

Commensal Bacteria Maintain a Qa-1^b-restricted Unconventional CD8⁺ T Population in Gut Epithelium

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Abstract

Intestinal intraepithelial lymphocytes (IELs) are characterized by an unusual phenotype and developmental pathway, yet their specific ligands and functions remain largely unknown. Here by analysis of QFL T cells, a population of CD8⁺ T cells critical for monitoring the MHC I antigen processing pathway, we established that unconventional Qa-1^b-restricted CD8⁺ T cells are abundant in intestinal epithelium. We found that QFL T cells showed a Qa-1^b-dependent unconventional phenotype in the spleen and small intestine of naïve wild-type mice. The splenic QFL T cells showed innate-like functionality exemplified by rapid response to cytokines or antigen, while the gut population was refractory to stimuli. Microbiota was required for the maintenance, but not the initial gut homing of QFL T cells. Moreover, monocolonization with *Pediococcus pentosaceus*, which expresses a peptide that cross-activated QFL T cells, was sufficient to maintain QFL T cells in the intestine. Thus, microbiota is critical for shaping the Qa-1^b-restricted IEL landscape.

eLife assessment

This is an **important** study that investigates the role of commensal microbes and molecules in the antigen presentation pathway affecting the development and phenotype of an unusual population of T lymphocytes. The authors provide **compelling** evidence to identify a population of unconventional T cells that exist in the small intestinal epithelium, which appear to depend on commensal microbes, and show that a single commensal microbe (that encodes an antigen capable of weakly stimulating these cells) is sufficient to maintain this T cell population.

The display of peptides by MHC class I (MHC I) molecules on the cell surface is critical for CD8⁺ T cell immune surveillance ([Shastri et al., 2002](#)). Generation of a peptide repertoire which accurately reflects intracellular events, such as viral infection or mutations, relies on a functional antigen processing and presentation pathway. After cytosolic cleavage of protein precursors and transport of peptides through the peptide transporter associated with antigen processing (TAP) into the endoplasmic reticulum (ER), the peptide intermediates are further customized by ER aminopeptidase associated with antigen processing (ERAP1) until ‘ideal’ peptides that fit the groove of MHC I molecules are eventually shuttled and displayed on the cell surface ([Serwold et al., 2002](#); [Shastri et al., 2005](#)). Disruption of each step of the pathway can lead to immunological dysfunction ([Grande et al., 2000](#); [Van Kaer et al., 1994](#); [Van Kaer et al., 1992](#)). The critical role of ERAP1 in the antigen processing pathway has been established through extensive studies of ERAP1 deficient (ERAP1-KO) cells and mice ([Blanchard et al., 2008](#); [Guan et al., 2021](#); [Hammer et al., 2007b](#)). Loss of ERAP1 severely disrupts the peptide repertoire presented by both the classical MHC Ia and the nonclassical MHC Ib molecules ([Hammer et al., 2006](#); [Hammer et al., 2007a](#)). Qa-1^b, a nonclassical MHC Ib molecule, has been shown to present a significantly increased number of peptides on the cell surface of ERAP1-KO cells as compared with wild-type (WT) cells ([Nagarajan et al., 2016](#)). One peptide presented by Qa-1^b, FYAEATPML (FL9) (with the Qa-1^b-FL9 complex termed QFL), was identified as an immunodominant ligand uniquely presented on ERAP1-deficient cells ([Nagarajan et al., 2012](#)). The CD8⁺ T cells that specifically recognize this ligand are thus named QFL-specific T (QFL T) cells.

Early analysis of QFL T cells revealed the unusual nature of these CD8⁺ T cells. Unlike conventional antigen-specific CD8⁺ T cells which are typically detected at a frequency of 1 in 10⁵–10⁶, QFL T cells are present at a frequency 10-fold higher in the spleen of naïve mice with the bulk splenic QFL T population displaying a CD44^{hi}CD122⁺ antigen-experienced phenotype ([Nagarajan et al., 2012](#)). T cell receptor (TCR) analysis of QFL T cells revealed that a large proportion of the QFL T population expresses an invariant TCRα-chain Va3.2Ja21 ([Guan et al., 2017](#)). These traits of QFL T cells indicate their potential similarity to other unconventional T cells, such as invariant NKT (iNKT) and mucosal associated invariant T (MAIT) cells, which are typically characterized by recognition of ligands presented by non-classical MHC Ib, expression of invariant TCRs, residence in nonlymphoid tissues and innate-like functions ([Godfrey et al., 2015](#); [Salio et al., 2014](#)). The tissue distribution and functions of QFL T cells remains to be fully elucidated.

The gut mucosa is an immunologically complex niche with abundant lymphoid populations ([Faria et al., 2017](#)). The small intestinal intraepithelial lymphocyte (siIEL) compartment is populated by unconventional T cells of both TCRγδ⁺ and TCRαβ⁺ lineage which express CD8αα but lack CD4, CD8αβ, CD5 and CD90 expression. The development of the CD8αα⁺CD4[−]CD8αβ[−] TCRαβ⁺ population (CD8αα⁺ IEL), categorized as natural IELs (natIELs) because they acquire their activated phenotype in the thymus, has been extensively studied ([Cheroutre et al., 2011](#)). Yet little is known about their antigen specificity and TCR repertoire. Emerging evidence shows that nonclassical MHC Ib molecules are important for the development and effector function of CD8αα⁺ IELs ([Das et al., 2003](#)). For instance, while loss of classical MHC Ia molecules showed little impact on the CD8αα⁺ IEL population, these cells were decreased in mice deficient for Qa-2 ([Das et al., 2000](#); [Das et al., 1999](#); [Park et al., 1999](#)). Furthermore, a population of CD8αα⁺ IEL precursors which preferentially expresses Va3.2 was shown to be decreased in number in the absence of CD1d ([Ruscher et al., 2017](#)). However, whether Qa-1^b is involved in shaping the CD8αα⁺ IEL population remains to be studied. In addition to MHC molecules, gut microbiota plays a critical role in the establishment and shaping of the gut immune system. Studies of germ-free (GF) mice have shown extensive defects in gut-associated lymphoid tissues together with morphological changes in the intestine associated with the absence of gut microbiota (Round et al., 2009). Notably,

despite the critical role of gut microbiota in the establishment of the gut immune system, natIELs that do not rely on cognate antigens in the periphery are believed to home to the gut independent of microbes ([Mota-Santos et al., 1990](#)).

Here we found abundant unconventional QFL T cells in both the spleen and siIEL compartment of naïve WT mice. The splenic Va3.2⁺QFL T cells expressed high level of CD44 and showed typical innate-like functions including hyperresponsiveness to cytokines and rapid IFN- γ production in response to antigen. In contrast, the populations of the same antigen specificity in the gut phenotypically resembled natIEL and were likewise functionally quiescent. Analysis of mice deficient of molecules associated with Qa-1^b-FL9 antigen presentation revealed that TAP was required for the presence of the splenic or siIEL QFL T cells, whereas Qa-1^b was essential for imprinting their unconventional phenotype. Analysis of GF mice showed that gut microbiota was needed for the long-term maintenance of QFL T cells. Furthermore, we found that maintenance of the gut Va3.2⁺QFL T was associated with colonization by the commensal bacterium *Pediosoccus pentosaceus* (*P. pentosaceus*) which expresses an FL9 homologue that could cross-activate QFL T cells. Overall, these results establish Va3.2⁺QFL T cells as a unique population of unconventional CD8⁺ T cells that rely on nonclassical MHC Ib to acquire their phenotype, and gut microbiota to be properly maintained in the intestine.

Results

Va3.2⁺QFL T cells are abundant in the spleen and gut of naïve wild-type mice

To identify QFL T cells in tissues of naïve WT mice, we generated Qa-1^b-FL9 dextramers (QFL-Dex) based on the ‘dextran-doping’ technique ([Bethune et al., 2017](#)). Prior work of our lab showed that unlike the Qa-1^b-Qdm tetramer which stains NK cells, the Qa-1^b-FL9 tetramer (QFL-Tet) does not bind to the NKG2A receptor but specifically stains CD8⁺ T cells ([Nagarajan et al., 2012](#)). Because the same QFL monomers were used for generating the QFL-multimers in this study, we reasoned that it is unlikely that the QFL-Dex will exhibit shifted specificity. Moreover, in comparison with QFL-Tet, QFL-Dex showed improved sensitivity and specificity for QFL T cell detection (**Supplementary Fig. 1**), and further allowed enrichment of QFL T cells using magnetic beads. The average number of QFL T cells – defined as CD45⁺CD19[−]TCR β ⁺CD4[−]QFL-Dex-PE⁺APC⁺ (**Fig. 1 a**) – detected in the siIEL compartment of naïve WT mice was comparable to the population in the spleen. Additionally, QFL T cells were present at a frequency of ~1 in 1,000 of the siIEL CD8⁺ T cells, which was 10 times more frequent than in the spleen (**Fig. 1 b, c**). Both the splenic and siIEL QFL T cells were essentially all CD8⁺ T cells, as these cells were barely detectable within the CD4⁺ T population (**Supplementary Fig. 2**). Consistent with prior studies, ~80% of the splenic QFL T cells expressed the ‘invariant’ TCR Va segment Va3.2 which was significantly higher than the proportion of Va3.2-expressing cells within the total CD8⁺ T population in the spleen (<5%) (**Fig. 1 d, e**) ([Guan et al., 2017](#)). Notably, QFL T cells in the siIEL compartment also contained a relatively larger proportion of Va3.2⁺ cells (20.1%) compared to the total CD8⁺ TCR $\alpha\beta$ siIELs (<10%) (**Fig. 1 f, g**). The average percentage of Va3.2⁺ cells within the QFL T population (20.1%) was lower in the siIEL compartment than in the spleen (63.3%). Va3.2⁺ QFL T cells were nevertheless abundantly present in both the spleen and gut of naïve WT mice.

The unconventional phenotype of Va3.2⁺QFL T cells

We have previously observed a high percentage of CD44^{hi} cells within the bulk splenic QFL T population of naïve WT mice ([Guan et al., 2017](#); [Nagarajan et al., 2012](#)). Here we further investigated the association between preferred Va3.2 usage by QFL T cells and their phenotype. We observed that Va3.2⁺QFL T cells contained a significantly larger proportion of CD44^{hi} cells

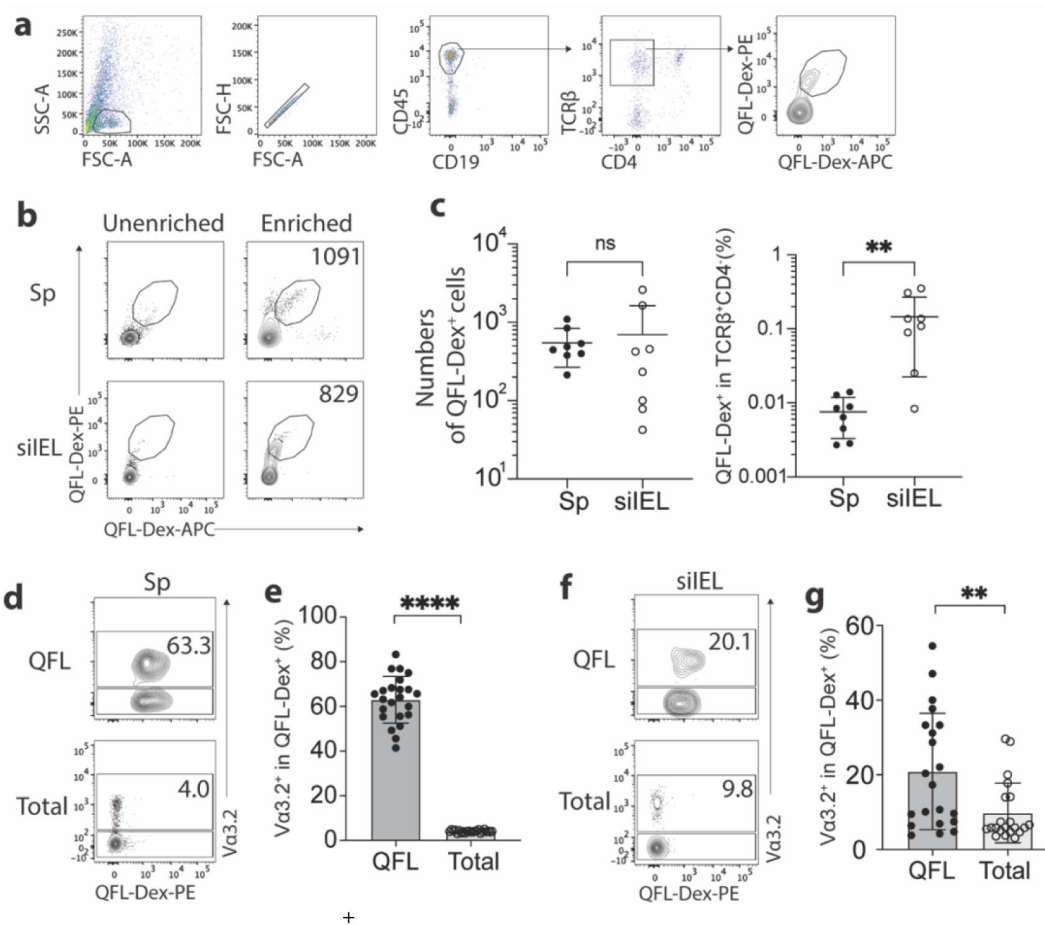


Figure 1

Abundant Va3.2⁺ QFL T cells in both the spleen and siIEL compartment of naïve WT mice.

