Abstract

MURRAY, SABRINA L. Peptide-MHC Class I Cytotoxic Tetramers Inhibit Specific CTL Activity and Alter Immunodominance Hierarchies in the HY Antigen System. (Under the direction of Drs. Paul Hess and Susan Tonkonogy).

The $CD8^+$ cytotoxic T lymphocyte (CTL) response is a major effector mechanism in allograft rejection. In this study, we used the well-established murine HY-reactive immune response as a model for allograft rejection at the minor histocompatibility level. We first sought to characterize the relationship between the D^b-restricted Uty and Smcy-specific $CD8^+$ T-cell populations known to respond to male-antigen within our model. We found that sensitization of B6 females with male bone marrow generated robust D^b-Uty and-Smcy –specific pMHC class I tetramer-positive CTLs that produced similar levels of IFN-γ, TNF-α, and granzyme B but exhibited different kinetics of expansion and peptide-pulsed target cell killing efficiency. To investigate whether this was due to differences in naïve CTL precursor frequency, we attempted to directly enumerate D^b-Uty and-Smcy reactive T-cell numbers in naïve female mice. However, despite several attempts we were unable to enumerate either CTL precursor population and are awaiting results from an alternative method. Once our mode of alloreactivity induction was established we investigated hypothesis that treatment of female mice with D^b-Uty or-Smcy specific pMHC class I tetramers coupled to the ribosome-inactivating toxin, saporin, would selectively delete naïve cognate $CD8^+$T cells and inhibit CTL activity through the prevention of CTL priming. As expected, administration of the cytotoxic pMHC class I tetramers prior to immunization dramatically reduced the specific CTL population 14 days following immunization and resulted in the specific inhibition of CTL activity. Interestingly, elimination of the D^b-Uty-specific CTL population magnified T-cell reactivity against the remaining epitope, D^b-Smcy. These findings imply that the relative amplitudes of the D^b-restricted H-Y responses depend, in part, on competition between $CD8^+$ T cells during priming. These data also suggest that cytotoxic pMHC class I tetramers could be useful to dissect or manipulate T-cell immunodominance hierarchies, as well as serve as a therapeutic agent to prevent priming of CTL that mediate graft injury and rejection in allotransplantation.

Peptide-MHC Class I Cytotoxic Tetramers Inhibit Specific CTL Activity and Alter Immunodominance Hierarchies in the HY Antigen System

by
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DEDICATION

This thesis is dedicated to Mr. Bowman, who introduced science to me as critical, imaginative curiosity. That introduction made all the difference.

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ABSTRACTS

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LIST OF ACRONYMS AND ABBREVIATIONS

[A]
AAALAC: Association for Assessment and Accreditation of Laboratory Animal Care
ACAD: activated T cell autonomous death
ADDC: antibody-dependent cell-mediated cytotoxicity
AICD: activation-induced cell death
AF647: Alexa Fluor® 647
APCs: antigen-presenting cells
APC: allophycocyanin
AT: adoptive transfer
ATG: anti-thymocyte globulin
[B]
BALB.B:C.B10-H2b/LiMcdJ
BM: bone marrow
BMDC: bone marrow dendritic cell
B6:C57BL/6
B6 GFP: C57BL/6-Tg(H2K^{b}-GFP)/Fre
B6 Thy1.1: B6.PL-*Thy1*^{a}/CyJ
[C]
CDR3: complementarity determining region 3
CFSE: 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester
CTL: cytotoxic T lymphocyte
CXBRI: CXB recombinant inbred strains
C9M: gp33 altered peptide ligand
C9M-SAP: D^{b}-C9M-cytotoxic tetramer
[E]
EDTA: ethylenediaminetetraacetic acid
ELISA: enzyme-linked immunosorbent assay
ELISPOT: enzyme-linked immunosorbent spot
[F]
FacsWash: 2% FBS and 0.1% NaN_3 in PBS
FacsFix: FacsWash containing 1% buffered formaldehyde
FasL: Fas ligand
FBS: fetal bovine serum
FITC: fluorescein isothiocyanate
[G]
GFP: green fluorescent protein
GrB: granzyme B
GVHD: graft-versus-host disease

[H]
HLA: Human Leukocyte Antigen
HY-CD8^{1nt}: C57BL/6-*Cd8a*^{tm1Mak}
HY-TCR: C57BL/6-TgN (TcrHY)
H3K4: lysine 4 on histone 3
H3K27: lysine 27 on histone 3
[I]
IACUC: Institutional Animal Care and Use Committee
IFN-γ: interferon-gamma
IP: intraperitoneal
IV: intravenous
[L]
LCMV: lymphocytic choriomeningitis virus
LDA: limiting dilution assay
[M]
M: molar
mAb: monoclonal antibody
MFI: mean fluorescence intensity
mHag: minor histocompatibility antigen
MHC: major histocompatibility complex
MiHC: minor histocompatibility complex
mL: milliliter
mm: millimeter
mM: millimolar
mTor: mammalian target of rapamycin
µL: microliter
µm: micrometer
[N]
nM: nanomolar
NOD: non-obsese diabetic/ShiLtJ
NOD CL4: NOD.Cg-Tg(Tcra^{C14}, Tcrb^{C14})1Shrm/Tisch
NOD scid: NOD.Cg-Prkdc^{scid}
NOD 8.3: NOD.Cg.Tg(TcraTcrb$^{NY8.3}$)1Pesa/DvsJ
[O]
OVA: ovalbumin
[P]
PBL: peripheral blood lymphocyte
PBS: phosphate buffered saline

PBSE: Pacific Blue succinimidyl ester
PE: phycoerythrin
pfu: plaque forming units
p.i.: post-immunization
pmol: picomole
pM: picomolar
PMA: phorbol myristate acetate
pMHC: peptide-major histocompatibility complex
P14: B6.D2TgN(*Tcr-Lcmv*)327Sdz/Fre
[R]
RB: round bottom
RBP2: retinoblastoma binding protein 2
Ref.: reference
rpm: rotations per minute
R-10: RPMI 1640 medium supplemented with 10% FBS, $5x10^{-5}$M 2-mercaptoethanol, 2mM L-glutamine, 100µg/mL streptomycin, and 100IU/mL penicillin
[S]
SA: streptavidin
sALT: serum alanine transaminase
SAP: saporin
SEM: standard error of the mean
Smcy: selected mouse cDNA on the Y chromosome
Smcy-SAP: D^b-Smcy-cytotoxic tetramer
[T]
TCR: T-cell receptor
TCR tg: T-cell receptor transgenic
TNF-α: tumor necrosis factor-alpha
TRAIL: tumor necrosis factor-related apoptosis
TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling
[U]
UNC-CH: The University of North Carolina at Chapel Hill
Uty: ubiquitously transcribed tetratricopeptide repeat gene on the Y chromosome
Uty-Sap: D^b-Uty-cytotoxic tetramer
[W]
WT: wild type
7AAD: 7-aminoactinomycin D
11P9: HY-specific CTL line

Chapter 1: Background information and project rationale

1.1 Introduction to allograft rejection

Early characterization of allograft rejection

T-cell tolerance is regulated by the immune system through a complex partnership between central and peripheral tolerance.[1] Any self-reactive T-cells that manage to escape the strict confines of negative selection within the thymus are removed or controlled within the periphery through the interconnected mechanisms of peripheral T-cell tolerance.[2] Despite the highly protective measures involved in T-cell tolerance, certain situations still occur where further regulation of T-cell activity is needed. Autoimmune disease and allograft rejection are two major examples of the deleterious consequences of a break in T-cell tolerance. Graft rejection that occurs between genetically distinct (allogeneic) individuals is known as allograft rejection, and is a situation that highlights the importance of T-cell tolerance in the clinical setting.[3] The immune response involved in allograft rejection has been well characterized and major immune mediators have been defined. A wide variety of methods for the induction of T-cell tolerance have also been attempted in this setting, with additional means suggested as more is discovered about the nature of allograft rejection. Although T-cell tolerizing methods continue to advance, antigen-specific T-cell tolerance remains an important hurdle for the prevention of allograft rejection and is of particular interest to our study.

The immune response involved in allograft rejection was appreciated as early as 1944 by Sir Peter Medawar through his work with skin transplantation. [4-5] In this early study, Medawar described the survival of skin autografts and homografts. [5] Autografts, using the same rabbit as the donor and recipient, and homografts, between rabbits acquired from different suppliers and thus considered to be genetically heterogeneous, were compared, and the cellular conditions involved in acceptance or rejection were meticulously detailed. Medawar described the process of allograft rejection in his rabbit homograft model as being composed of a primary and a secondary cycle. During the primary cycle a massive invasion of leukocytes was observed and was described as being of native origin. Inflammation was then said to reach a peak of violent intensity, after which point “every living element of the graft breaks down.” The secondary cycle included re-invasion of the graft dermis by capillary vessels and a re-population of the graft by leukocytes of native origin. Finally the epithelium overgrew or undermined the graft and inflammation subsided into a chronic state. Medawar also compared second set

homografts to single homografts and noted a more rapid breakdown of the second set homograft. He deemed the histological damage that he observed to occur as a result of an immune response and he further implicated invading lymphocytes in the rejection process.[5] Further research has expanded the characterization of allograft rejection and the immune response involved in this process.

Levels of genetic disparity

Allograft rejection occurs as result of genetic disparity between donor and host.[3] This disparity, or mismatch, can occur at two levels that include differences at the major histocompatibility (MHC) level (Human Leukocyte Antigen [HLA] in humans; H-2 in mice) and at the minor histocompatibility (MiHC) level. [6] Major Histocompatibility Complex molecules are well known for their crucial role in antigen presentation of foreign antigens, but were originally discovered because of their role in allograft rejection. [7]Although MHC mismatch is in most cases the primary cause of allograft rejection, the immune response against minor histocompatibility antigens (mHags) is also a clinically significant problem, resulting in graft rejection as well as graft-versus-host disease (GVHD).[8-9] The importance of mHags in allograft rejection is evidenced by the rejection of skin grafts, and the induction of GVHD following hematological stem cell transplants between MHC-matched siblings. [10] Allograft rejection occurring at the MiHC level is of particular importance to this study and will be discussed in detail in Chapter 2.

Damage to the allograft resulting in eventual rejection occurs directly through antigen-specific cell injury, as well as indirectly through non-specific inflammatory mediators that affect the physiological function of the graft. [11] Direct allograft damage is currently understood to result from a combination of innate and adaptive immune responses. Rejection has been described to occur in three patterns: hyper-acute, acute and chronic. [6,12-13] Hyper-acute rejection happens immediately following transplantation as the result of pre-formed antibodies against donor antigens and most commonly occurs in recipients of grafts from ABO blood group incompatible donors.[12,14-15] Acute rejection, also known as cellular rejection, typically occurs 1-2 weeks after transplantation and is characterized by various donor-directed lymphocyte effector mechanisms. Chronic rejection may occur months to years later and is the result of a donor-directed antibody response and lymphocyte effector mechanisms. [6,12-13,15-16]

1.2 T cells and allograft rejection

T lymphocytes (T cells) are principle contributors to acute and chronic allograft rejection; both $CD8^+$ and $CD4^+$ T cells are principle effectors, especially at the level of mHag mismatch, and cytotoxic lymphocytes (CTLs) are considered to constitute a principle immunological effector mechanism in allograft rejection as a whole. [6-7,11] The role of T cells in allograft rejection, as well as the specific parameters for activation, continue to raise questions, as these recognition and activation requirements tend to be reliant upon many factors including species type, strain (in several species, including mice and rats), graft tissue type, and other experimental parameters. Nonetheless, antigen specific $CD8^+$ and $CD4^+$ T cells are crucial to allograft rejection, and their activation and expansion requirements will be summarized in the following paragraphs.

CTL contribution to allograft rejection

The role of $CD8^+$ and $CD4^+$ T cells in allograft rejection has been confirmed by many researchers. Pivotal work includes characterization studies in which CTLs could be identified at or near the site of allograft rejection,[17] and experiments in which $CD4^+$ and $CD8^+$ T cells were depleted rejection was prevented thus highlighting their necessity in allograft rejection. In 1975 Strom *et al.* identified T lymphocytes within a heterogeneous population of cells recovered from rejected human renal allografts, and confirmed their cytolytic ability through chromium release CTL assays using peripheral blood lymphocytes (PBLs) that expressed donor antigens. Cytolysis was correlated with the histological grade of cellular rejection.[17] Depletion of CTLs was shown to prevent allograft rejection in mice treated with anti-$CD4^+$- and anti-$CD8^+$- depleting antibodies.[18] These immunocompromised mice were unable to reject fully MHC-mismatched skin grafts.[18]

T-cell activation and expansion

Because T cells are involved in allograft rejection, understanding how they are activated and regulated would be important in designing therapies to dampen or disable this pathologic response. T-cell activation and expansion requires three signals: T-cell receptor (TCR) recognition, co-stimulation, and inflammatory cytokines.[19-20] Signal 1 occurs when the TCR recognizes its cognate peptide and is known as TCR recognition. [6] In allograft rejection, termed allorecognition, this occurs through three different pathways.[6] Direct allorecognition is mainly associated with acute rejection; in this pathway intact MHC molecules (with peptide present in the binding cleft) are presented on the allograft and on donor passenger leukocytes acting as donor antigen presenting cells (APCs) and are recognized directly by host TCRs. [21] Through this pathway, disparities between MHC

molecules can be directly recognized by host TCR interaction, and disparities at the MiHC level can be recognized through the presentation of minor histocompatibility antigens by donor MHC molecules. The migration of the donor APCs is triggered through the trauma of graft retrieval and implantation, which leads to tissue inflammation and chemokine release. [6,22] The indirect allorecognition pathway has been suggested to play a greater role in chronic rejection, because over time donor APCs are eliminated. [21] In indirect allorecognition, host APCs present allograft-derived foreign antigens (alloantigens) through host MHC class I molecules ($CD8^+$ T-cell recognition) and MHC class II molecules ($CD4^+$ T-cell recognition).[4] Less is known about the semi-direct allorecognition pathway and its relative significance to allograft rejection. [6] In the semi-direct allorecognition pathway, host T cells directly recognize intact donor MHC molecules; however, in this pathway donor MHC molecules are presented on the surface of host APCs. [6] Host APCs acquire MHC molecules through exosomal transfer[23] or through cell-to-cell contact. [24] It is often a combination of all three allorecognition pathways that initiates T-cell activation.[6]

Co-stimulation (signal 2) is generally necessary for full activation and proliferation of naïve T cells.[19] Co-stimulation is acquired from host APCs through several different pathways. The best characterized co-stimulation pathways include the CD80/86-CD28 pathway and the CD40-CD40L pathway. [25] CD80/86(B7-1/B7-2) and CD40 are cell surface glycoproteins that act as receptors for CD28 and CD40L (CD154), respectively, located on the surface of T cells.[26] The receptors for CD80/86 and CD40 upon ligation induce T-cell production of IL-2 and additional signals needed for optimal T-cell proliferation and differentiation. [26-27]

Inflammatory cytokines constitute the third signal that is involved in directing T-cell differentiation. Following TCR recognition and co-stimulation, $CD8^+$ and $CD4^+$ T cells differentiate into different effector subsets, based on the cytokines in their microenvironment.[20] Interleukin-2, in addition to interferon-gamma (IFN-γ), is important for $CD8^+$ T-cell differentiation, for differentiation of a specific $CD4^+$ T-cell effector subset, while other cytokines such as IL-4 and IL-5 play a role in directing differentiation of additional $CD4^+$ T-cell effector subsets. [20]

Following activation, proliferation and differentiation, T cells then cause damage to the allograft through delayed-type hypersensitivity and direct cytotoxicity by previously naïve T cells and memory T cells. [13] Memory T cells have a lower activation threshold and thus pose a particular problem in allograft rejection; they may have been primed as a result of prior antigen exposure, such as blood transfusion, transplantation and pregnancy, or

even in the absence of prior antigen exposure, by bystander proliferation owing to immunosuppression induced lymphopenia or cross-reactivity from earlier infections. [13,28-29] Delayed-type hypersensitivity is primarily mediated by $CD4^+$ T cells which produce pro-inflammatory cytokines (IFN-γ), and tumor necrosis factor-alpha (TNF-α) that activate monocytes and macrophages.[11] These activated cells amplify cytokine and chemokine production which leads to immune cell infiltration and non-specific damage to the physiological function of the graft. [11] Direct cytotoxicity is largely mediated by $CD8^+$ T cells via the Fas-dependent pathway and granzyme exocytosis pathway.[11]

1.3 The role of $CD8^+$ T cells in allograft rejection

The role of $CD8^+$ T cells in allograft rejection has been a long debated topic, and has been overshadowed by the generally accepted notion that $CD4^+$ T cells are sufficient to promote rejection without $CD8^+$T cells.[30-32] It has also been suggested that any role that $CD8^+$ T cells may have in allograft rejection is dependent upon $CD4^+$ T cell help.[31] There are many studies, however, that have highlighted the rather crucial and sufficient role of $CD8^+$ T cells, in the absence of $CD4^+$ T cells in allograft rejection.[33-35] These studies mainly took advantage of adoptive transfer (AT) procedures, as well as $CD8^+$ T cell deficient states, to underscore $CD8^+$ CTL activity as a major effector mechanism in allograft rejection.

An early study addressing the question of the relative contributions of $CD8^+$ and $CD4^+$ T cells in allograft rejection used AT of $CD8^+$ T cells and $CD4^+$ T cells into B10nu/nu (athymic) mice.[33] In this experiment Rosenberg *et al.* found that B10 nude mice rapidly reject allogeneic skin grafts after receiving splenocytes from normal B10 mice, which was contrasted to the lack of rejection seen when B10 nude mice received splenocytes from T-cell deficient B10 mice. $Lyt.2^+$ ($CD8^+$) T cells or $L3T4^+$ ($CD4^+$) T cells were then transferred into B10 nude mice, who were monitored for allogeneic skin graft survival; both subsets were found to mediate skin graft rejection.[33] $CD8^+$ T cells were thus implicated as effectors of allograft rejection, and as AT experiments became more sophisticated, their role was further confirmed.

Several experiments have been carried out using AT of transgenic $CD8^+$ T cells to evaluate their role in allograft rejection. In 1999, Gilot *et al.* performed an adoptive transfer study that followed the fate of alloreactive $CD8^+$ TCR transgenic (TCR tg) T cells specific for the murine H-2 K^b MHC class I molecule *in vivo* after transplantation of an allogeneic heart.[34] The H-2 K^b specific 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE)-labeled $CD8^+$ T cells were first adoptively transferred into a syngeneic host, and 24 hours

later, the host received a cardiac transplant from either H-2K^{b+} or H-2K^{b-} mice. Five days later the heart function was evaluated, and single cell suspensions from the spleen, lymph nodes and heart infiltrating cells were assessed by intracellular and surface staining via flow cytometry. Proliferation of the TCR tg CD8^{+} T cells was assessed by CFSE dilution. Compared to the H-2 K^{b-} cardiac transplant mice, the tg CD8^{+} T cells were found to proliferate in response to their cognate H-2K^{b+} transplant, and following *in vitro* stimulation with phorbol myristate acetate (PMA)/ionomycin, to produce IFN-γ and IL-2. Transgenic CD8^{+} T cells that were infiltrating the graft were also found to have an activated phenotype (CD62L^{low} and CD69^{high}). CD8^{+} T cells were thus shown to proliferate and produce pro-inflammatory cytokines in an antigen-specific manner.

Another experiment, also performed in 1999, utilized the AT of H-2K^{b}-specific CD8^{+} T cells to evaluate the CD8^{+} T cell response after transplantation; however, this experiment additionally evaluated CD8^{+} T cell memory populations and was carried out in an immunocompromised host. [35] Jones *et al.* adoptively transferred CFSE-labeled H-2 K^{b} specific CD8^{+} T cells into syngeneic mice that had been thymectomized as adults, and T-cell-depleted by anti-CD4 and anti-CD8 monoclonal antibodies; 24 hours after transfer, the mice received an H-2 K^{b+} cardiac allograft or no allograft (AT only). T-cell depleted mice that received TCR tg CD8^{+}T cells rejected the allograft after an average of 18 days. Seven days following transplantation H-2 K^{b}-specific TCR tg CD8^{+} T cells were found to be dividing in the spleen and lymph nodes. In contrast to the AT-only mice, T cells from the allograft recipients were found to have upregulated CD44 and CD69 expression and downregulated CD45RB and CD62L, consistent with their activated status. Fifty days after transplantation, the memory H-2 K^{b}-specific TCR tg CD8^{+} T cells (now 3-5 fold decreased in number compared to control AT only mice) were assessed for *in vitro* cytokine production. As in the Gilot *et al.* study, the tg CD8^{+} T cells were found to produce IFN-γ and IL-2, in contrast to the AT only controls [34-35].

In addition to showing that CD8^{+} T cells do play a role in allograft rejection, AT experiments have also been used to further characterize the individual roles of CD8^{+} T cell subsets in allograft rejection. In a 2001 study by Delfs *et al.*, H-2^{b} RAG1-/- mice received allogeneic H-2 ^{d} cardiac transplants after which H-2^{d} reactive CD8^{+} T cells were adoptively transferred into the host; because of the deficiency in RAG1; allograft rejection could be attributed to the transferred CD8^{+} T cells in this model system. [36] The two CD8^{+} T-cell subsets employed in this experiment were TC1 CD8^{+} T cells that produce IFN-γ, and TC2 CD8^{+} T cells that produce IL-4,-5,-10 and low levels of IFN-γ. Histological scoring of the graft

at days 7, 14 and 28 following transplantation revealed the TC1 $CD8^+$ T cells to mediate vasculitis, while the TC2 $CD8^+$ T cells seemed to promote the recruitment of secondary effector cells into the graft, such as eosinophils. This study found that both subsets of $CD8^+$ T cells mediated different elements of the inflammatory response involved in allograft rejection. [36]

The crucial role of $CD8^+$ T cells in the allograft rejection has also been confirmed through studies employing $CD8^+$ T cell deficient mice. In 1993, Desai *et al.* transplanted allogeneic islets into β2 microglobulin deficient mice that had been rendered hyperglycemic via streptozotocin. [37] β2 microglobulin deficient mice do not express intact MHC class I molecules and as a result do not develop mature $CD8^+$ T cells.[37] Because of their hyperglycemic state prior to transplantation, survival of the transplanted islets was monitored by the glucose levels of the host mice. In this experiment, transplanted islets survived significantly longer in the $CD8^+$ T cell deficient mice compared to the $CD8^+$T cell intact hosts (49 vs. 15 days).[37] When $CD8^+$ T cell responses were blocked *in vivo* through anti-MHC class I antibodies or fragments of the anti-MHC class I antibodies (F(ab)2) in streptozotocin-induced hyperglycemic mice, allogeneic islets also survived longer than islets grafted into PBS treated recipients.[38] In rats treated with anti-CD8 monoclonal antibody and grafted with aortic allografts, $CD8^+$ T cells were found to play a pivotal role in rejection.[39] At 20 days following transplantation, the apoptotic index (measured by terminal dUTP nick end labeling) was significantly lower in $CD8^+$ T-cell depleted host rats compared to untreated hosts. Sixty days after transplantation, histological comparison of the grafts revealed greater graft survival in the $CD8^+$ T-cell depleted hosts than in untreated hosts. [39] However, experimental parameters including allograft tissue type have been shown to contribute to the role of $CD8^+$ T cells in allograft rejection; allogeneic skin grafts were rejected at similar rates in $CD8^+$T-cell deficient mice and $CD8^+$ T-cell intact mice in the β2 microglobulin deficient model previously described.[37] Although the role of $CD8^+$ T cells in allograft rejection remains to be completely elucidated, $CD8^+$ T cells are certainly overall an integral component that must be targeted to prolong allograft survival.

