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The Development of an Unconventional Qa1^{b} Restricted T cell Population

By

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Abstract

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CD8 T cells restricted to the nonclassical MHC 1 (MHC 1b) molecule MHC-E show great promise in vaccine settings, but the development and specificity of MHC-E restricted T cells remains poorly understood. Here we focus on a CD8 T cell population reactive to a self-peptide (FL9) bound to mouse MHC-E (Qa1^b) that is presented in response to loss of the classical (MHC 1a) 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 circulating memory phenotype CD8$\alpha\beta$ T cells. QFL T cells require the MHC 1 subunit β-2 microgloblin (β2m), but do not require Qa1^b or epithelial MHC 1 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.

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Dedication

Se lo dedico a todos los Boricuas que han luchado por un Puerto Rico libre y soberano. A todo Boricua que han dejado su isla por busca de mejores oportunidades. A todo los seres queridos perdidos por injusticias. A todo Producto de la UPR que han puesto a Puerto Rico en alto con sus logros. A cada persona que sueña indagar en la ciencia pero no cree en su valor.

Puerto Rico resistirá a todos los corruptos y los venceremos con dignidad y pasión.

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To all my friends that have been the best support system in my darkest times.

Chapter 1

The thymic development and function of conventional and unconventional αβ T cells

1.1 Conventional αβT cell function and development

The mammalian immune system is generally separated into the innate and adaptive compartments. The innate compartment is armed with germline encoded "sensor" proteins that recognize conserved features of pathogens and signs of cellular damage. The failure of the innate compartment to clear the pathogen triggers the adaptive compartment. This secondary component tailors its response to the specific pathogen/cellular damage afflicting the organism. A key component of the adaptive branch of the immune system is the T cell. The primary function of T cells is to recognize an infected/damaged cell and mount an appropriate response to clear out the pathogen or infected/damaged cell.

The T cell is a type of leukocyte that is characterized by a unique surface protein called the T cell receptor (TCR). Through this TCR, T cells can survey for signs of a pathogen or damaged cell. In order to fulfill their function, T cells require an antigen presenting cell (APC) that displays peptides derived from pathogens or damaged cells. These immunogenic peptides are loaded on a protein called the major histocompatibility complex (MHC). These proteins are located in one of the most polymorphic loci in the human genome (Fernandez Vina et al., 2012). Interestingly, these polymorphisms are generally where peptide binding sites are encoded. The diversity of MHC alleles provide differential responses to the same pathogen by binding different peptide sequences derived of a single pathogen derived protein. Since T cell responses are dependent on the reactivity of a TCR to a particular MHC+ peptide complex, this diversity of MHC+ peptide complexes can elicit a myriad of T cells that have varying rates of efficiency in clearance of a pathogen.

T cells are generally categorized based on the expression of their co-receptor CD4 or CD8. The former are known as $CD4^+$ T helper and the latter $CD8^+$ cytotoxic cells. These co-receptors assist in binding to specific classes of MHC molecules. The CD8 co-receptor binds MHC-I, which is expressed on the surface of all nucleated cells and loads peptides derived from cytosolic proteins. The CD4 co-receptor bind MHC-II, which is expressed on specialized "professional" APCs and loads peptides derived from extracellular proteins. In a healthy cell, MHC-1 displays peptides derived from cytosolic self-proteins and are generally non-immunogenic. In an unhealthy or infected cell, the repertoire of cytosolic proteins is enriched for pathogen encoded proteins or damage associated proteins. This leads to immunogenic peptides loaded onto MHC-I that provoke a CD8+ T cell response and eventual death of the unhealthy cell. This ability of T cells to discriminate between self and non-self-peptides is an essential component of their thymic development, a process called central tolerance.

T cell development occurs in the thymus, where they are referred to as thymocytes. The different stages of thymocyte development are categorized by the expression of CD4 and CD8 co-receptors. The goal of a developing thymocyte is to complete rearrangement of a functional TCR. The first stages of thymocyte development are named by their lack of CD4 and CD8 expression, these are called double negative (DN) thymocytes. During these stages of development notch dependent signals are critical for the maturation and survival of DN thymocytes(Li and von Boehmer, 2011). The DN phases of thymocyte development can be further segregated into 4 different stages based on the expression of CD44 and CD25: DN1 (CD44+CD25-), DN2 (CD44+CD25+), DN3(CD44-CD25+) and DN4 (CD44-CD25-). During the DN3-DN4 stages, thymocytes begin to arrange their TCR through a process called V(D)J recombination. This stochastic process leads to highly diverse set of potential TCR arrangements, with an estimated 10^{15} possibilities (Davis and Bjorkman, 1988). In reality many of this TCR arrangements are non-functional or potentially dangerous to the host and are eliminated. The first filtering of TCR arrangements occurs during β selection. A newly arranged β chain of the TCR is complexed with a germline encoded pre$T\alpha$. The ability of this complex to receive stimulation leads to survival and continue maturation of thymocytes (Murga and Barber, 2002). Completion of this developmental benchmark initiates the upregulation of CD4 and CD8 leading the thymocytes to the CD4+CD8+ double positive stage (DP) of development. The α chain of the TCR is rearranged during this stage and a complete $\alpha\beta$ T cell receptor is presented on the surface. The newly arranged TCR is tested for "self" reactivity by interacting with MHC+ peptides (pMHC) derived from self. These pMHC are presented by specialized cortical thymic epithelial cells (cTEC) (Kondo et al., 2017) (Figure1A). The thymocytes with a TCR that is not overly self-reactive, but is able to be stimulated proceeds development and it is positively selected (Hogquist et al., 1994; Lucas et al., 1999; Merkenschlager et al., 1997). The self pMHC-I or pMHC-II that provides a positive selecting signal to thymocytes determine whether thymocytes will become a CD8+ or CD4+ single positive (SP) thymocytes. The SP thymocytes that have been positively selected are tested once more for their self-reactivity by medullary thymic epithelial cells (mTEC) (Cosway et al., 2020; Kurd and Robey, 2016). These specialized APC display peptides derived from self- proteins that are specific to certain tissues , termed tissue restricted antigens (TRA). This final check on self-reactivity focuses on filtering out TCR clones that would react to immune-privileged sites, like the eyes and brain(Anderson et al., 2002). The combination of these rigorous checkpoints lead to a "central" tolerance that avoids autoimmunity and ensures a functional TCR repertoire.

1.2 Unconventional $\alpha\beta$T cell function and development

The family of TCR bearing cells has been slowly expanding over the past years. The termed "unconventional" $\alpha\beta$ T cells have been of particular interest. These unconventional T cells are poised to rapidly respond to target cells (Legoux et al., 2017), migrate to non-lymphatic tissues (Chandra and Kronenberg, 2015; Crosby and Kronenberg, 2018), possess a limited repertoire of TCRs (Boudinot et al., 2016; Salio et al., 2014) and recognize non-polymorphic non-classical MHC (MHCb) (Adams and Luoma, 2013; Souza et al., 2019; Sullivan et al., 2008). In contrast to classical MHC (MHCa), MHCb molecules have less than 4-5 alleles in a population (Arnaiz et al., 2022; Ohtsuka et al., 2008).The ligands recognized by these unconventional $\alpha\beta$ T cells are not restricted

peptides, but also include glycolipids, vitamin metabolites and others (Adams and Luoma, 2013; Legoux et al., 2017). The development of unconventional T cells has been extensively studied for two unconventional populations. The developmental pathway of these unconventional T cells diverges early on from the conventional T cells described above. Overall, unconventional T cells blur the line that separates innate and adaptive immunity.

iNKT

The MHC 1b molecule CD1d binds lipid molecules as well as hydrophobic peptides derived from microbes and host (Adams and Luoma, 2013; Brutkiewicz, 2006). The T cell population that recognizes CD1d is called a natural killer T cell (NKT), since it expressed natural killer cell markers and a TCR (Pasman and Kasper, 2017; Salio et al., 2014). The NKT cell population is divided into the Type 1 invariant NKT(iNKT) and the type 2 NKT. The iNKT population has a limited TCR repertoire with an almost invariant TCRα chain and a preferred set of TCRβ chains (Bezbradica et al., 2005; Chandra and Kronenberg, 2015). The type 2 NKT cell have a diverse TCR repertoire and recognize not only lipids, but also hydrophobic peptides (Nishioka et al., 2018). I will be focusing on the type 1 iNKT population.

iNKT are generally tissue resident lymphocytes defined by their semi-invariant TCR of Vα14-Jα18 (in rodents) and Vβ8, Vβ7 or Vβ2 (Kwon and Lee, 2017). Identifying iNKT cells in mice and humans has been facilitated by using tetramers of CD1d loaded with α-GalactosylCeramide, a potent ligand for this TCR. Interestingly, iNKT have been subcategorized based on their Th-like cytokine production: iNKT1, iNKT2, iNKT17, iNKT10 and iNKT$_{FH}$ (Hogquist and Georgiev, 2020). iNKT are also resident of diverse tissues and have been found to have protective or pathological effects in response to infection (Crosby and Kronenberg, 2018). The fact that iNKT respond to the non-polymorphic CD1d, their ability to respond to a wide range of pathogens and their diverse effector programs, has poised iNKT to be an interesting cellular therapy option (Godfrey et al., 2018; Painter et al., 2021).

The thymic development of iNKT begins to deviate from conventional T cells at the DP stage (Benlagha et al., 2005). During this stage, iNKT receive their positively selecting signal by interacting with other DP thymocytes presenting CD1d loaded a self-lipid (Bendelac, 1995) (Figure1B). iNKT thymocytes receive a strong self-reactive signal during positive selection termed “agonist selection” (Joseph et al., 2019; Kumar et al., 2020; Stritesky et al., 2012). This alternate outcome of positive selection has been observed in other unconventional $\alpha\beta$T cells and in T regulatory cells (Grandjean et al., 2017; Oh-Hora et al., 2013; Stritesky et al., 2012). Aside from CD1d+ self-lipid, iNKT thymocytes require homotypic interaction between selecting DP and selected DP thymocyte through signaling lymphocytic activation molecule (SLAM) receptors (Griewank et al., 2007). This interaction relays intracellular signals through the SLAM associated protein (SAP). The disruption of SAP mediated signaling alters iNKT development significantly (Chen et al., 2017). This SLAM/SAP axis has been proposed to dampen TCR signaling strength (Lu et al., 2019), which is an important component of iNKT development. In fact, it has been proposed that the strength of TCR signaling of a developing iNKT plays a role into the differentiation to: (in

descending order of signaling stength) iNKT2>iNKT17> iNKT1 (Tuttle et al., 2018). A direct consequence of iNKT TCR signaling is the expression of the transcription factor promyelocytic leukemia zinc finger (PLZF). This transcription factor is the master regulator for iNKT development and has been shown to impart innate-like characteristics to thymocytes (Kwon and Lee, 2017; Lee et al., 2020; Savage et al., 2008). In a similar pattern to TCR signaling, the levels of PLZF expression have been proposed to play a role in the differentiation to the different subsets. In contrast to conventional $\alpha\beta$ T cells, iNKT in general leave the thymus fully differentiated and ready to respond to antigen. The recently described iNKT10 and $iNKT_{FH}$ require some peripheral maturation events to gain their respective programming (Crosby and Kronenberg, 2018; Hogquist and Georgiev, 2020). Neither of these subsets have been identified in the thymus. This suggest an added level of plasticity in iNKT programming that should be considered in future studies.

MAIT CELLS

MR1 is an MHC 1b molecule that binds metabolites of vitamin B, a non-mammalian synthesized molecule (Kjer-Nielsen et al., 2012). The mucosal activated invariant T cells (MAIT) are the major T cell population that responds to MR1. These unconventional $\alpha\beta$ T cells possess a semi-invariant TCR comprised of TRAV 1(Vα19 in mice and Vα7.2 in humans) and TRAJ 33(Jα33) paired with limited set of TCRβ chains (le Bourhis et al., 2011). This highly conserved MHC 1b molecule has been shown to co-evolve with TRAV1 and TRAJ33, where mammalian species that express MR1 also express this specific pair of TCRα chain genes (Boudinot et al., 2016).

MAIT cells are defined by the expression of their invariant TCRα chain and binding to 5-OP-RU loaded MR1 tetramers. In general, MAIT cells show an effector programming similar to Th1 and Th17, termed MAIT1 and MAIT17. The MAIT cell population has been reported to play a role in the response to numerous bacterial, fungal, viral and parasitic infections (Godfrey et al., 2018, 2019; Pasman and Kasper, 2017). Interestingly, MAIT cells have also been implicated in several autoimmune diseases, allergic reactions and inflammatory disorders (Godfrey et al., 2019; Lin et al., 2020). MAIT cells can also be activated in a TCR independent manner. Curiously, this TCR independent activation requires at least two cytokine mediated signals (Godfrey et al., 2019). This alternate activation mechanism may explain MAIT responses to viruses and other pathogens that do not synthesize vitamin B. The versatility of MAIT cells may explain their high frequency in humans as they comprise around 1-10% of the blood circulating T cell (le Bourhis et al., 2011) .

Similar to iNKT cells, the thymic development of MAIT cells deviate from conventional T cells at the DP stage (Lee et al., 2020). MAIT cells are agonist selected by other DP thymocytes expressing MR1 loaded with an unknown ligand (Seach et al., 2013)(Figure1C). It has been proposed that microbial metabolites are transported to the thymus and these are presented to developing MAIT cells. In support of this, MAIT cell development is impaired in germ free mice, but rescued when the germ free mice are exposed vitamin b producing microbes (Legoux et al., 2019; Pellicci et al., 2020). However, it is still unclear how DP thymocytes uptake the proposed microbial metabolite and present it to developing MAIT cells. MAIT cell development requires PLZF expression, but not SLAM/SAP homotypic interactions (Koay et al., 2016) . Before exiting the

thymus, MAIT cells acquire their MAIT1 or MAIT17 programming. However, it is unknown what factors drive this differentiation. MAIT cells enter circulation with a defined effector programing and poised to respond to their antigen.