(a) Definition of QFL T cells by flow cytometry. Cells from the spleen or siIEL compartment were stained with Qa-1^b-FL9 (QFL) dextramers labeled with phycoerythrin (QFL-Dex-PE) or allophycocyanin (QFL-Dex-APC) and analyzed for before (Unenriched) or after (Enriched) magnetic enrichment of dextramer-positive cells. QFL T cells are defined as the CD45⁺CD19⁺TCRβ⁺CD4⁻QFL-Dex-PE⁺APC⁺ population. Plots representing siIELs from naïve WT mice after enrichment for QFL-Dex⁺ cells. (b) Flow cytometry of cells from the spleen (Sp) and siIEL compartment of naïve WT mice 'Unenriched' or 'Enriched' for dextramer-positive cells. Numbers in plots indicate absolute numbers of QFL-Dex⁺ cells detected after enrichment. (c) Absolute numbers (left) and frequencies (right) of QFL-Dex⁺ cells detected (as in b) among TCRβ⁺CD4⁻ population in the spleen or siIEL compartment. **P=0.0069 (d, f) Analysis of Va3.2 expression on QFL-Dex⁺ (QFL) or total CD8⁺ T (Total) cells from the spleen (d) or the siIEL compartment (f). Numbers in plots indicate average percentages of Va3.2⁺ cells within the indicated populations. (e) Frequencies of Va3.2⁺ cells detected as in d. ****P<0.0001 (g) Frequencies of Va3.2⁺ cells detected as in f. **P=0.0058 Representative data are shown in flow plots (a, b, d, f) and the number of replicates is specified in the bar graphs (c, e, g). Each symbol represents data collected from the indicated tissue isolated from an individual mouse. Data from ≥ 5 independent experiments were pooled for statistical analysis. P values were calculated with Student's *t* test. 'ns' indicates the comparison was not significant.

(~90%) than the Va3.2⁻ QFL T cells, suggesting that the antigen experienced phenotype of the QFL T population was mainly contributed by the Va3.2-expressing subpopulation (**Fig.2 a, b**). In contrast, only around 20% of the total Va3.2⁺CD8⁺ T population expressed high CD44. It is worth noting that the percentage of CD44^{hi} cells within the total Va3.2⁺CD8⁺ T population was slightly higher compared to the Va3.2⁻ population (**Supplementary Fig. 3 a**), which was in line with a previous report (*Prasad et al., 2021*). We thus conclude that although both Va3.2⁺ and Va3.2⁻ cells were detected within the splenic QFL T population, a memory phenotype was specifically enriched in Va3.2⁺ QFL T cells.

Given the high frequency of QFL T cells in the gut, we further investigated their phenotype. We assessed CD8α expression, a hallmark of the unconventional phenotype of natIELs, on Va3.2⁺ and Va3.2⁻ QFL T cells in the siIEL compartment of naïve WT mice. A relatively larger fraction of Va3.2⁺ QFL T cells lacked CD4 and CD8αβ expression but expressed CD8α compared to the Va3.2⁻ subpopulation suggesting that the majority of Va3.2⁺ QFL T cells were CD8α⁺ natIELs (**Fig.2 c, d**). No such difference was observed between the total Va3.2⁺ and Va3.2⁻ CD8⁺ IEL populations (**Supplementary Fig. 3 b**). These observations indicate that the Va3.2⁺ QFL T cells in both the spleen and siIEL compartment are unconventional CD8⁺ T cells which likely are derived from the same precursors in thymus.

The impact of Qa-1^b, ERAP1, and TAP on Va3.2⁺ QFL T cells

We further investigated what drives the unconventional phenotype of Va3.2⁺ QFL T cells by analyzing mice deficient of molecules that impact Qa-1^b-FL9 antigen presentation including Qa-1^b, ERAP1 or TAP (Qa-1^b-KO, ERAP1-KO, TAP-KO). While we consistently observed loss of both the splenic and siIEL Va3.2⁺ QFL T cells in TAP-KO mice, the populations were detectable in both tissues of Qa-1^b-KO or ERAP1-KO mice with a slightly reduced population size in the spleen of Qa-1^b-KO mice (**Fig.3 a**). Va3.2⁺ QFL T populations showed reduced percentages of CD44^{hi} cells in the spleen of Qa-1^b and ERAP1 deficient mice with the reduction being more significant in Qa-1^b-KO mice. In contrast, the phenotype of splenic Va3.2⁻ QFL T cells was not significantly affected (**Fig.3 b, c**). Similar pattern of phenotype change was observed for the siIEL Va3.2⁺ QFL T cells which showed almost complete loss of the unconventional natIEL phenotype including CD8α expression in Qa-1^b-KO mice (**Fig.3 d, e**, **Supplementary Fig. 4**). These results demonstrate that TAP and Qa-1^b both play a role in the establishment of the unconventional Va3.2⁺ QFL T population, with TAP being required for the presence of the population, and Qa-1^b being required for the unconventional phenotype imprinting of these cells. Paradoxically, loss of ERAP1, which would be expected to increase the presentation of FL9, also led to a reduction of memory and unconventional phenotypes in QFL T cells, perhaps due to the loss of certain QFL T clones as a result of tolerance mechanisms, such as negative selection. It is also worth noting that total splenic Va3.2⁺CD8αβ⁺ T cells showed a similar loss of memory phenotype in Qa-1^b-KO and ERAP1-KO mice, whereas Va3.2⁻CD8αβ⁺ T cells did not (**Supplementary Fig. 3 c**). Thus, the unconventional Va3.2⁺ QFL T cells specifically, and a substantial proportion of total Va3.2⁺ splenocytes, are impacted by Qa-1^b and ERAP1.

Va3.2⁺ QFL T cells are functionally innate-like in the spleen but are quiescent in the gut

To further investigate the functional features of Va3.2⁺ QFL T cells, we utilized transgenic mice (QFLTg) expressing the predominant invariant TCR found on QFL T cells (Va3.2Ja21, Vβ1Dβ1Jβ2-7), referred to hereafter as QFLTg cells. Abundant QFLTg cells or control OT-1 cells were detected in both the spleen and siIEL compartment of the respective TCR transgenic mice (**Supplementary Fig. 5 a**). Phenotypically, the QFLTg cells recapitulated the unconventional phenotype of polyclonal Va3.2⁺ QFL T cells with high CD44 expression in the spleen and CD8α expression in the siIEL

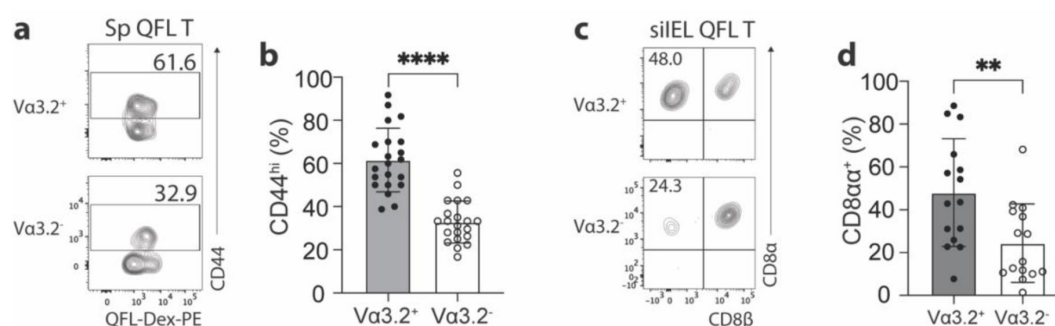


Figure 2

The unconventional phenotype of Va3.2⁺ QFL T cells.

(a) Analysis of CD44 expression on Va3.2⁺ or Va3.2⁻ QFL-Dex⁺ cells enriched from the spleen of naïve WT mice. Numbers in plots indicate average percentages of CD44^{hi} cells detected among the Va3.2⁺ or Va3.2⁻ QFL-Dex⁺ populations. (b) Frequencies of CD44^{hi} cells detected as in a. ****P<0.0001 (c) Analysis of CD8α and CD8β expression on Va3.2⁺ or Va3.2⁻ QFL-Dex⁺ cells enriched from the siEL compartment of naïve WT mice. Numbers in plots indicate average percentages of CD8α⁺ cells detected among the Va3.2⁺ or Va3.2⁻ QFL-Dex⁺ populations. (d) Frequencies of CD8α⁺ cells detected as in c. **P=0.0065 Samples of <20 cells in the Va3.2⁺QFL-Dex⁺ gate were excluded in the phenotype analysis. Representative data are shown in flow plots (a, c) and the number of replicates is specified in the bar graphs (b, d). Each symbol represents data collected from the indicated tissue isolated from an individual mouse. Data from ≥11 independent experiments were pooled for statistical analysis. P values were calculated with Student's *t* test. 'ns' indicates the comparison was not significant.

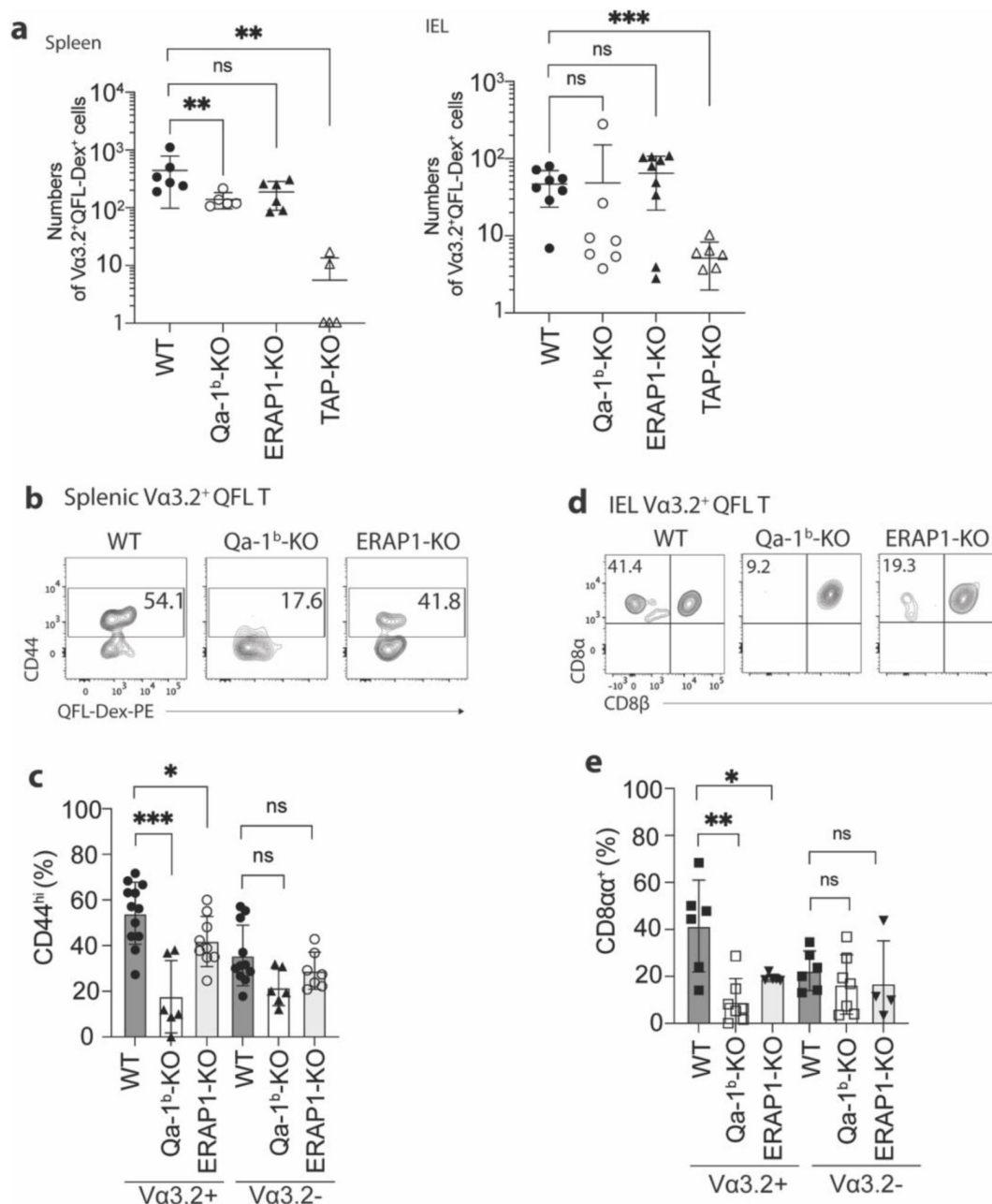


Figure 3

Phenotype of Va3.2⁺ QFL T cells in mice of various genotypes.

