Studies on the Molecular Mechanism of T cell Triggering and Detection and Characterization of Antigen-Specific T cells

By
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B.S. Chemical Engineering
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Submitted to the Department of Chemistry in
Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY
In Biological Chemistry

at the

Massachusetts Institute of Technology
February, 2005

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ABSTRACT

T cells recognize cognate peptide antigen in complex with major histocompatibility complex (MHC) proteins; however, the molecular events which trigger T cells upon binding of MHC-peptide to the T cell receptor (TCR) are unclear. To gain a better understanding of this mechanism, $CD4^+$ T cells were treated with soluble class II MHC-peptide monomers and oligomers instead of antigen-presenting cells, and the activation response was monitored. These experiments showed a requirement for multivalent TCR engagement to induce activation. Mathematical modeling of oligomeric equilibrium binding states indicates that the level of the T cell response correlates with the predicted number of receptor cross-links formed by soluble MHC oligomers. Treatment of $CD8^+$ T cells with class I MHC monomers and oligomers revealed a confusing process whereby peptide derived from soluble MHC reagents was loaded on to endogenous MHC complexes on the T cell surface and re-presented to other cells. When this method of stimulation was circumvented, multivalent TCR engagement was found to be required for $CD8^+$ T cell activation, similar to $CD4^+$ T cells. In both types of cells, monomeric MHC-peptide binding can compete off activation responses induced by MHC-peptide oligomers in the same mixture, further emphasizing the non-productive nature of monovalent TCR engagement. However, exquisite antigen sensitivity might be achieved on the surface of an APC due to the contribution of non-activating MHC-peptide complexes. Even though solubme monomeric MHC does not cause activation, soluble heterodimeric MHC-peptide ligands with only one activating peptide induce T cell activation responses.

Identification and characterization of T cell epitopes derived from infectious agents or vaccines can greatly enhance the ability to study and eventually direct the immune response. This work contains a description of a novel technique for identifying and characterizing specific T cell responses in parallel. The system involves incubating heterogeneous T cell mixtures with artificial antigen-presenting microarrays, which include immobilized cytokine capture antibodies, co-stimulatory molecules, and MHC complexes presenting many different potential T cell epitope peptides. These microarrays can rapidly, conveniently, and sensitively detect antigen-specific T cells and characterize the functional responses to many different epitopes in parallel in a location-dependent manner.

Thesis Supervisor: Lawrence J. Stern
Title: Associate Professor

ACKNOWLEDGEMENTS

There are many people I would like to thank for making this thesis possible.

Larry Stern, my advisor, has been an excellent resource for ideas and for helping me work through my own ideas. He has allowed me the freedom to direct my work, and given me the opportunity to try all kinds of approaches to address questions in my research. I really appreciated learning from and working with him.

I would also like to thank the members of my thesis committee, Professor Bruce Tidor, Professor Alexander Klibanov, and Professor Douglas Lauffenburger from Massachusetts Institute of Technology.

I have been part of several helpful collaborations, including Qing Ge in Jianzhu Chen's lab at MIT who provided expert transgenic mouse work, Herman Eisen at MIT who was available for many discussions on T cell activation, Phillip Norris at Blood Systems Research Institute in San Francisco who has characterized some interesting HIV-specific T cell reactivity, Melissa Precopio from Katherine Luzuriaga's lab at UMass Medical School who worked on Epstein Barr Virus T cell reactivity, Kenya Prince-Cohane, Joyce Pepe, and Robin Brody from the UMass Medical School MHC tetramer facility who collaborated on class I MHC protein production, John Cruz and Alan Rothman provided T cell lines and peptides for studying the response to vaccinia infection, and Airiel Davis from Cynthia Chambers' lab at UMass Medical School who provided expert help in mouse dissection and cell isolation.

The members of the Stern lab have been extremely helpful throughout my graduate experience. Mia Rushe was a great resource on every experimental protocol. Jenny Cochran and Tom Cameron brought to light many of the issues I have pursued in my own projects. Iwona Strug has helped me with all my synthetic chemistry issues. Sriram Chitta has been a great resource for issues related to cell culture and cellular assays, and Alexander Sigalov was always available for extended discussion of T cell activation mechanisms. Zarixia Zavala-Ruiz and Greg Carven, my fellow graduate students from MIT, were always there for discussing ideas and protocols—or anything else. Liying Lu and Guoqi Li have helped with development of large scale class II MHC and class I MHC production, respectively. Walter Demkowicz provided some greatly needed help with T cell culture at a critical point in my research. I have also benefited from the companionship of Dikran Aivazian, Tanyel Kiziltepe, Todd Thompson, Sulabha Pathak, Marcia Ross, Corrie Painter, Tina Nguyen, Walter Kim, Prasanna Venkatraman, Lauren Angelo, Christian Walker, and many others, past and present.

Finally, I would like to thank my family. My extended family in Connecticut was a valuable supportive resource for me, and I'd like to specifically mention my Grandmother and my Aunt Linda who are not able to see this accomplishment. My parents and brother in Ohio have been endlessly patient and understanding, and invaluable for my mental health. I would like to give the most thanks to my husband, Steve, who was not only a resource for me on any programming concerns I had, but also put up with me more than anyone else.

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Abbreviations used in this thesis

APC	Antigen-Presenting Cell or Allophycocyanin (fluorescent label)
BCR	B Cell Receptor
BSP	Biotin ligase Substrate Peptide
DTT	1,4-Dithiothreitol
ELISA	Enzyme-Linked Immunoorbent Assay
EBV	Epstein Barr Virus
FITC	Fluorescein Isothiocyanate
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
IFN-γ	Interferon-gamma
IL-2, -4, etc.	Interleuken-2, -4, etc.
ITAM	Immunoreceptor Tyrosine Activation Motif
MALDI	Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry
MHC	Major Histocompatibility Complex
MW	Molecular Weight
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-Buffered Saline
PBST	Phosphate-Buffered Saline containing 0.01% Tween-20 detergent
PE	R-Phycoerythrin
SA	Streptavidin
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
TCR	T Cell Receptor
TNF-α	Tumor Necrosis Factor alpha

I. Introduction

The immune system consists of a conglomeration of defenses that serves to protect an organism from invasion by foreign material. In this chapter, I will give an overview of the different aspects of the immune system in mammals. I will touch upon the strategy of the adaptive immune system and specifically focus on the role of T cells in responding to infection or disease. I will discuss the mechanism of antigen recognition by T cells in the context of antigen-presenting cells (APCs) and major histocompatibility proteins (MHCs). Finally, I will discuss the importance of T cell epitope identification in the study of diseases and the methods employed to address this issue. For more information on these and other general topics in immunology, I would direct the reader to consult a textbook on the subject (Janeway, 2004).

I.A. The Immune System

The immune system consists of many layers, each designed to protect an organism from foreign invasion. These layers span from very crude to extremely specific and specialized, and the system includes organs, cells, and proteins. All of these components are designed to segregate "self" from "non-self." A would-be pathogen must be able to avoid all of these defenses in order to cause stable infection and disease. I have outlined some of the layers of defense here.

I.A.1 Anatomical and Physiological Barriers

The first line of defense protecting any organism includes elements that serve to disallow entry and survival of anything foreign to the organism. For example, skin and mucous membranes form physical barriers against entry, while enzymes present in tears and saliva act to terminate potential infections. Additional factors such as low pH in the stomach and partial

pressure of oxygen in the blood (oxygen tension) create a hostile environment for invaders that do enter the organism. These primary defenses do an excellent job of excluding most environmental threats.

I.A.2 Innate Immunity

Should a pathogen surpass the physical barriers, the body possesses fast-response cellular and protein-based defenses that target general molecular patterns not present in vertebrates. These molecules include particular lipids, carbohydrates, peptidoglycans present in bacterial or yeast cell walls, or polynucleotides. Cells called phagocytes, including macrophages and dendritic cells, recognize these molecular patterns and ingest and break down the pathogens that carry them, and also secrete cytokines to recruit other immune cells to the site of infection. In addition, a series of soluble proteins referred to collectively as complement have the ability to augment the destruction of pathogens either directly or with the aid of antibody binding or by enhancing phagocytic cell recognition.

While these responses are rapid, they are often not effective enough to eliminate an infection, and can only contain the infection until the slower adaptive immune response can begin.

I.A.3. Adaptive Immunity

The strategy of the adaptive immune response is to create cells that can recognize tremendously diverse molecular patterns, taking care to eliminate any immune cells that target "self" patterns. These cells are slow to be activated upon their first encounter with their cognate molecule or antigen because of the need for selection of the appropriate specificities, but eventually the recognition results in proliferation of cells that specifically recognize the problematic pathogen and induction of a variety of effector functions that hopefully lead to

clearance of the infection. After the challenge, some of the cells that specifically recognize that pathogen remain in the body as memory cells. If the same pathogen should ever try to re-infect, the memory cells would respond much more quickly, fighting the infection before it can gain a foothold. This memory response is extremely valuable, and memory-like responses to certain diseases can be induced by vaccination without ever allowing an actual infection to take place.

The method by which this extraordinary diversity, specificity, and memory is achieved is through genetic recombination. Immune cells, specifically B cells and T cells, express surface receptors that recognize antigen called the B cell receptor (BCR) and T cell receptor (TCR), respectively. A developing B cell or T cell rearranges a unique antigen receptor from a set of genes by splicing different sections together, often with hypervariation at the junctions between sections (see Figure I.1). This process leads to ~10^{15} possible T cell receptors with different specificities (reviewed in (Marrack and Kappler, 1988)). The recombined receptor is then expressed at the surface, where it can recognize its unique antigen by binding to it.

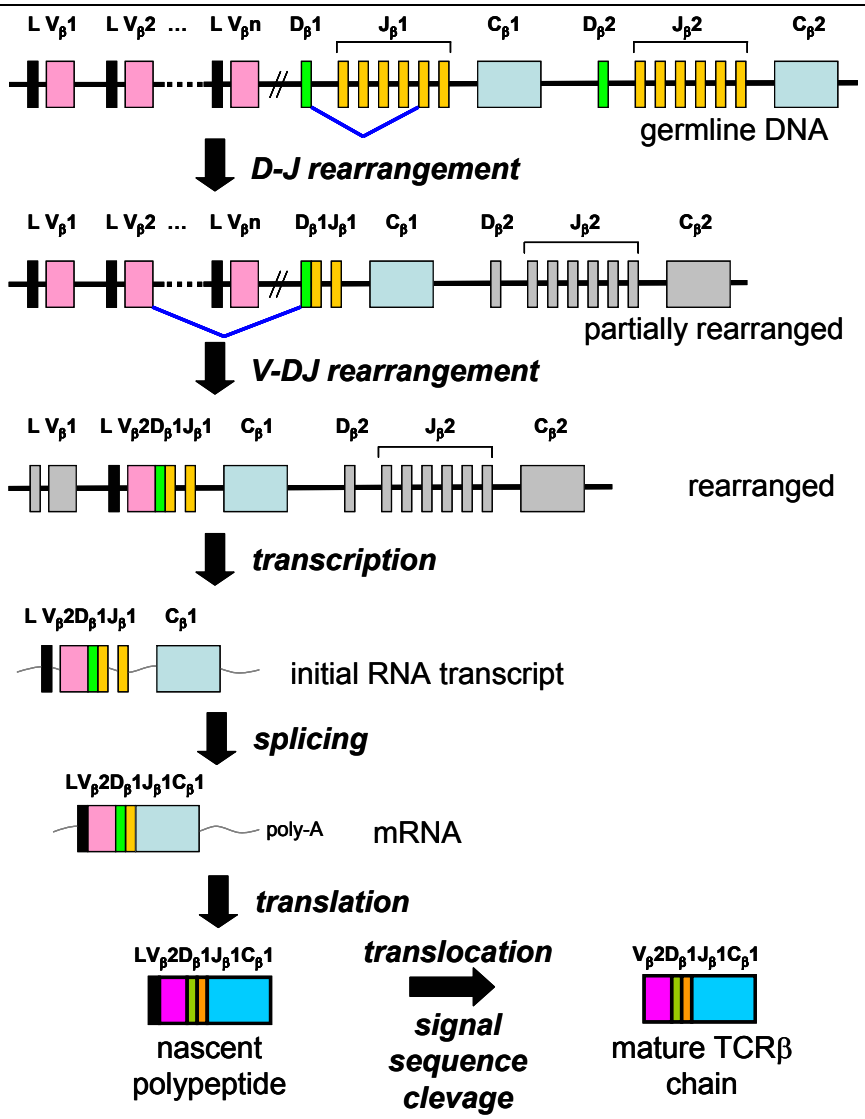


FIGURE I.1 DNA rearrangement creates great diversity in antigen receptors. Developing B and T cells rearrange their chromosomal DNA to combine different variable (V), joining (J), diversity (D) genes, and a single constant (C) gene is spliced into a final polypeptide. An example of this rearrangement is shown for the T cell receptor beta chain. The germline DNA is shown at the top. First, D-J rearrangement occurs, followed by V-DJ rearrangement, excising and discarding the intervening DNA sequences at each step. Then, the sequence is transcribed into RNA, where further splicing results in a final mRNA sequence. That sequence is then translated into a polypeptide, which is finally trimmed of its leader (L) sequence to give the final beta chain. Similar rearrangements occur for the T cell receptor alpha chain and for the B cell receptor heavy and light chains. In addition, further diversity arises from N-nucleotides added at junctions increasing randomness. For B cells, somatic hypermutation occurs after activation leading to affinity maturation of the progeny's B cell receptor for the recognized antigen.

B cells are an important part of the adaptive immune response. These cells originate in the bone marrow where they develop and individually rearrange their receptors. The structure of

the B cell receptor contains two identical binding sites for antigen, each composed of the variable regions of one heavy chain and one light chain, respectively. The receptor also contains associated subunits that do not bind antigen called Igα and Igβ which exist as disulfide-linked heterodimers, and have large intracellular immunoreceptor tyrosine activation motif (ITAM) domains, which can become phosphorylated and initiate signaling cascades upon receptor triggering. A schematic representation of the B cell receptor can be seen in Figure I.2 A. Once the B cell has rearranged and expressed its receptor, it is checked for autoreactivity. B cell clones that respond to autoantigens are deleted. The clones that are not self-reactive are released to monitor the body for their specific foreign antigens. If a B cell encounters a soluble antigen that it recognizes by binding specifically to its receptor, it becomes activated and proliferates, during which a process called somatic hypermutation occurs, resulting in further changes to the BCR sequence. The subsequent population contains clones with a higher affinity for the antigen, a phenomenon known as affinity maturation. The B cells can further differentiate into plasma cells which secrete soluble versions of the B cell receptor, known as antibodies (Figure I.2 B).

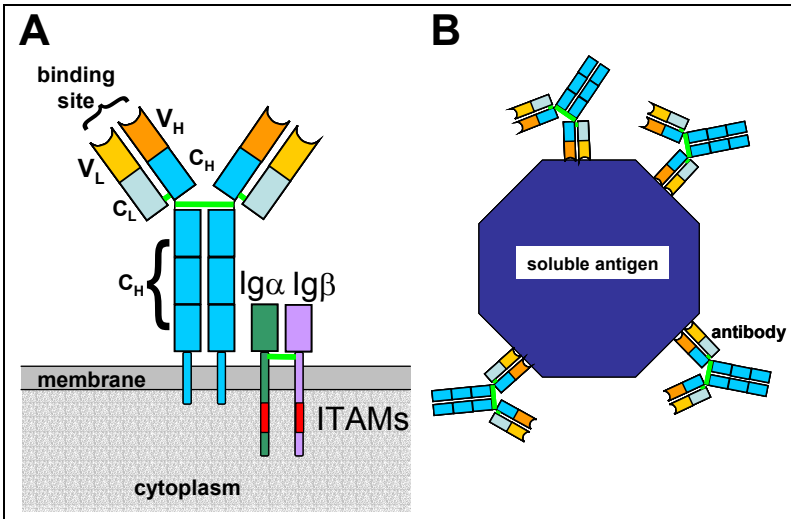


FIGURE I.2 The B cell antigen receptor. (A) The B cell receptor consists of two transmembrane heavy chains linked to each other by disulfide bonds (green), and two light chains, each linked to one heavy chain by a disulfide bond. The two identical antigen binding sites are comprised of the variable regions (V_H and V_L) of both the heavy chain and the light chain. The antigen binding portion of the receptor is associated with signaling subunit heterodimers containing disulfide-linked Igα and Igβ. These subunits have intracellular ITAM domains which can interact with cytoplasmic signaling proteins. The B cell becomes activated when the receptor binds to soluble antigen expressing multiple binding epitopes. (B) Upon activation of the B cell, it can produce an alternatively spliced BCR without transmembrane domains. This results in a soluble, secreted version known as an antibody. These antibodies bind to their antigens, labeling them for immune clearance.

T cells also play an important role in adaptive immunity. They originate in the bone marrow like B cells; however, they then migrate to the thymus for further development and differentiation. Unlike B cells which recognize soluble antigens, T cells recognize small antigens presented in complex with cell-surface major histocompatibility complex (MHC) proteins on the surface of other cells. During development, each T cell clone recombines its receptor similarly to B cells, creating a unique specificity, that must be within certain limits of affinity for self-antigen to avoid deletion. High affinity for self antigen results in autoreactivity, while low affinity suggests that the T cell receptor can not recognize the antigen-presenting MHC protein itself. T cells will be the focus of the following sections.

I.B. Antigen Recognition by T cells

I.B.1 The T cell receptor

The T cell receptor contains an alpha (α) and beta (β) chain that together form a single binding site for antigen. The alpha and beta chains are associated with two membrane heterodimers (delta-epsilon, δε, and gamma-epsilon, γε) that contain one cytoplasmic ITAM on each chain, and a zeta-zeta (ζ_2) homodimer which contains three intracellular ITAM domains on each chain and a very small extracellular portion. (Figure I.3). The signaling subunits of the TCR complex are collectively known as CD3. The exact stoichiometry of the different subunits of the T cell receptor has been in dispute for many years. Convincing evidence has been shown that the complex assembles with the stoichiometry $\alpha\beta\delta\varepsilon\gamma\varepsilon\zeta_2$ (Call et al., 2002); however, the possibility of altered arrangements based on activation or developmental state is not ruled out (Fahmy et al., 2001; Fernandez-Miguel et al., 1999; Garcia, 1999).

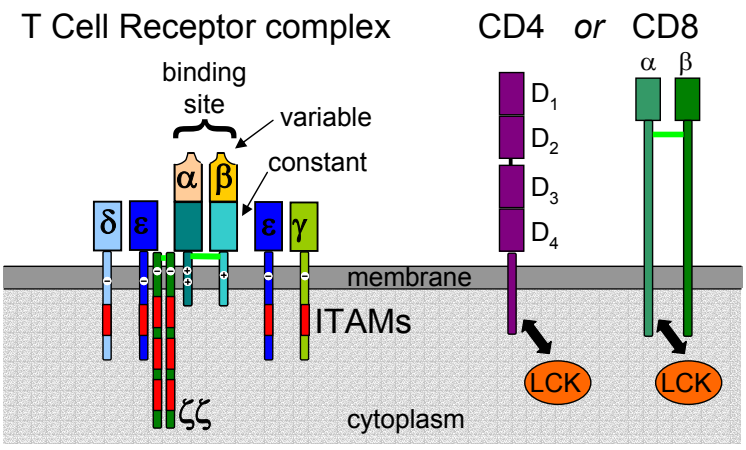


FIGURE I.3 The T cell antigen receptor. The T cell receptor consists of two transmembrane chains, α and β linked to each other by a membrane-proximal disulfide bond (green), which form the antigen-specific binding site. The binding chains are associated with two heterodimers δε and γε and a disulfide-linked ζζ homodimer, collectively referred to as CD3. These subunits contain signaling ITAM domains. The complex is held together by conserved transmembrane charge associations—positive charges on the αβ chains associate with negative charges on the CD3 chains. T cells also express a co-receptor for the MHC, either CD4 or CD8, which can associate with the cytoplasmic kinase LCK.

T cells recognize small peptides derived from antigen in complex with MHC molecules on the surface of APCs. When T cells detect specific antigen-MHC complexes through binding, cytoplasmic signaling cascades are initiated, leading to T cell receptor subunit phosphorylation, increased calcium flux, proliferation and other effector functions specific to the type of T cell

(Cantrell, 1996; Qian and Weiss, 1997; Wange and Samelson, 1996). Some common indicators used to measure T cell activation are TCR downregulation (Valitutti et al., 1997), and upregulation of cell-surface proteins such as CD69 (Testi et al., 1994), CD25 (Waldmann, 1989), and CD71 (Ponka and Lok, 1999).

T cells also express a co-receptor, either CD8 or CD4, which binds to MHC proteins in an antigen non-specific manner. There are two main classes of MHC molecules: class I MHCs which present primarily intracellular antigens to $CD8^+$ T cells, and class II MHCs which present primarily extracellular antigens to $CD4^+$ T cells. The co-receptor, which associates with the intracellular kinase Lck, can bind to its class of MHC regardless of the peptide antigen is presented. $CD8^+$ T cells, usually cytotoxic T lymphocytes (CTLs), recognize 8-11 amino acid long peptides in complex with class I MHCs, which are present on the surface of all nucleated cells. When a $CD8^+$ T cell becomes activated by a specific class I MHC-peptide complex on the surface of an antigen-presenting cell (APC), it initiates processes to kill or lyse the cell with which it is interacting. This activity is useful in eliminating virally infected cells or possibly mutated or cancerous cells. $CD4^+$ T cells, usually T helper cells (T_H), recognize 9-25 amino acid long peptides presented in complex with class II MHCs, which are present on the surface of professional antigen presenting cells, including macrophages, dendritic cells, and B cells. Upon recognition of its specific MHC-peptide complex, a $CD4^+$ T cell initiates effector functions such as cytokine secretion and cell-surface protein upregulation that can recruit and activate other immune cells or induce B cells to produce antibodies.

In addition to these main two types of T cells, there are further delineations between members of these sets. For example, T-helper cells can secrete different panels of cytokines if they are T_H1 cells (IL-2, IFN-γ, and TNF-α) as opposed to T_H2 cells (IL-4, IL-5, IL-6, and IL-

10). Also, an inhibitory subset called T-regulatory cells has been identified that downregulates an immune response in an antigen-specific manner (O'Garra and Vieira, 2004). Some T cells have also been identified that express a γδ antigen-binding receptor instead of αβ, and exhibit specificity to non-classical MHC-bound antigens (Holtmeier, 2003; Kabelitz et al., 2000; Kabelitz and Wesch, 2003). Finally, a novel lymphocyte subset related to innate immune cells, Natural Killer T cells, has been identified to play a role in cancer, autoimmunity, infection, and transplantation. These cells use a semi-invariant T cell receptor to recognize non-typical antigens such as glycolipids in complex with the non-classical class I-like molecule CD1d (Seino and Taniguchi, 2004; Swann et al., 2004).

I.B.2 Antigen Presentation by the Major Histocompatibility Complex (MHC)

The Major Histocompatibility Complex (MHC) is a polymorphic cell-surface glycoprotein expressed on antigen-presenting cells. These proteins form complexes with short peptides derived from self or antigenic proteins which can be recognized by T cells through TCR binding. There are two main classes of MHC proteins: class I and class II (see Figure I.4 A-B). The molecules are structurally related to one another, consisting of immunoglobulin-like lower domains and an upper domain that binds peptides between two alpha helices atop a beta-sheet floor, orienting residues for TCR recognition (reviewed in (Stern and Wiley, 1994)). Class I MHCs have a single heavy chain that crosses the cell membrane and associates with the β_2-microglobulin (β_2M) light chain. Short peptides are anchored in conserved N-terminal and C-terminal pockets in the peptide binding groove, disallowing very long peptides from binding (Figure I.4 C). Class II MHCs have two roughly equal sized chains, both with transmembrane spans, that form the complex. Longer peptides (9-25 amino acids long (Chicz et al., 1992; Rudensky et al., 1991)) can be bound, and the termini may extend out from both ends of the

peptide binding groove (Figure I.4 D). The peptides in class II MHCs are held in a polyproline type II conformation by conserved hydrogen bond interactions with the peptide backbone, and the binding specificity is defined by pockets in the protein at positions P1, P4, P6, and P9. Each type of MHC can bind a wide variety of peptides, leading to many potential T cell epitopes being presented by a single molecule. Different MHC alleles have slightly different anchor residue specificities, leading to a different spectrum of epitopes being presented. Peptide binding prediction algorithms have been developed in an attempt to determine which pieces of an antigenic sequence may be presented by a given MHC allele with varying success (Hammer et al., 1994; Rammensee et al., 1995; Southwood et al., 1998; Sturniolo et al., 1999).

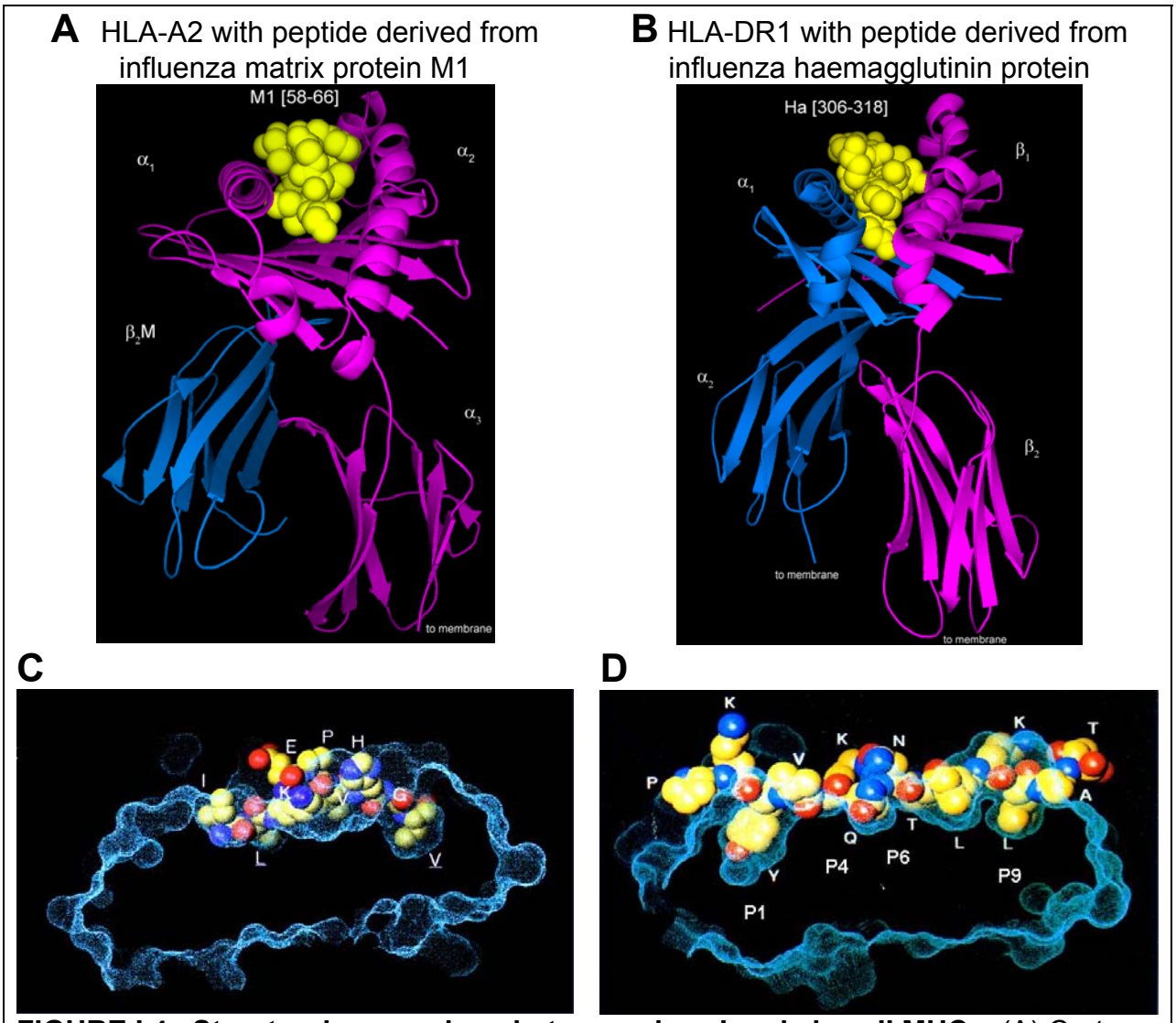


FIGURE I.4. Structural comparison between class I and class II MHCs. (A) Cartoon representation of the structure of human class I MHC HLA-A2 in complex with a peptide derived from the influenza matrix protein, M1. Heavy chain is shown in magenta, light chain is shown in blue, and peptide is shown in yellow as spheres. (B) Cartoon depiction of the structure of human class II MHC HLA-DR1 in complex with a peptide derived from the influenza haemagglutinin protein. Alpha chain is shown in blue, beta chain is shown in magenta, and peptide is shown in yellow as spheres. (C-D) Side view surface topology of the peptide-binding domain is shown with space-filled representations of bound peptide. (C) The class I MHC HLA-A2 bound to a peptide derived from the reverse transcriptase protein of HIV. Note that the peptide termini are buried in the MHC. (D) HLA-DR1 is shown bound to hemagglutinin-derived peptide from above. In contrast to class I, the class II MHC peptide binding groove is open-ended, and peptide binding specificity is determined by residues in several distinct pockets along the groove, labeled P1, P4, P6 and P9. (C-D) are reproduced from Stern & Wiley, Structure, 1994.

Class I MHC proteins are synthesized in the endoplasmic reticulum (ER) of all nucleated cells, where they are loaded with short peptides, 8-11 amino acids long. These peptides are brought into the ER from the cytoplasm by the ATP-dependent transporter associated with antigen processing (TAP). Often these peptides are derived from proteosomally degraded proteins, and can come from intracellular antigens such as viral components. Once the complex has loaded peptide, it is translocated through the golgi apparatus to the surface of the cell for recognition by $CD8^+$ T cells (Figure I.5 A). The process is reviewed in (Pamer and Cresswell, 1998).

Class II MHC proteins are also synthesized in the ER and immediately associate with the invariant chain (Ii), which places a loop in the peptide binding groove of the MHC. The two proteins are trafficked together to the endosome, where the invariant chain is trimmed to leave a small, loosely bound peptide called CLIP in the MHC binding groove. Peptides derived from extracellular antigens that have been endocytosed and degraded in lysosomes are also present in the endosome. With the help of the MHC homologue protein HLA-DM, the class II MHC exchanges CLIP for other peptides in the endosome. The resulting complex is then sent to the cell surface for recognition by $CD4^+$ T cells (Figure I.5 B). This process is reviewed in (Watts, 1997).

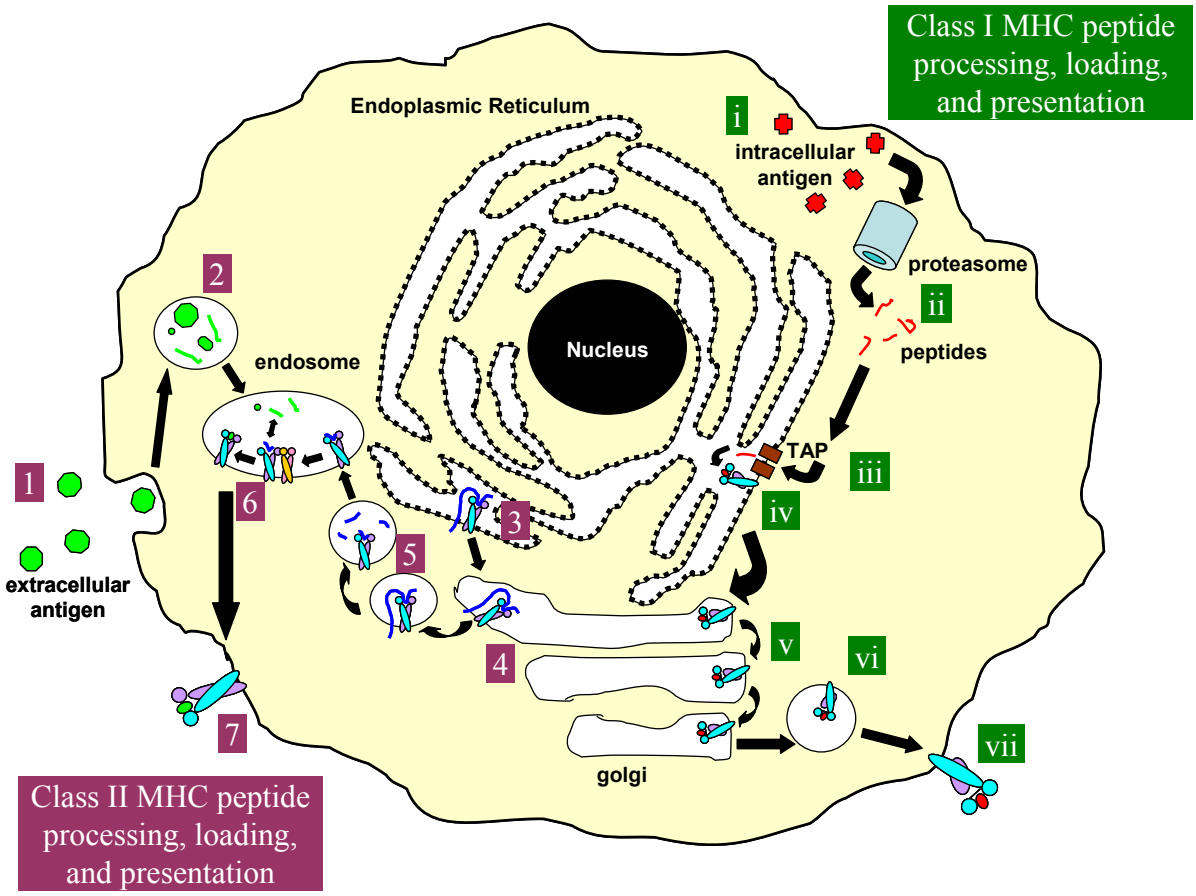


FIGURE I.5 The antigen processing, loading, and presentation pathways for class I and class II MHCs.

Class II MHC processing pathway:

1. Extracellular antigen is internalized via receptor-mediated endocytosis, phagocytosis, or pinocytosis.
2. Antigenic particles are broken down in low pH compartments by proteases.
3. Class II is synthesized in the ER with the Invariant Chain (Ii), which places a loop in the peptide binding groove of the MHC.
4. The MHC-Ii complex is trafficked through the golgi into endosomes.
5. The Ii protein is cleaved, leaving a small peptide fragment called CLIP in the MHC binding site.
6. The MHC-CLIP complex and antigen fragments meet in the endosome. HLA-DM, an MHC homologue, aids in peptide exchange. CLIP is released and antigenic peptide is bound.
7. The MHC-peptide complex is trafficked to the surface of the cell for presentation to $CD4^+$ T cells

Class I MHC processing pathway:

i. Intracellular antigen in the cytoplasm is tagged for degradation. These may include viral components or self proteins.
ii. Antigenic proteins are broken down into peptides by the proteasome.
iii. Peptides are pumped from the cytosol into the ER by TAP.
iv. Peptide is loaded onto newly synthesized class I MHC.
v. The class I MHC-peptide complex is trafficked to the golgi.
vi. The MHC-peptide buds off of the golgi in a vesicle.
vii. The MHC-peptide is sent to the cell surface for presentation to $CD8^+$ T cells

I.B.3 Other interactions between T cells and APCs (antigen non-specific)

While antigen-specific recognition is controlled by TCR binding to MHC-peptide complexes on the surface of APCs, it is not the only protein-protein interaction involved in the interface between the two cells. Other interactions contribute to both binding and signaling regardless of the antigenicity of the MHC-peptide.

Adhesion molecule interactions are some of the first interactions to form between the T cell and the APC. Molecules on the T cell including LFA-1 (CD11a) and LFA-2 (CD2) bind to ICAMs and LFA-3 (CD58) on the surface of APCs (Pribila et al., 2004). These interactions aid in T cell stimulation in response to antigenic stimuli, but do not cause T cell triggering without the presence of stimulatory MHC-peptide complexes.

Second, the MHCs on the surface of the APC which are in complex with non-antigenic peptide still seem to have some effect on the activation of the T cell. The precise contribution of these non-stimulatory complexes is not clear, but the presence of excess null MHC-peptide complexes seems to aid in the recognition of stimulatory MHC-peptide complexes (Irvine et al., 2002; Wulfing et al., 2002).

Third, the co-receptor expressed by the T cells is able to bind to the MHC in a distal site to the peptide binding groove. The co-receptor, CD4 or CD8, can associate intracellularly with the kinase Lck, which is thought to be involved in signaling cascades. It is unknown precisely what the contribution of the co-receptor is to the overall signaling mechanism. However, binding of co-receptor to the MHC can increase the overall affinity of the T cell for the complex, and may lower the threshold for antigenic MHC-peptide complexes required for T cell responses, or it may play a more direct role by bringing the co-receptor cytoplasmic domain associated with

Lck into the proximity of the TCR complex. (Delon et al., 1998; Garcia et al., 1996; Irvine et al., 2002; Luescher et al., 1995; Purbhoo et al., 2001; Renard et al., 1996; Zamoyska, 1998).

Finally, the T cell receives a number of co-stimulatory signals through cell-surface molecules such as CD28 and CTLA-4. These molecules bind to a class of co-stimulatory molecules called B7 on the surface of APCs, and the resultant signals are important for amplifying and then later downregulating the T cell response (Chambers, 2001; McAdam et al., 1998; Schwartz, 1992; Wulfing et al., 2002).

These interactions together make it difficult to dissect out the specific molecular events leading to T cell triggering by antigenic MHC-peptide complexes.

I.B.4 The Molecular Mechanism of T cell antigen recognition

The molecular trigger that causes T cell activation by foreign antigen presented by an APC has been problematical to determine. In the case of B cells which recognize soluble antigens, it has been possible to study the mechanism of activation by supplying antigen with defined epitope valencies; in those systems, multivalent antigen engagement has been shown to be crucial (Metzger, 1992; Siraganian et al., 1975). However, in the case of T cell stimulation by an antigen-presenting cell, antigen receptor binding to MHC-peptide complex alone does not define the interaction. The T cell can also interact with other non-stimulatory MHC-peptide complexes (either with the TCR or co-receptors CD4 or CD8) as well as co-stimulatory and adhesion molecules. Thus, it is difficult to determine specifically what features of the antigenic MHC-peptide binding cause the T cell to become activated.

In an attempt to uncover the mechanism of TCR signaling, many different techniques have been applied. It had been shown that a specific T cell clone can be activated by adding APCs pulsed with the appropriate chemically synthesized peptide (Lamb et al., 1982), but it was

initially difficult to produce soluble MHC-peptide reagents. Experiments done using peptide on APCs to stimulate T cells gave some insight into sensitivity (Brower et al., 1994; Reay et al., 2000; Sykulev et al., 1995; Sykulev et al., 1996), but were ultimately complicated by both the myriad of antigen non-specific interactions present between the cells and also the uncertainty of how (and how many) MHC-peptide complexes were being presented. Many studies were conducted treating T cells with antibodies against the T cell receptor (Kappler et al., 1983; Kaye et al., 1983; Yoon et al., 1994), or by creating chimeric receptors expressing TCR signaling subunit intracellular domains with unrelated extracellular domains that could be crosslinked with other ligands (Irving and Weiss, 1991; Letourneur and Klausner, 1991). Together, these studies suggested that crosslinking of T cell receptor complexes or subunits via multivalent ligand binding was sufficient to initiate signaling processes.

As soluble MHC-peptide reagents became more easily available, studies were conducted using soluble monovalent and multivalent arrangements of MHC-peptide complexes to study T cell clones or TCR transgenic mouse T cells (reviewed in (Cochran et al., 2001a)). Soluble MHC-peptide monomers were found to bind weakly to T cell receptors ($K_d \sim 10^{-6}$-10^{-4}M), and exhibited short half-lives (1-100 sec.) (Crawford et al., 1998; Davis et al., 1998). Oligomeric MHC-peptide reagents of defined sizes were used to show that for both $CD4^+$ and $CD8^+$ T cells, dimers or higher order oligomers were able to stimulate responses (Abastado et al., 1995; Boniface et al., 1998; Casares et al., 1999; Cochran et al., 2000; Daniels and Jameson, 2000; Doucey et al., 2001), although monomeric class I MHC reagents were shown in some studies to activate CD8 T cells in a co-receptor dependent manner (Delon et al., 1998).

Another technique applied to this issue has been confocal microscopy of the real-time immune response using fluorescently-labeled proteins. Dramatic images of a large

supramolecular activation complex (SMAC), termed the immunological synapse, were observed between T cells and APCs or supported bilayers containing molecules found on the APC cell surface (Dintzis et al., 1976; Grakoui et al., 1999; Monks et al., 1998). These supramolecular clusters consisting of a central region (C-SMAC) of TCR and MHC and a peripheral region (P-SMAC) containing adhesion molecules were thought to be related to T cell activation. However, signaling events have been seen without formation of the immunological synapse (Zaru et al., 2002), and CTL activity in $CD8^+$ cells doesn't require formation of these supramolecular structures (Purbhoo et al., 2004).

The high sensitivity of $CD8^+$ T cells suggested by target lysis without formation of an immunological synapse and at extremely low peptide antigen levels approaching one per cell (Sykulev et al., 1996), creates an apparent sensitivity difference between them and $CD4^+$ T cells, which can recognize a single antigenic peptide on an APC, but require higher antigen levels for functional activity (Irvine et al., 2002). It is unknown whether some these differences are due to differential co-receptor involvement or potentially even a different signaling mechanism.

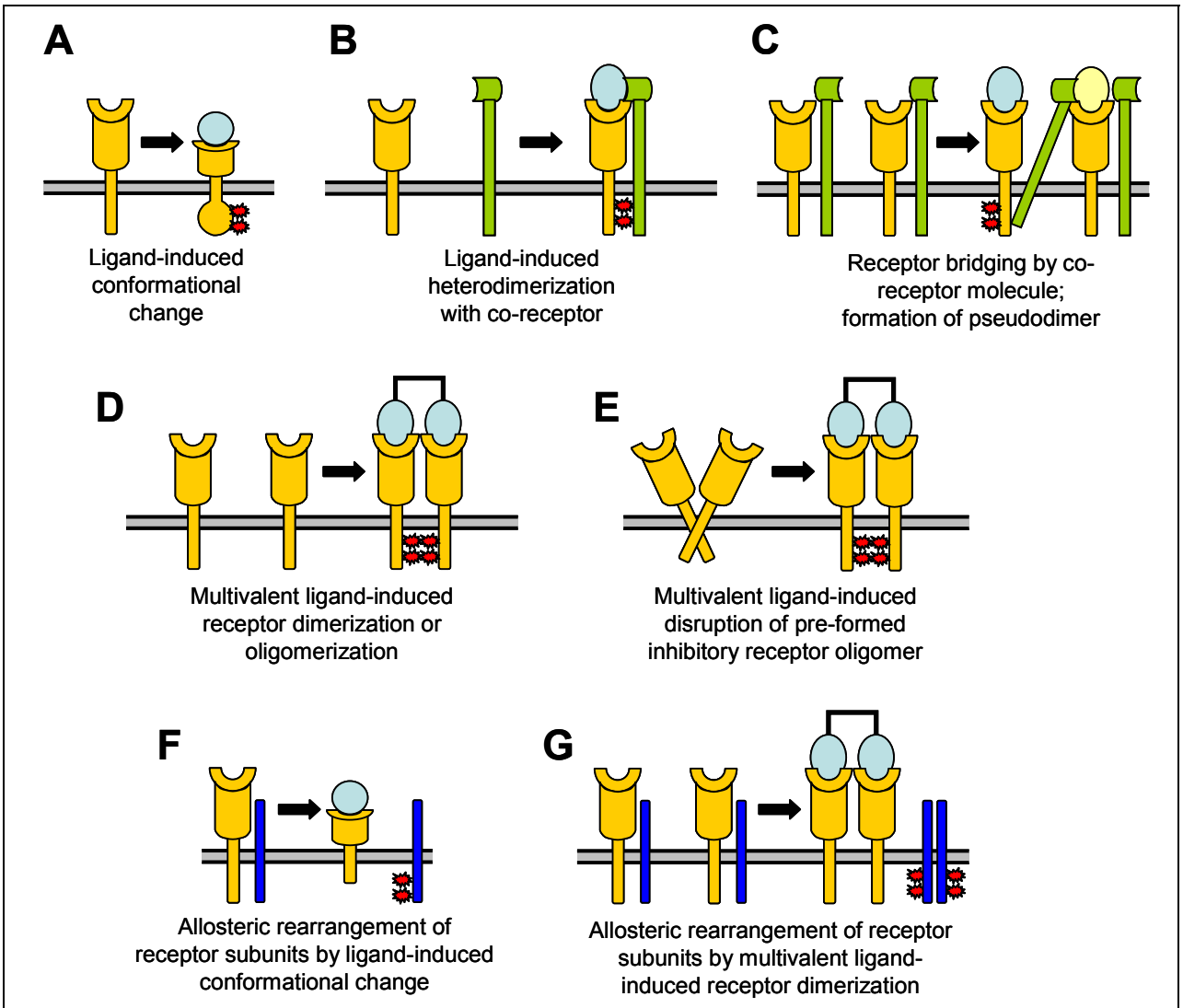


FIGURE I.6. Possible mechanisms for antigen-specific activation through the T cell receptor. The T cell antigen-binding receptor is shown in gold. Activating MHC-peptide complex is shown as a light blue oval, while non-activating MHC-peptide complex is shown in yellow. Co-receptor is shown in green as a tall stalk with a binding site near the top. TCR complex subunits are shown where relevant to the mechanism as dark blue stalks.

Many possible models for T cell triggering through the TCR have been proposed. These are outlined in Figure I.6. First, a mechanism common to seven transmembrane helix proteins which activate G proteins (Wess et al., 1997) describes a conformational change upon extracellular ligand binding that is transmitted through the membrane (Figure I.6 A). This mechanism has seemed unlikely for T cell triggering, as it is difficult to imagine a single

conserved conformational change resulting from diverse MHC-peptide stimuli binding to genetically dissimilar receptor binding domains. Additionally, the intracellular domains of the antigen binding chains (α and β) are very small, and signaling through the receptor is carried out through non-covalently associated CD3 chains (ε, δ, γ, and ζ) rather than the binding chains. In fact, multiple crystal structures of MHC-peptides bound to T cell receptors have failed to show structural differences between complexes with agonist versus antagonist peptides (Ding et al., 1999). However, it has been suggested that tight-binding immunodominant MHC-peptide complexes may induce a conformational change in the AB loop of the Cα chain of the TCR, which forms part of the putative interaction site with the CD3ε subunit (Kjer-Nielsen et al., 2003).

An alternative model suggests that T cell signaling is initiated when the T cell receptor is clustered with the MHC co-receptor by both binding to a single MHC-peptide complex, holding the intracellular kinase (Lck) associated with the co-receptor close to the TCR signaling subunit ITAM domains (Figure I.6 B). A variation on this model has also been proposed (Irvine et al., 2002; Krogsgaard et al., 2003) where an activating MHC-peptide bound TCR is associated with a co-receptor molecule that can bind to a non-activating MHC-peptide bound to an adjacent TCR, causing activation of the first TCR via a co-receptor linked pseudodimer (Figure I.6 C). This model was proposed in part due to structural studies showing that soluble CD4 bound to an MHC forms a sharp angle with the MHC-TCR axis, making it difficult to imagine a CD4 molecule and a TCR bound to the same MHC complex bringing their cytoplasmic domains into close proximity (Wang et al., 2001). Co-receptor models might explain how activation could occur from a single activating peptide on an APC. However, studies showing that signaling does not depend on co-receptor involvement (Cho et al., 2001; Kerry et al., 2003 and Chapter III of

this thesis) seem to suggest that this is not the main trigger for T cells, and may simply alter sensitivity levels or contribute to binding energy.

Another mechanism involves multivalent ligand binding which promotes receptor dimerization or oligomerization (Figure I.6 D). This type of mechanism is found in receptor tyrosine kinases which can transphosphorylate one another when brought together by ligand (Hubbard and Till, 2000). The T cell receptor subunits do not contain any enzymatic activity that might act in a trans manner upon receptor dimerization; however, there is much evidence that crosslinking of TCR or TCR complex subunits by soluble MHC-peptide reagents is sufficient for triggering activation responses (Abastado et al., 1995; Cochran et al., 2000; Daniels and Jameson, 2000; Doucey et al., 2001; Maile et al., 2001). Signaling occurs even for antibody-induced crosslinking or clustering of chimeric receptors which would not involve the MHC co-receptor (Irving and Weiss, 1991; Kappler et al., 1983; Kaye et al., 1983; Letourneur and Klausner, 1991). However, an oligomerization model does not explain how responses to single activating peptide-MHC complexes could occur.

Other researchers have suggested disruption of an inhibitory pre-defined oligomeric receptor structure (Figure I.6 E)(Cameron et al., 2001b; Reth, 2001). This mechanism can be seen with the bacterial aspartate receptor (Falke et al., 1997; Ottemann et al., 1999). However, the T cell receptor has been shown to assemble with a single antigen binding receptor per complex (Call et al., 2002). There is also evidence showing a lack of orientation dependence for T cell stimulation by multivalent MHC-peptide ligands (Cochran et al., 2001b). A different but related model proposes a rearrangement of the TCR subunits, possibly exposing novel epitopes for adaptor and signaling proteins (Gil et al., 2002; Sigalov, 2004). Evidence has been shown that T cell receptor signaling subunits homooligomerize through their unstructured cytoplasmic

domains (Sigalov et al., 2004), and are present on the cell surface unassociated with the larger TCR complex after activation (La Gruta et al., 2004). It is unsure whether this kind of mechanism would be initiated by a binding-induced conformational change (Figure I.6 F) or a clustering of receptors into close proximity (Figure I.6 G).

Further discussion of the antigen-specific trigger for CD4 and CD8 T cells can be found in Chapters II-IV of this thesis.

I.C. Detection and characterization of antigen-specific T cells

I.C.1 Importance for study of infection and vaccination

The population of T cells in the body is diverse. Different types of T cells exhibit different effector functions that may be more or less effective at controlling particular pathogens. For example, while some diseases are effectively controlled by antibody responses, other infections such as hepatitis C are not cleared despite the presence of neutralizing antibodies (Logvinoff et al., 2004). Likewise, the balance between T_H1 and T_H2 responses or particular protein epitope recognition in HIV-1 infection has been related to disease control or progression (Becker, 2004; Rosenberg et al., 1997), where the virus has developed mechanisms for evading CTL responses (Collins, 2003). Improper immune responses to certain infections can even lead to immunopathology or autoimmunity (Brehm et al., 2004; Christen and von Herrath, 2004; Mongkolsapaya et al., 2003; Terajima et al., 2004). One goal of health care is to create safer vaccines and immunotherapies that elicit appropriate adaptive immune responses for disease control and clearance.

An important step in studying the T cell response to a given infection is to identify T cell epitopes, or the portions of pathogenic proteins that are presented in complex with MHCs on the

surface of APCs that are then recognized by clonotypic T cells present in the overall repertoire. The low frequency of T cells that can recognize a given epitope, combined with the large number of peptides that may be derived from a single pathogen for presentation on MHC molecules, create challenges to routine identification of T cell epitopes from novel or emergent diseases.

I.C.2 Methods for identifying and quantifying antigen-specific T cells

Screening epitopes in cellular assays is usually accomplished by synthesizing 20-22 amino acid long peptides which cover the sequence of a pathogenic protein, keeping an overlap of 10-12 amino acids between adjacent peptides, and then testing each peptide for the ability to elicit a T cell response when added to a mixture of T cells and APCs. It is costly to synthesize the peptides, and many valuable cells from limited *ex vivo* samples must be used for the tests. When using recombinant MHCs to screen epitopes, the problem is further complicated. Class I MHC molecules can only bind short peptides of 8-10 amino acid lengths, requiring approximately 30 times as many peptides to be synthesized and screened as for class II. Attempts to reduce the pool of potential peptides by use of positional scanning prediction methods have thus far failed to be sensitive and predictive enough to reduce the number of peptides to a more reasonable number (Hammer et al., 1994; Rammensee et al., 1995; Southwood et al., 1998; Sturniolo et al., 1999).

There are several techniques which have been applied to screening T cell populations for specific reactivities. Many functional assays involve cellular presentation elements (APCs) that process and present overlapping peptides derived from a given pathogenic sequence to a responding bulk T cell population. The T cells can be monitored for functional activation responses such as proliferation in bulk (Strong et al., 1973) or by limiting dilution analysis (Merkenschlager et al., 1988), lysis of labeled target cells (Engers et al., 1975; Koenig et al.,

1990; Kurokohchi et al., 2001), or cytokine secretion by bulk enzyme-linked immunosorbent assay (ELISA), intracellular cytokine staining (Kern et al., 1998; Maecker et al., 2001; Prussin and Metcalfe, 1995), or ELISPOT (Czerkinsky et al., 1988). These techniques can utilize overlapping peptides for both class I and class II MHC presentation, since APCs process and load the peptides. However, large sample sizes are needed to screen a reasonable number of peptides, and only a few functional aspects of the cells can be monitored simultaneously. Also, it can be difficult to establish the frequency of the T cells of interest in the *ex vivo* sample, and determination of the MHC restriction of an observed response is non-trivial since most APCs express multiple MHC alleles.

Another group of techniques do not depend upon functional responses, but identify responding populations by their binding to recombinant oligomeric MHC in complex with cognate peptide. One such technique is to stain mixed T cell populations with MHC-peptide complexes linked to fluorescently-labeled streptavidin molecules via biotin incorporated into the MHC molecule either chemically or enzymatically—so-called "tetramer staining" (Altman et al., 1996; Cameron et al., 2002; Dunbar and Ogg, 2002; Kwok et al., 2002). The cells are then analyzed using flow cytometry, and the cells which are bound to the specific MHC-peptide complexes are fluorescently stained. This technique, popularized first for class I MHCs and then extended to class II MHC reagents, has not only provided much information about the frequencies of antigen-specific T cells over the course of infections and vaccinations, but has also become somewhat ubiquitous in recent years, with MHC oligomers becoming available from various core facilities (National Institutes of Health) or companies (Becton Dickinson "DimerX", Beckman Coulter "iTag Tetramers", or ProImmune "Pro5" MHC pentamers). Several disadvantages exist, however. Recombinant peptide-MHC complexes are not available

for every possible allele, and a relatively large number of cells is required to find a low-frequency response in a diverse population. In addition, no functional information is attained from this technique, and staining must be combined with other techniques to obtain that information. A related screening technique involves adhesion of cells to elements of a microarray containing recombinant MHC-peptides (Soen et al., 2003). This technique allows multiple peptide-MHC complexes to be tested with a smaller sample size, and can also be used to detect transient calcium flux in adhering cells.

Chapter V of this thesis reports progress made on a technique which can be used to screen small T cell samples for specific MHC-peptide binding and for induced functional responses using microarrays of artificial antigen-presenting cells containing recombinant MHC-peptide complexes and co-stimulatory molecules. These arrays can detect responses such as binding, cell-surface molecule expression, and cytokine secretion in a location-dependent manner.

I.D. Overview of this thesis

This thesis contains work directed at increasing our understanding of the molecular mechanism of $CD4^+$ and $CD8^+$ T cell activation as well as work on a method for screening potential T cell epitopes and characterizing the antigen-specific responses induced. Background related to these topics has been summarized in this chapter.

Chapter II describes the application of a mathematical model of equilibrium multivalent binding to $CD4^+$ T cell responses induced by MHC-peptide monomers and oligomers. $CD4^+$ T cells had previously been shown to respond to soluble dimers, trimers, and tetramers of MHC-peptide complexes, but not soluble monomers (Cochran et al., 2000). The concentration

dependence of these responses showed characteristic curves with responses rising at lower concentrations for higher valency oligomers, and the responses decreasing at high concentrations for all oligomers. This work shows that the level and concentration dependence of activation responses can be explained by the number of multivalently-bound T cell receptors. The number of crosslinked receptors can be predicted by two parameters: a monovalent binding constant, K_D, and a crosslinking constant, K_X, which can be fit from activation parameter curves. Data generated using these same parameters can be fit to multiple different activation read-outs as well as direct binding data. The association found between the predicted number of multivalently-engaged TCR and the level of T cell activation by multivalent MHC ligands argues that activation corresponds to TCR clustering or multivalent ligation. Jennifer R. Cochran, a former member of the Stern lab, contributed to these efforts by developing the experimental system and gathering much of the data modeled. This work was published in November, 2001 in Biophysical Journal (Stone et al., 2001).

Chapter III presents a confounding phenomenon uncovered in attempts to study $CD8^+$ T cell activation using soluble MHC-peptide monomers and oligomers. $CD8^+$ T cells, unlike $CD4^+$ T cells, were found to be activated when soluble syngeneic MHC-peptide monomers were added to the cells, and were even able to respond to free cognate peptide. Further investigation revealed that peptides derived from soluble complexes were able to be loaded onto endogenous MHC complexes on the surface of T cells and re-presented to other T cells in the assay. However, MHC monomers did not cause stimulation under conditions where peptide re-presentation was not possible; for example, allospecific MHC-peptide monomers yielded peptides that did not form stimulatory complexes with cell surface MHCs, and T cells engineered not to express class I MHCs on their surface did not respond to soluble syngeneic MHC

monomers. However, in both cases, immobilizing the stimulatory MHC complex on assay well surfaces resulted in T cell recognition. This work showed that observed monomer activation in $CD8^+$ T cells could be explained by multivalent peptide re-presentation on the surface of other T cells. Qing Ge in Jianzhu Chen's lab at the Massachusetts Institute of Technology Cancer Center contributed equally to myself in these endeavors, and other contributors include M. Todd Thompson, Jennifer R. Cochran, and Mia Rushe in the Stern lab, and Herman Eisen helped with extensive discussions. This work was published in October, 2002 in the Proceedings of the National Academy of Sciences, USA (Ge et al., 2002).

Chapter IV includes further investigations into the requirements for T cell activation by soluble MHC-peptide monomers and oligomers. $CD8^+$ T cells were presented with allogeneic MHC monomers and oligomers, which do not cause activation through peptide re-presentation at the T cell surface. Under these conditions, $CD8^+$ T cells were shown to have similar responses to the soluble complexes as $CD4^+$ T cells. For both $CD4^+$ and $CD8^+$ T cells, addition of soluble MHC-peptide monomers can antagonize activation induced by MHC-peptide oligomers. However, T cell responses could be induced by a single activating MHC-peptide complex when linked to a non-activating MHC-peptide complex in a heterodimer, consistent with studies showing that a single activating peptide can cause T cell signaling (Irvine et al., 2002; Sykulev et al., 1996), and that non-activating MHC-peptide complexes can contribute to activation responses (Wulfing et al., 2002). Jennifer R. Cochran contributed to this work by performing the monomer antagonism experiment for $CD4^+$ T cells. This work has been submitted for publication.

Chapter V illustrates a technique for mapping pathogen-derived T cell epitopes and characterizing functional antigen-specific T cell responses using microarrays of synthetic

antigen-presenting cells. Each element of the microarray presents a distinct potential peptide epitope derived from an infectious agent in complex with recombinant MHC co-immobilized with costimulatory molecules that allow full activation responses in the T cells. Effector responses to the arrayed proteins, including cytokine secretion, can be detected in a location-specific manner by including cytokine capture antibodies into the array elements where the cytokines are captured locally and are detected by fluorescently-labeled detection antibodies. Other facets of T cell activation such as adhesion or surface molecule modulation can be analyzed using this type of microarray as well. Responses from $CD8^+$ and $CD4^+$ T cells are detected specifically and at a sensitivity level similar to ELISPOT, but for many more antigens in parallel. Multiple responses can be measured simultaneously; for example, IFN-γ and IL-4 can both be detected at once. This technique shows potential for multidimensional specificity and functional information to be collected conveniently and rapidly, increasing our understanding of the immune response to novel infectious agents. Walter Demkowicz in the Stern lab contributed to this study by growing several T cell lines and clones. This work has been submitted for publication, and there is a patent pending on this technique (Pending patent application 10/823,866 "Major Histocompatability Complex (MHC)-Peptide Arrays").

Chapter VI contains overall conclusions and a discussion of the significance of this work. The Appendices contain detailed protocols from this work as well as information about constructs, peptides, and materials.

II. T cell activation by soluble MHC oligomers can be described by a two-parameter binding model[1]

SUMMARY

T cell activation is essential for initiation and control of immune system function. T cells are activated by interaction of cell-surface antigen receptors with major histocompatibility complex (MHC) proteins on the surface of other cells. Studies using soluble oligomers of MHC-peptide complexes and other types of receptor cross-linking agents have supported an activation mechanism that involves T cell receptor clustering. Receptor clustering induced by incubation of T cells with MHC-peptide oligomers leads to the induction of T cell activation processes, including downregulation of engaged receptors and upregulation of the cell-surface proteins CD69 and CD25. Dose-response curves for these T cell activation markers are bell-shaped, with different maxima and midpoints depending on the valency of the soluble oligomer used. In this study, we have analyzed the activation behavior using a mathematical model that describes the binding of multivalent ligands to cell-surface receptors. We show that a simple equilibrium binding model accurately describes the activation data for T cells treated with MHC-peptide oligomers of varying valency. The model can be used to predict activation and binding behavior for T cells and MHC oligomers with different properties.

[1] The research presented in this chapter has been published:
J.Stone, J.R. Cochran, and L.J.Stern. *Biophys J.* 2001 Nov;81(5):2547-57.

A. Introduction

$CD4^+$ T cells have an important role in the immune system in recognition and response to foreign antigens. $CD4^+$ T cell activation is triggered upon specific interaction of T cell surface receptors (TCR) with foreign antigens bound to class II major histocompatibility complex (MHC) proteins found on the surface of B cells, macrophages, and other antigen-presenting cells (Davis et al., 1998; Germain, 1994). MHC-TCR engagement triggers a cascade of signaling events, including phosphorylation of receptor subunits, docking of receptor-associated signaling and adapter proteins, activation of cytoplasmic signaling cascades, and up-regulation of several gene products (Cantrell, 1996; Qian and Weiss, 1997). The complete activation program also requires participation of antigen-independent adhesion and costimulatory molecules from both the T cell and antigen-presenting cell (Chambers, 2001), which can lead to formation of cell-surface supramolecular activating clusters or "immune synapses" (van der Merwe et al., 2000), and eventually cytokine secretion, clonal proliferation, and induction of other T cell effector functions required to help clear the foreign antigen from the host.

The precise molecular events that induce T cell triggering upon TCR ligation are not well understood, but substantial evidence points to receptor clustering as an important component of the signaling in this system (Germain, 1997). Early studies showed that antibody-mediated clustering of TCR (Janeway, 1995), or clustering of chimeric TCR cytoplasmic domains (Irvine et al., 2002; Letourneur and Klausner, 1991) could trigger T cell activation processes. More recently, soluble MHC-peptide oligomers have been used as reagents to investigate T cell activation processes (reviewed in (Cochran et al., 2001a)). These reagents include antibody-linked MHC dimers (Abastado et al., 1995), dimers created through chimeric fusions of MHC-peptide complexes to antibody Fc domains (Appel et al., 2000; Casares et al., 1999; Hamad et

al., 1998), streptavidin-linked oligomers of biotinylated MHC-peptide complexes (Boniface et al., 1998; Crawford et al., 1998), and a series of chemically-defined MHC dimers, trimers, and tetramers prepared using a flexible peptide-based cross-linkers (Cochran and Stern, 2000). These studies demonstrated that multivalent TCR engagement is necessary for T cell triggering, with an MHC dimer as the minimal activating unit (Boniface et al., 1998; Cochran et al., 2000). T cell activation induced by such soluble oligomeric reagents exhibits non-saturating, bell-shaped dose-response curves (Cochran et al., 2000), but these activation relationships have not been related to binding constants or other molecular properties of the system. Moreover, fluorescent MHC oligomers increasingly are used to track antigen-specific T cell populations in clinical samples (Ferlin et al., 2000; McMichael and O'Callaghan, 1998), and understanding the correlation between binding levels and molecular properties such as MHC-TCR affinity or TCR clustering is urgently needed.

To gain insight into the binding behavior of MHC oligomers, and the relationship between MHC-TCR binding and the resultant activation response, we have applied a simple receptor cross-linking model developed originally for characterization of equilibrium binding of multivalent ligands to receptors on mast cells (Perelson, 1981). Here, we show that the model accurately describes the behavior of soluble MHC oligomers in inducing activation processes in T cells for a variety of oligomer valencies, MHC-TCR affinities, and cross-linking strategies. The striking correlation of the model with the experimental data in this system show that several T cell responses are directly related to the number of multivalently engaged receptors. The behavior of the model under different experimental conditions suggests possible mechanisms for the cellular regulation of antigen sensitivity in T cells.

B. Materials and Methods

1. Preparation of class II MHC-peptide oligomers

HLA-DR1 α and β extracellular domains (Cochran and Stern, 2000) were expressed in *E. coli* cells as inclusion bodies, solubilized in 8M urea, purified by ion exchange, and refolded by dilution of the denaturant under redox-controlled conditions in the presence of peptide, as previously described (Frayser et al., 1999 and Protein Expression and Purification protocols in Appendix). Cysteine residues introduced into the α or β subunit C-termini (α_{cys}, β_{cys}, α_{Lcys}, and β_{Lcys}) were used for cross-linking. In some experiments, the cysteine was introduced immediately after the membrane proximal immunoglobulin domain (α_{cys}, β_{cys}); in others, the 5-10 residue connecting peptide region was included before the cysteine (α_{Lcys}, β_{Lcys}). Antigenic peptide Ha[306-318] (PKYVKQNTLKLAT) derived from influenza hemagglutinin (Lamb et al., 1982), control peptide A2[103-117] (VGSDWRFLRGYKQYA) (Chicz et al., 1992), and cross-linkers X3X (fβEK′SGSK′G) and X14X (fβEK′SGSGESGSEGSSEGK′G) (Cochran et al., 2001b) and related trivalent and tetravalent peptide-based cross-linkers (Cochran and Stern, 2000), where fβ is fluoresceinyl–β-alanine and K′ is N(ε)aminocaproylbenzylmaleimide lysine, were synthesized using FMOC chemistry, purified by reverse phase high performance liquid chromatography, and verified using mass spectrometry. The refolded HLA-DR1-peptide complexes carrying a cysteine on either the α or β subunit were oligomerized by reaction of the introduced thiols with maleimidyl groups on the peptide-based cross-linkers. Cross-linker was added in small aliquots to freshly-reduced MHC protein at room temperature over a period of five hours to a final molar ratio of MHC:cross-linker of 2:1 for dimers, 3:1 for trimers, and 4:1 for tetramers (Cochran and Stern, 2000 and Cross-linking Proteins Using Pep2x, Pep3x, and Pep4x (and relatives) protocol in Appendix). Purified MHC-peptide oligomers were isolated

using two Superdex 200 HR 10/30 columns (Pharmacia, Peapack, New Jersey) in series, and further characterized by SDS-PAGE (Cochran and Stern, 2000). For binding assays, FITC-labeled MHC-peptide monomers were prepared by reaction of the HLA-DR1 introduced cysteine residue with fluorescein-malemide (Pierce, Rockford, Illinois) followed by purification by gel filtration chromatography (Cochran and Stern, 2000).

2. T cell activation and binding assays

The T cell clone HA1.7 (Lamb et al., 1982) used in many of the experiments presented herein is specific for the Ha peptide bound to HLA-DR1, and was maintained by biweekly stimulation with peptide-pulsed irradiated antigen presenting cells and rested seven days before activation assays (Cochran et al., 2000 and T Cell Maintenance—CD4+ Clones and Lines protocol in Appendix). Two HLA-DR1-restricted, Ha-peptide specific, T cell clones, Cl-1 (Sette et al., 1994) and HaCOH8 (gift of Corrine Moulon, Warner-Lambert, Paris), and a short-term polyclonal T cell line, HA03 (Cameron et al., 2001a), were maintained similarly. T cell activation assays were performed as previously described (Cochran et al., 2000). Briefly, MHC-peptide oligomers were added to 7.5 x 10^4 T cells in round bottom 96 well plates and incubated at 37°C, 5% CO_2. After the desired incubation time, cells were placed on ice and stained concurrently with fluorescent monoclonal antibodies against T cell surface markers: R-phycoerythrin (PE) labeled anti-CD3 (UCHT-1) and allophycocyanin (APC) labeled anti-CD69 (FN50) or APC-anti-CD25 (M-A251) (all from Pharmingen, San Diego, California). Cells were washed with phosphate-buffered saline (0.01 M KH_2PO_4, 0.1 M Na_2HPO_4, 1.37 M NaCl, 0.027 M KCl, pH 7.4) containing 1% fetal bovine serum and 0.1% sodium azide and analyzed by flow cytometry. Fluorescence data were obtained with a Becton-Dickinson FACS Calibur flow

cytometer and analyzed using Cell Quest software. The number of MHC-peptide complexes bound during the course of the T cell activation assay was simultaneously measured with multicolor flow cytometry, using the fluorescein molecules incorporated into the cross-linkers (Cochran et al., 2000). The number of CD3 molecules downregulated upon oligomer treatment, and the number of MHC-peptide complexes bound per cell, were converted from mean fluorescence using SPHERO Rainbow calibration particles (Spherotech, Libertyville, Illinois) containing known amounts of PE and fluorescein equivalents (See Quantitation of Cell Surface Receptors by Flow Cytometry protocol in Appendix).

3. Generation of calculated cross-linking and binding curves

For a given K_X, K_D, and R_{tot}, the implicit equation for R_{eq} (Perelson, 1981) was solved numerically for each valency of oligomer and each concentration using the secant method (Kreyszig, 1993). That value was then used to calculate the number of oligomers bound per cell with each possible valency. The calculations were performed using programs created in FORTRAN 77 and MAPLE V. Φ_{xlink}, R_{multi}, R_{dimer}, and L_{bound} were calculated from binding distributions as described in the model section of this paper.

4. Fitting experimental activation and binding data

Fits of the model to the experimental sets were solved by a three-parameter minimization of K_X, K_D, and scale factor, using known R_{tot}. Minimization reduced the total χ^2 by iterative testing of combinations of parameter values one interval above and below the current values of K_D, K_X, and scale factor, and adopting the combination with the lowest χ^2 as the new values. The interval was reduced until convergence. A wide range of initial guesses was tried for each

data set to ensure uniqueness of the fit parameters. The uncertainty for each parameter, δ_{aj}^2, was determined by the following equation:

$$\delta_{aj}^2 = \frac{2}{(N-n)(\chi_{p+\sigma}^2 - 2\chi_p^2 + \chi_{p-\sigma}^2)}$$

where N is the number of measurements used for the fit, n is the number of parameters being fit by the program, and the χ^2 values correspond to the values calculated with the parameter p varying about the best fit value by the interval σ (Bevington, 1969).

C. Model

1. Distribution of bound states for a multivalent ligand of a cell-surface receptor

A simple equilibrium model that describes the interaction of multivalent ligands with cell surface receptors was used to simulate activation dose-response curves. Binding and cross-linking parameters were obtained by least-squares fitting of curves to experimental binding and activation data. This model was originally developed to describe general receptor binding by multivalent ligands, and has been applied to the release of histamine from basophils (Perelson, 1981), a response which requires receptor cross-linking (DeLisi and Siraganian, 1979). Similar models have been used to fit data for IgE-Fcε receptor clustering (Hlavacek et al., 1999), dissociation of insulin and nerve growth factor from their cross-linked receptors (DeLisi and Chabay, 1979), viral attachment to cell-surface receptors (Wickham et al., 1990), and dimeric MHC-peptide complexes binding to $CD8^+$ T cells (Fahmy et al., 2001).

