The promiscuous development of an unconventional Qa1^{b}-restricted T cell population

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Abstract:

MHC-E restricted CD8 T cells show promise in vaccine settings, but their development and specificity remains poorly understood. Here we focus on a CD8 T cell population reactive to a self-peptide (FL9) bound to mouse MHC-E (Qa-1b) that is presented in response to loss of the MHC I processing enzyme ERAAP, termed QFL T cells. We find that mature QFL thymocytes are predominantly CD8$\alpha\beta$+CD4-, show signs of agonist selection, and give rise to both CD8$\alpha\alpha$ and CD8$\alpha\beta$ intraepithelial lymphocytes (IEL), as well as memory phenotype CD8$\alpha\beta$ T cells. QFL T cells require the MHC I subunit β-2 microglobulin (β2m), but do not require Qa1^{b} or classical MHC I for positive selection. However, QFL thymocytes do require Qa1^{b} for agonist selection and full functionality. Our data highlight the relaxed requirements for positive selection of an MHC-E restricted T cell population and suggest a CD8$\alpha\beta$+CD4- pathway for development of CD8$\alpha\alpha$ IELs.

Introduction:

Thymic development of conventional CD8 T cells requires low affinity, but specific recognition of self-peptides bound to MHC I molecules expressed by cortical thymic epithelial cells and gives rise to naïve circulating CD8 T cells. Conventional CD8 T cells recognize peptides bound to classical MHC I (called MHC Ia) molecules, in contrast to unconventional T cell populations that recognize a diverse set of non-classical MHC I (called MHC Ib)[1,2]. MHC Ib molecules are structurally homologous to MHC Ia, and often associate with β2m, but are generally non-polymorphic, and can bind peptides or non-peptidic ligands [3]. The two most prominent and well-studied examples of unconventional αβTCR-expressing T cells are mucosal associated invariant T cells (MAIT cells), that recognize vitamin B metabolites presented by MR1, and invariant natural killer T cells (iNK T cells), that recognize lipid metabolites presented by CD1d. MAIT cells and iNK T cells, like conventional T cells, require their cognate MHC ligand to develop in the thymus[4,5]. However, unlike conventional T cells, they recognize ligands presented by thymic antigen presenting cells (APCs) of hematopoietic origin [4,5], and show signs of strong TCR signaling, termed agonist selection signaling [6]. The development of T cells restricted to other MHC Ib molecules remains understudied [7–12].

While MHC Ib restricted T cells are relatively rare in circulation, they contribute substantially to the intraepithelial lymphocyte (IEL) compartment of the small intestine [13–15]. αβTCR+ IEL are generally classified as either induced or natural IELs, which differ in their specificity and developmental pathways. Induced IELs, which express the CD8αβ heterodimer, are specific for classical MHC Ia molecules and are derived from conventional CD8 T cells following antigen encounter in the periphery[16,17]. On the other hand, natural IEL, which predominantly express the CD8αα homodimer, can recognize a variety of different MHC ligands and are programmed for an IEL fate by strong recogniton of self ligands in the thymus[18,19]. Studies of natural IEL development have largely focused on populations of αβTCR+CD4-CD8- (double negative or DN) thymocytes, which can give rise to CD8αα IEL upon transfer into T cell deficient mice [20,21]. However, it is unclear whether all natural IEL develop via an αβTCR+DN stage. Moreover, while it is known that many natural IEL require β2m, but not MHC Ia molecules, for their development[13–15,18,19], the specifity of IEL for particular MHC Ib molecules remains largely unknown. As a result, no studies to date have focused on the development of IELs specific for defined MHC Ib molecules.

The MHC Ib molecule MHC-E (called Qa1 in mouse) is best known for its role in regulating NK cell responses, however, recent attention has focused on its function as a restricting MHC molecule for CD8 T cells[22,23] . In healthy cells, MHC-E molecules predominantly display a self-peptide derived from an MHC Ia leader peptide (called QDM peptide in mouse), which serves as a ligand for NK receptors and provides an inhibitory signal to NK cells [24,25]. However, under conditions of impaired MHC Ia presentation, such as deficiency in ERAAP (endoplasmic reticulum aminopeptidase associated with antigen processing) or TAP (transporter associated with antigen processing), $Qa1^b$ is loaded with an alternative set of peptides that can be recognized by CD8 T cells[10,26–28]. MHC-E restricted T cells responsive to TAP and ERAAP deficient cells have been proposed to play a role in monitoring defects in MHC Ia presentation

induced by transformation or stress[27,28]. In addition, pathogen-specific MHC-E restricted CD8 T cells can be activated upon infection with a variety of viruses and bacteria [29–33]. Recent studies of a CMV-vectored anti-HIV vaccine showed that MHC-E restricted CD8 T cells can produce responses that are extremely broad, with an unusually large proportion of the potential epitopes being targeted for recognition, and which provide strong immune protection[34,35]. Altogether, the ability of MHC-E restricted T cells to respond broadly to both microbial antigens and abnormal self, suggests an unusual mode of T cell recognition with significant therapeutic potential. However, our limited understanding of the specificity and development of MHC-E restricted CD8 T cells hampers our ability to harness these responses for therapeutic purposes.

Perhaps the best characterized example of an MHC-E restricted CD8 T cell response are QFL T cells, which recognize Qa1^b loaded with a self-peptide FYAEATPML (FL9) derived from Fam49a/b proteins[27]. QFL T cells were discovered as part of the mouse T cell response against cells deficient for the antigen processing enzyme ERAAP, and they expand and exert effector functions upon immunization of wild type mice with ERAAP deficient splenocytes. Interestingly, QFL T cells display hybrid characteristics of both conventional and unconventional T cells. Like conventional MHC Ia-restricted T cells, QFL T cells are found in the spleen and express the CD8$\alpha\beta$ heterodimer. However, reminiscent of MAIT and iNK T cells, the majority use a semi-invariant TCR with a fixed TCRα and limited TCRβ usage [36]. Splenic QFL T cells display an antigen experienced phenotype in wild type, unimmunized mice, reminiscent of conventional CD8 T cells that acquire a memory phenotype following homeostatic proliferation to self, termed "memory phenotype" or "virtual memory" T cells [37,38]. While QFL T cells can be detected using FL9-Qa1^b tetramers (called QFL tetramers) in wild type and Qa1^b deficient mice[27], their development in the thymus, and their contribution to the IEL compartment have not yet been examined.

Here we use both QFL tetramers and mice expressing rearranged QFL-specific $\alpha\beta$TCR transgenes to probe the development of QFL T cells in wild type and MHC I deficient mice. QFL T cells can be readily detected in the spleen, thymus, and IEL compartment, with QFL T cells in the IEL compartment comprised of both CD8$\alpha\alpha$ and CD8$\alpha\beta$ phenotypes. Our data indicate that Qa1^b expression, predominantly by hematopoietic cells, drives the agonist selection of QFL T cells in the thymus, leading to mature CD8+CD4- thymocytes that exhibit signs of strong TCR signals. However, QFL T cells also recognize an alternative MHC I ligand, which can allow for positive selection of QFL CD8SP thymocytes with a more conventional phenotype in the absence of Qa1^b. Our data highlight the promiscuous recognition and development of QFL T cells, confirm their hybrid conventional/unconventional characteristics, and suggest an alternative pathway for the development of natural IELs.

Results

Characterization of QFL T cells in wild type and TCR transgenic mice

To investigate the development of QFL specific T cells, we used tetramer enrichment of lymphocytes using Qa1^{b} tetramers loaded with the FL9 peptide[27](hereafter called QFL tetramers). To increase the specificity of detection, we co-stained using both the QFL tetramer and an antibody specific for Vα3.2, which recognizes the invariant TCRα chain used by the majority of QFL T cells (Fig. 1A) [36]. In this study, we focused on QFL tetramer^{+} and Vα3.2^{+} cells, hereafter called QFL T cells. The majority of QFL T cells in the thymus, spleen and small intestine (SI) intraepithelial lymphocyte (IEL) compartment of wild type mice were CD8α^{+}CD4- (Fig. 1A). Interestingly, while mature QFL T cells in thymus and spleen predominantly expressed the CD8αβ heterodimer, QFL T cells in the IEL compartment were a mixture of cells expressing CD8αα or CD8αβ (Supplementary Fig. 1A-B). As previously reported[27], QFL T cells were relatively abundant in the spleen of wild type mice (~ 1/6197 of CD8 T cells or ~1325/spleen/mouse, Fig. 1B-C). For comparison, a study of conventional CD8 T cell frequencies reported a range of 1/30,000 to 1/160,000 [39]. Additionally, a substantial number of QFL T cells were identified in the thymus and IEL compartment of the small intestine, with an average of 245 QFL T cells and 1,174 QFL T cells respectively (Fig. 1B). The frequency of QFL T cells out of mature CD8 T cells was higher in the SI IEL compared to the thymus and spleen (Fig. 1C), suggesting that they undergo selective recruitment and/or expansion in this compartment.

Previous studies showed that QFL T cells respond to a self-peptide presented by Qa1^{b}[27]. In addition, splenic QFL T cells from wild type mice display an antigen experienced phenotype, suggesting that they may receive strong TCR signals during their development in the thymus. To test this notion, we examined expression of CD5, a marker which positively correlates with self-reactivity [40–43]. As predicted, we observed that CD5 is elevated in QFL CD8SP thymocytes compared to conventional CD8 single positive (SP) T cells (Fig. 1D). In addition, QFL CD8SP thymocytes showed slight but detectable downregulation of CD8β compared to conventional CD8SP T cells (Fig. 1E), a phenotype that has been associated with thymocyte self-reactivity and agonist selection[44,45].

As a complimentary method to characterize QFL T cells, we developed a TCR transgenic mouse that expresses the semi-invariant QFL TCR α-and β-chain (*Vα3.2Jα21, Vβ1Dβ1Jβ2-7*) used by a predominant clone [36], henceforth referred to as QFLTg. The mature QFL CD8SP thymocytes in these mice displayed elevated expression of CD5, similar to QFL thymocytes from non-transgenic mice (Fig. 1D, Supplementary Fig. 1C). Mature QFL CD8SP thymocyte from QFLTg mice also showed downregulation of CD8β, which was more pronounced than that observed in QFL CD8SP from non-transgenic mice (Fig. 1E). QFL CD8SP thymocytes from QFLTg mice also showed elevated levels of several markers associated with agonist selection, such as the transcription factors PLZF [46,47] and Tbet (Supplementary Fig. 1D). On the other hand, splenic, but not thymic, QFL CD8SP T cells express elevated levels of CD44, a marker associated with

antigen experience (Fig. 1F) (Supplementary Fig. 1D). In addition, QFL CD8SP thymocytes from QFLTg mice did not show detectable upregulation of PD1 or $\alpha4\beta7$, markers that are expressed by a subset of thymic IEL precursors (Supplementary Fig. 1D) [21]. Taken together these data suggest that QFL T cells experience relatively strong TCR stimulation and undergo agonist selection during their development in the thymus.

