Consistency in Polyclonal T-cell Responses to Gluten Between Children and Adults With Celiac Disease

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BACKGROUND & AIMS: Developing antigen-specific approaches for diagnosis and treatment of celiac disease requires a detailed understanding of the specificity of T cells for gluten. The existing paradigm is that T-cell lines and clones from children differ from those of adults in the hierarchy and diversity of peptide recognition. We aimed to characterize the T-cell response to gluten in children vs adults with celiac disease. **METHODS:** Forty-one children with biopsy-proven celiac disease (median age, 9 years old; 17 male), who had been on strict gluten-free diets for at least 3 months, were given a 3-day challenge with wheat; blood samples were collected and gluten-specific T cells were measured. We analyzed responses of T cells from these children and from 4 adults with celiac disease to a peptide library and measured T-cell receptor bias. We isolated T-cell clones that recognized dominant peptides and assessed whether gluten peptide recognition was similar between T-cell clones from children and adults. **RESULTS:** We detected gluten-specific responses by T cells from 30 of the children with celiac disease (73%). T cells from the children recognized the same peptides that were immunogenic to adults with celiac disease; deamidation of peptides increased these responses. Age and time since diagnosis did not affect the magnitude of T-cell responses to dominant peptides. T-cell clones specific for dominant α- or ω-gliadin peptides from children with celiac disease had comparable levels of reactivity to wheat, rye, and barley peptides as T-cell clones from adults with celiac disease. The α-gliadin–specific T cells from children had biases in T-cell receptor usage similar to those in adults. **CONCLUSIONS:** T cells from children with celiac disease recognize similar gluten peptides as T cells from adults with celiac disease. The findings indicate that peptide-based diagnostics and therapeutics for adults may also be used for children.

Keywords: Food Intolerance Mechanisms; Immune Response; Immunity; Pediatric; T-Cell Epitope.

Detailed understanding of the function and specificity of T cells responsible for autoimmunity is widely expected to translate to more effective strategies to diagnose, treat and prevent autoimmune disease.[1] Among all the autoimmune diseases, celiac disease (CD) is the only one for which there is broad consensus on the identity of immunodominant epitopes consistently recognized by pathogenic CD4^{+} T cells.[2] However, little is known about the CD4^{+} T-cell response in children close to the time when the immunological events responsible for CD are initiated.[3] Understanding these early events is important, given the rising incidence of CD[4,5] and recent failures of large intervention studies aimed at reducing the development of CD in high-risk infants.[6,7]

Celiac disease is characterized by the presence of gluten-dependent intestinal damage, autoantibodies to tissue transglutaminase (tTG), and the presence of human leukocyte antigen (HLA) genes that encode HLA-DQ2.5, HLA-DQ2.2, and/or HLA-DQ8, responsible for presenting gluten peptides to pathogenic CD4^{+} T cells.[8] CD4^{+} T cells isolated from intestinal tissue or circulating in blood after oral wheat challenge in adult CD patients preferentially recognize gluten peptides post-translationally modified (deamidated) by tTG that include highly conserved epitopes.[9,10] Approximately half or more of these wheat gluten-reactive CD4^{+} T cells in HLA-DQ2.5^{+} CD patients recognize 1 of 2 overlapping epitopes derived from partially deamidated wheat α-glaidin (PFPQPELPY: DQ2.5-glia-α1a or PQPELPYPQ: DQ2.5-glia-α2).[11,12] Several other epitopes, including 2 from partially deamidated ω-gliadin (PFPQPEQPF: DQ2.5-glia-ω1 and PQPEQPFPW: DQ2.5-glia-ω2), are also commonly recognized by a substantial proportion of wheat gluten-reactive T cells from the intestinal mucosa and in blood after oral wheat challenge in adults with CD.[13,14]

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Although CD is most often diagnosed in adulthood, prospective observational studies indicate the onset of CD is typically between 1 and 6 years of age.[3] The only detailed study to address the specificity of the T-cell response to gluten in children concluded that there were fundamental differences to adults.[15] Intestinal T-cell lines and clones from children revealed far greater diversity of the gluten-specific T-cell response, and included native gluten peptides not dependent on deamidation. This has established the prevailing dogma that the T-cell response to gluten in children with CD nearer disease onset is fundamentally different than that in adults with CD in terms of the hierarchy and diversity of peptide recognition. This dogma has remained unchallenged until the present study.

Our aim was to determine the hierarchy of gluten peptides responsible for activating T cells freshly isolated from blood after wheat challenge in children with CD and determine whether T-cell recognition of gluten differed between children and adults. Here we established the specificity and hierarchy of the polyclonal T-cell response to gluten in HLA-DQ2.5[+] children with CD in vivo and determined the redundancy of peptide recognition and T-cell receptor (TCR) gene usage to compare the specificity, magnitude, maturity, and clonality of T-cell responses to gluten between children and adults.

Materials and Methods

Participants and Oral Wheat Challenge

The study was approved by the Human Research Ethics Committees of The Royal Children's Hospital (RCH, no. 32018), the Walter and Eliza Hall Institute (no. 03/04) and the Royal Melbourne Hospital (no. 2003.009) and by the Carlo Romano Ethical Committee of the University of Naples, Federico II (no. 04/04/2012). All volunteers or their parents provided written informed consent. Volunteers had biopsy-proven CD diagnosed according to ESPGHAN criteria[16] and were HLA-DQ2.5[+] and HLA-DQ8^{-}. Volunteers followed a strict gluten-free diet for at least the previous 3 months and were recruited by advertisement in the Coeliac Victoria and Tasmania newsletter or during attendance at RCH or the Section of Pediatrics, DISMET, Italy. The Australian cohort consisted of 41 CD volunteers (3–17 years of age; median, 9 years; 17 M, 24 F) split into 3 age groups: 3–5 years old, 6–10 years old, and 11–18 years old. An additional 4 CD adults (18+) were recruited for T cell comparisons (Supplementary Table S2). The Italian cohort consisted of 10 HLA-DQ2.5[+] CD volunteers (10–17 years of age; median, 13 years old; 4 M, 6 F). Short-term (3-day) wheat challenge and blood collection on day 6 (D6) after challenge was performed as previously described,[13] with daily bread intake reduced for children (see Supplementary Materials and Methods).

Antigens

To optimize assessment with a limited blood volume, a modified library containing wild-type and in silico-deamidated versions of the most immunogenic wheat gliadin and glutenin peptides described previously[13] was assessed (n = 70, 37 wild-type, and 33 deamidated) (Supplementary Table S3). When blood volume allowed, an additional series of deamidated peptides immunogenic in a large group of CD adults were also assessed in the Australian cohort: barley hordein (n = 22), rye secalin (n = 30), and oat avenin (n = 2; 1 wild-type).[13,17] Also assessed were 7 sequences implicated in pediatric CD on the basis of intestinal T-cell line (TCL) reactivity[15] as wild-type and deamidated versions at a 50% mixture of leucine and isoleucine (Supplementary Table S3, indicated by "X"), and wild-type and deamidated versions of peptides recognized by intestinal TCL derived from adults with villous atrophy or potential CD in an Italian cohort, containing γ-gliadin epitopes and length variants[18,19] (Supplementary Table S3) (additional information on screening libraries and protein antigens is available in Supplementary Materials and Methods).

Immunological Assays

IFN-γ ELISpot, IFN-γ secretion assay, T-cell cloning, and TCR sequencing methods are described in Supplementary Materials and Methods.

Statistical Analysis

Simpson's diversity index was used to measure clonotype diversity within individuals (details are shown in Supplementary Materials and Methods). Two-tailed Mann-Whitney U test or Kruskal-Wallis test was performed for comparisons between 2 or more groups of paired data, respectively. Contingency analysis was performed using either the Fisher's exact test or chi-square test. Statistical tests were performed using Prism version 6 software (GraphPad, LaJolla, CA). P values $\leq$ .05 were considered significant.

Results

Clinical Response to Oral Wheat Challenge

Forty-one Australian children (17 M: 24 F) with confirmed CD participated (Supplementary Table S1). Most subjects presented with symptoms at diagnosis, with only 7 detected following screening based on positive family history. At the time of this study, children were between 3 and 17 years of age (median, 9 years) (Supplementary Table S2). Each participant undertook a 3-day wheat challenge consisting of 1 to 3 slices of wheat bread daily depending on age. Most volunteers (31 of 41) had normal baseline CD serology immediately prior to undertaking the challenge (Supplementary Table S2). The wheat challenge induced symptoms in 29 of 41 (71%) volunteers, which were mainly gastrointestinal (such as nausea, bloating, abdominal pain and vomiting) and comparable to that experienced by CD adults after short-term wheat challenge.[20] Four volunteers (3–5 years of age, n = 1; 6–10 years of age, n = 2; 11–18 years of age, n = 1) did not consume the entire serving of gluten over 3 days due to vomiting (n = 3) or poor tolerability (n = 1), but analysis on day 6 (D6) was still performed. Symptoms were fully resolved in 26 of 29 (90%) of children by D6 with conservative management. The proportion of symptomatic individuals did not vary by age grouping (P = not significant [NS], chi-square test) or by HLA-DQ2.5 zygosity (P = NS, Fisher's exact test). The presence of symptoms did not correlate with a significant

T-cell response (see below) to gliadin, gluten or any gluten peptide (*P* = NS, chi-square test), although all volunteers who experienced vomiting mounted a positive response to gluten peptide(s). There were no differences in age or degree of villous atrophy (Marsh 3A, 3B, or 3C) at time of CD diagnosis between HLA-DQ2.5 homozygous and heterozygous volunteers (*P* = NS, chi-square test).

