Characterisation of the murine non-classical MHC I molecule, H2-Q10

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Signature: ……

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Ad astra per aspera

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Abbreviations

aa	amino acid
Ab	antibody
ACK	ammonium chloride potassium
Ag	antigen
AIRE	autoimmune regulator
APC	allophycocyanin
APC	antigen-presenting cell
BCA	bicinchoninic acid
BCR	B cell receptor
bp	base pair
BSA	bovine serum albumin
CD(n)	cluster of differentiation (n), e.g. CD8
cDNA	complementary deoxyribonucleic acid
CDR	complementarity determining region
CMV	cytomegalovirus
CO2	carbon dioxide
CRISPR	clustered regularly interspaced short palindromic repeats
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DP	double positive
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
g	relative centrifugal force
GFP	green fluorescent protein
HeBS	HEPES-buffered saline
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
IEL	intraepithelial lymphocyte

IL-(n)	interleukin-(n), e.g. interleukin 10: IL-10
IP	immunoprecipitation
ITAM	immunoreceptor tyrosine-based activation motif
kDa	kilodalton
KIR	killer immunoglobulin-like receptor
KO	knockout
LB	Luria Broth
LIR	leukocyte immunoglobulin-like receptor
mAb	monoclonal antibody
MAIT	mucosal-associated invariant T cell
MAL II	*Maackia amurensis* lectin II
MCMV	murine cytomegalovirus
MGAT	monoacylglycerol acyltransferase
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NK	natural killer
ºC	degrees Celsius
PAM	protospacer associated motif
PAMP	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PHA	phytohaemagglutinin
pMHC	peptide-MHC
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute 1640 Medium
RT	room temperature
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sgRNA	short guide RNA
SLC	solute carrier
SNA	*Sambucus nigra* lectin

SPR	surface plasmon resonance
SULT	sulfotransferase
TAP	transporter associated with antigen processing
TBE	Tris-Borate-EDTA
TCR	T cell receptor
TE	Tris-EDTA
TLR	toll-like receptor
WT	wild-type
αMEM	Alpha Minimum Essential Medium

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Thesis Abstract

The major histocompatibility complex (MHC) is an essential genomic region containing the encoding genes for some of the most important protein in the immune system, MHC molecules, which are fundamental for the elicitation of immune responses. Understanding the biology and interactions of these molecules is key in understanding immunity as a whole. MHC class I molecules have been extensively studied for their role in antigen presentation to CD8 T cells, however, a subclass of MHC class I, known as non-classical MHC I or class Ib MHC, have been shown to exhibit characteristics outside of antigen presentation. Despite the evolutionary conservation and expansion of these molecules in the mouse, the function of many class Ib MHC molecules remains unknown. This thesis aims to characterise one of these molecules, H2-Q10, which is overexpressed in the liver. Firstly, through the generation of a novel knockout mouse model, the effect of H2-Q10 in immunity is explored whereby loss of H2-Q10 reduces specific immune cell subsets. Despite this, H2-Q10 does not appear to play a role in the reduction of viral titre burden against a natural mouse viral infection, murine cytomegalovirus (MCMV). In an exploratory approach to define the role of H2-Q10, transcriptomic analysis indicates that it may play a more important role in physiological, rather than immunological, processes. Secondly, this thesis defines a novel interaction that occurs between H2-Q10 and the CD8αα homodimer and shows that this interaction is controlled by post-translational modifications, specifically sialylation and N-linked glycosylation, of CD8αα. In addition, mutagenesis studies reveal critical residues on CD8αα and H2-Q10 which mediate this binding interaction. Finally, the role of post-translational modifications of human CD8αα is explored to show differences in its ability to bind human class Ib MHC molecules compared to interactions of H2-Q10 in the mouse. Overall, this thesis provides new insights into H2-Q10 biology to shed light on this class of enigmatic molecules.

Chapter One

Literature Review

This chapter contains content from the following published review:

Goodall, K.J., **Nguyen, A**., Sullivan, L.C., and Andrews, D.M. (2018). The expanding role of murine class Ib MHC in the development and activation of Natural Killer cells. Molecular Immunology.

1.1. INTRODUCTION TO THE IMMUNE SYSTEM

1.1.1. INNATE IMMUNITY

The immune system is broadly comprised of two branches, innate and adaptive, which both contribute towards protection of the host through elimination of foreign pathogens and malignancies. The innate immune system encompasses physical barriers and cells which are able to immediately respond to pathogenic challenge. As an evolutionarily-conserved defence mechanism in vertebrates, the importance of innate immunity in survival is fundamental (Chaplin, 2010). The physical barriers of innate immunity include the gut epithelial barrier, effector proteins such as complement, cytokines, chemokines as well as immune cells. These cells are mainly of the myeloid lineage and include monocytes, macrophages, neutrophils and dendritic cells (DCs). These innate cells are programmed to use generic molecular cues for their recognition of non-self, such as Toll-like receptors (TLR). TLRs recognise relatively microbial stimuli, such as pathogen-associated molecular patterns (PAMPs) or common bacterial products such as lipopolysaccharide. These molecules activate the TLR-dependent response, resulting in rapid detection of a foreign insult and the initiation of clearance. This is achieved through a diverse range of effector functions which include phagocytosis, production of cytokines/chemokines, complement activation and, in the case of antigen-presenting cells (APCs), processing and presentation of stimulatory antigen to cells of the adaptive immune system.

1.1.2. NATURAL KILLER (NK) CELLS

Natural killer (NK) cells are components of the innate immune system which are able to rapidly respond to infection or abnormal cellular transformation (e.g. tumours). The ability of an NK cell to recognise and respond to target cells is maintained by a fine balance between engagement of inhibitory and activating cell-surface receptors (Vivier et al., 2008). Activating NK cell receptors are able to recognise ligands expressed by 'stressed' cells; some examples include NK cell recognition of RAE-1 on target cells through NKG2D (Diefenbach et al., 2001) and Ly49H recognition of m157 glycoprotein encoded by cytomegalovirus (CMV)-infected cells (Smith et al., 2002; Arase et al., 2002). Furthermore, disengagement of NK cell inhibitory receptors with constitutively-expressed ligands can also lead to target cell lysis. For example, the lectin-like Ly49 receptors on NK cells are MHC class Ia-specific and thus absence of surface MHC, such as in the case of virus-mediated shutdown of MHC biosynthesis, leads to a 'shift in balance' resulting in NK cell activation (He and Tian, 2017) – this phenomenon is termed the 'missing self' response. NK cells are able to respond rapidly during an immune response and exhibit cytotoxicity through granzyme/perforin and cytokine release, a quality which is similar to cells of the adaptive immune system.

1.1.3. GAMMA DELTA (γδ) T CELLS

Within the last decade, the identification of "innate-like" T cells has defined populations of cells which encompass characteristics of both innate and adaptive immunity. γδ T cells, named after their non-conventional TCR comprised of a TCRγ and TCRδ chain, represent a class of these innate-like T cells which are important in mucosal immunity (Vantourout and Hayday, 2013). These cells are primarily activated by microbial compounds, given their abundance in the intraepithelial layer of the small intestine, but have also recently been shown to recognise

host-derived antigens (Bennett et al., 2015; Vavassori et al., 2013). Interestingly, γδ T cells have been shown to recognise non-classical MHC molecules (discussed in Section 1.4.3.3) such as H2-T10 and H2-T22 (Crowley et al., 2000; Fahl et al., 2018; Goodall et al., 2018a). Upon activation, γδ T cells can directly mediate target killing via production of pro-inflammatory cytokines, including IL-17 and IFNγ, and can also provide help by activating cells of the adaptive immune system (Bennett et al., 2015; Vantourout and Hayday, 2013).

1.1.4. ADAPTIVE IMMUNITY

In contrast to innate immunity, adaptive immunity is defined by its specificity to a particular antigen. The exquisite discrimination of antigen by adaptive lymphocytes is attributed to their antigen receptors. These include the TCR and B cell receptor (BCR), which unlike TLRs found on innate cells, undergo editing and somatic recombination throughout their development and maturation. This receptor diversity ensures that the host maintains an arsenal of lymphocytes that can respond to a plethora of different antigens. Using the TCR as an example, diversity is generated through the random rearrangement of one of many Variable (V) and Joining (J) genes at the TCR α locus, and V, Diverse (D), and J genes at the TCR β locus (Jung and Alt, 2004). Further diversity is generated via the template-independent addition or removal of nucleotides at the VDJ junctions. TCR α and β chains have three regions of hypervariability, referred to as complementarity determining regions (CDR1, CDR2 and CDR3), which form the antigen-binding site (Bassing et al., 2002). The CDR1 and CDR2 loops are encoded by the germline V gene segment, whilst the CDR3 loop is positioned at the V(D)J junction. As a consequence of this random gene rearrangement, the TCR repertoire has a theoretical diversity of ~10^{15} - 10^{20} different clonotypes. While this is theoretical, biological selection processes in the thymus reduce the number of peripheral T cells to approximately 2 x 10^{6} in mice and around 2.5 x 10^{7}

in humans (Arstila et al., 1999; Casrouge et al., 2000; Davis and Bjorkman, 1988) leading to the high probability of a T cell recognising a specific foreign pathogen upon first encounter.

As a defining characteristic of adaptive immunity, both B and T cells can generate immunological memory. During a primary infection, B and T cells specific for a particular antigen expand in number and frequency and become the active effectors of clearance. However, once the infection is resolved, having an expanded population of antigen specific cells becomes redundant. As such, a large proportion (>90%) of these cells die with the remaining pool becoming quiescent. Upon subsequent infection by the same pathogen, these memory cells become activated much more rapidly resulting in more effective control of infection, limiting the morbidity and mortality of the host. Indeed, the principal of immune memory underscores the program of vaccination and disease prevention.

Adaptive immunity is limited by its inability to directly 'see' antigen. This occurs as adaptive lymphocytes do not possess TLRs and cannot become activated until they recognise their specific/cognate antigen being presented to them by APCs. In the context of a primary immune response, presentation must arise through the activity of dendritic cells as these are the only cells in the immune system capable of initiating this response. Dendritic cells, possess all the necessary machinery to capture foreign pathogens, process them into their biochemical constituents and present small fragments of these pathogens to adaptive lymphocytes. The most widely recognised pathway for this activation, and thus the mechanism that governs T cell activation, occurs through the major histocompatibility complex (MHC).

1.2. THE MAJOR HISTOCOMPATIBILITY COMPLEX - INTRODUCTION

1.2.1. DISCOVERY OF MHC

In 1980, the Nobel Prize in Medicine and Physiology was awarded to George Snell, Baruj Benacerraf and Jean Dausset "for their discoveries concerning genetically-determined structures on the cell surface that regulate immunological reactions" (Marx, 1980). These "genetically-determined structures" are now well-known as MHC. Originally co-discovered by Peter Gorer and Snell, murine MHC was first implicated in tumour transplantation models as a genetic basis for tumour rejection (Gorer, 1937); (Snell, 1948). This work would later be supported by Dausset who drew parallels of murine MHC to those in humans and eventually coined the term Human Leukocyte Antigen (HLA) (Dausset, 1958). The role of MHC outside of tumour context was then shown by Benacerraf who identified its role in controlling antibody-mediated immune responses (Mcdevitt and Benacerraf, 1969).

1.2.2. GENETICS OF MHC

In humans, HLA genes and are located on chromosome 6 whereas MHC in mice, commonly denoted as "H-2", are located on chromosome 17. The MHC family is comprised of three classes (MHC I, MHC II and MHC III). Due to its primary role in encoding components of the complement system, discussion of MHC III is outside the scope of this review.

MHC diversity arises from its polygenicity, polymorphic nature and codominant expression. Firstly, MHC is polygenic in that it is encoded by a diversity of genes (Table 1.1). Secondly, each gene is polymorphic; indeed, genes encoding mouse and human MHC are the most polymorphic genes reported to date (Shiina et al., 2017; Norman et al., 2017). It has also been shown that more than 10,000 different MHC alleles exist within the human population (Rock et al., 2016). In order to characterise these gene sets, each particular set of alleles present on a

single MHC chromosome is termed the MHC haplotype. An individual inherits two MHC haplotypes: one each from maternal and paternal origin. The MHC molecules produced by the inherited alleles of the two parental haplotypes are both produced equally and are codominant in expression (Williams et al., 2002). Therefore, the substantial number of permutations that arise due to the polygenicity and polymorphism of MHC molecules increases diversity. Thus, excluding identical twins, no two individuals are exactly alike with respect to MHC expression. Indeed, this diversity was a barrier for a long time to successful transplantation and led to many instances of rejection, where a transplanted organ was recognised as foreign and rejected by the immune cells of the organ recipient (Ingulli, 2010). Fortunately, with advances in MHC haplotyping and immunosuppressive therapies, transplant success is increasing (Petersdorf et al., 2013; Ayala García et al., 2012).

1.2.3. MHC RESTRICTION

In 1975, Peter Doherty and Rolf Zinkernagal defined one the seminal theories in adaptive immunity, MHC restriction. With the original work being based on lymphocytic choriomeningitis virus (LCMV) infections, Doherty and Zinkernagal observed that specific cytotoxicity to the virus was not dependent solely on T cell recognition of antigen (Peter C. Doherty, 1975; Peter C. Doherty and Rolf M. Zinkernagel, 1976) but rather required additional compatibility of MHC haplotype. This was shown elegantly through an experiment whereby cells expressing strain-specific MHC haplotypes (L cells (H-2_k), P815 cells (H-2_d) or DBA/2 fibroblasts (H-2_d)) were uninfected or LCMV-infected were cultured with splenocytes from CBA/H (H-2_k) mice, BALB/c (H-2_d) mice or A/J (H-$2_{k/d}$) mice and analysed for cell lysis by chromium-release assay (Peter C. Doherty, 1975). It was found that shared H-2 haplotype of splenocytes and target cells, at least at one loci, was required for successful cell lysis of LCMV-infected cells. For example, L cell targets (expressing H-2_k) were only lysed by splenocytes with a matching H-2_k haplotype (i.e. CBA/H and A/J mice) and not those with mismatched haplotype (i.e. BALB/c). This pivotal finding would be central in understanding the requirement of both cognate antigen recognition and MHC haplotype matching in successful T cell recognition of infected cells (Figure 1.1).

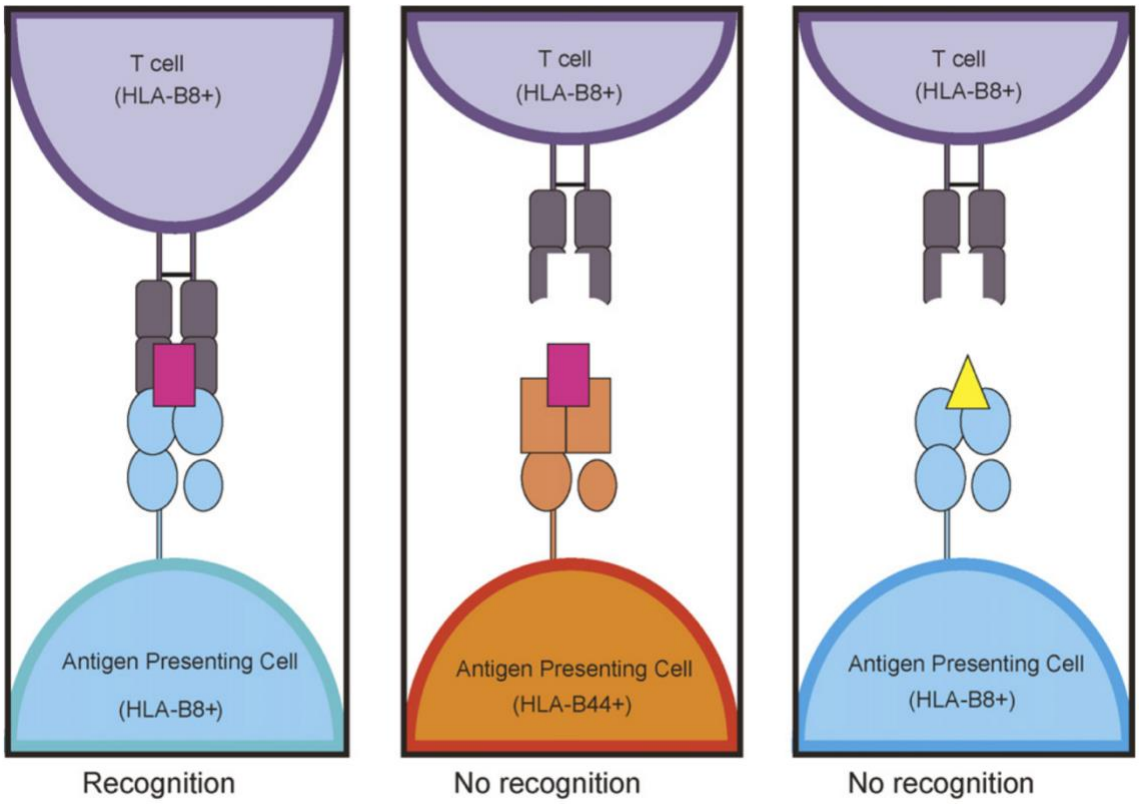


Figure 1.1. Example of Doherty and Zinkernagal's theory of MHC restriction. T cells are only able to recognise and kill infected cells if the MHC molecule on the APC is from the same MHC background and is presenting the specific antigen for that particular T cell. If the cognate antigen is presented on an MHC molecule with a different haplotype than the T cell, there is no interaction and no killing occurs. If the MHC molecule on the APC is of the same MHC background as the T cell, but is not presenting the cognate antigen for that T cell, no interaction and killing occurs (Archbold et al., 2008).

1.2.4. MHC IN THYMIC DEVELOPMENT

The primary lymphoid organ responsible for the development and maturation of T cells is the thymus (Miller, 2011), with the expression of MHC in the thymus controlling the fate of developing thymocytes. The development of T cells prior to expression of a TCR is largely independent of antigen however expression of the pre-TCR means that thymocytes undergo checkpoints dependent on MHC interactions within specific locations of the thymus (Figure 1.2).

To briefly summarise what has been decades' worth of work on T cell development, stem cells undergo successive differentiation in different anatomical compartments within the thymus with the acquisition of different cell surface markers as they progress. Firstly, stem cells are committed to T cell lineage and undergo gene rearrangement to form a TCR; these TCRs can be of an αβ or γδ configuration (Klein et al., 2014). As outlined above, the development of TCRs arising from somatic recombination, generates an enormous diversity of receptors with differing potential specificities (Anderson and Takahama, 2012). As such, checkpoints must occur to filter out T cells whose TCRs do not recognise self MHC or react too strongly to self MHC. This last interaction is potentially devastating to the host as it risks autoimmunity (St-Pierre et al., 2015). Following TCR expression, thymocytes advance through four double negative (DN) stages (DN1-4), designed to identify those cells that will become committed to the αβ or γδ lineage. Importantly, there is no expression of the CD4 or CD8 co-receptors at the DN stage (discussed later). Cells destined for the γδ lineage depart the thymus between DN2 and DN4 while those destined for the αβ lineage continue into the double positive (DP) stage where they express CD4 and CD8 co-receptors simultaneously. At this point, fate decisions are made via the ability and affinity for a TCR to bind MHC expressed by the selecting thymic stromal cells.

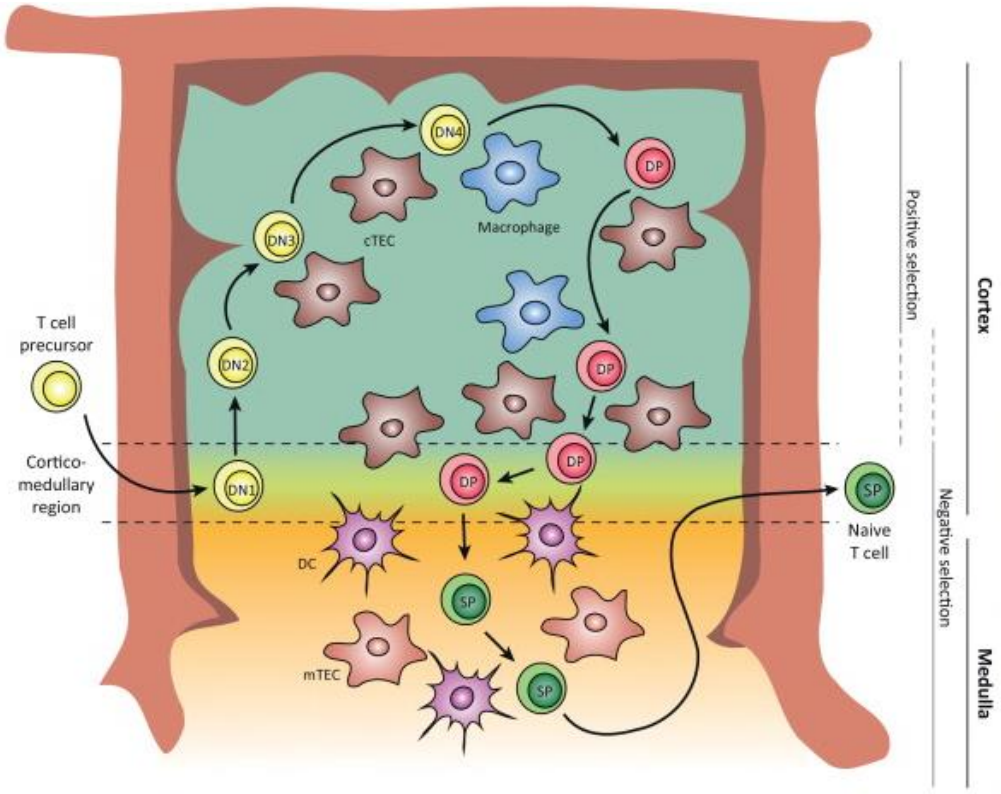


Figure 1.2. Thymic development of T cells. The thymus can be divided into two major regions representing distinct functional zones in T cell differentiation. Upon entry into the thymus from the bone marrow, T cells enter the cortico-medullary region and migrate to the cortex whereby positive selection occurs. As thymocytes develop into DP cells, they enter into the medulla whereby negative selection occurs to delete self-reactive cells prior to egress as naïve SP T cells into the periphery. Abbreviations: DC, dendritic cell; DN, double negative thymocytes; DP, double positive thymocytes; SP, single positive thymocytes; cTEC, cortical thymic epithelial cell; mTEC, medullary thymic epithelial cell. Figure adapted from Nunes-Alves et al., 2013.

The expression of self-peptides on self-MHC (self-pMHC) is crucial for positive and negative selection. Firstly, thymocytes that do not recognise self-pMHC at all undergo death by neglect; only those T cells that have a low affinity for self pMHC are positively selected. T cells which are positively selected are also committed to a specific lineage at this stage; if the thymocyte's TCR is positively selected by a MHC I molecule, it commits to a CD8 lineage and, conversely, if it recognises a MHC II molecule, it commits to a CD4 lineage. Negative selection occurs when single positive (SP) thymocytes binds to self pMHC with a high affinity. As aforementioned, T cells must be able to recognise self pMHC to become positively selected but if this interaction occurs too strongly, it could result in activation of T cells in the periphery to self antigens, leading to an unwanted autoimmune response. Therefore, negative selection

occurs when thymic APCs express self-pMHC which then interact with thymocytes; a high affinity interaction will lead to the activation of apoptotic signals leading to death of the T cell. In conclusion, the ability and strength of a T cell to bind self MHC via its TCR will ultimately dictate its survival and its lineage commitment.

The nature of the thymus stroma includes its ability to present peptides that are both spatially and temporally removed from this organ. When you consider mammalian development, mammary gland and testicular tissue is not yet formed in early life but the MHC is capable of eliminating T cells that could recognise antigens from these organs 10-15 years in the future. This remarkable ability arises from the autoimmune regulator (AIRE) gene, which allows for the transcription of tissue-specific antigens away from their original site (Anderson et al., 2002; Takada and Takahama, 2015). Thus, antigens of the eye, testes, breast and many other peripheral organs are expressed in the thymus and allows the negative selection of those cells that could initiate autoimmunity in these organs.

1.3. THE CO-RECEPTOR, CLUSTER OF DIFFERENTIATION 8 (CD8)

1.3.1. CD8

The cytotoxic T cell coreceptor, CD8, is a glycoprotein which is crucial in MHC class I-mediated responses. It is a relatively non-polymorphic molecule and shows a high degree of conservation across species (Gao and Jakobsen, 2000). The original discoveries concerning CD8 acknowledged its ability to bind directly to MHC class I during TCR engagement. However, it was only originally proposed to serve as an adhesion molecule which increased avidity and supported stability of the pMHC-TCR interaction (Garcia et al., 1996; MacDonald

et al., 1981; Moretta et al., 1984; Norment et al., 1988). Today, we appreciate the important role of CD8 as a coreceptor which is critical for T cell signalling following pMHC engagement; this is possible as CD8 is able to bind to the same pMHC molecule as the TCR is contacting.

1.3.2. CD8-MHC I INTERACTIONS IN DEVELOPMENT

Interestingly, the CD8 coreceptor has also been shown to play a role in regulating positive and negative selection. Owing to the ability for CD8 to also bind MHC, it has been shown that changes in the affinity of this interaction can alter positive selection by increasing the interaction between target cells and developing thymocytes (Daniels et al., 2002). Indeed, Grebe and colleagues showed that ligation of CD8 on a subset of immature DP thymocytes can lead to direct apoptosis independently of the TCR-pMHC interaction (Grebe et al., 2004). As this phenomenon is CD8-dependent, changes to the CD8 molecule itself can impact thymic selection. As such, changes occurring to CD8 throughout T cell development has been well-characterised, with one of the most important processes being post-translational modification (Daniels et al., 2002; Grebe et al., 2004; Marth and Grewal, 2008; Moody et al., 2001; Priatel et al., 2000).

The requirement for CD8 in enhancing antigen sensitivity is lost in the context of high affinity TCRs. Indeed, studies have shown that for high affinity pMHC-TCR interactions, the activation of $p56_{lck}$ can occur independently of CD8 engagement (Kerry et al., 2003). In a study by Kerry and colleagues, it was found that low/moderate affinity pMHC-TCR interactions required CD8 coreceptor help for early signalling events leading to proliferation, cytokine production and acquisition of a full effector T cell phenotype; high affinity interactions showed a lack of absolute requirement for coreceptor signalling (Kerry et al., 2003).

The form by which a CD8 molecule exists dictates its function as it can either be found in homodimeric form (CD8αα) or as a heterodimer (CD8αβ). In a manner similar to antibody-antigen interactions, it has been shown that one CD8 molecule binds one MHC molecule; the affinity of this interaction is weaker than TCR-pMHC interactions. Furthermore, the engagement of CD8 to pMHC is characterised by exceptionally fast kinetics; this is thought to ensure that peptide presentation by APCs to T cells is dominated by pMHC-TCR engagement which is critical for specificity of the response (Gao et al., 2002b).

In its homodimeric form, CD8 is comprised of two α chains covalently joined by a disulphide bridge whereas its heterodimeric form consists of the association between one α chain and one β chain. The CD8α chain (32-37 kD) consists of an extracellular element, made up of the Ig-like domain (122aa) and stalk region (48aa), and a transmembrane domain (28aa) connecting the complex to the cytoplasm (Kern et al., 1999a). The cytoplasmic tail of CD8α contains two binding domains for the Src-family kinase, $p56_{lck}$, and therefore is primarily responsible for mediating signalling in lymphocytes, specifically T cells (Marth et al., 1985; Veillette et al., 1988). The β chain (23 kD) shares approximately 20% amino acid similarity with the α chain (Gao et al., 1997), however, its stalk domain is relatively shorter and does not contain the $p56_{lck}$ binding domain, rendering it unable to initiate signalling alone. The α chain initiates the signalling cascade via $p56_{lck}$ phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on the δ, γ, ε and ζ subunits of the CD3 complex which acts to support the TCR signalling. The phosphorylation of these ITAMs results in recruitment of zeta-chain-associated protein kinase 70 (Zap70) which itself requires phosphorylation by $p56_{lck}$ (Rossy et al., 2012). Subsequently, a series of phosphorylation, activation and recruitment events occur leading to the ultimate consequence of activation of transcription factors, signal transduction to the nucleus and altered gene expression (Huse, 2009).

1.3.3. THE CD8 HETERODIMER (CD8αβ)

Of the CD8 forms, the CD8αβ heterodimer is expressed at a higher frequency than CD8αα within the conventional TCRαβ T cell population (Gao and Jakobsen, 2000). Earlier studies examining the involvement of the CD8β chain in coreceptor function showed that it was required for CD8α to adopt a conformation which would promote stable association with $p56_{lck}$ and thus mediate T cell signalling (Irie, 1995). To support and further explore this phenomenon, later studies involving the chimeric form of CD8α, whereby the stalk region was substituted with that of CD8β, showed that T cell activation is more effective as O-linked glycosylation of CD8β mediates its ability to stabilise its own conformation, as well as that of CD8α, during pMHC engagement (Gao and Jakobsen, 2000). Furthermore, the 19aa cytoplasmic tail of CD8β is critical as deletion of this domain lead to a significant impairment in the accessibility of $p56_{lck}$ to CD8α and thus compromises downstream signalling (Irie et al., 1998). The importance of CD8β is further exemplified in CD8β knockout mice which show a significant reduction in peripheral CD8+ T cells, indicating its importance in development and maturation (Nakayama et al., 1994).

1.3.4. THE CD8 HOMODIMER (CD8αα)

Although CD8αα has been shown to also be expressed on αβ T cells, it is predominantly found on intraepithelial lymphocytes (IELs), some NK cells and γδ T cells (Goodall et al., 2018 (Zamoyska, 1994). CD8αα has been shown to be a less efficient coreceptor despite the increased presence and localisation of $p56_{lck}$ binding sites. In addition, although the binding affinities to MHC I by CD8αα and CD8αβ have been reported to be similar, CD8αα is still recognised as a relatively inferior T cell coreceptor, with some groups observing a 100-fold decrease in its effectiveness (Gao et al., 2000). Furthermore, unlike CD8αβ, CD8αα has been

shown to not support positive selection of thymocytes during development. As mentioned earlier, the first discovered ligand of CD8αα was the MHC Ib molecule, H2-TL. Leishman and colleagues showed that the binding of CD8αα to H2-TL was of high affinity and was independent of the pMHC-TCR interaction which was not seen when H2-TL bound CD8αβ (Leishman et al., 2001a). This was supported by their findings that lymphocytes from CD8β-/- mice were still able to bind significantly to H2-TL tetramers. Cells which bound to H2-TL, however, did not show increased proliferation nor did they enhance their cytotoxicity. The discovery of this interaction which did not promote a strong effector response catalysed the study of CD8αα as an immunomodulatory, instead of stimulatory, coreceptor.

Earlier studies using CD8α-transfected cell lines indicated that CD8αα served to increase overall binding to MHC molecules independently of specificity (Miceli et al., 1991). This notion was contested later on with the dawn of surface plasmon resonance (SPR) techniques which disproved the hypothesis as the interaction is weak (low K_D/binding affinity) and extremely fast (Gao and Jakobsen, 2000). Thus, the physiological role of the CD8 homodimer was not attributable to physically enhancing pMHC-TCR contact. In conclusion, little is established about the CD8 homodimer and therefore understanding its interaction partners, regulators of these interactions and subsequent consequences will shed light on its immunological relevance.

1.4. THE MAJOR HISTOCOMPATIBILITY COMPLEX – CLASSIFICATION AND FUNCTION

1.4.1. ROLE OF MHC IN THE PERIPHERY

MHC molecules are crucial in eliciting humoral and cellular immune responses as they represent key components required in antigen presentation. In the context of foreign pathogen attack, such as bacterial, viral or parasitic invasion, MHC molecules serve to relay information about the specific antigen to effector cells of the adaptive immune system. For example, during bacterial infection, intracellular antigens which have been endocytosed are processed to activate CD4 T cells and initiate mainly T helper 1 (TH1) responses such as interferon production (Campbell and Shastri, 1998). CD4 T cells, once recognition of peptide-MHC II occurs, can also be polarised into T helper 2 (TH2) cells producing IL-4, IL-5 and IL-13 in order to expel large organisms, such as parasites, and recruit effector cells to mediating killing (Allen and Sutherland, 2014). During viral infection, antigenic peptides are also processed to allow for recognition of infected cells by CD8 T cells and elicitation of cytotoxic functions such as granzyme/perforin-mediated lysis (Rosendahl Huber et al., 2014; Koszinowski et al., 1991). Although many cancers downregulate surface MHC class I to avoid T cell recognition and lysis, the absence of MHC triggers NK cells to initiate ‘missing self’ responses to destroy malignant cells (Sharma et al., 2017) (Wu and Lanier, 2003).

1.4.2. MHC CLASS II

MHC II molecules are formed by the association of two α and β subunits. These proteins are constitutively expressed on professional APCs, including macrophages, dendritic cells and B cells. MHC II molecules present exogenous/lysosome-derived peptides to CD4 T helper cells

(Blum et al., 2013). In contrast to MHC I, MHC II molecules are loaded in antigen-containing endosomes which provide the compartment for peptide processing. Following synthesis in the Golgi, MHC II molecules bind to a polypeptide known as the invariant chain. The invariant chain and class II complex contain motifs that direct them to the cell membrane and initiates endocytosis. Following endocytosis, via processes outside the scope of this review, the endosomal MHC II molecules are dissociated from the invariant chain and loaded with peptides from the endosome milieu (Roche and Furuta, 2015). In contrast to the requirement for shorter peptide fragments by MHC I, the peptide-binding groove of MHC II can accommodate longer peptides of about 13-17 amino acids (Blum et al., 2013).

