Development and technical validation of tetramer staining for use as a biomarker for assessing gluten-specific T cells in clinical studies

Will McAuliffe,[1] Lynn Kisselbach,[2] Angelina Bisconte,[2] William Kwok,[3] Deborah Phippard,[2] Jose Estevam,[1] Glennda M. Smithson[1]

[1]Takeda Pharmaceuticals International Co., Cambridge, MA, USA;
[2]Precision for Medicine; [3]Benaroya Research Institute at Virginia Mason, Seattle, WA, USA

P4-17

Introduction

- Gluten-challenge in subjects with celiac disease results in a transient upregulation of gliadin-specific CD4+ T cells in the blood. Despite the increase, these cells are quite rare requiring a selective and sensitive assay for detection.

Method and results

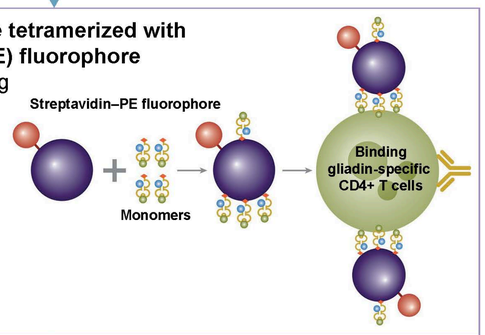
- We have developed a 12-colour tetramer flow assay to enable detection and immunophenotyping of the gliadin α-I/α-II CD4+ T cells in the blood (**Figure 1**).

Figure 1. Experimental procedure

Step 1. Separation and storage of PBMCs
- For clinical application, trial sites can collect whole blood
- Centralized PBMC isolation using SepMate™ tubes – Ficoll
- PBMCs frozen to maintain viability and allow batch testing
- Longitudinal samples from each subject to be tested together

↓

Step 2. Gliadin monomers are tetramerized with streptavidin phycoerythrin (PE) fluorophore
Tetramers are pooled for staining
- *Tetramer 1: gliadin α-I (QLQPFPQPELPY)*
- *Tetramer 2: gliadin α-II (PQPELPYPQPE)*



↓

Step 3. Isolation of CD4+ T cells from thawed PBMCs
- PBMCs are thawed and CD4+ cells 'isolated' via magnetic bead based separation
- Post purity >95% prior to staining

↓

Step 4. Stain isolated CD4+ T cells
Controls for staining include:
- Unstained samples to measure auto-fluorescence
- Single-colour controls, entire antibody panel minus 1 stain for each marker and gliadin α-I/II tetramers to set compensation, to evaluate impact of cross-reactivity between reagents and to objectively set gates

↓

Step 5. T cell tetramer full antibody panel
This includes CD3, CD4, CD38, B7, tetramer, CD45RA, CD62L, Alpha4, CCR7, CD39, lineage depletion (CD11c, CD14, CD19, CD56) and live/dead viability dye