H2-M3 restricted T cells

H2-M3 is a non-classical MHC 1b that binds peptides with a formylated methionine at the n-terminus (N-formylated) (Legoux et al., 2017), a common post-translational modification in prokaryotic cells. Therefore, it is of no surprise that H2-M3 presents peptides derived from bacterial and the mitochondrial proteins (Colmone and Wang, 2006; Lindahl et al., 1997). The CD8 T cells that are restricted to H2-M3 show an activated phenotype, have been identified in barrier sites (Linehan et al., 2018) and possess a diverse TCR repertoire (Legoux et al., 2017). H2-M3 has not been identified in humans, but there have been T cell responses directed at N-formylated peptides (Ristori et al., 2001).

The development of H2-M3 restricted T cells has been primarily described using a TCR transgenic of a listeria specific H2-M3 restricted clone (D7) (Berg et al., 1999). The D7 thymocytes requires thymic expression of H2-M3 loaded with an unknown N-formylated peptide. This selecting ligand can be presented by either epithelial cells or hematopoietic cells (Cho et al., 2011). However D7 thymocytes selected by hematopoietic cells express Eomes and show an innate like phenotype, but the epithelial selected D7 do not express Eomes and resemble conventional T cells. In contrast to iNKT and MAIT, D7 thymocytes do not express PLZF (Cho et al., 2011), but appear to gain their innate like phenotype from Eomes directed activity. Interestingly, D7 thymocytes require SLAM/SAP interactions for their activated phenotype, but not for their thymic development (Bediako et al., 2012). This implies that D7 thymocytes require a lymphocyte-lymphocyte homotypic interaction for their innate phenotype. It would be reasonable to suspect other DP thymocytes as the selecting APC, since these are the selecting APCs for MAIT and iNKT, this hypothesis has not been tested.

MHC-E restricted T cells

The non-classical peptide binding HLA-E (Human), Qa1 (Murine) and Mamu-E(Rhesus Macaque) are functional orthologs which are grouped under the term MHC-E (Ruibal et al., 2020). The dominant peptide bound to MHC-E molecules in steady state is derived from the leader sequence of classical MHC 1 molecules: VL9 in humans and Qa1 determinant modifier (QDM) in mice (Aldrich et al., 1994; Braud et al., 1997). This complex is recognized as an inhibitory signal by NK cells and activated cytotoxic lymphocytes (Braud et al., 1998; Vance et al., 1998). These inhibitory MHC-E complexes require a functional MHC 1 processing pathway. Additionally, MHC-E complexes are relatively unstable on the cell surface and are recycled back into the cell (Kambayashi et al., 2004). In order to provide inhibitory signals to cytotoxic immune cells, MHC-E must be frequently loaded and presented to the surface. Interestingly, many pathogens and cancers target the MHC 1 processing pathway as a form of immune evasion (Doorduijn et al.,

2016; Hislop and Taylor, 2015; Jhunjhunwala et al., 2021; Jia et al., 2015). Successful disruption of this pathway leads to a myriad of immunogenic peptides loaded on MHC-E that elicit diverse T cell responses (Table 1). These T cells responses are not limited to peptides derived from the pathogen, but also self-peptides that would normally be displaced by QDM or VL9. This secondary function of MHC-E has led to the hypothesis that MHC-E molecules monitor the MHC 1 processing pathway. In support of this, MHC-E restricted populations have been reported to respond to cells that lack transporter associated with antigen presentations (TAP) (Doorduijn et al., 2018) or endoplasmic reticulum aminopeptidase associated with antigen presentation (ERAAP) function (Nagarajan et al., 2012), which are key components of the MHC 1 processing pathway (Cifaldi et al., 2012; Hammer et al., 2007; Kotsias et al., 2019). Interestingly, both of these populations were found to be semi-invariant T cells (Table 1) (Doorduijn et al., 2018; Guan et al., 2017).

MHC-E appears to have alternate routes of peptide loading that are independent of the endoplasmic reticulum, the location of conventional MHC 1 peptide loading (Camilli et al., 2016; Smith et al., 2009). Aside from utilizing this conventional pathway, MHC-E appears to be able to load peptides from autophagosomes (Camilli et al., 2016). The constant recycling of MHC-E into the cell primes it to be introduced to phagosomal vesicles and be loaded with peptides. This alternate route of peptide loading can account for the responses to vesicle residing microbes like *M. Tuberculosis* (Grotzke et al., 2009; Harriff et al., 2017; Shang et al., 2016). In support of this, *M. Tuberculosis* infected dendritic cells are enriched for MHC-E compared to MHC 1a molecules (Grotzke et al., 2009) and there are over 60 HLA-E binding immunogenic peptides derived from *M. Tuberculosis* (McMichael and Picker, 2017). This alternate pathway has also been proposed to be a source for T cell derived peptides (Smith et al., 2009). This model states that endophagosomes containing apoptotic T cells are used to load recycled MHC-E molecules. It would be interesting to survey thymic phagocytic cells for this MHC-E pathway, as this would be an interesting source of self-peptides induced by cellular stress.

MHC-E restricted T cells have become the targets for vaccination strategies against HIV (McMichael and Picker, 2017; Sharpe et al., 2019). The proposed strategy was tested on rhesus macaques and their response to the simian immunodeficiency virus (SIV), the simian equivalent of HIV. They observed a broad response that was mediated mostly by MHC-E restricted CD8 T cells and that for every 100 amino acids of SIVgag approximately 4 epitopes were loaded on MHC-E (Walters et al., 2018). These numerous epitopes generated a substantial response in all of the subjects, where >50% of animals showed SIV replication arrest. Recently, the immunodominant peptide of this response was used to isolate human T cells that are capable of recognizing it. Interestingly, these MHC-E restricted T cells were able to eliminate HIV infected CD4+ T cells *in-vitro* (Yang et al., 2021).This observation brings new possibilities to vaccination strategies for elusive pathogens. The human population contains approximately 2 functional HLA-E alleles, which differ at a single amino acid (Arnaiz et al., 2022). This restricted diversity overcomes a pitfall that has bedeviled other vaccination strategies, where different MHC alleles present different peptides that elicit T cell responses of varying efficacies.

The literature on the development of MHC-E restricted T cells is limited. The bulk of the studies are centered on a $Qa1^b$ restricted T cell clone (6C5) that recognizes a peptide from beef insulin

(Sullivan et al., 2002; Tompkins et al., 1998) (Figure1D). A TCR transgenic model was developed utilizing the 6C5 TCR (Vα3.2 and Vβ5.1/2) and used to investigate thymic development of this MHC-E restricted T cells. Thymocytes bearing the 6C5 TCR showed high self-reactivity, required $Qa1^b$ for positive selection and could be selected by thymic hematopoietic cells. Interestingly, they showed that DP thymocytes were not responsible for presenting the positive selecting ligand (Sullivan et al., 2002). In contrast to 6C5, the thymic development of the semi-invariant $Qa1^b$ restricted T cell clone that monitors TAP deficiency was found to be $Qa1^b$ independent (Doorduijn et al., 2018), hinting at multiple thymic developmental pathways for MHC-E restricted T cells. This suggest that the development MHC-E restricted T cell differs from classical T cells as well as from the unconventional iNKT, MAIT and H2-M3 restricted cells. Further studies are required to dissect the development of MHC-E restricted T cells.

1.3 αβTCR Intraepithelial lymphocytes

The gut epithelium serves as a physical barrier between the internal body and the environment. Diverse microbes colonize the gut and form a symbiotic relationship with the host body. In this context, immune cells must be able to distinguish between potential pathogenic microbes and so called" commensal microbes". The epithelial lining of the gut and other mucosal surfaces are embedded with T cells called intraepithelial lymphocytes (IEL) that help maintain gut homeostasis. The thymic origins of these IELs is still being investigated. The IELs are generally classified as "natural" or "induced". The former are conventional αβT cells that migrated to the gut from peripheral tissues following antigen stimulation, while the latter are unconventional T cells that migrated to the gut directly from the thymus (Cheroutre et al., 2011; Gapin et al., 1999a). In general, natural IELs express the CD8αα homodimer, do not express activation markers and show an antigen experienced phenotype. The induced IEL are predominantly CD8αβ and show markers of recent activation. I will be focusing on the development of natural IELs

During positive selection some thymocytes that receive a strong agonist signal instead of being deleted undergo a lineage diversion to an IEL precursor (IELp) (Stritesky et al., 2012). Indeed, it has been shown that many negatively selected TCRs can give rise to IELs (McDonald et al., 2014, 2015). Following the strong TCR signal IELp downregulate CD4 and CD8 and continue to mature as DN thymocytes (Cheroutre et al., 2011; Mayans et al., 2014). Further characterization has led to the categorization of IELp as type A and type B (Ruscher et al., 2017). The Type A are highly self-reactive, reside in the thymic cortex, and are selected by MHC 1a. The type B are less self-reactive, prominent in the medulla and have a preference of MHC 1b. Interestingly, type B IELs are enriched for TCRα chain Vα3.2 and emigrate from the thymus with expression of Tbet, presumably with a Tbet induced effector program. These IELp may not be the only thymocytes that can become natural IELs. It has been shown that agonist selected CD8SP thymocytes can give rise to IELs when transferred to a recipient mouse (Kurd et al., 2021; Yamagata et al., 2004). Overall, IELp are heterogeneous group of thymocytes that utilize MHC 1a or MHC 1b, share the usage of self-reactive TCR clones and undergo lineage diversion to avoid negative selection

1.4 Virtual memory CD8 T cells

Memory T cells are an essential component of the adaptive immune system. Following the exposure to a pathogen a portion of the T cells that responded convert to a "memory" T cell. If re-exposure to the pathogen occurs, these memory T cells hasten the respond and poise the immune system to clear out the infection. The terms "virtual" memory (VM) or memory precursor T cells refers to T cells that display memory markers, but have not been primed by a foreign antigen (Jameson and Masopust, 2018; Kwesi-Maliepaard et al., 2021; Liu et al., 2015; Thiele et al., 2020). In fact, 15-20% of foreign antigen specific CD8 T cells are VM (Lee et al., 2013). These VM T cells respond faster to antigen than naïve and conventional memory T cells (Thiele et al., 2020; White et al., 2017). Interestingly, VM T cells were shown to be enriched for the TCRα chain Vα3.2 (Prasad et al., 2021). The thymic development of VM T cells is still unclear, but studies have shown that thymocytes that become VM T cells are strongly self-reactive (Drobek et al., 2018).

1.5 Concluding remarks

The innate immune system utilizes germline encoded receptors that recognize conserved motifs of distinct pathogens. These receptors are constantly surveying the environment and respond quickly to threats. On the other hand, the adaptive immune systems tailors its response to a particular pathogen and excels in its specificity. The trade-off is that the adaptive immune system is relatively slow and must design a response for each specific pathogen. The unconventional T cells provide the best of the adaptive and innate immune system. Their non-polymorphic MHC 1b proteins bind molecules that are present in multiple pathogens or self-molecules that are enriched in damaged cells. This approach to specificity positions unconventional T cells to respond to a broad set of pathogens and punishes pathogens for enacting their immune evasion strategies. Additionally, unconventional T cells can respond to their antigen rapidly, overcoming the compromise of the adaptive immune system. These properties have led to the hypothesis that unconventional T cells are an evolutionary bridge between the innate immune cells and the adaptive conventional T cells. Understanding the development of unconventional T cells can enhance our potential therapeutic uses for them and also inform on the evolution from innate to the adaptive immunity. The thymic development of MR1 and CD1d restricted T cells differs from conventional T cell selection at the critical DP stage and subsequently diverges into a separate pathway that employs different APCs and co-receptors. The development of MHC-E restricted T cells beckons further study. MHC-E is evolutionarily closer to classical MHC 1 than some other MHC 1b proteins, like CD1d and MR1. MHC-E restricted T cells may represent a population that is intermediate between conventional and iNKT/MAIT, and therefore may undergo a selection process that employs aspects of conventional and unconventional selection.