The model describes the distribution of different bound states of a multivalent ligand interacting with a monovalent receptor (Figure II.1). For example, a ligand dimer may be bound monovalently (2_1) or divalently (2_2), assuming both ligand units can bind simultaneously, or it may not be bound at all (2_0, Figure II.1, top). Similar states can be described for a ligand

tetramer (Figure II.1, bottom). The model can be used to describe the relative amounts of these states under different conditions. The monovalent binding of a ligand to its receptor is characterized by the equilibrium dissociation constant K_D (units of molarity). The tendency for multivalent binding is assigned to the cross-linking constant K_X (units of $(\#/cell)^{-1}$), with the assumption that binding of each additional monomer within the oligomer is described by the same constant. The actual independent modeling parameter is κ, the unitless product of the receptor density (R_{tot}/A, units of $(area)^{-1}$, where A is the surface area of the cell) and a 2-dimensional equilibrium binding constant (K_X*A, units of area). Since the receptor density is less convenient to measure experimentally than the number of receptors per cell, we report the cross-linking constant $K_X = \kappa / R_{tot}$. The model also assumes that rapid binding equilibrium is attained, and that the free ligand concentration is not significantly depleted by binding to cell surface receptors. At the cell densities conventionally used in these experiments (1-5 x 10^6 cells/mL), ligand is not significantly depleted for ligand concentrations greater than 10^{-10} M, even at 100% receptor occupancy.

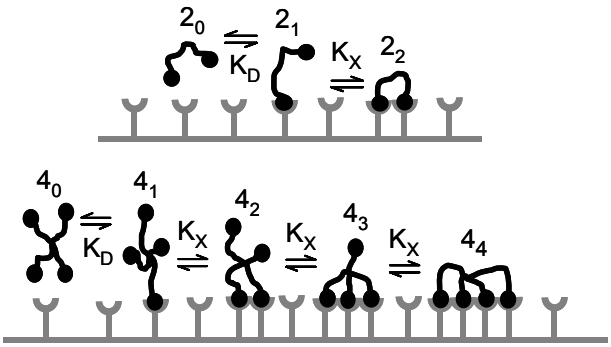


FIGURE II.1 Schematic description of oligomer binding for a dimer (top) and a tetramer (bottom). The top row shows a dimer binding sequentially to monomeric cell surface receptors. The terminology for the binding state is shown above each oligomer. The bottom panels show the same scheme for a tetramer binding sequentially to monomeric cell surface receptors.

Calculation over a range of concentrations yields the distinctive distribution of the various bound states, which is sensitive to changes in K_D, K_X, and the total receptor number

(R_{tot}). At equilibrium, the amount of oligomer with valency (v) that is bound using i ligands can be found by the following equation (Perelson, 1981):

$$\nu_{i,eq} = \frac{v!}{i!(v-i)!}(K_X)^{i-1}\frac{L_0}{K_D}(R_{eq})^i \tag{II.1}$$

where $\nu_{i,eq}$ is the number of oligomers of valency v bound i times per cell at equilibrium, L_0 is the bulk concentration of oligomer, and R_{eq} is the number of unbound receptors per cell at equilibrium. The value of R_{eq} must be found from the numerical solution of the implicit equation:

$$R_{tot} = R_{eq}(1 + v\frac{L_0}{K_D}(1 + K_X R_{eq})^{v-1}) \tag{II.2}$$

and R_{bound} is found by:

$$R_{bound} = R_{tot} - R_{eq} \tag{II.3}$$

Several related parameters describing the system can be extracted from this type of calculation. For example, the number of cross-links (Φ_{xlink}) formed by oligomers bound multivalently can be calculated as follows:

$$\Phi_{xlink} = \sum_{i=2}^{v}(i-1)\nu_{i,eq} \tag{II.4}$$

This is the original measure that was employed in previous studies (Hlavacek et al., 1999; Perelson, 1981). A tetramer bound divalently is considered to have one cross-link, whereas one bound trivalently has two cross-links. In addition, the number of cross-linked receptors, i.e., those associated with oligomers bound multivalently (R_{multi}), can be found by:

$$R_{multi} = \sum_{i=2}^{v} i\nu_{i,eq} \tag{II.5}$$

The number of discrete receptor dimers per cell (R_{dimer}) per cell can be found using the following equation:

$$R_{dimer} = \sum_{i=2,4,6,...}^{v} \frac{i}{2} \nu_{i,eq} \qquad \text{(II.6)}$$

A tetramer bound tetravalently forms two discrete receptor dimers, while one bound trivalently forms only one. Finally, the total number of oligomers bound per cell (L_{bound}) can be calculated using the following equation:

$$L_{bound} = \sum_{i=1}^{v} \nu_{i,eq} \qquad \text{(II.7)}$$

2. Theoretical distribution curves

The predicted distribution of bound states as a function of concentration for fixed K_X, K_D, and R_{tot} values is shown for a dimeric ligand in the upper panels of Figure II.2 (panels A, B, and C) and for a tetrameric ligand in the lower panels (Figure II.2 D, E, and F). The plots in Figure II.2 were calculated using the same R_{tot} (24,000 per cell) and K_D (1.4 μM) values, but with three different values for K_X. When the cross-linking tendency (K_X) is low (2.0×10^{-5} per cell^{-1}), as in Figure II.2A and D, the fraction of oligomers bound multivalently (using two, three, or four ligands) is low. The number of ligands bound monovalently rises rapidly to saturation with increasing ligand concentration. In the tetramer plot (Figure II.2D), the dashed line representing the sum of ligands bound multivalently (R_{multi}) is similar to the symmetric plot of divalently bound tetramers (4_2), as little or no trivalently (4_3) or tetravalently (4_4) bound oligomers are present. When the tendency to cross-link (K_X) is ten-fold higher, as in Figure II.2B and E, significant amounts of di-, tri-, and tetramerically bound ligands accumulate at intermediate concentrations, and decrease at higher concentrations. The decrease at high concentrations can

be understood by considering that mass action drives formation of monovalently bound oligomers at high concentrations of ligand. In the tetramer plot (Figure II.2E, dashed line), the curve describing the total number ligands bound multivalently (R_{multi}) begins to show some discernible asymmetry with a shallower slope at low ligand concentration and a sharper slope at high concentration. In Figure II.2C and F, the cross-linking tendency (K_X) is again raised by 10-fold, and the asymmetry of the multivalently bound curve in Figure II.2F is even more pronounced. The curves representing oligomers bound with their maximum valency rise rapidly with concentration. These oligomers remain maximally bound with increasing concentration, and are only slowly replaced by oligomers bound with lower valency, and then by monovelently bound oligomers. The curve describing monovalently bound oligomer rises at an even higher concentration than for lower K_X, as it is even more difficult for a monovalent interaction to compete with multivalent interactions of bound oligomers.

The effect of varying parameters other than K_X also can be determined. If the K_D is reduced by a factor of ten, the result is a linear shift of the curves to lower concentrations. Increasing K_D shifts the curves in the opposite direction. Alternatively, if R_{tot} is changed ten-fold, an identical effect on the shape of the curves is observed as for the corresponding change in K_X; however, the height of the curve will be scaled to correspond to the increased number of receptors per cell.

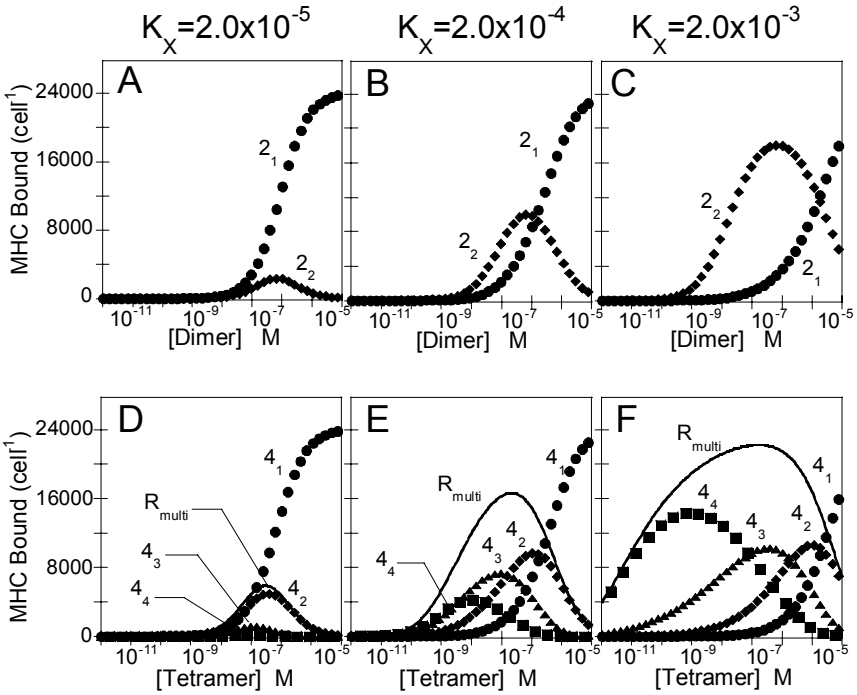


FIGURE II.2 Binding distribution plots. The distribution of variously bound oligomers as a function of oligomer concentration is shown in terms of the number of MHC molecules bound. (A-C) show the behavior of a dimeric ligand binding to monovalent cell surface receptors as the cross-linking capacity K_X increases in each panel as shown above the plots. (D-F) show the behavior of a tetrameric ligand binding to monovalent cell surface receptors. Oligomers bound monomerically (●), dimerically (◆), trimerically (▲), and tetramerically (■), are shown. The dashed line on the tetramer plots (D)-(F) represents the sum of the multivalently bound oligomers, R_{multi}.

D. Results

1. Analysis of experimental dose-response curves for activation of a T cell clone by a series of MHC oligomers

To investigate the molecular triggering mechanism of T cell activation, we have previously developed a series of chemically-defined MHC-peptide oligomers of the human class II protein HLA-DR1 (Cochran and Stern, 2000) and used these to trigger activation processes in the well characterized, influenza-specific human T cell clone HA 1.7 (Lamb et al., 1982). This series of MHC dimers, trimers, and tetramers was prepared using synthetic peptide-based cross-

linking reagents that were designed to be flexible and to allow simultaneous binding of multiple MHC molecules to the T cell surface. Activation processes induced by these oligomers were measured as changes in cell surface expression of activation markers detected by multicolor flow cytometry. The dose-response of T cell activation induced by MHC dimers, trimers, and tetramers was measured for three activation markers: downregulation of T cell receptor subunits (CD3) (Valitutti et al., 1997), upregulation of a T-cell associated lectin-like protein conventionally used as an early T-cell activation marker (CD69) (Testi et al., 1994), and upregulation of the interleukin-2 receptor α subunit involved in the autocrine proliferative response (CD25) (Waldmann, 1989). The dose-response for T cell activation triggered by each of the oligomers displayed a bell-shaped curve (Cochran et al., 2000) similar to those predicted by the simple equilibrium binding model. Increasing oligomer size caused a shift in the activation maximum to lower concentration and an increase in the maximum amplitude, behavior characteristic of the model applied here (Perelson, 1981).

The dose-response curves were fit using the model described above, using an experimentally determined value (24,000) for R_{tot}, the number receptors per untreated T cell. Figure II.3 shows the fits of the predicted R_{multi} values to experimental data for CD3 downregulation in the HA1.7 T cell clone (Cochran et al., 2000) measured at 12 and 27 hours, respectively, after addition of MHC oligomers to the T cell culture. Filled symbols indicate the experimental data (dimers, diamonds; trimers, triangles; tetramers, squares), and smooth curves represent the predicted number of TCR multivalently bound (R_{multi}) using best-fit values for K_D, K_X, and a scale factor relating R_{multi} to the experimental measure of internalized TCR. The curves obtained from a single experiment with different oligomers were fit simultaneously. The predicted curves fit well to the experimental data to within the expected uncertainty of the

measurements. Best-fit parameters for data collected at different times after oligomer addition are similar: $K_D = 1.4 \pm 0.1$ μM (12 hr) or $K_D = 1.7 \pm 0.4$ μM (27hr), and $K_X = 1.92 \pm 0.05 \times 10^{-4}$ per cell^{-1} (12 hr) or $K_X = 3.15 \pm 0.01 \times 10^{-4}$ per cell^{-1} (27hr) (Table II.1). The scale factor relating the predicted number of multivalently-engaged receptors to the experimental number of downregulated receptors was 0.776 ± 0.001 at 12 hr and 0.996 ± 0.001 at 27 hr. The value of the scale factor approaches one at long incubations, indicating that all engaged receptors eventually become downregulated as part of the T cell activation program, as suggested by cellular studies (Germain, 1997; Valitutti et al., 1997). The extracted values for K_D are within the range of affinities reported for other class II MHC-TCR systems determined by direct binding measurements using soluble receptors and ligands (Davis et al., 1998). The extracted values for K_X, and for the dimensionless value $\kappa = K_X * R_{tot}$, are comparable to those observed in other systems (Fahmy et al., 2001; Hlavacek et al., 1999; Wickham et al., 1990). For example, the K_X determined for IgE-Fcε receptor cross-linking by multivalent antigen was 1.35×10^{-5} per cell^{-1} (Hlavacek et al., 1999), and for the attachment of adenovirus to HeLa cells, a K_X of 5×10^{-3} per cell^{-1} was calculated (Wickham et al., 1990). These values correspond to κ values of 13 and 30, respectively. The κ values determined for binding of class I MHC-IgG fusion proteins to $CD8^+$ T cells range from 1-73 for cells in different activation states (Fahmy et al., 2001). Our values of $\kappa = 4$-8 are within the range observed in these systems.

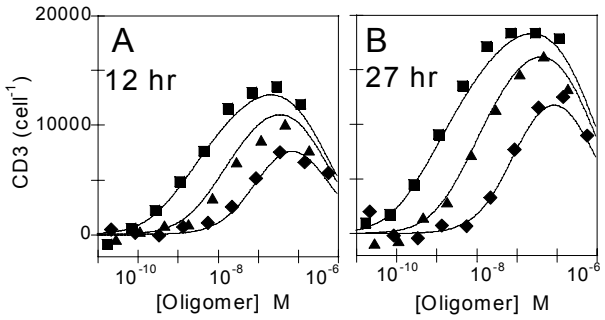


FIGURE II.3 Comparison of experimental CD3 downregulation data and model predictions. The predicted sum of multivalently bound oligomers at each concentration was fit to experimental CD3 downregulation data for treatment with a dimer (◆), trimer (▲), and tetramer (■) (Cochran et al., 2000). (A) shows the response of HA1.7 T cells at 12 hours of incubation with the oligomers, and (B) shows the response at 27 hours of incubation with the oligomers. Best fit parameters shown in Table II.1.

TABLE II.1 Binding and cross-linking parameters for several experimental markers of T cell activation for the clone HA1.7*

Experiment	K_D (μM)	K_X (x10^{-4}cell)
CD3 (12 hrs)	1.40 (0.12)	1.92 (0.05)
CD3 (27 hrs)	1.70 (0.40)	3.15 (0.01)
CD69 (12 hrs)	1.40 (0.60)	2.30 (0.60)
CD25 (27 hrs)#	1.80 (0.30)	3.00 (0.01)
Direct Binding	1.60 (0.30)	0.95 (0.01)

*R_{tot} was measured at 24,000 in these experiments, MHCs were linked through β subunit cysteines (β_{Lcys}) using peptide-based cross-linkers (Cochran et al., 2000). Values in parentheses represent parameter uncertainties from the least squares fit (see Methods).
#CD25 upregulation was fit with this scale: $CD25 = 4 \times 10^{-6} \times (R_{multi})^2 - 0.0073 \times R_{multi}$

Activation of HA1.7 T cells measured using other markers could also be described by the model. Upregulation of the early activation marker CD69 also was described by the model, although the data are noisier (Figure II.4A). This fit yielded a K_D of 1.4 ± 0.6 μM, and a K_X of 2.3 ± 0.6 x 10^{-4} per cell^{-1} (Table II.1), values similar to those obtained for CD3. If the CD3 downregulation and CD69 upregulation data are correlated, a linear relationship is seen with a slope of roughly 0.035 between the two (Figure II.4B). This corresponds well with the ratio of the fit scale factors (0.027). Upregulation of CD25, the low-affinity IL-2 receptor, was not

described well by the model using a linear scale factor (not shown). However, the plot of CD25 (IL-2R) upregulation versus CD3 downregulation did not exhibit a linear relationship as observed for CD69 with CD3, but rather a distinct curve (Figure II.4C). A best-fit quadratic function was found for the relationship of CD3 and CD25, and that function was applied to the model predictions to better represent the behavior of the data. After this application, the CD25 upregulation data were fit well by the model (Figure II.4D), giving best-fit values for K_D (1.8 ± 0.3 μM) and K_X (3.00 ± 0.01 per cell^{-1}) (Table II.1). Again, these values were similar to those observed for the other markers.

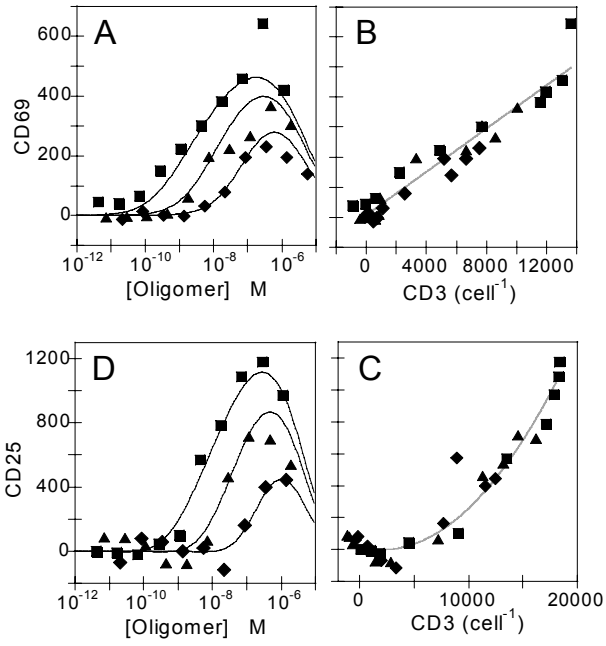


FIGURE II.4 CD69 (A,B) and CD25 (C,D) upregulation data and model predictions for a T cell clone. (A) The predicted sum of multivalently bound oligomers at each concentration was fit to experimental HA1.7 CD69 upregulation data for treatment with a dimer (◆), trimer (▲), and tetramer (■) (Cochran et al., 2000). (B) shows the relationship between CD69 and CD3 responses with a best fit line. The linear relationship suggests that a single scale factor could be used to relate CD69 response to R_{multi}. (C) shows the relationship between CD3 and CD25 responses with a best fit quadratic curve. The curved relationship suggests that CD25 response is not directly related to R_{multi}, and a quadratic filter was used to relate the model predictions to the data. (D) shows the experimental HA1.7 CD25 response to the dimer (◆), trimer (▲), and tetramer (■) treatments fit using a quadratic filter based on the relationship in (C). Parameters for the fits are shown in Table II.1.

Thus, a good correlation was observed between the predicted number of cross-linked receptors (R_{multi}) and the scaled experimental data ($R^2 = 0.99$) as shown in Figures II.3 and II.4. We also fit each of the data sets using the number of cross-links (Φ_{xlink}), or the number of distinct

dimers formed (R_{dimer}), instead of the number of cross-linked receptors. While these fits were slightly less good (not shown), the experimental data are not sufficiently precise to be able to distinguish definitively between the different measures.

2. Analysis of other T cell clones and MHC cross-linking strategies

Additional tests of this model were performed using two other influenza-specific T cell clones, Cl-1 and HACoH8, and also a short-term polyclonal influenza-specific T cell line raised from the peripheral blood of a $DR1^+$ homozygous individual, HA03 (Figure II.5). The dose-response of CD3 downregulation for the MHC oligomer series was measured for Cl-1 and HACoH8 after 24 hr (Figure II.5A and B) and for HA03 after 12 hr (Figure II.5C). For Cl-1, the CD3 downregulation dose-response curves were best-fit using $K_D = 5.00 \pm 0.01 \times 10^{-5}$ M and $K_X = 3.10 \pm 0.01 \times 10^{-4}$ per $cell^{-1}$ (Table II.2). These values are significantly different from those observed for HA1.7, particularly for K_D. The other T cell clone, HACoH8, exhibited $K_D = 1.80 \pm 0.07 \times 10^{-6}$ M, and $K_X = 42.2 \pm 1.4 \times 10^{-4}$ per $cell^{-1}$ (Table II.2). In this case, the K_X value is substantially different from those observed for the other clones. Finally, the polyclonal line HA03 exhibited best-fit values of $K_D = 1.60 \pm 0.04 \times 10^{-5}$ M and $K_X = 0.96 \pm 0.04 \times 10^{-4}$ per $cell^{-1}$ (Table II.2). These data show that K_D and K_X values can vary among different T cell lines, and that the model can describe these differences.

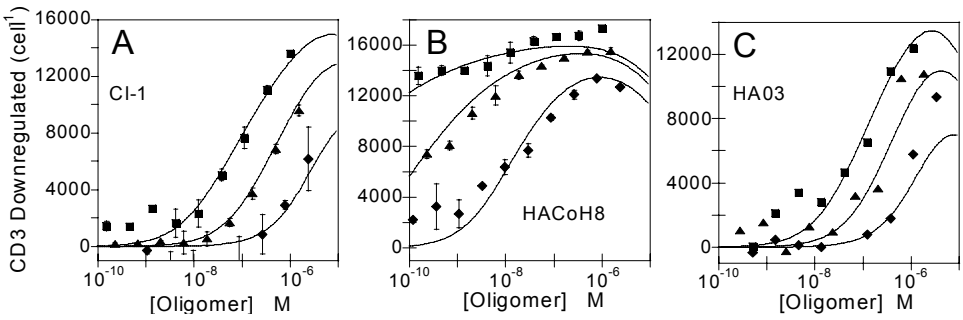


FIGURE II.5 CD3 response in other T cells. These plots show the CD3 downregulation response to dimer (◆), trimer (▲), and tetramer (■) for the T cell clones Cl-1 (A) and HACoH8 (B) and for the polyclonal cell line HA03 (C). The smooth curves show the model fits for these data sets. Model parameters for these fits are shown in Table II.2.

TABLE II.2 Comparison of binding parameters between different T cell clones*

T cell	R_{tot} (per cell)	K_D (μM)	K_X (x10^{-4}cell)
HA1.7	24,000	1.70 (0.40)	3.15 (0.01)
Cl-1	16,700	50.00 (0.02)	3.10 (0.01)
HACoH8	19,300	1.80 (0.07)	42.2 (1.4)
HA03#	27,000	16.00 (0.42)	0.96 (0.04)

*Parameters obtained by fitting CD3 downregulation data for HA1.7 (27 hrs), Cl-1 (24 hrs), HACoH8 (24 hrs), and HA03 (12 hrs). MHCs were linked through β subunit cysteines using peptide-based cross-linkers (Cochran et al., 2000). R_{tot} was measured directly by flow cytometry. Values in parentheses represent parameter uncertainties from the least squares fit (see Methods).
#Polyclonal T cell line

Another set of class II MHC oligomers has been used to investigate the effect of receptor proximity and orientation on T cell activation (Cochran et al., 2001b). In this series, MHC dimers were linked using a direct disulfide bond between cysteines introduced at the end of the α or β subunit, or using peptide-based synthetic cross-linkers of varying length. Dimers linked through either the α or β subunits, using either a disulfide bond (S-S) or a long cross-linker (X14X), were tested for their ability to induce CD3 downregulation in the HA1.7 T cell clone

(Figure II.6). Dose-response curves for these dimers exhibited characteristic bell-shaped curves, with the S-S dimers (closed symbols) activating more potently than the X14X dimers (open symbols) with more activation induced at lower concentrations and a higher maximum response. The activation data were described well by the binding model (lines). Since the cross-linking site is remote from the peptide-binding region, the cross-links are not expected to interfere with the TCR interaction, and a single K_D value was globally fit to the curves. The K_X was allowed to vary between the curves. The K_D extracted from these curves (1.61 ± 0.31 μM) was consistent with other K_D values obtained for the HA1.7 T cell clone. However, the best-fit K_X varied significantly within the series, with values (per cell)$^{-1}$ of 18.6 ± 0.8 x 10^{-4} (αS-S), 10.2 ± 0.2 x 10^{-4} (βS-S), 3.15 ± 0.02 x 10^{-4} (αX14X), and 3.79 ± 0.03 x 10^{-4} (βX14X) (Table II.3). The K_X was greater for the S-S dimers, in which the MHC monomer units are positioned close together, and lower for the X14X dimers, which are more loosely tethered by the long, flexible linker. Thus, the K_X parameter appears to reflect more facile receptor cross-linking for the more compact dimers, as expected by their geometrical constraints.

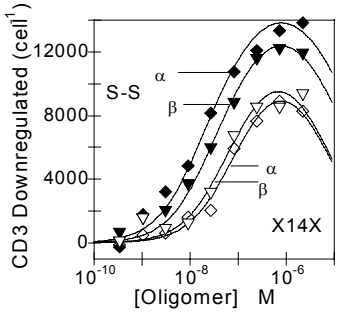


FIGURE II.6 CD3 response to MHC dimers with different length cross-links. This plot shows the CD3 downregulation in response to directly disulfide-bonded S-S dimers linked through the α_{cys} (◆) or the β_{cys} (▼) cysteine, and X14X dimers connected by a long flexible linker between the α_{cys} (◇) or β_{cys} (▽) cysteine. The smooth lines show the model fit to the data. Best-fit parameters are shown in Table II.3.

TABLE II.3 Comparison of K_X parameters obtained with differently linked MHC dimers*

MHC linkage#	Cross-linker Length§ (Å)	K_X (x 10^{-4}cell)
S-S (α)	2	18.7 (0.8)
S-S (β)	2	10.2 (0.2)
X14X (α)	90	3.15 (0.02)
X14X (β)	90	3.78 (0.03)

*CD3 downregulation for HA1.7 at 20 hours. A single K_D value was globally fit to the data set as 1.6 μM. R_{tot} was measured at 21,300 per cell.
#MHC's were linked by disulfide bonds (S-S) or long peptide-based cross-linkers (X14X) through cysteine residues on either the α or β subunit.
§The length represents the predicted spacing of the linked Cα atoms when the cross-linker is in an extended conformation.

3. Analysis of binding and competition data

In addition to the number of multivalently-bound receptors R_{multi}, the model can be used to predict the number of bound ligands, L_{bound}. This parameter is a sum over all of the variously bound states, and would be expected to correspond to the experimental ligand binding behavior. Figure II.7 (A-C) shows the predicted binding behavior for a series of oligomers of total valency 1 to 8, using values for K_D (1.4 μM) and K_X (0.2 –20 x 10^{-4} $cell^{-1}$) similar to those observed experimentally (and identical to those of Figure II.2). For the lowest K_X value (2 x 10^{-5} $cell^{-1}$), the curves are closely spaced and similarly shaped, with slightly more shallow slope and closer spacing for the oligomers with increased valency (Figure II.7A). At saturating concentration, distribution plots indicate that each oligomer is bound predominately monovalently (not shown). With a ten-fold increase in K_X, striking differences in shape are observed, with a strong asymmetry and greater spacing at lower oligomer concentrations as compared to the behavior at lower K_X (Figure II.7B). At high concentration, the multivalent curves cross below the curve for monomeric binding. A high-valency oligomer will compete very effectively for binding sites

relative to a monomeric ligand, and will occupy a given number of receptors using a smaller total number of bound oligomers (L_{bound}) as compared to a monomer. With another ten-fold increase in K_X (Figure II.7C), this behavior is even more pronounced. Higher valency leads to a rapid rise in the number of oligomers bound at lower concentration, as seen by the wide spacing of the curves at low concentration. At higher concentration, the strength of the multivalent binding is a significant impediment to binding of additional oligomers, and increasing oligomer size results in fewer total oligomers bound as compared to smaller oligomers. Thus, the predicted binding behavior is very sensitive to the parameter K_X. Changes in the parameter K_D do not affect the shape of the curves, but simply shift them to different concentrations, with lower K_D resulting in a linear shift to lower concentrations (not shown). This is the same behavior as observed for the binding distribution plots (Figure II.2). As before, changes in R_{tot} resulted in changes identical to the corresponding change in K_X, but with changes in the saturation value.

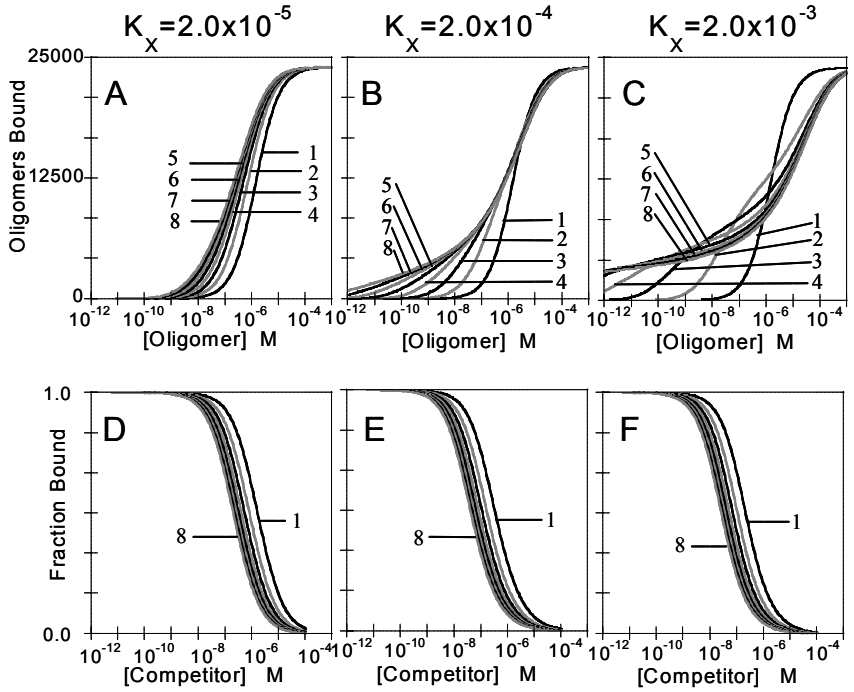


FIGURE II.7 Simulation of binding and competition. (A-C): Direct binding of multivalent ligands to a cell surface in terms of number of oligomers bound, L_{bound}. Each curve shows the behavior of a given valency of oligomer binding to the cell. K_X increases as shown from panel (A) to (C). (D-F): Competition of a labeled tetramer (35nM) by different valency unlabeled oligomers of the same ligand. K_X increases as shown from (D) to (F). R_{tot} = 24,000 per cell in all panels.

Experimental oligomer binding data were described well by the model. Binding of the MHC oligomers can be measured concurrently with T cell activation markers using fluorescent labels incorporated into the oligomer cross-linking reagents and multicolor flow cytometry (Cochran and Stern, 2000), although internalization of bound oligomer occurs during the extended incubations used for the activation assays (Cameron et al., 2001a). The MHC oligomer series used earlier in the activation experiments was tested for direct binding to the HA 1.7 clone (Figure II.8). The experimental binding curves exhibit some of the characteristics of the predicted binding curves, including variable spacing depending on ligand concentration and convergence at high concentration (Figure II.8). These data were fit to the modeled total number

of oligomers predicted to bind per cell at equilibrium (L_{bound}). The best fit to this data (Figure II.8) gives a K_D of 1.6 ± 0.3 μM and a K_X of 0.950 ± 0.001 x 10^{-4} per cell^{-1} (Table II.1). These values are similar to the K_D and K_X values obtained independently from the T cell activation data (Table II.1). The scale factor from the direct binding fit was 0.806 ± 0.002, which is somewhat below unity perhaps because of partial quenching of the fluorescein labels in acidic compartments after endocytosis (Cameron et al., 2001a).

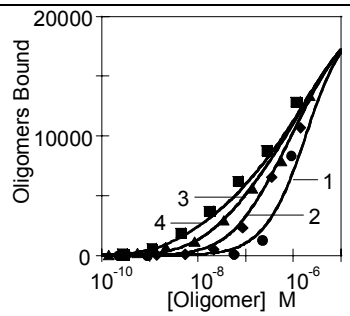


FIGURE II.8 Direct binding data and fit. These curves show the experimental results for direct binding of labeled monomer (●), dimer (◆), trimer (▲), and tetramer (■) (Cochran et al., 2000), and the model fit to these data (lines). Parameters shown in Table II.1.

The model can be used also to simulate competition experiments, in which a constant labeled "probe" oligomer is incubated in the presence of a variable concentration of unlabeled "competitor" oligomer or monomer. Figure II.7 (D-F) shows a series of predicted competition curve for oligomers of various valency, in each case using a constant concentration of probe tetramer (35nM). These conditions are similar to those that have been used to evaluate relative oligomer binding (Cochran et al., 2000; Reichstetter et al., 2000). As before, three different values of K_X in the range of the experimental values are shown in panels (D-F), with each panel varying by a factor of ten from the adjacent panel. In contrast to the predicted binding curves, the shape of the competition curves are quite similar for oligomers of different valency, with only a small decrease in curve spacing with increasing valency. Moreover, changes in K_X result only in small shifts on the concentration axis rather than substantial changes in curve shape or spacing. A similar effect can be seen for changes of K_D. Thus, although the predicted curves fit

well to experimental data (not shown), much less information about binding and cross-linking parameters can be extracted from this type of competition binding experiment.

E. Discussion

We have applied a simple two-parameter binding model to the activation of antigen-specific T cells by oligomeric class II MHC proteins. We find that the model accurately describes important features of the T cell responses to soluble MHC oligomers, including the bell-shaped, non-saturating concentration dependence and the variation of response maxima with oligomer size. Similar dissociation constants (K_D) and cross-linking constants (K_X) were extracted from different assays, including direct oligomer binding data and measurement of the T cell activation markers CD3 downregulation, CD69 upregulation, and CD25 upregulation, indicating that these parameters reflect intrinsic properties of the system. The strong correlation observed throughout the dose response between the levels of cellular activation and the predicted number of multivalently-bound receptors suggests that these activation markers simply report the number of suitably engaged receptors. Elaborate multi-step cytoplasmic signaling pathways have been elucidated for T cell signaling pathways that lead to upregulation of gene expression, including receptor phosphorylation, assembly of multi-component signaling complexes on receptor cytoplasmic domains, activation of various tyrosine and serine/threonine kinase cascades, changes in intracellular Ca^{++} concentration, and activation and nuclear translocation of transcription factors (Cantrell, 1996). The CD3 downregulation (receptor internalization) pathway is less completely understood, but appears to involve many of the same early processes (Itoh and Germain, 1997). Despite the apparent complexity of these signaling cascades, they do not appear to substantially modify the original binding signal and the final cellular readout

essentially reports the number of multivalently engaged receptors. For one marker (CD25), the relationship between binding and response was best described by a quadratic rather than linear relationship. Other activation responses may show other dependences and may incorporate information from other signaling pathways. Nonetheless, the simple correspondence between the number of engaged receptors and the degree of cellular activation observed many hours after stimulation is striking.

Implicit in the model is the notion that multivalent engagement of receptors leads to signaling, but that monovalent engagement does not. Although this is supported by recent experimental work, particularly in $CD4^+$ T cells (Boniface et al., 1998; Cochran et al., 2000), the biochemical basis of signal initiation is not yet clear. Receptor clustering is known to lead to phosphorylation of receptor cytoplasmic domains, but currently it is not clear how clustering results in kinase activation or in exposure of cytoplasmic domains, although several models have been proposed (Aivazian and Stern, 2000; Chan et al., 1994; Shaw and Dustin, 1997). The modeling performed here cannot definitively distinguish between a generic clustering mechanism where R_{multi} is the parameter that scales with T cell activation, or a specific dimerization mechanism where R_{dimer} describes the triggering.

An individual's T cell repertoire includes many different T cell clones of varying MHC-TCR affinity and signaling capacity (Janeway and Travers, 1994). Moreover, a T cell in different developmental and activation states has different sensitivities to antigenic stimulation. Parameters derived from least-squares fitting of the model to experimental activation data can be used to understand and predict these functional differences between T cells. We applied the model to several different T cell clones and to a polyclonal cell line representative of a subpopulation present in blood. The K_D and K_X parameters extracted from activation data

differed for the different T cells, with the differences consistent with behavior observed in cellular assays. For example, the HACoH8 clone has a K_X value 13-fold higher than for than HA1.7, indicating an increased tendency to form receptor cross-links. This clone can be stained by class II MHC oligomers even at 4°C, while most clones, including HA1.7 and Cl-1, require incubation at increased temperature to stain with those reagents (Cameron et al., 2001a). The temperature dependence has been attributed to membrane and/or cytoskeletal rearrangements that are necessary for monomeric TCR to co-localize sufficiently to allow multivalent binding of MHC oligomers, and which are inhibited at low temperature (Cameron et al., 2001a). The increased cross-linking tendency of HACoH8 would increase its ability to multivalently engage MHC oligomers, allowing oligomer staining under conditions where other clones are not stained.

With the increasing use of MHC tetramers in detection and analysis of specific T cells in clinical samples (McMichael and O'Callaghan, 1998), it is important to understand the parameters that govern the multivalent MHC-TCR interaction. Our analysis suggests that this relationship can be complex, with substantial non-linear contributions from the receptor number (R_{tot}) and cross-linking propensity (K_X). In an early description of the use class II MHC oligomers, a linear correlation was observed between oligomer staining intensity and binding affinity K_D, for several T cell hybridomas after correction for the total receptor number (Crawford et al., 1998). Our analysis suggests that this relationship will only hold for a restricted range of R_{tot}, and only for cells with similar K_X values. Finally, it is important to note that IC_{50} values determined from competition analysis cannot be related to MHC-TCR K_D values unless the relevant K_X values are known.

The sensitivity of the model to changes in K_X suggests a novel mechanism by which T cell could regulate their activation state. A naïve T cell, which has never previously seen

antigen, is much more difficult to activate than the corresponding memory T cell, which is the long-lived product of a prior encounter to antigen (Janeway and Travers, 1994). Certain treatments with high antigen dose or with partial antigenic stimuli are known to "anergize" T cells, i.e. to drive them to non-antigen responsive state (Schwartz, 1997). In both cases, the responsive and non-responsive T cells express the same receptor, and so have the same MHC-TCR affinity. It has generally been thought that these changes in activation sensitivity are due to changes in the intracellular signaling pathways. However, it is possible that T cells could regulate their activity by changing their ability to cluster TCR in the plane of the membrane, without any change in cytoplasmic signaling processes. In our model, this would correspond to a change in K_X. Such changes could be effected by alteration of receptor-cytoskeletal interactions (Viola et al., 1999), by receptor localization to membrane raft microdomains (Xavier and Seed, 1999), or by alteration of the receptor oligomeric state (Fernandez-Miguel et al., 1999). Recently, differences in the binding avidity of naïve as compared to activated T cells have been observed, and attributed to differences in TCR oligomeric state (Fahmy et al., 2001); these could as well be due to differences in the dynamic cross-linking propensity rather than the static oligomeric state. The pronounced effects on ligand sensitivity that we have observed for relatively small changes in K_X (Figures II.2, II.5) suggest that substantial changes in activation potential can be realized without any change in the intracellular signaling pathways. In principle, this possible mechanism is similar to one hypothesized to regulate cellular sensitivity to soluble monomeric antigen through changes in receptor organization (Bray et al., 1998).

There are some shortcomings of this approach in modeling the activation of T cells. We assume that all cross-linking events are equivalent, but it is possible that sequential cross-linking interactions are governed by different K_X values. The model assumes a constant receptor

number R_{tot}, although activated receptors become downregulated as part of the activation response (Liu et al., 2000), thus altering R_{tot} during the course of the experiment. We used the initial R_{tot} in calculating K_X values, since the T cell response to soluble MHC oligomers is rapid ((Boniface et al., 1998), and JRC and LJS, unpublished results). Using a constant R_{tot}, similar K_X values were obtained at different times in the response with only a change in scale factor. However, for derivation of an actual thermodynamic association constant, a more sophisticated analysis might be warranted. Finally, since this is an equilibrium model, it does not account for kinetic features of the interaction which may play a role in triggering; for example, the off-rate of the MHC-TCR complex has been proposed to be more important in regulating activation behavior than the affinity (Matsui et al., 1994).

Despite the simplifications made by the model, it correctly predicts T cell binding and activation behavior within observed experimental error. This model should prove useful in guiding experimental detection of T cells using MHC oligomers, and it provides a quantifiable measure of cellular parameters that appear to regulate antigen sensitivity in T cells.

F. Acknowledgements

We thank Corinne Moulon for the HACoH8 clone, A. Sette for Cl-1, J. Lamb for HA1.7, and Bader Yassine-Diab and Tom Cameron for providing the HA03 polyclonal line. This work was supported by NIH-NO1-AI48833 (LJS) and an NIH Biotechnology Training Grant NIH-T32-GM08334 (JDS, JRC).

III. Soluble peptide-MHC monomers cause activation of $CD8^+$ T cells through re-presentation of the peptide on T cell MHC molecules[2]

SUMMARY

T cell receptor (TCR)-mediated activation of $CD4^+$ T cells is known to require multivalent engagement of the TCR by, for example, oligomeric peptide-MHC complexes. In contrast, for $CD8^+$ T cells, there is evidence for TCR-mediated activation by univalent engagement of the TCR. We have here compared oligomeric and monomeric L^d and K^b peptide-MHC complexes, and free peptide, as stimulators of $CD8^+$ T cells expressing the 2C TCR. We found that the monomers are indeed effective in activating naïve and effector $CD8^+$ T cells, but through a novel mechanism that involves transfer of peptide from soluble monomers to the T cells' endogenous MHC (K^b) molecules. The result is that T cells, acting as antigen-presenting cells, are able to activate other naïve T cells.

[2] This research presented in this chapter has been published:
Ge Q, Stone JD, Thompson MT, Cochran JR, Rushe M, Eisen HN, Chen J, and Stern LJ. *Proc Natl Acad Sci USA*. 2002 Oct 15; 99(21):13729-34.

A. Introduction

Antigen-driven activation of cells of the immune system generally involves multivalent antigen engagement that leads to clustering or "cross-linking" of cell-surface receptors. This concept arose largely from observations of antigen-induced capping of surface immunoglobulins on B cells (Dintzis et al., 1976; Roelants et al., 1973), and multivalent antigen triggering of histamine release from mast cells (Metzger, 1992). Whether multivalent engagement of T cell antigen receptors (TCR) is required to initiate T cell activation has been difficult to determine, primarily because the antigens are peptide-MHC complexes normally found as integral membrane proteins on the surfaces of other cells. However, experiments using soluble recombinant MHC molecules lacking transmembrane domains and carrying defined synthetic peptides have shown clearly that peptide-MHC oligomers, but not monomers, can activate $CD4^+$ T cells (Abastado et al., 1995; Boniface et al., 1998; Casares et al., 1999; Cochran et al., 2000). Thus, for $CD4^+$ T cells, multivalent engagement of TCR is sufficient to initiate cellular activation, whereas monovalent engagement is apparently non-productive.

For $CD8^+$ T cells, however, the issue remains unclear. Several studies have found that multivalent engagement is required to activate these cells (Abastado et al., 1995; Daniels and Jameson, 2000; Doucey et al., 2001; Maile et al., 2001), but some evidence suggests the sufficiency of monovalent TCR engagement to trigger a cytolytic response, such as the requirement for such a small number of peptide-MHC complexes per target cell in cytolytic assays as to imply that a single complex can trigger the cytolytic response in CD8 T cells (Sykulev et al., 1996). Activation of $CD8^+$ T cells by peptide-MHC monomers has been clearly observed in the presence of CD8 molecules (Delon et al., 1998; Goldstein et al., 1997). It was suggested from that result that monovalent engagement of TCR can serve as the initiating event

when it results in TCR association with CD8 (Delon et al., 1998). However, in another study, TCR-CD8 hetero-dimerization induced by peptide-MHC monomers was observed, but found not to activate the cells (Doucey et al., 2001).

In this study, we investigated the requirements for initiating signaling processes in naïve and activated $CD8^+$ T cells carrying the 2C TCR. The 2C TCR recognizes various peptide-MHC complexes, including i) the potent allogenic activator QLSPFPFDL-L^d (QL9-L^d), which binds with the highest affinity so far measured for any TCR/peptide-MHC interaction (K_d ~ 0.07-0.1 μM, (Sykulev et al., 1994)), and ii) an agonist isolated from a random peptide library (Udaka et al., 1996) SIYRYYGL-K^b (SIY-K^b), which binds almost as strongly if cell-surface $CD8^+$ is present (K_d ~ 0.3 μM), but more weakly if cells do not express CD8 (K_d > 3 μM, (Cho et al., 2001)). Using soluble monomeric and oligomeric forms of these class I MHC-peptide complexes, we find that monomers can induce a variety of characteristic T cell activation processes in naïve and activated $CD8^+$ T cells, but only if the T cells express a class I MHC molecule able to form peptide-MHC complexes with peptide released from the offered peptide-MHC complexes. In addition to clarifying how MHC monomers activate CD8 T cells, these results show that $CD8^+$ T cells can act as antigen-presenting cells able to stimulate naïve T cells to undergo a variety of activation and development processes.

B. Materials and Methods

1. Soluble peptide-K^b complexes

Soluble murine class I MHC proteins H-2 K^b and L^d were produced by expression in *E. coli* and folded *in vitro* in the presence of peptide, as described for human class I MHC proteins (Garboczi et al., 1992 and Protein Expression and Purification Protocols in Appendix), except

that the heavy chains carried a C-terminal biotinylation signal peptide (bsp,(Altman et al., 1996; Chapman-Smith and Cronan, 1999)). *E. coli* expression vectors coding for K^b-bsp, L^d-bsp, and human β_2M, were a gift from J. Lippolis and J. Altman (NIH Tetramer Facility). Peptides were synthesized using 9-fluorenylmethoxycarbonyl chemistry and verified using mass spectrometry: OVA (SIINFEKL), SIY (SIYRYYGL), and QL9 (QLSPFPFDL). For preparation of oligomers, folded peptide-MHC complexes were biotinylated at the bsp sequence using birA enzyme (Avidity, Denver, CO, see Biotinylation of MHC Proteins Using the BirA Enzyme protocol in Appendix). Biotinylated complexes were isolated by gel filtration and oligomerized by stepwise addition of streptavidin to ¼ the molar ratio of the MHC (Crawford et al., 1998 and Staining CD4+ T Cells With DR Tetramers protocol in Appendix). Oligomers were characterized by gel filtration and non-denaturing SDS-PAGE. Protein concentration was estimated by UV absorbance (ε_{280nm}=90,000 $M^{-1}cm^{-1}$ for K^b, 108,000 $M^{-1}cm^{-1}$ for L^d). Immediately before use, peptide-MHC complexes were re-purified by gel filtration chromatography (Superdex 200) to remove aggregates and any free peptide released during storage.

2. Fluorescent labeling of class I MHC complexes

In some experiments, the association of MHC complexes with cells was monitored by flow cytometry, using fluorescein labels incorporated into the K^b and L^d heavy chain sequences at the free cysteine present at position 122. Cysteine 122 is not very reactive and generally was found in the reduced state after conventional isolation, so that treatment with reducing agent was not required to generate a free thiol prior to labeling. For labeling, purified MHC (100 μM) in PBS, pH 7.4, was treated with a 20-fold molar excess of fluorescein maleimide (Molecular Probes, 20 mM in DMSO). The mixture was incubated for 1 hour at room temperature, with

peptide-MHC complexes re-isolated by gel filtration to remove unreacted fluorescein. Labeling efficiency, determined by UV absorbance, was ~99%. For details, see Modifying Free Cysteines in a Protein Using Maleimide Reactive Groups protocol in the Appendix.

3. T cell clones

Murine $CD8^+$ T cell clones 4G3 (specific for OVA-K^b) (Walden and Eisen, 1990) and L3.100 (expressing the 2C TCR, specific for SIY-K^b and QL9-L^d) (Sykulev et al., 1998) were cultured at 37ºC, 5% CO_2, in RPMI medium with 10% fetal calf serum; cells were used 6-7 days after weekly stimulation using irradiated antigen presenting cells (EG7OVA and P815, respectively). See T Cell Maintenance—Murine $CD8^+$ T Cell Clone L3.100 for more details.

4. Naïve T cell purification

$Rag^{-/-}$ 2C TCR transgenic mice (H-2^b) (Manning et al., 1997) were used as a source of naïve T cells. Lymph nodes were isolated, and naïve T cells were purified from antigen presenting cells by depletion of $CD44^+$ cells using magnetic sorting (see Single Cell Suspensions from Mouse Organs protocol in Appendix). At least 99% of the resultant cell population expressed the 2C TCR, with 60-70% of the 2C TCR positive population $CD8^+$ and 30-40% $CD8^-$. In some experiments, $Rag^{-/-}$ 2C TCR transgenic mice crossed onto a constitutive GFP transgenic background, were used as the source of naïve T cells (Cho et al., 1999; Okabe et al., 1997). Naïve H-$2K^{b-/-}D^{b-/-}$ 2C T cells were obtained by adoptive transfer of 10^7 bone marrow cells from mice carrying the 2C transgene on an H-$2K^{b-/-}D^{b-/-}$ background (Maurice et al., 2001) into sublethally irradiated $RAG^{-/-}$, H-2^{b+} C57BL/6 recipients. This procedure allows the H-$2K^{b-/-}D^{b-/-}$ T cells to undergo positive selection in an H-2^{b+}environment. Eight weeks after transfer, T

cells isolated from the recipients were mostly 2C TCR^+ H-$2K^{b-/-}D^{b-/-}$, and were further purified to approximately 98% by depletion of (host) $CD44^+$ cells using magnetic sorting.

5. T cell activation assays

Activation assays were carried out in 96-well plates with 50,000-100,000 cells per well in 50 μl. Plates were pre-blocked by overnight incubation with 1% BSA in PBS solution at 4ºC. Cells were incubated with dilutions of peptide-MHC complexes or free peptide in culture medium for 2, 3, or 6 hours at 37ºC, then washed in cold FACS buffer (PBS with 1% BSA and 0.1% sodium azide), and stained with fluorescent antibodies (Pharmingen) to cell surface markers such as TCR (α-pan-TCRβ), CD69, CD25, and CD8, prior to analysis using a Becton-Dickinson FACS Calibur flow cytometer. Data from flow cytometry experiments is shown in units of mean fluorescence intensity (MFI). For experiments with soluble or plate-bound QL9-L^d complexes, an excess of purified QL9 peptide was included to prevent degradation or peptide loss from the complex. QL9 peptide alone does not activate 2C (H-2^b) cells up to concentrations of at least 10 μM. For antigen re-presentation assays, naïve 2C T cells (GFP^-) were incubated with peptide-MHC complexes or free peptide for 3 hours at 37ºC, washed four times, and re-suspended in media. Naïve GFP^+ 2C T cells were added, and the GFP^+ and GFP^- cells were incubated together for an additional 3 hours at 37ºC before staining as described above. Responses by GFP^+ and GFP^- cells were separated by gating on GFP fluorescence. For proliferation assays, naïve 2C T cells were incubated in the presence of soluble or plate-bound stimuli in 96-well plates for 3 days at 37ºC, after which ^{3}H-thymidine (1 μCi/well) was added, and the cells were incubated at 37ºC for an additional 12 hours, before harvest and assay for ^{3}H incorporation. For IFN-γ assay, naïve 2C T cells were treated with immobilized anti-CD3,

soluble peptide-MHC complex, or peptide alone for 3 days at 37ºC, then re-stimulated with SIY peptide in the presence of brefeldin-A. After 6 hours, the cells were stained for CD8 and TCR, fixed with paraformaldehyde, and stained with anti-IFN-γ antibodies in the presence of 0.1% saponin, before analysis by flow cytometry as described above.

C. Results

1. Activation of CD8$^+$ T cell clones by class I MHC monomers

Soluble peptide-MHC complexes carrying a biotinylation signal sequence at the heavy chain C-terminus were produced by a standard *in vitro* folding method (Garboczi et al., 1992). Streptavidin-mediated oligomerization (Altman et al., 1996) yielded primarily tetramers (~70%), with small amounts of trimers (~20%), dimers (~3%), and monomers (7%) (as detected by high-resolution gel filtration, see Figure III.1). We prepared monomers and oligomers of the 2C agonist SIY-K^b and the non-activating control complex OVA-K^b, and tested their ability to stimulate the CD8$^+$ T cell clone L3.100, which carries the 2C TCR (Sykulev et al., 1998). Several conventional activation markers were observed after incubation of L3.100 cells with SIY-K^b complexes, including downregulation of TCR (Figure III.2A) (Valitutti et al., 1997), upregulation of the early activation marker CD69 (Figure III.2B) (Testi et al., 1994), and upregulation of the high-affinity IL-2 receptor α subunit CD25 (Figure III.2C) (Waldmann, 1989). SIY-K^b oligomers (Figure III.2A-C, squares) were more potent than monomers (closed circles), and consistently induced equivalent activation responses at lower concentrations than the corresponding monomer. Activation was dependent on the specific peptide-MHC combination, as OVA-K^b complexes, which carry a different peptide, did not activate L3.100

cells (Figure III.2D), although they did activate 4G3 T cells, which are specific for OVA-K^b (Figure III.2E) (Walden and Eisen, 1990).

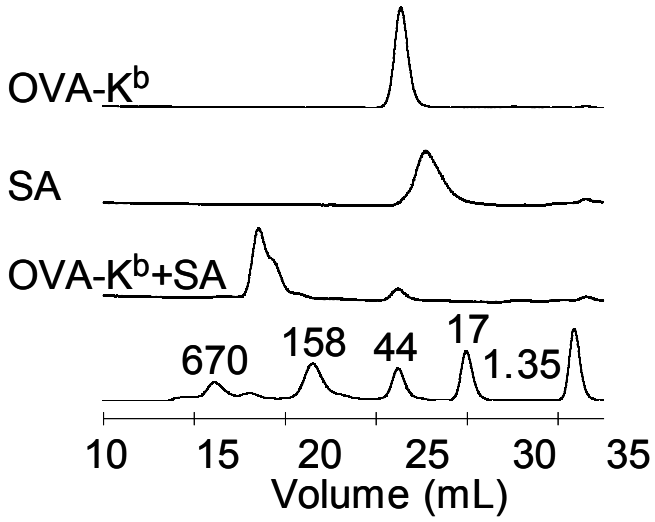


FIGURE III.1 High resolution gel filtration of MHC monomers and oligomers. High resolution gel filtration (Superdex 200) of OVA-K^b monomer, streptavidin (SA), and oligomer (OVA-K^b + SA). SIY-K^b and QL9-L^d complexes behaved similarly. Oligomers were prepared using biotinylated peptide-MHC complexes mixed with streptavidin. The peptide-MHC oligomers produced by this method are predominately tetramers, with some trimers, dimers, and monomers also present. Gel filtration of standard molecular weight markers is shown at the bottom.

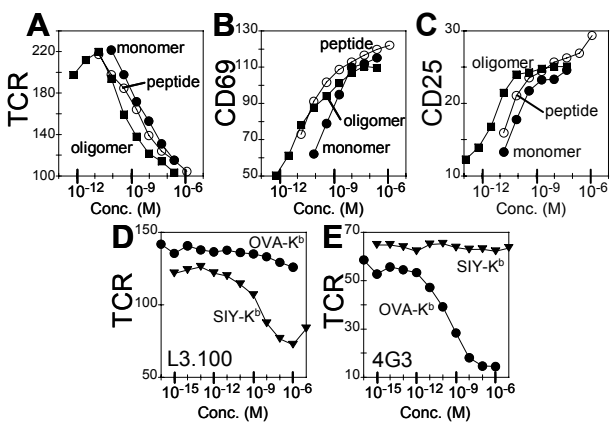


FIGURE III.2 Activation of $CD8^+$ T cell clones by class I MHC monomers and oligomers. (A-C) Response of a T cell clone expressing the 2C TCR (L3.100) to MHC monomers, oligomers, and free peptide. (A) TCR downregulation in response to 3 hour incubation with SIY-K^b oligomer (filled squares), SIY-K^b monomer (filled circles), and SIY peptide alone (open circles). Mean Fluorescence Intensity (MFI) shown on y-axis. (B) CD69 upregulation (MFI) and (C) CD25 upregulation (MFI) in response to the same treatment as (A). (D-E) Specificity of the response. (D) TCR downregulation (MFI) is observed for L3.100 incubated for 3 hours with SIY-K^b monomers (circles), but not for non-specific OVA-K^b (inverted triangles). (E) Response of a T cell clone specific for OVA-K^b complex (4G3) shows TCR downregulation (MFI) after 3 hours of incubation with OVA-K^b monomers (inverted triangles), but not with SIY-K^b (circles).

We have previously used these same activation markers in studies of the response of $CD4^+$ T cells, where we observed activation by class II MHC oligomers but not by monomers

(Cochran et al., 2000). In contrast, the $CD8^+$ T cells clearly were activated in the presence of class I MHC monomers. Given this result, we performed several control experiments to verify that no multivalent protein was present in the experiments. Since immobilized monomeric SIY-K^b is a potent T cell stimulus, we verified that the activation was not due to adventitious immobilization of the soluble molecules on the BSA-blocked assay wells. Removal of the medium containing SIY-K^b before addition of T cells completely abrogated activation (not shown). Activation by the monomeric preparations was not likely to be the result of contaminating MHC oligomers or aggregates, as none were observable by gel filtration (detection limit ~0.5%, Figure III.1) or by native gel electrophoresis (not shown), and a miniscule amount of oligomer contamination would not be expected to account for the observed activation, because oligomers were only ~6-fold more potent activators than the monomers (1.5-fold per peptide-MHC complex, Figure III.2A-C, filled symbols).

Since SIY peptide alone was able to induce activation processes in the T cells (Figures III.2A-C, open circles), we wanted to evaluate any contribution of released peptide to the observed monomer activation. Peptide-MHC monomers and oligomers routinely were separated from free peptide by gel filtration immediately before the activation assay. We incubated the peptide-MHC preparations under assay conditions, and separated free peptide by centrifugal ultrafiltration. Bioassay of the filtrates using peptide-induced CTL-mediated lysis of target cells indicated that very little peptide was released during the 3-hour assay period (the approximate half-time for peptide release was 27 hrs, consistent with earlier studies (Gakamsky et al., 1999)). Moreover, in many experiments the response to peptide-MHC monomers was significantly greater than that induced by an equivalent amount of peptide alone (see below). These details

suggest that the observed activation induced by MHC monomers predominately was not due to release of peptide into the solution.

2. Activation of naïve T cells by peptide-MHC monomers

To investigate whether naïve T cells could be activated by incubation with monomeric peptide-MHC complexes, we used T cells obtained from 2C $TCR^{+/+}$ $RAG^{-/-}$ ($H\text{-}2^b$) transgenic mice. We observed that the purified naïve 2C T cells exhibited TCR downregulation (Figure III.3A), CD69 upregulation (Figure III.3B), and CD25 upregulation (not shown), in response to peptide-K^b monomers and oligomers. Activation levels and half-maximal activation concentrations were similar to those observed with the T cell clones, with tetramers activating substantially more potently than monomers. In addition to these activation markers, we also observed naïve 2C cells to proliferate in response to soluble SIY-K^b (Figure III.3C). In this assay, the SIY-K^b monomers were substantially more active then free peptide (compare open and closed circles), giving further indication that the monomer activation was not due to simple peptide release into the media. The T cell proliferation due to denatured (boiled) SIY-K^b activated T cell proliferation was similar to the corresponding concentration of purified peptide alone, suggesting that endotoxins or other bacterial products potentially present in the SIY-K^b preparations were not contributing significantly to the observed activation (Figure III.3C).

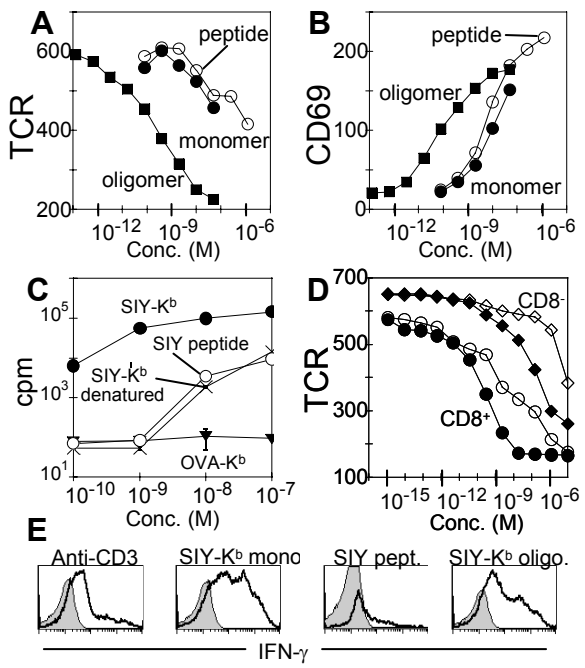


Figure III.3 Activation of naïve CD8$^+$ T cells by class I MHC monomers and oligomers. (A-B) Response of naïve T cells expressing the 2C TCR to MHC monomers, oligomers, and free peptide. (A) TCR downregulation in response to 2 hr. treatment with SIY-K^b oligomer (filled squares), SIY-K^b monomer (filled circles), and SIY peptide alone (open circles). Mean Fluorescence Intensity (MFI) shown on y-axis. (B) CD69 upregulation (MFI) in response to the same treatment. (C) Proliferation of naïve 2C T cells measured by ^{3}H-thymidine incorporation 3 days after treatment in response to SIY-K^b monomer (filled circles), SIY peptide alone (open circles), denatured (boiled) SIY-K^b monomer (X), and non-specific OVA-K^b monomer (inverted triangles). (D) TCR downregulation (MFI) of CD8$^+$ (circles) or CD8$^-$ (diamonds) populations present in purified naïve 2C T cells in response to SIY-K^b monomers (filled symbols) or SIY peptide alone (open symbols). (E) Maturation of naïve 2C T-cells in response to MHC monomers, oligomers, and free peptide. Naïve 2C T cells were stimulated with immobilized anti-CD3 antibody (10 μg/ml), SIY-K^b oligomers, SIY-K^b monomers, or free SIY peptide for 3 days. Intracellular IFN-γ production was measured after 6-hr restimulation by SIY peptide (bold line). The shaded area corresponds to isotype control staining.

To evaluate the importance of CD8 in the 2C-K^b system, we analyzed the substantial CD8$^-$ subpopulation present in naïve T cells isolated from 2C TCR transgenic mice, and compared it to the corresponding CD8$^+$ population. Both CD8$^+$ and CD8$^-$ subpopulations exhibited activation responses after treatment with specific SIY-K^b but not control OVA-K^b monomers, although the CD8$^-$ population required ~10^3-fold greater concentration to induce comparable activation levels (Figure III.3D and Figure III.4).

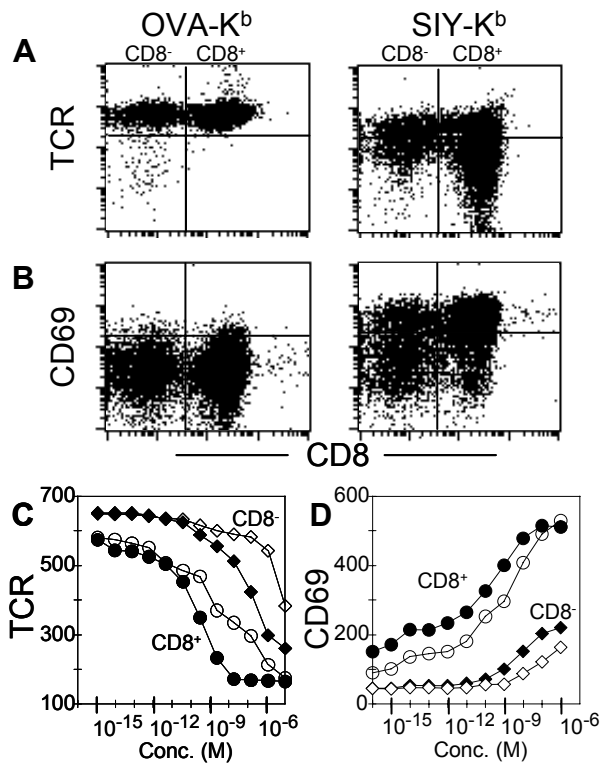


FIGURE III.4 Activation of naïve T cells by MHC monomers does not require CD8. (A-B) 2C T cells treated for 6 hours with 1 μM SIY-K^b monomers or non-specific OVA-K^b monomers were assayed by multicolor flow cytometry simultaneously for (A) TCR downregulation, (B) CD69 upregulation, and CD8 expression (horizontal scale). Both $CD8^+$ (60-70%) and $CD8^-$ (30-40%) were obtained from $Rag^{-/-}$ 2C transgenic mice. (C-D) shows the concentration dependence of the $CD8^+$ and $CD8^-$ 2C T cell response to 6 hour treatment. (C) TCR downregulation in the titration response to SIY-K^b monomers (filled symbols) or SIY peptide alone (open symbols), for $CD8^+$ (circles) or $CD8^-$ (diamonds) cells. (D) CD25 upregulation in response to the same stimuli.

Finally, we evaluated the ability of K^b monomers and oligomers to drive conversion of the naïve cells to effector cells. Effector but not naïve cells are able to produce IFN-γ in response to antigenic stimulation, and we used this as an assay of conversion from naïve to effector cells. After treatment with SIY-K^b monomers or oligomers, the 2C cells responded to antigenic stimulation with production of IFN-γ (Figure III.3E). Although less effective than SIY-K^b, free SIY peptide alone also was able to elicit a small degree of conversion to effector cells (Figure III.3E and data not shown), a response that conventionally is believed to require the participation of co-stimulatory molecules.

3. Activation mechanism: direct engagement or uptake/re-presentation?

The results presented above indicate that addition of peptide-MHC monomers to $CD8^+$ T cells is sufficient to induce T cell activation. Such activation could occur by direct monovalent

engagement of TCR by soluble MHC (Figure III.5A), and conventionally this is the interpretation given to such results (Delon et al., 1998). However, another process could also lead to the observed activation. Peptide-MHC complexes that bind to TCR on one cell could release peptides that are then transferred onto endogenous MHC proteins for presentation to other T cells (Figure III.5B). While T cells generally are not thought to be antigen presenting cells, we observed potent activation of such cells after treatment with peptide alone (Figure III.3), and T cell fratricide has been described previously (Su et al., 1993). Participation of T cell co-stimulatory and adhesion molecules in the activation process could help to explain the observed high sensitivity of the L3.100 and naïve 2C T cell response to soluble MHC monomers (ED_{50} ~ 1 nM), which contrasts with the relatively weak binding affinity measured for the corresponding MHC-TCR interaction (K_d ~300nM, (Cho et al., 2001)). To distinguish between these two mechanisms, we performed additional activation studies, as described below.

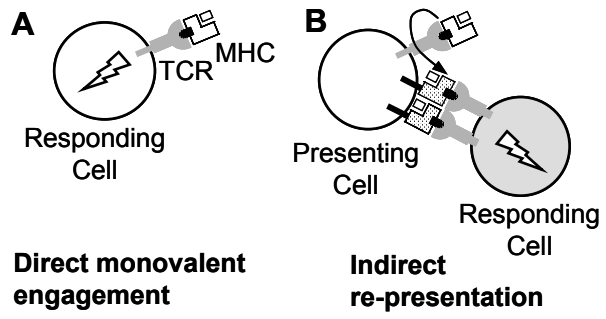


FIGURE III.5 Two potential mechanisms for activation of T cells by MHC monomers. (A) Binding of monomeric peptide-MHC complexes to cell-surface T cell receptors directly leads to activation (direct engagement model). (B) Transfer of peptide from the soluble MHC molecules to endogenous MHC molecules expressed by the T cell indirectly leads to activation of another T cell through interaction with peptide-MHC complexes on the surface of the first cell (re-presentation model). Binding of soluble peptide-MHC to cell-surface TCR (or CD8) could facilitate peptide transfer, although other mechanisms are possible.

4. Activation by soluble monomers requires the presence of endogenous MHC proteins

To evaluate the ability of a soluble monomer to activate 2C cells without contribution from the peptide re-presentation pathway, we made use of the allogeneic agonist (QL9-L^d) that has been described for the 2C TCR. QL9 peptide pulsed onto cell-surface L^d of antigen-

presenting cells (T2-L^d) was a potent stimulator for 2C cells (Figure III.6A), confirming previous work using other activation markers; and soluble QL9-L^d monomer bound tightly to 2C (Figure III.6B), consistent with previous measurements (Cho et al., 2001; Sykulev et al., 1994). The 2C T cells express K^b but not L^d molecules on their surface. By comparing the response to soluble SIY-K^b, which can access the re-presentation pathway, with that of QL9-L^d, which cannot, we were able to evaluate the role of re-presention by endogenous T cell MHC molecules to the observed activation processes. In contrast to SIY-K^b monomers, QL9-L^d monomers were not able to stimulate 2C TCR downregulation and CD69 upregulation (Figure III.6C,D). To demonstrate that the recombinant QL9-L^d complexes were functionally active, we used plate-bound QL9-L^d to stimulate 2C T cells: both TCR downregulation (Figure III.6E) and CD69 upregulation (not shown) were observed. Taken together, these results show that the allogeneic QL9-L^d monomer bound to the 2C TCR but did not induce activation processes, whereas the syngeneic SIY-K^b monomer induced substantial activation despite being an intrinsically weaker-binding ligand. Thus, only minimal activation is induced by direct binding of peptide-MHC monomers to TCR, and activation of 2C T cells induced in the presence of peptide-K^b monomers requires the participation of endogenous (K^b) MHC molecules.

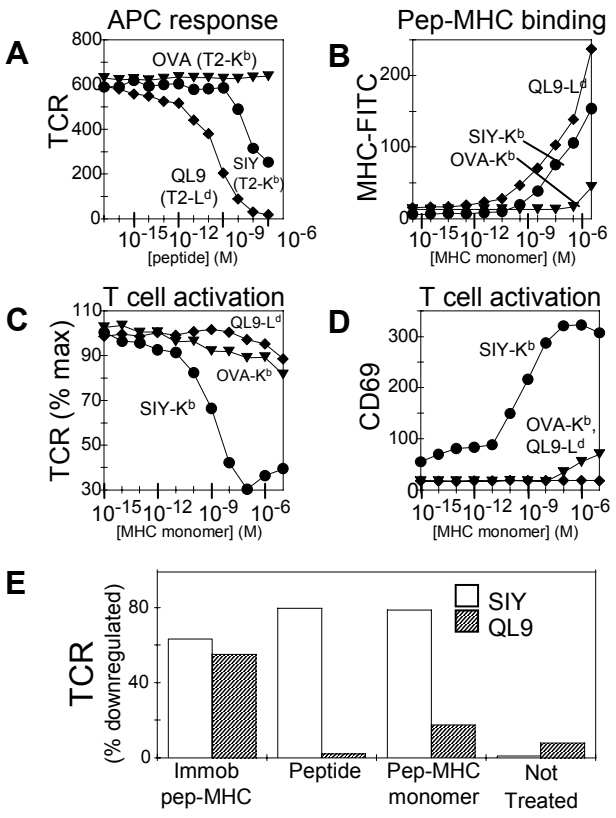


FIGURE III.6 CD8$^+$ T cell activation by MHC monomers requires endogenous MHC proteins. 2C T cells expressing K^b, but not L^d, are activated by soluble monomers of the syngeneic SIY-K^b but by not the potent alloantigen QL9-L^d. (A) Response of naïve 2C T cells to 3 hr of incubation with peptide-pulsed APCs. T2 cells expressing K^b were pulsed with OVA (inverted triangles) and SIY (circles) peptides, and T2 cells expressing L^d were incubated with QL9 peptide (diamonds). TCR Mean Fluorescence Intensity (MFI) shown on y-axis. (B) Binding of fluorescein-labeled SIY-K^b (circles), OVA-K^b (inverted triangles), or QL9-L^d (diamonds) to naïve 2C T cells. Cell-associated fluorescence was measured after incubation with MHC monomers for 30 min at 4°C, washing, and fixation with parafomaldehyde. (C-D) Response of naïve 2C T cells to 3 hr incubation with SIY-K^b (circles), OVA-K^b (inverted triangles), or QL9-L^d (diamonds) monomers. (C) TCR downregulation (percentage of maximum TCR expression), and (D) CD69 upregulation (MFI). (E) TCR downregulation of naïve 2C T cells in response to 6 hr incubation with plate-bound and soluble monomers or free peptide. Open bars represent SIY or SIY-K^b; shaded bars represent QL9 or QL9-L^d.