(a) Absolute numbers of Va3.2⁺QFL-Dex⁺ cells detected in the spleen (left) or siIEL compartment (right) of Qa-1^b, ERAP1 or TAP deficient (Qa-1^b-KO, ERAP1-KO or TAP-KO) mice in comparison with WT mice. ** P<0.009 ***P=0.0006 Symbols on x-axis indicate that the cells were undetectable in TAP-KO mice. (b) Analysis of CD44 expression on splenic Va3.2⁺QFL-Dex⁺ cells enriched from naïve WT, Qa-1^b-KO or ERAP1-KO mice. Numbers in plots indicate average percentages of CD44^{hi} cells. (c) Frequencies of CD44^{hi} cells detected among Va3.2⁺(as in b) or Va3.2⁻ QFL T cells. ***P=0.0004 *P=0.0388 (d) Flow cytometry analysis of CD8α and CD8β expression on the Va3.2⁺QFL-Dex⁺ cells enriched from the siIEL compartment of naïve WT, Qa-1^b-KO or ERAP1-KO mice. Numbers in plots indicate average percentages of CD8αα⁺ cells. (e) Percentages of CD8αα⁺ cells detected among Va3.2⁺(as in d) or Va3.2⁻ QFL T cells. **P=0.0082 *P=0.0335 Representative data are shown in flow plots (b, d) and the number of replicates is specified in the bar graphs (a, c, e). Each symbol represents data collected from the indicated tissue isolated from an individual mouse. Data from ≥4 independent experiments were pooled for statistical analysis. P values were calculated with Student's *t* test. 'ns' indicates the comparison was not significant.

compartment. In contrast, OT-1 cells were phenotypically similar to conventional naïve CD8⁺ T cells, as they expressed low to intermediate levels of CD44 in the spleen and lacked CD8αα expression in gut (**Supplementary Fig. 5 b, c**).

Cytokines including IL-15 and the combination of IL-7/IL-18 have been implicated in the maintenance and survival of both ‘virtual’ memory T cells and IELs ([Klose et al., 2014](#); [Okazawa et al., 2004](#); [Prasad et al., 2021](#); [Schluns et al., 2003](#)). We thus compared the proliferation of QFLTg and OT-1 cells in response to these cytokines. In keeping with their virtual memory surface marker phenotype, splenic QFLTg cells were significantly more responsive to IL-15 or IL-7/IL-18 stimulation than OT-1 cells (**Fig.4 a**). At 72hs, ~80% of QFLTg cells had proliferated in response to the cytokines, compared with only ~50% of OT-1 cells (**Fig.4 b**). In contrast, T cells isolated from the siIEL compartment of QFLTg or OT-1 mice showed a distinct pattern of cytokine responsiveness from the splenic T cells. First, both QFLTg and OT-1 cells from siIEL were less responsive to IL-15 than the splenic population. Second, siIEL QFLTg cells showed significantly reduced proliferation in response to the cytokines, especially to the combination of IL-7/IL-18, as compared with OT-1 cells (**Fig.4 c, d**).

To ensure that altered effector function was not skewed by the high specific TCR frequency in TCR transgenics, we generated partial hematopoietic chimeric mice with physiological precursor frequencies (QFLTg_WT) by transferring bone marrow cells from GFP⁺QFLTg mice into nonirradiated naïve WT neonates ([Ladi et al., 2008](#)). OT-1_WT chimera mice were generated in parallel as representative of conventional CD8⁺ T cells (**Fig.4 e**). This experimental design allowed us to track the GFP⁺ donor-derived T cells (GFP⁺CD45⁺CD19⁺TCRβ⁺CD4⁺Vα3.2⁺) separately from the recipient’s endogenous T cells (**Fig.4 f**). Based on the hyperresponsiveness of splenic QFLTg cells to cytokines, we hypothesized that these cells would exert effector functions more rapidly than conventional CD8 T cells, similar to the known innate-like T cells such as iNKT. We measured IFN-γ production by QFLTg or OT-1 cells from splenocytes or siIEL cells of naïve QFLTg_WT or OT-1_WT chimera mice stimulated with 2μM FL9 or SIINFEKL(SL8) peptide respectively for 4.5 hours directly *ex vivo*. A large fraction (~60%) of splenic QFLTg cells expressed high levels of IFN-γ under these conditions, while only 6% of OT-1 cells did so (**Fig.4 g, h**). Notably, despite the low responsiveness to epitopes, siIEL QFLTg cells showed relatively higher basal levels of IFN-γ expression than OT-1 cells (**Fig.4 i, j**). In contrast to the hyperresponsiveness of splenic QFLTg cells to their ligand, neither QFLTg nor OT-1 cells from the siIEL compartment showed significant IFN-γ production at 4.5 hours. The low IFN-γ production of IEL QFLTg cells was in keeping with their natIEL phenotype - antigen-experienced yet functionally quiescent ([Cheroutre et al., 2011](#); [Denning et al., 2007](#)). We conclude that Vα3.2⁺QFL T cells in the spleen are functionally innate-like, whereas the population with the same TCR specificity in the small intestine shows functional features of natIEL.

Gut microbiota is associated with retention but not homing of QFL T cells

The establishment and function of the intestinal immune system is intimately associated with homeostasis of the gut microbial community, as evidenced by an altered gut immune cell composition in germ-free (GF) mice ([Belkaid et al., 2014](#); [Macpherson et al., 2004](#); [Round and Mazmanian, 2009](#)). To investigate if an association exists between gut microbiota and gut QFL T cells, we compared the numbers and frequencies of QFL T cells in the spleen and siIEL compartment of naïve specific-pathogen-free (SPF) and GF WT mice of various ages. First, we found the absolute number of QFL T cells in the spleen was unaffected by the absence of gut microbiota in either young or old mice. The frequency of QFL T cells was relatively higher in GF than in SPF mice, as a result of a decreased number of non-QFL CD8⁺ T cells in the spleen of GF mice (**Fig.5 a, b**). However, the percentage of CD44^{hi} cells within the total CD8⁺ T population was proportionally higher in the spleen of GF WT than in SPF WT mice regardless of their Vα3.2 expression (**Supplementary Fig. 6 a**). This observation suggested that gut microbiota might be

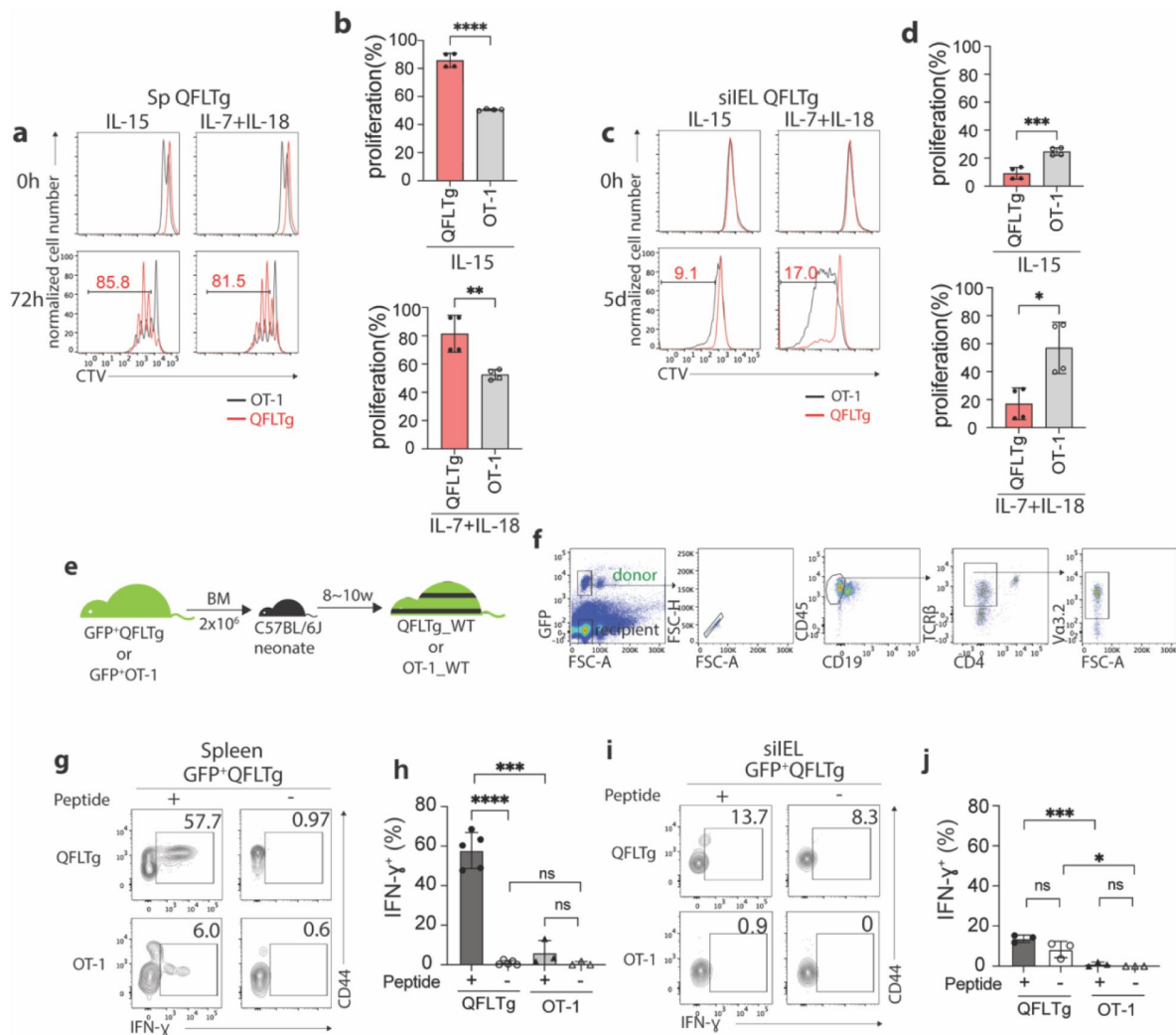


Figure 4

Distinct functional features of QFLTg cells in the spleen and gut.