QFL T cell development in absence of Qa1^b or classical MHC I

Previous reports showed that QFL T cells are detectable in the spleen of mice lacking Qa1^b, but undetectable in mice lacking $\beta2m$[27], a subunit of MHC I which is required for proper folding and surface expression of both classical MHC Ia and Qa1 [48]. This suggested the possibility that QFL T cells undergo positive selection on classical MHC Ia. To test this hypothesis, we generated K^{b}D^{b}KO mice and compared the number of QFL T cells in the thymus and spleen to that of WT and Qa1^{b}KO mice (Fig. 2A, Supplemental Fig. 2A-B). The QFL CD8SP thymocytes were slightly reduced in both K^{b}D^{b}KO and Qa1^{b}KO relative to wild type mice but were undetectable in $\beta2m$KO mice (Fig. 2A, and data not shown). Similar results were obtained with QFLTg mice, with substantial numbers QFL thymocytes found in the absence of Qa1^b or K^{b}D^b, but not in the absence of $\beta2m$ (Fig. 2B, and data not shown). These data suggest that neither classical MHC Ia, nor Qa1^b, are required for QFL T cell positive selection, although both may contribute to the efficiency of the process. Interestingly, CD8SP T cells in spleens of K^{b}D^{b}KO mice exhibit a higher frequency of V$\alpha3.2^+$ cells compared to WT or Qa1^{b}KO mice (Supplementary Fig. 2D), indicating that this V segment is preferentially used by T cells reactive to MHC Ib molecules.

Because Qa1^b presents agonist FL9 peptide to QFL T cells, we hypothesized that expression of Qa1^b might lead to agonist and negative selection of QFL thymocytes. In support of this, DP thymocytes in QFLTg (Qa1^b sufficient) mice exhibit reduced cellularity and a "DP^{lo}" phenotype associated with strong TCR signals [19,49–51] whereas QFLTg Qa1^{b}KO mice express normal levels of CD4 and CD8α (Fig. 2C). In addition, QFL CD8SP thymocytes from Qa1^b sufficient, but not Qa1^{b}KO mice, displayed CD8β downregulation, PLZF expression, and elevated CD5 expression compared to conventional mature CD8SP thymocytes (Fig. 2D-F). In contrast, in the absence of classical MHC I (K^{b}D^{b}KO mice) QFL CD8SP T cells showed strong downregulation of CD8β expression, maintained PLZF expression and showed a slight reduction in CD5 expression compared to WT mice (Fig. 2D-F). In the periphery, QFL T cells lost their antigen experienced phenotype in absence of Qa1^b, but not in absence of classical MHC I (Supplemental Fig. 2C). These data suggest that Qa1^b is required for agonist selection, but not the positive selection, of QFL T cells.

QFL T cells recognize an alternative ligand on Qa1^{b}KO APCs

To further explore the ligand-specificity of the QFL TCR we took advantage of the observation that MHC-naïve DP thymocytes are highly sensitive to in vitro TCR stimulation [52,53]. We examined expression of activation markers on pre-selection QFLTg (preQFLTg) thymocytes from a $\beta2m$KO background after co-culture with bone marrow derived dendritic cells (BMDC) isolated from mice lacking either Qa1^b, K^{b}D^b or $\beta2m$. PreQFLTg thymocytes showed stronger upregulation of the activation markers CD69 and CD5 upon 24 hour co-culture with WT and

K^bD^bKO, compared to $Qa1^bKO$, BMDC (Fig. 2G). This is consistent with the hypothesis that transient ERAAP downregulation can lead to low levels of agonist peptide displayed by $Qa1^{b27}$. Interestingly, preQFLTg thymocytes showed a modest activation when co-cultured with $Qa1^bKO$ BMDCs; this activation was significantly more compared to co-culture with β2mKO BMDCs (Fig. 2G). A similar pattern of reactivity was observed when preQFLTg were cultured in thymic slices derived from WT, $Qa1^bKO$ and β2mKO mice (Supplementary Fig. 3). This is consistent with the development of QFL T cells in $Qa1^bKO$ mice, and suggests that the QFL TCR is cross-reactive with an alternative β2m-utilizing molecule, most likely classical MHC I.

QFL T cell selection by hematopoietic and non-hematopoietic cells

While conventional αβT cells undergo positive selection by recognition of MHC molecules on thymic epithelial cells, MAIT cells and iNKT cells undergo selection via interactions with hematopoietic cells [4,5]. To investigate the cell type requirements for selection of QFL T cells, we generated reciprocal bone marrow chimeric mice in which either the donor cells or the host cells are β2mKO, and therefore lack surface expression of Qa1, as well as the classical MHC I molecules H2-D, H2-K (Supplementary Fig. 4). Interestingly, comparable numbers of QFL T cells were found in the thymus and spleen of the β2mKO>WT and WT>β2mKO chimeric mice (Supplementary Fig. 4B-C), implying that QFL T cell development could occur efficiently on either non-hematopoietic or hematopoietic cells. To confirm these results, we generated reciprocal β2mKO chimeras using donor cells that expressed the QFL TCR transgene (Fig. 3A). While there was some reduction in QFL T cell number in the thymus of β2mKO>WT compared to WT>β2mKO and wild type control chimeras (Fig. 3B), similar numbers of QFL T cells were found in the spleen (Fig. 3C). Thus, QFL T cell development is not strictly dependent on either non-hematopoietic or hematopoietic expression of MHC I.

We also examined whether QFL T cells that are selected exclusively by hematopoietic or non-hematopoietic cells retained their agonist selected phenotype. QFL thymocytes exhibited comparable CD8β downregulation but decreased PLZF expression when MHC I was restricted to non-hematopoietic cells (Fig. 3D-E, Supplementary Fig. 4D). Similarly, expression of CD5 was decreased when MHC I was restricted to non-hematopoietic cells (Fig. 3F, Supplementary Fig. 4E). In the periphery, QFL T cells in chimeric mice that lacked MHC I on hematopoietic cells did not display elevated expression of CD44 (Fig. 3G, Supplementary Fig. 4F). Overall, the thymic phenotype of QFL T cells in β2mKO>WT chimeras is similar, but not identical, to that observed in $Qa1^bKO$ mice (Fig. 2D-F). These data suggest that $Qa1^b$ on both hematopoietic and non-hematopoietic cells contribute to agonist selection, with hematopoietic cells playing the predominant role.

Impact of agonist selection on QFL T cell function

To test how agonist selection impacts the functionality of QFL T cells, we compared QFL T cells that arose in the presence or absence of $Qa1^b$ for their ability to respond in vitro to ERAAPKO splenocytes. QFL T cells from QFLTg showed more extensive upregulation of activation markers and increased proliferation in response to stimulation compared to QFL T cells from QFLTg $Qa1^bKO$ mice (Fig. 4A-B). This implies that agonist selection on $Qa1^b$ led to greater functional

responsiveness. Since agonist selection partially correlates with selection on hematopoietic cells (Fig. 3, Supplementary Fig. 4), we also examined QFL splenocytes from reciprocal β2mKO and wild type bone marrow chimeric mice as a further test of the impact of agonist selection on function. QFL T cells from QFLTg>β2mKO mice (Hematopoietic cell (HC) selected) responded more robustly to ERAAPKO APCs compared to cells from QFLTg β2mKO>WT mice (non- HC selected). Thus, QFL T cells that develop in the absence of Qa1^{b}, or in the absence of hematopoietically expressed MHC I, exhibit reduced functionality.

Agonist selected QFL thymocytes can populate the intestinal epithelial compartment

QFL thymocytes display an agonist selected phenotype that is enhanced by hematopoietic expression of MHC I. Given that agonist selection in the thymus can give rise to natural intraepithelial lymphocytes, we considered that QFL thymocytes might represent IEL precursors. To test this idea, we injected Rag2KO neonatal mice with QFL thymocytes from HC selected (QFLTg>β2mKO), non-HC selected (QFLTg β2mKO>WT) or both HC and non-HC selected (QFLTg>WT) chimeric mice (Fig. 4C). Both the HC selected QFL T cells and HC+ non-HC QFL T cells were readily detectable in the spleen and IEL compartment of the SI (Fig. 4D). Surprisingly, non-HC selected QFL thymocytes were not detected in the spleen or IEL compartment in this transfer system, although they were readily detectable in the spleen of intact bone marrow chimeric mice (Fig. 3C).

Although HC selected QFL T cells and HC+ non-HC selected QFL T cells were recovered from the IEL compartment in similar numbers (Fig. 4D), HC selected QFL T cells showed more pronounced downregulation of CD8β (~50%) when compared to HC+ non-HC selected QFL T cells (~10%)(Fig. 4E). Interestingly, the CD8β downregulation observed in the IEL compartment of HC+ non-HC transferred Rag2KO mice is less pronounced that that observed in intact QFLTg mice (Supplementary Fig. 1B). This suggests that QFL T cells may initially migrate to the gut with a CD8$\alpha^{+}\beta^{+}$ phenotype, and then gradually downregulate CD8β once resident in the IEL compartment.

Discussion

Most studies of unconventional T cells have focused on 2 prominent populations, MAIT (MR1-restricted) and iNKT (CD1d restricted) cells, and much less is known about the development of T cells restricted to other MHC Ib molecules. Moreover, while it is known that non classical MHC molecules contribute substantially to the CD8αα natural IEL compartment[13,15,53], and there is evidence that thymic mature CD4-CD8- cells contain IEL precursors [20,21,54,55] , it is unclear whether all natural IEL develop via a mature DN pathway. Here we have used both QFL TCR transgenic mice and FL9-Qa1^{b} tetramer staining of non-transgenic mice to investigate the development of a population of self-reactive Qa1^{b} restricted cells known as QFL T cells. QFL T cells are found in circulation as both naïve and memory phenotype CD8αβ T cells, and in the IEL compartment as both CD8αα and CD8αβ cells, whereas mature QFL thymocytes are predominantly CD8αβ+CD4- and show signs of agonist selection. QFL T cells have a more

relaxed requirement for positive selection compared to conventional CD8 T cells, requiring β2m on either hematopoietic or non-hematopoietic cells, but neither the restricting molecule Qa1^b, nor MHC Ia for positive selection. However, QFL thymocytes do require Qa1^b for agonist selection and full functionality. Our data highlight the promiscuous requirements for positive selection of a Qa1 restricted T cell population and identify an alternative CD8αβ+CD4- pathway for development of CD8αα IELs.

The flexible thymic development of QFL T cells parallels their ability to give rise to T cells with both conventional and unconventional properties. Unconventional MAIT and iNKT cells require selection by their restricting MHC molecules on hematopoietic cells, giving rise to T cells that migrate directly to tissues and exhibit preformed effector program. On the other hand, conventional CD8 T cells require selection by their restricting MHC Ia molecules on thymic epithelial cells, producing circulating naïve T cells that lack effector programing. QFL T cells appear to have the option to develop by either of these pathways, with selection by Qa1^b on hematopoietic cells leading to a more unconventional phenotype, and selection via an alternative MHC I ligand giving rise to T cells that resemble conventional CD8 T cells. Interestingly, the ability to be selected on either hematopoietic or non-hematopoietic cells in the thymus has been reported both for another Qa1 restricted T cell population [9], as well as a T cell population restricted to the MHC Ib molecule H2-M3 [8]. While the M3 restricted cells required M3 expression for thymic selection, hematopoietic selection led to T cells with more unconventional functional properties compared to non-hematopoietic selection. Thus, a flexible pattern of thymic selection leading to alternative functional programs may be a general feature of T cell reactive to some MHC Ib molecules.