To further evaluate the role of endogenous MHC in activation by soluble peptide-MHC monomers, we used 2C T cells that lacked the classical class I MHC molecules K^b and D^b (Maurice et al., 2001) (see Methods). We compared the activation of 2C TCR$^+$ $K^{b-/-}D^{b-/-}$ cells and normal (K^{b+}) 2C T cells after incubation with soluble peptide-MHC monomers. Binding curves for fluorescent SIY-K^b monomers were nearly identical for normal naïve and $K^{b-/-}D^{b-/-}$ 2C cells (Figure III.7A, open and closed circles), as were TCR expression levels. Activation by SIY-pulsed T2-K^b antigen-presenting cells was nearly indistinguishable between the two cell types, as measured by TCR downregulation (Figure III.7B). However, activation by soluble SIY-K^b

monomer was severely reduced for the $K^{b-/-}D^{b-/-}$ 2C cells (Figure III.7B). The response was not completely eliminated, and the low level of monomer-induced TCR downregulation observed for the $K^{b-/-}D^{b-/-}$ cells (Figure III.7C), probably resulted from the small fraction of contaminating K^{b+} cells in the T cell preparations. Nonetheless, these results show clearly that T cell activation by soluble SIY-K^b relies largely or completely on the participation of endogenous K^b.

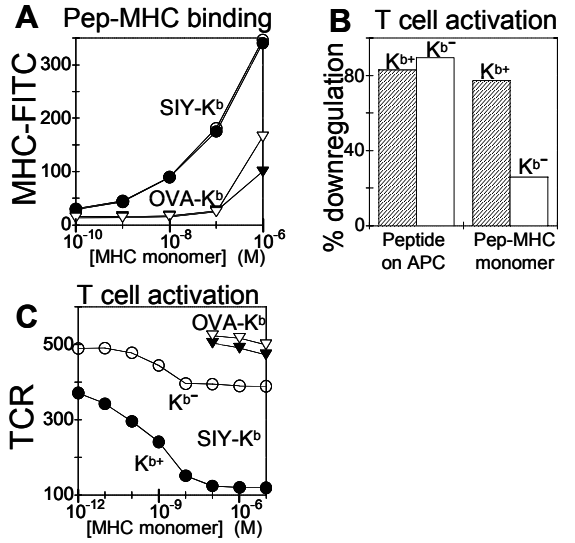


FIGURE III.7 Lack of activation of H2-K^b/$D^{b-/-}$ T cells by soluble MHC monomers. T cells expressing the 2C TCR, but not H2-K^b/D^b, are unable to be efficiently activated by soluble MHC monomers. (A) Binding of fluorescein-labeled MHC monomers to naïve 2C T cells (closed symbols) and $2C^+$, $K^{b-/-}D^{b-/-}$ T cells (open symbols). Binding is shown for SIY-K^b (circles) and OVA-K^b (inverted triangles). Mean Fluorescence Intensity (MFI) shown on y-axis. (B) Bar graph showing the % TCR downregulation for treatment of naïve 2C T cells (shaded bars) and $2C^+$, $K^{b-/-}D^{b-/-}$ T cells (open bars) with peptide-pulsed T2-K^b cells and soluble SIY-K^b monomer. (C) Concentration dependence of TCR downregulation (MFI) response in $K^{b+/+}D^{b+/+}$ (closed symbols) and $K^{b-/-}D^{b-/-}$ (open symbols) naïve 2C T cells after 3 hr incubation with soluble SIY-K^b (circles) and OVA-K^b (inverted triangles).

5. Triggering by soluble MHC monomers does not require direct exposure

In the proposed re-presentation mechanism, T cells that have not been directly exposed to antigen are activated by contact with other T cells that present peptide antigens on their surface. To determine whether or not this behavior was present in our system, we used naïve 2C T cells expressing green fluorescent protein (Cho et al., 1999; Okabe et al., 1997) to track antigen exposure in a mixed T cell population. The presence of GFP does not interfere with T cell activation, and GFP^+ 2C T cells exhibited K^b monomer and peptide activation profiles that were essentially indistinguishable from their GFP^- counterparts (see Figure III.8). In the experiments

shown in Figure III.9, GFP$^-$ 2C cells were incubated with specific K^b monomers for 3 hours at 37ºC, then washed and cultured with untreated GFP$^+$ 2C cells for an additional three hours before analysis of activation markers by flow cytometry. Untreated GFP$^+$ cells, as well as GFP$^-$ cells that had been directly exposed to antigen, both exhibited characteristic TCR downmodulation (Figure III.9A) and CD69 upregulation (Figure III.9B) responses. Thus, activation of naïve 2C T cells did not require direct exposure to monomeric SIY-K^b complexes. To evaluate the efficiency of the uptake/re-presentation mechanism, we compared the titration profiles for GFP$^+$ T cells that were directly exposed to monomer or peptide (Figure III.9C,D), with the profiles for GFP$^+$ T cells that had been co-cultured with GFP$^-$ monomer- or peptide-pulsed counterparts for 3 hours (Figure III.9E,F). The profiles were similar, with only ~10-fold decreased sensitivity for the indirectly exposed cells. The difference is likely due to more effective activation in the continuous presence of stimulators for the directly exposed GFP$^+$ cells (Figure III.9C,D), as compared to the indirectly exposed GFP$^+$ cells (Figure III.9E,F). These results indicate that uptake/re-presentation can provide an efficient antigenic stimulus able to activate untreated T cells, and that such a process is sufficient to account for all or nearly all of the T cell activation induced by soluble class I peptide-MHC monomers (and perhaps also for soluble oligomers, to some extent).

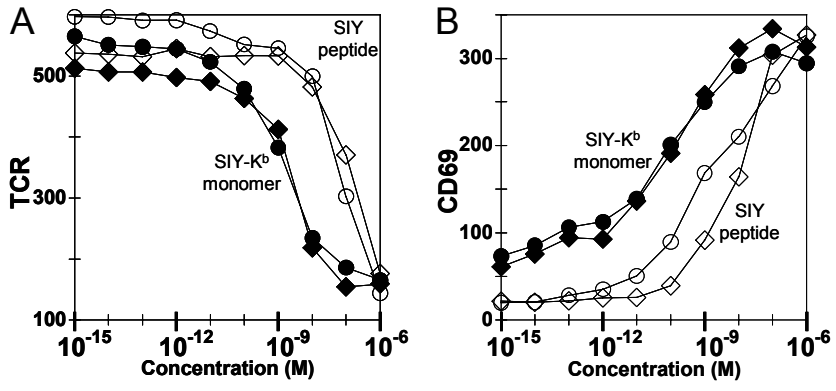


FIGURE III.8 Activation profiles for GFP$^+$ and GFP$^-$ naïve 2C T cells are similar. Activation response of GFP$^+$ (diamonds) and GFP$^-$ (circles) naïve 2C T cells in response to SIY-K^b monomer (closed symbols) or SIY peptide (open symbols) incubated at 37˚C for 3 hours. (A) TCR downregulation, and (B) CD69 upregulation.

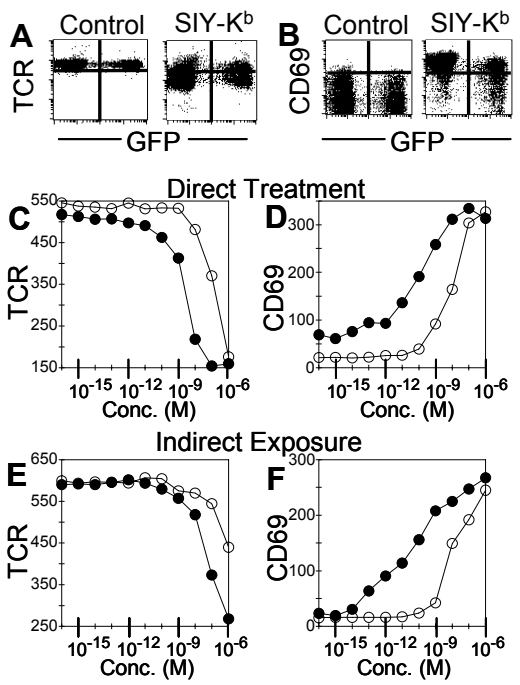


FIGURE III.9 T cell activation by uptake and re-presentation of antigen. Naïve GFP^+ 2C T cells were activated by exposure to GFP^- 2C T cells that previously had been treated with MHC monomers. (A-B) GFP^- cells were treated with control media or SIY-K^b for three hours, washed, and then GFP^+ cells were added to the GFP^- cells in fresh media. The cells were incubated together for an additional 3 hours at 37˚C. (A) TCR downregulation and (B) CD69 upregulation. The horizontal scale is GFP fluorescence level. (C-D) Response of directly treated GFP^+ 2C T cells. (C) TCR downregulation by GFP^+ 2C T cells in response to 3 hr incubation with SIY-K^b (filled circles) or SIY peptide (open circles). Mean Fluorescence Intensity (MFI) shown on y-axis. (D) CD69 upregulation (MFI) by GFP^+ 2C T cells in response to the same treatment. (E-F) Response of indirectly exposed GFP^+ 2C T cells. (E) TCR downregulation (MFI) by GFP^+ 2C T cells incubated for 3 hours with GFP^- 2C T cells that had been pre-treated with SIY-K^b (filled circles) or SIY peptide (open circles). (F) CD69 upregulation (MFI) of GFP^+ 2C T cells in response to the same stimuli.

D. Discussion

The results presented here demonstrate that $CD8^+$ cytolytic T cells and freshly isolated naïve $CD8^+$ T cells can be activated effectively by cognate soluble peptide-MHC monomers. Rather than arising directly from monomeric engagement of the TCR, however, the activation apparently stems from a mechanism that involves transfer of peptide from monomer to T cell MHC molecules. Support for this mechanism rests upon two independent lines of evidence. Firstly, T cells were stimulated effectively only when they expressed the same MHC as the activating monomer: 2C cells, which express K^b but not L^d, were activated by the SIY-K^b monomer but not by the more strongly binding QL9-L^d monomer; and 2C T cells that lacked K^b were not activated efficiently by the SIY-K^b monomer. Thus, T cell expression of the

appropriate class I MHC molecule is required for activation by soluble peptide-MHC monomers. Secondly, 2C cells that were not themselves exposed to the SIY-K^b monomer were activated in *trans* by 2C cells that had been previously exposed to this monomer. Thus, T cell class I MHC molecules can present peptides derived from soluble peptide-MHC monomers. Both of these independent results are consistent with a peptide re-presentation mechanism in which cognate peptide derived from soluble MHC monomers are presented on the T cells' own MHC molecules.

Although we found no evidence for an activation response triggered by monovalent engagement of the TCR, we cannot rule out the possibility that some intracellular signals result from monomeric engagement. Any such response, however, would have to be very small relative to that induced by re-presentation of peptide, and would not contribute significantly to the activation processes observed here. Since monomeric TCR engagement is ineffective in activating $CD8^+$ T cells, it seems reasonable to conclude that $CD8^+$ T cell triggering is due to TCR clustering, as seen for $CD4^+$ T cells. However, we do not know how many re-presented peptides are needed per cell for effective re-presentation, and there remain important differences between the activation of $CD8^+$ and $CD4^+$ T cells, particularly with regard to the minimal peptide-MHC density required on conventional presenting cells to induce T cell activation processes (Brower et al., 1994; Reay et al., 2000; Sykulev et al., 1995; Sykulev et al., 1996; Valitutti et al., 1995).

Precisely how peptide is transferred from soluble monomers to T-cell MHC molecules is unclear. The possibility that transfer results from quantitative dissociation of complexes in culture medium to yield free peptide that binds to T cell MHC is incompatible with estimates of the SIY-K^b complex's lifetime under conditions of the assay, and the observation that free

peptide usually induced less response than an equivalent concentration of peptide-MHC monomer. MHC-bound peptide is protected from proteolytic degradation in culture medium, and, by being slowly released, could be more effective than free peptide in loading onto T cell MHC molecules. The efficiency of peptide loading also might be enhanced by a high local concentration of monomers at the cell surface after binding to TCR and/or CD8 molecules. Still another possibility is that TCR-bound monomers are internalized into T-cell endocytic compartments where dissociating peptide is reloaded onto endogenous class I MHC molecules for recycling to the cell surface. Some evidence suggests that live cells are required for efficient re-presentation of monomer-derived peptides (Figure III.10). Further studies are needed to evaluate these possibilities.

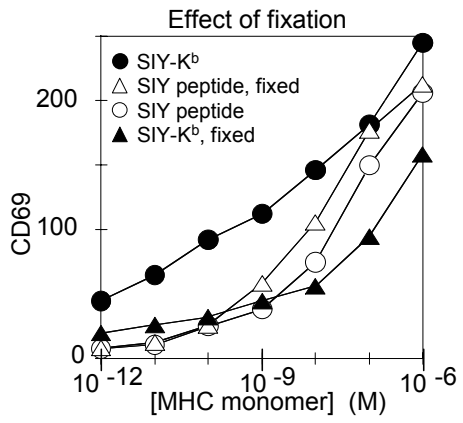


FIGURE III.10 Fixation reduces indirect T cell activation induced by pep-MHC monomer but not by peptide alone. Naïve GFP$^-$ 2C T cells treated with soluble peptide or peptide-MHC complexes are able to induce activation in GFP$^+$ 2C T cells that have not been exposed to soluble antigen. GFP$^-$ cells were either fixed with 0.5% paraformaldehyde for 20 minutes at room temperature or kept in media at 4˚C. Both populations were then washed and incubated with SIY-K^b or SIY peptide at 37˚C. After three hours, the cells were washed four times, and then GFP$^+$ cells were added to the GFP$^-$ cells in fresh media. The GFP$^+$ and GFP$^-$ cells were incubated together at 37˚C for an additional 3 hours. CD69 upregulation by GFP$^+$ 2C T cells is shown in response to GFP$^-$ 2C T cells that were either pre-fixed (triangles) or alive (circles) when treated with SIY-K^b monomers (closed symbols) or SIY peptide (open symbols).

T cell adhesion to antigen-presenting cells is known to sometimes result in the T cell's acquisition of antigen from the presenting cell. This phenomenon has been observed with CD4$^+$ and CD8$^+$ T cells and even with B cells (Hudrisier and Bongrand, 2002). The system described here is distinctive, however, in that antigen-presenting cells are absent: it consists only of

purified T cells and soluble peptide-MHC monomers. It resembles most closely the situation in which addition of cognate peptide at relatively high concentration to cultured CTL clones can, in the absence of any other cells, result in specific T cell-T cell interactions and extensive cell lysis ("fratricide") (Su et al., 1993; Walden and Eisen, 1990). Here, however, the resulting specific T cell-T cell interaction leads not to fratricide of activated cells, but to activation of naïve cells.

Much evidence indicates that, in general, activation of naive T cells requires that they recognize peptide-MHC complexes on professional antigen-presenting cells, usually dendritic cells (Mellman and Steinman, 2001). Our finding that naïve T cells can act as antigen-presenting cells able to induce maturation of naïve T cells is unexpected. In connection with a possible physiologic role of soluble peptide-MHC monomers in this unusual T cell behavior, it should be noted that soluble MHC proteins are found in human serum and synovial fluid, most likely as soluble peptide-MHC complexes (Munoz-Fernandez et al., 2001).

In conclusion, $CD8^+$ T cells are activated by soluble MHC monomers via a mechanism that involves T cell presentation of peptide derived from MHC-peptide complexes. The ability of $CD8^+$ T cells to acquire and present antigenic peptide derived from soluble molecules and/or presenting cells could be important in maturation of naïve T cells *in vivo*.

E. Acknowledgements

We thank John Altman and John Lippolis for K^b-bsp and L^d-bsp constructions, Carol McKinley for T cell maintenance, Madelon Maurice and Hidde Ploegh for $K^{b-/-}D^{b-/-}$ mice, and Glenn Paradis for help with flow cytometry. We would also like to thank Hidde Ploegh, Eckart Schott, and Nicolas Bertho for helpful discussions. This work was supported by NIH N01-AI96361 (LJS), NIH-AI44477 and CA60686 (HNE), NIH-AI44478, NIH-AI50631 (JC), Cancer Center

Core grant CA-14051 (Richard Hynes), Biotechnology training grant NIH-T32-GM08334 (JDS), Anna Fuller postdoctoral fellowship (QG), and support for the MIT Flow Cytometry Core Facility.

IV. On the valency requirements for T cell activation by soluble MHC-peptide complexes.[3]

SUMMARY

Soluble MHC-peptide complexes are useful tools for studying valency requirements for T cell activation. In several studies, CD4 T cells have been shown to respond to soluble oligomers, but not monomers, of activating class II MHC-peptide complexes. For CD8 T cells, however, some studies have reported that monomers can activate while others find a requirement for multivalent engagement. The studies reported here use soluble, allogeneic class I MHC-peptide complexes, to avoid potential re-presentation of antigenic peptide by cellular MHCs. In the absence of such representation, a dimer is necessary and sufficient to stimulate downstream activation in murine CD8 T cells. Soluble MHC-peptide monomers bind, but do not activate. For both murine CD8 and human CD4 T cells, monomeric MHC-peptide complexes antagonize activation induced by oligomers, providing further evidence that monomeric engagement is not productive. However, MHC dimers can be active even if only one of the MHC-peptide complexes is stimulatory.

[3] The research presented in this chapter has been submitted for publication.

A. Introduction

Determination of the molecular event(s) detected by a T cell upon interaction with a cognate APC has been problematic. For B cells and mast cells, which recognize soluble particles, activation requirements have been investigated using monomeric and oligomeric ligands, and multivalent receptor engagement has been observed to be crucial (Metzger, 1992). T cells, by contrast, are triggered by antigenic peptides in complex with MHC molecules on the surface of APCs. This signal is coupled with a multitude of non-antigen specific interactions including MHC co-receptor and adhesion molecule binding, co-stimulation, and possible contributions of non-stimulatory peptide-MHC complexes, which have made it difficult to deconvolute the antigen-specific signal recognized by the T cell.

One method that has been employed to investigate the molecular trigger for T cell activation is to treat T cells with soluble, recombinant MHC-peptide monomers and oligomers. This type of study in CD4 T cells showed that dimers and larger oligomers of cognate class II MHC-peptides were able to stimulate many conventional T cell markers while monomers were unable to induce a response (Boniface et al., 1998; Casares et al., 1999; Cochran et al., 2000). In the context of an APC, however, the contribution of non-activating MHC-peptide complexes may lower the threshold for specific MHC-peptide complexes (Irvine et al., 2002; Wulfing et al., 2002).

Conflicting results have been reported for similar studies of CD8 T cells (Abastado et al., 1995; Daniels and Jameson, 2000; Delon et al., 1998; Doucey et al., 2001; Schott et al., 2002 and Chapter III of this thesis). CD8 T cells generally are more sensitive to low antigen doses than are CD4 T cells. Calcium flux has been observed for both CD8 and CD4 T cells at a single activating peptide per APC, but only CD8 T cells exhibit functional behavior at that level (Irvine

et al., 2002; Sykulev et al., 1996), and CTL responses can occur without the formation of a stable immunological synapse associated with full activation in CD4 cells (Purbhoo et al., 2004). Several studies with soluble MHC complexes have seen triggering for oligomeric but not monomeric class I MHCs (Abastado et al., 1995; Daniels and Jameson, 2000; Doucey et al., 2001; Purbhoo et al., 2004), while another study reported CD8-dependent triggering by monomers (Delon et al., 1998). One complication of this approach for CD8 cells is that peptide from soluble MHC monomers can be presented on endogenous T cell class I MHC, causing activation through cell-mediated presentation (Schott et al., 2002 and Chapter III). Still, some evidence suggests that soluble class I MHC monomers are sufficient to induce signaling in T cells which have been "adhesion primed" by antigen non-specific interactions on an APC or a surface coated with antibodies to T cell surface molecules (Randriamampita et al., 2003).

In this study, we addressed these issues by taking advantage of the murine 2C TCR which reacts specifically to the allogeneic class I MHC-peptide complex L^dQL9 (Sykulev et al., 1994). Soluble monomers and oligomers of L^dQL9 were used to treat CD8 T cells to observe the response without the complication of peptide re-presentation (Schott et al., 2002 and Chapter III). The results showed that like CD4 cells (Cochran et al., 2000), CD8 T cells are stimulated by dimers and higher-order oligomers of soluble peptide-MHC complexes, but are not activated by soluble monomers. In fact, binding of soluble monomers blocks the activation induced by oligomers, further highlighting the non-productive nature of monomeric engagement. However, T cells can be activated by an MHC heterodimer carrying a single activating MHC-peptide linked to an irrelevant MHC-peptide complex.

B. Materials and Methods

1. Antibodies and peptides

Fluorescent monoclonal antibodies to cell-surface molecules were obtained from BD Pharmingen (San Diego, CA), calibration beads for flow cytometry from Spherotech (Libertyville, IL), and unlabeled anti-CD11a monoclonal antibody from Leinco Technologies (St. Louis, MO). Peptides: SIY (SIYRYYGL), OVA (SIINFEKL), QL9 (QLSPFPFDL,), Ha (PKYVKQNTLKLAT), and A2 (VGSDWRFLRGYHQYA), were synthesized using FMOC chemistry and verified using mass spectrometry.

2. T cells

The murine $CD8^+$ T cell clone L3.100 (expressing the 2C TCR, specific for K^bSIY and L^dQL9 complexes) and the human $CD4^+$ T cell clone HA1.7 (specific for the HLA-DR1 – Ha complex), were maintained in culture as previously described (Cochran et al., 2000; Ge et al., 2002), and were used for assays after resting 7-10 days post-stimulation (see T Cell Maintenance protocols in Appenix). Lymph nodes cells from 2C TCR transgenic mice ($H\text{-}2^b$ (Manning et al., 1997)) were depleted of APCs and $CD4^+$ T cells using magnetic beads (Dynal Biotech, Brown Deer, WI), and were at least 99% TCR^+ after purification (75-80% of T cells were $CD8^+$, 20-25% were $CD8^-$ in 2C transgenic T cell preparations). For details, see Single Cell Suspensions from Mouse Organs protocol in Appendix.

3. Soluble MHC-peptide complex monomers and oligomers

Soluble murine class I MHC heavy chain $H2\text{-}K^b$ and $H2\text{-}L^d$ extracellular domains and β_2M were expressed in *E. coli* and refolded *in vitro* as described previously for human class I

MHC complexes (Garboczi et al., 1992 and Protein Expression and Purification protocols in Appendix). For tetramerization, L^d heavy chain included a C-terminal signal peptide for biotinylation using birA (Avidity, Denver, CO) and incorporation into streptavidin-linked tetramers as previously described (Crawford et al., 1998 and Staining CD4+ T Cells with DR Tetramers in Appendix for rough protocol). Fluorescein maleimide was used to label Cys122 (Molecular Probes, Eugene, OR, see Modifying Free Cysteines in a Protein Using Maleimide Reactive Groups protocol in Appendix). For dimerization, heavy chains carried a C122R mutation and an introduced C-terminal cysteine. Disulfide bonds were formed by oxidation as previously described (Cochran and Stern, 2000 and Dimerizing Proteins Through Free Cysteines by Creating a Disulfide Bond protocol in Appendix).

Soluble human class II MHC HLA-DR1 extracellular domains were produced as inclusion bodies and refolded *in vitro* as previously described (Frayser et al., 1999). To make homodimers, a cysteine introduced at the C-terminus of either the alpha or beta subunit was oxidized as above (Cochran and Stern, 2000). For heterodimer formation, cysteine-containing MHCs were separately labeled with either 3-bromopyruvate (Sigma) or N-(ß-maleimidopropionic acid) hydrazide (BMPH, Pierce, Rockford, IL). Excess reagent was removed, and labeled MHCs were combined 1:1 in 30 mM acetate, pH 4.5 for 1 hr., 4˚C to promote hydrazone formation. The mixture was then rapidly transferred to 50 mM phosphate, pH 7.4, and incubated with 5 mM DTT for 30 min. at room temperature to cleave any disulfide-linked dimers. All monomeric and oligomeric MHC complexes were purified by size exclusion chromatography. For details, see Heterogeneous Crosslinking protocol in Appendix.

4. T cell activation and binding assays

T cell assays were carried out as previously described (Cochran et al., 2000 and Chapter III). Briefly, T cells and soluble protein were incubated in BSA-blocked, 96-well plates at 37˚C for 3-6 hours for CD8 T cells or 24 hours for CD4 T cells. After incubation, the samples were chilled on ice, stained for 30 minutes with fluorescent antibodies, then washed, fixed, and analyzed by flow cytometry. For adhesion-priming assays, wells were first incubated with anti-CD11a in PBS overnight at 4˚C, then blocked as above. Fluorescent MHC binding assays were carried out for 30 min. at 4˚C. Flow cytometry was performed on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA).

C. Results

1. Allogeneic MHC-peptide complexes activate CD8 T cells as oligomers, but not monomers.

The murine 2C TCR has well-characterized responses to the syngeneic complex K^bSIY (Udaka et al., 1996), and the allogenic complex L^dQL9 (Sykulev et al., 1994). These complexes do not directly activate 2C T cells as a soluble monomers (Chapter III), although peptide from K^bSIY can cause activation via peptide transfer to T cell K^b and cellular re-presentation. We used the L^dQL9 complex, which cannot be presented by H-2^b cells, to investigate whether murine $CD8^+$ T cells were activated by multivalent TCR engagement by soluble MHCs, as was seen in human $CD4^+$ T cells (Cochran et al., 2000). L3.100, a $CD8^+$ T cell clone carrying the 2C TCR, was treated with soluble L^dQL9 monomers, dimers, and tetramers (Figure IV.1A-B). The soluble MHC-peptide complex L^dQL9 was able to induce TCR downregulation (Figure IV.1A) and CD69 upregulation (Figure IV.1B) when present as a dimer or tetramer, but not as a soluble monomer. Soluble K^bSIY monomer was able to activate due to re-presentation of SIY on L3.100 K^b, as previously described (Schott et al., 2002 and Chapter III). The lack of activation

seen for the L^dQL9 monomer is not due to a lack of specific binding to the TCR, since the fluorescently-labeled monomer was able to bind to the T cells as well as K^bSIY (Figure IV.1C-D), consistent with previous studies (Chapter III). Cross-correlation of CD69 upregulation and TCR downregulation shows that MHC dimers, tetramers, and immobilized monomers all activate qualitatively similar responses (Figure IV.1E). To address the possible significance of the activation signals observed at the highest monomer concentrations (Figure IV.1A,B), in a separate experiment we examined the relationship between the amount of T-cell activation observed and the number of associated MHC. L^dQL9 monomers show no significant T cell activation response at levels of MHC bound that give maximal response for tetramers (Figure IV.1F). Similar results are seen for *ex-vivo* $CD8^+$ 2C transgenic T cells, although with a different dose-response (Figure IV.1G). These results show that while monomers of class I MHC-peptide complexes bind, dimers or higher-order oligomers are required to activate CD8 T cells, similar to CD4 T cells (Cochran et al., 2000).

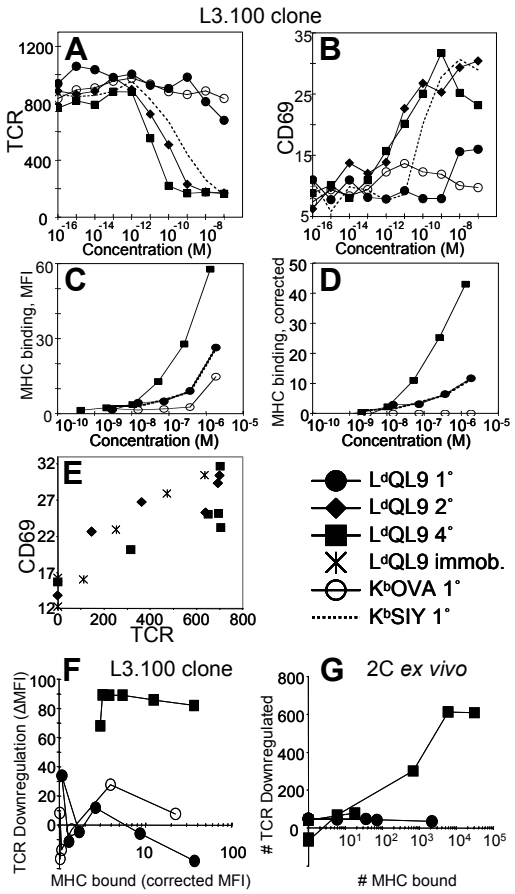


FIGURE IV.1 CD8$^+$ T cells are activated by allogeneic MHC oligomers, but not monomers. (A-B) L3.100, a murine CD8$^+$ T cell clone, was stimulated with soluble allospecific L^{d}QL9 monomers (closed circles), dimers (closed diamonds), or tetramers (closed squares), or soluble syngeneic K^{b}SIY monomers (dashed line), or immobilized K^{b}OVA (open circles). (A) TCR downregulation and (B) CD69 upregulation responses are shown. (C-D) Binding of fluorescently-labeled soluble K^{b}OVA (open circles), K^{b}SIY (dashed line), or L^{d}QL9 (closed circles) monomers, or L^{d}QL9 tetramers (closed squares). (C) Scaled MFI values reflecting relative number of MHCs bound. (D) MFI values with non-specific binding of K^{b}OVA subtracted. (E) Correlation between CD3 downregulation and CD69 upregulation in L3.100 stimulated by soluble L^{d}QL9 tetramers (closed squares), dimers (closed diamonds), or coated on a surface (black stars). (F-G) Relationship between MHC binding and TCR downregulation when treated with soluble L^{d}QL9 (closed circles), K^{b}OVA (open circles), or K^{b}SIY monomers (dashed line), or L^{d}QL9 tetramers (closed squares). (F) L3.100 T cells. (G) Murine CD8$^+$ 2C transgenic T cells.

2. Adhesion priming does not lead to sustained T cell signaling from a MHC-peptide monomer.

In the context of an adherent T cell, a soluble MHC-peptide monomer may be sufficient to trigger some activation directly. CD8$^+$ T cells allowed to adhere to a surface coated with antibodies against cell-surface molecules have showed rapid calcium flux responses to soluble syngeneic MHC-peptide monomer, possibly too quickly for peptide re-presentation (Randriamampita et al., 2003). We tested the ability of allogeneic MHC-peptide monomers and oligomers to induce activation in the presence of a similar adhesion signal. Assay wells were either blocked as normal or coated with antibody against the adhesion molecule CD11a (LFA-1),

which is expressed highly on the surface of 2C TCR transgenic T cells. Soluble monomeric MHC-peptide complexes did not induce TCR downregulation (Figure IV.2A-B) or CD69 upregulation (not shown), regardless of whether immobilized adhesion molecules were present. Responses induced by L^dQL9 tetramers and dimers also were essentially identical with or without adhesion molecules. We conclude that while some signaling from an MHC-peptide monomer cannot be ruled out, TCR engagement by a soluble monomer does not lead to sustained signaling and modulation of cell-surface molecules typical of an activation response.

To evaluate a possible role for CD8 in activation by MHC oligomers, we analyzed the relatively large population of $CD8^-$ T cells naturally occurring in 2C transgenic mice. Similar responses were observed for both $CD8^+$ and $CD8^-$ populations, but with a shifted dose-response for activation of $CD8^-$ cells (Figure IV.2C-D). These results show that MHC-peptide dimers are sufficient for activation without help from the CD8 co-receptor.

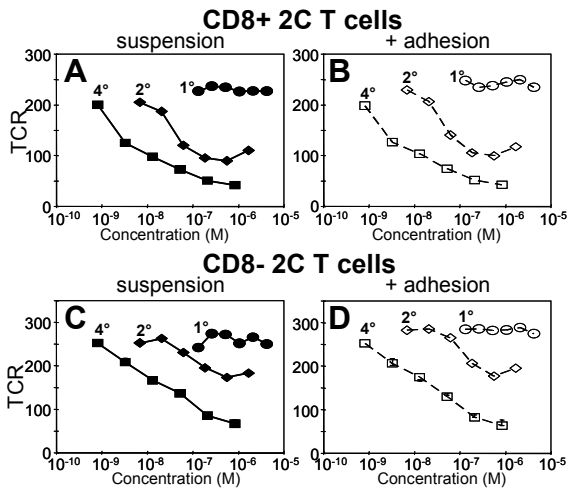


FIGURE IV.2 Adhesion priming does not cause allogeneic MHC complexes to activate downstream processes. $CD8^+$ and $CD8^-$ 2C transgenic T cells were stimulated with soluble L^dQL9 monomer (circles), dimer (diamonds), or tetramer (squares), with coated anti-CD11a antibody (open symbols) or without (closed symbols). (A-B) TCR downregulation for $CD8^+$ cells (A) in suspension or (B) with coated anti-CD11a. (C-D) TCR downregulation for $CD8^-$ cells (C) in suspension or (D) with coated anti-CD11a.

3. Monomeric MHC-peptide binding antagonizes activation induced by MHC-peptide oligomers.

To further evaluate the functional consequences of monovalent TCR engagement by soluble MHC-peptide complexes, we performed a competition experiment. L3.100 cells were

incubated with a constant activating concentration of L^dQL9 tetramer (10 nM) together with various concentrations of L^dQL9 or control K^bOVA monomer (Figure IV.3A). TCR downregulation was reduced by L^dQL9 monomer in a dose-dependent manner (*top panel*), with the inhibition occurring in the same concentration range as MHC-peptide binding (*bottom panel*). The ability of MHC-peptide monomers to inhibit oligomer-induced activation was observed also for CD8+ and CD8- 2C cells from transgenic mice (Figure IV.3B), and for the human $CD4^+$ T cell clone HA1.7, which is specific to HLA-DR1 bound to the Ha peptide (Figure IV.3C).

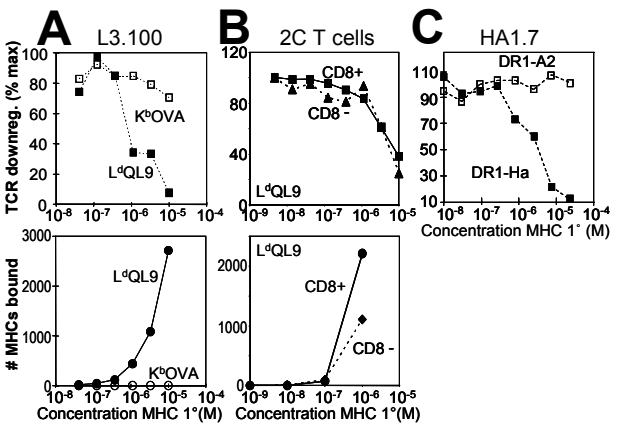


FIGURE IV.3 Monovalent MHC binding antagonizes T cell responses to MHC oligomers. (A) *top panel*, TCR downregulation in L3.100 stimulated with 10 nM L^dQL9 tetramer and increasing soluble L^dQL9 (closed squares) or K^bOVA (open squares) monomer. (A) *bottom panel*, Binding for L^dQL9 (closed circles) and K^bOVA (open circles) monomers. (B) *top panel*, TCR downregulation in $CD8^+$ (squares) and $CD8^-$ (triangles) 2C transgenic T cells stimulated with 10 nM L^dQL9 tetramer and increasing soluble L^dQL9 monomer. (B) *bottom panel*, Binding of L^dQL9 monomer to $CD8^+$ (circles) and $CD8^-$ (diamonds) 2C transgenic T cells. (C) $CD4^+$ HA1.7 T cells (specific to DR1-Ha) were incubated with 100 nM DR1-Ha dimer with increasing soluble monomeric DR1-Ha (closed squares) or non-specific complex DR1-A2 (open squares).

4. T cell activation induced by soluble heterodimers containing one activating and one non-activating MHC-peptide complex

Evidence that a single activating peptide on a cell can cause signaling (Irvine et al., 2002; Sykulev et al., 1996), combined with indications that non-activating MHC-peptide complexes play an important supporting role in T cell stimulation (Wulfing et al., 2002), caused us to investigate whether a single activating MHC-peptide complex could activate in the context of a

soluble MHC oligomer. We devised a method for specifically linking an activating MHC-peptide complex to a non-activating MHC-peptide complex using a unidirectional chemical crosslinking strategy (Figure IV.4A-B). This approach disallows any spontaneous disulfide-linked homodimers by reduction (see Heterogeneous Crosslinking protocol in Appendix). We were then able to compare the responses of the human $CD4^+$ T cell clone HA1.7 to a soluble homodimer of DR1-Ha vs. a heterodimer of DR1-Ha linked to DR1-A2 (Figure IV.4C). TCR downregulation was evident for both soluble dimeric complexes, although the heterodimer was significantly less potent than was the homodimer of DR1-Ha. We were not able to extend this approach to class I MHC proteins because of instability under the crosslinking conditions.

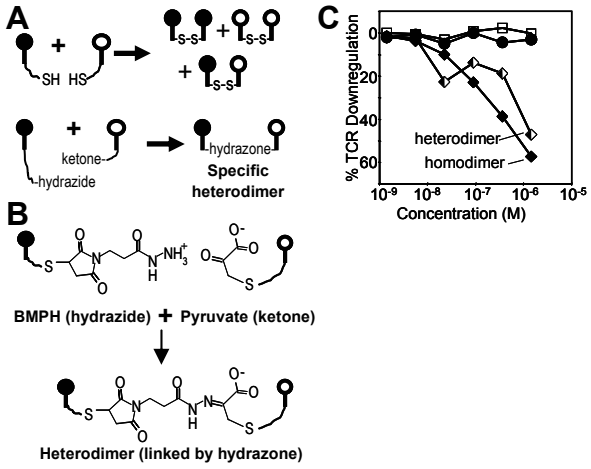


FIGURE IV.4 Soluble dimers consisting of activating and non-activating MHC complexes can activate T cells. (A) Mixing activating (closed) and non-activating (open) MHC complexes, each with a reactive cysteine, results in a mixture of products. Mixing a hydrazide-tagged MHC with a ketone-tagged MHC results in a specific heterodimer. (B) Chemistry used to create specific heterodimer. Free cysteine on one MHC was modified with BMPH, and free cysteine on the other MHC was modified with 3-bromopyruvate. Combining at low pH under reducing conditions results in only the specific heterodimer. (C) The human $CD4^+$ T cell clone HA1.7 (specific to DR1-Ha) was stimulated with DR1-Ha monomer (closed circles), DR1-A2 tetramer (open squares), DR1-Ha dimer (closed diamonds), or a heterodimer (half-open diamonds) of one complex DR1-Ha and one complex DR1-A2.

D. Discussion

In this study, we have shown that CD8 T cell triggering by soluble MHC-peptide complexes follows the same pattern as CD4 T cell triggering (Cochran et al., 2000) when not obscured by peptide re-presentation on endogenous cell-surface MHC molecules. A dimer of

MHC-peptide complexes is necessary and sufficient to induce TCR downregulation and CD69 upregulation, with or without expression of the MHC co-receptor CD8. Adhesion priming by immobilized antibodies to T cell adhesion molecules does not allow allogeneic MHC monomers to activate these processes. Activation by an oligomer of MHC-peptide complexes is antagonized by high concentrations of soluble MHC-peptide monomer. These results taken together suggest that the molecular trigger for CD8 T cells and CD4 T cells is likely to be the same: multivalent TCR engagement.

The nature of the antigen-specific signal transmitted through the TCR is still not completely understood. The fact that a soluble MHC-peptide monomer binding to the TCR does not result in productive signaling argues for a model where TCR clustering is a critical event. However, a soluble heterodimer consisting of only one activating MHC-peptide was able to induce T cell activation. This triggering may be due to the ability of some non-activating complexes to weakly bind the TCR when held in close proximity (extremely high local concentration) by a covalently-linked, bound, activating complex. When a T cell is stimulated by an APC, a single activating peptide could bring about a signal if endogenous MHC-peptide complexes could contribute to binding and promote TCR clustering, with the activating peptide serving as an anchor around which weaker MHC-TCR interactions can play a larger role. The threshold for these interactions could be lowered by the involvement of MHC co-receptors CD4 and CD8, either by simply increasing the affinity of non-stimulatory MHC-peptide complexes, or by a complex bridging model where a co-receptor bound to one MHC is associated with the cytoplasmic domain of a separate MHC-bound TCR (Irvine et al., 2002). However, we note that class I MHC dimers were stimulatory in the absence of CD8 (Figure IV.2D), indicating that any such co-receptor bridging process is not required for activation.

There has been discussion of a potential conformational change, or allosteric re-arrangement of subunits, induced in the TCR upon MHC-peptide binding, which could initiate signaling (Alarcon et al., 2003). Productive activation in T cells correlates with the very early exposure of an intracellular binding epitope for Nck (Gil et al., 2002). Whether the exposure of this epitope is a result of interaction with a single stimulatory MHC-peptide complex propagated through some conformational change, or a result of appropriate TCR clustering, is not known. It is known that the cytoplasmic domains of several components of the TCR complex tend to homo-oligomerize at high concentrations (Sigalov et al., 2004); perhaps ligand-induced clustering of the TCR drives the cytoplasmic domains of proximal receptors to rearrange, exposing the Nck binding epitope and propelling other signaling cascade processes. These results are intriguing for further study.

E. Acknowledgements

We would like to thank Jonathan Lamb (University of Edinburgh Medical School, Edinburgh, UK) for the HA1.7 T cell clone, Herman Eisen and Carol McKinley (MIT, Cambridge, MA) for the L3.100 T cell clone, Cynthia Chambers and Airiel Davis (UMass Medical School, Worcester, MA) for 2C transgenic mice and lymph nodes, and John Altman and John Lippolis (NIH Tetramer Facility) for soluble class I MHC constructs. This work was supported by NIH–U19AI57319 and NSF-NCB0091072.

V. HLA-restricted epitope identification and detection of functional T cell responses using MHC-peptide and co-stimulatory microarrays[4]

SUMMARY

Identification of T cell epitopes is a vital but often slow and difficult step in studying the immune response to infectious agents and autoantigens. We report a spatially-addressable technique for screening large numbers of T cell epitopes both for specific antigen recognition and for functional activity induced. This system uses microarrays of immobilized, recombinant MHC-peptide complexes, co-stimulatory molecules, and cytokine capture antibodies. The array elements act as synthetic antigen-presenting cells, and specifically elicit T cell responses including adhesion, secretion of cytokines, and modulation of surface markers. The method allows facile identification of pertinent T cell epitopes out of a large number of candidates and simultaneous determination of the functional outcome of the interaction. Using this method, we have characterized the activation of human $CD4^+$ and $CD8^+$ T cells responding to vaccinia, influenza, HIV-1, and Epstein Barr Viruses.

[4] This work has been submitted for publication.

A. Introduction

T cells recognize specific antigenic peptides bound to major histocompatibility complex (MHC) proteins on the surface of antigen-presenting cells (APCs), triggering intracellular signaling and T-cell effector functions. The particular functions expressed determine how successfully the infection can be eliminated, and insufficient or inappropriate responses can result in lack of clearance (Collins, 2003; Levitsky and Masucci, 2002), immunopathology (Terajima et al., 2004), or autoimmunity (Christen and von Herrath, 2004). An important step in studying T cell responses is to identify the relevant epitopes, usually short peptides derived from pathogenic molecules that are recognized by clonotypic T cells present in the overall repertoire. The typically low frequency of specific T cells within the overall circulating T cell population and the large number of potential epitopes present in a pathogenic or autoimmune proteome, present challenges to facile and routine identification of T cell epitopes.

T cell epitopes currently are identified by testing overlapping peptides from pathogenic sequences in cellular assays such as T cell proliferation (Yamada et al., 1985), target lysis (Engers et al., 1975), bulk or intracellular cytokine secretion (Maecker et al., 2001), or ELISPOT (Czerkinsky et al., 1988) assays. Screening synthetic peptides that span the entire length of a pathogenic sequence in cellular assays is time- and reagent-intensive, particularly consuming large numbers of T cells, which are often a limiting factor when testing clinical samples. This normally tedious and expensive process can be simplified by using an array format that can both identify pathogen-derived epitopes out of many candidates, and give broad functional information about the nature of the T cell response induced.

B. Materials and Methods

1. Recombinant production of human MHC proteins

Recombinant MHC proteins were prepared by adaption of standard methods for multiple parallel small-scale preparations. Soluble, extracellular portions of HLA-DR1 (HLA-DRB1*0101 and HLA-DRA1*0101) including a uniquely reactive cysteine engineered at the C-terminus of the alpha chain were produced in S2 Schneider cells, chemically-modified with PEO-maleimide–biotin (Pierce Chemical), and loaded with peptide as previously described (Cameron et al., 2002). HLA-A2 (A*0201) extracellular heavy chain carrying a biotinylation signal peptide (Altman et al., 1996), and β_2 microglobulin light chain were produced in *E. coli* as inclusion bodies, refolded in the presence of peptide, biotinylated, and purified by size exclusion chromatography as previously described (Garboczi et al., 1992). For details, see Protein Expression and Purification and Protein Modification and Cross-linking sections of the Appendix.

2. Peptides

Ha[306-318] (PKYVKQNTLKLAT) from influenza hemagglutinin, PP16 (PEVIPMFSALSEGATP) and PG13 (PEVIPMFSALSEG) from the HIV-1 p24 gag protein, QHY (QHYREVAAAKSSE) from the EBV protein BZLF-1, TT (QYIKANSKFIGITE) from tetanus toxin, MVA-74A (CLTEYILWV) and MVA-165 (KVDDTFYYV) from vaccinia, and null peptides A2[103-117] (VGSDWRFLRGYKQYA) from HLA-A2, and Tfr (RVEYHFLSPYVSPKESP) from transferrin receptor, were chemically synthesized and verified using mass spectrometry.

3. T cell clones and lines, growth and maintenance

The $CD4^+$ HA1.7 human T_{H0} clone specific to the influenza Ha peptide bound to the class II MHC protein HLA-DR1 (Lamb et al., 1982), $CD4^+$ CTL cell clone AC25 specific to the HIV-1 gag peptide PP16 in complex with HLA-DR1 (Norris et al., 2004), the $CD8^+$ T cell lines VA55 3.13 and VA49 3.12 specific to the vaccinia peptides MVA-74A and MVA-165, respectively, in complex with HLA-A2 (Terajima et al., 2003), and a short term polyclonal $CD4^+$ T cell line specific to the EBV-derived peptide QHY in complex with HLA-DR1 (Precopio et al., 2003) were maintained in RPMI 1640 medium (Invitrogen), 10% fetal bovine serum, with biweekly stimulation with irradiated allogeneic peripheral blood mononuclear cells (PBMC), 12F6 α-CD3 antibody (from Johnson Wong, Massachusetts General Hospital) and IL2 (BD Biosciences). A murine T cell hybridoma transfected with T cell receptor and CD4 binding domains derived from HA1.7 was maintained in S-MEM (Invitrogen) containing 10% fetal calf serum. See Media Recipes and T Cell Maintenance protocols in the Appendix.

4. Tetramer production and staining

Fluorescently-labeled streptavidin (SA) tetramers used for staining T cells were produced by stepwise addition of SA-phycoerythrin (SA-PE, Biosource) or SA-allophycocyanin (BD Pharmingen) to purified biotinylated MHC samples to a final molar ratio of 1:4, as previously described (Altman et al., 1996; Cameron et al., 2002). Cells were stained at 37°C, fixed with 1% paraformaldehyde, and measured using a BD FacsCalibur flow cytometer.

5. Production of artificial antigen-presenting arrays

Polystyrene, Permanox, and LabTek II CC2 slides were from Nalge Nunc Inc.. MHC-peptide monomers (50 μg/mL) and unlabeled SA-linked tetramers (50 μg/mL bio-MHC with 14μg/mL SA) were immobilized by spotting onto the surface in phosphate-buffered saline (PBS), pH 7.4. We investigated several direct and indirect immobilization strategies and found simple adsorption to plastic or treated glass to be straightforward and reproducible. Co-stimulatory or adhesion antibodies (α-CD11a, α-CD2, and α-CD28, Leinco) were included at 5 μg/mL in the same solution. For cytokine capture chips, the capture antibody (α-IFN, α-TNF, α-IL4, or α-granzyme B, BD Pharmingen) was first spotted at 40 μg/mL and allowed to dry, and the MHC/adhesion antibody solution was spotted onto the dry spots. The arraying was accomplished by either hand-spotting of 0.1-0.5 μL of solution, by a Cartesian Microsystems (Genomic Solutions) automatic non-contact array printer, or by using a Xenopore Manual Microarrayer (Xenopore Corp.). After air-drying, the chips can be stored for more than 3 months at 4 degrees without loss of activity. See Making MHC and Co-stimulatory Array Chips protocol in the Appendix.

6. Cytokine capture detection using artificial antigen-presenting arrays

Spotted, dry chips were blocked by incubation with serum-containing T cell medium for 30 minutes at room temperature. Then cells were added to the chip and incubated 4-16 hours at 37°C, contained within individual chamber slides (Nalge Nunc Inc.) or within a hydrophobic barrier (Ted Pella, Inc.). Typically ~10^6 cells per slide were used, although this was varied as noted. After removal of the supernatant, the chip was rinsed with fresh medium and examined for cell adhesion by eye or microscope. T cells were removed by washing with distilled water twice and with tris-buffered saline solution containing 0.1% Tween 20, pH 7.6 (TBST) three

times. Biotinylated detection antibody and fluorescently-labeled SA (Alexa 555 or Alexa 647, Molecular Probes) were pre-incubated at high concentration (>1mg/mL), and then diluted (1:250 final antibody dilution, 2 μg/mL final concentration SA) into TBST containing 0.3% BSA and 100 μM free biotin. Labeled MHC-detection antibodies were used at a final concentration of 1 μg/mL. Chips were stained for 1.5 hours at room temperature; then the slide was washed 3 times with TBST, 2 times with ddH_2O, and then dried. The chips were scanned using an Affymetrix 428 array scanner, and data were analyzed using Jaguar 2.0 software (Affymetrix Inc.). A single-pixel wide median averaging was performed to remove speckle noise from the images (Adobe Photoshop 7.0.1), and surface plots were prepared using ImageJ 1.30v (National Institutes of Health, USA). Spots exhibiting average fluorescence intensities significantly above no-MHC controls were considered positive. Typically, duplicate or triplicate spots were examined. For precipitating substrate analysis, SA-HRP (Sigma-Aldrich) was used in place of fluorescently-labeled SA, and the spots were developed using an AEC precipitating substrate (BD Pharmingen), and were observed by eye and under a dissection microscope. See the T-Cell Epitope Detection Experiment Using MHC and Co-stimulatory Arrays protocol in the Appendix.

7. Fluorescence microscopy

After incubation as above, the chips were chilled to 4°C and fluorescent antibodies and Hoechst stain were added on ice for 40 minutes. The chip was then fixed with 1% paraformaldehyde for 5 minutes at room temperature, rinsed twice with PBS, and dehydrated once with 70% ethanol. A coverslip was applied with Vectashield, and the spots were observed using a Nikon Eclipse E800 fluorescence microscope. Images were obtained using a Spot RT Slider camera with SpotBasic version 4.0.2 software (Diagnostic Instruments, Inc.).

C. Results

1. Artificial Antigen-Presenting Element Arrays can be used to detect T cell activation.

We prepared microarrays of soluble, recombinant MHC-peptide complexes immobilized together with cytokine capture antibodies (see Methods). When T cells are incubated with such arrays, antigen-specific activation occurs in the particular array elements carrying appropriate MHC-peptide complexes, and this interaction leads to secretion of cytokines and effector molecules. These molecules are captured locally by capture antibodies immobilized along with the MHC-peptide complexes, and are detected in a position-specific manner using fluorescent anti-cytokine detection antibodies (Figure V.1A). An example of such a microarray is shown**,** consisting of a polystyrene slide with a hydrophobic barrier surrounding the arrayed MHC-peptide complexes (Figure V.1B). Small volumes of cell suspension or staining antibody solutions (0.5-1.0 mL) are contained within the barrier. Up to ~1000 individual elements can be arrayed on a slide with each element maintaining its ability to function as a complete antigen presenting entity. Both class I and class II MHC complexes maintain their native conformation after immobilization as detected by conformationally specific antibodies (Figure V.1C, left and Figure V.2). Incubation of these arrays with T cell samples leads to T cell activation and expression of measurable responses. In this example, a T cell hybridoma specific for HLA-DR1 in complex with an influenza peptide (Ha) was incubated with an array carrying various DR1-peptide complexes. While native DR1 conformation was detected in all the array spots, antigen-induced secretion of IL-2 was detected only on the spots that contain the specific DR1-Ha complex (Figure V.1C, right). Devices such as these allow parallel testing of many potential T cell epitopes, conservation of T cells and other reagents, mass-production, and convenient use.

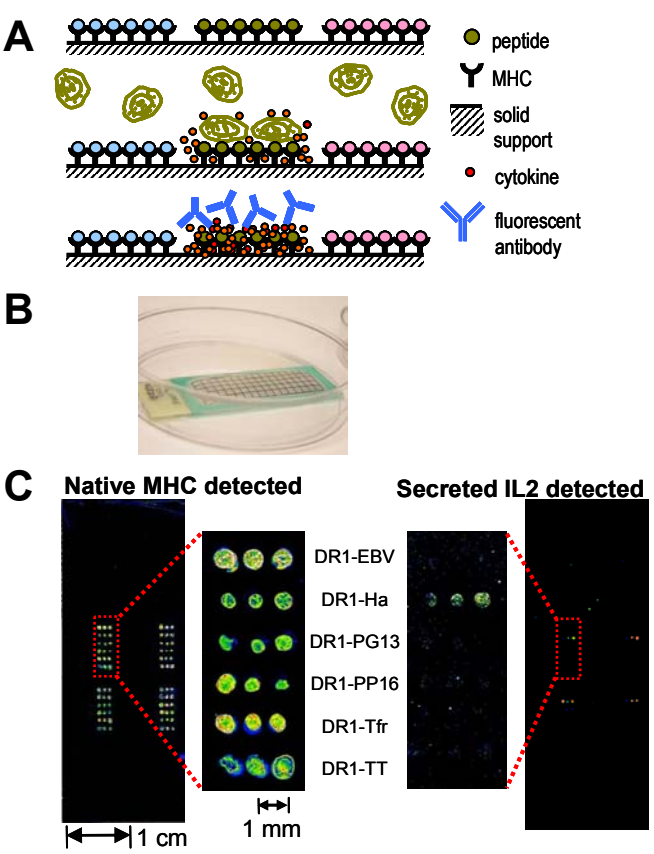


Figure V.1 Artificial antigen-presentation chips. (A) Schematic representation of artificial antigen-presenting microarray technology. Top: MHC-peptide complexes immobilized with different peptide antigens in distinct areas. Costimulatory and cytokine capture antibodies can be co-immobilized (not shown). Center: T cells are incubated with the array. Only specific MHC-peptide complexes induce T cell responses. Cytokines are captured locally by immobilized anti-cytokine antibodies. Bottom: Captured cytokines are detected by labeled antibodies in the locations where they were secreted, identifying the activating epitopes. (B) Photograph of a microarray with solution held in place by a hydrophobic barrier. (C) Microarray carrying different DR1-peptide complexes on a polystyrene slide, incubated with 10^6 murine hybridoma cells specific to DR1-Ha for 16 hours, and stained for native MHC with LB3.1-CY5 (left), and for captured mouse IL2 using biotinylated α-mouse-IL2 pre-incubated with SA-Alexa 555 (right). The pattern was repeated in four areas.

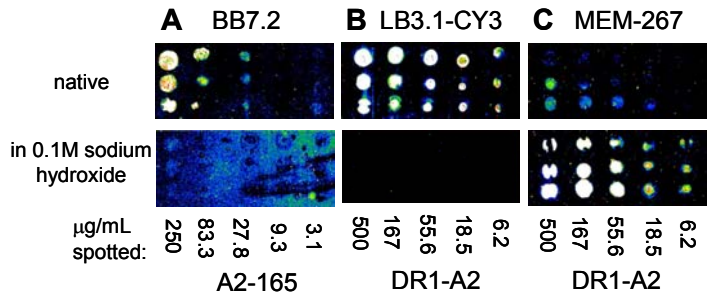


Figure V.2 Native MHC is detected on the arrays. The numbers below each fluorescent image indicate the concentration of MHC in the solution spotted onto the array in μg/mL. (A) HLA-A2 is detected with BB7.2; an antibody which recognizes properly folded A2 associated with light chain. Antibody binding was detected using goat anti-mouse conjugated to Alexa-555. (B) HLA-DR1 is detected using CY3-labeled LB3.1; an antibody which recognizes natively-folded empty and peptide-loaded DR1. (C) Denatured HLA-DR1 is detected using MEM-267, an antibody which recognizes empty or denatured but not peptide-loaded HLA-DR1. Antibody binding was detected using goat anti-mouse conjugated to Alexa-555.

2. The effect of co-immobilized co-stimulatory molecules on MHC-peptide arrays

Most non-transformed and primary T cells require non-specific co-stimulatory and/or adhesion signals in addition to specific MHC-peptide stimulus for induction of full activation responses. Engagement of conventional costimulatory molecules on the T cell surface such as CD28, or integrins such as CD2 (LFA-3) and CD11a (LFA-1), serves to amplify and/or stabilize signals that lead to cytokine secretion (Davis et al., 2003). In the absence of costimulatory signals, T cells adhere to the MHC-peptide coated surfaces in a peptide-specific manner, as has been previously described for murine T cells (Soen et al., 2003). VA55 3.13, a $CD8^+$ human T cell line isolated from a vaccinia-vaccinated individual, specifically recognizes HLA-A2 bound to MVA-74A, a peptide derived from the vaccinia 189R gene product (Terajima et al., 2003). VA55 T cells adhere to array elements containing the specific A2-74A complex, but not the non-specific A2-165 complex (Figure V.3A, left panels). When we included costimulatory signals in the form of antibodies to cell-surface molecules together with the MHC-peptide complexes,

VA55 adhesion was more robust, and T cell spreading indicative of productive interaction is induced in the area containing the specific complex (shown for α-CD11a in Figure V.3A, lower right panel). Although adhesion to non-specific areas is observed in the presence of α-CD11a (Figure V.3A, upper right panel), cytokine secretion is induced only in the specific areas (see below).

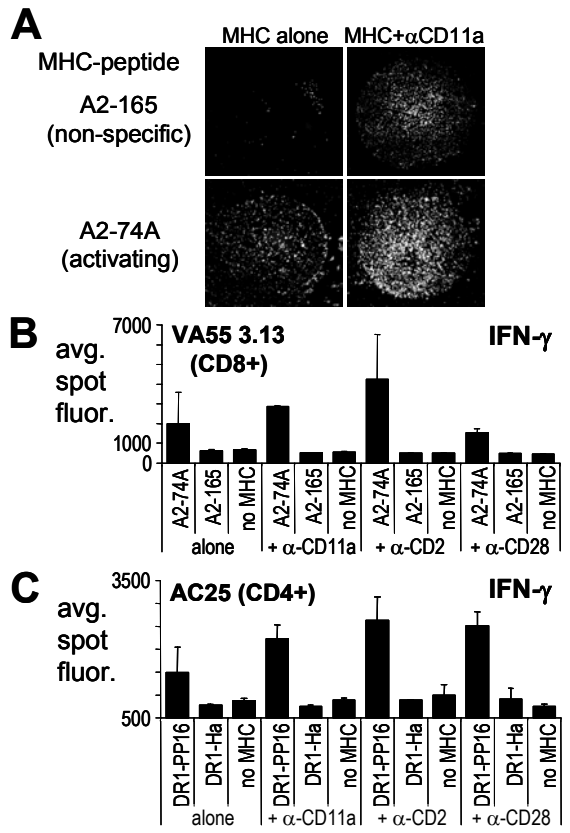


Figure V.3 The effect of co-stimulation on T cell detection. (A) HLA-A2$^+$ MVA-74A peptide specific T cells, incubated for 5 hours on a MHC-peptide array at ~10 cells/mm^2 (3x10^5/chamber on a 4-chamber slide), and stained with Hoechst nuclear stain. Left: MHC complexes were arrayed alone at 50 μg/mL. Right: α-CD11a adhesion antibodies were co-immobilized with MHCs. The top panels each show a non-activating MHC-peptide array element, while the bottom panels each show an activating MHC-peptide spot. The single spot images shown are characteristic of such spots on replicate arrays. (B-C) Average spot intensities (in arbitrary fluorescence units) for IFN-γ secretion by 10^6 cells in response to 6 hours incubation with array elements incorporating different types of co-stimulatory antibodies. (B) Response of VA55 3.13, a CD8$^+$ line specific to A2-74A. (C) Response of AC25, a CD4$^+$ clone specific to DR1-PP16.

Cytokine secretion is greatly enhanced by inclusion of co-immobilized costimulatory molecules in the microarray elements. VA55 3.13 T cells were tested for IFN-γ secretion in response to activating and non-activating MHC-peptide array elements, with and without various co-immobilized adhesion and costimulatory antibodies. IFN-γ secretion was observed in response to A2-74A immobilized on the array, but not to non-specific complexes or in the

absence of MHC's (Figure V.3B). The IFN-γ detected was significantly enhanced in the presence of each of the costimulatory antibodies α-CD11a, α-CD2, and α-CD28 without the large increase in background as for adhesion (Figure V.3A). Similar results were obtained for AC25, a $CD4^+$ T cell clone, raised from an individual infected with HIV-1, that recognizes HLA-DR1 bound to PP16, a peptide from HIV-1 gag p24 (Norris et al., 2004). AC25 also secretes IFN-γ specifically in spots containing the appropriate MHC-peptide complex, and again the signal is enhanced in the presence of the costimulatory molecules tested (Figure V.3C). The combination of specific MHC-peptide complexes and costimulatory antibodies allow the array elements to act as artificial antigen presenting cells with a single epitope localized to each area, and enhances detection of relevant specific T cell responses.

3. Specificity of the response

Identical MHC-peptide chips were created with specific and non-specific MHC-peptide complexes, and tested for IFN-γ induction. Incubations of both $CD4^+$ AC25 (Figure V.4A) and $CD8^+$ VA55 3.13 (Figure V.4B) on these chips result in clear, specific reactions with the correct complex, with little or no detection on non-specific spots. Tetramer staining experiments performed for these same cells indicate the same specificity as observed by the microarrays (Figure V.5).

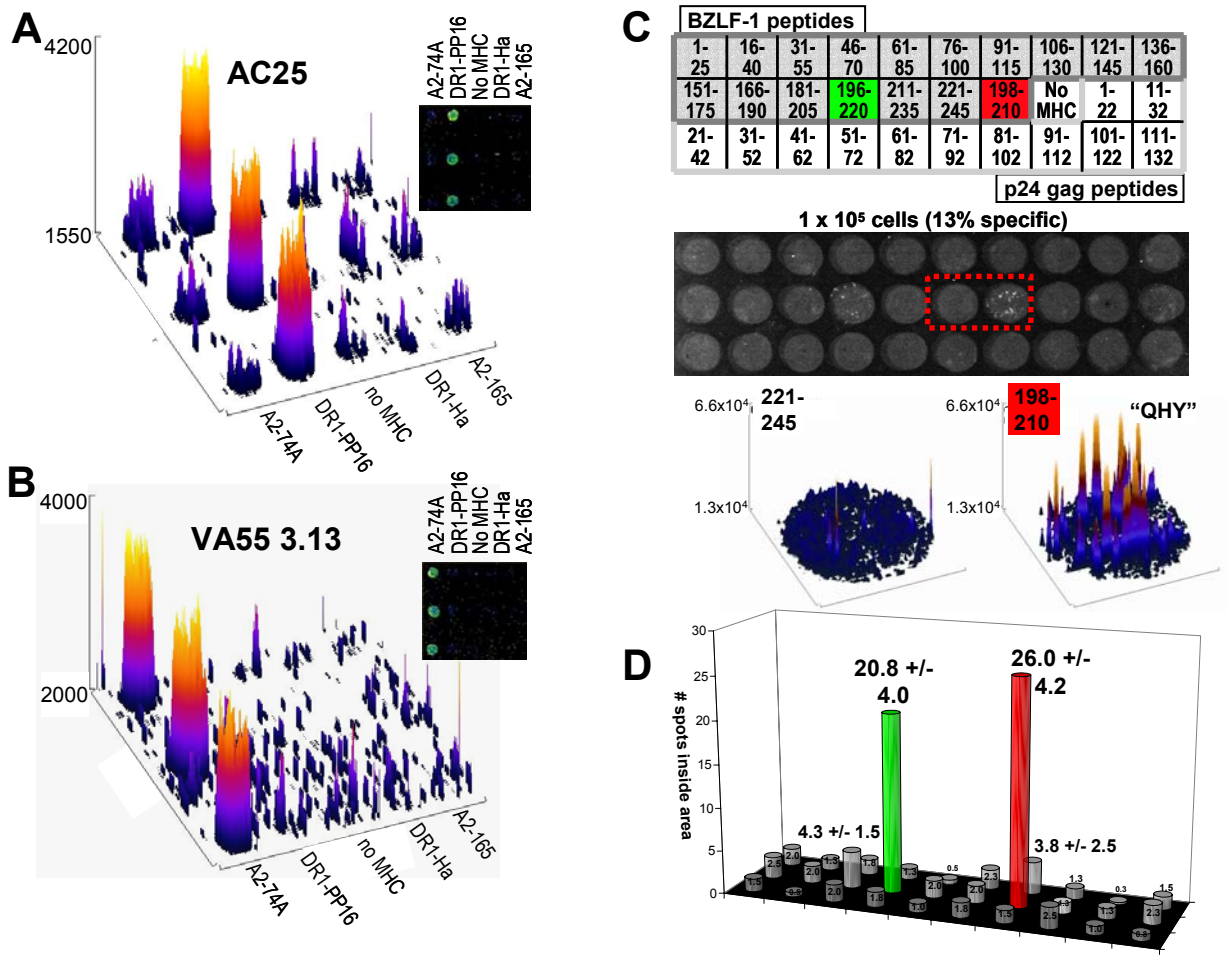


Figure V.4 Arrayed Class I and Class II epitopes are specifically recognized by human CD8+ and CD4+ T cells. (A-B) MHC-peptide complexes and α-CD11a antibody were immobilized with α-IFN-γ capture antibody. The chips were incubated with 1x10^6 T cells and IFN-γ was detected. Fluorescence intensity on the chip is shown as a 3-D surface plot of the array area; raw fluorescence image is shown as an inset. (*A*) CD4$^+$ clone AC25, specific to DR1-gag. (B) CD8$^+$ line VA55 3.13, specific to A2-74A. (C) Response of a short-term polyclonal human T cell line against the EBV protein BZLF-1 (Precopio et al., 2003) was analyzed using overlapping peptides covering the sequence of the protein, each in complex with HLA-DR1. Additional spots contained the previously identified minimal epitope peptide QHY (BZLF-1 [198-210]), a series of HIV p24 gag overlapping peptides, and other control complexes, for a total of 50 spots in an area of 192 mm^2. A partial map of the array is shown on top. The array was incubated with 100,000 T cells, of which ~13% were specific to BZLF-1 by intracellular cytokine staining (about 13,000 specific cells, or 60/mm^2). The secreted IFNγ detected after 6-hours incubation is shown below the map. Small spots, corresponding to cytokines secreted by single responding cells, can be seen within the overall spot areas for two specific array elements that contain BZLF-1 [196-220] (green) from the overlapping peptide series, and the previously identified minimal epitope BZLF-1 [198-210] ("QHY," red). Surface plots for the boxed array elements BZLF-1 [221-245] (non-activating) and BZLF-1 [198-210] (QHY, activating) are shown below the array. Intensity spikes from individual secretion sources can be seen. (D) The average number of such spots observed for each element of the microarray in (C) is shown as a bar graph, with the same layout (n=4).

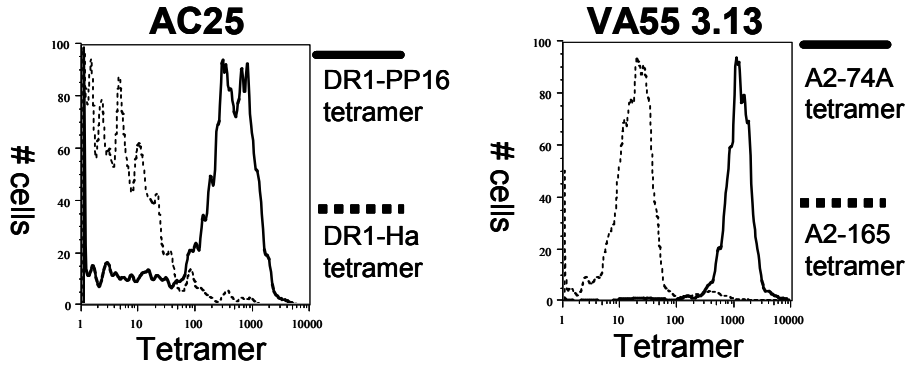


Figure V.5 Tetramer staining of AC25 and VA55 3.13 T cells. Left panel: The $CD4^+$ DR1-PP16 specific T cell clone AC25 binds to DR1-PP16 SA-PE tetramers (solid line) but not DR1-Ha SA-PE tetramers (dashed line). Right panel: The $CD8^+$ A2-74A specific T cell line VA55 3.13 binds to A2-74A SA-allophycocyanin tetramers (solid line), but not A2-165 SA-allophycocyanin tetramers (dashed line).