Gluten-Specific T Cells Have Similar Specificity in Children and Adults with CD

After oral wheat challenge, significant IFN-γ ELISpot responses were detectable in most CD volunteers (30 of 41 [73%]) to at least 1 wheat gluten peptide (Figure 1 [peptide details are shown in Supplementary Table S3]). Responses to whole protein (gliadin and/or gluten) were less consistent, present in 23 of the 30 wheat gluten peptide responders and in an additional 2 participants who did not respond to any wheat gluten peptides. Each age group contained a similar proportion of gluten peptide/protein responders, 3–5 years of age; 10 of 13, 76.9%; 6–10 years of age; 10 of 12, 83.3%; 11–18 years of age; 12 of 16, 75% (*P* = NS, chi-squared test). Significant responses to deamidated gliadin, an α-gliadin peptide (LQPFPQPELPYPQPQ) containing the T-cell epitopes DQ2.5-glia-α1a and α2 (hereafter named W02), and an ω-gliadin peptide (QPFPQPEQPFPWQ) containing the T-cell epitopes DQ2.5-glia-ω1 and ω2 (hereafter named W03) were seen on day 6 (D6) following wheat challenge but not prior to D0 (Figure 2A [n = 28]). A low positive response to deamidated gliadin was detected in 1 volunteer on D0. Responses to the positive control tetanus toxoid did not differ between D0 and D6 (Figure 2A). The presence of a T-cell response to gluten or gluten peptide was not affected by positive CD serology at baseline or DQ2.5 zygosity status (*P* = NS for both, Fisher's exact test).

There was a clear preference for deamidated antigens compared to their native counterparts (Figure 1 and Figure 2B). The highest IFN-γ ELISpot responses were commonly noted to a gluten peptide compared to whole-protein antigen (gliadin or gluten) in most cases (22 of 32 [69%], including 2 protein-only responders), suggesting

	3-5yrs										6-10yrs									11-18yrs										
Subject	C2	C1	C4	C6	C3	C7	C12	C11	C13	C9	C14	C22	C23	C24	C15	C19	C20	C16	C25	C33	C38	C39	C27	C35	C28	C36	C40	C34	C30	C41
Age (Years)	4 H	3	4	5	4	5	5	5	5 H	5 H	6 H	10	10	10	6	9	9 H	6	10	14	17 H	17	11 H	16	11	16	17	15 H	13 H	17 H
> 10 SFU (cut-off 1)	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
No Antigen	0	0	0	0.5	0	2	1	2.5	0	1	3.5	1	3	9.5	2	2.5	0	0.5	3.5	0.5	1	1	0.5	0	0.5	7.2	0	2.5	0.5	0.5
3 x No Antigen (cut-off 2)	0	0	0	1.5	0	6	3	7.5	0	3	10.5	3	9	28.5	6	7.5	0	1.5	10.5	1.5	3	3	1.5	0	1.5	21.6	0	7.5	1.5	1.5
Tetanus Toxoid positive control	ND	ND	11	3.5	15	54	4.5	13	10	21	104	14	31	30	54	30	13	112	45	ND	47	674	47	222	55	309	210	34	1	17
Deamidated gliadin (100µg/ml)	51.5	8	21.5	1.5	1	9	78.5	13.5	4	7.5	70.5	42	5	6	26	15.5	3	1	15	10	44.5	138	78.5	7.5	27.5	14	13	6.5	2.5	8
Gliadin (100µg/ml)	44.5	3	17.5	1.5	1.5	32	87	12.5	3	5.5	21	13	4.5	11	15.5	34	20	8	24.5	6	38.5	57	12.5	8.5	16	28	50	17	4	23
Deamidated Gluten (100µg/ml)	77.5	4	20	3.5	8	43	75.5	11.5	3	8	70	17.5	13.5	16.5	17.5	27	25	18.5	15.5	8	37	127	51	3	12.5	37.5	56.5	26.5	4.5	48
Gluten (100µg/ml)	47.5	4.5	10.5	1	2	30	86.5	21.5	4.5	13	12	8	8	16.5	16.5	24.5	5.5	5.5	23.5	5	25.5	60	8.5	2.5	6	21	40	9.5	3	12.5
Max peptide	48	19	11	13	60	44	13	14.5	12	16.5	196	53	35	30	16	15	48	57	27	20	30.5	178	224	15	17	64	97	81	10	95
QPFPQPEQPFPWQ	48	19	1		60	18		8	9		113	26	34	1	8	4	47	6	4	5	17	77	61	5	6	43	54	34	2	83
LQPFPQPELPYPQP	45	7	11		36	24		6	8		146	34	30	14	11	10	44	38	6	6	18	178	142	4	10	64	97	81	0	89
LQPFPQPELPFPQP	41	4	4		24	26		12	10		112	16	22	15	6	11	15	43	15	5	13	107	103	7	16	33	65	54	3	59
LQPFPQPELPYSQP	36	3	2		13	22		11	7		71	9	19	25	12	15	20	39	6	3	6	81	104	5	15	37	29	25	2	53
LPYPQPELPYPQP	33	6	11		33	44		9	5		196	53	35	15	16	11	33	45	3	2	12	161	111	7	10	36	78	44	1	62
QPFPQPEQPFPWQP	27.5	7	3.5	13	52.5	28.5	10	14.5	5.5	9	95	18.5	25	9	10	11	40.5	20.5	63	3.5	30.5	84.5	44	8	8.5	20	63	17	3.5	67.5
LQPFPQPELPYPQPQ	31	5.5	8	10	29	40.5	13	14	12	16.5	99.5	24	30	6.5	10.5	8.5	42	57	8	2.5	27	167	152	10	8	41.5	67.5	45	2	66.5
QPFPQPEQPIPVQ	29	2	2		53	10		11	4		126	12	27	1	9	5	46	9	9	5	20	103	76	3	10	42	51	27	1	95
PFPQQPQQPFPQP	4	2	2		3	0		12	1		5	3	3	6	6	6	1	3	1	0	2	1	0	0	2	14	9	2	2	0
PEQPFPEQPEQPF	4	0	0		9	4		11	0		24	22	3	24	2	2	4	12	5	2	1	2	1	2	9	14	11	5	10	15
PEQTFPEQPQLPF	2	1	3		1	0		4	1		179	47	4	12	1	4	1	0	4	3	0	1	19	15	9	4	2	1	0	0
FPQPEQEFPQPQQ	13	3	0		13	10		5	5		158	35	9	21	3	13	15	1	11	6	7	21	49	4	9	11	27	15	3	26
YEVIRSLVLRTLPN	0	2	0		1	1		3	9		4	2	3	30	1	2	0	1	10	20	0	2	1	1	3	49	2	3	0	1
PFPLQPEQPFPQP	24	7	1		24	22		8	10		52	37	21	29	9	13	34	8	27	6	15	35	30	2	11	27	43	23	5	28
LPYPQQLPYPQP	13	3	1		14	23		7	5		75	24	18	21	16	11	18	25	8	3	5	120	61	2	13	17	53	25	1	49
LQPFPQPQLPYPQP	22	4	3		21	17		5	1		93	10	10	10	14	12	33	29	5	1	10	64	86	3	14	25	38	35	0	51
QPFPQPQQPFSQQ	13	3	1		9	12		3	2		13	21	5	12	6	12	18	1	6	3	9	12	16	1	2	10	28	22	2	30
FPQPQQQFPQPQQ	6	1	3		0	5		4	2		1	6	2	19	6	11	2	1	2	2	5	4	12	1	5	4	10	4	0	1
PFPEQPEQPYPQQ	11	1	1		6	4		5	3		9	5	7	14	5	11	13	3	3	0	2	8	14	1	1	15	19	2	1	14
PIPQQPQQPFPLQ	0	1	1		2	1		7	7		1	3	9	12	5	11	3	4	14	6	1	5	5	2	10	9	13	4	3	3
QFIQPEQPFPQQ	8	6	2		7	6		6	1		30	17	19	5	3	11	9	0	8	0	6	28	31	2	3	8	25	13	2	21
GQSGYYPTSPQQS	2	0	4		2	3		5	0		6	1	3	16	0	11	1	4	11	5	2	10	0	1	4	10	5	4	1	1
QPFPQPQQPIPVQ	9	5	0		17	9		9	9		136	26	11	8	3	7	48	4	13	1	12	40	51	4	4	21	37	30	2	65
TIPEQPEQPFPLQ	2	3	1		11	3		5	3		18	6	0	7	2	5	6	3	1	0	1	14	224	2	4	7	8	5	2	6
PFPEQPEQPFPQP	19	2	3		24	6		5	2		51	25	16	13	4	8	18	4	9	4	11	32	30	5	17	8	35	13	0	24
GIIQPQQPAQL					0	0		5	3		3	22	5	19	2	11		1	3				0	1	7	0	4			2
FLQPEQPFPEQPEQPYPEQPEQPFPQ					12	10		3	3		22	20	12	8	6	1		15	20				1	6	11	18	26			23
PQQPQQSFPQQQQPA					0	0		7	2		1	23	10	12	3	8		0	10					11	1	4	1	2		3
GLERPWQEQPLPPQ	0	0	2		1	6		5	6		3	0	7	23	9	3	0	1	11	4	2	0	0	0	16	7	22	1	1	1