The IEL compartment harbors 2 distinct types of αβTCR+CD8+ T cells: "induced" CD8αβ T cells that are derived from conventional CD8 T cells following encounter with foreign antigen and differentiation into tissue resident memory T cells, and "natural" CD8αα IEL that are directed into an IEL program in the thymus due to their high self-reactivity[16,17]. The observation that the same TCR clone can give rise to both types of IEL blurs the distinction between these two types of cells. Moreover, the blurred distinction between induced and natural QFL IELs may be reflected in their pathway of thymic development. Previous studies of natural IEL development have suggested a pathway in which some DP thymocytes that receive strong TCR signals escape clonal deletion by downregulating CD4 and CD8 to give rise to mature CD4-CD8- IEL precursors (IELp), that can eventually migrate to the gut and upregulate CD8αα [17,21]. Our data are consistent with an alternative pathway for IEL development in which agonist selection leads to a mature CD8αβ+CD4- thymic IELp. Moreover, the partial downregulation of CD8β observed on mature QFL thymocytes and on some QFL IEL T cells, suggests that CD8β expression may be unstable in QFL cells, leading to partial or full downregulation once they arrive in the IEL compartment. This pathway is consistent with earlier studies of thymocytes agonist selection in organ culture that also implicated mature CD8SP as a thymic precursor to natural IEL[44,45]. A detailed understanding of the developmental pathways and signals involved in QFL IEL T cell development awaits further investigation.

Our data, together with published observations, support the notion that MHC-E restricted CD8 T cells are generally cross-reactive. We observed that QFL thymocytes are positively selected in the absence of Qa1^b and respond to Qa1^{b}KO APCs implying cross-reactivity to an alternative β2m-dependent molecule. The MHC-E restricted response to a CMV-vectored HIV vaccine showed extremely broad reactivity, with detectable responses to 4 epitopes for every 100 amino acids [56]. In addition, another Qa1^b restricted clone was shown to cross react with an MHC Ia molecule [57], although it was dependent on Qa1^b for its positive selection [9]. In this regard, it is intriguing that QFL T cells show strong preferential use of Vα3.2 (encoded by TRAV9N/D-3) [36]. Vα3.2 is preferentially used by CD8 compared to CD4 T cells [58] and has been suggested to be inherently reactive to MHC I [59]. In addition, Vα3.2 is enriched in a subset of natural IELs [21], and is used by another Qa1^b-restricted CD8 T cell clone [9]. Moreover, we found that the frequency of Vα3.2+ CD8 T cells is substantially increased in K^{b}D^{b}KO mice (Supplementary Figure 2D). Altogether, these observations suggest that Vα3.2 may work together with Qa1^b, and perhaps other non-classical MHC I molecules, to generate self-reactive T cells with a propensity to give rise to memory phenotype and natural IEL T cells.

If MHC-E reactive CD8 T cells are inherently cross-reactive, how do they escape negative selection in the thymus? While thymocyte intrinsic mechanisms, such as downregulation of CD4 and CD8 may contribute [51], it is interesting to consider how properties of the MHC molecules may also play a role. In particular, MHC-E molecules tend to be expressed at lower levels on the cell surface compared to MHC Ia molecules [9,60,61], a property that may be linked to their atypical peptide presentation pathway [62,63] and/or low surface stability [60] In addition, MHC-E molecules predominantly express a single self-peptide derived from MHC Ia leader peptides [64,65], and may not present a large array of self-peptides in healthy cells. Indeed, it has been proposed that MHC-E molecules may monitor alterations in the MHC Ia peptide presentation pathway that occur upon viral infection or cellular transformation[10,27], changes which may be mimicked by conditions of cellular stress. According to this notion, MHC-E restricted T cells may undergo rare or transient encounters with high affinity self-peptide-MHC-E complexes on stressed cells during their development in the thymus, allowing them to experience agonist selection signals while avoiding negative selection.

Methods

Mice

B6 (C57BL/6), B6 Ly5.1 (B6.SJL-*Ptprca Pepcb*/BoyJ) and Rag2-/- (B6(Cg)-Rag2tm1.1Cgn/J) mice were from Jackson Labs. β2M-/- (B6.129-B2mtm1Jae N12) mice were from Taconic. Qa1^{b}KO mice [66] were obtained from the Shastri lab. TCR transgenic mice specific for FL9-Qa1^{b} (QFL) and H-2K1/H-2D1-/- (K^{b}D^{b}KO) mice were generated in our lab (described below). All mice were bred in the UC Berkeley animal facility and all procedures were approved by the Animal Care and Use Committee (ACUC) of the University of California.

Generation of the QFLTg mouse:

The TCR alpha and beta chain sequences from the QFL specific BEKo8z hybridoma[27,36] were cloned and amplified from the genomic DNA of the BeKoz Hybridoma. The TRAV9N/D-3 TCR alpha chain was cloned with the forward primer (5' AAAACCCGGGCCAAGGCTCAGCCATGCTCCTGG) with an added XmaI cutting site at 5' end of the DNA sequence and a reverse primer for TRAJ21 (5' AAAAGCGGCCGCATACAACATTGGACAAGGATCCAAGCTAAAGAGAACTC) with an added Not1 cutting site at the 5' end of the DNA sequence. The TCR beta chain was cloned with the forward primer (5' AAAACTCGAGCCCGTCTGGAGCCTGATTCCA) with and added Xho1 cutting site at the 5' end of the DNA and a reverse primer for TRBJ2-7 (5' AAAACCGCGGGGGACCCAGGAATTTGGGTGGA) with a SacII cutting site flanking the 5' end of the DNA sequence. The cloned TCR alpha chain was cloned into pTα cassette vector by inserting it between the XmaI and Not1 sites, while the TCR beta chains were cloned into pTβ cassette vector in between the XhoI and SacII sites [67]. The ampicillin resistance gene was removed from pTα and pTβ cassette by EarI enzyme digest. The QFL transgenic mice were generated on the B6 background in the Cancer Research Laboratory Gene Targeting Facility at UC Berkeley under standard procedures. The QFL mice were maintained on the B6 background and bred once with B6 5.1 mice to generate (QFLTgxB65.1/2) background mice for use in experiments. Founder mice were identified by flow cytometry and PCR genotyping of tail genomic DNA using primers mentioned above.

Generation of K^{b}D^{b}KO mice

K^{b}D^{b}KO were generated by the Gene Targeting Facility at UC Berkeley using Cas9/CRISPR-mediated gene targeting. The H-2K1 gene was targeted using an sgRNA (5' GTACATGGAAGTCGGCTACG 3') that aligned with the sense strand and the H-2D1 gene was targeted using an sgRNA (5' AGATGTACCGGGGCTCCTCG 3') that aligned with the antisense strand. Wild-type C57BL/6J mice were originally obtained from the Jackson Laboratories. Zygotes were obtained from super ovulated C57BL/6J females for CRISPR/Cas9 targeting knockout experiment. In brief, CRISPR mix (i.e., Cas9 protein and sgRNAs) was introduced to zygotes by

electroporation as previously described [68] .The embryos were then transferred to 0.5dpc pseudo pregnant females (CD-1, Charles River Laboratories) with oviduct transfer. When the pups were born, tails samples were collected for DNA extraction and genotyping. The resulting founder mice were identified by flow cytometry. The H-2K1 gene had a 2bp deletion (5' TGCCTGGGCTTTCTGTGTCTCCCGCTCCCAATACTCGGGCCCCTCCTGCTCCATCCACCGC GCCCGCGGCTCATATCTCGGATTCTCCGCGTCGCTGTCGAAGCGCACGAACTCCGTGTCGTCCACGT-- CCGACTTCCATGTACCGGGGCTCCCCGAGGCCGGGCCGGGACACGGCGGTGACGAAATACCTCAA 3') where the sgRNA targeted. The H-2D1 gene had a 15bp deletion where the sgRNA targeted (5 ' CCGTNGGGTCGTTCTGTTCCAAACCTCGGACTTGGGACCCGGGACGTCAGCGTCCCTGTGTCGGGAAGT GGAGGGGCCTGACCTCCCACGCGGGGTCACTCACCGCCCGCGCTCTGGTTGTAGTAACCNAGCAGGTTC CTCAGGCTCACTCGGAACCACTGCTCTTGGGCCTTGGNTTTCTGTGTTTCCCGCTCCCAATACTCCGGCCC CTCCTGCTCCATCCACGGCGCCCGCGGCTCATATCTCGGATTCTCCGCGTCGCTTTCGAAACGCACGAAC TCCTTGTTGTCCACATAGCCAACAGAGATGTACCGGGGC---------- CGGGACACGGCGGTCTCGAAATACCGCATCGAGTGTGGGCCTGGGGACGGCGCGCGGTGAGACCCCG ACCTCCTCACCAAACCCCGGGCGGCTGCGCACGCCGGGAGGGGATCTGGGCGCGGGGCTCAGGTGGA GAAGGGGCGGAGGGTCCGNGGGGGCGACGA 3').

Preparation of Cell Suspension

Thymi, and spleens were mechanically dissociated in FACS buffer (0.5% BSA in PBS) or complete RPMI (10% FBS) to generate single-cell suspensions that were then passed through a 70µm filter. Intraepithelial lymphocytes (IELs) were isolated from the small intestine as previously described[69] . Briefly, small intestine was cut to 1cm pieces and washed with cold CMF. Tissue pieces were allowed to settle and CMF was poured off. The tissue was then digested with DTE solution for 30 min at 37C in a 50mL conical tube. Tissue pieces were centrifuged at 1,500rpm for 5 min at 4C. Supernatant was collected and centrifuged at 1,500rpm for 5min at 4C. Lymphocytes were isolated by percoll separation utilizing 40% and 80% percoll [21]. The percoll solution was centrifuged at 2000rpm with no brake for 20 min at room temperature. Lymphocyte layer was then washed with PBS and collected. Splenocytes were then RBC lysed using ACK lysis buffer (0.15M NH_4Cl, 1mM $KHCO_3$, 0.1mM Na_2EDTA) for 5 minutes at room temperature.