Often to determine T cell epitopes, one must test the T cells with overlapping peptides covering the entire sequence of a known protein immunogen. We prepared microarrays carrying overlapping peptides covering the sequence of the lytic stage antigen BZLF-1 from Epstein-Barr virus, and used them to screen for reactivity in a short-term T cell line raised from the PBMCs of a HLA-$DR1^+$ patient presenting with acute infectious mononucleosis (Precopio et al., 2003). In addition to the BZLF-1 overlapping 25-mer peptide series, the arrays also included the known minimal peptide epitope QHY (BZLF-1 [196-210]), and a second overlapping peptide series covering the sequence of HIV-1 gag p24.[5] Each peptide epitope was allowed to bind to peptide-receptive DR1, and the resulting complexes were spotted onto the array, resulting in 50 possible spots with which the cells could react. The spots were created using 0.1 μL MHC-peptide solution at 50 μg/mL per spot, with a resultant area of ~0.44 mm^2 for each spot. The short-term T cell line, which is about 13% specific for BZLF-1 by intracellular cytokine staining (not shown), was incubated on the chips at a density of 96,000 total cells per array (approximately 60

[5] Note that for class I MHC proteins, the peptide termini typically bind within the MHC binding site, and each potential epitope must be present as a separate peptide. For class II MHC proteins, the peptide termini can extend from the site, and a single long peptide can be used to evaluate multiple potential epitopes. Thus, many more individual peptides must be evaluated to screen a protein sequence for class I as compared to class II epitopes.

specific cells per mm^2) (Figure V.4C). IFN-γ was specifically detected in the spots with previously identified EBV epitopes: peptide 196-220 in the overlapping peptide series (Figure V.4C, green), and the minimal peptide epitope QHY (Figure V.4C, red). At these low cell densities, the inherent granularity of the assay is apparent, and the chips are more accurately analyzed by counting individual spots within the larger array spot areas, as in an ELISPOT analysis (Czerkinsky et al., 1988), instead of analyzing overall intensities. Surface plots for a non-activating array element (BZLF-1 [221-245]) and the adjacent activating array element (QHY) clearly show spikes corresponding to individual secreting cells, as opposed to higher density cell incubations where a more continuous peak is observed (compare surface plots in Figure V.4C to Figures V.4A-B). The spots within each area were enumerated, and the average values for four experiments are shown in a bar graph with the same layout as the array (Figure V.4D). The positive elements are readily apparent even at a low responding cell density. An example of an identical array incubated with the EBV-specific T cell line at higher density can be seen in Figure V.6.

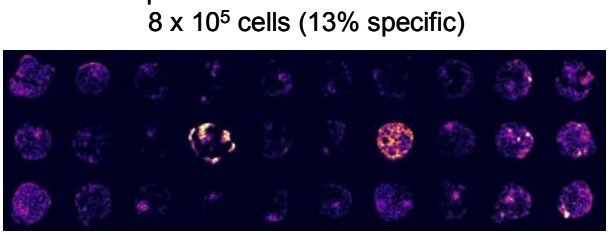


Figure V.6 EBV-specific response from a short-term line can be detected by a mapping chip. A short-term T cell line raised from a patient with acute infectious mononucleosis, which is about 13% specific for the EBV protein BZLF-1 by intracellular cytokine staining (not shown), was analyzed using a series of overlapping peptides covering the sequence of the protein, each in complex with HLA-DR1. Cells were incubated on a chip at a density of 500 specific cells per mm^2, or about 8 x 10^5 total cells per array. A map of the chip can be seen in Fig. 3C. The fluorescence map obtained after 6-hour incubation with the polyclonal T cell line and detection of secreted IFNγ is shown. IFN-γ was specifically detected most intensely in the spots with the previously identified EBV epitopes: BZLF-1 [196-220] in the overlapping peptide series and the minimal peptide epitope QHY (BZLF-1 [198-210]).

4. Sensitivity of cytokine secretion response

T cells responding to infection or vaccination often are present at low frequencies in the overall T cell population. As a test of sensitivity of this technique, the VA55 3.13 T cell line was diluted into non-specific (allogeneic) PBMCs and tested on an array with specific and non-specific HLA-A2 complexes. Secretion of granzyme B, a cytotoxic T cell marker, was detected. Fig. 4 shows VA55 3.13 tested at dilutions ranging from 10% to 0.1% of the total cells, using in each case $2.5x10^6$ cells, a sample size corresponding to 1-3 mL of blood. A specific secretion signal was observed even after dilution to 0.1% of the total cells, a frequency of ~1000 specific cells per million (Fig. 4, 0.1% panel), or roughly 20-100 per spot assuming even distribution of cells around the array; however, at this level, interpretation is complicated by the relatively high non-specific background that approaches the sample-to-sample variability. This sensitivity is in the range of other current T cell antigen screening assays such as MHC tetramer staining (Dunbar and Ogg, 2002) or ELISPOT assay (Czerkinsky et al., 1988); however, using the microarray assay, many epitopes can be assayed in a single well.

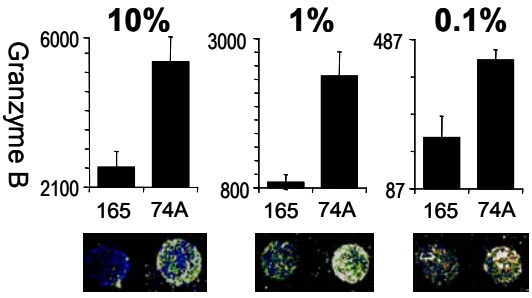


Figure V.7 Detection of low-frequency responses. VA55 3.13 $CD8^+$ T cells (specific to HLA-A2 in complex with MVA-74A) were diluted into allogeneic PBMCs, and granzyme B secretion was detected on the chips after incubation. In each panel, the total number of cells per chamber was $2.5x10^6$. The spots are ~1 mm in diameter, for an average spot area of 0.75 mm^2. Average intensities of spots (*n*=2) and raw fluorescence image is shown after dilution of VA55 3.13 (left to right panels) to 10% of the total cells (100,000 per million), 1% of the total cells (10,000 per million), and 0.1% of the total cells (1000 per million). At the lowest level, the average spot intensity is less straightforward to interpret due to relatively high background, and more representative values may be obtained by counting individual ELISPOT-like secretion spots within the array areas.

5. Multiple cytokines can be tested simultaneously

The cytokine capture chips can be designed for simultaneous detection of multiple cytokines from a single responding T cell population. In Figure V.8A, VA55 3.13 was screened in a four-chambered slide, with different capture antibodies immobilized in each chamber. Specific secretion of IFN-γ, granzyme B, and TNF-α, but not IL4, was observed. These results replicate those observed in conventional bulk cytokine secretion ELISA experiments performed on the same cells (Figure V.8A). In another approach, multiple cytokines can be distinguished using anti-cytokine antibodies labeled with different fluorescent probes. Figure V.8B shows an array carrying both IFN-γ and IL4 capture antibodies along with specific and non-specific MHC-peptide complexes. Using two differently labeled cytokine detection antibodies, both IL4 and IFN-γ were detected in only the appropriate regions (Figure V.8B). The two strategies of spatial separation and multiple fluorescent labels for detection could be applied concurrently to obtain large amounts of information from a single experiment. Thus, very broad screens for functional responses could be carried out simultaneously with parallel screens of many different T cell epitopes.

6. Non-fluorescent detection of cytokine capture

In some cases, it may be advantageous to detect the cytokine secretion in a manner visible to the naked eye by using enzyme-linked antibody and precipitating substrate, as in a traditional ELISPOT assay, rather than fluorescent detection. Figure V.8C shows an array which was incubated with the $CD8^+$ T cell line VA49 3.12, isolated from a vaccinia-immunized individual, which responds specifically to HLA-A2 in complex with the MVA-165 peptide (from

the vaccinia gene product "018L"), but not the MVA-74A peptide (Terajima et al., 2003). The array was analyzed using biotinylated α-IFN-γ and peroxidase-coupled SA. Visually apparent development can be seen in areas where specific A2-165 peptide complex or positive control α-CD3 stimulus was immobilized, but not in areas with non-activating A2-74A complex (Figure V.8C).

7. Other activation markers

MHC-peptide microarrays may be used to detect other activation markers in T cells. For example, specific detection of transient calcium flux in cells on peptide-MHC tetramer arrays has been reported in murine T cells (Soen et al., 2003). We investigated whether T cell surface markers, conventionally assayed in flow cytometry experiments, also could be evaluated in parallel on MHC-peptide and costimulatory microarrays. Figure V.8D shows a 60x view of a single element of a MHC-peptide array with human $CD4^+$ HA1.7 T cells specific for DR1-Ha (Lamb et al., 1982) adhering. These cells have been stained with Hoechst nuclear stain, α-CD3-PE, and α-CD69-FITC. CD69 is a common early activation marker that is upregulated upon T cell activation (Testi et al., 1994), and CD3 is a component of the T cell receptor which is downregulated upon activation (Valitutti et al., 1997). The HA1.7 T cells adhering to the specific DR1-Ha region are more numerous and clustered as seen by the blue Hoechst stain, and exhibit lowered CD3 expression (red) and substantial CD69 upregulation (green) as compared to cells in the non-specific DR1-PP16 region. This technique could be applied to detect the expression of other cell-surface activation markers, or markers of intracellular signaling pathways.

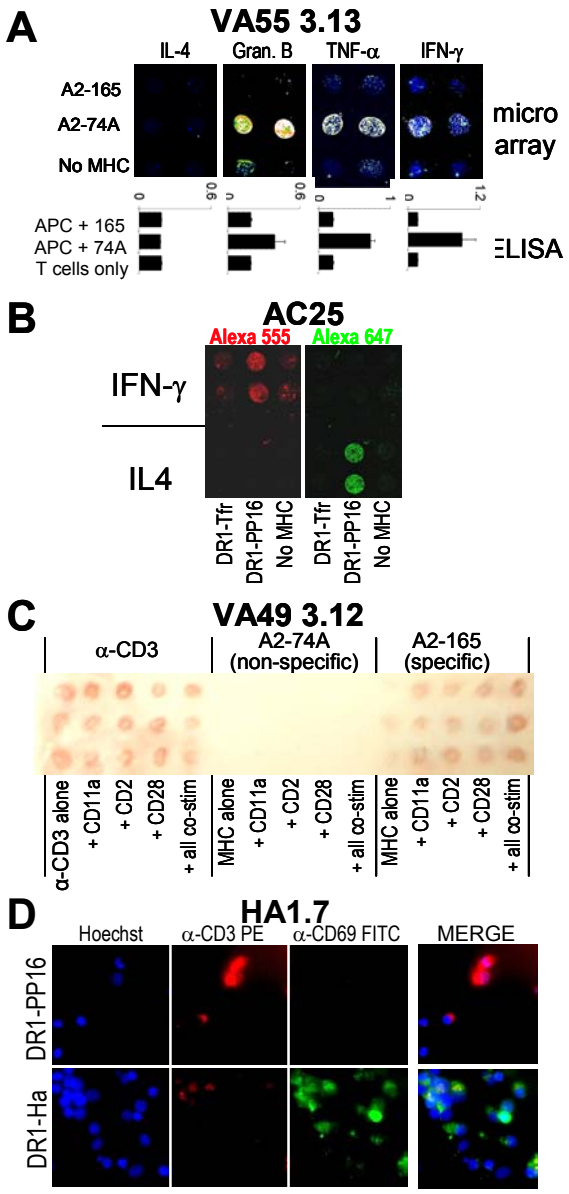


Figure V.8 Analysis of multiple T cell functions. (A) A multi-chamber LabTek II CC2 slide was prepared with a different capture antibody in each chamber, but identical MHC-peptide and α–CD11a patterns. $2.5x10^5$ VA55 3.13 cells (specific to A2-74A) were incubated in each chamber, and then cytokine secretion was analyzed using the appropriate detection antibody. Below each image is a bar graph showing the same cytokine detected by a bulk ELISA where VA55 3.13 T cells are stimulated with peptide-pulsed, HLA-$A2^+$ APCs. (B) A chip was spotted with α–IFN-γ and α-IL4 capture antibodies in different areas with the same MHC-peptide and α–CD11a stimuli co-immobilized. The chip was incubated with $1x10^6$ AC25 T cells (specific to DR1-PP16), and then stained with a mixture of biotinylated α–IFN-γ pre-incubated with SA-Alexa 555 and biotinylated α–IL4 pre-incubated with SA-Alexa 647. The chip was scanned at both wavelengths: Alexa 555 is shown in red, and Alexa 647 is shown in green. (C) Cytokine capture can be detected on the arrays using precipitating substrate. The array shown was incubated with VA49 3.12, a $CD8^+$ T cell line specific to A2-165. IFN-γ secretion was detected in the regions containing α–CD3 and A2-165, but not A2-74A. (D) Cell-surface protein upregulation in response to array elements can be detected using fluorescently labeled antibodies. HA1.7, a $CD4^+$ T cell clone specific to DR1-Ha, was incubated on a microarray containing DR1-PP16 (null, top row) and DR1-Ha (activating, bottom row). Hoechst nuclear stain shows the presence of adhering cells, while α–CD3-PE and α–CD69-FITC were used to show the relative levels of those activation-linked cell surface markers.

D. Discussion

We have described a new technique that can be used to screen small samples of T cells for specific peptide-MHC binding as well as for functional responses in a microarray format.

Each array element consists of recombinant peptide-MHC complexes co-immobilized with co-stimulatory antibodies and anti-cytokine antibodies to locally detect T cell adhesion and activation responses; these elements act as individual artificial antigen-presenting cells (Oelke et al., 2003) with each element presenting a different T cell epitope candidate. The arrays are convenient to use with small volumes of cell suspension or staining solutions, allowing for reagent conservation, and are mass-producible with commercially available array spotters. The arrayed MHC-peptide complexes are largely native and remain spatially distinct after multiple washes, incubations, and stainings. The dry MHC-peptide arrays are stable for long periods in excess of 3 months at 4°C. The functional responses induced when T cells recognize array elements, such as cytokine secretion or activation of cytolysis, are detected in a location-dependent manner. In previous work, MHC-peptide arrays were used to observe adhesion and calcium flux in murine $CD4^+$ T cells (Soen et al., 2003). Here, the combination of specific MHC-peptide complexes and costimulatory antibodies greatly enhances the detection of relevant specific functional responses.

The advantages of array technology that have revolutionized gene expression research have already begun to be applied to important clinical problems of immunology and infection. Protein microarrays have been used to detect specific antibody responses in human serum (Bacarese-Hamilton et al., 2003), which may be applied conveniently to allergy testing, diagnosis of some diseases, and vaccine design for certain pathogens. However, many infections are not eliminated by antibody responses (Aasa-Chapman et al., 2004; Khanna et al., 1995; Logvinoff et al., 2004), and in those cases, the ability to use array technology to analyze the cellular immune response would be beneficial. Using antigen-presentation microarrays with live immune cells allows broad, multidimensional information to be obtained quickly and easily. The

ability to conveniently track T cells for specificity and functional response for many epitopes in parallel in small clinical samples has the potential to lend new levels to our understanding of the rise and fall of T cell populations during vaccination or infection.

Artificial antigen-presenting arrays show promise as convenient tools with which to discover T cell epitopes and characterize T cell responses. This technology is able to sensitively detect low-frequency responses as well as give extensive information on the nature of the response to different T cell epitopes. This sort of data, conveniently and rapidly obtained, can be utilized to accelerate studies of the cellular immune response to novel pathogens and to promote development of safe, effective vaccines or other innovative immunotherapies.

E. Acknowledgements

We thank Liying Lu, Iwona Strug, and Joyce Pepe for reagents, John Cruz, Masanori Teramija, Melissa Precopio, Phillip Norris, Jonathan Lamb, and Jerome Bill for T cells, and Alan Rothman for helpful comments. Supported by NIH-N01-AI95361, U19-AI57319, and the UMass Genomics Core and Fluorescent Microscopy facilities.

VI. Conclusions and Future Directions for Research

A. T Cell Activation Mechanism

1. Multivalent T cell receptor binding is sufficient for activation processes

Chapters II-IV of this thesis showed that for both $CD4^+$ and $CD8^+$ T cells, simple cross-linking of T cell receptors is adequate to induce activation processes. This cross-linking may be achieved by cognate MHC-peptide complexes on the surface of an antigen-presenting cell, or by various soluble MHC-peptide reagents capable of binding to multiple T cell receptors simultaneously. Cross-linking by non-physiological ligands such as antibodies to the T cell receptor or signaling subunits of the TCR complex can also trigger T cell signaling.

Chapter II presented a simple model based on multivalent binding equilibria that relates properties of the $CD4^+$ T cell response such as MHC-ligand binding, TCR downregulation, CD69 upregulation, and CD25 upregulation using the binding parameter K_D to describe monovalent MHC-TCR affinity, and K_X to describe TCR cross-linking by subsequent MHC binding to TCR from the same oligomeric MHC complex. The parameters K_D and K_X could be fit to the observed T cell activation after treatment with a series of soluble MHC-peptide oligomers of increasing size. Different T cell clones recognizing identical MHC oligomers exhibit different binding and cross-linking parameters that could correlate with their sensitivity to the ligand. These results suggest that information about the number of cross-linked receptors on the surface of the T cell induced by MHC oligomer binding is maintained through signaling cascades, and that cross-links can be directly converted into activation signals.

Chapter III discussed an important confounding feature in the activation of $CD8^+$ T cells by soluble recombinant MHC-peptide molecules. Peptide derived from soluble MHC complexes

added to $CD8^+$ T cells can be loaded onto endogenous class I MHC molecules on the T cell surface, with cell-cell mediated presentation of the peptide-MHC complex sensitively triggering activation processes. This phenomenon may be at play in all T cell triggering by soluble molecules, depending on the stability of the MHC-peptide complex and the relative proteolytic stability of released peptides. Without contributions of cell-surface MHCs, $CD8^+$ T cells were shown not to respond to soluble MHC monomer binding, as analyzed by allogeneic MHC-peptide stimulation or syngeneic MHC-peptide stimulation of T cell chimeras that do not express cell-surface class I MHCs.

Chapter IV showed that $CD8^+$ T cells respond to soluble oligomers of allogeneic MHC-peptide complexes in a mode similar to $CD4^+$ T cells. Stimulation was seen by a soluble allogeneic class I dimer in naïve T cells with or without expression of the CD8 co-receptor. For both $CD4^+$ and $CD8^+$ T cells, a soluble MHC-peptide monomer can antagonize responses to soluble MHC-peptide oligomers in activation assays, suggesting that monomeric binding cannot contribute to cross-link induced T cell signaling. However, T cell triggering was observed when $CD4^+$ T cells were treated with a heterodimer of MHCs including one cognate MHC-peptide and one non-specific MHC-peptide, whereas neither treatment with soluble cognate monomers or treatment with non-specific oligomers was able to induce a response. These results show that T cells can respond very sensitively to specific MHC-peptide when it is accompanied by other interactions.

These results taken together argue for a T cell triggering mechanism involving multivalent ligation and cross-linking of T cell receptors. However, questions regarding the manner in which receptor cross-linking results in an activation signal remain. Recent studies have pointed to involvement of the TCR signaling subunits carrying intracellular ITAM

domains. The soluble cytoplasmic domain of the TCR-zeta (ζ) subunit has shown a tendency to bind to acidic phospholipids; when TCRζ is lipid-bound, it is less accessible for phosphorylation by soluble tyrosine kinases (Aivazian and Stern, 2000). Binding of activating antibody ligands to T cells has been shown to result in the rapid exposure of a new intracellular binding epitope for Nck on CD3-ε, an event which correlates well with productive signaling (Gil et al., 2002). At high concentrations, soluble TCRζ has been shown to reversibly homooligomerize, as have other ITAM-containing cytoplasmic domains (Sigalov et al., 2004). The geometry of the TCRζ homooligomerization interface is not known, and the interaction between cytoplasmic domains may or may not be possible in covalently-linked homodimers. In addition, signaling subunits such as TCRζ homodimers have been observed on the T cell surface unassociated with the larger TCR complex after T cell stimulation in a process dependent upon the presence of src-family tyrosine kinases $p56^{lck}$ (Lck) and $p59^{fyn}$ (Fyn) known to be involved in T cell signaling (La Gruta et al., 2004). A model may be synthesized from these data taken together, represented in Figure VI.1, whereby in resting T cell receptors, TCRζ cytoplasmic domains are held in a largely inaccessible arrangement, possibly lipid-bound. T cell receptor clustering by multivalent ligand brings cytoplasmic signaling domains into high local concentration which drives release from the inhibited arrangement and homooligomerization of soluble domains. These domains may then be substrates for cytoplasmic kinases and adaptor molecules. The signaling complex may then separate from the antigen-binding chains, which become degraded, and remain on the surface as a scaffold for continued signal transduction. A similar model has been presented as a general mechanism for all multichain ITAM-containing antigen receptors (Sigalov, 2004). Continuing studies would focus on the ability to distinguish the order and causality for observed steps in the activation mechanism.

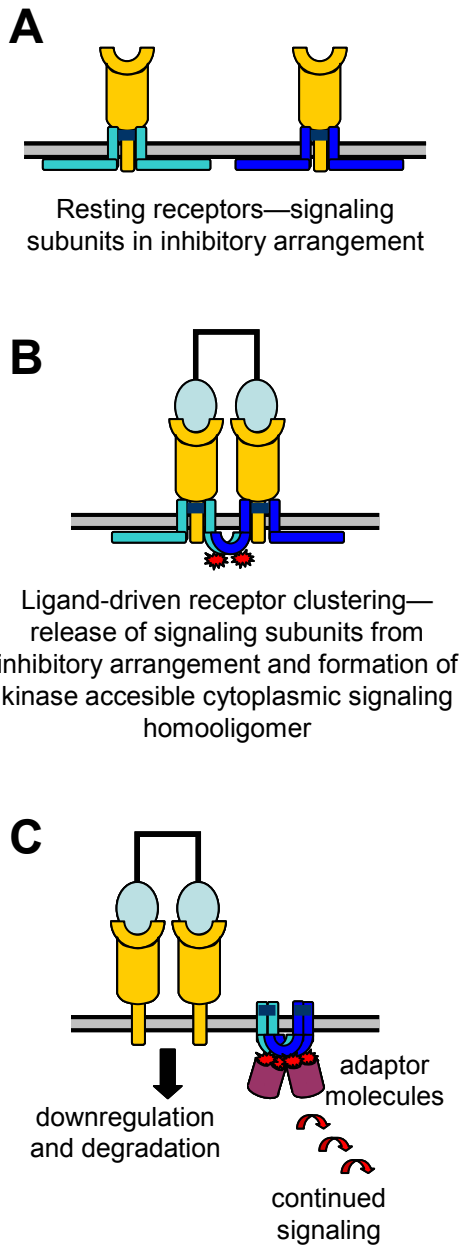


FIGURE VI.1 Model for T cell activation by multivalent ligand binding. (A) Resting T cell receptors consisting of antigen-binding chains (gold) and signaling chains such as disulfide-linked homodimer ζ-ζ (aqua on one receptor and blue on the other). Signaling domains are held in an arrangement that has low accessibility for cytoplasmic tyrosine kinases—potentially bound to the acidic lipids in the membrane. (B) Binding to multivalent ligand, whether soluble or presented on the surface of an APC, leads to receptor cross-linking or clustering. This allows interaction of the cytoplasmic domains of TCRζ, including release from the inaccessible arrangement and homodimerization/ homooligomerization. In this arrangement, the ITAMs on the cytoplasmic tails of TCRζ may expose a novel binding epitopes and become substrates for src family kinases Lck and Fyn. This results in tyrosine phosphorylation which may disallow re-binding to the lipid bilayer. (C) The signaling chain homooligomers may dissociate from the antigen-binding chains of the TCR. These chains are targeted for internalization and degradation. The phosphorylated TCRζ complexes may stay on the surface, allowing adaptor molecules to bind and acting as a scaffold for signal transduction.

2. Involvement of MHC co-receptor

While the MHC co-receptor has been shown both to bind extracellularly to MHC molecules and to associate intracellularly with kinases involved in TCR signaling, it is not certain what the role of the co-receptor is in T cell triggering. CD4 has been shown to move away from TCRζ in stable immunological synapses in activated T cells (Krummel et al., 2000),

and activation has been seen in T cells that do not express MHC co-receptor; although these cells are less sensitive than T cells which do express the co-receptor (Ge et al., 2002). T cells that bind their cognate MHC-peptide with high affinity do not appear to require co-receptor engagement for activation (Kerry et al., 2003). It is unknown whether the main contribution lies in rapidly recruiting cytoplasmic kinases to engaged T cell receptors before MHC dissociation (Li et al., 2004; Xiong et al., 2001), or whether co-receptor binding is most helpful in increasing the affinity and half-life for binding of weak MHC-peptide agonists (Cho et al., 2001). However, the ability of $CD8^-$ T cells to be activated by a soluble MHC-peptide dimer (Chapter IV) shows that receptor cross-linking without involvement of co-receptor can initiate signal transduction.

3. Does monovalent MHC-peptide binding to TCR initiate any signaling processes?

While monomeric MHC-peptide binding may not induce sustained signaling, there may still be certain events that are triggered when binding occurs. Crystallographic studies have showed that for immunodominant epitopes, a conformational change can occur in the putative CD3-ε interface of the T cell receptor upon MHC-peptide binding. Perhaps this change disturbs the association between TCR αβ and CD3-ε, exposing intracellular epitopes that can initiate signaling cascades. Further studies on the potential for soluble MHC-peptide monomers to induce activation-linked makers such as exposure of the Nck-binding epitope (Gil et al., 2002) or incomplete phosphorylation of signaling subunits must be conducted in a system taking care to avoid the complication of peptide re-presentation at the T cell surface.

4. Role of co-stimulation and adhesion signals

It is known that co-stimulatory and adhesion signals contribute to full T cell activation (Chambers, 2001; McAdam et al., 1998; Pribila et al., 2004). The presence of these antigen non-specific interactions allows T cell activation to occur at lower specific peptide levels, resulting in increased apparent sensitivity (Irvine et al., 2002; Wulfing et al., 2002). However, the specific mechanism by which these interactions contribute to specific recognition and signaling through the T cell receptor is not known. The technique of cross-linking heterogeneous molecules, used in Chapter IV to attach an activating MHC-peptide complex specifically to a non-activating MHC-peptide complex, may be applied to linking MHC-peptide complexes to co-stimulatory molecules to further investigate the minimal requirements for T cell recognition.

5. K_D and K_X differences between T cells subsets

Modeling of T cell binding and activation response to treatment with a soluble MHC-peptide oligomer series could be extended to determine the parameter values for many different types of T cells. For example, $CD8^+$ T cells, thought to be more sensitive than $CD4^+$ T cells, may express different values for these binding parameters. Memory and effector T cells respond more sensitively to antigen than naïve T cells; perhaps the ability to cross-link receptors has changed during development, and higher avidity binding is seen after T cell maturation (Slifka and Whitton, 2001). Anergized T cells, conversely, may have lowered the ability for their receptors to be cross-linked. Measuring binding and activation using soluble MHC-peptide oligomer series and using mathematical modeling to derive the binding parameters related to the simple model studied in Chapter II may help us to understand how T cells with similar

monovalent affinity for MHC-peptide (or even identical receptors) respond very differently when presented with cell-bound or multivalent stimuli.

B. Screening for T cell epitopes derived from vaccines or infectious agents

1. Large-scale production of class I and class II MHC-peptide complexes

Chapter V in this thesis describes a technique for screening T cell reactivity to pathogen-derived epitopes using MHC and co-stimulatory microarrays. Efficient production of these chips involves the unique challenge of producing many recombinant biotinylated MHC-peptide complexes easily in parallel. While only a small amount is required of each MHC-peptide complex for producing microarrays, a large number of different MHC-peptide complexes must be produced simultaneously. Some development of these techniques has already begun.

Soluble class II MHC complexes such as HLA-DR1 can be recombinantly produced by insect cells without specific peptide loaded. The MHCs can be made with a uniquely reactive cysteine at the C-terminus of either the α or β chain (Cameron et al., 2002; Cochran and Stern, 2000). The empty MHCs can be isolated using immunoaffinity chromatography, and the cysteine of the empty molecule can be modified using biotin maleimide (Sigma). Large batches of empty, biotinylated MHC complexes can be obtained in this manner. This material can then be split into small aliquots, and each aliquot can be loaded with a different peptide antigen by prolonged incubation at 37°C (Frayser et al., 1999). Thus, the goal of small batches of many different peptide complexes is achieved. Figure VI.2 shows a schematic for the production of these complexes with yields.

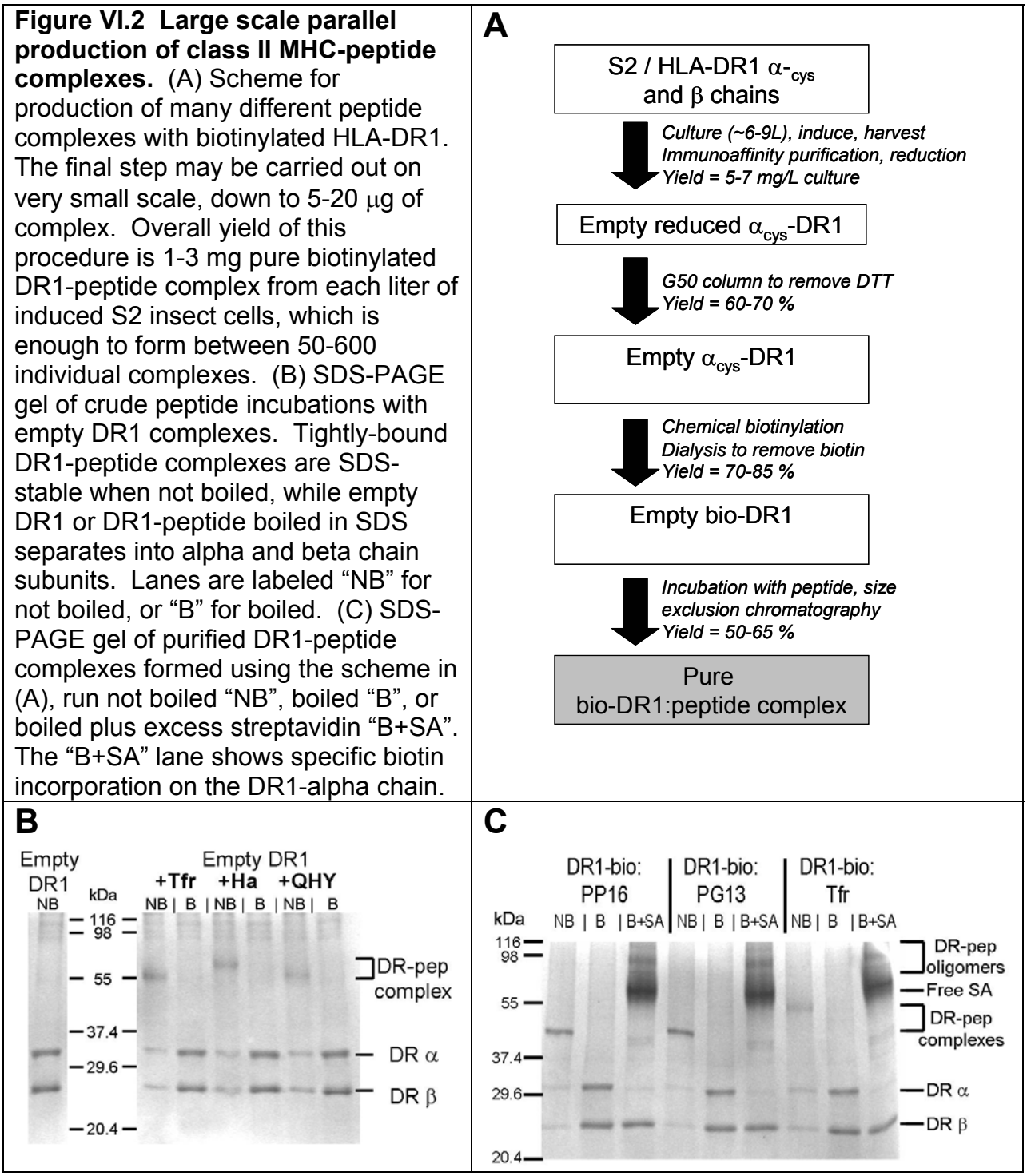
Class I complexes such as HLA-A2 are not conveniently produced without peptide loaded, and are generally refolded from urea-solubilized inclusion bodies in the presence of

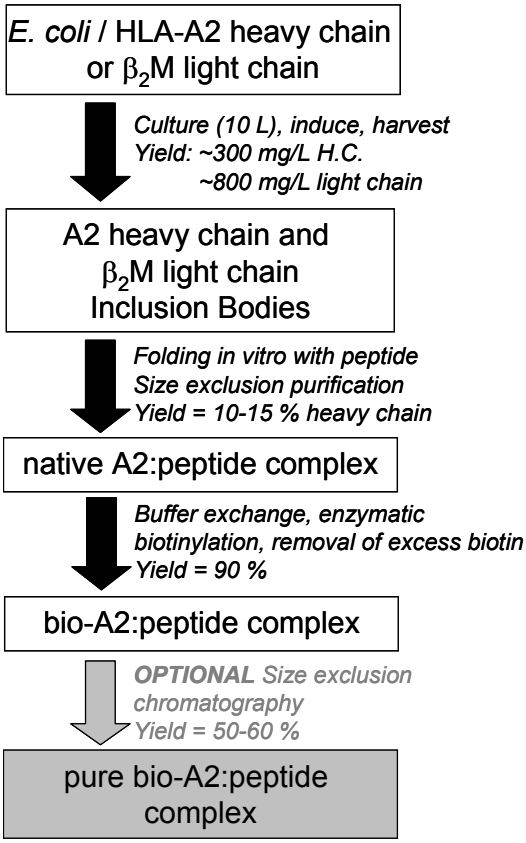
peptide (Garboczi et al., 1992). In order to produce class I MHC complexes with many different peptides, each on a small scale, the protocol has been tweaked such that small-scale refoldings are possible with good yield. Figure VI.3 shows the method for making and purifying many class I MHC complexes in parallel.

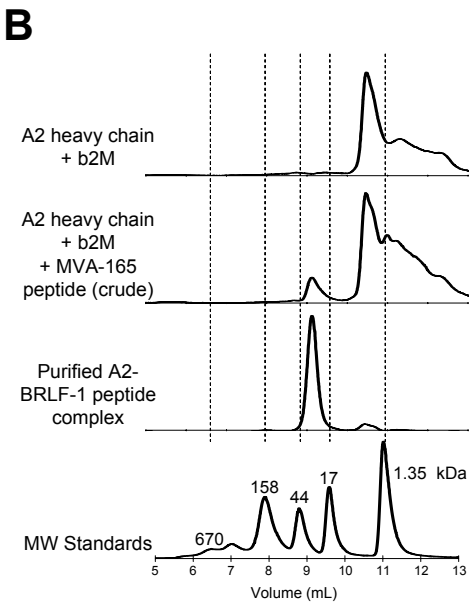
2. Applications of MHC microarrays

The technology discussed in Chapter V may be applied to the study of acute infection or vaccination. Chips may be arrayed with MHC-peptide complexes produced as above, where the peptides are derived from the primary sequences of proteins from either a pathogen or from a vaccine. Patient samples that are MHC-matched can be placed on the chips to determine which epitopes induce different responses, including adhesion, cell surface molecule modulations, and cytokine and effector molecule secretion. The responses induced by different epitopes may be matched to the ability to control or clear infection. This information can then be fed back into the design of new vaccines that elicit the most helpful responses.

Figure VI.2 Large scale parallel production of class II MHC-peptide complexes. (A) Scheme for production of many different peptide complexes with biotinylated HLA-DR1. The final step may be carried out on very small scale, down to 5-20 μg of complex. Overall yield of this procedure is 1-3 mg pure biotinylated DR1-peptide complex from each liter of induced S2 insect cells, which is enough to form between 50-600 individual complexes. (B) SDS-PAGE gel of crude peptide incubations with empty DR1 complexes. Tightly-bound DR1-peptide complexes are SDS-stable when not boiled, while empty DR1 or DR1-peptide boiled in SDS separates into alpha and beta chain subunits. Lanes are labeled "NB" for not boiled, or "B" for boiled. (C) SDS-PAGE gel of purified DR1-peptide complexes formed using the scheme in (A), run not boiled "NB", boiled "B", or boiled plus excess streptavidin "B+SA". The "B+SA" lane shows specific biotin incorporation on the DR1-alpha chain.







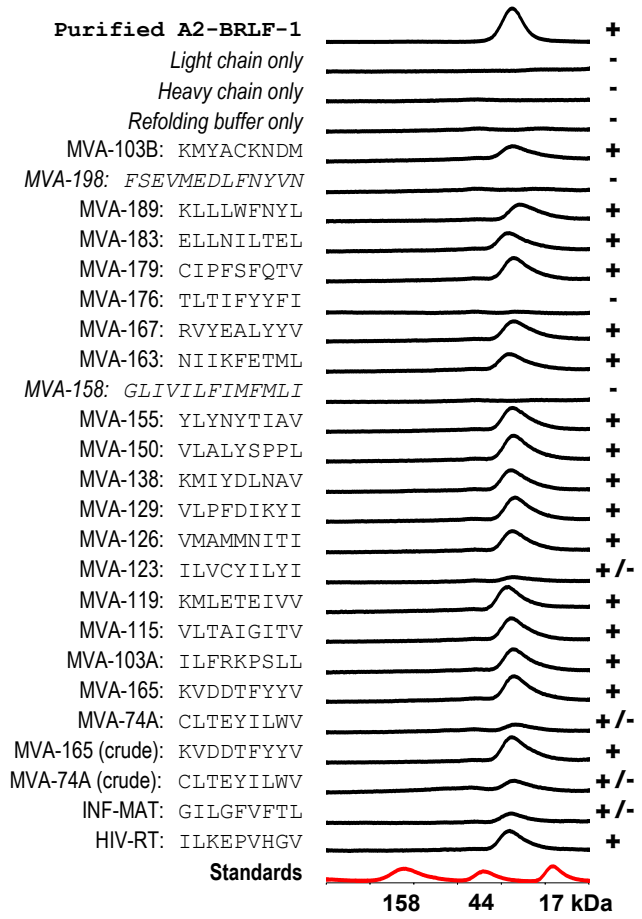


Figure VI.3 Large scale parallel production of class I MHC complexes. (A) Scheme for production of biotinylated HLA-A2 in complex with peptide from *E. coli* expression. Overall yield of purified complex is 13-25 mg per liter of culture induction based on heavy chain, or 27-40 mg if final purification is not required. (B) Refolding can be tested by size exclusion chromatography. The heavy chain and light chain in refolding mix do not refold without peptide (top trace). The second trace shows a crude refolding mix with a binding peptide. The third trace shows purified A2-peptide complex. Molecular weight markers are shown at the bottom. (C) Size exclusion chromatography may be automated using a robotic HPLC, allowing the purification of many A2-peptide complexes in parallel with little operator involvement. A screen of different potential A2-binding peptides was performed. The appearance of a peak between 44 and 17 kDa when crude refolding mix is injected indicates refolded complex.

Another benefit of the development of this technology will be the accumulation of data regarding MHC-peptide binding for different MHC alleles as well as which peptides create effective T cell epitopes. This type of data may be used to improve and extend current prediction algorithms for MHC binding and T cell stimulation, which may in turn accelerate discovery of novel epitope, inhibitor, and antagonist peptides.

3. Toward immobilized antigen-presenting cell arrays

Future development of microarray techniques may involve a modification of the artificial antigen-presenting cell design to take advantage of a patient's own endogenous antigen-presenting cells. Peptide epitopes in some sort of gel or matrix could be microarrayed onto a surface coated with cytokine capture antibodies. Antigen-presenting cells from the patient would then be cultured on top of this surface, which may include additional molecules to help immobilize the APCs in a single location. The APCs would take up the peptide from the matrix, process and load it onto their MHC molecules, and present these molecules at their surface for recognition by the patient's T cells. This solution avoids the requirement for large production of recombinant MHC-peptide molecules, and expands the applicability, since only a small fraction of the total MHC alleles can be produced recombinantly. There may be many technical challenges in setting up this system; however, evidence exists for the ability of cells cultured on top of a DNA microarray to take up and express the genes in a location-dependent manner (Ziauddin and Sabatini, 2001), giving hope for the eventual development of cell-based expression or presentation arrays.

VII. References

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VIII. APPENDICES
CONTENTS

A.1.a GROWING BL21 IN A 10 L FERMENTOR

Protocol from Mia Rushe

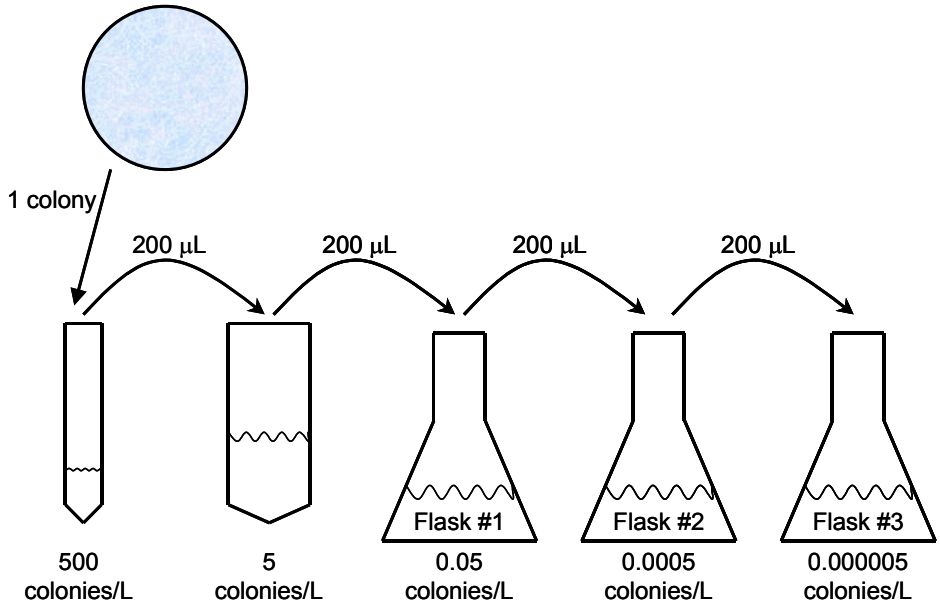
***Procedure*:**

Day 1:

1. Streak an LB-amp (or kan if kanamycin resistant strain, chlor also if cells are $pLys^S$) plate in the evening with BL21 cells of interest from frozen stocks. Incubate at 37˚C overnight.

Day 2:

1. Put streaked plate at 4˚C in morning, sealed with parafilm.
2. Autoclave assembled fermentor filled with 10 L of 1 x LB broth, 1 L of distilled H_2O, 1 L of 1 x LB broth, at least 150 mL 20 % glucose, and 3-125 mL Erlenmeyer flasks.
3. In the early evening, add 83 mL sterilized LB broth to one of the sterile flasks. Add 83 µL Ampicillin (50 mg/mL) and 1.2 mL 20 % glucose. **IF the cells are kanamycin resistant, substitute 83 µL of a 50 mg/mL stock of kanamycin sulfate for ampicillin. If cells are $pLys^S$, add 83 µL chloramphenicol (35 mg/mL).**
4. Mix well and take a 1 mL blank sample for OD_{600} measurements. You may want to add 1 µL 20 % NaN_3 to prevent growth in the blank sample. Keep this blank in a plastic cuvette.
5. The remaining LB solution will be split into different containers. Pipet 2 mL into a sterile 15 mL conical tube, 20 mL into a 50 mL conical tube, and 20 mL into each of the remaining sterile Erlenmeyer flasks. Label the flasks 1, 2 and 3.
6. Pick one colony from your plate. Add it to your 15 mL tube and vortex vigorously. Take 200 µL of that tube and add it to the 50 mL conical tube and vortex vigorously. Take 200 µL of that tube and add it to Erlenmeyer flask #1 and swirl a lot. Take 200 µL from that flask and add it to Erlenmeyer flask #2 and swirl a lot. Take 200 µL of that flask and add it to Erlenmeyer flask #3.
7. Grow the 3 Erlenmeyer flasks on a shaker table at 37˚C overnight.



A.1.a GROWING BL21 IN A 10 L FERMENTOR (continued)

Protocol from Mia Rushe

Day 3:

1. Measure the absorbance at 600nm of each flask compared to the blank you saved. Pick the one that is less than 1.0 OD_{600}, but still has measurable growth in it. (0.7 is about ideal.)
2. Set up the fermentor: Add 10 mL of Ampicillin, 50 mg/mL, 100 mL 20 % glucose (sterile), and 200 μL antifoam A. **IF the cells are kanamycin resistant, substitute 10 mL of a 50 mg/mL stock of kanamycin sulfate for ampicillin. If the cells are $pLys^S$, also add 10 mL 35 mg/mL chloramphenicol.** Mix well. Remove 1 mL as a blank and store in a plastic cuvette, possibly with 1 μL 20 % NaN_3.
3. Run the mixer on the fermentor at ~700 rpm, and run the air at 15 psi and 5 LPM. Watch for excessive foaming and add antifoam if it is necessary. **If you have never set the fermentor up before, ask for help!!
4. Seed the fermentor with 10 mL of the overnight flask you selected.
5. Take samples and check the OD_{600} every hour until you get close to 1.0, and then check more frequently. Do not overgrow!
6. When the OD_{600} reaches 1.0, take a 1 mL sample "not induced." Spin down and re-suspend in 1x urea-SDS loading buffer. Freeze at –20˚C.
7. Add IPTG to 0.75 mM final concentration. I use 10 mL of 0.75M IPTG stock (1000 x). Allow to grow for 2-5 hours with IPTG. (Longer for class I MHC subunits)
8. Take a 1 mL sample "induced." Spin down and re-suspend in 1x urea-SDS loading buffer. Freeze at –20 ˚C until you are ready to check the "not induced" and "induced" samples on a gel by SDS-PAGE.
9. Spin down all the cells at 8000 x g and discard the supernatant. For inclusion bodies, you may freeze the pellet at –20˚C, or proceed with the inclusion body prep through the DNAse step and freeze at –20˚C.

NOTES: The above protocol is the "safe" protocol. In addition to this, you may try a high density fermentation, where Terrific Broth is used instead of LB, and the cells are allowed to grow to 5-10 OD_{600} before induction. For this protocol, take care when the cells become more dense that the temperature does not spike and kill your cells or make them shed their plasmid—cooling water should be used.

A.1.a BL21 MEDIA RECIPES

Protocol from Lauren Angelo

Modified LB
10 g/L tryptone
5 g/L yeast extract
0.4-1% glucose
5 g/L NaCl
5 g/L K_2HPO_4
10 mM $MgSO_4$
1 mL/L Trace Elements

2x YT + Salts
16 g/L tryptone
10 g/L yeast extract
0.4-1% glucose
100 mL/L 10x Salts*

Terrific Broth
12 g/L tryptone
25 g/L yeast extract
0.4-1% glucose
100 mL/L 10x Salts*

*10x Salts
23.1 g/L KH_2PO_4
125.4 g/L K_2HPO_4
filter-sterilize—do not autoclave. Add to media after other ingredients have been autoclaved

A.1.b INCLUSION BODY PREPARATION FOR HLA-DR1 SUBUNITS

Protocol from Mia Rushe

Procedure (for pellet from 10 L culture):

1. Spin down cells at 5000 x g—collect supernatant into a container, sterilize with 1% Wescodyne for 10-20 minutes, and then dump down the sink.
2. With a rubber spatula, re-suspend fresh bacteria into a single plastic container with ~200 mL Sucrose Solution.
3. Chop the solution briefly in a homogenizer or polytron. **Do not sonicate!
4. If cells are not $pLys^s$, add 1 mg dry lysozyme per mL suspension (0.2 g) and stir for 10 minutes. If cells are $pLys^s$, just stir for 10 minutes.
5. While stirring, add 500 mL Deoxycholate-Triton Solution. Solution will become very viscous due to cell lysis and DNA release.
6. Add 1 mL of 4 M $MgCl_2$ Solution to make 5 mM final concentration.
7. Add 2 mL DNAse Solution. Stir until the solution is the viscosity of water.
8. Freeze overnight at –20˚C, or until ready to complete the prep.
9. Thaw solution in warm water bath.
10. Stir an additional 10 minutes after thawing to allow the DNAse to work again.
11. Spin down in 2 centrifuge bottles at 8000 x g for 20 minutes. Discard supernatant.
12. Re-suspend pellets in 300 mL or more each Triton Solution. Chop briefly, keeping the pellets on ice as much as possible. Spin down at 8000 x g for 20 minutes and discard supernatant.
13. Repeat step 12 three or more times.
14. Re-suspend pellets into 300 mL or more each Tris Solution. Chop briefly, keeping the pellets on ice as much as possible. Spin down at 8000 x g for 20 minutes and discard supernatant.
15. Repeat step 14 two or more times.
16. Re-suspend/dissolve the pellets and chop in ~ 200 mL Urea Solution.
17. Spin down at 20˚C, 15,000 x g for 30 minutes. Filter through a 0.2 μm filter.
18. Freeze at –70˚C until ready to purify by Urea HQ.

A.1.b INCLUSION BODY PREPARATION FOR HLA-DR1 SUBUNITS

Protocol from Mia Rushe

*Solutions (**NOTE: Do not add DTT until just before use!**)*:

Name	Stock	For 1 L, 1x	For 1 L, 10x
Sucrose Solution			
50 mM Tris, pH 8.0	1 M Tris, pH 8.0	50 mL	N/A
25 % sucrose	dry, FW = 342.3	250 g	
1 mM EDTA	0.5 M EDTA	2 mL	
0.1 % NaN_3	20 % NaN_3	5 mL	
10 mM DTT	dry, FW = 154.04	1.54 g	
Deoxycholate-Triton Solution			
1 % Deoxycholic Acid	dry, FW = 414.6	10 g	100 g
1 % Triton X-100	100% solution	10 mL	100 mL
20 mM Tris, pH 7.5	1 M Tris, pH 7.5	20 mL	200 mL
100 mM NaCl	5 M NaCl	20 mL	200 mL
0.1 % NaN_3	20 % NaN_3	5 mL	50 mL
10 mM DTT	dry, FW = 154.04	1.54 g	N/A
Triton Solution			
0.5 % Triton X-100	100% solution	5 mL	50 mL
50 mM Tris, pH 8.0	1 M Tris, pH 8.0	50 mL	500 mL
100 mM NaCl	5 M NaCl	20 mL	200 mL
1 mM EDTA	0.5 M EDTA	2 mL	20 mL
0.1% NaN3	20 % NaN3	5 mL	50 mL
1 mM DTT	dry, FW = 154.04	0.154 g	N/A
Tris Solution			
50 mM Tris, pH 8.0	1M Tris, pH 8.0	50 mL	500 mL
1 mM EDTA	0.5 M EDTA	2 mL	20 mL
0.1% NaN3	20 % NaN3	5 mL	50 mL
1 mM DTT	dry, FW = 154.04	0.154 g	N/A
Urea Solution			
8 M Urea	dry, FW = 60.06	480.48 g	N/A
20 mM Tris, pH 8.0	1M Tris, pH 8.0	20 mL	
0.5 mM EDTA	0.5 M EDTA	1 mL	
10 mM DTT	dry, FW = 154.04	1.54 g	
DNAse Solution (make 50 mL total, not 1L**)**			
75 mM NaCl	5 M NaCl	0.75 mL	N/A
50 % glycerol	100 % glycerol	25 mL	
2 mg/mL DNAse (Sigma D-5025 or DN25)	dry	100 mg	

A.1.c UREA HQ INCLUSION BODY PURIFICATION FOR HLA-DR1

Protocol from Mia Rushe

Column: Perseptives HQ resin, bed volume ~17 mL

Buffers: **Solution A**: 8 M urea, 20 mM tris, 1 mM DTT added just before use
Solution B: 8 M urea, 20 mM tris, 1 M NaCl, 1 mM DTT added just before use
For purifying A1S, both solutions should be pH 8.0
For purifying B1S, both solutions should be pH 9.0

Procedure:

1. Make approximately 3 L **solution A** and 1 L **solution B**, using the protocol for *Making Urea Solutions*. Use the appropriate pH for the inclusion body you will be purifying. If not using immediately, store at -20°C. Do not add the DTT until just before use.
2. Thaw urea-solubilized inclusion bodies. For B1S, adjust the pH to 9.0 using NaOH. (pH should be 8.0 in frozen inclusion bodies)
3. Equilibrate the HQ column in 1-5 column volumes **solution A**.
4. Flow the inclusion body solution over the column. If it is very concentrated, it may bind better if it is diluted 1:2 in **solution A**. **NOTE: Save flow-through of the column, as the protein is very concentrated and may saturate the column.**
5. Run a gradient of 0-30% **solution B** (1-300 mM NaCl) over 6 column volumes. Collect fractions.
6. Analyze fractions from all major peaks by SDS-PAGE. **NOTE: If the protein peak of interest looks different when run on reducing vs. non-reducing SDS-PAGE, add more DTT to the final inclusion body pool to completely reduce the protein.**
7. Pool peak fractions and measure the concentration of protein using absorbance at 280 nm against a blank of **solution A**. **NOTE: For A1S: 1.3 OD_{280} = 1 mg/mL. For B1S: 1.7 OD_{280} = 1 mg/mL.
8. For B1S, adjust the pH back to 8.0. Add EDTA to a final concentration of 5 mM (10 μL 0.5M EDTA stock / mL inclusion body solution).
9. Store in convenient aliquots at -80°C.

NOTES: A new HQ column has a protein binding capacity of about 5-10 mg / mL resin. The capcity becomes significantly lower over time with this procedure, possibly in part due to EDTA. Regeneration does not seem to be particularly helpful. Thus, when loading protein, do it slowly and monitor the absorbance of the flow-through to determine when the protein is no longer binding to the resin. The chromatograms will be very messy and inconsistent, but the refolding yield for inclusion bodies purified in this manner is much better.

A.1.d REFOLDING HLA-DR1 FROM E. coli INCLUSION BODIES

Protocol from Mia Rushe

***Procedure*:**

1. Chill Refolding Mix without glutathione to 4˚C. Refolding should be done in a container close to the size of the volume of liquid to reduce exposure to air. De-gas solution by sparging with argon for about 20 minutes.
2. Add glutathione (oxidized and reduced) and stir until just dissolved.
3. Stir as rapidly as possible
4. Add peptide—about 5-fold molar excess in a 1x refolding is good, so you should end up with a final peptide concentration of 0.4 μM. You can use the same concentration peptide for a 2x re-folding. **NOTE: If you want to re-fold empty DR1, simply omit the peptide.**
5. Slowly (dropwise) add HQ-purified inclusion bodies in urea. Add 2 (1 x) or 4 (2x) mg of each inclusion body (alpha and beta) per liter of Refolding Mix.
6. After everything is well mixed, cover tightly and store at 4˚C for at least 36 hours.

Refolding Mix:

20 mM Tris, pH 8.5
0.5 mM EDTA
25 % glycerol
2 mM glutathione, reduced ** Add just before use!
0.2 mM glutathione, oxidized ** Add just before use!

Example 2 x re-folding, 4 L total volume:

80 mL of 1 M Tris, pH 8.5
4 mL of 0.5 M EDTA
1000 g glycerol
2.45 g glutathione, reduced
0.488 g glutathione, oxidized
16 mg DRA1S inclusion bodies (8 mL of 2 mg/mL stock, Urea HQ pure)
16 mg DRB1S inclusion bodies (16 mL of 1 mg/mL stock, Urea HQ pure)
1.6 μmoles Ha peptide (800 μL of 2 mM Ha peptide stock)

A.1.e PREPARATION OF ANTIBODY-COUPLED MATRIX

Protocol from Mia Rushe

1. Spin Immobilized Protein A (RepliGen) 2000 rpm for 5 minutes, remove supernatant, and wash with Binding Buffer. Your volume will be 50% of the original volume after the wash.
2. Mix 1 mL Antibody Stock Solution per 1 mL Immobilized Protein A beads (or 1 mL Antibody Stock Solution per 2 mL original 50% Immobilized Protein A slurry). ****Take 20 μL sample of mixture: "TOTAL"**
3. Incubate on nutator at room temperature for 1 hour or longer.
4. Spin mixture at 2000 rpm for 5 minutes. Save the supernatant in the fridge in case you got low binding. ****Take 20 μL sample of supernatant: "NON-BOUND"**
5. Re-suspend pellet in 50 mL Borate Solution. Spin 2000 rpm for 5 minutes, remove and discard supernatant.
6. Re-suspend pellet to original volume of antibody/slurry mixture with fresh Borate Solution. ****Take 20 μL sample of mixture: "BOUND"**
7. Add a 1/10 dilution Dimethylpimelimidate Stock Solution (200 mM) to 20mM final. Incubate on nutator at room temperature for 30 minutes.
8. Spin 2000 rpm, 5 minutes, remove supernatant and check pH—should be above 8.0!
9. Re-suspend pellet in 50 mL Ethanolamine Solution and incubate at room temperature on nutator for 30 minutes or more.
10. Spin 2000 rpm, 5 minutes, remove and discard supernatant.
11. Perform the following washes:
    a. 50 mL Ethanolamine Solution
    b. 50 mL Borate Solution
    c. 50 mL Binding Buffer ****Take 20 μL sample of mixture: "WASH"**
    d. 50 mL CAPS Solution
    e. 50 mL Binding Buffer—repeat until supernatant has neutral pH.
12. Re-suspend pellet to original volume of antibody/slurry mixture in fresh Binding Buffer. ****Take 20 μL sample of mixture: "NON-COUPLED"**
13. Store matrix at 4°C—making sure that Binding Buffer contains 0.02% NaN_3.
14. Analysis of coupling: Boil all samples with 5 μL 5x Reducing Laemlli Load Buffer and spin down any matrix. Run 10% SDS-PAGE gel of samples. You should be able to see heavy and light chains in "TOTAL" and "BOUND" and nothing in "NON-BOUND", "WASH", or "NON-COUPLED". If there is any protein in "NON-BOUND", try a different binding buffer. If there is any protein in "NON-COUPLED", cycle the matrix back through all the binding and elution conditions that will be used for the affinity purification procedure. **Make sure to use fresh Dimethylpimelimidate!

A.1.e PREPARATION OF ANTIBODY-COUPLED MATRIX

Protocol from Mia Rushe

Materials:

Borate Solution: 200 mM Borate, pH 9.0 **MAKE FRESH!!
Ethanolamine Solution: 200 mM Ethanolamine, pH 8.0 **MAKE FRESH!!
Antibody Stock Solution: 2 mg/mL antibody, will add 10 g antibody per 5 mL matrix
Immobilized Protein A (RepliGen): use 5 mL beads/column—comes as a 50% slurry
Binding Buffer: PBSZ, 1x PBS with 0.02% NaN_3, make at least 500 mL
Dimethylpimelimidate Stock Solution: 200 mM, use fresh 250 mg vials (MW = 259)

Borate Solution	Dimethylpimelimidate Stock Solution	Ethanolamine Solution
1.86 g Borate	250 mg Dimethylpimelimidate	1.2 mL Ethanolamine
to 150 mL ddH_2O	4.83 mL ddH_2O	to 100 mL ddH_2O
to pH 9.0 with NaOH		to pH 8.0 with HCl

A.1.f IMMUNOAFFINITY PURIFICATION OF DR1

Protocol from Mia Rushe

Procedure:

1. Concentrate folding mix and switch buffers into 1x PBS, pH ~7. Make sure there is no glutathione, as that will ruin the antibody column! If the DR1 is from an insect cell prep instead of an *E.coli* refolding, make sure the supernatant has been filtered through a 0.2 μM filter.
2. Flow the protein solution over a Protein A sepharose pre-column and then the LB3.1 or L243 column by gravity. Be sure to use a safety loop so that the columns don't dry out. Save the flow-through to check on a gel.
3. Wash with at least 10 column volumes of cold PBS
4. Elute using high pH for DR1. Use ~5 column volumes of 50 mM CAPS, pH 11.5 and collect 1 mL fractions into tubes with 300 μL of 300 mM NaPi, pH 6.0 to neutralize them. Make a blank fraction for zeroing the UV-VIS. (**Note: low pH may be used to elute, using 5 column volumes 50 mM glycine, pH 2.6, and collecting 1 mL fractions into tubes containing 300 μL of 300 mM Tris, pH 8.0 in them. This will give a lower yield for DR1, but may be better for other alleles.)
5. Clean the column with pH swings:
    i. 3 column volumes 50 mM glycine, pH 2.6
    ii. 3 column volumes 50 mM CAPS, pH 11.5
    iii. 3 column volumes 50 mM glycine, pH 2.6
6. Wash the column with at least 10 column volumes of PBSZ—until the pH is neutral.
7. Check the Abs_{280} of the fractions and pool the protein-containing fractions. You may add azide to 0.02%, add peptide if the complex is empty and you wish to load a particular peptide, or dialyze/concentrate into 20 mM tris, pH 8.0 if you wish to further purify by ion exchange.

A.1.g LOADING PEPTIDE ONTO EMPTY MHC CLASS II

Protocol from Zarixia Zavala-Ruiz

***Procedure*:**

1. Obtain the empty MHC at a concentration of ~ 1 mg/mL, about 20 μM, in a suitable buffer such as PBS pH 7.0 with 0.02% sodium azide and 5 mM EDTA as protease inhibition.
2. Add the peptide of choice to a final concentration of at least 100 μM, or 5-fold molar excess.
3. Incubate the mixture at 37°C for 3 days.
4. Purify the peptide-loaded complex by gel filtration. **NOTE: Make sure to save a sample of the unloaded complex to test by SDS-PAGE and analytical gel filtration. Peptide loading will shift the MHC complex to a later time on a gel filtration column, and it will often cause SDS stability in a gel, as opposed to the empty MHC, which will break down into subunits.**

A.1.h INCLUSION BODY PREPARATION FOR CLASS I MHC SUBUNITS

Protocol from Jenny Cochran

***Procedure (for pellet from 10 L culture)*:**

1. Spin down cells at 5000 x g—collect supernatant into a container, sterilize with 1% Wescodyne for 10-20 minutes, and then dump down the sink.
2. With a rubber spatula, re-suspend fresh bacteria into a single plastic container with ~200 mL Sucrose Solution.
3. Chop the solution briefly in a homogenizer or polytron. **Do not sonicate!
4. If cells are not $pLys^s$, add 1 mg dry lysozyme per mL suspension (0.2 g) and stir for 10 minutes. If cells are $pLys^s$, just stir for 10 minutes.
5. While stirring, add 500 mL Deoxycholate-Triton Solution. Solution will become very viscous due to cell lysis and DNA release.
6. Add 1 mL of 4 M $MgCl_2$ Solution to make 5 mM final concentration.
7. Add 2 mL DNAse Solution. Stir until the solution is the viscosity of water.
8. Freeze overnight at –20˚C, or until ready to complete the prep.
9. Thaw solution in warm water bath.
10. Stir an additional 10 minutes after thawing to allow the DNAse to work again.
11. Spin down in 2 centrifuge bottles at 8000 x g for 20 minutes. Discard supernatant.
12. Re-suspend pellets in 300 mL or more each Triton Solution. Chop briefly, keeping the pellets on ice as much as possible. Spin down at 8000 x g for 20 minutes and discard supernatant.
13. Repeat step 12 three or more times.
14. Re-suspend pellets into 300 mL or more each Tris Solution. Chop briefly, keeping the pellets on ice as much as possible. Spin down at 8000 x g for 20 minutes and discard supernatant.
15. Repeat step 14 two or more times.
16. Re-suspend/dissolve the pellets and chop in ~ 200 mL Urea Solution.
17. Spin down at 20˚C, 15,000 x g for 30 minutes. Filter through a 0.2 μm filter.
18. Measure protein concentration by absorbance at 280nm. Aliquot inclusion bodies and freeze at -80°C.

A.1.h INCLUSION BODY PREPARATION FOR CLASS I MHC SUBUNITS

Protocol from Jenny Cochran

***Solutions (**Note: Do not add DTT until just before use!)*:**

Name	Stock	For 1 L, 1x	For 1 L, 10x
Sucrose Solution			
50 mM Tris, pH 8.0	1 M Tris, pH 8.0	50 mL	N/A
25 % sucrose	dry, FW = 342.3	250 g	
1 mM EDTA	0.5 M EDTA	2 mL	
0.1 % NaN_3	20 % NaN_3	5 mL	
10 mM DTT	dry, FW = 154.04	1.54 g	
Deoxycholate-Triton Solution			
1 % Deoxycholic Acid	dry, FW = 414.6	10 g	100 g
1 % Triton X-100	100% solution	10 mL	100 mL
20 mM Tris, pH 7.5	1 M Tris, pH 7.5	20 mL	200 mL
100 mM NaCl	5 M NaCl	20 mL	200 mL
0.1 % NaN_3	20 % NaN_3	5 mL	50 mL
10 mM DTT	dry, FW = 154.04	1.54 g	N/A
Triton Solution			
0.5 % Triton X-100	100% solution	5 mL	50 mL
50 mM Tris, pH 8.0	1 M Tris, pH 8.0	50 mL	500 mL
100 mM NaCl	5 M NaCl	20 mL	200 mL
1 mM EDTA	0.5 M EDTA	2 mL	20 mL
0.1% NaN3	20 % NaN3	5 mL	50 mL
1 mM DTT	dry, FW = 154.04	0.154 g	N/A
Tris Solution			
50 mM Tris, pH 8.0	1M Tris, pH 8.0	50 mL	500 mL
1 mM EDTA	0.5 M EDTA	2 mL	20 mL
0.1% NaN3	20 % NaN3	5 mL	50 mL
1 mM DTT	dry, FW = 154.04	0.154 g	N/A
Urea Solution (adjust pH to 6.0)			
8 M Urea	dry, FW = 60.06	480.48 g	N/A
25 mM MES, pH 6.0	dry, FW = 213.3	5.33g	
10 mM EDTA	0.5 M EDTA	20 mL	
0.1 mM DTT	dry, FW = 154.04	15.4 mg	
DNAse Solution (make 50 mL total, not 1L**)**			
75 mM NaCl	5 M NaCl	0.75 mL	N/A
50 % glycerol	100 % glycerol	25 mL	
2 mg/mL DNAse (Sigma D-5025 or DN25)	dry	100 mg	

A.1.i REFOLDING OF CLASS I MHC PROTEINS

Protocol modified from NIH Tetramer Facility

Procedure:

1. Pre-chill 1 L of **Folding Buffer** in a 1.5 L beaker to 10°C.
2. Add 5 mM reduced glutathione, 0.5 mM oxidized glutathione, and 0.2 mM PMSF to cold **Folding Buffer**, stirring quickly. **NOTE: Avoid foaming!
3. Calculate 1 μM heavy chain urea-solubilized subunit and add to 4 mL of **Injection Buffer**.
4. Calculate 2 μM β_2M urea-solubilized subunit and add to 4 mL of **Injection Buffer**.
5. Weigh out 30 mg of peptide and dissolve in 0.5 mL DMSO or water, depending on sequence.
6. Add peptide solution dropwise to rapidly stirring **Folding Buffer**.
7. Forcefully inject heavy chain and β_2M to the stirring reaction through a 26-gauge needle as close to the stir-bar as you can. There may be some precipitation or light foaming.
8. Replace the mixture at 10°C and incubate overnight.
9. In the morning, inject heavy chain ONLY in **Injection Buffer** into the cold, quickly-stirring mixture as above (1 μM more, now 2 μM total concentration heavy chain). Replace at 10°C.
10. In the evening, once again inject heavy chain ONLY in **Injection Buffer** into the cold, quickly-stirring mixture as above (1 μM more, now 3 μM total concentration heavy chain). Replace at 10°C and incubate overnight

Solutions:

Folding Buffer (pH 8.0)
400 mM L-Arginine (Sigma A5949)
100 mM Tris, pH 8.0
2 mM EDTA

Injection Buffer (pH 4.2)
3 M Guanidine-HCL
10 mM Sodium Acetate
10 mM EDTA

Other Materials
Urea-solubilized heavy chain and light chain (β_2M)
Peptide of choice
Reduced glutathione (Sigma G4705)
Oxidized glutathione (Sigma G4501)
100 mM Phenylmethylsulfonyl fluoride (PMSF—Sigma P7626)

A.1.j PURIFICATION OF FOLDED CLASS II MHC-PEPTIDE COMPLEXES USING DEAE BEADS

Procedure from M. Rushe and J. Cochran

Reagents:

20 mM Tris, pH 8.5 (add 5 mM DTT for cys-modified protein) **make 1 L**
20 mM Tris, pH 8.5, 0.5 M NaCl (add 5 mM DTT for cys-modified protein) **make 500 mL**
20 mM Tris, pH 8 (add 5 mM DTT for cys-modified protein)

Buffer A: 20 mM Tris (pH 8)
Buffer B: 20 mM Tris (pH 8), 1 M NaCl

Protocol:

1. Add DEAE Sephadex A-50 beads (A-50-120 Sigma-Aldrich) to 2 g / L refolding mix. Allow the beads to swell and adsorb refolded protein for at least 12 hours.
2. Collect the beads on a large Büchner funnel using vacuum filtration.
3. Rinse the beads with 1 L of 20 mM Tris, pH 8.5 (containing 5 mM DTT for cys-modified protein).
4. Elute protein from the beads by adding 20-30 mL aliquots of 20 mM Tris, pH 8.5, 0.5 M NaCl (containing 5 mM DTT for cys-modified protein). Allow the beads to sit in the buffer for a few minutes before applying vacuum.
5. Repeat the elution using 20-30 mL salt-containing buffer at a time until you have added all of the solution. **NOTE: The beads will shrink quite a bit with water loss the first few times you add the salt. This is normal.**
6. Filter the eluate through a 0.2 μm filter unit.
7. Concentrate the eluate as far as you can go—to about 20 mL. To achieve this, you may use a Pellicon-30 XL Filter (PXC03050 Millipore), although any concentration method should work.
8. Dilute the mixture 1:10-1:12 with 20 mM Tris, pH 8.0 (containing 5 mM DTT for cys-modified protein) and re-concentrate back down to the minimal volume. Repeat this for 2 full washes. **NOTE: This step is required to remove salt from the solution, as the salt will prevent binding of the protein to the anion exchange column later.**
9. Rinse the concentrator membrane with ~50 mL 20 mM Tris, pH 8.0 (containing 5 mM DTT for cys-modified protein) and pool the concentrated protein solution and the membrane rinse.
10. Filter the protein pool through a 0.2 μm filter unit.
11. Purify concentrated folding reaction by anion-exchange (HQ column) using Buffers A and B (Do not include DTT in these buffers!). Folded Class II will elute with ~ 200 mM NaCl.

NOTE: This procedure is not used for empty class II MHCs or weak-binding peptide complexes. In those cases, use a combination of immunoaffinity purification and size exclusion chromatography to purify the protein.

A.1.j PURIFICATION OF FOLDED CLASS II MHC-PEPTIDE COMPLEXES—ALTERNATIVE PROCEDURE

Procedure adapted from M. Rushe

Reagents:

20 mM Tris, pH 8 (add 5 mM DTT for cys-modified protein)

Buffer A: 20 mM Tris (pH 8)
Buffer B: 20 mM Tris (pH 8), 1 M NaCl

Protocol:

1. Concentrate the refolding mix as far as you can go—to about 200 mL. To achieve this, you may use an Amicon Spiral Concentrator, although other concentration methods should work.
2. Dilute the mixture 1:10-1:12 with 20 mM Tris, pH 8.0 (containing 5 mM DTT for cys-modified protein) and re-concentrate back down to the minimal volume. Repeat this for 2-3 full washes. **NOTE: This step is required to remove glycerol and glutathione from the solution, as these components are bad for the anion exchange column later.**
3. Filter the protein through a 0.2 μm filter unit.
4. Purify concentrated folding reaction by anion-exchange (HQ column) using Buffers A and B (Do not include DTT in these buffers!). Folded Class II will elute with ~ 200 mM NaCl.