Figure 1. Wheat-derived T-cell epitope hierarchy in pediatric celiac disease. Numbers are raw spot-forming units (SFU) from IFN-γ ELISpot assays performed on peripheral blood mononuclear cells (PBMC) from wheat-challenged celiac disease (CD) patients. Color coding represents responses above cutoff as a percentage of maximal peptide SFU (*red*, >70%; *orange*, 41–70%; *yellow*, 21–40%; *green*, 11–20%; and *blue*, 6–10%). Assay cutoffs are shown in *purple*, negative protein results in *gray*, and protein responses greater then peptide responses in *yellow*. Only peptides that reached >70% in at least 1 individual are shown. H, HLA-DQ2.5 homozygous individuals. Defined or predicted peptide sequence cores are in **bold**; ND, not done.
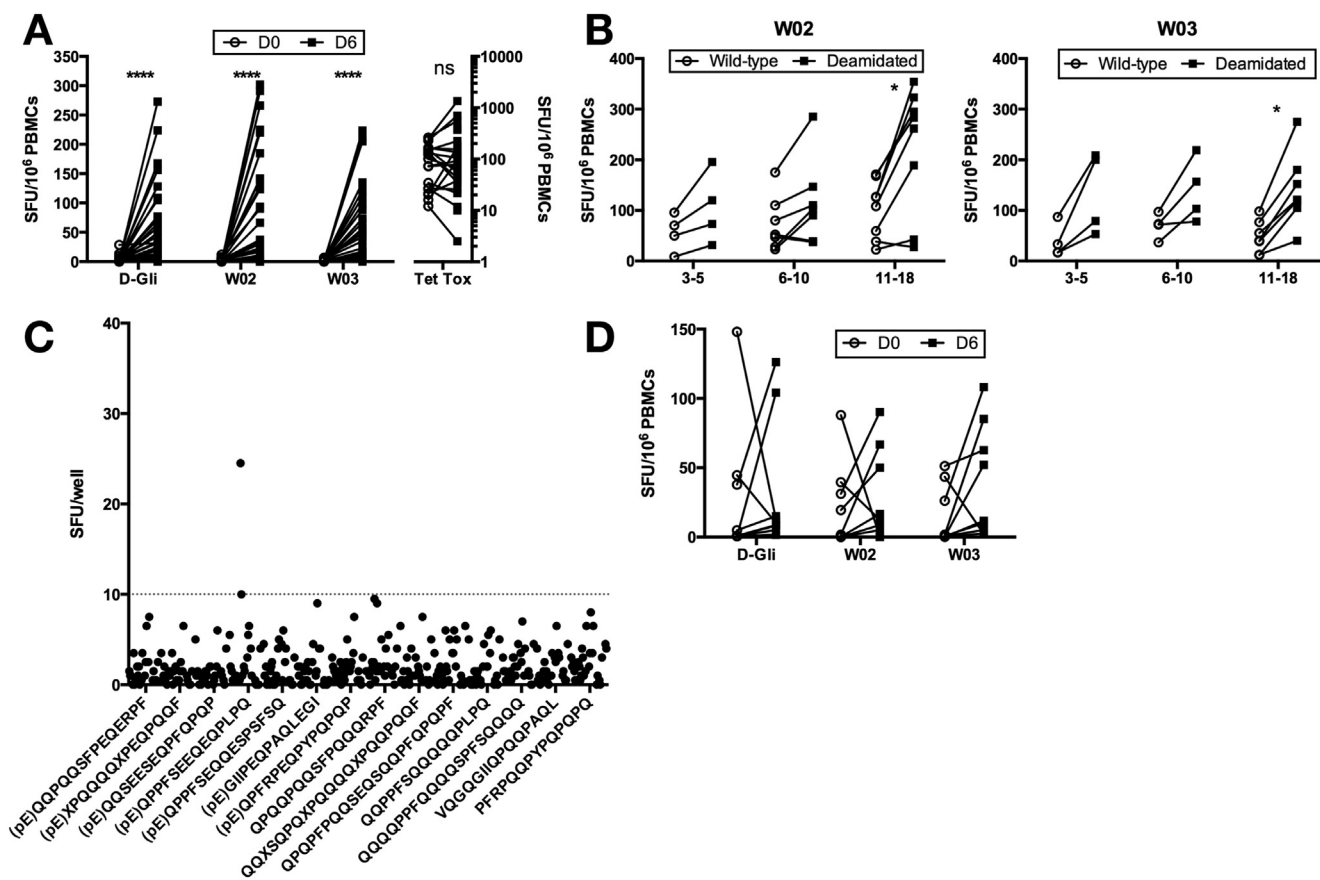
Figure 2. Wheat challenge induces gluten-specific T-cell responses in children with CD. Pediatric CD volunteers undertook 3-day oral wheat challenge, and T-cell responses to wheat-derived proteins and peptides were assessed by IFN-γ ELISpot. (*A*) Responses to deamidated gliadin, peptide W02 containing DQ2.5-glia-α1a/α2, and peptide W03 containing DQ2.5-glia-ω1/ω2 prior to (D0) and after (D6) wheat challenge (background subtracted) are shown. Gluten-specific responses were observed on D6 only and were significantly higher than D0 responses (*P* < .05, Kruskal-Wallis), whereas no differences were seen for tetanus toxoid. (*B*) Peptides W02 and W03 were tested in native and deamidated forms. Deamidation enhanced the response to peptide in all ages (n = 4, 3–5 years of age; n = 7, 6–10 years of age; n = 8, 11–18 years of age), some statistically significant (*P* < .05, Kruskal-Wallis). (*C*) Polyclonal responses to peptides described by Vader et al[15] as immunogenic to TCLs isolated from children. *Line* depicts response cutoff. (*D*) IFN-γ ELISpot responses of n = 9 Italian pediatric CD volunteers to deamidated gliadin, W02, and W03 (background subtracted).

these peptides were dominant and responsible for most of the immune response to whole protein. Overall, participants responded to a number of wheat gluten-derived peptides (Figure 1). Raw results were normalized by expressing them as a percentage of the maximal peptide response for that individual, and a response >70% was taken to be dominant.[13] Responses >70% of the maximal peptide response were seen in the majority of individuals against peptides containing the immunodominant DQ2.5-glia-α1/2 and ω1/2 epitopes or peptides with native or homologous versions of these epitopes. Dominant responses to additional unrelated wheat-derived peptide sequences were also detected in a small number of individuals, including the gliadin-derived sequence YEVIRSLVLRTLPN and the glutenin-derived sequences GQSGYYPTSPQQS and GLERPWQEQPLPPQ (Figure 1). Non-response to gluten, gliadin and any gluten peptide was significantly associated with a poor response to tetanus toxoid (*P* < .05, Mann-Whitney *U* test) (Supplementary Figure 1). Following wheat challenge, in vivo polyclonal T-cell responses against the novel immunogenic gluten peptides described by Vader et al[15]

were poor and detected in only 2 of 30 volunteers (6.7%) to the sequence QPPFSEEQEQPLPQ (Figure 2*C*).