The general $CD8^+$ T cell effector pathways involved in allograft rejection include the perforin/granzyme pathway and the Fas ligand pathway; these pathways are triggered as a result of CTL activation following the formation of the immunological synapse between the TCR and the peptide-loaded MHC class I molecule on the target cell. [11] The perforin/granzyme pathway, also referred to as the granzyme exocytosis pathway, causes eventual apoptosis of the target cells through cytotoxic granule production that includes

perforin, granzymes, granulysin, serglycin and other cytotoxic components. [11] Perforins assemble together to form poly-perforins that facilitate the insertion of granzymes, a family of serine proteases, through the cell membrane and into the target cell cytosol.[11] Granzymes, such as granzyme B and granulysin, trigger apoptosis of the target cell through various caspase cascades. [11,40] The Fas ligand (FasL) pathway likewise induces apoptosis; Fas ligand, either packaged into cytotoxic granules or expressed on the $CD8^+$ T cell surface, triggers caspase cascades upon binding of Fas receptors on the target cells. [11]

Additional components of CTL activity involved in allograft rejection include pro-inflammatory cytokines such as IFN-γ and TNF-α and apoptosis-inducing molecule TRAIL.[7,37-39] TRAIL, or tumor necrosis factor-related apoptosis-inducing ligand, is a member of the TNF superfamily, and similar to TNF-α, induces apoptosis of target cells through binding of its death ligands with death receptors on the target cell surface.[41] To summarize, in allograft rejection, CTL activity kills allogeneic target cells in a myriad of ways, and consequently, many attempts have been made, and are currently being implemented, to curb this activity and prolong allograft survival.

1.4 Current treatments for allograft rejection

Treatment overview and potential risks

Current treatments for allograft rejection include a combination of immunosuppressive therapies and tolerance induction methods. In general, current allograft rejection treatments include co-stimulation blockade, mixed hematopoietic chimerism, and T-cell depletion. [42] These treatments are commonly bolstered by calcineurin inhibitors, anti-metabolites (e.g. mycophenolic acid), mTOR inhibitors (e.g. sirolimus) and corticosteroids (e.g. prednisolone). [42-43] The use of such immunosuppressive drugs has been linked to many adverse side effects such as an increased risk of infection and certain malignant conditions. [44-45] Calcineurin inhibitors, specifically, have additional risks such as neurotoxicity, nephrotoxicity, with cyclosporine being linked to hypertension and hyperlipidemia, and tacrolimus to post-transplantation diabetes mellitus. [46-48] Corticosteroids can also lead to an increased risk of cardiovascular disease and adverse metabolic effects. [47] Because of the many adverse effects associated with prolonged use of immunosuppressive treatment, high levels of these drugs cannot be tolerated long-term in post-transplantation patients and their dosage must be tapered over time. [45] Because of this necessary tapering, long term allograft survival (5-10 years following transplantation) is severely compromised; a 2004 study that compared graft survival at 1, 5, and 10 years after

transplantation found kidney transplant survival rates to be reduced from 94.3% after 1 year to 76.8% and 55.2% after 5 and 10 years respectively. [45] Pancreas, liver, heart and lung transplant survival rates revealed even more rapid rejection. [45] The chronic allograft dysfunction resulting after treatment with an immunosuppressive regimen can be considered another important side effect of many immunosuppressive therapies. Antigen-specific tolerance induction without the use of immunosuppressive drugs would be an ideal alternative for allograft rejection treatments.

Co-stimulation blockade

Tolerance induction aimed at blocking co-stimulation attempts to change the efficiency by which immune activation, specifically T-cell activation, occurs. [42] Co-stimulation blockade treatments target such co-stimulation pathways as the CD80/86-CD28 pathway and the CD40L-CD40 pathway.[42] CTLA-4, an inhibitory surface protein that acts as a "counter balance" by competing with CD28 for CD80/86 ligation, is often used as a fusion protein (CTLA-4-Ig) to prevent co-stimulation.[42] A major obstacle for co-stimulation blockade as a treatment for allograft rejection is the population of memory T cells that require a much lower threshold for activation and may negate the effects of co-stimulation blockade [42,49].

Mixed hematopoietic chimerism

Mixed hematopoietic chimerism has shown great promise as a method for tolerance induction in the context of allograft rejection.[42] This method utilizes the mechanisms involved in central tolerance induction, allowing for clonal deletion of donor-specific T cells. In this treatment, the recipient receives total lymphoid irradiation, and in some cases antibody-mediated T-cell depletion, prior to transplantation. The recipient then receives both donor and self hematopoietic cells that influence thymic selection of newly produced T cells. Because central tolerance is achieved through this method, continual treatment with immunosuppressive drugs should be unnecessary. However, while tolerance can be induced via mixed hematopoietic chimerism in rodents, this method is less effective in non-human primates and humans. Also, the total lymphoid irradiation regimens can be very rigorous and carry with them their own dangerous side effects.[42]

T-cell depletion

Another way to inhibit allograft rejection is the depletion of T cells. This non-specific method for reducing the number of alloreactive T cells within the recipient is achieved through treatment with monoclonal antibodies (non-conjugated or toxin-conjugated) specific for T cell markers. [42] The non-conjugated monoclonal antibodies elicit T-cell

depletion through complement-dependent cytotoxicity, by antibody-dependent cell-mediated cytotoxicity (ADCC), and by activation-induced cell death (AICD). [50] Common non-conjugated monoclonal antibodies include anti-thymocyte globulin (ATG), anti-CD3, anti-CD2, anti-TCRαβ, anti-CD4, anti-CD8 and monoclonal antibodies specific for CD52, a surface glycoprotein that is expressed on healthy lymphocytes. [42,50-51] These methods are effective at depleting T cells; however, in addition to leaving the host vulnerable to opportunistic infections and certain cancers, homeostatic activation and proliferation is induced in the existing immunocompetent population of T cells that were resistant to antibody-mediated depletion. [42,50]

Attempts have also been made to deplete T-cell populations by targeting other constitutively expressed surface markers using anti-CD28 and anti-CD25 monoclonal antibodies. [50] While these depletion methods target and deplete reactive T-cells by inducing AICD, they can also target (as with the other T-cell depleting methods) T regulatory cells that are also needed for the maintenance of tolerance. [50,52] Another disadvantage to targeting such surface markers is the generation of adverse effects resulting from inadvertent T-cell activation.[50] A tragic example of this effect occurred in a human clinical trial in 2006 when anti-CD28 mAb was administered in hopes of reversing the T-cell deficiency found in B cell chronic lymphocytic leukemia. Administration of the anti-CD28 mAb unexpectedly led to multi-organ failure in 6 healthy volunteers. [53] Organ failure occurred as a result of a cytokine storm induced by T-cell activation.[53] T-cell depletion is a very attractive method for tolerance induction, but broadly depleting large T-cell populations and manipulating T-cell activation can cause severe side effects.

1.5 Peptide-MHC tetramers

Tetramer-mediated immune modulation

A more attractive method for tolerance induction in the context of allograft rejection would be to eliminate only allograft specific $CD8^+$ CTLs. This depletion of antigen-specific $CD8^+$ T cells would greatly improve allograft survival; donor-specific $CD8^+$ T cells could be removed prior to transplant, and the recipient's immune system would remain otherwise intact. Continual reliance upon immunosuppressive therapy would be unnecessary and their many adverse side effects could be avoided. Tetramer technology has provided an effective means of selectively depleting antigen-specific $CD8^+$ T cells and can be applied to the scenario of allograft rejection as a possible treatment option.

Peptide-MHC (pMHC) tetramers are soluble multimers consisting of four recombinant peptide-loaded MHC class I or class II monomers biotinylated and bound together by streptavidin. [54] Tetramers were devised for the identification and quantification of antigen-specific T cells and have been successfully used for that purpose, and also for modulating the immune response. [54-57] Originally, pMHC monomers were created; however, these monomers were found to exhibit only a moderate affinity and association rate with the target TCRs and to have a rapid off rate. [58] Tetramers enhance TCR binding avidity through a "bonus effect" of providing additional pMHC: TCR interactions should one pMHC disassociate. [55] Attaching a streptavidin-conjugated fluorophore to MHC class I and II tetramers has since yielded great success in identifying, quantifying and phenotypically characterizing antigen-specific T cells through flow cytometry.[55] Viral, tumor-specific, and autoreactive T cells have all been further enumerated and characterized as a result of tetramer technology. [10] In addition to the various characterization tools that tetramer technology has provided, tetramers have also been used to interact with the immune system through both activating and tolerizing mechanisms.

Peptide-MHC tetramers have been used to modulate the immune system by triggering activation, and, more recently, T-cell tolerance. Tetramers can trigger T-cell activation resulting in lytic granule release as well as release of cytokines such as INF-γ; tetramer exposure can also lead to expansion of antigen-specific $CD8^+$T cell populations. [55,59] However, of greater relevance is the possibility that tetramer-mediated immune modulation may contribute to the prevention of allograft rejection when used to induce tolerance. In 2001, Maile *et al.* explored the potential of tetramer-mediated tolerance through overstimulation, because it was known that exposure to high antigen concentrations could lead to $CD8^+$ T cell tolerance in transgenic mice. [56,60] Studies by Maile *et al.* using the HY antigen system, relied specifically on an H-2 D^b restricted anti-HY/anti-male immunogenic response in females exposed to HY/male antigen.[56] The effects of various HY pMHC class I tetramer injections were evaluated for their ability to activate or tolerize HY-specific $CD8^+$ T cells. It was found that one injection of HY-pMHC class I tetramer primed the female anti-HY response, as detected by *in vitro* proliferation of purified $CD8^+$ splenocytes. Two injections greatly diminished the anti-HY proliferative response, and three injections resulted in an undetectable response and enhanced survival of male skin grafts on female hosts. [56] In subsequent studies, Maile *et al.* correlated tetramer-mediated tolerance induction to pMHC/TCR-CD8 avidity. [57] In this study, also within the HY antigen-system, high and low avidity HY pMHC class I tetramers were created, and their effect on T-cell tolerance or activation was correlated to the ratio of $CD8^{high}$ and $CD8^{low}$ T-cell

populations that they elicited.[57] The high avidity tetramer actually enhanced male skin graft rejection. Thus, there is a fine balance between T-cell activation and tolerance that must be taken into consideration when using tetramers as tolerance inducers. Nonetheless, tetramers can be used to modulate the immune response in a manner that extends the survival of allogeneic skin grafts.

Additional tetramer treatments have been employed that may prove fruitful in modulating the immune system. Following the 2001 Maile study that showed induction of T-cell tolerance through soluble pMHC class I tetramers, an *in vitro* and *ex vivo* study was carried out using transgenic $CD8^+$ T cells that confirmed the potential of pMHC class I tetramers to indirectly eliminate CTLs through targeting of activation pathways [56,61]. In 2005, Cebecauer *et al.* compared pMHC class I tetramers created with short linkers (25Å) and those with long linkers (88Å) for their ability eliminate transgenic $CD8^+$ T cells in an antigen-specific manner. [61] The results of this study showed that tetramers with short linkers induced extensive CTL death, compared to the modest level of CTL removal elicited by the long linker tetramers. This death was most likely due to necrosis and the activated T-cell autonomous death (ACAD), as opposed to AICD. Activated T-cell autonomous death induced by tetramers with short linkers was implicated because of strong annexin V staining, but low TUNEL staining, as well as cytochrome c release (which are hallmarks of apoptosis). Cebecauer went on to postulate that the shorter linkers allowed for CTL activation, which then led to ACAD-mediated CTL death. [61]

Despite the potential for CTL inhibition that these tetramer mediated immune-modulating experiments have shown, manipulation via $CD8^+$T cell activation parameters warrants caution. Inadvertent activation is a possible disadvantage of the use of tetramers to induce T-cell tolerance through overstimulation; severe immunopathological damage has been found to occur through attempted overstimulation with agonist peptide. [62] As discussed above, AICD and ACAD are important pathways for the maintenance of tolerance and prevention of inadvertent immunopathology; a perhaps safer use of tetramers as CTL inhibitors would involve the maintenance of these pathways and hindrance of CTL effector pathways. In 2001, Xu *et al.* found that α3 domain mutant pMHC class I tetramers could be used to selectively inhibit CTL activity by disrupting CD8 co-receptor contact, while leaving FASL up regulation unaffected, and consequently, the occurrence of AICD unchanged.[63] The use of mutant pMHC class I tetramers could avoid the dangers of T-cell activation but still take advantage of the death pathways triggered by antigen exposure. These different experiments have established immunomodulation as an important use for tetramers in the

prevention of alloreactive and autoreactive T-cell activity. However, tetramers have since been used in an increasingly aggressive way to not only modulate the T-cell response, but to directly eliminate antigen-specific T-cell populations.

Ex vivo tetramer mediated depletion

In addition to over-stimulating antigen-specific T-cell populations, tetramers have also been used as a means to remove alloreactive donor T cells prior to engraftment. Two studies have been recently performed in a model for GVHD involving *ex vivo* tetramer mediated-depletion. In 2006, Kappel *et al.* performed transplants of T-cell depleted bone marrow and $CD3^+$ splenocytes, devoid of antigen-specific T-cell populations, to delay the onset of GVHD and to increase the median survival time of the recipient. [64] $CD3^+$ splenocytes from B6 mice were transferred into irradiated BALB.B mice, which differ from B6 mice by more than 29 mHags. Prior to transfer, splenocytes were incubated with fluorophore-conjugated tetramers specific for the immunodominant mHag H60. [64] Magnetic purification was then used to remove tetramer bound K^b-H60-specific $CD8^+$ T cells; this treatment resulted in a significantly increased survival time of host BALB.B mice and retention of the transplanted splenocyte anti-tumor activity. [64] A similar experiment was also performed by de Witte *et al.* with different results.[65] In this study tetramers were used to deplete B6 splenocytes of alloreactive $CD8^+$ T cells prior to transfer into irradiated BALB.B recipient mice.[65] While the results show a decrease in alloreactive $CD8^+$ T cell IFN-γ intracellular production compared to control splenocytes, the incidence and severity of GVHD was unaltered in mice receiving tetramer-depleted splenocytes. [65] A perhaps crucial difference between the protocols was that de Witte *et al.* used tetramers to deplete K^b-H60-specific $CD8^+$ T cells, along with K^b-H4-specific $CD8^+$T cells (another immunodominant epitope), while Kappel *et al.* only depleted K^b-H60-specific $CD8^+$T cells. [64-65] The number and specific epitope of $CD8^+$ T cell removed from the pool of 29 or more mHag reactive T-cell populations may have affected the immunodominance hierarchy present in this mHag model in such a way as to produce these varied results. Nevertheless, these studies highlight the potential for *ex vivo* tetramer-mediated treatment of grafts to prevent GVHD.

Ex vivo tetramer depletion may become a valuable treatment for GVHD, but it is unlikely to be helpful as a means to prevent allograft rejection, as the recipient would have to be completely purged of alloreactive T cells. Hence, In the case of allograft rejection tetramers must instead be used to selectively delete T-cell populations *in vivo*. This treatment has been used in several alloreactive and autoreactive settings and has shown great promise for the treatment of allograft rejection.

Peptide-MHC class I cytotoxic tetramers

The first description of selective deletion or killing of T cells was carried out by Yuan *et al.* in 2004. [66] Yuan *et al.* developed pMHC class I tetramers conjugated to actinum ^{225}, an alpha-emitting atomic nanogenerator; these tetramers were referred to as alpha-emitting suicide pMHC class I tetramers. The alpha-emitting suicide pMHC class I tetramers were used to selectively delete human and murine $CD8^+$ T cells specific for Epstein Barr virus latency membrane protein 1 (lmp1) and *Listeria monocytogenes* peptide LLO, respectively. These *in vitro* experiments demonstrated specific deletion of T-cell populations, but also resulted in low levels of non-specific T-cell killing. [66] Bystander radioactivity emission has been suggested as the causative agent for the observed non-specific T-cell killing in the experiment, as actinium ^{225} does not require internalization to kill target cells and can work through short-range killing. [66-67] A safer, more specific means for selectively deleting alloreactive T-cell populations would be an ideal alternative.

It was discovered that coupling a toxin to a pMHC class I tetramer could provide a more specific alternative for selective T-cell deletion. [68] In 2007, Hess *et al.* coupled pMHC class I tetramers with a type I ribosome-inactivating protein, saporin, to selectively delete $CD8^+$ T cell populations *in vivo*. [68] Upon recognition by the target TCR, the pMHC class I cytotoxic tetramer is taken into the cell. Following lysosomal degradation, saporin is released into the cytoplasm of the cell, where it prevents ribosomal activity, resulting in death of the target T cell. [68] Hess *et al.* began by showing *in vitro* killing of murine TCR tg HY and P14 $CD8^+$ T cells, following incubation with the appropriate pMHC class I cytotoxic tetramer. The investigators then transferred HY antigen-specific and P14 –green fluorescence protein (GFP) $CD8^+$ T cells into B6 recipients, and treated the recipients 24 hours later with pMHC class I cytotoxic tetramers or pMHC class I tetramers specific for P14 $CD8^+$ T cells.[68] Treatment with pMHC class I cytotoxic tetramers resulted in more than 75% depletion of P14-GFP^+ $CD8^+$T cells and an unchanged HY antigen-specific $CD8^+$ T cell population compared to the tetramer-treated group. [68] These results show, for the first time, that cytotoxic tetramers can be used to selectively delete a T-cell population *in vivo*.

Following the report by Hess and colleagues, two additional studies have been carried out using pMHC class I cytotoxic tetramers to selectively delete $CD8^+$ T cell populations. In 2009, Penaloza-MacMaster *et al.* used pMHC class I cytotoxic tetramers to prevent $CD8^+$T-cell mediated tissue destruction in a meningitis hepatitis model. [67] In the first model, irradiated recipient mice received an adoptive transfer of P14 $CD8^+$ T cells along with T cells of other specificities. The mice were then treated with pMHC class I cytotoxic

tetramers specific for gp33 and infected the following day with Lymphocytic choriomeningitis virus (LCMV). Infection typically causes lethal meningitis, but as a result of pMHC class I cytotoxic tetramer treatment, death of the recipient mice could be prevented. In the second model, ALB gp33 mice, which express gp33 in the liver under the albumin promoter were irradiated and received P14 $CD8^+$ T cells. The mice were then treated with gp33-specific pMHC class I cytotoxic tetramers and infected with LCMV. In this experiment pMHC class I cytotoxic tetramer treatment resulted in decreased liver damage, as measured by serum alanine transaminase (sALT activity). [67] Peptide-MHC class I cytotoxic tetramers were shown through these experiments to selectively delete specific $CD8^+$ T-cell populations, and in so doing, prevent $CD8^+$ T-cell mediated damage.

The final study to date that has employed delivery of pMHC class I cytotoxic tetramers to selectively delete $CD8^+$ T cell populations and to affect the immune response *in vivo*, and like the Hess *et al.* study, provides promise for translation to an allograft rejection study.[69] Vincent *et al.* used pMHC class I cytotoxic tetramers to selectively delete a $CD8^+$ T-cell population implicated in the pancreatic β-cell destruction of type I diabetes. [69] In this study non-obese diabetic (NOD) 8.3 T cells (TCR tg for IGRP peptide expressed by the β-cells) were transferred into NOD scid recipients, which were then treated with cognate pMHC class I cytotoxic tetramers. Seven days later, greater than 75% reduction of circulating 8.3 $CD8^+$ T cells was found in pMHC class I cytotoxic tetramer-treated mice, compared to mice given phosphate buffered saline (PBS) or an antigenically unrelated pMHC class I cytotoxic tetramer. Vincent *et al.* then targeted a heterogeneous pool of IGRP reactive $CD8^+$T cells in NOD mice. Non-obese diabetic mice will develop type 1 diabetes spontaneously over time. [69] NOD mice were treated with pMHC class I cytotoxic tetramers specific for IGRP $CD8^+$T cells and were then monitored for onset of hyperglycemia. Peptide-MHC class I cytotoxic tetramer-treated mice developed diabetes with a significant delay compared to PBS-treated mice (24.5 weeks vs 16.5 weeks). [69] Table 1 summarizes the aforementioned *in vivo* and *ex vivo* experiments involving pMHC class I tetramers and the induction of antigen-specific tolerance.

Table 1. Summary of *ex vivo* and *in vivo* studies employing pMHC class I tetramers towards the induction of antigen-specific $CD8^+$ T-cell tolerance.

Year	pMHC class I tetramer description	Mouse strain/cell line	Goal of Study	Outcome	Ref.
2001	pMHC class I tetramer	HY-TCR, B6	Tolerance induction via overstimulation	Multiple injections prolonged syngeneic male skin graft survival	56
2004	α-emitting suicide pMHC class I tetramer	BALB/c, human antigen- specific $CD8^+$T-cell populations	Selectively delete antigen-specific $CD8^+$T-cell populations *ex vivo*	Antigen-specific $CD8^+$T-cell populations deleted, with low levels of bystander killing.	63
2005	High and low avidity pMHC class I tetramer	HY-TCR, HY-$CD8^{Int}$, B6	Investigated relationship between pMHC/TCR-CD8 avidity and tolerance induction.	Tolerance correlated to ratio of $CD8^{high}$:$CD8^{low}$ T-cell populations elicited. High avidity decreased tolerance.	57
2005	pMHC class I tetramers; short(25Å) and long(88Å) linkers	T1 TCR Tg(RAG-$2^{-/-}$)	Compared effect on CTL inhibition.	pMHC class I tetramers with short linkers inhibited CTL activity to greatest extent; ACAD implicated	61
2006	Fluorochrome-conjugated pMHC class I tetramer	BALB.B, B6	Remove K^b-H60-specific $CD8^+$T-cells *ex vivo*, prior to transplant.	GVHD decreased over time, recipient survival time increased.	64
2007	Fluorochrome-conjugated class I tetramers	BALB.B, B6	Remove K^b-H60 and –H4 specific $CD8^+$T cells *ex vivo*, prior to transplant.	Antigen-specific IFN-γ production reduced, GVHD severity unaltered.	65
2007	pMHC class I cytotoxic tetramer	B6, P14, HY-TCR	Remove antigen-specific $CD8^+$ T-cell populations *in vivo*.	>75% of antigen-specific $CD8^+$T cells eliminated, with no long term toxicity effects on recipient.	68
2009	pMHC class I cytotoxic tetramer	B6, P14 $Thy1.1^+$, and OT-I $CD45.1^+$tg	Remove antigen-specific $CD8^+$ T-cell populations *in vivo*, prior to AT.	Antigen-specific $CD8^+$T cell mediated pathology reduced, recipient survival increased.	67
2010	pMHC class I cytotoxic tetramer	NOD, NOD CL4,NOD 8.3 and NOD scid	Remove antigen-specific $CD8^+$T-cell population *in vivo* from heterogeneous pool.	Delayed onset of type 1 diabetes in NOD mice.	69

Abbreviations: pMHC-peptide-Major Histocompatibility Complex, HY-TCR- C57BL/6-TgN (TcrHY), B6- C57BL/6, HY-$CD8^{Int}$- C57BL/6-*Cd8a*^{tm1Mak},BALB.B- C.B10-H2b/LiMcdJ, P14- B6.D2TgN(*Tcr-Lcmv*)327Sdz/Fre, NOD-NOD/ShiLtJ, NOD CL4- NOD.Cg-Tg(Tcra^{C14}, Tcrb^{C14})1Shrm/Tisch, NOD 8.3- NOD.Cg.Tg(TcraTcrb$^{NY8.3}$)1Pesa/DvsJ, NOD scid- NOD.Cg-Prkdc^{scid}, CTL-cytotoxic T lymphocyte, AT- adoptive transfer, ACAD- activated T cell autonomous death, GVHD- graft-versus-host disease, IFN-γ- interferon-gamma and Ref-Reference.