1.4.3. MHC CLASS I

The role of MHC class I (MHC I) molecules is to present endogenous/cytosol-derived peptides to CD8+ cytotoxic T cells. MHC I can be further subdivided into two groups (Table 1.1): classical (MHC Ia) and non-classical (MHC Ib).

	Humans	**Mice**
MHC Ia (classical MHC I)	HLA-A HLA-B HLA-C	H-2D H-2K H-2L
MHC Ib (non-classical MHC I)	HLA-E HLA-F HLA-G	H2-Q H2-T H2-M
MHC II	HLA-DR HLA-DQ HLA-DP	H-2A H-2E H-2O

Table 1.1. Subclasses of MHC molecules in the human and mouse.

1.4.3.1. CLASSICAL MHC (MHC IA)

MHC Ia molecules are expressed on almost all nucleated cells. As aforementioned, the classical MHC repertoire includes HLA-A, -B and -C molecules in humans and H-2K, -D and -L in mice (Klein et al., 1981; Ploegh et al., 1981).

The structure of MHC Ia molecules consists of a membrane-spanning heavy chain (3 alpha domains) non-covalently associated to a light chain (beta-2-microglobulin, β2m) (Klein et al., 1981) (Figure 1.3). The α1 and α2 domains comprise the peptide-binding cleft whereby peptides 8-10 amino acids in length are able to be presented to CD8 T cells (Gao et al., 2002a). The α3 domain contains the transmembrane region which anchors the entire complex to the cell membrane as well as providing a contact site to the CD8 coreceptor for stabilisation of the complex following engagement with the TCR. β2m does not contain a transmembrane region but is necessary for stable cell surface expression of the MHC class I complex; its importance is shown in β2m$^{-/-}$ mice, where CD8^{+} T cells are unable to develop as surface expression of MHC on selecting cells in the thymus is absent (Zijlstra et al., 1990).

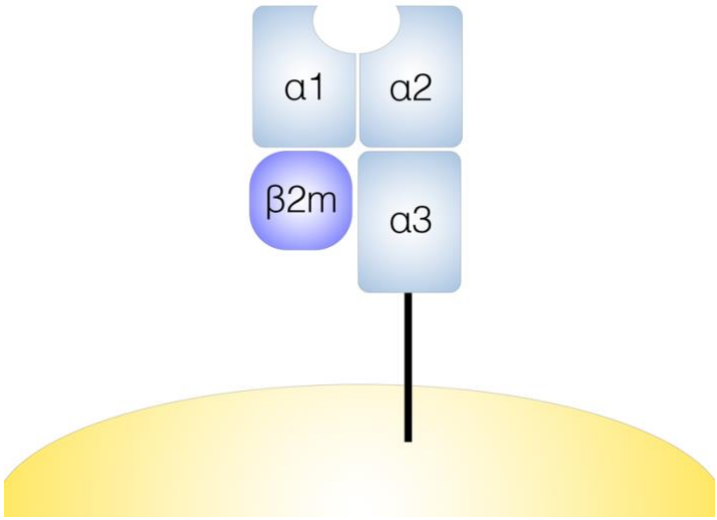


Figure 1.3. Schematic of MHC I structure anchored to the cell membrane associated with beta-2-microglobulin (Goodall et al., 2018a).

As aforementioned, the main role of MHC Ia is presentation of short peptide fragments to CD8 T cells. The process of peptide generation occurs through an ordered sequence beginning with pathogen capture and ending with presentation of the peptide fragments within the groove of the MHC. Following phagocytosis and pathogen degradation, self and non-self cytosolic proteins are marked for degradation following labelling with ubiquitin. The addition of a ubiquitin tag forces these proteins to undergo degradation by the proteasome. This is a multimeric structure which cleaves proteins via its catalytic core and produces small fragments that are transported to the endoplasmic reticulum (ER) (Coux et al., 1996). This stage of the process occurs via the transporter associated with antigen processing (TAP) protein which actively transports these small protein fragments into the ER. At this point, peptidases such as ER aminopeptidase associated with antigen processing 1 (ERAP1) further trim the protein fragments into smaller peptides and ER localised chaperone molecules (ERp57, calnexin, calreticulin and tapasin) load them onto newly-synthesised, ER-esident MHC monomers (Sadasivan et al., 1996) (Jensen, 2007). The addition of the peptide to the MHC groove and subsequent structural change arising from this loading allows the MHC molecule to non-covalently associate with β2m and triggers the release of the peptide chaperone molecules (Leone et al., 2013). At this point, the peptide MHC complex is freed from its ER localisation and then migrates through the Golgi complex before embedding on the cell surface (Figure 1.4). Here it becomes a focal point for immunosurveillance by cells of the immune system.

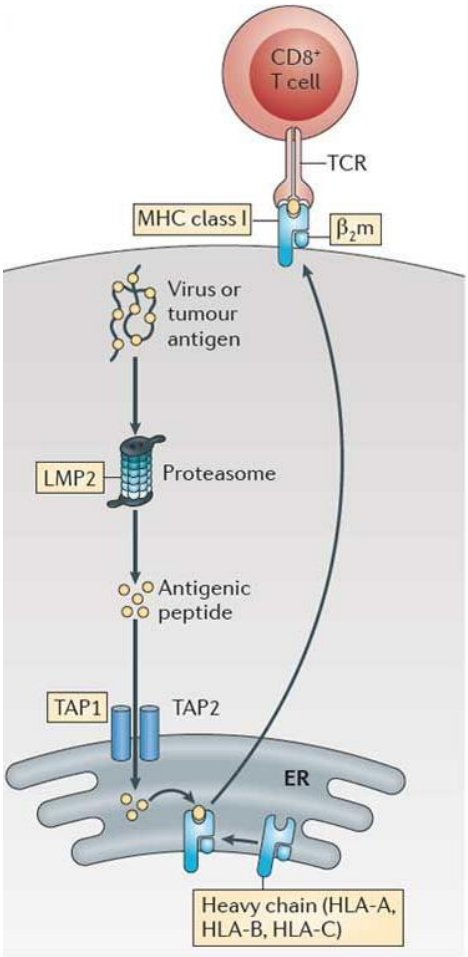


Figure 1.4. MHC class I processing pathway and presentation in humans. Intracellular antigens are processed via the catalytic function of the proteasome producing smaller peptide fragments which enter the endoplasmic reticulum (ER), via tapasin-associated (TAP) molecules. Antigenic peptides are loaded into empty HLA before shuttling to the cell surface and interacting with CD8+ T cells (Kobayashi and van den Elsen, 2012).

Cells expressing pMHC complexes that present self-derived peptides are ignored by CD8 T cells whereas presented peptides derived from viruses or other intracellular pathogens are recognised as non-self by cognate TCR and consequently destroyed (Kloetzel, 2001). Self-peptide antigen presentation is also crucial for NK cell immune responses; in the absence of pMHC on the cell surface, NK cells initate the “missing self” response and target the cell for killing. In conclusion, classical MHC Ia molecules are responsible for antigen presentation as they represent highly polymorphic proteins which can process a significant number of peptides to CD8 T cells.

1.4.3.2. MHC I-LIKE MOLECULES

In addition to MHC Ia and Ib molecules, an added layer of complexity is added when taking into consideration the existence of "MHC I-like" molecules. Genes encoding these proteins are unlinked from the MHC locus on chromosome 17 and, in mice, are actually found on chromosomes 3, 5, 7, 10 and 20 (Adams and Luoma, 2013). MHC-like molecules encoded by these genes across different chromosomes share similar properties such as antigen presentation but differ in their antigen specificity and crosstalk with other immune cells (Gleimer and Parham, 2003).

One example of an MHC I-like molecule is the major histocompatibility complex class I-related (MR1) gene located on chromosome 6 in mice, outside of the MHC-encoding chromosome 17 (Kjer-Nielsen et al., 2012). The MR1 gene is relatively monomorphic, similar to MHC Ib molecules, but its function and antigen specificity is restricted to the presentation of vitamin B metabolites derived from microbes. Like MHC Ia, MR1 molecules have been shown to be ubiquitously expressed but at relatively low levels due to its constant recycling from cell surface to cytosol (Lamichhane and Ussher, 2017). The presentation of vitamin B metabolites occurs to a subset of T cells named mucosal-associated invariant T (MAIT) cells (Treiner et al., 2003). These MAIT cells represent an "innate-like" population of T cells as they are able to respond quickly to bacterial challenge due to their semi-invariant TCR which recognises the vitamin B metabolites presented by MR1. The activation of MAIT cells leads to classical T cell effector functions, including the production of pro-inflammatory cytokines and direct cytotoxicity by perforin and granzyme release (McWilliam et al., 2015).

Similarly, CD1 represents a family of MHC I-like molecules which are comprised of differing individual proteins (CD1a-e) (Rossjohn et al., 2012). The role of these CD1 molecules is to

present glycolipid antigens to natural killer T (NKT) cells. Type I NKT cells express a semi-invariant TCR, with an invariant α chain usage coupled with a limited range of β chains. CD1 is monomorphic and is able to bind to a diverse range of lipid-based antigens and, upon engagement to NKT TCRs, can induce proinflammatory cytokine production and promote cellular immunity (Kinjo et al., 2005).

Thus, MHC I-like molecules are not technically defined as non-classical as their primary function is peptide presentation to T cells with no investigated functions independent of TCR engagement. Their exclusion from being classical MHC I, however, arises from their monomorphic nature and restrictive peptide-binding repertoire to innate-like lymphocytes.

1.4.3.3. NON-CLASSICAL MHC (MHC IB)

In contrast to human MHC Ib which consists of only three molecules HLA-E, -F and -G (O'Callaghan and Bell, 1998), murine MHC Ib are a highly expanded group of genes broadly divided into the H2-Q, -T and –M families (Figure 1.5). In the mouse, more than 30 genes exist across these three main families (Goodall et al., 2018a). Although some of these have been characterised, the biological function of many of these genes still remains unclear. Despite their structural similarity to classical MHC I molecules, MHC Ib family members display limited polymorphism, tissue-restricted expression and functional diversity existing outside of peptide presentation (Ohtsuka et al., 2008; Braud et al., 1997). Some of the better-characterised MHC Ib molecules are summarised in this section.

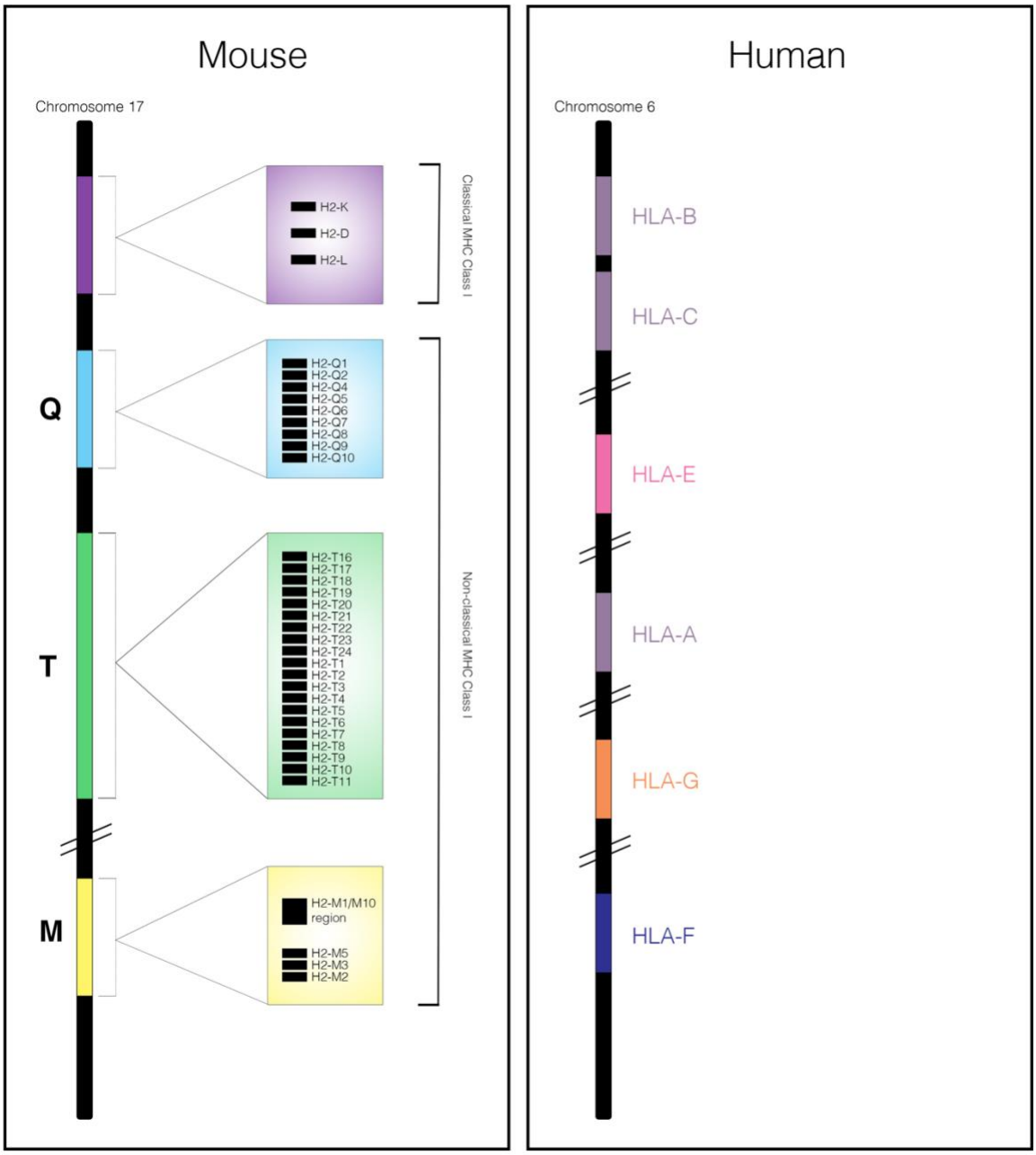


Figure 1.5. (left) Murine class Ia and class Ib MHC loci on mouse chromosome 17 with MHC class I families being localised in 'clusters' (not to scale). The class Ia MHC region, comprising the H2-K, H2-D and H2-L encoding genes, are followed by Q (blue), T (green) and M (yellow) family members, respectively. (right) Human class Ia and class Ib MHC loci on chromosome 5 (not to scale)(Goodall et al., 2018a).

1.4.3.3.1. HLA-F

HLA-F expression has been shown to be correlated with some autoimmune diseases, cancers, neuronal pathogenesis and pregnancy (Afroz et al., 2017; Dulberger et al., 2017; Santos et al., 2018; Wu et al., 2017; Zhang et al., 2013; Song et al., 2016; Burrows et al., 2016). It has also been shown that HLA-F is able to directly communicate to NK cells via ligation with the surface killer-immunoglobulin-like receptor (KIR), KIR3DS1, and consequently limiting replication of human immunodeficiency virus type 1 (HIV-1) (Garcia-Beltran et al., 2016). Although it was previously thought to exist in an open conformation due to a failure to identify endogenous loaded peptides (Goodridge et al., 2010), a recent study by Dulberger and colleagues has provided evidence of stable, peptide-loaded HLA-F which can be recognised at the cell surface by inhibitory receptors on NK cells, such as leukocyte immunoglobulin-like receptor 1 (LIR-1) (Dulberger et al., 2017). This study introduced the hypothesis that binding of peptide to HLA-F, and subsequent binding to inhibitory LIR-1, acts to balance NK cell reactivity by hindering open conformer HLA-F binding to activating KIR3DS1. Therefore, HLA-F represents a class Ib molecule which exhibits archetypal characteristics such as peptide-independence but also acts in a class Ia manner through its ability to bind peptides and directly interact with NK cells eliciting immunological function in various diseases.

1.4.3.3.2. HLA-G

The HLA-G gene shows very limited sequence variability across individuals consistent with the class Ib characteristic of low polymorphism (Nückel et al., 2005). HLA-G expression was first identified at high levels in cytotrophoblasts from placentas during early gestation which generally lack class Ia HLA expression (Clements et al., 2005; Kovats et al., 1990).

Interestingly, HLA-G proteins have been shown to exist in various membrane (mHLA-G; HLA-G1, HLA-G2, HLA-G3, HLA-G4) and soluble isoforms (sHLA-G; HLA-G5, HLA-G6, HLA-G7) (Lin and Yan, 2018), with the latter being detectable in serum (Hviid, 2006).

One of the non-immunological functions that HLA-G has been implicated to serve is during cytotrophoblast differentiation and invasion into the uterus (Ferreira et al., 2017). Increased expression of HLA-G correlates with the ability for cytotrophoblasts to acquire an invasive capacity which is necessary during early gestation; as foetal development progresses, the requirement for cytotrophoblasts to differentiate and invade becomes more redundant and thus HLA-G expression decreases in differentiated/"term" cells (McMaster et al., 1995; Kovats et al., 1990). The reduction of class Ia HLA expression to protect the developing foetus from T cell-mediated recognition and lysis could potentially enhance NK cell "missing self" killing, therefore, it has been suggested that HLA-G compensates for this by eliciting immune tolerance and reducing the risk of semi-allogeneic rejection (Gonen-Gross et al., 2003). The invasive nature of these cytotrophoblasts in early pregnancy was hypothesised to share characteristics of invasive tumour cells and therefore the role of HLA-G in cancer has been widely studied.

Immunosuppression has also been linked to the expression of HLA-G mainly in context of tumour development. Firstly, membrane-bound HLA-G has been detected on lymphomas, melanoma and various tissue carcinomas (Davies et al., 2001; Ibrahim et al., 2001; Rebmann et al., 2003; Seliger et al., 2003; Singer et al., 2003; Wagner et al., 2000). It has been suggested that expression of HLA-G on cancerous cells correlates with production of interleukin-10 (IL-10), an immunosuppressive cytokine which can be found in tumour microenvironments and results in the dampening of infiltrating inflammatory immune cells, such as macrophages and interleukin-17 (IL-17) T cells (Oft, 2014). In addition, production of IL-10 promotes

homeostasis of regulatory T cells (TREG) in the tumour microenvironment leading to enhanced anti-inflammatory conditions favourable to the tumour (Oft, 2014; Urosevic et al., 2001). IL-10 production has also been linked to the differentiation of a subset of tolerogenic DCs, known as DC-10 cells, which can produce more IL-10 and are characterised by acquisition and maintenance of high HLA-G expression; DC-10 cells can also negatively regulate the anti-tumour response by promoting suppressive CD4 T cell migration and maintenance (Gregori et al., 2010). Furthermore, similar to HLA-F, HLA-G is able to directly inhibit NK cell function through ligation with ILT2 which limits NK cell lysis particularly in uterine NK cells (Lin and Yan, 2018) . In summary, HLA-G represents a class Ib HLA molecule which has been predominantly associated with protection against allogeneic rejection of the developing foetus in pregnancy,

1.4.3.3.3. H2-M3

The well-studied MHC Ib molecule, H2-M3, has been reported to bind *N*-formylated peptides (Wang et al., 1995). These antigens are derived during bacterial infections, as N-formylation is a by-product of microbial protein synthesis (Colmone and Wang, 2006), or from mitochondrial peptides. This was first identified as H2-M3 was found to be maternally-transmitted to offspring and, as mitochondrial DNA is also inherited from mothers at birth, this would result in co-transmission (Ferris et al., 1983; Loveland et al., 1990). Given this capacity to present a specific peptide, H2-M3 is a restriction element for a specific subset of CD8 T cells. (Wang et al., 1995; Fischer Lindahl et al., 1997). Under homeostatic conditions H2-M3 is largely kept intracellularly but, upon infection, it is rapidly translocated to the cell surface. This is particularly advantageous as rapid peptide presentation to CD8 T cells facilitates faster T cell recognition and thus initiation of an immune response. One of the initial discoveries

pertaining to the role of H2-M3 in mediating cytotoxic responses was its role in *Listeria monocytogenes* (LM) infection. This was shown through observations that H-2K$_b$/H-2D$_b$ double KO mice infected with LM still possessed a small pool of CD8 T cells which were found to be H2-M3 restricted (Seaman et al., 1999; Colmone and Wang, 2006). The response of these restricted CD8+ T cells peaks rapidly, compared to class Ia-restricted CD8+ T cells, and results in the robust production of IFNγ (Berg et al., 1999). In addition, outside of its ability to present antigen, H2-M3 has also been shown to bind the inhibitory NK cell receptor, Ly49A, with this interaction being reported to mediate NK cell responsiveness and decrease tumour burden (Andrews et al., 2012).

1.4.3.3.4. H2-T23 (Qa-1b)/HLA-E

Arguably the most well-studied MHC Ib molecule, H2-T23 (Qa-1b) is a key protein involved in presenting self-peptides as an indicator of normal functioning in healthy cells. In humans, HLA-E has been described as the homologue of Qa-1b and shows very limited polymorphism with only 2 different alleles being predicted to exist within the human population (Grimsley et al., 2002).

Upon first encounter, Qa-1b/HLA-E appears to be much like class Ia MHC. It is ubiquitously expressed and presents endogenous peptides. However, a deeper examination demonstrated that, rather than being loaded by cytosolic peptides, Qa-1b/HLA-E presents small peptide fragments derived from the leader sequence of MHC I molecules synthesised in the ER (Kurepa et al., 1998). At least for Qa-1b these fragments were termed the Qa-1 determinant modifier, or Qdm. As such, the presentation of Qdm appears to allow for monitoring of proper functioning of the MHC pathway. This is an important physiological process as specific points

in the MHC biosynthesis pathway, such as proteasomal degradation or TAP transportation into the ER, have been shown to be dysregulated by various stimuli such as viral infections and malignant transformation (Oliveira et al., 2010). Thus, cells with these types of dysregulation are capable of expressing MHC on the cell surface but it is not informative of the overall health of the cell. As such, Qa-1b/HLA-E measures the "pulse" of the MHC pathway and acts to alert the immune system to any defects.

Under normal homeostasis, loading of Qa-1b/HLA-E with Qdm results in stable cell surface expression. This provides a reference point for surveying NK cells which engage Qa-1b via the inhibitory complex CD94/NKG2A. The resultant inhibitory signal ensures that NK cells do not target functionally healthy cells (Miyada and Wallace, 1986; Ying et al., 2017; Sullivan et al., 2015) and provides an extra layer of protection for healthy cells.

The correlation between Qa-1b and HLA-E has been established in the context of disease, with the basic understanding behind HLA-E engagement with CD94/NKG2 observed in clinical studies. It was found that human cytomegalovirus (CMV) encodes a mimic peptide which loads into HLA-E and thus CMV-infected cells are able to bypass NK cell-mediated lysis (Sullivan et al., 2015). This finding has provided insight into potential modes of rejection following organ transplantation by understanding the important interaction between class Ib MHC molecules and CMV-infected cells, the inhibition of which may improve transplantation outcomes.

CMV is able to manipulate HLA-E expression as UL40, a CMV-specific glycoprotein, has been identified as a peptide for HLA-E and thus can enhance its cell surface expression. This increase in surface HLA-E allows for the CMV-infected cell to evade NK cell lysis is

complemented by the ability of to downregulate HLA expression and activate NK cell "missing self" responses (Sullivan et al., 2015).

1.4.3.3.5. H2-TL

Thymic leukemia antigen (TL), originally discovered and identified as an antigen in tumours, is encoded by the H2-T3 gene in mice. Unlike MHC Ia, or some of its MHC Ib cousins, H2-TL is peptide-independent and thus can be freely expressed on the cell surface (Weber et al., 2002). This peptide independence was revealed in a study by Liu and colleagues who generated the crystal structure of H2-TL and found a unique α1-α2 domain which adopted a closed conformation, distinguishing it from many MHC I molecules (Liu et al., 2003). H2-TL was discovered to be expressed mainly on intestinal epithelial cells thus implicating it in mucosal immunity (Anderson and Brossay, 2016). Indeed, following its identification and characterisation, the ligand of H2-TL was found to be the CD8 coreceptor, specifically in the homodimeric form (CD8αα). This finding was supported by the discovery that CD8αα was expressed at a higher frequency on intraepithelial lymphocytes (IELs) than other cell subsets. Again, unlike most MHC I molecules, the α3 domain of H2-TL was able to bind to CD8αα with a higher affinity than the heterodimeric form, CD8αβ (Leishman et al., 2001; Tsujimura et al., 2004). The consequence of this higher affinity interaction is somewhat peculiar, as it has been shown to promote T cell cytokine release but also impede proliferation and acquisition of effector function (Cheroutre and Lambolez, 2008).

1.4.3.3.6. H2-Q10

H2-Q10 is a MHC Ib molecule which was first identified more than 30 years ago (Kress et al., 1983; Lew et al., 1986) as a liver-specific MHC I protein. The structure of H2-Q10 is unique

amongst MHC I molecules as it contains the archetypal alpha domains associated with β2m, however unlike classical MHC I, the α3 domain contains a 13bp deletion in exon 5, which encodes the transmembrane region, leading to a frameshift mutation and premature truncation at the C-terminus (Mellor et al., 1984). The lack of a transmembrane domain has been hypothesised to result in H2-Q10 being synthesised and secreted from cells. Indeed, earlier studies detected H2-Q10 in soluble form as a 38-40kD; these studies also suggested that H2-Q10 could be detected at high concentrations of 20-60 µg/ml in some mouse strains (Lew et al., 1986). In addition to its premature truncation, its solubility is further supported as the remaining amino acid residues at the C-terminus are mostly polar and would not support cell membrane expression (Glaberman et al., 2008). The suggested human homologue of H2-Q10 is HLA-G as it is also a soluble MHC I molecule, however, HLA-G is restricted to the maternal-foetal interface and is implicated mainly during pregnancy as an effector molecule which induces tolerance (Hunt and Langat, 2009).

Our group has previously shown that H2-Q10 binds the NK cell inhibitory receptor, Ly49C (Sullivan et al., 2016) and this interaction was hypothesised to regulate NK cell responses. However, despite the novel discovery, this study showed that in normal conditions, the MHC Ia molecule, H2-K_b, binds Ly49C with a higher affinity than H2-Q10 thus putting the physiological relevance of the H2-Q10-Ly49C interaction into question.

The study of MHC and its interactions with cells of the immune system has largely focused on biochemical recognition. However, these interactions do not occur in a binary system and can be modulated by the presence of other moieties on the MHC molecule or the interacting receptor, such as CD8. Like all surface exposed molecules, proteins of the immune system can be subject to post-translational modification affecting their function.

1.5. POST-TRANSLATIONAL MODIFICATION AND THE IMMUNE RESPONSE

Post-translational modification (PTM) of proteins can be defined as a process through which amino acids are covalently modified; as a consequence, these modifications can alter their function via changes to their biophysical properties, structure, stability and interactions with other proteins (Prabakaran et al., 2012). PTMs add an extra layer of complexity to the proteome; indeed, the ability for proteins to become modified from their original transcriptional material generates the diversity required for the constant evolution of the host. A vast amount of processes are classified under the PTM group (Duan and Walther, 2015), however, the focus of this section will be on glycosylation and sialylation.

1.5.1. GLYCOSYLATION

Glycosylation can be simply defined as a process by which saccharides are covalently attached to proteins (Spiro, 1973). Glycosylation is arguably better-defined as a co-translational modification whereby translation of the polypeptide and its modification occurs simultaneously in the Golgi complex. In mammalians, this enzymatic process is highly complex, with an estimated 700 proteins (approximately 200 of which are glycosyltransferases) involved in creating the entire diversity of glycans (Moremen et al., 2012). This huge diversity arises from only ten monosaccharides: fucose (Fuc), galactose (Gal), glucose (Glc), N-acetylgalactosamie (GalNAc), N-acetylglucosamine (GlcNAc), glucuronic acid (GlcA), iduronic acid (IdoA), mannose (Man), sialic acid (SA) and xylose (Xyl) (Boscher et al., 2011) (Cummings, 2009) (Marth and Grewal, 2008) (Moremen et al., 2012). The combination of these simple building blocks to generate glycan structures can have a significant impact on the final glycoprotein. Glycosylation can be broadly divided into two types depending on the

location of the glycan addition: N-linked glycosylation and O-linked glycosylation (not explored in this review).

1.5.1.1. N-GLYCOSYLATION

N-linked glycosylation involves the linkage of onto asparagine (Asn) residues contained within the specific sequon Asn-X-Ser/Thr, where X cannot be Pro. The initiating stages of N-glycosylation occurs in the endoplasmic reticulum (ER). Lipid-linked glycans are initially formed on the cytoplasmic side of the ER through the gradual addition of Man, GlcNAc and Glc monosaccharides onto ER membrane-embedded dolichol molecules (Moremen et al., 2012). Following this, translocation, or 'flipping', of these structures to the luminal side of the ER occurs, allowing oligosaccharyltransferases (OSTs) to catalyse the transfer of these glycans ($Glc_3Man_9GlcNac_2$) to Asn residues of nascent proteins (Easton, 2011) (Moremen et al., 2012) (Spiro, 2002). As a checkpoint for proper protein folding, eukaryotic cells subject these proteins to a repeated process whereby glucosidases and glucosyltransferases remove and add glucose monosaccharides, respectively, from attached glycan structures (Schwarz and Aebi, 2011). This process allows for interaction with chaperone molecules which promote proper folding of glycoproteins. This cycle ensures that proteins which have correctly folded are able to continue through the glycosylation pathway, via transport towards the Golgi complex, and those which are misfolded are targeted for proteasomal degradation. Thus, the earlier stages of N-glycosylation not only provide the structural backbone upon which glycan structures are further added in the Golgi complex but also act as a checkpoint for protein quality.

The level of modification following addition of the $Glc_3Man_9GlcNac_2$ structure onto the polypeptide by OSTs dictates its classification. Firstly, removal of Glc subunits by α-glucosidases I and II occurs followed by removal of Man residues by a number of different α-

mannosidases. The resultant glycan structure is of the configuration $Man_5GlcNac_2$ and is termed a 'high mannose' glycan, an important intermediate for the formation of hybrid and complex glycans. The subsequent editing steps are catalysed by a family of N-acetylglucosaminyltransferases, the first of which is α-1,3-mannosyl-glycoprotein 2-β-N-acetylglucosaminyltransferase (MGAT1, otherwise known as GLCNAC-TI, GLCT1, GLYT1, GNT-1, GNT-I, MGAT, GnT-I). MGAT1 first adds a GlcNAc residue to the $Man_5GlcNac_2$ core which triggers the activity of α-mannosidases to trim Man residues and produce $GlcNAcMan_3GlcNAc_2$. Following this, another N-acetylglucosaminyltransferase, MGAT2, catalyses the addition of GlcNAc to the glycan structure which will subsequently act as a foundation upon which complex N-glycans are created. The actions of MGAT3, MGAT4a, MGAT4b, MGAT5, MGAT5b and MGAT6 all contribute to the branching of larger antennae on the glycan structure (Aebi, 2013; Aebi et al., 2010; Easton, 2011; Ohtsubo and Marth, 2006; Shrimal et al., 2015; Spiro, 1973; Stanley et al., 2015) (Figure 1.6). Further modification of these N-glycans can arise from 'capping' with Fuc, Gal, GlcNAc and sialic acids.

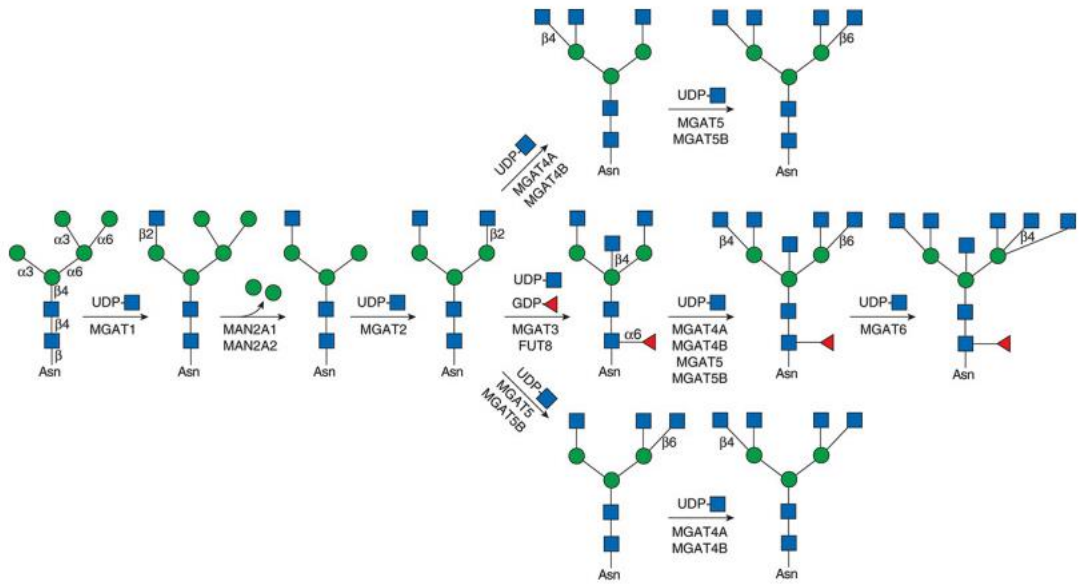


Figure 1.6. Modification of the $Man_5GlcNac_2$ N-glycan core by α-mannosidases and N-acetylglucosaminyltransferases to produce hybrid and complex structures (Stanley et al., 2015).

1.5.2. SIALYLATION

Sialylation occurs as one of the final modifications of N-glycans on proteins. Sialyltransferases catalyse the addition of sialic acid residues onto the end of N-glycans in different configurations, predominantly in an α2,3 or α2,6 linkage to terminal Gal residues by ST3GalX and ST6GalX enzymes, respectively (Bhide and Colley, 2017). Sialic acids are negatively charged and thus can have significant effects on protein-protein interactions; indeed, the presence of sialic acids on erythrocytes ensures repulsion between cells to avoid unwanted interactions between circulating cells (Varki, 2008).