Staining for Flow Cytometry

Thymi, spleens, and IELs were stained in 2.4G2 supernatant for 30 minutes at 4°C with the following antibodies: (BD Biosciences) CD4 (RM4-4), CD8α (53-6.7), CD5 (53-7.3), PLZF (R17-809), Runx3 (R3-5G4), (Biolegend) TCRβ (H57-597), CD90.2 (30-H12), TCR Vβ5.1/ 5.2 (MR 9-4), CD8β (YTS156.7.7), KLRG1 (2F1/KLRG1), PD-1 (29F.1A12), CXCR3 (CXCR3-173), Vα3.2 (RR3-16), Vα2 (B20.1), CD25 (PC61), CD45.2 (104), T-bet (4B10),(Invitrogen) CD8β (H35-17.2), Ki67 (SolA15), CD24 (M1/69), B220 (RA3-6B2), integrin α4β7 (DATK32), CD69 (H1.2F3), Qa-2 (69H1-9-9), Eomes (Dan11mag), Nur77 (12.14), (Tonbo) CD44 (IM7), and CD45.1 (A20). Cells were then washed in PBS and stained in Ghost Dye Violet 510 as described above. For intracellular staining, cells were

fixed and permeabilized using the eBioscience FoxP3/ Transcription Factor Staining Buffer Set (ThermoFisher) according to manufacturer's instructions. Biotinylated peptide-MHC monomers were obtained from the NIH Tetramer Facility (Atlanta, GA). Tetramers were assembled by conjugating the biotin-labeled monomers with PE-labeled streptavidin (Agilent, #PJRS27-1) according to NIH Tetramer Facility protocols. Cell numbers were calculated using AccuCheck Counting Beads for count and pipetting accuracy (Life Technologies #PCB100) according to manufacturer's instructions. All antibodies were from BD Biosciences, Biolegend, Invitrogen, or Tonbo Biosciences. Samples were processed using a Fortessa X20 (BD Biosciences) and analyzed using FlowJo software.

CFSE labeling

Cells were labeled with CFSE proliferation dye (ThermoFisher #C34554). PBS and cRPMI were pre-warmed in a 37°C water bath. Cells were resuspended in diluted CFSE dye and stained at 37°C for 9 minutes. Cells were first washed with pre-warmed cRPMI added to the labeled cells while vortexing. Cells were then washed again with pre-warmed PBS and resuspended at the desired concentration.

Tetramer Enrichment

Single-cell suspensions of thymi and spleens were generated as described above. Splenocytes were RBC lysed using ACK lysis buffer. Cells were incubated with Datsatinib (Sigma Aldrich, CDS023389-25MG) for 30 minutes at 37°C and then stained with tetramer in 2.4G2 supernatant for 1 hour at room temperature. After staining, cells were washed and incubated with Anti-PE MicroBeads (Miltenyi Biotec, #130-048-801) in MACS buffer (0.5% BSA) for 30 minutes at 4°C. Cells were then positively enriched for tetramer+ T cells using a magnetic column (Miltenyi Biotec.) according to manufacturer's instructions and washed before extracellular staining.

Bone Marrow Chimeras

Host mice were depleted of NK cells by I.P. injecting anti-NK1.1 (PK136, Leinco Technologies, #N123) at 100ug/100uL every 24Hrs for two days, for a total of 200ug of depleting antibody. Mice were irradiated in two doses of 600 rads (total of 1,200rads), with a resting period of 16hrs between doses. Mice were maintained on antibiotic water (TMS/ or full name Trimethoprim / Sulfamethoxazole?) 4 weeks following irradiation. Bone marrow was harvested from the femur of donor mice using standard techniques. Red blood cells were lysed using ACK lysis buffer (0.15M NH_4Cl, 1mM $KHCO_3$, 0.1mM Na_2EDTA) for 5 minutes at room temperature. Cells were depleted of CD4+ T cells by staining with CD4 PE-conjugated antibody (RM4-4) for 20 minutes at 4°C and then with Anti-PE MicroBeads (Miltenyi Biotec.) as described above. The labeled cells were washed, resuspended in MACS buffer, and then passed through a magnetic column (Miltenyi Biotec.). Flow-through (CD4-depleted bone marrow cells) was washed, resuspended at ($4x10^6$

cells) in 100uL of PBS and i.v. injected into recipient mice. Bone marrow chimeras were analyzed 8-11 weeks following reconstitution.

Bone Marrow Dendritic Cell Culture *In Vitro* Stimulation

Bone marrow cells were harvested as described above and RBC lysed using ACK lysis buffer (0.15M NH4Cl, 1mM KHCO3, 0.1mM Na2EDTA) for 5 minutes at room temperature. Bone marrow cells were resuspended in cRPMI and seeded at 5 x 10^6 cells per milliliter in 24 well plates. Cells were supplemented with GM-CSF (Peprotech, #315-03-20UG) until day 4 and adhering cells were harvested on day 6 using EDTA. 6 x 10^5 CD11c+MHC-II+ bone marrow cells per milliliter were seeded in 24 well plates. Preselection QFL thymocytes were generated by crossing QFL TCR transgenic mice onto a non-selecting, MHC-I deficient background (β2M-/-). Thymic single-cell suspensions were generated as described above. Thymocytes were resuspended in cRPMI and seeded at 4 x 10^6 cells per milliliter.

In Vitro Stimulation with Splenocytes

Single cell-suspensions of splenocytes were generated and RBC lysed as described above. Antigen presenting cells (APCs) were prepared by depleting splenocytes of CD4, CD8, and NK1.1 expressing cells using a magnetic column as described above. APCs were seeded at 4x10^5 cells per well in a 48 well plates. Responding splenocytes were depleted of CD4, NK1.1, B220, and CD19 expressing cells using a magnetic column as described above. Cells were then CFSE (ThermoFisher #C34554) labeled as described above and seed at 1x10^5 cells per well.

Thymic Tissue Slice Cultures

Thymic lobes were gently isolated and any connective tissue was removed. Lobes were embedded into 4% agarose with a low melting point (GTG-NuSieve Agarose, Lonza) and sectioned into 400-500mm slices using a vibratome (VT1000S, Leica). Thymic slices were overlaid onto 0.4mm transwell inserts (Corning, Cat. No.: 353090) in 6 well tissue culture plates with enough cRPMI under the insert to reach the slices. (2.5x10^5) thymocytes were overlaid onto each slice and allowed to migrate for 3 hours, after which excess thymocytes were removed by gently washing with PBS. Slices were cultured at 37°C 5% CO_2 until harvested for analysis. For flow cytometry, thymic slices were mechanically dissociated into single-cell suspensions prior to staining.