Immunogenic peptides were ranked by magnitude of response to establish the hierarchy of gluten peptides (Figure 1). Furthermore, "dominance scores" within each age grouping and overall were calculated (Table 1). Twelve of seventy wheat gluten peptides were associated with dominance scores greater than or equal to 30 (an arbitrary level) for all age groups. Notably, the most dominant 4 peptides across all age groups corresponded closely to those observed in adults following wheat challenge[13] and included W02 (LQPFPQPELPYPQPQ) and W01 (LPYPQ PELPYPQP), both of which contain the DQ2.5-glia-α1a or 1b and α2 epitopes and were the 2 most immunogenic peptides in adults,[13] W06 (LQPFPQPELPFPQP) containing a homolog of DQ2.5-glia-α1a, and W03 (QPFPQPEQPFPWQP) containing the DQ2.5-glia-ω1/ω2 epitopes. The dominance hierarchy also contained the native versions of W01, W02, and W04 (QPFPQPQQPIPVQ) but their deamidated equivalents were ranked higher in all cases (Table 1). An additional 3 peptides had dominance scores greater than 30 in the

Table 1. Dominance Scores for Wheat-Derived Peptides Measured in Children With Celiac Disease After Wheat Challenge

Peptide name	Peptide sequence (core in bold)	Gliadin source	Defined/predicted T-cell epitopes	Dominance scores			
				3–5 y	6–10 y	11–18 y	Mean for all ages
W02	LQ**PFPQPELPYPQ**PQ	α	DQ2.5-glia-α1a/α2	77	57	60	64
W01	L**PYPQPELPYPQ**P	α	DQ2.5-glia-α1b/α2	65	72	51	62
W06	LQ**PFPQPELPFPQ**P	α	PFPQPELPF; PQPELPFPQ	58	53	54	54
W03	Q**PFPQPEQPFPW**QP	ω	DQ2.5-glia-ω1/ω2	65	51	46	54
W26	PFP**LQPEQPFPQ**P	ω	LQPEQPFPQ	46	62	35	47
W08	LQ**PFPQPELPY**SQP	α	DQ2.5-glia-α1a	45	52	40	45
W04	Q**PFPQPEQPIPV**Q	ω	PFPQPEQPI; PQPEQPIPV	43	44	48	45
W01 (WT)	L**PYPQPQLPYPQ**P	α	DQ2.5-glia-α1b/α2	31	49	35	39
W02 (WT)	LQ**PFPQPQLPYP**QP	α	DQ2.5-glia-α1a/α2	30	43	35	37
W04 (WT)	Q**PFPQPQQPIPV**Q	ω	PFPQPQQPI; PQPQQPIPV	33	43	31	36
W16	F**PQPEQEFPQ**PQQ	γ	PQPEQEFPQ	23	47	26	33
W13	LQ**PFPQPELPY**LQP	α	DQ2.5-glia-α1a	34	32	30	32
W32	PFP**EQPEQPFPQ**P	γ	DQ2.5-glia-γ4c	26	34	28	30
W05	Q**PFPQPEQPFSQ**Q	γ	DQ2.5-glia-ω1; PQPEQPFSQ	23	33	27	28
W07	Q**PFPQPEQPFCQ**Q	γ	DQ2.5-glia-ω1; DQ2.5-glia-γ4d	40	20	16	24

NOTE. Dominance scores were calculated for each peptide as the mean score for each age group and the mean of all subjects (percentage of maximal peptide response from Figure 1). Scores >30 are shaded in *gray*. Top 15 peptides dominant in one subgroup are shown.

3- to 5-year-old and 6- to 10-year-old groups, and these contained sequences homologous to W03.

To further test the relevance of the immunogenic wheat gluten peptides, wheat challenges were undertaken in a group of Italian children (n = 10, 10–17 years of age) with CD. As lower blood volumes were collected, T-cell responses were measured against a limited selection of the same wheat derived peptides as above. Nine of ten volunteers responded to at least 1 peptide (Supplementary Table 4) with only a single CD child not responding to any peptides or proteins (data not shown). Six children had a positive response to wild-type or deami-dated gluten proteins, and 4 to wild-type gliadin, 2 of which also responded to deamidated gliadin (Supplementary Table 4). Four of nine volunteers (44%) and 3 of 9 (33%) responded to W02 and W03, respectively (Figure 2D). Strong positive responses >40% of the maximal peptide response were mainly against peptides containing DQ2.5-glia-α1/2 and -ω1/2 homologs (Supplementary Table 4). Some volunteers also responded to unrelated gliadin and glutenin-derived peptides (Supplementary Table 4). Consistent with the Australian cohort of the same age range, bioactivity was enhanced with deamidation, although some native peptides, such as GQPGYYPTSPQQIGQ and IQVDPSGQVQWPQQ, induced responses in some individuals. Background responses were generally high (>10 spot forming units/well) (Supplementary Table 4) and 2 individuals responded to either gluten protein or peptide at D0 before oral wheat challenge (Figure 2D).

### Age and Time From Diagnosis Do Not Affect the Gluten-Specific T-Cell Response

Dose-response curves and half maximal effective concen-tration (EC50) values were calculated in 17 children (n = 4,

3–5 years of age; n = 8, 6–10 years of age; and n = 5, 11–18 years of age) and 4 adults (18–70 years of age) based on T-cell responses to W02 and W03 (Figure 3). Median EC50 were similar across each age group for W03 (Figure 3A). EC50 values for W02 were similar between children aged 6–10 years, 11–18 years, and adults, but differences were observed between 3–5 and 6–10 years of age (Figure 3A). EC50 values were not statistically different between children who were heterozygous and those who were homozygous for HLA-DQ2.5 (Figure 3B), but a trend toward a lower EC50 in homozygotes was observed. The overall magnitude of T-cell responses to W02 and W03 was significantly greater for volunteers who were HLA-DQ2.5 homozygotes than for heterozygotes (Figure 3C). This difference was not seen when comparing age groups (P = NS, Kruskal-Wallis test) (Figure 3D).

We also compared EC50 values based on the years since diagnosis of CD to assess its impact on T-cell responses. As expected, the elapsed time from diagnosis was lowest in the youngest age group (3–5 years of age; 0.4–4.4 years of age; median, 1 year, 6–10 years of age; 0.6–7.7 years of age; median, 3.3 years; and 11–18 years of age; 1–9.9 years of age; median, 3.6 years; P < .05, Kruskal-Wallis). Given the variability in disease presentation and clinical diagnosis, true disease duration was not possible to determine. If we used an arbitrary cut-off of wheat challenge occurring less or more than 2 years from diagnosis, then median EC50 values calculated for both W02 and W03 were not statistically different (Figure 3E).

Gluten-Specific T Cells Cross-React With Hordein and Secalin Peptides

We sought to determine the level of barley and rye grain cross-reactivity in wheat-specific T cells from CD children
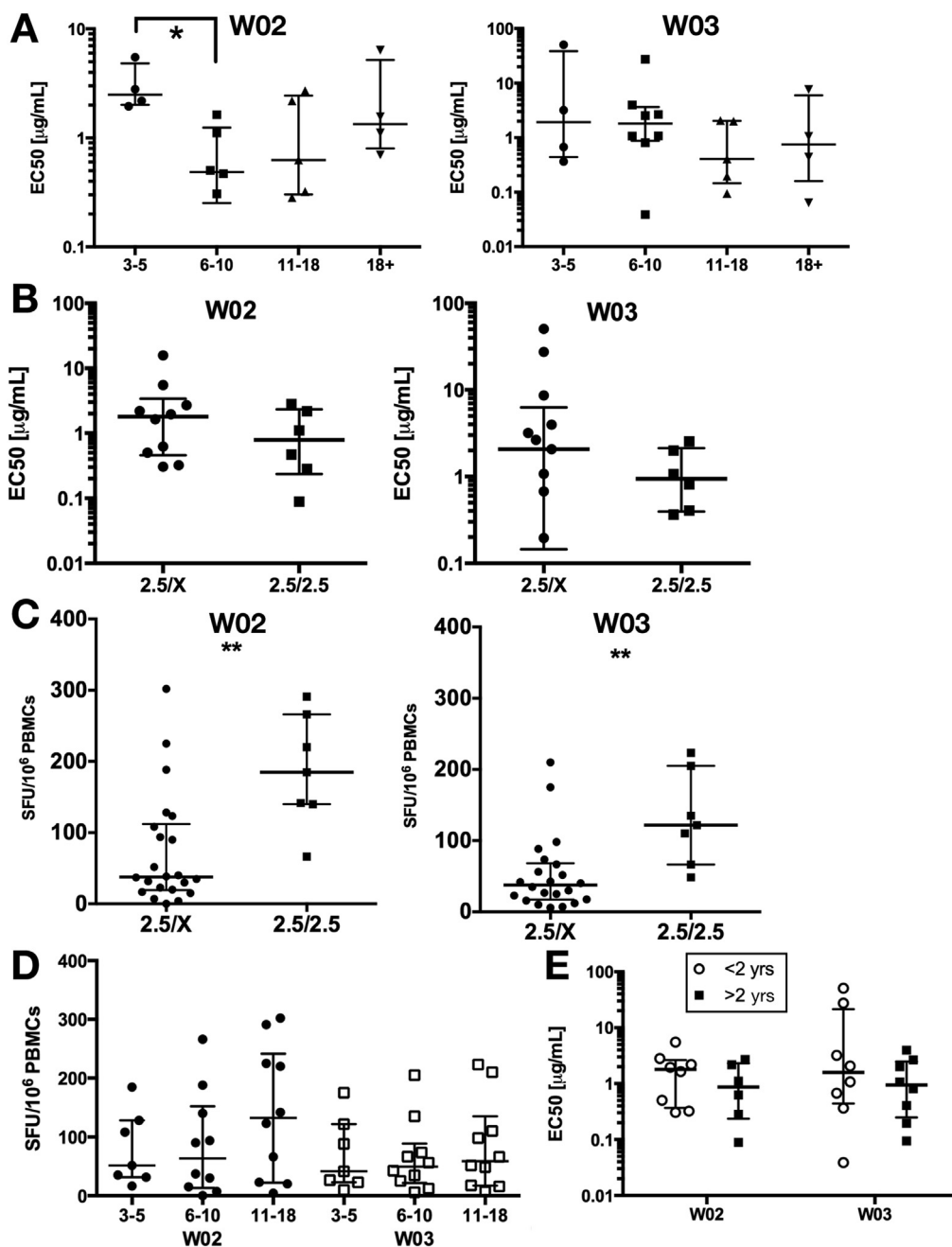
Figure 3. Effects of age, HLA-DQ2.5 zygosity, and time since diagnosis on gluten peptide T-cell responses. Peptides W02 and W03 were assessed in a dose-ranging study in pediatric and adult CD volunteers following oral wheat challenge. EC50 values were calculated and compared: (A) between age groups, (B) between HLA-DQ2.5 homozygous (n = 8) and heterozygous individuals (n = 10–13), and (E) between volunteers with diagnoses less than 2 years prior to gluten challenge (n = 8) or more than 2 years (n = 6–8). (C) ELISpot response magnitude is shown in homozygous (n = 7) and heterozygous (n = 22) individuals. (D) ELISpot response magnitude was categorized by age (n = 7, 3–5 years of age; n = 10, 6–10 years of age; and n = 10, 11–18 years of age). Median and interquartile ranges are shown (*$P < .05$, Kruskal-Wallis or Mann-Whitney U test).

