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Circulating gluten-specific FOXP3⁺CD39⁺ regulatory T cells have impaired suppressive function in patients with celiac disease



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Background: Celiac disease is a chronic immune-mediated inflammatory disorder of the gut triggered by dietary gluten. Although the effector T-cell response in patients with celiac disease has been well characterized, the role of regulatory T (Treg) cells in the loss of tolerance to gluten remains poorly understood.

Objective: We sought to define whether patients with celiac disease have a dysfunction or lack of gluten-specific forkhead box protein 3 (FOXP3)⁺ Treg cells.

Methods: Treated patients with celiac disease underwent oral wheat challenge to stimulate recirculation of gluten-specific T cells.

Peripheral blood was collected before and after challenge. To comprehensively measure the gluten-specific CD4⁺ T-cell response, we paired traditional IFN- γ ELISpot with an assay to detect antigen-specific CD4⁺ T cells that does not rely on tetramers, antigen-stimulated cytokine production, or proliferation but rather on antigen-induced coexpression of CD25 and OX40 (CD134).

Results: Numbers of circulating gluten-specific Treg cells and effector T cells both increased significantly after oral wheat challenge, peaking at day 6. Surprisingly, we found that approximately 80% of the *ex vivo* circulating gluten-specific CD4⁺ T cells were FOXP3⁺CD39⁺ Treg cells, which reside

within the pool of memory CD4⁺CD25⁺CD127^{low}CD45RO⁺ Treg cells. Although we observed normal suppressive function in peripheral polyclonal Treg cells from patients with celiac disease, after a short *in vitro* expansion, the gluten-specific FOXP3⁺CD39⁺ Treg cells exhibited significantly reduced suppressive function compared with polyclonal Treg cells. **Conclusion:** This study provides the first estimation of FOXP3⁺CD39⁺ Treg cell frequency within circulating gluten-specific CD4⁺ T cells after oral gluten challenge of patients with celiac disease. FOXP3⁺CD39⁺ Treg cells comprised a major proportion of all circulating gluten-specific CD4⁺ T cells but had impaired suppressive function, indicating that Treg cell dysfunction might be a key contributor to disease pathogenesis. (J Allergy Clin Immunol 2017;140:1592-603.)

Key words: Regulatory T cells, CD39, forkhead box protein 3, celiac disease, gluten, OX40

Celiac disease is a chronic inflammatory disorder with features of autoimmune disease that results from a loss of gluten tolerance.¹ It is characterized by villous atrophy and the presence

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Coeliac Research Fund; is Chief Scientific Officer of ImmusanT; is a coinventor of patents pertaining to the use of gluten peptides in therapeutics, diagnostics, and nontoxic gluten; and is a shareholder of Nexpep and ImmusanT. J. J. Zaunders is named inventor on a patent for the use of CD39 and the OX40 assay to identify antigen-specific regulatory T cells held by St Vincent's Hospital, Sydney, Australia. J. A. Tye-Din has received a grant from the Coeliac Research Fund; has consultant arrangements with ImmusanT; is coinventor of patents pertaining to the use of gluten peptides in therapeutics, diagnostics, and nontoxic gluten; and is a shareholder in Nexpep. A. D. Kelleher has received grants from the Australian Government Department of Health and Ageing, the National Health and Medical Research Council (510448 and 1085875), and the Coeliac Research Fund; has received a Practitioner Fellowship from the Australian Government Department of Health and Ageing; and is named inventor on a patent for the use of CD39 and the OX40 assay to identify antigen-specific regulatory T cells held by St Vincent's Hospital, Sydney, Australia. The rest of the authors declare that they have no relevant conflicts of interest.

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Abbreviations used

APC:	Antigen-presenting cell
CTV:	CellTrace Violet
FOXP3:	Forkhead box protein 3
GARP:	Glycoprotein A repetitions predominant
LAP:	Latency-associated peptide
SEB:	Staphylococcal enterotoxin B
Tconv:	Conventional T
Treg:	Regulatory T
TSDR:	Regulatory T cell–specific demethylated region
tTG:	Tissue transglutaminase 2

of autoantibodies to tissue transglutaminase 2 (tTG), an enzyme that deamidates gluten. Intestinal damage is caused by CD4⁺ T cells, which recognize deamidated gluten peptides presented in complex with HLA-DQ2.5, HLA-DQ2.2, and/or HLA-DQ8,^{2,3} and the immunodominant hierarchy of wheat gliadin T-cell epitopes in HLA-DQ2.5⁺ patients with celiac disease has been comprehensively mapped.⁴ Although HLA susceptibility haplotypes are expressed by 30% to 40% of the general population, celiac disease affects only approximately 1%, indicating that immune tolerance to gluten is the norm. However, the mechanisms that underpin maintenance of gluten tolerance remain poorly described.

Gluten-responsive effector CD4⁺ T cells can be detected in the peripheral blood of patients with celiac disease on a gluten-free diet 6 to 8 days after a 3-day oral gluten challenge.⁵ On activation, these cells secrete high levels of IFN- γ ,^{6,7} support B cell–mediated production of antibodies to tTG and modified gluten peptides, and enhance lysis of stressed epithelial cells by CD8⁺ T cells.^{4,6} Studies of total regulatory T (Treg) cells in patients with celiac disease have provided evidence for both normal suppressive function^{8–10} and impaired function,^{11,12} as well as suggesting that effector T cells have become resistant to Treg cell suppression.^{10,13,14} However, although forkhead box protein 3 (FOXP3)⁺ Treg cells have an important role in maintaining peripheral tolerance, until now, the frequency and function of gluten-specific FOXP3⁺ Treg cells in patients with celiac disease have not been studied.

For the first time, this study exploited acute *in vivo* gluten challenge in patients with celiac disease to interrogate both effector and regulatory components of the recall response to gluten. Specifically, we aimed to estimate the frequency of Treg cells within gluten-specific CD4⁺ T-cell recall responses; identify changes in the frequency of peripheral gut-homing memory CD4⁺ T-cell populations after gluten challenge; and phenotypically and functionally characterize gluten-specific Treg cells.

METHODS

Subjects and samples

Patients with celiac disease were recruited after provision of informed consent (Human Research Ethics Committees: Royal Melbourne Hospital ID 2003.009; Walter and Eliza Hall Institute of Medical Research ID 03/04). Enrollment criteria were biopsy-proved disease conforming to European Society for Paediatric Gastroenterology Hepatology and Nutrition guidelines,¹⁵ HLA-DQ2⁺, and compliance with a gluten-free diet for 6 months or more. Healthy donor blood was obtained from the Australian Red Cross Blood Service and volunteers (St Vincent's Hospital Human Research Ethics Committee ID HREC/13/SVH/145). Peripheral blood was collected into lithium

heparin vacutainers (BD, San Jose, Calif), transported at ambient temperature, and processed within 8 hours of collection. Mononuclear cells were obtained by means of centrifugation over Ficoll-Paque (GE Healthcare, Fairfield, Conn).

Serology and HLA typing

Serum titers of tTG IgA and deamidated gliadin peptide IgG were evaluated with commercial kits (INV 708760 and 704525; INOVA Diagnostics, San Diego, Calif) by a diagnostic laboratory (Gribbles-Healthscope, Clayton, Australia). The presence of alleles encoding HLA-DQ2.5, HLA-DQ2.2, and HLA-DQ8 was determined by detecting 5 single nucleotide polymorphisms (rs2187668, rs2395182, rs4713586, rs7454108, and rs7775228), as previously described.^{16,17} HLA-DQB1 and HLA-DQA1 alleles were determined by using PCR sequence–specific oligonucleotide hybridization (Victorian Transplantation and Immunogenetics Service, Victoria, Australia).

Oral gluten challenge

All participants undertook a gluten challenge⁵ from days 1 to 3 by consuming 4 slices of commercial white bread daily (approximately 10 g/d wheat gluten) and recorded symptoms daily to day 6, grading them as mild, moderate, or severe.¹⁸

Reagents

We used 2 HLA-DQ2.5–restricted 15mers that encompass the immunodominant deamidated wheat gliadin T-cell epitopes DQ2.5-glia- α 1/ α 2 (LQPFQPELPYPQPO) and DQ2.5-glia- ω 1/ ω 2 (QPFQPEQFPWQP).⁴ Gluten peptide mix contained an equimolar mixture of these two 15mers. An HLA-DQ2.5–restricted 15mer that encompasses the immunodominant barley hordein T-cell epitope DQ2.5-hor-3 (PEQPIPEQPQYPYQQ) acted as a specificity control.⁴ Peptides were synthesized to 95% purity or greater, as confirmed by means of high-performance liquid chromatography (Pepsan, Lelystad, The Netherlands); dissolved in dimethyl sulfoxide (Sigma-Aldrich, St Louis, Mo); and stored at -80°C until use.^{5,18} Chymotrypsin digestion and deamidation of gliadin (#101778; ICN Biomedicals, Cost Mesa, Calif) were performed, as previously described.^{5,18} Tetanus toxoid was from CSL (Parkville, Australia), and staphylococcal enterotoxin B (SEB) was from Sigma-Aldrich.

IFN- γ secretion assays

Antigen-stimulated IFN- γ secretion from PBMCs was assessed by using either ELISpot (Mabtech, Nacka Strand, Sweden) or ELISA (Mabtech), assays that are equivalent in their ability to detect gluten-specific responses.¹⁸ Gluten peptide mix and deamidated gliadin were used at 100 $\mu\text{g}/\text{mL}$ and tetanus toxoid was used at 10 LfU/mL, and assays were performed in triplicate, as previously described.^{5,18,19}

OX40 assay

The OX40 assay was performed, as previously described,^{20,21} with either fresh whole blood (diluted 1:1 with RPMI 1640 media, Invitrogen) or PBMCs at 2×10^6 cells/mL and incubation with antigen for 44 hours at 37°C (5% CO_2). PBMC assays were performed in RPMI-1640 media (Invitrogen) supplemented with 10% human heat-inactivated AB serum (Sigma-Aldrich), 1% L-glutamine and 1% penicillin/streptomycin (Invitrogen). Antigen concentrations were as follows: SEB, 1 $\mu\text{g}/\text{mL}$; tetanus toxoid, 2 LfU/mL; deamidated gliadin, 100 $\mu\text{g}/\text{mL}$; and DQ2.5-hor-3m 50 $\mu\text{g}/\text{mL}$. Optimal concentrations of gluten antigens were determined in a pilot study (see Fig E1, B, in this article's Online Repository at www.jacionline.org). DQ2.5-glia- α 1/ α 2 and DQ2.5-glia- ω 1/ ω 2 were used separately at 50 $\mu\text{g}/\text{mL}$ and in an equimolar gluten peptide mix. Assay cutoffs were as follows: greater than 0.02% of CD4⁺ T cells (mean + 3 SDs of unstimulated wells) and greater than 20 cells.

Population tracking within the OX40 assay

Postchallenge PBMCs were purified by fluorescence-activated cell sorting into CD4⁺ T-cell populations: (1) CD45RO⁺ T cells and

(2) CD45RO⁺CD127^{high}CD25^{low} conventional T (Tconv) cells, with the CD45RO⁺CD127^{low}CD25^{high} Treg cells subdivided into (3) CD39⁺ and (4) CD39⁻ cells. Portions of the 3 CD45RO⁺ populations were stained with CellTrace Violet (CTV) to enable cell tracking. In some experiments PBMCs were stained with DQ2.5-glia α 21/ α 2 tetramer and sorted into tetramer-negative and tetramer-positive CD4⁺ T cells that were then labeled with CTV. PBMCs were reconstituted, maintaining *ex vivo* cell ratios, and supplemented with CD3-depleted autologous PBMCs (Dynabeads CD3, Invitrogen) to one sixth of the total cell number. Each well contained a single CTV-labeled population, and the OX40 assay was performed and analyzed, as described above.