↓

Step 6. Detection with BD LSRFortessa™ 5-laser 18-colour system

PBMC, peripheral blood mononuclear cell

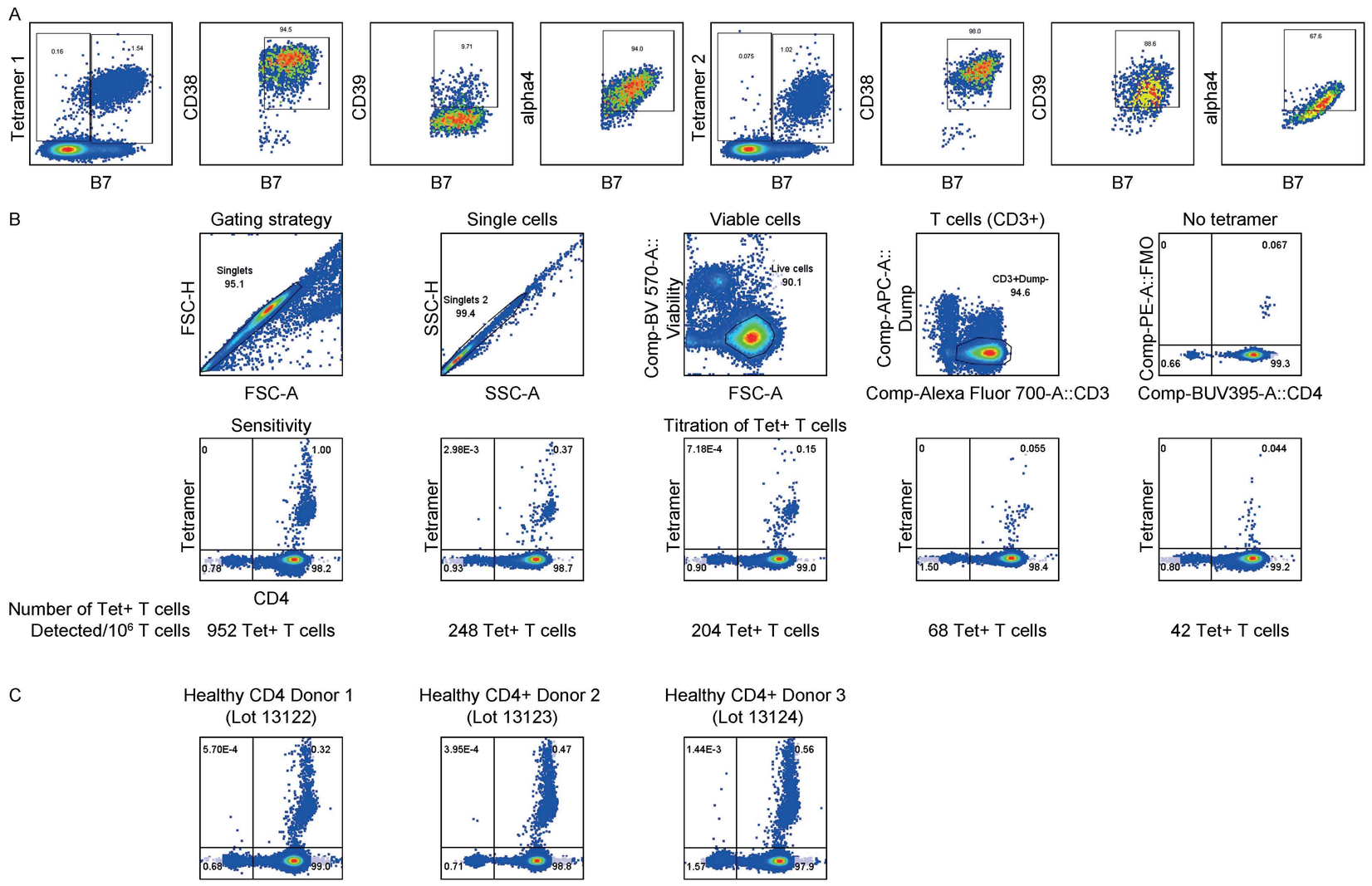
- The assay was validated using cryopreserved AccuCell™ peripheral blood mononuclear cells (PBMCs) and CD4+ T cells from healthy subjects spiked with cloned T cells isolated from subjects with celiac disease that were specific for gliadin α-I (QLQPFPQPELPY) or gliadin α-II (PQPELPYPQPE).
- The assay conditions were optimized for sensitivity, optimal signal:noise ratio and detection of the gliadin α-I/α-II tetramers. The assay identifies gluten-specific (Tet+) T cells and immunophenotypes the cells as either naïve or memory (CD4/CD3/CD45RA/CD62L/CCR7), activated (CD38), regulatory (CD39) and gut-homing (β7/α4). For validation, pre-set criteria were used to assess inter-assay, intra-assay, and inter-operator precision and post-staining stability (**Table 1**).