raised in vivo and in vitro. We found polyclonal T cells induced by oral wheat challenge cross-reacted to a series of peptides derived from barley hordein and rye secalin (n = 22) (Figure 4). Fifteen of 22 CD children (68%) responded to W03 containing DQ2.5-glia-ω1/ω2 (Figure 4) and, in most cases, also responded to other peptides containing homologous sequences within hordein and secalin, consistent with the presence of a homologous T-cell epitope in barley (DQ2.5-hor-1: PFPQPEQPF).

A T-cell clone (TCC) specific to DQ2.5-glia-α2 and 1 specific to DQ2.5-glia-ω1/ω2 were raised from 2 different children with CD, and their recognition of comprehensive wheat gliadin, barley hordein, and rye secalin peptide libraries were assessed (Figure 5). Peptide responses were compared to those obtained from previously isolated TCC from adults with CD who were tested against the same libraries.[13] TCC 3007.28 (specific to DQ2.5-glia-α2) showed minimal reactivity to hordein and secalin peptides, consistent with the observation that peptides containing the immunodominant wheat T-cell epitopes DQ2.5-glia-α1a and DQ2.5-glia-α2 are infrequent in barley or rye (Figure 5, positive response to 575 of 3329 peptides is shown by color; peptide sequences and nonreactive peptides are not shown). In contrast, TCC specific to DQ2.5-glia-ω1/2 showed substantially more immunoreactivity to a range of hordein and secalin peptides that encompassed both epitopes. Notably, secalin and hordein cross-reactivity patterns of the TCC from children were very similar to those raised

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Figure 4. Cross-reactive T-cell responses in children with celiac disease after wheat challenge. Numbers are raw spot-forming units (SFU) from IFN-γ ELISpot assays performed on peripheral blood mononuclear cells (PBMC) from wheat-challenged CD patients. Color coding represents significant responses as percentages of maximal peptide SFU (*red*, >70%; *orange*, 41–70%; *yellow*, 21–40%; *green*, 11–20%; and *blue*, 6–10%). Defined or predicted peptide sequence cores are in **bold**.

Subjects	3-5yrs			6-10yrs								11-18yrs										
	C3	C12	C11	C18	C14	C22	C23	C24	C15	C20	C25	C33	C38	C39	C27	C35	C28	C36	C40	C34	C41	C30
Wheat																						
QPFPQPEQPFP**WQP**	52.5	10	14.5	5.5	95	18.5	25	9	10	40.5	11	3.5	30.5	84.5	44	8	8.5	20	63	17	67.5	3.5
Barley																						
QPFPQPEQPFP**LQ**	65	12	13	13	88	29	22	8	9	45	6	5	16	64	70	10	4	21	83	18	94	0
N**PLQPEQPFPLQ**PQPP	10	13	11	1	38	19	6	7	3	14	4	3	12	25	21	1	14	6	23	9	33	1
QPFPQPEQP**IPYQ**	31	11	9	5	124	21	22	11	11	30	8	8	23	84	86	2	3	33	58	55	84	0
PEQP**FPEQPQPY**PQQP	1	5	12	2	36	6	1	9	1	1	4	2	2	6	13	2	6	10	2	1	0	1
PEQP**FPEQPFPQQ**	6	2	12	1	31	8	7	3	1	3	7	2	1	6	12	5	3	9	7	6	11	0
PEQPQP**FPEQPVPQQP**	3	2	11	0	7	4	3	4	2	0	4	3	3	6	4	1	0	8	6	1	5	0
QPFPQPEQP**FSWQ**	26	8	0	2	79	20	25	3	8	42	6	0	16	19	34	6	1	14	29	18	41	1
QPFPQPEQPF**RQQ**	24	5	6	4	15	21	20	8	8	37	6	1	15	21	37	3	0	16	44	21	58	0
QEFPQPEQPFPQQ	30	6	8	8	93	21	11	3	2	36	2	4	15	38	30	4	1	7	44	23	67	1
Rye																						
QPFPQPEQP**IPQQ**	24	12	4	3	132	11	17	5	7	44	7	3	29	62	87	4	1	48	64	41	87	1
QPFPQPEQP**TPIQ**	20	12	8	0	86	7	5	0	4	38	6	6	11	73	63	1	1	23	39	21	61	2
QPFPQPEQ**QLPLQ**	2	11	6	3	79	9	3	3	5	11	11	1	5	5	30	1	1	11	11	4	27	3
QPFPQPEQ**ELPLQ**	9	5	15	6	57	13	7	0	0	33	8	4	7	31	46	1	5	17	14	12	36	1
Q**LFPLPEQPFPQP**	3	0	14	1	26	5	4	4	2	7	1	2	2	2	2	0	0	11	13	9	13	1
FPQ**TEQPEQPFPQP**	3	4	13	0	67	19	6	5	3	5	5	1	0	2	3	2	1	3	10	5	7	0
QPF**PQPEQPFPQS**	44	7	12	2	57	18	25	6	4	32	11	4	15	35	46	5	4	13	55	27	70	1
PFPLQPEQPVPEQPQ	5	3	11	1	3	5	7	11	2	1	1	2	9	20	3	0	3	6	5	10	21	1
LPFFQPEQPFVVV	18	8	8	7	29	18	13	9	7	34	3	1	20	15	16	3	3	20	31	8	42	1
QPEQ**PFPLQPEQPVP**	6	2	8	1	11	8	4	4	1	3	19	1	0	27	9	2	3	12	20	9	16	0

from adults,[13] showing the same level of high cross-reactivity for TCC specific for DQ2.5-glia-ω1/2 and more restricted cross-reactivity for TCC specific for DQ2.5-glia-α1a/α2 (Figure 5).

Gluten-Specific TCR Repertoires Are Similar in Children and Adults with CD

We used T-cell receptor (TCR) diversity as a measure of the maturation of the effector T-cell response and compared between age groups. We sequenced the CDR3 region in gluten-specific CD4⁺ T cells that were single-cell sorted based on their secretion of IFN-γ in response to gluten peptide stimulation (Figure 6). Polyclonal T cells responding to W02 were assessed in an 11-year-old (C27), a 6-year-old (C14), and an adult (A1) subject with CD. There was clear bias in both Vβ and Vα subunit usage for all 3 volunteers with an emphasis on *TRBV 7-2* or *TRBV 7-8* and *TRAV 26-1* for C27, *TRBV 7-2* and *TRAV 26-1* for C14, and *TRBV 7-2* and *TRBV 29-1* and *TRAV 26-1* for A1 (Figure 6A). Simpson's diversity index values for the CDR3 Vα and CDR3 Vβ chain sequences, which takes into account both the number and distribution of sequences within samples, were all close to 1 (Figure 6B), indicating similar clonal diversity between samples. Most CDR3 sequences collected for TRBV 7-2 contained a conserved arginine (Arg) residue (Supplementary Table 5), consistent with previous reports.[21] Clonal expansion within the TRBV 7-2 population was evident due to repetitive sequences within the polyclonal population for each individual. Moreover, 1 public clonotype was evident between volunteers A1 and C14, and 1 between C14 and C27. Sharing of TRBV7-2 CDR3 sequences was also evident between our volunteers and the CD cohorts examined by Qiao et al[21] and Han et al,[22] both of whom used tetramer-based sorting. TCR sequences were obtained for 2 TCC, 1 isolated from C27 (DQ2.5-glia-α2-specific) and 1 from a 17-year-old CD volunteer C40 (DQ2.5-glia-α1a-specific). The DQ2.5-glia-α2-specific TCC 3007.28 expressed *TRBV 7-2* and the