Flow cytometry

Staining was performed, as previously described,²² with anti-CD3–peridinin-chlorophyll-protein complex–Cy5.5 (SK7), CD4–Alexa Fluor 700 (RPA-T4), CD25–allophycocyanin (APC; 2A3), OX40 (CD134)–phycoerythrin (PE; L106), cytotoxic T lymphocyte-associated antigen 4–PE (BNI3), glycoprotein A repetitions predominant (GARP) GARP-BV711 (7B11), integrin β 7–PE (FIB504; BD); CD45RO-ECD (UCHL1; Beckman Coulter, Fullerton, Calif); CD127-eFluor450 (eBioRDR5), CD39-PECy7 (A1); latency-associated peptide (LAP)-PECy7 (FNLAP; eBioscience), Helios–Alexa Fluor 488 (22F6), and anti-FOXP3–Alexa Fluor 488 (259D; BioLegend, San Diego, Calif). FOXP3 staining was performed with the FOXP3 buffer kit (BD), and the IgG1 κ -FITC antibody (BD) was used to set analysis gates. All mAbs were used at manufacturers' recommended dilutions. Cell labeling was performed with the CTV Cell Proliferation Kit (Invitrogen) and PKH26 Red Fluorescent Cell Linker Kit for General Cell Membrane Labelling (Sigma-Aldrich) per the manufacturers' recommendations by using 5 μ mol/L dye concentrations. HLA-DQ2 proteins were synthesized with bound DQ2.5-glia- α 1, DQ2.5-glia- α 2, or the HLA class II invariant peptide by using previously described constructs and methods.²³ Tetramers were produced by means of addition of either NeutrAvidin R-APC or R-PE conjugate (Invitrogen) to biotinylated protein, as previously described.²⁴ Staining was performed with 50 μ g/mL tetramer in complete media at 37°C for 1 hour. A 4-laser LSRII flow cytometer (BD) was used, and analysis was performed with FlowJo software (v8.8.7; TreeStar, Ashland, Ore).

In vitro expansion of T-cell populations

The protocol used to expand Treg cells, Tconv cells, and DQ2.5-glia- α 1/ α 2-specific CD39⁺ T cells was adapted from Gregori et al,²⁵ as previously described²⁰; T-cell cloning was performed by plating cells at 1 cell/well. Irradiated feeder cell mix consisted of 5 \times 10⁵ cells/mL of mixed PBMCs (equal mix of autologous PBMCs and PBMCs from 2 allogeneic healthy donors) and 5 \times 10⁴ cells/mL of an autologous EBV-transformed B-cell line generated and maintained, as previously described.²⁶

Quantitative RT-PCR and T-cell receptor clonotype analysis by 5' Rapid Amplification of cDNA Ends

Quantitative RT-PCR was performed on resting T-cell populations, and relative expression levels to β -actin were calculated, as previously described²⁰: FOXP3 forward, 5'-TCACCTACGCCACGTCAT-3'; FOXP3 reverse, 5'-TCATTGAGTGTCCGCTGCTT-3'; TGF- β forward, 5'-CCCTGGACACCAACTATTGC-3'; and TGF- β reverse, 5'-CAGA AGTTGGCATGGTAGCC-3'. T-cell receptor clonotypes were analyzed by using 5' Rapid Amplification of cDNA Ends (Clontech, Mountain View, Calif) PCR, as previously described.²⁷ Sequences were analyzed by using the ImmunoGenetics V-quest database.²⁸

Suppression assay

Suppression assays were performed, as previously described.²⁰ CD4⁺CD127⁺CD25⁻ responder T cells were labeled with CTV (Invitrogen), and suppressor cells were labeled with PKH26 (Sigma-Aldrich). Wells contained 50,000 irradiated autologous antigen-presenting cells (APCs) and

20,000 responder T cells, with suppressor T cells added at the ratios indicated. Assays were stimulated for 4 days with 0.25 μ g/mL soluble anti-CD3 (Invitrogen). CD39 enzyme activity was blocked in some assays with 250 μ mol/L ARL67156 (Sigma-Aldrich). The division index was used to calculate the percentage of suppression.²⁹

Treg cell-specific demethylated region analysis

Genomic DNA was isolated, and bisulfite conversion was performed with the EZ DNA Methylation-Direct kit (Zymo Research, Irvine, Calif). PCR was performed with the PyroMark PCR kit (Qiagen, Hilden, Germany) and Human FoxP3 Methylation Assay ADS783FS2 (EpigenDx, Ashland, Mass), which reports the methylation of 8 representative CpG sites in the regulatory T cell–specific demethylated region (TSDR). Pyrosequencing was performed on a PyroMark Q96 ID (Qiagen) with PyroMark Gold Q96 reagents (Qiagen) and Streptavidin Sepharose (GE Healthcare). All kits/reagents were used according to the manufacturer's instructions. Analysis was performed on female subjects, and the levels of methylation have not been adjusted to account for X-inactivation.

Statistics

Mann-Whitney *U* tests or 1-way ANOVA were used unless samples were matched, and then Wilcoxon signed-rank tests were performed. Correlation analyses used Spearman rho (r_s). *P* values were considered significant at less than .05. Prism 6.0 software (GraphPad Software, La Jolla, Calif) was used for all statistical analyses.

RESULTS

Numbers of circulating gluten-specific FOXP3⁺CD39⁺ Treg cells are significantly increased after gluten challenge

To investigate CD4⁺ T-cell recall responses to gluten, we recruited a cohort of 17 treated patients with celiac disease (see Tables E1 and E2 in this article's Online Repository at www.jacionline.org). We used our previously developed OX40 assay, which detects antigen-specific CD4⁺ T cells through antigen-induced coexpression of CD25 and OX40,²¹ to measure changes in the frequency of circulating gluten-specific CD4⁺ T cells in patients with celiac disease after gluten challenge (Fig 1, A). In a pilot study we found the optimal time for detecting responses was 6 to 8 days after gluten challenge (termed days 6 and 8; see Fig E1, A). At day 6, we observed significantly increased responses to deamidated gliadin ($n = 15$, $P = .007$) and gluten peptide ($n = 9$, $P = .008$; Fig 1, B). The overall peak response to gluten antigens occurred at day 6 (median response 0.27% of CD4⁺ T cells). There were no detectable responses to the barley hordein peptide DQ2.5-hor-3 (Fig 1, A), indicating that the wheat peptide responses are specifically induced by oral wheat gluten challenge. Patients with detectable gluten peptide responses also had a significant increase in numbers of both total and gut-homing CD39⁺FOXP3⁺ Treg cells at day 6 (Fig 1, C), a trend not seen in gluten peptide nonresponders (see Fig E2, A and B, in this article's Online Repository at www.jacionline.org). There were no significant changes observed within numbers of total or gut-homing CD4⁺ Tconv cells (data not shown).

We assessed the presence of gluten-specific CD4⁺ T cells in 6 healthy volunteers (HLA-DQ genotypes were known for 4, and only 1 carried HLA-DQ2.5). The median deamidated gliadin-specific response for the non-HLA-DQ2.5 subjects was 0.09% of CD4⁺ T cells (range, 0% to 0.15%), and the response

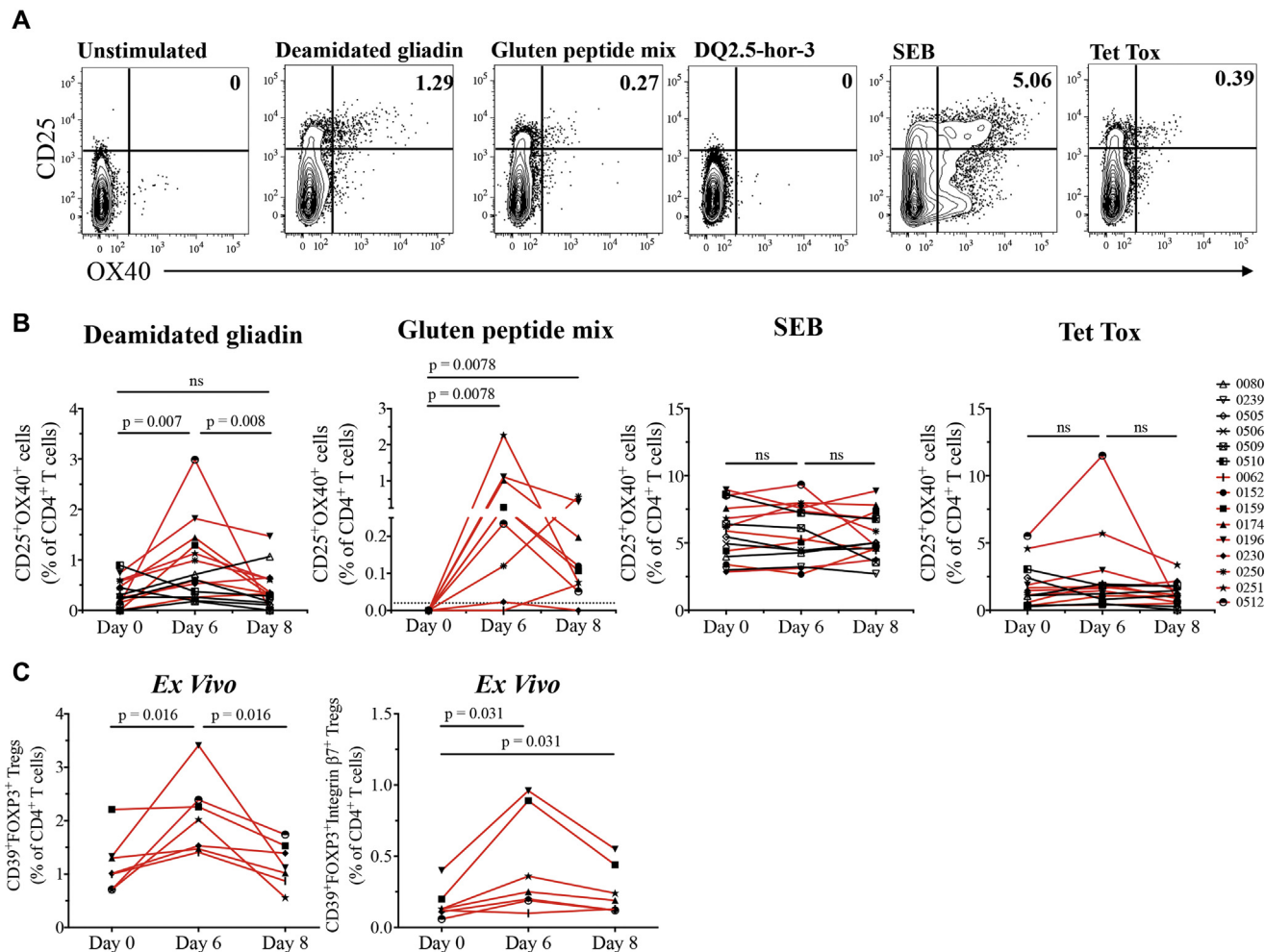


FIG 1. OX40 assay responses to gluten antigen peak at day 6 after gluten challenge. **A**, Representative OX40 assay responses from patients with celiac disease at day 6. **B**, For 15 patients with celiac disease, the percentage of CD4⁺ T cells responding to deamidated gliadin, gluten peptide mix, SEB, or tetanus toxoid (*Tet Tox*) are shown. Dotted line, Assay cutoff. **C**, Flow cytometric analysis of total and integrin $\beta 7^{+}$ CD39⁺ FOXP3⁺ Treg cell (CD45RO⁺ CD127^{low} CD25^{high}) frequencies in *ex vivo* peripheral blood at days 0, 6, and 8 for patients with celiac disease and detectable gluten peptide responses ($n = 7$). Red lines, Patients with gluten peptide mix responses ($n = 9$); black lines, patients with undetectable gluten peptide responses ($n = 7$). Statistical analyses used Wilcoxon signed-rank tests.

for all 6 subjects was 0.19% (range, 0% to 0.77%; see Fig E1, C). No deamidated gluten peptide responses were detected in these subjects (data not shown), indicating that CD4⁺ T-cell responses to deamidated gluten peptides are a more specific marker of celiac disease.