1.6 Study rationale

The results of the pMHC cytotoxic tetramer studies, especially the *in vivo* experiments, suggest a new treatment method for allograft rejection. Applying pMHC class I cytotoxic tetramer technology to an allograft rejection setting may result in the removal of specific populations of alloreactive $CD8^+$ T cells. This removal is instrumental in the establishment of operational tolerance meaning an allograft may survive indefinitely and maintain its function without the need for chronic immunosuppression.[70]

Chapter 2: Experimental model, hypothesis, and specific aims

2.1 HY antigen system

The rationale of using pMHC class I cytotoxic tetramers to promote allograft tolerance without the potentially dangerous side effects of immunosuppressive treatments can be further explored using a model of allograft rejection that is restricted to rejection at the MiHC level of genetic disparity. Applying pMHC class I cytotoxic tetramer technology to allograft rejection at this level would narrow the extent of potential alloreactive $CD8^+$ T cell populations involved and allow for comparison of specific CTL activities between distinct groups following treatment and transplantation. The HY antigen system would provide a means of further restricting the potential pMHC class I cytotoxic tetramer targets, and is therefore an ideal model to investigate the potential of pMHC class I cytotoxic tetramer mediated allograft tolerance.

Minor histocompatibility antigens

The HY antigen system is a well characterized model for allograft rejection that takes advantage of the female immune response against male derived minor histocompatibility antigens.[7] As briefly alluded to in Chapter 1, a disparity of mHags between donor and recipient can result in allograft rejection in the absence of immunosuppressive therapy.[9] Minor histocompatibility antigens, although associated with slower rejection responses than MHC mismatch, remain a formidable barrier for allograft survival. [71] Minor histocompatibility antigens have been found to play an important role in hematopoietic stem cell rejection between MHC-matched donor and recipient, and are considered to be a serious problem for solid organ transplantation, as is the case with renal and corneal transplants. [9,72] Mismatch at the MiHC level is particularly cumbersome, because, unlike MHC mismatch that is preventable by typing donors prior to transplant, the number of identified mHags that donors can be screened for represents only a narrow range of the variability present between donor and recipient.[9-10] While mHags with high stimulatory activity may be feasibly identified, in an outbred population, such as humans, mismatches between mHags cannot be wholly avoided. Mismatch at the MiHC level is therefore a major contributor to allograft rejection, and because rejection is mainly T-cell mediated, a model for allograft rejection taking place at this level of genetic disparity could accurately address the efficiency and merit of selectively deleting a $CD8^+$ T cell population.

Minor histocompatibility antigenic epitopes are small peptides derived from polymorphic cellular proteins encoded by both autosomal and Y chromosome genes and

expressed on cell surfaces in complex with MHC molecules.[7,73] These antigens can be recognized by $CD8^+$ and $CD4^+$ T cells respectively.[10] Additionally, mHags such as mitochondrially encoded minor histocompatibility peptides can also be recognized by non-classical MHC-restricted cells.[74-75] The immune response against mHags evoked in the HY antigen system triggers well characterized MHC class I and class II restricted responses.

The HY response

The HY antigen system refers to the female anti-male response, or the HY response. Over the past 55 years this response against male mHags has been well studied and manipulated in hopes of achieving tolerance induction within this system. HY antigens are male mHags that are recognized as foreign by female recipients upon transplantation.[76] The known murine HY epitopes have been mapped to the ΔSxrb deletion interval of the short arm of the Y chromosome.[10,77] HY antigens have been found to be ubiquitously expressed in many different tissues including skin, testis, pancreas, lung, spleen, liver, thymus, kidney and the brain.[7,78-79] HY antigens often differ considerably from their X chromosome-encoded homologues, and this degree of difference in turn determines the strength of immune response that they are capable of inducing; generally, the more an HY antigen differs from its X chromosome homologue the greater the elicited immune response.[75] Compared to other autosomal and mitochondrially derived mHags, HY antigens are generally recognized as the most foreign mHags, with the exception of the immunodominant H60 and H28 autosomal mHags that have no allelic homologue and are thus completely foreign.[75] The immune response resulting from the recognition of HY antigens as foreign has been thoroughly investigated especially in inbred mouse strains.

It was first noted in 1955 that female mice rejected male syngeneic skin grafts; since that early discovery, the HY response has been implicated in other cases of allograft rejection such as renal, cardiac, liver and bone marrow transplants, and in GVHD as well.[73,75,80] The HY response is considered to be an immunologic response of the classical transplantation reaction type in that a second set rejection can be observed to occur in an accelerated fashion following an initial syngeneic male graft rejection.[76] Most relevant to the studies presented here, the HY response was originally characterized as being restricted by immune response genes which were later found to be mapped within the MHC loci and finally to be MHC class I and II molecules.[76,81-84] Among the MHC class I and II restricted HY responses a great degree of strain variation has been noted in mice.[85] This variation in HY responsiveness is partially dependent upon the MHC haplotype; $H2^b$ haplotypes (such as the B6 strain) have been found to be particularly strong responders and within this haplotype

the major $CD8^+$ T cell response is D^b-restricted.[84-86] Only certain $H2^k$ strains are capable of an HY response and most $H2^d$ strains are non- responders to HY antigens; it is important to note however, that these differences in addition to being H2 haplotype dependent, are also reliant upon the type of tissue being transplanted and the route of delivery.[76,82,84-85,87-89] Because of the strong $CD8^+$ T cell restricted HY response known to occur in $H2^b$ strains, many studies have taken advantage of this D^b-restricted response including our own.

H2 D^b HY response

The H2 D^b restricted $CD8^+$ T cell HY response has been the subject of many studies. While the results of these experiments are partially dependent upon the experimental parameters, in addition to the particular $H2^b$ mouse strains used, rejection trends have been noted and confirmed by several later studies. Skin grafting is perhaps the predominant method of allotransplantation used in these studies. It has been found that females reject an initial syngeneic male skin graft approximately 30 days following transplant, while second set skin grafts are rejected within 13-15 days following transplant.[85] The results of pancreatic islet transplant have been more variable.[85,90] Studies evaluating the kinetics of response for both $CD8^+$ and $CD4^+$ T cells of the $H2^b$ strains have revealed T-cell expansion to peak at approximately 14 days following transplantation.[85,91] In a 2001 study, Millrain *et al.* observed T-cell expansion beginning at day 14 following immunization of female recipients with male dendritic cells, and Tyznik *et al.* observed T-cell expansion 9-10 days following immunization with male splenocytes, with a peak of expansion at 14 days.[85,91] These same studies also revealed the D^b-restricted $CD8^+$ T cell response to be dependent upon $CD4^+$ Th cells. Priming CTL responses requires both direct and indirect antigen presentation.[85,89,91] Millrain *et al.* also noted a variation of T-cell response between individual responders of the same strain.[85]

H2 D^b epitopes

Two D^b restricted epitopes have been identified to date and are known as Uty and Smcy.[10] The *Uty* gene encodes for a protein Uty, from which the immunogenic peptide epitope WMHHNMLDI, designated Uty (ubiquitously transcribed tetratricopeptide repeat gene on the Y chromosome) is derived. The Uty peptide can be correlated with a UTY peptide homologue associated with HLA-B60 and HLA-B8 MHC class I molecules in humans.[10,85] The *Smcy* gene encodes for a Smcy protein from which the peptide KCSRNRQYL referred to as Smcy (selected mouse cDNA on the Y chromosome) is derived; in humans an SMCY homologue is associated with HLA-A2 and HLA-B7 MHC class I molecules.[10] Uty and Smcy are strongly conserved across mammalian species.[92] While these proteins are best

known for their role in allograft rejection, Uty and Smcy proteins normally play a role in transcription regulation. [7] Both Uty and Smcy share homology with homologues encoded by the X chromosome that escape x-inactivation; Utx and Smcx respectively.[7,73] Smcy was isolated in 1994 and shares 80% homology with retinoblastoma binding protein 2 (RBP2);[7,93] RBP2 along with the X chromosome homologue Smcx are histone demethylases specific for lysine 4 of histone 3(H3K4) that act as transcription factors during Hox gene regulation, cellular differentiation and development. [94] Uty was isolated in 1996 and shares 84% homology with Utx, which is a transcription factor responsible for demethylation of lysine 27 on histone 3 (H3K27).[95-96]

Tolerance induction within HY antigen system

Tolerance, which is defined in this setting as the lack of T-cell response to allopeptides, has been suggested to be more readily achieved in an MiHC level mismatch model of allograft rejection, compared to a major mismatch, based on the observation that a smaller dose of immunosuppressive agents are generally needed to maintain solid organs differing at the MiHC level. [71] Furthermore, as the number of mHags differing between host and recipient decreases, tolerance is more likely. Thus tolerance induction has been well explored in the limited confines of the HY antigen system with a fair amount of success. Major methods of tolerance induction that have been investigated include male antigen exposure during pregnancy or at the neonatal stage, and the administration of soluble HY peptides. Most recently, manipulation of T regulatory cell populations have also been considered as a means of tolerance induction within the HY antigen system.[97]

Studies in the late 1950's and early 1960s by Billingham *et al.* achieved tolerance in the HY antigen system by exposing neonatal female pups to male antigen.[98-99] By injecting neonatal female pups with syngeneic male splenocytes, syngeneic male skin grafts performed after pups had reached maturity were able to survive for more than 100 days following transplantation, compared to 33 day survival on unexposed female mice. This enhanced survival was shown to depend on the number of male splenocytes, exact age of the neonates, and route of delivery.[98-99] Similarly, Weissman *et al.* achieved tolerance to male syngeneic skin grafts following exposure of female neonates to male antigen, and under certain circumstances, was able to transfer this tolerance to other neonatal pups by injecting them with splenocytes from the tolerized mature females.[100] Billingham was then able to prevent syngeneic male skin graft rejection when female recipients had undergone multiple syngeneic pregnancies (multiparous).[101] Building on this study, in 1977, Smith and Powell were able to transfer tolerance by injecting thymus-dependent lymphocytes

(unfractionated splenocytes, splenic T cells, and thymocytes) from multiparous females into naïve female recipients, which could then accept syngeneic male skin grafts.[102] These early tolerance studies were carried out before the epitopes of HY were identified. Since their identification and characterization, more sophisticated studies have taken place using HY peptides, rather than unfractionated male antigen, to induce tolerance.

In 2002, James *et al.* injected female bone marrow derived immature dendritic cells (BMDCs) that had been pulsed with D^b-restricted Uty peptide into female mice of the $H2D^b$ haplotype.[103] Treated female mice were grafted with syngeneic male skin 21 days later; grafts survived for 100 days compared to 45 day survival in control mice. However, when immature BMDCs that were peptide-pulsed with MHC class II restricted HY antigen, Dby, were injected into female recipients, rapid second set skin rejection was observed. Although Dby peptide induced second set skin rejection in the hands of James *et al.*, when Chai *et al.* administered Dby, Uty, or both peptides in soluble form intranasally to female recipients, syngeneic male skin grafts survived indefinitely when transplanted 10 days following treatment with Dby, or with Uty and Dby. Intranasal administration of Uty alone was less effective, but still extended allograft survival compared to PBS treated female recipients.[92] When LPS was combined with Dby peptide during intranasal administration, a second set skin rejection was observed. Both the James and Chai studies were in part taking advantage of tolerance induction when an antigen is presented by immature DCs, as evidenced by the effects of LPS in the Chai study; if DCs are activated this tolerance may be forfeited. HY peptides have the potential to induce tolerance to allograft transplantation, although this appears to be dependent upon the activation status of DCs. While this situation is more easily predicted in laboratory mouse models such strategies prove harmful as a means of tolerance induction in humans, where the immunogenic response to HY peptides may be less predictable.[92,103-104]

In addition to the use of immature DCs, T regulatory cell populations have also been implicated as a means of tolerance induction in the HY antigen system.[97] T regulatory cells comprise an important tolerance induction mechanism currently under investigation in the field of transplantation tolerance. In a recent 2008 study, Yoon *et al.* found that inducing T regulatory cell expansion in female recipients could allow for survival of syngeneic male pancreatic islets and secondary syngeneic male skin grafts.[97] However, these studies do not directly deplete antigen-specific $CD8^+$ effector T cells; they only leave the effector T-cell populations anergic. This anergy is often highly conditional.[105] A safer and more reliable method of tolerance induction would be to selectively eliminate antigen-specific effector T

cells. In this model, pMHC class I cytotoxic tetramers have tremendous potential to induce tolerance by selectively eliminating antigen-specific $CD8^+$ T-cell populations, and, in so doing, induce stable tolerance of male allografts.

2.2 Immunodominance

Immunodominance: a CTL response pattern

Because it is well known that $CD8^+$ T-cell responses are generated in a predictable, limited, and hierarchical fashion, it is conceivable that selective elimination of dominant naïve HY reactive T-cell precursors may also skew or modulate T-cell responses. By this manner, Peptide-MHC class I cytotoxic tetramers can be used to manipulate the immunodominance hierarchies present between the Uty and Smcy epitopes of the HY antigen system. The HY immunodominance hierarchy, specifically among the D^b restricted epitopes, is thus another important aspect of selective deletion via pMHC class I cytotoxic tetramers

When a protein antigen is processed for antigen presentation and results in several different peptide epitopes presented to T cells via MHC molecules, T-cell responses are generally specific for only one or a few of these peptide epitopes.[106] This restriction of the T-cell response occurs with MHC class I and class II restricted T cells and is known as immunodominance.[106] Immundominance of MHC class I restricted T cells is understood to be a common pattern of the CTL response and is believed to occur as an attempt to minimize the diversity of the immune response in an effort to avoid autoimmunity.[107] This pattern of limited specificity of $CD8^+$T cells has been greatly appreciated in the context of viral responses, but is also known to occur in the $CD8^+$ T cell response to mHags.[107-110]

Several studies evaluating the mHags involved in the B6 anti-BALB.B response in mice have expanded the understanding of immunodominance occurring at the MHC class I restricted MiHC level.[111-114] Building on the finding that skin graft rejection at the MiHC level is dominated by two immunodominant epitopes (H60 and H4), and that one antigenic disparity can interfere with rejection of a different antigenic disparity, Wettstein *et al.* performed several studies in the 1980s that confirmed and further characterized the anti-BALB.B immunodominant response.[111-114] In these experiments CXB recombinant inbred strains (CXBRI) were created by inbreeding B6 mice with BALB/C mice to differentially express individual subsets of mHags.[113-114] In these studies, two immunodominant epitopes were identified, and it was further shown that, when these epitopes were eliminated from the B6 anti-BALB.B response, a second order of dominance could be seen.[114] The exact mechanism(s) underlying immunodominance is unknown, but immunodominance most

likely results from a combination of factors. Wettstein *et al.* reasoned that the immunodominance hierarchy observed in their model was a result of differences in antigen presentation; that is, certain epitopes were more successfully presented by APCs than others, and thus better able to activate $CD8^+$ T cells.[114]

Since the work of Wettstein *et al.*, the role of APCs in establishing immunodominance hierarchies has been supported by several other studies. Other factors that have been identified include $CD8^+$ T cell precursor frequency and negative selection within the thymus.[75,107,115-118] Additionally, work by Yin *et al.* has suggested that $CD8^+$T cell immunodominance may also depend upon weak $CD4^+$ T helper epitopes or differences in binding affinity between MHC class I bound peptide epitopes and antigen-specific $CD8^+$ TCRs.[119] The immunodominant epitope generally means the epitope(s) that elicit an antigen-specific T-cell response in a multiple antigenic system, such as exposure to the whole protein antigen.[120] Cryptic peptide epitopes do not induce a T-cell response in the presence of other epitopes, but can effectively stimulate T cells when presented alone.[120] Within the HY antigen system, specifically the D^b restricted model, evidence supports an immunodominance hierarchy between the Uty and Smcy peptide epitopes.[85]

D^b restricted HY immunodominance hierarchy

While immunodominant hierarchies are a well-established feature of polyclonal T-cell responses, the terminology that describes the relative rank of effector population can be less clear. Studies of the D^b-restricted HY antigen system have predominately identified Uty as the immunodominant epitope, and Smcy as the sub-dominant epitope; however there have been studies that suggest otherwise.[91,121] In 2005, a study by Wettstein *et al.* compared the ability of Uty and Smcy peptides to prime female recipients for an HY response when given to mice along with certain adjuvants including lipid A-aqueous formulation and CpG oliognucleotides.[121] In this experiment, 10 days following priming, splenocytes from the female recipients were incubated with Uty or Smcy-pulsed target cells and compared for IFN-γ production by ELISPOT. Wettstein *et al.* found that Smcy could prime a stronger HY response than Uty peptide, and further, that this was due to a difference in residue charge of the peptide tails, Smcy is more positively charged than Uty.[121] This observation may be dependent upon the use of the adjuvants, especially given that, in these experiments, H60, the immunodominant B6 anti-BALB.B epitope, could not elicit IFN-γ production in B6 mice.[121] Smcy was also labeled the immunodominant epitope following kinetics experiments by Tyznik *et al.*, where Smcy specific $CD8^+$ T cells were found to produce more IFN-γ and to exhibit greater lytic activity in an *in vivo* CTL assay than Uty.[91]

Although these two studies found Smcy to elicit the major effector response, a majority of studies comparing the Uty and Smcy D^b restricted $CD8^+$ T cell response indicate Uty as the immunodominant epitope. [56,85-86,90,122]

Early studies using the Smcy-specific HY TCR transgenic mouse line, have implicated Uty as the immunodominant epitope.[56,86,90,122] In 1993, Bassiri *et al.* found that syngeneic male skin grafts were rejected by normal female recipients, but not by HY TCR-tg (Smcy reactive) females, suggesting that such T cells are not important anti-graft effectors.[90] However, an alternative explanation is that CTL responses are disabled by the lack of $CD4^+$T-cell help in the TCR-tg mice. In one study, $CD8^+$ HY tg T cells were found capable of producing IFN-γ by enzyme-linked immunosorbent assay (ELISA), but were unable to lyse male cells *in vitro*, [122] supporting the Bassiri findings. To circumvent the potential need for $CD4^+$ T-cell help in the HY TCR-tg mice, Arsov *et al.* added exogenous IL-2 to their *in vitro* cultures and still found inadequate lysis of male cells, potentially due to the fact that the Smcy TCR had a lower avidity for the antigen-MHC class I complex.[122] This may be a reason for the natural sub-dominant characteristics often described for the Smcy epitope. However, in a later study Maile *et al.* found that HY tg female recipients could reject syngeneic male skin grafts; therefore observations from the Smcy TCR- tg model only weakly supported the immunodominance of Uty at best.[56]

Stronger support was obtained in 1994 when Gavin *et al.*, in seeking to derive the sequence of the D^b restricted HY epitope, screened the D^b library with CTLs specific for the B6-associated HY antigen.[86] Several peptide epitopes were isolated and found to be capable of inducing an HY response in female recipients. This immunogenic degenerate nonamer peptide that was generated by Gavin *et al.* was later found to share homology with the natural peptide, Uty, isolated in 1996.[86,95]

Perhaps the strongest evidence for Uty as the immunodominant epitope in the D^b restricted HY response comes from the 2001 study by Millrain *et al.*.[85] Millrain *et al.* examined many parameters of the D^b restricted HY response in their 2001 study; most notable were their findings concerning the immunodominance hierarchy between the Uty and Smcy peptide epitopes.[89] Using fluorochrome-conjugated tetramers, Millrain *et al.* compared D^b- Uty and -Smcy-specific $CD8^+$ T-cell responses following immunization of female recipient mice with male dendritic cells. Although a first and second set syngeneic male skin graft rejection was observed, as well as the expansion of $CD8^+$ T cell subsets, no clear immunodominance was noted.[85] To identify the immunodominance hierarchy between D^b- Uty- and -Smcy-specific $CD8^+$ T cell populations, Millrain *et al.* performed both

in vivo and *in vitro* comparisons following chronic antigen stimulation. For an *in vivo* comparison, female mice were immunized with male splenocytes, and 18 days later were sub-lethally irradiated and re-constituted with male bone marrow. Five weeks following re-constitution, the female recipients received male skin grafts. After the male skin grafts were rejected, female recipient splenocytes were analyzed via flow cytometry for D^b-Uty and-Smcy specific tetramer-positive populations. This experiment revealed the D^b-Uty specific $CD8^+$ T cell population to expand 3-18-fold over the D^{b-}Smcy-specific $CD8^+$ T-cell population. For an *in vitro* comparison, female mice were immunized with male dendritic cells and given male skin grafts. Following allograft rejection, recipient splenocytes were stimulated *in vitro* with Uty or Smcy peptide, and proliferation was measured by CFSE dilution. The D^b-Uty-specific population was found to undergo more divisions than the D^b-Smcy-specific $CD8^+$ T cell populations. Additionally the D^b/Uty interaction was shown to have a stronger TCR affinity than the D^b/Smcy interaction.[85] It is therefore generally believed that Uty is the immunodominant epitope in the D^b-restricted HY response, and that Smcy is the sub-dominant epitope. It is important to note that Millrain *et al.* only implicated Uty as the immunodominant D^b-restricted HY epitope under cases of chronic antigenic stimulation, and found no difference in responses in a typical anti-HY response.[85] Overall, the hierarchical relationship between D^b-Uty-and-Smcy-specific $CD8^+$ T cells remains ambiguous. Table 2 summarizes studies that have directly or indirectly implicated a D^b-restricted immunodominant epitope in the HY antigen system.

Table 2. Summary of studies implicating Uty or Smcy as the D^b-restricted immunodominant epitope within the HY antigen system.

Year	Immunodominant epitope	Direct or indirect support?	Mouse strain/cell line	Evaluation method	Ref.
1993	Uty	Indirect	B6, HY-TCR	Compared male syngeneic allograft (skin and pancreatic islets) survival between B6 mice and HY TCR mice	90
1994	Uty	Direct	B6, 11P9 and RMA-S lymphoma cell lines.	Screened random peptide libraries for D^b and K^b restricted CTL mimotopes.	86
1997	Uty	Indirect	B6, HY-TCR	Evaluated HY TCR (D^b-Smcy specific) $CD8^+$T-cell functional response through IFN-γ (ELISA), serine-release assay and a chromium release CTL assay.	122
2001	Uty	Direct	B6, $\beta 2_m$-/-B6, RMA-S lymphoma cell line	Compared D^b-Uty and-Smcy specific $CD8^+$T-cell expansion via fluorochrome-conjugated pMHC class I tetramers and CFSE divisions, and TCR binding affinity via flow cytometry.	85
2005	Smcy	Direct	B6, BALB.B	Compared D^b-Uty- and –Smcy specific $CD8^+$T cell IFN-γ production via ELISPOT	121
2007	Smcy	Direct	B6	Compared D^b-Uty and-Smcy specific $CD8^+$T-cell IFN-γ production via intracellular staining, expansion via fluorochrome-conjugated pMHC class I tetramers, and specific lysis via *in vivo* cytotoxic assay.	91

Abbreviations: Uty- ubiquitously transcribed tetratricopeptide repeat gene on the Y chromosome(WMHHNMLDI), Smcy-selected mouse cDNA on the Y chromosome (KCSRNRQYL), B6- C57BL/6 , HY-TCR- C57BL/6-TgN (TcrHY), 11P9-HY-specific CTL line, BALB.B- C.B10-H2b/LiMcdJ, CTL-cytotoxic T lymphocyte, IFN-γ-interferon-gamma, ELISA-enzyme-linked immunosorbent assay, pMHC-peptide-major histocompatibility complex, ELISPOT-enzyme-linked immunosorbent spot and Ref.-Reference.