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Figures

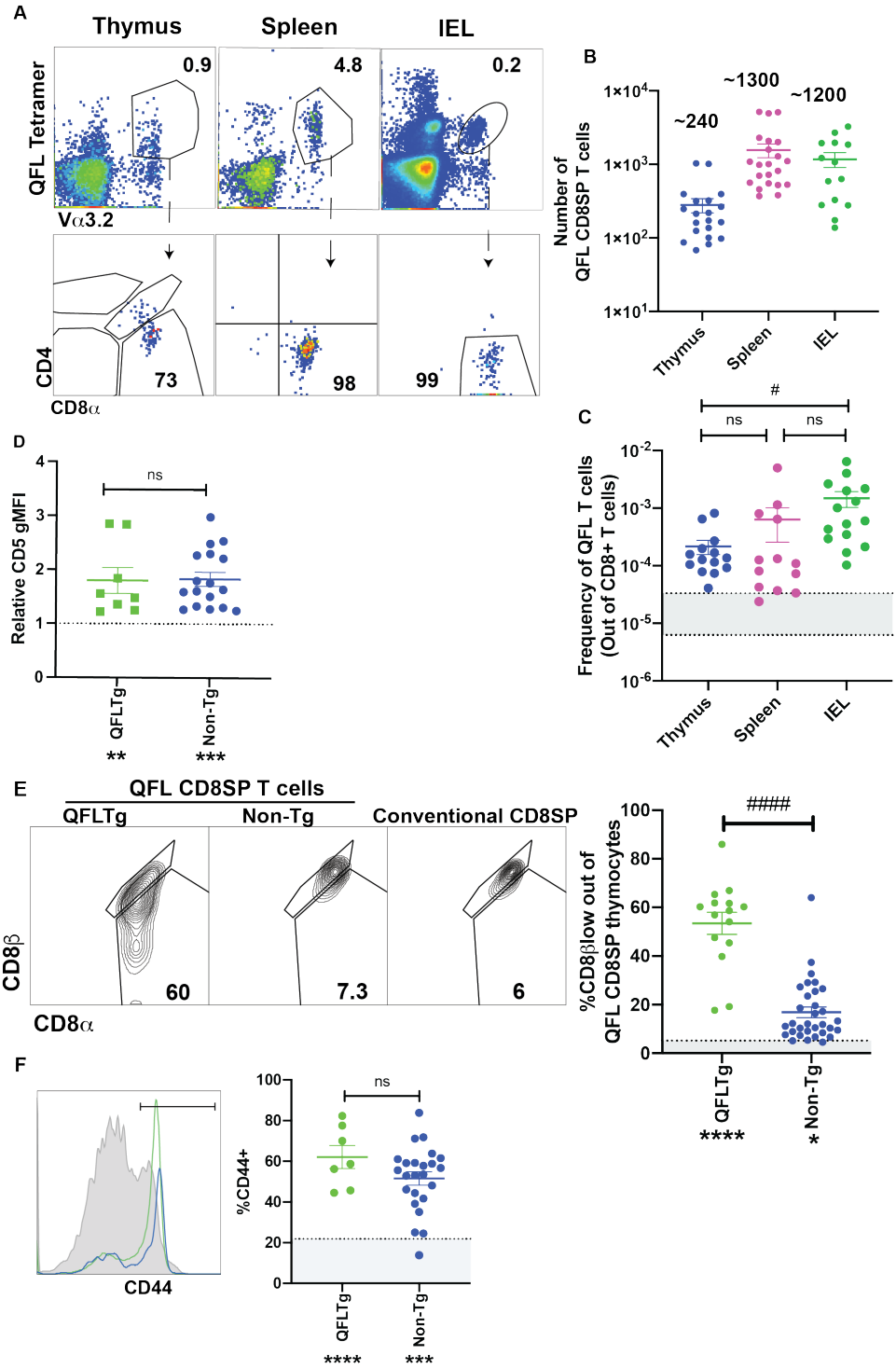


Figure 1

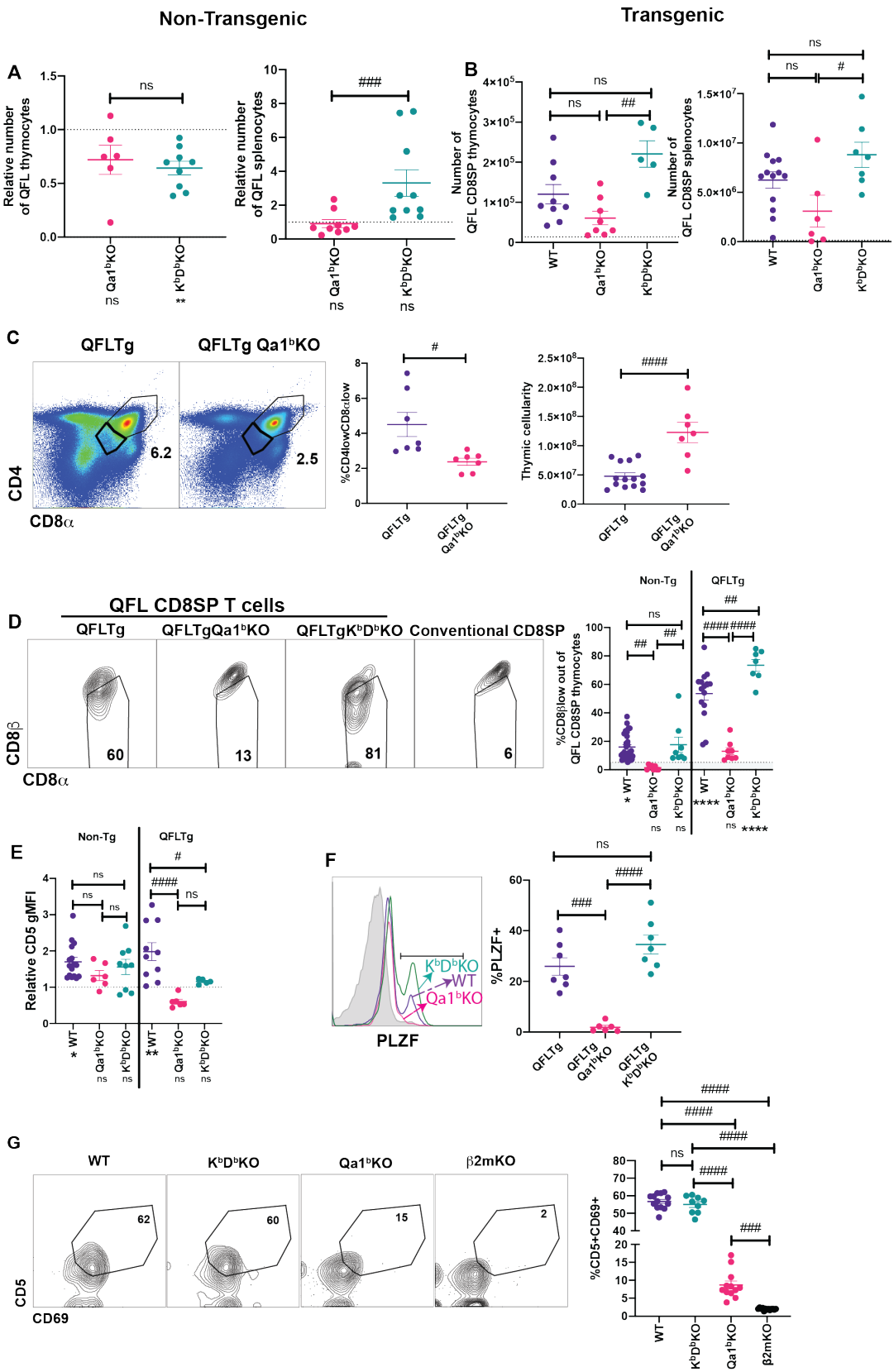


Figure 2