The majority of gluten peptide-specific CD4⁺CD25⁺OX40⁺ T cells express CD39

We have previously shown that within antigen-responsive CD25⁺OX40⁺ T cells, a subset of Treg cells can be identified on the basis of CD39 expression, providing a sensitive and specific way to measure and isolate viable antigen-specific FOXP3⁺ Treg cells.²⁰ We used this approach to determine the contribution of CD39⁺FOXP3⁺ Treg cells to gluten-specific OX40 assay responses (Fig 2, A). On average, 72% of deamidated gliadin-specific T cells were CD39⁺ (75% coexpressed FOXP3⁺), and 89% of gluten peptide-

specific T cells were CD39⁺, with 82% of these cells expressing FOXP3 (Fig 2, B).

We found similar CpG methylation patterns within the TSDR in DQ2.5-glia- $\alpha 1/\alpha 2$ -specific CD39⁺ T cells sorted directly after a 44-hour OX40 assay compared with *ex vivo* Treg cells isolated from both healthy subjects and patients with celiac disease (Fig 2, C). These data are consistent with gluten-specific CD25⁺OX40⁺CD39⁺ T cells being highly enriched for Treg cells.

We confirmed OX40 assay specificity through several experiments using DQ2.5-glia- $\alpha 1/\alpha 2$ tetramer reagents. We costained DQ2.5-glia- $\alpha 1/\alpha 2$ peptide-stimulated OX40 assays with DQ2.5-glia- $\alpha 1/\alpha 2$ tetramer and observed that of all quadrants in the CD25 versus OX40 plot, the CD25⁺OX40⁺ quadrant had the highest proportion of tetramer-positive cells (Fig 2, D). We confirmed that, similar to OX40 responses, tetramer staining was only observed in day 6 postchallenge PBMCs (not prechallenge PBMCs) and that the majority of the

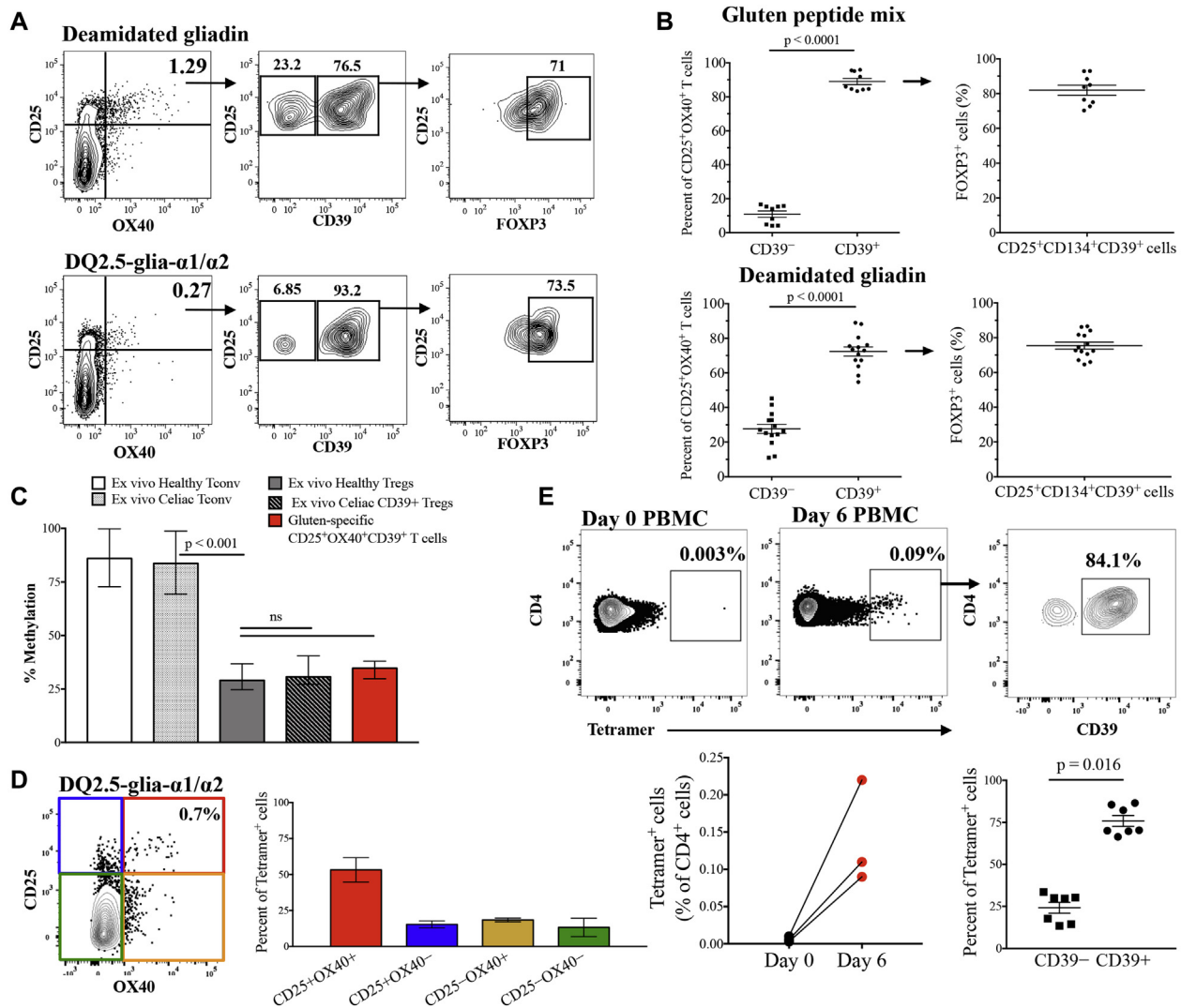


FIG 2. The majority of circulating gluten peptide-specific CD4⁺ T cells are CD39⁺FOXP3⁺. **A** and **B**, CD39 and FOXP3 expression within OX40 assay responses: representative data (Fig 2, A) and within responses to gluten peptide mix (n = 9) and deamidated gliadin (n = 14; mean ± SEM; Fig 2, B). **C**, Percentage methylation averaged from 8 CpG sites in the TSDR of *FOXP3* within *ex vivo* Treg and Tconv cells (n = 3 healthy female subjects and n = 3 female patients with celiac disease) and DQ2.5-glia-α1/α2-specific CD39⁺ cells (n = 3; median ± interquartile range). *ns*, Not significant. **D**, The frequency of DQ2.5-glia-α1/α2 tetramer-positive cells is shown within each quadrant of the CD25/OX40 plot for DQ2.5-glia-α1/α2-stimulated OX40 assays (n = 3; mean ± SEM). **E**, Representative DQ2.5-glia-α1/α2 tetramer and CD39 staining of unstimulated PBMCs at days 0 and 6 post-gluten challenge. For 3 patients, tetramer staining is shown at both day 0 and day 6. The proportion of tetramer-positive cells that are CD39⁺ is shown for 7 patients with celiac disease at day 6 after gluten challenge (mean ± SEM). Statistical analyses in Fig 2, B and D, used Mann-Whitney *U* tests, and those in Fig 2, E, used Wilcoxon signed-rank tests.

CD4⁺ tetramer-positive cells were CD39⁺ (median, 70.3%; n = 7; Fig 2, E). Finally, we sorted CD4⁺CD25⁺OX40⁺CD39⁺ T cells and labeled with cell proliferation dye before restimulating with a panel of antigens in the presence of autologous APCs. We observed that stimulation with cognate antigen generated substantially more proliferation (87%) than DQ2.5-glia-ω1/ω2 (29%), DQ2.5-hor-3 (29%), or no antigen (21.7%). DQ2.5-glia-α1/α2 restimulation of matched CD25⁻OX40⁻ T cells did not stimulate cell proliferation (see Fig E3, D, in this article's Online Repository at www.jacionline.org).

Importantly, although CD39 expression varies between subjects, in each subject the proportion of CD39⁺ cells within

CD4⁺ T cells did not vary within the timeframe of the OX40 assay (see Fig E4, C, in this article's Online Repository at www.jacionline.org). The proportion of CD39⁺ cells within recall responses also did not significantly vary from before challenge through day 8 after challenge (see Fig E2, C, Online Repository). Responses to the mitogen SEB were similar between the celiac cohort (n = 15; mean response, 5.8% CD4⁺ T-cells) and healthy subjects (n = 15; mean age, 37; 60% female; mean response, 7.2% of CD4⁺ T cells) and consisted of less than 30% CD39⁺ T cells (20.7% in healthy subjects vs 29.1% in patients with celiac disease; see Fig E2, D and E). This suggests that a high proportion of CD39⁺ cells within antigen-specific responses is not an inherent feature of the OX40 assay but is instead

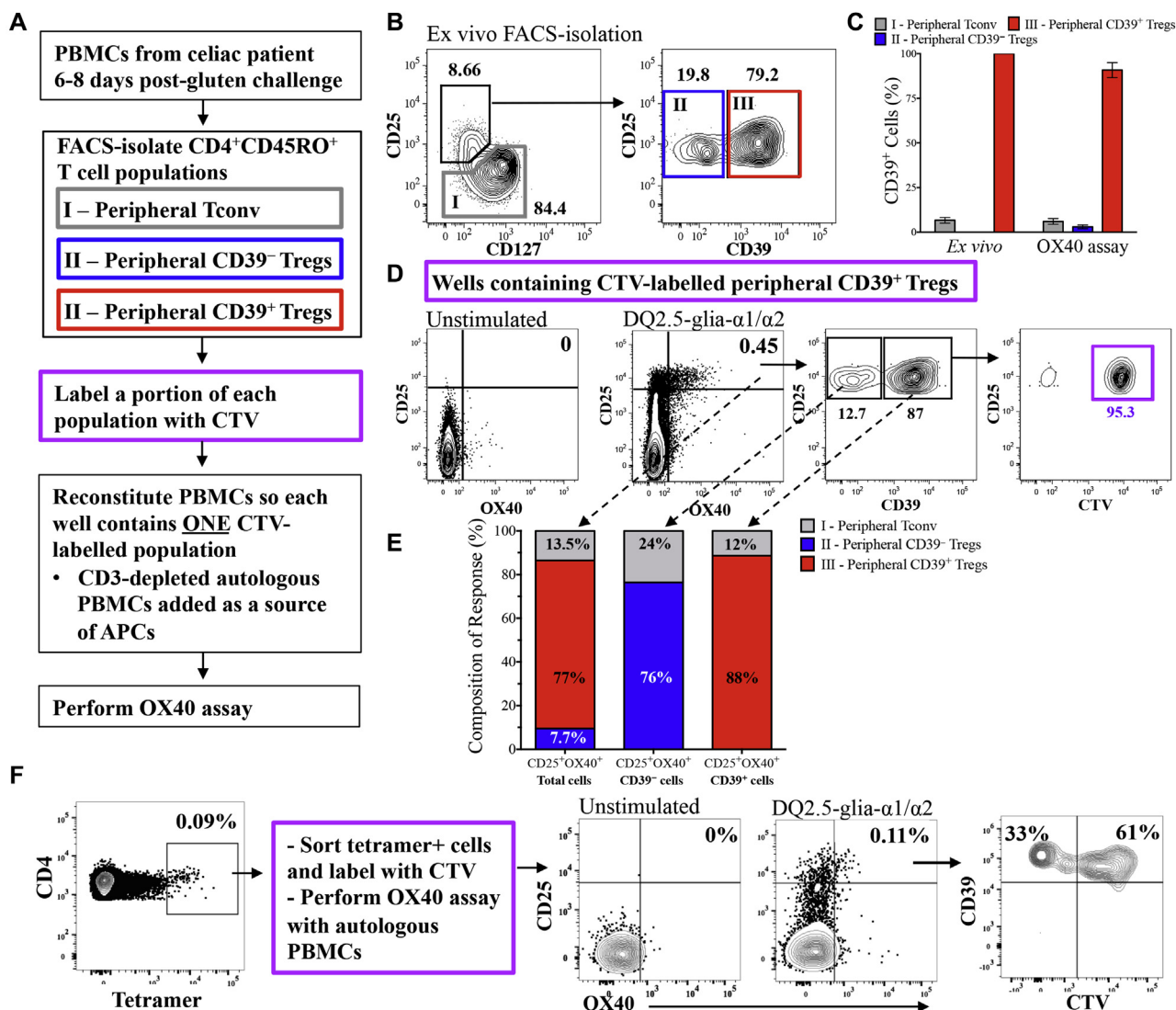


FIG 3. DQ2.5-glia- α 1/ α 2-specific CD25⁺OX40⁺CD39⁺ T cells originate from peripheral CD39⁺ Treg cells. **A** and **B**, Overview of the method and gating strategy used to conduct population tracking within an OX40 assay. **C**, Changes in CD39 expression between *ex vivo* isolation and after a 44-hour OX40 assay for Tconv cells, CD39⁻ Treg cells, and CD39⁺ Treg cells (mean \pm SEM). **D**, Representative plots for unstimulated wells and DQ2.5-glia- α 1/ α 2-stimulated wells that contained CTV-labeled CD39⁺ Treg cells. **E**, Proportion of Tconv cells (gray), CD39⁻ Treg cells (blue), and CD39⁺ Treg cells (red) within the total DQ2.5-glia- α 1/ α 2-specific CD25⁺OX40⁺ T-cell response and the CD39⁻ and CD39⁺ fractions of this response. Data in Fig 3, **C** and **E**, are the mean of 5 independent experiments with 4 patients with celiac disease. **F**, Tetramer-positive cells were sorted from day 6 PBMCs, labeled with CTV, and either left unstimulated or stimulated for 44 hours with DQ2.5-glia- α 1/ α 2 peptide in the presence of autologous APCs. The proportion of CD39⁺CTV⁺ cells within the CD25⁺OX40⁺ quadrant is representative of 2.

a unique feature of the recall response to gluten antigen in patients with celiac disease.