1.6 Figures

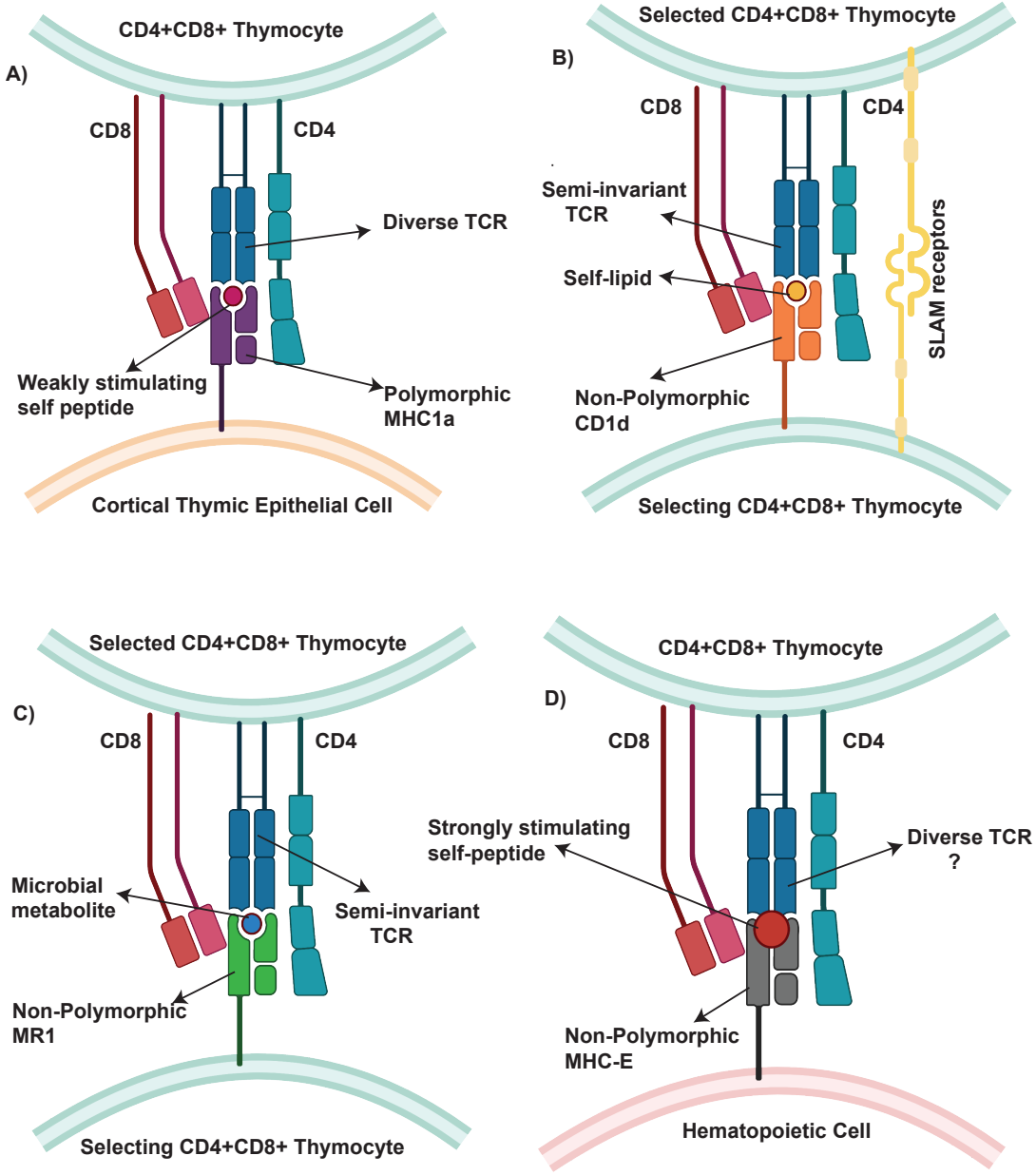

Figure 1: Positive Selection of αβ T cells. **A) Conventional:** DP(CD4+CD8+) thymocytes undergo positive selection by interacting with cortical thymic epithelial cells. The DP thymocytes can arrange a diverse set of TCR that can interact with MHC loaded with a weakly stimulating self-peptide. **B) iNKT:** selected DP thymocytes undergo positive selection by interacting with a selecting DP thymocytes. The semi-invariant TCR interacts with CD1d loaded with a self-lipid that strongly stimulates the selected DP thymocyte. The SLAM/SLAM homotypic interactions help dampen the strength of TCR stimulation, in order to prevent negative selection. **C) MAIT:** selected DP thymocytes undergo positive selection by interacting with a selecting DP thymocytes. The semi-invariant TCR interacts with MR1 loaded with a microbial derived metabolite that strongly stimulates the selected DP thymocyte. **D) MHC-E restricted:** DP thymocytes undergo positive selection by interacting with an unknown hematopoietic cell. The DP thymocytes can arrange a TCR of unknown diversity that can interact with MHC-E loaded with a strongly stimulating self-peptide.

Table1: MHC-E Restricted Responses

Pathogen /Deficiency	Host	Peptide Source	Peptide	T cell tracking method	TCR	References
S. typhimurium	Human/ Mouse	Salmonella HSP60	GMQFDRGYL	SalTc 1.69 clone established by limited dilution	Unknown	(Lo et al., 1999, 2000)
M. tuberculosis	Human/ Mouse	Mtb44/mtb32 and others	P55, P68	Isolated from infected host	Diverse	(Bian et al., 2017; Grotzke et al., 2009; Harriff et al., 2017)
TAP	Mouse	Med15	RLIIHFRDI	TCR transgenic	Semi-invariant (TRAV13-D4)	(Doorduijn et al., 2018)
ERAAP	Mouse	Fam49a/b	FYAEATPML	Tetramer enrichment	Semi-invariant (TRAV9-D3)	(Guan et al., 2017; Nagarajan et al., 2012)
Insulin	Mouse	Pork Insulin B chain	Unknown	TCR transgenic	Diverse	(Sullivan et al., 2002; Tompkins et al., 1998)
HIV/SIV	Human/ Rhesus Macaque	SIV/HIV Gag	SIV- RMYNPTNIL HIV- RMYSPTSIL	Isolate from infected host/ PBMCs exposed to RL9	Diverse	(Hansen et al., 2016; Yang et al., 2021)
Allogenic CD4 T cells	Mouse	TCR Vβ	Unknown	T cells mediating Heart graft rejection	Unknown	(Choi et al., 2020)
L. monocytogenes	Mouse	Unknown	Unknown	Isolated from infected host	Unknown	(Seaman et al., 1999)
Hepatitis C	Human	HCV core	YLLPRRGPRL	Isolated from infected host	Unknown	(Schulte et al., 2009)
EBV	Human	BZLF1	SQAPLPCVL	HLA-E expressing RMA-S loaded with synthetic peptides/Tetramers	Unknown	(Jørgensen et al., 2012)

Chapter 2

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

2.1 Introduction

Thymic development of conventional CD8 T cells requires low affinity, but specific recognition of self-peptides bound to MHC 1 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 1 (called MHC 1a) molecules, in contrast to unconventional T cell populations that recognize a diverse set of non-classical MHC 1 (called MHC 1b) (Legoux et al., 2017; Rodgers and Cook, 2005). MHC 1b molecules are structurally homologous to MHC 1a, and often associate with β2m, but are generally non-polymorphic, and can bind peptides or non-peptidic ligands (Adams and Luoma, 2013). 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. However, unlike conventional T cells, they recognize ligands presented by thymic antigen presenting cells (APCs) of hematopoietic origin (Bendelac, 1995; Seach et al., 2013), and show signs of strong TCR stimulation during development, termed agonist selection signaling (Stritesky et al., 2012). The development of T cells restricted to other MHC 1b molecules remains understudied (Bediako et al., 2012; Berg et al., 1999; Chiu et al., 1999; Cho et al., 2011; Doorduijn et al., 2016; Sullivan et al., 2002).

While MHC 1b restricted T cells are relatively rare in circulation, they contribute substantially to the intestinal epithelial lymphocyte (IEL) compartment (Das et al., 2000; Gapin et al., 1999; Park et al., 1999). 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 1a molecules and are derived from conventional CD8 T cells following antigen encounter in the periphery. 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 (Mayans et al., 2014; McDonald et al., 2014). Studies of the development of natural IELs 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 (Pobezinsky et al., 2012; Ruscher et al., 2017). However, it is unclear whether all natural IEL develop via an αβTCR+DN population. Moreover, while it is known that many natural IEL require β2m, but not MHC 1a molecules, for their development (Das et al., 2000; Gapin et al., 1999; Mayans et al., 2014; McDonald et al., 2014; Park et al., 1999), the specifity of IEL for particular MHC 1b molecules remains largely unknown. As a result, no studies to date have focused on the development of IELs specific for defined MHC 1b molecules.

The MHC 1b 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 (Godfrey et al., 2018; van Hall et al., 2010; Sharpe et al., 2019). In healthy cells, MHC-E molecules predominantly display a self-peptide derived from an MHC 1a leader

peptide (called QDM peptide in mouse), which serves as a ligand for NK receptors and provides an inhibitory signal to NK cells (Braud et al., 1998; Vance et al., 1998). However, under conditions of impaired MHC 1a presentation, such as deficiency in ERAAP (endoplasmic reticulum aminopeptidase associated with antigen processing) or TAP (transporter associated with antigen processing), the QDM peptide is replaced by an alternative set of peptides that can be recognized by CD8 T cells (Doorduijn et al., 2016, 2018; Hammer et al., 2007; Nagarajan et al., 2012). MHC-E restricted T cells responsive to TAP and ERAAP deficient cells have been proposed to play a role in monitoring defects in MHC 1a presentation induced by transformation or stress (Doorduijn et al., 2018; Nagarajan et al., 2012). In addition, pathogen-specific MHC-E restricted CD8 T cells can be activated upon infection with a variety of viruses and bacteria (Anderson et al., 2019; Bian et al., 2017; Lo et al., 1999; Seaman et al., 1999; Shang et al., 2016). 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 (Hansen et al., 2016; Yang et al., 2021). 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 called FL9 derived from Fam49a/b proteins (Nagarajan et al., 2012). QFL T cells were discovered as part of the mouse T cell response against cells deficient for the MHC 1a processing enzyme ERAAP, and they expand and acquire 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 1a-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, they use a semi-invariant TCR with a fixed TCRα and limited TCRβ usage (Guan et al., 2017). 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 (Jameson and Masopust, 2018; White et al., 2017). While QFL T cells can be detected using FL9-Qa1^b tetramers (called QFL tetramers) in wild type and Qa1^b deficient mice (Nagarajan et al., 2012), 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 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 1 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.

2.2 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 (Nagarajan et al., 2012) (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 (Guan et al., 2017). The double stained populations were considered "bonafide" 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- (Figure 2.1A). Interestingly, while mature QFL T cells in thymus and spleen predominantly expressed the CD8$\alpha\beta$ heterodimer (Supplementary Figure 2.1A-B), QFL T cells in the IEL compartment were a mixture of cells expressing CD8$\alpha\alpha$ or CD8$\alpha\beta$ (Supplementary Figure 2.1A-B). As previously reported (Nagarajan et al., 2012), QFL T cells were relatively abundant in the spleen of wild type mice (~ 1/6197 of CD8 T cells or ~1325/spleen/mouse, Figure 2.1B, C). For comparison, a previous study reported a range of 1/30,000 to 1/160,000 for conventional CD8 T cell populations (Obar et al., 2008). Additionally, 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 (Figure 2.1B). The frequency of QFL T cells out of mature CD8 T cells was higher in the SI IEL compared to the thymus and spleen, 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 $Qa1^b$ (Nagarajan et al., 2012). 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 (Azzam et al., 2001; Fulton et al., 2015; Mandl et al., 2013). As predicted, we observed that CD5 is elevated in QFL CD8SP thymocytes compared to conventional CD8 single positive (SP) T cells (Figure 2.1D). In addition, QFL CD8SP thymocytes showed slight but detectable downregulation of CD8β compared to conventional CD8SP T cells (Figure 2.1E), a phenotype that has been associated with thymocyte self-reactivity and agonist selection (Kurd et al., 2021; Yamagata et al., 2004).

As a complementary method to characterize QFL T cells, we developed a TCR transgenic mouse that expresses rearranged TCR transgenes encoding the semi-invariant QFL TCR α and β genes (Vα3.2 and Vβ1) from a QFL T cell clone (Guan et al., 2017), 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 (Figure 2.1D, Supplementary Figure 2.1C). 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 (Figure 2.1E). CD8SP thymocytes from QFLTg mice also show elevated levels of several markers associated with agonist selection, such as the transcription factors PLZF (Koay et al., 2016; Savage et al., 2008) and Tbet (Supplementary Figure 2.1D). On the other hand splenic, but not thymic, QFL CD8SP T cells express elevated levels of CD44, a marker associated with T cell proliferation (Figure 2.1F) (Supplementary Figure 2.1D). In addition, QFL CD8SP thymocytes from QFLTg mice did not show detectable upregulation of PD1 or α4β7, markers that are expressed by a subset of thymic IEL precursors (Supplementary Figure 2.1D) (Ruscher et al., 2017). 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 1

Previous reports showed that QFL T cells are detectable in the spleen of mice lacking Qa1^b, but undetectable in mice lacking β2m (Nagarajan et al., 2012), a subunit of MHC 1 which is required for proper folding and surface expression of both classical MHC 1a and Qa1^b (Robinson et al., 1998). This suggested the possibility that QFL T cells undergo positive selection on classical MHC 1a. 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 (Figure 2.2A). 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 β2mKO mice (Figure 2.2A). 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 β2m (Figure 2.2B). These data suggest that neither classical MHC 1a, 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 Vα3.2$^+$ cells compared to WT or Qa1^{b}KO mice (Supplementary Figure 2.2D), indicating that this V segment is preferentially used by T cells reactive to MHC 1b 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 (Kreslavsky et al., 2013; Lee et al., 2021; McDonald et al., 2014, 2015) whereas QFLTg Qa1^{b}KO mice express normal levels of CD4 and CD8α (Figure 2.2C). 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 (Figure 2.2D-F). In contrast, in the absence of classical MHC 1 (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 (Figure 2.2D-F). In the periphery, QFL T cells lost their antigen experienced phenotype in absence of Qa1^b, but not in absence of classical MHC 1 (Supplemental Fig. 2.2C). 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 (Davey et al., 1998; Lucas et al., 1999). We examined expression of activation markers on pre-selection QFLTg (preQFLTg) thymocytes from a β2mKO background after co-culture with bone marrow derived dendritic cells (BMDC) isolated from mice lacking either Qa1^b, K^{b}D^b or β2m. PreQFLTg thymocytes showed stronger upregulation of the activation markers CD69 and CD5 upon 24 hour co-culture with WT and K^{b}D^{b}KO, compared to Qa1^{b}KO BMDC (Figure 2.2G). This is consistent with the hypothesis that ERAAP sufficient cells can present low levels of the FL9 peptide on Qa1^b (Nagarajan et al., 2012). Interestingly, preQFLTg thymocytes showed a modest activation when co-cultured with Qa1^{b}KO BMDCs; significantly more compared to co-culture with β2mKO BMDCs (Figure 2.2G). A similar pattern of reactivity was observed when preQFLTg were cultured in thymic slices derived from WT, Qa1^{b}KO and β2mKO mice (Supplementary Figure 2.3). This is consistent with the development of QFL T cells in Qa1^{b}KO mice, and suggests that the QFL TCR is cross-reactive with an alternative β2m-utilizing molecule, most likely classical MHC 1.

Non-hematopoietic versus hematopoietic selection of QFL T cells

While conventional αβT cells undergo positive selection by recognition of MHC molecules on thymic epithelial cells, MAIT cells and iNKT cells undergo positive selection via interactions with hematopoietic cells (Bendelac, 1995; Seach et al., 2013). 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 classical MHC 1 molecules H2-D, H2-K, as well as Qa1 (Figure 2.3 and Supplementary Figure 2.4). Interestingly, comparable numbers of QFL T cells were found in the thymus and spleen of the β2mKO>WT and WT>β2mKO chimeric mice (Supplementary Figure 2.4B-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 (Figure 2.3A). 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 (Figure 3B), similar numbers of QFL T cells were found in the spleen (Figure 2.3C). Thus QFL T cell development is not strictly dependent on either non-hematopoietic or hematopoietic expression of MHC 1.