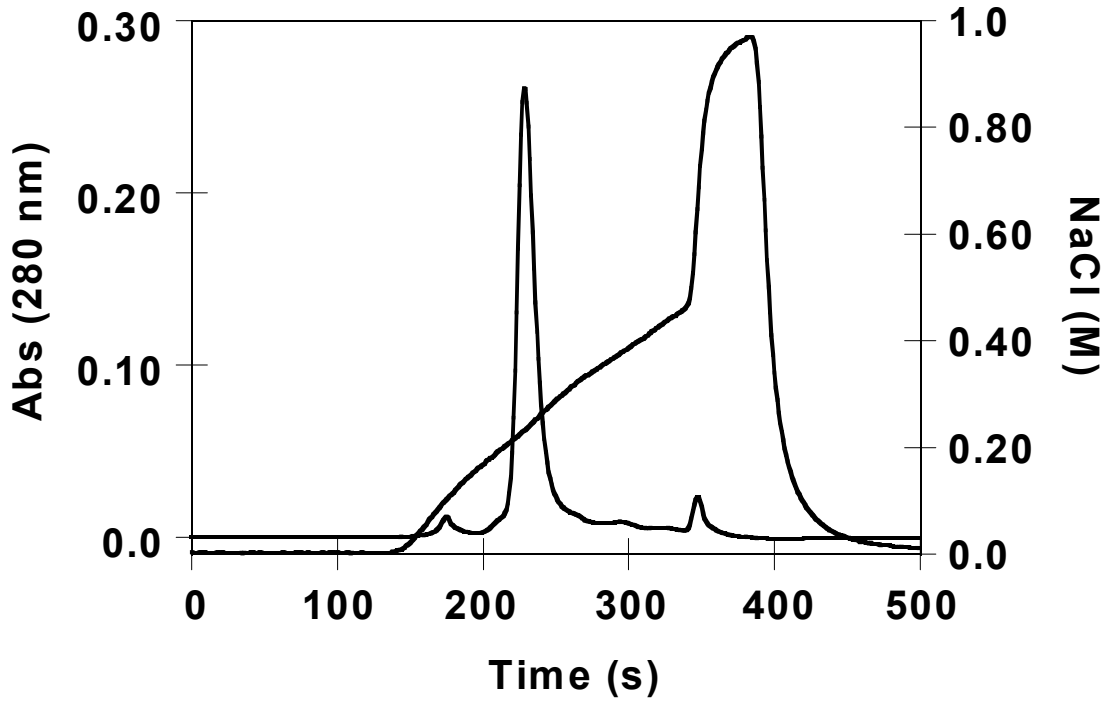
NOTE: This procedure gives much better overall yield from the refolding; however, the lifetime of the anion exchange column seems to be diminished by components in the refolding mix which are not fully removed. It may be advantageous to use less expensive disposable columns from Pharmacia such as HiTrap columns for this purification, especially since the protein is fairly pure already and does not require very high resolution.

NOTE: This procedure is not used for empty class II MHCs or weak-binding peptide complexes. In those cases, use a combination of immunoaffinity purification and size exclusion chromatography to purify the protein.

A.1.j PURIFICATION OF FOLDED CLASS II MHC-PEPTIDE COMPLEXES (continued)

Procedure from M. Rushe and J. Cochran

Sample trace from anion-exchange purification of DR1-Ha with a NaCl gradient using POROS 20 HQ resin



A.1.k BIOTIN LIGASE PURIFICATION

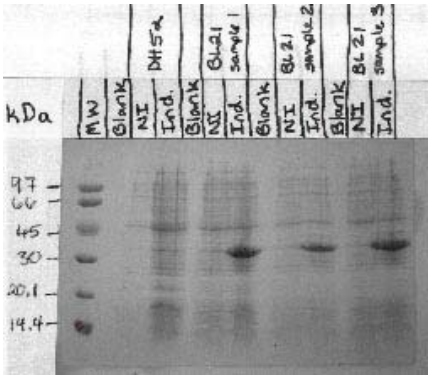
Protocol from Kai Wucherpfennig

1. Inoculate a 50 mL culture (LB/Amp 50μg/mL) with *E. coli* (BL21 (DE3)) transformed with pET22b-BirA-(His) tag. Grow O.N, 37ºC, shaker.
2. Inoculate 2 liters of LB/Amp (50μg/mL) with the O/N culture. Grow at 37ºC, 225 rpm, to an OD_{600} of ~0.6 (start monitoring OD after 3 hours).
3. IPTG induction: add IPTG to 1mM. Grow O/N at RT on shaker. The yield of soluble biotin ligase is greatly increased by growth at RT rather than 37ºC.
4. Harvest 2L culture; Spin 3000 rpm, 4ºC, 30'.
5. Resuspend in ~25mL sonication buffer: 30 mM Tris, pH 8.0
   150 mM NaCl
   1 mM PMSF
6. Sonicate resuspended pellet on ice. Settings for sonication: Timer: Hold
   Output cycle: 5
   Input cycle: 50

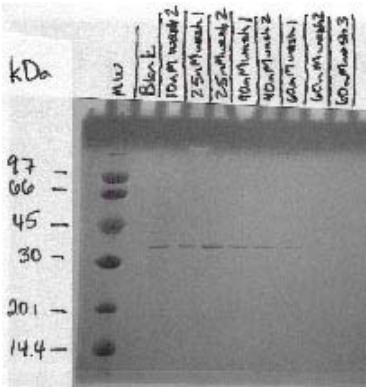
   Sonicate 4-5 times for 1'
7. Spin in ultracentrifuge to remove all insoluble material: 25,000rpm, 4ºC, 30', SW28 rotor. Set temperature well in advance; it takes a while to reach 4ºC. Make sure that the tube (Beckman Ultra-Clear™, Cat # 344058) is filled almost all the way to the top or else it will collapse!
8. Save supernatant on ice
9. Repeat resuspension, sonication, and centrifugation on the pellet from the ultracentrifugation spin.
10. Equilibrate Ni-NTA column (7.5 mL bed volume) with 30 mM Tris, pH 8.0, 150 mM NaCl.
11. Load supernatant onto column (0.8 mL/min, cold room)
    Wash column with ~2 bed volumes: 30 mM Tris, pH 8.0
    150 mM NaCl
    Imidazole wash (30 mL): 30 mM Tris, pH 8.0
    150 mM NaCl
    10 mM Imidazole
    The imidazole wash removes a lot of contaminating proteins

Elution:	40 mL	30 mM Tris, pH 8.0 150 mM NaCl 25 mM Imidazole
	40 mL	30 mM Tris, pH 8.0 150 mM NaCl 40 mM Imidazole
	30 mL	30 mM Tris, pH 8.0 150 mM NaCl 60 mM Imidazole
12. Fractions eluted with 60 mM Imidazole are quite pure. Fractions from 25 and 40 mM elutions still have contaminating proteins. These can be removed using a cation exchange HPLC column.

13. Buffer exchange using PD-10 column prior to HPLC. Add 2.5 mL of fraction to the column and load by gravity flow.
    To elute, add 3.5 mL of 30 mM Tris (HPLC buffer A), elute by gravity flow.
14. HPLC Poros HS column:
    Buffer A: 30 mM Tris, pH 8.0
    Buffer B: 30 mM Tris, pH 8.0 + 1.0 M NaCl
    Load sample using 5mL loop
    Gradient of buffer B from 0-100% in 20 minutes (protein elutes at ~12 minutes)
15. Exchange buffer using PD-10 columns; elute in 50 mM Bicine, pH 8.3 (storage buffer)
16. Aliquot and store at -80ºC (glycerol can be added to 5%)



Induction gel of Bir A enzyme



Gel of Ni-NTA column fractions

A.1.l S2 CELL CULTURING

Protocol from Tom Cameron

S2 cells, also called Schneider Cells, are a Drosophila melanogaster cell line available from ATCC, CRL-1963. They are smaller than Sf9 cells. They double every 24-48 hours, and they are somewhat adherent to plastic. They can usually be removed by pipetting up and down. **Incubate cells at 25ºC or room temperature.

Serum Media, 100 mL:	Serum-Free Media, 100 mL:	Freezing Media:
Schneider's or Grace's	Sf-900, Ex-cell, or BaculoGold	90% Serum Media
Pre-made media	Pre-made media	10% DMSO
1 mL Penn-Strep	1 mL Penn-Strep	**freeze cells at
1 mL Glutamine	1 mL Glutamine	3-4 million cells
1 mL Fungizone	1 mL Fungizone	per mL.
10 mL FBS		

Split cells 1:2 or 1:3 every 3-4 days, keeping density between 0.5-4 million cells/mL in plates or flasks. In order to pass from flasks to spinners, let the cells overgrow two T-75 flasks or one T-150 flask to make sure plenty are not sticking to the plastic. Pipet vigorously to remove adherent cells, and transfer to a 100 mL spinner flask. In spinner flasks, the cells can grow happily up to 10 million cells/mL, and can reach up to 20 million cells/mL.

A.1.m MATERIALS FOR S2 CELL TRANSFECTION

From Invitrogen

Calcium Phosphate Transfection

Material (kept sterile)	Amount required per construct transfected
Thawed, growing, untransfected S2 cells	3 million
S2 media with serum	~ 30 mL
2 M $CaCl_2$	18 µL
2x Hepes Buffered Saline (HBS)	150 µL
Construct plasmid	5 µg per plasmid
pNeo (G418 resistance)	0.25 µg
S2 media with 1.5 mg/mL G418	>30 mL
0.5 M $CaSO_4$ solution (1000x, sterile)	1 mL per liter induction

Lipofectin Transfection

Material (kept sterile)	Amount required per construct transfected
Thawed, growing, untransfected S2 cells	3 million
S2 media with 10% serum, PSFQ	0.3 mL for overnight plating, ~30 mL other
Sf-900 II media with PSFQ	2.7 mL
Sf-900 II media with Q only	6.0 mL, no antibiotic, for transfection
Construct plasmid	3 µg per plasmid
pNeo	0.2 µg
Lipofectin Reagent	20 µL (diluted into 80 µL Sf-900 II + Q)

A.1.n CALCIUM PHOSPHATE S2 CELL TRANSFECTION

Protocol from Tom Cameron and Invitrogen

1. Buy Invitrogen Calcium Phosphate Transfection Kit (K2780-01).
2. Plate 3 million cells per well untransfected S2 cells into 6-well plates and allow to sit overnight.
3. In the morning, spin the 6-well plates at 1500 rpm for 5 minutes, aspirate out the media, and add 5 mL per well fresh media. Allow to sit another two hours or more while you set up your DNA solutions.
4. Sterilize your DNA with sterile Spin-X eppendorf filters. You will need approximately 5 μg of each plasmid that will produce protein and 0.25 μg pNeo for a selection tag in each transfection well.
5. In the tissue culture hood, add plasmid and sterile water to 132 μL. Add 18 μL 2 M $CaCl_2$ Solution to give 150 μL total volume. Label this with the transfection name and "Solution A."
6. In a separate 15 mL conical tube, add 150 μL 2x Hepes Buffered Saline (HBS), and label this with the transfection name and "Solution B."
7. While vortexing "Solution B" (still in the tissue culture hood), add "Solution A" dropwise slowly over the course of 1-2 minutes. This will ensure the finest possible precipitate.
8. Allow the mixture to sit at room temperature for 30 minutes.
9. Add the mixture to the cells in the 6-well plate dropwise while swirling. Allow to sit for 24 hours.
10. The next day, pipet the cells up and down well and transfer them to 15-mL conicals and wash 1 time with fresh media before re-suspending in fresh media.
11. Meanwhile, rinse the plate wells out with fresh media several times, swirl and aspirate.
12. Re-plate the washed cells into the same 6-well plate (same wells).
13. Allow cells to sit 2 days at room temperature to incorporate DNA.
14. After 2 days, add 1.5 mg/mL Geneticin, or G418. This antibiotic will cause cells that have not incorporated pNeo to die over the course of 3-4 weeks.
15. Maintain cells in G418-containing media, rinsing often into fresh media for 3 weeks in the same wells. **At some point during this time, you may wish to do a test induction of protein, growing up some of the cells in $CuSO_4$-containing media for several days and testing the supernatant for protein expression using an ELISA protocol.
16. At the end of 3 weeks, start expanding the lines, maintaining the G418 until you get to a 100 mL spinner flask. **At this point, make multiple freezes at 3-4 million/mL. **Once you are getting past the 100 mL spinner flask, you may start phasing out the G418 because the cell line should be stable with the resistance.

**NOTE: If you are only doing a transient tranfection, you can add 0.5 mM sterile $CuSO_4$ after step 12 to induce protein production. Maintain cells in $CuSO_4$-containing media for 4-5 days before harvesting.

A.1.o LIPOFECTIN S2 CELL TRANSFECTION

Protocol from Tom Cameron and Invitrogen

1. Buy Lipofectin Reagent from Invitrogen (18292-011).
2. Grow nice, untransfected S2 cells in serum-containing media to 3 million per transfection well.
3. Spin cells down and aspirate media. Resuspend in an appropriate volume to give 3 mL per transfection well of 90% Sf-900 II with PFSQ and 10% S2 media with PFSQ, 10% FBS (giving 1% serum final). Allow cells to sit overnight.
4. Prepare 3 μg of each transfection plasmid and 0.2 μg of pNeo for each transfection and sterilize using a sterile eppendorf Spin-X filter. Dilute to 100 μL total volume in Sf-900. Label with construction name and "Solution A." Allow to stand at room temperature for 30-45 minutes.
5. Prepare 20 μL of Lipofectin Reagent and 80 μL Sf-900 (1:5 dilution). Label as "Solution B."
6. Add "Solution A" to "Solution B" and incubate at room temperature for 15 minutes.
7. Spin cell plates at 1500 rpm for 5 minutes and aspirate media. Add Sf-900 and swirl. Spin plates again, aspirate media, and add 3 mL/well Sf-900 + Q and swirl.
8. Add the mixture of Solutions A and B to the plate. Swirl vigorously or pipet-mix.
9. Allow transfections to sit overnight at 25˚C
10. The next day, spin cell plates at 1500 rpm, 5 minutes, aspirate media, and replace with 5 mL of S2 media with serum and PSFQ. Allow to sit for 48 hours.
11. After 48 hours, spin cell plates at 1500 rpm, 5 minutes, aspirate media, and replace with 5 mL S2 media with serum, PFSQ, and 1.5 mg/mL Geneticin (G418).
12. One week after the transfection, spin plates and replace old media with fresh G418-containing media.
13. At some point (perhaps weekly between weeks 1 and 4) do some test protein expressions (ELISA).
14. Continue to simply replace the media with fresh media until the cells are starting to grow dense and happy (low death). Then begin to expand the cells. The lines should be stable and G418 resistant at 4 weeks. Make many freezes at 3-4 million cells/mL.
15. After you have plenty of freezes, you can begin to reduce the G418 and passage into Sf-900 II + PSFQ (no media), expand up to spinner flasks, and begin serious protein production.

**NOTE: If you are only doing a transient tranfection, you can add 0.5 mM sterile $CuSO_4$ after step 10 to induce protein production. Maintain cells in $CuSO_4$-containing media for 4-5 days before harvesting.

A.1.p PROTEIN PRODUCTION IN S2 CELLS

Protocol from Tom Cameron, Liying Lu, and J. Stone

NOTE: This protocol is for constructs using a metallothionine promoter, e.g. pRMHA-3

Protocol:

1. Grow the S2 cell line in a plate or T flask, maintaining selection marker if applicable, until they are sufficiently dense to switch to a shake flask. Grow the cells between 24-27°C with adequate aeration (no CO_2 required). They can grow to ~4-6 x 10^6/mL in the T flask.
2. Split the cells into a sterile, capped Erlenmeyer flask (can use disposable flasks or autoclaved glass flasks provided they are only washed with 7x detergent, not Alconox!!). Use a volume ~1/3 of the total flask volume for cells and media, e.g. 160 mL in a 500 mL flask. Do not split the cells so they are below 1 x 10^6/mL!
3. Grow these flasks on a shaker table at 100-120 rpm at 27°C, making sure the cap is loose to allow for aeration. **NOTE: there may be a lag at this stage before the cells begin doubling again, but once they take off, they will grow VERY QUICKLY!!**
4. Increase the volume of the shaking culture by splitting the cells into larger flasks or multiple flasks, keeping the cell density between 1-6 x 10^6/mL. They can grow denser, but since the idea is to increase the culture, there is no need to wait until they are very dense. During this phase, it is possible to stop adding the selecting agent (e.g. G418). You should check the cells every day, since they will double every 30 or so hours.
5. When the culture has reached the size you would like (e.g. 1.5 L in a 4 L flask), allow the cells to reach an appropriate density. A good induction density is 6-10 x 10^6/mL, with an optimum around 8 x 10^6/mL.
6. Add a 1:1000 dilution of 1 M $CuSO_4$ sterile stock solution to the culture. The media should look dark green.
7. Replace the induced cells on the shaker table. Incubate another 2-5 days. Optimal expression time may vary, but will generally be within this range. 3 days is usually good.
8. After induction period, spin down the cells at 5000 x g and harvest the supernatant for soluble proteins.
9. Check the supernatant by ELISA or Western Blot if possible to ensure production was successful.
10. Concentrate the supernatant down to 200-500 mL unless a large amount of precipitation is found. (Generally this is due to excess glutamine in the media.) Filter the supernatant through a 0.2 μm filter.
11. Add the following components: sodium azide to 0.2%, EDTA to 5 mM, PMSF* to 1 mM, and Iodoacetamide* to 1 mM. Stock solutions are described on the next page.
12. Store the supernatant in the fridge or freezer until ready to purify by immunaffinity.

<u>1 M $CuSO_4$, 50 mL (1000x)</u>**:**
7.98g $CuSO_4$ (Sigma C1297)
fill to 50 mL with ddH_2O
filter-sterilize with 0.2 μm filter

<u>Additions to supernatant:</u>
NaN_3 to 0.02% (1:1000 dilution of 20% stock)
5 mM EDTA (1:100 dilution of 0.5 M stock)
*PMSF to 1 mM (Sigma P7626, make a 200 mM stock solution in ethanol, dilute 1:200)
*Iodoacetamide to 1 mM (Sigma I6125, make a 500 mM stock solution, dilute 1:500)

* If the protein being produced has a free cysteine, do not add PMSF or Iodoacetamide to the supernatant.

A.2.a FLUORESCEIN-LABELING OF PROTEINS AND ANTIBODIES

Protocol modified from J. Cochran and Molecular Probes

Reagents:

Fluorescein-isothiocyanate (FITC) 3 mg/ml in DMSO
50 mM NH_4Cl
0.1 M sodium carbonate, pH 9

Protocol:

1. Exchange buffer in protein of interest to 0.1 M sodium carbonate, pH 9 and concentrate to ~2.5 mg/ml.
2. Add 10 µl of FITC stock to each sample and incubate overnight at 4 °C in the dark.
3. Add NH_4Cl to 50 mM. Incubate for an additional 2 hours.
4. Separate FITC-labeled protein from unreacted FITC by size exclusion chromatography (G50 column)
5. Measure OD of fractions at 280nm and 495nm. Calculate the ratio of 495nm/280nm and pool fractions containing a similar ratio.

Estimation of degree of FITC labeling in an antibody by UV absorbance scanning of the labeled complex:

$$\frac{\#\text{FITC}}{\text{Ab}} = \frac{\left(\frac{A_{495}\text{labeled protein}}{\varepsilon_{495}\text{FITC}}\right)}{\left(\frac{A_{280}\text{labeled protein} - \left(A_{495}\text{labeled protein} * \frac{\varepsilon_{280}\text{FITC}}{\varepsilon_{495}\text{FITC}}\right)}{\varepsilon_{280}\text{unlabeled protein}}\right)}$$

Assume: ε_{495}FITC= 69,000 M^{-1} cm^{-1}
ε_{280}FITC= (0.274)(69,000) M^{-1} cm^{-1}
ε_{280}Ab= 210,667 M^{-1} cm^{-1}
ε_{280}MHC class II= 54,000 M^{-1} cm^{-1}

A.2.b MODIFYING FREE CYSTEINES IN A PROTEIN USING MALEIMIDE REACTIVE GROUPS

Protocol synthesized from experience and Jennifer Cochran, Liying Lu, Molecular Probes and Pierce

Procedure:

1. If the protein is not freshly reduced, add DTT to 5 mM and incubate the protein for 1 hr at room temperature. **Note: If the protein has been stored in DTT and is freshly purified by anion exchange, there is no need to re-reduce. Skip to step 3.
2. Either column-purify, dialyze, or concentrate/switch buffers to remove DTT and put protein in reaction buffer (PBS, tris, or some buffer with pH 7-8--if the pH is too high, the maleimide reagent will also modify amines such as lysine).
3. Immediately check the concentration of the protein—should be no higher than 20 μM.
4. Add the maleimide reagent (commonly used reagents: fluorescein maleimide or biotin maleimide) to a final concentration of 100 μM. Your stock solution should be in DMSO, since the maleimide groups are water-labile, and the stock concentration should be such that you can dilute 1:10 or 1:20 at least into the protein solution (1 mM or more concentrated).
5. Mix well and store the solution at room temperature for 1-2 hours. If the probe is fluorescent, store the solution in the dark to prevent quenching.
6. After the incubation, add DTT to a final concentration of 5 mM to quench any remaining unreacted maleimide.
7. Either column-purify, dialyze, or concentrate/switch buffers to remove the maleimide probe and the excess DTT.
8. Measure the final concentration and, if possible, the labeling stoichiometry.

A.2.c BIOTINYLATION OF MHC PROTEINS USING THE BIRA ENZYME

Adapted from instructions from Avidity (Denver, Colorado)

Reagents:

BirA enzyme (1mg/ml stock) in:
50 mM imidazole (pH 6.8), 50 mM NaCl, 5% glycerol, 5 mM mercaptoethanol
5000 Units/μg. 1 Unit is the amount of enzyme that will biotinylate 1 pmol of peptide substrate in 30 min at 30 °C using the provided buffers and 38 μM of substrate.

Biomix A:
0.5 M Bicine buffer (pH 8.3)

Biomix B:
100 mM ATP
100 mM MgOAc
500 μM biotin

NOTES: Biomix A and B can be stored at –20 °C with repeated thawing and refreezing BirA should be stored at –80 °C. It retains >90% of its activity for > 3 months when stored at 4 °C.

It is recommended for every 10 nmol of substrate (at 40 μM), to use 2.5 μg of birA enzyme to complete the biotinylation in 30-40 minutes at 30 °C. Final reaction mixture should contain 1 part Biomix A, 1 part Biomix B, and 8 parts protein.

NaCl, glycerol, and ammonium sulfate inhibit the activity of the birA.

Substrate should be as concentrated as possible (up to 40 μM).

1. Dilute 1 mg of MHC-peptide complex containing the biotinylation sequence to a concentration of 1.8 mg/ml (1 mg per 560 μL). Protein should be in 10 mM Tris (pH 8).
2. Add 70 μL of Biomix A and 70 μL of Biomix B to the protein solution.
3. Add 7 μg of birA enzyme (7 μL of 1 mg/ml stock) to the mixture from step 2.
4. Incubate at room temperature 16-20 hours or 1 hour at 30°C (reduces aggregation and protein loss).
5. Concentrate and purify by size exclusion chromatography.
6. Pool fractions, and freeze at –80 °C in aliquots to help prevent proteolysis.

To estimate extent of biotinylation, use the protocol “Biotinylation Yield Test” in this thesis.

A.2.d BIOTINYLATION YIELD TEST

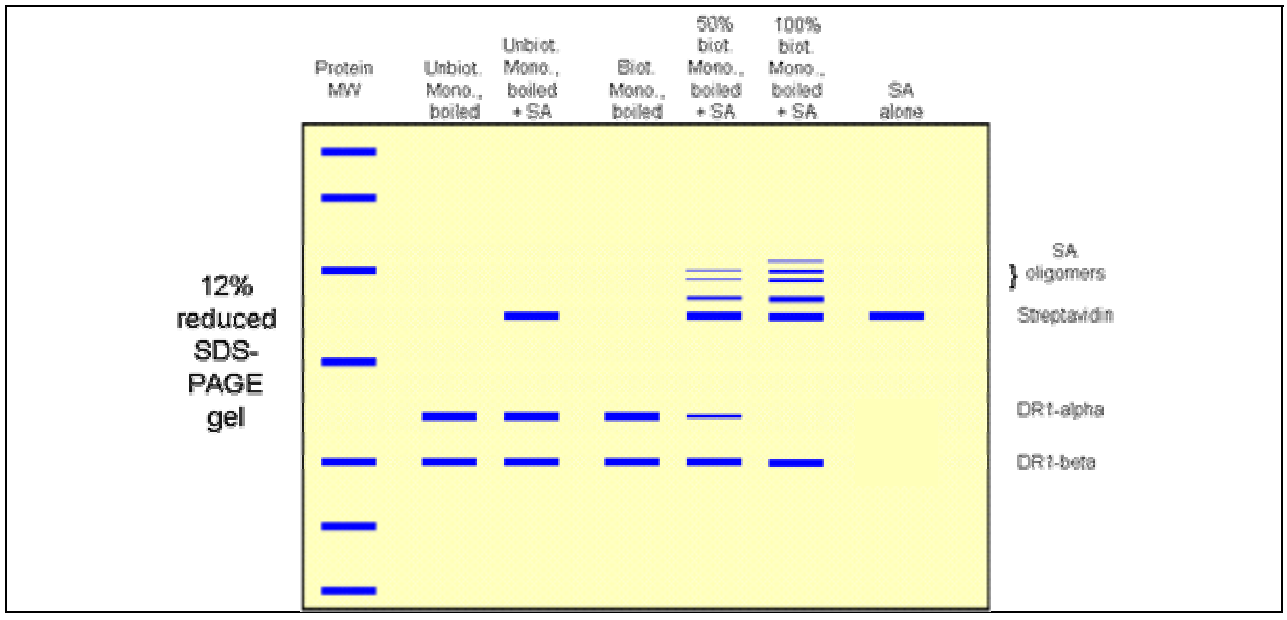
Protocol worked out with Tom Cameron and Liying Lu

Procedure:

1. Set up to do reducing SDS-PAGE using a 12% polyacrylamide gel (or other percentage if it is appropriate).
2. Assemble the following samples in reducing SDS laemlli loading buffer—your protein should be boiled if required to separate into individual subunits (such as HLA-DR1 with tight-binding peptide). Samples b-f should be brought to the same volume:
    a. Protein MW standards
    b. Unbiotinylated monomer, 2 µg
    c. Unbiotinylated monomer, 2 µg
    d. Biotinylated monomer, 2 µg
    e. Biotinylated monomer, 2 µg
    f. 1x PBS or similar buffer
3. Cool all boiled samples to at least room temperature. Then add 2µg of streptavidin to samples c, e, and f, and an equivalent volume of PBS or similar buffer to samples b and d. Allow samples to sit at least 10-15 minutes to allow streptavidin-biotin interaction to occur.
4. Run samples on gel, then stain with Coomassie. The band corresponding to the biotinylated chain of your monomer should disappear in sample e, and larger bands will appear indicating binding to streptavidin. The yield of biotinylation can be determined by determining the fraction of monomer chain remaining after streptavidin was added.

**NOTE: make sure your reducing laemilli load buffer in which the samples are boiled in step 2 is concentrated enough to run the samples after the additional volume of streptavidin or PBS is added in step 3.

Schematic of results of HLA-DR1 with a biotinylation site on the alpha chain:



A.2.e DIMERIZING PROTEINS THROUGH FREE CYSTEINES BY CREATING A DISULFIDE BOND

Protocol from Jennifer Cochran

Procedure:

1. If the protein is not freshly reduced, add DTT to 5 mM and incubate the protein for 1 hr at room temperature.
2. Either column-purify, dialyze, or concentrate/switch buffers to remove DTT and put protein in PBS or HEPES reaction buffer, pH ~ 7.4.
3. Make sure the protein is concentrated—at least 1 mg/mL.
4. Add HEPES to a concentration of 50 mM if another buffer was used.
5. Add 1,10-phenanthroline to 1.3 mM.
6. Add $CuSO_4$ to 0.25 mM.
7. Incubate for at least 1 hour at room temperature or 30 minutes at 37°C.
8. Either column-purify, dialyze, or concentrate/switch buffers to remove copper and phenanthroline and put protein into desired buffer. **Note: Reducing agents will un-do the dimerization.
9. Assay the extent of dimerization by size exclusion chromatography or SDS-PAGE gel (non-reducing). If purified dimer is desired, usually size exclusion chromatography can be used.

A.2.f CROSS-LINKING PROTEINS USING Pep2x, Pep3x, and Pep4x (and relatives)

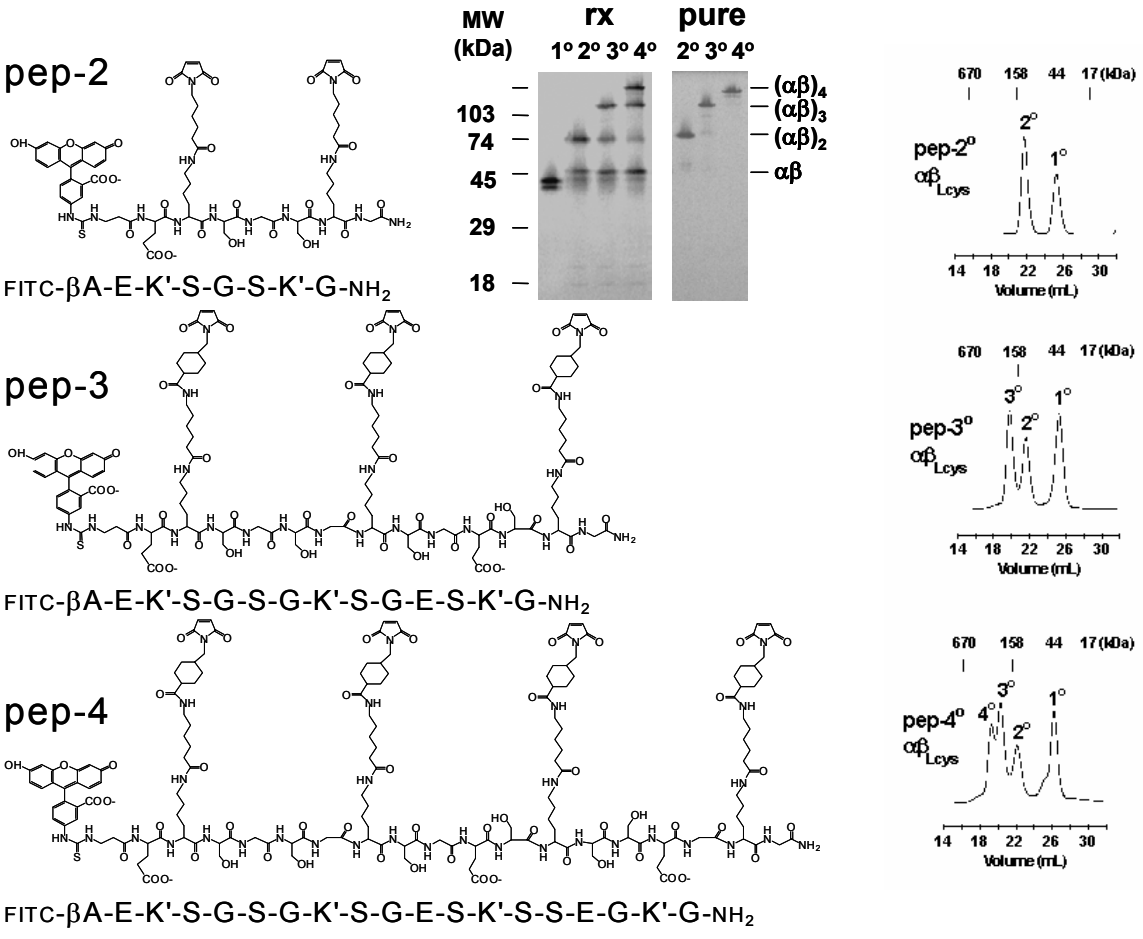
Protocol by J. Stone, based on method used by J. Cochran

***Procedure*:**

1. *Immediately before use*, dissolve lyophilized peptide-based cross-linker in small volume 1x PBS, pH 7.0 (around 50µL). Store on ice to minimize hydrolysis of maleimide before reaction with cysteine from protein. Take absorbance scan of 1µL diluted 1:100 at 495nm.

   [cross-linker]=100*Abs_{495}/69,000
2. Calculate amount to add to freshly-purified cysteine-containing protein. ****There can be no DTT present in the protein sample at this stage.**** Save a sample of unreacted protein to run on a gel.

   moles cross-linker = moles protein / valency of cross-linker
3. Divide the volume of cross-linker that must be added into 4 equal-volume aliquots. Add ¼ of total cross-linker to the protein and allow the mixture to react in the dark at room temperature for 1 hour. Repeat with the other three aliquots.
4. Take a small sample of the reaction mixture to run on a gel.
5. Purify by size exclusion chromatography. For DR1 MHC protein, use two Superdex 200 columns in series to achieve required resolution.



A.2.g HETEROGENEOUS CROSSLINKING

Protocol by J. Stone

**NOTE: To use this protocol, your protein must be stable for short periods at low pH at 4°C.

Procedure:

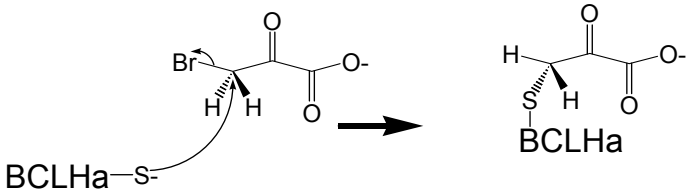
1. Store small aliquots (~ 20 μL) stock solutions of cross-linker reagents 3-bromopyruvate (3-BP, Aldrich Cat. #238341) and N-(ß-Maleimidopropionic acid) hydrazide (BMPH, Pierce Cat. #22297) at 300 mM in DMSO at -20°C.
2. Prepare both freshly reduced, cysteine containing samples (***A*** and ***B***) separately into either 1x PBS, pH 7.4 or 20 mM Tris, pH 8.0. ****There can be no DTT present in the protein sample at this stage.**** Save a sample of unreacted protein to run on a gel. Calculate the concentration of the protein—it should be between 0.1-0.5 mg/mL, or 5-10μM.
3. Calculate the amount of each cross-linker to add to the proteins. Do not allow the final volume of cross-linker to be greater than 10% of the total volume.
   moles 3-BP = (20-30)* moles protein ***A***
   moles BMPH = (20-30)* moles protein ***B***
4. Add the appropriate amount of 3-BP to sample ***A*** and BMPH to sample ***B*** (keep separated). Allow to react at room temperature for approximately 1 hour at room temperature. Take a small sample of each reaction mixture to run on a gel.
5. Rinse both samples (separately) well in concentrators (Amicon Ultra-4, for example) to remove excess cross-linker reagent. Usually 3 washes (200 μL → 4 mL in PBS and concentrated back down) are enough to reduce the concentration of free cross-linker down far below the concentration of the protein (2 orders of magnitude).
6. Check the concentration of each protein.
7. Add equal molar amounts of ***A*-3BP** and ***B*-BMPH** together in the same concentrator and dilute to 4 mL with 30 mM acetate, pH 4.5, 150 mM NaCl. Concentrate in a centrifuge for 1.5 hours at 4°C.
8. Dilute back to 4 mL with 50 mM phosphate, pH 7.4, 150 mM NaCl. Concentrate in a centrifuge for 40 minutes at 4°C.
9. Add DTT to 5 mM and incubate 20-30 minutes at room temperature to reduce any disulfide-linked dimers from unreacted cysteines on ***A*** or ***B*** (or both).
10. Filter the sample and purify by size exclusion chromatography. For DR1 MHC protein, may wish to use two Superdex 200 columns in series to achieve required resolution.

A.2.g HETEROGENEOUS CROSSLINKING (continued)

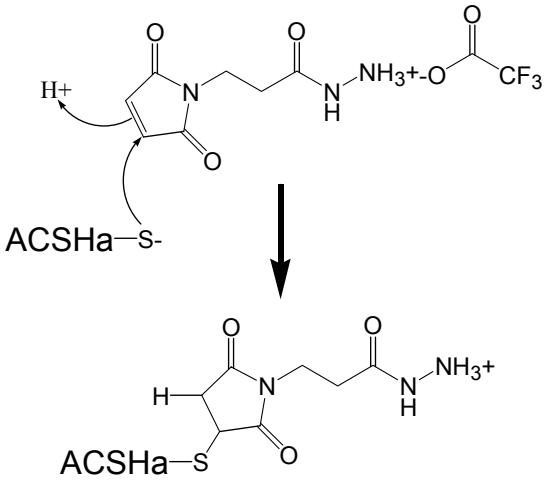
Protocol by J. Stone

Label each protein by the uniquely-reactive cysteine with a different cross-linker

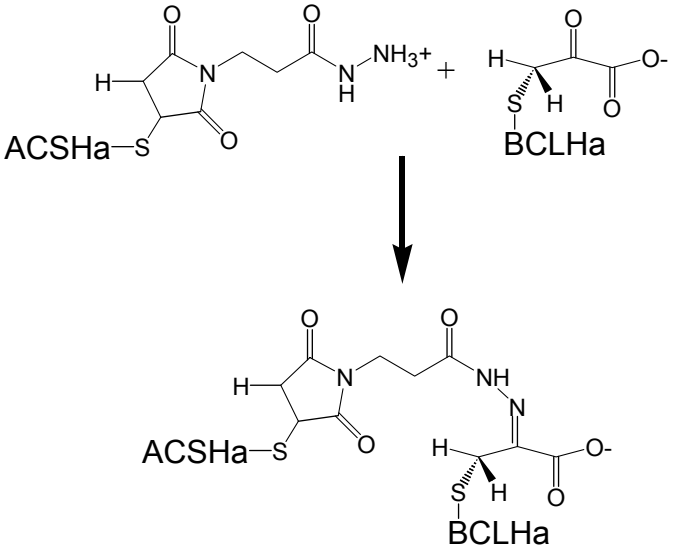
3-Bromopyruvic Acid from Sigma



N-(β-Maleimidoproprionic acid) hydrazine (trifluoroacetic acid salt)
BMPH from Pierce

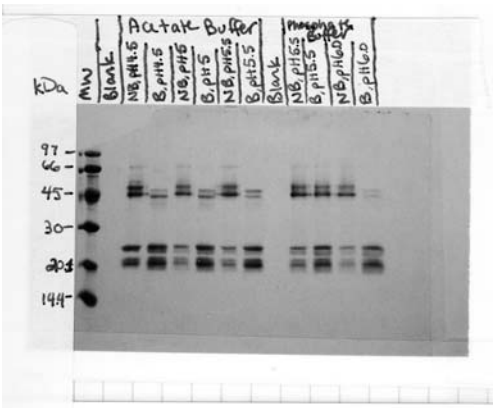


Combine the two cross-linker labeled proteins at high concentration, pH 4.5 for 1.5 hours at 4 °C

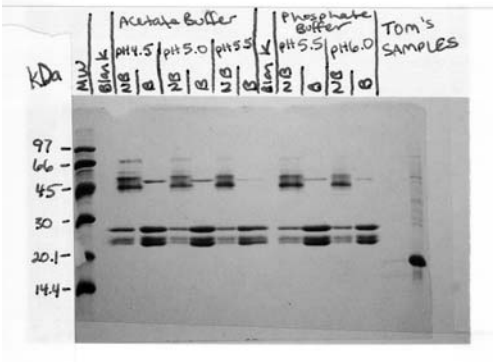


A.2.g HETEROGENEOUS CROSSLINKING (continued)

Protocol by J. Stone

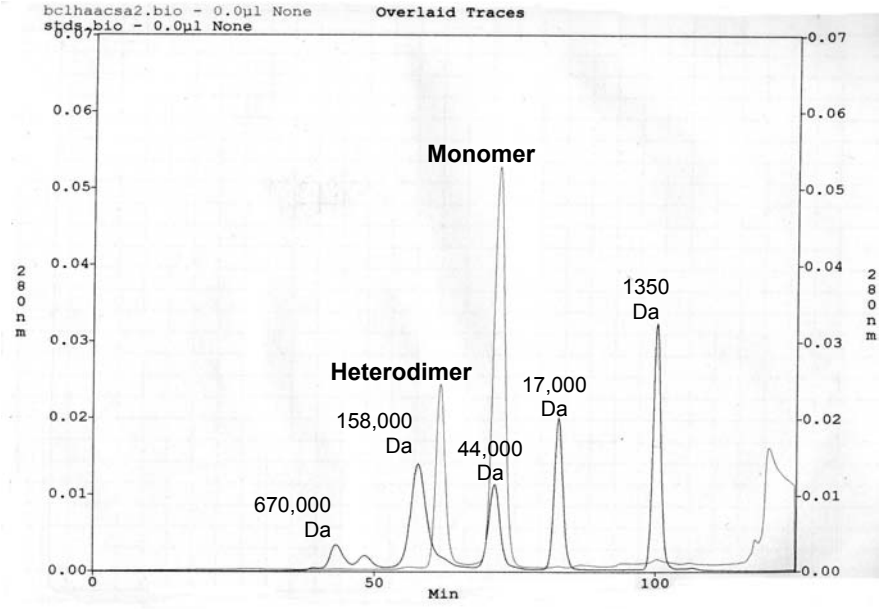


Non-reduced gel of reaction mixtures between ACS-A2-3BP and BCL-HA-BMPH. Both Alpha and Beta chains should be present when the complex is boiled, but the cross-linked chains should hold together.



Reduced gel of the reaction mixture. The dimer is not DTT-sensitive, and forms in the largest amount at pH 4.5

Gel filtration trace of reaction mixture on double Superdex 200 columns. Heterodimer is around 94 kDa and monomer is around 47 kDa—easily separable.



A.2.h SYNTHESIS OF PEPTIDE-BASED CROSSLINKERS

Protocol modified from J. Cochran

Peptide synthesis:

1. Synthesize peptides by FMOC chemistry on a solid phase peptide synthesizer. TentaGel S RAM Fmoc resin (Advanced ChemTech) should be used to give a C-terminal amide upon cleavage. **NOTE: If crosslinkers are to be fluorescently-labeled, β-alanine should be used as the N-terminal amino acid to prevent thiazolidone formation and release of fluorescein during peptide deprotection.**
2. Cap peptides at their N-termini by reaction with excess fluorescein isothiocyanate (FITC) or EZ-link™ NHS-LC-LC-biotin (Pierce) dissolved in DMF.
3. Cleave peptides from the resin and deprotect side-chains with 88% trifluoroacetic acid (TFA), 2% triisopropylsilane, 5% dithiothreitol, shaking for at least 4 hours at RT.
4. Evaporate off as much TFA as possible, heating at 37 °C and gently blowing with a continuous stream of nitrogen.
5. Precipitate the peptide with cold ether (overnight in freezer is best). Wash the peptide twice with cold ether to remove cleavage products and cleavage reagent.
6. Purify peptides by reverse phase HPLC using a shallow acetonitrile gradient in 0.1% TFA and a C18 column (Vydac). Verify molecular weight of peptide by mass spectrometry. This purification may seem like an extra step, but it is important to remove all traces of DTT before addition of modification reagents.

Modification of cysteine residues to include aldehyde groups (for heterocrosslinkers):

1. Dissolve purified peptides (2-5 mg) in 500 ml of 20 mM Na-phosphate buffer (pH 7), 150 mM NaCl.
2. Prepare a 5-fold molar excess solution of 3-bromopyruvate (3BP).
3. Mix together peptide and crosslinker and incubate at RT for approximately 1.5 hours (in the dark). Crosslinker will react through an SN2 reaction with the sulfhydryl, eliminating bromide.
4. Purify modified peptides by reverse-phase HPLC as above. Confirm appropriate number of aldehyde functional groups with mass spectrometry.

   NOTE: If making heterocrosslinkers, modification of cysteines must be done before modification of lysines, since free sulfhydryl groups can also react with the lysine modification reagents. When using heterocrosslinker, react first through the maleimide to attach to a free cysteine on the protein, then couple the crosslinker and protein to a second, BMPH-modified protein. For details, see the protocol for "Heterogeneous Crosslinking"

A.2.h SYNTHESIS OF PEPTIDE-BASED CROSSLINKERS (continued)

Protocol modified from J. Cochran

Modification of lysine residues to include maleimide groups (all peptide-based crosslinkers):

1. Dissolve purified peptides (2-5 mg) in 500 ml of 20 mM Na-bicarbonate buffer (pH 9), 150 mM NaCl.
2. Prepare a 5-fold molar excess solution of either *N*-[ε-maleimidocaproyloxy] succinimide ester (EMCS, Pierce) or succinimidyl-4-[*N*-maleimidomethyl]-cyclohexane-1-carboxy-[6-amidocaproate] (LC-SMCC, Pierce) in *N,N* -dimethylformamide (DMF).
3. Mix together peptide and crosslinker and incubate at RT for approximately 1.5 hours (in the dark). Crosslinker will react through the succinimide ester to the lysine ε-amino groups on the peptide. Solution may turn a bit cloudy during the course of the reaction, as crosslinker is not very soluble in buffer. This is not cause for alarm as the reaction should take place very quickly after mixing reagents.
4. Purify modified peptides by reverse-phase HPLC as above. Confirm appropriate number of maleimide functional groups with MALDI-TOF.

Peptide sequences used for making crosslinkers: (can also be made without fluorescein)

Name	*Sequence*
pep2[*]	fluorescein-βA-E-K-S-G-S-K-G-NH_2
pep3	fluorescein-βA-E-K-S-G-S-G-K-S-G-E-S-K-G-NH_2
pep4	fluorescein-βA-E-K-S-G-S-G-K-S-G-E-S-K-S-S-E-G-K-G-NH_2
het2[#]	fluorescein-βA-E-K-S-G-S-C-G-NH_2
het3a	fluorescein-βA-E-K-S-G-S-G-K-S-G-E-S-C-G-NH_2
het3b	fluorescein-βA-E-K-S-G-S-G-C-S-G-E-S-K-G-NH_2
het4a	fluorescein-βA-E-K-S-G-S-G-K-S-G-E-S-K-S-S-E-G-C-G-NH_2
het4b	fluorescein-βA-E-K-S-G-S-G-K-S-G-E-S-C-S-S-E-G-K-G-NH_2
het4c	fluorescein-βA-E-K-S-G-S-G-K-S-G-E-S-C-S-S-E-G-C-G-NH_2
het4d	fluorescein-βA-E-K-S-G-S-G-C-S-G-E-S-K-S-S-E-G-C-G-NH_2

[*]"pep" crosslinkers are homogeneous. Modified peptide crosslinkers are called pep(n)x, where (n) is the valency of the crosslinker

[#]"het" crosslinkers are heterogeneous. Modified peptide crosslinkers are called het(n)(arr)x, where (n) is the total valency of the crosslinker, and (arr) is an optional letter corresponding to the arrangement of different moieties in higher-order oligomers.

A.3.a MEDIA RECIPES FOR TISSUE CULTURE

Complete Tumor Medium (CTM)

Used for HA1.7 Hybridoma cells

30 mL **Tumor Cocktail (TC)**
50 mL Fetal Calf Serum (heat inactivated) Fill to 500 mL with S-MEM
***Filter sterilize (0.2 μm) and store at 4°C.*

Tumor Cocktail (TC)

351 mL S-MEM (Gibco 11380-037)
7.5 g Dextrose (Fisher BP350)
3.361 g L-Glutamine (Sigma G-8540)
75 mL Essential Amino Acids (50x, Gibco 11130-051)
140 mL Non-Essential Amino Acids (100x, Gibco 11140-050)
100 mL Sodium Pyruvate (100x, Gibco 11360-070)
***Mix and adjust pH to 7.0 with 10N NaOH*
Then, add:
8.5 g Sodium Bicarbonate (Fisher S233)
500 mg Gentamycin Sulfate (Sigma G-3632)
600 mg Penicillin G Sodium Salt (Sigma P-3032)
1 g Streptomycin Sulfate (Sigma S-9137)
34 μL 2-mercaptoethanol (Sigma M-7522)
***Filter sterilize (0.2 μm), aliquot into 30 mL, and store at -20°C.*

EBV Medium

Used for EBV-transformed B cell lines such as LG2, Priess, and EBV 1.24

50 mL Fetal Calf Serum (heat inactivated)
5 mL Hepes
5 mL Penn-Strep
5 mL L-Glutamine
Fill to 500 mL with RPMI 1640
***Filter sterilize (0.2 μm) and store at 4°C.*

A.3.a MEDIA RECIPES FOR TISSUE CULTURE (continued)

751P Medium

Used for fibroblasts such as 751P

50 mL Fetal Calf Serum (heat inactivated)
5 mL Glutamine
5 mL Penn-Strep
Fill to 500 mL with DMEM
***Filter sterilize (0.2 μm) and store at 4°C.*

"K" Medium

Used for P815 cells

500 mL EBV Medium
5 μL 2-mercaptoethanol
***Filter sterilize (0.2 μm) and store at 4°C.*

"C" Medium

Used for murine CD8+ 2C clone L3.100

5 mL Rat ConA Sup (equivalent to 30 U/mL mouse rIL2)
3 mL a-methylmannoside (Sigma M6882, 20% stock in Hank's, Invitrogen 14175095)

THP-1 Medium

Used for THP-1 macrophage pre-cursor line

5 mL **100x Sodium Bicarb/Gluc Sol'n***
5 mL Hepes
5 mL L-Glutamine
5 μL 2-mercaptoethanol
50 mL Fetal Calf Serum (heat inactivated)
Fill to 500 mL with RPMI-1640
***Filter sterilize (0.2 μm) and store at 4°C.*

A.3.a MEDIA RECIPES FOR TISSUE CULTURE (continued)

100x Sodium Bicarb/Gluc Sol'n

75 g sodium bicarbonate
225 g glucose
Fill to 500 mL with ddH_2O
***Filter sterilize (0.2 μm) and store at 4℃.*

Drosophila (S2) Medium
For newly thawed insect cells

5 mL Penn-Strep-Fungizone (also called Antibiotic/Antimycotic)
5 mL L-Glutamine
50 mL Fetal Calf Serum (heat inactivated)
Fill to 500 mL with Schneider's or Grace's
****NOTE:** you can also use Sf-900 II as a base medium and add these supplements for a very rich medium
***Filter sterilize (0.2 μm) and store at 4℃.*

Serum-Free S2 Medium
Used for insect cells that will be secreting protein

5 mL Penn-Strep-Fungizone (also called Antibiotic/Antimycotic)
5 mL L-Glutamine
Fill to 500 mL with Sf-900 II medium
***Filter sterilize (0.2 μm) and store at 4℃.*

Serum-Free S2 Selecting Medium
Used for insect cells that have been transfected with the pNeo selection marker for Geneticin (G418) resistance

5 mL Penn-Strep-Fungizone (also called Antibiotic/Antimycotic)
5 mL L-Glutamine
5 mL 50-150 mg/mL Geneticin (G418)
Fill to 500 mL with Sf-900 II medium
***Filter sterilize (0.2 μm) and store at 4℃.*

A.3.a MEDIA RECIPES FOR TISSUE CULTURE (continued)

Freezing Medium (generic)

Can be used for almost any tissue culture cells

9 mL Fetal Calf Serum (heat inactivated)
1 mL DMSO
***Freeze cells at ~4-5 million/mL at least*

A.3.b T CELL MAINTENANCE—$CD4^+$ CLONES AND LINES

Protocol from Jennifer R. Cochran

***Procedure*:**

1. Pool T cells in a conical tube. **If you are freshly thawing, wash 1 time to remove DMSO.
2. Count a small aliquot to determine T cell density. You will need to re-stimulate at a density of 7 x 10^5-10^6 cells per well in a 24-well plate. Spin cells down and re-suspend to the proper density in T cell medium (7 x 10^5-10^6 per mL). Keep cells on ice.
3. Count HLA-matched EBV-immortalized B cells (maintain in B cell medium, keeping density between 0.3-1.0 x 10^6/mL). You will need 1-2 x 10^6 per well, so you must take enough to match the number of T cell wells you will have. Spin down the appropriate volume of cells and re-suspend in plain RPMI to the proper density (1-2 x 10^6 per mL).
4. Add sterile peptide to a concentration of 1 μM for tight binders, higher for weak binders. Incubate the B cells with the peptide at 37°C for 1-2 hours.
5. Spin the peptide-pulsed B cells at 1500 rpm for 5-10 minutes and re-suspend in fresh T cell medium at 1-2 x 10^6 per mL.
6. Thaw feeder cells (PBMC's—Peripheral Blood Mononuclear Cells) from three different donors. You need 1-2 x 10^6 per well. Wash the feeders 1 time to remove DMSO, count, spin down cells, and re-suspend to the appropriate volume in fresh T cell medium (final density 1-2 x 10^6 per 100 μL).
7. Irradiate B cells and feeder cells:
    a. PBMC's = 5000 rads
    b. B cells = 10,000 rads
8. Set up wells in 24-well plate for re-stimulation. Add 1 mL T cell suspension (7 x 10^5-10^6 cells), 1 mL peptide-pulsed, irradiated B cell suspension (1-2 x 10^6 cells), and 100 μL of irradiated PBMC's (1-2 x 10^6 cells).
9. Incubate plate at 37°C, 5-7% CO_2 overnight.
10. The next day, supplement each well with 20 Cetus units (120 IU) per mL of recombinant human IL-2. (240 IU per well).
11. Leave cells at 37°C, 5-7% CO_2 for approximately 7 days or until T cells are resting (appear round, not elongated, under the microscope). **If T cells look overcrowded on day 4 or 5, split the wells 1:2, adding 1 mL fresh T cell medium to 1 mL re-suspended T cell solution without adding IL-2.
12. Once the T cells are resting, they can be used in assays, frozen, or re-stimulated.

Media:

<u>T cell Medium</u>
5% Human Serum (heat inactivated)
5% Fetal Calf Serum (heat inactivated)
5 mL 100x HEPES, pH 7.0
5 mL 100x Glutamine
2.5 mL 100x Penn-Strep
fill to 500 mL with RPMI medium
Filter and store at 4°C

<u>B cell Medium</u>
10% Fetal Calf Serum (heat inactivated)
5 mL 100x HEPES, pH 7.0
5 mL 100x Glutamine
5 mL 100x Penn-Strep
fill to 500 mL with RPMI medium
Filter and store at 4°C

**sera from Sigma, other media ingredients from Gibco

A.3.b T CELL MAINTENANCE—CD4$^+$ CLONES AND LINES

Protocol from John Cruz, University of Massachusetts Medical School

***Procedure*:**

1. Pool T cells in a conical tube. **If you are freshly thawing, wash 1 time to remove DMSO.
2. Count a small aliquot to determine T cell density. You will re-stimulate at a density of 1 x 10^6 cells per mL. Spin cells down and re-suspend to the proper density in **PHA medium**. Keep cells on ice until you are ready to plate them out.
3. Thaw 3 x 10^6 allogeneic feeder cells (can use PBMC's from 2-3 different donors) per mL of T cells being stimulated. Spin them down and re-suspend them to 3 x 10^7 cells per mL in **Maintenance medium**.
4. Irradiate the feeder cells for 5,000 rads (about 1 hour in an 80 rad/min machine).
5. Plate out the T cells and feeders in a 24-well plate. Add 1 mL of T cells in PHA medium and 100 μL of feeder cells in Maintenance medium to each well. **Make sure surrounding wells are filled with sterile RPMI or PBS to prevent excess evaporation.
6. Incubate plate at 37°C, 5-7% CO_2 overnight.
7. On day 4, expand 1:2 using **2x Maintenance medium**.
8. On days 7 and 11, count the cells. If they are dense (>2 x 10^6 per mL), expand them 1:2 into new wells, using 2x Maintenance medium. If they are not very dense, then simply remove 1 mL of medium from each well and replace with 2x Maintenance medium. **T cells can be used for assays or for freezing on days 7-14.
9. Re-stimulate on day 14.

Media:

PHA Medium
50% 2x Maintenance Medium
3 μg per mL PHA (1:100 dilution of stock sol'n)
To 100% with AIM V medium (Gibco)

2x Maintenance Medium
30% Fetal Calf Serum (heat inactivated)
To 100% with AIM V medium (Gibco)
Filter and store at 4°C
**Before use, add 100-300 units/mL rIL2 (BD Collaborative Research)

**For 1x Maintenance Medium, dilute complete 2x Maintenance Medium 1:2 with AIM V medium (no addititives).
**AIM V medium contains strep, gent, and glutamine

A.3.b T CELL MAINTENANCE—CD4$^+$ CLONES AND LINES

Protocol from Melissa Precopio, University of Massachusetts Medical School

***Procedure*:**

1. Pool T cells in a conical tube. **If you are freshly thawing, wash 1 time to remove DMSO.
2. Count a small aliquot to determine T cell density. You will re-stimulate at a density of 1 x 10^6 cells per mL. Spin cells down and re-suspend to the proper density in **OKT3 medium**. **Another anti-CD3 antibody such as 12F6 (from Johnson Wong, Massachusetts General Hospital) or UCHT-1 (available from BD Pharmingen) can be used instead. Keep cells on ice until you are ready to plate them out.
3. Thaw 3 x 10^6 allogeneic feeder cells (can use PBMC's from 2-3 different donors) per mL of T cells being stimulated. Spin them down and re-suspend them to 3 x 10^7 cells per mL in **Growth medium**.
4. Irradiate the feeder cells for 5,000 rads (about 1 hour in an 80 rad/min machine).
5. Plate out the T cells and feeders in a 24-well plate. Add 1 mL of T cells in OKT3 medium and 100 μL of feeder cells in Growth medium to each well. **Make sure surrounding wells are filled with sterile RPMI or PBS to prevent excess evaporation.
6. Incubate plate at 37°C, 5-7% CO_2 overnight.
7. On day 4, expand 1:2 using **2x Growth medium**.
8. On days 7 and 11, count the cells. If they are dense (>2 x 10^6 per mL), expand them 1:2 into new wells, using 2x Growth medium. If they are not very dense, then simply remove 1 mL of medium from each well and replace with 2x Growth medium. **T cells can be used for assays or for freezing on days 7-14.
9. Re-stimulate on day 14.

Media:

<u>OKT3 Medium</u>
50% 2x Growth Medium (including rIL2)
1-5 μg/mL OKT3
To 100% with RPMI 1640 medium (Gibco)

<u>2x Growth Medium</u>
20% Fetal Calf Serum (heat inactivated)
2x Glutamine
2x HEPES
2x Gent
To 100% with RPMI 1640 medium (Gibco)
**Before use, add 100-300 units/mL rIL2 (BD Collaborative Research)

**For 1x Growth Medium, dilute complete 2x Growth Medium 1:2 with RPMI 1640 medium (no addititives).

A.3.c T CELL MAINTENANCE—MURINE $CD8^+$ 2C CLONE L3.100

Protocol from Carol McKinley, Herman Eisen's Lab

***Procedure*:**

1. Count P815 cells (maintained by splitting frequently in "K" medium). Pool 6 x 10^6 cells in a tube, spin down, and re-suspend in 10 mL "C" medium.
2. Irradiate P815 cells on ice → 20,000 rads.
3. In a 50 mL centrifuge tube, pool several wells of day 7 post stimulation L3.100 cells. Count cells, and take 6 x 10^6 cells. Centrifuge cells at 1000 x g for 5 minutes at room temperature. Aspirate all but 5 mL of old medium.
4. Combine P815 cells with L3.100 cells in the 50 mL tube. Bring volume up to 48 mL with "C" medium and distribute 2 mL per well in a 24-well plate.
5. Incubate L3.100 cells in 24 well plate for 7 days at 37°C, 5% CO_2.

Media:

<u>"K" Medium</u>
10% Fetal Calf Serum (heat inact.)
10 mM HEPES, pH 7.0
2 mM L-glutamine
100 U/mL Penicillin
100 μg/mL Streptomycin
50 mM of β-mercaptoethanol
in RPMI 1640 Medium

<u>"C" Medium</u>
"K" medium containing
5% Rat ConA Sup (equivalent to
30 U/mL recombinant mouse IL2)
3% a-methylmannoside (Sigma M6882)
(20% stock in Hank's, Invitrogen 14175095)

A.3.d LEUKOPACK PROCESSING

Protocol from Jennifer R. Cochran

***Procedure*:**

1. Set the temperature on the centrifuge to 25˚C (room temperature).
2. Add 15 mL Ficoll to Accuspin tubes
3. Centrifuge for 2 minutes at 500 RPM to get the Ficoll below the frit.
4. Aspirate off residual Ficoll remaining on top of the frit.
5. Add a 35 mL or less of leukopack blood (undiluted) per tube.
6. Centrifuge at room temperature at 1800 RPM for 20 minutes. **Turn the brake off on the centrifuge for this spin to avoid disrupting your gradient.
7. **After removing the Accuspin tubes from the centrifuge, turn the brake back on, and set the temperature back down to 4˚C and allow it to begin cooling.
8. Remove the leukocyte layer from the Accuspin tubes. The leukocytes from 2 Accuspin tubes can go into a single 50 mL conical tube. The bottom layer will be red blood cells (red), followed by Ficoll (colorless), leukocytes (cloudy thin layer), and serum (yellow).
9. Fill the remaining space in the conical tube with RPMI (without any additives). Centrifuge at 1500 RPM at 4˚C to pellet the cells.
10. Wash a second time with RPMI.
11. Resuspend pellet in a small volume (around 5 mL). Count a 1:100 dilution of this mixture. Your final concentration will be 1-2 x 10^7 cells/mL.
12. Dilute concentrated cells in RPMI to appropriate concentration in Freezing Media. (Note: If your dilution will be less than 1:8, re-pellet the cells and take up directly into Freezing Media.)
13. Aliquot cells into cryovials, adding 1 mL cells per vial.
14. Freeze vials of cells slowly at -70˚C overnight.
15. Transfer vials to liquid nitrogen dewar.

***25 mL of leukopack blood will yield 200-500 million cells.

Materials:

Accuspin Tubes – Sigma Diagnostics (A2055)
Ficoll-Paque – Pharmacia Biotech (17-0840-02)
Freezing Media – 90% Fetal Calf Serum, 10% DMSO, filtered

A.3.e QUICK FICOLL GRADIENT PURIFICATION

Protocol from Sriram Chitta

Procedure:

1. Set centrifuge to 21°C, 1500 x g (rcf), brake = 0
2. Resuspend cells 3 mL (10 mL) in RPMI, 10% FCS in a 15 mL (50 mL) tube. Count live cells with Trypan Blue.
3. Add 7 mL (30 mL) sterile Hanks Balanced Salt Solution or PBS and mix.
4. Add 2 mL (10 mL) of sterile Ficoll-Paque SLOWLY at the bottom of the tube. Should form a discrete layer.
5. Spin cells (balanced) for 20 minutes at 20°C, 1500 x g, NO BRAKE.
6. After the spin, there should be a buffy layer of leukocytes around the 2.5 mL (mL) mark on the tube. With a pipet, carefully recover that layer and dilute into medium. Try not to get much Ficoll, as that will spin down when you wash the cells.
7. Wash cells twice with medium to remove remaining Ficoll.
8. Resuspend and count cells. Debris and dead cells should be absent by Trypan Blue.

A.3.f SINGLE CELL SUSPENSIONS FROM MOUSE ORGANS

Protocol from Airiel Davis

**Note: save the supernatants from every step of this protocol until you are finished and know you have enough cells.

***Procedure*:**

1. Collect the mouse organs (lymph nodes, spleen, etc.) in a Petri dish with 3-5 mL RPMI, 2% FCS.
2. Crush the organs between the frosted handles of two glass slides (sterilized). Start with the smallest organs, as the solution will become cloudy and the small organs will be difficult to see later.
3. Spin down cells at 1500 rpm, 10 minutes. Ensure that a pellet has formed! Remove supernatant and retain in a separate tube.
4. Resuspend cells in 2-3 mL room temperature **RBC lysis buffer** per mouse. Allow to sit 1-5 minutes—no longer! **NOTE: If you have trouble with losing cells in this step, try a Ficoll gradient to isolate the white blood cells instead of lysing the RBC's.
5. Dilute with 10 mL/mouse RPMI, 2% FCS and spin down at 1500 rpm, 10 minutes. Ensure that a pellet has formed! Remove supernatant and retain in a separate tube.
6. Resuspend cells in RPMI, 2% FCS and count total number.

To purify TCR transgenic CD8 T cells with Dynal Magnetic Beads:

1. Calculate from the total cell number the number of B cells/APCs expected and determine the volume of B220 beads (Dynal cat. # 114.01) to add such that there are at least four beads per cell. Do the same calculation for CD4+ T cells to determine the volume of CD4 beads (Dynal cat # 114.05) to add.
2. Add the appropriate volume of beads into PBS, 1% FCS in a tube to rinse away azide.
3. Place the tube into the magnetic concentrator (Dynal cat. # 120.01) and allow the beads to stick to the side of the tube. Remove the PBS solution.
4. Pipet the resuspended lymph node cells into the tube with the beads. Remove the tube from the concentrator, cap tightly, and agitate at 4°C for 20 minutes.
5. Place the tube back into the magnetic concentrator and allow the beads and cells bound to them to stick to the side of the tube, depleting $CD4^+$ T cells and B cells/APCs.
6. Remove the cells still in solution with a pipet, place into a new tube, and count.

from Lymph Nodes (20% B cells/APCs, 2-5% $CD4^+$)

μL B220 beads = 2*(# million cells total)
μL CD4 beads = 0.5*(# million cells total)

from Spleen (up to 60% B cells/APCs, up to 30% $CD4^+$)

μL B220 beads = 6*(# million cells total)
μL CD4 beads = 3*(# million cells total)

A.3.f SINGLE CELL SUSPENSIONS FROM MOUSE ORGANS (continued)

Protocol from Airiel Davis

Solutions:

160 mM NH_4Cl
8.45 g. NH_4Cl
to 1 L with ddH2O
sterile filter

170 mM Tris, pH 7.65
20.6 g. Tris base
115 mL 1M HCl
pH to 7.65 in 1L total
sterile filter

RBC lysis buffer, pH 7.2
90 mL 160 mM NH_4Cl
10 mL 170 mM Tris, pH 7.65
sterile filter

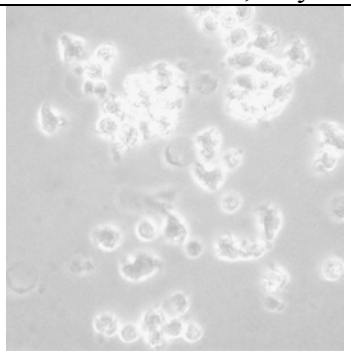
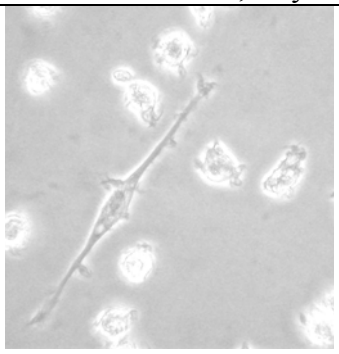
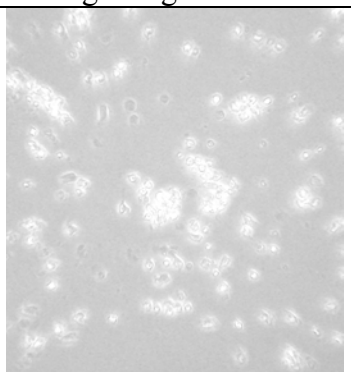
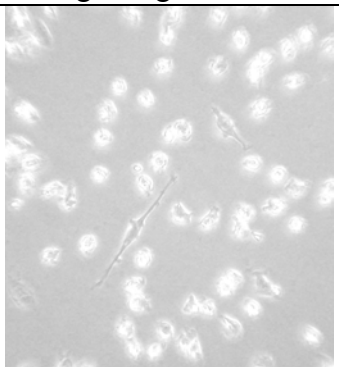
**Since the RBC lysis is a very sensitive step (as I have lost all my cells multiple times at this point), it may be worth A) testing a small part of the cells before lysing all of them and/or B) purchasing the RBC lysis buffer from Roche (cat. # 1814389).

A.3.g MAKING MACROPHAGES OUT OF PBMC MONOCYTES

Protocol from Sriram Chitta

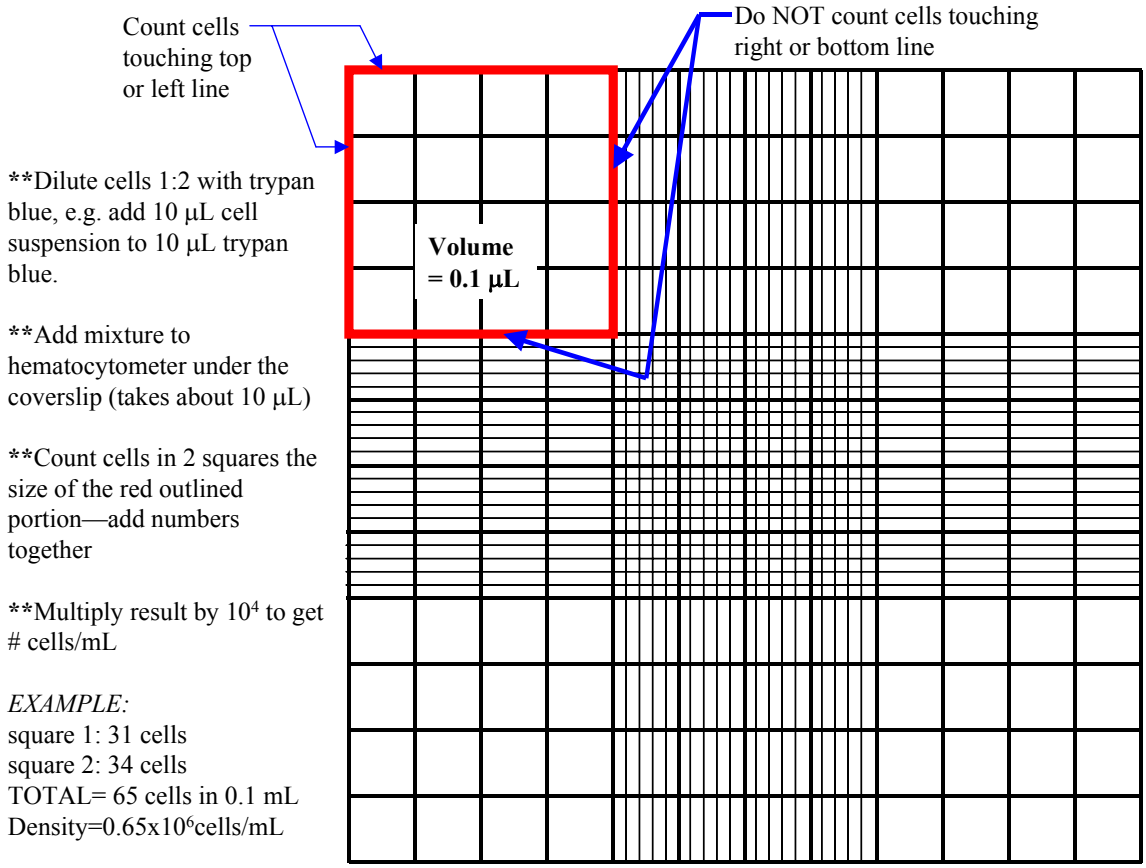
Procedure:

1. Take PBMC's in RPMI-based medium with 10% serum, Penn-Strep, Glutamine, and Hepes. Plate them onto a cell-culture polystyrene surface at high density.
2. Allow cells to sit at 37°C for 2 hours.
3. After incubation, swirl medium and remove non-adherent cells. **You may wish to keep these in a separate tube, depending upon what you are going to do.
4. Wash the adherent cells 2x with sterile PBS, swirling each time.
5. Add fresh RPMI-based medium (described above) with 25 ng/mL M-CSF.
6. On 3rd day, remove ½ medium from the plated cells and replace with new RPMI medium with 25 ng/mL M-CSF.
7. On day 6, the monocytes should have transformed into macrophages. To remove, soak in PBS with 1 mM EDTA for 10 minutes at room temperature, use a cell lifter to gently remove cells, and wash the cells well to remove the EDTA.
8. The cells can be re-plated on a new surface—they should re-adhere within 2 hours at 37°C.