To confirm that gluten-specific CD25⁺OX40⁺CD39⁺ T cells originated from the pool of circulating Treg cells, we used our previously described method for population tracking within the OX40 assay (Fig 3, **A** and **B**).²⁰ We found that CD39⁺ memory Treg cells comprised an average of 88% of DQ2.5-glia- α 1/ α 2-specific CD25⁺OX40⁺CD39⁺ T cells, with CD39⁻ Treg cells also the dominant population (average, 76%; n = 4) within the CD25⁺OX40⁺CD39⁻ T cells (Fig 3, **E**). Again, we observed

that during a 44-hour OX40 assay, CD39 expression was not affected by cell activation (Fig 3, **C**). For 2 subjects, we tracked sorted, CTV-labeled CD4⁺DQ2.5-glia- α 1/ α 2 tetramer-positive cells within OX40 assays stimulated with DQ2.5-glia- α 1/ α 2 peptide. We observed that approximately 60% of the CD25⁺OX40⁺ cells were composed of CD39⁺CTV⁺ cells (Fig 3, **F**). These data support our findings that peripheral CD4⁺CD45RO⁺CD25^{high}CD127^{low} Treg cells constitute more than 80% of the total DQ2.5-glia- α 1/ α 2-specific CD25⁺OX40⁺ T-cell response after gluten challenge.

DQ2.5-glia- α 1/ α 2-specific CD39⁺ T cells have impaired suppressive function

DQ2.5-glia- α 1/ α 2-specific CD4⁺CD25⁺OX40⁺CD39⁺ T cells were expanded *in vitro* from post-gluten challenge PBMCs (sort purities, >90%) to further investigate the phenotype and function of gluten-specific FOXP3⁺CD39⁺ Treg cells. For female patients with celiac disease and healthy donors, we also expanded non-gluten-specific CD25⁻OX40⁻ T cells and, from unstimulated PBMCs, CD25^{high}CD127^{low} Treg cells and CD25⁻CD127⁺ Tconv cells. We generated 3 T-cell clones, 2 from patient #0174 (C1 and C2) and 1 from patient #0251 (C3). Clonality was confirmed through sequencing the T-cell receptor β chain variable region. Both T-cell clones from patient #0174 expressed the same TRBV7-2 clonotype (CASSLRVYTDQYF), which might be a public clonotype, as it has been previously identified in another celiac cohort.³⁰

Suppression assays used soluble anti-CD3 stimulus for 4 days in the presence of autologous APCs with a 1:1 ratio of suppressor to responder cells (Fig 4, A). Suppressive function of *ex vivo* celiac Treg cells (CD25^{high}CD127^{low}; median, 73.4%; n = 5) before oral gluten challenge was comparable with that of healthy Treg cells (median, 70.5%; n = 6) but significantly greater than that of Tconv cells (CD25⁻CD127⁺; median, 12%; n = 5), as expected ($P = .016$; Fig 4, B). Interestingly, the CD39⁺ subset of *ex vivo* CD25^{high}CD127^{low} Treg cells that was isolated before gluten challenge had reduced suppressive function (median, 46.3%; n = 5; $P = .016$) that was not further affected by the CD39 inhibitor ARL67156 (median, 45.5%). Similarly, 56-day expanded DQ2.5-glia- α 1/ α 2-specific CD39⁺ T cells had slightly reduced suppression compared with *ex vivo* Treg cells from patients with celiac disease (mean, 55.8%; range, 30.1% to 74.8%), as did the CD39⁺ T-cell clones C1 and C2 (62% and 52% suppression, respectively; Fig 4, C). Addition of the CD39 inhibitor ARL67156 had a minimal effect (mean reduction in suppression, 11%; Fig 4, C).

We assessed *in vitro* suppressive function of 14 day-expanded: DQ2.5-glia- α 1/ α 2-specific CD39⁺ T cells (n = 4), a T-cell clone (C3), and, for 4 healthy subjects and patients with celiac disease, Treg cells (CD25^{high}CD127^{low}) and Tconv cells (CD25⁻CD127⁺). Suppression assays were performed with suppressor/responder cell ratios of 1:1 to 1:32. The celiac Treg cells exerted suppression across all cell ratios comparable with that of Treg cells from healthy subjects, whereas the gluten-specific CD39⁺ T-cell clone C3 began to exhibit markedly lower suppressive function at a 1:8 cell ratio (Fig 4, D). The expanded gluten-specific CD39⁺ T cells had significantly reduced suppressive function compared with that of polyclonal Treg cells from both healthy subjects and patients with celiac disease across all ratios tested (Fig 4, D). These data indicate that in patients with celiac disease after gluten challenge, the expanded subset of peripheral gluten-specific CD39⁺ Treg cells, but not polyclonal Treg cells, has impaired suppressive function.

Approximately 50% of expanded gluten-specific CD25⁺OX40⁺CD39⁺ T cells stained positive for DQ2.5-glia- α 1/ α 2 tetramer compared with less than 2% tetramer-positive cells within non-gluten-specific CD25⁻OX40⁻ T cells (see Fig E3, B). These cells had substantial expression of CD39, CD25, cytotoxic T lymphocyte-associated antigen 4, and integrin β 7, but FOXP3 expression was low or absent (see Table E3 in this article's Online Repository at www.jacionline.org).

Interestingly, gluten-specific CD39⁺ T cells were Helios negative, suggesting they originate from a peripherally derived Treg cell population (see Fig E3, C). Quantitative RT-PCR confirmed that expanded gluten-specific CD39⁺ T cells had very low levels of FOXP3 and moderate-to-high levels of TGF- β expression that corresponded to increased surface expression of GARP and LAP, which tether latent TGF- β to the cell membrane (see Fig E3, A and E). Loss of FOXP3 protein expression corresponded to increased CpG methylation in the TSDR of expanded cells. Compared to *ex vivo* analysis (Fig 2, C), expanded CD39⁺ T cells had an average 2.8-fold increase in methylation, whereas Treg cells from healthy subjects and patients with celiac disease had 1.5- and 1.4-fold increases in methylation, respectively (see Fig E3, F).

Antigen-stimulated expression of CD25 and OX40 detects significantly more gluten-specific T cells than conventional IFN- γ secretion assays

We performed correlation analyses to compare the sensitivity of the OX40 assay with that of conventional IFN- γ ELISpot assays. We observed a positive correlation between the IFN- γ ELISpot assay at day 6 and peak OX40 assay responses to gluten peptide stimulus (n = 13, $r_s = 0.876$, $P = .0002$; Fig 5, A, and Table I). For the 12 HLA-DQ2.5 patients with celiac disease and detectable IFN- γ responses to gluten peptide antigen, 10 (83%) also had detectable OX40 assay responses. A linear regression analysis of responses to gluten peptide antigen detected by each assay generated a line of best fit with a slope (m) of 4.806. This indicates the OX40 assay detects approximately 5 times the number of gluten peptide-specific CD4⁺ T cells than the IFN- γ ELISpot (Fig 5, B).

A high proportion of memory Treg cells from patients with celiac disease express CD39

Flow cytometry was used to measure the frequency of peripheral lymphocyte populations (see gating in Fig E4, B) in healthy volunteers (n = 13; mean age, 47; 69% female) and patients before gluten challenge (n = 13; mean age, 58; 69% female). The proportion of CD39⁺ cells within memory Treg cells was significantly higher in patients (mean, 74.65%; range, 59.7% to 83.7%) than healthy control subjects (mean, 48.07%; range, 11.10% to 73.30%; $P < .0001$; Fig 5, C). Patients with celiac disease also had significantly reduced total memory Treg cell numbers at day 0 (mean, 3.14% of CD4⁺ T cells) compared with healthy control subjects (mean, 7.97%; $P < .0001$; Fig 5, D), which persisted at days 6 and 8 after challenge (data not shown), and significantly more CD39⁺ memory Treg cells within CD4⁺ T cells at day 0 ($P = .037$; Fig 5, E).

Symptom severity associated with stronger gluten peptide recall responses in the OX40 assay

Patients with celiac disease were split into 2 groups based on their symptom severity to identify associations between immunologic variables and clinical symptoms (Table I). No significant differences were observed for the frequency of total CD45RO⁺CD39⁺ Treg cells at day 0 or for ELISpot responses