(a, c) Proliferation of splenic (a) or siIEL (c) QFLTg or OT-1 cells from QFLTg or OT-1 mice in response to IL-15 (left) or a combination of IL-7 and IL-18 (right) stimulation. Cells were tracked using Cell Tracing Violate (CTV). Numbers in plots indicate average percentages of proliferated QFLTg cells. (b) Percentages of proliferated splenic QFLTg or OT-1 cells as detected in a. ****P<0.0001 **P=0.0052 (d) Percentages of proliferated siIEL QFLTg or OT-1 as detected in c. ***P=0.0009 *P=0.0101 (e) Generation of QFLTg_WT or OT-1_WT partial hematopoietic chimera mice. 2×10^6 of the bone marrow (BM) cells from GFP⁺QFLTg or GFP⁺OT-1 mice were transferred into WT neonates at 3-5 days of age. The chimera mice were analyzed at 8-10 weeks of age. (f) Gating strategy for the QFLTg cells originated from donor bone marrow cells in QFLTg_WT chimera mice. Plots representing donor-derived QFLTg population in the spleen being gated as GFP⁺CD45⁺CD19⁺TCRβ⁺CD4⁺Vα3.2⁺ cells. (g, i) Flow cytometry measurement of IFN-γ production by QFLTg or OT-1 cells isolated from the spleen (g) or siIEL compartment (i) of naïve chimera mice stimulated with or without 2μM FL9 or SL8 peptide respectively for 4.5h. Numbers in plots indicate average percentages of IFN-γ⁺ cells. (h) Percentages of IFN-γ⁺ cells detected as in g. ****P<0.0001 ***P=0.0001 (j) Percentages of IFN-γ⁺ cells detected as in i. ***P=0.0004 *P=0.0247 Representative data are shown in flow plots (a, c, f, g, i) and the number of replicates is specified in the bar graphs (b, d, h, j). Data from 2(b, d) or 3(h, j) independent experiments were pooled for statistical analysis. Each symbol represents data collected from the indicated tissue isolated from an individual mouse. P values were calculated with Student's *t* test. 'ns' indicates the comparison was not significant.

more closely associated with the presence of conventional naïve CD8⁺ T cells rather than the memory phenotype CD8⁺ T population in the spleen. Second, while the number and frequency of QFL T cells in siIEL compartment showed no significant difference between GF and SPF mice of relatively young age (8~17-week), the population was gradually lost in old GF mice (18~22-week) (**Fig.5 c, d**), with the decrease in GF mice starting from around 16 weeks of age (data not shown). In addition, the QFL T cells detected in the spleen or siIEL compartment of young SPF or GF mice showed similar expressions of Va3.2, CD44 and CD8αα (**Supplementary Fig. 7**). We conclude that although QFL T cells could home to gut in the absence of microbiota, microbe-derived antigens or signals are required for their maintenance there. Additionally, we observed that the average percentage of CD8αα⁺ cells among the total TCRβ⁺CD4⁺ T population was proportionally larger in GF mice than SPF mice regardless of Va3.2 expression, although the Va3.2⁺ population showed a more significant increase (**Supplementary Fig. 6 b**). This was mainly a result of a decreased number of CD8αβ⁺ T cells in the siIEL of GF mice (data not shown). Nevertheless, here we showed that Qa-1^b restricted T cells could home to the small intestine epithelium and display a natiEL phenotype independent of gut microbiota.

QFL T cells cross-react with a microbial antigen

We next investigated how the QFL T population was retained in the gut of SPF WT mice, hypothesizing that the presence of specific antigen might play a role. QFL T cells specifically recognize the Qa-1^b-FL9 complex which is uniquely presented on ERAP1 deficient cells. Because it is unlikely this ligand is constantly presented in gut epithelium under homeostatic conditions (**Supplementary Fig. 8 a**), we reasoned that QFL T cells might be retained in the gut through exposure to FL9 homologue peptide(s) expressed by commensal bacteria that colonize small intestine.

To identify potentially cross-reactive peptides, we first determined the key residues of the FYAEATPML(FL9) peptide that could potentially affect activation of QFL T cells. Due to the lack of structural information on QFL-TCR:FL9:Qa-1^b ternary complex, we started by predicting the structure using AlphaFold2 ([Mirdita et al., 2022](#)). The sequences of the α1 and α2 domain of Qa-1^b α-chain, FL9 and Va3.2Ja21, Vβ1Dβ1Jβ2-7 were submitted as input. Most of the residues in the predicted structure have high to intermediate confidence scores as was measured by the predicted local distance difference test (pLDDT). The output structure model of the top ranked pLDDT score ('rank_1' = 90.8) was selected for further visualization (**Supplementary Fig. 8 b**). The simulated QFL-TCR:FL9:Qa-1^b complex model suggests a similar TCR docking as observed in TCRαβ complexed with classical MHC I (**Fig.6 a**). While we fully appreciated the limitations in accurately predicting TCR docking, prediction of the pMHC complex is nevertheless relatively reliable based on the well-defined principles ([Nielsen et al., 2020](#)). Superimposition of simulated FL9:Qa-1^b model onto the crystallographic structure of Qdm:Qa-1^b (PDB 3VJ6) suggested similar main chain conformations between FL9 and Qdm (**Fig.6 b**). We thus refer to the reported structure of Qdm:Qa-1^b whose anchoring residues were P2, P3, P6, P7 and P9 of AMAPRTLTL(Qdm) for determination of putative key residues on FL9 ([Zeng et al., 2012](#)). Among the rest of the non-anchoring residues, we hypothesized that the glutamic acid at P4 of FL9 may be critical for interacting with the QFL TCR. Notably, the importance of P4 for TCR interaction was also highlighted in recently published studies where crystal structures of several disease-related TCR:pMHC I complexes were reported including an αβTCR complexed with an HIV peptide loaded in HLA-B5301 ([Li et al., 2023](#)) and a pre-TCR complexed with vesicular stomatitis virus octapeptide(VSV8) loaded in H-2K^b ([Li et al., 2021](#)). To test the hypothesis, we used the QFL T hybridoma cell line BEko8Z which expresses TCR-induced β-galactosidase (LacZ) to test whether the mutant FL9 peptide with the P4 residue substituted by alanine altered T cell responses. The P6 threonine whose side chain basically bends towards to the binding groove with relatively limited access to TCR engagement was tested in parallel as a putative anchoring residue. By measuring the LacZ production of BEko8Z cells in response to Qa-1^b-expressing Lmtk⁻ (L-Qa-1^b) cells pulsed with FYAAATPML (FL9-P4A) or FYAEAAPML (FL9-P6A), we found that in agreement with the prediction,

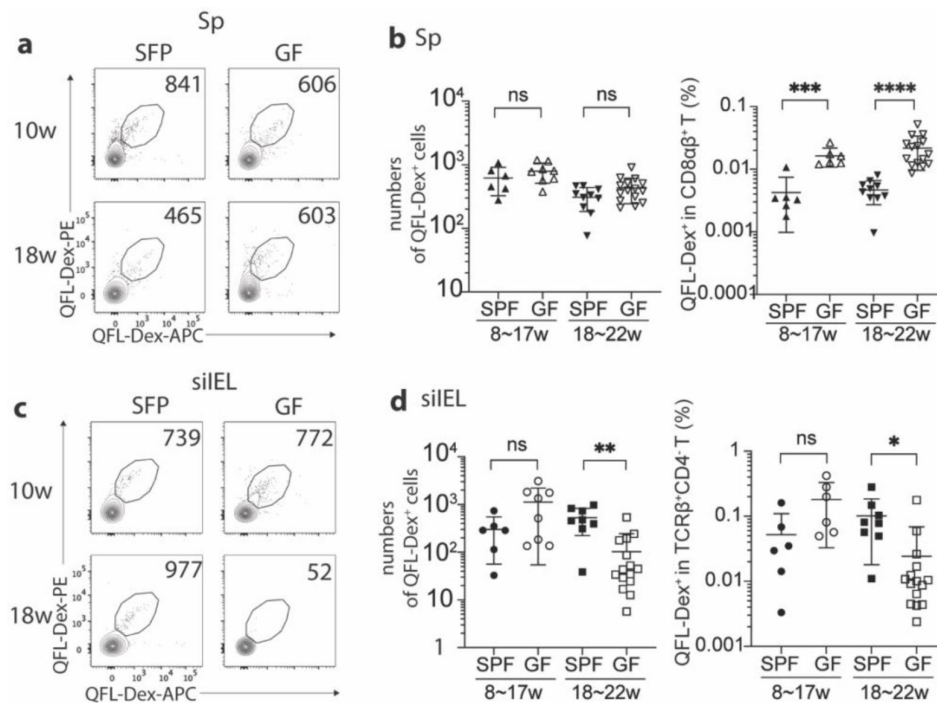


Figure 5

Gut microbiota is associated with retention but not homing of QFL T cells in the siIEL compartment.

(a, c) Flow cytometry of QFL-Dex⁺ cells enriched from the spleen (a) or siIEL compartment (c) of specific-pathogen-free (SPF) or germ-free (GF) WT mice at 10 or 18 weeks of age. Numbers in plots indicate absolute numbers of QFL-Dex⁺ cells. (b) Absolute numbers (left) and frequencies (right) of QFL-Dex⁺ cells detected within the total splenic CD8⁺ population in SPF or GF WT mice of 8~17 weeks or 18~22 weeks of age. ***P=0.009 ****P<0.0001 (d) Absolute numbers and frequencies of QFL-Dex⁺ cells detected among the total siIEL TCRβ⁺CD4⁺ population in SPF or GF WT mice of 8~17 weeks or 18~22 weeks of age. **P=0.005 *P=0.037 Representative data are shown in flow plots (a, c) and the number of replicates is specified in the bar graphs (b, d). Each symbol represents data collected from the indicated tissue isolated from an individual mouse. Data from ≥5 independent experiments were pooled for statistical analysis. P values were calculated with Student's *t* test. 'ns' indicates the comparison was not significant.

replacement of the P6 threonine reduced TCR recognition, while replacement of the P4 glutamic acid led to the complete loss of the BEko8Z response (**Fig. 6c**). Additionally, we performed Qa-1^b binding predictions for various FL9 mutant peptides using NetMHCpan4.1 ([Reynisson et al., 2020](#)). The predicted binding scores suggested that substitution of the P9 Leu with Ala should decrease binding of FL9 peptide to Qa-1^b, whereas mutation of the rest of the residues were less likely to have such effect. Hence, Leu at P9 position may serve as a key anchoring residue (**Supplementary Fig. 8 c**). Thus, P4, P6 and C-terminus positions were considered the key residues with the P4 Glu being particularly critical for QFL T recognition. Based on these data, the presence of glutamic acid at P4 was considered to be important, while the C-terminus leucine and P6 threonine were considered secondary. Although it is possible that the residues that were not tested in the T cell assays are involved in peptide binding or TCR interaction, data on the P4, P6 and P9 residues of FL9 peptide were sufficient for determining peptide homology and establishing a testable library of bacterial FL9 homologs.

To identify potential bacterial FL9 homologous peptides, we aligned the FL9 peptide sequence with proteomes of the five dominant gut commensal bacterial phyla, including Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, Verrucomicrobia from the NCBI Swissprot Non-redundant UniProtKB database ([Donaldson et al., 2016](#)). The alignment result was further curated to 30 candidate peptides based on their homology with FL9 peptide as described above (**Fig. 8 d, Supplementary Table.1**). By measuring LacZ production of BEko8Z cells in response to L-Qa-1^b cells pulsed with 50nM of each candidate peptide, we identified a single stimulatory peptide FYAEDGTPIL (FL10) that is expressed in *P. pentosaceus*, a gram-positive lactic acid bacteria (LAB) that colonizes in the small intestine (**Fig. 6 e**). While sensitivity to FL10 was ~1,000 folds less than FL9 peptide, BEko8Z cells could recognize FL10 at concentrations as low as 1.5nM. In contrast, FYAEDDTPIV (FV10), a FL10 homologous peptide expressed in *Lactobacillus johnsonii* which is a common LAB that colonize GI tract, failed to activate BEko8Z cells (**Fig. 6 f**, **Supplementary Fig. 8 d**).