Sialic acids are involved in a diverse range of interactions but of interest to this review is its role in immunity. Sialic acids are key mediators of lectin binding to ligands, for example, selectins are cell adhesion molecules which bind sialylated ligands on endothelial cells to mediate leukocyte rolling and extravasation (Lehmann et al., 2006).

1.5.3. POST-TRANSLATIONAL MODIFICATIONS IN IMMUNITY

Sialylation has been well-documented to be developmentally regulated in thymic T cells. Daniels (Daniels et al., 2001b) and Moody (Moody et al., 2001) each reported that pMHC tetramers were able to bind DP thymocytes however this ability was lost as maturation occurred. It was discovered that sialylation of O-glycans on CD8αβ was developmentally regulated via upregulation of the sialylytransferase, ST3Gal-I, throughout T cell maturation. This increase in sialylation is hypothesised to decrease the binding interaction of T cells with MHC to reduce strong non-specific binding following positive selection (Merry et al., 2003). The importance of this binding is evident as the inability for ST3Gal-I to modify developing thymocytes, such as in a genetic knockout, results in a significant loss of CD8 T cells (Van

Dyken et al., 2007; Varki, 2008). Outside of development, sialylation also plays an important role in the contractile phase of an immune response. It has been suggested that the downregulation of ST3Gal-I, which occurs following antigen clearance, is a primary mechanism that induces apoptosis in CD8+ T cells involved in the response. Van Dyken and colleagues showed that inactivation of ST3Gal-I resulted in an overall reduction in sialylation of CD8αβ which rendered affected CD8 T cells susceptible to apoptosis by cross-linking of O-glycosylated proteins leading to the activation of caspase (Van Dyken et al., 2007) and the perpetuation of cell death signalling. It is evident that sialylation plays an important biological function in mediating T cell development and downstream protein interactions, specifically in CD8+ T cells.

While the role of PTM in many aspects of immune development and function has been assessed, the importance of this change in MHC biology requires further exploration. In particular, the role of PTM in the recognition of class Ib MHC has not been determined and requires attention.

1.6. STATEMENT OF THESIS AIMS

Non-classical MHC molecules represent a largely underappreciated group of proteins which display clear immunological relevance. Of these, H2-Q10 represents one of the more intriguing subjects due to its uniqueness as a soluble MHC I molecule and its overexpression in the liver. Characterisation of its expression and interactions with other proteins will broaden our understanding of its physiological role which will provide fundamental insight for potential translation into human studies.

1. Characterisation of the novel H2-Q10 knockout mouse and identification of an immunological and physiological role for H2-Q10.
2. Defining the biochemical signature regulating the interaction of H2-Q10 to its ligand.
3. To compare binding interactions between H2-Q10 and HLA.

Chapter Two

Materials and Methods

2.1. MICE

C57BL/6 mice were bred at Alfred Medical Research and Education Precinct (AMREP) Animal Services, Clayton Animal Resource Laboratory or the University of Western Australia Animal Services. All mice were used between 6 and 8 weeks of age. C57BL/6.H2-Q10$_{tm1.1(KOMP)Mbp}$ mice were generated by the trans-NIH Knock-Out Mouse Project (KOMP) and obtained from the KOMP repository (www.komp.org). NIH grants to Velocigene at Regeneron INC (U01HG004085) and the CSD Consortium (U01HG004080) funded the generation of gene-targeted ES cells for 8500 genes in the KOMP Program and archived and distributed by the KOMP Repository at UC Davis and CHORI (U42RR024244). A LacZ and Neomycin construct was inserted into the coding region of H2-Q10. Cre recombinase was then used to excise the neomycin gene leaving LacZ in the second exon of H2-Q10. A breeding colony of homozygous C57BL/6.H2-Q10$_{tm1.1(KOMP)Mbp}$ knockouts were established at Monash University Animal Services (Clayton). Genotyping of breeding pairs was performed by Transnetyx (Memphis, Tennessee, USA) using real-time PCR (RT-PCR). All mice were used between 6 and 8 weeks of age. All experiments were in accordance with the Animal Ethics Guidelines of the National Health and Medical Research Council of Australia. All experiments were approved under the project number E/1533/2015/M by the AMREP Animal Ethics Committee.

2.1.1. MCMV INFECTION

Experiments were performed by Dr. Iona Schuster and Mr. Peter Fleming (Lion's Eye Institute/University of Western Australia/Monash University) using established protocols (Schuster et al., 2014).

2.2. CELL CULTURE

2.2.1. CELL LINES

Cell lines were cultured in Alpha Minimum Essential Medium (αMEM), Roswell Park Memorial Institute 1640 Medium (RPMI) or Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, California, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), L-glutamine (2mM), 100µg/ml streptomycin and 100U/ml penicillin (Sigma Aldrich, Massachusetts, USA). Cells were maintained at 37°C in 5% CO2. For adherent cell lines, dissociation from tissue culture flasks was performed by first washing cells with warmed Phosphate Buffered Saline (PBS) (8g/L NaCl, 0.2g/L KCl, 1.44g/L Na_2HPO_4, 0.24g/L $KH_2PO_ß$ adjusted to pH 7.4) followed by addition of TrypLE (Thermo Fisher Scientific, Massachusetts, USA) for 5 minutes at 37°C. Following confirmation of cell detachment using a light microscope, TrypLE was deactivated by the addition of complete media before centrifugation (300*g*, 5 minutes).

Name	Species Of Origin	Research Resource Identification (RRID)	Characteristics	Growth Conditions
RMA-S	Mus musculus (C57Bl/6)	CVCL_2180	• Mouse leukemia • TAP-deficient cell line • Deficient in antigen processing	Complete RPMI
Chinese Hamster Ovary (CHO)	Cricetulus griseus	CVCL_0213	• Spontaneously immortalised cell line	Complete αMEM
Jurkat	Homo sapiens	CVCL_0065	• Childhood T acute lymphoblastic leukemia	Complete RPMI
293T	Homo sapiens	CVCL_0063	• Embryonic kidney cell line	Complete DMEM

Table 2.1. Cell line characteristics.

2.2.2. GENERATING VIRAL SUPERNATANTS USING 293T TRANSFECTION

Retroviral or lentiviral particles were produced using standard calcium phosphate transfection of 293T cells. Briefly, 1 x 10^6 293T cells were plated the day before transfection in complete DMEM media. For each transfection, cells were transfected with 50µl 2.5M $CaCl_2$, 450µl sterile water, 500µl of 2.5mM HeBS (280mM NaCl, 50mM HEPES, 1.5mM Na_2HPO_4, pH 7.05), 10µg of plasmid DNA and 10µg pCL-Ampho retrovirus packaging vector (for retrovirus production) or 2.4µg pMDL, 2.4µg pRSV-Rev and 4.8µg pVSV-G packaging vectors (for lentivirus production). Transfection mixtures were vortexed and added dropwise onto 293T cells. 16 hours after transfection, 293T cell supernatant was removed and cells were washed twice with PBS prior to addition of 4ml fresh complete media specific for tranfection target cell. Viral supernatants were harvested at 48 hours or 72 hours post-transfection and passed through a 0.45µm filter prior to use. Viral supernatant stocks were stored at -80°C.

2.2.3. GENERATION OF STABLE CELL LINES

For retroviral transductions, non-tissue culture treated 6-well plates were coated with Retronectin (Takara, Shiga Prefecture, Japan) prior to addition of 5ml viral supernatant. Plates were centrifuged for 2 hours (32°C, 1500*g*) and 5 x 10^4 cells were seeded per well.

Transduced cells were cultured and expanded prior to cell sorting based first on reporter fluorescence (GFP or mCherry) followed by multiple rounds of antibody-based sorting. For CD8α-expressing cell lines, multiple rounds of sorting based on CD8α antibody (clone 53-6.7) were performed. Prior to analysis, cells were sorted based on equalised CD8α expression.

2.2.4. GENERATION OF KO CELL LINES USING CRISPR/CAS9

The Harvard CHOPCHOP database (http://chopchop.cbu.uib.no) was utilised for design of short guide RNAs (sgRNAs). Two 20bp sgRNAs per target were selected based on minimal predicted off-targets (see Table 2) and early exon targeting. Overhangs on the forward (5'-TCCC-3') and complementary reverse (5'-AAAC-3') were added onto sgRNAs for cloning into the FgH1tUTG lentiviral vector (Marco Herold, Addgene Plasmid 70183). Oligomers were annealed and phosphorylated prior to cloning using BsmBI restriction digestion. Briefly, 3µl of 100µM oligomers (sense and antisense) were added to 0.5µl 3M NaCl, 2µl 1M $MgCl_2$, 2µl 1M Tris-HCl (pH 7.5) and 9.5µl Tris-EDTA (TE) buffer. Samples were annealed using a thermocycler under the following run conditions: 96°C for 5 minutes and 80°C for 60 minutes. Annealed oligomers were phosphorylated by adding 5µl annealed oligomers, 2µl T4 DNA Ligase Buffer (Promega, Wisconsin, USA), 1µl PNKinase (New England Biolabs, Massachusetts, USA) and 12µl RNAse-free water and incubating samples for 20 minutes at 30°C followed by heat-inactivation (10 minutes, 70°C). Ligated plasmids were subjected to Sanger sequencing to confirm correct orientation and sequence of sgRNA.

sgRNA	Species	Sequence (5'-3')	NCBI Reference	Target Exon
MGAT1 SG1 Sense	Mus musculus	TGAGGTCATCCACCTGGCCG	NM_001110148.1	4
MGAT1 SG1 Antisense	Mus musculus	CGGCCAGGTGGATGACCTCA		
MGAT1 SG2 Sense	Mus musculus	GGTCGCAAGGGTGTGAGCCA		
MGAT1 SG2 Antisense	Mus musculus	TGGCTCACACCCTTGCGACC		
SLC35A1 SG1 Sense	Mus musculus	GGTAGAAAGAACAATGGCTG	NM_011895.3	7
SLC35A1 SG1 Antisense	Mus musculus	CAGCCATTGTTCTTTCTACC		
SLC35A1 SG2 Sense	Mus musculus	TCTTAAAGCTACGGTGTAAG		2
SLC35A1 SG2 Antisense	Mus musculus	CTTACACCGTAGCTTTAAGA		

MGAT1 SG1 Sense	Homo sapiens	AAGTCGGGGGTTCCGAGAGC	NM_001114617	3
MGAT1 SG1 Antisense	Homo sapiens	GCTCTCGGAACCCCCGACTT		
MGAT1 SG2 Sense	Homo sapiens	ACGATGACCTTTGGCCGCAA		
MGAT1 SG2 Antisense	Homo sapiens	TTGCGGCCAAAGGTCATCGT		

Table 2.2. Short guide (SG) RNA sequences used for targeted knockout in CRISPR/Cas9 experiments.

For lentiviral transductions, 1 x 10^6 target cells were resuspended in 2ml viral supernatant and 10µg/ml polybrene (Sigma Aldrich). Cells were centrifuged for 2 hours (32°C, 1500*g*) and cultured overnight in standard culturing conditions. Cells were then washed and centrifuged (5 minutes, 300*g*) to remove viral supernatant and resuspended in complete media.

Following subsequent transduction into Cas9-expressing target cells which were validated using Western Blot. Cells were bulk sorted based on GFP^+ (FgH1tUTG-sgRNA) and $mCherry^+$ (Cas9) (Section 4.2). For induction of gene knockout, cell lines were treated with 1µg/ml doxycycline hyclate (Sigma Aldrich) for 72 hours. Cells were analysed for efficient gene knockout using flow cytometric analysis of lectin binding followed by FACS sorting.

2.3. PRIMARY CELL ISOLATION

2.3.1. MOUSE TISSUE COLLECTION AND PREPARATION

Following euthanasia, organs were harvested from mice into ice-cold PBS. Single cell suspensions were prepared using mechanical dissociation of organs through 70-100µm filters with wash steps performed using 1X PBS and centrifugation at 300 g for 5 minutes. For spleens, red blood cell lysis was performed using Ammonium Chloride Potassium (ACK) lysis buffer (150mM NH_4Cl, 10mM $KHCO_3$, 0.1mM Na_2EDTA) for 1 minute at room temperature prior to downstream analysis. For livers, isolation of hepatocytes and leukocytes were performed using previously published protocols

(Goodall et al., 2018b). Briefly, single cell suspensions were prepared from mouse livers using mechanical dissociation through metal sieves. Cells were washed in PBS and pelleted (300*g*, 5 minutes) prior to resuspension in 15ml 37.5% Percoll and gradient separation by centrifugation (690*g*, 12 minutes, no acceleration or brake). Hepatocytes were collected from the top layer and leukocytes pelleted at the bottom of the Percoll gradient. Both fractions were washed three times in PBS before downstream use. For isolation of cells in the gut, small intestines were minced and digested in PBS containing 5-10% FCS, 15mM HEPES and 5mM EDTA for one hour at 37$_{o}$C with agitation. Following digestion, supernatant was collected and centrifuged to pellet released cells. Cell pellets were resuspended in 40% Percoll, overlaid on 80% Percoll and subjected to gradient separation by centrifugation as previously stated. Lymphocytes within the interface were collected and washed prior to preparation for flow cytometric analysis.

2.3.2. ISOLATION OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCS)

Venous blood was collected and diluted 1:1 with 1X PBS. Gradient separation was performed by adding 12.5ml Ficoll-Paque (GE Healthcare, Illinois, USA) to a 50ml tube and layering diluted blood (10-20ml) carefully on top before centrifugation (400*g*, 25 minutes, no brake). The PBMC layer at the interface was collected and transfer to a fresh tube. PBMCs were washed with 1X PBS and centrifuged (823*g*, 10 minutes).

2.4. MOLECULAR BIOLOGY

2.4.1. CLONING

2.4.1.1. RESTRICTION DIGEST

Restriction digestion was performed on MSCV-GFP or MSCV-mCherry as well as gene constructs using EcoRI and XhoI in Buffer 2.1 (New England Biolabs). Digestion was performed at 37°C for 2 hours. Digested products were run on a 1% (w/v) agarose gel (in 0.5X Tris/Borate/EDTA (TBE) buffer; 89mM Tris, 2mM EDTA, 80mM boric acid) at 100V for 60 minutes and required bands excised. Gel cleanup was performed on excised gel pieces using AccuPrep Gel Purification Kit (Bioneer, South Korea).

2.4.1.2. LIGATION

Purified digested vectors and gene products were combined at a 1:1 or 3:5 ratio using ligase buffer (Promega) for 16 hours in an ice bath.

2.4.1.3. CHEMICAL TRANSFORMATION

Ligated products were added to chemically-competent Top10 *E.coli* bacteria (Life Technologies) and incubated for 30 minutes on ice before heat shocking for 45 seconds at 42°C and incubating for 2 minutes on ice. 200µl of Luria Broth (LB) was added to the transformed Top10 *E.coli* and incubated with shaking (60 minutes, 225rpm, 37°C). Transformed Top10 *E.coli* were spread onto ampicillin-containing agar plates generated using imMedia Growth Medium (Thermo Fisher Scientific) followed by incubated overnight (37°C). Colonies were picked and cultures were grown overnight in ampicillin-containing LB with 100mg/ml ampicillin. Plasmids were purified using ZymoPURE Plasmid Miniprep Kit (Zymo Research, California, USA) as per manufacturer's instructions. Orientation and correct sequence of gene insert was confirmed by Sanger DNA sequencing using established pipelines (Monash Micromon Genomics). For plasmids using the MCMV backbone, the following sequencing primers were used (5'→3'): forward TTGAACCTCCTCGTTCGACC and

reverse <CATATAGACAAACGCACACC> For plasmids using the FgH1tUTG backbone, the following sequencing primer was used (5'→3'): CAGACATACAAACTAAAGAAT.

2.4.2. GENERATION OF N-GLYCOSYLATION MUTANTS

Prediction of N-glycosylation sequons in mouse CD8α (NM_001081110.2) was performed using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). N-glycosylation sites were predicted at amino acid positions 69, 97 and 150 of mouse CD8α. To generate mutants, asparagine (N) residues in predicted sequons were substituted to alanine (A). Genes encoding full-length and single-point mutated mouse CD8α protein were purchased using GeneArt Strings DNA Fragments (Thermo Fisher Scientific). Mutated CD8α proteins were denoted as N69A, N97A, N150A. Cell lines expressing these mutated constructs were generated as per Section 2.3.

2.4.3. GENERATION OF TETRAMERS

Tetramers were generated using published protocols (Goodall et al., 2018) in conjunction with Dr Lucy Sullivan (Peter Doherty Institute, Melbourne, Australia).

Briefly, cDNA encoding residues of the MHC tetramer of interest were generated by Genscript (Nanjing, China) or Life Technologies and cloned into a pUC57 vector. Sequences were sub-cloned into a pET-30-based vector (Peter Doherty Institute) that allowed for an in-frame fusion of a substrate peptide for the enzyme BirA. The heavy chains of each molecule and mouse beta-2-microglobulin (ß2m) were expressed separately in *E. coli*, purified from inclusion bodies and refolded without peptide (in instances of peptide independence) or with known peptide ligands. Monomers were tetramerized with streptavidin conjugated to allophycocyanin (SA-APC) and stored at 4°C. Tetramers generated are listed in Table 2.3

Tetramer	Peptide	Mutation Notes
H2-Q10 TGT	T cell receptor (TCR) V-beta chain, TGTETLYF	n/a (native form)
H2-Q10 VGI	Ribophorin, VGITNVDL	n/a (native form)
H2-Q10 T187A	TCR V-beta chain, TGTETLYF	Threonine (T) at position 187 of H2-Q10 protein sequence (without leader sequence) was substituted with alanine (A)
H2-Q10 G194R	TCR V-beta chain, TGTETLYF	Glycine (G) at position 194 of H2-Q10 protein sequence (without leader sequence) was substituted with A
H2-Q10 T187A G194R	TCR V-beta chain, TGTETLYF	T at position 197 of H2-Q10 protein sequence A and G at position 194 of H2-Q10 protein sequence was substituted with A
H2-Q10 SRP	TCR V-beta chain, TGTETLYF	Proline/glycine/serine (PGS) at position 193-195 of H2-Q10 protein sequence was substituted with serine/arginine/proline (SRP)
H2-Q4	Influenza nucleoprotein (NP), NP_{366}	n/a (native form)
H2-Q9		n/a (native form)
H2-TL	n/a	n/a (native form)
Qa-1b	Qa-1 determinant modifier (QDM), AMAPRTLLL	n/a (native form)
H2-D_b	NP_{366}	n/a (native form)
H2-K_b	Ovalbumin, SIINFEKL	n/a (native form)
H2-K_bα3Q10	Ovalbumin, SIINFEKL	Substitution of native H2-K_b α3 region with H2-Q10
H2-Q10α3K_b	NP_{366}	Substitution of native H2-Q10 α3 region with H2-K_b
HLA-G		n/a (native form)
HLA-E	HLA-A2 leader sequence, VMAPRTLVL	n/a (native form)
HLA-Eα3Q10	HLA-A2 leader sequence, VMAPRTLVL	Substitution of native HLA-E α3 region with H2-Q10

Table 2.3. Summary of generated tetramers.

2.4.4. RNA EXTRACTION

RNA from cell lines was prepared using the RNeasy Mini Kit (Qiagen) as per manufacturer's instructions.

2.4.5. RNA EXTRACTION – TRIZOL

Mouse livers were isolated, sectioned and snap-frozen in liquid nitrogen. 1ml of TRI Reagent (Sigma Aldrich) was added per 100mg of frozen tissue followed by homogenisation (50Hz oscillation, 60 seconds) using a TissueLyser LT (Qiagen). Samples were then centrifuged (10,000g, 5 minutes) and the RNA containing supernatants collected. Phase separation of the supernatants was then performed by adding 200µl chloroform followed by gentle mixing (1 minute) and incubation at room temperature for 3 minutes. Samples were then centrifuged (12,000, 15 min) and the aqueous phase collected and transferred to new tubes. 500µl isopropanol was then added to these samples, incubated at room temperature for 10 min and centrifuged (12,000g, 10 min) to pellet the RNA. The pellet was then de-salted by washing thoroughly using 1ml ice-cold ethanol (75% v/v) followed by centrifugation (12,000g, 10 min). This step was performed a minimum of three times. The de-salted and purified RNA pellets were then air-dried for 15 min at room temperature before being resuspended in RNAse-free water. RNA concentrations were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific) and samples were stored at -80°C.

2.4.6. COMPLEMENTARY DNA (CDNA) SYNTHESIS

cDNA was synthesised using either a iScript reaction kit (Bio-Rad, California, USA) or a FIREScript reaction kit (Solis Biodyne, Tartu, Estonia). For iScript, 500-1000ng of RNA was added to 5x iScript Reaction Mix and iScript Reverse Transcriptase as per the iScript cDNA Synthesis Kit manufacturer's instructions. cDNA was synthesised by 5 min at 25°C, 20 min at 46°C and 1 min at 95°C. For FIREScript, 500-1000ng of RNA was added to 1µl random primers, 0.5µl dNTP mix, 2µl 10X RT

reaction buffer with DTT, 1µl FIREScript reverse transcriptase, 0.5µl RiboGrip RNase Inhibitor and made up to 20µl with nuclease-free water as per the manufacturer's instructions. The reaction protocol consisted of running the following steps on a MultiGene OptiMax thermal cycler (Labnet International, New Jersey, USA): priming (5 minutes, 25°C), reverse transcription (30 minutes, 46°C), transcriptase inactivation (1 minute, 85°C) and hold (4°C).

2.4.7. QUANTITATIVE POLYMERASE CHAIN REACTION (QPCR)

cDNA was diluted 1:10 and 1µl was added to 0.5µl of forward primer (10µM), 0.5µl of reverse primer (10µM), 0.05µl QN Rox Reference Dye, 5µl QN SYBR Green PCR Master Mix and made up to a final volume of 10µl with 3.95µl RNase-free water. qPCR was performed on a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific) under the following conditions: activation (2 minutes, 95°C), denaturation (5 seconds, 95°C), annealing/extension (10 seconds, 60°C) for 40 cycles. Melting curve analysis was performed to ensure amplification of only single products. Raw data was analysed using the delta-delta Ct ($2^{-\Delta\Delta Ct}$) quantification method using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping/normalisation control.

2.4.8. POLYMERASE CHAIN REACTION (PCR)

2µl of neat cDNA template was mixed with 10µl 2x GoTaq Green Master Mix, 0.5µM forward and reverse primer and made up to 20µl with nuclease-free water. The reaction protocol was as follows: one cycle of initial denaturation (95°C, 5 minutes, 1 cycle), 40 cycles of denaturation (95°C, 30 seconds)/annealing (60°C, 30 seconds)/extension (72°C, 15 seconds), one cycle of final extension (72°C, 5 minutes) and hold (4°C).

Name	Strand	Sequence	Species	Exon	Amplicon Size	Tm
H2-Q10	Forward	CGATTATCACCCGACGCAAG	Mus musculus	3	181	61.4
	Reverse	GTGACATCACCTTCAGATCCTGG		4		60.8
GAPDH	Forward	TGGCCTTCCGTGTTCCTAC	Mus musculus	5	178	61
	Reverse	GAGTTGCTGTTGAAGTCGCA		6		61
Sult2a1	Forward	CAAACCTTGTGAAGAATCCAGG	Mus musculus	3	121	58.97
	Reverse	TCCCATTCTCTCATGGACAGC		4		59.65

Table 2.4. Summary of designed primers used in PCR or qPCR.

2.4.9. GEL ELECTROPHORESIS

1% (w/v) agarose gel was prepared with 0.5X TBE (Tris/Borate/EDTA) buffer and 1:10,000 SYBR Safe DNA gel stain (Invitrogen, California, USA). Gel electrophoresis was performed at 100V for 30-60 minutes in 0.5X TBE buffer. Gels were imaged with EpiChemi II Darkroom imager (UVP, California, USA).

2.4.10. RNA SEQUENCING AND ANALYSIS

RNA samples from livers of four wild-type and three H2-Q10 KO mice were prepared by Trizol extraction (as per Section 2.4.8). Samples underwent quality control analysis (as per Monash Micromon Genomics protocols) and selected samples underwent poly-A enrichment prior to subsequent sequencing by Illumina NextSeq500 (Illumina, California, USA) using the mm10 mouse reference genome. Previously established pipelines from the Monash Bioinformatics Platform were used for data analysis. Raw data was uploaded and visualised using Degust (Monash University, Melbourne, Australia). Pathway analysis was performed on differentially-expressed genes using PANTHER (http://www.pantherdb.org). Differential gene expression was confirmed using qPCR as per 2.4.10.

2.5. FLOW CYTOMETRY

2.5.1. FLOW CYTOMETRIC ACQUISITION AND ANALYSIS

Cells were blocked with 2% (v/v) goat serum (Thermo Fisher Scientific) in PBS for 20 min and washed with 2% (v/v) FCS/PBS. Cells were stained with antibody (Section 2.7) or biotinylated lectin (Section 2.6) for 20 min on ice before pelleting and resuspending in 2% FCS/PBS. For lectin-stained cells, samples were further stained with fluorophore-conjugated streptavidin for 20 minutes (Section 2.7) before pelleting and resuspending in 2% FCS/PBS For samples requiring tetramer staining, cells were co-stained with CD8α antibody (1:100) and tetramer (final concentration 0.1mg/ml) for 20 minutes on ice to promote stabilisation of the bound tetramer. Tetramer stained cells were then fixed in 4% (v/v) paraformaldehyde/10% FCS/PBS immediately following antibody staining, washed and resuspended in 2% FCS/PBS prior to analysis. Flow cytometry experiments were performed with a BD LSR-Fortessa X-20 or BD LSR II (BD Biosciences, New Jersey, USA) and data was analysed using FlowJo software (LLC, Oregon, USA, Version 10). Tetramers and antibodies used in publications are summarised as previously reported (Goodall et al., 2018).

Tetramer	Conjugate	Species Reactivity	Dilution
H2-Q10 TGT	APC	Mouse	2mg/ml tetramers were used at 1:200 dilution. 1mg/ml tetramers were used at 1:200 dilution.
H2-Q10 VGI	APC	Mouse	
H2-Q10 T187A	APC	Mouse	
H2-Q10 G194R	APC	Mouse	
H2-Q10 T187A G194R	APC	Mouse	
H2-Q10 SRP	APC	Mouse	
H2-K_b	APC	Mouse	
H2-K_bα3Q10	APC	Mouse	
H2-Q10α3K_b	APC	Mouse	
Qa-1b	APC	Mouse	
HLA-E	APC	Human	
HLA-Eα3Q10	APC	Human	
HLA-G	APC	Human	

Table 2.5. Tetramers used for flow cytometric analysis.

Lectin	Conjugate	Species Reactivity	Clone Number	Catalogue Number	Company	Dilution
Phaseolus Vulgaris Leucoagglutinin (PHA-L)	Biotin	n/a	n/a	B-1115	Vector Labs	1:2000
Maackia Amurensis Lectin II (MAL II)	Biotin	n/a	n/a	B-1265	Vector Labs	1:1000
Sambucus Nigra Lectin (SNA)	Biotin	n/a	n/a	B-1305	Vector Labs	1:2000

Table 2.6. Lectins used for flow cytometric analysis.

Antibody	Conjugate	Species Reactivity	Clone Number	Company	Dilution
CD8β	PE-Cy7	Human	SIDI8BEE	eBioscience	1:100
CD8α	BV421	Human	RPA-T8	Biolegend	1:100
CD3	FITC	Human	UCHT1	BD Biosciences	1:100
CD8α	BV421	Mouse	53-6.7	BioLegend	1:100
NKp46	PE/BV421	Mouse	29A1.4	BioLegend	1:100
CD49a	APC	Mouse	HMα2	BioLegend	1:100
CD49b	PE	Mouse	HMα2	BioLegend	1:100
CD94	FITC/PE	Mouse	18d3	BioLegend	1:100
Ly49C/I	BV607	Mouse	14B11	BD Biosciences	1:100
TCRγδ	PE	V	GL3	BioLegend	1:100
CD3	APC/BV605	Mouse	17A2	BioLegend	1:100
CD8β	PE	Mouse	53-5.8	BioLegend	1:100
CD27	APC/Pacific Blue	Mouse	LG.3A10	BioLegend	1:100
CD11b	APC/PE	Mouse	M1/70	BioLegend	1:100
CD96	BV421	Mouse	3.3	BioLegend	1:100
Streptavidin (SA)	PE	n/a	n/a	Life Technologies	1:100
Streptavidin (SA)	APC	n/a	n/a	eBioscience	1:100

Table 2.7. Antibodies used for flow cytometric analysis.

2.5.2. FLUORESCENCE-ACTIVATED CELL SORTING (FACS)

Cells were resuspended in Zombie Near Infrared (1:10000) or Zombie Violet (1:1000) Fixable Dye (Biolegend) in PBS for 15 minutes at room temperature and protected from light. Cells were washed (300*g*, 5 minutes), blocked with 2% (v/v) goat serum in PBS and incubated for 20 minutes on ice. Cells were washed after blocking (300*g*, 5 minutes), resuspended in antibodies diluted in sort buffer

(2% FCS, 1mM EDTA in PBS) and incubated (20 minutes, on ice). Cells were resuspended at a final concentration of 1 x 10^7 cells/ml in sort buffer and passed through a 70µm filter prior to transfer into polypropylene tubes. Samples were sorted using a BD FACSAria or BD Influx (BD Biosciences) into complete growth media (cell type dependent, see Table 2.1).

2.6. PROTEIN BIOCHEMISTRY

2.6.1. MOUSE SERUM PREPARATION

Following euthanasia, a cardiac bleed was performed using a 25-gauge needle. Blood samples were incubated at room temperature for 30 min to clot before centrifugation at 1000*g* for 10 min) and the serum layer collected. SIGMAFAST™ Protease Inhibitor (1.62mg/ml) (Sigma-Aldrich) was added to the serum prior to aliquoting and storage at -80°C.

2.6.2. PROTEIN ISOLATION

Cell pellets were washed once with ice-cold PBS and centrifuged (300 g, 5 minutes). Cell pellets were resuspended in CytoBuster Protein Extraction Reagent (Novagen) containing 1X SIGMAFAST™ Protease Inhibitor (Sigma-Aldrich) and incubated at room temperature for 10 minutes. Samples were centrifuged (15,000*g*, 5 minutes, 4°C) and supernatant collected.

2.6.3. PROTEIN QUANTIFICATION

Protein determination was performed using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) as per manufacturer's instructions. Absorbance was measured spectrophotometrically at 562nm wavelength on a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific).

2.6.4. SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Protein samples were denatured (70°C, 10 minutes) with 1X NuPAGE LDS Sample Buffer (Invitrogen) and 50mM DTT. Samples and SeeBlue Plus2 Pre-Stained Protein Standard (Invitrogen) were loaded into pre-cast 4-12% Bolt Bis-Tris gels (Invitrogen) loaded in a Mini Gel Tank (Invitrogen) in the presence of 1X NuPAGE MES SDS Running Buffer (50 mM MES, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.3) (Invitrogen). Electrophoresis was performed at 100-120V for 60-90 minutes.

2.6.5. WESTERN BLOTTING

SDS-PAGE gels were transferred onto polyvinylidene fluoride (PVDF) membranes using the iBlot 2 Dry Blotting System (Invitrogen). Transfer steps were performed as follows: 20V (1 minute), 23V (4 minutes), 25V (2 minutes). Membranes were blocked for 1 hour at room temperature in 5% (w/v) skim milk powder in 0.1% (v/v) Tween-20/PBS. Membranes were probed with primary antibodies (1:3000) diluted in 5% skim milk in PBS-Tween (Table 2.8). Primary antibody probing was performed overnight at 4°C. Membranes were washed three times (10 minutes per wash) in 0.1% PBS-Tween. Horseradish peroxidase (HRP)-conjugated secondary antibodies (Table 2.8) were used (1:3000 in 5% skim milk in PBS-Tween) and incubated at room temperature for one hour. Membranes were washed three times (10 minutes per wash) in 0.1% PBS-Tween. Chemiluminescence was observed using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) on Amersham Hyperfilm (GE Healthcare)

Antibody	Clone Number	Catalogue Number	Company
Anti-mouse H2-Q10	N/A	N/A	Developed by Monash Antibody Technologies Facility (MATF)
Mouse anti-mouse beta-actin	AC-15	A1978-100UL	Sigma Aldrich
Sheep anti-mouse IgG F(ab')2 (HRP conjugated)	Polyclonal	GEHENA9310-1ML	GE Healthcare

Table 2.8. Antibodies used for western blot analysis.

2.6.6. ENYZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISA was performed using Mouse Dehydroepiandrosterone sulfate (DHEA-S) ELISA Kit (Abkine, Wuhan, China) as per manufacturer's instructions. Briefly, standards were prepared (2.5 pg/mL – 80 pg/mL) and 50µl were loaded into the pre-coated 96-well plate. 1:5 dilutions of serum samples were prepared and 50µl added to the plate followed by incubation for 45 minutes at 37°C. Plates were washed three times using provided Wash Buffer. Supplied HRP-conjugated detection antibody was added and plate was incubated for a further 30 minutes at 37°C. Plates were washed three times. 100µl of Chromogen solution was added to each well and incubated for 15 minutes at 37°C. 50µl of Stop solution was added to each well. Absorbance was measured spectrophotometrically at 450nm wavelength on a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific).