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 1 was restricted to non-hematopoietic cells (Figure 2.3D-E, Supplementary Figure 2.4D). Similarly, expression of CD5 was decreased when MHC 1 was restricted to non-hematopoietic cells (Figure 2.3F, Supplementary Figure 2.4E). In the periphery, QFL T cells in chimeric mice that lacked MHC 1 on

hematopoietic cells did not display elevated expression of CD44 (Figure 2.3G, Supplementary Figure 2.4F). Overall, the thymic phenotype of QFL T cells in β2mKO>WT chimeras is similar, but not identical, to that observed in Qa1^{b}KO mice (Figure 2.2D-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^{b}KO mice (Figure 2.4A-B), implying that agonist selection on Qa1^{b} led to greater functional responsiveness. Since agonist selection partially correlates with selection on hematopoietic cells (Figure 2.3, Supplementary Figure 2.3), we also examined QFL splenocytes from reciprocal β2mKO and wild type bone marrow chimeras mice as an further test of the impact of agonist selection on function. QFL T cells from QFLTg>β2mKO mice (HC selected) responded more robustly to ERAAPKO APCs compared to cells from QFLTg β2mKO>WT mice (non-HC selected) (Figure 2.4A,B). Thus QFL T cells that develop in the absence of Qa1^{b}, or in the absence of hematopoietically expressed MHC 1, 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 1, implying that they can give rise to intraepithelial lymphocytes. 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 (Figure 2.4C). 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 (Figure 2.4D). 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 (Figure 2.3C).

Although HC selected QFL T cells and HC+ non-HC selected QFL T cells were recovered from the IEL compartment in similar numbers (Figure 2.4D), HC selected QFL T cells showed more pronounced downregulation of CD8β (~50%) when compared to HC+EC selected QFL T cells (~10%)(Figure 2.4E). Interestingly, the CD8β downregulation observed in the IEL compartment of transferred Rag2KO mice is less pronounced that that observed in intact QFLTg mice (Supplementary Figure 2.1B). 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.

2.3 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 1b molecules. Moreover, while it is known that non classical MHC molecules contribute substantially to the CD8αα natural IEL compartment(Das et al., 2000; Gapin et al., 1999; Park et al., 1999) , and there is evidence that thymic mature CD4-CD8- cells contain IEL precursors (Golec et al., 2017; Klose et al., 2014; Pobezinsky et al., 2012; Ruscher et al., 2017) , 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 epithelial cells, but neither the restricting molecule Qa1^b, nor MHC 1 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 to tissues and exhibit preformed effector program. On the other hand, conventional CD8 T cells require selection by their restricting MHC 1a 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 1 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 (Sullivan et al., 2002), as well as a T cell population restricted to the MHC 1b molecule H2-M3 (Cho et al., 2011). While the M3 restricted cells required M3 expression for thymic selection, hematopoietic selection led to T cells with more unconventional functional properties compared to epithelial 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 1b molecules.

The IEL compartment harbors 2 distinct types of αβTCR+CD8+ T cells: CD8αβ T cells that are derived from conventional CD8 T cells following encounter with foreign antigen and differentiation into tissue resident T cells, and “natural” CD8αα IEL that are directed into an IEL program in the thymus due to their high self-reactivity. The observation that the same TCR clone can give rise to both types of IEL blurs the distinction between these two types of IEL.

Previous studies of natural IEL development have suggested a pathway in which some DP thymocytes that receive strong TCR signals escape clonal deletion and downregulate CD4 and CD8 to give rise to mature CD4-CD8- IELp that can eventually migrate to the gut and upregulate CD8αα (McDonald et al., 2018; Ruscher et al., 2017). Our data are consistent with an alternative pathway for natural IEL development in which agonist selection leads to a mature CD8αβ+CD4- thymic IELp, and complete downregulation of CD8β occurs after migration to 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 precursors to natural IEL (Kurd et al., 2021; Yamagata et al., 2004).

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 an CMV-vectored HIV vaccine showed extremely broad reactivity, with detectable responses to 4 epitopes for every 100 amino acids (Hansen et al., 2016b). In addition, another $Qa1^b$ restricted clone was shown to cross react with an MHC 1a molecule (Reed-Loisel et al., 2005), although it was dependent on $Qa1^b$ for its positive selection (Sullivan et al., 2002). In this regard, it is intriguing that QFL T cells show strong preferential use of Vα3.2 (encoded by TRAV9N-3) (Guan et al., 2017). Vα3.2 is preferentially used by CD8 compared to CD4 T cells(Sim et al., 1998) and has been suggested to be inherently reactive to MHC 1 (Prasad et al., 2021). In addition, Vα3.2 is enriched in a subset of natural IELs (Ruscher et al., 2017), and is used by another $Qa1^b$-restricted CD8 T cell clone (Sullivan et al., 2002). Moreover, we found that the frequency of Vα3.2+ CD8 T cells is substantially increased in K^bD^bKO mice (Supplementary Figure 2D). Altogether, these observations suggest that Vα3.2 may work together with $Qa1^b$, and perhaps other non-classical MHC 1 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 (McDonald et al., 2015), 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 1a molecules (Kambayashi et al., 2004; Sullivan et al., 2002, 2008), a property that may be linked to their atypical peptide presentation pathway (Grotzke et al., 2009; Smith et al., 2009) and/or low surface stability (Kambayashi et al., 2004) In addition, MHC-E molecules predominantly express a single self-peptide derived from MHC 1a leader peptides (Braud et al., 1997; Zeng et al., 2012), 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 1a peptide presentation pathway that occur upon viral infection or cellular transformation(Doorduijn et al., 2016; Nagarajan et al., 2012). According to this notion, MHC-E restricted T cells may undergo rare or transient encounters with high affinity self-peptide-MHC-E complexes during their development in the

thymus, allowing them to experience agonist selection signals while avoiding negative selection.

2.4 Figures

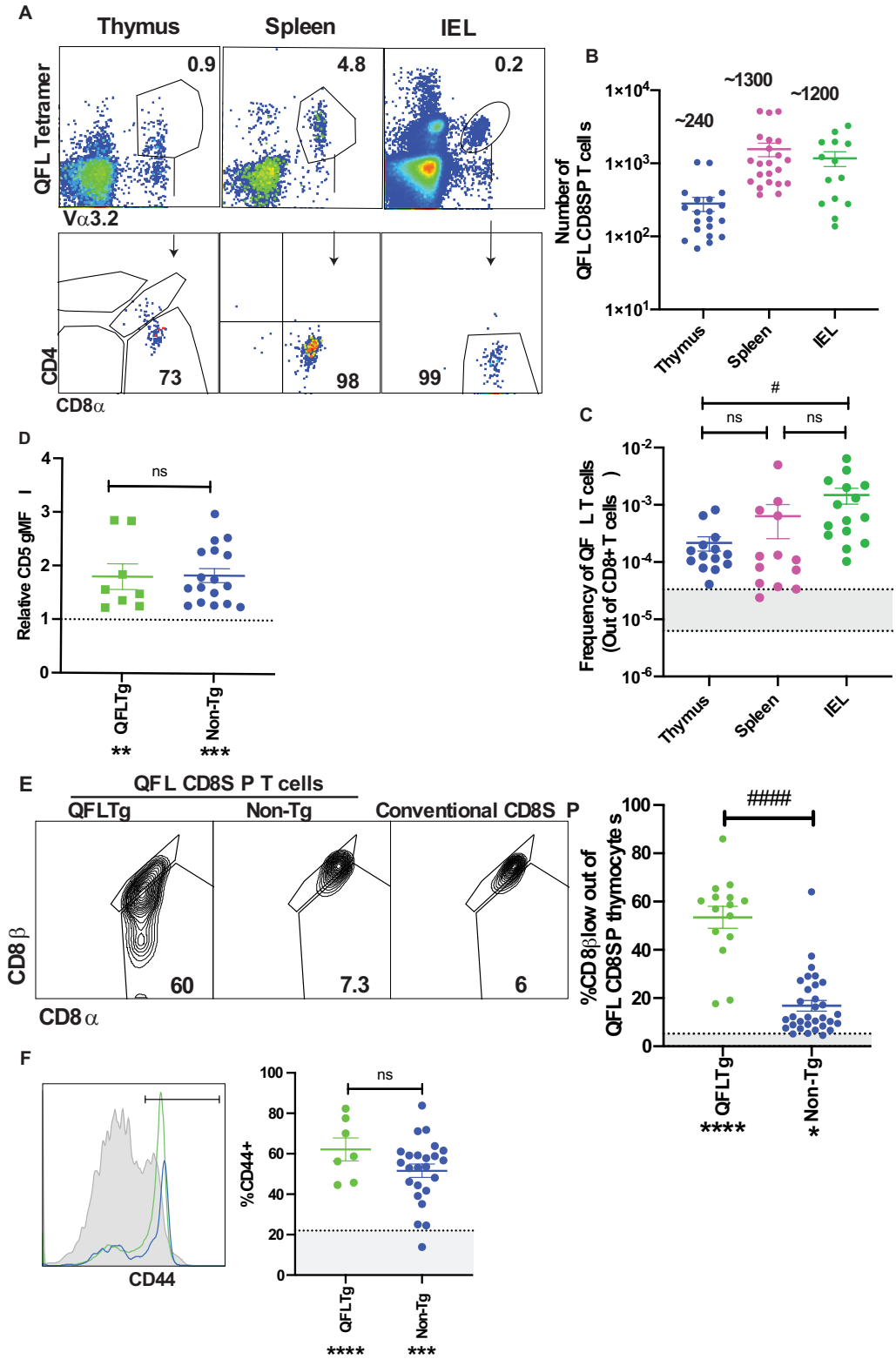
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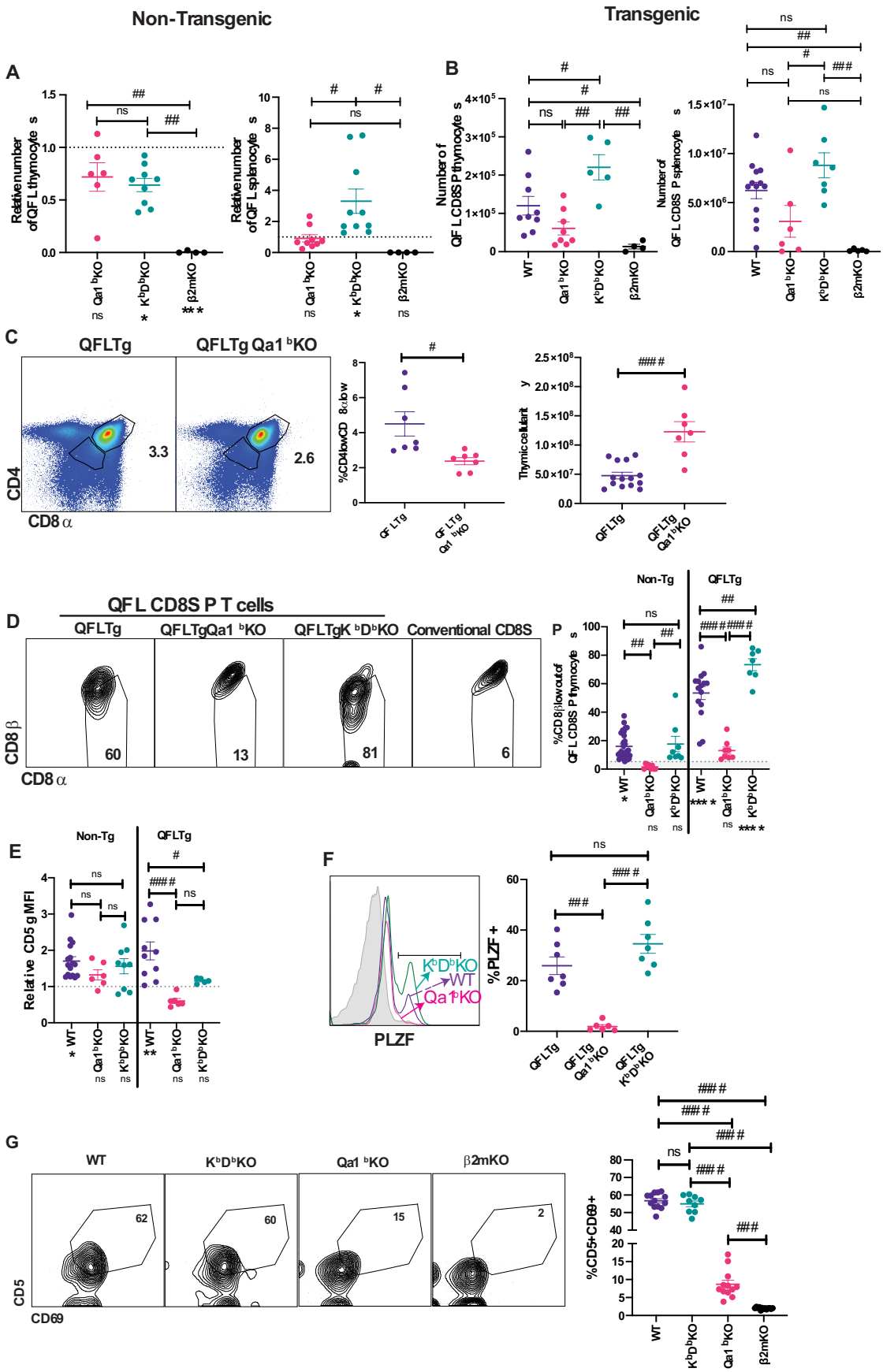
Figure 2.1: Characterization of QFL T cells in non-transgenic and transgenic mice. **A-C)** QFL T cells were identified by flow cytometry from wild type mice. **A)** Representative plots of QFL tetramer and Vα3.2 TCRα from tetramer enriched thymocytes, tetramer enriched splenocytes, and unenriched small 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 based on (Obar et al., 2008). **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 Figure 1C), non-transgenic tetramer enriched (Gated: QFL tetramer$^+$ and Vα3.2$^+$ CD8α$^+$CD4$^-$) thymi and 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


Non-Transgenic
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CD5
CD69
PLZF
3.3
2.6
60
13
81
6
62
60
15
2

Figure 2.2: 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. **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. **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 represents 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: 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 wild type samples (panel A), or to conventional CD8SP (panel B) (shown below sample name) * P value <0.05, ** P value <0.005 *** P value<0.0005.