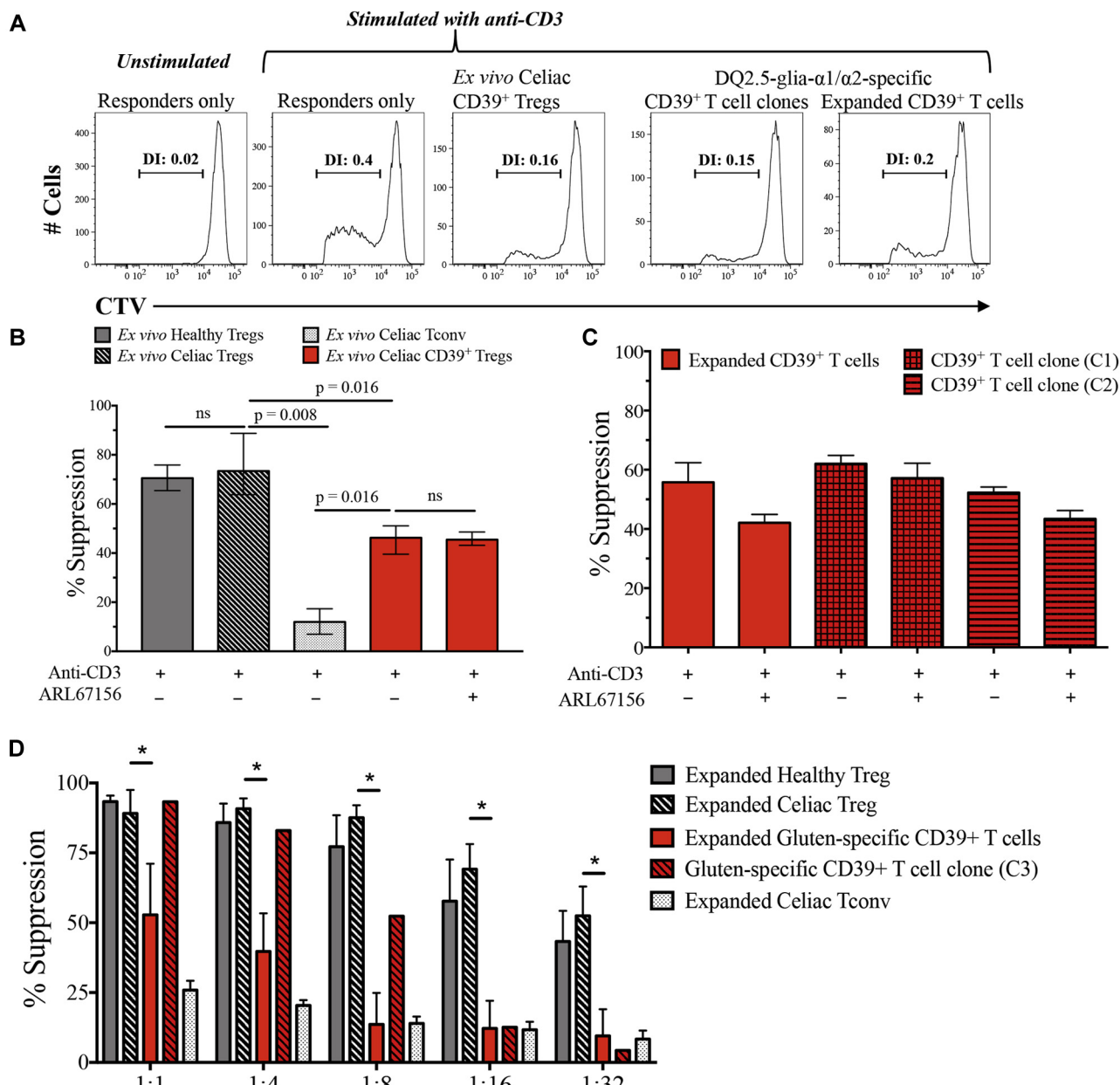


FIG 4. *In vitro*-expanded DQ2.5-glia- α 1/ α 2-specific T cells have impaired suppressive function. **A**, Representative responder cell proliferation showing division index (DI). **B**, Percentage of suppression of *ex vivo* Treg cells from healthy subjects (n = 6), Treg cells from patients with celiac disease (n = 5), CD39⁺ Treg cells from patients with celiac disease (n = 5), and Tconv cells from patients with celiac disease (n = 5) at a 1:1 ratio with responder T cells. ns, Not significant. **C**, Percentage suppression of 56-day expanded DQ2.5-glia- α 1/ α 2-specific CD39⁺ T-cell populations (n = 2) and CD39⁺ T-cell clones (C1 and C2). The CD39 inhibitor ARL67156 was added as indicated. **D**, Percentage suppression of 14-day expanded Treg cells (n = 4), Tconv cells (n = 4), and DQ2.5-glia- α 1/ α 2-specific CD39⁺ T cells (n = 4) from healthy subjects and patients with celiac disease and T-cell clone C3 for 1:1 to 1:32 suppressor/responder cell ratios. Data in Fig 4, B-D, are medians \pm interquartile ranges of 1 to 3 independent experiments, and statistical analyses used Mann-Whitney U tests.

to gluten peptide mix (see Fig E5, A and C, in this article's Online Repository at www.jacionline.org). However, celiac disease patients with more severe symptoms had significantly more CD39⁺ cells within deamidated gliadin-specific CD25⁺OX40⁺ T cells ($P = .014$) and significantly larger OX40 assay responses to gluten peptides ($P = .011$; see Fig E5, B and D).

DISCUSSION

These data are the first report of the contribution of FOXP3⁺CD39⁺ Treg cells to gluten-specific CD4⁺ T-cell responses in patients with celiac disease after *in vivo* gluten challenge. Surprisingly, we observed that FOXP3⁺CD39⁺ Treg cells comprised more than 80% of circulating gluten

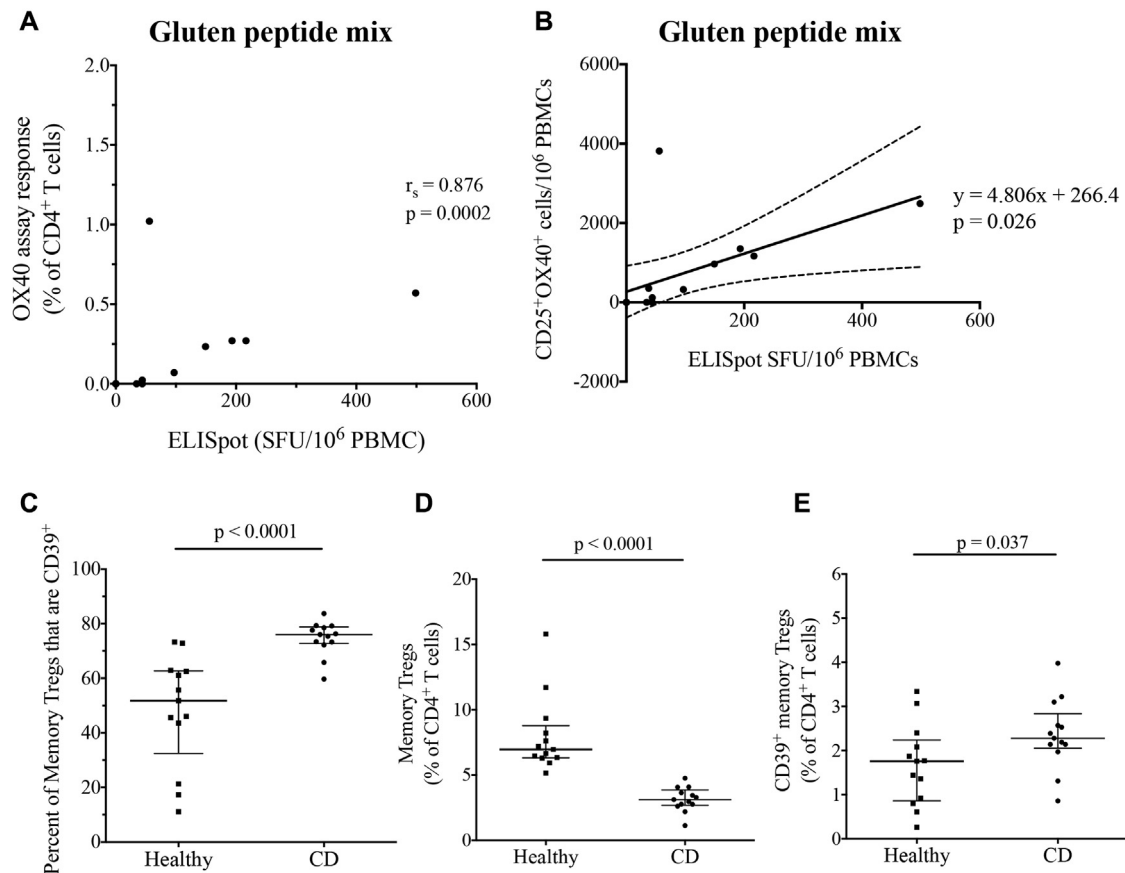


FIG 5. Analysis of assay correlation and peripheral Treg cell frequency and phenotype. **A** and **B**, Gluten peptide mix IFN- γ ELISpot responses (SFU/10⁶ PBMCs; $n = 13$) are correlated with OX40 assay responses ($n = 13$) expressed as either CD25⁺OX40⁺ cells as a percentage of CD4⁺ T cells (Fig 5, **A**) or CD25⁺OX40⁺ cells/10⁶ PBMCs (Fig 5, **B**). In Fig 5, **A**, the calculated Spearman rho (r_s) and P values are shown, and in Fig 5, **B**, the line of best fit (solid line) and 95% CIs (dashed line) are shown. **C-E**, Flow cytometric phenotyping data from 13 patients with celiac disease were compared with those in healthy subjects ($n = 13$) for the proportion of CD4⁺CD45RO⁺CD127^{low}CD25^{high} Treg cells that expressed CD39 (Fig 5, **C**), the proportion of CD45RO⁺ memory Treg cells within CD4⁺ T cells (Fig 5, **D**), and the proportion of CD4⁺ T cells that were CD45RO⁺CD39⁺ Treg cells (Fig 5, **E**). Error bars represent medians \pm interquartile ranges, and statistical analyses used Mann-Whitney U tests.

peptide-specific CD4⁺ T cells in patients with celiac disease after gluten challenge. We confirmed that greater than 85% of the gluten peptide-specific FOXP3⁺CD39⁺ T cells originate from the peripheral pool of CD39⁺ Treg cells and that the extent of CpG methylation in the TSDR within the FOXP3 loci of these cells is similar to that seen in CD127^{low}CD25^{high} Treg cells from healthy subjects.

Because the majority of FOXP3⁺ Treg cells do not secrete IFN- γ , our data indicate that IFN- γ -based methods only detect approximately 20% of the total CD4⁺ T-cell response to gluten antigen (supported by our linear regression analysis). The OX40 assay correlated with the IFN- γ ELISpot for the detection of gluten-specific CD4⁺ T-cell responses. This concurs with previous studies that found the OX40 assay has strong agreement with IFN- γ release assays for *Mycobacterium tuberculosis*^{31,32} and concordance with serology, proliferation, and cytokine responses to HIV-1,²¹ hepatitis C virus,³³ human papillomavirus,³⁴ *Mycobacterium avium* complex, varicella zoster virus, EBV, CMV, *Candida albicans*, and *Streptococcus pneumoniae*.^{21,35} For the first time, we also show that the OX40

assay corresponds with class II tetramer staining for responses to the DQ2.5-glia- α 1/ α 2 epitopes in patients with celiac disease after gluten challenge.

These data complement our previous study showing that CD39⁺ Treg cells comprise a substantial proportion of CD4⁺ T-cell recall responses to viral and bacterial antigens.²⁰ Previous studies of FOXP3⁺ Treg cells within *in vivo* recall responses to varicella zoster virus in human subjects^{36,37} and within secondary immune responses to influenza virus in mice^{38,39} identified a key role for pathogen-specific Treg cells in controlling the cellular immune response. Of particular interest is the recent discovery that particle-associated antigens drive a Treg cell response, whereas distinct soluble antigens instead drive an effector T-cell response.⁴⁰ In addition to the type of antigen, the balance between Treg and Tconv cells within antigen-specific responses is also influenced by chronicity of antigen exposure.⁴¹ This study is a large contribution to the relatively underexplored area of antigen-specific human Treg cells and shows that, for patients with celiac disease on a gluten-free diet, the CD4⁺ T-cell response to acute dietary gluten re-exposure is skewed toward

TABLE I. Celiac disease cohort symptoms and CD4⁺ T-cell responses to gluten antigens

ID	Symptoms after gluten challenge	Post-gluten challenge gluten peptide mix response, OX40 assay‡	Post-gluten challenge gluten peptide mix response, IFN-γ secretion
0062*	Mild depressed mood and lethargy (days 4-6)	Detected	Detected
0077*	Asymptomatic	Not detected§	Detected
0080*	Mild nausea (days 1-3)	Not detected	Not detected
0152†	Severe vomiting, lethargy, and diarrhea (days 1-3); moderate nausea, bloating, and abdominal pain (days 1-3)	Detected	Detected
0159*	Mild bloating, abdominal pain, and lethargy (days 2-3)	Detected	Detected
0174†	Severe nausea, vomiting, abdominal pain, and diarrhea (days 1-3); moderate lethargy, hot flushes, cold sweats, and flatulence (days 2-4)	Detected	Detected
0196†	Moderate-to-severe abdominal pain, nausea, and lethargy and mild diarrhea (days 1-3)	Detected	Detected (ELISA)
0230*	Mild nausea and diarrhea (days 1-3)	Detected	Detected
0239*	Asymptomatic	Not detected	Detected (at assay limit of detection)
0250†	Severe vomiting, nausea, abdominal pain, lethargy, and cold sweats (days 1-3); mild bloating and diarrhea (days 2-4)	Detected	Detected
0251†	Moderate-to-severe abdominal pain, bloating, nausea, and lethargy	Detected	Detected (ELISA)
0505*	Severe lethargy and mild diarrhea (days 1-3)	Not detected	Not detected
0506*	Mild constipation (days 1-3)	Not detected	Not detected
0509*	Mild bloating, abdominal pain, and diarrhea (day 1)	Not detected	Not detected
0510†	Severe bloating, abdominal pain, and constipation (days 203)	Not detected	Not detected
0512*	Asymptomatic	Detected	Detected
0072†	Moderate-to-severe nausea, bloating, and vomiting (day 1); moderate abdominal pain and lethargy (day 1)	Detected	Detected

*Nonsevere group: reported mild or no symptoms.