Donor C PBMC's, Day 1	Donor C PBMC's, Day 4
 High magnification	 High magnification
 Medium magnification	 Medium magnification

A.3.h COUNTING TISSUE CULTURE CELLS

Protocol from Jennifer Stone



SPLITTING TISSUE CULTURE CELLS

1. Count your cell suspension and find the density (D_i)
2. Determine the final cell density you would like (D_f)
3. Determine the final volume of suspension you would like (V_f)
4. Calculate the volume of current suspension to add using the following equation:

$$V_i = \frac{D_f V_f}{D_i}$$

   Where V_i is the volume you will keep from your initial cell suspension
5. The cells should be split using V_i from the initial cell suspension and V_m of fresh media, where $V_m = V_f - V_i$

A.3.i GENERAL T CELL ACTIVATION ASSAY

Protocol and notes by J. Stone

NOTE: ***PLANNING* is the most crucial part of a successful T cell assay. Overdocument everything in advance so you are not grasping for materials or performing last-minute calculations—that WILL result in errors. For long assays (24 hours or more), it is important to keep everything sterile-filter all protein solutions and buffers and perform everything in the biosafety cabinet. For a shorter assay, sterility is less crucial, especially since the T cell stimulation is performed in media that generally contains antibiotics.**

Protocol:

1. Select a plate or plates that will hold all of your samples. In general, polystyrene round bottom 96-well plates are useful, although flat-well plates may be better for immobilized stimuli. For ELISPOT assays, you will normally perform the assay in commercially available plates with wells (usually with a nitrocellulose membrane bottom) pre-coated with cytokine or chemokine capture antibody, although you can replicate this yourself.
2. Plan your assay, including concentrations and assay volumes. An assay volume for a T cell stimulation may be as low as 20 μL per well if reagent conservation is a high priority, although larger volumes (50 μL or so) may be more convenient to manipulate. For later ELISA analysis of the supernatant to determine cytokine secretion, even larger volumes (100-200 μL/well) may be desired so there is enough supernatant at the end of the incubation to perform the ELISA experiment.
3. Perform any surface coating for immobilized stimuli first. Put the proteins at the appropriate concentrations in the well in PBS and incubate 2 hours at room temperature or overnight at 4°C.
4. Block the plate using 1% BSA or 4% FBS in PBS or RPMI. Fill each well with 200-300 μL of the block solution and incubate 2 hours at room temperature or overnight at 4°C.
5. Remove the block solution by aspiration or flicking. (Flicking is a useful technique that is fast and results in low cell loss during wash steps. I recommend it!)
6. Plate out soluble stimuli and control wells with media alone. Perform dilutions very carefully. Replicates of different conditions are always useful if you have enough of the cells and stimulus. If cells are to be used as a stimulus, make sure they are pulsed with any peptides or chemicals in advance (usually incubated 2 hours at 37°C) and washed into fresh media.
7. Count your T cells and determine what volume of fresh media to resuspend them into so that at the volume of T cell you will add (V_T) will contain the appropriate number of T cells. Generally, you will want to make sure your assay wells each contain 50,000-200,000 T cells, with a clonal population of T cells requiring fewer cells per condition and a mixed T cell population requiring more.
8. Add the T cells to the wells, taking care not to carry stimulus from one well to another. If you have difficulty here, use fresh pipet tips for each well. Do not lose your place—check carefully to make sure you added stimulus or media and T cells to each well.
9. Incubate the T cells with the stimulus at 37°C, 5% CO_2 for an appropriate time to detect your desired activation marker. If fluorescently labeled stimulus is being used, take care to keep the assay plate in the dark. For $CD8^+$ T cells, many cell surface proteins detected by flow cytometry are modulated in the first 3-6 hours (CD3 and CD69 are more rapid,

and CD25 and CD71 are more slow), while for $CD4^+$ T cells, those proteins are more slowly modulated—on the order of 12-24 hours. Cytokine secretion assays (ELISA or ELISPOT) may be detected between 16-72 hours of incubation, depending on the cells and the cytokine desired. For your particular marker, you may need to perform a time course to determine the optimal incubation time, or consult the literature.

FOR ELISPOT:

After incubation, lyse and remove cells, and detect cytokines following the protocol for your kit (or your own protocol that you have worked out), developing spots for secreting cells with precipitating substrate.

FOR ELISA:

After incubation, spin down cells, remove and retain supernatants for detection of bulk cytokine secretion, usually by sandwich ELISA with matched pairs of monoclonal antibodies.

FOR PROLIFERATION USING ^{3}H-THYMIDINE:

After incubation for several days, add 1μCi of ^{3}H-thymidine to each well and allow cells to continue incubating for another 12 hours (overnight). Harvest cells onto a glass fiber filter and count associated radioactivity (see separate protocol for ^{3}H-Thymidine assay).

FOR FLOW CYTOMETRY:

After incubation, chill cells on ice for 5 minutes or so to halt membrane turnover. Prepare your antibodies to add to the cells, using the same strategy for planning as you did to resuspend your T cells for the assay—always plan enough antibody mixture for 5-10 more samples than you need, as you are likely to run short after many samples. You may use as many differentially labeled fluorescent antibodies as you can detect on your flow cytometer simultaneously in each sample. For small incubation volumes, you can add fluorescent antibodies directly to the assay media, pipet-mixing well to ensure adequate staining. For larger volumes, you may wish to spin down the cells, flick out the media, and resuspend each well in an antibody cocktail made up in FACS buffer (0.02% azide, 1% FBS in 1x PBS). You may have to titrate your fluorescent antibodies to determine the proper amount to add to each well, although generally it will be 1-5 μL. Allow the cells and antibodies to sit on ice in the dark for 30-60 minutes. Then, spin down and wash the cells 2-3 times (resuspend, pipet-mix, spin down, and flick out supernatant) with FACS buffer and finally resuspend in a small volume (50-100 μL—the higher the volume, the more slowly the flow cytometer will analyze the sample) of FACS buffer (may add very small concentration of ethidium bromide if you will be using that channel to gate out dead or lysed cells) or Fixing buffer (1% paraformaldehyde in PBS—all cells are dead, so you can't use ethidium bromide, but the cells may sit for up to a week at 4°C before analysis). Move the samples into labeled FACS tubes which can be used on your flow cytometer.

A.3.i GENERAL T CELL ACTIVATION ASSAY (continued)

Protocol and notes by J. Stone

Sample calculation for plating stimulus and cells:

$$V_f = V_{stim} + V_T \quad (1)$$

where V_f is the final volume in the assay well, V_{stim} is the volume of the solution containing the stimulus in each well, and V_T is the volume of T cell suspension added to each well. In general, I add the T cells to the assay well last before the incubation. To calculate what volume to resuspend your T cells into so that you can add V_T to each well and have the appropriate number of cells added:

$$V_{resuspend} = V_T * \frac{N_{tot}}{N_{well}} \quad (2)$$

where $V_{resuspend}$ is the volume of fresh media to resuspend all your T cells into, N_{tot} is the total number of T cells you have, and N_{well} is the total number of T cells you will add to each well. **NOTE: N_{tot}/N_{well} MUST be greater than the number of samples you are planning by at least 5-10. ALWAYS calculate enough cell suspension that you will be left with enough volume for 5-10 extra samples at the end. When pipetting samples such as these, often using a multipipettor, you will never end up with the exact right volume, and you will always come up short if you don't plan for extra.** Then, to calculate the concentration of stimulus to plate out in the stimulus solution before adding the T cells:

$$C_{stim} = C_f * \frac{V_f}{V_{stim}} \quad (3)$$

where C_{stim} is the concentration of the reagent in the stimulus solution before T cells are added, V_{stim} is the volume of the stimulus solution without T cells, and C_f and V_f refer to the final concentration of the stimulus reagent and final volume of the assay with T cells added, respectively. Then, using those values and the concentration of your stock solution, you can concentrate the volume of stock solution to add to the well:

$$V_{stock} = V_{stim} * \frac{C_{stim}}{C_{stock}} \quad (4)$$

where V_{stock} is the volume stock solution to add, and C_{stock} is the concentration of the stimulus in the stock solution. To find the amount of media, V_{media}, to add to the stimulus solution:

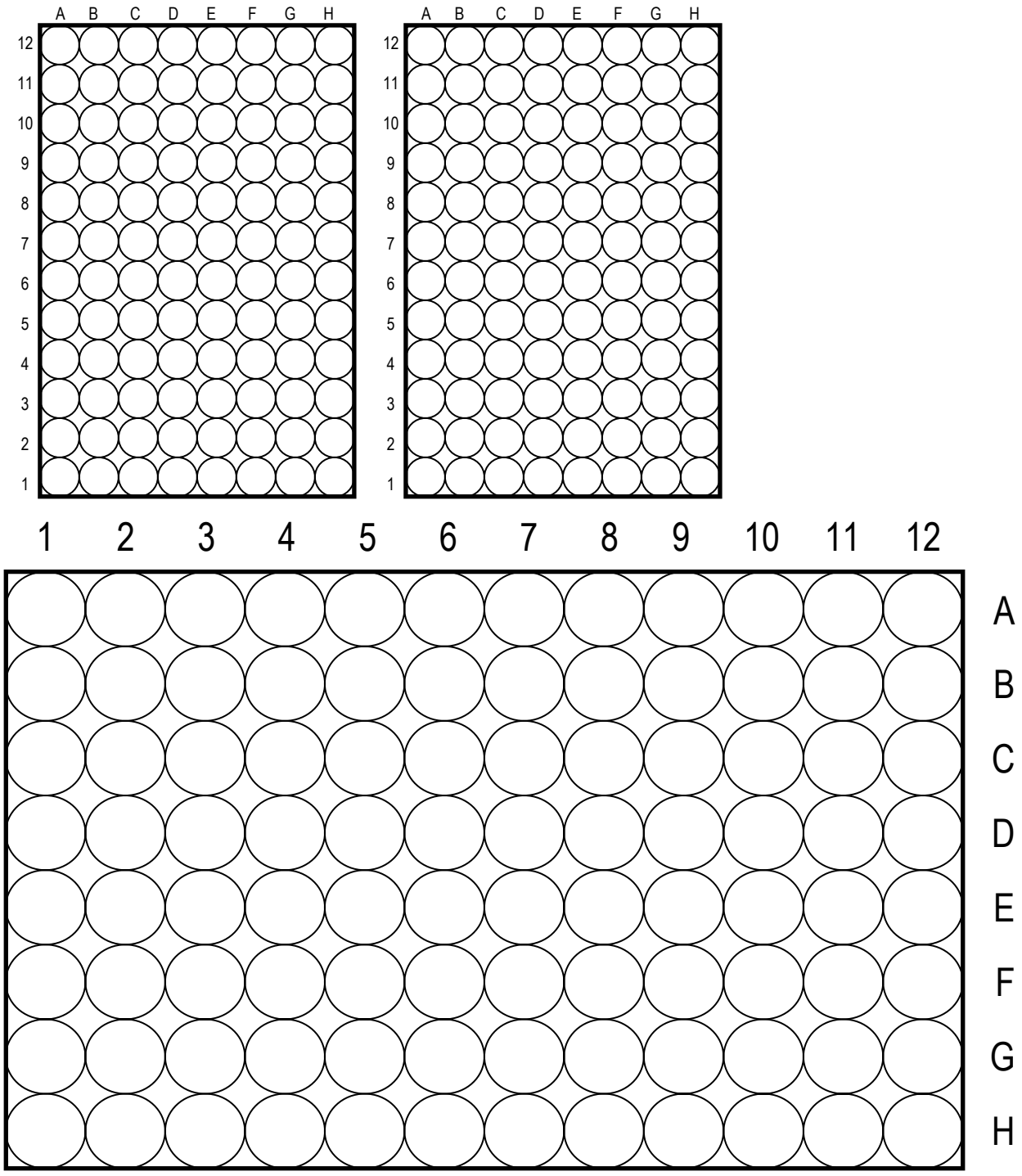
$$V_{media} = V_{stim} - V_{stock} \quad (5)$$

**NOTE: If you plan to do serial dilutions of some top concentration of stimulus, you will calculate your C_{stim} the same way for the top concentration. However, for making your stimulation solution in equations (4) and (5), use a V'_{stim} that is larger—enough that you will have the original V_{stim} after you remove a portion to dilute into fresh media for the first dilution. For example, if you will do 1:3 dilutions of stimulus, add the volume of your V_{stim} in fresh media to all but the top well. Your V'_{stim} (top well) will be equal to $3/2*V_{stim}$ in equations (4) and (5). Then, you can take a volume equal to $1/2V_{stim}$ from the top concentration well (leaving V_{stim}) and dilute it into the next well for a 1:3 dilution, and mix well. Repeat this for as many dilutions as you plan to make. Finally remove a volume of $1/2V_{stim}$ from the lowest concentration well after the dilution so that you are left with V_{stim} in that well. Put the excess solution in the waste.

A.3.i GENERAL T CELL ACTIVATION ASSAY (continued)

Protocol and notes by J. Stone

For planning assays in a 96-well plate:



A.3.j ANERGIZING T CELLS

Protocol by T. Cameron, based on experiment of 4/9/01

Procedure:

Day 1:

Plan experiment and calculate the number of wells and cells you will need. (Plan triplicate samples for proliferation assays.)

To anergize using OKT3 on a plate, dilute Ab in PBS to 5 μg/ml and add 150 ml/well in a flat-bottomed 96-well plate. Incubate 2 hrs at room temperature. Wash several times with PBS or RPMI, making sure wells do not dry out.

Wash T cells and resuspend in T cell media to 1.333 x 10^6 cells/ml. Add 150 μl/well.

Add Anergizing Stimulus (5 μM control or specific peptide) or activating stimulus (EBV + peptide, PMA/Ionomycin, or PHA/IL-2) for wells without OKT3.

Incubate overnight or ~24 hours at 37°C with 7% CO_2.

Day 2:

Warm some T cell media.

In a flat-bottomed 96-well plate, prepare the activation stimuli (peptide, PMA/Ionomycin, PHA/IL-2) at 3x concentration. Prepare 50 μl for each final stimulated well you will have of that stimulus.

Collect healthy EBV 1.24 (DR1+) cells in T cell media to 2 x 10^6 cells/ml. Irradiate with 10 krads.

Collect anergized T cells in 15 ml conicals, spin down, aspirate (leave some liquid, and do not suck up the pellet), and resuspend to 2 x 10^6 cells/ml. Add 50 μl T cells and 50 μl EBV's to each well in 96-well round-bottom plate.

Add 50 μl stimulation reagent to each well. Pipet-mix briefly.

Incubate for desired time at 37°C with 7% CO_2.

A.3.j ANERGIZING T CELLS

Protocol by T. Cameron, based on experiment of 4/9/01

***Solutions/Reagents*:**

Ha and Tfr: Specific and null peptides. Use at 5 μM (15mM in preparation well).

PMA: 0.05 mg/ml aliquots, 10,000x. Best results when adding 0.25 μl/ml, or 0.75 μl/ml in preparation well (12.5 ng/ml final).

Ionomycin: 0.4 mg/ml aliquots, 1000x. Best results when adding 2.5 μl/ml or 7.5 μl/ml in preparation well (1000 ng/ml final).

PHA: 0.2 mg/ml aliquots. Add 10 μl/ml or 30 μl/ml in the preparation well (2 μg/ml final).

IL-2: 22,000 units in 50 μl RPMI aliquots. Add 0.091μl/ml, or 0.273 μl/ml in preparation well (40 units/ml final).

OKT3: α-CD3 mAb, used clinically.

A.3.k INTRACELLULAR CYTOKINE STAINING

Protocol by T. Cameron, synthesized from protocols at BD, Caltag, Zoran, and websites

***Procedure and approximate time scale*:**

9:00 AM

Wash cells, resuspend in T cell media, add ~100,000 cells/well into a round-bottom 96-well plate with stimulus (EBV's, PMA/Ionomycin, PHA/IL-2). Incubate at 37°C with 7% CO_2 for 2-3 hours.

11:30 AM

Add 46.7 µl T cell media to 20 µl aliquot of 5 mg/ml BFA. Add 1 µl per well (150 ml stimulated cells) to final concentration of 10 µg/ml. Incubate at 37°C with 7% CO_2 for 4 more hours.

3:30 PM

Spin down (5 min at 1500 rpm) and flick out supernatant. Resuspend in 20-50 µl cold FACS buffer WITH EDTA containing α–CD4-APC at 1:50 dilution. Stain for 15 minutes at 4°C. Add 150 µl Fixing Solution per well after 15 minutes and incubate 15 more minutes at 4°C.

4:00 PM

Spin down, flick, and resuspend in 200 µL FACS buffer (without EDTA). Spin down again, flick, and resuspend in 20 µl Permealization Solution. Incubate 10 minutes at 4°C. Split samples into 2 wells (10 µl and 10 µl) each—to one set, add 20 µl α-cytokine (α-IL-2) antibody, and to the other set, add 2.5 µl of a 1:4 dilution (200 µg/ml stock, 50 µg/ml dilution, 10 µg/ml final in well) the isotype control. Incubate 30 minutes at 4°C.

5:00 PM

Add 150 µl Permealization Solution. Spin down, flick, resuspend in 200 µl Permealization Solution, spin down, and flick again. Resuspend in 50 µl FACS buffer. Analyze on FACSCalibur Flow Cytometer.

A.3.k INTRACELLULAR CYTOKINE STAINING

Protocol by T. Cameron, synthesized from protocols at BD, Caltag, Zoran, and websites

***Solutions/Reagents*:**

BFA: Brefeldin A, Sigma B7651. Make stock solution 5 mg/ml in DMSO. Freeze at –20 or -80°C in 20 µl aliquots. Use at a final concentration of 10 µg/ml.

Fixing Solution: 4% Paraformaldehyde in 1x PBS, stored at 4°C, less than 1 month old. To make, add dry paraformaldehyde with PBS at 56-66°C stirring for 30-60 minutes. Cool, adjust pH to 7.2-7.4, and filter.

Permeabilizing Solution: 1% FBS, 0.1% NaN_3, and 0.1% Saponin (Sigma S-4521) in 1x PBS, adjust pH to 7.2-7.4, filter, and store cold.

FACS Buffer (without EDTA): 1% FBS and 0.1% NaN_3 in 1x PBS.

FACS Buffer WITH EDTA: 1% FBS, 0.1% NaN_3, and 5 mM EDTA in 1x PBS.

α-cytokine mAb: Use BD Cat # 340448, Clone 5344.111, α-Hu-IL-2-FITC

Isotype control mAb: Use Sigma IgG1-FITC Isotype Control F-6397. Comes at 200 µg/ml—dilute 1:4 in permeabilizing solution to 50 µg/ml, and add 2.5 µl/well to a final concentration of 10 µg/ml.

α-CD4 mAb: Use Diatec α-CD4-APC Cat # 3023

A.3.l PROLIFERATION ASSAY FOR T CELLS (^{3}H-Thymidine Incorporation)

Protocol by Jennifer Cochran

***Procedure*:**

Day 1:

Set up T cell assay in 96-well plate. Stimulate with stimulus of choice. (See Day 2 of "ANERGIZING T CELLS" Procedure.) Incubate plate 36-48 hours at 37°C with 7% CO_2.

Day 3:

Add 1 microcurie of ^{3}H-Thymidine to each well. For a 1 mCi/ml stock, this will be 20 µl of a 1:20 dilution. Rinse out pipet tips in wells to quantitatively dispense all the reagent.

Incubate overnight (~ 12 hours) at 37°C with 7% CO_2.

Day 4:

Harvest cells onto glass fiber filters and count radioactivity.

A.3.m COUNTING ^{3}H-THYMIDINE INCORPORATION INTO CELLS

Protocol by Sriram Chitta

***Procedure*:**

1. Get plates that have been incubated with ^{3}H-thymidine for at least 12 hours. Take them to the radioactivity room with the scintillation counters (door code 1-3-5).
2. To harvest cells, first turn on the two vacuum pumps (grey), and then turn on the harvester itself (switch on right). **Make sure the water reservoir is at least half-full before you begin (white plastic tank to the right of the harvester).
3. Turn toggle switch on the trap (on the left near sink) to the "TRAP" setting—a green light will display.
4. Place a dummy glass filter in the filter press and use a deep tray full of water on the plate tray with the wash/aspirator tips. **A1 goes in the upper left, and the filter should touch the rubber gaskets at the top and right. Pull the press down and lock it over the filter by pulling the metal bar toward you until it is horizontal.
5. Press pulse wash—it will wash once, and then the light will flash. Press it again, and it will continue with three more washes. The red light will go off when it is done.
6. Remove the tray of water and replace it with a dummy 96-well plate (empty), and place it up around the wash/aspirator tips.
7. Press pulse wash—it will wash once, and then the light will flash. Press it again, and it will continue with three more washes. The red light will go off when it is done.
8. Remove the dummy filter and replace it with your assay glass filter—make sure it is marked with indelible ink or by notching the filter so you can identify it later. **A1 goes in the upper left, and the filter should touch the rubber gaskets at the top and right. Pull the press down and lock it over the filter by pulling the metal bar toward you until it is horizontal.
9. Press pulse wash—it will wash once, which will serve to pre-wet your experimental filter. DO NOT PRESS PULSE WASH THE SECOND TIME—leave it flashing.
10. Remove the dummy plate and replace it with your experimental 96-well plate. Raise it up around the wash/aspirator tips.
11. Once the experimental plate is in place, press the pulse wash button again, which should be flashing. It will harvest the cells from the experiment and then wash. The red light will go off when it is done.
12. Remove the filter onto which you harvested the cells when the pulse wash is done. Keep it to the side and allow it to completely dry while you clean the harvester. Replace the dummy filter into the filter press. Close and lock the press.
13. Press pulse wash—it will wash once, and then the light will flash. Press it again, and it will continue with three more washes. The red light will go off when it is done.
14. Remove the experimental plate and replace it with a dummy 96-well plate (empty), and place it up around the wash/aspirator tips.
15. Press pulse wash—it will wash once, and then the light will flash. Press it again, and it will continue with three more washes. The red light will go off when it is done.
16. Remove the dummy plate and replace the water tray. Make sure it is raised so the wash/aspirator tips are in the water so they do not dry out.
17. Remove the dummy filter from the filter press. Leave the metal bar vertical (unlocked) so the gaskets are not under pressure.

18. Toggle the switch on the trap to the "DRAIN" position. Stand away from the sink to avoid any splashing when the water begins to drain. Once the trap has stopped draining, place the switch on the trap to the neutral position—directly in the center.
19. Turn off the harvester.
20. Turn off both vacuum pumps.
21. Make sure your experimental filter paper is dry. If it is not, use a hair dryer to finish drying it.
22. Place the filter paper in a plastic sample bag and seal the edges close to, but not right on top of, the filter. Make sure there is enough room for you to cut and re-seal one corner!
23. Cut one corner off the plastic bag and add 5 mL of BetaScint solution. Spread it around evenly such that the entire filter paper is wet inside. Make sure there is no excess solution and there are no bubbles.
24. Re-seal the corner of the plastic bag. Make sure it is not leaking scintillation fluid.
25. Place the filter in the plastic bag into a carriage for reading in the Microbeta Trilux scintillation counter. Make sure the 96-well demarcations line up with the holes in the carriage.
26. Use the bar code for protocol 08 on the first carriage and no bar codes for subsequent carriages that you will also count.
27. Place the carriages inside the Microbeta from the bottom shelf (1) upwards. Place an empty carriage with a "STOP" protocol bar code in the shelf immediately following your sample carriages.
28. Open the Microbeta Workstation window on the computer. Click on the large "Protocols" icon. Select "General" in the menu that pops up. Then, select "sriram" (not "sriram Cr"). Use protocol "8" on the screen. Check the plate map to make sure it will count all the wells in which you had samples. When the plate map is appropriate, click on start next to the protocol number. It will then say "READY" on the bottom of the screen. Then, click the large "Start" icon with the green light.
29. If you are counting a plate, it will usually give a live display. If you would like to check a previous plate, you may by clicking the "Prev" button to the right of the plate display.
30. MAKE SURE YOU DISPOSE OF ALL RADIOACTIVE MATERIAL PROPERLY!!

A.3.n STAINING CD4+ T CELLS WITH DR TETRAMERS

Protocol from Tom Cameron

Reagents:

DR1-pep: Biotinylated DR1-peptide of choice in PBS.
SA-PE: R-phycoerythrin conjugated Streptavidin from BioSource, Inc
PBS, 1x
RPMI
T cell Media
T cells and/or **PBMCs**
FACS buffer: 1x PBS, 1% BSA or FBS, 0.02% sodium azide
Fixing Solution: 1x PBS, 1% paraformaldehyde (must be dissolved at 60°C)
Fluorescent antibodies to cell-surface markers:
- α-CD4-APC
- α-CD14-PerCP (optional, good for PBMCs),
- α-CD3-Fitc (optional, but good, esp for clones).

Make the Oligomer Reagent (for 50 μl):

NOTE: this procedure is generally completed on ice, although it may be done at room temperature. The reagent should be made up fresh for each stain.

1. Aliquot 1.5 μg **DR1-pep** into an eppendorf tube.
2. In a separate tube, dilute 2 μL **SA-PE** with 4 μL **PBS**
3. Add 2 μL of diluted **SA-PE** mixture to the tube with **DR1-pep** and pipet-mix well
4. Wait 2 minutes
5. Add 2 μL of diluted **SA-PE** mixture to the tube with **DR1-pep** and pipet-mix well
6. Wait 2 minutes
7. Add the rest of the diluted **SA-PE** mixture to the tube with **DR1-pep** and pipet-mix well
8. Wait 2 minutes → complex should form between biotinylated **DR1-pep** and **SA-PE**: **DR1-SAPE oligomer**
9. Dilute **DR1-SAPE oligomer** with **RPMI** or **T cell media** to a final volume of 50 μL.

NOTES: This is called a 60 ug/ml reagent, approximately half **DR1-pep and half **SA-PE** (by weight). Since **SA-PE** is a poorly characterized heterogeneous reagent, that isn't very precise. Different lots of SA-PE should be titrated with DR1-pep to find the optimal ratio between the two reagents. For Lot 1401 from BioSource, Tom Cameron found experimentally that the optimal ratio was 1.3 μL SA-PE per 1 μg DR1-pep. In general, approximately 1 μL SA-PE per 1 μg DR1-pep should give decent staining. **

A.3.n STAINING CD4+ T CELLS WITH DR TETRAMERS (continued)

Protocol from Tom Cameron

Staining $CD4^+$ T Cells:

1. Collect cells in a 15 ml conical tube and spin down 5 minutes, 1500 rpm
2. Aspirate as much of the media as you can.
3. Resuspend the cells in a very small volume of media. You will want to have a final volume such that you use 10 μL cell suspension for each stain you would like to do. (example: staining with one specific tetramer and one control tetramer → resuspend cells in 20 μL)
4. Aliquot 10 μl cell suspension into one well of a 96-well round-bottom plate for each staining experiment. **NOTE: avoid outer wells and if possible, fill the wells surrounding the experiment with 200 μL RPMI to prevent evaporation during the assay.**
5. Add 5 μl of **DR1pep-SAPE oligomer** reagent (tetramer) to the cell suspension in appropriate wells.
6. Incubate the plate at 37°C for 3-5 hours. In general, use a hydrated 37°C incubator with 5% CO_2.
7. Chill plate on ice 5 minutes.
8. Prepare a **cocktail** of desired **fluorescent antibodies**. Typically use 1 or 2 μl of each antibody per well and dilute with **T cell media**, **RPMI** or **PBS** so that you will add 5 μL **cocktail** per sample.
9. Add 5 μl of **fluorescent antibody cocktail** to each sample. Pipet mix well.
10. Incubate on ice for 20-45 minutes.
11. Add 200 μl cold **FACS buffer** to each sample. Spin plate at 4°C for 5 minutes, 1500 rpm. **NOTE: it can be reassuring to look at the bottom of the plate to make sure cell pellets have formed in your sample wells.
12. Aspirate supernatant with pipet tip or flick plate into sink.
13. Wash with 200 μl cold FACS buffer and pipet mix well. Spin plate at 4°C for 5 minutes, 1500 rpm.
14. Aspirate or flick supernatant
15. Resuspend samples in either **FACS buffer** or **Fixing Solution**.
16. Analyze by flow cytometry.

NOTES: Remember to include plenty of controls including single-stained cells for compensation adjustment, control tetramers, control cells if available. Try to keep samples cold after the 37° staining incubation is over, since α-CD3 antibody can stimulate the cells at RT or 37°C, resulting in odd results (downregulated TCR, maybe CD4, etc.).

A.3.o T CELL—T CELL ACTIVATION ASSAY
Used for 2C T cell assays using Class I MHC monomers or peptide alone

Protocol by J. Stone and Q. Ge

***Procedure and approximate time scale*:**

Night Before

Block enough V-bottom FACS plates for your experiment with 150μL/well of 1%BSA in PBS—incubate overnight at 4°C.

6:30 AM

Kill naïve 2C mice with and without GFP and purify cells for low CD44 expression with magnetic beads (keeping + and – GFP cells separate). Count both sets of cells, and re-suspend each set in T cell media to a volume that gives 50,000-100,000 in 24μL. These cells should be ready around 11:00AM-12:00PMish. Store cells on ice until needed.

7:00 AM

Check monomer by analytical gel filtration, and check concentration of monomer and peptide by UV absorbance. Calculate dilutions for assay—you will need to start with 1.857x of your first concentration in the first well—made up by dilution in T cell media just before the assay begins.

11:30 AM

On ice, add 32μL of 1.857x of your first concentration in T cell media to the first well, and perform 1:8 dilutions of 4μL into 28μL down the row, mixing well at each stage. Throw 4μL away at the end of the row to leave 28μL in each well. Add 28μL media alone to any control wells you have planned. Add 24μL GFP- cells to each well. Incubate at 37°C for 3 hours.

2:30 PM

Spin cells down in plate, flick, and wash 4x by resuspending in wash buffer and/or media. After 4 washes, resuspend all wells in 28μL. Add 24μL GFP+ cells to assay wells, making sure you have some control wells for these cells, as well. Incubate another 3 hours at 37°C.

5:30 PM

Chill cells on ice. Stain with aCD8-APC and either αTCR-PE or αCD69-PE at a 1:100 final dilution. (Make up 48μL/well, 1μl Ab/well, in FACS buffer with azide.) Stain on ice for 1 hour.

6:30 PM

Spin down cells, and wash in FACS buffer twice. After last wash, resuspend in FACS buffer containing Propidium Iodide, about 60μL/well, and transfer into FACS tubes. Analyze on the FACSCalibur Flow Cytometer.

A.3.p ASSAY OF ERK PHOSPHORYLATION

Protocol from Leslie Berg's lab, from papers by Karen Liu and Steve Bunnel

Reagents:

PMA and Ionomycin: Final concentrations 2.5ng/mL and 375ng/mL, respectively.
RPMI
Stopping Solution: 20 mM NaF and 1 mM Na3VO4 in 1x PBS. Make solution fresh using 15 mL PBS, 75 μL **Na_3VO_4 stock solution**, 600 μL **NaF stock solution**.
NaF Stock Solution: 0.5 M in DMSO, store aliquots at -20°C
Na_3VO_4 Stock Solution: 0.2 M in DMSO, store aliquots at -20°C
Lysis Buffer: 25 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM PMSF, 10 μg/mL leupeptin, and 1 mM Na_3VO_4. Make 15 mL aliquots of solution without Na_3VO_4 and store at -20°C. When ready to use, thaw aliquot and add 75 μL **Na_3VO_4 stock solution** fresh.
Phospho-p44/42 MAP Kinase: detects phosphorylated ERK, from Cell Signaling 9101S
p44/42 MAP Kinase: detects total ERK, from Cell Signaling 9102
HRP-secondary antibody: choose any polyclonal antibody reagent against the proper species. For ERK, use goat anti-rabbit HRP.
NOTE: Can also use Phospho SAPK/JNK or other markers in this protocol.

Procedure:

1. Plan experiment to study T cell stimuli, being sure to include a positive control such as **PMA and Ionomycin** and negative controls including T cells with no stimulus and **lysis buffer** alone.
2. Incubate 3-5 x 10^6 T cells with each of the stimuli you plan to study at 37°C for 0 sec., 30 sec., 2 min., 5 min., 10 min., and 15 min. Volume should be ~120 μL/sample.
3. Add 1 mL/sample ice-cold **stopping solution** to stop reactions.
4. Spin cells and remove supernatant.
5. Resuspend in a small volume of cold **lysis buffer** (~100 μL) and incubate for 15 minutes on ice.
6. Clear lysates by spinning 10,000 x g for 10 minutes.
7. Analyze by SDS-PAGE, and transfer to an Immobilon-P membrane (Millipore)
8. Block, blot for phospho-p44/42 MAP Kinase, detect with an HRP-labeled goat anti-rabbit reagent, and develop using ECL reagents (for example, Chemicon 2600: Chemiluminescent Blot Detection System). A sample protocol can be seen in "Western Blot—ECL Detection"
9. After detection, strip using an ECL-approved stripping protocol (for example, Chemicon 2504: Re-Blot Plus Strong Ab. Stripping Solution) and re-probe the same membrane for total p44/42 MAP Kinase.

A.3.q MAKING MHC AND CO-STIMULATORY ARRAY CHIPS

Protocol by Jennifer D. Stone, Modified from B.D. Pharmingen ELISPOT Protocol

Procedure:

1. Take a 25 mm x 75 mm slide (works best on polystyrene or LabTek II CC2 chamber slides). For hand-spotting, mark the array pattern on the back using a pre-printed template, leaving the frosted/rough handle area free. **LABEL YOUR SLIDE WELL.
2. If you are not using a chamber slide, surround the array area on the front of the slide with your hydrophobic barrier of choice; for example, paint hydrophobic ink around the array area and allow to dry (works best on glass), or use an adhesive-backed rubber gasket cut to surround the area (Molecular Probes P-24745). Place this type of slide into a secondary container such as a petri dish to catch any spills that may occur.
3. Dilute your monoclonal cytokine capture antibody (not biotinylated) 1:25 (40 μg/mL final) into 1x PBS, pH 7.4.
4. If hand-spotting, place one spot of the capture antibody solution (0.1-0.5 μL) in each location. If automatically spotting, spot one layer of the pattern (0.05-0.1 μL per spot). Allow to dry completely at room temperature.
5. Repeat the spotting of the capture antibody solution in the same pattern 3 more times, allowing to dry each time between layers. Total layers of capture antibody solution = 4 in each spot.
6. Spot your MHC and/or antibody solutions onto the slide in the pattern of your choice on top of the dry capture antibody spots.
    a. As a control, it is good to use anti-CD3 in some spots, which should stimulate all T cells.
    b. Combining the anti-CD3 or MHC-peptide complex with a co-stimulatory antibody such as anti-CD11a, anti-CD28, or anti-CD2 will enhance the activation seen, but will not cause activation by non-specific peptide-MHC complexes.
    c. Concentrations should be 1 μM or approximately 50 μg/mL of MHC-peptide complex, and/or 5 μg/mL of anti-CD3 or co-stimulatory antibody.
    d. It is generally beneficial to tetramerize MHC-peptide complexes by biotinylating the soluble monomers and adding them to streptavidin in a 4:1 ratio. The final MHC concentration should remain 50 μg/mL in the spot.
7. Allow the spots to dry completely.
8. Store the array slide at 4°C in a dessicator until you are ready to use it.

A.3.r T-CELL EPITOPE DETECTION EXPERIMENT USING MHC AND CO-STIMULATORY ARRAYS

Protocol by Jennifer D. Stone, Modified from B.D. Pharmingen ELISPOT Protocol

Procedure:

1. Take a dry, arrayed slide with the appropriate MHC-costimulatory-capture antibody pattern and allow to come to room temperature. **Note: it is useful to keep the array inside the petri dish if it is not a chambered slide.
2. Block the slide by incubation with T cell medium containing 10% FCS for 30 minutes at room temperature.
3. Re-suspend and count your T cell sample. You will want to add enough T cells that you would expect at least 1 x 10^4 specific cells to be present on the array. Take the appropriate amount of the T cell solution and spin down the cells at 1500RPM for 10 minutes at 4°C.
4. Aspirate the supernatant.
5. Re-suspend the pellet in 1-2 mL T cell medium. **Note: for some cell lines or for precipitating substrate development, you may wish to use 5 ng/mL PMA to enhance the antigen-specific activation you will see. The PMA alone will not cause activation in the T cells. Generally, this is not required.
6. Aspirate the T cell medium from the MHC-peptide array that was blocking the surface. Add the freshly re-suspended T cells in medium to the surface of the array inside the hydrophobic barrier, making sure the entire area is covered.
7. Carefully place the array with the T cells inside a 5% CO_2, 37°C incubator, being careful not to spill the T cell solution, and making sure that the array is level inside the incubator. **Note: Make sure the incubator is well-humidified to reduce evaporation during the incubation.
8. Incubate the array at 37°C, 5% CO_2 for 4-16 hours, depending on the cytokine you wish to analyze and the type of T cell ($CD4^+$ or $CD8^+$, murine or human, etc.). For IFN-γ response, 6 hours is best for fluorescent detection, and 16 hours is best for precipitating substrate detection.
9. Pre-incubate your monoclonal cytokine detection antibody (biotinylated) with either fluorescent streptavidin (SA-CY3, -CY5, -Alexa 555, or -Alexa 647, 2 mg/mL, for fluorescent detection) or enzyme-linked streptavidin (SA-HRP, 2 mg/mL, for precipitating substrate detection) at high concentration for ≥ 30 minutes. Your final dilution will be 1:250 for the detection antibody and 1:1000 for the streptavidin at 2 mg/mL in Dilution Solution containing excess biotin.
10. After the incubation, carefully remove the array from the incubator. Aspirate off the cell solution and observe for cell adhesion to the spotted pattern. **Note: it is possible to analyze the array for adhesion only under a microscope—see protocol for Detecting Adherent Cells on MHC-costimulatory Arrays.
11. Spread ddH_2O inside the hydrophobic barrier. Allow the array to sit for 5 minutes at room temperature. This will help lyse any remaining cells.
12. Aspirate the ddH_2O and repeat with another 5 minute ddH_2O wash.
13. Aspirate the ddH_2O and wash the slide 3 times with TBST, aspirating the buffer off after each wash.

14. Dilute your pre-incubated detection antibody and streptavidin mixture into Dilution Solution containing 100 μM d-biotin to prevent non-specific binding to streptavidin-containing spots. Apply this solution (1.0-1.5 mL/array) inside the hydrophobic barrier. Allow the array to incubate with the detection antibody solution for 2 hours at room temperature.
15. After the incubation, aspirate the detection antibody solution and wash 3 times with TBST.

16. *FOR FLUORESCENT DETECTION:* Wash 2 additional times with ddH_2O, then dry completely. Remove the chamber if using a chambered slide array (LabTek II CC2). Scan using an array scanner.

17. *FOR PRECIPITATING SUBSTRATE DETECTION:* No more than fifteen minutes before you are ready to use it, prepare the AEC substrate solution by adding 20 μL of substrate per milliliter of PBS and mixing well.
18. Wash 2 additional times with PBS—NO AZIDE. Azide will inhibit the enzyme.
19. Spread 1.0-1.5 mL of the AEC substrate solution on the array, making sure the entire area is covered evenly. Allow this reaction to proceed in the dark for 5-60 minutes, checking the progress periodically.
20. To stop the reaction, aspirate the AEC solution and wash 2 times with ddH_2O. Allow to dry completely in the dark.
21. Observe the spots by eye and under a dissection microscope. The AEC precipitate photographs very well, but will fade over time, especially if exposed to light.

A.3.r T-CELL EPITOPE DETECTION EXPERIMENT USING MHC AND CO-STIMULATORY ARRAYS (continued)

Protocol by Jennifer D. Stone, Modified from B.D. Pharmingen ELISPOT Protocol

***Solutions and Reagents*:**

T cell medium (for assay)
10% Fetal Bovine Serum, heat inact.
1:100 Penn-Strep solution (Gibco)
1:100 Glutamine solution (Gibco)
1:100 Hepes solution (Gibco)
Fill with 1x RPMI with phenol red (Gibco)
Filter and store at 4°C

TBST:
50 mM Tris, pH 7.5
150 mM NaCl
0.1% Tween-20
filter and store at 4˚C

Dilution Solution:
TBST
0.3% BSA
100 μM d-biotin
filter and store at 4˚C

Company	*Catalogue #*	*Description*
Nalge Nunc International	160004	Polystyrene Cell Culture Slide—25 mm x 75 mm, one end frosted, 20/pack. Buy through VWR or Fisher
Nalge Nunc International	154739 (1 chamber) 154917 (4 chambers)	LabTek II CC2 Chamber Slides with cover, 16/pack. Buy through VWR or Fisher.
Molecular Probes	P-24742	Press-to-Seal silicone isolator with adhesive, 24 wells, 2.5 mm diameter, 2.0 mm deep, 25/pack
Molecular Probes	P-24745	Press-to-Seal silicone sheet with adhesive, 13 cm x 18 cm, 0.5 mm thick, 5/pack
Molecular Probes	S32355	Streptavidin-Alexa 555
Molecular Probes	S32357	Streptavidin-Alexa 647
Sigma	S5512	Streptavidin-HRP
Sigma	B3399	d-biotin
Ted Pella, Inc.	22309	Super Pap Pen Liquid Blocker—hydrophobic ink
Avery (Office Supply)	8665	Clear Full Sheet Labels
B.D. Pharmingen	551884	Anti-human IL2 monoclonal antibody pair, detection antibody biotinylated
B.D. Pharmingen	551873	Anti-human IFNγ monoclonal antibody pair, detection antibody biotinylated
B.D. Pharmingen	551881	Anti-mouse IFNγ monoclonal antibody pair, detection antibody biotinylated
B.D. Pharmingen	551950	Avidin-HRP, 100x, 1 mL
B.D. Pharmingen	551951	AEC precipitating substrate set, to use with Avidin-HRP

A.3.s QUANTITATION OF CELL SURFACE RECEPTORS BY FLOW CYTOMETRY

Protocol from J. Cochran

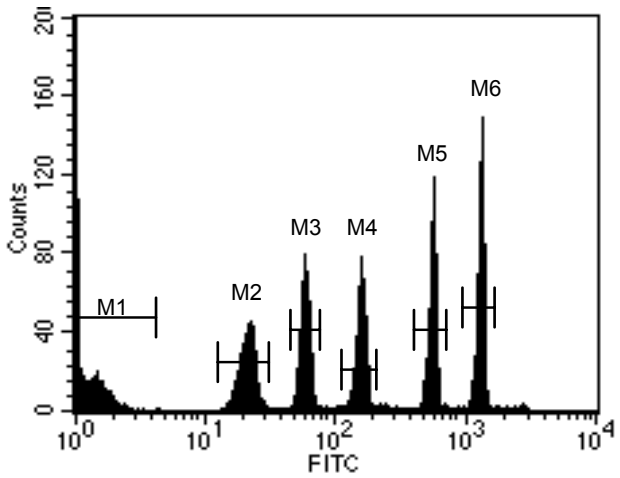
NOTE: In order to quantitate receptor number you must know how many FITC or PE molecules are present per antibody molecule. For FITC, you can estimate this value using the protocol "Fluorescein labeling of proteins and antibodies" in this thesis. For PE you can try to get this number from the manufacturer, or calculate a value from comparison to the levels obtained with a FITC labeled version of the same antibody.

1. After performing FACS assay and running tubes through the flow cytometer, run a sample containing diluted SPHERO™ Rainbow Calibration Particles (RCR-30-5; Spherotech, Inc.) through the flow cytometer.
2. Plot up histograms of mean FITC and PE. You should see 6 peaks on your histogram (see next page for an example). Obtain mean fluorescence values for each peak.
3. Plot up a standard curve of Mean FITC versus Mean equivalents of fluorescein (MEFL). Repeat for Mean PE versus Mean equivalents of PE (MEPE).

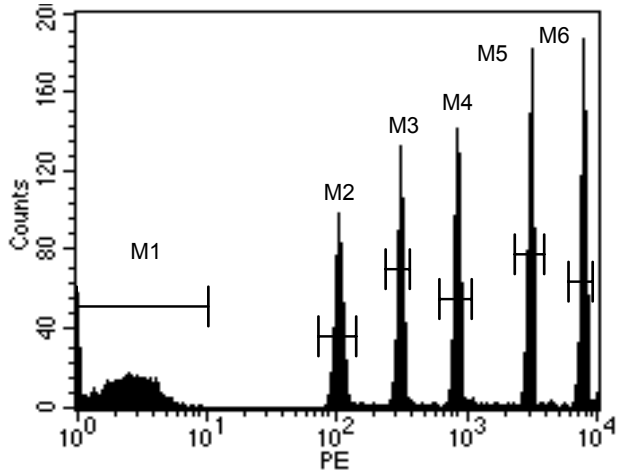
Peak	MEFL	MEPE
1	100	70
2	4700	3800
3	15000	12000
4	40000	34000
5	140000	124000
6	330000	300000

4. Obtain mean fluorescence values for each samples of interest. Using linear regression of your standard curve, calculate Mean equivalents of fluor present on the cell surface.
5. Divide the Mean equivalents by the number of fluors present per antibody to obtain number of cell surface receptors present.

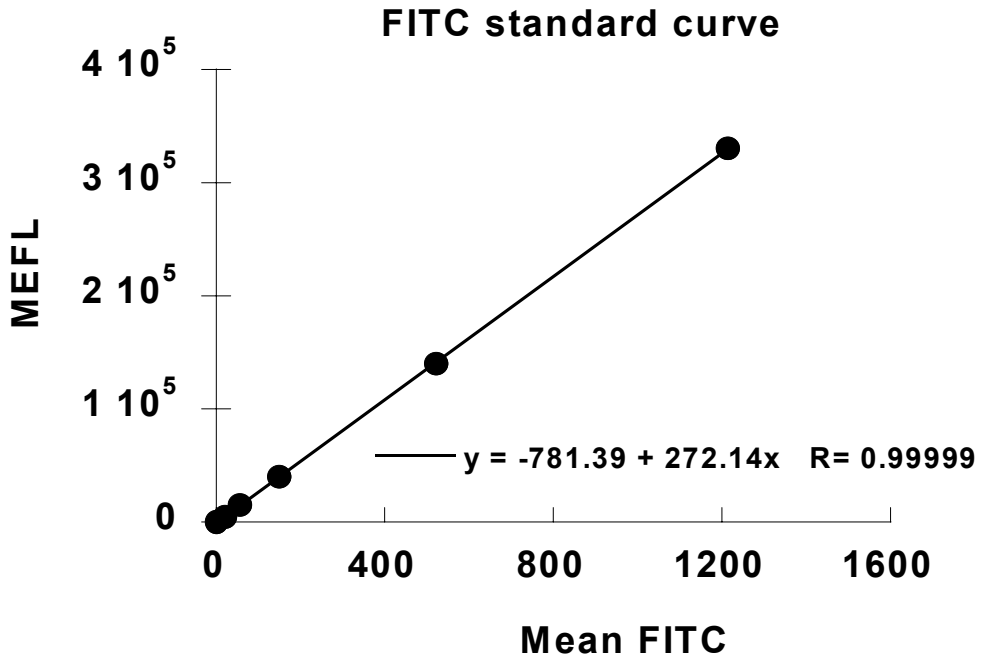
See the following page for an example of flow cytometry data and standard curve



FITC	
Marker	**Mean FITC**
M1	1.23
M2	20.45
M3	56.76
M4	150.17
M5	521.86
M6	1213.52



PE	
Marker	**Mean PE**
M1	2.53
M2	99.10
M3	291.64
M4	791.48
M5	2864.38
M6	7169.17



A.4.a AGAROSE GEL ELECTROPHORESIS

Protocol from Applied Molecular Biology at the University of Maryland and Tom Cameron

***Procedure*:**

1. Prepare 100 ml of a 1% agarose solution. (Measure 1 g agarose into a glass beaker or flask and add 100 ml 1X TBE or TAE.)
2. Microwave or stir on a hot plate until agarose is dissolved and solution is clear. **NOTE: the solution may boil rapidly after microwaving, so be careful handling it.
3. Allow solution to cool to about 55˚C before pouring. (Ethidium bromide can be added at this point to a concentration of 0.5 µg/ml.)
4. Prepare clean gel tray by sealing ends with tape or other custom-made dam.
5. Place comb in gel tray about 1 inch from one end of the tray and position the comb vertically such that the teeth are about 1-2 mm above the surface of the tray.
6. Pour 50˚C gel solution into tray to a depth of about 5 mm. Allow gel to solidify about 20 minutes at room temperature. Excess agarose can be stored at room temperature and re-melted in a microwave.
7. To run, gently remove the comb, place tray in electrophoresis chamber, and cover (just until wells are submerged) with electrophoresis buffer (the same buffer used to prepare the agarose).
8. To prepare samples for electrophoresis, add 1 µl of 6x gel loading dye for every 5 µl of DNA solution. Mix well. Load 5-12 µl of DNA per well (for minigel).
9. Electrophorese at 50-150 volts until dye markers have migrated an appropriate distance, depending on the size of DNA to be visualized.
10. If the gel was not stained with ethidium during the run, stain the gel in 0.5 µg/ml ethidium bromide until the DNA has taken up the dye and is visible under short-wave UV light, if the DNA will not be used further, or with a hand-held long-wave light if the DNA is to be cloned.

Materials:

agarose solution in TBE or TAE (generally 0.7-1%)
1X TBE or TAE (same buffer as in agarose)
gel loading dye
10 mg/ml ethidium bromide

50x TAE (fill to 1L with ddH_2O)
- 242 g Tris base
- 57.1 g glacial acetic acid
- 100 ml 0.5 M EDTA

10x TBE (fill to 1L with ddH_2O)
- 108 g Tris base
- 55 g boric acid
- 40 ml 0.5 M EDTA, pH=8

6x gel loading buffer
- 0.25% Bromophenol blue
- 0.25% Xylene cyanol FF
- 33% glycerol
- 18% 50x TAE
- 49% ddH_2O

A.4.b TRANSFORMING COMPETENT *E. coli* (DH5α, BL21)

Procedure from Tom Cameron and Lawrence Stern

Make sure you have enough LB-agar plates for all of your samples, containing the antibiotic you will be using to screen (for example, ampicillin or kanamycin). You will also need roughly sterile eppendorf tubes, pipet tips, and a spreader or autoclaved spreading beads.

Thaw an aliquot of competent cells on ice.

Pre-heat a water bath to 42°C.

For each sample, add 1-100 ng plasmid to 25 μL competent cells in a roughly sterile eppendorf tube. **Whenever doing a transformation, make sure to perform at least two controls: a known plasmid with the antibiotic resistance tag you will be using (such as pLM1 for ampicillin resistance), and cells with no DNA added.

Incubate on ice 5-15 minutes.

Place tubes in the water bath at 42°C for 45-90 seconds. **Do not allow the bath to get too hot!

Return the tubes to ice for another 2 minutes.

Add ~9 x volume of sterile LB media to each tube and mix by inverting. There should be no antibiotic in the media at this stage.

Incubate the cells at 37°C for 10-30 minutes. This allows recovery from the heat shock in the water bath.

Plate the cells onto LB plates containing the antibiotic you are using to screen (for example, ampicillin or kanamycin). Use a spreader or autoclaved spreading beads. If using a spreader, sterilize it well between each sample by rinsing with 70% EtOH and flaming over a Bunsen burner. Be careful not to get any bacteria from one sample onto another plate.

Incubate plate overnight at 37°C. **You should have colonies on your positive control plasmid plate (such as pLM1), and no colonies on your "no DNA" plate. **Note: If you get too many colonies or a "lawn" on your transformation plates, use a smaller volume of the transformation mixture to spread onto your plates.

To test protein induction in BL21 or other production strain, grow several colonies from the transformed plate in 5 mL of 1x LB medium, 50 μg/mL ampicillin (or appropriate amount of kanamycin or other selective agent), 0.2-0.5% glucose until the cultures reach an OD_{600} of ~0.7. Take a "Not Induced" sample from each culture. Add IPTG to 0.75 mM final concentration and allow to grow another 1-3 hours at 37°C. Take an "Induced" sample of the same volume from each culture. Run an SDS gel of the samples against molecular weight markers to test protein induction in each culture. Freeze down the cultures that induce in 1x LB with 30% glycerol. Store at -80°C.

A.4.b TRANSFORMING COMPETENT *E. coli* (DH5α, BL21, continued)

Procedure from Tom Cameron and Lawrence Stern

Calculating Efficiency of Transformation:

for your positive control plasmid,

$$Efficiency_{max}\ (colonies/\mu g) = \frac{\#\ colonies/plate}{(\mu L\ plasmid\ added)\ \ (\mu g/\mu L\ plasmid)}$$

for your plasmid of interest,

$$Efficiency_{actual}\ (colonies/\mu g) = \frac{\#\ colonies/plate}{(\mu L\ plasmid\ added)\ \ (\mu g/\mu L\ plasmid)}$$

relative efficiency

$$Efficiency_{rel} = \frac{Actual\ transformation\ (colonies/\mu g)}{Maximum\ transformation\ (colonies/\mu g)}$$

A.4.c VARIOUS AND SUNDRY NOTES AND TIPS ON MOLECULAR BIOLOGY

Collected from Tom Cameron, Lawrence Stern, and personal experience

Cloning Genes into Plasmids:

Vector Piece:

- Grow a large amount of plasmid and purify using something like Qiagen midi prep. If the plasmid is not clean enough, the digestions will not work well, and you may have to clean the prep using a Qiagen mini prep or Qiaquick kit.
- Run test digestions to find what concentrations of enzyme completely cut the plasmid. You will want to use about a 3-fold excess of each enzyme in your actual reaction mix to try to ensure complete cleavage. It can be useful if the plasmid you are starting with has a gene in it so the doubly cleaved band can be easily identified on a gel. **NOTE: the New England Biolabs (NEB) catalogue has tons of useful information about setting up digestions.**
- Digest about 3 μg of vector for your cloning experiment. Keep your DNA concentration around 0.2 μg/μL, and do not allow the enzyme volume to be more than 10% of the final reaction volume, as the glycerol will inhibit the reaction. **NOTE: to reduce recyclization of vector, you can add a phosphatase (CIP) into the reaction.
- For reaction volumes greater than 30 μL, clean up the plasmid using a Qiaquick kit so that your sample can be loaded into a single well on an agarose gel.
- Run the digested vector on a 1% agarose gel, making sure everything is clean. Keep the voltage low (~70 volts), and run for 3-4 hours until you get clear separation. **NOTE: Do not use a bright or short wavelength UV source to look at the gel, as it will cause mutations in the fragment. Use a dim, long wavelength source, and keep the exposure time short.**
- Identify the vector band and cut it out using a brand-new razor blade.
- Purify the digested vector using the Qiaquick kit gel extraction directions. Check a small aliquot of the product on an agarose gel to estimate the concentration and confirm that it is the right size.
- Yield will be about 0.5 μg from an initial 3 μg of uncut vector.

Gene Insert:

- From PCR Product:
  - Estimate the concentration of your PCR product on an agarose gel.
  - Digest the PCR product if necessary to create appropriate sticky ends. If the digestion does not work in the PCR reaction mixture, run a Qiagen PCR clean-up kit before the digestion.
  - Gel-purify 3-10 μg using a Qiaquick kit as above.
- From another vector:
  - Digest 5-8 μg DNA, keeping concentration around 0.2 μg/μL. Use excess enzyme as described above (Vector Piece). **NOTE: This may require a large reaction volume.**
  - Use Qiaquick to concentrate the sample before loading on gel.
  - Gel Purify the reaction using a Qiaquick kit as above.
- Cloning from Primers:

- If you used CIP in your vector prep, kinase the primers each separately and heat inactivate the kinase when done.
- Mix the primers at an equimolar ratio at ≥ 10 μM in a restriction enzyme buffer (e.g. NEB buffer 1). Heat up to 90-100°C and allow to cool slowly to room temperature using the PCR machine or bring to boiling in a water bath and allow to cool. Store in the freezer.
- When ready to use, dilute to 1-100 fM (1-100 nmol/μl) and use 1 μl in ligation reaction.

Ligation Reaction:

- Use about 200 ng for a new vector, about 50 ng for an already tested/proved vector.
- Use between 1-5 fold molar excess insert (about 100 ng is good—the insert is often around 1/10 the weight of the vector).
- Set up control samples including vector without insert and insert alone along with your samples of vector with (various) inserts.
- Plan the ligation reaction in 12-20 μL usint ~1 μL T4 DNA Ligase. Leave at 16°C for 12-24 hours. **NOTE: faster ligations are possible at room temperature using fast ligation enzymes from NEB.**
- Use 2-4 μL to transform 25 μL competent DH5α cells. (See method for "Transforming Competent *E. coli*")

Picking colonies, Growing, and Sequencing:

- If you have 2-fold more colonies on your plates with vector plus insert vs. your vector alone sample, your cloning may have worked. (The higher the ratio, the better chance that you got the appropriate ligation.)
- Pick between 2-10 colonies (including two from the vector alone plate). Grow 5 mL overnight cultures of each colony in LB/selecting agent (e.g. ampicillin).
- Freeze down a small aliquot of each sample (0.5 mL culture with 0.5 mL autoclaved 30% glycerol in LB) and store at -80°C.
- Do a Qiagen Mini-prep kit to isolate plasmid.
- Digest about 1 μg of each plasmid (assume about 1 μg plasmid per mL of culture) with the cloning enzymes and check on an agarose gel. You should be able to see your insert at the right size. You may want to also do a digest that cuts in the middle of the insert.
- If you have clones that look right, Qiagen purify 5-100 μg and send out for DNA sequencing.

A.4.d SDS-PAGE GEL

Protocol from Mia Rushe

***Procedure*:**

1. Assemble the gel apparatus, being careful to avoid leakage.
2. Select the percentage of Separating Gel you would like to pour. (Lower percentage gels allow higher molecular weight proteins to separate further.)
3. Add the ingredients in the order listed—mix well between each step.
4. After the temed and APS, move quickly, since the acrylamide will try to begin polymerizing.
5. Pour about 2.5-3 mL of acrylamide mixture into the space between the gel plates.
6. Slowly layer a small amount of water on top of the acrylamide mixture using a syringe. The acrylamide polymerizes faster in anaerobic conditions.
7. Allow the Separating Gel to polymerize for about 45 minutes.
8. Remove the water layer from the top of the gel by tilting and using a paper towel. **Note: if the gel itself moves when you tilt the apparatus, allow it to polymerize for longer.
9. Mix the ingredients for the Stacking Gel (shown below) in order, mixing after each one.
10. Layer the Stacking Gel mixture on top of the Separating Gel. Fill all the way to the top.
11. Insert a comb into the stacking gel to create lanes for loading your samples.
12. Allow the Stacking Gel to polymerize for about 45 minutes.
13. Remove the comb.
14. At this point, you can either place the gel into the electrophoresis chamber covered with SDS Running Buffer, load samples in 1x SDS Loading Buffer, and run the gel, or you can store the gel at 4˚C by wrapping in several soaked paper towels and then surrounding it with Saran Wrap. The gel should keep stored this way for a week or two.
15. After running your samples, disassemble the apparatus and place the gel into Coomassie Stain (or use other stain protocol). Allow to shake for >1 hour at room temperature.
16. Remove the stain and place gel into Destain on shaker. Observe on light box.

SDS-PAGE Gel Recipes:

Separating Gel

	7.5%	10%	12.5%	15%
30% Acrylamide	1 mL (4)	1.3 mL (5.2)	1.7 mL (6.8)	2 mL (8)
ddH_2O	2 mL (8)	1.7 mL (6.8)	1.3 mL (5.2)	1 mL (4)
4x Lower Buffer	1 mL (4)	1 mL (4)	1 mL (4)	1 mL (4)
temed	10 µL (40)	10 µL (40)	10 µL (40)	10 µL (40)
10% APS	20 µL (80)	20 µL (80)	20 µL (80)	20 µL (80)

Stacking Gel

30% Acrylamide	0.3 mL (2)
ddH_2O	1.2 mL (8)
4x Upper Buffer	0.5 mL (4)
temed	5 µL (20)
10% APS	20 µL (80)

A.4.d SDS-PAGE GEL

Protocol from Mia Rushe, 2x SDS Sample Buffer from Alistair Easterfield

***Solutions*:**

4x Lower Buffer
181.7 g Tris base
4.0 g SDS
Fill to 1L with ddH_2O
Adjust pH → 8.8

4x Upper Buffer
60.6 g Tris base
4.0 g SDS
Fill to 1L with ddH_2O
Adjust pH → 6.8

10x Running Buffer
30.3 g Tris Base
10.0 g SDS
144.0 g glycine
Fill to 1L with ddH_2O
Do Not Adjust pH!

5x Laemlli Load Buffer
60 mM Tris, pH 6.8
100 mM DTT (if reducing)
1 % SDS
10 % glycerol
0.001% bromophenol blue
in ddH2O
***Freeze in 1 mL aliquots at –20˚C*

Coomassie Blue Stain
0.25% CBBR-250
15% Methanol
10% Acetic Acid
in ddH_2O
***Note: Dissolve CBBR in Methanol first, then add acid and water. Filter.*

Destain
15% Methanol
10% Acetic Acid
in ddH_2O

2x SDS Sample Buffer
6.67 g SDS
20 g Sucrose
0.3 g Tris base
1.03 g DTT
***Note: Dissolve in 80 mL ddH_2O, pH to 7.5 with HCl, then make up to 100 mL with ddH_2O*

A.4.e FOLDED DR1 ELISA

Protocol from Mia Rushe

Procedure:

1. Coat Immulon IV plate (from Dynex) with 100 μL per well α-DR monoclonal antibody LB3.1 or L243, diluted 1:1000 in plain PBS. Stock is 1.8 mg/mL in PBS, 0.02% NaN_3. Incubate plate on nutator for 2 hours at 37˚C or overnight at 4˚C.
2. Wash each well 3 times with PBST Solution.
3. Block the plate by filling each well to the top with Block Solution. Incubate at least one hour on nutator at 37˚C. The plate can be stored at this point if the Block Solution is replaced by Dilution Solution. Store plates at 4˚C, covered in packing tape and surrounded by plastic wrap.
4. Remove Block Solution or Dilution Solution in stored plates. Add 50 μL fresh Dilution Solution to each well.
5. Make up standards of folded DR1 in dilution solution. Make high concentration of 2 ng/μL as the high concentration, and dilute 1:2 down to 0.002 ng/μL. Add 50 μL of standard per well, giving 100-0.1 ng/well—make sure to do duplicate measurements of the standards. Also, include a blank measurement.
6. Add samples and diluted samples into wells. A good way to test most refolding mixes is to add 50 μL, 5 μL, and 2.5 μL of sample to give the dilutions, making up the difference to 50 μL with dilution solution (0 μL, 45 μL, and 47.5 μL).
7. Incubate plate on nutator for 1 hour at 37˚C, 2 hours at room temperature, or overnight at 4˚C.
8. Carefully wash each plate 3 times with PBST, avoiding spillover from neighboring wells.
9. Add 100 μL/well of rabbit CHAMP polyclonal α-DR1 antibody diluted 1:25,000 (or 1:500 of a 1:50 stock solution) in Dilution Solution. Incubate plate on nutator for 1 hour at 37˚C, 2 hours at room temperature, or overnight at 4˚C.
10. Wash wells 3 times with PBST. ** Follow Alternate Procedure after this point for Europium Staining (following page)
11. Add 100 μL goat α-rabbit IgG peroxidase (HRP) conjugate (from Roche) diluted 1:4000 in dilution solution. No azide should be present at this step, as it inhibits the enzyme. Incubate on nutator 0.5-2 hours at room temperature or 37˚C. **Background increases if this step is allowed to sit overnight at 4˚C.
12. Wash wells 3 times with PBST.
13. Add 200 μL ABTS Solution to each well.
14. Read absorbance in ELISA plate reader at 405 nm.
15. To stop plate from developing, add 50 μL Stopping Solution to each well.
16. Try several absorbance readings to catch the solutions at the most informative stage of developing—before saturation.
17. Use a 4-parameter curve fit to the standards to determine the MHC class II concentration of the samples.

A.4.e FOLDED DR1 ELISA—EUROPIUM PROCEDURE

Protocol from Mia Rushe

Follow steps 1-10 on the previous page—FOLDED DR1 ELISA

11. Add 100 μL/well of 1:1000 diluted goat α-rabbit Europium conjugate (from Perkin Elmer Life Sciences, AD0105). Incubate on the nutator for 1 hour at 37˚C or 2 hours at room temperature. Don't leave this step overnight, as background will increase.
12. Wash 5x with PBST.
13. Add 100 μL/well of DELFIA Assay Buffer (from Perkin Elmer Life Sciences, 1244-111). Incubate on nutator for 30 minutes at 37˚C. Meanwhile, thaw DELFIA Enhancement Solution (from Perkin Elmer Life Sciences, 1244-105) and allow to reach room temperature.
14. Wash 7x with PBST.
15. Add 200 μL/well of DELFIA Enhancement Solution and allow to sit at room temperature for 15-20 minutes.
16. Read on Victor 1420 Multilabel Counter using standard Europium protocol.

A.4.e FOLDED DR1 ELISA

Supplies and Solutions--Protocol from Mia Rushe

PBST:
1x PBS
0.05% Triton X100
filter and store at 4˚C

Dilution Solution:
1x PBS
0.3% BSA
0.1% Triton X-100
filter and store at 4˚C

Block Solution:
1x PBS
3% BSA
0.02% NaN_3
filter and store at 4˚C

ABTS Solution:
5 mL 10x ABTS Solution
(From Roche—stored at –20˚C, 5mL aliquots)
45 mL ddH_2O
1 ABTS tablet (Roche)—allow to dissolve completely
**unused portion may be stored in dark at 4˚C, ~ 2 weeks

Stopping Solution:
20% SDS
50% DMF
in ddH_2O

A.4.e FOLDED DR1 ELISA

Data Treatment--Protocol from Mia Rushe

Equations to fit ELISA standards:

$$y = \frac{m1 - m2}{1 + (\frac{x}{m3})^{m4}} + m2$$

where m1-m4 are constants that will be fit to the data. When inputting this fit into Kaleidograph, the following format should be used, including guesses for each constant, substituting "m0" for "x", and adding partial derivatives to the fitting algorithm.

In the "General Curve Fit" window, type:

((m1-m2)/(1+(m0/m3)^m4))+m2; m1=0.7; m2=0; m3=2; m4=-0.5

In the "dF/dM1" window, type:

1/(1+(m0/m3)^m4)

In the "dF/dM2" window, type:

1-(1/(1+(m0/m3)^m4))

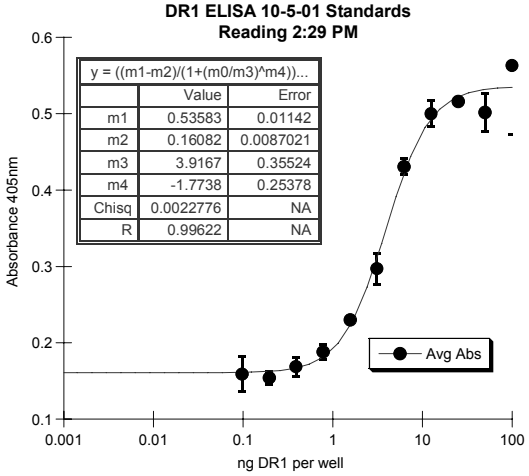
In the "dF/dM3" window, type:

(m1-m2)*(1+(m0/m3)^m4)^(-2)*(m4*(m0/m3)^(m4-1))*m0/m3^ 2

In the "dF/dM4" window, type:

(m2-m1)*(1+(m0/m3)^m4)^(-2)*(m0/m3)^m4*ln(m0/m3)

Sample ELISA standards curve fit:



A.4.f WESTERN BLOT—ALKALINE PHOSPHATASE DETECTION

Procedure from L. Stern

**Note: This procedure will detect human DR1 proteins. If using another protein, the antibodies used will vary, but the procedure should still work.

Run an SDS gel using pre-stained molecular weight markers with two copies of each lane (including the markers). The duplicates should be arranged so you can cut the gel in half and get identical lanes on each half.

After running the gel, cut off part of the loading gel so that only nubs from the comb region remain.

Wet a gel-sized rectangle of transfer membrane with 70% EtOH.

Soak the membrane and 6 gel-sized rectangles of filter paper in transfer buffer.

Place the transfer membrane flat on the gel. Remove any bubbles gently.

Sandwich the transfer membrane and the gel between the filter paper rectangles soaked in Transfer Buffer—3 sheets on each side. Remember which side has the transfer membrane!

Place the sandwich flat on the blotter with the membrane on the cathode (+) side, and the gel on the anode (-) side. Once again, gently remove any bubbles.

Close the blotter and use screws to create even, light pressure between the plates. Do not over tighten screws.

Run the blotter at constant amperage: 400mamps for about ½ hour, or until most of the protein is transferred from the gel to the membrane (watch the pre-stained MW markers). When it is done, turn off the power source and open the blotter.

Remove the gel and the membrane from the blotter. Cut the transfer membrane into the 2 identical halves.

Place ½ of the membrane into Coomassie stain in a clean petri dish.

Place the other ½ of the membrane into Block Solution in a clean petri dish. Leave both on a shaker table at room temperature for 1 hour to overnight.

Place the membrane from the Coomassie into 50% methanol or isopropanol and 50% normal gel de-stain. Leave this membrane on the shaker table at room temperature, changing the de-stain mixture periodically, until it has de-stained properly. **This may take a long time!

Rinse the membrane in the Block Solution with TBST.

Add a 1:25,000 dilution of Champ in TBST to the petri dish and membrane. Leave on the shaker table for 1 hour at room temperature.

Wash WELL with TBST—4 times, 5 minutes each on the shaker table.

Add a 1:1000 dilution of anti-rabbit polyclonal antibody linked to alkaline phosphatase in TBST to the petri dish and membrane. Leave on the shaker table at room temperature for ½ hour.

Wash WELL with TBST—4 times, 5 minutes each on the shaker table.

Add 10 mL Alkaline Phosphatase Buffer with 70 μL NBT and 70 μL BCIP. The color should begin to develop on the membrane almost immediately. **Do not overdevelop!

Stop the reaction by rinsing with ddH_2O. Enjoy!