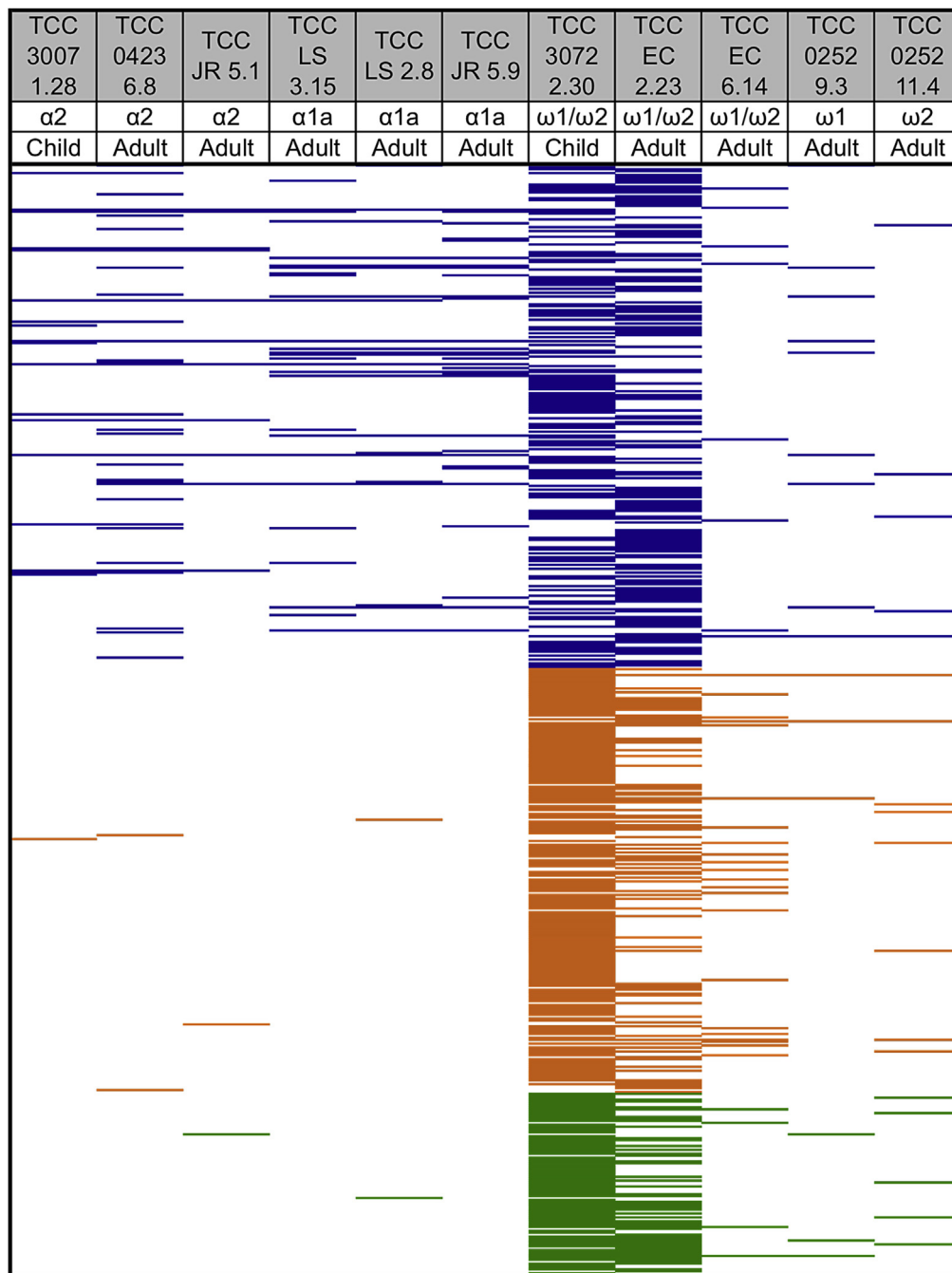conserved Arg residue in the CDR3β region (sequence SIRTTDTQ). The DQ2.5-glia-α1a-specific TCC 3072 1.14 expressed *TRBV 29-1* (CDR3β sequence GTVWFTDTQ). Both TCC expressed *TRAV 26-1*. There were no conserved sequence motifs within the TRAV CDR3 region within the polyclonal or clonal gluten-specific T-cell populations, but 2 CDR3α sequences were shared between C27 and C14 (data not shown).

Discussion

Our assessment of gluten-specific T cells in HLA-DQ2.5⁺ children with CD indicates remarkable consistency in T-cell recognition of immunodominant wheat gluten epitopes, specifically DQ2.5-glia-α1/α2 and DQ2.5-glia-ω1/ω2, and confirms the importance of deamidation in enhancing bioactivity of most immunogenic peptides. The findings are comparable to those in adults with CD from England, Italy, Norway, and Australia.[10,13,14,23] Although our focus on immunogenic peptides in adults means this is not an unbiased study of gluten T-cell epitopes in pediatric CD, our finding that T-cell responses to select gliadin peptides were generally greater than to whole gluten antigen suggests that the contribution of other untested peptides to the total gluten immune response is minimal. As the T-cell epitope dominance hierarchy or strength of response based on EC50s was not affected by age or time from diagnosis, our results suggest that the recall T-cell response to specific gluten peptides develops early in disease pathogenesis, is well established when CD is eventually diagnosed, and remains consistent over time.

Many features of the immunopathology of CD are common to adults and children. For instance, chronic ingestion of gluten is associated with elevated levels of serum immunoglobulin A (IgA) specific for tTG and both IgG and IgA specific for highly conserved deamidated 5-mer peptide motifs derived from gliadin.[24,25] In contrast, in vitro data from CD children suggested a lower rate of response to dominant T-cell epitopes, a lower rate of dependence on

TCC 3007 1.28	TCC 0423 6.8	TCC JR 5.1	TCC LS 3.15	TCC LS 2.8	TCC JR 5.9	TCC 3072 2.30	TCC EC 2.23	TCC EC 6.14	TCC 0252 9.3	TCC 0252 11.4
α2	α2	α2	α1a	α1a	α1a	ω1/ω2	ω1/ω2	ω1/ω2	ω1	ω2
Child	Adult	Adult	Adult	Adult	Adult	Child	Adult	Adult	Adult	Adult

Figure 5. T-cell clone promiscuity. T-cell clones specific to DQ2.5-glia-α1a/α2 or DQ2.5-glia-ω1/ω2 were tested against wheat, barley, and rye peptide libraries by IFN-γ ELISpots. Positive responses are shaded as follows: wheat, *blue*; barley, *orange*; and rye, *green*. Peptide sequences are not shown. Nonreactive peptides were removed.

deamidation for bioactivity, and implicated a series of novel immunogenic peptides.[15] Studies assessing peripheral blood mononuclear cells (PBMC) from untreated children with CD using in vitro culture (and no oral gluten challenge) detected responses to DQ2.5-glia-α1a and α2 in 30% or less of children[26] and proliferation was not detected.[27] Vader et al[15] showed that only 8 of 16 TCL from 25 Dutch children (1–12 years of age; average age, 4 years) with CD responded to DQ2.5-glia-α1a/α2 epitopes regarded as immunodominant in adults with CD. However, in the same study only 2 of 4 TCL isolated from adults with CD responded to these α-gliadin epitopes, contrasting with 17 of 17 (100%) TCLs isolated from adult Norwegian