2.3 Hypothesis and specific aims

Hypothesis

We hypothesized that pMHC class I cytotoxic tetramers could selectively delete, *in vivo*, male-specific $CD8^+$ T cells (Uty and Smcy) in the B6 HY antigen system. We further hypothesized that selective deletion of D^b-Uty and or -Smcy specific $CD8^+$ T cells would 1) inhibit epitope-specific CTL activity, 2) allow for enhanced syngeneic male allograft survival in female recipients and 3) alter the existing immunodominance hierarchy between D^b- Uty and -Smcy-specific $CD8^+$ T cells. The HY antigen system, with its well-characterized, distinct male-specific D^b-restricted $CD8^+$T-cell epitopes, provides an ideal model to test our hypothesis.

Specific aims

To investigate our hypothesis, it was first necessary to characterize the B6 HY antigen system in our own hands. To accomplish this, we sought to determine the kinetics of expansion of both the D^b-Uty and -Smcy specific $CD8^+$ T cells following exposure to male antigen. Additionally, we assessed the functional responses of D^b-Uty and -Smcy specific $CD8^+$ T cells by comparing IFN-γ, TNF-α, granzyme B, perforin, FasL and TRAIL production. Finally because relative naïve precursor frequency has been suggested as an indicator of the $CD8^+$ T cell immunodominance hierarchy, we attempted to quantify the D^b- Uty and -Smcy specific $CD8^+$ T-cell precursor frequency.[123]

After characterizing both the D^b-Uty and -Smcy $CD8^+$ T-cell responses of the B6 HY antigen system, we then turned to our hypothesis. We first compared *in vivo* CTL inhibition of D^b-Uty and -Smcy specific $CD8^+$ T cells following an initial treatment regimen of pMHC class I cytotoxic tetramers, followed by priming of female recipients will male antigen. In these CTL inhibition experiments, we not only evaluated the success of CTL inhibition for each epitope, but we assessed the effect of selective deletion of each epitope-specific T cell population on the number and function of the surviving T cell population. We then tested the effects of deleting both D^b-Uty and -Smcy specific $CD8^+$ T cells in actual allograft survival scenario.

Caveat consideration

A potential caveat to consider in attempts to induct allograft tolerance by selective deletion of donor-specific $CD8^+$ T cells is the possibility of newly emerging thymic emigrants following treatment and transplantation. The danger of donor-specific $CD8^+$ T cells emerging

from the thymus following treatment has been recognized by several investigators of allograft tolerance.[92,124] However, while donor-specific $CD8^+$ thymic emigrants are a valid concern for the induction of allograft tolerance, several studies downplay their potential. These studies, concerning acquired systemic tolerance, suggest that new donor-specific $CD8^+$ thymic emigrants will instead emerge as tolerant cells following treatment and transplantation.

Acquired systemic tolerance, or, the ability of the thymus to recapitulate central tolerance mechanisms such as positive and negative selection following exposure to donor antigen and induce antigen-specific peripheral tolerance, has been well studied as an additional treatment option for allograft rejection.[125] Allopeptides, MHC class I complexed allopeptides and allopeptide-pulsed host lymphoid and myeloid dendritic cells have all been found to induce transplant tolerance when injected intrathymically into recipient animals.[125-128] It has also been suggested that allograft derived antigen shed in the periphery can migrate to the thymus and induce acquired systemic tolerance, possibly through presentation by thymic dendritic cells or thymic epithelial cells.[125,129]

In addition to exposure of the thymus to donor antigen presented by various dendritic cells types and thymic epithelial cells, activated T cells have also been suggested to bring about acquired systemic tolerance through two possible mechanisms.[129] First, it has been found that activated T cells can emigrate to the thymus and that this reentry can affect the induction of acquired systemic tolerance.[130-131] In a 2001 study by Gopinathan *et al.*, *in vivo* activated T cells were confirmed to migrate to the thymus and upon intravenous (IV) administration of allopeptide activated T cells along with transient immunosuppression, permanent cardiac graft survival could be achieved.[131] This tolerance was not observed in rats that received a thymectomy prior to activated T-cell administration; the authors thus concluded that the activated T cells may be responsible for the induction of transplant tolerance in this setting.[131] Donor T cells from the graft may also contribute to tolerance induction, should they too become activated through various stimuli and migrate to the host thymus; indeed Chowdburry *et al.* induced tolerance and acceptance of small bowel and cardiac transplants in rats by intrathymically injecting donor T cells into the recipient rats along with total body irradiation prior to transplantation.[132] Chowdhurry postulated that presentation of antigen (donor T cells) to developing thymocytes can lead to a reemergence of antigen-specific tolerant T cell clones.[132] Both donor-specific recipient T cells and donor T cells have therefore been found to contribute to the establishment of acquired systemic tolerance in a transplant setting.

The necessity of the thymus in achieving acquired systemic tolerance is critical when considering the potential outcomes following transplantation. These experiments taken together suggest the emergence of tolerant $CD8^+$ thymic emigrants and do not warrant concerns regarding the potential dangers $CD8^+$ thymic effector cells. However, if long-term graft survival is the goal, and the thymus is understood to be critical for support of a peripheral state of tolerance following the removal of donor-specific $CD8^+$ T cells, would there not be negative consequences as the recipient begins to age and the thymus to involute?[133] Oluwole *et al.* tested this hypothesis within their intrathymic MHC class I peptide exposure model and found that removing the thymus after 3 weeks of tolerance establishment did not reverse the acquired systemic tolerant state; the thymus in this scenario can therefore be considered necessary for the establishment of tolerance but not for tolerance maintenance.[134] Selective deletion of donor-specific $CD8^+$ T cells via pMHC class I cytotoxic tetramers could thus be used to inhibit specific CTL activity and thereby prolong allograft survival. The inevitable reemergence of $CD8^+$ thymic emigrants can be predicted to aid the in the establishment of allograft tolerance and not allograft rejection.

Chapter 3: Peptide-MHC class I cytotoxic tetramers inhibit specific CTL activity and alter existing immunodominance hierarchies

3.1 Introduction

Peptide-MHC class I cytotoxic tetramers are soluble multimers consisting of four peptide-loaded MHC class I molecules biotinylated and bound together by toxin-coupled streptavidin.[54,68] Peptide-MHC class I tetramers coupled to saporin, a type I ribosome-inactivating protein, cause rapid cell death upon lysomal degradation within the target $CD8^+$ T cell. [68] In mice, cytotoxic tetramers have been used *in vivo* and *in vitro* to selectively delete $CD8^+$ T-cell populations that mediate β-cell destruction and other forms of $CD8^+$ T cell-mediated tissue damage; this antigen-specific $CD8^+$ T-cell depletion was also found to inhibit CTL activity and to prevent or delay respective disease progression.[67,69]

Antigen-specific $CD8^+$ T cells play a major effector role in allograft rejection; the selective deletion of donor-specific $CD8^+$ T cells could contribute to allograft survival without the adverse effects of immunosuppressive treatment.[11,46] *Ex vivo* MHC tetramer mediated depletion of donor allogeneic T cells has been used to prevent graft-versus -host disease with variable results, but cytotoxic tetramer technology has not yet been applied to a transplantation setting towards the enhancement of allograft survival.[64-65]

Working in the well characterized murine HY antigen system as a model for allograft rejection at the minor histocompatibility level, we have used pMHC class I cytotoxic tetramers to selectively delete donor-specific $CD8^+$T cell populations *in vivo*, resulting in the inhibition of epitope-specific CTL activity. Furthermore, we have found that cytotoxic tetramers are capable of altering the immunodominance hierarchy between the two major D^b restricted epitopes, Uty and Smcy.

3.2 Materials and methods

Mice

C57BL/6J (B6) and B6.PL-*Thy1*^{a}/CyJ (Thy1.1+ B6) mice were purchased from The Jackson Laboratory. B6.D2TgN(*Tcr-Lcmv*)327Sdz/Fre (P14),[135] *Yeti,* [136] and C57BL/6-Tg(H2K^{b}-GFP)/Fre(GFP)[137]mice were bred in house. All mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited specific pathogen-free laboratory animal facility. The mice were typically used at 6 to 8 weeks of age in experiments approved by the Institutional Animal Care and Use Committee (IACUC) of The University of North Carolina at Chapel Hill (UNC-CH).

Magnetic-bead enrichment

Magnetic-bead enrichment was performed as previously described.[68] Briefly, spleens from donor B6 or P14 mice were aseptically harvested into PBS containing fetal bovine serum (FBS) at 0.5%. Following disaggregation, splenocytes were erythrocyte depleted using ammonium chloride lysis buffer (0.15 molar (M) NH_4Cl_4, 1 millimolar (mM) $KHCO_3$, and 0.1mM Na_2 ethylenediaminetetraacetic acid(EDTA), and strained with a 40 micrometer (μm) nylon cell strainer (BD Biosciences, Franklin Lakes, NJ). B6 splenocytes were B220 depleted by negative selection using anti- phycoerythrin (PE) microbeads over a quadroMACS magnetic separator (Miltenyi Biotec, Auburn, CA), following staining with anti-B220 (RA3-6B2)-PE (BD Biosciences). P14 splenocytes were enriched for CD8^{+} T cells via negative selection using a CD8^{+}T-cell isolation microbead cocktail and the quadroMACS magnetic separator (Miltenyi Biotec).

Immunization

Female mice received an erythrocyte-depleted, single-cell suspension of fresh syngeneic male cells in 200 microliters (μL) of sterile PBS (B6 bone marrow [(BM)] or B220-depleted splenocytes) intraperitoneally (IP) or IV via the lateral tail vein.

Peptide and tetramer preparation

As previously described, H2-D^{b}-restricted pMHC class I tetramers were produced to present the following peptides: Uty (WMHHNMLDI), Smcy (KCSRNRQYL), and the gp33 altered peptide ligand C9M (KAVYNFATM).[68] Streptavidin (SA)-saporin (SAP) (Advanced Targeting

Systems, San Diego, CA), phycoerythrin (PE) or allophycocyanin (APC)-conjugated streptavidin (Leinco Technologies, St Louis, MO) were added also as previously described.[68]

Peptide-MHC class I cytotoxic tetramer administration

Mice received 2 doses (33 pmol each) of pMHC class I cytotoxic tetramer 5-6 days apart via the lateral tail vein.

LCMV infection

A B6 female mouse was infected IP with 20,000 plaque forming units (pfu) of LCMV-Armstrong in 200μL sterile PBS 7 days prior to spleen harvest.

Peripheral blood lymphocyte acquisition

Venous blood was collected via submandibular bleeding using a 5 milimeter (mm) Goldenrod lancet (MEDIpoint Inc, Mineola, NY). Blood was collected in PBS containing 4mM EDTA, layered over Histopaque-1083 (Sigma-Aldrich, St. Louis, MO) and centrifuged at 1800 rotations per minute (rpm) for 20 minutes. The interface was collected in fluorescence-activated cell sorter buffer (2% FBS and 0.1% NaN_3 in PBS) (FacsWash) and stained with fluorochrome-conjugated tetramers or monoclonal antibodies (mAbs) and analyzed via flow cytometry.

Flow cytometry

Single-cell suspensions were stained with fluorochrome-conjugated mAbs or tetramers in Facs Wash for 45 minutes at 4°C in 96 well round bottom (RB) plates, following blocking with Fc Block (anti-mouse CD16/CD32 [eBioscience, San Diego, CA]). For intracellular IFN-γ and TNF-α staining, splenocytes were incubated in 96 well flat bottom plates, at 37°C in RPMI 1640 medium supplemented with 10% FBS $5x10^{-5}$M 2-mercaptoethanol, 2mM L-glutamine, 100μg/mL streptomycin, and 100IU/mL penicillin (R-10) with fluorochrome-conjugated tetramer (5 hours); brefeldin A (eBioscience, San Diego, CA) was added after the first hour. Cell permeabilization and staining were performed according to the Cytofix/Cytoperm manufacturer's protocol (BD Biosciences). For granzyme B intracellular staining, fluorochrome-conjugated tetramer and surface marker staining was carried out at 4°C for 45 minutes in Facs Wash. Following surface staining, cells were then permeabilized and subjected to intracellular staining (Cytofix/Cytoperm, BD Biosciences). For *in vivo* CTL experiments, syngeneic female B6 splenocytes were peptide-pulsed with 10μg/mL of Uty, Smcy, both peptides or left unpulsed for 1 hour at 37°C in sterile R-10. Peptide-pulsed

target cells were then labeled with Pacific Blue succinimidyl ester (PBSE) (~30 micromolar [μM]) and with differing concentrations of CFSE (0, 0.05, 0.5 or 2.5μM) (Invitrogen, Carlsbad, CA) for 10 minutes at 22°C followed by FBS quenching. Labeled, pulsed targets were adoptively transferred into B6 female recipients IV after mixing in a 1:1:1:1 ratio. For analysis via flow cytometry, cells were typically fixed in FacsWash containing 1% buffered formaldehyde (FacsFix). The data were collected with a FACSCalibur (BD Biosciences) or CyAn ADP cytometer (Beckman Coulter, Brea, CA) and analyzed with Summit software (version 4.3, Dako, Carpinteria, CA). The following fluorochrome-conjugated mAbs (Ebioscience, Invitrogen, or Biolegend, San Diego, CA) were used: anti-CD8α (53-6.7), anti-CD19 (eBioID3 or 6D5), anti-CD4 (GK1.5), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-human/anti-mouse GrB (GRB05), anti-IFNγ (XMG1.2), anti-TCR Cβ (H57), anti-TNF-α (MP6-XT22), anti-Thy1.1 (H1551 or OX-7), and anti-Thy1.2 (53-2.1).

Adoptive transfer

Thy1.2^{+} B6 female mice received ATs of male and female splenocytes IV via the lateral tail vein. Splenocytes from Thy1.1^{+} B6 and GFP mice of opposite genders were harvested and erythrocyte-depleted as described earlier. Splenocytes were then re-suspended at a 1:1 ratio in 200μL of sterile PBS at varying cellular concentrations.

Statistical analysis

Data were analyzed with GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). Difference in means of T-cell population numbers were calculated by a 2-tailed *t* test. Statistical significance was set at a *P* value of less than .05.

3.3 Results and discussion

Characterization of anti-HY response

Within the HY antigen system, the two dominant D^{b}-restricted T-cell epitopes are Uty and Smcy.[10] While Uty is generally considered the immunodominant of the two, [85-86] other studies have alternatively implicated Smcy.[91,121] Because our objective was to deplete the major HY-reactive effector population, using the smallest doses of cytotoxic tetramer, we first sought to compare the strength of both T-cell responses. As a first step, we sought to

find an efficient immunization protocol. Earlier experiments performed in the lab showed that administration of male splenocytes yielded variable priming, and thus we evaluated other preparations of male cells. Splenocytes depleted of B220-positive cells were considered a potentially strongly immunogenic candidate, as B cells can turn off naïve T cells, and in the HY antigen system, have been shown to induce long-lasting tolerance to male skin grafts. [138] In another study, male BM was shown to be the most effective tissue immunogen, compared to DCs and spelnocytes.[85] Accordingly, we immunized female B6 mice with male BM or B220-dpeleted splenocytes, and found that the former preparation gave the most consistent responses (Figure 1Ai); thus, we used BM cells in all subsequent experiments described below.

Following immunization, we observed the numbers of circulating D^b-Smcy and –Uty-specific $CD8^+$ T cells to peak at 14 and 20 days respectively. Although Uty-specific $CD8^+$ T cells expanded more slowly, the overall population reached significantly greater numbers 20 days after immunization, and beyond (Figure 1Aii-iii). These observations differed from the results of previous anti-HY kinetics studies, which found both epitope-specific populations to peak at 14-15 days after exposure to male antigen. Tyznik *et al.* also reported that a greater number of Smcy specific $CD8^+$ T cells could be detected at day 15 post-immunization (p.i.). [85,91] We then compared the $CD8^+$ T-cell functional responses to both epitopes within the same mouse. We chose 14 days following immunization as the time point for this comparison, as both epitope-specific $CD8^+$ T cell responses were measurable and the population sizes were not significantly different at this time. We found that both T-cell populations produced similar levels of granzyme B, TNF-α and IFN-γ (Figure 1B-D). D^b-Uty and D^b-Smcy specific $CD8^+$ T cells were also compared for their production of perforin, FasL, and TRAIL through surface and intracellular staining. Despite multiple attempts we were unable to detect perforin, FasL or TRAIL production in our model system (data not shown). However, despite the lack of significant difference in functional molecule production, we have generally found D^b-Uty-specific $CD8^+$ T cells more efficiently eliminate peptide-pulsed target cells in our *in vivo* CTL assays (See Figure 3, below).

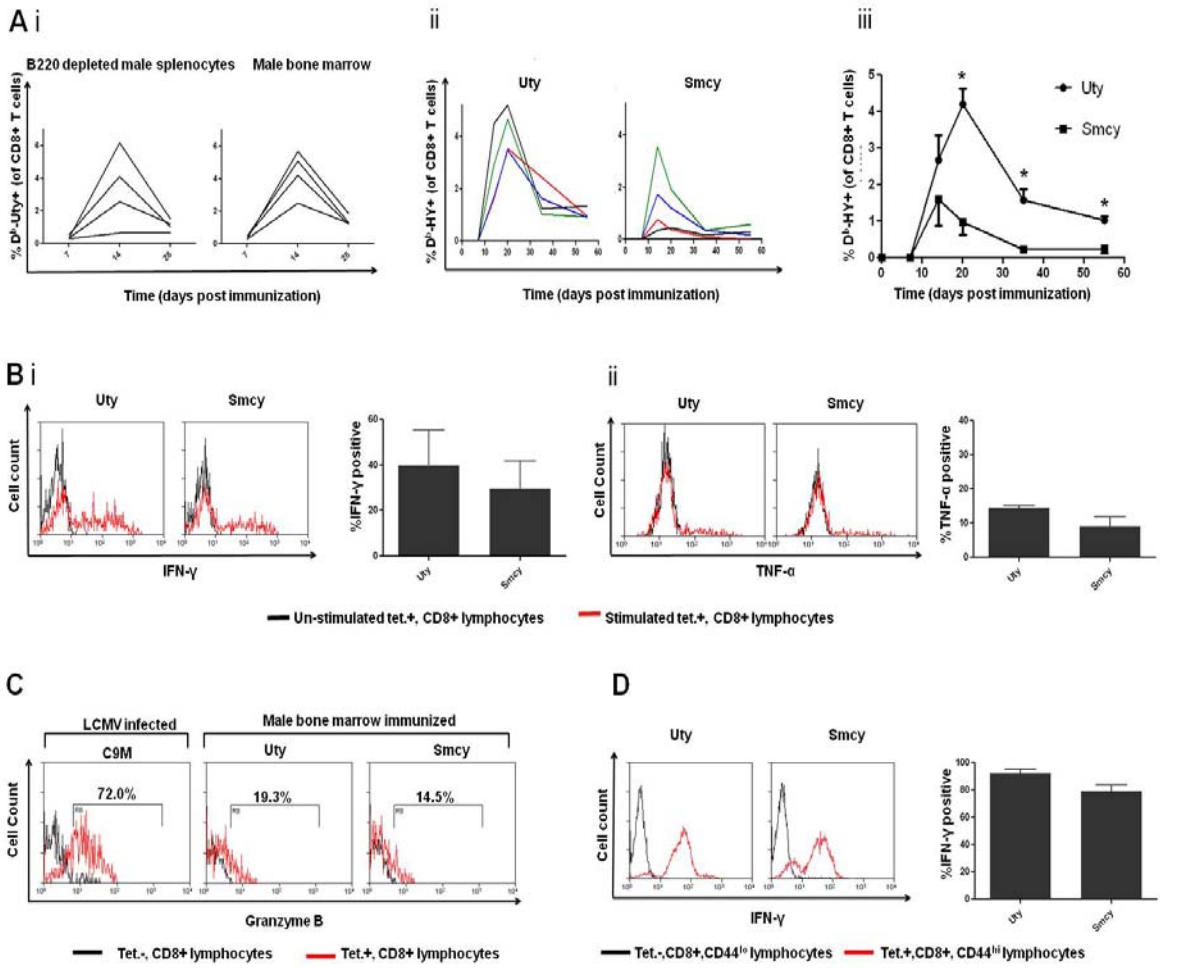

Figure 1. D^b-Uty and –Smcy –specific $CD8^+$ T cells differ in their kinetics of expansion but exhibit similar functional responses. (Ai) Female B6 mice PBLs were compared for D^b-Uty-specific $CD8^+$T-cell population number by fluorochrome-conjugated tetramer staining at various time points following IP immunization with $5x10^6$ syngeneic male BM or $5x10^6$ B220 depleted male splenocytes. BM immunization provided the most consistent anti-HY response. (Aii) T-cell populations were longitudinally compared following BM immunization, by fluorochrome-conjugated tetramer staining of PBLs; each color represents an individual mouse. (Aiii) Population numbers significantly differed at days 20 ,35, and 55 with P-values of 0.0094, 0.0348 and 0.0162 respectively, n=4. (B-C) T-cell populations were compared for IFN-γ (Bi), and TNF-α (Bii) and granzyme B (C) production by intracellular staining of splenocytes 14 days following immunization. D^b-C9M-specific $CD8^+$ T cell production 7 days post infection (B6 female) with LCMV served as a positive control. (D) IFN-γ production was also compared 14 days p.i. by surface staining B6 yeti reporter mice splenocytes, whose cells fluoresce in the fluorescein channel upon production of IFN-γ. Functional response experiments were n=2. Error bars represent SEM.

Treatment with cytotoxic tetramers

As a means of further evaluating the individual contributions of D^b-Uty and –Smcy specific $CD8^+$ T cells to the anti-HY response, we employed an *in vivo* CTL assay. By this method we were able to compare the survival of different peptide-pulsed female target cells 18-24 hours later in the spleens of syngeneic female recipients (Figure 2Ai). We first confirmed that Uty, Smcy, or Uty & Smcy peptide-pulsed targets were diminished following adoptive transfer into immunized female recipients compared to naïve females (Figure 2Aii). Supporting D^b-Uty as the epitope eliciting the major HY response, Uty and Uty & Smcy peptide-pulsed targets were eliminated to the greatest extent (Figure 2B). It was apparent that this assay would be useful to evaluate the efficiency of cytotoxic tetramers in selectively deleting either the Uty or Smcy-reactive T-cell population. Moreover, this depletion could provide insight into the physiological relationship between the two epitopes.

We hypothesized that pre-treatment with D^b-Uty or -Smcy cytotoxic tetramers prior to immunization would inhibit antigen-specific CTL activity and allow for selective target survival within our *in vivo* CTL assay. Cytotoxic tetramers were administered in 2 doses, 5-6 days apart. This dosing interval was selected based on the finding that naïve $CD8^+$ T cells transiently lose tetramer binding ability following activation;[139] and our own experiments that showed tetramer binding by the TCR was largely restored by 5 days after tetramer administration (Figure 3). As expected, cytotoxic tetramer pre-treatment was capable of enhancing male-peptide pulsed target cell survival in an epitope specific manner. The target recovery results along with tetramer staining highlight the ability of antigen-specific cytotoxic tetramers to selectively delete D^b-Uty or –Smcy specific $CD8^+$ T cells and allow for the survival of their respective cognate peptide-pulsed target cells *in vivo* (Figure 2B-C).