2.6.7. IMMUNOPRECIPITATION

Immunoprecipitation was performed using Pierce Co-Immunoprecipitation Kit (Thermo Fisher Scientific) as per manufacturer's instructions. Briefly, 10µg of affinity-purified H2-Q10 antibody was covalently coupled to AminoLink Plus Coupling Resin (room temperature, 2 hours) and washed extensively in supplied wash buffers (1000*g*, 1 minute). 1mg of serum was first pre-cleared using supplied Control Agarose Resin and subsequently incubated with antibody-coupled beads (4°C, overnight). Following immunoprecipitation, resin columns were centrifuged and flow-through was

collected for analysis of immunoprecipitation efficiency. Low pH buffer was applied to the resin columns for 10 minutes at room temperature to elute captured proteins followed by neutralisation with 1M Tris (pH 9.5).

2.6.8. MASS SPECTROMETRY

Experiments were performed in a collaborative effort with Monash Proteomics Facility using established protocols and data analysis pipelines. Briefly, using a Dionex UltiMate 3000 RSLCnano system equipped with a Dionex UltiMate 3000 RS autosampler, an Acclaim PepMap RSLC analytical column (75 µm x 50 cm, nanoViper, C18, 2 µm, 100Å; Thermo Scientific) and an Acclaim PepMap 100 trap column (100 µm x 2 cm, nanoViper, C18, 5 µm, 100Å; Thermo Scientific), the tryptic peptides were separated by increasing concentrations of 80% ACN / 0.1% formic acid at a flow of 250 nl/min for 98 min and analysed with a Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific) using in-house optimized parameters to maximize the number of peptide identifications. The raw files were searched with Byonic v3.0.0 (ProteinMetrics, California, USA) to obtain sequence information. Only proteins falling within a predefined false discovery rate (FDR) of 1% based on a decoy database were considered for further analysis.

2.6.9. SURFACE PLASMON RESONANCE (SPR)

Experiments were performed as previously published (Goodall et al., 2018b). Biotinylated MHC were captured on the surface of a ProteOn NLC neutravidin chip (Bio-Rad, ~150 RU of each). An empty flow cell with neutravidin alone served as a control. Serially diluted CD8α was injected simultaneously over the control and test surfaces. After subtraction of data from the control flow cell, K_D was calculated by kinetic analysis using a Langmuir 1:1 binding model with the ProteOn Manager software (Bio-Rad).

2.7. STATISTICAL ANALYSIS

Mann-Whitney U tests (for non-parametric distributions) were performed to assess statistical significance (Prism 7, GraphPad Software, California, USA). Where more than two groups were being analysed, a non-parametric, one-tailed Kruskal-Wallis ANOVA was performed. Statistical significance was defined as $p<0.05$.

Chapter Three

A non-immunological role for H2-Q10 in mouse physiology?

Sections of this chapter have been published in the following article:

Goodall, K.J., **Nguyen, A**., Matsumoto, A., McMullen, J.R., Eckle, S.B., Bertolino, P., Sullivan, L.C., and Andrews, D.M. (2018). Multiple receptors converge on H2-Q10 to regulate NK & γδT cell development. Immunology & Cell Biology.

3.1. ABSTRACT

The murine non-classical MHC I molecule, H2-Q10, is overexpressed in the liver however its function has not been defined. Previous work from our group has shown that H2-Q10 is a ligand for the NK cell receptor, Ly49C, however, this interaction does not occur in the presence of class Ia MHC molecules. To attempt to understand the function of H2-Q10 outside of Ly49C binding, this chapter provides supporting data for its high expression in the liver, as well as implicating hepatocytes as the main producing cell type, which is also altered with age. Furthermore, the solubility of H2-Q10 is explored through generation of a novel antibody and subsequent mass spectrometric analysis. In the pursuit of understanding its function, H2-Q10 knockout (H2-Q10 KO) mice were generated and immunophenotyped, showing a reduction in specific NK and γδ T cell frequencies. This chapter also shows that H2-Q10 deficiency, and its impact on immune cell numbers, did not have a significant effect in reducing viral titres in an viral infection (MCMV) model. Owing to subtle immunological changes in the context of H2-Q10 deficiency, RNASeq analysis was performed on H2-Q10 KO mice and revealed differential gene expression which implicates H2-Q10 in mediating physiological processes. Taken together, this chapter provides insight into the expression, immunological and physiological roles of H2-Q10.

3.2. INTRODUCTION

The major histocompatibility complex plays a central role in innate and adaptive immunity. The archetypal role of MHC is in antigen presentation, a process which elicits protective and sterilising immunity against a range of pathogens. The MHC family is typically divided into class I (MHC I) and class II (MHC II). These molecules present peptides to CD8 and CD4 T cells respectively, and guide the development and activation of cellular and humoral immunity.

MHC I can be further subdivided into two groups: classical (MHC Ia) and non-classical (MHC Ib). MHC Ia genes are highly polymorphic and MHC proteins encoded by these genes are almost ubiquitously expressed on all nucleated cells. Class Ia MHC molecules in mice are represented by H2-K, -D and -L families of genes. In humans, this same family of genes is known as HLA-A/B/C. The primary role of class Ia MHC molecules is to present antigen from infected cells to cells in the arm of the adaptive immune system. An example of this process can be found during the response to viral infection. Following infection, peptides, of approximately nine amino acids in length, are processed via the immunoproteasome, loaded onto class Ia MHC molecules in the endoplasmic reticulum and then displayed on the surface of the virally-infected cell. In addition, antigens which have been internalised by some dendritic cells are able to be processed and presented by MHC class I molecules in a process known as cross-presentation (Embgenbroich and Burgdorf, 2018).

The capacity of MHC Ia to respond to a large number of viruses is reflected by the polymorphism in the peptide binding groove of these molecules. This allows them to present a wide array of peptides to CD8 T cells, resulting in activation, expansion and the generation of memory.

Despite their structural similarity to class Ia MHC molecules, MHC Ib family members display limited polymorphism, tissue-restricted expression and functional diversity existing outside of peptide presentation (Ohtsuka et al., 2008). The MHC Ib molecules in mice are contained within distinct families defined by grouping on chromosome 17; these families are H2-Q, H2-T and H2–M (Goodall et al., 2018a). Murine MHC Ib, in contrast to the human MHC Ib HLA-E, HLA-F and HLA-G, are a highly expanded group of molecules with more than 30 genes existing across the three main families (Goodall et al., 2018a). Although some of these molecules have been characterised with respect to their expression pattern and biological function, the vast majority of these class Ib family proteins remain a "black box". Of the class Ib MHC families, the Q family proteins have been described as being the most closely related to class Ia MHC; this is supported by Q family proteins such as H2-Q7/9, H2-Q10 and H2-Q4 (unpublished data) presenting classical peptide repertoires (He et al., 2001; Zappacosta et al., 2000). Despite their evolutionary similarity to class Ia MHC, and therefore their potential to share similar characteristics, the Q family proteins remain understudied.

H2-Q10 is a member of the Q family of proteins which shows liver-specific expression (Kress et al., 1983; Lew et al., 1986). It has previously been shown to be soluble owing to its polar C-terminus, suggestive of an inability to support cell membrane insertion, as well as the lack of a translated transmembrane domain, thus resulting in secretion from expressing cells (Mellor et al., 1984); indeed, the soluble form of H2-Q10 has been measured in earlier studies in mouse sera (Lew et al., 1986).

Our group has previously shown that H2-Q10 binds the NK cell inhibitory receptor, Ly49C (Sullivan et al., 2016). While this suggested that H2-Q10 could be involved in regulating NK cell responses it was further shown that under normal physiological conditions, the MHC Ia

molecule, H2-K_b, outcompeted H2-Q10 for binding to Ly49C. Thus, the physiological relevance of the interaction between H2-Q10 and Ly49C remains unclear. To further explore the biological role of H2-Q10, this chapter aimed to define the potential physiological and/or immunological function of this class Ib MHC molecule.

3.3. RESULTS

H2-Q10 expression is expressed highly in the liver and increases with age

To support previous findings of H2-Q10 expression in the liver, and expand this analysis to other peripheral organs, qPCR was used to detect H2-Q10 mRNA transcript levels in the liver, thymus, spleen and gut. It was found that the liver was a rich source of H2-Q10 when compared to the thymus, spleen and gut, with a median increase of approximately 47-fold compared to the thymus (Figure 3.1a). To determine which specific cell type in the liver expresses H2-Q10, livers were subjected to gradient separation and RNA was prepared from hepatocyte or non-hepatocyte cells. Of the expression of H2-Q10 in whole liver, there was no significant difference compared to transcript levels in hepatocytes, indicative of its major contribution to total liver H2-Q10 expression (Figure 3.1b). In contrast, non-hepatocytes provided little to overall liver expression. These data demonstrate that the liver, specifically hepatocytes, represents a site of high H2-Q10 expression.

The expression of MHC class I molecules has been shown to be associated with age (Janick-Buckner et al., 1991; Sidman et al., 1987). Owing to its similarities to class Ia MHC, H2-Q10 was analysed for its expression in early neonatal life (week 1) and adulthood (week 8). It was found that there was a significantly higher H2-Q10 mRNA transcript expression in adult mice compared to neonates (Figure 3.1c). Thus, H2-Q10 appears to show an age-dependent expression pattern in the liver.

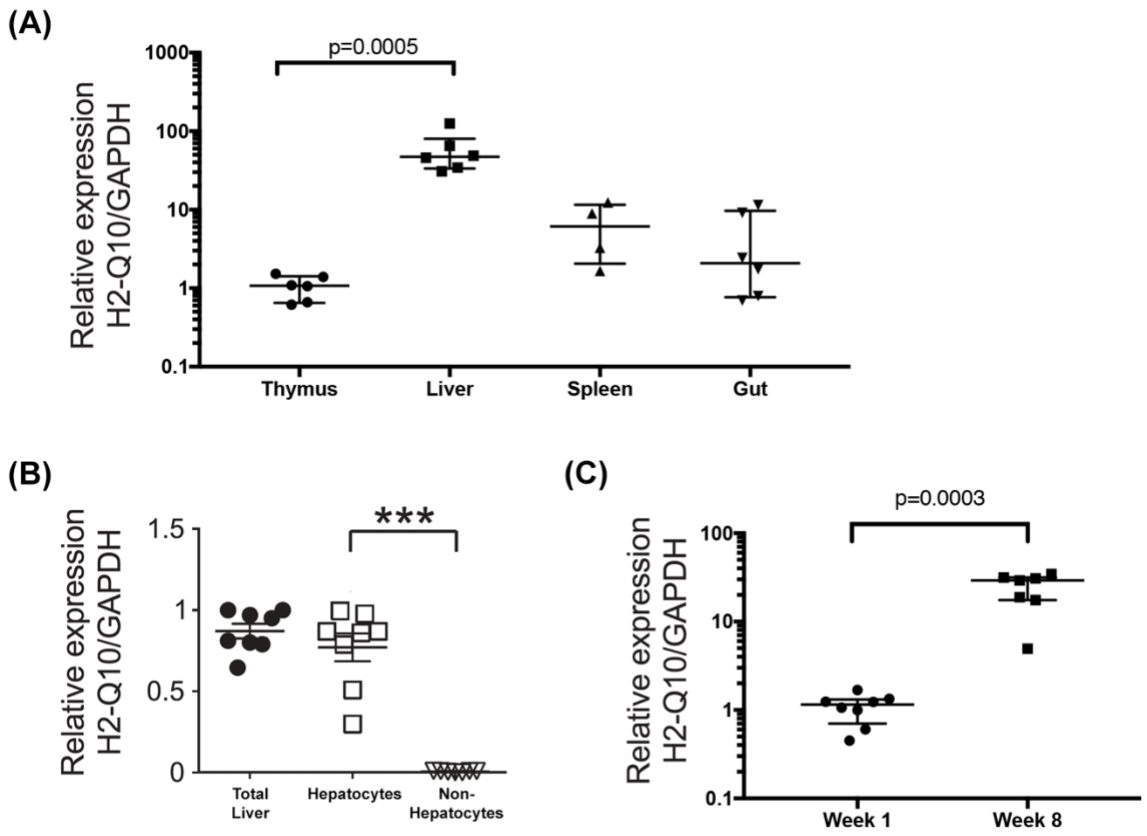


Figure 3.1. H2-Q10 transcripts are highly expressed in the liver, specifically in hepatocytes, and increases with age. (A) Whole organs were isolated and RNA extracted for real-time PCR analysis. H2-Q10 expression is normalised to housekeeping gene, GAPDH, and results are expressed as a fold change relative to the mean ddCt of 'Thymus' group. (B) Liver samples were divided into hepatocyte and non-hepatocyte compartments by gradient separation prior to extraction of RNA and subsequent qPCR analysis. H2-Q10 expression is normalised to GAPDH and results are expressed as a fold change relative to the mean "Total Liver" group. (C) Whole livers were isolated and RNA extracted for real-time PCR analysis. H2-Q10 expression is normalised to GAPDH and data are expressed as fold change relative to the mean of week 1 samples. n = 8 (Week 1) and n = 7 (Week 8) samples were pooled from two independent experiments. . Symbols represents individual mice and the median ± interquartile range (IQR) is shown. Data is representative of at least two independent experiments. Data was analysed using a non-parametric Mann-Whitney U t-test (B and C) or a non-parametric Kruskal-Wallis one-way ANOVA with multiple comparisons (A). Significance is defined as shown or *** $p<0.001$.

Generation of a H2-Q10 monoclonal antibody

To determine the effect of H2-Q10 deficiency *in vivo*, H2-Q10 knockout (H2-Q10 KO) mice were generated, validated and phenotyped. Briefly, no abnormalities in gross anatomy were observed between WT and H2-Q10 KO mice (data not shown); further differences between the phenotype of these mice are discussed later in this chapter.

While a polyclonal antibody to H2-Q10 has been generated (Lew et al., 1986), this antibody is not specific for H2-Q10 and does interact with other class Ib MHC (unpublished data). In order to develop a monoclonal antibody, H2-Q10 KO mice were immunised and boosted with H2-Q10 monomers prior to the generation of multiple hybridomas. Supernatants from these cell lines were screened in an ELISA-based assay against a panel of class I MHC monomers with a hybridoma producing a monoclonal antibody against H2-Q10 selected (data not shown; experiments performed by Monash Antibody Technologies Facility). Due to the sequence similarity of Q family proteins to class Ia molecules, the generated antibody was first tested for cross-reactivity with class Ia monomers. Western blot analysis of MHC monomers indicated that the antibody only detected H2-Q10 monomers and did not react with H-2D$_b$ or H-2K$_b$ (Figure 3.2a). Furthermore, cross-reactivity of the H2-Q10 antibody to other class Ib monomers, Q9 and TL, was tested by Western Blot. It was found that H2-Q10 monomers folded around the three most common peptides, VGI, TGT and HGT, were all detected by the H2-Q10 antibody whereas Q9 and TL were not detected (Figure 3.2b).

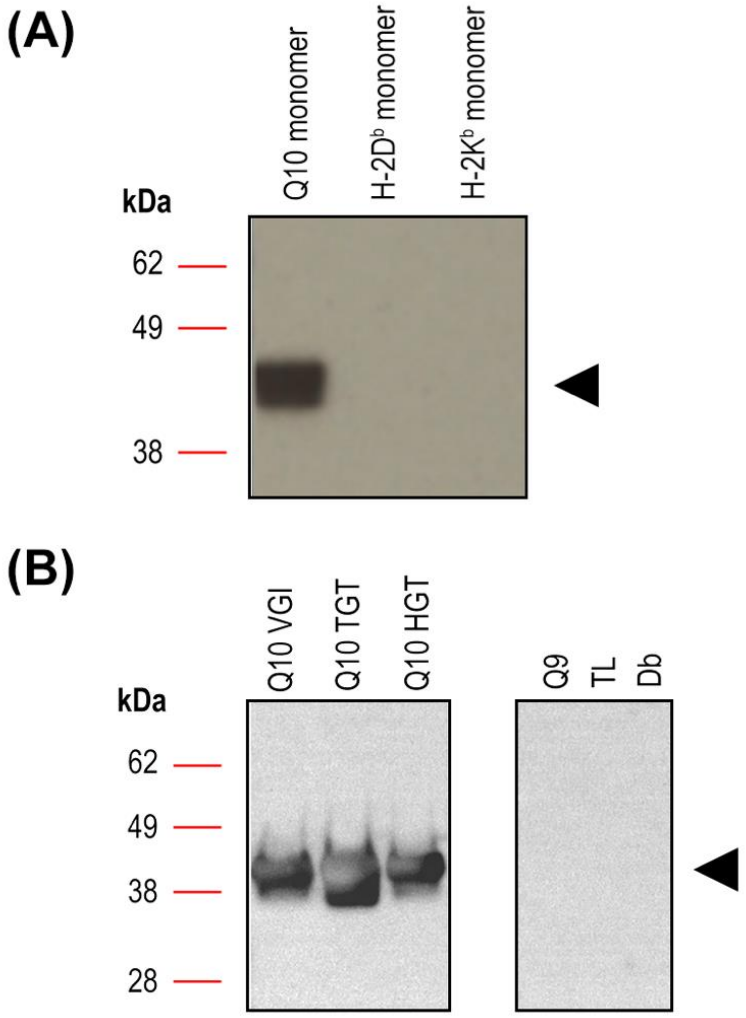
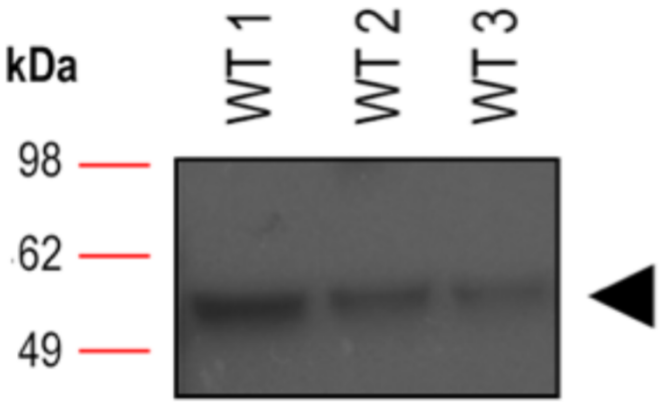


Figure 3.2. Monoclonal antibody generated is reactive to H2-Q10 and not class Ia MHC proteins. (A) 10µg of total protein from Q10, H-2Db and H-2Kb were run on an SDS-PAGE and Western blotting was performed using purified H2-Q10 antibody followed by sheep anti-mouse secondary conjugated to HRP. (B) 10µg of Q10, Q9, TL and Db monomers were loaded and Western blotting performed using H2-Q10 antibody. Chemiluminescence was measured following treatment of membrane with ECL and developed on Amersham Hyperfilm for 30 seconds. Arrows indicate position of H2-Q10 or labelled monomer. Blots are representative of two independent experiments.

H2-Q10 exists as a soluble molecule in the serum of WT mice

To determine whether the antibody was able to detect H2-Q10 from primary samples, serum was isolated from WT mice and subjected to Western Blotting. A slightly larger (50kDa) than recombinant H2-Q10 protein (~38kDa) band was detected in serum following probing with H2-Q10 antibody (Figure 3.3a). To support the identification of H2-Q10 protein in serum, mass spectrometry was performed on WT serum samples immunoprecipitated with the H2-Q10 antibody (Figure 3.3b). These results demonstrated positive identification of H2-Q10.

(A)



(B)

Protein Rank	Species	Name	Gene Name	Log Prob	Number of Amino Acids in Protein
1	Mus musculus	Thrombospondin-1	*Thbs1*	622.65	1170
2	Mus musculus	Serum albumin	*Alb*	569.19	608
3	Mus musculus	Ig gamma-2A chain C region secreted form	-	522.50	335
4	Mus musculus	Ig mu chain C region	*Ighm*	455.97	454
5	Mus musculus	Transitional endoplasmic reticulum ATPase	*Vcp*	451.63	806
6	Mus musculus	Complement C3	*C3*	386.37	1663
7	Mus musculus	Fibronectin	*Fn1*	371.93	2477
8	Mus musculus	Ig gamma-2B chain C region	*Igh-3*	324.22	404
9	Mus musculus	Ig kappa chain C region	-	253.10	106
10	Mus musculus	IGHG1_MOUSE Ig gamma-1 chain C region secreted form	*Ighg1*	210.63	324
11	Mus musculus	CD5 antigen-like	*CD5l*	168.41	352
12	**Mus musculus**	**H-2 class I histocompatibility antigen, Q10 alpha chain**	***H2-Q10***	**162.74**	**325**
13	Mus musculus	Ig kappa chain V-II region 26-10	-	159.75	113

Figure 3.3. H2-Q10 can be detected in the serum of C57Bl/6 mice. (A) 1µg of serum from three WT mice (WT1, WT2, WT3) were run on an SDS-PAGE and Western blotting was performed using purified H2-Q10 antibody followed by sheep anti-mouse secondary conjugated to HRP. Chemiluminescence was measured following treatment of membrane with ECL and developed on Amersham Hyperfilm for 30 seconds. Arrows indicate position of H2-Q10 or labelled monomer. Blots are representative of two independent experiments. (B) Mass spectrometry performed on WT mouse serum sample immunoprecipitated with the H2-Q10 monoclonal antibody displaying the top 13-ranked identified proteins. Samples were run on a Fusion Mass Spectrometer (Thermo Scientific) and analysed using the Byonic (ProteinMetrics) search engine. "Log Prob" represents the absolute value of the $\log_{10}$ protein p-value. Highlighted in green is the H2-Q10 protein (bait protein).

H2-Q10 deficiency reveals reduced Qa-1b expression

To determine the impact of H2-Q10 deletion *in-vivo*, the novel H2-Q10 deficient mice were phenotyped for the impact of H2-Q10 loss on the expression of other MHC molecules which may compensate for H2-Q10 deficiency. Firstly, analysis of other class Ib MHC expression in H2-Q10 KO mice was performed to determine whether a compensatory increase of other molecules results from H2-Q10 deficiency. It was observed that Qa-1b surface levels were significantly reduced in purified hepatocytes of H2-Q10 KO mice (Figure 3.4a) however there was no significant different observed in the spleen (Figure 3.4b). As Qa-1b has been reported to present the leader peptide sequence from H2-Q10, the effect of Qa-1b reduction in the liver was explored further in relation to changes in immune cell subsets.

Qa-1b is a ligand for CD94/NKG2 on NK cells and ILCs and therefore the absence of H2-Q10 may alter these cell frequencies. Therefore, NK cells and ILCs were isolated from the liver, and the number and frequencies of cells were determined. Indeed, although the overall ILC compartment (NKp46+ CD3-) did not show any difference in absolute cell number between WT and H2-Q10 KO mice, upon subdivision of this compartment into of CD49b+ conventional NK (cNK) cells and CD49a+ ILC-1 cells, a small but significant reduction could be seen in CD49b+ cNK cells (Figure 3.5). Thus, H2-Q10 may play a role in the development of liver cNK cells. Interestingly though, although there was an effect of H2-Q10 deficiency in absolute cell number, there was no effect on cNK cell maturation or functionality, suggesting that H2-Q10 is required in the generation of these cells and not their maturation (Figure 3.6).

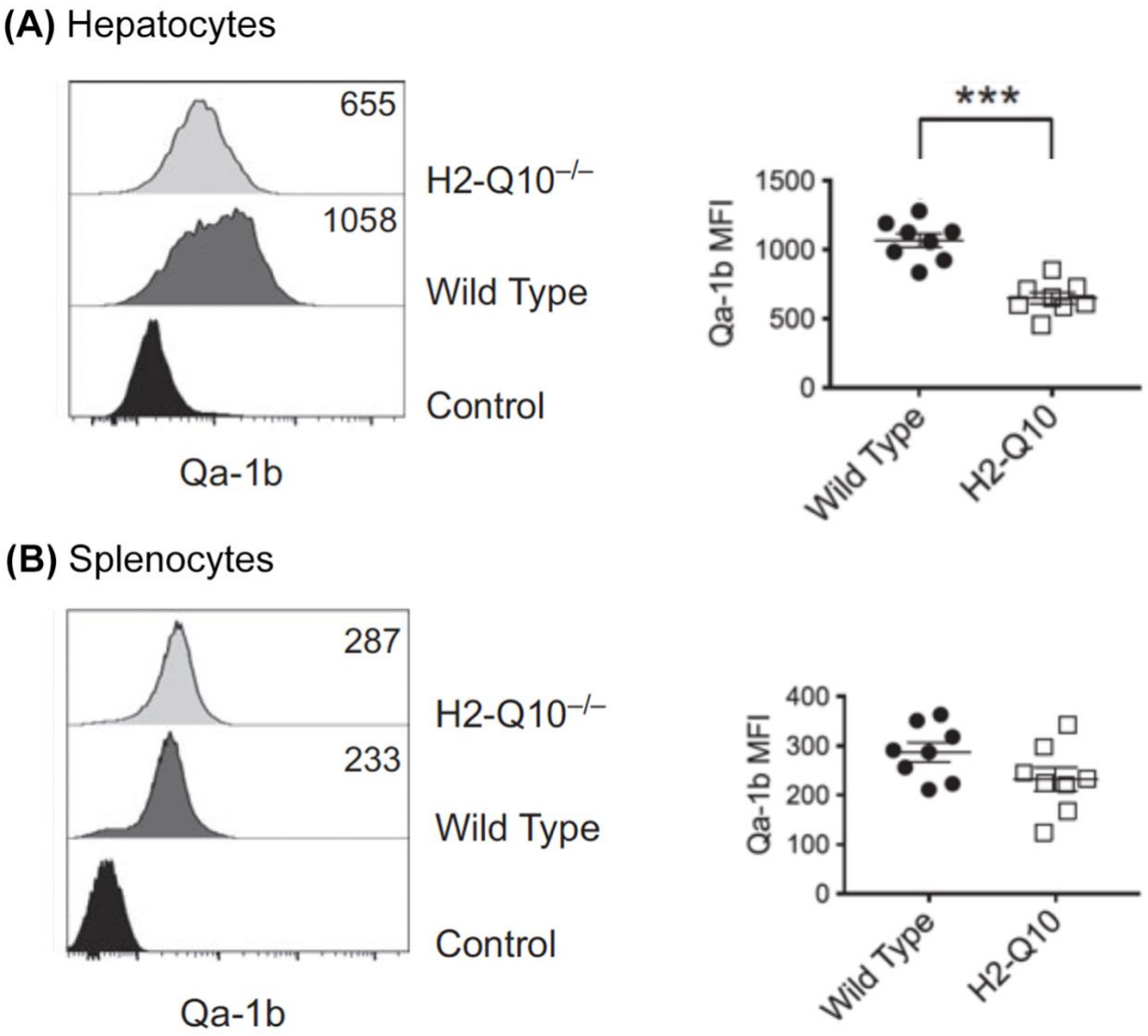


Figure 3.4. **Qa-1b expression is reduced in hepatocytes, and not splenocytes, in H2-Q10-deficient mice.** WT (filled black circles) and H2-Q10 KO (H2-Q10; open white squares) hepatocytes (A) and splenocytes (B) were analysed by flow cytometry for surface expression of Qa-1b as measured by median fluorescence intensity (MFI) (left panels) with summarised data of n =8 WT and n=8 H2-Q10 KO mice pooled from two independent experiments (right panels). Statistical significance was determined using a non-parametric Mann-Whitney U test and significance is defined as *** $p < 0.001$.

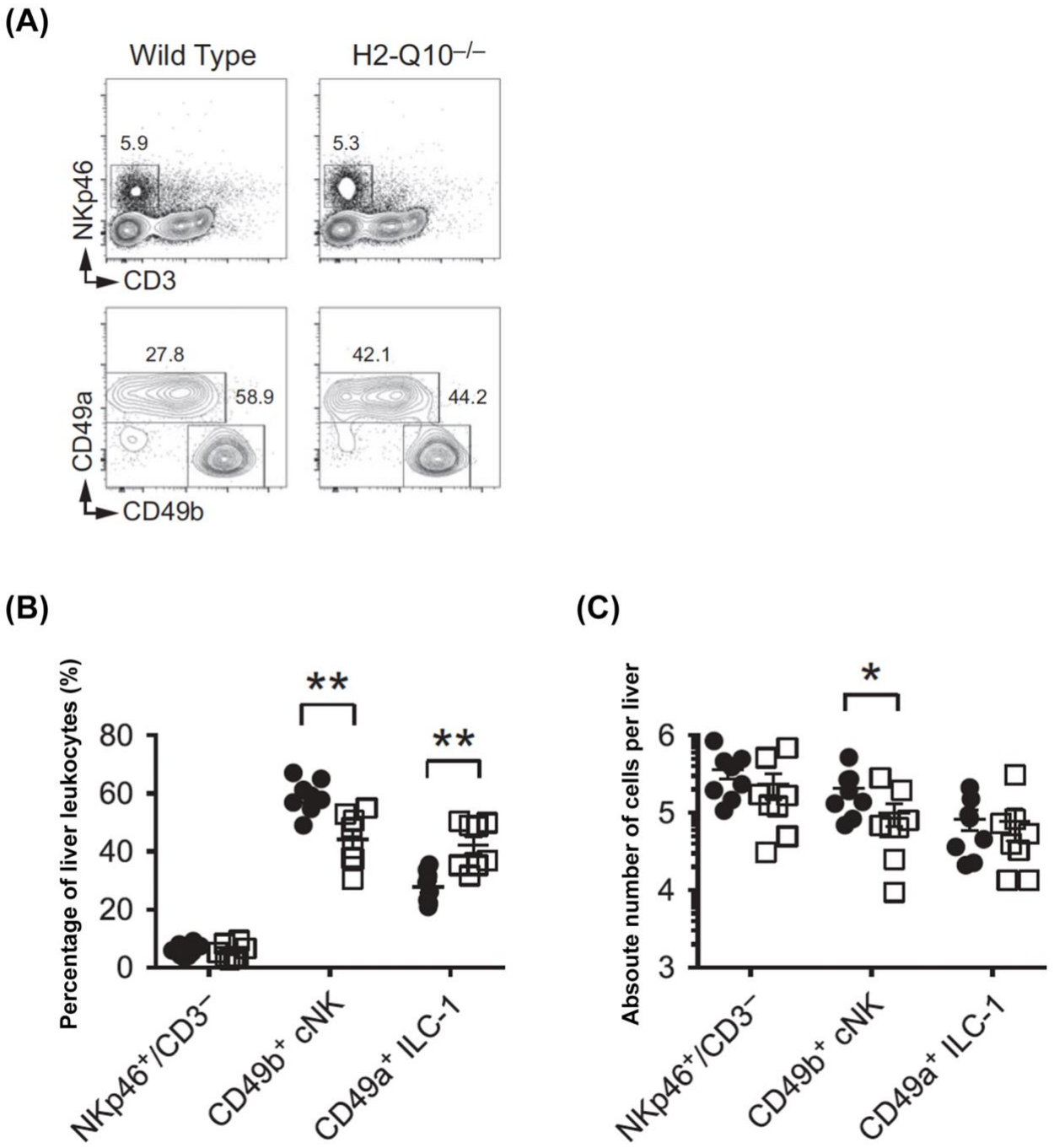


Figure 3.5. CD49b+ conventional NK (cNK) cell numbers are significantly reduced in the absence of H2-Q10. (A) Total ILCs (NKp46+/CD3-) and its subgroups (CD49b+ cNK cells and CD49a+ ILC-1 cells) were quantitated from WT and H2-Q10 KO (H2-Q10-/-) livers via flow cytometry. Summary data of percentages (B) and absolute number (C) are shown and represent pooled data from two independent experiments with n=8 mice per group. Absolute number is presented on a logarithmic scale on the y-axis. Each symbol represents an individual mouse with filled black circles representing WT mice and open white squares representing H2-Q10 KO mice. Statistical significance was determined using a non-parametric Mann-Whitney U test and significance is defined as *$p < 0.05$ or ** $p < 0.01$.