A.4.f WESTERN BLOT—ALKALINE PHOSPHATASE DETECTION

Procedure from L. Stern

Solutions/Materials:

70% EtOH	SDS gel and equipment	Filter paper
Coomassie Stain	Clean petri dishes	Methanol/Isopropanol
De-stain	Blotter/power supply	Shaker table
NBT stock	BCIP stock	

Transfer Buffer:

50 mM CAPS, pH 11.0
20% MeOH

Transfer Membrane:

Millipore Immobilon-P Transfer membranes, IPUH 304 FO

TBST:

50 mM Tris, pH 7.5
150 mM NaCl
0.1% Tween-20

Block Solution:

3% BSA
0.02% NaN_3
in 1 x PBS

Alkaline Phosphatase Buffer:

100 mM Tris, pH 9.0-9.5
100 mM NaCl
5 mM $MgCl_2$

Antibodies for HLA-DR1 detection:

CHAMP – rabbit anti-HLA-DR1 polyclonal antibody serum. Use at 1:25,000 dilution

Anti-Rabbit Polyclonal linked to Alkaline Phosphatase enzyme

A.4.g WESTERN BLOT—ECL DETECTION

Procedure from Alistair Easterfield

1. Run an SDS gel using pre-stained molecular weight markers.
2. After running the gel, cut off part of the loading gel so that only nubs from the comb region remain.
3. Wet a gel-sized rectangle of transfer membrane with 70% EtOH.
4. Soak the membrane and 6 gel-sized rectangles of filter paper in transfer buffer.
5. Place the transfer membrane flat on the gel. Remove any bubbles gently.
6. Sandwich the transfer membrane and the gel between the filter paper rectangles soaked in Transfer Buffer—3 sheets on each side. Remember which side has the transfer membrane!
7. Place the sandwich flat on the blotter with the membrane on the cathode (+) side (up), and the gel on the anode (-) side (down). Once again, gently remove any bubbles.
8. Close the blotter and use screws to create even, light pressure between the plates. Do not over tighten screws.
9. Run the blotter at constant amperage: 400mamps for about 25-30 minutes, or until most of the protein is transferred from the gel to the membrane (watch the pre-stained MW markers). When it is done, turn off the power source and open the blotter.
10. Remove the gel and the membrane from the blotter.
11. Place the membrane into freshly-made Block Solution (5% dry milk, 0.1% Tween-20 in 1x PBS) in a clean petri dish. Leave on a shaker table at room temperature for 1 hour to overnight.
12. Rinse the membrane in the Block Solution with TBST.
13. Add your primary antibody at an appropriate dilution in dilution solution. Leave on the shaker table at room temperature for 2 hours to overnight.
14. Wash WELL with TBST—4 times, 5 minutes each on the shaker table.
15. Add your secondary antibody at an appropriate dilution in dilution solution. Leave on the shaker table at room temperature for ½ hour.
16. Wash WELL with TBST—6 times, 5 minutes each on the shaker table.
17. Add freshly-prepared ECL detection solution (4-10 mL, 1:1 mixture of supplied reagents). Incubate for 1-3 minutes in this solution, and then drain excess, tapping corner on a paper towel.
18. Sandwich the membrane between 2 sheets of transparency film, cut to size. Remove any bubbles carefully.

PERFORM FOLLOWING IN DARK ROOM WITH SAFETY RED LIGHT ON ONLY!!

19. Open film box and remove one sheet. Mark the sheet so you can tell the orientation in which you use it (i.e. clip one corner). DO NOT TRY TO DEVELOP THE CARDBOARD! Make sure the package with the film is closed again when you have removed your sheet.
20. Lay the film and the membrane in an ECL development cartridge in defined spots for pre-set periods of time, i.e. 10 seconds, 1 minute, 2 minutes, 5 minutes, 20 minutes, 1 hour. Move the membrane between each exposure. DO NOT TURN THE LIGHT ON DURING THIS TIME!!

21. Replace the film in the package and close it again. You may then turn on the light, making sure to turn off the safety red light.

TAKE THE EXPOSED FILM TO ROOM S5-126. SIGN UP ON SHEET, THEN ENTER DARK ROOM.

22. Take the film in the closed box to the development room. TURN ON THE SAFETY RED LIGHT AND TURN OFF THE WHITE LIGHT!!
23. Remove your film and place it into the developer with the long side of the film along the left side of the tray. Push it all the way forward and the rollers should pull it in. If not, try turning the machine off and on several times.
24. Once the film is completely within the developer, a beep will sound, indicating you can place your next film in OR turn on the white light.
25. Before leaving the dark room, turn on the white light and turn off the red light.
26. Your developed films will appear right outside the dark room on the developer tray.

A.4.g WESTERN BLOT—ECL DETECTION

Procedure from Alistair Easterfield

Solutions/Materials:

70% EtOH	SDS gel and equipment	Filter paper
Immobilon Membrane	Clean petri dishes	Methanol/Isopropanol
Antibodies	Blotter/power supply	Shaker table
Dilution Solution	TBST	Block Solution
ECL Reagents	BioMax XAR film	Light-blocking cartridge
Transparency film		

Transfer Buffer:

50 mM CAPS, pH 11.0
20% MeOH

Transfer Membrane:

Millipore Immobilon-P Transfer membranes, IPUH 304 FO

TBST:

50 mM Tris, pH 7.5
150 mM NaCl
0.1% Tween-20

Block Solution:

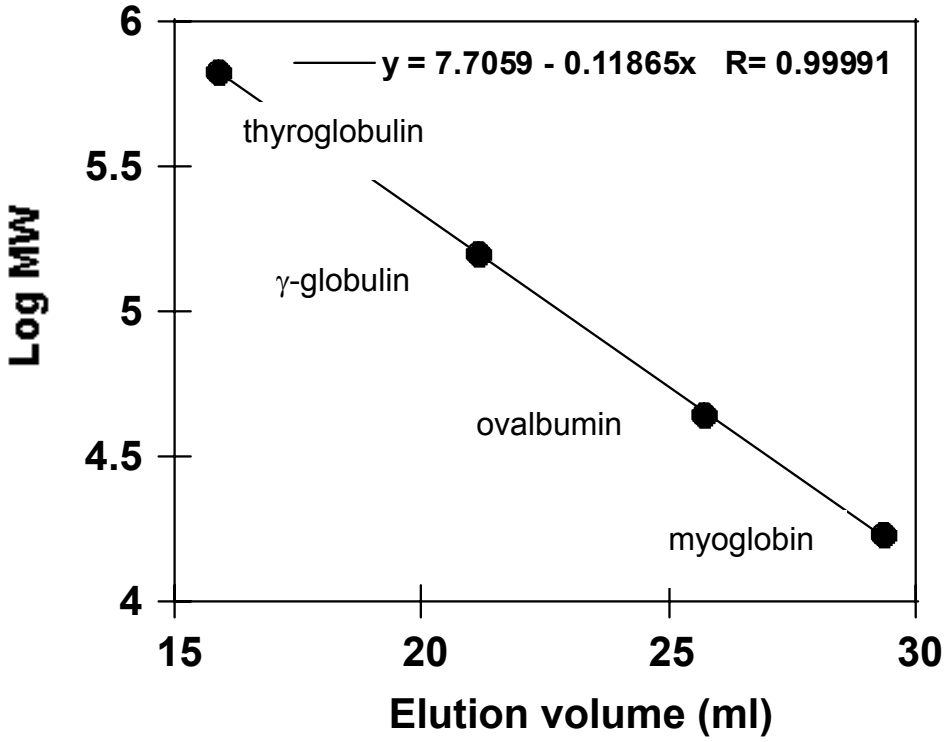
5% Non-Fat Dry Milk
0.1% Tween-20
in 1 x PBS

A.4.h DETERMINATION OF APPARENT MOLECULAR WEIGHT FROM SIZE EXCLUSION CHROMATOGRAPHY

Procedure:

1. Run molecular weight standards (Bio-rad) containing thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa) under the same conditions as you would your sample.
2. Run the samples of interest. **NOTE: if you take the column off the HPLC for any reason, or introduce air in to the lines you cannot compare the samples or the standard curve and need to start over.**
3. Determine the elution volume (mL) of the peak for both your standards and samples.
4. For the standards, make a plot of elution volume versus log MW.
5. Determine the log MW for the samples by linear regression, and convert to MW to obtain the apparent molecular weight (MW_{app}).

Sample standard curve for Bio-Rad molecular weight markers



A.4.i HYDRODYNAMIC CALCULATIONS FOR MHC OLIGOMERS

from J. A. Zarutskie
Calculations may be found in:
Zarutskie et. al. (1999) A conformational change in the human major histocompatibility complex protein HLA-DR1 induced by peptide binding. *Biochemistry* 38*:5878-87*

Calculations:
The partial specific volume of DR1-Ha was calculated as the weighted average of the partial specific volumes of the amino acids composing DR1-Ha.

The hydrated volume V_H of DR1-Ha was calculated using the equation:

$$V_H = \frac{MW_{app}(V_{ps} + (hyd_{protein} * \rho_{water}))}{N}$$

where MW_{app} is the apparent molecular weight of the protein, V_{ps} is the partial specific volume (0.736 cm^3/g), $hyd_{protein}$ is the estimated extent of hydration of the protein (0.35 g of water/g of protein), ρ_{water} is the density of water at 20 °C (0.998 g/cm^3), and N is Avogadro's number (6.02 x 10^{23}). **NOTE: There is an error in this equation in the reference cited above (Zarutskie et. al., 1999).**

The Stokes radius, R_s, was calculated using the hydrated volume, V_H:

$$R_s = \left(\frac{3V_H}{4\pi}\right)^{\frac{1}{3}}\left(\frac{f}{f_o}\right)$$

where f/f_o is the frictional coefficient, a measure of how much the protein's shape deviates from a sphere. From the DR1-Ha crystal structure, the monomer can be approximated as a prolate ellipsoid with a 76 Å major axis and a 38 Å minor axis, for an axial ratio of p ~2. The frictional efficient was calculated as follows:

$$\frac{f}{f_o} = \frac{\left(p^2 - 1\right)^{\frac{1}{2}}}{p^{\frac{1}{3}}\left(p + \left(p^2 - 1\right)^{\frac{1}{2}}\right)}$$

Using the calculated frictional coefficient (1.044), the Stokes radius of the DR1-Ha monomer in the crystal structure was calculated to be 27.9 Å.

For the crystallographic DR1-Ha dimer (assuming a spherical model), Jennifer Zarutskie calculated an axial rato if 1.04, a frictional coefficient of 1.00, and a R_s of 34.2 Å.

A.4.j FREEZING CELLS (PBMC's, T cells, B cells)

Procedure from T. Cameron and J. Ogrodnick-Cochran

Prepare suspension media and freezing media as specified below and cool to 4°C.

Spin cells down at 2000 RPM for 10 minutes and aspirate supernatant.

Suspend cells to 1 x 10^{-7} cells/ml in cold suspension media.

Aliquot into cryovials kept on ice (~0.5 ml/vial).

Add an equal volume dropwise (~0.5 ml/vial) cold freezing media into each vial and mix.

Place in Styrofoam and keep in –70°C freezer overnight.

Move into liquid nitrogen cell dewar within 24 hours.

Suspension Media:	**Freezing Media:**
20-50% Serum (human for PBMC's) in RPMI Media	20-50% Serum (human for PBMC's) 14-20% Dimethyl Sulfoxide (DMSO) in RPMI Media

A.4.k FREEZING BACTERIA (*E. coli*)

Procedure from T. Kiziltepe

Prepare freezing media as specified below.

Grow cells in liquid culture as usual to OD_{600} ~0.7-1.0.

Add 0.5 ml liquid culture to cryovials and mix with 0.5 ml freezing media.

Keep in –70°C freezer.

Freezing Media:
30% Glycerol
in 1x LB Media
AUTOCLAVE and cool completely before use

A.4.l MAKING UREA SOLUTIONS

Protocol from Mia Rushe

***Procedure*:**

1. Add concentrated buffer stock solution, any additives (such as EDTA or NaCl), and dry urea (FW = 60.06). Add ddH_2O to an appropriate volume to make 8 M Urea.
2. Stir slowly—you can warm in a bucket of water, but do not warm too quickly or too much.
3. After urea is dissolved, add Sigma mixed bed resin TMD-8, ½ teaspoon at a time until the beads stop turning yellow in the solution (i.e. there are a lot of blue beads).
4. Stir slowly another 10 minutes, and then let the beads settle.
5. Filter using a 0.2 μm membrane.
6. Store at –20˚C if you are not going to use immediately.

A.4.m STOCK SOLUTIONS AND BUFFERS

Keep a supply filtered to use in making other solutions
Add ddH_2O to 1 L for recipes unless otherwise indicated

1 M Tris, pH 8.0
40.46 g Tris Base
104.95 g Tris-HCl

1 M Tris, pH 8.5
74.26 g Tris Base
60.99 g Tris-HCl

1 M Tris, pH 9.0
101.03 g Tris Base
18.28 g Tris-HCl

10 x PBS
80 g NaCl
2 g KCl
11.5 g Na_2HPO_4
2 g KH_2PO_4

20 % NaN_3
200 g Sodium Azide

0.5 M EDTA
168.1 g EDTA
NaOH pellets (~ 20)
to pH 8.0-8.5

5 M NaCl
292.2 g NaCl

1 N NaOH
40 g NaOH pellets
(very hygroscopic)

10 % SDS
100 g SDS

4 M $MgCl_2$
381.2 g $MgCl_2$

20 % glucose (sterile)
200 g glucose

LB broth (sterile)
10 g tryptone digest
5 g yeast extract
10 g NaCl

Ethidium Bromide
1 g ethidium bromide
** in 100 mL ddH_2O

NBT—freeze –20˚C
0.5 g nitro blue
tetrazolium chloride
** in 10 mL 70% DMF

BCIP—freeze –20˚C
0.5 g 5-bromo-4-
chloro-3-indolyl
phosphate disodium
** in 10 mL DMF

5 mM DTT
0.77 g DTT
(FW = 154.04)

8 M Urea—freeze, -20˚C
480.48 g urea

Gene Construct	HLA-DR1 AS (α chain, short) and AL (α chain, long)
Plasmid	Full length in pHN1 (T7 promoter) called pDRA10 Short version in pLM1 (T7 promoter) Amp resistance gene for plasmid production in *E. coli*
Made by	Mia Rushe in Larry Stern's lab
Sites used for cloning	EcoRI and HindIII
Insert size	~580bp AS, ~600bp AL
For production in	*E. coli* BL21 Induce with IPTG
Gene Sequence	gaattcaggaggaatttaaaatgATCAAAGAAGAACATGTGATCATCCAGGCCGAGTTC TATCTGAATCCTGACCAATCAGGCGAGTTTATGTTTGACTTTGATGGTGATGAGATTTTC CATGTGGATATGGCAAAGAAGGAGACGGTCTGGCGGCTTGAAGAATTTGGACGATTTGCC AGCTTTGAGGCTCAAGGTGCATTGGCCAACATAGCTGTGGACAAAGCCAACTTGGAAATC ATGACAAAGCGCTCCAACTATACTCCGATCACCAATGTACCTCCAGAGGTAACTGTGCTC ACGAACAGCCCTGTGGAACTGAGAGAGCCCAACGTCCTCATCTGTTTCATCGACAAGTTC ACCCCACCAGTGGTCAATGTCACGTGGCTTCGAAATGGAAAACCTGTCACCACAGGAGTG TCAGAGACAGTCTTCCTGCCCAGGGAAGACCACCTTTTCCGCAAGTTCCACTATCTCCCC TTCCTGCCCTCAACTGAGGACGTTTACGACTGCAGGGTGGAGCACTGGGGCTTGGATGAG *short:* CCTCTTCTCAAGCACTGGGAGTTTGATGCTtaaaagctt *long:* CCTCTTCTCAAGCACTGGGAGTTTGATGCTCCAAGCCCTCTCCCAGAGACTACAGAGAAC taaaagctt
Protein Sequence	*iqeefk*MIKEEHVIIQAEFYLNPDQSGEFMFDFDGDEIFHVDMAKKETVWRLEEFGRFAS FEAQGALANIAVDKANLEIMTKRSNYTPITNVPPEVTVLTNSPVELREPNVLICFIDKFT PPVVNVTWLRNGKPVTTGVSETVFLPREDHLFRKFHYLPFLPSTEDVYDCRVEHWGLDE *short:* PLLKHWEFDAEND *long:* PLLKHWEFDAPSPLPETTENEND
Protein MW	AS: 21,264; AL: 22,330 Da
Protein pI	AS: 4.75; AL: 4.67
Protein extinction coefficient	28,000 at 280nm (unfolded)

Gene Construct	HLA-DR1 ACS (α chain, short) and ACL (α chain, long) Uniquely reactive cysteine at C-terminus of protein chain
Plasmid	pLM1 (T7 promoter) Amp resistance gene for plasmid production in *E. coli*
Made by	Jody Lubetsky in Larry Stern's Lab
Sites used for cloning	EcoRI and HindIII
Insert size	~580bp ACS, ~600bp ACL
For production in	*E. coli* BL21 Induce with IPTG
Gene Sequence	xgaattcaggaggaatttaaaatgATCAAAGAAGAACATGTGATCATCCAGGCCGAGTTC TATCTGAATCCTGACCAATCAGGCGAGTTTATGTTTGACTTTGATGGTGATGAGATTTTC CATGTGGATATGGCAAAGAAGGAGACGGTCTGGCGGCTTGAAGAATTTGGACGATTTGCC AGCTTTGAGGCTCAAGGTGCATTGGCCAACATAGCTGTGGACAAAGCCAACTTGGAAATC ATGACAAAGCGCTCCAACTATACTCCGATCACCAATGTACCTCCAGAGGTAACTGTGCTC ACGAACAGCCCTGTGGAACTGAGAGAGCCCAACGTCCTCATCTGTTTCATCGACAAGTTC ACCCCACCAGTGGTCAATGTCACGTGGCTTCGAAATGGAAAACCTGTCACCACAGGAGTG TCAGAGACAGTCTTCCTGCCCAGGGAAGACCACCTTTTCCGCAAGTTCCACTATCTCCCC TTCCTGCCCTCAACTGAGGACGTTTACGACTGCAGGGTGGAGCACTGGGGCTTGGATGAG *short:* CCTCTTCTCAAGCACTGGGAGTTTGATGCATGCTAAaagctt *long:* CCTCTTCTCAAGCACTGGGAGTTTGATGCTCCAAGCCCTCTCCCAGAGACTACAGAGAAC gcatgctaaaagctt
Protein Sequence	*iqeefk*MIKEEHVIIQAEFYLNPDQSGEFMFDFDGDEIFHVDMAKKETVWRLEEFGRFAS FEAQGALANIAVDKANLEIMTKRSNYTPITNVPPEVTVLTNSPVELREPNVLICFIDKFT PPVVNVTWLRNGKPVTTGVSETVFLPREDHLFRKFHYLPFLPSTEDVYDCRVEHWGLDE *short:* PLLKHWEFDACEND *long:* PLLKHWEFDAPSPLPETTENACEND
Protein MW	ACS: 21,367; ACL: 22,504 Da
Protein pI	ACS: 4.75; ACL: 4.67
Protein extinction coefficient	28,000 at 280nm (unfolded)

Gene Construct	HLA-DR1 AL (α chain, long) Also called plasmid #196
Plasmid	pRMHA-3 (metallothionine promoter) Amp resistance gene for plasmid production in *E. coli* no resistance for S2; co-transfect with pNeo (G418 resistance)
Made by	Elizabeth Mellins at Stanford University
Sites used for cloning	BamHI symmetrically (new EcoRI site in sequence)
Insert size	~670bp AL
For production in	S2 Drosophila cells Induce with $CuSO_4$
Gene Sequence	GGATCCTATAAATATGGCCATAAGTGGAGTCCCTGTGCTAGGATTTTTCATCATAGCTGT GCTGATGAGCGCTCAGGAATCATGGGCTATCAAAGAAGAACATGTGATCATCCAGGCCGA GTTCTATCTGAATCCTGACCAATCAGGCGAGTTTATGTTTGACTTTGATGGTGATGAGAT TTTCCATGTGGATATGGCAAAGAAGGAGACGGTCTGGCGGCTTGAAGAATTTGGACGATT TGCCAGCTTTGAGGCTCAAGGTGCATTGGCCAACATAGCTGTGGACAAAGCCAACTTGGA AATCATGACAAAGCGCTCCAACTATACTCCGATCACCAATGTACCTCCAGAGGTAACTGT GCTCACGAACAGCCCTGTGGAACTGAGAGAGCCCAACGTCCTCATCTGTTTCATCGACAA GTTCACCCCACCAGTGGTCAATGTCACGTGGCTTCGAAATGGAAAACCTGTCACCACAGG AGTGTCAGAGACAGTCTTCCTGCCCAGGGAAGACCACCTTTTCCGCAAGTTCCACTATCT CCCCTTCCTGCCCTCAACTGAGGACGTTTACGACTGCAGGGTGGAGCACTGGGGCTTGGA TGAGCCTCTTCTCAAGCAtTGGGAaTTcGATGCTCCAAGCCCTCTCCCAGAGACTACAGA GAACtaaGGATCC
Protein Sequence	*dpin*MAISGVPVLGFFIIAVLMSAQESWAIKEEHVIIQAEFYLNPDQSGEFMFDFDGDEI FHVDMAKKETVWRLEEFGRFASFEAQGALANIAVDKANLEIMTKRSNYTPITNVPPEVTV LTNSPVELREPNVLICFIDKFTPPVVNVTWLRNGKPVTTGVSETVFLPREDHLFRKFHYL PFLPSTEDVYDCRVEHWGLDEPLLKHWEFDAPSPLPETTENENDgs
Protein MW	AL: 24,819 Da
Protein pI	AL: 4.63
Protein extinction coefficient	33,690 at 280nm (unfolded)

Gene Construct	HLA-DR1 ACL (α chain, long) Also called X16-3
Plasmid	pRMHA-3 (metallothionine promoter) Amp resistance gene for plasmid production in *E. coli* no resistance for S2; co-transfect with pNeo (G418 resistance)
Made by	Tom Cameron in Larry Stern's Lab
Sites used for cloning	EcoRI (? may be BamHI) to BamHI (new EcoRI site in sequence)
Insert size	~680bp ACL
For production in	S2 Drosophila cells Induce with $CuSO_4$
Gene Sequence	gaattctataaatATGGCCATAAGTGGAGTCCCTGTGCTAGGATTTTTCATCATAGCTGT GCTGATGAGCGCTCAGGAATCATGGGCTATCAAAGAAGAACATGTGATCATCCAGGCCGA GTTCTATCTGAATCCTGACCAATCAGGCGAGTTTATGTTTGACTTTGATGGTGATGAGAT TTTCCATGTGGATATGGCAAAGAAGGAGACGGTCTGGCGGCTTGAAGAATTTGGACGATT TGCCAGCTTTGAGGCTCAAGGTGCATTGGCCAACATAGCTGTGGACAAAGCCAACTTGGA AATCATGACAAAGCGCTCCAACTATACTCCGATCACCAATGTACCTCCAGAGGTAACTGT GCTCACGAACAGCCCTGTGGAACTGAGAGAGCCCAACGTCCTCATCTGTTTCATCGACAA GTTCACCCCACCAGTGGTCAATGTCACGTGGCTTCGAAATGGAAAACCTGTCACCACAGG AGTGTCAGAGACAGTCTTCCTGCCCAGGGAAGACCACCTTTTCCGCAAGTTCCACTATCT CCCCTTCCTGCCCTCAACTGAGGACGTTTACGACTGCAGGGTGGAGCACTGGGGCTTGGA TGAGCCTCTTCTCAAGCAtTGGGAaTTcGATGCTCCAAGCCCTCTCCCAGAGACTACAGA GAACCAACCTGCATGCtaaGGATCC (underlined sequence is uncertain)
Protein Sequence	*dpin*MAISGVPVLGFFIIAVLMSAQESWAIKEEHVIIQAEFYLNPDQSGEFMFDFDGDEI FHVDMAKKETVWRLEEFGRFASFEAQGALANIAVDKANLEIMTKRSNYTPITNVPPEVTV LTNSPVELREPNVLICFIDKFTPPVVNVTWLRNGKPVTTGVSETVFLPREDHLFRKFHYL PFLPSTEDVYDCRVEHWGLDEPLLKHWEFDAPSPLPETTENQPACEND*gs*
Protein MW	ACL: 25,218 Da
Protein pI	ACL: 4.63
Protein extinction coefficient	33,690 at 280nm (unfolded)

Gene Construct	HLA-DR1 B1S (β chain, short) and B1L (β chain, long)
Plasmid	Full length in pHN1 (T7 promoter) called pDRB10 Short version in pLM1 (T7 promoter) Amp resistance gene for plasmid production in *E. coli*
Made by	Mia Rushe in Larry Stern's Lab
Sites used for cloning	EcoRI and HindIII
Insert size	~600bp B1S, ~620bp B1L
For production in	*E. coli* BL21 Induce with IPTG
Gene Sequence	xgaattcaggaggaatttaaaatgGGGGACACCCGACCACGTTTCTTGTGGCAGCTTAAG TTTGAATGTCATTTCTTCAATGGGACGGAGCGGGTGCGGTTGCTGGAAAGATGCATCTAT AACCAAGAGGAGTCCGTGCGCTTCGACAGCGACGTGGGGGAGTACCGGGCGGTGACGGAG CTGGGGCGGCCTGATGCCGAGTACTGGAACAGCCAGAAGGACCTCCTGGAGCAGAGGCGG GCCGCGGTGGACACCTACTGCAGACACAACTACGGGGTTGGTGAGAGCTTCACAGTGCAG CGGCGAGTTGAGCCTAAGGTGACTGTGTATCCTTCAAAGACCCAGCCCCTGCAGCACCAC AACCTCCTGGTCTGCTCTGTGAGTGGTTTCTATCCAGGCAGCATTGAAGTCAGGTGGTTC CGGAACGGCCAGGAAGAGAAGGCTGGGGTGGTGTCCACAGGCCTGATCCAGAATGGAGAT TGGACCTTCCAGACCCTGGTGATGCTGGAAACAGTTCCTCGGAGTGGAGAGGTTTACACC *short:* TGCCAAGTGGAGCACCCAAGTGTGACGAGCCCTCTCACAGTGGAATGGAGAGCAtaaaagctt *long:* TGCCAAGTGGAGCACCCAAGTGTGACGAGCCCTCTCACAGTGGAATGGAGAGCACGGTCT GAATCTGCACAGAGCAAGtaaaagctt
Protein Sequence	*iqeefk*MGDTRPRFLWQLKFECHFFNGTERVRLLERCIYNQEESVRFDSDVGEYRAVTELGRP DAEYWNSQKDLLEQRRAAVDTYCRHNYGVGESFTVQRRVEPKVTVYPSKTQPLQHHNLLVCSV SGFYPGSIEVRWFRNGQEEKAGVVSTGLIQNGDWTFQTLVMLETVPRSGEVYTCQVEHPSVTS *short:* PLTVEWRAEND *long:* PLTVEWRARSESAQSKEND
Protein MW	B1S: 22,185; B1L: 23,059 Da
Protein pI	B1S: 5.98; B1L: 6.23
Protein extinction coefficient	38,930 at 280nm (unfolded)

Gene Construct	HLA-DR1 BCS (β chain, short) and BCL (β chain, long) Uniquely reactive cysteine at C-terminus of protein chain
Plasmid	Full length in pHN1 (T7 promoter) called pDRB10 Short version in pLM1 (T7 promoter) Amp resistance gene for plasmid production in *E. coli*
Made by	Jody Lubetsky in Larry Stern's Lab
Sites used for cloning	EcoRI and HindIII
Insert size	~600bp BCS, ~625bp BCL
For production in	*E. coli* BL21 Induce with IPTG
Gene Sequence	xgaattcaggaggaatttaaaatgGGGGACACCCGACCACGTTTCTTGTGGCAGCTTAAG TTTGAATGTCATTTCTTCAATGGGACGGAGCGGGTGCGGTTGCTGGAAAGATGCATCTAT AACCAAGAGGAGTCCGTGCGCTTCGACAGCGACGTGGGGGAGTACCGGGCGGTGACGGAG CTGGGGCGGCCTGATGCCGAGTACTGGAACAGCCAGAAGGACCTCCTGGAGCAGAGGCGG GCCGCGGTGGACACCTACTGCAGACACAACTACGGGGTTGGTGAGAGCTTCACAGTGCAG CGGCGAGTTGAGCCTAAGGTGACTGTGTATCCTTCAAAGACCCAGCCCCTGCAGCACCAC AACCTCCTGGTCTGCTCTGTGAGTGGTTTCTATCCAGGCAGCATTGAAGTCAGGTGGTTC CGGAACGGCCAGGAAGAGAAGGCTGGGGTGGTGTCCACAGGCCTGATCCAGAATGGAGAT TGGACCTTCCAGACCCTGGTGATGCTGGAAACAGTTCCTCGGAGTGGAGAGGTTTACACC *short:* TGCCAAGTGGAGCACCCAAGTGTGACGAGCCCTCTCACAGTGGAATGGAGAGCATGCTAA aagctt *long:* TGCCAAGTGGAGCACCCAAGTGTGACGAGCCCTCTCACAGTGGAATGGAGAGCACGGTCT GAATCTGCACAGAGCAAGgcatgctaaaagctt
Protein Sequence	iqeefkMGDTRPRFLWQLKFECHFFNGTERVRLLERCIYNQEESVRFDSDVGEYRAVTEL GRPDAEYWNSQKDLLEQRRAAVDTYCRHNYGVGESFTVQRRVEPKVTVYPSKTQPLQHHN LLVCSVSGFYPGSIEVRWFRNGQEEKAGVVSTGLIQNGDWTFQTLVMLETVPRSGEVYTC *short:* QVEHPSVTSPLTVEWRACEND *long:* QVEHPSVTSPLTVEWRARSESAQSKACEND
Protein MW	BCS: 22,288; BCL: 23,233 Da
Protein pI	BCS: 5.98; BCL: 6.23
Protein extinction coefficient	39,050 at 280nm (unfolded)

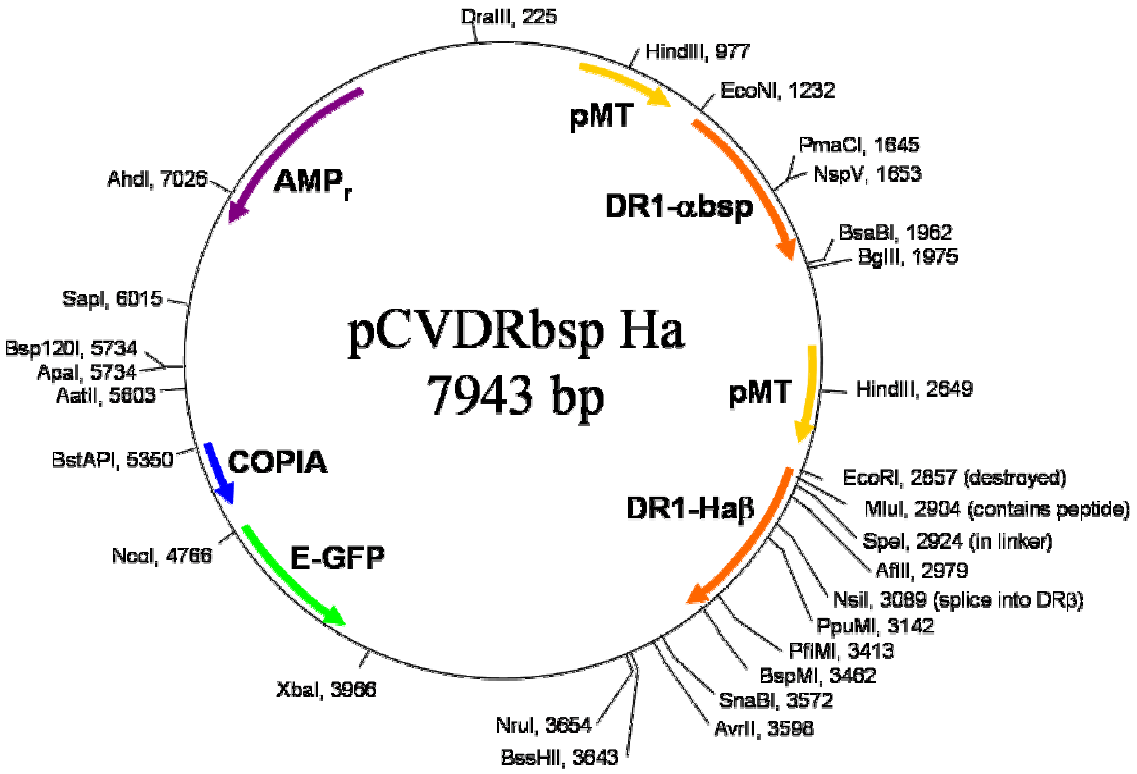
Gene Construct	HLA-DR1 Ha-B1S (Ha peptide: "KYVKQNTLKLAT" and linker at the N-terminus of the β chain, short)
Plasmid	pLM1 (T7 promoter) Amp resistance gene for plasmid production in *E. coli*
Made by	J. Stone based on Souheil Younes' construct
Sites used for cloning	XbaI and HindIII (peptide cloned into MluI, SpeI site in the linker, MaeII site after linker, spliced from Souheil Younes' construct into B1S at NsiI site)
Insert size	~710bp Ha-B1S
For production in	*E. coli* BL21 Induce with IPTG
Gene Sequence	tctagaaataattttgttgaattgctgagcaggaggaatttaaaATGGGGGACACGCGTG TCAAGTATGTCAAACAGAATACCCTAAAACTGGCGACGCGTGGAGGTGGAGGCTCACTAG TGGGAGGTGGCTCTGGTGGTGGTGGTTCGCGTCCACGTTTCTTGTGGCAGCTTAAGTTTG AATGTCATTTCTTCAATGGGACGGAGCGGGTGCGGTTGCTGGAAAGATGCATCTATAACC AAGAGGAGTCCGTGCGCTTCGACAGCGACGTGGGGGAGTACCGGGCGGTGACGGAGCTGG GGCGGCCTGATGCCGAGTACTGGAACAGCCAGAAGGACCTCCTGGAGCAGAGGCGGGCCG CGGTGGACACCTACTGCAGACACAACTACGGGGTTGGTGAGAGCTTCACAGTGCAGCGGC GAGTTGAGCCTAAGGTGACTGTGTATCCTTCAAAGACCCAGCCCCTGCAGCACCACAACC TCCTGGTCTGCTCTGTGAGTGGTTTCTATCCAGGCAGCATTGAAGTCAGGTGGTTCCGGA ACGGCCAGGAAGAGAAGGCTGGGGTGGTGTCCACAGGCCTGATCCAGAATGGAGATTGGA CCTTCCAGACCCTGGTGATGCTGGAAACAGTTCCTCGGAGTGGAGAGGTTTACACCTGCC AAGTGGAGCACCCAAGTGTGACGAGCCCTCTCACAGTGGAATGGAGAGCAtaaaagctt
Protein Sequence	*k-fc-iaeqeefk*MGDTRVKYVKQNTLKLATRGGGGSLVGGGSGGGGSRPRFLWQLKFEC HFFNGTERVRLLERCIYNQEESVRFDSDVGEYRAVTELGRPDAEYWNSQKDLLEQRRAAV DTYCRHNYGVGESFTVQRRVEPKVTVYPSKTQPLQHHNLLVCSVSGFYPGSIEVRWFRNG QEEKAGVVSTGLIQNGDWTFQTLVMLETVPRSGEVYTCQVEHPSVTSPLTVEWRAEND*kl*
Protein MW	Ha-B1S: 25,086 Da
Protein pI	Ha-B1S: 8.26 ** very high—no refolding at normal DR1 conditions
Protein extinction coefficient	40,210 at 280nm (unfolded)
Notes	No refolding seen under normal refolding conditions for DR1-pep from *E.coli*. Perhaps pH must be raised due to very high pI

Gene Construct	HLA-DR1 B1L (β chain, long) Also called plasmid #197
Plasmid	pRMHA-3 (metallothionine promoter) Amp resistance gene for plasmid production in *E. coli* no resistance for S2; co-transfect with pNeo (G418 resistance)
Made by	Elizabeth Mellins at Stanford University
Sites used for cloning	BamHI symmetrically
Insert size	~700bp BCL
For production in	S2 Drosophila cells Induce with $CuSO_4$
Gene Sequence	GGATCCTATAAATATGGTGTGTCTGAAGCTCCCTGGAGGCTCCTGCATGACAGCGCTGAC AGTGACACTGATGGTGCTGAGCTCCCCACTGGCTTTGGCTGGGGACACCCGACCACGTTT CTTGTGGCAGCTTAAGTTTGAATGTCATTTCTTCAATGGGACGGAGCGGGTGCGGTTGCT GGAAAGATGCATCTATAACCAAGAGGAGTCCGTGCGCTTCGACAGCGACGTGGGGGAGTA CCGGGCGGTGACGGAGCTGGGGCGGCCTGATGCCGAGTACTGGAACAGCCAGAAGGACCT CCTGGAGCAGAGGCGGGCCGCGGTGGACACCTACTGCAGACACAACTACGGGGTTGGTGA GAGCTTCACAGTGCAGCGGCGAGTTGAGCCTAAGGTGACTGTGTATCCTTCAAAGACCCA GCCCCTGCAGCACCACAACCTCCTGGTCTGCTCTGTGAGTGGTTTCTATCCAGGCAGCAT TGAAGTCAGGTGGTTCCGGAACGGCCAGGAAGAGAAGGCTGGGGTGGTGTCCACAGGCCT GATCCAGAATGGAGATTGGACCTTCCAGACCCTGGTGATGCTGGAAACAGTTCCTCGGAG TGGAGAGGTTTACACCTGCCAAGTGGAGCACCCAAGTGTGACGAGCCCTCTCACAGTGGA ATGGAGAGCACGGTCTGAATCTGCACAGAGCAAGtaaGGATCC
Protein Sequence	*dpin*MVCLKLPGGSCMTALTVTLMVLSSPLALAGDTRPRFLWQLKFECHFFNGTERVRLL ERCIYNQEESVRFDSDVGEYRAVTELGRPDAEYWNSQKDLLEQRRAAVDTYCRHNYGVGE SFTVQRRVEPKVTVYPSKTQPLQHHNLLVCSVSGFYPGSIEVRWFRNGQEEKAGVVSTGL IQNGDWTFQTLVMLETVPRSGEVYTCQVEHPSVTSPLTVEWRARSESAQSKEND*gs*
Protein MW	B1L: 25,831 Da
Protein pI	B1L: 6.53
Protein extinction coefficient	39,050 at 280nm (unfolded)

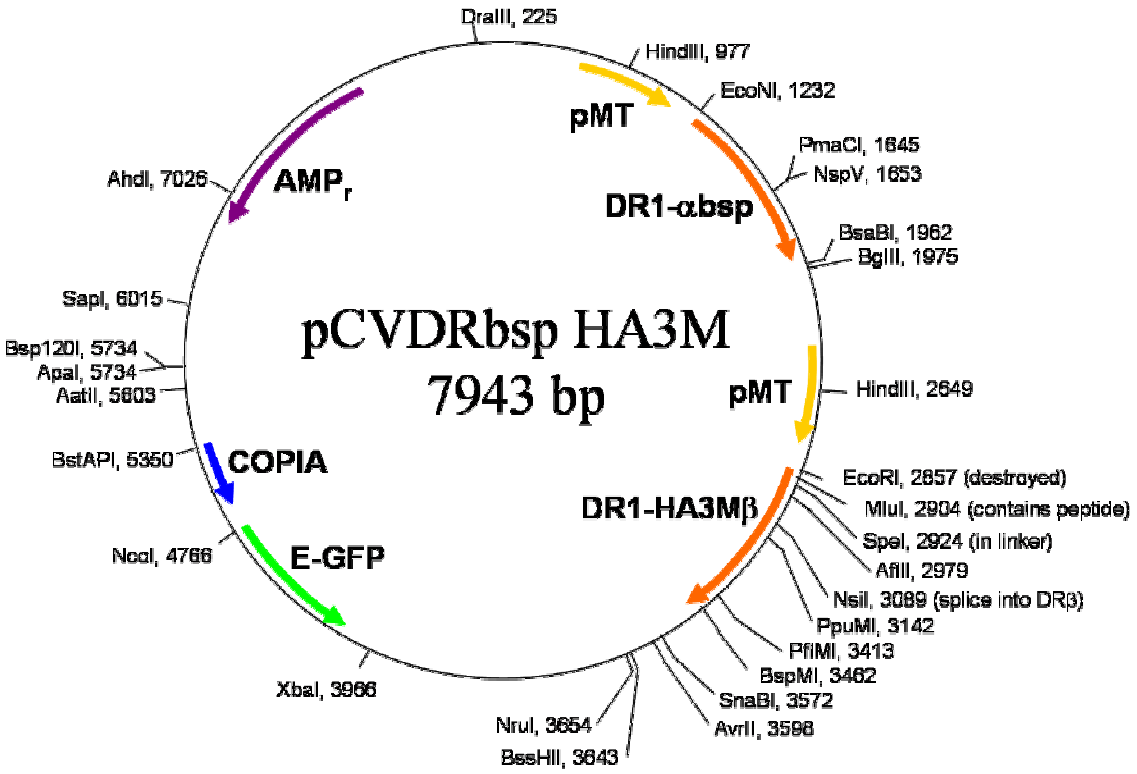
Gene Construct	HLA-DR1 HaB (Ha peptide: "KYVKQNTLKLAT" and linker at the N-terminus of the β chain, long)
Plasmid	pRMHA-3 (metallothionine promoter) Amp resistance gene for plasmid production in *E. coli* no resistance for S2; co-transfect with pNeo (G418 resistance)
Made by	J. Stone
Sites used for cloning	Cloned from upstream HindIII in promoter from pCVDR1-Ha plasmid to NsiI (ATGCAT) site in plasmid #197 (β in pRMHA-3). BamHI (GGATCC) downstream. SacI (GAGCTC) in signal sequence, MluI (ACGCGT) before and after peptide.
For production in	S2 Drosophila cells Induce with $CuSO_4$
Gene Sequence	ggtcctgtcctgttctccagcATGGTGTGTtTGAAGCTCCtTGGAGGCTCCTGCAT GACAGCGCTGACAGTGACACtGATGGTGCTGAGCTCCCCACTGGCTTTGGCTGGGG ACACGCGTGTCAAGTATGTCAAACAGAATACCCTAAAACTGGCGACGCGTGGAGGT GGAGGCTCACTAGTGGGAGGTGGCTCTGGTGGTGGTGGTTCGCGTCCACGTTTCTT GTGGCAGCTTAAGTTTGAATGTCATTTCTTCAATGGGACGGAGCGGGTGCGGTTGC TGGAAAGATGCATCTATAACCAAGAGGAGTCCGTGCGCTTCGACAGCGACGTGGGG GAGTACCGGGCGGTGACGGAGCTGGGGCGGCCTGATGCCGAGTACTGGAACAGCCA GAAGGACCTCCTGGAGCAGAGGCGGGCCGCGGTGGACACCTACTGCAGACACAACT ACGGGGTTGGTGAGAGCTTCACAGTGCAGCGGCGAGTTGAGCCTAAGGTGACTGTG TATCCTTCAAAGACCCAGCCCCTGCAGCACCACAACCTCCTGGTCTGCTCTGTGAG TGGTTTCTATCCAGGCAGCATTGAAGTCAGGTGGTTCCGGAACGGCCAGGAAGAGA AGGCTGGGGTGGTGTCCACAGGCCTGATCCAGAATGGAGATTGGACCTTCCAGACC CTGGTGATGCTGGAAACAGTTCCTCGGAGTGGAGAGGTTTACACCTGCCAAGTGGA GCACCCAAGTGTGACGAGCCCTCTCACAGTGGAATGGAGAGCACGGTCTGAATCTG CACAGAGCAAGtaaGGATCC
Protein Sequence	*signal sequence, cleaved:* *gpvlfssMVCLKLLGGSCMTALTVTLMVLSSPLALA* *final protein sequence:* GDTRVKYVKQNTLKLATRGGGGSLVGGGSGGGGSRPRFLWQLKFECHFFNGTERV RLLERCIYNQEESVRFDSDVGEYRAVTELGRPDAEYWNSQKDLLEQRRAAVDTYCRHNYG VGESFTVQRRVEPKVTVYPSKTQPLQHHNLLVCSVSGFYPGSIEVRWFRNGQEEKAGVVS TGLIQNGDWTFQTLVMLETVPRSGEVYTCQVEHPSVTSPLTVEWRARSESAQSKENDgs
Protein MW	Ha-B: 25,829 Da
Protein pI	Ha-B: 8.59
Protein extinction coefficient	40,210 at 280nm (unfolded)
Notes	Since this was cloned very far upstream, and multiple attempts at sequencing have yielded poor results, the upstream sequence (toward the promoter and HindIII site) is uncertain. It looks to be different than the pRMHA-3 construct, but I'm not sure how far it is different—could just be the polyclonal site. However, the resulting protein is able to bind to LB3.1 antibody and can activate DR1-Ha specific T cells and hybridomas.

Gene Construct	HLA-DR1 HA3MB (HA3M peptide: "KYVKQ**A**TL**A**LAA**A**" and linker at the N-terminus of the β chain, long)
Plasmid	pRMHA-3 (metallothionine promoter) Amp resistance gene for plasmid production in *E. coli* no resistance for S2; co-transfect with pNeo (G418 resistance)
Made by	J. Stone
Sites used for cloning	Cloned from upstream HindIII in promoter from pCVDR1-HA3M plasmid to NsiI (ATGCAT) site in plasmid #197 (β in pRMHA-3). BamHI (GGATCC) downstream. SacI (GAGCTC) in signal sequence, MluI (ACGCGT) before and after peptide.
For production in	S2 Drosophila cells Induce with $CuSO_4$
Gene Sequence	ggtcctgtcctgttctccagcATGGTGTGTTTGAAGCTCCTTGGAGGCTCCTGCATGACA GCGCTGACAGTGACACTGATGGTGCTGAGCTCCCCACTGGCTTTGGCTGGGGACACGCGT GTCAAGTATGTCAAACAGGCAACCCTCGCACTGGCGGCGCGTGGAGGTGGAGGCTCACTA GTGGGAGGTGGCTCTGGTGGTGGTGGTTCGCGTCCACGTTTCTTGTGGCAGCTTAAGTTT GAATGTCATTTCTTCAATGGGACGGAGCGGGTGCGGTTGCTGGAAAGATGCATCTATAAC CAAGAGGAGTCCGTGCGCTTCGACAGCGACGTGGGGGAGTACCGGGCGGTGACGGAGCTG GGGCGGCCTGATGCCGAGTACTGGAACAGCCAGAAGGACCTCCTGGAGCAGAGGCGGGCC GCGGTGGACACCTACTGCAGACACAACTACGGGGTTGGTGAGAGCTTCACAGTGCAGCGG CGAGTTGAGCCTAAGGTGACTGTGTATCCTTCAAAGACCCAGCCCCTGCAGCACCACAAC CTCCTGGTCTGCTCTGTGAGTGGTTTCTATCCAGGCAGCATTGAAGTCAGGTGGTTCCGG AACGGCCAGGAAGAGAAGGCTGGGGTGGTGTCCACAGGCCTGATCCAGAATGGAGATTGG ACCTTCCAGACCCTGGTGATGCTGGAAACAGTTCCTCGGAGTGGAGAGGTTTACACCTGC CAAGTGGAGCACCCAAGTGTGACGAGCCCTCTCACAGTGGAATGGAGAGCACGGTCTGAA TCTGCACAGAGCAAGtaaGGATCC
Protein Sequence	*signal sequence, cleaved:* *gpvlfssMVCLKLLGGSCMTALTVTLMVLSSPLALA* *final protein sequence:* GDTRVKYVKQATLALAARGGGGSLVGGGSGGGGSRPRFLWQLKFECHFFNGTERVRLLE RCIYNQEESVRFDSDVGEYRAVTELGRPDAEYWNSQKDLLEQRRAAVDTYCRHNYGVGES FTVQRRVEPKVTVYPSKTQPLQHHNLLVCSVSGFYPGSIEVRWFRNGQEEKAGVVSTGLI QNGDWTFQTLVMLETVPRSGEVYTCQVEHPSVTSPLTVEWRARSESAQSKEND*gs*
Protein MW	HA3M-B: 25,699 Da
Protein pI	HA3M-B: 8.30
Protein extinction coefficient	40,210 at 280nm (unfolded)
Notes	Since this was cloned very far upstream, and multiple attempts at sequencing have yielded poor results, the upstream sequence (toward the promoter and HindIII site) is uncertain. It looks to be different than the pRMHA-3 construct, but I'm not sure how far it is different—could just be the polyclonal site. However, the resulting protein is able to bind to LB3.1 antibody and does not activate DR1-Ha specific T cells and hybridomas, despite looking identical to the HaB protein from the similar S2 construct by SDS-PAGE and size exclusion chromatography.

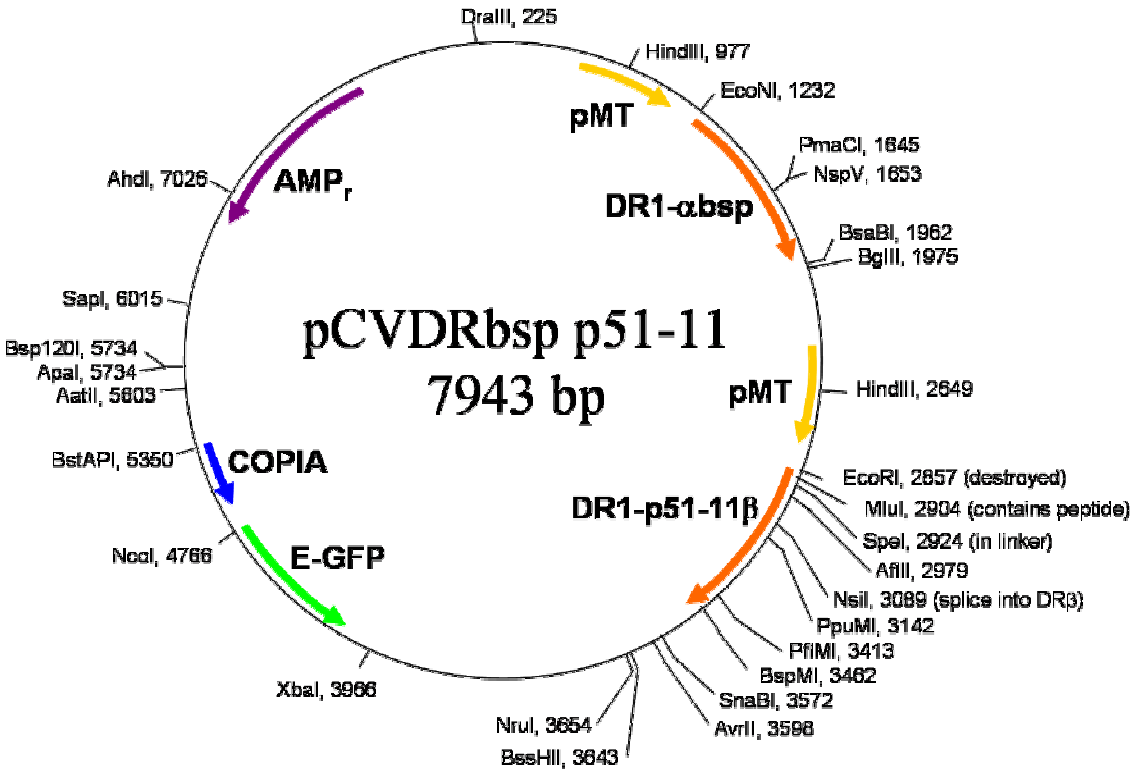
Gene Construct	HLA-DR1 αbsp and Ha-β chains ("bsp" biotinylation substrate peptide at the C-terminus of the α chain, and Ha peptide: "KYVKQNTLKLAT" and linker at the N-terminus of the β chain)
Plasmid	pCV (metallothionase promoters for each DR chain) Amp resistance gene for plasmid production in *E. coli* no resistance for S2; co-transfect with pNeo (G418 resistance) Constitutive GFP production (under COPIA promoter)
Made by	Souheil Younes
Sites used for cloning	MluI used for peptide upstream—unsure about downstream
For production in	S2 Drosophila Induce with $CuSO_4$
Gene Sequence	`Similar to DR1 AL in pRMHA-3 but with a bsp tag, and DR1 HaB in pRMHA-3`
Protein Sequence	`Similar to DR1 AL in pRMHA-3 but with a bsp tag, and DR1 HaB in pRMHA-3`
Notes	Construct from Souheil Younes, not much information and difficult to obtain good sequencing data. Lower protein production than pRMHA-3 constructs.



Gene Construct	HLA-DR1 αbsp and HA3Mβ chains ("bsp" biotinylation substrate peptide at the C-terminus of the α chain, and HA3M peptide: "KYVKQ**A**TL**A**L**A**A" and linker at the N-terminus of the β chain)
Plasmid	pCV (metallothionase promoters for each DR chain) Amp resistance gene for plasmid production in *E. coli* no resistance for S2; co-transfect with pNeo (G418 resistance) Constitutive GFP production (under COPIA promoter)
Made by	Souheil Younes
Sites used for cloning	MluI used for peptide upstream—unsure about downstream
For production in	S2 Drosophila Induce with $CuSO_4$
Gene Sequence	`Similar to DR1 AL in pRMHA-3 but with a bsp tag, and DR1 HA3MB in pRMHA-3`
Protein Sequence	`Similar to DR1 AL in pRMHA-3 but with a bsp tag, and DR1 HA3MB in pRMHA-3`
Notes	Construct from Souheil Younes, not much information and difficult to obtain good sequencing data. Lower protein production than pRMHA-3 constructs.



Gene Construct	HLA-DR1 αbsp and p51-11β chains ("bsp" biotinylation substrate peptide at the C-terminus of the α chain, and p51-11 peptide and linker at the N-terminus of the β chain—not sure of p51-11 sequence; unrelated to Ha)
Plasmid	pCV (metallothionase promoters for each DR chain) Amp resistance gene for plasmid production in *E. coli* no resistance for S2; co-transfect with pNeo (G418 resistance) Constitutive GFP production (under COPIA promoter)
Made by	Souheil Younes
Sites used for cloning	MluI used for peptide upstream—unsure about downstream
For production in	S2 Drosophila Induce with $CuSO_4$
Gene Sequence	`Similar to DR1 AL in pRMHA-3 but with a bsp tag, and DR1 p51-11 in pRMHA-3`
Protein Sequence	`Similar to DR1 AL in pRMHA-3 but with a bsp tag, and DR1 p51-11 in pRMHA-3`
Notes	Construct from Souheil Younes, not much information and difficult to obtain good sequencing data. Lower protein production than pRMHA-3 constructs.



Gene Construct	H2-K^{b}bsp (mouse heavy chain with bsp tag)
Plasmid	JA3 (T7 promoter) Kanamycin resistance gene for plasmid production in *E. coli*
Made by	NIH tetramer facility
Sites used for cloning	NdeI to HindIII
Insert size	~900bp
For production in	*E. coli* BL21 Induce with IPTG
Gene Sequence	CATATGGGACCACATTCGCTGAGGTATTTCGTCACCGCCGTGTCCCGGCCCGG CCTCGGGGAGCCCCGGTACATGGAAGTCGGCTACGTGGACGACACGGAGTTCG TGCGCTTCGACAGCGACGCGGAGAATCCGAGATATGAGCCGCGGGCGCGGTGG ATGGAGCAGGAGGGGCCCGAGTATTGGGAGCGGGAGACACAGAAAGCCAAGGG CAATGAGCAGAGTTTCCGAGTGGACCTGAGGACCCTGCTCGGCTACTACAACC AGAGCAAGGGCGGCTCTCACACTATTCAGGTGATCTCTGGCTGTGAAGTGGGG TCCGACGGGCGACTCCTCCGCGGGTACCAGCAGTACGCCTACGACGGCTGCGA TTACATCGCCCTGAACGAAGACCTGAAAACGTGGACGGCGGCGGACATGGCGG CGCTGATCACCAAACACAAGTGGGAGCAGGCTGGTGAAGCAGAGAGACTCAGG GCCTACCTGGAGGGCACGTGCGTGGAGTGGCTCCGCAGATACCTGAAGAACGG GAACGCGACGCTGCTGCGCACAGATTCCCCAAAGGCCCATGTGACCCATCACA GCAGACCTGAAGATAAAGTCACCCTGAGGTGCTGGGCCCTGGGCTTCTACCCT GCTGACATCACCCTGACCTGGCAGTTGAATGGGGAGGAGCTGATCCAGGACAT GGAGCTTGTGGAGACCAGGCCTGCAGGGGATGGAACCTTCCAGAAGTGGGCAT CTGTGGTGGTGCCTCTTGGGAAGGAGCAGTATTACACATGCCATGTGTACCAT CAGGGGCTGCCTGAGCCCCTCACCCTGAGATGGGAGCCTCCTCCATCCGGATC CCTGCATCATATTCTGGATGCACAGAAAATGGTGTGGAATCATCGTTAAAAGC TT
Protein Sequence	*h*MGPHSLRYFVTAVSRPGLGEPRYMEVGYVDDTEFVRFDSDAENPRYEPRARWM EQEGPEYWERETQKAKGNEQSFRVDLRTLLGYYNQSKGGSHTIQVISGCEVGS DGRLLRGYQQYAYDGCDYIALNEDLKTWTAADMAALITKHKWEQAGEAERLRA YLEGTCVEWLRRYLKNGNATLLRTDSPKAHVTHHSRPEDKVTLRCWALGFYPA DITLTWQLNGEELIQDMELVETRPAGDGTFQKWASVVVPLGKEQYYTCHVYHQ GLPEPLTLRWEPPPSGSLHHILDAQKMVWNHRENDkl
Protein MW	34,271.4 Da
Protein pI	5.80
Protein extinction coefficient	78,900 at 280nm (unfolded)

Gene Construct	H2-K^{b}CS (short) and K^{b}CL (long, with connecting peptide) Mouse heavy chain with uniquely reactive cysteine at the C-terminus
Plasmid	pLM1 (T7 promoter) Amp resistance gene for plasmid production in *E. coli*
Made by	Mia Rushe in Larry Stern's lab
Sites used for cloning	EcoRI to HindIII
Insert size	~860bp (short) and 880bp (long)
For production in	*E. coli* BL21 Induce with IPTG
Gene Sequence	gaattcAGGAGGAATTTAAAATGGGACCACATTCGCTGAGGTATTTCGTCACCG CCGTGTCCCGGCCCGGCCTCGGGGAGCCCCGGTACATGGAAGTCGGCTACGTGG ACGACACGGAGTTCGTGCGCTTCGACAGCGACGCGGAGAATCCGAGATATGAGC CGCGGGCGCGGTGGATGGAGCAGGAGGGGCCCGAGTATTGGGAGCGGGAGACAC AGAAAGCCAAGGGCAATGAGCAGAGTTTCCGAGTGGACCTGAGGACCCTGCTCG GCTACTACAACCAGAGCAAGGGCGGCTCTCACACTATTCAGGTGATCTCTGGCT GTGAAGTGGGGTCCGACGGGCGACTCCTCCGCGGGTACCAGCAGTACGCCTACG ACGGCcGtGATTACATCGCCCTGAACGAAGACCTGAAAACGTGGACGGCGGCGG ACATGGCGGCGCTGATCACCAAACACAAGTGGGAGCAGGCTGGTGAAGCAGAGA GACTCAGGGCCTACCTGGAGGGCACGTGCGTGGAGTGGCTCCGCAGATACCTGA AGAACGGGAACGCGACGCTGCTGCGCACAGATTCCCCAAAGGCCCATGTGACCC ATCACAGCAGACCTGAAGATAAAGTCACCCTGAGGTGCTGGGCCCTGGGCTTCT ACCCTGCTGACATCACCCTGACCTGGCAGTTGAATGGGGAGGAGCTGATCCAGG ACATGGAGCTTGTGGAGACCAGGCCTGCAGGGGATGGAACCTTCCAGAAGTGGG CATCTGTGGTGGTGCCTCTTGGGAAGGAGCAGTATTACACATGCCATGTGTACC *short:* ATCAGGGGCTGCCTGAGCCCCTCACCCTGAGATGGGAGCCTtgctaaaagctt *long:* ATCAGGGGCTGCCTGAGCCCCTCACCCTGAGATGGGAGCCTCCTCCATCCGGAT CCtgctaaaagctt
Protein Sequence	*iqeefk*MGPHSLRYFVTAVSRPGLGEPRYMEVGYVDDTEFVRFDSDAENPRYEPRARWME QEGPEYWERETQKAKGNEQSFRVDLRTLLGYYNQSKGGSHTIQVISGCEVGSDGRLLRGY QQYAYDGRDYIALNEDLKTWTAADMAALITKHKWEQAGEAERLRAYLEGTCVEWLRRYLK NGNATLLRTDSPKAHVTHHSRPEDKVTLRCWALGFYPADITLTWQLNGEELIQDMELVET *short:* RPAGDGTFQKWASVVVPLGKEQYYTCHVYHQGLPEPLTLRWEPCENDkl *long:* RPAGDGTFQKWASVVVPLGKEQYYTCHVYHQGLPEPLTLRWEPPPSGSCENDkl
Protein MW	32,122 (short) and 32,547.4 (long) Da
Protein pI	5.57 (short) and 5.57 (long)
Protein extinction coefficient	73,210 (short) and 73,210 (long) at 280nm (unfolded)

Gene Construct	H2-L^{d}bsp (mouse heavy chain with bsp tag)
Plasmid	JA3 (T7 promoter) Kanamycin resistance gene for plasmid production in *E. coli*
Made by	NIH tetramer facility
Sites used for cloning	NdeI to HindIII
Insert size	~900bp
For production in	*E. coli* BL21 Induce with IPTG
Gene Sequence	CATATGGGCCCACACTCGATGCGGTATTTCGAGACCGCCGTGTCCCGGCCCGGC CTCGGGGAGCCCCGGTACATCTCTGTCGGCTATGTGGACAACAAGGAGTTCGTG CGCTTCGACAGCGACGCGGAGAATCCGAGATATGAGCCGCAGGCGCCGTGGATG GAGCAGGAGGGGCCGGAGTATTGGGAGCGGATCACGCAGATCGCCAAGGGCCAG GAGCAGTGGTTCCGAGTGAACCTGAGGACCCTGCTCGGCTACTACAACCAGAGC GCGGGCGGCACTCACACACTCCAGTGGATGTACGGCTGTGACGTGGGGTCGGAC GGGCGCCTCCTCCGCGGGTACGAGCAGTTCGCCTACGACGGCTGCGATTACATC GCCCTGAACGAAGACCTGAAAACGTGGACGGCGGCGGACATGGCGGCGCAGATC ACCCGACGCAAGTGGGAGCAGGCTGGTGCTGCAGAGTATTACAGGGCCTACCTG GAGGGCGAGTGCGTGGAGTGGCTCCACAGATACCTGAAGAACGGGAACGCGACG CTGCTGCGCACAGATTCCCCAAAGGCACATGTGACCCATCACCCCAGATCTAAA GGTGAAGTCACCCTGAGGTGCTGGGCCCTGGGCTTCTACCCTGCTGACATCACC CTGACCTGGCAGTTGAATGGGGAGGAGCTGACCCAGGACATGGAGCTTGTGGAG ACCAGGCCTGCAGGGGATGGAACCTTCCAGAAGTGGGCATCTGTGGTGGTGCCT CTTGGGAAGGAGCAGAATTACACATGCCGTGTGTACCATGAGGGGCTGCCTGAG CCCCTCACCCTGAGATGGGAGCCTCCTCCGTCCGGATCCCTGCATCATATTCTG GATGCACAGAAAATGGTGTGGAATCATCGTTAAAAGCTT
Protein Sequence	*h*MGPHSMRYFETAVSRPGLGEPRYISVGYVDNKEFVRFDSDAENPRYEPQAPWM EQEGPEYWERITQIAKGQEQWFRVNLRTLLGYYNQSAGGTHTLQWMYGCDVGSD GRLLRGYEQFAYDGCDYIALNEDLKTWTAADMAAQITRRKWEQAGAAEYYRAYL EGECVEWLHRYLKNGNATLLRTDSPKAHVTHHPRSKGEVTLRCWALGFYPADIT LTWQLNGEELTQDMELVETRPAGDGTFQKWASVVVPLGKEQNYTCRVYHEGLPE PLTLRWEPPPSGSLHHILDAQKMVWNHRENDkl
Protein MW	34,359.4 Da
Protein pI	5.61
Protein extinction coefficient	91,560 at 280nm (unfolded)

Gene Construct	H2-L^{d}CS (short) and L^{d}CL (long, with connecting peptide) Mouse heavy chain with uniquely reactive cysteine at the C-terminus
Plasmid	pLM1 (T7 promoter) Amp resistance gene for plasmid production in *E. coli*
Made by	Mia Rushe in Larry Stern's lab
Sites used for cloning	EcoRI to HindIII
Insert size	~860bp (short) and 880bp (long)
For production in	*E. coli* BL21 Induce with IPTG
Gene Sequence	gaattcaggaggaatttaaaATGGGCCCACACTCGATGCGGTATTTCGAGACCG CCGTGTCCCGGCCCGGCCTCGGGGAGCCCCGGTACATCTCTGTCGGCTATGTGG ACAACAAGGAGTTCGTGCGCTTCGACAGCGACGCGGAGAATCCGAGATATGAGC CGCAGGCGCCGTGGATGGAGCAGGAGGGGCCGGAGTATTGGGAGCGGATCACGC AGATCGCCAAGGGCCAGGAGCAGTGGTTCCGAGTGAACCTGAGGACCCTGCTCG GCTACTACAACCAGAGCGCGGGCGGCACTCACACACTCCAGTGGATGTACGGCT GTGACGTGGGGTCGGACGGGCGCCTCCTCCGCGGGTACGAGCAGTTCGCCTACG ACGGCcGtGATTACATCGCCCTGAACGAAGACCTGAAAACGTGGACGGCGGCGG ACATGGCGGCGCAGATCACCCGACGCAAGTGGGAGCAGGCTGGTGCTGCAGAGT ATTACAGGGCCTACCTGGAGGGCGAGTGCGTGGAGTGGCTCCACAGATACCTGA AGAACGGGAACGCGACGCTGCTGCGCACAGATTCCCCAAAGGCACATGTGACCC ATCACCCCAGATCTAAAGGTGAAGTCACCCTGAGGTGCTGGGCCCTGGGCTTCT ACCCTGCTGACATCACCCTGACCTGGCAGTTGAATGGGGAGGAGCTGACCCAGG ACATGGAGCTTGTGGAGACCAGGCCTGCAGGGGATGGAACCTTCCAGAAGTGGG CATCTGTGGTGGTGCCTCTTGGGAAGGAGCAGAATTACACATGCCGTGTGTACC *short:* ATGAGGGGCTGCCTGAGCCCCTCACCCTGAGATGGGAGCCTtgctaaaagctt *long:* ATGAGGGGCTGCCTGAGCCCCTCACCCTGAGATGGGAGCCTCCTCCGTCCGGAT CCtgctaaaagctt
Protein Sequence	*iqeefk*MGPHSMRYFETAVSRPGLGEPRYISVGYVDNKEFVRFDSDAENPRYEP QAPWMEQEGPEYWERITQIAKGQEQWFRVNLRTLLGYYNQSAGGTHTLQWMYGC DVGSDGRLLRGYEQFAYDGRDYIALNEDLKTWTAADMAAQITRRKWEQAGAAEY YRAYLEGECVEWLHRYLKNGNATLLRTDSPKAHVTHHPRSKGEVTLRCWALGFY PADITLTWQLNGEELTQDMELVETRPAGDGTFQKWASVVVPLGKEQNYTCRVYH *short:* EGLPEPLTLRWEPCENDkl *long:* EGLPEPLTLRWEPPPSGSCENDkl
Protein MW	32,210 (short) and 32,635.4 (long) Da
Protein pI	5.37 (short) and 5.37 (long)
Protein extinction coefficient	85,870 (short) and 85,870 (long) at 280nm (unfolded)

Gene Construct	OVA-β_2M-K^{b}bsp, Y84A (Single-chain class I MHC, Y84A to allow peptide to lay in groove more easily, position 121 is C, bsp peptide at C-terminus)
Plasmid	pET21a (T7 promoter) Amp resistance gene for plasmid production in *E. coli*
Made by	Ted Hansen's lab at Washington University Medical School
Sites used for cloning	NdeI and HindIII
Insert size	~1326bp
For production in	*E. coli* BL21 Induce with IPTG
Gene Sequence	GANTGTGAGCGGaTAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATA TAcatatgAGTATCATTAATTTCGAAAAACTTGGAGGAGGTGCTAGCGGTGGTGGTGGTAGC GGAGGTGGAGGCAGCATCCAGAAAACCCCTCAAATTCAAGTATACTCACGCCACCCACCGGA GAATGGGAAGCCGAACATACTGAACTGCTACGTAACACAGTTCCACCCGCCTCACATTGAAA TCCAAATGCTGAAGAACGGGAAAAAAATTCCTAAAGTAGAGATGTCAGATATGTCCTTCAGC AAGGACTGGTCTTTCTATATCCTGGCTCACACTGAATTCACCCCCACTGAGACTGATACATA CGCCTGCAGAGTTAAGCATGCCAGTATGGCCGAGCCCAAGACCGTCTACTGGGATCGAGACA TGGGCGGTGGTGGTTCCGGTGGAGGCGGTTCCGGAGGTGGTGGATCCGGTGGTGGAGGTAGT GGCCCACACTCGCTGAGGTATTTCGTCACCGCCGTGTCCCGGCCCGGCCTCGGGGAGCCCCG GTACATGGAAGTCGGCTACGTGGACGACACGGAGTTCGTGCGCTTCGACAGCGACGCGGAGA ATCCGAGATATGAGCCGCGGGCGCGGTGGATGGAGCAGGAGGGGCCCGAGTATTGGGAGCGG GAGACACAGAAAGCCAAGGGCAATGAGCAGAGTTTCCGAGTGGACCTGAGGACCCTGCTCGG CGCCTACAACCAGAGCAaGGGCGGCTCTCACACTATTCAGGTGATCTCTGGCTGTGAAGTGG GGTCCGACGGGCGACTCCTCCGCGGGTACCAGCAGTACGCCTACGACGGCTGCGATTACATC GCCCTGAACGAAGACCTGAAAACGTGGACGGCGGCGGACATGGCGGCGCTGATCACCAAACA CAAGTGGGAGCAGGCTGGTGAAGCAGAGAGACTCAGGGCCTACCTGGAGGGCACGTGCGTGG AGTGGCTCCGCAGATACCTGAAGAACGGGAACGCGACGCTGCTGCGCACAGATTCCCCAAAG GCCCATGTGACCCATCACAGCAGACCTGAAGATAAAGTCACCCTGAGGTGCTGGGCCCTGGG CTTCTACCCTGCTGACATCACCCTGACCTGGCAGTTGAATGGGGAGGAGCTGATCCAGGACA TGGAGCTTGTGGAGACCAGGCCTGCAGGGGATGGAACCTTCCAGAAGTGGGCATCTGTGGTG GTGCCTCTTGGGAAGGAGCAGTATTACACATGCCATGTGTACCATCAGGGGCTGCCTGAGCC CCTCACCCTGAGATGGGAGCCTCCTCCATCCACTGGTGGCGGTCTGAACGACATCTTCGAGG CTCAGAAAATCGAATGGCACGAATAAaagcttGCGGCCGCACTCGAGCACCACCACCACCAC CACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTCC
Protein Sequence	*xvsgstopqfpsrnnfv-l-egdih*MSIINFEKLGGGASGGGGSGGGGSIQKTPQIQVYS RHPPENGKPNILNCYVTQFHPPHIEIQMLKNGKKIPKVEMSDMSFSKDWSFYILAHTEFT PTETDTYACRVKHASMAEPKTVYWDRDMGGGGSGGGGSGGGGSGGGGSGPHSLRYFVTAV SRPGLGEPRYMEVGYVDDTEFVRFDSDAENPRYEPRARWMEQEGPEYWERETQKAKGNEQ SFRVDLRTLLG**A**YNQSKGGSHTIQVISGCEVGSDGRLLRGYQQYAYDG**C**DYIALNEDLKT WTAADMAALITKHKWEQAGEAERLRAYLEGTCVEWLRRYLKNGNATLLRTDSPKAHVTHH SRPEDKVTLRCWALGFYPADITLTWQLNGEELIQDMELVETRPAGDGTFQKWASVVVPLG KEQYYTCHVYHQGLPEPLTLRWEPPPSTGGGLNDIFEAQKIEWHEEND*klaaalehhhhh* *hstopdpaankarkeaelaa*
Protein MW	48,973 Da
Protein pI	5.75
Protein extinction coefficient	95,520 at 280nm (unfolded)