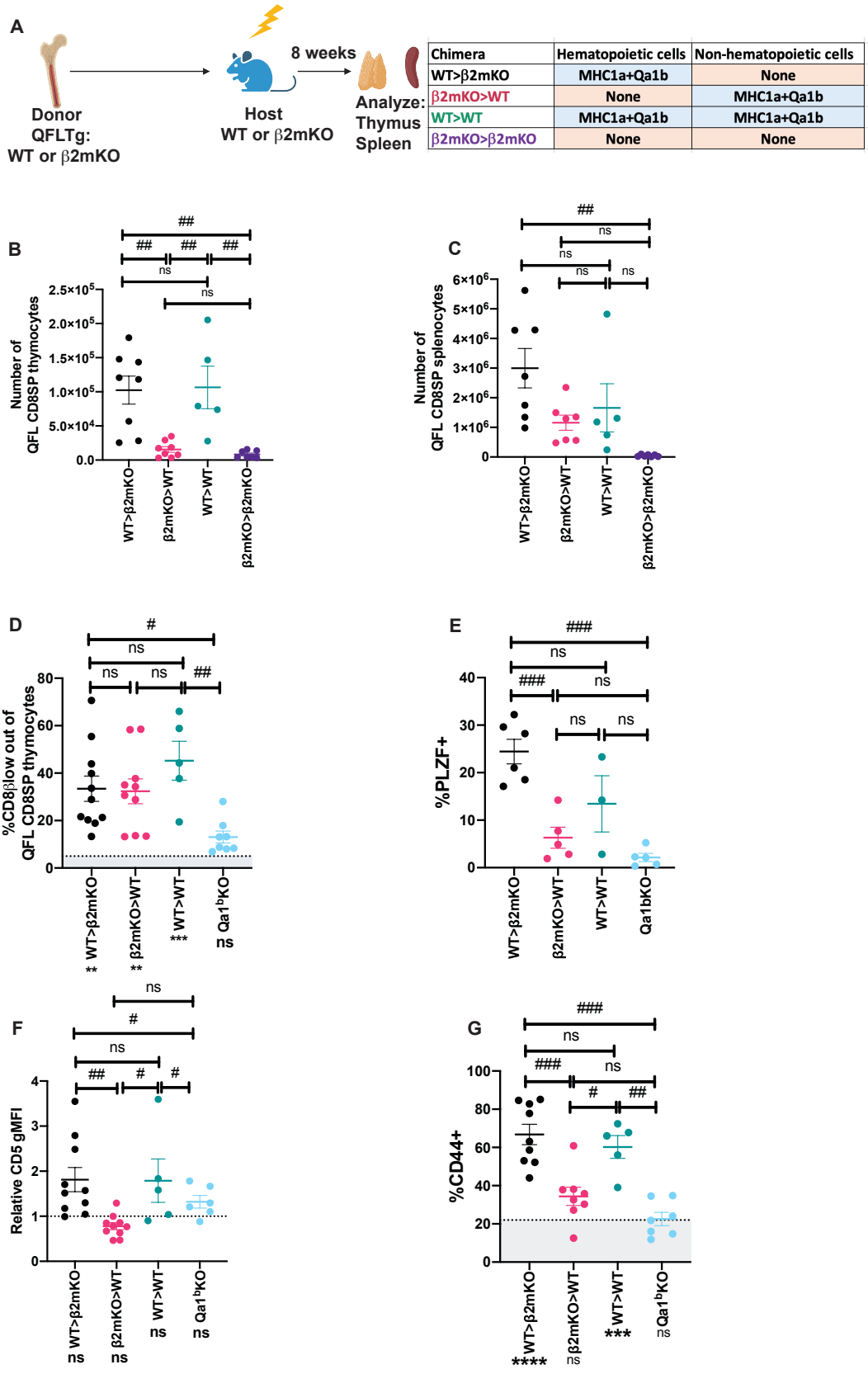

A
Donor
QFLTg:
WT or β2mKO
8 weeks
Host
WT or β2mKO
Analyze:
Thymus
Spleen
Chimera | Hematopoietic cells | Non-hematopoietic cells
WT>β2mKO | MHC1a+Qa1b | None
β2mKO>WT | None | MHC1a+Qa1b
WT>WT | MHC1a+Qa1b | MHC1a+Qa1b
β2mKO>β2mKO | None | None
B
Number of QFL CD8SP thymocytes
C
Number of QFL CD8SP splenocytes
D
%CD8βlow out of QFL CD8SP thymocytes
E
%PLZF+
F
Relative CD5 gMFI
G
%CD44+
WT>β2mKO
β2mKO>WT
WT>WT
β2mKO>β2mKO
Qa1bKO
Qa1bKO

Figure 2.3: Requirement for epithelial cell or hematopoietic cell in QFL 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 1 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$^-$). **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. Data from QFLTg Qa1^{b}KO mice is from Figure 2D and is included for comparison. 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%). 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. 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.

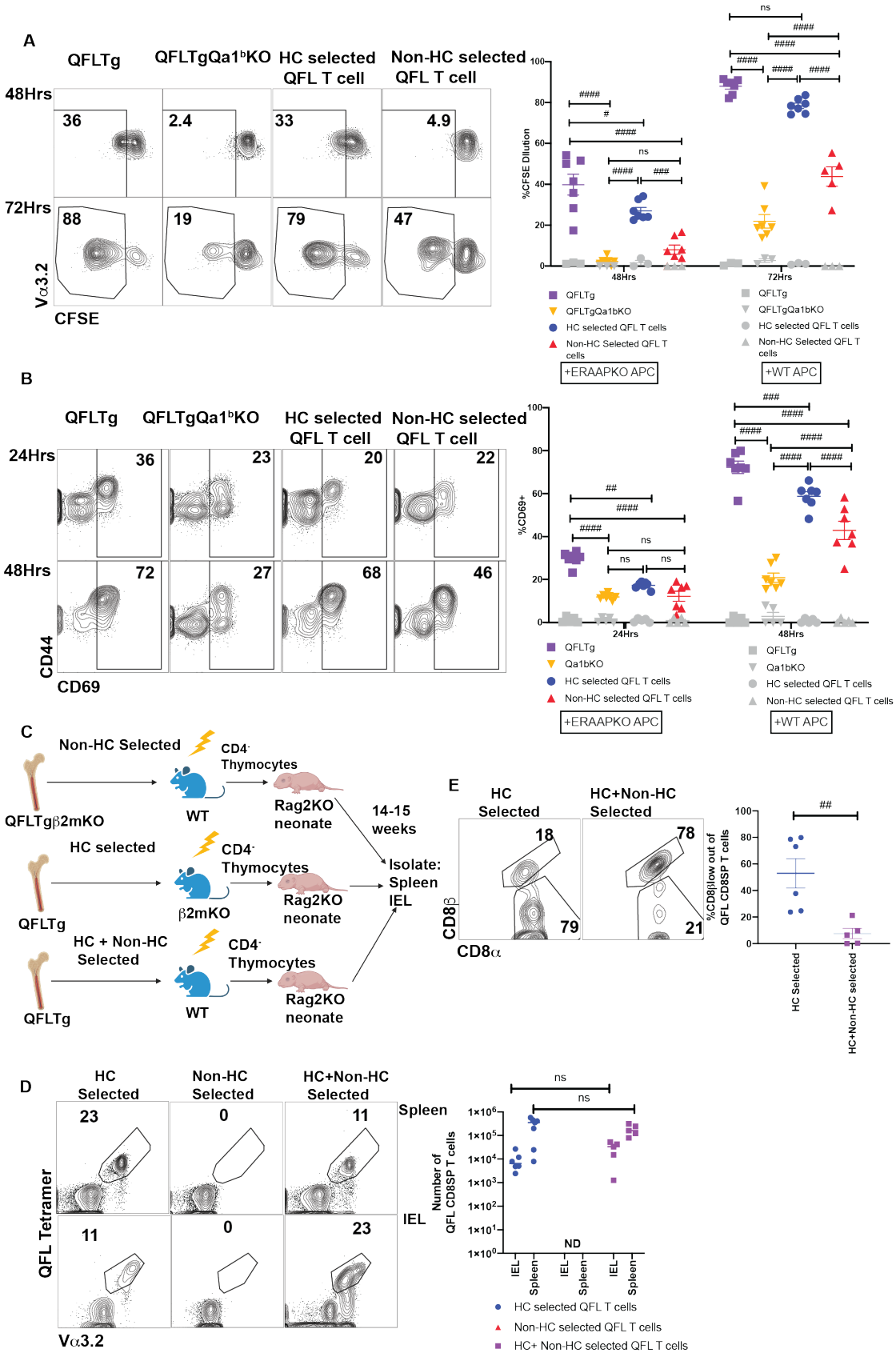
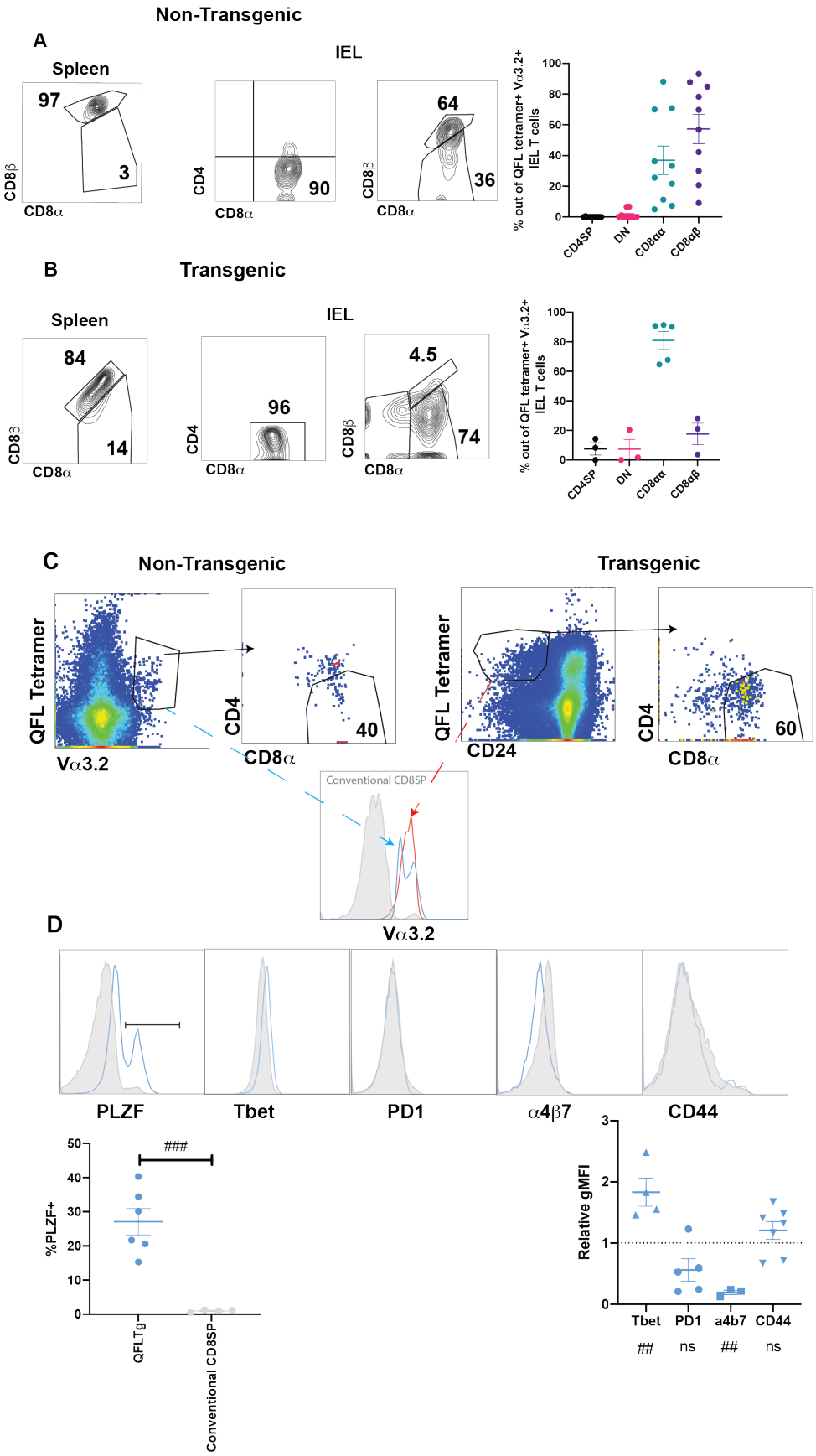

A
QFLTg
QFLTgQa1bKO
HC selected QFL T cell
Non-HC selected QFL T cell
48Hrs
72Hrs
36
2.4
33
4.9
88
19
79
47
Vα3.2
CFSE
%CFSE Dilution
48Hrs
72Hrs
QFLTg
QFLTgQa1bKO
HC selected QFL T cells
Non-HC Selected QFL T cells
+ERAAPKO APC
+WT APC
B
QFLTg
QFLTgQa1bKO
HC selected QFL T cell
Non-HC selected QFL T cell
24Hrs
48Hrs
36
23
20
22
72
27
68
46
CD44
CD69
%CD69+
24Hrs
48Hrs
QFLTg
Qa1bKO
HC selected QFL T cells
Non-HC selected QFL T cells
+ERAAPKO APC
+WT APC
C
Non-HC Selected
QFLTgβ2mKO
CD4- Thymocytes
WT
Rag2KO neonate
14-15 weeks
HC selected
QFLTg
β2mKO
Isolate: Spleen IEL
HC + Non-HC Selected
QFLTg
WT
E
HC Selected
HC+Non-HC Selected
18
79
78
21
CD8β
CD8α
%CD8βlow out of QFL CD8SP T cells
HC Selected
HC+Non-HC selected
D
HC Selected
Non-HC Selected
HC+Non-HC Selected
23
0
11
11
0
23
QFL Tetramer
Vα3.2
Spleen
IEL
Number of QFL CD8SP T cells
ND
IEL
Spleen
HC selected QFL T cells
Non-HC selected QFL T cells
HC+ Non-HC selected QFL T cells