†Severe group: reported moderate-to-severe symptoms.

‡Post-gluten challenge OX40 assay responses were only listed as detected if they were greater than the baseline response.

§Cohort analyses were performed with 15 patients; patient #0077 was not included because day 8 OX40 assays used cryopreserved PBMCs, and patient #0072 was not included because day 8 analysis was not performed.

Treg cells. Furthermore, we demonstrate that functional defects can be unmasked by studying the relevant disease antigen-specific population rather than polyclonal Treg cells.

On average, maximal OX40 responses to gluten antigens occurred at day 6 after gluten challenge, a similar time course to that previously noted for IFN-γ responses.^{7,18} These dynamic changes were restricted to the gluten-derived antigens because there was little change in responses to mitogen or control antigens. Importantly, CD39⁺ proportions within all OX40 assay responses were not significantly altered after gluten challenge, indicating the high CD39⁺ proportions observed within gluten peptide responses occur independently of immune activation. For those patients with celiac disease who did not respond to the gluten peptides in this study, there is no evidence of durable immune tolerance because the gluten challenge still caused clinical symptoms for these patients. It is more likely that the immune response is present but either below the assay's limit of detection or not detectable in peripheral blood. The severity of gastrointestinal symptoms also corresponded to larger immune responses to gluten and a higher frequency of CD39⁺ cells within these responses. This is similar to a previous observation that the density of intestinal FOXP3⁺ Treg cells positively correlated with the severity of histologic damage.¹⁰

Both expanded DQ2.5-glia-α1/α2-specific CD39⁺ T-cell lines and clones and total *ex vivo* CD39⁺ Treg cells from patients with celiac disease had reduced suppressive function in response to a polyclonal stimulus that was not dependent on CD39 function but corresponded to a loss of stable FOXP3 expression, indicating that gluten-specific CD39⁺ Treg cells from patients with celiac

disease might have an inherent functional defect. These data contrast with a previous observation that found expanded IL-10-secreting gluten-specific T regulatory type 1 (Tr1) cell clones had normal *in vitro* suppressive function.⁸ Our *in vitro*-expanded DQ2.5-glia-α1/α2-specific CD39⁺ T cells had a CD25⁺FOXP3⁻TGF-β⁺GARP⁺LAP⁺ phenotype that most closely resembles that of human regulatory T_H3 cells.⁴² In contrast to the previously observed stable FOXP3 expression seen in expanded CMV-P1-specific T-cell clones,²⁰ expanded gluten-specific CD39⁺ T-cell populations lost FOXP3 expression *in vitro* within 14 days, which corresponded to increased CpG methylation in the TSDR. This might indicate that gluten-specific CD39⁺ Treg cells retain a high degree of plasticity,⁴³ although expanded healthy Treg cells also acquired methylation *in vitro*, albeit to a lesser extent.

Compared with healthy control subjects, patients with celiac disease before challenge had significantly more CD39⁺ cells within CD45RO⁺ Treg cells yet lower absolute numbers of CD45RO⁺ Treg cells. This is likely due to a single nucleotide polymorphism in the CD39 gene that determines CD39 expression levels in Treg cells, and future work should investigate the association of such CD39 single nucleotide polymorphisms with celiac disease.⁴⁴ CD39⁺ Treg cells have been shown to be potent suppressors of IFN-γ and IL-17 and to be increased in the synovia of patients with juvenile arthritis.⁴⁴ Therefore CD39⁺ Treg cells might be preferentially expanded, yet their numbers are insufficient to control inflammation after gluten exposure in patients with celiac disease. CD39 expression on Treg cells might also be useful in predicting clinical outcomes

because low CD39 expression has been associated with better CD4⁺ T-cell recovery after antiretroviral therapy in HIV⁺ patients²⁰ and methotrexate resistance in patients with rheumatoid arthritis.⁴⁵

Together, the data presented here indicate that in patients with celiac disease after gluten challenge, FOXP3⁺CD39⁺ Treg cells dominate peripheral recall responses to gluten and can be readily expanded following *in vivo* antigen challenge, yet exhibit impaired *in vitro* suppressive function. Therefore, one interpretation of these data is that, in response to gluten challenge, FOXP3⁺CD39⁺ Treg cells are induced *in vivo* in an attempt to restore homeostasis. However, the generated cells have impaired suppressive function, possibly as a result of generation under inflammatory conditions *in vivo*. A key area for further investigation is whether *in vivo* challenge conditions could be manipulated to drive expansion of functional Treg cells.

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Key messages

- In patients with celiac disease, 6 days after gluten challenge *in vivo*, a surprisingly large proportion of circulating gluten-specific CD4⁺ T cells are FOXP3⁺CD39⁺ Treg cells.
- In patients with celiac disease after gluten challenge, gluten-specific Treg cells exhibit impaired polyclonal suppressive function *in vitro*, suggesting that an intrinsic dysfunction of expanded CD39⁺ Treg cells might contribute to the loss of tolerance to gluten.
- Detection of gluten-specific CD4⁺ T cells based on antigen-induced coexpression of CD25 and OX40 is more sensitive than traditional methods relying on antigen-induced cytokine production and, for the first time, allows detailed characterization of antigen-specific Treg cells in patients with this disease.

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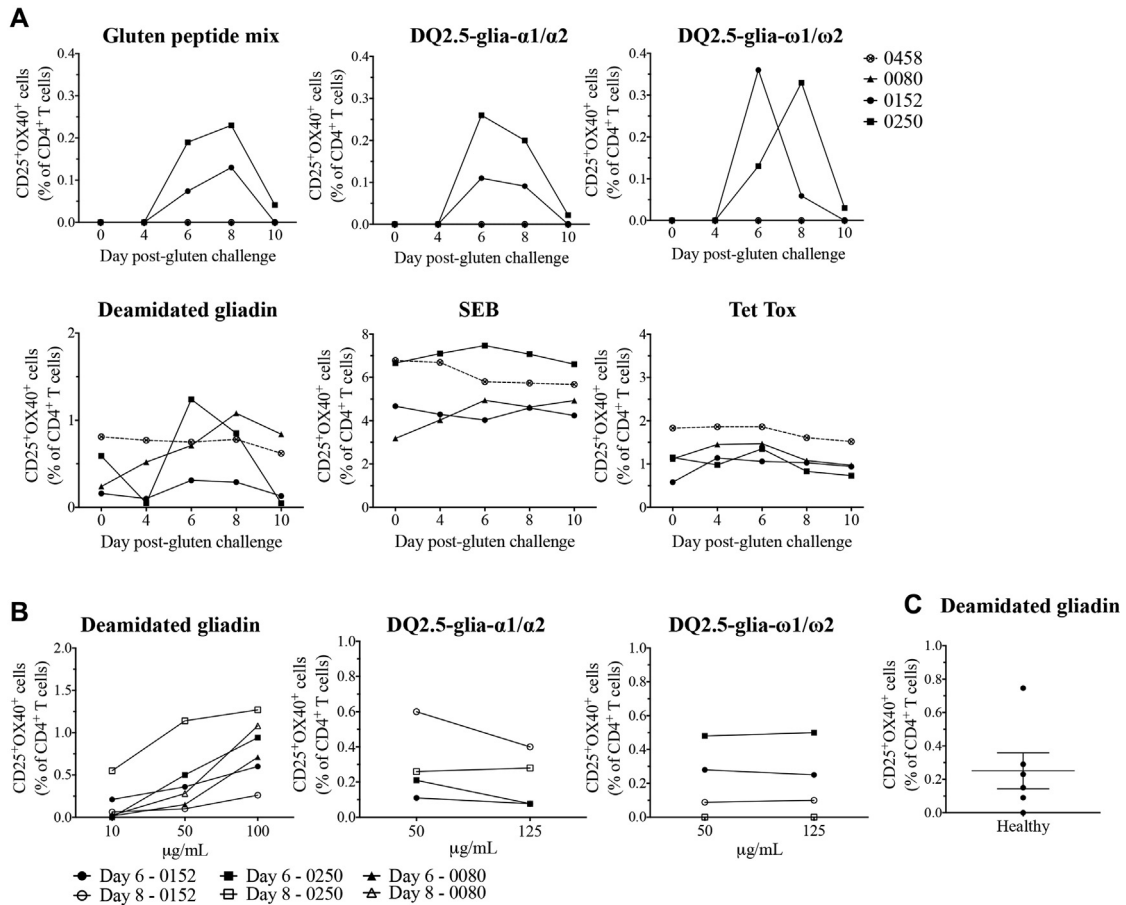


FIG E1. Optimization of OX40 assay time course and gluten antigen concentration. **A**, In a pilot study OX40 assays were performed with peripheral blood from 3 patients (#0080, #0152, and #0250) and 1 HLA-DQ2⁺ subject without celiac disease (#0458). Blood was collected before gluten challenge (day 0) and at days 4, 6, 8, and 10 after challenge. OX40 assay responses (CD25⁺OX40⁺) are shown for gluten peptide mix, DQ2.5-glia- α 1/ α 2, DQ2.5-glia- ω 1/ ω 2 deamidated gliadin, SEB, or tetanus toxoid (*Tet Tox*). **B**, OX40 assay responses are shown at days 6 and 8 to 10, 50, and 100 μ g/mL deamidated gliadin; 50 and 125 μ g/mL DQ2.5-glia- α 1/ α 2; or 50 and 125 μ g/mL DQ2.5-glia- ω 1/ ω 2. **C**, OX40 assays were performed with peripheral blood of 6 healthy subjects. The deamidated gliadin responses are shown for a single experiment with each subject. Error bars represent means \pm SEMs.