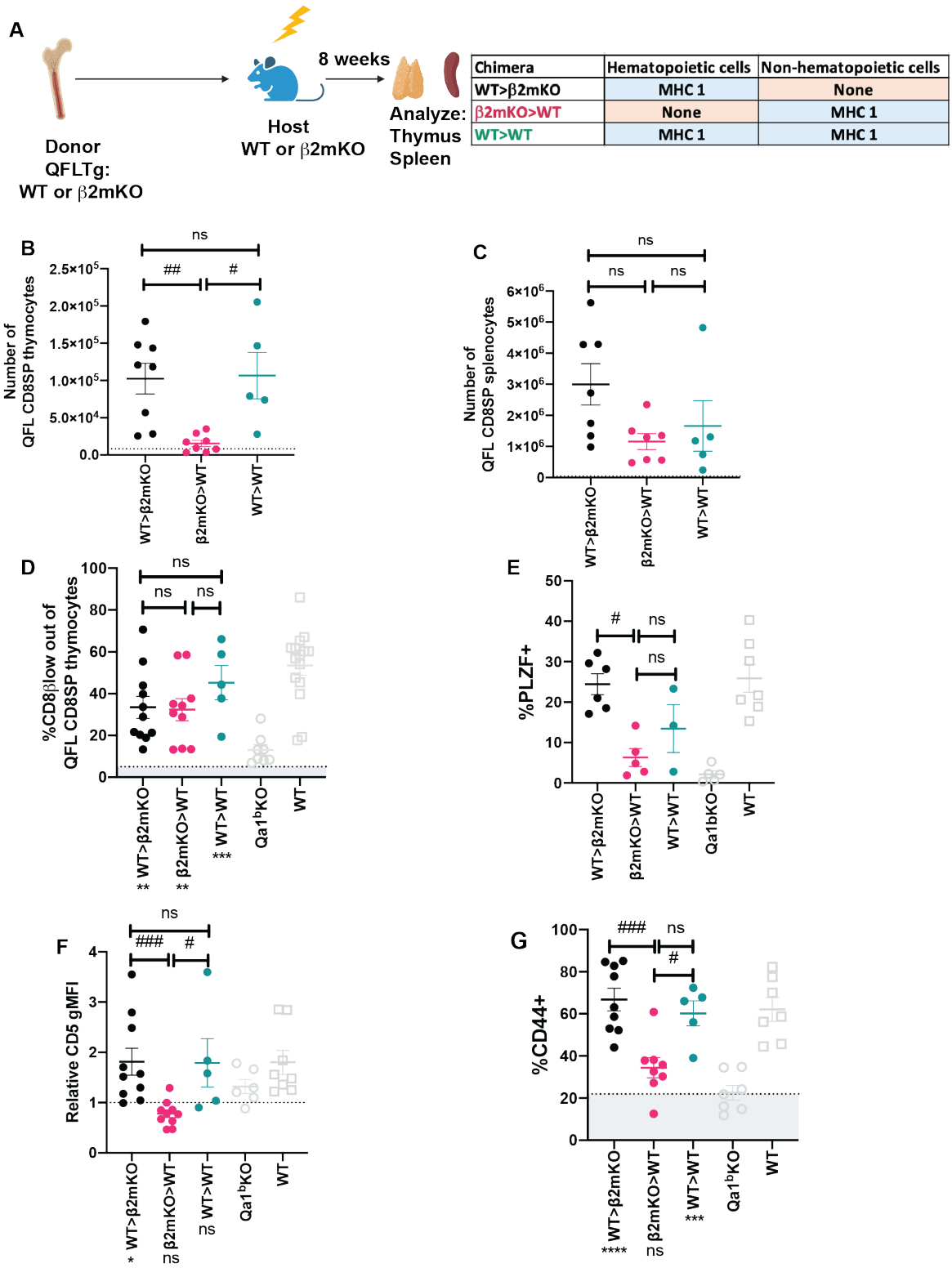


Figure 3

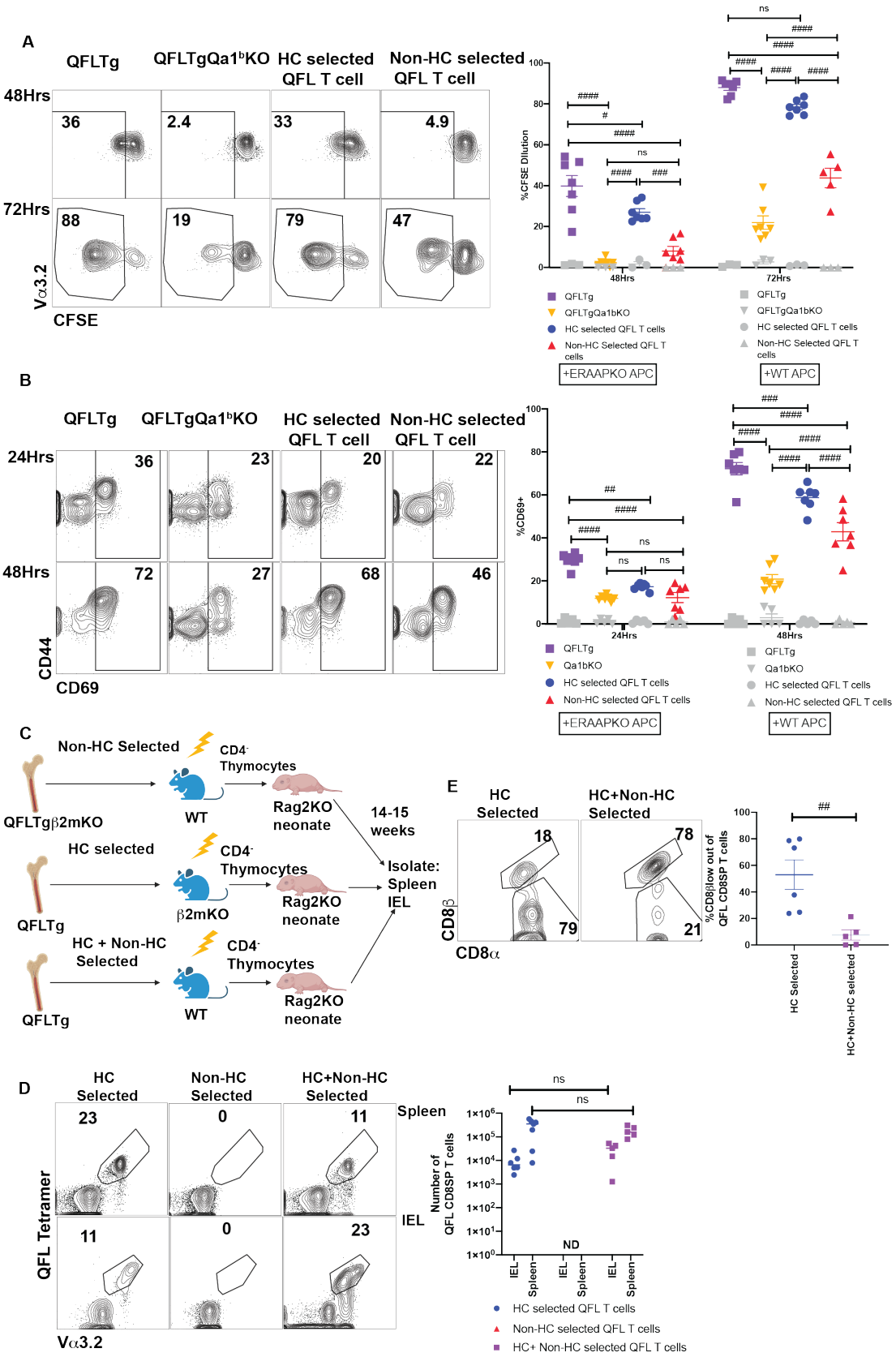
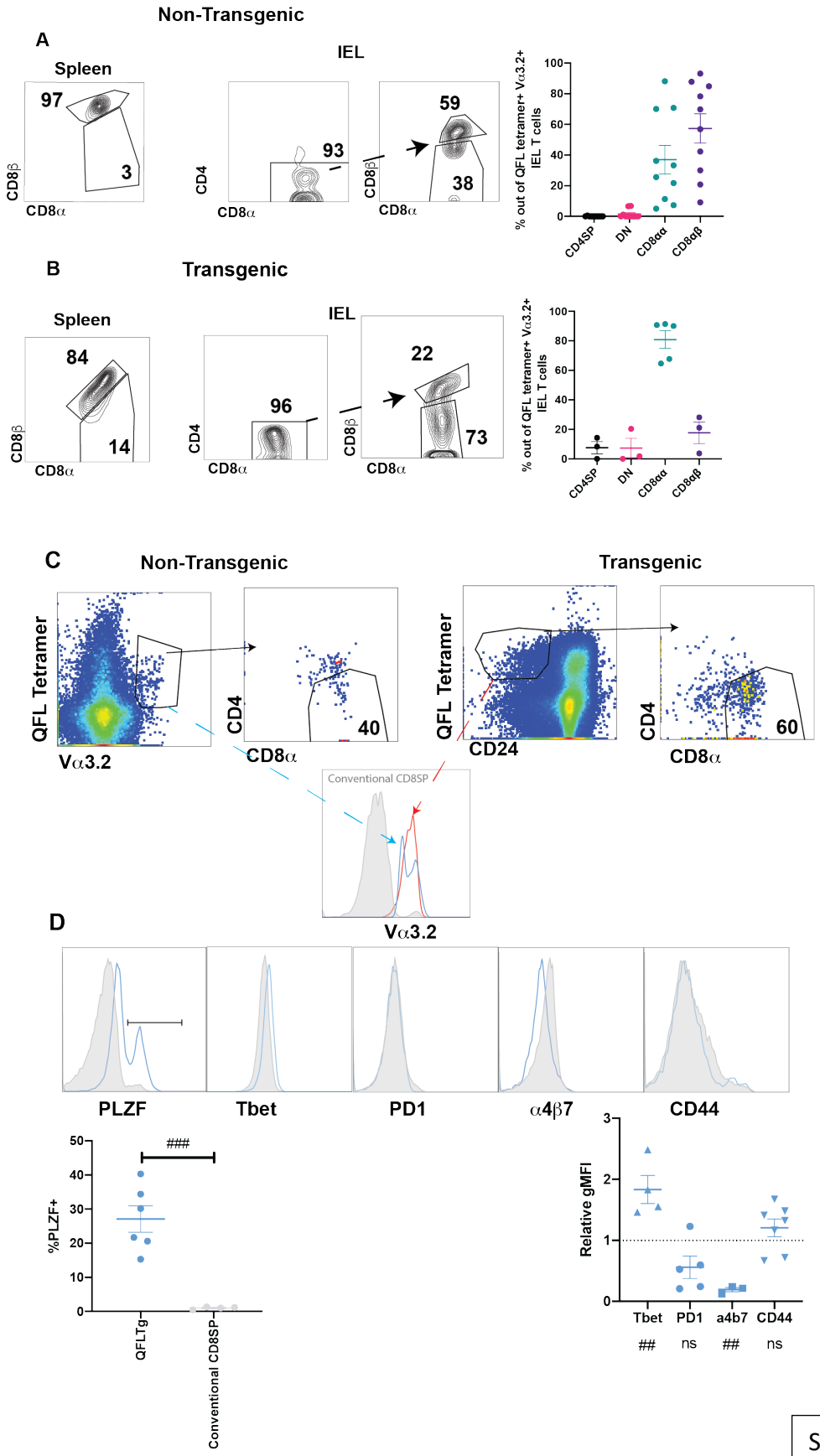
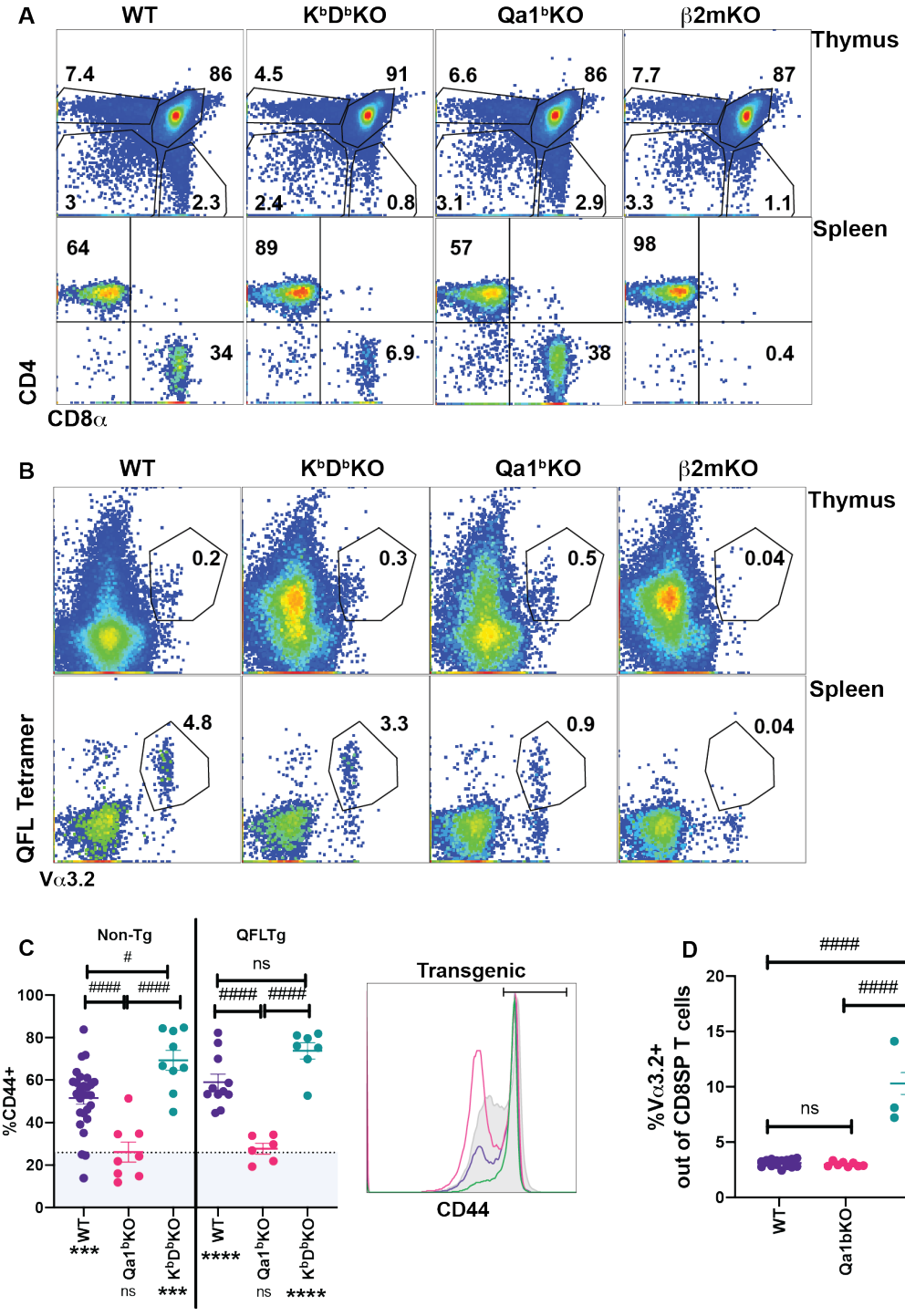