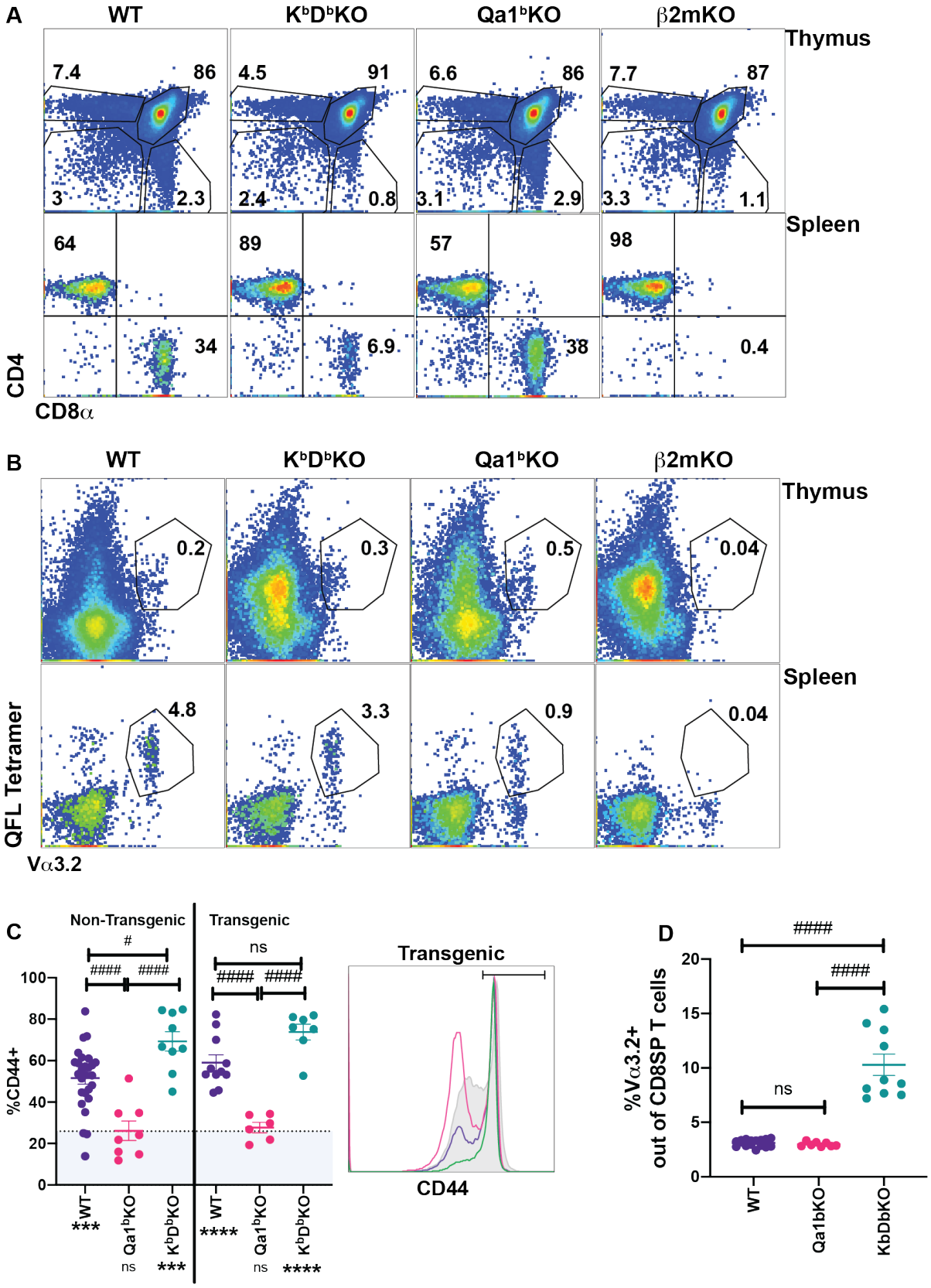
Figure 2.4: Agonist selected QFL T cells respond rapidly to antigen exposure and home to the IEL compartment.

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. ND=non detectable Plots on the right show the number of QFL tetramer^{+}Vα3.2^{+}CD8α^{+} cells recovered from each sample. **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

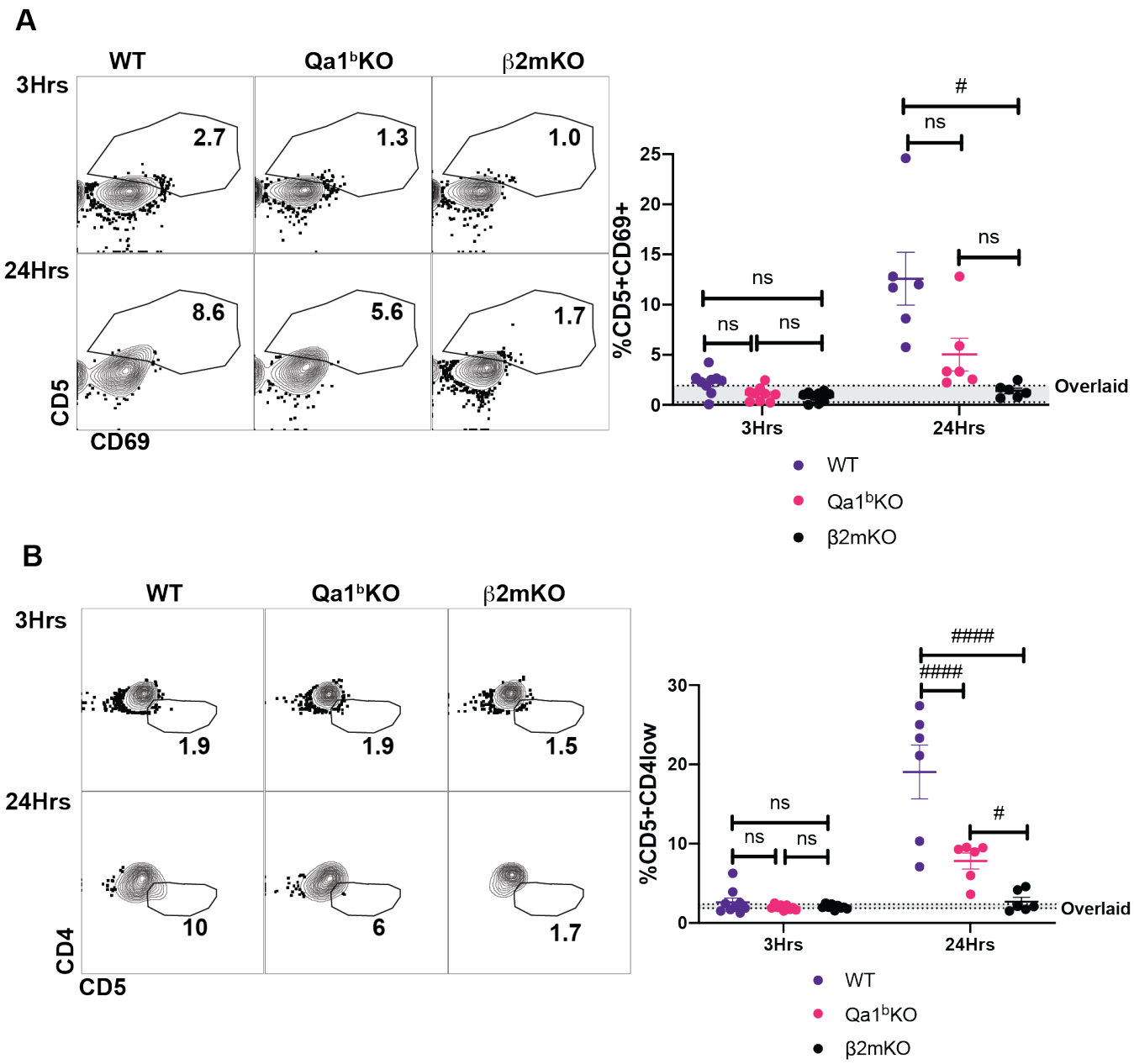

Non-Transgenic
A
Spleen
IEL
97
3
CD8β
CD8α
CD4
90
64
36
% out of QFL tetramer+ Vα3.2+ IEL T cells
CD4SP
DN
CD8αα
CD8αβ
B
Transgenic
84
14
96
4.5
74
C
Non-Transgenic
Transgenic
QFL Tetramer
Vα3.2
40
CD24
60
Conventional CD8SP
D
PLZF
Tbet
PD1
α4β7
CD44
%PLZF+
###
QFLTg
Conventional CD8SP
Relative gMFI
Tbet
PD1
a4b7
CD44
##
ns
##
ns

Supplemental Figure 2.1: QFL T cell characterization. **A-B) (Left)** Representative plots of CD8a and CD8b of QFL CD8SP splenocytes **(Right)** Representative plots CD4, CD8α and CD8β of QFL T cells in the SI IEL compartment. Quantification of CD4, CD8α and CD8β surface expression on QFL T cells (Gated: TCRβ^+QFLtetramer$^+$Va3.2$^+$) in the SI IEL compartment of non-transgenic **(A)** and QFLTg **(B)** mice. CD4SP (CD4$^+$CD8α^-) (Black dots), DN (CD4-CD8α^-)(Magenta dots), CD8$\alpha\alpha$(CD4$^-$CD8α^+CD8β^-) (Teal dots), CD8$\alpha\beta$ (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 $\alpha4\beta7$ 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, $\alpha4\beta7$ 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.

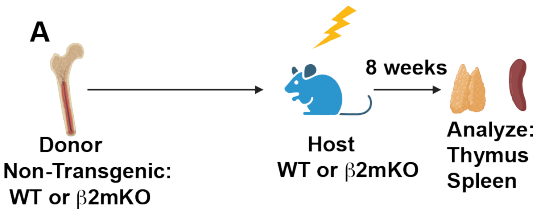

A
WT
KbDbKO
Qa1bKO
β2mKO
Thymus
Spleen
7.4
86
3
2.3
4.5
91
2.4
0.8
6.6
86
3.1
2.9
7.7
87
3.3
1.1
64
34
89
6.9
57
38
98
0.4
CD4
CD8α
B
WT
KbDbKO
Qa1bKO
β2mKO
Thymus
Spleen
0.2
0.3
0.5
0.04
4.8
3.3
0.9
0.04
QFL Tetramer
Vα3.2
C
Non-Transgenic
Transgenic
%CD44+
WT
Qa1bKO
KbDbKO
Transgenic
CD44
D
%Vα3.2+ out of CD8SP T cells
WT
Qa1bKO
KbDbKO
ns

Supplemental Figure 2.2: Phenotype of KbDbKO and Qa1bKO thymus and spleen

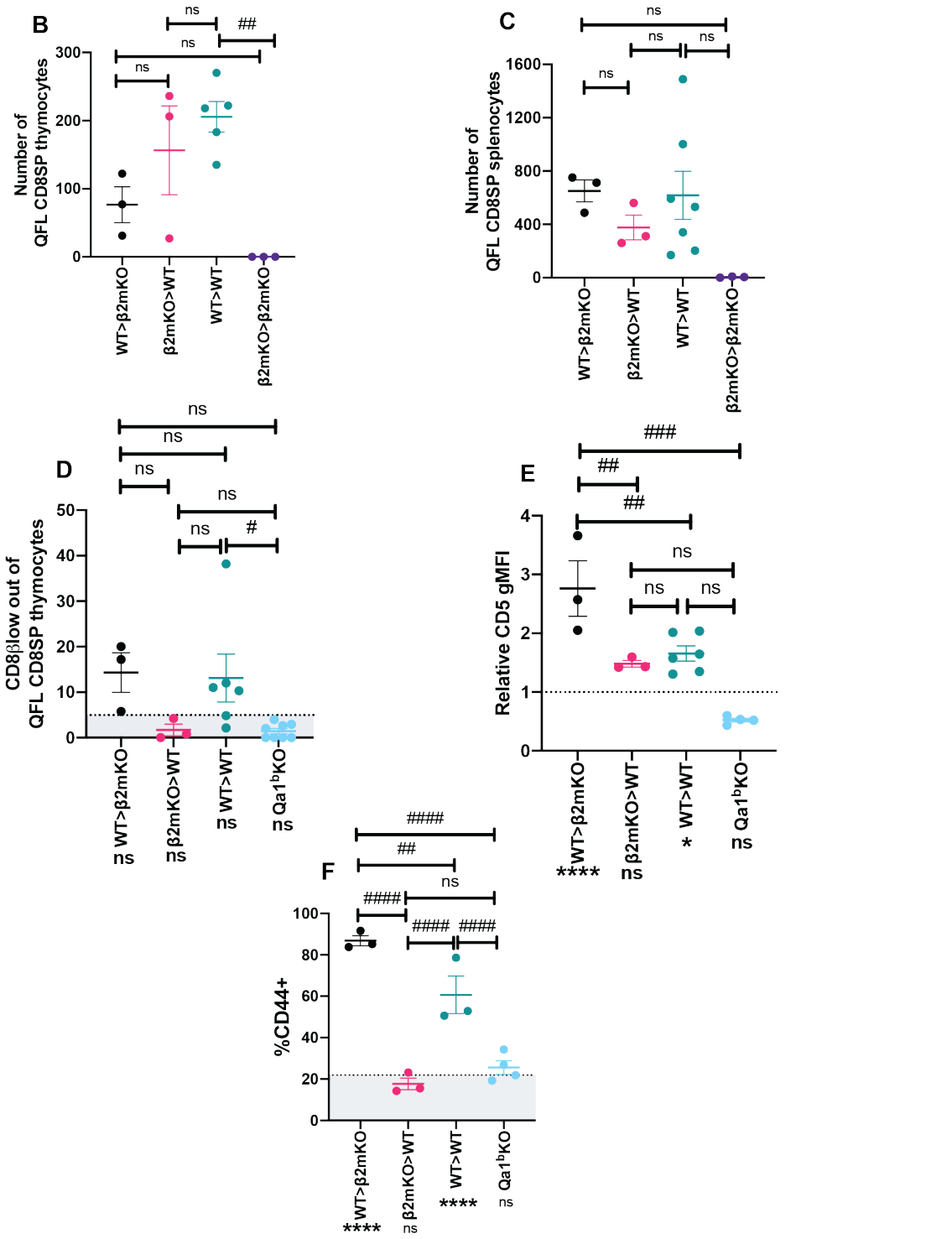
A) Representative flow plots of CD4 and CD8α expression on unenriched WT, K^bD^bKO, Qa1^{b}KO and β2mKO thymocytes (Gated: Live cells) and splenocytes (Gated: TCRβ^+). **B)** Representative flow plots of QFL tetramer and Vα3.2 expression on tetramer enriched thymocytes (Gated: Live) and splenocytes (Gated: TCRβ^+) of B6, K^bD^bKO, 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^bD^bKO 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 2.3: 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



Chimera	Hematopoietic cells	Non-hematopoietic cells
WT>β2mKO	MHC1a+Qa1b	None
β2mKO>WT	None	MHC1a+Qa1b
WT>WT	MHC1a+Qa1b	MHC1a+Qa1b
β2mKO>β2mKO	None	None



Supplemental Figure 2.4: Requirement of Hematopoietic cells and Non-hematopoietic cells 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 1 expression to hematopoietic or non-hematopoietic cells. **B-C)** Absolute numbers of QFL CD8SP T cells in chimeras generated using non-transgenic β2mKO or wild type donors and irradiated β2mKO or wild type hosts. **(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 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)** 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: 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

2.5 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(Hu et al., 2004) 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 (Guan et al., 2017; Nagarajan et al., 2012) were cloned and amplified from the genomic DNA of the BEko8z Hybridoma. The TRAV9D-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 Xmal and Not1 sites, while the TCR beta chains were cloned into pTβ cassette vector in between the Xhol and SacII sites (Kouskoff et al., 1995). 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 Ly5.1 mice to generate (QFLTgxB6 Ly5.1/2) 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 superovulated 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 (Chen et al., 2016) .The embryos were then transferred to 0.5dpc pseudopregnant 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 (1% 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 (Lefrançois and Lycke, 2001). 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 (Ruscher et al., 2017). 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.