Also, as hypothesized, pMHC class I cytotoxic tetramer treatment did not damage a bystander $CD8^+$T cell population. In fact, removal of D^b-Uty-specific $CD8^+$ T cells resulted in an enhanced D^b-Smcy-specific population, and further decreased Smcy-pulsed target cell survival (Figure 2 B-C). These results, along with our earlier characterization comparisons, suggest that under normal physiological circumstances D^b-Uty-specific $CD8^+$ T cells suppress D^b-Smcy-specific $CD8^+$ T cells. [123] Because Uty and Smcy-reactive T cells cannot compete for killing of Smcy peptide-pulsed targets, the enhanced killing of these cells with D^b-Uty-SAP treatment implies that the normal inhibition of Smcy-reactive $CD8^+$T cells by Uty-reactive $CD8^+$T cells occurs during the priming phases. Potential mechanisms for this suppression might include competition for antigen or antigen-presenting cells. Our cytotoxic tetramer

findings thus reveal a promising option for the induction of allograft tolerance and may prove beneficial to other scenarios of $CD8^+$ T cell-mediated damage, such as autoimmune and virally induced disease states. Additionally, when combined with our *in vivo* CTL assay, cytotoxic tetramers become a valuable new tool for probing the precarious interplay between epitope-specific $CD8^+$T cell populations that results in the phenomenon of immunodominance.

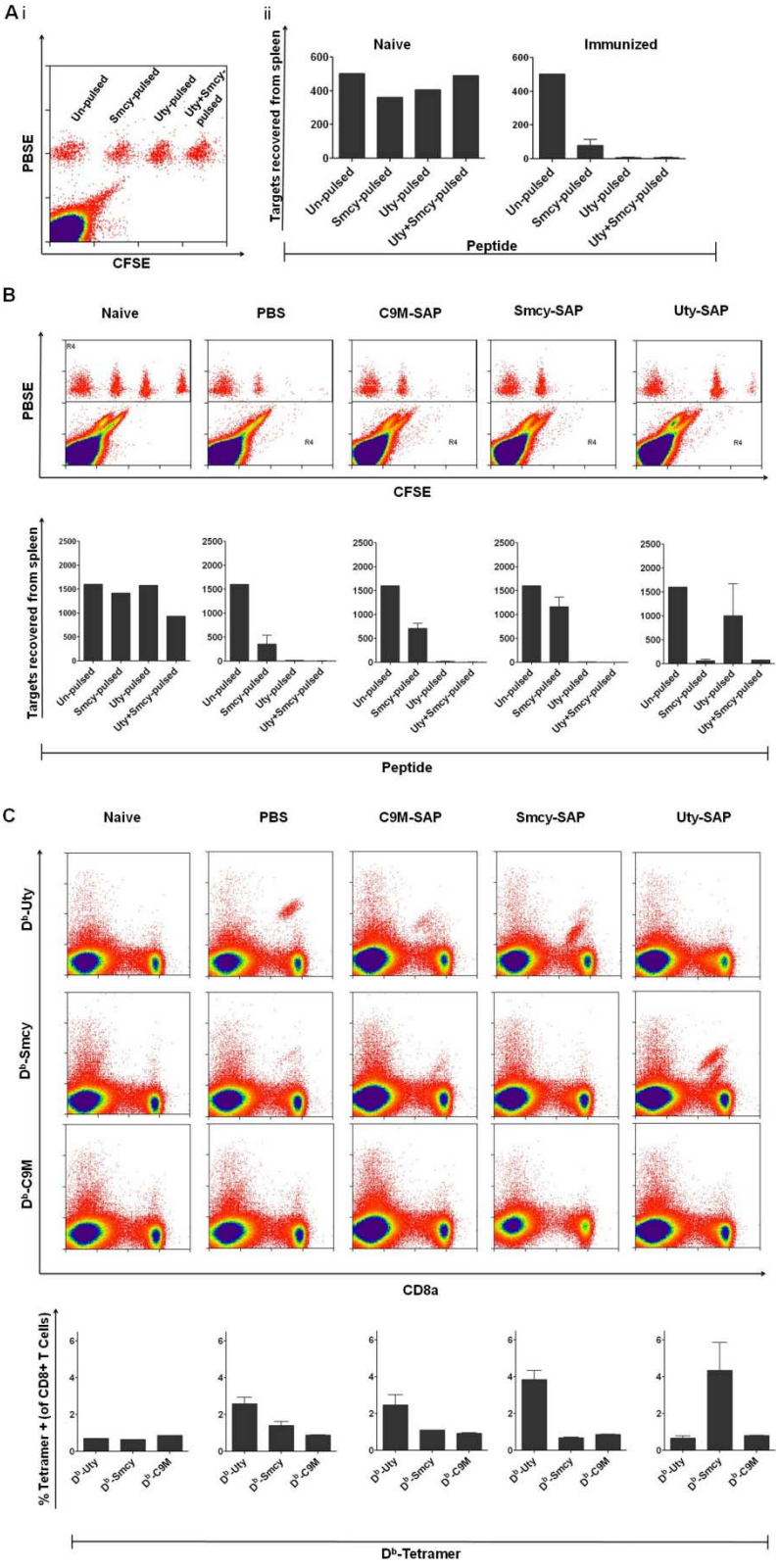

Figure 2. Cytotoxic tetramers inhibit specific CTL and alter the existing immunodominance hierarchy. In the *in vivo* CTL assay, different peptide-pulsed syngeneic female target cells were recovered 18-24 hours later in the spleens of female recipients. (Ai) Target populations were distinguished from one another by differential PBSE and CFSE labeling concentrations. (Aii) Male peptide-pulsed target cells were diminished following transfer into immunized females (14 days p.i.) compared to transfer into naïve females; by this model D^b-Uty-specific $CD8^+$ T cells display stronger CTL activity than D^b-Smcy specific $CD8^+$ T cells. (B) Pre-treatment with cytotoxic tetramers inhibit specific CTL activity, allowed for increased survival of cognate peptide-pulsed targets. Specific inhibition of D^b-Uty specific CTL also resulted in increased D^b-Smcy-specific CTL activity. D^b-C9M-cytotoxic tetramers served as control for specificity. (C) Staining splenocytes from different treatment groups with tetramer conjugated to fluorophore confirmed the results of the *in vivo* CTL assay. Cytotoxic tetramers specifically reduced D^b-Uty or –Smcy-specific $CD8^+$T-cell populations. Consistent with target recovery results, reduction of D^b-Uty-specific $CD8^+$ T cells coincided with a rise in D^b-Smcy-specific $CD8^+$T cells. Target recovery numbers were normalized to unpulsed targets recovered. Each experiment used 2 or 3 mice per group. Error bars represent SEM. Data exclude 1 out of the 3 D^b-Uty-cytotoxic tetramer treated mice due to treatment failure. Uty-Sap: D^b-Uty-cytotoxic tetramer, Smcy-SAP: D^b-Smcy-cytotoxic tetramer, and C9M-SAP: D^b-C9M-cytotoxic tetramer.

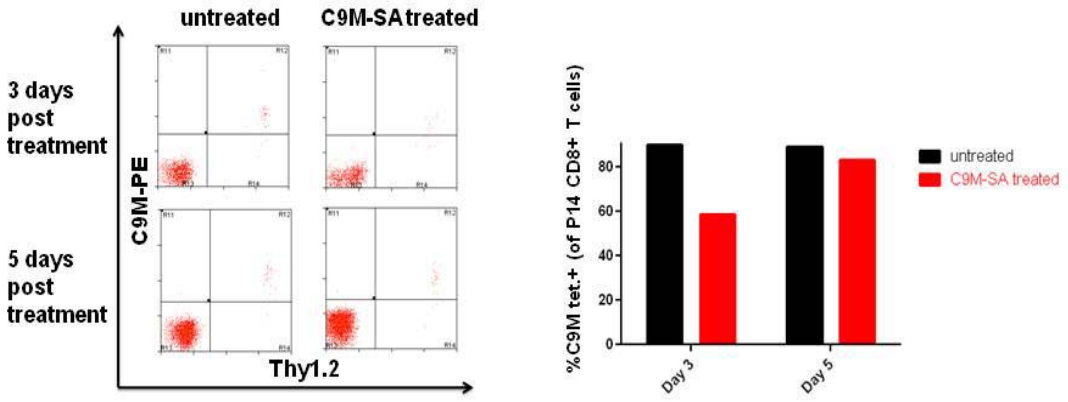

Figure 3. CD8$^+$ T cells regain MHC class I tetramer binding ability 5 days following exposure to tetramer. 2 B6 Thy1.1 female mice received 5x10^6 Thy1.2 P14 CD8$^+$ T cells (splenocytes purified via magnetic-bead enrichment) IV. P14 CD8$^+$ T cells recognize the gp33 epitope. The following day, one mouse received 5µg D^b-C9M tetramer IV and the other mouse remained untreated. PBLs were then collected from both mice 3 and 5 days following tetramer administration. Percentages of Thy1.2$^+$, D^b-C9M-specific CD8$^+$ T cells were then compared between the mice following staining with fluorochrome-conjugated D^b-C9M specific tetramer and flow cytometry analysis. At day 5, tetramer exposed P14 T cells achieved tetramer binding capability equivalent to that of unexposed P14 T cells. Histograms gated on CD8$^+$T lymphocytes

Adoptive transfer of male cells

After treatment with individual pMHC class I cytotoxic tetramers was found to enhance peptide-pulsed target survival, we next asked whether treatment with both D^b-Uty and –Smcy specific cytotoxic tetramers could enhance syngeneic male cell survival when adoptively transferred into female recipients. For the first attempt, B6 Thy1.1$^+$ male splenocytes and GFP$^+$ female B6 splenocytes were adoptively transferred into B6 female recipients that were pre-treated with both D^b-Uty and-Smcy specific cytotoxic tetramers. Female splenocytes were transferred along with male cells as a negative control for rejection; a male B6 recipient was also included as a negative control. The male: female target ratio was monitored longitudinally by analysis of PBLs via flow cytometry. In this particular experiment, although the male: female target ratio did decrease over time, male target cells were not completely eliminated, which cannot be readily explained. Also, while there was a slight difference in ratio rate of decrease between the tetramer and cytotoxic tetramer treated mice and the PBS control mice, treated mice exhibited similar rates of ratio decrease (Figure 4A).

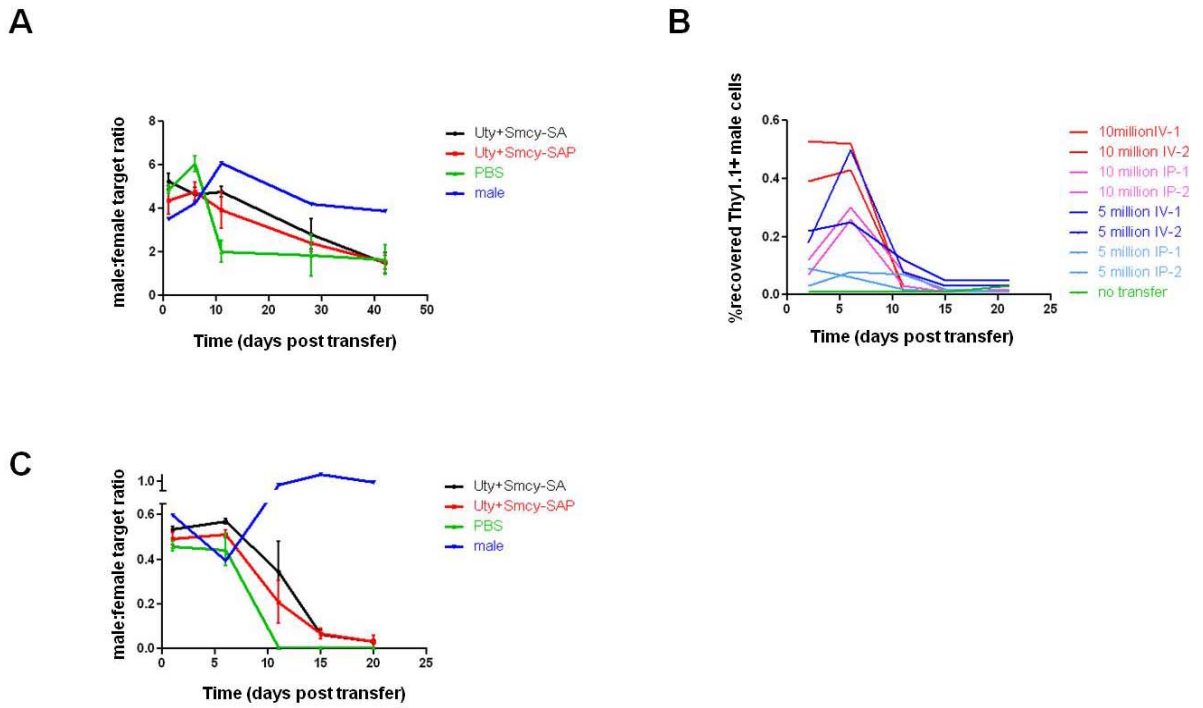

Figure 4. Pre-treatment with combined D^b-Uty and-Smcy cytotoxic tetramers delays but does not prevent rejection of syngeneic male cells. (A) Female B6 mice received a 1:1 ratio of male Thy1.1+ splenocytes and female GFP+ splenocytes at ~$5x10^6$ cells each, IP. Recipients (n=3/group) were treated with cytotoxic (Uty+ Smcy-SAP) or non-cytotoxic (Uty+Smcy-SA) tetramers 8 and 3 days prior to adoptive transfer. Other recipients remained untreated (PBS). The recovery ratio of male: female targets was then calculated through analysis of PBLs via flow cytometry. (B) A comparison of $10x10^6$ and $5x10^6$ male Thy1.1+ splenocytes via an IP or IV route of transfer revealed IV transfer of $10x10^6$ cells induced a more readily detectable rejection response compared to the other routes evaluated. Each color represents an individual mouse. (C) Pre-treatment with cytotoxic and non-cytotoxic tetramers (n=3/group), did not however prevent rejection of male splenocytes when transferred at a 1:1 ratio with female splenocytes, IV at $10x10^6$ cells each. (A and C) Male recipients were included as a positive control for male cell survival. Error bars represent SEM.

Because the efficiency for priming an anti-male response within the HY antigen system has been shown to be affected by the route of antigen presentation (priming via direct antigen presentation or cross-priming via indirect antigen presentation)[85,89], we reasoned that an alternate antigen presenting route or parameter would possibly provide a more robust response and more interpretable results. We therefore compared IP and IV (lateral tail vein) adoptive transfers of 5 million or 10 million male splenocytes each. We found that 10 million male cells transferred IV induced the most rapid and measurable rejection response (Figure 4B). Using this result, we then sought to again determine whether treatment with HY-reactive cytotoxic tetramers could prolong the survival of male cells. To investigate this, we transferred 10 million male and female splenocytes IV into

female recipients, once again pre-treated with both cytotoxic tetramers, and monitored the male: female target ratio. Transferring 10 million male and female cells did indeed yield a clear elimination of male cells compared to female cells, however, tetramer-treated control mice and cytotoxic tetramer-treated mice continued to demonstrate similar rates of ratio decrease, rates that were only slightly less than the PBS-treated control mice. Furthermore, all treatment regimens resulted in the eventual rejection of male cells (Figure 4C).

There are several reasons that could account for the inability of cytotoxic tetramers to prolong allograft survival within this experimental setting. A CTL response against GFP has been reported in mice, including the C57BL/B6 strain, however, an anti-GFP $CD8^+$ T cell response is not likely to be the reason for the lack of cytotoxic tetramer protection resulting in the rejection of male splenocytes. When $Thy1.2^+GFP^+$ female splenocytes and $Thy1.1^+$male splenocytes were transferred into cytotoxic tetramer treated female recipients, the GFP^+ female splenocytes were not rejected; only the $Thy1.1^+$ male cell counts decreased (Figure 4A). A more plausible explanation could be the contribution of non-H2 alloantibodies specific for HY antigens; an anti-HY antibody response has in fact been found to develop in human female patients receiving male kidney transplants.[140] In addition to anti-HY alloantibodies, it is also possible that in the absence of D^b-Uty and –Smcy specific $CD8^+$ T cells, $CD8^+$ T cells specific for currently unknown D^b or K^b restricted subdominant epitopes may arise. Another possible explanation is that $CD4^+$ T cells are also playing a part in the rejection of the male target cells in our model. In support of this hypothesis, within the B6 strain, $CD4^+$ T cells specific for the I-A^b restricted HY antigen, Dby, have been found to contribute to allograft rejection through specific lysis of Dby-pulsed target cells, as well as male target cells.[91,141]

Conclusions

Without additional experimentation it is difficult to pinpoint the exact effectors involved in the rejection of male target cells in this model system. It may be that pMHC class I cytotoxic tetramers need to be administered along with pMHC class II cytotoxic tetramers specific for Dby. Additionally, if subdominant anti-HY $CD8^+$ T cell epitopes are involved their identification would be necessary and a multiple-cytotoxic tetramer treatment method using all relevant epitopes should be employed. Nevertheless, we were able to achieve specific CTL inhibition through the use of cytotoxic tetramers and this has tremendous potential towards the mitigation of allograft rejection.

Chapter 4: Naïve CTL precursor frequency estimation within the HY antigen system

4.1 Introduction

Our studies in the HY minor histocompatibility model compared and manipulated the immunodominance hierarchy between D^b-restricted Uty- and -Smcy specific $CD8^+$ T cells through pMHC class I tetramer technology. To further characterize the D^b-Uty and Smcy $CD8^+$ T cell responses and to more definitively identify an immunodominant epitope, naïve D^b-Uty and –Smcy $CD8^+$ T cell populations should be enumerated. In fact, naïve CTL precursor frequencies have been suggested to act along with MHC class I binding affinity to shape $CD8^+$ T cell immunodominance.[123,142]

The diversity of the T-cell repertoire primarily results from TCR α and β chain genetic recombination that occurs by V, D and J region rearrangement resulting in a variable complementarity-determining region, CDR3.[143-144] The T-cell repertoire has been theoretically proposed to consist of ~10^{15} different TCR combinations[145], while the mouse spleen has been estimated to actually contain only $2x10^6$ distinct TCR combinations.[144] Several attempts to enumerate antigen-specific T-cell populations in the naïve mouse have been made over the past 20 years. The earliest studies that employed indirect methods, such as limiting dilution assays (LDAs), estimated frequencies of several virus-specific CTL precursors at 1 in 100,000 $CD8^+$ T cells.[146-147] Given the fact that an average mouse is considered to have $2x10^7$ $CD8^+$ T cells, these early studies enumerated splenic naïve $CD8^+$ T-cell populations that were estimated to be ~ 200 cells per T-cell population.[146-148] Additional indirect methods include analysis of TCR β chain repertoire diversity through various CDR3 sequencing techniques, which have collectively found naïve antigen-specific T-cell populations ranging from 10-1500 T cells per population,[143-144,149-151] and the titration of adoptively transferred TCR transgenic T cells, which has yielded estimates from 100-3,000 T cells per population.[152-154] Most recently, naïve T-cell precursor frequencies have been counted through a combination of fluorochrome-conjugated pMHC class I or II tetramer staining and magnetic-bead enrichment to range from 15- 1070 T cells per population (pioneered by Moon *et al.*).[123,142,155-156]

One study by Obar *et al.* directly estimated naïve $CD8^+$ T-cell precursor frequencies for several D^b-or K^b- restricted viral epitopes.[142] Through the use of magnetic-bead enrichment following staining with dual fluorochrome-conjugated pMHC class I tetramers of

the same specificity, Obar *et al.* found that various CTL precursor populations ranged from 120-603 $CD8^+$ T cells in a B6 mouse.[142] As an indirect means of establishing that the rare, tetramer-stained populations did not consist entirely of artifactual events, Obar demonstrated that no naïve ovalbumin (OVA)-reactive T cells could be isolated from mice that exhibit central deletion of these T cells due to transgenic expression of OVA. In our study, we applied the method of Obar *et al.* to the HY antigen system to investigate the relative numbers of Uty-reactive precursors. Because the fluorescence of D^b-Uty-stained populations is low compared to background noise, modifications of the original protocol, as well as development of our own method, proved necessary. While we were indeed able to use these techniques to isolate Uty-specific effector/memory cells in pilot experiments, we were ultimately unable to visualize the naïve counterparts of these populations using these strategies. Given this limitation, we will again use the sequence diversity of TCR Vβ CDR3 loops in sorted Uty-and Smcy-reactive CTL to estimate the differences in precursor frequency between these T-cell populations

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4.2 Materials and methods

Mice

B6 mice were obtained from The Jackson Laboratory. The mice were housed in an AAALAC accredited specific pathogen-free laboratory animal facility and were typically used at 6 to 8 weeks of age in experiments approved by the IACUC of UNC-CH.

Magnetic-bead enrichment

Magnetic-bead enrichment was performed as described in Chapter 3 with the following exception: in these experiments, enrichment with anti-PE microbeads (Miltenyi Inc.) was carried out to achieve positive selection of tetramer-PE labeled splenocytes. $CD8^+$ T cells were enriched from splenocytes and lymph nodes including: popliteal, periaortic, mesenteric, and superficial lymph nodes. Tissue was disaggregated, erythrocyte-depleted, and cell-strained as previously described in Chapter 3.

Immunization

Female mice received an erythrocyte-depleted, single-cell suspension of fresh syngeneic male cells in 200µL of sterile PBS (B6 BM or splenocytes) IP.

PBL acquisition

Venous blood was collected and PBLs analyzed via flow cytometry as described in Chapter 3.

Cell staining and tetramer preparation

H2-D^b-restricted pMHC class I tetramers were produced to present the following peptides: Uty (WMHHNMLDI), Smcy (KCSRNRQYL), and the gp33 altered peptide ligand C9M; tetramers were then combined with PE, Alexa Fluor 647 (AF647), or APC-conjugated streptavidin as previously described in the literature.[68] Single-cell suspensions were stained with fluorochrome-conjugated mAbs or pMHC class I tetramers in 5 mL RB polypropylene tubes or in 96-well RB plates, in FacsWash or R-10 in the presence of dasatinib (LC Laboratories, Woburn, MA) at a 50 nanomolar (nM) concentration. Single-cell suspensions were stained at varying cellular concentrations at 4°C, or 37°C for time periods ranging from 35 minutes to 1 hour. Cells were incubated with Fc Block (2.4G2 [HB-197^{TM}]: ATCC, Manassas, VA) or anti-mouse CD16/CD32 (eBioscience), at 4°C before or during cell-staining for various time periods. Cells were typically fixed in FacsFix prior to analysis via flow cytometry. The following fluorochrome-conjugated mAbs (eBioscience and Biolegend) were used in these experiments: anti-CD8α (53-6.7), anti-CD19 (eBioID3 or 6D5), anti-CD4 (GK1.5), anti-Thy1.2 (53-2.1), anti-CD11c (N418), anti-F4/80 (BM-8), and anti-CD49b (DX5).

Flow cytometry and cell sorting

Data were acquired using a FACScalibur (BD Bioscience), CyAn ADP Analyzer (Beckman Coulter, Brea, CA), or iCyt Visionary Bioscience Reflection (iCyt, Champaign, IL), and analyzed with Summit (Version 4.3, Dako, Carpinteria, CA) or WinList (Version 6.0, Verity Software House, Topsham, ME). Data were also analyzed using Prism 5.0 (GraphPad Software, San Diego, CA). Cells were sorted using an iCyt Visionary Bioscience Reflection, or a MoFlo cell sorter (Beckman Coulter). Sorted D^b-Uty and –Smcy specific $CD8^+$ effector T cells for 454 sequencing were lysed and stored in TRIzol (Invitrogen) at -80°C.

