at www.clinicaltrials.gov (NCT0246415). All patients gave written informed consent.

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AUTHOR CONTRIBUTIONS

A.C., L.F.R., L.M.E., K.E.A.L. and L.M.S. designed the study. K.E.A.L. and S.Z. recruited patients and performed the clinical challenges, and provided patient material. L.F.R., S.D.K., L.M.E. and S.Z. collected and processed the patient material. L.F.R., L.M.E. and S.Z. acquired the data. R.S.N. developed the bioinformatics tools. L.F.R., L.M.E., A.C. and L.M.S. analysed the data and wrote the manuscript. All the authors revised and approved the manuscript.

ADDITIONAL INFORMATION

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Ethics declaration: K.E.A.L. and L.M.S., either privately or as representatives of their employer, have been consultants during the two last years for Amyra Biotech AG (K.E.A.L.), Bioniz Therapeutics (K.E.A.L., L.M.S.), Chugai Pharmaceutical (K.E.A.L., L.M.S.), Dr. Falk Pharma GMBH (K.E.A.L.), Immusant Therapeutics (K.E.A.L., L.M.S.), Interexon Actobiotics (K.E.A.L., L.M.S.), UCB Biopharma (L.M.S.), Merck (L.M.S.) and GSK (L.M.S.). The other authors declare no conflicts of interest.

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REFERENCES

1. Lebwohl, B., Sanders, D. S. & Green, P. H. R. Coeliac disease. *Lancet* **391**, 70–81 (2018).
2. Sollid, L. M. Molecular basis of celiac disease. *Annu Rev. Immunol.* **18**, 53–81 (2000).
3. Bodd, M. et al. Direct cloning and tetramer staining to measure the frequency of intestinal gluten-reactive T cells in celiac disease. *Eur. J. Immunol.* **43**, 2605–2612 (2013).
4. Risnes, L. F. et al. Disease-driving CD4$^+$ T cell clonotypes persist for decades in celiac disease. *J. Clin. Invest* **128**, 2642–2650 (2018).
5. Lundin, K. E. et al. Gliadin-specific, HLA-DQ(α1*0501,β1*0201) restricted T cells isolated from the small intestinal mucosa of celiac disease patients. *J. Exp. Med.* **178**, 187–196 (1993).
6. Christophersen, A. et al. Tetramer-visualized gluten-specific CD4$^+$ T cells in blood as a potential diagnostic marker for coeliac disease without oral gluten challenge. *U. Eur. Gastroenterol. J.* **2**, 268–278 (2014).
7. Raki, M. et al. Tetramer visualization of gut-homing gluten-specific T cells in the peripheral blood of celiac disease patients. *Proc. Natl Acad. Sci. USA* **104**, 2831–2836 (2007).
8. Zuhlke, S. et al. CD38 expression on gluten-specific T cells is a robust marker of gluten re-exposure in coeliac disease. *U. Eur. Gastroenterol. J.* **7**, 1337–1344 (2019).

9. Anderson, R. P., Degano, P., Godkin, A. J., Jewell, D. P. & Hill, A. V. In vivo antigen challenge in celiac disease identifies a single transglutaminase-modified peptide as the dominant A-gliadin T-cell epitope. *Nat. Med.* **6**, 337–342 (2000).
10. Han, A. et al. Dietary gluten triggers concomitant activation of CD4$^+$ and CD8$^+$ αβ T cells and γδ T cells in celiac disease. *Proc. Natl Acad. Sci. USA* **110**, 13073–13078 (2013).
11. Mazzarella, G. et al. Gliadin activates HLA class I-restricted CD8$^+$ T cells in celiac disease intestinal mucosa and induces the enterocyte apoptosis. *Gastroenterology* **134**, 1017–1027 (2008).
12. Picascia, S. et al. Gliadin-specific CD8$^+$ T cell responses restricted by HLA Class I A*0101 and B*0801 molecules in celiac disease patients. *J. Immunol.* **198**, 1838–1845 (2017).
13. Meresse, B. et al. Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. *Immunity* **21**, 357–366 (2004).
14. Hüe, S. et al. A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease. *Immunity* **21**, 367–377 (2004).
15. Meresse, B. et al. Reprogramming of CTLs into natural killer-like cells in celiac disease. *J. Exp. Med.* **203**, 1343–1355 (2006).
16. Sarna, V. K. et al. HLA-DQ:gluten tetramer test in blood gives better detection of coeliac patients than biopsy after 14-day gluten challenge. *Gut* **67**, 1606–1613 (2018).
17. Eggesbo, L. M. et al. Single-cell TCR sequencing of gut intraepithelial γδ T cells reveals a vast and diverse repertoire in celiac disease. *Mucosal Immunol.* **13**, 313–321 (2020).
18. Jabri, B. et al. TCR specificity dictates CD94/NKG2A expression by human CTL. *Immunity* **17**, 487–499 (2002).
19. Mayassi, T. et al. Chronic inflammation permanently reshapes tissue-resident immunity in celiac disease. *Cell* **176**, 967–981.e919 (2019).
20. Lopez-Palacios, N. et al. Evaluation of T cells in blood after a short gluten challenge for coeliac disease diagnosis. *Dig. Liver Dis.* **50**, 1183–1188 (2018).
21. Sollid, L. M. & Thorsby, E. HLA susceptibility genes in celiac disease: genetic mapping and role in pathogenesis. *Gastroenterology* **105**, 910–922 (1993).
22. Bhagat, G. et al. Small intestinal CD8$^+$TCRγδ$^+$NKG2A$^+$ intraepithelial lymphocytes have attributes of regulatory cells in patients with celiac disease. *J. Clin. Invest* **118**, 281–293 (2008).
23. Jarry, A., Cerf-Bensussan, N., Brousse, N., Selz, F. & Guy-Grand, D. Subsets of CD3$^+$ (T cell receptor α/β or γ/δ) and CD3$^-$ lymphocytes isolated from normal human gut epithelium display phenotypical features different from their counterparts in peripheral blood. *Eur. J. Immunol.* **20**, 1097–1103 (1990).
24. Balk, S. P. et al. Oligoclonal expansion and CD1 recognition by human intestinal intraepithelial lymphocytes. *Science* **253**, 1411–1415 (1991).
25. Kerckhove, C. Van et al. Oligoclonality of human intestinal intraepithelial T cells. *J. Exp. Med.* **175**, 57–63 (1992).
26. Fonseca, R. et al. Developmental plasticity allows outside-in immune responses by resident memory T cells. *Nat. Immunol.* **21**, 412–421 (2020).
27. Vander, J. A. et al. pRESTO: a toolkit for processing high-throughput sequencing raw reads of lymphocyte receptor repertoires. *Bioinformatics* **30**, 1930–1932 (2014).