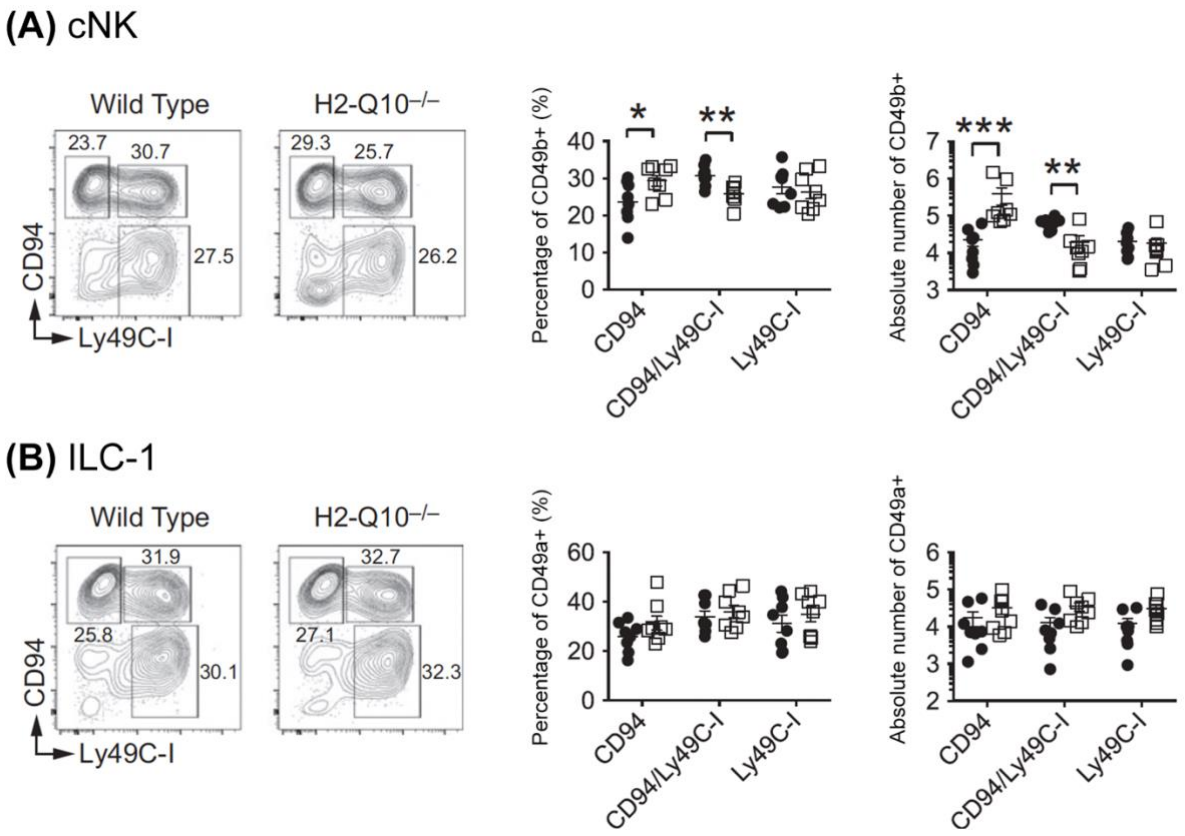


Figure 3.6. **H2-Q10 deficiency does not impact the maturation of liver cNK cells.** (A) Representative plots of CD94 and Ly49C-I-expressing subsets of cNK cells in the liver of WT and H2-Q10 KO (H2-Q10-/-) mice (left panels) and summary data of percentage and absolute number of CD49b-expressing cells pooled from two independent experiments (right panels). (B) Representative plots of CD94 and Ly49C-I-expressing subsets of ILC-1 cells in the liver of WT and H2-Q10 KO (H2-Q10-/-) mice (left panels) and summary data of percentage and absolute number of CD49a-expressing cells pooled from two independent experiments (right panels) with n=8 mice per group. Absolute number is presented on a logarithmic scale on the y-axis. Each symbol represents an individual mouse with filled black circles representing WT mice and open white squares representing H2-Q10 KO mice. Statistical significance was determined using a non-parametric Mann-Whitney U test and significance is defined as *$p < 0.05$ or ** $p <0.01$ or ***$p<0.001$.

As CD94/NKG2 heterodimers can also be found on other cells, including CD8 T cells, NKT cells and γδ T cells, the role of H2-Q10 in the development of these cell subsets was analysed. It was found that H2-Q10 deficient mice displayed statistically similar number and percentage of conventional CD8 T cells and NKT cells (data not shown), however, the absolute number of γδ T cells were impaired in the H2-Q10 deficient mouse (Figure 3.7a). γδ T cells can be broadly divided based on their expression of CD8 (CD8αα or CD8αβ). Upon analysis of their subpopulations, the reduction in γδ T cells was attributed to the CD8αα-expressing, and not CD8αβ-expressing, γδ T cells (Figure 3.7b and 3.7c).

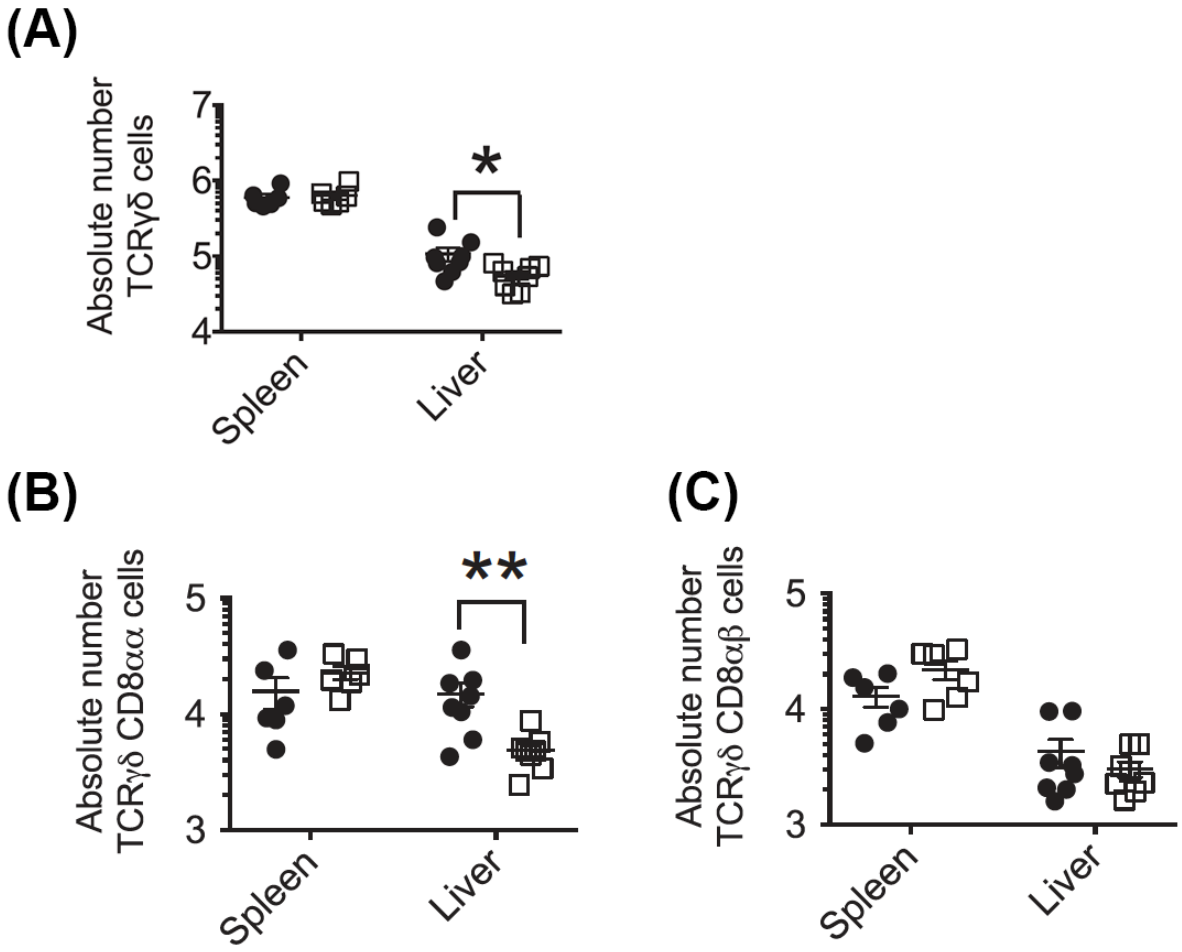


Figure 3.7. CD8αα γδ T cells are significantly reduced in the liver of H2-Q10 KO mice. (A) Absolute number of TCRγδ+ CD3+ cells in WT and H2-Q10 KO spleen and liver. **(B)** Absolute number of TCRγδ+ CD3+ CD8α+ CD8β- cells in WT and H2-Q10 KO spleen and liver. **(C)** Absolute number of TCRγδ+ CD3+ CD8α+ CD8β+ cells in WT and H2-Q10 KO spleen and liver. Each symbol represents an individual mouse. Filled black circles represent WT mice and open white squares represent H2-Q10 KO mice. Data is pooled from two independent experiments with n=4 mice per experiment. Y-axes are displayed on a logarithmic scale. Statistical significance was determined using a non-parametric Mann-Whitney U test and significance is defined as **$p < 0.01$.

Control of murine cytomegalovirus in C57Bl/6 mice is not altered by H2-Q10 deficiency

Owing to the decrease in NK cells and γδ T cells in the context of H2-Q10 deficiency, we next sought to determine whether this loss could impact the adaptive immune response to infection. We utilised murine cytomegalovirus (MCMV) as a representative model for viral infection owing to its liver tropism, control by NK cells as well as its relationship with other class Ib MHC molecules in a previous study (Anderson et al., 2019). Following inoculation with K181 MCMV, WT (C57Bl/6) and H2-Q10 KO mice were analysed for viral burden at different peripheral sites during the course of infection. Although H2-Q10 KO mice showed a loss in effector cells involved in the adaptive response against MCMV, this did not result in any significant changes to viral titre during early or late timepoints in the affected organs of the spleen, liver, lung or salivary gland (Figure 3.8). These results suggest that the impact of H2-Q10 deficiency on NK cells and γδ T cell frequencies is not sufficient to drive a change in the reduction of viral burden in MCMV infection.

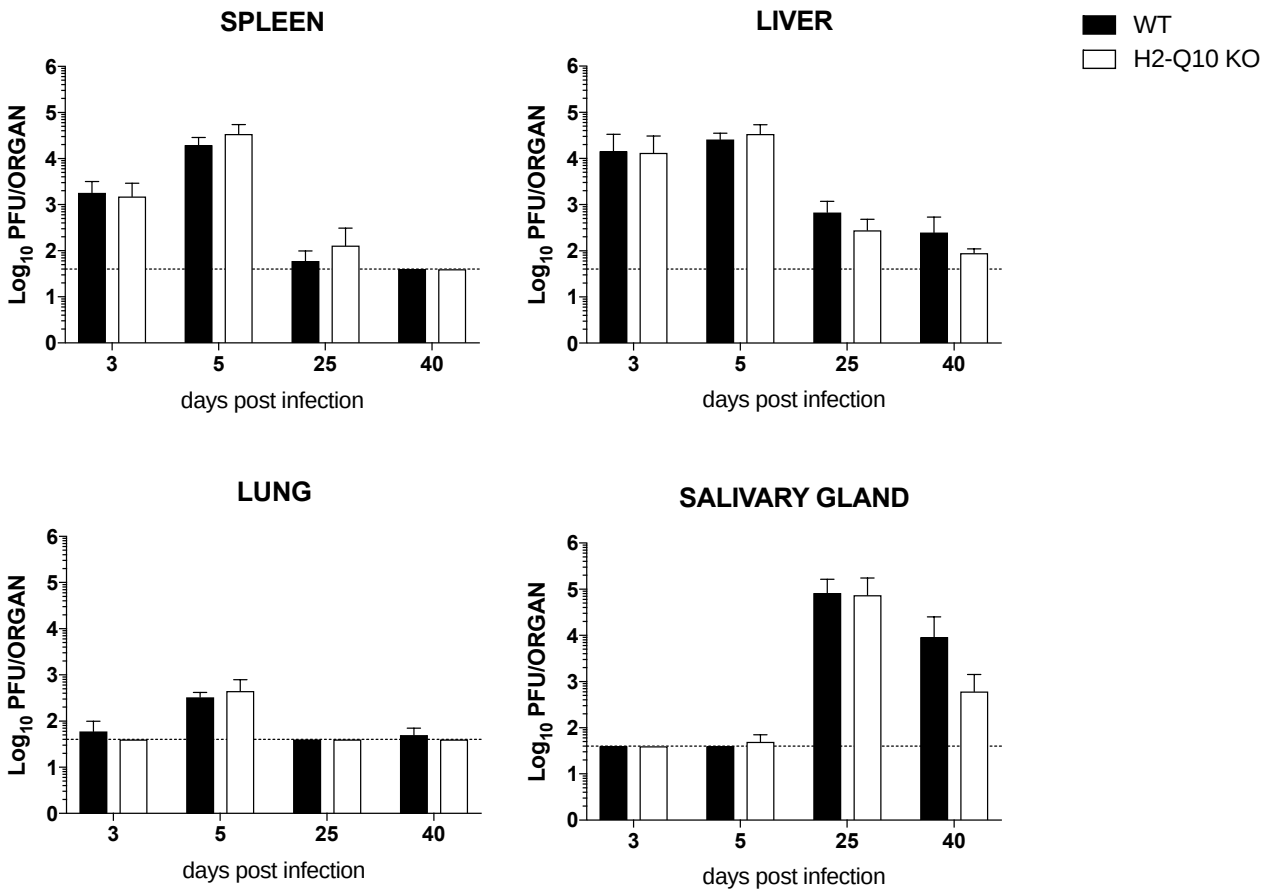


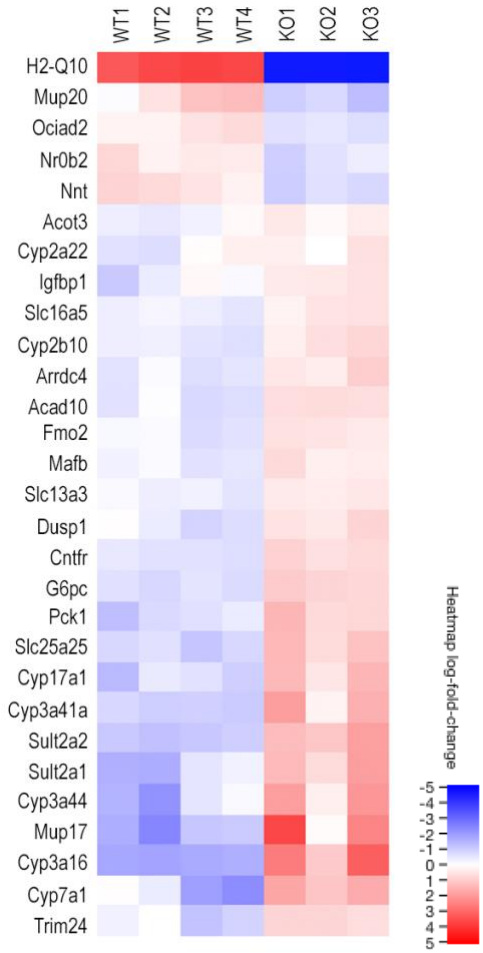
Figure 3.8. H2-Q10 does not contribute significantly in viral titre loads in MCMV infection.8-14 week old C57Bl/6 (WT, black bars) and C57Bl/6 H2-Q10 KO (H2-Q10 KO, open bars) were infected intraperitoneally with 5×10^3 plaque-forming units (PFU) of $K181_{m157}$ MCMV and organs harvested at 3, 5, 26 and 40 days post-infection. Spleens, livers, lungs and salivary glands were analysed for viral titers (PFU) determined by plaque assay. Data is represented as mean ± SEM.

Exploring non-immunological functions of H2-Q10 using RNA sequencing

Despite its high expression in the liver (Figure 3.1a), H2-Q10 did not show any major involvement in MCMV infection (Figure 3.8) and had only minimal impact on the development of liver specific leukocytes (Figures 3.5, 3.6 and 3.7), thus, it is possible that H2-Q10 may be redundant during immune development and activation. To determine whether there was any potential for H2-Q10 to act in a non-immunological manner, we employed RNAseq analysis to identify differential gene expression in WT and H2-Q10 KO hepatocytes. Using a well-established and characterised data analysis pipeline (Monash Bioinformatics Platform), 29 genes were identified which showed significant differential expression between WT and H2-Q10 KO mice (Figure 3.9a). Pathway analysis was then performed to categorise these differentially-expressed genes into gene ontology (GO) processes (Figure 3.9b). In line with a potential non-immunological function for H2-Q10, pathway analysis demonstrated that almost half of the differentially-expressed genes were related to metabolic processes. The top differentially-expressed protein-coding gene, Sulfotransferase Family 2A Member 1 (Sult2a1), was selected for further analysis and validation.

Sult2a1 is an enzyme involved in adding sulfate groups onto various proteins. Sult2a1 is primarily responsible for the sulfination of dehydroepiandrosterone (DHEA) into DHEA-S which has been implicated as a biommarker for viral and parasitic infections. To determine whether the enhanced transcript levels of Sult2a1 corresponded to elevated levels of DHEA-S, a sandwich ELISA was performed to detect DHEA-S in WT and H2-Q10 KO mouse serum samples. Despite qPCR validation of RNASeq data indicating upregulating of Sult2a1 (Figure 3.10a) sandwich ELISA results revealed no significant changes in DHEA-S serum levels between WT and H2-Q10 KO mice (Figure 3.10b). Therefore, although Sult2a1 mRNA is upregulated in the context of H2-Q10-deficiency, this does not translate into a difference in its function in sulfination of serum DHEA.

(A)



Gene Name	Biotype	Log2 FC (KO)
H2-Q10	protein_coding	-12.68517618
Sult2a1	protein_coding	5.778377272
Sult2a2	protein_coding	5.243264628
Cyp3a16	protein_coding	7.721993946
Cntfr	protein_coding	3.493672715
Slc25a25	protein_coding	1.928758355
Nnt	protein_coding	-1.87290098
Cyp3a44	protein_coding	3.900099091
G6pc	protein_coding	1.651833409
Pck1	protein_coding	2.067381313
Ociad2	protein_coding	-1.29761501
Cyp7a1	protein_coding	1.863294384
Cyp2b10	protein_coding	1.286550368
Acad10	protein_coding	1.738435049
Fmo2	protein_coding	1.116921984
Trim24	protein_coding	1.10669826
Cyp17a1	protein_coding	2.301602319
Cyp2a22	protein_coding	1.084296128
Slc16a5	protein_coding	1.256066819
Acot3	protein_coding	1.247413885
Mup20	protein_coding	-1.557701904
Igfbp1	protein_coding	1.350026047
Cyp3a41a	protein_coding	4.771041686
Mup17	protein_coding	5.371445224
Nr0b2	protein_coding	-1.558688675
Arrdc4	protein_coding	1.553244924
Slc13a3	protein_coding	0.995030809
Mafb	protein_coding	1.187207825
Dusp1	protein_coding	1.089581784

(B)

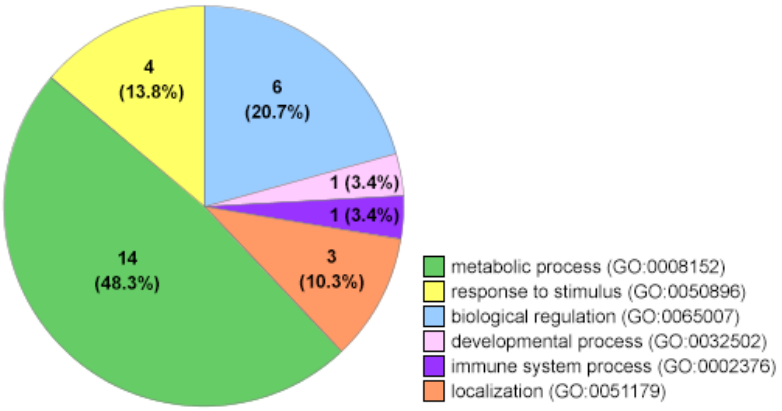
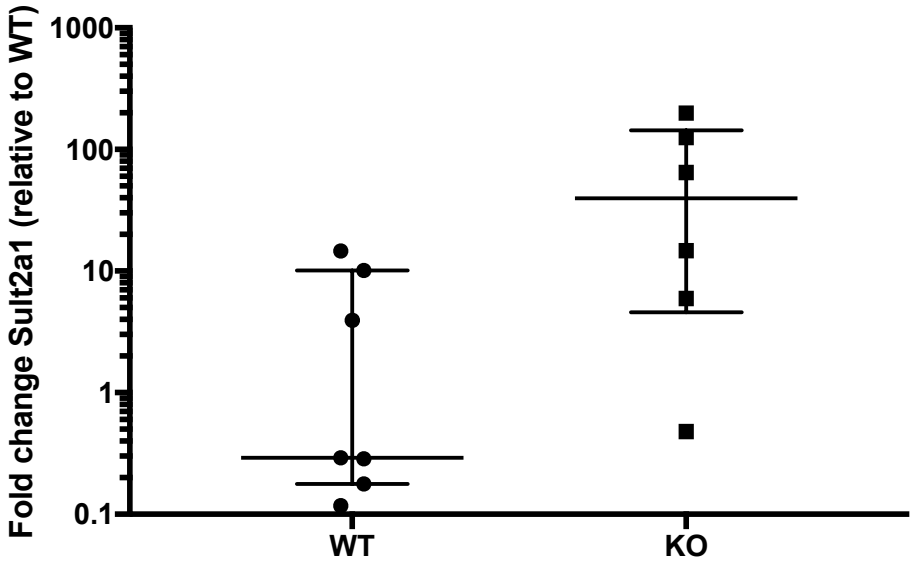


Figure 3.9. Differentially expressed genes identified in RNA sequencing between WT and H2-Q10 KO liver RNA samples. RNA was prepared from WT and H2-Q10 KO mice and subjected to bulk RNA sequencing as per established pipelines provided by Monash Micromon Genomics. **(A)** Heatmap of significantly (FDR>0.05) differentially-expressed genes. Log_2 (fold change) values of significantly differentially-expressed genes in KO mice are summarised in the table. Downregulated genes are highlighted in red and upregulated genes are highlighted in green. **(B)** Pathway analysis of differential gene expression using PANTHER and Gene Ontology (GO) processes database.

(A)



(B)

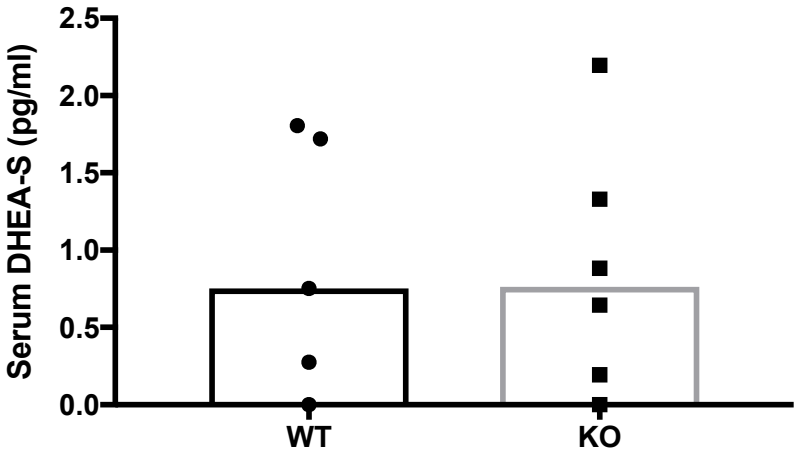


Figure 3.10. Sult2a1 is upregulated in H2-Q10 KO mice but does not correlate with increased production of DHEA-S. (A) Livers were isolated and RNA extracted for real-time PCR analysis. Sult2a1 expression is normalised to GAPDH and results are expressed as a fold change relative to the mean ddCt of 'WT' group. **(B)** Serum was isolated from WT and H2-Q10 KO mice and subjected to DHEA-S sandwich ELISA. Data is representative of two independent experiments. Symbols represents individual mice (n≥5 samples per group) and the median ± interquartile range (IQR) is shown. Statistical testing was analysed using a non-parametric Mann-Whitney U test.

3.4. DISCUSSION

This findings from this chapter validate expression patterns of H2-Q10 and the immunological and physiological role for H2-Q10 deficiency using a novel knockout mouse model. Firstly, we show H2-Q10 expression in the liver increases with age. Previous studies have indicated that H2-Q10 expression is largely restricted to the liver, however these data sets were generated using non-quantitative analysis which has reduced sensitivity when compared to real-time PCR (Ohtsuka et al., 2008). To determine the expression pattern of H2-Q10 using a more sensitive real-time PCR, H2-Q10 mRNA was measured using neonatal and adult mice as well as a variety of tissues from adult mice. In a study by Friedman and colleagues, mouse cDNA libraries were compared across various age groups to determine whether any genes showed age-related changes in expression. Although not formally shown, the authors predicted that one of their identified age-dependent cDNA clones was most likely H2-Q10 due to sharing 98.9% sequence identity (Friedman et al., 1990). Furthermore, another study has been shown that serum levels of H2-Q10, measured using rabbit polyclonal antisera, increases with age starting at less than 10µg/ml at birth and plateauing at approximately 30µg/ml at 12 weeks (Lew et al., 1986). These conclusions, in addition to our findings, consolidate our understanding of an age-dependent control of H2-Q10 expression. It will be of interest to study the determinants which may govern this change in expression, such as activation or repression of specific transcription factors with age, concurrent development of a functional immune system or perhaps the role of the developing microbiota in altering H2-Q10 expression. In addition, the role of sex has not been explored in this thesis, as only female mice were used, thus further studies into potential sex-differences may be warranted.

Existing studies of H2-Q10 have relied on the measurement of mRNA transcripts to analyse expression. Although H2-Q10 monomers have been generated previously (Sullivan et al., 2016), there have been no specific antibodies generated to H2-Q10 itself, preventing detection of the protein. This

is an important consideration because the identification of transcript production is not always directly correlated with protein levels. As such, it is possible that high levels of H2-Q10 mRNA can be detected in the liver but whether this is reflected by a complete translation to protein is unknown. In an attempt to produce novel reagents to better study H2-Q10 biology, we have shown via generation of a monoclonal antibody that H2-Q10 exists in a soluble form detectable in the serum. Unpublished data from our group indicates that upon immunoblotting of H2-Q10 KO serum samples, using our monoclonal antibody, an unexpected similar-sized product to that seen in WT samples could be identified. Although mass spectrometry confirmed the presence of H2-Q10 protein in immunoprecipitated samples using the H2-Q10, this analysis was not performed on H2-Q10 KO samples which will be necessary in identifying a potential new soluble MHC molecule. Furthermore, it would be of interest to determine whether the presence of H2-Q10, such as in the WT context, acts to limits the ability of the potential unidentified soluble MHC molecule to be produced or secreted into the serum. Therefore, perhaps in the context of H2-Q10 deficiency, delimitation can occur allowing for expression and detection of another soluble MHC molecule. Previous studies have shown that another Q family member, H2-Q4, may exist in soluble form due to a premature stop codon located in exon 5 which is responsible for translation of the transmembrane domain (Robinson, 1985; Robinson et al., 1988); as aforementioned, this feature is also shared with H2-Q10. It is intriguing to note there is high similarity (72.3% identity) between the amino acid sequences for H2-Q10 and H2-Q4; this could suggest that there is potential for shared antibody epitopes between H2-Q4 and H2-Q10 and supports possible cross-reactivity (Figure 3.11). Unpublished data from our group has shown that indeed H2-Q4 may represent a novel class Ib MHC molecule with the potential to be secreted following synthesis. This hypothesis could potentially be explored further through immunoprecipitation using β_2m in both WT and H2-Q10 KO serum followed by mass spectrometric analysis to detect all soluble MHC class I molecules.

Our findings support previous observations of enriched H2-Q10 in the liver and furthers this understanding by implicating hepatocytes as the main cell type responsible for this overexpression (Cosman et al., 1982; Ohtsuka et al., 2008). The liver has been well-established as a site of immunological importance whereby immune and parenchymal cells are constantly colocalised with various systemic molecules introduced via the portal vein. The liver is able to sample the contents of the gastrointestinal tract through the portal vein (Racanelli and Rehermann, 2006). It can be hypothesised that microbial products, derived from the gut microbiota, may enter circulation and pass through the highly vascularised liver and alter H2-Q10 expression. Unpublished data from our group has shown that in germfree mice, H2-Q10 expression is significantly reduced in the liver, however, the mechanism by which the microbiota is able to control this expression has yet to be elucidated. Preliminary studies of this potential crosstalk have been undertaken in our lab where we have shown that short chain fatty acids, a microbial byproduct produced from gut bacteria, as well as toll-like receptor (TLR) agonists, such as LPS, were unable to directly upregulate H2-Q10 expression in a hepatocyte cell line (AML-12); further examination of other potential microbial stimuli, or other consequences of a germfree environment, will be necessary to explore this crosstalk. The interaction of the microbiota with MHC, and its subsequent effect on liver-resident immune cells, has been elegantly described by Li and colleagues; this study showed that the activation, survival and proliferation of liver-resident, IL-17A-production γδ T cells (γδT-17) was dependent on lipid antigens produced by the microbiota and presented in the context of the MHC-like molecule, CD1d, expressed by hepatocytes (Li et al., 2017). It is interesting to note in this study that although deletion of CD1d resulted in a significant decrease in γδT-17 cells, a residual population of these cells were maintained in the liver. Although H2-Q10 has not been shown to bind lipid-based antigens, it is enticing to speculate a non-antigen presenting role of H2-Q10 in mediating γδT-17 numbers in the liver which may in turn be influenced by the microbiota. This understanding could provide a mechanism by which γδT-17 populations can be manipulated for disease contexts such as cholestatic liver disease,

inflammatory diseases and non-alcoholic fatty liver disease (NAFLD) (Khairallah et al., 2018; Li et al., 2017; Tedesco et al., 2018).

```
                                        20                                  40
                                         |                                   |
Q10 Protein  MGAMAPRTLL  LLLAA-----  ----------A  LAPTQTQAGS  ----------  26
 Q4 Protein  MAS----TML  LLLVAVAQTL  IEIRAAPRPA  PLPARSPAGG  PGRSSGSHRA  46
                     60                                  80                                 100
                      |                                   |                                   |
Q10 Protein  -----HSMRY  FETSVSRPGL  GEPRFIIVGY  VDDTQFVRFD  SDAETPRMEP  71
 Q4 Protein  LPPGPHLLSY  FYTSVSRPGL  GEPRFISVGY  VDNTEFVRFD  SDAENPRYEP  96
                                    120                                 140
                                     |                                   |
Q10 Protein  RAPWMEQEGP  EYWERETQRA  KGNEQSFHVS  LRTLLGYYNQ  SESGSHTIQW  121
 Q4 Protein  RAPWMEQEGP  EYWERETQKA  KGNEQIFRVN  LRTLLSYYNQ  SAGGSHTIQV  146
                    160                                 180                                 200
                      |                                   |                                   |
Q10 Protein  MYGCKVGSDG  RFLRGYLQYA  YDGRDYIALN  EDLKTWTAAD  VAAIITRRKW  171
 Q4 Protein  ISGCEVGSDG  RLLRGYQQFA  YDGRDYIALN  EDLKTWTAAD  MAAQITRRKW  196
                                    220                                 240
                                     |                                   |
Q10 Protein  EQAGAAEYYR  AYLEAECVEW  LLRYLELGKE  TLLRTDPPKT  HVTHHPGSEG  221
 Q4 Protein  EQAGATEKSK  AYLEGACVQS  LRRYLELGKE  TLLRTDPPKA  HVTCHHRSDG  246
                    260                                 280                                 300
                      |                                   |                                   |
Q10 Protein  DVTLRCWALG  FYPADITLTW  QLNGEELTQD  MELVETRPAG  DGTFQKWASV  271
 Q4 Protein  DVTLRCWALG  FYPANIILTW  QLNGEELTQD  MELVETRPSG  DGTFQKWASV  296
                                    320                                 340
                                     |                                   |
Q10 Protein  VVPLGKEQNY  TCHVYHEGLP  EPLTLRWEPP  PSTDSIMSHI  ADLL----WP  317
 Q4 Protein  VVPLGKEQNY  TCHVHHEGLP  EPLTLRWEPP  PSTVSNMANV  AVLVVLGAWP  346

Q10 Protein  SLKLWWYL  325
 Q4 Protein  SLQLWWLL  354
```

Figure 3.11. H2-Q10 shares a high sequence identity with another Q family protein, H2-Q4. Sequence identity was calculated following alignment using EMBOSS (72.3% sequence identity). Differing residues are highlighted in red.

γδ T cells have been previously shown to confer protection from MCMV infection in the absence of conventional αβ T cells through limiting viral load, expanding in peripheral organs, such as the liver, spleen and lungs, and generating memory-like populations at these sites (Khairallah et al., 2015). Although our data indicates that H2-Q10 deficiency leads to a significant reduction in CD8αα γδ T cell frequencies in the liver, this loss did not limit the ability of the remaining cells to control viral titres. As the reduction in CD8αα γδ T cell frequencies in H2-Q10 KO mice was modest, it is likely that H2-Q10 represents only one factor amongst many which contribute towards control of these cells. Furthermore, a reduction in CD49a+ NK cells in H2-Q10 KO mice did not have an impact on MCMV pathogenesis measured by viral titre burden. The decreased expression of Qa-1b is attributable to the leader sequence provided by H2-Q10 which serves as a peptide for loading into empty Qa-1b, the absence of which would lead to reduced Qa-1b surface expression (Goodall et al., 2018b). The lack

of a significant effect on MCMV viral titre clearance may be due to the idea that although a statistically significant decrease in CD49a+ NK cells was found, the biological consequence may not be as severe due to a sufficient pool of these cells remaining to combat infection. NK cells are also critical in the early control of MCMV infection via potent Ly49H-mediated activation (Brown et al., 2001; Daniels et al., 2001a; Lee et al., 2001) as well as during late stages via maintenance of CD8+ DCs through IL-12 and IL-18 (Andrews et al., 2003). Their significance in MCMV is supported further by a study whereby NK cell-deficient mice (Rag2−/− × Ilr2g−/−) containing a very small residual NK cell pool (0.2% in liver and 0.05% in spleen) were able to expand more than 300-fold within only 7 days post-infection (Sun et al., 2009). This finding indicates that even at barely detectable levels of NK cells, vigorous expansion of these cells still occurs rapidly in response to MCMV infection such that sufficient protection is still provided. Thus, although H2-Q10 reduces the number of CD49a+ NK cells, expansion of the residual population can understandably compensate and provide adequate protection against MCMV.

The hypothesis that MHC molecules can play a role in non-immunological processes has been supported in previous literature. In humans, HLA expression was shown to alter parenchymal cell metabolism, positioning and differentiation (Edidin, 1983). Furthermore, HLA-B has been implicated in interacting with antibiotics, such as penicillin, which Claas and colleagues showed could prevent binding to antigen (Claas et al., 1982). Thus, owing to our findings of only very subtle immune differences between WT and H2-Q10 KO mice, analysis into the role of H2-Q10 outside of immunological processes is warranted. Our findings showed that the absence of H2-Q10 increases mRNA expression levels of Sult2a1 in hepatocytes. As mentioned, Sult2a1 belongs to the family of sulfotransferases and is involved in liver-specific metabolic processes, namely the conjugation of sulfate groups onto bioactive proteins. A well-studied function of these sulfotransferase members is the sulfonation of the steroid dehydroepiandrosterone (DHEA) to produce DHEA sulfate (DHEA-S). Owing to high expression of H2-Q10 in hepatocytes, and its co-localisation with Sult2a1, we

proposed that perturbations in H2-Q10 would effect the expression and subsequent activity of Sult2a1 and other sulfotransferases. Interestingly, our findings showed that despite increased Sult2a1 mRNA expression in hepatocytes, there was no corresponding increase in its sulfonation activity as measured by serum DHEA-S levels. This could potentially be explained by either a lack of DHEA substrate or overall reduced sulfate levels which would limit DHEA-S production.