Neonatal Chimeras

Donor bone marrow cells were isolated as described above and RBC 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+, CD19+, and NK1.1+ cells using a magnetic column as described above. Bone marrow cells were resuspended in PBS and $3x10^5$-$4x10^5$ cells were intrahepatically injected into 5-7 day old recipient mice.

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 NH_4Cl, 1mM $KHCO_3$, 0.1mM Na_2EDTA) 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.

Chapter 3

Addendum and Future Directions

3.1 Characterization of the alternate MHC 1 ligand and its role in QFL T cell development

Role of alternate MHC 1 ligand in QFL IEL development

In the previous chapter, we showed that QFL T cells are flexible in their requirements for positive selection. The presence or absence of Qa1^b did not impair the development of QFL T cells, but played a role in their phenotype and overall functionality. Importantly, the high self-reactivity and downregulation of CD8β of QFL thymocytes was dependent on Qa1^b. The loss of agonist selection may alter the QFL T cell lineage commitment to IEL precursors. Interestingly, QFL T cells were identifiable in the IEL compartment of non-transgenic Qa1^{b}KO mice in comparable numbers to WT mice (Figure 3.1A). QFL IEL T cells were also detectable in QFLTg Qa1^{b}KO and QFLTg K^{b}D^{b}KO mice in similar numbers to QFLTg mice(Figure 3.1B). In our hands, the proportion of CD8$\alpha\alpha$ and CD8$\alpha\beta$ QFL IEL T cells was also unaffected by the presence or absence of Qa1^b or K^{b}D^b(Figure 2.1C). Our collaborators observed conflicting results, where CD8$\alpha\alpha$ QFL T cells were reduced in non-transgenic Qa1^{b}KO mice (unpublished results). These data suggest that QFL T cells can populate the small intestine of Qa1^{b}KO mice and that QFL CD8$\alpha\alpha$ a may be affected in absence of Qa1^b.

Influence of the alternate MHC 1 ligand in hematopoietic or non-hematopoietic driven QFL T cell development

The QFL T cells selected by the non Qa1^b alternative MHC 1 ligand display a conventional phenotype. Since conventional T cells are selected by MHC 1a on thymic epithelial cells, we reasoned that phenotypically conventional QFL T cells would follow the same pattern. Therefore, we hypothesized that MHC 1a on non-hematopoietic cells might be required for positive selection of conventional QFL T cells. To investigate the role of the alternative MHC 1 ligand on hematopoietic or non-hematopoietic cells for QFL T cell selection, we generated reciprocal bone marrow chimeric mice in which Qa1^b was absent and the alternative MHC 1b ligand was present either on hematopoietic cells (QFL transgenic Qa1^{b}KO donors into β2mKO hosts) or non-hematopoietic cells (QFL transgenic β2mKO donors into Qa1^{b}KO hosts) (Fig 3.2A). Interestingly, QFL T cells were recovered in similar numbers in both thymus (Figure 3.2B) and spleen (Figure 3.2C) regardless of MHC 1a expression on hematopoietic or non-hematopoietic cells. Additionally, QFL T cells did not show extensive CD8β downregulation (Figure 3.2D) and maintained CD5 levels comparable to conventional CD8SP, confirming the requirement for Qa1^b for these phenotypes. Taken together these data suggest that QFL T cells can be positively selected by an alternate MHC 1 ligand presented by either hematopoietic or non-hematopoietic cells.

Identifying the alternate MHC 1 Ligand

The QFL thymocyte alternative MHC 1 ligand remains to be identified. We hypothesized that a MHC 1a molecule could provide a positive selecting signal to pre-selection QFL thymocytes. This

was tested by co-culturing pre-selection QFL thymocytes with Qa1^{b}KO BMDCs pre-treated with αK^{b}, αD^{b} or $\alpha K^{b}D^{b}$ blocking antibodies. Following 24 hours of co-culture, QFL thymocytes were assessed for upregulation of activation markers CD5 and CD69. Surprisingly, pre-treatment with blocking antibodies did not significantly alter the upregulation of activation markers of QFL thymocytes (Figure 3.3). A slight reduction was observed when BMDCs were pre-treated with αK^{b}, but it was not statistically significant. This result should be interpreted with the caveat of observable background staining and spread of data points in the blocking antibody conditions. This assay may not have the resolution to properly identify subtle differences in activation marker expression. Alternative approaches should be employed to address this question.

Concluding remarks

QFL T cells can develop in absence of their cognate Qa1^{b} ligand. Albeit, the QFL T cells that are selected on this alternative MHC 1 ligand are less functional compared to their Qa1^{b} selected counterparts. Interestingly, QFL T cells can populate the IEL compartment regardless of Qa1^{b} exposure. Additionally, the alternative MHC 1 ligand can be presented by hematopoietic or non-hematopoietic cells. The QFL population may avoid deletion by possessing a promiscuous TCR. Unfortunately, the degree of cross-reactivity of this TCR remains to be determined. Future studies need to be made to pinpoint the identity of the alternative MHC 1 ligand or ligands. Overall, QFL T cells represent an unconventional T cell population that challenge our understanding of “requirements” for thymocyte positive selection.

3.2 Figures

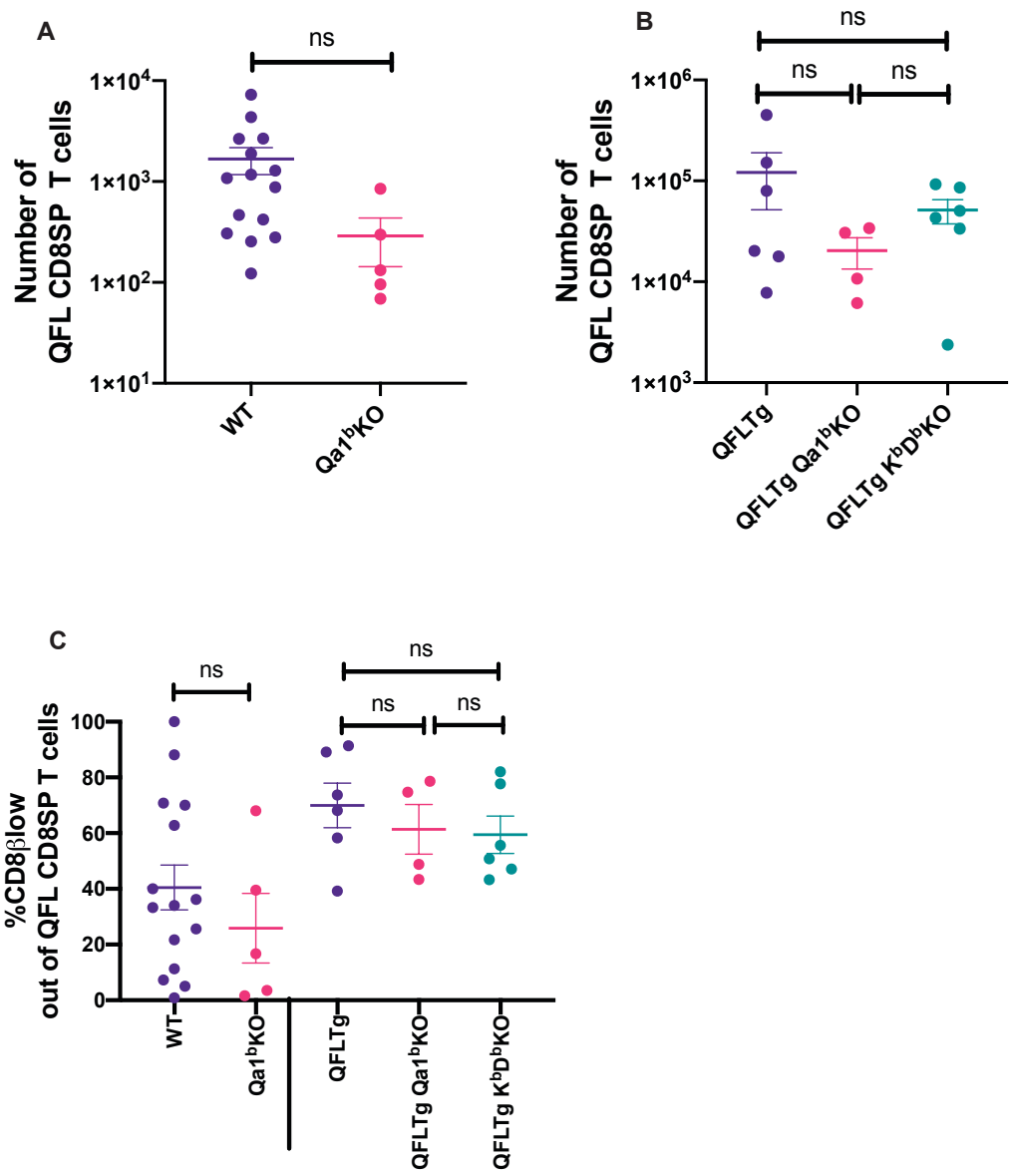
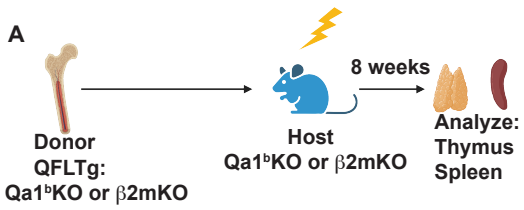
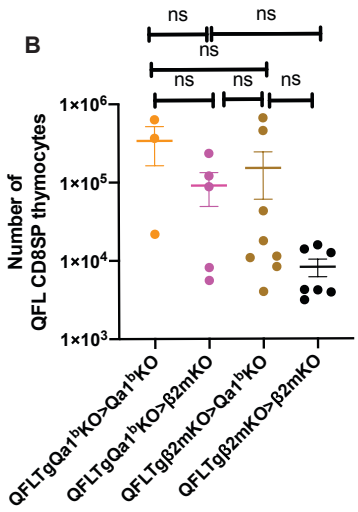


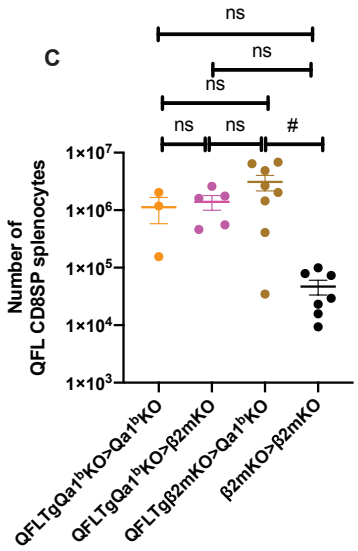
Figure 3.1: MHC requirements for QFL T cells in the IEL compartment of the small intestine

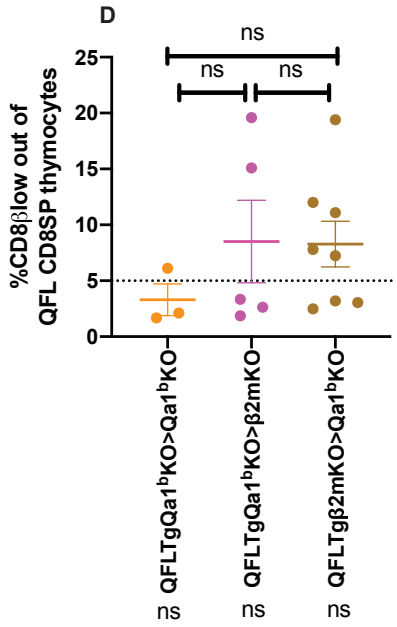
A-B) Number of QFL CD8SP T cells in the IEL compartment of the small intestine in **A)** non-transgenic of indicated genotype or **B)** QFLTg crossed to the indicated genotype (Gated: QFLtetramer$^+$Vα3.2$^+$TCRβ^+CD8α^+CD4$^-$). **C)** Graph shows % of CD8β low out of QFL CD8SP T cells in the IEL compartment of the small intestine from **(Left of the line)** non-transgenic of the indicated genotype or **(right of the line)** QFLTg crossed to the indicated genotypes (Gated: QFLtetramer$^+$Vα3.2$^+$TCRβ^+CD8α^+CD4$^-$). Statistical analysis: 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.