CD donors,[9] suggesting that methodological differences could contribute to these differences. Artifacts can result from prolonged in vitro culture and the use of mitogens, which may expand naïve T cells and affect the composition and function of the T-cell population of interest.[23] Although epitope spreading was postulated to account for the heterogeneous gluten peptide responses they observed in children,[15] our findings suggest the repertoire of gluten-specific T cells is well established by the time CD is diagnosed. As epitope spreading typically occurs within weeks, not years, of an initial immune response, it is possible that the gluten-specific immune response at the earliest phase of CD development is different from the stable
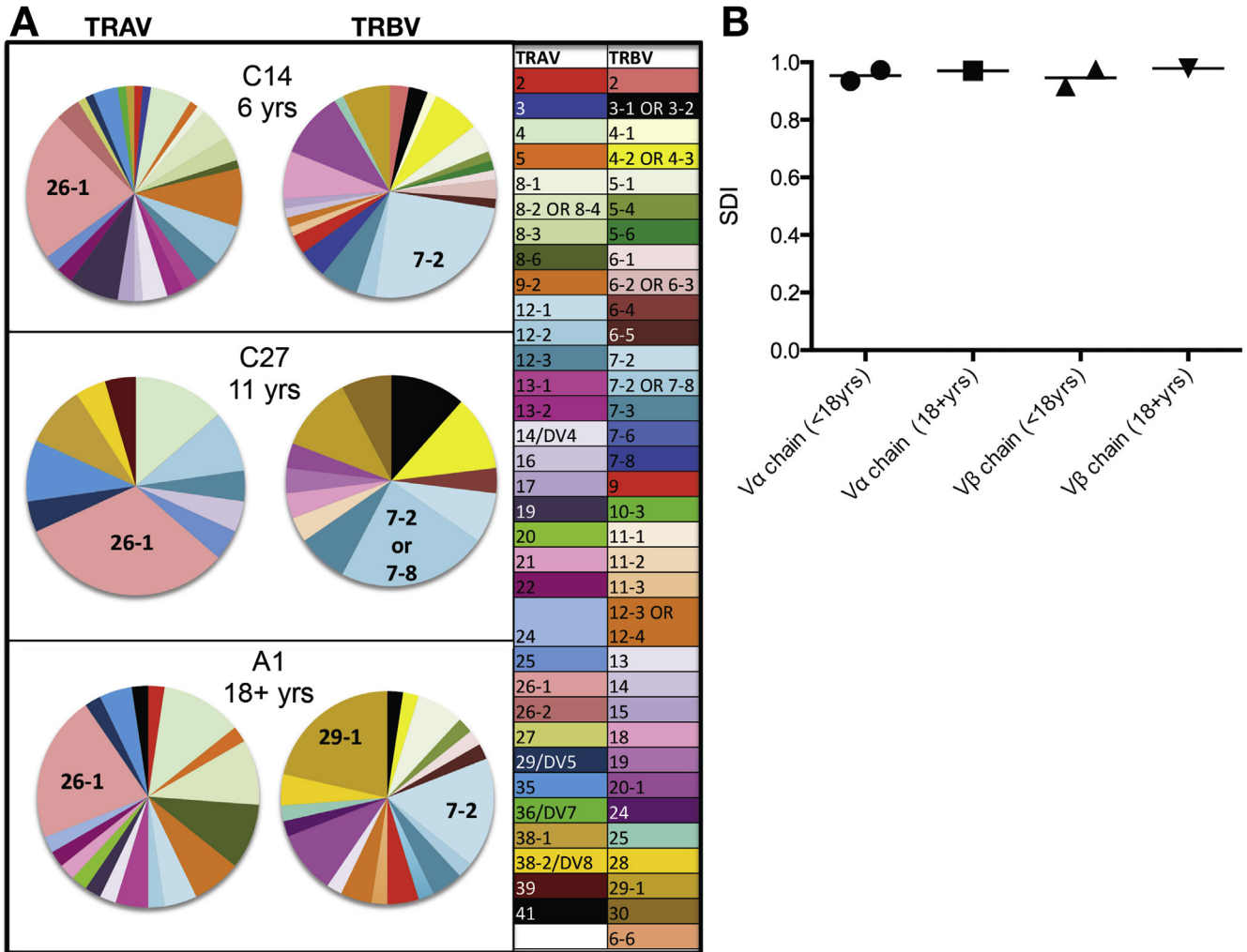
Figure 6. Similar TCR bias occurred in gluten-specific T cells from children and adults with CD. (*A*) Single IFN-γ–producing DQ2.5-glia-α1a/α2-specific CD4$^+$ T cells were isolated by fluorescence-activated cell sorting for multiplex PCR. Sequencing analysis of the TCR Vα (TRAV) and Vβ chains (TRBV) expressed by this population was performed in 3 wheat-challenged volunteers with CD (subjects C14, C27, and A1). The compositions of the TRAV and TRBV usage are shown in separate pie charts, with individual chains represented by different colors. The most commonly used chains are listed within the pie charts. (*B*) Simpson's diversity index for Vα and Vβ CDR3 sequences were calculated for the same individuals as in *A*.

recall response. However, identifying and assessing children who have CD prior to clinically recognizable disease is difficult, and we have shown previously that the successful induction of an in vivo recall response requires participants to be following a gluten free diet for at least several weeks prior to an oral wheat challenge.[12] This means, in practice, only the stable memory T-cell response can be interrogated. Nevertheless, native peptides may still be important for disease development. In a humanized HLA-DQ8 transgenic mouse model,[28] immunization with a native gluten peptide comprising the DQ8-glia-α1 epitope (QGSFQPSQQ) can recruit a T-cell population that is cross-reactive and substantially more reactive against the corresponding deamidated peptides. It is possible in early disease T cells are recruited by native peptide, triggering inflammation and tTG release, and then continue to be activated by more immunogenic deamidated peptides.

In Italian children with CD peptides containing DQ2.5-glia-α1/2, DQ2.5-glia-ω1/2 or homologs commonly elicited

a significant IFN-γ response after wheat challenge. Although bioactivity was enhanced by deamidation and peptides were typically more immunogenic than whole protein, in contrast to the Australian cohort, W02 and W03 (containing DQ2.5-glia-α1/2 and DQ2.5-glia-ω1/2) did not induce >70% of the maximal peptide response in the majority of children tested. Possible explanations include the smaller sample size, higher background responses (generally >10 spot forming units/well) that elevated the cutoff level required to establish a positive response, and potential differences in the amount and composition of the bread flour used for the wheat gluten challenge. Collectively, the data supports the importance of a focused immune response after wheat ingestion to dominant α- and ω-gliadins, and the importance of deamidation for gluten peptide bioactivity in children with CD.

It has been reported that the HLA-DQ2.5 gene dose has a strong quantitative effect on the magnitude of gluten-specific T-cell responses.[29] Although the overall

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magnitude of T-cell responses to dominant peptides were higher in HLA-DQ2.5 homozygotes the EC50s were not significantly lower. Responses to subdominant peptides in gliadin, hordein, and secalin appeared broader in homozygotes, possibly due to more efficient presentation of these peptides on the surface of antigen presenting cells, or to more efficient priming/expansion of cognate T cells in HLA-DQ2.5 homozygous CD. In some reports, HLA-DQ2.5 homozygosity is associated with a younger age and more severe phenotype at diagnosis.[30] We did not find an association between homozygosity and earlier disease onset or more severe histology at diagnosis, although the sample of HLA-DQ2.5 homozygous volunteers was relatively small.

High redundancy of gluten peptide recognition by T cells specific for a small selection of dominant peptides underpins the feasibility of peptide-based applications in CD.[13] In adults with CD, we have shown the polyclonal T-cell response induced after gluten challenge specific for W02, W03, and B08 (from barley; EPEQPIPEQPQPYPQQ) were equivalent to as much as 90% of that elicited by optimal concentrations of deamidated wheat gliadin, barley hordein, or the most immunogenic rye secalin fraction (ω-secalin). Moreover, TCCs isolated from adults with CD specific for DQ2.5-glia-α1a/α2, DQ2.5-glia-ω1/ ω2, DQ2.5-hor-3, and a rye epitope (DQ2.5-sec-1) recognize almost 90% of the T-cell stimulatory gluten peptides from all of the toxic cereals in CD. Here we showed that TCC specific for the immunodominant wheat T-cell epitopes in CD children share the same degree of cross-reactivity as TCC isolated from CD adults. The findings suggest a discrete number of dominant peptides can replicate the whole gluten response in children with CD as they can in adults.

Biased TCR gene usage has previously been observed in DQ2.5-glia-α2-specific T cells,[2] with overusage of the TRAV26-1 and TRBV7-2 gene segments and conservation of a non-germline-encoded Arg residue in the CDR3β loop, suggesting structural constraints are involved during in vivo TCR selection.[21,31,32] However, it is unclear whether selective outgrowth as a consequence of extensive antigen exposure prior to diagnosis of CD aids in the selection of cells expressing an optimal TCR. We detected identical CDR3β sequences within the DQ2.5-glia-α1a/α2-specific T-cell populations in pediatric and adult CD volunteers. Sequences were also shared with those previously described in a tetramer-selected T-cell population isolated from adults with CD.[21] Sorting by IFN-γ secretion enables selection of functional gluten-specific T cells that is not possible in tetramer-based assays.[33] Although we selected high-gluten–responding donors that could potentially bias TCR selection, the public sequences obtained suggest this was not the case. Diversity scores were similar between the pediatric and adult CD donors, suggesting that the level of TCR diversity had reached similar levels and that disease had progressed to a similar level in each individual. Importantly, there seemed to be no continued repertoire narrowing or affinity maturation in adults that had not yet occurred in children, despite additional chances of repeated exposure to antigen. Collectively, these data

further validated the public usage of these CDR3 sequences in the DQ2.5-α2-specific T-cell response in CD donors, confirmed the importance of immunodominant epitopes shaping the selection of TCRs, and supported consistency of T-cell responses to dominant wheat gluten peptides across all ages.