4.3 Results and discussion

Dual tetramer staining and magnetic-bead enrichment

We began by focusing on the enumeration of naïve D^b-Uty-specific $CD8^+$ T cells. Because we had also observed D^b-Uty-specific $CD8^+$ T cells to reach greater numbers than Smcy-reactive $CD8^+$ T-cell populations, we reasoned that D^b-Uty-specific $CD8^+$ T cells may have the higher precursor frequency and that they could be more readily identified in a naïve animal. For our first attempt, we used a B6 female mouse that had an established anti-HY memory response to verify that we could use our modified isolation method to identify a $CD8^+$T-cell population specific for D^b-Uty via dual tetramer staining. This first attempt used the spleen and lymph nodes; the single-cell suspension was stained with D^b-Uty- PE and D^b-Uty-AF647, followed by magnetic-bead separation using anti-PE microbeads. Staining with two tetramers of the same specificity but with different fluorochromes allowed for strict parameters with which to determine true tetramer-positive T-cell populations. The sample was also stained for CD8a and with the following mAbs including anti-CD4, CD19, CD11c and F4/80 for gate exclusion during analysis. Gate exclusion refers to the removal during software analysis of certain cell populations based on their surface marker staining. Unfortunately, despite gating on $CD8a^+$ lymphocytes and excluding all CD4, CD19, CD11c and $F4/80^+$ lymphocytes, a clear dual tetramer-positive population could not be identified (Figure 5).

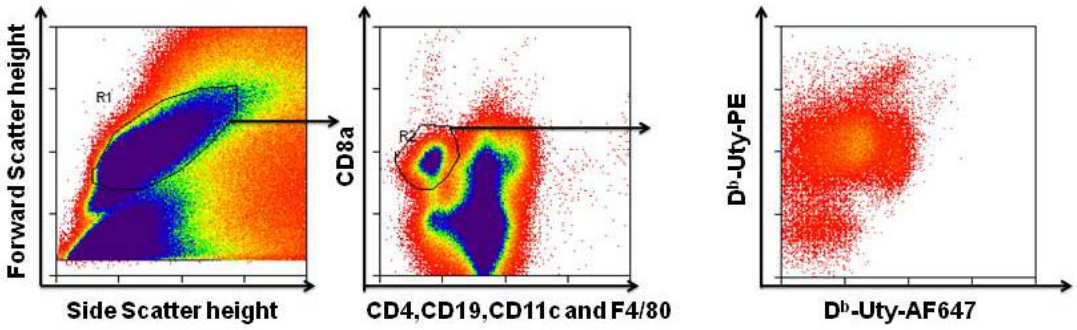

Figure 5. Exclusion gating, dual D^b-Uty tetramer staining and magnetic-bead enrichment does not reveal a dual tetramer$^+$ D^b-Uty specific $CD8^+$ T cell population. Spleen and lymph nodes from B6 female previously immunized with B6 male splenocytes were aseptically harvested, disaggregated, erythrocyte-depleted, and stained with D^b-Uty-PE and-AF647 tetramers, both at 25nM, along with anti-CD8a, 45 minutes at 4°C at [$5x10^7$cells/mL]. Cells were then magnetically enriched via anti-PE microbeads and stained with cell surface exclusion markers for 15 minutes at 4°C at a cellular concentration of [$20x10^6$/mL] and analyzed via flow cytometry.

The results of our first attempt at isolating a dual tetramer-positive $CD8^+$ T-cell population indicated a crucial need for optimization of this adapted method. We therefore performed a series of optimization experiments that included titrating all fluorochrome-conjugated mAbs used, determining the best fluorochrome to use for anti-CD8a surface staining, blocking with streptavidin to prevent non-specific tetramer binding, and testing other reagents to block non-specific binding, (2.4G2 , anti-mouse CD16/CD32) (data not shown). We found that blocking with streptavidin had no effect on dual tetramer-positive cell isolation and that anti-mouse CD16/CD32 had the greatest effect at reducing non-specific binding (data not shown). We also titrated D^b-Uty-PE and –APC- conjugated tetramers individually, using $CD8^+$T-cell enriched splenocytes from anti-HY responding B6 female mice. From these titration experiments, we calculated a signal-to-noise ratio for each staining dilution, and further determined that individually, D^b-Uty-PE and-APC-conjugated tetramers optimally highlight D^b-Uty specific $CD8^+$ T cell populations when used at a concentration range of 6.25-12.5nM (Figure 6). D^b-Uty-APC tetramers fluoresce in the same channel as D^b-Uty-AF647 tetramers, and because we found D^b-Uty-APC tetramers to exhibit better tetramer staining than D^b-Uty-AF647 tetramers (data not shown), we began to use D^b-Uty-APC tetramers in place of our original D^b-Uty-AF647 tetramers.

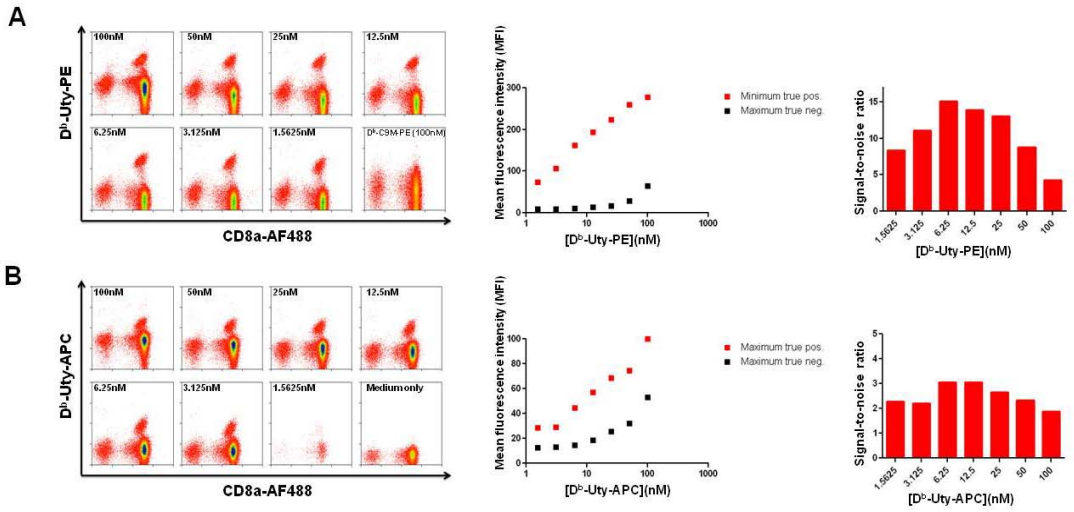

Figure 6. D^b-Uty-PE and –APC tetramers exhibit similar optimal staining concentrations when used individually. Erythrocyte-depleted splenocytes from a B6 female previously immunized with B6 male splenocytes were enriched for $CD8^+$T cells by negative selection via magnetic-bead enrichment. The splenocytes were then stained for CD8a expression and with (A) D^b-Uty-PE or (B) APC tetramers at various dilutions at a cellular concentration of [200,000 /100µL], for 1 hour at 4°C and analyzed via flow cytometry. The mean fluorescence intensity (MFI) for the maximum truly tetramer-positive population and the maximum truly negative populations were determined and plotted. From these values a signal to noise ratio was calculated (true positive/true negative), and optimal staining concentrations for each tetramer individually were determined by considering the signal to noise ratio and the MFI of the truly tetramer negative population.

After determining the optimal staining concentration ranges for each tetramer individually, we then performed a series of titration competition experiments, where we varied both D^b-Uty-PE and –APC tetramer staining concentrations to determine the staining parameters that yielded the most distinct dual tetramer-positive populations with the most separation from the background population. As before, we used B6 females stimulated to produce an anti-HY response so that sufficient numbers of cognate T cells could be easily visualized. We found optimal detection of dual tetramer-positive populations using D^b-Uty-PE and –APC concentrations as follows: D^b-Uty-PE (6.25nM) and D^b-Uty-APC (25nM) or D^b-Uty-PE (3.125nM) and D^b-Uty-APC (12.5nM) (Figure 7Ai-ii). Generally the PE-conjugated tetramers stained at higher MFIs than the APC-conjugated tetramers and when the PE-conjugated tetramers were used at equivalent concentrations with the APC-conjugated tetramers, the MFI for the APC-conjugated tetramers was decreased (data not shown). We then set D^b-Uty-PE at a fixed low concentration (~4nM) and varied D^b-Uty-APC concentrations; we found the best dual tetramer-positive population to be observed when D^b-Uty-APC was

used at 25nM (Figure 7B). Finally we applied these parameters to CD8$^+$T-cell enriched splenocytes from an anti-HY responding female, followed by magnetic-enrichment with anti-PE beads according to Obar *et al.*.[142] Although a dual tetramer population was visible compared to our first attempt with an entire spleen (Figure 5), the separation of the dual tetramer-positive population from the background was not robust enough to apply this method to a naïve mouse. Further optimization was needed.

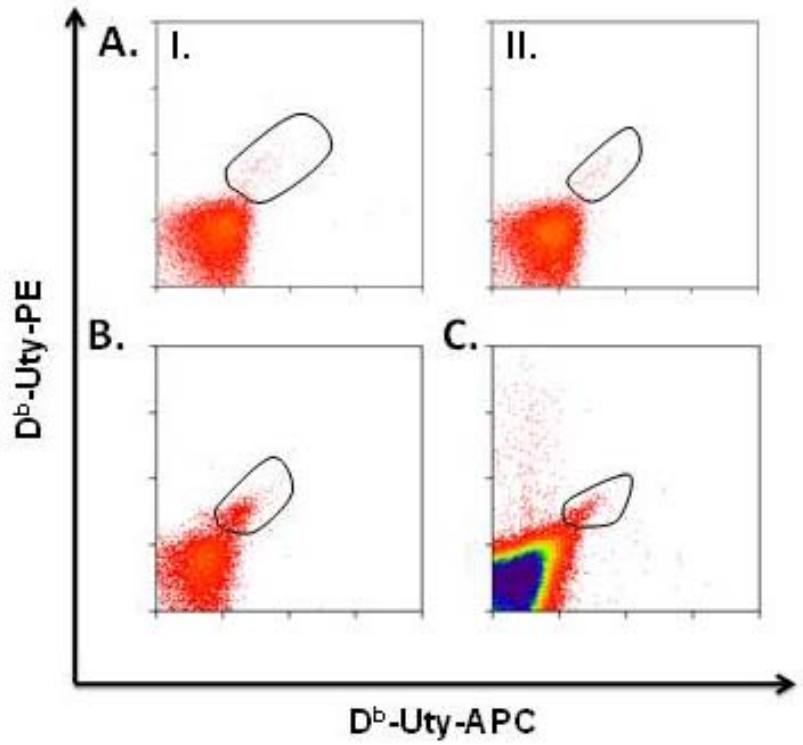

Figure 7. D^b-Uty-PE tetramers out-compete–APC tetramers for TCR binding and must be used at a lower concentration. Splenocytes from a B6 female previously immunized with male B6 splenocytes were enriched for CD8$^+$T cells via magnetic separation. (Ai) Splenocytes were stained for CD8 surface expression and with D^b-Uty-PE and-APC tetramers at varying concentrations at cellular concentrations of [250,000 /100µL] for 1 hour at 4°C. PE- and APC conjugated tetramer concentrations of D^b-Uty-PE (6.25nM) and D^b-Uty-APC (25nM) or (Aii) 3.125nM and 12.5nM respectively, provided the most distinct dual tetramer-positive populations. (B) CD8$^+$T-cell enriched splenocytes were stained for CD8a expression and D^b-Uty-PE at ~4nM and varying concentrations of D^b-Uty-APC tetramer at a cellular concentration of [400,000 /100µl] for 45 minutes at 4°C in the presence of FC block. Db-Uty-APC at 25nM revealed the most distinct dual tetramer population. (C) CD8$^+$T-cell enriched splenocytes stained for CD8 expression, D^b-Uty-PE (5nM) and D^b-Uty-APC (25nM) with Fc block (anti-mouse CD16/CD32) at a cellular concentration of 80x10^6 cells/500µL for 45 minutes at 4°C followed by magnetic-bead separation with anti-PE microbeads. All samples were analyzed via flow cytometry; results are gated on CD8$^+$ lymphocytes, dual tetramer-positive populations indicated.

We then hypothesized that because D^b-Uty-PE had been found to out-compete D^b-Uty-APC for TCR binding, additional measures had to be taken to ensure more equivalent binding between the two tetramers. We therefore performed another series of experiments to titrate D^b-Uty-PE concentrations against a fixed D^b-Uty-APC concentration of 50nM (Figure 8A, upper panel). Additionally, we compared the effects of adding D^b-Uty-PE at different time points after adding D^b-Uty-APC (Figure 8A, lower panel). We also titrated D^b-Uty-PE following a 45 minute incubation with D^b-Uty-APC (50nM) at both 4°C and 37°C (Figure 8B). As in previous experiments, we used $CD8^+$T-cell enriched splenocytes from an anti-HY responding B6 female. For these studies we determined the optimum staining conditions to be 45 minutes at 37°C with D^b-Uty-APC (50nM), followed by 30 minutes at 37°C with D^b-Uty-PE (~5nM) (Figure 8B).

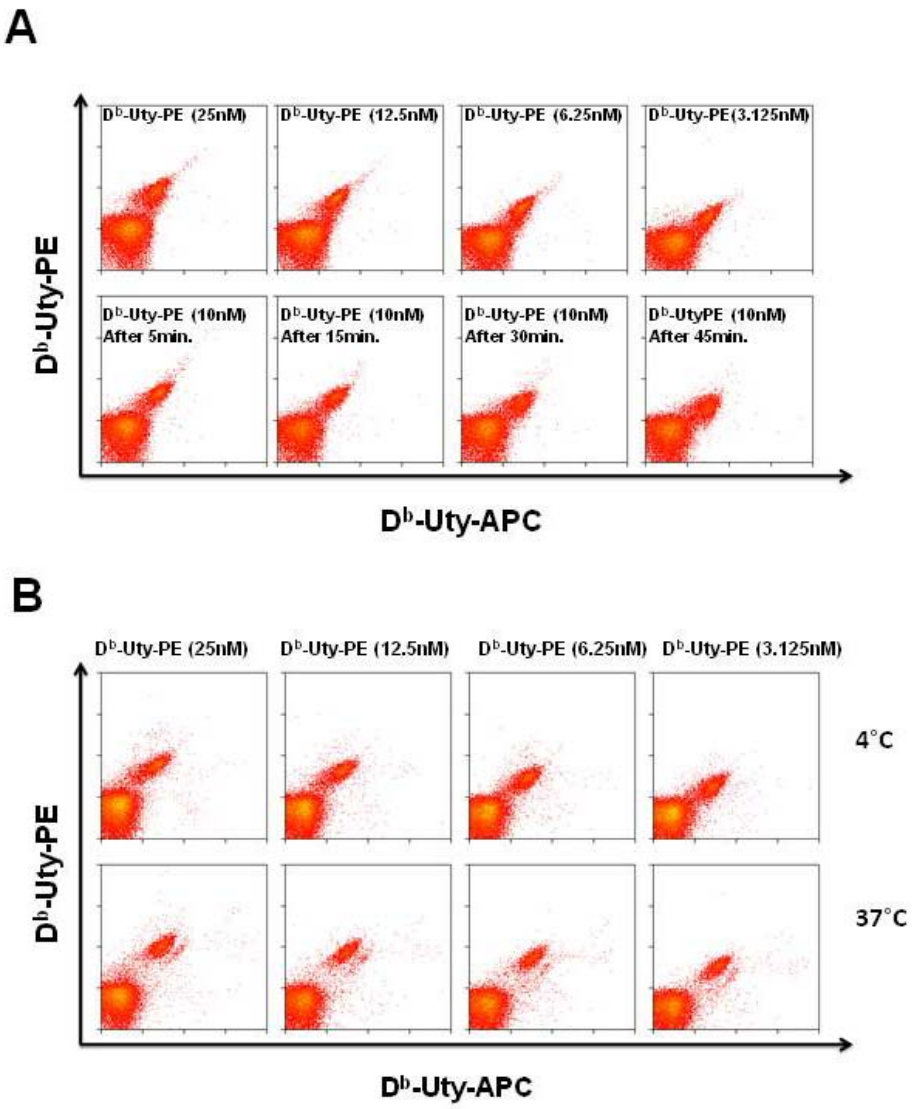

Figure 8. Successive staining with D^b-Uty-APC (50nM) and -PE (3.125 or 6.25nM) at 37°C yields the most distinct dual tetramer-positive population. (A, upper panel) Splenocytes from an anti-HY responding B6 female were enriched for $CD8^+$T-cells and stained for CD8a expression, and with D^b-Uty-APC(50nM) and varying concentrations of D^b-Uty-PE tetramers at a cellular concentration of [200,000 /100µL] at 4°C for 45 minutes. (A, lower panel) $CD8^+$ T-cell enriched splenocytes were stained for CD8a expression and with D^b-Uty-APC (50nM) followed by the addition of D^b-Uty-PE (5nM final dilution) after varying time points under same conditions as the above panel. (B, upper panel) $CD8^+$T-cell enriched splenocytes were stained for CD8a expression and with D^b-Uty-APC (50nM) for 45 minutes at 4°C or 37°C at a cellular concentration of 200,000 cells/100µL followed by a 30 minute incubation with D^b-Uty-PE at varying concentrations at 4°C or (B lower panel) 37°C. All samples stained following incubation with FC block (anti-mouse CD16/CD32) and analyzed by flow cytometry; results are gated on $CD8^+$ lymphocytes.

We then applied our optimized protocol of dual tetramer staining with anti-PE magnetic-bead enrichment and gate exclusion analysis. Although we were able to isolate a distinctly dual tetramer-positive population using $CD8^+$T-cell enriched splenocytes from an anti-HY responding B6 female (Figure 9A), we also found that combining negative and positive selection via magnetic-bead enrichment before and after tetramer staining resulted in too much cell death, as detected by 7-aminoactinomycin D (7AAD) live/dead staining (data not shown). We therefore removed the $CD8^+$ T-cell enrichment step; Obar *et al.* only included anti-PE magnetic-bead enrichment.[142] Successive tetramer staining at our optimized concentrations, along with gate exclusion and anti-PE magnetic-bead enrichment, resulted in a very clear dual tetramer-positive population in an immunized female mouse (Figure 9B). We then applied this protocol to cells obtained from a naive B6 female, but unfortunately could not identify a dual tetramer-positive naïve precursor population specific for D^b-Uty (data not shown).

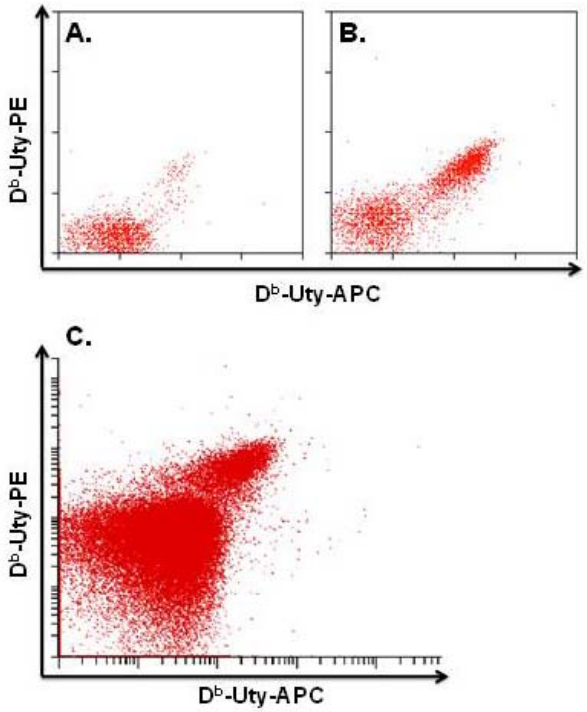

Figure 9. Successive D^b-Uty-APC and –PE tetramer staining protocol along with high-speed cell sorting allows for detection of dual tetramer-positive population in an-HY responsive mouse. (A) $CD8^+$T-cell enriched splenocytes, from anti-HY responding B6 female, were successively stained at a cellular concentration of [$5x10^6$ /500µL] with D^b-Uty-APC (50nM) for 45 minutes and D^b-Uty-PE (5nM) for 30 minutes, both at 37°C. Splenocytes were then magnetically enriched with anti-PE microbeads, stained for CD8a, and FITC conjugated: CD19, CD11C, CD49b, CD4 and F4/80, and analyzed via flow cytometry (CyAn) at 4°C for 15-30 minutes. (B) Splenocytes treated in same manner as (A) excluding $CD8^+$T cell enrichment, and tetramer stained instead at a cellular concentration of ~[$60x10^6$/mL]. (C) Female B6 (male BM immunized) splenocytes stained for CD8a, Thy1.2, CD19, Cd11c, CD49b, CD4, and F4/80 at cellular concentration of ~[$90x10^6$/mL] at 4°C for 30 minutes. Cells then sorted via iCyt Reflection for $Thy1.2^+$, $CD8a^+$ T-cell population. Sorted cells stained at a cellular concentration of ~[$5x10^6$/mL] with D^b-Uty-APC (50nM) for 45 minutes at followed by D^b-Uty-PE (5nM) tetramer incubation for 30 minutes, both at 37°C. Cells then analyzed via flow cytometry (iCyt Reflection). All samples stained following incubation with Fc block at 4°C. Results are gated on $CD8a^+$ lymphocytes (A-B) or $Thy1.2^+$, $CD8a^+$ lymphocytes(C).

Cell sorting and dasatinib treatment

In an attempt to further reduce cell death and thereby isolate a greater number of naïve CTL precursor cells we further modified our isolation protocol. Instead of removing unwanted cell populations via gate exclusion and by $CD8^+$T-cell magnetic-bead enrichment, we adopted a 2-phase high speed cell sorting protocol using the iCyt Visionary Bioscience Reflection instrument. In addition to the added fluorescence capabilities and increased acquisition rates, this instrument allowed for a higher percentage purity for $CD8^+$T-cell enrichment and the physical removal of cell populations that do not express CD8 in place of removal through analysis techniques (gate exclusion) (data not shown). Using previously determined concentrations of fluorochrome-conjugated mAbs (courtesy of Adam Buntzman), we first sorted $Thy1.2^+$, $CD8a^+$ splenocytes from an HY-reactive B6 female mouse. We then dual stained the sorted population with our D^b-Uty-PE and D^b-Uty-APC successive tetramer staining protocol, and applied gate exclusion to the low level of unwanted cell populations that had escaped the stringent sorting protocol. By this method we were able to identify a dual tetramer-positive population (Figure 9C).

Utilizing the sorting capabilities of the iCyt Reflection instrument, we had achieved identification of a dual tetramer-positive population of D^b-Uty specific $CD8^+$T cells in an anti-HY responding female. However, this population was not as distinct as the cells observed after optimizing the method, using only analysis but not sorting by flow cytometry (Figure 8), although it did yield higher cell viability (data not shown). To improve the separation between the dual tetramer-positive population and background, we then investigated the effects of the protein kinase inhibitor, dasatinib. Dasatinib has been found to enhance tetramer staining of antigen-specific $CD8^+$ and $CD4^+$ T cells, without an increase in background staining, by preventing TCR downregulation and tetramer internalization; dasatinib has also been shown to reduce tetramer-induced cell death.[157] We found dasatinib to dramatically enhance fluorochrome-conjugated D^b-Uty tetramer binding, which we tested with splenocytes from B6 females that had been previously immunized once, twice, or three times with male B6 BM (Figure 10).