QFL T cells are retained in gut by a commensal bacterium

To further investigate the association between *P. pentosaceus* and Va3.2⁺QFL T cells in gut, we first generated QFLTg_WT partial hematopoietic chimeras using germ-free WT mice (GF QFLTg_WT) and monocolonized the GF chimera mice with *P. pentosaceus* at 8 weeks of age. The mice were analyzed 8 weeks later (**Fig. 7 a**). The donor-derived QFLTg cells and the recipient-derived Va3.2⁺QFL T cells were distinguished by GFP expression and further defined as the CD45⁺CD19⁻TCRβ⁺CD4⁻Va3.2⁺QFL-Dex-PE⁺ population on flow cytometry as was described earlier. While the donor-derived QFLTg populations were present and phenotypically unaltered in the spleen and siIEL compartment of GF QFLTg_WT chimera mice at 8 weeks of age, they were barely detectable in the GF chimera mice at 16 weeks of age (**Supplementary Fig. 9, Fig. 7 b**). Notably, monocolonization of the GF QFLTg_WT chimera mice with *P. pentosaceus* completely restored the donor-derived QFLTg population in both tissues in 16-week GF chimera mice (**Fig. 7 b**). We then further tested whether restoration of the gut QFL T population was specifically associated with *P. pentosaceus* by monocolonizing GF WT mice with *P. pentosaceus* or *L. johnsonii* (**Fig. 7 a**). Analysis of the microbiome composition of fecal pellets showed that both species efficiently colonized the GF mice (**Fig. 7 c**). While the endogenous Va3.2⁺QFL T population in the siIEL compartment was retained by *P. pentosaceus* colonization of GF mice at 16 weeks of age, *L. johnsonii* colonization failed to restore the population to level comparable with SPF or *P. pentosaceus* colonized mice. On the other hand, maintenance of the endogenous splenic QFL T population was independent of microbes, in line with our earlier observations (**Fig. 7 d**). Donor-derived QFLTg and endogenous Va3.2⁺QFL T cells in the spleen likely showed discrepant results due to different sources of precursors for the two populations. Unlike the endogenous populations which were constantly replenished, the donor-derived QFLTg cells rose from a fixed number of bone marrow cells, and gradually decreased in number as the animal age (**Supplementary Fig. 10**). Thus, presence of the ligand which was supplied by peptide derived from *P. pentosaceus* might be required for the donor-derived QFLTg cells to be retained in the spleen, whereas the

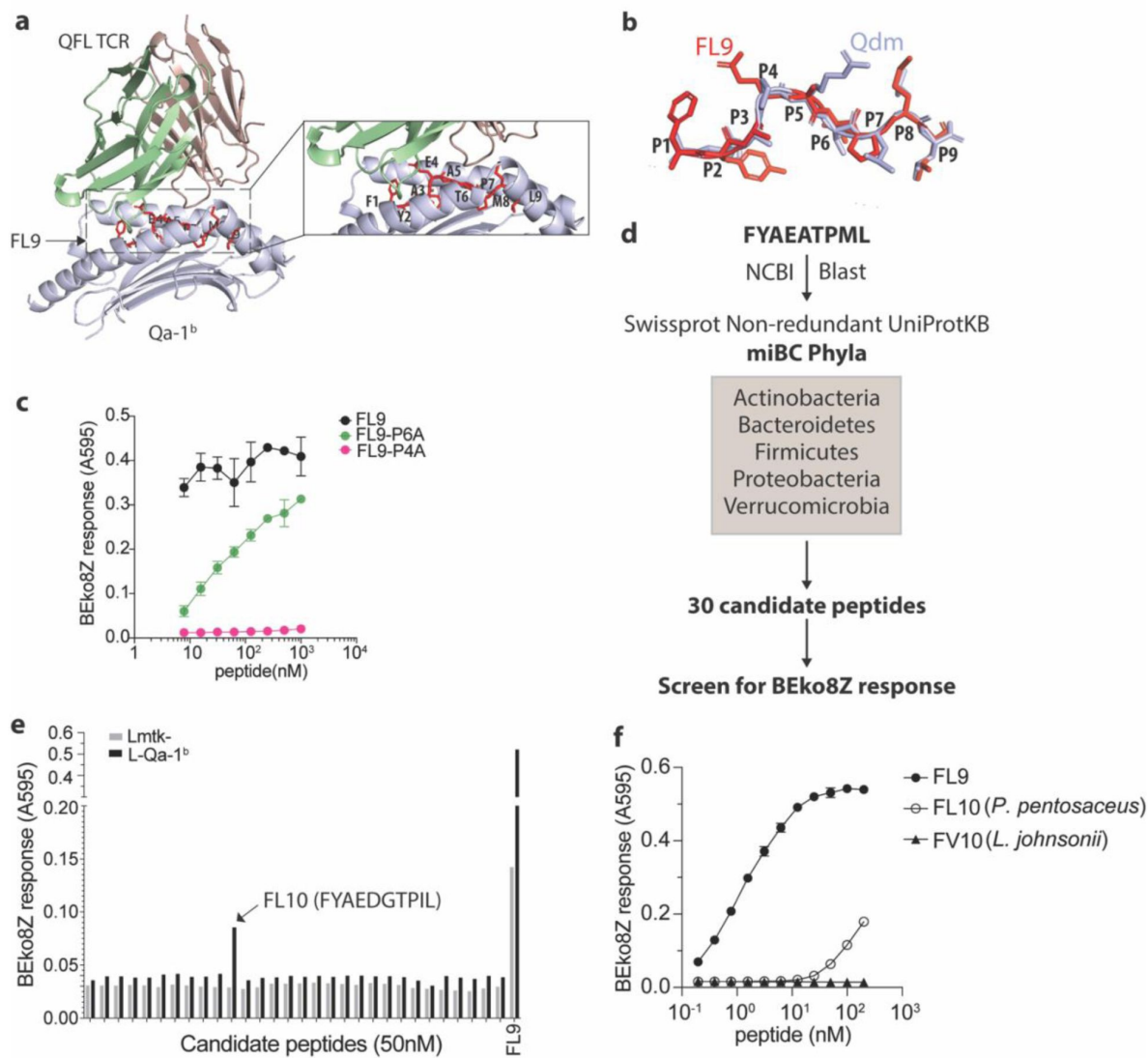


Figure 6

Identification of a QFL T cross-reactive FL9 homolog peptide expressed in commensal bacterium *P. pentosaceus*.

(a) Structure model of the QFL TCR:FL9:Qa-1^b complex predicted by AlphaFold2. Detailed view of the binding groove is shown in the right panel. (b) Conformation alignment of FL9 (red) and Qdm (periwinkle). (c) Alanine screen of the predicted key residues (P4 and P6) in FL9. Activation of QFL T cells was assessed by measuring the LacZ response of BEko8Z hybridoma cells to the Qa-1^b-expressing Lmtk⁻ (L-Qa-1^b) cells pulsed with mutant FL9 peptides including FYAATPML (FL9-P6A) and FYAAATPML (FL9-P4A) or the wild-type FYAATPML (FL9) using spectrophotometry. (d) Workflow for identification of commensal bacterial FL9 homolog peptide which cross-activate QFL T cells. (e) Identification of the QFL T cross-reactive peptide FYAEDGTPIL (FL10). BEko8Z response to the L-Qa-1^b cells or the non-Qa-1^b-expressing Lmtk⁻ cells pulsed with 50nM of the FL9 or the candidate homolog peptides. (f) Response of BEko8Z hybridoma to L-Qa-1^b cells pulsed with FL9, FL10 or FYAEDTPIV (FV10) peptide of various concentrations. (c, e, f) Data are from one experiment representative of three independent experiments.

endogenous splenic population was unaffected by microbes. Phenotype analysis revealed that both the donor-derived QFLtg cells and the endogenous Va3.2⁺QFL T cells from the colonized GF chimera mice showed a memory phenotype identical to that seen in the SPF chimera mice (**Supplementary Fig. 11**). We thus conclude that the late-life maintenance of Va3.2⁺QFL T cells in the small intestine was dependent on the commensal bacterium *P. pentosaceus* which expresses a FL9 homologue peptide. While the presence of the bacterial FL10 peptide likely plays an important role in retention of gut QFL T cells, we do not exclude the possible contributions of other commensal bacterial factors. In addition, the unconventional phenotype of QFL T cells was likely acquired through microbe-independent mechanisms.

Discussion

The small intestine epithelium harbors a highly heterogeneous intraepithelial lymphocyte population which is comprised of a large proportion of CD4⁺CD8αβ⁺CD8αα⁺ T cells. Due to the heterogeneity, it has been challenging to identify or study a particular antigen-specific T cell clone from the population. Here we found that QFL T cells, a Va3.2-expressing CD8⁺ T cell population which specifically recognizes the Qa-1^b-FL9 ligand presented on ERAP1-KO cells, naturally resided in both the spleen and small intestine epithelium of naïve WT mice. Further characterization of QFL T cells revealed their unconventional phenotype, functionality and intimate association with gut microbiota (**Table 1**).

Unlike conventional memory T cells which are generated upon encounter with their cognate peptide antigens on classical MHC molecules, ‘innate’ memory T cells arise under homeostatic conditions in response to neonatal lymphopenia, high levels of IL-4 or exposure to self-antigens in naïve mice ([Jameson et al., 2015](#); [Sprent et al., 2011](#)). Here, we found that splenic Va3.2⁺QFL T cells display a memory phenotype and the corresponding rapid effector function, but acquire this phenotype in a Qa-1^b dependent manner. Given the unique dependence on Qa-1^b expression for the phenotype imprinting of QFL T cells, it is possible that QFL T cells are exposed to transiently induced QFL epitope on cells due to various intracellular stressors that might affect ERAP1 function. Alternatively, QFL T cells might cross-react with Qa-1^b presented FL9 homologue peptide(s). Indeed, we found that Va3.2⁺QFL T cells cross-reacted with one such variant of FL9 peptide (FL10) expressed in a commensal bacterium and presented by Qa-1^b. However, the Qa-1^b-FL10 ligand itself is unlikely to be directly associated with the memory imprinting for QFL T cells as these cells showed unaltered phenotype in germ-free mice. There might be other unidentified self-peptide(s) presented by Qa-1^b that are involved in the process. It is also possible that there is some level of degeneracy in the TCR-Qa-1^b interaction, so the precise peptide being presented is less critical than the presence of any peptide-Qa-1^b complex. It is worth noting that the predicted structure of QFL-TCR:FL9:Qa-1^b complex indicated that the FL9 peptide binds to Qa-1^b in a manner potentially similar to Qdm. Because the binding groove of Qa-1^b and its human homolog HLA-E display remarkable structural homology ([Zeng et al., 2012](#)), it is possible that HLA-E can present FL9 or its homologue peptides and further support the establishment of a QFL T-like CD8 T population. Studies on cytomegalovirus-vectored vaccines have shown that HLA-E-restricted CD8 T cells could recognize a broad repertoire of peptides presented upon vaccination and provide protection against HIV-1 infection ([Hansen et al., 2016](#); [Yang et al., 2021](#)). Thus, analysis of the HLA-E peptide repertoire and investigation on the presence of HLA-E-restricted human version of QFL T cells will be promising and meaningful future directions.

In line with a previous study, we observed that the Va3.2-expressing CD8αβ⁺ T population was comprised of a relatively larger proportion of memory phenotype cells in the spleen than the non-Va3.2 expressing population ([Prasad et al., 2021](#)). In addition, these memory phenotype non-QFL Va3.2⁺CD8αβ⁺ T cells were lost in Qa-1^b-KO mice. These results indicate that Va3.2⁺QFL T cells are likely only one prototypical example of Qa-1^b restricted Va3.2⁺CD8⁺ T cells clones with memory phenotype and innate-like function. We detected abundant Va3.2⁺QFL T cells in small