Chimera	Hematopoietic cells	Non-hematopoietic cells
Qa1bKO>Qa1bKO	MHC1a	MHC1a
Qa1bKO>β2mKO	MHC1a	None
β2mKO>Qa1bKO	None	MHC1a
β2mKO>β2mKO	None	None







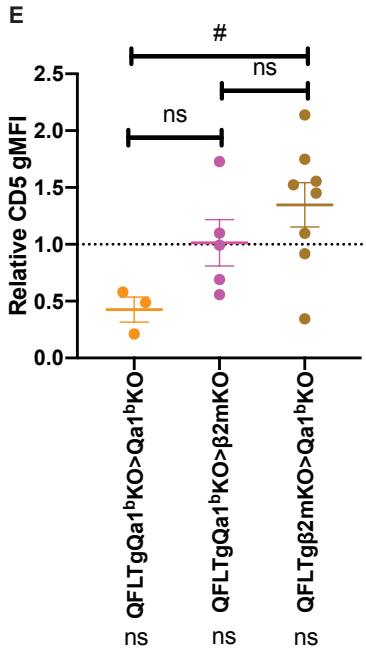


Figure 3.2: Requirement for MHC 1a on Non-hematopoietic cell or hematopoietic cell in QFL selection **A)** Diagram of experimental design. QFLTg Qa1^{b}KO or QFLTg β2mKO mice were used as bone marrow donors to reconstitute irradiated β2mKO or Qa1^{b}KO hosts in order to restrict MHC 1a expression to 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^{-}). **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)** Graph shows gMFI of CD5 expression of QFL CD8SP thymocytes normalized to the gMFI of conventional CD8SP thymocytes from wild type mice analyzed in the same experiment. 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. 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.

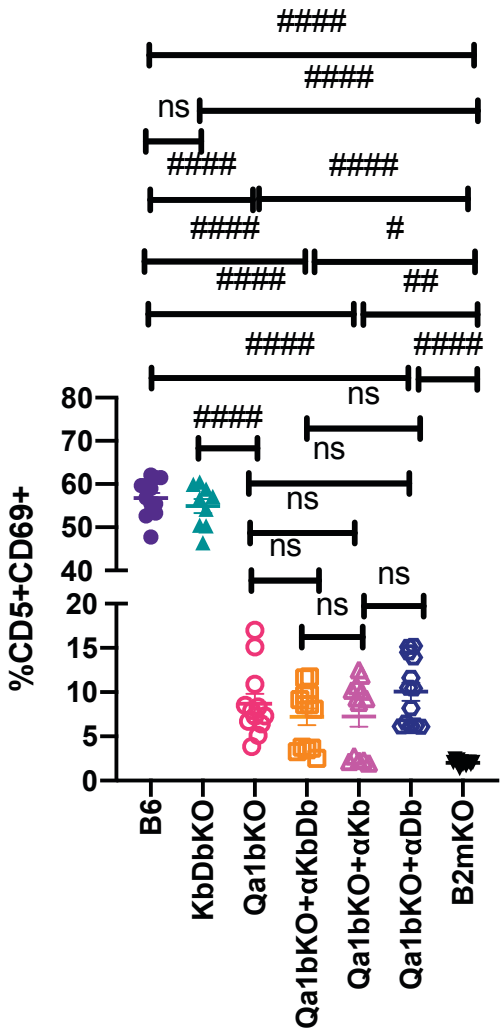


Figure 3.3: Identifying alternative QFL T cell ligand by utilizing blocking antibodies

Pre-selection QFL thymocytes (from QFLTg β2mKO mice) were co-cultured with Bone Marrow Derived Dendritic cells (BMDC) from the indicated mouse strains. Where indicated BMDCs were pre-treated with blocking antibodies specific for K^b(Clone:AF6-88.5), D^b (Clone:KH95) or a mixture of both antibodies at 0.1mg/mL for 30mins at 37C prior to co-culture with pre-selection QFL thymocyte. Dot plots show compiled data of CD5 and CD69 expression on QFL DP thymocytes (Gated:QFLtetramer$^+$Vα3.2$^+$CD4$^+$CD8α$^+$) after 24 hours of co-culture, with each dot representing a sample from an individual culture well. Statistical analysis: 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.

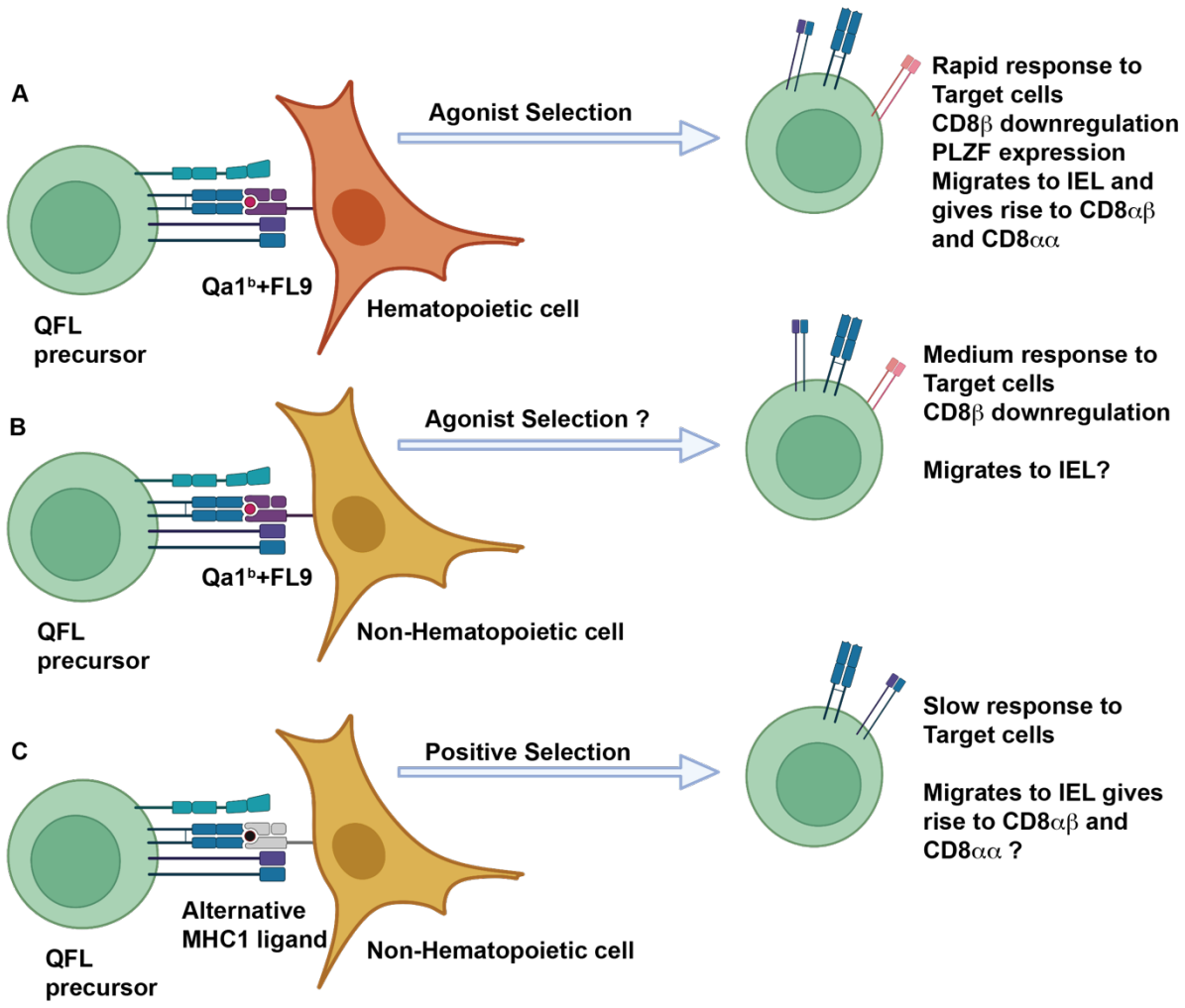


Figure 3.4: Model for QFL T cell development

QFL precursors at the DP(CD4+CD8+) stage audition their semi-invariant TCR (Vα3.2+Jα21+Vβ1) by interacting with: **A)** a hematopoietic cell presenting Qa1^{b}+FL9 providing an agonist selecting signal. The resulting QFL T cell acquires an innate-like phenotype and exits the thymus fully differentiated. These QFL T cells can also migrate to the small intestine and give rise to QFL CD8αα and CD8αβ IELs. **B)** a non-hematopoietic cell presenting Qa1^{b}+FL9 providing a somewhat strong signal. The resulting QFL T cell shows downregulation of CD8b and mount a limited response to target cells. It is unclear if these QFL T cells migrate to the small intestine. **C)** a non-hematopoietic cell presenting an alternative MHC 1 ligand providing a weak positive selecting signal. The resulting QFL T cell exits the thymus, can mounts a slow response to target cells and migrate to the small intestine. It is unclear if these QFLT cells can give rise to CD8αα and CD8αβ QFL IELs.

3.3 Future Directions

In the previous sections and chapters, I have shown that the thymic context of QFL T cell selection determines the type of QFL T cell that develops (Figure 3.4). The QFL thymocyte can undergo agonist selection by encountering Qa1^{b}+FL9 in the thymus or positive selection by encountering an unknown alternate MHC 1 ligand. If the QFL thymocyte is agonist selected by a hematopoietic cell, it acquires innate like characteristics and phenotypically resembles an unconventional $\alpha\beta$T cell. If the QFL thymocyte is selected by a non-hematopoietic cell presenting Qa1β+FL9, it shows a phenotype that is intermediate between unconventional and conventional $\alpha\beta$ T cells. If the QFL thymocyte is positively selected by an alternate MHC 1 ligand presented by a non-hematopoietic cell, it resembles a conventional $\alpha\beta$ T cell. The QFL TCR has an affinity for the IEL compartment, but it is unclear if there is a specific or multiple QFL developmental pathways that gives rise to QFL IELs. Additionally, the degree of cross-reactivity of the QFL TCR remains to be determined. Finally, the transcriptional program that commit a QFL T cells to an innate-like or conventional like T cell remains to be understood.

Defining the degree of QFL TCR cross-reactivity

In chapter 2, I showed that the QFL TCR is cross-reactive with a non-Qa1^{b} MHC 1 molecule. I hypothesize that it is cross reactive with MHC 1a (H2-K/D). I attempted to test this hypothesis utilizing αK and αD blocking antibodies in a BMDC co-culture system. Unfortunately, the results were inconclusive and a better method is needed to test this hypothesis. A pitfall of the previous system was that it relied on almost complete blocking of H2-K/D, which is not guaranteed. Additionally, the QFL TCR could be cross-reactive with multiple MHC molecules and this approach would only block classical MHC 1a. Therefore, an alternate system that assures the expression of a single MHC molecule would have better resolution in defining the QFL alternate ligand or ligands. In collaboration with the Coscoy lab we're generating DC cells lines that are Qa1^{b}+ K^{b} and/or D^{b} deficient. This strategy will limit the potential source of MHC 1 presentation to a single MHC 1a or other MHC 1b molecules. Once these lines are generated, we will co-culture them with pre-selection QFL thymocytes for 24Hrs and by flow cytometry measure upregulation of activation markers CD5 and CD69. I predict that both K^{b} and D^{b} can provide a weak stimulation to QFL thymocytes. An interesting outcome could be that QFL thymocytes can be stimulated by MHC 1a and/or multiple MHC 1b molecules. This would imply that the QFL TCR is extremely cross-reactive. This strategy of limiting MHC 1a expression could be applied to test if QFL T cells can be stimulated by differing haplotypes. The QFL T cells have been identified in B6 mice which are H2^{b}, but it is possible that H2^{k,q,d} bearing mice could harbor QFL T cells, especially since Qa1^{b} is non-polymorphic. In fact, 6C5 T cells which were discovered in B6 mice and use the same Vα3.2 as QFL T cells, were shown to be cross reactive with H2^{q} (Reed-Loisel et al., 2005). This extensive cross-reactivity maybe a general property of Vα3.2 and/or MHC-E restricted T cells.

Dissecting the source of QFL specific IELs

In the previous chapters we found that QFL T cells can be isolated in the small intestine regardless of Qa1^b expression. This was an unexpected observation considering that QFL T cells require Qa1^b for agonist selection and IEL precursors are highly self-reactive (Kurd et al., 2021; Yamagata et al., 2004). Positive selected QFL T cells were shown to functionally limited and perhaps not fully differentiated compared to agonist selected QFL T cells(Figure 2.4). It would be reasonable to think that positive selected QFL T cells require an additional maturation event in order to gain their effector phenotype. Furthermore, it is unclear if QFL IELs migrate directly from the thymus as committed IEL precursors or whether they require a peripheral differentiation event before migrating to the small intestine. I hypothesize that agonist selected QFL IEL precursors migrate directly from the thymus to the small intestine, while positive selected QFL T cells migrate to a peripheral organ, undergo a differentiation event, and then migrate to the small intestine. In order to test this hypothesis, neonatal Rag2KO mice will be injected with CD4 depleted QFLTg or QFLTg Qa1^{b}KO thymocytes. I predict that QFLTg thymocytes will populate both spleen and small intestine, while QFLTg Qa1^{b}KO thymocytes will only populate the spleen. In parallel, I will transfer CD4 and B cell depleted QFLTg or QFLTg Qa1^{b}KO splenocytes to neonatal Rag2KO mice. I predict that both splenocyte populations will populate the spleen and small intestine of the host mice. If my hypothesis is right then QFL T cells will have a distinct pathways that lead to migration to the small intestine.

Transcriptional difference between agonist and positive selected QFL thymocytes

The QFL T cells undergo agonist selection in presence of Qa1^b, but can be positively selected by an alternative MHC 1 molecule. Furthermore, the agonist selected QFL T cells upregulate PLZF, rapidly proliferate and respond to target cells more efficiently than positive selected QFL T cells. Both of these populations may co-exist in the same thymic environment and possess the same TCR. Thus QFL T cells could provide an excellent model to dissect the transcriptional differences between and agonist selected and a positively selected T cell. I propose to utilize single cell RNA-seq of QFLTg thymocytes and QFLTg Qa1^{b}KO thymocytes. Comparison of these samples will confirm the coexistence of agonist and positive selected QFL T cells in the QFLTg thymus. The resolution of single cell RNA-seq can reveal subtle changes in transcripts that determine the lineage differentiation of an agonist selected T cell. Furthermore, it might reveal novel mechanisms that allow QFL T cells to avoid negative selection, considering their cross-reactivity. These samples may also reveal the transcriptional differences between an innate-like thymocyte and conventional thymocyte, utilizing a single TCR clone.

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