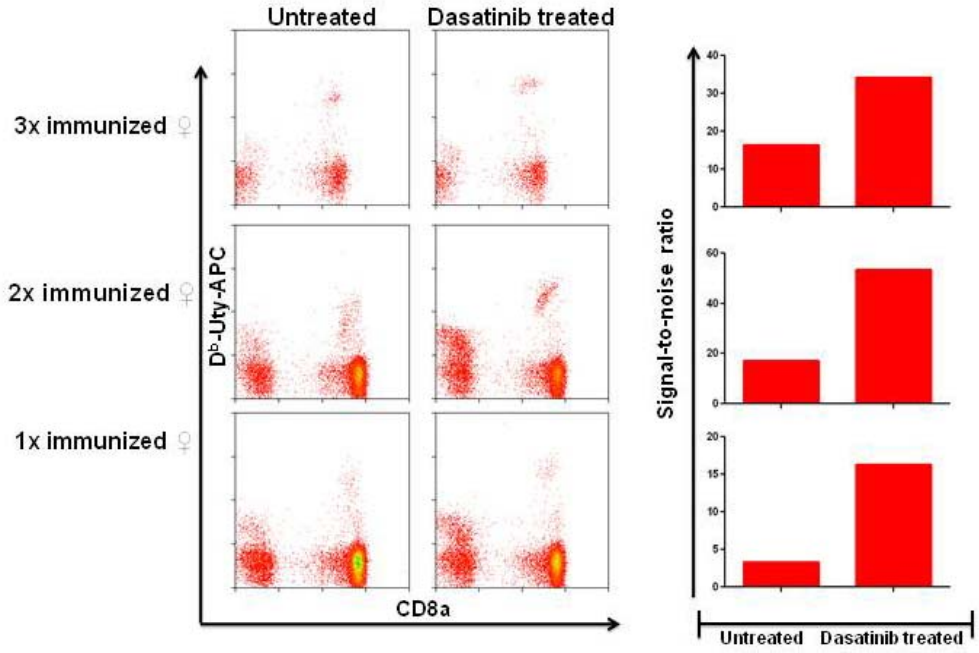

Figure 10. The protein kinase inhibitor, dasatinib, enhances D^b-Uty-APC tetramer staining. PBLs were collected from B6 female mice previously immunized with $5x10^6$ male B6 BM. The cells were split into two aliquots; one aliquot was incubated in 1mL R-10 and the other was treated with dasatinib (50nM) in 1mL R-10, both for 30 minutes at 37°C. The dasatinib treated cells were then incubated in 100uL R-10 at a 50nM concentration of both dasatinib and D^b-Uty-APC at 37°C for 45 minutes, while the untreated cells were stained with D^b-Uty-APC at 37°C for 45 minutes. The cells were then stained for CD8a, CD4, and CD19 expression for 45 minutes at 4°C and analyzed via flow cytometry; CD19+ and CD4+ cells were removed from analysis via gate exclusion. The signal-to-noise ratios were calculated as described in Figure 2. Results are gated on $CD8a^+$, $CD4^-$, $CD19^-$ lymphocytes.

After finding that dasatinib could be used to enhance D^b-Uty-APC tetramer staining in a memory T-cell population, we then added dasatinib to our successive tetramer staining protocol and again attempted to isolate a dual tetramer-positive population in a previously immunized B6 female using the iCyt Reflection instrument. We also added a second sorting step to our protocol. We reasoned that sorting the dual tetramer population and re-analyzing the cells using iCyt Reflection, would allow for further discrimination between the truly dual tetramer-positive population and any background populations through the re-distribution of true negative and positive populations. We were able to isolate a dual tetramer-positive population with an acceptable amount of separation from the background. As seen in Figure 11A, there also appeared to be a D^b-Uty-APC^+, PE^- population, in addition to the dual tetramer-positive population. We did not include these cells in our subsequent sort, as they could not reliably be considered actual naïve D^b-Uty-specific

$CD8^+$T-cells; only dual tetramer-positive populations were considered to be naïve CTL precursor cells under the criteria that we had defined. Re-analysis of the sorted dual tetramer-positive population did reveal a re-distribution between the truly dual tetramer-positive population and the background; 14.54% of the originally dual-positive sorted cells fell outside of the dual positive gate (Figure 11A), and presumably represented artifacts. Nonetheless, 85.46% retained their dual staining characteristics; consistent with true positive D^b-Uty-reactive CTL, suggesting our sorting method was robust.

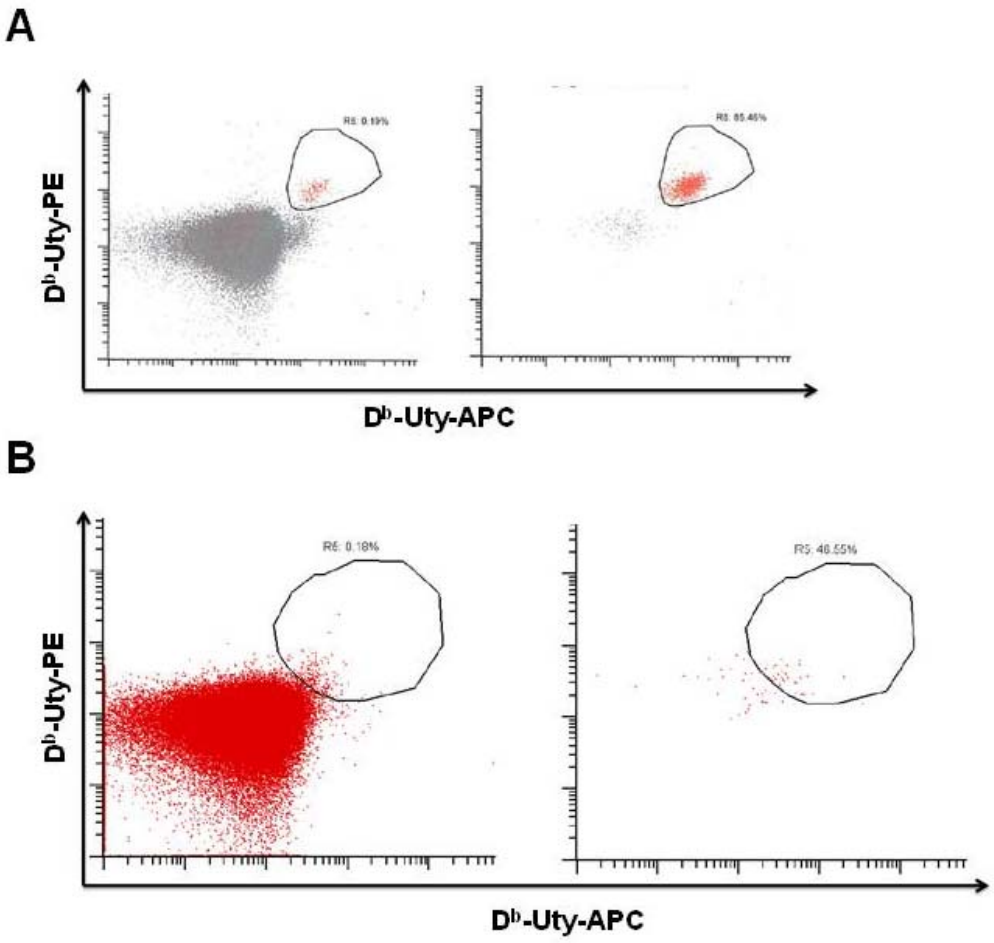

Figure 11. Improved isolation method allows for clear detection of dual tetramer-positive memory population but not a naïve population. (A, left panel) Splenocytes from a B6 female previously immunized with $5x10^6$ male B6 BM IP were treated with dasatinib (50nM) in R-10 for 45 minutes at 37°C. Following a 15 minute incubation period at 4°C with Fc block, the cells were then stained for CD8a, and Thl.2 expression and with FITC conjugated mAb specific for CD19, CD11C, CD49b, CD4 and F4/80 at a cellular concentration of ~[$100x10^6$/mL] for 35 minutes at 4°C. $CD8a^+$,Thy1.2+ lymphocytes were then sorted by the iCyt Reflection and stained with successive D^b-Uty-APC (50nM) and D^b-Uty-PE(5nM) tetramers in the presence of dasatinib (50nM), both at 37°C at a concentration of [300,000 /100µL]. The dual tetramer-positive population was detected by flow cytometry. (A, right panel) Dual positive cells were then sorted and re-analyzed (iCyt Reflection). (B) Splenocytes from a naïve B6 female were sorted and analyzed via flow cytometry according to the protocol used in (A), cellular staining concentrations were [$70x10^6$/mL]for fluorochrome-conjugated mAb staining and [300,000 /100µL] for tetramer staining.

Finally, we applied our dual D^b-Uty-PE and-APC tetramer staining protocol to a naïve B6 female mouse. Despite the promising results of our improved protocol with HY reactive B6 female mice, we were unable to identify a distinct tetramer-positive population among splenocytes of a naïve B6 female (Figure 11B). Furthermore, the putative naïve precursor cells that were collected during the second sort did not re-distribute upon re-analysis in the same manner as the D^b-Uty specific memory $CD8^+$ T cell population (Figure 11). There was no clear divide between background and dual tetramer-positive cells, with 53.45% of the sorted cells falling outside of the dual tetramer-positive gate, providing further evidence that we had not successfully isolated a naïve dual tetramer-positive population (Figure 11B).

Our method for detecting a dual tetramer-positive population was subjective and therefore did not allow for complete confirmation of the naïve nature of any isolated dual tetramer-positive cells. In deciphering all of our experimental results, we evaluated the separation of dual tetramer-positive populations from background populations by means of aesthetic qualification, meaning we evaluated the degree of separation of the dual tetramer-positive population from the negative populations visually. To confirm that our sorted dual tetramer-positive population taken from a naïve mouse was in fact naive, additional experiments are required. Such experiments would include staining the sorted cells for markers such as CD44 and CD62L to identify a naïve phenotype, and exposing the naïve $CD8^+$T cells to cognate peptides, along with other control peptides such as OVA, to show antigen-specific activation and CTL activity. We could also apply our techniques to a male mouse and perhaps verify that our method does not identify non-specific dual tetramer-positive cells. However, our results for the naïve CTL precursor isolation did not warrant these additional experiments. The dual tetramer-positive cells from naïve mice that were identified were not considered to be true dual tetramer-positive $CD8^+$ T cells by means of aesthetic evaluation, and based on the comparison of their re-distribution after sorting with memory dual tetramer-positive $CD8^+$T cells (Figure 11). Although we do not consider the isolated cells are in fact naïve $CD8^+$ T cells specific for D^b-Uty, without these additional experiments, we cannot completely exclude this possibility. Based on the failure to isolate naïve D^b-Uty-specific $CD8^+$T cells, we did not attempt to enumerate a naïve D^b-Smcy-specific $CD8^+$T-cell population.

454 sequencing

In addition to direct enumeration of naïve CTL precursor frequency via fluorochrome-conjugated tetramer staining, comparing CDR3 diversity is an indirect method of determining CTL precursor frequency. Dyson *et al.* have recently compared the diversity of

naïve D^b-Smcy specific $CD8^+$T cells before and after thymic selection, through a RT-PCR, cloning, and sequencing strategy.[158] Thymocytes from TCRα-/- mice do not undergo thymic selection (positive or negative) owing to their lack of mature αβTCR expression; D^b-Smcy-specific $CD8^+$T cell repertoires from these mice were compared to wild type (WT) B6 females. By this strategy, Dyson *et al.* analyzed ~100 CDR3 sequences per repertoire and found decreased D^b-Smcy-specific TCR diversity after selection, compared to pre-thymic selection CDR3 diversity.[158] Though this study provided some insight into the size of the D^b-Smcy specific $CD8^+$T cell precursor repertoire based on the diversity observed, the limited number of CDR3 sequences compared could possibly have underestimated the population number.

CD3R sequencing technology has recently been revolutionized by 454 sequencing. This method allows for greater sequencing volume, without the normally required sub-cloning into bacteria, through emulsion-based DNA amplification and pyrophosphate-based sequencing.[159-160] Although we were unable to directly enumerate the naïve CTL precursor frequency for D^b-Uty and-Smcy-specific $CD8^+$T cells our protocol, a comparison of the of size, diversity and amount of public CDR3 sequences for each epitope would be very valuable toward a more definitive identification of the immunodominant epitope for HY D^b-restricted B6 strain. To make this estimation, we therefore stained splenocytes from male immunized B6 females with fluorochrome-conjugated tetramers and sorted D^b-Uty and –Smcy- tetramer-positive $CD8^+$T cells (MoFlo). Females were immunized IP with $5x10^6$ male B6 BM 14 days prior to spleen harvesting. The cells were lysed and are currently stored at -80°C, awaiting RNA extraction and 454 sequencing. The sequencing results are expected to provide a minimum number of naïve CTL precursors that must have been present in the mouse at the naïve stage, prior to antigen-exposure. This minimum diversity of CTL precursor frequencies could then be compared between D^b-Uty- and Smcy-specific $CD8^+$T cell populations.

Chapter 5: Concluding remarks

The results of our study have provided further characterization and understanding of the relationship between D^b-Uty and Smcy-specific $CD8^+$T cell responses in the HY antigen system. They have also confirmed the potential of MHC class I cytotoxic tetramers to inhibit specific CTL activity and have provided another beneficial use for cytotoxic tetramer technology.

In Chapter 3 we compared the kinetics of expansion and functional responses of D^b-Uty and Smcy-specific $CD8^+$ T cells following female immunization with syngeneic male BM. We found that the epitope-specific T-cell populations differ in their kinetics of expansion, with D^b-Smcy specific $CD8^+$ T cells peaking at 14 days p.i. and D^b-Uty-specific $CD8^+$T cells peaking at 20 days p.i. Despite this difference, functional responses between the two populations did not differ (granzyme B, IFN-γ, and TNF-α production) at 14 days p.i. We also found D^b-Uty-specific $CD8^+$T cells to significantly outnumber the D^b-Smcy-specific $CD8^+$T cell population in number at 20 days p.i. and beyond, and the D^b-Uty-specific $CD8^+$T cells to overall exhibit more robust activity in our *in vivo* CTL assay experiments. Once we established that we were capable of reliably eliciting CTL responses against these two major epitopes, we then sought to determine whether administration of D^b-Uty-or-Smcy-SAP tetramers could delete the naïve precursors, and abolish the epitope specific responses to immunization. As we hypothesized, treatment with cytotoxic tetramers selectively deleted D^b-Uty and –Smcy specific-$CD8^+$T cells as seen through direct analysis of splenocyte populations with fluorochrome-conjugated tetramers. This selective deletion enhanced survival of target cells pulsed with the correlated cognate peptide. Most interestingly, we observed a decreased survival of Smcy peptide-pulsed targets when D^b-Uty-specific $CD8^+$T cells were deleted; this decreased survival occurred as a result of D^b-Smcy-specific $CD8^+$T-cell expansion in the absence of D^b-Uty-specific $CD8^+$T cells and was confirmed by fluorochrome-conjugated tetramer staining.

In Chapter 4 we sought to further characterize the relationship between D^b-Uty and –Smcy specific $CD8^+$T cells by enumerating the naïve CTL precursor population for each epitope. We began with a protocol modified from Obar *et al.* that employed a combination of dual staining with PE and APC-conjugated MHC class I tetramers of the same specificity and magnetic-bead enrichment.[142] We used tetramers of the same specificity but conjugated with two different fluorochromes further restrict the parameters by which CD8+T-cell populations are considered tetramer population. By this method only dual

tetramer-positive $CD8^+$ T cells are considered truly specific for pMHC in question. We optimized our adapted protocol by attempting to isolate a dual D^b-Uty tetramer-positive population in an HY responding B6 female. As a result of many optimization and titration experiments we formulated a protocol involving CD8+ T-cell enrichment via high-speed sorting, followed by successive tetramer staining in the presence of the protein kinase inhibitor, dasatinib, to allow for the successful isolation of a dual tetramer-positive population. However, when we applied this optimized protocol to cells obtained from an unimmunized B6 female, we were unable to enumerate a naïve D^b-Uty-specific CTL population. We instead decided to enumerate a minimum number of D^b-Uty and –Smcy specific precursor CTLs based on CDR3 region sequence. We therefore sorted both epitope-specific $CD8^+$ T-cell populations at 14 days p.i. and are planning to sequence their CDR3 regions using 454 sequencing. The sequence diversity within each population, averaged over four mice, should provide an estimate of the functionally-responsive precursor number.

Collectively, our results substantiate the potentially therapeutic role of MHC class I cytotoxic tetramers through the selective deletion of alloreactive antigen-specific $CD8^+$T cells. Our results also highlight a new role for cytotoxic tetramers. Cytotoxic tetramers can be used to selectively delete dominant epitope-specific $CD8^+$T-cell populations and thereby enable the characterization of subdominant epitope responses, both previously identified epitopes and epitopes yet to be discovered. A comparison of epitope-specific responses with and without the presence of certain $CD8^+$T-cell populations can provide insight into the physiological relationship between antigen-specific T cells during a $CD8^+$T-cell response. Indeed, by this method we found that D^b-Uty specific $CD8^+$T cells suppress D^b-Smcy-specific $CD8^+$T-cell number and CTL activity. This finding, in conjunction with our earlier characterization studies, has led us to designate D^b-Uty as the immunodominant (major) epitope and D^b-Smcy as the subdominant (minor) epitope. Our designation is in agreement with the results reported by Millrain and by Simpson.[85,161] Pending results concerning the CDR3 sequence diversity for each epitope will provide additional insight into this relationship. The results may further address the question of immunodominance in the HY antigen system, possibly through correlation of the CTL precursor frequency of D^b-Uty and-Smcy specific $CD8^+$T cells with the degree of response each epitope evokes, and through public TCR comparisons between each epitope-specific $CD8^+$T-cell repertoire.

References

1. Kruisbeek AM, Amsen D. Mechanisms underlying T-cell tolerance. Curr Opin Immunol. 1996;8:233-244.

2. Redmond WL, Sherman LA. Peripheral tolerance of CD8 T lymphocytes. Immunity. 2005;22:275-284.

3. Arakelov A, Lakkis FG. The alloimmune response and effector mechanisms of allograft rejection. Semin Nephrol. 2000;20:95-102.

4. Trivedi HL. Immunobiology of rejection and adaptation. Transplant Proc. 2007;39:647-652.

5. Medawar PB. The behaviour and fate of skin autografts and skin homografts in rabbits: A report to the War Wounds Committee of the Medical Research Council. J Anat. 1944;78:176-199.

6. Issa F, Schiopu A, Wood KJ. Role of T cells in graft rejection and transplantation tolerance. Expert Rev Clin Immunol. 2010;6:155-169.

7. Scott DM, Ehrmann IE, Ellis PS, Chandler PR, Simpson E. Why do some females reject males? The molecular basis for male-specific graft rejection. J Mol Med. 1997;75:103-114.

8. Game DS, Lechler RI. Pathways of allorecognition: implications for transplantation tolerance. Transpl Immunol. 2002;10:101-108.

9. Dierselhuis M, Goulmy E. The relevance of minor histocompatibility antigens in solid organ transplantation. Curr Opin Organ Transplant. 2009;14:419-425.

10. Simpson E, Scott D, James E, *et al.*. Minor H antigens: genes and peptides. Transpl Immunol. 2002;10:115-123.

11. Rocha PN, Plumb TJ, Crowley SD, Coffman TM. Effector mechanisms in transplant rejection. Immunol Rev. 2003;196:51-64.

12. Hayry P. Mechanisms of rejection. Curr Opin Immunol. 1989;1:1230-1235.

13. Clayberger C. Cytolytic molecules in rejection. Curr Opin Organ Transplant. 2009;14:30-33.

14. Gallon LG, Leventhal JR, Kaufman DB. Pretransplant evaluation of renal transplant candidates. Semin Nephrol. 2002;22:515-525.

15. Hubscher SG. Transplantation pathology. Semin Diagn Pathol. 2006;23:170-181.

16. Libby P, Pober JS. Chronic rejection. Immunity. 2001;14:387-397.

17. Strom TB, Tilney NL, Carpenter CB, Busch GJ. Identity and cytotoxic capacity of cells infiltrating renal allografts. N Engl J Med. 1975;292:1257-1263.

18. Jones ND, Van Maurik A, Hara M, *et al.*. CD40-CD40 ligand-independent activation of CD8+ T cells can trigger allograft rejection. J Immunol. 2000;165:1111-1118.

19. Martinez OM, Rosen HR. Basic concepts in transplant immunology. Liver Transpl. 2005;11:370-381.

20. Glimcher LH. Lineage commitment in lymphocytes: controlling the immune response. J Clin Invest. 2001;108:s25-s30.

21. Bueno V, Pestana JO. The role of CD8+ T cells during allograft rejection. Braz J Med Biol Res. 2002;35:1247-1258.

22. el-Sawy T, Fahmy NM, Fairchild RL. Chemokines: directing leukocyte infiltration into allografts. Curr Opin Immunol. 2002;14:562-568.

23. Morelli AE, Larregina AT, Shufesky WJ, *et al.*. Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells. Blood. 2004;104:3257-3266.

24. Game DS, Rogers NJ, Lechler RI. Acquisition of HLA-DR and costimulatory molecules by T cells from allogeneic antigen presenting cells. Am J Transplant. 2005;5:1614-1625.

25. Thomson AW, Fairchild RL. The last 5 years of basic science investigation in transplant immunology. Am J Transplant. 2006;6:1768-1773.

26. Chen L. Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. Nat Rev Immunol. 2004;4:336-347.

27. Appleman LJ, Berezovskaya A, Grass I, Boussiotis VA. CD28 costimulation mediates T cell expansion via IL-2-independent and IL-2-dependent regulation of cell cycle progression. J Immunol. 2000;164:144-151.

28. Oldstone MB. Molecular mimicry and autoimmune disease. Cell. 1987;50:819-820.

29. Tough DF, Borrow P, Sprent J. Induction of bystander T cell proliferation by viruses and type I interferon *in vivo*. Science. 1996;272:1947-1950.

30. Krieger NR, Yin DP, Fathman CG. CD4+ but not CD8+ cells are essential for allorejection. J Exp Med. 1996;184:2013-2018.

31. Krieger NR, Fathman CG. The use of CD4 and CD8 knockout mice to study the role of T-cell subsets in allotransplant rejection. J Heart Lung Transplant. 1997;16:263-267.

32. Dalloul AH, Chmouzis E, Ngo K, Fung-Leung WP. Adoptively transferred CD4+ lymphocytes from CD8 -/- mice are sufficient to mediate the rejection of MHC class II or class I disparate skin grafts. J Immunol. 1996;156:4114-4119.

33. Rosenberg AS, Mizuochi T, Sharrow SO, Singer A. Phenotype, specificity, and function of T cell subsets and T cell interactions involved in skin allograft rejection. J Exp Med. 1987;165:1296-1315.

34. Gilot BJ, Hara M, Jones ND, *et al.*. *In vivo* differentiation of alloreactive CD8+ T cells after murine cardiac allograft transplantation. Transplant Proc. 1999;31:130.

35. Jones ND, Van Maurik A, Hara M, Gilot BJ, Morris PJ, Wood KJ. T-cell activation, proliferation, and memory after cardiac transplantation *in vivo*. Ann Surg. 1999;229:570-578.

36. Delfs MW, Furukawa Y, Mitchell RN, Lichtman AH. CD8+ T cell subsets TC1 and TC2 cause different histopathologic forms of murine cardiac allograft rejection. Transplantation. 2001;71:606-610.

37. Desai NM, Bassiri H, Kim J, *et al.*. Isl*et al.*lograft, islet xenograft, and skin allograft survival in CD8+ T lymphocyte-deficient mice. Transplantation. 1993;55:718-722.