Overall, these findings have provided further detailing into the patterns of expression of H2-Q10 which are dependent on age and tissue restriction. In addition, we have shown that although H2-Q10 deficiency results in statistically significant reductions in CD8αα γδ T cells and CD49a+ NK cells in the liver, this does not translate into a clear biological consequence such as in the case of MCMV viral infection. Furthermore, the loss of the highly expressed H2-Q10 protein in the liver drives changes in mRNA expression related primarily metabolic processes however the functional consequence of these changes in transcript has yet to be shown. This is the first time that H2-Q10 has been investigated in non-immunological functions and may potentially expand this function to other class Ib MHC. Future studies ascertaining the non-immunological roles of H2-Q10 will provide necessary insight into its evolutionary selection and conservation and will support the need to expand these findings to other class Ib MHC molecules.

Chapter Four

Post-translational modifications regulate H2-Q10 binding to CD8

This chapter contains content from the following published research article:

Goodall, K.J., **Nguyen, A**., Matsumoto, A., McMullen, J.R., Eckle, S.B., Bertolino, P., Sullivan, L.C., and Andrews, D.M. (2018). Multiple receptors converge on H2-Q10 to regulate NK & γδT cell development. Immunology & Cell Biology.

4.1. ABSTRACT

Class Ib MHC molecules have been shown to bind various immunological ligands thus providing insights into their functions. Although H2-Q10 has been shown to bind Ly49C, this interaction has yet to be explored due to the preferentially binding of Ly49C with classical MHC I. This chapter aims to identify and define another ligand for H2-Q10. Here, a novel interaction between H2-Q10 and CD8, specifically in the CD8αα homodimeric form, is defined. The 'biochemical signature' of this interaction is defined as being dependent on the post-translational modifications of CD8αα, specifically sialylation and N-linked glycosylation. These findings are further dissected by identifying key sites in the Ig-like domain of CD8αα, where N-glycosylation can occur, which impacts binding to H2-Q10. In addition this chapter also explores the role of the α3 domain of H2-Q10 in mediating contact with CD8αα, with critical specific binding residues being defined. Finally, the biological relevance of post-translational modifications of CD8αα are explored in this chapter by showing that γδ T cells are naturally able to bind H2-Q10 due to their low sialylation profile. Altogether, these results have provided insight into the biochemical signature governing the novel interaction between H2-Q10 and CD8αα which provides a biochemical rationale for the exploration of CD8αα T cell biology.

4.2. INTRODUCTION

Major histocompatibility complex class I (MHC I) molecules are central for eliciting immune responses due to their primary function of peptide antigen presentation to CD8+ T cells. Structurally, MHC I molecules consist of three alpha domains non-covalently linked to β2m. The α1 and α2 domain comprises the peptide binding cleft where 8-12er peptides are presented to the TCR on CD8 T cells, while the α3 domain binds β2m and helps provide stability to the complex. MHC I can be subdivided into two groups: MHC Ia (classical) and MHC Ib (non-classical).

A well-characterised interaction of a class Ib MHC molecule to its ligand can be seen with H2-TL which has been previously shown to bind CD8αα primarily in the small intestine (Attinger et al., 2005; Godfrey et al., 2015; Olivares-Villagomez et al., 2008, 2011; Van Kaer et al., 2014). CD8 is a glycoprotein which is crucial in MHC Ia-mediated T cell development as well as T cell activation following recognition of cognate antigen presented by class Ia to the T cell receptor (TCR). CD8 exists as either a homodimer (CD8αα) or as a heterodimer (CD8αβ). Unlike CD8αβ, which shows a relatively higher frequency of expression due to its presence predominantly on TCRαβ T cells, CD8αα has only been shown to be expressed mainly on IELs, some human NK and TCRγδ T cells (Goodall et al., 2018a). CD8αβ primarily interacts with class Ia molecules with a higher affinity compared to CD8αα. As mentioned, the only recognised ligand for CD8αα is the MHC Ib molecule, H2-TL, (Attinger et al., 2005) (Leishman et al., 2001b). As CD8αα-expressing cells preferentially reside in the liver (Ohtsuka et al., 1996), in which H2-TL is not located, we speculated that H2-Q10 may be an alternative ligand for CD8αα.

CD8 T cells are susceptible to post-translational modification of their coreceptor; perhaps the best documented processes are sialylation and N-linked glycosylation. N-linked glycosylation involves

the addition of N-glycan branches, comprised of a large diversity of constituent monosaccharides, to asparagine (Asn) residues contained within the specific sequon Asn-X-Ser/Thr, where X cannot be proline ((Boscher et al., 2011; Cummings, 2009; Marth and Grewal, 2008; Moremen et al., 2012). Sialylation occurs as one of the final modifications of N-glycans on proteins. Sialyltransferases catalyse the addition of negatively charged sialic acid residues onto the end of N-glycans in different configurations, predominantly in an α2,3 or α2,6 linkage to terminal residues (Bhide and Colley, 2017), and thus can have significant effects on protein-protein interactions.

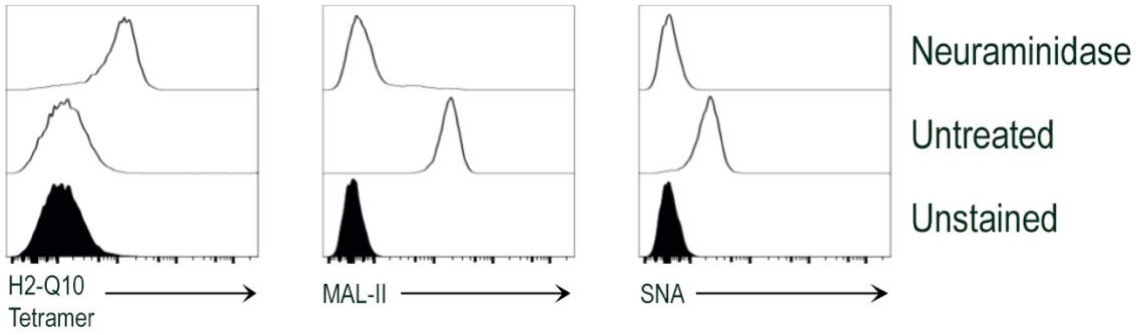
Sialylation has been well-documented to be developmentally regulated in thymic T cells. Daniels (Daniels et al., 2001b) and Moody (Moody et al., 2001) each reported that pMHC tetramers were able to bind DP thymocytes however this ability was lost as maturation occurred. It was discovered that sialylation of O-glycans on CD8αβ was developmentally regulated via upregulation of the sialylytransferase, ST3Gal-I, throughout T cell maturation. This increase in sialylation is hypothesised to decrease the binding interaction of T cells with MHC to reduce strong non-specific binding following positive selection (Merry et al., 2003), essential for the specific nature of TCR-pMHC-I recognition. In line with this work, here we show that H2-Q10 is a high affinity ligand for the CD8αα homodimer and implicate sialylation and N-linked glycosylation as the regulators of this interaction. Furthermore, building upon this identification, we demonstrate that sialylation is differentially regulated between T cell subsets, facilitating the class Ib MHC binding characteristics of CD8αα TCRγδ T cells.

4.3. RESULTS

Chemical inhibition of sialylation and N-glycosylation enhances binding of CD8αα to H2-Q10

During the search for potential H2-Q10 ligands, CD8αα was selected as a candidate owing to its binding to H2-TL, another class Ib molecule. Upon expression of CD8αα in cell lines, extremely low levels of H2-Q10 binding was observed (Figure 4.1a). As mentioned, post-translational modifications of CD8 have previously been shown to impact binding to class I MHC. Therefore, to determine whether sialylation of the CD8αα homodimer could alter its binding to H2-Q10, CD8αα-expressing cells were treated with neuraminidase, an enzyme originating in viruses which cleaves sialic acid residues from α(2,3), α(2,6) and α(2,8) linkages at the termini of glycan structures on proteins. To show successful inhibition of sialylation by neuraminidase, treated cells were stained with biotinylated lectins (SNA and MAL-II) and analysed by flow cytometry to detect a loss in binding. Neuraminidase treatment reduced lectin binding to CD8αα-expressing cells indicating a reduction in sialylation at the cell surface (Figure 4.1a). Following treatment, cells were stained with H2-Q10 tetramer to observe changes in binding as a result of decreased sialylation. It was found that inhibition of sialylation on neuraminidase-treated to CD8αα-expressing cells enhanced binding to H2-Q10 (Figure 4.1a). N-linked glycans are sugar 'scaffolds' upon which sialic acids are added, thus, to explore the role of N-linked glycosylation in mediating the interaction between H2-Q10 and CD8αα, tunicamycin was used to inhibit hybrid and complex N-glycan synthesis. The cells were validated for a reduction in N-glycosylation using the lectin PHA (Figure 4.1b). In a dose-dependent manner, we showed that loss of hybrid and complex N-glycans (as detected by PHA) on CD8αα cells enhanced binding to H2-Q10 tetramer (Figure 4.1b). The enhanced binding of H2-Q10 to tunicamycin-treated cells appears to occur over a broad range; this may coincide with the range observed in loss of PHA binding and might suggest a 'threshold' requirement of N-glycan loss prior to enhanced tetramer binding. Overall, inhibition of sialylation and N-glycosylation by neuraminidase and tunicamycin respectively, enhances binding of H2-Q10 to CD8αα.

(A)



(B)

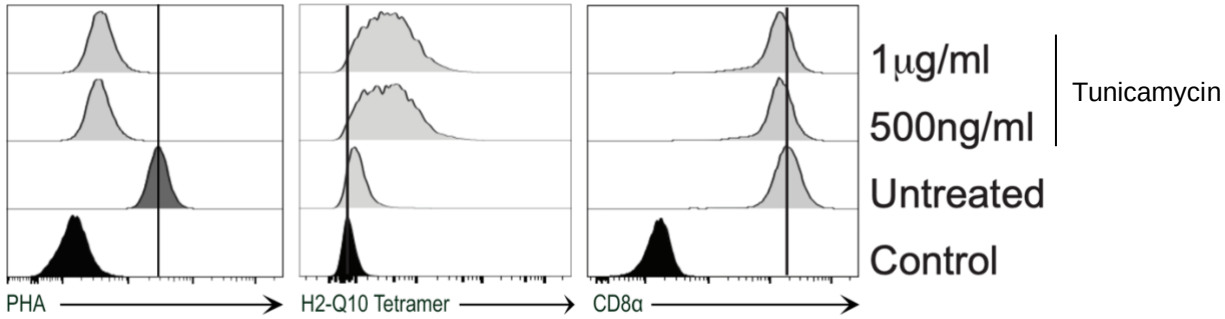


Figure 4.1. Sialylation and N-linked glycosylation mediates H2-Q10 binding to CD8αα. CHO cells expressing CD8αα were left untreated or treated with 0.032 units of type II neuraminidase (**A)** or 500-1000ng/ml tunicamycin (**B**) and analysed by flow cytometry for loss of surface sialylation or N-glycosylation, respectively, via decreased binding of SNA and MAL-II (for sialylation) or PHA (for N-linked glycosylation). Treated cells were also stained with H2-Q10 tetramer to assess changes in CD8αα binding. Representative histogram summaries are shown of at least two independent experiments and are presented as normalised to mode.

Genetic knockout of sialylation and N-glycosylation enhances binding to H2-Q10

To eliminate the possibility that chemical treatment of cells could be facilitating H2-Q10 binding in a manner apart from inhibition of sialylation and N-glycosylation, we sought to determine whether the genetic deletion of these pathways would also result in binding of H2-Q10 to CD8αα. Using CRISPR/Cas9, we firstly generated RMA-S cells which constitutively expressed the endonuclease, Cas9, with validation performed using Western blot for FLAG expression (Figure 4.2a), prior to introducing the sgRNA sequence of the desired gene knockout. The generation of this "parental" cell line (RMA-S Cas9) was essential for subsequent generation of the "offspring" cells where the genes controlling post translational modification were targeted for disruption. The use of a single cell line carrying the Cas9 endonuclease provided a control for Cas9 levels and ensured all gene targeted cells came from the same parental line.

Expression of the *Slc35a1* gene, which encodes for the sialic acid transporter in the Golgi was targeted first. This transporter controls sialic acid availability in the Golgi (Eckhardt et al., 1996), indicating genetic disruption should reduce sialylation of proteins. Incorporation of targeting sgRNA into the RMA-S Cas9 cells, and subsequent induction of gene knockout, resulted in the generation of a pool of sialic acid-deficient cells henceforth known as RMA-S SLC35A1-/- cells which were analysed and purified by cell sorting based on loss of lectin binding (Figure 4.2b). RMA-S SLC35A1-/- cells were transduced to express CD8αα and, following validation and sorting based on CD8αα expression, we performed H2-Q10 tetramer binding analysis. Similar to the results observed with neuraminidase, these cells showed a marked increase in H2-Q10 binding (Figure 4.3a). In conjunction with our results from chemical inhibition studies (Figure 4.1a), these data provide the first evidence that sialic acids control the recognition of the class Ib MHC, H2-Q10, by CD8αα.

To confirm the role of N-glycosylation observed following chemical inhibition (Figure 4.1b), a cell line lacking MGAT-1 and expressing CD8αα was generated (RMA-S MGAT-/- CD8αα). MGAT-1 is the master enzyme controlling hybrid and complex N-glycan additions and deletion of this molecule has been shown to prevent the adduction of these sugars to proteins (Ioffe and Stanley, 1994). Similar to the cells lacking sialic acids, RMA-S MGAT-/- CD8αα cells were purified by flow cytometry through selection of cells showing decreased binding to PHA (Figure 4.2c). Knockout of MGAT1, similar to knockout of SLC35A1, enhanced the binding H2-Q10 to CD8αα (Figure 4.3c). The broad ramge/heterogeneity in H2-Q10 binding in these knockout cell lines may be attributable to a 'threshold' whereby cells with high expression of CD8αα may be more susceptible to enhanced tetramer binding following loss of N-glycosylation. These data suggest that hybrid and complex N-glycans provide the scaffolding for sialic acids on CD8αα and that these moieties critically influence H2-Q10 binding.

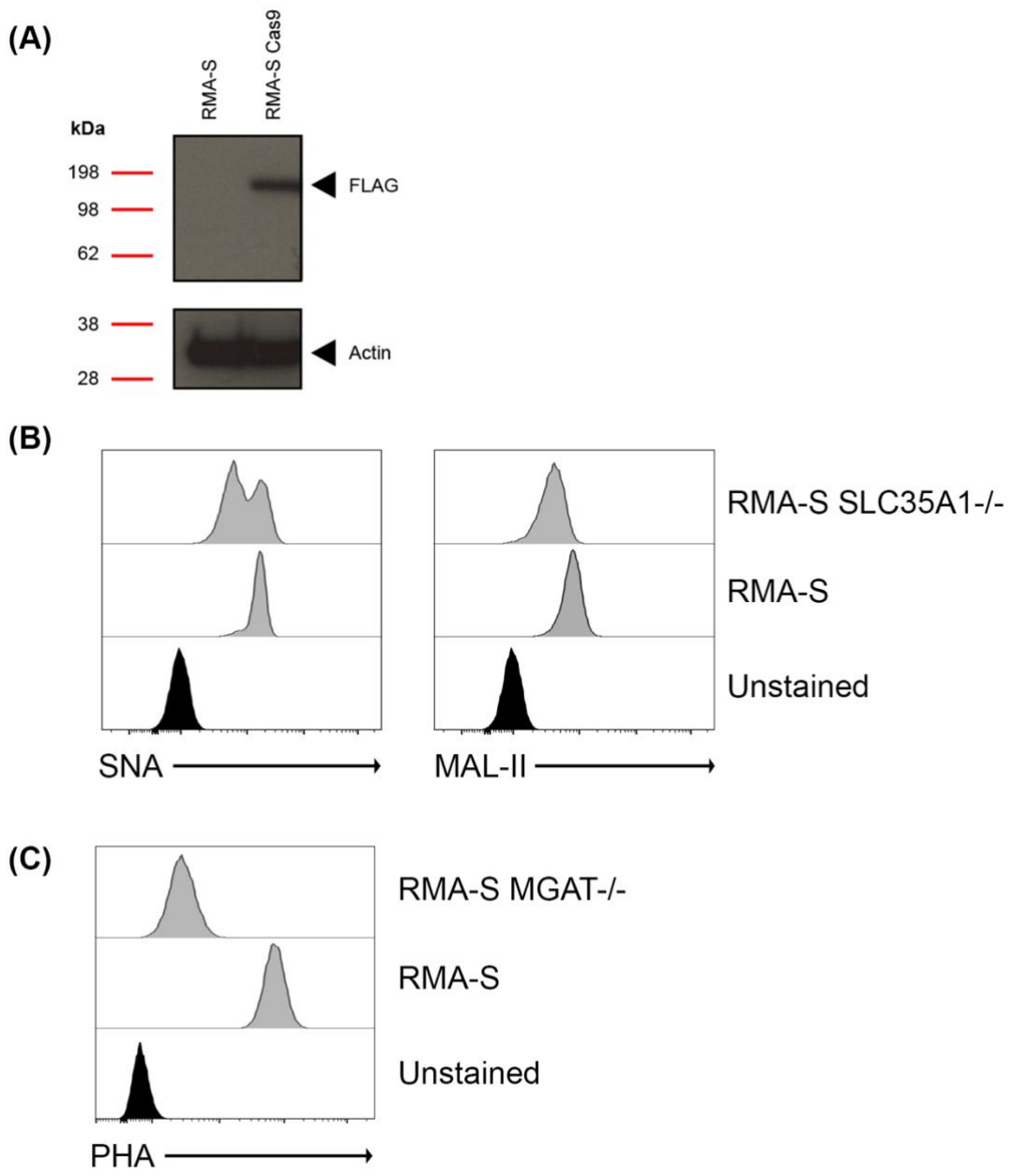


Figure 4.2. Generation and validation of SLC35A1 and MGAT1 knockout in RMA-S cells. (**A**) 10µg of total cell lysate from parental (RMA-S) and Cas9-transduced (RMA-S Cas9) cells were separated by SDS-PAGE and transferred to PVDF membranes followed by primary antibody incubation with FLAG or beta-actin as a loading control. Ladder is shown on the left with displayed in kilodaltons (kDa). (**B**) Representative histogram summary of decreased sialylation in SLC35A1 knockout cells (RMA-S SLC35A1-/-) as indicated by loss of lectin (SNA and MAL-II) binding. (**C**) Representative histogram summary of decreased N-glycosylation in MGAT1 knockout cells (RMA-S MGAT-/-) as indicated by loss of lectin (PHA) binding. Histograms are representative of data collected from at least three independent experiments and are presented as offset and modal.

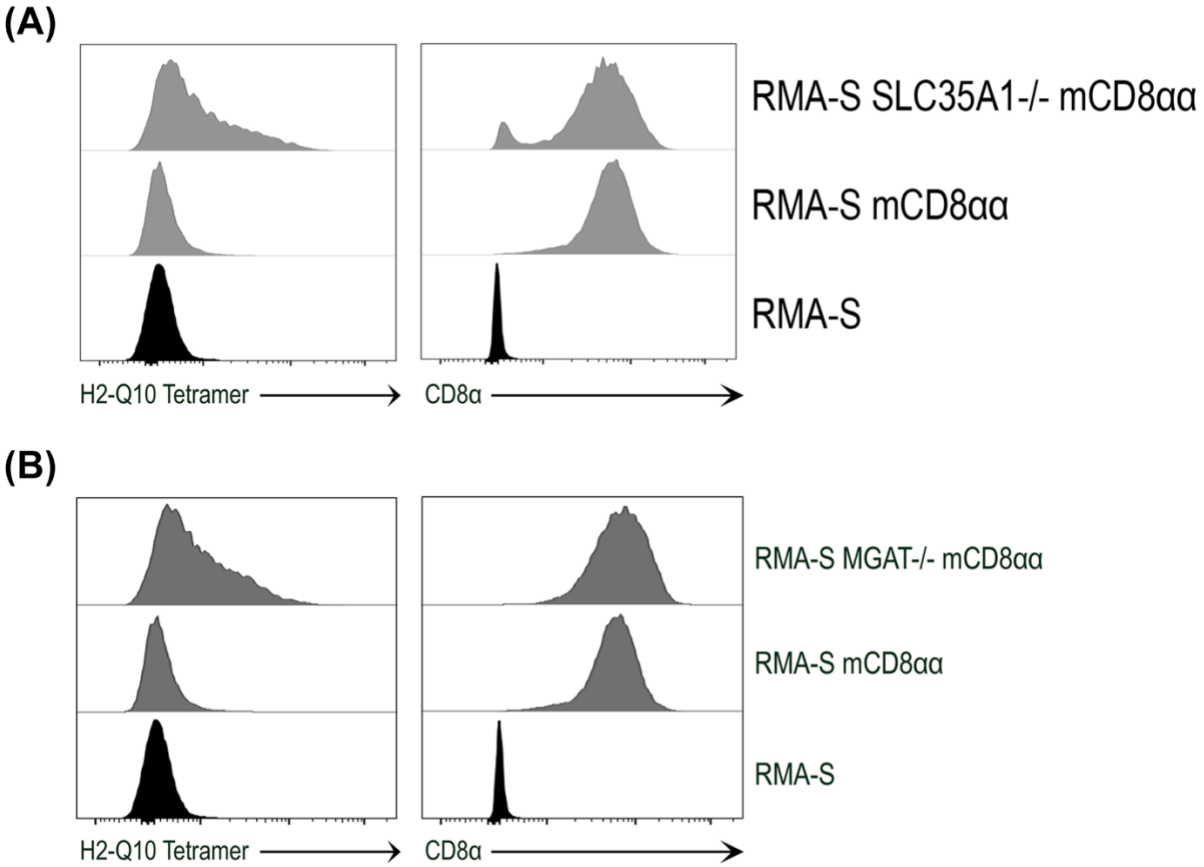
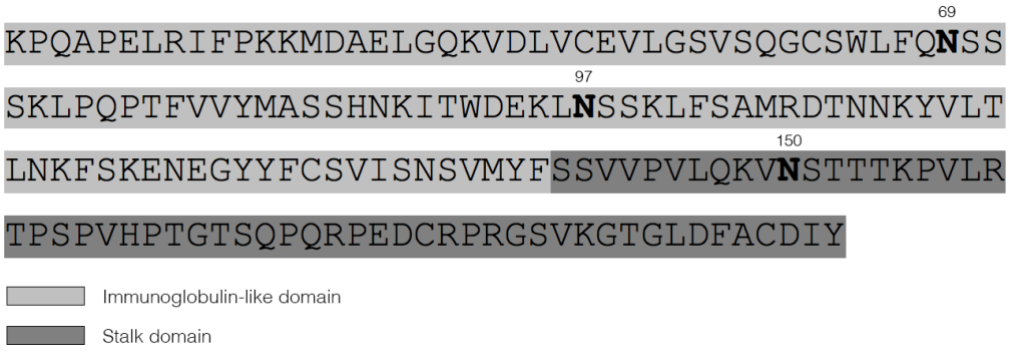


Figure 4.3. Loss of sialylation and N-glycosylation enhances binding of H2-Q10 to CD8αα. **(A)** Flow cytometric analysis of CD8αα expression levels and binding of H2-Q10 tetramer in sialic-acid deficient cells expressing mCD8αα (RMA-S SLC35A1-/- mCD8αα. **(B)** Flow cytometric analysis of CD8αα expression levels and binding of H2-Q10 tetramer in hybrid-complex N-glycosylation deficient cells expressing mCD8αα (RMA-S MGAT-/- mCD8αα). Histograms are representative of data collected from at least three independent experiments and are presented as offset and modal.

N-glycosylation of the CD8α Ig-like domain regulates binding to H2-Q10

To more precisely map the role of N-glycosylation during the interaction between H2-Q10 and CD8αα, a N-glycosylation prediction database (see Methods) was used to locate Asp-X-Ser/Thr consensus sequences within CD8α. This approach indicated that two N-glycosylation sites, N69 and N97, are located in the Ig-like domain of CD8α whilst one was site was located in the stalk domain at N150 (Figure 4.4a). To map the N-glycan sites controlling H2-Q10 binding to murine CD8α (mCD8αα), cell lines were generated which expressed mutant forms of CD8α and validated by Sanger sequencing (data not shown). Using alanine scanning mutagenesis of the asparagine residue at these predicted N-glycosylation sites (N69A, N97A, N150A) (Cunningham and Wells, 1989), binding of H2-Q10 tetramers was increased when sites within the Ig-like domain (N69A and N97A) were mutated (Figure 4.4b). In contrast, changes to the site within the stalk domain (N150A) did not affect binding (Figure 4.4b). In the case of cell lines which expressed slightly lowered surface expression of CD8αα, such as N69A N97A mCD8αα, H2-Q10 tetramer binding appeared to be similar or higher compared to other CD8αα-expressing cell lines, thus, small variations in surface CD8αα expression do not appear to have significant effects on H2-Q10 binding. Altogether, these results suggest that N-linked glycosylation of the Ig-like domain mediates binding of CD8αα to H2-Q10.

(A)



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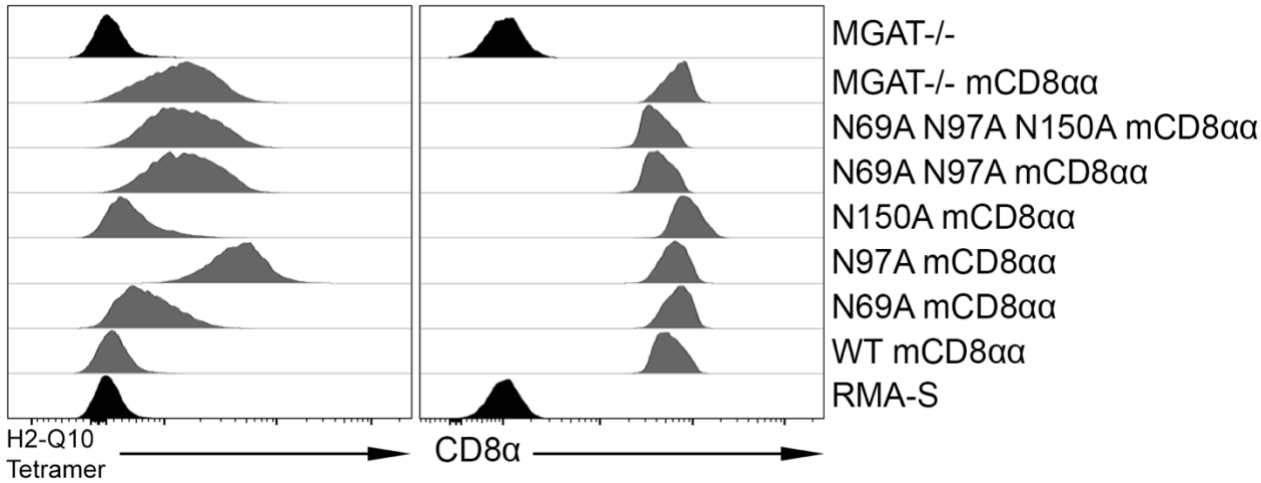


Figure 4.4. Loss of hybrid and complex N-glycan branching on the Ig-like domain of CD8αα facilitates binding to H2-Q10. (A) Protein sequence of CD8α extracellular domain with predicted asparagine targets of N-glycosylation (contained within consensus sequence Asp-X-Ser/Thr) highlighted in bold. Numbers above bolded asparagine residues represent position within full-length CD8α protein sequence. (B) Representative histogram summary of H2-Q10 tetramer binding to RMA-S cells expressing either wild-type (WT) murine CD8αα (WT mCD8αα), single Asp→Ala substitutions at predicted sites of N-glycosylation (N69A mCD8αα/N97A mCD8αα/N150A mCD8αα), double substitutions (N69A N97A mCD8αα), triple substitutions (N69A N97A N150A mCD8αα) or global MGAT knockout (MGAT-/- mCD8αα). Data is representative of three independent experiments (n=3). Histograms are presented as offset and modal.

H2-Q10 contacts CD8αα via its α3 domain

TL, a class Ib molecule which binds to CD8αα , contacts CD8αα through key residues located within the α3 domain (Leishman et al., 2001b). To determine whether this interaction is specific for TL or was more widely associated with class Ib MHC binding to CD8αα, mutant tetramers were generated in which the native α3 domain of H2-Q10 was substituted to that of H-2K_b (termed H2-Q10α3K_b). This recombination was chosen as H-2K_b is a class Ia molecule which weakly binds CD8αα (Kern et al., 1998). Complementary studies were performed where substitution of the α3 domain of H2-Q10 into H-2K_b was performed (K_bα3H2-Q10). To more efficiently demonstrate the effect of substitution, analysis was performed using RMA-S MGAT-/- CD8αα, as these cells bind H2-Q10 with high affinity (Figure 4.5), making quantifiable differences easier to observe. Indeed, staining of these cells with the H2-Q10α3K_b tetramer showed a loss in the ability to bind CD8αα compared to native H2-Q10. In contrast, K_bα3H2-Q10 tetramer binding to mutant CD8αα cell lines was increased compared to H2-Q10α3K_b binding but did not reach the same levels as native H2-Q10. Together, these results show that the α3 domain of H2-Q10 controls the interaction with CD8αα. This indicates that the interaction between CD8αα and class Ib MHC is likely a conserved process and provides a rationale for targeted deletion of specific amino acids in this region.

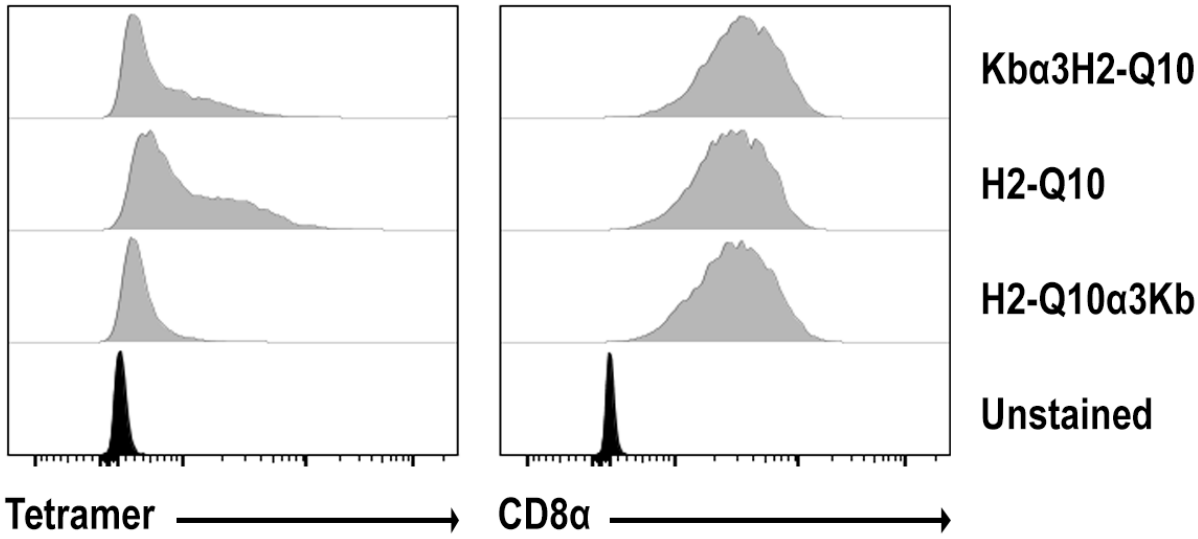


Figure 4.5. **The α3 domain of H2-Q10 is critical in binding CD8αα**. RMA-S MGAT-/- CD8αα cells were stained with native H2-Q10 tetramer (Q10) or mutant tetramers (K_bα3Q10, Q10α3K_b) and analysed for changes in tetramer binding (left panel) and equal surface CD8αα expression (right panel). Histograms are presented as offset and modal and are representative of two independent experiments.

Critical H2-Q10 α3 residues involved in binding CD8αα

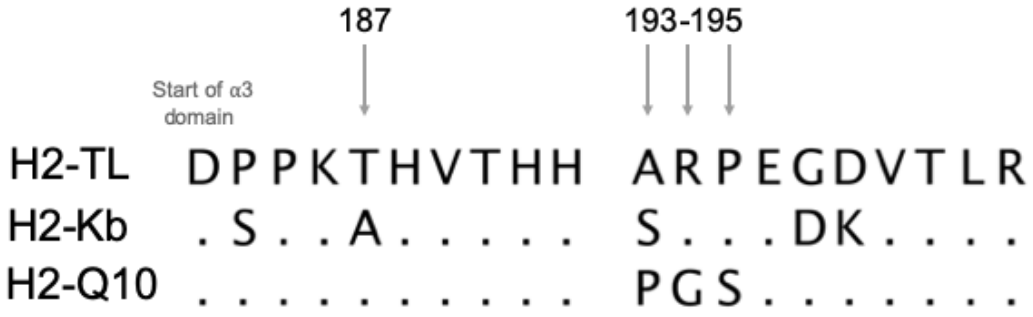
To build upon the observations that the α3 domain of H2-Q10 controls the interaction with CD8αα, the key residues which facilitate this interaction were examined. Crystal structures of H2-K_b and H2-TL bound to CD8αα identified key residues controlling binding and provided a template to identify those resides which may control the interaction between H2-Q10 and CD8αα. Initial examination focused on the residues at positions 193-195 as these play an important role in the interaction of H2-TL with CD8αα (Attinger et al., 2005) (Figure 4.6a). For these studies we generated a mutant where the amino acids in H2-Q10 were substituted for H-2K_b (H2-Q10: P193/G194/S195, K_b: S193/R194/P195). Mutation of these residues (Q10 SRP) demonstrated a reduction in tetramer binding to CD8αα (Figure 4.6b), suggesting that positions 193-195 represent a hotspot for the interaction between H2-Q10 and CD8αα.