Future studies are required to assess the consistency of immune responses to immunodominant rye secalin and barley hordein T-cell epitopes, previously identified in adults,[13] after consumption of these grains in children with CD. Furthermore, this study confirms previous observations that the presence and severity of symptoms after gluten challenge correlates poorly with the T-cell response and greater insight into the mechanisms responsible for gluten-induced symptoms in CD are necessary.

We show the specificity of the gluten-specific T-cell response reactivated by oral wheat challenge in children with CD does not differ from adults. Stability of epitopes recognized by gluten-reactive CD4$^+$ T cells after diagnosis of CD whether in childhood or adult life indicates clinical applications of gluten-derived T-cell epitopes should be relevant to CD patients of all ages.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2015.07.013.

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Supplementary Material and Methods

Pediatric Wheat Challenge and Blood Collection

The amount of bread consumed daily was modified for younger ages: 3–5 years, 1 slice of bread ($\sim$3 g of gluten/day); 6–10 years, 2 slices ($\sim$ 6 g of gluten/day); and 11–18 years, 3 slices ($\sim$ 9 g of gluten/day). This corresponded to a similar amount of daily gluten intake across all age groups ($\sim$ 0.2 g/kg gluten based on median weight using a weight-for-age percentile chart). Six- to 10-year-old Italian children ate 3 slices of bread ($\sim$9 g of gluten/day), and 11- to 18-year-olds ate 4 slices of bread ($\sim$12 g of gluten/day). Blood for T-cell studies was collected in lithium heparin Vacutainers (Becton-Dickinson, Hunt Valley, MD) before (Day 0 [D0]) and 6 days after (D6) commencing the oral challenge, and volume was determined by weight based on World Health Organization guideline.[S1] Volunteers completed symptom diaries, recording symptom type and severity (mild, moderate, or severe) daily until D6.

Antigens

The screening library was custom synthesized, and the identity of each peptide was confirmed by liquid chromatography-mass spectrometry (GL Biochem, Minhang, China). Additional high-quality (>80%) peptides were synthesised by Pepscan (Lelystad, the Netherlands) or GL Biochem. Comprehensive gliadin ($n = 1535$), hordein ($n = 1444$), and secalin ($n = 350$) peptide libraries consisting of wild-type and in silico-deamidated sequences[13] were used to screen T-cell clones (TCC). Whole gluten (a generous gift from A. Tatham) was assessed in addition to gliadin (catalog 101778; ICN Biomedicals, Aurora, OH) to determine whether untested glutenin peptides contributed to the whole gluten response. Gluten and gliadin were digested with chymotrypsin and deamidated with tTG as previously described.[10,12]

IFN-γ ELISpot Assay

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by using Ficoll-Paque Plus density-gradient centrifugation (GE Healthcare, Buckinghamshire, UK). IFN-γ ELISpot (Mabtech, Cincinnati, OH) assays were performed and analyzed as previously described.[13] Briefly, PBMC were incubated overnight with or without individual peptides (50 μg/mL), and with positive controls Tetanus toxoid (CSL, Behring, Australia) and phytohemagglutinin-L (PHA-L; Sigma-Aldrich, St. Louis, MO). Spot-forming units (SFU) in individual wells were counted using an automated ELISPOT reader (AID ELISpot reader system; Autoimmun Diagnostika GmbH, Strassberg, Germany; or in Italy, using an Aelvis ELISpot reader, Hannover, Germany). Wells showing more than 10 SFU and >3$\times$ the SFU counted in wells containing PBMC incubated with medium alone were regarded as positive. Dominance scores for each peptide were defined using the IFN-γ response elicited as a proportion of the most active peptide screened and then averaged across each volunteer group. SFU were adjusted to 1 million PBMC plated to enable comparisons. Median effective concentration (EC50) values, representing the half maximal peptide concentration, were calculated using Prism version 6.0 software (GraphPad, LaJolla, CA) on a dose curve containing 8 peptide concentrations ranging from 0.1 to 50 μg/mL.

T-Cell Cloning

TCC were generated as previously described.[13] Briefly, carboxyfluorescein succinimidyl ester (CFSE)-labeled PBMC were incubated with antigen for 7 days, after which proliferating single cells were incubated in the presence of IL-2, IL-4, anti-CD3 monoclonal antibody, irradiated allogeneic PBMC, and JY-EBV (an Epstein-Barr virus-immortalized B cell line), followed by expansion. TCC were tested by ELISpot for specificity and to determine HLA-DQ2.5 restriction. TCC also were tested in the presence of anti-human HLA-DQ or HLA-DR blocking antibodies. TCC clonality and TCR Vbeta usage was tested by PCR or IOTest Beta Mark TCR V kit (Beckman Coulter, Sykesville, MD), and minimal epitopes were defined with lysine scans.[S2]

IFN-γ Secretion Assay

Either fresh or cryopreserved PBMC from known T-cell responders were rested overnight, and CD4$^+$ T cells were enriched using an EasySep negative selection human CD4$^+$ T-cell enrichment kit (Stem Cell Technologies, Vancouver, BC, Canada), following manufacturer's recommendations. CD4$^+$ T cells were stimulated or not with 50 μg/mL peptide, in addition to 10 μg/mL purified anti-CD28 antibody and 1.25 μg/mL anti-CD49d antibody (both from Biolegend, San Digo, CA), and autologous PBMC at a 1:1 ratio, in 96-well round-bottom plates in replicate wells containing a final volume of 150 μL of media containing 20 μg/mL DNase I (Roche, Basel, Switzerland) for 4 hours. The MACS IFN-γ secretion assay detection kit (fluorescein isothiocyanate) human (Miltenyi Biotec, Bergisch Gladbach, Germany) was used to enable cell sorting of IFN-γ secreting, gluten-specific CD4$^+$ T cells, following manufacturer's recommendations. Cells were stained with IFN-γ-FITC (in kit), CD4-APC and CD14-PerCP (BD Biosciences), CD69-PECy7 (Biolegend), and propidium iodide (Sigma). Gluten-specific (IFN-γ^+CD69$^+$CD4$^+$) cells were single-cell sorted into 96-well PCR (Eppendorf) plates up to 80 wells and 1 column left empty as nontemplate controls, on a FACS Aria (Becton-Dickinson) machine. Wells were capped with strip lids and stored frozen for later processing.

TCR sequencing

cDNA sequences encoding the alpha and beta chains of the TCR on sorted single cells were determined using a nested multiplex PCR as previously described.[S3] Simpson's diversity index (SDI) is calculated as $D = \Sigma i \, [(ni \, \{ni - 1\})/(N \, \{N - 1\})]$ where ni is the number of sequences in the ith clonotype, and N is the total number of sequences in the whole population.[S4] This is a measure of diversity, which is defined as a combination of richness (the number of different clonotypes within a population) and evenness (the distribution of the clonotypes) and is expressed as 1-D so that a higher number corresponds to greater diversity.

Supplementary References

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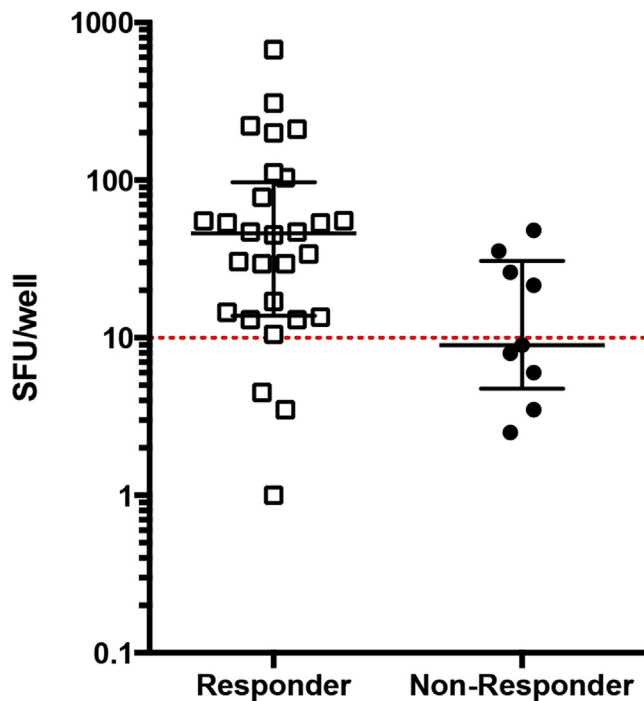
Supplementary Figure 1. Poor response to tetanus toxoid predicts lack of response to gluten peptides. Volunteers were separated into responders (n = 31) or non-responders (n = 9) based on the IFN-γ ELISpot response to gluten after oral wheat challenge. Responses to the positive control antigen tetanus toxoid were compared on D6 following wheat challenge. *Line* depicts response cut-off. Median response with interquartile range is shown.