Gene Construct	SIY-β_2M-K^{b}bsp, Y84A (Single-chain class I MHC, Y84A to allow peptide to lay in groove more easily, position 121 is C, bsp peptide at C-terminus)
Plasmid	pET21a (T7 promoter) Amp resistance gene for plasmid production in *E. coli*
Made by	Vinay Mahajan in Jinazhu Chen's lab at MIT
Sites used for cloning	NdeI and HindIII
Insert size	~1326bp
For production in	*E. coli* BL21 Induce with IPTG
Gene Sequence	GANTGTGAGCGGaTAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATA TAcatatgAGTATCTACCGCTACTACGGCCTGGGAGGAGGTGCTAGCGGTGGTGGTGGTAGC GGAGGTGGAGGCAGCATCCAGAAAACCCCTCAAATTCAAGTATACTCACGCCACCCACCGGA GAATGGGAAGCCGAACATACTGAACTGCTACGTAACACAGTTCCACCCGCCTCACATTGAAA TCCAAATGCTGAAGAACGGGAAAAAAATTCCTAAAGTAGAGATGTCAGATATGTCCTTCAGC AAGGACTGGTCTTTCTATATCCTGGCTCACACTGAATTCACCCCCACTGAGACTGATACATA CGCCTGCAGAGTTAAGCATGCCAGTATGGCCGAGCCCAAGACCGTCTACTGGGATCGAGACA TGGGCGGTGGTGGTTCCGGTGGAGGCGGTTCCGGAGGTGGTGGATCCGGTGGTGGAGGTAGT GGCCCACACTCGCTGAGGTATTTCGTCACCGCCGTGTCCCGGCCCGGCCTCGGGGAGCCCCG GTACATGGAAGTCGGCTACGTGGACGACACGGAGTTCGTGCGCTTCGACAGCGACGCGGAGA ATCCGAGATATGAGCCGCGGGCGCGGTGGATGGAGCAGGAGGGGCCCGAGTATTGGGAGCGG GAGACACAGAAAGCCAAGGGCAATGAGCAGAGTTTCCGAGTGGACCTGAGGACCCTGCTCGG CGCCTACAACCAGAGCAaGGGCGGCTCTCACACTATTCAGGTGATCTCTGGCTGTGAAGTGG GGTCCGACGGGCGACTCCTCCGCGGGTACCAGCAGTACGCCTACGACGGCTGCGATTACATC GCCCTGAACGAAGACCTGAAAACGTGGACGGCGGCGGACATGGCGGCGCTGATCACCAAACA CAAGTGGGAGCAGGCTGGTGAAGCAGAGAGACTCAGGGCCTACCTGGAGGGCACGTGCGTGG AGTGGCTCCGCAGATACCTGAAGAACGGGAACGCGACGCTGCTGCGCACAGATTCCCCAAAG GCCCATGTGACCCATCACAGCAGACCTGAAGATAAAGTCACCCTGAGGTGCTGGGCCCTGGG CTTCTACCCTGCTGACATCACCCTGACCTGGCAGTTGAATGGGGAGGAGCTGATCCAGGACA TGGAGCTTGTGGAGACCAGGCCTGCAGGGGATGGAACCTTCCAGAAGTGGGCATCTGTGGTG GTGCCTCTTGGGAAGGAGCAGTATTACACATGCCATGTGTACCATCAGGGGCTGCCTGAGCC CCTCACCCTGAGATGGGAGCCTCCTCCATCCACTGGTGGCGGTCTGAACGACATCTTCGAGG CTCAGAAAATCGAATGGCACGAATAAaagcttGCGGCCGCACTCGAGCACCACCACCACCAC CACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTCC
Protein Sequence	*xvsgstopqfpsrnnfv-l-egdih*MSIINFEKLGGGASGGGGSGGGGSIQKTPQIQVYS RHPPENGKPNILNCYVTQFHPPHIEIQMLKNGKKIPKVEMSDMSFSKDWSFYILAHTEFT PTETDTYACRVKHASMAEPKTVYWDRDMGGGGSGGGGSGGGGSGGGGSGPHSLRYFVTAV SRPGLGEPRYMEVGYVDDTEFVRFDSDAENPRYEPRARWMEQEGPEYWERETQKAKGNEQ SFRVDLRTLLG**A**YNQSKGGSHTIQVISGCEVGSDGRLLRGYQQYAYDG**C**DYIALNEDLKT WTAADMAALITKHKWEQAGEAERLRAYLEGTCVEWLRRYLKNGNATLLRTDSPKAHVTHH SRPEDKVTLRCWALGFYPADITLTWQLNGEELIQDMELVETRPAGDGTFQKWASVVVPLG KEQYYTCHVYHQGLPEPLTLRWEPPPSTGGGLNDIFEAQKIEWHEENDklaaalehhhhh *hstopdpaankarkeaelaa*
Protein MW	48,972 Da
Protein pI	5.75
Protein extinction coefficient	95,570 at 280nm (unfolded)

Gene Construct	OVA-β_2M-K^{b}CL, Y84A, C121R (Single-chain class I MHC, Y84A to allow peptide to lay in groove more easily, C121R to allow unique Cys at C-terminus)
Plasmid	pET21a (T7 promoter) Amp resistance gene for plasmid production in *E. coli*
Made by	J. Stone
Sites used for cloning	NdeI and HindIII
Insert size	~1281bp
For production in	*E. coli* BL21 Induce with IPTG
Gene Sequence	GANTGTGAGCGGaTAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATA TAcatatgAGTATCATTAATTTCGAAAAACTTGGAGGAGGTGCTAGCGGTGGTGGTGGTAGC GGAGGTGGAGGCAGCATCCAGAAAACCCCTCAAATTCAAGTATACTCACGCCACCCACCGGA GAATGGGAAGCCGAACATACTGAACTGCTACGTAACACAGTTCCACCCGCCTCACATTGAAA TCCAAATGCTGAAGAACGGGAAAAAAATTCCTAAAGTAGAGATGTCAGATATGTCCTTCAGC AAGGACTGGTCTTTCTATATCCTGGCTCACACTGAATTCACCCCCACTGAGACTGATACATA CGCCTGCAGAGTTAAGCATGCCAGTATGGCCGAGCCCAAGACCGTCTACTGGGATCGAGACA TGGGCGGTGGTGGTTCCGGTGGAGGCGGTTCCGGAGGTGGTGGATCCGGTGGTGGAGGTAGT GGCCCACACTCGCTGAGGTATTTCGTCACCGCCGTGTCCCGGCCCGGCCTCGGGGAGCCCCG GTACATGGAAGTCGGCTACGTGGACGACACGGAGTTCGTGCGCTTCGACAGCGACGCGGAGA ATCCGAGATATGAGCCGCGGGCGCGGTGGATGGAGCAGGAGGGGCCCGAGTATTGGGAGCGG GAGACACAGAAAGCCAAGGGCAATGAGCAGAGTTTCCGAGTGGACCTGAGGACCCTGCTCGG CGCCTACAACCAGAGCAaGGGCGGCTCTCACACTATTCAGGTGATCTCTGGCTGTGAAGTGG GGTCCGACGGGCGACTCCTCCGCGGGTACCAGCAGTACGCCTACGACGGCTGCGATTACATC GCCCTGAACGAAGACCTGAAAACGTGGACGGCGGCGGACATGGCGGCGCTGATCACCAAACA CAAGTGGGAGCAGGCTGGTGAAGCAGAGAGACTCAGGGCCTACCTGGAGGGCACGTGCGTGG AGTGGCTCCGCAGATACCTGAAGAACGGGAACGCGACGCTGCTGCGCACAGATTCCCCAAAG GCCCATGTGACCCATCACAGCAGACCTGAAGATAAAGTCACCCTGAGGTGCTGGGCCCTGGG CTTCTACCCTGCTGACATCACCCTGACCTGGCAGTTGAATGGGGAGGAGCTGATCCAGGACA TGGAGCTTGTGGAGACCAGGCCTGCAGGGGATGGAACCTTCCAGAAGTGGGCATCTGTGGTG GTGCCTCTTGGGAAGGAGCAGTATTACACATGCCATGTGTACCATCAGGGGCTGCCTGAGCC CCTCACCCTGAGATGGGAGCCTCCTCCATCCGGATCCTGCTAAaagcttGCGGCCGCACTCG AGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTG GCTGCTCC
Protein Sequence	*xvsgstopqfpsrnnfv-l-egdih*MSIINFEKLGGGASGGGGSGGGGSIQKTPQIQVYS RHPPENGKPNILNCYVTQFHPPHIEIQMLKNGKKIPKVEMSDMSFSKDWSFYILAHTEFT PTETDTYACRVKHASMAEPKTVYWDRDMGGGGSGGGGSGGGGSGGGGSGPHSLRYFVTAV SRPGLGEPRYMEVGYVDDTEFVRFDSDAENPRYEPRARWMEQEGPEYWERETQKAKGNEQ SFRVDLRTLLG**A**YNQSKGGSHTIQVISGCEVGSDGRLLRGYQQYAYDG**R**DYIALNEDLKT WTAADMAALITKHKWEQAGEAERLRAYLEGTCVEWLRRYLKNGNATLLRTDSPKAHVTHH SRPEDKVTLRCWALGFYPADITLTWQLNGEELIQDMELVETRPAGDGTFQKWASVVVPLG KEQYYTCHVYHQGLPEPLTLRWEPPPSGSCEND*klaaalehhhhhhstopdpaankarke* *aelaa*
Protein MW	47,247 Da
Protein pI	6.04
Protein extinction coefficient	89,830 at 280nm (unfolded)

Gene Construct	SIY-β_2M-K^{b}CL, Y84A, C121R (Single-chain class I MHC, Y84A to allow peptide to lay in groove more easily, C121R to allow unique Cys at C-terminus)
Plasmid	pET21a (T7 promoter) Amp resistance gene for plasmid production in *E. coli*
Made by	Vinay Mahajan in Jianzhu Chen's lab at MIT
Sites used for cloning	NdeI and HindIII
Insert size	~1281bp
For production in	*E. coli* BL21 Induce with IPTG
Gene Sequence	GANTGTGAGCGGaTAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATA TAcatatgAGTATCTACCGCTACTACGGCCTGGGAGGAGGTGCTAGCGGTGGTGGTGGTAGC GGAGGTGGAGGCAGCATCCAGAAAACCCCTCAAATTCAAGTATACTCACGCCACCCACCGGA GAATGGGAAGCCGAACATACTGAACTGCTACGTAACACAGTTCCACCCGCCTCACATTGAAA TCCAAATGCTGAAGAACGGGAAAAAAATTCCTAAAGTAGAGATGTCAGATATGTCCTTCAGC AAGGACTGGTCTTTCTATATCCTGGCTCACACTGAATTCACCCCCACTGAGACTGATACATA CGCCTGCAGAGTTAAGCATGCCAGTATGGCCGAGCCCAAGACCGTCTACTGGGATCGAGACA TGGGCGGTGGTGGTTCCGGTGGAGGCGGTTCCGGAGGTGGTGGATCCGGTGGTGGAGGTAGT GGCCCACACTCGCTGAGGTATTTCGTCACCGCCGTGTCCCGGCCCGGCCTCGGGGAGCCCCG GTACATGGAAGTCGGCTACGTGGACGACACGGAGTTCGTGCGCTTCGACAGCGACGCGGAGA ATCCGAGATATGAGCCGCGGGCGCGGTGGATGGAGCAGGAGGGGCCCGAGTATTGGGAGCGG GAGACACAGAAAGCCAAGGGCAATGAGCAGAGTTTCCGAGTGGACCTGAGGACCCTGCTCGG CGCCTACAACCAGAGCAaGGGCGGCTCTCACACTATTCAGGTGATCTCTGGCTGTGAAGTGG GGTCCGACGGGCGACTCCTCCGCGGGTACCAGCAGTACGCCTACGACGGCTGCGATTACATC GCCCTGAACGAAGACCTGAAAACGTGGACGGCGGCGGACATGGCGGCGCTGATCACCAAACA CAAGTGGGAGCAGGCTGGTGAAGCAGAGAGACTCAGGGCCTACCTGGAGGGCACGTGCGTGG AGTGGCTCCGCAGATACCTGAAGAACGGGAACGCGACGCTGCTGCGCACAGATTCCCCAAAG GCCCATGTGACCCATCACAGCAGACCTGAAGATAAAGTCACCCTGAGGTGCTGGGCCCTGGG CTTCTACCCTGCTGACATCACCCTGACCTGGCAGTTGAATGGGGAGGAGCTGATCCAGGACA TGGAGCTTGTGGAGACCAGGCCTGCAGGGGATGGAACCTTCCAGAAGTGGGCATCTGTGGTG GTGCCTCTTGGGAAGGAGCAGTATTACACATGCCATGTGTACCATCAGGGGCTGCCTGAGCC CCTCACCCTGAGATGGGAGCCTCCTCCATCCGGATCCTGCTAAaagcttGCGGCCGCACTCG AGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTG GCTGCTCC
Protein Sequence	*xvsgstopqfpsrnnfv-l-egdih*MSIYRYYGLGGGASGGGGSGGGGSIQKTPQIQVYS RHPPENGKPNILNCYVTQFHPPHIEIQMLKNGKKIPKVEMSDMSFSKDWSFYILAHTEFT PTETDTYACRVKHASMAEPKTVYWDRDMGGGGSGGGGSGGGGSGGGGSGPHSLRYFVTAV SRPGLGEPRYMEVGYVDDTEFVRFDSDAENPRYEPRARWMEQEGPEYWERETQKAKGNEQ SFRVDLRTLLG**A**YNQSKGGSHTIQVISGCEVGSDGRLLRGYQQYAYDG**R**DYIALNEDLKT WTAADMAALITKHKWEQAGEAERLRAYLEGTCVEWLRRYLKNGNATLLRTDSPKAHVTHH SRPEDKVTLRCWALGFYPADITLTWQLNGEELIQDMELVETRPAGDGTFQKWASVVVPLG KEQYYTCHVYHQGLPEPLTLRWEPPPSGSCEND*klaaalehhhhhhstopdpaankarke aelaa*
Protein MW	47,247 Da
Protein pI	6.04
Protein extinction coefficient	89,830 at 280nm (unfolded)

Gene Construct	p2Ca-β_2M-K^{b}CL, Y84A, C121R (Single-chain class I MHC, Y84A to allow peptide to lay in groove more easily, C121R to allow unique Cys at C-terminus)
Plasmid	pET21a (T7 promoter) Amp resistance gene for plasmid production in *E. coli*
Made by	Vinay Mahajan in Jianzhu Chen's lab at MIT
Sites used for cloning	NdeI and HindIII
Insert size	~1281bp
For production in	*E. coli* BL21 Induce with IPTG
Gene Sequence	GANTGTGAGCGGaTAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATA TAcatatgCTGAGCCCGTTCCCGTTCGACCTGGGAGGAGGTGCTAGCGGTGGTGGTGGTAGC GGAGGTGGAGGCAGCATCCAGAAAACCCCTCAAATTCAAGTATACTCACGCCACCCACCGGA GAATGGGAAGCCGAACATACTGAACTGCTACGTAACACAGTTCCACCCGCCTCACATTGAAA TCCAAATGCTGAAGAACGGGAAAAAAATTCCTAAAGTAGAGATGTCAGATATGTCCTTCAGC AAGGACTGGTCTTTCTATATCCTGGCTCACACTGAATTCACCCCCACTGAGACTGATACATA CGCCTGCAGAGTTAAGCATGCCAGTATGGCCGAGCCCAAGACCGTCTACTGGGATCGAGACA TGGGCGGTGGTGGTTCCGGTGGAGGCGGTTCCGGAGGTGGTGGATCCGGTGGTGGAGGTAGT GGCCCACACTCGCTGAGGTATTTCGTCACCGCCGTGTCCCGGCCCGGCCTCGGGGAGCCCCG GTACATGGAAGTCGGCTACGTGGACGACACGGAGTTCGTGCGCTTCGACAGCGACGCGGAGA ATCCGAGATATGAGCCGCGGGCGCGGTGGATGGAGCAGGAGGGGCCCGAGTATTGGGAGCGG GAGACACAGAAAGCCAAGGGCAATGAGCAGAGTTTCCGAGTGGACCTGAGGACCCTGCTCGG CGCCTACAACCAGAGCAaGGGCGGCTCTCACACTATTCAGGTGATCTCTGGCTGTGAAGTGG GGTCCGACGGGCGACTCCTCCGCGGGTACCAGCAGTACGCCTACGACGGCTGCGATTACATC GCCCTGAACGAAGACCTGAAAACGTGGACGGCGGCGGACATGGCGGCGCTGATCACCAAACA CAAGTGGGAGCAGGCTGGTGAAGCAGAGAGACTCAGGGCCTACCTGGAGGGCACGTGCGTGG AGTGGCTCCGCAGATACCTGAAGAACGGGAACGCGACGCTGCTGCGCACAGATTCCCCAAAG GCCCATGTGACCCATCACAGCAGACCTGAAGATAAAGTCACCCTGAGGTGCTGGGCCCTGGG CTTCTACCCTGCTGACATCACCCTGACCTGGCAGTTGAATGGGGAGGAGCTGATCCAGGACA TGGAGCTTGTGGAGACCAGGCCTGCAGGGGATGGAACCTTCCAGAAGTGGGCATCTGTGGTG GTGCCTCTTGGGAAGGAGCAGTATTACACATGCCATGTGTACCATCAGGGGCTGCCTGAGCC CCTCACCCTGAGATGGGAGCCTCCTCCATCCGGATCCTGCTAAaagcttGCGGCCGCACTCG AGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTG GCTGCTCC
Protein Sequence	*xvsgstopqfpsrnnfv-l-egdih*MLSPFPFDLGGGASGGGGSGGGGSIQKTPQIQVYS RHPPENGKPNILNCYVTQFHPPHIEIQMLKNGKKIPKVEMSDMSFSKDWSFYILAHTEFT PTETDTYACRVKHASMAEPKTVYWDRDMGGGGSGGGGSGGGGSGGGGSGPHSLRYFVTAV SRPGLGEPRYMEVGYVDDTEFVRFDSDAENPRYEPRARWMEQEGPEYWERETQKAKGNEQ SFRVDLRTLLG**A**YNQSKGGSHTIQVISGCEVGSDGRLLRGYQQYAYDG**R**DYIALNEDLKT WTAADMAALITKHKWEQAGEAERLRAYLEGTCVEWLRRYLKNGNATLLRTDSPKAHVTHH SRPEDKVTLRCWALGFYPADITLTWQLNGEELIQDMELVETRPAGDGTFQKWASVVVPLG KEQYYTCHVYHQGLPEPLTLRWEPPPSGSCEND*klaaalehhhhhhstopdpaankarke aelaa*
Protein MW	47,219 Da
Protein pI	5.94
Protein extinction coefficient	89,830 at 280nm (unfolded)

Gene Construct	dEV8-β_2M-K^{b}CL, Y84A, C121R (Single-chain class I MHC, Y84A to allow peptide to lay in groove more easily, C121R to allow unique Cys at C-terminus)
Plasmid	pET21a (T7 promoter) Amp resistance gene for plasmid production in *E. coli*
Made by	Vinay Mahajan in Jianzhu Chen's Lab at MIT
Sites used for cloning	NdeI and HindIII
Insert size	~1281bp
For production in	*E. coli* BL21 Induce with IPTG
Gene Sequence	GANTGTGAGCGGaTAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATA TAcatatgGAACAATACAAATTCTACAGCGTGGGAGGAGGTGCTAGCGGTGGTGGTGGTAGC GGAGGTGGAGGCAGCATCCAGAAAACCCCTCAAATTCAAGTATACTCACGCCACCCACCGGA GAATGGGAAGCCGAACATACTGAACTGCTACGTAACACAGTTCCACCCGCCTCACATTGAAA TCCAAATGCTGAAGAACGGGAAAAAAATTCCTAAAGTAGAGATGTCAGATATGTCCTTCAGC AAGGACTGGTCTTTCTATATCCTGGCTCACACTGAATTCACCCCCACTGAGACTGATACATA CGCCTGCAGAGTTAAGCATGCCAGTATGGCCGAGCCCAAGACCGTCTACTGGGATCGAGACA TGGGCGGTGGTGGTTCCGGTGGAGGCGGTTCCGGAGGTGGTGGATCCGGTGGTGGAGGTAGT GGCCCACACTCGCTGAGGTATTTCGTCACCGCCGTGTCCCGGCCCGGCCTCGGGGAGCCCCG GTACATGGAAGTCGGCTACGTGGACGACACGGAGTTCGTGCGCTTCGACAGCGACGCGGAGA ATCCGAGATATGAGCCGCGGGCGCGGTGGATGGAGCAGGAGGGGCCCGAGTATTGGGAGCGG GAGACACAGAAAGCCAAGGGCAATGAGCAGAGTTTCCGAGTGGACCTGAGGACCCTGCTCGG CGCCTACAACCAGAGCAaGGGCGGCTCTCACACTATTCAGGTGATCTCTGGCTGTGAAGTGG GGTCCGACGGGCGACTCCTCCGCGGGTACCAGCAGTACGCCTACGACGGCTGCGATTACATC GCCCTGAACGAAGACCTGAAAACGTGGACGGCGGCGGACATGGCGGCGCTGATCACCAAACA CAAGTGGGAGCAGGCTGGTGAAGCAGAGAGACTCAGGGCCTACCTGGAGGGCACGTGCGTGG AGTGGCTCCGCAGATACCTGAAGAACGGGAACGCGACGCTGCTGCGCACAGATTCCCCAAAG GCCCATGTGACCCATCACAGCAGACCTGAAGATAAAGTCACCCTGAGGTGCTGGGCCCTGGG CTTCTACCCTGCTGACATCACCCTGACCTGGCAGTTGAATGGGGAGGAGCTGATCCAGGACA TGGAGCTTGTGGAGACCAGGCCTGCAGGGGATGGAACCTTCCAGAAGTGGGCATCTGTGGTG GTGCCTCTTGGGAAGGAGCAGTATTACACATGCCATGTGTACCATCAGGGGCTGCCTGAGCC CCTCACCCTGAGATGGGAGCCTCCTCCATCCGGATCCTGCTAAaagcttGCGGCCGCACTCG AGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTG GCTGCTCC
Protein Sequence	*xvsgstopqfpsrnnfv-l-egdih*MEQYKFYSVGGGASGGGGSGGGGSIQKTPQIQVYS RHPPENGKPNILNCYVTQFHPPHIEIQMLKNGKKIPKVEMSDMSFSKDWSFYILAHTEFT PTETDTYACRVKHASMAEPKTVYWDRDMGGGGSGGGGSGGGGSGGGGSGPHSLRYFVTAV SRPGLGEPRYMEVGYVDDTEFVRFDSDAENPRYEPRARWMEQEGPEYWERETQKAKGNEQ SFRVDLRTLLG**A**YNQSKGGSHTIQVISGCEVGSDGRLLRGYQQYAYDG**R**DYIALNEDLKT WTAADMAALITKHKWEQAGEAERLRAYLEGTCVEWLRRYLKNGNATLLRTDSPKAHVTHH SRPEDKVTLRCWALGFYPADITLTWQLNGEELIQDMELVETRPAGDGTFQKWASVVVPLG KEQYYTCHVYHQGLPEPLTLRWEPPPSGSCEND*klaaalehhhhhhstopdpaankarke* *aelaa*
Protein MW	47,346 Da
Protein pI	6.04
Protein extinction coefficient	92,390 at 280nm (unfolded)

Gene Construct	E1-β_2M-K^{b}CL, Y84A, C121R (Single-chain class I MHC, Y84A to allow peptide to lay in groove more easily, C121R to allow unique Cys at C-terminus)
Plasmid	pET21a (T7 promoter) Amp resistance gene for plasmid production in *E. coli*
Made by	Vinay Mahajan in Jianzhu Chen's lab at MIT
Sites used for cloning	NdeI and HindIII
Insert size	~1281bp
For production in	*E. coli* BL21 Induce with IPTG
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Protein Sequence	*xvsgstopqfpsrnnfv-l-egdih*MEIINFEKLGGGASGGGGSGGGGSIQKTPQIQVYS RHPPENGKPNILNCYVTQFHPPHIEIQMLKNGKKIPKVEMSDMSFSKDWSFYILAHTEFT PTETDTYACRVKHASMAEPKTVYWDRDMGGGGSGGGGSGGGGSGGGGSGPHSLRYFVTAV SRPGLGEPRYMEVGYVDDTEFVRFDSDAENPRYEPRARWMEQEGPEYWERETQKAKGNEQ SFRVDLRTLLG**A**YNQSKGGSHTIQVISGCEVGSDGRLLRGYQQYAYDG**R**DYIALNEDLKT WTAADMAALITKHKWEQAGEAERLRAYLEGTCVEWLRRYLKNGNATLLRTDSPKAHVTHH SRPEDKVTLRCWALGFYPADITLTWQLNGEELIQDMELVETRPAGDGTFQKWASVVVPLG KEQYYTCHVYHQGLPEPLTLRWEPPPSGSCENDklaaalehhhhhhstopdpaankarke aelaa
Protein MW	47,289 Da
Protein pI	5.94
Protein extinction coefficient	89,830 at 280nm (unfolded)

Gene Construct	GNY-β_2M-K^{b}CL, Y84A, C121R (Single-chain class I MHC, Y84A to allow peptide to lay in groove more easily, C121R to allow unique Cys at C-terminus)
Plasmid	pET21a (T7 promoter) Amp resistance gene for plasmid production in *E. coli*
Made by	Vinay Mahajan in Jinazhu Chen's lab at MIT
Sites used for cloning	NdeI and HindIII
Insert size	~1281bp
For production in	*E. coli* BL21 Induce with IPTG
Gene Sequence	GANTGTGAGCGGaTAACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATA TAcatatgGGCAACTACAGCTTCTACGCCCTGGGAGGAGGTGCTAGCGGTGGTGGTGGTAGC GGAGGTGGAGGCAGCATCCAGAAAACCCCTCAAATTCAAGTATACTCACGCCACCCACCGGA GAATGGGAAGCCGAACATACTGAACTGCTACGTAACACAGTTCCACCCGCCTCACATTGAAA TCCAAATGCTGAAGAACGGGAAAAAAATTCCTAAAGTAGAGATGTCAGATATGTCCTTCAGC AAGGACTGGTCTTTCTATATCCTGGCTCACACTGAATTCACCCCCACTGAGACTGATACATA CGCCTGCAGAGTTAAGCATGCCAGTATGGCCGAGCCCAAGACCGTCTACTGGGATCGAGACA TGGGCGGTGGTGGTTCCGGTGGAGGCGGTTCCGGAGGTGGTGGATCCGGTGGTGGAGGTAGT GGCCCACACTCGCTGAGGTATTTCGTCACCGCCGTGTCCCGGCCCGGCCTCGGGGAGCCCCG GTACATGGAAGTCGGCTACGTGGACGACACGGAGTTCGTGCGCTTCGACAGCGACGCGGAGA ATCCGAGATATGAGCCGCGGGCGCGGTGGATGGAGCAGGAGGGGCCCGAGTATTGGGAGCGG GAGACACAGAAAGCCAAGGGCAATGAGCAGAGTTTCCGAGTGGACCTGAGGACCCTGCTCGG CGCCTACAACCAGAGCAaGGGCGGCTCTCACACTATTCAGGTGATCTCTGGCTGTGAAGTGG GGTCCGACGGGCGACTCCTCCGCGGGTACCAGCAGTACGCCTACGACGGCTGCGATTACATC GCCCTGAACGAAGACCTGAAAACGTGGACGGCGGCGGACATGGCGGCGCTGATCACCAAACA CAAGTGGGAGCAGGCTGGTGAAGCAGAGAGACTCAGGGCCTACCTGGAGGGCACGTGCGTGG AGTGGCTCCGCAGATACCTGAAGAACGGGAACGCGACGCTGCTGCGCACAGATTCCCCAAAG GCCCATGTGACCCATCACAGCAGACCTGAAGATAAAGTCACCCTGAGGTGCTGGGCCCTGGG CTTCTACCCTGCTGACATCACCCTGACCTGGCAGTTGAATGGGGAGGAGCTGATCCAGGACA TGGAGCTTGTGGAGACCAGGCCTGCAGGGGATGGAACCTTCCAGAAGTGGGCATCTGTGGTG GTGCCTCTTGGGAAGGAGCAGTATTACACATGCCATGTGTACCATCAGGGGCTGCCTGAGCC CCTCACCCTGAGATGGGAGCCTCCTCCATCCGGATCCTGCTAAaagcttGCGGCCGCACTCG AGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTG GCTGCTCC
Protein Sequence	*xvsgstopqfpsrnnfv-l-egdih*MGNYSFYALGGGASGGGGSGGGGSIQKTPQIQVYS RHPPENGKPNILNCYVTQFHPPHIEIQMLKNGKKIPKVEMSDMSFSKDWSFYILAHTEFT PTETDTYACRVKHASMAEPKTVYWDRDMGGGGSGGGGSGGGGSGGGGSGPHSLRYFVTAV SRPGLGEPRYMEVGYVDDTEFVRFDSDAENPRYEPRARWMEQEGPEYWERETQKAKGNEQ SFRVDLRTLLG**A**YNQSKGGSHTIQVISGCEVGSDGRLLRGYQQYAYDG**R**DYIALNEDLKT WTAADMAALITKHKWEQAGEAERLRAYLEGTCVEWLRRYLKNGNATLLRTDSPKAHVTHH SRPEDKVTLRCWALGFYPADITLTWQLNGEELIQDMELVETRPAGDGTFQKWASVVVPLG KEQYYTCHVYHQGLPEPLTLRWEPPPSGSCEND*klaaalehhhhhhstopdpaankarke* *aelaa*
Protein MW	47,217 Da
Protein pI	6.03
Protein extinction coefficient	92,390 at 280nm (unfolded)

Gene Construct	HLA-A2bsp (heavy chain with C-terminal bsp sequence)
Plasmid	pHN1+ (T7 promoter) Amp resistance gene for plasmid production in *E. coli*
Made by	from University of Massachusetts Medical School Tetramer Facility
Sites used for cloning	SacI and HindIII
Insert size	~900bp (not certain)
For production in	*E. coli* BL21 or XA90 Induce with IPTG
Gene Sequence	not checked *HLA-A*020101 sequence from IMGT:* ATGGCCGTCATGGCGCCCCGAACCCTCGTCCTGCTACTCTCGGGGGCTCTGGCCCTGA CCCAGACCTGGGCGGGCTCTCACTCCATGAGGTATTTCTTCACATCCGTGTCCCGGCC CGGCCGCGGGGAGCCCCGCTTCATCGCAGTGGGCTACGTGGACGACACGCAGTTCGTG CGGTTCGACAGCGACGCCGCGAGCCAGAGGATGGAGCCGCGGGCGCCGTGGATAGAGC AGGAGGGTCCGGAGTATTGGGACGGGGAGACACGGAAAGTGAAGGCCCACTCACAGAC TCACCGAGTGGACCTGGGGACCCTGCGCGGCTACTACAACCAGAGCGAGGCCGGTTCT CACACCGTCCAGAGGATGTATGGCTGCGACGTGGGGTCGGACTGGCGCTTCCTCCGCG GGTACCACCAGTACGCCTACGACGGCAAGGATTACATCGCCCTGAAAGAGGACCTGCG CTCTTGGACCGCGGCGGACATGGCAGCTCAGACCACCAAGCACAAGTGGGAGGCGGCC CATGTGGCGGAGCAGTTGAGAGCCTACCTGGAGGGCACGTGCGTGGAGTGGCTCCGCA GATACCTGGAGAACGGGAAGGAGACGCTGCAGCGCACGGACGCCCCCAAAACGCATAT GACTCACCACGCTGTCTCTGACCATGAAGCCACCCTGAGGTGCTGGGCCCTGAGCTTC TACCCTGCGGAGATCACACTGACCTGGCAGCGGGATGGGGAGGACCAGACCCAGGACA CGGAGCTCGTGGAGACCAGGCCTGCAGGGGATGGAACCTTCCAGAAGTGGGCGGCTGT GGTGGTGCCTTCTGGACAGGAGCAGAGATACACCTGCCATGTGCAGCATGAGGGTTTG CCCAAGCCCCTCACCCTGAGATGGGAGCCGTCTTCCCAGCCCACCATCCCCATCGTGG GCATCATTGCTGGCCTGGTTCTCTTTGGAGCTGTGATCACTGGAGCTGTGGTCGCTGC TGTGATGTGGAGGAGGAAGAGCTCAGATAGAAAAGGAGGGAGCTACTCTCAGGCTGCA AGCAGTGACAGTGCCCAGGGCTCTGATGTGTCTCTCACAGCTTGTAAAGTGTGA
Protein Sequence	not checked *HLA-A*020101 sequence from IMGT:* MAVMAPRTLVLLLSGALALTQTWAGSHSMRYFFTSVSRPGRGEPRFIAVGYVDDTQFVRF DSDAASQRMEPRAPWIEQEGPEYWDGETRKVKAHSQTHRVDLGTLRGYYNQSEAGSHTVQ RMYGCDVGSDWRFLRGYHQYAYDGKDYIALKEDLRSWTAADMAAQTTKHKWEAAHVAEQL RAYLEGTCVEWLRRYLENGKETLQRTDAPKTHMTHHAVSDHEATLRCWALSFYPAEITLT WQRDGEDQTQDTELVETRPAGDGTFQKWAAVVVPSGQEQRYTCHVQHEGLPKPLTLRWEP SSQPTIPIVGIIAGLVLFGAVITGAVVAAVMWRRKSSDRKGGSYSQAASSDSAQGSDVSL TACKVEND
Protein MW	~40,922 Da (full length from IMGT)
Protein pI	~6.50
Protein extinction coefficient	~87,720 at 280nm (full length from IMGT, unfolded)

Gene Construct	HLA-A2cys (heavy chain with C-terminal cysteine)
Plasmid	pLM1 (T7 promoter) Amp resistance gene for plasmid production in *E. coli*
Made by	J. Stone
Sites used for cloning	EcoRI and HindIII
Insert size	~863bp
For production in	*E. coli* BL21 Induce with IPTG
Gene Sequence	TTTTGTTgaattcAGGAGGAATTTAAAATGGGCTCTCACTCCATGAGGTATTTCTTCACATCC GTGTCCCGGCCCGGCCGCGGGGAGCCCCGCTTCATCGCAGTGGGCTACGTGGACGACACGCAG TTCGTGCGGTTCGACAGCGACGCCGCGAGCCAGAGGATGGAGCCGCGGGCGCCGTGGATAGAG CAGGAGGGTCCGGAGTATTGGGACGGGGAGACACGGAAAGTGAAGGCCCACTCACAGACTCAC CGAGTGGACCTGGGGACCCTGCGCGGCTACTACAACCAGAGCGAGGCCGGTTCTCACACCGTC CAGAGGATGTATGGCTGCGACGTGGGGTCGGACTGGCGCTTCCTCCGCGGGTACCACCAGTAC GCCTACGACGGCAAGGATTACATCGCCCTGAAAGAGGACCTGCGCTCTTGGACCGCGGCGGAC ATGGCAGCTCAGACCACCAAGCACAAGTGGGAGGCGGCCCATGTGGCGGAGCAGTTGAGAGCC TACCTGGAGGGCACGTGCGTGGAGTGGCTCCGCAGATACCTGGAGAACGGGAAGGAGACGCTG CAGCGCACGGACGCCCCCAAAACGCATATGACTCACCACGCTGTCTCTGACCATGAAGCCACC CTGAGGTGCTGGGCCCTGAGCTTCTACCCTGCGGAGATCACACTGACCTGGCAGCGGGATGGG GAGGACCAGACCCAGGACACGGAGCTCGTGGAGACAAGGCCTGCAGGGGATGGAACCTTCCAG AAGTGGGCGGCTGTGGTGGTGCCTTCTGGACAGGAGCAGAGATACACCTGCCATGTGCAGCAT GAGGGTTTGCCCAAGCCCCTCACCCTGAGATGGGAGGCGTGCTAATAGTGAaagctt
Protein Sequence	*fc-iqeefk*MGSHSMRYFFTSVSRPGRGEPRFIAVGYVDDTQFVRFDSDAASQRMEPRAPWIE QEGPEYWDGETRKVKAHSQTHRVDLGTLRGYYNQSEAGSHTVQRMYGCDVGSDWRFLRGYHQY AYDGKDYIALKEDLRSWTAADMAAQTTKHKWEAAHVAEQLRAYLEGTCVEWLRRYLENGKETL QRTDAPKTHMTHHAVSDHEATLRCWALSFYPAEITLTWQRDGEDQTQDTELVETRPAGDGTFQ KWAAVVVPSGQEQRYTCHVQHEGLPKPLTLRWEAC**END**--*kl*
Protein MW	32,114 Da
Protein pI	6.01
Protein extinction coefficient	75,060 at 280nm (unfolded)

Gene Construct	HLA-B7bsp (heavy chain with C-terminal bsp sequence)
Plasmid	pET9a (T7 promoter) Amp resistance gene for plasmid production in *E. coli*
Made by	from University of Massachusetts Medical School Tetramer Facility
Sites used for cloning	?
Insert size	~900bp (not certain)
For production in	*E. coli* BL21 or XA90 Induce with IPTG
Gene Sequence	not checked *HLA-B*070201 sequence from IMGT:* ATGCTGGTCATGGCGCCCCGAACCGTCCTCCTGCTGCTCTCGGCGGCCCTGGCCCTGACC GAGACCTGGGCCGGCTCCCACTCCATGAGGTATTTCTACACCTCCGTGTCCCGGCCCGGC CGCGGGGAGCCCCGCTTCATCTCAGTGGGCTACGTGGACGACACCCAGTTCGTGAGGTTC GACAGCGACGCCGCGAGTCCGAGAGAGGAGCCGCGGGCGCCGTGGATAGAGCAGGAGGGG CCGGAGTATTGGGACCGGAACACACAGATCTACAAGGCCCAGGCACAGACTGACCGAGAG AGCCTGCGGAACCTGCGCGGCTACTACAACCAGAGCGAGGCCGGGTCTCACACCCTCCAG AGCATGTACGGCTGCGACGTGGGGCCGGACGGGCGCCTCCTCCGCGGGCATGACCAGTAC GCCTACGACGGCAAGGATTACATCGCCCTGAACGAGGACCTGCGCTCCTGGACCGCCGCG GACACGGCGGCTCAGATCACCCAGCGCAAGTGGGAGGCGGCCCGTGAGGCGGAGCAGCGG AGAGCCTACCTGGAGGGCGAGTGCGTGGAGTGGCTCCGCAGATACCTGGAGAACGGGAAG GACAAGCTGGAGCGCGCTGACCCCCCAAAGACACACGTGACCCACCACCCCATCTCTGAC CATGAGGCCACCCTGAGGTGCTGGGCCCTGGGTTTCTACCCTGCGGAGATCACACTGACC TGGCAGCGGGATGGCGAGGACCAAACTCAGGACACTGAGCTTGTGGAGACCAGACCAGCA GGAGATAGAACCTTCCAGAAGTGGGCAGCTGTGGTGGTGCCTTCTGGAGAAGAGCAGAGA TACACATGCCATGTACAGCATGAGGGGCTGCCGAAGCCCCTCACCCTGAGATGGGAGCCG TCTTCCCAGTCCACCGTCCCCATCGTGGGCATTGTTGCTGGCCTGGCTGTCCTAGCAGTT GTGGTCATCGGAGCTGTGGTCGCTGCTGTGATGTGTAGGAGGAAGAGTTCAGGTGGAAAA GGAGGGAGCTACTCTCAGGCTGCGTGCAGCGACAGTGCCCAGGGCTCTGATGTGTCTCTC ACAGCTTGA
Protein Sequence	not checked *HLA*020101 sequence from IMGT:* MLVMAPRTVLLLLSAALALTETWAGSHSMRYFYTSVSRPGRGEPRFISVGYVDDTQFVRF DSDAASPREEPRAPWIEQEGPEYWDRNTQIYKAQAQTDRESLRNLRGYYNQSEAGSHTLQ SMYGCDVGPDGRLLRGHDQYAYDGKDYIALNEDLRSWTAADTAAQITQRKWEAAREAEQR RAYLEGECVEWLRRYLENGKDKLERADPPKTHVTHHPISDHEATLRCWALGFYPAEITLT WQRDGEDQTQDTELVETRPAGDRTFQKWAAVVVPSGEEQRYTCHVQHEGLPKPLTLRWEP SSQSTVPIVGIVAGLAVLAVVVIGAVVAAVMCRRKSSGGKGGSYSQAACSDSAQGSDVSL TAEND
Protein MW	~40,460 Da (full length from IMGT)
Protein pI	~5.57
Protein extinction coefficient	~77,740 at 280nm (full length from IMGT, unfolded)

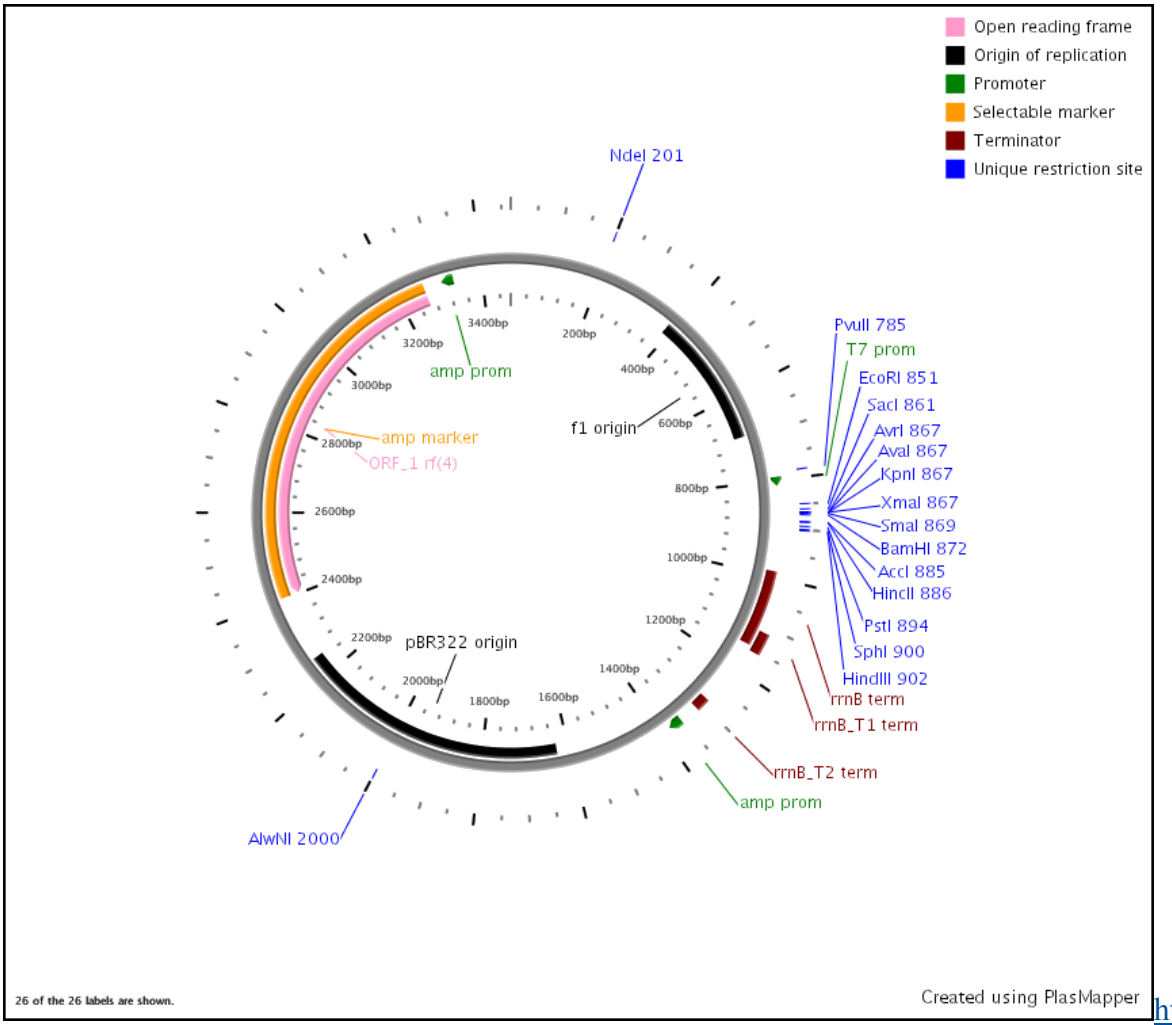
Gene Construct	HLA-B7cys (heavy chain with C-terminal cysteine)
Plasmid	pLM1 (T7 promoter) Amp resistance gene for plasmid production in *E. coli*
Made by	J. Stone
Sites used for cloning	EcoRI and HindIII
Insert size	~863bp
For production in	*E. coli* BL21 Induce with IPTG
Gene Sequence	TTTTGTTgaattcAGGAGGAATTTAAAATGGGCTCCCACTCCATGAGGTATTTCTACACCTCC GTGTCCCGACCCGGCCGCGGGGAGCCCCGCTTCATCTCAGTGGGCTACGTGGACGACACCCAG TTCGTGAGGTTCGACAGCGACGCCGCGAGTCCGAGAGAGGAGCCGCGGGCGCCGTGGATAGAG CAGGAGGGGCCGGAGTATTGGGACCGGAACACACAGATCTACAAGGCCCAGGCACAGACTGAC CGAGAGAGCCTGCGGAACCTGCGCGGCTACTACAACCAGAGCGAGGCCGGGTCTCACACCCTC CAGAGCATGTACGGCTGCGACGTGGGGCCGGACGGGCGCCTCCTCCGCGGGCATGACCAGTAC GCCTACGACGGCAAGGATTACATCGCCCTGAACGAGGACCTGCGCTCCTGGACCGCCGCGGAC ACGGCGGCTCAGATCACCCAGCGCAAGTGGGAGGCGGCCCGTGAGGCGGAGCAGCGGAGAGCC TACCTGGAGGGCGAGTGCGTGGAGTGGCTCCGCAGATACCTGGAGAACGGGAAGGACAAGCTG GAGCGCGCTGACCCCCCAAAGACACACGTGACCCACCACCCCATCTCTGACCATGAGGCCACC CTGAGGTGCTGGGCCCTGGGTTTCTACCCTGCGGAGATCACACTGACCTGGCAGCGGGATGGC GAGGACCAAACTCAGGACACTGAGCTTGTGGAGACCAGACCAGCAGGAGATAGAACCTTCCAG AAGTGGGCAGCTGTGGTGGTGCCTTCTGGAGAAGAGCAGAGATACACATGCCATGTACAGCAT GAGGGGCTGCCGAAGCCCCTCACCCTGAGATGGGAGGCGTGCTAATAGTGAaagctt
Protein Sequence	MGSHSMRYFYTSVSRPGRGEPRFISVGYVDDTQFVRFDSDAASPREEPRAPWIEQEGPEYWDR NTQIYKAQAQTDRESLRNLRGYYNQSEAGSHTLQSMYGCDVGPDGRLLRGHDQYAYDGKDYIA LNEDLRSWTAADTAAQITQRKWEAAREAEQRRAYLEGECVEWLRRYLENGKDKLERADPPKTH VTHHPISDHEATLRCWALGFYPAEITLTWQRDGEDQTQDTELVETRPAGDRTFQKWAAVVVPS GEEQRYTCHVQHEGLPKPLTLRWEACEND
Protein MW	32,223.4 Da
Protein pI	5.36
Protein extinction coefficient	70,650 at 280nm (unfolded)

Gene Construct	B_2M (human light chain, beta-2 microglobulin)
Plasmid	(T7 promoter) Amp resistance gene for plasmid production in *E. coli*
Made by	NIH tetramer facility
Sites used for cloning	
Insert size	~300bp
For production in	*E. coli* BL21 Induce with IPTG
Gene Sequence	not checked
Protein Sequence	not checked *sequence from Reid et.al., J Exp Med v. 184 (2279) 1996:* IQRTPKIQVYSRHPAENGKSNFLNCYVSGFHPSDIEVDLLKNGERIEKVEHSDLSFSK DWSFYLLYYTEFTPTEKDEYACRVNHVTLSQPKIVKWDRDM
Protein MW	11,731 Da
Protein pI	6.07
Protein extinction coefficient	19,180 at 280nm (unfolded)

Gene Construct	Biotin Ligase enzyme (BirA) containing his-tag for purification
Plasmid	pET22b Amp resistance gene for plasmid production in *E. coli*
Made by	Kai Wucherpfennig
Sites used for cloning	NdeI to XhoI
Insert size	~967bp
For production in	*E. coli* BL21 Induce with IPTG **protein is soluble—not in inclusion bodies**
Gene Sequence	CATATGAAGGATAACACCGTGCCACTGAAATTGATTGCCCTGTTAGCGAACGGTGAATTT CACTCTGGCGAGCAGTTGGGTGAAACGCTGGGAATGAGCCGGGCGGCTATTAATAAACAC ATTCAGACACTGCGTGACTGGGGCGTTGATGTCTTTACCGTTCCGGGTAAAGGATACAGC CTGCCTGAGCCTATCCAGTTACTTAATGCTAAACAGATATTGGGTCAGCTGGATGGCGGT AGTGTAGCCGTGCTGCCAGTGATTGACTCCACGAATCAGTACCTTCTTGATCGTATCGGA GAGCTTAAATCGGGCGATGCTTGCATTGCAGAATACCAGCAGGCTGGCCGTGGTCGCCGG GGTCGGAAATGGTTTTCGCCTTTTGGCGCAAACTTATATTTGTCGATGTTCTGGCGTCTG GAACAAGGCCCGGCGGCGGCGATTGGTTTAAGTCTGGTTATCGGTATCGTGATGGCGGAA GTATTACGCAAGCTGGGTGCAGATAAAGTTCGTGTTAAATGGCCTAATGACCTCTATCTG CAGGATCGCAAGCTGGCAGGCATTCTGGTGGAGCTGACTGGCAAAACTGGCGATGCGGCG CAAATAGTCATTGGAGCCGGGATCAACATGGCAATGCGCCGTGTTGAAGAGAGTGTCGTT AATCAGGGGTGGATCACGCTGCAGGAAGCGGGGATCAATCTCGATCGTAATACGTTGGCG GCCATGCTAATACGTGAATTACGTGCTGCGTTGGAACTCTTCGAACAAGAAGGATTGGCA CCTTATCTGTCGCGCTGGGAAAAGCTGGATAATTTTATTAATCGCCCAGTGAAACTTATC ATTGGTGATAAAGAAATATTTGGCATTTCACGCGGAATAGACAAACAGGGGGCTTTATTA CTTGAGCAGGATGGAATAATAAAACCCTGGATGGGCGGTGAAATATCCCTGCGTAGTGCA GAAAAACTCGAGCACCACCACCACCACCACTGA
Protein Sequence	*h*MKDNTVPLKLIALLANGEFHSGEQLGETLGMSRAAINKHIQTLRDWGVDVFTVPGKGYS LPEPIQLLNAKQILGQLDGGSVAVLPVIDSTNQYLLDRIGELKSGDACIAEYQQAGRGRR GRKWFSPFGANLYLSMFWRLEQGPAAAIGLSLVIGIVMAEVLRKLGADKVRVKWPNDLYL QDRKLAGILVELTGKTGDAAQIVIGAGINMAMRRVEESVVNQGWITLQEAGINLDRNTLA AMLIRELRAALELFEQEGLAPYLSRWEKLDNFINRPVKLIIGDKEIFGISRGIDKQGALL LEQDGIIKPWMGGEISLRSAEKLEHHHHHHHEND
Protein MW	36,450 Da
Protein pI	6.73
Protein extinction coefficient	47,510 at 280nm (unfolded)

Plasmid	pLM1 (T7 promoter) Amp resistance gene for *E. coli*
Size	3467 bp
For production in	*E. coli*—Induce with IPTG Contains Ampicillin resistance gene
Multicloning site	EcoRI (850) to HindIII (901) including Ecl135II (858, blunt), SacI (860), Acc65I (862), AvaI, KpnI, and XmaI (866), SmaI (868, blunt), BamHI (871), SalI (883), AccI (884, 2bp overhang), HincII (885, blunt), PstI (893), SbfI (893), BspMI (896), and SphI (899). Number indicates bp before the cut.
Gene Sequence	ACTTTGTCTTCCCCCTTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGC AGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCG TCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCA GAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAA GGAGAAAATACCGCATCAGGCGAAATTGTAAACGTTAATATTTTGTTAAAATTCGCGT TAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCC TTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAG AGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGG GCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTGCCG TAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAG CCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGC TGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCC GCTACAGGGCGCGTCGCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGA TCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGCGAAATTAATACGACTCACTATAGG GAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTT**GAATTC**GAGCTCGGTACCCGG GGATCCTCTAGAGTCGACCTGCAGGCATGC**AAGCTT**GGCTGTTTTGGCGGATGAGAGA AGATTTTCAGCCTGATACAGATTAAATCAGAACGCAGAAGCGGTCTGATAAAACAGAA TTTGCCTGGCGGCAGTAGCGCGGTGGTCCCACCTGACCCCATGCCGAACTCAGAAGTG AAACGCCGTAGCGCCGATGGTAGTGTGGGGTCTCCCCATGCGAGAGTAGGGAACTGCC AGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTT GTTTGTCGGTGAACGCTCTCCTGAGTAGGACAAATCCGCCGGGAGCGGATTTGAACGT TGCGAAGCAACGGCCCGGAGGGTGGCGGGCAGGACGCCCGCCATAAACTGCCAGGCAT CAAATTAAGCAGAAGGCCATCCTGACGGATGGCCTTTTTGCGTTTCTACAAACTCTTT TGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGAT AAATGCTTCAATAATCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGC GTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCT GCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGG GATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAA AGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAA TCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTT CCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACC TGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTA TCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTT CAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGAC ACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGT AGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACA GTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCT CTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCA GATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCT GACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAA GGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTAT ATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCA GCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTA

	CGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACG CTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGA AGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTA GAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCAT CGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCA AGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTC CGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACT GCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTAC TCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGT CAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAA ACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATG TAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTG GGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAA ATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTAT TGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTC CGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGAC ATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCCCCT



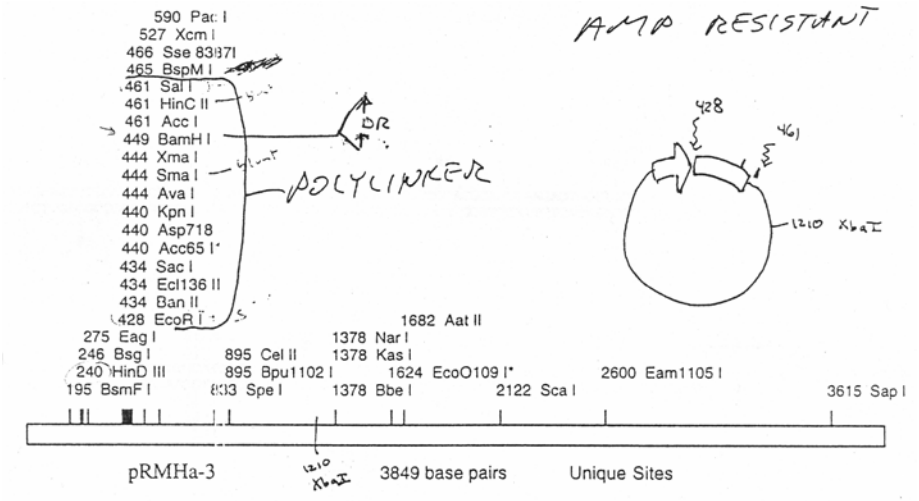
http://wishart.biology.ualberta.ca/PlasMapper.

Notes

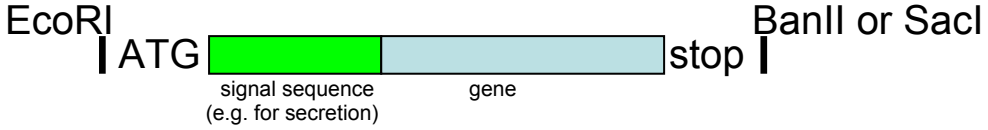
Enzyme	#Cuts	Site position (Fragment length)			
AatII	1	3393	(3467)		
Acc65I	1	861	(3467)		
AccI	1	882	(3467)		
AflIII	1	1582	(3467)		
AhdI	1	2470	(3467)		
AlwNI	1	1993	(3467)		
AvaI	1	865	(3467)		
BamHI	1	870	(3467)		
BpmI	1	2544	(3467)		
BsaAI	1	476	(3467)		
BsmBI	1	61	(3467)		
BspMI	1	886	(3467)		
BstAPI	1	194	(3467)		
DraIII	1	473	(3467)		
Ecl136II	1	855	(3467)		
EcoO109I	1	3450	(3467)		
EcoRI	1	849	(3467)		
HincII	1	882	(3467)		
HindIII	1	900	(3467)		
KpnI	1	861	(3467)		
NaeI	1	579	(3467)		
NdeI	1	198	(3467)		
NgoMIV	1	579	(3467)		
PciI	1	1582	(3467)		
PsiI	1	348	(3467)		
PstI	1	888	(3467)		
PvuII	1	781	(3467)		
SacI	1	855	(3467)		
SalI	1	882	(3467)		
SapI	1	1459	(3467)		
SbfI	1	887	(3467)		
ScaI	1	2953	(3467)		
SmaI	1	865	(3467)		
SphI	1	894	(3467)		
XmaI	1	865	(3467)		
BanII	2	549	(306)	855	(3161)
BcgI	2	2979	(12)	2991	(3455)
BglI	2	720	(1869)	2589	(1598)
BmrI	2	1136	(1384)	2520	(2083)
BsmFI	2	997	(2449)	3446	(1018)
BsrDI	2	2529	(180)	2709	(3287)
BsrFI	2	579	(1976)	2555	(1491)
BtsI	2	2868	(26)	2894	(3441)
EaeI	2	1421	(1442)	2863	(2025)
Eco57I	2	2109	(1032)	3141	(2435)
FspI	2	731	(1964)	2695	(1503)
PvuI	2	751	(2091)	2842	(1376)
SspI	2	268	(3009)	3277	(458)
TatI	2	182	(2771)	2953	(696)
TfiI	2	1417	(140)	1557	(3327)
XbaI	2	831	(45)	876	(3422)
XmnI	2	926	(2144)	3070	(1323)

	No Sites found for the following Restriction Endonucleases AfeI AflII AgeI ApaI AscI AvrII BaeI BbeI BbsI BbvCI BclI BglII BlpI BplI Bpu10I BsaBI BseRI BsgI BsiWI BsmI BspEI BsrGI BssHII BstBI BstDSI BstEII BstXI BstZ17I Bsu36I BtgI BtrI ClaI EagI EcoNI EcoRV FseI HpaI KasI MfeI MluI MscI NarI NcoI NheI NotI NruI NsiI PacI PflMI PmeI PmlI Ppu10I PpuMI PshAI PspOMI RsrII SacII SanDI SexAI SfiI SfoI SgfI SgrAI SnaBI SpeI SrfI StuI StyI SwaI TliI Tth111I XcmI XhoI

Plasmid	pRMHA-3 (metallothionine promoter) Amp resistance gene for plasmid production in *E. coli* no resistance for S2; co-transfect with pNeo (G418 resistance)
Size	3849 bp
From	Chris Garcia at Stanford. Reference: Bunch et. al., Nucleic Acids Research, Feb. 1988, 16(3):1043.
Promoter	Metallothionine promoter—sequence starts at position 2: GTTGCAGGACAGGATGTGGTGCCCGATGTGACTAGCTCTTTGCTGCA...
For production in	S2 Drosophila cells Induce with $CuSO_4$
Gene Sequence	**ONLY HAVE HARD COPY OF THIS SEQUENCE** Polclonal site reads: GAATCATCTCAGTGCAACTAAAGGGGGGAATTCGAGCTCGGTACCCGGGGATCCTCTAG AGTCGACCTGCAGGCATGCAATTCGATGCACACTCACATTCTTCTCCTAATACGATAAT AAAACTTTCCATGAAAAATATGGAAAAATAT Which includes EcoRI (GAATTC), SacI (GAGCTC), KpnI (GGTACC), AvaI (CCCGGG), SmaI (CCCGGG, blunt), Xma I (CCCGGG), BamHI (GGATCC), SalI (GTCGAC) unique sites. XbaI (TCTAGA) is also present, but NOT unique (there is another at pos 1210). Absent sites from vector include: AflII, AgeI, AscI, AvrII, BglII, BspeI, ClaI, DsaI, EcoRV, MluI, NcoI, NheI, NotI, SacII, SnaBI, SplI, XhoI.
Notes	Contains an Ampicillin resistance gene for production in *E. coli*.



Model Cloning Scheme:



C.1 PEPTIDE SEQUENCES

Name	Sequence
Starting peptides for making peptide-based crosslinkers	
pep2	fluorescein-βA-E-K-S-G-S-K-G-NH_2
pep3	fluorescein-βA-E-K-S-G-S-G-K-S-G-E-S-K-G-NH_2
pep4	fluorescein-βA-E-K-S-G-S-G-K-S-G-E-S-K-S-S-E-G-K-G-NH_2
het2	fluorescein-βA-E-K-S-G-S-C-G-NH_2
het3a	fluorescein-βA-E-K-S-G-S-G-K-S-G-E-S-C-G-NH_2
het3b	fluorescein-βA-E-K-S-G-S-G-C-S-G-E-S-K-G-NH_2
het4a	fluorescein-βA-E-K-S-G-S-G-K-S-G-E-S-K-S-S-E-G-C-G-NH_2
het4b	fluorescein-βA-E-K-S-G-S-G-K-S-G-E-S-C-S-S-E-G-K-G-NH_2
het4c	fluorescein-βA-E-K-S-G-S-G-K-S-G-E-S-C-S-S-E-G-C-G-NH_2
het4d	fluorescein-βA-E-K-S-G-S-G-C-S-G-E-S-K-S-S-E-G-C-G-NH_2
Binds to DR1, from influenza haemagglutinin	
Ha	P-K-Y-V-K-Q-N-T-L-K-L-A-T-NH_2
Binds to DR1, from human class I MHC HLA-A2	
A2	V-G-S-D-W-R-F-L-R-G-Y-H-Q-Y-A-NH_2
Bind to DR1, from HIV-1	
gag_s (PG13, p24(34))	P-E-V-I-P-M-F-S-A-L-S-E-G-NH_2
gag_L (PP16)	P-E-V-I-P-M-F-S-A-L-S-E-G-A-T-P-NH_2
Binds to DR1, from BZLF-1 protein of Epstein Barr Virus	
EBV	Q-H-Y-R-E-V-A-A-A-K-S-S-E
Binds to DR1, from Nuclear Protein	
NP [206-217]	N-F-W-R-G-E-N-G-R-K-T-R-N
Binds to DR1, from Transferrin Receptor	
Tfr	R-V-E-Y-H-F-L-S-P-Y-V-S-P-K-E-S-P
Binds to DR1, from Tetanus Toxin	
TT	Q-Y-I-K-A-N-S-K-F-I-G-I-T-E
Binds to DR1, from another strain of influenza (Flu strain B)	
FluB	P-Y-Y-T-G-E-H-A-K-A-I-G-N
Binds to DR1, from influenza matrix protein	
MA	S-G-P-L-K-A-E-I-A-Q-R-L-E-D
Binds to L^d--stimulatory to 2C T cells	
QL9	Q-L-S-P-F-P-F-D-L
Binds to L^d and K^b weakly—weak agonist for 2C in complex with both	
p2Ca	L-S-P-F-P-F-D-L
Binds to L^d—does not stimulate 2C	
MCMV	Y-P-H-F-M-P-T-N-L
Binds to K^b—stimulates 2C T cells	
SYRGL	S-I-Y-R-Y-Y-G-L

C.1 PEPTIDE SEQUENCES

Name	Sequence
Binds to K^b—stimulates OT-1 T cells	
OVA	S-I-I-N-F-E-K-L
Binds to K^b—does not stimulate 2C T cells	
SEV	F-A-P-G-N-Y-P-A-L
Binds to K^b—2C weak agonist	
dEV8	E-Q-Y-K-F-Y-S-V
Binds to K^b—2C antagonist	
GNY	G-N-Y-S-F-Y-A-L
Binds to K^b—OT-1 antagonist	
E1	E-I-I-N-F-E-K-L
Bind to A2, from Modified Vaccinia Ankara	
MVA-165	K-V-D-D-T-F-Y-Y-V
MVA-74A	C-L-T-E-Y-I-L-W-V
Binds to B7, from EBNA protein of Epstein Barr Virus	
B7 EBNA 3A	R-P-P-I-F-I-R-R-L

C.2 EBV BZLF-1 OVERLAPPING PEPTIDES

Overlapping peptides from Melissa Precopio from BZLF-1. This protein is an immediate early gene product of the Epstein Barr Virus which functions as a transcription factor. These peptides are 25-mers that overlap by 10 amino acids.

#	Peptide Sequence	MW
1	MMDPNSTSEDVKFTPDPYQVPFVQA	2844.16
2	DPYQVPFVQAFDQATRVYQDLGGPS	2799.05
3	RVYQDLGGPSQAPLPCVLWPVLPEP	2762.19
4	CVLWPVLPEPLPQGQLTAYHVSTAP	2717.18
5	LTAYHVSTAPTGSWFSAPQPAPENA	2600.83
6	SAPQPAPENAYQAYAAPQLFPVSDI	2645.91
7	APQLFPVSDITQNQQTNQAGGEAPQ	2639.82
8	TNQAGGEAPQPGDNSTVQTAAAVVF	2430.57
9	TVQTAAAVVFACPGANQGQQLADIG	2430.72
10	NQGQQLADIGVPQPAPVAAPARRTR	2611.95
11	PVAAPARRTRKPQQPESLEECDSEL	2808.12
12	ESLEECDSELEIKRYKNRVASRKCR	3042.44
13	KNRVASRKCRAKFKQLLQHYREVAA	3001.55
14	LLQHYREVAAAKSSENDRLRLLLKQ	2952.41
15	NDRLRLLLKQMCPSLDVDSIIPRTP	2894.44
16	MCPSLDVDSIIPRTPDVLHEDLLNF	2840.26
min	QHYREVAAAKSSE	1475.58

C.3 HIV-1 GAG OVERLAPPING PEPTIDES

Overlapping peptides from Phillip Norris from gag p24, which is a major core protein of the human immunodeficiency virus encoded by the HIV gag gene. HIV-seropositive individuals mount a significant immune response to p24 and thus detection of antibodies to p24 is one basis for determining HIV infection by ELISA and Western blot assays. The protein is also being investigated as a potential HIV immunogen in vaccines. These peptides are 22-mers overlapping by 12 residues.

#	Peptide Sequence	MW
p24-109	PIVQNIQGQMVHQAISPRTLNA	2415.80
p24-110	VHQAISPRTLNAWVKVVEEKAF	2522.93
p24-111	NAWVKVVEEKAFSPEVIPMFSA	2478.89
p24-112	AFSPEVIPMFSALSEGATPQDL	2307.60
p24-113	SALSEGATPQDLNTMLNTVGGH	2213.40
p24-114	DLNTMLNTVGGHQAAMQMLKET	2403.77
p24-115	GHQAAMQMLKETINEEAAEQDR	2500.74
p24-116	ETINEEAAEWDRVHPVHAGPIA	2441.64
p24-117	DRVHPVHAGPIAPGQMREPRGS	2364.67
p24-118	IAPGQMREPRGSDIAGTTSTLQ	2286.54
p24-119	GSDIAGTTSTLQEQIGWMTNNP	2321.50
p24-120	LQEQIGWMTNNPPIPVGEIYKR	2583.99
p24-121	NPPIPVGEIYKRWIILGLNK	2320.81
p24-122	KRWIILGLNKIVRMYSPTSILD	2617.19
p24-123	IVRMYSPTSILDIRQGPKEPFR	2604.07
p24-124	LDIRQGPKEPFRDYVDRFYKTL	2757.14
p24-125	FRDYVDRFYKTLRAEQASQEVK	2750.07
p24-126	TLRAEQASQEVKNWMTETLLVQ	2575.92
p24-127	VKNWMTETLLVQNANPDCKTIL	2531.97
p24-128	CQNANPDCKTILKALGPAATLE	2271.63
p24-129	ILKALGPAATLEEMMTACQGVG	2204.64
p24-130	LEEMMTACQGVGGPGHKARVL	2184.97
p24-131	VGGPGHKARVLAEAMSQVTNTATIM	2539.95
min-“gagL”	PEVIPMFSALSEGATP	1645.89
antag-“gagS”	PEVIPMFSALSEG	1376.59

C.4 SOME MODIFIED VACCINIA ANKARA PEPTIDES

Peptides from John Cruz in Alan Rothman's lab which gave some response in an ELISPOT test or proliferation assay. These peptides are derived from Modified Vaccinia Ankara, and they were tested for binding to HLA-A2.

Peptide Name	protein	Amino Acid number	Amino Acid Sequence
MVA074A	B22R	079-087	CLTEYILWV
MVA165	C7L	074-082	KVDDTFYYV
MVA103A	A44L	292-300	ILFRKPSLL
MVA115	I5L	007-015	VLTAIGITV
MVA119	J4R	141-149	KMLETEIVV
MVA123	A36R	016-024	ILVCYILYI
MVA126	F12L	050-058	VMAMMNITI
MVA129	F12R	196-204	VLPFDIKYI
MVA 138	F12R	552-560	KMIYDLNAV
MVA150	A17L	070-078	VLALYSPPL
MVA155	AAB96557	020-028	YLYNYTIAV
MVA158	H3L	292-304	GLIVILFIMFMLI
MVA163	A7L	152-160	NIIKFETML
MVA167	D12L	251-259	RVYEALYYV
MVA176	L2R	078-086	TLTIFYYFI
MVA179	A41L	011-019	CIPFSFQTV
MVA183	F14L	039-047	ELLNILTEL
MVA189	I8R	197-205	KLLLWFNYL
MVA198	I4L	094-106	FSEVMEDLFNYVN
MVA103B	A44L	281-289	KMYACKNDM

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EDUCATION

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HONORS AND AWARDS

NIH Biotechnology Training Grant fellowship, MIT, August 2001-July 2003
Teaching Assistant awards in Biochemisrty 5.10 and General Chemistry Lab 5.310, MIT, June 2000
Magna cum laude, Chemical Engineering, The Ohio State University, June 1999
Dean's list, The University Honors Scholarship, Women in Chemical Engineering Scholarship, Chemical Engineering Class of '41 Scholarship, The Ohio State University, 1994-1999
The Robert C. Byrd Scholarship, The Batelle Scholarship, and The Ohio Space Grant Consortium Scholarhip, 1994-1998

RESEARCH EXPERIENCE

Graduate research associate, *Jan. 2000-Feb. 2005*, Prof. Lawrence J. Stern, MIT Department of Chemistry
Investigating the molecular mechanism of T cell activation and detecting antigen-specific T cell responses
Undergraduate research assistant, Mar. 1998-Mar. 1999, Prof. Gary E. Means, OSU Department of Biochemistry
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Undergraduate research assistant, Mar. 1997-Mar. 1998, Prof. James F. Rathman, OSU Department of Chemical Engineering. Production and properties of magnetorheologic fluids
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Quality control and defect monitoring of high-performance film.

PUBLICATIONS

Stone, J.D., Cochran, J.R., and Stern, L.J. On the valency requirements of T cell activation by soluble MHC-peptide complexes. *J. Immunol.* submitted.
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PATENT APPLICATION

Stern, L.J., Stone, J.D., Carven, G.J., Chitta, S., and Strug, I. Major Histocompatability Complex (MHC)-Peptide Arrays. Pending Patent Application # 10/823,866, filed April, 2004.