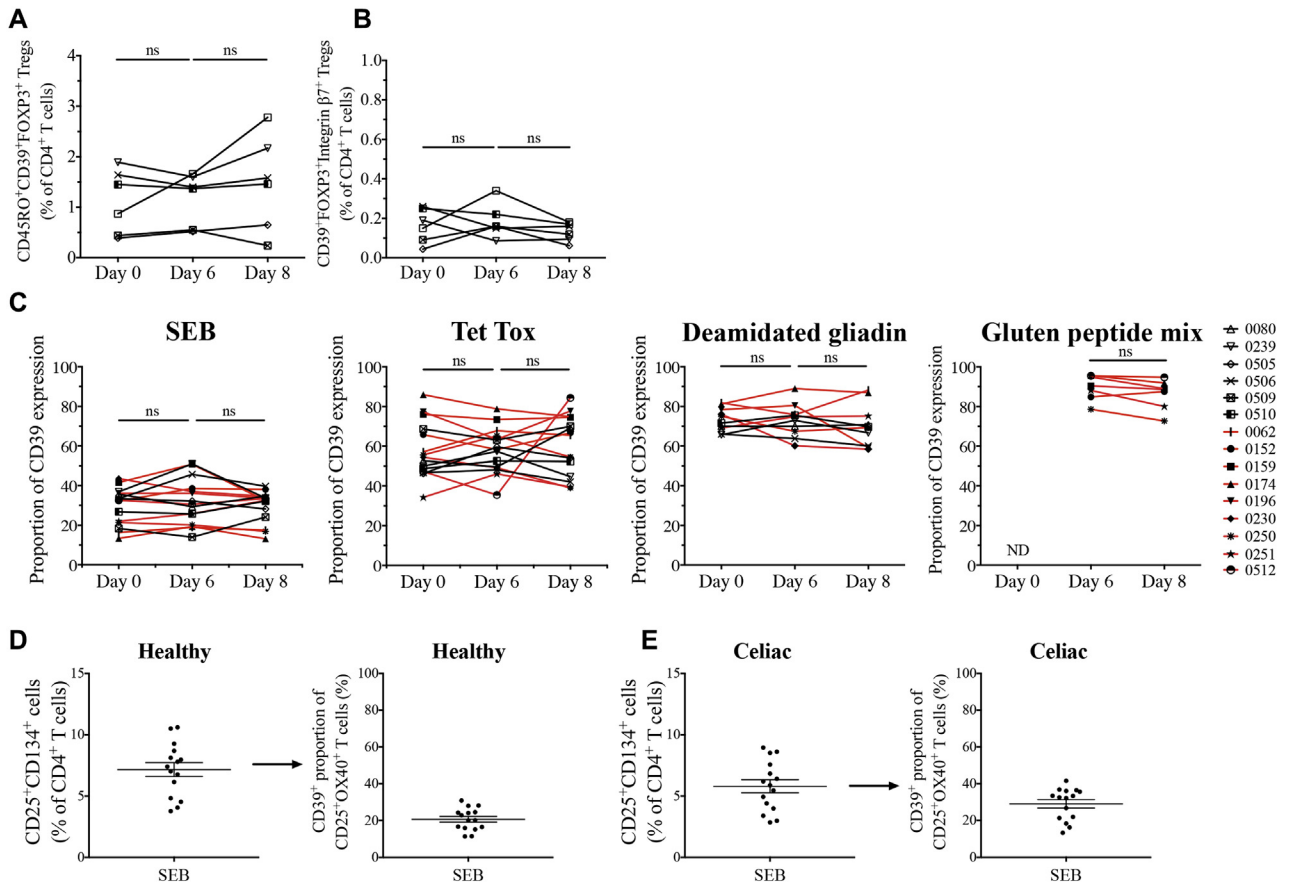


FIG E2. Changes in peripheral Treg cell frequencies and OX40 assay CD39⁺ proportions after gluten challenge. **A** and **B**, Frequencies of CD4⁺ T cells that were memory CD39⁺FOXP3⁺ Treg cells (Fig E2, A) and integrin β 7⁺CD39⁺ memory Treg cells (Fig E2, B) are shown for patients who did not have detectable gluten peptide responses (n = 6). **C**, The proportion of CD39 expression within OX40 assay responses is plotted for assays stimulated with SEB (n = 15), tetanus toxoid (*Tet Tox*; n = 15), deamidated gliadin (n = 10), and gluten peptide mix (n = 7). Subjects were only included in analyses if they had detectable responses at all 3 time points or, for gluten peptide mix, if they had detectable responses at both day 6 and day 8. Statistical analyses were performed with Wilcoxon signed-rank tests. *ND*, Not detected; *ns*, not significant. Each subject is represented by a different symbol, and *red connecting lines* indicate those subjects with gluten peptide responses. **D** and **E**, Total CD4⁺CD25⁺OX40⁺ T-cell responses to SEB, and the proportion of CD39⁺ cells within these responses, are shown for healthy subjects (n = 15; Fig E2, D) and patients with celiac disease (n = 15; Fig E2, E). Means \pm SEMs are shown.

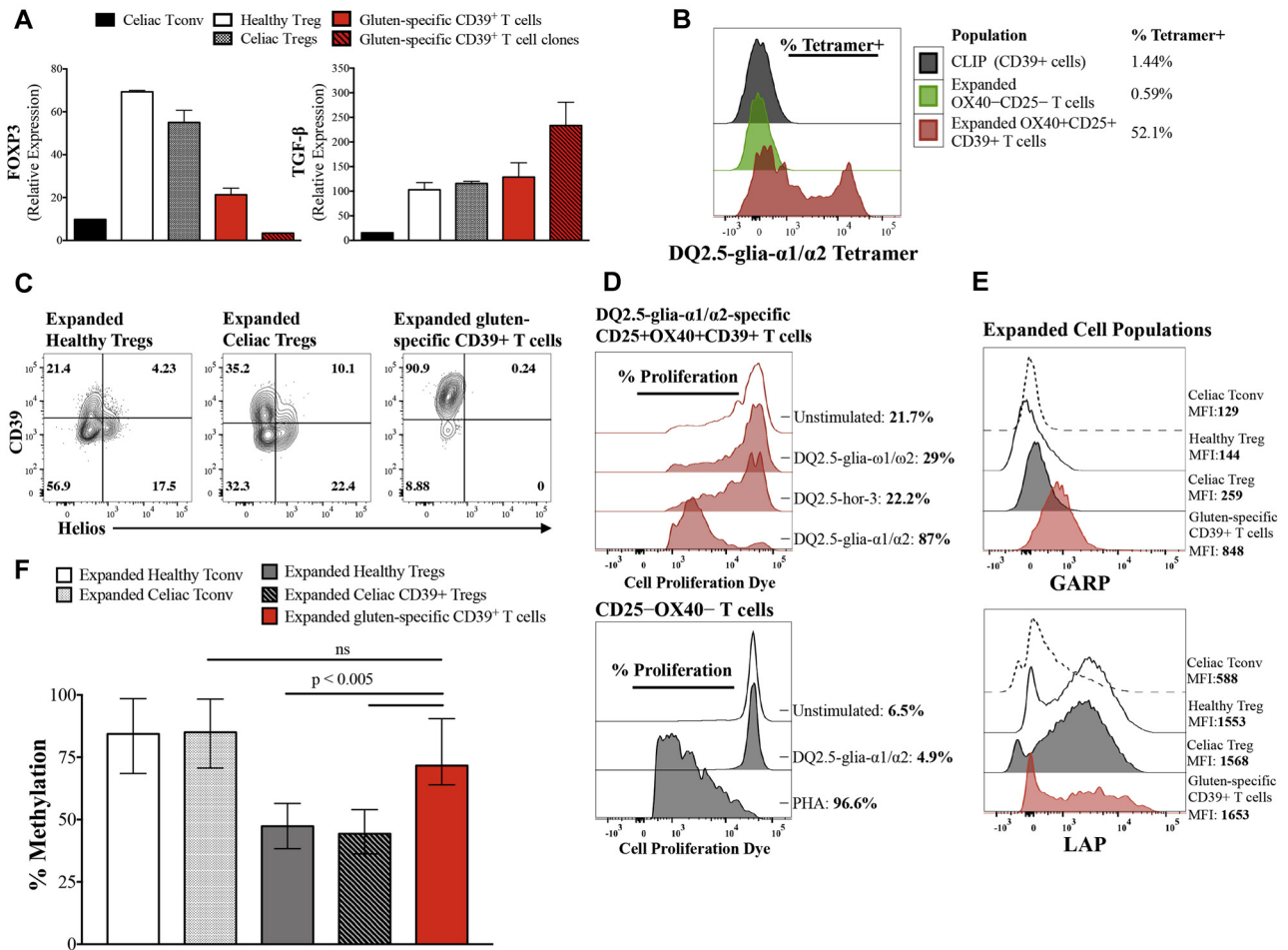


FIG E3. Characterization of expanded DQ2.5-glia- α 1/ α 2-specific CD39⁺ T cells. **A**, Quantitative RT-PCR was used to compare FOXP3 and TGF- β mRNA expression levels in 14-day expanded gluten-specific CD39⁺ T cells to levels in expanded CD127^{low}CD25^{high} Treg cells from healthy subjects ($n = 3$) and expanded Treg and CD127⁺CD25⁻ Tconv cells from patients with celiac disease ($n = 2$). Relative expression is shown after normalization to β -actin. Means + SEMs are shown for 2 independent experiments. **B**, DQ2.5 tetramer (loaded with HLA class II invariant peptide or DQ2.5-glia- α 1/ α 2) staining of 14-day expanded DQ2.5-glia- α 1/ α 2-specific CD25⁺OX40⁺ and non-gluten-specific CD25⁻OX40⁻ T cells. Frequencies of tetramer-positive cells are representative of expanded CD39⁺ cells and clones from 2 subjects. **C**, CD39 and Helios staining (representative of $n = 3$) of expanded CD127^{low}CD25^{high} Treg cells and gluten-specific CD39⁺ T cells from healthy subjects and patients with celiac disease. **D**, DQ2.5-glia- α 1/ α 2-specific CD25⁺OX40⁺CD39⁺ (red) and non-gluten specific CD25⁻OX40⁻ (black) T cells were isolated by fluorescence-activated cell sorting directly after an OX40 assay and restimulated for 5 days with the antigens indicated. Percentage proliferation shown is representative of 3 subjects. **E**, Histograms showing expression of GARP and LAP in cells expanded from patient #0251 with celiac disease and a healthy control subject. Median fluorescence intensity (MFI) is shown in the legend. **F**, Percentage methylation in the TSDR of *FOXP3* is shown for 14-day expanded CD127^{low}CD25^{high} Treg cells from healthy subjects ($n = 3$) and patients with celiac disease ($n = 3$) and gluten-specific CD39⁺ T cells ($n = 3$; median \pm interquartile range; all donors were female). *ns*, Not significant.