38. Osorio RW, Ascher NL, Melzer JS, Stock PG. beta-2 Microglobulin gene disruption prolongs murine isl*et al.*lograft survival in NOD mice. Transplant Proc. 1994;26:752.

39. Legare JF, Issekutz T, Lee TD, Hirsch G. CD8+ T lymphocytes mediate destruction of the vascular media in a model of chronic rejection. Am J Pathol. 2000;157:859-865.

40. Krensky AM, Clayberger C. Granulysin: a novel host defense molecule. Am J Transplant. 2005;5:1789-1792.

41. Mirandola P, Ponti C, Gobbi G, *et al.*. Activated human NK and CD8+ T cells express both TNF-related apoptosis-inducing ligand (TRAIL) and TRAIL receptors but are resistant to TRAIL-mediated cytotoxicity. Blood. 2004;104:2418-2424.

42. Bruno DA, Dhanireddy KK, Kirk AD. Challenges in therapeutic strategies for transplantation: where now from here? Transpl Immunol. 2005;15:149-155.

43. Pillai AA, Levitsky J. Overview of immunosuppression in liver transplantation. World J Gastroenterol. 2009;15:4225-4233.

44. Denton MD, Magee CC, Sayegh MH. Immunosuppressive strategies in transplantation. Lancet. 1999;353:1083-1091.

45. Sayegh MH, Carpenter CB. Transplantation 50 years later--progress, challenges, and promises. N Engl J Med. 2004;351:2761-2766.

46. Golshayan D, Pascual M. Tolerance-inducing immunosuppressive strategies in clinical transplantation: an overview. Drugs. 2008;68:2113-2130.

47. Pascual M, Theruvath T, Kawai T, Tolkoff-Rubin N, Cosimi AB. Strategies to improve long-term outcomes after renal transplantation. N Engl J Med. 2002;346:580-590.

48. Kasiske BL, Snyder JJ, Gilbertson D, Matas AJ. Diabetes mellitus after kidney transplantation in the United States. Am J Transplant. 2003;3:178-185.

49. Valujskikh A. Memory T cells in allograft rejection. Adv Exp Med Biol. 2007;601:247-256.

50. Haudebourg T, Poirier N, Vanhove B. Depleting T-cell subpopulations in organ transplantation. Transpl Int. 2009;22:509-518.

51. Dumont FJ. T-cell depletion for transplant tolerance induction: promises and hurdles. Curr Opin Investig Drugs. 2003;4:1275-1278.

52. Golovina TN, Mikheeva T, Suhoski MM, *et al.*. CD28 costimulation is essential for human T regulatory expansion and function. J Immunol. 2008;181:2855-2868.

53. St Clair EW. The calm after the cytokine storm: lessons from the TGN1412 trial. J Clin Invest. 2008;118:1344-1347.

54. Altman JD, Moss PA, Goulder PJ, *et al.*. Phenotypic analysis of antigen-specific T lymphocytes. Science. 1996;274:94-96.

55. Wooldridge L, Lissina A, Cole DK, van den Berg HA, Price DA, Sewell AK. Tricks with tetramers: how to get the most from multimeric peptide-MHC. Immunology. 2009;126:147-164.

56. Maile R, Wang B, Schooler W, Meyer A, Collins EJ, Frelinger JA. Antigen-specific modulation of an immune response by *in vivo* administration of soluble MHC class I tetramers. J Immunol. 2001;167:3708-3714.

57. Maile R, Siler CA, Kerry SE, Midkiff KE, Collins EJ, Frelinger JA. Peripheral "CD8 tuning" dynamically modulates the size and responsiveness of an antigen-specific T cell pool *in vivo*. J Immunol. 2005;174:619-627.

58. Corr M, Slanetz AE, Boyd LF, *et al.*. T cell receptor-MHC class I peptide interactions: affinity, kinetics, and specificity. Science. 1994;265:946-949.

59. Savage P, Millrain M, Dimakou S, Stebbing J, Dyson J. Expansion of CD8+ cytotoxic T cells *in vitro* and *in vivo* using MHC class I tetramers. Tumour Biol. 2007;28:70-76.

60. Racke MK, Critchfield JM, Quigley L, *et al.*. Intravenous antigen administration as a therapy for autoimmune demyelinating disease. Ann Neurol. 1996;39:46-56.

61. Cebecauer M, Guillaume P, Hozak P, *et al.*. Soluble MHC-peptide complexes induce rapid death of CD8+ CTL. J Immunol. 2005;174:6809-6819.

62. Aichele P, Bachmann MF, Hengartner H, Zinkernagel RM. Immunopathology or organ-specific autoimmunity as a consequence of virus infection. Immunol Rev. 1996;152:21-45.

63. Xu XN, Purbhoo MA, Chen N, *et al.*. A novel approach to antigen-specific deletion of CTL with minimal cellular activation using alpha3 domain mutants of MHC class I/peptide complex. Immunity. 2001;14:591-602.

64. Kappel BJ, Pinilla-Ibarz J, Kochman AA, *et al.*. Remodeling specific immunity by use of MHC tetramers: demonstration in a graft-versus-host disease model. Blood. 2006;107:2045-2051.

65. de Witte MA, Toebes M, Song JY, Wolkers MC, Schumacher TN. Effective graft depletion of MiHAg T-cell specificities and consequences for graft-versus-host disease. Blood. 2007;109:3830-3838.

66. Yuan RR, Wong P, McDevitt MR, *et al.*. Targeted deletion of T-cell clones using alpha-emitting suicide MHC tetramers. Blood. 2004;104:2397-2402.

67. Penaloza-MacMaster P, Masopust D, Ahmed R. T-cell reconstitution without T-cell immunopathology in two models of T-cell-mediated tissue destruction. Immunology. 2009;128:164-171.

68. Hess PR, Barnes C, Woolard MD, *et al.*. Selective deletion of antigen-specific CD8+ T cells by MHC class I tetramers coupled to the type I ribosome-inactivating protein saporin. Blood. 2007;109:3300-3307.

69. Vincent BG, Young EF, Buntzman AS, *et al.*. Toxin-coupled MHC class I tetramers can specifically ablate autoreactive CD8+ T cells and delay diabetes in nonobese diabetic mice. J Immunol. 2010;184:4196-4204.

70. Battaglia M, Roncarolo MG. Induction of transplantation tolerance via regulatory T cells. Inflamm Allergy Drug Targets. 2006;5:157-165.

71. Simpson E. Minor transplantation antigens: animal models for human host-versus-graft, graft-versus-host, and graft-versus-leukemia reactions. Transplantation. 1998;65:611-616.

72. Boisgerault F, Anosova N, Fedoseyeva EV, Tam RC, Benichou G. Analysis of T-cell response using altered peptide ligands. Methods Mol Biol. 2001;156:211-218.

73. Simpson E, Scott D, Chandler P. The male-specific histocompatibility antigen, H-Y: a history of transplantation, immune response genes, sex determination and expression cloning. Annu Rev Immunol. 1997;15:39-61.

74. Loveland B, Wang CR, Yonekawa H, Hermel E, Lindahl KF. Maternally transmitted histocompatibility antigen of mice: a hydrophobic peptide of a mitochondrially encoded protein. Cell. 1990;60:971-980.

75. Roopenian D, Choi EY, Brown A. The immunogenomics of minor histocompatibility antigens. Immunol Rev. 2002;190:86-94.

76. Simpson E. The Role of H-Y as a Minor Transplantation Antigen. Immunology Today. 1982;3:97-106.

77. McLaren A, Simpson E, Epplen JT, *et al.*. Location of the genes controlling H-Y antigen expression and testis determination on the mouse Y chromosome. Proc Natl Acad Sci U S A. 1988;85:6442-6445.

78. Johnson LL, Bailey DW, Mobraaten LE. Genetics of histocompatibility in mice. IV. Detection of certain minor (non-H-2) H antigens in selected organs by the popliteal node test. Immunogenetics. 1981;14:63-71.

79. Xu J, Burgoyne PS, Arnold AP. Sex differences in sex chromosome gene expression in mouse brain. Hum Mol Genet. 2002;11:1409-1419.

80. Eichwald EJ, Silmser CR. Skin. Transplant Bull. 1955;2:148-149.

81. Zaalberg OB. An analysis of the Eichwald-Silmser effect. Transplant Bull. 1959;6:433-435.

82. Bailey DW, Hoste J. Gene Governing Female Immune Response to Male Antigen in Mice. Transplantation. 1971;11:404-7.

83. Gordon RD, Simpson E. Immune-response gene control of cytotoxic T-cell responses to H-Y. Transplant Proc. 1977;9:885-888.

84. Gordon RD, Samelson LE, Simpson E. Selective response to H-Y antigen by F1 female mice sensitized to F1 male cells. J Exp Med. 1977;146:606-610.

85. Millrain M, Chandler P, Dazzi F, Scott D, Simpson E, Dyson PJ. Examination of HY response: T cell expansion, immunodominance, and cross-priming revealed by HY tetramer analysis. J Immunol. 2001;167:3756-3764.

86. Gavin MA, Dere B, Grandea AG, 3rd, Hogquist KA, Bevan MJ. Major histocompatibility complex class I allele-specific peptide libraries: identification of peptides that mimic an H-Y T cell epitope. Eur J Immunol. 1994;24:2124-2133.

87. Fierz W, Brenan M, Mullbacher A, Simpson E. Non-H-2 and H-2-linked immune response genes control the cytotoxic T-cell response to H-Y. Immunogenetics. 1982;15:261-270.

88. Bailey DW. Allelic forms of a gene controlling the female immune response to the male antigen in mice. Transplantation. 1971;11:426-428.

89. Millrain M, Scott D, Addey C, *et al.*. Identification of the immunodominant HY H2-D(k) epitope and evaluation of the role of direct and indirect antigen presentation in HY responses. J Immunol. 2005;175:7209-7217.

90. Bassiri H, Markmann JF, Desai NM, Kim JI, Teh HS, Barker CF. Allograft-Rejection by T-Cell Receptor Transgenic Mice. J Surg Res. 1993;54:437-444.

91. Tyznik AJ, Bevan MJ. The surprising kinetics of the T cell response to live antigenic cells. J Immunol. 2007;179:4988-4995.

92. Chai JG, James E, Dewchand H, Simpson E, Scott D. Transplantation tolerance induced by intranasal administration of HY peptides. Blood. 2004;103:3951-3959.

93. Agulnik AI, Mitchell MJ, Lerner JL, Woods DR, Bishop CE. A mouse Y chromosome gene encoded by a region essential for spermatogenesis and expression of male-specific minor histocompatibility antigens. Hum Mol Genet. 1994;3:873-878.

94. Christensen J, Agger K, Cloos PA, *et al.*. RBP2 belongs to a family of demethylases, specific for tri-and dimethylated lysine 4 on histone 3. Cell. 2007;128:1063-1076.

95. Greenfield A, Scott D, Pennisi D, *et al.*. An H-YDb epitope is encoded by a novel mouse Y chromosome gene. Nat Genet. 1996;14:474-478.

96. Xu J, Deng X, Watkins R, Disteche CM. Sex-specific differences in expression of histone demethylases Utx and Uty in mouse brain and neurons. J Neurosci. 2008;28:4521-4527.

97. Yoon IH, Choi SE, Kim YH, *et al.*. Pancreatic islets induce CD4(+) [corrected] CD25(-)Foxp3(+) [corrected] T-cell regulated tolerance to HY-mismatched skin grafts. Transplantation. 2008;86:1352-1360.

98. Billingham RE, Silvers WK. Induction of tolerance of skin isografts from male donors in female mice. Science. 1958;128:780-781.

99. Billingham RE, Silvers WK. Studies on tolerance of the Y chromosome antigen in mice. J Immunol. 1960;85:14-26.

100. Weissman IL. Transfer of tolerance. Transplantation. 1973;15:265-269.

101. Billingham RE, Silvers WK, Wilson DB. A Second Study on the H-Y Transplantation Antigen in Mice. Proc R Soc Lond B Biol Sci. 1965;163:61-89.

102. Smith RN, Powell AE. The adoptive transfer of pregnancy-induced unresponsiveness to male skin grafts with thymus-dependent cells. J Exp Med. 1977;146:899-904.

103. James E, Scott D, Chai JG, Millrain M, Chandler P, Simpson E. HY peptides modulate transplantation responses to skin allografts. Int Immunol. 2002;14:1333-1342.

104. Sireci G, Barera A, Macaluso P, *et al.*. A continuous infusion of a minor histocompatibility antigen-immunodominant peptide induces a delay of male skin graft rejection. Immunobiology. 2009;214:703-711.

105. Boussiotis VA, Gribben JG, Freeman GJ, Nadler LM. Blockade of the CD28 co-stimulatory pathway: a means to induce tolerance. Curr Opin Immunol. 1994;6:797-807.

106. Adorini L, Appella E, Doria G, Nagy ZA. Mechanisms influencing the immunodominance of T cell determinants. J Exp Med. 1988;168:2091-2104.

107. Perreault C, Roy DC, Fortin C. Immunodominant minor histocompatibility antigens: the major ones. Immunol Today. 1998;19:69-74.

108. Zinkernagel RM, Doherty PC. MHC-restricted cytotoxic T cells: studies on the biological role of polymorphic major transplantation antigens determining T-cell restriction-specificity, function, and responsiveness. Adv Immunol. 1979;27:51-177.

109. Van Bleek GM, Nathenson SG. Isolation of an endogenously processed immunodominant viral peptide from the class I H-2Kb molecule. Nature. 1990;348:213-216.

110. Deng Y, Yewdell JW, Eisenlohr LC, Bennink JR. MHC affinity, peptide liberation, T cell repertoire, and immunodominance all contribute to the paucity of MHC class I-restricted peptides recognized by antiviral CTL. J Immunol. 1997;158:1507-1515.

111. Johnson LL, Bailey DW, Mobraaten LE. Genetics of histocompatibility in mice. II. Survey for interactions between minor (non-H-2) antigens by skin grafting. Immunogenetics. 1980;11:363-372.

112. Johnson LL, Bailey DW, Mobraaten LE. Antigenic competition between minor (non-H-2) histocompatibility antigens. Immunogenetics. 1981;13:451-455.

113. Wettstein PJ, Bailey DW. Immunodominance in the immune response to "multiple" histocompatibility antigens. Immunogenetics. 1982;16:47-58.

114. Wettstein PJ. Immunodominance in the T cell response to multiple non-H-2 histocompatibility antigens. III. Single histocompatibility antigens dominate the male antigen. J Immunol. 1986;137:2073-2079.

115. Grufman P, Wolpert EZ, Sandberg JK, Karre K. T cell competition for the antigen-presenting cell as a model for immunodominance in the cytotoxic T lymphocyte response against minor histocompatibility antigens. Eur J Immunol. 1999;29:2197-2204.

116. Kedl RM, Rees WA, Hildeman DA, *et al.*. T cells compete for access to antigen-bearing antigen-presenting cells. J Exp Med. 2000;192:1105-1113.

117. Kedl RM, Schaefer BC, Kappler JW, Marrack P. T cells down-modulate peptide-MHC complexes on APCs *in vivo*. Nat Immunol. 2002;3:27-32.

118. Choi EY, Christianson GJ, Yoshimura Y, *et al.*. Immunodominance of H60 is caused by an abnormally high precursor T cell pool directed against its unique minor histocompatibility antigen peptide. Immunity. 2002;17:593-603.

119. Yin L, Poirier G, Neth O, Hsuan JJ, Totty NF, Stauss HJ. Few peptides dominate cytotoxic T lymphocyte responses to single and multiple minor histocompatibility antigens. Int Immunol. 1993;5:1003-1009.

120. Wolpert E, Franksson L, Karre K. Dominant and cryptic antigens in the MHC class I restricted T cell response across a complex minor histocompatibility barrier: analysis and mapping by elution of cellular peptides. Int Immunol. 1995;7:919-928.

121. Wettstein PJ, Borson ND, Park JG, McNallan KT, Reed AM. Cysteine-tailed class I-binding peptides bind to CpG adjuvant and enhance primary CTL responses. J Immunol. 2005;175:3681-3689.

122. Arsov I, Vukmanovic S. Altered effector responses of H-Y transgenic CD8+ cells. Int Immunol. 1997;9:1423-1430.

123. Kotturi MF, Scott I, Wolfe T, *et al.*. Naive precursor frequencies and MHC binding rather than the degree of epitope diversity shape CD8+ T cell immunodominance. J Immunol. 2008;181:2124-2133.

124. Iwakoshi NN, Markees TG, Turgeon N, *et al.*. Skin allograft maintenance in a new synchimeric model system of tolerance. J Immunol. 2001;167:6623-6630.

125. Chowdhury NC, Murphy B, Sayegh MH, *et al.*. Acquired systemic tolerance to rat cardiac allografts induced by intrathymic inoculation of synthetic polymorphic MHC class I allopeptides. Transplantation. 1996;62:1878-1882.

126. Griesemer AD, Sorenson EC, Hardy MA. The Role of the Thymus in Tolerance. Transplantation. 2010; 90:465-74.

127. Saborio DV, Chowdhury NC, Hardy MA, Oluwole SF. Maintenance of acquired thymic tolerance to rat isl*et al.*lografts by regulatory/suppressor T cells. Transplant Proc. 1999;31:897.

128. Garrovillo M, Ali A, Depaz HA, *et al.*. Induction of transplant tolerance with immunodominant allopeptide-pulsed host lymphoid and myeloid dendritic cells. Am J Transplant. 2001;1:129-137.

129. Houssaint E, Flajnik M. The role of thymic epithelium in the acquisition of tolerance. Immunol Today. 1990;11:357-360.

130. Agus DB, Surh CD, Sprent J. Reentry of T cells to the adult thymus is restricted to activated T cells. J Exp Med. 1991;173:1039-1046.

131. Gopinathan R, DePaz HA, Oluwole OO, *et al.*. Role of reentry of *in vivo* alloMHC peptide-activated T cells into the adult thymus in acquired systemic tolerance. Transplantation. 2001;72:1533-1541.

132. Chowdhury NC, Fawwaz RA, Oluwole SF. Induction of donor-specific tolerance to rat cardiac and small bowel allografts by intrathymic inoculation of donor T-cells. J Surg Res. 1993;54:368-374.

133. Awong G, LaMotte-Mohs R, Zuniga-Pflucker JC. Key players for T-cell regeneration. Curr Opin Hematol. 2010;17:327-332.

134. Oluwole SF, Chowdhury NC, Fawwaz RA. Induction of donor-specific unresponsiveness to rat cardiac allografts by pretreatment with intrathymic donor MHC class I antigens. Transplantation. 1993;55:1396-1402.

135. Wang B, Maile R, Greenwood R, Collins EJ, Frelinger JA. Naive CD8+ T cells do not require costimulation for proliferation and differentiation into cytotoxic effector cells. J Immunol. 2000;164:1216-1222.

136. Stetson DB, Mohrs M, Reinhardt RL, *et al.*. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. J Exp Med. 2003;198:1069-1076.

137. Wu YP, McMahon E, Kraine MR, *et al.*. Distribution and characterization of GFP(+) donor hematogenous cells in Twitcher mice after bone marrow transplantation. Am J Pathol. 2000;156:1849-1854.

138. Fuchs EJ, Matzinger P. B cells turn off virgin but not memory T cells. Science. 1992;258:1156-1159.

139. Drake DR, 3rd, Ream RM, Lawrence CW, Braciale TJ. Transient loss of MHC class I tetramer binding after CD8+ T cell activation reflects altered T cell effector function. J Immunol. 2005;175:1507-1515.

140. Tan JC, Wadia PP, Coram M, *et al.*. H-Y antibody development associates with acute rejection in female patients with male kidney transplants. Transplantation. 2008;86:75-81.

141. Scott D, Addey C, Ellis P, *et al.*. Dendritic cells permit identification of genes encoding MHC class II-restricted epitopes of transplantation antigens. Immunity. 2000;12:711-720.

142. Obar JJ, Khanna KM, Lefrancois L. Endogenous naive CD8+ T cell precursor frequency regulates primary and memory responses to infection. Immunity. 2008;28:859-869.

143. Kedzierska K, Day EB, Pi J, *et al.*. Quantification of repertoire diversity of influenza-specific epitopes with predominant public or private TCR usage. J Immunol. 2006;177:6705-6712.

144. Casrouge A, Beaudoing E, Dalle S, Pannetier C, Kanellopoulos J, Kourilsky P. Size estimate of the alpha beta TCR repertoire of naive mouse splenocytes. J Immunol. 2000;164:5782-5787.

145. Davis MM, Bjorkman PJ. T-Cell Antigen Receptor Genes and T-Cell Recognition. Nature. 1988;334:395-402.

146. Selin LK, Nahill SR, Welsh RM. Cross-reactivities in memory cytotoxic T lymphocyte recognition of heterologous viruses. J Exp Med. 1994;179:1933-1943.

147. Selin LK, Vergilis K, Welsh RM, Nahill SR. Reduction of otherwise remarkably stable virus-specific cytotoxic T lymphocyte memory by heterologous viral infections. J Exp Med. 1996;183:2489-2499.

148. Seedhom MO, Jellison ER, Daniels KA, Welsh RM. High Frequencies of Virus-Specific CD8(+) T-Cell Precursors. J Virol. 2009;83:12907-12916.

149. Bousso P, Casrouge A, Altman JD, *et al.*. Individual variations in the murine T cell response to a specific peptide reflect variability in naive repertoires. Immunity. 1998;9:169-178.

150. Pewe L, Heard SB, Bergmann C, Dailey MO, Perlman S. Selection of CTL escape mutants in mice infected with a neurotropic coronavirus: quantitative estimate of TCR diversity in the infected central nervous system. J Immunol. 1999;163:6106-6113.

151. Pewe LL, Netland JM, Heard SB, Perlman S. Very diverse CD8 T cell clonotypic responses after virus infections. J Immunol. 2004;172:3151-3156.

152. Butz EA, Bevan MJ. Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. Immunity. 1998;8:167-175.

153. Blattman JN, Antia R, Sourdive DJ, *et al.*. Estimating the precursor frequency of naive antigen-specific CD8 T cells. J Exp Med. 2002;195:657-664.

154. Whitmire JK, Benning N, Whitton JL. Precursor frequency, nonlinear proliferation, and functional maturation of virus-specific CD4+ T cells. J Immunol. 2006;176:3028-3036.

155. Moon JJ, Chu HH, Pepper M, *et al.*. Naive CD4(+) T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. Immunity. 2007;27:203-213.

156. Haluszczak C, Akue AD, Hamilton SE, *et al.*. The antigen-specific CD8+ T cell repertoire in unimmunized mice includes memory phenotype cells bearing markers of homeostatic expansion. J Exp Med. 2009;206:435-448.

157. Lissina A, Ladell K, Skowera A, *et al.*. Protein kinase inhibitors substantially improve the physical detection of T-cells with peptide-MHC tetramers. J Immunol Methods. 2009;340:11-24.

158. Furmanski AL, Ferreira C, Bartok I, *et al.*. Public T cell receptor beta-chains are not advantaged during positive selection. J Immunol. 2008;180:1029-1039.

159. Margulies M, Egholm M, Altman WE, *et al.*. Genome sequencing in microfabricated high-density picolitre reactors. Nature. 2005;437:376-380.

160. Rothberg JM, Leamon JH. The development and impact of 454 sequencing. Nat Biotechnol. 2008;26:1117-1124.

161. Rice J, Buchan S, Dewchand H, Simpson E, Stevenson FK. DNA fusion vaccines induce targeted epitope-specific CTLs against minor histocompatibility antigens from a normal or tolerized repertoire. J Immunol. 2004;173:4492-4499.