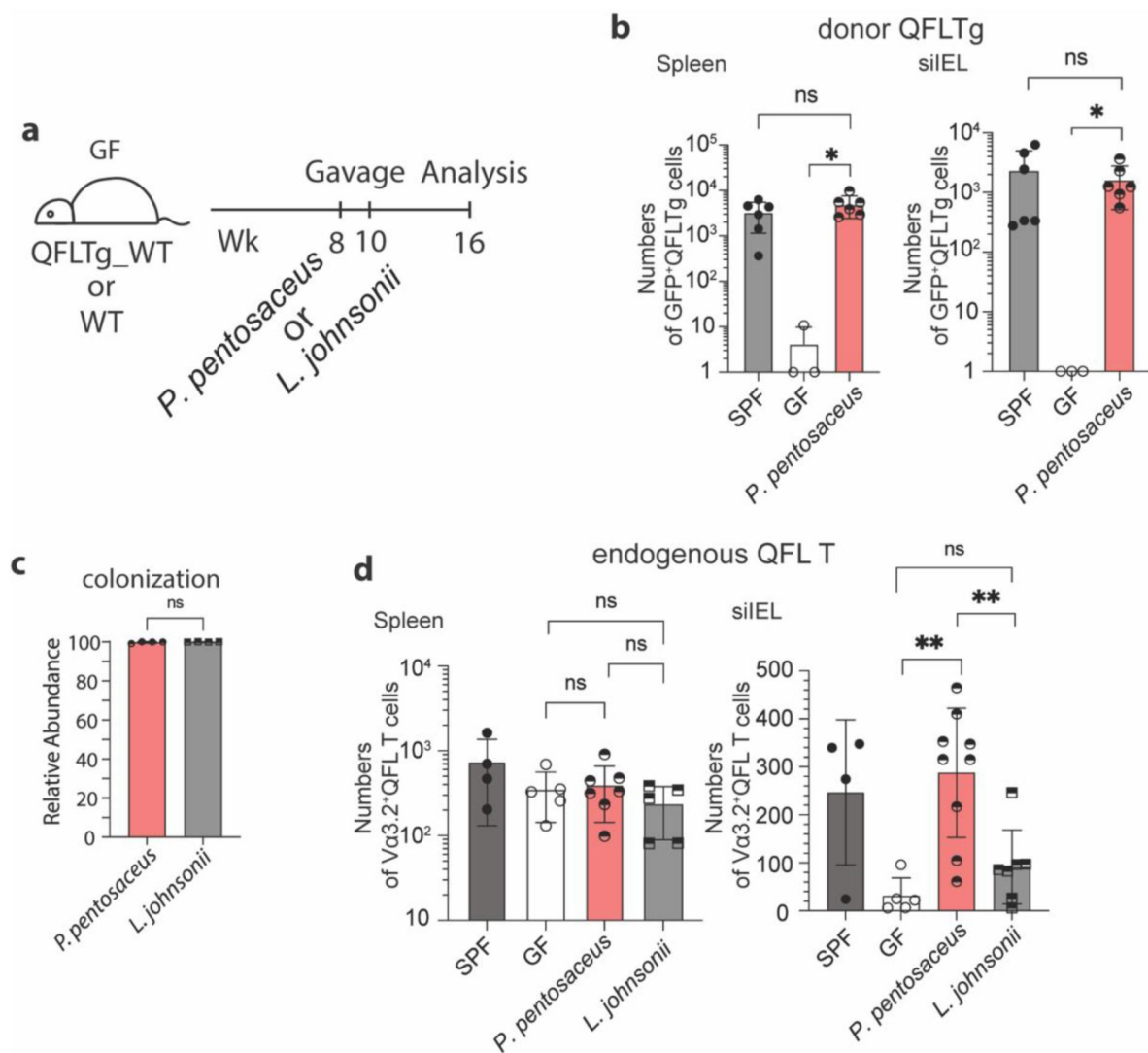


Figure 7

Association between Va3.2⁺QFL T cells and the commensal bacterium *P. pentosaceus*.

(a) Colonization of GF mice with commensal bacteria. GF QFLTg_WT chimera mice received oral gavage of *P. pentosaceus* at 8 weeks of age and were analyzed for both the donor-derived QFLTg cells and the endogenous Va3.2⁺QFL T cells at 16 weeks of age. GF WT mice received oral gavage of *P. pentosaceus* or *L. johnsonii* at 8 and 10 weeks of age and were analyzed for the endogenous Va3.2⁺QFL T cells at 16 weeks of age. (b) Absolute numbers of donor-derived QFLTg cells in the spleen (left) or siIEL compartment (right) of 16-week GF QFLTg_WT chimera mice colonized with *P. pentosaceus* in comparison with SPF or uncolonized GF chimera mice. Symbols on x-axis indicate that QFLTg cells were undetectable in the indicated group. * $P < 0.03$ (c) Relative abundance of *P. Pentosaceus* or *L. Johnsonii* determined by whole shotgun metagenomic sequencing of microbiota composition in pooled fecal pellets collected from each cage. (d) Absolute numbers of endogenous Va3.2⁺QFL T cells in the spleen (left) or siIEL compartment (right) of 16-week colonized GF mice compared with SPF or uncolonized GF chimera mice. Endogenous Va3.2⁺QFL T data collected from the GF QFLTg_WT chimeric mice and the non-chimeric WT mice were pooled for this analysis. ** $P < 0.008$ The number of replicates is specified in the bar graphs with each symbol representing data collected from the indicated sample/tissue isolated from an individual cage (c) or mouse (b, d). Data from ≥ 3 independent experiments were pooled for statistical analysis. P values were calculated with Student's *t* test. 'ns' indicates the comparison was not significant.

Tissue	Cell type	Phenotype	Microbiota	Critical molecules
Spleen	Innate-like T	CD44 ^{hi}	Independent	Population establishment: Qa-1 ^b (partially required), TAP Phenotype imprinting: Qa-1 ^b , ERAP1 (partially required)
IEL	Natural IELs	CD8αα ⁺ CD5 ⁻ CD90 ⁻	Homing: Independent Retention: Dependent	

Table 1.

Characteristics of Vα3.2⁺ QFL T cells in the spleen and IEL

Summary of the characteristic features of the splenic and IEL Vα3.2⁺ QFL T cells including their cell type, expression of cell surface markers, dependence on microbiota for tissue homing/retention and the critical molecules required for population establishment or phenotype imprinting.

intestine epithelium which showed the signature phenotype of natIELs including expression of CD8 $\alpha\alpha$ and lack of CD4, CD8 $\alpha\beta$, CD5 and CD90. Similar to the splenic population, while TAP is required for the presence of the population, the unconventional phenotype of Va3.2⁺QFL T cells in the gut is strongly Qa-1^b-dependent. It is worth noting that compared with the other Qa-1^b-restrict T cells reported, such as a population of semi-invariant T cells which recognize TAP-independent peptides ([Doorduyn et al., 2018](#)) or an insulin peptide-specific CD8 T population ([Sullivan et al., 2002](#)), QFL T is likely a distinct population which undergoes alternative developmental stages which requires TAP-dependent presentation of FL9 ([Nagarajan et al., 2012](#)) (or other alternative essential peptides) for positive selection and Qa-1^b for acquiring their unconventional features. Additionally, it is so far reported that CD8 $\alpha\alpha$ ⁺ IELs can be restricted to K^bD^b, CD1d or even unknown MHC Ib molecules ([Mayans et al., 2014](#); [Ruscher et al., 2017](#)). Here by characterization of the gut Va3.2⁺QFL T cells, we showed evidence of abundant Qa-1^b-restricted TCR $\alpha\beta$ ⁺ natIELs being present in the gut.

Functionally, the gut Va3.2⁺QFL T cells showed features consistent with their CD8 $\alpha\alpha$ ⁺ natIEL phenotype. Notably, although the siIEL Va3.2⁺QFL T cells showed delayed and reduced IFN- γ production in response to the cognate peptide, they displayed higher basal levels of intracellular IFN- γ than CD8 $\alpha\beta$ ⁺ IELs. It has been proposed that spontaneous IFN- γ secretion by IELs may be an important component of immunosurveillance at the mucosal surface, capable of identifying and eliminating transformed cells ([Carol et al., 1998](#); [Roberts et al., 1993](#)). Thus, siIEL QFL T cells may likewise be poised for immediate elimination of abnormal cells.

CD8 $\alpha\alpha$ ⁺ IELs are intrinsically programmed for innate functionality, as was shown by PMA/ionomycin induced production of high level of IFN- γ , CXCL2 etc. ([Van Kaer et al., 2014](#)). However, our observation of the relative hyporesponsiveness of siIEL Va3.2⁺QFL T to stimuli indicated that under physiological conditions, the activation of IELs was more complex and highly regulated as TCRs and inhibitory coreceptors such as CD8 $\alpha\alpha$ were engaged ([Cheroutre et al., 2008](#); [Madakamutil et al., 2004](#)). It is not surprising that the gut QFL T cells were functionally quiescent as these cells are constantly challenged by the microbial or dietary antigens from gut lumen ([Denning et al., 2007](#)). Cross-reaction between QFL T cells and peptide expressed in the commensal bacterium *P. pentosaceus* further supports this notion. Surprisingly, monocolonization of the GF mice with *P. pentosaceus* was sufficient to restore gut Va3.2⁺QFL T cells that were lost in old GF mice. Of note, the control bacterium *L. johnsoni*, which lack FL10 expression, did not restore gut Va3.2⁺QFL T cells. Despite the evidence we remain cautious of other possible signals generated from colonization of the commensal bacterium, such as secretion of bacteriocins.

In conclusion, Va3.2-expressing QFL T cells represent an unconventional population of Qa-1^b-restricted CD8⁺ T cells which naturally reside at high frequency in small intestine epithelium. These cells have memory phenotype in the spleen and reveal the characteristics of natIELs in the gut. In addition, the fact that Va3.2⁺QFL T cross-reacts with FL9 homologue peptide expressed in a commensal bacterium suggests that these cells might be functionally versatile. Although we have not yet detected steady-state FL9 presentation, it remains to be assessed whether FL9 peptide can be transiently presented under certain conditions at particular locations or within certain time windows. Further study of the biological significance of these cells could potentially reveal the delicate equilibrium between gut microenvironment and immune system.

Methods

Mice

SPF wild-type C57BL/6J, H-2T23-deficient Qa-1^b-KO, C57BL/6-Tg (Tcr α Tcr β)1100Mjb/J OT-1, or C57BL/6-Tg (UBC-GFP)30Scha/J B6.GFP mice were obtained from the Jackson Laboratory. QFL T TCR transgenic mice QFLTg were generated in the laboratory of E. Robey (University of California

Berkeley, Berkeley, CA) and housed in our facility. GFP⁺QFLTg mice were generated in our facility by crossing B6.GFP mice with QFLTg mice. SPF QFLTg_WT, SPF OT-1_WT and GF QFLTg_WT partial hematopoietic chimera mice were generated as previously described ([Ladi et al., 2008](#)). GF wild-type C57BL/6J and GF QFLTg_WT chimera mice were generated and maintained in the Johns Hopkins Germ-free Mouse Core Facility. Mice were housed and all procedures were done in accordance with protocols approved by Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

Generation of the QFLTg mouse

The QFLTg TCR α - and β -chain sequences were cloned and amplified from the genomic DNA of BEko8Z hybridoma ([Guan et al., 2017](#); [Nagarajan et al., 2012](#)). The TCR α -chain was amplified with the forward primer 5'-AAAACCCGGGCCAAGGCTCAGCCATGCTCCTGG-3' and the reverse primer 5'-AAAAGCGGCCGCATACAACATTGGACAAGGATCCAAGCTAAAGAGAACTC-3'. The TCR β -chain was cloned with the forward primer 5'-AAACTCGAGCCCGTCTGGAGCCTGATTCCA-3' and the reverse primer 5'-AAAACCGCGGGGACCCAGGAATTTGGGTGGA-3'. The TCR α -chain DNA fragment was cloned into pTa cassette vector by inserting it between the XmaI and NotI sites, while the TCR β -chain DNA fragment was cloned into pT β cassette vector in between the XhoI and SacII sites ([Kouskoff et al., 1995](#)). The ampicillin resistance gene was removed from pTa and pT β cassette by EarI enzyme digest. The QFLTg mice were generated on C57BL/6J background in the Cancer Research Laboratory Gene Targeting Facility at UC Berkeley under standard procedures. Founder mice were identified by flow cytometry and PCR genotyping of tail genomic DNA using primers mentioned above.

Antibodies, cell lines and peptides

Antibody for flow cytometry were from BioLegend (anti-CD45(30-F11), anti-CD19(1D3/CD19), anti-TCR β (H57-597), anti-CD8 β (53-5.8), anti-Va3.2(RR3-16), anti-Va2(B20.1), anti-CD90.2(53-2.1), anti-CD5(53-7.3), anti-IFN- γ (XMG1.2), anti-CD62L(MEL-14) and BD Biosciences (anti-CD4(RM4-5), anti-CD8 α (53-6.7), anti-CD44(IM7)). BEko8Z, L-Qa-1^b or Lmtk⁻ cells were maintained as previously described ([Nagarajan et al., 2012](#)). Peptides were obtained from GenScript. The purity of SL8, FL9 and FL10 peptides were $\geq 98\%$.

Generation of the QFL-dextramer

Qa-1^b-FL9 (QFL) monomers were synthesized by the Tetramer Core Facility of the US National Institutes of Health. Phycoerythrin (PE)- or allophycocyanin (APC)-conjugated streptavidin were obtained from Agilent. The QFL dextramers were generated following the 'dextran doping' technique ([Bethune et al., 2017](#)). The QFL monomers were incubated with PE or APC conjugated streptavidin at a molar ratio of 3:1 at 4°C for 10min followed by addition of biotinylated dextran molecular weight 500kDa at a molar ratio of 1:20 with respect to streptavidin. The mixture was further incubated at 4°C for ≥ 1 h before being used in experiments.