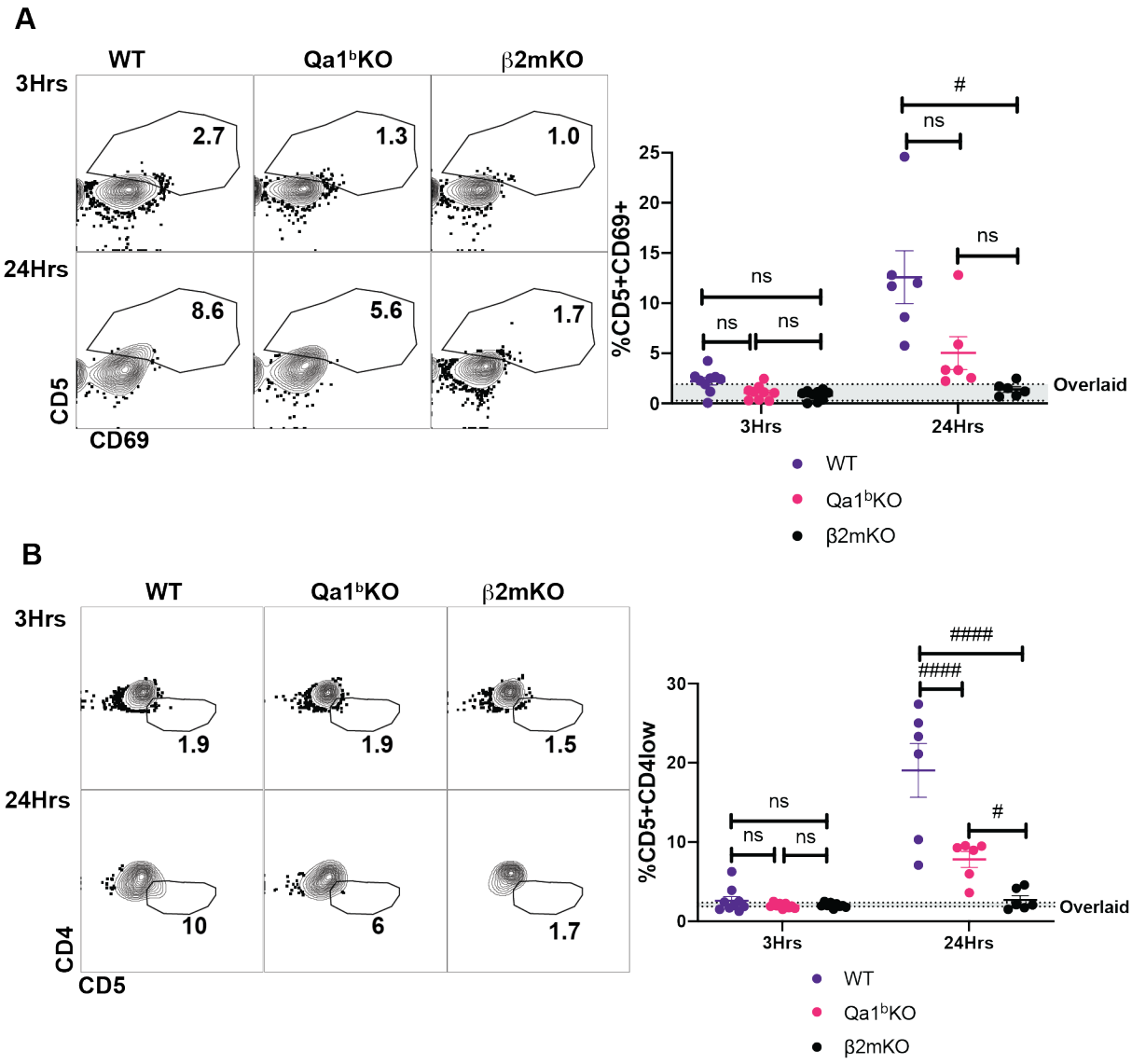
Figure 4



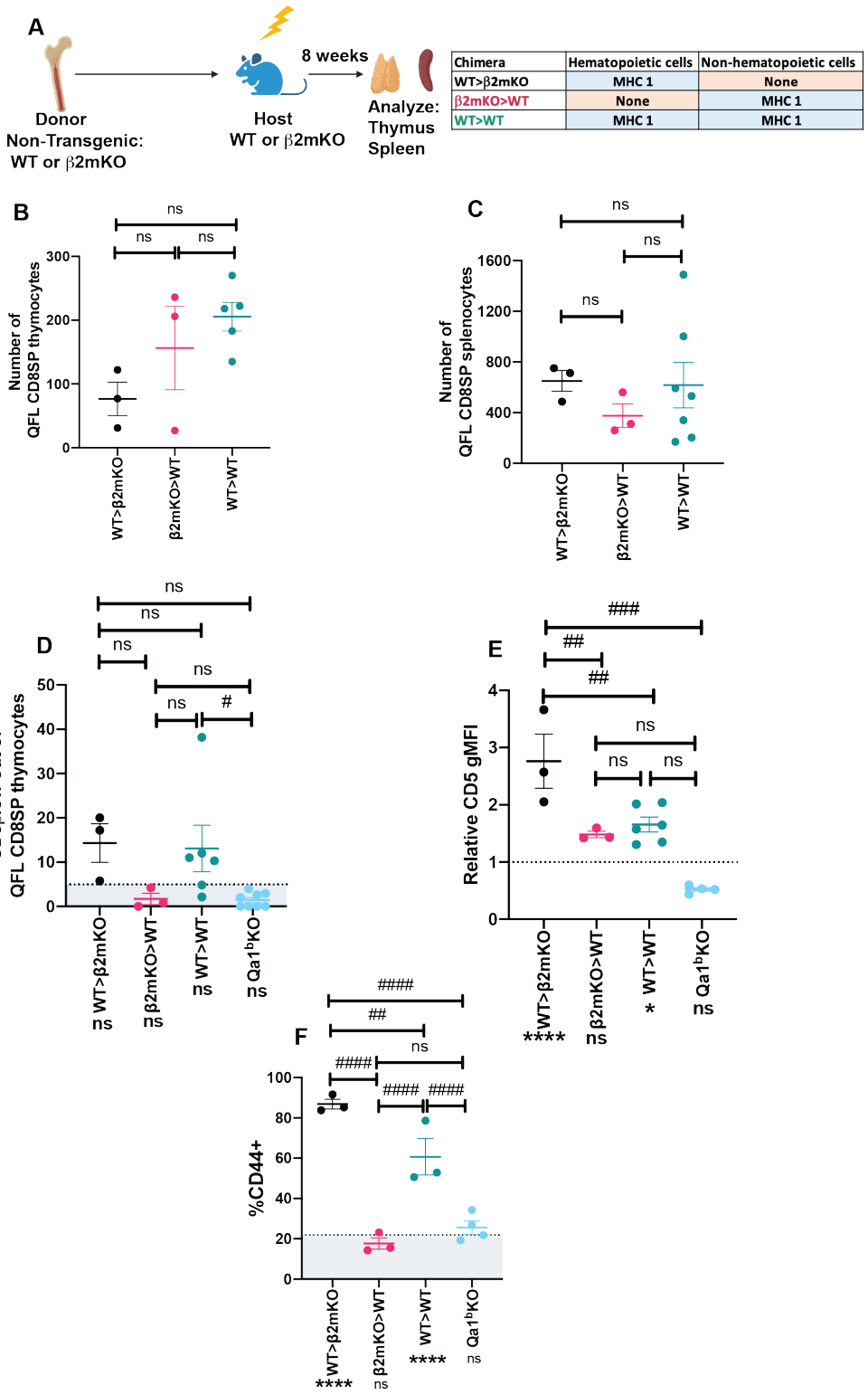
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Chimera	Hematopoietic cells	Non-hematopoietic cells
WT>β2mKO	MHC 1	None
β2mKO>WT	None	MHC 1
WT>WT	MHC 1	MHC 1

Supplementary Figure 4

Figure Legends

Figure 1: Characterization of QFL T cells in non-transgenic and transgenic mice.

A-C) QFL T cells were identified by flow cytometry from wild type mice. **A)** Representative plots of QFL tetramer and Vα3.2 TCR from tetramer enriched thymocytes, tetramer enriched splenocytes, and unenriched small intestine intraepithelial lymphocytes. Splenocytes and IEL were gated for TCRβ+ cells. CD4 and CD8α expression on the indicated gated populations are shown below. **B)** Absolute numbers of QFL CD8SP T cells in the indicated compartments of wild type mice. **C)** Frequency of QFL CD8SP T cells out of total CD8SP T cells in the indicated compartments of wild type mice. For tetramer enriched samples, frequencies out of CD8 T cells were determined by back-calculating to the unenriched samples. Greyed area represents the range of frequencies observed for naïve conventional CD8SP T cells[39]. **D)** Ratio of CD5 gMFI of QFL CD8SP T cells from QFLTg mice (Green) (Gated: QFL tetramer$^+$CD24$^-$ CD8α$^+$CD4$^-$) and non-transgenic mice (Blue) (tetramer enriched and gated: QFL tetramer$^+$ Vα3.2$^+$ CD8α$^+$CD4$^-$) over conventional CD8SP CD5 gMFI. **E)** Representative plots of CD8β and CD8α expression on mature QFL CD8SP thymocytes from QFL TCR transgenic mice (QFLTg) (Gated: QFL tetramer$^+$CD24$^-$CD8α$^+$CD4$^-$, as in Supplementary Fig. 1C), non-transgenic tetramer enriched (Gated: QFL tetramer$^+$ and Vα3.2$^+$ CD8α$^+$CD4$^-$) thymi. Conventional CD8SP (Gated: CD8α$^+$CD4$^-$) from unenriched non-transgenic thymi are shown for comparison. Dot plots show compiled data from QFLtg (green) and non-transgenic QFL tetramer$^+$ thymocytes (blue). The average value for conventional CD8SP is indicated by dashed line. **F)** Representative histogram of CD44 expression on QFL CD8SP T cells from splenocytes of QFLTg mice (Green), and non-transgenic mice (Blue) (QFL tetramer enriched and gated: TCRβ$^+$B220$^-$QFL tetramer$^+$Vα3.2$^+$CD8α$^+$CD4$^-$), and conventional CD8SP T cells (Grey histogram). Dot plots show compiled data for QFL T cells from transgenic and non-transgenic spleen. The average value for conventional CD8+ splenocytes is indicated by dashed line.
Statistical analyses: One way ANOVA, followed by Tukey's multiple comparison test. # P value <0.05, ## P value <0.005, ### P value <0.0005, #### P value <0.00001. For data that are normalized to conventional CD8SP, significance for comparisons to conventional CD8 T cells are shown below sample name.

Figure 2: MHC requirements for QFL T cell development

A) Relative number of QFL T cells in thymi (tetramer enriched and gated: QFLtetramer$^+$Vα3.2$^+$CD8α$^+$CD4$^-$) or spleen (tetramer enriched and gated: TCRβ$^+$QFLtetramer$^+$Vα3.2$^+$ CD8α$^+$CD4$^-$) of non-transgenic mice of the indicated genotype. To correct for variation in the efficiency of tetramer enrichment, data are normalized to the number of QFL T cells recovered from a wild type mouse analyzed in the same experiment (represented by dotted line). **B)** Number of QFL CD8SP T cells in the thymi (Gated: QFLtetramer$^+$ CD24$^-$CD8α$^+$CD4$^-$) or spleens (Gated:

TCRβ$^+$QFLtetramer$^+$Vα3.2$^+$ CD8α$^+$CD4$^-$) of QFLTg mice crossed to the indicated gene knock out strains. Dotted line represents the limit of detection which was based on β2mKO control. **C)** Representative plots of CD4 and CD8α expression on QFL thymocytes from QFLTg and QFLTg Qa1^{b}KO mice. Dot plot shows compiled data for % of CD4lowCD8αlow in individual mice (Gated: live cells). Panel to the right shows thymus cellularity from QFLTg and QFLTg Qa1^{b}KO mice. Each dot represents an individual mouse. **D)** Representative plots of CD8β and CD8α expression on QFL CD8SP thymocytes (Gated: QFL tetramer$^+$CD24$^-$CD8α$^+$CD4$^-$) from QFLTg, QFLTg Qa1^{b}KO and QFLTg K^{b}D^{b}KO mice. Conventional CD8+ thymocytes from wild type mice (Gated:CD8α$^+$CD4$^-$) are shown for comparison. Graph shows % of CD8β low out of QFL CD8SP T cells in thymi of non-transgenic or QFLTg mice of the indicated genotypes. Dotted line indicated the average value (5.2%) for conventional CD8SP (Gated: CD8α$^+$CD4$^-$) from wild type mice. **E)** CD5 expression on QFL CD8SP thymocytes from either non transgenic or QFLTg mice of the indicated genotypes. Graph shows gMFI of CD5 expression of QFL T cells normalized to the gMFI of conventional CD8SP thymocytes from wild type mice analyzed in the same experiment. **F)** Representative histogram of PLZF expression in QFL CD8SP T cells from QFLTg mice of the indicated genotypes. Grey histograms represent conventional CD8SP (Gated:CD8α$^+$CD4$^-$). **G)** Pre-selection QFL thymocytes (from QFLTg β2mKO mice) were co-cultured with Bone Marrow Derived Dendritic cells (BMDC) from the indicated mouse strains. Representative flow cytometry plots of CD5 and CD69 expression on QFL DP thymocytes (Gated: QFLtetramer$^+$Vα3.2$^+$CD4$^+$CD8α$^+$) after 24 hours of co-culture. Dot plots show compiled data, with each dot representing a sample from an individual culture well. Statistical analysis: Kruskal-Wallis test followed by Dunn's test multiple comparison test. For comparisons across displayed samples (shown above dots) # P value, <0.0332, ## P value <0.0021, ### P value<0.0002, #### P value<0.00001. For comparisons to wild type samples (panel A), or to conventional CD8SP (panel B) (shown below sample name) * P value, <0.0332, ** P value <0.0021, *** P value<0.0002, **** P value<0.00001. One way ANOVA followed by Tukey's multiple comparison test. For comparisons across displayed samples (shown above dots) # P value, <0.05, ## P value <0.005, ### P value<0.0005, #### P value<0.00001. For comparisons to conventional CD8SP (panel D and E) (shown below sample name) * P value <0.05, ** P value <0.005 *** P value<0.0005.