Structural analysis of H2-TL and CD8αα demonstrated the importance of arginine at position 194 (R194) as it increases the number of hydrogen bonds formed with nearby amino acid residues, therefore increasing the 'closeness' of CD8αα to the MHC molecule. This residue is present in the weakly-binding K_b and in the strong-binding H2-TL, but is a glycine in H2-Q10. Given that H2-Q10 binds CD8αα with high affinity, an examination of the importance of G194 was warranted (Goodall et al., 2018b). Thus, a mutant tetramer of H2-Q10 was generated where the glycine at 194 was substituted with arginine (G194R). Interestingly, this G194R tetramer binding showed a decreased ability for H2-Q10 to bind CD8αα, suggesting that G194 plays an important role in the efficient binding of H2-Q10 (Figure 4.6b).

Sequence alignment of H2-K_b, T H2-TL and H2-Q10 also revealed differences at position 187 (K_b A187, TL T187, H2-Q10 T187), another key binding site between H2-TL and CD8αα. To determine whether threonine or alanine at this position was a determinant in the binding of H2-Q10 to CD8αα,

a second H2-Q10 mutant was generated (T187A). Similar to G194R, T187A tetramer binding revealed a reduction in H2-Q10 binding to CD8αα, (Figure 4.6b), suggesting that T197 is a probable determinant for high avidity binding of class Ib MHC, as mirrored by TL and H2-Q10. To further explore these interactions, G194 and T187 were mutated together (Q10 G194R T187A). Interestingly, the loss of both of these sites resulted in a synergistic reduction of H2-Q10 binding to CD8αα reinforcing the importance of both G194 and T187 in determining H2-Q10 binding avidity.

(A)



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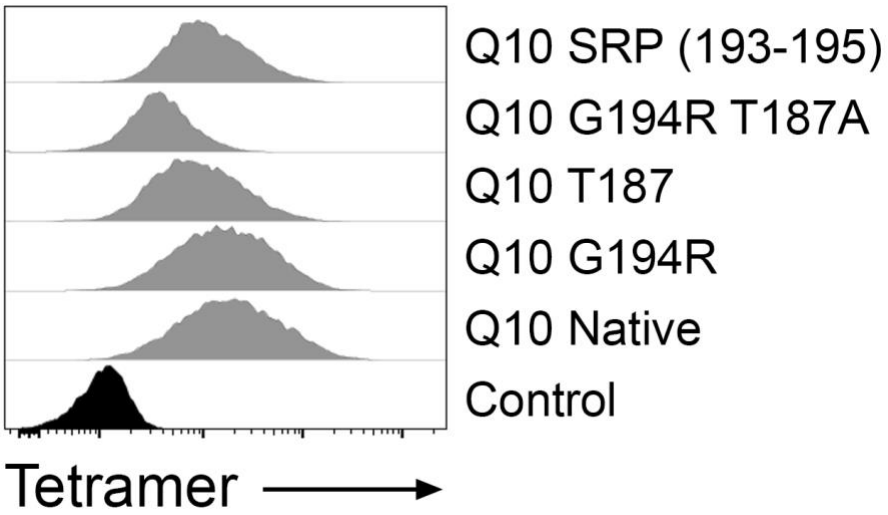
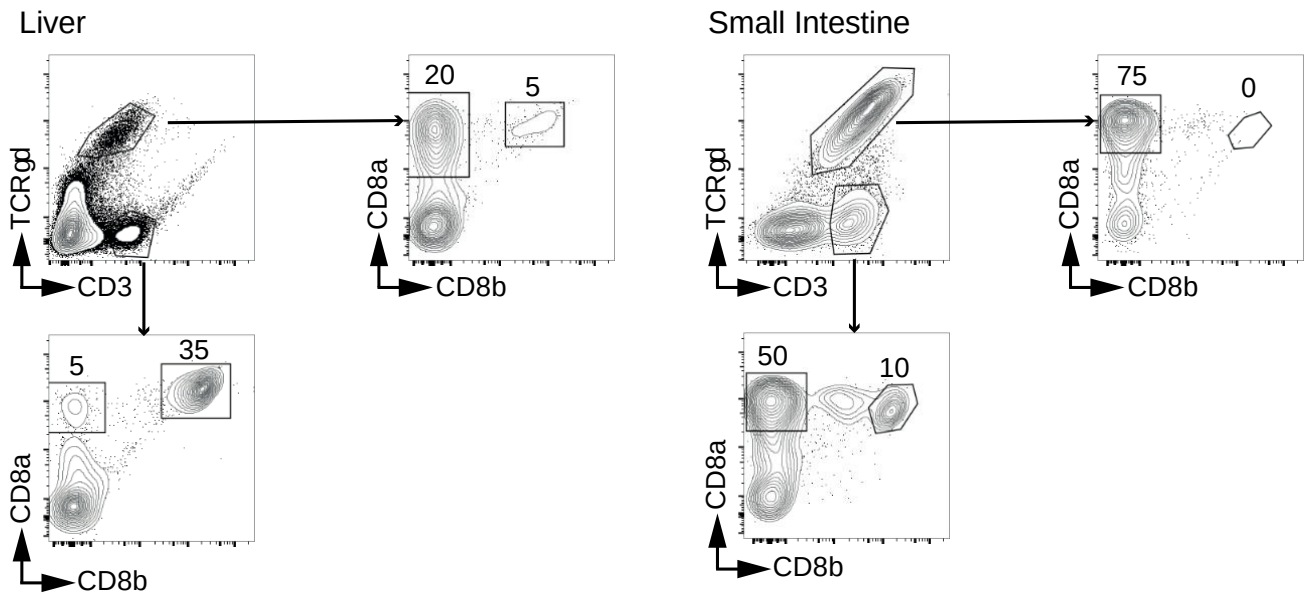


Figure 4.6. Recognition sites within the α3 domain of H2-Q10 regulate binding to CD8αα. (**A**) Key residues found in the α3 domain of H2-Q10 mediate binding to CD8αα. Sequence alignment of α3 domain section, which contains sites known to control binding to CD8αα, of H2-TL (moderate binder of CD8αα), H2-Kb (weak binder of CD8αα) and H2-Q10. Position 187 in H2-Q10 (T) was mutated to alanine (A) of H2-Kb. Positions 193-195 of H2-Q10 was mutated to that found in H2-Kb (SRP). Position 194 of H2-Q10 (G) was mutated to arginine (R) of H2-TL. (**B**) Native H2-Q10 tetramer (Q10) was subject to various single residue substitutions (Q10 G194R, Q10 T187A), double residue substitution (Q10 G194R T187A) or triple residue substitution at position 193-195 (Q10 SRP). Mutants were used to stain RMA-S MGAT-/- mCD8αα cells. Histograms are modal and offset and are representative of data collected from two independent experiments.

H2-Q10 is a high affinity ligand for CD8αα on γδ T cells due to differential sialyation

Having established the biochemical determinants controlling the recognition of H2-Q10 by CD8αα, the next step was to determine the biological consequences resulting from these interactions. Firstly, a determination of *ex vivo* CD8αα cells binding to H2-Q10 was performed. Although CD8αα cells are overall not as abundant as conventional CD8αβ cells, the liver and small intestine are two major sites where these cell populations are enriched. Leukocytes were subdivided based on TCR expression (TCRαβ or TCRγδ) as well as CD8 expression (CD8αβ or CD8αα)(Figure 4.7a). Populations were analysed using SNA and MAL-II lectins to determine levels of sialylation as described. We found that CD8αα cells, which comprises most of the TCRγδ population**,** had reduced sialylation as indicated by reduced binding to SNA and MAL-II in both the liver and small intestine (Figure 4.7b). This suggested that CD8αα TCRγδ T cells represent likely candidates as binders to H2-Q10. To explore this, we specifically analysed CD8αα TCRγδ T cells for binding to both H2-Q10 and H2-TL and showed that they were capable of binding to both tetramers (Figure 4.8a). SPR analysis of this interaction indicated that although CD8αα TCRγδ T cells could bind both tetramers, H2-Q10 had a much higher affinity for CD8αα (K_D ~ 300nM, Figure 4.8b) than H2-TL (K_D ~ 781nM, Figure 4.8b). We further showed the dependence of CD8αα in this interaction by performing antibody blockade (CT-CD8α) and showed that binding to H2-Q10 was diminished when TCRγδ T cells were pre-treated with blocking antibody (Figure 4.8c). Thus, we conclude that the natural reduced sialic acid content of CD8αα TCRγδ T cells allows for a high affinity interaction with H2-Q10.

(A)



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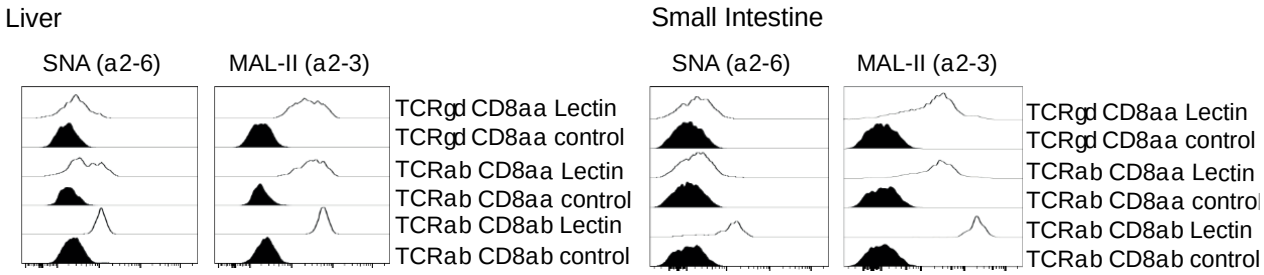

Figure 4.7. **Differential sialylation profiles of CD8αα and CD8αβ T cells.** (**A**) Cells were isolated from liver and small intestine and analysed for CD8αα and CD8αβ population frequencies. (**B**) TCRγδ and TCRαβ cells were subdivided based on CD8αα or CD8αβ expression. Cells were profiled via flow cytometry for sialic acid content using SNA and MAL-II lectin binding (Lectin) compared to unstained (Control).

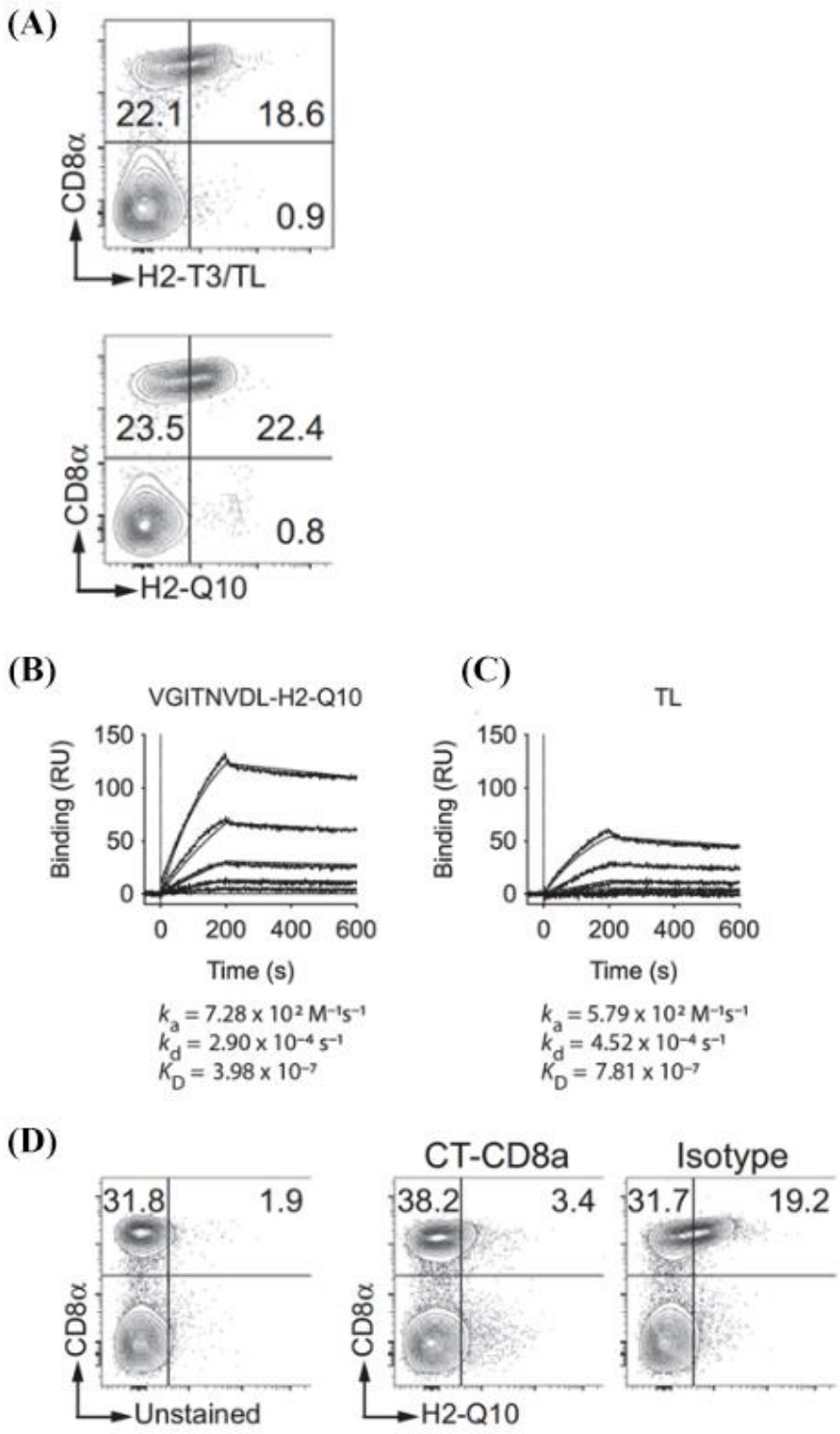

Figure 4.8. H2-Q10 is a high affinity ligand for CD8αα-expressing γδ T cells. (**A**) Flow cytometric analysis of H2-Q10 and H2-TL tetramer binding to TCRγδ+ CD3+ CD8β- cells. Population percentages are displayed as black text within gates. Binding of decreasing concentrations of CD8αα (4000, 1600, 640, 256 and 102.4 nM; solid lines, top to bottom) to neutravidin-immobilized H2-Q10 refolded with VGITNVDL (**B**) or H2-TL (no peptide, **C**). Results are presented in response units (RU) after subtraction of baseline values. Plots are representative of at least two independent experiments. Dashed vertical lines at 0 s indicate injection start. Irregular lines represent raw data and solid lines indicate data fit using a 1:1 Langmuir binding model. (**D**) Pre-treatment of CD8αα γδ T cells with blocking antibody (CT-CD8α) prevents H2-Q10 tetramer binding. Data is representative of at least two independent experiments using 3-4 mice per experiment.

4.4. DISCUSSION

The MHC class Ib molecule H2-Q10, despite its overabundance in the liver and uniqueness as a soluble MHC protein, is still poorly understood with respect to its biological function. Our current work has added to our understanding of this enigmatic molecule by identifying a novel interaction with CD8αα that is dependent on sialylation and N-linked glycosylation.

We have demonstrated that the absence of terminal sialic acids enhances binding of CD8αα to H2-Q10, however, a more profound effect is observed when hybrid and complex N-glycans, the scaffolding upon which sialic acids are supported, are absent from CD8αα. Sialylation has previously been implicated in mediating other protein-protein interactions due to the presence of a negative charge on sialic acids causing repulsion of interacting molecules (Margraf-Schönfeld et al., 2011). Indeed, we have shown that chemical inhibition and genetic interference of sialylation shows a positive effect on CD8αα binding to H2-Q10 which might be hypothesised to be attributable to diminished steric hindrance.

The inhibition and knockout of MGAT1 in cells results in an arrest of the N-glycosylation pathway at the hybrid and complex stages of branching with resultant N-glycosylated proteins being limited to the early oligomannose (Man_{5-9}) stage. The role of N-glycosylation in the CD8αα interaction is more speculative than the role of sialylation. We showed a significant enhancement of H2-Q10 binding to CD8αα in the absence of N-glycosylation, specific to CD8αα. As shown, H2-Q10 binds CD8αα with high avidity when the Ig-like domain of CD8αα is not N-glycosylated. N-glycosylation is known to impact protein binding in various ways, from altering tertiary protein structure to the involvement of N-glycans in epitope creation and stabilisation (Demetriou et al., 2001; Grigorian and Demetriou, 2010; Johnson et al., 2013; Margraf-Schönfeld et al., 2011). As sites N69 and N97 are found within the Ig-like domain of CD8αα, the usual site of contact with the α3 domain of MHC I,

it is possible that these N-glycosylation sites under normal conditions impede H2-Q10 binding. The loss of N-glycosylation on CD8αα could also potentially cause conformational changes leading to enhanced binding to H2-Q10. As the structure of H2-Q10 binding to CD8αα has yet to be discovered, the precise binding contact site remains unknown but will facilitate our understanding of how N-glycosylation is involved in this interaction.

The role of sialylation and N-glycosylation of the CD8 protein is relevant *in vivo.* Previous studies in mice have reported that changes in sialylation of both the CD4 and CD8 co-receptors during T cell development is responsible for changes in binding with MHC Ia. Observations of CD8αβ throughout T cell development has shown that differences in sialylation between thymic T cell precursors and peripheral T cells may be responsible for the changes in MHC Ia binding during development (Moody et al., 2001). This change in sialylation is required to allow for promiscuous binding of MHC Ia to developing T cells during thymic selection as well as the subsequent limitation of binding following maturation. Our work has extended this body of knowledge by implicating a similar role for sialylation and N-glycosylation in regulating class Ib MHC binding to the CD8αα homodimer. It will be of interest for future studies to determine whether the developmental regulation of CD8αα-expressing T cells during thymic maturation and selection is also controlled by interactions dependent on sialylation and N-glycosylation. Furthermore, various stimuli in the periphery, such as cytokine stimulation or activation of T cells, have also been reported to alter glycosylation of CD8 T cells (Boscher et al., 2011; Comelli et al., 2006; Smith et al., 2018) and thus might be implicated in mediating binding to class Ib MHC.

This chapter demonstrates that sialylation and N-glycosylation profiles are biologically relevant as differences indeed exist within the CD8αα T cell population. The exploration of a 'biochemical signature' which enables specific cell subsets to bind H2-Q10 is supported by the observations of lowered sialylation and N-glycosylation of liver-resident CD8αα γδ T cells. γδ T cells have been

shown to exit the thymus at the double negative 4 (DN4) stage (Cheroutre and Lambolez, 2008) where sialic acids have yet to cap the CD4 or CD8 co-receptors. This could indicate developmental 'priming' of these cells to bind MHC Ib molecules. As other CD8αα-expressing cells exist outside of γδ T cells, it will be interesting to determine whether cells such as NK cells and MAIT cells are also able to bind H2-Q10 dependent on their sialylation or glycosylation profiling.

The role of various stimuli known to alter N-glycosylation, such as cytokine release, pathogen-derived stimuli or disease contexts, and whether they are able to directly affect N-glycosylation of specific cell types may be interesting to determine whether this glycosylation profile shift can enhance or impede their ability to bind MHC Ib. This hypothesis is supported by previous studies showing that, in the context of B cells, signals induced by toll-like receptor stimulation and released cytokines, are able to modulate N-glycosylation of immunoglobulins which can then directly impact their binding and effector functions (Wang et al., 2011).

Outstanding questions relate to the downstream consequence of the H2-Q10-CD8αα interaction. The transmembrane domain of CD8α is associated with the tyrosine kinase, $p56_{lck}$ which initiates the signaling cascade in T cells (Veillette et al., 1988). Despite this, CD8αα has been described as a poor co-receptor as its co-expression alongside CD8αβ reportedly diminishes T cell activation, due to sequestering of signaling components away from CD8αβ (reviewed by Cheroutre and Lambolez, 2008), and could potentially associate with inhibitory molecules (Zhu et al., 2006). This suggests that ligation of CD8αα is unlikely to cause signalling. Furthermore, as a soluble monomeric MHC Ib molecule, the hypothesis that H2-Q10 may be directly causing signaling responses following CD8αα ligation is unlikely. However, it is of interest to note that the average binding affinity of pMHC Ia to CD8αβ is K_D ~ 145µM (Wooldridge et al., 2010)and that a reduction of ~50µM has been reported to translate to an almost 100-fold increase in antigen sensitivity of a CD8 T cell (Cole et al., 2012).

Our current work has reported a $K_D \sim 300nM$ high affinity interaction of CD8αα and H2-Q10. Despite its potential inability to directly alter cellular function, the high affinity ligation of H2-Q10 to CD8αα may be playing an antagonistic role whereby binding blocks the ability for other MHC Ib molecules, particularly surface-bound molecules, to induce a functional response. Our results here emphasise the need for exploration of high-affinity MHC Ib molecules as ligands for CD8 T cells and their functions, with these findings being critical in understanding how effector functions can be manipulated to improve immune responses.

Chapter Five

Exploring the biochemical signature of class Ib MHC binding to human CD8αα

5.1. ABSTRACT

Sialylation and N-glycosylation of murine CD8αα impacts its ability to bind H2-Q10. This chapter aims to determine whether human CD8αα can also be controlled by post-translational modifications and whether this could control binding to non-classical HLA. HLA-G is a non-classical MHC molecule present in humans which is considered to be similar to H2-Q10 due to its solubility and ability to present leader sequences for other class Ib MHC molecules. Of interest, HLA-G has been defined as a ligand for human CD8αα. Firstly, this chapter shows that HLA-G binding to CD8αα, is different to that of H2-Q10, and is not significantly impact by N-linked glycosylation as human CD8α appears to be non-N-glycosylated. Furthermore, the predicted lack of N-linked glycosylation sites on human CD8αα allows for 'natural' recognition by H2-Q10 both in cell lines and primary peripheral blood mononuclear cells (PBMCs). Furthermore, similar to Chapter 4, this chapter has defined key binding residues of H2-Q10 which participate in this strong binding interaction. Therefore, although H2-Q10 and HLA-G do share common characteristics, the control mechanisms defining their respective interactions with CD8αα are different.

5.2. INTRODUCTION

Arguably the most well-studied human MHC Ib molecule, the function of HLA-E is presentation of self-peptides as an indicator of normal functioning in healthy cells (Braud et al., 1997; O'Callaghan and Bell, 1998). HLA-E appears to behave similarly to class Ia MHC in that it is also ubiquitously expressed and presents endogenous peptides. HLA-E presents small peptide fragments derived from the leader sequence of MHC I molecules synthesised in the ER (Kurepa et al., 1998) which is an important indicator of a properly functioning MHC biosynthesis pathway. This pathway can be dysregulated by viral infections and malignant transformations making cells resistant to CD8 T cell killing (Oliveira et al., 2010). Under normal homeostasis, loading of HLA-E with Qdm results in stable cell surface expression to engage inhibitory receptors on natural killer (NK) cells; the result from this engagement ensures that NK cells do not target functionally healthy cells (Miyada and Wallace, 1986; Ying et al., 2017; Sullivan et al., 2015). HLA-E is highly conserved across species, with the mouse homologue Qa-1b presenting a very similar peptide and fulfilling the same role as its human counterpart (Goodall et al., 2018a).

HLA-G is another class Ib HLA molecule which shows very limited sequence variability across individuals and was first identified in placental cytotrophoblasts. This was an important observation as these cells which generally lack class Ia HLA expression during early gestation (Kovats et al., 1990) and suggested HLA-G played an important role in foetal immunology (Clements et al., 2005). Interestingly, HLA-G molecules have been shown to exist in various membrane (mHLA-G; HLA-G1, HLA-G2, HLA-G3, HLA-G4) and soluble isoforms (sHLA-G; HLA-G5, HLA-G6, HLA-G7) (Lin and Yan, 2018), with the latter being detectable in serum (Hviid, 2006). HLA-G is thought to be involved in immunosuppression during tumour development, with expression being induced by interleukin-10 (IL-10) (Oft, 2014). Like HLA-E, HLA-G can directly inhibit NK cell function through ligation of ILT2 to limit NK cell lysis, particularly in uterine NK cells (Lin and Yan, 2018). Unlike

HLA-E, a mouse homologue for HLA-G has yet to be identified. We have recently shown that H2-Q10 is a high-affinity ligand for CD8 (Goodall et al., 2018b) with its binding being controlled by post-translational modifications (Chapter Four). As mentioned, H2-Q10 is a soluble murine MHC Ib molecule which is expressed at a high level in the liver (Lew et al., 1986). Chapter Four has shown that H2-Q10 is a high-affinity ligand for CD8 (Goodall et al., 2018b) with its binding being controlled by post-translational modifications, namely sialylation and N-glycosylation, of specifically the CD8 homodimer. Despite their distinct expression patterns, H2-Q10 has been proposed as a functional analogue to be HLA-G as they are both soluble MHC I molecules. The differences between H2-Q10 and HLA-G would suggest that these molecules are encoded by gene homologues, particularly the observations that reductions in HLA-G expression can result in preterm birth, spontaneous abortion and miscarriage while H2-Q10 deficient mice do not demonstrate these issues (Ferreira et al., 2017; Goodall et al., 2018b). However, the similarities between H2-Q10 and HLA-G, with respect to CD8αα binding, prompted a biochemical analysis of the whether these molecules interacted in the same manner.

5.3. RESULTS

Human CD8α is not predicted to be N-glycosylated and binds to murine H2-Q10

To determine whether N-linked glycosylation can mediate class Ib HLA interactions with CD8α, as evident in mice, prediction software was employed to determine whether human CD8α (hCD8α) contained any potential N-glycosylation motifs (as per Chapter Four). As previously shown, murine CD8α contains three N-glycosylation sites at 69, 97 and 150. In contrast, human CD8α did not contain any N-glycosylation sequons (Figure 5.1), although at position 49-51 an Asn-X-Thr sequence exists where X is occupied by Pro and is therefore highly unlikely to be N-glycosylated (Breitling and Aebi, 2013; Lizak et al., 2011).

Owing to our previous data demonstrating that N-glycosylation inhibits binding of murine CD8α to class Ib MHC, this suggested that the existence of hCD8αα in a naturally non-N-glycosylated state would allow this molecule to be 'primed' to bind to class Ib MHC. To test this hypothesis, cell transfectants expressing human CD8αα (hCD8αα) or CD8αβ (hCD8αβ) were stained with murine and human class Ib MHC tetramers. Similar to previous data demonstrating that HLA-G is a ligand for hCD8αα (Sanders et al., 1991), the results demonstrated strong binding to hCD8αα but not hCD8αβ-expressing cells (Figure 5.2).

Observations from our group (unpublished data) have shown that loss of N-linked glycosylation not only impacts H2-Q10 binding to murine CD8αα but also other class Ib MHC molecules, such as Qa-1b. As such, it was possible that the lack of N-glycosylation of hCD8αα might broaden its ability to interact with other class Ib HLA. However, despite the lack of N-glycosylation of CD8α, HLA-E does not bind this molecule, or hCD8αβ (Figure 5.2).

Interestingly, cross-reactivity between murine tetramers and hCD8αα and hCD8αβ was observed, with Qa-1b binding hCD8αβ but not hCD8αα; the inability of Qa-1b to bind hCD8αα contrasts observations in the mouse where Qa-1b represents a high-affinity ligand for murine CD8αα (unpublished data). Furthermore, substantial binding of H2-Q10 to hCD8αα and hCD8αβ was observed (Figure 5.2). Therefore, the lack of N-glycosylation of CD8α does not necessarily permit binding to HLA-E but does allow for cross-reactivity with Qa-1b and H2-Q10. Due to the predicted lack of N-glycosylation of hCD8α, hCD8αβ heterodimers are able to bind class Ib MHC, an observation not seen in mice. Although murine class Ib MHC binding to hCD8 does not represent a natural/physiological phenomenon, it suggests that human CD8 interactions to non-classical HLA occurs through a different mechanism to mice.

(A) Mouse CD8αα

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MASPLTRFLSLNLLLLGESIILGSGEAKPQAPELRIFPKKMDAELGQKVDLVCEVLGSVSQG
      69                                97
CSWLFQNSSSKLPQPTFVVYMASSHNKITWDEKLNSSKLFSAMRDTNNKYVLTLNKFSKENE
                                150
GYYFCSVISNSVMYFSSVVPVLQKVNSTTTKPVLRTPSPVHPTGTSQPQRPEDCRPRGSVKG

TGLDFACDIYIWAPLAGICVALLLSLIITLICYHRSRKRVCKCPRPLVRQEGKPRPSEKIV
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(B) Human CD8αα

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MALPVTALLLPLALLLHAARPSQFRVSPLDRTWNLGETVELKCQVLLSNPTSGCSWLFQPRG

AAASPTFLLYLSQNKPKAAEGLDTQRFSGKRLGDTFVLTLSDFRRENEGYYFCSALSNSIMY

FSHFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIW

APLAGTCGVLLLSLVITLYCNHRNRRRVCKCPRPVVKSGDKPSLSARYV
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Figure 5.1. Human CD8α does not contain any predicted N-linked glycosylation sequons. Protein sequences from mouse CD8α (NP_001074579.1, top panel) and human CD8α (NP_001759.3, bottom panel) were inputted into NetNGlyc 1.0 Server (R. Gupta et al., 2004) for analysis of asparagine (N) – any amino acid (X) – threonine (T) sequences. Red numbers indicate the position of asparagine residues (bold) predicted to be glycosylated.

Knockout of MGAT1 does not significantly impact binding of HLA-G or H2-Q10

Based on computational prediction, it is possible that CD8α does not have N-glycosylation sequons, and thus is not N-glycosylated, however to confirm this experimentally, knockout studies of MGAT1 were performed to remove any possible N-glycosylation on these receptors. It was predicted this would not have a significant impact on H2-Q10 binding to CD8α, as the receptor does not contain sites for MGAT1-mediated N-glycosylation to occur in normal conditions.

Using the Jurkat human T cell line, which naturally expresses no CD4 or CD8 proteins, we transduced Cas9-mCherry and two sgRNA-GFP constructs specific to human MGAT1 and induced knockout with 1µg/ml doxycycline hyclate for 72 hours owing to the tetracycline-inducible expression vector used for sgRNA expression. Following multiple rounds of FACS sorting and generation of a stable PHA_{lo} cell population, we then transduced these cells to express hCD8αα and sorted on equal expression (Figure 5.3a and 5.3b).

H2-Q10 bound almost all cells expressing hCD8αα only (80.3%) (Figure 5.2b). The loss of N-linked glycosylation alone (Jurkat MGAT-/-) did not result in non-specific binding of H2-Q10, however, binding to MGAT-/- cells expressing hCD8αα showed an increased percentage of cells bound to H2-Q10 (99.1%); these observations were also reflected in HLA-G binding with an increase from 2.13% on hCD8αα-expressing cells to 9.81% H2-Q10 binding in MGAT-/- hCD8αα cells (Figure 5.3b). Complementing the computational prediction, the loss of MGAT1 in CD8αα-expressing Jurkats did not lead to any additional binding of H2-Q10 compared to CD8αα-expressing cells with an intact N-linked glycosylation pathway. Therefore, although loss of N-linked glycosylation slightly increased the percentage of cells bound to H2-Q10 and HLA-G, this level of tetramer binding was still comparable to cells expressing hCD8αα only. In conclusion, we show that hCD8αα is not significantly affected by loss of cellular N-glycosylation.