Isolation of IELs

Small intestinal IELs were isolated following an established protocol with minor modifications ([Qiu et al., 2018](#)). In brief, the small intestine with Payer's patches removed were cut into appropriate length. The fecal content and mucus were removed by expelling with the flat side of forceps followed by flushing with PBS. The intestine was cut open longitudinally to reveal the epithelium and further cut laterally into ~ 2 cm pieces. The intestine pieces were then placed in 25ml warmed dithioerythritol (DTE) solution (Ca²⁺- and Mg²⁺-free Hanks balanced salt solution, HEPES-bicarbonate buffer, 10% FCS) in 50ml conical tube and were shaken at 75rpm 37°C for 20min. The tube was vortexed for 10s before the supernatant was transferred into a new 50ml conical tube through a 70 μ M cell strainer. The cells were pelleted and resuspend in 44% Percoll solution (44% Percoll in RPMI 1640). Density gradient was generated by underlaying the cell

suspension with 67% Percoll solution (67% Percoll in RPMI 1640). The gradient cell suspension was further centrifuged at $1600 \times g$ for 20 min at RT without using the brake. The layer of cells at the 44% and 67% interphase were thus collected as IELs.

Enrichment for dextramer-positive cells

Splenic dextramer-positive cells were enriched as previously described ([Nagarajan et al., 2012](#)). IELs were resuspended in 100 μ l sorter buffer (0.1% sodium azide and 5% FCS in PBS). PE or APC labeled QFL dextramers were added at a final dilution of 1:100. Cells were incubated at room temperature for 45 min, then were washed twice with 3ml of sorter buffer. 20 μ l of anti-PE and 20 μ l of anti-APC microbeads (Miltenyi Biotec) were added into cells resuspended in 450 μ l of sorters buffer, followed by incubation of 20 min at 4°C. Cells were washed twice with 3ml sorter buffer. The PE- and APC-labeled cells were positively selected by passing through LS magnetic columns (Miltenyi Biotec). The entire isolated population was stained with anti-CD45, anti-CD19, anti-TCR β , anti-CD4, anti-CD8 α and anti-CD8 β . QFL T cells were gated as CD45⁺CD19⁻TCR β ⁺CD4⁻QFL-Dex-PE⁺QFL-Dex-APC⁺ population. The absolute numbers and frequencies of the cells were calculated based on a fixed number of CountBright Beads (Thermo Fisher) added into each sample.

Cytokine stimulation and proliferation assay

Splenocytes or small intestinal IELs isolated from QFLTg or OT-1 mice were depleted of non-T cells using Pan T isolation Kit II (Miltenyi Biotec). The negatively selected T cells were then labeled with 5 μ M Cell Tracing Violet (CTV) (Thermo Fisher) and adjusted to the concentration of 10^6 /ml. 10^6 of the CTV labeled T cells were stimulated *in vitro* for 3 or 5 days in cultures containing 100ng/ml IL-15 (BioLegend) or a combination of 100ng/ml IL-7 (BioLegend) and 100ng/ml IL-18 (BioLegend) in 24-well plate at 37°C. Proliferation of cells were measured by dilution of CTV on flow cytometer.

CTL assay

Lymphocytes isolated from the spleen or siIEL compartment of naïve QFLTg_WT or OT-1_WT chimera mice were cultured *in vitro* with 2 μ M of FL9 or SL8 peptide respectively together with GolgiPlug (BD Biosciences) for 4.5h. WT splenocytes were supplemented into siIEL cultures as APCs. Cells were then washed and stained for surface markers. For measurement of IFN- γ production, cells were fixed and permeabilized using Fixation/Permeabilization Kit (BD Biosciences) and stained for intracellular IFN- γ .

Hybridoma assay

10^5 of BEko8Z hybridoma cells were cocultured with 10^5 of L-Qa-1^b or Lmtk-cells in medium containing peptides of indicated concentrations in 96-well plates at 37°C for 12 hours. T cell response was determined by cleavage of the chromogenic LacZ substrate chlorophenol red- β -D-galactopyranoside (CPRG, Millipore Sigma) by TCR-induced LacZ and measure by spectrophotometry as presented by absorbance at 595 nm (A595) ([Sanderson et al., 1994](#)). Background signals were subtracted by measurement of absorbance at 655nm.

Commensal culture and colonization

Pediococcus pentosaceus Mees (33314) and *Lactobacillus johnsonii* (33200) were obtained from ATCC. The bacteria were grown overnight at 37°C in 416 Lactobacilli MRS Broth (BD Biosciences). GF QFLTg_WT chimera mice were gavaged with 200 μ l of 2×10^8 /ml CFU of *P. pentosaceus* Mees at 8 weeks of age. GF WT mice were gavaged with 200 μ l of 2×10^8 /ml CFU of *P. pentosaceus* Mees or *L. johnsonii* at 8 and 10 weeks of age. Fecal pellets collected from the colonized mice were homogenized in 416 Lactobacilli MRS Broth, and the associated mice were determined by plating

the appropriate dilution of homogenate on 416 Lactobacilli MRS agar plates. Bacterial colonization efficiency was determined by whole shotgun metagenomic sequencing of fecal pellets collected from the colonized mice at 16 weeks of age (TransnetYX Microbiome).

AlphaFold2 structure and Qa-1^b binding predictions

The predicted structure of QFL TCR:FL9:Qa-1^b complex was obtained by submitting the sequences of the $\alpha 1$ and $\alpha 2$ domain of Qa-1^b α -chain, FL9 and V α 3.2Ja21, V β 1D β 1J β 2-7 as ‘hetero-oligomer’ to ColabFold v1.5.2-patch: AlphaFold2 using MMseqs2 at <https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb>. The prediction was conducted using the alphafold2_multimer_v3 model on default settings. Visualization and alignment of the protein structures was conducted on the PyMOL software.

The Qa-1^b binding prediction was conducted by submitting the sequences of Qdm, FL9 or mutant FL9 peptides and Qa-1^b (UniProt: P06339) to NetMHCpan4.1 at <https://services.healthtech.dtu.dk/services/NetMHCpan-4.1/>.

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Reviewer #1 (Public Review):

Guan et al. explored the mechanisms responsible for the development, maintenance, and functional properties of a specific subset of unconventional T cells expressing a Va3.2 T cell receptor that recognizes a peptide, QFL, presented by the class Ib protein Qa-1. Prior studies from this group showed that cells from mice deficient in the ER protease ERAAP elicit responses in wild-type animals enriched for Qa-1-restricted CD8 T cells. They further showed that a significant proportion of these responses were directed against the QFL peptide derived from a conserved protein with incompletely understood functions. Many of these so-called QFL T cells expressed Va3.2-Ja21, were present in the spleen of wild-type mice, and exhibited a memory-like phenotype. Due to their relatively low frequency and weak staining with Qa-1 tetramers, analyzing QFL T cells has been challenging. Therefore, the authors generated dextramers, which permitted them to more rigorously identify these cells. They confirmed some of their previous findings and further showed that Va3.2+ and Va3.2- QFL T cells were present in the intestinal epithelium, where they also express CD8alpha homodimers, a characteristic of most small intestinal intraepithelial lymphocytes (siIELs), and most similar to the so-called natural siIELs that acquire their innate functions in the thymus. The authors show that TAP but not Qa-1 or ERAAP expression are required for the development of these cells, and both Qa-1 and ERAAP are required for the natural siIEL phenotype. Some of these findings were confirmed using a new TCR transgenic mouse expressing the QFL TCR. They further show that retention but not homing of QFL T cells to the intestinal epithelium involves commensal microorganisms, and using in silico approaches, they identify a commensal that contains a peptide similar to QFL that can activate QFL T cells. Finally, they show that this organism, *P. pentosaceus*, can promote gut retention of QFL T cells when it is introduced into germ-free mice. From these findings, the authors conclude that the microbiota influence the maintenance of Qa-1-restricted T cells.

Comments:

1. The authors employ a number of new reagents and elegant approaches to explore the development, maintenance and functional properties of QFL T cells.
2. Generally, conclusions made are well supported by the data presented.
3. One limitation of the work is that the immunological functions of QFL T cells remain unclear.
4. In their revised manuscript, the authors present additional data that have appropriately addressed the reviewer comments.

Reviewer #2 (Public Review):

Summary: CD8+ QFL T cells recognize a peptide, FYAEATPML (FL9), presented on Erap1-deficient cells. QFL T cells are present at a high frequency in the spleen of naïve mice. They express an antigen-experienced phenotype, and about 80% express an invariant TCRα chain Va3.2Ja21.

Here, Guan and coll. report that QFL T cells are present not only in the spleen but also in the intestinal epithelium, where they display several phenotypic and functional peculiarities. The establishment of spleen and gut Va3.2+ QFL T cells is TAP-dependent, and their phenotype is regulated by the presence/absence of Qa-1b and Erap1. Maintenance of gut Va3.2+ QFL T cells depends on the gut microbiota and is associated with colonization by *Pediococcus pentosaceus*.

Strengths:

This article contains in-depth studies of a peculiar and interesting subset of unconventional CD8 T cells, based partly on generating two novel TCR-transgenic models.

The authors discovered a clear relation between the gut microbiome and the maintenance of gut QFL T cells. One notable observation is that monocolonization of the gut with *Pediococcus pentosaceus* is sufficient to sustain gut QFL T cells.

Weaknesses:

In the absence of immunopeptidomic analyses, the presence or absence of the FL9 peptide on various cell types is inferred based on indirect evidence. Hence, whether the FL9 peptide is presented by some cells that express Qa-1b but not Erp1 remains unknown.

Analyses of the homology between the FL9 and bacterial peptides were limited to two amino acid residues (P4 and P6). This limitation is mitigated in part by the justifications provided by the authors in the revised preprint.

The potential function of QFL T cells remains elusive. The present article should provide an incentive for further functional studies.

Reviewer #3 (Public Review):

The authors investigate the role of commensal microbes and molecules in the antigen presentation pathway in the development and phenotype of CD8 T cells specific for the Qa-1b-restricted peptide FL9 (QFL). The studies track both endogenous QFL-specific T cells and utilize a recently generated TCR transgenic model. The authors confirm that QFL-specific T cells in the spleen and small intestine intraepithelial lymphocyte (IEL) pool show an antigen-experienced phenotype as well as unique phenotypic and innate-like functional traits, especially among CD8⁺ T cells expressing Va3.2⁺ TCRs. They find that deficiency in the TAP transporter leads to almost complete loss of QFL-specific T cells but that loss of either Qa1 or the ERAAP aminopeptidase does not impact QFL⁺ T cell numbers but does cause them to maintain a more conventional, naïve-like phenotype. In germ-free (GF) mice, the QFL-specific T cells are present at similar numbers and with a similar phenotype to SPF animals, but in older animals (>18w) there is a notable loss of IEL QFL-specific cells. This drop can be avoided by neonatal colonization of GF mice with the commensal microbe *Pediococcus pentosaceus* but not a different commensal, *Lactobacillus johnsonii*, and the authors show that *P. pentosaceus* encodes a peptide that weakly stimulates QFL-specific T cells, while the homologous peptide from *L. johnsonii* does not stimulate such cells.

This study provides new insights into the way in which the differentiation, phenotype, and function of CD8⁺ T cells specific for Qa-1b/FL9 is regulated by peptide processing and Qa1 expression, and by interactions with the microbiota. The approaches are well designed, the data compelling, and the interpretation, for the most part, appropriate.

The response to several of my concerns involved reference to a different manuscript from the authors (which has not been through peer review), and for point #3, it would have been useful to provide experimental evidence (e.g., competitive inhibition assays) to justify their hypothesis that P4 serves as a TCR contact while P6 may be a Qa-1b contact residue. Nevertheless, the authors have made considerable efforts to clarify their approaches and interpretation, which strengthens the manuscript.

Author Response

The following is the authors' response to the original reviews.

We appreciate the reviewers' detailed corrections and insightful comments. We have revised our manuscript per reviewers' recommendations by including new data and clarifications/expansion of the discussion on our findings. Please see below for details.