Table 1. Assay validation parameters with pre-set acceptance criteria

Validation parameters	Definition	Acceptance criteria
Intra-assay precision	Comparison of replicates using the same test on the same sample in the same experiment	≤30%
Inter-assay precision	Comparison of reproducibility of the same test on the same sample across separate experiments	≤40%
Intra-operator precision	Comparison of same test on the same sample by different operators	≤40%
Post-staining stability	Comparison of staining stability on the day of staining to 24 hours post-staining	≤40%

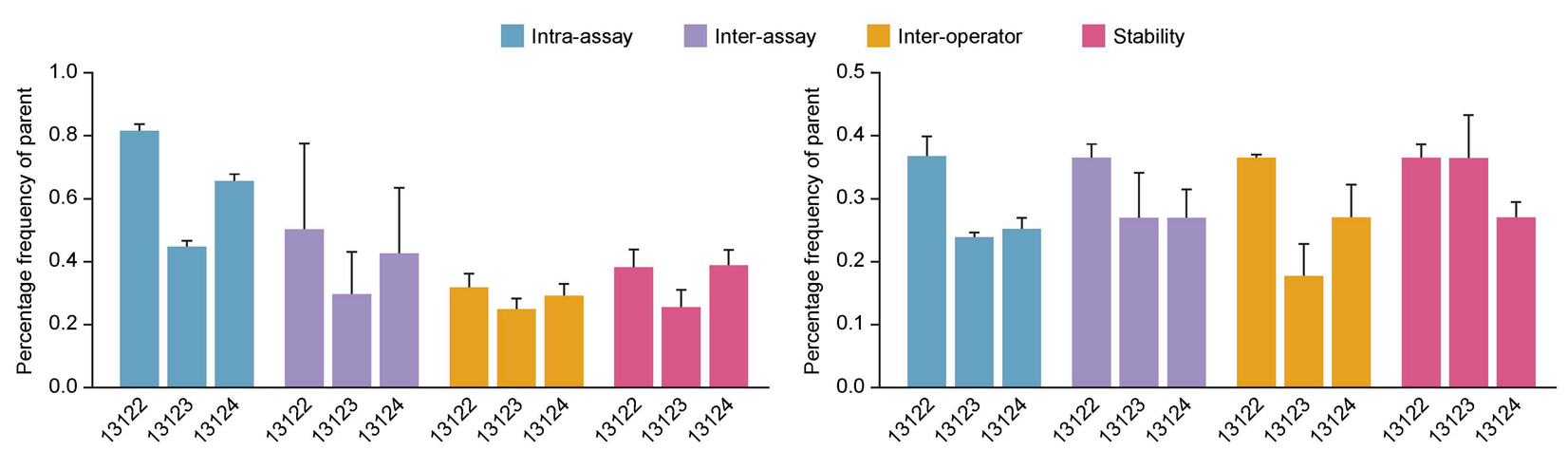
- Primary T cell clones isolated from the peripheral blood of subjects with celiac disease were used as positive controls for validation and were specific for tetramer 1 (gliadin α-I; QLQPFPQPELPY) or tetramer 2 (gliadin α-II; PQPELPYPQPE) (**Figure 2A**). Cells that bound tetramer 1 did not bind tetramer 2 and those that bound tetramer 2 did not bind tetramer 1 (data not shown). Cells were stained with the antibody panel described in **Figure 1** step 5, gated on CD4+ cells, and show staining characteristics similar to what has been described.[1,2]

Figure 2. Evaluating sensitivity of gliadin α-I/α-II tetramers. (A) Primary gluten-specific CD4+ T cell clones used for validation have a phenotype consistent with gluten-specific T cells. (B) Detection of gliadin α-II tetramers+ T cells spiked in CD4+ T cells from a subject without celiac disease. (C) CD4+ T cells from different healthy donors does not impact detection of Tet+ T cells.



Tet+, gliadin α-I/α-II tetramer positive

Figure 3. Tetramer staining is reproducible and stable. (A) The frequency of α-I tetramer+ CD4+ T cells (Gliadin 1 Clone 4) spiked into three different CD4+ T cell samples isolated from individual healthy controls (13122, 13123 and 13124). (B) The frequency of α-II tetramer+ CD4+ T cells (Gliadin 2 Clone 2) spiked into three different CD4+ T cell samples isolated from individual healthy controls (13122, 13123 and 13124).