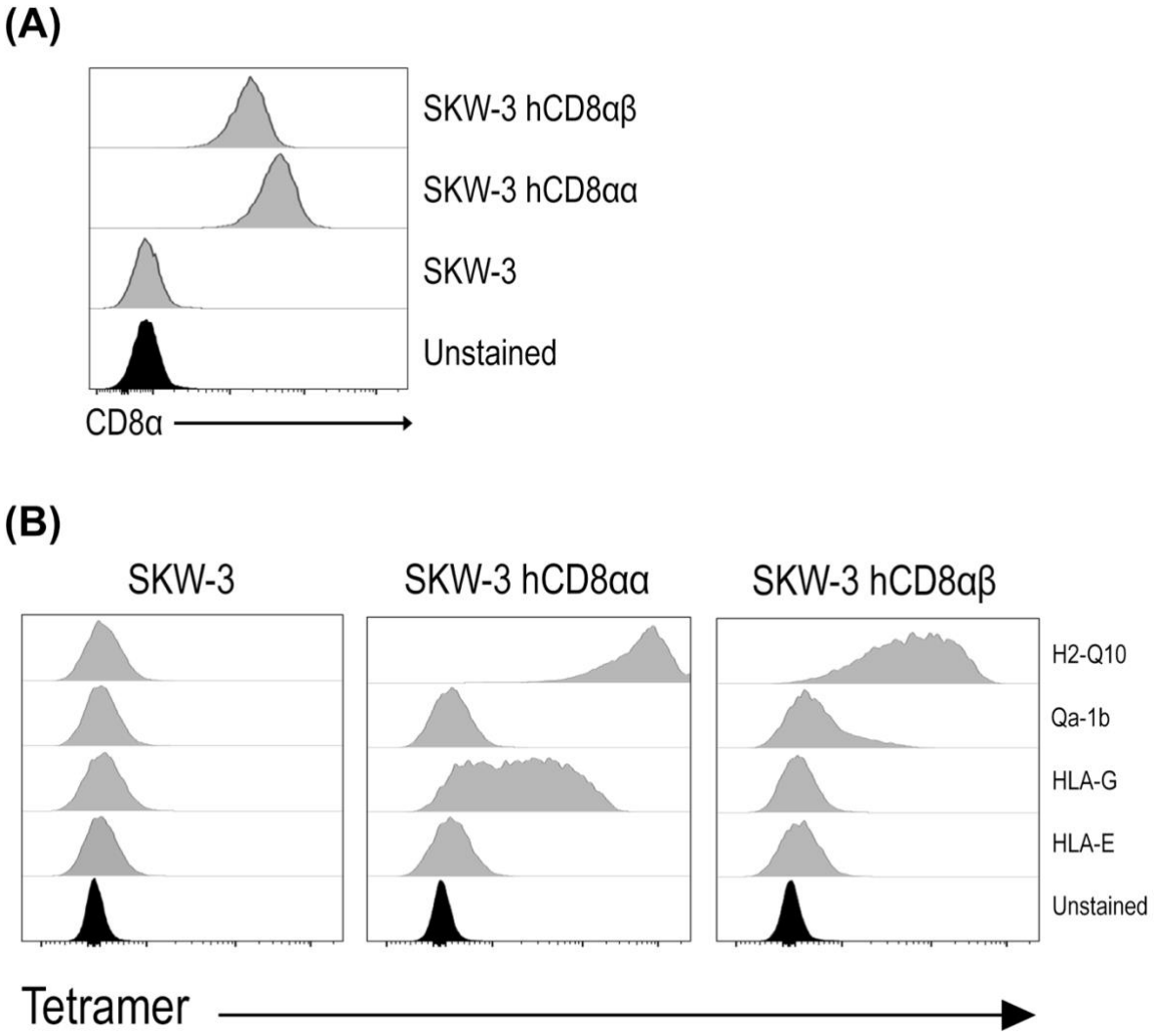
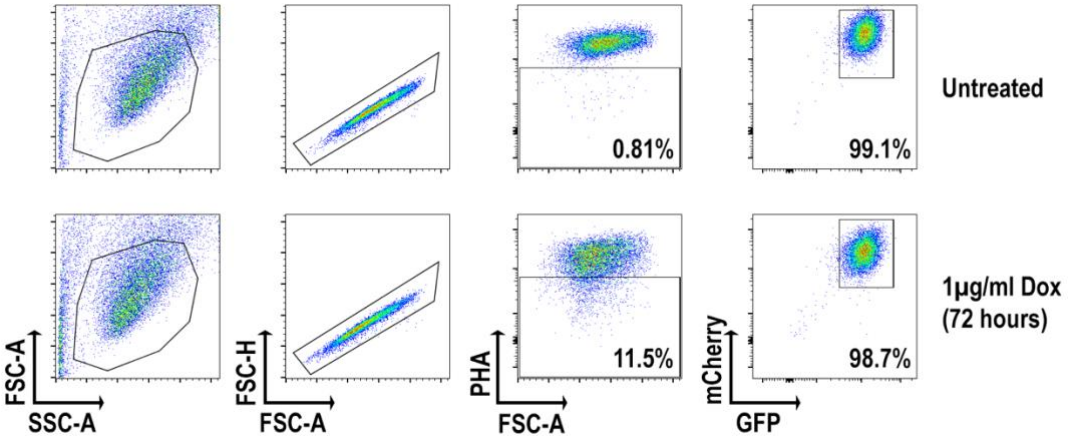


Figure 5.2. H2-Q10 is able to bind human CD8α. Parental cell lines (SKW-3) or cell transfectants expressing human CD8αα (SKW-3 hCD8αα) or human CD8αβ (SKW-3 hCD8αβ) were stained with human class Ib MHC tetramers (HLA-E and HLA-G) and murine class Ib tetramers (Qa-1b and H2-Q10) and analysed for binding via flow cytometry. Median fluorescence intensities are shown as red values. Histograms are modal and offset. Data is representative of two independent experiments.

(A)



(B)

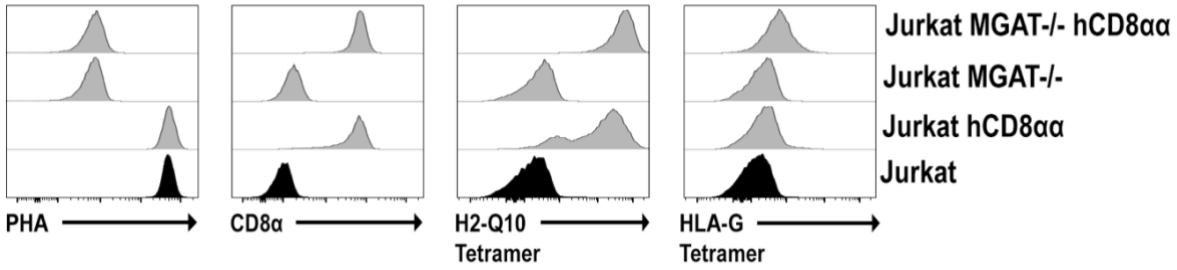


Figure 5.3. Knockout of MGAT1 does not significantly impair human CD8αα binding to H2-Q10 or HLA-G. (**A**) Jurkat cells were transduced with Cas9 and MGAT1-specific sgRNA and treated with 1μg/ml doxycycline hyclate for 72 hours. Cells were analysed via flow cytometry for loss of N-linked glycosylation through diminished binding to the lectin, PHA. (**B**) Jurkat cells (Jurkat), Jurkat cells expressing human CD8αα (Jurkat hCD8αα), Jurkat cells with MGAT1 knockout (Jurkat MGAT-/-) and MGAT1-deficient Jurkat cells expressing human CD8αα (Jurkat MGAT-/- hCD8αα) were analysed via flow cytometry for binding to H2-Q10 and HLA-G tetramers. Bottom panel indicates total loss of N-glycosylation in respective cell lines through diminished binding to the lectin, PHA. Population frequencies are displayed as percentage values within corresponding gates. Data is representative of two independent experiments.

H2-Q10 binds CD8α+ cells in human blood

Our previous findings show that binding of H2-Q10 to primary murine cells is dependent on the sialylation profile of the cell which alters how CD8αα is presented at the cell surface. As previous data has suggested that hCD8α is non-glycosylated, and thus glycosylation may not dictate its binding to class Ib MHC, it was predicted that there would be an ability of H2-Q10 to 'universally' bind hCD8α which would not be dependent on the sialylation/glycosylation characteristics of different cell types. To show this, human peripheral blood mononuclear cells were isolated from venous blood and analysed the binding ability of HLA-E, HLA-G, H2-Q10 and Qa-1b tetramers which would also support our earlier *in vitro* data. We found that HLA-E, unlike our observations *in vitro*, was able to bind a small proportion of cells (1.16%); of these tetramer-positive cells, 41.8% were CD3- CD8α- and 43.2% were CD3+, with the CD3+ compartment being further subdivided into 21.1% CD8α+ and 32.1% CD8α- (Figure 5.4, second column). Of the HLA-E+ CD3+ CD8α+ population, the majority of cells were also expressing CD8β (84.9%) compared to CD8α alone (7.91%). These observations remained consistent across HLA-G and Qa-1b binding with only 0.4% and 2.22% total tetramer binding, respectively (Figure 5.4, third and fourth columns). Of these tetramer-positive cells, the majority of cells were CD3+ CD8α- (48.9% for HLA-G and 51.6% for Qa-1b) and of the small CD8α+ population, almost all co-expressed CD8β. Of particular interest was the binding pattern of H2-Q10 given its strong binding to hCD8α *in vitro*. In contrast to staining with other tetramers, H2-Q10 showed a clear tetramer-positive population (17.1%) (Figure 5.4, fifth column). Upon analysis of these H2-Q10 positive cells, we observed that almost all cells, unlike HLA-E, HLA-G and Qa-1b, were in the CD3+ CD8α+ population (92.9%) with the majority of these cells co-expressing CD8β (98.5%) compared to CD8α alone (1.43%). In conclusion, we showed that H2-Q10 could convincingly bind human PBMCs and nearly all H2-Q10 tetramer-positive cells are able to be found within the bulk CD8α+ population without needing to scrutinise based on different cell subsets.

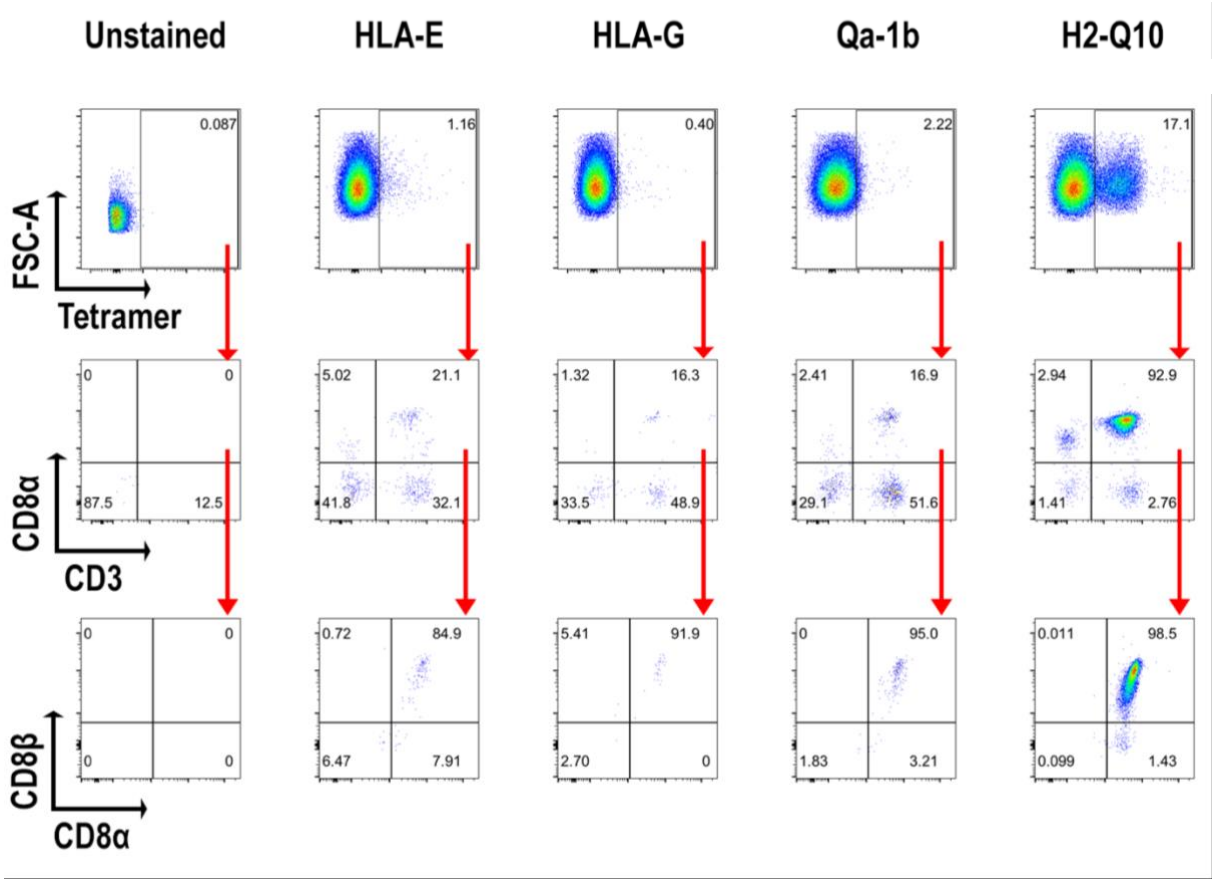


Figure 5.4. H2-Q10 binds to human peripheral blood mononuclear cells. Human peripheral blood mononuclear cells were isolated and stained with HLA-E, HLA-G, Qa-1b and H2-Q10 tetramers for flow cytometric analysis of binding. Analysis of tetramer-positive cells was performed using CD3 and CD8α followed by subdivision of CD3+CD8α+ populations based on CD8β expression. Black values represent percentages within populations.

Contact residues within the α3 domain of H2-Q10 are critical for binding to human CD8αα

In line with our observations that H2-Q10 binding human CD8αα is not dictated by N-glycosylation, we sought to determine the key residues on H2-Q10 regulating its binding with hCD8αα by using the same mutated H2-Q10 tetramers as shown in Chapter 4. hCD8αα strongly binds native H2-Q10 tetramer despite a small reduction in CD8α expression (Figure 5.5). This high level of binding was lost upon staining with Q10 G194R and T187A single mutant tetramers with an additive effect in the G194R T187A Q10 double mutant tetramers. In addition, the Q10 SRP mutant tetramer, which is a mutant form of H2-Q10 that contain sites known to mediate classical H2-K_b binding to mCD8αα, showed reduced binding capability to hCD8αα comparable to that of the G194R/T187A single mutants. Therefore, the strong binding ability of H2-Q10 to human CD8αα is governed by shared contact residues also critical in mediating the murine CD8 αα-H2-Q10 interaction.

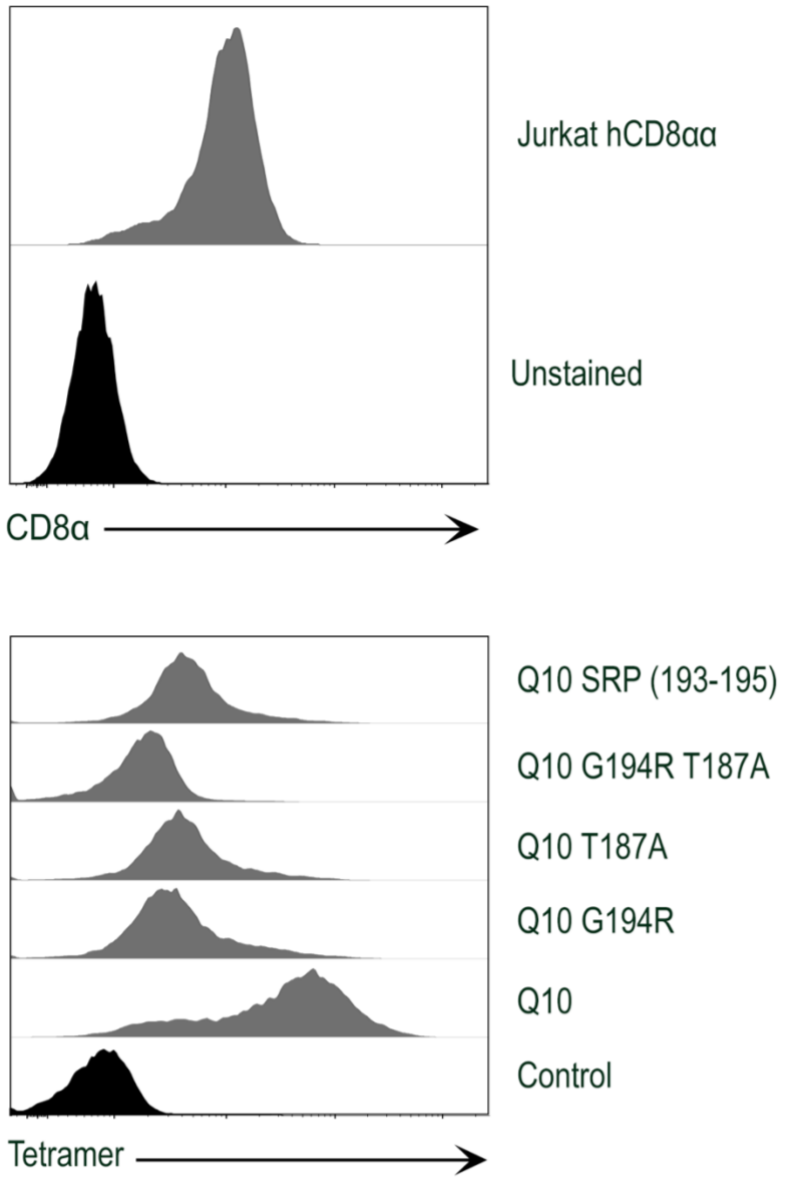


Figure 5.5. Contact sites mediating binding of H2-Q10 to murine CD8αα are also critical in binding to human CD8αα. Tetramers containing mutations in critical contact residues governing H2-Q10 binding to murine CD8αα (G194R, T187A, G194R/T187A, SRP 193-195) were used to stain Jurkat cells expressing human CD8αα. Histograms are presented as offset and modal. Data is representative of two independent experiments.

5.4. DISCUSSION

Mouse and human class Ib MHC molecules have typically been analysed to identify similarities which may define them as homologues. Although H2-Q10 has been predicted to share similarities with HLA-G which may support their homology, this has not been explored in great detail. In an attempt to compare the binding interaction between HLA-G and H2-Q10 binding to CD8α, this chapter attempts to apply the findings from Chapter Four relating to the biochemical signature which mediates this interaction. Findings from this section reinforces previous findings that N-linked glycosylation regulates binding of class Ib MHC to CD8α in mice and extends these findings by implicating this post-translational modification in human CD8α interactions. Firstly, we show that loss of the master regulator of hybrid and complex N-glycan structures, MGAT1, does not have a substantial effect on hCD8α interactions with human or murine class Ib MHC; this is supported by the prediction that the CD8α chain does not contain N-glycosylation sequons. Although the minimal impact of MGAT1 loss on hCD8α binding to class Ib MHC tetramers may support its natural non-N-glycosylated status, deeper analyses, perhaps through mass spectrometry, should be used to validate our findings.

We showed that the enhanced ability of H2-Q10 to bind to mCD8α in the absence of N-glycosylation could also be observed in cell lines and primary PBMCs expressing hCD8α. Although HLA-G is a known ligand for CD8αα, obvious binding of HLA-G in human CD8α+ PBMCs was not observed. This could possibly be due to competition between CD8αα and other receptors found on PBMCs, namely inhibitory receptors Ig-like transcript 2 (ILT2) and ILT4, for HLA-G binding. ILT2 and ILT4 are expressed on a wide range of cells, including monocytes, B cells, dendritic cells, some NK cell subsets and T cells (Borges et al., 1997; Colonna et al., 1998; Cosman et al., 1997; Samaridis and Colonna, 1997). ILT2 has been implicated in mediating NK cell responses as engagement with HLA-G initiates an inhibitory signalling cascade resulting in supresses NK cell function (Shiroishi et al.,

2003). In a study by Shiroishi and colleagues, it was found that ILT2 and ILT4 shared similar contact residues for HLA-G when compared to CD8αα, however, these inhibitory receptors bound with a higher affinity and thus were selected preferentially to bind HLA-G (Shiroishi et al., 2003). Therefore, in relation to our data, co-expression of both CD8αα and ILT2/ILT4 on PBMCs will favour engagement of the latter with HLA-G. This is further supported by our findings showing strong H2-Q10 binding to CD8αα. Previous studies have shown that ILT2/ILT4/CD8αα contacts HLA-G via critical residues within the α3 domain at E198, T214, T225-E232, K242, K248 and Q262 (Gao et al., 2000). Our data shows that the critical residues for H2-Q10 binding to human CD8α exist outside of these sites (T187, G194, P193-S195) and thus is not likely to compete with other HLA-G ligands such as ILT2/ILT4.

The ability of murine class Ib MHC tetramers to bind hCD8α is supported by previous studies showing H2-TL tetramer reactivity to a subset of CD8αα-expressing human thymocytes (Attinger et al., 2005; Huang et al., 2011; Madakamutil et al., 2004; Verstichel et al., 2017). We showed that H2-Q10 was able to bind hCD8αα and hCD8αβ. Owing to our observations of considerably strong binding between H2-Q10 and hCD8α, it would be of interest to determine binding affinity through SPR analysis to understand whether the strength of this interaction can provide insight into the potential for a functional outcome of this interaction. Indeed, future studies should attempt to stimulate hCD8αα-expressing cells through cross-linking with H2-Q10 to examine activation or induction of effector function. This hypothesis is supported by an earlier study by Wooldridge and colleagues show that "superenhanced" ligation of murine MHC to CD8αα, via generation of chimeric high affinity class Ia tetramers, can non-specifically (i.e. in the absence of TCR/pMHC interaction) activate cytotoxic T cells and induce cytokine release, degranulation and proliferation (Wooldridge et al., 2010). The binding affinity of the chimeric tetramer used in this study was $K_D \sim 10\mu M$. Findings from Chapter 4 indicate a binding affinity of H2-Q10 to murine CD8αα as $K_D \sim 300nM$. Unpublished data from our group has also shown that although H2-Q10 binds murine CD8αα with high affinity,

its binding to hCD8αα appears to be greater and thus we speculate that the binding affinity of this interaction could exceed K_D ~ 300nM. It has also been shown that ligation of high-affinity MHC tetramers to PBMCs can cause non-specific expansion of CD8 T cells (Wooldridge et al., 2010). This might be especially advantageous in a clinical setting, such as adoptive T cell therapy, whereby the T cells of a patient can be induced to proliferate, potentially via non-specific ligation with H2-Q10, prior to reinfusion back into the patient.

A potential interesting application of our finding that H2-Q10 shows enhanced binding to hCD8αα is the generation of chimeric HLA tetramers. In a previous study, a mutation in the known CD8α binding domain of HLA-A2 (Q115E) (Devine et al., 1999) was able to slightly enhance TCR/pMHCI stability duration but importantly improved pro-inflammatory cytokine production (IFN-γ and MIP-1β) and proliferation of CD8 T cells (Wooldridge et al., 2007). Furthermore, naïve CD8 T cells isolated from PBMCs were primed much more efficiently with this high-affinity mutant tetramer compared to WT HLA-A2 without substantial compromise of antigen specificity; the authors claimed that this mutant, in tetrameric form, performed better than pulsed APCs expressing the same molecule, alluding to the advantage of using soluble tetramerised complexes. This effect on CD8 T cells was largely explained by improved early signal transduction events afforded by the increased CD8/pMHCI affinity but also by other studies showing that the ability of high-affinity CD8α ligands to induce T cell signalling cascades may be due to the extensive cross-linking of CD8α molecules at the cell surface (Artyomov et al., 2010; Grebe et al., 2004; Wooldridge et al., 2003, 2007). The ability to generate chimeric HLA molecules, with substitution of the α3 domain of H2-Q10, might show potential clinical applications in adoptive T cell therapy as this moderate increase in CD8-HLA/pMHCI interaction could lead to a substantial improvement in overall CD8 T cell responses. It will be interesting to support these findings with antigen-specific classical and non-classical HLA molecules. Indeed, we have generated HLA-E tetramers with an α3 domain substitution to that of H2-Q10 to determine whether CD8 T cells specific to HLA-E, such as those specific to the UL40 protein found in human cytomegalovirus

(Jouand et al., 2018; Sullivan et al., 2017), may be induced to proliferation or become activated as previously mentioned.

Overall, our findings show, for the first time, that a mouse MHC-Ib molecule can bind with high affinity to hCD8αα. We have shown, that H2-Q10 may be a strong ligand for hCD8α with the potential to induce the functional consequences of enhanced CD8 T cell proliferation and improved effector functionality. Future studies examining the strength of H2-Q10 binding to hCD8α and the downstream signalling events and functional output of this interaction will be useful for future clinical applications especially in adoptive T cell therapy settings.

Chapter Six

General Discussion and Concluding Remarks

The discovery and exploration into the fundamental biology of MHC has undeniably shaped our understanding of immunity, however, due to the complexity and expansion of non-classical MHC, these potentially important molecules have remained largely understudied and underappreciated. This thesis has provided novel insights into one of these enigmatic molecules through focused analysis of H2-Q10.

Through the generation of a H2-Q10 knockout mouse model, this chapter has explored the immunological and physiological effects of H2-Q10 deficiency. Firstly, it has been demonstrated that the loss of H2-Q10, through its subsequent effect on other class Ib MHC molecules, impairs the development and maturation of conventional hepatic NK cells as well as CD8αα γδ T cells. Despite this reduction in two effector arms of anti-viral immunity, the absence of H2-Q10 itself did not have a significant impact on MCMV pathogenesis. Interestingly, a recent study has revealed the role of class Ib MHC in mediating CD8 T cell frequencies and functions during MCMV infection in the absence of class Ia MHC whereby a residual number of class Ib-restricted CD8 T cells acted in a compensatory role to protect against lethality (Anderson et al., 2019). Despite the majority of these cells being Qa-1/H2-T23 restricted, there remained a pool of CD8 T cells involved in this protective response which were non-Qa-1 restricted. This hints towards a potential contribution from another class Ib MHC molecule, however this was left unexplored by the authors. In furthering these insights, data from this thesis suggests that H2-Q10 is most likely not the restricting element for these CD8 T cells, due to its soluble nature and thus inability to engage TCR and activate T cells, prompting potential future studies into other class Ib molecules as candidates.

Further pathogenesis studies, potentially involving other viruses or even bacteria, may yield a more exciting role for H2-Q10 in the development of immunity. An exploration into the role of H2-M3 during *Mycobacterium tuberculosis* infection demonstrated that although H2-M3-restricted CD8 T cells could respond during infection, a non-M3 class Ib-restricted CD8 T cell pool contributed a larger

proportion of these protective cells (Shang et al., 2016). Although Qa-2-restricted cells comprised the majority of these non-M3-restricted cells, there still remains a small fraction of non Qa-2-restricted cells which may be restricted by another class Ib MHC molecule. Although non-self peptides have yet to be identified for H2-Q10, it is worth exploring whether in contexts of bacterial or viral infection, H2-Q10 may be able to present antigen to a small, but present, pool of CD8 T cells or is able to elicit non-specific expansion of these cells as discussed in Chapter 4. Preliminary experiments for future studies may involve computational approaches, to determine sites of MCMV proteins which can be targeted by known H2-Q10 peptide-binding motifs, as well as mass spectrometric analysis of infected hepatocytes to identify pathogen-specific peptides which may be loaded into H2-Q10.

In general, as a highly overexpressed protein in the liver, it is unlikely that H2-Q10 would be evolutionarily conserved to only produce such a relatively minor biological effect as evidenced by its impact on NK and γδ T cell frequencies (Chapter 3). Therefore, using RNA sequencing and adopting an exploratory approach, the non-immunological role of an otherwise prototypical immunological molecule provided a glimpse into the potential physiological role of class Ib MHC. Indeed, my data demonstrated that H2-Q10 may play an important role in the detoxification processes of the liver, specifically altering the expression of enzymes regulating steroids and scents. As such, this thesis bridges the fields of immunology and physiology and builds upon ideas proposed more than 40 years ago through discovery of upregulated gene expression of metabolic enzymes, such as the sulfotransferase family, and odour-related proteins such as the major urinary proteins (MUPs). Although relatively understudied, the role of MHC in impacting phenomena such as metabolism and even social communication has been previously explored by other groups (Overath et al., 2014; Sturm et al., 2013) whereby MHC-dependent generation of olfactory signals impacted animal behaviour and mating preference (Yamazaki et al., 1976). A potential future study might involve the setup of male mice expressing various ‘doses’ of H2-Q10 – full (WT), half (H2-Q10 heterozygotes) or deficient (H2-Q10 homozygous KO). Co-housing these mice with females followed by analysis of

sulfotransferase expression in conjunction with various olfactory proteins, such as MUP20, might provide further evidence into H2-Q10's ability to have an effect on mating outcomes. Therefore, despite the role of the liver in immune processes, as outlined by Chapter 3, it may be just as intriguing to provide supporting evidence into immunophysiological crosstalk.

The only known ligand for H2-Q10, prior to the findings of this thesis, was the NK cell inhibitory receptor, Ly49C. Despite the initial discovery of this interaction, the physiological relevance remained questionable as membrane-bound recognition of classical MHC I in *cis* to Ly49C abolished H2-Q10 recognition (Sullivan et al., 2016). Chapter 4 defines a novel interaction of H2-Q10 with another ligand, CD8αα, which is a higher affinity interaction (300nM) compared to both binding with Ly49C (5μM) and the previously only other identified ligand of CD8αα, H2-TL (800nM) (Goodall et al., 2018b; Liu et al., 2003). The existence of another ligand for CD8αα permits potential future studies into the development and regulation of CD8αα-expressing cells present beyond the gut-restricted H2-TL. Therefore, this finding raises the hypothesis that multiple members within the class Ib MHC family may be serving as ligands for CD8αα. As Chapter 4 has defined key interacting motifs of both of H2-Q10 and CD8αα in this interaction, it will be interesting to determine whether binding of CD8αα is a shared feature of other class Ib MHC molecules where it might be speculated that class Ib MHC binding to CD8αα may occur in a hierarchical manner depending on the availability of class Ib MHC at each site.

Furthermore, for the first time, this thesis has also implicated differential sialylation profiles between primary αβ and γδ T cells. These data have shown that this defines their ability to bind H2-Q10 which implicates non-classical MHC molecules for the dissection of γδ T cell biology. Despite the characterised interaction with H2-Q10 and CD8αα homodimers expressed on γδ T cells, there exists other immune cells apart from the highly sialylated αβ T cells which express CD8αα and thus are potential targets for H2-Q10 ligation. Future studies into the localisation of these cells, their

sialylation and N-glycosylation profiles, and their subsequent binding to H2-Q10 will be of significant interest in understanding how CD8αα-expressing cells are controlled. In addition, other CD8αα-expressing cells exist outside of T cells, such as NK cells and MAIT cells (Kern et al., 1999b; Zamoyska, 1994). Owing to some shared functionality with these cells and T cells, such as perforin/granzyme-mediated cytotoxicity, it will be of interest to determine whether they are able to bind to H2-Q10 and if interaction can be harnessed to control effector responses.

In vivo studies of H2-Q10 will provide excellent insight into its ability to interact with CD8αα and whether a functional consequence arises from this interaction. As most of the work presented in this thesis was performed *in vitro* and utilising tetrameric forms of H2-Q10, it has not been formally shown whether H2-Q10 can exist as a multimer *in vivo* (Contini et al., 2003; Fournel et al., 2000; Lew et al., 1986; Rebmann et al., 2003). The potential for H2-Q10 to elicit a functional consequence will be very much dependent on its ability to crosslink CD8αα as it is highly unlikely to provide stimulus in its soluble and monomeric form (Doucey et al., 2001; Luo and Sefton, 1990; Wooldridge et al., 2003).

It has long been presumed that activation of CD8 T cells leads to the up-regulation of CD8αα owing to the ability of activated CD8 T cells to bind H2-T3/TL tetramers (Madakamutil, 2004). Data presented in this thesis raises an interesting possibly that CD8αα homodimers on activated T cells become more recognisable not just due to increase surface upregulation but owing to a change in sialylation following their activation which consequently affects binding to MHC. This is supported by the dual observations that H2-Q10 only binds to CD8αα when it is unsialylated and that the activation of CD8 T cells results in a significant decrease in SNA expression (Comelli, 2006). Given that CD8 preferentially forms homodimers (Pellicci, 2000), it is more likely that CD8 T cells continuously express CD8αα homodimers constitutively, but this only becomes obvious when sialic acids are removed and the MHC class Ib epitopes become available. Given the non-lymphoid tissue

restricted expression pattern of most class Ib MHC (Ohtsuka, 2008), it is possible that overexpression of CD8αα ligands at these sites provides a means by which to control the activation and long-term survival of CD8αα T cells in these areas, independent of the effects occurring within the lymphoid organs.

Non-classical MHC molecules in mice and humans have been studied in parallel to define potential homologues. Previous literature has attempted to draw parallels between H2-Q10 and HLA-G despite their differential tissue localisation in the liver and trophoblasts, respectively. These data, in conjunction with the findings in Chapter 3, indicate that H2-Q10 and HLA-G are synthesised and secreted as soluble molecules. Furthermore, both molecules display more of a class Ia MHC-like peptide repertoire, due in part to the reduced number of anchor residues in these molecules as opposed to the majority of the class Ib MHC members. Since the discovery of the ability of HLA-G to bind CD8αα (Sanders et al., 1991), there have been limited studies exploring the control mechanisms and consequences of this interaction, however, Chapters 4 and 5 have shown that H2-Q10 appears to bind mCD8αα in a sialylation and N-glycosylation-dependent manner whereas HLA-G binding to hCD8αα may occur via another independent mechanism. By comparing murine and human class Ib MHC interactions with CD8, this thesis has provided evidence for murine class Ib MHC interactions with human ligands. The potential avenue for the findings of Chapter 5 mainly lie in the possibility of developing reagents for studies of human CD8αα biology. In the era of adoptive T cell therapy and generation of chimeric receptors, understanding how to manipulate the strength and avidity of ligation between immune receptors, as well as the functional output of these interactions, may be a useful tool. Understanding the key residues controlling the interaction between H2-Q10 and human CD8αα could allow for manipulation of these critical binding sites that can be used to generate high-affinity HLA tetramers to be used in human research. It will be of interest to build upon the findings of Chapter 5 by first understanding the functional consequence of H2-Q10 and CD8αα ligation and whether mutant HLA tetramers may be able to induce similar effects such as non-specific activation.

James Allison, upon receipt of his shared Nobel Prize for the discovery of PD-1 and subsequent development of targeted cancer immunotherapy, stated "*I'm a basic scientist. I didn't get into these studies to cure cancer, I wanted to understand how T cells work*…". This appreciation for the requirement of fundamental basic science as a foundation for potential clinical applications resonates with the primary goal of this thesis. Careful dissection of the basic biology of these understudied class Ib MHC molecules will provide the necessary building blocks for potential future applications.

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