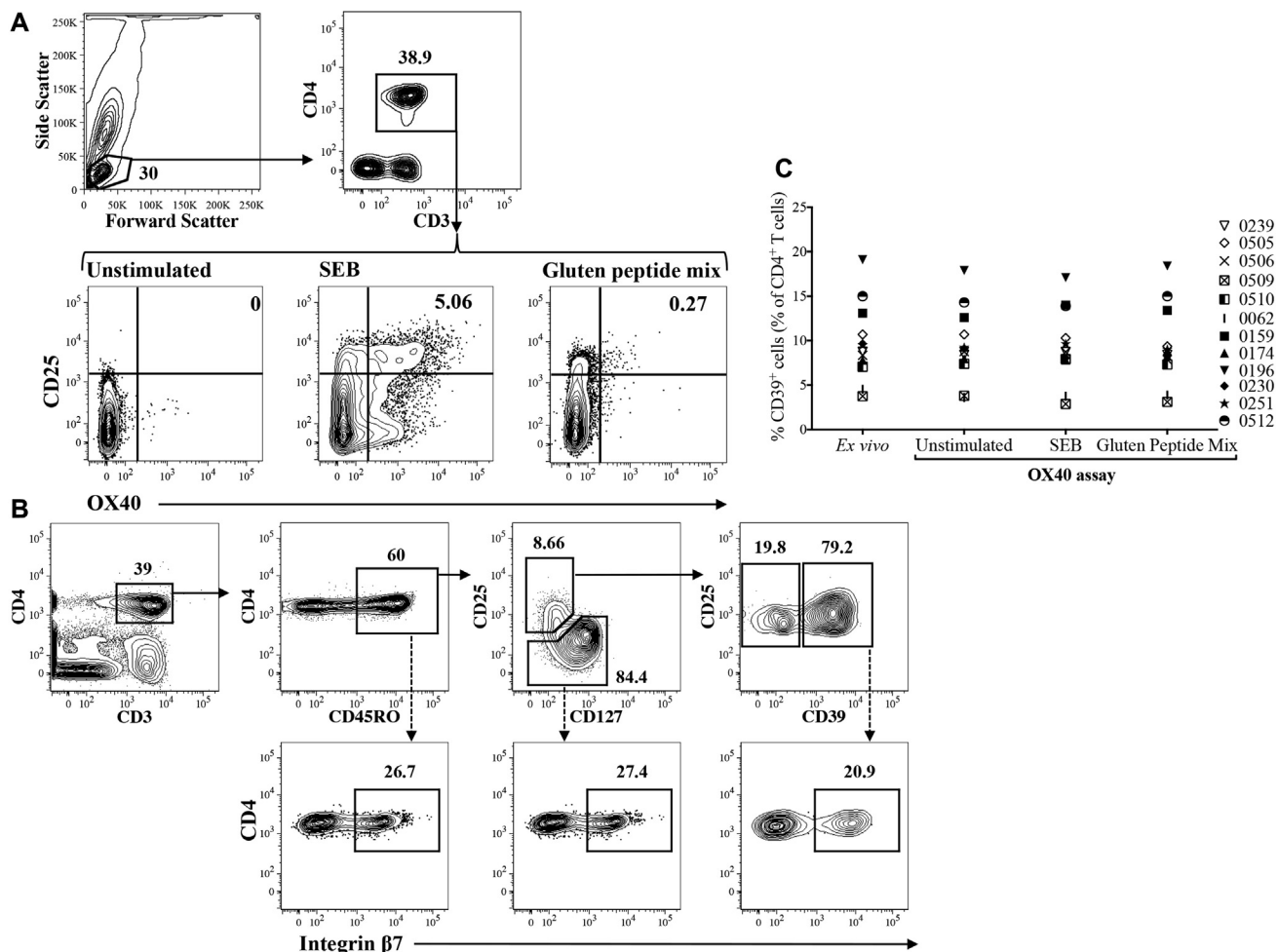


FIG E4. Flow cytometric gating strategies. **A**, Gating strategy for OX40 assay analysis. Lymphocytes were gated based on their forward- and side-scatter profiles, followed by gating CD3⁺CD4⁺ T cells. The crosshatch gates used to define the antigen-specific CD25⁺OX40⁺ T cells were set initially on unstimulated wells and refined by using responses in SEB-stimulated positive control wells. **B**, Gating strategy shown for isolation and phenotypic analysis of peripheral CD4⁺ memory T-cell populations. After gating on lymphocytes and CD3⁺CD4⁺ T cells, the CD45RO⁺ memory cells were divided into Treg cells (CD127^{low}CD25^{high}) and the remaining non-Treg cells, with the Treg cells separated further into CD39⁺ and CD39⁻ populations. Expression of phenotypic markers, such as the gut-homing marker integrin β 7, were analyzed within each gated T-cell population. **C**, For 12 patients with celiac disease, the percentage of CD39⁺ cells within CD4⁺ T cells is shown for PBMCs sourced directly *ex vivo* and from OX40 assay wells after 44 hours of stimulation with no antigen, SEB, or gluten peptide mix.

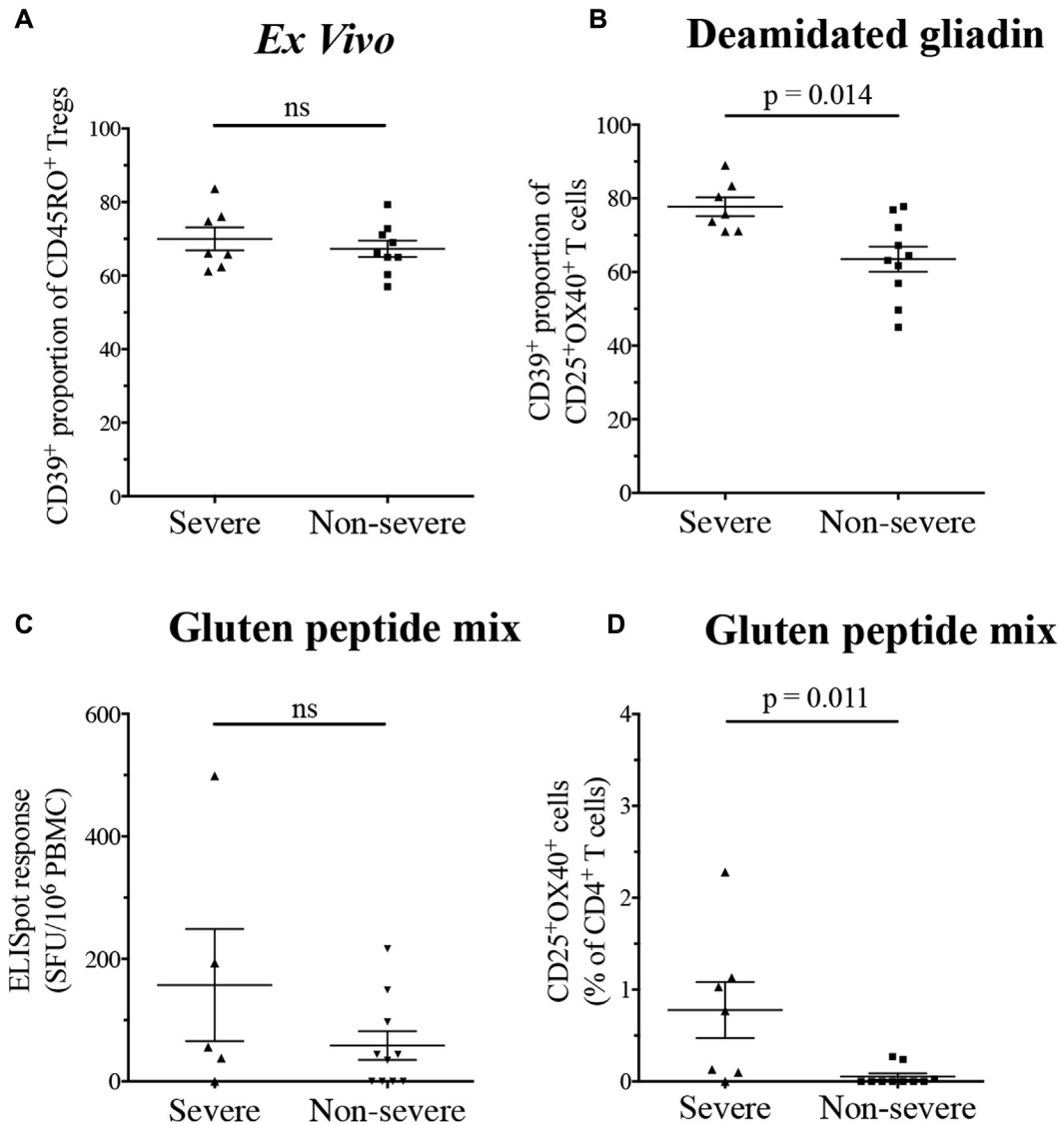


FIG E5. Symptom severity is associated with greater responses to gluten peptide antigen. Patients with celiac disease were divided into groups based on symptom severity: severe, $n = 7$; nonsevere, $n = 10$. Groups were compared by identifying differences in **(A)** the proportion of CD39⁺ cells within *ex vivo* memory Treg cells **(B)**, the proportion of CD39 expression within deamidated gliadin-specific CD25⁺OX40⁺ T cells **(C)**, the magnitude of day 6 postchallenge IFN- γ ELISpot response to gluten peptide mix ($n = 5$ in severe group because $n = 2$ only had ELISA data; **(D)**, the maximal OX40 assay response to gluten peptide mix. Error bars represent means \pm SEMs. Mann-Whitney U tests were performed. *ns*, Not significant.

TABLE E1. Celiac disease cohort characteristics

ID	Age (y)	Sex	Time since diagnosis	tTG IgA (U/mL)*	DGP IgA (U/mL)*	HLA-DQ Haplotype
0062	66	F	5 y, 9 mo	<20	<20	HLA-DQ2.5
0077	48	M	21 y	<20	<20	HLA-DQ2.5
0080†	68	F	31 y	<20	<20	HLA-DQ2.5
0152†	54	M	1 y, 9 mo	<20	<20	HLA-DQ2.5
0159	67	F	14 y, 9 mo	<20	<20	HLA-DQ2.5 (homozygous)
0174	51	F	15 y, 7 mo	<20	<20	HLA-DQ2.5
0196	60	F	9 y, 3 mo	<20	<20	HLA-DQ2.5
0230	53	F	12 y, 9 mo	<20	<20	HLA-DQ2.5
0239	71	F	39 y, 3 mo	<20	<20	HLA-DQ2.5 (homozygous)
0250	62	M	8 y, 5 mo	<20	<20	HLA-DQ2.5
0251†	53	F	8 y, 2 mo	<20	<20	HLA-DQ2.5
0505	35	F	34 y, 11 mo	<20	<20	HLA-DQ2.2
0506	69	M	2 y, 1 mo	<20	<20	HLA-DQ2.5 (heterozygous)
0509	29	F	7 y, 11 mo	<20	<20	HLA-DQ2.5 (heterozygous)
0510	38	M	4 y, 11 mo	62 (strongly positive)	27 (weakly positive)	HLA-DQ2.5 and HLA-DQ2.2
0512	52	F	1 y, 4 mo	<20	29 (weakly positive)	HLA-DQ2.5 (heterozygous)
0072	57	F	20 y, 2 mo	<20	<20	HLA-DQ2.5 (heterozygous)
Mean	55	71% F 29% M	14 y, 1 mo	<20	<20	

All data were collected at the time of gluten challenge. All subjects have been on a gluten-free diet since diagnosis.

DGP, Deamidated gliadin peptide; F, female; M, male.

*Normal serology is less than 20 U/mL.

†Indicates subjects analyzed in the pilot study.

TABLE E2. HLA-DQ alleles detected in the celiac disease cohort

ID	HLA-DQA	HLA-DQB
0062	<i>DQA1*05</i>	<i>DQB1*02</i> <i>DQB1*03</i>
0077	<i>DQA1*01</i> <i>DQA1*05</i>	<i>DQB1*02</i> <i>DQB1*05</i>
0080*	<i>DQA1*05:01</i>	<i>DQB1*02:01</i> <i>DQB1*02:02</i>
0152*	<i>DQA1*01</i> <i>DQA1*05</i>	<i>DQB1*02</i> <i>DQB1*06</i>
0159	<i>DQA1*05:01</i>	<i>DQB1*02:01</i>
0174	<i>DQA1*05:01</i>	<i>DQB1*02:01/02</i> <i>DQB1*05:02</i>
0196	<i>DQA1*05:01</i>	<i>DQB1*02:01/02</i> <i>DQB1*06:02</i>
0230	<i>DQA1*05:01</i>	<i>DQB1*02:01</i> <i>DQB1*05:02</i>
0239	<i>DQA1*05:01</i>	<i>DQB1*02:01</i>
0250*	<i>DQA1*02</i> <i>DQA1*05</i>	<i>DQB1*02</i>
0251	<i>DQA1*01</i> <i>DQA1*05</i>	<i>DQB1*02:01</i> <i>DQB1*05:01</i>
0505	<i>DQA1*02</i> <i>DQA1*04/06</i>	<i>DQB1*02</i> <i>DQB1*04</i>
0506	N/A	N/A
0509	N/A	N/A
0510	<i>DQA1*02:01</i> <i>DQA1*05:01</i>	<i>DQB1*02:01</i> <i>DQB1*02:02</i>
0512	NA	NA
0072	NA	NA

HLA-DQ2.5 refers to the *DQA1*0501:DQB1*0201* haplotype, and HLA-DQ2.2 refers to the *DQA1*0201:DQB1*0202* haplotype.

NA, HLA-DQB1 and HLA-DQA1 alleles were not assessed, and instead only the presence of alleles encoding HLA-DQ2.5, HLA-DQ2.2, and HLA-DQ8 were detected.

*Indicates subjects analyzed in the pilot study.

TABLE E3. Phenotype of *in vitro*-expanded DQ2.5-glia- α 1/ α 2-specific CD4⁺CD25⁺OX40⁺CD39⁺ T cells

Molecule	Clone C1 (#0174)	Clone C2 (#0174)	Clone C3 (#0251)	CD39 ⁺ cells (#0174)	CD39 ⁺ cells (#0230)	CD39 ⁺ cells (#0196)	CD39 ⁺ cells (#0251)
CD25	+++	+++	+++	+++	+++	+++	+++
CD39	+++	+++	+++	+++	+++	+++	+++
FOXP3	+	–	++	+	–	+	+
CTLA-4	+++	+++	++	+++	+++	++	+++
CD45RO	+++	+++	+++	+++	+++	+++	+++
Integrin β 7	++	++	++	++	+++	+	+
LAP	NA	NA	+++	NA	NA	+	+
GARP	NA	NA	++	NA	NA	++	++
Helios	NA	NA	–	NA	NA	–	–

CTLA-4, Cytotoxic T lymphocyte-associated antigen 4; *NA*, not assessed; +++, expression on greater than 90% of cells; ++, expression on 50% to 90% of cells; +, expression on 10% to 50% of cells; –, expression on less than 10% of cells.