The error bars are the %CV for the intra-assay (n=3), inter-assay (n=7) and inter-operator (n=4) precision, and 24-hour post-staining stability (n=4).
%CV, percentage coefficient of variation

Table 2. Assay criteria were met for the validation parameters

Tetramer (Tet+ T cell clone)	Healthy CD4 Donor#	Intra-assay Mean	Intra-assay %CV	Inter-assay Mean	Inter-assay %CV	Inter-operator Mean	Inter-operator %CV	Stability Mean	Stability %CV
Gliadin α-I tetramer (Gliadin 1 Clone 4)	13122	0.8	2.4	0.5	54.0	0.3	12.1	0.4	14.7
	13123	0.5	3.4	0.3	43.8	0.3	12.6	0.3	20.6
	13124	0.7	3.0	0.4	47.5	0.3	12.0	0.4	11.7
Gliadin α-II tetramer (Gliadin 2 Clone 2)	13122	0.4	8.0	0.4	5.6	0.4	0.9	0.4	5.6
	13123	0.3	2.2	0.3	24.5	0.2	27.1	0.4	17.7
	13124	0.3	6.2	0.3	15.1	0.3	18.0	0.3	8.2

%CV, percentage coefficient of variation; Tet+, gliadin α-I/α-II tetramer positive

- Titrating primary, α-II tetramer positive T cell clone (Gliadin 2 Clone 14) into CD4+ T cells from a healthy subject. Included was a No Tetramer (no tetramer + full antibody panel) control for objective gating. All other samples were stained with the full antibody panel including the gliadin α-II tetramer. One million CD4+ T cells from heathy subject were spiked with five cellular concentrations of the Tet+ T cells (bottom panel, L to R: 12.5K, 3.1K, 781, 195, 48 [not shown]) and no cells as a negative control) (**Figure 2B**).
- CD4 T cells from three different healthy controls were spiked with 3.1K of Gliadin 2 Clone 14 primary T cell clone and stained with gliadin α-II tetramer (**Figure 2C**). The cells were also stained with the gliadin α-I tetramer to demonstrate specificity of the Gliadin 2 Clone 14 primary T cells to the gliadin α-II tetramer (not shown).
- This study established the sensitivity of the tetramer staining on the three CD4 lots spiked with Gliadin 2 Clone 14 primary T cells used in the validation study.
- The data shown in **Figure 3** for the gliadin α-I and gliadin α-II tetramers are summarized in **Table 2**. The Z-score test was used to identify outliers for the inter-assay precision for G1C4. Based on the Z-score test and the inter-assay precision, the results for the CD4+ tetramer+ G1C4 T cell clone are within the validation acceptance criteria.

Conclusions

- This gliadin α-I/α-II DQ2 tetramer flow cytometric assay was developed and validated to be sensitive and selective. Technical validation of the assay was successfully met and the assay performs within the precision parameters.
- This assay will enable characterization of the gliadin α-I/α-II CD4+ T cells in the blood for celiac patients, providing a more comprehensive evaluation of response to new therapies and may reduce invasive biopsy-based measurements.

References

1. Han A *et al. Proc Natl Acad Sci USA* 2013;110:13073–8.
2. Sarna VK *et al. Gastroenterology* 2018;154:886–96.

Disclosures

GMS, WM and JE are employees of Takeda. LK, AB and DP are employees of Precision for Medicine. WK has no disclosures to report.

Acknowledgements

We would like to extend our thanks and appreciation to the teams at Takeda Pharmaceuticals, the Benaroya Research Institute and Precision for Medicine for their substantial contributions, compilation of information, and ongoing commitment to quality.

Presented at the 18th International Celiac Disease Symposium • 4–7 September 2019 • Paris, France