Figure 3: Requirement for hematopoietic versus non-hematopoietic cell MHC I expression for QFL thymic selection

A) Diagram of experimental design. QFLTg or QFLTg β2mKO mice were used as bone marrow donors to reconstitute irradiated β2mKO or wild type hosts in order to restrict MHC I expression to hematopoietic or non-hematopoietic cells. **B-C)** Absolute numbers of QFL CD8SP T cells in the indicated chimeric mice in **B)** Thymus (gated: QFLtetramer$^+$CD24$^-$CD8α$^+$CD4$^-$) and **C)** Spleens (gated: TCRβ$^+$QFLtetramer$^+$Vα3.2$^+$CD8α$^+$CD4$^-$). Dotted line represents the limit of detection which was based on β2mKO>β2mKO control. **D)** Downregulation of CD8β on QFL CD8SP

thymocytes of the indicated chimeric mice. Dotted line represents the average for conventional CD8SP (Gated: CD8α^+CD4-) from unenriched non-transgenic thymi (5.2%). **E)** Quantification of PLZF expression in QFL CD8SP thymocytes from the indicated chimeric. **F)** CD5 expression on QFL CD8SP thymocytes of the indicated chimeric mice. Graph shows gMFI of CD5 expression of QFL thymocytes normalized to the gMFI of conventional CD8SP thymocytes from wild type mice analyzed in the same experiment. **G)** Quantification of CD44 expression of QFL CD8SP T cells (Gated: TCRβ^+QFLtetramer$^+$Vα3.2$^+$CD8α^+CD4$^-$) from: WT>β2mKO (Black dots), β2mKO>WT (Magenta dots), WT>WT (Teal dots) chimeric spleens and Qa1^{b}KO (Light blue dots) spleens. Dotted line represents the average value for conventional CD8SP (Gated: TCRβ^+CD8α^+CD4$^-$) (22%). In D-G, data from intact QFLTg and QFLTg Qa1^{b}KO mice from Figure 2D is included for comparison. Statistical analysis: Kruskal-Wallis test followed by Dunn's test multiple comparison test (Panel B and C). For comparisons across displayed samples (shown above dots) # P value, <0.0332, ## P value <0.0021, ### P value<0.0002, #### P value<0.00001. One way ANOVA followed by Tukey's multiple comparison test. # P value, <0.05, ## P value <0.005, ### P value<0.0005, #### P value<0.00001. One way ANOVA comparing samples to conventional CD8 T cells (shown below sample name). * P value <0.05, ** P value <0.005 *** P value<0.0005.

Figure 4: Agonist selected QFL T cells respond rapidly to antigen exposure and home to the IEL compartment.

A-B) QFL CD8SP T cells from QFLTg or QFLTgQa1^{b}KO mice or from QFLTg>β2mKO (Hematopoietic cell (HC) Selected) or QFLTgβ2mKO>WT (non-HC selected) bone marrow chimeric mice were labeled with CFSE and co-cultured with splenocytes from ERAAP KO or wild type mice, and analyzed after 24, 48, or 72 hours of co-culture. Representative plots and quantification of CFSE dilution **(A)** or CD69 surface expression **(B)** on QFL CD8SP T cells (Gated: TCRβ^+B220$^-$QFL tetramer$^+$ Vα3.2$^+$CD8α^+). **C)** Experimental design: Bone marrow chimeric mice using QFLTg bone marrow donors and lacking β2m on either donor or host were generated as in Figure 3A. After 8-11 weeks of reconstitution, CD4 depleted thymocytes from chimeric mice were injected into Rag2KO neonates, and spleen and small intestinal IEL compartments were analyzed 14-15 weeks post injection. **D)** Left-hand panels show representative flow cytometry plots of QFL tetramer and Vα3.2 from spleen or IEL of the indicated transferred Rag2KO mice. Plots on the right show the number of QFL tetramer$^+$Vα3.2$^+$CD8α^+ cells recovered from each sample. ND denotes not detected. **E)** Surface expression of CD8α and CD8β on QFL T cells (Gated: TCRβ^+QFL tetramer$^+$Vα3.2$^+$CD8α^+) isolated from the IEL compartment of the indicated transferred Rag2ko mice. Statistical analyses: Two-way ANOVA followed by Tukey's multiple comparison test comparing samples to each other in their respective time point. # P value, <0.05, ## P value <0.005, ### P value<0.0005, #### P value<0.00001. student's T test. * P value, <0.05, **P value <0.005, *** P value<0.0005, ****P value<0.00001

Supplemental Figure 1: QFL T cell characterization.

A-B) (Left) Representative plots of CD8α and CD8β on QFL CD8SP splenocytes **(Right)** Representative plots CD4, CD8α and CD8β of QFL T cells in the SI IEL compartment. Quantification of CD4, CD8α (Gated: TCRβ$^+$QFLtetramer$^+$Vα3.2$^+$) and CD8β (Gated: TCRβ$^+$QFLtetramer$^+$Vα3.2$^+$CD8α$^+$CD4$^-$) surface expression on QFL T cells in the SI IEL compartment of non-transgenic **(A)** and QFLTg **(B)** mice. CD4SP (CD4$^+$CD8α$^-$) (Black dots), DN (CD4-CD8α$^-$) (Magenta dots), CD8αα(CD4$^-$CD8α$^+$CD8β$^-$) (Teal dots), CD8αβ (CD4$^-$CD8α$^+$CD8β$^+$) (Purple dots. **C)** Representative plots showing the gating strategy to identify QFL CD8SP T cells in non-transgenic (tetramer enriched) and QFLTg thymocytes. Note that virtually all QFL tetramer$^+$CD24$^-$ from QFLTg mice express Vα3.2 (lower histogram). **D)** Representative histograms of PLZF, Tbet, PD1 and α4β7 in QFL CD8SP thymocytes (Light Blue curve/dots) (Gated: QFL tetramer$^+$CD24$^-$CD8α+CD4$^-$) and conventional CD8SP T cells (Grey histogram/dots) (Gated: TCRβ$^+$ CD8α$^+$CD4$^-$). Quantification of PLZF reported as represented as %PLZF$^+$ out of QFL CD8SP thymocytes or conventional CD8SP thymocytes. Quantification of Tbet, PD1, α4β7 and CD44 are expressed as a ratio of gMFI on QFL CD8SP thymocytes over conventional CD8SP thymocytes. Statistical analysis: One way ANOVA followed by Tukey's multiple comparison test. # P value, <0.05, ## P value <0.005, ### P value<0.0005, #### P value<0.00001. student's T test. * P value, <0.05, **P value <0.005, *** P value<0.0005, ****P value<0.00001.

Supplemental Figure 2: Phenotype of K^{b}D^{b}KO and Qa1^{b}KO thymus and spleen

A) Representative flow plots of CD4 and CD8α expression on unenriched WT, K^{b}D^{b}KO, Qa1^{b}KO and β2mKO thymocytes (Gated: Live cells) and splenocytes (Gated: TCRβ$^+$). **B)** Representative flow plots of QFL tetramer and Vα3.2 expression on tetramer enriched thymocytes (Gated: Live) and splenocytes (Gated: TCRβ$^+$) of B6, K^{b}D^{b}KO, Qa1^{b}KO and β2mKO mice (Gated: Live cells). **C)** Representative histogram and compiled data of CD44 expression on QFL CD8SP T cells (Gated: TCRβ$^+$QFLtetramer$^+$Vα3.2$^+$CD8α$^+$) in Non-Transgenic **(Left of the line)** and QFL Transgenic **(Right of the line)**: WT (Purple dots), Qa1^{b}KO (Magenta dots) and K^{b}D^{b}KO spleens. Dotted line represents the average value for conventional CD8SP (Gated: TCRβ$^+$CD8α$^+$) (22%). **D)** Frequency of Vα3.2+ cells out of CD8SP splenocytes from the indicated mouse strains. Statistical analyses: One way ANOVA comparing samples to conventional CD8 T cells (shown below sample name). * P value <0.05, ** P value <0.005 *** P value<0.0005. One way ANOVA followed by Tukey's multiple comparison test. # P value, <0.05, ## P value <0.005, ### P value<0.0005, #### P value<0.00001

Supplemental Figure 3: QFL thymocyte stimulation in thymic tissue slice culture

A-B) Pre-selection QFL thymocytes (from QFLTg β2mKO mice) were overlaid onto thymic tissue slices from the indicated mouse strains. Representative flow cytometry plots of **(A)** CD5 and CD69 expression or **(B)** CD5 and CD4 expression on QFL DP thymocytes (Gated: QFLtetramer$^+$Vα3.2$^+$CD4$^+$CD8α$^+$) after 3 and 24 hours of co-culture. Dot plots show compiled data of two experiments, with each dot representing a sample from an individual thymic slice.Statistical analysis: Two-way ANOVA followed by Tukey's multiple comparison test. For comparisons across displayed samples (shown above dots) # P value, <0.05, ## P value <0.005, ### P value<0.0005, #### P value<0.00001

Supplemental Figure 4: Requirement for hematopoietic cells versus non-hematopoietic cell MHC I expression in QFL T cell development in non-transgenic mice

A) Diagram of experimental design. Non-transgenic WT or β2mKO mice were used as bone marrow donors to reconstitute irradiated β2mKO or wild type hosts in order to restrict MHC I expression to hematopoietic or non-hematopoietic cells. **B-C)** Absolute numbers of QFL CD8SP T cells in **(B)** thymus (tetramer enriched and gated: QFLtetramer$^+$Vα3.2$^+$CD8α$^+$) and **(C)** spleens (tetramer enriched and gated: TCRβ$^+$QFLtetramer$^+$Vα3.2$^+$CD8α$^+$) from the indicated chimeric mice. **D)** Downregulation of CD8β o QFL CD8SP thymocytes of the indicated chimeric mice. Dotted line represents the average for conventional CD8SP (Gated: CD8α$^+$CD4-) from unenriched non-transgenic thymi (5.2%). **E)** CD5 expression on QFL CD8SP thymocytes of the indicated non-transgenic chimeric mice and Qa1^{b}KO mice. Graph shows gMFI of CD5 expression of QFL thymocytes normalized to the gMFI of conventional CD8SP thymocytes from wild type mice analyzed in the same experiment. **F)** Quantification of CD44 expression of QFL CD8SP T cells (Gated: TCRβ$^+$QFLtetramer$^+$Vα3.2$^+$CD8α$^+$CD4$^-$) from tetramer enriched non-transgenic splenocytes from: WT>β2mKO (Black dots), β2mKO>WT (Magenta dots), WT>WT (Teal dots) chimeric spleens and Qa1^{b}KO (Light blue dots) spleens. Dotted line represents conventional CD8SP (Gated: TCRβ$^+$CD8α$^+$) (22%-26%). Statistical analysis: Kruskal-Wallis test followed by Dunn's test multiple comparison test (Panel B and C). For comparisons across displayed samples (shown above dots) # P value, <0.0332, ## P value <0.0021, ### P value<0.0002, #### P value<0.00001. One way ANOVA comparing sample to Conventional CD8SP. * P value <0.05, ** P value <0.005 *** P value<0.0005. One way ANOVA followed by Tukey's multiple comparison test. # P value, <0.05, ## P value <0.005, ### P value<0.0005, #### P value<0.00001