Reviewer #1 (Recommendations For The Authors):

1. The introduction notes that CD1d KO mice show reduced levels of Va3.2 T cells (Ruscher et al.), which is interesting because innate memory T cell development in the thymus often requires IL-4 production by NKT cells. Have the authors explored QFL T cells in CD1d KO and/or IL-4 KO mice? Since their QFL TCR Tg mice still develop QFL T cells (and these animals likely have very few thymic NKT cells), NKT cells may not be required for the intrathymic development of QFL T cells?

Answer: We agree that investigation on the role of NKT cells or IL-4 in QFL T cell development will greatly further our understanding of these cells.

We validated the finding that expression of the QFL TCR transgene largely repressed the expression of endogenous TCR α , as indicated by the low levels of endogenous Va2 on mature CD8SP T cells in both thymus and spleen. However, the frequencies of Va2 usage in CD4 SP thymocytes and splenocytes from QFL transgenic mice were similar to non-transgenic mice, confirming that they underwent positive selection using endogenous TCR rather than the QFL TCR. We thus do not exclude the possible presence of NKT cells in QFLTg mouse and their potential involvement in the QFL T cells development. Our manuscript here is mainly focused on investigating the peripheral phenotype of QFL T cells and their association with the gut microbiota environment. Investigations into the role of CD1d/IL-4 will be best addressed in our future studies.

1. The finding that Qa-1 expression is not required for the development of QFL T cells raises questions about other MHC products that may be involved. In this context, it is interesting that TAP-deficient mice develop few QFL T cells, for reasons that are unclear, but the authors may speculate a bit. In this context, it may be helpful for the authors to note whether TAP is required for QFL presentation to QFL T cells. Since Qa-1 is not required, and CD1d is still expressed in TAP KO mice, what then could be responsible for their defect in QFL T cell development?

Answer: This is a great point. Figure 2 (from (Valerio et al., 2023) on the development of QFL T cells) tested whether QFL TCR cross-react with other MHC I molecules.

We assessed the activation of pre-selection QFLTg thymocytes in response to various MHC I deficient DC2.4 cell lines. While the QFL thymocytes showed partially reduced activation when stimulated with Qa-1b deficient APCs, triple knock-out (KO) of Qa-1b, Kb, and Db in DC2.4 cells reduced activation close to background levels. However, double knock-out of Qa-1b with either Kb, or Db led to stimulation that was intermediate between the triple KO and Qa-1b-KO cell lines. These data suggest that Kb and Db may contribute to the positive selection of QFL T cells in Qa-1b-KO mice.

TAP is required for FL9 peptide presentation and is very likely needed for presentation of the yet unidentified MHC Ia presented peptide(s) that are essential to QFL T positive selection. While CD1d/NKT cells/IL-4 may be involved in supporting the maturation of QFL T cells, we think in the TAP-KO mice the absence of TAP led to deletion/alteration of the QFL T population at early developmental stage. We have added clarification on this point in the revised manuscript (line 412~418).

1. It may be worthwhile for the authors to note that Qa-1 was also dispensable for the intrathymic selection of another Qa-1-restricted TCR (Doorduyn et al. 2018. *Frontiers Immunol.*), although this is presumably not the case for others (Sullivan et al. 2002. *Immunity* 17, 95).

Answer: We appreciate this recommendation. We have noted this point in the resubmitted manuscript (line 412~418).

1. Lines 122-124: The sentence "Interesting ..." seemed confusing to me; are the numbers (60 and 30%) correct?

Answer: The numbers 60% and 30% were referring to the largest number we have detected for percentages of Va3.2 QFL T cells and Va3.2 CD8 T cell respectively. Here in the revised version, we replaced these numbers with average percentages (20.1% and <10%) to avoid confusion (line 134).

1. Qa-1/peptide complexes may also be recognized by CD94/NKG2 receptors, which may complicate the interpretation of the data (e.g., staining of the dextramers). From their previous work, it appears that Qa-1/QFL does not bind CD94/NKG2, which would be helpful to note in the text.

Answer: We have noted this point in the revised manuscript (line 117~121).

1. It would be helpful to add a few comments about the potential relevance to HLA-E.

Answer: We have included discussion on this point (line 391~401).

1. Figure legends: Most legends note the total number of replicates, which is usually quite high. It would also be helpful to indicate the total number of independent experiments performed and, when relevant, that the data are pooled from multiple independent experiments.

Answer: Thank you for raising the concern. We have clarified the experimental repeats in figure legends.

Reviewer #2 (Recommendations For The Authors):

1. The work of Nilabh Shastri was the foundation of the present study. Unfortunately, he passed away in 2021. Since he can no longer assume the responsibilities of a senior author, I wonder if it would be more appropriate to dedicate this paper to him than to list him as a co-author.

Answer: We have removed Dr. Shastri's name as a co-senior author and have dedicated this work to his memory.

1. The official symbol for ERAAP is Erap1.

Answer: We have replaced ERAAP with ERAP1.

1. Please refrain from editorializing. For example, "strikingly" appears eight times and "interestingly" 9 times in the manuscript. Most readers believe they do not need to be said when something is striking or interesting.

Answer: We appreciate the Reviewer's suggestion and have removed 'strikingly' and 'interestingly' from the manuscript.

1. *In WT mice, are there some cell types that express Qa-1b but not Erap1 and could therefore present the FL9 peptide?*

Answer: This is a great question. Using our highly sensitive QFL T cell hybridoma line BEko8Z (sensitivity shown in Fig. 6b), we have so far not been able to detect steady-state FL9 presentation by cells isolated from the spleen, lymph nodes, various gut associated lymphoid tissues or intestinal epithelial cells (Supplementary Fig. 8 a left panel). However, we do not exclude the possibility of FL9 peptide being transiently presented under certain conditions (i.e. ER stress/transformed cells) at particular locations or within certain time windows, which is of great importance for understanding the function of these cells but is beyond the scope of this study.

1. *Since you have not tested substitutions at other positions, could you explain your reasoning that P4 and P6 are the critical residues (lines 271-272)?*

Answer: Thank you for raising the concern. We have expanded on explanation of our strategy for determining peptide homology (line 272~313) in the revised manuscript. We have also included data on the structure the QFL TCR: FL9-Qa-1b complex predicted by AlphaFold2, conformation alignment of FL9 and Qdm (Figure 6. a, b) and the NetMHCpan prediction of Qa1b binding of Qdm, FL9 and various FL9 mutant peptides (Supplementary Fig. 8 c) to help readers visualize the reasoning behind our strategy.

1. *Readers might appreciate having a Figure summarizing the differences between spleen and gut QFL T cells.*

Answer: This is a great suggestion. We have added a table summarizing the characteristic features of the splenic and IEL QFL T cells (Table 1).

1. *In the discussion, readers would like to know what plan you might have to elucidate the function of QFL T cells.*

Answer: We appreciate the recommendation. We have elaborated on our opinions and future directions in the resubmitted manuscript (line 393~401, 446~455).

Reviewer #3 (Public Review):

1. *For most of the report, the authors use a set of phenotypic traits to highlight the unique features of QFL-specific CD8⁺ T cells - specifically, CD44^{high}, CD8aa⁺ve, CD8ab⁻ve. In Supp. Fig. 4, however, completely distinct phenotypic characteristics are presented, indicating that IEL QFL-specific T cells are CD5^{low}, Thy-1^{low}. No explanation is provided in the text about whether this is a previously reported phenotype, whether any elements of this phenotype are shared with splenic QFL T cells, what significance the authors ascribe to this phenotype (and to the fact that Qa1-deficiency leads to a more conventional Thy-1⁺ve, CD5⁺ve phenotype), and whether this altered phenotype is also seen in ERAAP-deficient mice. At least some explanation for this abrupt shift in focus and integration with prior published work is needed. On a related note, CD5 expression is measured in splenic QFL-specific CD8⁺ T cells from GF vs SPF mice (Supp. Fig. 9), to indicate that there is no phenotypic impact in the GF mice - but from Supp. Fig. 4, it would seem more appropriate to report CD5 expression in QFL-specific cells from the IEL, not the spleen.*

Answer: Expression of CD8 $\alpha\alpha$ and lack of CD4, CD8 $\alpha\beta$, CD5 and CD90 expression was indeed reported as the characteristic phenotype of natIELs. We have clarified this point in the resubmitted manuscript (line 80). The CD8 $\alpha\alpha$ + IEL QFL T cells have consistently showed CD5CD90- phenotype. While CD8 $\alpha\alpha$ expression was sufficient to describe their natIEL phenotype, we showed the CD5-CD90- data in Supplementary figures only to provide additional evidence.

The CD5 molecule by itself reflects the TCR signaling strength and high CD5 level is associated with self-reactivity of T cells (Azzam et al., 2001; Fulton et al., 2015). The implication of CD5 expression on QFLTg cells is discussed in our other manuscript where we investigate the development of these cells (Valerio et al., 2023). In Supplementary Fig. 9, because the donor splenic QFLTg cell have consistently showed comparable CD5 level between the GF and SPF group, we reasoned that it would not interfere with our interpretation of the CD44 expression.

1. The authors suggest the finding that QFL-specific cells from ERAAP-deficient mice have a more "conventional" phenotype indicates some form of negative selection of high-affinity clones (this result being somewhat unexpected since ERAAP loss was previously shown to increase the presentation of Qa-1b loaded with FL9, confirmed in this report). It is not clear how this argument aligns with the data presented, however, since the authors convincingly show no significant reduction in the number of QFL-specific cells in ERAAP-knockout mice (Fig. 3a), and their own data (e.g. Fig. 2a) do not suggest that CD44 expression correlates with QFL-multimer staining (as a surrogate for TCR affinity/avidity). Is there some experimental basis for suggesting that ERAAP-deficient lacks a subset of high affinity QFL-specific cells?

Answer: We think the presence of QFL T cells in ERAAP-KO mice is a result of the unconventional developmental mechanism of these cells which is better addressed in our complementary manuscript on the development of QFL T cells (Valerio et al., 2023). Valerio et al. found that the most predominant QFL T clone which expresses Va3.2Ja21, V β 1D β 1J β 2-7 received relatively strong TCR signaling and underwent agonist selection during thymic development, indicating that the QFL ligand is involved in selection of the innate-like QFL T population.

We agree that there is so far no direct evidence showing the QFL T cells that were absent in the ERAAP-KO mice were high-affinity clones. We have removed 'high-affinity' from the manuscript (line 180). While CD44 expression has been associated the antigen-experiences phenotype of T cells, it is yet unclear whether expression level of this molecule directly reflects TCR affinity/avidity. Identification of clones of different affinities/avidities require high precision technologies that are not currently available to the research community. While we do have zMovi, a newly developed (developing) technology, in the lab claimed to measure relative avidity/affinity of different cell types for ligands, during the past two years working with this instrument has taught us that the technology is not yet advanced enough; it can only produce reliable data on extreme differences of single clones, i.e., high numbers of homogeneous cell types expressing very high affinity receptors.

1. *The rationale for designing FL9 mutants, and for using these data to screen the proteomes of various commensal bacteria needs further explanation. The authors propose P4 and P6 of FL9 are likely to be "critical" but do not explain whether they predict these to be TCR or Qa-1b contact sites. Published data (e.g., PMID: 10974028) suggest that multiple residues contribute to Qa-1b binding, so while the authors find that P4A completely lost the ability to stimulate a QFL-specific hybridoma, it is unclear whether this is due to the loss of a TCR- or a Qa-1-contact site (or, possibly, both). This could easily be tested - e.g., by determining whether P4A can act as a competitive inhibitor for FL9-induced stimulation of BEko8Z (and, ideally, other Qa-1b-restricted cells, specific for distinct peptides). Without such information, it is unclear exactly what is being selected in the authors' screening strategy of commensal bacterial proteomes. This, of course, does not lessen the importance of finding the peptide from *P. pentosaceus* that can (albeit weakly) stimulate QFL-specific cells, and the finding that association with this microbe can sustain IEL QFL cells.*

Answer: Thank you for raising the concern. We have expanded on explanation of our strategy for determining peptide homology (line 272~313) in the revised manuscript. We have also included data on the structure the QFL TCR: FL9-Qa-1b complex predicted by AlphaFold2, conformation alignment of FL9 and Qdm (Figure 6. a, b) and the NetMHCpan prediction of Qa1b binding of Qdm, FL9 and various FL9 mutant peptides (Supplementary Fig. 8 c) to help readers visualize the reasoning behind our strategy.

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